RAD51 and DMC1 Form Mixed Complexes Associated with Mouse Meiotic Chromosome Cores and Synaptonemal Complexes

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Abstract. The eukaryotic RecA homologues RAD51 and DMC1 function in homology recognition and formation of joint-molecule recombination intermediates during yeast meiosis. The precise immunolocalization of these two proteins on the meiotic chromosomes of plants and animals has been complicated by their high degree of identity at the amino acid level. With antibodies that have been immunodepleted of cross-reactive epitopes, we demonstrate that RAD51 and DMC1 have identical distribution patterns in extracts of mouse spermatocytes in successive prophase I stages, suggesting coordinate functionality. Immunofluorescence and immunoelectron microscopy with these antibodies demonstrate colocalization of the two proteins on the meiotic chromosome cores at early prophase I. We also show that mouse RAD51 and DMC1 establish protein–protein interactions with each other and with the chromosome core component COR1(SCP3) in a two-hybrid system and in vitro binding analyses. These results suggest that the formation of a multiprotein recombination complex associated with the meiotic chromosome cores is essential for the development and fulfillment of the meiotic recombination process.

Key words: meiosis • genetic recombination • RAD51 • DMC1 • synaptonemal complex

GENETIC recombination, pairing, and synapsis of homologous chromosomes are events common to the meiotic prophase I of most sexually reproducing organisms, whereby they ensure the correct chromosome segregation at the end of the first meiotic division. In mammals, DNA replication is followed by alignment of the homologous chromosomes at early stages of prophase I (leptotene and zygotene). Simultaneously, a proteinaceous core assembles along the length of each homologue. COR1(SCP3) (Dobson et al., 1994; Lammers et al., 1994) and SCP2 (Offenberg et al., 1998) are two components of the chromosomal cores with well-characterized distribution patterns during meiosis. Synapsis is achieved at the subsequent stage, pachytene, by connecting the two protein cores with each other through a central region containing the synaptic protein SYN1(SCP1) (Meuwissen et al., 1992; Dobson et al., 1994). The chromosomal cores become part of the two lateral regions into which the chromatin loops are anchored (Heng et al., 1994). This specialized tripartite structure formed of proteins and nucleic acids that ensures chromosome cohesion is termed the synaptonemal complex (SC[1]; von Wettstein et al., 1984).

Studies of the homotypic and heterotypic interactions involving protein components of the SC have contributed to the understanding of SC assembly on the chromosomes. COR1(SCP3) has the ability to form strong homotypic interactions (Tarsounas et al., 1997) and to assemble into aggregates with the features of intermediate filaments when heterologously expressed in somatic cells (Yuan et al., 1998). These filaments exhibit transverse striations similar to those reported in the lateral elements of some fungi and insects (Moens, 1969; Zickler, 1973). The central region contains transverse filaments that close like a zipper at synapsis. The major component of these filaments is the SYN1(SCP1) protein which self-interacts through its NH$_2$-terminal domain (Liu et al., 1996) located in the middle of the central region (Dobson et al., 1994; Schmekel et al., 1996). A similar central region architecture has been proposed for the SCs of the yeast *Saccharomyces cerevisiae*, with Zip1 protein forming the transverse connections between homologues (Tung and Roeder, 1998).

The relationship between the SC and meiotic recombination is complex and controversial, and seems to be highly variable from one species to another (reviewed in

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1. *Abbreviations used in this paper:* DSBs, double strand breaks; SC, synaptonemal complex; ssDNA, single-stranded DNA.

Roeder, 1997). Homologous recombination generates genetic variability during meiosis and the SC may facilitate its accomplishment by intimately connecting the homologous chromosomes. For example, a fully assembled SC is required for the late steps of meiotic recombination in mice (Stack, 1984), when maturation of recombination intermediates into chiasmata occurs. Also, certain yeast mutants defective in SC assembly show less than normal levels of meiotic recombination (Sym et al., 1993; Rockmill et al., 1995), lack of cross-over interference (Sym and Roeder, 1994), and reduced interhomologue recombination intermediates (Schwacha and Kleckner, 1997). Conversely, SC formation may require at least some of the recombination steps to be correctly completed (Bishop, 1994; Rockmill et al. 1995; Nairz and Klein, 1997).

Genes of the *RAD*52 epistasis group (*RAD*51, *RAD*52, *RAD*54, *RAD*55, and *RAD*57) promote repair of double strand breaks (DSBs) by homology-directed mechanisms (i.e., single-strand annealing and homologous recombination) in mitotic cells (Critchlow and Jackson, 1998; Haber, 1998) and by homologous recombination in meiotic cells (Shinohara and Ogawa, 1995; Kleckner, 1996). The eukaryotic *RAD*51 (Shinohara et al., 1992, 1997; Yoshimura et al., 1993; Benson et al., 1994) and *DMC*1 (Bishop et al., 1992; Kobayashi et al., 1993; Morita et al., 1993; Terasawa et al., 1995; Habu et al., 1996) genes encode the structural homologues of *Escherichia coli* strand transfer enzyme RecA (West, 1992). Human RAD51 (Baumann et al., 1996; Baumann and West, 1997; Sung and Robberson, 1995) and DMC1 (Li et al., 1997) proteins exhibit DNA-dependent ATPase activity and posses the ability to promote homologous DNA pairing and strand transfer reactions in vitro. These characteristics indicate significant functional similarities of the two proteins to the RecA recombinase.

Although they share a high degree of identity at the amino acid level and exhibit similar catalytic activities in vitro, the RAD51 and DMC1 proteins may play distinct roles in vivo. Eukaryotic RAD51 is expressed in both mitotic and meiotic cells, while DMC1 is meiosis-specific (Bishop et al., 1992; Habu et al., 1996). In yeast, the meiotic phenotypes of the *rad*51 and *dmc*1 single mutants appear to be similar in several respects: they accumulate DNA DSBs to levels higher than normal (Bishop et al., 1992; Shinohara et al., 1992; Schwacha and Kleckner, 1997) and exhibit significant reduction in homologue pairing and delayed synapsis (Rockmill et al., 1995). However, the critical difference is that the absence of *dmc*1 prevents interhomologue interactions, which illustrates the role of Dmc1 in promoting interhomologue recombination during meiosis (Schwacha and Kleckner, 1997). In mice, the *Rad*51 null mutation is lethal during embryogenesis (Lim and Hasty, 1996; Tsuzuki et al., 1996), while the *Dmc*1 null mutation causes meiotic arrest at zygotene without homologue synapsis (Pittman et al., 1998) or with occasional synapsis between non-homologues (Yoshida et al., 1998). This characteristic phenotype substantiates the role of DMC1 in promoting interactions between homologous chromosomes, which appears to be conserved from yeast to mammals.

In yeast and lily, Rad51 and Dmc1 proteins colocalize on foci along the early prophase I chromosomes (Bishop, 1994; Terasawa et al., 1995; Anderson et al., 1997; Dresser et al., 1997). Assembly of the Rad51 foci in yeast requires the Rad52, Rad55, and Rad57 proteins (Gasior et al., 1998), and in mammalian cell lines the RAD51 homologue XRCC3 (Bishop et al., 1998). Using an antibody that recognizes both Rad51 and Lim15 proteins, Anderson et al. (1997) have demonstrated in lily that Rad51/Lim15 are the components of some early recombination nodules (RNs). The early RNs are structures positioned in the central region of the SC and postulated to be involved in synaptic initiation (von Wettstein et al., 1984; Albini and Jones, 1987; Anderson and Stack, 1988; Zickler et al., 1992).

Here we present evidence for the colocalization of mouse RAD51 and DMC1 proteins in spermatocyte chromosome spreads. Using immunofluorescence and EM with immunogold labeling, we show that they colocalize through the early to middle stages of the meiotic prophase I. Both proteins are detected in Western blots of testis fractions containing early prophase I cells, consistent with the timing of their presence on the chromosomes. These observations are supported by protein–protein interaction analyses performed in vivo and in vitro, which suggest that RAD51 and DMC1 may form mixed recombination complexes by direct interactions between the two proteins. We show that RAD51 and DMC1 interact with the meiotic core component COR1(SCP3), and that only RAD51 interacts with the synaptic protein SYN1(SCP1). We propose that the SC provides the structural frame that may facilitate the formation of recombination complexes and that some steps of meiotic homologous recombination occur in the context of the SC.

Materials and Methods

Plasmids for the Two-Hybrid System

PCR reactions were performed with the *Pfu* polymerase (Stratagene) according to the manufacturer's instructions. The nucleotide sequences of the DNA primers used are available upon request. The full-length hamster *COR1(SCP3)* cDNA was PCR-amplified and inserted in the EcoRI-BamHI sites of pGAD424 and pGBT9 as described previously (Tarsounas et al., 1997). The full-length mouse *SYN1(SCP1)* cDNA was excised from a pET28a construct (Tarsounas et al., 1999) with NcoI, ends were filled in with the Klenow fragment of *E. coli* DNA polymerase I and further digested with XhoI. This fragment was subcloned into the SmaI-SalI sites of pGAD424 and pGBT9 vectors.

The full-length mouse *DMC*1 cDNA was PCR-amplified from a pET3 construct (Habu et al., 1996) and subcloned into the EcoRI-BamHI sites of pGBT9 and pGAD424. The full-length mouse *RAD*51 cDNA was PCR-amplified from a pET3 construct (Habu et al., 1996) and subcloned into the SmaI-BamHI sites of pGBT9 and pGAD424. The fragments of mouse DMC1 and RAD51 proteins used in two-hybrid and in vitro protein–protein interaction analyses are shown in Fig. 1. The cDNA fragment encoding DMC1N was PCR-amplified and inserted into the EcoRI-SalI sites of pGBT9 and pGAD424. The cDNA fragment encoding DMC1C was obtained by removing the 5′ StuI-EcoRI fragment from the construct containing full-length DMC1 cDNA in pGAD424. After digestion with these two enzymes, the ends were blunted with the Klenow fragment of *E. coli* DNA polymerase I and religated, rendering the pGAD424 vector plus a cDNA fragment that encodes DMC1C. The cDNA fragment encoding RAD51N was PCR-amplified and subcloned into the SmaI-SalI sites of pGBT9 and pGAD424. The cDNA fragment encoding Rad51C was obtained by removing the 5′ EcoRI fragment from the construct of full-length *RAD*51 cDNA in pGAD424. After EcoRI digestion, the ends were religated, rendering the pGAD424 vector with a cDNA fragment that encodes RAD51C. All constructs were sequenced with MATCHMAKER5′ primer (Clontech) to verify the correct open reading frames.

Two-Hybrid Interaction Analyses

The method for cotransformation of the GAL4 fusion constructs into the yeast strains HF7c and SFY526 (kindly provided by O. Kovalenko, Yale University, New Haven, CT) was described by Chen et al. (1998). The selective media required as well as the methods for determining β-galactosidase activity using the filter assay have been described previously (Tarsounas et al., 1997). Each one of the interactions reported was tested in at least three independent experiments.

Bacterial Plasmids for Protein Expression

For the in vitro protein–protein interaction experiments and polyclonal antibody generation we expressed the proteins involved as $(His)_6$ or HA fusions. The $(His)_6$-tagged proteins were generated in pET29b as COOH-terminal or in pET28a/pET3a as NH_2-terminal fusions. The COOH-terminal fusion of COR1(SCP3) with the $(His)_6$ tag was generated by PCR amplification of the coding region of hamster cDNA and cloning into the EcoRI-XhoI sites of pET29a. An NH_2-terminal $(His)_6$ fusion of the SYN1(SCP1)/SCP1 was generated in pET28a as previously described (Tarsounas et al., 1999) from a construct containing the full-length cDNA in pBluescript (provided by J. Sage, University of Nice). The pET3-derived plasmids used for expression of the DMC1 and RAD51 proteins $(His)_6$-tagged at the NH_2 terminus were described previously (Habu et al., 1996).

The HA fusions were generated in *E. coli* from a modified pET29a vector generated in our laboratory that we named pET29aHA. To introduce the HA tag into the pET29a vector we synthesized two complementary 45-bp oligonucleotides with the following DNA sequences: 5′-GGC CAT ATG TAC CCA TAC GAT GTT CCA GAT TAC GCT GGT ACC CGC-3′ and 5′-GCG GGT ACC AGC GTA ATC TGG AAC ATC GTA TGG GTA CAT ATG GCC-3′. After annealing and digestion with KpnI, the resulting product was subcloned into the pET29a (Novagen) expression vector that had been digested with NdeI, ends filled in with the Klenow fragment of *E. coli* DNA polymerase I and further digested with KpnI. With this procedure, we replaced the NH_2-terminal S-Tag™ (Novagen) normally present in the pET29 vector, with the HA epitope. The new tag can be removed by thrombin digestion (Fig. 2): 20 μg of soluble extract from bacteria induced to express HA-COR1(SCP3) was incubated for 15 min at room temperature with 0.25 U thrombin and thrombin buffer (20 mM Tris-HCl, pH 8.3, 150 mM NaCl, and 2.5 mM $CaCl_2$), or with thrombin buffer alone. The HA-tagged COR1(SCP3) was detected with an anti-HA antibody, while no major band is visible in the Coomassie staining of the same gel. Thrombin treatment abolishes the anti-HA staining (Fig. 2), indicating complete removal of the HA tag from the protein. All the constructs made in pET29aHA rendered NH_2-terminal in-frame fusions of the desired proteins with the HA tag and lacked the $(His)_6$ tag, as determined by DNA sequencing.

The full-length hamster *COR1(SCP3)* cDNA was excised as an EcoRI-SalI fragment from the pGBT9 construct (Tarsounas et al., 1997) and subcloned into the EcoRI-XhoI sites of the pET29aHA. The full coding region of mouse *SYN1(SCP1)* cDNA was PCR-amplified with *Pfu* polymerase (Stratagene) from a construct in pBluescript provided by J. Sage (University of Nice) and subcloned into the BamHI-XhoI sites of pET29aHA. The full-length mouse *DMC1* and *RAD51* cDNAs were excised as EcoRI-SalI fragments from the corresponding pGAD424 constructs (described below) and subcloned into the EcoRI-XhoI sites of pET29aHA.

To express the RAD51 and DMC1 NH_2-terminal fragments (DMC1N and RAD51N; Fig. 1) as HA fusions, the corresponding sequences were PCR-amplified from constructs of the full-length cDNAs in pGAD424 and subcloned into the EcoRI-XhoI sites of pET29aHA. The cDNA fragments encoding RAD51C and DMC1C were excised with EcoRI and SalI from the corresponding constructs in pGAD424 and subcloned into the EcoRI-XhoI sites of pET29aHA.

In Vitro Protein–Protein Interactions

To test protein–protein interactions in vitro, proteins were expressed as $(His)_6$ or HA fusions. The $(His)_6$-tagged protein was expressed in *E. coli* strain BL21(DE3) upon induction with 1 mM IPTG. After lysis and sonication in sonication buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 10 mM imidazole), the soluble fraction was incubated with Ni-NTA agarose (Qiagen) and the bound protein was washed three times in sonication buffer with 50–100 mM imidazole. The remaining unbound sites on the beads were blocked by incubation in storage buffer (Fuchs et al., 1997; 40 mM Hepes, pH 7.6, 150 mM NaCl, 10 mM EDTA, 1 mM DTT, 0.5 mM PMSF,

and 0.2% Nonidet P-40) for 2 h at room temperature. The protein bound to the Ni-NTA agarose was stored in 100% glycerol at −20°C. The HA-tagged proteins were expressed in *E. coli* strain BL21(DE3) after induction with 0.5 mM IPTG. After lysis under nondenaturing conditions and sonication the soluble fraction was isolated by centrifugation at 50,000 *g* for 1 h and stored at −20°C.

The protein-binding reaction contained 1× reaction buffer (Kovalenko et al., 1998; 20 mM Tris-HCl, pH 7.2, 25 mM NaCl, and 2 mM $MgCl_2$), 0.05% Tween 20, 50 μg BSA, 20 μl slurry with $(His)_6$-tagged protein preequilibrated in reaction buffer (∼10 μg of protein) and 50 μg soluble fraction of the bacterial extract containing the HA-tagged protein. The proteins were incubated for 1 h at room temperature on a Nutator. The beads were sedimented, washed three times with 500 μl reaction buffer with 50 mM imidazole, and resuspended in 2× SDS sample buffer (Laemmli, 1970) supplemented with 4 M urea. Samples were boiled for 5 min and separated on 10% or 12% SDS–polyacrylamide gels. The $(His)_6$-tagged protein was detected in the control reaction using an antibody specific for this protein. The presence of the HA-tagged protein in the eluted samples was detected with a rat monoclonal anti-HA antibody (Boehringer-Mannheim).

Antibodies

The mouse DMC1 and RAD51 full-length proteins were overexpressed in *E. coli* using the pET3 expression system (Habu et al., 1996). The $(His)_6$-tagged proteins were purified on a Ni-NTA agarose column (Qiagen) and injected into mice and rabbits, respectively. The preimmune sera gave no staining when tested on meiotic prophase chromosomes. The polyclonal anti-DMC1 serum was depleted of anti-RAD51 cross-reactive components by adsorption on Sepharose-bound (Pharmacia) mouse RAD51 protein. The depleted antiserum was used at a 1:200 dilution in blocking buffer (5% skim milk powder, 0.05% vol/vol Triton X-100 in PBS) for Western blotting and 1:2 dilution in antibody dilution buffer (10% vol/vol goat serum, 3% BSA wt/vol, 0.05% vol/vol Triton X-100 in PBS) for immunocytochemistry. Similarly, the rabbit anti-RAD51 polyclonal serum was immunodepleted of components recognizing DMC1 epitopes by adsorption on a DMC1-bound Ni-NTA agarose column (Qiagen). This procedure abolished the cross-reactivity with the DMC1 protein initially observed on Western blots. As an additional specificity test, the two polyclonal sera were each reacted separately with either the mouse DMC1 or RAD51 proteins. The immune complexes were pelleted by centrifugation, and the supernatant used in immunofluorescent staining of spermatocyte nuclei.

COR1(SCP3) was overexpressed in an *E. coli* expression system and purified using a $(His)_6$-Tag. The full-length protein was injected into mice to generate a polyclonal serum. COR1(SCP3) localization on meiotic chromosomes was described previously (Dobson et al., 1994; Tarsounas et al., 1999).

Cell Preparation for Immunofluorescence and EM

The localization of RAD51 and DMC1 antigens was determined on spread rat spermatocyte nuclei prepared as described in Tarsounas et al. (1999). Human CREST sera were used to identify the centromeres. The fluorochromes were visualized with a Polyvar epifluorescent microscope (200 W mercury-vapor light source) and recorded on 400ASA black/white or color slides. For EM, nuclei were attached to a 50-nm-thick plastic carrier film and treated for 5 min at room temperature with DNaseI at a final concentration of 1 μg/ml in MEM (GIBCO BRL). The surface-spread nuclei were incubated with primary antibody for 2 h at 37°C and with secondary antibody (goat anti–mouse conjugated with 5 nm gold, goat anti–rabbit conjugated with 10 nm gold and goat anti–human conjugated with 15 nm gold, 1/50 dilution) for 1 h at 37°C. The immunogold-labeled preparations were postfixed in 4% OsO_4 before observing under the EM. The electron microscope images were recorded at 8,500× magnification on 35-mm film.

Isolation of Spermatocyte Fractions

Testis preparation was performed as described by Tarsounas et al. (1999). Separation of the spermatocyte fractions by elutriation was performed following the procedure described by Grabske et al. (1975) and Meistrich (1977), with modifications by Heyting et al. (1985). 100 ml were collected from each fraction at increasing flow rates from 15 to 30 ml/min (5-ml/min increase at every step). The composition of these fractions in cells at various meiotic stages was assayed by immunofluorescent staining for the
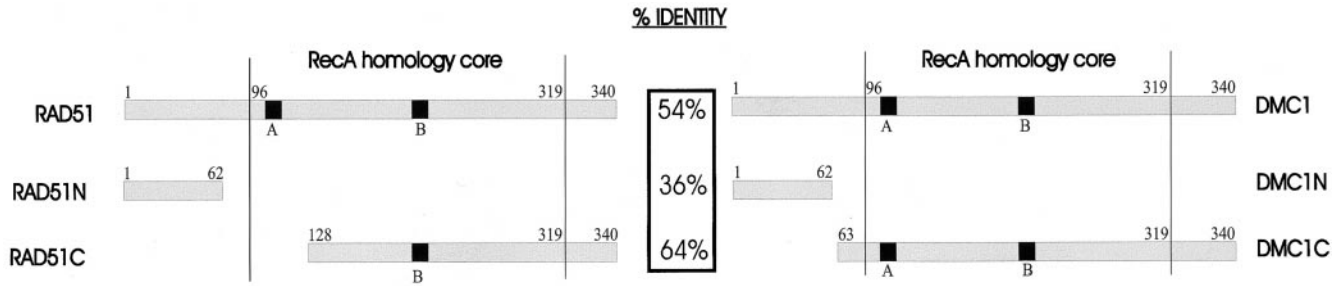
% IDENTITY

Figure 1. Truncation derivatives of the mouse RAD51 and DMC1 proteins used in two-hybrid and in vitro binding analyses. We divided the 340 amino acids into two regions with different degrees of identity between the two proteins. The black boxes depict nucleotide binding motifs (Story et al., 1993; Habu et al., 1996) located in the RecA homology core.

COR1(SCP3), SYN1(SCP1), and centromeric marker proteins. The composition varies from early to late prophase I stages (i.e., fraction I contains 45% leptotene cells, 45% zygotene cells, and 10% pachytene cells; fraction IV contains 85% pachytene cells and 15% leptotene/zygotene cells; fraction VI contains 80% diplotene cells and 20% pachytene cells). The number of spematogonia observed was too small to be taken into consideration. The cells in each fraction were centrifuged and resuspended in sample buffer (4 M urea, 10% SDS, 0.25 M Tris-HCl, pH 6.8, 20% glycerol, 0.015% bromophenol blue, 1 mM EDTA, and 10% vol/vol β-mercaptoethanol) to a concentration of 10^6 cells/ml. For Western blot analysis, protein corresponding to equal numbers of cells were loaded in each lane.

Results
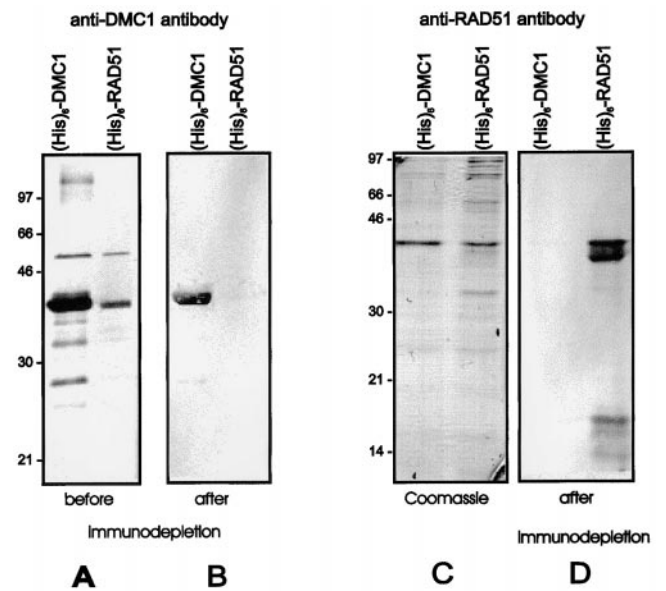
Specificities of the Polyclonal Antibodies

To determine the chromosomal distribution of RAD51 and DMC1, we generated antibodies against the full-length mouse proteins. As the overall degree of identity at the amino acid level between the two proteins is 54%,

each antibody displayed immunoreactivity with the homologous protein on a Western blot (e.g., the anti-DMC1 antibody recognized the RAD51 protein; see Fig. 3 A). To eliminate the cross-reactivity of the anti-DMC1 antibody with the RAD51 protein, we produced a Sepharose matrix with covalently bound RAD51. The polyclonal anti-DMC1 serum was adsorbed on this matrix twice; the immunodepleted antibody reacted with DMC1 but showed no reactivity with the RAD51 protein (Fig. 3 B). Similarly, the anti-RAD51 antibody was immunoadsorbed twice on a matrix containing the mouse DMC1 protein covalently attached to it. This treatment rendered a specific anti-RAD51 antibody (Fig. 3 D). Equal amounts of proteins
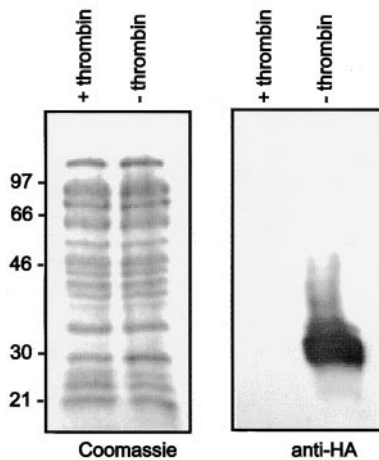

Figure 2. Thrombin removal of the HA-tag. The pET29a[HA] vector engineered here was tested for solubility of the target protein expressed and retention of the thrombin digestion site. COR1(SCP3) expressed in this system with an HA-tag at the NH$_2$ terminus, was detected in large amounts in the soluble fraction of the bacterial extract with an anti-HA antibody. The thrombin treatment removed the HA tag, indicating that the expression vector functions according to our design.

Figure 3. Specificity of the anti-RAD51 and anti-DMC1 antibodies determined on Western blots of affinity-purified proteins. The anti-DMC1 and anti-RAD51 polyclonal antibodies recognize both proteins on Western blot analyses (shown here for anti-DMC1 in A). To ensure specificity for their own antigen, each of the two antibodies was immunodepleted of components cross-reacting with the homologous protein. This procedure renders an antibody with specificity for its antigen (B and D). Equal amounts of the purified protein were loaded into the gel as shown in the Coomassie-stained gel (C). The second band in the RAD51 doublet in D corresponds to a degradation product.
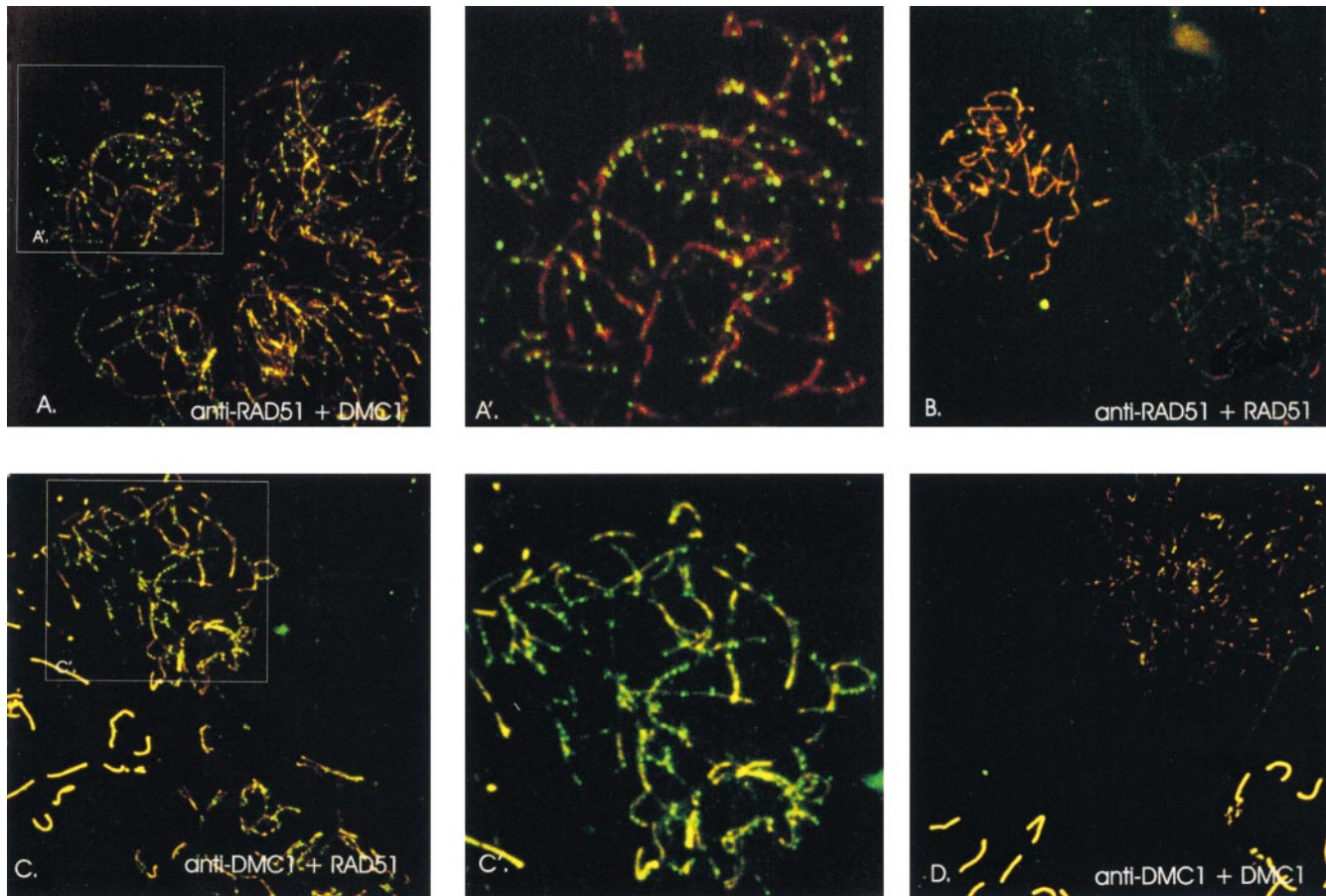
Figure 4. Specificity of anti-RAD51 and anti-DMC1 antibodies determined using an immunocytological approach. Anti-RAD51 and anti-DMC1 purified antibodies were each reacted with one and the other of the two proteins, the immune complexes removed by centrifugation, and the resulting antibodies analyzed on spermatocyte chromosome spreads. (A, A′, and B) Anti-DMC1 antibody (green) blocked with RAD51 recognizes the expected number of foci at early stages (A and A′; A′ is an enlargements of the area marked in A), while the same antibody blocked with DMC1 does not produce any green fluorescence signal (B). (C, C′, and D) Similarly, the signal generated with the anti-RAD51 antibody (green) is not affected by reaction with DMC1 protein (C and C′), while RAD51 blocks it completely (D). Note that the green fluorescence is enhanced in B and D to detect any possible signal associated with the chromosomes. Chromosomal cores are stained in red with mouse (A, A′, and B) or rabbit (C, C′, and D) anti-COR1 antibodies.

purified on Ni-agarose column were loaded in each lane, as shown in the Coomassie staining of the gel (Fig. 3 C). The purified antibodies were further used in immunostaining preparations for light and EM, and Western blotting of the spermatocyte fractions.

As an additional method for determining the specificity of the antibodies, we reacted each of them with the RAD51 and DMC1 proteins separately, discarded the immune complexes by centrifugation, and used the depleted sera in immunofluorescence staining of rat meiotic chromosomes (Fig. 4). Normal numbers and position of the RAD51 foci on the chromosome cores were observed when the anti-RAD51 antibody was reacted with the mouse DMC1 protein (Fig. 4, A and A′), and when the anti-DMC1 antibody was reacted with the mouse RAD51 protein (Fig. 4, C and C′). Fig. 4, A and C, encompass several nuclei of early prophase I spermatocytes, where the RAD51 and DMC1 proteins are abundant. In Figs. 4, A′ and C′, a section of the A and C figures is enlarged. When the anti-RAD51 antibody was reacted with the RAD51

protein, the ability of this antibody to stain discrete foci on the chromosomes was completely blocked (Fig. 4 B). Similarly, reacting the anti-DMC1 antibody with DMC1 protein abolished the staining by this antibody (Fig. 4 D). These experiments established that each purified antibody recognized only the protein against which it was generated.

EM Reveals That RAD51 and DMC1 Colocalize on Mouse Meiotic Chromosomes

Surface-spread mouse meiotic chromosomes were reacted with the purified rabbit anti-RAD51 and mouse anti-DMC1 antibodies, each visualized with EM of immunogold particles of different sizes (10 and 5 nm, respectively) attached to the secondary antibodies. The 15-nm gold particles identify the centromere stained with a human CREST serum. The majority of foci observed at various prophase I stages contained a mixture of 5- and 10-nm gold particles (Fig. 5 B′). We counted 150 of these EM foci
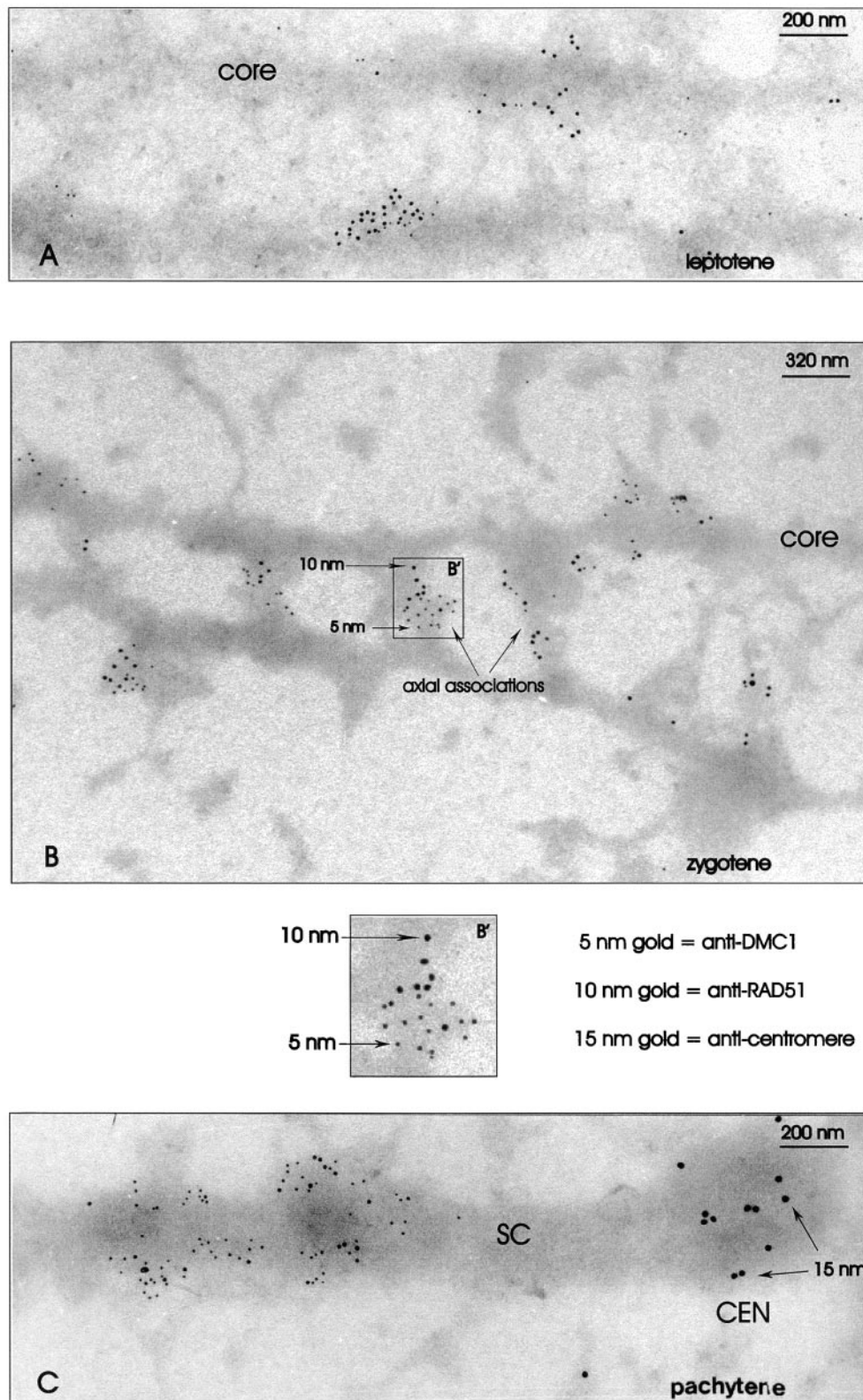
Figure 5. Colocalization of RAD51 and DMC1 proteins with immunogold EM. DMC1 is visualized with a goat anti–mouse secondary antibody conjugated with 5-nm-gold particles. RAD51 is visualized with a goat anti-rabbit secondary antibody conjugated with 10-nm-gold particles. Centromeres are stained with a human CREST serum visualized here with 15-nm-gold grains. (A) In the leptotene configuration, the grains identify mixed RAD51/DMC1 foci attached to individual chromosome cores. (B) At zygotene, the RAD51/DMC1 foci colocalize with axial associations established between homologous chromosomes, the sites of synapsis initiation. The B′ section is enlarged to point out the differences in size between the gold particles. (C) At pachytene, the RAD51/DMC1 foci localize along the fully synapsed chromosomes.

present in leptotene, zygotene, and pachytene cells, and 11% of them contained only one size of grains. This is consistent with the null class estimate predicted from the Poisson distribution of the results (data not shown). It seems reasonable to conclude that the foci contain both sizes of grains, and the presence of foci with a single grain size is due to the random binding of the antibody to the available sites. This distribution pattern of the gold grains indicates that the two proteins colocalize on the chromosomal cores before they synapse and in the confines of the SC, suggest-

ing that they might act in a concerted manner during meiosis. Interestingly, at leptotene, when the cores are in the process of alignment, the gold grains are physically associated with the cores (Fig. 5 A). Later on, at zygotene, the grain distribution coincides extensively with interstitial connections between the homologues (Fig. 5 B), cytologically similar to the axial associations reported in yeast to be the synapsis initiation sites (Rockmill et al., 1995). In lily and mice, these sites most likely identify the early recombination nodules (Anderson et al., 1997; Moens et al., 1997). At pachytene (Fig. 5 C), the grains label electron-dense structures localized in the central region of the SC.

Stage-specific Distribution of the RAD51/DMC1 Complexes

Because the foci observed with EM contained both RAD51 and DMC1 recombination proteins, we refer to them as recombination complexes. We attempted to show that at the level of fluorescence microscopy the numbers and relative positions of DMC1 and RAD51 foci are also identical. The distribution of RAD51 foci during successive stages of the meiotic prophase I in mammalian oocytes and spermatocytes has been reported previously (Moens et al., 1997; Barlow et al., 1997a). Using double

staining of the DMC1 and RAD51 proteins (Fig. 6) and superimposing the two colors as described in Gasior et al. (1998), it can be seen that RAD51 and DMC1 foci coincide. A correspondence of 95% was recorded for 10 prophase nuclei. This is in agreement with the colocalization results of EM where 89% of the foci are mixed. As the distribution of COR1(SCP3) during meiosis is well characterized (Dobson et al., 1994; Lammers et al., 1994), we used a mouse anti-COR1(SCP3) antibody (red) to identify the prophase I stages and sub-stages. This antibody was added in trace amounts to merely mark the cores, without interfering with the staining of the anti-DMC1 antibody also generated in a mouse. In Fig. 6 A the leptotene nucleus was identified based on the fact that the cores are just beginning to form, while in the adjacent zygotene nucleus, the cores are extensive (Fig. 6 C). The coincident staining with anti-RAD51 and anti-DMC1 antibody shows that the foci are associated with the cores (Fig. 6, C and F) and that the number of foci per nucleus is high at leptotene/zygotene (250–300 foci per nucleus) and low at pachytene (∼100 foci or less per nucleus).

To monitor the presence of the two recombinases at progressive stages of meiotic prophase, we have isolated fractions of testicular cells by centrifugal elutriation (Grabske et al., 1975). In general, this method allows cell
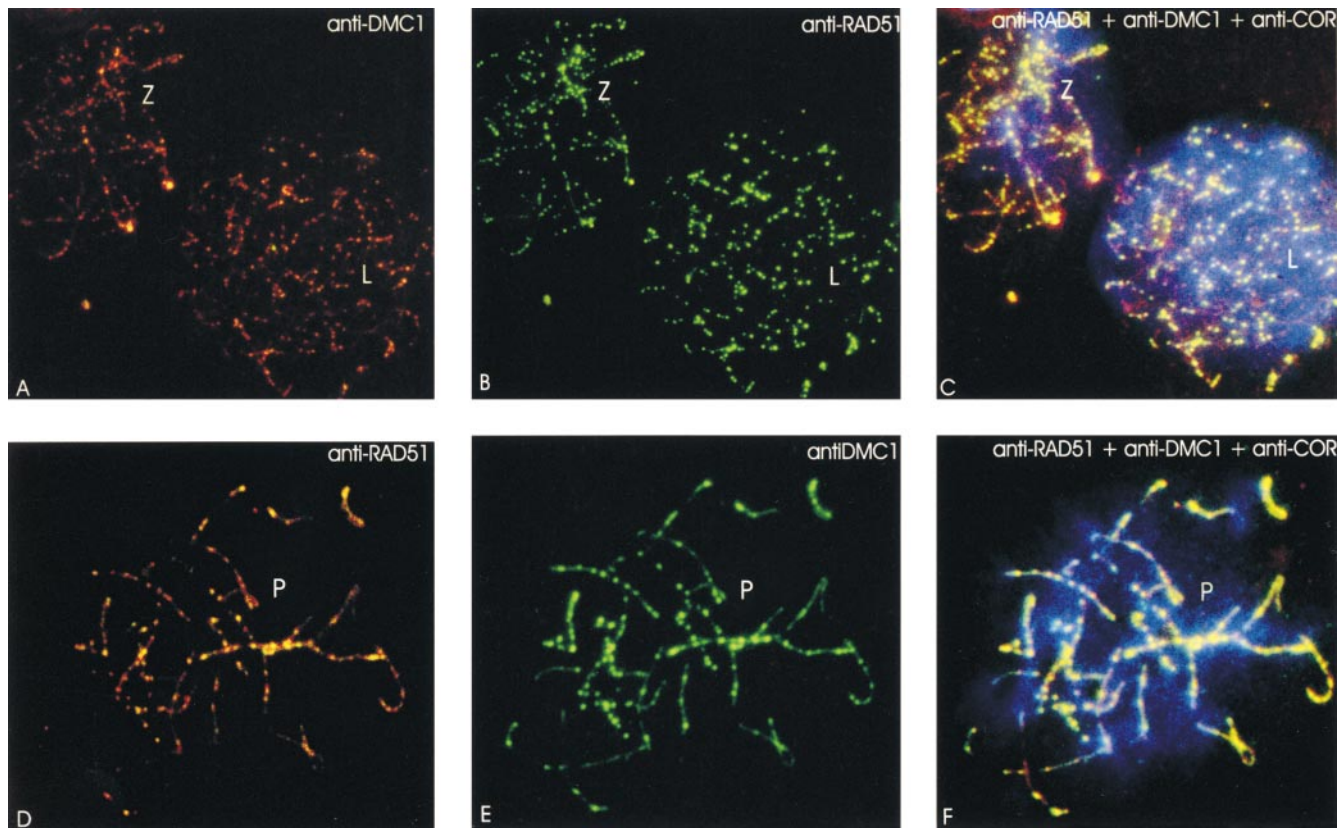

Figure 6. Colocalization of RAD51 and DMC1 proteins with immunofluorescence labeling. Immunodepleted mouse and rabbit antibodies were used to detect DMC1 and RAD51, respectively, on rat surface-spread spermatocytes. The cores are stained with trace amounts of a mouse anti-COR1(SCP3) antibody, visible in C and F. DNA is stained with DAPI (blue). (A–C) In early prophase I stages (leptotene, L, and zygotene, Z) RAD51 and DMC1 foci are positioned along the cores of homologous chromosomes. To a red-labeled focus in A corresponds a green-labeled focus in B in 95% of the cases. (D, E, and F) At pachytene (P) the RAD51/DMC1 foci are positioned along the SCs and are reduced in number as compared with previous stages.

fractionation by size. As the size of spermatocytes increases with their progression through meiosis, it is possible to separate prophase I cells in fractions enriched for cells at specific meiotic stages (Tarsounas et al., 1999). A sample of each fraction was fixed in 2% paraformaldehyde and used to determine the composition in cells at various meiotic stages, using immunofluorescence staining with anti-SYN1(SCP1), anti-COR1(SCP3), and anti-centromeric antibodies. The prophase I spermatocytes increased from 90% leptotene/zygotene cells in fraction I to 80% diplotene cells in fraction VI (data not shown). To demonstrate that the protein extracts prepared from these fractions give an accurate representation of the stages identified with immunofluorescence staining, we demonstrate the distribution of marker proteins COR1(SCP3) and SYN1(SCP1) in these fractions (Fig. 7). In accordance with its distribution on the meiotic chromosomes from early prophase to metaphase I, COR1(SCP3) was detected in all fractions as a constantly present 30-kD polypeptide. A higher molecular mass form of the protein was also detected, which changed from 35 kD in early prophase I to 33 kD in the late stages. Previously, COR1(SCP3) was being referred to as the 30/33-kD polypeptide, because of its constant detection on Western blots of testicular cells as a doublet of these sizes. The results presented here indicate that the COR1(SCP3) protein (estimated M_r 28,000) most likely corresponds to the 30-kD polypeptide, while the slower migrating band (35 or 33 kD) is possibly the result of posttranslational modifications such as phosphorylation (Lammers et al., 1995), which vary with the meiotic stage. SYN1(SCP1) was used as a second marker for the protein composition of the testicular fractions collected. This protein is known to be present on the chromosomes during zygotene when synapsis initiates, and at pachytene in synapsed chromosomes. The protein disappears at the end of pachytene/onset of diplotene when the homologues separate (Meuwissen et al., 1992; Dobson et al., 1994). Consistent with its presence in the meiotic chromosomes, the SYN1(SCP1) protein was only detected in fractions II–IV, and not in the fractions where the initial and final prophase I stages were predominant. The presence of RAD51 and DMC1 (Fig. 7) was only detected in the first three fractions containing cells in early prophase I stages, in accordance with the immunocytological observation of abundant RAD51/DMC1 foci on the chromosomal cores of early stages of prophase I.

Protein–Protein Interactions Involving RAD51 and DMC1 Proteins

We used the yeast two-hybrid system to test possible protein–protein interactions involving mouse RAD51 and DMC1 (Table I A). Homotypic interactions were detected for both mouse proteins, as shown previously for the yeast and human homologues (Donovan et al., 1994; Kovalenko et al., 1996, 1997; Dresser et al., 1997). We tested heterotypic interactions with each protein expressed either as a fusion to the GAL4 activation domain or to the GAL4 DNA-binding domain. Compared with their ability to self-interact, the heterotypic interactions between RAD51 and DMC1 were weaker in the sense that the reporter gene expression (*HIS*3 and *lacZ*) was
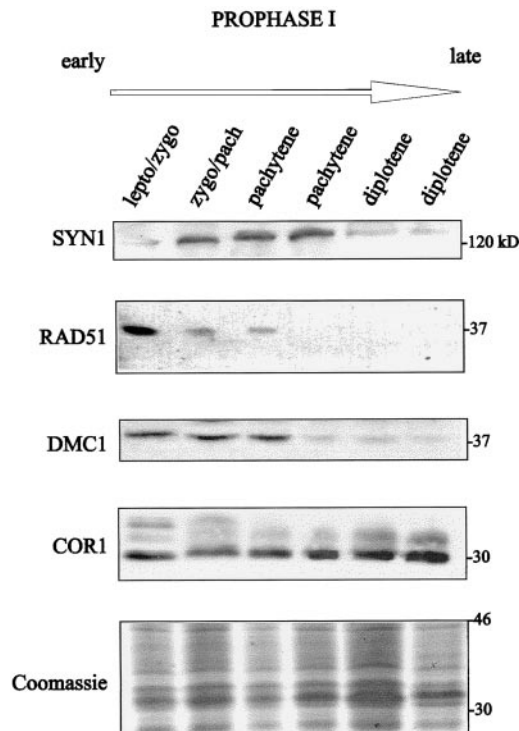
Figure 7. Western blot detection of RAD51, DMC1, and meiotic markers COR1(SCP3) and SYN1(SCP1) in rat spermatocyte fractions isolated by centrifugal elutriation. The composition of each fraction was estimated from the distribution of COR1(SCP3), SYN1(SCP1), and centromeres with immunofluorescence staining. Protein amounts corresponding to equal numbers of cells were loaded into each lane. The presence of COR1(SCP3) and SYN1(SCP1) in this Western blot follows the expected temporal pattern (see Discussion). RAD51 and DMC1 are detected only in fractions enriched in early prophase I spermatocytes, consistent with fluorescence and EM observations.

delayed. In the negative controls no expression was detected under identical conditions.

The interaction between RAD51 and DMC1 was also tested using an in vitro assay in which the RAD51 protein was expressed in *E. coli* as a (His)$_6$-fusion and bound to Ni-NTA agarose column. The HA-tagged DMC1 from a soluble *E. coli* extract was incubated with the RAD51 matrix, precipitated, and detected with an anti-HA antibody (Fig. 8 A). These data indicate that the formation of the RAD51/DMC1 complexes can be mediated by direct interactions between these two proteins. As a positive control for this experiment, precipitation of HA-DMC1 with a (His)$_6$-DMC1 fusion confirms the homotypic interaction detected in the in vivo two-hybrid assay.

Next, we wished to determine which region of the mouse RAD51 and DMC1 proteins was essential for homotypic and heterotypic interactions. We investigated whether the NH$_2$-terminal domains (Fig. 1), which show the lowest identity between the two proteins, could have distinct abilities in establishing protein–protein interactions. In a two-hybrid assay, we show that the DMC1 COOH-terminal fragment (DMC1C; M_r 31,000) containing amino acids 63–340 interacts with the full-length pro-

Table I. Protein–Protein Interactions Analyzed in the Two-Hybrid System

A. Interactions involving RAD51 and DMC1 proteins and their derivatives

		pGAD424						
		RAD51	RAD51N	RAD51C	DMC1	DMC1N	DMC1C	No insert
pGBT9	RAD51	+*	−	+	+/−	−	+/−	−
	DMC1	+/−‡	−	+/−	+	−	+	−

B. Interactions involving RAD51, DMC1, and SC components

		pGAD424			
		COR1(SCP3)	SYN1(SCP1)	SCP2C	No insert
pGBT9	RAD51	+	+	−	−
	DMC1	+	−	−	−
	COR1 (SCP3)	+	−	+	−

* + indicates a strong interaction, i.e., a His+ phenotype was detected within 3 d of growth at 30°C on minimal medium supplemented with Ade and Lys, and a LacZ+ phenotype was detected within 1 h of incubation at 30°C in the presence of X-gal.

‡ +/− indicates a weak interaction, i.e., under the conditions described above, the His+ phenotype is detectable after 3 d of growth at 30°C, and detection of the LacZ+ phenotype requires 8 h incubation. With identical growth conditions and incubation times, neither *HIS*3 nor *lacZ* are expressed in the negative controls.

tein (340 amino acids; M_r 37,800), while the NH$_2$-terminal fragment (DMC1N; M_r 7,200) containing amino acids 1–62 does not. This indicates that the COOH-terminal region, or a portion of it, is required for the DMC1 homotypic interaction to occur. Similarly, the COOH-terminal RAD51 fragment (RAD51C; M_r 23,000), or a portion of it, mediates RAD51 self-interacting capacity. In addition, the COOH-terminal region of RAD51 is able to interact weakly with the full-length DMC1 in a two-hybrid assay and conversely, the COOH terminus of DMC1 establishes weak interactions with the full-length RAD51 (Table I A). These data suggest that the COOH-terminal regions of the two proteins may be involved in mediating heterotypic RAD51/DMC1 interactions as well.

The homotypic interactions observed in the two-hybrid assays were confirmed by the in vitro binding experiments. Fig. 8 B shows that only DMC1C, and not DMC1N, interacts with the full-length DMC1 protein. The truncated derivatives of RAD51 act similarly in the presence of the full-length RAD51 protein. These data suggest that the NH$_2$- and COOH-terminal regions of the two proteins behave similarly in mediating self-interactions.

### RAD51 and DMC1 Interact with SC Components In Vivo and In Vitro

To gain further insight into the relationship between the SC and the RAD51/DMC1 recombinases, we tested possible interactions between the SC proteins and the components of recombination complexes. We performed in vivo two-hybrid assays with DMC1, RAD51, and each of the SC components, SYN1(SCP1) and COR1(SCP3), the results of which are presented in Table I B. COR1(SCP3) is a component of the chromosomal cores from early prophase I when the cores start to assemble, to pachytene when the cores synapse and form the lateral domains of the SC, and until late diplotene when the homologues separate from each other (Dobson et al., 1994; Lammers et al., 1994; Tarsounas et al., 1999). Both RAD51 and DMC1 interact with COR1(SCP3) in the in vivo two-hybrid system and in the in vitro coprecipitation assays. This is consistent with the presence of the recombination complexes in association with the cores from leptotene to pachytene, as detected

with electron and light microscopy (Figs. 5 and 6). SYN1 (SCP1) is pachytene-specific, possibly identifying the protein that establishes and maintains synapsis between homologues (Meuwissen et al., 1992; Dobson et al., 1994; Tarsounas et al., 1999). RAD51 interacts with this synaptic protein, while DMC1 does not (Fig. 8 B).

The third component of the SC tested for interaction with the RAD51/DMC1 recombinases was SCP2. This protein localizes in the cores/lateral domains of the SC and has a temporal and spatial distribution very similar to that of COR1(SCP3) (Offenberg et al., 1998). In a two-hybrid screen of a hamster testis cDNA library for proteins interacting with COR1(SCP3) (Tarsounas et al., 1997), we have isolated a 50-kD polypeptide from the COOH-terminal region of the SCP2 protein (M_r 190,000). We tested this fragment in a two-hybrid assay for its ability to participate in direct interactions with the DMC1/RAD51 proteins. No interaction was detected, suggesting that the COOH-terminal SCP2 region which mediates the interaction between the two core components (COR1(SCP3) and SCP2) is not required for the binding of the recombination complexes to the cores. This finding does not exclude the possibility that another region of the large SCP2 protein may be functional in this respect.

Discussion

A major difficulty in studying the distribution of RAD51 and DMC1 in mammalian meiotic chromosomes is that sera raised against the full-length proteins recognize both proteins in immunoblots and presumably in cytological preparations. A polyclonal serum raised against a 15–amino acid NH$_2$-terminal peptide of RAD51, while showing a high specificity for RAD51 on Western blots, does not render any signal on meiotic chromosome spreads (data not shown), possibly because the NH$_2$ terminus of this protein is not available to the antibody. To differentiate between these two proteins, we have used sera that were depleted of the cross-reactive antibodies. The high specificity of the resulting antibodies was apparent in Western blot analyses of bacterially expressed proteins (Fig. 3) and in spermatocyte immunostaining in which
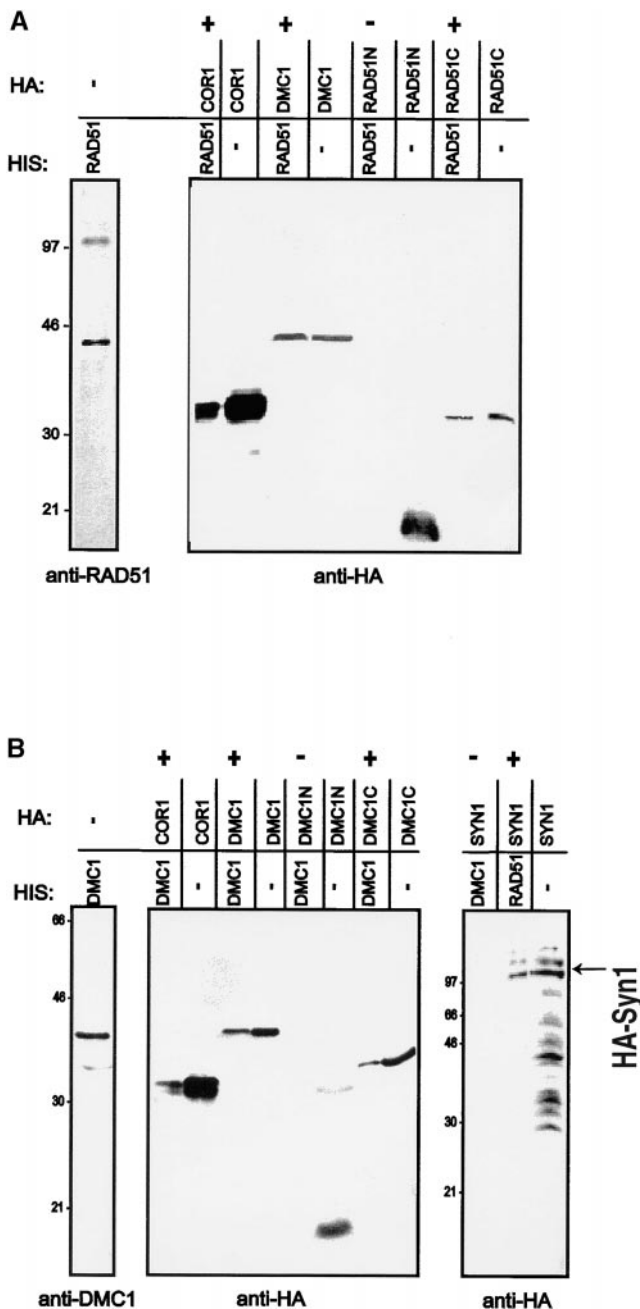
Figure 8. Protein–protein interactions determined in vitro with a pull-down assay. The two His-tagged proteins are detected with specific anti-RAD51 (A) and anti-DMC1 (B) antibodies. The same amount of His-tagged protein bound to a Ni-NTA agarose column was incubated with a soluble bacterial extract expressing the HA-tagged protein (in the amounts shown in the lanes containing HA-tagged protein only). Interacting proteins were coprecipitated. After washes and elution from the beads, the HA-tagged proteins engaged in an interaction with the His-tagged protein were detected with an anti-HA antibody, as described in the Materials and Methods. + Indicates interaction, and − lack of interaction.

each antibody was prereacted with the antigen or with the homologous protein (Fig. 4). Data presented here, therefore, accurately reflect the distribution of the two proteins on the chromosomes.

Mouse RAD51 and DMC1 Proteins Colocalize on the Cores of Mouse Meiotic Chromosomes at Early Prophase Stages

In yeast, Rad51 and Dmc1 proteins form discrete foci along the meiotic chromosome cores with variable degrees of colocalization (Bishop, 1994; Dresser et al., 1997). We report here the results of a similar study performed on mammalian meiotic chromosomes. The larger size of these chromosomes, as well as the spreading techniques available for their fixation allow a more detailed analysis of the proteins associated with the chromosomal cores. The RAD51 and DMC1 foci detected by immunofluorescence staining of rat spermatocyte nuclei with antibodies against each of the two proteins overlap in 95% of the cases. This observation does not necessarily imply that the two proteins colocalize on the chromosomes (Freire et al., 1998). Thus, we attempted to define the relative position of the RAD51 and DMC1 foci using EM with immunogold visualization. RAD51 was visualized with 10-nm gold grains and DMC1 with 5-nm gold grains. The two types of particles occurred in mixed groups in 89% of the cases, while only 11% of the groups were composed of single-size particles. According to the Poisson analysis of our numeric data, the latter focus type is expected to occur by chance alone if the number of labeled proteins is small relative to the total number of protein molecules per focus. Similarly, in immunofluorescence staining of meiotic nuclei a small percentage of foci (~5%) were composed of only one or the other type of protein. We conclude that RAD51 and DMC1 colocalize in mixed foci on the mouse meiotic chromosomes.

The number of RAD51/DMC1 complexes is most abundant at early meiotic stages and decreases thereafter, becoming undetectable at late pachytene/early diplotene. Consistent with this is the detection of the two proteins in spermatocyte fractions containing predominantly early meiotic stages (Fig. 7). The downregulation of DMC1/RAD51 during meiotic prophase I is correlated with the progression of recombination. The DMC1/RAD51 complexes are most abundant at the leptotene/zygotene stages, during which homologous recombination is initiated, and less numerous at pachytene, when maturation of the recombination intermediates is postulated to occur. This is consistent with a role for these proteins in the early steps of meiotic recombination, presumably in homology recognition and initiation of strand exchange reactions as previously proposed (Baumann et al., 1996).

The Relevance of RAD51 and DMC1 Homotypic and Heterotypic Interactions for the Assembly of the Two Proteins on the Meiotic Chromosomes

We addressed the question whether the RAD51/DMC1 mixed foci observed with immunocytological methods are formed by direct interactions between the mouse RAD51 and DMC1 proteins. The interaction detected between RAD51 and DMC1 in an in vivo two-hybrid assay was weak, as estimated from the delayed expression of the *HIS*3 and *lacZ* reporter genes. One possibility is that the interaction between RAD51 and DMC1 is indirect and requires an adaptor protein. A strong candidate for such an adaptor protein is the yeast Tid1 (Dresser et al., 1997), a

Rad54 homologue which interacts with both Rad51 and Dmc1 in a two-hybrid screen. It is possible that the yeast Tid1 protein can mediate the interaction between the two mouse homologues when they are expressed in the same yeast cell in the two-hybrid assay. Its recruitment, however, may delay the establishment of an interaction between the two proteins and, consequently, the expression of the reporter genes. The second possibility is that each of the two proteins (RAD51 and DMC1) form homotypic interactions very easily, especially in the presence of the DNA substrates as may be the case in vivo, and, therefore, little protein is left available for the heterotypic interaction to occur (Dresser et al., 1997). Using biochemical assays, we showed that RAD51 and DMC1 can be coprecipitated in vitro (Fig. 8). This suggests that the mixed RAD51/DMC1 foci detected immunocytologically may assemble through direct interactions between the two proteins.

Determining the regions of RAD51 and DMC1 involved in homotypic interactions will help understand the assembly of the recombination complexes on the chromosomes. We used truncated derivatives of the two proteins in two-hybrid and in vitro binding experiments, and showed that a version of RAD51 or DMC1 bearing a deletion at the NH_2 terminus still allows establishment of heterotypic and homotypic interactions.

Biochemical studies have shown that RAD51 assembles nucleoprotein filaments with single- and double-stranded DNA (Benson et al., 1994; Baumann and West, 1997) and DMC1 may have similar properties (Li et al., 1997). Our EM data indicating colocalization of these proteins on the meiotic chromosomes and their ability to establish direct heterotypic protein–protein interactions suggest the possibility that RAD51/DMC1 form mixed filaments associated with DNA at meiosis. Moreover, we scored 150 EM foci containing >1,500 gold grains and found that 46% of the grains correspond to RAD51 and 54% to DMC1 (data not shown). Therefore, the possibility that these filaments have a constant stoichiometry of RAD51 to DMC1 molecules is not excluded.

The Synaptonemal Complex Functions as a Protein Frame for Attachment of Recombination Complexes

We show here that interactions between RAD51/DMC1 complexes and SCs are established by direct protein–protein interactions between their components. These are detected in an in vivo two-hybrid system and in vitro binding analyses using bacterially expressed proteins. Coimmunoprecipitation of these proteins from testis extracts is technically unattainable due to the high insolubility of the SC components in these extracts (Tarsounas, M., P. Moens, and R.E. Pearlman, unpublished data). The interactions detected between SC components and the two recombinases support the cytological observations from immunofluorescence and EM, where the RAD51/DMC1 complexes are detected in association with the chromosomal cores at the very early prophase I stages or with the SCs at pachytene (Barlow et al., 1997a; Moens et al., 1997; this study). Based on these data, it is possible that the SC components may provide the structural frame that stabilizes complexes formed of recombination proteins and DNA (Yuan et al., 1998).

Several lines of evidence support this hypothesis. In mammals, SC formation is obstructed in the absence of Atm (Barlow et al., 1996; Keegan et al., 1996; Xu et al., 1996). This structural defect may cause the mislocalization of RAD51/DMC1 complexes detected in the Atm−/− spermatocytes (Barlow et al., 1997b, 1998). In yeast, the core-associated Red1 protein with a meiotic distribution similar to that of COR1/SCP3 (Smith and Roeder, 1997) is necessary for recombination (Rockmill and Roeder, 1990; Storlazzi et al., 1996) and specifically for normal levels of interhomologue joint molecule formation (Schwacha and Kleckner, 1997).

Using genetic analyses and EM of spread yeast chromosomes, Rockmill et al. (1995) have shown that Rad51 and Dmc1 proteins are required to establish the axial associations between homologues, which represent the synapsis initiation sites. Here we show that in rat chromosome spreads the RAD51/DMC1 complexes coincide with cytologically similar axial associations. The gold grains may detect a nucleoprotein filament formed at the site of a DSB after resection of the ends as predicted (Szostak et al., 1983; Bishop, 1994). This nucleoprotein filament bridges the two homologues and possibly plays a role in homology recognition as well. By these criteria, a RAD51/DMC1-coated ssDNA visualized in association with single cores at leptotene/early zygotene (Fig. 5 A) may recognize homologous DNA sequences on another chromosome and establish the axial associations visualized in Fig. 5 B, as previously suggested (reviewed by Baumann and West, 1998). This hypothesis is also supported by the recent observation that RAD51 foci are associated with ssDNA in meiotic cells (Raderschall et al., 1999).

At pachytene, the gold grains appear in the central region of the SC (Fig. 5 C). The recombination complexes may be anchored there by a direct interaction of RAD51 with the synaptic protein SYN1(SCP1) which also localizes in the central region of the SC (Meuwissen et al., 1992; Dobson et al., 1994). We detected such a direct interaction between RAD51 and SYN1(SCP1) in the two-hybrid and in vitro binding analyses. The significance of this is unclear at present. It is possible, however, that the few recombination complexes present at pachytene are anchored in the central region of the SC through a direct interaction with SYN1(SCP1).

In addition to the RAD51/DMC1 recombinase, other proteins involved in DNA repair (e.g., MLH1, BRCA1, BRCA2), as well as sensors of DNA damage (e.g., HRAD1, ATR) have been detected in association with the meiotic chromosome cores and/or the SCs (Baker et al., 1996; Keegan et al., 1996; Scully et al., 1997; Barlow et al., 1998; Chen et al., 1998; Freire et al., 1998; Moens et al., 1998). This suggests a concerted action of all these proteins in the repair of meiotic DSBs and maintenance of genomic integrity in the germ line. Determining the molecular details of this process requires further experimentation.

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