

Meiotic cohesin REC8 marks the axial elements of rat synaptonemal complexes before cohesins SMC1 β and SMC3

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In meiotic prophase, the sister chromatids of each chromosome develop a common axial element (AE) that is integrated into the synaptonemal complex (SC). We analyzed the incorporation of sister chromatid cohesion proteins (cohesins) and other AE components into AEs. Meiotic cohesin REC8 appeared shortly before premeiotic S phase in the nucleus and formed AE-like structures (REC8-AEs) from premeiotic S phase on. Subsequently, meiotic cohesin SMC1 β , cohesin SMC3, and AE proteins SCP2 and SCP3 formed dots along REC8-AEs, which extended and fused until they lined REC8-AEs along their length. In metaphase I, SMC1 β , SMC3, SCP2, and SCP3 disappeared from the

chromosome arms and accumulated around the centromeres, where they stayed until anaphase II. In striking contrast, REC8 persisted along the chromosome arms until anaphase I and near the centromeres until anaphase II. We propose that REC8 provides a basis for AE formation and that the first steps in AE assembly do not require SMC1 β , SMC3, SCP2, and SCP3. Furthermore, SMC1 β , SMC3, SCP2, and SCP3 cannot provide arm cohesion during metaphase I. We propose that REC8 then provides cohesion. RAD51 and/or DMC1 coimmunoprecipitates with REC8, suggesting that REC8 may also provide a basis for assembly of recombination complexes.

Introduction

Cohesion between sister chromatids is essential for proper chromosome disjunction and homologous recombination in the mitotic cycle and in meiosis (Nasmyth, 2001). It depends on a protein complex, cohesin, which consists of four proteins (cohesins) (Guacci et al., 1997; Michaelis et al., 1997; Losada et al., 1998). The four cohesins, named Smc1, Smc3, Scc1, and Scc3 in yeast, contribute in stoichiometric amounts to the cohesin complex and each is essential for cohesion (for reviews see Hirano, 2000; Koshland and Guacci, 2000; Nasmyth, 2001; Jessberger, 2002). In the mitotic cycle of yeast, cohesins bind to chromatin before S phase, whereas cohesion is established during S phase (Uhlmann and Nasmyth, 1998). Cohesion is maintained until the metaphase to anaphase transition, when it is released upon cell cycle-regulated proteolytic cleavage of Scc1 (Uhlmann et al., 1999). Proteins homologous to the yeast

cohesins occur in all analyzed eukaryotes. Higher eukaryotes may possess more than one variant of one or more mitotic cohesins, whereas yeast has only one of each (Losada et al., 2000; Waizenegger et al., 2000).

In meiosis, cohesins also function in chromosome segregation, but in a modified way. In the meiotic cell cycle, two nuclear divisions (meioses I and II) follow a single S phase. Sister chromatid cohesion can ensure proper chromosome segregation in both meiotic divisions, because it is released in two steps (Buonomo et al., 2000). At anaphase I, cohesion between sister chromatid arms is lost, which leads to disjunction of homologous chromosomes, and at anaphase II, centromeric cohesion is lost so that sister chromatids can separate (see discussion in Buonomo et al., 2000).

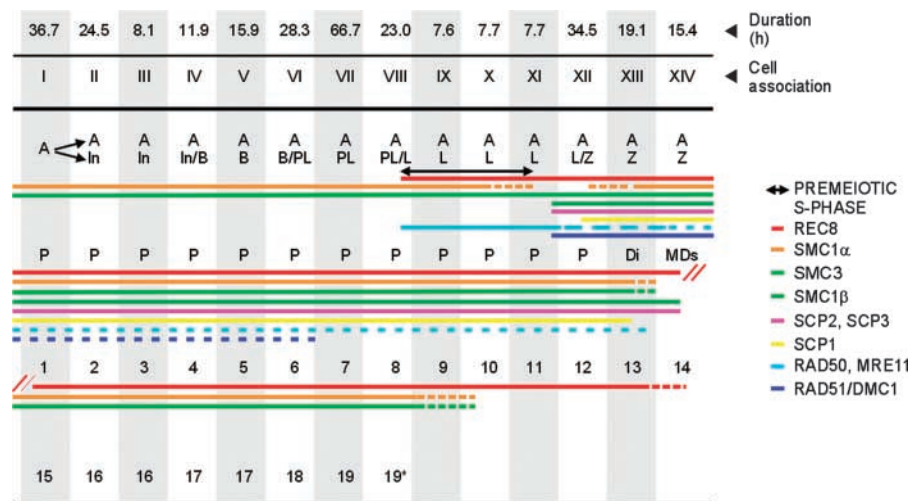
Cohesins not only function in chromosome segregation, but also in DNA repair by homologous recombination (Jessberger et al., 1996; Hirano, 2000; Jessberger, 2002). In the mitotic cycle, this role may include the assembly of recombinational repair complexes (Hartsuiker et al., 2001) and the direction of recombinational repair toward the sister chromatid rather than the homologous chromosome, if there is one (Grossenbacher-Grunder and Thuriaux, 1981). In somatic mammalian cells, recombination between sister chromatids is a prominent pathway of DNA repair (Johnson and Jasin, 2000). In meiosis,

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Figure 1. The 14 successive cell associations (numbered I–XIV) in testicular tubules of the rat. Each column represents one cell association, with the cell types found in that association. A, In, and B, A-type, intermediate, and B-type spermatogonia; PL, L, Z, P, and Di, preleptotene, leptotene, zygotene, pachytene and diplotene spermatocytes; MDs, meiotic divisions; 1–19, successive differentiation stages of spermatids; 19*, mature spermatozoa. The top of the figure shows the life span of each cell association in Wistar rats (Hilscher and Hilscher, 1969). The horizontal lines indicate when various proteins appear and when they disappear. For RAD50 and MRE11, the continuous line indicates when these proteins are abundant throughout the nuclei, whereas the broken line shows in which stages of meiosis these proteins are only abundant in sex vesicles (XY bivalents) (Eijpe et al., 2000b). REC8 is present until anaphase II and reappears in spermatids.



cohesins are also required for homologous recombination (Klein et al., 1999), but their role has been modified in such a way that recombination occurs preferentially between non-sister chromatids of homologous chromosomes rather than sister chromatids (Schwacha and Kleckner, 1997).

A third aspect of the altered role of cohesins in meiosis is their contribution to the assembly of synaptonemal complexes (SCs).^{*} SCs are zipper-like protein structures that are assembled between homologous chromosomes (homologues) during meiotic prophase. They play an only partly understood role in adapting recombination and cohesion for meiosis (Roeder, 1995; Kleckner, 1996). SCs consist of two axial elements (AEs), which are connected by transverse filaments. Each AE supports the two sister chromatids of one homologue. Cohesins are required for the assembly of AEs and constitute part of AEs (Klein et al., 1999; Eijpe et al., 2000a; Pelttari et al., 2001).

Given these specific roles of cohesins in meiosis, it is not surprising that meiotic variants of cohesins exist. Meiotic cohesin Rec8 replaces Scc1 in all species analyzed thus far. Rec11 of *Schizosaccharomyces pombe* (Krawchuk et al., 1999) and mammalian STAG3 (Pezzi et al., 2000) are meiotic variants of Scc3, and SMC1 β is a mammalian meiotic variant of SMC1 (further denoted as SMC1 α) (Revenkova et al., 2001).

Previously, using Mabs 462 (anti-SMC3) and β 70 (anti-SMC1 β), we found that in rat, SMC1 β (Eijpe et al., 2000a) and SMC3 (Revenkova et al., 2001) colocalized with meiotic AE components SCP2 (Offenberg et al., 1998) and SCP3 (Lammers et al., 1994). This agreed with the colocalization of Smc3 with AE component Red1 in yeast (Klein et al., 1999). However, according to our first approximation, SCP2, SCP3, SMC1 β , and SMC3 appeared simultaneously in AEs in leptotene, after premeiotic S phase (Offenberg et al., 1998; Eijpe et al., 2000a; Revenkova et al., 2001). This was unexpected for SMC1 β and SMC3, because the cohesin

complex as a whole is thought to bind to chromatin before S phase and to establish cohesion during S phase (Uhlmann and Nasmyth, 1998; Ciosk et al., 2000). Furthermore, SMC1 β and SMC3 had virtually disappeared from the chromosome arms at metaphase I (Revenkova et al., 2001), when arm cohesion is most needed for proper disjunction of homologues (Buonomo et al., 2000). In this study, we analyzed therefore in detail the presence and localization of cohesins in successive stages of meiosis of the male rat. We focused on REC8, which is a target of the cell cycle-regulated protease that releases cohesion in yeast meiosis I and II (Buonomo et al., 2000). Furthermore, we included SMC1 α in the analysis and a new anti-SMC3 serum.

Results

Experimental system

We studied the order of appearance of REC8, other cohesins, and AE components in testis sections and spread spermatocytes of rat. The sections were essential for the staging of the cells. Cross sectioned tubules of the rat testis display well-defined cellular associations, which consist of four to five cell layers (Fig. 1), with the earliest stages of spermatogenic differentiation in the outer cell layers and the latest stages near the lumen of the tubules. In rat, 14 cell associations have been defined, numbered I–XIV, based on spermatid morphology (Leblond and Clermont, 1952). Within a given association, cells differentiate coordinately so that each association as a whole develops into the next. This process is cyclic, because association XIV develops into association I. The life span of each cell association is precisely known for Wistar rats (Hilscher and Hilscher, 1969) and is given at the top of Fig. 1. In previous studies, we found that SCP2 and SCP3 first appeared in AEs in the outer layer of spermatocytes in tubules containing cell association XI (stage XI tubules) (Offenberg et al., 1991, 1998; Lammers et al., 1994), whereas transverse filament protein SCP1 (a marker for synapsis) appears slightly later, in stage XII (Scherthan et al., 1996). SMC3 (as detected by M α SMC3) and SMC1 β ap-

^{*}Abbreviations used in this paper: AE, axial element; AP, alkaline phosphatase; RN, recombination nodule; SC, synaptonemal complex.

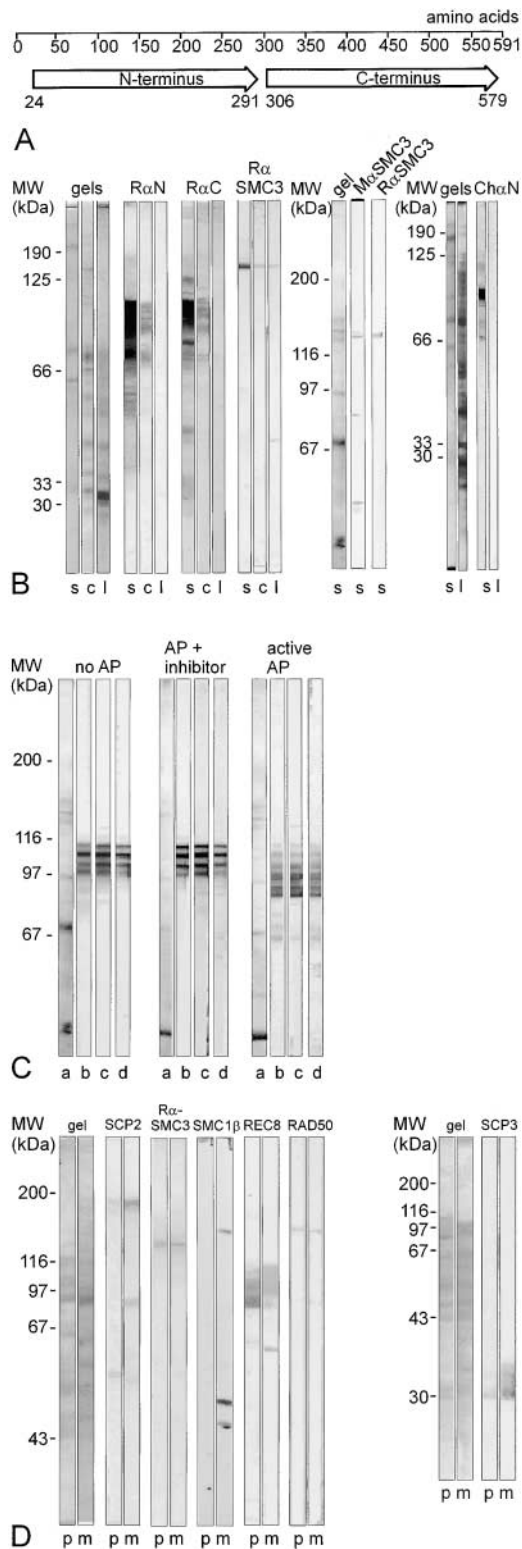


Figure 2. Western blot analyses using anti-REC8 antibodies. (A) Peptides of mouse REC8 that were used for immunization. (B) Binding of M α SMC3 and affinity-purified antibodies from R α N, R α C, Ch α N, or R α SMC3 (see Materials and methods) to immunoblot strips carrying proteins from purified SCs (s), spermatocyte nuclei (c), or liver nuclei (l). (C) Dephosphorylation of REC8; isolated SCs were treated with AP (right panel), AP and a specific inhibitor of this enzyme (middle panel), or AP buffer only (left panel) and analyzed by Western blot. Strips of the resulting blot were stained with Ponceau S (a) and probed with the affinity-purified anti-REC8

peared in AEs simultaneously with SCP2 and SCP3 (Revenkova et al., 2001), in stage XI tubules.

In this study, we focused on meiotic cohesin REC8. We immunized a rabbit and a chicken with the NH₂-terminal half of REC8 to obtain R α N and Ch α N, and a rabbit with the COOH-terminal half of REC8 to get R α C (Fig. 2 A). R α N, Ch α N, and R α C recognized a series of 80–100-kD bands on Western blots of spermatocyte nuclei (Fig. 2 B), whereas pre-immune sera did not recognize these bands (unpublished data). We thought it unlikely that the anti-REC8 antibodies bind to these multiple bands due to cross reactivity, because the antibodies originate from three different immunizations against two nonoverlapping fragments of REC8. Therefore, we assumed that the 80–100-kD bands represent various forms of REC8. These bands are not recognized in liver nuclear extracts (Fig. 2 B), which agrees with the testis-specific expression of REC8 (Parisi et al., 1999; Lee et al., 2002), whereas they are strongly enriched in purified SCs (Fig. 2 B), which fits with the localization of Rec8 along AEs in yeast (Klein et al., 1999). The REC8 bands migrated more slowly in SDS gels than expected, based on the predicted molecular mass of REC8 (67 kD), which could be due to the high proline content of this protein (Hames, 1990) or to posttranslational modifications. Because posttranslational modifications could also explain that anti-REC8 antibodies recognize multiple bands on Western blots, we analyzed REC8 phosphorylation in rat. Fig. 2 C shows Western blot strips of isolated SCs probed with anti-REC8 antibodies. The electrophoretic mobility of the REC8 bands increased if the SCs had been dephosphorylated with alkaline phosphatase (AP), but not if the SCs had been treated with AP and a specific inhibitor of this enzyme. We conclude, therefore, that REC8 is phosphorylated in SCs. Dephosphorylation did not reduce the pattern of REC8 bands to a single band, possibly because REC8 within SCs could not be dephosphorylated completely, and/or because REC8 carries other modifications besides phosphate groups. R α N, Ch α N, and R α C produced essentially the same results in this experiment, which confirms that these three antibodies recognize the same protein, REC8.

Presence of cohesins during premeiotic S phase

We used R α N antibodies in most immunofluorescence experiments. R α C and Ch α N were used to verify the results obtained with R α N. First, we analyzed whether REC8 is present during premeiotic S phase, when cohesion is thought to be established. We examined sets of two adjacent testicular sections of a rat that had been injected with BrdU to mark the cells in S phase (Fig. 3, A–C). One section was labeled with anti-REC8, anti-BrdU, and DAPI, and one with anti-REC8, anti-SCP3, and DAPI. Premeiotic S phase starts in preleptotene, ~10 h before the end of stage VIII, and ends just before the transition from stage X to stage XI (indicated in Fig. 1; Hilscher and Hilscher, 1969). Mitotic (sper-

antibodies R α N (b), R α C (c), or Ch α N (d). (D) Abundance of various proteins in premeiotic S phase/preleptotene (p) or midprophase (= pachytene and diplotene) (m), analyzed by Western blot, using antibodies recognizing SCP2 (serum 493), R α SMC3, SMC1 β (Mab 76), REC8 (R α N), RAD50, or SCP3 (serum A1). MW, molecular weight markers.

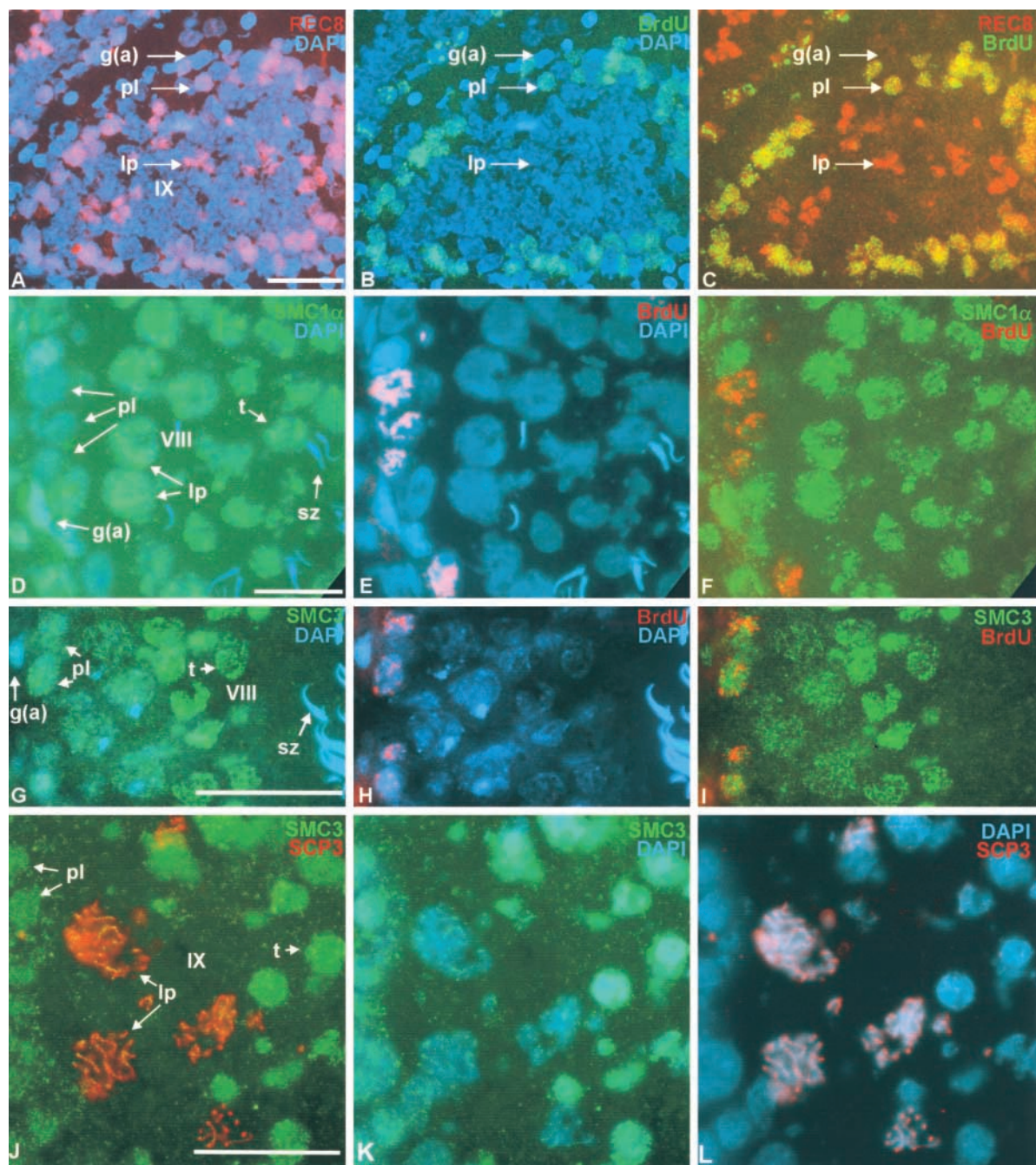


Figure 3. Appearance of cohesins in premeiotic S-phase cells. Immunofluorescence double labeling of frozen sections of rat testis with anti-BrdU and an antibody against a cohesin. The Roman numerals refer to the cell associations present in the tubules (Fig. 1). The three panels in each row represent the same section. We have indicated in the upper right corners what the different colors represent. SMC3 was detected by α SMC3. g(a), spermatogonium type A; lp, late pachytene; pl, preleptotene; pl(S), preleptotene (premeiotic S phase); t, spermatid; sz, spermatozoa. Bars: (A) 50 μ m; (D, G, and J) 25 μ m.

matogonial) S phases occur in stages III, V–VI, XI, XIII, and XIV (Hilscher and Hilscher, 1969), which differ from stage VIII–X tubules by the SCP3 labeling pattern and the morphology of the spermatid nuclei (Leblond and Clermont, 1952). We found 33 stage VIII–X tubules with REC8 in the preleptotene cell layer, and in 29 of these tubules, the preleptotene cells were also labeled with BrdU. The four tubules with REC8-positive but BrdU-negative preleptotene cells were all in stage VIII, which differs from stages IX and X by the presence of mature spermatozoa (indicated as 19* in Fig.

1). REC8 thus appears briefly (at most 3 h) before premeiotic S phase. SCC1, in contrast, was abundant in spermatogonia, but not detectable in premeiotic S phase and later stages of meiosis (Fig. 4, J–L). This fits observations in yeast (Klein et al., 1999) and mouse (Lee et al., 2002). Thus, REC8 replaces SCC1 from premeiotic S phase on.

SMC1 α was diffusely distributed through the nucleus of somatic cells and all stages of spermatogenesis, including premeiotic S phase (Fig. 3, D–F), except leptotene and zygotene (cell associations XI–XIII) and the meiotic divisions (Revenkova et

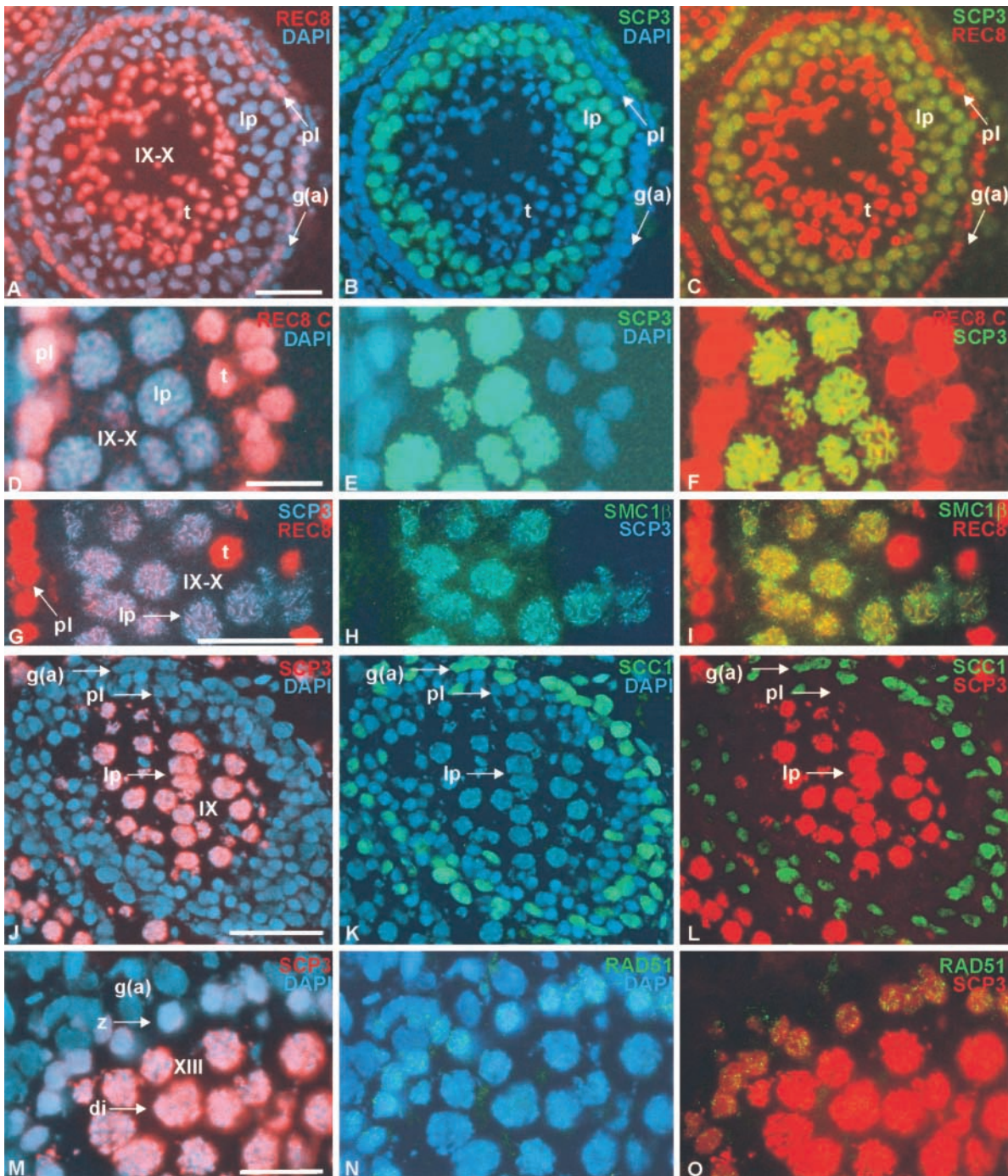


Figure 4. **Appearance of AE components and RAD51 in premeiotic S-phase cells.** Immunofluorescence double and triple labeling of frozen sections of rat testis. The Roman numerals refer to the cell associations (Fig. 1). The three panels in each row represent the same section. We have indicated in the upper right corners what the different colors represent. In D–F, we detected REC8 using R α C, which recognizes the COOH terminus of REC8 (REC8 C); in panels A–D, we used R α N. di, diploctene; g(a), spermatogonium type A; lp, late pachytene; pl, preleptotene; pl(S), preleptotene (premeiotic S phase); t, spermatid; z, zygote. Bars: (A and J) 50 μ m; (D, G, and M) 25 μ m.

al., 2001). Meiotic cohesin SMC1 β appeared after premeiotic S phase, simultaneously with AE components SCP2 and SCP3. These proteins were absent from (pre)leptotene cells in stage VIII–X tubules (shown for SMC1 β and SCP3 in Fig. 4, A–L). For SCP3, we also showed directly that it was absent from premeiotic S-phase cells by double labeling testis sections with anti-BrdU and anti-SCP3. We found 33 stage VIII–X tubules containing BrdU-labeled preleptotene cells, and SCP3 was absent in all of the preleptotene cells in these tubules.

For labeling of SMC3, we used monoclonal (M α SMC3) and polyclonal (R α SMC3) antibodies, which recognize the same band on Western blots, which is enriched in SCs (Fig. 2 B). In previous studies, we found that M α SMC3 specifically labeled the AEs and did not label somatic or premeiotic S-phase cells (Eijpe et al., 2000a; Revenkova et al., 2001). The new anti-SMC3 serum R α SMC3 predominantly produced a diffuse nuclear labeling in all cell types, including somatic and premeiotic S-phase cells (Fig. 3, G–I); in Triton X-100–

treated sections, the diffuse labeling was largely lost from spermatocyte nuclei, whereas a weak labeling remained along the AEs (Fig. 3, J–L) (also see next paragraph). To summarize, during premeiotic S phase, all three analyzed types of cohesin (SCC1/REC8, SMC1 α /SMC1 β , and SMC3) are represented and are diffusely distributed through the nucleus.

Assembly of AEs

Before we analyzed the incorporation of proteins in AEs, we tested the antibodies on various types of preparations under various conditions. Anti-SMC1 α and R α SMC3 antibodies could produce both a diffuse labeling throughout spermatocyte

nuclei and labeling of the AEs; which labeling pattern prevailed depended on the conditions. Pretreatments that were likely to extract proteins and disrupt structures resulted in loss of the overall nuclear labeling but enhanced the labeling of AEs by anti-SMC1 α (Eijpe et al., 2000a) and R α SMC3 (see above and Fig. 3, J–L). Because isolated SCs are enriched in SMC1 α and SMC3 (Eijpe et al., 2000a; Fig. 2 B), we think that these proteins make part of AEs and are rather inaccessible to anti-SMC1 α or R α SMC3 but accessible to M α SMC3 within these structures. We therefore used M α SMC3 for detection of SMC3 within AEs.

We analyzed the incorporation of REC8, SMC1 β , SMC3,

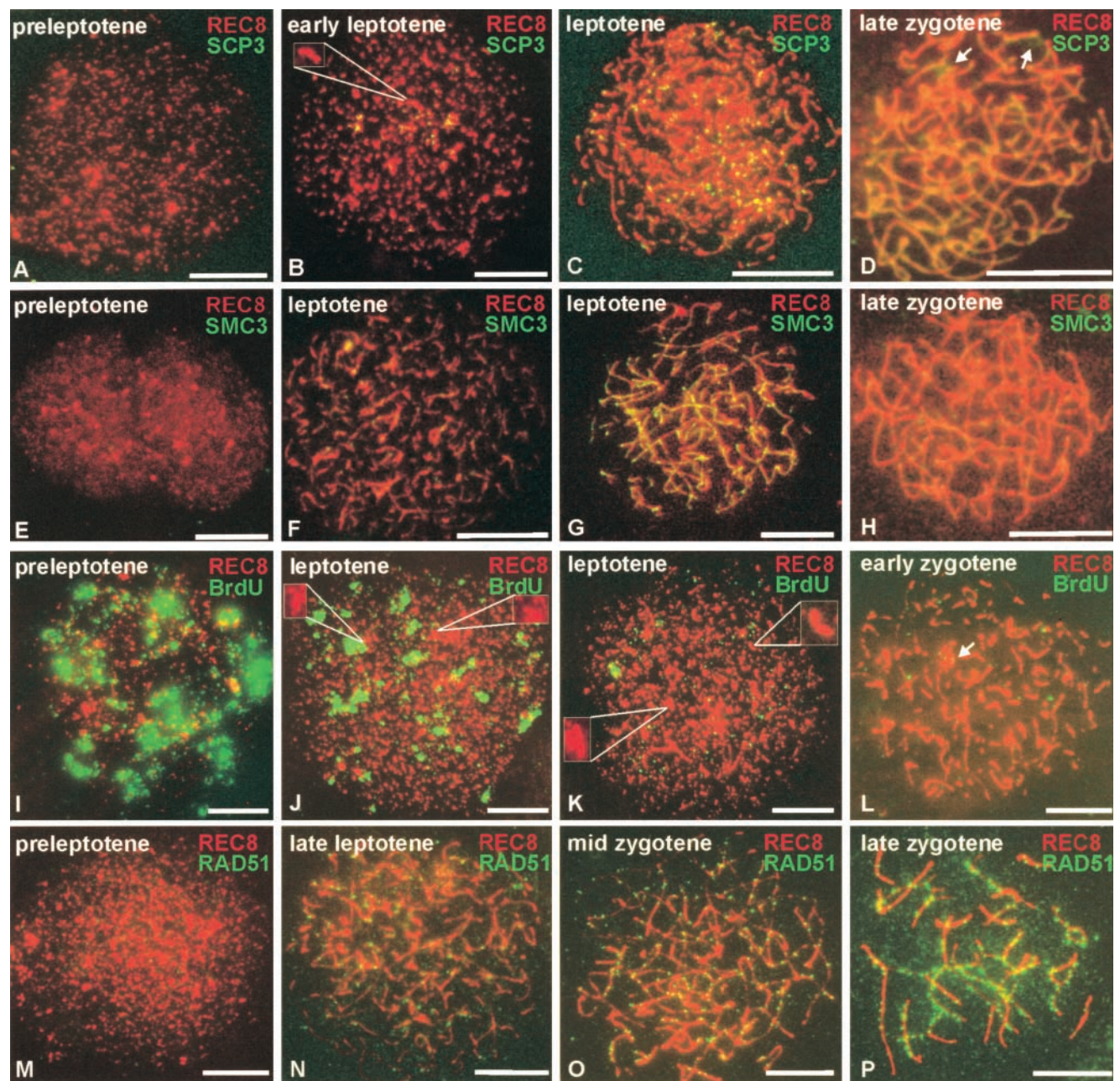


Figure 5. REC8 in early spermatocytes. Spread early rat spermatocytes were labeled with anti-REC8 (R α N) in combination with (A–D) anti-SCP3 (II52F10), (E–H) anti-SMC3 (M α SMC3), (I–L) anti-BrdU, and (M–P) monoclonal anti-RAD51/DMC1. The cells in I–L originate from a BrdU-treated rat (see Materials and methods). In B, J, and K, some of the first short stretches of REC8-AEs have been enlarged. They appear already during premeiotic S phase (J and K). Arrows in D point at sites where SCP3 has a broader distribution along AEs than REC8. Arrow in L points at BrdU incorporation along an AE in early zygotene. Bars, 10 μ m.

SCP2, and SCP3 in AEs using primarily dried down preparations (Fig. 5). REC8 formed short axial structures (REC8-AEs) before SMC1 β , SMC3, SCP2, and SCP3; the first short REC8-AEs appeared already during premeiotic S phase (Fig. 5, B, J, and K). After premeiotic S phase, the leptotene cells assembled increasingly longer REC8-AE fragments (Fig. 5, C, F, and N). SCP3 appeared in leptotene and localized along already formed REC8-AEs from its first appearance on; it formed dots along REC8-AEs (Fig. 5, B and C), which extended and fused until they lined REC8-AEs along their length (Fig. 5 D). At some sites in leptotene and zygotene nuclei, it appeared as if the chromatin surrounding the REC8-AEs also contained some SCP3 (Fig. 5 D, arrows). Using M α SMC3 and Mab β 70 (anti-SMC1 β), we got corresponding results for SMC1 β and SMC3, as is shown for SMC3 in Fig. 5 (E–H). R α SMC3 did not label AEs detectably in (pre)leptotene (unpublished data). Because we found previously that SCP2, SCP3, SMC1 β , and SMC3 appear along AEs simultaneously and colocalize with each other from their first appearance on (Offenberg et al., 1998; Schalk et al., 1998; Eijpe et al., 2000a; Revenkova et al., 2001), we conclude that these four proteins are all deposited along already existing REC8-AE fragments in leptotene spermatocytes.

We also compared the abundance of cohesins and AE components in preleptotene and midpachytene–diplotene cells using Western blot (Fig. 2 D). REC8 was more abundant in preleptotene than in midpachytene–diplotene spermatocytes, whereas SMC3 (detected by R α SMC3) was equally abundant in both cell populations. In contrast, we detected little SCP2 and SCP3 and no SMC1 β on the preleptotene blots. Thus, SMC1 β , SMC3, SCP2, and SCP3 are not present on AE-like fragments in preleptotene, but appear after REC8 on REC8-AEs in leptotene nuclei. SCC1 was absent from all cell types containing AEs (Fig. 4, J–L).

Bridges between desynapsed AEs

Fig. 6 shows REC8 and other AE components in later stages of meiosis. In pachytene, REC8 colocalized with other SC components (Fig. 6, A and B). However, in late diplotene, SCP2, SCP3, SMC1 β , and SMC3 started to accumulate in the centromeric and telomeric regions, whereas REC8 did not (Fig. 6, C–E). Furthermore, in nuclei with almost complete desynapsis, some bivalents showed one or two bridges between AEs, which were labeled by anti-SCP3 (Fig. 6, G, J, and L). We found such bridges before by immunofluorescence labeling of SMC3 (using M α SMC3), SCP2 (Schalk, 1999), or SMC1 β (Revenkova et al., 2001). These bridges do not contain REC8 however (Fig. 6, F and H). Cdk2, which marks the position of crossovers on the AEs until late pachytene/diplotene (Ashley et al., 2001), is still present at the position of part of the bridges (Fig. 6, K and L), which indicates that the bridges represent crossover sites. Thus, SMC1 β , SMC3, SCP2, and SCP3 fulfill functions at crossover sites that do not require the presence of REC8.

REC8, but not SMC1 β , SMC3, SCP2, and SCP3, persists along chromosome arms until anaphase I

In previous studies, we found that SMC1 β , SMC3, SCP2, and SCP3 had virtually disappeared from the chromosome arms at metaphase I, whereas SMC1 α was not detectable at all (Eijpe et al., 2000a; Revenkova et al., 2001). Because arm cohesion is needed for proper chromosome disjunction in

metaphase I, we compared in this study the localization of REC8 with that of SMC1 β , SMC3, SCP2, and SCP3 in meiosis I and II. SCP2 and SCP3 colocalize almost perfectly (Schalk et al., 1998); in this paper, we show the results for SCP3. At the end of diplotene, SMC1 β , SMC3 (as detected by M α SMC3), SCP2, and SCP3, but not REC8, started to accumulate in the telomeric and centromeric regions (shown for SCP3 and SMC1 β in Fig. 6, D and E). In diakinesis, SMC1 β , SMC3, SCP2, and SCP3 accumulated further in the centromeric regions and gradually disappeared from the telomeric ends and chromosome arms (shown for SCP3 in Fig. 6, M–Q; Schalk, 1999; Revenkova et al., 2001). Apparently, these four proteins first lose binding sites from the chromosome arms and then from the telomeres. In most metaphase I cells, they are not detectable anymore along the chromosome arms (shown for SCP3 and SMC1 β in Fig. 6, X–AE). R α SMC3 (Fig. 6, R–W) and anti-SMC1 α (Revenkova et al., 2001) did not label, or hardly labeled, metaphase I or later stages of meiosis. It is therefore unlikely that SMC1 α , SMC1 β , SMC3, SCP2, or SCP3 is responsible for arm cohesion until the end of metaphase I. REC8, in contrast, persists in appreciable amounts along the chromosome arms in metaphase I (Fig. 6, X–AE) and does not accumulate in the centromeric regions. Thus, only the localization of REC8 is consistent with a role in arm cohesion until anaphase I.

Disappearance of cohesins and AE components from the centromeric regions at anaphase II

At the metaphase I to anaphase I transition, all SMC1 β , SMC3 (as detected by M α SMC3), SCP2, and SCP3 had concentrated in the centromeric region (shown for SCP3 in Fig. 7, A–E; Revenkova et al., 2001). REC8 had disappeared from the distal regions and persisted in the proximal regions of the chromosome arms so that two groups of REC8-labeled dots remained, which flanked the centromeric regions (Fig. 7, C and D). In anaphase I, REC8 was confined in most bivalents to two spots, which flanked the kinetochores (Fig. 7, F and H), whereas SCP3 stayed accumulated in a broad area around the kinetochores (Fig. 7, F and G). This area became more compact and needle shaped in metaphase II (Fig. 7, L and M; see also the metaphase II/anaphase II nucleus in Fig. 7, I and J). We found this previously for SMC3, SMC1 β (Revenkova et al., 2001), and SCP2 (Schalk, 1999). At the metaphase II to anaphase II transition, some of these needle-shaped aggregates were still associated with REC8, whereas others were not. The colocalization of REC8 and SCP3 with kinetochores was also lost at the metaphase II–anaphase II transition (Fig. 7, I and J).

Association of RAD50 and RAD51/DMC1 with REC8

Because REC8 has a role in meiotic recombination (Klein et al., 1999), we analyzed whether it associates with other proteins involved in meiotic recombination, namely RAD50 and RAD51/DMC1. RAD50 functions in early steps of meiotic recombination (Smith and Nicolas, 1998) and becomes abundant throughout (pre)leptotene nuclei, but does not concentrate visibly along AEs in any stage of meiosis (Eijpe et al., 2000b); RAD51 and DMC1 are involved in later steps of meiotic recombination (Smith and Nicolas,

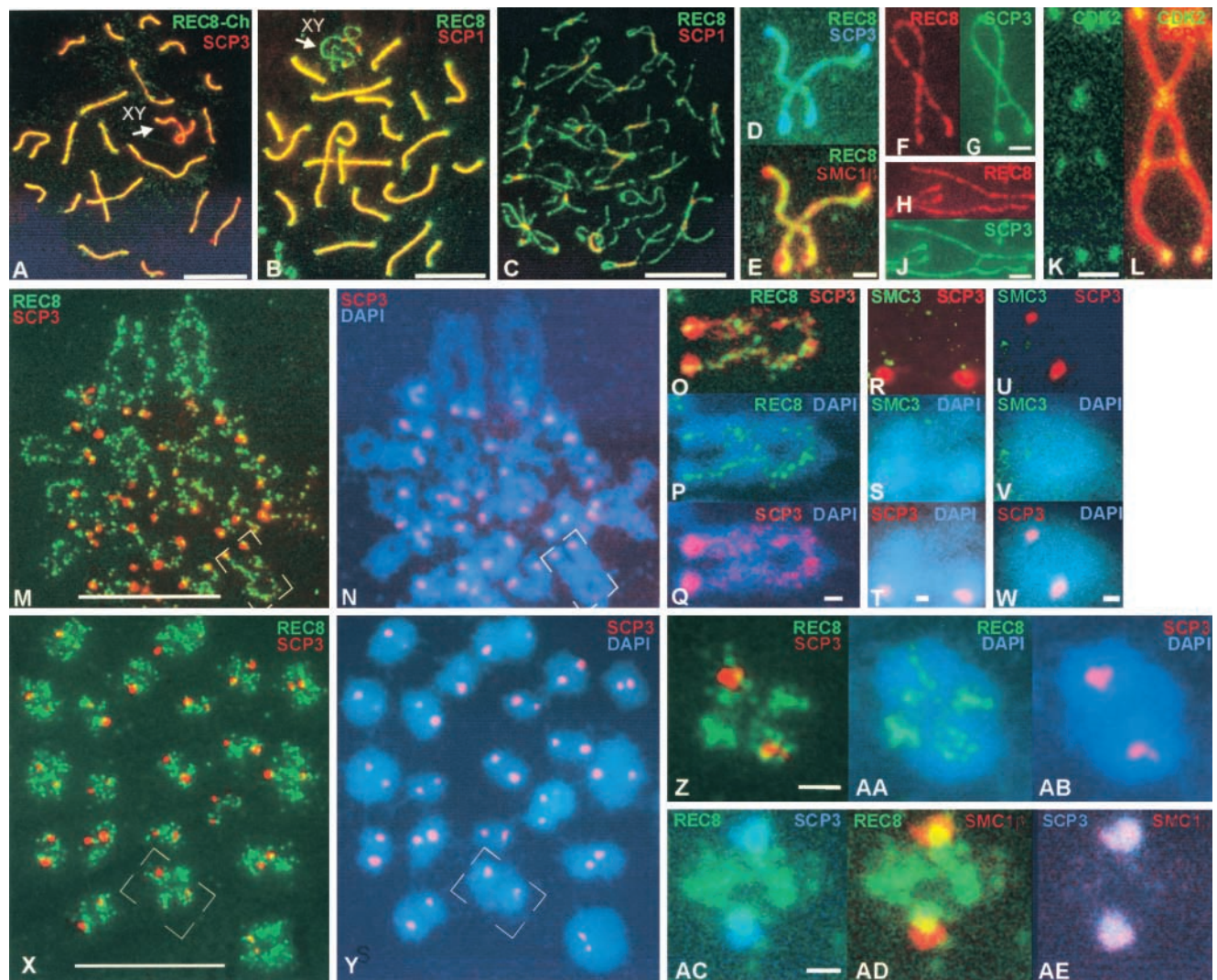


Figure 6. REC8 in spread spermatocytes in pachytene to metaphase I. We detected REC8 using affinity-purified antibodies from R α N, except in panel A, where we used affinity-purified antibodies from Ch α N. (A and B) Pachytene nuclei (XY, XY bivalent). (C–L) Diplotene SCs. (F–L) Diplotene SCs with bridges between desynapsed AEs (the bridges contain SCP3, but not REC8). (K and L) Cdk2 marks the position of these bridges on the AEs. (M and N) Diakinesis; the boxed bivalent is enlarged in O–Q to show that SCP3 and REC8 do not colocalize precisely anymore. (R and S) Metaphase I bivalents labeled with R α SMC3 and anti-SCP3 (II52F10). (X and Y) Metaphase I cell; the boxed bivalent is shown in detail in Z–AB to show that SCP3 is absent from the chromosome arms. (AC–AE) Bivalent from another metaphase I cell to show absence of SCP3 and SMC1 β , but not REC8, from the chromosome arms. Bars: (A–C, M, and X) 10 μ m; (all other panels) 2 μ m.

1998) and are incorporated in early recombination modules (RNs), which associate with AEs (Bishop, 1994; Terasawa et al., 1995; Anderson et al., 1997; Plug et al., 1998). Immunofluorescence labeling of RAD51/DMC1 revealed two types of foci, namely numerous weakly labeled foci throughout meiotic prophase nuclei and fewer intensely labeled foci, which were localized along REC8-AEs (Fig. 4, M–O, and Fig. 5, M–P) and correspond to early RNs (Anderson et al., 1997). A small proportion of RAD51/DMC1 coimmunoprecipitated with REC8 from spermatocyte lysates (Fig. 8 A), and a small proportion of REC8 coprecipitated with RAD51/DMC1 (Fig. 8 B), which indicates that protein complexes exist in spermatocytes that contain both REC8 and RAD51/DMC1. Probably such complexes occur in early RNs. Also, a small proportion of RAD50 coimmunoprecipitated with REC8 from spermatocyte lysates (Fig. 8 A), which possibly indicates that some RAD50 occurs in

protein complexes in spermatocytes that also contain REC8; however, we could not coprecipitate REC8 with RAD50. AE components SCP2 and SCP3 did not coprecipitate with REC8 (Fig. 8 C).

Discussion

Fig. 1 provides an overview of the appearance and disappearance of proteins, whereas Fig. 9 summarizes their localization patterns. For the localization of most proteins, including cohesins SMC3, SMC1 β , and REC8, we applied more than one antibody, various preparational techniques, and, if possible, Western blot controls. All results were consistent with the following conclusions: the analyzed AE components can be divided into three groups according to their localization, and this classification divides the cohesins; only one cohesin, REC8, showed localization patterns that were

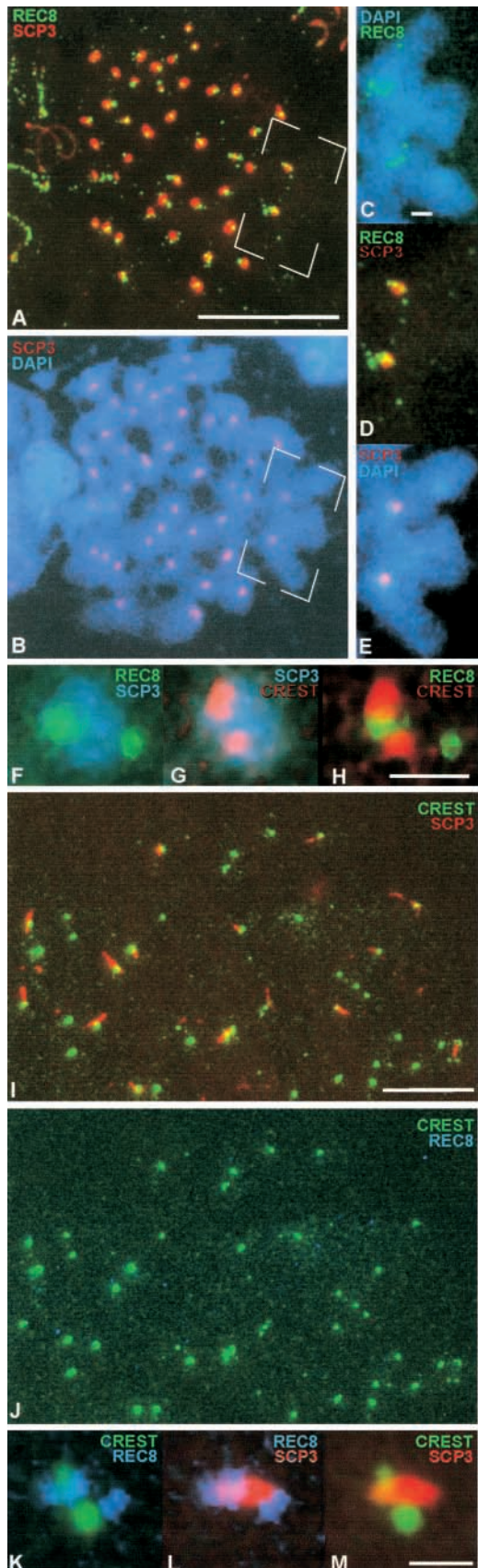


Figure 7. REC8 in spread metaphase I/anaphase I to anaphase II spermatocytes. For all panels, we used affinity-purified rabbit anti-REC8 antibodies (R α N). (A and B) Cell at the metaphase I to anaphase I transition; the homologues of at least one bivalent (boxed) have separated; this bivalent is shown in detail in C–E. (F–H) Detail of the centromeric region of a chromosome from another anaphase I cell (for an interpretation, see Fig. 9). (I and J) Anaphase II cell. (K–M) Centromeric region of one of the chromosomes in a metaphase II cell (for an interpretation, see Fig. 9). Bars: (A and I) 10 μ m; (all other panels) 2 μ m.

consistent with an essential role in sister chromatid cohesion throughout meiosis; two cohesins, SMC1 α and SMC3, occurred along AEs and throughout the nuclei of somatic and spermatogenic cells, but were absent during metaphase I; and one cohesin, SMC1 β , together with AE proteins SCP2 and SCP3, appeared too late along AEs and disappeared too early from the chromosome arms to be essential for cohesion in all stages of meiosis.

Establishment of meiotic sister chromatid cohesion

One reason for the present investigations was our observation that SMC1 β appeared well after premeiotic S phase in spermatocytes of rat (Revenkova et al., 2001). That appeared contrary to the idea that cohesion is established during S phase (Uhlmann and Nasmyth, 1998) and that the four cohesins act in a complex to provide cohesion (Ciosk et al., 2000; Haering et al., 2002). However, the experiments in this paper show that of each analyzed type of cohesin, at least one representative is found in premeiotic S-phase nuclei. These cohesins (REC8, SMC1 α , and SMC3) are diffusely distributed through the nuclei of premeiotic S-phase cells, and it is possible that they establish meiotic sister chromatid cohesion. Although this may solve the original question, new questions arise with respect to the role of cohesins in AE assembly and maintenance of cohesion during the meiotic divisions.

REC8 and AE assembly

Several investigations (Klein et al., 1999; Eijpe et al., 2000a; Pezzi et al., 2000; Pelttari et al., 2001; Revenkova et al., 2001) indicate that cohesins provide a basis for AEs. Our results support this idea and allow for distinguishing between the role of REC8 and other cohesins and AE components. We think that REC8 provides a basis for AE assembly because it forms AE-like structures before SMC1 β , SMC3, SCP2, and SCP3. It is not known what triggers the formation of REC8-AEs, but one possibility is phosphorylation of REC8, because the pattern of REC8 bands in preleptotene and midprophase (pachytene/diplotene) suggests that the degree of REC8 phosphorylation increases between these stages of meiosis (Fig. 2 D). Within REC8-AEs, REC8 molecules are initially not associated with SMC1 (α or β) and SMC3. SMC1 α disappears temporarily in late leptotene/early zygotene (Eijpe et al., 2000a), and SMC1 β appears in midleptotene. SMC3 is present throughout meiotic prophase but does not associate with AEs until late leptotene. Possibly, cohesin complexes are gradually rebuilt during meiotic prophase as the AEs are assembled, and this could be accompanied by phosphorylation of REC8 (Fig. 2 D). SMC1 β appears too late to contribute to the establishment of cohesion; possibly, it contributes to maintenance of cohesion, and/or it functions primarily in recombination.

(F–H) Detail of the centromeric region of a chromosome from another anaphase I cell (for an interpretation, see Fig. 9). (I and J) Anaphase II cell. (K–M) Centromeric region of one of the chromosomes in a metaphase II cell (for an interpretation, see Fig. 9). Bars: (A and I) 10 μ m; (all other panels) 2 μ m.

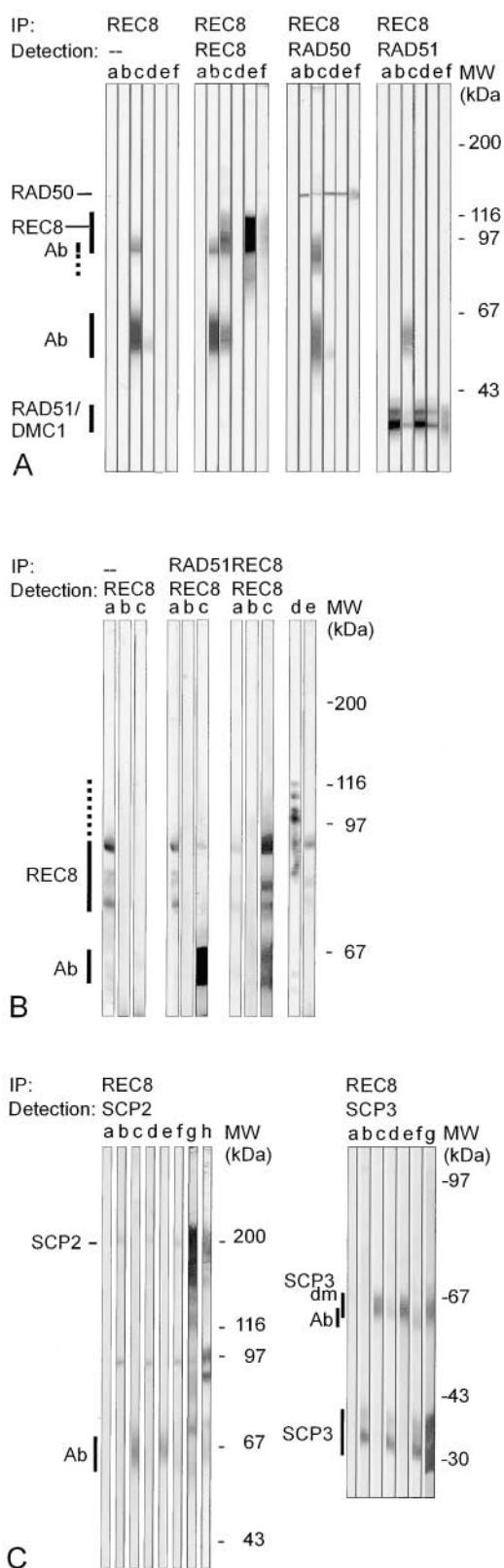


Figure 8. Coimmunoprecipitation of proteins with REC8. (A) Coimmunoprecipitation of RAD50 and RAD51/DMC1 with REC8. REC8 was precipitated from spermatocyte extract by affinity-purified R α N antibodies, and the immunoprecipitate, in parallel with various controls, was analyzed by immunoblotting. Strips of the blots were probed with blocking buffer (–; negative control) and affinity-purified antibodies recognizing REC8 (R α N; positive control for the immunoprecipitation), RAD50, or RAD51/DMC1 (serum

AE components and meiotic sister chromatid cohesion at meiosis I

Of the analyzed AE components, only REC8 persisted along the chromosome arms until the metaphase I to anaphase I transition. In part of the metaphase I cells, we found some SMC1 β , SMC3 (using M α SMC3), SCP2, and SCP3 along the chromosome arms. Because the abundance of these proteins along the arms was negatively correlated with the degree of chromosome condensation, we assume that these proteins detach from the arms as condensation proceeds and thus cannot contribute to cohesion until the metaphase I to anaphase I transition. Anti-SMC1 α and R α SMC3 did not label metaphase I chromosomes. Thus, of the analyzed cohesins, only REC8 can ensure chromosome arm cohesion until metaphase I. It remains to be investigated whether proteins other than REC8 are required for arm cohesion maintenance, and if so, which. In anaphase I, REC8 disappeared from the chromosome arms, as expected for a cohesion protein. REC8 and the other analyzed proteins displayed an interesting pattern between anaphase I and II: whereas SMC1 β , SMC3, SCP2, and SCP3 occurred throughout the centromeric chromatin, REC8 occupied two spots, which flanked the kinetochores (Fig. 7, F–H, and Fig. 9). This is consistent with REC8 being part of the physical link between centromeric regions of sister chromatids. The two REC8 spots furthermore reconcile two apparently contradictory observations on mammalian chromosomes. On the one hand, cohesion is specifically retained at the centromeres in mitotic metaphase chromosomes (for review see Rieder and Cole, 1999), and this is correlated with persistence of SCC1 in the centromeric region (Waizenegger et al., 2000). On the other hand, the central domain of the centromere undergoes microtubule-dependent elastic deformation.

2308). The strips in each subpanel carry (a) control immunoprecipitate (obtained without anti-REC8 [R α N] antibodies), (b) the supernatant of the control immunoprecipitate, (c) the REC8 immunoprecipitate, (d) supernatant of the REC8 immunoprecipitate, (e) purified SCs, or (f) spermatocyte lysate. (B) Coimmunoprecipitation of REC8 with RAD51/DMC1. We precipitated RAD51/DMC1 from spermatocyte extract using affinity-purified antibodies from serum 2308 and analyzed the immunoprecipitate on Western blots, using affinity-purified anti-REC8 antibodies (R α N). For comparison, we precipitated in parallel REC8 from the same spermatocyte extract using R α N. In each subpanel, the strips carry (a) the unbound fraction of the spermatocyte lysate, (b) the last wash of the immunoprecipitate, and (c) the RAD51/DMC1 or REC8 immunoprecipitate. Strip d carries synaptonemal complex proteins, analyzed in parallel with the immunoprecipitates, and strip e carries proteins of the spermatocyte extract that was used for immunoprecipitation. (C) No detectable coimmunoprecipitation of SCP2 and SCP3 with REC8. The strips in each subpanel carry (a) control immunoprecipitate (obtained without R α N or R α C), (b) the supernatant of the control immunoprecipitate, (c) immunoprecipitate obtained with R α N, (d) supernatant of the R α N immunoprecipitate, (e) immunoprecipitate obtained with R α C, (f) supernatant of the R α C immunoprecipitate, (g) proteins of purified SCs, and (h) (left subpanel only) spermatocyte lysate. The position of various proteins on the blots is indicated to the left of the strips. SCP3 dm indicates the position of SCP3 dimers on the strips (Lammers et al., 1994). The fuzzy bands indicated by Ab result from reaction of the goat anti-rabbit IgG (conjugated to AP) with monomers or dimers of the heavy chain of the rabbit anti-REC8 antibodies that were used for immunoprecipitation. MW, molecular weight markers.

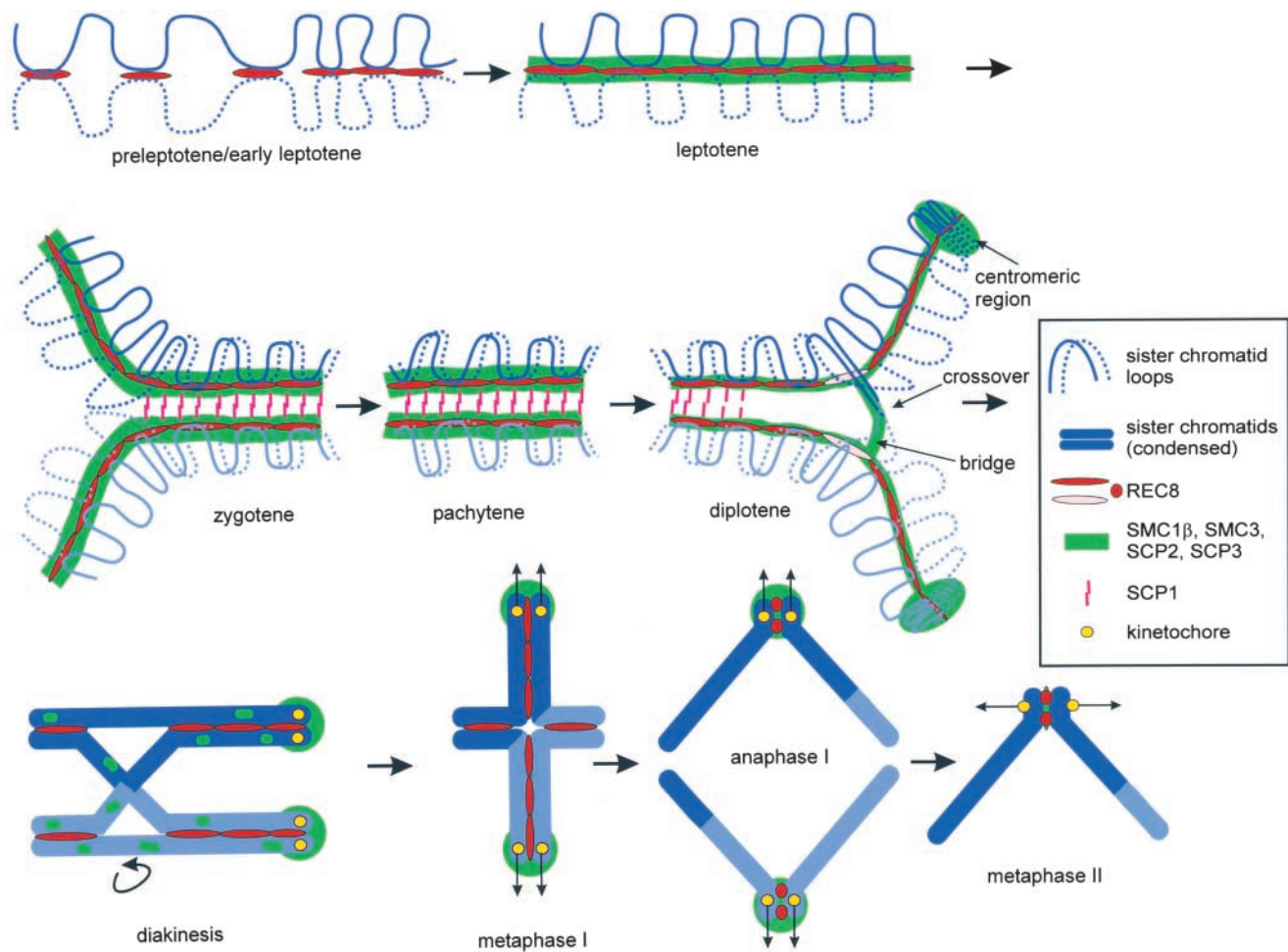


Figure 9. Interpretative drawing showing the localization of REC8, SMC1 β , SMC3, SCP2, and SCP3 along AEs and chromosomes throughout meiosis. Diffusely distributed protein has not been indicated.

mations during mitotic metaphase (Shelby et al., 1996), and it has been suggested that this could be due to transient local separations of sister chromatids (He et al., 2000). Two cohesion sites that flank the kinetochores can explain these observations. It should be noted, however, that we found two supposed cohesion sites per centromeric region in meiosis, whereas elastic deformation and transient separation of centromeric domains have been found in mitosis (Shelby et al., 1996; Waizenegger et al., 2000).

The role of SMC1 β , SMC3, SCP2, and SCP3 in the centromeric region in metaphase I to anaphase II remains to be investigated. It is possible that these proteins stabilize REC8-mediated arm cohesion until metaphase I and centromeric cohesion until anaphase II. After anaphase I, they might furthermore contribute to a change in the orientation of kinetochores. The change in the shape of the SCP3-labeled domain between anaphase I and metaphase II (Fig. 7, compare F and G with L and M) suggests a conformational change of the chromatin around the centromeres.

Role of REC8 in recombination

Cohesins, including REC8 (de Veaux et al., 1992; Klein et al., 1999), also function in homologous recombination. We proposed (van Heemst and Heyting, 2000) that after S

phase, cohesins attract protein complexes that are involved in the early steps of homologous recombination. In mitotic G2, the cohesion proteins would then direct the homology search of broken DNA ends toward the corresponding segment of the sister chromatid. In meiosis, specific proteins, including AE components (Schwacha and Kleckner, 1997), would block the homology search on the sister chromatid and direct it toward the homologous chromosome. Here, one prediction of this model is confirmed, namely the association of recombination proteins (RAD51/DMC1 and possibly RAD50) with a cohesion protein, REC8 (Fig. 8, A and B). Interaction of Rad50 with Rad21 (homologous to Scc1) in the mitotic cycle of *S. pombe* has been proposed before (Hartsuiker et al., 2001).

One observation points to a different role in recombination of REC8 on the one hand and SMC1 β , SMC3, SCP2, and SCP3 on the other hand, namely the “bridges” between AEs in late diplotene. The persistence of Cdk2 at these bridges (Fig. 6 L) indicates that they mark sites of crossing over (Ashley et al., 2001). SMC1 β , SMC3, SCP2, and SCP3 are constituents of the bridges (Fig. 6, G, J, and L; Schalk et al., 1998; Revenkova et al., 2001), whereas REC8 is not. Bridges are found in only a small proportion of the bivalents (Schalk et al., 1998; Revenkova et al., 2001), and

Cdk2 marks only some of the bridges. In male mouse meiosis, Cdk2 is lost from most crossover sites before desynapsis (Ashley et al., 2001). Possibly, Cdk2 monitors a late step in recombination at the DNA level, whereas the bridges represent an even later step, for instance, the start of the formation of recombinant chromatid axes. SMC1 β , SMC3, SCP2, and SCP3 might stabilize crossover intermediates until recombinant chromatid axes have been formed. Apparently, the latter step is usually, but not always, completed before desynapsis.

To summarize, the localization of REC8 differs from that of other analyzed cohesins in various stages of meiosis. Probably, REC8 provides a basis for AEs and RNs and ensures cohesion throughout meiosis. The role of SMC1 α , SMC1 β , and SMC3 is less clear, apart from an essential role of SMC3 in cohesion and recombination in yeast meiosis (Klein et al., 1999) and a likely role of SMC1 β and SMC3 in centromeric cohesion (Revenkova et al., 2001). SMC1 α and diffusely distributed SMC3 might contribute to the establishment of cohesion, whereas SMC1 β and AE-associated SMC3, together with SCP2 and SCP3, possibly further support REC8-mediated cohesion, promote recombinational interactions with the homologous chromosome, stabilize crossover intermediates, and provide a basis for the formation of recombinant chromatid axes.

Materials and methods

Antibodies

We cloned two nonoverlapping cDNA fragments of REC8, obtained by PCR on the full-length mouse REC8 cDNA (Human Science Research Resources Bank; ID AU080225), in the pGEMT-easy vector (Promega) and then in pET24a (Novagen). The encoded peptides were produced in *Escherichia coli* from the pET24a constructs, purified on a Ni²⁺-NTA resin (QIAGEN), and used for immunization as previously described (Offenberg et al., 1991). This yielded rabbit serum 602 (R α N) and chicken antibody SN11 (Ch α N) against the NH₂-terminal peptide, and rabbit serum 610 (R α C) against the COOH-terminal peptide of REC8. By the same procedure, using mouse testis cDNA as template for PCR, we elicited rabbit antiserum 624 (R α SMC3) against a peptide covering the 411 COOH-terminal amino acids of mouse SmcD (homologous to yeast Smc3). We affinity purified the anti-REC8 and anti-SMC3 antibodies on columns that carried the peptides that we had used for immunization. Mouse monoclonal antibodies IX5B2 (anti-SCP1; Offenberg et al., 1991), I152F10 (anti-SCP3; Offenberg et al., 1991), β 70 and β 76 (anti-SMC1 β ; Revenkova et al., 2001), and 462 (anti-full-length bovine SMC3; further denoted as M α SMC3; Stursberg et al., 1999); hamster serum H1 (anti-SCP3; Eijpe et al., 2000b); rabbit sera A1 (anti-SCP3; Lammers et al., 1994), 493 (anti-SCP2; Schalk et al., 1998), and 526 (anti-RAD50, Eijpe et al., 2000b); and the rabbit anti-SCC1 serum (Waizenegger et al., 2000) have been described. To label kinetochores, we used a human autoimmune serum from a patient with CREST syndrome (Moens et al., 1987). The mouse monoclonal antibodies against RAD51 (catalog no. MS-988; NeoMarkers), BrdU (catalog no. RPN202; Amersham Biosciences), or Cdk2 (catalog no. SC-6248; Santa Cruz Biotechnology, Inc.) have been described by the suppliers. Rabbit serum 2308 was elicited against full-length human RAD51 by C. Beerends and R. Kanaar (Erasmus University, Rotterdam, Netherlands). The monoclonal and polyclonal anti-RAD51 antibodies also recognize DMC1 and are therefore denoted as anti-RAD51/DMC1.

Immunocytochemistry

Paraffin sections (Heyting et al., 1983) and frozen sections (Eijpe et al., 2000a) of testes from Wistar rats were prepared as described. Rat spermatocytes were spread by the dry-down technique (Peters et al., 1997) or by agar filtration (Heyting and Dietrich, 1991). We performed immunofluorescence labeling according to Heyting and Dietrich (1991) and mounted the slides in Vectashield (Vector Laboratories) containing 2 μ g DAPI per ml. For detection of BrdU, we treated preparations with 70% formamide, 1

mM EDTA in 2 \times SSC at 55°C for 2 min and washed them in 70, 90, 100% cold ethanol before incubating them with anti-BrdU antibodies. All monoclonal antibodies were diluted 1:1. Affinity-purified antibodies from rabbit serum 602 (R α N), 610 (R α C), and 624 (R α SMC3) and chicken SN11 (Ch α N) were diluted 1:50. All secondary antibodies, conjugated to fluorescent dyes, were diluted according to the instructions of the suppliers. We used immunodepleted serum fractions as negative controls for R α N and R α C. Immunofluorescent preparations were analyzed as described by Revenkova et al. (2001).

Cell separation

We separated cells from rat testes by elutriation and density centrifugation in Percoll (Amersham Biosciences) (Heyting and Dietrich, 1991). For purification of preleptotene spermatocytes, we collected cells from a BrdU-labeled rat at 1,800 rpm and 15–25 ml/min during elutriation, and then centrifuged these cells in 29% Percoll. The cell band with the highest density was enriched in preleptotene spermatocytes (10% Sertoli cells, 23.3% spermatogonia, 46% preleptotene [BrdU and REC8 positive; no SCP3], 15.6% leptotene [BrdU negative and REC8 positive; REC8-AEs with some SCP3; no synapsis], 4.1% zygotene, 1% spermatids). Pachytene and diplotene spermatocytes were purified from rat testis as described by Lammers et al. (1995).

Immunoprecipitation

We performed immunoprecipitations according to Goedecke et al. (1999), with some modifications. We incubated the cell lysates overnight at 4°C with affinity-purified primary antibodies (from rabbits), and then we added paramagnetic beads coupled to sheep anti-rabbit antibodies (Dynal A.S.) and incubated the mixture with the beads for another 4 h at 4°C. We performed all incubations and washes in the presence of the complete mini-protease inhibitor cocktail (Roche Diagnostics GmbH). After incubation, we collected the beads, washed them with 1% NP-40, 50 mM NaCl, 10 mM Tris-HCl (pH 8.0), eluted the bound proteins from the beads by boiling them in SDS-PAGE sample buffer (Lammers et al., 1994), and applied the eluted proteins to a 1-cm-wide slot of a polyacrylamide SDS gel. In parallel with the immunoprecipitates, we loaded one slot with total lysate of 5 \times 10⁵ purified spermatocytes and one slot with protein from 10⁷ purified SCs. After electrophoresis, the gel was blotted onto nitrocellulose, and the resulting blot was stained with Ponceau S. The blot of each lane was cut into four strips, which were probed with various antibodies.

Other procedures

For BrdU incorporation, we injected rats intraperitoneally with 60 mg BrdU (catalog no. B3002; Sigma-Aldrich) per kilogram body weight at 3 and 2 h before sacrifice. SCs (Heyting et al., 1985) and nuclei from spermatocytes (Meistrich, 1975) or liver (Blobel and Potter, 1966) were isolated by described procedures. SDS-PAGE (Laemmli, 1970) and immunoblotting (Dunn, 1986) were performed as previously described. For immunoblot analysis of nuclei, we loaded 20 μ g of protein per 2-cm-wide slot of a 7–18% linear gradient polyacrylamide SDS gel. After electrophoresis, we stained 0.5 cm of each lane with Coomassie blue and blotted the remainder onto nitrocellulose. For immunoblot analysis of whole cells, we loaded proteins from 10⁶ cells per 2-cm slot of a 7.5% or 10% polyacrylamide gel. SCs were dephosphorylated according to Lammers et al. (1995); samples containing 4 \times 10⁷ isolated SCs were treated with AP, AP pretreated with 8-hydroxyquinoline-5-sulfonic acid (a specific inhibitor of AP; Simpson and Vallee, 1968), or AP buffer only. After treatment, each sample was dissolved in electrophoresis sample buffer, loaded onto a 1-cm-wide slot of a 7.5% SDS gel, electrophoresed, and blotted onto nitrocellulose. Immunoblots stained with Ponceau S were scanned using an Agfa Snapscan 1212 flatbed scanner before they were probed with antibodies. Binding of antibodies to the blots was detected by secondary antibodies conjugated to AP (Promega) and incubation in NBT/BCIP; the Ponceau S stain is lost during these steps. After incubation in NBT/BCIP, we scanned the blots again and processed the obtained images using the Corel Photopaint and CorelDraw software packages.

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