

Novel Meiosis-Specific Isoform of Mammalian SMC1

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Structural maintenance of chromosomes (SMC) proteins fulfill pivotal roles in chromosome dynamics. In yeast, the SMC1-SMC3 heterodimer is required for meiotic sister chromatid cohesion and DNA recombination. Little is known, however, about mammalian SMC proteins in meiotic cells. We have identified a novel SMC protein (SMC1 β), which—except for a unique, basic, DNA binding C-terminal motif—is highly homologous to SMC1 (which may now be called SMC1 α) and is not present in the yeast genome. SMC1 β is specifically expressed in testes and coimmunoprecipitates with SMC3 from testis nuclear extracts, but not from a variety of somatic cells. This establishes for mammalian cells the concept of cell-type- and tissue-specific SMC protein isoforms. Analysis of testis sections and chromosome spreads of various stages of meiosis revealed localization of SMC1 β along the axial elements of synaptonemal complexes in prophase I. Most SMC1 β dissociates from the chromosome arms in late-pachytene-diplotene cells. However, SMC1 β , but not SMC1 α , remains chromatin associated at the centromeres up to metaphase II. Thus, SMC1 β and not SMC1 α is likely involved in maintaining cohesion between sister centromeres until anaphase II.

The eukaryotic, evolutionarily highly conserved SMC (Structural Maintenance of Chromosomes) proteins are involved in several key DNA and chromatin dynamic processes (for recent reviews, see references 11, 21, 26, 27, 31, 48, 60, and 62). The best-documented processes are chromosome condensation and sister chromatid cohesion. Evidence is also accumulating for a function in DNA recombination and repair. A fourth role of SMC proteins is in gene dosage compensation in *Caenorhabditis elegans*. The phylogenetic tree comprises five subfamilies (32): SMC1 to SMC4 and an ancestral family that includes the recently defined SMC5 and SMC6 groups with the Rad18 and Spr18 proteins of *Schizosaccharomyces pombe* (16), which act in recombinational repair.

SMC proteins share a characteristic design. Coiled-coil domains are flanked by globular N- and C-terminal domains and are divided in the central region by a flexible hinge domain of about 150 aa. The N- and C-terminal domains of about 100 to 150 aa are highly conserved and carry important motifs. The N-terminal domain includes an NTP binding motif (Walker A box [68]), which has been shown to bind the ATP analogue azido-ATP (1). The C-terminal domain contains a DA box (68). The C-terminal and second coiled-coil domains, but not the N terminus, bind DNA (1, 2). It has been proposed that the antiparallel, heterodimeric SMC1-SMC3 protein with an N and C terminus at each end may connect two DNA molecules, such as sister chromatids, and may directly contribute to their alignment in cohesion and to recombination between sister chromatids (2, 26, 62).

In eukaryotes the SMC1-SMC3 or SMC2-SMC4 heterodimers form large multiprotein complexes. One of these complexes is condensin, which, besides the SMC2-SMC4 heterodimer, contains several non-SMC subunits. Condensin is necessary for mitotic chromosome condensation in *Saccharo-*

myces cerevisiae (61), *S. pombe* (64), and *Xenopus laevis* egg extracts (25) and has also been described in human cells (57). The MIX-1 and DPY-27 proteins of *C. elegans*, proteins homologous to SMC2 and SMC4, are present in a different multiprotein complex, which regulates gene dosage compensation on the X chromosomes of the hermaphrodite nematode (8, 39).

The other pair of SMC proteins, SMC1 and SMC3, is present in at least two protein complexes with distinct, albeit partially connected, functions. Genetic studies of *S. cerevisiae* revealed a requirement for Smc1p and Smc3p in mitotic sister chromatid cohesion (19, 45). The respective protein complex is called cohesin, contains two other polypeptides besides Smc1p and Smc3p, and interacts with several other factors required for sister chromatid cohesion and its release (reviewed in references 11, 48, and 62). One of the non-SMC cohesin subunits is the *S. cerevisiae* Scc1p (Mcd1p) protein, homologous to Rad21 in *S. pombe* (4, 19, 45). The *rad21-45* mutation (in *S. pombe*) also causes X-ray sensitivity and a mitotic hyperrecombination phenotype (4, 18). A similar cohesin complex was identified from *X. laevis* cell extracts, extensively characterized, and found to be required for sister chromatid cohesion in this system (41, 42). We have identified the SMC1 and SMC3 proteins as constituents of the mammalian recombination complex RC-1, which is present in a variety of somatic cells (29, 30, 63). This complex catalyzes SMC protein-dependent cell-free transfer of duplex DNA molecules, which mimics recombinational repair of gaps and deletions (29, 30). The presence of the SMC1 and SMC3 proteins in these multiprotein complexes furthered speculations about an SMC-mediated functional link between sister chromatid cohesion and recombinational repair (26, 31, 62). Recent evidence from studies of yeast supports this concept. Klein et al. (36) reported that *S. cerevisiae* Smc3p is required not only for meiotic sister chromatid cohesion, but also for meiotic DNA recombination.

Sister chromatid cohesion and DNA recombination are both essential for meiosis (for reviews, see references 35, 54, and 66). In mitotic cells, DNA recombination is primarily a means to repair DNA damage, and the role of cohesins may include

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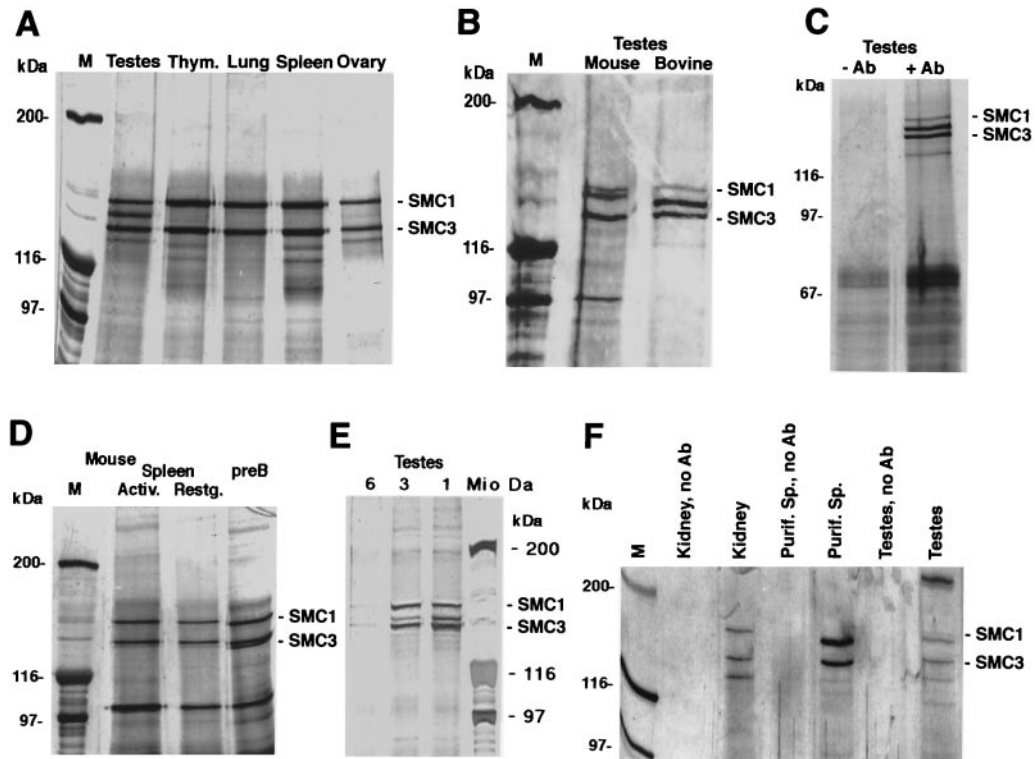


FIG. 1. Immunoprecipitation of SMC3 and associated SMC1 proteins with anti-SMC3 antibodies. (A) Immunoprecipitation from various bovine tissue nuclear extracts. Thym., thymus. (B) Immunoprecipitation from mouse and bovine testis nuclear extracts. (C) Control immunoprecipitation from bovine testis nuclear extract, with (+Ab) and without (–Ab) anti-SMC3 antibody included. (D) Immunoprecipitation from nuclear extracts prepared from actively proliferating (lipopolysaccharide-induced; Activ.) and resting (Restg.) mouse spleen cell cultures and from an actively growing mouse pre-B-cell line. (E) Immunoprecipitation from bovine testis nuclear extract gel filtration (BioGel A15m resin) fractions (20 μ g of protein each) representing 1-, 3-, and 6-MDa molecular-mass positions. (F) Immunoprecipitation from mouse kidney, rat testis, and purified rat spermatocyte (Purif. Sp.) nuclear extracts (25 μ g of protein each). Controls without antibody (no Ab) are included. All precipitates were analyzed by SDS-polyacrylamide gel electrophoresis and silver staining. M, molecular mass marker.

the direction of recombinational repair towards the sister chromatid rather than the homologous chromosome (if there is one) (33, 58). In meiosis, recombination and sister chromatid cohesion are essential, but the relationship between the two processes has been modified. Whereas meiotic recombination has to be directed towards the homologue rather than the sister chromatid, cohesion between sister chromatids has to be maintained to ensure the proper orientation and disjunction of homologues at meiosis I (66). During the meiotic prophase, a characteristic, zipperlike protein structure, the synaptonemal complex (SC), is formed between homologues and likely plays an important but not entirely clarified role in adapting recombination and cohesion for meiosis (20, 23). SCs consist of two axial elements (AEs), which are connected by numerous transverse filaments along their lengths. Each AE structurally supports the two sister chromatids of one homologue.

In budding yeast, Smc3p colocalizes with an AE component during the meiotic prophase (36). In meiotic yeast cells, the cohesin protein Scc1p (Mcd1p) is largely replaced by its meiosis-specific homologue, Rec8p, or its homologues in other organisms (36, 47, 69). In *S. cerevisiae*, Rec8p localizes along the AEs of SCs (36).

These observations also suggest for mammalian meiotic cells an association of cohesion proteins with the SC. In mammals, two AE components have been identified, SCP2 (49) and SCP3

(37), which are specifically expressed in the meiotic prophase. Recently, we have shown that mammalian SMC1 is present in meiotic nuclei throughout prophase I. Upon permeabilization of spermatocytes in the presence of Triton X-100, SMC1 is specifically retained in a dotlike pattern along the AEs of SCs. We also showed that mammalian SMC1 and SMC3 proteins associate with AE components, e.g., SCP2 and SCP3 (13). Thus, it is intriguing to hypothesize about an essential role of mammalian SMC proteins in meiotic sister chromatid cohesion—its establishment, maintenance, and resolution—and in meiotic DNA recombination. Differences in mitotic sister chromatid cohesion, however, extend beyond meiotic prophase I. Sister chromatids separate only in the chromosome arms, but not at the centromeres, during meiosis I, leaving centromeric cohesion intact until anaphase II, when all cohesion is finally removed. An important question is whether there is specific adaptation of SMC proteins and their complexes to their specific meiotic functions. The differences mentioned above in the interplay of sister chromatid cohesion and DNA recombination between meiosis and mitosis render such adaptation likely. One way to achieve this adaptation is through expression of a meiosis-specific homologue or isoform of a somatic protein.

Here, we demonstrate the existence of a meiosis-specific isoform of mammalian SMC1, named SMC1 β , which we describe in this report.

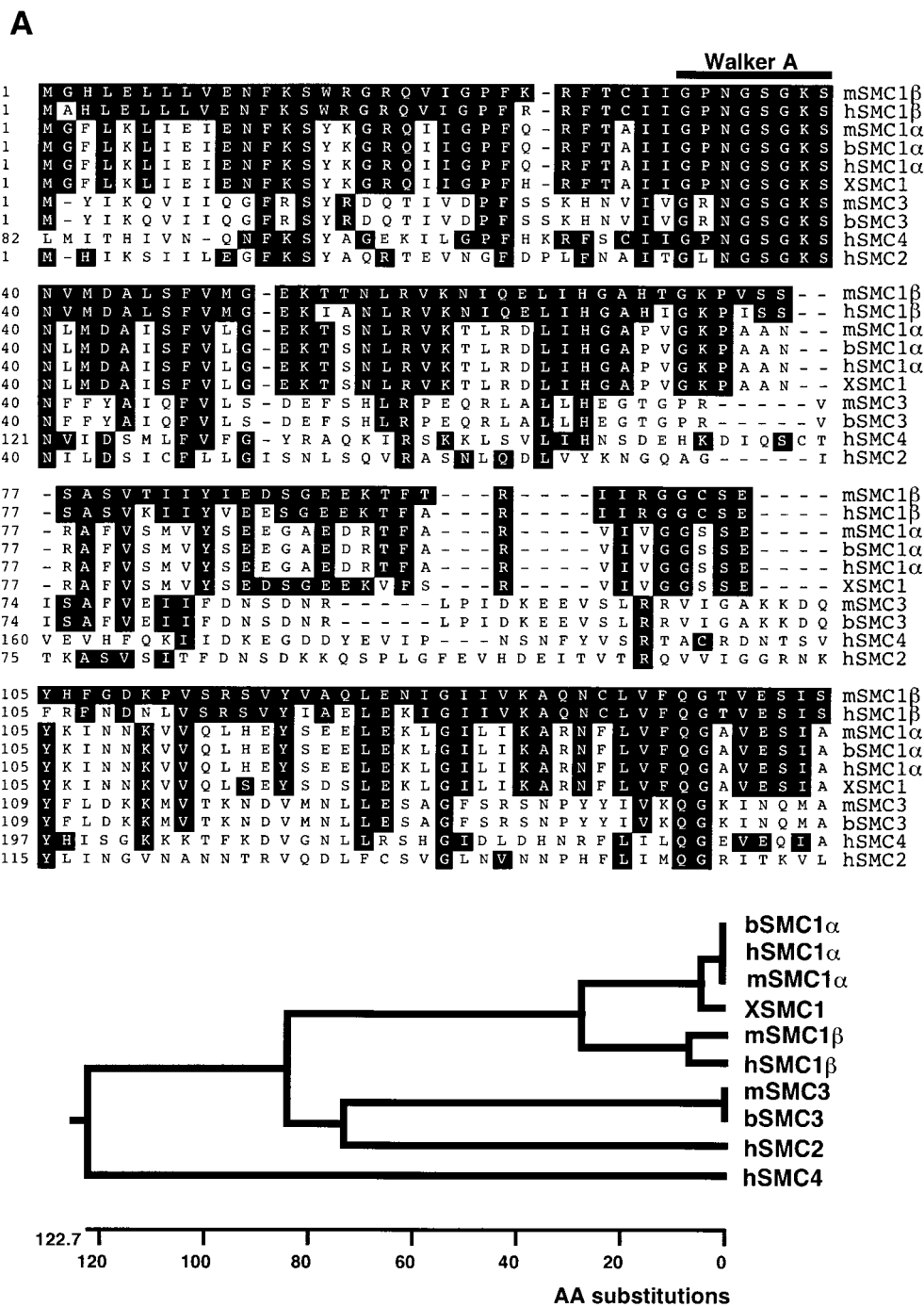


FIG. 2. Amino acid sequence comparison and dendrogram of SMC1β. (A) N-terminal domains of SMC1β and mammalian SMC proteins representing the four most closely related SMC subfamilies. (B) C-terminal domains of SMC1β and mammalian SMC proteins representing the most closely related SMC subfamilies. The program Megalign (DNASar) was used. The accession numbers for the related SMC proteins are as follows: mSMC1α (mouse SMCB; AF047600), bSMC1α (bovine SMC1; AF072712), hSMC1α (human SB1.8; S78271), XSMC1 (*X. laevis*; AF051784), mSMC3 (mouse SMCD; AF047601), bSMC3 (bovine; AF072713), hSMC4 (human CAP-C; AB019987), and hSMC2 (human CAP-E; AF092563). mSMC1β, mouse SMC1β; hSMC1β, predicted human protein (CAB41703; partial sequence). Identical residues are shaded. Dashes indicate gaps in alignment.

MATERIALS AND METHODS

Cloning of SMC1β cDNA. The 150-kDa protein was isolated from testis nuclear extracts by immunoprecipitation with anti-SMC3 antibody (63) and separation by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Sequences from seven peptides, generated by tryptic digest of the protein, were

determined. Based on this sequence information, oligonucleotides were designed for the screening of a mouse testis 5'-STRETCH cDNA library (Clontech Inc.). Several overlapping clones were isolated, which covered a 1,735-bp fragment at the 5' end of the cDNA. To recover the missing 3' end, rapid amplification of cDNA ends (RACE) (15) was performed using a SMART RACE cDNA am-

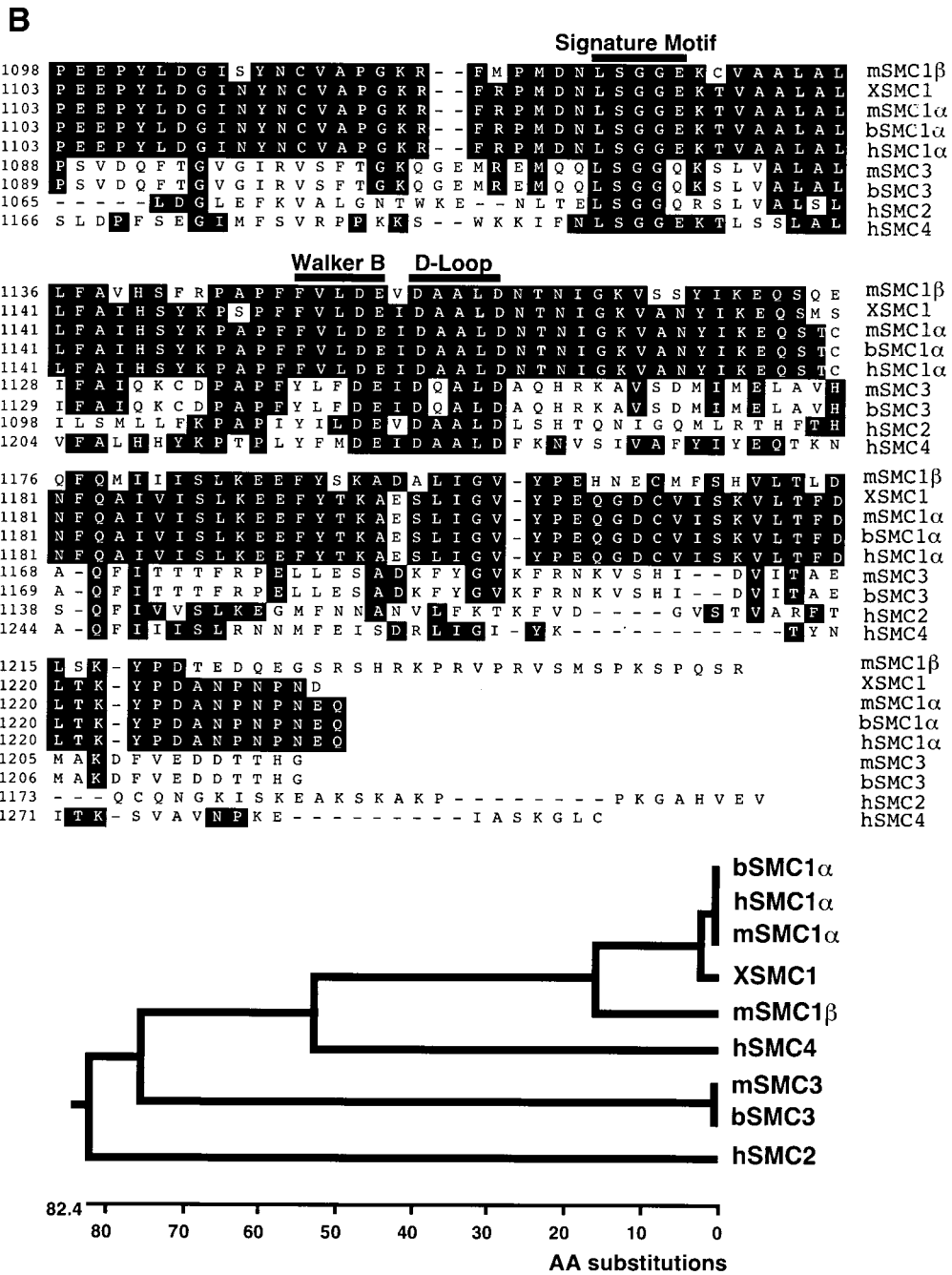


FIG. 2—Continued.

plification kit (Clontech Inc.). Several independent clones have been obtained and sequenced. The sequence of the C terminus was further verified by performing nested PCR on 3' RACE PCR products from testis RNA. The PCR used a gene-specific primer located at the positions corresponding to aa 1171 to 1179 of SMC1β, and the Nested Universal Primer (Clontech Inc.), located downstream of poly(A) within the region defined by the RACE universal primer. Twenty cloned PCR products were analyzed by restriction analysis, and five clones were sequenced. All belonged to the same gene and encoded identical C termini. The 4,056-bp full-length cDNA was assembled using standard cloning protocols.

Northern blot analysis. Eight-microgram aliquots of total RNA from various mouse tissues (Ambion) were separated electrophoretically and transferred to a nylon membrane (Hybond N; Amersham) using standard protocols (55). Hy-

bridization was performed as described previously (9). The probe corresponded to the first 616 bp of the SMC1β cDNA.

Protein purification and antibody generation. The C-terminal domain of SMC1β (SMC1β-C) was subcloned into the *Escherichia coli* expression vector pQE32 (Qiagen Inc.). The His₆-tagged protein, with a molecular mass of 33 kDa, was overexpressed and purified on Ni-nitrilotriacetic acid agarose resin (Qiagen) and eluted in steps with increasing imidazole. The yield was 200 to 600 μg per liter of *E. coli* culture. About 80% of the total SMC1β-C protein was found to be insoluble under native conditions and had to be dissolved in 8 M urea. The remaining 20%, native soluble protein, was mixed with an equal amount of denatured protein and injected into mice. Monoclonal antibodies were generated by standard hybridoma techniques. Hybridoma tissue culture supernatants

were screened by enzyme-linked immunosorbent assay using 96-well plates that were coated with the C-terminal protein. Subsequently, the positive supernatants were tested for their specificities by Western blotting on testis versus somatic cell extracts and by immunofluorescence on testis versus liver sections. At least six independent hybridoma clones produced antibodies specific in both procedures for SMC1 β .

The rabbit anti-SMC1 serum SMC1-C1 and the anti-SMC3 serum (13, 63), the hamster anti-SCP3 serum H1 (14), the rabbit anti-SCP3 serum 175 (37), and the rabbit anti-SCP2 serum (49) have been described. For labeling of kinetochores, we used a human autoimmune serum from a patient with CREST (calcinosis, Raynaud syndrome, esophageal dysmotility, sclerodactyly, and telangiectasia) syndrome; this serum reacts with kinetochore proteins and has been described by Moens et al. (46).

Immunoprecipitation. Nuclear extracts from tissue or cells were prepared as described previously (29), with the concentration of dithiothreitol (DTT) lowered to 0.1 mM. Five micrograms of affinity-purified anti-SMC3 antibody was incubated for 4 to 16 h at 4°C with nuclear extract (50 μ g of protein unless otherwise indicated), which was diluted 1:5 in buffer IP (phosphate-buffered saline plus 0.75% Brij 58, and 500 mM NaCl). A 20- μ l slurry of protein A beads was added, and the mixture was further incubated for 1 h. The beads were washed six times with buffer IP or variations of it as described in the text and were boiled with SDS gel sample buffer before being loaded on reducing 5% polyacrylamide SDS gels. Detection of proteins was done by silver staining according to a published protocol (29).

Preparation of spreads and agar filtrates. Spreads of mouse spermatocytes were prepared by the dry-down technique of Speed (59), as modified by Peters et al. (51). Agar filtrates of lysed rat spermatocytes were prepared as described by Heyting and Dietrich (22).

Immunofluorescence labeling. Immunofluorescence labeling of dry-down preparations and agar filtrates was performed as described previously (22). The slides were mounted in Vecta Shield (Vector Laboratories Inc., Burlingame, Calif.). The monoclonal anti-SMC1 β antibodies in tissue culture supernatant (from two independent hybridomas) were diluted 1:1, serum 175 (rabbit anti-SCP3) was diluted 1:500, CREST serum (anti-kinetochore) was diluted 1:1,000, and serum H1 (hamster anti-SCP3) was diluted 1:50. Goat anti-rabbit immunoglobulin G (IgG) conjugated with aminomethylcoumarin acetate (AMCA) (Vector) or with Texas red (Jackson ImmunoResearch Laboratories, West Grove, Pa.), goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) (Jackson), goat anti-hamster IgG conjugated with AMCA, and goat anti-human IgG conjugated with Texas red were used as secondary antibodies and were diluted according to the instructions of the suppliers.

Microscopy. Spread preparations were examined with a Zeiss Axioplan research microscope equipped with epifluorescence illumination and Plan-Neofluar optics. Selected images were directly photographed on an ISO 400 color negative film using single-band-pass emission filters (for DAPI [4',6'-diamidino-2-phenylindole]-AMCA, FITC, and Texas red fluorescence) with separated excitation filters. Negatives were scanned at high resolution, and their computer images were processed and combined using the CorelDraw and Corel Photopaint software packages.

Isolation of meiotic cells. Spermatocytes were isolated from rat testes by cell elutriation and density centrifugation according to the method of Heyting and Dietrich (22), and the composition of the isolated cell fraction was analyzed by differential counts of Giemsa-stained preparations, as described previously (38). The cell fraction that was used in this study had the following composition: Sertoli cells, 0.3%; spermatogonia, 0.7%; and spermatocytes, 99%, of which 1.2% was in leptotene-zygotene, 29% was in early-mid pachytene, 54% was in late pachytene or prediffuse diplotene, and 16% was in diffuse or postdiffuse diplotene.

DNA interaction assays. DNA concentrations are expressed as nucleotide equivalents. The assay for retention of double-stranded DNA on nitrocellulose filters through binding to protein (filter binding assay) was performed essentially as described elsewhere (3).

Linear double-stranded DNA fragments were generated by digestion of pBluescript SK(+) (Stratagene Inc.) with *AluI* and were labeled at their 5' ends with ³²P. Reactions were performed in 20- μ l mixtures containing 25 mM Tris-HCl (pH 7.5), 10 mM KCl, 0.2 mM EDTA, 1 mM DTT, 10 ng of linear DNA, and various amounts of peptide. After 20 min at room temperature, the reaction mixtures were diluted by adding 1 ml of reaction buffer containing 10 mM sodium pyrophosphate. The reaction mixtures were filtered through prewashed 0.1- μ m-pore-size nitrocellulose filters (Whatman Inc.). The filters were washed thrice with 1 ml of the reaction buffer containing sodium pyrophosphate, and the radioactivity retained on the filters was measured in a liquid scintillation counter.

The gel shift (gel retardation) assay was performed as described previously (1,

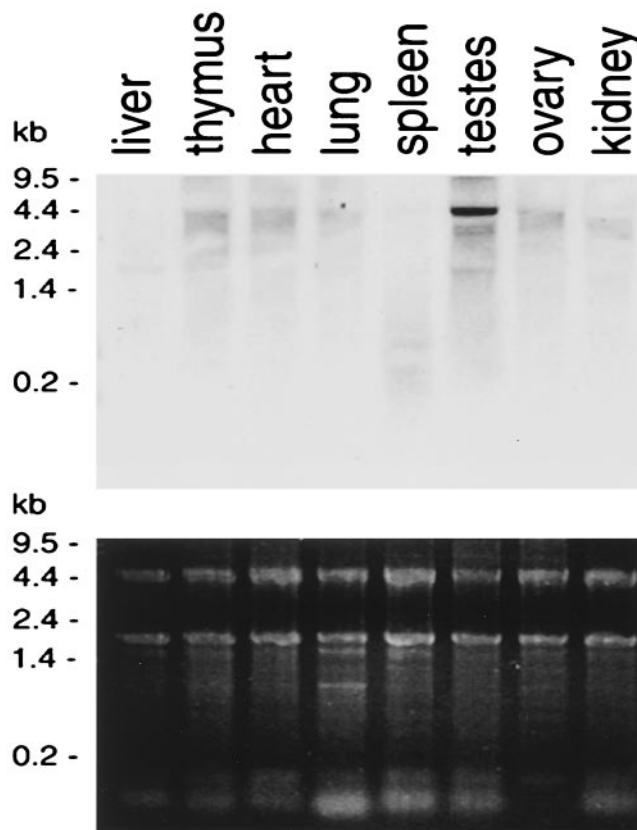


FIG. 3. Tissue specificity of SMC1 β RNA expression. (Top) Northern blot of total RNA extracted from different mouse tissues and hybridized to an SMC1 β -specific probe. (Bottom) Corresponding agarose gel stained with Radiant Red fluorescent RNA stain prior to transfer for loading control.

2). For a DNA substrate, we used a 200-bp DNA fragment of the 5S rRNA gene or a 230-bp DNA fragment derived from the double-stranded form of M13mp8, which we know are good binding substrates for SMC protein domains (1, 2). The reaction buffer contained 0.8 pmol of ³²P 3'-end-labeled DNA (5,000 to 10,000 cpm) in 20 mM HEPES (pH 7.5), 1 mM DTT, and 0.1 mg of bovine serum albumin (ultrapure; Amersham-Pharmacia Inc.)/ml. The SMC1 β peptide consisted of the C-terminal 28 aa (TEDQEGSRSHRKPRVPRVSMSPKQPSQR; theoretical pI, 11.4). The positive control peptide was derived from mouse Rad54L (34), aa 152 to 181 (KVCPRHQREGVKFLWECVTSRRIPGSHGLI; theoretical pI, 10.09). The negative control peptide was derived from human Rad21 (44), aa 612 to 631 (TQEEPYSIIATPGPRFHII; theoretical pI, 4.65).

The assay for network formation was done as described for RecA (7) or mammalian DNA binding proteins (17), with some modifications. The assay measures coaggregation of DNA into DNA-protein complexes that sediment rapidly. The DNA substrate was *AluI*-digested (blunt-ended), 5'-³²P-labeled plasmid DNA as used for the filter binding assay. Various amounts of peptide were incubated with the DNA (1.5 pmol) in a volume of 50 μ l for 20 min at room temperature in the DNA binding buffer also used for gel shift experiments. The reaction mixture was then centrifuged for 3 min at 14,000 \times g. The supernatant was transferred to a scintillation vial, and the pellet was solubilized in 100 μ l of 0.1% SDS and also transferred to a scintillation vial. The supernatant (nonaggregated) and pellet (aggregated) DNAs were measured. All experiments were done in triplicate.

Nucleotide sequence accession numbers. The GenBank accession number of mSMC1 β is AF303827.

RESULTS

A novel SMC protein complex from testis. Earlier, we used affinity-purified polyclonal anti-SMC3 antibodies, raised against

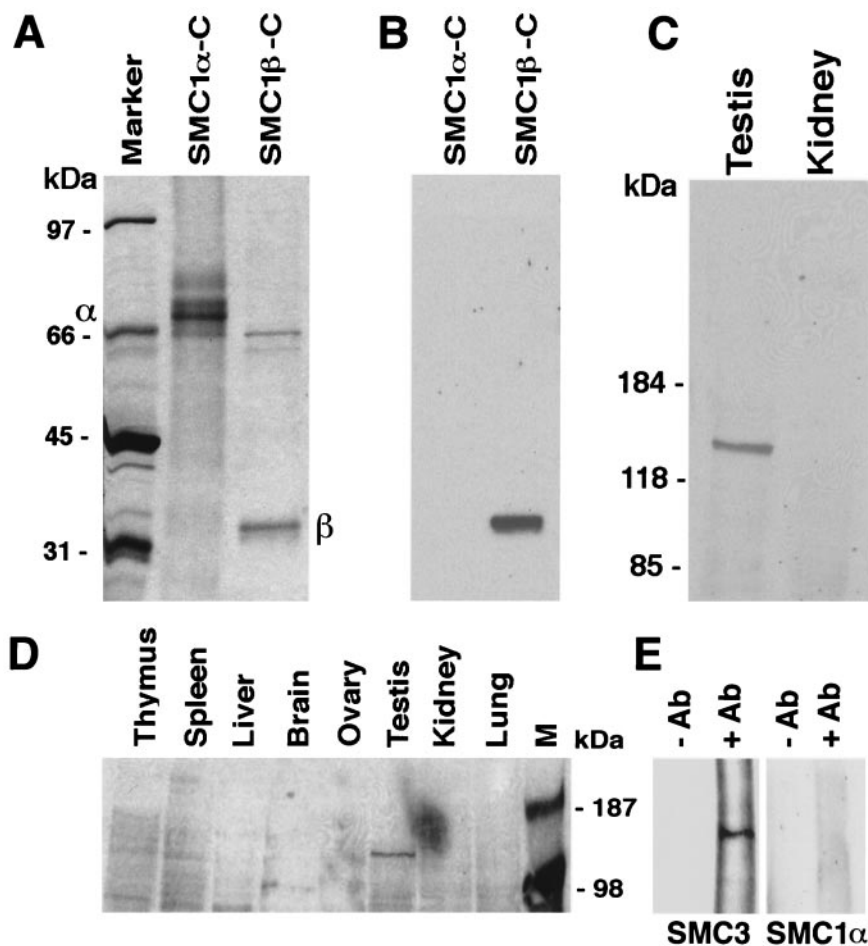


FIG. 4. Generation and specificity of anti-SMC1 β monoclonal antibodies. (A) Coomassie blue-stained SDS-polyacrylamide gel loaded with the C-terminal domains of SMC1 α (approximately 67 kDa) and SMC1 β (approximately 33 kDa), with their positions indicated by α and β . (B) Immunoblot of a gel identical to that in panel A probed with a monoclonal anti-SMC1 β antibody. (C) Immunoblot of nuclear extracts from mouse testis and kidney probed with a monoclonal anti-SMC1 β antibody. (D) Immunoblot of nuclear extracts from a variety of bovine tissues probed with a monoclonal anti-SMC1 β antibody. M, marker. (E) Anti-SMC1 β immunoprecipitates from testis nuclear extracts probed in Western blotting with anti-SMC3 or anti-SMC1 α antibodies. -AB, no anti-SMC1 β ; +AB, with anti-SMC1 β .

the C-terminal domain of bovine SMC3, in immunoprecipitation experiments with nuclear extracts of mitotically dividing cells of human, mouse, hamster, and bovine origin (reference 63 and unpublished observations). Under stringent precipitation conditions, the SMC1 and SMC3 proteins were observed as the only strong bands in silver-stained SDS polyacrylamide gels, used to analyze the precipitates. A weaker 120-kDa band was often visible and probably represented the Rad21 protein. Immunoprecipitation experiments with nuclear extracts prepared from a variety of mouse and bovine tissues, however, revealed a marked difference between extracts from testes and all other extracts. From testes, we coimmunoprecipitated a hitherto undescribed protein that migrates at an approximately 145- to 155-kDa position between SMC1 and SMC3, depending on the particular gel electrophoresis conditions (Fig. 1). The 150-kDa protein was also not observed in immunoprecipitates from various human, mouse, and hamster cell lines, nor was it present in cytoplasmic extract fractions (63; data not shown). However, a faint 150-kDa band was visible in immunoprecipitates from ovary extracts (Fig. 1A). Comparable re-

sults were obtained with mouse tissues (Fig. 1B and F). The coprecipitated protein from mouse testis extract migrates at 155 kDa, a position slightly higher than that of the bovine protein. The 150-kDa protein is present neither in resting nor in activated, proliferating somatic mouse cells (Fig. 1D).

Incubation of the precipitates with bacterial alkaline phosphatase or the inclusion of ATP (1 mM) or of the phosphatase inhibitor *o*-vanadate (0.5 mM) in the extracts and all buffers did not alter the result (not shown). The characteristic band pattern also did not change upon variation of the precipitation and wash conditions, e.g., the use of different detergents, differently pretreated protein A or protein G beads, or protein G-precleared extracts (not shown). Association of the 150-kDa protein with SMC3 was found to be as resistant to stringent precipitation reaction conditions as that of SMC1 with SMC3. The 150-kDa protein was also immunoprecipitated from testis nuclear extract fractions that had been obtained by gel filtration of the extract through a large BioGel A15m chromatography column. Similar chromatography experiments were done before for either purification of RC-1 from thymus or other

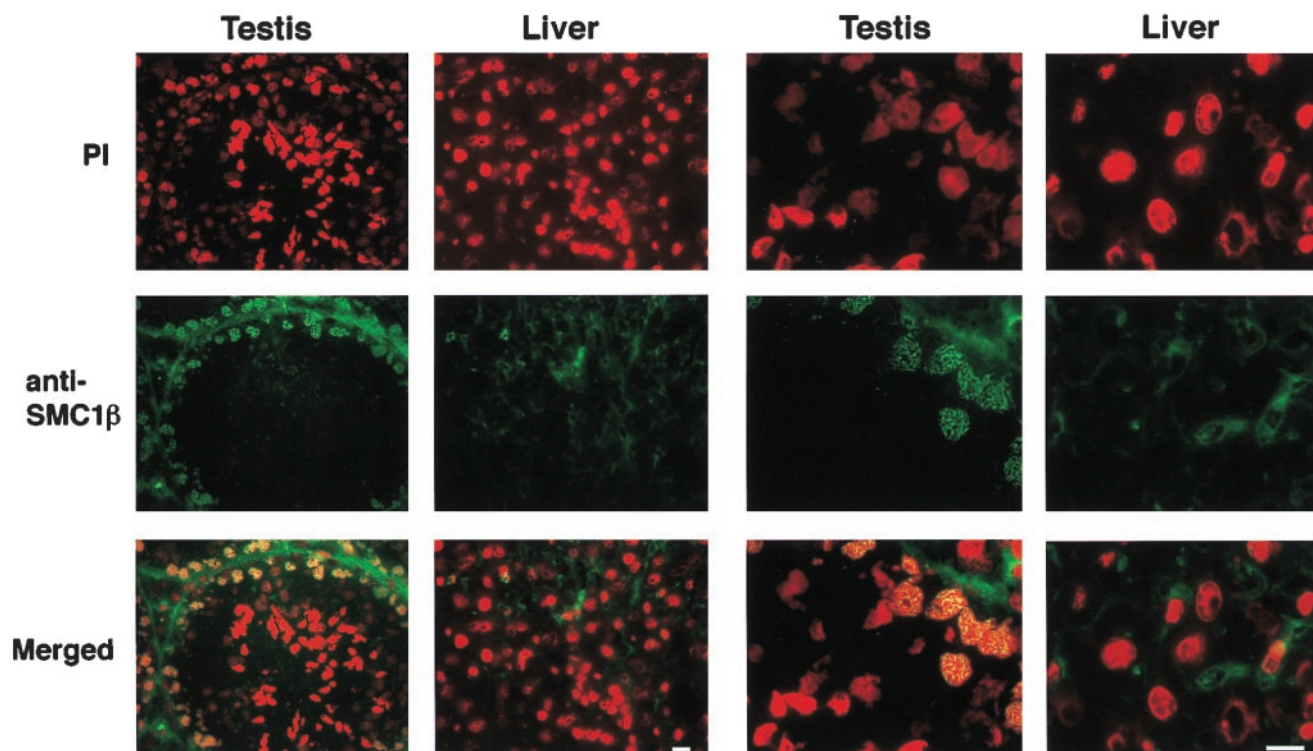


FIG. 5. Testis and liver section stained with anti-SMC1 β . Sections of mouse testis or liver were incubated with either propidium iodide (PI)-RNase or anti-SMC1 β , FITC-labeled, to visualize DNA or SMC1 β . The merged images are at the bottom, and two magnifications are shown as indicated. Bar = 10 μ m.

analysis of testis extract fractions (13, 30) (Fig. 1E). The protein was found together with SMC3 in fractions that represent molecular masses of globular proteins of around 1 MDa and—albeit in smaller amounts—at 3 MDa. At 3 MDa, more of SMC1-SMC3 was visible, possibly indicating differences in masses between complexes based on SMC1-SMC3 or the 150-kDa protein and SMC3. Thus, the 150-kDa protein coprecipitates and copurifies with SMC3 and is likely a component of a large multiprotein complex, similar but not identical to that containing the SMC1-SMC3 heterodimer (13).

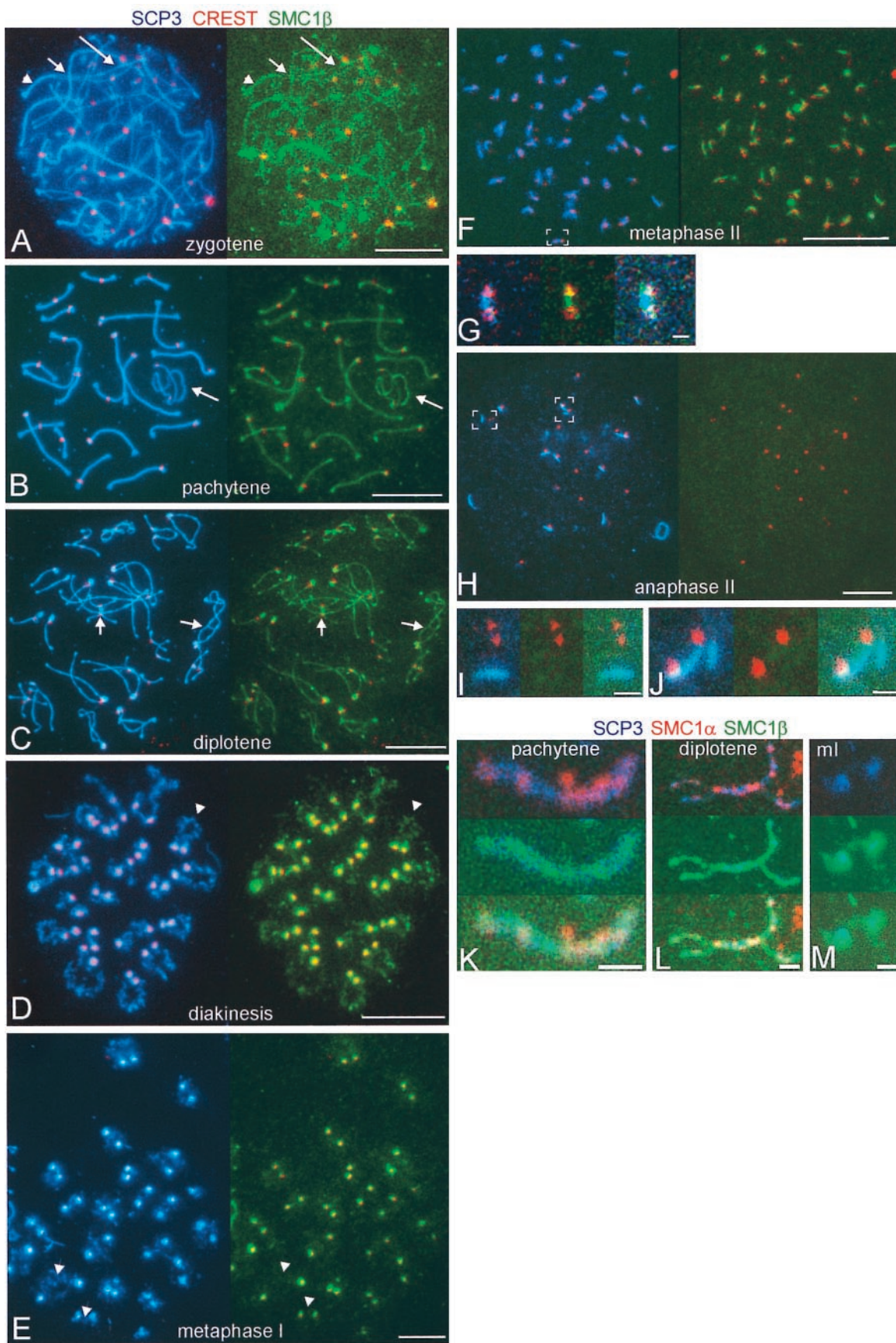
In immunoblotting, neither a monoclonal nor an affinity-purified polyclonal antibody, both raised against the C-terminal domain of SMC1, recognizes any protein migrating between the SMC1 and SMC3 proteins (13, 63). Likewise, anti-SMC3

antibodies do not react with the protein in immunoblotting. Thus, the 150-kDa protein is not a degradation product of SMC1 or very homologous to SMC3.

From immunoblotting and immunoprecipitation experiments, we estimate an approximate relative abundance of SMC1 α , 150-kDa protein, and SMC3 of 1:2:3 in total testis nuclear extracts. In extracts from purified spermatocyte preparations, which consist of >99% meiotic cells (70% late pachytene-diplotene [22, 38]), only a small amount of SMC1 α was seen, while the 150-kDa protein and SMC3 precipitated in an approximately equimolar ratio (Fig. 1F).

Large-scale immunoprecipitation followed by SDS-polyacrylamide gel electrophoresis and amino acid sequencing of the 150-kDa protein allowed us to deduce seven peptide se-

FIG. 6. Immunolocalization of SMC1 β and SMC1 α in successive stages of meiosis. (A to J) Images of immunofluorescence triple labeling of SCP3 (blue), SMC1 β (green), and kinetochores (red) in dry-down preparations of rat spermatocytes. (A to F and H) Shown on the left are the merged images of SCP3 (blue) and kinetochores (red), and shown on the right are the merged images of the same cell of SMC1 β (green) and kinetochores (red). (A) zygotene (the long arrows indicate asynapsed segments of AEs, the short arrows point to regions of presynaptic alignment, and the arrowheads designate paired segments of AEs); (B) pachytene (the XY bivalent is indicated by arrows); (C) diplotene (the arrows point to connections containing SCP3 and SMC1 β between AEs of homologous chromosomes); (D) diakinesis (the arrowheads point to partial splitting of AEs); (E) metaphase I (the arrowheads indicate a weak signal for SCP3 in the chromosome arms, whereas SMC1 β is hardly detectable); (F) metaphase II; (H) anaphase II. (G) Enlargement of the area indicated in panel F, with the merged images of SCP3 (blue) and kinetochores (red) (left); SMC1 β (green) and kinetochores (red) (middle); and SCP3 (blue), SMC1 β (green), and kinetochores (red) (right). (I and J) Enlargements of areas indicated in panel H, with the merged images of SCP3 (blue) and kinetochores (red) (left); SMC1 β (green) and kinetochores (red) (middle); and SCP3 (blue), SMC1 β (green), and kinetochores (red) (right). (K to M) Images of immunofluorescence triple labeling of SCP3 (blue), SMC1 β (green), and SMC1 α (red). Shown are a pachytene SC (K), a diplotene SC (L), and a metaphase I bivalent (M) in a dry-down preparation of rat spermatocytes. The tops of panels K to M show the merged images of SCP3 (blue), SMC1 α (red), and SMC1 β (green); the middles show SCP3 (blue), SMC1 β (green), and SMC1 α (red); and the bottoms show SCP3 (blue), SMC1 β (green), and SMC1 α (red). Bars = 10 μ m (A to F and H) and 1 μ m (G, I, J, and K to M).



quences 6 to 12 aa in length. This information was used to generate oligonucleotides with which a mouse testis library was screened. By a combination of further screening and reverse transcription-PCR from mouse testis RNA, the entire cDNA was cloned and then sequenced. This cDNA encodes a protein that has not been reported previously and that shows a high level of homology to mammalian SMC1 (Fig. 2A). The homology is highest in the conserved functional domains of SMC proteins, the N-terminal, C-terminal, and hinge domains. Lower degrees of homology were found with SMC4 and SMC3. Dendrogram analyses of the N- and C-terminal domains confirmed the close relationship to SMC1 (Fig. 2B). Therefore, we call the protein SMC1 β , indicating an SMC1 variant or isoform. The "classical" SMC1 may be termed SMC1 α . SMC1 β bears a unique C-terminal sequence of 28 aa that has been found neither in any other SMC protein nor in the databases. Among the 28 aa are 4 proline residues and 7 arginine and lysine residues. This C-terminal peptide is very basic, with a theoretical pI of 11.4. In contrast, the entire C-terminal domain of SMC1 β , with 186 aa, has a theoretical pI of 6.9. This C-terminal motif was present in all independently isolated clones and was confirmed by RT-PCR of mouse testis and subsequent sequencing of PCR products. SMC1 β shows all motifs characteristic of SMC proteins, including the N-terminal Walker A box, the C-terminal Walker B box, and the signature motif typical of ABC-ATPases (28, 68), as well as the extended coiled-coil domains with heptad repeats and the hinge region.

Specific expression of SMC1 β in meiotic cells. Northern blotting of RNA from a variety of mouse tissues was performed using a 616-bp 5' DNA fragment of SMC1 β as a probe (Fig. 3). This experiment confirmed testis-specific expression of the gene. The specific signal of about 4.5 kb was not seen in RNA from any of the other tissues. We also used the same probe to analyze RNA prepared from purified spermatocytes. The same 4.5-kb signal was observed, and no other band was detected (not shown).

Next, we generated monoclonal antibodies for the analysis of protein expression. The antibodies were raised in mice against a C-terminal 33-kDa fragment of mouse SMC1 β , expressed in *E. coli*, and purified by affinity chromatography on Ni-Sepharose (Fig. 4A). The C-terminal protein was partially soluble under native conditions, and the insoluble precipitates were solubilized in 8 M urea. A mixture of native and denatured protein was used for immunization. We obtained several hybridoma lines that produce antibodies that specifically recognize SMC1 β but not SMC1 α (Fig. 4B). No cross-reaction with even large amounts of the purified C-terminal domain of SMC1 α , or with SMC proteins present in somatic cell nuclear extracts, was observed (Fig. 4B and C). The tissue specificity of SMC1 β was also confirmed for a large variety of bovine tissues (Fig. 4D). We obtained several antibodies that recognize SMC1 β of mouse, rat, and bovine origin. Immunoprecipitation using the anti-SMC1 β antibody with testis extracts confirmed the association of SMC1 β with SMC3 (Fig. 4E). We never observed SMC1 α to coimmunoprecipitate with anti-SMC1 β antibodies.

We then used these monoclonal antibodies to investigate expression and localization of SMC1 β in testis. Mouse testis sections and, for control, liver sections were prepared and

immunoprobed with FITC-labeled anti-SMC1 β and stained with propidium iodide (after RNase treatment) to visualize nuclear DNA (Fig. 5). No specific anti-SMC1 β signal was obtained with liver sections. In testis, however, strong staining of prophase I nuclei was observed. The antibodies stained the compact chromosomal axes within the meiotic nuclei, indicative of the presence of SMC1 β along the SCs. In these sections, only weak staining was observed in cells of later stages. These results were confirmed with three other anti-SMC1 β antibodies (not shown).

By immunoblotting, SMC1 β was also found in preparations of SCs from rat spermatocytes (13) (not shown). As these preparations contain only a very limited number of proteins (<20) and are prepared under stringent conditions, this indicates a close association of SMC1 β with SCs.

Chromosomal localization of SMC1 β throughout meiosis. High-resolution analysis of rat spermatocyte nuclear spreads confirmed and significantly extended our initial observations (Fig. 6 and 7). Several different anti-SMC1 β antibodies were used in immunofluorescence, all yielding identical results. Spreads of cells in consecutive stages of meiosis up to anaphase II were analyzed for SMC1 β , the AE-specific protein SCP3, and kinetochores (Fig. 6). SMC1 β tightly colocalizes with SCP3 from early prophase I (leptotene and zygotene) on along the entire AEs of the chromosomes in presynapsed (leptotene-zygotene [Fig. 6A]), synapsed (pachytene), unsynapsed (XY bivalent [Fig. 6B]), and desynapsed (diplotene [Fig. 6C]) regions. The distribution of SMC1 β appears rather uniform along the AEs, with occasional more intense dots. Until diplotene, there is no concentration of SMC1 β around the centromeres (Fig. 6A and B). SCP3 and SMC1 β remain tightly associated with the AEs throughout pachytene. Upon desynapsis of the homologous chromosomes in diplotene, SCP3 and SMC1 β start to accumulate around the centromeres and to dissociate from the chromosome arms (Fig. 7C to E). Staining for both proteins is also visible at sites of bridges between the homologues, which possibly represent sites of crossover (Fig. 6C).

The further meiosis I continues, the less SCP3 and SMC1 β we find in the chromosome arms, but intense staining remains present at the centromeres. This is obvious in diakinesis (Fig. 6D), metaphase I (Fig. 6E), and up to metaphase II (Fig. 6F). In anaphase II, SMC1 β is not visibly associated with the chromosome anymore. Aggregates containing SCP3 can still be seen in anaphase II, but most of these have detached from the kinetochores (Fig. 6H to J). The colocalization of SCP3 and SMC1 β is also not perfect in other respects. The SMC1 β signal on the AEs has a more granular appearance (though far less dotted than that of SMC1 α [13]), and there is a limited SMC1 β but no SCP3 signal present on peripheral chromatin loops from leptotene until metaphase II. Moreover, some SCP3, but very little if any SMC1 β , persists in the chromosome arms in metaphase I (Fig. 6E).

To compare the chromosomal localization of SMC1 β with that of SMC1 α , we performed additional immunofluorescence experiments on agar filtrates of rat spermatocytes (13), using anti-SMC1 β , anti-SMC1 α , and anti-SCP3 (Fig. 6K to M). As described by us earlier (13), SMC1 α is present throughout spermatocyte nuclei in frozen sections (Fig. 6A). However, if spermatocytes are lysed in buffers containing Triton X-100 (as

is done for agar filtration), or if they are permeabilized in such buffers (as we have done previously with frozen sections [13]), SMC1 α is preferentially retained in intensely labeled dots along the AEs. Thus, the localization of SMC1 α differs from that of SMC1 β , which is almost uniformly distributed along the AEs. Also, the SMC1 α dots appear not to be as close to the AEs as the SMC1 β staining (Fig. 6K and L). Thus, SMC1 β seems to localize most closely to the SC, while SMC1 α also appears SC associated, albeit not centered as much towards the AEs. Strikingly, and unlike SMC1 β , SMC1 α did not accumulate around the centromeres in diakinesis and metaphase I (Fig. 6 M).

We also analyzed the localization of SMC3 in spermatocytes by triple labeling of SMC3, SCP3, and SMC1 α , using frozen sections that had not been exposed to Triton X-100 (Fig. 7A to C) or agar filtrates (Fig. 7D), and by triple labeling of SMC3, SCP3, and kinetochores, using dry-down preparations (Fig. 7E to K). Along the AEs, SMC3, like SMC1 β , occurred mainly homogeneously distributed on the AEs. From late diplotene up to metaphase II, SMC3 was concentrated at the kinetochores, very similar to the localization seen for SMC1 β (Fig. 7F to H and J), whereas SMC3—like SMC1 β —was not detectable anymore at the kinetochores in anaphase II (Fig. 7I and K). In SMC1 α -SMC3-SCP3 triple labelings of frozen sections, SMC3 does not colocalize with SMC1 α , indicating yet another difference between SMC1 α and SMC1 β . Unexpectedly, we could not demonstrate the tight colocalization of SMC3 with AEs if we labeled the AEs with anti-SCP2 rather than anti-SCP3 (not shown and reference 13). Apparently, the anti-SCP2 antibodies interfere with the immunolabeling of SMC3 in AEs, perhaps implying a close association of SMC3 with SCP2.

Initial analysis of the C-terminal motif. As noted above, SMC1 β carries an unusual, basic C-terminal amino acid sequence of 28 aa that has not been found in any other SMC protein. Analyzing this sequence, we found a nuclear localization signal (NLS) sequence (RKPR [24]), and therefore, the C-terminal motif may contribute to nuclear import of the protein. In addition, the basic pI of the peptide and two consecutive SP motifs (10) renders interaction with DNA likely. We tested both hypotheses.

To test for an NLS function, we cloned the 28-aa motif in frame with the enhanced green fluorescent protein (EGFP) gene into a mammalian expression vector. The construct was transfected into 293 cells, and the intracellular distribution of EGFP was monitored by fluorescence microscopy. For a positive control, we used the EGFP-Nuc protein, a variant of EGFP fused to three copies of the NLS of the simian virus 40 large T antigen (Clontech Inc.), and the unaltered EGFP was used for a negative, cytoplasmic control. Screening several thousand cells, we did not detect EGFP expression in the nucleus (not shown). Thus, the 28-aa motif does not confer NLS activity on EGFP and therefore is not likely to decisively contribute to the nuclear import of SMC1 β .

Preliminary evidence for an interaction of the 28-aa peptide with DNA was obtained in filter binding and gel shift (gel retardation) assays. For a positive control in these assays, we used a peptide derived from the Rad54L protein (34); for a negative control, we used a Rad21-derived peptide from a non-DNA binding region (44).

In filter binding assays (43, 53), we used *AluI*-digested

(blunt-ended) plasmid DNA, radioactively labeled at the 5' ends. The DNA was efficiently retained by the peptide and the Rad54 control peptide but not by the Rad21 control peptide. The amount of DNA retained on the nitrocellulose filters was directly proportional to the amount of peptide used in the assay (not shown).

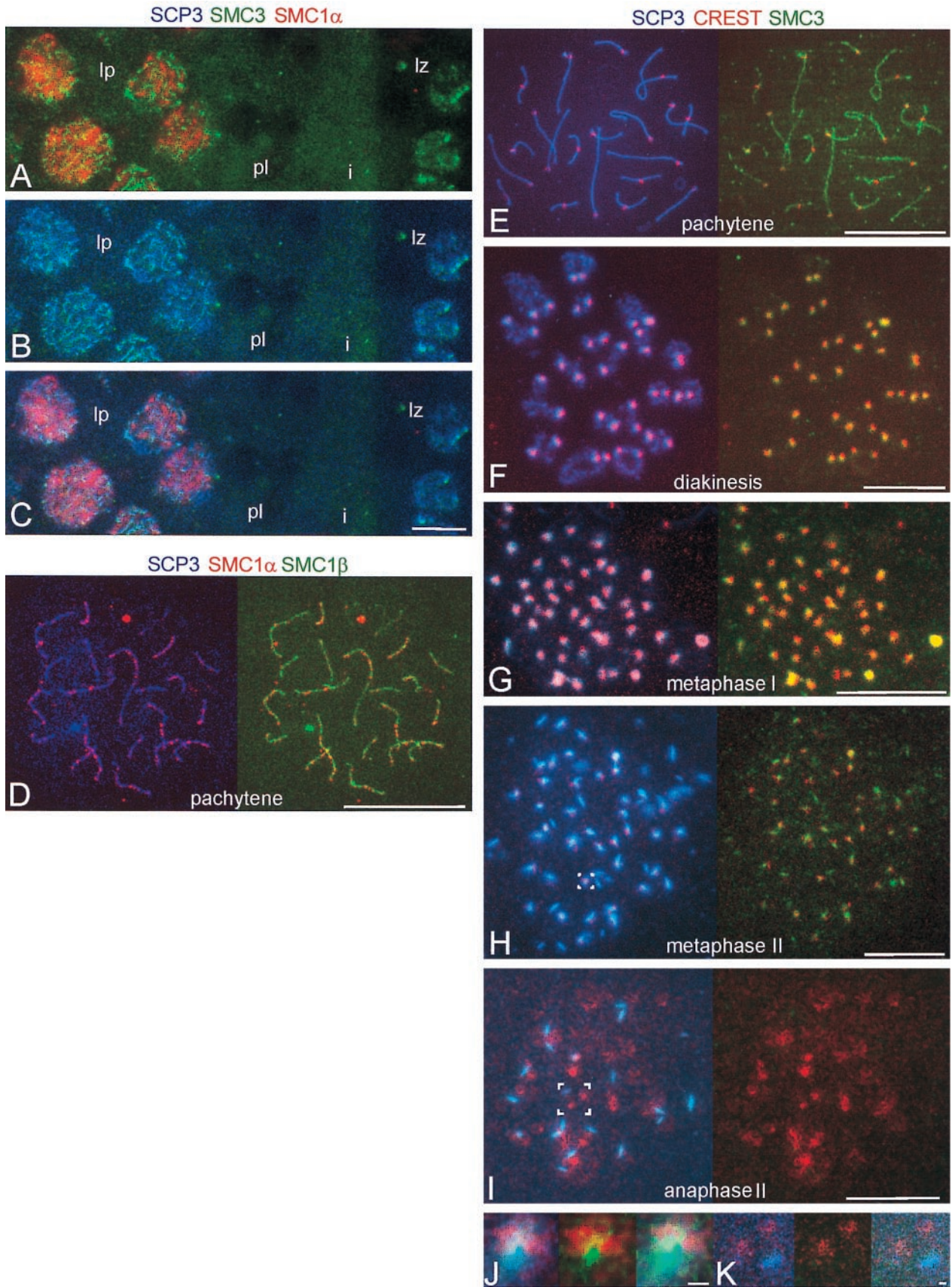
In gel shift experiments, end-labeled DNA fragments, either from M13mp18 or from the 5S rRNA gene, were used (1, 2, 40) and incubated with increasing amounts of the peptide. The results (Fig. 8A) show efficient binding of the SMC1 β -C peptide to the DNA substrate. The migration distance from the start position linearly decreased with increasing amounts of peptide added, with no indication of cooperative binding. Surprisingly, almost all DNA was shifted to a higher position even with submolar amounts of peptide (the ratio of peptide to DNA was 1:12 in nucleotide equivalents or 1:2 in DNA molecules). This may indicate binding of one peptide molecule to more than one DNA molecule, i.e., network formation.

To further test this hypothesis, we used an assay for nucleoprotein network formation that was used primarily in studies of DNA recombination proteins, such as *E. coli* RecA or mammalian proteins (7, 17). Nucleoprotein network formation activity was found to be required for homologous DNA pairing (7). Radioactively labeled DNA is incubated with protein, and aggregates are pelleted by centrifugation. The pellet (bound DNA) and supernatant (unbound) are measured in a scintillation counter. The results (Fig. 8B) show network formation activity of the SMC1 β peptide. The Rad21 peptide did not yield a signal above background, and the Rad54L peptide was active but eightfold less efficient than the SMC1 β -C terminal peptide (not shown). Network formation as seen in this assay starts at a minimal molar ratio of peptide to DNA of 1:1 (in nucleotides). However, to be efficiently pelleted, DNA-protein aggregates of considerable mass have to be created. Therefore, the true minimum ratio required for one peptide molecule to bind several DNA molecules may be significantly lower. Future molecular studies will have to determine the details of the reaction requirements, specificities, and mechanism, as well as the function of the motif in the context of the entire protein or protein complex.

Together, these initial results demonstrate DNA binding activities of the unique SMC1 β C-terminal motif.

DISCUSSION

In this report, we demonstrate for the first time the existence of a tissue- and cell-type-specific isoform of a mammalian SMC protein, SMC1 β . Specific expression of SMC1 β was found in meiotic cells and tissue from several mammalian species, including mouse, rat, and cow. This, and the high degree of evolutionary conservation generally found among SMC proteins, renders SMC1 β likely to exist in humans as well. Indeed, the working draft sequence of human chromosome 22 contains a gene highly homologous to the mouse SMC1 β gene (GenBank accession number NT011522). This gene was mapped to the region 22q13.31. Currently, only one locus in this region is known to be associated with human disease, i.e., methemoglobin reductase deficiency, which apparently has no direct connection to chromatin dynamics. We could not, however, identify a corresponding gene in the *S. cerevisiae* genome. While



there exist a few open reading frames with low homology, no certain assignment to SMC1 β could be made. Likewise, no *C. elegans* or *S. pombe* orthologues were detected in the database. Thus, the SMC1 gene may have diversified in higher eukaryotes throughout evolution into a universally and a meiotically expressed isoform. Swapping of SMC proteins in somatic cells has been reported for the gene dosage compensation complex in *C. elegans* (39), but no SMC protein isoform, and no meiosis-specific SMC protein, has been described.

While up to 70% homologous to SMC1 α in the globular domains, the new SMC1 β displays a characteristic feature that distinguishes it from SMC1 α : the highly basic C-terminal domain of 28 aa. Distinct C-terminal protein sequences, albeit not of such unusual composition, that are specific for meiotic isoforms of somatic proteins have been described, for example, for mammalian DNA ligase III (6). We predicted that this unique C-terminal sequence would contribute important functions to SMC1 β . It may, for example, affect interactions of the protein with DNA, or it may act as an NLS. The latter proved to be unlikely, since the peptide on its own did not confer nuclear localization on EGFP. An NLS function may also not be necessary: in SMC1 β , there are seven more predicted NLSs distributed all over the protein. The C-terminal peptide, however, interacts with DNA. The basic pI and the presence of two SP motifs, known to constitute DNA binding motifs (e.g., in histone H1 [10]), rendered this likely. Indeed, initial evidence from three independent assays, filter binding, gel shift, and protein-DNA network formation, suggests that the peptide efficiently binds DNA. The gel shift experiments indicated binding of one peptide molecule to more than one DNA molecule, i.e., possible network formation. Preliminary experiments with network formation confirmed the capacity of the peptide molecules to link several DNA molecules, i.e., to form DNA-peptide aggregates. The ability of short peptides to promote networking-related reactions, such as homologous DNA pairing, has been reported, for example, for a 20-aa peptide derived from the *E. coli* RecA protein (67). A detailed molecular analysis of these DNA interactions, and the demonstration of their significance in vivo, are important subjects for future studies. Together, the unique C-terminal sequence of SMC1 β is likely to codetermine the DNA binding properties of the protein.

The new evidence for specialization of mammalian SMC proteins is reminiscent of what has been reported for a limited number of other proteins that are also involved in DNA dynamics. Examples in yeast are the Scc1-type proteins—also collectively called gordin proteins (48)—and Rec8p proteins, as well as the Rad51p and Dmc1p proteins (5, 47, 49, 65, 69).

Recently, a meiosis-specific homologue of the Scc3 protein, STAG 3, has been described (52). Whereas Rad51p and MCD1p-Scc1p-RAD21 exist in both somatic and meiotic cells, Dmc1p and Rec8p of yeast and of higher eukaryotes are restricted to meiotic cells (although human Rec8p transcripts were also found in spermatids and the thymus [50]) and post-meiotic cells (47). In meiosis, the relationship between cohesion and recombination is modified, and apparently this is accompanied by replacement of components of the protein complexes involved, like the gordins. Our results suggest that in mammals one of the SMC components of cohesin, SMC1 α , is replaced by a meiosis-specific isoform, SMC1 β . As had been found for the gordins and Rad51 in yeast, SMC1 α is only partially replaced in mammals: SMC1 α is present in both somatic and meiotic cells, whereas SMC1 β exists only in meiotic cells.

Earlier, we demonstrated the association of SMC1 α with meiotic chromatin in rat spermatocytes, and we have shown its presence in preparations of SCs (13). Although SMC1 α and SMC1 β are coimmunoprecipitated from total testis extracts by anti-SMC3 antibodies, a fraction of SMC1 α in spermatocytes does not colocalize with SMC3. Thus, two different SMC3-containing complexes exist in testis. The slightly different behavior of SMC1 α -SMC3 and SMC1 β -SMC3 in gel filtration, with the latter eluting predominantly at a lower-molecular-mass position around 1 MDa, further indicates two different higher-order complexes of different masses or stabilities that share SMC3 but contain either one of the two SMC1 isoforms. Furthermore, very little SMC1 α , but an equimolar amount of SMC1 β , is coimmunoprecipitated with SMC3 from late-prophase I spermatocytes. More evidence for this hypothesis originated from chromosomal localization studies.

As for SMC1 α , expression of SMC1 β is regulated during meiotic cell development, i.e., most protein is observed in prophase I of meiosis. There are, however, important differences in chromosomal localization between SMC1 α and SMC1 β . While SMC1 α is distributed throughout the meiotic prophase nucleus and—upon permeabilization or lysis of cells in the presence of Triton X-100—is preferentially retained in a dotlike pattern along AEs of SCs (reference 13 and this paper), SMC1 β is more tightly associated and more uniformly distributed along the AEs. In late prophase I (pachytene-diplotene), SMC1 β also associates with bridges between the AEs of homologues, which possibly represent the sites of crossover. This has not been observed for SMC1 α . In addition, the association of SMC1 β with the AEs of SCs seems to be even closer than that of SMC1 α with the SC. Thus, there may be a structural organization in layers, with SMC1 β constituting the inner and

FIG. 7. Immunolocalization of SMC3 and SMC1 α in successive stages of meiosis. (A to C) Images of immunofluorescence triple labeling of SMC1 α (red), SMC3 (green), and SCP3 (blue) in a frozen section of rat testis. (A) Merged images of SMC1 α (red) and SMC3 (green). (B) Merged images of SCP3 (blue) and SMC1 α (red). (C) Merged images of SMC1 α (red), SMC3 (green), and SCP3 (blue). The pictures show parts of two testicular tubules. lp, late-pachytene spermatocytes; pl, preleptotene spermatocytes; i, interstitial zone between the two tubules; lz, late-zygotene spermatocytes. (D) Images of immunofluorescence triple labeling of SMC1 α (red), SMC3 (green), and SCP3 (blue) in a rat pachytene nucleus, spread by agar filtration; on the left, the merged images of SCP3 (blue) and SMC1 α (red) are shown, and on the right, the merged images of SMC3 (green) and SMC1 α (red) are shown. (E to K) Images of immunofluorescence triple labeling of SCP3 (blue), SMC3 (green), and kinetochores (red) in dry-down preparations of rat spermatocytes. (E to I) On the left are the merged images of SCP3 (blue) and kinetochores (red), and on the right are SMC3 (green) and kinetochores (red). (J) Enlarged images of the area indicated in H. (K) Enlarged images of the area indicated in I. On the left are the merged images of SCP3 (blue) and kinetochores (red), in the middle are SMC3 (green) and kinetochores (red), and on the right are SCP3 (blue), SMC3 (green), and kinetochores (red). Bars = 10 μ m (A to I) and 1 μ m (J and K).

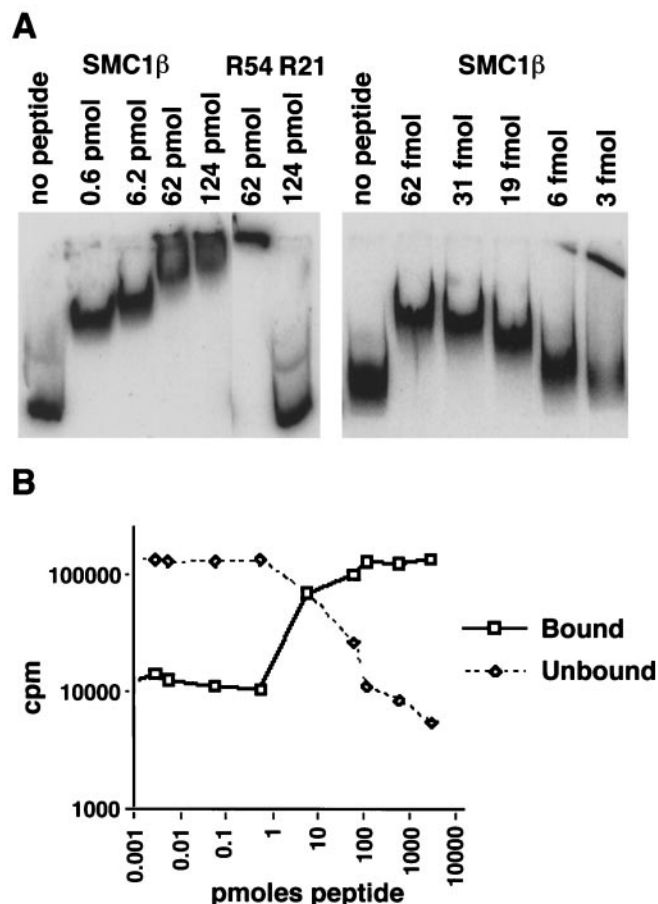


FIG. 8. DNA interaction of the 28-aa SMC1 β C-terminal motif. (A) In a gel shift assay, increasing amounts of the 28-aa peptide were incubated with 0.8 pmol of a 5'-³²P-labeled 200-bp ribosomal DNA fragment as indicated. R54 and R21, Rad54 and Rad21 control peptides. (B) Assay for protein-DNA network formation. Bound (pelleted) and unbound (supernatant) DNA was measured after incubation of 1.5-pmol of DNA substrate with increasing amounts of the 28-aa peptide.

SMC1 α an outer SMC-containing layer at the SC. Finally, in diplotene and diakinesis, SMC1 β and SMC3 accumulate around the centromeres, where the proteins persist until anaphase II, whereas SMC1 α does not concentrate at the centromeres in any stage of meiosis. This strongly suggests that SMC1 β , and not SMC1 α , is involved with SMC3 in the maintenance of centromere cohesion during the first meiotic division in mammals. In *S. cerevisiae*, a similar conclusion was reached for another meiosis-specific component of cohesin, Rec8 (36). SCP3 (reference 12 and this paper) and SCP2 (56) also accumulate at the centromeres from late diplotene until anaphase II. It remains to be investigated whether these SC proteins contribute directly to maintenance of centromere cohesion. Most likely, the dissociation of SMC1 α and SMC1 β from the chromosome arms in late prophase contributes to the release of sister chromatid arm cohesion, while centromeric cohesion is further supported by SMC1 β .

In addition, we observed that preparations of nuclear extracts and total cell lysates, especially those from purified pachytene-diplotene spermatocytes (22), contained a charac-

teristic 85-kDa degradation product of SMC1 β but no SMC1 α or SMC3 degradation products (not shown). Northern blotting showed the same 4.5-kb transcript in RNA from purified spermatocytes as in testis RNA, rendering alternative splicing unlikely. Thus, SMC1 β appears to be more sensitive to proteolysis than the other SMC proteins. One may speculate that such proteolysis may be required for the release of arm cohesion, similar to degradation of Rec8. The SMC1 β degradation product does not coprecipitate with SMC3, indicating that the 85-kDa fragment of SMC1 β is not present in a complex with SMC3. Alternatively, it may also be cleaved quickly after synthesis and thus prevented from associating with SMC3. The nature of the protease that cleaves SMC1 β , and whether such cleavage is necessary for meiotic progression, are among the questions now to be addressed.

In summary, we propose the existence of two multiprotein complexes in meiotic cells that are based on two different SMC1-SMC3 cores: SMC1 α -SMC3 and SMC1 β -SMC3. Both complexes associate with meiotic chromatin and should contribute to meiotic sister chromatid cohesion. The " α -complex," however, appears more loosely chromosome associated, in a punctate pattern, and also in the chromatin loops. This complex dissociates and is released from the chromatin in late prophase I. The " β -complex" closely localizes to the SC and remains chromosome associated at the centromeres beyond prophase I until metaphase-anaphase II. Therefore, the β -complex, and not the α -complex, is likely responsible for centromeric cohesion until anaphase II. The interplay among meiotic sister chromatid cohesion, DNA recombination, and the new meiosis-specific SMC1 β is now amenable to future studies.

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