IDENTIFICATION AND CHARACTERIZATION OF SYNAPTONEMAL COMPLEX PROTEINS OF THE RAT

Identificatie en karakterisering van
synaptonemale complex eiwitten van de rat

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IDENTIFICATION AND CHARACTERIZATION OF SYNAPTONEMAL COMPLEX PROTEINS OF THE RAT

Proefschrift

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Contents

Abbreviations

AP	attachment plaques
CE	central element
HU	hydroxy urea
LE	lateral element
Mab	monoclonal antibody
M_r	relative electrophoretic mobility
RN	recombination nodule
SC	synaptonemal complex
SCP1	synaptonemal complex protein 1
SCP2	synaptonemal complex protein 2

STELLINGEN

1 De chromatine herrangschikkingen tijdens de meiotische profase vinden plaats door reorganisatie van het chromatine op nieuw-geassembleerde structuren (de synaptonemale complexen) die geheel of grotendeels bestaan uit meiose-specifieke componenten (dit proefschrift).

2 Het uitblijven van een aantoonbare kruisreactie van één monoclonaal antilichaam met overeenkomstige eiwitten uit verschillende organismen betekent niet noodzakelijk dat de desbetreffende eiwitten slecht geconserveerd zijn (Hollingsworth and Byers, 1989, Genetics, 121, 445-462)

3 De bewering: "SC components are absent in *hop1* mutants, this raises the intriguing possibility that with the HOP1 protein a component of the SC that interacts directly with DNA has been discovered...." is verwarrend doordat het woord "component" eest als morfologische en vervolgens als biochemische term wordt gebruikt (Loidl, J., 1991, Chromosoma, 100, 289-292).

4 Elke poging om met antilichamen geselecteerde cDNA's te bevestigen met behulp van antilichamen die opgewekt zijn tegen het translatieproduct van de betreffende cDNA's houdt een cirkelredenering in.

5 De excessieve groei van het aantal uitzendbureau's heeft veel jongeren tijdelijk werk gegeven, maar heeft de kans op een vaste baan voor velen verminderd.

6 De kwaliteit van een voetbalploeg wordt niet afgemeten aan de voetbaltechnische en tactische kwaliteiten maar aan het aantal doelpunten dat gescoord wordt.

7 Men kan zich bij een technische vinding als high definition television (HDTV) afvragen of de noodzaak voor de industrie tot produceren niet groter is dan de behoefte in de maatschappij.

8 De trend om studies die een direct economische rol spelen met geldelijke middelen te bevoordelen leidt tot intellectuele verarming.

9 Het feit dat alkohol-vrij bier veelal wordt ingedeeld bij alkohol-houdende dranken heeft meer te maken met het maatschappelijk acceptabel maken van het product dan met de werkelijke chemische samenstelling.

10 Het veelvuldig opduiken van steeds nieuwere wapens in gebieden van strijd en de moeilijkheden de strijd aldaar te stoppen wijst op een grotere macht van de wapenhandel dan van de Verenigde Naties.

11 Millitaire dienstplicht voor afgestudeerden is een vorm van kapitaalvernietiging.

12 Voorkeursbeleid voor etnische minderheden en vrouwen leidt waarschijnlijk tot grotere maatschappelijke kansen voor deze groepen, maar kan ook leiden tot verlies van zelfrespect.

13 Competitie in de wetenschap kan leiden tot versnelde vooruitgang maar ook tot verlamming.

14 Oorzaken van problemen tussen verschillende bevolkingsgroepen moeten niet alleen bij betrokken groepen zelf gezocht worden, maar ook bij de wetgeving.

Stellingen behorend bij het proefschrift "Identification and characterization of synaptonemal complex proteins of the rat" door H.H. Offenberg, te verdedigen op 26 januari 1993 te Wageningen.

CHAPTER 1

INTRODUCTION

Introduction

The life cycle of sexually reproducing eukaryotes is characterized by the alternation of haploid and diploid generations of cells. Haploid cells have a single set of chromosomes, diploid cells have two such sets (Figure 1). The transition from the haploid to the diploid phase takes place at fertilization, when two haploid cells (gametes) fuse to form a diploid zygote; the diploid phase switches to the haploid phase at meiosis.

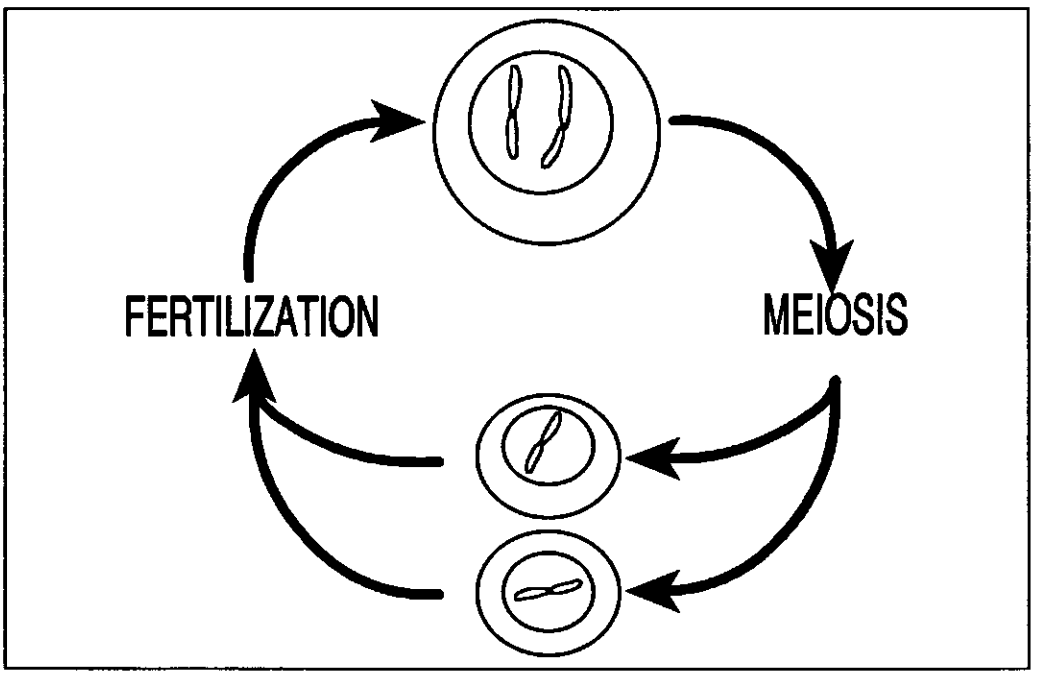

Figure 1. Life cycle of sexually reproducing eukaryotes (from Alberts *et al.*, 1983).

Meiosis consists of two successive cell divisions, meiosis I and II. After premeiotic S-phase, during the prophase of meiosis I, a series of chromatin rearrangements takes place by which homologous chromosomes condense, pair, recombine and segregate; the result is that at meiosis I diploid cells divide to produce haploid cells with new combinations of genes. Subsequently, at meiosis

II, the chromatids of each chromosome segregate; this division is very similar to a mitotic division.

It is of fundamental importance for eukaryotic genetics to analyze the chromatin rearrangements of meiotic prophase at the molecular level. Not only is this essential for the interpretation of genetic crosses, but it may also provide insight into the evolutionary origin of meiosis. Nevertheless, only during recent years the molecular analysis of meiosis has got into its stride. This thesis describes some preliminary investigations to allow the analysis of meiotic prophase at the molecular level.

Synaptonemal complexes and the rearrangements of chromatin during meiotic prophase.

In almost all eukaryotes analyzed thus far, the chromatin rearrangements of meiotic prophase are accompanied by the assembly and disassembly of nuclear structures that are specific for meiotic prophase nuclei: the synaptonemal complexes or SCs (Moses, 1968). These are flat, zipper-like structures (Figure 2) which appear between paired homologous chromosomes. They consist of two compact proteinaceous axes, one along each homologue. These are connected by thin transverse filaments. On the transverse filaments, between the axes, there is another longitudinal structure, the central element or CE. Both LEs together with the CE make up the tripartite structure of the SC; homologues are called *synapsed* if they are connected by this tripartite structure. The assembly and disassembly of the SC closely correlates with the chromatin rearrangements of meiotic prophase: early in meiotic prophase (leptotene) proteinaceous axes are formed along the chromosomes; the axes of homologous chromosomes (homologues) are subsequently connected (during zygotene) by the transverse filaments, and the CE appears on the transverse filaments. The chromosomal axes are called lateral elements (LEs), where they make part of the tripartite structure, i.e. where chromosomes are synapsed (see Figure 3). In some species, for instance the tomato, the process of synapsis is preceded by so-called presynaptic alignment (reviewed by Loidl, 1990); this is a rough align-

Figure 2. Detail of an ultrathin section of the nucleus of a pachytene spermatocyte of the rat. SC, synaptonemal complex; LE, lateral element; TF, transverse filament; CE, central element; Bar represents 150 μm (from K. Schmekel, with permission).

ment of homologous chromosomes at a larger distance (about 300 nm) than the width of the SC (100 nm) (see Figure 4). During the pachytene stage of meiotic prophase homologues are synapsed along their entire length, and the SC extends from telomere to telomere. During diplotene the SCs are disassembled, and the now recombined chromosomes condense further (diakinesis) in preparation of the first meiotic division. During diakinesis chiasmata show up as the visible results of the reciprocal exchanges between non-sister chromatids of

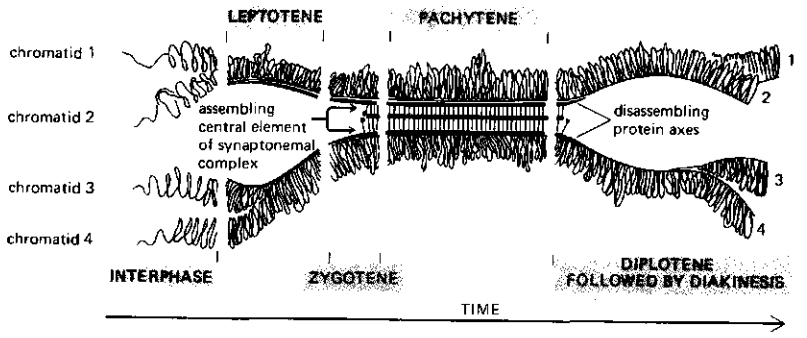

Figure 3. The successive stages of meiotic prophase can be defined on the basis of morphological changes of the SC: leptotene (formation of proteinaceous axes along the homologues), zygotene (the start of the actual synapsis of homologous chromosomes), pachytene (synapsis is complete; the SC extends along the homologues from telomere to telomere), and diplotene (disassembly of the tripartite structure) and diakinesis (SCs completely disassembled; further condensation of chromosomes, which are still connected by chiasmata; not shown) (From Alberts *et al.*, 1983).

homologues.

The assembly and disassembly of SCs are accompanied by the appearance and disappearance of recombination nodules (RNs); these are small, electron dense structures which appear on the central element of the SC in zygotene and pachytene. In some species there are two classes of morphologically distinct RNs: early RNs, which are present in zygotene and early pachytene; and late RNs which occur in early and mid pachytene (reviewed by Carpenter, 1987). The distribution of late RNs along the bivalents (pairs of homologous chromosomes) is similar to that of chiasmata (Albini and Jones, 1988), and the number of late RNs correlates with the number of reciprocal recombination events (Carpenter, 1975; Stack and Anderson, 1986) or chiasmata (Albini and Jones, 1988). For early RNs no such correlations have been found (reviewed in Von Wettstein *et al.*, 1984; Carpenter, 1987 and 1989): in general the number of early RNs is larger than the number of reciprocal recombination events (Stack

Figure 4. Early zygotene SC of the tomato; the axes of homologous chromosomes are aligned at a distance of about 300 nm, a phenomenon called presynaptic alignment (from J.H. de Jong, with permission).

and Anderson, 1986; Albini and Jones, 1987). Most investigators tentatively agree that late RNs are located at crossover sites, and may be involved in the mechanism of crossing over (Carpenter, 1987). It is possible that early RNs have a function in homology search and/or homology testing, and that they give rise to gene conversions as by-product of these activities (Rasmussen and Holm, 1978; Carpenter, 1987, 1989) (see below).

It seems likely that SCs are essential for the proper progress of meiotic prophase, because morphological alterations of SCs closely match the successive rearrangements of chromatin, and because the SC-structure has been conserved almost universally among eukaryotes. However, as yet no functions have been assigned with certainty to SCs. The analysis of SC functions is the major subject of the research project of which this thesis makes part.

Hypotheses about SC functions.

Information about possible SC functions comes from four different experimental approaches, namely (1) ultrastructural analysis of SC assembly in individuals with normal or aberrant karyotypes; (2) analysis of mutants with a defect in meiosis; (3) determination of the order of events during meiotic prophase and (4) biochemical analysis of purified SCs.

Ad (1). From the ultrastructural analysis of SC assembly we know that the chromosomal segments that are synapsed are not always homologous: in zygotene, short segments of non-homologous synapsis can be observed with low frequency (Rasmussen and Holm, 1978); in early pachytene, synapsis appears to be largely homologous. The specificity of synapsis appears to relax in later stages of meiotic prophase: in late pachytene pairs of homologous chromosomes (bivalents) are synapsed along their entire length, irrespective (within limits) of structural differences such as inversions, duplications, translocations or deletions. This relaxation of specificity of synapsis has been called synaptic adjustment (Moses and Poorman, 1981). The observations of non-homologous synapsis shed doubt on a direct role of the SC in the recognition of homology.

Ad (2). Analysis of mutants with a defect in meiosis has been performed in several species (Baker *et al.*, 1976; Esposito and Klapholtz, 1981; Roeder, 1990; Zickler, 1991; Maguire and Riess, 1991; Curtis and Doyle, 1991; Gulobovskaya, 1989), and many of these mutants have a defect in SC assembly. As yet, for none of the mutations it has been proven with certainty that they directly affect the SCs, since the observed defects in SC (dis)assembly could be a cause as well as an effect of the disturbance of meiosis. However, what we have learned from these analyses is, that the SC is not essential for meiotic levels of recombination: certain mutants of yeast with reduced spore viability display normal or appreciable levels of correct meiotic reciprocal recombination, but fail to assemble SCs (Rockmill and Roeder, 1990; Engebrecht and Roeder, 1989).

Ad (3). The order of events during meiotic prophase at the DNA-level and at the level of chromatin organization was studied by Padmore *et al.* (1991) in synchronized cultures of yeast. From this study it appears that the tripartite structure has no function in the initial event at the DNA-level preceding recombination, namely site specific double strand scission (Nicolas *et al.*, 1989), because the resulting double strand breaks occur prior to or concomitant with the first appearance of the tripartite structure of SCs (Padmore *et al.*, 1991).

Ad (4). We have chosen to approach the question about SC functions by biochemical analysis of SCs. For this purpose we have developed a procedure to isolate SCs from rat spermatocytes (Heyting *et al.*, 1985; Heyting and Dietrich, 1991) and to isolate monoclonal anti-SC antibodies (Heyting *et al.*, 1987, 1989; Heyting and Dietrich, 1991). In this thesis, the isolation of several monoclonal as well as polyclonal anti-SC antibodies is described (Chapter 2). These antibodies have been used for the identification and characterization of SC-components (Chapter 3 and Chapter 6) and for the isolation of cDNAs encoding SC proteins (Chapter 4; Meuwissen *et al.*, 1992, Chapter 5; Lammers *et al.*, in preparation). These cDNAs now provide the means to perform targeted mutagenesis of genes encoding SC-components, and to study meiosis in mutants for which it has been proven that the primary defect concerns the SCs.

To summarize our present knowledge about SC function: it is doubtful whether SCs play a direct role in the recognition of homology; SCs are not essential for meiotic levels of recombination, and the tripartite structure probably has no function in the initiation of recombination. What then could be the role of SCs? Several suggestions have been made, of which I will discuss four: (1) SCs help to resolve tangles of chromosomal axes; (2) SCs control the number and distribution of chiasmata; (3) SCs perform a test for long-range homology of chromosomes or (4) SCs help to prevent ectopic recombination.

Ad (1). The need for a mechanism to prevent or resolve tangles of chromosomal axes during meiotic prophase is obvious, unless some specific chromosome arrangement already exists at the onset of meiosis (see discussion Loidl, 1990;

Heslop-Harrison and Bennett, 1990). Interlocking axial elements have been observed in zygotene nuclei of various organisms (Rasmussen and Holm, 1980). Kleckner *et al.* (1991) suggested that obstacles in the assembly of the tripartite structure can be sensed by the meiotic prophase cells. Progression through meiotic prophase is blocked until these obstacles have been removed or circumvented and complete tripartite SCs have been formed. Obstacles can be circumvented at later stages of pachytene, when the specificity of synapsis is relaxed or abandoned, and heterologous chromosomes (reviewed in Von Wettstein *et al.*, 1984) or chromosomal segments (Moses and Poorman, 1981) are synapsed (see above). Meiotic prophase cells which succeed, with or without heterologous synapsis or synaptic adjustment, to form a complete set of entirely synapsed bivalents, have a better chance of producing viable meiotic products than cells which do not (e.g. De Boer and De Jong, 1989). According to this hypothesis mutation of genes encoding structural components of SCs will lead to a block in meiotic prophase.

Ad (2). The need for at least some control of chiasma number and distribution is also clear: a minimum of one chiasma per bivalent is required to ensure proper disjunction, although there are exceptions to this rule (discussed by Hawley, 1988). It is possible that the same control mechanism that might ensure the resolution of tangles, also ensures a minimum of one chiasma per bivalent: if the assembly of a stable tripartite structure starts at sites where reciprocal exchange has been initiated, a minimum of one chiasma per bivalent is ensured, provided that progress to diplotene is blocked until all bivalents are completely synapsed. This proposal implies that the cell can discriminate between initiated reciprocal and non-reciprocal recombination events (as has been suggested by Carpenter, 1987).

The need for other aspects of chiasma distribution, like specific chiasma localization and chiasma interference (reviewed by Jones, 1984, 1987) is less clear. Egel (1978) proposed that possible sites of reciprocal exchange are established before synapsis, and that the formation of the tripartite structure starts at these sites; the tripartite structure then prevents the establishment of

further possible sites of reciprocal exchange. This will result in positive chiasma interference. According to this view chiasma interference is simply the consequence of the way in which the tripartite structure is nucleated and extended. In the context of this hypothesis it is conceivable that mutations in genes encoding certain SC components eliminate positive chiasma interference, without affecting other aspects of meiosis.

Ad (3). Whether there is a mechanism to test long-range homology as a precondition for reciprocal exchange is still a matter of debate (see for instance discussion in Carpenter, 1987). In various objects, for instance mice (De Boer and De Jong, 1989), reciprocal exchange between a translocated segment and the homologous segment at its original position occurs frequently, so apparently telomere-to-telomere homology is not required. However, in most cases the chance for such an ectopic reciprocal exchange (i.e. an exchange between homologous segments at non-homologous positions) is small if the translocated segment is short. Does this mean that at least long-range homology is required, or that the chance is small that short homologous segments find each other? Information from studies on maize (Maguire, 1977) argues against a test for long-range homology, but is compatible with nucleation of tripartite SC at sites where recombination has already been initiated: Maguire observed a 1:1 relation between homologous synapsis of certain chromosomal segments with inversion heterozygosity and crossing over within those segments. Also in yeast, ectopic recombination occurs frequently between short gene duplications which had been generated by transplacement; this indicates that pairing of extensive regions of homology is not required for recombination events (Lichten *et al.*, 1987). What seems to be the rate limiting step in meiotic recombination is the activation of a locus to become an initiation site for recombination (by double strand scission); once activated, a locus can search the entire genome for a homologous partner with which to recombine (Haber *et al.*, 1991). The efficiency of a locus to serve as a donor for allelic or ectopic recombination depends possibly on a meiosis-specific chromosome organization by which some sequences are preferentially exposed for (homology search and) initiation of recombination (i.e. are hot spots of meiotic recombination, Lichten and

Haber, 1989). It is unlikely that the intact tripartite structure is required for such an effect, because in yeast initiation of recombination occurs before synapsis (see above, and Padmore *et al.*, 1991). However, it is possible that certain components of SCs, for instance of the lateral elements, contribute to the meiosis-specific chromatin organization by which certain loci become hot spots.

Ad (4). Are the observations on ectopic recombination in yeast also valid for eukaryotes with larger genomes, for instance in mice? What then prevents ectopic recombination between the numerous repeated sequences in mice? Is recombination inhibited in regions containing repeats, or is only a limited number of sequences selected for homology search and initiation of recombination (Stern *et al.*, 1975)? If so, what determines this selection? Or is there a test for long-range homology mice, although there is no evidence for this in yeast? Recently, Kricker *et al.* (1992) presented an interesting hypothesis how ectopic recombination could be prevented: what is required is (a) a mechanism by which homologous sequences of 20-200 nucleotides, depending on the species, in a heterologous environment (duplications) are accurately recognized, (b) a mechanism by which duplications are diversified and (c) a mechanism which can detect mismatches in heteroduplexes of imperfect repeats and eliminate the heteroduplexes containing these mismatches.

The mechanism by which duplications are recognized is not yet known, but it seems likely that recognition involves base pairing (Selker, 1990; Faugeron *et al.*, 1990). Diversification mechanisms have been identified in some fungi (*Neurospora crassa* and *Ascobolus immersus*; Selker, 1990; Faugeron *et al.*, 1990), and are suspected in other organisms including humans (Kricker *et al.*, 1992). In *N. crassa* and *A. immersus*, after recognition of a duplication, cytosines are probably methylated and C→T transition occurs by demethylation of 5-methylcytosine to thymine (Selker, 1990; Kricker *et al.*, 1992). At least in *Neurospora* and *Ascobolus* the diversification takes place prior to meiosis (Selker, 1990; Faugeron *et al.*, 1990).

Radman (1988, 1989) suggested that a long patch mismatch repair mechanism

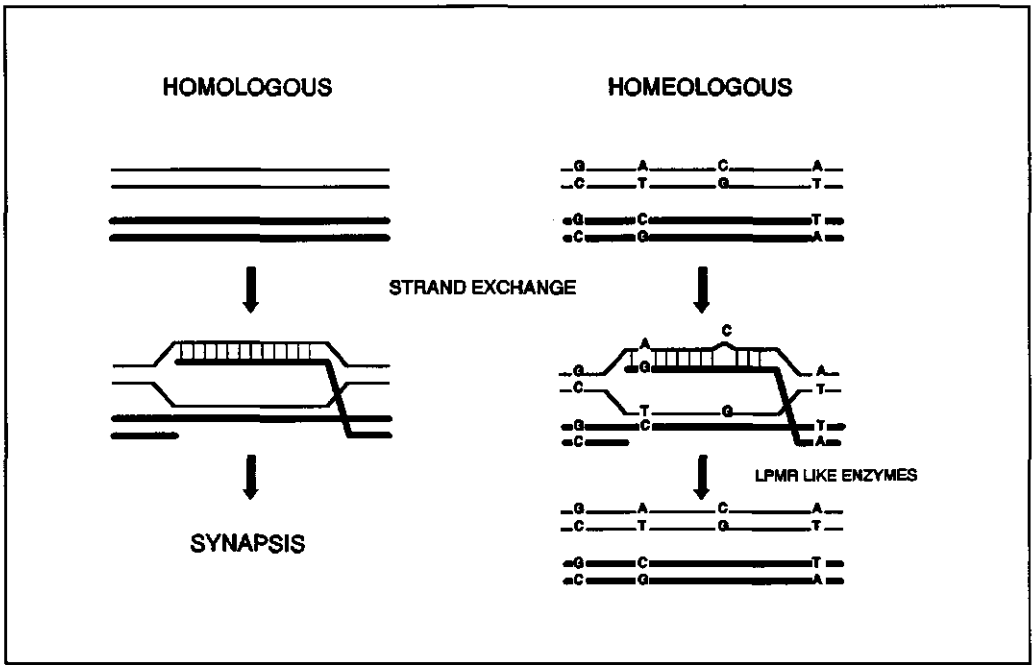

Figure 5. Molecular model of homology search by heteroduplex formation. Heteroduplex formation between homologues leads to synapsis. Heteroduplex formation between homeologous (diversified) sequences due to mismatches or unpaired residues will be resolved by LPMR like enzymes (see text for explanation). Only base-pair differences between the parental molecules are indicated: the hybrid region in this sketch (shown with dashes indicating hydrogen bonding) contains one A.G mismatch plus one unpaired C residue (as in a frameshift mutation) (according to Rayssiguier *et al.*, 1989).

(LPMR), analogous to the mutL/mutS system in *E.coli* (Jones *et al.*, 1987; Rayssiguier *et al.*, 1989; Petit *et al.*, 1991) could serve to detect mismatches in heteroduplexes and eliminate the heteroduplexes containing these mismatches. He proposed that such a system contains gene products capable of recognizing a single mismatch in a 20-200 nucleotide-long stretch and an enzyme (helicase) capable of unwinding a heteroduplex in which a mismatch has been detected. Homeologous or non-homologous pairing attempts can be aborted by such a system (see Figure 5). If this hypothesis is correct, a mismatch repair-like system should be active during early meiotic prophase, when homologous chromosomes align, and recombination is initiated (Padmore *et al.*, 1991).

It is unlikely that components of a mismatch repair-like system make part of the SC, because the tripartite structure itself seems to be insensitive to homology: non-homologous synapsis is often observed (see above). However, it is possible that early recombination nodules contain mismatch repair enzymes, because the stages where a mismatch repair-like system is expected to be active correspond to the stages where early recombination nodules are observed. In zygotene, heterosynapsis could be the result of homeologous heteroduplex formation which is not yet recognized as such; in late pachytene, synaptic adjustment could result from heterosynapsis, which is not recognized anymore because the supposed mismatch repair-like system is no longer active.

Summarizing, at present the function of the SC is not at all clear; most investigations discussed above exclude possible functions of SCs. Several questions concerning SCs remain unanswered, including their evolutionary and ontogenetic origin, their role in meiotic chromosome pairing and recombination, and the regulation of their (dis)assembly. In this thesis, I describe how we have elicited several antibodies directed against SC components. Using these antibodies we have isolated cDNAs encoding four SC proteins (Offenberg *et al.*, in preparation, Chapter 4 this thesis; Meuwissen *et al.*, 1992, Chapter 5 this thesis; Lammers *et al.*, in preparation). For the analysis of the function of these proteins and of the function of SCs it is now possible to perform targeted mutagenesis of the corresponding genes and study meiosis in these mutants. I expect that the outcome of these mutagenesis experiments will bring along a series of surprises.

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CHAPTER 2

SYNAPTONEMAL COMPLEX PROTEINS

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Summary

Synaptonemal complexes were isolated from rat spermatocytes for the purpose of biochemical and morphological analysis. Several monoclonal antibodies were elicited against purified synaptonemal complexes to study the composition and assembly of these structures. Four classes of antibodies could be discriminated according to the polypeptides that they recognize on Western blots of purified synaptonemal complexes, namely antibodies recognizing (i) a 190-kDa[1] polypeptide; (ii) a 30- and a 33-kDa polypeptide; (iii) two polypeptides with molecular weights of about 120 kDa; and (iv) polypeptides with molecular weights of 66-55 kDa. The localization of these antigens within spermatocytes was analyzed light microscopically, by means of the immunoperoxidase technique and ultrastructurally, by immunogold labelling of surface-spread spermatocytes. The 66- to 55-kDa polypeptides are not confined to synaptonemal complexes; rather, these polypeptides appear to be chromosomal components. The 190-, 30-, and 33-kDa polypeptides make part of the lateral elements of paired as well as unpaired segments of synaptonemal complexes. The distribution of the 190-, 120-, 30-, and 33-kDa polypeptides within the testis was analyzed by immunofluorescence staining of cryostat sections. All these polypeptides turned out to be specific for nuclei of zygotene up to and including diplotene spermatocytes. Only in some early spermatids could the 190-, 120-, 30-, and 33-kDa polypeptides be detected, presumably in remnants of synaptonemal complexes. We conclude that the lateral elements of synaptonemal complexes do not arise by rearrangement of pre-existing components in the nucleus, but that their major components are newly synthesized during meiotic prophase.

[1] This paper was published in (1989) Genome, **31**, 81-87. The molecular weights reported for the identified proteins have been estimated on basis of their relative electrophoretic mobilities: in later Chapters these proteins are designated by their relative electrophoretic mobilities. For instance, the 30 kDa protein in this Chapter corresponds to the M_r 30,000 protein in later Chapters.

Introduction

The first meiotic division is a specialized cell division during which the transition from the diploid to the haploid phase of the life cycle of sexually reproducing organisms takes place. A series of complex chromatin rearrangements precedes this division: after premeiotic S-phase the chromosomes condense, and homologous chromosomes pair, recombine, and segregate. All of these rearrangements appear to be mediated by synaptonemal complexes (SCs). These nuclear structures, characteristic for meiotic prophase cells, undergo a series of morphological alterations that correlate with the successive rearrangements of chromatin (Gillies, 1975; Lu, 1984; Moses *et al.*, 1984): at the beginning of meiotic prophase, an axial core is formed along each chromosome; the axial cores of homologous chromosomes are then aligned, and transversal filaments are formed between them (zygotene); when chromosome pairing is complete (pachytene), the structural elements of SCs include two lateral elements (LEs, the former axial cores), attachment plaques (APs) at the end of LEs by which SCs appear to be connected to the nuclear wall, transversal filaments between the LEs, and a central element (CE), which is formed on the transversal filaments between the LEs.

We want to study the structure and composition of SCs, the origin of their components as well as the regulation of their assembly-disassembly, to obtain more insight into the mechanisms of chromatin rearrangements during meiotic prophase. For this purpose we developed a procedure to isolate SCs (Heyting *et al.*, 1985) and elicited monoclonal antibodies (Mabs) against purified SCs (Heyting *et al.*, 1987). In this paper we summarize the results, obtained up till July 1988.

Results

Isolation of SCs from rat spermatocytes.

Figure 1 summarizes the protocol for the isolation of SCs. Two problems had to be solved for the isolation of these structures, namely to detach the SCs from the nuclear matrix (Comings *et al.*, 1976; Ierardi *et al.*, 1983; Raveh and Ben-Zeev, 1984) and to separate them from other subcellular structures. We began with purified spermatocytes, to avoid contamination of the final preparation with nuclear laminae (which are lacking from spermatocytes (Fawcett, 1966; Stick and Schwarz, 1983)), and sperm heads and tails. The effects of a variety of lysis conditions on spermatocytes were monitored by phase-contrast microscopy and electron microscopical analysis of agar filtrates of lysed spermatocytes. Agar filtration is a very useful technique to study the effects of successive steps of an isolation procedure, because it allows the inspection of the complete composition of a suspension. Those lysis conditions that appeared to cause aggregation, as indicated by an increased contrast in the nuclei of lysed spermatocytes, and clumping of cellular material in the agar filtrates were avoided. Ionic detergents, NaCl, and Mg^{2+} ions had to be omitted from the lysis medium to prevent aggregation. Lysis of spermatocytes in Triton X100, EDTA, and DTT at neutral pH yields swollen nuclei with thin SC-like structures. The SCs can be liberated from these swollen nuclei by digestion with DNAse II, which does not require Mg^{2+}. After DNAse II digestion the SCs can be separated from other nuclear components by centrifugation through 1.5 M sucrose. The resulting preparation consists of clean 60-80% pure SCs. Figure 2 shows an agar filtrate of SCs, purified from late pachytene spermatocytes. In these SCs only fragments of the CE are present. SCs, isolated from early pachytene SCs have thinner LEs, and an apparently intact CE (not shown).

Polypeptide composition of purified SCs.

Figure 3 shows an SDS-polyacrylamide gel electropherogram of purified SCs from zygotene, early-mid pachytene, late pachytene, and diplotene SCs. The

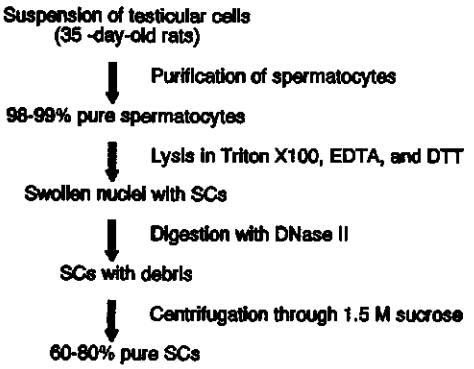


Figure 1. Protocol for the isolation of SCs.

Figure 2. Agar filtrate of SCs, isolated from late pachytene spermatocytes; the SCs have thick LEs and few remnants of the CE. Bar, 1 μm.

spermatocyte fractions were obtained after synchronization of spermatogenesis with hydroxyurea (HU), which kills spermatogonia in S-phase (Oud *et al.*, 1979). By purification of spermatocytes at successive points of time after release from a HU block (Dietrich and Mulder, 1981), spermatocyte populations enriched in zygotene (47% purity), early-mid pachytene (70% purity), or diplotene (90% purity) were obtained (R.J. Dettmers, C.Heyting, A.J.J. Dietrich, E.J.W. Redeker, and A.C.G. Vink, in preparation). SC preparations 60-80% pure could be isolated from early-mid or late pachytene SCs, with thick LEs, and only fragments of the CE present. The major polypeptides in these preparations have relative electrophoretic mobilities (M,s) corresponding to molecular masses of, respectively, 190, 130-120, 66-65, 55-53, 48, 45, 33, 30 and 26 kDa (Figure 3, lanes c and d). The LEs of SCs from diplotene spermatocytes were often observed to disassemble into long sub-fibrils (not shown); the polypeptide composition of these preparations is more complex than that of pachytene SCs

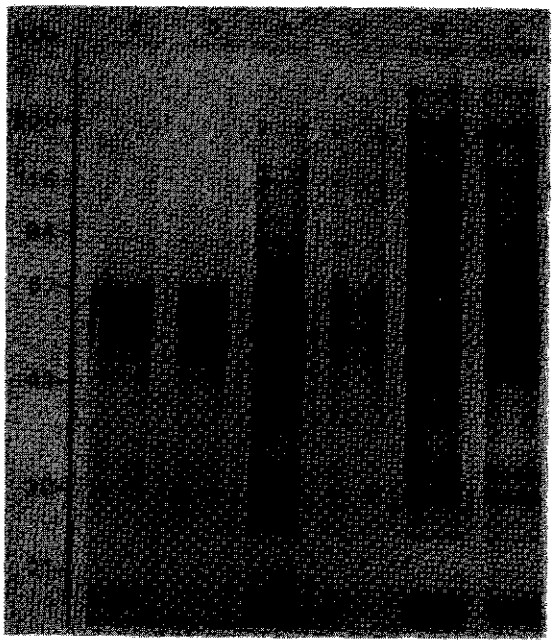


Figure 3. SDS-polyacrylamide gel electrophoresis of SCs, isolated from spermatocytes in successive stages of meiotic prophase. *Lanes* ***a*** and ***b***, SCs from zygotene spermatocytes, isolated 20 days after release from the hydroxyurea (HU) block; *lane* ***c***, SCs, isolated from late pachytene spermatocytes, isolated 26 days after release from the HU block; *lane* ***d***, SCs from early-mid pachytene spermatocytes, isolated 24 days after release from the HU block; *lanes* ***e*** and ***f***, SCs from diplotene spermatocytes, isolated 31 days after release from the HU block. Molecular mass markers used are myosin, 200 kDa; ß-galactosidase, 116 kDa; phosphorylase B, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 21 kDa. 7 to 18% linear gradient polyacrylamide gradient slab gel, stained with silver.

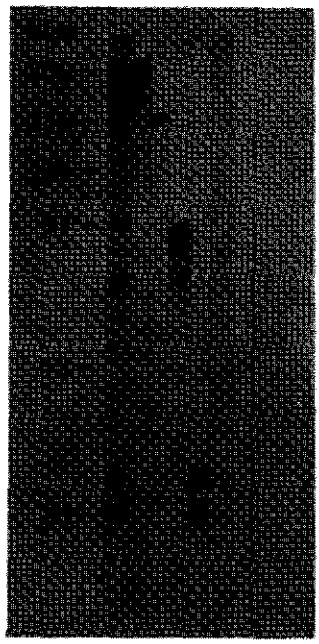


Figure 4. Immunoblot analysis of anti-SC monoclonal antibodies. *Lane* ***a***, polypeptides of purified SCs, separated on a 10% SDS-polyacrylamide slab gel and stained with Coomassie blue; *lane* ***b***, immunoblot after incubation in Mab IX9D5; *lane* ***c***, immunoblot after incubation in Mab IX5B2; *lane* ***d***, immunoblot after incubation in Mab IX8E11; *lane* ***e***, immunoblot after incubation in Mab II52F10; *lane* ***f***, immunoblot after incubation in a control hybridoma supernatant, elicited against fish brain homogenate. Molecular mass markers, see legend to Figure 3.

(Figure 3, lanes e and f); this may be ascribed in part to proteolytic breakdown of SC components, as the SCs are falling apart in diplotene cells; however, proteolytic breakdown cannot provide an explanation for the presence of some high

molecular mass polypeptides in diplotene SC preparations.

The few SCs that could be isolated from the zygotene spermatocyte cell fraction were fully paired early pachytene SCs; it is doubtful whether zygotene SCs can stand the isolation procedure. About 70% of the "zygotene" SC preparations consisted of contaminating chromatin and (or) nuclear matrix material. These preparations contain a relatively large amount of polypeptides with M_rs of 66-55 kDa (Figure 3, lanes a and b); probably, (some of) these polypeptides do not originate from SCs, but from SC-associated material (see below).

Monoclonal anti-SC antibodies.

To find out which of the polypeptides in SC preparations make part of SCs, we elicited monoclonal antibodies against purified SCs and localized their antigens within spermatocytes. After immunization of mice, and fusion of their lymphocytes with myeloma cells, we screened the resulting hybridomas for the production of anti-SC antibodies by means of the immunoperoxidase technique and phase-contrast microscopy, applied to agar filtrates of lysed spermatocytes. We thus found 75 different anti-SC antibody producing clones, 47 of which react with polypeptides on Western blots of purified SCs. These 47 clones can be divided into four classes, according to the polypeptides with which they react (Table 1). Examples of these four classes will be discussed below.

Localization of antigens.

30- and 33- kDa polypeptides.

Eighteen anti-SC Mabs react with a 30- and a 33-kDa polypeptide on immunoblots of SCs. The best characterized Mabs of this class are II52F10 (Figure 4, lane e) and IX8G9. Their antigens have been localized ultrastructurally on the LEs of SCs of zygotene up to and including diplotene SCs (Moens *et al.*, 1987; Heyting *et al.*, 1987; Figure 6a). Both polypeptides are specific for meiotic prophase nuclei and are absent from mitotic chromosomes (Heyting *et al.*,

1988). Immunocytochemical staining of lysed spermatocytes with IX8G9 is shown in Figures 5d-5f.

120- to 130-kDa polypeptides.

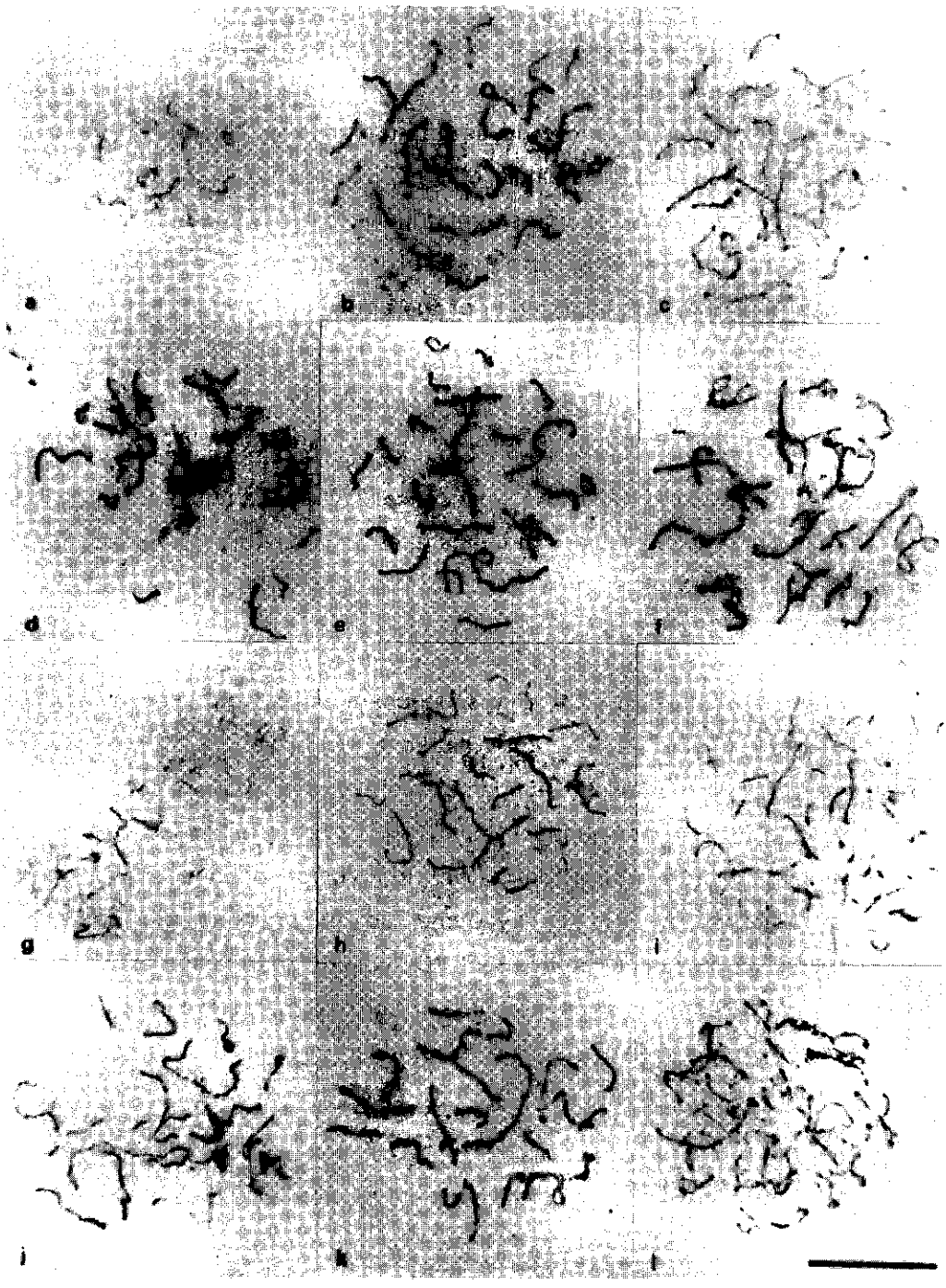
We found 13 Mabs that react with polypeptides with M_rs of 120-130 kDa on Western blots of purified SCs. The best characterized Mab of this class is IX5B2

Table 1. Reaction pattern of Mabs elicited against purified SCs

Prototype	Immunoblot (kDa)	Reaction with: SCs of lysed spermatocytes (LM) paired	SCs of lysed spermatocytes (LM) unpaired	cryostat sections of rat testis (LM)[a]	Surface-spread spermatocytes (EM)[a]	No. of clones
II52F10 and IX8G9	30 and 33	+	+	Spermatocyte nuclei (SCs)	LEs (paired and unpaired)	18
IX5B2	120-130	+	-/+	Spermatocyte nuclei (SCs or SC fragments)	Inner edge of LEs (paired)	13
IX9D5	190	+	+	Spermatocyte nuclei (SCs), sperm heads	LEs	14
IX8E11	66-55	+[b]	+[b]	not determined	SC- associated material	2

Note: LM, light microscopy; EM, electron microscopy.
[a]Localization of antigens on cryostat sections and in surface-spread spermatocytes has been performed with prototype antibodies only.
[b]Series of dots on SCs.

Figure 5. Immunoperoxidase staining of lysed spermatocytes with anti-SC monoclonal antibodies (a-c) Mab IX9D5; (d-f) Mab IX8G9; (g-i) Mab IX5B2; (j-l) Mab IX8E11. (a,d,g, and j) Zygotene; (b,e,h, and k) pachytene; (c,f,i and l) diplotene. Bright-field illumination. Bar, 20 μm.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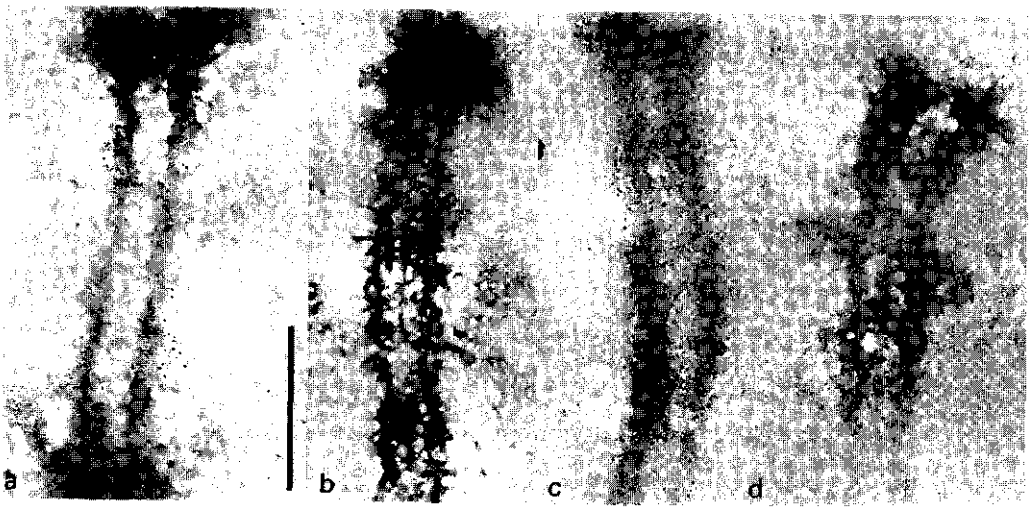

Figure 6. Ultrastructural localization of the antigens of anti-SC monoclonal antibodies by immunogold staining of surface-spread pachytene spermatocytes. (a) Mab IX8G9; (b) Mab IX5B2; (c) IX9D5; (d) Mab IX8E11. Bar represents 0.5 μm.

to 130-kDa polypeptides (Moens *et al.*, 1987); unfortunately the antigen of III15B8 could not be identified.

The SC components that we have now identified all appear to be newly synthesized during meiotic prophase (Heyting *et al.*, 1988; this paper). Apparently, the LEs of SCs do not originate from pre-existing structures in the nucleus. Thus, the chromatin rearrangements of meiotic prophase appear not to be affected by rearrangements of chromatin-supporting structures; rather, it appears that the chromatin has to detach from the lamina and (or) the nuclear matrix and to reorganize on the SCs.

With respect to the further analysis of chromatin rearrangements during meiotic prophase, the following steps now appear feasible: the assembly of SCs can be analyzed by detailed immunocytochemical studies; the composition of SCs may be compared with that of mitotic chromosomes; changes in the pattern of

modifications of SC proteins in successive stages of meiotic prophase (if any) can be analyzed; and the genes coding for the newly identified SC components can be isolated by screening expression libraries of the rat testis with the anti-SC Mabs; this should allow us to obtain more information about the amino acid sequence of SC polypeptides and possibly also about their function.

Acknowledgements

We thank W. van Raamsdonk (Zoological Laboratory, University of Amsterdam) and R. van Noorden and I. Vogels (Laboratory for Histology and Cell biology, University of Amsterdam) for facilities and many practical advises; R. Lutgerhorst and W. van Est for photographical assistance; and R. Wekker for expert technical assistance.

Materials and Methods

Most of the procedures mentioned in this paper have been described before: the isolation of spermatocytes and SCs, and the procedures of agar filtration and one-dimensional SDS-polyacrylamide gel electrophoresis by Heyting *et al.* (1985); the preparation of anti-SC Mabs and the screening of hybridomas by Heyting *et al.* (1987); the immunogold staining of surface-spread spermatocytes by Moens *et al.* (1987); and the procedures of immunofluorescence staining of cryostat sections and of immunoblotting by Heyting *et al.* (1988). The procedures of fractionation of spermatocytes will be described in detail later by R.J. Dettmers, C. Heyting, A.J.J. Dietrich, E.J.W. Redeker, and A.C.G. Vink (in preparation).

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CHAPTER 3

TISSUE DISTRIBUTION OF TWO MAJOR COMPONENTS OF SYNAPTONEMAL COMPLEXES OF THE RAT

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Summary

In this paper we describe an analysis of the tissue distribution of two recently identified components of synaptonemal complexes (SCs), an M_r 125,000 and an M_r 190,000 protein, in the male rat by immunoblot analysis and immunocytochemical techniques. We compared the tissue distribution of these antigens with that of two earlier identified SC components, an M_r 30,000 and an M_r 33,000 polypeptide. For this purpose we used monoclonal antibodies (Mabs), that react exclusively with SCs in lysed spermatocytes, and that recognize the above mentioned antigens specifically in immunoblots of SC proteins or of nuclear proteins from spermatocytes; these were Mab IX9D5 (anti-190,000), Mab IX5B2 (anti-125,000), Mab II52F10 (anti-30,000+33,000), and Mab IX8G9 (anti-30,000+33,000). In the immunoblot experiments, we could detect the M_r 190,000 and M_r 125,000 antigens exclusively in blots of SC proteins or nuclear proteins from spermatocytes; these antigens were not detectable in blots of nuclear proteins from liver, brain, spermatogonia of spermatids or in blots of proteins from mitotic chromosomes or nuclear laminae. With the anti-30,000+33,000 Mabs we obtained essentially the same result, except that Mab IX8G9, but not II52F10, recognizes a small amount of M_r 30,000 antigen in blots of nuclear proteins from spermatids and spermatogonia. Although this might be ascribed to contamination of the isolated spermatids and spermatogonia, we cannot exclude that a small amount of 30 kDa antigen is present in these cells. In the immunofluorescence analysis, the testis was the only tissue that reacted detectably with the above antibodies. Within the testis, spermatocytes and some early spermatids were the only cell types which contained detectable amounts of antigen. The M_r 125,000 antigen was exclusively observed in nuclei of spermatocytes, from zygotene up to and including diplotene, in paired segments of SCs. The M_r 30,000+33,000 and 190,000 antigens were present in paired as well as unpaired segments of SCs in nuclei of spermatocytes, from zygotene up to and including diplotene and in the nuclei of some early spermatids in presumed remnants of SCs. We conclude that SCs largely consist of meiosis-specific proteins.

Introduction

During the prophase of the first meiotic division homologous chromosomes pair, and recombination takes place between non-sister chromatids of homologous chromosomes. These processes appear to be mediated by specific structures of the meiotic prophase nucleus, the synaptonemal complexes (SCs) (Von Wettstein *et al.*, 1984). As yet, little is known about the ontogenetic and phylogenetic origin of these structures and about the regulation of their (dis)assembly. Elucidation of the ontogenetic origin of SCs is required to obtain insight into the mechanism of chromatin rearrangements in meiotic prophase: SCs might arise either from preexisting chromatin supporting structures in the nucleus like the nuclear matrix or the nuclear lamina, or from newly synthesized products. The latter possibility implies more drastic chromatin rearrangements. Elucidation of the phylogenetic origin of SCs or SC components might provide clues to the evolutionary origin of the whole process of meiosis.

In this paper we concentrate on the ontogenetic origin of SCs. For this purpose, we developed a procedure to isolate SCs from spermatocytes of the rat, and elicited monoclonal antibodies (Mabs) against purified SCs (Heyting *et al.*, 1985, 1987, 1989, Chapter 2 this thesis). In a previous publication we reported that two major components of the lateral elements (LEs) of SCs with M_rs of 30,000 and 33,000 are specific for meiotic prophase nuclei (Heyting *et al.*, 1988). In this paper we show by immunohistochemical and immunoblot analyses, that two recently identified SC antigens, with M_rs of 190,000 and 125,000 respectively, also occur exclusively in meiotic prophase cells. One of these antigens (M_r 190,000) forms part of the LEs of paired as well as unpaired segments of SCs; the other antigen (M_r 125,000) is localized specifically on the inner edge of the LEs in paired segments (Heyting *et al.*, 1989; Moens *et al.*, 1987). Thus, with respect to their ontogenetic origin, it is gradually becoming clear that SCs are not derived from other chromatin supporting structures of the nucleus but that they are assembled from newly synthesized components during the prophase of the first meiotic division.

Results

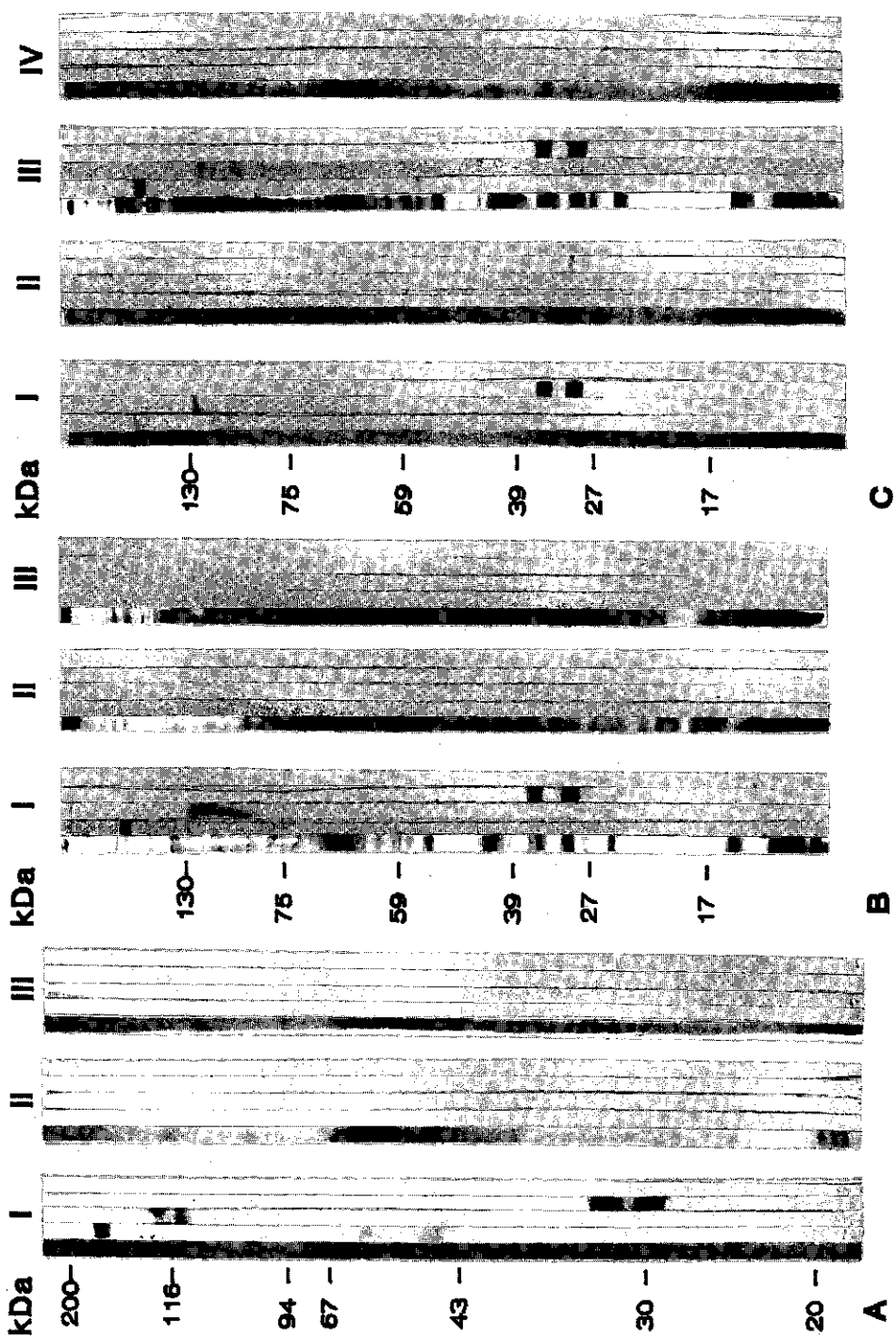
Immunoblot analysis.

Table 1 shows the composition of the testicular cell fractions that were used for isolation of nuclei and immunoblot analysis. The conditions mentioned in the Materials and Methods section allow the isolation of almost spermatocyte free spermatogonia and spermatid fractions. The spermatid fraction contained

Table 1. Composition of the cell fractions used for proten immunoblot analysis

Cell fraction	Composition (% of identifiable cells)			
	Spermato-gonia	Spermato-cytes	Spermatids	Non-sperma-togenic
Spermatogonia	93.1	0	6.9	0
Spermatocytes	0.6	98.5	0.6	0.2
Spermatids	0.9	4.3	94.0	0.9

Figure 1. Immunoblot analysis of proteins of various nuclei and nuclear fractions with monoclonal antibodies (Mabs) IX9D5 (anti-M, 190,000), IX5B2 (anti-M, 125,000) and IX8G9 (anti-M, 30,000 + 33,000). Samples containing about 60 μg of soluble protein or of $3x10^7$ SCs were loaded onto 2 cm wide slots of 16x20x15 cm 7-18% linear gradient SDS-polyacrylamide gels. 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 these filters 0.3 cm wide strips were cut for incubation in hybridoma supernatants. The strips shown in **A, B** and **C** are from different gels. In **A**, the following samples were layered: *lane I*, synaptonemal complexes (SCs); *lane II*, rat liver nuclear laminae; *lane III*, rat mitotic chromosomes. **B**: *Lane I*, spermatocyte nuclei; *lane II*, liver nuclei; *lane III*, brain nuclei.**C**: *lane I*, SCs; *lane II*, spermatogonial nuclei; *lane III*, spermatocyte nuclei; *lane IV*, spermatid nuclei. Each lane shows from left to right: Coomassie blue stained gel, and immunoblots incubated in respectively, Mab IX9D5 (anti-M, 190,000), Mab IX5B2 (anti-M, 125,000), Mab IX8G9 (anti-M, 30,000 + 33,000) and a control hybridoma supernatant. Molecular weight markers used are for **A**: myosin, 200 kDa; ß-galactosidase, 116 kDa; phosphorylase B, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20 kDa; for **B** and **C** we used prestained molecular weight markers (BioRad), which were blotted together with the other lanes of the same gel onto nitrocellulose; the relative electrophoretic mobilities as specified by the manufacturer are shown.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primarily round spermatids and did not contain mature sperm heads; it was not possible to differentiate between all types of spermatogonia in the Giemsa-stained preparations, thus, we cannot exclude that some early resting spermatocytes were scored as B-type spermatogonia.

The reaction pattern of the anti-SC Mabs with immunoblots of nuclear proteins from various sources is shown in Figure 1. Like Mab II52F10 (Heyting *et al.*, 1988), Mab IX8G9 exclusively recognizes M_r 30,000 and 33,000 proteins on blots of SCs or spermatocyte nuclei (Figure 1A, lane I, Figure 1B, lane I and Figure 1C, lanes I and III). Because Mab IX8G9 produces a severalfold stronger signal on blots than Mab II52F10 (compare Heyting *et al.*, 1988), Mab IX8G9 was used for further immunoblot experiments. Mab IX8G9 does not recognize any proteins on immunoblots of nuclei from liver or brain (Figure 1B, lanes II and III) or on blots of purified liver nuclear laminae (Figure 1A, lane II) or of mitotic chromosomes (Figure 1A, lane III). This is consistent with results obtained earlier with Mab II52F10 (Heyting *et al.*, 1988). Mab IX8G9 produces a weak signal at M_r 30,000 on blots of purified spermatogonia (Figure 1C, lane II) or spermatids (lane V). From scans of the nitrocellulose strips we estimate that the intensity of this signal is at most 2 % of the intensity of the signal at M_r 30,000 on the blot of spermatocyte nuclei (Figure 1C, lane III).

The anti-M_r 190,000 Mab IX9D5 exclusively recognizes an M_r 190,000 protein in immunoblots of SCs or spermatocytes (Figure 1C, lanes I and III, Figure 1A, lane I and Figure 1B, lane I). It does not detectably recognize any protein on blots of nuclei from other sources (Figure 1B, lanes II and III and Figure 1C, lanes II and IV) or of nuclear laminae (Figure 1A, lane II) or mitotic chromosomes (lane III). Similarly, Mab IX5B2 (anti-M_r 125,000) only recognizes an M_r 125,000 protein on immunoblots of SCs or spermatocyte nuclei (Figure 1A, lane I, Figure 1B, lane I, Figure 1C, lanes I and III) and does not react with any proteins from nuclei from other sources, liver nuclear laminae or mitotic chromosomes (other lanes in Figure 1).

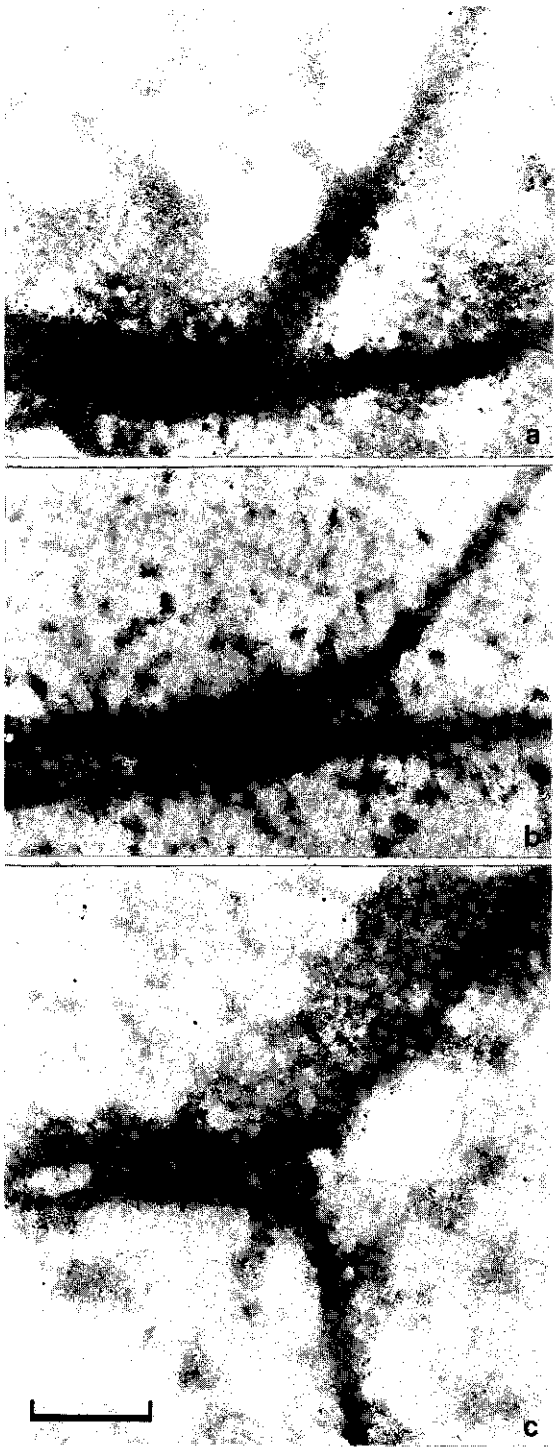


Figure 2. Ultrastructural localization of the antigens of the Mabs IX9D5 (anti-M, 190,000) **a**, IX5B2 (anti-M, 125,000) **b** and IX8G9 (anti-M, 30,000 + 33,000) **c**, by immunogold staining of surface-spread diplotene spermatocytes. Bar represents 0.5 μm.

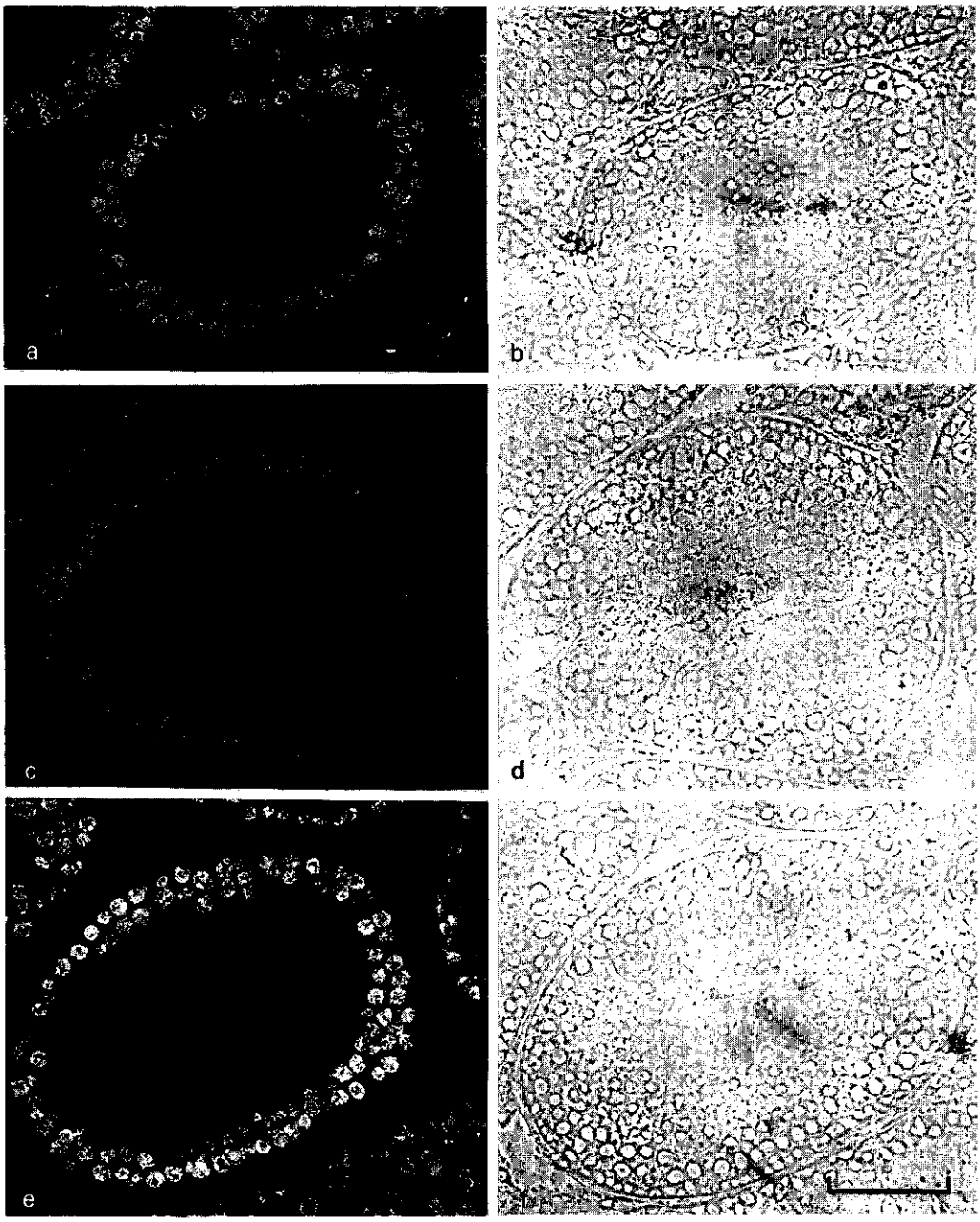


Figure 3. Frozen sections of rat testis after immunofluorescence staining with Mabs IX9D5 (a), IX5B2 (b) and II52F10 (c); a, c and e, immunofluorescence; b, d and f, phase contrast of the same sections. Bar represents 50 μm.

Immunolocalization of antigens.

Figure 2 shows electron micrographs of pre-diffuse diplotene SCs after immunogold staining with Mabs IX8G9, IX5B2 and IX9D5, respectively. The M_r 30,000+33,000 antigens as well as the M_r 190,000 antigen are localized specifically on the LEs of SCs in paired as well as unpaired segments (Figure 2a, c). The M_r 125,000 antigen is confined to the inner edge of the LE in paired segments (Figure 2b). In light micrographs of zygotene or diplotene cells, stained by the indirect immunoperoxidase technique with Mab IX5B2, SCs show up as a series of interrupted lines (Heyting *et al.*, 1989), while after immunoperoxidase staining with Mab II52F10 (Heyting *et al.*, 1987), IX8G9 or IX9D5 (Heyting *et al.*, 1989) SCs are visible as forked structures.

In the immunofluorescence analysis of tissue sections, the testis was the only tissue that contained detectable amounts of antigen of Mab IX9D5, II52F10 or IX5B2 (not shown).

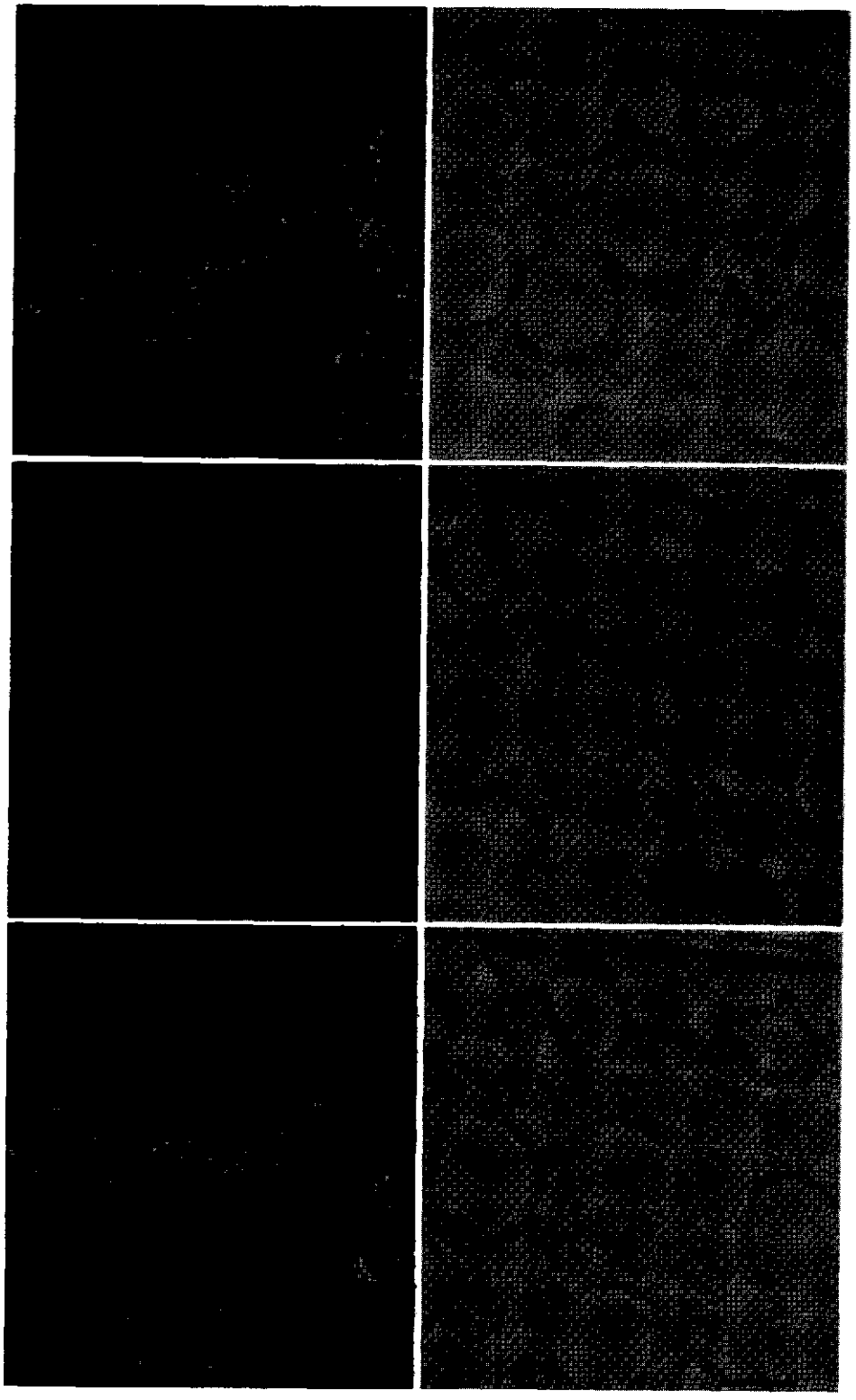
Figures 3 to 6 show the result of immunofluorescence staining of frozen sections of the rat testis with these Mabs. As has been reported earlier for the M_r 30,000+33,000 antigens (Heyting *et al.*, 1988), the M_r 125,000 and M_r 190,000 antigens are confined to nuclei of spermatocytes and associated with the axes of SCs. The identity of the antigen-containing cells could be deduced from the stages of the seminiferous epithelium (Leblond and Clermont, 1952). The central tubules in Figure 3a to f are in stage VII to XI because of the presence of the relatively large spermatocytes with fully paired SCs and of round spermatids. All three tested Mabs react exclusively with the layer of spermatocyte cells in these tubules. Figure 3e (top left) also shows part of a tubule where two layers of cells react with Mab II52F10. These are stage XIII tubules which contain two layers of spermatocytes, one consisting of zygotene cells, the other of diplotene cells (Leblond and Clermont, 1952). Figures 4 to 6 show details of such tubules. In Figure 4 the inner layer of spermatocytes is in diplotene and has partially unpaired SCs, while the outer layer is in zygotene (Leblond and Clermont, 1952). In the zygotene cells the anti-M_r

30,000+33,000 Mab II52F10 recognizes very short pieces of presumed SC axes, particularly in the outer rim of the nucleus (Figure 4e). The anti-M_r 190,000 Mab IX9D5 produces an almost identical staining pattern (Figure 4a), while the anti-M_r 125,000 Mab recognizes paired segments of diplotene SCs, but does not react detectably with the early zygotene nuclei (Figure 4c).

In Figures 5 and 6, the results of double staining experiments are shown, with polyclonal rabbit anti-M_r 30,000+33,000 and monoclonal mouse anti-M_r 125,000 (IX5B2) as first antibodies, and horse anti-rabbit-TRITC and goat anti-mouse-FITC conjugates as second antibodies. In Figure 5, the layer of diplotene spermatocytes has partially unpaired SCs (Figure 5b), which show up as interrupted lines after staining with the anti-M_r 125,000 Mab (Figure 5c). The layer of zygotene cells contains short fragments of axial cores or LEs containing the M_r 30,000+33,000 antigen (Figure 5b), and very little M_r 125,000 antigen (Figure 5c). In Figure 6, the layer of diplotene cells has almost entirely unpaired SCs, still containing the M_r 30,000+33,000 antigens (Figure 6b), but not the M_r 125,000 antigen (Figure 6c). In the layer of zygotene cells the M_r 30,000+33,000 as well as the M_r 125,000 antigens are detectable, particularly in the periphery of the nucleus. Thus, in immunofluorescence studies the M_r 125,000 antigen becomes detectable at slightly later stages of zygotene than the M_r 30,000+33,000 (and M_r 190,000) antigens, while it disappears from earlier stages of diplotene cells.

We also looked for the presence of antigens in frozen sections of rat liver and brain. However, in cell types other than spermatocytes we could not detect any SC antigens (not shown). The only exception is a reaction of Mab IX9D5 with

Figure 4. Details of frozen sections of testicular tubules (stage XIII) with partially unpaired SCs in the layer of diplotene spermatocytes; immunostaining with Mabs IX9D5 (a), IX5B2 (c) and II52F10 (e); **a**, **c** and **e**, immunofluorescence; **b**, **d** and **f**, phase contrast of the same section. **z**, zygotene; **p** prediffuse diplotene; **s**, Sertoli cell; **t**, spermatid; **i**, interstitial cell; **g**, spermatogonium; Bar represents 10 μm.



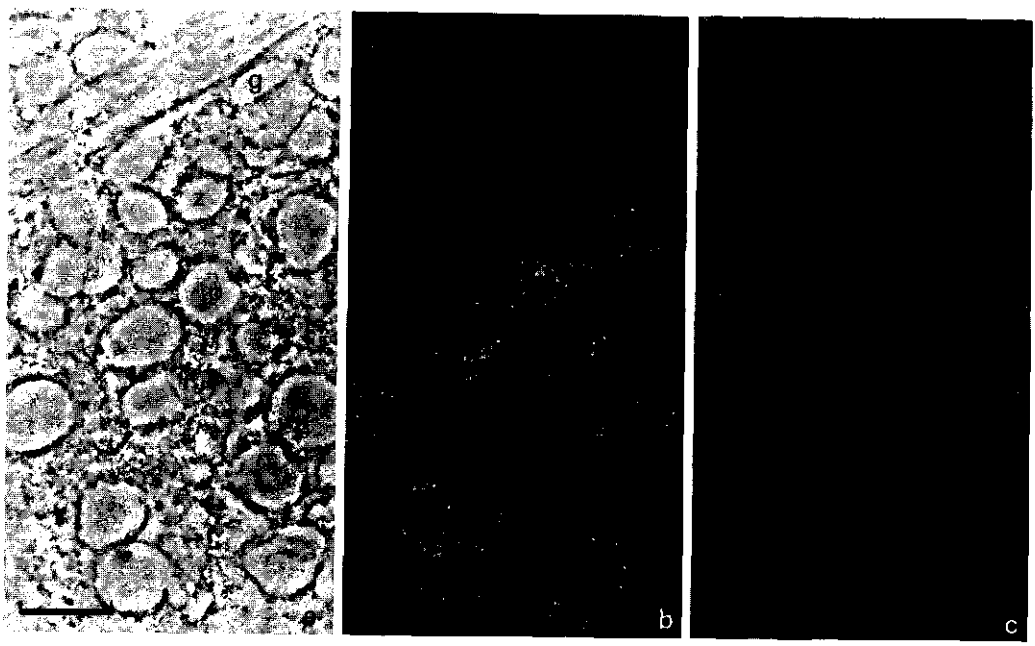


Figure 5. Details of a frozen section of a testicular tubule (stage XIII) with partially unpaired SCs in the diplotene spermatocytes, stained with polyclonal rabbit anti-M, 30,000 + 33,000 and monoclonal mouse anti-M, 125,000 (IX5B2) as first antibodies and horse anti-rabbit-TRITC and goat anti-mouse-FITC conjugates as second antibodies; **a**, phase contrast; **b**, TRITC fluorescence; and **c**, FITC fluorescence of the same section Bar represents 20 μm.

sperm heads (Figure 3a). However, we doubt whether IX9D5 recognizes the same protein in sperm heads as in spermatocytes. Spermatids do not react with IX9D5, neither on immunoblots (Figure 1C, lane IV), or on frozen sections (Figures 3a, 4).

Discussion

The experiments described in this paper show that two recently identified components of SCs, with M_rs of 125,000 and 190,000 respectively, are

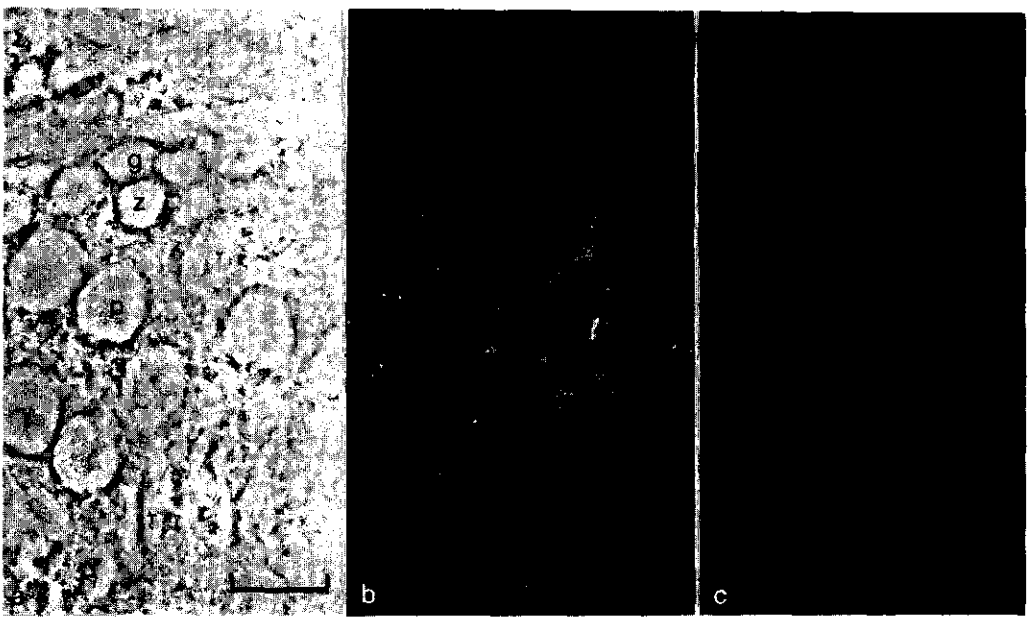


Figure 6. Details of a frozen section of a testicular tubule (stage XIII), with unpaired SCs in the diplotene spermatocytes. The section was immunostained as described in the legend of Figure 5. a, Phase contrast; b, TRITC fluorescence; c, FITC fluorescence of the same section. Bar represents 20 μm.

specifically detected in nuclei of meiotic prophase cells. We had drawn the same conclusion earlier for two other major components of SCs, with M_rs of 30,000 and 33,000 (Heyting *et al.*, 1988); to this latter conclusion we should now add the proviso that we cannot exclude that a small amount of the M_r 30,000 component is present in spermatogonia and/or spermatids. As we reported earlier (Heyting *et al.*, 1988), the anti-M_r 30,000+33,000 Mab II52F10 does not react detectably with immunoblots of nuclear proteins from spermatogonia and spermatids; however, Mab IX8G9, which produces a far stronger signal on immunoblots than Mab II52F10, reacts detectably with an M_r 30,000 component of nuclei from spermatogonia or spermatids, although the

intensity of the signal is such that it can be explained from a possible contamination of spermatogonia or spermatids with spermatocytes. After immunofluorescence staining we did not detect M_r 30,000+33,000 antigens in spermatogonia, but a small amount of evenly distributed antigen could have gone undetected. We did detect small spots of M_r 30,000+33,000 antigens in some early round spermatids (Heyting *et al.*, 1988), presumably in remnants of SCs; these could provide an explanation for the small amount of 30,000 antigen detected by Mab IX8G9 on blots of spermatid nuclei. Strictly spoken, we should keep the same proviso for the M_r 190,000 and M_r 125,000 components of SCs as for the M_r 30,000+33,000 components, namely that small amounts of these antigens (less than 2% of the spermatocyte level) in non-spermatocyte nuclei may not have been detected. Like the M_r 30,000+33,000 antigens, the M_r 190,000 and M_r 125,000 polypeptides are major components of SCs, and show up as heavy bands in silver or Coomassie blue stained SDS-polyacrylamide gels of purified SCs (see Heyting *et al.*, 1989, Figure 3). Thus, from these as well as experiments described earlier SCs emerge as structures composed of proteins that are largely or entirely specific for meiotic prophase cells.

Other information concerning the ontogeny of SCs comes from molecular genetic analysis of meiosis-defective mutants of yeast: several genes, though not all (Alani *et al.*, 1990), that are required for the normal assembly of SCs are expressed specifically during meiotic prophase and some of these might encode SC components (Hollingsworth *et al.*, 1990; Engebrecht *et al.*, 1990).

One of the implications of the meiosis specifity of SCs is that the characteristic chromatin rearrangements of the meiotic prophase, namely folding of chromatin fibers into loops, as observed in Bombyx (Rattner *et al*, 1980 and 1981), condensation of chromosomes, pairing, recombination and segregation, are not accomplished by rearrangement of the structures to which chromatin is already attached, but by detachment of chromatin from supporting structures like the nuclear lamina and re-attachment to a new, meiosis-specific structure.

The identification of SC components is far from complete: the composition of the transversal filaments has not yet been elucidated and several polypeptides, with M_rs of 90,000, 48,000, 45,000 and 26,000, consistently copurify with SCs (see Heyting *et al.*, 1989, Figure 3); we think it likely that there are SC components among them. It is thus still possible that certain (minor) components of SCs are not newly synthesized.

Although the main purpose of this investigation concerns the analysis of the tissue distribution of SC components, the immunofluorescence experiments presented here also provide some information about the assembly of SCs.

The M_r 30,000+33,000 and 190,000 components of the axial elements/LEs appear at the nuclear wall in early spermatocytes; the M_r 125,000 component of the central region appears later. This fits the observation that, at least in mouse spermatocytes, relatively long unpaired axial elements are formed during zygotene, often before any paired segments are detected (Dietrich and De Boer, 1983, Figure 2a); it also fits the observation of Moens *et al.* (1987), who found that in rat spermatocytes long segments of axial elements are labeled by an anti-M_r 30,000+33,000 Mab before they are paired. The M_r 125,000 component disappears earlier than the M_r 30,000+33,000 and 190,000 components. This is in agreement with morphological observations of diplotene SCs of the mouse, which first unpair almost completely before the axial elements fall apart (Solari, 1970; Dietrich and De Boer, 1983). The immunofluorescence staining by anti-M_r 30,000+33,000 and anti-M_r 190,000 Mabs is virtually indistinguishable. The antigens of both classes of antibodies are detectable as soon as and as long as fragments of SCs can be morphologically discerned, and possibly even longer, up to the early spermatid stage. This can be considered as an indication that these antigens do not serve some stage-specific process, but fulfil a structural function (cf. Moens *et al.*, 1987). The M_r 125,000 antigen, however, might have a specific function in chromosome pairing.

Materials and methods

Purification of spermatogonia, spermatocytes and spermatids.

Testicular cell suspensions were prepared by a modification of the procedure of Romrell *et al.* (1976), as described earlier (Heyting *et al.*, 1985). For purification of spermatogonia we started from 21-day-old rats, for purification of spermatocytes we used 27-day-old rats, and for spermatids we used 49-day-old rats. The cells were separated on the basis of their sedimentation velocity by centrifugal elutriation (Bucci *et al.*, 1986) in a Beckman JE 6.1 rotor at 10° C in Spermatocyte Isolation Medium (SIM, Heyting and Dietrich, 1991) containing 0.1% bovine serum albumin (BSA). Spermatids and spermatocytes were isolated at 1800 rpm and flow rates of 15 to 17.5 ml/min; spermatogonia at 2500 to 1800 rpm and 15 ml/min and spermatocytes at 1800 rpm and 20 to 35 ml/min. The cell fractions were washed once in SIM, and then further purified by density centrifugation in Percoll. The cells were resuspended in 25 ml 27% Percoll in SIM and the refractive index (ND_{20}) was adjusted with SIM or 80% Percoll in SIM to 1.3398 (spermatids), 1.3400 (spermatogonia) or 1.3399 (spermatocytes). The volume was then adjusted to 28 ml with a Percoll suspension in SIM with the same refractive index, and the cells were centrifuged for exactly 20 min. at 10,000 rpm and 20° C in siliconized Corex glass tubes in a Beckman JA21 rotor. Under these (non-equilibrium) conditions a shallow density gradient forms in the middle of the tube, with steep parts at the bottom and the top. In such a gradient cells tend to form two bands, one in each of the two steep parts of the gradient, while they are separated on the basis of tiny density differences in the shallow middle part of the gradient (see Heyting and Dietrich, 1991). For purification of spermatogonia or spermatocytes the (more dense) lower band was collected and for purification of spermatids the (less dense) upper band.

The purified cell fractions were analyzed by differential counts of Giemsa-stained preparations (Oud and Reutlinger, 1981). At least 200 cells were scored per preparation.

Isolation procedures.

Nuclei from liver and brain were prepared according to Blobel and Potter (1966). Nuclei from spermatogenic cells were prepared according to the hypotonic lysis/Triton method, described by Meistrich (1975). Mitotic chromosomes were isolated from synchronized rat glioma cells according to Gooderham and Jeppesen (1983) as described by Heyting *et al.* (1988). SCs were isolated from rat testes as described earlier (Heyting *et al.*, 1985, 1987). Nuclear laminae were isolated from rat liver nuclei according to Kaufman *et al.* (1983).

Antibodies.

Mab II52F10 (anti-M_r 30,000+33,000) has been described by Heyting *et al.* (1987). Mabs IX8G9 (anti-M_r 30,000+33,000), IX5B2 (anti-M_r 125,000) and IX9D5 have been described preliminary by Heyting *et al.* (1989). These three Mabs were obtained after immunization of a BALB/c mouse according to the following scheme: day 0: 5.1×10^8 SCs, mixed with complete Freund's adjuvant; day 14, 27 and 41: 2.5×10^8 SCs, mixed with incomplete Freund's adjuvant. The immunizations were performed by intraperitoneal injection. At 76 h after the last injection the spleen cells were isolated and fused with SP2 mouse myeloma cells as described by Moorman *et al.* (1984). Screening and selection of antibodies was performed as described by Heyting *et al.* (1988).

Other procedures.

Electrophoresis, immunoblotting and immunofluorescence staining were carried out as described by Heyting *et al.* (1988) and Dunn (1986). After immunoblotting and staining nitrocellulose strips were scanned with a Cybertech CS1 image documentation system (Cybertech, Berlin). Ultrastructural localization of antigens was performed by immunogold labelling of surface spread rat spermatocytes essentially as described by Moens *et al.* (1987).

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CHAPTER 4

A MAJOR COMPONENT OF THE AXIAL CORES OF MEIOTIC PROPHASE CHROMOSOMES WITH FEATURES OF A DNA-BINDING PROTEIN

H.H. Offenberg, R.L.J. Meuwissen, M. van Aalderen, H. Kester, and C. Heyting

Summary

During meiotic prophase, as chromosomes condense, proteinaceous axes are formed along each chromosome. These axes become later the lateral elements (LEs) of the meiosis-specific structures that connect homologous chromosomes, the synaptonemal complexes or SCs. Using monoclonal anti-SC antibodies, we isolated cDNAs most probably encoding a major component of the lateral elements, the M_r 190,000 SC protein.

The protein predicted from the nucleotide sequence of a full length cDNA, called SCP2, consists of 1294 amino acids and has a predicted molecular weight of 148 kDa and an isoelectric point of 8.36. SCP2 is proline-rich, and contains several S/T-P-X-X (15x) and S/T-S/T-X-X (38x) motifs that are supposed to contribute to DNA binding by interaction with the minor groove. It shares these features with some recently characterized nuclear matrix components that bind to the minor groove of matrix attachment regions (MARs). Furthermore, SCP2 has several potential phosphorylation sites, including target sites for $p34^{cdc2}$ and cAMP/cGMP dependent protein kinases, that could play a role in the regulation of the assembly and disassembly of SCs. SCP2 has a limited amino acid sequence similarity to the RED1 protein of yeast, which is involved in the assembly of SCs.

The gene encoding SCP2 is exclusively or predominantly transcribed in meiotic prophase cells. We tentatively conclude, that we have cloned the cDNA encoding a major component of the LEs of SCs, and speculate that the predicted protein, SCP2, is involved in the structural organization of meiotic prophase chromosomes.

Introduction

During the prophase of the first meiotic division the chromatin undergoes a complicated series of rearrangements as chromosomes condense, pair, recombine and segregate. How the rearrangements are accomplished is still largely unknown. However, a meiosis-specific nuclear structure has been identified, the synaptonemal complex (SC), which undergoes a series of morphological alterations which correlate with the successive rearrangements of chromatin (reviewed in Von Wettstein *et al.*, 1984): after completion of premeiotic S-phase, thin, proteinaceous axes are formed along each chromosome, presumably between the sister chromatids (leptotene); subsequently, the axes of homologous chromosomes are connected (synapsed) by transverse filaments, and a third longitudinal structure, the central element is formed between both axes. The axes are called lateral elements (LEs) where chromosomes are synapsed. Both LEs, together with the central element make up the tripartite structure of the SC. During pachytene homologous chromosomes are synapsed along their entire length, and within each bivalent the SC extends from telomere to telomere. Despite the correlation between SC morphology and chromatin rearrangements, it is not clear which role SCs fulfil precisely and how their assembly is regulated. Particularly with respect to the lateral elements there are several unanswered questions: are LEs comparable to the axes of mitotic chromosomes? How are the LEs assembled? Do they play any role in recombination (see reviews from Kleckner *et al.*, 1991; Loidl, 1990; Roeder, 1990)? If so, is this an inhibiting or an enhancing role? Or do LEs have a function in chiasma maintenance (Rockmill and Roeder, 1988) or sister chromatid cohesion (Maguire, 1990)?

In order to address these questions, we developed a procedure to purify SCs from rat spermatocytes (Heyting *et al.*, 1985) and elicited monoclonal and polyclonal antibodies against purified SCs (Heyting *et al.*, 1987, 1989, Chapter 2 this thesis). Four major components of SCs were identified by means of these antibodies, and localized within the SC (Heyting *et al.*, 1989): one component (M_r 125,000) was found specifically between the lateral elements in synapsed

regions of SCs (Heyting *et al.*, 1989; Offenberg *et al.*, 1991, Chapter 3 this thesis; Meuwissen *et al.*, 1992, Chapter 5 this thesis); and three components (M_r 30,000, 33,000 and 190,000) were located in the LEs/axial elements in synapsed as well as unsynapsed segments of SCs (Heyting *et al.*, 1987, 1988, 1989; Moens *et al.*, 1987; Offenberg *et al.*, 1991). All these SC components occur specifically in meiotic prophase cells; they are absent from other cell types and from mitotic chromosomes (Heyting *et al.*, 1988; Offenberg *et al.*, 1991).

In this paper we describe the isolation and characterization of a cDNA encoding the M_r 190,000 protein of SCs. The polypeptide predicted from the nucleotide sequence, called SCP2, shares features with some DNA binding proteins, which make part of the nuclear matrix. In addition, SCP2 has target sites for $p34^{cdc2}$ and cAMP/cGMP protein kinases, which could play a role in the regulation of SC assembly and disassembly. Furthermore, SCP2 is has a limited amino acid sequence similarity to the yeast RED1 protein, which plays a role in the assembly of yeast SCs (Thompson and Roeder, 1989; Rockmill and Roeder, 1990). We speculate that SCP2 is involved in the organization of meiotic prophase chromosomes.

Results

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

In the experiments described in this paper we used six independently isolated monoclonal antibodies each of which recognizes exclusively the M_r 190,000 SC component (see Figure 1); this protein is a major component of the lateral elements (Heyting *et al.*, 1989; Offenberg *et al.*, 1991). Using a pool of these antibodies, we screened an expression cDNA library, that had been constructed from poly(A)$^+$ RNA isolated from testes of 28-day-old rats (see Materials and Methods section). Among $3x10^5$ recombinant phage of this library we found 10 weakly reacting clones. For rescreening, lysed colonies were probed with the same pool of monoclonal antibodies; this yields a stronger signal than im-

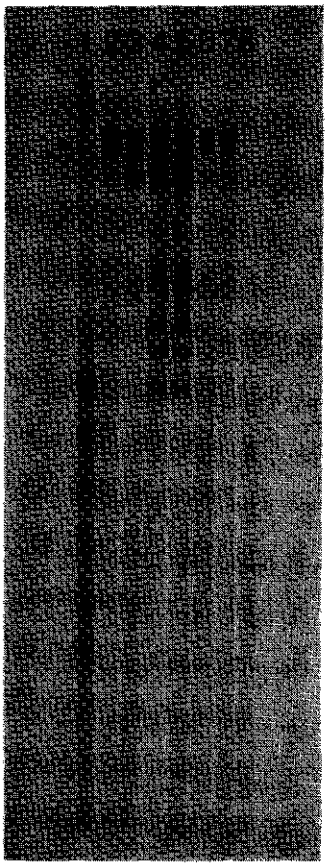


Figure 1. Immunoblot analysis of SC proteins with anti-M, 190,000 monoclonal antibodies. $3x10^7$ SCs were loaded onto 2 cm wide slots of a 16x20x15 cm 10% SDS polyacrylamide gel. The position of the M_r 190,000 SC protein is indicated. *Lane 1*: 0.4 cm wide strip of the lane stained with Coomassie blue; *lanes 2* to *8*: immunoblots incubated in respectively Mab IX9D5, Mab IX8F1, Mab IX1H9, Mab IX3E4, Mab IX2G11, Mab IX8B11 and a control hybridoma supernatant.

munological screening of plaques. All cDNA clones analyzed had overlapping restriction enzyme maps. In order to isolate a full length clone we screened the cDNA library with the 5′ EcoRI fragment of clone 3C1 (see Figure 2). This yielded clone 5 (see Figure 2), with an insert size of 4400 bp, which extended 500 bp further in the 5′ direction than clone 3C1. No clones with longer inserts could be detected in the cDNA library, either by screening with the 5′ EcoRI-HindIII fragment of clone 5 (see Figure 2) or by polymerase chain reaction (PCR). In search of longer inserts by means of PCR we used a clone-specific 5′ primer, a vector-specific 3′ primer (see Materials and Methods section), and the cDNA library as a template. No clones extending further in the 5′ direction than clone 5 were detected by this procedure (see Figure 3).

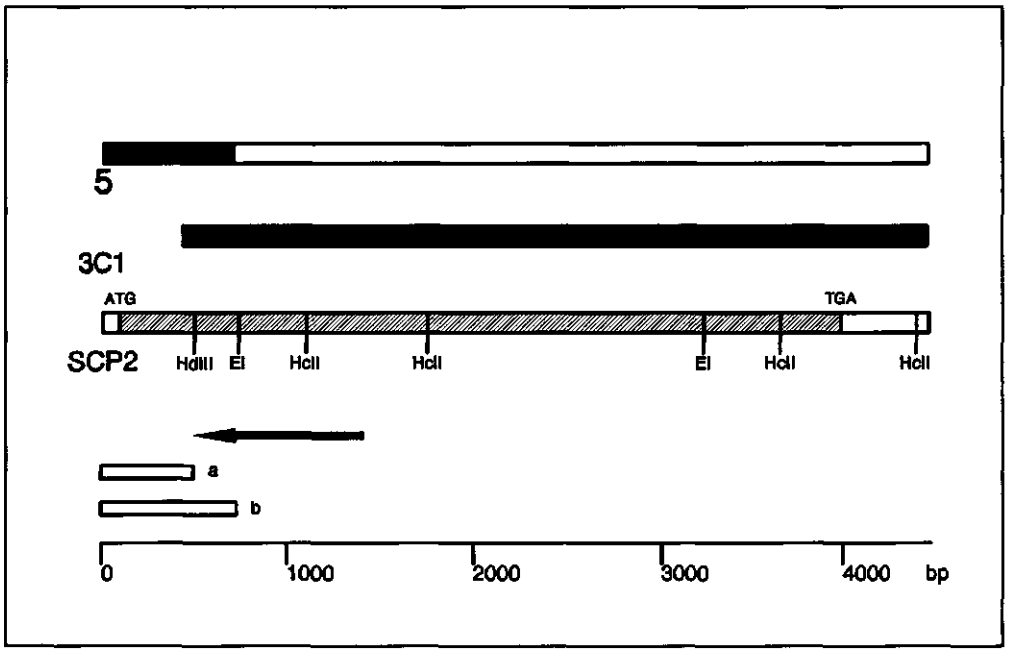


Figure 2. Schematic map of cDNA clone 5, encoding SCP2. The hatched area represents the open reading frame. The solid bars in clone 3C1 and clone 5 indicate the sequenced parts. The arrow indicates the position of the RNA probes used for Northern analysis and *in situ* hybridization. Bar **a** indicates the probe used for secondary screening; bar **b** indicates the probe used for analysis of PCR products (see text). EI, *EcoRI*; *HcII*, *HincII*; *HdIII*, *HindIII*.

The nucleotide sequence of clone 3C1 and parts of the sequence of clone 5 were determined as described in Figure 2 and the Materials and Methods section. No discrepancies were found between the sequences of these clones. Clone 5 contains a single open reading frame of 3885 nucleotides, encoding a protein of 1294 amino acids, with a calculated molecular weight of 148 kDa, and a calculated isoelectric point of 8.36.

We think that clone 5 represents the complete cDNA encoding the M_r 190,000 SC protein for the following reasons: first, the recombinant gene product is recognized by four of the six independently isolated monoclonal antibodies that were used for screening (not shown), each of which specifically recognizes the

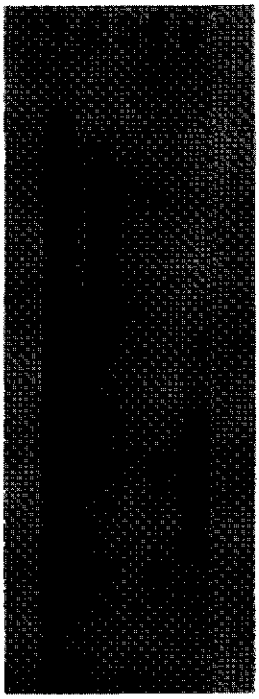


Figure 3. Southern blot analysis of PCR products, with 3′ EcoRI probe from clone 5. *Lane 1*: positive control, amplification with clone 5 in pBluescript as a template; *lane 2*: negative control, no DNA added; *lane 3*: negative control, no primers added; *lane 4*: amplification with cDNA A3 as a template; *lane 5*: amplification with cDNA A4 as a template (see Materials and Methods section).

M_r 190,000 SC component (Heyting *et al.*, 1989; Offenberg *et al.*, 1991). Second, the predicted pI (8.36) is in good agreement with the observed pI of the M_r 190,000 SC component (Offenberg *et al.*, Addendum to Chapter 4, this thesis) and third, the gene is predominantly or exclusively expressed in those cells which contain the M_r 190,000 SC component (see below). We therefore tentatively conclude that we have cloned the cDNA encoding a major component of the LEs of SCs, for which we propose the name SCP2 (synaptonemal complex protein 2). However, conclusive evidence has to come from comparison of the amino acid sequence of the M_r 190,000 SC component with that of SCP2; these analyses are in progress.

The discrepancy between the relative electrophoretic mobility of the M_r 190,000 SC component and the predicted molecular weight (148,000) of SCP2 is probably due to the fact that SCP2 is a basic protein with a high percentage of proline residues. Proteins with these characteristics will migrate more slowly in SDS-polyacrylamide gels than can be expected on the basis of their molecular weight (Hames, 1990). A similar discrepancy between predicted molecular

weight and observed electrophoretic mobility has been found for other proteins with similar characteristics, for instance, the proline-rich protein RAP1 (Shore and Nasmyth, 1987).

SCP2 has features of a DNA-binding protein.

The predicted amino acid sequence of SCP2 was deduced from the nucleotide sequence of clone 5, and is shown in Figure 4. Predictions of the secondary structure of the polypeptide by means of the algorithm of Chou and Fasman (1978) did not reveal large-scale structural motifs. The protein is rich in ß-turns. It contains several motifs that are of potential interest: the amino acid sequence is enriched in the S/T-P-X-X motif (15x), which is supposed to contribute to DNA binding, presumably by interaction with the phosphodeoxyribose backbone in the minor groove (Suzuki, 1989). In addition, SCP2 contains 38 so-called S/T-S/T-X-X motifs (some of which overlap with each other or with the S/T-P-X-X motifs), which, according to Suzuki (1989), could mimic the conformation of the S/T-P-X-X motif. These motifs are localized in two clusters: one between amino acid residues 316 and 791, with 7 S/T-P-X-X motifs and 16 S/T-S/T-X-X motifs and one cluster between residues 874 and 1272, with 8 S/T-P-X-X and 21 S/T-S/T-X-X motifs (see Figure 4; the S/T-P-X-X motifs are shaded). The region including these two clusters and the amino acid sequence between them is hydrophilic and basic, with a calculated pI of 9.52.

The predicted amino acid sequence contains several protein kinase target sites: there is one $p34^{cdc2}$ kinase target site which satisfies the consensus Z-S/T-P-X-Z (where X is a polar and Z a basic residue; Moreno and Nurse, 1990), at position 655 and there are two $p34^{cdc2}$ kinase target sites which satisfy the relaxed consensus S/T-P-X-Z (Draetta, 1990), at positions 381 and 413. There are eight potential cAMP/cGMP-dependent protein kinase target sites (consensus: K/R-K/R-X-S/T; Fremisco *et al.*, 1980), all of which are present in the central basic region of the protein. In addition, 48 protein kinase C target sites (consensus S/T-X-R/K, where X is any amino acid; Kemp and Pearson, 1990), 14 potential N-glycosylation sites (consensus N-{P}-S/T-{P}; Marshall, 1972; Miletich and

```
MIKQGLVQKM VSWFENSKEI ILSQRQSKDE AVMNMIEDLF DLLNVVYDVN DEGKNQVLES FIPHICALVI DSRVNFCIQQ  80

EALKKMNLML DRIPQDANKI LCNQEILTLM SNMGERILDV GDYELQVGIV EALCRMTTEK RRQELAYEWF SMDFIANAFK 160

KIKDCEFETD CRIFLNLVNG MLGDRRRVFT FPCLSAFLGK YELQIPSDEK LEEFWIDFNL GSHTLSFYIA GDDDDHQWEA 240

VTVPEEKVDM YNIEVRESKK LLTLTLKNIV KISKKEGKEL LLYFDAALEI TNVTKKLFGG NKYKEFTRKQ DISVAKTSIH 320

VLFDASGSQI LVPESQPSPV KENLIHLKEK SNLQKKLTNP LEPDNSSSQR DRKNSQDEIT FPSRKKMSEA SMIVPDTDRY 400

TVRSPILLIN TSTPRRSRAP LQAIHSAEKA VSKTSESGVD YAVSLKSRQS DGRNRGNNRA NHNKTATVQN KGHEHHESPD 480

QTFNEIEETL SDAYAVEKVD KPVLPGVLDI SKNKAHSRWA CWTPVTTIKL CNNQRSCALP GDTFTQDTGV NKKCTKQKSV 560

SDDDSEETQR VKYSKDVIKC NKSEEAEVCE RNIQEQNHPK YSQKKNTANA KKNDWHIESE TTYKSVLLNK TTEESLIYKK 640

TCVLSKDVNT TICDKSPSRK SMRSHTKSRK ELMSEVTSCE LDEIPVRENS KGKRFTGTAE SLINLINKRY NSSDDMISTR 720

KLKEPRDGSG FSKKPELQFN KVQRKSYRKL KTVVNVTSEC PLNDVYNFSL NGADEPVIKL GIQEFQATTR EASMDNSIKL 800

VDVRNRDERD LSLKTKDERI LSHERKTLFS DTETECGWDD SKTDISWLRK PKSKRQWIIV EINTKKCKSI KSRSSTEKGQ 880

PRSTVVLSKN IAKNDYEVIV DGRTRLPRRA TKTKKNYKDL STSGSESESE KEISYLFKDK LPTKEETVHS SAQTKKLPKK 960

QQKVFNTEAL KGQPSEEQKN SSTLRNGRED SLYLSSASVS GSSSSVEVMR CTEKITERDF TQDYDYITKS LSPTPKAASP 1040

EFLNRSNRVV GHGKSPRISE TSAVCVRKSC SPASGLPFSP RHTTKNNSVM NIKNTNSVIN NQRTQHCNSY SDVSSNSSEK 1120

LYMEPESPDS CENHVQSKRE ENHAASPTSL SSEKIEKIWF DMPNDNTHVS GPSQRGSKRR MYLEEDELSN PSEAEVQEAE 1200

EREHLVSKKL CQREHFDQHT SETSLSTPEF SVPKDWQQEL QGAGMFYDNI NSDYKRKTDT QHKIMDDFTT KTLKLTQQHL 1280

LAWPVKLGDT GMKY*                                                                      1294
```

Figure 4. Deduced amino acid sequence of SCP2. The single letter designations for amino acids are used. The S/T-P-X-X motifs are shaded.

Broze, 1990) and 7 myristoylation sites (consensus G-{E/D/R/K/H/P/F/Y/W}-X-X-S/T/A/G/C/N-{P}, where X is any amino acid; Towler, 1988) are dispersed all over the amino acid sequence of SCP2 (not indicated). Potential nuclear target-

ing signals (consensus K-R/K-X-R/K, where X is any amino acid; Chelsky *et al.*, 1989) are located at amino acid positions 160 and 865.

Searching libraries of known sequences with the programs FASTA, BLAST, TFASTA and BLASTP (Pearson, 1990; Altschul *et al.*,1990) did not reveal any large-scale similarities at either the nucleotide or the amino acid sequence level. However, a limited similarity was detected at the amino acid sequence level with the yeast RED1 protein (Thompson and Roeder, 1989; see Figure 5). Yeast red1 mutants have a defect in SC-assembly (Rockmill and Roeder, 1990). The similarity includes only a small part of both proteins. In other aspects (pI, size) the RED1 protein and SCP2 are totally different.

```
SCP2 335 SQPSPVKENLIHLKEKSNLQKKLTNPLEPDNSSSQRDRKNSQDEITTPSRKKMS 387
         :||:: |::  | ||| : ||||||     : :||  |:   :  | |   |:|
RED1 564 GQPPSKKQKQFHKKEKKKQQKKLTNFKPIIDVPSQDKRNLRSNAPTKPKSIKVS 617
```

Figure 5. Amino acid sequence comparison of SCP2 and the RED1 protein. The numbers to the left indicate for each protein the first amino acids where the similarity begins. Connecting lines indicate identical amino acid residues; colons indicate similar amino acid residues.

<u>The gene encoding SCP2 is expressed predominantly or exclusively during meiotic prophase.</u>

The antigens recognized by the anti-M_r 190,000 monoclonal antibodies occur exclusively in meiotic prophase cells (see Figure 1, and Offenberg *et al.*, 1991). Northern blot analysis, performed with anti-sense RNA probes (see Figure 2) revealed a single transcription product in poly(A)$^+$ RNA from the testis, but not in RNA from other organs (Figure 6). Within the testis, we found that the expression of the gene encoding SCP2 occurs predominantly in the meiotic prophase cells, as determined by *in situ* hybridization (Figure 7).

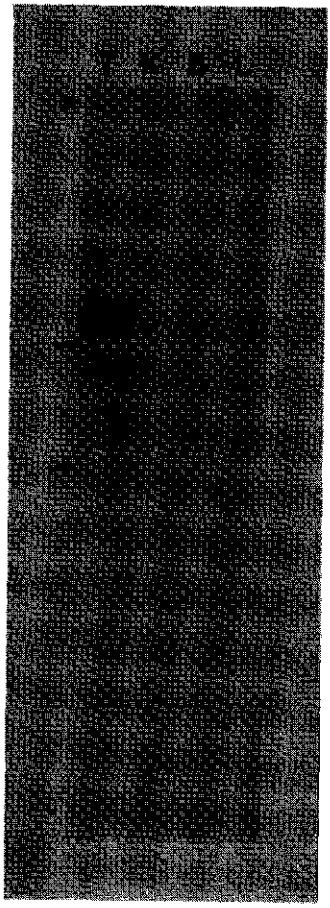
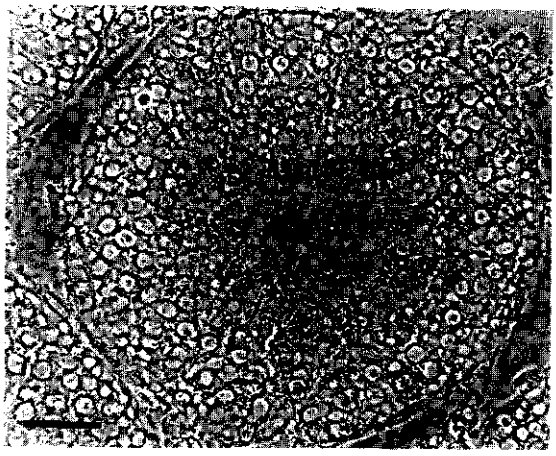


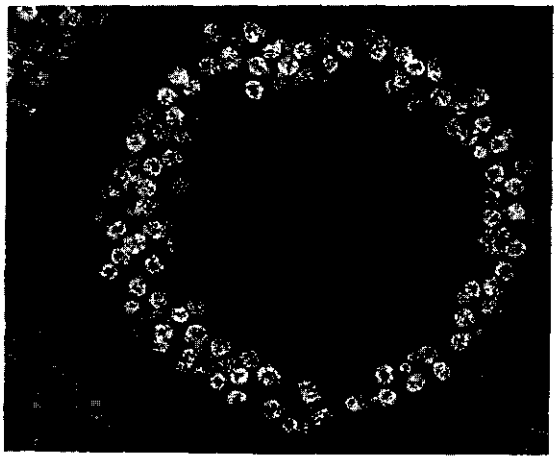
Figure 6. Transcription of the gene encoding SCP2, analysed by Northern blot analysis. 15 μg of poly(A)$^+$ RNA from respectively, testis (**T**), kidney (**K**), brain (**B**), and liver (**L**) was layered per 0.5 cm wide slot of a 1% agarose gel. After electrophoresis and blotting hybridization was performed with an RNA transcript of an 850 bp fragment of cDNA clone 3C1 (see Figure 2). Bars indicate the position of the 28 S and 18 S ribosomal RNA of the rat. The arrowhead indicates the top of the gel.

Discussion

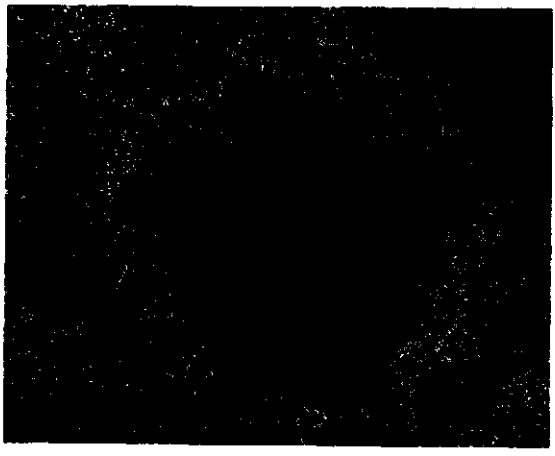
The assembly and disassembly of synaptonemal complexes closely matches the successive rearrangements of chromatin during meiotic prophase. Nevertheless, it is still not clear which role SCs fulfil precisely in these rearrangements, and what triggers their assembly and disassembly. For the lateral elements several functions have been proposed. LEs could play an enhancing or inhibititing role in recombination (see reviews by Kleckner *et al.*, 1991; Loidl, 1990; Roeder, 1990); they might be involved in chiasma maintenance (Rockmill and Roeder, 1988) or in sister chromatid cohesiveness (Maguire, 1990). In order to study the nature and possible functions of LEs we have elicited Mabs that recognize specifically components of the LEs of SCs. In this paper we describe the isola-



A



B



C

Figure 7. Localization of SCP2 transcripts in the testis by *in situ* hybridization. **(A)** Phase contrast micrograph of a transverse section of a testicular tubule; **(B)** localization of SCP2 in the same section by indirect immunofluorescence staining with Mab IX1H9 as primary antibody: this antibody made part of the pool of anti-M, 190,000 Mabs that was used for screening of the cDNA library; it recognizes the fusion protein of cDNA clones 3C1 and 5. **(C)** Localization of SCP2 transcript in the adjacent section by *in situ* hybridization; a ^{35}S-labelled anti-sense RNA transcript was used as a probe (see Figure 2). Note that the transcripts are present in the cytoplasm: nuclei are visible as "black holes". Bar represents 50 μm.

tion and characterization of cDNAs encoding one of these proteins, the M_r 190,000 SC protein, called SCP2, which is a major component of the lateral elements of SCs.

SCP2 transcription is confined to meiotic prophase cells.

The M_r 190,000 SC component occurs exclusively in meiotic prophase cells, i.e. spermatocytes (Offenberg *et al.*, 1991) and oocytes (Dietrich *et al.*, 1992). the experiments described in this chapter show that expression of SCP2 is regulated at the transcriptional level (Figures 6 and 7). A similar conclusion was reached earlier with respect to another SC protein, SCP1 (Meuwissen *et al.*, 1992). As far as we can judge from the *in situ* hybridization experiments, the transcripts of the genes encoding SCP1 and SCP2 occur in the same cells that contain the translation products. These results corroborate our earlier conclusion (Heyting *et al.*, 1988; Offenberg *et al.*, 1991) that SCs originate by assembly from newly synthesized components rather than by rearrangement of preexisting nuclear structures.

Sequence of the SCP2 gene.

The amino acid sequence of the predicted protein SCP2 contains several motifs that are of potential interest: SCP2 contains one target site for $p34^{cdc2}$ protein kinase which fulfils the consensus K-S/T-P-X-Z (Moreno and Nurse, 1990) and two sites which satisfy the relaxed consensus S/T-P-X-Z (Draetta, 1990). These sites could be important for regulation of the assembly and disassembly of the SC: mutation of CDC28, which encodes the *S. cerevisiae* analogue of $p34^{cdc2}$, causes an arrest in pachytene (Shuster and Beyers, 1989); this suggests that the $p34^{cdc2}$ protein kinase might play a role in the regulation of SC disassembly. The major component of the transverse filaments of SCs, SCP1, also has a target site for $p34^{cdc2}$ protein kinase (Meuwissen *et al.*, 1992). Besides the $p34^{cdc2}$ kinase target sites, SCP2 contains eight target sites for cAMP/cGMP dependent protein kinase (Fremisco *et al.*, 1980). It is possible that this protein kinase is also involved in the regulation of SC (dis)assembly: inhibition of

phosphorylation of nuclear lamins by cAMP/cGMP-dependent protein kinase plays a key role in the regulation of the disassembly of the nuclear lamina (Lamb *et al.*, 1991). However, it still has to be sorted out whether the potential phosphorylation sites on SCP2 are actually phosphorylated *in vivo*, and whether this plays any role in the regulation of SC (dis)assembly.

A major component of the lateral elements of SCs has features of a DNA binding protein.

SCP2 is a structural component of the LEs of SCs. Its predicted amino acid sequence shares features with other proteins that have a function in chromatin organization. Several nuclear matrix proteins also contain S/T-P-X-X sequences, for instance yeast NUF1 (Mirzayan *et al.*, 1992), mammalian nuclear matrix protein NUMA (Compton *et al.*, 1992; Young *et al.*, 1992), lamins (chicken lamin A, B1 and B2, Human lamin A and C; Peter *et al.*, 1989; Vorburger *et al.*, 1989; McKeon *et al.*, 1986; Fisher *et al.*, 1986), repressor/activator site binding protein RAP1 (Shore and Nasmyth, 1987) and SAR DNA-binding protein SATB1 (Dickinson *et al.*, 1992). The nuclear matrix proteins RAP1 (Shore and Nasmyth, 1987) and SATB1 (Dickinson *et al.*, 1992) have been shown to bind to DNA, although no obvious similarity with conserved features of DNA binding proteins could be identified in these proteins besides the S/T-P-X-X motifs (Shore and Nasmyth, 1987; Dickinson *et al.*, 1992). It is possible that SCP2 evolved by specialization of such a nuclear matrix protein.

Possible functions of SCP2.

The LEs of SCs differ from mitotic chromosome scaffold in several respects: first, the lateral elements of SCs largely consist of newly synthesized, meiosis specific proteins (Offenberg *et al.*, 1991; this paper). Second, LEs are longer than fully condensed mitotic chromosomes. Third, their staining properties are different from mitotic chromosome scaffolds (Earnshaw and Laemmli, 1984). Fourth, topoisomerase II, which is a major component of the scaffold of mitotic chromosomes (Gasser and Laemmli, 1987), is localized adjacent to, not in the

LEs (Klein *et al.*, 1992; Moens and Earnshaw, 1989). At the end of meiotic prophase, the SCs, including the LEs, are shed from the chromosomes (Zickler and Olson, 1975; Sherman *et al.*, 1989; Kehlhoffner and Dietrich, 1983). In this respect the LEs are more similar to the nuclear matrix on which the chromatin is organized, than to the axes of mitotic chromosomes, which are axially localized within the chromatin. From cytological studies we know that the chromatin is organized on the LEs in loops (Weith and Traut, 1980), but how the chromatin is organized on the LEs is still not clear.

It seems likely that the meiosis-specific components of SCs have a function that is not required during mitosis. For SCP2, with its features of a DNA-binding protein, we would like to consider a role in one or more of the following processes: attachment of chromatin to the lateral elements, homology search, initiation of recombination, sister chromatid cohesiveness and/or chiasma maintenance.

It is possible that SCP2 organizes the chromatin in loops on the SC. SCP2 could be positioned on the loops by other factors that regulate the accessibility of chromatin. The S/T-P-X-X motifs are frequently found in gene regulatory proteins (transcription factors), and often surround a specific DNA binding motif, like a zinc finger or a helix-turn-helix motif. Whether this is also the case for SCP2 is not known because it is not yet clear whether the sequences between the two S/T-P-X-X clusters have any DNA binding properties. It is possible that the S/T-P-X-X clusters allow SCP2 to interact with accessible DNA; competition with transcription factors could then direct the association of SCP2 with DNA.

It is also possible that SCP2 has a function in homology search; for instance, SCP2 could selectively bind to certain sequences (analogous to SATB1, Dickinson *et al.*, 1992), and make these sequences accessible for homology recognition proteins. If this hypothesis is correct, mutations in the gene encoding SCP2 will inhibit homologous chromosome pairing, and possibly also homologous recombination.

SCP2 could also play a role in the initiation of recombination. In yeast, several hot spots have been identified, where meiotic recombination is initiated by double-strand scission with a relatively high frequency (Sun *et al.*, 1989; Cao *et al.*, 1990); many but not all of these hot spots are localized within promotor regions (Nicolas *et al.*, 1989). Furthermore, there are indirect as well as direct indications that DNA in hot spots is relatively accessible (Schultes and Szostak, 1991; Shenkar *et al.*, 1991). In this respect SCP2 could play a role by locally influencing chromatin structure, and preferentially exposing certain sequences for initiation of recombination. According to this hypothesis, mutations in the gene encoding SCP2 will lead to an altered recombination frequency and/or an altered distribution of recombination events.

Another possible function of SCP2 concerns sister chromatid cohesiveness. For chiasma maintenance and the proper disjunction of chromosomes at meiosis I sister chromatid cohesiveness is thought to be essential (Maguire, 1990; Roeder, 1990; Von Wettstein *et al.*, 1984). SCP2 has a limited amino acid sequence similarity to the yeast RED1 protein (Thompson and Roeder, 1989); this protein is essential for meiosis in *S. cerevisiae*, and is required for proper chromosome segregation at meiosis I (Rockmill and Roeder, 1988). *S. cerevisiae* red1 mutants fail to assemble normal SCs during meiotic prophase, while meiotic recombination is not severely affected (Rockmill and Roeder, 1990). The amino acid sequence of RED1 protein also contains S/T-S/T-X-X (18x) and S/T-P-X-X (8x) motifs. Nevertheless, we do not think that the RED1 protein is the *S. cerevisiae* homologue of SCP2: there is a lack of overall sequence similarity and both proteins differ in several respects: the RED1 protein has a much lower molecular weight (95.5 kDa) and isoelectric point (6.79) than SCP2 (Thompson and Roeder, 1989). If SCP2 has a role in sister chromatid cohesiveness, mutations in SCP2 will lead to non-disjunction or a block in meiotic prophase.

Summarizing, we speculate that a major component of the LEs of SCs directly interacts with DNA. The interaction with DNA is possibly enhanced by S/T-P-X-X and S/T-S/T-X-X motifs, that are supposed to contribute to binding to the minor groove of DNA (Suzuki, 1989).

Materials and Methods

Antibodies.

The Mabs recognizing the M, 190,000 SC protein were obtained after immunization of mice with rat SCs as described by Offenberg *et al.* (1991). Of these antibodies, Mab IX9D5 has been described in detail by Heyting *et al.* (1989) and Offenberg *et al.* (1991).

Isolation of cDNAs encoding SCP1.

For the isolation of cDNAs encoding the M, 190,000 SC protein we constructed 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). The synthesized cDNA (cDNA A) was packaged in five fractions; the resulting libraries were designated as cDNA library A1 to A5. Each of these five libraries consisted of 1.4×10^6 independent clones. The average insert size was approximately 2 kb, as determined by excision of a fraction of the total libraries and subsequent digestion with XbaI. We screened one of the libraries (cDNA A1) with a pool of six independently isolated Mabs, each of which recognizes the M, 190,000 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 (Heyting *et al.*, 1988; Dunn, 1986). Among 3×10^5 phage screened, 10 positive clones were found and purified. The purified clones had overlapping restriction enzyme maps and inserts ranging in length from 1100 to 3900 bp. The 5′ EcoRI fragment of clone 3C1 (see Figure 2) was used for a secondary screening of cDNA library A5, in order to search for clones extending further in the 5′ direction. This yielded clone 5 with an insert size of 4400 bp (see Figure 2). 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).

PCR.

In search of cDNA clones extending further in the 5′ direction than clone 5, we performed a PCR incubation with the cDNA libraries A1-A5 as a template, and oligonucleotides complementary to 5′ sequences of clone 5 and to vector sequences (pBluescript SK) located at the 5′ end of the cDNA inserts as primers. For this purpose, we excised pBluescript with inserts from the complete λzap® library according to the manufacturer (Stratagene). The PCR incubation involved 1 cycle 5 min. 95 °C, and 30 cycles 2 min. 51 °C, 1.5 min. 72 °C and 1 min. 95 °C, followed by 15 min. 72 °C, and was performed with $Vent_R^{TM}$DNA polymerase (New England Biolabs Inc.). The amplification products were analyzed by Southern blotting and subsequent hybridization with a 5′ EcoRI fragment of clone 5 as a probe (see Figure 2).

Sequence analysis.

The insert of cDNA clone 3C1 was subcloned into the pBluescript vector (SK^+) according to the instructions of the manufacturer (Stratagene). From both ends of the insert of clone 3C1 we generated unidirectional sets of deletions by partial digestion with exonuclease III and S1 nuclease using the erase-a-base kit of Promega (Madison, Wisconsin, USA). In addition we subcloned several restriction enzyme fragments of the independently isolated cDNA clone 5 (see Figure 2) 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 Corp.), Taq polymerase (GIBCO/BRL, or Promega) and oligonucleotide primers complementary to the polylinker sequences of pBluescript. The sequence was assembled by means of the Wisconsin GCG sequence analysis package. Sequence similarity searches of the Gen EMBL, Swissprot and PIR data banks were carried out with FASTA, tFASTA (Pearson, 1990), BLAST, BLASTP and TBLASTN (Altschul *et al.*, 1990). Prediction of secondary structure was performed by means of a program based on Chou-Fasman algorithms (1978).

Immunocytochemical staining.

Immunofluorescence staining of frozen sections of the rat testis was carried out with a 1:1 dilution of hybridoma supernatant containing Mab IX1H9 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.

RNA isolation and Northern blot hybridization.

RNA was isolated from various tissues of 37-day-old rats by the GuTC/LiCl method of Cathala *et al.* (1983); poly(A)$^+$ RNA was purified by affinity chromatography on oligo(dT)-cellulose (Aviv and Leder, 1972). 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 3x SSC, dried and fixed with UV light (312 nm; 200 J/m^2) for two minutes. As probes for Northern blot hybridization we used RNA transcripts produced with a 3' deletion clone, linearized with HindIII, as template (see Figure 2). Transcription was performed from the T7 promotor in the presence of [α-^{32}P]rATP (3000 Ci/mmole). The Northern blot membranes were prehybridized in 50% formamide, 5x SSC, 50 mM Tris-HCl pH 7.5, 0.1% sodiumpyrophosphate, 1% SDS, 0.2% PVP, 0.2% Ficoll, 5 mM EDTA and 150 μg/ml sheared herring sperm DNA for 6 hrs at 60°C. Hybridization was performed in the same mixture with 0.07 μg/ml probe (36x10^6 cpm/μg) for 17 hr at 60°C. Subsequently the blots were washed for 30 minutes at 65°C in successively 2x SSC 0.1% SDS, 2x SSC 0.1% SDS, 0.1x SSC 0.1% SDS and 0.1x SSC 0.1% SDS.

In situ hybridization.

For *in situ* hybridization 10 μm frozen sections were cut from testes of 3-months-old rats at -19°C. The sections were mounted on 3-aminopropyl-triethoxysilane (Aldrich Chemical Corp.) coated slides, quickly air-dried at room temperature, and heated for 2 min. at 50°C on a hot plate. After further drying

for 1-2 hr. 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 analyze the expression of SCP2, consecutive sections were, respectively, stained immunocytochemically with Mab IX1H9, and 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 0.2M HCl, 5 min. wash in deionized water, 7.5 min. at 70°C in 2x 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, 2x 30 sec. wash 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 hr at room temperature. Hybridization was performed for 18 hr. at 55°C in 50% formamide, 0.3 M NaCl, 20 mM Tris-HCl pH 8.0, 5 mM EDTA pH 8.0, 10 mM NaH_2PO_4/Na_2HPO_4 pH 8.0, 10% dextrane sulfate, 1x Denhardts (Denhardt, 1966), 0.5 μg/ml yeast tRNA and $2x10^5$ cpm/μl ^{35}S-labeled RNA probe ($18x10^3$ cpm/μg). The RNA probe was obtained by transcription from the T7-promotor of a 3' deletion clone which was linearized by digestion with HindIII (see Figure 2); probe synthesis was performed in the presence of [α-^{35}S] rUTP (3000 Ci/mM, Amersham Corp.). After hybridization the sections were washed as follows: 30 min. in 5x SSC 10 mM DTT at 55°C; 1 hour at 50°C in 50% formamide 5x SSC 20 mM DTT; 3x 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; 2x 15 min. in 2x SSC and 2x 15 min. in 0.1x 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 analyzed by dark field microscopy.

Other procedures.

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

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ADDENDUM TO CHAPTER 4

H.H. Offenberg, A.H.F.M. Peters, M. van Aalderen, and C. Heyting

Introduction

This addendum describes the analysis of the antigens of six independently isolated Mabs, each of which recognizes an M_r 190,000 SC component. To find out whether these Mabs recognize the same or different SC components, SC proteins were separated by 2D electrophoresis, and blotted onto nitrocellulose. Incubation of the blots in the anti-M_r 190,000 Mabs showed that all Mabs recognize the same, basic M_r 190,000 component, and that different Mabs recognize different domains of this protein.

Results

Figure 1 of Chapter 4 shows immunoblots of SC proteins separated on a 1-dimensional (1D) polyacrylamide-SDS gel, after incubation in different Mabs which recognize an M_r 190,000 SC component. In addition to the M_r 190,000 protein, several bands of higher electrophoretic mobility are recognized by these Mabs. We interpret the "extra" bands as breakdown products of the M_r 190,000 SC component. The other possible interpretation, namely cross reaction of the Mabs with other SC proteins seems less likely, because the intensity of these bands relative to the M_r 190,000 band varies with the SC preparation. Cross reaction with contaminating proteins in the SC preparations cannot provide an explanation, because the Mabs bind exclusively to SCs in tissue sections or surface-spread spermatocytes (Heyting *et al.*, 1989, Chapter 2 this thesis; Offenberg *et al.*, Chapter 3 this thesis). Figure 1 of Chapter 4 shows that different anti-M_r 190,000 Mabs recognize different breakdown products. Three classes of anti-M_r 190,000 Mabs can be discriminated on the basis of the breakdown products that are recognized: class I antibodies (IX9D5 and IX8F1) almost exclusively recognize the M_r 190,000 band, and weakly some lower molecular weight bands. Class II antibodies (IX1H9 and IX3E4) recognize besides the major M_r 190,000 band several weaker bands with M_rs between $\approx$60,000 and $\approx$70,000. Class III antibodies (IX2G11 and IX8B11) recognize a similar but slightly different pattern of bands compared to class I antibodies (see Figure 1, Chapter 4).

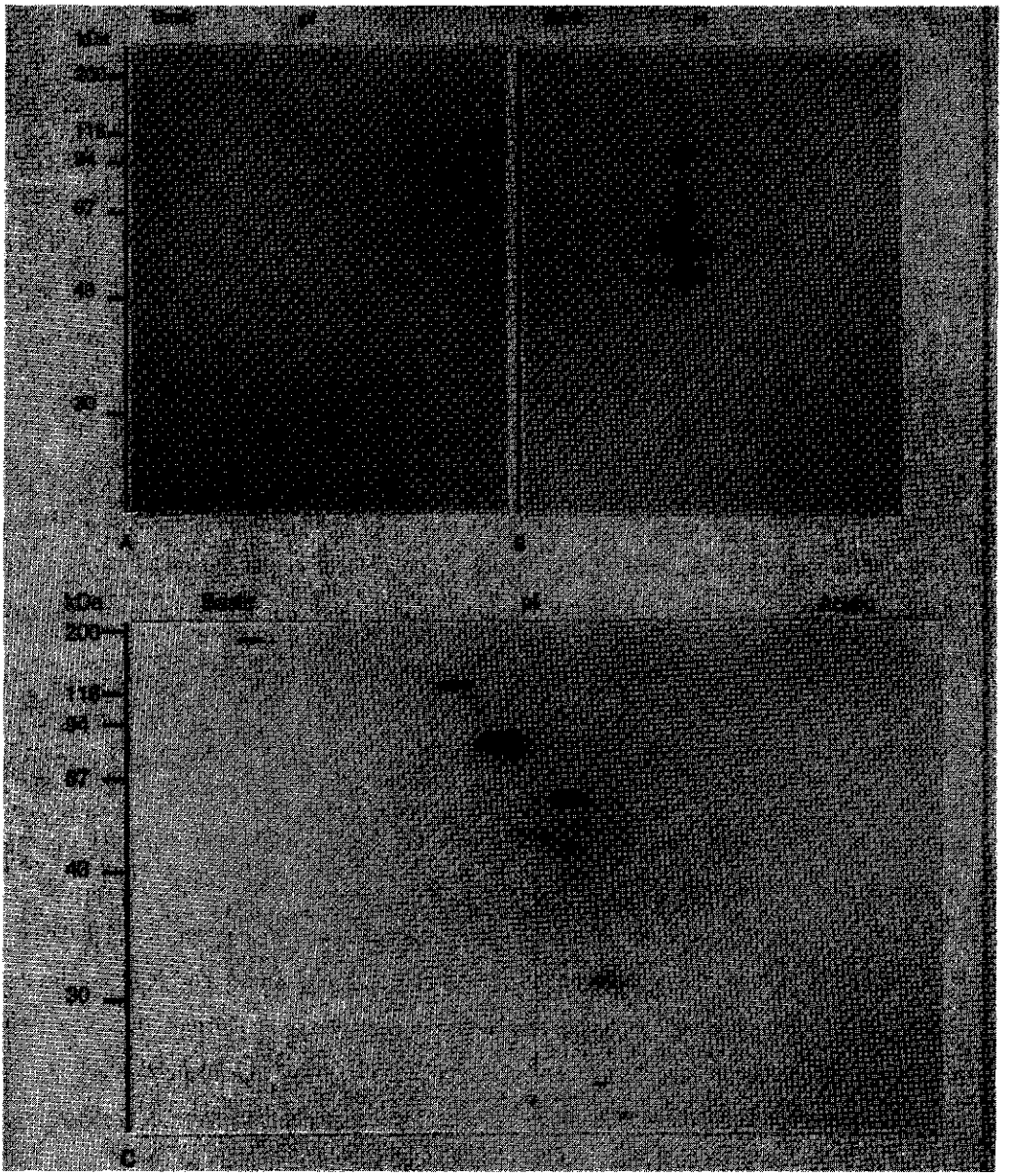

Figure 1. Immunoblots of proteins of isolated SCs, separated on 2D gels after incubation in anti-M, 190,000 SC protein monoclonal antibodies. Samples containing $3x10^7$ SCs were layered on a tube gel. Separation in the first dimension was performed by isoelectric focusing, with ampholines pH 3.5-10 and pH 5-8 mixed 1:4. Separation in the second dimension was performed on 7-18% linear gradient SDS-polyacrylamide gels. The gels were blotted onto nitrocellulose filters and incubated in hybridoma supernatants. **A** Immunoblot incubated in Mab IX9D5; **B** Immunoblot incubated in Mab IX1H9; **C** Immunoblot incubated in Mab IX2G11 (Of immunoblots **A** and **B** the basic part is shown; immunoblot **C** is shown completely. Arrow indicates the M, 190,000 spot).

The reactivity of the anti-M_r 190,000 antibodies with SC-proteins was further analyzed on blots of 2D gels (Figure 1). All anti-M_r 190,000 monoclonal antibodies probably recognize the same M_r 190,000 protein, but again, different Mabs recognize different patterns of presumed breakdown products. Class I antibodies only weakly react with 2D blots, the M_r 190,000 spot is hardly visible (see Figure 1A); this is probably due to the small amount of protein on this blot; class I antibodies react relatively weakly with the M_r 190,000 SC component, compared to the other two classes. Class II monoclonal antibodies recognize an M_r 190,000 protein at the same position as the spot recognized by class I antibodies (pI ≈ 9) and a series of spots with higher electrophoretic mobility and a higher pI (≥ 9) (see Figure 1B). Class III monoclonal antibodies recognize the same M_r 190,000 spot, but the recognized spots of higher electrophoretic mobility have lower pI-values (see Figure 1C). Some of the lower molecular weight spots recognized by class I antibodies are also recognized by class III antibodies.

Discussion

The experiments described in this addendum show that the independently isolated anti-M_r 190,000 Mabs recognize the same, basic SC protein. The results obtained with blots of 2D gels can be explained if the M_r 190,000 SC component is a polypeptide with a "basic end" and an "acidic end". The "basic end" of the protein is then recognized by class II antibodies, which only bind to degradation products at the basic end of the 2D gel. The "acidic end" (pI ≈ 7) of the protein is recognized by class III monoclonal antibodies. Immune incubations of fusion proteins produced by subclones of cDNA clone 3C1 (which encodes SCP2, see Chapter 4 this thesis) revealed that the translation product of 1 kb of the 3′ end of this clone can only be recognized by antibodies of class III; the part of SCP2 encoded by 1 kb of sequences derived from the 5′ end of clone 3C1 could only be recognized by class II antibodies (data not shown). This fits the observation that the C-terminus of SCP2, which is encoded by sequences at the 3′ end of the cDNA, is more acidic than the N-terminal part.

We conclude that the SCs probably contain only one M_r 190,000 polypeptide. The epitopes recognized by the three different classes of monoclonal antibodies are probably localized in three different domains on this polypeptide. The pI of the M_r 190,000 SC component is fairly basic (pI $\approx$ 9).

Materials and Methods

2D gel electrophoresis and immunoblotting were performed according to the procedures described in the Materials and Methods section of Chapter 6.

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CHAPTER 5

A COILED-COIL RELATED PROTEIN SPECIFIC FOR SYNAPSED REGIONS OF MEIOTIC PROPHASE CHROMOSOMES

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Summary

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 for 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 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.

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-specific 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; Roeder, 1990; Zickler, 1991; Maguire and Riess, 1991; Curtis and Doyle, 1991; Gulobovskaya, 1989). 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 (Rockmill and Roeder, 1990; Engebrecht and Roeder, 1989).

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, Chapter 2 this thesis) and, using these antibodies, identified four major components of SCs (Heyting *et al.*, 1987, 1989; Moens *et al.*, 1987; Offenberg *et al.*, 1991, Chapter 3 this thesis). 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 of 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 an M_r 125,000 SC protein (Heyting *et al.*, 1989), we screened about 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

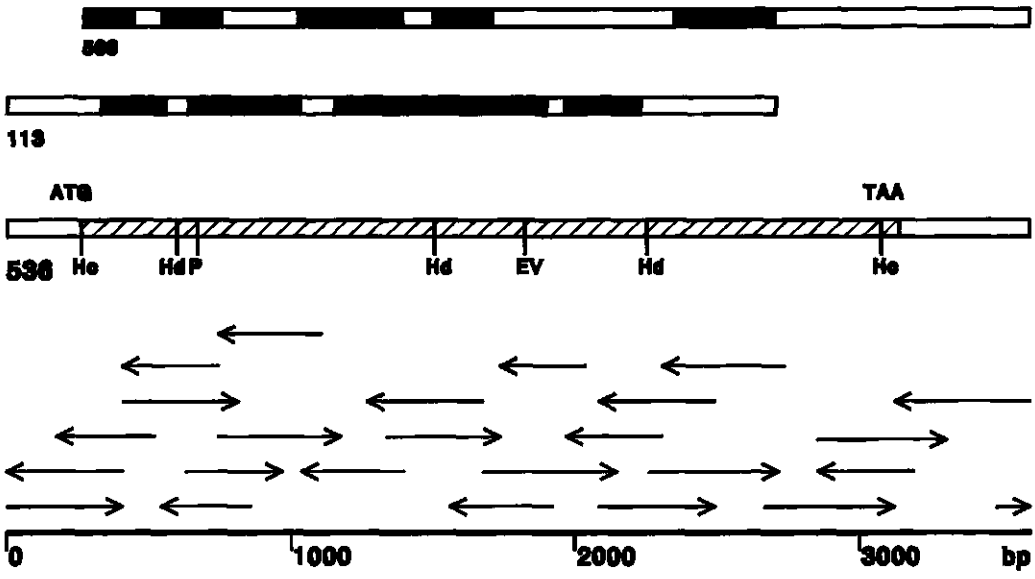
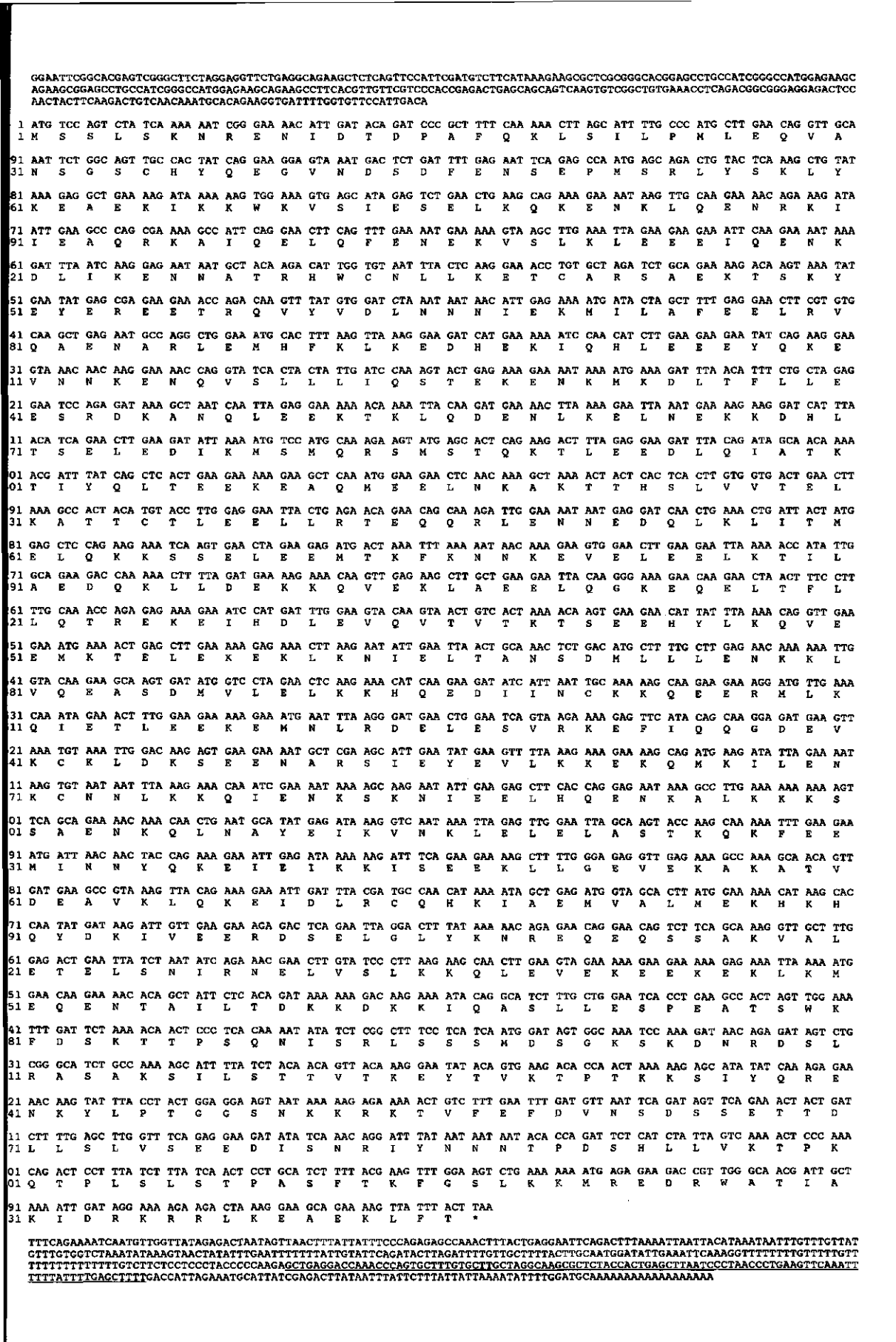


Figure 1. *Restriction map of SCP1 cDNA and sequencing strategy. Arrows indicate the length and direction of the sequence determined of clone 536. For clone 536, the hatched segment indicates the coding region. For clone 566 and 113, the black segments were sequenced as controls for the sequence of clone 536. Hc, HincII; Hd, HindIII; EV,* EcoRV; P, PstI.

selected for further analysis (Figure 1). A secondary screening with the 5′ PstI fragment (between the PstI site of the insert and 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 the Materials and Methods section. 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

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



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 SCP1 is only transcribed in the testis, and within the testis only in those cells that contain the antigen recognized by Mab IX5B2 (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 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 (synaptonemal complex protein 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 (nucleotide 3380 to 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 residue 52 to 752 shows amino

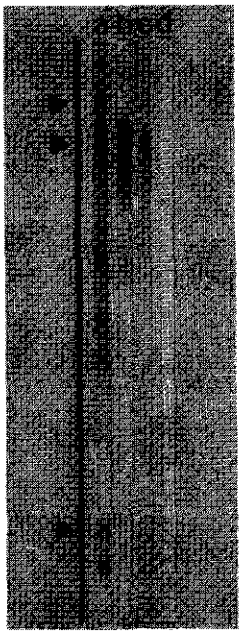


Figure 3. Immunoblot of proteins of rat SCs, probed with Mab IX5B2 (*lane b*), a polyclonal antiserum, elicited against the fusion protein of cDNA clone 536 (*lane c*) and the pre-immune serum (*lane d*). *Lane a* shows the Coomassie-blue stained gel. The M, 190,000 and M, 125,000, M, 33,000 SC proteins are indicated. Samples containing $3x10^7$ 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 in anti-SC antibodies.

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 which 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 position 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 which shows similarity to myosin (residue 52 to 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

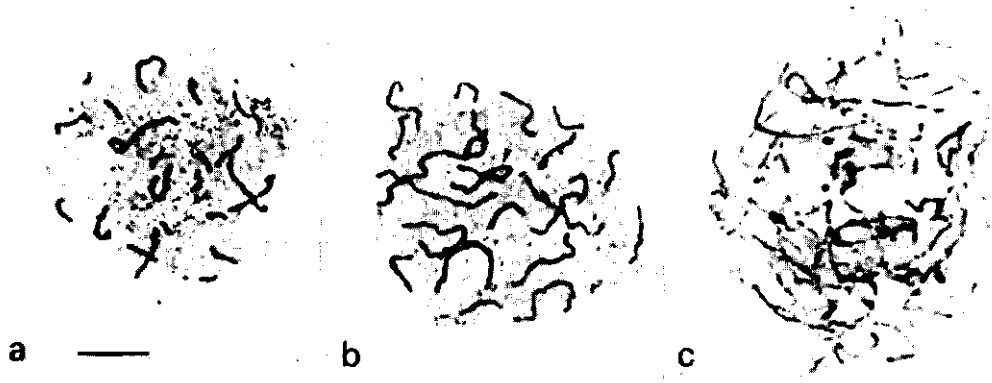


Figure 4. **Indirect immunoperoxidase staining of agar filtrates of lysed spermatocytes with a polyclonal antiserum elicited against the fusion protein of cDNA clone 536. (a) Zygotene stage; note short interruptions in the labelling of the SCs; (b) pachytene stage, arrowhead indicates the XY-bivalent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

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 752 is expected to be capable of forming a coiled-coil structure. This stretch would be about 100 nm long (Creighton, 1984).

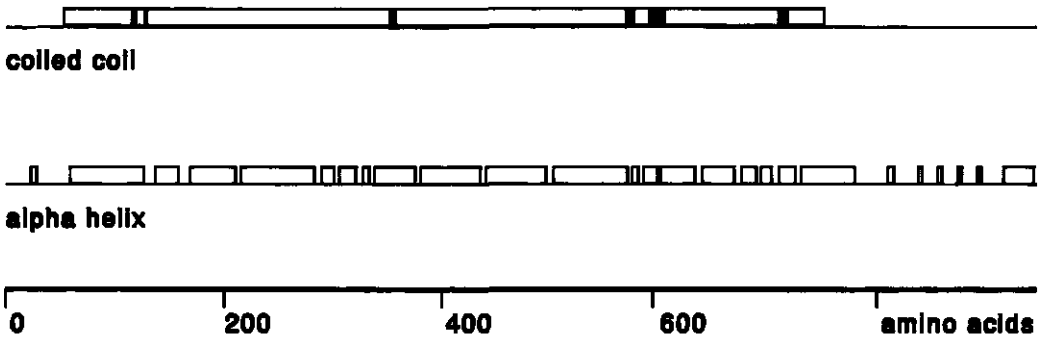


Figure 5. Secondary structure predictions of SCP1. On the upper bar the predicted coiled-coil domain (amino acid 52 - 752) is indicated, with the interruptions in the heptad repeat shown in black; these interruptions do not affect the frame of the heptad repeat. The lower bar shows the regions capable 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.

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 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 position 851 to 854 and 931 to 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 (residue 1 to 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 position 67-70, 560-563 and 851-854. Furthermore, there are several potential target sites for protein kinases. For instance, the sequence K-T-P-T-K (position 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

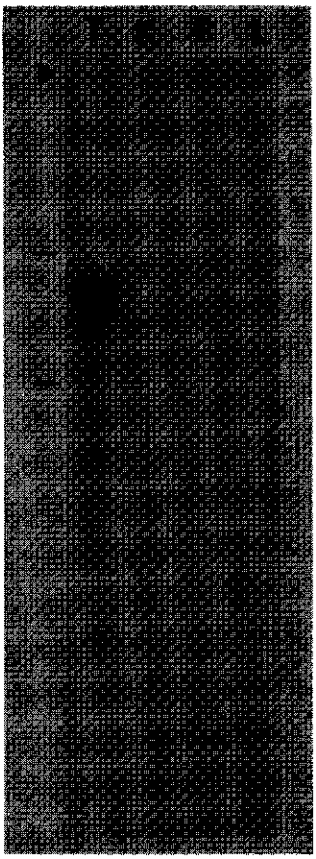


Figure 6. Transcription of SCP1, analyzed by Northern blot hybridization. 20 μg of poly(A)$^+$ RNA from respectively, testis (**T**), kidney (**K**), brain (**B**) and liver (**L**) was layered per ½ cm wide slot. An RNA transcript of a 900 bp HindIII fragment of cDNA clone 536 was used as a probe. Bars indicate the position of the 28 S (4700 nucleotides) and 18 S (1900 nucleotides) ribosomal RNA of the rat. Arrowhead indicates top of the gel.

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 position 341 to 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 HindIII 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 about 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.

Localization of SCP1 within SCs.

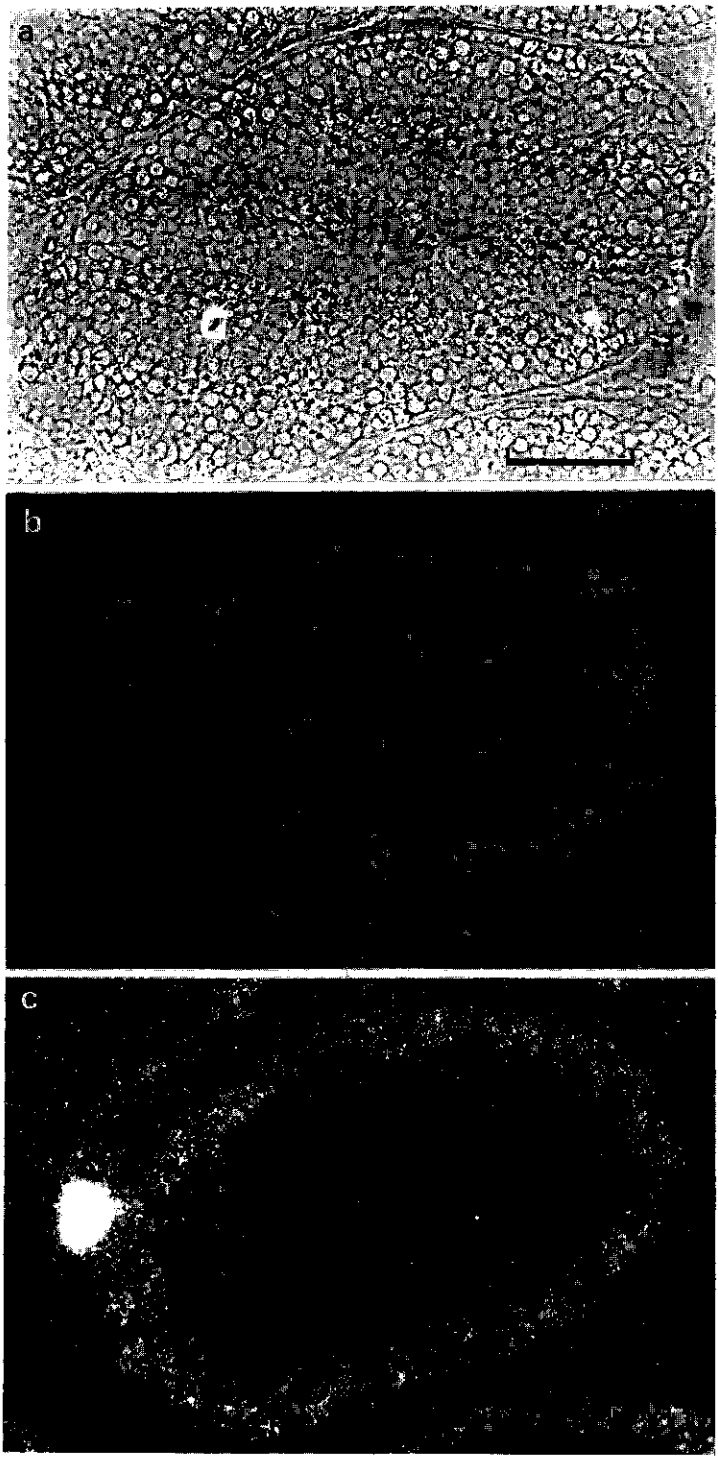
Figure 4 a to c show agar filtrates of lysed spermatocytes after indirect immunoperoxidase staining with a polyclonal anti-SCP1 antiserum. Late zygotene SCs show up as interrupted lines, the interruptions probably representing unpaired segments of SCs (Figure 4a). In some zygotene cells the unpaired 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 chromosome are also stained weakly. Ultrastructural localization of SCP1 by means of immunogold labelling gave similar results (Figure 8): the paired segments of SCs are labeled 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

Figure 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 aggrgation of nuclear contents, and thus of nuclear antigen, against the nuclear wall. This effect is not seen in other experiments (Offenberg *et al.*, 1991) where fixation has been optimized for the immunolocalization of SC components; (c) localization of the SCP1 transcript in the adjacent section by *in situ* hybridization; an ^{35}S-labeled RNA transcript of a 450 bp HincIIxPstI-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.

IX5B2. First, labelling of unpaired segments of SCs with Mab IX5B2 has never or only rarely 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 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


a
b
c

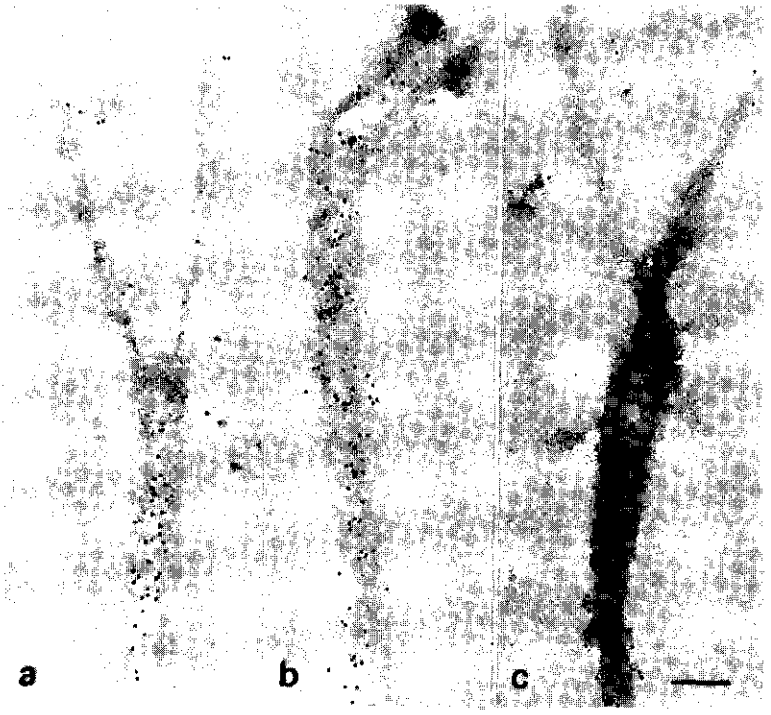


Figure 8. Ultrastructural localization of SCP1 by indirect immunogold labelling of surface spread spermatocytes with the polyclonal antiserum against the fusion protein of cDNA clone 536 as primary antiserum, and goat-anti-rabbit IgG conjugated to 10 nm gold as secondary antibody; a) zygotene, b) pachytene,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

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.

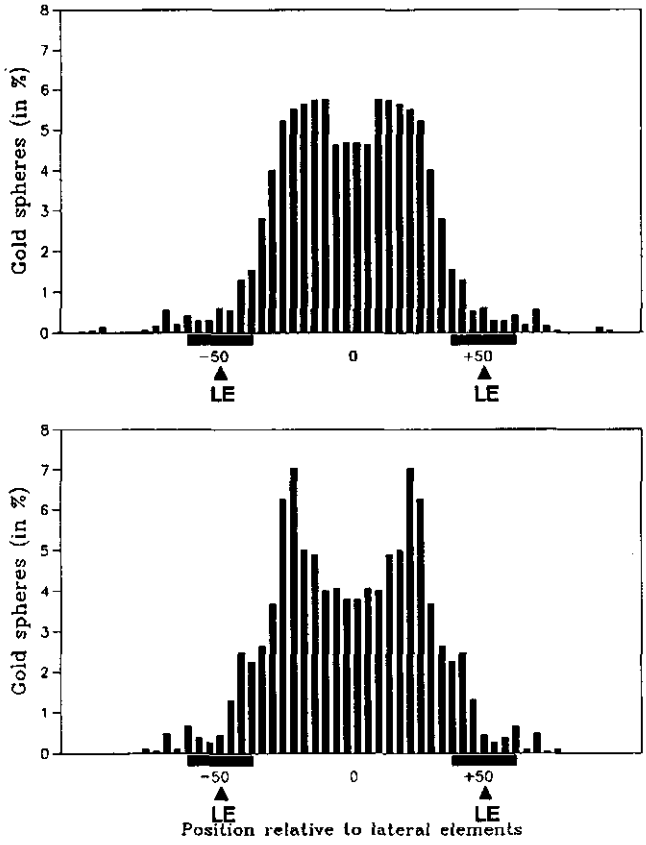


Figure 9. Distance of immunogold grains to the center of the nearest LE after indirect labelling with the primary antibody being being either the polyclonal antifusion protein antiserum (panel **A**, 2503 grains), or Mab IX5B2 (panel **B**, 909 grains). For each grain we measured first the distance between 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 has been indicated schematically on the horizontal axis. In each panel, the vertical bars add up to 100%.

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 components occur specifically in nuclei of meiotic prophase cells, i.e. spermatocytes (Heyting *et al.*, 1988; 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 and 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 (Mirzayan *et al.*, 1992; Young *et al.*, 1992; Compton *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 (Peter *et al.*, 1989; Vorburger *et al.*, 1989; McKeon *et al.*, 1986; Fisher *et al.*, 1986). 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 (Cao *et al.*, 1990; Alani *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 dissasembly 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 as well as 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 position 341 to 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 itself is 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 SCs 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). Because Mab IX5B2 probably recognizes a single epitope, and the polyclonal anti-SCP1 antiserum several on the SCP1

molecule, this 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 about 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 one 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 (Heyting *et al.*, 1988, Dunn, 1986). 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 PstI 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 Wis-

consin 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 92,000-120,000x). 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 GuTC/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) by standard procedures (Sambrook *et al.*, 1989). After transfer the membranes were washed in 3x 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 HindIII-fragment (see Figure 1) which had been subcloned into pBluescript. Transcription was performed from the T3 promotor in the presence of [α-^{32}P]rATP (3000 Ci/mmole). The Northern blot membranes were prehybridized in 50% formamide, 5x 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 hr at 65°C. Hybridization was performed in the same mixture with 1.5 μg/ml probe (15x10^6 cpm/μg) for 17 hr at 65°C. Subsequently the blots were washed for 30 min at 65°C in, succesively, 2x SSC, 0.1% SDS; 2x SSC, 0.1% SDS; 0.1x SSC, 0.1% SDS; and 0.1x SSC, 0.1% SDS.

In situ hybridization.

For *in situ* hybridization, 10 μm tissue sections were cut from testes of 3-months-old rats at -19°C. The sections were mounted on slides coatred 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, respectively, stained immunocytochemically with Mab IX5B2, and 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 2x 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 hr at room temperature. Hybridization was performed for 18 hr. 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, 1x Denhardt's (Denhardt, 1966), 0.5 μg/ml yeast tRNA and $2x10^5$ cpm/μl ^{35}S-labelled RNA probe ($18x10^3$ cpm/μg). The RNA probe was obtained by transcription from the T3-promotor of a 450 bp HincII x PstI 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 Corp.). After hybridization the sections were washed as follows: 30 min. in 5x SSC 10 mM DTT at 55°C; 1 hour at 50°C in 50% formamide, 5x SSC, 20 mM DTT; 3x 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; 2x 15 min. in 2x SSC and 2x 15 min. in 0.1x SSC at room temperature. Dehydration by quick transfer through 50, 70, 96, 100 and 100% ethanol each including 0.3M 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.

Other procedures.

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 6

ANALYSIS OF M_r 30,000 AND 33,000 COMPONENTS OF SYNAPTONEMAL COMPLEXES OF THE RAT BY TWO-DIMENSIONAL GEL ELECTROPHORESIS

H.H. Offenberg, A.H.F.M. Peters, M. van Aalderen, and C. Heyting

Summary

After two-dimensional electrophoresis of synaptonemal complex (SC) proteins, the M_r 30,000 - 33,000 SC components are resolved in at least 24 spots with pI values ranging from 6 to)9. These spots represent closely related proteins, because two independently isolated anti-M_r 30,000 - 33,000 Mabs and one polyclonal anti-M_r 30,000 - 33,000 antiserum recognize almost all these spots. Two other anti-M_r 30,000 - 33,000 Mabs recognize a subset of the spots. The nature of the differences between the pI variants has not yet been elucidated.

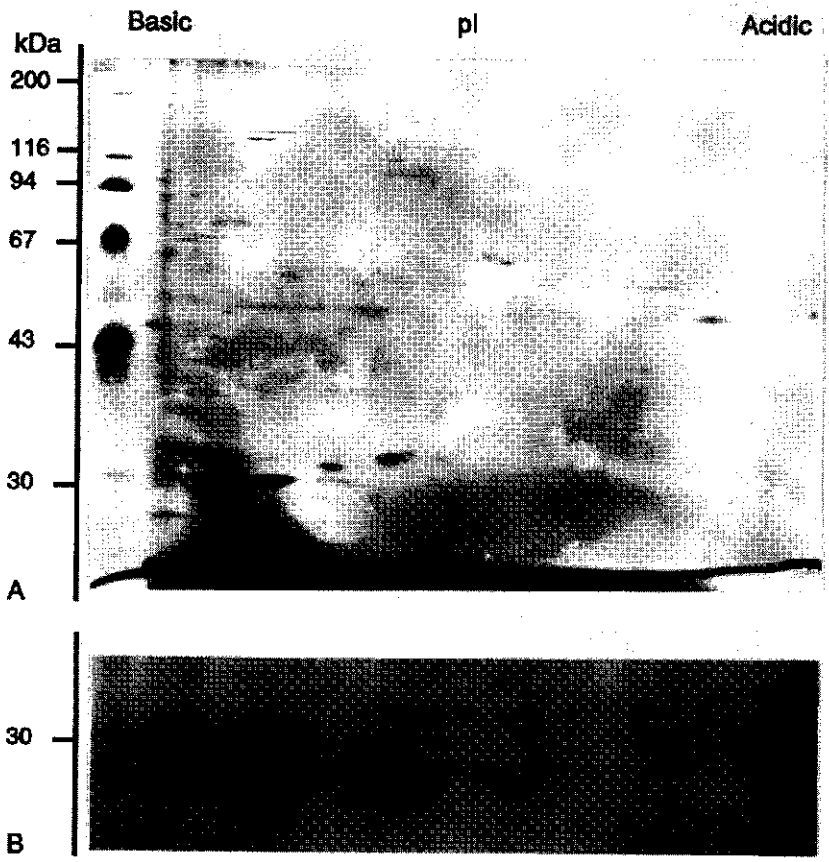


Figure 1. Two dimensional separation of SC proteins. Samples containing $3x10^7$ SCs were dissolved and separated as described in the Materials and Methods section. Figure 1A and Figure 1B show separations of proteins from different SC preparations. Figure 1A shows the entire 2D gel, Figure 1B shows the M, 30,000 - 33,000 region only.

Introduction

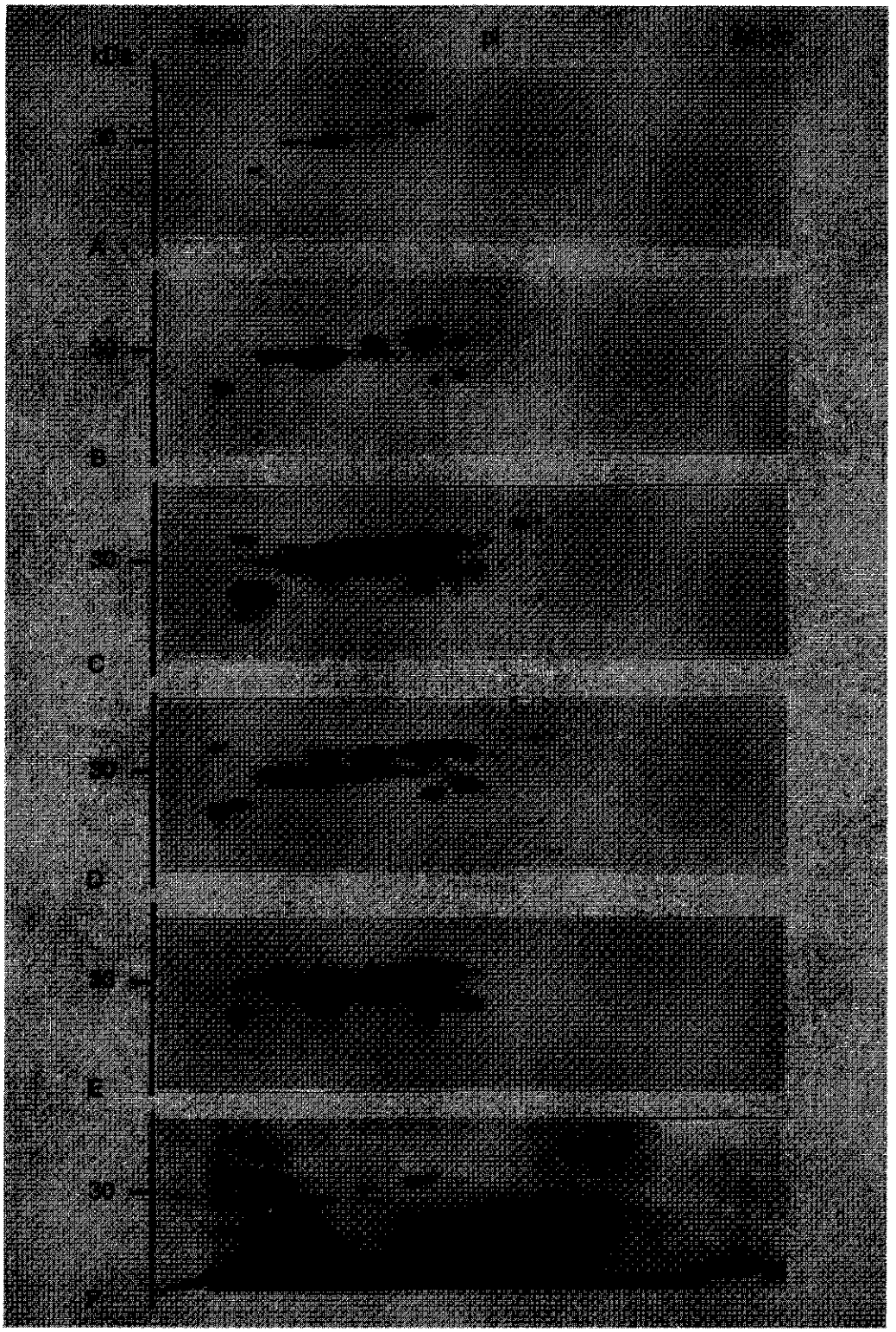
Synaptonemal complexes (SCs) are meiosis specific structures that mediate pairing of homologous chromosomes during meiotic prophase (Von Wettstein *et al.*, 1984). Biochemical analysis of SCs is possible since these structures can be

purified from spermatocytes of the rat (Heyting *et al.*, 1985). One-dimensional (1D) SDS-polyacrylamide gel electrophoresis of proteins from purified SCs show two prominent bands with relative electrophoretic mobilities (M_rs) of 30,000 and 33,000 respectively (Heyting *et al.*, 1985, 1987). These bands contain protein components of SCs, because several monoclonal antibodies (Mabs) that recognize these bands bind specifically to SCs (Heyting *et al.*, 1987, 1988, 1989 (Chapter 2 this thesis); Moens *et al.*, 1987; Offenberg *et al.*, 1991 (Chapter 3 this thesis)). The M_r 30,000 and M_r 33,000 SC components are immunologically related, because 18 independently isolated anti-SC Mabs recognize both components, whereas as yet no anti-SC Mabs have been identified that can discriminate between these components. In this Chapter we analyse the M_r 30,000 and M_r 33,000 SC components in detail by two-dimensional gel electrophoresis and immunoblot analysis.

Results and Discussion

Figure 1A and B show silver stained gels, obtained after 2D separation of proteins from two different SC preparations. In both gels the M_r 30,000 and M_r 33,000 protein bands are resolved in a large number of spots with pI values ranging from 6 to)9. The patterns of spots in Figure 1A and 1B are not identical; this is probably due to variation among SC-preparations, because different 2D separations of proteins from the same SC-preparation result in the same pattern of spots (Figure 2). To analyse which of the SC spots correspond to the M_r 30,000 and M_r 33,000 SC components, we performed immunoblot analyses

Figure 2. Immunoblot analysis of the M_r 30,000 - 33,000 SC proteins. Six 2D separations of proteins from the same SC preparation were performed. One of the resulting 2D gels was stained with silver (panel 2F; this is a detail of the same gel that is shown in Figure 1A), or blotted onto nitrocellulose (panel 2A to E). The resulting immunoblots were probed with the following anti-M_r 30,000 - 33,000 antibodies: **A**, Mab IX8G9; **B**, Mab IX7B12; **C**, Mab IX3H3; **D**, Mab IX4D4; **E**, polyclonal antiserum "Knuf". In panel **F** the spots are indicated that are *not* recognized by any of the anti-Mr 30,000 - 33,000 antibodies (indicated with an asterisk). The spots indicated with an asterisk in panel **C** and **D** are due to a reaction with an anti-M_r 190,000 Mab with which these immunoblots were probed before incubation in the anti-M_r 30,000 - 33,000 Mabs.


pI
A
B
C
D
E

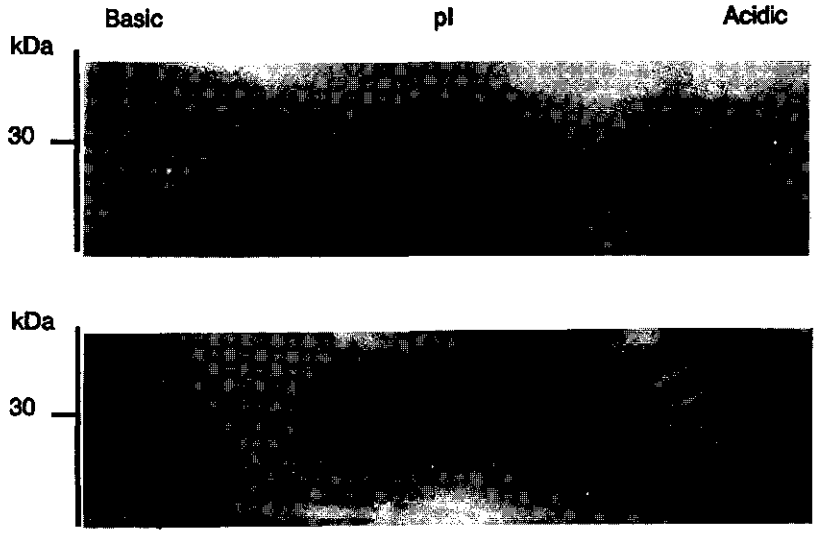


Figure 3. Two-dimensional separation of SC proteins after treatment with alkaline phosphatase (A) or buffer only (B). Samples containing $3x10^7$ SCs were treated with alkaline phosphatase or buffer, and subsequently subjected to 2D gel electrophoresis. After treatment with alkaline phosphatase the relative intensity of spot a has increased compared to that of spot b.

of SC proteins that had been separated on 2D gels. For this purpose we performed six 2D separations of proteins of the same SC preparation. One of the resulting 2D gels was stained with silver (Figure 1A and 2F); the other five were blotted onto nitrocellulose, and probed with anti-M_r 30,000 - 33,000 Mabs (Figure 2A to D) or with a polyclonal anti-M_r 30,000 - 33,000 antiserum (Figure 2E). Most of the spots are recognized by all antibodies tested; the immunoblots in Figure 2B to E show even more spots than the silver stained gel. Mabs IX3H3 and IX4D4 recognize 24 spots (Figure 2C and D); the polyclonal antiserum recognizes 22 of these 24 spots (Figure 2E); and Mabs IX7B12 and IX8G9 recognize 14 and 13 of these 22 spots respectively. Only a few minor spots in the M_r 30,000 - 33,000 region of the silver stained gel (indicated in Figure 2F) are *not* recognized by any of the antibodies tested. We conclude that most of the spots in the M_r 30,000 - 33,000 region contain immunologically

closely related proteins. Because the only structures that are recognized by the anti-M_r 30,000 - 33,000 antibodies are the SCs (Heyting *et al.*, 1988, 1989; Offenberg *et al.*, 1991), all these related proteins must be components of SCs.

What could be the nature of the differences between the pI variants? Particularly in Figure 1B it is clear that the spots are arranged in rows that slightly slant down towards the basic side of the gel. Such a pattern can arise if a protein can have different numbers of the same acidic residue. From the slope of the rows we estimate the molecular weight of such a residue at about 0.1 kDa. This corresponds to the molecular weight of a phosphate group or an amino acid residue. We have tried to analyse whether the M_r 30,000 - 33,000 SC components are phosphorylated by treatment of intact SCs with alkaline phosphatase. This resulted in a shift of part of some spots to a more basic position in the gel; compare for instance spots a and b in Figure 3A with the same spots in Figure 3B. If this shift is due to dephosphorylation, the alkaline phosphatase treatment has been incomplete. It is possible that the phosphatase could not remove all phosphate groups from the intact SC structure. SCs can only be dissolved in the presence of an ionic detergent such as SDS; alkaline phosphatase is not active on SDS-treated SC proteins, however (not shown).

We have also considered differences in poly-ADP-ribosylation as a possible explanation for the occurrence of pI variants of the M_r 30,000 - 33,000 SC components. Several nuclear proteins can be poly-ADP-ribosylated (Hayaishi and Ueda, 1977), and poly-ADP-ribosylation of histones seems to play a role in several chromatin functions, including DNA repair (Althaus *et al.*, 1990). Poly-ADP-ribosyl moieties can be hydrolysed to AMP monomers and *iso* ADP-ribose by treatment with alkali or phosphodiesterase (Hayaishi and Ueda, 1977). However, treatment of intact SCs with either 0.2 N NaOH or snake venom phosphodiesterase did not cause any change in 2D electropherograms of SC proteins (not shown).

To summarize: it is possible that differences in phosphorylation provide an explanation for part of the pI-variants of the M_r 30,000 - 33,000 SC com-

ponents. It is unlikely that the variants differ with respect to poly-ADP-ribosylation. There are several other possible explanations for the existence of variants of the M_r 30,000 - 33,000 SC components. The variants might represent members of a protein family; translation products of differentially spliced messenger RNAs, or proteolytic breakdown products. Whether one of these explanations could be valid can be sorted out by isolation of the cDNA(s) encoding the M_r 30,000 - 33,000 SC components, and analysis of the predicted amino acid sequence(s). Such analyses are in progress (Lammers *et al.*, in preparation).

Materials and Methods

Antibodies.

The monoclonal antibodies IX3H3, IX4D4, IX7B12 and IX8G9 which recognize the M_r 30,000 - 33,000 SC components were obtained after immunization of mice with rat SCs as described by Offenberg *et al.* (1991). The polyclonal antiserum "Knuf" was elicited by subcutaneous and intramuscular injection of a rabbit according to the scheme described by Offenberg *et al.* (1991) for the immunization of mice. 20 ml bleedings were collected from the ear-veins with two-week intervals, starting one week after the third injection.

Gel electrophoresis and immunoblotting.

Electrophoresis was carried out as described by Heyting *et al.* (1988). Two-dimensional gel electrophoresis was performed as described by O'Farrell (1975). After intensive vortexing and boiling of SCs in 5 mM EDTA, 2% SDS and 1% DTT, the sample buffer was adjusted to 2.67 % ampholine mixture (Pharmacia-LKB), 10 % Nonidet P-40, 1 mM PMSF and 9.0 M Urea and subsequently layered on the tube gels. Isoelectric focusing in the first dimension was performed in 4% acrylamide gel containing 2% ampholines (one part pH 3.5-10, four parts pH 5-8), 2% Nonidet P-40, 9.16 M urea for approximately 4000 Vh at a maximum voltage of 2000 V. The isoelectric focusing tube gels were in-

cubated in SDS equilibration buffer (10% glycerol, 125 mM Tris-HCl pH 6.8, 2% SDS, 0.4% DTT, 1 mM PMSF) for 10 min. at room temperature. SDS-PAGE in the second dimension was performed on a 7 to 18% linear gradient acrylamide gel as described by Heyting *et al.* (1987).

After gel electrophoresis, proteins were transferred to nitrocellulose by electroblotting in 10 mM $NaHCO_3$, 3 mM Na_2CO_3, 25 mM Tris-HCl pH 9.9, 20% Methanol (Dunn, 1986) at 4 °C for 1400 Vh at a maximum of 0.5 A. After blotting, the filters were washed in deionized water and stored under vacuum at 4 °C. Immune incubations were performed as described by Heyting *et al.* (1988) and Offenberg *et al.* (1991).

Modification studies.

Isolated rat SCs were treated with alkaline phosphatase as described by Gerace *et al.* (1984) for the nuclear lamina. SCs were incubated with 12 U alkaline phosphatase (Sigma type III, Bacterial E. coli suspension in 2.5 M $(NH_4)_2SO_4$) in 0.1 M Tris-HCl pH 8.0, 5 mM $MgCl_2$, 2 µg/ml leupeptine (Boehringer Mannheim), 10 µg/ml aprotinine (Boehringer Mannheim), 10 µg/ml trypsin inhibitor (Boehringer Mannheim) and 1 mM PMSF for 3 hr. at 37 °C.

Poly-ADP-ribosylation of SC proteins was studied by incubating SCs with 0.2 U snake venom phosphodiesterase per mg protein (Mullins *et al.*, 1977; Wong *et al.*, 1983) or by incubation in 0.2 M NaOH, for 30 min. at 25 °C (Yoshihara *et al.*, 1975; Mullins *et al.*, 1977).

Other procedures.

SCs were isolated as described by Heyting *et al.* (1985) and Heyting and Dietrich (1991). Silver staining was performed as described by Oakley *et al.* (1980).

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CHAPTER 7

GENERAL DISCUSSION

General Discussion

This thesis describes the isolation of antibodies which recognize SC-specific proteins, and the identification and characterization of the major SC components by means of these antibodies. Using the antibodies described in Chapter 2 and Chapter 3 of this thesis, we isolated a cDNA clone which probably encodes a major component of the LEs, the M_r 190,000 SC component (Offenberg *et al.*, in preparation, Chapter 4, this thesis). In addition, we recently isolated a cDNA clone encoding a major component of the transverse filaments of SCs (the M_r 125,000 SC component; Meuwissen *et al.*, 1992, Chapter 5 this thesis) by means of these antibodies, and probably also a cDNA encoding the M_r 30,000 and/or 33,000 components of SCs (Lammers *et al.*, in preparation). In this final chapter, I would like to consider what we have learned from the experiments described in this thesis with respect to the questions that have been posed in Chapter 1: what is the possible evolutionary and ontogenetic origin of SCs? How is their (dis)assembly regulated? And what is their role in meiotic chromosome pairing and recombination?

What is the evolutionary and ontogenetic origin of SCs?

Two different mechanisms can be envisaged with respect to the ontogenetic origin of SCs and the mechanism of chromatin rearrangements of meiotic prophase (Figure 1): either the chromatin detaches from supporting structures like the nuclear lamina and nuclear matrix, and reorganizes onto a new, meiosis specific structure, the SC (Figure 1A); or chromatin-supporting structures rearrange to form axial elements which are then connected to form SCs (Figure 1B). To discriminate between these two mechanisms, we have analyzed the assembly of SCs immunocytochemically by means of the anti-SC antibodies (Chapter 3, this thesis; Heyting *et al.*, 1988); we found that all SC components analyzed thus far occur exclusively in meiotic prophase cells, on the SCs (Heyting *et al.*, 1987, 1988, 1989 (Chapter 2, this thesis); Offenberg *et al.*, 1991 (Chapter 3, this thesis); see also Smith and Benavente, 1991). These identified SC components are absent from mitotic chromosomes (Heyting *et al.*, 1988;

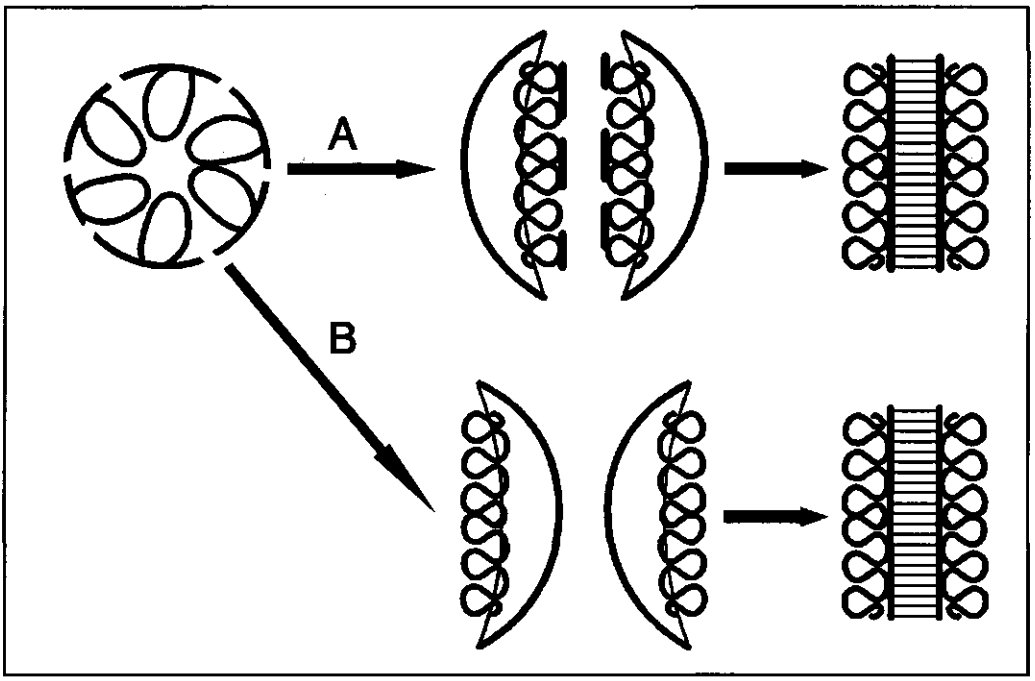

Figure 1. Two possible mechanisms of chromatin rearrangement during meiotic prophase. Explanation, see text (From Heyting and Dietrich, 1992, with permission).

Offenberg *et al.*, 1991 (Chapter 3, this thesis). Furthermore, nuclear lamins could not be detected in rat SCs (Heyting *et al.*, unpublished). Thus, though it is still possible that components of the interphase nuclear matrix are (minor) components of SCs, it seems likely that SCs consist largely of meiosis-specific components, and that during meiotic prophase the chromatin reorganizes onto a newly synthesized structure (Figure 1A).

The amino acid sequences of SCP1 (the M, 125,000 SC component) and SCP2 (the M, 190,000 component) also provide some information about the possible evolutionary origin of SCs: both SC-components show structural, though not sequential similarity to nuclear matrix proteins: SCP1 has a large domain capable of forming an amphipathic α-helix; furthermore, it has a cluster of S/T-

P-X-X motifs outside this domain. It shares these features with nuclear lamins (Peter *et al.*, 1989; Vorburger *et al.*, 1989; Mckeon *et al.*, 1986; Fisher *et al.*, 1986), and some recently characterized components of the nuclear matrix, such as mammalian NUMA and yeast NUF1, that are supposed to fulfil chromatin supporting functions (Compton *et al.*, 1992; Young *et al.*, 1992; Mirzayan *et al.*, 1992; Luderus *et al.*, 1992). We speculate that SCP1 has evolved by specialization of such a nuclear matrix protein, although as yet no sequence similarity has been detected with any of such proteins. The α-helical domain of SCP1 has significant sequence similarity to the S2-domain of myosin heavy chain (Meuwissen *et al.*, 1992, Chapter 5 this thesis). This has also been found for two nuclear proteins of yeast, namely the RAD50 protein (Alani *et al.*, 1989) and the ZIP1 protein (M. Sym *et al.*, unpublished observations). It is possible that the α-helical domains of SCP1, myosin heavy chain, RAD50 protein and ZIP1 protein have a common progenitor.

SCP2 has one structural feature in common with proteins that are supposed to be involved in chromatin loop organization, such as the gene products of SATB1 and RAP1 proteins, namely a high content of S/T-P-X-X and S/T-S/T-X-X motifs (see Chapter 4 this thesis). However, SCP2 has no amino acid sequence similarity to any of these proteins except the RED1 protein of yeast. This protein plays a role in the assembly of yeast SCs. The similarity covers only limited segments of SCP2 and RED1 proteins, and does not extend to the region of RED1 that is enriched in the S/T-P-X-X and S/T-S/T-X-X motifs. Thus, it is possible that SCP2 has evolved by specialization of a nuclear protein that plays a role in chromatin loop organization, but as yet no candidate progenitor proteins have been identified.

How is the (dis)assembly of SCs regulated?

Because antibodies recognizing different SC components, and cDNAs encoding these components, are now available, the regulation of the assembly and disassembly of SCs has become accessible for analysis at the molecular level. The amino acid sequences of SCP1 and SCP2 contain several protein kinase target

sites that could play a role in this respect. Both SCP1 and SCP2 contain target sites for $p34^{cdc2}$ protein kinase. It has been shown that phosphorylation of such sites on nuclear lamins A and C is important for the disassembly of the lamina at mitosis (Heald and McKeon, 1990; Peter *et al.*, 1990; Ward and Kirschner, 1990). $p34^{cdc2}$ function is also required for disassembly of SCs and progression through meiotic prophase in yeast: mutation of CDC28 (which is the *Saccharomyces cerevisiae* gene which corresponds to CDC2 of *Schizosaccharomyces pombe*) prevents disassembly of SCs and causes an arrest in pachytene (Shuster and Byers, 1989). It thus appears that some of the controls that function at the transition from G2 to mitosis, are also active at the exit from pachytene. Other phosphorylation sites in SCP1 and SCP2 that are of potential interest for the regulation of SC (dis)assembly are the cAMP/cGMP dependent protein kinase target sites. It has been shown that inhibition of phosphorylation of such sites on nuclear lamins is essential for the disassembly of the lamina during mitosis (Lamb *et al.*, 1991). However, it remains to be determined whether the potential phosphorylation sites of SCP1 and SCP2 are actually phosphorylated *in vivo*, and if so, whether this has any biological significance.

What is the function of SCs?

The two major components of SCs that have now been analyzed, SCP1 and SCP2, have characteristics of nuclear matrix proteins. SCP1 shares features with components of the nuclear lamina and with proteins that could make part of a fibrillar network in the nucleus, the nuclear matrix (Bereznev and Coffey, 1977). SCP2 has structural similarities to proteins that are involved in the organization of chromatin in loops (see above). These results suggest a structural function for SCs: SCs might organize the chromatin in such a way that homology search, recombination and/or chromosome segregation can proceed properly. Rather than speculating further about this, I would like to make two remarks: first of all, a structural function of SCs does not exclude involvement in other functions; and second, different SC components can be involved in totally different functions. The most direct approach to the analysis of SC

function(s) includes the identification of conserved domains in SC proteins, and the targeted mutagenesis of the genes encoding these proteins. For this purpose, we have isolated the yeast genes that are homologous to the rat genes encoding SCP1 and SCP2. Mutagenesis of the yeast SC-genes, and analysis of the meiotic phenotype of the mutants should not only provide more information about possible functions of SCs, but also about the regulation of their (dis)assembly.

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SUMMARY

Summary

Synaptonemal complexes (SCs) are structures that are formed between homologous chromosomes during meiotic prophase. They undergo a series of morphological alterations which closely correlate with the successive rearrangements of meiotic prophase chromatin, namely chromosome condensation, pairing, recombination and segregation. Despite this correlation, as yet no functions have been assigned with certainty to SCs (Chapter 1). The work described in this thesis is focused on the identification and characterization of components of SCs of the rat with the purpose of biochemical and functional analysis of SCs. For the identification of SC components we elicited monoclonal and polyclonal antibodies against isolated SCs.

Chapter 2 describes the isolation of SC-specific monoclonal antibodies and the identification of four major components of the SC by means of these antibodies. Three major components of the lateral elements (LEs) were identified, with relative electrophoretic mobilities (M_rs) of 30,000, 33,000 and 190,000 respectively. All 18 monoclonal antibodies that recognize the M_r 30,000 SC component also recognize the 33,000 component; apparently these components are related. One M_r 125,000 component was localized at the inner edge of the LEs, specifically where chromosomes are paired (synapsed). Furthermore, we found that M_r 66,000 - 55,000 antigens were localized in clusters in the vicinity of the SCs. It is possible that these antigens are SC-associated proteins, rather than real SC components.

Chapter 3 describes an analysis of the tissue distribution of the M_r 30,000, 33,000, the M_r 125,000 and the M_r 190,000 SC antigens. All antigens could only be detected in meiotic prophase cells by means of immunofluorescence. The M_r 30,000 and 33,000 components were observed in all stages of meiotic prophase where LEs are present, i.e. from zygotene up to and including diplotene, in paired as well as unpaired segments of SCs. It could not be excluded that traces of both proteins, in a form not recognizable as SCs, were present in spermatogonia and spermatids. The tissue distribution of the M_r 190,000 com-

ponent, as detected by immunofluorescence, was indistinguishable from the distribution of the M_r 30,000 and 33,000 antigens. The M_r 125,000 component was exclusively present in meiotic prophase nuclei, in paired segments of SCs. Thus, SCs largely consist of newly synthesized proteins and the chromatin rearrangements of the meiotic prophase involve the reorganization of chromatin onto newly assembled, meiosis-specific structures, the SCs.

Chapter 4 describes the isolation of cDNA clones encoding SCP2, a major component of the LEs of SCs, by means of Mabs that recognize the M_r 190,000 SC component. The gene encoding SCP2 is exclusively expressed in the testis, predominantly in spermatocytes. SCP2 has a predicted molecular weight of 148 kDa. It shares some features with DNA binding proteins that are involved in chromatin organization, such as the SATB1 and RAP1 proteins: it is rich in ß-turns, it has a high content of proline residues, and it has several S/T-P-X-X and S/T-S/T-X-X motifs. We speculate that SCP2 interacts directly with DNA and has a chromatin organizing function.

Chapter 5 describes the isolation of cDNA clones encoding SCP1, a major component of transverse filaments of SCs, by means of a Mab that recognizes the M_r 125,000 SC component. The transcription of the gene encoding SCP1 is restricted to zygotene till diplotene spermatocytes. A polyclonal antiserum raised against the fusion protein produced by one of the cDNA clones recognizes a single protein on Western blots of isolated SCs, with identical electrophoretic mobility as the antigen recognized by the anti-M_r 125,000 Mab. SCP1 has a predicted molecular weigth of 111 kDa. It shares several features with nuclear lamins and some nuclear matrix proteins. A major part of SCP1 is capable of forming an amphipathic α-helix. This region shows amino acid similarity to the coiled-coil region of myosin heavy chain. We speculate that SCP1 has evolved by specialization of a nuclear matrix protein.

Chapter 6 describes the analysis of the M_r 30,000 and 33,000 SC components by two-dimensional gel electrophoresis. These SC components are resolved in at least 24 spots, with pI values between 6 and)9. Some of the anti-M_r 30,000

and 33,000 Mabs recognize all 24 spots, which indicates that the spots represent related proteins or variants or breakdown products of the same protein.

Samenvatting

Synaptonemale complexen (SC's) zijn structuren die tijdens de meiotische profase tussen homologe chromsomen gevormd worden. Ze ondergaan een serie morfologische veranderingen die correleren met de opeenvolgende chromatine herrangschikkingen van de meiotische profase, namelijk chromosoom condensatie, paring, recombinatie en segregatie. Ondanks deze correlatie heeft men tot nu toe nog geen functies met zekerheid aan SC's kunnen toeschrijven (Hoofdstuk 1). Het werk dat in dit proefschrift beschreven wordt is gericht op de identificatie en karakterisering van componenten van SC's van de rat, met het doel om de biochemie en de functie van SC's te analyseren. Voor de identificatie van SC componenten hebben wij monoclonale en polyclonale anti-SC antilichamen bereid.

In Hoofdstuk 2 wordt de isolatie van specifieke anti-SC antilichamen beschreven, en de identificatie van vier kwantitatief belangrijke componenten van SC's door middel van deze antilichamen. Er werden drie componenten van de laterale elementen (LE's) geïdentificeerd, met relatieve electroforetische mobiliteiten (M_r's) van, respectievelijk, 30.000, 33.000 en 190.000. Alle 18 monoclonale antilichamen (Mabs) die de M_r 30.000 component herkennen, herkennen ook de M_r 33.000 component; kennelijk zijn deze componenten verwant. Een M_r 125.000 component werd gelocaliseerd aan de binnenkant van de LE's, specifiek in de gepaarde segmenten van de chromosomen. Bovendien bleken M_r 66.000 - 55.000 antigenen in clusters voor te komen in de buurt van SC's. Het is mogelijk dat deze antigenen SC-geassocieerde eiwitten zijn en geen echte SC componenten.

In Hoofdstuk 3 wordt de weefselverdeling van de M_r 30.000, 33.000, 125.000 en 190.000 SC antigenen geanalyseerd. Alle antigenen waren door middel van immuunfluorescentie uitsluitend detecteerbaar in cellen die in de meiotische profase verkeerden. De M_r 30.000 en 33.000 componenten kwamen voor in alle stadia van de meiotische profase waarin LE's aanwezig waren, van zygoteen tot en met diploteen, in gepaarde zowel als ongepaarde segmenten

van SC's. Het is niet uitgesloten dat sporen van beide eiwitten, in een vorm die niet herkenbaar is als SC, aanwezig zijn in spermatogoniën en spermatiden. De weefsel-verdeling van de M_r 190.000 SC component was door middel van immuunfluorescentie niet te onderscheiden van de verdeling van de M_r 30.000 en 33.000 antigenen. De M_r 125.000 component was uitsluitend aanwezig in meiotische profase kernen, in gepaarde segmenten van SC's. Samenvattend, SC's bestaan voor een groot deel uit nieuw gesynthetiseerde eiwitten; de chromatine herrangschikkingen van de meiotische profase houden een reorganisatie in van het chromatine op nieuw geassembleerde, meiose-specifieke structuren, de SC's.

In Hoofdstuk 4 wordt de isolatie beschreven van cDNAs die coderen voor SCP2, één van de voornaamste componenten van de LE's van SC's, door middel van Mabs die de 190,000 SC component herkennen. Het gen dat codeert voor SCP2 komt uitsluitend tot expressie in de testis, vooral in de spermatocyten. Het voorspelde molecuulgewicht voor SCP2 is 148 kDa. SCP2 heeft enkele kenmerken gemeen met DNA bindende eiwitten die betrokken zijn bij de organisatie van het chromatine, zoals de SATB1 en RAP1 eiwitten: het bevat veel β-turns, het heeft een hoog proline-gehalte en het heeft een aantal S/T-P-X-X en S/T-S/T-X-X motieven. Wij speculeren dat SCP2 directe interactie met DNA kan aangaan en een chromatine-organiserende functie heeft.

In Hoofdstuk 5 wordt de isolatie beschreven van cDNAs die coderen voor SCP1, één van de voornaamste componenten van de transversale filamenten van SC's, door middel van een Mab dat de M_r 125,000 SC component herkent. Het gen dat codeert voor SCP1 komt alleen tot expressie in spermatocyten van zygoteen tot diploteen. Met behulp van het fusie eiwit, dat geproduceerd werd door één van de cDNAs, werd een polyclonaal antiserum opgewekt. Dit antiserum herkent een eiwit op westernblots van geïsoleerde SC's, met eenzelfde electroforetische mobiliteit als het eiwit dat herkend wordt door de anti-M_r 125,000 Mabs. Het voorspelde molecuulgewicht van SCP1 is 111 kDa. SCP1 heeft een aantal kenmerken gemeenschappelijk met nucleaire lamina en enkele nucleaire matrix eiwitten. Een groot deel van SCP1 is in staat om een

amfipatische α-helix te vormen. Dit deel vertoont overeenkomsten met het coiled-coil deel van de zware keten van myosine. Wij speculeren dat SCP1 geëvolueerd is door specializatie van nucleaire matrix eiwitten.

In Hoofdstuk 6 worden de M_r 30.000 en 33.000 SC componenten geanalyseerd door middel van 2-dimensionale gel electroforese. Deze SC componenten worden in meer dan 24 spots gescheiden, met isoelectrische punten tussen 6 en)9. Sommige van de anti-M_r 30.000 en 33.000 Mabs herkennen 24 spots, wat erop duidt dat de meeste spots verwante eiwitten bevatten, varianten van hetzelfde eiwit, of afbraakproducten van hetzelfde eiwit.

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Curriculum Vitae

Hildo Offenberg werd op 10 december 1963 geboren in Enkhuizen. Na het behalen van het VWO diploma aan de Rijks Scholen Gemeenschap te Enkhuizen begon hij in 1982 de studie Scheikunde aan de Vrije Universiteit te Amsterdam. In augustus 1983 werd het propedeuse diploma gehaald. De doctoraalfase omvatte een hoofdvak Biochemie aan de Vrije Universiteit en een bijvak Enzymologie aan de Universiteit van Amsterdam. In december 1987 werd de studie afgesloten met het doctoraal examen. Aansluitend in januari 1988 werd hij aangesteld als onderzoeker in opleiding bij de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO), onder leiding van Prof. Dr. C. Heyting. Van januari 1988 tot april 1989 werd het onderzoek uitgevoerd op de vakgroep Antropogenetica van de Universiteit van Amsterdam. Het project werd vervolgd op de vakgroep Erfelijkheidsleer van de Landbouwuniversiteit Wageningen, tot januari 1992. In januari 1992 heeft hij een Shell studiereis gewonnen, waarmee een werkbezoek aan Harvard University (USA) werd bekostigd. Hier heeft hij gewerkt op het laboratorium van Prof. Dr. N. Kleckner aan het heteroloog kloneren van enkele ratte genen met behulp van beschikbare gistgenen.