SCP1, a major protein component of synaptonemal complexes of the rat

SCP1, een belangrijke eiwit-component van synaptonemale complexen van de rat

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SCP1, a major protein component of synaptonemal complexes of the rat

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Stellingen

1. SCP1 is een hoofdcomponent van de transversale filamenten van het synaptonemale complex.

dit proefschrift

2. Onderzoek waarbij polyklonale antisera, opgewekt tegen overlappende SC eiwitfragmenten, worden gebruikt voor immunogoud labeling experimenten met SC preparaten van gespreide spermatocyten, leveren onbetrouwbare resultaten op die niet zonder meer gebruikt kunnen worden voor de lokalisatie bepaling van dit eiwit in het SC.

Dobson et al. *J. Cell Sci.* **107:** 2749-2760

dit proefschrift

3. De afzonderlijke SC eiwitten van *S. cerevisae* en zoogdieren kunnen een grotere structurele en functionele overeenkomst vertonen dan men op grond van hun gelijkenis in de primaire aminozuurvolgorde zou verwachten.

4. De vraag of een intacte SC structuur verantwoordelijk is voor het verschijnsel van "crossover interferentie" kan beter in een *red1* dan een *zip1red1* achtergrond in *S. cerevisae* worden bestudeerd.

Storlazzi et al. *Proc. Natl. Acad. Sci. USA* **93:** 9043-9048

5. Het afdwingen van sluitingstijden door het gebruik van semi-professionele bewakingsapparatuur in een universitair laboratorium leidt af van de primaire doelstelling als faciliteit voor het optimaal kunnen uitvoeren van wetenschappelijk onderzoek. Deze maatregel werkt hierdoor volledig contra-productief.

6. Machtswellust is vaak gebaseerd op pure angst.

7. Niet alleen Jezus droeg zijn eigen kruis.

8. De opmerking van westerse sociaal-historici dat Afrika een "verloren continent" zou zijn met het oog op stabiele welvaartsgroei, gaat voorbij aan het feit dat de deplorabele economische toestand in menig Afrikaanse staat juist in de hand wordt gewerkt door het actief steunen van incompetente en corrupte regimes door westerse regeringen.

9. Een te hoog aandeel van spelletjes en loterij-programma's in het aanbod van de commerciële televisie ontaardt al gauw in plee-TV.

10. DNA heb je voor 't leven.

11. Een slappe haan kraait niet meer.

12. Een dispuut brengt weinig vrienden.

13. Intellectuelen zijn vaak te slim voor het oplossen van eenvoudige problemen.

Stellingen behorend bij het proefschrift 'SCP1, a major component of synaptonemal complexes of the rat' van Ralph Meuwissen
Wageningen, 4 februari 1997

Voor mijn ouders

Contents

CHAPTER 1

Introduction

INTRODUCTION

Meiosis is an essential process in the sexual reproduction cycle of eukaryotes. It brings about the transition from diploid to haploid generations of cells. At fertilization, the diploid state is restored when two haploid gametes fuse to form a diploid zygote.

Meiosis consists of two successive nuclear divisions, meiosis I and II, which follow a single round of DNA replication. After premeiotic S-phase, during the prophase of meiosis I, a series of characteristic chromatin rearrangements take place by which homologous chromosomes condense and pair, and the non-sister chromatids of homologous chromosomes (homologues) recombine with each other (von Wettstein *et al.*, 1984; Loidl, 1994; Griffiths *et al.*, 1996). These events are followed by segregation of the homologues (Figure 1).

The segregation of the homologues causes the reduction of the chromosome complement from diploid to haploid: meiosis I is therefore called a reductional division. Meiosis II, in contrast, is an equational division because the ploidy level is maintained: the two sister chromatids of each chromosome are then separated, as in a mitotic division (Fig.1). The characteristic aspects of chromosome behaviour in meiosis are summarized in Table I, which shows that many meiosis-specific events take place during meiotic prophase. These events, namely the pairing and recombination of homologous chromosomes, are accompanied by the assembly and disassembly of meiosis-specific structures, the synaptonemal complexes (SCs) (Moses, 1969).

Table I **Chromosome behaviour during eukaryotic cell divisions**

Meiosis I	Meiosis II	Mitosis
Pairing of homologous chromosomes	n.a. [1)]	Usually no pairing between homologous chromosomes
High frequency of recombination, preferably between non-sister chromatids	n.a.	Low frequency of recombination, preferably between sister chromatids
Sister chromatid cohesion until anaphase I	no cohesion	cohesion until anaphase
The two sister-centromeres act as a unit	two sister-centromeres act independently from each other and are separated	two sister-centromeres act independently from each other and are separated

1) n.a., not applicable

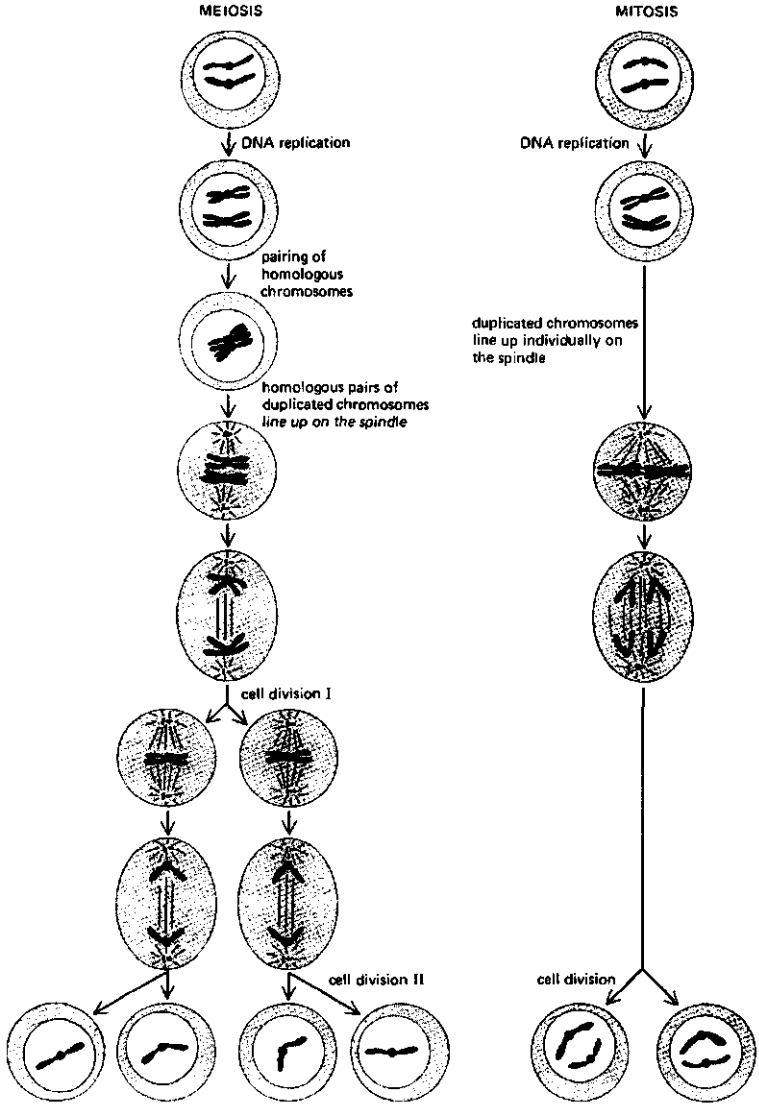

Figure 1. The process of meiosis compared to mitosis. Only one set of homologous chromosomes is shown. The pairing of the duplicated homologous chromosomes is unique to meiosis. During meiosis, two nuclear divisions are needed to produce haploid gametes. Each diploid cell entering meiosis thus produces four haploid cells (from Alberts *et al.*, 1989).

Synaptonemal complexes (SCs)

The SC is a proteinaceous, zipper-like structure which holds the homologues in close apposition along their entire length during meiotic prophase (von Wettstein *et al.*, 1984) (Figure 2a). At the beginning of meiotic prophase, after premeiotic S-phase, the two sister chromatids develop a single proteinaceous axis, called axial element. As meiotic prophase proceeds, these axial elements are connected along their entire length by numerous thin, transverse filaments (TFs). Between the axial elements resides a third longitudinal structure, referred to as the central element (CE). The two axial elements together with the CE make up the so-called tripartite SC structure. Within the context of the SC, the axial elements are also called lateral elements (LEs) of the SC. Homologues are called synapsed if they are connected by the tripartite structure. The tripartite SC structure is conserved among sexually reproducing eukaryotes (Dresser and Giroux, 1988; Schmekel *et al.*, 1993; Schmekel and Daneholt, 1995), although there is much variation in both CE structure and LE shape (von Wettstein *et al.*, 1984).

A schematic overview of the (dis)assembly of SCs and the concurrent chromosome rearrangements is shown in Figure 2a and 2b. The successive stages of meiotic prophase are defined by the stages of SC (dis)assembly (Figure 2a): during leptotene, a single axial element is formed between the sister chromatids; during zygotene, the axial elements of homologues are connected by TFs, and the CE appears in synapsed regions. During pachytene, the homologues are synapsed from telomere to telomere. In diplotene, the tripartite structure starts to disassemble; in mammals the TFs disappear first (von Wettstein *et al.*, 1984). At the end of diplotene and the onset of diakinesis, the LEs have disappeared and the two scaffolds of the individual sister chromatids (shown in Figure 2b) become discernible as the now recombined chromosomes condense further. The chiasmata become visible as the results of reciprocal exchange between two non-sister chromatids of homologues.

In metaphase I, the nuclear envelope disappears and the chromosomes condense further while the centromeres are attached to the spindle and chromosome pairs (bivalents) orient themselves in preparation of chromosome disjunction.

Recombination nodules

During the assembly of SCs, electron-dense spherical or ellipsoidal structures with a diameter of 100-200 nm are formed, the so-called recombination nodules (RNs)

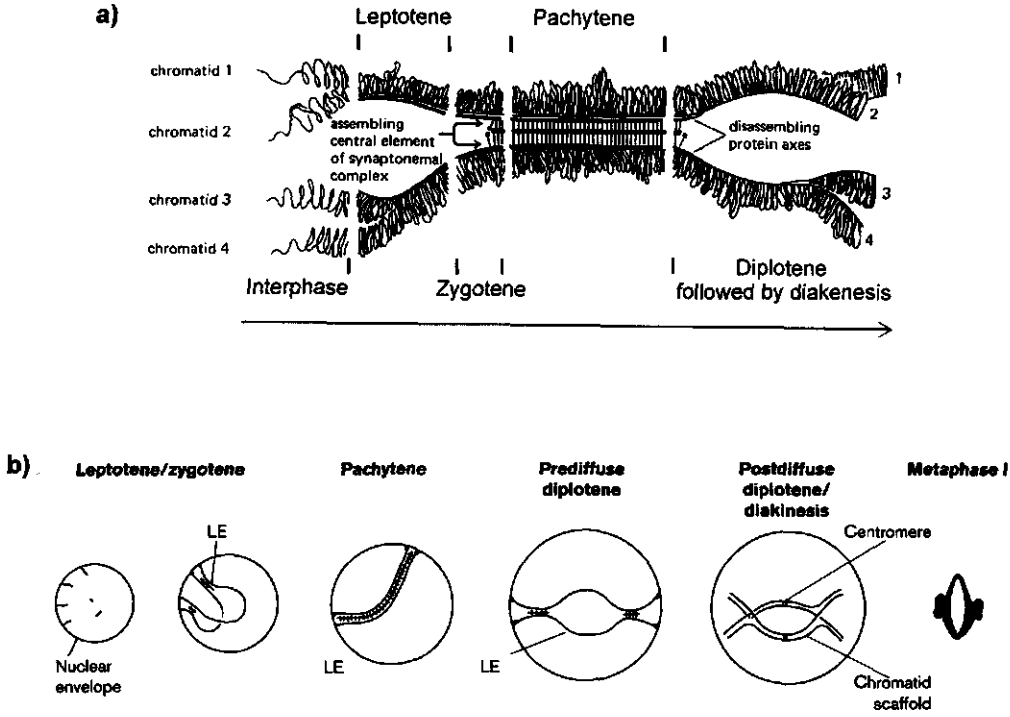

Figure 2. **a)** A schematic figure showing the successive stages of meiotic prophase as defined on the basis of morphological changes of the SC: leptotene (proteinaceous axes start to form along the homologous chromosomes), zygotene (start of synapsis of the homologues), pachytene (synapsis is completed and the SC extends from telomere to telomere), diplotene (SC disassembles) and diakinesis (SCs are completely disassembled) (from Alberts *et al.*, 1989). **b)** Schematic diagram showing the events in a single chromosome pair in a meiotic prophase nucleus. The circles represent the nuclear envelope. In leptotene/zygotene, pachytene and prediffuse diplotene, the axial cores/LEs of the SC are represented (indicated by LE); the axial cores/LEs are probably localized between the sister chromatids (not shown). In the postdiffuse nucleus, the scaffolds (longitudinal supporting structures) of the sister chromatids are shown. In leptotene phase, the assembly of the axial cores begins mainly at the nuclear envelope. In the zygotene phase, axial cores are connected by TFs to form SCs, which are represented in this figure by zipper-like stretches. Synapsis is complete in the pachytene phase. The telomeric ends of the axial cores are attached to the nuclear envelope and in the zygotene phase these ends are clustered in a so-called bouquet arrangement. During pachytene, the telomeric ends have spread out but remain attached to the nuclear envelope. In the prediffuse diplotene phase the SCs are disassembled and in postdiffuse diplotene/diakinesis phase, the axial cores/LEs have completely disappeared whereas the scaffolds of the sister chromatids become discernible. In addition to this, the telomeres are detached from the nuclear envelope. In metaphase I, the nuclear envelope has disassembled and the chromosomes have condensed further. Moreover, the chromosomes are deformed by the attachment of the centromeres to the spindle (the spindle is not shown in this figure), and due to the compaction of the chromosomes the scaffolds of the sister chromatids are no longer discernible (from Heyting, 1996).

(Carpenter, 1975; 1994). Two types of RNs can be distinguished: early and late RNs. Early RNs appear during leptotene and zygotene along unpaired axial cores of the homologues and on top of the tripartite structure in late zygotene/early pachytene (Figure 3). The late RNs occur in early and mid-pachytene (Carpenter, 1994) and are located on top of the tripartite SC structures. The early nodules are formed in greater numbers than late nodules (Stack and Anderson, 1986; Carpenter, 1987). The frequency and distribution of the late nodules correlates with those of reciprocal exchange (crossing-over events) and chiasmata (Zickler *et al.*, 1992; Carpenter, 1994; Sherman and Stack, 1995). In several organisms, including *Sordaria* (Zickler *et al.*, 1992) and tomato (Havekes *et al.*, 1994), the position of late nodules almost completely coincides with the synapsis initiation sites. In other species, however, this is not always observed (Rasmussen *et al.*, 1978). The late nodules are presumed to mark the sites of crossing-over and to contain the protein complexes responsible for the reactions that result in reciprocal recombination.

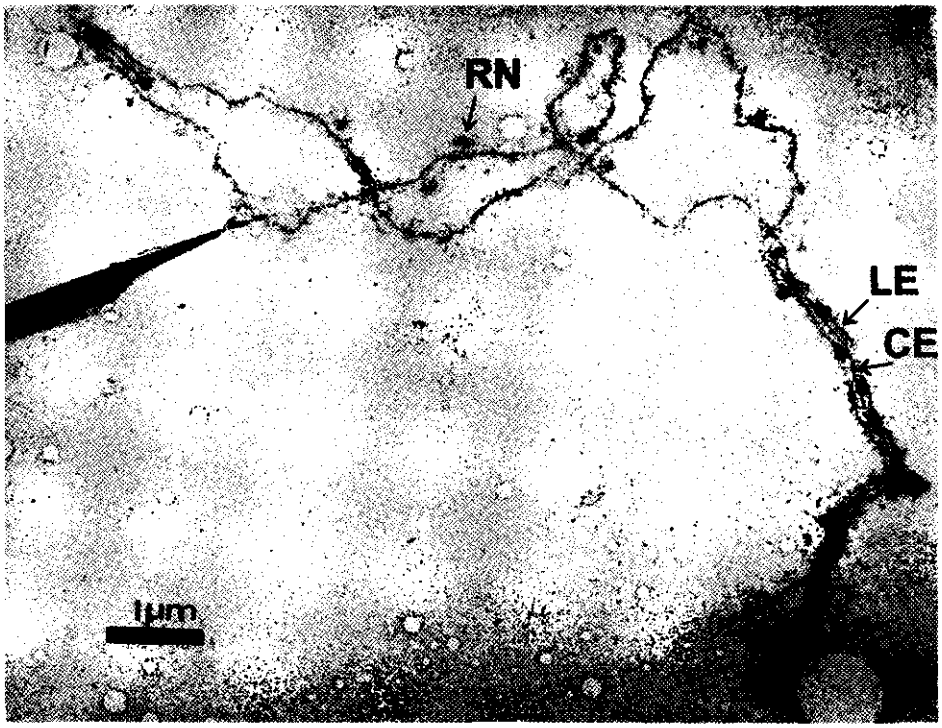

Figure 3. Agar filtrate of an isolated zygotene SC from tomato. CE, central element; LE, lateral element; RN, recombination nodule. Bar represents 1 µm. (From Heyting *et al.*, 1994).

The early RNs are supposed to be involved in homology search (Carpenter, 1994). It is possible that they leave gene conversions as footprints of their activity (Carpenter, 1987). It has been proposed that at the prospective crossover sites, the early nodules turn into late nodules (Sherman and Stack, 1995), but this still has to be established.

Composition of SCs

Several structural components of SCs have recently been identified and characterized. Table II summarizes the meiosis-specific structural components of SCs. SC components from rodents (Heyting *et al.*, 1987; 1989) and from lilly (Anderson *et al.*, 1994) were identified in preparations of isolated SCs and localized within SCs by immunofluorescence and immunogold labeling. In *Saccharomyces cerevisae*, several putative SC components were identified by cytological analysis of meiotic mutants and tentatively localized to the SC by immunofluorescence (Roeder, 1995).

In rodents, two components of the LEs have been characterised so far, namely SCP2 (synaptonemal complex protein 2) and SCP3(COR1) (Offenberg, 1993; Lammers *et al.*, 1994); and two components of the central region, namely SCP1(SYN1) (chapter 2 of this thesis) and an unnamed 48 kDa protein (Smith and Benavente, 1994). The cDNAs encoding SCP1 (this thesis), SCP2 (Offenberg, 1993) and SCP3 (Lammers *et al.*, 1994) have been cloned. In yeast, Red1 and Hop1 proteins are probably associated with the axial elements (Rockmill and Roeder, 1990; Hollingsworth *et al.*, 1990) but this still has to be established by immunogold labeling. The yeast Zip1 protein is most likely a component of the TFs (Sym *et al.*, 1993; Sym and Roeder, 1995). SCP1, SCP2 and SCP3 show no homology with already known yeast SC components; however SCP1 and Zip1p are similar at the secondary structural level.

One other meiosis-specific protein which is found in close contact with the SC is the yeast Dmc1 protein. Dmc1p is homologous to the RecA protein of *E. coli* (Story *et al.*, 1992). Dmc1p is exclusively localized in the nucleoplasm of yeast meiotic prophase cells; it occurs in foci which often colocalize with Zip1p (Bishop, 1994). The lilly protein Lim15p, which is homologous to Dmc1p of yeast, shows a similar localization pattern (Terasawa *et al.*, 1995). The meiosis-specific yeast Msh4p, a homologue of the *E. coli* MutS-protein, localizes on discrete foci on top of the synapsed tripartite SC structures (Ross-Macdonald and Roeder, 1994).

Table II **Meiosis-specific synaptonemal complex components**

Component	First identified in	Gene or cDNA cloned ?	Mol weight*	Localization within SC	Remark	References
SCP1(SYN1)	Rat	Yes	117 000 Da	Central region	TF component ?	a) b)
SCP2	Rat	Yes	170 000 Da	Axial core	Associated with synapsed + unsynapsed axial cores	c)
SCP3(COR1)	Rat	Yes	30 000 Da	Axial core	Associated with synapsed + unsynapsed axial cores	b) d)
Unnamed	Rat	No	48 000 M_r	Central region	ND[0]	e)
Unnamed	*Lilium*	No	52 000-70 000 M_r	Axial core	Synapsed + unsynapsed	f)
Zip1p	Yeast	Yes	110 000 Da	Central region	TF component ?	g)
Hop1p	Yeast	Yes	70 000	Axial core	Associated with core	h)
Red1p	Yeast	Yes	90 000 Da	Axial core	Associated with core	i)

[0] ND, no published data. * Shown as M_r (relative elctrophoretic mobility) or Da (molecular weight predicted from nucleotide sequence of cDNA or gene). References are: a) Chapter 1, this thesis; b) Dobson *et al.*, 1994; c) Offenberg, 1993; d) Lammers *et al.*, 1994; e) Smith and Benavente, 1994; f) Anderson *et al.* 1994; g) Sym *et al.*, 1993; h) Hollingsworth *et al.*, 1989; i) Thompson and Roeder, 1989.

Several proteins that are not meiosis-specific also localize in the vicinity of SCs. Topoisomerase II is associated with SCs, particularly in the late stages of meiotic prophase (Moens and Earnshaw, 1989); Rap1p (Klein *et al.*, 1992) can be detected at the telomeric ends of yeast meiotic prophase chromosomes; the yeast Rad51p (a RecA homologue which is not meiosis specific) colocalizes with Dmc1p (Bishop, 1994) and the lilly Rad51p colocalizes with Lim15p (Terasawa *et al.*, 1995). Recently, it has been demonstrated that Rad51 makes part of early nodules (Anderson *et al.*, in prep.).

Especially the RecA-like proteins such as Rad51p and Dmc1p could play an active role in the DNA recombination process within the context of the SC structure.

Subject of this thesis

This thesis deals with the structure and function of SCP1, a TF protein of rat SCs. It describes the isolation of the cDNA and the characterization of the gene product, SCP1. The main question on which this thesis is focused, concerns the role of SCP1 within the SCs. However, before we can address this question, we need to discuss the current understanding of the possible functions of the SC structure.

Possible functions of the SC

Traditionally, SCs were considered as structures which create the preconditions for correct recombination between homologous chromosomes during meiosis I (von Wettstein *et al.*, 1984). According to this view, pairing of the homologues depends upon SC formation, and recombination is exclusively initiated in the context of the tripartite SC-structure. However, three major lines of evidence have emerged against this view:

I) *Pairing of homologues and presynaptic alignment occur in the absence of an SC.*

In situ hybridization experiments have shown that in yeast homologous chromosome pairing is initiated before SC assembly (Scherthan *et al.*, 1992; Weiner and Kleckner, 1994; Xu and Kleckner, 1995). Furthermore, pairing of homologous chromosomes occurs to some extent in yeast mutants that are defective in SC formation (Loidl *et al.*, 1994; Weiner and Kleckner, 1994; Nag *et al.*, 1995). In man and mouse, combined FISH analysis and immunolabeling of SC components in the same meiotic prophase cells also demonstrated that the first stages of pairing of homologous chromosomes precede SC-formation (Scherthan *et al.*, 1996).

II) *Recombination in yeast is initiated before the onset of SC assembly.*

In yeast, most or all of the meiotic recombination events are initiated by double strand DNA scission (Nicolas *et al.*, 1989; Sun *et al.*, 1989; Cao, *et al.*, 1990; Wu and Lichten, 1994). At the so-called hotspots of meiotic recombination in the yeast genome, meiotically induced double-strand DNA breaks (DSBs) can be detected at the beginning of meiotic prophase (Zenvirth *et al.*, 1992; Lichten and Goldman, 1995). Careful analysis of meiotic events at the DNA and chromosomal level in synchronized

populations of meiotic yeast cells showed that the DSBs are induced before the onset of synapsis (Padmore *et al.*, 1991).

III) *Meiotic levels of recombination can occur in the absence of an SC.*

This is illustrated by at least two species, namely *Schizosaccharomyces pombe* (the fission yeast) and *Aspergillus nidulans* (a filamentous fungus), which do not assemble a morphological recognizable SC, but display a high level of meiotic recombination (Egel-Mitani *et al.*, 1982; Munz, 1994; Kohli and Bähler, 1994; Egel, 1995). Furthermore, *zip1* mutants of *S. cerevisae* assemble full-length aligned axial cores, which are not connected by TFs (Sym *et al.*, 1993). In some yeast strains, these mutants produce viable spores with a nearly wildtype frequency of recombination (Sym and Roeder, 1994; Storlazzi *et al.*, 1996). However, in contrast to wildtype yeast and other species, neither *zip1* mutants nor *S. pombe* nor *A. nidulans* display crossover interference (Kafer, 1977; Egel-Mitani *et al.*, 1982; Sym and Roeder, 1994; Munz, 1995). The *red1* mutant of yeast is another example of a yeast mutant which does not assemble SCs, but still displays meiotic levels of recombination (Rockmill and Roeder, 1990).

It was thus concluded, that SCs are not required for the initial stages of homology recognition and recombination (Kleckner *et al.*, 1991). However, if SCs are not involved in these processes, what else could be their function ?

Recent evidence combined with already known genetic data from various organisms, have thoroughly changed the view on SC functions during the meiotic prophase (Hawley and Arbel, 1993). It now seems more likely that SCs serve to adapt the homologous recombination mechanism in such a way that it contributes to proper chromosome disjunction at metaphase I. SCs are supposed to do this in the following ways:

(a) They regulate the number and distribution of reciprocal exchanges (crossovers) along bivalents (paired homologous chromosomes) and guarentee at least one crossover per bivalent. Crossover interference can be considered as a manifestation of such a regulatory mechanism (Egel, 1978; King and Mortimer, 1990; Egel, 1995).

(b) They convert reciprocal exchanges at the DNA level into functional chiasmata, i.e., structures that provide the tight physical contact between the homologues which enables the balancing of poleward forces on both homologues, and thus ensures the

proper orientation of bivalents in the metaphase I spindle (Figure 4) (Maguire, 1974; Maguire, 1995).

(c) They prevent recombination between sister chromatids and/or enhance recombination with a chromatid of the homologue (Schwacha and Kleckner, 1994).

Ad (a) SCs are involved in crossover interference

Studies in various eukaryotes revealed that the distribution of meiotic crossovers along homologues is non-random in two respects (Mortimer and Fogel, 1974). Firstly, two crossovers rarely occur close together on one chromosome arm. This reduction in the probability of a nearby second crossover, which is distance-dependent, is often referred to as positive interference (Muller, 1916). Interference can be quantitated by calculation of the coefficient of coincidence (C.O.C.), which equals the ratio of observed to expected number of COs in two linked chromosomal regions (Weinstein, 1918). Interference equals 1- C.O.C. Secondly, each pair of homologous chromosomes performs at least one reciprocal exchange, the so-called obligate crossover (Carpenter, 1994).

Several models have been proposed to explain crossover interference (Stam, 1979; King and Mortimer, 1990; Foss *et al.*, 1993; Kleckner, 1996), and at least one of these models assumes the involvement of SCs in crossover interference (King and Mortimer, 1990). Some of these models will be discussed in detail in Chapter 6. The obligate crossover in each bivalent is probably due to a meiotic checkpoint at which meiotic cells are stalled until at least one initiated recombination event on each bivalent has been resolved as a crossover (Roeder, 1995; Tishkoff *et al.*, 1995).

Ad (b) SCs contribute to the formation of stable chiasmata

Reciprocal recombination has a dual role in meiosis: it contributes to the genetic diversity among the products of meiosis, and it is indispensable for chromosome disjunction (Figure 4).

However, recombination *per se* is not sufficient to ensure chromosome disjunction.

From Figure 4 it is clear that a crossover by itself cannot hold the homologues together. It is also necessary to prevent that the connection provided by the crossover is lost because of sliding of the sister chromatids along each other. Two mechanisms have been proposed to accomplish this (Carpenter, 1994; Maguire, 1995): either the sliding of the sister chromatids is prevented at the position of the chiasma by a

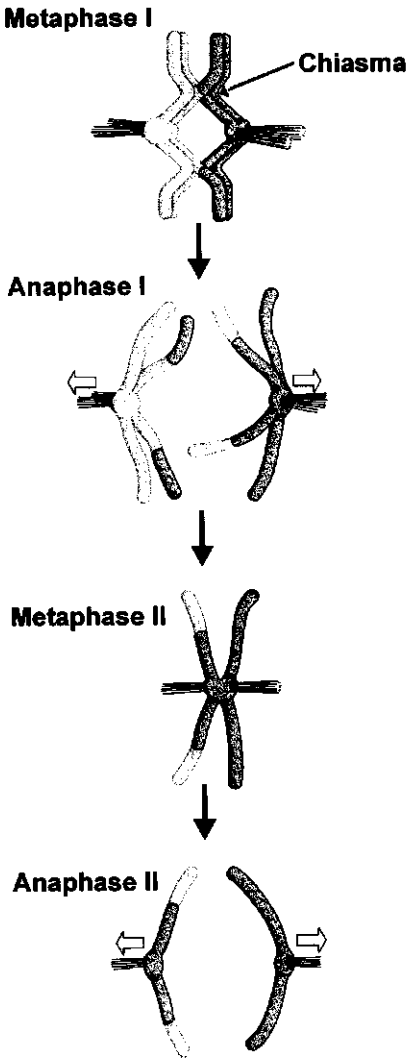

Figure 4. Schematic overview showing the chromosome alignment (at metaphase) and segregation (anaphase) during meiotic division I and II. In metaphase I, the homologous chromosomes are aligned and still connected due to two crossovers. The chiasma is the visible result of a crossover event between two non-sister chromatids of both homologues. Besides the chiasmata, the sister chromatids and the centromeres are attached to each other whereas the kinetochore fibers of both centromeres point in the same direction. Note that in this situation a stable chiasma is needed to withstand the poleward forces on the centromeres, and thus enables the proper alignment of both homologous in the metaphase I spindle, which then can result in a correct chromosome disjunction. In anaphase I, the sister chromatids of each homologue are only connected by their centromeres while each homologue is separated towards an opposite pole. In metaphase II, the kinetochores are reoriented and the kinetochore fibres of the sister chromatids point in opposite directions. Like in mitosis, the centromeres hold the sister chromatids together and ensure their alignment in the metaphase II spindle. After this, disjunction of the sister chromatids towards opposite poles can be performed in anaphase II like in mitosis. (Adapted from Alberts *et al.*, 1989).

"chiasma binder"; or the sister chromatids cohere distal to the chiasma (Figure 4). It is possible that components of the lateral elements of SCs have a role in chiasma maintenance, by providing sister chromatid cohesion. In rodents, the lateral element component SCP3 (COR1) persists between the sister chromatids long after the SCs have disassembled, until anaphase I (Dobson *et al.*, 1994). SCP3 thus behaves as a protein involved in sister chromatid cohesion (Moens and Spyropoulos, 1994).

The *ord1* mutant of *Drosophila* has a high level of meiotic recombination, but nevertheless, it shows chromosome non-disjunction and premature sister chromatid separation at metaphase I (Miyazaki and Orr-Weaver, 1992; 1994). It is thus possible that the Ord1 gene product is also involved in chiasma maintenance by sister chromatid cohesion (Sekelsky *et al.*, 1995; Bickel *et al.*, 1996). The *rec8* mutant of the fission yeast *Schizosaccharomyces pombe* provides another example of a mutant which is possibly disturbed in meiotic sister chromatid cohesion: *S. pombe* does not assemble tripartite SCs, but instead axial core-like linear elements are formed on the meiotic chromosomes (Bähler *et al.*, 1993). The *rec8* mutant is disturbed in linear element assembly, and although recombination is somewhat reduced, chromosome non-disjunction due to the lack of crossovers is very limited (Molnar *et al.*, 1995). Most of the chromosomal missegregations in *rec8* mutants are caused by premature sister chromatid separation (Lin *et al.*, 1992; Molnar *et al.*, 1995). It is thus possible that the rec8 gene product is essential for linear element formation and for establishment of sister chromatid cohesiveness during meiotic prophase of *S. pombe* (Molnar *et al.*, 1995).

Support for the existence of a local "chiasma binder" is provided by desynaptic *dy1* and *dsy1* mutants of maize, which assemble normal SCs and display (near) normal levels of meiotic crossing over, but display a high level of non-disjunction at meiosis I (Maguire, 1978; Maguire *et al.*, 1991; 1993). Both mutants have SCs with normal morphology except for a greater width of the central region of the SC compared to wildtype controls (Maguire *et al.*, 1991; 1993). It is not known whether the two mutants are allelic (Maguire, 1995).

In the *med1* (*dmc1*) mutants of yeast, meiotic crossing over is reduced to 20-50% of the wildtype level (Bishop *et al.*, 1992; Rockmill and Roeder, 1994). Chromosome non-disjunction and precocious sister chromatid separation are both increased in these mutants (Rockmill and Roeder, 1994).

Furthermore, the increased non-disjunction level cannot be completely accounted for by the decreased number of crossovers in the *med1* mutant: even recombined chromosomes fail to disjoin (Rockmill and Roeder, 1994). The product of MED1 (DMC1) is a RecA homologue (see above) which is involved in heteroduplex formation (Nag *et al.*, 1995). It is possible that heteroduplex formation by Dmc1p enhances initiation of synapsis and contributes to the proper instalment of LEs to provide sister chromatid cohesion. In such a model, Dmc1p functions both as a local chiasma binder and an enhancer of sister chromatid cohesion.

Furthermore, YACs containing human genomic fragments often display a low recombination frequency and premature separation of sister chromatids at meiosis I of yeast (Sears *et al.*, 1992). Implantation of a yeast recombination hotspot into such a YAC had two effects: recombined YACs disjoined properly, and premature sister chromatid separation decreased. Possibly the recombination initiation sites serve as nucleation sites for the assembly of stable LEs, which then provide sister chromatid cohesion (Sears *et al.*, 1994).

Ad (c) SCs prevent recombination between sister chromatids.

Meiotic reciprocal recombination occurs preferentially between non-sister chromatids of homologous chromosomes, whereas the sister chromatid is preferred for mitotic exchange (Kadyk and Hartwell, 1992). It has been proposed (Lammers *et al.*, 1994; 1995) that components of the lateral elements of SCs regulate meiotic recombination between sister chromatids. SCP3, which is a component of the lateral elements of rat SCs, is a phosphoprotein, of which the phosphorylation pattern changes in mid-pachytene (Lammers *et al.*, 1995). Presumably, this event marks a functional switch of the LEs. Possibly, LEs prevent intersister interactions in the first half of pachytene, whereas this control is relaxed in the second half of pachytene, in order to make the sister chromatid available as a template for the repair of those initiated recombination events for which the homologous chromosome did not provide a template.

Furthermore, the *hop1* mutant of yeast does not assemble LEs or SCs, and has a strongly reduced frequency of meiotic recombination between homologues (Hollingsworth *et al.*, 1989; Byers and Hollingsworth, 1994). In contrast, the formation of recombination intermediates between sister chromatids is increased in this mutant (Schwacha and Kleckner, 1994). The Hop1 protein has been localized to

synapsed and unsynapsed segments of the meiotic prophase chromosomes (Hollingsworth *et al.*, 1990), and is possibly a lateral element component.

To summarize, synaptonemal complexes appear to modify the homologous recombination process in such a way that a correct reductional chromosome segregation is enhanced. It is possible that components of the lateral elements convert crossovers into stable chiasmata, provide sister chromatid cohesiveness, and prevent recombination between sister chromatids. The transverse filaments are possibly involved in a regulatory mechanism that causes crossover interference, and ensures at least one crossover per bivalent.

At the Department of Genetics (WAU), the function of SCs is analysed by a biochemical approach, with rat and tomato as major research organisms. Surprisingly, none of the SC-components that have been identified by this approach show homology to putative SC-components in other organisms (Table I), although one component, SCP1 of the rat, is similar at the secondary structural level to Zip1p of yeast.
The experiments presented in this thesis are focused on the structure and function of SCP1, a TF component of rat SCs. The cytological and biochemical characterization of this protein provides a basis for speculations about how SCP1 could function within the SC.

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CHAPTER 2

A coiled-coil related protein specific for synapsed regions of meiotic prophase chromosomes

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A coiled-coil related protein specific for synapsed regions of meiotic prophase chromosomes

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Synaptonemal complexes (SCs) are structures that are formed between homologous chromosomes during meiotic prophase. They are probably involved in chromosome pairing and recombination. Using a monoclonal anti-SC antibody we isolated cDNAs encoding a major component of SCs which is localized specifically in synapsed segments of meiotic prophase chromosomes. The protein predicted from the nucleotide sequence of a full-length cDNA, named SCP1, consists of 946 amino acid residues and has a molecular weight of 111 kDa. It shares several features with nuclear lamins and some recently identified nuclear matrix proteins. The major part of SCP1 consists of long stretches capable of forming amphipathic α-helices. This region shows amino acid sequence similarity to the coiled-coil region of myosin heavy chain. A leucine zipper is included in this region. The carboxy-terminus has two small basic domains and several S/T-P-X-X motifs, which are characteristic of DNA-binding proteins. One of these motifs is a potential target site for p34^{cdc2} protein kinase. The amino-terminus is acidic and relatively proline-rich, but does not contain the S/T-P-X-X motif. The transcription of the gene encoding SCP1 is restricted to zygotene–diplotene spermatocytes. A polyclonal antiserum raised against the fusion protein of one of the cDNA clones recognizes a single protein on Western blots of isolated SCs, with an electrophoretic mobility identical to that of the antigen recognized by the original monoclonal antibody (mAb), IX5B2. From a detailed comparison of the immunogold labelling of rat SCs by mAb IX5B2 and the polyclonal anti-fusion protein antiserum respectively, we tentatively infer that the carboxy-terminus of SCP1 is orientated towards the lateral elements and that the other domains of the protein extend towards the central region between the lateral elements. We conclude that SCP1 is the major component of the transverse filaments of SCs, and speculate that it has evolved by specialization of a nuclear matrix protein.

Key words: cDNA/meiosis/protein/synaptonemal complex

Introduction

Meiosis plays a central role in the life cycle of sexually reproducing eukaryotes. Its two major effects are to reduce the ploidy level and to generate new combinations of genes. Both effects are accomplished during the first of two meiotic divisions through a series of chromatin rearrangements by which homologous chromosomes pair, recombine, condense and segregate. The rearrangements of meiotic prophase chromatin are accompanied by the assembly and disassembly of meiosis-specifc nuclear organelles, the synaptonemal complexes (SCs) (reviewed in Von Wettstein *et al.*, 1984). Early in meiotic prophase (leptotene), proteinaceous axes are formed along the chromosomes; the axes of homologous chromosomes (homologues) are subsequently connected by thin, transverse filaments, and another longitudinal structure, the central element, appears on the transverse filaments between the axes (zygotene). The central element together with both axes make up the tripartite structure of the SC. Axes are called lateral elements (LEs) where they make part of this tripartite structure. During the pachytene stage of meiotic prophase, homologues are connected (synapsed) by the tripartite structure along their entire length, and the SC extends as a flat, zipperlike structure from telomere to telomere. During diplotene the SCs are disassembled and the now recombined (Padmore *et al.*, 1991) chromosomes condense further in preparation of metaphase I.

It seems likely that SCs are essential for the proper progress of meiotic prophase, because the morphological alterations of SCs closely match the successive rearrangements of chromatin, and because the SC structure has been conserved almost universally among sexually reproducing eukaryotes. Nevertheless, as yet no functions have been assigned with certainty to SCs. Information about possible SC functions comes from three different experimental approaches, namely the analysis of mutants with a defect in meiosis, the determination of the order of events during meiotic prophase, and the biochemical analysis of purified SCs.

Several mutants with a defect in meiosis have been described, and many of these also have a defect in SC assembly or disassembly (Baker *et al.*, 1976; Esposito and Klapholtz, 1981; Gulobovskaya, 1989; Roeder, 1990; Curtis and Doyle, 1991; Maguire and Riess, 1991; Zickler, 1991). It has not been possible to identify SC functions from the analysis of these mutants because the observed defects in SC (dis)assembly could be a cause as well as an effect of the disturbance of meiosis. However, what we do know from these analyses is that SCs are not essential for meiotic levels of recombination. Certain mutants of yeast with reduced spore viability display normal or appreciable levels of correct meiotic reciprocal exchange, but fail to assemble the tripartite structure (Engebrecht and Roeder, 1989; Rockmill and Roeder, 1990).

Padmore *et al.* (1991) analysed the order of events at the DNA level and at the level of chromatin organization in cultures of yeast, which progressed carefully synchronized through meiotic prophase. From this study it appears that the tripartite structure has no function in the initial events

at the DNA level antecedent to recombination. One of the presumed earliest events at the DNA level, namely site-specific double-strand scission (Nicolas *et al.*, 1989), occurs prior to or concomitant with the appearance of the tripartite structure of SCs (Padmore *et al.*, 1991). From earlier cytological work it even appears that the tripartite structure itself is insensitive to homology in early as well as later stages of meiotic prophase (see reviews by Von Wettstein *et al.*, 1984; Carpenter, 1987; Loidl, 1990 and Kleckner *et al.*, 1991).

We have chosen to approach the question about SC functions by biochemical analysis of SCs. For this purpose we developed a procedure to isolate SCs from rat spermatocytes (Heyting *et al.*, 1985; Heyting and Dietrich, 1991), elicited monoclonal anti-SC antibodies (Heyting *et al.*, 1987, 1989) and, using these antibodies, identified four major components of SCs (Heyting *et al.*, 1987, 1989; Moens *et al.*, 1987; Offenberg *et al.*, 1991). From these analyses we know that SCs are not assembled by rearrangement of the nuclear matrix or nuclear lamina, but that their major components are newly synthesized during meiotic prophase. Apparently, the chromatin has to reorganize at the onset of meiosis onto a new, meiosis specific structure, but for what purpose this occurs we still do not know.

Not only the function of SCs but also the regulation of their (dis)assembly and their phylogenetic origin are enigmas. Elucidation of the regulation of SC (dis)assembly may provide insight into the coordination of events at different levels of integration in the meiotic prophase cell. The phylogenetic origin of SCs or SC components may provide clues to the evolutionary origin of the whole process of meiosis. In this paper we describe the isolation cDNAs encoding a M_r 125 000 protein of rat SCs. This protein is localized specifically in tripartite segments of SCs, between the lateral elements (Heyting *et al.*, 1989; Dietrich *et al.*, 1992). The protein predicted from the nucleotide sequence, called SCP1, has several interesting features. The major part is similar to proteins that are capable of forming α-helical coiled-coil dimers, such as the S2 domain of myosin heavy chain, or tropomyosin. The carboxy-terminus is enriched in the S/T-P-X-X-motif, which is characteristic of DNA-binding proteins, and has two small basic domains. The protein has several potential phosphorylation sites that could play a role in SC assembly and disassembly. SCP1 is probably the major component of transverse filaments of SCs.

Results

Isolation of cDNAs encoding a major component of SCs

Using a pool of five independently isolated monoclonal antibodies (mAbs), each of which recognizes a M_r 125 000 SC protein (Heyting *et al.*, 1989), we screened $\sim 10^6$ recombinant phage of an expression cDNA library of poly(A)$^+$ RNA from testes of 28 day-old rats. About 1 in 10^4 clones were recognized by this pool. For 55 of these clones we analysed which of the mAbs could recognize their recombinant gene product, and in all cases this turned out to be a single mAb, IX5B2 (described in Heyting *et al.*, 1989 and Offenberg *et al.*, 1991). Three clones with colinear restriction enzyme fragment maps were selected for further analysis

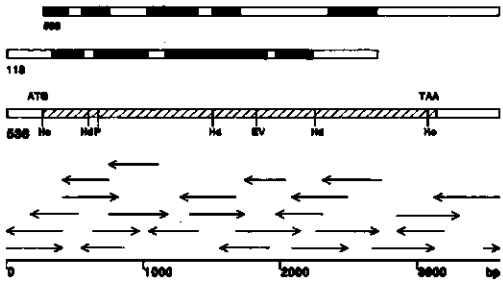

Fig. 1. Restriction map of SCP1 cDNA and sequencing strategy. Arrows indicate the length and direction of the sequence determined of clone 536. For clone 536, the hatched segment indicates the coding region. For clones 566 and 113, the black segments were sequenced as controls for the sequence of clone 536. Hc, *Hin*cII; Hd, *Hin*dIII; EV, *Eco*RV; P, *Pst*I.

(Figure 1). A secondary screening with the 5′ *Pst*I fragment (between the *Pst*I site of the insert and that of the polylinker of pBluescript at the 5′ end, see Figure 1) of the insert of clone 536 as a probe did not yield any clones that extended further in the 5′ direction than did clone 536 itself. The nucleotide sequence of clone 536 was determined as described in Figure 1 and in Materials and methods. In addition, parts of the sequence of the independently isolated clones 566 and 113 were determined for verification. No discrepancies between the sequences of different clones were found, except for apparent deletions and duplications at the 3′ end of some cDNA clones, which we interpret as cloning artefacts. Clone 536 contains a single complete open reading frame which is capable of encoding a 111 kDa protein consisting of 946 amino acids (Figure 2). We think that this protein is identical to the SC protein recognized by mAb IX5B2 for the following reasons: first, the predicted molecular weight (111 kDa) and pI (5.5) are in reasonable agreement with the M_r (125 000) and pI (5.5, unpublished observations) of the SC protein recognized by mAb IX5B2. Second, the gene encoding SCP2 is only transcribed in the testis, and within the testis only in those cells that contain the antigen recognized by mAb IX 5B2 (see below). Third, a polyclonal antiserum raised against the fusion protein of clone 536 recognizes a single band on immunoblots of purified SCs; the M_r of the recognized protein is identical to that of the antigen recognized by mAb IX5B2 (Figure 3). Fourth, the SCs are the only structures that this antiserum recognizes in frozen sections of the testis (not shown) or in agar filtrates of lysed spermatocytes (Figure 4) are the SCs. Because the M_r 125 000 antigen that is recognized by mAb IX5B2 and by the polyclonal anti-fusion protein antiserum is a major component of SCs (Heyting *et al.*, 1989; Offenberg *et al.*, 1991; Figure 3, lane a), we conclude that we have isolated cDNAs encoding a major component of SCs of 111 kDa. For this we propose the name SCP1 (*s*ynaptonemal *c*omplex *p*rotein 1).

SCP1 contains a large domain with similarity to coiled-coil segments of other proteins

The complete nucleotide sequence of cDNA clone 536 is shown in Figure 2, together with the amino acid sequence of the encoded protein. The first ATG codon is found at nucleotide 262. At the 3′ end there is a sequence (nucleotides

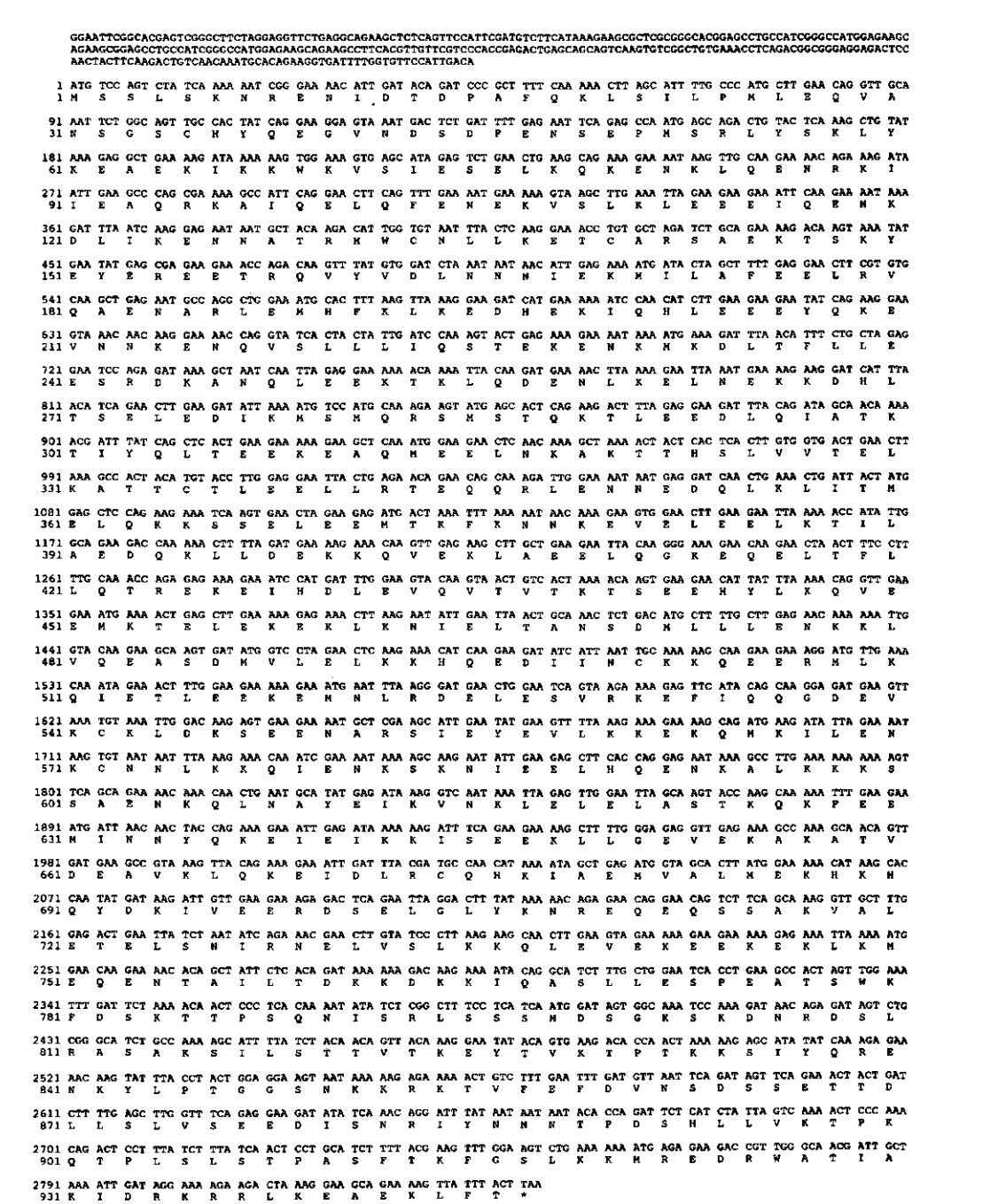

Fig. 2. Nucleotide sequence and predicted amino acid sequence of SCP1. The predicted translation product is shown below the nucleotide sequence.

3380–3480) that is specific for the rat (Barta *et al.*, 1981; Yavachev *et al.*, 1986) and that occurs adjacent to several diverse rat genes. No other homologies were detected at the nucleotide sequence level. At the 3′ end there is an untranslated region of 459 nucleotides, with a potential polyadenylation signal, AATAAA, at position 3206.

The protein encoded by the open reading frame is rich in charged amino acids, particularly lysine (14.5%) and glutamine (16.3%). A large segment of the predicted protein, SCP1, namely amino acid residues 52–752, shows amino acid sequence similarity to the S2 fragment of several types of myosin heavy chain. The highest similarity was observed with myosin heavy chain of human embryonic fast skeletal muscle (16.5% identity in a 689 amino acid overlap). Other proteins that showed sequence similarity to SCP1 include keratins, lamin B2, dystrophin and tropomyosin. All of these proteins are (predicted to be) capable of forming long coiled-coil dimers consisting of two amphipathic α-helices which associate through hydrophobic interaction. The amphipathic character of the α-helices arises by the regular spacing of hydrophobic amino acid residues in a so-called heptad repeat pattern; this is a repeat of a seven amino acid residue sequence with a strong enrichment of hydrophobic residues at the first and fourth of the seven positions. Because seven consecutive residues will form two turns of an α-helix, the residues at positions 1 and 4 will form a hydrophobic ridge on one side of the molecule (McLachlan and Karn, 1982). Figure 5 shows the α-helical regions of SCP1, predicted according to Chou and Fasman (1978). The region that shows similarity to myosin (residues 52–752) is predicted to have an α-helical structure, with some interruptions. Within this region the heptad repeat pattern is clear: if single residues are skipped at positions 192, 543 and 709, the pattern strength for the heptad repeat, P7, for this domain is 28.55 (McLachlan, 1977); this value is highly significant ($P << 0.001$) and is comparable to values obtained for other coiled-coil proteins (Beavil *et al.*, 1992). The heptad repeat has a number of small interruptions between residues 344 and 358, 361 and 372, 569 and 579, 590 and 607 and 710 and 720; these do not contain proline residues, however, and do not disturb the heptad repeat frame. They will probably not interrupt the coiled-coil structure. Thus, a 700 amino acid stretch of SCP1, from residue 52 to residue 752, is expected to be capable of forming a coiled-coil structure. This stretch would be ~100 nm long (Creighton, 1984).

The amino- and carboxy-terminal domains that flank this structure are 52 and 194 amino acids long, respectively. All of the proline residues fall into these two domains. In the carboxy-terminal domain, seven out of eight proline residues are preceded by a serine or threonine residue. This S/T-P

Fig. 3. Immunoblot of proteins of rat SCs, probed with mAb IX5B2 (lane b), a polyclonal antiserum, elicited against the fusion protein of cDNA clone 536 (lane c) and the pre-immune serum (lane d). Lane a shows the Coomassie-blue stained gel. The M_r 190 000, 125 000 and 33 000 SC proteins are indicated. Samples containing 3×10^7 SCs were loaded onto 2 cm wide slots of a 10% SDS polyacrylamide gel. A 0.4 cm wide strip of each lane was stained with Coomassie blue; the remaining 1.6 cm was blotted onto a nitrocellulose filter. From this filter 0.25 cm wide strips were cut for incubation with anti-SC antibodies.

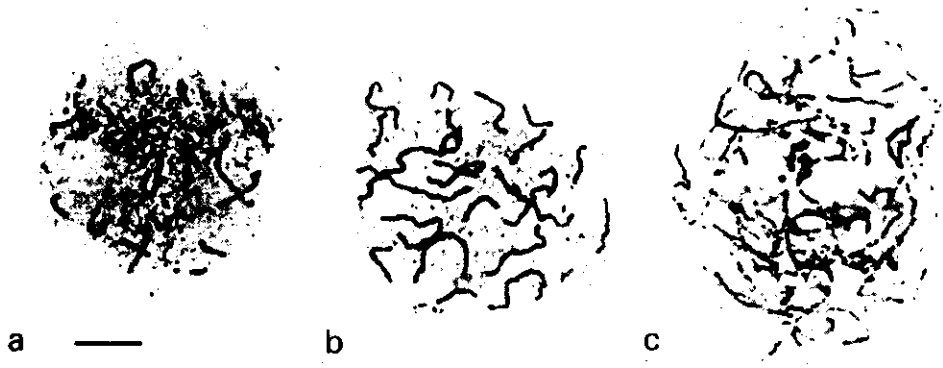

Fig. 4. Indirect immunoperoxidase staining of agar filtrates of lysed spermatocytes with a polyclonal antiserum elicited against the fusion protein of cDNA clone 536. (a) Zygotene stage; note short interruptions in the labelling of the SCs; (b) pachytene stage, arrowhead indicates the XY bivalent with weakly stained unpaired axes; (c) diplotene stage. Bar represents 10 μm.

sequence makes part of the so-called S/T-P-X-X-motif, which is enriched in all sorts of DNA-binding proteins (Suzuki, 1989). It has been proposed that this motif functions as a DNA-binding unit (Suzuki, 1989). The carboxy-terminus is also enriched in S/T-S/T-X-X motifs, which, according to Suzuki (1989), could mimic the conformation of the S/T-P-X-X-motif. Furthermore, the carboxy-terminus contains small basic domains at positions 851–854 and 931–939. At the amino-terminus, none of the proline residues is preceded by serine or threonine. In contrast to the carboxy-terminus, the amino-terminus (residues 1–50) has an excess of acidic residues. The amino acid sequence of SCP1 contains several other interesting motifs. Potential nuclear location signals (consensus K-R/K-X-R/K, where X is any amino acid; Chelsky *et al.*, 1989) are located at positions 67–70 and 560–563 and 851–854. Furthermore, there are several potential target sites for protein kinases. For instance, the sequence K-T-P-T-K (positions 929–934) fits the consensus for the target of $p34^{cdc2}$ protein kinase (Z-S/T-P-X-Z, where X is polar and Z is generally basic; Langan *et al.*, 1989); phosphorylation sites for cAMP/cGMP dependent protein kinases (consensus K/R-K/R-X-S/T, Fremisco *et al.*, 1980) occur at positions 364–367, 597–600, 642–645 and 852–855. In addition there are 19 target sites for protein kinase C (consensus S/T-X-R/K, Kemp and Pearson, 1990) dispersed all over the amino acid sequence of SCP1 (not indicated). Furthermore, there is a leucine zipper motif at positions 341–369, which is out of frame with respect to the heptad repeat, with the leucines at the fifth rather than the first or fourth position of the heptad.

To summarize, SCP1 consists of three domains: a large central domain, capable of forming an α-helical coiled-coil structure; a carboxy-terminal domain that shares features with DNA binding proteins, and has some small, basic domains, and an amino-terminal domain which contains some proline residues and is acidic.

The gene encoding SCP1 is transcribed specifically during meiotic prophase

The M_r 125 000 antigen of mAb IX5B2, which we think is identical to SCP1, occurs exclusively in meiotic prophase cells (Offenberg *et al.*, 1991; Dietrich *et al.*, 1992). We analysed whether expression of the gene encoding SCP1 is regulated at the level of transcription by Northern blot analysis and *in situ* hybridization. Northern blot analysis with the 900 bp *Hind*III fragment of clone 536 (the left *Hind*III fragment in Figure 1) as a probe revealed a single transcription product of 4.2 kb in poly(A)$^+$ RNA from the testis, but not in RNA from other organs (Figure 6). Within the testis the transcript occurs exclusively in zygotene– diplotene spermatocytes (Figure 7). This corresponds to a time span of ~11 days in the development of spermatocytes in the rat. The intensity of the *in situ* hybridization signal correlates with the intensity of the immunofluoresence staining (Figure 7). Apparently there is no long term storage of SCP1 mRNA before meiotic prophase.

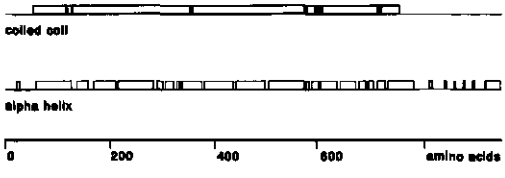

Fig. 5. Secondary structure predictions of SCP1. On the upper bar the predicted coiled-coil domain (amino acids 52–752) is indicated, with the interruptions in the heptad repeat shown in black; these interruptions do not affect the frame of the heptad repeat. The lower bar shows the regions capable of forming α-helices, as predicted by the algorithm of Chou and Fasman (1978). The predicted α-helical domain has short interruptions which also do not affect the frame of the heptad repeat.

Localization of SCP1 within SCs

Figure 4 panels a–c show agar filtrates of lysed spermatocytes after indirect immunoperoxidase staining with a polyclonal anti-SCP1 antiserum. Late zygotene SCs show up as interrupted lines, the interruptions probably representing unpaired segments of SCs (Figure 4a). In some zygotene cells the unpaired axes are also stained weakly (not shown). In pachytene, SCs react more strongly and along their entire length (Figure 4b). In diplotene, paired segments of SCs react as strongly as in pachytene, while the unpaired segments react weakly with this antiserum (Figure 4). The heterologous segments of the X and Y bivalent are also stained weakly. Ultrastructural localization of SCP1 by means of immunogold labelling gave similar results (Figure 8): the paired segments of SCs are labelled heavily from zygotene until diplotene; the unpaired axial elements of zygotene (Figure 8a) and diplotene (not shown) SCs have dispersed clusters of immunogold label; the heterologous segments of the X and Y bivalent also have some dispersed immunogold label on the inner side of the axes (not shown). These results differ in the following aspects from those obtained with mAb IX5B2. First, labelling of unpaired segments of SCs with mAb IX5B2 has never or only rarely

Fig. 6. Transcription of SCP1, analysed by Northern blot hybridization. 20 μg of poly(A)$^+$ RNA from respectively, testis (T), kidney (K), brain (B) and liver (L) was layered per 0.5 cm wide slot. An RNA transcript of a 900 bp *Hind*III fragment of cDNA clone 536 was used as a probe. Bars indicate the position of the rat 28S (4700 nucleotides) and 18S (1900 nucleotides) ribosomal RNA. The arrowhead indicates the top of the gel.

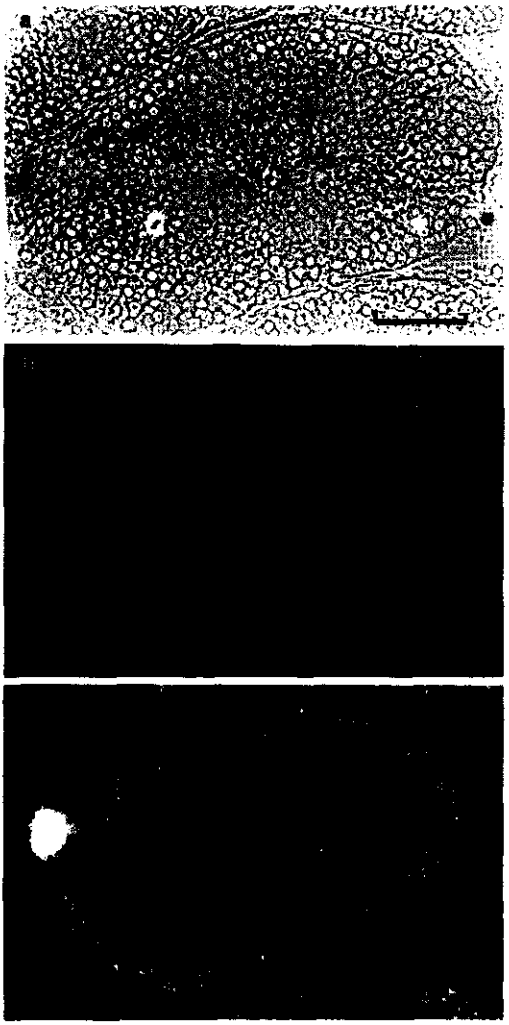

Fig. 7. Localization of SCP1 transcripts in the testis by *in situ* hybridization. (a) Phase contrast micrograph of a transverse section of a testicular tubule; (b) localization of SCP1 in the same section by indirect immunofluorescence staining with mAb IX5B2 as primary antibody; in this experiment serial sections were cut from the rat testis and pretreated for *in situ* hybridization. Alternate sections were then subjected to *in situ* hybridization or immunofluorescence staining; the pretreatment for *in situ* hybridization (heating at 50°C and transfer through graded ethanol) may have caused aggregation of nuclear contents, and thus of nuclear antigen, against the nuclear wall. This effect was not seen in other experiments (Offenberg *et al.*, 1991) in which fixation was optimized for the immunolocalization of SC components; (c) localization of the SCP1 transcript in the adjacent section by *in situ* hybridization; a ^{35}S-labelled RNA transcript of a 450 bp *Hin*cII–*Pst*I fragment of cDNA clone 536 was used as a probe; note that the transcripts are localized in the cytoplasm: nuclei are visible as 'black holes'. Bar represents 50 μm.

been observed (see Offenberg *et al.*, 1991, Figure 3 and Dietrich *et al.*, 1992, Figure 4); this can be ascribed to the relatively low intensity of labelling by mAb IX5B2. And second, in immunogold labelling experiments, mAb IX5B2

Fig. 8. Ultrastructural localization of SCP1 by indirect immunogold labelling of surface spread spermatocytes with the polyclonal antiserum against the fusion protein of cDNA clone 536 as primary antiserum, and goat anti-rabbit IgG conjugated to 10 nm gold as secondary antibody; (a) zygotene, (b) pachytene, (c) diplotene. Bar represents 0.2 μm.

tends to label the inner edge of the lateral elements (Heyting *et al.*, 1989, Figure 6; Dietrich *et al.*, 1992, Figure 4), while the polyclonal anti-SCP1 fusion protein antiserum labels the space between the lateral elements more evenly (Figure 8). The distributions of immunogold grains over the tripartite SC differ significantly (Figure 9; $P < 0.001$). This may be due to the difference between monoclonal and polyclonal antibodies: mAb IX5B2 probably recognizes a single epitope on SCP1, while the polyclonal antiserum probably recognizes several.

Discussion

The mechanism and role of meiotic chromosome synapsis have been the subject of intensive studies for several decades, but the vital questions about the process still remain unanswered. It is unknown how initiation of synapsis is accomplished, whether initiation depends upon DNA sequence homology and whether sequence homology is important for the zippering process (the spreading of synapsis from initiated sites). How synapsis and desynapsis are regulated, and whether synapsis has any role in meiotic recombination, are also open questions. An essential step in the study of such questions is the identification and characterization of the molecules involved. In previous publications we reported the identification of four major components of SCs by means of specific anti-SC antibodies (Heyting *et al.*, 1987, 1989). In this paper we describe the isolation and characterization of the cDNA encoding one of these components, called SCP1, and we present evidence that SCP1 is the major component of transverse filaments.

SCP1 is expressed specifically during meiotic prophase

Until now SC components have been identified only in rodents, particularly mice and rats (Heyting *et al.*, 1987, 1989; Smith and Benavente, 1992). All identified

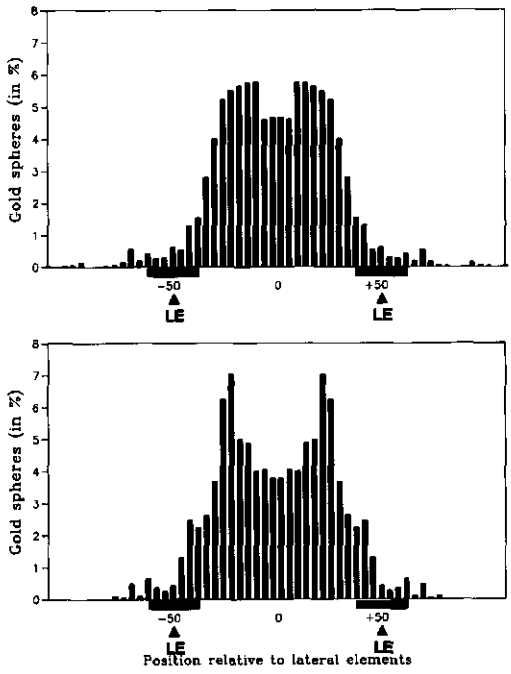

Fig. 9. Distance of immunogold grains to the centre of the nearest LE after indirect labelling with the primary antibody being either the polyclonal antifusion protein antiserum (panel **A**, 2503 grains), or mAb IX5B2 (panel **B**, 909 grains). For each grain we measured first the distance between the centre of both LEs at the site of the grain; this distance was set at 100 arbitrary units; and second, the distance of that grain to the centre of the nearest LE; this distance was expressed in the same units. For presentation we mirrored the obtained distribution, so that 'zero' (0) on the horizontal axis represents the middle between both LEs at any given point, and +50 and −50 represent the position of the centre of the right and left LE at that point; the position of the LEs is indicated schematically on the horizontal axis. In each panel, the vertical bars add up to 100%.

components occur specifically in nuclei of meiotic prophase cells, i.e. spermatocytes (Heyting *et al.*, 1988; Smith and Benavente *et al.*, 1992; Offenberg *et al.*, 1991) and oocytes (Dietrich *et al.*, 1992). The experiments in this paper show, at least for SCP1, that the expression is regulated at the transcriptional level (Figures 6 and 7). As far as we can judge from the *in situ* hybridization experiments, the mRNA and the gene product occur in the same cells i.e. the spermatocytes, while earlier developmental stages (the spermatogonia) do not contain detectable amounts of mRNA or gene product. This confirms our earlier conclusion (Heyting *et al.*, 1988; Offenberg *et al.*, 1991) that SCs do not arise by rearrangement of existing chromatin supporting structures, like the nuclear matrix or nuclear lamina, but that they are assembled during meiotic prophase from newly synthesized proteins and that the chromatin is reorganized onto these newly assembled structures. Another intriguing aspect of Figure 7, and of results presented earlier (see Offenberg *et al.*, 1991, Figures 3 and 4), is the remarkable synchrony of spermatocytes in transverse sections of testicular tubules. Developmental synchrony in testicular tubular segments of rats and mice has been described in detail on the basis of haematoxylin- and Feulgen-stained sections (Leblond and Clermont, 1952; Clermont, 1972). We also observe this synchrony at the level of transcription and SC assembly (Figure 7): spermatocytes within the same segment of a testicular tubule are even synchronous with respect to the degree of synapsis or desynapsis (Figure 7; Offenberg *et al.*, 1991, Figures 4−6). This may be due to the fact that spermatocytes are organized in large syncytia (Moens and Hugenholtz, 1975). Apparently, *trans*-acting factors are active in these syncytia to synchronize the transcription of SC-genes, and the assembly and disassembly of SCs in the separate nuclei.

The predicted SCP1 protein sequence contains a large domain with similarity to coiled-coil proteins

The amino acid sequence similarities of SCP1 and the secondary structure analyses indicate that this predicted protein has a large central domain which is capable of forming coiled-coil structures. The predicted secondary structure of SCP1 is similar to that of intermediate filament proteins, which also (are predicted to) consist of a central coiled-coil domain flanked by nonhelical ends (Steinert and Roop, 1988). This similarity may account for the cross-reaction of various anti-myosin and anti-intermediate filament antibodies with the region between the lateral elements of SCs (DeMartino *et al.*, 1980; Dresser, 1987).

The predicted secondary structure is also similar to that of two recently characterized nuclear matrix proteins, namely yeast NUF1 (Mirzayan *et al.*, 1992) and mammalian NUMA (also called centrophilin; Tousson *et al.*, 1991; Compton *et al*, 1992; Young *et al.*, 1992). Like SCP1, lamins as well as NUMA and NUF1 have short, non-helical ends that are enriched in the S/T-P-X-X-motif (Compton *et al.*, 1992; Mirzayan *et al.*, 1992; Young *et al.*, 1992). This motif is common in gene regulatory proteins and is supposed to contribute to DNA binding (Suzuki, 1989). In SCP1 the carboxy-terminus, in NUF1 the amino-terminus and in NUMA both termini are enriched for this motif. The lamins have two (chicken lamin B1) or three (chicken lamins A and B2, and human lamins A and C) S/T-P-X-X motifs in their 30 residue amino-terminus (Fisher *et al.*, 1986; McKeon *et al.*, 1986; Peter *et al.*, 1989; Vorburger *et al.*, 1989). SCP1 may thus belong to a class of nuclear proteins that are capable of forming coiled-coil filaments, and of attaching to DNA with one or both ends; we speculate that SCP1 may have evolved from such a nuclear matrix protein. As has been suggested earlier (Wu *et al.*, 1986; Diffley and Stillman, 1989; Chen *et al.*, 1992), the coiled-coil domains of such proteins may function not only in homotypic interactions to assemble large structures which support the chromatin, but also to interact heterotypically with similar domains in proteins with functions other than structural ones, such as SIR4 (Diffley and Stillman, 1989), the REP1 protein (Wu *et al.*, 1986) and possibly also the yeast RAD50 protein, which is involved in the initiation of meiotic recombination and chromosome synapsis (Alani *et al.*, 1990; Cao *et al.*, 1990) and has two large amphipathic α-helical domains (Alani *et al.*, 1989).

SCP1, NUF1, NUMA and the nuclear lamins have one or more target sites for $p34^{cdc2}$ protein kinase (Compton *et al.*, 1992; Mirzayan *et al.*, 1992; Young *et al.*, 1992). For nuclear lamins A and C it has been shown that phosphorylation of these sites is important for the disassembly of the lamina during mitosis (Heald and

McKeon, 1990; Peter *et al.*, 1990; Ward and Kirschner, 1990). It is possible that phosphorylation of the $p34^{cdc2}$ kinase target site of SCP1 is required for desynapsis at the onset of diplotene: mutations in the *Saccharomyces cerevisiae CDC28* gene (which is the equivalent of the *Schizosaccharomyces pombe cdc2* gene) cause an arrest in pachytene (Davidow and Beyers, 1984). Other phosphorylation sites of SCP1 that are of potential interest are the cAMP/cGMP dependent protein kinase target sites. Inhibition of phosphorylation by this kinase plays a key role in the disassembly of the nuclear lamina at mitosis (Lamb *et al.*, 1991). However, for these and all other potential phosphorylation sites it still has to be determined whether they are actually phosphorylated, and whether this plays any role in the (dis)assembly of SCs.

SCP1 also contains a leucine zipper motif, at positions 341–369, which is out of frame with respect to the heptad repeat on both sides of the motif, with the leucine residues at position five rather than at positions one or four of the heptad; the heptad repeat is itself interrupted at this position. It is possible that this will cause a distortion of the coiled-coil structure, and/or that these leucine residues are available for interactions other than homotypic ones.

Localization of SCP1

It seems likely that SCP1 is a major component of transverse filaments: it is a major component of SC which colocalizes with these filaments, and its predicted secondary structure is similar to that of filamentous proteins. The detailed localization of SCP1 epitopes will thus provide information about how the transverse filament is integrated in the tripartite structure. The experiments in this paper provide some information on this, although they have not been particularly designed for that purpose.

After immunogold labelling of SCs with mAb IX5B2, the gold grains are located somewhat closer to the lateral elements than after labelling with the polyclonal anti-SCP1 antiserum (Figure 9). That mAb IX5B2 probably recognizes a single epitope, and the polyclonal anti-SCP1 antiserum several, on the SCP1 molecule, is an indication that the SCP1 molecules have a fixed orientation within the SC, with the epitope of mAb IX5B2 orientated towards the LEs. The shortest cDNA that we have isolated using mAb IX5B2 had a 1100 bp fragment of the 3′ terminus; this includes ~640 bp of the coding sequence; it is possible that mAb IX5B2 recognizes even shorter clones, but we have not analysed this. Thus, the epitope recognized by mAb IX5B2 lies within 215 amino acids from the carboxy-terminus of the SCP1 protein, and, therefore, the SCP1 molecule is probably oriented with its carboxy-terminus towards the LE. How far the remainder of the molecule extends towards the opposite LE cannot be said from Figure 9. It is possible that the amino-terminus of the SCP1 molecule reaches the opposite LE, because the length of the expected coiled-coil domain (100 nm) is about equal to the distance between the LEs in tripartite SCs. In that case the dip in the distribution of grains in Figure 9a needs to be explained; for instance, it is possible that the central element has covered part of the SCP1 molecule. It is also possible, however, that the SCP1 molecules do not connect the LEs directly, but that they interdigitate between the LEs (see Steinert and Roop, 1988, for a discussion of possible arrangements of intermediate filament-like proteins). This can be sorted out by a precise localization of the amino-terminus of SCP1 within the SC.

Materials and methods

Antibodies

The mAbs recognizing the M_r 125 000 SC protein were obtained after immunization of mice with rat SCs as described by Offenberg *et al.* (1991). Of these antibodies, mAb IX5B2 has been described in detail by Heyting *et al.* (1989) and Offenberg *et al.* (1991). One polyclonal antiserum was elicited by injection of a rabbit with the fusion protein of cDNA clone 536. 1.5 mg of inclusion bodies containing the fusion protein was injected subcutaneously and intramuscularly at 2 weekly intervals. For the first injection the antigen was mixed with complete Freund's adjuvant; for all later injections it was mixed with incomplete Freund's adjuvant. 20 ml bleedings were collected from the ear-veins at 2 week intervals, starting 1 week after the third injection.

Isolation of cDNAs encoding SCP1

To isolate cDNAs encoding the M_r 125 000 SC protein, we contructed a λzap expression cDNA library of poly(A)$^+$ RNA from the testes of 28 day-old rats, using a cDNA library construction kit (Stratagene, San Diego, USA). We screened the library with a pool of six mAbs that recognize the M_r 125 000 SC protein as primary antibodies, and a goat anti-mouse alkaline phosphatase conjugate (Promega, Madison, USA) as secondary antibody, using the Western blot incubation procedure described earlier (Dunn, 1986; Heyting *et al.*, 1988). Among 10^6 phage screened, 110 positive clones were identified, 55 of which were purified. All purified clones had overlapping restriction enzyme maps and inserts ranging in length from 1100 to 3600 bp. The *Pst*I fragment of clone 536 (see Figure 1) was used for a secondary screening of the library in search for clones extending further in the 5′ direction; labelling of the probe with [α-^{32}P]dATP was performed by random primed labelling; labelling of the probe and screening were performed according to procedures described in Sambrook *et al.* (1989).

Sequence analysis

The insert of cDNA clone 536 was subcloned into the pBluescript vector (SK$^+$) according to the instructions of the manufacturer (Stratagene). From both ends of the insert of clone 536 we generated unidirectional sets of deletions by partial digestion with exonuclease III and S1 nuclease using an Erase-a-base kit (Promega, Madison, WI, USA). In addition, we subcloned several restriction enzyme fragments of the independently isolated cDNA clones 566 and 113 (see Figure 1) in pBluescript. We determined the nucleotide sequences by the dideoxy chain termination method of Sanger *et al.* (1977), using [α-^{35}S]dATP (650 Ci/mmol; Amersham), *Taq* polymerase (Gibco/BRL or Promega) and oligonucleotide primers complementary to the polylinker sequences of pBluescript. The sequence was assembled by means of the University of Wisconsin GCG sequence analysis package. Sequence similarity searches of the GenBank, EMBL, Swissprot and PIR data banks were carried out with FASTA and tFASTA programs (Pearson, 1990). Prediction of secondary structure was performed by means of a program based on Chou–Fasman algorithms (Chou and Fasman, 1978).

Immunocytochemical staining

Immunoperoxidase staining of agar filtrations of lysed spermatocytes was performed with a 1:500 dilution of the polyclonal anti-SCP1 antiserum as described by Heyting *et al.* (1987) and Heyting and Dietrich (1991). Immunofluorescence staining of frozen sections of the rat testis was carried with a 1:1 dilution of hybridoma supernatant containing mAb IX5B2 as described by Heyting *et al.* (1988) and Heyting and Dietrich (1991), except that the sections were mounted and fixed as described below for in situ hybridization. Immunogold labelling was performed in surface-spread spermatocytes, according to Moens *et al.* (1987), as described by Heyting and Dietrich (1991). 1:1200 diluted polyclonal anti-fusion protein antiserum was used as primary antibody, and goat anti-rabbit IgG conjugated to 10 nm colloidal gold (Amersham) as secondary antibody. After immunogold labelling and washes the preparations were stained for 5 min in 1% uranyl acetate in deionized water, rinsed in deionized water and air-dried.

Analysis of the distribution of immunogold grains

The distance of individual gold grains to the nearest LE was measured on a digitizer from electron micrographs (magnification 92 000–120 000×). The distributions of grains relative to the LEs were compared by a Kolmogorov-Smirnov two-sample test.

RNA isolation and Northern blot hybridization

RNA was isolated from various tissues of 37 day-old rats by the guanidinium thiocyanate/LiCl method of Cathala *et al.* (1983); poly(A)$^+$ RNA was purified by affinity chromatography on oligo(dT)–cellulose (Aviv and Leder, 1972). RNA was electrophoresed on formaldehyde–agarose gels and transferred to Hybond-N$^+$ membranes (Amersham Corp.) by standard procedures (Sambrook *et al.*, 1989). After transfer the membranes were washed in 3×SSC, dried and fixed with UV light (312 nm; 200 J/m^2) for 2 min. As probes for Northern blot hybridization we used RNA transcripts of the 900 bp *Hin*dIII fragment (see Figure 1) which had been subcloned into pBluescript. Transcription was performed from the T3 promoter in the presence of [α-^{32}P]rATP (3000 Ci/mmol). The Northern blot membranes were prehybridized in 50% formamide, 5×SSC, 50 mM Tris–HCl pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 0.2% PVP, 0.2% Ficoll, 5 mM EDTA and 150 μg/ml yeast tRNA for at least 3 h at 65°C. Hybridization was performed in the same mixture with 1.5 μg/ml probe (15×10^6 c.p.m./μg) for 17 h at 65°C. Subsequently the blots were washed for 30 min at 65°C in, successively, 2×SSC, 0.1% SDS; 2×SSC, 0.1% SDS; 0.1×SSC, 0.1% SDS; and 0.1×SSC, 0.1% SDS.

In situ hybridization

For *in situ* hybridization, 10 μm tissue sections were cut from testes of 3 month-old rats at −19°C. The sections were mounted on slides coated with 3-aminopropyl-tri-ethoxysilane (Aldrich), quickly air-dried at room temperature and heated for 2 min at 50°C on a hot plate. After further drying for 1–2 h at room temperature the sections were transferred through graded ethanol (50–70–100%) air-dried, wrapped in aluminum foil and stored at −80°C. To analyse the expression of SCP1, consecutive sections were stained immunocytochemically with mAb IX5B2 or subjected to *in situ* hybridization. Before hybridization or immunocytochemical staining the sections were allowed to assume room temperature while still in the foil. For *in situ* hybridization they were pretreated as follows: 20 min at room temperature in 0.2 M HCl, 5 min wash in deionized water, 7.5 min at 70°C in 2×SSC, 5 min wash in deionized water, 20 min at 37°C in 0.5 U/ml self-digested pronase (Koch-Light, 41 U/ml), two rinses in 0.2% glycine in PBS, two 30 s washes in PBS, fixation for 20 min at room temperature in 4% PFA in PBS, dehydration through graded ethanol and air-drying for at least 1 h at room temperature. Hybridization was performed for 18 h at 55°C in 50% formamide, 0.3 M NaCl, 20 mM Tris–HCl pH 8.0, 5 mM NaEDTA pH 8.0, 10 mM NaH_2PO_4/Na_2HPO_4 pH 8.0, 10% dextran sulfate, 1×Denhardt's (Denhardt, 1966), 0.5 μg/ml yeast tRNA and 2×10^5 c.p.m./μl ^{35}S-labelled RNA probe (18×10^3 c.p.m./μg). The RNA probe was obtained by transcription from the T3 promoter of a 450 bp *Hin*cII–*Pst*I fragment (see Figure 1), which had been subcloned in pBluescript; probe synthesis was performed in the presence of [α-^{35}S]rUTP (3000 Ci/mM, Amersham). After hybridization the sections were washed as follows: 30 min in 5×SSC, 10 mM DTT at 55°C; 1 h at 50°C in 50% formamide, 5×SSC, 20 mM DTT; 3×10 min at 37°C in 0.5 M NaCl, 10 mM Tris, 5 mM EDTA pH 8.0; 30 min at 37°C in the same buffer with 20 μg/ml RNase A; 2×15 min in 2×SSC and 2×15 min in 0.1×SSC at room temperature. Dehydration by quick transfer through 50, 70, 96, 100 and 100% ethanol each including 0.3 M ammonium acetate, two rinses in 100% ethanol without ammonium acetate and air-drying. The sections were dipped in Ilford K5 nuclear track emulsion, exposed for 3 weeks at 4°C, developed in Kodak developer D19 and analysed by dark field microscopy.

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SCs were isolated as described by Heyting *et al.* (1985) and Heyting and Dietrich (1991); SDS–PAGE 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).

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CHAPTER 3

Human synaptonemal complex protein 1 (SCP1): isolation and characterization of the cDNA and chromosomal localization of the gene

Ralph L.J. Meuwissen, Ilonka Meerts, Jan M.N. Hoovers, Nico J. Leschot and Christa Heyting

Genomics, in press

Abstract

Synaptonemal complexes (SCs) are structures which are formed between homologous chromosomes (homologs) during meiotic prophase. They consist of two proteinaceous axes, one along each homolog, which are connected along their length by numerous transverse filaments (TFs). The cDNA encoding one major component of TFs of SCs of the rat, rnSCP1, has recently been isolated and characterized. In this paper we describe the isolation and characterization of the cDNA encoding the human protein homologous to rnSCP1, hsSCP1.

hsSCP1 and rnSCP1 have 75% amino acid identity. The most prominent structural features and amino acid sequence motifs of rnSCP1 have been conserved in hsSCP1. Most probably, hsSCP1 is functionally homologous to rnSCP1. The hsSCP1 gene was assigned to human chromosome 1p12-p13 by fluorescence in situ *hybridization.*

Introduction

The life cycle of sexually reproducing organisms is characterized by the alternation of diploid and haploid generations of cells. The transition from the diploid to the haploid state is accomplished at meiosis, which in mammals immediately precedes gametogenesis. At meiosis, a single round of DNA replication is followed by two successive nuclear divisions, meiosis I and II. During the prophase of meiosis I, homologous chromosomes (homologs) condense, pair, recombine and disjoin. At meiosis II, the chromatids of each chromosome segregate, as in a mitotic division.

The chromatin rearrangements of meiotic prophase are accompanied by the assembly and disassembly of synaptonemal complexes (SCs) (reviewed by von Wettstein *et al.*, 1984). SCs consist of two proteinaceous axial cores or lateral elements (LEs), one along each homolog, which are connected along their length by numerous transverse filaments (TFs); a third longitudinal structure, the central element (CE), exists on the TFs, between both LEs (Gillies, 1975; Schmekel *et al.*, 1993).

In order to analyze the function of SCs, we have started to study their composition. Several protein components of SCs of rodents (Heyting *et al.*, 1987, 1989; Smith *et al.*, 1992; Chen *et al.*, 1992) and yeast (reviewed in Roeder, 1995) have been identified. Among these components were the putative TF proteins Zip1p of yeast (Sym *et al.*, 1993, 1995) and synaptonemal complex protein 1 (SCP1) of the rat (in this paper referred to as rnSCP1) (Meuwissen *et al.*, 1992). The cDNA encoding rnSCP1 was isolated and sequenced (Meuwissen *et al.*, 1992), and the predicted amino acid sequence was analyzed: rnSCP1 consists of an α-helical stretch of 700 amino acid residues, flanked by N- and C-terminal globular domains. rnSCP1 molecules are highly organized within SCs: the C-terminal domains are located in the inner half of the LEs, whereas the N-terminal domains lie in the vicinity of the CE (Chapter 5; Schmekel *et al.*, 1996). The C-terminal domain of rnSCP1 contains S/T-P (Ser/Thr-Pro) motifs, which are characteristic of DNA-binding proteins (Suzuki, 1989a; Churchill and Travers, 1991). The DNA-binding capacity of the C-terminal domain was recently demonstrated (Meuwissen *et al.*, Chapter 4 of this thesis). The gene encoding rnSCP1 is transcribed exclusively in meiotic prophase cells (Meuwissen *et al.*, 1992). If, as seems likely, rnSCP1 is functionally homologous to Zip1p of yeast

(Sym *et al.*, 1993, 1995), it probably has a function in the regulation of reciprocal crossing-over and chromosome segregation (Sym *et al.*, 1994).

We cloned and characterized the cDNA encoding the human homolog of rnSCP1 (hsSCP1) in order to identify conserved domains within the protein, and obtain a better insight in the importance of structural features and amino acid sequence motifs in SCP1. The predicted amino acid sequence of hsSCP1 has 75% identity to rnSCP1; all prominent predicted structural features and most amino acid sequence motifs were conserved in hsSCP1. The gene encoding hsSCP1 was localized on human chromosome 1p12-p13 by fluorescent *in situ* hybridization (FISH). No human meiotic phenotype could be correlated with abnormalities in the chromosome 1p12-p13 region.

Materials and methods

Isolation of cDNAs encoding human SCP1. A human testis cDNA library in λgt11 (Huynh *et al.*, 1985) was screened with a polyclonal anti-rnSCP1 antiserum (Meuwissen *et al.*, 1992) and the monoclonal anti-rnSCP1 antibody IX5B2 (Offenberg *et al.*, 1991). Screening of 4×10^5 phage yielded two positive clones with inserts of 1.5 kb and 1.8 kb, respectively. A 5' end probe of 350 bp derived from the 1.8 kb insert was used for screening a human testis cDNA library in λgt10 (Huynh *et al.*, 1985) (Clontech Laboratories Inc., CA, USA). This yielded a clone with a 2.7 kb insert which overlapped with the cDNAs from the λgt11 library. We obtained the missing 5' end of the human cDNA from the λgt10 library by means of PCR, for which we used two nested oligonucleotides homologous to the 2.7 kb cDNA insert and one oligonucleotide homologous to the λgt10 vector as primers: about 2×10^8 plaque forming units of the λgt10 library were resuspended in 75 µl deionized water and heated for 5 min. at 70° C. Subsequently, the sample was adjusted to 1.5 mM $MgCl_2$, 0.2 mM dATP, dGTP, dCTP and dTTP, and 50 pmol of both primers and 2.5 units Taq polymerase (Pharmacia Biotech Europe) were added. The sample was incubated according to the following schedule: 1 cycle 5 min. 95° C; 30 cycles 1 min. 95° C, 1 min. 52° C, 2 min. 72° C; 1 cycle 1 min. 95° C, 1 min. 52° C, 15 min. 72° C. The PCR reaction yielded a 750 bp fragment which after southern blot analysis (Sambrook *et al.*, 1989), hybridized with a 5' end probe of rnSCP1 cDNA. The 750 bp fragment was gel purified and sequenced with the use of oligonucleotide primers derived from the 5' end of the human 2.7 kb insert sequence and the λgt10 vector. The nucleotide sequence of the 2.7 kb insert was determined as follows: we subcloned the 2.7 kb cDNA insert into the pBluescript SK(+) (Stratagene Inc., CA, USA) vector, and generated unidirectional sets of deletions from both ends of

the 2.7 kb insert by partial digestion with exonuclease III and S1 nuclease, using an Erase-a-base kit (Promega, Madison, WI, USA). We performed the sequencing reactions on the deletion clones and the PCR products, using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer Inc., CA, USA) and analyzed the nucleotide sequences on an ABI 373A automatic DNA sequencer (Applied Biosystems Inc., CA, USA). The nucleotide sequence of human SCP1 (hsSCP1) was assembled by means of the GCG sequence analysis software package (University of Wisconsin, WI, USA). Sequence similarity searches of Genbank, EMBL, Swissprot and PIR databases were carried out with several BLAST programs (Altschul *et al.*, 1990), FASTA and tFASTA (Pearson, 1990). Prediction of secondary structure was performed by means of a program based on Chou-Fasman algorithms (Chou and Fasman, 1978). Amino acid sequence alignments were determined using the Pile-Up program from the GCG sequence analysis software package (University of Wisconsin, WI, USA).

Fluorescence in situ hybridization analysis A 2700 bp fragment of the human SCP1 cDNA was labeled with biotine-11-dUTP, whereas a human subtelomeric repeat probe, D1Z2, specific for chromosome 1 (Buroker *et al.*, 1987), was labeled with digoxigenin-11-dUTP. Both labeling reactions were performed by nick-translation and both probes were mixed together for hybridization with the metaphase chromosomes. The metaphases were accumulated in EBV transformed human lymphocytes by the thymidine synchronization method (Viegas-Péquignot, 1993), and spread onto slides as described before (Dauwerse *et al.*, 1992). *In situ* hybridization was performed as described by Dauwerse *et al.* (1992). Briefly, the procedure was as follows: the metaphase chromosomes on slides were aged overnight at 60° C. After this, they were incubated for 1 hour at 37° C in 100 mg/ml RNase in 2 x SSC, washed for 3 x 2 min. in 2 x SSC at 37° C, incubated for 10 min. in 0.01% pepsin in 10 mM HCl at 37° C, washed for 2 x 5 min. with PBS at room temperature, fixed for 10 min. in 3.7% formaldehyde in PBS, washed for 2 x 5 min. in PBS, dehydrated in successively 70%, 96% and 100% ethanol (2 min. per step), and air-dried. The chromosomes were then denatured for 5 min. at 80° C in 60% formamide, 2xSSC, 50 mM NaH_2PO_4/Na_2HPO_4 pH 7.0, dehydrated and fixed by incubation for 2 x 5 min. in 70% ethanol at -20° C, 5 min. in 96% ethanol at room temperature and 5 min. in 100% ethanol at room temperature, and air-dried. The D1Z2 probe was dissolved in 50% formamide, 50 mM Na_2HPO_4/NaH_2PO_4 pH7.0, 10% dextran sulfate and a 50-fold excess of Cot-1 DNA, denatured for 5 min. at 70° C, and prehybridized by incubation for 30 min. at 37° C. The hsSCP1 probe was dissolved in 50% formamide, 50 mM Na_2HPO_4/NaH_2PO_4 pH7.0 and 10% dextran sulfate, and denatured for 5 min. at 70° C before it was mixed with the prehybridized D1Z2 probe. After mixing, the final concentration of D1Z2 was 5 ng/ml, and of hsSCP1 10ng/ml. The hybridization mixture was added to the pretreated slides for an overnight hybridization at 37° C in a moist chamber. After hybridization the slides were washed: 3 x 5 min. at 42° C in 50% formamide, 2 x SSC pH 7.0; 3 x 5 min. at 60° C in 0.1 x SSC; 5 min. at room temperature in 4 x SSC, 0.05% Tween. Hybridized human SCP1 probe was detected by successive incubation rounds in avidin-FITC

conjugate and biotinylated goat anti-avidin antibodies. The hybridized subtelomeric repeat probe D1Z2 was detected by successive incubation rounds in mouse anti-digoxigenin, sheep anti-mouse antibodies and anti-sheep IgG-TRITC conjugates. Counter staining of the chromosomes was performed with DAPI in a Vectashield (Vecta Laboratories Inc., USA) antifade solution. Slides were examined in a Zeiss Axioplan epifluorescence microscope. For digital imaging microscopy the Cytovision Probe System (Applied Imaging Inc., UK) was used.

Results

Isolation and sequencing of human SCP1 cDNAs

A mixture of an affinity-purified polyclonal anti-rnSCP1 antiserum (Meuwissen *et al.*, 1992) and a monoclonal anti-rnSCP1 antibody, IX5B2 (Offenberg *et al.*, 1991), was used for screening 4 x 10^5 recombinant phage of a human testis λgt11 cDNA library. This yielded two clones with insert sizes of 1.5 kb and 1.8 kb, respectively. Both cDNA inserts had overlapping restriction enzyme maps, and displayed nucleotide sequence homology with the rat SCP1 cDNA sequence. We performed a secondary screening of a human testis λgt10 cDNA library in order to isolate the remaining part of the human cDNA (see Materials and Methods). Extensive screening of the independent λgt10 and λgt11 cDNA libraries yielded only a single type of cDNA clones, i.e. we found only one type of human cDNA clones homologous to the rat SCP1 cDNA. The complete human SCP1 (hsSCP1) cDNA sequence contained an open reading frame of 2928 nucleotides, which encoded a protein of 976 amino acids (Fig. 1). The translation initiation codon in the human cDNA sequence (Fig. 1, nucleotide position 95-97) was the first ATG in the open reading frame and is preceded by a consensus sequence for eukaryotic translation initiation (Kozak, 1986). Furthermore, in the amino acid sequence alignment of hsSCP1 and three rodent SCP1 proteins (Fig. 2) the first 6 amino acid residues are identical.

Amino acid sequence homology between the different SCP1 proteins.

The amino acid identity between hsSCP1 and SCP1 of the mouse (Sage *et al.*, 1995), hamster (Dobson *et al.*, 1994) or rat (Meuwissen *et al.*, 1992) is 74.6%, 74.4% and 75.7% respectively.The amino acid identity is distributed evenly over hsSCP1, except

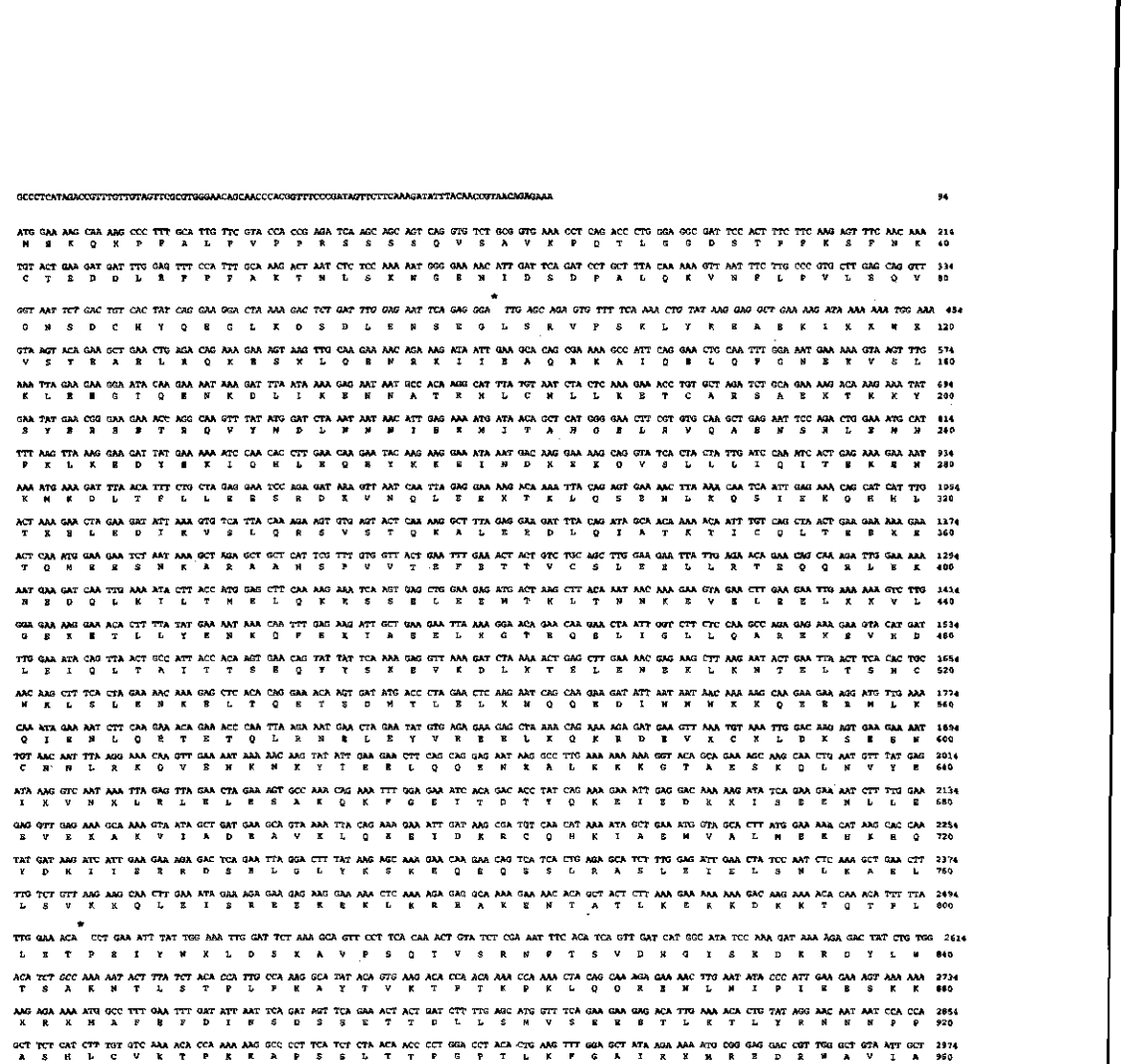


Figure 1. Nucleotide sequence and derived amino acid sequence of the cDNA encoding hsSCP1. The predicted translation product is presented below the first nucleotide of each codon. The putative poly(dA) addition signal and addition site are underlined. The ends of the coiled-coil region as predicted according to Lupas *et al.* (1992) with a window of 28 amino acids, are indicated by asterisks. The name and accession number for the human SCP1 cDNA sequence in the EMBL database are: hsSCP1, accession number: X95654.

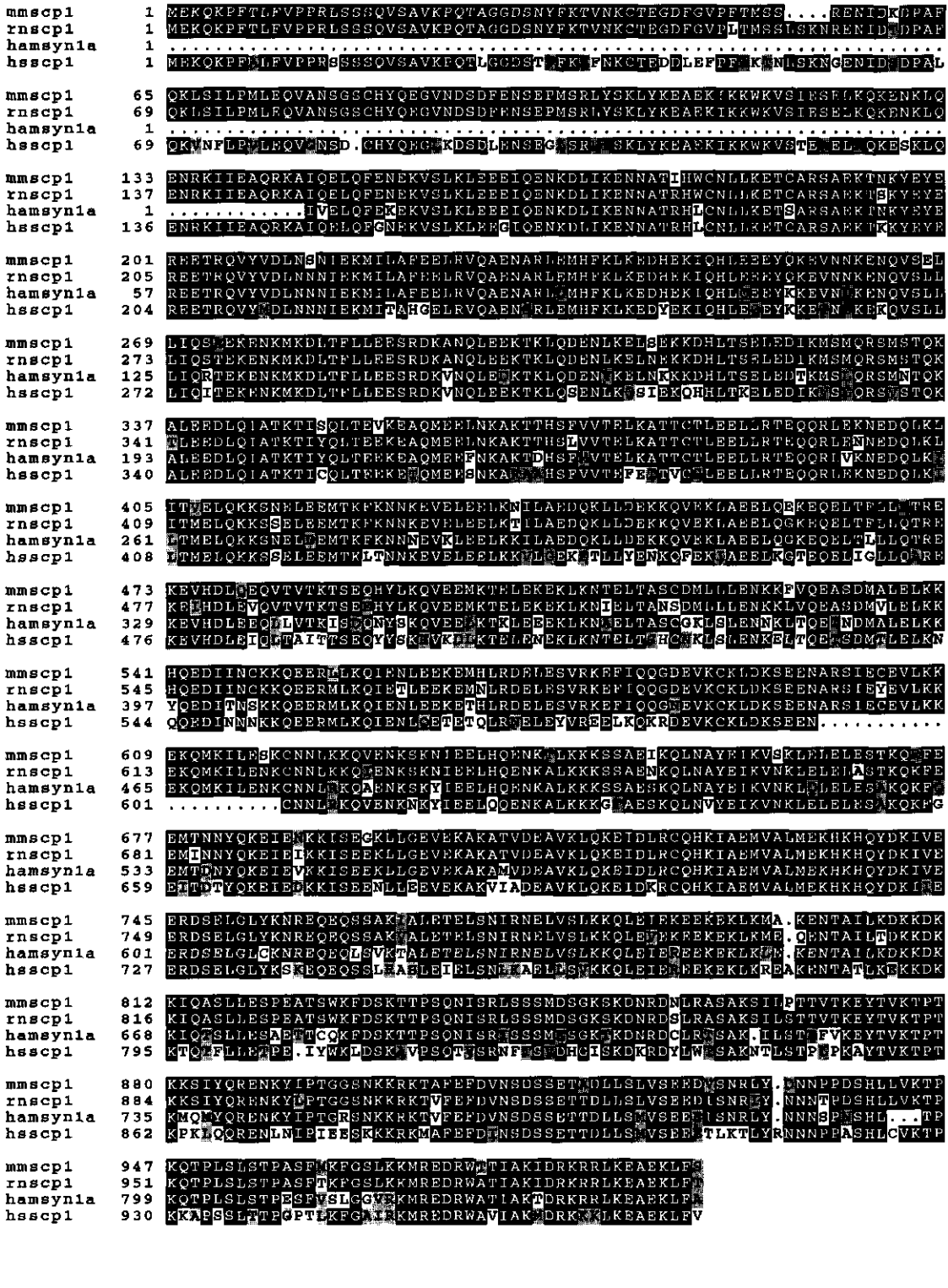
for the C-terminal 174 amino acid residues, from hsSCP1 position 802 to 976, which show only 64.5% amino acid identity.

However, the level of amino acid sequence identity in the hsSCP1 alignment (Fig. 2) is sufficient to imply a structural and functional similarity between hsSCP1 and the rodent SCP1 proteins (Sander and Schneider, 1991).

Amino acid sequence features of SCP1

The structural organization of the SCP1 proteins is very similar. Like the rodent SCP1s, hsSCP1 has three distinct domains, each having its own characteristic secondary structure as predicted by the algorithm of Chou and Fasman (1978). In hsSCP1, the N-terminal domain includes amino acid residues 1-102. It is rich in acidic amino acids, and does not contain any structural motifs. From position 102 to 802, hsSCP1 has a predicted amphipatic α-helical domain; within this domain hsSCP1 has a deletion of 21 amino acid residues compared to rodent SCP1s (Fig. 2). We found the corresponding deletion in three independently isolated cDNA clones, two of which originated from the human testis cDNA library in λgt11 and one from the cDNA library in λgt10. The deletion of 21 amino acids does not disturb the heptad repeat frame of the predicted amphipatic α-helix. The C-terminal domain of hsSCP1 extends from position 803 to 976, is enriched in basic amino acids and has a predicted pI of 9.8, compared with a pI of 5.9 for the entire hsSCP1 protein.

Figure 2. Amino acid sequence alignment of the known rodent SCP1 proteins with the hsSCP1 protein. Alignments were performed by means of the Pile-Up program (GCG software package, University of Wisconsin, WI, USA) and the results are presented using the Boxshade program (Bioinformatics Group, ISREC, Lausanne, Switzerland). Identical amino acids are highlighted in black, similar amino acids in gray. The following amino acids were considered similar: (M, V, I, L), (D, E, Q, N), (S, A, T, G) and (H, R, K). Abbreviations: mmscp1, mouse SCP1 (Sage *et al.*, 1995); rnscp1, rat SCP1 (Meuwissen *et al.*, 1992); hamsyn1a, hamster SCP1 (Dobson *et al.*, 1994); hsscp1, human SCP1. The complete amino acid sequence of the hamster SCP1, is not known (Dobson *et al.*, 1994). The dots at the N-terminal part of the hamster SCP1 represent the unknown amino acid sequence. The remaining dots represent the gaps in the amino acid sequence alignment. The predicted amino acid sequence of rnSCP1 in this figure extends 51 amino acids further in the N-terminal direction than the originally published sequence (Meuwissen *et al.*, 1992). Due to a sequencing error, the codon CCC for a proline residue was read as CC. This resulted in a frame-shift by which the first 6 amino acid residues of rnSCP1 were missed. Thereupon the startcodon was erroneously located at amino acid position 52 of rnSCP1 (Sage *et al.*, 1995; Meuwissen, unpublished results).



motifs are at exactly corresponding positions. The S/T-P and S/T-S/T motifs are thought to cause β-turns in peptide chains so that these chains get a "kinky" conformation; such chains are thought to fit into the minor groove of DNA, and to make contact with the phosphoribose backbone at the β-turns (Suzuki, 1989a; Churchill and Suzuki, 1989).

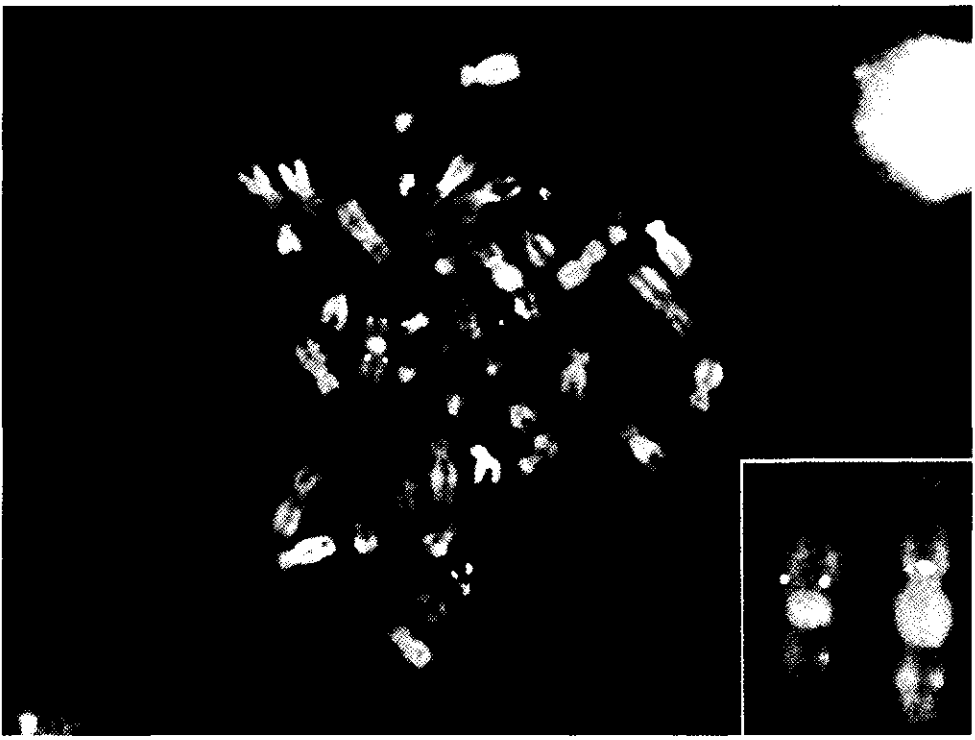


Figure 4. Localization of the human SCP1 gene to chromosome 1p12-p13. Metaphase chromosomes from human lymphocytes were hybridized with a mixture of two probes: a biotin-dUTP labeled probe derived from hsSCP1 cDNA (detected with FITC, yellow pseudo-color and a digoxigenin-dUTP labeled human subtelomeric repeat probe D1Z2 (detected with TRITC, red pseudo-color). The chromosomes were counter-stained with DAPI (blue). We considered the presence of a paired signal as a positive localization. **Inset**: enlargement of the individual chromosomes #1 of the same metaphase.

The precise position of the S/T-P and S/T-S/T motifs is not very crucial for that (Suzuki, 1989a). Some S/T-S/T motifs in rnSCP1 are S/T-P motifs in hsSCP1 (e.g. at position 850 in hsSCP1), which indicates that the presence of the β-turn is important. *In vitro*, the C-terminus of rnSCP1 binds to DNA (Meuwissen *et al.*, unpublished experiments).

Another conserved amino acid sequence motif which deserves attention is the $p34^{cdc2}$ protein kinase target site. This site also occurs in nuclear lamins A and C, where it is involved in the regulation of the disassembly of the nuclear lamina at mitosis (Heald and McKeon, 1990). In budding yeast (*Saccharomyces cerevisiae*), mutations of *CDC28*, which is the gene equivalent to *cdc2* of fission yeast (*Schizosaccharomyces pombe*), results in a block in the pachytene stage of meiosis: SCs are not disassembled in these mutants (Davidow and Byers, 1984; Shuster and Byers, 1989). Two other conserved potential phosphorylation sites are the cAMP/cGMP dependent protein kinase (PKA) target sites. Inhibition of phosphorylation by PKA is important for the disassembly of the nuclear lamina at mitosis (Lamb, 1991). It will therefore be of interest to discover if the potential $p34^{cdc2}$ and PKA phosphorylation sites in SCP1 are phosphorylated *in vivo*.

Mutation of SCP1 will most probably result in defects in meiosis, such as chromosomal nondisjunction (Sym *et al.*, 1993; Sym and Roeder, 1994) and infertility. As yet, no human phenotypes have been identified that are linked with defects in meiosis, and that are correlated with chromosomal abnormalities in human chromosome 1p12-p13 (Weith *et al.*, 1996). Further analysis of the human 1p12-p13 chromosomal area together with mutational analysis of SCP1 in rodents should provide more information about the meiotic function of SCP1.

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CHAPTER 4

Binding of DNA by SCP1, a major component of synaptonemal complexes

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Submitted

of the transverse filaments (25). SCP1 and SCP2 have several S/T-P (25,27) motifs, which are common in a variety of DNA-binding proteins, and allow non-sequence specific interactions of the protein with the minor groove of DNA. These proteins are therefore of interest for the analysis of the attachment of chromatin to SCs. In this paper we concentrate on SCP1, which is localized between the LEs (4,25). The major part of this protein, a stretch of 700 amino acid residues, is predicted to form an amphipathic α-helix capable of forming coiled-coil structures (25). This stretch is flanked by a predicted globular N-terminal domain of 102 amino acids, and a predicted globular C-terminal domain of 195 amino acids (25). This predicted secondary structure is similar to that of Zip1p of yeast (20); however, no amino acid sequence similarity between SCP1 and Zip1p has been found beyond that expected between two coiled coil proteins (20,25). All seven S/T-P motifs of SCP1 are concentrated in the C-terminal domain. The C-terminus is also enriched in S/T-S/T motifs, which, according to Suzuki (28), can mimic the conformation of S/T-P motifs. Furthermore, there are two basic α-helical domains near the C-terminal end of SCP1, one of which is similar to the DNA-binding amino acid sequence motif of protein tyrosine phosphatase of the rat (29). Within the SC, the SCP1 molecules are oriented with their C-terminus towards the LEs (4).

The predicted secondary structure of SCP1 and its orientation within SCs prompted us to investigate the DNA-binding capacity of SCP1. Because SCP1 is insoluble in the absence of strong detergents (Meuwissen, R. unpublished experiments), the analyses were performed on south-western blots. From these analyses we conclude that SCP1 is capable of binding to DNA *in vivo* and *in vitro*, that its DNA-binding capacity resides in its C-terminal domain, that the C-terminus interacts with the minor groove of DNA, and that SCP1 has a preference for binding rat genomic DNA rather than *E. coli* DNA. Furthermore, we concluded from *in vivo* crosslinking experiments with paraformaladehyde (PFA), that SCP1 interacts with DNA *in vivo*.

Materials and Methods

Expression and purification of full-length SCP1 and the peptide fragments N, M and C. We subcloned three fragments of SCP1 cDNA encoding, respectively, the 549 N-terminal amino acid residues (fragment N), the amino acid residues 415-549 (fragment M) and the 221 C-terminal residues (fragment C). These three fragments were cloned into pQE3X vectors (Qiagen, Chatsworth, CA, USA) in frame with the fragment of the *LacI* gene and six successive histidine codons in these vectors. *E. coli* SG 13009 cells (Qiagen) were transformed with the resulting constructs and synthesis of the encoded fusion proteins was induced by means of IPTG. The fusion proteins were then purified under strong denaturing conditions from bacterial lysates by affinity chromatography on nickel columns according to the instructions of the supplier of the columns (Qiagen). To obtain sufficient amounts of full-length SCP1, we cloned the complete coding region of the SCP1 cDNA in a baculovirus expression vector. Briefly, the construction was as follows. A 36 bp *Bam*HI-*Not*I fragment, encoding a heptamer peptide consisting of a methionine followed by six successive histidine residues, was ligated between the *Bam*HI and *Not*I sites of the pBluescript SK(-) vector in frame with the 5' end of the cDNA insert for SCP1. The new clone, pRM180, provided a 2.9 kb *Bam*HI fragment encoding the full-length SCP1 protein. This fragment was cloned into the unique *Bam*HI site of the polyhedrin-based baculovirus expression vector pAcDZ1 (30) giving pAcRLM1. *Spodoptera frugiperda* Sf9 insect cells were cotransfected with transfer vector pAcRLM1 and *Bsu*36I-linearized *Autographa californica* nucleopolyhedrovirus (AcMNPV) PAK6 DNA (31). Recombinant virus was plaque-purified by standard techniques, essentially as described by Summers and Smith (32) and Vlak *et al.* (33). For SCP1 expression and purification, 1.3×10^{10} Sf9 cells were infected with AcMNPV/RLM1 with a multiplicity of infection of $4 \times TCID_{50}$ (Tissue Culture Infective Dose 50%) units per cell. AcMNPV/RLM1-infected cells, harvested 60 hours post infection, were washed three times with phosphate-buffered saline and lysed at 0-4°C in lysis buffer (6 M guanidine hydrochloride, 100 mM NaH_2PO_4 (pH=8.0), 10 mM Tris-Cl (pH=8.0)). The SCP1 protein was purified by nickel chelate chromatography under denaturing conditions as described above. For the preparation of the 19.7 kDa Oct-1 POU domain (34), we used a construct consisting of the expression vector pET 15b (34) which contained the sequences encoding the Oct-1 POU domain in frame with six consecutive histidine codons. This construct was expressed in *E. coli* BL21 (DE_3) plysS cells by means of the T7 expression system (35). The His-tagged Oct-1 POU domain was purified under denaturing conditions by nickel chelate affinity chromatography as described above.

South-western blot analysis The procedures for south-western blot analysis used in this paper are based on Miskimins *et al.* (36) and von Kries *et al.* (37). The full-length SCP1 protein and fragment N, M and C peptides were separated on preparative polyacrylamide-SDS gels (38). SC proteins were separated by two-dimensional electrophoresis according to O'Farrell (39), with minor modifications (40). The proteins were blotted onto nitrocellulose filters (BA85, 0.45 mm, Schleicher & Schuell) as described by Heyting and Dietrich (41), but with a different blotting buffer (10 mM $NaHCO_3$, 3 mM

Na_2CO_3, 25 mM Tris-HCl pH=9.9, 20% (v/v) methanol). The blots of the preparative gels were cut into exactly 0.4 cm wide strips, so that each individual strip contained the same amount of protein. These blot strips and also the complete blot of the two-dimensional gel, were blocked overnight at room temperature in an ample volume of preincubation buffer (10 mM Tris-HCl (pH=7.5), 10% (v/v) glycerol, 2.5% (v/v) NP-40, 150 mM NaCl, 0.1 mM DTT, 5% non-fat dry milk), and subsequently preincubated for 1 hour in binding buffer (10 mM Tris-HCl (pH=7.5), 8% (v/v) glycerol, 50 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 0.125% non-fat dry milk). Subsequently, each filter strip was incubated separately in fresh binding buffer containing various amounts of end-labeled [^{32}P]DNA probe (about 10^4-10^6 cpm/ml), with or without various amounts of unlabeled competitor DNA (see Results). Binding was carried out overnight at room temperature, with gentle agitation. The volume of the binding buffer was 1 ml per cm^2 nitrocellulose filter. The filters were washed four times for 15 min. in 300-500 ml of binding buffer and the DNA-protein complexes were visualized by autoradiography. The amount of [^{32}P]DNA bound to the protein was determined for each separate filter strip by scintillation counting and phosphorimager analysis (ImageQuant, version 3.3, Molecular Dynamics Ltd., Kensing, England). After phosphorimager analysis, SCP1 was visualized by incubation of the nitrocellulose filter in, successively, affinity-purified rabbit anti-SCP1 antibodies, goat anti-rabbit alkaline phophatase conjugate and BCIP/NBT alkaline phosphatase substrate according to described procedures (41).

Preparation of DNA probes Total rat genomic DNA was isolated according to Sambrook *et al.* (43), completely digested with *Eco*RI and then sonicated. DNA fragments with an average length of 1500 bp were end-labeled with [α-^{32}P]dATP by the Klenow polymerase fill-in reaction (43). This [^{32}P]DNA was used as a probe in the south-western blot assays. Unlabeled rat genomic DNA, fragmented as described above, was used as competitor DNA in some of the assays. *E. coli* DNA was isolated following the procedure of Marmur and Doty (44). The purified *E. coli* DNA was sonicated and a DNA fraction with an average size of 1500 bp was used as competitor DNA. The SAR DNA fragments were all gel-purified and end-labeled with [α-^{32}P]dATP (43). We used one SAR fragment in our study, namely the 1000 bp *Hin*dIII-*Pst*I SAR fragment from the Drosophila histone gene repeat (45). Double stranded oligonucleotides containing the Oct-1 POU domain binding site were end-labelled with T4 polynucleotide kinase and purified by preparative polyacrylamide gel electrophoresis. The oligonucleotide sequence was: 5' TGTCGTATGCAAATCACTAGAA 3', in which the Oct-1 octamer binding site (46) is underlined.

Distamycin treatment of DNA For the experiments with distamycin A, the DNA probes were pretreated as follows: 10 mg rat genomic or *Drosophila* SAR DNA, end-labeled with ^{32}P, or 10 ng oct-1 linker [^{32}P]DNA together with 10 mg unlabeled rat genomic DNA, were mixed with 1 ml binding buffer. Distamycin A, freshly diluted in binding buffer from a 1 mM stock solution in 96% ethanol, was added to the DNA probes so that the final drug concentrations ranged from 25 nM to

100 μM. The resulting mixtures were incubated for 15 min. at room temperature before they were added to the western blot strips. The strips were then incubated and washed as described above.

***In vivo* crosslinking of SCP1 to DNA** To analyse whether SCP1 binds to DNA *in vivo*, we performed *in vivo* crosslinking experiments following a procedure described by Solomon *et al.* (57) and Orlando and Paro (58). Primary spermatocytes were purified from testicular cell suspensions from C57Bl/6 mice by means of elutriation, as described before for the isolation of rat spermatocytes (41), except that mouse spermatocytes were collected at slightly lower flow rates (18-30 ml/min) than rat spermatocytes. The harvested cells were washed twice with prewarmed PBS at 33 °C, and resuspended in prewarmed PBS (33 °C) to a final concentration of 5 x 10^6 cells/ml PBS. The suspension was mixed with an equal volume of prewarmed 2% paraformaldehyde in PBS (33 °C) and incubated for 8 min at 33 °C. Subsequently, an equal volume of ice-cold PBS was added, the cells were pelleted at 200 x *g* for 5 min at 4 °C,washed for 10 min in PBS at 0 °C, and again pelleted. The cells were then lysed by resuspension in 0.25% Triton X-100, 10 mM NaEDTA, 0.5 mM NaEGTA, 10 mM Tris.HCl (pH 8.0) to a final concentration of 1 x 10^6 nuclei/ml and incubation for 10 min. at room temperature. Subsequently, the nuclei were pelleted and washed for 10 min. in 0.2 M NaCl, 1 mM NaEDTA, 0.5 mM NaEGTA, 10 mM Tris.HCl (pH 8.0). After pelleting, the nuclei were resuspended in 1 mM NaEDTA, 0.5mM NaEGTA, 10 mM Tris.HCl (pH 8.0) to a final concentration of 1 x 10^7 nuclei/ml. 750 ml portions of the nuclear suspension were sonicated on ice in a MSE soniprep 150 sonicator, using the microtip at an amplitudo of 14 mm, for three burts of 30 sec. each. The volume of the sonicated nuclei (about 5 x 10^7) was adjusted to 12 ml with 1 mM NaEDTA, 0.5 mM NaEGTA, 10 mM Tris.HCl (pH 8.0) (room temperature), and 0.6 ml 10% sarkosyl was added. After a 10 min incubation at room temperature, the suspension was centrifuged at 16,000 x *g* for 10 min at room temperature to remove undissolved nuclear debris. The density of the supernatant was adjusted to 1.42 g/ml with CsCl, divided in portions of 5 ml/tube, and centrifuged in a Beckman SW50 rotor at 40,000 rpm for 72 hrs at 20 °C. Fractions of 250 ml were collected, and the position of the DNA in the gradient was determined by measuring Hoechst 33258-fluorescence in a DNA fluorometer according to the manufacturers instructions (fluorometer TKO 100, Hoefer Scientific Instr., San Francisco). The density of the gradient fractions was determined by refractometry. SCP1 was assayed in each fraction as follows: The fractions were dialysed overnight at 4 °C against 5% glycerol, 1 mM NaEDTA, 0.5 mM NaEGTA, 10 mM Tris.HCl (pH 8.0). Subsequently, 100 μl of undiluted fractions and of serial dilutions (1:10 to 1:10.000) in dialysis buffer were spotted onto nitrocellulose filter. SCP1 and actin in the spots were visualized after successive incubations of the filters in affinity-purified rabbit anti-SCP1 or anti-actin antibodies, goat anti-rabbit alkaline phosphatase conjugate and BCIP/NBT alkaline phosphatase substrate as described before for western blots (41). The intensity of the purple precipitate was determined by a flat bed scanner Agfa Atras II, and quantitated (ImageQuant, version 3.3, Molecular Dynamics Ltd., Kensing, England).

Other procedures

SCs were isolated as described by Heyting *et al.* (22) and Heyting and Dietrich (41). The anti-SCP1 antibodies used in this study were described in Meuwissen *et al.* (25), and the anti-actin antibodies were described in Pool *et al.* (42).

Results

In vitro DNA-binding by SCP1

One-dimensional south-western blot analyses, performed on SC proteins with rat genomic [^{32}P]DNA as a probe, revealed several proteins that are capable of binding DNA. One of these proteins had a relative electrophoretic mobility (M_r) of 125,000 (not shown); this protein could be identical to SCP1, because SCP1 has about the same electrophoretic mobility, and has several so-called S/T-P motifs which are characteristic of DNA-binding proteins (25) (not shown).

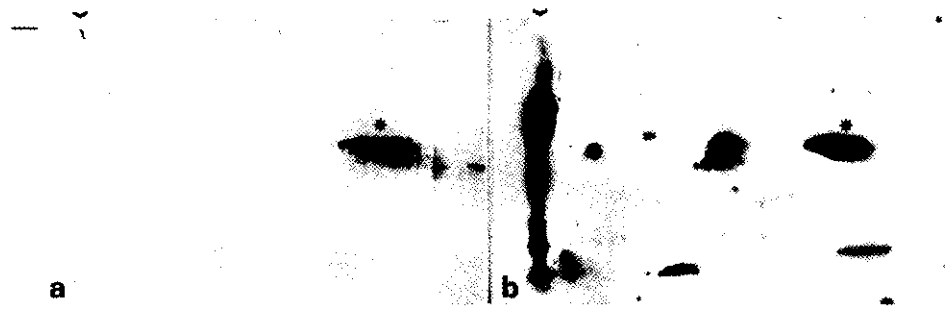

FIGURE 1. Two-dimensional south-western blot analysis of synaptonemal complex proteins. Proteins from $4x10^7$ SCs were separated by two-dimensional gel electrophoresis, transferred to nitrocellulose and probed successively with rat genomic [^{32}P]DNA and anti-SCP1 antibodies. (*a*) The blot after incubation in affinity-purified rabbit anti-SCP1 antibodies and detection of the bound antibodies by goat-anti-rabbit alkaline phosphatase conjugate; (*b*), autoradiogram obtained after probing the same blot with rat genomic [^{32}P]DNA under standard conditions. The position of SCP1 is indicated by an *asterisk*. *Arrowheads* indicate the top of the isoelectric-focussing gel and *horizontal bar* indicates the top of the polyacrylamide-SDS gel.

In a set of preliminary experiments, the optimal NaCl concentration for assaying binding of rat genomic DNA to this 125,000 M_r protein by south-western analysis was assessed at 50 mM. At lower salt concentrations, aspecific binding of DNA to the nitrocellulose filter and to the proteins increased; at higher concentrations, binding of DNA to the 125,000 M_r protein band decreased (not shown).

We therefore used 50 mM NaCl in all further experiments (see Materials and Methods, standard assay). To ascertain that the DNA-binding 125,000 M_r protein was identical to SCP1, we performed a two-dimensional south-western blot analysis on SC proteins with rat genomic [^{32}P]DNA as a probe (Fig. 1). Two distinct spots on the resulting autoradiograph (Fig. 1B), a 125,000 M_r and a 115,000 M_r spot, comigrated with the two spots that were recognized by affinity-purified anti-SCP1 antibodies (Fig. 1A). These spots probably represent the intact SCP1 protein (125,000 M_r) and a proteolytic breakdown product (115,000 M_r). SCP1 is thus capable of binding DNA.

To localize the DNA-binding domain within SCP1, we subcloned three parts of SCP1 cDNA encoding the N-terminal domain (fragment N), a peptide from the middle of SCP1 (fragment M), and the C-terminal domain (fragment C), respectively (see Materials and Methods, and Fig. 2A). These peptides, together with full-length SCP1 (see Materials and Methods, and Fig. 2B), were analysed for their capacity of binding a number of different [^{32}P]DNA probes in the presence of various competitor DNAs by means of south-western blot analysis. As is shown in Fig. 2B, our preparation of SCP1 contains besides the full-length protein a truncated peptide or breakdown product of SCP1, which does not bind DNA. For quantitation of DNA-binding by SCP1, we only measured the amount of [^{32}P]DNA at the position of the full-length protein.

As is shown in Fig. 3, the DNA-binding capacity of SCP1 resides in the C-terminal domain; this was to be expected because the S/T-P motifs are localized in this domain (25); see also Introduction). However, fragment C is a basic peptide (pI=9.7), and it was therefore possible that aspecific electrostatic interactions rather than the S/T-P motifs were responsible for binding of DNA by fragment C. We therefore compared binding of rat genomic [^{32}P]DNA by cytochrome *c* (pI=9.6) with binding by fragment C (pI=9.7) and by full-length SCP1 (pI=6.3). Under none of the conditions tested, cytochrome *c* bound more than 10% of the amount of probe bound by equimolar amounts of fragment C or SCP1 (Fig. 4). Furthermore, binding of rat genomic DNA

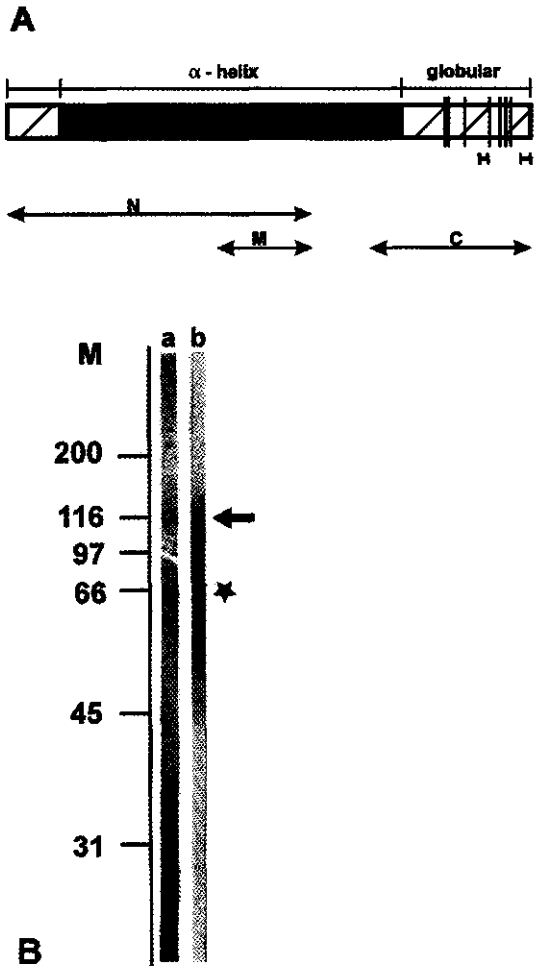

FIGURE 2. Fragments of SCP1 used in this study. (*A*) Schematic representation of the SCP1 molecule. The predicted coiled coil domain is indicated in black, and the predicted non-coiled coil N- and C-terminal domains are hatched. The positions of the fragments N, M and C are indicated by *horizontal arrows*. The small *vertical bars* represent the S/T-P motifs; the positions of the two small basic domains near the C-terminus are underlined. (*B*) Western blot analysis of full-length SCP1, purified from SF9 cells carrying recombinant baculovirus containing the full-length SCP1 cDNA (see Materials and Methods). Lane *a*, coomassie blue stained gel; lane *b*, western blot of the same gel, incubated with mAb IX5B2, which recognizes specifically fragment M of SCP1 (24,25). The *arrow* indicates the full-length protein and the *asterisk* indicates a presumed breakdown product of SCP1 which probably lacks the C-terminus (see text). M indicates the protein standards, which are, from *top* to *bottom*: myosin (M_r=200,000), b-galactosidase (M_r=116,000), phosphorylase B (M_r= 97,400), bovine serum albumin (M_r=66,000), ovalbumin (M_r=45,000) and carbonic anhydrase (M_r=31,000).

by cytochrome *c* did not sustain in the presence of a 1 to 2-fold molar excess *E. coli* DNA, whereas binding by fragment C or SCP1 was hardly affected under these conditions (Fig. 5). This suggests that fragment C and full-length SCP1 have a preference for binding rat genomic DNA rather than *E. coli* DNA, and makes it unlikely that binding of rat genomic DNA to fragment C is exclusively the result of aspecific electrostatic interactions.

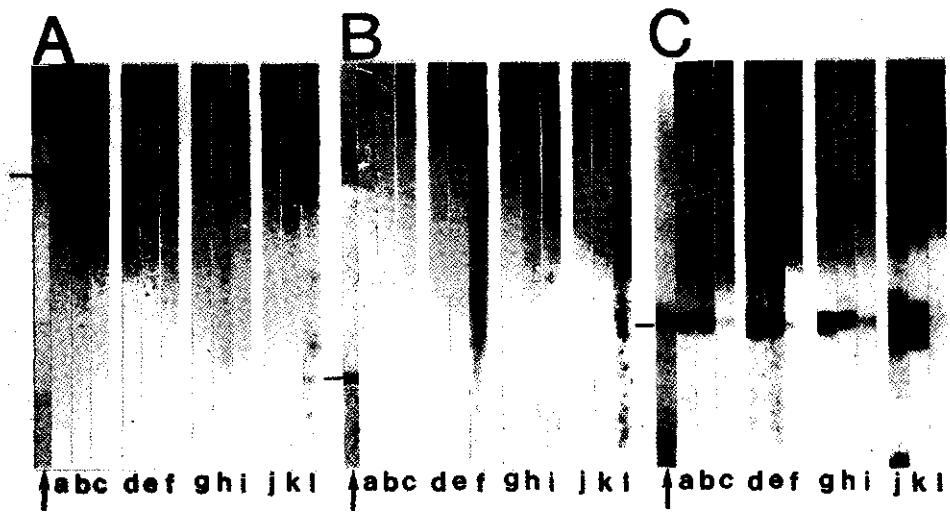

FIGURE 3. Localization of the DNA-binding capacity of SCP1 on the C-terminus. Preparative polyacrylamide-SDS gels were loaded with 2.5 mg of fragment N, M or C per cm slot. After electrophoresis, a 0.5 cm wide strip of each gel was stained with coomassie blue; the remainder of the gels was blotted onto nitrocellulose filters. From these filters, 0.4 cm wide strips were probed with various concentrations of rat genomic [^{32}P]DNA or *Drosophila* SAR [^{32}P]DNA (specific activity: 2.5×10^7 cpm/μg) in the presence of a 1000-fold or a 250-fold excess of unlabeled *E. coli* DNA. All strips were incubated under standard conditions (see Materials and Methods). Legends: panel (*A*) fragment N, analysed on a 10% gel; (*B*) fragment M, analysed on a 12.5% gel; (*C*), fragment C, analysed on a 12.5% gel. Each panel shows from left to right: coomassie blue stained gel (lanes indicated by *arrow*) and 12 autoradiograms of western blot strips of the same gel, incubated in, respectively: strips of lanes *a*, *d*, *g* and *j*: 20 ng/ml; *b*, *e*, *h* and *k*: 10 ng/ml; *c*, *f*, *i* and *l*: 5 ng/ml [^{32}P]DNA probe. Lanes *a-c* were incubated in: rat genomic [^{32}P]DNA + 1000-fold excess *E. coli* DNA; lanes *d-f*: rat genomic [^{32}P]DNA + 250-fold excess *E. coli* DNA; lanes *g-i*: *Drosophila* SAR [^{32}P]DNA + 1000-fold excess *E. coli* DNA; lanes *j-l*: *Drosophila* SAR [^{32}P]DNA + 250-fold excess *E. coli* DNA. The *horizontal bars* show the positions of fragments N, M and C.

Further support for the specificity of the interaction between fragment C and rat genomic DNA is provided by the experiment shown in Fig. 4, which shows the relationship between the amounts of added and bound rat genomic DNA for fragment C (Fig. 4A) and SCP1 (Fig. 4B).

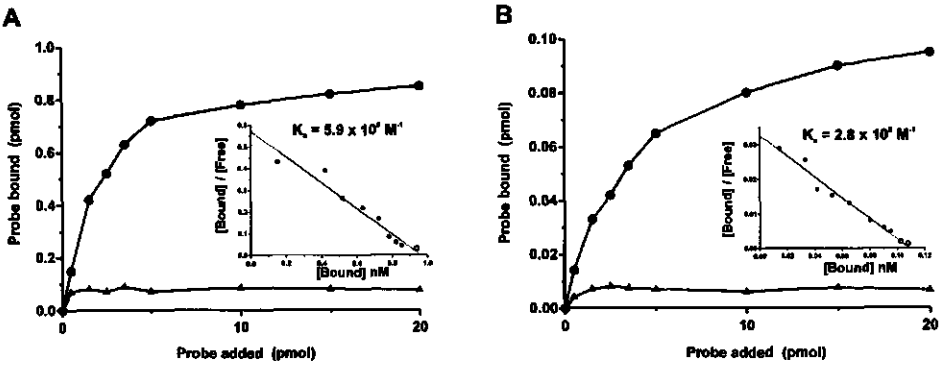

FIGURE 4. Binding of rat genomic DNA to full-length SCP1 and to fragment C. (*A*) Binding of rat genomic [^{32}P]DNA to fragment C (●) or cytochrome *c* (▲). 0.4 cm wide western blot strips, each containing 25 pmol of fragment C or 5 pmol of cytochrome *c*, were separately incubated in 1 ml of binding buffer containing the indicated amount of rat genomic [^{32}P]DNA probe (spec. act. 5 x 10^3 cpm/ pmol). After incubation and washes, the amount of probe bound to the protein was determined by means of a phosphor-imager. (*B*) Binding of rat genomic [^{32}P]DNA to full-length SCP1 (●) or cytochrome *c* (▲). 0.4 cm wide western blot strips, each containing 2.1 pmol of full-length SCP1 or 5 pmol cytochrome *c*, were separately incubated in 1 ml of binding buffer containing the indicated amount of rat genomic [^{32}P]DNA probe (specific activity, 5x10^3 cpm/pmol). Insets: Scatchard plots (47) of the data presented in panel *A* and *B* (●) and Fig. 5A and 6A (O). The association constants (K_a) as measured in these particular experiments, are shown in the insets.

The Scatchard plots (47) of these data (insets in Fig. 4A and B) are linear for the whole range of DNA probe concentrations tested. This implies that in the south-western blot assay, DNA-binding by SCP1 or fragment C follows second order kinetics, and that only one type of binding site (with respect to the association constant K_a) on the protein is involved in the binding of rat genomic DNA. Because protein molecules are fixed on a filter in the south-western blot assay, and cannot form dimers

or multimers, one protein molecule will probably bind one DNA molecule in this assay. The horizontal intercepts in Fig. 4A and B correspond to the maximum amount of DNA that can be bound in this assay.

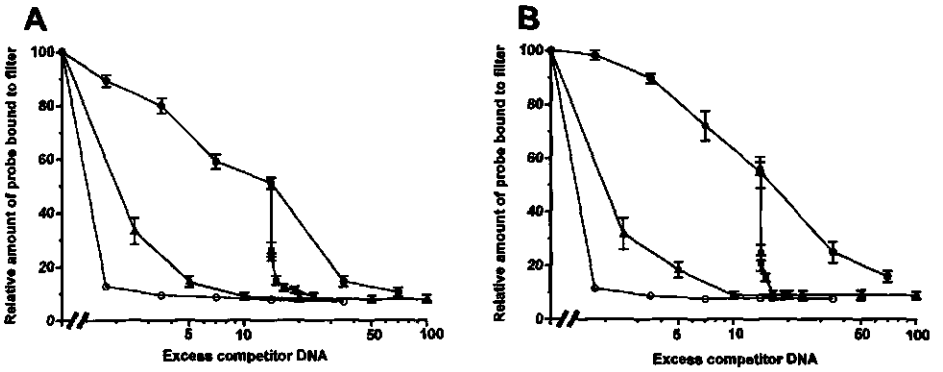

FIGURE 5. Competition of various DNAs for binding to fragment C. (*A*) Competition of unlabeled *E. coli* DNA with rat genomic [^{32}P]DNA for binding to fragment C or cytochrome *c*. Identical 0.4 cm wide western blot strips, each containing 25 pmol of fragment C or 5 pmol cytochrome *c*, were separately incubated in 1 ml of binding buffer containing 20 pmol of rat genomic [^{32}P]DNA probe ($8x10^3$ cpm/pmol) plus the indicated molar excesses of competitor DNAs. As competitor DNA was added: (▲), unlabeled rat genomic DNA; (●), *E. coli* DNA; and (■), a constant amount of *E. coli* DNA (280 pmol, which is a 14-fold molar excess) supplemented with an increasing amount of unlabeled rat genomic DNA. Only *E. coli* DNA was used as competitor DNA (O) for the incubations with cytochrome *c*. (*B*) Competition of unlabeled *E. coli* DNA or rat genomic DNA with *Drosophila* SAR [^{32}P]DNA for binding to fragment C. The incubation conditions are the same as in panel A, except that *Drosophila* SAR [^{32}P]DNA ($7x10^3$ cpm/pmol) was used as a probe. *Vertical bars* indicate the range of three measurements which were performed in parallel. For cytochrome *c* (O), only a single measurement per amount of competitor DNA was performed.

For SCP1, this is 0.11 pmol and for fragment C 0.95 pmol. If, as seems likely (see above), there is a 1:1 stoichiometry of protein and DNA molecules in the protein-DNA complexes on the filter, this means that 5% of the SCP1 molecules and 4% of the molecules of fragment C on the filter strips were capable of binding rat genomic DNA.

The K_a values deduced from Fig. 4A and B are $3x10^8$ M^{-1} (binding of rat genomic DNA to SCP1) and $6x10^8$ M^{-1} (binding of rat genomic DNA to fragment C) (Table I). To analyse the relationship between added and bound DNA for amounts of added rat genomic DNA larger than 20 pmol, we used the data presented in Fig. 5A and 6A. The insets in Fig. 4A and 4B show that for these larger amounts of probe the Scatchard plots are still linear, and that essentially the same conclusions can be drawn with respect to kinetics, K_a values and specificity as were drawn for smaller amounts of probe.

TABLE I ***Association constants for binding of various DNA probes to SCP1 or fragment C***

Protein	**Probe**	**K_a (M^{-1})**	
		Independent measurements	Average
Fragment C	Rat genomic DNA	3	6.0×10^8
	Drosophila SAR DNA	3	4.7×10^8
	E. coli DNA	3	3.8×10^7
SCP1	Rat genomic DNA	2	3.0×10^8
	Drosophila SAR DNA	2	2.8×10^8

The DNA probes are described in Materials and Methods. The K_a values were determined by Scatchard analysis (47).

To analyse the specificity of DNA-binding by fragment C and SCP1 further, we performed the competition experiments presented in Fig. 5 and 6. Fig. 5A shows that fragment C has a considerably higher affinity for rat genomic DNA than for *E. coli* DNA. The association constants are $3.8x10^7$ M^{-1} for binding of *E. coli* DNA, and $6.0x10^8$ M^{-1} for binding of rat genomic DNA (Table I). Fig. 5B shows that fragment C also has a higher affinity for a scaffold attachment region (SAR) of *Drosophila melanogaster* than for *E. coli* DNA. The K_a values for binding of rat genomic DNA and *Drosophila* SAR DNA to fragment C are similar (Table I). Even in competition experiments involving rat genomic DNA and *Drosophila* SAR DNA (Fig. 6) we could

not detect a significant difference between these two types of DNA with respect to their affinities for SCP1.

It has been suggested that the S/T-P motifs contribute to DNA-binding by interaction with the minor groove of DNA, preferably in AT-rich stretches (48,49). We therefore tested whether fragment C interacts with DNA through contacts with the minor groove, by analysis of DNA-binding in the presence of distamycin A, a drug which binds specifically to the minor groove of AT-rich DNA (50-53).

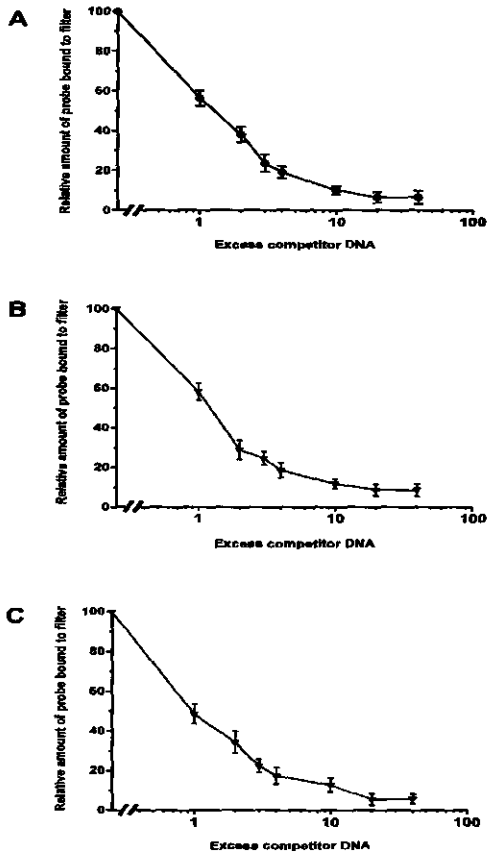

FIGURE 6. Competition between rat genomic DNA and *Drosophila* SAR DNA for binding to full-length SCP1. (*A*) Competition of unlabeled rat genomic DNA with rat genomic [^{32}P]DNA for binding to full-length SCP1. 0.4 cm wide western blot strips, each containing 2.1 pmol full-length SCP1 were separately incubated in 1 ml binding buffer containing 20 pmol rat genomic [^{32}P]DNA probe and the indicated amounts (given in molar excess) of unlabeled rat genomic competitor DNA. (*B*) As panel A, except that unlabeled *Drosophila* SAR DNA was used as competitor DNA. (*C*) As panel A, except that 20 pmol *Drosophila* SAR [^{32}P]DNA was used as a probe. *Vertical bars* indicate the range of two measurements which were performed in parallel.

Fig. 7 shows that at high concentrations of distamycin A, the binding of both rat genomic DNA and *Drosophila* SAR DNA are quantitatively inhibited. However, at lower distamycin A concentrations, binding of *Drosophila* SAR DNA is inhibited

more effectively than binding of rat genomic DNA. Similar results were obtained under identical conditions for SCP1 (data not shown). As a control for the specificity of distamycin A for the minor groove of DNA, we analysed whether this drug inhibited binding of the Oct-1 POU domain to its target sequence under the same conditions as were used for fragment C (see Materials and Methods). The 19.7 kDa Oct-1 POU domain (34) consists of two separate DNA binding subdomains: the N-terminal 71 amino acid POU-specific domain, unique to the POU domain family (54) and a C-terminal 59 amino acid POU homeodomain related to the classical homeodomains (55). Both subdomains are connected by a 23 amino acid linker region. The Oct-1 POU domain recognizes the octamer 5' ATGCAAAT 3' (56) and binds specifically to the major groove. Distamycin A can only inhibit the interactions of the Oct-1 POU domain with its target sequence at concentrations higher than 50 μM (Fig. 7). This indicates that the specificity of distamycin A for the minor groove is maintained under our experimental conditions.

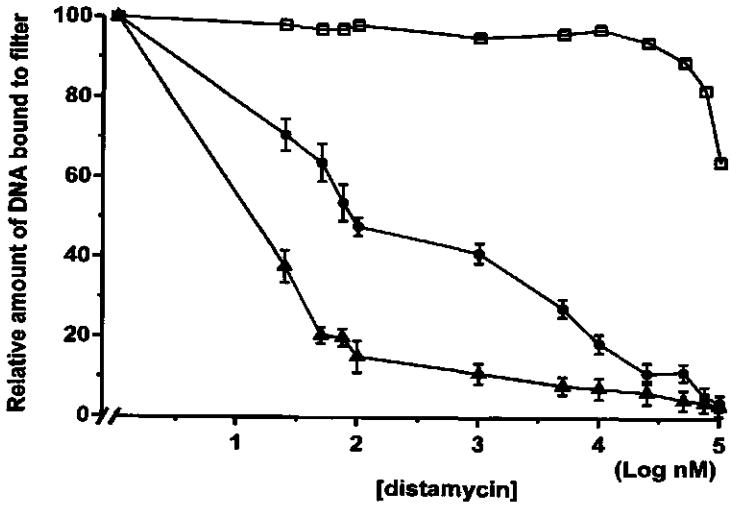

FIGURE 7. Distamycin A inhibits binding of DNA to fragment C. 0.4 cm wide western blot strips, each containing 2.5 pmol of fragment C, were separately incubated in 1 ml of binding buffer containing either 10 μg of rat genomic [^{32}P]DNA ($1.5x10^4$ cpm/μg) (●), or 10 μg of *Drosophila* SAR [^{32}P]DNA fragment ($2.4x10^4$ cpm/μg) (▲).0.4 cm wide strips containing 2.5 pmol Oct-1 POU domain were incubated in 1 ml binding buffer containing 10 ng oct1 linker [^{32}P]DNA ($3.1x10^7$ cpm/μg) (□). Distamycin A was added to the binding buffer containing the [^{32}P]DNA to the indicated final concentrations 15 min before incubation with a western blot strip (see Materials and Methods). Incubation and washing steps were performed according to the standard assay (see Materials and Methods). *Vertical bars* indicate the range of two measurements which were performed in parallel.

Binding of SCP1 to DNA *in vivo*

To analyse whether SCP1 binds to DNA *in vivo*, we treated isolated, intact spermatocytes with paraformaldehyde (PFA), using a procedure described by Solomon *et al.* (57) and Orlando and Paro (58). PFA crosslinks can span a distance of at most 0.2 nm (57). Under the crosslinking conditions employed, a large fraction of the histones is expected to bind to DNA, whereas intracellular protein-protein crosslinking is minimal (57). As a consequence of this crosslinking, DNA from PFA-treated cells bands in isopycnic CsCl gradients at a density of about 1.4 g/cm^3, which is the density of PFA-fixed DNA-protein complexes (57). In contrast, DNA from untreated cells is found at the bottom of the CsCl gradient tubes, at a density of ≥1.62 g/cm^3 (Fig. 8B); this corresponds to the density of free or nearly free rat genomic DNA. Although the bulk of SCP1 from PFA-treated cells floats on top of the isopycnic CsCl gradients (Fig. 8A), and was thus not crosslinked to DNA, a small but significant fraction of SCP1 (about 2%) bands at the same density as PFA-fixed DNA-protein complexes (Fig. 8A). This was found reproducibly in three independent experiments (not shown). In contrast, SCP1 from untreated cells floats quantitatively on top of the CsCl gradients (Fig. 8A). As a negative control we analysed the distribution of actin, which is not supposed to bind to DNA, throughout the isopycnic gradients. We found that actin, both from PFA-treated and from untreated spermatocytes, does not band at the density of PFA-fixed DNA-protein complexes, but floats quantitatively on top of the gradients (Fig. 8C)

Discussion

Characterization of DNA-binding by SCP1

The experiments presented in this paper show that a major component of synaptonemal complexes, SCP1, is capable of binding DNA, and that the DNA-binding capacity resides in the C-terminal domain of the protein. Furthermore, they show that SCP1 interacts with the minor groove of DNA, because binding can be completely inhibited by distamycin A, a drug which binds exclusively to the minor groove, and has very little effect on the secondary structure of DNA (59,60).

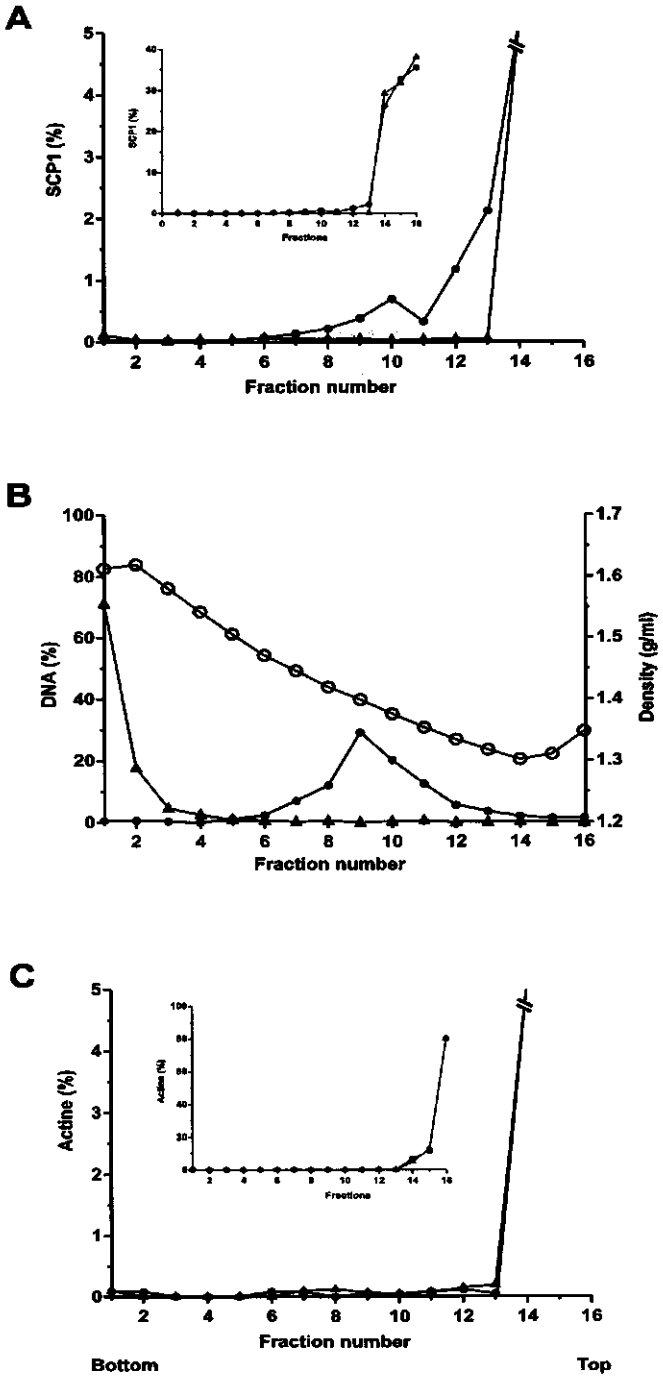

FIGURE 8. *In vivo* crosslinking of SCP1 to DNA by paraformaldehyde (PFA) treatment. Nuclei from PFA treated (●) or untreated (▲) spermatocytes were sonicated, dissolved in sarkosyl and centrifuged to equilibrium in sarkosyl-containing CsCl gradients. The gradients were fractionated and the SCP1 content (panel *A*), DNA content (panel *B*, closed symbols), density (panel *B*, open symbols) and actin content (panel *C*) were determined in each fraction. The vertical axes to the left represent the percentage of total SCP1, DNA or actin in the gradient that is present in each fraction. The vertical axis to the right in panel *B*, represents the density (O) for each fraction as determined by refractometry. The densities of fractions 15 and 16 are probably overestimated by refractometry because of the high concentrations sarkosyl and proteins in this fractions. The graphs in panel *A* and *C* show in detail the relative amounts of SCP1 and actin at the density of DNA-protein complexes (about 1.4 g/ml); the insets of these graphs show the distribution of SCP1 and actin throughout the gradients.

The S/T-P motifs are probably involved in this interaction, because the DNA-binding domain lies within the C-terminal fragment, which contains all S/T-P motifs, and because other DNA-binding proteins carrying these motifs interact with the minor groove of DNA (49,61). Other amino acid sequences that possibly contribute to the interaction of SCP1 with the minor groove are the two predicted basic α-helices, between amino acid residues 897 to 909, and 970 to 992. One of these helices (residues 970 to 992) has significant homology with the DNA-binding domain of a murine protein tyrosine phosphatase (29,62).

In the south-western blot assay used in this study, binding of DNA to SCP1 follows second order kinetics. We therefore suppose that one molecule of SCP1 binds one DNA molecule. The Scatchard plots in Fig. 4A and B also suggest that only one type of binding site is involved in the binding of DNA. Apparently, the C-terminal domain as a whole serves as a single binding site, and cooperative binding does not occur, at least not in this assay. Generally, proteins binding exclusively to the minor groove, such as SATB1 (63,64) or HMG-I/Y (48,49,65), do not recognize specific nucleotides or nucleotide sequences, although they may have a preference for certain structural characteristics (64,66,67). This is to be expected because the minor groove provides little opportunity for distinguishing between nucleotides, although one protein, SRY, can recognize nucleotides in the minor groove (68). The competition experiments in Fig. 5 and Fig. 6 provide evidence for some specificity of SCP1 and fragment C, because the association constant, K_a, for binding of rat genomic DNA to fragment C is 10 to 15-fold higher than for binding of *E. coli* DNA (Table I). The competition experiments with *Drosophila* SAR DNA (Fig. 6) and distamycin A (Fig. 7) provide further information about the specificity of SCP1; although SCP1 has the same affinity for *Drosophila* SAR DNA as for rat genomic DNA, distamycin A competes more effectively with SCP1 for binding to *Drosophila* SAR DNA than for binding to rat genomic DNA (Fig. 7). Apparently, SCP1 has a preference for a broader range of sequences than distamycin A, which binds almost exclusively to poly-dAT stretches (50,69,70). It is possible that on *Drosophila* SAR DNA, which is rich in poly-dAT stretches (45,71), SCP1 binds predominantly to these stretches, where distamycin A can effectively compete with SCP1 for binding. On rat genomic DNA, however, it is possible that SCP1 binds with equal affinity to sequences that are not pure poly-dAT, so that distamycin A will compete less effectively with SCP1 for binding to rat

genomic DNA than for binding to SAR DNA. Because in mitotic chromosomes, SARs are supposed to constitute the basis of the chromatin loops (72,73), it will be of interest to sort out whether SCP1 binds to other DNA sequences than SARs *in vivo*.

Biological relevance

To judge the biological relevance of DNA-binding by SCP1, it is important to know whether SCP1 binds to DNA *in vivo*, and, if so, to which sequences. We therefore started to study these questions, following an *in vivo* crosslinking approach (57,58).

The experiment in Fig. 8 shows that about 2% of SCP1 from PFA-fixed spermatocytes is found at the density of DNA-protein complexes in sarkosyl-containing isopycnic CsCl gradients, whereas SCP1 from unfixed cells is not found at this position (Fig. 8A). This result was reproducibly found in three independent experiments (not shown). Actin, a protein which is not supposed to bind to DNA, is not found at the position of DNA- protein complexes after PFA fixation (Fig. 8C). Because PFA-induced crosslinks can span not more than 0.2 nm (57), we conclude that the PFA-produced SCP1-DNA crosslinks are due to sarkosyl-sensitive interactions between SCP1 and DNA *in vivo*. Under the fixation conditions employed, most of the histones are crosslinked to the DNA (Fig. 8B; 57), whereas only 2% of SCP1 is crosslinked. There are several possible reasons why SCP1 is crosslinked less effectively to DNA than histones: first, it is possible that histones make more contacts with DNA than SCP1, and/or that they make contacts that are more easily crosslinked by PFA than the *in vivo* contacts between SCP1 and DNA. Second, it is possible that only a small fraction of SCP1 molecules interacts with DNA at any time during meiotic prophase. Third, it is possible that SCP1 interacts with DNA during a short period of meiotic prophase, or during a substage of meiotic prophase that is not well represented in the population of purified spermatocytes that was used for crosslinking. These possible explanations will be subjects of further investigations.

The K_a value for binding of rat genomic DNA to SCP1, as measured in the south-western blot assay, is about $3x10^8$ M^{-1} (Table I). This is high compared to the values measured for non sequence-specific interaction of other proteins with DNA, but at least one order of magnitude lower than what has been measured for binding of proteins to their specific target sequence. For instance, the association constant for non sequence-specific interaction of human TATA binding protein (TBP) with DNA is

$2.7x10^6$ M^{-1}, whereas the K_a value for binding to its specific target sequence, the TATA box, is $2x10^9$ M^{-1} (74). However, it should be kept in mind that the K_a value measured for binding of SCP1 to DNA, $3x10^8$ M^{-1}, is a minimum estimate for native SCP1, because it is uncertain whether SCP1 molecules renature completely after SDS-electrophoresis and blotting. Furthermore, it seems likely that SCP1 molecules form dimers *in vivo* (25), which will possibly bind with a higher affinity to DNA than the SCP1 monomers on the south-western blots.

It is difficult to consider the biological significance of DNA-binding by SCP1 without considering the function of the protein. One possible clue with respect to its function is the work on the yeast Zip1 protein. SCP1 and Zip1p have similar predicted secondary structures, and a similar localization within meiotic prophase nuclei (4,20,24,25,75). Zip1p is essential for chromosome synapsis (20) and crossover interference (21). Presumably, Zip1p associates with protein-DNA complexes containing early recombination intermediates (76). It is possible that Zip1p brings these intermediates within the context of the SC by interaction with the LEs, and that from these sites synapsis proceeds along the bivalents by association of more Zip1 protein molecules with each other and with the LEs. The correlation between synapsis and interference can be explained if crossing over is prevented in those parts of the bivalents where synapsis has been established (21). DNA-binding by Zip1p could play a role in the association of Zip1p with either the recombination intermediates, or the LEs, or both. The same can be hypothesized for SCP1. However, it is still an open question whether SCP1 and Zip1p are functional homologs. Although both proteins have a similar predicted secondary structure, there are also differences: Zip1p has a shorter predicted coiled coil domain than SCP1, and, in contrast to SCP1, Zip1p has S/T-P motifs at both ends (20). The characterization of the DNA sequences bound to SCP1 *in vivo*, and the analysis of the fate of these sequences throughout meiotic prophase should provide more information about the functional relationship between Zip1p of yeast and SCP1 of the rat.

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CHAPTER 5

Organization of SCP1 protein molecules within synaptonemal complexes of the rat

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Abstract

SCP1, a major protein component of synaptonemal complexes (SCs), is probably a constituent of the transverse filaments (TFs). The protein consists of three domains: a short, proline-rich N-terminal part, a stretch of 700 amino acid residues capable of forming an amphipathic α-helix, and a C-terminal domain of 240 amino acid residues which is capable of binding to DNA. To analyze the orientation of SCP1 molecules within SCs, we elicited polyclonal antibodies against three non-overlapping fragments of SCP1, which comprise, respectively, the N-terminus, the C-terminus and a fragment from the middle of the SCP1 molecule. Using these antibodies, we performed immuno-electron microscopy on SCs in two types of preparations, namely surface-spread spermatocytes and ultrathin sections of Lowicryl-embedded testicular tissue of the rat. For each of the three antibodies used, the distribution of immunogold label on surface-spread spermatocytes differed significantly from the distribution of label on sections. Masking of SCP1 epitopes within the lateral elements (LEs) and the central element (CE) of SCs in surface-spread preparations and the influence of the surface morphology of the spreads on the labeling pattern were considered as possible explanations for these differences. We therefore relied on the results from sections for the localization of epitopes. On the basis of the distributions of immunogold label in Lowicryl sections and the predicted secondary structure and dimensions of SCP1 molecules, we present the following model: the C-terminus of SCP1 molecules lies in the inner half of the LE, the molecules protrude from the LE through the central region into the CE, and end up with their N-terminus between the centre of the CE and the opposite LE, so that the N-termini of SCP1 molecules from opposite LEs overlap. The model has several implications for the assembly of SCs and the possible functions of SCP1.

Introduction

The condensation and pairing of meiotic prophase chromosomes are generally accompanied by the assembly and disassembly of synaptonemal complexes (SCs) [1]. SCs consist of two compact axial cores, one along each homologue, the lateral elements (LEs), which appear to be directly connected by 100-nm-long transverse filaments (TFs) [2]. On the TFs, between the LEs, there is a third longitudinal structure, the central element (CE). The LEs together with the CE make up the tripartite structure of the SC [1]. In most species, (stretches of) LEs are assembled first, and are later brought together [1, 3] by fine fibres, which are attached to each LE and seem to fuse to form TFs [4]. This process of synapsis seems to be reversible, because the pattern of synapsis can change during progression through meiotic prophase [1, 5, 6].

Several protein components of SCs of rodents [7, 8, 9-12] and yeast [13] have been identified, including putative components of TFs [7, 8, 13], and genes or cDNAs encoding these components have been cloned [8, 10, 11, 13]. In this paper we concentrate on SCP1, a protein which seems likely to be a major component of TFs. The SCP1 protein [7] and the corresponding cDNA [8] were first identified and characterized in the rat. SCP1 colocalizes with TFs and its predicted secondary structure is similar to that of filamentous proteins: the major part of SCP1, a stretch of 700 amino acid residues, is predicted to form an amphipathic α-helix capable of forming coiled-coil structures [8]; the predicted length of the α-helix is 100 nm, which corresponds to the distance between the inner edges of the two LEs. This stretch is flanked by a small, predicted globular N-terminal domain, and a predicted globular C-terminal domain containing S/T-P motifs (a serine or threonine residue followed by a proline residue), which are common in a variety of DNA-binding proteins, and which allow non-sequence-specific interactions of the protein with the minor groove of DNA [14]. The C-terminal domain is capable of binding to DNA (Meuwissen, unpublished experiments).

Elucidation of the organization of SCP1 molecules within SCs will provide information about how SCP1 is involved in the assembly of SCs, in particular how the TFs are integrated in the tripartite structure. First of all, a perpendicular orientation of SCP1 molecules relative to the LEs will further support the idea that SCP1 makes part of the TFs [8]. Second, the localization of the C-terminal domain with its DNA-binding characteristics can provide information about the attachment of meiotic prophase

chromatin to the tripartite SC. And third, the position of the N-terminus relative to the C-terminus is relevant to several questions concerning the mechanism of synapsis: can individual SCP1 molecules make direct connections between opposite LEs, or is synapsis established by interaction between SCP1 molecules that protrude from opposite LEs, or are other proteins than SCP1 required for the establishment or maintenance of synapsis?

The predicted dimensions and features of SCP1, together with the regular, repetitive structure of the SC, allow the analysis of the organization of SCP1 molecules by the ultrastructural immunolocalization of SCP1 domains within SCs. On the basis of preliminary immunogold labeling experiments, we [8] and others [16] have tentatively suggested that SCP1 molecules are oriented with their C-terminus towards the LEs. These experiments were performed with polyclonal antisera elicited against largely overlapping fragments of SCP1, with considerable parts of both the C- and N-terminus excluded [16], or with a combination of a polyclonal antiserum against the whole SCP1 molecule and a monoclonal antibody [8]. Furthermore, these experiments were performed on surface spreads of spermatocytes, which, as it appears from this study, are not suitable for a precise comparison of immunogold labeling patterns. For the analysis of the organization of SCP1 molecules within SCs, we therefore prepared three antisera which recognize specifically limited, non-overlapping fragments of SCP1, which cover the N-terminus, the C-terminus and a fragment from the middle of the SCP1 molecule. Using these antisera, we analyzed the organization of SCP1 molecules within SCs by means of immunogold labeling. We performed the labeling on two types of preparations: surface-spread spermatocytes, to allow the correlation of this study with earlier work [7, 8, 16], and ultrathin sections of Lowicryl-embedded testicular tissue. We obtained different labeling patterns on surface spreads than on sections. In the surface-spread preparations, the label intensity was locally low over the regions of the LEs and the CE, creating sharp dips in the distributions of label, while in the sections the label over these regions showed smooth peaks. We explain the dips in the labeling pattern of surface spreads by effects of the surface morphology on the distribution of gold label and/or masking of epitopes on the SCP1 molecule by LE or CE components. We therefore consider the results obtained on sections more reliable. From the labeling patterns on sections we conclude that the C-terminus of the SCP1 molecules lies in the inner half of the LEs, and that the SCP1 molecules extend from the LE into the CE, so that the N-

terminal domains overlap; the SCP1 molecules end up with their N-terminus between the centre of the CE and the opposite LE.

Materials and Methods

Antibodies. For the preparation of antisera against fragments of SCP1, we subcloned three fragments of SCP1 cDNA, encoding, respectively, the 371 N-terminal amino acid residues (the 5' fragment), the 221 C-terminal residues (the 3' fragment), and amino acid residues 415 - 549 of SCP1 (the middle fragment). The 5' fragment includes the codons for 51 amino acid residues N-terminal to what was originally considered the first methionine of SCP1 [8]; it thus encodes the 371 utmost N-terminal residues of SCP1 [17]. The 5' fragment was subcloned in pBluescript (Stratagene, San Diego, California, USA), in frame with the fragment of the lacI gene on this vector, and E. coli XL1-Blue cells (Stratagene) were transformed with the resulting construct. Synthesis of the fusion protein encoded by this construct was then induced by the addition of isopropyl-b-D-thiogalactopyranoside (IPTG) to a culture of the transformed cells, and inclusion bodies containing the fusion protein were isolated from these cells according to Sambrook et al. [18], and washed according to Harlow and Lane [19]. A rabbit was immunized with these inclusion bodies according to the immunization protocol described by Meuwissen et al. [8], to yield serum 458 (further indicated as "anti-N"). The middle fragment and the 3' fragment were subcloned in the pQE30 vector (Qiagen, Chatsworth, CA, USA), in frame with the fragment of the lacI gene and six successive histidine codons on this vector. E. coli SG 13009 cells (Qiagen) were transformed with the resulting constructs, and synthesis of the encoded fusion proteins was induced by means of IPTG. The fusion proteins were then purified from bacterial lysates by affinity chromatography on nickel columns according to the instructions of the supplier of the columns (Qiagen), and antisera against the middle fragment (serum 461 or "anti-M") and the C-terminal fragment (serum 471 or "anti-C") of SCP1 were elicited by immunization of rabbits according to the schedule described by Meuwissen et al. [8]. We affinity-purified the anti-N and anti-M antibodies from serum 461 and 458 respectively, using strips of western blots containing the N-terminal or middle fragment of SCP1, by a procedure described earlier [10]. The anti-C antibodies bound so strongly to affinity columns or western blot strips containing the C-terminal fragment of SCP1, that they could not be eluted without losing most of their reactivity; these antibodies were therefore not affinity-purified from serum 471.

Immunogold labeling. Cell suspensions from rat testes were prepared and processed for surface spreading as described before [20]; the surface-spreading procedure includes treatment of the surface-spread cells with DNAse I and SDS [20]. For the preparation of ultrathin sections, testicular material was fixed and embedded in Lowicryl K11M as previously described [21], and 80- to 100-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 [22], except that E. coli lysate was added to a final concentration of 1 mg E. coli protein/ml to the antibody dilution buffer ("holding buffer" [22]). Affinity-purified anti-N and anti-M antibodies were used undiluted; the anti-C antiserum was diluted 1:5; goat-anti-rabbit antibodies conjugated to 10-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 followed by lead citrate [20]. Specimens were photographed in a Philips EM 420 electron microscope operated at 80 or 100 kV, at 15,000 or 18,000x magnification.

Analysis of the labeling patterns. After incubation of sections or surface spreads in preimmune serum, we found a low level of background labeling, which was not concentrated above the SCs, but was distributed evenly over the preparations (experiments not shown). The immunogold labeling patterns obtained after incubation in the immune serum were analyzed as follows: grains were collected within an area corresponding to three LE-LE distances with the SC in the centre. At the position of each grain, the distance between the centres of the LEs (one LE-LE distance) was determined, and this distance was set at 100 arbitrary units (on average, one unit corresponds to 1.8 nm); the distance of the grain to the centre of the nearest LE (as defined by the uranyl acetate/lead citrate staining, see Fig. 3) was then measured, and expressed in the same units. Subsequently, the distribution of the collected grains over 30 classes, each comprising 10 distance units, was assessed. As the level of background label we took the average number of grains in the 5 outer classes on each side, i.e., further than 50 distance units (90 nm) from the centre of the nearest LE. Thus, we obtained an estimate of the background level using the same serum bleeding in the same immune incubation on the same section as we used for the specific immunogold labeling of SCP1.We performed the measurements on electron micrographs which were digitized by means of a CCD-camera, using a Quantimet 500 computer and the Quantimet 15 QUIN 2.0 program (Leica, Cambridge, UK). After reduction of the background labeling, the distributions of grains were compared by the Kolmogorov-Smirnov two-sample test [23] (Tables 1 and 2). The data obtained on sections were further analyzed as follows: we assumed that the SC is symmetrical relative to the CE, and that the distribution of gold grains over the SC should therefore also be symmetrical relative to the CE. Thus, the obtained distribution of grains should be mirrored relative to the centre of the CE (as is done in Fig. 4) in order to correlate it with the structure of the SC. We then tested for each antiserum whether, after reduction of the background, these mirrored distributions could be considered as the sum of two normal distributions with equal values, but with opposite sign, for μ (the average distance to the centre of the CE) and identical values for the standard deviation σ (see also Results section and legends of Fig. 4). First, μ and σ of these distributions were estimated by reiteration of the least square method for values of y (the number of grains at a given distance from the CE). Subsequently, the observed distributions of grains were compared with the distributions expected on the basis of the estimated values of μ and σ, by means of the Kolmogorov-Smirnov two-sample test. For all three antisera, there was no significant difference between the observed and expected distributions. The

95% confidence interval of m equals $\mu \pm 1.96 \times \sigma/\sqrt{n}$, where n is the number of grains after reduction of the background in the original, unmirrored distribution.

Other procedures. SDS-polyacrylamide gel electrophoresis of proteins [24, 25] and western blotting [20, 26] were performed according to described procedures.

Results

Antibodies

For the experiments in this paper, we prepared antisera against three non-overlapping fragments of SCP1, termed N, M and C (Fig. 1). On western blots, these antisera recognize the fragments against which they were raised, and also the whole SCP1 protein of synaptonemal complexes (Fig. 1). Most importantly, they do not recognize the fragments against which they were not raised (Fig. 1). At the light microscopic level, the immunoperoxidase labeling patterns obtained with anti-N, anti-M and anti-C antisera on agar filtrates of lysed spermatocytes are indistinguishable: within spermatocytes, all three antisera label specifically the SCs, in particular the synapsed regions (not shown).

Comparison of two preparational techniques for immunogold labeling

For the analysis of the labeling pattern at the ultrastructural level, we performed immunogold labeling of two types of preparations: surface-spread spermatocytes and ultrathin sections of Lowicryl-embedded testicular tissue. The results of these experiments are shown in Figs. 2-4. The labeling patterns on surface spreads and sections differ in several respects. First, the labeling intensity is much lower on sections than on surface spreads (compare Figs. 2 and 3), and therefore, the numbers of grains collected on sections are much smaller than those collected on spreads (Fig. 4). This was to be expected, because immunogold labeling of ultrathin sections is essentially a surface-labeling [27-29], and very few epitopes are exposed at the surface of the sections. In surface spreads, more epitopes will be exposed per length unit of SC than in sections, because the material is not embedded, and because the chromatin surrounding the SCs is much more expanded and disrupted than in sections. Furthermore, we have enhanced the accessibility of epitopes in spreads by pretreatments with DNAse I and SDS [20].

position of the LEs (Fig. 4H). A similar difference between the distributions of label on spreads and sections occurs when anti-N or anti-M are used as primary antisera: on spreads there are

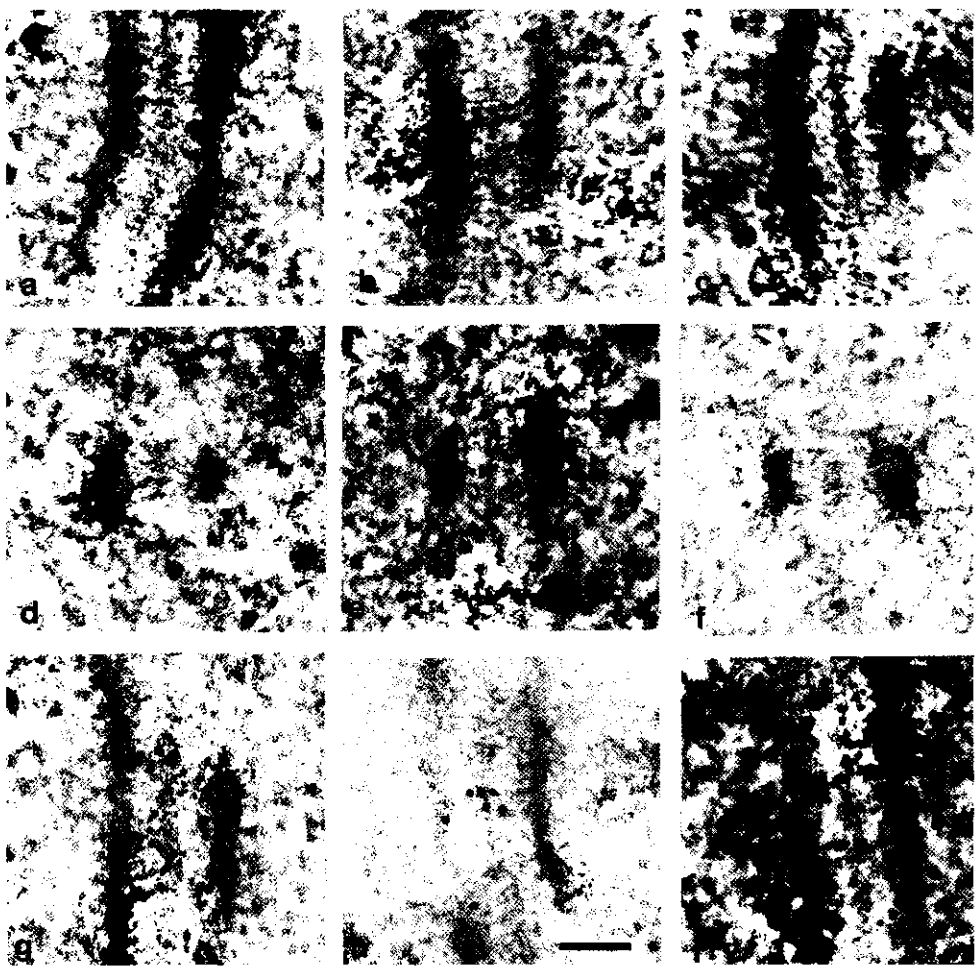

Figure 3. Ultrastructural localization of the fragments N, M and C of SCP1 by indirect immunogold labeling of ultrathin sections of Lowicryl-embedded testicular tissue of the rat, with the antisera anti-N (panels a, d and g), anti-M (panels b, e and h) and anti-C (panels c, f and i) as primary antisera and goat-anti-rabbit IgG conjugated to 10 nm gold as secondary antibodies. Panels a, b, c, g, h and i show frontal sections of SCs, panels d, e and f show cross sections. Note the presence of immunogold label throughout the LEs (panel i and g), CE (panels g and h) and cross-sectioned SCs (panel e). Bar represents 200 nm.

dips at the position of the LEs, whereas such dips are absent or less pronounced on sections (Fig. 4C and F). Using the anti-N and anti-M sera, we also found a more

pronounced dip at the position of the CE on spreads than on sections (Fig. 4C and F). Most likely, the differences between the labeling patterns on surface spreads and on sections are due to some artifacts on the surface spreads. In spreads, the level of immunogold labeling

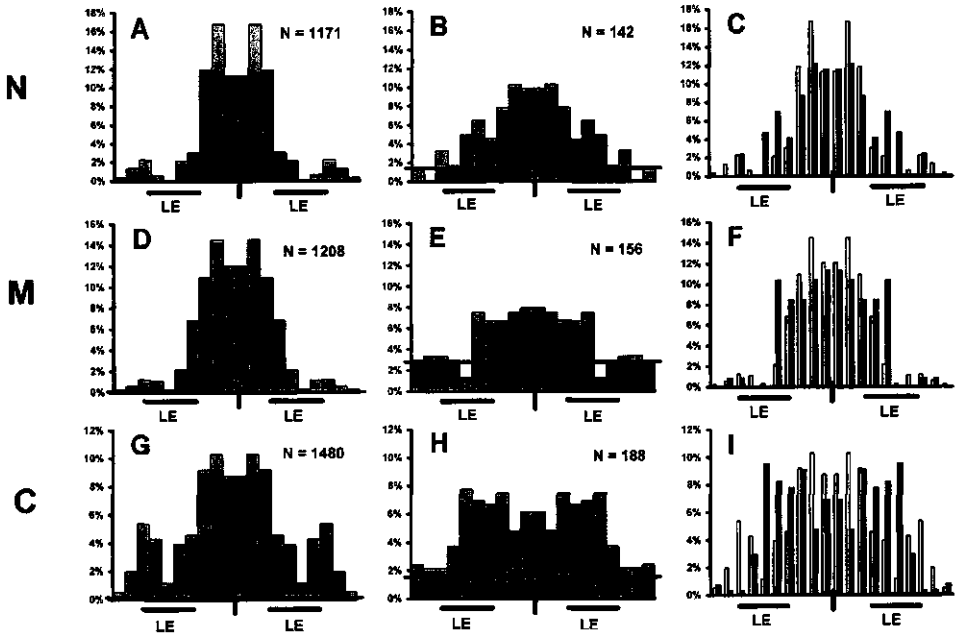

Figure 4. Distribution of immunogold label over SCs in surface spreads and ultrathin sections of Lowicryl-embedded testicular tissue. Panels A, D and G show the distributions of grains on surface spreads, and panels B, E and H the distributions on sections. Panels A, B and C show the results obtained with anti-N; panels D, E and F the results with anti-M, and panels G, H and I the results with anti-C. In panels C, F and I, the distributions of grains on spreads (white bars) are compared with those on sections (black bars), after subtraction of the background. The distributions of label were assessed by measuring the distance of the gold grains to the centre of the CE as described in the Materials and methods section. To correlate the obtained distributions with the structure of the SC, we mirrored them relative to the centre of the CE (indicated by the small, vertical bar on the horizontal axis). Each unit on the horizontal axis represents 1/10th of the distance between the centres of the LEs at the site of the gold grain. The vertical axis represents the percentage of total grains in each class of the mirrored distribution. The lines above the horizontal axes represent the background level (see Materials and methods). In panels A, D and G, these lines almost coincide with the horizontal axes. The bars below the horizontal axes represent the position of the lateral elements as defined by the uranyl acetate/lead citrate staining. N represents the number of grains, including the background, in the original, unmirrored distribution.

is relatively low at the position of the LEs and the CE. Possible explanations for this, which are not mutually exclusive, are the following: first, certain epitopes in surface spreads could be relatively inaccessible to antibodies because of the compactness of these structures; and second, it is possible that the very uneven surface of the spreads [16, 22] has influenced the distribution of gold label [28] (see also Discussion). In sections, such artifacts are unlikely to occur, because immunogold labeling of plastic-embedded sections is exclusively a surface labeling [27-29]. Most likely, the epitopes on the surface of a section represent a random selection of epitopes, each being exposed in relation to its abundance in the sample. Furthermore, the surface of plastic sections is smooth [28], so that the surface morphology of sections will probably not influence the labeling pattern. For the localization of SCP1 domains within SCs, we will therefore rely on the results from sections rather than those from spread preparations.

Table 1 Comparison of the distributions of gold grains found on surface spreads with those found on sections, after immunogold labeling with anti-N, anti-M or anti-C antiserum[a]

Antiserum	Surface spreads versus sections[b]
Anti-M	$0.01 < P < 0.02$
Anti-M	$0.025 < P < 0.05$
Anti-C	$0.001 < P < 0.005$

[a] The fragments of SCP1 against which these antisera are directed are depicted in Fig. 1.

[b] After reduction of the background, the distributions of gold grains were compared by the Kolmogorov-Smirnov two-sample test (see Materials and methods section).

Comparison of the distribution of immunogold label, obtained with anti-N, anti-M and anti-C

Table 2 shows the results of pairwise comparisons, in a parameter-free test, of the distributions of label obtained with anti-N, anti-M and anti-C. For surface spreads, significant differences between distributions were found in all pairwise comparisons. On sections, the distribution of label obtained with anti-C differed significantly from the

distributions obtained with anti-N or anti-M. The distributions found with anti-M and anti-N were not significantly different at the 5% level in this test.

As argued above, we will rely on the results from sections to determine the localization of SCP1 epitopes within SCs. For this purpose we reasoned as follows: we assume that the SC is symmetrical relative to the centre of the CE, and that SCP1 molecules can integrate in only one possible position and orientation relative to the LEs. An antiserum against a fragment of SCP1 will then produce two peaks of gold grains, positioned symmetrically relative to the centre of the CE.

Table 2 Comparison of the distributions of gold grains, obtained by immunogold labeling with anti-N, anti-M or anti-C as primary antiserum[a]

Comparison[b]	Surface spreads	Sections
Anti-C versus anti-M	$P < 0.001$	$0.025 < P < 0.05$
Anti-C versus anti-N	$P < 0.001$	$0.001 < P < 0.005$
Anti-M versus anti-N	$0.01 < P < 0.025$	$0.05 < P < 0.10$

[a] The fragments of SCP1 against which these antisera are directed are depicted in Fig. 1.

[b] After reduction of the background, the distributions of gold grains were compared by the Kolmogorov-Smirnov two-sample test (see Materials and Methods section).

For a monoclonal antibody, the width of each individual peak is determined by the size of the complex of the first and the second antibody and the attached colloidal gold particle, which is about 30 nm [30, 31], and the irregularity of the SC structure. For the polyclonal antisera that we used in this study, the width the peak is further influenced by the size of the fragment that was used for immunization, and the position of recognized epitopes on that fragment. We assume that there are no sharp bends or kinks in the predicted coiled coil domain of SCP1. The N-terminal fragment then covers 39 nm of the predicted coiled coil domain of SCP1 plus the small N-terminal globular domain; the middle fragment covers 19 nm of coiled coil domain, and the C-terminal fragment covers 4 nm of the coiled coil plus the C-terminal globular domain. We expect therefore that labeling with the anti-N antiserum will produce a peak of gold grains at each side of the CE, and that the individual peaks will extend at least about 70 nm to both sides; the anti-C and anti-M antisera were elicited against shorter fragments of SCP1, so for these antisera we expect that the individual peaks will extend slightly less far, but still at least

about 50 nm. Thus, for antisera against components of TFs, the individual peaks on both sides of the centre of the CE will probably overlap in the region of the CE; therefore, the position of epitopes in the SC is not represented by the position of the peaks in the overall distribution of grains in Fig. 4, but by the position of the peaks of the symmetrically positioned individual distributions. The positions of the peaks in the individual distributions were estimated as follows: for all three antisera, the overall distribution of label could be fitted to the sum of two normal distributions, positioned symmetrically relative to the CE and overlapping in the region of the CE (see Materials and Methods). For anti-C, the estimations of m for the individual normal distributions are +65 and -65 nm, and of s 41 nm; for anti-M, the estimations of m are +40 nm and -40 nm and of s 40 nm; and for anti-N the estimations of m are +13 and -13 nm, and for s 59 nm. m corresponds to the average distance of gold grains to the centre of the CE; thus, unless the epitopes on N, M or C are clustered at one side of the fragment, the centre of fragment C (C_M) lies 65 nm from the centre of the CE, M_M 40 nm, and N_M 13 nm. At the bottom of Fig. 5, these estimated positions of N_M, M_M, and C_M within the SC have been indicated, together with their 95% confidence intervals. These intervals do not overlap, so that M_M should lie further from the centre of the CE than N_M, and C_M further than M_M. In order to fit the SCP1 molecules into Fig. 5, we have taken into account some shrinkage of SCP1 molecules during fixation, dehydration, embedding and viewing of the sections [32-34]. The estimation of shrinkage is not critical, because our conclusions will be essentially the same for shrinkage in the range between 0% and 30%. In Fig. 5, we have assumed a linear shrinkage of about 12% [32-34], so that the coiled coil part of SCP1 is represented by bars of 88 nm rather than the 100 nm that is predicted for the native protein. On these bars, we have indicated in black the positions of the coiled coil parts of N, M and C on SCP1, as they are predicted from the amino acid sequence, again assuming that shrinkage was about 12%. The middle of these fragments is indicated by small vertical lines; these lines should fall within the 95% confidence intervals of the positions of N_M, M_M, and C_M, as estimated from the immunogold labeling. This is possible if (i) the C-terminus is located in the inner edge of the LE, which is in agreement with suggestions made earlier [8, 16]; (ii) the SCP1 molecules extend with their N-termini into the CE, where they overlap; and (iii) the N-termini do not reach the opposite LE. To summarize, Fig. 5 shows the model that we propose for the organization of SCP1 molecules in SCs. The assumptions that we have

made for this model are that (i) SCP1 molecules integrate in only one orientation and position relative to the LEs, (ii) the predicted coiled coil domain of SCP1 does not have sharp bends or kinks, (iii) the epitopes on fragments N, M and C are not clustered at one side of the fragment, and (iv) linear shrinkage of SCP1 molecules in Lowicryl sections is in the range of 0-30%. Another uncertainty with respect to the model that we have not yet mentioned explicitly is the conformation and position of the non-coiled coil parts relative to the coiled coil domain of SCP1.

The immunogold labeling of sections also demonstrates one other aspect of the localization of SCP1 epitopes that could not be studied on spreads. SCs of the rat [2] and

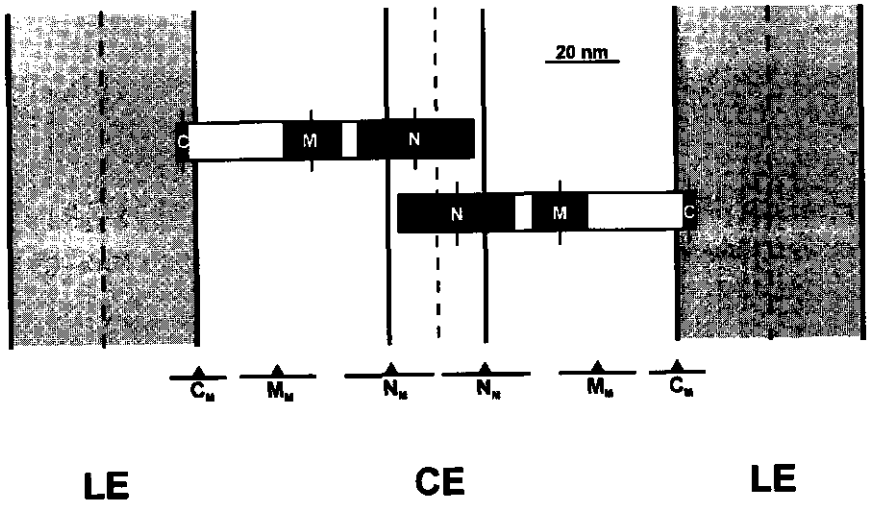

Figure 5. Organization of SCP1 molecules within the SC. The figure is drawn to scale with respect to the Lowicryl sections. The solid vertical lines represent the borders of the LEs and CE, as defined by the zone of intense uranyl acetate/lead citrate staining. The dashed lines represent the positions of the centres of the LEs and the CE. At the bottom of the figure, the arrowheads indicate the positions of the centres of the of fragments N, M and C (N_M, M_M, and C_M), as estimated from the immunogold labeling. The horizontal bars below the arrowheads indicate the 95% confidence intervals for the estimated positions of N_M, M_M and C_M. To fit the SCP1 molecules into this figure, we assumed a shrinkage of the tissue in Lowicryl sections of about 12%. The length of the predicted coiled coil part of SCP1 (represented by the large horizontal bars) in this figure is thus 88 nm rather than the 100 nm that is predicted for the primary amino acid sequence of SCP1. The position of the coiled coil parts of fragments N, M and C on SCP1 are indicated in black. The small vertical lines indicate the position of the middle of the coiled coil part of these fragments. The globular domains at the N and C terminus of SCP1 have not been indicated. The scale bar represents a distance of 20 nm in the Lowicryl sections.

of other organisms [2, 35] are multilayered structures with several layers of TFs on top of each other. The immunogold labelings of sections show that SCP1 epitopes occur throughout the SCs (see cross sections in Fig. 4). This confirms the colocalization of SCP1 with the TFs, and further supports the idea [8] that SCP1 is a major component of the TFs.

Discussion

Methodology

For the analysis of the organization of SCP1 molecules within SCs, antibodies are required that recognize specifically non-overlapping, relatively small fragments of the termini and the middle of SCP1 (Fig. 1), and preparational techniques that do not influence the immunogold labeling patterns. The results in this paper illustrate how important the choice of the preparational technique is: for all three antisera tested, the labeling pattern on surface spreads differs considerably from that on sections (Fig. 4 and Table 1). The difference is most pronounced in the region of the LE, where the relative amount of immunogold label is higher on sections than on surface spreads for all three antibodies. One possible explanation for the differences is, that the reactivity of the antisera depends upon the experimental conditions; for instance, it is possible that another part of fragment C is recognized on spreads than on sections. However, this cannot result in large shifts in the labeling patterns, because the three antisera recognize relatively short fragments within SCP1, and do not recognize other proteins (Fig. 1). Furthermore, there are several reasons to consider the labeling patterns on surface spreads with more caution than those on sections: in surface-spread preparations, the three-dimensional [2, 35] structure of the SC has collapsed on a flat support. These preparations therefore have an uneven surface [22], and probably also local differences in compactness [35]. Both characteristics can influence the labeling pattern: in surface spreads, the LEs and the CE are relatively dense, and it is therefore possible [27] that epitopes within these structures are inaccessible to antibodies. This provides one possible explanation for the relatively low level of labeling of LEs and CEs in surface spreads as compared to sections (Fig. 4 C, F and I; see also Fig. 7 in Ref. 16). Furthermore, the LEs and the CEs become ridges in surface spreads, whereas the regions

between the CE and the LEs form two clefts, as is evident in shadow cast preparations [16, 22]. By indirect immunogold labeling, the gold grain is deposited within a radius of about 30 nm from the epitope to which the first antibody has bound [31]. It is possible that within this radius the gold grain settles preferably at those sites where it can make several contacts with the surrounding preparation [28]. Ridges in the preparation, like LEs and CEs in surface spreads, are at a disadvantage in this respect. This could provide another explanation for the presence of more pronounced dips in the distribution of label at the position of LEs and CEs in surface spreads than in sections. Other factors that can influence the results from surface spreads but not from sections are the deformation of the SC structure by surface spreading, the use of low concentrations of SDS [20], and the fact that the uneven surface of spread SCs is projected on flat electron micrographs; this causes an overestimation of the labeling intensity of all surfaces in the preparation that were not parallel to the photographic film. It is also possible that combinations of these factors have caused the differences between the labeling patterns of surface spreads and sections. Thus, although the number of grains collected on sections is much smaller than on spreads, we relied on sections rather than spreads for the localization of SCP1 domains within SCs.

Organization of SCP1 molecules within SCs

According to the model in Fig. 5, SCP1 molecules protruding from opposite LEs will overlap in the region of the CE. We speculate that the CE is the region where SCP1 molecules, extending from opposite LEs, overlap and join to form continuous TFs. Several details of the ultrastructure of rat SCs can be explained by the model in Fig. 5: (i) at least part of the TFs run continuously from one LE through the CE to the opposite LE [2, 35]. Within the CE, these TFs are more heavily stained by uranyl acetate and lead citrate, and thus probably contain more material than outside the CE [2, 35]. This extra material could (partly) originate from the overlap of SCP1 molecules protruding from opposite LEs. (ii) In *Drosophila* SCs, part of the TFs do not run continuously from one LE to the other one [2], and this is probably also true for rat SCs. Possibly these TFs have not (yet) joined with TFs from the opposite LE. (iii) It is possible that the N-terminal globular domain of SCP1 makes part of the "heavily stained globular or pillarlike junction structures" that have been observed between TFs and longitudinal CE components at the borders of CEs [2], or serve as anchorage sites for these structures.

(iv) A row of C-terminal globular domains could give the impression of a thin longitudinal structure at the inner edge of the LEs [2, 21].

Two other models for the organization of presumptive TF components have been presented. Dobson *et al.* [16], working with surface-spread spermatocytes and antibodies against largely overlapping parts of the hamster protein homologous to SCP1, found a dip in all distributions of immunogold label at the position of the CE; they therefore proposed that molecules extending from opposite LEs do not overlap or touch each other. However, given the predicted size of the SCP1 molecule, this is incompatible with our immunoelectron microscopy results (Fig. 5). Furthermore, as we have shown in this paper, the dip at the position of the CE is not found if sections rather than surface spreads are labeled with antibodies against the N-terminal part of the protein. Sym *et al.* [38] introduced insertions into the *ZIP1* gene of yeast, so that the length of the coiled coil domain of Zip1p increased. As a result, the distance between the LEs increased with once rather than twice the predicted increase in length of Zip1p, and Sym *et al.* therefore proposed that individual Zip1p molecules connect opposite LEs directly. This model cannot be true for SCP1, because it implies that N_M is much closer to the LE than M_M, which is not supported by our immunogold data: the 95% confidence intervals for N_M and M_M do not overlap, and M_M should therefore lie closer to the LE than N_M (Fig. 5). However, the observations of Sym *et al.* can also be explained if there are two different types of coiled coil protein molecules in the central region of the SC, one going all the way across between the LES, as has been proposed for Zip1p [38], and one with overlapping N-termini, as has been proposed for SCP1 (this paper). That would explain why the distance between the LEs is not decreased in yeast mutants producing Zip1p molecules with a 40 nm shorter coiled coil domain than wildtype [38]. It is thus possible that Zip1p is functionally unrelated to SCP1, although it seems likely that both proteins make part of SCs. Although there are similarities between the predicted secondary structures of SCP1 and Zip1p, alignment of the predicted amino acid sequences did not reveal homology beyond that expected between two coiled coil proteins [8]. Furthermore, there are also differences between Zip1p and SCP1: Zip1p has a shorter coiled coil domain than SCP1, and in contrast to SCP1, Zip1p has S/T-P motifs at both ends [13].

SCP1 and the assembly of the SC

The model in Fig. 5 has implications for the assembly of TFs, the attachment of TFs to the lateral elements, and the process of synapsis and synaptic adjustment.

It seems likely that SCP1 is a major component of TFs: it colocalizes with TFs, and its secondary structure is similar to that of intermediate filament proteins [8, 39]. The results in this paper further support this idea, because the immunogold localization of SCP1 subdomains can be brought into line with the predicted dimensions of the SCP1 molecule, and with a perpendicular orientation of SCP1 molecules relative to the LE. Possibly, as a first step in the assembly of TFs, SCP1 molecules form homodimeric coiled coils, like intermediate filament proteins do [39]. Given the narrow localization of C_M in the inner edge of the LEs (Fig. 5), such dimers should be parallel, i.e., both monomers have the same orientation. Presumably, coiled coil dimers of SCP1 have to associate side by side to form TFs: TFs have a diameter of 2-10 nm, and appear to consist of subfibres with the same diameter as coiled coils (2nm) [2, 35]. How such a side-by-side association is accomplished, and whether extra proteins are required for this is not known. However, a staggered organization of dimers, such as occurs in intermediate filaments [39], is, at least with respect to SCP1, not compatible with the immunoelectron microscopy results presented in this paper. It seems likely that the TFs make contact with the homologous chromosomes by direct interaction of SCP1 with DNA, because the C-terminus lies at the inner edge of the LE, contains amino acid sequence motifs that occur in certain DNA-binding proteins, and is capable of binding to DNA ([8], Meuwissen *et al.*, Chapter 4 of this thesis). This does not exclude the possibility that protein-protein interactions also contribute to the attachment of TFs to the LEs. It is not known how the association and dissociation of SCP1 to the LEs is regulated, but we think it likely that the potential $p34^{cdc2}$ protein kinase target site in the C-terminal domain is important for this (see Discussion in Meuwissen *et al.* [8], Chapter 2 of this thesis). This site has been conserved in the mouse [17], hamster [40] and human (Meuwissen *et al.*, Chapter 3 of this thesis) homologs of SCP1. The proposed organization of SCP1 molecules (Fig. 5) raises the possibility that continuous TFs between LEs can arise by association of the overlapping N-terminal parts of SCP1 molecules extending from opposite LEs. Whether extra proteins are required for this, and whether the association of N-termini, if any, is regulated, is unknown. Another unsolved question is, how synapsis is established: are SCP1 molecules or dimers first

attached to opposite LEs, and is synapsis subsequently established by interaction of, for instance, the N-termini, or do full length TFs first assemble on one LE, and do the free C-termini subsequently make contact with the opposite LE? Maybe both sequences of events can occur: TFs that do not reach the opposite LE have been observed in *Drosophila* [2]. On the other hand, apparently complete central regions can assemble on one unpaired LE (e.g.,[41]). In any case, the associations considered above provide little opportunity for sensitivity to homology. Given the numerous observations of non-homologous synapsis [1, 3, 42], there is no need to consider associations that provide such a sensitivity. It is possible that a similar spacing of SCP1 molecules on homologous LEs enhances homologous synapsis. However, it is also possible that synapsis is entirely insensitive to homology, and that other factors, such as a specific spatial organization of chromosomes in the nucleus and/or the initiation of recombination are required for homologous synapsis to occur.

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CHAPTER 6

General Discussion

General Discussion

Characterization of SCP1

This thesis describes the analysis of the structure and function of SCP1, a major protein component of synaptonemal complexes of the rat. Using monoclonal anti-SCP1 antibodies, we isolated the cDNA and characterized the gene product.

SCP1 has the following features:

1) The predicted secondary structure of SCP1 is similar to that of filamentous proteins. SCP1 is predicted to consist of three distinct domains: a major central domain of about 700 amino acid residues, which is predicted to form an amphipathic α-helix, flanked by smaller predicted globular or randomly coiled C- and N-terminal domains. The C-terminal domain is enriched in Ser/Thr-Pro (S/T-P) motifs, which are characteristic of DNA-binding proteins (Suzuki, 1989) (Chapter 2). These features have been conserved in SCP1 from other rodents and man (Chapter 3).

2) SCP1 is capable of binding to DNA *in vitro* and *in vivo*. The DNA-binding capacity resides in its C-terminal domain (Chapter 4).

3) SCP1 is localized between the LEs of the SC; the SCP1 molecules are probably integrated in only one possible position and orientation relative to the LEs. The C-termini lie in the inner half of the LE, the SCP1 molecules protrude from the LE through the central region into the CE, and end up with their N-terminus between the CE and the opposite LE, so that the N-termini of SCP1 molecules from opposite LEs overlap (Chapter 5).

Given these features, SCP1 is most probably a major component of TFs. In the following paragraphs I will consider what these features could tell us about possible roles of SCP1 during meiotic prophase. Before I can do this, I first have to compare the results obtained for SCP1 molecules with information about TFs and TF components in other species.

TFs and TF components in other species than rat

A detailed electronmicroscope tomography study of SCs in rat, *Drosophila*, and *Blaps* (a beetle) showed that the central region of SCs is organized in a similar fashion in these three species (Schmekel *et al.*, 1993; 1995). The structural unit of the central

region consists of a single TF which spans the distance between the LEs, and carries two symmetrically placed thickenings. These thickenings are organized in two longitudinal rows, which together constitute the CE (Chapter 1). There are three to five layers of TFs on top of each other. We assume that the N-terminal domains of SCP1 molecules from opposite LEs overlap and interact in the region of the CE to form continuous TFs (Chapter 5 and Liu *et al.*, 1996).

TFs appear to consist of 2-5 subfibres, each of which has a diameter of about 2 nm, which corresponds to the diameter of a coiled-coil dimer (Steinert *et al.*, 1985). It is thus possible that TFs are composed of 4-10 SCP1 dimers (2-5 dimers from each LE).

In budding yeast (*Saccharomyces cerevisae*), the tripartite SC structure is also conserved, although the structural features are less pronounced than in *Drosophila* or rat. TFs of yeast SCs can hardly be visualized by electromicroscopy (Dresser and Giroux, 1988).

One putative component of yeast TFs has been identified, namely Zip1p. The predicted secondary structure of Zip1p is similar to that of SCP1: both proteins have a long central coiled-coil stretch bordered by globular or randomly coiled N- and C-terminal domains. However, there is also a difference: Zip1p is enriched in S/T-P motifs at both the N- and C-terminus, whereas SCP1 carries these motifs at the C-terminus only.

The following observations suggest that Zip1p is a component of TFs in yeast:

a) *zip1* mutants assemble full-length LEs, but these do not synapse. Instead, the LEs align at a distance of about 300 nm (Sym *et al.*, 1993; Sym and Roeder, 1995).

b) If the length of the coiled-coil domain of Zip1p is artificially increased by insertions in the ZIP1 gene of yeast, the distance between the LEs increases with about once the predicted increase in length of Zip1p (Sym and Roeder, 1995).

An important question is, whether Zip1p of yeast corresponds to SCP1 of the rat. Despite their similar structure and position within SCs, SCP1 and Zip1p do not show amino acid sequence similarity beyond that expected to be found between two coiled-coil proteins. However, SCP1 is not a conserved protein (Chapter 3) and it is still possible that SCP1 and Zip1p are at least functionally homologous. Because an increase of the length of the coiled-coil domain of Zip1p results in an increase of the distance between the length of the LEs (Sym and Roeder, 1995), it seems likely that

Zip1p is a TF component. However, the LE-LE distance increases with once rather than twice the predicted increase in length of Zip1p, and it was therefore proposed that Zip1p molecules traverse the entire central region. If Zip1p molecules were organized within SCs in the same way as SCP1 molecules (Chapter 5, Figure 5), the distance between the LEs would have increased by twice the predicted increase in length of Zip1p. However, it is also possible that the Zip1p molecules are organized in the same way as SCP1 molecules, but that they are bent or kinked, so that the increase of the LE-LE distance is less than expected. Alternatively, there are two coiled-coil proteins in the central region of SCs: one corresponding to SCP1, and one corresponding to Zip1p. To summarize, it is as yet undecided whether Zip1p and SCP1 are functional homologues. In this discussion I will assume that the data obtained about Zip1p function(s) are relevant for SCP1.

TFs, synapsis and genetic interference

In at least two species, namely *Aspergillus nidulans* (a filamentous fungus) and *Schizosaccharomyces pombe* (fission yeast) synapsis does not occur, but nevertheless meiosis is completed successfully (Egel-Mitani *et al.*, 1982; Kohli and Bähler,1994; Egel, 1995). However, there are two differences between meiotic recombination in these two species and in all other species analysed thus far: (i) there is no crossover interference (see Chapter 1), and (ii) the number of crossovers per bivalent is very high (Clutterbuck, A.J., (1992); Swart, K., (1996); Kohli and Bähler, 1994).

In some strains of *Saccharomyces cerevisae* (budding yeast), *zip1* mutants have a similar phenotype: there is no synapsis in these mutants, but still meiosis is completed. However, although the frequency of meiotic crossing over approaches the wildtype level, there is no crossover interference. It thus seems likely that TF-components are not only involved in synapsis, but also influence meiotic crossing over.

Storlazzi *et al.* (1996) have analysed in detail the role of Zip1p in meiotic recombination in yeast. The *zip1* mutation has the following effects on the level of meiotic recombination (Storlazzi *et al.*, 1996):

-Meiotic recombination (crossover plus non-crossover) reaches nearly wildtype levels.

-The level of crossover events is decreased to about 25% of the wildtype level.

-The level of non-crossovers is about the same as in wildtype.

In addition, the *zip1* mutation has an effect on the kinetics of the resolution of recombination intermediates (double Holliday junctions; Schwacha and Kleckner, 1995): in *zip1* mutants, the formation of all crossovers is delayed, whereas formation of part of the non-crossovers is delayed. The frequency of the delayed non-crossover events corresponds to the frequency of crossover events. Storlazzi *et al.* (1996) therefore proposed that in wildtype yeast, Zip1p converts part of the recombination intermediates into crossovers. They proposed that in *zip1* mutants, the resolution of **these** intermediates is delayed, and only about 50% of them is converted into a crossover. Storlazzi *et al.* further observed that the *zip1* mutation still resulted in a decrease in crossover frequency in *red1* mutants, which do not assemble any recognizable SC substructures. Apparently, the context of an SC is not required for Zip1p to exert its influence on the commitment to crossover formation.
How can this possible influence of Zip1p on the predestination of recombination intermediates be related to its effect on crossover interference ?

In order to consider this question, the following features of meiotic recombination should also be taken into account:

- interference has only been observed between crossovers; non-crossovers (gene conversions) do not interfere with each other or with crossovers (Mortimer and Fogel, 1974).
- the physical distance (in bp) over which interference extends varies between organisms by several orders of magnitude. In contrast, the map distance (in centimorgans) over which intereference extends is of the same order of magnitude in various organisms (Stam, 1979): the map distance is the most important variable that determines the degree of interference between two loci (Stam, 1979; Foss *et al.*, 1993).

With respect to the role of SCs and SC-components in crossover interference, two types of models have been proposed:

Model I: Crossover interference is a consequence of the polymerization of the tripartite SC structure.

The most elaborate model of this type has been proposed by King and Mortimer (1990).

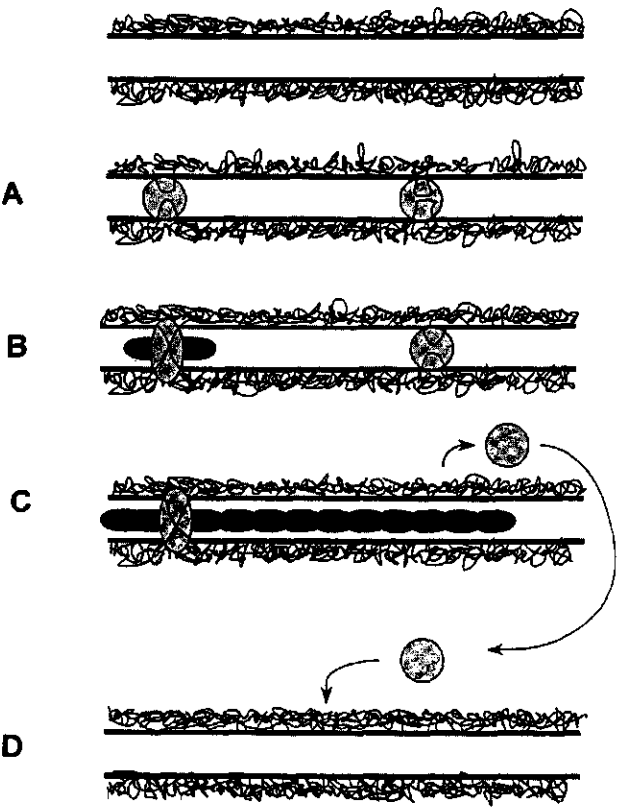

Figure 1 Schematic diagram of Model I.
Early structures (spherical structures) bind randomly to the SC. Some initiate polymerization reactions thus becoming recombination nodules (ellipsoidal). The growing polymers eject early strcutures. The ejected early structures are either degraded or bind to SC that is free of polymers. (After King and Mortimer, 1990)

This model assumes that there are several stages in the establishment of crossover events. First, early recombination intermediates are formed randomly along the paired homologues (Figure 1a). As these early intermediates are processed to become more advanced recombination intermediates (Figure 1b), a bi-directional polymerization reaction along the paired homologues is initiated. Each "late" structure has an equal chance per unit time for initiation of bi-directional polymerization. The ongoing polymerization reaction then blocks new initiations (Figure 1c), or the not yet evolved early recombination structures are released and cannot evolve into late structures.
A meiotic checkpoint would guarantee that the whole process continues (Figure 1d) until a polymerized structure is present along each bivalent. A computer simulation of this model generated crossover distributions along chromosomes which were in accordance with experimental data from *S. cerevisae* and *Drosophila*. The early and late events were suggested to correspond to early and late recombination nodules, and the polymerization could correspond to SC synapsis. Note that only late events evolving into crossovers can give rise to the initiation of SC synapsis. Another feature of this model is, that it predicts at least one crossover per chromosome, also on the smaller chromosomes. This will result in lower crossover rates (centimorgans per kbp) in larger chromosomes and higher crossover rates in small chromosomes. Exactly this was found for meiotic recombination in *S. cerevisae* (Kaback *et al.*, 1992).

The second model takes into account an observation of Storlazzi *et al.* (1996), namely that Zip1p enhances the resolution of recombination intermediates into crossovers, even in the absence of any recognizable SC substructure:

Model II: The formation and distribution of crossovers is independent of the intact tripartite SC structure.

According to this model, which is presented in Figure 2, Zip1p regulates the number of crossovers **before** synapsis. First, meiotic recombination is initiated by random double strand breaks (Figure 2A), and the resulting DNA ends are processed and stabilized. Rad51p and Dmc1p are probably involved in these steps (Bishop *et al.*,1992; Shinohara *et al.*, 1992; Ogawa *et al.*, 1993). Storlazzi *et al.* further propose that the ends have to be "activated" to commit to crossover (Figure 2B).

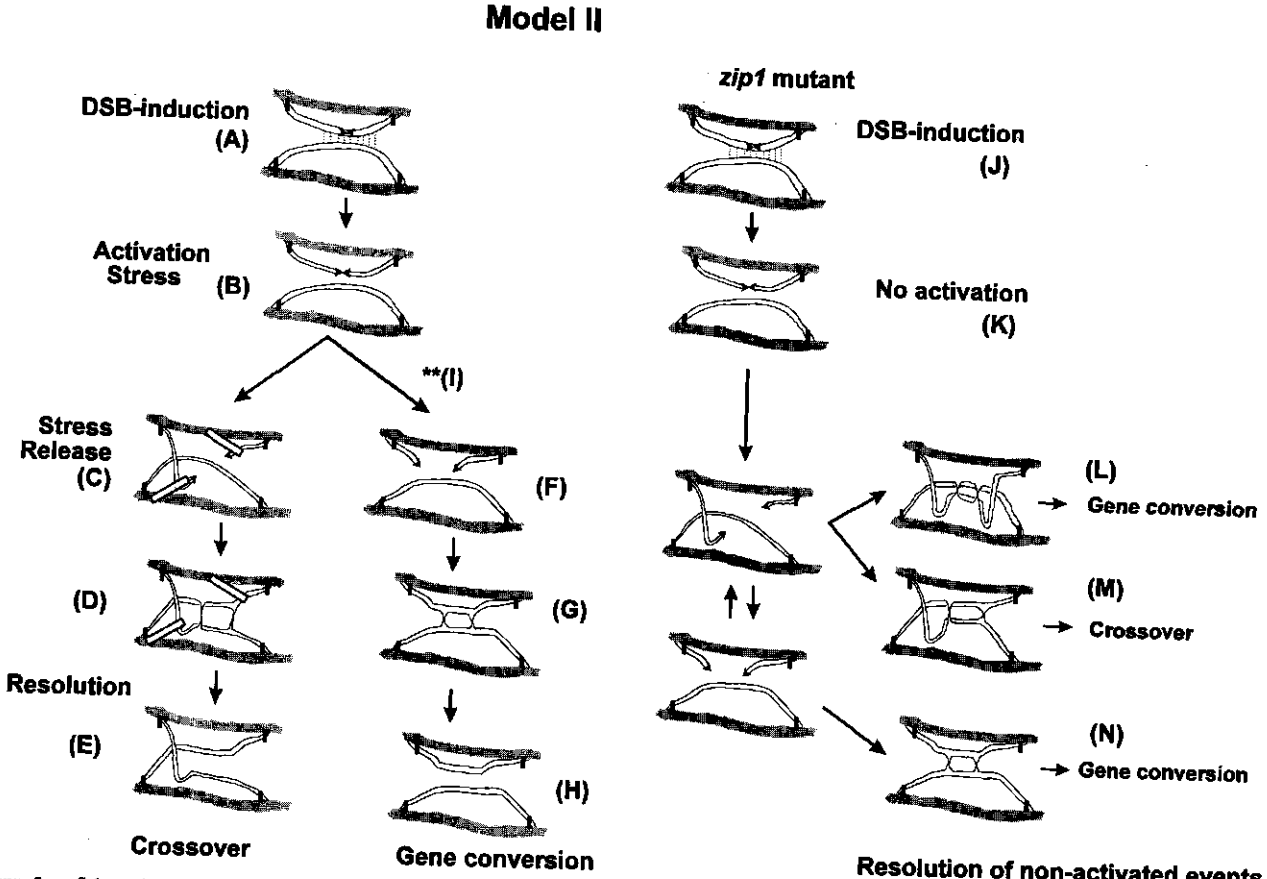

Figure 2 Schematic overview of Model II
The individual steps and mechanisms are explained in the text. The chromatin scaffold is indicated by *grey bars*. The scaffold attachment sites of the chromatin are indicated by *black vertical bars*. The activated DSB ends are indicated by *arrowheads*. The Zip1p molecules are indicated by long *grey rectangular bars*. (Adapted from Storlazzi *et al.*, 1996)

They think that mechanical stress, generated by chromosome condensation, could be the activating factor. Zip1p then recognizes the "activated ends" binds to them, and pulls one end to the axis (or LE) of the homologous chromosome (Figure 2C). The double Holliday structure which is generated in this context (Figure 2D) can only be resolved into a crossover (Figure 2E). Resolution of an intermediate into a crossover would release the mechanical stress in its immediate vicinity; this would cause de-activation of DNA ends, so that crossover formation cannot occur (Figure 2F-H). This would explain crossover interference. In *zip1* mutants, no activation of DNA ends takes place (Figure 2J-N). 50% of the "non-activated" events are resolved into crossovers (Figure 2L), and 50% into non-crossovers. The model further supposes implicitely a mechanism (Figure 2I) which prevents de-activated, but not non-activated intermediates to form a crossover.

I am in favour of a combination of both models to explain crossover interference:

Model III: TF-components have a dual function: before synapsis, they are involved in crossover commitment, and, at a later stage, they contribute to crossover interference by the process of synapsis.

This model is presented in Figure 3.

I propose that SCP1 molecules associate by their N-terminus with the stabilized ends of the double-strand DNA break and with each other (Figure 3B). The C-termini bind to DNA at the basis of the chromatin loops. Because C-termini are diametrically opposed, they cannot bind both to the axis of the same homologue (Figure 3B). This has two effects: (i) a pre-crossover configuration is generated (Figure 3C), and (ii) synapsis is triggered, either because of side-by-side association of more SCP1 molecules, or because the pre-crossover configuration has pulled the chromosomal axes (or LEs) sufficiently close together for further synapsis, or both (Figure 3D). Within synapsed regions, no more pre-crossover configurations can be generated because all binding sites for the C-termini of SCP1 are occupied. This explains interference. It is possible that individual Zip1p molecules act in the same way as two SCP1 molecules that are associated by their N-termini, because Zip1p molecules have DNA-binding S/T-P motifs at both ends.

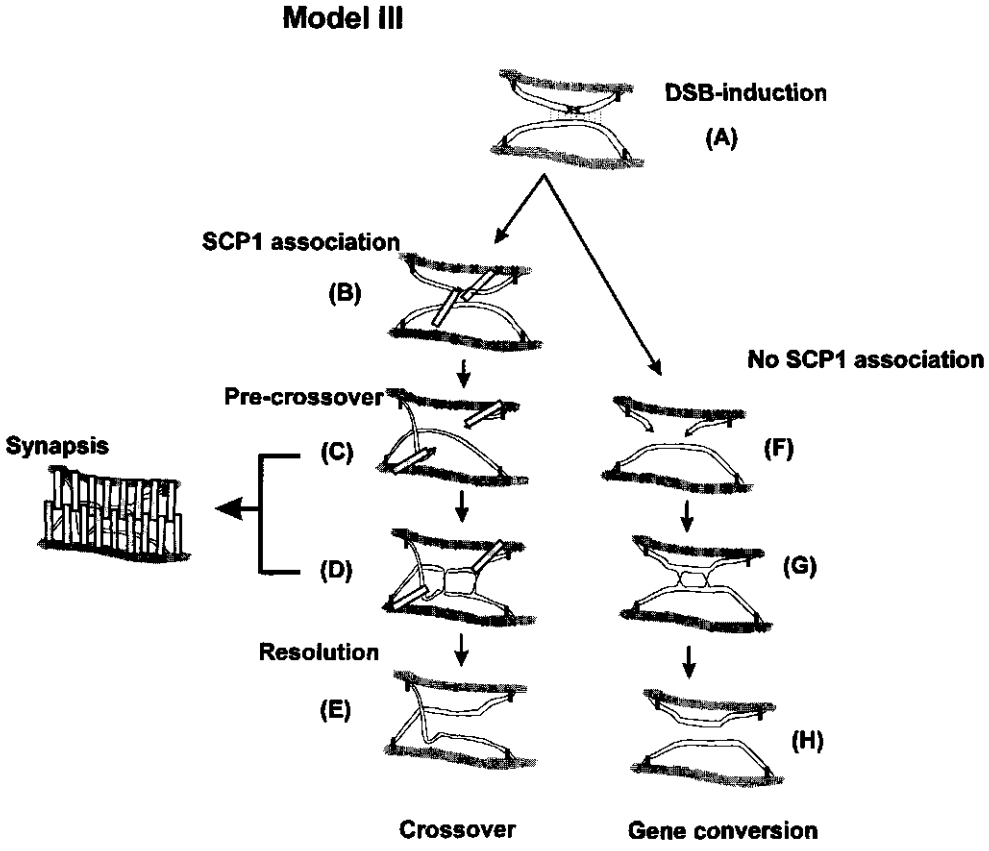

Figure 2 Schematic overview of Model III
The individual steps and mechanisms are explained in the text. The chromatin scaffold is indicated by *grey bars*. The scaffold attachment sites of the chromatin are indicated by *black vertical bars*. The DSB ends are indicated by *arrowheads*. The SCP1 molecules are indicated by long *grey rectangular bars*. *Crosses* represent binding sites for SCP1 on the LEs or chromosomal axes. In (D), synapsis is not shown, in order to show the crosover inter mediate.

A meiotic checkpoint can halt meiosis until all chromosomes are synapsed (Roeder, 1995). Because synapsis is supposed to initiate at prospective crossover sites, this will guarantee at least one crossover per bivalent. The model predicts that SCP1 molecules are already present early in meiotic prophase at the sites of stabilized double-strand breaks, possibly at the sites of early meiotic nodules. Furthermore, it predicts that synapsis is initiated at the sites of crossover. The model does not preclude that synapsis is also initiated at other sites, but if this occurs, synapsis should be reversible. It is possible that the chance of synapsis to initiate at other than crossover sites is species-dependent, and varies from very small to large. The model further predicts, that commitment to crossover resolution can occur in the absence of tripartite SC, and even in the absence of recognizable LEs (Storlazzi *et al.*, 1996), but crossover interference cannot.

Experiments to further investigate the functions of SCP1

The model presented above predicts that early in meiotic prophase, SCP1 molecules colocalize with proteins that associate with the DNA ends generated by the meiotically induced double-strand DNA breaks, for instance Rad51p and Dmc1p. Colocalization of Dmc1p and Zip1p has already been demonstrated by immunofluorescence in yeast (Bishop, 1994). These studies should be extended by immunogold labeling experiments, preferably in an organism with well defined recombination nodules and SC (sub)structures. Furthermore, proteins with wich SCP1 possibly interacts should be identified, for instance by two-hybrid analyses. I predict that there will be recombination proteins among them.The model additionally predicts that SCP1 binds to DNA at the basis of chromosomal loops. The isolation and characterization of the DNA sequences to wich SCP1 binds is therefore important. In particular, it will be interesting to compare the positions of SCP1 binding sites with those of scaffold- or matrix- attachment regions (SARs or MARs).

These detailed analyses of a single transverse filament protein, SCP1, may seem trivial. However, if SCP1 is involved in the decision whether a recombination intermediate will be resolved as crossover or as conversion, elucidation of its function will largely contribute to our understanding of meiosis and in particular of meiotic recombination in mammals.

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Summary

Synaptonemal complexes (SCs) are structures that are formed between homologous chromosomes during meiotic prophase. SCs consist of two proteinaceous axes, one along each homologue, that are connected along their length by numerous transverse filaments (TFs). The assembly and disassembly of SCs closely correlates with the successive events at the chromosomal level: the condensation, pairing, recombination and segregation of homologous chromosomes. In **Chapter 1**, recent advances in the analysis of the structure and composition of SCs are described, and possible roles of SCs in meiotic chromosome pairing and recombination are considered. The regulation of meiotic recombination and the formation and maintenance of stable chiasmata are now considered as possibly the principal functions of SCs.

The experimental work described in this thesis is focused on the structure and function of SCP1, a major protein component of rat SCs (**Chapters 2-5**).

Chapter 2 describes how cDNA clones encoding SCP1 of the rat were isolated by screening a cDNA library with monoclonal antibodies that recognize a 125,000 M_r SC component. A polyclonal antiserum raised against the translation product of one of the isolated cDNA clones recognizes a single protein on Western blots of proteins of isolated SCs with identical electrophoretic mobility as the protein recognized by the monoclonal antibody that was used for screening. The protein encoded by the cDNAs (called SCP1: synaptonemal complex protein 1) has a predicted molecular weight of 117 kDa. The central part of SCP1 is capable of forming an amphipathic α-helix, whereas the C-terminal domain carries motifs that are characteristic of DNA-binding proteins (Ser/Thr-Pro motifs). In immunogold labeling experiments, the monoclonal antibody that was used for screening and the polyclonal antiserum that was elicited against the translation product of the cDNA both labeled the central region of the SC. We conclude that SCP1 is most probably a major component of the transverse filaments of SCs, and speculate that SCP1 has evolved by specialization of a nuclear matrix protein.

In order to identify conserved features of SCP1, we isolated and characterized cDNAs encoding human SCP1 (**Chapter 3**). Human SCP1 and rat SCP1 have 75% amino acid identity. Most of the prominent structural features of rat SCP1 are conserved in human

SCP1. The human SCP1 gene was localized on human chromosome 1p12-p13. We conclude that human SCP1 is most probably the functional homologue of rat SCP1, and that SCP1 is not a very conserved protein.

Because SCP1 contains amino acid sequence motifs which are characteristic of DNA-binding proteins, we analysed the DNA binding capacity of SCP1, by means of a quantitative south-western blot assay (**Chapter 4**). The DNA binding capacity was confined to the C-terminal domain. This domain has about the same affinity for total rat genomic DNA as for a *Drosophila* SAR DNA probe, but its affinity for *E. coli* DNA is significantly lower. Similar results were obtained for full-length SCP1.
Analysis of the kinetics of DNA binding revealed that there is probably one type of DNA binding site on SCP1. Distamycin A could completely inhibit binding of DNA by the C-terminal domain. We therefore concluded that the C-terminal domain, and thus also full-length SCP1, binds to DNA through interaction with the minor groove. The binding of SCP1 to DNA *in vivo* was shown by paraformaldehyde crosslinking of living spermatocytes. The DNA-binding capacity of the C-terminal domain of SCP1 is in agreement with the localization of this domain in the LEs of SCs, where most of the DNA is situated. We speculate about the DNA sequences to which SCP1 binds *in vivo* and about the implications of this for the role of SCP1 in the assembly and function(s) of SCs.

Chapter 5 describes the analysis of the organization of SCP1 molecules within SCs. Using polyclonal antibodies elicited against non-overlapping fragments of SCP1, we performed immunogold of SCs. Two types of SC preparations were used for this purpose, namely surface-spread spermatocytes and ultrathin sections of Lowicryl-embedded testicular tissue of the rat. The distribution of immunogold label on surface-spread spermatocytes differed significantly from the distribution of label on sections. This difference is probably due to masking of SCP1 epitopes within the SCs in surface-spread preparations and/or the surface morphology of the surface spreads. The results obtained on sections were therefore used for the localization of subdomains of SCP1 within the SC structure. We present the following model for the organization of SCP1 molecules within SCs: the C-terminus of SCP1 lies in the inner half of the lateral element (LE), and the molecules protrude from the LE through the central region into

the central element (CE), so that N-termini of SCP1 molecules from opposite LEs overlap. The implications of this model for the assembly of SCs and for the possible functions of SCP1 are discussed.

In **Chapter 6**, the cytological and biochemical features of SCP1 are compared with data about TF components in other organisms. Especially the possible function(s) of a putative TF component of yeast, Zip1p, are discussed, and I consider the relevance of the Zip1p data for the possible function(s) of SCP1. Finally, I present a model for the function(s) of SCP1 during meiotic prophase.

Resumen

Los complejos sinaptonémicos (CSs) son estructuras que se forman entre cromosomas homólogos durante la profase meiótica. Los CSs constan de dos ejes proteínicos, uno a lo largo de cada homólogo, que están conectados a lo largo de su longitud por numerosos filamentos transversales (FTs). El montaje y desmontaje de los CSs se correlaciona con los sucesivos acontecimientos a nivel cromosómico: condensación, apareamiento, recombinación y segregación de cromosomas homólogos. En el **Capítulo 1** se describen los avances recientes en el análisis de la estructura y composición de los CSs y se consideran los posibles papeles de los CSs en el apareamiento cromosómico meiótico y en la recombinación. La regulación de la recombinación meiótica y la formación y mantenimiento de quiasmas estables son consideradas en la actualidad como posiblemente las principales funciones de los CSs.

El trabajo experimental descrito en esta Tesis se enfoca en la estructura y función de SCP1, una de las principales proteínas componentes de los CSs de rata (**Capítulos 2-5**).

En el **Capítulo 2** se describe como fueron aislados clones de DNAc que codifican para SCP1 de rata, por medio del análisis de una genoteca de DNAc con anticuerpos monoclonales que reconocen un componente del CS de 125.000 M_r. Un anticuerpo policlonal obtenido frente al producto de traducción de uno de los clones de DNAc aislados reconoce mediante Western blot de CSs aislados una única proteína de movilidad electroforética similar a la proteína reconocida por el anticuerpo monoclonal utilizado en el análisis de la genoteca. La proteína codificada por el DNAc (llamada SCP1: proteina 1 del complejo sinaptonémico) tiene un peso molecular estimado de 117 kDa. La parte central de SCP1 es capaz de formar una hélice-α anfipática, mientras que el dominio C-terminal contiene motivos que son característicos de proteínas que se unen al DNA (motivos Ser/Thr-Pro). En experimentos de inmunotinción con oro tanto el anticuerpo monoclonal que se usó para el aislamiento del clon de DNAc como el anticuerpo policlonal que fue obtenido contra el producto de traducción del DNAc marcan la región central de el CS. Se concluye que SCP1 es un componente principal de los filamentos transversales de los

CSs y se especula que SCP1 ha evolucionado por especialización de una proteína de la matriz nuclear.

Con el fin de identificar características de SCP1 se ha aislado y caracterizado el DNAc que codifica la SCP1 en humanos (**Capítulo 3**). La SCP1 de humanos y la SCP1 de rata tienen un 75% de identidad de aminoácidos. La mayoría de las características estructurales importantes de la SCP1 de rata están conservadas en la SCP1 de humanos. La SCP1 humana ha sido localizada en el cromosoma humano 1p12-p13. Se concluye que lo mas probable es que la SCP1 humana es la homóloga funcional de la SCP1 de rata y que SCP1 no es una proteína muy conservada.

Debido a que la secuencia de aminoacidos de SCP1 contiene motivos que son característicos de proteínas que se unen al DNA, se ha analizado la capacidad de unión al DNA de SCP1 por medio de un ensayo cuantitativo de "south-western blot" (**Capítulo 4**). La capacidad de unión al DNA se localizó en el dominio C-terminal. Este dominio tiene aproximadamente la misma afinidad por DNA total genómico de rata como por una sonda de DNA SAR de *Drosophila*, pero su afinidad por DNA de *E. coli* es significativamente mas baja. Resultados similares se obtuvieron con la longitud completa de SCP1.

El análisis de la cinética de unión al DNA reveló que probablemente hay un tipo de sitio de unión al DNA en SCP1. Distamicina A pudo inhibir completamente la unión del dominio C-terminal al DNA. Por lo tanto, se concluye que el dominio C-terminal de SCP1, y de este modo también la proteína completa, se unen al DNA a través de la interacción con el surco menor. La unión de SCP1 al DNA *in vivo* se mostró por medio de la unión cruzada en espermatocitos vivos en paraformaldehido. La capacidad de unión al DNA del dominio C-terminal de SCP1 concuerda con la localización de este dominio en el elemento lateral de los CSs donde se situa la mayoría del DNA. Especulamos acerca de las secuencias de DNA a las que SCP1 se une *in vivo* y acerca de las implicaciones de esto en el papel de SCP1 en la organización y función(es) de los CSs.

En el **Capítulo 5** se describe el análisis de la organización de las moléculas de SCP1 dentro de los CSs. Por medio de anticuerpos policlonales obtenidos frente a fragmentos no solapantes de SCP1 se realizaron inmunotinciones con oro de los CSs.

Para esto se usaron dos tipos de preparaciones de CSs, esto es, espermatocitos extendidos en superficie y secciones ultrafinas de tejido testicular de rata incluidas en Lowicryl. La distribución de las partículas de oro sobre los espermatocitos extendidos en superficie se diferenció significativamente de la distribución del marcaje en las secciones ultrafinas. Esta diferencia en distribución es debida probablemente al ocultamiento de epitopos de SCP1 dentro de los CSs de las preparaciones de espermatocitos extendidas en superficie, y/o a la morfología superficial de los extendidos. Por lo tanto, los resultados obtenidos en secciones fueron utilizados para la localización de subdominios de SCP1 dentro de la estructura del CS. Se presenta el siguiente modelo de organización de las moléculas de SCP1 dentro del CS: el C-terminal de SCP1 está situado en la mitad interior del elemento lateral (EL), las moleculas sobresalen del EL a través de la región central en el elemento central (EC) de forma que el N-terminal de las moleculas de SCP1 procedentes de ELs opuestos se solapan. Se discuten las implicaciones de este modelo para el montaje de los CSs y las posibles funciones de SCP1.

En el **Capítulo 6** se comparan las características citológicas y bioquímicas de SCP1 con los datos de los de FTs en otros organismos. Se discuten especialmente la funciones de un componente del FT de levadura, Zip1p, y se considera la importancia de estos datos de Zip1p para la(s) posible(s) función(es) de SCP1. Finalmente se presenta un modelo de las funciones de SCP1 durante la profase meiótica.

Samenvatting

Synaptonemale complexen (SC's) zijn structuren die tijdens de meiotische profase tussen homologe chromosomen gevormd worden. SC's bestaan uit twee eiwitachtige assen, één langs elk chromosoom, die over hun gehele lengte met elkaar worden verbonden door talrijke transversale filamenten (TF). De assemblage en de-assemblage van SC's is nauw gecorreleerd aan de opeenvolgende gebeurtenissen op het chromosomale niveau: de condensatie, paring recombinatie en segregatie van homologe chromosomen. In **Hoofdstuk 1** worden recente vorderingen in de analyse van de structuur en samenstelling van SC's beschreven en wordt de mogelijke rol van SC's in de meiotische chromosoom paring en recombinatie besproken. Tegenwoordig worden de regulatie van de meiotische recombinatie en de vorming en handhaving van stabiele chiasmata beschouwd als de mogelijke hoofdfuncties van SC's.

Het experimentele werk dat in dit proefschrift wordt beschreven, is gericht op de structuur en functie van SCP1, een belangrijke eiwit-component van SC's van de rat (**Hoofdstukken 2-5**).
In **Hoofdstuk 2** wordt beschreven hoe cDNA klonen die coderen voor SCP1 van de rat werden geïsoleerd door een cDNA bank te screenen met behulp van monoclonale antilichamen die een M_r 125.000 SC component herkennen. Een polyklonaal antiserum, opgewekt tegen het translatie produkt van één van de cDNA klonen, herkende een enkel eiwit op westernblots van eiwitten van geïsoleerde SC's, met eenzelfde electroforetische mobiliteit als het eiwit dat herkend wordt door het monoklonale antilichaam dat gebruikt werd voor screening. De cDNA klonen coderen voor een eiwit (SCP1 genaamd: synaptonemale complex proteïne 1) met een voorspeld molecuulgewicht van 117 kDa. Het centrale gedeelte van SCP1 is in staat tot het vormen van een amfipatische α-helix. Het monoklonale antilichaam dat werd gebruikt voor de cDNA isolatie en het polyklonale antiserum dat werd opgewekt tegen het translatie produkt van het cDNA markeerden beiden het centrale gebied van het SC in immuungoud-labelings experimenten. Wij concluderen dat SCP1 hoogstwaarschijnlijk een hoofdbestanddeel is van de transversale filamenten van SC's. Tevens speculeren wij dat SCP1 geëvolueerd is door specialisatie van kernmatrix eiwitten.

Teneinde geconserveerde eigenschappen van SCP1 te identificeren, isoleerden en karakteriseerden wij cDNAs die coderen voor het humane SCP1 (**Hoofdstuk 3**). 75% van de aminozuren in humaan SCP1 en ratte SCP1 is identiek. De meeste opvallende structurele kenmerken van ratte SCP1 zijn in humaan SCP1 geconserveerd. De ligging van het humane SCP1 gen werd bepaald op chromosoom 1p12-p13. Wij concluderen dat humaan SCP1 hoogst waarschijnlijk functioneel homoloog is met ratte SCP1, en dat SCP1 een niet erg geconserveerd eiwit is.

Omdat SCP1 aminozuur-sequentie motieven bevat die kenmerkend zijn voor DNA-bindende eiwitten, hebben wij het DNA-bindend vermogen van SCP1 onderzocht, door middel van een kwantitatieve south-western blot bepaling (**Hoofdstuk 4**). Het bleek dat het DNA-bindende vermogen zich beperkt tot het carboxyl-terminale domein. Dit domein heeft ongeveer dezelfde affiniteit voor totaal rat genomisch DNA als voor een *Drosophila* SAR DNA fragment, maar de affiniteit voor *E. coli* DNA is beduidend lager. Vergelijkbare resultaten werden verkregen met het totale SCP1 eiwit.
Analyse van de DNA-bindingskinetiek maakte duidelijk dat er waarschijnlijk maar één type DNA-bindingsplaats aanwezig is is op SCP1. Distamycine A kon de binding van het carboxy-terminale domein aan DNA volledig remmen. We concluderen daarom dat SCP1 aan DNA bindt door middel van interactie met de "minor groove". De binding van SCP1 aan DNA *in vivo* werd aangetoond door middel van paraformaldehyde crosslinking van levende spermatocyten. Het DNA-bindend vermogen van het carboxyl-terminale domein is in overeenstemming met de localisatie van dit domein in de laterale elementen van SC's, waar zich het meeste DNA bevindt. Tenslotte speculeren wij over de mogelijke DNA sequenties waaraan SCP1 *in vivo* bindt en bovendien over de implicaties hiervan voor de rol van SCP1 in de assemblage en functie(s) van SC's.

In **Hoofdstuk 5** wordt de analyse besproken van de organisatie van SCP1 moleculen in SC's. Wij gebruikten polyklonale antisera opgewekt tegen niet-overlappende SCP1 fragmenten voor de immunogoud labelling van SC's. Twee soorten SC preparaten werden hiervoor gebruikt, namelijk gespreide spermatocyten en ultra-dunne coupes van in Lowicryl ingebed testis weefsel van de rat. De verdeling van de immunogoud korrels op gespreide spermatocyten verschilde aanmerkelijk met die op coupes. Dit verschil valt waarschijnlijk te wijten aan het maskeren van de SCP1 epitopen in de SC's in spreid-

preparaten en/of aan de oppervlakte-morfologie van de spreid-preparaten. De resultaten verkregen op coupes werden daarom gebruikt voor de localisatie van de subdomeinen van SCP1 in de SC structuur. Wij presenteren het volgende model voor de organisatie van SCP1 moleculen in SC's: het carboxyl-uiteinde van SCP1 ligt in de binnenste helft van het laterale element (LE) en de moleculen steken uit het LE door het centrale gebied heen tot in het centrale element (CE), zodat de amino-uiteinden van SCP1 moleculen van tegenoverliggende LE's overlappen. De implicaties van dit model voor de assemblage van SC's en de mogelijke functies van SCP1 worden besproken.

In **Hoofdstuk 6** worden de cytologische en biochemische eigenschappen van SCP1 vergeleken met gegevens over TF componenten uit andere organismen. Speciale aandacht wordt besteed aan de mogelijke functie(s) van Zip1p, een vermoedelijke TF component van gist, en aan de relevantie van de gegevens over Zip1p voor de functie(s) van SCP1. Tot slot presenteer ik een model voor de functie(s) van SCP1 in de meiotische profase.

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Nu het proefschrift is voltooid en het Wagingse 'station' is gepasseerd, wil ik toch nog een paar opmerkingen plaatsen als dank voor hetgeen ik in de afgelopen jaren heb mogen meemaken. Zoals velen onder U weten, kan een proefschrift vaak alleen van de grond komen door de steun en inspiratie van de omgeving waarin je goed kan functioneren. Welnu, in mijn geval was dat zeker zo.
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Curriculum Vitae

Ralph Meuwissen werd op 11 juni 1964 geboren te Sittard. Na het behalen van het VWO-B diploma aan het Bisschoppelijk College te Echt begon hij in 1982 aan zijn studie Scheikunde aan de Rijksuniversiteit Leiden. Na een hoofdvak in de vakgroep Moleculaire Genetica en een bijvak in de vakgroep Medische Biochemie, werd de studie in mei 1987 afgerond met het doctoraal examen. Hierop volgde een aanstelling als tweejarige AiO bij de vakgroep Plantevirussen onder leiding van Prof. dr. J. Bol. In dit projekt heeft hij transgene tabaksplanten vervaardigd voor de bestudering van de constitutieve expressie van PR-genen. Na afloop van deze periode volgde een aanstelling per 1 augustus 1990, als OiO (gesubsidieerd door GMW/NWO) bij de vakgroep Erfelijkheidsleer onder leiding van Prof. dr. C. Heyting met als doel de isolatie en karakterisatie van de toen nog onbekende genen coderend voor SC eiwitten van de rat. Dit heeft uiteindelijk geleid tot het schrijven van dit proefschrift. In 1992 heeft Ralph de vakgroep tijdelijk verlaten voor het werken aan de meiose in gist en een poging tot het kloneren van een gist homoloog van SCP1, in het laboratorium van Prof. dr. N. Kleckner aan de Harvard University. Hiertoe werd hij in staat gesteld door een Fullbrigth Fellowship van de Amerikaanse overheid. De laatste anderhalf jaar was Ralph in dienst als gastmedewerker aan de Vakgroep Erfelijkheidsleer. Per 1 november 1996 is hij in dienst getreden van het Nederlands Kanker Instituut bij de vakgroep Moleculaire Genetica onder leiding van Prof. dr. A. Berns. In dit nieuwe projekt gaat hij meewerken aan de vervaardiging van conditionele tumor suppressor 'knock-out' en oncogen-expressie muizen die als model zullen dienen voor de bestudering van het mechanisme van de aanzet tot tumorigenese.