The Mammalian Process of Meiotic Synapsis

by

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The Mammalian Process of Meiotic Synapsis

Abstract

by

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The synaptonemal complex (SC) provides the structural framework for synapsis and recombination of homologous chromosomes during meiotic prophase. The relationship between the SC and other prophase events differs among species but studies of model organisms clearly demonstrate that the completion of prophase is dependent on the formation of mature SCs. Although the SC is essential to meiosis, little is known about the details involved in the construction of the mammalian SC.

Human testicular biopsy material provides an opportunity to study the meiotic process in the human male. The studies described in this thesis were designed to characterize synapsis and formation of the synaptonemal complex in spermatocytes of normal human males. Using current cytogenetic techniques, SCs were characterized at different stages of prophase to determine genome-wide synaptic patterns, patterns of individual chromosomes and variation within and among individuals.

We initially focused on the correlation between synapsis and recombination, by evaluating the temporal relationship between SC formation and the emergence of double strand breaks. Subsequent studies investigated the possibility of co-localization of synaptic initiation sites with sites of recombination. Our results indicated that
recombination is initiated before the formation of the SC, and that sites of synaptic initiation are not translated into sites of exchange.

We then characterized the process of synapsis in detail by determining the location and number of synaptic initiation events on specific chromosomes. Initial analysis showed that in the human male, synapsis predominantly begins at the distal region. We identified synaptic patterns based on chromosome structure, wherein acrocentric chromosomes harbor one synaptic initiation event on the long arm while nonacrocentric chromosomes may sustain two initiation sites, one per chromosome arm. Subsequent analysis of the synaptic process determined that the SC progresses to the centromeric region, which inhibits synapsis across chromosome arms. Further, similar synaptic patterns were observed in our analysis of several individuals suggesting that synapsis is a highly uniform process among individuals.
Chapter I: Introduction and Specific Aims

Meiosis – An overview

Meiosis is the crucial cellular process that yields the haploid gametes necessary for transmission of the genome from one generation to the next. It involves one DNA replication event and two rounds of chromosome segregation. The first division, or meiosis I (MI), is the reductional division in which homologous chromosomes separate, leaving the sister chromatids together. The second division (MII), the equational division, is essentially a haploid mitosis involving the segregation of sister chromatids (see Roeder, 1997; Lee and Amon, 2001 for review; see Figure I-1). At the end of MII, the DNA complement has been reduced from the diploid to the haploid state.

While the basics of meiosis are highly conserved among organisms, there are notable species- and sex-specific differences. For example, in many organisms, including mammals, there is sexual dimorphism in the chronology of events and the number of meiotic products. Male germ cells enter meiosis at the onset of sexual maturation, and each meiotic event yields four functional gametes. Stem cells undergo repeated mitoses to replenish the pool of available meiocytes; as a result, males continuously produce sperm from puberty throughout their lifespan (Hassold and Hunt, 2001). In contrast, in the female, germ cells enter meiosis during fetal development (~8 weeks in human fetuses, and ~13 days post coitus in mouse fetuses). Oocytes arrest at the end of prophase (dictyate arrest) and do not resume meiosis until the onset of puberty (see Mehlmann, 2005 for review). Directly preceding ovulation, the completion of MI produces the oocyte and a polar body. Upon fertilization the oocyte completes MII,
A. Meiosis-specific events of prophase: the representative homologues must pair, synapse, and recombine.

B. Representation of a homologous chromosome pair illustrating the meiosis-specific chromosomal behaviors at prophase essential to the first (reductional) and second (equational) divisions to produce four gametes.

**Figure I-1.** Schematic of human male meiosis. A. Meiosis-specific events of prophase: the representative homologues must pair, synapse, and recombine. B. Representation of a homologous chromosome pair illustrating the meiosis-specific chromosomal behaviors at prophase essential to the first (reductional) and second (equational) divisions to produce four gametes.
producing the fertilized egg and a second polar body (Tan et al., 2005). Thus, for each female meiotic event, only one functional gamete is produced.

These sexual dimorphic properties dictate different strategies in analyses of mammalian meiosis, especially studies involving humans. For example, in the human male, a single ejaculate contains millions of meiotic products, and acquisition of testicular biopsy material provides mature sperm in addition to all of the meiotic stages as the spermatocytes progress through the tubules. In contrast, analysis of the human female is much more complicated: oocytes at the earliest stage of meiosis must be obtained during fetal development and analysis of oocytes from adults requires invasive techniques. Thus, most analyses of human meiosis have been restricted to the male.

Meiotic prophase: a key to successful meiosis

Meiotic prophase may be the most important stage of meiosis, as it provides the proper organization of the chromosomes for the subsequent divisions. Arrangement of the chromosomes involves three unique events: pairing, the alignment or association of homologous chromosomes; synapsis, the physical association of homologues that occurs via the formation of a protein scaffold (the synaptonemal complex or SC) (Zickler and Kleckner, 1999); and recombination, the physical exchange of DNA material between homologues (crossovers).

Changes in SC morphology provide a convenient method to follow the progression of meiocytes through the substages of prophase. The SC is a tripartite, proteinaceous structure formed by two axial elements, bound to the DNA of the homologues, coalesced by the transverse filament of the central element (Moses, 1969;
von Wettstein et al., 1984; Zickler and Kleckner, 1999; see Figure I-2). At leptotene, the first stage of meiotic prophase, the homology search (pairing) is underway while axial elements begin to assemble on the chromatin as short fragments. Zygotene, the second stage, is marked by the elongation of axial elements and the initiation of synapsis as the transverse filament begins to polymerize; upon synapsis axial elements are referred to as lateral elements. By the pachytene stage, homologous synapsis is complete and the mature SC is now comprised of the two lateral elements joined by the transverse filament. Diplotene and diakinesis are characterized by desynapsis of bivalents (removal of SC proteins) except at sites of recombination, called chiasmata, and centromeres (see Heyting, 1996; Zickler and Kleckner, 1998; Cohen and Pollard, 2001 for review).

Abnormalities in pairing, synapsis, or recombination can severely disrupt the meiotic process (Tishkoff et al., 1995; Liu et al., 2004; Bellani et al., 2005; de Vries et al., 2005). These disturbances can trigger various meiotic checkpoints (Cohen et al., 2006) leading to meiotic arrest and apoptosis, and ultimately, subfertility or infertility (Edelmann et al., 1996; McKee, 1998; Yuan et al., 2000, 2002; Kolas et al., 2003). Alternatively, some meiocytes may “slip through” leading to an increase risk of nondisjunction at MI or MII (Eichenlaub-Ritter, 2005).

The temporal order of meiotic prophase

For most of the 20th century, it was thought that homologous chromosomes first paired, then synapsed, and lastly recombined before being “competent” for the first meiotic division (Giroux, 1988; Padmore et al., 1991; Smith and Benavente, 1992). This
Figure I-2. Schematic of synaptonemal complex (SC). Representation of the SC illustrating the attachment of the axial/lateral elements (comprised of SCP2 and SCP3 in humans) to the chromatin of each homologue and coalesced by the transverse filament (comprised of SCP1 in humans).
is indeed true for the fly, *Drosophila melanogaster*, and the worm, *Caenorhabditis elegans*, in which the alignment of homologues and the formation of the synaptonemal complex occur prior to the initiation of recombination. In fact, pairing and synapsis are essential for recombination and disruption of either prevents subsequent crossovers (Dernburg et al., 1998; McKim et al., 1998; Sherizen et al., 2005).

However, studies in the budding yeast, *Saccharomyces cerevisiae*, provide a surprising exception to this rule: double strand breaks (DSBs; the initiators of meiotic recombination) were identified in leptotene prior to homologous pairing and SC formation (Padmore et al., 1991; Bergerat et al., 1997; Keeney et al., 1997). Specifically, localization of SPO11, the enzyme responsible for catalyzing meiotic DSBs, is observed during leptotene, when axial elements are beginning to form (Padmore et al., 1991; Bergerat et al., 1997; Keeney et al., 1997). The single-stranded tails created by DSBs are involved in the homology search for pairing and chromosome alignment (Roeder, 1997) and inhibition of DSB formation by the introduction of mutations in the *spo11* gene prevent the formation of the SC (Giroux et al., 1989; Henderson and Keeney, 2004).

These findings indicate an important role for DSBs in the regulation of both pairing and synapsis; these processes are inter-dependent in yeast. That is, DSBs are required for pairing and SC formation and the completion of recombination is SC-dependent (Padmore et al., 1991; Baudat et al., 2000; Henderson and Keeney, 2004).

This difference in temporal sequence between flies, worms, and yeast indicates that at least two different “paradigms” can be utilized for pairing and synapsing homologous chromosomes. The first mechanism, employed by *Drosophila* and *C. elegans*, relies on the complete alignment and synapsis of homologues before
recombination can begin. The second mechanism, originally identified in *S. cerevisiae*, depends on DSBs as a tool to align and synapse homologues.

Following these initial studies, there was great interest to determine which, if either, of the known paradigms mammals follow. Mahadevaiah and colleagues (2001) used molecular cytogenetic analysis of $\gamma$-H2AX, a marker of DSBs, to investigate the timing of DSBs in relation to synapsis in mouse spermatocytes. They found that, at least superficially, mice were more similar to yeast than flies or worms. Specifically, DSBs were present at the leptotene stage, prior to the formation of the tripartite SC (Mahadevaiah et al., 2001).

Recently, it was observed that, similar to yeast and mice, human oocytes initiate meiosis by forming double strand breaks before completing synapsis (Lenzi et al., 2005). In studies of human oocytes from fetuses at 17-24 weeks gestation, $\gamma$-H2AX localization shows that DSBs are present during axial element formation but prior to the completion of synapsis (Lenzi et al., 2005). To investigate whether human spermatocytes follow the same paradigm, we initiated a study of synapsis in a series of males attending an infertility clinic. These studies are further described in Chapter II of this thesis.

Regardless of the differences among species in the chronology of prophase events, most organisms must faithfully pair homologous chromosomes, fully synapse them, and create chiasmata to successfully segregate homologous chromosomes at MI. Detailed below are the specifics of these events representative of different organisms.
Pairing

Despite its importance to the meiotic process, pairing remains poorly understood (Roeder, 1997; Page and Hawley, 2003; Pawlowski and Cande, 2005). Most of what is known about the initial events in pairing is based on studies of yeast, flies, and worms (Chikashige et al., 1994; Walker and Hawley, 2000). Thus far, two regions of the chromosome have been implicated: the telomeres and the centromere and different organisms use these chromosomal elements differently to facilitate the pairing process.

Pairing in *S. cerevisiae*: depends on the ends

In *S. cerevisiae*, the chromosomes undergo well choreographed movements at the onset of meiosis. Specifically, centromeres, which are clustered near the spindle pole body (SPB) mitotically, lose their association at the onset of meiosis (Hayashi et al., 1998; Jin et al., 1998; Trelles-Sticken et al., 2005). During the transition from leptotene to zygotene, chromosomes are reorganized so that their telomeres cluster in association with the SPB to form a “telomere bouquet” structure (Trelles-Sticken et al., 1999, see Figure I-3). Chikashige and associates (1994) hypothesized that these movements indicate a role of telomeres in pairing and recombination.

Other investigators confirmed this hypothesis. For example, using yeast strains with either an extra linear chromosome or a circular chromosome, Rockmill and Roeder (1998) demonstrated the importance of telomeres in the pairing process. Cells with an additional *linear* chromosome (comprised of telomere-like sequences) display meiotic delay, whereas strains with an extra *circular* chromosome (without telomere-like sequences) sporulate much like wild type (Rockmill and Roeder, 1998). The circular
Figure I-3. Formation of the telomeric bouquet. Representation of two pairs of homologous chromosomes, one metacentric and one acrocentric. Homologues are unpaired at pre-leptotene. At leptotene, homologues orient themselves to cluster the telomeres at the nuclear envelope. This results in the formation of the “bouquet,” which is best visualized at the leptotene/zygotene transition. The bouquet begins to break down at zygotene as synapsis begins (adapted from Scherthan, 2001).
chromosome fails to pair with its endogenous linear homologue, bypassing the homology recognition process. The ability of strains with an extra circular chromosome to continue through meiosis is believed to be the result of a lack of telomeric interactions (Rockmill and Roeder, 1998). It is hypothesized that the extra chromosome with telomeres attempts to pair with the endogenous homologue but cannot, causing sporulation delay. This suggests that homology recognition is initiated through telomeric interaction.

Disruption of these telomeric interactions, such as those caused by mutations in the ndj (nondisjunction) gene, delay pairing, synapsis and intermediate recombination events (Chua and Roeder, 1997; Conrad et al., 1997; Wu and Burgess, 2006). To date, Ndj is the only known protein involved in the formation of the S. cerevisiae bouquet (Wu and Burgess, 2006). Separate from its delayed pairing, ndjΔ cells are capable of catalyzing double strand breaks for recombination but show an increase in precocious sister chromatid segregation and achiastic bivalents (Chua and Roeder, 1997; Trelles-Sticken et al., 1999). More recent studies indicate a relationship between telomeric bouquets and recombination: ndjΔ mutants are delayed in the processing and repair of DSBs and the formation of crossover and noncrossover products (Wu and Burgess, 2006). This delay in meiotic events further delays the MI division, highlighting the importance of the clustering of telomeres at the onset of meiosis.

**Pairing in C. elegans: the center of it all**

Although chromosome movements and interactions with the nuclear envelope have been identified in the nematode, C. elegans, formation of a true bouquet has not been observed (Phillips et al., 2005). Instead, regions near one telomere of the
holocentric chromosomes that characterize this organism associate with the nuclear envelope during meiotic prophase (Phillips et al., 2005). These regions, called the “homolog recognition regions” or “pairing centers” (PC) (McKim, 2005) apparently facilitate chromosome movement and nuclear envelope interaction (MacQueen et al., 2005). However, as detailed below, the PC has a much more complicated role in prophase than simply being the site of nuclear envelope attachment.

Several studies suggest that the PC serves as the site of recombination (McKim et al., 1988; Herman and Kari, 1989), synaptic initiation (MacQueen et al., 2005), and homologous pairing (MacQueen et al., 2005; Phillips et al., 2005): i.e., a one-stop shop for meiotic prophase in *C. elegans*. Initial studies indicate that the PC of the X chromosome facilitate and maintain pairing in the absence of the transverse filament (MacQueen et al., 2002). Recently, the Dernburg group demonstrated that deletion of both PCs on a pair of autosomal chromosomes significantly reduces pairing and prevents synapsis; deletion of one PC allows synapsis but disrupts stabilized pairing in the absence of the transverse filament (MacQueen et al., 2005). A second study investigated the role of the meiosis-specific protein, HIM-8, in pairing (Phillips et al., 2005). An abnormal HIM-8 protein fails to interact with the PC of the X chromosome, disrupting pairing and synapsis of only the X chromosome. The major conclusions of these more recent studies are that PCs on all chromosomes are, at least in part, responsible for the homology search, synapsis, and synapsis-independent stabilization; further, they identify a protein involved in the pairing process of the X chromosome (MacQueen et al., 2002, 2005; Phillips et al., 2005). These studies provide evidence that disruption of the PC prevents
chromosome interactions essential to pairing and later synapsis, further illustrating the importance of the PC in early meiotic events (MacQueen et al., 2005).

**Pairing in *D. melanogaster*: chromosome and sex-specific differences**

Unlike yeast, *Drosophila* chromosomes do not show distinctive movements to form a telomeric bouquet (McKee, 1996). Further, in contrast to most sexually dimorphic organisms, only the female fly produces a synaptonemal complex and chiasmata (the physical manifestations of exchange) (McKee, 1996). In males, in which SCs are not formed, pairing is essential to homologous segregation and meiotic progression (McKim, 2005, for review). Therefore, in this discussion special attention will be given to understanding the mechanisms that allow for appropriate segregation of homologous chromosomes using different, sex-specific methods.

The most informative studies of pairing in *Drosophila* males involved deletion studies of the sex chromosomes. Serial deletions of the X and Y chromosomes provide a means to map the pairing site to a single area of the heterochromatic regions called the collochores (Cooper, 1964; Hawley, 2002). Further characterization of these regions demonstrates that the collochores at the base of the X chromosome and the middle of the short arm of the Y chromosome contain rRNA genes (Cooper, 1964; McKee and Karpen, 1990). By utilizing P-element transposons and deletion mapping, McKee and colleagues (1992) demonstrates that the rRNA transcription units and the intergenic spacer located in the heterochromatin of the X chromosome (*Xh*) are essential for pairing and segregation. This was further supported by the observation that mini-X chromosomes containing only
the rRNA genes and a small amount of centromeric sequence are capable of pairing (and disjoining) normally (Park and Yamamoto, 1995).

In contrast to the sex chromosomes, the mechanism(s) that facilitates autosomal pairing in the male fly remains unclear. Most research detailing the pairing sites of male autosomes has been conducted using compound chromosomes 2 and 2-Y transpositions. Heterochromatic homology alone may not be sufficient, as successful pairing is proportional to the amount of euchromatin on chromosome 2 (McKee et al., 1993). Later studies revealed that the histone locus, a transcriptionally active site located in the euchromatin of chromosome 2, might also function as a pairing site (McKee et al., 1996). Thus, transcriptional activity or the presence of an active promoter, rather than the amount of heterochromatin, seems to play a role in pairing of male autosomes (McKee et al. 1993).

Recently, three proteins involved in autosomal pairing or XY bivalent maintenance have been identified [Teflon (tef), Tomkiel et al., 2001; Stromalin in Meiosis (snm); and Modifier of Mdg4 in Meiosis (mnm), Thomas et al., 2005]. Mutations in the tef gene cause male specific autosomal nondisjunction at MI without affecting the pairing and segregation of the XY bivalent (Tomkiel et al., 2001). Cytological studies indicate that the disruption occurs during late prophase, suggesting that Tef is required for the maintenance, but not the initiation, of autosomal pairing. Likewise, mutations in snm and mnm cause MI-specific nondisjunction of autosomes. However, sex chromosome pairing is also affected by these mutations (Thomas et al., 2005). It remains unknown whether SNM and MNM are involved in initiation of pairing or later maintenance like Teflon. Thomas and colleagues (2005) reported that SNM,
MNM, and Tef localize to the homologous pair in a mutually dependent manner. They hypothesized that SNM and MNM localize to the XY bivalent via a different mechanism than to the autosomes; for autosomes, localization of SNM and MNM is thought to depend on the initial localization of Tef (Thomas et al., 2005). If this model is correct, SNM and MNM, like Tef, are involved in the maintenance of homologous pairing, leaving the components of pairing initiation unknown.

In contrast to the male, in female Drosophila mature SCs form and recombination occurs for all chromosomes except the fourth, which is always achiasmate. Because of its ability to maintain a stable association without known structural intermediates most pairing research has focused on chromosome 4, which utilizes the “homologous achiasmate system” to facilitate pairing during MI (Cooper, 1964; Hawley et al., 1993). Mini-chromosomes were used to demonstrate that meiotic pairing and disjunction are dependent on the amount of centric heterochromatin (approximately 430 – 1000 kb) (Hawley et al., 1993; Karpen et al., 1996). Later studies show that the heterochromatic regions of chromosome 4 are the sites of physical association before segregation (Dernburg et al., 1996).

Pairing in mammals: the bouquet as the centerpiece

Intuitively, it seems more likely that pairing of mammalian chromosomes would be more similar to other multicellular organisms (e.g., Drosophila and C. elegans) than yeast. However, Scherthan and associates (1996) observed chromosome movements more similar to budding yeast than Drosophila. Specifically, in mice a telomere bouquet
is formed in both sexes during prophase, although there are sex-specific differences in timing (Scherthan et al., 1996; Tankimanova et al., 2004).

Unlike yeast, pre-meiotic association between homologues is not known to occur in mice. Studies in the male mouse suggest that initial movements orient the centromeres to the nuclear envelope (NE) during mid-preleptotene, followed by the formation of the bouquet at the leptotene/zygotene transition (Scherthan et al., 1996). A chromosome-specific FISH probe was used to confirm that pairing (as measured by closely aligned or fused FISH signals) is initiated at the same time, presumably during the bouquet stage (Scherthan et al., 1996).

Similar chromosomal movements during early prophase have been documented in human spermatocytes. Using telomeric and pan-centromeric \( \alpha \)-satellite probes, spermatocytes at different stages of prophase were analyzed (Scherthan et al., 1996). As in mouse spermatocytes, telomeres in the human male appear to migrate to the NE to form the bouquet during early prophase (Scherthan et al., 1996). These results mirror those of Rasmussen and Holm (1978), who used three-dimensional reconstruction and electron microscopy to examine human spermatogenesis at early zygotene.

The prevailing thought is that the bouquet is the mechanism coordinating pairing and synapsis in mammalian spermatocytes; therefore understanding the bouquet may be important to understanding these earliest events. Much of our understanding of the bouquet stage and its structure comes from cytogenetic studies focused on pairing and synapsis, not the bouquet itself. The bouquet is a transient structure rarely observed, thus making it difficult to isolate and study directly; e.g. in wild type spermatocytes, cells at
the bouquet stage comprise only 0.2-0.8% of all prophase cells (Scherthan et al., 1996, 2000).

To date, three different approaches have provided insight into the regulation of the mammalian bouquet: 1) studies of the ATM kinase in mice (Pandita et al., 1999), 2) studies of the histone H2A variant, H2AX, in mice (Pandita et al., 1999; Fernandez-Capetillo et al., 2003), and 3) studies of the presence of an extra chromosome in human oocytes (Roig et al., 2005). Somatically, ATM is involved in telomeric maintenance during the mitotic cycle and, meiotically, it is required for telomere movement (Pandita, 2002). Atm-/- male mice are infertile possibly because telomeric organization is altered, prolonging the bouquet stage, and thus preventing progression through prophase (Pandita et al., 1999). During meiotic prophase in the mutants, telomeric association with the nuclear envelope is observed well into late zygotene and pachytene, much prolonged from wild-type (Pandita et al., 1999).

Similar results have been observed in mice homologous for the H2ax null mutation, which are also infertile (Celeste et al., 2002; Fernandez-Capetillo et al., 2003). The H2ax-/- phenotype includes an increase in observable bouquet stage nuclei and cells persistent in a bouquet-like state as late as pachytene (Fernandez-Capetillo et al., 2003). H2AX is an immediate target of the ATM kinase (discussed above), which is involved in homologous pairing by phosphorylating H2AX (Fernandez-Capetillo et al., 2003). Therefore, both ATM and H2AX are required for proper bouquet formation and subsequent prophase events.

Contrary to the transient nature of the bouquet stage in spermatocytes, mammalian oocytes exhibit prolonged bouquet structures lasting until mid-pachytene
(Pfeifer et al., 2003; Roig et al., 2004; Tankimanova et al., 2004). By definition, synapsis initiates at the leptotene/zygotene transition but the mammalian oocyte bouquet does not form until mid- to late zygotene and persists until pachytene (Pfeifer et al., 2003; Roig et al., 2004; Tankimanova et al., 2004). In human oocytes trisomic for chromosome 18, the bouquet stage is further prolonged (Roig et al., 2005). The comparatively late formation of the oocyte bouquet makes it unlikely that it has the same immediate role in pairing suggested in spermatocytes; however other possible roles have yet to be hypothesized.

**Synapsis and the Synaptonemal Complex**

Once chromosomes have paired, homologues remain intimately associated via the synaptonemal complex (SC). The SC, first identified in 1956 in crayfish spermatocytes by Montrose Moses, is an evolutionarily conserved structure composed of a tripartite “glue” that holds the homologous chromosomes together as recombination is completed (Moses, 1969; von Wettstein et al., 1984; Zickler and Kleckner, 1999; see Figure I-2).

Table I-1 shows the known SC proteins of *S. cerevisiae, Drosophila, C. elegans, and mammals*. The structural proteins that comprise the synaptonemal complex of many organisms share homologous function and protein structure, if not protein sequence (Yuan et al., 2000; Page and Hawley, 2001; Heyting, 2005). The known transverse filaments (Zip1, C(3)G, Syp-1/2, and SCP1) are arranged as long coiled-coil proteins that form parallel homodimers. Both ends have globular domains in which the N-termini interact in the central element while the C-termini are embedded within the lateral elements (reviewed by Heyting, 2005). Known components of the axial/lateral elements
<table>
<thead>
<tr>
<th>Organism</th>
<th>Axial/lateral elements</th>
<th>Transverse filament</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. cerevisiae</em></td>
<td>RED1</td>
<td>ZIP1</td>
</tr>
<tr>
<td></td>
<td>HOP1</td>
<td>ZIP2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ZIP3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ZIP4</td>
</tr>
<tr>
<td><em>D. melanogaster</em></td>
<td>Unknown</td>
<td>C(3)G</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C(2)M</td>
</tr>
<tr>
<td><em>C. elegans</em></td>
<td>HIM-3</td>
<td>SYP-1</td>
</tr>
<tr>
<td></td>
<td>HTP-1</td>
<td>SYP-2</td>
</tr>
<tr>
<td>Mouse/Human</td>
<td>SCP2</td>
<td>SCP1</td>
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<tr>
<td></td>
<td>SCP3</td>
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</tr>
</tbody>
</table>
Hop1, Red1, HIM-3, SCP2/3) are all predicted to have nucleotide-binding domains (reviewed by Page and Hawley, 2004). Hop1 in yeast and HIM-3 in C. elegans both contain HORMA structural domains, a conserved amino acid sequence predicted to form a globular domain that senses specialized chromatin states (Page and Hawley, 2004).

**Not just a scaffold**

Until recently, the SC was thought of more as “a means to an end”; the end being meiotic recombination. While the SC keeps paired homologues juxtaposed so that recombination can either begin (for flies and worms) or continue and finish (yeast, mice, and human oocytes), recent studies report that the SC functions as more than a scaffold (Page and Hawley, 2004). For example, Börner and colleagues (2004) suggested a role for the mature SC of S. cerevisiae in processing recombination intermediates, i.e., the formation of double Holliday junctions and their processing into mature crossovers. These studies provide a model in which the full-length SC “twists” to accommodate the DNA changes necessary to create the double Holliday junctions and crossovers (Börner et al., 2004). This twisting “mediates axial interchange” between homologues, meaning that the SC physically ensures recombination between homologues but not between nonhomologous chromosomes or sister chromatids, while its flexibility allows the DNA to be rearranged.

Individual components of the SC also have important functions in other processes. For example, in several organisms, axial elements have a role in chromosome pairing and recombination. Specifically, mutations in red1 or hop1 in budding yeast, him-3 in worms, and Scp3 in mice impair both synapsis and recombination (Loidl et al., 1994; Nag
et al., 1995; Yuan et al., 2000, 2002; Couteau et al., 2004; Liebe et al., 2004). The meiotic consequences of these mutations are profound: nondisjunction of homologues, reduced sporulation (in yeast), or meiotic arrest.

For many organisms, central elements are involved in DSB placement and crossover versus noncrossover decisions; i.e., which DSBs are resolved as exchange events and which are repaired by noncrossover generating mechanisms (Page and Hawley, 2004). For example, the *Drosophila* C(2)M protein, identified as a component of the transverse filament (Anderson et al., 2005), determines which DSBs will become crossovers via a C(3)G-dependent mechanism (Manheim and McKim, 2003). When C(2)M is mutated, recombination is reduced by 10%. The involvement of C(3)G in the formation of crossovers is even clearer; when mutated, recombination is eliminated (Manheim and McKim, 2003).

In organisms that initiate recombination before completing synapsis, SC formation and recombination seem to be parallel events. Whether these events are related in mammals remains unknown. Recent studies of the budding yeast illustrate a cascade of events linking the formation of DSBs to the formation of the SC to the sites of recombination (Pochart et al., 1997; Chua and Roeder, 1998; Nakagawa and Ogawa, 1999; Agarwal and Roeder, 2000; Perry et al., 2005). This relationship is discussed in detail below.

In some organisms, the SC forms before crossing over

*Drosophila* and *C. elegans* are often grouped together when discussing pairing and synapsis since, in both, synapsis is completed before recombination is initiated.
However, their commonalities are not limited to temporal order. Both flies and worms rely on “pairing sites” near centromeres to initiate pairing and further, to initiate synapsis (McKim, 2005). Once pairing has commenced, the homologous chromosomes of both synapse as in other organisms: localization of the axial elements to the chromatin of homologues followed by “zippering up” of the central element.

Temporally, prophase in fly and worm meiocytes occurs in such a way that each event is regulated by the preceding process: synapsis occurs after homologues are paired and recombination is initiated only in the context of a mature SC (Page and Hawley, 2001). Therefore, disruption of synapsis disrupts recombination (Page and Hawley, 2001; MacQueen et al., 2002) but disruption of recombination does not affect SC formation (McKim et al., 1998). For example, null mutations of syp-1 in *C. elegans* or *c(3)G* in *Drosophila* prevent the formation of the SC and remarkably decrease or eliminate recombination (Page and Hawley, 2001; MacQueen et al., 2002), but mutations of genes that encode early recombination proteins (*spo11* and *mei-P22* in flies) prevent crossing over and gene conversion but show no effect on the SC (McKim et al., 1998; McKim and Hayashi-Hagihara, 1998).

Of special note in the nematode, once synapsis is initiated at the pairing center it continues in a “highly processive” fashion regardless of homology. *C. elegans* heterozygous for balancer chromosomes (created by several reciprocal inversions) completely synapse with the wild-type homologue, i.e., synapsing through regions of nonhomology (MacQueen et al., 2005). This is in contrast to SCs involving translocated chromosomes in other animals, e.g. humans, in which synapsis occurs only at regions of
homology (Gabriel-Robez et al., 1986). In humans, this causes a delay in synapsis and may result in meiotic arrest (Gabriel-Robez et al., 1986).

In yeast and mice, double strand breaks precede synapsis

Unlike Drosophila and C. elegans, double strand breakage and early formation of the SC (the establishment of axial elements) are parallel events in budding yeast and commonly studied mammals (Bergerat et al., 1997; Keeney et al., 1997; Romanienko and Camerini-Otero, 2000; Mahadevaiah et al., 2001; Lenzi et al., 2005). In these organisms, DSBs have a role in SC formation, which in turn plays a role in deciding which DSBs mature into crossover or noncrossover events (Chua and Roeder, 1998; Agarwal and Roeder, 2000; Henderson and Keeney, 2004). Null mutations in genes that encode recombination proteins lead to disruptions of the SC, causing partial and nonhomologous synapsis to varying degrees (Yoshida et al., 1998; Edelmann et al., 1999; de Vries et al., 1999; Kneitz et al., 2000). One such example in budding yeast is that of the intermediate recombination protein encoded by the hop2 gene that when mutated leads to incomplete SC formation and extensive nonhomologous synapsis (Tsubouchi and Roeder, 2002; 2003). In mammals, mutations in genes that encode proteins involved in early recombination events (e.g., DMC1, required for single strand invasion [Li et al., 1997]) and later recombination events (e.g., MSH4 and MSH5) not only disrupt recombination, but also pairing and synapsis (Yoshida et al., 1998; de Vries et al., 1999; Edelmann et al., 1999; Kneitz et al., 2000). Likewise, mutations in components of the SC (e.g., components of the axial elements, hop1, red1, and Scp3, or components of the transverse filament, the zip genes and Scp1) reduce or eliminate recombination (Sym et al., 1993;
Sym and Roeder, 1994; Chua and Roeder, 1998; Agarwal and Roeder, 2000; Yuan et al., 2000, 2002; de Vries et al., 2005). Therefore, in these organisms, it becomes difficult to study the prophase events separately.

The SC in mammalian males

*Rat*

The major components of the mammalian SC were first identified in rat spermatocytes (Heyting et al., 1985, 1987). Two components of the rat lateral element, later to be named SCP2 and SCP3, were identified using antibodies against rat testicular extracts (Heyting et al., 1987; Schalk et al., 1998). It was observed that antibodies to these proteins react with the SC in spermatocytes from the zygotene stage to the diplotene stage and that they localize to both paired and unpaired regions of the SC (Heyting et al., 1987). SCP2 has a DNA-binding domain at the C-terminal end believed to interact with the DNA of the homologues (Offenberg et al., 1998). SCP3 also has a potential nucleotide-binding domain and is predicted to form coiled-coil motifs for protein-protein interaction (Lammers et al., 1994).

SCP1, a component of the mammalian transverse filament, was also identified first in the rat. Two groups (Meuwissen et al., 1992; Smith and Benavente, 1992) localized antibodies to the SC that only react to synapsed regions. Rat SCP1 is a large protein (111 kD) sharing structural features of nuclear matrix proteins and is initially identifiable in the meiocyte after pairing and synapsis has begun (Meuwissen et al., 1992).
Mouse

Mutational studies of the various SC components have elucidated their specific roles. Yuan and colleagues (2000) published the first molecular study investigating the role of the mammalian axial/lateral elements in meiotic progression. Using targeted mutagenesis, they generated a null mutation for the mouse \textit{Scp3} gene. They showed that SCP3 is a structural component of the axial/lateral elements and is necessary for the assembly of the murine SC. In male mice lacking SCP3, complete SCs are unable to form leading to meiotic arrest at the zygotene stage and infertility (Yuan et al., 2000). In 2002, Yuan and colleagues (2002) published a companion paper describing the \textit{Scp3}-/- female mouse. Although having a similar SC structural phenotype, \textit{Scp3}-/- females are subfertile, with reduce litter sizes, increase pup resorption, and increase aneuploidy in oocytes and one-cell zygotes (Yuan et al., 2002). These studies show that the establishment of SCs is essential for the normal mammalian meiotic process. These studies also highlight the significance of the sexual dimorphic phenotypes (complete infertility versus reduce fertility) associated with mammalian meiosis (Yuan et al., 2000, 2002). In many instances males are unable to bypass meiotic checkpoints leading to increase levels of apoptosis and infertility, conversely female meiosis is often less affected but profound consequences (e.g., aneuploidy) can be observed in the mature oocyte and resulting progeny.

In contrast to \textit{Scp3} -/- animals, sex-specific differences were not observed for animals with null mutations in the \textit{Scp1} gene (de Vries et al., 2005). \textit{Scp1}-/- mice, both male and female, are infertile. In the absence of the transverse filament, axial elements form and homologous alignment is observed but axial elements fail to coalesce (de Vries
et al., 2005). DSBs form but are not repaired resulting in little to no recombination as there is no evidence of MLH1, the mismatch repair protein essential to recombination (de Vries et al., 2005). de Vries and colleagues (2005) suggested that meiosis is disrupted because of the lack of recombination. This causes a significant increase in spermatoocyte apoptosis, explaining the infertility in males. Disruption of major SC components commonly produces two general outcomes: an inability to maintain association of homologues and a disruption of downstream events, i.e., recombination and chromosome segregation.

**Human**

Multiple studies have demonstrated the importance of the SC in model organisms, but little is known of the human SC. Current understanding of the human SC is provided by studies of individuals with apparent disruptions in synapsis and recombination (Gonsalves et al., 2004; Judis et al., 2004; Sun et al., 2005). Disruptions in synapsis have long been thought to be linked to, if not a cause of, human infertility (see Vallente et al., 2006, for review). Most studies have focused on the male, since the appropriate tissue can be acquired in adult males when fertility concerns arise.

Several studies have analyzed prophase progression and synaptic patterns for males diagnosed with idiopathic non-obstructive azoospermia (NOA) (Judis et al., 2004; Sun et al., 2005; Ma et al., 2006). One such case involved an individual with complete meiotic arrest caused by disruption in the formation of the SC (Judis et al., 2004). Meiosis is initiated and complete axial elements are formed but homologues fail to coalesce, suggesting an aberrant transverse filament protein(s). Two recent studies
compare individuals with NOA to individuals with obstructive azoospermia (individuals seen at infertility clinics for reasons unrelated to meiotic defects) (Sun et al., 2005; Ma et al., 2006). Both studies showed a significant increase in cells at the leptotene and zygotene stages of prophase in individuals with NOA (Sun et al., 2005; Ma et al., 2006). These findings suggest that there is a “block” in homologous pairing or synapsis at leptotene or zygotene, increasing the number of cells at the earliest stages (Sun et al., 2005). Sun and colleagues (2005) also identified a near-significant increase in SC discontinuities at pachytene, i.e., gaps in the SC or asynapsed regions. Overall, SC abnormalities were identified in 70% of pachytene cells from men with NOA. The authors suggested that SC anomalies can cause meiotic arrest as a result of a synapsis checkpoint that eliminates cells with such defects (Sun et al., 2005).

While analysis of individuals with NOA is a fairly recent endeavor and relatively few individuals known with meiotic abnormalities have been analyzed, there are fewer SC studies of control males (Rasmussen and Holm 1978; Barlow and Hultén, 1996). Barlow and Hultén (1996) presented a preliminary study examining the formation of the SC in one normal male using immunofluorescence technology. Their studies showed that pairing is initiated at the telomeres of homologues during the bouquet stage and that synapsis, once initiated, moves toward the centromere (Barlow and Hultén, 1996). The work of Barlow and Hultén (1996) agrees with results from an earlier electron microscopic study of three individuals (Rasmussen and Holm, 1978).

To characterize the synaptic process in normal human spermatogenesis, we used immunocytogenetic techniques to identify and describe the formation of the SC (Chapter II of this thesis). Spermatocytes were analyzed to detail the events from the formation of
axial elements at leptotene to the initiation of synapsis at zygotene. Lastly, specific chromosomes were identified to determine chromosome-specific synaptic patterns common among human males.

The chromosomal location of synaptic initiation

The telomeric region has been implicated as the site of synaptic initiation in males of several mammalian species, including humans (Holm and Rasmussen, 1977; Barlow and Hultén, 1996; Scherthan et al., 1996), mice (Scherthan et al., 1996), and cattle (Pfeifer et al., 2001). The early meiotic movement and reorganization of chromosomes shifts the orientation so that previously unassociated chromosomes become tethered at the telomeres to form the bouquet. This is hypothesized to be the time and site of the homology search (Barlow and Hultén, 1996; Scherthan et al., 1996).

In contrast, studies of oocytes in these same species suggest sex-specific differences in synaptic initiation. Studies by Holm and Rasmussen and R. M. Speed first demonstrated the sexual dimorphism in humans: males initiate synapsis predominantly at distal sites and females synapose first at interstitial regions with ends pairing last (Holm and Rasmussen, 1977; Speed, 1982, 1985). More recent studies in cattle have described synapsis in oocytes as occurring both distally and interstitially, contrasting with the predominantly distal initiation sites of the male (Pfeifer et al., 2001, 2003). Studies of fetal oocytes in the mouse demonstrate that, while the telomeres form bouquet structures, they apparently have little influence on synapsis, as synapsis is initiated prior to bouquet formation (Tankimanova et al., 2004). The mechanism(s) that facilitates synaptic initiation in mammalian oocytes remain unknown.
Synapsis and recombination

In mammals and budding yeast, the formation and processing of DSBs occur in parallel to the formation of the SC. Several studies investigated the inter-dependency of the two events, such that the formation of the SC depends on the presence of DSBs and processing of DSBs into mature crossovers depends on the SC (Henderson and Keeney, 2004; de Vries et al., 2005). However, the mechanistic relationship between these two events is still unknown. Evidence in the budding yeast and the fungus *Sordaria macrospore* suggests that sites of synaptic initiation are the location of recombination.

The evidence in support of this hypothesis relies on protein-protein interactions between SC components and recombination machinery. The linkage between synaptic initiation and recombination in *S. cerevisiae* can be followed by a cascade of protein-protein interactions that connect the placement of synaptic initiation events to the sites of later formed crossovers (Chua and Roeder, 1998; Agarwal and Roeder, 2000). The *S. cerevisiae* transverse filament is composed of at least four known proteins, Zip1, Zip2, Zip3, and, most recently identified, Zip4 (Spo22) (Chua and Roeder, 1998; Agarwal and Roeder, 2000; Perry et al., 2005). These four proteins are members of the ZMM protein group. The other members of this group are Mer3, Msh4, and Msh5, proteins involved in facilitation of meiotic crossovers (Pochart et al., 1997; Nakagawa and Ogawa, 1999).

Immunoprecipitation and immunolocalization studies of the four Zip proteins show physical interactions that suggest a stepwise pathway of recruitment and stabilization (see Figure I-4): Zip3 loads onto homologous chromosomes in discrete foci, recruiting the Zip2/Zip4 complex at particular sites (Agarwal and Roeder, 2000; Tsubouchi et al., 2006). Those sites of Zip2/Zip4 localization become synaptic initiation sites that cause
the polymerization of Zip1 along the length of the homologous chromosomes (Chua and Roeder, 1998; Agarwal and Roeder, 2000). Zip2 and Zip3 interact with proteins involved in recombination. Specifically, Zip2 colocalizes with proteins involved in DSB formation and repair: Rad50, Mre11, and Xrs2 (Chua and Roeder, 1998). Zip3 also interacts with Mre11, Rad51, and Rad57, as well as late recombination proteins Msh4 and Msh5 (Agarwal and Roeder, 2000). This pathway illustrates how the sites of synaptic initiation (Zip2 and Zip3) will become the sites of recombination (Msh4 and Msh5) in *S. cerevisiae*.

Further evidence that sites of synaptic initiation translate into sites of crossovers comes from studies of the Sgs1 protein, a protein involved in DNA damage repair and meiotic recombination (Rockmill et al., 2003). In yeast with null mutations in *sgs1*, synaptic initiation sites increase at the same frequency as recombination. Rockmill and colleagues (2003) hypothesized that Sgs1 is involved in the crossover versus non-crossover decision. Therefore, in its absence, DSBs that would have been resolved via the noncrossover pathway instead become recombination events and additional sites of synaptic initiation (Rockmill et al., 2003).

Other evidence includes the coupled disruption of both synapsis and recombination caused by aberrant proteins involved in either process. For example, null
Figure I-4. The ZMM pathway. The proteins of the transverse filament bind to the proteins of the lateral element. Proteins involved in the synaptic initiation process, Zip 2, 3, and 4 also interact with proteins involved in the recombination pathway, both early events (Zip3 interacts with Mre11, Rad51, Rad57; Zip2 interacts with Mre11, Rad50, and Xrs2) and late (Zip3 interacts with Msh4 and Msh5; ZMM). These protein-protein interactions suggest the sites of synaptic initiation colocalize with the sites of recombination.
mutations in the *zip* genes decrease the level of recombination and aberrant proteins involved in recombination disrupt the localization of Zip proteins (Chua and Roeder, 1998; Agarwal and Roeder, 2000; Fung et al., 2004).

The fungus *Sordaria macrospore* is a second organism hypothesized to place exchange events at sites of synaptic initiation (Zickler et al., 1992). Mutational studies of two *Sordaria* strains with reduced recombination show a parallel reduction in synaptic initiation sites (Zickler et al., 1992). However, there is a small excess of synaptic initiation events compared to the number of recombination events, meaning that not all sites of synaptic initiation become sites of exchange. Using electron microscopy, synaptic initiation sites were localized at various stages of pairing and sites of recombination were determined by genetic maps to be within the same regions along the chromosome (Zickler et al., 1992). The recombination phenotype of the two mutants suggests that recombination occurs at the sites of effective pairing and synaptonemal complex formation.

The possible colocalization of synaptic initiation sites and sites of exchange has yet to be studied in mammalian meiosis. Synaptic initiation is suggested to occur distally in mammalian spermatocytes of several species and distal sites of exchange are a common trend among male mammals: human genetic maps and studies of mouse spermatocytes demonstrate a preference for distal exchanges (Froenicke et al., 2002; Lynn et al., 2002). Since the location of recombination events and the hypothesized placement of synaptic initiation sites show a commonality, the association of the two events seems likely as is the case in *S. cerevisiae* and *Sordaria* (Zickler et al, 1992; Agarwal and Roeder, 2000). To investigate this possible relationship in human males,
sites of synaptic initiation and recombination were mapped on specific chromosomes.

These studies are described in Chapter III of this thesis.
Specific aims

The general process of meiotic prophase is understood, providing a template for the completion of recombination: pairing brings homologues together, synapsis keeps them intimately associated, and chiasmata facilitate proper segregation at anaphase I. In addition, several species-specific patterns have been elucidated: e.g., Drosophila homologues pair by utilizing both heterochromatic and euchromatic regions, and synapsis occurs before recombination is initiated, while yeast and mice apparently pair by means of telomeric associations and initiate recombination prior to the formation of the synaptonemal complex.

Many questions remain. For example, before this thesis research was initiated the temporal relationship between synapsis and recombination was not established for human spermatocytes. Further, we still have little general understanding of mammalian synapsis. Details regarding synaptic initiation, synaptic progression, and synaptic patterns of individual chromosomes are still needed for many organisms. The purpose of this thesis research was to fill in some of these gaps by characterizing synapsis in human spermatocytes.

Chapter II of this thesis examines the question of temporal order in human males, addressing whether, like yeast and mice, human spermatocytes initiate recombination before the completion of SC formation. Conventional methods of immunostaining were combined with fluorescence in situ hybridization (FISH) to visualize synapsis in zygotene cells. Components of the SC were monitored to determine the stage when DSBs form and how this is correlated with the formation of the SC. In a second set of studies, the number and chromosomal location of synaptic initiation sites were
investigated, examining the role of chromosomal elements (i.e., centromeres and
telomeres) in synapsis. Also, individual chromosomes were identified to further
characterize specific behaviors and synaptic patterns. This chapter was published in the
*American Journal of Human Genetics* (Brown et al., 2005).

In Chapter III, investigation of the possible association between the sites of
synaptic initiation and the location of exchanges in human spermatocytes was
undertaken. This chapter is a draft of the manuscript to be submitted for publication to
the *Proceedings of the National Academy of Sciences (PNAS)*. Human chromosomes 1
and 16 were identified by region specific FISH probes to map sites of synaptic initiation
at the leptotene/zygotene transition and recombination at pachytene. The results of these
studies were compared to determine whether, like yeast, sites of synaptic initiation are
translated into sites of crossovers in human spermatocytes.

Finally, Chapter IV summarizes the results and conclusions of this thesis and
considers the direction of possible future experiments to further characterize synapsis in
mammals, using both human spermatocytes and the mouse.
Chapter II: Characterization of Early Synaptic Events in the Human Male

Title of manuscript: Meiotic synapsis proceeds from a limited number of sub-telomeric sites in the human male

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ABSTRACT

The formation of the synaptonemal complex (SC) is a crucial early step in the meiotic process, but relatively little is known about the establishment of the human SC. Accordingly, we recently initiated a study of synapsis in the human male, combining immunofluorescence and fluorescence in situ hybridization methodologies to analyze prophase spermatocytes from a series of control individuals. Our results indicate that synapsis is a tightly regulated process, with relatively little variation among individuals. On nonacrocentric chromosomes there are two synaptic “initiation” sites, one on the distal short arm and one on the distal long arm, while acrocentric chromosomes exhibit a single site on the distal long arm. For both types of chromosomes, synapsis then proceeds towards the centromere, with little evidence that specific p- or q-arm sequences affect the process. However, the centromere appears to have an inhibitory effect on synapsis; i.e., when one arm of a nonacrocentric chromosome is “zippered up” before the other, the centromere acts as a barrier to further movement from that arm.
INTRODUCTION

Meiosis is the specialized cellular division that, after one round of DNA replication and two divisions, transforms diploid cells into haploid gametes. The first stage of meiosis I, prophase, involves a complex series of chromosomal interactions required for homologous chromosome pairing, synapsis, and recombination (e.g., see Zickler and Kleckner, 1999). Over the past 10-15 years, studies of model organisms have made it clear that mutations affecting any of these processes can lead to meiotic arrest or the generation of genetically abnormal gametes (Sym and Roeder, 1994; Yuan et al., 2000, 2002; Page and Hawley, 2001; Hunt and Hassold, 2002). However, the impact of these errors on human meiosis is much less certain. To be sure, altered levels or positioning of recombination events have been linked to human aneuploidy (Hassold and Hunt, 2001). However, the contribution of errors in either pairing or synapsis to meiotic arrest and/or chromosome malsegregation has yet to be fully documented.

As part of ongoing studies of human meiosis, our laboratory has been interested in characterizing the synaptic process, the physical association of homologous chromosomes that is mediated by a tripartite, proteinaceous structure: the synaptonemal complex (SC). The SC is a highly-conserved, meiosis-specific structure found in most eukaryotic organisms, including budding yeast, flies, worms, and mammals. The mature SC is composed of two axial elements, which are bound to the DNA of each homolog, and the central transverse filament, which connects the two axial elements (which are then referred to as “lateral elements”) (Holm and Rasmussen, 1977; Schmekel and Daneholt, 1998; Yuan et al., 2000).
Studies of model organisms indicate a link between disruption of synapsis and "downstream" meiotic abnormalities, including decreased levels of recombination and/or increases in nondisjunction. For example, in *D. melanogaster* mutants lacking a functional copy of c(3)G, a key component of the transverse filament, the SC fails to form and crossing-over is eliminated (Page and Hawley, 2001; Anderson et al., 2005). In mammals, mice homozygous for a null mutation in the gene encoding the axial element protein SCP3 (MIM 604759) are either infertile due to meiotic arrest at zygotene (males) or subfertile, with an increased incidence of aneuploid gametes (females); neither males nor females are capable of forming complete SCs (Yuan et al., 2000, 2002). Thus, in both organisms meiotic errors in synapsis have similar downstream consequences: either meiotic arrest and infertility or aneuploid products.

Presumably, synaptic defects contribute to infertility and aneuploidy in humans as well, but practical problems have impeded our ability to investigate this possibility. In part, this reflects the difficulties associated with acquisition of the appropriate meiotic material; i.e., fetal ovaries for females and testicular material for males. However, there have also been methodological limitations, including the inability to simultaneously visualize chromosomes and chromosome-associated proteins in meiotic prophase. Thus, while there have been several light and electron microscopic analyses of human male and female meiosis over the past 25 years (Holm and Rasmussen, 1977; Wallace and Hultén, 1983; Speed, 1984; Speed and Chandley, 1990; Barlow and Hultén, 1998), the basic meiosis I “program” of chromosome-chromosome and chromosome-protein interactions has yet to be detailed.
Fortunately, recent advances in molecular cytogenetic and immunofluorescence methodology now make it possible to overcome this limitation. Specifically, by utilizing appropriate antibodies the different substages of prophase can be distinguished and analyzed. Further, by coupling this approach with fluorescence *in situ* hybridization (FISH)-based analyses of individual chromosomes or chromosome regions (e.g., telomeres or centromeres), it becomes possible to characterize the temporal interactions between homologous chromosomes and chromosome-associated proteins.

In the present study, we have used this strategy to address three basic issues regarding synapsis in the human male – specifically, the extent of variation in synaptic patterns within and among individuals, the location and number of synaptic initiation sites per chromosome, and the kinetics of synapsis. Our analyses of over 300 leptotene or zygotene stage cells from seven control males indicate that synapsis is a highly regulated event that shows relatively little variation within or among individuals. Indeed, in all individuals and for all chromosomes, synapsis appears to initiate in subtelomeric regions, and proceeds proximally toward the centromere. However, against this background, there are clear chromosome-specific differences, with nonacrocentric chromosomes handling synapsis differently than the acrocentrics ones do. Further, chromosome regions influence the extent and kinetics of synapsis, since centromeric sequences appear to act as a barrier to synaptic progression across chromosome arms.
MATERIALS and METHODS

Sample population

Testicular samples were obtained from six individuals attending the University Hospitals of Cleveland’s Department of Urology or the Glickman Urological Institute of the Cleveland Clinic Foundation for treatment of infertility (Table II-1). The ages of the individuals ranged from 26-43 years. All had been diagnosed with obstructive azoospermia, three because of a previous vasectomy, two due to a congenital bilateral absence of the vas deferens (CBAVD [MIM 277180]) in association with mutations in the CFTR gene, and one because of extensive scarring attributable to multiple surgeries. Blood samples were available on five of the six individuals. Karyotypic analysis indicated a normal male (46, XY) chromosomal constitution in all instances, and routine histological analysis indicated normal levels of spermatogenesis in all individuals. STS-based assays for Yq microdeletions failed to detect any abnormalities. For all patients, informed consent was obtained following protocols established by the Institutional Review Boards (IRB) of the University Hospitals of Cleveland or the Cleveland Clinic Foundation.

In addition, a seventh testicular sample was received from the Pathology Department of the University Hospitals of Cleveland; this sample was obtained in accordance with IRB protocols on discarded tissue samples. Patient information was unavailable on this case, but histological examination indicated normal spermatogenic progression within the tubules.
Sample processing

Testicular samples were processed following modifications of the technique of Barlow and Hultén (1996), as previously described (Judis et al., 2004). Briefly, seminiferous tubules were gently teased apart, and the tissue transferred to freshly prepared buffer solution (30 mM Tris, pH 8.2; 50 mM sucrose; 17 mM citric acid; 5 mM EDTA; 0.5 mM DTT; 0.1 mM PMSF, pH 8.2-8.4), and incubated at room temperature for 45 to 60 minutes. Approximately 3-5 mm of the tissue was transferred into 20 µL of 100 mM sucrose for further teasing, and 10µL of the resultant germ cell slurry was deposited onto microscope slides coated with a 1% paraformaldehyde solution (Sigma, St. Louis, MO; pH 9.2; 0.14-0.20% Triton X) and incubated overnight in a humid chamber. Slides were air dried and placed in a 0.04% PhotoFlo solution (Kodak 200 solution, Eastman Kodak, Rochester, NY) for two minutes, drained, allowed to air dry, and immediately processed for immunostaining.

Immunostaining and fluorescence in situ hybridization (FISH)

Microscope slides were hydrated in 1 X ADB (1% normal donkey serum, Jackson ImmunoResearch, West Grove, PA; 0.3% BSA, and 0.005% Triton X in PBS) for 30 minutes at room temperature. 60 µL of the primary antibody cocktail – consisting of SCP3 (1:75), CREST (MIM 181750) antisera (1:1000), and either SCP1 (MIM 602162) (1:1000), γ-H2AX (1:100; UpState, Chicago, IL), Spo11 (MIM 605114) (1:75; NeoMarkers, Fremont, CA) or RAD51 (MIM 170617) (1:75) (Santa Cruz Biotechnology, Santa Cruz, CA) in 1 X ADB – was overlaid on slides, and the slides were coverslipped and incubated overnight at 37°C in a humid chamber. After the primary antibody
incubation, slides were washed in 1 X ADB for 20 minutes at room temperature followed
by a second wash in 1 X ADB at 4°C overnight. Slides were then overlaid with 60 µL of
the secondary antibody cocktail [Fluorescein Donkey Anti-Rabbit, Rhodamine Donkey
Anti-Goat, and AMCA Donkey Anti-Human (1:100; Jackson ImmunoResearch) in 1 X
ADB] for 60 minutes at 37°C in a humid chamber. Slides were washed three times in 1
X PBS for 10 minutes, drained, mounted in Antifade (BioRad Laboratories, Hercules,
CA), and coverslipped.

Leptotene and zygotene cells were identified and analyzed using a Zeiss Axiophot
epifluorescence microscope (Carl Zeiss, Thornwood, NY). Images were captured using
the Applied Imaging Quips Pathvision System (Applied Imaging, Santa Clara, CA) and
cell coordinates were noted for subsequent FISH analyses.

For chromosome-specific FISH studies, previously immunostained slides were
refixed in 2% formaldehyde solution (Fisher Scientific, Pittsburgh, PA) for
approximately 12 minutes, washed in PBS for 30 minutes at room temperature and in 2 X
SSC for 30 minutes. Slides were overlaid with 40µL of a probe cocktail consisting of
directly labeled DNA paint probes specific for particular human chromosomes [WCP 8,
WCP9, WCP21, and WCP22 (1:20)] in hybridization buffer (Vysis, Inc., Downers
Grove, IL), coverslipped, transferred to an 85°C hot plate for 8 minutes, and incubated
overnight at 37°C in a humid chamber. Coverslips were soaked off in 2 X SSC at 75°C
for 2 minutes; slides were then transferred to 2 X SSC at room temperature for 7 minutes,
washed in PN buffer for 10 minutes, stained with DAPI, rinsed in PN buffer, mounted in
Antifade, and coverslipped. Previously imaged cells were then relocated and analyzed.
To identify telomeric repeat sequences, previously immunostained and/or “FISHed” slides were rehydrated in 1 X PBS for 5 to 15 minutes, fixed for 2 minutes in 4% formaldehyde in PBS, washed in 1 X PBS three times for 5 minutes, and dehydrated in serial ethanol washes (70%, 90%, and 100%) for 5 minutes each and allowed to air dry. 60 µL of a telomere-detecting PNA probe cocktail (0.069% PNA Oligomer, Applied Biosystems, Framingham, MA; 0.5% blocking agent, and 0.1% Tris) in 70% formamide was added, coverslipped, and denatured on a 80°C heating block for 3 minutes. Slides were incubated in the dark at room temperature for at least 4 hours, washed twice in buffer (70% formamide, Tris (10 mM), and 0.1% BSA) for 15 minutes, washed three times in TBST buffer for 5 minutes, and dehydrated in serial ethanol washes. Slides were allowed to air dry, DAPI stained, rinsed in PBS, mounted in Antifade, and coverslipped. Previously identified cells were then relocated and analyzed.

Cytological analysis of synapsis

We analyzed leptotene and zygotene cells for initiation and progression of synapsis, using antibodies against SCP3 (detecting a component of the axial/lateral element of the SC) and/or SCP1 (detecting a component of the transverse filament of the SC) to monitor formation of the SC. Using this approach, the first three substages of prophase (leptotene, zygotene and pachytene) are easily distinguished. Leptotene is characterized by the appearance of short, SCP3 positive linear segments (axial elements) which coalesce as leptotene progresses; during zygotene, axial elements of homologous chromosomes continue to elongate and begin to synapse, with the points of synapsis identified by the appearance of SCP1 signals or the merger of SCP3 signals; by
pachytene, synapsis is complete, with SCP1 and SCP3 colocalizing along the entire length of each of the 22 autosomes and across the pseudoautosomal region of the XY bivalent.

Operationally, we defined synapsis as the merger of homologous axial elements (detected by the merger of SCP3 signals) or the appearance of the transverse filament (detected by SCP1). For cells in leptotene or early zygotene, we scored for synaptic “initiation sites”, defined as punctate regions in which we observed SCP1 signals or merged SCP3 signals. In mid- and late zygotene stage cells, we monitored progression of synapsis, visualized by the elongation of SCP1 signals and/or elongation of merged SCP3 signals.
RESULTS and DISCUSSION

We analyzed synapsis in seven individuals, scoring 8-71 leptotene or zygotene stage cells per individual (Table II-1). Since we were interested in the progression of synapsis, we chose cells in which bivalents were asynapsed or partially synapsed. At leptotene, we typically were able to score 3-4 bivalents per cell; at zygotene, we arbitrarily restricted our analyses to cells in which at least 5 nonacrocentric chromosomes and/or at least 2 acrocentric chromosomes were incompletely synapsed. In total, we analyzed 109 bivalents from 30 leptotene stage cells and 4800 bivalents from 286 zygotene stage cells. As discussed below, the general features of synapsis were similar among all individuals; therefore, except as indicated, analyses were based on pooled observations from all seven individuals.

The initial events of meiotic recombination precede synapsis in males

In our initial studies, we were interested in determining the temporal relationship between the recombination and synaptic pathways – in particular, asking which comes first, synapsis or recombination. Until recently, it has been assumed that the initial events of meiosis involve pairing and synapsis of homologs, with the synaptonemal complex providing the template for subsequent recombinational processes (e.g., the formation of double strand breaks [DSBs], strand invasion, and repair of DSBs). This view is supported by molecular studies of meiosis in D. melanogaster and C. elegans, which indicate that the initial event in meiiotic recombination – DSBs – occurs after the formation of the mature, tripartite SC, and that the SC can form in the absence of DSBs.
Table II-1. Summary of patient information.

| ID Number | Age | Reason for ascertainment | Chromosome constitution | Yq microdeletion | Number of cells scored at: | Leptotene | Zygotene |
|-----------|-----|--------------------------|--------------------------|-----------------|-----------------------------|-----------|
|           |     |                          |                          |                 |                             | 4         | 11       |
| Sp370     | 32  | Postvasectomy            | 46XY                     | No deletion     |                             | 8         |
| Sp389     | ?   | ?                        | Unknown                  | Unknown         |                             | 8         |
| Sp393     | 31  | CBAVD*                   | 46XY                     | No deletion     |                             | 36        |
| Sp401     | 43  | Postvasectomy            | 46XY                     | No deletion     |                             | 1         | 68       |
| Sp403     | 31  | Postvasectomy            | 46XY                     | No deletion     |                             | 7         | 41       |
| Sp407     | 26  | CBAVD*                   | 46XY                     | No deletion     |                             | 4         | 67       |
| Sp1006    | 39  | Epididymal obstruction assoc. with surgeries | Unknown | Unknown | 14 | 55 |
| Total     |     |                          |                          |                 |                             | 30        | 286      |

*Congenital bilateral absence of the vas deferens
(Dernburg et al., 1998; McKim et al., 1998; McKim and Hayashi-Hagihara, 1998). In contrast, in *S. cerevisiae*, DSBs occur before the formation of the SC and, indeed, are required for normal development of the SC (Sym and Roeder, 1994; Roeder, 1997). Similarly, recent studies of the mouse (Mahadevaiah et al., 2001) and the human female (Lenzi et al., 2005) indicate that DSBs precede SC formation, suggesting that mammals follow the yeast temporal paradigm.

Thus, we were interested in determining whether DSBs form in advance of the complete SC in the human male as well. Accordingly, we monitored localization patterns of proteins known to be involved in DSB formation (SPO11, responsible for catalyzing the initial DSB reaction [Keeney et al., 1997] or in DSB processing (RAD51, a strand invasion protein [McIlwraith et al., 2000], and γ-H2AX, a histone H2A variant that becomes phosphorylated immediately after DSB formation [Rogakou et al., 1998; Mahadevaiah et al., 2001]) with proteins associated with the formation of the SC (SCP1, a component of the transverse filament [Liu et al., 1996], and SCP3, a component of the axial elements [Lammers et al., 1995]). As shown in Figure II-1, all three markers of DSBs (SPO11, RAD51 and γ-H2AX) were present in early leptotene cells, indicating that the recombinational pathway had already been activated. In contrast, SCP1 was not detected in any early leptotene preparations (data not shown) and SCP3 was observable only as diffuse patterns of localization, with individual SCP3 signals being present as punctate foci or short linear fragments. Thus, neither component of the SC had been “built” by this point, despite the fact that the recombinational process had already begun. From this, we conclude that, in human males, as in human females, mice and budding
Figure II-1. Immunofluorescence images of human leptotene stage spermatocytes. SCP3 (which detects axial elements) is in red, and CREST (which detects kinetochores) is in blue. Three different markers of DSBs are shown in green: SPO11 (A), RAD51 (B) and γH2AX (C). All three are present in leptotene nuclei, despite the fact that the axial elements are not yet fully formed.
yeast, the initial events in the recombinational pathway do not depend on the presence of the mature, tripartite SC.

**Synapsis proceeds from the distal regions of human chromosomes**

In a second set of experiments, we were interested in determining whether specific chromosomal sites are important in the initiation of synapsis in human males. The telomere has long been suggested to be the site of synaptic initiation in both males and females (e.g., see Rasmussen and Holm, 1978; Speed, 1984; Wallace and Hultén, 1985; Speed and Chandley, 1990; Barlow and Hultén, 1996). As part of the meiotic rearrangement of chromosomes in prophase, mammalian telomeres cluster at the nuclear envelope to form a “bouquet” during the leptotene/zygotene stage (Zickler and Kleckner, 1998). Scherthan et al. (1996) showed that in male mice the bouquet facilitates pairing between chromosomes at the leptotene-zygotene transition. This suggests that synapsis might also be initiated at the telomeric regions shortly after homologs begin to pair.

We tested this hypothesis by examining the number and location of synaptic initiation sites, defined operationally as short regions of merged SCP3 signals (i.e., merged axial elements) or by the appearance of SCP1 signals (i.e., the appearance of the transverse element). For most individuals, the results were based solely on the SCP3 data. However, to be certain that these data were consistent with those obtained with SCP1, one individual (Sp1006) was analyzed using both SCP3 and SCP1. The data were compared and showed virtually identical results (Figure II-2), indicating that the two approaches are equivalent.
Figure II-2. Localization patterns of SCP1 and SCP3 on partially synapsed submetacentric (A-C), metacentric (D-F) and acrocentric (G-I) bivalents. SCP1 (which detects the transverse filament) is in green, SCP3 (which detects axial elements) is in red, and CREST (which detects kinetochores) is in blue. By comparing the merged images (left panels) with images highlighting either SCP3 (middle panels) or SCP1 (right panels), it is clear that SCP1 localizes only to chromosomal sites where the two axial elements have already merged.
Using this approach, we first examined the distribution of synaptic initiation sites on nonacrocentric autosomes (i.e., chromosomes 1-12 and 16-20). We analyzed 286 zygotene stage cells from the seven individuals, yielding a total of 3734 analyzable bivalents involving nonacrocentric chromosomes. Of these, 2191 consisted of a single uninterrupted linear SC; these were scored as being completely synapsed and were not further considered. We then focused on the remaining 1543 bivalents, for which the axial elements were separated from one another along at least a portion of the SC (see Table II-2; Figure II-3A). These bivalents fell into one of four categories, the vast majority of which were synapsed at both ends of the chromosomes but contained a single, asynaptic “bubble” located interstitially (Table II-2). Specifically: 1) in nearly 30% of cases (440/1543), SC formation was complete for part of the short and long arms, but there was a bubble encompassing both the p- and q-arm pericentromeric regions (Figure II-3B); 2) in over 40% of cases (682/1543), SC formation was complete for the p-arm, but a bubble extended from the centromere onto the q-arm (Figure II-3C); 3) in approximately 10% of cases (182/1543), a bubble was restricted to the interstitial region of the q-arm, with the p-arm and the proximal q-arm being completely synapsed (Figure II-3D); and 4) in about 15% of cases (239/1543), more complicated synaptic patterns were observed. These included bivalents that were paired but not synapsed (Figure II-3E), bivalents with asynapsed ends (Figure II-3F), and bivalents with two or more bubbles (Figure II-3G).

These results indicate that, at least for the human male, there are typically two synaptic initiation sites on nonacrocentric chromosomes. One is located distally on the p-arm and one distally on the q-arm, and the SC then “zippers up” proximally from these sites. This process appears to be constant for all chromosomes, as we saw no obvious
Table II-2. Synapsis of nonacrocentric chromosomes.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of partially synapsed bivalents N</th>
<th>N (%)</th>
<th>N (%)</th>
<th>N (%)</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sp370</td>
<td>44</td>
<td>10</td>
<td>24</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>(22.7)</td>
<td>(54.5)</td>
<td>(6.8)</td>
<td>(15.9)</td>
<td></td>
</tr>
<tr>
<td>Sp389</td>
<td>28</td>
<td>2</td>
<td>16</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>(7.1)</td>
<td>(57.1)</td>
<td>(10.7)</td>
<td>(25.0)</td>
<td></td>
</tr>
<tr>
<td>Sp393</td>
<td>107</td>
<td>17</td>
<td>47</td>
<td>13</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>(15.9)</td>
<td>(43.9)</td>
<td>(12.1)</td>
<td>(28.0)</td>
<td></td>
</tr>
<tr>
<td>Sp401</td>
<td>385</td>
<td>107</td>
<td>183</td>
<td>39</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>(27.8)</td>
<td>(47.5)</td>
<td>(10.1)</td>
<td>(14.5)</td>
<td></td>
</tr>
<tr>
<td>Sp403</td>
<td>231</td>
<td>73</td>
<td>101</td>
<td>26</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>(31.6)</td>
<td>(43.7)</td>
<td>(11.3)</td>
<td>(13.4)</td>
<td></td>
</tr>
<tr>
<td>Sp407</td>
<td>348</td>
<td>69</td>
<td>157</td>
<td>39</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>(19.8)</td>
<td>(45.1)</td>
<td>(11.2)</td>
<td>(23.8)</td>
<td></td>
</tr>
<tr>
<td>Sp1006</td>
<td>400</td>
<td>162</td>
<td>154</td>
<td>59</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>(40.5)</td>
<td>(38.5)</td>
<td>(14.7)</td>
<td>(6.2)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1543</td>
<td>440</td>
<td>682</td>
<td>182</td>
<td>239</td>
</tr>
<tr>
<td></td>
<td>(28.5)</td>
<td>(44.2)</td>
<td>(11.8)</td>
<td>(15.5)</td>
<td></td>
</tr>
</tbody>
</table>
**Figure II-3.** Synapsis of nonacrocentric chromosomes. SCP3 (which detects axial elements) is in red, CREST (which detects kinetochores) is in blue, and sequences detected by a pan-telomeric PNA probe is in yellow. (A) Representative zygotene stage cell with both synapsed and partially synapsed bivalents. (B-G) Various synaptic configurations observed in individual zygotene bivalents. The three common “bubble” structures (see Table II-2) are shown (B-D), a partially paired but not synapsed bivalent in (E) and more complicated synaptic configurations (F-G).
effect of chromosome shape (metacentric or submetacentric) or size on the number or location of initiation sites.

In contrast, acrocentric autosomes (i.e., chromosomes 13-15, 21, and 22) initiated synapsis only at the distal region of the q-arm. We analyzed 1066 acrocentric bivalents from the seven individuals; over 65% (729/1066) of these bivalents were completely synapsed. Of the remaining 337 partially synapsed bivalents one structure was repeatedly detected: asynaptic “forks” in which merged SCP3 signals were observed for varying segments of the long arm, with the proximal regions of the q-arm and the p-arm being asynapsed (Table II-3; Figure II-4A). This pattern was observed in over 85% (287/337) of the partially synapsed bivalents. In the remaining 50 instances more complex synaptic patterns were observed, including interstitial initiation sites in addition to the distal site and unsynapsed bivalents (Figure II-4B).

These analyses indicate that, for both nonacrocentric and acrocentric chromosomes, terminal regions play a crucial role in synaptic initiation in male meiosis. However, there is an important caveat to this interpretation. Specifically, the majority of our observations came from mid- or late-zygotene stage cells, and consequently were biased toward bivalents in which the synaptic process was nearing completion. Thus, it is possible that we failed to identify interstitial synaptic initiation sites because they had already “zippered up”. However, in subsequent studies of leptotene stage cells (described below) telomeric initiation sites again predominated, suggesting that interstitial regions are, indeed, relatively unimportant to human male synapsis.

Our results also make it clear that there are chromosome-specific differences in synapsis: nonacrocentric chromosomes invariably have two initiation sites, one per
### Table II-3. Synapsis of acrocentric chromosomes.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Number of partially synapsed bivalents</th>
<th>N</th>
<th>N (%)</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sp370</td>
<td></td>
<td>21</td>
<td>65.6</td>
<td>11</td>
</tr>
<tr>
<td>Sp389</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sp393</td>
<td></td>
<td>23</td>
<td>88.5</td>
<td>3</td>
</tr>
<tr>
<td>Sp401</td>
<td></td>
<td>55</td>
<td>83.3</td>
<td>11</td>
</tr>
<tr>
<td>Sp403</td>
<td></td>
<td>33</td>
<td>80.5</td>
<td>8</td>
</tr>
<tr>
<td>Sp407</td>
<td></td>
<td>87</td>
<td>91.6</td>
<td>8</td>
</tr>
<tr>
<td>Sp1006</td>
<td></td>
<td>66</td>
<td>88.0</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>287</td>
<td>85.2</td>
<td>50</td>
</tr>
</tbody>
</table>

The table shows the number of partially synapsed bivalents (%), and the number of other synapsed bivalents (%).
Figure II-4. Synapsis of acrocentric chromosomes. SCP3 (which detects axial elements) is in red, CREST (which detects kinetochores) is in blue, and sequences detected by a pan-telomeric PNA probe are in yellow. In almost all partially synapsed acrocentric chromosomes, synapsis proceeded from the q-arm terminus toward the centromere (A) although, rarely, interstitial asynaptic regions were observed (B).
chromosome arm, whereas acrocentric chromosomes have only one initiation site located on the q-arm. Indeed, synapsis of the p-arm without complete synapsis of the q-arm and centromere was never observed for any acrocentric bivalent, suggesting that acrocentric p-arms are incapable of seeding synapsis. This is consistent with previous electron microscopic studies of human fetal oocytes, in which synapsis was observed to begin at q-arm termini and proceed through the centromere to the p-arms (Wallace and Hultén, 1985). Possibly this helps to prevent synapsis between nonhomologous acrocentrics, thus reducing the likelihood of de novo Robertsonian translocations.

In general, our observations agree with results previously obtained in electron microscopic (e.g., see Rasmussen and Holm, 1978) and immunofluorescence analyses (e.g., see Barlow and Hultén, 1996) of human spermatocytes, both of which pinpointed telomeric regions as the sites of synaptic initiation. In addition, Rasmussen and Holm (1978) noted a delay in pairing of the short arms of acrocentric chromosomes, consistent with our observations; and Barlow and Hultén (1996) observed “ballooning axial elements”, similar to our “bubble” structures. Thus, the basic conclusions of all three are similar. However, the previous analyses were limited in at least two respects: first, by sample size, as the study of Rasmussen and Holm (1978) was based on three dimensional reconstructions of only 39 cells, and that of Barlow and Hultén (1996) was based on a single individual; and second, by study design, as neither analysis was intended to examine genome-wide patterns of synapsis. Thus, the present report is the first to demonstrate a “set” number of synaptic initiation sites on individual chromosomes, and the first to detail the differences between nonacrocentric and acrocentric chromosomes.
**Synapsis is initiated subtelomerically, not at the telomere proper**

Although our initial analyses implicated the telomere in synaptic initiation, it was unclear whether the telomere-proper or the subtelomeric region was the important determinant. Thus, a subsequent set of experiments sought to distinguish between these two possibilities.

We used pan-telomeric PNA probes to localize the telomeric repeat sequences (TTAGGG) of previously immunostained leptotene cells. At this stage, the axial elements are still incompletely formed, consisting of multiple short linear fragments; therefore, this represents the earliest stage at which synaptic initiation can be easily visualized (Figure II-5A). We analyzed 109 informative bivalents from 30 leptotene cells and in the vast majority of cases, we found that synaptic initiation sites were located proximal to the telomeric signals (PNA foci) (Figure II-5B-D). Specifically, in 44.9% (49/109) of cases we observed short regions of asynapsed axial elements between the merged SCP3 signals and two distinct telomeric signals (Figure II-5B-C), while in an additional 46.8% (51/109) of bivalents doublet telomeric signals were located immediately adjacent to merged SCP3 signals (Figure II-5D). In only 8.2% (9/109) of cases were initiation sites telomeric, without adjacent merged SCP3 signals (Figure II-5E-F).

These results indicate that synapsis proceeds from sites located in subtelomeric regions, and not at the telomeres proper. However, the way in which this occurs is not yet known, nor is it clear why there is variation in the distance between the telomere proper and the synaptic initiation sites. Possibly, there are chromosome-specific determinants, with each chromosome having specific sequences that “seed” synaptic initiation. If the
Figure II-5. Analysis of sites of synaptic initiation in leptotene spermatocytes. SCP3 (which detects axial elements) is in red, CREST (which detects kinetochores) is in blue, and sequences detected by a pan-telomeric PNA probe are in yellow. (A) Representative leptotene stage cell showing early stages of synapsis. In most informative bivalents, merged SCP3 signals were observed some distance from two distinct telomeric signals (B-C) or the telomere appeared as a doublet (D); rarely, synapsis appeared to begin at the telomere (E-F).
location of these sequences varies among chromosomes, it could explain the variation in telomere-initiation site distances that we observed. Alternatively, it may be that there is no sequence specificity to synaptic initiation; instead there may simply be a preference for the subtelomeric region, possibly attributable to chromatin modifications that make the region more accessible. Our study was not designed to distinguish between these possibilities, as we made no attempt to examine the location of synaptic initiation sites on specific chromosomes (i.e., by using FISH). However, this is clearly a straightforward exercise, and will be an important first step in the characterization of the genomic determinants that direct synapsis in humans.

The centromere impedes synapsis across chromosome arms

Our analysis of nonacrocentric chromosomes indicated that synapsis begins distally and proceeds toward the center of the chromosome. Assuming that synaptic initiation occurs relatively synchronously for long and short arms, and that synapsis proceeds at a constant rate, we would then expect to see two types of “bubbles” depending on the shape of the chromosome: for metacentric chromosomes, the centromeric region would be the last to synapse while for submetacentric chromosomes, the middle of the chromosome (and hence the bubble) would be positioned somewhere on the long arm.

As described above, we did indeed identify both types of bubbles (Table II-2; Figure II-3). Approximately one-third of all bubbles included the centromere (Figure II-3A), as expected since 5 of the 17 nonacrocentric chromosomes are metacentric, or nearly so (i.e., chromosomes 1, 3, 16, 19, and 20). However, surprisingly, only 12% of
nonacrocentric bivalents exhibited a bubble on the long arm (Figure II-3C). Instead, we observed a third, more common type of bubble, in which the p-arm was completely synapsed, but in which there was a bubble that extended distally from the q-arm pericentromeric region (Figure II-3B). That is, in these instances it appeared that synopsis had proceeded from the distal p-arm to the centromere, but that the centromere had acted as a “stop sign”, prohibiting further progression onto the proximal q-arm.

As this third type of bubble appeared to be restricted to submetacentric chromosomes we decided to investigate it further by focusing on two specific C-group chromosomes, one with (chromosome 9) and one without (chromosome 8) a large block of pericentromeric heterochromatin. We used FISH to identify 43 partially synapsed chromosome 9 bivalents from either of two individuals (Sp401 and Sp407) and 36 chromosome 8 bivalents from two individuals (Sp403 and Sp1006) and examined the location of asynaptic bubbles. For each chromosome, the results were similar to our previous observations. For chromosome 9, in almost all instances (39/43) we observed fully synapsed p-arms, with asynaptic regions of variable lengths extending from the pericentromeric region of 9q. In each of the remaining four cases we observed a relatively large bubble encompassing both proximal 9p and much of 9q; while this could represent a different pattern of synopsis, it may also be that the bivalents were simply at an earlier stage of synopsis, and would have later adopted the bubble configuration observed for the other 39 bivalents. Similarly, 23/36 chromosome 8 bivalents demonstrated complete synopsis of the p-arm with the asynaptic bubble extending distally from the centromeric region of the q-arm and in six other cases the bubble encompassed both the p- and q-arms, possibly indicating a bivalent at an early stage of
synapsis. Only 7 of the 36 bivalents showed the morphology “expected” for partially
synapsed submetacentric chromosomes; i.e., a bubble located in the middle of 8q.

Taken together, our observations of nonacrocentric chromosomes in general, and
of chromosomes 8 and 9 in particular, suggest that the centromere acts as a barrier to
synapsis. As our evidence for this effect is limited to observations on submetacentric
chromosomes, it is formally possible that this phenotype is restricted to certain
chromosomes. However, we think this is unlikely. Instead, we propose that all
centromeres share this property, but that it is simply more difficult to document on other
chromosomes. That is, the p-arms of acrocentric chromosomes are apparently unable to
seed synapsis, making it impossible to visualize movement from the p telomere toward
the centromere; and on metacentric chromosomes, we anticipate that synapsis occurs
relatively synchronously for both p- and q-arm pericentromeric regions, meaning that the
centromeric region is the last to synapse.

If our conclusions are correct, they indicate a basic difference between synapsis
and crossing over in the way in which signals are propagated. That is, they indicate that
the centromere interferes with the spread of synapsis, at least across part of the
chromosome. In contrast, there is little evidence that crossover interference operates
across the centromere in humans. For example, in a genome-wide genetic linkage
analysis, Broman and Weber (2000) were unable to find any impact of the centromere on
recombination levels and, in our immunofluorescence analyses of pachytene stage
spermatocytes, we have observed no effect of the centromere on the location and
distribution of MLH1 foci, a marker of crossovers (Lynn et al., 2002; Lynn and Hassold,
unpublished observations). Thus, it appears that, at least in the pericentromeric region,
there are separate determinants for synaptic progression and transmission of recombination pathway signals.

Synapsis occurs similarly in different individuals

Tables II-2 and II-3 provide information on the synaptic configurations observed in each of seven individuals in our series. We observed highly significant among-individual variation in the frequency of the different types of configurations for both nonacrocentric ($\chi^2 = 103.4$, $p<0.001$) and for acrocentric chromosomes ($\chi^2 = 14.7$; $p<0.05$). For nonacrocentric chromosomes, the effect was largely attributable to variation in the frequency of the “other” category, and to the high proportion of the first type of bubble in one individual (Sp1006). For the acrocentric chromosomes, the $\chi^2$ value was almost entirely due to the high proportion of “other” configurations in Sp370. As the “other” category included bivalents that were difficult to analyze or unanalyzable, as well as those that were atypical, we think that much of the individual variation was artifactual in nature. Indeed, as there was relatively little among-individual variation in the distribution of the “typical” configurations, we suggest that synapsis occurs relatively uniformly among males.

This conclusion is supported by our observations on other aspects of synapsis. That is, we saw no evidence for temporal differences in synaptic progression among individuals; in all individuals, synapsis was initiated subtelomERICally, with two initiation sites for nonacrocentric chromosomes and one for acrocentric chromosomes; and, in each individual, the centromere acted as an impediment to synaptic progression. Thus, while
we observed individual variation in the frequency of synaptic configurations, the general features of synapsis were remarkably preserved among individuals.
CONCLUSIONS

Our examination of early prophase spermatocytes from seven males demonstrates the importance of the subtelomeric region to synapsis. This is not particularly unexpected, since previous analyses of many organisms, including humans (e.g., Rasmussen and Holm, 1978; Speed, 1984; Wallace and Hultén, 1985; Barlow and Hultén, 1996; Scherthan et al., 1996), have implicated the telomere in this process. However, our results indicate that sites of synaptic initiation are in subtelomeric regions, not at the telomeres themselves. Further, our results suggest that this process may be linked to recombination-associated events. That is, the number and location of synaptic initiation sites appears to be tightly regulated and, at least superficially, mimics the distribution of crossovers in the human male. Similar to crossovers (e.g., see Broman and Webber, 2000; Lynn et al., 2002): there appears to be a single “obligatory” synaptic initiation site for p- and q-arms of nonacrocentric chromosomes and for q-arms of acrocentric chromosomes; the p-arms of acrocentric chromosomes rarely, if ever, exhibit synaptic initiation sites; and synaptic initiation sites are preferentially distally located. However, our results also make it clear that the synaptic and recombinational pathways are regulated differently. Recombination-associated proteins are visualized in leptotene spermatocytes prior to the initiation of synapsis, and the number of DSBs (as judged by the number of SPO11 foci; see Figure II-1A) far exceeds the number of synaptic initiation sites. Further, the number of synaptic initiation sites seems under tighter control than is the number of crossovers. Recent linkage and cytological studies of meiotic exchanges indicate substantial variation within and among individuals, with a range of approximately 45-60 exchanges/cell in normal males (Lynn et al., 2002; Tease
and Hultén, 2004, Sun et al., 2005). That is, in addition to the obligatory crossovers on all chromosome arms (other than 13p, 14p, 15p, 21p, 22p and XqYq), there are generally at least 5-15 “optional” exchanges per cell. In contrast, our results imply an extremely tight range per cell for synaptic initiation sites. We found little evidence for interstitially-located sites, suggesting that, over the entire complement, most spermatocytes contain approximately 40 synaptic initiation sites. Further, this appears to be a feature of most, if not all, males, as we found a surprising lack of variation in the number or distribution of initiation sites in our series. Finally, our results indicate differences in the “spreading” of recombination and synaptic signals. Our observations indicate that the centromere acts as a barrier to the spread of synapsis from one chromosome arm to the other, while recent analyses of crossing over provide little evidence that the centromere similarly affects recombinational events.

Thus, our results provide an initial characterization of the synaptic process in human males, and make it clear that sites of synaptic initiation are not equivalent to sites of crossovers. However, it still may be that some, or even a majority of synaptic initiation sites eventually mature into crossovers. By combining immunofluorescence and FISH to localize synaptic initiation sites and crossovers on individual chromosomes, we should be able to address this possibility.
ACKNOWLEDGEMENTS

This research was supported by research grants HD21341 and HD42720 (to TH). We are grateful to Dr. Sue Varmuza for providing the CREST anti-sera, Dr. Terry Ashley for providing the SCP3 antibodies, and Dr. Peter Moens for providing the SCP1 (SYN1) antibody. We appreciatively acknowledge the helpful comments of Jodi Jackson, Sheila Cherry and Patricia Hunt in the preparation of this manuscript.
WEB RESOURCES

The URLs for data presented herein are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for SCP3, CBAVD, CREST, SCP1, SPO11, and RAD51)
Chapter III: Synaptic Initiation and Recombination

Title of manuscript: Sites of synaptic initiation do not translate into sites of recombination in the human male

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ABSTRACT

In most organisms, it is essential that each homologous chromosome pair have at least one exchange for faithful segregation at the first meiotic division. However, what regulates the placement of exchange events is not known. A possible mechanism has been suggested in the budding yeast, *Saccharomyces cerevisiae*, as synaptic initiation events appear to be translated into sites of exchange. Hence, we initiated a study that combines immunofluorescence and fluorescence in situ hybridization (FISH) to localize both the sites of synaptic initiation and recombination on the short arms of two chromosomes (1 and 16) in normal human spermatocytes. Our results suggest that although both synaptic initiation and recombination occur distally, the two events do not occur within the same chromosome region. Instead, synapsis is generally initiated within the first ten megabases from the telomere and exchange events are typically proximal to sites of synaptic initiation by at least 5 Mbs.
INTRODUCTION

From studies of many organisms it is clear that crossing-over is a prerequisite for proper segregation at the first meiotic division (Smith and Nicolas, 1998; Whitby, 2005; Keeney and Neale, 2006). Further, placement of exchanges is also important as crossovers placed too distally or too proximally increase the risk of nondisjunction (Tease et al., 2002; Lamb et al, 2005). Indeed, altered recombination events (i.e., in number or placement) are associated with an increased risk of malsegregation at either meiotic division in many organisms (Zitron and Hawley, 1989; Orr-Weaver, 1996; Nicolaidis and Petersen, 1998; Tepperberg et al., 1999). The result of nondisjunction in humans is profound – aneuploid conceptuses, which are the foremost cause of miscarriage, congenital birth defects, and mental retardation (Tease et al., 2002; Lynn et al., 2004) -- and all human trisomies yet studied show an association with aberrant recombination (Hassold et al., 1987, 1991, 1995; Warren et al., 1987; Fisher et al., 1995; Griffin, 1996; Lamb et al., 1996; Sherman et al., 2006).

The formation of double strand breaks (DSB) is the initiating event in the formation of crossovers (Ohta et al., 1994). However, there is a ten-fold excess of DSBs compared to exchange events (Moens et al., 1997, 2002). How and where these DSBs are chosen to become exchanges remain unknown (Cartlon et al., 2006), but there is growing evidence linking meiotic synapsis to recombination. For example, in Saccharomyces cerevisiae mutational studies of the genes encoding the synaptonemal complex proteins Zips 1-4 indicate that disruptions in the formation of the SC prevent recombination. Null mutations of the zip1 gene, which encodes the major component of
the transverse filament, reduce the number of exchanges threefold and abolish crossover interference (Tung and Roeder, 1998). zip4 mutants are similarly unable to polymerize Zip1, inhibiting SC formation and diminishing crossover interference, resulting in clustered exchange events (Tsubouchi et al., 2006). Immunoprecipitation, immunofluorescence, and two-hybrid screen studies indicate that other transverse filament proteins (Zip2 and Zip3) that localize to sites of synaptic initiation interact with proteins involved in both early and late recombination events (Chua and Roeder, 1998; Agarwal and Roeder, 2000; Perry et al., 2005; Tsubouchi et al., 2006).

Similarly, in the fungus, *Sordaria macrospore*, two mutant strains with reduced recombination show a parallel reduction in synaptic initiation events (Zickler et al., 1985, 1992). Further, synaptic initiation, as determined by electron microscopy, occurred within the same regions as did recombination, as determined by genetic mapping data (Zickler et al., 1992). However, there was an excess of synaptic initiation sites, suggesting that not all sites of synaptic initiation become sites of exchange but that recombination is dependent on effective pairing and SC formation.

Hints of this association have been found in mammals, since several species show a sex-specific correlation between the sites of exchange and synaptic initiation (Scherthan et al., 1996; Pfeifer et al., 2001, 2003; Froenicke et al., 2002). In human males, studies have shown that synapsis is initiated distally on the chromosomes (Rasmussen and Holm, 1977; Barlow and Hultén, 1996; Brown et al., 2005) similar to the location of recombination events (Broman and Weber, 2000). To further investigate the possible association of synaptic initiation and placement of exchanges in human spermatocytes, we used unique sequence FISH probes to map these events on individual human
chromosomes. For these analyses we chose two chromosomes: chromosome 16 because it is the most frequently nondisjoining human chromosome and one that typically has a single exchange event on each arm and, for contrast, chromosome 1, which is genetically longer, generally having two exchanges on both arms. For each chromosome, we focused on the short arms, asking three questions: Can synaptic initiation events be mapped to a particular site? Do sites of synaptic initiation and recombination coincide? Is there variation within or among individuals regarding the placement of the two events?

Our observations on partially synapsed zygotene spermatocytes suggest that the initiation of synapsis is restricted to the distal region, but we were unable to identify a specific site on either chromosome. Furthermore, synaptic initiation was limited to a single distal site that did not typically occur within the same region as recombination events, with synaptic initiation generally occurring distal to exchanges. Similar results were observed among the individuals in our sample population, suggesting that there is little variation among human males with regard to the placement of synaptic initiation and recombination events. Thus, our studies suggest that, unlike the budding yeast and fungus, sites of synaptic initiation are not translated into sites of recombination, providing the first evidence that the two events are “uncoupled” in human males.
MATERIALS and METHODS

Sample Population

Testicular samples were obtained from three individuals diagnosed with obstructive azoospermia in whom meiosis appeared normal based on routine histological analysis. The three individuals attended the Glickman Urological Institute of the Cleveland Clinic Foundation for treatment of infertility. The ages of the individuals were 36, 39 and 51 years at the time of tissue collection. STS-based assays for Yq microdeletions failed to detect any abnormalities. For all patients, informed consent was obtained in accordance with protocols established by the institutional review board (IRB) of the Cleveland Clinic Foundation.

Sample Processing

Testicular samples were processed following modifications of the technique of Barlow and Hultén (1996). Briefly, tissue was minced and put into a hypotonic solution, incubated for approximately one hour, and transferred to a 1% paraformaldehyde solution for subsequent slide preparation, as previously described (Judis et al., 2004; Brown et al., 2005).
Immunostaining and FISH

Microscope slides were hydrated in 1X antibody dilution buffer (ADB), incubated overnight with a primary antibody cocktail consisting of SCP3, CREST antisera, and either SCP1 or MLH1 (Calbiochem), washed overnight, and incubated for approximately one hour with the secondary antibody cocktail (fluorescein donkey anti-rabbit, rhodamine donkey antigoat, and AMCA donkey anti-human) as previously described (Brown et al., 2005). Images were captured using the Applied Imaging Quips Pathvision System or the Carl Zeiss Vision AxioVision LE version 4.3. Cell coordinates were noted for subsequent FISH analyses.

For BAC mapping studies, three BAC probes were used to identify chromosome 16 (RP11-139i12, ~0.8 Mb from 16pter; RP11-61e13, ~1.7 Mbs from 16pter; and RP11-18g19, ~10 Mbs from 16pter based on the UCSC Genome Browser, March 2006 assembly) and one commercially available BAC probe was used to identify chromosome 1 (~3.5 Mbs from 1pter; Vysis, Des Plaines, IL). Previously immunostained slides were dehydrated in serial ethanol washes (concentrations 70%, 90%, and 100%) for 2 min each and then allowed to air-dry. Slides were then denatured in 70% formamide in 2X SSC at 73°C for 5-7 min and again dehydrated in serial ethanol washes and air-dried. Previously labeled BAC probes were denatured at 73°C for 5 min. After the slides were completely dried, the BAC probes were overlaid, slides were coverslipped, and incubated overnight in a humid chamber at 37°C. Coverslips were removed and slides were washed in 0.4X SSC for 10 sec, 2X SSC/0.1% NP-40 for 3 sec, rinsed in H2O, and allowed to air-dry. Once dry, slides were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride.
(DAPI), rinsed in PBS, mounted in Antifade, and coverslipped. Previously imaged cells were then relocated and analyzed.

To identify telomeric repeat sequences, slides that were previously immunostained and/or analyzed by FISH were re-stained using a quantitative FISH (Q-FISH) protocol as previously described (Brown et al., 2005). Previously imaged cells were then relocated and analyzed.

Cytological Analysis of Synapsis and Scoring Criteria

Cells at either of two substages of prophase were analyzed: early zygote cells to identify synaptic initiation events and pachytene cells to determine the sites of meiotic exchange. Antibodies against SCP1 (detecting a component of the central element of the SC) and/or SCP3 (detecting a component of the axial/lateral elements of the SC) were used to monitor formation of the SC. Antibodies against MLH1 (a DNA mismatch repair protein detecting sites of exchange) were used to identify the chromosomal location of recombination events. For cells in early zygotene, synaptic initiation sites were defined as short linear regions of SCP1 localization and/or short regions of merged SCP3 signals. MLH1 foci were scored in pachytene cells defined as cells with complete synapsis between the 23 bivalents.

In all analyses, we focused on one of two chromosome arms: 1p or 16p. In a large proportion of cells, we observed either complete synapsis or complete asynapsis of 1p or 16p; since these cells provide no information on the site(s) of synaptic initiation, they were not further processed. The remaining cells, which contained “partially synapsed” segments on either 1p or 16p, were then examined. We focused on
chromosomes with the shortest possible regions of synapsis; arbitrarily we decided to exclude all cells with greater than 15 megabases (Mbs) of synapsis. In these cells, the partially synapsed regions were measured using the MicroMeasure version 3.3 software program (Reeves and Tear, 2000) to determine, in Mbs, the size and proximity of the synaptic initiation sites to the telomere of the respective chromosome. In zygotene cells, the synapsed portions (“SCP3 tracts”) were measured in microns, and the BAC signals were used as landmarks to convert the measurements from microns to Mbs. For example, on chromosome 16p BAC RP11-18g19 is approximately 10 Mbs from the p-telomere based on the UCSC Genome Browser, therefore MicroMeasure measurements in microns for each cell were equal to 10 Mbs, this conversion factor was used to convert the measurement of the synapsed region into Mbs for each respective cell. For 16p, the BAC signal furthest from the telomere or the centromere, when visible, was used as the conversion factor. For 1p, the BAC signal or the centromere, when visible, was used. Under the assumption that the central element of the SC forms bidirectionally, the midpoint of each SCP3 tract was used to estimate the site of synaptic initiation. In pachytene cells, the locations of the MLH1 foci were converted from microns to megabases using similar logic. For example, the physical distance between the 1p telomere to the centromere is approximately 125 Mbs based on the UCSC Genome Browser, therefore the measured length of the short arm in microns was equated to 125 Mbs and the distance of each MLH1 focus from the telomere was calculated based on this conversion factor.
Statistics

The comparison of estimated synaptic initiation sites among individuals was tested with simple goodness of fit tests. Similarly, for comparison of the location of recombination events goodness of fit tests were performed among individuals.
RESULTS

Spermatocytes from three individuals (see Table III-1) were analyzed to identify the synaptic initiation sites and sites of recombination events on chromosomes 1p and 16p. Based on chi square analysis, the individuals showed similar positioning of synaptic initiation events for both chromosomes 1p ($\chi^2=15.41; p>0.05$, see Table III-2) and 16p ($\chi^2=8.06; p>0.8$, see Table III-3). The chromosome locations of MLH1 foci on chromosome 16p were also similar among individuals ($\chi^2=12.40; p>0.5$, see Table III-4). For chromosome 1p, recombination events were categorized based on the number of MLH1 foci localized to the chromosome arm. For single, double, and triple exchange events the location of the foci were similar among individuals (E1, $\chi^2=6.92$, p>0.5; E2, $\chi^2=10.29$, p>0.2; E3, $\chi^2=5.85$, p>0.5, see Table III-5). Since there was no evidence for differences among individuals, all subsequent analyses were based on pooled observations from all three individuals.

Chromosome 16

Synaptic initiation is limited to one event on 16p

Spermatocytes at the earliest stage of synapsis were identified as large cells with short segments of synapsed or asynapsed axial elements (see Figure III-1). The short arm of chromosome 16 (16p) is approximately 38.2 Mbs in physical length (USCS Genomics, Santa Cruz, CA). With the intent of localizing and mapping the synaptic initiation site(s)
Table III-1. Summary of patient information.

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<th>Number of Cells:</th>
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<td></td>
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<td>Chromosome 16p</td>
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<td></td>
<td></td>
<td>Zygotene</td>
<td>Pachytene</td>
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<td></td>
<td></td>
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<td>Pachytene</td>
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<tr>
<td>Total</td>
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### Table III-2. Position of synaptic initiation sites on chromosome 1p

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<th>75 – 100 Mbs</th>
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**Table III-3.** Position of synaptic initiation sites on chromosome 16p

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### Table III-4. Position of MLH1 foci on chromosome 16p

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Table III-5. Position of MLH1 foci on chromosome 1p

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</table>
Figure III-1. Representative early zygotene-stage spermatocyte. The earliest stages of synapsis are identified as regions of merged SCP3 signals (darker red staining) or as localized SCP1 signals among asynapsed axial elements, also SCP3 (lighter red staining). Also labeled are kinetochores (identified by CREST in blue) and telomeric sequences (identified by a pan-telomeric PNA probe in yellow). The short arm of chromosome 16 is identified by three unique-sequence BAC probes (insert A). The two distal BACs localized to the forked region of 16p (insert B).
on 16p, two distal BAC probes and one interstitial probe were localized to the short arm (see Figure III-2A). Initially, we analyzed early zygotene cells from the three individuals with the aim to localize the synaptic initiation site. However, we were unable to identify a single site associated with synaptic initiation (see Figure III-3). Instead, telomeric forks (see Figure III-1) in the synapsed region were identified both proximal and distal to the two distal BACs, indicating that synapsis was limited to the distal region of the chromosome arm, not a particular sequence.

In subsequent analyses, we observed only one synaptic initiation event, as each cell (n = 88) had a single SCP3 tract (see Figure III-3). Further, as Figure III-3 shows, there was no single site at which all of the SCP3 tracts aligned. Therefore, to determine whether any region of 16p were more prone to initiate synapsis, the distribution of the estimated synaptic initiation sites (SCP3 tract midpoints) was determined by dividing 16p into eight (8) segments of 5 Mbs. Nearly 70% of all synaptic initiation events were positioned between 0-5 Mbs from the telomere and the remaining initiation sites were 5-10 Mbs from the telomere (see Figure III-4).

Sites of synaptic initiation did not localize with sites of meiotic recombination on 16p

Using the same BAC probes as in the analyses of synapsis, sites of meiotic recombination were mapped on 16p. The sites of recombination were localized using an antibody against MLH1, a mismatch repair protein that localizes to sites of exchange on the SC during pachytene (see Figure III-2B). As predicted by previous genetic linkage studies (Broman and Weber, 2000) MLH1 foci were typically distally located. In
virtually all cells there was only one recombination event per meiosis on 16p, with two

Figure III-2. Immunostaining of zygote (A) and pachytene (B) spermatocytes and
FISH of chromosome 16p. The distal BAC probes are labeled in magenta, white and
blue, which identify the short arm of chromosome 16 (insert). Also labeled are the
kinetochores (identified by CREST in blue), telomeric sequences (identified by a pan-
telomeric PNA probe in yellow, A), and recombination events (identified by MLH1 in
yellow, B).
Figure III-3. SCP3 tracts on chromosome 16p. The synapsed regions of the chromosome arm are illustrated as colored bars, each individual is represented as a different color, Sp1018 are the red bars, Sp1006 the green bars, and Sp1005 the blue bars. Each bar represents the size and location of synapsis. Following the ideogram at the bottom (red line representative of the 16p SC) the bars are positioned from the p-telomere (green focus) to the centromere (blue focus). The three BACs are relatively positioned on the ideogram, BAC 1 is the magenta star, BAC 2 the white star, and BAC 3 the blue star.
Figure III-4. The distribution pattern of MLH1 and estimated synaptic initiation sites on chromosome 16p. The occurrence of exchange events (blue line) or synaptic initiation events (pink line) are illustrated for 5 Mb segments of the chromosome arm from the telomere (0 Mbs) to the centromere (40 Mbs). The region of synaptic initiation (0-5 Mbs) does not colocalize with the region of preferred exchange (10-15 Mbs).
exchanges occurring in just 1 of 243 cells. The distribution pattern of exchange events was determined by again dividing 16p into eight 5 Mbs segments. MLH1 foci mapped along the entire short arm of chromosome 16, however as shown in Figure III-4, nearly 80% of recombination events occurred between 0-20 Mbs from 16pter. The highest incidence of recombination occurred within the 10-15 Mbs segment. The distribution patterns of both the sites of recombination and synaptic initiation were compared (see Figure III-4) to determine that, in general, the preferred region of recombination (between 10-15 Mbs) did not colocalize with the sites of synaptic initiation (between 0-10 Mbs).

**Chromosome 1**

*Synaptic initiation is limited to one event on 1p*

To further investigate individual chromosome synaptic initiation and the association of synaptic initiation and exchange placement, the short arm of the genetically longer chromosome 1 (1p) was investigated. Studies similar to those for 16p were conducted for 1p, which is approximately 125 Mbs in physical length (USCS Genomics, Santa Cruz, CA). Using a distal FISH probe, 1p was identified in both zygotene and pachytene cells (see Figure III-5). The initial results of these experiments were similar to those of 16p (see Figure III-6): a single synaptic initiation event was observed in the distal region of chromosome 1p for all cells. Similar to 16p, the identified SCP3 tracts on 1p failed to align to one particular site. To determine the distribution pattern of calculated synaptic initiation sites, 1p was divided into 5 Mb segments for a total of 25 segments and as illustrated by Figure III-7, greater than 90% of synaptic initiation events fell within the
Figure III-5. Immunostaining of zygotene (A) and pachytene (B) spermatocytes and FISH of chromosome 1. The distal BAC probe, in magenta, identifies the short arm of chromosome 1 (insert, arrow). The interstitial BAC probe, in white, identifies the long arm of chromosome 1 (insert, arrowhead). Also labeled are the kinetochores (identified by CREST in blue), telomeric sequences (identified by a pan-telomeric PNA probe in yellow, A, or white, B), and recombination events (identified by MLH1 in yellow, B).
**Figure III-6.** SCP3 tracts on chromosome 1p. The synapsed regions of the chromosome arm are illustrated as colored bars, each individual is represented as a different color, Sp1018 are the red bars, Sp1006 the green bars, and Sp1005 the blue bars. Each bar represents the size and location of synapsis. Following the ideogram at the bottom (red line representative of the 1p SC) the bars are positioned from the p-telomere (green focus) to the centromere (blue focus). The BAC (magenta star) used to identify 1p is relatively positioned on the ideogram.
first two segments (0-10 Mbs). The majority of calculated synaptic initiation sites occurred between 5-10 Mbs.

*Synaptic initiation events were not translated into recombination events on 1p*

The location of recombination events was determined for 1p. In contrast to 16p, the short arm of chromosome 1 typically had two recombination events (>80%). Therefore, the placement distribution for the MLH1 foci was determined separately for those chromosomes with one exchange (E1), two exchanges (E2), or three exchanges (E3). In the event of one exchange, MLH1 foci were located in interstitial regions of the chromosome arm (see Figure III-7A) but a single region of preferred exchange was not evident. The regions with the highest incidence of exchange occurred equally between 25-30 Mbs and 55-60 Mbs from the telomere, which are both proximal to the region of synaptic initiation. The distribution pattern of single exchange events was compared to the sites of synaptic initiation (Figure III-7A). Similar to the results observed for 16p, synaptic initiation events were distal to sites of recombination when there was a single exchange event on 1p.

When there were three exchanges, the comparison between synaptic initiation events and the distribution of exchange position (Figure III-7C) also indicated that sites of synaptic initiation were distal to the sites of recombination, with fewer than 20% of exchanges occurring within the first 10 Mbs of 1p. Therefore, in instances of one or three exchange events on 1p, there appeared to be no concordance between the location of those exchanges and the sites of synaptic initiation.
Figure III-7 (previous page). The distribution pattern of MLH1 and estimated synaptic initiation sites on chromosome 1p. The occurrence of exchange events (blue line) or synaptic initiation events (pink line) are illustrated for 5 Mb segments of the chromosome arm from the telomere (0 Mbs) to the centromere (125 Mbs). MLH1 distribution patterns are separated corresponding to the number of exchange events; single events, E1s (A); double events, E2s (B); and triple events, E3s (C).
Conversely, in the event of two exchange events, the peak number of MLH1 foci coincided with the peak number of estimated synaptic initiation sites (see Figure III-7B). However, the incidence of the most distal exchanges, 20% of all exchanges, was much reduced compared to the incidence of synaptic initiation sites in the most distal region of the chromosome arm (92%). Furthermore, for all interstitial and proximal exchanges, synaptic initiation events never colocalized within these regions.
DISCUSSION

In a previous study of the human male, we asked several basic questions regarding synapsis and SC formation in human spermatocytes (Brown et al., 2005). Our results from these initial studies indicated that, like other mammals (Mahadevaiah et al., 2001; Lenzi et al., 2005), initiation of recombination via the formation of DSBs preceded the formation of the SC. Once the SC began to form, synapsis was initiated on both chromosome arms of nonacrocentric chromosomes and only from the long arm of acrocentric chromosomes. In all individuals analyzed, synapsis began in the distal region but not at the telomere proper and did not proceed across the centromere from the short arm.

These studies suggested that synapsis is a highly homogeneous process among individuals. However, many questions remained. In particular, our analyses relied on observations of late zygotene cells, preventing us from determining whether distal sites of synaptic initiation were the result of a single event or of multiple events that polymerized before we were able to observe them. Therefore, the studies discussed here were intended to further characterize the initiation of synapsis. We used probes to the subtelomeric region of the frequently nondisjoining chromosome 16, to investigate the number and more precise location of synaptic initiation events at the earliest stages of synapsis on the short arm. Our first observation was that synapsis was a result of a single initiation event from the distal region of the chromosome arm. Secondly, as expected from our previous studies, forks at the distal region of 16p were frequently observed. However, the forks varied in size regardless of the amount of synapsis on the
chromosome arm. This provided evidence that there was no single site of synaptic initiation and that the subtelomere may not be the region of interest, since telomeric fork sizes ranged from approximately 1 Mb to nearly 5 Mbs, clearly outside the subtelomeric region of 10-300 kb (Mefford and Trask, 2002; Linardopoulou et al., 2005). Therefore, we decided to identify the region(s) most commonly associated with synaptic initiation on the short arms of chromosomes 16 and 1.

Similar to the initial findings of 16p, the synaptic initiation events of 1p did not localize to a specific site, suggesting that synapsis is localized to the distal region but not to a single site within that region. As indicated from our results, the distal region that initiates synapsis on both chromosomes 16p and 1p is located no further than 10 Mbs from the p-telomere. The mechanism(s) that initiates synapsis is still unknown. However, of particular interest was the growing evidence from model organisms that suggests that synaptic initiation sites are translated into sites of recombination (Zickler et al., 1992; Chua and Roeder, 1998). We focused on this suggested association as a potential mechanism in human males.

Immunostaining of the *S. cerevisiae* SC suggests that synapsis is capable of initiating anywhere along the chromosome as axial associations between the Zip2 and Zip3 transverse filament proteins (Chua and Roeder, 1998; Agarwal and Roeder, 2000). These transverse filament proteins interact with proteins involved in the formation of exchange events. Additionally, previous studies demonstrated that the DSBs that are translated into mature exchange events are also involved in SC formation (Henderson and Keeney, 2004). At least in the budding yeast, it appears that sites of synaptic initiation are associated with sites of recombination.
The human male presents an analogous association between distal sites of synaptic initiation and recombination. Previous observations of recombination events using immunofluorescence with an antibody against the MLH1 protein demonstrated that exchanges occurred preferentially within the distal region, but were capable of occurring anywhere along the chromosome arms (Lynn et al., 2002; Hassold et al., 2004). However, synaptic initiation appeared limited to one event per arm and restricted to the distal region (Brown et al., 2005). Not surprisingly, when the localization of synaptic initiation events was compared to the sites of recombination in this study, the two events did not correlate within the same distal region(s). This is in contrast to what appears to be the mechanism in the budding yeast.

In conclusion, synaptic initiation was localized to a single initiation event on the distal region of chromosomes 1p and 16p in human spermatocytes. Unlike the observations in budding yeast, these sites of synaptic initiation do not colocalize with sites of recombination, uncoupling the two events in human males. Mechanisms that regulate either the placement of synaptic initiation events or recombination remain elusive.
Chapter IV: Summary and Future Directions

Summary

Molecular and cytogenetic studies have established the role of the synaptonemal complex (SC) in meiotic prophase. However, many of the details regarding specific dynamics of the SC remain ambiguous. The studies described in this thesis examined the formation of the SC in human spermatocytes, providing information on the temporal relationship between synapsis and recombination, identifying chromosome regions involved in synapsis, and distinguishing synaptic initiation from the placement of exchange events. In addition, spermatocytes from several individuals were analyzed to investigate variation in synaptic patterns within and among human males.

Studies in model organisms have provided evidence of at least two temporal “relationships” between synapsis and recombination. In flies and worms, homologous chromosomes pair at leptotene, build the SC at zygotene, and initiate and complete recombination at pachytene. In budding yeast and mice, there is a different sequence: recombination is initiated at leptotene, and in the context of DSBs, homologous chromosomes pair and synapse, and recombination is completed at pachytene. The first analysis presented in this thesis (Chapter II) demonstrated that human spermatocytes initiate recombination at the earliest stage of prophase – leptotene, prior to the formation of the SC.

Our second set of studies (also discussed in Chapter II) focused on the formation of the SC itself, addressing the number and location of synaptic initiation events and chromosome regions involved in the formation of the SC. In general, synapsis was found
to be a highly homogeneous process initiated at the distal regions of chromosomes and “zippering” to the centromere. Nonacrocentric chromosomes had two initiation events; one per chromosome arm. Acrocentric chromosomes initiated synapsis only at the distal region of the long arm and synapsed through the centromere to the short arm. Further analysis of nonacrocentric chromosomes demonstrated that the centromeric region acted as a barrier to synapsis, frequently impeding synapsis across chromosome arms.

The short arms of acrocentric chromosomes are incapable of initiating synapsis, which may be related to their highly repetitive and homologous sequences (Choo, 1990). It is unknown if the inability to initiate synapsis at the acrocentric p-arm is a result of the DNA sequence or the physical association of the short arms with the nucleolus during meiotic prophase (Tres, 1975). Subsequently, synapsing from the long arms to the short arms may prevent nonhomologous interactions associated with the formation of Robertsonian translocations.

Finally, studies of individual chromosomes (1 and 16) provided an opportunity to investigate synapsis of individual chromosomes and its association with recombination (Chapter III). Chromosome 16 was chosen based on its clinical importance since trisomy 16 is the most common trisomy in human conceptuses (Hassold et al., 1995). In preliminary studies, we found that chromosome 16p typically had a single recombination event on the short arm. Thus, we chose to compare it with the short arm of chromosome 1 since 1p typically has two recombination events.

For both 1p and 16p, our results suggested that synapsis is usually initiated subtelomERICALLY, within 0 – 10 Mbs of the telomere. We compared the location of synaptic initiation events with sites of exchange and in general the two events appear to
be distinct processes as synaptic initiation sites are not translated into recombination events.

In conclusion, formation of the human synaptonemal complex is a highly homogeneous event. Two chromosome elements play important roles: the distal regions are typically the site of synaptic initiation and the centromeric region displays an unforeseen role in the progression of synapsis – that of an impediment. Finally, while sites of synaptic initiation are distal and recombination events are preferentially distal, the two apparently occur at different chromosomal sites.
Future Directions

Human studies

Synaptic role of the centromere

Studies of nonacrocentric chromosomes indicate that at least in the human male, synapsis was impeded across one arm to the other within the centromeric region. Whether this is a result of centromeric sequences or centromere-associated proteins that make up the kinetochore remains unknown. To further investigate this question, individuals heterozygous for centromeric polymorphisms could be analyzed. For example, human chromosomes 1, 9, and 16 contain large blocks of centric heterochromatin (Madon et al., 2005). It has been suggested that these heterochromatic regions pose difficulties in meiotic synapsis. Both meiotically normal individuals and those with azoospermia related to meiotic anomalies have a higher incidence of SC discontinuities and unsynapsed regions at pachytene at these heterochromatic regions, particularly at the centric heterochromatic regions on chromosomes 1 and 9 (1qh and 9qh) (Sun et al., 2005; Codina-Pascual et al., 2006). Codina-Pascual and colleagues (2006) concluded that these heterochromatic regions are the last to synapse, an observation that may be attributable to polymorphisms within the population. In their study, an infertile individual with a polymorphism on the 1qh region had the highest incidence of 1qh SC discontinuities and unsynapsed regions of all individuals (Codina-Pascual et al., 2006). In a recent study by Sun and colleagues (2005), gaps at the 9qh region were reported as often as 91% of analyzed cells (n = 72). Further, Antonelli and colleagues (2000) hypothesized that chromosome abnormalities, including
heterochromatic polymorphisms, cause spermatogenetic arrest because of synaptic anomalies.

The chromosome 9 polymorphisms could be used to further investigate the importance of heterochromatic variation to the synaptic process. Individuals ascertained for obstructive azoospermia could be genotyped at the 9qh region to identify alleles associated with different sized polymorphisms. MI spermatocytes could be analyzed for chromosome-9-specific synaptic patterns in individuals heterozygous for these heterochromatic polymorphisms and compared to spermatocytes from homozygous individuals. Consistently larger presynaptic bubbles observed from heterozygotes would suggest that two alleles of different size affect synapsis within this region. Likewise, consistently observed smaller bubbles in individuals with similar sized, but sequence divergent, alleles would suggest that sequence influences synapsis of this region. However, presynaptic bubbles observed in spermatocytes from homozygous individuals would suggest that synapsis at this region is influenced by something other than the sequence around the centromere or the centromere itself, perhaps the proteins associated with the kinetochore at prophase.

*What initiates synapsis?*

Detailed analysis of early synaptic events of chromosomes 1p and 16p indicate that synapsis is initiated distally but not at the telomere proper. The distal portion of human chromosomes can be roughly divided into three regions; i.e., the telomere proper, the subtelomeric region, and unique sequence. The most distal region is the telomere, which is composed of TTAGGG repeats (3-20 kb) and capped by telomere-associated
proteins shared by all human telomeres (Moyzis et al., 1988; Slijepcevic, 1998; Pandita et al., 1999). The subtelomere, mosaic stretches of variable repeat sequences arranged in a “patchwork” of blocks that extend between 10-300 kb, forms the transition from the telomeric repeats to the chromosome-specific sequence (Mefford and Trask, 2002). These mosaic blocks are <2 kb in length and are evolutionarily conserved (Flint et al., 1997; Mefford and Trask, 2002). The arrangement and sequence in humans are polymorphic in copy number and location. Some chromosomes, e.g., human chromosome 16, are significantly polymorphic in size and sequence (Figure IV-1) (Wilkie et al., 1991; Mefford et al., 2001). On the short arm of 16, three common variants have been thus far identified: the A allele is 165 kb in size and is present in 76% of the Caucasian population, allele B is 415 kb and is present in 24% of the Caucasian population, and the C allele is 495 kb in size and is present in <1% of the Caucasian population (see Figure IV-1) (Wilkie et al., 1991).

These large-scale polymorphisms on 16p could be screened among individuals with previously determined synaptic patterns. For example, the synaptic patterns of 16p in individuals heterozygous for the two polymorphisms with the greatest size difference (i.e., AB or AC individuals) would test whether the size of the distal region (at ~ 100kb magnitude) affects synapsis. In contrast, individuals heterozygous for two polymorphisms of similar size but different sequence (alleles BC) could be used to investigate the effect of sequence on synapsis of the most distal region.

Two possible outcomes might be observed: (1) If heterozygosity causes synaptic delays, heterozygous individuals would have more subtelomeric forks than observed for homozygous individuals. (2) If heterozygosity causes synaptic “difficulties” dependent
Figure IV-1. Schematic of the three polymorphic alleles of the subtelomeric region in 16p, included are the allele frequencies. (Adapted from M. Johnson)
on the sequence or region size, the frequency and size of synaptic forks could be correlated to allelic variation. It can be hypothesized that if the size of the subtelomeric region affects synapsis, the incidence of larger forks will be observed in later staged spermatocytes from individuals with two different sized alleles, e.g., AB or AC individuals. However, if sequence heterozygosity affects synapsis then the incidence of forks will be observed equally for BC individuals (relatively equal size alleles) and AB (or AC) individuals (significantly different size alleles). Homozygous individuals should have the lowest incidence of subtelomeric forks.
Mouse studies

As discussed in Chapter III of this thesis, the sites of synaptic initiation and recombination apparently do not colocalize in human spermatocytes. The uncoupling of synaptic initiation events and exchange may be a human-specific phenomenon as evidence from cattle and yeast suggests that synaptic initiation sites are translated into sites of recombination in these species (Chua and Roeder, 1998; Agarwal and Roeder, 2000; Pfeifer et al., 2001, 2003). To determine the method in which synapsis proceeds in other mammals, it would be useful to examine other species. The mouse provides an attractive, genetically defined, organism and accordingly, we have already initiated studies of synapsis in both males and females.

Our preliminary results show that, unlike the tightly regulated synaptic pattern of the human male, formation of the SC is not uniform in the mouse. Various synaptic patterns are commonly observed including initiation sites located distally, interstitially, and proximally. Synapsis in the male mouse does not seem to be regulated by chromosome location (i.e., proximity to the distal region), as preliminary studies indicate that any region of the autosomal chromosomes is capable of initiating synapsis (see Figure IV-2).

Additionally, sites of synaptic initiation were compared with sites of recombination on mouse chromosomes 1 and 10. Our preliminary data strongly suggest that in male mice, sites of synaptic initiation colocalize with sites of recombination, at least in the proximal half of the chromosomes. As shown in Figure IV-3, interstitial and proximal sites of synaptic initiation and exchange events occur at similar frequency within common regions. To complete these studies more individuals should be analyzed.
Figure IV-2. SCP3 tracts on individual mouse chromosomes. Diagram of synapsed regions (red bars) oriented from the telomere (green focus on ideogram) to the centromere (blue focus on ideogram) on mouse chromosomes 1 (A) and 10 (B). The chromosomes are 195 Mbs and 130 Mbs in physical size, respectively.
**Figure IV-3.** Distribution pattern of MLH1 and estimated synaptic initiation sites. Frequency of exchange events and synaptic initiation events on mouse chromosomes 1 (A) and 10 (B) from the telomere (0 Mbs) to the centromere (195 Mbs and 130 Mbs, respectively).
While the 129 mouse strain was used in these preliminary investigations because of its high frequency of exchange events per cell, analysis of other mouse strains should be undertaken. Further study will be needed to address specific questions: e.g., whether the synaptic initiation and exchange events colocalize at the distal region of the chromosome and whether there is protein-protein interaction between proteins involved in the respective events.
Works Cited:


