Centromere Protein B Null Mice are Mitotically and Meiotically Normal but Have Lower Body and Testis Weights

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Abstract. CENP-B is a constitutive centromere DNA-binding protein that is conserved in a number of mammalian species and in yeast. Despite this conservation, earlier cytological and indirect experimental studies have provided conflicting evidence concerning the role of this protein in mitosis. The requirement of this protein in meiosis has also not previously been described. To resolve these uncertainties, we used targeted disruption of the Cenpb gene in mouse to study the functional significance of this protein in mitosis and meiosis. Male and female Cenpb null mice have normal body weights at birth and at weaning, but these subsequently lag behind those of the heterozygous and wild-type animals. The weight and sperm content of the testes of Cenpb null mice are also significantly decreased. Otherwise, the animals appear developmentally and reproducitively normal. Cytogenetic fluorescence-activated cell sorting and histological analyses of somatic and germ-line tissues revealed no abnormality. These results indicate that Cenpb is not essential for mitosis or meiosis, although the observed weight reduction raises the possibility that Cenpb deficiency may subtly affect some aspects of centromere assembly and function, and result in reduced rate of cell cycle progression, efficiency of microtubule capture, and/or chromosome movement. A model for a functional redundancy of this protein is presented.

The protein components of the mammalian centromere can be broadly classified into two groups. Proteins from the first group are constitutively present on the centromere throughout the cell cycle, and include CENP-A, CENP-B, and CENP-C. The second group of proteins has been referred to as passenger proteins, since these proteins undergo complex relocations to other cellular organelles during the cell cycle, appearing on the centromere only during specific stages of the cycle (Brinkley et al., 1992; Earnshaw and Mackay, 1994). Examples of passenger proteins are INCENPs, MCAK, CENP-E, CENP-F, 3F3/2 antigens, and cytoplasmic dynein (reviewed by Earnshaw and Mackay, 1994; Pluta et al., 1995; Choo, 1997a). The proposed biological roles for these passenger proteins have included centromere formation and maturation, motor movement of chromosomes, sister chromatid cohesion and release, modulation of spindle dynamics, nuclear organization, intercellular bridge structure and function, and cytokinesis (reviewed by Choo, 1997a).

Amongst the constitutive centromere proteins, CENP-A has been localized to the outer kinetochores domain, and is a member of a growing class of proteins referred to as histone H3-like proteins whose members also include the S. cerevisiae homologue of CENP-A, CSE4p (Sullivan et al., 1994; Wilson et al., 1994; Stoler et al., 1995). Since CENP-A is found in association with histone H4 and the other core histones in particles that copurify with nucleosome core particles (Palmer and Margolis, 1985; Palmer et al., 1987), the protein is thought to act as a histone H3 homologue, replacing one or both copies of histone H3 in a certain set of centromeric nucleosomes, and is thought to serve to differentiate the centromere from the rest of the chromosome at the most fundamental level of chromatin structure: the nucleosome (Sullivan et al., 1994). CENP-C is located at the inner kinetochore plate, and has been shown to have an essential although yet undetermined centromere function as seen from its association with the active, but not the inactive centromeres of human dicentric chromosomes (Earnshaw et al., 1989; Page et al., 1995; Sullivan and Schwartz, 1995), arrest of mitotic progression after microinjection of anti-CENP-C antibodies into cul-
Human CENP-B is an 80-kD polypeptide that has been localized throughout the heterochromatin or central domain of the centromere (Earnshaw and Rothfield, 1985; Earnshaw et al., 1987; Cooke et al., 1990; Sullivan and Glass, 1991; Saïtoh et al., 1992). The protein is encoded by an intronless gene present in a single copy within the genome (Sugimoto et al., 1993; Seki et al., 1994). The number of CENP-B protein molecules has been estimated to be ~20,000 per diploid genome in HeLa cells (Cooke et al., 1990; Muro et al., 1992). On different human chromosomes, variable but generally detectable levels of the protein have been observed (Earnshaw et al., 1987). A notable exception is the Y chromosome, which has been shown to consistently lack this protein (Earnshaw et al., 1987). Through the recognition of a 17-bp PyTTCGTTGGAA-CENP-B box sequence known as the CENP-B box motif, CENP-B protein has been demonstrated to bind human centromeric α-satellite DNA directly (Masumoto et al., 1989; Muro et al., 1992; Pluta et al., 1992; Yoda et al., 1992).

Comparison of cloned human and mouse CENP-B gene sequences (Earnshaw et al., 1987; Sullivan and Glass, 1991) reveals a high degree of homology between the two species, with the coding regions showing an overall 96% sequence similarity and substantial stretches demonstrating 100% nucleotide identity between the two species (Sullivan and Glass, 1991). Of particular importance, both the NH2-terminal DNA-binding and COOH-terminal dimerization domains are totally conserved. Surprisingly, even the 5′ and 3′ untranslated sequences demonstrate an unusually high level (95% and 83%, respectively) of homology that is suggestive of possible posttranscriptional regulatory mechanisms (Mullner and Kühn, 1988; Caput et al., 1986). Like its human counterpart, the mouse gene is single-copy and intronless. Although the mouse genome does not contain recognizable α-satellite DNA, CENP-B binding occurs through the 17-bp consensus CENP-B box motif that is found in the mouse centromeric minor satellite DNA (Pietras et al., 1983; Rattner, 1991). In addition to mouse and humans, the CENP-B gene is conserved in hamster, African green monkey, great ape, tupaias, calf, Indian muntjac, and sheep (Sullivan and Glass, 1991; Haaf and Ward, 1995; Yoda et al., 1996; Bejarano and Valdivia, 1996; EMBL accession no. U35655). Significant homology is also found between CENP-B and two S. pombe centromere DNA-binding proteins Cbh+ and Abp1p, where cbh+ has been shown to be an essential gene (Lee et al., 1997), while abp1-deleted strains exhibit slower growth and a pronounced meiotic defect (Halverson et al., 1997). The CENP-B box motif has been found in the centromeric satellite DNA of species as diverse as primates, Mus musculus, Mus caroli, tree shrews, giant panda, gerbils, and ferrets (Pietras et al., 1983; Masumoto et al., 1989; Rattner, 1991; Muro et al., 1992; Pluta et al., 1992; Yoda et al., 1992; Haaf and Ward, 1995; Kipling et al., 1995; Kipling et al., 1994; Wu et al., 1990; Volobouev et al., 1995; Choo et al., 1991; Laursen et al., 1992; Haaf et al., 1995). Based on this observed conservation of CENP-B and its DNA-binding motif, it may be speculated that CENP-B is a functionally important component of the mammalian centromere.

Through its CENP-B box-binding and dimerization properties, the protein has the hallmark of a cross-linking protein that is involved in assembly of the large arrays of centromeric α-satellite or minor satellite DNA (Yoda et al., 1992; Muro et al., 1992). However, the absence of this protein on the Y chromosome in humans and mouse (Earnshaw et al., 1987), and on the centromeres of African green monkeys, which are known to be composed largely of α-satellite DNA containing little or no binding sites for CENP-B (Goldberg et al., 1996), suggests that this role may not be universal. In other studies, the protein has been shown to be present on both the active and inactive centromeres of mitotically stable pseudocentric human chromosomes (Earnshaw et al., 1989; Page et al., 1995; Sullivan and Schwartz, 1995), suggesting that CENP-B binding does not immediately translate into centromere activity. Furthermore, an increasing number of stable human neocentromeric marker chromosomes (Voulaire et al., 1993; Ohashi et al., 1994; Choo, 1997b; Depinet et al., 1997; du Sart et al., 1997) have now been described that are capable of normal mitotic division in the absence of CENP-B binding, indicating that CENP-B is nonessential for mitotic chromosome segregation, at least for these marker chromosomes. Earlier attempts at defining the role of CENP-B in mammals have yielded conflicting results. Microinjection of polyclonal anti-CENP-B antibodies into human and mouse cells resulted in disruption of centromere assembly during interphase, and led to inhibition of kinetochore morphogenesis and function in mitosis (Bernat et al., 1990; Simerly et al., 1990; Bernat et al., 1991). However, a different study has indicated that expression of truncated versions of CENP-B in HeLa cells does not lead to a mitotic or cell cycle arrest phenotype (Pluta et al., 1992). To date, the role of CENP-B in meiosis has not been investigated.

To better understand the role of CENP-B in centromere function, we used gene targeting in mouse embryonic stem cells to derive animals with a null mutation in the CENP-B gene. A major advantage of such mouse mutants is that it enables us to study CENP-B functions not only in mitosis, but also in meiosis. We report that CENP-B–deficient mice appear to be mitotically and meiotically normal, but develop lower body and testis weights. We discuss the implications of these results and propose a model in favor of a redundancy of CENP-B in centromere function.

Materials and Methods

Construction of Targeting Vectors

A hybridization probe spanning the coding region of Cenpb was prepared from genomic DNA of mouse embryonic stem (ES) cell line E14 by PCR using primers Bprot-1 (5′-GGCGAGATCTTGGCGGCCCCAGGCCGC-3′) and Bprot-3 (5′-TCAAGATTCATGTTGCAAAGGAGACC-3′). Screening of mouse genomic phage libraries with this probe led to the isolation of a Cenpb fragment of the correct size (2350 ± 50 bp) from a 12-bph MalF::Phage library (kindly provided by M. A. Piñol-Roma, University of California, San Francisco). The fragment was subcloned into Smal/EcoRV-digested pUC13, yieldi...
probe resulted in the identification of a positive clone (designated E1) from a 129/OLA library (gift of M. Kennedy) of E14 cells, and a second clone (designated D1) from a 129/SV library (Stratagene) of B1 mouse ES cells. An E1-derived fragment spanning nucleotides 920–2676 of mouse Cenpb gene (Sullivan and Glass, 1991, EMBL accession no. X55038) was ligated with a D1-derived fragment spanning 2676–5800 and cloned into a modified pSP72 vector (Promega Corp., Madison, WI). An oligonucleotide linker sequence designated D/TAA (5′-GATCCATCAAGATCTAC-3′) (Fig. 1) was inserted at position 1207, which is 72 amino acids downstream of the ATG start site of the 1.8 kb-coding sequence of Cenpb (Fig. 1b). This linker introduced a Dral site, a frame-shift mutation, and three stop codons in all three reading frames, of which TAA was in frame with Cenpb translation, disrupting not only the critical NH2-termini of amino acid centromere DNA-binding domain (Yoda et al., 1992; Kitagawa et al., 1995), but also removing all remaining CENP-H-terminal regions including the dimerization domain (Yoda et al., 1992; Kitagawa et al., 1995). The IRES-neomycin (Mountford et al., 1994) and IRES-hygro- mycin (A. Smith, personal communication) markers were separately cloned into an AvrII site at position 3202 in the 3′ untranslated region of Cenpb-null ES cell line designated R1–189N/H, in which both the Cenpb alleles were disrupted. For disruption of the mouse Cenpb gene in ES cells, a promoterless targeting vector was constructed that incorpo- rated a translation frame-shift linker, designated D/TAA, containing stop codons in all three reading frames, and the IRES-neomycin or IRES-hygro-mycin marker (Fig. 1b and Methods). The D/TAA linker inserted 72 amino acids

Southern Blot and PCR Analyses of Targeting Events
Correct gene targeting in ES cells and mouse tail genomic DNA was determined by Southern analysis using a 5′ genomic probe generated from the E1 mouse phage clone with NheI (position 564) and SacII (position 920) situated outside the targeting construct sequence (see Fig. 1a). For PCR genotyping, the following primers flanking the D/TAA linker were used: Fd-1 (5′-ACCATCCGAGAAGAACAAGG-3′) and Rev-2 (TGGAACCGATCGAGAGAAG), which gave 128-bp and 154-bp products for wild-type and targeted alleles, respectively; and Fd-1 and Rev-3 (5′-TGGAAACGAGATCGAGAGAAG-3′), which gave a 173-bp and 199-bp product for wild-type and targeted alleles, respectively (see Fig. 1, d and g). PCR conditions were as follows: 95°C for 30 s, 55°C for 1 min, and 72°C for 1.5 min for a total of 35 cycles using a 50-μl vol containing 50–200 ng genomic tail DNA, 1 U Taq polymerase, 200 μM dNTPs, and 300 ng of each primer in 1 X Taq PCR buffer (Perkin-Elmer Corp., Norwalk, CT).

Generation of Targeted ES Cells and Mouse Chimeras
For transfection, 50 μg of the IRES(neo) construct was linearized at the 3′ end with AatII or SspI, or at the 5′ end with AatII or XbaI, and electroporated into approximately 105 ES cells in 800 μl vol using a single pulse from a Bio-Rad Gene Pulser at 800 V, 3 μF, 650 μF. The ES cell lines used in this study were R1 (Nagy et al., 1993), W9.5, and W9.8 (Buzin et al., 1994). Trans- ferred cells were plated onto mitomycin C-treated, neomycin-resistant, STO fibroblasts (Robertson, 1987) plus 10 μg/ml LIF (Amrad-Pharmacia) and selected in G418 (Gibco-BRL) active at 300 μg/ml to allow integration of the targeting construct sequence (see Fig. 1, b and c) plus 10 μg/ml colcemid for 2 h. Immunofluorescence staining was performed as previously described (Jeppeson et al., 1992; du Sart et al., 1997). After the antibody binding, the cells were postfixed in 1% formalin, washed, and counterstained with DAPI and DABCO mounting. Images were analyzed using an Axioskop fluorescence microscope equipped with a 100× objective (Carl Zeiss, Inc., Thornwood, NY) and a cooled CCD camera (Photometrics Image Point) linked to a Power-Mac computer.

Histology, Advanced Sperm Count (ASC), and Stereology
The organs analyzed for histology were dissected from 10-wk-old and 6-mo-old mice using standard techniques. Testicular determination of homogenization-resistant ASC was performed as previously described (Robby et al., 1978) on 10-wk-old animals. Stereological analysis was performed on testicular materials from 10-wk-old mice using the optical dissector (sic) technique (Wroeford et al., 1995) to investigate the efficiency of meiosis by determining the ratio of pachytene spermatocytes to round spermatids associated with stages I–VIII of spermatogenesis. This ratio has an expected value of 4.1 if the efficiency of division through meiosis I and II is 100% and there is no loss of round spermatids after meiosis.

Cytogenetics and Flow Sorting
For karyotyping, –/– R1-189 N/H cell line as well as spleen and bone marrow cell cultures (with and without phytohaemagglutinin) were isolated from –/– mice and compared with the +/+ cell line and +/+ animal. Cells were treated with colcemid and GFT-stained using standard cytogenetic techniques. For flow sorting, 50,000 cells from spleen, bone marrow, or testis were isolated from 30-wk-old mice. These were analyzed by two-parameter analysis of DNA content vs. cell diameter using a FAC-Scan™ (Becton Dickinson & Co., Sparks, MD) cell sorter equipped with an argon laser at 488 nm. Signals were collected by a FL2 detector with a 585-nm band pass filter. Tests cells were profiled into five main regions corresponding to the different steps in maturation from elongated and round haploid spermatids to diploid, S-phase, and tetraploid cells.

Results

Generation of Cenpb-null ES Cells and Mice
For disruption of the mouse Cenpb gene in ES cells, a promotorless targeting vector was constructed that incorpo- rated a translation frame-shift linker, designated D/TAA, containing stop codons in all three reading frames, and the IRES-neomycin or IRES-hygro-mycin marker (Fig. 1b and Methods). The D/TAA linker inserted 72 amino acids

Reverse Transcription (RT)-PCR Analysis and Sequencing
Total genomic DNA was extracted from wild-type R1 cells (+/+) and the IRES(neo)-targeted cell line R1–26 (+/–), and the double-targeted cell line R1–189N/H (+/–). 0.5–1.0 μg total RNA was reverse-transcribed us-
downstream of the translation start site not only disrupted the critical NH2-terminal 125-amino acid centromere DNA-binding domain (Kitagawa et al., 1995; Yoda et al., 1992), but also removed all remaining COOH-terminal regions including the dimerization domain (Kitagawa et al., 1995; Yoda et al., 1992). The IRES-neo and IRES-hygro selectable markers were placed in the 3' untranslated region before the polyadenylation signal. For correct targeting and gene disruption, two homologous recombination events external to the linker and IRES(neo/hygro) regions were required (Fig. 1 b, solid-cross regions). When the IRES(neo) construct was linearized at the 3' end with AatII or SspI and transfected into R1 and W9.5 cells, 2% (or 2 out of 103 neomycin resistant colonies) of R1 cells, 1.3% (1 out of 72 colonies) of W9.8, and 3.5% (12 out of 344 colonies) of W9.5 cells gave the desired targeted gene disruption (Fig. 1 e, lane 5). Interestingly, a significantly higher frequency (90% for R1, 67% for W9.8, and 92% for W9.5) of an undesired targeting event was detected (Fig. 1 e, lanes 2, 4, 6, and 7) where incorporation of the IRES-neo element at the Cenpb locus had not been accompanied by the D/TAA linker. This result was due to recombinations occurring within the region between the IRES-neo cassette and the D/TAA linker (Fig. 1 b, broken-cross region) instead of in the region 5' of the linker. The observation of a higher recombination frequency in this region was perhaps not surprising in view of the fact that 1995 bp of homologous DNA was present in this region compared with only 287 bp of homologous DNA between the D/TAA linker and the 5' end of the construct. In subsequent experiments, it was further demonstrated that use of construct DNA linearized at the 5' end using KspI to expose the Cenpb DNA end, as distinct from the plasmid vector DNA end using AatII or SspI, gave a 2.5-fold increase in the frequency of the desired targeting in R1 cells and a 3.8-fold increase in W9.5 cells (data not shown).

From the above screening, 21 heterozygous ES cell colonies with a disrupted Cenpb allele were obtained from the R1, W9.5, and W9.8 cell lines. Two of these colonies, R1-26 from the R1 line and W-190 from the W9.8 cell line, were retransfected with the IRES(hygro) construct to obtain a Cenpb null cell line. Selection of the transfected cells in neomycin and hygromycin gave rise to one double-targeted colony (out of five resistant colonies screened) designated R1-189N/H from R1-26, and two double-targeted colonies (out of 76 colonies screened) from W-190. All three colonies showed normal cell morphology and apparently normal growth rates. No desired double-targeted event (0 out of 81 colonies) were seen for both W9.8 and R1 when the transfected cells were selected in hygromycin alone, due presumably to a direct replacement of the IRES(neo)-targeted allele with the IRES(hygro) cassette. Furthermore, as with the IRES(neo) construct, a much higher frequency (three out of five colonies for R1-26, and 57 out of 76 colonies for W-190) of the undesired targeting event involving the loss of the D/TAA linker was observed.

The heterozygous R1-26 cell line was injected into C57 bl/a/6 blastocysts to produce germline chimeras, from which heterozygous (+/− neo) and homozygous (−/− neo) mice carrying the IRES(neo)-targeted allele were produced (Fig. 1 f). The double-targeted R1-189N/H cell line was similarly injected into C57 bl/a/6 blastocysts and, through selective breeding, heterozygous (+/− hygro) and homozygous (−/− hygro) mice carrying the IRES(hygro) allele were generated (data not shown). These mice, together with the various cell lines created above, were subjected to further detailed studies.

**Abolition of Cenpb Gene Expression in the Targeted Cell Lines**

Cenpb gene disruption was determined by RNA analysis using PCR performed with primers designed across the D/TAA linker region. The results (Fig. 1 g) indicated the presence of normal Cenpb transcripts in the wild-type (Fig. 1 g; lanes 1 and 4) and heterozygous cell lines (lanes 2 and 5), but not in the double-targeted R1-189N/H cell line (lanes 3 and 6). This result suggested that transcription of both copies of the wild-type Cenpb alleles in the −/− cell line had been abolished and replaced by that of the targeted alleles. In addition, we wished to determine whether the D/TAA linker had incorporated correctly into the NH2-terminal centromere DNA-binding domain of the targeted Cenpb gene, and that no unforeseen sequence re-arrangement undetected by the Southern or RT-PCR analyses had occurred. This was done by purifying and cloning the 199-bp fragment corresponding to the targeted allele (Fig. 1 g, lane 6) and direct sequencing analysis. The results (not shown) confirmed the correct insertion of the D/TAA linker and therefore the stop codons.

**Absence of Cenpb Binding on Centromeres by Direct Immunofluorescence Staining**

Immunofluorescence staining of metaphase chromosomes was used to detect specific centromere-binding proteins. Fig. 2 shows results obtained with the +/+ R1 and −/− R1-189N/H cell lines; the results for the +/− R1-26 cell line were similar to those of the +/+ R1 cell line, and are not shown. The anti-Cenpb monoclonal antibody clearly demonstrated the presence of Cenpb on the centromeres of the +/+ cell line, but not on those of the −/− cell line. The intensity of the Cenpb signals in the +/+ cells varied considerably on different chromosomes, reflecting the intrinsic quantitative variation in the amount of Cenpb boxes and Cenpb binding on different centromeres (Earnshaw et al., 1987). When these cell lines were tested with a CREST antibody and antibodies for Cenpc and CENP-E, uniform staining of the centromere was observed (Fig. 2). Similar results (not shown) were obtained with cells established from the +/+, +/−, and −/− IRES(neo) and IRES(hygro) mice. These data therefore provided direct evidence that the Cenpb gene has been disrupted in the −/− neo and −/− hygro knockout mice. They also demonstrated that Cenpb is not essential for centromeric binding of Cenpc and Cenpe and for CREST antibody binding on active centromeres.

**Cenpb Null Mice Have Lower Body Weight and Testis Size, but Are Otherwise Developmentally Normal**

Cenpb null mice appeared phenotypically normal, and routinely gave normal litter size and the expected Mendelian ratios of offspring, suggesting that Cenpb deficiency
did not drastically affect cell division, development, and reproduction of the mice. This phenotype is in stark contrast to that of another mouse model we have recently created with a disruption of Cenpc, where null mutants display severe mitotic disarray and die during early embryogenesis (Kalitsis et al., 1998). To determine if Cenpb gene disruption has a more subtle effect on growth, we measured the body weight of the IRES(neo) animals over an 8-mo period (Fig. 3). The −/− mice as a group appeared uniform in size at birth, and presented with normal weights at weaning (3 wk), but subsequent weight gain in this group lagged behind those of sex-matched +/+ and +/- animals.
with the difference reaching a level of significance ($P < 0.05$) after 22 wk in males and 12 wk in females (see Table I for representative weight data for 26-wk-old animals). When the weight data were collected from the IRES(hygro) animals, similar trends as those obtained for the IRES(neo) mice were seen for the different genotypes in the two sexes (data not shown).

**Histological Analysis Reveals No Gross Abnormality**

To investigate the reasons for the observed weight difference, various organs from the IRES(neo)-targeted animals were subjected to histological examination. The organs analyzed were stomach, duodenum, descending colon, liver, hairy skin, ear flap, salivary gland, spleen, pancreas, kidney, thymus, brain, lung, adrenal, seminal vesicle, ovary, uterus, and pituitary. When organs from 10-wk- and 6-mo-old male and female $+/-$ mice were directly compared with those derived from age- and sex-matched $+/+$ and $+/-$ animals, the results indicated no obvious abnormality in any of these organs. During this analysis, the testes of $+/+$ mice were found to be markedly smaller (29%; $P < 0.01$) than those of the wild-type mice (Table I). Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels were measured and found to be normal. When the testes of 10-wk-old animals were assessed for sperm content (ASC), a 39.5% reduction ($P = 0.0007$) was seen in the $+/-$ cell line compared with the $+/+$ cell line ($N = 12$). When the efficiency of meiosis was determined using the more comprehensive stereological analysis on sectioned testicular materials from $+/+$ ($N = 5$), $+/-$ ($N = 6$), and $+/-$ ($N = 4$) mice, values of $3.9 \pm 0.2$ (mean ± SEM), $3.8 \pm 0.1$,
and 3.6 ± 0.2, respectively, were obtained, which were not significantly different from the expected 4:1 ratio. Thus, although it appears that reduction in germ cell content is correlated with testicular weight reduction, results of the stereological analysis have revealed no substantial difference in the efficiency of either mitotic or meiotic division.

The question of whether CENP-B is essential for chromosome segregation has been intensely debated in recent years. Conservation of the protein in different mammalian species and in lower eukaryotes and its demonstrated centromere DNA-binding property attest to a significant functional role. However, various cytological observations have hinted at CENP-B not being critical for mitosis, although such evidence are often indirect and open to interpretation. For example, detection of CENP-B on both the active and inactive centromeres of mitotically stable pseudodiplocentric human chromosomes (Earnshaw et al., 1989; Page et al., 1995; Sullivan and Schwartz, 1995), rather than indicating a lack of functional importance for CENP-B, can be interpreted to mean that additional centromere proteins are necessary to make the inactive centromere fully active. Similarly, the finding that the protein is absent on the Y chromosome in both humans and mouse (Earnshaw et al., 1987) is intriguing but needs to be interpreted in light of the unresolved peculiarity that this observed absence is associated with the only centromere in these genomes that does not undergo sister centromere pairing in meiosis. The absence of CENP-B on various analphoid neocentromeres (Choo, 1997b) also does not per se exclude functions for CENP-B (or α-satellite DNA) on normal centromeres.
since these neocentromeres may have gained centromere function through some epigenetic modifications (Karpen and Allshire, 1997). Finally, the observation that the α-satellite DNA-containing centromeres of African green monkey lacks binding sites for CENP-B (Goldberg et al., 1996) could be because monkeys have evolved a different and functionally equally important way to compensate for their CENP-B deficiency.

In addition to the uncertainty on mitotic functions, the requirement of CENP-B in meiosis has also not previously been investigated. Such an investigation is especially important in light of recent evidence indicating a specific role of centromeric heterochromatin in meiotic chromosome segregation in Drosophila (Dernburg et al., 1996; Karpen et al., 1996), and in view of the fact that CENP-B binds directly to centromeric heterochromatic DNA and is thought to be involved in the higher order organization of this DNA (Yoda et al., 1992; Muro et al., 1992). The production and characterization of Cenpb null mice allows several conclusions regarding the functional significance of this protein in mitosis and meiosis to be drawn. The apparently normal growth and reproductive characteristics of these mice indicate that Cenpb is not essential for either of these cell division processes. This result is confirmed by direct cytogenetic and FACS analyses of chromosomes, which have not detected any karyotypic abnormality in the −/− mice. A closer look at the different stages of male meiosis in these animals has similarly not revealed any obvious defect. The protein also appears not to be required for the structural integrity of the centromere–kinetochore complex since the centromeres of Cenpb-deficient cells continue to show clear association with at least two of the functionally important centromere proteins: Cenpc and Cenpe. The observation that Cenpb is not essential for mitosis or meiosis therefore drastically contrasts the severe phenotypes previously reported for the cbh+ and abp1 null yeast strains (Halverson et al., 1997; Lee et al., 1997), suggesting that the functions of these homologues have diverged significantly.

Despite the lack of any detectable mitotic and meiotic phenotype, adult Cenpb null mice are significantly smaller in body weight compared with age- and sex-matched wild-type or heterozygous mice. In addition, the −/− male testes show a pronounced reduction both in weight (by 30%) and in total sperm count (by 39.5%) compared with wild-type animals. Extensive histological analysis of many different organs and direct measurement of FSH and LH hormones have not revealed any abnormality. It is possible that the absence of CENP-B may have a subtle effect on centromere assembly and function, and result in a slight reduction in the rate of progression through one or more phases of the cell cycle, in the efficiency of chromosome capture by microtubules, and/or chromosome movement. Alternatively, a small number of cells beyond our detection ability may not enter mitosis at all, or carry severe chromosomal abnormality, resulting in loss of valuable cells from the cycling cell population sufficiently to cause a significant weight reduction over time. The possibility that the weight phenotype is caused by some as yet unidentified hormonal or metabolic factors cannot be discounted at present.

In formulating any model on the role of CENP-B, the following observations need to be taken into consideration: (a) the protein is highly conserved in divergent mammals; (b) the protein binds centromeric repetitive DNA via the CENP-B-box motif and possesses dimerization properties that allow the protein to cross-link centromeric repeats (Yoda et al., 1992; Muro et al., 1992); (c) CENP-B box and CENP-B protein are not detected on human and mouse Y chromosomes, and are poorly represented on centromeric subdomains of certain human chromosomes (e.g. α13-II, α14-II, and α21-II domains of chromosomes 13, 14, and 21; Trowell et al., 1993; Ikino et al., 1994; Choo, 1997a); (d) the centromeres of African green monkey are composed largely of α-satellite DNA containing few if any binding sites for CENP-B (Goldberg et al., 1996); (e) the protein is found on both active and inactive centromeres of dicentric chromosomes (Earnshaw et al., 1989); (f) despite the lack of CENP-B binding, human neocentromeres derived from noncentromeric chromosomal regions display full mitotic functions (Voullaire et al., 1993; Depinet et al., 1997; du Sart et al., 1997; Choo, 1997b); and (g) the protein is neither essential for mitosis nor meiosis in Cenpb knockout mice.

Based on the sequence similarity between CENP-B and certain transposases, Kipling and Warburton (1997) suggested that CENP-B may share the DNA strand cleavage function of transposases and promote nicks adjacent to CENP-B boxes to facilitate the evolution and maintenance of satellite DNA. This model does not, however, take into consideration the dimerization property of CENP-B, offers no direct evidence for the proposed strand cleavage function, and cannot explain the absence of CENP-B on human and mouse Y chromosomes or the paucity of this protein on the α-satellite–containing centromeres of African green monkey and centromeric subdomains of at least some human chromosomes. Here we present a different model that satisfies all the reported observations. Simply stated, we propose that the role of CENP-B is to organize structurally the great abundance of repetitive DNA found in the centromere, with this role neither being exclusive to CENP-B nor directly essential or sufficient for centromere function. Our model further implicates the existence of a functionally related but perhaps lower-affinity protein, arbitrarily designated CENP-Z, that can perform a similar function to CENP-B in its absence (Fig. 4). The proposal of a role for CENP-B in organizing centromeric repeats is based on the biochemical (observation b above), cytogenetic (observation e above), and, indirectly, evolutionary (observation a above) properties of the protein. The suggestion that this role is not exclusive to CENP-B is based on the fact that centromeric repetitive DNAs with little or no CENP-B binding (observations c, d and g above) are nonetheless organized in a way that is compatible with centromere function. The suggestion that CENP-B is neither essential nor sufficient for centromere function is evident from the fact that the protein can be totally absent on centromeres without detrimental effects on chromosome segregation (observations c, d, f, and g above), and that its mere presence on some centromeres does not immediately lead to centromere activity (observation e above).

A nuclear protein, plα (Gaff et al., 1994), has previously been described that binds a 9-bp sequence motif, GTG(G/A)AAAAG, that is present as an alternative nucleotide
tosomes and X chromosomes. (c) In the absence of CENP-B-binding, a functionally related protein CENP-Z (blue circle) assumes the role of CENP-B to cross-link CENP-Z box–containing monomers. This mode of organization is suggested for the human and mouse Y chromosomes, various CENP-B box-poor centromeric subdomains, and the centromeres of African green monkey and Cenpb null mice.

configuration to the CENP-B-box motif on a significant proportion (~14%) of α-satellite monomers, including those that constitute the centromere of the human Y chromosome and the various CENP-B box-poor human centromeric subdomains (Tyler-Smith and Brown, 1987; Alexandrov et al., 1993; Vissel and Choo, 1992; Romanova et al., 1996). A recent study has further demonstrated that pJα box-containing α-satellite monomers are the primordial DNA from which the CENP-B box-containing monomers arose (Romanova et al., 1996). In preliminary studies, we have detected pJα proteins in the nuclear extracts of the +/+ , +/-, and −/− Cenpb knockout mice, and in that of the African green monkey (D.F. Hudson and K.H.A. Choo, unpublished data). In addition, the consensus sequence for the CENP-B box-poor α-satellite DNA of African green monkey has been shown to contain a perfectly conserved pJα-box motif GTGAAAAAG (Yoda et al., 1996). These analyses therefore suggest that pJα may be a suitable candidate for the proposed CENP-Z protein. The availability of the Cenpb null mice should provide an amenable system to allow the further study of the role of Cenpb, as well as investigation of the proposed CENP-Z protein.

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