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CENP-B Binds a Novel Centromeric Sequence in the Asian Mouse *Mus caroli*

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Minor satellite DNA, found at *Mus musculus* centromeres, is not present in the genome of the Asian mouse *Mus caroli*. This repetitive sequence family is speculated to have a role in centromere function by providing an array of binding sites for the centromere-associated protein CENP-B. The apparent absence of CENP-B binding sites in the *M. caroli* genome poses a major challenge to this hypothesis. Here we describe two abundant satellite DNA sequences present at *M. caroli* centromeres. These satellites are organized as tandem repeat arrays, over 1 Mb in size, of either 60- or 79-bp monomers. All autosomes carry both satellites and small amounts of a sequence related to the *M. musculus* major satellite. The Y chromosome contains small amounts of both major satellite and the 60-bp satellite, whereas the X chromosome carries only major satellite sequences. *M. caroli* chromosomes segregate in *M. caroli* × *M. musculus* interspecific hybrid cell lines, indicating that the two sets of chromosomes can interact with the same mitotic spindle. Using a polyclonal CENP-B antiserum, we demonstrate that *M. caroli* centromeres can bind murine CENP-B in such an interspecific cell line, despite the absence of canonical 17-bp CENP-B binding sites in the *M. caroli* genome. Sequence analysis of the 79-bp *M. caroli* satellite reveals a 17-bp motif that contains all nine bases previously shown to be necessary for in vitro binding of CENP-B. This *M. caroli* motif binds CENP-B from HeLa cell nuclear extract in vitro, as indicated by gel mobility shift analysis. We therefore suggest that this motif also causes CENP-B to associate with *M. caroli* centromeres in vivo. Despite the sequence differences, *M. caroli* presents a third, novel mammalian centromeric sequence producing an array of binding sites for CENP-B.

Centromeres have a crucial role in both mitotic and meiotic chromosome segregation (reviewed by Pluta et al. [39], Rattner [40], Schulman and Bloom [42], Brinkley et al. [5], and Bloom [4]). Seen as the primary constriction of a metaphase mammalian chromosome, the centromere is the site of attachment of the spindle microtubules. This interaction is mediated by a specialized structure, termed the kinetochore, which appears as a trilaminar plate when observed by electron microscopy. Detailed biophysical experiments indicate that at least one of the mechanochemical motors responsible for poleward chromosome movement is located at, or very close to, the kinetochore (34). The centromere is also the last point at which sister chromatids remain attached to each other prior to the commencement of poleward chromosome movement. The centromere is therefore presumed to be one target for the cell cycle signal that triggers the metaphase-anaphase transition (39).

The centromeres of the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are among the best-studied eukaryotic centromeres (8, 16). Genomic fragments cloned from both species can confer full centromere function when reintroduced into the cell, and a number of interacting proteins have been identified (16, 42). The functionally important sequences at mammalian centromeres are less clearly defined. Human centromeres consist, at least in part, of α-satellite sequences (reviewed by Tyler-Smith and Willard [48]). This repetitive DNA family is organized as large tandem repeat arrays, often over 1 Mb in size, of an underlying 171-bp monomer. *Mus musculus* centromeres, as defined by the location of centromere antigens detected by CREST autoimmune sera, colocalize with arrays of a repetitive DNA sequence family called the minor satellite (6, 12, 20, 21, 23, 31, 33, 38, 53, 54). In *M. musculus*, the minor satellite is organized as tandem repeat arrays of a 120-bp monomer sequence, with an average of approximately 300 kb of minor satellite per chromosome (21, 23, 38, 54).

The ability of human chromosomes to segregate successfully in somatic cell hybrids suggests that human centromeres can function, at least to a certain extent, in rodent cells. Despite this apparent functional interchangeability, there is only limited similarity between the known centromeric sequences of humans and mice. Some monomers of both the human α-satellite and mouse minor satellite contain a 17-bp motif which has been shown in vitro to be the binding site for the centromere-associated CENP-B protein (27, 32, 56). A deletion derivative of human chromosome 17 with reduced amounts of α-satellite has reduced amounts of bound CENP-B detectable by immunocytochemistry, which would be consistent with CENP-B also binding α-satellite in vivo (52).

The immunocytochemical localization of the CENP-B protein to human centromeres is consistent with this protein having some role in centromere function. Cell transfection studies indicate that α-satellite can confer at least some centromere-like properties to its site of integration in the genome, although the role of CENP-B in this phenomenon has not been addressed (15, 26). Nevertheless, microinjection of CREST sera containing antibodies predominantly against CENP-B can result in disrupted kinetochore formation and mitosis (2). One hypothesis is that an array of α-satellite or minor satellite,

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because of its ability to bind CENP-B, is sufficient to result in kinetochore assembly at this chromosomal site. Although attractive, there are a number of observations that challenge this simple model of mammalian centromere function. For example, the centromere of the *M. musculus* Y chromosome appears to contain no minor satellite DNA sequences (6, 31), and its centromere does not detectably bind the CENP-B protein (28a). Furthermore, there are examples of human marker chromosomes which are mitotically stable and CREST positive yet contain no detectable α-satellite (3, 37, 50). In one case in which it was tested, the marker chromosome did not label with anti-CENP-B serum even though it was CREST positive (50). While the presence of minor satellite or α-satellite, together with the binding of the CENP-B protein, may be a component of an active centromere under normal circumstances, these data suggest that neither is absolutely essential for centromere function. Conversely, the observation that minor satellite can be present at chromosomal sites without formation of a centromere (6) argues that centromere formation is not an obligatory consequence of the presence of this DNA sequence. A similar problem is posed by one example of a cell line in which a dicentric human chromosome shows detectable amounts of CENP-B at the α-satellite arrays of both the active and the inactive centromere (10). This finding suggests that even if a satellite array binds CENP-B, this binding does not necessarily result in the formation of a centromere. Such lines of evidence argue that a simple model of centromere organization involving an array of CENP-B binding sites might be fundamentally wrong. It should be emphasized that although the minor and α-satellites appear to be the predominant DNA sequence families at mouse and human centromeres, respectively, no exhaustive sequence analysis of any mammalian centromere has been undertaken. It therefore remains possible that either additional structural features or DNA sequence elements (or both) may be required to determine kinetochore location.

The Asian mouse *Mus caroli* is an extreme example of the challenges to a simple model of centromere function involving CENP-B, as it does not appear to have any binding sites for this protein in its genome (40). *M. caroli* separated from *M. musculus* about 5 to 7 million years ago, predating the separation of *Mus spretus* and *M. musculus* (approximately 3 to 4 million years ago). Unlike the latter two species, *M. caroli* has little if any detectable minor satellite in its genome (12, 38, 53). Furthermore, oligonucleotide hybridizations do not detect any canonical 17-bp CENP-B binding sites in the *M. caroli* genome (see below). *M. caroli* does contain a small amount of a sequence family which cross-hybridizes to the mouse major satellite, the latter localizing to the pericentric heterochromatin of *M. musculus* (9, 12, 36, 38, 43). Despite the lack of minor satellite, *M. musculus* × *M. caroli* interspecific hybrids can nevertheless be produced (51). Although the embryos usually die in utero, cell lines have been developed from such fetuses, and these cells contain and segregate chromosomes from both species (7). Therefore, despite apparently having different centromeric DNA sequences, the chromosomes of both species can interact successfully with the same mitotic spindle.

This report presents a possible resolution of this paradox by the analysis of two novel but related sequence families found at *M. caroli* centromeres and also demonstrates that *M. caroli* centromeres bind mouse CENP-B in vivo in cells which express detectable amounts of this protein. A variant form of the canonical CENP-B binding site is present in the sequence of one of the *M. caroli* satellites. CENP-B from HeLa nuclear extracts specifically bound this sequence motif in vitro, strongly suggesting that this sequence motif is responsible for CENP-B binding to *M. caroli* centromeres in vivo. This evidence strengthens the hypothesis that CENP-B has a role in mammalian centromere function and contributes to defining the specific sequences involved in CENP-B binding.

MATERIALS AND METHODS

Mice. *M. caroli* mice were obtained from V. Chapman (Roswell Park, Buffalo, N.Y.) and maintained in Edinburgh.

Cell culture. The interspecific hybrid cell line was line B, derived by Chapman and Shows (7) from the minced tissue of a 16-day female *M. musculus* × *M. caroli* fetus, produced by artificial insemination, and was generously provided by V. Chapman. It was grown in RPMI 1640 supplemented with 10% fetal calf serum in a 10% CO_2 atmosphere. HeLa cells were cultured in Dulbecco modified Eagle medium (Nissui, Tokyo, Japan) supplemented with 10% calf serum (ICN Biomedicals) in 5% CO_2.

Oligonucleotides for hybridization. Oligonucleotide C86 (5′-ATTCGTTGGAAACGGGA-3′) corresponds to the CENP-B box found within the mouse minor satellite (27). Oligonucleotide 203 (5′-GGAAAATGATAAAAACCACACTGTACAACATATTA-3′) hybridizes to positions 1 to 35 of the minor satellite (numbering as in reference 54). The major satellite (18) was detected with oligonucleotide 204 (5′-CACTTTAGGACGTGAAATATGGCGAGGAAAACTGA-3′). The oligonucleotides used to detect the 60- and 79-bp *M. caroli* satellites are R334 (5′-CTGAAAAACCCGCAT-3′), R335 (5′-CTTAGAATATAAGAAACGCA-3′), and R336 (5′-ATATGAGTGAGTTGCACTG-3′).

Complementary oligonucleotide pairs for gel mobility shift assays. CB30a and CB30b (together designated CB30a/b) have the sequences 5′-ACTGAGGCCTTCGTTGGAAACGGGATTTCC-3′ and 3′-CTCCGGAAGCAACCTTTGCCCTAAAGGTGA-5′. CB30c and CB30d (together designated CB30c/d) are the same as CB30a/b except that two nucleotides in the CENP-B box were altered (underlined): 5′-ACTGAGGCCTTAGTTGGAAACTGGATTTCC-3′. CB30k and CB30l (together designated CB30k/l) are the same as CB30a/b except that the nucleotides in the CENP-B box were altered to the *M. caroli* motif (5′-TTTCGTCTAATGCGGGT-3′). ^{32}P-labelled CB30a/b and CB30k/l were generated by an end-filling reaction using the Klenow fragment of DNA polymerase (Takara Shuzo, Kyoto, Japan).

Gel mobility shift assays. Preparation of 0.5 M NaCl HeLa cell nuclear extract was carried out as described by Masumoto et al. (27). The DNA-binding reaction using this extract was performed essentially as described by Muro et al. (32). Nuclear extract corresponding to 2×10^4 nuclei was mixed with 20 μl of binding buffer (10 mM Tris-HCl [pH 8.0], 10% glycerol, 1 mM EDTA, 0.05% Nonidet P-40, 2 mM dithiothreitol, 150 mM NaCl [final concentration]), supplemented with end-labelled CB30k/l or CB30a/b (0.6 ng), 5 μg of sonicated salmon sperm DNA, and 5 μg of poly(dI-dC) poly(dI-dC), and incubated at 25°C for 1 h. Antibody or serum (0.1 μl) was then added to the mixture, and in such cases the mixture was incubated for a further 30 min. After incubation, the mixture (5 μl) was electrophoresed through 5% polyacrylamide gels in TBE buffer (12.5 mM Tris-HCl [pH 8.0], 12.5 mM boric acid, 0.5 mM EDTA) at 20 V/cm at 4°C. Gels were dried and exposed to Kodak XAR-5 X-ray film.

Isolation and sequencing of *M. caroli* satellite clones. Genomic DNA from the kidneys of a male *M. caroli* mouse was digested to completion with *Rsa*I and separated through 1.2% low-melting-point agarose. The 60/79-bp doublet was visualized by ethidium bromide staining, excised from the gel, and recovered by using β-agarase (New England Biolabs) as instructed by the manufacturer. This DNA was cloned into the *Sma*I site of pGEM7 (Promega) by using *Escherichia coli* SURE (Stratagene). Six random insert-containing clones were sequenced by using standard double-strand protocols and Sequenase II (U.S. Biochemical) as specified by the manufacturer. Plasmids R397 and R398 are the clones which carry the 60-bp satellite insert C3 (see Fig. 2A) and 79-bp satellite insert C10, respectively.

*Dde*I fragments were cloned in a similar fashion following filling-in of cohesive ends by the Klenow fragment of DNA polymerase.

Pulsed-field gel electrophoresis and filter hybridization. High-molecular-weight genomic DNA embedded in agarose plugs was prepared from *M. caroli* kidneys as described previously (22). Restriction enzyme digestions were performed in the manufacturer's recommended buffer as described by Allshire et al. (1). Samples were separated though a 0.9% agarose gel in 0.25 × TBE for 40 h, using a Biometra Rotaphor apparatus with an electrode angle of 120°, voltage ramped logarithmically from 200 to 150 V, and switch time ramped logarithmically from 60 to 10 s. The size markers were *S. cerevisiae* chromosomes from strain YPH148. Gels were neutral blotted to nylon membranes (Hybond; Amersham) by standard procedures. Figure 1 was obtained by conventional X-ray film autoradiography, whereas Fig. 3 is a PhosphorImager (Molecular Dynamics) image.

Oligonucleotide hybridization probes were prepared by labelling 20 pmol of oligonucleotide with [γ-^{32}P]ATP, using T4 polynucleotide kinase. Oligonucleotide hybridizations were performed in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–2× Denhardt's solution–0.1% sodium dodecyl sulfate (SDS) at 45°C. Filters were washed twice for 20 min in 5× SSC–0.1% SDS at 45°C. Filters were stripped of probe between hybridizations by washing in 0.1% SDS at 95°C.

The *M. musculus* major satellite probe used was the insert of pMR196 (38). It

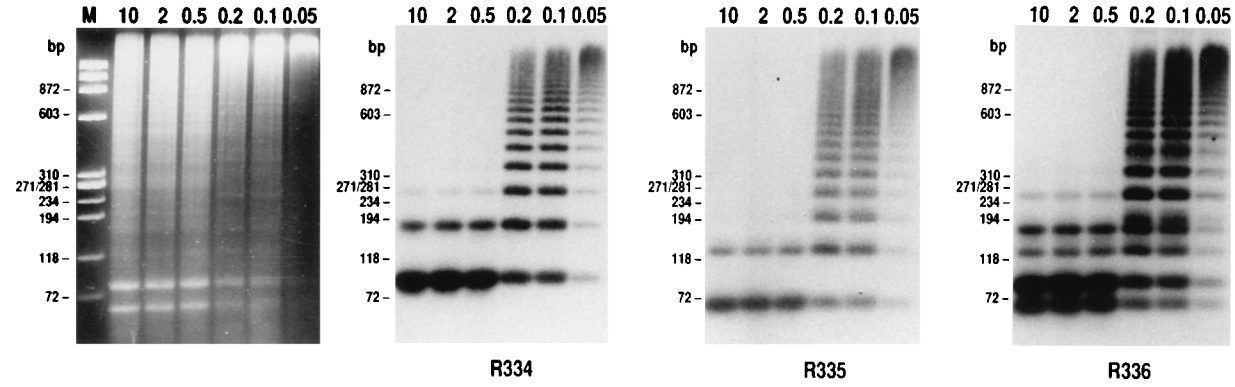

FIG. 1. Southern analysis of partial digests of *M. caroli* genomic DNA. A 1.5-μg sample of *M. caroli* genomic DNA (male kidneys) was digested overnight with various amounts of *Rsa*I, separated on a 4% agarose gel (3% NuSieve [FMC], 1% Sigma type II-A), and stained with ethidium bromide. The numbers of units of *Rsa*I used are indicated for each lane. The gel was blotted to a nylon membrane and successively probed with oligonucleotides R334, R335, and R336. M, size marker (ϕX174/*Hae*III).

was labelled by the random-priming protocol (11). Hybridization was in 0.5 M Na_2HPO_4 (pH 7.2)–7% SDS–1 mM EDTA–0.5% dried milk at 65°C. Final washes were in 0.1× SSC–0.1% SDS at 65°C. The *M. musculus* minor satellite probe used was the cloned trimer and contains three full-length monomers (23). Labelling and hybridization were as for the major satellite probe except that the final wash was in 5× SSC for 5 min at room temperature.

Chromosome preparation and primed in situ hybridization (PRINS). *M. caroli* mice were sacrificed by cervical dislocation, and spleens were removed under sterile conditions. Cells were removed by perfusing the spleens with 10 ml of RPMI 1640 medium (lacking serum), using a 25-gauge hypodermic needle. The resulting cell suspension was pelleted at 1,000 rpm and gently resuspended in 2 ml of RPMI 1640 before adjustment of the volume to 5 ml with phosphate-buffered saline. The cell suspension was loaded onto a 3-ml Lymphopaque gradient (Nyegaard Diagnostica, Oslo, Norway) and centrifuged at 2,600 rpm for 20 min, using a swing-out rotor. The lymphocyte layer was carefully removed and washed in RPMI 1640 supplemented to 15% with fetal calf serum and to 0.03% with glutamine. The cells were pelleted at low speed and resuspended in 10 ml of the same medium. Ten-milliliter cultures were set up at 10^6 cells per ml and supplemented with lipopolysaccharide (Sigma) to 25 mg/ml. Cells were cultured for 72 h at 37°C.

Cells were accumulated in mitosis and spread following fixation in 3:1 methanol-acetic acid (20). The PRINS technique (14, 25, 29) was used with the following modifications. Chromosomal DNA was denatured in 30 mM NaOH–1 M NaCl at 0°C for 45 min followed by a brief rinse in 10 mM Tris (pH 7.6). PRINS annealing was at 50°C for 10 min, and extension was for 20 min at 63°C. Digoxygenin-11-dUTP or biotin-16-dUTP (Boehringer Mannheim) was incorporated during the PRINS reaction and detected with anti-digoxigenin-fluorescein Fab fragment (Boehringer Mannheim) or with Texas red avidin D (Vector Laboratories). Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) or propidium iodide. Double PRINS reactions were carried out as described by Mitchell et al. (30). Figures 4 and 8 were obtained by using a Zeiss Axioplan fluorescence microscope equipped with a 100-W mercury source and a Photometrics CCD camera. The images were acquired and processed by using software from Digital Scientific (Cambridge, England). Slides were imaged by using a Chroma P1 filter set with excitation filters mounted in a rotating wheel, giving exact registration between images from different fluorochromes.

Metaphase chromosome preparation for indirect immunofluorescence microscopy. Native chromosome spreads were prepared as described by Jeppesen et al. (19). CREST anticentromere serum (a gift from G. Nuki, Rheumatology Unit, Northern General Hospital, Edinburgh, Scotland) was diluted 1:1,000, and the rabbit polyclonal CENP-B antiserum (a gift from W. C. Earnshaw, Johns Hopkins University, Baltimore, Md.) was diluted 1:300 for immunocytochemistry as described by Jeppesen et al. (19).

Immunoblotting. Nuclear proteins were isolated from mouse livers by the following modification of Strauss and Varshavsky (44). Livers were minced finely with scissors and homogenized in a Teflon-glass motor-driven homogenizer. After 10 to 15 strokes with buffer A in 1.5 M sucrose, the nuclei were separated by centrifugation over a 5-ml cushion of buffer A in 2 M sucrose at 20,000 rpm for 30 min. The pellet was carefully rehomogenized and centrifuged as described above. The nuclear pellet was then washed with buffer A and pelleted at low speed. All the manipulations described above were done at 4°C. Nuclear proteins were extracted in buffer A containing 0.5 M NaCl for 2 h at 4°C. Finally, the protein extract was stored at −80°C in the buffer described above supplemented with glycerol to 15%.

SDS–15% polyacrylamide gel electrophoresis was carried out as described by Gooderham and Jeppesen (13), and the products were transferred to Immobilon polyvinylidene difluoride transfer membranes (Millipore) as described by Towbin et al. (47), with methanol omitted from the transfer buffer. Antibody labelling with rabbit polyclonal CENP-B antiserum (1:300 dilution) and with human autoimmune and control sera was carried out as described by Nicol and Jeppesen (35).

Nucleotide sequence accession numbers. The sequences in Fig. 2A have been submitted to the EMBL database and assigned the following accession numbers: Z32555 (clone C10), Z32556 (clone C2), Z32557 (clone C11), Z32558 (clone C4), Z32559 (clone C8), and Z32560 (clone C3).

RESULTS

Cloning of *M. caroli* satellite sequences. Buoyant density centrifugation and reassociation kinetics (41, 46) indicate that the *M. caroli* genome contains a number of highly abundant satellite DNA sequences, which would be good candidates for centromeric repetitive DNA sequences. The minor and major satellites of *M. musculus* do not detect highly abundant sequence families in *M. caroli* by Southern blotting or fluorescence in situ hybridization (9, 12, 36, 38, 43, 46). This finding suggests that the *M. caroli* satellite sequences are distinct from the major and minor satellites of *M. musculus.*

A prominent doublet of bands approximately 60 and 80 bp in size is observed when male *M. caroli* genomic DNA is digested with *Rsa*I, separated by agarose gel electrophoresis, and stained with ethidium bromide (Fig. 1). *Dde*I digestion also produces prominent bands of similar size (data not shown). This doublet was not observed when *M. caroli* DNA was digested with *Alu*I, *Bst*NI, *Hae*III, *Hin*fI, *Mbo*I, *Msp*I, or *Taq*I (data not shown). A similar doublet was not seen when either *M. spretus* or *M. musculus* genomic DNA was digested with any of these enzymes (data not shown). Prominent bands on ethidium bromide-stained gels are characteristic of a sequence family present at high copy number.

The prominent *Rsa*I doublet was isolated from an agarose gel and cloned into a plasmid vector. The sequences of six independent, randomly chosen insert-containing clones are shown in Fig. 2A. Three clones (C3, C4, and C11) have 60-bp inserts, and the other three (C2, C8, and C10) have 79-bp inserts. Both 60- and 79-bp sequences contain a *Dde*I site (Fig. 2A), suggesting that these two sequence families might also be responsible for the similar-size *Dde*I doublet. A number of similarly cloned *Dde*I fragments were therefore obtained. Their sequence reveals them to be members of the same two repeat families isolated from the *Rsa*I digest except that they start and terminate at the *Dde*I site (Fig. 2A) and circularly permute around the *Rsa*I site. This finding demonstrates that

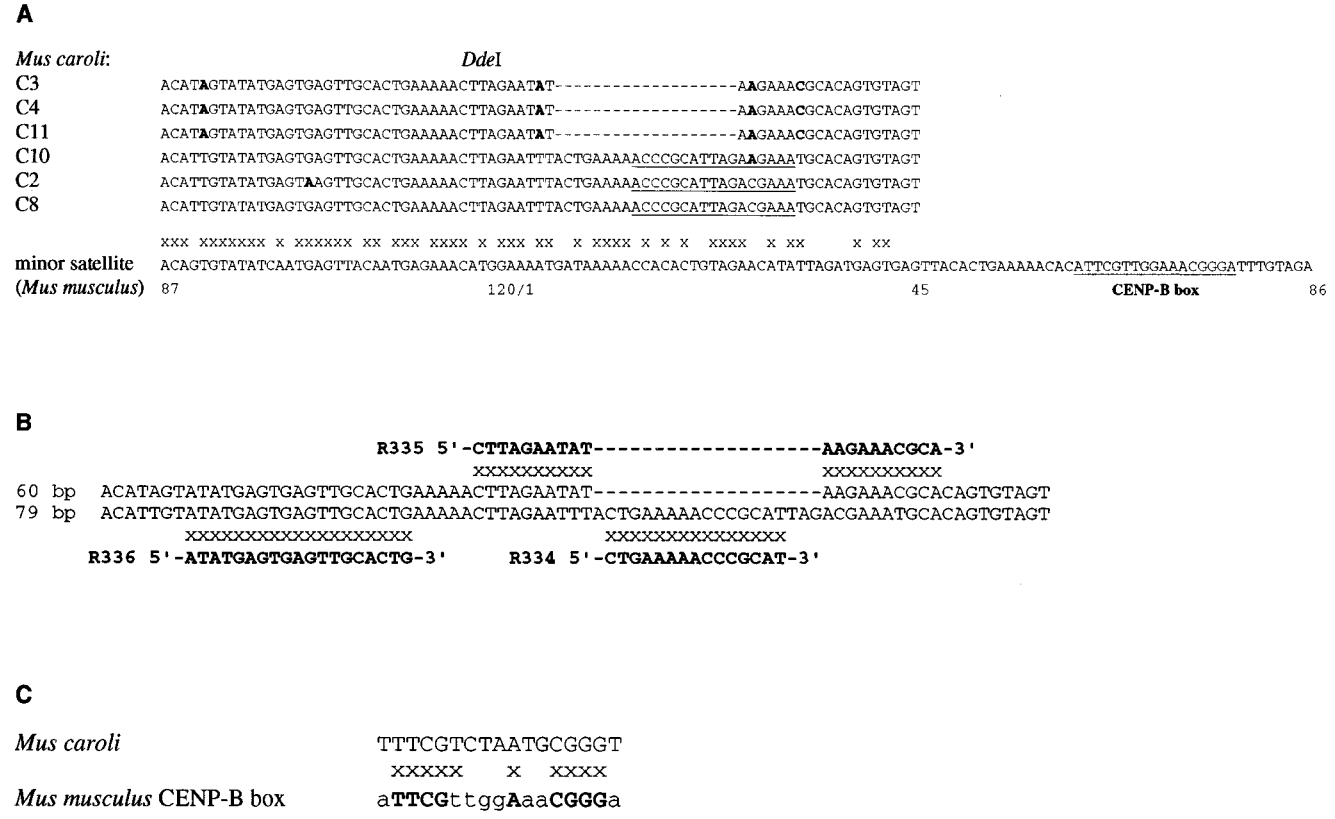

FIG. 2. Sequences of the 60- and 79-bp *M. caroli* satellites. (A) Alignment of the sequences of three 60-bp and three 79-bp *M. caroli* satellite *Rsa*I clones. Dashes indicate spaces introduced to permit the alignment. Bases which vary between the 60- and 79-bp satellites are indicated in boldface, as are two additional point mutations. The *M. caroli* satellites are shown aligned to the consensus sequence of the *M. musculus* minor satellite determined by Wong and Rattner (54) (numbering is based on that reference). The *M. musculus* and *M. caroli* CENP-B boxes are underlined. (B) Oligonucleotides R334, R335, and R336 aligned with the 60- and 79-bp satellites. (C) The *M. caroli* CENP-B box aligned with the canonical 17-bp *M. musculus* CENP-B box. The nine positions in the *M. musculus* CENP-B box identified as essential for binding to the CENP-B protein in vitro are indicated by boldface uppercase letters (28). Note that in panel C, the *M. caroli* CENP-B box is shown as the complement of the sequence in panel A.

the prominent *Rsa*I and *Dde*I bands in *M. caroli* genomic digests reflect the same two repetitive DNA sequence families, and the circular permutation of the *Dde*I and *Rsa*I clones suggests that they are organized as tandem repeat arrays. These repetitive DNA sequence families are named the 60- and 79-bp *M. caroli* satellites.

The 60- and 79-bp *M. caroli* satellites are distinct but very closely related in sequence. All cloned monomers of the 60-bp *M. caroli* satellite have four single-base changes and a 19-bp deletion with respect to monomers of the 79-bp satellite (Fig. 2A). Neither satellite shows significant sequence similarity to any entry in the EMBL DNA database, although there is some similarity between the 60- and 79-bp *M. caroli* satellites and the 120-bp *M. musculus* minor satellite (Fig. 2A). Southern analysis revealed that the *M. caroli* doublet cross-hybridizes to cloned *M. musculus* minor satellite under low-stringency conditions (washing in 5× SSC, room temperature; data not shown), which is consistent with this sequence similarity.

Physical organization of the 60- and 79-bp *M. caroli* satellites determined by using family-specific oligonucleotide probes. To analyze the organization of the *M. caroli* satellites further, we designed oligonucleotide probes which distinguish between the 60- and 79-bp satellites (Fig. 2B). Oligonucleotide R334 is predicted to hybridize only to the 79-bp satellite, oligonucleotide R335 is predicted to hybridize specifically to the 60-bp satellite, and oligonucleotide R336 is predicted to hybridize to both satellites. Southern analysis indicated that these oligonucleotides hybridized to plasmid clones containing the 60- or 79-bp *M. caroli* satellite as predicted and with a high degree of specificity (data not shown).

To determine whether the 60- and 79-bp satellites are arranged as tandem repeat arrays and, if so, to what extent they are interspersed with each other, Southern analysis of *Rsa*I partial digests of *M. caroli* genomic DNA was performed. As shown in Fig. 1, oligonucleotide R334, which detects the 79-bp satellite, hybridizes to a ladder of bands which are multiples of 79 bp, while R335, which detects the 60-bp satellite, hybridizes to a ladder of fragments which are multiples of 60 bp. As expected, oligonucleotide R336, which hybridizes to both satellites, produces a signal that is a composite of the two individual patterns. This shows that the 60- and 79-bp satellites are arranged as tandem repeat arrays, as was initially suggested by the cloned sequences circularly permuting around the *Rsa*I and *Dde*I sites (Fig. 2). The observation that R335 and R334 detect ladders which are multiples of 60 and 79 bp, respectively, also argues that there is little local interspersion of the two satellites, with the majority of 60-bp satellite monomers being adjacent to another 60-bp satellite monomer, and most 79-bp satellite monomers being adjacent to another 79-bp satellite monomer. At a local level, by analysis of domains approximately 1 kb in size, the two *M. caroli* satellites appear organized as homogeneous tandem repeat arrays, with little if any interspersion of 60- and 79-bp satellite monomers.

To determine the extent of these tandem repeat arrays,

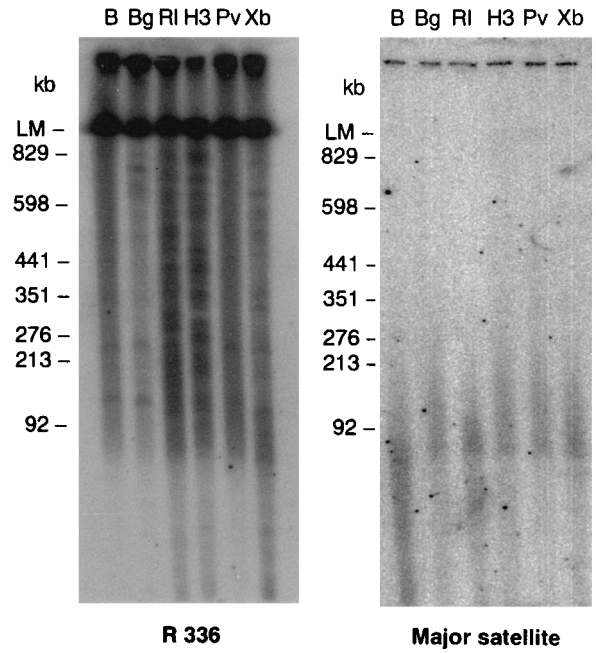

FIG. 3. Long-range physical organization of *M. caroli* satellite sequences. Shown is Southern analysis of *M. caroli* genomic DNA digested with *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (RI), *Hin*dIII (H3), *Pvu*II (Pv), and *Xba*I (Xb) and separated by pulsed-field gel electrophoresis. The probes used were oligonucleotide R336, which detects both the 60- and 79-bp *M. caroli* satellites, and cloned *M. musculus* major satellite. These images were obtained by using a Molecular Dynamics PhosphorImager with ImageQuant and Adobe Photoshop software. Size markers are based on *S. cerevisiae* chromosomes. LM, limit mobility DNA.

Southern analysis was performed on *M. caroli* genomic DNA digested with restriction enzymes such as *Bam*HI, which are predicted not to have a recognition sequence within either of the two satellites (Fig. 2A). Following separation by pulsed-field gel electrophoresis, the majority of the R336-hybridizing signal was found at the position of limit mobility DNA (Fig. 3). This result indicates that the majority of these two *M. caroli* satellites are present on restriction fragments greater than 1 Mb in size. This finding, together with the partial digest data (Fig. 1), argues that the 60- and 79-bp *M. caroli* satellites are found as tandem repeat arrays which are at least 1 Mb in size and are largely uninterrupted by nonsatellite sequences. An analogous sequence organization has been argued for repetitive DNA families such as α-satellite and *M. musculus* major satellite (49).

Chromosomal distribution and organization of the 60- and 79-bp satellites. Oligonucleotides 204, R334, R335, and R336 were used to determine the physical organization of the 60- and 79-bp *M. caroli* satellites and the major satellite sequences by PRINS to metaphase *M. caroli* chromosomes. Figure 4 shows results of PRINS using methanol-acetic acid-fixed *M. caroli* chromosomes. With oligonucleotide 204 (corresponding to the *M. musculus* major satellite), the X chromosome and all autosomes are labelled (Fig. 4A). Like the case for *M. musculus*, however, the *M. caroli* Y chromosome contains no detectable sequences homologous to the *M. musculus* major satellite (Fig. 4A, arrowhead). In *M. caroli*, the sequences homologous to oligonucleotide 204 localize to the boundary between the centromeric heterochromatin and the euchromatin of the chromosome arms, with most of the centromeric domain unlabelled. Southern analysis also detected a signal when *M. caroli* DNA was hybridized with a *M. musculus* major satellite probe (Fig. 3), which is in agreement with previous studies (9, 12, 36, 38, 43). The major satellite signal is distinct from that produced by R336 (Fig. 3) and thus does not reflect cross-hybridization of the *M. musculus* major satellite probe to the

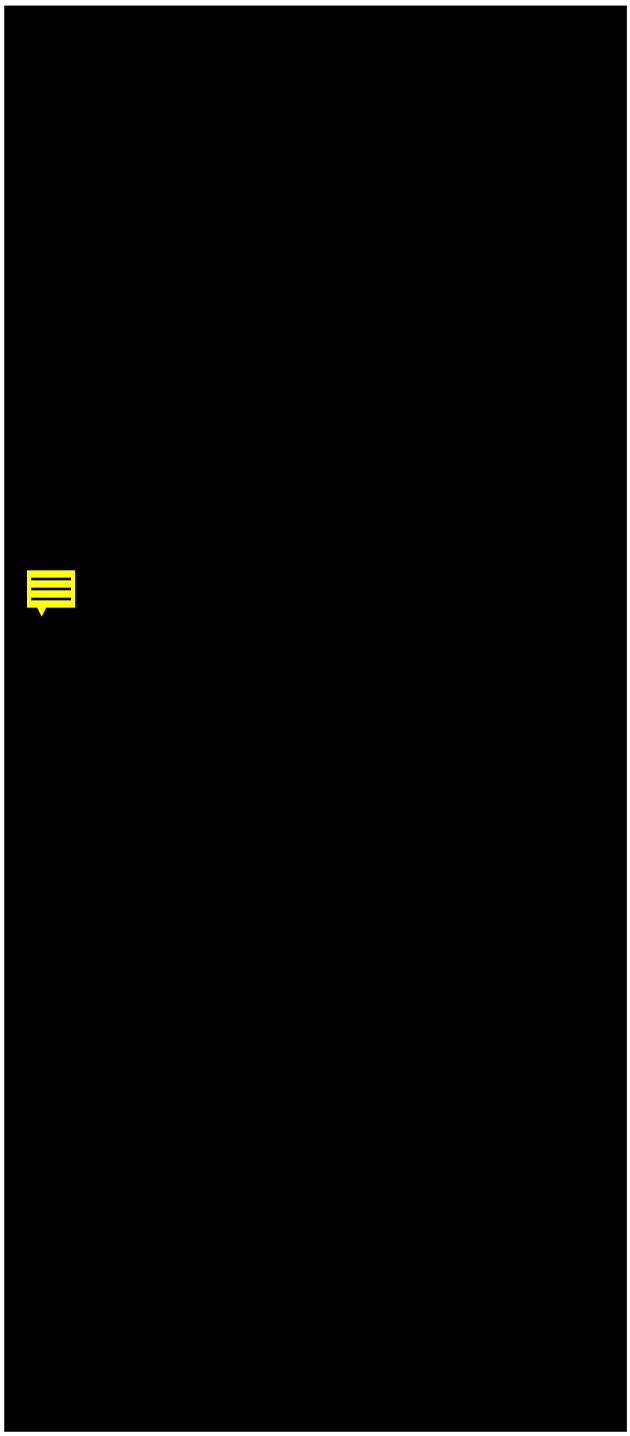

FIG. 4. PRINS to methanol-acetic acid (3:1)-fixed *M. caroli* chromosomes, using oligonucleotides 204 (A), R334 (B), and R335 (C). The Y (arrowhead) and X (arrow) chromosomes are indicated. Note the weak signal to the Y chromosome with R335 (C) and the apparent lack of signal with either R334 or R335 to the X chromosome. All images are merged, and the counterstain is propidium iodide.

60- and 79-bp *M. caroli* satellites, which is consistent with the in situ data. It is not clear whether the major satellite signal reflects a low-copy-number *M. caroli* sequence family which is very similar to the *M. musculus* major satellite or cross-hybridization to a more abundant but diverged DNA sequence family. The major satellite in situ signal is relatively weak and is restricted to a small domain, consistent with this sequence family being at lower copy number in the *M. caroli* genome than either of the *M. caroli* satellites.

Figures 4B and C show PRINS results with oligonucleotides R334 and R335, which correspond to the 79- and 60-bp *M. caroli* satellites, respectively. With R334, only the autosomes are labelled. However, with R335, the *M. caroli* Y chromosome shows a weak but clearly positive signal on every metaphase. The *M. caroli* X chromosome remains unlabelled with both R334 and R335. For the autosomes, both R334 and R335 produce identical in situ signals which decorate the entire pericentromeric heterochromatin domain of each chromosome. Thus, at the cytological level, the 60- and 79-bp satellites appear interspersed with each other, although further work is required to address this point given the limited resolution of in situ hybridization to metaphase chromosomes. If the satellites are interspersed, the partial digest data (Fig. 1) suggest that most arrays are homogeneous for domains of at least 1 kb in size.

Southern analysis using the cloned 60- and 79-bp *M. caroli* satellite monomers as probes detected little signal in either male *M. musculus* or *M. spretus* genomic DNA (data not shown), a result illustrated by in situ hybridization in Fig. 8c. This finding argues that the 60- and 79-bp *M. caroli* satellites do not constitute a major repetitive sequence family in either *M. musculus* or *M. spretus*, and in particular we have been unable to detect an array of the 60- and 79-bp *M. caroli* satellites at the *M. musculus* Y chromosome centromere (data not shown). The sequence of the Y centromere remains completely unknown; no cloned repetitive sequence which maps to this centromere has been identified.

In agreement with earlier studies (12, 38), no significant signal was detected when Southern filters of *M. caroli* genomic DNA were probed with cloned *M. musculus* minor satellite under stringent conditions (data not shown). Similarly, *M. musculus* minor satellite sequences could not be detected on *M. caroli* chromosomes by PRINS using either oligonucleotide C86 (canonical 17-bp CENP-B box) or oligonucleotide 203 (data not shown).

Location of the centromere in *M. caroli*. The centromeres of *M. caroli* chromosomes react positively with CREST anticentromere serum (Fig. 5B), giving a characteristic double-dot signal within the pericentromeric heterochromatin. This is the domain labelled by both the R334 and R335 in situ signals. Thus, centromere proteins appear to assemble at a site that is, at the limited level of resolution provided by cytogenetic analysis, coincident with the location of the *M. caroli* 60- and 79-bp satellites and adjacent to the domain containing the major satellite. However, it was not possible to detect a positive signal by using a rabbit polyclonal serum raised against bacterially expressed human CENP-B (Fig. 5D). In contrast, *M. musculus* chromosomes gave positive signals with both CREST (Fig. 6A) and CENP-B (Fig. 6B) antisera, although the latter is much weaker. Western blot (immunoblot) analyses (Fig. 7) largely support these results. CREST (Fig. 7, lane 1) detects an 80-kDa polypeptide in a *M. musculus* protein extract (arrow) that is not detected by normal human serum (lane 4). Although variation in the number of bands detected can occur, the CENP-B polyclonal antiserum reproducibly detects an 80-kDa band (lanes 2 and 5) not seen with preimmune serum (lane 3). Similar results have been reported by Sullivan and Glass (45). In contrast, no specific 80-kDa band could be confirmed with a similar extract of *M. caroli* proteins by using either the CREST (lane 9) or the CENP-B (lane 8) antiserum. The CENP-B antiserum used was raised against human CENP-B and produces a relatively weak reaction with *M. musculus* CENP-B (Fig. 6B), possibly reflecting reduced reactivity in cross-species experiments. Given the weak signal even with *M. musculus*, one explanation for the apparent lack of CENP-B signal in *M. caroli* is reduced amounts of CENP-B in this species, resulting in levels of bound protein below that which can be detected with this antiserum. Other possibilities include qualitative changes in the *M. caroli* protein, such as altered epitopes caused by gene polymorphism or differences in posttranslational modification, thus making the *M. caroli* protein undetectable by either serum. The CENP-B gene is highly conserved between mice and humans (45) and can be detected in *M. caroli* genomic DNA by PCR (data not shown and reference 55).

To further address this question, we took advantage of an interspecific *M. caroli* × *M. musculus* cell line (7) in which the *M. musculus* chromosomes provide an internal control for detection of CENP-B (Fig. 8d). The *M. caroli* chromosomes can be unambiguously identified by using oligonucleotide R336, which recognizes both of the *M. caroli* satellites (Fig. 8c; chromosome indicated by a closed arrow in Fig. 8a). The *M. musculus* chromosomes (Fig. 8c; chromosome indicated by an open arrow in Fig. 8a) are negative with this oligonucleotide. In Fig. 8d, the chromosomes are labelled with the polyclonal CENP-B antiserum (fluorescein isothiocyanate), demonstrating that in this hybrid cell line, chromosomes of both species give a positive signal with the CENP-B antiserum. The merged image (Fig. 8a) demonstrates that CENP-B binds to a site within the heterochromatic domain formed by the *M. caroli* satellites. From these data, we conclude that in a cell expressing detectable amounts of the protein, mouse CENP-B is able to bind to *M. caroli* centromeres.

A CENP-B box in the 79-bp *M. caroli* satellite. There is no detectable minor satellite in the *M. caroli* genome (see above). Furthermore, neither oligonucleotide C86 (which corresponds to the canonical 17-bp CENP-B binding site) nor oligonucleotide 203 (which corresponds to positions 1 to 35 of the minor satellite) detects anything in the *M. caroli* genome when used as a hybridization probe for either Southern analysis or PRINS (data not shown). In contrast, the canonical CENP-B box oligonucleotide hybridizes to *M. musculus* centromeres (30). The observation that *M. caroli* chromosomes are able to bind mouse CENP-B therefore came as a surprise.

Neither of the two *M. caroli* satellites contains a perfect match to the 17-bp *M. musculus* CENP-B box, although there is a 10-of-17-base match to the canonical CENP-B box in the 79-bp *M. caroli* satellite, underlined in Fig. 2A. The alignment shown in Fig. 2A, which maximizes the similarity between the two sequence families, does not align the 17-bp *M. caroli* motif with the *M. musculus* CENP-B box, and indeed the minor satellite is quite different in sequence in the region corresponding to the *M. caroli* motif (Fig. 2A). A full 17-bp *M. caroli* motif is not found anywhere in the minor satellite, and the *M. caroli* motif is located in a different local sequence environment than the CENP-B binding site in the minor satellite. The match between the two motifs is shown in Fig. 2C.

Masumoto et al. (28) have determined a consensus sequence for CENP-B binding on the basis of an in vitro assay. They identified nine positions in the canonical 17-bp CENP-B box where mutations caused significant loss of protein binding (Fig. 2C). In contrast, single-base changes in the remaining positions

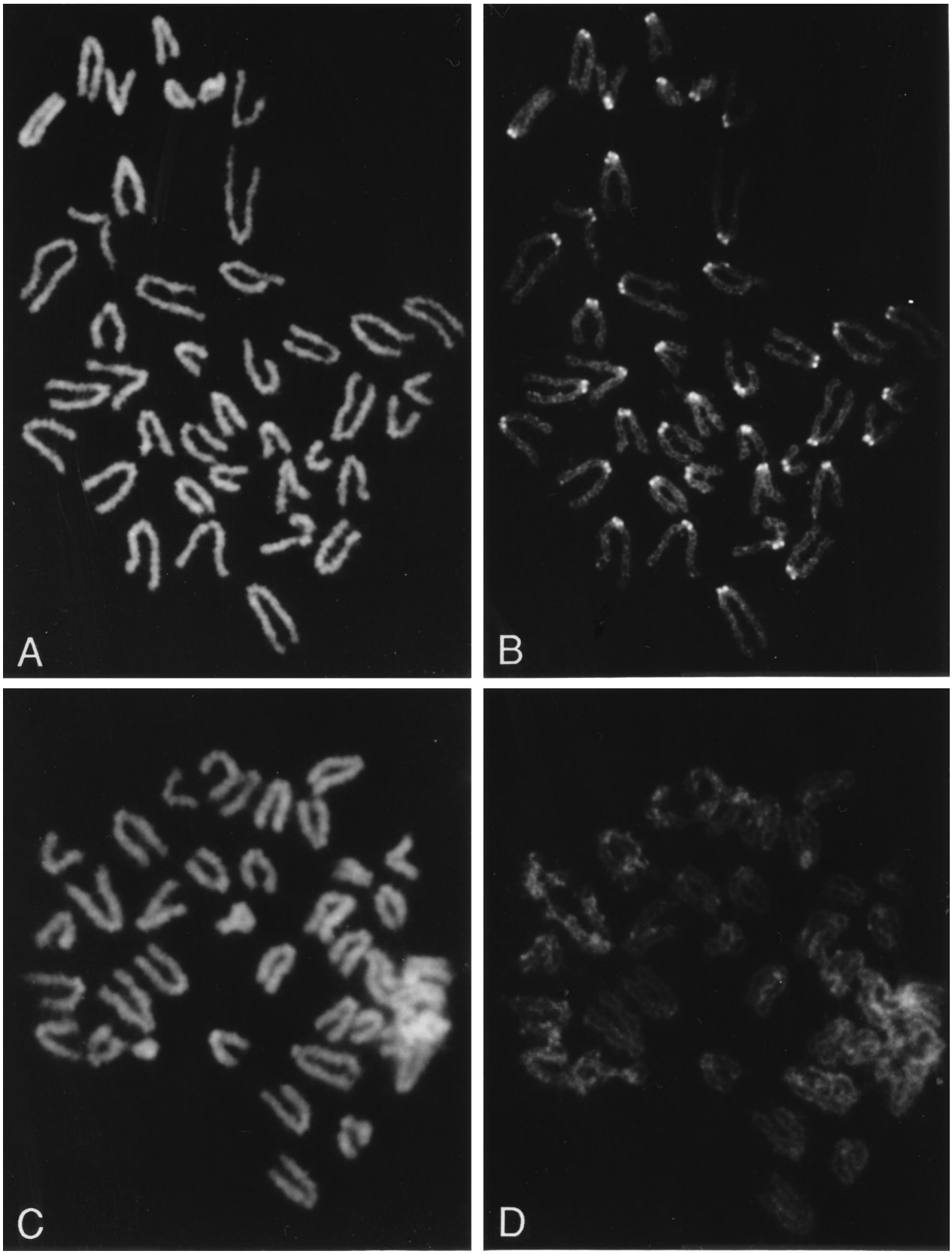

FIG. 5. Cytospin preparations of native *M. caroli* chromosomes. Shown are spreads stained with CREST antiserum (B), spreads stained with rabbit CENP-B antiserum (D), and the same spreads stained with DAPI (A and C).

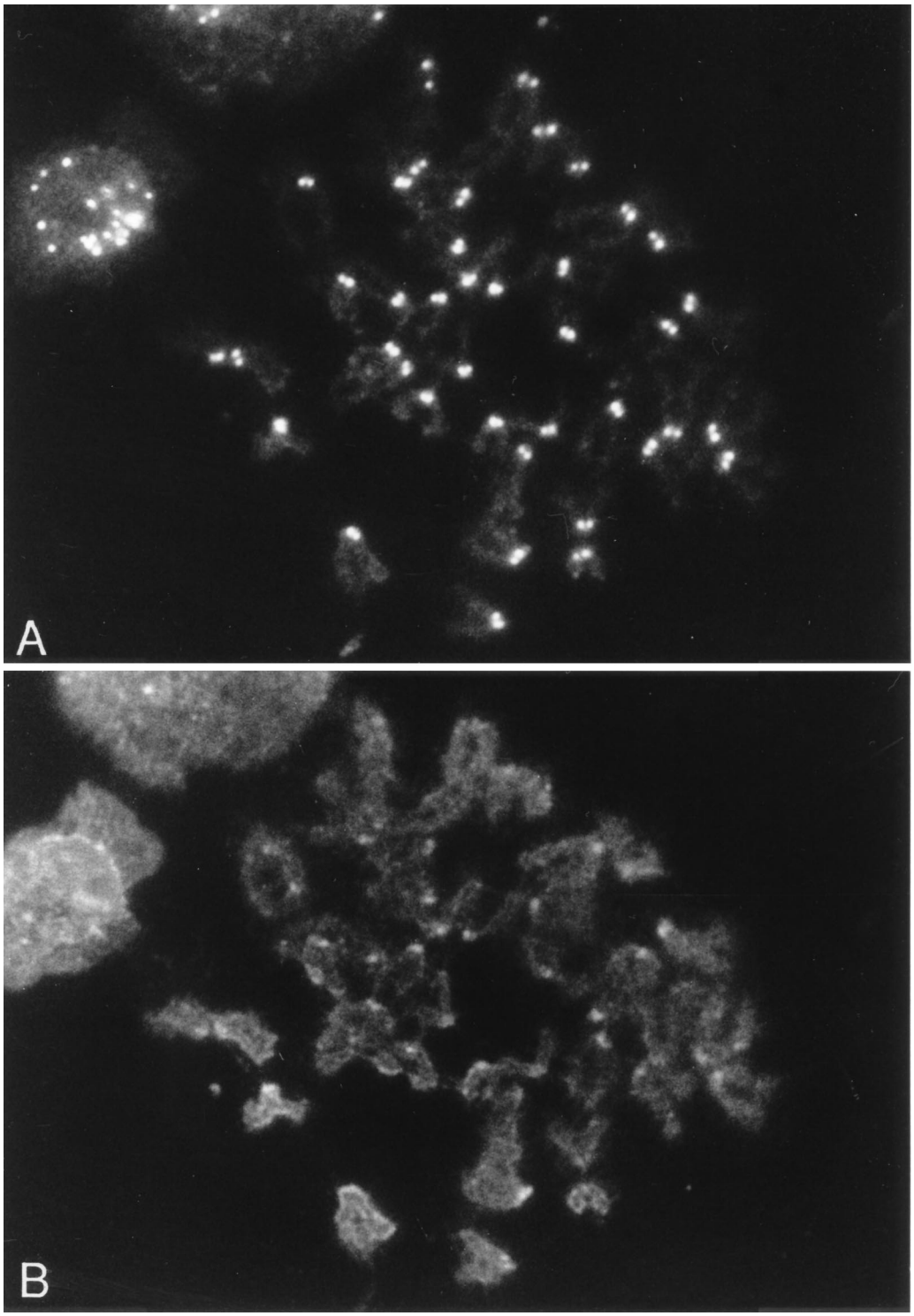

FIG. 6. Cytospin preparations of native *M. musculus* chromosomes stained with CREST antiserum (A) and rabbit CENP-B antiserum (B).

had little or no effect on in vitro binding (28). All of these nine conserved bases are present with the correct spacing in the 79-bp *M. caroli* satellite (Fig. 2C). We therefore speculated that this sequence motif within the 79-bp *M. caroli* satellite is a variant CENP-B binding site.

To test this possibility, interactions between the *M. caroli* motif and the CENP-B protein were analyzed in a gel mobility shift assay. The N- and C-terminal regions of CENP-B involved in specific DNA binding and dimer formation, respectively, are completely conserved between human and mouse genomes (24, 45). We therefore used CENP-B extracted from HeLa cell nuclear extracts in the assay. When a ^{32}P-labelled

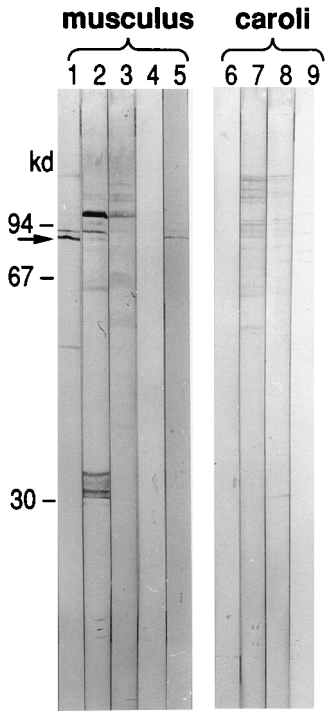

FIG. 7. Western blot of CENP-B antiserum to *M. musculus* and *M. caroli* protein extracts, using anticentromere autoantibody serum CP (lanes 1 and 9), control normal human serum JL (lanes 4 and 6), control rabbit preimmune serum (lanes 3 and 7), and rabbit polyclonal CENP-B antiserum (lanes 2, 5, and 8). Lanes 2 and 5 are from separate experiments.

oligonucleotide probe containing the *M. caroli* motif (CB30k/l) was incubated with HeLa CENP-B and electrophoresed under native conditions, a shifted band with a strong intensity was observed (Fig. 9A, lane 1, position d) at a position corresponding to that typical for the CENP-B dimer complex (Fig. 9B) (24), and a weaker monomer complex was also observed (Fig. 9A, lane 1, position m). These complexes disappeared upon competition with excess unlabelled oligonucleotides corresponding to either the *M. caroli* motif (CB30k/l; lane 2) or the human CENP-B box (CB30a/b; lane 3). In contrast, these complexes were not abolished by an oligonucleotide pair corresponding to the human CENP-B box but containing two point mutations which abolish its ability to bind human CENP-B (CB30c/d; lane 4). The bands were supershifted by incubation with an anti-CENP-B polyclonal antibody raised against the N-terminal DNA-binding domain (lane 5) (24) and were trapped at the gel origin with human serum containing anticentromere antibodies (lane 6). No such supershift was observed with either normal human serum (lane 7) or preimmune rabbit serum (lane 8). The band seen at a position between the monomer complex and the free DNA appears to be nonspecific; it was neither abolished with any of the competitors nor supershifted by any of the sera.

^{32}P-labelled CB30a/b (human CENP-B box) was used for a similar gel mobility shift assay (Fig. 9B). Increasing amounts of unlabelled CB30a/b (Fig. 9B, lanes 2 to 4) or CB30k/l (lane 5 to 7) were added to the mixture. The human CENP-B box and the *M. caroli* motif caused similar reductions in the intensity of the shifted bands. In contrast, the mutant CENP-B box pair CB30c/d (lane 8 to 10) was unable to abolish the complexes. In a similar competition experiment, excess amounts of plasmid R398, which contains the 79-bp satellite, caused the complete disappearance of the shifted bands (Fig. 9C, lane 2), whereas plasmid R397, which contains 60-bp satellite, had no effect (lane 3).

These results clearly indicate that the 17-bp motif in the 79-bp *M. caroli* satellite is a variant but functional CENP-B binding site and that it is similar in CENP-B binding affinity to the human or *M. musculus* CENP-B box. This finding strongly suggests that the binding of CENP-B to *M. caroli* centromeres in vivo (Fig. 8) reflects CENP-B binding to the 79-bp *M. caroli* satellite.

DISCUSSION

The sequences and physical organization of two novel centromeric satellite DNAs from the Asian mouse *M. caroli* are presented. These satellites are organized as tandem repeat arrays of either a 60- or a 79-bp monomer, with little interspersion of the two monomer sequences. They are highly abundant in the *M. caroli* genome, with most arrays being over 1 Mb in size as determined by pulsed-field gel electrophoresis. These satellites are not found in *M. musculus*. Sequences homologous to *M. musculus* major satellite DNA are present, albeit in a restricted area, within the centromeric chromatin of *M. caroli* chromosomes. They are found at the boundary between the heterochromatin and the euchromatin of the chromosome arm, a position similar to that found in *M. spretus* (unpublished data). The major satellite may be one of the oldest repeated DNA families within the genus *Mus*. Unlike *M. musculus*, in which the sex chromosomes contain no satellite DNAs, all of the chromosomes in the *M. caroli* genome contain at least one satellite DNA family.

Although there is no detectable CENP-B bound to *M. caroli* chromosomes isolated from an animal, it is possible to show that this protein can be present at *M. caroli* centromeres by using an *M. musculus* × *M. caroli* interspecific cell line in which this protein can be detected with a polyclonal serum. The 79-bp *M. caroli* satellite contains within its sequence a 17-bp motif which contains all nine base positions defined by Masumoto et al. (26a, 28) as essential for CENP-B binding in vitro. The specific DNA-binding and dimerization domains of CENP-B are conserved between mouse and human genomes (45), and we demonstrate that CENP-B isolated from HeLa cells binds this *M. caroli* motif in vitro with an affinity similar to that for the interaction between CENP-B and the canonical human or *M. musculus* CENP-B box. This *M. caroli* motif is therefore a variant but functional CENP-B binding site. We suggest that the ability of murine CENP-B to bind *M. caroli* centromeres reflects binding to this motif in the 79-bp *M. caroli* satellite.

It is not clear why we have been unable to detect CENP-B in cells from *M. caroli* animals. Copies of the CENP-B gene are present in the *M. caroli* genome (data not shown and reference 55). The CENP-B protein is highly conserved between human and mouse genomes, with 92% identity at the protein level (45), and antiserum raised against human CENP-B is able to detect the *M. musculus* protein (Fig. 6 and 7). However, such a CENP-B antiserum gives a weak signal even on *M. musculus* chromosomes (Fig. 6), possibly reflecting the antibody being raised against the human version of the protein. A modest relative reduction in the amount of CENP-B bound to *M. caroli* centromeres compared with *M. musculus* centromeres may therefore be sufficient to cause it to be below the detection limit of this serum. If so, the ability to detect CENP-B at *M. caroli* centromeres in the hybrid cell line might simply reflect an overall increase in the amount of CENP-B in this cell type. Resolution of this issue will require cloning of the *M. caroli*

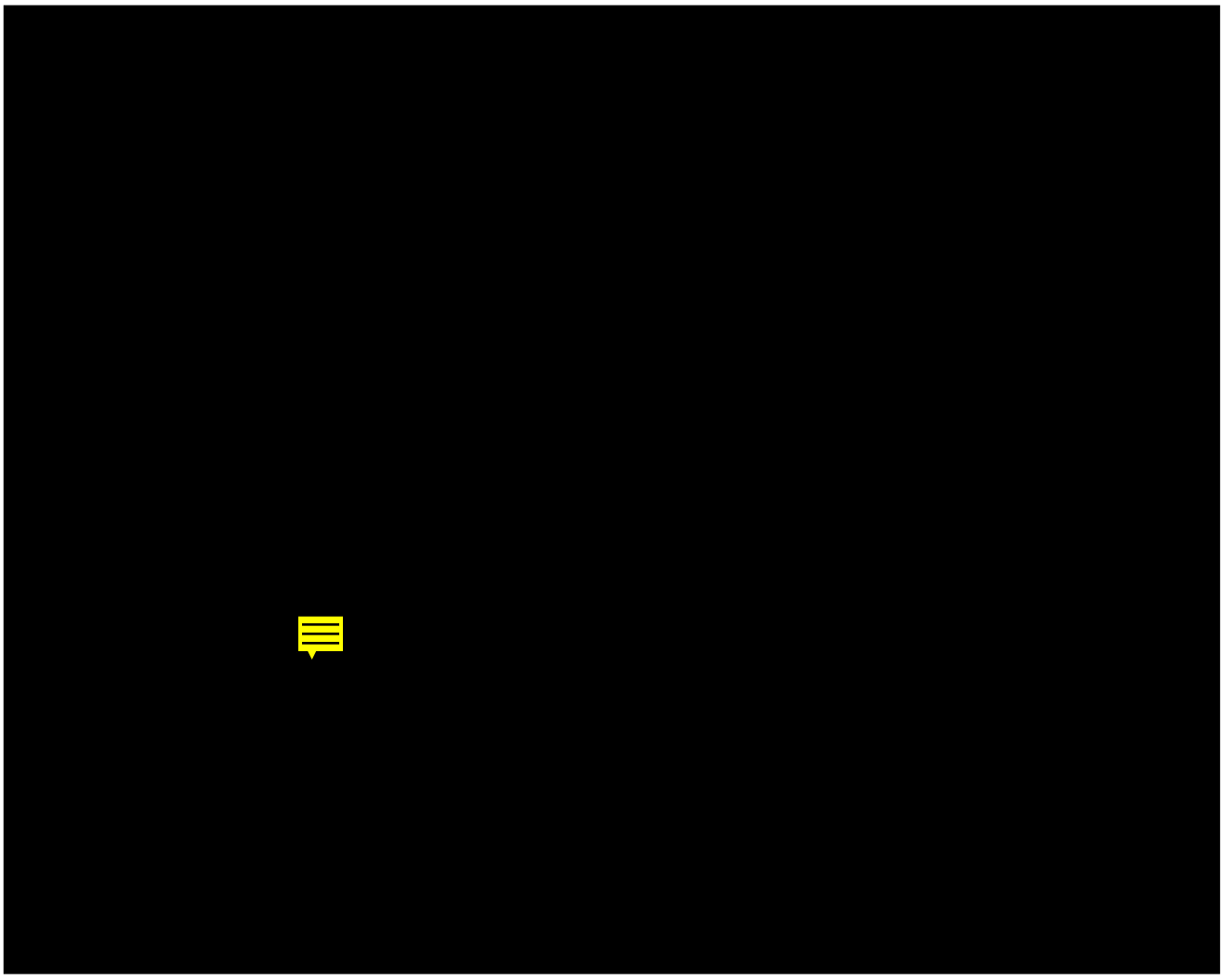

FIG. 8. Combined immunofluorescence and PRINS to native, cytospin preparations of chromosomes from an *M. musculus* × *M. caroli* interspecific hybrid cell line. (a) Merged image of panels b to d; (b) DAPI counterstain; (c) PRINS using oligonucleotide R336 (Texas red); (d) immunofluorescence using CENP-B antiserum (fluorescein isothiocyanate). *M. caroli* and *M. musculus* chromosomes are indicated by closed and open arrows, respectively.

CENP-B gene and production of antibodies against the expressed polypeptide.

The sequence organization of mammalian centromeres presents a paradox (4). Fully functional *S. cerevisiae* centromeres can be as small as 125 bp (16) and bind a single spindle microtubule (4). Mammalian centromeres bind only 20 to 30 microtubules yet are characterized by many hundreds of kilobases of repetitive sequences. If the human α-satellite and mouse minor satellite DNAs are functionally important for microtubule attachment, then at first sight there appears to be a massive excess of centromeric sequences for this particular function in mammals. An alternative hypothesis is that these mammalian repetitive DNA sequences have only an auxiliary role in centromere function, with other (as yet unidentified) DNA sequences being more closely involved in microtubule attachment via their interacting proteins. It should be emphasized that no mammalian centromere has yet been fully sequenced. The suggestion that α-satellite and minor satellite arrays are largely devoid of other sequences (48) is based mainly on physical maps which indicate a paucity of restriction enzyme sites within these arrays. Although it seems clear that these arrays are not generally interrupted by large domains of nonsatellite sequences, such analyses would not be able to detect relatively small amounts within the arrays of other sequence elements which might have functional importance. In this case, it would be possible to speculate that the α-satellite and minor satellite DNAs play a secondary role, such as providing an appropriate spacing between these other DNA sequence elements. Another possibility, based on the observation that transcription across an *S. cerevisiae* centromere can render it inactive (17), is that these satellites might contribute to centromere function by forming a domain of transcriptionally inactive heterochromatin.

Because *M. caroli* centromeres do not contain copies of the 17-bp CENP-B binding site motif that is found in the *M. musculus* minor satellite, analysis of *M. caroli* seemed to present a strong argument against a simple model whereby human α-satellite and mouse minor satellite DNAs have a role in centromere function by providing an array of potential binding sites for CENP-B. However, here we demonstrate that *M. caroli* centromeres are able to bind mouse CENP-B and demonstrate that this is due to a variant form of the canonical

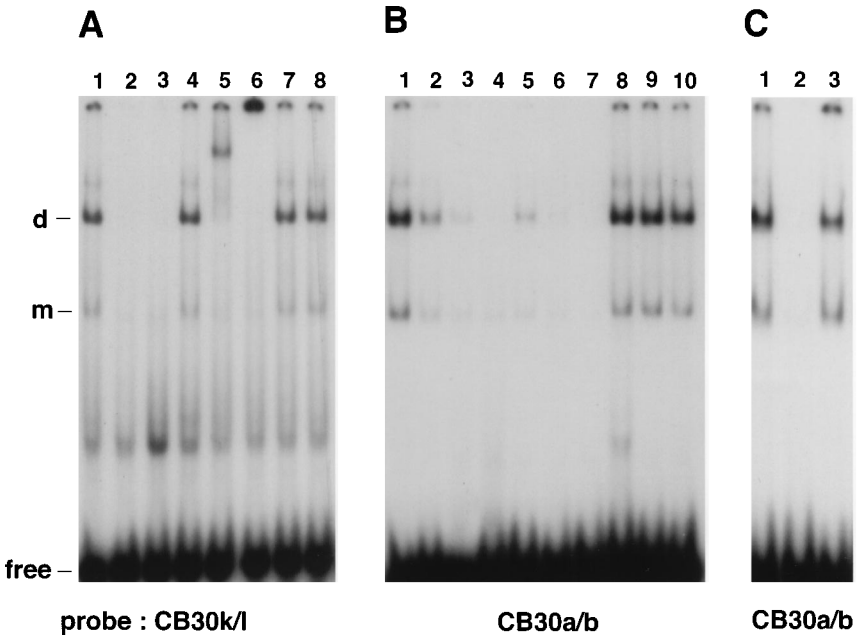

FIG. 9. Gel mobility shift analysis of complexes formed between the *M. caroli* motif and CENP-B. (A) DNA-binding reactions were carried out with end-labelled CB30k/l containing the *M. caroli* motif and a 0.5 M NaCl nuclear extract from HeLa cells (lane 1 to 8). One-hundred-fold excess amounts of unlabelled CB30k/l (lane 2), CB30a/b (lane 3), and CB30c/d (lane 4) compared with the amount of end-labelled CB30k/l were used to supplement the mixture; 0.1 μl of anti-CENP-B antibody (BN1; lane 5), anticentromere autoantibody (lane 6), normal human serum (lane 7), or preimmune rabbit serum (lane 8) was added to the reaction mixture. (B) DNA binding reactions carried out with end-labelled CB30a/b and 0.5 M NaCl nuclear extract. Excess amounts of unlabelled CB30a/b (0-, 5-, 25-, and 125-fold excess; lanes 1 to 4), CB30k/l (5-, 25-, and 125-fold excess; lanes 5 to 7), and CB30c/d (5-, 25-, and 125-fold excess; lanes 8 to 10) compared with the amount of end-labelled CB30a/b were used. (C) Fiftyfold molar excess amounts of plasmid R398 (lane 2), plasmid R397 (lane 3), and no plasmid (lane 1) compared with the molar amount of end-labelled CB30a/b were added to the binding mixture. d and m, the CENP-B dimer and monomer complexes, respectively; free, the position of unbound probe DNA.

CENP-B binding site that is present in the 79-bp *M. caroli* satellite, data which also contribute to defining the specific sequences involved in CENP-B binding. The observation that three largely unrelated centromeric sequences (α-satellite, minor satellite, and 79-bp *M. caroli* satellite) are all capable of binding the CENP-B protein strengthens the argument that this protein has a role in centromere function. Whether it is directly involved in promoting kinetochore formation or has some other function, such as formation of the altered chromosome structure characteristic of these regions of the mammalian genome, is currently under study.

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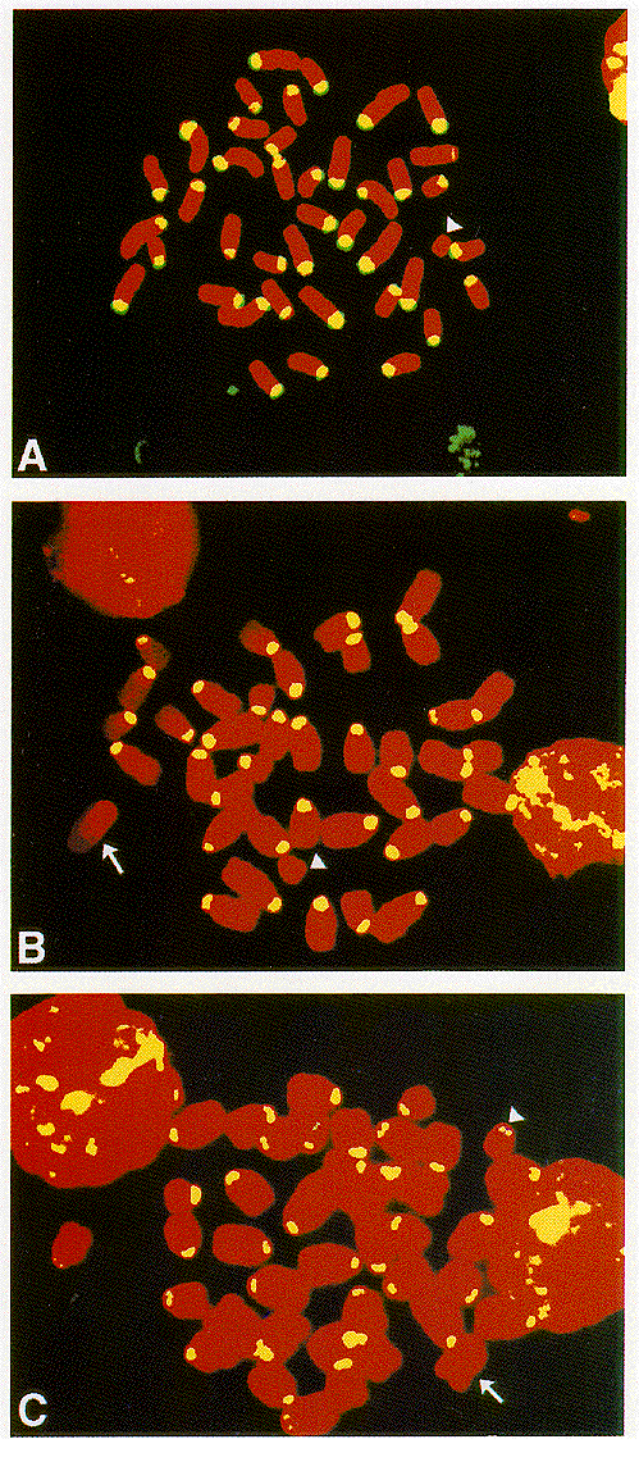
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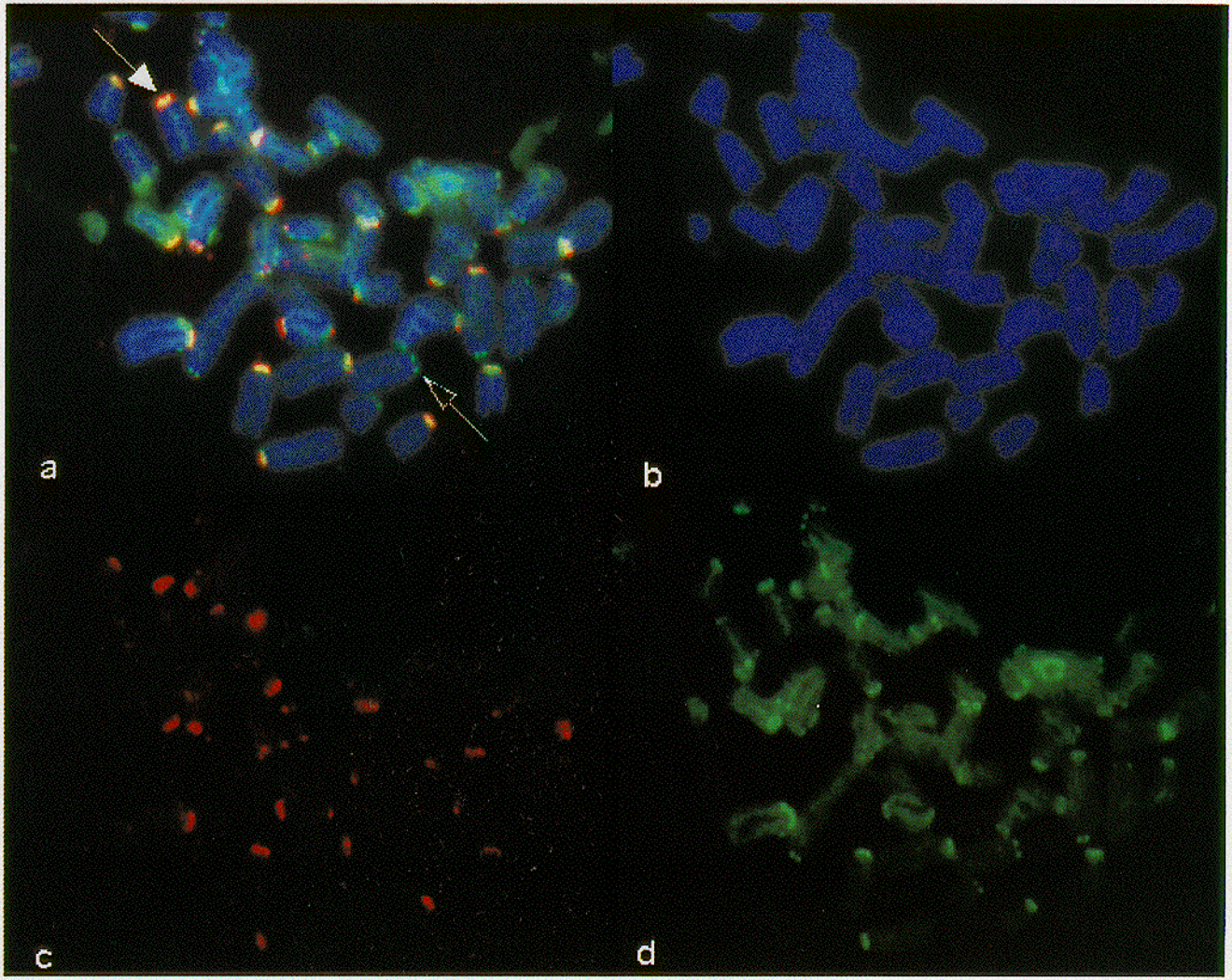
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