

## An Antigenic Determinant on Human Centromere Protein B (CENP-B) Available for Production of Human-Specific Anticentromere Antibodies in Mouse

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**ABSTRACT.** Centromere protein B (CENP-B) is one of the centromere DNA binding proteins constituting centromere heterochromatin throughout the cell cycles. Some components of mammalian centromeres including CENP-B are target antigens for autoimmune disease patients, often those with scleroderma. Recent isolations of CENP-B genes from human and mouse suggested that CENP-B was highly conserved among mammals. From the previous analysis of the reactivity of patient anticentromere sera, two autoepitopes have been located on the DNA binding domain at the amino-terminal region. The amino acid sequences for both the epitopes are perfectly conserved in the two species, human and mouse. In this study, to identify a human-specific antigenic determinant, the remaining two epitopes were further located in separate carboxyl-terminal regions of human CENP-B. Although the amino acid sequence of one epitope is identical to that of the corresponding region in mouse CENP-B, the other has a less homologous sequence. To confirm that the latter epitope was available for production of human-specific anticentromere antibodies, mice were immunized with the recombinant human CENP-B product. One serum that exclusively stained human centromere structure, but not that of other mammals, was identified in the immunofluorescence microscopic observation. The epitope analysis showed that the less conserved one was recognized by this serum. These results suggested that the corresponding region defines the antigenic determinants for the species specificity.

In connective tissue diseases, autoantibodies have been shown to be highly specific in the reaction with intracellular components of cells (see ref. 26 for a review). Some components of human centromere structure are also recognized as the target antigens in autoimmune disease patients who often suffer from scleroderma, as originally reported by Moroi *et al.* (16). So far, three centromere antigens have been consistently identified as human centromere proteins (CENPs); 17 kDa (CENP-A), 80 kDa (CENP-B) and 140 kDa (CENP-C) by the groups of Earnshaw and Rothfield (7), del Mazo *et al.* (10) and Muro *et al.* (18). Crossreactivity of affinity-eluted anticentromere antibodies suggested that these three antigens shared some antigenic determinants, although the precise epitope of each antigen remains unknown (7). In addition, many other mammalian centromeres such as hamster, mouse (16, 17), rat, muntjac, rat kangaroo (4), porcine, rabbit (13), and bovine (20) are also immunoreactive to the patient sera as well, suggesting the existence of another common antigenic determi-

nant among mammals. To clarify each centromere antigenic determinant, however, it is essential to obtain monospecific anticentromere antibodies that react only with a defined antigen, since these patient sera often contain polyclonal anticentromere antibodies which recognize two or more centromere antigen molecules (7, 18). Besides, the epitope analysis might help us to assign some domain structures to human CENP-B, since it has been demonstrated that autoepitopes are often localized on active sites or functioning regions (26).

We have recently isolated a human CENP-B cDNA and succeeded in expressing its gene product functionally in *Escherichia coli* (24). The biochemical analyses of human CENP-B demonstrated that it has specific DNA binding activity to a 17 bp motif on some families of human centromeric alphoid DNA repeats (14, 15, 24, Muro, Y. *et al.*, *J. Cell Biol.* 116: 585). The DNA binding domain has been precisely located within the N-terminal 134 amino acid residues, in which a helix-loop-helix structure could be predicted as in the case of some other DNA binding proteins (24, 25). Recently, a mouse homologue for CENP-B gene has been isolated by Sullivan and Glass (25), and the predicted amino acid se-

Abbreviations: CENPs, centromere proteins; CENP-B, centromere protein B; IPTG, isopropyl- $\beta$ -D(-)-thiogalactopyranoside.

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quence corresponding to the DNA binding domain has been shown to be perfectly conserved. Therefore, CENP-B was considered to be a centromere DNA binding protein common in mammals, sharing the common antigenic determinants for anticentromere patient sera.

In this study, to understand the organization of the antigenic determinants of CENP-B and to find a human-specific antigenic determinant, four autoepitopes are characterized on the recombinant human CENP-B polypeptides using a variety of anticentromere sera from autoimmune disease patients (18, 19). In addition to two epitopes located on the amino-terminal half of the DNA binding domain (24), the other two are located on the separate carboxyl-terminal regions with unknown function. We show that only one epitope has a less homologous sequence and is available for production of an antiserum specific to human centromere structure, not that of other mammals.

## MATERIALS AND METHODS

**Sera.** Patient anticentromere sera were previously described (18, 19). To obtain a mouse anti-CENP-B serum, a recombinant human CENP-B was expressed in *Escherichia coli* (*E. coli*) strain Y1-11 which had been constructed by lysogenizing the original recombinant lambda clone ( $\lambda$ 1-11), in which was inserted the 2.8 kb cDNA encoding the whole CENP-B region except the initiation codon (23, 24). Y1-11 cells were grown in LB medium and the expression of the fused CENP-B products was induced in the presence of isopropyl- $\beta$ -D(-)-thiogalactopyranoside (IPTG), as described elsewhere (11). Induced cells were solubilized in an SDS sample buffer and subjected to electrophoresis on 7.5% or 10% SDS-polyacrylamide gels. The detailed procedure for production of a mouse anti-CENP-B serum and a rabbit serum against T7 phage gene 10 product using the protein bands separated in the gel has been described elsewhere (1).

**Mammalian cell culture and indirect immunofluorescence assay.** African green monkey kidney cells (vero), bovine kidney cells (MDBK) and porcine kidney cells (PK15) were grown in Eagle's MEM supplemented with 10% fetal calf serum (FCS). Mouse thymus stroma cells (TS-1-47) were a generous gift from Dr. Y. Takamori (Department of Veterinary Radiology, University of Osaka Prefecture) and grown in DMEM supplemented with 10% FCS. Culture cells were grown on coverslips in 35 mm dishes and fixed in PLP (periodate-lysine-paraformaldehyde) solution, as described (14, 16). Human epitheloid cells (HEP-2) stabilized on slides were commercially available (Immuno Concepts, CA). Anticentromere sera were diluted to 100-fold and used for staining mammalian centromere structures. FITC-conjugated anti-human IgG antibodies and anti-mouse Ig antibodies were from Jackson Immuno-research Laboratories, Inc. (USA) and Amersham International plc (U. K.), respectively. Slides were analyzed using a Bio-Rad MRC-600 laser scanning confocal microscope.

**Plasmid DNA construction.** The parental plasmid pCENPB1 encoding the C-terminal region of human CENP-B was constructed by inserting the 1.2 kb DNA fragment of another human CENP-B cDNA clone ( $\lambda$ 1-1) isolated from a human cDNA library (23), as described (24, 27), into the *EcoRI*-*NotI* site of pGEMEX vectors (Promega). A series of plasmids, pCENPB4-1, pCENPB3-15, pCENPB4-4, pCENPB3-21, pCENPB4-8, pCENPB4-7, pCENPB4-5, pCENPB1-24, pCENPB1-42, pCENPB1-12, pCENPB2-16, pCENPB1-1, and pCENPB2-6, has been constructed by deleting the original plasmid pCENPB1-11 stepwisely from the C-terminal *NotI* site (see Fig. 3a in ref. 24). The coding region of each construct described below was schematically represented in the text figure (see Fig. 1). The pCENPB1/N was the same as pCENPB1 except the C-terminal deletion downstream of the *NcoI* site at the position 548. Plasmid pCENPB1/1-12 was a chimera construct between pCENPB1 and pCENPB1-12 at the *SacI* site at the position 468, possessing the limited coding region between 438 and 492. Plasmid pCENPB1/A had the C-terminal deletion downstream of the *ApaI* site (position 475), resulting in its possessing the coding region between 438 and 475. Plasmid pCENPBS/1-12 had the limited coding region between 468 and 492. Plasmid pCENPB/NL had the N-terminal deletion upstream of the *NcoI* site (position 548), retaining the coding region between 548 and 599. Three plasmids, pCENPBO/2-6, pCENPBO/1-1 and pCENPO/2-16 were constructed by ligating the oligonucleotides encoding the amino acid residues between 535 and 553, namely,

5'-AATTCAGATGGTGACGAGGTCCCTGTCCCGAG-  
3'-GTCTACCACTGCTCCAGGGACAGGGCTC-  
CTTTGGGGAGGCTATGGCTTACTTTGC-3'  
GAAACCCCTCCGATACCGAATGAAACGGTAC-5'

with the longer *EcoRI*-*NcoI* fragments of pCENPB2-6, pCENPB1-1 and pCENPB2-16, respectively. The gene manipulation was followed by the standard procedures (21).

**Immunoblotting.** Truncated recombinant CENP-B products were expressed in *E. coli* BL21 (DE3) strain harboring each plasmid construct, as described (22). Immunoblotting procedures were previously described (18), except that the transfer of proteins to PVDF membrane (Millipore) was done using the Trans-blot SD (Bio-Rad) according to their instructions. The membrane was incubated with anticentromere sera, which were then visualized with horseradish peroxidase-conjugated second antibodies (Bio-Rad) as described (12), or using ECL western blotting detection reagents (Amersham International plc).

## RESULTS

**Analysis of autoepitopes on human CENP-B.** Human centromere protein B (CENP-B) is a centromere DNA binding protein that has specific DNA binding activity to a 17 bp motif on certain families of human cen-

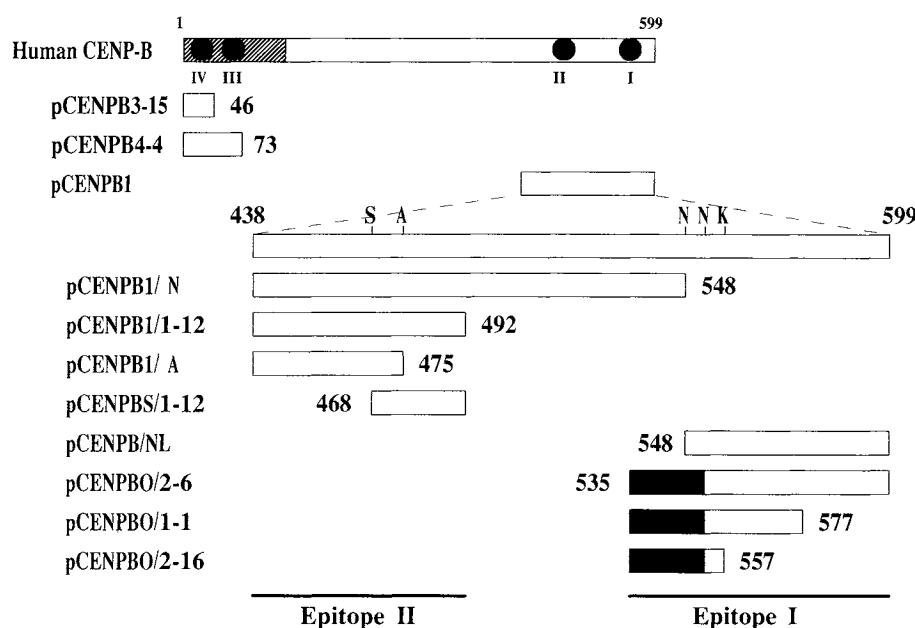
tromeric alphoid DNA repeats (14, 15, 24, Muro, Y. *et al.*, in press). The DNA binding domain has been finely located on the N-terminal 134 amino acid residues which possessed the predicted helix-loop-helix structure on the N-terminal half and a leucine zipper-like motif on the C-terminal half (24). Using a number of anticentromere autoimmune sera (18, 19), at least four autoepitopes from I to IV have been predicted on the human CENP-B by the previous preliminary epitope analysis (see Fig. 1 top). However, the precise location of epitopes I and II remained unknown, since we used truncated CENPB products which had been deleted exclusively from the C-terminus.

To characterize the latter two epitopes further, we here prepared another set of CENP-B constructs as shown in Fig. 1. We used a plasmid pCENPB1 as the parental plasmid, since both epitopes were anticipated to exist within this region. To dissect the two epitopes independently, we first divided this region into two portions at the *Nco*I site at position 548. The N-terminal part between positions 438 and 548 was retained in the plasmid pCENPB1/N and the remaining C-terminal part was retained in the plasmid pCENPB/NL.

truncated CENP-B products were expressed in *Escherichia coli* as a fusion protein to T7 phage gene 10 product under control of IPTG-inducible T7 RNA polymerase (22).

To examine whether the antigenicity was still retained within the separate regions, we tested the reactivity of a variety of anticentromere patient sera by immunoblotting procedure (18). As expected, the CENP-B product encoded in the parental plasmid pCENPB1 was immunoreactive to all the patients (Fig. 2, lane 1 in all panels). After the dissection of this region, the N-terminal portion encoded in the pCENPB1/N corresponding to epitope II, was indeed recognized by some patient sera (Fig. 2, lane 2 in panels *b*, *d*, and *f*). The C-terminal region encoded in the pCENPB/NL, however, was no longer immunoreactive (Fig. 2, lane 6 in all panels). This result indicated that the C-terminal antigenic determinant, probably for epitope I, was destroyed by the dissection at the *Nco*I site and that epitope II was located within the region between position 438 and 548.

The C-terminal boundary of epitope II was mapped using two additional plasmid constructs, pCENPB1/1-12 and pCENPB1/A. As shown in lane 4 in panels *b*, *d*,



**Fig. 1.** Plasmid constructions used for the dissection of the antigenic determinants on human CENP-B. Four autoepitopes are shown as closed circles with Roman numerals (from I to IV) on the whole human CENP-B polypeptide containing 599 amino acid residues, based on the reactivity of anticentromere sera from autoimmune disease patients (24). The shaded area indicates the minimum DNA binding domain, narrowed down to the N-terminal 134 amino acid residues (24). The CENP-B coding regions in each construct are presented as open boxes with the C-terminal amino acid residues to the right. The C-terminal region of human CENP-B between positions 438 and 599 was first dissected at the *Nco*I site at position 548, resulting in pCENPB1/N and pCENPB/NL. Antigenic determinant for epitope II was limited by using pCENPB1/1-12, pCENPB1/A and pCENPBS/1-12. For epitope I, the oligonucleotides encoding the polypeptide region between 535 and 553 were introduced into the other *Nco*I site at position 553. Antigenic determinant for epitope I was limited by using pCENPBO/2-6, pCENPBO/1-1 and pCENPBO/2-16. Closed boxes show the region supplemented by the synthetic oligonucleotides (see *Materials and Methods*). S, *Sac*I (position 468); A, *Apa*I (position 475); N, *Nco*I (positions 548 and 553); K, *Kpn*I (position 557). The limited antigenic determinants for epitopes I and II are shown underneath.

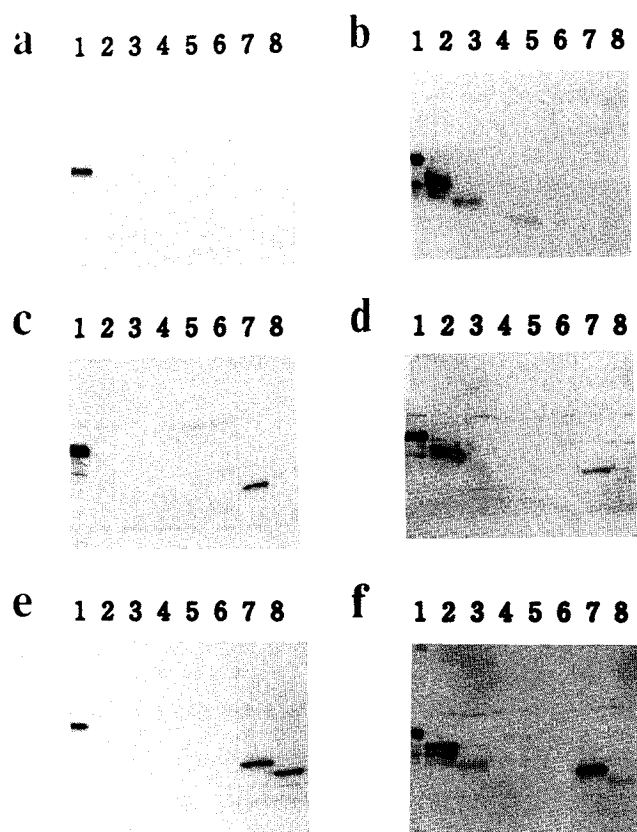
and *f* in Fig. 2, the CENP-B product encoded in the pCENPB1/A was no longer immunoreactive. In contrast, the 55 amino acid region encoded in the pCENPB1/1–12 was still immunoreactive to all the patient sera (panels *b*, *d* and *f*, Fig. 2). Therefore, the C-terminal boundary was limited to position 492. Similarly, the N-terminal boundary was examined with an additional pCENPBS/1–12 construct. The limited 25 amino acid peptide region was barely immunoreactive to the panel *b* patient serum, nor was it immunoreactive any longer to the other patient sera (lane 5 in panels *d* and *f* in Fig. 2). Therefore, we assigned the 55 amino acid residues between positions 438 and 492 to the common antigenic determinant for epitope II, although het-

erogeneity in the antigen recognition of patient sera was suggested.

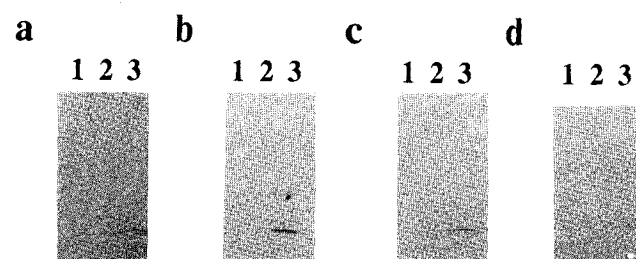
We then limited the antigenic determinant for epitope I. The absence of the antigenicity in the polypeptide encoded in the pCENPB/NL suggested that its antigenic determinant was extended further upstream of the *Nco*I site at position 548 (see Fig. 1). However, we could not find any suitable restriction site that could be used to supplement the lost upstream region on a plasmid construction. Instead, we synthesized the oligonucleotides covering the upstream CENP-B region between 535 and 553 (see *Materials and Methods* for details). In this way, the plasmid, pCENPBO/2–6 was obtained by inserting the DNA fragment at the *Nco*I site at position 553. In a similar immunoblotting analysis shown in Fig. 3, the truncated CENP-B product now became immunoreactive to all the patients. Therefore, the N-terminal boundary of epitope I was determined at position 535. Similarly, the C-terminal boundary was examined using two additional constructs, pCENPBO/1–1 and pCENPBO/2–16. However, antigenicity was no longer detected in the polypeptides encoded by these constructs (lanes 1 and 2 in Fig. 3). From these results, we concluded that the antigenic determinant for epitope I was within the C-terminal 65 amino acid residues between positions 535 and 599.

**Grouping of anticentromere positive patient sera.** The four epitopes have been finely located on the recombinant human CENP-B. According to the reactivity to each epitope, the forty anticentromere patient sera were now grouped into six categories, combining the reactivity to epitopes III and IV (lanes 7 and 8 in all panels, Fig. 2). The reactivity to each antigenic determinant was summarized in Fig. 4.

Those which only reacted with epitope I were assigned to group 1, although epitope I was recognized by



**Fig. 2.** Six reactive patterns of patient anticentromere sera to an epitope panel of human CENP-B. Antigenicity of the series of truncated CENP-B products presented in Fig. 1 was tested using forty anticentromere sera from autoimmune disease patients by immunoblotting after a separation in a 7.5% SDS-polyacrylamide gel (18, 19). The CENP-B products were expressed in *Escherichia coli* harboring each plasmid: pCENPB1 (lane 1), pCENPB1/N (lane 2), pCENPB1/1–12 (lane 3), pCENPB1/A (lane 4), pCENPBS/1–12 (lane 5), pCENPB/NL (lane 6), pCENPB4–4 (lane 7) and pCENPB3–15 (lane 8) in all the panels. According to these patterns, the patient sera were grouped into six categories: Nine sera in group 1 (panel *a*), one in group 2 (panel *b*), twelve in group 3a (panel *c*), three in group 3b (panel *d*), ten in group 4a (panel *e*), and five in group 4b (panel *f*).



**Fig. 3.** Determination of the antigenic determinant for epitope I. The truncated CENP-B products covering the C-terminal region were expressed in *E. coli* cells harboring the plasmids; pCENPBO/2–16 (lane 1 in common), pCENPBO/1–1 (lane 2 in common) and pCENPBO/2–6 (lane 3 in common). The reactivity of each CENPB product was tested with the sera of 40 patients, as described in the Fig. 2 legend except a 10% polyacrylamide gel was used. The representative patterns were with the patients from group 1 (panel *a*), group 2 (panel *b*), group 3 (panel *c*) and group 4 (panel *d*).

all the anticentromere sera. In contrast, the other three epitopes were not always recognized by all the patients. One serum that showed the reactivity only to epitopes I and II was assigned to group 2. Fifteen sera showing reactivity to epitope IV were assigned to group 4. Another fifteen sera with the reactivity to epitope III, but not to epitope IV, were assigned to group 3. The antigenic determinant for epitope II had a unique feature, in that it could be recognized not only by the group 2 patient, but by the minority in groups 3 and 4 as well. These patients were assigned to subgroups 3b and 4b, respectively.

*Production of anti-CENP-B serum with human specificity.* Recently, a mouse homologue to human CENP-B gene has been isolated and the human amino acid sequence for these epitopes could be compared with the mouse correspondences (25). The amino acid sequences identical to those for epitopes III and IV have been found in mouse CENP-B (24). As shown in Fig. 5, the amino acid sequence for epitope I was also perfectly conserved in this species, suggesting that at least one of them might be the antigenic determinant common to mammalian centromere proteins (4, 13, 16, 17, 20). In contrast, epitope II shared less homology, indicating that it would actually be the one that defines a human specificity. To examine this possibility further, we attempted to produce an anti-CENP-B serum only specific to human centromere structure by immunizing mice with the recombinant human CENP-B expressed in *E. coli*. Although production of polyclonal and monoclonal antibodies against human CENP-B has been reported, the species specificity was not the focus of inves-

	Epitopes			
	IV	III	II	I
group 1				+
group 2			+	+
group 3a		+		+
group 3b		+	+	+
group 4a	+	N.D.		+
group 4b	+	N.D.	+	+

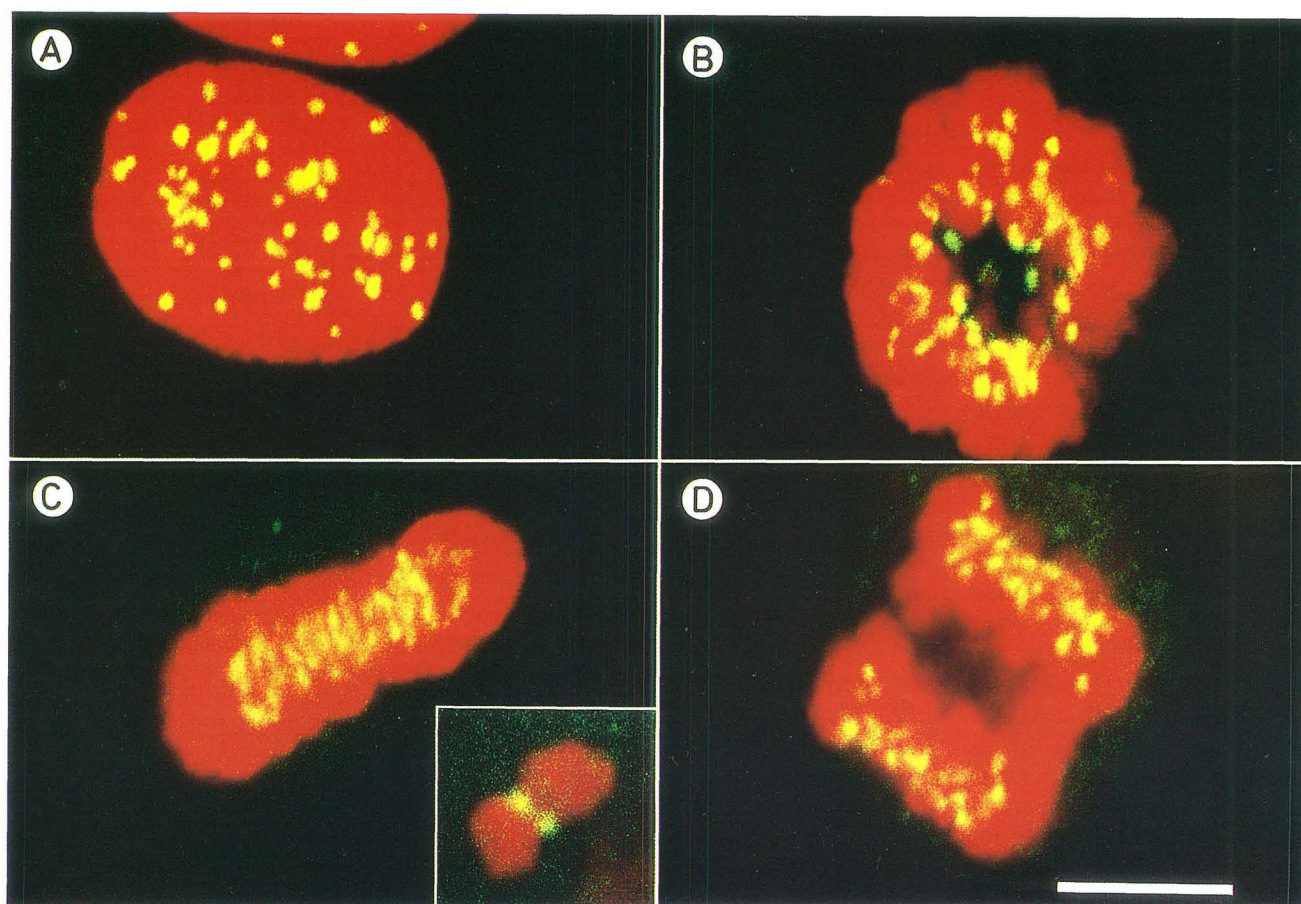
Fig. 4. Summary of the reactivity of anticentromere autoimmune sera to the four CENP-B epitopes. The forty anticentromere sera from autoimmune disease patients were grouped into 6 categories according to the reactivity patterns shown in Fig. 2. Minorities in groups 3 and 4 also reacting with epitope II were subdivided into groups 3b and 4b, respectively.

tigation (8). After several trials of mouse immunization, we obtained one serum which showed the speckled pattern typical of centromere staining in human cell nuclei by

		535
Epitope I	Human	DGDEV <sup>.</sup> PVPSFGEAMAY <sup>.</sup> FAMVKRYLT <sup>.</sup> SFPIDDRVQSH <sup>.</sup>
	Mouse	-----
		* ** 599
	Human	ILHLEHDLVHVTRKNHARQAGV <sup>.</sup> RGLGHQS <sup>.</sup>
	Mouse	-----
		438
Epitope II	Human	EGEELGEEEEVEEEGDV <u>DSDEEEEE</u> DEESS <sup>.</sup>
	Mouse	----E- -----E-D----- --
		492
	Human	SEGLEAEDWAQGVVEAGGSFGAYGA <sup>.</sup>
	Mouse	-----S-G--G-SV

Fig. 5. Amino acid sequences defining the antigenicity for epitopes I and II. Amino acid sequence for epitope I was from the pCENPBO/2-6 construct and that for epitope II was from the pCENPB1/1-12 construct. These amino acid sequences of human CENP-B were deduced from our human CENP-B cDNA clones. Corresponding sequences of mouse CENP-B have been recently reported by Sullivan and Glass (25), and the only substituted residues were shown under the human sequence. The three asterisked amino acid residues differ from the human CENP-B sequence previously reported by Earnshaw *et al.* (8). The conserved putative casein kinase II site is boxed (see Discussion).





**Fig. 6.** Immunofluorescent staining of human centromere by mouse anti-CENP-B serum. Human epitheloid cells (HEp-2) stabilized on slides were incubated with a 100-fold diluted mouse serum raised against human recombinant CENP-B. Centromere staining was obtained by further incubation of FITC-conjugated anti-mouse Ig antibodies (1: 40) and chromatin staining by propidium iodide. (A) interphase; (B) prophase; (C) metaphase, with a 2-fold magnified metaphase chromosome in inset; (D) anaphase. Bar, 10  $\mu$ m.

indirect immunofluorescence technique (16, 17). As shown in Fig. 6, the mouse serum indeed stained the centromere at the primary constriction of metaphase chromosome and the centromere staining was observed throughout the cell cycle. The same result was recently reported by Compton *et al.* (6).

The existence of the CENP-B homologues in other mammalian cells was expected, since the antigens with the same molecular weight as human CENP-B (80 kDa) have been previously identified in porcine, mouse and muntjac cells using anticentromere autoserum (2, 3). When we tested mouse, monkey, bovine and porcine cell preparations, the same speckled staining patterns were obtained with the patient anticentromere autoserum

(panels B to E in Fig. 7). Although this patient serum contained other polyclonal antibodies against CENP-A and CENP-C as well, the same staining pattern was observed with other patient sera with relatively few anti-CENP-A or anti-CENP-C antibodies (18) (data not shown). Therefore, we assumed that the antigenicity of the centromere proteins including CENP-B was preserved in these cell preparations.

We then examined whether the mouse anti-CENP-B serum was reactive to other mammalian centromeres. However, no centromere staining pattern was observed in any but human nuclei (panels a to e in Fig. 7). This result suggested the antigenic determinant recognized by this mouse serum should be specific to human centromere

**Fig. 7.** Human-specific centromere staining of mouse anti-CENP-B serum. Mammalian cells were grown and fixed on coverslips (see *Materials and Methods* for details). These cell preparations were stained with a representative anticentromere serum from group 4b (A to E) or with mouse anti-CENP-B serum (a to e). In each panel, the centromere staining patterns with FITC-conjugated anti-mouse Ig antibodies (1: 40) or anti-human IgG (1: 100) antibodies are shown at left, while staining of chromatin by propidium iodide in the same cells is shown at right. Human HEp-2 cells (A-a), mouse TS-1-47 cells (B-b), monkey vero cells (C-c), bovine MDBK cells (D-d) and porcine PK15 cells (E-e). Bar, 10  $\mu$ m.

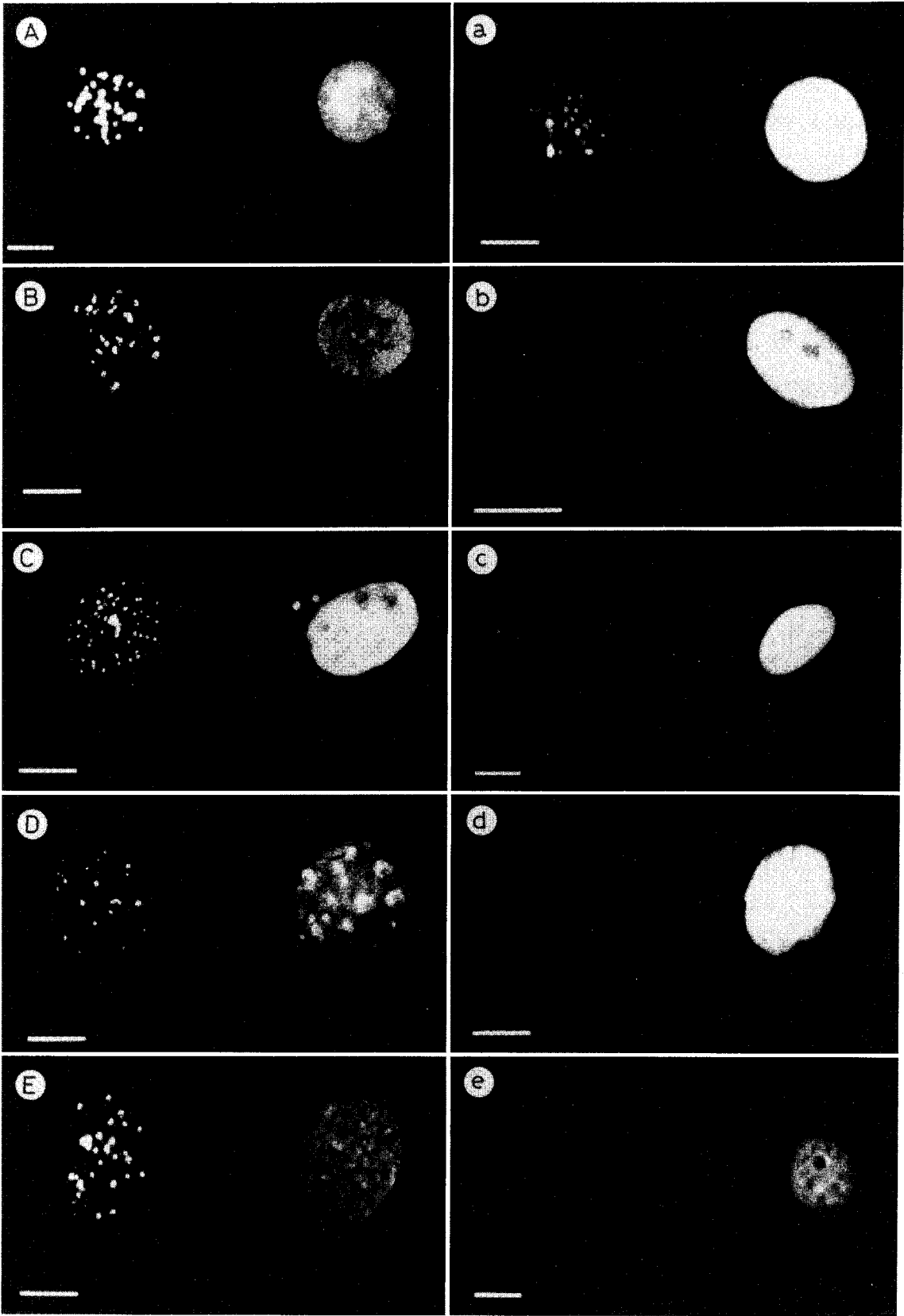


Fig. 7.

mere and be different from that of other mammals.

**Epitope mapping of human-specific antigenic determinant.** To identify the antigenic determinant recognized by the mouse anti-CENP-B serum, we did the epitope mapping again. To estimate its location roughly, we used the previous set of truncated CENP-B products which had been deleted from the C-terminus (24). As shown in panel *a* in Fig. 8, the C-terminal boundary was limited to position 492 using the pCENPB1-12 construct. This result suggested that the antigenic determinant was located within the C-terminal region encoded in the pCENPB1 construct, since no antigenicity was detected in the upstream region. We then used the same set of CENP-B products used for the analysis of epi-

topes I and II (see Fig. 1). The staining pattern was similar to that with the group 2 patient serum (panel *b*, Fig. 8). This result suggested that the antigenic determinant was within the 55 amino acid region encoded in the pCENPB1/1-12 construct, that was previously assigned to epitope II, corresponding to the only epitope with a less homologous amino acid sequence in mouse. To examine if it also reacted with epitope I, we tested the reactivity to the remaining set for epitope I. The expression of the recombinant products was confirmed by a rabbit serum raised against T7 phage gene 10 product (panel *d*, Fig. 8). The mouse anti-CENP-B serum also reacted with the CENP-B product encoded in pCENPBO/2-6 construct.

## DISCUSSION

The four antigenic determinants on human CENP-B have been characterized using anticentromere sera from autoimmune disease patients. Two of them have been located on the predicted helix-loop-helix structure of the N-terminal DNA binding domain (24). In this study, the remaining two were precisely located in separate regions on the C-terminus. The antigenic determinant for epitope I was limited to the 65 amino acid residues. We assumed it to be the "major epitope" on human CENP-B that was recognized by all the anticentromere sera from a variety of autoimmune disease patients (18, 19), as described by Earnshaw *et al.* (8, 9). In contrast, epitope II, limited to the 55 amino acid residues, was regarded as the so-called "minor epitope," which was

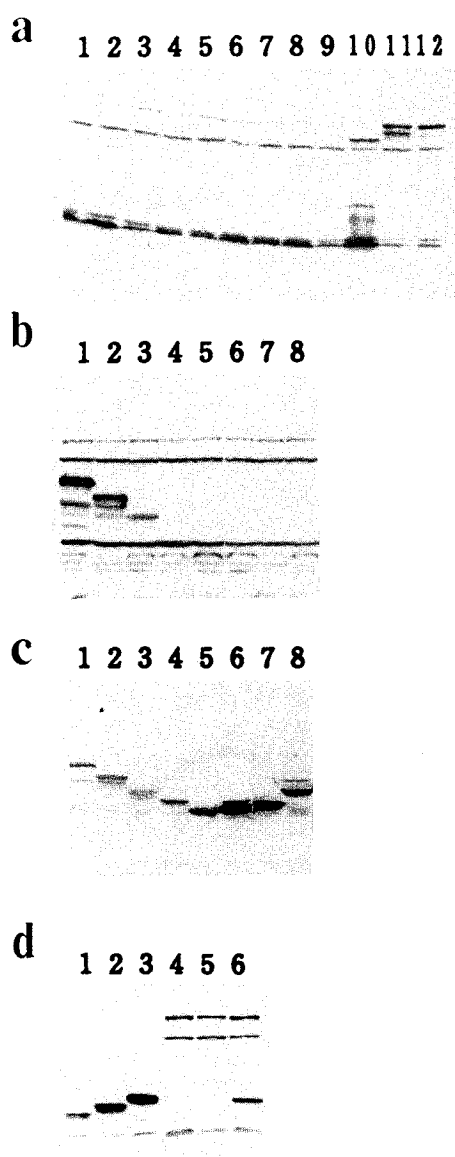


Fig. 8.

**Fig. 8.** Identification of the human-specific antigenic determinant defined by the mouse anti-CENP-B serum.

*a.* The location of the antigenic determinant recognized by the mouse anti-CENP-B serum was roughly estimated using the previous set of truncated CENP-B products which had been deleted from the C-terminus, as described in the legends to Figs. 2 and 3. The truncated products were expressed in *E. coli* harboring the plasmids: pCENPB4-1 (lane 1), pCENPB3-15 (lane 2), pCENPB4-4 (lane 3), pCENPB3-21 (lane 4), pCENPB4-8 (lane 5), pCENPB4-7 (lane 6), pCENPB4-5 (lane 7), pCENPB1-24 (lane 8), pCENPB1-42 (lane 9), pCENPB1-12 (lane 10), pCENPB1-1 (lane 11) and pCENPB2-6 (lane 12) (see Fig. 3a in ref. 24 for details of these plasmid constructs).

*b.* The antigenic determinant was limited using the same set of CENP-B constructs as used in Fig. 2; pCENPB1 (lanes 1), pCENPB1/N (lanes 2), pCENPB1/1-12 (lanes 3), pCENPB1/A (lanes 4), pCENPB3/1-12 (lanes 5), pCENPB/NL (lanes 6), pCENPB3-15 (lanes 7), pCENPB4-4 (lanes 8).

*c.* The expression of each CENP-B fusion product was analyzed by immunoblotting with the rabbit antiserum against T7 phage gene 10 product. The same set of CENP-B products as in panel *b* was used for each lane.

*d.* Epitope I was also recognized by the mouse anti-CENP-B serum. The CENP-B products were from the pCENPBO/2-16 (lanes 1 and 4), pCENPBO/1-1 (lanes 2 and 5) and pCENPBO/2-6 (lanes 3 and 6) constructs and the expression was confirmed by immunoblotting with the rabbit serum against T7 phage gene 10 product (lanes 1 to 3). The reactivity of the mouse anti-CENP-B serum was tested (lanes 4 to 6).



only immunoreactive in a minority of patients. The requirement of relatively large regions for the antigenicity suggested that the secondary structure, rather than the primary structure, was recognized by the autoimmune sera.

Although "epitopes 1 to 3" have been predicted on the recombinant human CENP-B by a pioneer study of Earnshaw *et al.* (8, 9), a detailed analysis has not been performed heretofore. We assumed that epitope I corresponded to "epitope 1," since the antigenicity was destroyed by the dissection of the CENP-B polypeptide at the *Nco*I site (position 548) or by the 22-amino-acid deletion from the C-terminus (see pCENPB/NL and pCENPBO/1-1 in Fig. 2, respectively). Compared with the amino acid sequence of the previous human CENP-B reported by Earnshaw *et al.* (8), we found three substituted amino acids at positions 583 (Met), 592 (Leu) and 593 (Leu) in this region (Fig. 5). The same substitutions have been predicted from the human genomic CENP-B sequence recently characterized by Sullivan and Glass (25). Epitope II should correspond to "epitope 2," since it was located within the 104 amino acid stretch previously identified as epitope 2. "Epitope 3" would have been located on the N-terminal DNA binding domain, since we could not detect any antigenicity in the region between 78 and 466 so far tested (data not shown).

The patient sera have been grouped into 6 categories according to their reactivities to the four epitopes on human CENP-B (Fig. 4). Each epitope seemed to be quite distinct: The patient sera from group 1 reacted with epitope I, but not with epitopes II, III or IV. Similarly, the group 2 patient serum did not react with epitopes III or IV. The group 3 patient sera did not recognize epitope IV. Moreover, epitope II was not always recognized by all the sera from groups 3 and 4. Although a minority of patient sera in groups 3b and 4b recognized epitope II, a monoclonal antibody which recognizes epitope II did not cross-react with the others (data not shown).

We have obtained the mouse anti-CENP-B serum which was only specific to human centromere (Fig. 7). Centromere antigens with the molecular weight of 80 kDa have been detected in other mammals using anticentromere sera from autoimmune disease patients by several groups of investigators (2, 3). The homologous DNA sequences to human CENP-B gene have been detected in other mammals and a chicken (data not shown) and the mouse CENP-B gene has actually been isolated (25). Therefore, we assumed that CENP-B was probably expressed among mammals. The epitope analysis of the mouse anti-CENP-B serum showed that the antigenic determinants were located at epitope II as well as epitope I. The amino acid sequence data of mouse CENP-B made it possible to predict that the difference within the 55 amino acid residues shown in Fig. 5 was enough to yield human specificity and, possibly, vice

versa.

Rabbit polyclonal antibodies to human CENP-B have been previously produced by Earnshaw *et al.* (8). Although the serum has been shown to recognize both epitopes 1 and 2 on human CENP-B, its failure "to stain some mammalian kinetochores or to recognize 80 kDa bands" in immunoblotting was also indicated in a recent review (unpublished observations in ref. 5). We do not know why other mammalian centromeres are unable to be stained by these sera raised against bacterially expressed human CENP-B. Mammalian CENP-B might interact with another molecule or have some protein modification at the epitope I region so that it would not react with anticentromere antibodies against epitope I.

Recently, we found that the bacterially expressed CENP-B could be phosphorylated in an *in vitro* reaction and that the phosphorylation site was at the putative casein kinase II site between positions 456 and 462 (Sugimoto and Himeno, *Biosci. Biotech. Biochem.* in press). As in the case of epitopes III and IV (24), another important function of CENP-B *in vivo* might be assigned to the epitope I and II regions.

Knowledge of the antiserum specific to human centromere will allow us to focus on the behavior of a human CENP-B when it is introduced into other mammalian cells, since it could not be distinguished from the host centromere proteins by the anticentromere autoimmune sera so far tested. It will enable us to analyze the expression and regulation profile of human CENP-B gene in the future.

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