The M_r 30,000-33,000 major protein components of the lateral elements of synaptonemal complexes of the rat

Promotor: Dr. C. Heyting, hoogleraar in de moleculaire en celgenetica

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יש דבר שיאמר ראה־זה חדש הוא

כבר היה לעלמים אשר היה מלפננו

Men zegt wel van iets:"Zie dat, dat is iets nieuws!",
maar het was er lang geleden al, ver voor ons.

(vrij vertaald naar Prediker 1, 10)

Aan mijn oma, Hubertina Josephina Wienhoven-Steegh

Voor mijn ouders

Stellingen

1. De M_r 30.000-33.000 componenten van het synaptonemale complex van de rat komen naast elkaar voor tijdens de gehele meiotische profase als verschillende iso-electrische varianten, die voornamelijk van elkaar verschillen in fosforyleringsgraad.
   Dit proefschrift

2. Geïsoleerde synaptonemale complexen hebben het vermogen om bepaalde DNA-sequenties, waaronder verschillende 'matrix attachment regions' (MARs), specifiek te binden *in vitro*.
   Dit proefschrift

3. Kennis over enkel de meiose-specifieke componenten van het synaptonemale complex is onvoldoende om de functie van deze structuur op te helderen.

4. Bij uitspraken over de aan- of afwezigheid van matrix attachment regions (MARs) in SC-geassocieerd DNA worden sequentiekenmerken van enkele specifieke MARs vaak ten onrechte gebruikt als consensussequenties.
   *Pearlman et al. (1992), Genetics, **130**, 865-872 en Karpova et al. (1995), Mol.Biol., **29**, 289-295.*

5. De stelling van Karl Rahner: "De economische Triniteit is de immanente Triniteit en omgekeerd" laat te weinig ruimte voor Gods vrijheid en Anders-zijn.
   Karl Rahner (1967) "Der Dreifaltige Gott als tranzendenter Urgrund der Heilsgeschichte", in: 'Mysterium salutis", Dl.2, p.317-401.

6. De uitspraak "Wat onze tijd mist is een geloofwaardige beeldvorming van de relatie tussen God en wereld", wijst op een belangrijke reden waarom het spreken over God voor veel mensen niet (meer) vanzelfsprekend is.
   naar Sallie McFague (1987) "Models of God: theology for an ecological, nuclear age", p. 46.

7. Een geneticus is vaak ge(e)n et(h)icus.

8. In onze drang naar kennis zijn wij bereid alles te onderzoeken, behalve onszelf.
   Paul van den Bergh

9. Veel mensen menen ten onrechte dat een wetenschappelijk stuk alleen in ingewikkelde bewoordingen kan worden geschreven.

10. Hoe meer communicatiemiddelen hoe minder echte communicatie.

11. Het overwaarderen van 'buitenlandervaring' wekt ten onrechte de indruk dat het onderzoek in het buitenland van een hoger niveau is dan het nederlandse.

12. De invoering van een 24-uurs economie levert voornamelijk één beroepsgroep meer werk op: die van psychologen en psychiaters.

13. Musica è.
Eros Ramazzotti

14. Beter één goede loodgieter dan tien tot IT-er omgeschoolden.
Moderne versie van een bekend spreekwoord

15. Het is met chromosomen net als met de bijbel: dezelfde volgorden van letters hebben voor iedereen een andere betekenis.

Stellingen behorende bij het proefschrift 'The M_r 30,000-33,000 major protein components of the lateral elements of synaptonemal complexes of the rat', Hans Lammers, Wageningen, 10 maart 1999.

Contents

Chapter 1

General Introduction

An overview of meiosis

In sexually reproducing organisms, the formation of gametes requires a reduction of the chromosomal complement from the diploid to the haploid level. Restoration of the diploid chromosomal complement occurs when two gametes fuse to form a zygote.
The halving of the number of chromosomes is achieved by a specialized type of division called meiosis. Meiosis differs from mitosis in several important respects. First, during meiosis, a single round of DNA replication is followed by two successive nuclear divisions designated meiosis I and meiosis II (Figure 1), so that four haploid cells are produced from a single diploid cell. Second, during mitosis, the two sister chromatids, which are produced by replication of each chromosome during S-phase, are aligned between two poles at metaphase and move to opposite poles at anaphase, whereas at meiosis I the two sister chromatids behave as a single unit. Instead of the two sister chromatids, the two versions of each chromosome, which each diploid cell contains (homologs), align to form a pair or bivalent. The homologs, each consisting of two sister chromatids, move to opposite poles at anaphase I. At anaphase II, the sister chromatids separate, as in mitosis. Third, during the prophase of meiosis I, the level of homologous recombination is several orders of magnitude higher than during mitosis. And fourth, during meiosis recombination occurs primarily between non-sister chromatids of homologous chromosomes, whereas during mitosis recombination occurs predominantly between sister chromatids. Some recombination events between non-sister chromatids result in crossing-overs, which at a later stage of meiotic prophase can be observed cytologically as chiasmata, i.e. sites where non-sister chromatids of homologs appear to be physically connected. Normally, at least one crossing-over between non-sister chromatids of homologs is found per bivalent.

Apart from these differences, the mitotic and meiotic division share several characteristics. It is therefore attractive to speculate that meiosis evolved from the mitotic division. It is of great interest to analyse whether and how the mitotic division has been adapted to provide a reliable machinery for the reduction of the ploidy level and the generation of genetic diversity. It is of fundamental importance for eukaryotic genetics to understand the meiosis-specific mechanisms at the molecular level. This thesis focuses on the molecular analysis of a meiosis-specific nuclear structure, the synaptonemal complex, which appears to be involved in meiotic chromosome pairing and recombination. The detailed molecular analysis of one protein component of this structure is described.

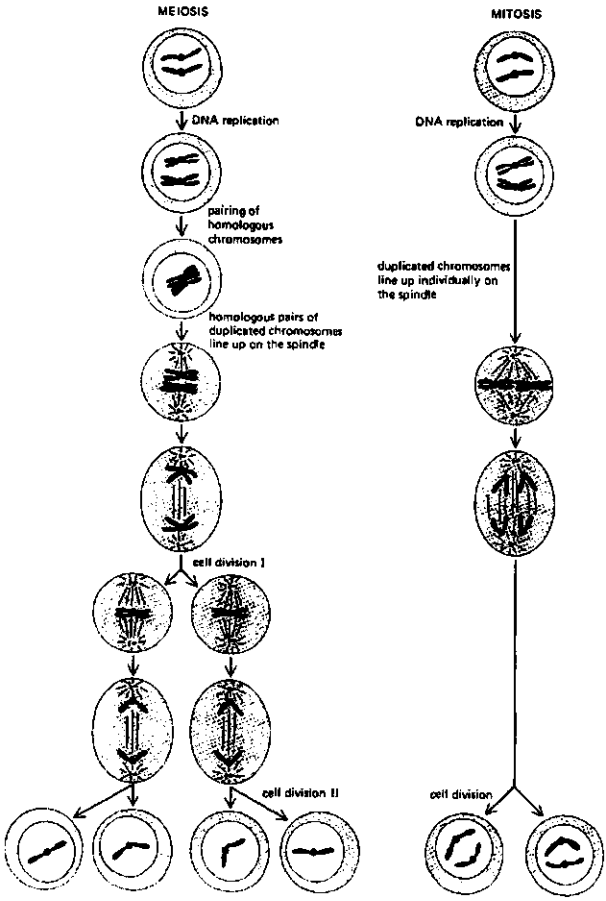

Figure 1: Schematic representation of meiosis compared to mitosis. Only one set of homologous chromosomes is shown. Pairing of (duplicated) homologous chromosomes is specific for meiosis. Meiosis consists of two successive, nuclear divisions. Each diploid cell which undergoes meiosis thus produces four haploid gametes (from Alberts *et al.*, 1983).

The synaptonemal complex (SC)

In almost all sexually reproducing eukaryotes analysed thus far, a proteinaceous structure, the synaptonemal complex (SC), is formed between homologous chromosomes (reviewed by von Wettstein *et al.*, 1984). At the beginning of meiotic prophase (leptotene), the two sister chromatids of each chromosome develop a single proteinaceous axis, the axial element. These axial elements are then connected by numerous transverse filaments (TFs) to form the structure of a SC. Between the axial elements, a third longitudinal structure, the central element (CE), is formed. The axial elements, together with the CE, make up the tripartite structure of the SC (Figure 2). Within the context of this tripartite structure, axial elements are also called lateral elements (LEs). At the pachytene stage, homologous chromosomes are connected by the tripartite structure along their length. At diplotene, the SCs are disassembled. In mammals, the TFs and CE disappear first and the axial elements later. At the end of diplotene, the scaffolds of the individual sister chromatids become discernable (Rufas *et al.*, 1992), whereas the axial elements disappear.

Molecular-genetic analysis of SCs and their components has mainly been carried out in the budding yeast *Saccharomyces cerevisiae* whereas biochemical analysis has been focussed on rodents. In yeast a large number of meiotic mutants have been analyzed and from these analyses several candidate SC components were identified (reviewed by Roeder, 1995). Major disadvantages of yeast as research organism are the small size of yeast chromosomes and the absence of protocols for isolation of SCs.

A procedure to isolate 60-80 % pure SCs from rat has been developed by Heyting *et al.* (1985, 1987). The polypeptide composition of isolated SCs is still quite complex (Heyting *et al.*, 1987, 1989). Three components of rat SCs were identified by means of monoclonal antibodies elicited against preparations of isolated SCs. Two of the identified components, a M_r 30,000-33,000 and a M_r 190,000 component were localized in the axial elements in synapsed and unsynapsed segments (Heyting *et al.*, 1987, Moens *et al.*, 1987, Heyting *et al.*, 1989), whereas one component was localized in the region between the LEs in synapsed segments of SCs (Meuwissen *et al.*, 1992). All proteins were specific for meiotic prophase nuclei and were not found in the nuclear lamina or in mitotic chromosomes (Heyting *et al.*, 1988; Offenberg *et al.*, 1991). Moreover, in spermatocytes they were all detected from the zygotene stage up to and including diplotene (Offenberg *et al.*, 1991). Despite the characterization of a number of putative SC components in yeast and an extensive search for yeast homologs of the rat SC components (Meuwissen *et al.*, 1992, Offenberg *et al.*, 1998 and Lammers *et al.*, 1994; Chapter 2), no SC proteins have been found that show conservation between yeast and rodents at the amino acid sequence level. Perhaps only structural characteristics of SC-proteins have been conserved, and not

the primary amino acid sequence.

As a first step in the analysis of the properties and functions of the identified SC proteins, cDNAs encoding these SC components were isolated by screening an expression cDNA library by means of the monoclonal antibodies. The cDNAs encoding the M_r 125,000 and M_r 190,000 proteins were isolated and sequenced. The translation product of the cDNA encoding the M_r 125,000 protein, which was designated *s*ynaptonemal *c*omplex *p*rotein 1 (SCP1, Meuwissen *et al.*, 1992), is a protein which contains a long domain capable of forming coiled coil structures, which is flanked by the N-terminal and C-terminal domain of the protein. The C-terminal domain can bind to DNA *in vitro* (Meuwissen *et al.*, submitted). The translation product of the cDNA encoding the M_r 190,000 protein, designated SCP2 (Offenberg *et al.*, 1998), contains several motifs that could be involved in DNA-binding. When I started this investigation, the cDNA encoding the M_r 30,000 and 33,000 proteins had not yet been been isolated. Insight into the properties and functions of these LE components together with those of SCP2 might provide a molecular basis for understanding the function of the lateral element.

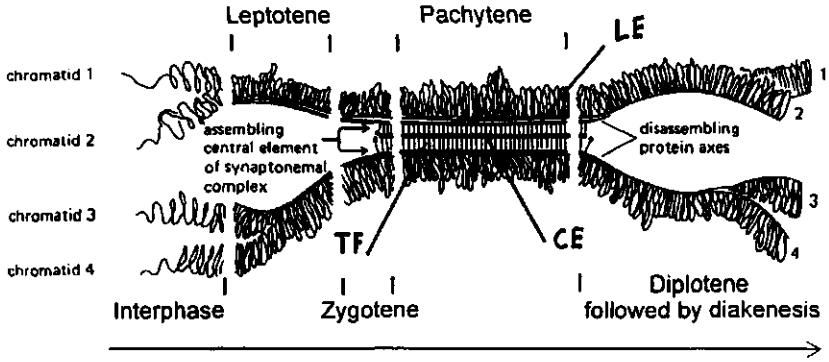

Figure 2: Schematic representation of the assembly and disassembly of the SC. During leptotene, proteinaceous axes begin to develop along homologous chromosomes. Zygotene begins when synapsis of the homologs starts. When synapsis is completed the stage of pachytene begins. During diplotene the SC starts to disassemble, after which, the SC disappears and the scaffolds of sister chromatids become discernible. LE: lateral element, TF: transverse filament and CE: central element.

Meiotic recombination and the assembly of synaptonemal complexes: a short overview

Before addressing the possible functions of the lateral element, I will briefly summarize current models concerning the events of meiotic prophase leading to pairing and recombination, which are mainly based on studies performed with yeast (for more detailed reviews, see e.g. Kleckner (1996), Roeder (1995) and Loidl (1994)).

In yeast, meiotic recombination is initiated, at the molecular level, by a DNA double-strand break (DSB). The 5'-termini are then quickly recessed, leaving 3' single-stranded tails. These tails invade a non-sister duplex of the homolog, generating heteroduplex DNA (Nag *et al.*, 1995). The intermediate thus formed is a double Holliday junction which is resolved to either a crossover or a non-crossover at the end of pachytene (Storlazzi *et al.*, 1995).

At the cytological level, an early alignment of homologs is observed. Alignment and subsequent synapsis of homologs is proposed to be guided by weak paranemic DNA-DNA interactions which become increasingly stable when homology is encountered. Concomitantly, the axial elements are formed. At a number of interstitial positions these approach one another till a distance of 200-300 nm (Albini and Jones, 1987). At these sites, called 'association sites' electron-dense particles, called nodules, are often seen. Nodules are believed to be involved in homology search at early stages of meiotic prophase (early nodules; Carpenter (1994)) and in the recombination process at later stages (late nodules; Zickler *et al.*, 1992, Carpenter, 1994, Sherman and Stack, 1995). Association sites are therefore proposed to be involved in homology search and/or the early steps in recombination (Rockmill *et al.*, 1995). After association sites have been formed, the axial elements of homologs approach one another more closely, up till 100 nm, and are connected along their length by the tripartite structure (Loidl, 1994). When the SC disassembles (at diplotene) homologs remain connected at positions where a crossover has occurred. These are the so-called chiasmata, which persist until homologs are separated at anaphase I.

Timing of the events observed at the molecular and cytological level has been extensively analysed in yeast (Table 1). Multiple interstitial interactions between homologs are already present before pre-meiotic S-phase, and lost during DNA replication (Weiner and Kleckner, 1994). Interhomolog interactions reappear prior to or concomitant with the formation of DSBs, at a time when association sites become visible. The formation of DSBs precedes the formation of tripartite SC (Padmore *et al.*, 1991), which is complete at the time that double Holliday junctions are observed (Schwacha and Kleckner, 1995). At the DNA-level, mature recombinants appear at late pachytene (Goyon and Lichten, 1993), when the SC starts to dissassemble.

An attractive model places a number of these events in a single progressive pathway (Kleckner, 1996). First, multiple interstitial interactions are promoted at selected positions along each chromosome pair, and these positions are later used for the initiation of recombination. These selected sites are organized at the bases of the chromatin loops, i.e. on the axial elements. Initiated recombination (DSBs) might trigger SC formation. Processed DSBs give rise to both crossover and non-crossover recombinants (Storlazzi *et al.*, 1995) which appear when the SC disassembles.

Table 1: Timing of events during meiotic prophase (adapted from Kleckner, 1996 and Padmore *et al.*, 1991).

	Pre-Lept.	Leptotene	Zygotene	Pachytene	Diplotene
'DNA'	pairing	DSBs		DHJs rec	
EM/LM		AEs	SC-form.	trip.SC	SC disass.

'DNA': events at the molecular level, EM/LM: events at the cytological level (electron and light microscopy), AEs: axial elements, DHJs: double Holliday junctions, rec: recombinants, trip.: tripartite.

Possible functions of the SC

The function of the SC is still unclear. Initially, SCs were considered necessary for pairing of homologous chromosomes and to provide the correct framework for recombination to occur. However, several recent findings argue against such a function. Pairing was shown to precede SC formation (Weiner and Kleckner, 1994) and even to take place to some extent in mutants that are defective in SC assembly (Rockmill and Roeder, 1990; Loidl *et al.*, 1994; Nag *et al.*, 1995). Furthermore, recombination is initiated before SC assembly (Table 1) and recombination can take place in the absence of tripartite SCs as exemplified by the fission yeast *Schizosaccharomyces pombe* and the fungus *Aspergillus nidulans* (Kohli and Bähler, 1994; Egel-Mitani *et al.*, 1982), two organisms that do not assemble SCs. When the function of the SC is considered, I would like to make a distinction between the lateral/axial elements and the central region (the transverse filaments and the central element). Axial elements are present throughout most of meiotic prophase. Together with their presence at the bases of the chromatin loops this points to a structural function for axial elements that is necessary during the greater part of meiotic prophase. A complete central region, however, is only present during pachytene, which might

suggest a function at a specific stage of meiotic prophase, presumably in the meiotic recombination process. The central region is believed to be involved in the regulation of the number and distribution of crossing-overs during the recombination process ensuring that at least one crossing-over occurs per bivalent (for a more detailed discussion see Meuwissen, 1997). This regulation would manifest itself as crossover interference, i.e. the presence of a crossover reduces the probability of a second crossover nearby (Mortimer and Fogel, 1974).
In this thesis, I will focus on the function of the lateral elements and some of its components.

Possible functions of the lateral elements of SCs

Several meiosis-specific functions have been suggested for SCs and their components. I will now discuss those that could be fulfilled by the LEs or (some of) their components. These include (1) the structural organization of the chromatin of sister chromatids such that recombination between non-sister chromatids of homologs is promoted, and (2) the establishment of sister chromatid cohesiveness.

Structural organization of sister chromatid chromatin such that recombination between non-sister chromatids of homologs is promoted

▪ *Does organization of sister chromatid chromatin on a single axis promote non-sister interactions?*
During mitotic prophase, replicated sister chromatids are closely associated along their entire length, and apparently form a single long cylinder, which can be visualized under special experimental conditions (Giménez-Abián *et al.*, 1995). As mitosis proceeds, this cylinder splits into two thin cylinders that remain closely apposed (Sumner, 1991; Giménez-Abián *et al.*, 1995). During meiotic prophase the sister chromatids appear to be organized in a similar fashion: initially they cannot be distinguished from one another, and develop a single axial element. At diakinesis, individual sister chromatids become visible as each sister chromatid is organized on its own scaffold. Both mitotic and meiotic chromatin are organized in loops that are attached to an axial structure (Paulson and Laemmli, 1977; Moens and Pearlman, 1988; Weith and Traut, 1988). Special DNA sequences called 'matrix attachment regions' (MARs), are bound to the nuclear matrix in interphase and many of these (called *s*caffold *a*ttachment *r*egions (SARs)) are probably also bound to the scaffold of mitotic chromosomes (Saitoh and Laemmli, 1994). A similar

organization might be expected for meiotic chromatin.
During mitosis recombination takes place preferentially between sister chromatids (Kadyk and Hartwell, 1992), whereas during meiosis recombination between non-sisters is predominant (Roeder and Stewart, 1988; Petes and Pukkila, 1995). The preferences observed could be the result of a different structural organization of the chromatin of sister chromatids during mitosis and meiosis. One difference between chromatin organization in mitosis and meiosis is that in meiotic prophase, the chromatin of sister chromatids lies at the same side of the shared axial element, whereas during mitotic prophase, each of the scaffolds is surrounded by the loops of one chromatid. A second difference is that in mitotic metaphase sister chromatids are coiled with opposite handedness, in contrast to meiotic sisters which are coiled with the same handedness (Boy de la Tour and Laemmli, 1988). Coiling with opposite handedness would allow a close approach of homologous sequences whereas similar handedness would prevent close association. Perhaps coiling with opposite handedness promotes homologous interactions between sisters during mitosis. The same could then be true for non-sister chromatids in meiosis which are also coiled with opposite handedness (Kleckner, 1996). Thus, modification of the structural, mitosis-like organization of the chromatin of sister chromatids during meiotic prophase might promote recombinational interactions between non-sister chromatids of homologous chromosomes. It is possible that components of the lateral elements are involved in such a meiosis-specific modification of the chromatin organization of sister chromatids.

▪ *Are non-sister interactions promoted by bringing DSB-containing sites in the context of the SC?*
In yeast, pairing of homologs involves multiple interstitial interactions (Weiner and Kleckner, 1994). The number of these interactions is similar to the number of meiotic recombinational interactions. The positions where DSBs are formed during meiotic prophase, are accessible to nucleases in mitotic cells (Wu and Lichten, 1994). Interhomolog interactions probably precede the formation of DSBs at the same site; a point mutation in a hotspot for meiotic recombination not only influences DSB formation *in cis*, but also *in trans* at the corresponding site on the homolog (Xu and Kleckner, 1995). On the basis of these observations a model was proposed in which multiple accessible sites in the chromatin are selected for pairing interactions. Subsequently, pairing results in the organization of such sites, in both homologs, at the bases of the chromatin loops. These bases then become incorporated in the axial elements. A subset of these sites is used for the formation of DSBs, which are then automatically positioned between the homologs; this would facilitate non-sister interactions (Xu and Kleckner, 1995). The resulting recombinational non-sister interactions might correspond to the cytologically observable association

sites seen in *Allium* (Albini and Jones, 1987) and in the yeast *zip1* mutant (Sym *et al.*, 1993).

This model was refined by Kleckner (1996) who suggested that sites having the potential for interhomolog (pairing) interactions tend to become located at the bases of the chromatin loops irrespective of the presence or absence of a homolog by a mechanism that is similar to the organization of mitotic chromatin which facilitates intersister interactions. Meiosis-specific factors, including components of the axial elements, then modify this basic organization so that non-sister interactions are preferred (as discussed earlier).

According to a slightly different model, the mitotic scaffold is structurally related to the meiotic axial element. Thus, according to this model SARs are associated with the axial elements. DSBs, which in this model can occur anywhere in the genome, are brought into the context of the axial element through interaction of specific proteins with DSB-sites and SARs or SAR-associated proteins. The recombination machinery then assembles with the DSBs at the SARs, like has been proposed for DNA-repair (Mullenders *et al.*, 1988; Figure 3). Bringing recombination intermediates in the context of the SC might be a function of axial element components. The yeast Red1 protein has been proposed to be involved in a such a function (Rockmill and Roeder, 1990). An important difference between this model and that proposed by Kleckner (1996) is that DSBs are not necessarily formed at sites with a potential for interhomolog interactions, but can be formed at any site within the genome.

▪ *Does selection of one chromatid of each pair of sisters to be active in recombinational interactions explain the preferential non-sister recombination during meiosis?*

An important question concerning meiotic recombination is how the cell prevents involvement of the same sites on the two sister chromatids in the recombination process at the same time. Schwacha and Kleckner (1994) proposed that one sister chromatid of each pair is selected to be active in recombinational interactions. Such a selection could take place during or after DNA replication (Petes and Pukkila, 1996). Binding of proteins (sequence-specific or nucleosomal) to one of the sisters might provide a mark, that allows it to be active in recombinational interactions. However, selection of one of the two sister chromatids for recombination cannot extend over long regions of the chromosome, because then four-strand double crossover events would be rare, which is not the case (Hawthorne and Mortimer, 1960; Fogel and Hurst, 1967).

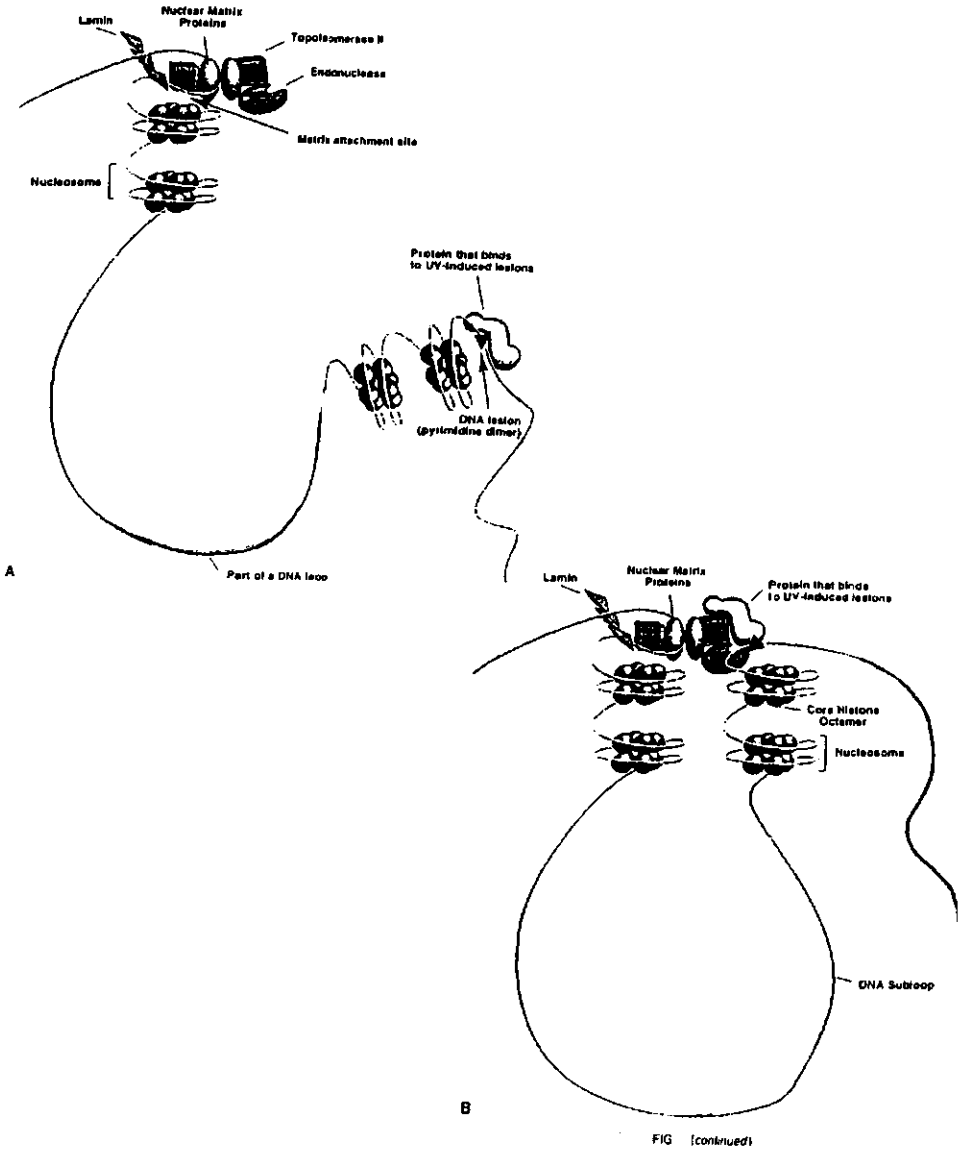

FIG (continued)

Figure 3: Schematic diagram showing how a DNA-lesion is recognized by a protein (A), which binds at/near the lesion and anchors the damaged DNA region to the nuclear matrix, where factors involved in DNA-repair can bind and repair takes place (from Mullenders *et al.*, 1988).

The establishment of sister chromatid cohesiveness

An important difference between the mitotic and the first meiotic division is that during the latter type of division sister chromatids move together towards the same pole, whereas during mitosis sister chromatids segregate to opposite poles. This means that whereas cohesion between sister chromatids is lost during anaphase of the mitotic division, cohesion must persist during meiosis to delay sister chromatid segregation until anaphase II. Sister chromatid cohesiveness is probably also necessary for the proper orientation of bivalents in the metaphase I spindle. Bipolar tension is essential for the stable attachment of kinetochores to opposite poles. This requires physical linkage of the homologs. Such linkage is provided by chiasmata, which are a prerequisite for proper disjunction at anaphase I (reviewed by Carpenter, 1994). However, chiasmata as such are not sufficient to resist the tension exerted on bivalents if the recombined chromatids do not cohere, but can slide along each other. Chiasmata must therefore be stabilized until the metaphase I/anaphase I transition (Bickel and Orr-Weaver, 1996). Such a stabilization of chiasmata, or chiasma maintenance, can be accomplished by cohesiveness of sister chromatids distal to the chiasmata or by the presence of proteins at or near the chiasma (the so-called 'chiasma binder' (Maguire, 1974; Carpenter, 1994)). However, the behavior of acentric fragments in maize, formed by crossovers between chromosomes heterozygous for a paracentric inversion could not be explained by the chiasma binder theory (Maguire, 1993). Moreover, no mutant has been characterized, as yet, that could have a defective chiasma binder (Carpenter, 1994). Therefore, it seems more likely that chiasmata are maintained by sister chromatid cohesiveness at least distal to the chiasmata. This also means that cohesion between sister chromatids during meiosis must be different along chromosome arms and at the centromere, because arm cohesion must be relieved at anaphase I, whereas cohesion at the centromere must persist until anaphase II (Bickel and Orr-Weaver, 1996). The mechanism underlying sister chromatid cohesiveness is still unknown (reviewed by e.g. Maguire, 1990, 1993 and 1995; Carpenter, 1994; Bickel and Orr-Weaver, 1996). A number of proposed mechanisms will be discussed below.

▪ *Can linkage proteins provide for sister chromatid cohesiveness?*

Koshland and Hartwell (1987) proposed that sister chromatids are held together by proteins which act as a glue. Although the SC is disassembled at diplotene and sister chromatid cohesiveness must last until anaphase I, it has been suggested that SC components or remnants of SCs might persist to maintain cohesiveness. In grasshoppers, structures that resemble SC remnants were detected between sister chromatids at metaphase I (Moens and Church, 1979). It has been proposed that Dmc1, a protein which in

early meiotic prophase is found in foci which colocalize with Zip1 (a putative SC-component) is required for sister chromatid cohesiveness (Rockmill and Roeder, 1994). A strain with a *dmc1* mutation forms apparently normal SC, but displays missegregation at meiosis I. Nondisjunction was the result of both reduced crossing-over and precocious sister chromatid separation. It was therefore suggested that the dmc1 mutation leads to an alteration of the chromatin structure, which affects both crossing-over and sister chromatid cohesiveness (Rockmill and Roeder, 1994). *Red1* mutants of yeast show considerable nondisjunction at meiosis I, which is correlated with reduced crossing-over but also by the inability to form functional chiasmata (Rockmill and Roeder, 1990). The Red1 protein, which is probably required for axial element formation, was proposed to play a role in the organization of the chromatin (see above) that is necessary for both crossover formation and sister chromatid cohesiveness.

In the fission yeast, *Schizosaccharomyces pombe*, no SCs are formed during meiosis, but so-called linear elements, which probably correspond to axial elements of SCs (Bähler *et al.*, 1993). The *rec8* mutant of *S. pombe* forms aberrant linear elements, has a defect in meiotic recombination, and shows precocious sister chromatid separation at meiosis I. It is possible that the *rec8*-mutation primarily affects an essential protein for linear element formation, and that the defects in recombination and sister chromatid cohesiveness are secondary effects of the mutation. Alternatively, the *rec8*-mutation primarily affects a recombination protein, whereas the defects in linear element formation and sister chromatid cohesiveness are secondary effects; in that case recombination would be required for sister chromatid cohesiveness, with linear element formation perhaps linking these two processes.

The hamster protein homologous to the rat M_r 30,000-33,000 LE components, COR1, (Dobson *et al.*, 1994) is localized along chromosome arms until metaphase I and at the centromeres until metaphase II (Dobson *et al.*, 1994; Moens and Spyropoulos, 1995). Dobson *et al.* suggested that the COR1 protein anchors the chromatin of the two sister chromatids to their common , single axial core. This would provide sister chromatid cohesiveness, and would thus stabilize chiasmata. Additionally, they proposed that COR1 inhibits the separation of the sister centromeres at anaphaseI. The dissociation of COR1 from the sister centromeres at anaphase II would then allow their separation. Analysis of the desynaptic (*dy*) mutant of maize also suggested that SC remnants or SC components are involved in sister chromatid cohesiveness. In this mutant synapsis and crossing-over seem to be normal, but chiasmata seem to be sporadically lost, which results in aberrant segregation at meiosis I (Maguire *et al.*, 1991 and 1993). At late pachytene to early diplotene the central region appears wider and disintegrates earlier than the lateral elements in the *dy*-mutant than in wildtype.

Several other proteins that are not or have not been shown to be SC components have also been suggested to play a role in sister chromatid cohesiveness. Two of these were identified in *Drosophila*. In *Drosophila*, meiosis proceeds through different pathways in males and females. Whereas in females crossing-overs, SCs and chiasmata are formed, these are missing in males. It was therefore surprising that mutations were identified that affected segregation in meiotic divisions in both sexes (Sekelsky and Hawley, 1995). The first, the *mei-S332* mutation, results in the premature separation of sister chromatids late in anaphase I. The MEI-S332-GFP (Green Fluorescent Protein) fusion protein localizes at the centromeric region of all chromosomes from prophase I up to anaphase II (Kerrebrock *et al.*, 1995). The MEI-S332 protein is thus an essential component that holds sister chromatid centromeres together until anaphase II. The second mutation, *ord*, results in reduced crossing-over in females and precocious sister chromatid separation before anaphase I. Precocious sister separation was not to entirely due to the failure to form crossovers (Mason, 1976). The *ord* mutation is not rescued by MEI-S332-mediated sister centromere cohesiveness and it is therefore possible that the ORD protein is necessary for cohesion along chromosome arms until anaphase I (Sekelsky and Hawley, 1995). Analysis of different mutant alleles of the *ORD* gene suggests that its role in sister chromatid cohesiveness requires interaction with other proteins, which could include SC proteins in females (Bickel *et al.*, 1996).

A mutant of the fungus *Sordaria macrospora*, *spo76*, has perhaps the most clear phenotype that is expected for a defect in sister chromatid cohesiveness (Moreau *et al.*, 1985). This mutant shows a block in meiosis after the first division, incomplete pairing and early centromere cleavage. Moreover, only segmented lateral elements are formed which are split into two smaller elements. This probably reflects the failure to form a single axis for both sister chromatids. Finally, in mutants heterozygous for the *spo76* mutation crossing-over was markedly decreased. An interesting observation is that on Western blots of total ascus protein from wildtype *Sordaria* several proteins, among which two with relative electrophoretic mobilities of about 30,000 and 33,000 are are recognized by antibodies elicited against isolated rat SCs. These proteins are not detected on similar blots containing protein from the *spo76* mutant (D. Zickler, personal communication). Importantly, the *spo76* mutant also exhibits increased sensitivity to UV-radiation. The product of the *SPO76* gene is thus not specific for meiosis.

▪ *Is sister chromatid cohesiveness promoted by sister chromatid exchange (SCE)?*
Sears *et al.* (1994) proposed the involvement of sister chromatid exchange (SCE) in sister chromatid cohesiveness. They observed a decrease in precocious sister chromatid separation after introduction of a yeast hot spot for meiotic recombination on a human DNA-derived YAC. This decrease could not be accounted for by the increase of reciprocal recombination only. Moreover, this effect was also observed when the hot spot was present on only one YAC (so that crossing-over events did not occur in the introduced region). It was therefore suggested that the formation of DSBs also leads to SCE, though probably at low levels due to an inhibiting effect of the SC. A relationship *in cis* between the formation of DSBs and sister chromatid cohesiveness was also found in the analysis of the effect of a 31 bp mutation in a region of centromeric yeast DNA (Sears *et al.*, 1995). Recombination was reduced and precocious sister chromatid separation was increased irrespective of the presence of a homolog carrying the same mutation. Sears *et al.* suggested that formation of DSBs leads to interhomolog recombination and also to SCE. The balance between these two types of processes was proposed to be regulated by components of the SC. In this scenario, interhomolog recombination is favoured during the early stages of meiotic prophase whereas at later stages (after pachytene) SCE might be required for sister chromatid cohesiveness. Components of the lateral elements can be involved in such a mechanism by influencing the organization of the chromatin as described before. If SCEs are involved in sister chromatid cohesiveness, they must be stabilized in some way, like has been suggested for chiasmata.

▪ *Is sister chromatid cohesiveness the result of catenation of DNA strands?*
The replication of chromosomes is accompanied by an activity of topoisomerases that disentangles sister chromatids, which would otherwise be wound around one another thousands of times. It appears, however, that topoisomerases are excluded from regions where replication forks meet, leaving multiple intertwinings (Figure 4). Murray and Szostak (1985) proposed that these residual intertwinings maintain the connection between sister chromatids until anaphase I. Before separation of homologs can take place these entanglements must be removed, probably with the help of topoisomerase II (topo II). Topo II is necessary for the segregation of recombined chromosomes in yeast meiosis I (Rose *et al.*, 1990). The protein is associated with SCs, though not exclusively (Moens and Earnshaw, 1989; Klein *et al.*, 1992). Topo II seems to congregate on the lateral elements from late pachytene and remains associated with these when they separate at diplotene. The untangling activity of topo II is reversible and seems to be driven to completion by chromosome condensation and chromosome movement (Holm, 1994).

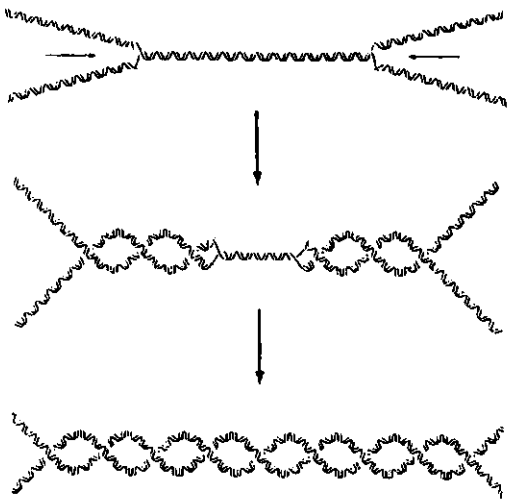

Figure 4: Schematic representation of DNA replication. When two replication forks approach one another, proteins required for resolution of intertwinings may be sterically excluded in the region between the two replication forks. This would result in a segment containing intertwined sister chromatids (from Holm, 1994).

If multiple intertwinings of sister chromatids contribute to sister chromatid cohesiveness one expectation is that the action of topo II is inhibited until metaphase I or anaphase I since chromosomes condensation continues after the disassembly of SCs. This would probably drive topo II action towards disentanglement before homologs are separated at anaphase I. Components of the SC that persist after SC disassembly might be involved in this inhibitory action. Another view is that entanglement of sister chromatids serves the cohesiveness of sisters only until the SC disassembles and chromatin condensation takes place. In the presence of the SC, maintenance of intertwinings of sister chromatids may then be either an effect of inhibition of topo II action by factors such as SC components or of the relatively decondensed state of the chromatin that is maintained in the presence of SCs. Chromatin condensation and/or SC disassembly might then allow the action of topo II, which would result in the separation of sister chromatids at anaphase I. The activity of topo II is necessary for the separation of chromatid cores during prometaphase of mitosis (Giménez-Abián *et al.*, 1995). Interestingly, replication forks (where topoisomerases are thought to be excluded after DNA replication) often cohabit with S/MARs (Boulikas, 1995), sequences that are expected to be present in the lateral elements. During the stages between SC disassembly and homolog segregation other processes must provide sister chromatid cohesiveness.

▪ *Is sister chromatid cohesiveness provided by small, unreplicated regions?*
Stern and Hotta (1987, review) found small regions in the genome of lily that are not replicated at premeiotic S-phase. Between 0.1 and 0.2% of the genome, designated zygDNA, was not replicated until zygotene. The ends of the zygDNA segments are not replicated until after pachytene. Stern and Hotta proposed that these segments maintain sister chromatid cohesiveness until anaphase I. Although it is doubtful whether the hydrogen-bonding within the small, unreplicated segments is sufficient to keep sister chromatids tightly linked, it is conceivable that such intersister connections are stabilized, e.g. by proteins. These proteins might include components of the lateral elements of SCs. The replication of zygDNA takes place predominantly along the lateral elements (Kurata and Ito, 1978), and therefore the supposed unreplicated segments are probably localized within the SCs.
These experiments have been in debate until now, because other investigators have not been able to reproduce the findings of Stern and Hotta.

To summarize: several functions have been proposed for the lateral elements of SCs and their components. What all of the proposed functions have in common is that they are structural. Lateral elements are either involved in the organization of sister chromatid chromatin, which promotes the high rate of recombination between non-sister chromatids which is specific for meiosis or in the establishment of cohesiveness between sister chromatids after recombination has occured to assure proper segregation of recombined chromosomes. Although I have discussed these two types of function separately, a combination of the two is also possible.

Aim and outline of this thesis

As outlined above, several possible functions have been suggested for LEs of SCs and for (some of) their components. The aim of the investigations presented in this thesis was the characterization of major protein components of the LEs of rat SCs with relative electrophoretic mobilities of 30,000 and 33,000, in order to define their function.
Chapter 2 describes the isolation of a cDNA encoding these proteins, their predicted features and the relationship between the M_r 30,000 and 33,000 components. Chapter 3 shows that these proteins are phosphorylated *in vivo*. Chapters 4 and 5 describe the DNA-binding characteristics of the proteins *in vivo* and *in vitro*. In Chapter 6, the results of these studies will be discussed in the context of the possible functions of the LEs of SCs that have been discussed in this chapter.

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Chapter 2

The gene encoding a major component of the lateral elements of synaptonemal complexes of the rat is related to X-linked lymphocyte-regulated genes

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The Gene Encoding a Major Component of the Lateral Elements of Synaptonemal Complexes of the Rat Is Related to X-Linked Lymphocyte-Regulated Genes

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The lateral elements of synaptonemal complexes (SCs) of the rat contain major components with relative electrophoretic mobilities (M_rs) of 30,000 and 33,000. After one-dimensional separation of SC proteins on polyacrylamide-sodium dodecyl sulfate gels, these components show up as two broad bands. These bands contain closely related proteins, as judged from their peptide maps and immunological reactivity. Using affinity-purified polyclonal anti-30,000- and anti-33,000-M_r component antibodies, we isolated a cDNA encoding at least one of the 30,000- or 33,000-M_r SC components. The protein predicted from the nucleotide sequence of the cDNA, called SCP3 (for synaptonemal complex protein 3), has a molecular mass of 29.7 kDa and a pI value of 9.4. It has a potential nucleotide binding site and contains stretches that are predicted to be capable of forming coiled-coil structures. In the male rat, the gene encoding SCP3 is transcribed exclusively in the testis. SCP3 has significant amino acid similarity to the pM1 protein, which is one of the predicted products of an X-linked lymphocyte-regulated gene family of the mouse: there are 63% amino acid sequence similarity and 35% amino acid identity between the SCP3 and pM1 proteins. However, SCP3 differs from pM1 in several respects, and whether the proteins fulfill related functions is still an open question.

Synaptonemal complexes (SCs) are nuclear structures that are formed between homologous chromosomes during prophase of the first meiotic division. They are assumed to contribute to the two major effects of meiosis, the reduction of the ploidy level and the generation of new combinations of genes. These effects are accomplished during the first of two meiotic divisions as homologous chromosomes condense, pair, recombine, and segregate. SCs consist of two proteinaceous axes, one along each homolog, that are connected by transverse filaments. On the transverse filaments, between the axial cores, there is a third longitudinal element, the central element; both axial cores together with the central element make up the tripartite structure of the SC (65). The axial cores are called the lateral elements (LEs) of SCs where they make part of the tripartite SC.

The assembly and disassembly of SCs correlate with the successive rearrangements of chromatin. Early in meiotic prophase (leptotene), the axial cores are formed along the chromosomes, presumably between the sister chromatids; during zygotene, they are connected by the transverse filaments, and a central element is formed between them. In pachytene, the homologous chromosomes are connected (synapsed) by the tripartite structure along their entire length. Subsequently, the SCs are disassembled (diplotene), the chromosomes condense (diakinesis), and chiasmata, which result from the physical exchange between non-sister chromatids of homologs (67), show up as physical connections between homologs. In metaphase I, bivalents (paired homologs) orient themselves in the spindle. The chiasmata are essential for this because they hold the bivalents together. There is circumstantial evidence that exchange between non-sister chromatids per se is not sufficient to generate a mature chiasma that can fulfill this function (46, 53). For the proper orientation of bivalents, sister chromatid cohesiveness, which persists until anaphase I, is also essential (24, 30, 41).

At the DNA level, the major events of meiotic prophase result in a high rate of homologous recombination and gene conversion. Meiotic and mitotic recombination differ in several respects. (i) The rate of meiotic reciprocal exchange (and of gene conversion) is several orders of magnitude higher than that of mitotic exchange. (ii) With few exceptions (62), there is positive interference between meiotic reciprocal exchanges (reviewed in references 50 and 67), whereas no evidence has been obtained for interference between mitotic reciprocal exchanges (32). (iii) In several organisms, hot spots for meiotic reciprocal recombination (and gene conversion) have been identified (6, 18, 22, 57), but most of these are not hot spots for mitotic recombination (reviewed in reference 50). (iv) Meiotic reciprocal recombination occurs preferentially between non-sister chromatids of homologous chromosomes (19, 64), whereas the sister chromatid is preferred for mitotic exchange (31). Thus, there is a meiosis-specific chromatin organization by which some sequences (hot spots) are preferentially exposed for homology searching and recombination, positive interference between reciprocal exchanges is effected, reciprocal exchanges between sister chromatids are avoided (or exchanges between non-sister chromatids are preferentially enhanced), sister chromatid cohesiveness is brought about, and reciprocal exchanges between non-sister chromatids result in the formation of functional chiasmata.

It seems likely that SCs, particularly the LEs, play a role in one or more of these aspects of meiosis-specific chromatin

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organization, because SCs are assembled from meiosis-specific components (25, 44, 48, 60, 63) and because the meiotic prophase chromatin appears to be organized on the LEs and axial cores. However, as yet, no direct evidence has been obtained for this.

In order to get more information about SCs, we developed a procedure to isolate these structures from rat spermatocytes (26, 28), elicited monoclonal and polyclonal anti-SC antibodies (27, 29, 48), and, using these antibodies, identified major components of SCs with M_rs of 30,000, 33,000, 125,000, and 190,000 (27, 29). In this paper, we concentrate on the 30,000- and 33,000-M_r SC components, which occur specifically in meiotic prophase cells, on the SCs (15, 25); they are localized on the LEs and axial cores, irrespective of whether these are synapsed or unsynapsed (29, 47). We describe the isolation of a cDNA encoding at least one of the 30,000- or 33,000-M_r proteins by means of affinity-purified anti-30,000 and anti-33,000-M_r protein antibodies. The protein predicted from the nucleotide sequence, called SCP3 (for synaptonemal complex protein 3), is basic (pI 9.4) and has a molecular mass of 29.7 kDa; it has a potential nucleotide binding site. SCP3 has sequence homology to an X-linked lymphocyte-regulated mouse protein called pM1 (20).

MATERIALS AND METHODS

Antibodies. The monoclonal antibodies (MAbs) used in this study were elicited and isolated as described by Offenberg et al. (48); they are described in detail by Heyting et al. (27) and Offenberg et al. (48). A polyclonal anti-30,000- and anti-33,000-M_r antiserum (serum 175) was elicited by immunization of a rabbit with rat SCs according to the same schedule used for the immunization of mice (48). Serum samples (20 ml each) were collected at 2-week intervals, starting 1 week after the third injection of antigen. Although this serum was elicited against whole rat SCs, it recognizes specifically the 30,000- and 33,000-M_r SC components (48). For the experiments in this study, we affinity purified the anti-30,000- and anti-33,000-M_r antibodies from this serum as follows: from preparative immunoblots of SC proteins, we excised strips containing the 30,000- and 33,000-M_r SC components; these strips were incubated in blocking buffer (10 mM Tris-HCl [pH 7.4], 250 mM NaCl, 0.05% [wt/vol] Tween 20, containing 5% [wt/vol] nonfat dry milk and 5% [vol/vol] normal goat serum) for 1 h at 37°C, washed three times for 5 min each in phosphate-buffered saline, and then incubated for 1 h at 37°C in the polyclonal anti-30,000- and anti-33,000-M_r antiserum, diluted 1:100 in blocking buffer supplemented with 0.02% (wt/vol) NaN_3, *Escherichia coli* lysate (1 mg of *E. coli* protein per ml), and 1 mM phenylmethylsulfonyl fluoride. Bound antibodies were eluted from the strips by two successive incubations for 1 h each at room temperature in 10 ml of 0.1 M glycine (pH 2.7). The glycine in the eluted fractions was neutralized with an equimolar amount of Na_2HPO_4, and normal goat serum was added to a final concentration of 10% (vol/vol). Only the second eluted fraction was used. A polyclonal antiserum against the fusion protein of clone 2A4.7 (serum 448) was raised by immunization of a rabbit with inclusion bodies from bacteria harboring clone 2A4.7, containing the fusion protein, exactly as described by Meuwissen et al. (44).

Isolation of cDNAs encoding SCP3. For the isolation of cDNAs encoding 30,000- and 33,000-M_r SC proteins, we subcloned the inserts of about 10^8 phage of an expression cDNA library of rat testis (44) into the pBluescript vector according to the instructions of the manufacturer (Stratagene, San Diego, Calif.). Colonies of *E. coli* XL1 Blue cells carrying these pBluescript vectors with inserts were transferred to nitrocellulose filters. Two replica filters were made, and expression of the cloned cDNA was induced as described by Sambrook et al. (55). Subsequently, the colonies were lysed on the filters by the following incubations at room temperature: 20 min in 5 mg of lysozyme per ml in 50 mM Tris-HCl (pH 7.6)–150 mM NaCl–0.1% Tween 20 (TBST), 1 min in 0.5 M NaOH–1.5 M NaCl, and 5 min in 1 M Tris-HCl (pH 7.4)–1.5 M NaCl. Filters were then washed in TBST, and bacterial debris was wiped off. We screened the lysed colonies by means of the affinity-purified anti-30,000- and anti-33,000-M_r SC protein antibodies as primary antibodies and a goat anti-mouse alkaline phosphatase conjugate as secondary antibody by using the Western blot (immunoblot) incubation procedure described before (16, 25). We performed a secondary screening of the cDNA library by plaque hybridization with the *Hin*cII fragment of clone 2A4 (see Fig. 3) as a probe. Labelling of the probe by random primed labelling and screening were performed according to procedures described by Sambrook et al. (55).

Sequence analysis. We generated unidirectional sets of deletions from both ends of the cDNA insert of clone 2A4 by partial digestion with exonuclease III and S1 nuclease with an Erase-a-base Kit (Promega, Madison, Wis.). In addition, fragments 2, 5, 6, 7, and 9 (see Fig. 3) were subcloned in pBluescript SK^- for nucleotide sequence determination. We determined the nucleotide sequences by the dideoxy-chain termination method of Sanger et al. (56) with the double-stranded DNA Cycle Sequencing System (GIBCO BRL, Gaithersburg, Md.), [γ-^{32}P]ATP (>5,000 Ci/mmol [Amersham]), and oligonucleotide primers complementary to the polylinker sequences of pBluescript. The sequence was assembled with the University of Wisconsin Genetics Computer Group sequence analysis package. Sequence similarity searches of the GenBank, EMBL, Swissprot and PIR data banks were carried out with the FASTA (49) and BLAST (3) programs. Prediction of secondary structure was performed with a program based on Chou-Fasman algorithms (10); coiled-coil regions were identified with an algorithm developed by Lupas et al. (40). Alignment of sequences was performed with the BESTFIT and GAP programs, which are included in the Genetics Computer Group sequence analysis package.

RNA isolation and Northern blot hybridization. RNA was isolated from various tissues of 37-day-old rats by the guanidinium-LiCl method of Cathala et al. (7). RNA was electrophoresed on formaldehyde-agarose gels and transferred to Hybond-N^+ membranes (Amersham) by standard procedures (55). After transfer, the membranes were washed in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), dried, and fixed by baking at 80°C for 2 h. We used five DNA probes (see Fig. 3) for Northern blot (RNA) hybridization. Labelling of the probes with [α-^{32}P]dATP was performed by random primed labelling according to the method of Sambrook et al. (55). The Northern blot membranes were prehybridized in 50% formamide–6× SSC–0.5% SDS–0.01% Na-pyrophosphate–5× Denhardt's solution–200 μg of denatured herring sperm DNA per ml for at least 6 h at 42°C. Hybridization was performed in the same mixture with 1.4 ng of probe (1.1×10^6 cpm/μg) per ml for 17 h at 42°C. The blots were washed subsequently for 30 min in, successively, 2× SSC–1% SDS at 42°C (twice) and 1×SSC–0.1% SDS and 0.1× SSC–0.1% SDS at 65°C.

Characterization of the protein encoded by the cDNA insert

of 2A4. The open reading frame of the insert of cDNA clone 2A4 had a shift of −2 with respect to the reading frame of the *Lac*I fragment to which it was fused. To characterize the protein encoded by the cDNA insert of clone 2A4, we put the insert in frame with respect to the *Lac*I fragment of the pBluescript vector as follows: we digested the vector containing the insert with *Xba*I and *Sma*I, both of which cut the polylinker sequence of the pBluescript vector between the *Lac*I fragment and the *Eco*RI site into which the cDNA was inserted; subsequently the *Xba*I site was filled by means of Klenow polymerase, and the blunt ends of the *Sma*I site and the filled *Xba*I site were ligated; this resulted in a deletion of 16 nucleotides and did not generate a stop codon. *E. coli* XL1-Blue cells were transformed with the resulting construct. The clone containing this construct was designated 2A4.7. A 10-ml culture of clone 2A4.7 was grown overnight at 37°C in Luria-Bertani medium containing 100 μg of ampicillin per ml. A total of 250 ml of prewarmed Luria-Bertani medium containing 100 μg of ampicillin per ml was inoculated with 2.5 ml of the overnight culture. After incubation at 37°C for 2 h, synthesis of the fusion protein was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After incubation for another 3 h at 37°C, the cells were harvested. The inclusion bodies were purified as described by Sambrook et al. (55) and washed according to the method of Harlow and Lane (23). A total of 2 mg of inclusion body protein was then loaded per 1-cm slot of a 7 to 18% linear gradient polyacrylamide–SDS gel; subsequently, electrophoresis, Western blotting, and immunodetection of translation products of the cDNA insert by means of the polyclonal anti-30,000- and anti-33,000-M_r antibodies were performed as described by Heyting et al. (25).

Peptide mapping. Peptide mapping of the 30,000- and 33,000-M_r SC proteins and of the full-length fusion protein was performed as described by Cleveland et al. (11). Subsequent blotting and immunodetection were performed as described by Heyting et al. (25).

Other procedures. SCs were isolated as described by Heyting et al. (28) and Heyting and Dietrich (26); SDS-polyacrylamide gel electrophoresis of proteins was performed according to the method of Laemmli (35) as described by Heyting et al. (28). Before electrophoresis, the protein samples were boiled in sample buffer (2 M urea, 5% SDS, 125 mM Tris-HCl [pH 6.8], 10% glycerol, 0.5 mM EDTA, 0.1 M β-mercaptoethanol) for 10 min; immunoblotting was carried out according to the method of Dunn (16) as described by Heyting and Dietrich (26).

Nucleotide sequence accession number. The EMBL accession number of the SCP3 cDNA is X75785.

RESULTS

The 30,000- and 33,000-M_r SC components are closely related. One-dimensional SDS-polyacrylamide gel electropherograms of proteins from purified rat SCs show two broad prominent bands with M_rs of 30,000 and 33,000 (28, 29 [Fig. 1A]). These bands contain protein components of SCs, because several MAbs that recognize these bands bind specifically to SCs (25, 27, 29, 48). The 30,000- and 33,000-M_r SC components are immunologically related, because 18 independently isolated anti-SC MAbs recognize both components (Fig. 1A [27]), whereas, as yet, no anti-SC MAbs that can discriminate between these components have been identified. The similarity of the 30,000 and 33,000-M_r SC components is also evident from their peptide maps.

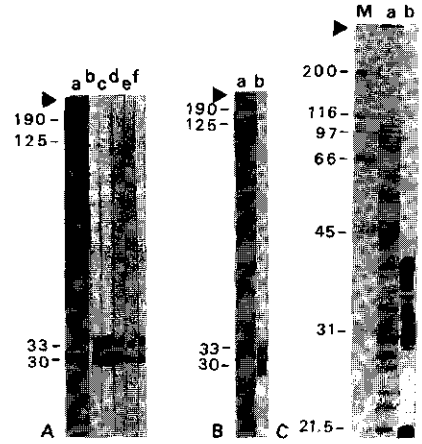

FIG. 1. Reaction of antibodies used in this study with SC proteins. Proteins of 2 × 10^7 SCs were loaded per cm slot of an SDS–10% polyacrylamide gel and stained with Coomassie blue or transferred to nitrocellulose. (A) Reaction of antibodies elicited against the 30,000- and 33,000-M_r components of SCs with SC proteins: Lanes: a, Coomassie blue-stained strip; b to f, immunoblot strips of the same gel incubated in MAb IX3H3 (b), MAb IX4D4 (c), MAb IX8G9 (d), MAb IX7B12 (e), and affinity-purified polyclonal anti-30,000- and anti-33,000-M_r antibodies from serum 175 (f). (B) Reaction of antibodies elicited against the fusion protein of cDNA clone 2A4.7 with SC proteins. Lanes: a, Coomassie blue-stained gel; b, immunoblot strip of the same gel probed with the antiserum elicited against the fusion protein of clone 2A4.7 (serum 448). In panels A and B, the positions of the 190,000-, 125,000-, 33,000-, and 30,000-M_r SC protein bands are indicated. (C) Immunoblot analysis of the translation products of cDNA clone 2A4.7; 2 mg of inclusion bodies of clone 2A4.7 was applied per cm slot of a 7 to 18% linear gradient polyacrylamide-SDS gel, electrophoresed, and stained with Coomassie blue (a) or blotted onto nitrocellulose and probed with affinity-purified anti-30,000- and anti-33,000-M_r antibodies from serum 175 (b). Lane M contains the following molecular mass markers: myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase B, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; and trypsin inhibitor, 21.5 kDa. The arrowheads indicate the tops of the gels.

Figure 2A shows the peptides that are produced from the 30,000- and 33,000-M_r protein bands by digestion with *Staphylococcus aureus* V8 protease (SV8) (11) and that are recognized by the antibodies directed against the 30,000- and 33,000-M_r SC components. Lanes a and e of Fig. 2A show the undigested input controls of the 30,000- and 33,000-M_r components. These lanes contain extra bands with M_rs of ~65,000 that are not detected on immunoblots of standard polyacrylamide-SDS gels (Fig. 1). We suppose that these extra bands contain dimers of 30,000- or 33,000-M_r molecules, because they originate from excised 30,000- or 33,000-M_r protein bands (see Materials and Methods) and because they are recognized by the anti-30,000- and anti-33,000-M_r antibodies. Apparently these dimers arise during the lengthy stacking gel phase of the peptide mapping procedure (11); it is possible that they are not easily formed from proteolytic cleavage products (Fig. 2A, lanes b to d and f to h).

Isolation of cDNAs encoding a 30,000- or 33,000-M_r component of SCs. For the isolation of cDNAs encoding 30,000-

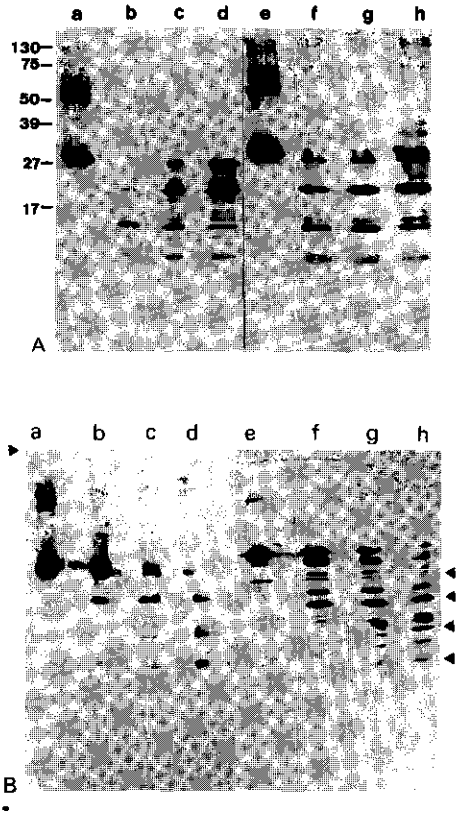

FIG. 2. Characterization of the 30,000- and 33,000-M_r SC components and of the translation products of cDNA clone 2A4.7 by peptide mapping. (A) Proteins of 10^8 SCs were loaded per cm slot of an SDS–10% polyacrylamide gel and electrophoresed. The 30,000- and 33,000-M_r protein bands were excised, digested with SV8, and electrophoresed according to the method of Cleveland (11) (see Materials and Methods); subsequently, the resulting peptides were transferred to nitrocellulose, and the immunoblot was probed with the polyclonal anti-30,000- and anti-33,000-M_r antibodies from serum 175. Lanes: a, undigested 30,000-M_r SC protein; b to d, peptides obtained by digestion of the 30,000-M_r protein with 1,050, 350, and 125 ng of SV8, respectively; e, undigested 33,000-M_r SC protein; f to h, peptides obtained by digestion of the 33,000-M_r SC protein with 1,050, 350, and 125 ng of SV8, respectively. The positions of the prestained marker proteins are indicated by their apparent molecular masses (in kilodaltons). (B) SV8 digests of the 33,000-M_r SC protein (lanes b to d) and the full-length fusion protein product of cDNA clone 2A4.7 (lanes f to h); the 33,000-M_r SC protein and the full-length fusion protein product of cDNA clone 2A4.7 were excised from one-dimensional SDS-polyacrylamide gels and digested with 125 (lanes b and f), 350 (lanes c and g), or 1,050 (lanes d and h) ng of SV8; lane a contains the undigested 33,000-M_r SC component, and lane e contains the undigested full-length fusion protein of cDNA clone 2A4.7. The undigested proteins and the digestion products were electrophoresed according to the method of Cleveland (11), transferred to nitrocellulose, and analyzed with the affinity-purified polyclonal anti-30,000- and anti-33,000-M_r antibodies from serum 175. Arrowheads to the left indicate the positions of the tops of the gels; arrowheads to the right indicate the bands that the digests of the 33,000-M_r SC component and the fusion protein of clone 2A4.7 have in common.

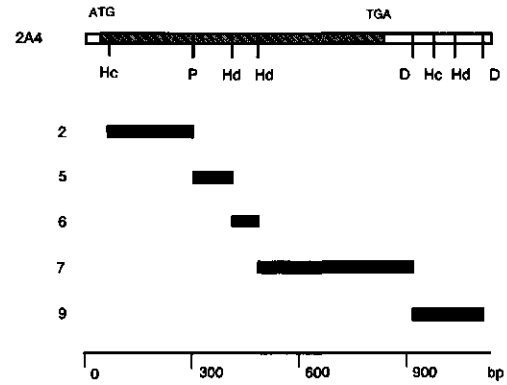

FIG. 3. Restriction map of cDNA clone 2A4. The shaded segment indicates the coding region. The black bars indicate the restriction fragments that were subcloned and used for Northern blot analysis and nucleotide sequence determination. Hc, *Hin*cII; P, *Pst*I; Hd, *Hin*dIII; D, *Dra*I.

and 33,000-M_r SC proteins, we screened an expression cDNA library of the rat testis with affinity-purified polyclonal anti-30,000- and anti-33,000-M_r antibodies. One colony, harboring clone 2A4, reacted relatively strongly with the antibodies. Figure 3 shows the restriction map of the cDNA insert of this clone. Using the *Hin*cII fragment of clone 2A4 as a probe, we screened about 2×10^5 recombinant phage of the cDNA library by plaque hybridization. This yielded 30 positive clones, all with cDNA inserts that were colinear with that of clone 2A4, as judged from their restriction maps. No clones extending further in the 5′ direction than clone 2A4 were found by this secondary screening.

The nucleotide sequence of clone 2A4 was determined as described in Materials and Methods (Fig. 4). Clone 2A4 contains a single open reading frame that encodes a 29.7-kDa protein consisting of 257 amino acids (counted from the first ATG codon) but which is out of frame with respect to the fragment of the *Lac*I gene of the pBluescript vector to which the cDNA insert was fused. The major translation product of clone 2A4 that is recognized by anti-30,000- and anti-33,000-M_r antibodies is a 19,000-M_r peptide (not shown). The synthesis of this peptide probably initiates at the ATG codon at positions 337 to 339, because this codon is preceded by an almost perfect Shine-Dalgarno consensus sequence (GGAGG, at positions 319 to 323). Only small amounts of longer translation products are detected in bacteria harboring clone 2A4 (not shown). However, if the insert of clone 2A4 is put in frame with the fragment of the *Lac*I gene (to produce clone 2A4.7 [see Materials and Methods]), the major translation product of the insert that is recognized by anti-30,000- and anti-33,000-M_r antibodies is a 37,000-M_r peptide (Fig. 1C). This is in good agreement with the expected size of the full-length translation product (4.2 kDa of the *Lac*I fragment plus 31.9 kDa from the cDNA insert). We think that the insert of clone 2A4 encodes at least one of the 30,000- and 33,000-M_r SC components for the following reasons. (i) The predicted molecular mass (29.7 kDa) is in good agreement with the M_rs (30,000 and 33,000) of the SC proteins that are recognized by the antibodies that were used for screening. (ii) The fusion protein encoded by the insert of

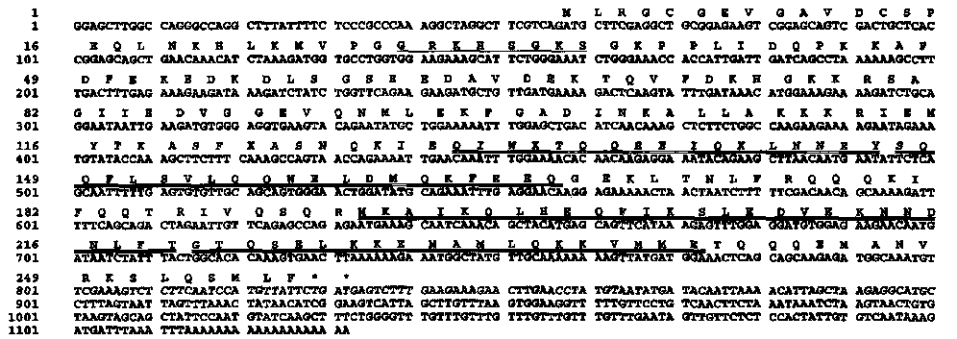

FIG. 4. Nucleotide sequence and predicted amino acid sequence of SCP3. The predicted translation product is shown above the nucleotide sequence. The purine nucleotide binding motif A is underlined. Double underlining indicates amino acid sequences that have a probability of >0.5 of forming coiled-coils, as calculated according to Lupas et al. (40) with a window of 14 amino acid residues. The asterisks indicate the first two stop codons.

clone 2A4 is recognized by at least four independently isolated anti-30,000- and anti-33,000-M_r MAbs that were not used for screening (not shown). (ii) The peptide maps of the supposed full-length fusion protein and of the 33,000-M_r SC component have four bands in common that are recognized by the polyclonal anti-30,000- and anti-33,000-M_r antibodies (Fig. 2B). The SV8 digests of the fusion protein also contain several peptides that do not occur in the digests of the 33,000-M_r SC component. These peptides probably consist of part of the *Lac*I fragment linked to part of the translation product of the cDNA. (iii) The antiserum elicited against the translation products of clone 2A4.7 (serum 448) recognizes specifically the 30,000- and 33,000-M_r SC components, like the antibodies that were used for screening (Fig. 1B), whereas the preimmune serum of serum 448 does not produce any signal (not shown). (iv) In frozen sections of the testis, serum 448 reacts specifically with the nuclei of meiotic prophase cells (spermatocytes); the immunofluorescence staining pattern obtained with serum 448 (Fig. 5A) is identical to the staining pattern obtained with antibodies against the 30,000- and 33,000-M_r SC components (see Fig. 1a in reference 25 and Fig. 3e in reference 48); the preimmune serum is negative in this test (not shown). (v) Within spermatocytes, serum 448 recognizes specifically the lateral elements of SCs (Fig. 6 and 7), like the antibodies against the 30,000- and 33,000-M_r SC components (compare Fig. 6 with Fig. 4a, c, and e in reference 29 and Fig. 5d to f in reference 27). (vi) The gene encoding the predicted 29.7-kDa protein is transcribed specifically in the testis; probes derived from SCP3 cDNA (Fig. 3) detect two transcripts of nearly equal size (about 1.1 kb) in testis RNA, whereas no transcripts are detected in RNA from other tissues and organs (Fig. 8). Thus, we conclude that we have isolated a cDNA encoding at least one of the 30,000- and 33,000-M_r SC components. For this, we propose the name SCP3.

Sequence of the SCP3 cDNA. The complete nucleotide sequence of cDNA clone 2A4, together with the amino acid sequence of the encoded protein, is shown in Fig. 4. The first ATG codon is found at nucleotide position 58. There is a polyadenylation signal (AATAAA) at nucleotide positions 1094 to 1099. The predicted protein SCP3 is rich in glutamine (11.6%) and lysine (13.6%). The glutamine residues are concentrated in the C-terminal half of the protein and often occur in clusters of two or three residues. For other proteins, it has been suggested that such glutamine clusters serve as spacers within the protein molecule (9).

Prediction of the secondary structure according to the method of Chou and Fasman (10) did not reveal large-scale structural motifs. However, analysis of the amino acid sequence according to the method of Lupas et al. (40) revealed four stretches in the C-terminal half of the protein that are likely to form coiled-coil structures (Fig. 4). These are associations of two or three amphipathic α-helices; the amphipathic character of the α-helices is the result of a heptad repeat pattern, with hydrophobic amino acid residues in the first and fourth positions of the heptad repeat. Because seven consecutive residues will form two turns of an α-helix, the hydrophobic residues at positions 1 and 4 will form a hydrophobic ridge on one side of the molecule (43). Such amphipathic α-helical stretches are capable of forming coiled-coil structures through (homotypic or heterotypic) hydrophobic interactions with other amphipathic α-helices (13). It is possible that such interactions were responsible for the appearance of presumed dimers of the 30,000- and 33,000-M_r SC components and, to a lesser extent, for the appearance of the full-length translation product of clone 2A4.7 on Cleveland gels (Fig. 2B).

The amino acid sequence of SCP3 has some other interesting features: there is a potential nuclear targeting signal (consensus, K-R/K-X-R/K, where X is any amino acid [8]) at amino acid positions 109 to 112, and there are potential cyclic AMP [cAMP]-cyclic GMP [cGMP]-dependent protein kinase target sites (consensus, K/R-K/R-X-S/T [17]) at positions 29 to 32 and 77 to 80. In addition, there are four potential target sites for protein kinase C(S/TX-R/K [33]) and five potential target sites for casein kinase II (S/T-X-X-D/E [51]) (not indicated). Furthermore, SCP3 shares the so-called "motif A" (G/AXXXXGKS/T [66]) with several nucleotide binding proteins (amino acid positions 28 to 35). This motif is found in a loop that is involved in the binding of phosphate groups of purine nucleotide molecules (5, 61). The secondary structure, as predicted by Chou-Fasman analysis (10), also displays other features that are supposed to be essential for nucleotide binding, namely a β-strand and an α-helix flanking the loop containing the A motif and a β-strand with a negatively charged amino acid at its C terminus (designated B motif by Walker et al. [66]). This negatively charged amino acid is probably involved in the binding of Mg^{2+}, which is

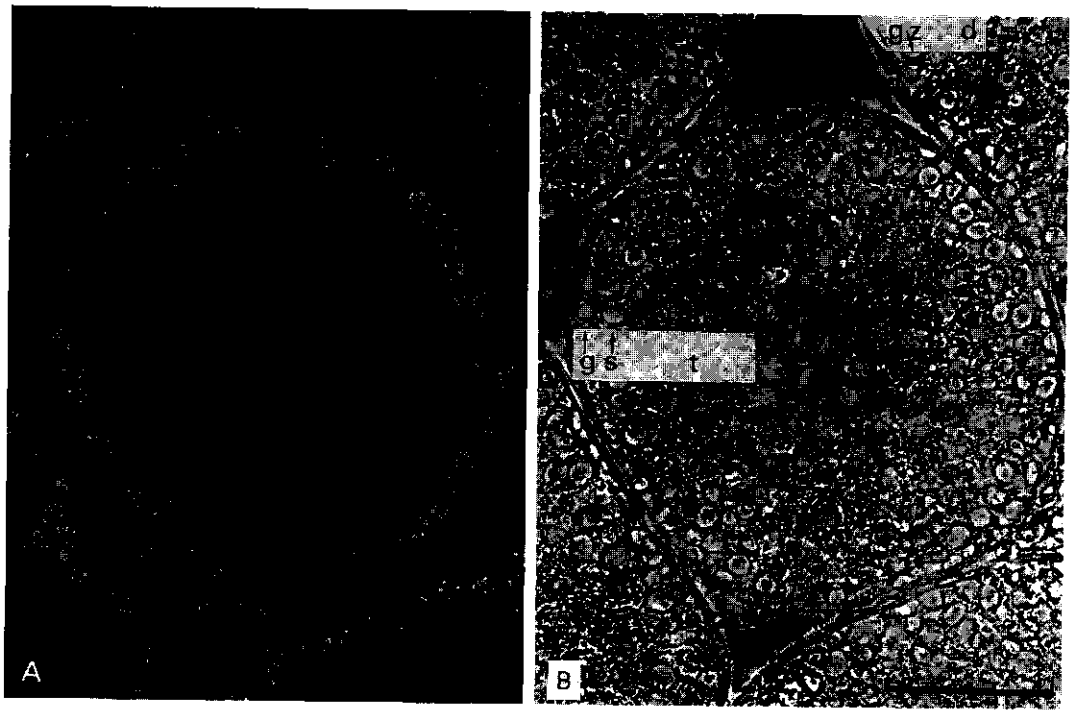

FIG. 5. Frozen section of rat testis after immunofluorescence staining with the polyclonal antiserum elicited against the fusion protein of clone 2A4.7 (serum 448). (A) Immunofluorescence; (B) phase-contrast micrograph of the same section. The centre of the micrograph shows a cross section of a testicular tubule. In panel B, the cell layers containing spermatogonia (g) and spermatocytes (s) are indicated. The seminiferous epithelium in the central tubule is in developmental stages VII to IX (37), because it contains a single layer of relatively large spermatocytes; these are in pachytene (25, 37); the cells inside the layer of spermatocytes are spermatids (t). The upper right corner shows part of a stage XIII tubule (37), with two successive layers of spermatocytes, which are in diplotene (the large cells to the right [d]) and zygotene (z) (25, 37); the layer of spermatogonia (g) is also indicated in this tubule. Bar, 50 μm.

thought to interact with β- and γ-phosphates (5, 61). In SCP3, the A motif is preceded by a segment predicted to form a β-strand (residues 21 to 25) and is followed by a region predicted to form an α-helical structure (residues 40 to 43). There are four additional segments capable of forming a β-strand, and two of these have a negatively charged amino acid at their C terminus (D at position 73 and E at position 96). Amino acid residues 73 to 76 match the consensus for a second sequence element that is characteristic for GTP-binding proteins, namely DXXG at a distance of 40 to 80 residues from motif A (14). However, SCP3 lacks the third consensus sequence that has been identified for GTP-binding proteins, namely NKXD at 40 to 80 residues from the DXXG sequence (14). Thus, although it seems likely that SCP3 can bind purine nucleotide molecules, the nucleotide specificity is uncertain.

Comparison of amino acid sequences with libraries of known sequences (see Materials and Methods) revealed a large-scale similarity to an X-linked lymphocyte-regulated mouse protein called pM1 (59). The amino acid sequence of pM1 can be aligned along its entire length with that of SCP3 in such a way that there is 35.3% identity between the aligned sequences (Fig. 9). Despite this overall similarity, there are also significant differences between pM1 and SCP3. SCP3 carries a stretch of 43 additional residues N terminal to the region of alignment with pM1. The motif A consensus sequence for nucleoside triphosphate-binding proteins and one of the cAMP-cGMP-dependent protein kinase target sites lie within this stretch; motif B and the other cAMP-cGMP-dependent protein kinase target site of SCP3 have not been conserved in pM1. Furthermore, pM1 has a much lower calculated pI (5.0) than SCP3 (9.4). The nuclear localization signal has been conserved in pM1, which is consistent with the nuclear localization of this protein (21), and pM1 has one amphipathic α-helical stretch that is likely to form a coiled-coil structure (pM1 residues 120 to 142). There is also significant nucleotide sequence similarity between the cDNAs encoding SCP3 and pM1 (54.6% identity, as determined after alignment of the nucleotide sequences by means of the GAP program). No other sequence similarities were detected at either the amino acid or nucleotide sequence level.

DISCUSSION

During meiotic prophase, chromosomes are organized in loops on proteinaceous axial cores that later become the LEs of SCs. These cores are distinct from mitotic or meiotic

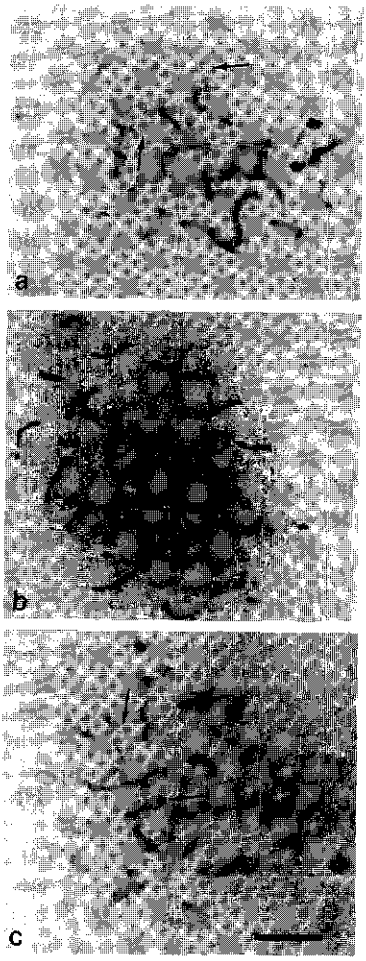

FIG. 6. Light micrographs (bright-field illumination) of agar filtrates of lysed spermatocytes after indirect immunoperoxidase staining with the polyclonal antiserum elicited against the fusion protein of cDNA clone 2A4.7 (serum 448). (a) Zygotene; (b) pachytene; (c) diplotene. Note the staining of the unpaired axes of the zygotene and diplotene bivalents (arrows); the arrowhead indicates the XY bivalent. Bar, 10 μm.

metaphase chromosome scaffolds and from interphase nuclear matrices because they consist largely of components that are specific for meiotic prophase (27, 29, 47). It is still largely unknown which functions are fulfilled by the LEs or their components, although several possible functions have been suggested. LEs could play an inhibiting or enhancing role in recombination (34, 39, 54); they might be involved in chiasma maintenance (53) or in sister chromatid cohesiveness (41). 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 show that two major components of the LEs, with M_rs of 30,000 and 33,000, are closely related, and we describe the isolation and characterization of a cDNA encoding at least one of these components, SCP3.

The 30,000- and 33,000-M_r SC components are closely related. The LEs of SCs contain 30,000- and 33,000-M_r components that, upon one-dimensional gel electrophoresis, are resolved in two broad bands with M_rs of 30,000 and 33,000. We think that these components are closely related because 18 of 18 independently isolated anti-30,000- and anti-33,000-M_r MAbs recognize both the 30,000- and 33,000-M_r protein bands and because the SV8 digestion patterns of the 30,000- and 33,000-M_r proteins are very similar to each other. What causes the difference with respect to M_r still has to be sorted out. It is possible that the M_r variants are translation products of different transcripts of the same gene, because various probes derived from SCP3 cDNA (Fig. 3) hybridize with two transcripts of nearly equal size on Northern blots of testis RNA (Fig. 8). Hybridization of the same probes with rat genomic blots indicate that these transcripts are encoded by a single gene (not shown). However, the relationship between the 30,000- and 33,000-M_r variants and the relationship between the two transcripts are still under investigation, and other possible explanations for the difference with respect to M_r, such as proteolytic breakdown, have not yet been excluded.

Sequence and predicted secondary structure of SCP3. The predicted amino acid sequence of SCP3 has several interesting features. First, there are two domains in the C-terminal half of the protein (residues 130 to 175 and 205 to 245 [Fig. 4]) that each contain two stretches that are likely to form coiled-coil structures. It is possible that these domains facilitate the formation of dimers of 30,000- and/or 33,000-M_r molecules. It is also possible, however, that the coiled-coil domains serve to anchor the 30,000- and 33,000-M_r SC components to coiled-coil proteins of the nuclear matrix or the SC (12, 44, 45, 63, 70). In that case, SCP3 might have similar functions, as has been suggested for another nuclear protein with a relatively short coiled-coil domain, namely the yeast Rep1 protein (69), which is involved in plasmid segregation. Another interesting feature of SCP3 is the potential purine nucleotide binding site; it seems likely that SCP3 can bind ATP. Numerous proteins involved in DNA repair and recombination bind ATP or at least have the consensus sequence for purine nucleotide binding, G/AXXXXGKS/T (66). Among the proven ATP-binding proteins are the *E. coli* RecA protein (42) and type II DNA topoisomerases (38, 68). For these proteins, it has been proposed that hydrolysis of bound ATP causes conformational changes that alter the DNA binding properties of the protein molecules (52, 61). The consensus for purine nucleotide binding has also been found in a number of yeast proteins that are involved in meiotic recombination and chromosome synapsis—for instance, the RecA-like Rad51 and Dmc1 proteins (4, 58) and the Rad50 protein (1). However, whether SCP3 binds ATP and whether it is capable of hydrolyzing it remain to be proven.

SCP3 has two cAMP-cGMP-dependent protein kinase target sites; one of these overlaps with the consensus sequence for nucleotide binding. It is possible that these potential phosphorylation sites are important for the regulation of the assembly and disassembly of the LEs of SCs. For nuclear lamins, it has been shown that inhibition of phosphorylation by cAMP-cGMP-dependent protein kinases is essential for the disassembly of the nuclear lamina at mitosis (36). cAMP-cGMP-dependent protein kinase target sites also occur in two other SC proteins, namely SCP1 and SCP2 (44, 48a).

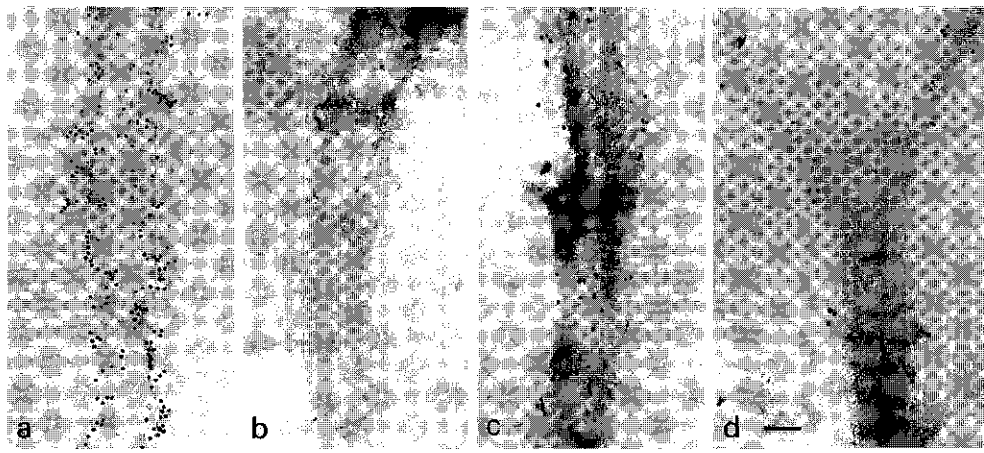

FIG. 7. Electron micrographs of surface-spread spermatocytes after immunogold labeling with antiserum against the 30,000- and 33,000-M_r SC components (serum 175 [a]), antiserum against the fusion protein of cDNA clone 2A4.7 (serum 448 [c and d]), or the preimmune serum of serum 448 (b). (a to c) Pachytene; (d) diplotene. Bar, 100 nm.

To summarize the main features of SCP3, it is a basic protein with a potential nucleotide binding site, it has two domains predicted to be capable of forming coiled-coil structures, and it has target sites for cAMP-cGMP-dependent protein kinases. Can these features be related to possible functions of SCP3? As was argued in the Introduction, the LEs and axial cores of SCs are probably involved in the structural organization of meiotic prophase chromatin and in transient, meiosis-specific modulations of chromatin function such as the preferential exposure of sequences for recombination, the establishment of sister chromatid cohesiveness, or the formation of functional chiasmata. As a component of the LEs and axial cores, SCP3 could be involved in any of these functions. It is possible that SCP3 is involved in the structural organization of meiotic prophase chromatin—for instance, the establishment of sister chromatid cohesiveness or the integration of the LEs in the nuclear matrix or in the tripartite structure of the SC. The predicted amphipathic α-helical stretches could play a role in this by allowing coiled-coil interactions between SCP3 and components of the SC or between the SCP3 molecules themselves. With respect to the possible structural functions of SCP3, it

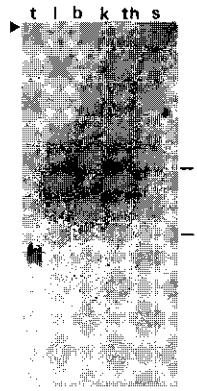

FIG. 8. Transcription of the gene encoding SCP3, analyzed by Northern blot hybridization. Thirty micrograms of total RNA from testis (t), liver (l), kidney (k), brain (b), thymus (th), and spleen (s) was loaded per 0.75-cm-wide slot. For this experiment, fragment 2 (see Fig. 3) was used as a probe; hybridization with probes 5, 6, 7, and 9 (see Fig. 3) yields essentially the same result (not shown). The bars indicate the positions of the rat 28S (4,700 nucleotides) and 18S (1,900 nucleotides) rRNA; the arrowhead indicates the top of the gel.

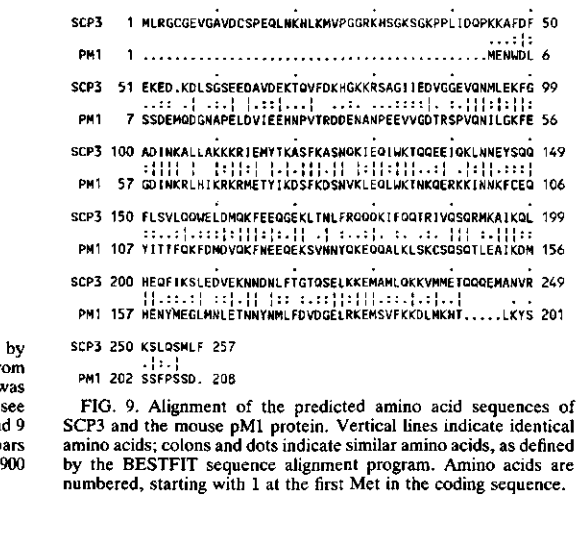

FIG. 9. Alignment of the predicted amino acid sequences of SCP3 and the mouse pM1 protein. Vertical lines indicate identical amino acids; colons and dots indicate similar amino acids, as defined by the BESTFIT sequence alignment program. Amino acids are numbered, starting with 1 at the first Met in the coding sequence.

should be kept in mind that SCP3 is no longer immunologically detectable after meiotic prophase (15, 25), apart from some clumps of antigenic material in part of the early spermatids (25). Thus, although it is possible that SCP3 establishes sister chromatid cohesiveness, other factors must be responsible for the maintenance of cohesiveness during dictyotene (for results with females, see reference 15), diakinesis, and metaphase I.

Similarity of SCP3 to the pM1 protein. SCP3 has considerable amino acid sequence homology to the pM1 protein, which is one of the predicted products of an X-linked lymphocyte-regulated (*Xlr*) gene family of the mouse (59). Mouse genomic DNA contains 50 to 75 *Xlr* sequences (59) that probably have arisen by amplification during recent evolution of the mouse; the closely related species *Coelomys pahari* and *Rattus norvegicus* have only one or two *Xlr*-related sequences (20). Most of the *Xlr* sequences of the mouse do not encode functional transcripts, and only two potentially functional transcripts have been identified. One, encoding the pM1 protein, is produced during the most mature stages of differentiation of T- and B-cell lines (20, 59); the other one, which has been described preliminarily (20), occurs specifically in the testis, but is shorter (0.9 kb) than the transcript encoding SCP3 (1.1 kb). The rat homolog of this transcript (if there is one) is not detected on Northern blots with any of the probes derived from SCP3 cDNA that we have tested (Fig. 8). pM1 is a nuclear protein (21), like SCP3, and part of the predicted amphipathic α-helical stretch of SCP3 has been conserved in the pM1 protein. However, there are also significant differences between the proteins; the pM1 protein can be extracted from the nucleus by chelating agents like EDTA (21), whereas SCP3 remains stably integrated in the LEs and axial cores in the presence of EDTA (28, 29). Furthermore, the pM1 protein lacks the potential nucleotide binding site and the potential cAMP-cGMP-dependent protein kinase target sites of SCP3, and it has a much lower pI value. Therefore, it seems doubtful that the proteins fulfill related functions. One function that could be considered for both proteins is the inhibition of recombination. The pM1 protein is expressed in the most mature stages of differentiation of B cells, where allelic exclusion has been established (2); SCP3 is localized in the LEs and axial cores of SCs and could have a role in the prevention of recombination between sister chromatids or in the inhibition of the initiation of recombination between non-sister chromatids in later stages of meiotic prophase. However, given the considerable differences between the pM1 protein and SCP3, it is quite possible that these proteins, although they are obviously evolutionarily related, are involved in completely different functions.

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Addendum to Chapter 2

The M_r 30,000 and 33,000 components of synaptonemal complexes of the rat are probably encoded by one gene and one messenger RNA.

J.H.M. Lammers, H. Kreuwel, I.C. Mey, W. Basuki, S. Maassen and C. Heyting

Abstract

The M_r 30,000-33,000 components of the lateral elements of synaptonemal complexes (SCs) of the rat are closely related according to their peptide maps and immunological reactivity (Lammers *et al.*, 1994; Chapter 2). We have tried to identify at which level differences between these proteins arise. We conclude that the M_r 30,000-33,000 SC proteins are most probably encoded by a single gene and a single messenger RNA. Mechanisms by which the difference between the M_r 30,000-33,000 SC proteins could arise during translation and posttranslational modification are discussed.

Introduction

The lateral elements (LEs) of synaptonemal complexes (SCs) of the rat contain two major components with relative electrophoretic mobilities (M_rs) of 30,000 and 33,000. From their nearly identical peptide maps and the fact that all of 18 monoclonal antibodies tested are unable to discriminate between the two proteins (Lammers *et al.*, 1994, Heyting *et al.*, 1989), we conclude that these are closely related. Recently, a cDNA encoding at least one of these components was isolated from a rat testis expression cDNA library. Polyclonal antibodies raised against the translation product of this cDNA (SCP3) recognized both the M_r 30,000 and 33,000 SC proteins on Western blots (Lammers *et al.*, 1994). We are interested in the nature of the difference between the M_r 30,000 and M_r 33,000 proteins, because this might provide clues to the possible function of these SC components.

Differences between related proteins can arise at various levels. First, the proteins can be encoded by related genes. Second, a single gene can yield two different transcripts, either by alternative splicing or by the usage of different promoters or terminators (reviewed e.g. by Smith *et al.*, 1989). Alternative splicing yields two proteins that differ in their amino acid sequence at internal positions, whereas the use of different promoters or terminators results in the production of two proteins that differ at their N- or C-terminus, respectively. Third, translation of a single messenger RNA (mRNA) can produce two closely related proteins when, under certain conditions, some ribosomes can reach a second AUG startcodon and initiate translation from there (Kozak, 1989). Moreover, certain parts of the sequence of an mRNA, called 'translational introns', can, occasionally, be bypassed during translation, which also allows the formation of two closely related proteins (Benhar and Engelberg-Kulka, 1993). Fourth, posttranslational modifications can give rise to different variants of the same protein. This includes the removal of one or more amino acids from the N- or C-terminal end of the protein and the modification of one or more amino acid residues.

In this addendum, we describe experiments, carried out to establish at which level differences between the M_r 30,000 and 33,000 SC proteins arise. We conclude that the two SC proteins are products of a single gene and propose that the difference between these two proteins arises during translation or by posttranslational modification.

Experimental procedures

Genomic Southern blots

Genomic DNA was isolated from rat liver according to Sambrook *et al.* (1989). For the preparation of genomic Southern blots, 10 μg of DNA was digested with either *Eco*RI, *Bam*HI, *Pst*I or *Hin*dIII and the resulting fragments were subsequently separated on 0.7% (w/v) agarose gels. After electrophoresis, gels were immersed twice for 15 min in, successively, 0.25 M HCl; 1.5 M NaCl, 0.5 NaOH; and 1.5 M NH_4Ac, 0.1 M NaOH. The DNA fragments were transferred to Hybond-N+ membranes (Amersham) by Southern blotting using 1.5 M NH_4Ac, 0.1 M NaOH as blotting buffer. After transfer, the membranes were washed in 3x SSC (0.45 M NaCl, 0.045 M sodium citrate), dried and fixed by baking for 2 h at 80°C. Four probes (designated 316, 24, 58 and 640; Figure 2) were used for genomic Southern blot hybridization (Figure 1). Labelling of the probes with [α-^{32}P]dATP was performed by random primed labelling according to Sambrook *et al.* (1989). Membranes were prehybridized for at least 6 h at 65°C in 6x SSC, 5x Denhardt's solution, 1% SDS, 0.01% sodium pyrophosphate, 150 μg/ml denatured herring sperm DNA. Hybridization was performed in fresh buffer of the same composition, to which 25 ng of radioactively labelled probe (10^8 cpm/μg) was added, for 20 h at 65°C. The membranes were subsequently washed for 30 min at 65°C in, successively, 2x SSC; 2x SSC, 1% SDS; 1x SSC, 0.1% SDS and 0.1x SSC, 0.1% SDS.

Isolation of a clone containing the presumptive 5'-region of the SCP3 gene

For the isolation of clones containing the 5'-end of the SCP3 gene, we screened a rat genomic library by plaque hybridization according to Sambrook *et al.* (1989), using fragment 24 (Figure 1) as a probe.

RNA isolation, oligo(dT) cellulose chromatography and Northern blot hybridization

RNA from various rat tissues was isolated according to Cathala *et al.* (1983). Isolation of poly(A)$^+$ RNA by oligo(dT) cellulose chromatography and RNA electrophoresis was carried out according to Sambrook *et al.* (1989). Northern blots were prehybridized for at least 3 h at 65°C in 0.5 M sodium phosphate buffer (pH 7,2), 7% SDS, 1 mM EDTA (Church and Gilbert, 1984). Hybridization was performed in the same mixture with 25 ng of radioactively labelled DNA probe (10^8 cpm/μg). The blots were washed 4 times for 15 min at 65°C in 40 mM sodium phosphate buffer (pH 7.2), 1% SDS, 1 mM EDTA.

5'-RACE of cDNAs encoding the M_r 30,000 and 33,000 SC components

For the amplification of 5'-ends of cDNA we adapted the basic protocol designed by Frohman *et al.* (1988) as follows: 1 μg of poly(A)$^+$ RNA (1 μg/μl) from rat testis and 10 pmol of Gene Specific Primer 1 (GSP1, 5'-TTCCTCTTGTTGTGTTTTCC-3'; 10 pmol/μl) were added to 10 μl of DEPC-treated MilliQ, heated to 70°C for 10 min and then placed at room temperature. To this mixture 1 μl of RNAse block II (Stratagene, La Jolla, Ca, USA), 2 μl of 0.1 M DTT, 1 μl of 10

mM dNTPs and 4 μl of 5x SuperScript reaction buffer (Gibco BRL, Grand Island, NY, USA) were added. After incubation at 37°C for 2 min, 200 Units of SuperScript II RNAse H$^-$ Reverse Transcriptase (Gibco BRL, 200 Units/μl) were added and first strand cDNA synthesis was carried out at 45°C for 1 h. The reaction was terminated by addition of RNAse H and RNAse A (2 Units each) and incubation at 55°C for 10 min.

GSP1 was removed from the single-stranded cDNA uby means of the Geneclean II kit (BIO 101). The cDNA was then dissolved in 11 μl of MilliQ. For tailing, the cDNA was heated to 70°C for 5 min, quenched on ice and added to 2 μl of 2 mM dCTP, 2 μl of 10x cacodylate buffer (Amersham) and 10 Units of terminal deoxynucleotidyl-transferase (Amersham). The mixture was incubated at 37°C for 20 min, the reaction terminated by incubation at 65°C for 15 min and the tailed cDNA precipitated by addition of NH_4Ac and ethanol. The cDNA was redissolved in 20 μl of MilliQ. For second strand synthesis and amplification, 5 μl of the recovered cDNA was added to a mixture with a final volume of 50 μl, containing 1x Vent reaction buffer (New England Biolabs, Beverly, MA, USA), 100 μg/ml of BSA, 200 μM of dNTPs, 10 pmol of anchor primer (5'-AAATGGATCCTTCTAGATGC$(G)_{17}$-3'), 25 pmol of adapter primer (5'AAATGGATCCTTCTAGATGC-3'), 25 pmol of GSP2 (5'-TTACTGGCTTTGAAAGAAGC-3') and 2 Units of Vent DNA Polymerase (New England Biolabs). The mixture was overlaid with 50 μl of mineral oil and heated to 95°C for 5 min in a PCR apparatus. The anchor primer was allowed to anneal at 51°C for 2 min, and was subsequently extended at 72°C for 40 min. cDNA was then amplified by 30 incubation cycles consisting of: 94°C, 40 sec; 51°C, 2 min and 72°C, 3 min, followed by a final extension of 15 min at 72°C.

PCR products were analysed by agarose gel electrophoresis and subsequent Southern blot hybridization.

PCR analysis of cDNAs encoding the M_r 30,000 and 33,000 SC components present in a rat testis expression cDNA library

The inserts of about 10^8 phage of the rat testis expression cDNA library were subcloned into the pBluescript SK- vector according to the instructions of the manufacturer (Stratagene). For PCR analysis a gene specific primer (GSP1) and a primer present in the vector immediately 5' with respect to the cDNA inserts (M13 reverse) were used. Reaction mixtures (50 μl) contained 1x Vent reaction buffer (New England Biolabs), 100 μg/ml BSA, 100 μM of dNTPs, 33 pmol of GSP1, 33 pmol of M13 reverse primer, 100 ng subcloned cDNA and 2 Units of Vent DNA Polymerase (New England Biolabs). Amplification involved incubation at 95°C for 5 min followed by 30 cycles consisting of: 95°C, 1 min; 51°C, 2 min and 72°C, 1.5 min. Subsequently a final extension of 15 min at 72°C was carried out.

PCR products were analysed by alkaline agarose gel electrophoresis and subsequent Southern blot hybridization.

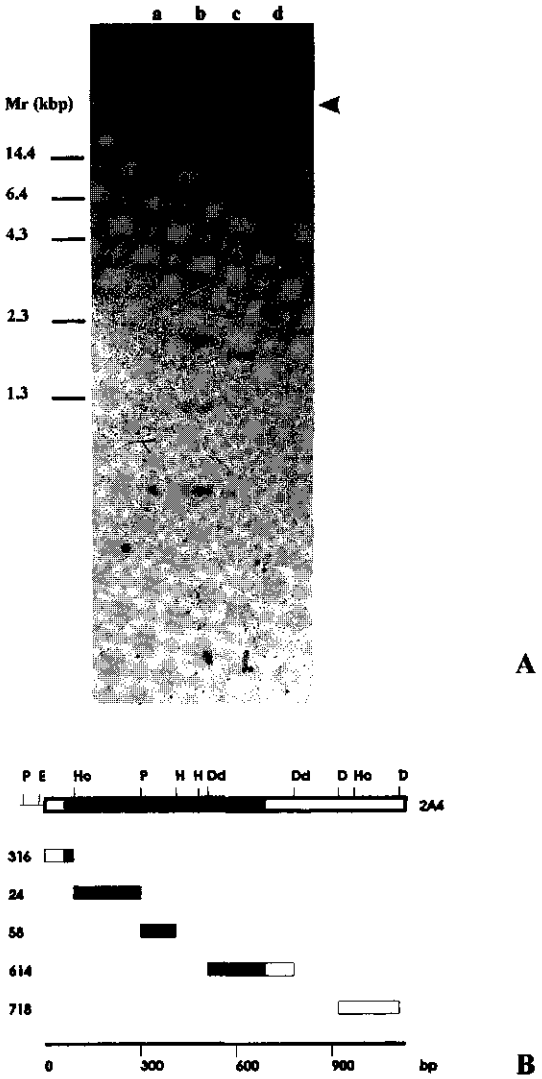

Figure 1: A: Genomic Southern blot analysis of genes encoding the M, 30,000 and 33,000 SC proteins. Ten μg of rat genomic DNA was digested with *Eco*RI (a), *Hin*dIII (b), *Pst*I (c) and *Bam*HI (d), and loaded onto a 0.7% agarose gel. Here only the hybridization pattern obtained with probe 614 is shown. The arrowhead indicates the top of the gel.
B: Restriction map of cDNA clone 2A4. The coding sequences are indicated in black. The bars below the map indicate the probes used for genomic Southern and Northern hybridization analysis. Restriction enzymes: P, *Pst*I; E, *Eco*RI; Hc, *Hinc*II; H, *Hin*dIII; Dd, *Dde*I; D, *Dra*I.

Results

The M_r 30,000 and 33,000 SC components are probably encoded by a single gene

In order to assess the number of genes homologous to cDNA 2A4, which is a full-length cDNA encoding at least one of the M_r 30,000-33,000 SC components, we probed genomic Southern blots of the rat with several probes derived from cDNA 2A4 (Figure 1). In most lanes a single band was detected within the genomic DNA. In only two cases (lanes b and c), where the largest probe (280 bp) was used two bands showed up. The most likely interpretation of this result is that only a single gene in the rat genome is homologous to cDNA 2A4. However, this result does not rule out the possible presence of two highly homologous genes that lie closely together in the genome. These genes would then show a different hybridization pattern on genomic blots with only one cDNA-derived probe (the 280 bp fragment).

Figure 2 shows that, on Northern blots, only a single transcript hybridizes with probes derived from cDNA 2A4. So, if there is only a single gene for the M_r 30,000-33,000 SC components, this gene encodes only a single mRNA or more mRNAs of about the same electrophoretic mobility. mRNAs differing only by 50 nucleotides or less would probably not be resolved on the gels used.

The presence of a single mRNA encoding the M_r 30,000-33,000 SC proteins is further corroborated by the 5'-RACE experiment shown in Figure 3. The RACE protocol used (see Experimental procedures) yields a product which migrates as single, sharp band of about 440 bp (not shown); this is somewhat shorter than expected (431 bp of cDNA plus about 35 bp of anchor primer). Similar results were obtained if first strand cDNA synthesis was performed at 48°C, 51°C and 53°C (Figure 3). As a control, PCR was performed on cDNA 2A4. Remarkably, this yielded two bands (Figure 3, lane e), one of the expected length (431 bp plus 113 bp of vector sequence) and a shorter one (see Discussion). We performed only 5'-RACE and not 3'-RACE because we expected potential differences at the mRNA level to be located at the 5'-end. The reasons for this were (i) that all cDNAs isolated in a secondary screening of the cDNA expression library with a fragment of clone 2A4 as a probe, were colinear with 2A4 at the 3'-end (see below) and (ii) that all monoclonal anti-M_r 30,000-33,000 antibodies that reacted with both the M_r 30,000 and the M_r 33,000 SC protein on Western blots, recognized epitopes in the C-terminal half of the cDNA-encoded protein SCP3 (experiments not shown).

The cDNA products of the 5'-RACE were subcloned in the pBluescript SK- vector and two of the resulting clones were sequenced. Both cDNA inserts were shorter at the 5'-end than cDNA 2A4, one clone reaching position 85 in the 2A4 sequence and the other position 102. Inspection of the sequence of clone 2A4 in this region revealed a palindro-

me consisting of 16 nucleotides (positions 83 to 98). Analysis of this palindrome by means of the Generunner program (Hastings Software, Inc.) showed that it can form hairpins with melting temperatures of 78.8°C and 73.2°C, and free energies of -6.3 and -6.6 kCal/Mol, respectively (calculated for the corresponding mRNA sequence). This could explain the failure to synthesize full-lentgh cDNA even when first strand synthesis is carried out at 53°C (see Discussion).

Most cDNAs in the expression cDNA library, that encode the M_r 30,000 and 33,000 SC components, are shorter than clone 2A4

From 24 cDNAs isolated in a secondary screening of the rat testis expression cDNA library with a *Hin*cII fragment of 880 bp of clone 2A4, 22 were shorter than the 2A4 cDNA. Only two clones were of the same length, whereas 7 clones were between 72 to 100 nucleotides shorter at the 5'-end than the 2A4 cDNA. The 5'-ends of these shorter cDNA clones are thus located within or near the 16 nucleotide palindrome sequence. The remaining 14 cDNA clones were 300 to 700 nucleotides shorter than clone 2A4 at their 5'-ends, which indicates a low efficiency of cDNA synthesis of this particular mRNA.
To get an overview of the length distribution of all M_r 30,000 and 33,000 SC-encoding cDNAs, in the cDNA library, that are longer than 600 nucleotides, PCR was carried out to amplify their 5'-ends. Figure 4 shows that most cDNAs are about 100 bp shorter than cDNA 2A4. In lanes a and d products of the same length as the 2A4-product can be detected.

The first ATG startcodon of clone 2A4 is preceded by a TAA stopcodon in genomic DNA

Because cDNA 2A4 does not contain a stopcodon 5' to the first ATG-codon, we inspected the genomic DNA upstream of this codon for the presence of a stopcodon. Using a 5'-probe of clone 2A4 (probe 24, Figure 1), we screened a rat genomic library and isolated one strongly reacting clone. We subcloned a 3.3 kbp *Sal*I fragment that hybridized to the most 5'-probe of 2A4 (fragment 316, Figure 1) and part of this fragment was sequenced from a primer complementary to sequences at the 5'-end of clone 2A4 (Figure 5). The frame which encodes the amino acid sequence of SCP3 contains a TAG stopcodon at 78 nucleotides 5' to the first ATG-codon of SCP3. No additional ATG-codon was found in between these two codons. Further analysis of the sequence revealed several features indicative for promoter regions of eukaryotic genes: two potential Sp1 transcription factor binding sites (GGGCGG, positions 410-416 and 471-477), three potential AP2 transcription factor binding sites (CCC(A/C)N(G/C)(G/C)(G/C), positions 327-334, 332-339 and 408-415) and two c-Myc binding sites (CACGTG, positions 440-445 and 461-466).

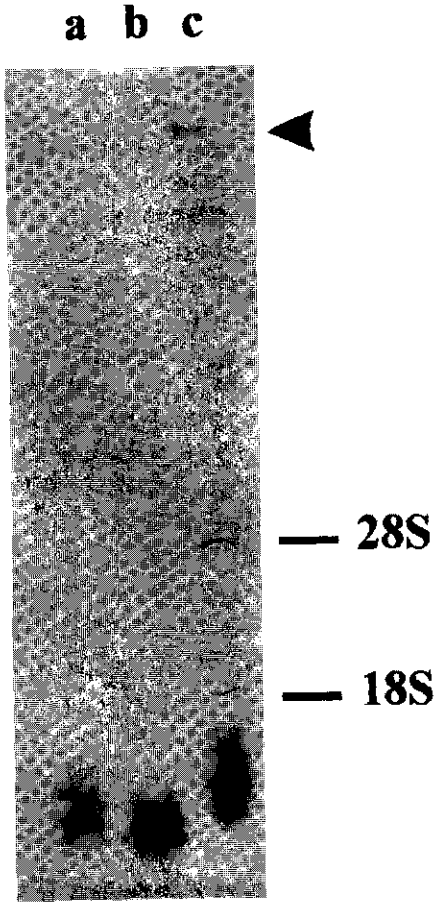

Figure 2: Northern blot analysis of transcripts of the gene encoding the M_r 30,000 and 33,000 SC proteins. Two μg of poly(A)$^+$ RNA, purified from total rat testis RNA by one (a) or two (b) rounds of oligo(dT) cellulose chromatography, or 15 μg of total rat testis RNA (c) were loaded onto 0.75 cm-wide slots. Probe 316 (see Figure 1) was used for hybridization. The bars indicate the positions of the rat 28S (4,700 nucleotides) and 18S (1,900 nucleotides) rRNA. The arrowhead indicates the top of the gel.

The GRAIL and GENE-ID programs were used to search for potential (first) exons within the sequence. Two small potential exons (postions 335-347 and 469-486) were found. If any of these would encode additional amino acids of SCP3 then the sequence between

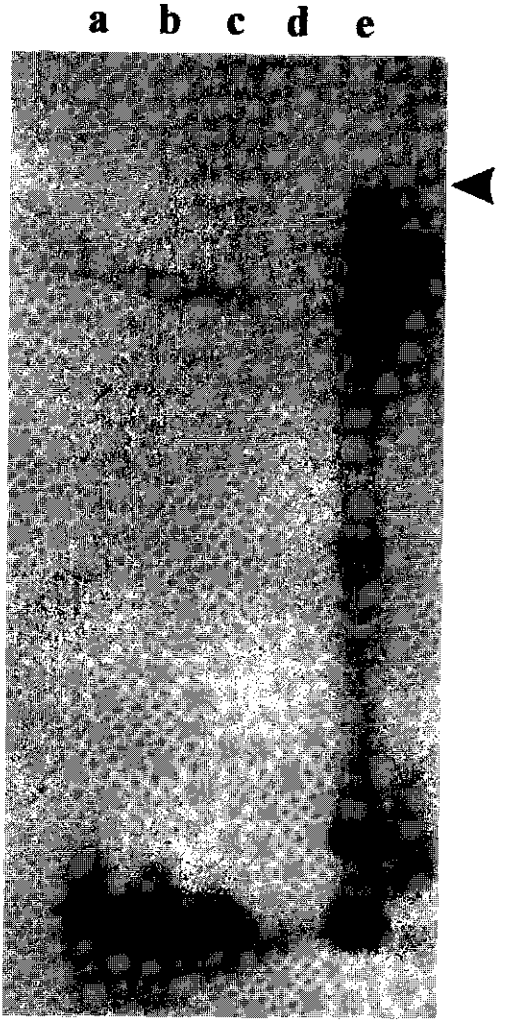

Figure 3: 5'-RACE of cDNAs encoding the M_r 30,000 and 33,000 SC components. 5'-RACE was performed with 1 μg of poly(A)$^+$ RNA from rat testis (see Experimental procedures). Products were analyzed by Southern blot hybridization with the 5'-*Pst*I-fragment of cDNA 2A4 (see Figure 1). First strand synthesis was performed at 48°C (lane a), 51°C (lane b) and 53°C (lane c). As a control for specificity, the PCR step was performed: with products from first strand synthesis at 48°C, without the addition of primers (lane d), or with cDNA 2A4 as a template (lane e). The arrowhead indicates the top of the gel.

these exons and the end of the TAA stopcodon at position 488-490 should be removed by splicing. The only potential 3'-splice acceptorsite that would serve this purpose is located

immediately in front of the first nucleotide of clone 2A4 (the conserved AG preceding the potential splice junction is located at postion 523-524). Such splicing events would, however, result in either the addition of about 8 kDa of amino acid sequence, or in the addition of sequences containing no extra startcodon in the right frame. However, it can not be excluded that exons encoding additional amino acids of SCP3 are present anywhere up to 10 kbp or more upstream of the 2A4 sequence.

Discussion

The M_r 30,000 and 33,000 SC components of the LEs of synaptonemal complexes of the rat are nearly identical as judged from their peptide maps and immunological reactivity (Lammers *et al.*, 1994; Chapter 2). The M_r 3,000 difference between the two SC components can be generated in two ways. In the first, the primary sequence of the two components is different. The experiments described in this addendum, make it likely (although they do not proof) that the M_r 30,000 and 33,000 proteins are encoded by a single gene and a single mRNA. Therefore a difference at the primary amino acid level could arise at either the translational or posttranslational level. The latter would then involve the removal or addition of amino acids. In this case the peptide maps foretell that such a difference is likely to be located at the N- or C-termini of the proteins. A difference at an internal position involving about 25-30 amino acids, would undoubtedly have resulted in a different banding pattern in the peptide maps. The second way in which a difference can be generated involves posttranslational modification, without affecting the amino acid sequence.

A difference at the amino acid level is generated during or after translation

Genomic Southern blot hybridization showed that the M_r 30,000 and 33,000 SC components are probably encoded by a single gene. Moreover, on Northern blots only one mRNA can be detected. This almost rules out alternative splicing or the use of alternative promoters or terminators as a mechanism by which the difference between the two SC proteins is caused, even though differences in size between two mRNAs of up to 50 nucleotides would probably not be noticed on Northern blots. If such a small difference would exist, then we would expect it to be located at the 5'-end, because analysis of 24 cDNA clones isolated in a secondary screening of the expression cDNA library, showed that there was no variation among the 3'-ends of the cDNAs whereas there was variation among the 5'-ends. Only two clones contained the same 5'-end as clone 2A4, whereas seven clones lacked between 72 and 100 nucleotides at the 5'-end when compared to 2A4.

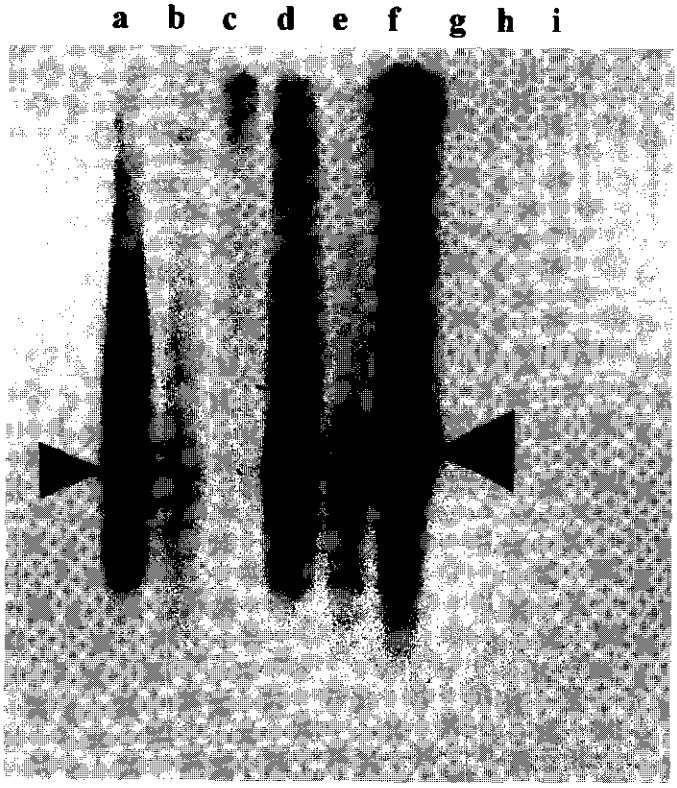

Figure 4: PCR analysis of the 5'-ends of cDNAs encoding the M_r 30,000 and 33,000 SC components, present in the expression cDNA library. Reactions were carried out with cDNA from each of five sublibraries designated A1 (lane a), A2 (lane b), A3 (lane c), A4 (lane d) and A5 (lane e) as well as with cDNA 2A4 (lane f) as a template. Control reactions contained either: no template (lane g), template but no primers (lane h) or template nor primer (lane i). PCR products were separated on alkaline agarose gels and analysed by Southern blot hybridization with the *Eco*RI-*Hin*dIII fragment of clone 2A4 as a probe (see Figure 1). The large arrow points to the the major product of the reaction with 2A4-cDNA as a template, whereas the small arrow points to the majority of products obtained with the sublibraries as templates. The latter are about 100 nucleotides shorter than the product obtained with 2A4.

This result agreed well with that obtained after amplification of the 5'-ends of all M_r 30,000 and 33,000 SC-encoding cDNAs present in the cDNA library. Whereas some products were of the same length as clone 2A4, most were about 100 nucleotides shorter at the 5'-end. 5'-RACE experiments also yielded products that were about 100 nucleotides shorter at the 5'-end than 2A4, even if a temperature of 53°C was used for first strand cDNA synthesis. It seems likely that the production of cDNAs lacking about 100 nucleotides when compared to 2A4 is an artifact of the first strand cDNA synthesis. A 16 bp perfect palindrome located at position 83-98 in the 2A4 sequence can form two small, but rather stable stem-loop hairpins (ΔG= - 6.3 or -6.6 kCal/Mol) that could interfere with full-length cDNA synthesis. Recently, it was shown that reverse transcription of ß-globin mRNA and synthetic RNAs derived from the *pol* gene region of human immunodeficiency virus-1 (HIV-1), by HIV-1 reverse transcriptase (RT) or by AMV (Avian myeloblastosis virus) RT was impaired by the presence of a stem-loop hairpin with a free energy of -5.7 kCal/Mol (Olsen *et al.*, 1994). Even the control PCR in the 5'-RACE experiment (Figure 4, lane e) with 2A4 cDNA as a template yields besides the expected full-length cDNA a truncated product which is probably the result of linear amplification terminated at or near this palindrome-containing region.

We think it unlikely that the 2A4-mRNA is an incompletely processed hnRNA, and that the mature mRNA is about 100 nucleotides shorter, because probe 316, which covers the utmost 5' sequences of 2A4, detects the same transcripts on Northern blots as other probes derived from cDNA 2A4. We can not exclude that there are small differences (up to 50 bp) at the 5'-end of two mRNAs, but these can not account for a M_r 3,000 difference between the translation products. Therefore we think it is likely that the difference between the M_r 30,000 and 33,000 SC components arises during or after translation.

Mechanisms by which a difference between the M_r 30,000 and 33,000 SC components could arise during translation

The general model for eukaryotic translation involves the binding of 40 S ribosomal subunits at the capped 5'-end of an mRNA, migration ('scanning') along the sequence of nucleotides until the first AUG codon is reached and initiation of translation starting from this 5' proximal AUG codon (Kozak, 1989). Altough this 'first AUG rule' is quite strict, some cases are known in which translation starts from a downstream AUG codon. 'Leaky scanning' has been observed in cases where the first AUG codon is in an unfavourable context (not a purine at position -3 or another nucleotide than a G at position +4 (where the position of the A in the AUG codon is denoted +1)) (Kozak, 1989). Some 40 S ribosomal subunits will then bypass the first AUG codon, reach the second AUG and start

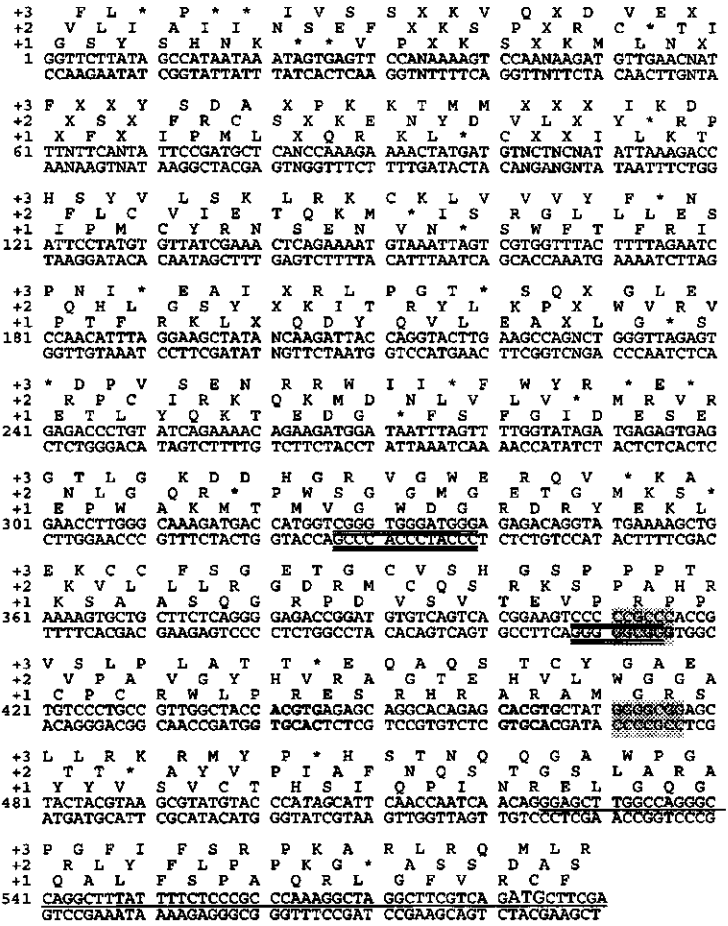

Figure 5: Nucleotide sequence of genomic DNA upstream of the SCP3 gene. Predicted products for translation in the three forward frames are shown above the nucleotide sequence. At the positions denoted 'X', the amino acid could not be determined because of ambiguities in the nucleotide sequence. '*' denotes a stopcodon. The 5'-end of the sequence obtained from cDNA clone 2A4 is underlined. The first ATG codon in the 2A4 sequence is printed in large letters. The TAG stopcodon in the frame encoding SCP3, preceding the 2A4 sequence is printed in italics. Potential binding sites for transcription factor Sp1 (shaded), AP2 (double underlined) and c-myc (bold letters) are indicated.

translation from there. Thus, 'leaky scanning' results in the production of two closely related proteins, differing at their N-termini, from a single mRNA (Kozak, 1989). The first AUG codon in the 2A4 cDNA lies in an unfavourable context, with a C at position -3 and a C at position +4. The second AUG codon is a much more potent initiation codon with an A at postion -3 and a G at position +4. A 'leaky scanning' mechanism would in

this case result in the production of two proteins differing 2.5 kDa in calculated molecular weight, with the smaller protein having an isoelectric point (IEP) that is 0.25 units higher than that of the larger protein. Recently, the production of the membrane-associated (MB) and soluble (S) forms of catechol *O*-methyltransferase (COMT) of the rat was shown to occur by 'leaky scanning'. The 5' proximal AUG codon from which the translation of MB-COMT started is in an unfavourable context, similar to that for the first AUG codon in 2A4 (a C at position -3 and also at position +4). The second AUG codon from which translation produced S-COMT was in a favourable context. In cell cultures transfected with the corresponding full-length cDNA both proteins were produced; the ratio of MB- to S-COMT (which was 0.3) could be increased by the introduction of point mutations that altered the context of the MB-COMT AUG codon in a favourable way (Tenhunen and Ulmanen, 1993). A similar situation was found for an mRNA encoding the mitochondrial (m) and peroxisomal (p) variants of the rat serine:pyruvate/alanine:glyoxylate aminotransferase (SPT/AGT). Although the downstream AUG codon from which SPT/AGT-p was formed was here also in a more favourable context than the 5' proximal AUG codon from which SPT/AGT-m was produced, translation started more from often the first AUG codon than from the second. This effect was attributed to the presence of a stable stem-loop structure (ΔG= -12.4 kCal/Mol) downstream of the first AUG codon (Funai and Ichiyama, 1995). It is assumed that a small amount of secondary structure downstream of a suboptimal AUG startcodon enhances initiation from that codon by preventing 40 S ribosomal subunits from scanning too fast or too far (Kozak, 1990). Such an effect might also be exerted by the stem-loop structure located downstream of the first AUG codon in clone 2A4 and upstream of the second AUG.

Another mechanism by which translation of a single mRNA results in the formation of more than one protein has been found in yeast, plants, birds and mammals and is referred to as 'reinitiation'; it involves the translation of a small 5' open reading frame (ORF) from an AUG startcodon that lies in a rather favourable context (Kozak, 1989). After translation of the first (invariably small) ORF (that is terminated by a stopcodon) the 40 S ribosomal subunits resume scanning and are capable of reinitiating translation from a downstream AUG codon. As the efficiency of reinitiation improves when the distance between the downstream AUG codon and the small 5'-ORF increases, translation can start from more than one internal AUG codon (Kozak, 1987, Calkhoven *et al.*, 1994). Inspection of the sequence upstream of the first AUG codon of clone 2A4 (Figure 6) shows one potential small 5'-ORF (position 495-506). Although the AUG startcodon of this small 5'-ORF lies in an unfavourable context, translation by means of a reinitiation mechanism could explain the formation of two SC proteins that are the same as considered above for the products of a 'leaky scanning' mechanism. The fact that the second

AUG codon (postion 127-129 in 2A4) lies in an optimal context could then explain the absence of products initiated from AUG codons further downstream.
A straightforward approach to check if the M_r 30,000 and 33,000 SC components indeed are the products of translation events initiated at different AUG startcodons would be to determine their N-terminal amino acid sequences. Unfortunately, two attempts to accomplish this failed, possibly because of the presence of N-terminally blocked amino acid residues. Therefore the N-terminal amino acid sequences of the M_r 30,000 and 33,000 SC proteins could not be compared to the predicted sequence of SCP3.
A third mechanism by which two closely related proteins can be obtained is bypassing of so-called 'translational introns'. In such a mechanism, translation starts at one AUG startcodon, but occasionally, the ribosomes bypass an internal stretch of nucleotides in the mRNA, after which translation is resumed (Benhar and Engelberg-Kulka, 1993). If such a mechanism would be adopted to produce the M_r 30,000 and 33,000 SC proteins, the bypassed region should be located near the 5'- or 3'-end of the mRNA, because otherwise a difference between the proteins would have been detected with respect to their peptide maps. Moreover, until nowthe bypassing mechanism has only been found in prokaryotic systems and is always associated with a frame-shift. Such a mechanism can therefore not explain the formation of the two SC proteins.

Posttranslational modification as the source of variability between the M_r 30,000 and 33,000 SC components

Posttranslational modification can involve the amino acid sequence itself as well as the addition of functional groups or entire molecules to amino acid residues. In the first case, the programmed removal of a stretch of amino acids can occur from the N- or C-terminal ends of a protein but also from internal positions. As pointed out before for the M_r 30,000 and 33,000 SC components, such a modification is only likely to occur at the N-terminal end. Removal of a stretch of amino acids from the N-terminal end of the larger of the two proteins (probably with the same amino acid sequence as SCP3) would yield essentially the same two products as those that would arise from 'leaky scanning' during translation. In both cases we assume that the difference between the calculated molecular weights (29.7 and 27.2 kDa) and the relative electrophoretic mobilities of these proteins is caused by their high content of basic amino acids, which can result in a slower migration through SDS-polyacrylamide gels than expected (Hames, 1990).
We have shown before that both the M_r 30,000 and 33,000 SC components are phosphorylated (Lammers *et al.*, 1995, Chapter 3). The major variant of the M_r 30,000 SC component probably carries 2 phosphate residues, and the major variant of the M_r 33,000 SC component 4 phosphate residues. Is it possible that a different degree of phosphoryla-

tion causes the proteins to migrate with different mobilities on SDS-gels? According to a model proposed by Lundahl *et al.* (1986), the addition of phosphate groups will interfere with SDS-binding, and this will result in a slower migration of the protein through a gel. The addition of two, three or four phosphate groups can therefore result in an unexpected increase in electrophoretic mobility. It is, however, unlikely that this is the case for the M_r 30,000 and 33,000 SC components, because dephosphorylation of the proteins does not result in a major change in the electrophoretic mobility of the two proteins as judged from two-dimensional Western blots (Lammers *et al.*, 1995, Chapter 4, Figure 2A).
Besides phosphorylation, various modifications can cause a change in electrophoretic mobility. As yet, only one of these, poly-ADP-ribosylation, has been studied and was found not to be involved (Offenberg, 1993).

To summarize: our results indicate that the M_r 30,000 and 33,000 SC proteins are probably encoded by a single gene and a single mRNA. As yet, it is not clear if the difference between the proteins arises during or after translation. 'Leaky scanning' is one possible mechanism by which the two proteins could be formed from translation initiation events involving the first and second AUG codon present in cDNA 2A4. A stem-loop secondary structure located between the two AUG codons could enhance translation from the first AUG codon that lies in a more unfavourable context than the second. As an alternative several posttranslational modifications could be responsible for the difference between the two SC proteins. However, as yet it is unclear what type of modification could be involved.

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Chapter 3

A change in the phosphorylation pattern of the 30,000-33,000 M_r synaptonemal complex proteins of the rat between early and mid-pachytene

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A change in the phosphorylation pattern of the 30000–33000 M_r synaptonemal complex proteins of the rat between early and mid-pachytene

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Abstract. The lateral elements (LEs) of synaptonemal complexes (SCs) of the rat contain major components with relative electrophoretic mobilities (M_rs) of 30000–33000, which are the products of a single gene. After one-dimensional separation of SC proteins on polyacrylamide-SDS gels, these components show up as two major bands, whereas upon two-dimensional electrophoresis they are resolved in at least 24 spots, which focus at pH 6.5 to 9.5. In this paper we show that these spots represent phosphorylation variants. For the analysis of the phosphorylation of the 30000- to 33000-M_r SC components during progression through meiotic prophase, we developed a procedure for isolation of fractions of testicular cells of the rat that are enriched in separate stages of meiotic prophase. Analysis of the 30000- to 33000-M_r SC components in these fractions by two-dimensional electrophoresis and immunoblotting showed that phosphorylated variants of the 30000- to 33000-M_r SC proteins occur throughout meiotic prophase. However, the extent of phosphorylation changes between early and mid-pachytene, when one phosphate group is probably added to each of the variants.

Introduction

During early meiotic prophase (leptotene), chromosomes are organized in loops on proteinaceous axial cores. These cores later become the lateral elements (LEs) of synaptonemal complexes (SCs). The axial cores/LEs are distinct from mitotic or meiotic metaphase chromosome scaffolds and from interphase nuclear matrices, because they consist largely of components that are specific for meiotic prophase nuclei (Heyting et al. 1988; Offenberg et al. 1991). Which roles LEs or their components fulfil is not known, but several possible functions have been considered. LEs could have an inhibiting or enhancing role in recombination (Lammers et al. 1994; Kleckner et al. 1991; Roeder 1990; Loidl 1990; Schwacha et al. 1994); they might be involved in sister chromatid cohesiveness and chiasma maintenance (Maguire 1991; Dobson et al. 1994; Carpenter 1994), or they might regulate other aspects of sister chromatid interactions (Schwacha et al. 1994). In order to obtain more insight into the structure and possible functions of LEs, we have elicited Mabs that recognize specifically LE components. Major components of LEs of the rat, with M_rs of 190000 and 30000–33000, have been identified by means of these antibodies (Heyting et al. 1987, 1989; Moens et al. 1987). In this article, we concentrate on the components with 30000–33000 M_rs; these are closely related proteins, as judged from their peptide maps and immunological reactivities (Heyting et al. 1989, Lammers et al. 1994): 18 independently isolated Mabs and one polyclonal antiserum recognize both the 30000 and the 33000 M_r proteins (Heyting et al. 1989; Lammers et al. 1994), and as yet, no antibody has been identified that can discriminate between these SC components. Using the anti-30000–33000 M_r antibodies, we have recently isolated a cDNA that encodes the 30000- to 33000-M_r components (Lammers et al. 1994). The encoded protein, called SCP3, is basic (pI=9.4) and has a molecular mass of 29.7 kDa. SCP3 has a potential ATP-binding site, and its C-terminal half contains stretches that are predicted to be capable of forming coiled-coil structures (Lammers et al. 1994). It has potential target sites for several protein kinases (Lammers et al. 1994). From hybridization of probes derived from SCP3 cDNA with Southern blots of rat genomic DNA, we concluded that there is only a single gene encoding the 30000- to 33000-M_r SC components (Lammers et al. 1994, and unpublished experiments). The nature of the difference between the 30000- and 33000 M_r SC components is not yet known; amino acid sequence analysis will be carried out to settle this point.

In this article we show that in vivo SCP3 not only displays variation with respect to M_r, but also with respect to pI. On two-dimensional (2D) gels, the 30000-

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to 33000- M_r components are resolved in at least 24 spots. These spots represent phosphorylation variants of the same protein (this paper). There are several reasons to speculate that protein phosphorylation plays a role in the coordination of events during meiotic prophase: (i), protein phosphorylation also plays a key role in the regulation of the mitotic cycle; (ii), in yeast, mutation of the *cdc28* gene, which encodes the *Saccharomyces cerevisiae* homologue of the $p34^{cdc2}$ protein kinase of *Schizosaccharomyces pombe*, causes an arrest in pachytene (Davidow and Byers 1984); (iii), the *MEK1/MRE4* gene of *S. cerevisiae* encodes a meiosis-specific protein kinase, which is directly or indirectly involved in SC assemply (Rockmill and Roeder 1991; Leem and Ogawa 1992); and (iv) other protein kinases which are not meiosis-specific, such as the products of the *HHR25* and *DBF2* genes in yeast, are also required for progression through meiotic prophase (reviewed in Hoekstra et al. 1991). We therefore analysed whether the phosphorylation patterns of the 30000- to 33000- M_r SC components changed during progression through meiotic prophase. We found that phosphorylated variants of the 30000- to 33000 M_r proteins occur throughout meiotic prophase. However, the extent of phosphorylation increases between early and mid-pachytene; we estimate that one phosphate group is then added to each of the variants. This change occurs at about the same time as the relaxation of the specificity of synapsis (Poorman et al. 1981) and ultrastructural alterations of the LEs (Dietrich and Boer 1983). We speculate that the 30000- to 33000 M_r components of SCs have a function in the regulation of sister chromatid interactions, and that a change in these interactions takes place at mid-pachytene.

Materials and methods

Purification of spermatocytes in successive stages of meiotic prophase. For the purification of spermatocytes in successive stages of meiotic prophase, we made use of vitamin A deficiency (VAD) to synchronize the development of spermatogenic cells. VAD causes an arrest of spermatogenic cells at the formtion of differentiating type A spermatogonia (van Pelt and de Rooij 1990a). Upon release from the VAD block by vitamin A suppletion, the spermatogenic cells resume their development synchronously (Morales and Griswold 1987; van Pelt and de Rooij 1990a, b).

Wistar rats were subjected to a vitamin A-deficient diet and were released from the VAD block as described before (van Pelt and de Rooij 1990a). On the basis of earlier observations (van Pelt and de Rooij 1990a), we decided to isolate spermatocytes at day 14, 15, 18, 20, 22, 26 and 30 after vitamin A supplementation. From all experimental animals, part of the testis was frozen for cryosectioning and immunostaining (Heyting et al. 1988), or fixed in Bouin's fixative for histological analysis (van Pelt and de Rooij 1990a). From the remainder of the testes, cell suspensions were prepared by a modification of the procedure of Romrell et al. (1976), as described earlier (Heyting et al. 1985). Spermatocytes were separated from other cell types by centrifugal elutriation (Bucci et al. 1986) in a Beckman JE6.1 rotor at 10°C, as described by Heyting et al. (1988) and Heyting and Dietrich (1991). In preliminary experiments (Heyting and Dietrich, unpublished) we had assessed the rotor speed and flow rates at which spermatocytes in successive stages of meiotic prophase could be isolated from the elutriator. The testicular cell suspensions were loaded at 2500 rpm and a flow rate of 15 ml/min; subsequently, the rotor speed was adjusted to 1800 rpm, and the flow rate was adjusted to the lowest value given in Table 1; elutriation was then continued until no more cells escaped from the rotor. Subsequently, the spermatocytes were collected by slowly adjusting the flow rate to the highest value given in Table 1.

The collected fractions were analysed by differential counts of Giemsa-stained preparations, as follows: pellets of harvested cell fractions were resuspended in as little phosphate-buffered saline as possible to yield a viscous suspension. With a drawn pasteur pipet, a microdroplet of cells was placed in the middle of a clean microscope slide. This drop, which had a diameter of less than 2

Table 1. Composition of cell fractions enriched in spermatocytes in successive stages of meiotic prophase

Day[a]	Flow rate (ml/min[b])	Composition of cell fraction[c]							
		Spermatogonia	Spermatocytes						
			Comp.	Exp.	EP	MP	M/LP	LP/D1	D	D2[d]
14	15–22.5	13.5	61.3	23.4	1.8	–	–	–	–	–
15	15–22.5	26.1	37.8	30.7	5.4	–	–	–	–	–
18	17.5–27.5	15.2	–	4.5	56.3	24.0	–	–	–	–
20	20–30	8.0	1.8	5.3	–	10.6	62.8	11.5	–	–
22	22.5–32.5	7.6	2.5	8.4	–	–	40.3	41.2	–	–
26	25–35	0.9	6.6	–	–	–	29.2	63.3	–	–
30	25–40	1.9	15.7	–	5.6	0.9	–	–	61.1	14.8
Contr.[e]	20–35	–	–	2.5	0.8	26.5	16.5	33.1	11.6	5.0

The composition of cell fractions was scored in Giemsa-stained preparations of cell nuclei, as described in the Materials and methods section. Unscorable/degenerated nuclei never exceeded 10% of the total cell fraction

[a] Day after release from vitamin A deficiency block, when cells were harvested

[b] Flow rates at which the cells were harvested from the elutriator

[c] Percentage of identifiable nuclei

[d] Comp., meiotic prophase stage with a small compacted nucleus; Exp., meiotic prophase stage with expanding nucleus. These stages most likely represent leptotene/zygotene. EP, presumptive early pachytene stage, sex vesicle not visible; MP, mid-pachytene stage, sex vesicle usually visible; M/LP, mid-late pachytene stage, sex vesicle always visible; LP/D1, late pachytene, pre-diffuse diplotene, sex vesicle always visible; D, diffuse diplotene, sex vesicle always visible; D2, late or post-diffuse diplotene, sex chromosomes condense from the sex vesicle. For illustrations of the stages, see Fig. 4

[e] Rats not subjected to vitamin A deficiency and vitamin A suplementation

mm, was touched with a larger drop of Carnoy's fixative. The cells were allowed to flatten, and two more drops of Carnoy's fixative were added. The slide was flame-dried and stained with 5% Giemsa in phosphate buffer, pH 6.8. With this treatment, nuclear stages from the spermatogenic line expand and the contours of the nuclei are revealed. Nuclei with an uninterrupted boundary were taken to be spermatogonial (Oud et al. 1979). For all experimental animals, the developmental stage of the spermatocytes was determined by immunocytochemical staining of frozen sections of the testes with monoclonal antibody II52F10. This antibody labels the lateral elements of synaptonemal complexes, and allows the distinction between the major substages of meiotic prophase (Heyting et al. 1987, 1988).

Antibodies. The monoclonal antibodies (Mabs) used in this paper were elicited and isolated as described by Offenberg et al. (1991); they are described in detail by Heyting et al. (1989) and Offenberg et al. (1991). The polyclonal anti- M_r 30000–33000 antiserum (serum 175) was elicited by immunization of a rabbit with rat SCs as described by Lammers et al. (1994). Although this serum was elicited against whole rat SCs, it recognizes predominantly the M_r 30000–33000 SC components (Offenberg et al. 1991). For the experiments in this paper we affinity-purified the anti- M_r 30000–33000 antibodies from this serum as described by Lammers et al. (1994).

Isolation of nuclei and synaptonemal complexes. Nuclei were isolated from the cell fractions by the hypotonic lysis/TritonX-100 method of Meistrich (1975). The isolated nuclei were pelleted, and per 2×10^7 nuclei we added: 7 µl DNAseI (400 µg/ml in Hanks' balanced salt solution), 1.5 µl RNAse A (250 µg/ml in 7 mM TrisHCl pH 7.0), and 1.5 µl trypsin inhibitor (10 mg/ml in 7 mM Tris-HCl pH 7.0). The nuclei were then resuspended by vortexing, and incubated for 1 h at room temperature. Subsequently, an equal volume of 10% SDS and 1/20 volume of deionized 0.5 M DTT were added, and the samples were stored at −80° C until 2D electrophoresis. SCs were isolated as described before (Heyting et al. 1985; Heyting and Dietrich 1991).

Electrophoresis and immunoblotting. 2D electroporesis of proteins from spermatocyte nuclei was performed according to O'Farrell (1975), with some minor modifications. Samples containing 10^7 nuclei (see above) were vortexed, heated for 10 min at 100° C and vortexed again for 5 min. Subsequently the sample was adjusted to 9.0 M urea, 10% Nonidet P-40, 2.67% ampholines. In most of the experiments we used a mixture of 4 parts ampholines pH 5–8 plus 1 part ampholines pH 3–10; this gives a good separation between pH 8.5 and 5.5; in some experiments we used a mixture of 1 part ampholines pH 5–8 and 1 part ampholines pH 8–10.5; this mixture provides a good resolution between pH 9.5 and 6.5. The samples were loaded onto isoelectric focusing tube gels under a solution of 8 M urea, 2% ampholines (the same mixture as was used for the tube gels), 2% Nonidet P-40, 1% DTT and 15 mM thioglycolic acid. The pH gradient after focusing was measured in four tube gels that were loaded with sample buffer only and run in parallel with the protein-loaded gels. After the isoelectric focusing run, the parallel tubes were divided in 0.5-cm pieces, which were extracted in a small volume of deionized water to measure the pH. Electrophoresis in the second dimension was performed on a 7–18% linear gradient SDS-polyacrylamide slab gel. 2D separation of SC proteins was performed in the same way, except that 4×10^7 SCs rather than 10^7 nuclei were used.

For the experiment in Fig. 2, dephosphorylation of SC proteins was performed by incubation of 4×10^7 SCs for 6 h at 37° C in 50 µl alkaline phosphatase buffer (0.1 M Tris-HCl, 5 mM $MgCl_2$ pH 8.0) containing 0.1 unit of *E. coli* alkaline phosphatase (Sigma cat. no. P4252) and protease inhibitors (0.02 mg/ml leupeptine, 0.1 mg/ml aprotinine, 0.1 mg/ml trypsin inhibitor and 1 mM PMSF). Prior to incubation the enzyme was dialysed overnight at 4° C against alkaline phosphatase buffer containing 1 mM PMSF. As controls we incubated SCs under otherwise the same conditions in alkaline phosphatase that had been inactivated by overnight dialysis against alkaline phosphatase buffer containing 11.25 mM of the specific inhibitor 8-hydroxyquinoline-5-sulfonic acid (Sigma cat. no. H1129) (Simpson and Vallee 1968) and 1 mM PMSF, and in alkaline phosphatase buffer with protease inhibitors, but without the enzyme. After incubation the SCs were washed twice in 8 mM TrisHCl pH 7.0, and dissolved for 2D electrophoresis as described above.

Immunoblotting was performed as described by Dunn (1986). Blots of 2D gels were probed with a mixture of Mabs IX3H3 and IX4D4 and affinity-purified anti-M_r 30000–33000 antibodies (Lammers et al. 1994). Binding of antibodies to the blots was detected by incubating the blots in goat-anti-mouse and goat-anti-rabbit alkaline phosphatase conjugate, and staining with BCIP and NBT, as described before (Heyting et al. 1988). 2D gels did not fit into our blotting apparatus, and were therefore cut in two halves before blotting. This is visible in Figs. 1B–E and 2D.

Results

SCs contain several phosphorylation variants of the 30000–33000 M_r *components*

In this study we make use of several independently isolated monoclonal antibodies (Mabs), which were elicited against purified SCs of the rat and recognize two broad protein bands on immunoblots of purified SCs, a 30000 M_r and a 33000 M_r band. On 1D Coomassie blue-stained SDS-gel electropherograms of SC proteins, these are the most prominent bands (Heyting et al. 1985). In addition, we make use of polyclonal anti-30000–33000 M_r antibodies isolated from an anti-SC antiserum by affinity purification (Lammers et al. 1994). In preparations of lysed spermatocytes, these antibodies bind specifically to the LEs/axial cores of SCs (Heyting et al. 1987, 1989; Moens et al. 1987). Because some variants of histone H1 comigrate with the 30000–33000 M_r components on 1D polyacrylamide SDS gels (Heyting et al. 1988), we also performed 2D separations of SC proteins (Fig. 1). For the experiment in Fig. 1 we performed six 2D separations of proteins from the same SC preparation. One of the resulting gels was stained with silver (Fig. 1F), and the other five were blotted onto nitrocellulose filters that were probed with different anti- M_r 30000–33000 antibodies (Fig. 1A–E). The 30000- to 33000- M_r SC components were resolved in a large number of spots, which focused at pH 6.5 to ≥8.5 (Fig. 1F). Most of the spots that show up on the silver-stained gel are recognized by one or more of the antibodies; the pattern in Fig. 1F is almost identical to that in Fig. 1A; only some spots at the basic side of the gel (indicated by arrows in Fig. 1F) are not recognized by the antibodies. These could represent variants of histone H1, or basic variants of the 30000- to 33000 M_r SC components that are not recognized by the antibodies. The immunoblots in Fig. 1B–E show even more spots than the silver-stained gel, presumably because the detection on Western blots is more sensitive than silver staining. Mabs IX3H3 and IX4D4 detect 24 spots (Fig. 1C, D); the polyclonal anti-30000- to 33000- M_r antibodies recognize 22 of these 24 spots; and Mabs IX7B12 and IX8G9 recognize 14 and 13 of

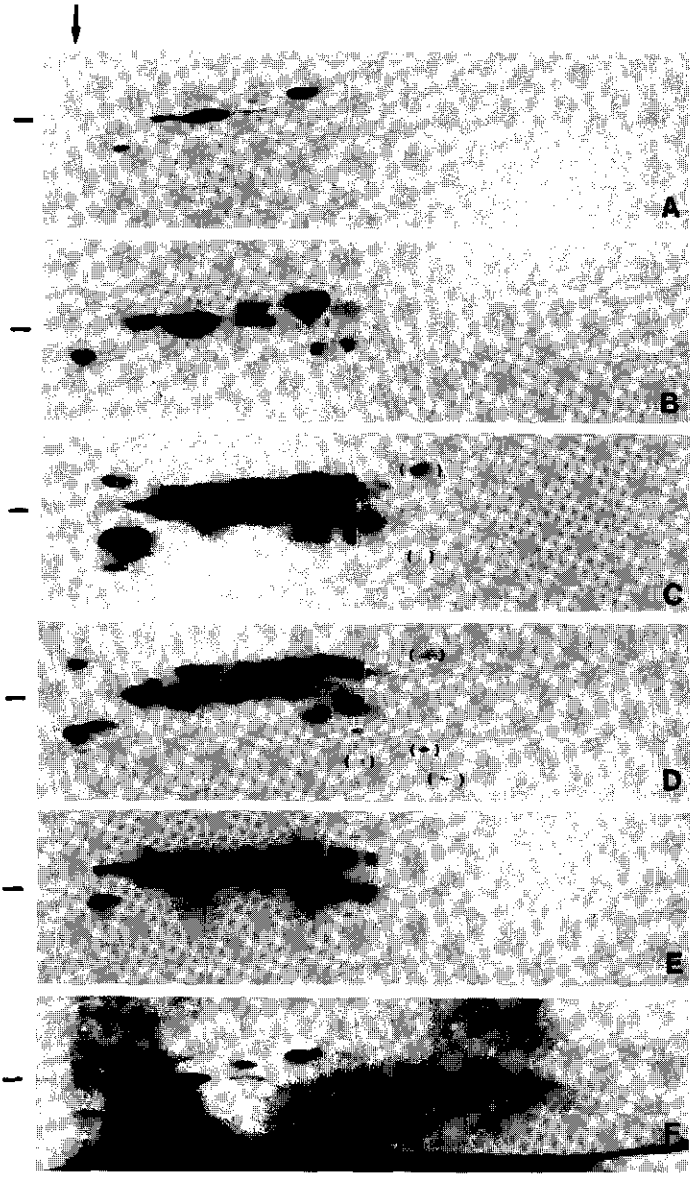


Fig. 1A–F. Immunoblot analysis of the 30000- to 33000-M_r SC components after 2D electrophoresis; six 2D separations of proteins of the same SC preparation were performed. Separation in the first dimension was performed in a single run on six parallel isoelectric focusing tubes. One of the 2D gels was stained with silver (F), the other five were blotted onto nitrocellulose. The resulting immunoblots were probed with the following anti-30000- to 33000-M_r antibodies: **A** Mab IX8G9, **B** Mab IX7B12, **C** Mab IX3H3, **D** Mab IX4D4, **E** polyclonal anti-30000- to 33000-M_r antibodies; the spots between brackets in **C** and **D** are due to a reaction with an anti-190000-M_r antibody (another SC component, Heyting et al. 1989), with which these immunoblots were probed before incubation in the anti-30000- to 33000 M_r Mabs. The *arrow* at the *top* of the figure indicates the position of the (basic) top of the isoelectric focusing gels. The *arrows* in **F** indicate spots that are not recognized by any of the anti-30000–33000 M_r antibodies. The position of the 31-kDa molecular weight marker (carbonic anhydrase) is indicated

these spots, respectively. On a more basic isoelectric focusing pH gradient (pH 9.7 to 6.5), we did not observe additional, more basic pI variants (experiments not shown). Thus, there are at least 24 variants of the 30000- to 33000- M_r SC components, differing from each other in pI value and/or electrophoretic mobility.

To find out whether the variation in pI was due to differences in phosphorylation, we treated synaptonemal complexes with alkaline phosphatase and then performed 2D separation of SC proteins. Figure 2 shows that after alkaline phosphatase treatment, most of the 30000- to 33000- M_r components focus at the basic side of the gel (pH≥8.5; cf. panels A and D). A straightforward interpretation of Fig. 2 is that SCs contain two major M_r variants in the 30000–33000 M_r range, a 30000 M_r and a 33000 M_r variant, each of which can be phosphorylated at 1–5 (30000 M_r) or 1–6 (3000 M_r) sites. Some of the more acidic 30000- to 33000- M_r variants were not completely dephosphorylated (Fig. 2A). This can be ascribed to the fact that whole SCs rather than

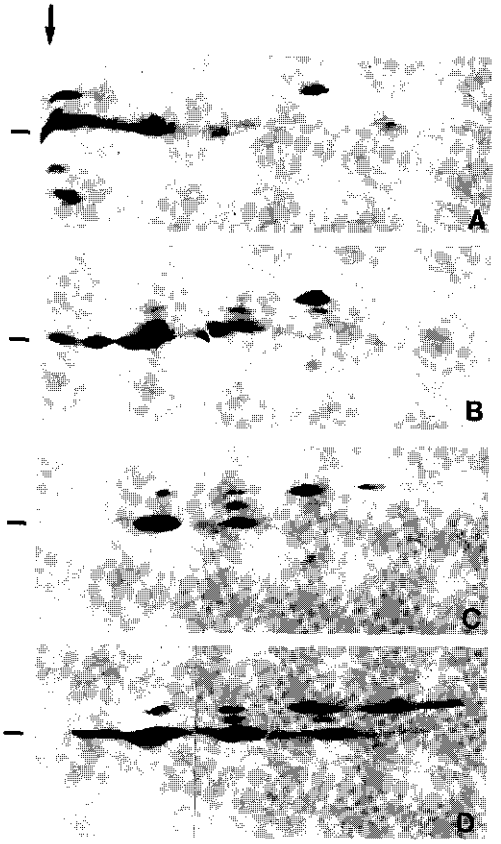


Fig. 2A–D. Immunoblot analysis of the 30000- to 33000-M_r components of SCs pretreated with **A** alkaline phosphatase, **B** alkaline phopshatase, inactivated with the specific inhibitor 8-hydroxyquinoline-5-sulfonic acid, or **C** alkaline phosphatase buffer only; **D** shows an immunoblot analysis of the 30000- to 33000- M_r SC components of SCs that had not been pretreated. Separation in the first dimension was performed in a single run on four parallel isoelectric focusing tubes. The immunoblots were probed with the polyclonal anti-M_r 30000–33000 antibodies. The *arrow* indicates the position of the (basic) top of the isoelectic focusing gel; *bars* indicate the position of the 31-kDa molecular weight marker (carbonic anhydrase)

isolated components were treated with alkaline phosphatase; this was necessary because we could only solubilize the 30000- to 33000- M_r components under conditions that inactivate alkaline phosphatase (experiments not shown). Particularly from Fig. 2D it appears that besides the major 30000- M_r and 33000- M_r components, SCs contain phosphorylation variants of at least two minor 30000- and 33000- M_r components.

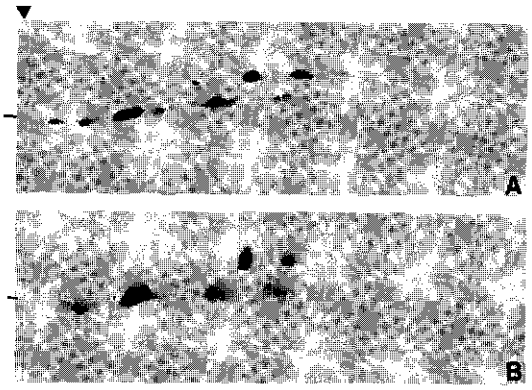


Fig. 3A, B. Immunoblot analysis of the 30000- to 33000-M_r SC components after 2D separation of proteins from isolated SCs (**A**) or from spermatocyte nuclei (**B**). Separation in the first dimension was performed in a single run on two parallel isoelectric focusing tubes. The *arrowhead indicates* the position of the (basic) top of the isoelectric focusing gel. *Bars* indicate the position of the 31-kDa molecular weight marker (carbonic anhydrase)

Preparation and analysis of cell fractions enriched in substages of meiotic prophase

Because it is possible that protein phosphorylation plays a role in the coordination of meiotic events (see Introduction), we decided to analyse the phosphorylation pattern of the 30000- to 33000- M_r SC components during progression through meiotic prophase. For this purpose we developed a procedure to obtain cell fractions from the rat testis that are enriched in separate stages of meiotic prophase. The procedure consists of the synchronization of spermatogenesis in the rat testis by release from a block in spermatogonial development, followed by purification of spermatocytes in specific stages of meiotic prophase from other cell types by elutriation (see Materials and methods section). Figure 5 shows micrographs of immunostained sections of rat testes, obtained at various days after release from the block (by vitamin A supplementation). At days 14 and 15, spermatocytes were in early zygotene (Fig. 5A), at day 18 in late zygotene/early pachytene (Fig. 5B), at day 20 and 22 in mid-pachytene (Fig. 5C), and at day 26 in late pachytene/early prediffuse diplotene (Fig. 5D); at day 30, most tubules contained two layers of spermatocytes: one consisting of zygotene, and one of diplotene spermatocytes (Fig. 5E, F). These results are in agreement with the analyses of the paraffin sections: at day 22 after vitamin A supplementation the spermatogenic epithelium was in stage II–VI (as defined by Leblond and Clermont 1952). In these stages, the spermatocytes are in early to mid-pachytene. At day 26, the epithelium was in stage VI–XI/XII (spermatocytes in late pachytene – early prediffuse diplotene); at day 30, the epithelium was in stage XIII–I (spermatocytes in zygotene/early pachytene, and in diplotene-metaphase I and II).

Combining the immunocytochemical and histological data with the nuclear images in the Giemsa-stained preparations yielded the subdivision of stages given in

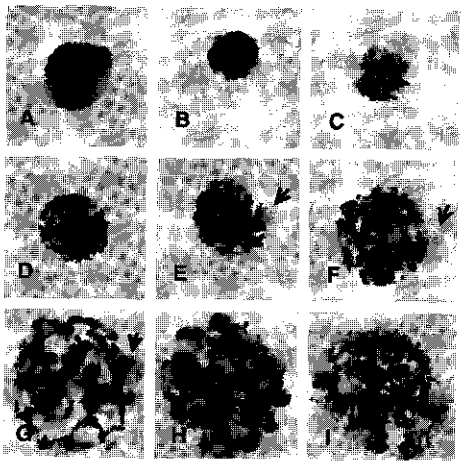


Fig. 4A–I. Photomicrographs of Giemsa-stained flame-dried spermatogenic nuclei, all at the same magnification. **A** Spermatogonium. **B** Meiotic prophase stage with compacted nucleus. **C** idem with expanding nucleus. **D** Presumptive early pachytene. **E** Mid–pachytene. **F** Mid-late pachytene. **G** Late pachytene/pre-diffuse diplotene. **H** Diffuse diplotene. **I** Post-diffuse diplotene. The *arrow* points at the sex chromosomes

Table 1. A detailed description of this analysis will be given elsewhere (de Boer et al., in preparation). Figure 4 shows micrographs of nuclei in Giemsa-stained preparations of the cell suspensions obtained after elutriation. The exact subdivision of leptotene, zygotene and early pachtene cannot be given, because in Giemsa-stained preparations only a gradual expansion of the nuclei can be observed during progression from zygotene to early pachytene. Therefore, the expansion of nuclei that occurs during these stages has been graded subjectively, and the designation „early pachytene" is a subjective one. Table 1 shows that the combination of synchronization and elutriation allows the isolation of relatively pure fractions of spermatocytes in separate stages of meiotic prophase. Although a relatively large percentage of the nuclei scored at days 14, 15 and 18 after vitamin A supplementation is spermatogonial, this does not interfere with the analysis of the 30000- to 33000- M_r SC components, because these proteins do not occur in spermatogonia (Heyting et al. 1988).

The phosphorylation pattern of the 30000- to 33000- M_r *proteins changes at mid-pachytene*

The phosphorylation of the 30000–33000 M_r SC components was analysed in whole spermatocyte nuclei rather than SCs, because SCs from zygotene and early pachytene spermatocytes are preferentially lost during the SC isolation procedure (Heyting et al. 1985; and unpublished experiments). Figure 3 shows that the pattern of 30000- to 33000- M_r spots is the same for proteins from whole spermatocyte nuclei and from isolated SCs. Figure 6 shows the immunoblot analysis of the 30000- to 33000- M_r proteins after 2D separation of proteins from

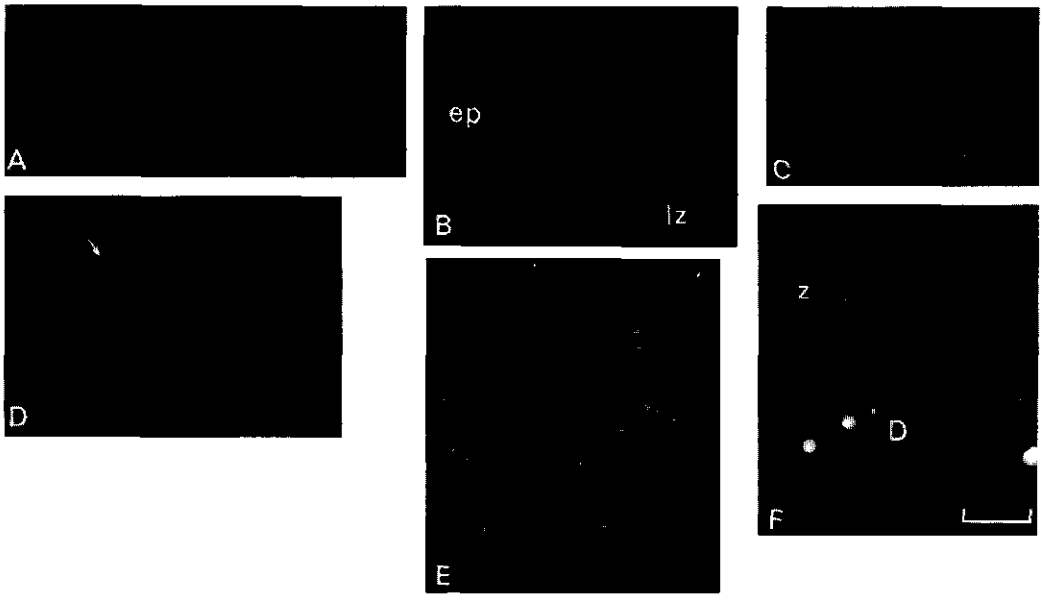
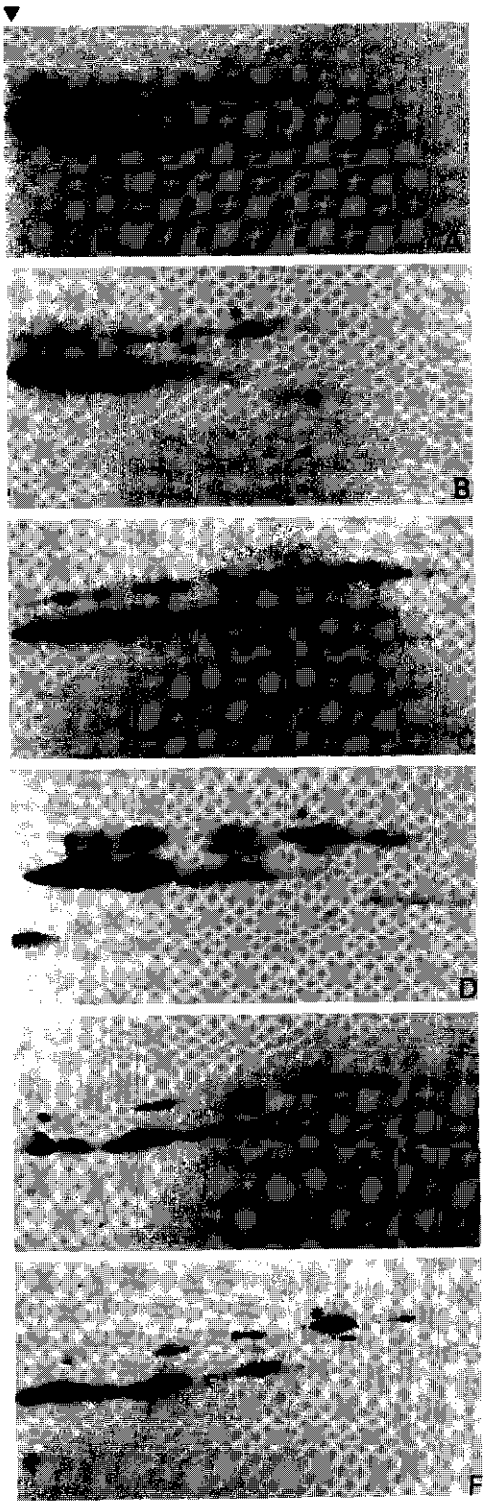


Fig. 5A–F. Immunofluorescence micrographs of spermatocytes in frozen sections of testes from the rats that were used for the experiment in Fig. 6. Immunofluorescence labelling was performed with a monoclonal antibody against the lateral elements of SCs. **A** Day 15 after release from the vitamin A deficiency block (early zygotene). **B** Day 18; late zygotene (*lz*), early pachytene (*ep*). **C** Day 22 (mid-pachytene). **D** Day 26 (late pachytene/early prediffuse diplotene; the *arrow* points at a site where desynapsis has already started). **E** Day 30, (diplotene). **F** Day 30; zygotene (*z*), diffuse diplotene (*D*). *Bar* 10 μm



spermatocyte nuclei in successive stages of meiotic prophase. Between day 18 (late zygotene/early pachytene) and day 20 (mid-pachytene) after release from the VAD block the phosphorylation pattern changes. The overall spot pattern remains the same, but for samples obtained on day 20 or later the spots shift further to the acidic side of the gel than for samples obtained on day 18 or earlier. In a separate experiment, we found that the spot pattern obtained on day 22 after release from the VAD block was identical to that obtained on day 26 (not shown). Most probably, this shift is due to a more extensive phosphorylation beyond day 18. For one easily recognizable spot (marked with asterisks in Fig. 6) we estimated the increase in the number of phosphate groups per protein molecule: between early (day 18) and late (day 26) pachytene, the pI value of the marked spot shifts from 6.9 to 7.2. From the predicted titration curve of SCP3 we estimate that a pI=7.2 variant of SCP3 carries three phosphate groups and a pI=6.9 variant four, provided that the pI variants to not carry other charged modifications than phosphate groups. Thus, the most straightforward interpretation of the results in Fig. 6 is that one phosphate group is added to each of the pI variants between early (day 18) and mid (day 20)-pachytene. As a control, we analysed the M_r 30000–33000 spots for a sample containing predominantly late pachytene and prediffuse diplotene spermatocytes (Table 1), which was obtained from rats that had *not* been subjected to the synchronization procedure. The pattern of spots is the same as the patterns obtained for day 26 and 30 after release from the VAD block; this indicates that the synchronization procedure does not influence the phosphorylation pattern.

Discussion

In this article we show that the major components of the lateral elements of SCs of the rat, the 30000- to 33000-M_r proteins, are phosphoproteins and that their phosphorylation pattern changes between early and mid-pachytene. For the analysis of the phosphorylation of these proteins throughout meiotic prophase, we developed a procedure to obtain cell fractions from the rat testis that are enriched in spermatocytes in successive stages of meiotic prophase.

←

Fig. 6A–F. Immunoblot analysis of the 30000- to 33000-M_r SC components after 2D separation of proteins from nuclei from spermatocytes in successive stages of meiotic prophase. Spermatocyte nuclei were isolated from vitamin A-deficient rats at successive days after release from the vitamin A deficiency block. **A** Day 15 (zygotene). **B** Day 18 (late zygotene/early pachytene). **C** Day 20 (mid pachytene). **D** Day 26 (late pachytene). **E** Day 30 (diplotene). **F** shows the 30000–33000 M_r components from spermatocytes, isolated from untreated rats; these spermatocytes were mainly late pachytene/prediffuse diplotene spermatocytes (see Table 1 for the composition of the cell fractions). Separation in the first dimension was performed in a single run on six parallel isoelectric focusing tubes. The *arrowhead* indicated the position of the (basic) top of the isoelectric focusing gels. *Asterisks* explained in the text

Purification of spermatocytes in successive stages of meiotic prophase

The rat testis is one of the best sources of meiotic prophase cells in quantities sufficient for biochemical analysis. This is particularly true for late pachytene and diplotene spermatocytes, which can easily be separated from other testicular cells by centrifugation (Meistrich 1975; Bucci et al. 1986). Zygotene and early pachytene cells are less easily purified, because the density and sedimentation behaviour of these cells is not very different from those of B-type spermatogonia on the one hand, and mid-pachytene cells on the other (Bucci et al. 1986). Nevertheless, for the biochemical analysis of chromosomal changes during progression through meiosis, it is essential to obtain cell fractions enriched in each of the successive stages of meiotic prophase. The combination of cell separation by centrifugation with the in vivo developmental synchronization of spermatogenic cells turned out to be useful for the isolation of such fractions (Table 1). The characterization of the cell fractions obtained in this way left us with one problem, namely the unequivocal correlation of the state of the synaptonemal complex, which is decisive for the classification of spermatocytes, with the well-known nuclear images in Giemsa-stained preparations. It is not possible to decide on the basis of Giemsa-stained preparations alone whether a nucleus originates from a leptotene, zygotene or early pachytene cell. The nuclei gradually expand during progression through these stages (see Fig. 4; Leblond and Clermont 1952; Oud et al. 1979; Dietrich and de Boer 1983; Heyting and Dietrich 1992), but for an individual nucleus it cannot be decided from which stage it originates, because other than developmental factors, for instance the local spreading conditions, may have influenced the expansion of that nucleus. The classification of the early spermatocyte stages (Comp., Exp., and EP in Table 1) is therefore a subjective one. This classification problem was met in part by the in vivo developmental synchronization of spermatogenic cells. From the immunofluorescence staining of sections, it was clear that the synchronization had been quite effective, and that in individual animals most or all of the spermatocytes were in a narrow developmental range. The width of this range, as estimated from the histological analyses, corresponds to 2–3 days. This is in agreement with earlier estimations of the degree of synchronization after release from the VAD block (van Pelt et al. 1990a). We therefore infer that on days 14 and 15 most of the spermatocytes in the collected cell fractions must have been in leptotene/early zygotene, and on day 18 in late zygotene/early pachtene, although no exact numbers can be given. This classification problem will be discussed in detail elsewhere (de Boer et al., in preparation).

The procedure for the isolation of cell fractions enriched in spermatocytes in successive stages of meiotic prophase will be indispensible for the dissection of the biochemical alterations at the DNA and protein level during progression through meiosis. In this article this is illustrated by the analysis of the phosphorylation pattern of the 30000- to 33000- M_r SC components.

Phosphorylation pattern of the 30000- to 33000- M_r SC proteins

In earlier publications (Heyting et al. 1988, 1989; Lammers et al. 1994) we presented evidence that the 30000- to 33000 M_r SC components are closely related; most probably, they are products of the same gene (Lammers et al. 1994). In this paper we show that several phosphorylation variants of these proteins occur in synaptonemal complexes. Interestingly, different phosphorylation variants coexist in spermatocytes of the same stage. In late pachytene/diplotene spermatocytes, the 33000- M_r protein carries 1–6 phosphate residues (major variant: 4 residues), and the 30000- M_r protein 1–5 residues (major variant: 2 residues). The relative intensity of the spots is fairly reproducible (cf. Figs. 2D, 3, 6F). We have not yet been able to identify the amino acid residues that are phosphorylated in vivo, because the 30000- to 33000- M_r SC components can only be purified in small amounts from synaptonemal complexes, which have to be isolated from pure rat spermatocytes (Heyting et al. 1985), and because in vivo labelling of these proteins with ^{32}P is practically impossible in the rat. However, the predicted amino acid sequence of SCP3 contains several potential protein kinase target sites, including two potential cAMP/cGMP-dependent protein kinase target sites, and several potential protein kinase target sites for protein kinase C, casein kinase II and DNA-activated protein kinase (DNA-PK) (Lammers et al. 1994). The DNA-PK sites, S/T-Q (serine/threonone-glutamine) (Lees-Miller et al. 1992; Bannister et al. 1993), are of particular interest: DNA-PK is activated by free DNA ends (Gottlieb and Jackson 1993), and by a variety of DNA structures containing double-to-single strand transitions that could be recombination intermediates (Morozov et al. 1994). Among the substrates for DNA-PK are various DNA-binding proteins that are involved in transcription, replication, repair or recombination [reviewed by Anderson (1993) and Gottlieb and Jackson (1994)]. It has been suggested that DNA-PK plays a role in coordinating the use of DNA segments as templates for these processes (Anderson et al. 1993). All known substrates of DNA-PK are phosphorylated at several sites (Anderson and Lees-Miller 1992). Because (i) SCP3 has six potential target sites for DNA-PK, (ii) SCP3 and the corresponding 30000- to 33000 M_r SC proteins are capable of binding to DNA (Lammers et al., unpublished experiments), (iii) the 30000- to 33000 M_r proteins are major components of a structure (the SC) which is supposed to have a regulatory role in meiotic recombination, and (iv) the 30000- to 33000- M_r SC components are phosphorylated at several sites (this paper), DNA-PK is a candidate kinase that might be responsible for the multiple phosphorylations of the 30000- to 33000- M_r SC components.

Other potential phosphorylation sites than S/T-Q that deserve attention are two potential target sites for cAMP/cGMP-dependent protein kinases (PKA). One of these sites overlaps with the consensus sequence for nucleotide binding (Lammers et al. 1994). Two other SC proteins, namely SCP1 and SCP2, also contain potential PKA target sites (Meuwissen et al. 1992; Offenberg et al., in preparation). For these potential PKA sites of

SCP3, it will be important to analyse whether they are phosphorylated in vivo, and, if so, when.

The change in the phosphorylation of the 30000- to 33000- M_r SC components is the first alteration observed at the molecular level in LEs. It correlates in time with structural alterations of the SC: first of all, synaptic adjustment starts in early pachytene of mice (Poorman et al. 1981); and second, Dietrich and de Boer (1983) observed, also in mice, that between zygotene/early pachytene and mid pachytene the LEs become thicker and morphologically better defined. In mice and rats, it is as yet impossible to assess the functional significance of these changes. However, on the basis of work done on yeast (Schwacha and Kleckner 1994; Collins and Newlon 1994) it is tempting to speculate that in the first half of meiotic prophase the axial cores/LEs of SCs have a role in the prevention of recombination between sister chromatids; it is possible that later in prophase, when initiation of recombination has stopped (Padmore et al. 1991), and DNA–DNA interactions between homologous non-sister chromatids have been established (Schwacha and Kleckner 1994), this control has to be relaxed, in order to make the sister chromatid available as a template for the repair of those initiated recombination events, for which there was no template on the homologous chromosome (see discussion in Schwacha and Kleckner 1994). The 30000- to 33000- M_r SC components could be involved in such a functional switch of the LEs.

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Chapter 4

Identification of an SC-associated region in the *Hsp70-Bat5* gene cluster of the mouse MHC III locus which colocalizes with a MAR/SAR

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Abstract

We have tried to identify the DNA-sequences which are bound to the mouse synaptonemal complex (SC) by means of immunoprecipitation of meiotic chromatin crosslinked *in vivo*, with specific anti-SC antibodies. We performed our search for SC-bound DNA-fragments in the *Hsp70-Bat5* gene cluster of the mouse MHC III region and identified an SC-associated region (SCAR) between genes *G7b* and *G7e*. Sequences within the identified SCAR behaved as a MAR/SAR in nuclear matrix binding experiments *in vitro*. Moreover, two known MARs from other organisms bound specifically to isolated SCs in binding assays *in vitro*. These results indicate that chromatin organization in meiotic prophase resembles those in mitosis and interphase.

Introduction

According to the scaffold-loop model, the chromatin in mitotic chromosomes is organized in loops, which are attached by special DNA-regions, the scaffold attachment regions (SARs), to a scaffold. In a mitotic chromosome each chromatid has its own scaffold. In meiotic prophase chromosomes, the chromatin is also organized in loops (Weith and Traut, 1980), but an important difference with mitotic chromosomes is, that the two chromatids of each chromosome share a single, proteinaceous axis, called axial core. As meiotic prophase proceeds, the axial cores of homologous chromosomes are connected along their length to form a synaptonemal complex (SC). In the SC the axial cores are referred to as lateral elements (LEs). There is ample evidence that SCs are not only involved in chromosome pairing, but also in meiotic recombination. Their precise role, however, still has to be elucidated. The differences between chromatin organization during mitosis and meiotic prophase might be related to meiosis-specific features such as cohesiveness of sister chromatids, preferential non-sister recombination and the regulation of the number and the position of recombinational events.

Until now, the relationship between the mitotic scaffold and the meiotic axial core has been unclear. Rufas *et al.* (1992) proposed that the scaffolds of sister chromatids make part of the axial cores. In rodents, the single axial core becomes less compact as meiotic prophase proceeds and displays a multistranded appearance in surface-spread preparations (Del Mazo and Gil-Alberdi, 1986). Sometimes two subelements, which might correspond to the separate scaffolds of the sister chromatids are discernible in association with the axial core. Two major components of meiotic axial cores, however, do not make part of the mitotic chromosome scaffold (Offenberg *et al.*, 1991). Moreover, it is unknown if the modes of attachment of chromatin to the scaffolding structure during mitosis and meiotic prophase are the same.

Sequences that are retained by mitotic chromosome scaffolds or the interphase nuclear matrix (after removal of the histone-depleted chromatin loops with DNAses or restriction enzymes) are termed scaffold attachment regions (SARs) or matrix attachment regions (MARs). SARs/MARs are therefore proposed to represent the bases of the mitotic and interphase chromatin loops (Laemmli *et al.*, 1992). These sequences have been implicated in various nuclear processes including replication, transcription, repair and recombination (reviewed by Boulikas (1995)). If the basic organization of meiotic prophase chromatin resembles that of mitotic chromatin, one would expect the presence of SARs/MARs at the bases of the meiotic chromatin loops.

Attempts to characterize the DNA-sequences which are associated with SCs (SC-associated DNA or SC-DNA; Karpova *et al.*, 1989 and 1995; Pearlman *et al.*, 1992) revealed

the possible enrichment of certain repetitive elements. Pearlman *et al.* (1992) observed no obvious resemblance between the analyzed SC-DNA sequences and SARs/MARs at the primary sequence level.

We have set out to identify the DNA-sequences bound to the mouse SC by means of immunoprecipitation with specific anti-SC antibodies, after crosslinking *in vivo* of meiotic chromatin. The isolated DNA sequences were used to identify SC-DNA within a defined region of the mouse genome, the *Hsp70-Bat5* gene cluster of the class III MHC locus. In this region a hotspot for meiotic recombination has been localized (Snoek *et al.*, 1991 and 1993), and the sequence of a large part of this region is available. Hotspots are regions along chromosomes at which meiotic recombination is more often initiated than at others. Several hotspots in yeast and higher eukaryotes have been characterized (reviewed by Lichten and Goldman, 1995). In yeast many of these lie within regions of accessible chromatin, as shown by their nuclease hypersensitivity (Shenkar *et al.*, 1991; Wu and Lichten, 1994; Ohta *et al.*, 1994; Liu *et al.*, 1995). This enhanced accessibility of these chromatin regions was not only found during meiotic prophase but also in mitosis (Wu and Lichten, 1994). Thus, chromatin structural features that are not specific for meiosis might be involved in determining where meiotic recombination is initiated.

One investigator (Kleckner, 1996) proposed that meiotic chromatin organization is established essentially as in mitosis but that meiosis-specific alterations promote interhomolog interactions. In this view, sites with a potential for interhomolog interactions end up near the bases of the chromatin loops as a result of an axis-mediated organization. A subset of these sites would, at the same time, be involved in pairing interactions that would modify chromatin organization in such a way that when homology is encountered these homologous sites become located at homologous positions in both chromosomes. Finally, the recombination machinery would, independently, assemble at/near these sites and recombination would be preferentially initiated near the bases of the chromatin loops. Therefore, it would be of great interest to determine the positions of recombination hotspots relative to SARs/MARs and to find out whether, and if so, when they are located at the bases of the chromatin loops during meiotic prophase.

We report the identification of an SC-associated region (SCAR) within the *Hsp70-Bat5* gene cluster of the MHC III region of the mouse, which colocalizes with a SAR/MAR. On the basis of *in vitro* SC-binding experiments, an additional number of potential SCARs has been identified within this gene cluster, most of which colocalize with a SAR/MAR. These observations will be discussed in the context of models that describe the role of chromatin organization in meiotic recombination.

Experimental procedures

Antibodies

Polyclonal anti-M_r 30,000-33,000 SC antibodies (serum 175) were elicited against purified SCs as described by Offenberg *et al.* (1991) and Lammers *et al.* (1994, Chapter 2). Serum 175 recognizes predominantly the M_r 30,000-33,000 SC components, but also other SC proteins. Affinity-purified anti-M_r 30,000-33,000 SC antibodies were obtained using Westernblot strips as described by Lammers *et al.* (1994, Chapter 2) and by affinity chromatography of serum 175 on a Sepharose 4B column carrying SCP3 (i.e. the bacterial translation product of a cDNA clone encoding the M_r 30,000-33,000 SC proteins), according to the instructions of the manufacturer (Pharmacia, Uppsala, Sweden). Immunoblotting and detection were carried out as described by Dunn (1986) and Heyting and Dietrich (1991).

Isolation of spermatocytes and formaldehyde crosslinking

Spermatocytes were isolated from male C57Bl mice by means of elutriation as described for rat spermatocytes by Heyting *et al.* (1988), except that mouse spermatocytes were collected at slightly lower flow rates (18-30 ml/min). About 10^8 spermatocytes were resuspended in PBS at 33°C to a final concentration of 5 x 10^6 cells/ml. Crosslinking *in vivo* was performed by addition of an equal volume of prewarmed 2% paraformaldehyde in PBS (33°C) and incubation for 8 min at 33°C, in a shaking waterbath. Subsequently, an equal volume of ice-cold PBS was added, the cells were pelleted at 200 x g for 5 min at 4°C, washed for 10 min in PBS at 0°C and again pelleted.

Isolation and purification of chromatin fragments crosslinked *in vivo*

Fragments of chromatin crosslinked *in vivo* were obtained by sonication and subsequent purification of protein-DNA complexes by CsCl density centrifugation essentially as described by Orlando and Paro (1993). Briefly, cells were lysed by incubation in 0.25% Triton X-100, 10 mM NaEDTA, 0.5 mM NaEGTA, 10 mM Tris-HCl (pH 8.0) to a final concentration of 2 x 10^7 nuclei/ml. Portions of 750 μl were sonicated on ice in a MSE soniprep 150 sonicator, using the microtip at an amplitudo of 14 μm, for three bursts of 30 sec each. This yielded DNA fragments with a length of 300-3000 bp (average length 1500 bp). The volume of the sonicated nuclei was adjusted to 12 ml with 1 mM NaEDTA, 0.5 mM NaEGTA, 10 mM Tris-HCl (pH 8.0) (room temperature), and 0.6 ml 10% sarcosyl was added. After a 10 min incubation at room temperature, the suspension was centrifuged at 16,000 x g for 10 min at room temperature to remove undissolved nuclear debris. The supernatant was adjusted to a density of 1.42 g/ml with CsCl, divided into portions of 5 ml/tube and centrifuged in a Beckman SW50 rotor, at 40,000 rpm for 72 h at 20°C. Fractions of 250 μl were collected and dialyzed overnight at 4°C against 5% glycerol, 1 mM EDTA, 0.5 mM EGTA, 10 mM Tris-HCl (pH 8). The concentration and size distribution of DNA fragments in these fractions was determined after reversal of crosslinks (see below) by measuring the fluorescence at 460 nm and electrophoresis on a 1% (w/v) agarose gel containing ethidium bromide. The presence of SC protein within the fractions was assayed after

addition of 25 μl of 400 μg/ml of DNAse I in TIM (104 mM NaCl, 45 mM KCl, 1.2 mM $MgSO_4$, 0.6 mM KH_2PO_4 (pH 7.3) and 1 μl of 1 M $MgCl_2$ to 100μl aliquots of all dialyzed gradient fractions, followed by incubation for 3 h at 37°C. Samples were then applied to a nitrocellulose membrane using a dotblot apparatus. SC proteins were visualized on the dot blots by immunodetection, as decribed before for Western blots (Heyting and Dietrich, 1991).

Immunoprecipitation of chromatin fragments crosslinked *in vivo*

Aliquots of 400 μl of purified DNA-protein complexes in RIPA-buffer (1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, 140 mM NaCl, 1 mM phenylmethylsulphonate fluoride (PMSF)), containing 0.5% of blocking agent (Boehringer, Mannheim), were precleared by adding 60-250 μl of 50% (v/v) protein A-Sepharose CL4B (Pharmacia) in RIPA-buffer, abd subsequently incubated for 2 h at 25°C. Protein A-Sepharose CL4B was removed by centrifugation at 200 x g for 5 min. For immunoprecipitation 1-3 μl of polyclonal anti-M_r 30,000-33,000 SC antibodies, 1.6 ml of anti-M_r 30,000-33,000 SC antibodies affinity-purified on Westernblot strips (Lammers *et al.* (1994; Chapter 2) or 1 ml of anti-M_r 30,000-33,000 SC antibodies purified on an affinity column (see above) were added and incubated on a rocker platform for 1 h at 25°C, followed by overnight incubation at 4°C. To the control reactions no antibodies were added. The same amount of protein A-Sepharose beads as was used for preclearing was then added and incubation was continued for 2 h at 25°C. Immunocomplexes were pelleted and washed as described by Orlando and Paro (1993).

Decrosslinking and amplification of immunoprecipitated DNA

Immunocomplexes were incubated for 1 h in 50 μg/ml of DNAse-free RNAseA, 10 mM Tris-HCl (pH 8), 1 mM EDTA at 37°C. SDS and proteinase K (Boehringer, Mannheim, Germany) were added to final concentrations of 0.25% and 0.25 mg/ml respectively, and incubated at 37°C for 3-6 h followed by overnight incubation at 56°C. Decrosslinking was completed by incubation at 65°C for at least 6 h. DNA was then isolated by phenol-chloroform extractions and precipitation as described by Orlando and Paro (1993).

The isolated DNA (typically 20-30 ng per immunoprecipitation) was amplified by adapter-mediated PCR, without the use of restriction enzymes in order to avoid preferential amplification of certain fragments within the sample. DNA fragments were blunted using T4 DNA polymerase for 30 min at 37°C, extracted with successively phenol-chloroform and chloroform, and precipitated in the presence of 2 μg of glycogen (Boehringer). DNA fragments were dissolved in a buffer containing 30 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP and 0.5 μM of adaptor. Ligation was performed overnight at 4°C, in a final volume of 10 μl, by the addition of 1.5 U of T4 DNA ligase (Promega, Madison, Wi, USA). Two adapters were used in these ligation experiments. The *Eco*RI adaptor consisted of oligonucleotides of sequences 5'-AATTCATGGTACCTGATCTAGACG-3' (Eco3) and 5'-CGTCTAGATCAGGTACCATG3' (Eco4) of which the latter was phophorylated at the 5'-end. The *Hin*dIII adaptor consisted of oligonucleotides of sequences 5'-CAAGCTTCACTCAGTCGACC-3' (*Hin*dIII-I) and

5'-GGTCGACTGAGTGAAGCTTG-3' (*Hin*dIII-II, phosphorylated at the 5'-end). Amplification of ligated products was carried out by adding 1-2.5 μl of ligation mixture to a final volume of 100 μl containing 0.4 μM of either the Eco3 or *Hin*dIII-I oligonucleotide and 1 U of Goldstar DNA polymerase (Eurogentec, Seraing, Belgium) for PCR. We performed PCR in a Pharmacia Gene Attaq apparatus using 1 cycle at 94°C for 5 min; 30 cycles at 94°C for 30 s, at 48°C for 30 s, and at 72°C for 3 min; and 1 cycle at 94°C for 30 s, at 48°C for 30 s, and at 72°C for 10 min. PCR products were analysed on 1% agarose gels. Unincorporated adaptors were removed from the PCR products by ethanol precipitation.

Isolation of SC-associated DNA

SCs were isolated as described before (Heyting *et al.*, 1985; Heyting *et al.*, 1987). During the course of the isolation procedure, the chromosomal DNA is degraded by means of an extensive DNAseII digestion. The DNA still contained by SCs after this procedure is designated SC-DNA. To isolate this DNA, the SC containing fraction obtained after sucrose gradient centrifugation was incubated for 1 h at 37°C in 50 μg/ml of RNAse A. Subsequently, SDS was added to a final concentration of 0.5%, followed by incubation at 65°C for 30 min. Finally, proteinase K was added to a final concentration of 25 μg/ml and incubation was continued overnight at 56°C. Samples were then extracted with phenol-chloroform and chloroform, and the DNA was precipitated with ethanol. DNA fragments were amplified by means of adapter-mediated PCR as described above.

Hybridization of dotblots and Southern blots

DNA probes were labelled with [α-^{32}P]dATP by means of random primed labelling according to Sambrook *et al.* (1989). For the preparation of blots Hybond-N+ membranes (Amersham) were used. Blots were prehybridized for at least 3 h at 65°C in 6x SSC, 5x Denhardt's solution, 1% SDS, 0.01% Na-pyrophosphate and 150 μg/ml of denatured herring sperm DNA. Hybridization was performed after addition of 25 ng of radioactively labelled probe (10^8 cpm/μg), for 20 h at 65°C. Blots were subsequently washed for 30 min at 65°C in, successively, 2x SSC; 2x SSC, 1% SDS; 1x SSC, 0.1% SDS and 0.1x SSC, 0.1% SDS.

For Southern blot analysis of the *Hsp70-Bat5* region, 20 x 20 cm 0.7% horizontal agarose gels were run overnight in Tris-borate buffer at 1.5 V/cm. Southern blots were prepared by standard procedures (Sambrook *et al.*, 1989) and the DNA was fixed by baking at 80°C for 2 h.

For the preparation of dotblots DNA samples were heated to 95°C and chilled on ice. One volume of 20x SSC was added and the desired amount of DNA was applied to each well of the dotblot apparatus. The membrane was prewetted in 10x SSC before DNA was applied. The DNA on the blot was denatured by immersion in 1.5 M NaCl, 0.5 M NaOH, for 5 min, followed by transfer to a neutralization buffer (1.5 M NaCl, 0.5 M Tris-HCl (pH 7.4), 1 mM EDTA). The DNA was fixed by baking at 80°C for 2 h.

Quantitative analysis dotblots was performed by phosphorimager analysis (Molecular Dynamics, Kensing, UK; ImageQuant program version 3.3).

Subtraction of immunoprecipitated DNA

Subtraction of immunoprecipitated DNA with mouse genomic DNA was performed essentially as described by Klickstein (1992). Briefly, PCR-amplified, immunoprecipitated DNA fragments were digested with *Xba*I, which cuts at least the adapters at both ends of the fragments, but possibly also at internal sites. The DNA was subsequently extracted with phenol-chloroform and chloroform and precipitated with ethanol. Genomic DNA was isolated from livers of C57Bl mice according to Sambrook *et al.* (1989) and digested with *Eco*RV and *Hin*cII, which resulted in blunt fragments with an average size of about 4 kb. Subtraction was performed with ratios of immunoprecipitated fragments to genomic DNA fragments of 1:5, 1:10 and 1:50 (w/w) with 200 ng of immunoprecipitated DNA in all cases. After subtraction the DNA was purified by means of phenol-chloroform extraction and ethanol precipitation. The DNA was dissolved in ligation buffer to which 100 ng of *Xba*I-digested and subsequently dephosphorylated pBluescript SK- vector (Stratagene, La Jolla, Ca, USA) was added. Ligation was performed overnight at 15°C and resulting constructs were used to transform *E. coli* XL1 Blue cells. One half of the transformation mixture was plated on LB plates containing 100 μg/ml of ampicillin and inspected for the number of white potential transformants. The other half was used to inoculate 50 ml of fresh LB containing 100 μg/ml of ampicillin. After overnight incubation at 37°C cells were harvested and DNA was isolated using the Qiagen Plasmid Midi Kit (Qiagen). Inserts were labelled by means of standard PCR, using oligonucleotides present in the multiple cloning site of the pBluescript vector, in the presence of [α-^{32}P]dATP.

Isolation of nuclear matrix and mapping of matrix attachment regions

Preparation of nuclear matrix and and mapping of MARs/SARs was performed essentially as described by Mirkovitch *et al.* (1984) and Ludérus *et al.* (1992). Briefly, nuclei from rat liver cells (isolated as described by Izaurralde *et al.*, 1988) were subjected to a lithium-3,5-diiodosalicylate (LIS) extraction protocol (Mirkovitch *et al.*, 1984). Nuclei of 10^7 cells were washed once in 10 ml of washing buffer (3.75 mM Tris-HCl (pH 7.4), 20 mM KCl, 0.5 mM EDTA, 0.125 mM spermidine, 0.05 mM spermine, 1% (v/v) thioglycol, 0.1% (w/v) digitonin and 20 μg/ml of aprotinin). After pelleting (300 x g for 10 min at 4°C) nuclei were gently resuspended in 0.5 ml of washing buffer and stabilized by incubation for 20 min at 42°C. Non-matrix protein was extracted by adding 10 ml of 10 mM LIS in extraction buffer (20 mM Hepes-KOH (pH 7.4), 100 mM lithium acetate, 1 mM EDTA, 0.1 mM PMSF, 0.1% (w/v) digitonin and 20 μg/ml aprotinin), followed by incubation for 15 min at 25°C. The resulting nuclear halos were collected by centrifugation (15,000 x g, for 5 min at 4°C) and washed four times with 10 ml of digestion buffer (20 mM Tris-HCl (pH7.4), 70 mM NaCl, 20 mM KCl, 10 mM $MgCl_2$, 0.125 mM spermidine, 0.05 mM spermine and 10 μg/ml aprotinin). For the MAR/SAR binding assay rat nuclear matrices were obtained by digestion of the genomic DNA of the halos in 1 ml of digestion buffer containing 1000 units each of *Eco*RI, *Hin*dIII and *Xho*I, for 2 h at 37°C. Matrix preparations were adjusted to a final concentration of 15 mM EDTA and 120 μg/ml of *E. coli* competitor DNA. To identify MARs/SARs, nuclear matrices from 10^6 cells were incubated overnight at 37°C

with 15 ng of the [^{32}P]-labeled DNA fragments tested. Fragments were labeled by end-labeling as described by Sambrook *et al.* (1989). After separation into pellet and supernatant fraction by centrifugation (15,000 x g for 30 min at 4°C), DNA was purified by incubation at 37°C for 60 min with 0.1% SDS and 50 μg/ml proteinase K, followed by phenol-chloroform extraction. Equal amounts of DNA from pellet and supernatant fractions together with input fragments were loaded on a 1% agarose gel. After electrophoresis the gel was dried on Whatman 3MM paper, and DNA fragments were visualized by overnight autoradiography.

Mapping of SC-associated regions

SC-associated regions were mapped mapped essentially as described for matrix attachment regions (above). In this case, isolated SCs from 10^6 spermatocytes were used in the binding reaction. SCs that were used for this purpose were submitted to an additional centrifugation step through 1.5 M of sucrose at the end of the SC isolation procedure (Heyting *et al.*, 1985) to remove DNAseII.

Results

A potential binding site for the M_r 30,000-33,000 SC components in the *Hsp70-Bat5* region of the MHC revealed after subtraction of immunoprecipitated DNA

To assess the DNA-binding *in vivo* of SC proteins we employed the procedure described by Orlando and Paro (1993) (experimental procedures). We isolated mouse spermatocytes and optimized the conditions for crosslinking *in vivo* so that sarkosyl-soluble DNA-protein complexes were formed, in which SC protein was immunologically detectable after CsCl density centrifugation (see below). Increasing the extent of the crosslinking yielded mainly insoluble complexes, whereas milder conditions resulted in failure to crosslink detectable amounts of SC protein to DNA (not shown).

After CsCl density centrifugation of chromatin crosslinked *in vivo* the DNA peak was found at a density ($\pm$ 1.4 g/ml, Figure 1A) close to that found by Solomon *et al.* (1988) for DNA-protein complexes (1.39 g/ml), whereas DNA in the uncrosslinked control sample was found at higher densities ($>$ 1.56 g/ml, Figure 1B). A small fraction of M_r 30,000-33,000 SC protein (about 2%, as visualized by immunodetection with affinity-purified anti-M_r 30,000-33,000 SC antibodies) sediments with the DNA peak in the crosslinked sample (Figure 1A), whereas virtually no M_r 30,000-33,000 SC protein is found at this density in the uncrosslinked control (Figure 1B). More than 50% of M_r 30,000-33,000 SC protein is detected at densities lower than 1.34 g/ml, whereas a considerable amount of these proteins was found floating at the top of the gradient. We concluded that this represents crosslinked protein-protein complexes and uncrosslinked protein as this is the only position at which M_r 30,000-33,000 SC protein is detected in the uncrosslinked

control (Figure 1B). Similar results were obtained for another SC protein, SCP1 (Meuwissen et al., 1997). Interestingly, a peak of M_r 30,000-33,000 SC protein is detected in the crosslinked sample at a density of about 1.5 g/ml, which might indicate the presence of complexes of protein with DNA which differs from the bulk of DNA detected at a density of 1.4 g/ml (Figure 1A). Finally, in the uncrosslinked sample the M_r 30,000-33,000 SC protein is not only detected at densities lower than 1.34 g/ml but seems to 'tail' towards higher densities, possibly due to the migration of the uncrosslinked DNA to high densities (> 1.53 g/ml, Figure 1B).

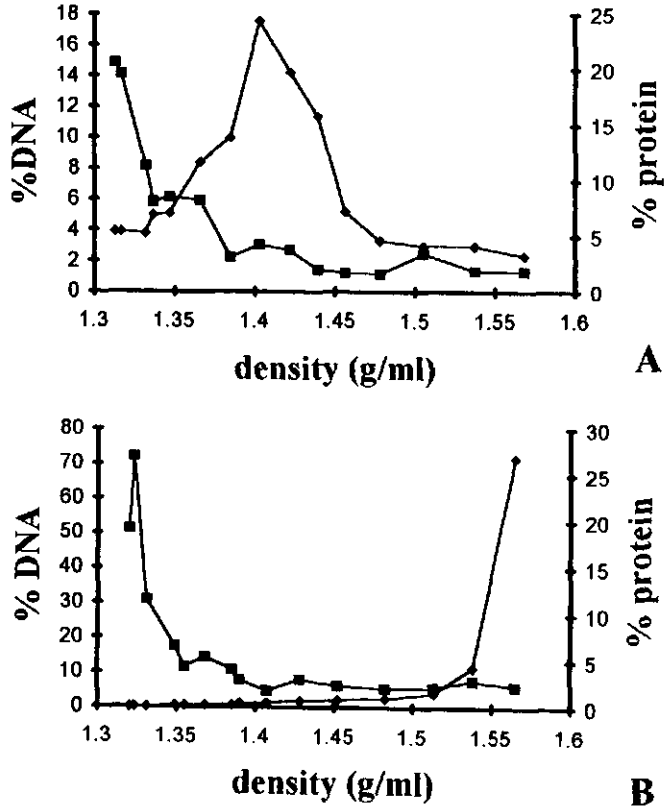


Figure 1: Crosslinking *in vivo* of the M_r 30,000-33,000 SC proteins.
Nuclei from formaldehyde treated (A) and untreated (B) spermatocytes were sonicated, dissolved in sarkosyl and centrifuged to equilibrium in sarkosyl-containing CsCl gradients. The gradients were fractionated and M_r 30,000-33,000 SC protein (□) and DNA content (♦) were assayed in each fraction. The vertical axes to the left represent the percentage of the total DNA in the gradient and the axes to the right represent the percentage of the total M_r 30,000-33,000 SC protein.

The immunoprecipitated and PCR-amplified fragments were hybridized to Southern blots of restriction enzyme digests of five cosmids, which together cover 120 kbp of the *Hsp70-Bat5* gene cluster in the MHC III region of the mouse genome (Figure 2). As a control, we hybridized the Southern blots with mouse genomic DNA. The hybridization patterns were similar for all probes used (compare e.g. Figures 3A and 3C). The hybridizing bands therefore most probably represent highly and moderately repetitive sequences and no unique sequence within the *Hsp70-Bat5* gene cluster was enriched detectably during immunoprecipitation. We therefore decided to use a subtraction step (see experimental procedures) to lower the background of highly repetitive sequences and to search for unique or low-copy number enriched sequences within the *Hsp70-Bat5* region, present in the immunoprecipitated sample. Hybridization of MHC Southern blots with the subtracted sequences as a probe (ratio of immunoprecipitated fragments to mouse genomic DNA fragments during subtraction for the probe used was 1:5) revealed three strongly hybridizing bands (Figure 3B), two of which were identical 3 kb fragments, whereas the third was an overlapping 850 bp fragment. This pointed to a potential binding site for the M_r 30,000-33,000 SC proteins in or near a 3 kb *Eco*RI-*Xba*I fragment located between the *G7b* and *G7e* genes (Figure 2).

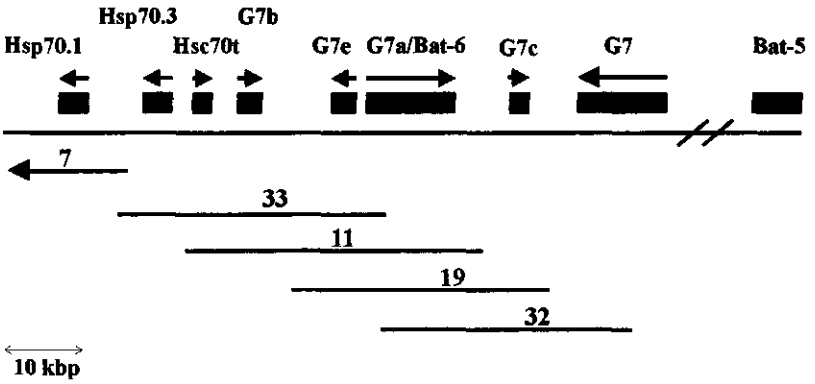


Figure 2: Map of the *Hsp70-Bat5* gene cluster of the mouse MHC-III locus (adapted from Snoek *et al.*, 1991 and 1996). Genes are indicated by black boxes. Arrows indicate the direction of transcription. Cosmids are represented by black lines and their numbers.

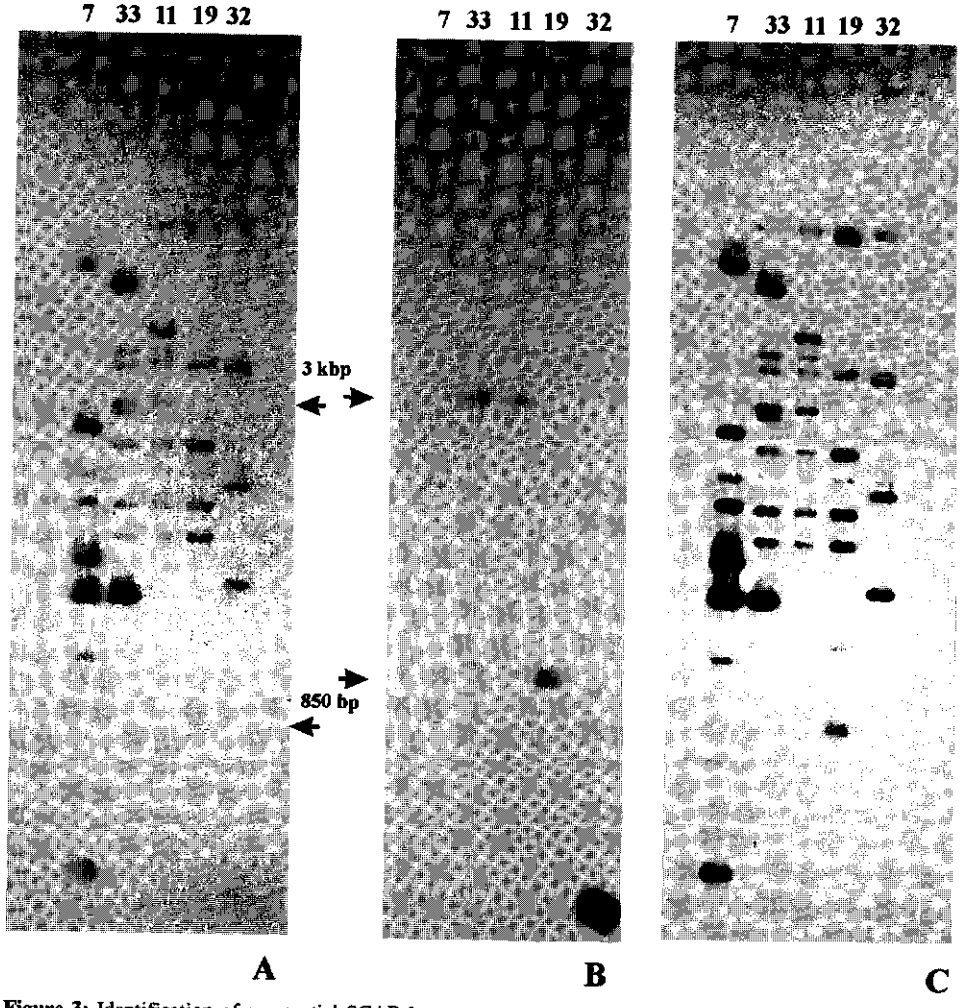


Figure 3: Identification of a potential SCAR between genes *G7b* and *G7e*.
Southern blots containing DNA from five cosmids covering the *Hsp70-Bat5* gene cluster were probed (see experimental procedures) with either PCR-amplified fragments obtained after immunoprecipitation of crosslinked chromatin with affinity-purified *anti*-M_r 30,000-33,000 SC antibodies (A), fragments obtained after subtraction of the fragments used in A with mouse genomic DNA (B) or mouse genomic DNA (C). The 3 kbp *Eco*RI-*Xba*I fragment and the 850 bp fragment are indicated by arrows. Cosmid numbers indicated on the top of the slots correspond to those in Figure 2. The blot shown in B was prepared from a different gel than blots shown in A and C.

Fine mapping of the SC-associated region in the *Hsp70-Bat5* gene cluster

The 3 kbp fragment identified in DNA immunoprecipitated with anti-M_r 30,000-33,000 SC antibodies affinity-purified on Western blots, could have been enriched either as a result of preferential crosslinking of these sequences compared to other sequences, or by specific immunoprecipitation. We therefore analysed the enrichment of short, unique probes within and outside the 3 kbp *Eco*RI-*Xba*I fragment in the immunoprecipitated DNA and in the non-specifically precipitated control DNA. The fragments tested (positions are indicated by the asterisks in Figure 5) showed 2- to 3-fold enrichments in DNA immunoprecipitated with anti-M_r 30,000-33,000 SC antibodies that were affinity-purified on Westernblot strips (Lammers *et al.* (1994; Chapter 2). With anti-M_r 30,000-33,000 SC antibodies purified on an affinity column (see Experimental procedures) a 3- to 4-fold enrichment was found for the probes tested.

Because we considered these levels of enrichment insufficient for the proper detection of a gradient of enrichment, the immunoprecipitation was repeated with complete serum 175. Fragments obtained by immunoprecipitation with serum 175 showed a stronger hybridization to the 850 bp fragment and for the overlapping 3 kb *Eco*RI-*Xba*I fragment than the fragments from the corresponding control DNA (Figure 4). By means of unique probes within and outside the 3 kbp *EcoRI-XbaI* fragment a peak of enrichment was found within a 2000 bp segment between genes *G7b* and *G7e* of the *Hsp70-Bat5* gene cluster (Figure 5). Enrichment was determined by dot blot hybridization (see Experimental procedures). Blots contained 50 ng per dot of mouse genomic DNA, $C_0.t=1$ DNA, DNA isolated from DNA-protein complexes obtained after CsCl density centrifugation of crosslinked chromatin, PCR-amplified DNA obtained by immunoprecipitation of protein-DNA complexes with serum 175 and PCR-amplified DNA from the control immunoprecipitation. Enrichment of a fragment is defined as the difference between hybridization of that fragment to immunoprecipitated DNA and control DNA. To standardize the values obtained, these were divided by the hybridization of the fragment to DNA isolated from DNA-protein complexes after CsCl density centrifugation. Enrichment is thus defined as the effect of the addition of specific antibodies in the immunoprecipitation step.

We termed this region SC-associated region (SCAR). This SCAR contained the 850 bp fragment identified earlier by Southernblot hybridization (Figure 3B). The sequence flanking the 850 bp at the side of the *G7e* gene shares 77% homology to a human retrovirus related cDNA encoding a POL polyprotein, as was reported by Snoek *et al.* (1996) and 76% homology with a clone designated masc76r, identified by Karpova *et al.* (1995) as an SC-associated sequence in the hamster.

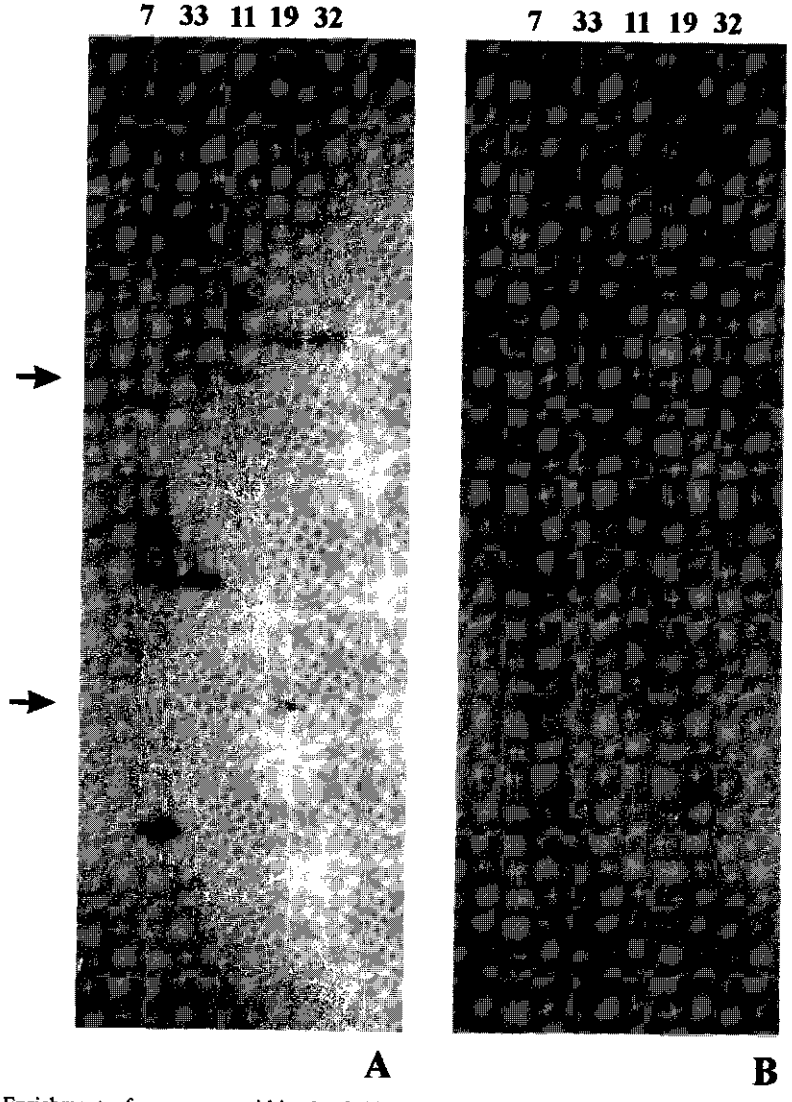
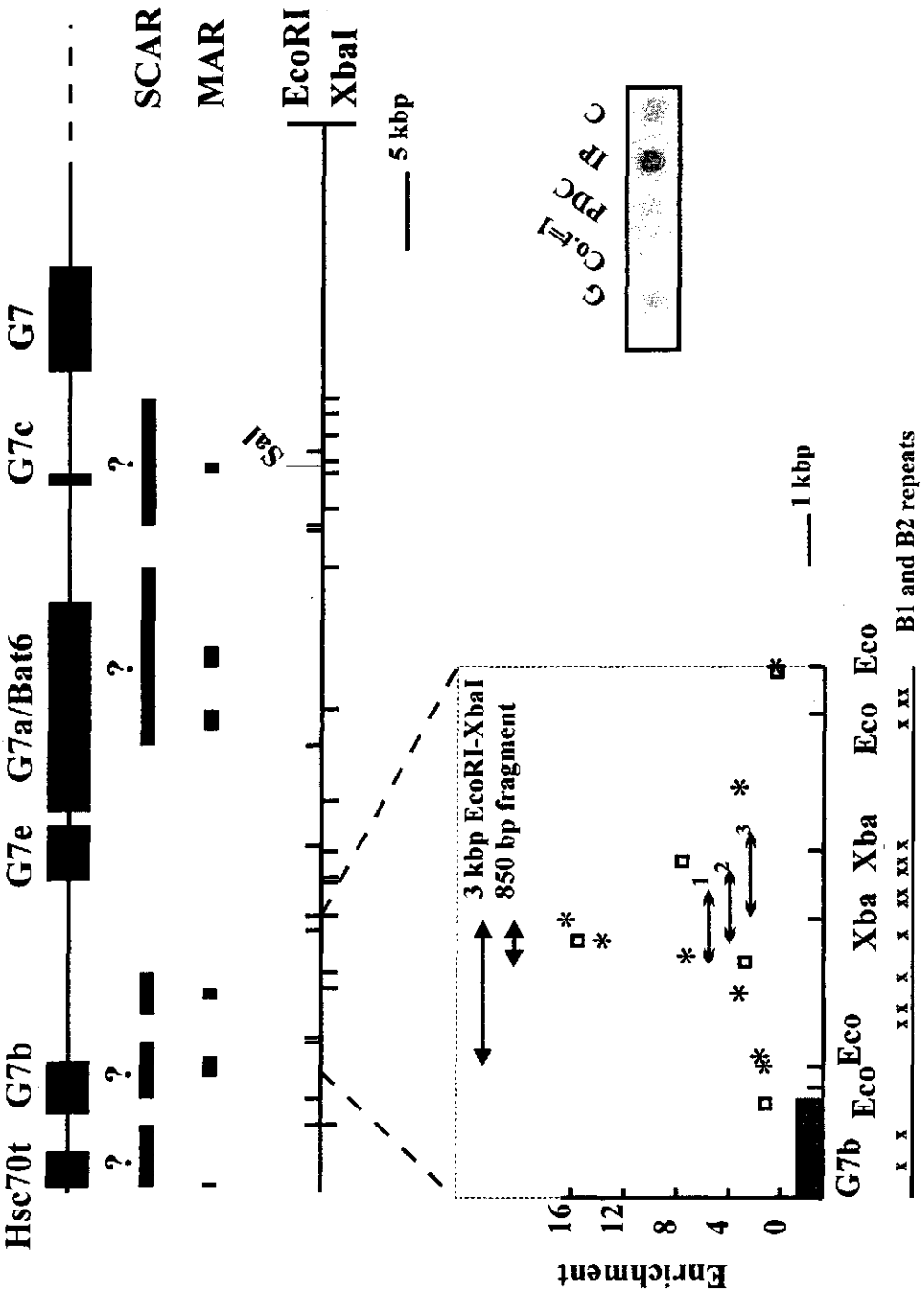


Figure 4: Enrichment of sequences within the 3 kbp *Eco*RI-*Xba*I fragment by immunoprecipitation with serum 175.
Southern blots containing DNA from the cosmids covering the *Hsp70-Bat5* gene cluster were probed with equal amounts of PCR-amplified, [^{32}P]-labelled fragments obtained after immunoprecipitation with (A) or without (B) serum 175. Enriched fragments are indicated by arrows.


Hsc70t
G7b
G7e
G7a/Bat6
G7c
G7
SCAR
MAR
EcoRI
XbaI
SalI
5 kbp
1 kbp
3 kbp EcoRI-XbaI
850 bp fragment
Enrichment
16
12
8
4
0
G7b
Eco Eco
Xba Xba
Eco Eco
B1 and B2 repeats

Sequences within the identified SCAR are also enriched in SC-associated DNA

If the identified region presents an attachment site of meiotic chromatin to the SC, an enrichment for these sequences should also be found in SC-associated DNA (SC-DNA; see Experimental procedures). We therefore analysed the enrichment of SC-DNA for these sequences by dotblot hybridization. Only probes located within the identified 2000 bp region are substantially enriched (Figure 5). This confirms the identification of a SCAR in a 2000 bp region between genes *G7b* and *G7e* in the *Hsp70-Bat5* gene cluster of the mouse MHC III.

The identified SCAR behaves as a MAR/SAR

To test if the identified SCAR was also a MAR, we performed matrix binding analyses with fragments spanning the identified SCAR (Figure 5). One fragment (nr.2) showed clear matrix binding (Figure 6). This means that the identified SCAR colocalizes with a MAR/SAR. Sequences located within 500 bp from both the 5'- and the 3'-end of the probe which showed the highest enrichment in immunoprecipitated DNA were required for binding to the nuclear matrix (Figure 6). Additionally, we tested fragments in and around the region which harbors the meiotic recombination hotspot (Snoek *et al.*, 1991; Snoek *et al.*, 1998) for matrix binding. A 426 bp *SalI-EcoRI* fragment showed detectable binding (Figure 6D). This fragment lies at the telomeric side of the *G7a/Bat6* gene (Figure 5), about 200 bp from a 2.3 kbp *XbaI*-fragment, to which several recombinational breakpoints in the *Hsp70-Bat5* gene cluster have been pinpointed (Snoek *et al.*, 1998). This 2.3 kbp *XbaI*-fragment was enriched by immunoprecipitation with serum 175 by a factor of about 4 (not shown).

Figure 5 (previous page): Fine-mapping of the SCAR located between genes *G7b* and *G7e*.
Part of the region between genes *Hsp70* and *Bat5* is shown. Coding sequences are indicated by black boxes. MARs (identified by matrix binding assays (Figure 6A and D) or by the presence of the MAR recognition signature (MRS)), the identified SCAR and potential SCARs (denoted by a question mark) mentioned in the text are indicated by boxes. The region in which the SCAR was mapped is shown in more detail in the lower part of this figure. B1 and B2 repeats are indicated by crosses.
Enrichment of selected probes (asterisks) was determined by dot blot hybridization (see Experimental procedures and Results). The inset shows a typical result of a dot blot hybridization experiment (G=mouse genomic DNA, $C_0.t=1$ = $C_0.t=1$ DNA, PDC=protein-DNA complexes, obtained after CsCl density centrifugation of crosslinked chromatin, IP=PCR-amplified, immunoprecipitated DNA, C=PCR-amplified DNA from the control immunoprecipitation). Enrichment of selected probes in SC-DNA (squares) compared to genomic DNA was also determined by dot blot hybridization.
The three overlapping probes (nr's 1, 2 and 3) used for nuclear matrix and SC-binding assays *in vitro* are also indicated, as well as the 3 kbp *EcoRI-XbaI* fragment and the 850 bp fragment which were initially found to be enriched.

SCs exhibit specific binding *in vitro* of MARs

To analyse the relationship between SCARs and MARs further, we tested whether sequences that were known as MARs bound to SCs by performing matrix binding assays in which nuclear matrices were replaced by SCs. In the *in vitro* binding assay (see Experimental procedures), isolated SCs bound the MAR of the *Drosophila* histone gene cluster (Mirkovitch *et al.*, 1984, Figure 7A), a recently identified MAR of *Arabidopsis* (van Drunen *et al.*, 1997; Figure 7B) and the SCAR identified in this paper (Figure 7C). Fragments containing the *Drosophila* MAR or the SCAR were bound under standard conditions, whereas the fragment containing the *Arabidopsis* MAR was only bound after overnight incubation with SCs. Under these conditions a second fragment, not flanking the MAR, was also bound. This fragment does not bind to the nuclear matrix under the same conditions (Figure 6C). All three overlapping fragments that cover the SCAR bound to SCs (Figure 7C), whereas only fragment no.2 bound to the nuclear matrix (Figure 6A). We also performed a DNA-binding assay with SCs and fragments obtained by digestion of cosmid 11 (see Figure 2) with *Eco*RI and *Xba*I. In addition to the fragment covering the SCAR, four, additional, non-adjacent fragments of cosmid 11 were bound (Figure 7D). All of these contain a recently identified sequence, the MAR recognition signature (MRS) which is characteristic for MARs (C.M. van Drunen, unpublished results). These potential SCARs are indicated in Figure 5.

Discussion

We have employed paraformaldehyde crosslinking *in vivo* and immunoprecipitation to identify DNA sequences that are attached to synaptonemal complexes of the mouse *in vivo*. We thus identified an SC-associated region (SCAR), which is located between the *G7b* and *G7e* genes in the mouse MHC III locus. This SCAR behaves as a MAR/SAR in *in vitro* matrix binding assays.

Technical considerations

The *in vivo* crosslinking approach that we have followed has the following technical limitations: First, the fraction of SC protein (SCP3) that is crosslinked to DNA *in vivo* is small (Figure 1). This also holds true for other non-histone proteins (Solomon *et al.*, 1985). This means that it is difficult to find conditions that guarantee a high level of crosslinking but do not result in the formation of large insoluble complexes.

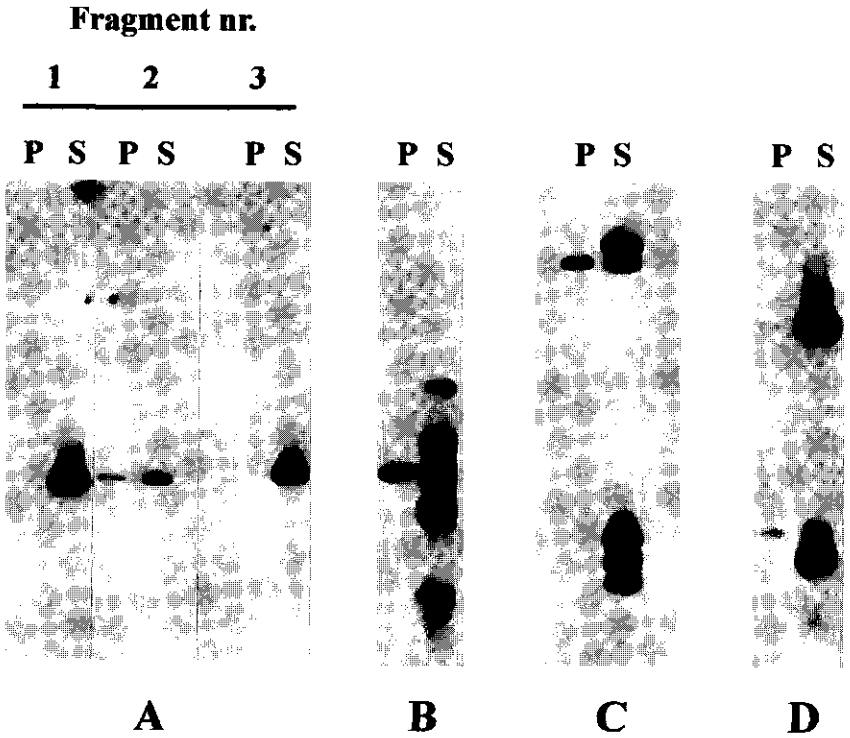


Figure 6: The identified SCAR colocalizes with a MAR.
Nuclear matrix binding was carried out with three overlapping PCR-fragments (Figure 5) covering the identified SCAR (A), fragments covering the region between the *H1* and *H3* genes in the *Drosophila* histone gene cluster (B), fragments containing sequences in the vicinity of the *plastocyanin* gene from *Arabidopsis* (C) and a 426 bp fragment (Figure 5) located at the telomeric side of the mouse MHC *G7a/Bat6* gene (D). P=pellet, S=supernatant. Matrix binding was considered relevant when at least 5% of a labeled fragment was detected in the pellet fraction.

Second, it is possible that *in vivo* protein is crosslinked more readily to DNA at/near the bases of the chromatin loops than in the periphery of the loops, because of the possible presence of larger amounts of tightly associated protein at the bases. In that case the DNA-protein complexes are already enriched in SC-associated sequences before immunoprecipitation. Therefore we have calculated enrichment of sequences *by immunoprecipitation*, i.e. we used DNA-protein complexes isolated from the CsCl-gradient as a standard.

Third, we only observed substantial enrichment of DNA sequences with polyclonal antiserum 175. Affinity-purified antibodies from this serum yielded only low levels of enrichment.

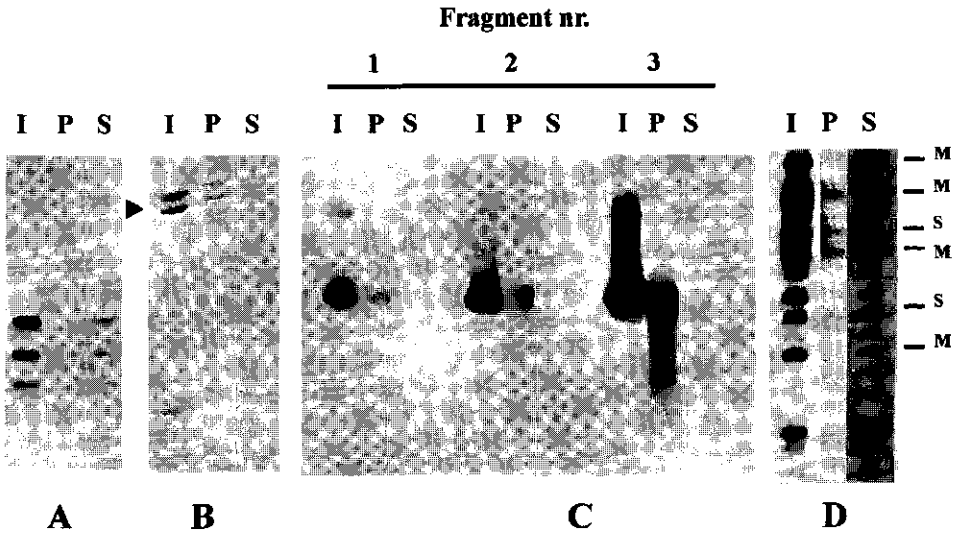


Figure 7: SCs exhibit specific MAR-binding *in vitro*.
Binding was carried out with fragments covering the region between the *H1* and *H3* genes in the *Drosophila* histone gene cluster (A), fragments containing sequences in the vicinity of the *plastocyanin* gene from *Arabidopsis* (B), three overlapping PCR-fragments covering the identified SCAR (C), and fragments produced by digestion of cosmid 11 (see Figure 2) with *EcoRI* and *XbaI* (D).
I=input, P=pellet, S=supernatant. In (B) the fragment which contains a MAR is indicated by an arrowhead. In (C) fragment numbers correspond to those in Figure 5. In the supernatant fraction in panel C some breakdown of probes is visible. This is caused by the presence of residual DNAseII in the isolated SCs. In (D) fragments containing MARs (based on the results shown in Figure 6A or on the presence of the MRS within the sequence) are indicated with 'M' and fragments covering the identified SCAR with 'S'.

Fourth, the amplification of immunoprecipitated DNA fragments by means of adapter-mediated PCR is a critical step. In the original protocol, Orlando and Paro (1993) used restriction enzymes to obtain DNA fragments of similar size; they reasoned that after ligation of adapters, these fragments would amplify with similar efficiency. However, only fragments with two restriction sites were efficiently amplified, and the representation of the fragments in the samples was changed (Strutt *et al.*, 1997). We therefore fragmented the crosslinked DNA by sonication, and used T4 DNA polymerase to create blunt-ended fragments, ligated adapters and then performed the amplification step. This procedure thus involves larger differences in fragment size. As smaller fragments are amplified more efficiently than larger fragments, the number of amplification cycles used must be limited to prevent large changes in the representation of the fragments in the sample.

Finally, we found that the use of different adapters for the amplification of immunoprecipitated fragments results in the production of pools of fragments that are not entirely the same. Selected probes hybridized to different extents to these different pools (H.H. Offenberg, unpublished results).
All factors mentioned might contribute to the fact that different, independent experiments give different enrichments for selected probes (H.H. Offenberg, unpublished results). It is therefore of great importance to optimize each of these factors so that these experiments become increasingly reproducible.

The M_r 30,000-33,000 SC proteins are components of meiotic chromatin that are probably directly bound to DNA

In this study, we obtained evidence that under the conditions we used, the M_r 30,000-33,000 SC proteins can be crosslinked *in vivo* to meiotic chromatin. We think it likely that the M_r 30,000-33,000 SC proteins are bound to DNA *in vivo* and were directly crosslinked to DNA for the following reasons: First, the crosslinker, paraformaldehyde, will only crosslink two molecules that are separated by less than 0.2 nm (Jackson, 1978), which means that they must be in close contact. Second, formation of complexes containing more than one crosslink per histone molecule requires extended incubation with the crosslinker (30 min to several hours, Jackson, 1978); the conditions used in this paper were found to minimize the number of crosslinks between individual histone molecules (Solomon *et al.*, 1988). Third, a protein that is known not to be bound to DNA *in vivo*, actin, is not found in the DNA-protein fraction after crosslinking *in vivo* (Meuwissen, 1997). Therefore, the presence of the M_r 30,000-33,000 SC proteins in the DNA-protein fraction is probably the result of a crosslink between these proteins and DNA, which would indicate that at least a fraction of these proteins is directly bound to DNA *in vivo*.
The fraction of M_r 30,000-33,000 SC protein that is crosslinked to DNA is rather low. This might be improved by changing the crosslinking conditions such that more crosslinks can be introduced, for instance by increasing the concentration of paraformaldehyde. However, care must be taken that no insoluble complexes are formed. On the other hand, *in vivo*, non-histone proteins are crosslinked far less effectively to DNA than histones (Solomon *et al.*, 1985). Another explanation for the small fraction of M_r 30,000-33,000 SC proteins that are crosslinked to DNA is that only a minor fraction of these proteins is bound to DNA *in vivo*. It is even possible that this depends on the stage of meiotic prophase.

The identification of a SCAR between the *G7b* and *G7e* genes

Using Southern blot analysis of the cosmid contig covering the *Hsp70-Bat5* gene cluster we were unable to identify enriched sequences among DNA fragments obtained by immunoprecipitation with affinity-purified anti-M_r 30,000-33,000 SC proteins. After subtraction of this material with fragments of mouse genomic DNA, sequences present within a 3 kbp *Eco*RI-*Xba*I fragment were enriched. These sequences must be unique or moderately repeated, and not make part of the numerous B1 or B2 repeats that are present in the analysed region, because otherwise we would have been unable to identify the fragment so clearly among others (Figure 3B). A possible explanation for these observations might be that the presence of B1 and B2 repeats in fragments that were enriched during immunoprecipiation made it impossible to score these as enriched by Southern blot hybridization. Several other fragments containing B1 and/or B2 repeats can also hybridize to the 3 kbp *Eco*RI-*Xba*I fragment and the 850 bp fragment under the conditions used, so that a clear enrichment is masked and an underestimated enrichment of only 2-3 is found using dot blot hybridization. During subtraction with a relatively low excess of genomic DNA fragments, enriched fragments containing B1 and/or B2 repeats will be lost from the sample allowing the detection of the SCAR by the (more) unique, enriched fragments that remain present in the sample.

A more detailed analysis of fragments within the 3 kbp *Eco*RI-*Xba*I region obtained by immunoprecipitation with serum 175 showed that their enrichment occurred during immunoprecipitation. The enriched DNA-region, which we have termed SC-associated region (SCAR), lies within a 2000 bp DNA-segment (Figure 5). Additional findings that support the identification of a SCAR are (1) the observed enrichment of sequences in this region in SC-DNA, (2) fragments covering the identified SCAR bind specifically to SCs in an *in vitro* binding assay and (3) a 126 bp segment present in the SCAR shares extensive homology to a sequence that was reported to be an SC-associated sequence in hamster (Karpova *et al.*, 1995). To our knowledge this is the first SCAR identified whose location has been mapped.

Before a conclusion can be drawn about the validity of the *in vivo* crosslinking and immunoprecipitation approach, one or two additional SCARs should be identified by this procedure, and these should be verified by binding *in vitro* to isolated SCs.

The relationship between SCARs and MARs/SARs

Our results indicate that SCARs might also be capable of binding to the nuclear matrix, which is in agreement with the models presented in the Introduction. If colocalisation with a MAR is a general feature of SCARs, it would mean that the basic organization of meiotic chromatin is similar to that of interphase and mitotic chromatin. An important

question that then remains is whether these sequences are also involved in similar functions during these different stages of the cell cycle. MARs are involved in the regulation of several different processes including transcription, replication and splicing. MARs have also been implicated in recombinational processes. In these cases, however, the presence of MARs was connected with illegitimate types of recombination as they were detected near the breakpoints of translocations such as in the mouse immunoglobin κ-chain gene (Sperry *et al.*, 1989) and in the human acute myeloid leukemia (*AML*) gene (Strissel Broeker *et al.*, 1996).

MARs are considered as sites where the machineries for different nuclear processes can enter the chromatin and perform their function. During mitosis and meiosis these sites may become located at the bases of the chromatin loops, i.e. become part of the chromosome scaffold. We think that it is therefore possible that during meiotic prophase, the recombination machinery assembles at/near MARs/SCARs as predicted by Kleckner (1996). We suggest that recombination is not necessarily initiated at/near MARs/SCARs, but also within the chromatin loops themselves. For recombination to proceed, however, sites where recombination has been initiated must be brought near the bases of the chromatin loops where the recombination machinery assembles. The recruitment of initiated recombination events could (at least in part) be mediated by meiosis-specific proteins.

Does this mean that during different stages of the cell cycle the same MAR sequences are bound to the same proteins? In our experiments sequences that were capable of binding to the SC were not always MARs. Moreover, even if SCARs colocalise with MARs, there seem to be differences in the requirements for binding to either the SC or the nuclear matrix (Figures 6 and 7). The binding assays *in vitro* only show the capacity of a sequence to bind to the SC or the nuclear matrix, but they do not tell us if this sequence is also bound to the SC or the nuclear matrix *in vivo*. It is conceivable that in different cells different sets of MARs become associated with the SC. If this is the case it is important to find out on which criteria this choice is based. Alternatively, a defined set of MARs (and other sequences) always becomes associated with SCs in every cell.

Nucleotide sequences surrounding the identified SCAR

For the identification of SCARs in a defined region of the genome we focused on a 50 kbp region of the *Hsp70-Bat5* gene cluster because this region contains a hotspot for meiotic recombination (Snoek *et al.*, 1991 and 1993). The SCAR identified in this study lies between genes *G7b* and *G7e* (Figure 2). The nucleotide sequence of this region has several interesting features (see also Snoek *et al.*, 1996): First, a large number of repetitive B1 and B2 sequences are clustered in this region, as well as 7 segments sharing

homology to retrovirus-like *pol* genes and 5 to retrovirus-like *gag* genes. One of the *pol* sequences maps within the identified SCAR. Additionally, the *G7e* gene itself shares homology to retroviral *env* genes. Between *G7e* and *G7a/Bat6* a solitary long terminal repeat (LTR) is found and in the second intron of the *G7a/Bat6* gene there is a sequence element highly homologous to the mouse MT family, surrounded by $(GT)_n$ repeats. These sequence elements hint to retroviral insertion events. This might be a consequence or the cause of a chromatin configuration that is prone to recombination events. The presence of retrovirus-derived sequences, simple repeats and LTR and MT elements was also reported for two other meiotic recombination hotspots in the mouse MHC region, namely near the *Eb* and the *Lmp2* genes (Zimmerer and Passmore, 1991; Uematsu *et al.*, 1986). Recently, a large number of breakpoints in the *Hsp70-Bat5* gene cluster has been localized to a relatively small region of about 2.5 kbp at the telomeric side of the *G7a/Bat6* gene (Snoek *et al.*, 1998). This hotspot for meiotic recombination is located at about 30 kbp from the identified SCAR, and within 1 kbp from a potential SCAR. Moreover, it lies within a region where no retrovirus-like elements are present. It therefore seems that there is no direct relation between the many retrovirus-like elements and the hotspot. Perhaps the proximity of a MAR/SCAR might be a characteristic of a recombination hotspot, possibly because recombination intermediates are resolved near MARs/SCARs.

Are the M_r 30,000-33,000 SC proteins bound to SCARs *in vivo*?

The first indication for the presence of the identified SCAR came from experiments in which we tried to enrich sequences that are bound *in vivo* to the M_r 30,000-33,000 SC components. After subtraction with fragments of mouse genomic DNA, we identified a 3 kbp region that was enriched and was shown to harbor the identified SCAR. These results thus indicate that, probably among other proteins, the M_r 30,000-33,000 SC proteins are bound to sequences within this SCAR *in vivo*. An important question for further research is if the M_r 30,000-33,000 SC proteins bind to all SCARs or only to a subset. This might help elucidate the function of these potential SCAR-binding proteins.

The fact that the potential second SCAR not only colocalizes with a MAR, but is also located close to the hotspot in this region might be useful to investigate the spatial relationship between initiation of recombination, the resolution of recombination intermediates and SCARs.

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Chapter 5

SCP3, a major protein component of the lateral elements of synaptonemal complexes of the rat, binds preferentially to single-stranded DNA *in vitro*

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Abstract

We have analysed the DNA-binding capacity of SCP3, the bacterial translation product of a cDNA encoding the M_r 30,000-33,000 components of the lateral elements of synaptonemal complexes (SCs) of the rat.

SCP3 binds preferentially to single-stranded DNA (ssDNA) *in vitro*. The DNA-binding domain of SCP3 is located in its N-terminal 8.8 kDa. The affinity of SCP3 for single-stranded DNA substrates is influenced by their nucleotide composition, which probably influences the secondary structure.

The M_r 30,000-33,000 SC proteins also exhibit preferential ssDNA-binding *in vitro*. The binding of ssDNA *in vitro* is influenced by the number of phosphate groups attached to these proteins *in vivo*.

Introduction

The prophase of the first meiotic division is characterized by the pairing of homologous chromosomes and a high rate of recombination. Pairing of homologous chromosomes (homologs) is initiated before the formation of a proteinaceous, intranuclear structure, the synaptonemal complex (SC) (Weiner and Kleckner, 1994). At the pachytene stage of meiotic prophase, the SC is found between the homologs along their entire length. It consists of two lateral elements (LEs), one along the chromatin of each homolog. The LEs are connected by transverse filaments. Between the LEs, a central element is discernible, which runs in parallel with the LEs along the entire chromosome. Recombination takes place predominantly between two non-sister chromatids of homologs. Some recombination events lead to crossovers, which become cytologically observable late in meiotic prophase as chiasmata. Currently, the SC is believed to promote meiotic interhomolog recombination and to influence the number and distribution of crossovers along a bivalent (e.g. Heyting, 1996). How this is achieved is still unclear.

We are interested in the function of the SC in general and in the organization of meiotic chromatin on the SC in particular. In this regard, two questions can be raised: How are recombinational events brought in the context of the SC? And how can the SC or its components influence the recombination processes?

We have isolated the cDNAs encoding three major protein components of the rat SC, namely SCP1 (Meuwissen *et al.*, 1992), SCP2 (Offenberg *et al.*, 1998) and SCP3 (Lammers *et al.*, 1994, Chapter 2). We are currently involved in the characterization of these components. In this report, we will focus on a component of the LEs of the rat SC, SCP3.

Early in meiotic prophase, the two sister chromatids of each chromosome are organized on a single, axial core; the axial cores of homologous chromosomes become the LEs of SCs. At the end of diplotene, two distinct axes become discernible (Rufas *et al.*, 1992), which possibly correspond to the scaffolds of the individual chromatids. In order to analyse how the sister chromatids are attached to their common axial core, we analysed possible interactions of SCP3 with DNA.

Using paraformaldehyde fixation of spermatocytes, we obtained evidence that SCP3 is bound to DNA *in vivo* (Chapter 4). If the protein binds to double-stranded or single-stranded DNA is unknown. The deduced amino acid sequences of SCP1 and SCP2 contain both several motifs which indicated that these might bind to AT-rich, double-stranded DNA. This was shown *in vitro* for SCP1 (Meuwissen, 1997). A DNA-binding motif, however, was not identified within the amino acid sequence of SCP3. We have therefore investigated the potential DNA-binding *in vitro* of SCP3 to double-stranded (ds)

as well as single-stranded (ss) DNA. Moreover, since several differentially phosphorylated variants of SCP3 occur in spermatocyte nuclei (Lammers *et al.*, 1995; Chapter 3), we analysed the influence of the degree of phosphorylation on the DNA-binding *in vitro*.
Here we show that the translation product of the cDNA encoding the M_r 30,000-33,000 components of the lateral elements of SCs of the rat, SCP3, is capable of binding to DNA *in vitro*. SCP3 has a 5000-fold higher capacity for binding ssDNA than for binding dsDNA. The DNA-binding domain of SCP3 is located in its N-terminal 8.8 kDa. The M_r 30,000-33,000 SC components also show a strong preference for binding to ssDNA *in vitro*. The ssDNA-binding capacity *in vitro* of these proteins is influenced by their degree of phosphorylation. The implications of the presence of several ssDNA-binding proteins near the bases of looped chromatin during meiotic prophase and the possible role of the M_r 30,000-33,000 SC proteins are discussed.

Experimental procedures

Production and isolation of peptides

For the production and isolation of SCP3 and several subfragments of this protein, the insert of the cDNA-clone encoding full-length SCP3, clone 2A4 (Lammers *et al.*, 1994; Chapter 2), was subcloned in the pQE32 His-tag vector (Qiagen, Hilden, Germany). *E.coli* SG13009 cells were transformed with the resulting construct. Expression of His-tagged SCP3 was induced by means of IPTG, as described before (Lammers *et al.*, 1994; Chapter 2). His-tagged SCP3 was isolated by means of FPLC (Pharmacia, Uppsala, Sweden) on a nickel-NTA (nitrilo-tri-acetic acid) column under denaturing conditions, according to the instructions of the manufacturer of the nickel column (Qiagen). To produce and isolate subfragments of SCP3 (see Figure 1), the corresponding fragments of the cDNA were obtained by digestion with restriction enzymes or by means of PCR with specific primers. These were subcloned in the appropriate pQE3X his-tag vector and his-tagged peptides were expressed and isolated as described. Isolated peptides were checked for purity on 10% SDS-polyacrylamide gels (Laemmli, 1970) or on 14.5% Tricine-SDS polyacrylamide gels (see below). Additionally, peptides were transferred onto nitrocellulose membranes (Schleicher and Schuell) and detected immunologically with polyclonal anti-M_r 30,000-33,000 SC antibodies as described before (Lammers *et al.*, 1994; Chapter 2). The concentration of the isolated peptides was determined according to Lowry *et al.* (1951).

Production of a peptide with a mutated ATP-binding motif

A N-terminal fragment of SCP3 with a non-functional ATP-binding site was generated as follows: the ATP-binding motif A comprises amino acid residues 28-35 in the SCP3 protein. In order to change the codon for the conserved lysine (K34) in one encoding an isoleucine, we made use of an oligonucleotide (K34I, 5'-TTCCCAGATATCCCAGAATG-3') which represents the com-

plementary sequence from nucleotide 148 to 167 of the SCP3 cDNA, with an A rather than a T at position 158. This changes the codon for lysine (K34) into isoleucine. PCR was carried out in a 100 μl-volume containing 20 pmol of this oligonucleotide and 20 pmol of SK-primer, 1 ng of cDNA 2A4 (in the pBluescript SK- vector) as a template, 200 μM of dNTPs, 1.5 mM of $MgCl_2$, 1 x Goldstar Reaction buffer (Eurogentec, Seraing, Belgium) and 2.5 Units of Goldstar DNA polymerase (Eurogentec). Amplification involved one cycle consisting of: 94°C, 5 min, 30 cycles consisting of: 94°C, 30 sec; 42°C, 50 sec; 72°C, 1.5 min, and a final cycle consisting of: 94°C, 30 sec; 42°C, 50 sec; 72°C, 10 min. This yielded a 200 bp fragment that was used as a 'megaprimer' in a second, linear PCR. As a template in this step the insert of cDNA clone 2A4 was used, as obtained from a standard PCR in which the M13-forward and -reverse primers were used for amplification in the presence of a dNTP mixture containing dCTP, dGTP, dATP and dUTP (instead of dTTP). Linear amplification was carried out in a 100 μl-volume, containing 1 x Goldstar Reaction buffer, 1.5 mM $MgCl_2$, 200 μM of dNTPs, 25 ng of dUTP-containing cDNA-insert, 50 ng of 'megaprimer' and 1 Unit of Goldstar DNA polymerase. The reaction involved one cycle consisting of: 94°C, 5 min and 5 cycles consisting of: 94°C, 30 sec; 55°C, 10 sec; 72°C, 3 min. Subsequently, 20 μl of reaction mixture was added to 1 Unit of Uracil DNA Glycosylase (UDG, Gibco) and incubated at 37°C for 1 h, to remove the dUTP-containing cDNA template. To this mixture were then added: 62.2 μl of MilliQ, 7.8 μl of 10 x Goldstar Reaction buffer, 4.7 μl of 25 mM $MgCl_2$, 2 μl of 10 mM dNTPs (without dUTP), 1 μl (20 pmol) of SK-primer, 1 μl (20 pmol) of a gene specific primer (5'-TTCCTCTTGTTGTGTTTTCC-3') and 1 Unit of Goldstar DNA polymerase. A third PCR was carried out that involved one cycle consisting of: 94°C, 5 min, 30 cycles consisting of: 94°C, 30 sec; 42°C, 30 sec; 72°C, 2 min, and a final cycle consisting of: 94°C, 30 sec; 42°C, 30 sec and 72°C, 10 min. We subsequently purified the PCR product by phenol-chloroform extraction and ethanol precipitation, and subcloned in the appropriate pQE3X his-tag vector, making use of a *Bam*HI-site in the SK-primer sequence and a *Hin*dIII-site in the cDNA sequence. The introduction of the mutation in the ATP-binding motif was confirmed by DNA sequence analysis. The corresponding peptide (SCP3-N-K34I) was produced and isolated as decribed in the previous section.

Preparation of DNA probes

For the analysis of DNA-binding by SCP3 or its subpeptides the following probes were used: single- and double-stranded fragments from cDNA subclones pHL58 and pHL614, from a subcloned 92 bp fragment of the mouse telomeric repeat sequence 5'-TTAGGG-3' and single-stranded poly(dA) and poly(dC). Clone pHL58 consists of the 118 bp *Pst*I-*Hin*dIII fragment of cDNA 2A4, subcloned in pBluescript SK- (Stratagene). Clone pHL614 contains the 280 bp *Dde*I-*Dde*I fragment of cDNA 2A4. Double-stranded probes were produced by PCR in a 100 μl-volume containing 1 x Goldstar Reaction buffer, 1.5 mM of $MgCl_2$, 200 μM of dNTPs, 0.825 μM of [α-^{32}P]-dATP (Amersham), 1 pg of cDNA template, 20 pmol of both M13-forward and -reverse primer and 1 Unit of Goldstar DNA polymerase. Amplification involved one cycle consisting of: 94°C, 5 min, 30 cycles of: 94°C, 30 sec; 51°C, 30 sec; 72°C, 1 min, and a final cycle consisting of:

94°C, 30 sec; 51°C, 30 sec; 72°C, 10 min.

Single-stranded probes were produced by means of linear amplification in a 100 μl-volume containing the same components as for the production of double-stranded probes except that 12.5 ng of template was used and 20 pmol of either the SK- or the KS primer. Essentially the same cycles were used as for the production of double-stranded probes except that as annealing temperatures 48°C and 42°C were used for the KS- and the SK-primer, respectively. Products obtained by either primer gave essentially the same results in the DNA-binding assay. Therefore in most experiments single-stranded probes were prepared with the KS-primer, because of a higher yield after PCR. To check if the small difference in length between the double-stranded and single-stranded substrates influenced the DNA-binding, double-stranded substrates were also prepared by means of PCR with the KS- and SK-primer (annealing temperature 42°C). The DNA-binding of this shorter DNA-fragment was the same as for the longer fragment produced with the M13-primers.

PCR-products were purified by means of phenol-chloroform extraction and subsequent precipitation by addition of NH_4Ac and ethanol. After incubation at room temperature for 5 min, the DNA was pelleted by centrifugation for 5 min at 10,000 x g at room temperature. Pellets were washed with 80% ethanol and the DNA was subsequently dissolved in MilliQ (Dorit *et al.*, 1991).

The concentration of double-stranded DNA probes was determined by measuring Hoechst 33258-fluorescence in a DNA fluorometer according to the manufacturer's instructions (fluorometer TKO 100, Hoefer Scientific, San Francisco); that of single-stranded probes by measuring the absorbance at 260 nm. Probes were routinely checked for their quality on 2% agarose gels.

Poly(dA) (M_r $8.9x10^4$) and poly(dC) (M_r $9.2x10^4$, Pharmacia Biotech, Uppsala) were end-labeled by means of T4 kinase and [γ-^{32}P]-ATP (Amersham), according to Sambrook *et al.* (1989).

DNA-binding assay

For analysis of the DNA-binding capacity of SCP3 and its subfragments, equimolar amounts of all peptides were applied to different slots of Tricine-SDS-polyacrylamide gels which consisted of a 14.5% T, 6% C separating gel, a 10% T, 3% C spacer gel and a 4% T, 3% C stacking gel. Electrophoresis was performed according to Schägger and von Jagow (1987). Protein was either stained with Coomassie Brilliant Blue or transferred onto nitocellulose membranes (BA-85, Schleicher and Schuell) according to Dunn (1986), as described by Heyting and Dietrich (1991) with 10 mM $NaHCO_3$, 3 mM Na_2CO_3, 25 mM Tris-HCl (pH 9.9), 10% (v/v) methanol as blotting buffer. Membranes were blocked overnight in 10 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 2.5% (w/v) Nonidet P-40, 150 mM NaCl, 0.1 mM DTT, 5% (w/v) non-fat dry milk and 0.05% (w/v) Na-azide. Subsequently, the membranes were washed and preincubated for at least 1 h, at room temperature, in binding buffer (10 mM Tris-HCl (pH 7.5), 8% (v/v) glycerol, 80 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$ and 0.125% (w/v) non-fat dry milk). Binding reactions were performed overnight at room temperature in fresh binding buffer (1 ml per cm^2) containing [^{32}P]-labeled DNA probes. Membranes were washed four times for 15 min at room temperature in binding buffer and DNA-binding was visualized by autoradiography.

For quantitative analysis, SCP3 was applied to a single 13-cm-wide slot of a 16 x 16 cm 10% SDS-polyacrylamide gel. Preparative blots were made as described above. The blot was cut into exactly 0.3 cm wide strips. Filter strips were used in DNA-binding assays, as described, with 0.26 ml of binding buffer per cm^2 of blot. DNA-binding was quantified by scintillation counting and phosphorimager analysis (Molecular Dynamics, Kensing, England). For quantitative analysis of aspecific electrostatic interactions between the DNA probes used in this study and a non-DNA-binding, basic protein (cytochrome *c*, pI=9.6), similar strips were prepared after electrophoresis of cytochrome *c* through a 15% SDS-polyacrylamide gel.

DNA-binding assay on 2D-blots

Two-dimensional separation of SC proteins was performed according to O'Farrell (1975) with some modifications (Lammers *et al.*, 1995; Chapter 3). Proteins were transferred to nitrocellulose membranes as described above. DNA-binding analysis was performed as described, with 0.2 ml of binding buffer per cm^2 of blot.

Mobility-shift assay

To study the DNA-binding of SCP3 in solution, increasing amounts of the N-peptide (Figure 1) were preincubated for 2 min. at room temperature in a buffer containing 50 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 mM $MgCl_2$, 10% glycerol, 0.1% Nonidet P-40 and 50 μg/ml of BSA. Subsequently, 2 ng of radio-actively labeled DNA-probe were added and mixtures were incubated for 30 min. at room temperature. DNA-protein complexes were then separated from unbound DNA-probe by electrophoresis through a 5% non-denaturing polyacrylamide gel. Gels were dried and DNA-binding was visualized by autoradiography.

Results

Purification of SCP3 and several subpeptides

Figure 1 shows the positions of the subpeptides of SCP3 used in this study.

The subpeptides (Figure 1) were designated N (a.a. 1-119), N1 (a.a. 1-81), C (a.a. 141-257), C1 (a.a 120-189) and C2 (a.a. 201-257). In addition, we purified N-K34I, which is identical to peptide N except that the lysine residue in the ATP-binding motif (residue 34) is replaced by an isoleucine. Of each peptide 45 pmol was analyzed on a 14.5% Tricine-SDS polyacrylamide gel, together with 45 pmol of cytochrome *c* (Figure 2A). Blots for analysis of DNA-binding and immunodetection of the peptides were prepared from gels run in parallel. Polyclonal anti-M_r 30,000-33,000 SC antibodies recognized all bands that were visible on the Coomassie-stained gel (not shown), which indicates that the peptides in the preparations were relatively pure and of the expected length.

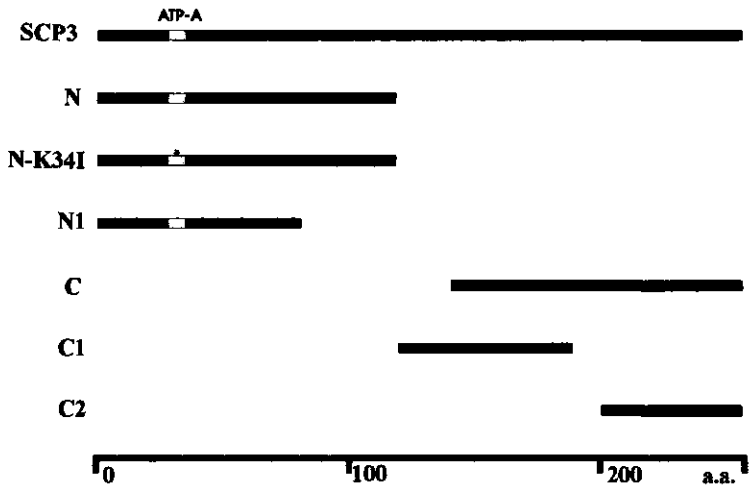


Figure 1: SCP3 and the subpeptides used in this study. The ATP-binding motif A (shaded region) is indicated. The asterisk indicates the point mutation in the ATP-binding motif A, in peptide N-K34I; a.a.: amino acids. See text for more details.

The DNA-binding domain is located near the N-terminus

We analysed the possible DNA-binding characteristics of SCP3 and its subpeptides by Southwestern blotting (see Experimental Procedures, Figure 2). Binding reactions were performed at 50 and 80 mM NaCl. At both salt concentrations two types of probe were tested: a double-stranded [^{32}P]-labeled fragment of cDNA-clone pHL58 (9.3 x 10^8 c.p.m./ml) and the same fragment denatured by boiling. The molar amounts of ss- and ds probe used in these assays were the same, assumed that no reannealing occurred. At both salt concentrations, SCP3 bound more ss- than dsDNA; at the more stringent conditions (80 mM NaCl) full-length SCP3 bound 4.4-fold more moles of ssDNA than of dsDNA. Furthermore, at 80 mM NaCl, the N-terminal peptides (N and NI) and N-K34I also still bound detectable amounts of ss- and dsDNA, albeit about 10-fold less than full-length SCP3. Like full-length SCP3, the N-terminal peptides bound more ss- than ds probe (Figure 2C and E). The substitution of the conserved lysine (K34) in the ATP-binding motif had an effect on DNA-binding. At 80 mM NaCl, the N peptide bound 4.4-fold more ssDNA than its mutated version. In these comparisons of binding of SCP3 and its subpeptides to ss- and dsDNA, the ratio of ss- to dsDNA-binding is possibly underestimated because of possible reannealing of the denatured strands of the cDNA probe.

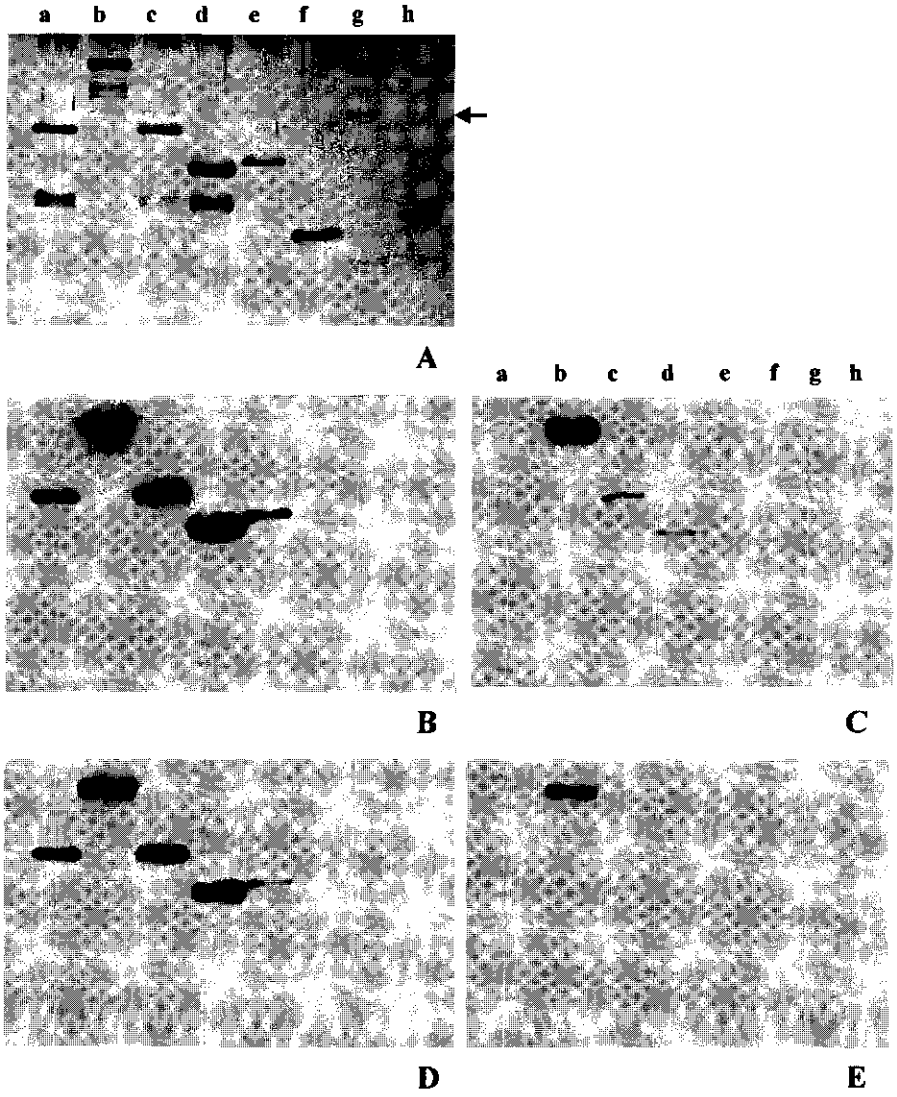


Figure 2: A: Coomassie Brilliant Blue-stained 14.5% Tricine-SDS-polyacrylamide gel containing the peptides analysed for DNA-binding *in vitro*: N-K34I (lane a), SCP3 (lane b), N (lane c), N1 (lane d), C (lane e), C1 (lane f), C2 (lane g), cytochrome *c* (lane h). The top of the 14.5% T, 6% C separating gel is indicated by an arrow. **B-E**: Autoradiographs obtained after Southwestern analysis of blots prepared from gels run in parallel to that shown in **A**; the Southwestern analyses were performed under the following conditions. (B): 50 mM NaCl, ssDNA probe, (C): 80 mM NaCl, ssDNA probe, (D): 50 mM NaCl, dsDNA probe, (E): 80 mM NaCl, dsDNA probe.

SCP3 binds preferentially to ssDNA *in vitro*

We analyzed the possible preference of SCP3 for binding to ssDNA by means of the mobility shift assay (MSA, Figure 3). For these experiments peptide N was used, which, in contrast to full-length SCP3, is soluble in aqueous solutions. We used a fragment containing telomeric repeats (directly added and added after denaturation by boiling) in these experiments because we had found that SCP3 was probably associated with telomeric DNA *in vivo*.

Whereas no shifted band could be detected when dsDNA probes were used, a clear shift was observed for various ssDNA (or more precisely: denatured; see Discussion) probes, whereas the band of unbound ssDNA decreased (Figure 3B). Although addition of more than 40 ng of peptide N seems also to result in some decrease of the intensity of the band of unbound dsDNA in the experiment in Figure 3B, this does probably not mean that dsDNA makes part of the shifted band because no such decrease is detected in Figure 3A. These results thus confirmed our earlier observations which showed a preference for binding of non-dsDNA by SCP3.

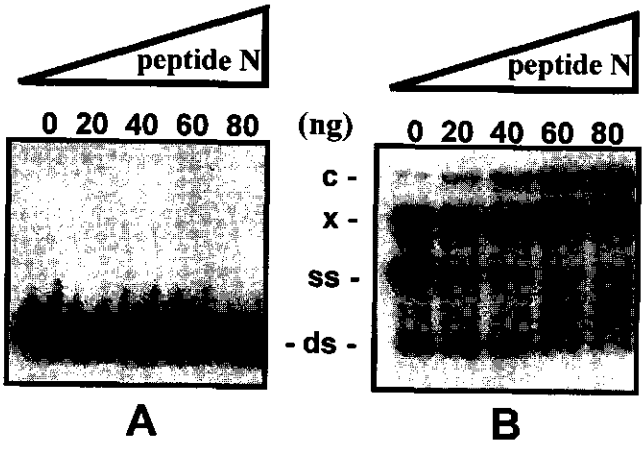


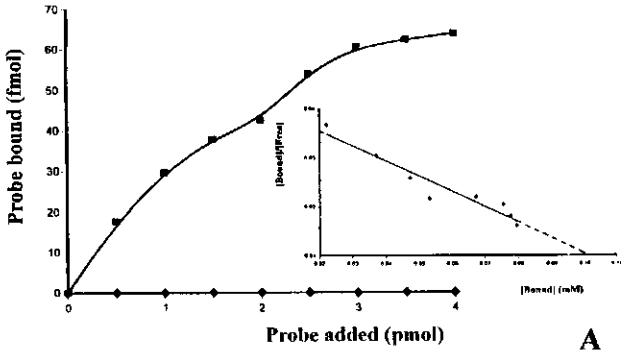
Figure 3: ssDNA-binding by peptide N. DNA-binding was analysed by means of the mobility-shift assay (MSA, see Experimental Procedures) with a DNA probe containing 50 telomeric repeats of 5'-TTAGGG-3'.**A**: MSA with dsDNA probe. **B**: MSA with the same probe denatured by boiling prior to addition to the binding mixture. Amounts of peptide N (in ng) in the binding reactions are indicated at the top of the lanes. ds: double-stranded DNA probe; ss: single-stranded DNA-probe; x: unresolved secondary structure of DNA-probe; c: DNA-protein complex.

To study the DNA-binding of SCP3 to ss- and dsDNA more specifically, we used the cDNA-fragment of clone pHL58 (which was regarded as a more suitable probe than telomeric DNA; see Discussion) as dsDNA probe, and derived a ss probe of it by linear amplification (see Experimental Procedures). Figure 4A shows the binding of ssDNA and Figure 4B of dsDNA by SCP3 at increasing probe concentrations. The DNA-binding by cytochrome *c* is also shown in Figure 4B. The binding capacity of SCP3 for ssDNA was several orders of magnitude higher than for dsDNA. The maximal amount of ssDNA that was bound by 3 pmol of SCP3 under our experimental conditions is about 100 fmol (estimated from the intercept of the horizontal axis of the Scatchard plot in Figure 4A). In experiments in which we used 3 pmol of SCP3, dsDNA-binding was hardly detectable and a plateau was not reached (Figure 4B). In a separate series of experiments where 15 pmol of SCP3 was used per Western blot strip, a plateau value was reached of about 0.1 fmol of dsDNA (Figure 4C). This means that about 5000-fold less dsDNA was bound per mole of SCP3 than ssDNA. From the intercept of the vertical axis of the Scatchard plot in Figure 4A (Scatchard, 1949), we estimated that about 3.6% of SCP3 on the blot was involved in the binding of ssDNA, at least if we assume that one molecule of SCP3 binds one molecule of ssDNA.

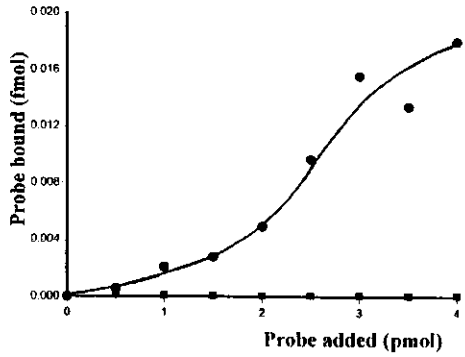
If we assume a 1:1 stoichiometry of SCP3 and DNA molecules on the filter, the association constant, K_a, for binding of SCP3 to ssDNA was 3.1×10^8 M^{-1}. A similar K_a-value was obtained when single-stranded probes of almost equal length were prepared from another cDNA clone (pHL614). An almost 10-fold lower K_a-value was estimated for the telomeric repeat sequences $(5'\text{-TTAGGG-}3')_{15/50}$ and $(5'\text{-CCCTAA-}3')_{15/50}$, based on mobility shift assays (Table 1). DNA-binding by cytochrome *c* under the conditions used was negligible, even compared to the binding of dsDNA by SCP3 (Figure 4B). Thus we conclude that SCP3 preferentially binds to ssDNA *in vitro*.

Table 1: Association constants for binding *in vitro* by SCP3 to different ssDNA-probes.

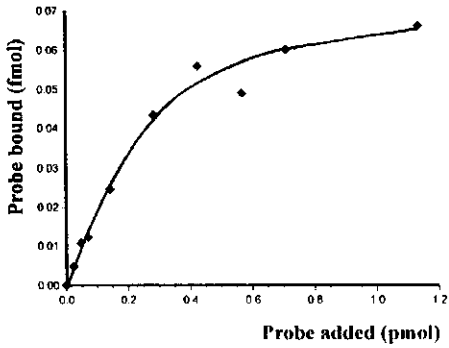
ssDNA-probe	K_a (M^{-1})	number of independent series
pHL58	3.1×10^8	n=3
pHL614	7.0×10^7	n=1
$(TTAGGG)_{15/50}$ or $(CCCTAA)_{15/50}$	3.4×10^7	n=3 data from MSA


Probe bound (fmol)
Probe added (pmol)

A


Probe bound (fmol)
Probe added (pmol)

B


Probe bound (fmol)
Probe added (pmol)

C

We next tested the capacity of SCP3 to bind two artificial ssDNA probes, which do not have the problem of reannealing, namely poly(dA) and poly(dC) (Figure 5). On the basis of the observed plateau values, we found that SCP3 bound ten times more poly(dC) than poly(dA). Furthermore, we found that the maximal amount of poly(dC) that could be bound by 3 pmol of SCP3 is approximately 20 times less than for the ssDNA probes described before. These results indicate that in addition to single-strandedness other factors determine the DNA-binding capacity of SCP3 for ssDNA. For these experiments, Scatchard analysis showed that binding events between SCP3 and the artificial ssDNA molecules were not independent (not shown).

Binding of the M_r 30,000-33,000 SC proteins to DNA

Within SCs several isolelectric variants of SCP3 have been found (Lammers *et al.*, 1995; Chapter 3) of which we also analysed the DNA-binding capacity *in vitro*. Figure 6 shows the result of a two-dimensional Southwestern blot analysis, performed with a ss- (Figure 6C) or dsDNA probe (Figure 6D). All variants of SCP3 that showed detectable DNA-binding displayed a preference for binding to ssDNA. Several variants of SCP3 show no detectable DNA-binding at all under these conditions. This analysis also revealed proteins within SC preparations which show only detectable binding to either ss- or to dsDNA (arrows in Figure 6C and D).

Discussion

SCP3 is a ssDNA-binding protein *in vitro*

The analyses performed in this study show that SCP3 apparently exhibits non-sequence specific, ssDNA-binding.

We think that the DNA-binding by SCP3 in the Southwestern blotting experiments is not simply the consequence of electrostatic interactions between the positively charged protein and the negatively charged DNA probe, because cytochrome *c*, which has a comparable pI (9.6) as SCP3, does not bind detectably to DNA (Figure 4B).

Figure 4: Binding of the insert of cDNA clone pHL58 by SCP3. DNA-binding by SCP3 was analyzed at 80 mM NaCl at increasing concentrations of radio-actively labeled probe to Western blot strips containing 3 pmol of SCP3. As a control, DNA-binding was performed under the same conditions on Western blot strips containing 3 pmol of cytochrome *c*. **A**: binding of the probe in a double-stranded (◆) and single-stranded (■) form. Inset: Scatchard plot of the data for the binding of the single-stranded probe. **B**: Binding of the probe in its double-stranded form (●) by SCP3 (same data as in A) compared to binding of the same probe in its double- or single-stranded form by an equimolar amount of cytochrome *c* (▪, combined data for single- and double-stranded probe). **C**: Binding of dsDNA probe by SCP3 with 15 pmol of protein per Western blot strip.
Note different scales on vertical axes in A, B and C.

In Southwestern blotting experiments, SCP3 preferentially binds to ssDNA (Figure 4A and 4B). One interpretation for this observation is that ssDNA is more flexible than dsDNA and therefore can interact more efficiently with the immobilized protein than dsDNA. This could even happen if the affinity of SCP3 for ssDNA and dsDNA would be the same. This interpretation, however, is not in agreement with the results of the mobility-shift assay, which also shows a clear preference for binding of SCP3 to ssDNA, under conditions where the protein is no longer immobilized (Figure 3). The observed preferential binding of ssDNA by SCP3 is thus caused by a higher affinity for this type of DNA probe.

An important factor which influences the affinity of SCP3 for ssDNA is the nucleotide composition of the probe. This is demonstrated by the binding of SCP3 to poly(dA) and poly(dC). Although both are ssDNA probes, SCP3 shows a clear preference for binding to poly(dC) (Figure 5). A factor which might influence the affinity of SCP3 for binding to these ssDNA probes is the occurence of certain types of secondary structure in these homopolynucleotides. Poly(dC), for instance, exhibits considerable stacking of bases which results in an altered threedimensional structure (Cantor and Schimmel, 1980).

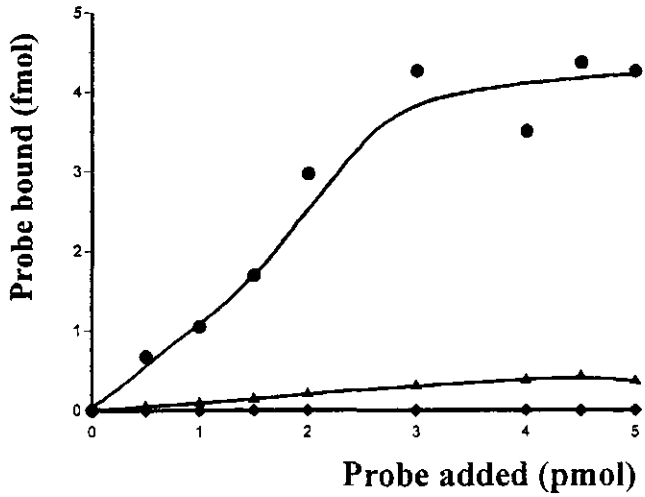
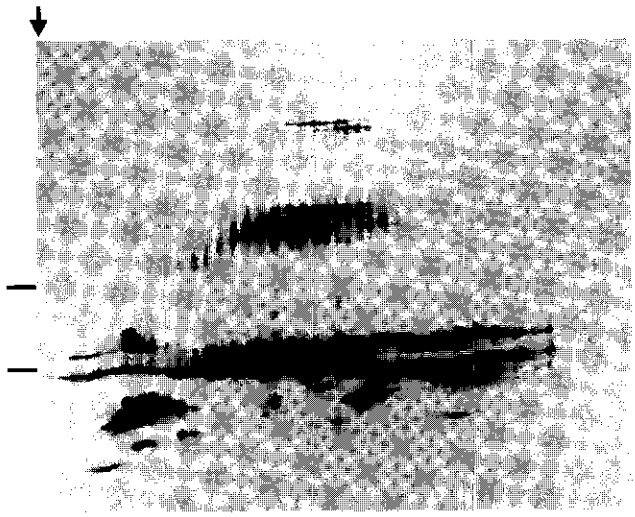


Figure 5: Binding of poly(dA) (▲) and poly(dC) (●) to SCP3 and to an equimolar amount of cytochrome c (◆, combined data for poly(dA) and poly(dC)).

Furthermore, the telomeric DNA-fragments used in the mobility shift assay are not only single-stranded but exhibit various types of intra- and interstrand interactions, which lead to the formation of several types of secondary and threedimensional structure (reviewed by Henderson, 1995). This possibly also holds true, though probably to a lesser extent, for the cDNA-fragment from clone pHL58. The observed 10-fold difference between K_a-values for binding by SCP3 to the cDNA-fragment of clone pHL58 and the telomeric repeats might result from denaturation of the telomeric repeat fragments by boiling prior to addition to the binding mixture. Under these conditions telomeric repeats form structures to which SCP3 might not bind or for which it has a lower affinity.
We thus propose that in addition to single-strandedness additional factors, possibly including the presence of certain secondary structures, determine the affinity of SCP3 for DNA probes. In the Southwestern blotting experiments, the N-terminal one-third of SCP3 appears to harbor the DNA-binding domain (Figure 2 and 3). Interestingly, the omission of the C-terminal half of the protein, which hardly shows DNA-binding itself, results in a considerable reduction of the level of DNA-binding. Possibly, the C-terminal half of SCP3 is needed for proper folding of the protein. This part of the protein contains two domains that can adopt an amphipatic α-helical structure and possibly contribute to the strong tendency of SCP3 to form dimers, even in the presence of SDS (Lammers *et al.*, 1994; Chapter 2). Such a dimerization might increase the DNA-binding affinity and could occur during the blotting of SCP3 from an SDS gel onto the nitrocellulose membrane.
Introduction of a point mutation at the conserved lysine residue (K34) within the ATP-binding motif A decreases the DNA-binding by SCP3. This could be the result of the loss of a positively charged amino acid that is involved in the binding of the negatively charged DNA but also of a changed folding of the protein that influences the (nearby) DNA-binding domain.

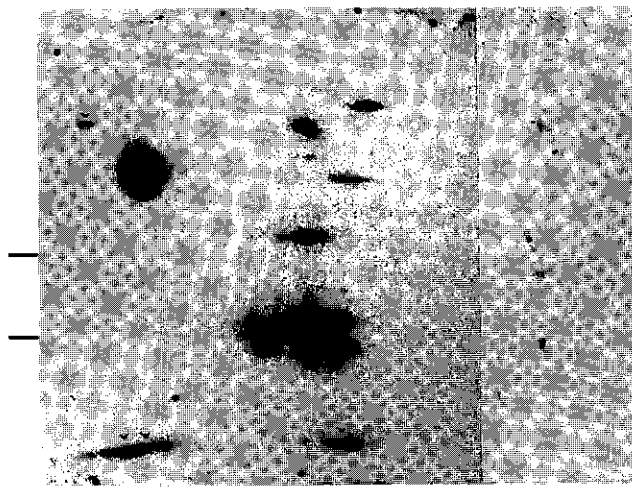
Binding of DNA by isoelectric variants of SCP3

Analysis of the DNA-binding *in vitro* of SCP3 from rat SCs also shows a preference for ssDNA, although ssDNA-binding seems to be restricted to a subset of the pI-variants detected on 2D-Western blots (Lammers *et al.*, 1995, Chapter 3; Figure 6C). We previously showed that rat SCs contain several pI-variants of SCP3. These variants differ in the number of attached phosphate groups. If we assume that phosphorylation alone is responsible for the observed differences in pI-values we estimate that SCP3 *in vivo* carries one to six phosphate groups (Lammers *et al.*, 1995, Chapter 3). We also showed that this heterogeneity is already present during the zygotene stage and that the phosphorylation pattern changes between early-and mid-pachytene, probably by the addition of one phosphate group to all variants.



A

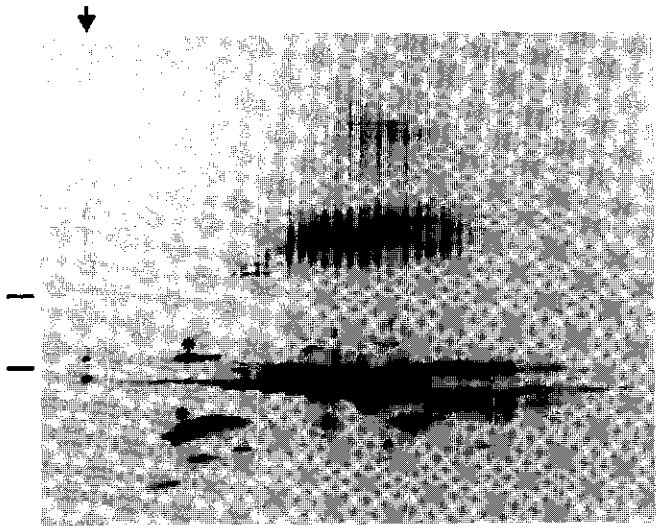
basic acidic



C

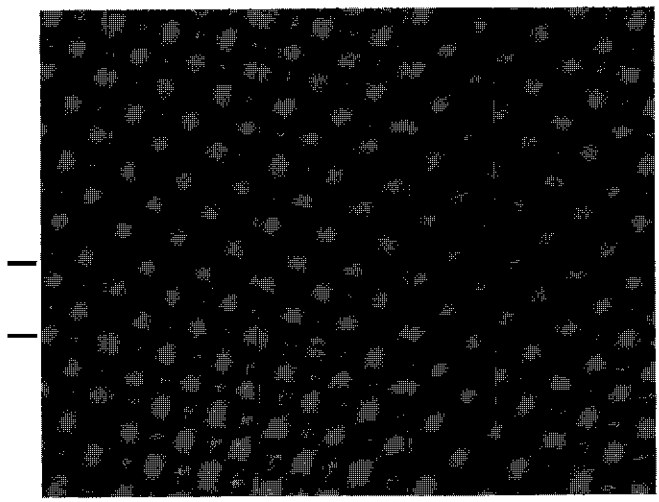
basic acidic

Figure 6: Two-dimensional Southwestern blot analysis of the M_r 30,000-33,000 SC components. **A** and **B**: Two-dimensional Western blots of SC proteins after detection of the M_r 30,000-33,000 components with an affinity-purified anti-M_r 30,000-33,000 SC antiserum (Lammers *et al.*, 1997a; Chapter 4). **C** and **D**: 2D-Southwestern analysis performed under stringent conditions (80 mM NaCl) with amounts of probe sufficient to reach about 70% of saturation for ssDNA-binding by SCP3.



B

basic **acidic**



D

basic **acidic**

(Figure 6 continued) **C**: autoradiogram obtained after probing the blot shown in **A** with [^{32}P]-labeled single-stranded insert of pHL58. **D**: autoradiogram obtained after probing the blot shown in **B** with [^{32}P]-labeled double-stranded insert of pHL58. Arrowheads indicate proteins that show detectable DNA-binding to only one type of probe. Asterisks indicate the SCP3 variants which have a similiar pI-value as bacterially expressed SCP3. The arrow indicates the (basic) top of the isolelectric focussing gel. Bars to the left of the blots indicate the positions of markers with relative electrophoretic mobilities of 37 and 31 kDa.

The 2D-Southwestern analysis shows no detectable ssDNA-binding for the most basic as well as the most acidic pI-variants of SCP3. One explanation for this observation is that no ssDNA-binding is detected due to to the presence of lower amounts of these pI-variants than of pI-variants that exhibit detectable ssDNA-binding on the Western blots (compare e.g. Figure 6A and 6C). This would explain why variants with a pI comparable to the bacterial SCP3 (asterisks in Figure 6A and B) show no detectable ssDNA-binding. Alternatively, the affinity of the different pI-variants of SCP3 for the ssDNA probe is not the same, which results in the detection of ssDNA-binding by only those pI-variants which have the highest affinity for the ssDNA-probe. This would then explain the dramatic loss of detectable ssDNA-binding by pI-variants of SCP3 going from the basic to acidic side of the blot (Figure 6C). The absence of detectable ssDNA-binding by some of the most basic variants might, in this case, be connected with their different relative electrophoretic mobilities (the variants indicated with an asterisk have either a relative electrophoretic mobility which is slightly higher than 33,000 or well below 30,000 in Figure 6A), which indicate that they might be distinct from the bulk of SCP3-variants.
The absence of detectable ssDNA-binding by the more acidic pI-variants of SCP3 might be the result of a decrease in the positive charge of the proteins which would weaken interactions with the negatively charged DNA. Alternatively, the presence of multiple phosphate groups may interfere with dimerization of the protein during the transfer of protein from the gel onto the blot. This might lower the affinity for binding to DNA (see above). Finally, a single phosphorylation event at a certain position within the SCP3 protein (possibly in the N-terminal one-third of the protein) might impair DNA-binding.
Another interesting observation is that in Figure 6C, next to the pI-variants of SCP3, several other proteins present in SC preparations show detectable binding to ssDNA. Most of these appear to bind ssDNA better than dsDNA or do not show detectable dsDNA-binding at all.

Is SCP3 bound to ssDNA *in vivo*?

We have obtained evidence that SCP3, which is a major protein component of the LEs of SCs is bound to DNA *in vivo* (Chapter 4). The DNA-binding studies *in vitro* presented here indicate a preference for binding of ssDNA rather than of dsDNA by these proteins. If this reflects the DNA-binding *in vivo* of SCP3, one might consider the existence of single-stranded DNA regions within the genome, during at least part of meiotic prophase. This is not unexpected since several other (putative) ssDNA-binding proteins, such as Rad51 (Sung and Robberson, 1995), Dmc1 and Rpa (Brill and Stillman, 1989) have been localized at or near the LEs (Plug, 1997). If such single-stranded regions exist these are expected to be located near the bases of the chromatin loops which are associated in some

way with the LEs. An interesting question is what causes the presence of these presumed single-stranded regions within meiotic chromatin.
First, single-stranded regions are often found at/near matrix attachment regions (MARs) or scaffold attachment regions (SARs) (Probst and Herzog, 1985; Bode *et al.*, 1992, Kay and Bode, 1994). We have obtained evidence that MARs/SARs are related to the association sites by which meiotic chromatin is attached to the LEs during prophase (Chapter 4). Therefore, single-stranded regions might be present at/near the bases of chromatin loops, in the vicinity of the LE, and could be bound by SCP3.
Second, these single-stranded regions might represent segments of the genome whose replication is delayed until zygotene, as was found in *Lillium* (Hotta and Stern, 1975) and in the male mouse (Hotta *et al.*, 1977a and Hotta *et al.*, 1977b). Replication of this so-called 'zygDNA' was observed to localize in regions of SC (Kurata and Ito, 1978). The ends of the zygDNA remain unreplicated until after pachytene (Hotta and Stern, 1976). Delayed zygDNA replication was supposed to be coupled to chromosome pairing, whereas the presence of unreplicated segments at the ends of the zygDNA was assumed to function in sister chromatid cohesiveness (Stern and Hotta, 1987). Such unreplicated DNA-segments might adopt a secondary structure in which single-stranded regions are present, which could be bound by SC-components such as SCP3.
Finally, another source of ssDNA segments is the initiated recombination event. In yeast, the 5'-ends of a DSB are resected leaving 3' single-stranded tails of considerable length (Sun *et al.*, 1991). For interhomolog recombination to occur these sequences must be brought within the context of the SC. Several ssDNA-binding proteins might associate with the generated single-stranded tails either to protect the DNA or to regulate subsequent steps in the recombination pathway. Binding of recA-like proteins Rad51 and Dmc1, and Rpa might precede invasion of the single-stranded tails into the homologous duplex, whereas other ssDNA-binding proteins remain bound when the DSB is not used for recombinational interactions and is repaired at a later stage. The latter function might be fulfilled by components of the LE, such as SCP3.

If SCP3 would bind to single-stranded DNA *in vivo*, could this DNA-binding then be regulated by phosphorylation? We previously hypothesized that SCP3 could be an *in vivo* target for DNA-dependent protein kinase (DNA-PK; Lammers *et al.*, 1995, Chapter 3). DNA-PK is probably involved in a checkpoint for DNA damage (Jackson and Jeggo, 1995). However, in mice with a defect in DNA-PK (SCID mice; Blunt *et al.*, 1995) meiosis and rates of meiotic recombination are unaffected (Heine *et al.*, 1996). This makes it unlikely that DNA-PK has a function in monitoring progression through meiotic prophase. Recently, however, two DNA-PK-related protein kinases, ATM and ATR,

were localized on meiotic chromosomes (Keegan *et al.*, 1996). ATR is first detected during the zygotene stage, in unsynapsed regions. ATM is also detected from the zygotene stage on and localized to synapsed regions of the chromosomes. Stretches of axial core were detected, by anti-COR1 antibodies, before ATR and ATM. Because of their localization it was assumed that ATR and ATM were involved in monitoring the processes of synapsis and recombination. ATR and ATM therefore might be responsible for the high degree of phosphorylation of SCP3 *in vivo*. The question is, however, if phosphorylation of SCP3 *in vivo* is related to its potential ssDNA-binding. If SCP3 is phosphorylated at an early stage (leptotene/early zygotene, Lammers *et al.*, 1995, Chapter 3, Figure 6A) by ATM or ATR, and the DNA-binding capacity *in vivo* of SCP3 would be similar as *in vitro*, it would render at least part of the protein incapable of binding to DNA. Possibly, DNA-binding by SCP3 protein is thus inactivated at sites where single-stranded regions are used in processes from which SCP3 is excluded. These could involve the invasion of a non-sister duplex by a single-stranded tail formed at the site where a DSB had been introduced or the replication of zygDNA at zygotene.

If the observed change in the phosphorylation pattern of SCP3 between early and mid-pachytene regulates ssDNA-binding, one might consider if this is related to a change on the DNA-level such as the transition from interactions between non-sister chromatids being promoted to interactions between sister chromatids (Schwacha and Kleckner, 1994). In such a scenario, SCP3 might act as an inhibitor of intersister interactions or be involved in temporal storage of initiated recombination events, which have not been selected for recombinational interactions. Alternatively, SCP3 might be removed from the ends of zygDNA when these are replicated after pachytene.

In addition to all of these considerations one has to keep in mind that the influence of phosphorylation on the ssDNA-binding by SCP3 observed in this study can be completely due to the experimental conditions. In that case the observed phosphorylation of SCP3 *in vivo* might also regulate interactions of SCP3 with itself and other proteins.

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Chapter 6

General Discussion

Introduction

In this chapter I want to discuss the possible function of the M_r 30,000-33,000 SC proteins. First, I will summarize what is known about the M_r 30,000-33,000 SC proteins, and second, I will describe my view of chromatin organization during meiotic prophase. This will be mainly based on the observation that the bases of meiotic prophase chromatin loops colocalize with MARs/SARs. Finally I will discuss possible roles of the M_r 30,000-33,000 SC proteins in chromatin organization.

■ *A summary of what is known about the M_r 30,000-33,000 SC proteins*

1) The M_r 30,000-33,000 SC proteins are components of the lateral elements of the rat (Heyting *et al.*, 1987; Moens *et al.*, 1987). They can be detected from zygotene up to and including diplotene (Offenberg *et al.*, 1991; Dietrich *et al.*, 1992).
2) We isolated a cDNA which probably encodes both the M_r 30,000 and 33,000 SC components (Lammers *et al.*, 1994; Chapter 2 and addendum to Chapter 2). What causes the difference between the M_r 30,000 and 33,000 proteins is still unknown, but the difference probably arises during or after translation. The predicted translation product of this cDNA, SCP3, has an ATP-binding motif A located near the N-terminus and two amphipatic alpha-helical regions in the C-terminal half. The amphipathic alpha-helices are predicted to be capable of coiled-coil interactions by which dimerization or polymerization could occur.
3) Both the M_r 30,000 and the M_r 33,000 SC components are represented by a number of phosphorylation variants, which co-exist during successive stages of meiotic prophase (Lammers *et al.*, 1995, Chapter 3).
4) The phosphorylation pattern of the M_r 30,000-33,000 SC components changes between early and mid-pachytene, presumably by the addition of one phosphate group to all variants (Lammers *et al.*, 1995, Chapter 3).
5) The M_r 30,000-33,000 SC components bind to DNA *in vitro* and *in vivo* (Chapter 4 and 5). *In vitro*, the M_r 30,000-33,000 SC components and SCP3 bind preferentially to single-stranded DNA, irrespective of the nucleotide sequence. The phosphorylation variants of the M_r 30,000-33,000 SC components differ in their DNA-binding capacity *in vitro*. The most acidic phosphorylation variants are no longer capable of binding to DNA *in vitro*. The DNA-binding domain is located in the N-terminal one-third of the protein. The M_r 30,000-33,000 SC proteins are probably associated with sequences that make up the bases of the chromatin loops. One of these sequences, for which we propose the term 'SCAR' (SC-associated region) colocalizes with a

MAR/SAR. MARs/SARs constitute the attachment regions of the chromatin to the interphase nuclear matrix and the mitotic scaffold, respectively. Moreover, several MARs exhibit specific binding to isolated SCs in an *in vitro* assay.

6) Proteins homologous to SCP3 have been identified in mouse (Klink *et al.*, 1997) and hamster (COR1, Dobson *et al.*, 1994).
7) During male meiosis, COR1 gradually concentrates in spots at centromeres between diplotene and meta/anaphase I (Moens and Spyropoulos, 1995). COR1 can be detected at centromeres until anaphase II (Dobson *et al.*, 1994; Moens and Spyropoulos, 1995). During female meiosis, the M_r 30,000-33,000 SC proteins are not detected in meiotic chromosome cores after diplotene (Dietrich *et al.*, 1992).
8) Overexpression of SCP3 in a heterologous system results in the formation of intermediate filament-like structures. The capacity to form these filaments lies in the C-terminal part of the protein and is probably based on coiled-coil interactions (Yuan *et al.*, 1998). It must be noted that in this system SCP3 might not be phosphorylated.

On the basis of these findings, I propose that the M_r 30,000-33,000 SC proteins are DNA-binding proteins *in vivo*, and that they probably bind to segments of single-stranded DNA. Furthermore, from the gradual concentration of SCP3 after SC-disassembly near centromeres, Moens and coworkers have suggested that these proteins are involved in the cohesion of sister chromatids along chromosome arms until anaphase I and at the centromere until anaphase II (Dobson *et al.*, 1994; Moens and Spyropoulos, 1995).

Before discussing this proposed function in more detail, I will first turn to the topic of chromatin organization during meiotic prophase. After presenting a general view of chromatin organization and its characteristics during meiotic prophase, I will return to the M_r 30,000-33,000 SC proteins and try to show how these fit into this view.

Meiosis requires a modified organization of the chromosome

Meiotic recombination is characterized by: (i) a high crossover frequency, (ii) a preference for recombination between non-sister chromatids, and (iii) positive interference between crossovers but not gene conversions. Which are the requirements to achieve this? Non-sister recombination requires a relationship between sister chromatids which is different from what is found during mitosis. Sister chromatid interactions should be (at least temporarily) inhibited and non-sister interactions promoted. For crossover interference to occur, a signal must be emitted from an event selected to become a crossover,

along the chromosomes, to inhibit the selection of other nearby events to become crossovers.
After formation of crossovers, which can be detected cytologically as chiasmata, cohesiveness between sister chromatids must be maintained distal to the chiasma to prohibit precocious sister separation and to supply for sufficient resistance against the forces that are exerted upon the chromosomes which are pulled towards opposite poles (reviewed by Bickel and Orr-Waever, 1996). Alternatively, a local 'chiasma binder' could provide for this resistance (Carpenter, 1994). If sister chromatid cohesiveness (rather than a chiasma binder) stabilises the chiasmata, then cohesiveness distal to the chiasmata must persist until anaphase I, and cohesiveness at the centromeres until anaphase II.

All of the above-mentioned requirements demand a specialized organization of the meiotic chromosome. I will now present a general view of meiotic chromosome organization which could account for the requirements mentioned. The assumption which is central to this view is that an SC-associated region (SCAR) is a matrix attachment region or scaffold attachment region (MAR/SAR). Although it is not necessary to assume that every MAR/SAR can also function also as a SCAR, but that certain requirements might make MARs/SARs candidates for functioning as a SCAR, I will not make such a distinction here for the sake of simplicity.

■ *Meiotic chromatin organization promotes non-sister interactions, provides a framework for crossover interference and provides the basis for sister chromatid cohesiveness*
During mitotic prophase, specialized AT-rich DNA sequences, called SARs associate with a chromosome scaffold in such a way that the chromatin becomes organized in loops. A similar organization of chromatin loops which are attached to a scaffolding structure is found during meiotic prophase (Weith and Traut, 1980; Moens and Pearlman, 1988; Heng *et al.*, 1994; Heng *et al.*, 1996). On the basis of the findings presented in Chapter 4, I propose that during meiotic prophase the same DNA sequences make up the bases of the chromatin loops as during mitotic prophase. Thus, I assume that SCARs are SARs/MARs. Kleckner (1996) proposed that during meiotic prophase the chromatin is organized by an axis-mediated process which is similar to that during mitotic prophase. Such a proposition is in agreement with the idea that SCARs and SARs/MARs are identical sequences, which constitute the bases of the chromatin loops. Moreover, it provides for a selection of sequences (the SCARs or adjacent sequences) that could be used in the processes of homology search and homolog pairing.
However, the association of SCARs and SARs/MARs with the meiotic lateral element and the mitotic scaffold respectively, might be different (Chapter 4). Most likely this reflects

the differences between the lateral elements of SCs and the scaffolds of mitotic sister chromatids. Therefore, one might expect meiosis-specific as well as (more) general nuclear matrix proteins to be components of lateral elements. Rufas *et al.* (1992) proposed that the chromatin of each sister chromatid is organized on a scaffold, and that a meiosis-specific lateral element would associate with the two scaffolds of the sister chromatids.

Schwacha and Kleckner (1994) proposed that formation of the axial/lateral element is a prerequisite for the substitution of intersister interactions by interhomolog interactions and thus for the formation of crossovers. Mutations in (putative) components of axial/lateral elements are thus predicted to result in a decrease of interhomolog recombination and increased non-disjunction at meiosis I (see below). The yeast *hop1Δ* mutant (Hollingsworth and Byers, 1989) fits this description. In the *hop1Δ* mutant interhomolog recombination is reduced to 1% of the wild-type level. Joint molecules are formed at late pachytene, but only between sister chromatids (Schwacha and Kleckner, 1994). Although DSBs are formed at 10% of the wild-type level during early prophase, interhomolog joint molecules are not formed. The Hop1 protein was proposed to function in the organization of the DNA at the bases of the chromatin loops (Friedman *et al.*, 1994).

As a next step I propose that (at least part of) the recombination machinery assembles at/near SCARs (see also Kleckner, 1996) and that initiated recombination events end up at/near these sites, irrespective of whether recombination is initiated here or in the chromatin loops. Moens *et al.* (1997) and Barlow *et al.* (1997) have shown that RAD51-foci associate with SCP3, as soon as SCP3-containing fragments are detectable. Possibly, this association occurs on top of already existing axial element fragments containing SCP2, another major component of the LEs, but not yet SCP3 (M. Schalk, unpublished observations).

The regulation of the number and the distribution of those initiated events that eventually become crossovers (crossover control) is poorly understood. In many organisms, the number of crossovers is limited to only a few per bivalent and crossovers are rarely found in each others neighbourhood (crossover interference, see for discussion Meuwissen (1997)). Of all initiated recombinational events only one or two per bivalent become crossovers, while the remainder become gene conversions (non-crossovers). In contrast to crossovers, gene conversions do not show interference (Mortimer and Fogel, 1974). The formation of crossovers and non-crossovers probably occurs via the same pathway (Storlazzi *et al.*, 1995) and it was proposed that initiated events are selected to become either a crossover or a non-crossover before synapsis (Storlazzi *et al.*, 1996). The Zip1 protein of yeast (Sym *et al.*, 1993) which is probably involved in this selection is possibly a

functional homolog of SCP1 (Meuwissen, 1997). In the rat, SCP1 might thus play an at least partly similar role. Synapsis might thus include binding of SCP1 to SCARs on both sides of the SC. An attractive model for crossover interference thus includes binding of SCP1 to AT-rich SCAR sequences, starting near an event selected to become a crossover, thus blocking the formation of crossovers nearby and constraining the number of crossover events to a few that are well separated.
During late pachytene and the earliest stages of diplotene the lateral element between two sister chromatids becomes thicker and less compact and sometimes two subelements, which probably correspond to the sister scaffolds, can be discerned (Heyting and Dietrich, 1992). Subsequently, the lateral elements are disassembled and the scaffolds of the sister chromatids can be distinguished. I propose that the separation of sister scaffolds takes place at/near SCARs. This probably requires the breakdown of the lateral element and the action of topoisomerase II (topo II). Topo II congregates onto the lateral elements, starting from late pachytene (Moens and Earnshaw, 1989) and was shown to be required for separation of sister scaffolds during mitosis (Giménez-Abián *et al.*, 1995). The phenomenon of sister chromatid cohesiveness might thus, among others, involve the persistence of lateral element components at/near SCARs and the inhibition of topo II at/near these sites.

The function of the M_r 30,000-33,000 SC proteins

As stated before in this chapter, I expect the M_r 30,000-33,000 SC proteins to be involved in the modification of the relationship between sister chromatids during meiotic prophase. More specifically, I propose that these proteins contribute to the meiosis-specific chromatin organization which inhibits intersister interactions and provides for (at least in part) sister chromatid cohesiveness. I will now discuss this function of the M_r 30,000-33,000 SC proteins in somewhat more detail against the background of what I have said about meiotic chromatin organization in the previous section.

The M_r 30,000-33,000 SC proteins are major components of the lateral elements of rat SCs (Heyting *et al.*, 1987; Moens *et al.*, 1987). On the basis of their immunolocalization (Heyting *et al.*, 1985, 1987 and 1988; Offenberg *et al.*, 1991) and their capability to polymerize (Yuan *et al.*, 1998), I regard these proteins as major structural building blocks of the lateral element, together with SCP2. This structural property resides in the C-terminal half of the SCP3 protein (Yuan *et al.*, 1998). The N-terminal half of the SCP3 protein is capable of binding to DNA *in vitro*. This DNA-binding was characterized by a

preference for single-stranded DNA, which was insensitive to the nucleotide sequence. I propose that SCP3, together with SCP2 (which on the basis of its DNA-binding motifs could bind to dsDNA), binds to DNA of both sister chromatids, which provides for a physical link between the two sister chromatids and the single lateral element. Attachment of both sisters to one lateral element takes place at/near SCARs. Additionally, it forces sister chromatids to act as a single unit. Together with additional modifications this could result in the meiosis-specific chromatin organization, which inhibits intersister interactions and provides for sister chromatid cohesiveness.

If DNA-binding *in vivo* of the M_r 30,000-33,000 SC proteins, like that *in vitro*, also involves single-stranded DNA is not yet known. If it does, it would mean that single-stranded DNA regions are present in/near SCARs. In view of the similarity between SCARs and MARs/SARs, this is not unlikely. Single-stranded DNA regions are often found in/near MARs/SARs (Probst and Herzog, 1985; Bode *et al.*, 1992 and 1995; Kay and Bode, 1994) and were required for binding to the nuclear matrix (Ludérus *et al.*, 1994).

After pachytene, when crossovers provide for a physical link between homologs, the relationship between sister chromatids changes in such a way that intersister interactions are no longer inhibited (Schwacha and Kleckner, 1994). This coincides with the disassembly of the SC. Although the SC disassembles and the lateral elements disappear, the M_r 30,000-33,000 SC proteins remain present at several positions along chromosome arms, where it colocalizes with SCP2 until anaphase I, and near centromeres until anaphase II (Dobson *et al.*, 1994; Moens and Spyropoulos, 1995; M. Schalk, unpublished observations). Moens and coworkers have proposed that the persistence of the M_r 30,000-33,000 SC proteins (as well as of SCP2) contributes to the phenomenon of sister chromatid cohesiveness (Dobson *et al.*, 1994; Moens and Spyropoulos, 1995). Although sister scaffolds become discernible after pachytene, the presence of the M_r 30,000-33,000 SC proteins at/near SCARs still provides for linkage of the two. Such a physical link might be one of the means to maintain chiasmata and prevent precocious sister separation (Bickel and Orr-Weaver, 1996). In this view it is interesting to note that near centromeres where sister chromatids should not separate until anaphase II, many MARs/SARs are found (Strissel *et al.*, 1996).

The mechanism by which the M_r 30,000-33,000 SC proteins might actually provide for (part of) sister chromatid cohesiveness might involve inhibition of the action of topoisomerase II (topo II). Topo II was identified as a component of the mitotic scaffold (reviewed by Laemmli *et al.*, 1992) and is capable of binding to MARs/SARs (Adachi *et al.*, 1989). It was shown to be required for proper segregation of chromatids at mitosis (Giménez-Abián *et al.*, 1995), and of homologs at meiois I (Rose *et al.*, 1990). Topo II

probably acts at/near SCARs in the process of sister scaffold separation. Linkage of sisters by the M_r 30,000-33,000 SC proteins (together with SCP2) could either block this action of topo II or prevent separation of sister scaffolds after topo II action. The question then is why the M_r 30,000-33,000 SC proteins remain present at these particular positions, whereas the majority of the protein (which made up the lateral element) is lost. I expect that the M_r 30,000-33,000 SC proteins that remain are protected against disassembly e.g. because they are not tagged for breakdown. These particular M_r 30,000-33,000 SC proteins could make part of a structure that does not allow close contact of a modifying enzyme with these. The observed interaction between COR1 protein and the ubiquitin-conjugating enzyme UBC9 in a two-hybrid screen (Tarsounas *et al.*, 1997) might indicate that addition of ubiquitin to the M_r 30,000-33,000 SC proteins sets the stage for their breakdown. When sister chromatid cohesiveness is no longer needed the structures containing the M_r 30,000-33,000 SC proteins might disassemble and the SC proteins are broken down.

An important point that remains concerns the phosphorylation of the M_r 30,000-33,000 SC proteins. Why do so many isoelectric variants of these proteins co-exist? And what is the consequence of the phosphorylation event which takes place between early and mid-pachytene (Lammers *et al.*, 1995; Chapter 3)? The presence of various isoelectric forms might permit different and dynamic interactions between M_r 30,000-33,000 SC proteins and other proteins such as SCP2. In yeast, the interaction between the axial element components Red1 and Hop1 seems to be regulated by the phosphorylation of these proteins (Hollingsworth and Ponte, 1997). In turn, this influences the composition of axial elements. Similarly, interaction of SCP3 and SCP2 (next to possible SCP2/SCP2 and SCP3/SCP3 interactions) might be regulated by phosphorylation of at least SCP3. The possible interaction of SCP3 and SCP2 however, has not yet been investigated.
The phosphorylation event at early-mid pachytene takes place when synapsis has just been completed. At this stage, the number of Rad51-foci (or early recombination nodules) decreases strongly (Barlow *et al.*, 1997; Moens *et al.*, 1997). This indicates that the recombination process probably enters a new stage, which does no longer require the early recombination nodules. Phosphorylation of SCP3 at this stage might regulate a function which this protein fulfills in either the early or the late steps of the recombination process. Xu *et al.* (1997) proposed that among other factors the yeast Red1 protein and the putative protein kinase Mek1 provide for the chromosomal context for recombination intermediates, which allows their progression through meiotic prophase to be monitored. Perhaps SCP3 is involved in providing such a chromosomal context at/near SCARs. After phosphorylation between early and mid-pachytene, the role of SCP3 might

be only to act as a structural component which provides for sister chromatid cohesiveness at centromeres and, together with SCP2, at/near SCARs along chromosome arms.

Future prospects

The model presented in this chapter is based on several findings from experiments carried out *in vitro*. It predicts certain aspects of chromatin organization during meiotic prophase which could be tested *in vivo*. Important questions to be adressed involve the presence of (transient) single-stranded regions at/near SCARs, the regulation of DNA-binding by the M_r 30,000-33,000 SC proteins, the association of initiated recombination events with SCARs and the interactions between SC components and SCARs and between SC components themselves. Further analysis of the chromatin organization during meiotic prophase might provide a better understanding of the molecular basis of the features that are specific for meiosis: the preferential recombination between non-sister chromatids and positive crossover interference. Additionally, I think it would be of interest to establish the difference between the M_r 30,000 and the M_r 33,000 proteins and the effect of the phosphorylation of all M_r 30,000-33,000 SC variants during early pachytene. This might reveal more about the function of these major protein components of the lateral elements of rat SCs.

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Summary

Summary

Synaptonemal complexes (SCs) are intranuclear structures which are formed during meiotic prophase between homologous chromosomes. The SC consists of two protein-rich axes, either of which is found at the basis of one of the homologous chromosomes. These axes, called lateral elements (LEs), are connected along their entire length by so-called transverse filaments. Between and parallel to the LEs runs a third element, called central element. The assembly and disassembly of SCs take place in a period during which a number of important events takes place at the chromosomal level: condensation, pairing, recombination and segregation of homologous chromosomes. The possible involvement of the SC in these events is an important topic in the research program of our section. This thesis focusses on the possible function of the LE and its components (Chapter 1). The experimental work described in this thesis (Chapters 2-5) concerns the characterization of the LE-components with relative electrophoretic mobilities of 30,000 and 33,000 (the M_r 30,000-33,000 components).

The isolation of cDNAs encoding the M_r 30,000-33,000 components is described in Chapter 2. The isolation took place by screening of an expression cDNA-library with an affinity-purified polyclonal antiserum. The largest cDNA, 2A4, encodes a protein with a predicted molecular mass of 29.7 kDa, which we have termed synaptonemal complex protein 3 (SCP3). A polyclonal antiserum raised against SCP3 recognizes only the M_r 30,000-33,000 components on a westernblot of SC-protein and exhibits a similar immunological localisation as monoclonal antibodies and a polyclonal antiserum raised against the M_r 30,000-33,000 components. The deduced amino acid sequence shows that SCP3 is a potential ATP-binding protein and that the C-terminal half of the protein is capable of forming an amphipatic α-helix. Moreover, part of the amino acid sequence exhibits considerable homology to the predicted protein products of two members of a gene familiy of X-linked lymphocyte-regulated (*Xlr*) genes. We conclude that cDNA 2A4 encodes at least one of the M_r 30,000-33,000 components and that SCP3 is a major component of the LEs of the rat. We speculate that the homology between SCP3 and two products of *Xlr* genes might be connected to a function of both types of protein in processes which share the common purpose of blocking certain recombination events.

The M_r 30,000- and the 33,000 component are closely related according to their almost identical peptide maps and the fact that all isolated antibodies always recognize both proteins. A first step to elucidate the difference between these two components and the level at which such a difference arises is described in the addendum to Chapter 2. We

conclude that the M_r 30,000-33,000 components are probably encoded by a single gene and a single messenger RNA and that the difference between the two components arises during or after translation. Mechanisms by which this could be achieved are discussed. A practical problem which arises here is that N-terminal sequencing of the two components by means of standard techniques is not possible.

When SC-protein is separated by means of two-dimensional gel electrophoresis (2D-electrophoresis) and the gel is subsequently blotted and the blot used for immunological detection of the M_r 30,000-33,000 components, a large number of variants, differing in isoelectric point and relative electrophoretic mobility can be discerned. In Chapter 3 experiments are described which were performed to determine (i) the nature of the differences between the variants and (ii) possible changes in the observed pattern on 2D-gels during subsequent stages of meiotic prophase. We conclude that differences in the number of attached phosphate groups are largely responsible for the existence of different variants of the M_r 30,000 as well as the M_r 33,000 component. Moreover, we observed a change in the phosphorylation pattern between early- and midpachytene, probably because of the addition of one phosphate group to all variants. We speculate that the phosphorylation pattern observed as early as zygotene is the result of the action of a kinase which responds to DNA-damage.

The presence of the LEs at the bases of the chromatin of the homologous chromosomes prompted us to investigate whether points of contact exist between LEs and the chromatin, and if so, which LE-components and DNA-sequences are involved (Chapter 4). Based on crosslinking experiments *in vivo* of chromatin from mouse spermatocytes, we assumed that the M_r 30,000-33,000 components are bound to DNA or at least associated with DNA. By immunoprecipitation of protein-DNA complexes, obtained after crosslinking *in vivo* of mouse spermatocytes, with antibodies raised against the M_r 30,000-33,000 components as well as against isolated SCs, a region was identified within a 120 kbp gene cluster as an SC-associated region (SCAR). This SCAR colocalizes with a so-called 'matrix attachment region' (MAR). We also developed a method by which specific binding of DNA-fragments to the SC can be tested *in vitro*. By means of this method we show that MARs from different organisms bind specifically to rat SCs. We conclude that the organisation of the chromatin during meiotic prophase shows a certain similarity to those during mitosis and interphase.

After it was determined that the M_r 30,000-33,000 components are probably bound to DNA *in vivo*, we have further analysed the possible DNA-binding of these proteins as

well as SCP3 *in vitro* (Chapter 5). Because of the insolubility of SCP3 in aqueous solutions, these studies were carried out by means of the Southwestern blotting technique. We conclude that SCP3 binds to DNA *in vitro* and shows a preference for single-stranded DNA. From a comparison of different single-stranded DNA substrates we conclude that SCP3 probably favours binding to single-stranded DNA which adopts a spatial conformation caused by intra- or intermolecular interactions. In similar experiments the M_r 30,000-33,000 components also exhibit a preference for single-stranded DNA, although the variants which carry the most phosphate residues show no detectable DNA-binding at all. Based on the assumption that in the situation *in vivo* the M_r 30,000-33,000 components would exhibit a similar type of DNA-binding as *in vitro*, we speculate on the (temporal) existence of single-stranded DNA during meiotic prophase.

Finally, I present a summary on what is known about the M_r 30,000-33,000 components at the beginning of the general discussion (Chapter 6). Then I discuss a model which describes the chromatin organization during meiotic prophase, in which I emphasize the modifications which, starting from a general organization pattern, are necessary for the proper progress of the processes of pairing, recombination and segregation of homologous chromosomes. Within this model I discuss the possible involvement of components of the SC. After that I focus on the function of the M_r 30,000-33,000 components in this model. I propose that the M_r 30,000-33,000 components play a role in the structural organization of the chromosomes, in such a manner that recombination between sister chromatids is inhibited temporarily and cohesion between sister chromatids is maintained as long as this is needed.

Samenvatting

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Synaptonemale complexen (SC's) zijn intranucleaire structuren die tijdens de meiotische profase worden gevormd tussen homologe chromosomen. Het SC bestaat uit twee eiwitrijke assen, die elk aan de basis van één van de homologe chromosomen is gelegen. Deze assen, die laterale elementen (LE's) worden genoemd, worden over hun gehele lengte verbonden door zogenaamde transversale filamenten. Tussen de LE's treft men een derde element aan dat parallel loopt met de LE's, het centrale element. De vorming en afbraak van SC's vinden plaats in een periode waarin een aantal belangrijke gebeurtenissen op chromosomaal niveau optreedt: condensatie, paring, recombinatie en segregatie van homologe chromosomen. De mogelijke betrokkenheid van het SC bij deze gebeurtenissen is een belangrijk onderwerp in het onderzoek dat in onze sectie plaatsvindt. In dit proefschrift wordt met name ingegaan op de mogelijke functie van het LE en componenten hiervan (Hoofdstuk 1). Het experimentele werk dat in dit proefschrift is beschreven (Hoofdstuk 2 t/m 5) betreft de karakterisering van de LE-componenten met relatieve electroforetische mobiliteiten van 30.000 en 33.000 (de M_r 30.000-33.000 componenten).

De isolatie van cDNA's coderend voor de M_r 30.000-33.000 componenten wordt beschreven in Hoofdstuk 2. De isolatie vond plaats door screening van een expressie cDNA-bank met een affiniteitsgezuiverd polyclonaal antiserum. Het grootste cDNA, 2A4, codeert voor een eiwit met een voorspelde molecuulmassa van 29,7 kDa, dat wij synaptonemaal complex proteïne 3 (SCP3) hebben genoemd. Een polyclonaal antiserum, opgewekt tegen SCP3, herkent enkel de M_r 30.000-33.000 componenten op een western-blot van SC-eiwit en vertoont eenzelfde immunologische localisatie als monoclonale antilichamen en een polyclonaal antiserum opgewekt tegen de M_r 30.000-33.000 componenten. Uit de afgeleide aminozuurvolgorde blijkt dat SCP3 een potentieel ATP-bindend eiwit is en dat de C-terminale helft van het eiwit in staat is een amfipatische α-helix te vormen. Bovendien blijkt een deel van de aminozuurvolgorde van SCP3 een aanzienlijke homologie te vertonen met de voorspelde eiwitproducten van twee leden van een genfamilie van 'X-linked lymphocyte-regulated' (*Xlr*) genen. Wij concluderen dat cDNA 2A4 tenminste één van de M_r 30.000-33.000 componenten codeert en dat SCP3 een hoofdcomponent is van de LE's van de rat. Wij speculeren dat de gelijkenis van SCP3 met twee producten van *Xlr* genen verband zou kunnen houden met een functioneren van beide type eiwitten in processen die elk tot doel hebben bepaalde recombinatiegebeurtenissen te blokkeren.

De M_r 30.000- en de 33.000 component vertonen een grote gelijkenis, zoals blijkt uit hun bijna identieke peptidekaarten en het feit dat alle antilichamen die zijn geïsoleerd altijd beide eiwitten herkennen. Een eerste aanzet om te ontdekken wat het verschil is tussen deze twee componenten en op welk niveau dat ontstaat is beschreven in het addendum bij hoofdstuk 2. Wij concluderen dat de M_r 30.000- en 33.000 component waarschijnlijk beide worden gecodeerd door eenzelfde gen en eenzelfde messenger RNA en dat het verschil tussen de eiwtten ontstaat tijdens of na de translatie. Enkele mechanismen hiervoor worden bediscussieerd. Een praktisch probleem bij het zoeken naar het verschil tussen de M_r 30.000- en 33.000 component is dat eiwitsequentiebepaling vanaf de N-terminale uiteinden van de eiwitten niet mogelijk is via standaardmethoden.

Wanneer de eiwitten van het SC worden gescheiden m.b.v. tweedimensionale gelelectroforese (2D-electroforese), de gel wordt geblot en de blot wordt gebruikt om de M_r 30.000-33.000 componenten te detecteren met antilichamen, blijkt dat een groot aantal varianten, van elkaar verschillend in iso-electrisch punt en relatieve electroforetische mobiliteit, kan worden onderscheiden. In Hoofdstuk 3 worden experimenten beschreven die ten doel hadden (i) de aard van de verschillen tussen deze varianten en (ii) eventuele veranderingen in het waargenomen patroon op 2D-gels tijdens opeenvolgende stadia van de meiotische profase, vast te stellen. Wij concluderen dat verschillen in de mate van fosforylering grotendeels verantwoordelijk zijn voor het voorkomen van verschillende varianten van zowel de M_r 30.000- als de M_r 33.000 component. Bovendien stellen we vast dat het fosforyleringspatroon verandert tussen vroeg- en midpachyteen, waarschijnlijk door de toevoeging van één fosfaatgroep aan alle varianten. Wij speculeren dat, voor het reeds in het zygoteen waargenomen fosforyleringspatroon, een kinerende activiteit verantwoordelijk is die wordt geactiveerd door DNA-schade.

De ligging van de LE's aan de bases van het chromatine van de homologe chromosomen bracht ons ertoe na te gaan of er contactpunten zijn tussen LE's en het chromatine en, indien dit het geval is, welke LE-componenten en DNA-sequenties hierbij betrokken zijn (Hoofdstuk 4). Daarbij gingen wij er, op grond van de resultaten van *in vivo* crosslinkingsexperimenten met het chromatine van muizen spermatocyten vanuit, dat de M_r 30.000-33.000 componenten aan het chromatine gebonden zijn of er op zijn minst mee zijn geassocieerd. Via immunoprecipitatie van DNA-eiwit complexen verkregen door *in vivo* crosslinking van het chromatine van muizen spermatocyten met zowel antilichamen opgewekt tegen de M_r 30.000-33.000 componenten als tegen gehele SCs, werd in een gekozen gencluster van 120 kbp. dezelfde regio geïdentificeerd als een SC-geassocieerde regio (SCAR). Deze SCAR colocaliseert met een zogenaamde 'matrix attachment region'

(MAR). Bovendien werd een methode opgezet waarin specifieke binding van DNA-fragmenten aan SCs *in vitro* kan worden getest. In dergelijke experimenten blijken MARs uit verschillende organismen specifiek aan het SC van de rat te binden. Wij concluderen dat de organisatie van het chromatine tijdens de meiotische profase overeenkomst vertoont met die tijdens de mitose en de interfase.

Nadat was vastgesteld dat de M_r 30.000-33.000 componenten waarschijnlijk *in vivo* aan DNA gebonden zijn, hebben wij de mogelijke DNA-binding van zowel deze eiwitten als SCP3 verder *in vitro* onderzocht (Hoofdstuk 5). Vanwege de onoplosbaarheid van SCP3 in waterige oplossingen zijn deze studies uitgevoerd m.b.v. Southwestern blotting. Wij concluderen dat SCP3 *in vitro* aan DNA bindt en wel met een voorkeur voor enkelstrengs DNA. Uit een vergelijking van verschillende enkelstrengs DNA substraten blijkt dat SCP3 waarschijnlijk een voorkeur heeft voor het binden van enkelstrengs DNA dat een bepaalde ruimtelijke conformatie aanneemt t.g.v. intra- of intermoleculaire interacties. In soortgelijke experimenten vertonen de M_r 30.000-33.000 componenten eenzelfde voorkeur voor binding aan enkelstrengs DNA, zij het dat varianten met een hoge mate van fosforylering geen detecteerbare DNA-binding meer vertonen. Uitgaande van de aanname dat de M_r 30.000-33.000 componenten *in vivo* eenzelfde type DNA-binding vertonen als in de *in vitro* experimenten, speculeren wij over het (tijdelijk) voorkomen van enkelstrengs DNA tijdens de meiotische profase.

Tenslotte geef ik in een algemene discussie (Hoofdstuk 6) een samenvatting van de belangrijkste gegevens die bekend zijn over de M_r 30.000-33.000 componenten. Daarna bespreek ik een model van de organisatie van het chromatine tijdens de meiotische profase, waarbij ik met name wijs op de modificaties die, uitgaande van een algemeen organisatiepatroon, een correct verloop van de processen van paring, recombinatie en segregatie van homologe chromosomen mogelijk maken. Ik geef hierbij aan op welke manier onderdelen van het SC een rol kunnen vervullen. Vervolgens concentreer ik mij op de functie van de M_r 30.000-33.000 componenten binnen dit alles. Ik stel hierbij voor dat de M_r 30.000-33.000 componenten een rol spelen in de structurele organisatie van de chromosomen, op zo'n wijze dat recombinatie tussen zusterchromatiden tijdelijk wordt geremd en de cohesie tussen zusterchromatiden wordt gehandhaafd zolang dit vereist is.

Dankwoord

Through these fields of destruction
Baptisms of fire
I've witnessed your suffering
as the battles raged higher
And though they did hurt me so bad
In the fear and alarm
You did not desert me
My brothers in arms

(Uit: Dire Straits: "Brothers in arms")

Na zeven jaar is nu het moment daar dat ik achter mijn promotie-onderzoek een punt kan zetten. Het heeft erg lang geduurd voordat het boekje dat u nu leest gedrukt werd. De weg die daarvoor afgelegd moest worden ging waarlijk niet over rozen. De eerlijkheid gebiedt mij te zeggen dat ik met gemengde gevoelens terugkijk op de afgelopen zeven jaar, voorzover het mijn promotie-onderzoek betreft. Wat ik heb meegemaakt in met name de laatste jaren van die periode zal altijd een smet blijven op de herinnering aan dit onderzoek. Dat het boekje er nu ligt en ik deze periode daarmee kan afsluiten geeft me in ieder geval wel de nodige voldoening. Daarom wil ik graag alle mensen bedanken, die mij in de afgelopen jaren hebben gesteund en aangemoedigd en er zo mede verantwoordelijk voor zijn geweest dat ik de moed niet heb opgegeven.
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Curriculum vitae

Johannes Hubertus Maria Lammers werd op 16 oktober 1967 in Wageningen geboren. Na het behalen van zijn VWO-diploma aan het toenmalige Heldring College te Zetten begon hij in 1986 aan de studie Moleculaire Wetenschappen aan de Landbouwuniversiteit. In de doctoraalfase koos hij twee afstudeervakken: Moleculaire genetica (constructie van een cDNA-bank waarin specifieke mRNA's verrijkt waren) bij prof.dr. Heyting en Entomologie (opzetten van een zuiveringsmethode en eerste karakterisering van diapauze-eiwit 1 van de Coloradokever) bij dr. de Kort. Voor beide afstudeerscripties ontving hij de Unilever Research Prijs in 1992. In januari 1992 studeerde hij cum laude af, waarna hij, als assistent in opleiding, aan zijn promotie-onderzoek begon bij de vakgroep Erfelijkheidsleer aan de LU, opnieuw in de groep van prof.dr. Heyting. De resultaten van dit onderzoek vindt u in dit proefschrift. Na het beëindigen van het praktische werk in het kader van dit onderzoek werd hij in februari 1997 aangesteld als postdoctoraal onderzoeker aan de vakgroep Biochemie van de Universiteit van Amsterdam waar hij in de groep van prof.dr. van Driel werkte aan het onderzoek naar het functioneren van matrix attachment regions (MARs). In augustus 1997 besloot hij de onderzoekswereld te verlaten. Sinds 1997 studeert hij theologie aan de Katholieke Theologische Universiteit te Utrecht; vanaf januari 1998 werkt hij daarnaast als parochiesecretaris te Wageningen. Last but not least is hij in 1995 getrouwd met Simone en sinds september 1998 de trotse vader van Joost.