SCP2, a major protein component of the axial elements of synaptonemal complexes

SCP2, een belangrijke eiwit component van de axiale elementen van synaptonemale complexen

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SCP2, a major protein component of the axial elements of synaptonemal complexes

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen,
dr. C.M. Karssen,
in het openbaar te verdedigen
op woensdag 10 maart 1999
des namiddags te half twee in de Aula.

The work presented in this thesis was performed at the Wageningen Agricultural University, Department of Genetics, Dreijenlaan 2, NL-6703 HA Wageningen, The Netherlands.
This research was financially supported by a grant from the Dutch Organization for Scientific Research (NWO), project no. 900-501-143.

ISBN 90-5485-996-2

Stellingen

1. SCP2 is betrokken bij zusterchromatiden-cohesie tot aan anafase I.
   dit proefschrift

2. SCP2 en SCP3 stabiliseren crossovers tussen homologe chromosomen tijdens laat diploteen en metafase I.
   dit proefschrift

3. Spreidpreparaten zijn niet geschikt voor nauwkeurige immunolocalisatiestudies wegens artefacten die ontstaan tijdens de spreidprocedure
   Schmekel *et al.*, Exp. Cell Res. **226:** 20-30
   dit proefschrift

4. SCP2 van de rat vertoont veel structurele en mogelijk ook functionele overeenkomsten met Red1 van gist.
   dit proefschrift

5. Microchip array technologie maakt de bestudering van het geheel van complexe biochemische pathways mogelijk. Daarmee krijgt de biologie naast reductionistische ook holistische gereedschappen ter beschikking.

6. De complexe wisselwerking tussen de biosfeer en de andere sferen van onze planeet (de geosfeer, de hydrosfeer en de atmosfeer) vertoont hoogstwaarschijnlijk een zelforganiserend karakter. De madeliefjesplaneet van James Lovelock vormt een goede aanzet voor een studie aan de cybernetische eigenschappen van de aarde.

7. Aangezien de prognose voor kanker aan de cervix gerelateerd is aan het type human papiloma virus aanwezig in de tumor, zal DNA-diagnostiek bij deze vorm van kanker een belangrijke rol gaan spelen in de ontwikkeling van specifieke behandelingen voor de patient.
   Lombard *et al.*, J. Clin. Oncol. **16:** 2613-2619

8. De aanstellingsduur van een OIO zou gecorreleerd moeten zijn aan de grootte van het eiwit dat hij/zij bestudeert.

9. De aarde verschaft genoeg om tegemoet te komen aan ieders behoefte, maar niet aan ieders hebzucht.
   Gandhi

10. Voor een goeie stelling gaat men naar de Gamma

Stellingen behorend bij het proefschrift "SCP2, a major protein component of the axial elements of synaptonemal complexes" in het openbaar te verdedigen op 10 maart 1999 door Marjolijn Schalk.

De feiten horen alleen maar tot de opgave, niet tot de oplossing.

Wittgenstein

Contents

Chapter 1

General introduction

Meiosis

Nearly all our body cells are diploid, i.e., they contain two sets of chromosomes, one of maternal and one of paternal origin. The gametes are exceptions to this rule, because they are haploid and contain only one set of chromosomes per cell. At fertilization, two haploid gametes fuse to form a single diploid cell, the zygote, which is the first cell of a new, diploid individual. Meiosis is the counterpart of fertilization because it marks the transition from the diploid to the haploid phase of the life cycle. It consists of two successive divisions, meiosis I and meiosis II, which follow a single round of DNA replication. Meiosis I is the reductional division by which the chromosome number is reduced from diploid to haploid, and meiosis II is an equational division (Fig. 1). The life cycles of all sexually reproducing eukaryotes display such an alternation of diploid and haploid generations of cells, and meiosis plays a provital role in the life cycle of all these organisms. The investigations described in this thesis make part of a research line that is focused on the events during meiosis I.

After premeiotic S-phase, during the prophase of meiosis I, homologous chromosomes pair, and between the paired chromosomes proteinaceous structures, the synaptonemal complexes (SCs), are formed. Two paired chromosomes constitute a bivalent. In a bivalent, the non-sister chromatids of homologous chromosomes exchange genetic material, which results in crossovers and gene conversions. At the end of prophase I, SCs disassemble; during diakinesis and metaphase I, the homologous chromosomes remain connected at the chiasmata (see Fig. 1), which represent the sites where a crossover between non-sister chromatids occurred. The chiasmata are required to connect the homologues, while these orient themselves in the metaphase I spindle. In anaphase I, the chiasmata are resolved and the homologous chromosomes disjoin. During the second meiotic division, the sister chromatids of each chromosome segregate. Thus, starting with one diploid cell, the two divisions of meiosis result in four haploid cells.

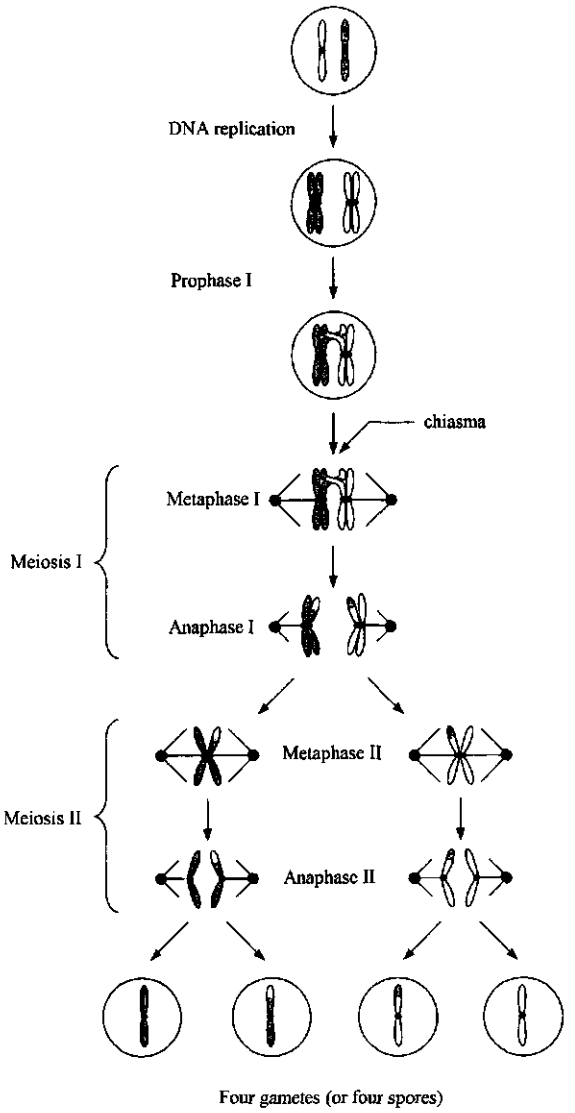

Figure 1. Meiosis. Meiotic chromosome behavior is schematically represented for a cell with two homologous chromosomes. One round of DNA replication precedes the two meiotic divisions so that each chromosome consists of two chromatids when meiosis starts. Subsequently the homologous chromosomes pair and non-sister chromatids of homologous chromosomes recombine during prophase I. At metaphase I, the homologous chromosomes stay connected at the chiasmata so that chromosome pairs rather then individual chromosomes orient themselves in the spindle. At anaphase I, the chiasmata are resolved and the homologous chromosomes disjoin. A second meiotic division follows in which the sister chromatids separate (From Murray and Hunt, 1993).

The synaptonemal complex

Meiotic prophase I is subdivided in several stages according to the state of assembly or disassembly of SCs. In leptotene, proteinaceous axes (axial elements) are formed along each chromosome; the two sister chromatids of each chromosome are associated by a single axial element. In zygotene, the axial elements of homologous chromosomes become connected by transverse filaments (TFs), a process called synapsis. In pachytene, synapsis along the length of the chromosomes is complete. Between the axial elements, a third longitudinal structure is formed, the central element (CE). The axial elements, TFs and CE, together constitute the SC (Fig. 2). In the context of the SC, the axial elements are also called lateral elements (LEs). During diplotene, the axial elements desynapse and at diakinesis the axial elements disassemble. In the first section of this introduction I will give an overview of the possible functions of the SC in chromosome pairing, recombination and segregation.

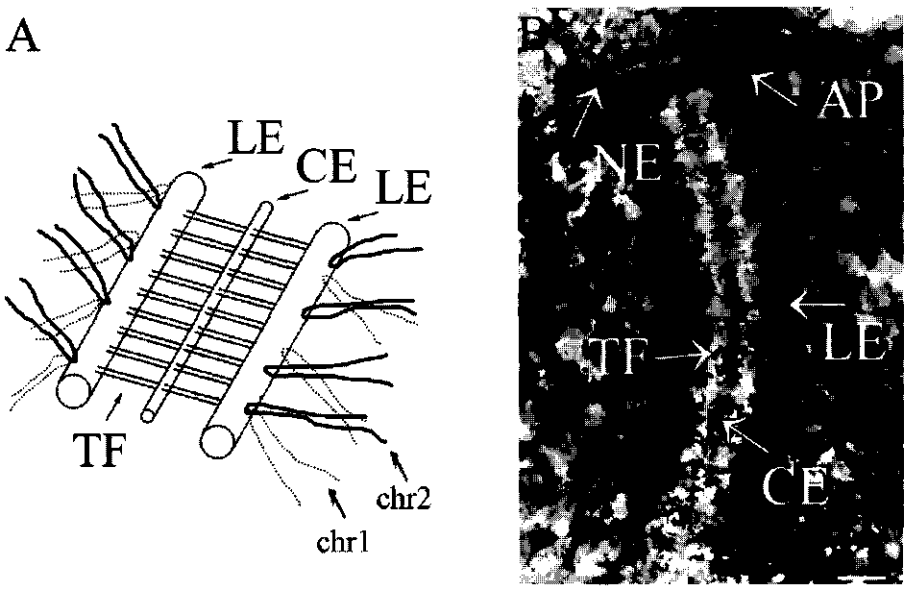

Figure 2. Structure of the synaptonemal complex. Panel A shows a schematic representation of the structure of the SC. Indicated are the two lateral elements (LE), the transverse filaments (TF) and the central element (CE), and the chromatin of the sister chromatids of each chromosome (resp. chr.1, chr.2, chr.3 and chr.4), which is attached in loops to the LEs. Panel B shows an SC in an ultrathin section of spermatocytes of *Blaps cribrosa* (from Schmekel, 1993). Indicated are the lateral elements (LE), the central element (CE), the transverse filaments (TFs), the attachments plaque (AP) at the end of the SC and the nuclear envelope (NE). Bar represents 100 nm.

Chromosome pairing

Since SCs are present between paired homologous chromosomes it seemed likely that they establish chromosome pairing. However, pairing of homologous chromosomes precedes SC formation (Scherthan *et al.*, 1992; 1996). In some organisms, homologous chromosomes are already paired in pre-meiotic cells (Weiner and Kleckner, 1994), so the SCs seem not to be required to establish chromosome pairing. Furthermore, at least two organisms do not display detectable SCs, but nevertheless perform meiosis (Egel-Mitani *et al.*, 1982; Bähler *et al.*, 1993). Possibly, the premeiotic and meiotic pairing of homologous chromosomes are established by unstable interactions (Kleckner and Weiner, 1993; Weiner and Kleckner, 1994), which are stabilized by SCs as meiosis proceeds.

Recombination

Meiotic recombination takes place between non-sister chromatids of homologous chromosomes. It is generally assumed that meiotic recombination occurs according to the double strand break repair model (Sun *et al.*, 1991; Szostak *et al.*, 1983) (Fig. 3). According to this model, recombination is initiated by a double strand break (DSB) in a single chromatid. Resection of the 5' ends of this DSB results in two 3' single stranded tails. One of these tails can invade one of the chromatids of the homologous chromosome and as a consequence a D-loop is formed at this chromatid (Fig. 3). Repair synthesis is primed from the 3' end of the invading strand and the D-loop is enlarged. This D-loop can anneal to the complementary ssDNA tail of the invading chromatid and also on this chromatid repair synthesis is initiated. This model predicts the presence of heteroduplex DNA on the two recombining chromatids as a consequence of the strand invasion and annealing of the D-loop. Branch migration produces two Holliday junctions which can be resolved as a crossover or a non-crossover event (gene conversion). In yeast, intermediates postulated by this model, like DSBs, heteroduplex DNA and Holliday junctions have been demonstrated (reviewed by Roeder, 1997).

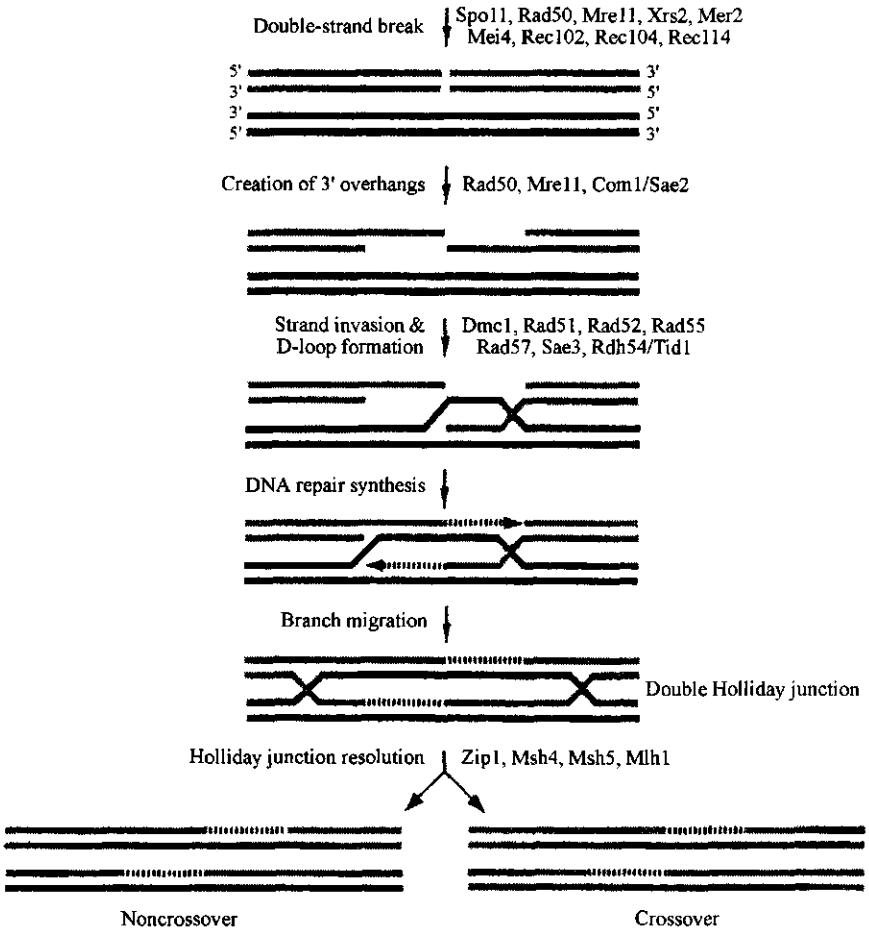

Figure 3. The double-strand break repair model of meiotic recombination. (From Roeder, 1997). The yeast genes that are supposed to be involved in each step are indicated. For further explanation see text.

Most of our knowledge of meiotic recombination comes from studies on mutants in yeast that are blocked at different steps in the meiotic recombination pathway (reviewed by Roeder, 1997) (Fig. 3). For example *mre11* and *rad50* mutants do not form DSBs (McKee and Kleckner, 1997; Alani *et al.*, 1990). *rad50S*, a specific allele of *rad50*, forms DSBs, but these are not processed, and accumulate (Alani *et al.*, 1990; Keeney and Kleckner, 1995). In *rad51* mutants, DSBs are resected but strand invasion by the single stranded DNA is abolished (Shinohara *et al.*, 1992; Nag *et al.*, 1995). In most yeast mutants that are affected in recombination, meiosis is arrested or results in non-viable spores.

In yeast, initiation of recombination by DSB formation precedes or occurs concomitantly with SC formation (Padmore *et al.*, 1991). Therefore, it seems unlikely that intact SCs are involved in the early steps of recombination. Recombination can occur independently from intact SCs as is obvious from studies with mutants that are defective in SC formation. For example the *zip1* and *red1* mutants in *S. cerevisiae* do not assemble SCs, but still display meiotic recombination (Sym and Roeder, 1994; Rockmill and Roeder, 1990). Furthermore, *Schizosaccharomyces pombe* and *Aspergillus nidulans*, which do not assemble SCs, perform a high level of meiotic recombination (Egel-Mitani *et al.*, 1982; Munz, 1994; Kohli and Bähler, 1994; Egel, 1995). SC formation seems even to be dependent on initiation of recombination since yeast mutants that are blocked in the early steps of the meiotic recombination pathway, like *spo11* and *rad50*, do not form SCs (Giroux *et al.*, 1989; Alani *et al.*, 1990). However, in the *mei-P22* and *mei-W68* mutants in *Drosophila*, which do not perform meiotic recombination, chromosomes still synapse (McKim *et al.*, 1998). Possibly, in more complex genomes, synapsis is not dependent on recombination, but alternative mechanisms for initiation of synapsis exist.

Although SCs seem not to be required for the initiation of recombination they probably influence recombination events; In the *zip1* mutant of *Saccharomyces cerevisiae*, in which synapsis is abolished, crossover interference is also abolished (Sym and Roeder, 1994). Crossover interference is the interaction between crossovers in adjacent chromosome regions. This interaction is possibly mediated through SCs (King and Mortimer, 1990).

Recombination nodules

In leptotene, zygotene and pachytene, electron-dense, spherical bodies, called recombination nodules (RNs), are observed on unsynapsed axial elements and on the SCs (Carpenter, 1988). In several organisms early and late RNs can be distinguished on the basis of morphology and time of appearance (Carpenter, 1988). Rad51 and/or DMC1, which are involved in strand exchange (Sung, 1994; Nag *et al.*, 1995), are components of early RNs (Anderson *et al.*, 1997). The number and position of late RNs corresponds with the number and position of crossovers (Carpenter, 1988), thus these late RNs probably represent the places of crossing-over events. The position of recombination nodules suggests that recombination occurs in the context of SCs.

Chromosome disjunction

Crossing-over events result in physical connections between homologous chromosomes, which can be seen in metaphase I as chiasmata. These chiasmata enable the two paired chromosomes to orient themselves to opposite poles during metaphase I; thus they ensure a proper disjunction of chromosomes at anaphase I. In mutants that do not form chiasmata, homologous chromosomes detach from one another prematurely, so that they cannot orient themselves properly at metaphase I, and therefore do not always move to opposite poles at anaphase I.

The axial element

The axial elements, which are present along the chromosomes from leptotene up till and including diplotene, probably play an important role in most of the functions of SCs. Because this thesis deals with a component of the axial elements, I will focus in this section on the structure and function of axial elements of SCs.

Structure of the axial elements

Ultrastructural analysis of axial elements in rat and mouse revealed that axial elements contain multiple strands, which are connected by a fibrous network (del Mazo and Gil-Alberdi, 1986; Heyting *et al.*, 1985). The axial elements differ from the chromatid cores of mitotic chromosomes in that axial elements are shared by the two sister

chromatids of meiotic chromosomes, whereas chromatid cores are present in each chromatid. Furthermore, the chromatid cores in mitotic chromosomes are located in the center of the chromatids (Rufas *et al.*, 1987), whereas the axial elements are peripherally located. However, the axial elements and the chromatid cores also share some features: topoisomerase II, which is a component of the cores in mitotic chromosomes (Earnshaw and Heck, 1985) gradually congregates onto the axial elements during the late stages of meiotic prophase (Moens and Earnshaw, 1989; Klein *et al.*, 1992). Furthermore, detailed ultrastructural analysis of axial elements revealed that they consist of two main sub-elements (Comings and Okada, 1971; Heyting *et al.*, 1985; Dietrich *et al.*, 1992). Silver-stained chromatid cores are detected in diplotene and diakinesis at the positions where the axial elements have disappeared in grasshopper (Rufas *et al.*, 1992) and in rye chromosomes (Fedotova *et al.*, 1989). In electron microscope observations, these cores appeared double (Rufas *et al.*, 1992). Possibly, the chromatid cores are present during meiosis and are associated through the axial elements. They become visible as two cores as soon as the axial elements disappear (Rufas *et al.*, 1992).

Axial elements contain several meiosis-specific proteins (Heyting *et al.*, 1988, 1989; Offenberg *et al.*, 1991). In rat, two axial element components, SCP2 and SCP3, with relative electrophoretic mobilities (M_r) of respectively 190,000 and 30,000-33,000 were identified with monoclonal antibodies (Mabs) against purified SCs (Heyting *et al.*, 1987, 1989). The cDNA encoding SCP3 has been isolated and sequenced (Lammers *et al.*, 1994) as also a partial cDNA encoding the hamster homologue of SCP3, called COR1 (Dobson *et al.*, 1994). In yeast, two meiosis-specific proteins, Red1 and Hop1, were identified that are associated with the axial elements of SCs (Smith and Roeder, 1997, Hollingsworth and Byers, 1989). And in lily, a meiosis-specific component of axial elements was identified by means of a Mab (Anderson *et al.*, 1994).

Possible functions of axial elements include: 1. Chromatin organization. 2. Sister chromatid cohesion. 3. Chiasma maintenance. 4. Regulation of recombination. Each of these functions will be considered here.

Chromatin organization

During meiosis the chromatin is organized in loops, which are attached at their base to the SCs. The size of the loops is species-dependent and can vary from 2 to 200 kb, depending on the species. Vazquez Nin *et al.* (1993) have shown that in meiotic chromosomes the DNA extends into the axial elements, while the central region of SCs is free of DNA. Possibly, the chromatin is organized in loops that run through the axial elements. The organization in loops resembles the mitotic chromatin organization, where loops are attached to chromosome scaffolds. In mitotic chromosomes, the sequences that are bound to the scaffold are called scaffold attachment regions (SARs). SARs are AT-rich and as a result they have a narrow minor groove (Nelson *et al.*, 1987). Preparations of SCs are enriched in GT/CA tandem repeats and LINE and SINE sequences (Pearlman *et al.*, 1992), so these sequences are possibly at the basis of meiotic chromatin loops. Whether SAR sequences are also at the basis of chromatin loops in meiotic prophase still has to be sorted out.

Sister chromatid cohesion

Cohesion between the sister chromatids ensures that they do not separate prematurely. Several hypotheses have been put forward how cohesiveness is established. Murray and Szostak (1985) proposed that sister chromatids are associated by DNA catenations, which remain from DNA replication, and that at the metaphase/anaphase transition, topoisomerase II is responsible for the decatenation of the intertwined sister chromatids. It is also possible that stable attachments between sister chromatids are mediated by proteins (Holloway *et al.*, 1993).

During meiosis sister chromatid cohesion is released in two steps; cohesion along the chromatid arms, which is released at the metaphase I/anaphase I transition, and cohesion at the centromeres, which is released at the metaphase II/anaphase II transition. In *Drosophila*, two proteins have been identified that are possibly involved in these two phases of sister chromatid cohesion; ord and mei-S332 (Miyazaki and Orr-Weaver, 1992; Goldstein, 1980). Flies which have a mutation in the *ord* gene are defected in sister chromatid cohesion during meiosis I and meiosis II as revealed by

cytological and genetical analysis (Bickel *et al.*, 1997). Mutations in the *mei-S332* gene cause a defect in sister chromatid cohesion in late anaphase I, which results in non-disjunction at meiosis II (Goldstein, 1980). Mei-S332 has been localized to the centromeric regions of meiotic chromosomes from prophase I until anaphase II (Kerrebock *et al.*, 1995). In hamster, the localization pattern of the COR1 protein, the homologue of SCP3, suggests a possible role of this protein in both modes of sister chromatid cohesion; it is present along the axial elements between the sister chromatids until diplotene and at the centromeres until anaphase II (Dobson *et al.*, 1994; Moens and Spyropoulos, 1995). COR 1/SCP3 may act as a binding substance between the two chromatids, or as an inhibitor of topoisomerase II, which prevents decatenation of the sister chromatids along the chromosome arms until metaphase I, and at the centromeres until anaphase II.

Axial element components are present between sister chromatids as long as cohesion along the chromatid arms exists. Therefore, a function of axial elements in sister chromatid cohesion seems likely. The *rec8* mutant in *S. pombe* does not form axial elements and in this mutant the sister chromatids separate prematurely (Molnar *et al.*, 1995). Since axial elements disappear at diakinesis, they cannot be involved in sister chromatid cohesion at the centromeres after anaphase I. However in grasshopper, strands can be detected between the two sister kinetochores by silver impregnation in metaphase II (Rufas *et al.*, 1989). Such centromeric filaments were also observed in mouse (Tandler and Solari, 1991) and several other organisms among which rat (Solari and Tandler, 1991). These centromeric filaments are possibly a remnant of axial elements, which provide sister chromatid cohesion at the centromeres until anaphase II.

Chiasma maintenance

As mentioned above, reciprocal recombination between non-sister chromatids provides connections between homologous chromosomes which can be seen in metaphase I as chiasmata, and which ensure a proper segregation of the chromosomes at the first meiotic division. Mutants that are defective in recombination, display a high frequency of chromosome non disjunction at meiosis I. However, many mutants exist that have a

normal or slightly reduced level of recombination but nevertheless show a high level of non-disjunction of homologous chromosomes. For example, in the *ord* mutant in *Drosophila*, crossing-over is only slightly affected, but at the first meiotic division both exchange and non-exchange chromosomes segregate improperly (Mason, 1976; Miyazaki and Orr-Weaver, 1992). As mentioned above, the *ord* mutant is affected in sister chromatid cohesion and this cohesion, distal from the chiasmata, probably stabilizes the chiasmata at metaphase I, as is shown in the model in Fig. 4A. It seems likely that axial element components that are involved in sister chromatid cohesion, also stabilize chiasmata; Red1 is possibly a component of the axial elements in *S. cerevisiae* (Smith and Roeder, 1997). In the *red1* mutant axial element formation is abolished, and both exchange and non-exchange chromosomes missegregate at anaphase I (Rockmill and Roeder, 1990).

As proposed by the alternative model in Fig. 4B, stabilization occurs by chiasma binders. However no possible candidate-chiasma binders have been identified yet (reviewed by Carpenter, 1994).

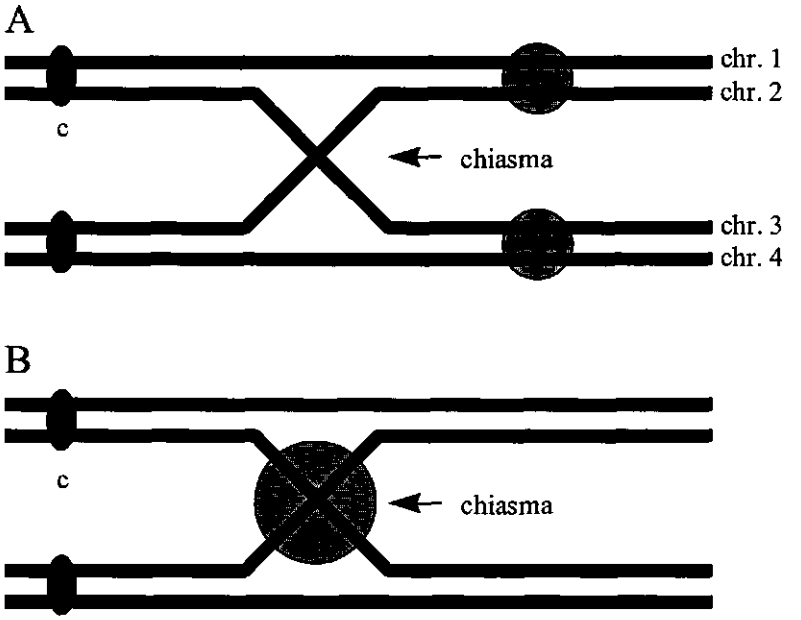

Figure 4. Two models for chiasma stabilization. Shown are two homologous chromosomes, which are connected by a chiasma. Indicated are the four chromatids in a bivalent (resp. chr. 1, chr. 2, chr. 3 and chr. 4), the centromeres (c) and the chiasma. In model A, the chiasma is stabilized by sister chromatid cohesion (shown in gray) distal to the chiasma. In model B, the chiasma is stabilized by chiasma binders (shown in gray). The models are not mutually exclusive.

Regulation of recombination

Knowledge about possible functions of axial element components in recombination comes from studies on yeast. In a *red1* mutant, the number of crossovers is decreased to 25% of the wild type level, whereas intrachromosomal recombination is not affected (Rockmill and Roeder, 1990). Because the *red1* mutation does not cause a meiotic arrest, and can even alleviate the meiotic arrest in *rad51*, *dmc1* and *zip1* mutants, Red1 is possibly involved in the signaling of recombination intermediates (Xu *et al.*, 1997). Red1 does probably not act in the intrachromosomal pathway (Mao-Draayer *et al.*, 1996). This view was supported by Schwacha and Kleckner (1997) who showed that in a *red1* mutant the formation of recombination intermediates between homologues is reduced to 25 % of wildtype level, whereas the formation of recombination intermediates between sister chromatids is not affected. They concluded that Red1 is responsible for the interhomologue bias that exists during meiosis and exerts its function prior or during DSB formation. Through Red1 the DSBs are directed into the interhomologue-only pathway (Schwacha and Kleckner, 1997).

At the time of DSB formation, SCs are not yet formed (Padmore *et al.*, 1991). However, it is possible that axial element components, like Red1, are already present and influence DSB formation so that recombination between homologues is enhanced. Possibly, axial element formation is nucleated from these recombination initiation sites.

In summary, the axial elements possibly are involved in the organization of the chromatin in loops and in sister chromatid cohesion, chiasma stabilization and in enhancing recombination between homologues rather than between sister chromatids.

Aim of this thesis

Most of our knowledge about the role of axial elements during meiosis comes from studies on mutants in yeast and several other organisms that are somehow disturbed in axial element formation (see above). Identification and analysis of individual components of axial elements will increase our knowledge of molecular mechanisms that underlie chromosome pairing and recombination. Offenberg (1993) has described

the identification of two axial element components of SCs in rat, SCP2 and SCP3. This thesis focuses on SCP2.

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Chapter 2

SCP2: a major protein component of the axial elements of synaptonemal complexes of the rat

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Published in Nucleic Acids Research, 1998, 26: 2572-2579

Abstract. *In the axial elements of synaptonemal complexes (SCs) of the rat, major protein components have been identified, with relative electrophoretic mobilities (M_rs) of 30,000-33,000 and 190,000. Using monoclonal anti-SC antibodies, we isolated cDNA fragments which encode the 190,000 M_r component of rat SCs. The translation product predicted from the nucleotide sequence of the cDNA, called SCP2 (for synaptonemal complex protein 2), is a basic protein (pI = 8.0) with a molecular mass of 173 kDa. At the C-terminus, a stretch of about 50 amino acid residues is predicted to be capable of forming coiled coil structures. SCP2 contains two clusters of S/T-P motifs, which are common in DNA-binding proteins. These clusters flank the central, most basic part of the protein (pI = 9.5). Three of the S/T-P motifs are potential target sites for $p34^{cdc2}$ protein kinase. In addition, SCP2 has eight potential cAMP/cGMP-dependent protein kinase target sites. The gene encoding SCP2 is transcribed specifically in the testis, in meiotic prophase cells. At the amino acid sequence and secondary structural level, SCP2 shows some similarity to the Red1 protein, which is involved in meiotic recombination and the assembly of axial elements of SCs in yeast. We speculate that SCP2 is a DNA-binding protein involved in the structural organization of meiotic prophase chromosomes.*

Introduction

During meiotic prophase, chromosomes are arranged in an orderly manner along proteinaceous axes called axial elements (Von Wettstein *et al.*, 1984). These elements differ from mitotic metaphase chromatid scaffolds because 1. the two chromatids of a meiotic prophase chromosome share a single axial element, whereas the chromatids of a metaphase chromosome have each their own scaffold; 2. the major protein components of axial elements are meiosis-specific and thus not found in chromatid scaffolds (Heyting *et al.*, 1989); and 3. axial elements are longer than metaphase chromatid scaffolds, and morphologically better defined. Axial elements and chromatid scaffolds can both be visualized by silver impregnation techniques, at least in certain types of microscopical preparations of spread cells (Howell and Hsu, 1979; Rufas *et al.*, 1982; Rufas *et al.*, 1992; Earnshaw and Laemmli, 1984; Stack, 1991).

As meiotic prophase proceeds, the axial elements are incorporated in zipperlike structures, called synaptonemal complexes (SCs), which keep homologous chromosomes in close apposition along their length (Von Wettstein *et al.*, 1984). Meiotic recombination probably initiates just before or simultaneously with axial element assembly, and the assembly of full length axial elements appears to depend upon the initiation of meiotic recombination by double-strand DNA scission, at least in yeast (Padmore *et al.*,1991). At the end of meiotic prophase, the SCs are disassembled at about the same time when recombination intermediates are resolved (Schwacha and Kleckner, 1994), and within each homolog the two separate scaffolds of the sister chromatids gradually become discernable (Rufas *et al.*, 1992). At the sites of reciprocal recombination between non-sister chromatids of homologous chromosomes, the scaffolds of the recombined chromatids "cross over" (Rufas *et al.*, 1992; Stack, 1991) to form chiasmata, which contribute to the physical connection between homologs. In most eukaryotes, such connections are essential for the proper orientation of bivalents at metaphase I.

The relation between axial elements and sister chromatid scaffolds remains to be elucidated. In mitotic chromosomes, the sister chromatids do not normally share one axis, although single axes of a similar length as axial elements have been demonstrated by silver impregnation along mitotic chromosomes that were forced to condense from

G2 in the presence of a topoisomerase II inhibitor (Giménez-Abián *et al.*, 1995). These single G2 axes probably represent the still unseparated sister chromatid scaffolds. Although axial elements of SCs are morphologically more similar to these single G2 chromosome axes than to metaphase chromatid scaffolds, there are also important differences, because axial element assembly is part of normal meiotic chromosome behaviour and does not require cell cycle drugs and topoisomerase II inhibitors, and because the major protein components of axial elements are specific for meiotic prophase (Heyting *et al.*, 1987, 1988, 1989; Offenberg *et al.*, 1991). In the rat, these meiosis-specific components have relative electrophoretic mobilities (M_rs) of 30,000-33,000 and 190,000 (Heyting *et al.*, 1987; 1988, 1989; Offenberg *et al.*, 1991), of which the 30,000-33,000 M_r components are most probably products of a single gene, *Scp3* (Lammers *et al.*, 1994). In yeast, at least two candidate components of axial elements have been identified, namely Hop1 and Red1 (Hollingsworth *et al.*, 1990; Rockmill and Roeder, 1990; Smith and Roeder, 1997), which are also meiosis-specific. It is possible that the axial element of a meiotic prophase chromosome is formed after premeiotic S-phase by association of meiosis-specific proteins with the still undivided chromosome scaffold (Rufas *et al.*, 1992). Alternatively, an entirely meiosis-specific axial element is assembled at the beginning of meiotic prophase, which is replaced by the two sister chromatid scaffolds when the axial element is disassembled. To distinguish between these possibilities, it is necessary to characterize the axial element components, and analyse their interaction with chromatin, in particular with the special DNA-regions called SARs (scaffold attachment regions, Laemmli *et al.*, 1992), by which chromatin is attached to chromatid scaffolds.

Why meiotic prophase chromosomes should be organized on single, at least partially meiosis-specific axial elements is another unresolved question, although several suggestions have been made: it is possible that the axial elements fix the chromosomes in an elongated state, and enhance the exposure of relevant pairing sites in an ordered, longitudinal array. This could facilitate the alignment of homologous chromosomes (Scherthan *et al.*, 1996). Other possible functions of axial elements include the inhibition of sister chromatid exchanges and/or the enhancement of recombination between non-sister chromatids of homologous chromosomes (Schwacha and Kleckner, 1994, 1996, 1997; Hollingsworth *et al.*, 1990; Xu *et al.*,

1997), the conversion of the products of interchromosomal reciprocal recombination into stable chiasmata that can ensure the proper orientation of bivalents at metaphase I (Rockmill and Roeder, 1990), and/or the generation of sister chromatid cohesiveness (Maguire, 1990; Dobson *et al.*, 1994).

In order to learn more about the nature and function(s) of meiotic axial elements/LEs, we study the composition of these structures. In this paper we describe the isolation and characterization of the cDNA encoding the 190,000 M_r component of rat SCs. The protein predicted from the nucleotide sequence of the cDNA, called SCP2, is basic (pI = 8.0) and has features of a protein which is capable of binding to the minor groove of AT-rich DNA. It shares these features with proteins that bind to SARs (Saitoh and Laemmli, 1994), including topoisomerase II (Laemmli *et al.*, 1992; Mirkovitch *et al.*, 1984), which is a major chromosome scaffold component (Earnshaw and Heck, 1985). We speculate that SCP2 is involved in the organization of chromatin during meiotic prophase, possibly by temporarily binding to SARs.

Materials and methods

The DDBJ/EMBL/GenBank accession number of the SCP2 cDNA sequence is Y08981.

Antibodies

The Mabs recognizing the 190,000 M_r SC protein in rat were elicited by immunization of mice with rat SCs as described by Offenberg *et al.* (1991). Of these antibodies, Mab IX9D5 has been described in detail by Heyting *et al.* (1989) and Offenberg *et al.* (1991). A polyclonal antiserum (serum 493) against amino acid residues 293 to 828 of the predicted translation product of the rat cDNA was prepared as follows: a 1600 bp *Pst*I fragment of cDNA clone 5 (which encodes a major part of SCP2 of the rat, see below) was cloned in the pQE31 expression vector (Qiagen, Chatsworth, CA, USA). Expression and isolation of the translation product were performed by means of the Qia expressionist system (Qiagen) according to the instructions of the manufacturer.

Antibodies were elicited in a rabbit by eight injections of 60 µg fusion protein in 750 µl PBS, mixed with 750 µl Freund's complete adjuvant (Sigma, St. Louis, MO, USA) (first injection), or 750 µl Freund's incomplete adjuvant (Sigma) (all following injections). The rabbit was injected subcutaneously at two-week intervals. One week after the fourth and the sixth injection, 20 ml bleedings were collected from the ear-veins. After the eighth injection a final bleeding of 80 ml was collected.

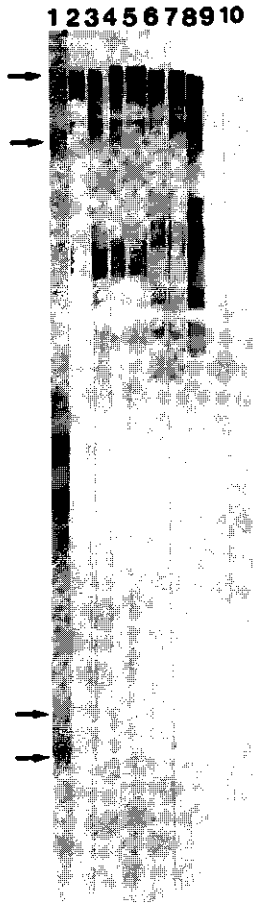

Figure 1. Reaction of antibodies used in this study with SC proteins. 1.5 x 10^7 SCs were loaded per cm slot of a SDS-10% polyacrylamide gel and stained with Coomassie blue or transferred to nitrocellulose. The arrows indicate from top to bottom the position of the 190,000, the 125,000 and the 30,000 and 33,000 M_r SC proteins. Lane 1: 0.4 cm wide strip of the gel stained with Coomassie blue; lanes 2 to 10: immunoblot strips of the same gel incubated in Mab IX8B11 (2), Mab IX2G11 (3), Mab IX9D5 (4), Mab IX8F1 (5), Mab IX1H9 (6), Mab IX3E4 (7), serum 493 (8), pre-immune serum 493 (9), control hybridoma supernatant (10).

Isolation of cDNAs encoding SCP2

For the isolation of cDNAs encoding the 190,000 SC M_r protein of the rat, we screened an expression cDNA library of the rat testis (Meuwissen *et al.*, 1992) in λzap® (Stratagene, San Diego, USA) with a pool of six independently isolated Mabs, each of which recognizes the 190,000 M_r SC protein, following described procedures (Meuwissen *et al.*, 1992). Among $3x10^5$ phage clones, 10 positive clones were found and purified. The purified clones had overlapping restriction enzyme fragment maps and inserts ranging in length from 1.1 to 3.9 kbp. The 5' *Eco*RI fragment of the longest clone, 3C1, was used for a secondary screening, and this yielded clone 5 with an insert size of 4.4 kbp. In search of rat cDNA clones extending further in the 5' direction than clone 5, we performed a 5' RACE experiment (Frohman *et al.*, 1988) exactly as described by Van Heemst *et al.* (1997), using oligonucleotides complementary to the most 5' sequences of clone 5 as primers and total rat testis RNA as a template. This yielded a DNA fragment which extended 302 nucleotides further in the 5' direction than clone 5. We then performed new RACE experiments, using primer sets complementary to this DNA fragment, a higher concentration of total testis RNA, and higher temperatures during cDNA synthesis. PCR performed on the cDNA-fragments obtained at 50°C and 52°C yielded two major products in each reaction.The longest product of each reaction was cloned in pGEM-T (Promega, Madison, WI, USA) and sequenced. Both (independently obtained) RACE products had identical sequences which extended 32 basepairs further in the 5' direction, and contained a stopcodon in frame with the first ATG codon. We therefore concluded that we had isolated and sequenced the full-length SCP2 cDNA.

Sequence analysis

The insert of cDNA clone 3C1 was subcloned into the pBluescript vector SK(-) according to the instructions of the manufacturer (Stratagene). From both ends of the insert of clone 3C1 we generated unidirectional sets of deletions by partial digestion with exonuclease III and S1 nuclease using the erase-a-base kit of Promega. In addition, we subcloned several restriction enzyme fragments of the independently isolated cDNA clone 5 in pBluescript. We determined the nucleotide sequences by the di-

deoxy chain termination method of Sanger *et al.* (1977), using [α-^{35}S]dATP (650 Ci/mmol; Amersham Corp., Buckinghamshire, UK), *Taq* polymerase (Gibco BRL Life Technologies, Paisley, UK or Promega) and oligonucleotide primers complementary to the polylinker sequences of pBluescript. The products of the RACE experiments (see above) were cloned into the pGEM-T vector according to the instructions of the supplier and sequencing reactions were performed in both directions from vector-specific primers, by means of the Dye Deoxy Terminator Cycle sequencing kit from Perkin-Elmer (Norwalk, Connecticut, USA) and the nucleotide sequence was analyzed on a 373A stretch 48 cm WAR DNA sequencer (Applied Biosystems, Inc., Foster City, CA, USA). The complete cDNA sequence encoding SCP2 was assembled by means of the Wisconsin GCG sequence analysis package (University of Wisconsin, WI, USA).

Immunocytochemical staining

Immunofluorescence staining of frozen sections of the rat testis was carried out as described by Heyting *et al.* (1988) and Heyting and Dietrich (1991). Ultrastructural localization of the antigen was performed by immunogold labeling of surface spread rat spermatocytes essentially as described by Moens *et al.* (1987) and Heyting and Dietrich (1991).

RNA isolation and northern blot hybridization

RNA was isolated from various tissues of 37-day-old rats by the GuTC/LiCl method of Cathala *et al.* (1983); poly(A)$^+$ RNA was purified by affinity chromatography on oligo(dT)-cellulose (Aviv and Leder, 1972). 15 μg of RNA per 0.5 cm slot was electrophoresed in the presence of ethidium bromide on formaldehyde/agarose gels, and transferred to Hybond-N$^+$ membranes (Amersham Corp.) by standard procedures (Sambrook *et al.*, 1989). After transfer, we verified on the basis of ethidium bromide fluorescence, that all lanes on the northern blot membranes contained a similar amount of RNA. The membranes were washed in 3x SSC, dried and fixed with UV light (312 nm; 200 J/m^2) for two minutes. As probes for northern blot hybridization we used RNA transcripts of a 3' deletion clone, which had been linearized with *Hin*dIII.

Transcription was performed from the T7 promotor in the presence of [α-^{32}P]rATP (3000 Ci/mmole). The northern blot membranes were prehybridized in 50% formamide, 5x SSC, 0.1% sodiumpyrophosphate, 1% SDS, 0.2% PVP, 0.2% Ficoll, 5 mM EDTA and 150 µg/ml sheared herring sperm DNA for 6 hrs at 60°C. Hybridization was performed in the same mixture with 0.07 µg/ml probe ($36x10^6$ cpm/µg) for 17 hrs at 60°C. Subsequently the blots were washed for 30 minutes at 65°C in successively 2x SSC 0.1% SDS, 1x SSC 0.1% SDS, 0.1x SSC 0.1% SDS and 0.1x SSC 0.1% SDS.

In situ *hybridization*

In situ hybridization was performed on 10 µm thick frozen sections of rat testes, as described by Meuwissen *et al.* (1992). As a probe we used RNA that was obtained by transcription from the T7-promoter of a linearized 3' deletion clone of clone 3C1; probe synthesis was performed in the presence of [α-^{35}S] rUTP (3000 Ci/mM, Amersham Corp.), as described by Meuwissen *et al.* (1992). After hybridization and washes, the slides were dipped in Ilford K5 nuclear track emulsion, exposed for 3 weeks at 4°C, developed in Kodak developer D19, and analyzed by dark field microscopy.

Other procedures

SCs were isolated as described by Heyting *et al.* (1985) and Heyting and Dietrich (1991); SDS-polyacrylamide gel electrophoresis of proteins was performed according to Laemmli (1970), as described by Heyting *et al.* (1985); immunoblotting was carried out according to Dunn (1986), as described by Heyting and Dietrich (1991).

Results

Isolation of cDNAs encoding the 190,000 M_r SC component (SCP2) of the rat

For the isolation of cDNAs encoding the 190,000 M_r component of rat SCs, we screened a rat testis cDNA expression library, using a pool of six independently

isolated Mabs, which had been elicited against isolated rat SCs. On Western blots carrying SC proteins, each of these Mabs recognizes specifically the 190,000 M_r SC component, and a series of smaller fragments. We interpret the smaller fragments as breakdown products of the 190,000 M_r protein, because different Mabs recognize the same pattern of peptide bands (compare Mab IX1H9 (Fig. 1, lane 6) and IX3E4 (Fig. 1, lane 7). Among $3x10^5$ recombinant phage clones, this pool of Mabs recognized 10 clones, containing cDNA inserts of 1100 to 3900 nucleotides with overlapping restriction enzyme maps. In order to isolate a full-length clone, we screened the cDNA library with the 5' *Eco*RI fragment of the clone with the longest insert, clone 3C1. This yielded clone 5, with an insert size of 4437 bp, which extended 500 bp further in the 5' direction than clone 3C1. By successive RACE experiments, performed on total testis RNA as a template, we identified and sequenced 334 additional nucleotides at the 5' end.

The nucleotide sequence of clone 3C1 and parts of the sequence of clone 5 were determined, and no discrepancies were found. The nucleotide sequence of the complete cDNA, as assembled from the sequences of clone 3C1, clone 5, and the products of the RACE experiments, contains a single open reading frame of 4515 nucleotides, which encodes a 173 kDa protein consisting of 1505 amino acids (Fig. 2). Nucleotide 154 to 156 is assigned as the translation start codon because it is the first in-frame ATG codon; 135 to 133 nucleotides upstream of this ATG codon (position 19 to 21 in Fig. 2) there is an in-frame stopcodon.

We think that the nucleotide sequence in Fig. 2 represents the full-length cDNA sequence encoding the 190,000 M_r SC protein of the rat, for the following reasons: (i) the recombinant gene product is recognized by four of the six independently isolated Mabs that were used for screening (not shown). (ii) The predicted pI (8.0) is in good agreement with the pI (8) of the 190,000 M_r component as observed in two-dimensional separations of SC proteins (Offenberg, 1993). (iii) A polyclonal antiserum, elicited against the translation product of part of the cDNA, serum 493, reacts specifically with the 190,000 M_r SC component on a Western blot, carrying SC proteins (Fig. 1), and a series of peptide bands, which are also recognized by two of the Mabs (compare Fig. 1 lane 8 with lane 6 and 7), and which we interpret as breakdown products of the 190,000 M_r SC component.

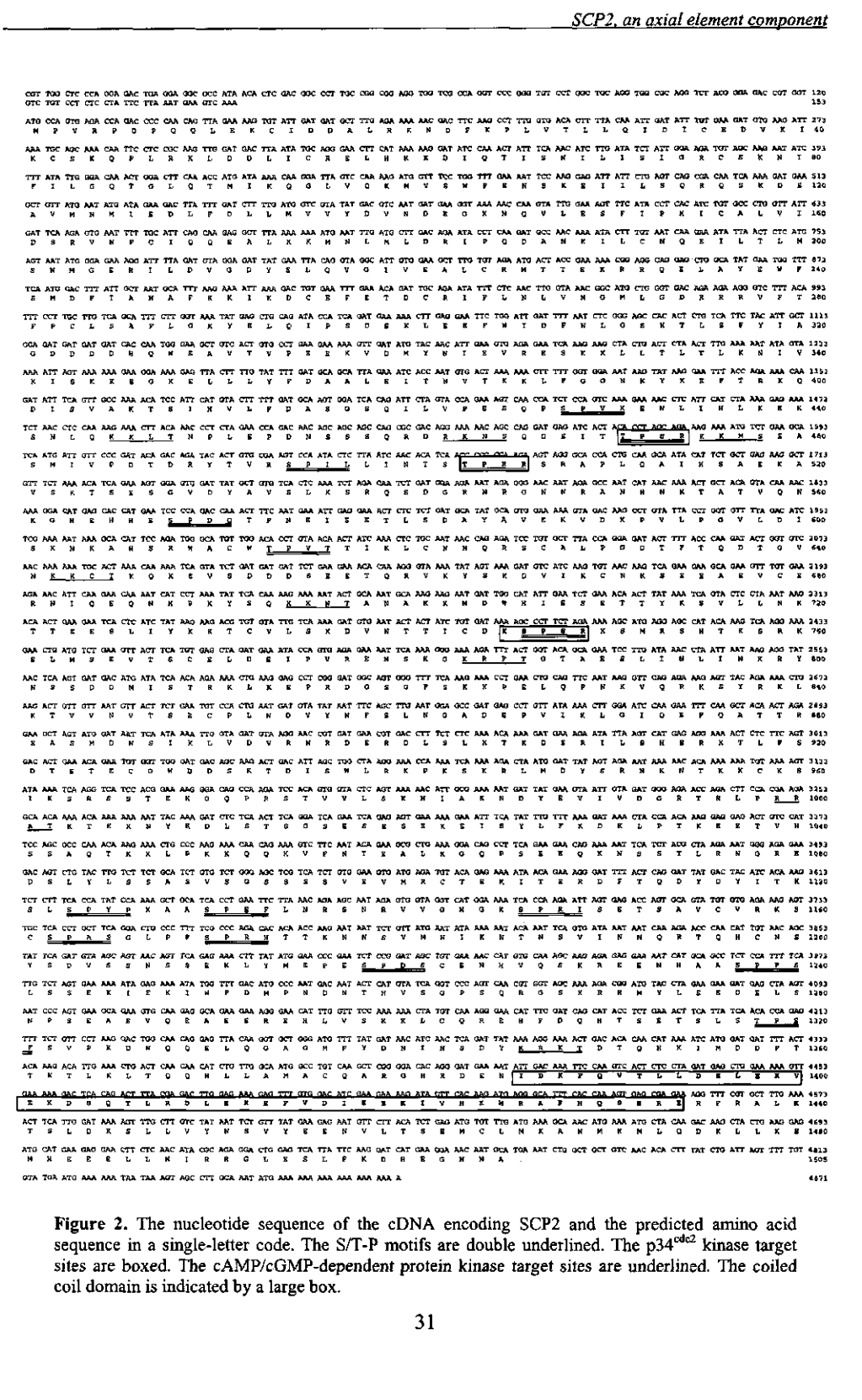

Figure 2. The nucleotide sequence of the cDNA encoding SCP2 and the predicted amino acid sequence in a single-letter code. The S/T-P motifs are double underlined. The p34^{cdc2} kinase target sites are boxed. The cAMP/cGMP-dependent protein kinase target sites are underlined. The coiled coil domain is indicated by a large box.

(iv) In frozen sections of the testis, serum 493 reacts specifically with nuclei of meiotic prophase cells (spermatocytes): the immunofluorescence pattern in Fig. 3 is virtually identical to the pattern obtained with the Mabs that were used for screening (compare Fig. 3 of this paper with Fig. 3a in Offenberg *et al.*(1991)).

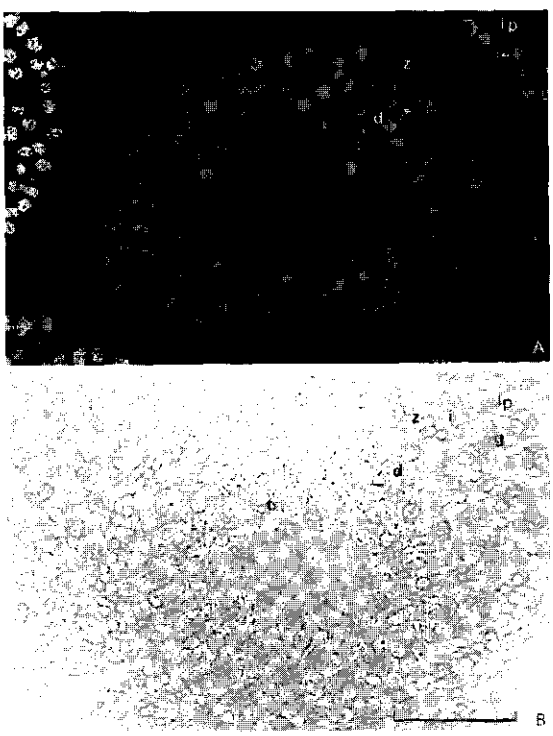

Figure 3. Frozen section of rat testis after immunofluorescence staining with serum 493 (elicited against the translation product of part of the SCP2 cDNA). (A), immunofluorescence, and (B), phase contrast of the same section. The centre of the micrographs shows a cross-sectioned tubule which is in developmental stage XIII because it contains two layers of spermatocytes; these are in zygotene (z) and diplotene (d) (Leblond and Clermont, 1952). The cell layer outside the spermatocyte layers contains spermatogonia (g); the cells inside the spermatocyte layers are spermatids (t); between the tubules, there are interstitial cells (i). The upper right corner of the micrographs shows part of a stage VII to IX tubule, because it contains a single layer of relatively large spermatocytes; these are in mid-late pachytene (lp) (Leblond and Clermont, 1952). Bar represents 150 μm.

(v) Within spermatocytes, serum 493 recognizes specifically the LEs of SCs, like the Mabs that were used for screening (Fig. 4).

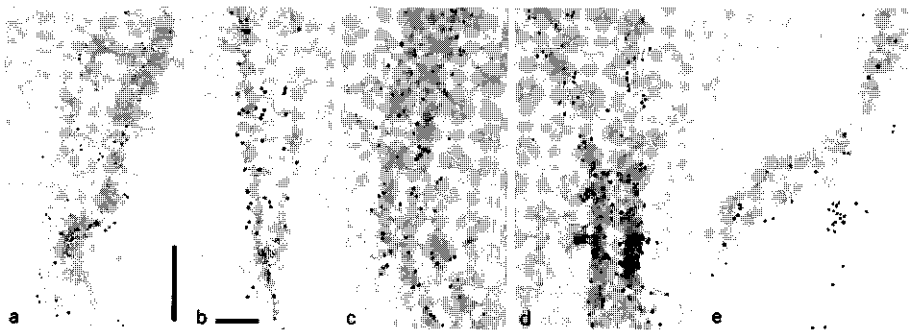

Figure 4. Ultrastructural localization of the antigens of serum 493 by immunogold labeling of surface spread spermatocytes. (b), zygotene, (c), pachytene, and (d), diplotene spermatocytes; (e), immunogold labeling of the XY chromosome pair by serum 493; (a), immunogold labeling of a pachytene SC by Mab IX9D5, one of the monoclonal antibodies that were used for screening. In (a), the immunogold grains have a diameter of 5 nm, in (b) to (e) 10 nm. Bars represent 200 nm. The magnification in (c) to (e) is the same as in (b).

We therefore conclude that we have cloned the cDNA encoding the 190,000 M_r component of the LEs of SCs, for which we propose the name SCP2 (*s*ynaptonemal *c*omplex *p*rotein 2). The discrepancy between the relative electrophoretic mobility of the 190,000 M_r SC component and the predicted molecular weight is probably due to the fact that SCP2 is a basic protein with a high percentage of proline residues. Proteins with these characteristics will migrate more slowly in SDS-polyacrylamide gels than can be expected on basis of their molecular weight (Hames, 1990). A similar discrepancy between predicted molecular weight and observed electrophoretic

mobility has been found for other proteins with these characteristics, for instance, the proline-rich protein RAP1 (Shore and Nasmyth, 1987). Searching libraries of known sequences by the BlastP program (Altschul *et al.*, 1990) revealed a limited amino acid sequence similarity between residue 425 to 478 of SCP2 and residue 564 to 617 of the Red1 protein of *S. cerevisiae* (Fig. 5), which has a role in SC-assembly and meiotic recombination (Rockmill and Roeder, 1990).

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SCP2   425 SQPSPVKENLIHLKEKSNLQKKLTNPLEPDNSSSQRDRKNSQDEITTPSRKKMS 478
           :||:: |::  | ||| : ||||||     : :||  |:   :  | |   |:|
RED1   564 GQPPSKKQKQFHKKEKKKQQKKLTNFKPIIDVPSQDKRNLRSNAPTKPKSIKVS 617
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Figure 5. Amino acid sequence comparison of SCP2 and the Red1 protein of yeast (*Saccharomyces cerevisiae)* by the BLASTP program (Altschul *et al.*, 1990). The numbers to the left indicate for each protein the first amino acid where the similarity begins. Connecting lines indicate identical amino acid residues, and colons similar amino acid residues.

SCP2 has features of a DNA-binding protein

Between amino acid residues 1364 and 1499, SCP2 contains a predicted amphipathic α-helical domain, of which, according to the algorithm of Lupas *et al.* (1991), the stretch between residue 1386 and 1434 is capable of forming a coiled coil structure. According to Chou-Fasman analysis (Chou and Fasman, 1978) SCP2 is rich in β-turns. No other readily identifiable secondary structural motifs were found in SCP2.

SCP2 contains several interesting small scale amino acid sequence motifs: the protein is enriched in S/T-P motifs, which are common in a variety of DNA-binding proteins (Suzuki, 1989), and allow non-sequence specific binding to DNA, presumably through interaction in the minor groove with the phosphodeoxyribose backbone (Churchill and Travers, 1991; Green *et al.*, 1993). SCP2 has 15 of these motifs and in addition 40 S/T-S/T motifs, which can mimick the conformation of the S/T-P motif (Suzuki, 1989). The S/T-P motifs occur in two clusters (Fig. 2), which are separated by a hydrophilic and basic domain of the protein with a calculated pI of about 9.5. SCP2 has several potential protein kinase target sites, including one target site for $p34^{cdc2}$ protein kinase which fulfils the consensus K-S/T-P-X-Z (Moreno and Nurse,

1990), and two sites which satisfy the relaxed consensus S/T-P-X-Z (Draetta, 1990), and eight cAMP/cGMP-dependent protein kinase target sites (K/R-K/R-X-S/T, where X is any amino acid (Feramisco *et al.*, 1980)), which are interspersed with the S/T-P motifs, or are located in the basic central part of the protein. Furthermore, SCP2 contains two nuclear targeting signals (K-R/K-X-R/K, where X is any amino acid (Chelsky *et al.*, 1989)).

The gene encoding SCP2 is transcribed specifically during meiosis

The antigens recognized by the anti-190,000 M_r monoclonal antibodies (Heyting *et al.*, 1989; Offenberg *et al.*, 1991) and the polyclonal anti-SCP2 serum (serum 493) (Fig. 3 and Fig. 7) are found exclusively in meiotic prophase cells. Northern blot analysis, performed with anti-sense RNA probes derived from SCP2, revealed a single transcript of about 4900 nucleotides in poly(A)$^+$ RNA from the testis, but not in RNA from other organs (Fig. 6). Within the testis, the gene encoding SCP2 is transcribed predominantly in meiotic prophase cells (Fig. 7).

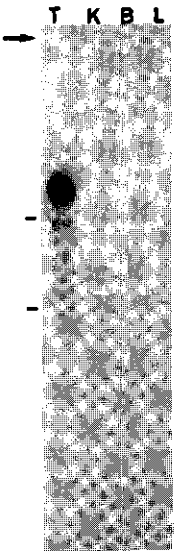

Figure 6. Transcription of the gene encoding SCP2, analysed by northern blot analysis. 15 µg of poly(A)$^+$ RNA from respectively, testis (T), kidney (K), brain (B), and liver (L) was layered per 0.5 cm wide slot of a 1% denaturing agarose gel. After electrophoresis and blotting, hybridization was performed with an RNA transcript of an 850 bp fragment of cDNA clone 3C1. Bars indicate the position of the 28S (4700 nucleotides) and 18S (1900 nucleotides) ribosomal RNA of the rat. The arrowhead indicates the top of the gel.

Discussion

Meiotic prophase chromosomes are organized in loops along proteinaceous axes, called axial elements, which are incorporated as lateral elements (LEs) in the tripartite structure of SCs. The axial elements are distinct from mitotic or meiotic metaphase chromosome scaffolds in that they largely consist of meiosis-specific components (Heyting *et al.*, 1989). Furthermore, each axial element is shared by the two sister chromatids of a meiotic prophase chromosome, whereas the sister chromatids of mitotic or meiotic metaphase chromosomes each have their own scaffold. Elucidation of the relation between axial elements and chromosome scaffolds will provide insight into the structure of chromosomes and the mechanisms of meiotic chromosome pairing and crossover formation. In mammals, major protein components of axial elements have been identified, with M_rs of 30,000, 33,000 and 190,000, respectively (Heyting *et al.*, 1987, 1989). The 30,000 and 33,000 M_r components are closely related and probably the products of a single gene, *Scp3* (Lammers *et al.*, 1994). The corresponding cDNA of the rat has been cloned, and encodes a 30 kDa protein called SCP3 (Lammers *et al.*, 1994); the cDNAs encoding the homologous proteins of the mouse (SYCP3, (Klink *et al.*, 1997)), and of the hamster (COR1, (Dobson *et al.*, 1994)) have also been cloned. In this paper we describe the isolation and sequencing of the cDNA encoding the 190,000 M_r component of the axial elements.

Expression of the gene encoding SCP2

The 190,000 SC M_r component occurs exclusively in meiotic prophase nuclei, in SCs (Heyting *et al.*, 1989; Offenberg *et al.*, 1991; Dietrich *et al.*, 1992; this paper, Fig. 3 and 4). The experiments in this paper show that expression of *Scp2* is regulated at the transcriptional level, because northern blot analysis of RNA from various tissues only revealed transcripts of the *Scp2* gene in testis RNA (Fig. 6). A similar result was obtained earlier with respect to two other major components of SCs, namely SCP1 (Meuwissen *et al.*, 1992) and SCP3 (Lammers *et al.*, 1994). These results corroborate our earlier conclusion (Heyting *et al.*, 1988; Offenberg *et al.*, 1991) that SCs originate predominantly by assembly from newly synthesized components rather than by

rearrangement of pre-existing nuclear structures. Within the testis, *Scp2* transcripts occurred predominantly in meiotic prophase cells, whereas a low level of transcripts appeared to persist in spermatids (Fig. 7).

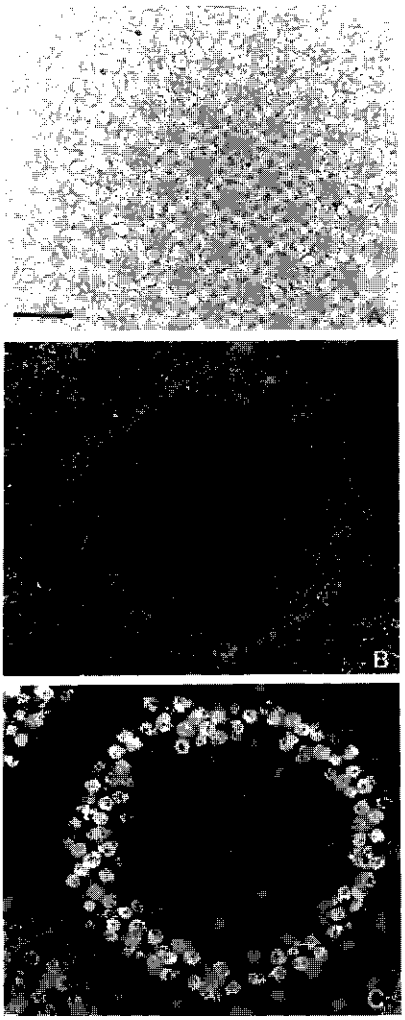

Figure 7. Localization of *SCP2* transcripts in the testis by *in situ* hybridization. (A), Phase contrast micrograph of a transverse section of a testicular tubule in developmental stage VII to IX spermatocytes (mid-late pachytene; Leblond and Clermont, 1952). (B), Localization of *SCP2* transcript in the adjacent section by *in situ* hybridization; a ^{35}S-labeled anti-sense RNA transcript was used as a probe. Note that the transcripts are present in the cytoplasm: nuclei are visible as "black holes". (C), Localization of SCP2 in the same section by indirect immunofluorescence staining with Mab IX1H9 as primary antibody: this antibody made part of the pool of anti-190,000 M_r Mabs that was used for screening of the cDNA library. Bar represents 50 μm.

We cannot exclude that the *Scp2* gene is transcribed at very low levels in other organs. Of the SCP1 gene, low levels of transcripts have been detected in the brain by means of PCR (Kerr *et al.*, 1996), although such transcripts were not detectable on northern blots of brain RNA (Meuwissen *et al.*, 1992). However, we doubt whether such very low levels of transcription are functionally significant, because no SCP1 or SCP2 protein has been detected in any other tissue than testis and ovary.

Sequence and predicted secondary structure of SCP2

The amino acid sequence of the predicted protein SCP2 contains several motifs of potential interest: the protein contains three potential $p34^{cdc2}$ kinase target sites, which could be important for regulation of the assembly and disassembly of the SC: mutation of the CDC28 gene, which encodes the *S. cerevisiae* protein homologous to $p34^{cdc2}$, causes an arrest in pachytene (Shuster and Byers, 1989); it is thus possible that the $p34^{cdc2}$ protein kinase plays a role in the regulation of SC disassembly. The major component of the transverse filaments of SCs, SCP1, also has a potential target site for $p34^{cdc2}$ protein kinase (Meuwissen *et al.*, 1992). Besides the $p34^{cdc2}$ kinase target sites, SCP2 contains eight target sites for cAMP/cGMP dependent protein kinase (Feramisco *et al.*, 1980). This protein kinase is possibly also involved in the regulation of SC (dis)assembly: inhibition of phosphorylation of nuclear lamins by cAMP/cGMP-dependent protein kinase plays a key role in the regulation of the disassembly of the nuclear lamina (Lamb *et al.*, 1991). Furthermore, axial element component SCP3 also has two potential target sites for cAMP/cGMP-dependent protein kinase (Lammers *et al.*, 1994). However, it still has to be sorted out whether the potential kinase target sites on SCP2 are actually phosphorylated *in vivo*, and whether this plays any role in the regulation of SC (dis)assembly.

SCP2 has features of a DNA binding protein

SCP2 shares features with several other proteins that have a function in chromatin organization. SCP2 has two large clusters of S/T-P and S/T-S/T motifs, which flank a basic domain of the protein. S/T-P and S/T-S/T motifs are common in a variety of DNA-binding proteins, and allow non-sequence-specific interaction with the minor

groove of DNA (Churchill and Travers, 1991; Green *et al.*, 1993). Transverse filament proteins SCP1 of the rat (Meuwissen *et al.*, 1992) and Zip1 of yeast (Sym *et al.*, 1993), and meiotic chromosome core component Red1 of yeast (Smith and Roeder, 1997; Thompson and Roeder, 1989) also contain clusters of S/T-P and S/T-S/T motifs. Several nuclear matrix proteins also contain S/T-P clusters, for example SAF-A, a protein for which *in vivo* binding to matrix attachment DNA-regions has been proven (Goehring and Fackelmayer, 1997). Other nuclear matrix proteins carrying clusters of S/T-P motifs include mammalian nuclear matrix protein NUMA (Compton *et al.*, 1992; Yang *et al.*, 1992), lamins (chicken lamin A, B1 and B2, human lamin A and C; Peter *et al.*, 1989; Vorburger *et al.*, 1989; McKeon *et al.*, 1986; Fisher *et al.*, 1986), repressor/activator site binding protein Rap1 of yeast (Shore and Nasmyth, 1987), and topoisomerase II (Austin *et al.*, 1993), which is found in chromosome scaffolds (Earnshaw and Heck, 1985). Lamin B-1 (Ludérus *et al.*, 1992; 1994), Rap1 (Shore and Nasmyth, 1987). SATB1 (Dickinson *et al.*, 1992) and SCP1 (Meuwissen, 1997) have been shown to bind to DNA *in vitro*, although no obvious similarity with conserved features of DNA binding proteins could be identified in these proteins besides the S/T-P and S/T-S/T clusters. Furthermore, Meuwissen (1997) showed that SCP1 binds to DNA through interaction with the minor groove, and that its DNA-binding activity is confined to the C-terminal domain, which contains all S/T-P and S/T-S/T motifs. In preliminary southwestern blot experiments, an expression product of a large part of the SCP2 cDNA was also capable of binding to DNA (K_a=3.6×10^9 M^{-1}, unpublished experiments). However, it remains to be demonstrated that SCP2 binds to DNA *in vivo*.

Possible functions of SCP2

The localization and predicted secondary structural features of SCP2 suggest that this protein is involved in the organization of meiotic prophase chromatin, possibly by temporarily binding to SARs. In detailed immunofluorescence studies (Schalk *et al.*, in preparation), we found that SCP2 first assembles into short stretches of axial element, which fuse to form long, linear, unsynapsed axial elements, which then shorten and thicken as synapsis proceeds. How the assembly of linear axes is accomplished is not

known. It is not necessary to suppose a specific function for SCP2 in this respect, because chromosomes that are forced to condense from G2 in somatic cells, which do not contain SCP2, also develop long, linear axes (Giménez-Abián *et al.*, 1995). However, (premature) condensation from somatic G2 results in the assembly of one axis for each chromatid; only if condensation proceeds in the presence of a topoisomerase II inhibitor (ICRF), a single, undivided axis is formed for the two chromatids of each chromosome (Giménez-Abián *et al.*, 1995); upon recovery from ICRF, the single axis splits, the centromeric regions being separated last. It is possible that in meiotic prophase, SCP2 has a comparable effect as a topoisomerase II inhibitor on the separation of sister chromatids, by competing with topoisomerase II for binding to SARs. The two sister chromatids would then remain unseparated until SCP2 is removed, possibly by phosphorylation, and replaced by topoisomerase II, which gradually congregates onto the axial elements in the second half of meiotic prophase (Moens and earnshaw, 1989). SCP2 persists in the centromeric region during meiotic metaphase I (Schalk *et al.*, in preparation), as has been described earlier for SCP3 (COR1) (Dobson *et al.*, 1994); such a localization would be consistent with a role of SCP2 in sister chromatid cohesion. The ultrastructural localization of SCP2 in the center of the axial element (Schalk *et al.*, in preparation) is also consistent with such a role.

One other possible clue to the function(s) of SCP2 is provided by the work on the yeast Red1 protein. SCP2 shows a limited sequence similarity to the Red1 protein of yeast. SCP2 and Red1 are also similar in that both proteins contain many S/T-P and S/T-S/T motifs (Red1 has 6 S/T-P motifs and 20 S/T-S/T motifs, (Thompson and Roeder, 1989)), and are predicted to form a short coiled-coil domain at their C-terminus. Although there are also considerable differences (Red1 has a much lower molecular weight and pI than SCP2), the similarities are of interest, because Red1 is a candidate component of the axial elements of yeast SCs (Smith and Roeder, 1997). The Red1 protein localizes to the cores of meiotic prophase chromosomes (Smith and Roeder, 1997). *red1* mutants do not assemble axial elements (Rockmill and Roeder, 1990), and display a decreased level of meiotic chromosome pairing, heteroduplex formation (Nag *et al.*, 1995), and interchromosomal gene conversion and crossing over (Rockmill and Roeder, 1990). Furthermore, Red1 is required for the formation of

crossovers that can ensure a proper disjunction of homologous chromosomes at metaphase I (Rockmill and Roeder, 1990) and has a role in monitoring the recombination process (Xu *et al.*, 1997). The involvement in such a variety of processes can be understood if Red1 is a structural component of the axial elements which interacts with several proteins that function in one or more of these processes. The same could be true for SCP2. The cloning of the cDNA encoding SCP2 will allow us to analyse this by searching for the proteins and DNA-sequences that interact with SCP2.

Acknowledgments

We thank A.C.G. Vink for expert technical assistance, and M. Nijland for her contribution during her undergraduate study. This work was supported in part by NWO Netherlands Organization for Scientific Research, grants no. 900-501-064 and 900-501-143.

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Chapter 3

Isolation and characterization of the human SCP2 cDNA and chromosomal localization of the gene

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Submitted for publication in Mammalian Genome

Abstract. *SCP2 is a meiosis-specific component of the axial elements of synaptonemal complexes, and was originally identified in the rat. The cDNA encoding SCP2 of the rat (rnSCP2) has recently been isolated and sequenced. The protein contains several S/T-P and S/T-S/T motifs, which are supposed to contribute to DNA binding by interaction with the minor groove of DNA. Furthermore, rnSCP2 contains a coiled-coil domain at its C-terminus and several potential phosphorylation sites. We isolated and sequenced the cDNA encoding the human SCP2 protein (hsSCP2) to study the conservation of these features. The predicted amino acid sequence of hsSCP2 showed 63 % identity with rnSCP2. Several structural features and amino acid sequence motifs were conserved; hsSCP2 contains S/T-P motifs in the same domains as rnSCP2, and has a predicted coiled-coil region at its C-terminus. The hsSCP2 gene was assigned to human chromosome 20q13.33 by fluorescence in situ hybridization.*

Introduction

Synaptonemal complexes (SCs) are proteinaceous structures, which are formed between homologous chromosomes during prophase I of meiosis. The formation of the synaptonemal complex starts in early prophase, when a single proteinaceous axial element is formed between the two sister chromatids of each chromosome. The chromatin loops of the two sister chromatids of each chromosome are connected at their bases to the axial elements. Later in meiotic prophase I, the axial elements of homologous chromosomes are connected by transverse filaments to form the synaptonemal complex structure; within the context of the SC, axial elements are called lateral elements (LEs).

The function of axial elements and SCs is still under investigation. From studies in fission yeast (*Schizosaccharomyces pombe*) and of certain mutants of budding yeast (*Saccharomyces cerevisiae*), it has been concluded that transverse filaments are not indispensable to meiotic recombination and the formation of functional chiasmata (Egel-Mitani *et al.*, 1982; Bähler *et al.*, 1993; Sym *et al.*, 1993), but that they are required for meiotic crossover interference (Munz, 1994; Sym and Roeder, 1994). Possible functions of the axial elements include the conversion of crossovers into functional chiasmata that contribute to the proper orientation of bivalents at metaphase I (Molnar *et al.*, 1995; Hollingsworth and Byers, 1989; Rockmill and Roeder, 1990), and the enhancement of recombination between homologous chromosomes rather than sister chromatids (Schwacha and Kleckner, 1994; Mao-Draayer *et al.*, 1996). Two candidate components of yeast SCs have been identified, namely Hop1 and Red1 (Hollingsworth and Byers, 1989; Rockmill and Roeder, 1988). *hop1* and *red1* mutants fail to assemble axial elements, but still display 10-20% of wildtype level of crossing-over (Hollingsworth and Byers, 1989; Rockmill and Roeder, 1990). These residual crossovers do not enhance the proper disjunction of homologous chromosomes (Rockmill and Roeder, 1990).

The structure of the SC has been largely conserved among eukaryotes and it seems likely that at least part of the functions of SCs have also been conserved. At the level of individual SC-proteins however, there are as yet little indications of

evolutionary conservation: SC-components have not only been identified in yeast, but also in rodents (Heyting *et al.*, 1987, 1989; Smith and Benavente, 1992; Chen *et al.*, 1992; Dobson *et al.*, 1994) and in lily (Anderson *et al.*, 1994). cDNAs encoding components of rodent SCs have now been cloned and sequenced, namely a component of the lateral elements, called SCP3 (COR1) (Lammers *et al.*, 1994; Dobson *et al.*, 1994) and a component of the transverse filaments, called SCP1 (SYN1) (Meuwissen *et al.*, 1992; Dobson *et al.*, 1994). At the amino acid sequence level, these proteins do not display homology to any of the yeast SC-components, although SCP1 shows some structural similarities to Zip1, a putative component of transverse filaments of yeast SCs (Sym *et al.*, 1993; Sym and Roeder, 1994, 1995). It is possible that SCP1 is funtionally homologous to Zip1 (Meuwissen *et al.*, 1992).

Recently, we isolated and sequenced the cDNA encoding the M_r 190,000 component of the axial elements of rat SCs (rnSCP2) (Offenberg *et al.*, 1998). The predicted amino acid sequence has some interesting structural and small scale amino acid sequence motifs; rnSCP2 contains several S/T-P and S/T-S/T motifs, which are localized in two clusters, and which might be involved in DNA-binding (Suzuki, 1989). Furthermore, rnSCP2 contains a coiled-coil region at its C-terminus and it has several potential phosphorylation sites (Offenberg *et al.*, 1998). It shares the structural features with the Red1 protein of yeast (Thompson and Roeder, 1989). Furthermore, rnSCP2 and Red1 share a small region of amino acid sequence similarity (Offenberg *et al.*, 1998). Possibly, rnSCP2 and Red1 are homologous proteins, or at least functional homologues. In order to learn more about the amino acid sequence conservation of SCP2, and the possible homology of SCP2 to Red1 of yeast, we also isolated and sequenced the human SCP2 cDNA. The results show that SCP2 is not a very conserved protein, and that there are no indications for amino acid sequence homology between hsSCP2 and Red1 of yeast. However it is possible that rnSCP2 and hsSCP2 have a similar function as the Red1 protein, since the organization of structural features within the proteins is similar.

Materials and methods

Isolation and sequence analysis of human cDNA clones encoding the rat SCP2 homologue

For the isolation of cDNA encoding the human homologue of SCP2, we screened 5×10^5 recombinant phage of a human testis cDNA library in λgt10 (Huynh *et al.*, 1985) (Clontech Laboratories Inc., Palo Alto, CA, USA, HL 1161a), using a 5' terminal fragment of 750 bp and a 3' terminal fragment of 730 bp of rnSCP2 cDNA as probes. Screening with the 5' probe yielded 3 positive clones, with inserts of 1.1, 1.2 and 2.1 kb respectively. Screening with the 3' probe yielded one clone with an insert of 2.5 kb. Restriction enzyme fragments of the 1.1 and 2.1 kb inserts of the 5' cDNA clones and of the 2.5 kb insert of the 3' cDNA clone were subcloned into the pBluescript SK(+) vector (Stratagene Inc., San Diego, CA, USA). We obtained the middle part of the human cDNA by means of PCR on the λgt10 library, using primers derived from the 2.1 kb insert of the 5' cDNA clone and the 2.5 kb insert of the 3' cDNA clone: 2×10^8 plaque-forming units of the human cDNA library were resuspended in 75 µl deionized water and heated for 5 minutes at 70°C. Subsequently the sample was adjusted to 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 50 pmol of each primer and 2.5 units *Taq* polymerase (Pharmacia Biotech, Uppsala, Sweden) were added. The sample was incubated according to the following schedule: 1 cycle: 5 minutes 94°C; 30 cycles: 1 minute 94°C, 2 minutes 51°C, 3 minutes 72°C; 1 cycle: 1 minute 94°C, 2 minutes 51°C, 15 minutes 72°C. Subsequently, we took 25 µl of the PCR-reaction mixture and added 1 µl of 1 mM dATP, 0.5 µl of a 10 mM dNTP mixture, 5 units T4 polynucleotide kinase (Gibco BRL Life Technologies, Paisley, UK) and 5 units T4 DNA polymerase (Gibco BRL), and incubated for 20 minutes at room temperature. This fragment was then subcloned into the pBluescript SK(+) vector (Stratagene). We amplified the 5' end of the 1.2 kb insert of the 5' cDNA clone by PCR, using a primer derived from the 2.1 kb cDNA insert and a λgt10 vector-specific primer. The PCR reaction was performed on isolated λgt10 phage DNA containing the desired cDNA sequence, in reaction buffer which contained 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 20 pmol of each primer and 2.5 units of *Taq* polymerase

(Goldstar DNA polymerase, Eurogentec, Seraing, Belgium), according to the following schedule: 1 cycle: 1 minute 94°C; 30 cycles: 20 seconds 94°C, 20 seconds 51°C, 1 minute 72°C; 1 cycle: 20 seconds 94°C, 20 seconds 51°C, 15 minutes 72°C. The obtained product was subcloned into the pGEM-T vector (Promega, Madison, Wisconsin, USA). We obtained fragments of the human cDNA extending further in the 5' direction by PCR on 1250 ng of isolated λgt10 library DNA, using a 5'-oriented primer derived from the human cDNA and a 3'-oriented primer derived from rnSCP2 cDNA, which contained the first ATG startcodon. The PCR-reaction was performed as described above. In order to obtain cDNA fragments that extend beyond the first ATG, a linear PCR was performed on 1250 ng isolated λgt10 DNA with a primer derived from the human SCP2 cDNA followed by a PCR with a nested human cDNA-specific primer in combination with a λgt10 vector-specific primer. PCR products of the above described experiments were cloned into the pGEM-T vector (Promega). We performed sequencing reactions on the resulting clones, using the Dye Deoxy Terminator Cycle sequencing kit from Perkin-Elmer (Norwalk, Connecticut, USA) and determined the nucleotide sequence on a 373A stretch 48 cm WAR DNA sequencer (Applied Biosystems, Inc., Foster City, CA, USA). The obtained sequences were assembled by means of the University of Wisconsin GCG sequence analysis package (University of Wisconsin, WI, USA). Sequence similarity searches of the Genbank, EMBL, Swissprot and PIR data bases were carried out with BLASTX, BLASTN and TBLASTN (Altschul *et al.*, 1997). Amino acid sequence alignments were determined by means of the Pile-Up program (GCG software package) and the results were presented in the Boxshade program. The EMBL accession number of the complete hsSCP2 cDNA sequence is Y08982.

Fluorescence in situ *hybridization analysis*

A genomic PAC clone (Ioannou *et al.*, 1994), containing the *hsSCP2* gene was isolated by screening a human PAC library (library RPCI1, 3-5 Human PAC0, constructed by Ioannou and de Jong, Roswell Park Cancer Institute, Buffalo, New York, was obtained from the Resource Center Primary Database of the German Human Genome Project, Berlin-Charlottenburg, Germany) with a pool of five hsSCP2

cDNA clones, which together cover most of the hsSCP2 coding sequence. The presence of the *hsSCP2* gene on the PAC clone was confirmed by two Southern blot hybridizations with two non-overlapping hsSCP2 cDNA probes (not shown). Fluorescence *in situ* hybridization was performed with the PAC DNA on human metaphase chromosomes according to standard protocols (Hoovers *et al.*, 1992). A chromosome 20-specific alpha satellite probe, pBS20Z (Baldini *et al.*, 1992) was used as centromeric probe to recognize chromosome 20. Probes were labeled with biotin and detected with avidin-FITC (Vector, Burlingame, CA, USA). Counterstaining of the chromosomes was performed with propidium iodide.

Results

Isolation and Sequencing of Human SCP2 cDNAs

For the isolation of cDNA encoding the human homologue of rnSCP2, we screened 5×10^5 recombinant phage of a human testis cDNA library in λgt10, using a 5' and a 3' terminal fragment of rnSCP2 cDNA as probes. Screening with the 5' probe yielded 3 clones with inserts of 1.1, 1.2 and 2.1 kb respectively. Screening with the 3' probe yielded one clone with an insert of 2.5 kb. Restriction enzyme fragments of the 1.1 and 2.1 kb 5' cDNA clones and the 2.5 kb 3' cDNA clone were subcloned into the pBluescript SK(+) vector and sequenced. The missing middle part of the human cDNA was obtained by means of PCR on the library, with the use of two oligonucleotides homologous to the 2.1 kb insert of the 5'cDNA clone and the 2.5 kb insert of the 3'cDNA clone as primers (see Materials and methods). The thus obtained cDNA fragments displayed nucleotide sequence homology with rnSCP2 cDNA, although some discrepancies were found between the 5' end of the 1.1 and 2.1 kb cDNA inserts; the 2.1 kb insert contained an Alu repeat at its 5' terminus, which probably resulted from a ligation of independent fragments during the construction of the library. The 5' end of the 1.1 kb insert showed a sudden drop in nucleotide sequence homology to rnSCP2 cDNA and we supposed that this insert was also a scrambled clone, generated during the construction of the library. The 5' end of the remaining human 5' cDNA

clone, with an insert of 1.2 kb, was amplified by PCR and subcloned into the pGEM-T vector. The 5' end of the 1.2 kb insert showed a considerable level (77 %) of nucleotide sequence homology to rnSCP2 cDNA. Since this clone did not cover the position of the first ATG codon of rnSCP2 cDNA, we performed a series of PCR experiments on the λgt10 library (see Materials and methods), and identified and sequenced 49 additional nucleotides at the 5' end, which contained an in-frame ATG startcodon at the same position as in rnSCP2 cDNA. We did not obtain longer cDNA fragments by PCR on the library, using more 5' positioned primers. We believe that the isolated cDNA fragments together cover the complete human SCP2 cDNA, because they cover the positions of both the start- and the stopcodon in rnSCP2 cDNA. The complete sequence (Fig. 1) was assembled from the sequences of the PCR products and the cDNA subclones; it contains an open reading frame of 4593 nucleotides, encoding a protein of 1530 amino acids, called hsSCP2, with a predicted molecular weight of 176 kDa and a pI of 8.9.

Comparison of hsSCP2 and rnSCP2

The overall homology between the predicted amino acid sequence of hsSCP2 and rnSCP2 is 63 % (Fig. 2).The homology is especially high at the N-terminus where amino acid residues 1-441 of hsSCP2 and rnSCP2 show 81% of amino acid identity. Homology is considerably lower in the rest of the protein where some regions show only 50% of identity. The highly conserved N-terminal part of hsSCP2 does not contain any obvious structural motifs. hsSCP2 contains 14 S/T-P motifs and 36 S/T-S/T motifs (Fig. 1). These motifs are enriched in DNA-binding proteins (Suzuki, 1989). The S/T-P and S/T-S/T motifs form β-turns, which can interact with the minor groove of DNA (Suzuki, 1989). These DNA-binding motifs have also been found in rnSCP2 (Offenberg *et al.*, 1998). Eight of the 15 S/T-P motifs and 18 of the 40 S/T-S/T motifs in rat are conserved in human (Fig. 3A and B). Three S/T-S/T motifs in rat are S/T-P motifs in human, and one S/T-P motif in rat is an S/T-S/T motif in human (Fig. 3A). Although only half of the motifs in hsSCP2 appear at exactly corresponding positions in rnSCP2, the organization of these motifs in two clusters is similar in rat and human (Fig. 3). The region between these clusters is very basic, with a pI of 9.6 in

hsSCP2 and 9.5 in rnSCP2. hsSCP2 contains two potential $p34^{cdc2}$ kinase target sites, namely at amino acid residue 472 and 994 (consensus S/T-P-X-Z (Draetta, 1990)), whereas rnSCP2 has three such sites; one of these is found at a conserved position in hsSCP2 (a.a. residue 471). Furthermore, hsSCP2 contains four potential cAMP/cGMP dependent phosphorylation sites (consensus R/K(2)-X-S/T (Feramisco *et al.*, 1980)) (Fig. 1) of which three are found at the corresponding position in rat. rnSCP2 has six potential tyrosine kinase phosphorylation sites (a.a. residue 206, 230, 231, 365, 720, 1076) (consensus (R/K-X(2)-D/E-X(3)-Y (Cooper *et al.*, 1984)), of which three are conserved in human (a.a. residue 206, 365, 731). Both rnSCP2 and hsSCP2s contain two nuclear targeting signals (a.a. residue 250 and 956 in rnSCP2 and a.a. residue 250 and 810 in hsSCP2) (consensus K-R/K-X-R/K (Chelsky and Jonak, 1989)). At the C-terminus, hsSCP2 contains a region which is predicted to be capable of forming coiled-coil structures (Lupas *et al.*, 1991), at the corresponding position as the coiled-coil region in rnSCP2 (residue 1386 to 1434), eventhough the homology in this region is only 50%.

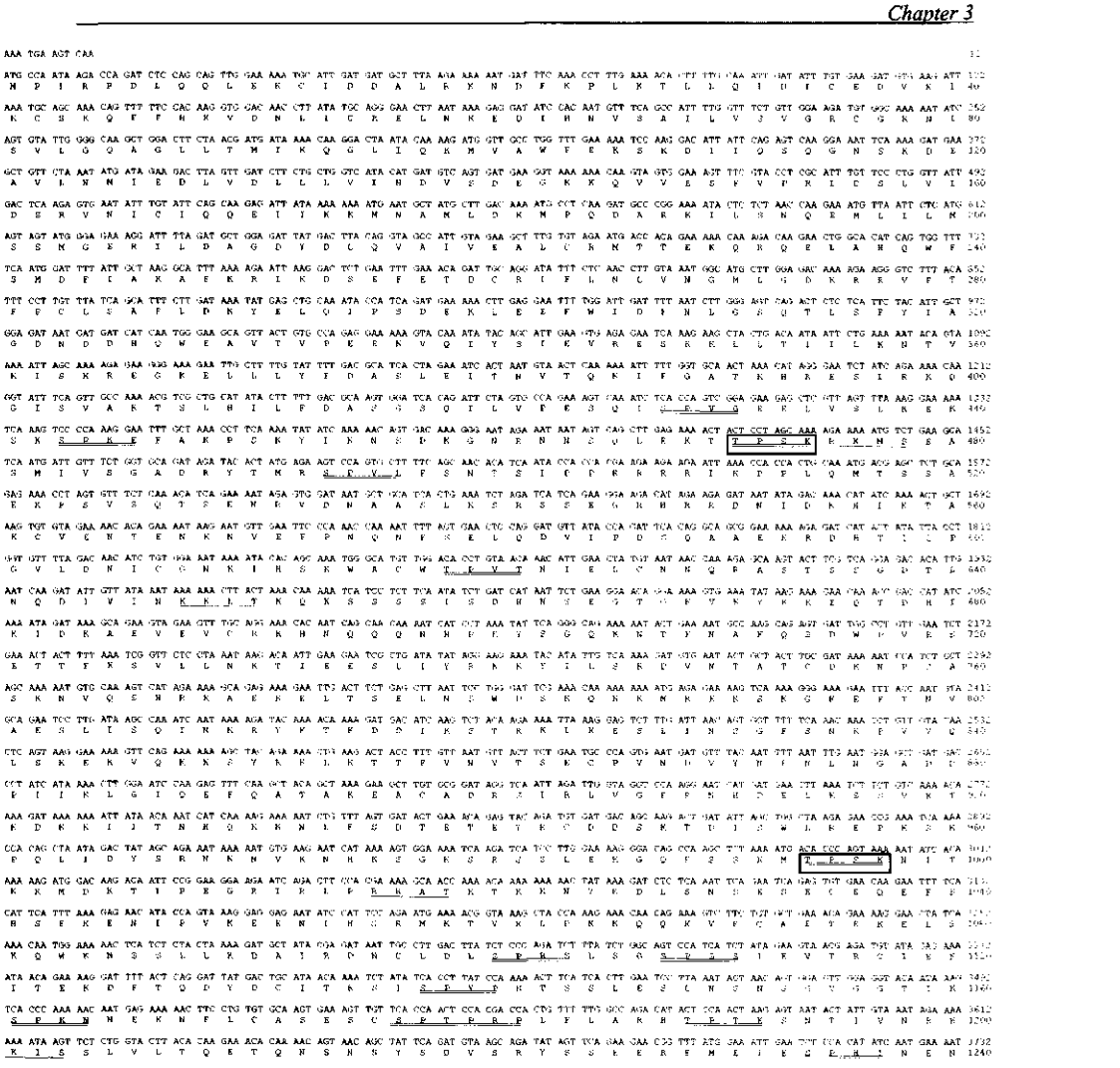

Figure 1. Nucleotide sequence and predicted amino acid sequence of hsSCP2. The predicted translation product is shown below the nucleotide sequence. The S/T-P motifs are double underlined. The potential p34^{cdc2} protein kinase target sites are boxed. The putative cAMP/cGMP-dependent protein kinase target sites are underlined.

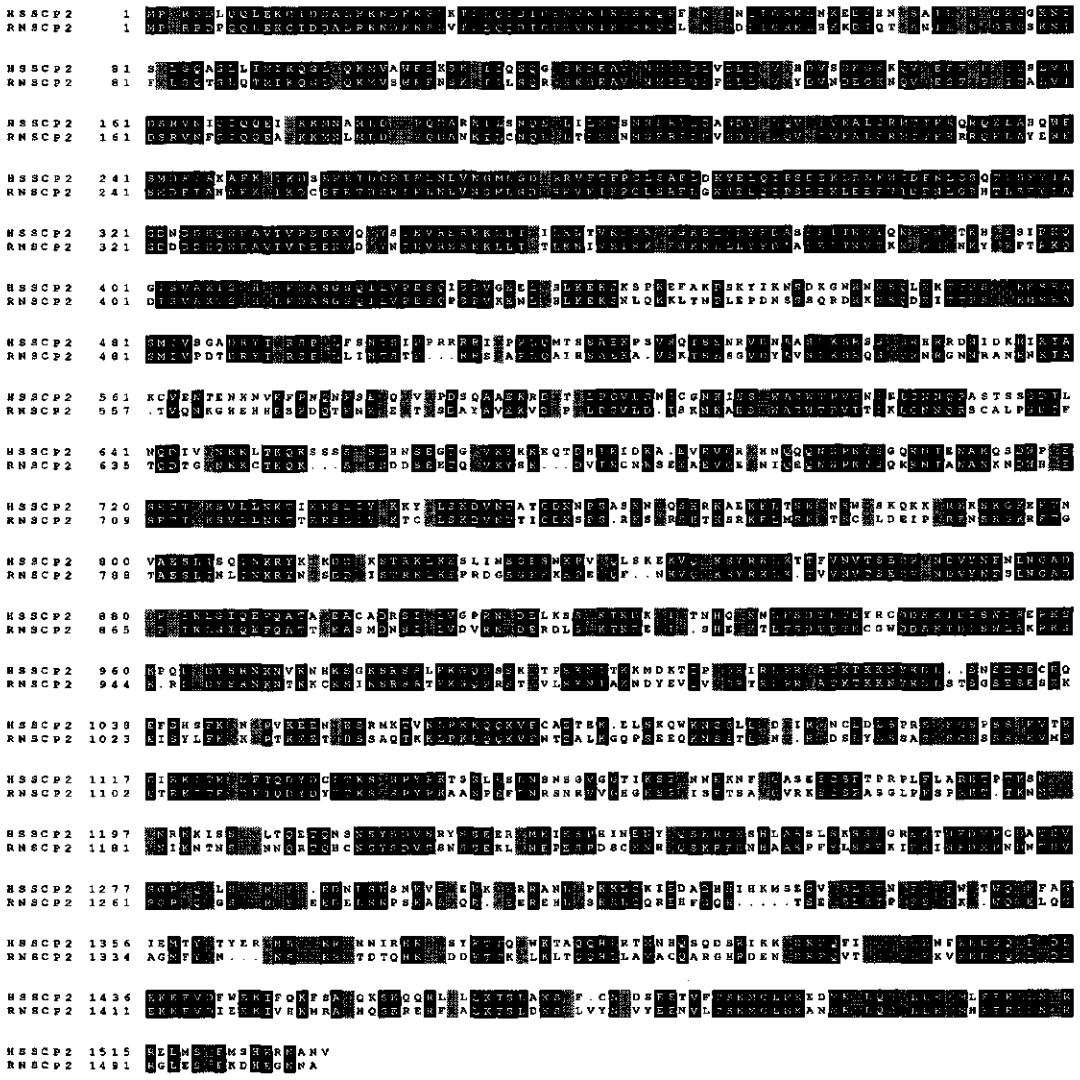

Figure 2. Amino acid sequence alignment of hsSCP2 and rnSCP2. Amino acid sequence alignment was determined by means of the Pile-Up program (GCG software package, University of Wisconsin, WI, USA) and the results are presented in the Boxshade program. Identical amino acids are highlighted in black, functionally conserved amino acids are highlighted in gray. Conserved amino acids are taken as follows: M,V,I,L and F,Y,W and H,R,K and D,E and N,Q and G,A and T,S.

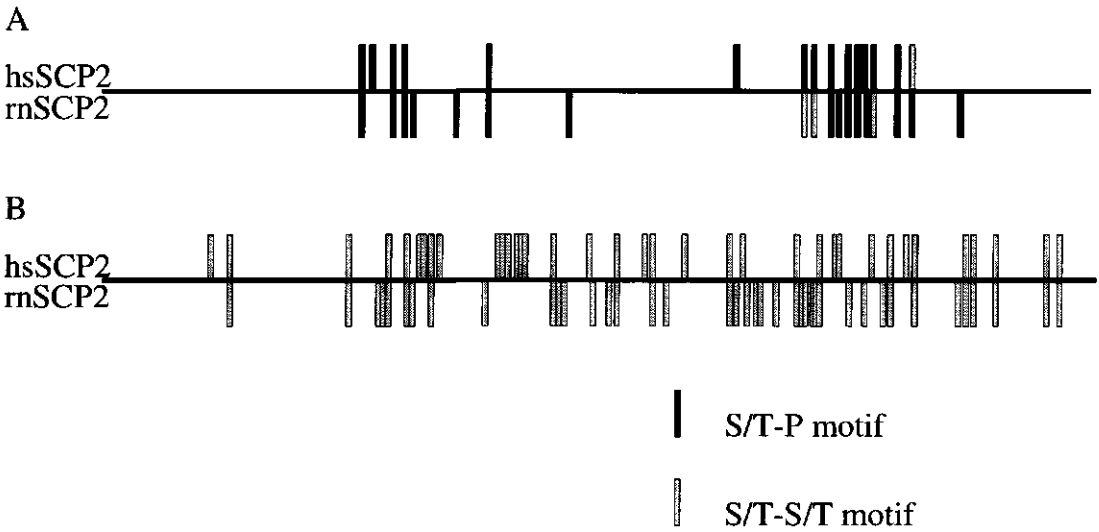

Figure 3. Comparison of the position of the S/T-P and S/T-S/T motifs in SCP2 of rat and human. **(A)** Position of the S/T-P motifs in hsSCP2 and rnSCP2; only those S/T-S/T motifs are shown for which an S/T-P motif is present at the exactly corresponding position of the homologous protein. **(B)** Position of the S/T-S/T motifs in hsSCP2 and rnSCP2.

Homology with other proteins

Screening of databases with the hsSCP2 cDNA sequence revealed three ESTS from a human testis cDNA library, one EST from a heart and four ESTs from a placenta cDNA library, which represented part of the hsSCP2 cDNA. No homology to other proteins than rnSCP2 was detected.

Chromosomal Localization of the Human SCP2 *Gene*

The human *SCP2* gene was localized by fluorescence *in situ* hybridization (Fig. 4). A genomic PAC clone, containing the *hsSCP2* gene, and a centromere-specific probe, which recognizes chromosome 20 (pBS20Z) (Baldini *et al.*, 1992), were used as probes. Ten metaphases with two signals on both chromosomes were analysed and the *hsSCP2* gene was assigned to chromosome 20q13.33.

Figure 4. Localization of the human *SCP2* gene to chromosome 20q13.33. Metaphase chromosomes from human lymphocytes were hybridized with a mixture of two biotin labeled probes: a PAC clone, containing the *hsSCP2* gene and a human centromeric probe specific for chromosome 20, pBS20Z (Baldini *et al.*, 1992). The probes were detected with avidin-FITC. The chromosomes were counterstained with propidium iodide.

Discussion

In this paper we describe the isolation of cDNA fragments which together encode the human homologue of rnSCP2. The overall amino acid identity between hsSCP2 and rnSCP2 is 63%. The most conserved domain of the protein is the N-terminal part (81% amino acid identity); other parts in the protein display a much lower level of homology (50-65%). The conserved N-terminal part contains no obvious secondary structures or small-scale amino acid motifs. Possibly, this part of the protein is involved in specific protein-protein interactions. hsSCP2 contains numerous structural features and amino acid sequence motifs in the rest of the protein, of which several have been conserved between hsSCP2 and rnSCP2.

Comparison of SCP2 from human and rat

hsSCP2 and rnSCP2 both contain several S/T-P and S/T-S/T motifs. The S/T-P motifs are thought to form β-turns which contribute to DNA-binding through interaction with the minor groove, preferably of AT-rich DNA (Suzuki, 1989; Churchill and Suzuki, 1989). S/T-S/T motifs can mimick the conformation of the S/T-P motifs (Suzuki, 1989). Both in hsSCP2 and rnSCP2, the S/T-P motifs are organized in two clusters (Fig. 3A); the clustering of S/T-S/T motifs is less pronounced (Fig. 3B), although these sequences are rare in the N-terminal domain outside the S/T-P clusters. Half of the S/T-P motifs and S/T-S/T sequences in hsSCP2 appear at corresponding positions in rnSCP2. Three S/T-P motifs in human are S/T-S/T motifs in rat, and one S/T-P motif in rat is an S/T-S/T motif in human (Fig. 3A). Thus, although the exact number and position of S/T-P motifs is not well-conserved in SCP2, their organization in two clusters is similar in human and rat. Meuwissen *et al.* (1997) reached a similar conclusion with respect to SCP1, a transverse filament protein of SCs with one cluster of S/T-P and S/T-S/T sequences in its C-terminal domain (Meuwissen *et al.*, 1992). S/T-P motifs often flank a specific DNA-recognizing structure (Suzuki, 1989). The two clusters of S/T-P motifs in rnSCP2 and hsSCP2 surround a very basic region. It is possible that the clusters with S/T-P motifs bind to DNA in a non-specific way and that this basic region is responsible for strong DNA binding, possibly to specific sequences.

Two of the S/T-P motifs in hsSCP2 are also potential target sites for $p34^{cdc2}$ kinase; one of these sites has been conserved in rat. It is possible that these sites are important for the regulation of the assembly and disassembly of SCs (discussed in Offenberg *et al.*, 1998). Four cAMP/cGMP dependent phosphorylation sites are dispersed throughout hsSCP2. The position of three of these sites is conserved between rat and human. These sites are possibly also important for the regulation of SC (dis)assembly by phosphorylation. Furthermore, hsSCP2 contains three tyrosine kinase sites, which are conserved between rat and human. Recently, a meiosis-specific protein-tyrosine phosphatase was identified (Ohsugi *et al.*, 1997), which possibly plays an important role in the regulation of meiosis by (de)phosphorylation of certain proteins. SCP1, the major protein component of the transverse filaments of SCs also contains such a potential tyrosine kinase target site, which has been conserved in human, rat and mouse (Meuwissen *et al.*, 1997; Meuwissen *et al.*, 1992; Sage *et al.*, 1995).

Both hsSCP2 and rnSCP2 contain a domain at their C-terminus which is predicted to be capable of forming a coiled-coil structure (Lupas *et al.*, 1991). Although the homology in this region is low (50% amino acid identity), the position of this coiled-coil domain in hsSCP2 corresponds with that of the coiled-coil domain in rnSCP2. It is possible that SCP2 interacts with other proteins or with itself through this domain.

Apart from differences at the end of some cDNA clones, which we interpret as cloning artefacts, the screening and PCR experiments yielded only one type of cDNA clones with homology to rnSCP2. Although the amino acid identity between hsSCP2 and rnSCP2 is not very high, we think that we have isolated the functional homologue of rnSCP2 because the organization of several structural motifs within hsSCP2 is identical to rnSCP2, and no other human cDNAs were detected by screening or by PCR.

Expression of the hsSCP2 *gene*

Screening of databases with hsSCP2 cDNA sequence revealed ESTs from a human testis cDNA library, but also from a human heart and a human placenta library. The identified ESTs were fully identical to the hsSCP2 cDNA. Offenberg *et al.* (1998)

analyzed the transcription of the *rnSCP2* gene by northern blotting and *in situ* hybridization. On northern blots, SCP2 transcripts were only detected in mRNA from testis, and *in situ* hybridization showed that the *rnSCP2* gene was transcribed predominantly in meiotic cells (spermatocytes), although some SCP2 transcripts were detected in spermatids. Possibly, the ESTs identified in cDNA libraries of human heart and placenta result from very low levels of transcription of the *SCP2* gene. A similar situation was encountered with respect to the *rnSCP1* gene: SCP1 transcripts could be detected in mouse brain tissue by means of PCR (Kerr *et al.*, 1996) although northern blotting revealed no *SCP1* transcripts in this tissue (Meuwissen *et al.*, 1992).

Comparison with the RED1 protein

In BLASTP sequence alignments, a small domain of rnSCP2 displayed some amino acid sequence homology to the yeast Red1 protein; this similarity was of interest because Red1 is a putative component of the lateral elements of SCs in *S. cerevisiae* (Smith and Roeder, 1997). Like rnSCP2 and hsSCP2, Red1 contains a domain at its C-terminus, which can form a coiled-coil structure (Lupas *et al.*, 1991). Furthermore Red1 contains six S/T-P motifs and 19 S/T-S/T sequences dispersed throughout the protein and several potential phosphorylation sites. However, in alignments of rnSCP2, hsSCP2 and Red1 by MACAW (Schuler *et al.*, 1991) this similarity did not sustain. Eventhough no amino acid homology between Red1 and hsSCP2 was detected, it is very suggestive that several structural features of SCP2 are also found in Red1, and that the organization of structural features is similar in SCP2 and Red1. Possibly, this organization is more important for the function of the proteins than the amino acid sequence. Because the organization of structural motifs within Red1 and SCP2 is comparable, we think it is still possible that these proteins are at least partially functionally homologous.

In yeast, the Red1 protein promotes interchromosomal recombination and enhances the formation of functional chiasmata. A defect in Red1 leads to a decrease of crossing-over frequency (10% of wildtype) and only 1% spore-viability due to chromosome nondisjunction at anaphase I (Rockmill and Roeder, 1990). Since Red1 and SCP2 possibly share some functions and SCP2 makes part of meiotic prophase chromosomes, defects in SCP2 will probably cause meiotic chromosome

nondisjunction and infertility. By fluorescence *in situ* hybridization *hsSCP2* was assigned to chromosome 20q13.33 (Fig. 4). As yet no infertility problems are known to be correlated with chromosomal abnormalities in this chromosomal area, but the localization of *SCP2* offers some prospects for further research.

Acknowledgments

We thank M. van Aalderen for expert technical assistance; T. van Kampen for performing most of the sequencing reactions and R.L.J. Meuwissen and J.H.M. Lammers for discussions and practical advice. This research was supported by the Netherlands Organization for Scientific Research (grant no. 900-501-143).

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Chapter 4

Localization of SCP2 and SCP3 protein molecules within synaptonemal complexes of the rat

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Chromosoma, in press.

Abstract. *SCP2 and SCP3 are major protein components of the lateral elements (LEs) of synaptonemal complexes (SCs) of the rat, with molecular masses of 173 kDa and 30 kDa. In this paper we perform a detailed immunocytochemical comparison of the localization of SCP2 and SCP3 within SCs at the EM level. The ultrastructural localization of SCP2 and SCP3 was analyzed by immunogold labeling of two types of preparations, namely surface-spread spermatocytes and ultrathin sections of Lowicryl-embedded testicular tissue of the rat. For each of the antisera used, the distribution of immunogold label over SCs in surface-spread spermatocytes differed significantly from the distribution of label on sections. We attributed this difference to artifacts caused by the surface-spreading technique, and therefore we relied on sections for the precise localization of epitopes. On sections, the distributions of label obtained with two antisera against non-overlapping, widely separated fragments of SCP2 did not differ significantly. There was a small but significant difference between the labeling pattern obtained with an anti-SCP3 serum and the pattern obtained with either of the two antisera against fragments of SCP2; although for all three antisera the peak of the immunogold label coincided with the center of the LE, the distributions of label obtained with the antisera against fragments of SCP2 were asymmetrical, with a shoulder at the inner side of the LE, whereas the distribution of label obtained with anti-SCP3 antibodies was symmetrical. Furthermore, we observed fuzzy connections between the LEs, which were labeled by anti-SCP2 but not anti-SCP3 antibodies. It is possible that labeling of these "fuzzy bridges" caused the shoulder in the gold label distributions obtained with anti-SCP2 antibodies.*

Introduction

Ultrastructural studies of meiotic prophase cells have revealed zipper-like, proteinaceous structures, the synaptonemal complexes (SCs), between paired homologous chromosomes (Moses, 1968). The formation of SCs starts at the leptotene stage of prophase I, when single protein axes are laid along the two sister chromatids of each chromosome. At zygotene, the axial elements of two homologous chromosomes are connected by transverse filaments (TFs) and a central element is formed on these TFs. These axial elements, transverse filaments and central element together form the tripartite structure of the SC. Within the context of the tripartite SC, the axial elements are called lateral elements (LEs). At pachytene, the formation of SCs is complete and homologous chromosomes are connected by SCs along their entire length. At diplotene, the SCs fall apart, but the homologous chromosomes remain connected at the chiasmata, which result from reciprocal exchanges between non-sister chromatids. At metaphase I, the SC is no longer ultrastructurally recognizable, and at anaphase I the homologous chromosomes segregate.

In the rat, four major protein components of SCs have been identified, with relative electrophoretic mobilities (M_rs) of 30,000-33,000, 125,000 and 190,000 (Heyting *et al.*, 1989). In this paper we focus on the 30,000-33,000 and 190,000 M_r SC proteins. The 30,000-33,000 M_r proteins are closely related, and are most probably products of a single gene, *SCP3* (Lammers *et al.*, 1994). The cDNA encoding the 190,000 M_r component of rat SCs has recently been cloned and sequenced (Offenberg *et al.*, 1998); it encodes a 173 kDa protein, called SCP2, which has features of a protein which can bind to DNA, particularly to AT-rich sequences. In surface-spread preparations of spermatocytes and oocytes, SCP2 and SCP3 have both been localized to the axial elements, within and outside tripartite segments of SCs, from leptotene up till and including the diplotene stage of meiotic prophase (Heyting *et al.*, 1987, 1989; Moens *et al.*, 1987; Offenberg *et al.*, 1991; Dietrich *et al.*, 1992a).

The functions of axial element components are still under investigation. On the basis of immunofluorescence studies, Dobson *et al.* (1994) proposed that SCP3 (COR1) has a role in the regulation of sister chromatid cohesion, because this protein

persists in the chromosome arms until metaphase I, and in the centromeric region until anaphase II.

The localization of SCP2 and SCP3 to the axial elements was confirmed ultrastructurally by immunogold labeling of agar filtrates and surface spreads of spermatocytes (Heyting *et al.*, 1987, 1989; Offenberg *et al.*, 1991; Moens *et al.*, 1987; Dobson *et al.*, 1994) and oocytes (Dietrich *et al.*, 1992a). However, the results were not unambiguous in all respects. Moens *et al.* (1987) performed immunogold labeling of surface-spread rat and mouse spermatocytes, using a monoclonal anti-SCP3 antibody, and found most of the label on the LEs, with the peak of the distribution of gold label above the center of the LEs. This would be consistent with a role of SCP3 in sister chromatid cohesion. However, Dobson *et al.* (1994), using a polyclonal antiserum against the hamster homologue of SCP3 (COR1) on surface-spread hamster spermatocytes, reported a broad distribution of gold label around the LEs, with the peak of the label outside the LEs. That would suggest that SCP3 is not an axial element component, but a component of a subfraction of the chromatin that is attached to the LEs. Immunogold labeling of surface-spread spermatocytes with anti-SCP2 antibodies also produced a broad distribution of label around the LEs, rather than a narrow distribution on top of the LEs (Heyting *et al.*, 1989; Offenberg *et al.*, 1998). One possible explanation for this is, that small variations in the spreading technique may influence the localization of SC proteins, and thus the distribution of immunogold label (see also discussion in Schmekel *et al.*, 1996).

For several reasons the precise localization and orientation of SCP2 and SCP3 within the SCs is of interest. First, it is important to make a distinction between structural components of LEs and LE-associated proteins. Components of the LEs contribute to the structure of the LE and would therefore show a narrow colocalization with the LE. They do not (re)distribute from/to the chromatin before or after prophase I. Components that are just associated with the LE are expected to be loosely organized around the LE and to be redistributed over the chromatin when the SC assembles and/or disassembles. Second, a precise colocalization of SCP2 and SCP3 would suggest that these proteins interact *in vivo*, whereas a different localization would provide clues to distinguish between possible functions of these two proteins. Third, the orientation of these proteins within SCs is of interest, particularly of SCP2,

which is a large protein; analysis of its orientation by immunogold labeling with antibodies against widely separated domains should be feasible. We compared the precise localization of SCP2 and SCP3 within SCs by immunogold labeling, using monoclonal and polyclonal antibodies against SCP2 and SCP3. The experiments were performed on surface-spread spermatocytes, for comparison with earlier work (Heyting *et al.*, 1987, 1989; Offenberg *et al.*, 1991; Moens *et al.*, 1987; Dobson *et al.*, 1994), and on ultrathin sections of Lowicryl-embedded material. Schmekel *et al.* (1996) argue that Lowicryl sections allow a more precise localization of epitopes relative to SC substructures than surface spreads; in spreads, the surface morphology can influence the distribution of immunogold label, whereas in Lowicryl sections the surface is smooth (Stierhof *et al.*, 1986) and will not affect the distribution. Furthermore, masking of epitopes can occur in spreads, while immunogold labeling of Lowicryl sections is a surface labeling, with all epitopes on the surface being equally accessible to antibodies. The harsh conditions of the surface spreading procedure (SDS and DNase I treatment) might affect the structure of the SC whereas gross rearrangements are less likely to occur in Lowicryl sections. On sections, we furthermore analyzed whether SCP2 has a fixed orientation within LEs, using two antisera elicited against widely separated domains of the protein.

We found a considerable discrepancy between the immunogold labeling patterns of surface spreads and of sections. On sections, the peaks of immunogold label obtained with anti-SCP2 or anti-SCP3 antibodies always coincided with the center of the LE. We found a small but significant difference between the immunogold labeling patterns obtained with anti-SCP2 and anti-SCP3 antibodies. The experiments did not provide evidence for a fixed orientation of SCP2 molecules within LEs.

Materials and methods

Antibodies

For the preparation of antisera against fragments of SCP2, we subcloned two fragments of SCP2 cDNA, which encode amino acid residues 293-828 (fragment P) and amino acid residues 1236-1505 (fragment D) of SCP2 (Fig. 1). The fragments were subcloned in PQE31 (Qiagen, Chatsworth, CA), in frame with an ATG startcodon and 6 successive histidine codons on this vector. *E.coli* SG13009 cells (Qiagen) were transformed with the resulting construct. Synthesis of the fusion proteins encoded by these constructs was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a culture of transformed cells. The fusion proteins were purified from bacterial cell lysates by affinity chromatography on nickel columns according to the instructions of the supplier of the columns (Qiagen) and dialyzed against PBS (140 mM NaCl, 10 mM sodium phosphate pH 7.3). Both fragment P and fragment D precipitated during the dialysis. Antisera against SCP2 fragments P and D were elicited by immunization of rabbits; 60 μg of fusion protein was injected subcutaneously and intramuscularly at 2-week intervals. For the first injection, the antigen was mixed 1:1 with complete Freund's adjuvant (Sigma, St. Louis, MO, USA); for all later injections it was mixed with incomplete Freund's adjuvant. 20 ml bleedings were collected from the ear-veins at 4-week intervals, starting 1 week after the fourth injection. We thus obtained serum 493 (anti-SCP2 fragment P antiserum; further indicated as anti-P) and serum 509 (anti-SCP2 fragment D antiserum; further indicated as anti-D). We affinity-purified the anti-P antibodies from serum 493 using strips of Western blots containing full-length fragment P by a procedure described earlier (Lammers *et al.*, 1994). The mouse monoclonal antibodies (Mabs) II52F10 and IX9D5 were elicited and prepared as described by Offenberg *et al.* (1991); they are described in detail by Heyting *et al.* (1989) and Offenberg *et al.* (1991). Polyclonal antiserum 175 was elicited by immunization of a rabbit with whole SCs from rat. The serum recognizes predominantly the M_r 30,000 and 33,000 SC components (SCP3) (Lammers *et al.*, 1994) and will therefore be further indicated as anti-SCP3 serum. We purified anti-SCP3 antibodies from this serum by affinity chromatography on columns

carrying the full-length translation product of SCP3 cDNA (Lammers *et al.*, 1994), by a protocol adapted from Harlow and Lane (1988). The columns were washed with 0.1 M NaH_2PO_4/Na_2HPO_4 pH 7.0 (NaPi) supplemented with 0.01% (wt./vol.) NaN_3 and blocked with 10% goat control serum in 0.1 M NaPi, 0.01% NaN_3. Subsequently, the antiserum, diluted 1:10 in 0.1 M NaPi, 0.01% NaN_3, was loaded onto the columns and allowed to bind overnight at room temperature. After extensive washes with 0.1 M NaPi, 0.01% NaN_3, bound antibodies were eluted from the columns with 0.1 M glycine-HCl pH 2.5, 0.01% NaN_3. Fractions were collected and neutralized with 1/10th volume of 1 M Na_2HPO_4. Serum 493 and 509 were depleted from the anti-SCP2 fragment P or anti-SCP2 fragment D antibodies by affinity chromatography on columns carrying SCP2 fragments P or D.

Immunogold labeling

Cell suspensions from rat testes were prepared and processed for surface spreading as described before (Moens *et al.*, 1987); the surface spreading includes treatment of the surface spread cells with DNAse I and SDS (0.1%). For the preparation of ultrathin sections, rat testicular tissue was fixed and embedded in Lowicryl K11M as previously described (Dietrich *et al.*, 1992b), and 70 nm thick sections were cut on a Reichert Ultracut E microtome. Immunogold labeling of the surface spreads and sections was performed according to described procedures (Moens *et al.*, 1987; Schmekel *et al.*, 1996). We used the following dilutions of antibodies: IX9D5 and II52F10, 1:1; serum 175 (anti-SCP3), 1:1000; serum 493 (anti-P), 1:100; serum 509 (anti-D), 1:50; the affinity-purified anti-SCP3 antibodies, 1:20; the affinity-purified anti-P antibodies, 1:1. The preimmune sera and the depleted sera (described above) were used in the same dilution as the immune sera. Incubations of surface spreads with the preimmune sera and of sections with the preimmune sera and the depleted sera gave some background labeling although no concentration of label above the SCs was observed. Goat-anti-rabbit antibodies conjugated to 10-nm gold particles and goat anti-mouse antibodies conjugated to 5-nm gold particles were used as secondary antibodies and were diluted according to the instructions of the supplier (Amersham, Buckinghamshire, UK). After immunogold labeling, the preparations were contrasted with uranyl acetate and lead citrate (Heyting and Dietrich, 1991). Specimens were

photographed in a Philips EM 420 electron microscope operated at 80 kV at 15000 or 18000 X magnification.

Analysis of the immunogold labeling patterns

We compared the immunogold labeling pattern on pachytene SCs in spreads with the labeling of synapsed SC segments in sections. In the sections, cells with incompletely synapsed SCs were excluded from the comparison. The immunogold labeling patterns were analyzed as follows: grains were collected within an area corresponding to 3 LE-LE distances (one LE-LE distance is the distance between the centers of the LEs at the position of the grain) with the SC in the center. The position of the grain was defined as its distance to the center of the nearest LE, divided by one LE-LE distance. If the grain was between the centers of the LEs, the relative distance was given a positive value; if the grain was outside the centers of the LEs, the distance was given a negative value. The distances were expressed in units comprising 1/20th of the LE-LE distance. We did not discriminate between left and right LE since the two alternate at every twist of the SC. We performed the measurements on electron micrographs which were digitized by means of a CCD camera, using a Quantimet 15 QUIN 2.0 program (Leica, Cambridge, UK). The distributions of gold grains were compared by the Kolmogorow-Smirnow two-sample test and the statistical software package SPSS, version 7.5.

Other procedures

SDS-polyacrylamide gel electrophoresis of proteins (Laemmli, 1970; Heyting *et al.*, 1985) and Western blotting (Heyting and Dietrich, 1991; Dunn, 1986) were performed according to described procedures.

Results

Antibodies

Fig. 1 shows an immunoblot analysis of the antibodies used in this study.

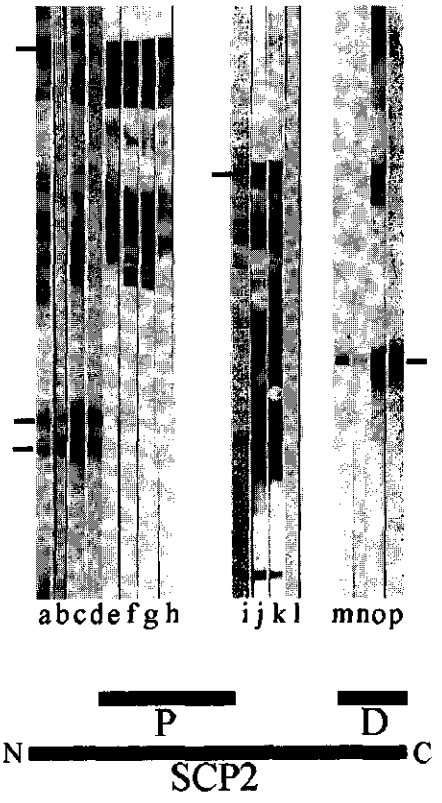

Figure 1 a-p. Immunoblot analysis of antibodies used in this study. **a**, strip of a preparative polyacrylamide-SDS gel, which is loaded with 2 x 10^7 SCs/cm slot (about 3 µg protein) ,stained with Coomassie blue; **b-h**, strips of an immunoblot of the same gel as shown in **a**; **i**, strip of a preparative polyacrylamide-SDS gel, which is loaded with 0.1 µg of SCP2 fragment P/cm slot, stained with Coomassie blue; **j-l**, strips of an immunoblot of the same gel as shown in **i**; **p**, strip of a preparative polyacrylamide-SDS gel, which is loaded with 0.5 µg of SCP2 fragment D/cm slot, stained with Coomassie blue; **m-o**, strips of an immunoblot of the same gel as shown in **p**; The strips were incubated in the following antibodies or antisera: **b**, Mab II52F10 (anti-SCP3); **c**, anti-SCP3 serum (serum 175); **d**, anti-SCP3 antibodies, affinity-purified from serum 175; **e**, Mab IX9D5 (anti-SCP2); **f, j and m**, anti-P serum (serum 493); **g, k and n**, anti-P antibodies, affinity-purified from serum 493; **h, l and o**, anti-D serum (serum 509). For lanes **a-h**, the positions of SCP2 (M_r 190,000) and SCP3 (M_r 30,000-33,000) are indicated by horizontal bars. For lanes **i-l**, the position of the full-length fragment P is indicated by a horizontal bar. For lanes **m-p**, the position of the full-length fragment D is indicated by a horizontal bar.

Mab II52F10, serum 175, and an affinity-purified fraction from serum 175 were used to label the M_r 30,000-33,0000 SC-components. Because these proteins are most probably products of a single gene, *SCP3* (Lammers *et al.*, 1994), these antibodies will further be designated as anti-SCP3 antibodies. On Western blots of SC-proteins, serum 175 recognizes predominantly the M_r 30,000-33,000 SC components, and more weakly a M_r 125,000 component, called SCP1 (Meuwissen *et al.*, 1992), some M_r 65,000 SC proteins and a M_r 190,000 SC component (Fig. 1c). Mab II52F10 and an affinity-purified fraction from serum 175 recognize exclusively the M_r 30,000-33,000 SC components; most importantly, they do not recognize the M_r 190,000 component (Fig. 1 b and d). The full-length cDNA encoding the M_r 190,000 SC protein has recently been cloned (Offenberg *et al.*, 1998); it encodes a 173 kDa protein called SCP2. For the immunogold labeling of SCP2, we prepared two antisera against non-overlapping fragments of SCP2, called fragment P and fragment D (bottom of Fig. 1). On western blots of SCs, the anti-P serum binds to SCP2 and to a series of more rapidly migrating peptides which we interpret as proteolytic breakdown products of SCP2 (Fig. 1f), because affinity-purified anti-P antibodies from this serum and two monoclonal anti-SCP2 antibodies display exactly the same reaction pattern on western blots of SC proteins (Fig. 1g and Offenberg *et al.*, 1998). The anti-D serum also binds specifically to SCP2 on western blots of SC proteins (Fig. 1h). Fig. 1 furthermore shows that the anti-D serum does not crossreact with fragment P of SCP2 (Fig. 1l). Unexpectedly, the anti-P antiserum and also the affinity-purified anti-P antibodies crossreact with fragment D (Fig. 1 m and n). Depletion of the anti-P serum on a column carrying fragment P not only eliminated the reactivity of the serum with fragment P, but also with fragment D (not shown). Apparently, fragment P and D share some epitope(s). For the detection of SCP2 we further used a mouse monoclonal antibody, Mab IX9D5, which has been described before (Heyting *et al.*, 1989; Offenberg *et al.*, 1991). This antibody does not recognize fragments P or D on Western blots (not shown); on immunoblots of SC-proteins it specifically recognizes the M_r 190,000 SC-component (lane 1e).

Immunolocalization experiments

At the light microscopic level, the labeling pattern obtained with anti-P serum on testicular sections or agar filtrates of lysed spermatocytes is indistinguishable from the pattern obtained with Mab IX9D5 (Heyting *et al.*, 1989; Offenberg *et al.*, 1991; Offenberg *et al.*, 1998): the anti-P serum labels specifically spermatocyte nuclei, and within these nuclei both paired and unpaired segments of SCs (Offenberg *et al.*, 1998). Anti-SCP3 antibodies produce a similar labeling pattern in light microscopic preparations (Heyting *et al.*, 1987, 1989; Moens *et al.*, 1987; Offenberg *et al.*, 1991).

Choice of preparational technique

To compare the ultrastructural localization of SCP2 and SCP3, we performed immunogold labeling experiments on two types of preparations: surface-spread spermatocytes and ultrathin sections of Lowicryl-embedded testicular tissue of the rat. Surface spreads had been used in earlier experiments by us (Heyting *et al.*, 1987, 1989; Moens *et al.*, 1987; Offenberg *et al.*, 1991; Dietrich *et al.*,1992a) and others (Dobson *et al.*, 1994); large numbers of cells can be analyzed relatively easily in these preparations, and the intensity of immunogold labeling is usually high. However, the risk of artifacts is also high for these preparations, because the spreading technique involves the use of DNAse I and SDS (Moens *et al.*, 1987). Furthermore, as Schmekel *et al.* (1996) have argued, the surface morphology of spreads may affect the immunogold distribution and epitope masking may occur. We therefore also performed immunogold labeling of ultrathin sections of Lowicryl-embedded paraformaldehyde-fixed testicular tissue.

The results obtained on surface-spreads are presented in Fig. 2, and the results obtained on sections in Fig. 3. Anti-SCP3 and anti-P were applied both to surface-spreads and to sections. The immunogold distributions obtained with these antisera on sections differ significantly from those on spreads (Fig. 4 and Table 1). For both antisera the distributions of label are much broader on spreads than on sections; on spreads, the immunogold distributions show a dip at the position of the center of the LE, whereas such a dip is almost absent in the distributions obtained on sections (Fig. 4).

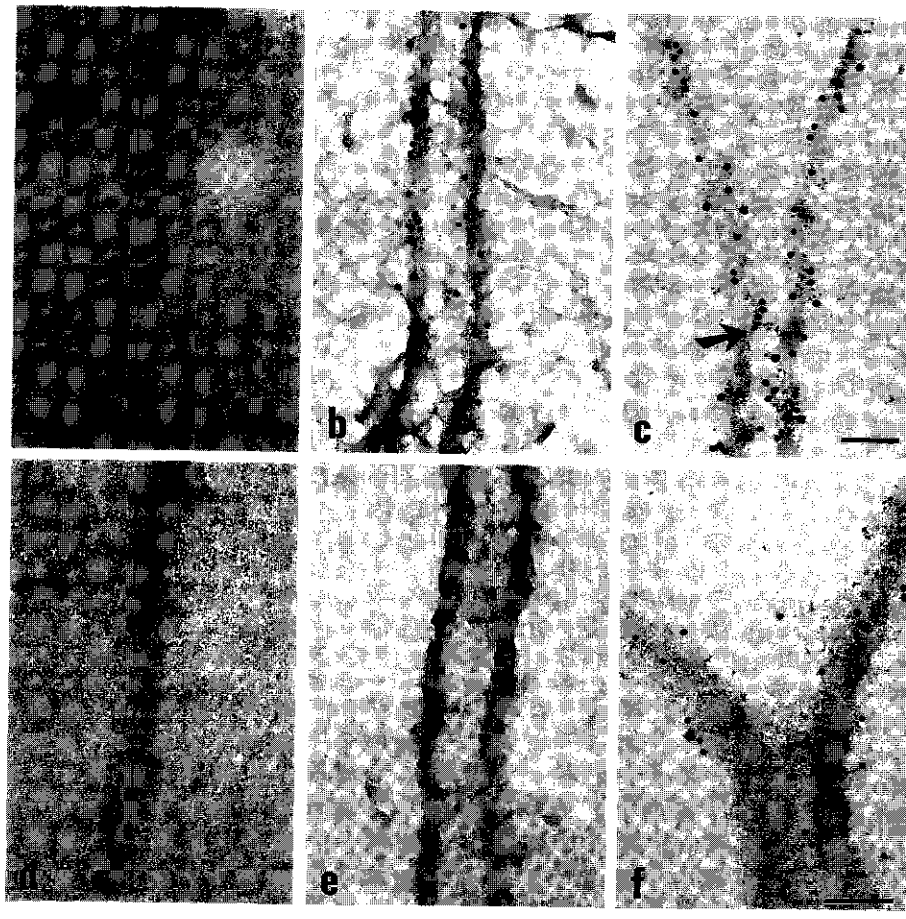

Figure 2 a-f. Ultrastructural localization of SCP3 and fragment P of SCP2 by indirect immunogold labeling of surface-spread spermatocytes of the rat. **a,b** and **c**, double-labeling performed with rabbit anti-SCP3 serum (serum 175) and mouse Mab IX9D5 (anti-SCP2) as primary antibodies; **d,e** and **f**, double-labeling performed with mouse Mab II52F10 (anti-SCP3) and anti-P serum (serum 493) as primary antibodies. As secondary antibodies were used: goat anti-rabbit IgG, conjugated to 10 nm gold, and goat-anti-mouse IgG, conjugated to 5 nm gold. **a** and **d**, zygotene; **b** and **e** pachytene; **c** and **f** diplotene. Arrow indicates a "bridge" between the LEs, which is labeled by anti-SCP2. The magnifications for **a, b** and **c** are similar. The magnifications for **d, e** and **f** are similar. Bars represent 200 nm.

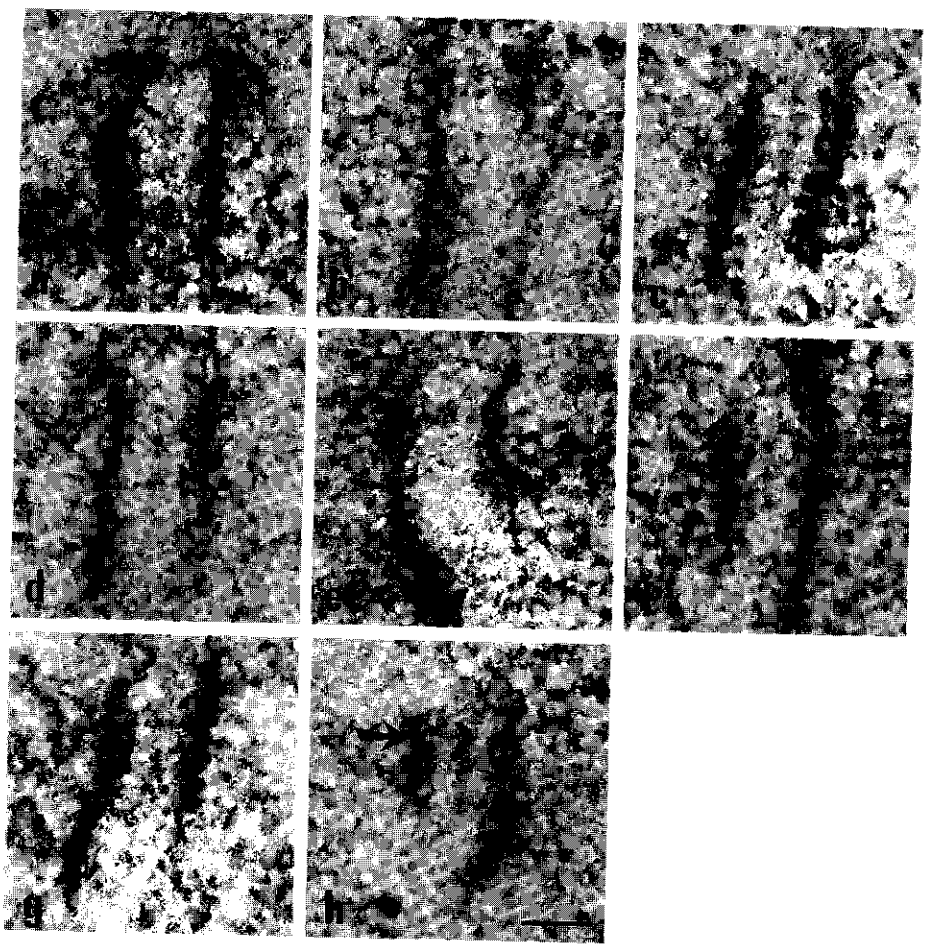

Figure 3 a-f. Ultrastructural localization of SCP3 and the fragments P and D of SCP2 by indirect immunogold labeling of ultrathin sections of Lowicryl-embedded testicular tissue of the rat. Labeling was performed with: **a** and **b**, anti-SCP3 serum (serum 175); **c**, anti-SCP3 antibodies, affinity-purified from serum 175; **d** and **e**, anti-P serum (serum 493); **f**, anti-P antibodies, affinity-purified from serum 493; **g** and **h**, anti-D serum (serum 509) as primary antibodies. Goat anti-rabbit IgG conjugated to 10 nm gold was used as secondary antibody. Arrows indicate bridges between the LEs, which are labeled by anti-P and anti-D. Bar represents 200 nm.

Table 1. Comparison of the distributions of gold grains on surface spreads with those on sections after immunogold labeling with anti-SCP3 or anti-SCP2 serum.

Antiserum	Surface spreads vs. sections[1)]
Anti-SCP3 (serum 175)	P<0.001
Anti-P (serum 493)	P<0.001

[1)]The distributions of gold grains were compared by the Kolmogorow-Smirnow two-sample test (see Materials and methods).

We think that the surface-spreading technique affects the structure of the LEs, and causes their broadening. The width of the LE as seen with electron-dense stains was in surface-spread preparations indeed wider (about 80 nm) than in sections (about 50 nm), whereas the width of the SC (center LE-center LE) is narrower in spreads (about 145 nm) than in sections (about 175 nm). Because gross morphological rearrangements are less likely to occur in fixed and embedded material, we relied on sections for the precise localization of epitopes, eventhough the level of labeling was lower in this type of preparation.

Comparison of the localization of SCP2 and SCP3 in surface spreads

Although surface-spreads are not useful for the precise localization of epitopes, we performed double-labeling experiments on these preparations, as a first screen for gross differences (if any) in the localization of SCP2 and SCP3 (Fig. 2). On sections, such experiments were not possible, because we could not combine gold labels of different sizes; 5 nm gold grains could not be identified with certainty within the structure of a section, while the level of labeling with 15 nm gold grain was too low, presumably because of steric hindrance.

We did not observe any obvious differences between SCP2 and SCP3 in these double-labeling experiments with one possible exception: some gold grains of the anti-SCP2 label were localized in the central region. These grains occurred in groups, which partly or entirely spanned the central region (see arrow Fig. 2c). In some instances fuzzy bridges were visible underneath these grains. Although some anti-SCP3 gold grains were observed in the central region, these did not occur in clusters as seen after labeling with anti-SCP2.

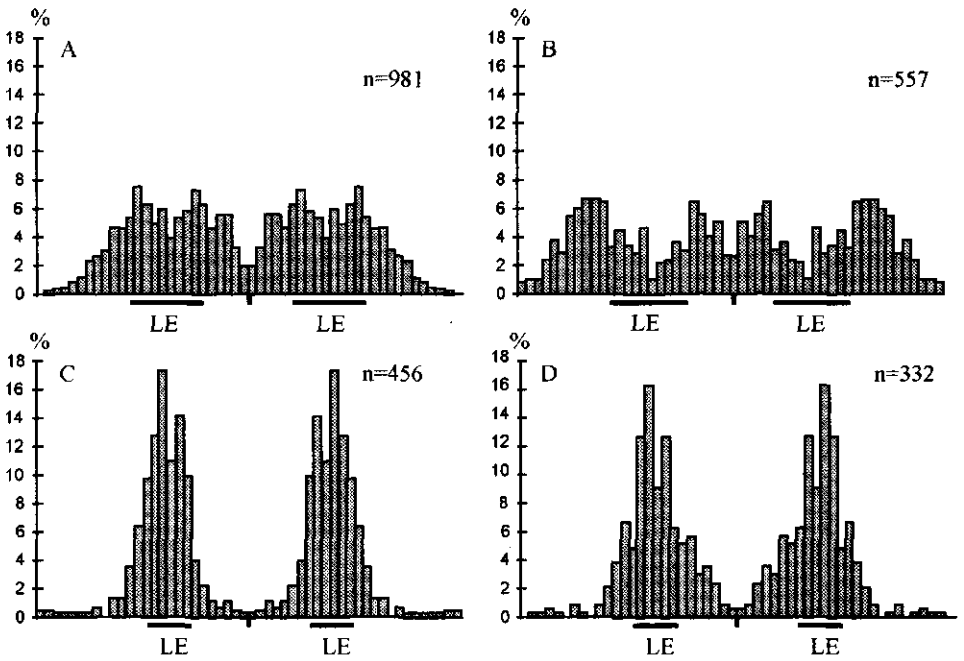

Figure 4 A-D. Distributions of immunogold label over SCs in surface spreads of rat spermatocytes (**A, B**), and ultrathin sections of Lowicryl-embedded testicular tissue (**C, D**). Anti-SCP3 serum (serum 175) (**A, C**) or anti-P serum (serum 493) (**B, D**) were used as primary antibodies and goat-anti rabbit IgG conjugated to 10 nm gold as secondary antibody. One unit on the horizontal axis represents 1/20 th of the distance between the centers of the LEs at the site of the grain. To correlate the obtained distributions with the structure of the SC, we mirrored the distributions relative to the center of the CE (indicated by the small vertical bar on the horizontal axis). The vertical axis represents the percentage of total grains in each class of the original, unmirrored distribution. The bars below the horizontal axis represent the position of the LE as defined by uranyl acetate/lead citrate staining. n represents the number of grains including the background in the original, unmirrored distribution.

Localization of SCP2 and SCP3 on sections

Figs. 3 and 5 show the immunolocalization of SCP2 and SCP3 on sections. Anti-SCP3 and the two anti-SCP2 sera (anti-P and anti-D) produced narrow distributions of gold grains, with the peaks positioned at the center of the LE (Fig. 5). This narrow colocalization with the LE strongly suggests that SCP2 and SCP3 are structural components of the LEs, rather than components of SC-associated chromatin. The distribution of anti-SCP3 label was virtually symmetrical (Fig. 5A), whereas both distributions of anti-SCP2 label (from anti-P and anti-D), had a shoulder at the inner side of the LE (see arrows in Fig. 5B and 5C). The differences between the distribution of anti-SCP3 label and either of the two distributions of anti-SCP2 label were significant (Table 2). The distributions of anti-P and anti-D label did not differ significantly from each other (Table 2). Thus our data do not provide evidence for a fixed orientation of SCP2 molecules within LEs. However, it is possible that the crossreactivity of anti-P antibodies with fragment D has hampered the detection of a small difference (if any) between the localization of fragment P and D. In sections labeled with anti-P or anti-D, we found some examples of groups of grains, which bridged the central region and appeared to label some fuzzy connections between the LEs (see arrows Fig. 3e and 3h). Such bridges were not observed after labeling with anti-SCP3.

Table 2. Comparison of the distributions of immunogold grains obtained with anti-SCP3, anti-P or anti-D antibodies[1].

Comparison	P-value[5]
anti-SCP3[2] vs anti-P[3]	P<0.001
anti-SCP3[2] vs anti-D[4]	P <0.05
anti-P[3] vs anti-D[4]	not significant

[1] experiments performed on Lowicryl-embedded sections
[2] affinity-purified antibodies from serum 175
[3] affinity-purified antibodies from serum 493
[4] serum 507 (see Materials and methods)
[5] The distributions of gold grains were compared by the Kolmogorow-Smirnow two-sample test (see Materials and methods).

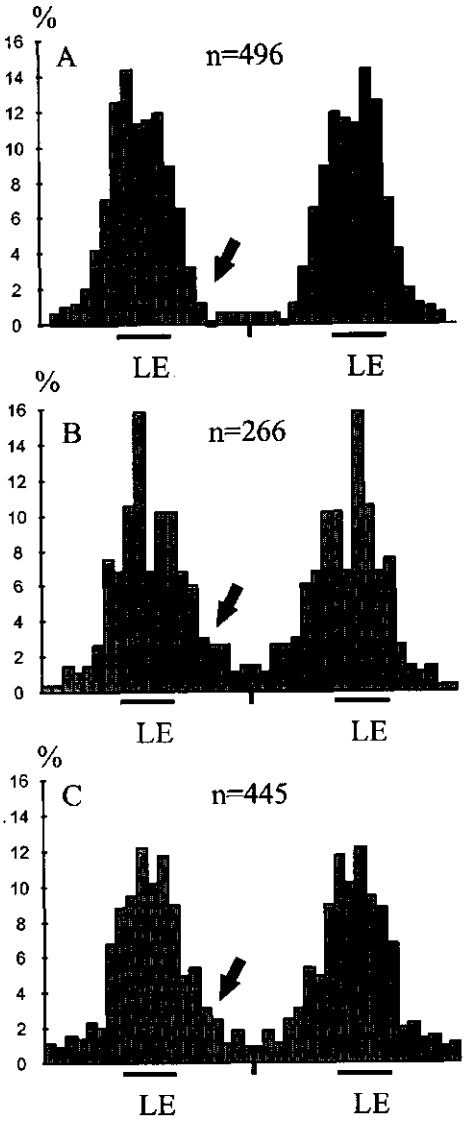

Figure 5 A-C. Distributions of immunogold label over SCs in ultrathin sections of Lowicryl-embedded testicular tissue obtained after labeling with anti-SCP3 antibodies, affinity-purified from serum 175 (**A**), anti-P antibodies, affinity-purified from serum 493 (**B**), or anti-D serum (serum 509) (**C**). The arrows in B and C indicate the shoulder in the distribution at the inner side of the LE. The arrow in Fig 5A, at the same position as the arrows in Fig 5B and 5C, indicates that this shoulder is absent in this distribution. For further explanation, see legends Fig. 4.

Discussion

Preparational techniques

We performed immunogold labeling experiments on surface-spread spermatocytes and ultrathin sections of Lowicryl-embedded testicular tissue of the rat to define the localization and organization of SCP2 and SCP3 within SCs. Our experiments revealed a dramatic difference between the immunogold distribution on surface spreads and sections with both anti-SCP2 and anti-SCP3 sera; the distribution of immunogold over the LEs was much broader on spreads than on sections and the distribution on spreads showed a dip at the center of the LE, which was almost absent in sections. A similar difference was observed before with antibodies against another SC-component, SCP1 (Schmekel *et al.*,1996). We attributed the broad distribution of anti-SCP2 and anti-SCP3 label in spreads to structural changes in the SC caused by the surface-spreading technique. Surface-spreading is a harsh technique which includes the use of SDS and DNase I (Moens *et al.*, 1987) and may cause deformation of the SC; the LEs in spreads, as detected by uranyl-acetate, were much broader than in sections whereas the width of the SC was smaller in spreads than in sections. Possibly, the surface morphology of spreads and the masking of epitopes in spreads also influenced the distribution of the immunogold grains over the SCs (see discussion Schmekel *et al.*, 1996), and caused a dip in the gold label distribution at the center of the LE.

The possible effects of the spreading technique and the surface morphology of spreads on the immunogold distribution, might also explain the variations in immunogold distribution as observed for SCP3; Moens *et al.* (1987), applying a monoclonal anti-SCP3 antibody to surface spreads, found an immunogold distribution with a peak at the center of the LE; Dobson *et al.* (1994), performing immunogold labeling of COR1 (the hamster homologue of SCP3) in surface spreads, found that the immunogold label was widely distributed over the LE with a peak at the outer edge of the LE, whereas we found a broad distribution of anti-SCP3 label over the LE, with a dip at the center of the LE (Fig. 4, this paper). In all these studies surface spreading was performed according to Moens *et al.* (1987), with the exception that Dobson *et al.* (1994) did not use SDS. The differences in the results are probably created by

small variations in the spreading procedure. Liu *et al.* (1996) analyzed the ultrastructural localization of SCP3 in cryosections of isolated mouse spermatocytes which were fixed with methanol/acetone. They found most of the immunogold label at the inner side of the LE. In order to minimize preparational artifacts, we fixed testicular tissue rather than isolated cells in paraformaldehyde/glutaraldehyde, and embedded the tissue in Lowicryl before sectioning. Although shrinkage can occur during fixation and embedding in Lowicryl (Luther, 1992; Braunfeld, 1994), this technique will probably reduce the chance of gross rearrangements or deformations of SCs. Lowicryl sections have a flat surface, so that the surface morphology will not influence the labeling pattern. Furthermore, immunogold labeling of Lowicryl sections is essentially a surface labeling, so that the labeling intensity is proportional to the amount of epitope at the surface. Because of this surface labeling it is unlikely that differences in accessibility of epitopes within SCs will influence the distribution of label on Lowicryl sections (see discussion in Schmekel *et al.*, 1996). We therefore think that distribution of immunogold label on Lowicryl sections provides the best estimation of the position of SCP2 and SCP3 epitopes within SCs.

Localization of SCP2 and SCP3 within the LE

Since immunogold labeling of both SCP2 and SCP3 produces a narrow distribution of label on the LE in sections it seems likely that SCP2 and SCP3 are structural components of the LEs rather than SC-associated proteins. Possible functions of LEs include anchoring of chromatin loops to the SC (Weith and Traut, 1980), generation of sister chromatid cohesiveness (Maguire, 1990; Dobson *et al.*, 1994), conversion of recombination intermediates into stable chiasmata (Rockmill and Roeder, 1990), and inhibition of sister chromatid exchange and/or promotion of recombination between non-sister strands (Hollingsworth *et al.*, 1990; Schwacha and Kleckner, 1997; Xu *et al.*, 1997); probably SCP2 and SCP3 have a role in at least one of these processes.

The distribution for SCP2 has a shoulder at the inner side of the LE whereas the distribution for SCP3 is symmetrical (Fig. 5) This shoulder can be explained by the "bridges" between the LEs, which were found in both spread preparations and sections and were labeled by anti-SCP2 but not by anti-SCP3 (Fig. 2 and 3). Vazquez Nin *et al.*, (1993) showed by immunogold labeling experiments with anti-DNA antibodies

that DNA is located within the LE, but not within the central region of SCs, with the exception of DNA threads, which spanned the central region. Since SCP2 has features of a DNA-binding protein (Schalk, unpublished observations), it is possible that it interacts with these DNA threads and that this shows up in our preparations as bridges between the LEs. Although we did not find clear examples of bridges that were labeled by SCP3, we cannot exclude that these structures also contain SCP3; in spreads, the distribution of label over the LEs is so broad that many grains cover the central region, and immunogold labeled bridges are difficult to discern. In sections, the intensity of labeling is lower, so that immunogold labeled bridges are easily missed. To summarize, we observed bridges consisting of fuzzy material between the LEs, which are labeled by anti-SCP2; whether these structures also contain SCP3, still has to be sorted out. If these bridges contain SCP2 only, this might account for the shoulder in the immunogold distribution of SCP2 but not SCP3. Thus, it is possible that SCP2 is not only a structural component of the LE, but also a component of these bridges within the central region. Alternatively, SCP2 is more often located at the inner side of the LE, for instance because it interacts with transverse filament proteins.

Acknowledgments

We would like to thank J. van Marle and H. van Veen for use of EM facilities and C. Alonso Blanco for help with statistical analysis.

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Chapter 5

SCP2 and SCP3, their localization in successive stages of meiosis of the rat

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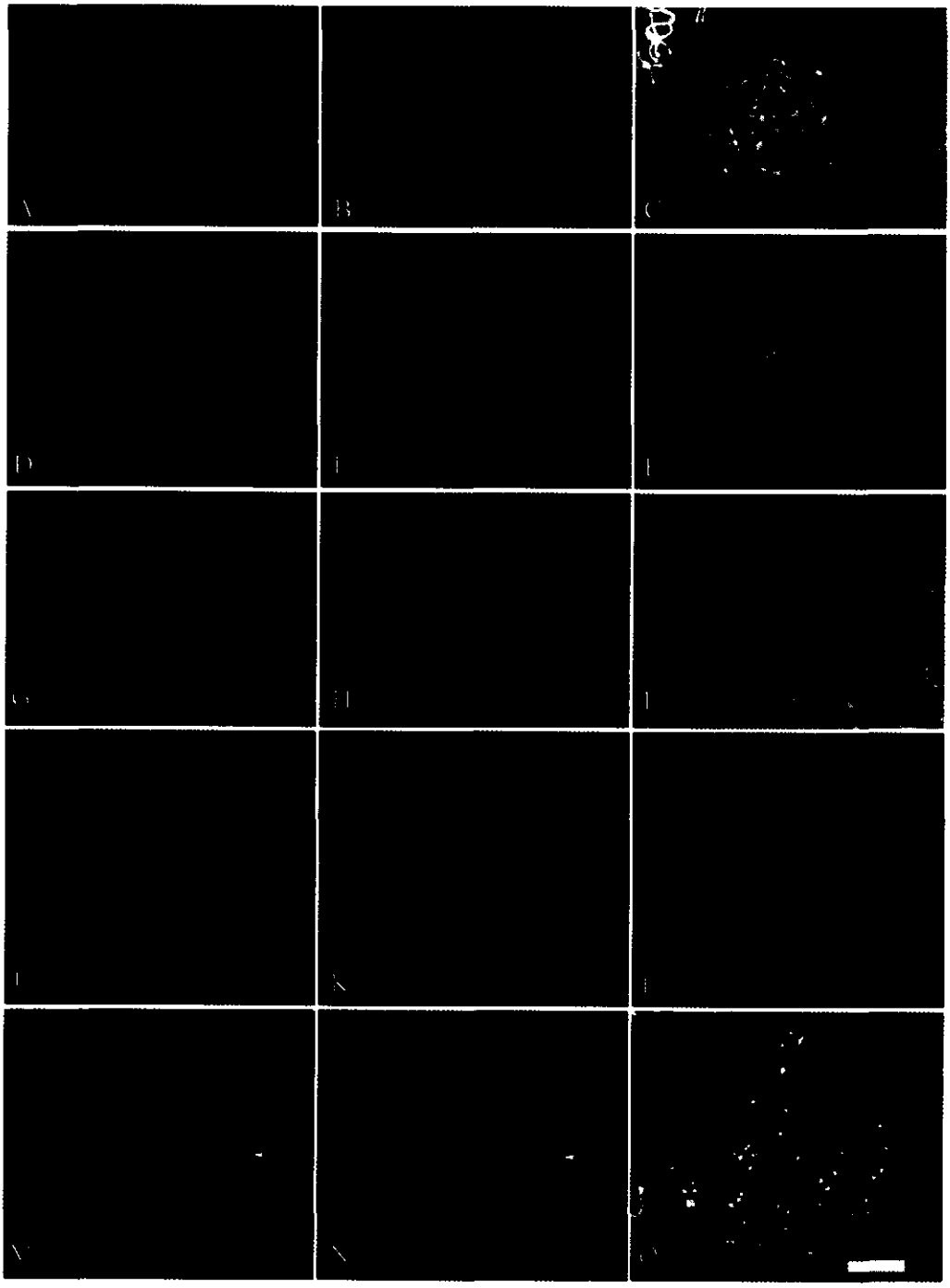
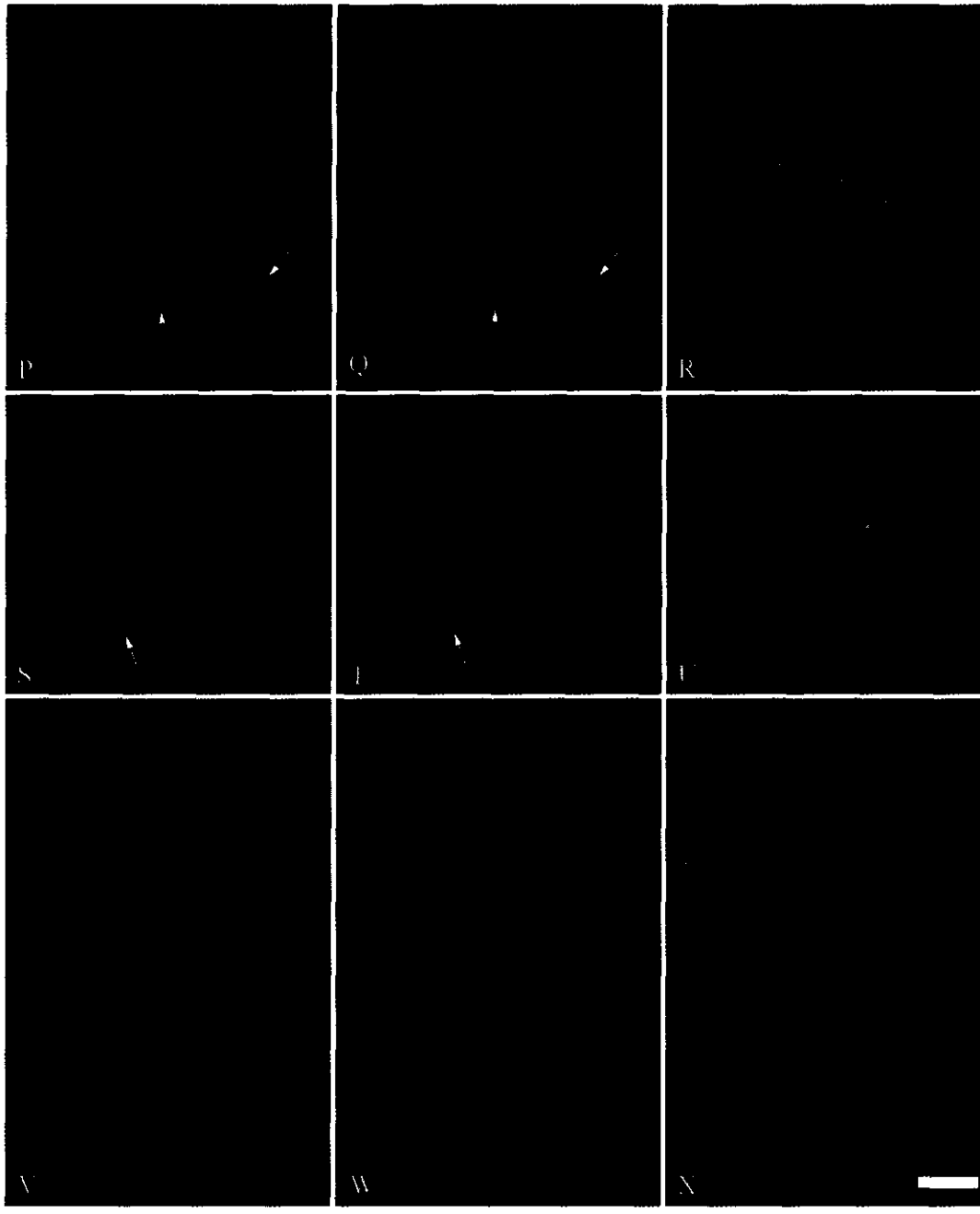
Abstract. *SCP2 and SCP3 are major protein components of the axial elements of synaptonemal complexes of the rat. In this paper we analyze the localization of SCP2 and SCP3 in successive stages of meiosis by immunofluorescence double labeling, using combinations of monoclonal (Mabs) and polyclonal (Pabs) antibodies against these proteins. SCP2 and SCP3 colocalized in most stages of meiotic prophase I, although some differences were observed: (1) in pachytene, axial cores of XY bivalents are labeled more intensely by anti-SCP3 than by anti-SCP2 antibodies. (2) In all stages of prophase I, we found aggregates of SCP3, but not of SCP2 outside the SCs. (3) SCP2 gradually disappears after metaphase I, whereas SCP3 persists longer in late meiotic cells. In part of the diplotene bivalents, we found that the desynapsing axial elements remained connected by one or two thin fibers, which were labeled by anti-SCP2 and anti-SCP3 antibodies. The cohesin proteins SMC1 and SMC3, which we recently localized along the axial elements of SCs (Eijpe et al., in preparation), were also found along these connections. This indicates that these connections could represent sites of crossing-over. In late diplotene, when the axial elements disintegrate, small amounts of SCP2 and SCP3 are retained along the chromosome arms, where they persist until metaphase I. Furthermore, during late diplotene, SCP2 and SCP3 concentrate at the centromeres, where SCP2 persists until at least metaphase I, and SCP3 until anaphase II. These immunolocalization patterns support the hypotheses that axial elements have a regulatory role in meiotic sister chromatid cohesion and contribute to the formation of functional chiasmata.*

Introduction

Meiotic chromosome pairing and recombination are accompanied by the formation of synaptonemal complexes (SCs), which are assembled between homologous chromosomes during the prophase of the first meiotic division. Meiotic prophase I can be subdivided in stages according to the state of (dis)assembly of the SCs. During leptotene, a single axial element is formed along each chromosome, which is shared by the two sister chromatids. Zygotene starts when axial elements of homologous chromosomes start to pair and are connected ("synapsed") by transverse filaments. In pachytene, the axial elements of homologous chromosomes are synapsed along their length, and in diplotene and diakinesis the SCs are disassembled. In rat and mouse, the transverse filaments disappear first, so that full-length desynapsed axial elements remain, which fall apart during late diplotene and diakinesis (Heyting and Dietrich, 1992).

Figure 1 A-X. Immunolocalization of SCP2 and SCP3 in successive stages of meiosis. A triple labeling was performed on dry-down preparations of rat spermatocytes with Mab II52F10 (anti-SCP3), serum 493 (anti-SCP2) and a CREST serum (anti-kinetochores). The antibodies were detected with respectively goat-anti-mouse-Texas Red, goat-anti-rabbit-AMCA and goat-anti-human-FITC. The left two panels represent the unmerged images for SCP3 (red) and SCP2 (blue), the right panel represents the merged images for SCP3, SCP2 and the CREST antigen (green). A,B,C, early zygotene; D,E,F, zygotene; G,H,I, early pachytene; J,K,L, late pachytene; M,N,O, diplotene; P,Q,R, late diplotene/diakinesis; S,T,U, diakinesis/metaphase I; V,W,X, metaphase I. Arrows in panel M, N, P and Q indicate connections between desynapsed axial elements, which are labeled by anti-SCP2 and anti-SCP3. Arrowhead in panel P and Q indicates converging axial elements, without a connection at that position. Arrows in panel S and T indicate the XY axes. Bars represent 10 μm.

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The origin of axial elements and their roles in meiosis are still under investigation. So far, two axial element proteins have been identified in the rat, namely 30,000 - 33,000 M_r components, which are products of a single gene called *SCP3*, (Heyting *et al.*, 1987, Lammers *et al.*, 1994), and a 190,000 M_r component encoded by a gene called *SCP2* (Heyting *et al.*, 1989; Offenberg *et al.*, 1991, 1998). The SCP2 and SCP3 proteins make part of the axial elements of synapsed and unsynapsed segments of SCs from leptotene up to and including diplotene (Heyting *et al.*, 1987, 1989; Offenberg *et al.*, 1991, 1998; Dietrich *et al.*, 1992). Immunogold labeling of sections of Lowicryl-embedded tissue showed that in pachytene, SCP2 and SCP3 are both localized within the axial elements as defined by uranyl acetate staining (Schalk *et al.*, 1998). Dobson *et al.* (1994) have analyzed the localization of the hamster protein homologous to SCP3 (COR1) in later stages than diplotene by immunofluorescence labeling of surface-spread spermatocytes. They found that SCP3 (COR1) concentrates at the centromeres during diakinesis and metaphase I and persists there until anaphase II; along the chromosome arms, it is retained in small amounts until anaphase I. The localization of SCP2 in later stages of meiosis had not yet been established.

SCP2 and SCP3, which are major protein components of axial elements, are expressed exclusively in meiosis (Offenberg *et al.*, 1991, 1998; Lammers *et al.*, 1994). Axial elements are thus not derived from pre-existing chromatin-supporting structures in the nucleus, but consist largely or entirely of newly synthesized, meiosis-specific components. Recently, we found that the SMC1 and SMC3 proteins, which are involved in mitotic sister chromatid cohesion and chromosome condensation, are localized in a characteristic "beads-on-a-string" arrangement along, but not within the axial elements of SCs (Eijpe *et al.*, in preparation). Most likely these rows of beads mark the chromosomal axes before they have divided into the two sister chromatid axes. That would set the chromosomal axes apart from the axial elements of SCs (Rufas *et al.*, 1992), and strongly suggests that meiotic sister chromatid cohesion is at least co-mediated by SMC1 and SMC3. It is possible however, that components of the axial elements of SCs influence the extent and duration of meiotic sister chromatid cohesion. Dobson *et al.* (1994) proposed on the basis of the localization of SCP3 (COR1) during meiosis I and II, that this protein contributes to the cohesion of sister

kinetochores and sister chromatid arms during meiosis.

Other possible functions that have been proposed for axial elements include the creation of a bias for reciprocal recombination between homologous chromosomes rather than sister chromatids (Schwacha and Kleckner, 1997), the monitoring of the meiotic recombination process (Xu *et al.*, 1997) and the production of meiotic crossovers in such a way that they can be converted into stable chiasmata (Rockmill and Roeder, 1990). It is possible that different axial element components are involved in different combinations of such functions.

In this paper, we compare the localization of SCP2 and SCP3 throughout meiosis by immunofluorescence double-labeling experiments; a difference in localization could be indicative of a difference in function. However, we found an almost complete colocalization of the two proteins along the length of the axial elements until diplotene and at the centromeres after diplotene. After metaphase I, SCP2 disappears from the centromeres, whereas SCP3 does not. In part of the bivalents in diplotene cells we found thin connections between the separating axial elements, which contained both SCP2 and SCP3. SMC1 and SMC3 were localized in dots along these connections. Possibly, these connections represent sites of crossing-over.

We hypothesize that SCP2 and SCP3 both have a regulatory role in meiotic sister chromatid cohesion, and that they stabilize certain recombination intermediates until diplotene.

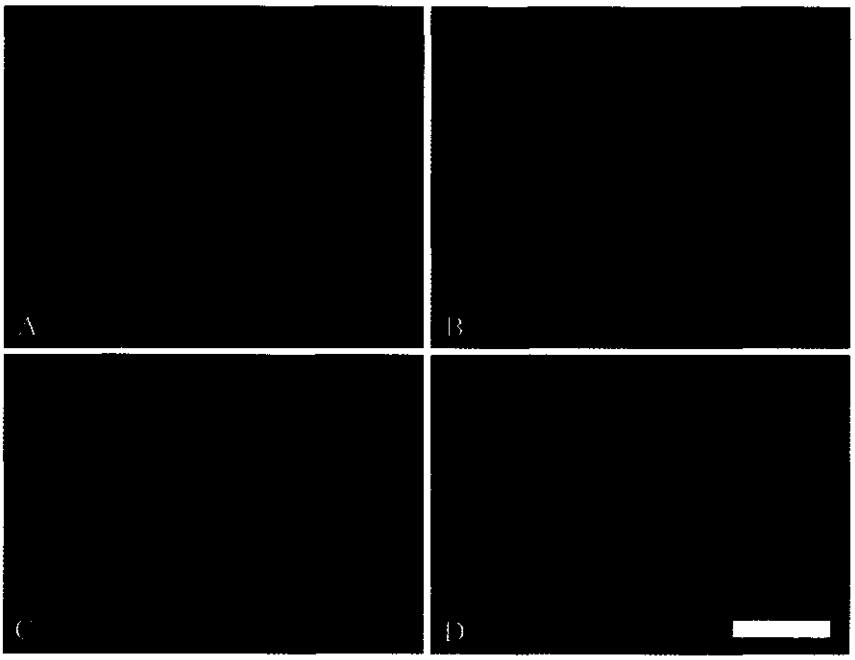

Figure 2. Immunolocalization of SCP2 and SCP3 in anaphase II. A triple labeling was performed on dry-down preparations of rat spermatocytes with Mab II52F10 (anti-SCP3), serum 493 (anti-SCP2) and CREST (anti-centromeres). The antibodies were detected with respectively goat-anti-mouse Texas Red, goat-anti-rabbit-AMCA and goat-anti-human-FITC. The left panels represent the merged images for SCP3 (red) and CREST (green), the right panels represent the unmerged image for SCP2 (blue). Panel A and B show two closely associated anaphase II nuclei, panel C and D show one anaphase II nucleus. Bar represents 10 μm.

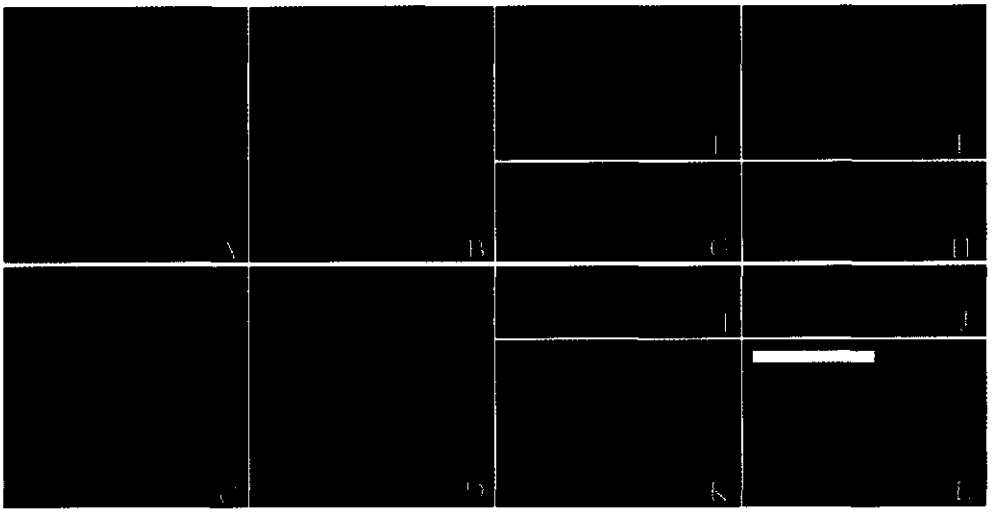

Figure 3. Examples of SCP2 and SCP3-containing connections between axial elements in late diplotene. Double labeling of dry-down preparations of rat spermatocytes with Mab II52F10 (anti-SCP3) and serum 493 (anti-SCP2). The antibodies were detected with goat-anti-mouse-Texas Red and goat-anti-rabbit-AMCA. The left panels (A, C, E, G, I and K) show the localization of SCP3 (red), and the right panels (B, D, F, H, J and L) show the localization of SCP2 (blue). Bar represents 10 μm.

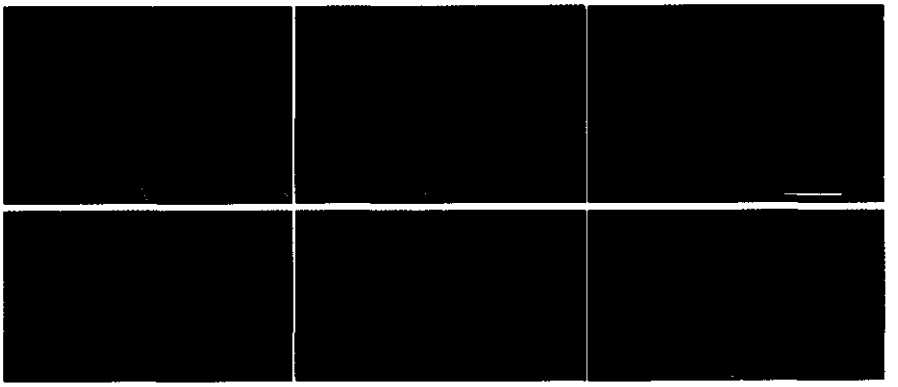

Figure 4. Immunolocalization of SMC1 and SCP2 in late diplotene SCs. A double labeling was performed on agar filtrates of lysed spermatocytes with anti-SMC1 Mab and serum 493 (anti-SCP2). The antibodies were detected with respectively goat-anti-mouse-FITC and goat-anti-rabbit-Texas Red. The left panels (A, C, E, G, I and K) represent the merged images for SMC1 (green) and SCP2 (red); the right panels (B, D, F, H, J and L) represent the unmerged images for SCP2. Bar represents 4 μm.

Materials and methods

Antibodies

Polyclonal antiserum 493 was raised in a rabbit against an expression product of a fragment of the rat SCP2 cDNA, it has been described by Offenberg *et al.* (1998). Polyclonal antiserum 175 was elicited by immunization of a rabbit with rat whole SCs as has been described by Lammers *et al.* (1994). It recognizes predominantly the 30,000 and 33,000 M_r SC components (SCP3). The mouse monoclonal antibodies (Mabs) II52F10 and IX1H9 were elicited and isolated as described by Offenberg *et al.* (1991); they are described in detail by Heyting *et al.* (1987; 1989) and Offenberg *et al.* (1991). On Western blots, Mab II52F10 recognizes the 30,000 and 33,000 M_r SC components (SCP3), and Mab IX1H9 the 190,000 M_r SC component (SCP2). For labeling of kinetochores, we used a human autoimmune serum from a patient with CREST (calcinosis, Raynaud syndrome, esophageal dismobility, sclerodactyly, and telangiectasia) syndrome; this serum reacts with kinetochore proteins and has been described by Moens *et al.* (1987). For labeling of the SMC1 and SMC3 proteins we used two monoclonal antibodies, which were raised against bovine SMC1 and SMC3, and have been described by Eijpe *et al.* (in preparation).

Preparation of spreads and agar filtrates

Spreads of rat spermatocytes were prepared by the dry-down technique of Speed (1982), as modified by Peters *et al.* (1997). Agar filtrates of lysed spermatocytes were prepared as described by Heyting and Dietrich (1991).

Immunofluorescence labeling

Immunofluorescence labeling of dry-down preparations and agar filtrates was performed as described by Heyting and Dietrich (1991). The slides were mounted in Vecta Shield (Vector Laboratories Inc., Burlingame, CA, USA). The Mabs were diluted 1:1; serum 175 (anti-SCP3) 1:500; serum 493 (anti-SCP2) 1:400; CREST-serum 1:1000. Goat-anti-rabbit Immunoglobuline G (IgG) conjugated with aminomethylcoumarin acetate (AMCA) (Vector) or Texas Red (Jackson

ImmunoResearch laboratories, Pennsylvania, USA), goat-anti-mouse IgG conjugated with Texas Red (Jackson) or fluorescein isothiocyanate (FITC) (Jackson) and goat-anti-human IgG conjugated with FITC (Jackson) were used as secondary antibodies and were diluted according to the instructions of the suppliers. As a negative control for the Mabs, dry-down preparations of rat spermatocytes were incubated with the secondary antibodies only. As a control for serum 175 and 493 we used the preimmune sera in the same dilutions as the corresponding sera, and immune-depleted serum fractions. Furthermore, we incubated the slides with Mabs as primary antibodies and subsequently with goat-anti-rabbit-AMCA or goat-anti-human-FITC as secondary antibodies as a control on cross-reactivity of the secondary antibodies. Similarly, we checked the goat-anti-human-FITC and goat-anti-mouse-Texas Red conjugates for cross-reactivity with the rabbit polyclonal antibodies and the goat-anti-mouse-Texas Red and goat-anti-rabbit-AMCA conjugates for cross-reactivity with the human CREST antibodies. The goat-anti-human-FITC displayed some cross-reactivity with rabbit serum 175. The crossreacting antibodies were depleted from the goat-anti-human-FITC by affinity chromatography on a column carrying Igs from serum 175 (Harlow and Lane, 1988).

Microscopy

Spread preparations were examined with a Zeiss Axioplan research microscope equipped with epifluorescence illumination and Plan-Neofluar optics. Selected images were directly photographed on a 400 ISO color negative film using single band-pass emission filters (for DAPI, FITC and Texas Red fluorescence) with separated excitation filters. Negatives were scanned at high resolution and their computer images were processed and combined using the Adobe Photoshop software package.

Results

For immunolocalization of SCP2 and SCP3 in successive stages of meiosis we used dry-down spread-preparations of spermatocytes of the rat (see Materials and methods). Most stages of meiosis are preserved by this procedure (Peters *et al.*, 1997). We performed double-labeling experiments on dry-down preparations using combinations of monoclonal (Mabs) and polyclonal (Pabs) antibodies against SCP2 and SCP3. Mab II52F10 (anti-SCP3) and serum 175 (anti-SCP3) produced identical labeling patterns (not shown). The patterns obtained with Mab IX1H9 (anti-SCP2) and serum 493 (anti-SCP2) were almost identical, except that Mab IX1H9 hardly reacted with the axial cores of the XY bivalent, whereas serum 493 produced a clear signal.

SCP2 and SCP3 colocalized along the length of the axial elements until diplotene

The earliest leptotene/zygotene cells that we could identify contained axial element fragments, which were labeled by anti-SCP2 and anti-SCP3 (not shown). In these cells, we could not identify synapsed segments. Furthermore, we found several nuclei with long thin axial elements, which stained with both anti-SCP2 and anti-SCP3. These nuclei also contained very short synapsed or aligned segments (Fig. 1A-C). In pachytene (Fig. 1 G-L) and diplotene (Fig. 1 M-O), SCP2 and SCP3 were present along the length of the axial elements.

Fig. 1 D-F show a nucleus in mid-zygotene with an aggregate containing SCP3 but not SCP2. Such SCP3-containing aggregates were found in all stages of meiosis (Fig. 1P and S). Fig. 1 G-I show an early pachytene nucleus and Fig. 1 J-L a late pachytene nucleus. In these nuclei the axial elements of the XY bivalents are not paired and are therefore easily recognized. The axial elements of the XY bivalent do not pair until early pachytene and separate again in late pachytene (Joseph and Chandley, 1984). Although the labeling of the axial elements of the autosomes with anti-SCP2 and anti-SCP3 was identical, the axial elements of the XY bivalent were stained more intensely with anti-SCP3 than with anti-SCP2 (Fig. 1 G-L).

SCP2 and SCP3 accumulate at the centromeres during diplotene/diakinesis

SCP2 and SCP3 concentrated at the centromeres in diplotene when the SCs desynapse, as can be seen in Fig. 1 M-O. In late diplotene/diakinesis, as the axial elements fall

apart, SCP2 and SCP3 further concentrated between the kinetochores (Fig. 1P-R) and in diakinesis/metaphase I, SCP2 and SCP3 were mainly present at the centromeres, although small amounts of SCP2 and SCP3 were retained along the chromosome arms (Fig. 1S-U). In the nucleus in Fig. 1S-U (see arrow) the axes of the XY bivalent are still heavily labeled by anti-SCP2 and anti-SCP3 antibodies. In metaphase I, almost all SCP2 and SCP3 material had disappeared from the chromosome arms and SCP2 and SCP3 were retained at the 42 centromeric dots (Fig. 1V-X).

We observed several nuclei that contained more than 21 and less than 42 centromeric dots (Fig 2 A and C). The only stages in which between 21 and 42 centromeric dots are to be expected, are leptotene, zygotene, diplotene and anaphase II. Since no axial elements were present in the nuclei in Fig. 2 A-D we interpreted them as anaphase II nuclei in which the centromeric dots represent the dividing or still undivided sister centromeres. In most cases SCP3 was present between two centromeric dots or adjacent to a single dot. However, we also observed centromeric dots with no SCP3 in the vicinity. In these anaphase II nuclei, immunofluorescence for SCP2 was very faint. If SCP2 was detectable, it colocalized with SCP3 (Fig. 2D). In some instances the whole nucleus was diffusely labeled by anti-SCP2 (Fig 2B and D).

We interpreted small nuclei with 21 kinetochore dots as spermatid nuclei. Many of these nuclei contained aggregates of SCP3, some of which were associated with kinetochores. Some spermatids contained small amounts of SCP2, which colocalized with SCP3 (not shown), but in most spermatids SCP2 was not detectable.

SCP2 and SCP3 are present on connections between the axial elements in late diplotene

In diplotene nuclei with almost completely desynapsed axial elements, some of the bivalents displayed one or two connections between the axial elements, which were labeled both by anti-SCP2 and anti-SCP3 (Fig 1 M-O and Fig. 3). To study whether chromatin is organized along these connections, we performed double-labeling experiments with anti-SMC1 or anti-SMC3 antibodies and anti-SCP2 or anti-SCP3 antibodies. SMC1 and SMC3 are cohesins, which play a role in sister chromatid cohesion during the mitotic cell cycle (Michaelis *et al.*, 1997). During meiosis, SMC1 and SMC3 are localized in dots along the axial elements (Eijpe *et al.*, in preparation).

Fig. 4 shows that the SMC1 and SMC3 proteins are also present along the connections. Possibly, these connections represent the sites of crossing-over.

In diplotene/diakinesis, the possible sites of chiasmata could be recognized by the convergence of the remnants of the axial elements (Fig. 1P-R). However, only at part of these positions we found connections containing SCP2 and SCP3 (see arrow in Fig. 1Q). This is in agreement with observations of Dobson *et al.* (1994) and Moens and Spyropoulos (1995) who found no SCP3 (COR1) between the converging axial elements in late diplotene/diakinesis.

Discussion

A possible role for SCP2 in sister chromatid cohesion and chiasma stabilization

We performed immunofluorescence double-labeling of spermatocytes, using combinations of Mabs and Pabs against SCP2 and SCP3, in order to compare in detail the localization of these two axial element components in all successive stages of meiosis. SCP2 and SCP3 colocalized in most stages of meiosis. They were present all along the axial elements from leptotene unto and including diplotene. During diplotene SCP2 and SCP3 concentrate at the centromeres. In diakinesis, as the axial elements fall apart, both proteins further concentrate at the centromeres and small amounts of SCP2 and SCP3 are retained along the chromosome arms. A similar localization pattern has been observed earlier for COR1 (Dobson *et al.* 1994), the hamster homologue of SCP3. Dobson *et al.* (1994) argued that SCP3 (COR1) stabilizes chiasmata at metaphase I by providing sister chromatid cohesion along the chromosomal axes, distal to the chiasmata. From our observations we conclude that not only SCP3 but also SCP2 could play such a role in sister chromatid cohesion and chiasma stabilization.

SCP2 gradually disappears from the centromeres after metaphase I, whereas COR1 (SCP3) is retained at the centromeres until anaphase II (Dobson *et al.*, 1994; this paper). Possibly, SCP2 exerts its function until metaphase I, while SCP3 holds the sister chromatids together at the centromeres until anaphase II, when the sister kinetochores separate. However, it is also possible that remnants of SCP3 aggregate at the centromeres after metaphase I, but are not functional anymore. This is supported

by the fact that aggregates containing SCP3 also occur in spermatids; many of these aggregates still colocalized with centromeric dots, although cannot have a role in sister chromatid cohesion anymore.

Differences between the localization patterns of SCP2 and SCP3

Besides the persistence of SCP3 in later meiotic stages than SCP2, we observed some other differences between the localization patterns of SCP2 and SCP3. Aggregates of SCP3 within the nucleus are present in most stages of meiosis, while this is not observed for SCP2. The SCP3 aggregates have also been observed in female meiosis of rat (Dietrich *et al.*, 1992), in preleptotene stages of mouse (Scherthan *et al.*, 1996) and in hamster spermatocytes (Dobson *et al.*, 1994). The aggregates are not present in every nucleus, but it is possible that they are easily washed away during the spreading procedure. We think that these aggregates contain excess of SCP3 and have no function.

Another difference between the immunolocalization of SCP2 and SCP3 shows up in XY bivalents: anti-SCP3 labels the axial elements of pachytene XY bivalents more intensely than anti-SCP2. Joseph and Chandley (1984) observed that prior to pairing, the X and Y axes are very thick and more darkly stained compared with uranyl acetate than autosomes. On completion of synapsis at mid pachytene, the unpaired axis of X remains thick, whereas the Y axis is much thinner (Joseph and Chandley, 1984). Possibly, the thickening of the XY axes is accompanied by an increase in the amount of SCP3 but not SCP2 on these axes.

Connections between desynapsed axial elements in late diplotene

In late diplotene, we found cross connections between desynapsed axial elements. Although such connections were found in only part of the bivalents, we think that they could represent the sites of crossing-over. First, remnants of such cross connections can be seen in some diakinesis bivalents at sites of convergence of the two homologues (Fig. 1P-R). Such sites of convergence are supposed to represent the sites of chiasmata (Moens and Spyropoulos, 1995). Second, foci containing SMC1 and SMC3 are localized on these connections. SMC1 and SMC3 are involved in mitotic sister chromatid cohesion, and because they are localized along the axial elements of

SCs (Eijpe *et al.*, in preparation), they are probably also involved in meiotic sister chromatid cohesion. Presumably, SMC1 and SMC3 mark the chromatid axes, which would mean that the chromatid axes follow the cross connections between desynapsed axial elements. As has been pointed out by several authors (Rockmill and Roeder, 1990; Engebrecht *et al.*, 1990), a crossover at the DNA-level is not sufficient to create a functional chiasma that can keep homologous chromosomes together in the metaphase I spindle. The continuity of the recombined chromatids must also be restored at the level of the chromatid axes. We hypothesize that axial element components like SCP2 and SCP3 temporarily stabilize crossovers at the DNA level, and guide the axes of the recombining chromatids, so that these axes are also cross-connected. As soon as this has been accomplished, the SCP2 and SCP3 containing cross connections are no longer required and disappear. That would explain why these cross connections are not seen in all bivalents.

Acknowledgments

We thank R. Jessberger who kindly provided the monoclonal anti-SMC1 and anti-SMC3 antibodies and P. Moens for a gift of the human CREST serum. We thank E. Roos for his help with computer image processing.

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Chapter 6

Discussion

Discussion

SCP2 is a 173 kDa component of the axial elements of synaptonemal complexes (SCs) of the rat. Fig. 1 shows a schematic representation of the organization of structural features within SCP2 among which the two domains with S/T-P motifs, which are common in DNA-binding proteins. SCP2 is present along the axial elements from leptotene up to and including diplotene. During diplotene, SCP2 concentrates at the centromeres where it persists until at least anaphase I. Furthermore, small amounts of SCP2 are retained along the chromatid arms until metaphase I. On the basis of the localization pattern of SCP2 we proposed that SCP2 is involved in sister chromatid cohesion and chiasma stabilization (chapter 5). In late diplotene, we found connections between the axial elements that contained SCP2. We presented arguments that these connections represent the sites of crossing-over (chapter 5). In this chapter we propose a model for the relationship between chromatid axes and axial elements of SCs and we speculate about possible functions of axial elements.

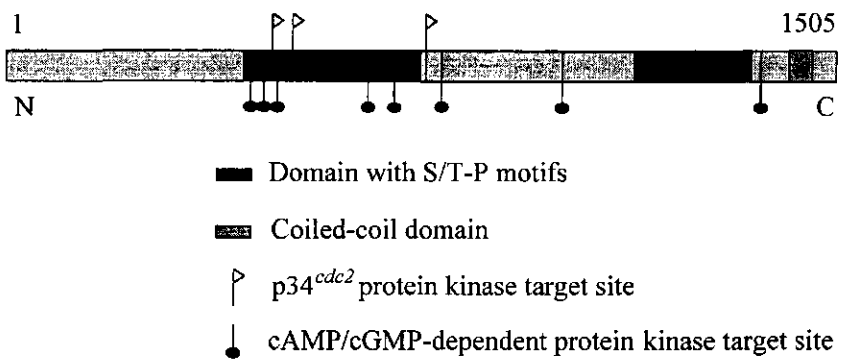


Figure 1. Schematic representation of SCP2 of the rat. Shown are the coiled-coil domain at the C-terminus, the two domains with S/T-P motifs (DNA-binding motifs), the $p34^{cdc2}$ kinase target sites and the cAMP/cGMP-dependent protein kinase target sites.

Relationship between axial elements and chromatid axes

The axial elements differ in several respects from the chromatid axes that are observed along the chromatids in mitotic chromosomes (also mentioned in chapter 2); 1.The two chromatids of a meiotic prophase chromosome share a single axial element, whereas the chromatids of a metaphase chromosome each have their own scaffold; 2. The major protein components of axial elements are meiosis-specific and thus not found in chromatid scaffolds (Heyting *et al.*, 1989); 3. Axial elements are longer than metaphase chromatid axes and morphologically better defined.

However, chromatid axes and axial elements also share some features; 1. Axial elements and chromatid axes can both be visualized by silver-staining (Fletcher, 1979; Howell and Hsu, 1979) 2.Topoisomerase II (Topo II), which is a major component of the chromatid axes (Berrios *et al.*, 1985; Earnshaw and Heck., 1985; Gasser *et al.*, 1986), is also present along the axial elements (Moens and Earnshaw, 1989; Klein *et al.*, 1992), particularly in later stages of meiotic prophase. 3. Furthermore, SMC1 and SMC3, which are present along the chromatid axes in mitotic chromosomes (Losada *et al.*, 1998) are localized in dots along the axial elements (Eijpe *et al.*, in preparation). 4. Chromatid axes are observed during meiosis, in diplotene/diakinesis, when the axial elements have disappeared (Rufas *et al.*, 1992). Rufas *et al.* (1992) proposed that the chromatid axes are not replaced by the axial elements at the onset of meiosis, but that they are present during meiotic prophase in close association with the axial elements.

Several researchers have observed that the axial elements consist of multiple strands (Heyting *et al.*, 1985; Dietrich *et al.*, 1992; Comings and Okada, 1971; del Mazo and Gil-Alberdi, 1986). del Mazo and Gil-Alberdi (1986) proposed that the multiple strands within the axial element are clustered into two sub axial elements, each of which is connected with one of the two sister chromatids. Taking into account that the axial elements consist of two sub axial elements, we think that the chromatid axes and axial elements are organized as shown in Fig. 2. According to this model, one sub-axial element and one chromatid axis are present along each sister chromatid. The two sub-axial elements of one chromosome are tightly associated. The components of the chromatid axes can still fulfill their functions during meiosis. However, we think that these functions are influenced by the axial elements;

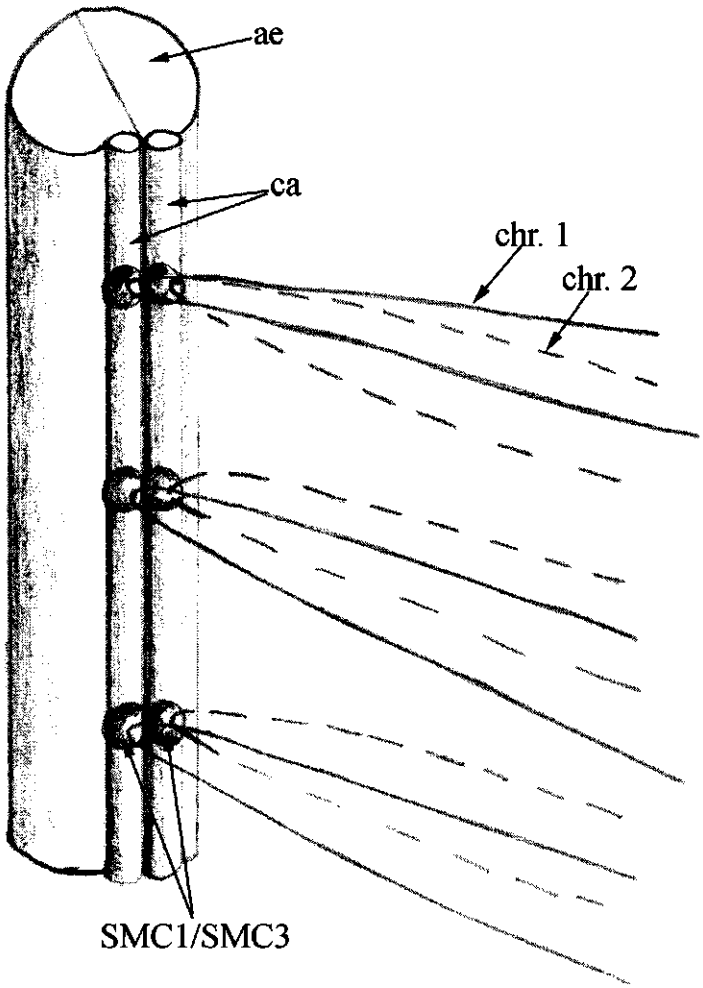


Figure 2. Schematic representation of the organization of the chromatid axes and axial elements within meiotic chromosomes (adapted from Rufas *et al.*, 1992). Shown are the chromatin of the two sister chromatids (chr.1 and chr.2), the chromatid axes (ca), the axial element (ae) and subunits, containing SMC1 and SMC3.

Topo II, which is a component of the chromatid axes, is probably involved in the organization of the chromatin in loops on the chromosome scaffold. The organization of the chromatin in loops separates the DNA in domains, which can behave independently with respect to gene expression. The bases of the loops are called scaffold attachment regions (SARs) and are AT-rich. Topo II, which is a DNA-binding protein, indeed binds preferentially to SAR-DNA in competition experiments (Adachi *et al.*, 1989). The organization of the chromatin in meiotic chromosomes resembles the organization of mitotic chromosomes (reviewed by Moens and Pearlman, 1988). The main differences between mitotic and meiotic chromosomes is that in mitotic chromosomes the looped domains are more tightly packaged (Saitoh and Laemmli, 1994), whereas in prophase I of meiosis, the chromosomal loops are organized in a linear array. SCP2 and Topo II both contain S/T-P motifs, which can interact with the minor grooves of AT-rich DNA, for example SAR-DNA. Possibly, SCP2 can compete with Topo II for binding sites on the DNA and inhibit the function of Topo II. Hereby condensation of the DNA can be prevented and the linear packaging of chromatin loops is maintained during meiosis. This is probably necessary to establish homologue recognition, chromosome pairing and recombination.

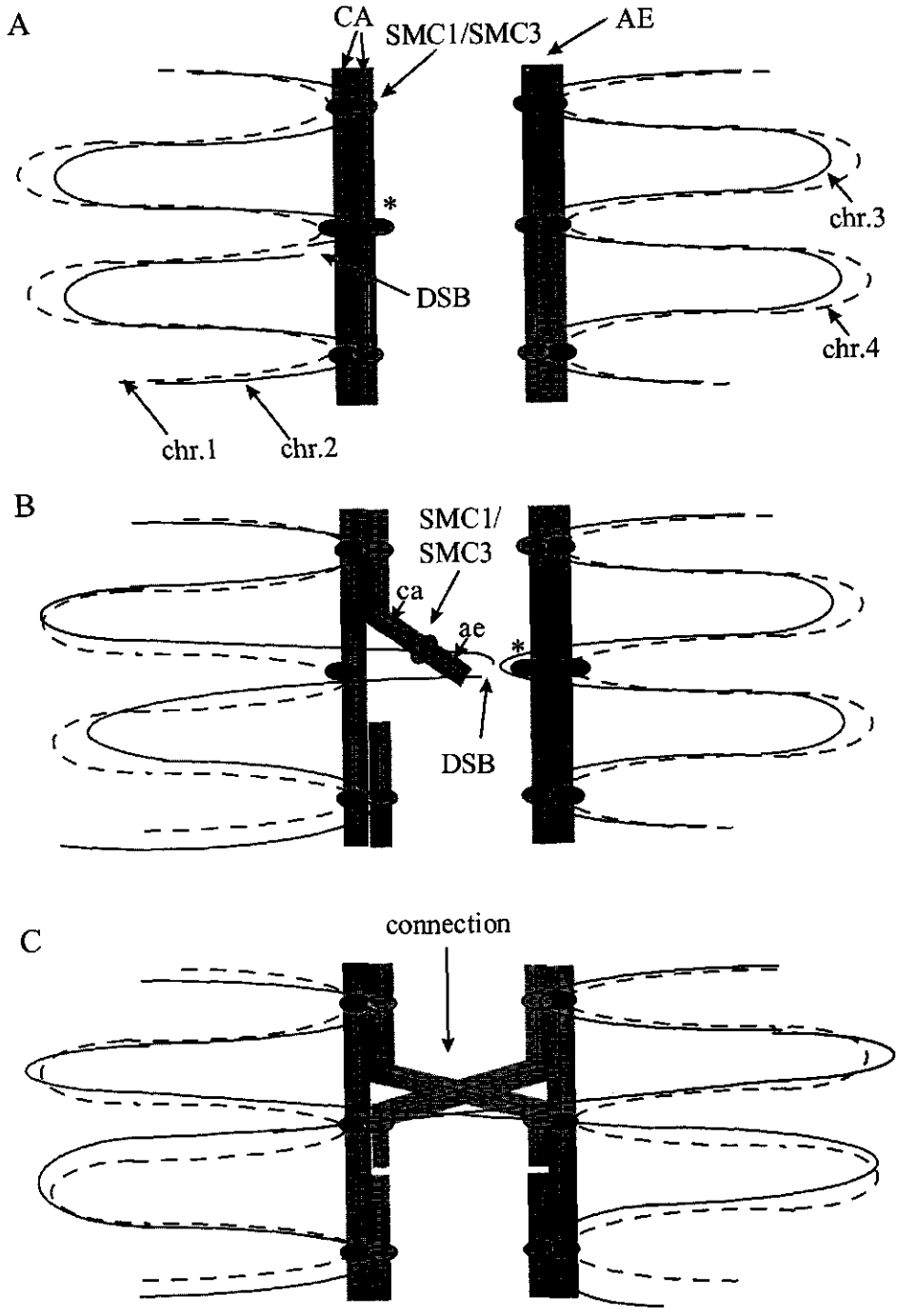
Topo II furthermore decatenates interlocked DNA strands after replication. This decatenation occurs at the metaphase-anaphase transition during mitosis. Murray and Szostak (1985) proposed that the catenation is required for the maintenance of the association of the sister chromatids until this stage. SMC1 and SMC3 are involved in sister chromatid cohesion during mitosis (Michaelis *et al.*, 1997). They constitute complexes that hold the sister chromatids together (Michaelis *et al.*, 1997). At the metaphase-anaphase transition, SMC1 and SMC3 are replaced by SMC2 and SMC4 (Losada *et al.*, 1998), which are required for chromatin condensation (Hirano and Mitchison, 1994). The sister chromatid cohesion during meiosis differs from sister chromatid cohesion during mitosis. In mitosis, the sister chromatid arms and the sister centromeres lose their cohesion almost simultaneously, at the metaphase-anaphase transition. In meiosis, however, the chromatid arms lose their cohesion at the metaphase I - anaphase I transition, whereas the cohesion between the sister centromeres is not lost before the metaphase II-anaphase II transition. Since SCP2 and SCP3 are present at the places where sister chromatid cohesion is required, it seems

likely that these proteins are involved in the regulation of the sister chromatid cohesion. Possibly, these proteins influence the behavior of SMC1 and SMC3 at these places (chapter 5). For instance, it is possible that SCP2 and/or SCP3 prevent SMC2 and SMC4 to replace SMC1 and SMC3. I therefore expect many interactions between components of the axial elements and the chromatid axes. In order to learn more about the relationship between axial elements and chromatid axes these interactions should be analyzed.

Possible roles of axial element components in recombination

Recombination between homologous chromosomes is a meiosis-specific phenomenon. Intact axial elements are probably not required for recombination since the *red1* mutant of *S. cerevisiae* does not assemble axial elements, but still displays high levels of interhomologue recombination (Rockmill and Roeder, 1990). However, it seems likely that the axial elements influence the interhomologue recombination. For example, the Red1 protein contributes to the meiotic bias for interhomologue recombination (Schwacha and Kleckner, 1997), and has a role in monitoring the progress of the meiotic recombination process (Xu *et al.*, 1997). In late diplotene, we found fibrous connections between the axial elements, which contained SCP2 and SCP3. SMC1 and SMC3 are also present on these connections, which probably means that the chromatid axes follow these connections. Therefore we think that these connections represent the sites of crossing-over (chapter 5). I propose that SCP2 establishes the continuity of the chromatid axes at the position of the crossing-overs. Possibly, this is accomplished as shown in the model in Fig. 3.

Figure 3. Schematic representation of the behavior of axial elements and chromatid axes at the position of a homologous recombination event. Chr.1 and chr. 2 represent the two sister chromatids of a chromosome, chr.3 and chr.4 represent the two sister chromatids of the homologous chromosome. In this diagram chr. 2 and chr. 3 recombine. **A.** A double strand break (DSB) in chromatid 2 is brought to the chromatid axis (ca). At this position sister chromatid cohesion, established by SMC1 and SMC3 complexes, is released (asterisk). **B.** The DNA-strands of the broken chromatid invade an homologous chromatid; this has two consequences: (i) sister chromatid cohesion is locally lost in the invaded chromatid (asterisk), and (ii) the recombining DNA-strands pull the locally detached chromatid axes/axial element halves into the central region of the SC (in B this is shown for one of the two chromatids). **C.** The chromatid axes and axial elements of the recombining chromatids are crosswise connected whereby continuity of the axes of the recombined chromatids is established.


A
CA
SMC1/SMC3
AE
*
chr.3
DSB
chr.4
chr.1
chr.2
B
SMC1/
SMC3
ca
ae
*
DSB
C
connection

In this figure the successive events that occur at the position of a crossover are drawn; Recombination is initiated by a DSB and we think that this DSB is brought somehow to the axial element (Fig. 3A). At the position of the DSB, sister chromatid cohesion, presumably established by the SMC1 and SMC3 proteins, is temporarily released (Fig. 3A). Next, the protruding 3' end of the DSB can invade a non-sister chromatid of the homologous chromosome. At the site of invasion, sister chromatid cohesion would also be lost (Fig. 3B). Probably, together with the invading DNA strand, part of the chromatid axis and the axial element "halves" of the recombining chromatids are pulled into the central region. The chromatid axis and the axial element of the invading strand can then make a connection with the chromatid axis and the axial element of the invaded strand (Fig. 3C). This ensures that the continuity of the chromatid axes is re-established at the sites of crossover before resolution of the chiasmata at anaphase I.

Furthermore, it is possible that the connections also stabilize the recombination intermediates between homologues. Thereby they would provide a bias towards recombination with the homologue rather than the sister chromatid. In this way, SCP2 possibly enhances interhomologue recombination, as has been found for Red1 in *S. cerevisiae* (Schwacha and Kleckner, 1997).

Are the functions of the axial elements regulated by phosphorylation ?

SCP2 contains several potential phosphorylation sites. Some of these sites are found in one of the two domains with DNA-binding motifs (Fig. 1). The $p34^{cdc2}$ protein kinase target sites are also S/T-P motifs. Phosphorylation of these motifs causes loss of a crucial hydrogen bond that is required for stabilization of the β-turn structure of the S/T-P motif (Churchill and Travers, 1991). Possibly, DNA-binding activities of SCP2 are influenced by phosphorylation so that during meiosis disassembly of the axial elements and condensation of the chromatin can be regulated by phosphorylation, like in mitosis.

Phosphorylation sites are also found in other SC components; SCP1, which is a component of the transverse filaments in rat (Meuwissen *et al.*, 1992), contains one $p34^{cdc2}$ protein kinase target site. SCP3, which is a component of the axial elements of

SCs in rat, contains two potential target sites for cAMP/cGMP-dependent protein kinase (Lammers *et al.*, 1994). Other evidence that phosphorylation is possibly involved in the regulation of SC disassembly comes from observations in yeast: A mutation in CDC28, which is the *S. cerevisiae* homologue of $p34^{cdc2}$, causes an arrest in pachytene (Davidow and Beyers, 1984).

SCP3 is phosphorylated *in vivo*. In early pachytene, one phosphate group is added to the protein (Lammers *et al.*, 1995). This change in phosphorylation of SCP3 correlates in time with the thickening of the axial elements between zygotene/early pachytene and mid pachytene (Dietrich and de Boer, 1983). Lammers *et al.* (1995) speculate that in the first half of meiotic prophase the axial elements of SCs have a role in the prevention of recombination between sister chromatids and that later in prophase, when initiation of recombination has stopped and DNA-DNA interactions between homologous non-sister chromatids have been established, this control has to be relaxed to make the sister chromatid available as a template for the repair of those initiated recombination events, for which there was no template on the homologous chromosomes.

Evolutionary conservation of the axial elements

Axial element components seem not to be very conserved; attempts to isolate proteins homologous to SCP2 in organisms like *S. cerevisiae*, *A. nidulans* and *C. elegans* have failed (unpublished results). Analysis of the cDNA encoding the human SCP2 (hsSCP2), revealed that the amino acid sequence of SCP2 is not very conserved (63% amino acid identity) between rat and human. However, the organization of the structural domains within the proteins is similar (chapter 3). Therefore we think that SCP2 has an important structural function within the axial elements. We think that SCP2 maintains the structural integrity of the chromosomes, and influences chromatin organization, sister chromatid cohesion and recombination.

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Summary

Synaptonemal complexes (SCs) are ladderlike protein structures, which are formed between homologous chromosomes during the prophase of the first meiotic division. SCs consist of two axial elements, one along each chromosome, and transverse filaments (TFs), which connect the axial elements. On the TFs, between the axial elements, there is a third longitudinal structure, the central element (CE). Possible functions of the axial elements include organization of the chromatin in loops, providing sister chromatid cohesion, stabilization of chiasmata and regulation of recombination. These possible functions are explained in **chapter 1**. In axial elements of SCs of rat, two major protein components have been identified, named SCP2 and SCP3, with relative electrophoretic mobilities (M_r) of 190,000 and 30,000-33,000. This study mainly focused on the analysis of the function of SCP2.

In **chapter 2** we describe how we isolated and sequenced the cDNA encoding SCP2 of the rat (rnSCP2). The protein predicted from the nucleotide sequence is basic and has a mass of 173 kDa. At the C-terminus, SCP2 contains a region which is predicted to be capable of forming a coiled-coil structure. Furthermore, SCP2 contains two clusters of S/T-P motifs, which are common in DNA-binding proteins, and several potential phosphorylation sites. The *SCP2* gene is expressed predominantly in meiotic prophase cells.

In order to identify conserved domains within SCP2, we isolated and analyzed the human SCP2 cDNA (hsSCP2). This is described in **chapter 3**. Although there is only 63% identity at the amino acid level between hsSCP2 and rnSCP2, several structural features and amino acid sequence motifs are conserved; hsSCP2 contains S/T-P motifs, which are commonly found in DNA-binding proteins, in the same domains as rnSCP2 and has a predicted coiled-coil region at its C-terminus. The structural organization of hsSCP2 and rnSCP2 resembles the structural organization of Red1, a protein component of the axial elements in *S. cerevisiae*. We speculate that Red1 and SCP2 are functional homologues. The *hsSCP2* gene was assigned to chromosome 20q13.33 by fluorescence *in situ* hybridization.

The ultrastructural organization of SCP2 and SCP3 within SCs was analyzed by immunogold labeling of surface-spread spermatocytes and sections of testicular tissue as described in **chapter 4**. The immunogold-labeling patterns of spreads and sections differed significantly and we attributed this difference to artifacts, caused by the surface-spreading technique. Therefore we relied on sections for the precise localization of SCP2 and SCP3. The immunogold-label distribution of anti-SCP2 and anti-SCP3 antibodies coincided with the axial elements and therefore we conclude that both SCP2 and SCP3 are structural components of the axial elements. The distribution of anti-SCP2 label had a shoulder at the innerside of the axial element, which was not observed with the anti-SCP3 label. Because we observed fuzzy connections between the axial elements that were labeled by anti-SCP2 but not anti-SCP3 antibodies, we attributed the shoulder in the gold label distribution of anti-SCP2 antibodies to the labeling of these connections.

To study the localization of SCP2 and SCP3 in successive stages of meiosis, we performed immunofluorescence labeling of dry-down spread preparations of spermatocytes with anti-SCP2 and anti-SCP3 antibodies (**chapter 5**). SCP2 and SCP3 colocalize along the axial elements from leptotene up till and including diplotene. After diplotene, SCP2 and SCP3 concentrate at the centromeres and small amounts of SCP2 and SCP3 are retained along the chromosome arms until metaphase I. From these results we conclude that both SCP2 and SCP3 are involved in sister chromatid cohesion and chiasma stabilization. SCP2 and SCP3 are furthermore present on connections between desynapsed axial elements in late diplotene. SMC1 and SMC3, which are involved in sister chromatid cohesion during mitosis, and are present in dots along the axial elements during meiosis, are also present along these connections. We think that these connections represent the sites of crossing-over and that they stabilize recombination intermediates between homologous chromosomes, and ensure the continuity of the cores of recombining chromatids.

In **chapter 6** we consider possible functions of SCP2 and of axial elements.

Samenvatting

Synaptonemale complexen (SCs) zijn eiwitstructuren die worden gevormd tussen de gepaarde homologe chromosomen tijdens de meiose. Het SC bestaat uit twee axiale elementen, één langs elk chromosoom, en transversale filamenten, die de axiale elementen verbinden. Een derde langwerpige structuur, het centrale element, bevindt zich in het midden van het SC, evenwijdig aan de axiale elementen. Mogelijke functies van het axiale element zijn organisatie van het chromatine in loops, waarborgen van zuster chromatide cohesie, stabilisatie van chiasmata en regulatie van recombinatie. Deze mogelijke functies worden besproken in **hoofdstuk 1**. In de axiale elementen van SCs van de rat zijn twee componenten geïdentificeerd, SCP2 en SCP3, met relatieve electrophoretische mobiliteiten (M_r) van 190,000 en 30,000-33,000. Deze studie richt zich voornamelijk op de analyse van de functie van SCP2.

In **hoofdstuk 2** beschrijven we hoe het cDNA dat codeert voor SCP2 van de rat (rnSCP2) is geïsoleerd en geanalyseerd. Het rnSCP2 cDNA codeert voor een basisch eiwit met een molecuul gewicht van 173 kDa. Aan de C-terminus bevat SCP2 een gebied dat mogelijk een coiled-coil structuur kan vormen. Verder bevat het SCP2-eiwit twee clusters met S/T-P motieven, die veel worden aangetroffen bij DNA-bindende eiwitten, en verschillende mogelijke phosphorylerings sites. Het *SCP2* gen komt voornamelijk tot expressie in meiotische profase cellen.

Met de bedoeling om geconserveerde domeinen in SCP2 te identificeren hebben wij het humane SCP2 cDNA (hsSCP2) geïsoleerd en geanalyseerd. Dit staat beschreven in **hoofdstuk 3**. De aminozuursequenties van hsSCP2 en rnSCP2 vertonen slechts 63% homologie. Toch zijn verschillende voorspelde structurele eigenschappen en aminozuur sequenties geconserveerd; hsSCP2 bevat S/T-P motieven in dezelfde domeinen als rnSCP2 en aan de C-terminus van het eiwit bevindt zich een coiled-coil domein. De structurele organisatie van hsSCP2 en rnSCP2 vertoont overeenkomsten met de structurele organisatie van Red1, een eiwit dat onderdeel uitmaakt van de axiale elementen in *S. cerevisiae*. Wij vermoeden dat Red1 en SCP2 functionele homologen zijn. Het *hsSCP2* gen bevindt zich op chromosoom 20q13.33, zoals gebleken is uit fluorescentie *in situ* hybridisatie.

De ultrastructurele organisatie van SCP2 en SCP3 in het SC is geanalyseerd door middel van immunogoud labeling van oppervlakte-gespreide spermatocyten en coupes van testis weefsel, zoals beschreven in **hoofdstuk 4**. Er was een aanzienlijk verschil tussen de verdelingen van immunogoud op SCs in spreidpreparaten en in coupes. Waarschijnlijk werd dit verschil veroorzaakt door artefacten, ontstaan tijdens de oppervlakte-spreiding van spermatocyten. Daarom zijn wij uitgegaan van de resultaten op coupes voor de preciese localisatie van SCP2 en SCP3. In coupes vielen de immunogoud verdelingen verkregen met anti-SCP2 en anti-SCP3 antilichamen samen met de structuur van het axiale element en beide eiwitten maken dus hoogst waarschijnlijk deel uit van het axiale element. De verdeling van het anti-SCP2 label vertoonde een schouder aan de binnenzijde van het axiale element, terwijl dit niet het geval was voor het anti-SCP3 label. Omdat we tussen de axiale elementen dwarsverbindingen waarnamen die gelabeld waren met anti-SCP2, maar niet met anti-SCP3 antilichamen veronderstellen wij dat de schouder in de immunogoud verdeling van anti-SCP2 is toe te schrijven aan de labeling van deze dwarsverbindingen.

Door middel van immunofluorescentie labeling van dry-down preparaten van spermatocyten hebben we de localisatie van SCP2 en SCP3 in opeenvolgende stadia van de meiose bestudeerd (**hoofdstuk 5**). Beide eiwitten colocaliseren met de axiale elementen van leptoteen tot en met diploteen. Na diploteen concentreren SCP2 en SCP3 zich op de centromeren terwijl kleine hoeveelheden SCP2 en SCP3 achter blijven langs de chromosoom armen tot aan metaphase I. Uit deze resultaten hebben wij geconcludeerd dat zowel SCP2 als SCP3 mogelijk betrokken zijn bij zuster chromatide cohesie en chiasma stabilisatie. SCP2 en SCP3 zijn verder ook aanwezig op dwarsverbindingen tussen de gedesynapste axiale elementen in laat diploteen. SMC1 en SMC3, die betrokken zijn bij zuster chromatide cohesie in mitose en tevens aanwezig zijn langs de axiale elementen in meiose, bevinden zich ook langs deze verbindingen. Wij denken dat deze verbindingen de posities van crossovers aangeven en dat ze recombinatie intermediairen tussen homologe chromosomen stabiliseren en de continuiteit van de assen van recombinerende chromatiden waarborgen.

In **hoofdstuk 6** beschouwen wij mogelijke functies van SCP2 en van axiale elementen.

Nawoord

Hè, hè, de klus is geklaard. Rest mij nog slechts het schrijven van de laatste twee bladzijden van mijn proefschrift. En dat is eigenlijk wel de meest plezierige klus: het bedanken van alle mensen die mij hebben geholpen bij het tot stand komen van dit boekje.

Als eerste wil ik graag Christa Heyting bedanken, mijn promotor die mij de mogelijkheid heeft geboden om dit promotieonderzoek uit te voeren. Christa, bedankt voor je dagelijkse begeleiding en voor de bemoedigende woorden als het af en toe eens tegen zat en bedankt voor het zeer zorgvuldig nakijken van dit manuscript.

Op het lab werd ik vaak bijgestaan door Hildo Offenberg. Toch handig, zo'n collega die Maniatis van buiten kent! Hildo, je was altijd bereid om te helpen en voor elk probleem wist jij wel weer een oplossing! Ik heb je collegialiteit altijd erg gewaardeerd. Nog bedankt voor die laatste 32 nucleotiden!!

Mirjam, als jij niet had geholpen met het uitvoeren van talloze immuunincubaties en antilichaam zuiveringen dan had dit onderzoek nog wel wat jaartjes langer geduurd. Bedankt voor je hulp en veel succes in de toekomst.

Ralph en Hans waren tijdens een groot deel van mijn OIO-tijd lotgenoten in zware tijden. Want promoveren valt niet altijd mee. Maar samen met jullie was het een gezellige tijd! En Hans, kijk jij ook zo uit naar 11 maart?

Lorrie Anderson and Karin Schmekel, I enjoyed our time on the lab together very much. I hope we will meet again in the near future!

Wolfgang, thanks for a lot of helpful suggestions and being a nice colleague.

Maureen, slechts korte tijd heb ik samen met jou op het lab gestaan. Toch prijken een aantal van jouw plaatjes nu ook in dit proefschrift. Ik wens je veel succes met het vervolg van je promotie. En het is even wennen, maar Wageningen is écht net zo leuk als Amsterdam!

Erik Peters, Miriam Nijland en Bas Janssen, jullie hebben als student een bijdrage geleverd aan dit onderzoek. Bedankt voor jullie inzet.

Ik kan onmogelijk alle collega's van Erfelijkheidsleer bij naam noemen. Ik wil eenieder van jullie die op welke wijze dan ook heeft bijgedragen aan mijn onderzoek hartelijk bedanken. In het bijzonder wil ik nog even Hans de Jong noemen voor zijn hulp bij lastige computer-, statistieke- en imaging-problemen.
Een deel van mijn onderzoek heb ik uitgevoerd op de vakgroep Antropogenetica van het AMC in Amsterdam. Axel Dietrich, bedankt dat ik ruim zes maanden bij je op het lab heb kunnen werken. Het heeft mijn promotie een heel andere wending gegeven. Bedankt voor de goede samenwerking! Agnes Vink, bedankt voor je geduldige instructies voor het werken met de electronen-microscoop. Dankzij jou heb ik geleerd dat cytologie eigenlijk heel erg leuk is.
Jan Hoovers en Nancy Groot, bedankt voor jullie bijdrage aan hoofdstuk 3.
Magda Usmany, Douwe Zuidema, en Just Vlak van de vakgroep Virologie in Wageningen hebben mij geholpen bij het (op de valreep) full-length tot expressie brengen van SCP2 via het bacoluvirus. Helaas zijn de resultaten hiervan niet opgenomen in dit proefschrift, maar ze vormen een basis voor vervolgonderzoek. Bedankt voor jullie hulp!

Naast werken bestaat er ook nog een ander leven.
Pa en Ma, jullie hebben me daar gelukkig vaak genoeg aan herinnerd. Bedankt voor jullie steun en vertrouwen.
Johan, Monique, Gijs, Corrie, Sylvia, Wim, Maaike, Bertjan, Giedo, Mirjam, Jeroen en Monique, bedankt voor jullie belangstelling.
Inge, bedankt dat ik voor één dag jouw "aanstaande" mag lenen!
Elisabeth, Onno, Bert, Karin en Nathalie, de gezellige maaltijden samen waren vaak een welkome afleiding tussen al het gestress door!
Belinda, Monique, Arie, Anita, Erik en Nathalie, hè hè, kunnen we eindelijk over iets anders praten! Marnix, zet hem op!
Eelco, bedankt voor het ontwerpen van de omslag van dit proefschrift. Bedankt voor de kopjes koffie en warme maaltijden die je voor me maakte terwijl ik achter de computer zat. Maar bovenal: Bedankt voor wie je bent!

Onno en Klaaseric, bedankt dat jullie op 10 maart aan mijn zijde willen staan!

Curriculum Vitae

Marjolijn Schalk werd geboren op 27 april 1969 te Prinsenbeek. Zij verhuisde in 1973 met haar ouders en haar broer naar Etten-leur. In 1987 behaalde ze haar VWO diploma aan de Katholieke Scholengemeenschap Etten-Leur en in datzelfde jaar startte ze haar studie Moleculaire Wetenschappen aan de Landbouwuniversiteit te Wageningen. In het kader van haar studie liep zij stage aan de afdeling Inwendige Geneeskunde van het Dijkzigtziekenhuis te Rotterdam en aan de vakgroep Environmental Toxicology van de University of California te Davis, USA. In augustus 1993 behaalde ze haar bul. Van september 1993 tot februari 1998 verrichtte zij promotieonderzoek als onderzoeker in opleiding (OIO) aan de vakgroep Erfelijkheidsleer te Wageningen. De resultaten van dit onderzoek staan beschreven in dit proefschrift. Een deel van haar onderzoek voerde zij uit aan de vakgroep Antropogenetica van het Academisch Medisch Centrum te Amsterdam in de periode februari 1996 tot en met augustus 1996. In maart 1998 trad zij in dienst als R&D medewerker bij KREATECH Diagnostics te Amsterdam.