To the Graduate Council:

I am submitting herewith a dissertation written by Timothy John Wiltshire entitled "Mammalian Meiosis: Events of Meiotic Prophase I in Spermatogenesis." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Science.

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We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor and
Dean of the Graduate School

MAMMALIAN MEIOSIS: EVENTS OF MEIOTIC PROPHASE I IN SPERMATOGENESIS

A Dissertation

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Doctor of Philosophy

Degree

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Timothy John Wiltshire

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ABSTRACT

The unique events of meiosis include the pairing and recombination of chromosomes during prophase of meiosis I; still little is known about these events in mammalian meiosis. In this study some of the basic mechanisms governing the structure and behavior of chromosomes, and the timing of events during male mammalian meiotic prophase are investigated in mouse spermatogenic cells. This has developed as three main areas of work; defining chromatin structure and function during prophase I, cell culture to establish a system for experimental analysis of meiosis, and cell cycle analysis.

During the pachytene stage of meiotic prophase in male mammals the X and Y chromosomes become transcriptionally inactive and establish a chromatin domain, the sex body, that is visually distinct from the transcriptionally active autosomes. We sought objective criteria to describe chromatin differences by assessing DNase-I sensitivity of sex chromosome and autosomal sequences. Results support the hypothesis that in pachytene spermatocytes the chromatin configuration of the X-Y pair in the sex body is more condensed and inaccessible to enzymatic digest, while the autosomal chromatin is in a more open configuration. We consider the possibility that this configuration may well be related to recombinational status.

This study also reports the use of a simple system for the short-term culture of pachytene spermatocytes, in which we have assayed parameters of cell function pertinent to meiotic prophase, namely chromosome pairing and synapsis. Little is known about the control of events ending the lengthy prophase of meiosis I and leading to the G2/M-phase transition in mammalian spermatocytes. We have utilized short-term cultures of pachytene spermatocytes to study events of the G2/M cell-cycle transition induced by the protein phosphatase inhibitor okadaic acid (OA). Treatment of cultured pachytene spermatocytes with OA induced a rapid and premature onset of events leading to the M phase. These

results suggest that OA treatment overrides a cell-cycle checkpoint control that normally keeps pachytene spermatocytes in a lengthy prophase, and that this control may be exerted by regulation of protein phosphorylation status.

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PART I

GENERAL INTRODUCTION

CHAPTER I

Spermatogenesis and Meiosis

The focus of this study is primarily on events of prophase of the first meiotic division in mouse spermatogenesis. There were three specific aims for this work:

- The investigation of chromatin configuration and chromosome behavior during the pachytene stage of spermatogenesis, and how this relates to meiotic function.
- The development of primary cultures of spermatocytes for experimental analysis of meiotic events.
- An investigation of the cell cycle controls involved in the G2/M meiotic progression.

This general introduction presents relevant background underlying the research, including mammalian testis structure, the general features of mammalian spermatogenesis, and, most importantly, the meiotic processes, and dynamic changes that are occurring at a number of different levels during the early stages of spermatogenesis.

The Process of Spermatogenesis

Gametogenesis is a fundamental process in the reproduction of eukaryotes and spermatogenesis is at the same time one of the most complex differentiation processes in higher eukaryotes. The morphological events have been extensively reviewed previously (Fawcett, 1975; Bellvé, 1979; Bellvé and O'Brien, 1983), and a brief description of some aspects central to this research is presented here.

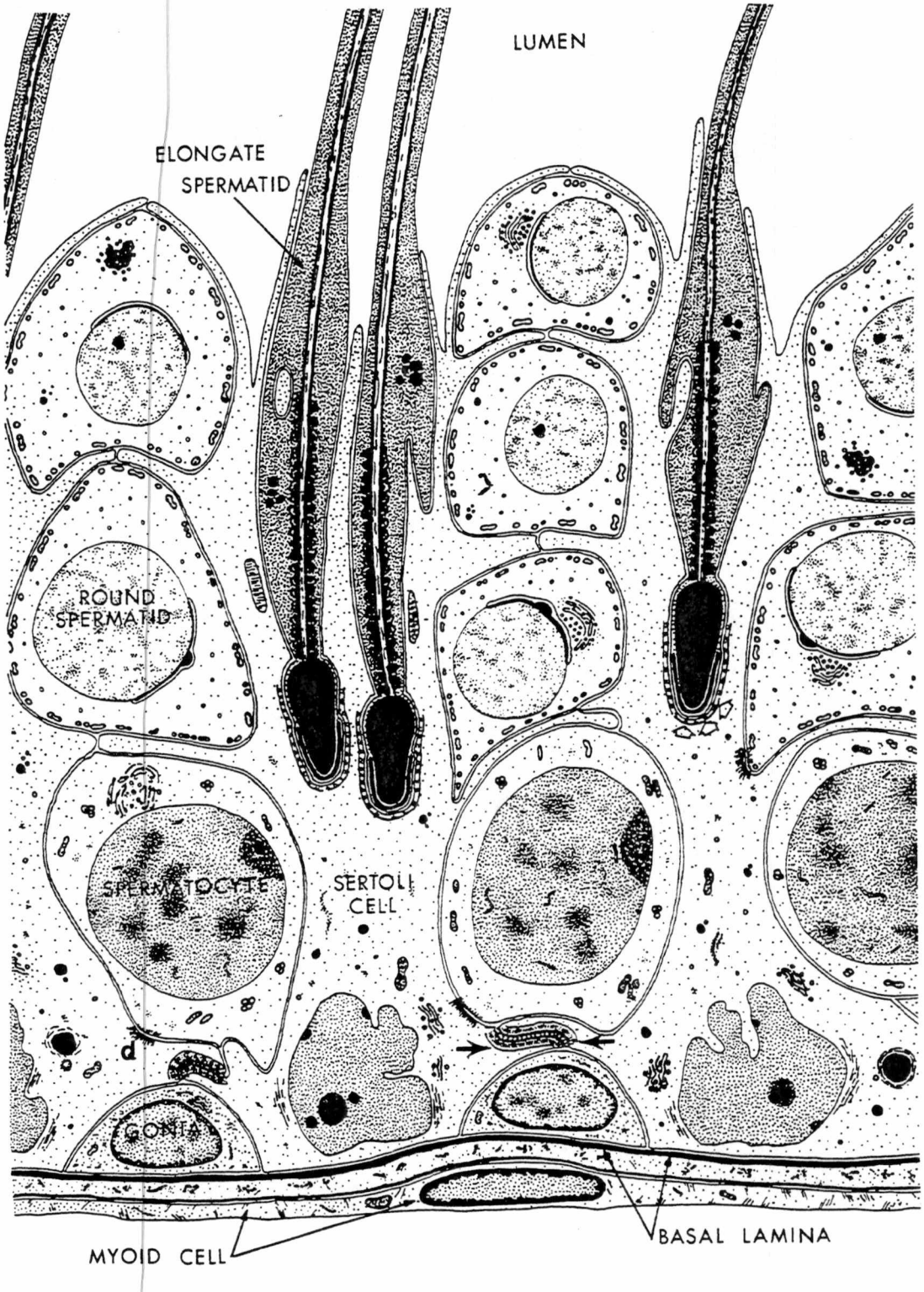
Although the process of spermatogenesis is a developmental continuum from undifferentiated stem cells to the highly specialized spermatozoa, spermatogenesis occurs in

three distinct phases: a mitotic phase, a meiotic phase, and a post-meiotic phase. The meiotic phase is the focus of this work. During the mitotic phase, spermatogonial stem cells give rise to proliferating spermatogonia. These cells undergo a series of doublings which results in a substantial expansion in the number of germ cells, as well as a replenishment of the stem cell pool. Since the proliferating spermatogonia undergo incomplete cytoplasmic divisions, clones of pre-meiotic spermatocytes are interconnected by cytoplasmic bridges in a syncytium (Dym and Fawcett, 1970). Fully differentiated spermatogonia (type B) undergo a single mitotic division to give rise to preleptotene spermatocytes, which then undergo a round of DNA synthesis before entering meiotic prophase.

In the meiotic phase spermatocytes accomplish the unique events of pairing of chromosomal homologs, genetic recombination, and the reductional division of meiosis to form secondary spermatocytes which undergo the equational division to form spermatids; now haploid in genome content. During the post-meiotic phase the initially round spermatid is extensively remodelled into the terminally differentiated spermatozoon. The acrosomal cap and flagellum form and previously existing structures are reshaped and redistributed to produce the male gamete.

Spermatogenesis in mammals takes place within the seminiferous epithelium lining the seminiferous tubules (Fig 1). The tubule walls consists of several layers of myoid cells enclosed by collagen and other extracellular matrix materials (De Kretser and Kerr, 1988). This is surrounded by the interstitium. The interstitium is the vascularized region of the testis and is populated exclusively by somatic cells. The major somatic cell of the interstitium is the Leydig cell. These cells produce testosterone which is essential for development of primary and secondary male sex characteristics, as well as for normal spermatogenesis. The epithelium of the seminiferous tubules is composed solely of two

Fig. 1. A portion of the tubule wall is diagrammed to show the organization of cells within the bounds of the seminiferous tubule. Spermatogonia (or gonia), spermatocytes and round spermatids are seen at succesively higher levels within the epithelium. Elongating and elongate spermatids occupy crypts with deep apical recesses of the Sertoli cell, and thus often appear to be between the other germ cell types. Spermatogonia and Sertoli cells are the only cells shown that contact the basal lamina. The Sertoli cell extends from the base of the tubule to the lumen and makes contact with all of the germinal cells. Of special note is the region of Sertoli-Sertoli contact (paired arrows) occurring around the entire tubule as the basolateral regions of Sertoli cell meet. This is the area of the occluding junctions of the Sertoli cell barrier. Desmosome-like junctions (d) are seen between most germ cell types and the Sertoli cell. Taken from Russell, L.D. (1983). Normal testicular structure and methods for evaluation under experimental and disruptive conditions. In: Reproductive and Developmental Toxicity of Metals, Plenum Press, N.Y., pp 227-252.


LUMEN
ELONGATE
SPERMATID
ROUND
SPERMATID
SPERMATOCYTE
SERTOLI
CELL
d
GONIA
MYOID CELL
BASAL LAMINA

cell types: spermatogenic cells and the somatic Sertoli cells which surround them. The Sertoli cells apparently provide a structural framework and the correct developmental environment for spermatogenesis. Specialized tight junctions between Sertoli cells separate the seminiferous epithelium into two compartments, the basal and adluminal compartments, and this creates a blood-testis barrier (Dym and Fawcett, 1970). The basal compartment contains spermatogonia and the earliest spermatocytes, while the adluminal compartment contains later stage spermatocytes and spermatids. The transition from pre-meiotic spermatogonia to the meiotic spermatocytes is accompanied by crossing the blood-testis barrier to the adluminal compartment. Here germ cells are isolated from the immune system and differentiate in a fluid environment of unusual ionic and molecular composition (Fritz, 1978).

The duration of spermatogenesis, from the precursor spermatogonial stem cells to spermatozoon formation is about 35 days. The duration of the meiotic part of this development, from the preleptotene spermatocytes through two meiotic divisions to the haploid round spermatids, is about 9 days (Oakberg, 1956).

Meiotic Events of Spermatogenesis

The biological function of meiosis for an individual organism is the halving of the chromosome complement during gametogenesis. This is accomplished by a process similar to the mitotic cell cycle, but with major modifications. During meiosis a single round of DNA replication is followed by two divisions involving chromosome segregation, instead of one as in mitosis. It is the first division, the reductional division, that is unique to meiosis; at this time replicated homologs segregate to opposite poles. Correspondingly, much of meiosis is devoted to the complex series of events leading up to this first division. A summary of some of these events is shown in Figure 2. Most of

Figure 2. Summary of prophase I stages of spermatogenesis showing cell stages used in this study, the meiotic substages and meiotic events. The timing of events of SC formation and differential conformation of the X-Y pair are known in the mouse, but the timing of the other meiotic events have not been precisely determined and are based on studies in yeast. Cell drawings are taken from Bellve, A.R., Cavicchia, J.C., Millette, C.F., O'Brien, D.A., Bhatnagar, Y.M., Dym, M. (1977). Spermatogenic cells of the prepuberal mouse. J Cell Biol 74:68-85.



leptotene spermatocyte | zygotene spermatocyte | pachytene spermatocyte | secondary spermatocyte | round spermatid

Cell Types spermatogonia → primary spermatocytes → secondary spermatocytes → spermatids → spermatozoa

Mitosis | **Meiosis I** (prophase I, metaphase I, anaphase I) | **Meiosis II** | spermiogenesis

Meiotic Stage leptotene, zygotene, pachytene, diplotene, telophase I

S C forms → SC fully formed → SC separates

X and Y show different chromatin configuration

Meiotic Events homology search; initiation of synapsis; full synapsis; desynapsis; homologs separate; chromatids separate → haploid

double strand breaks; initiation of recombination; recombination resolved?

these events occur during the period between the end of the DNA replication phase and the time of formation of a spindle, at metaphase I; this period defines meiotic prophase. The most fundamental requirement for successful chromosome segregation at metaphase I is thought to be the formation of stable physical connections between homologous chromosomes (Kleckner *et al.*, 1991). This unique feature of meiosis demands that a maternal and paternal homolog become paired. It is not clear how this process occurs. The physical connection between homologs is associated with a proteinaceous structure, the synaptonemal complex (SC), the function of which is still not clear from a genetic viewpoint (reviewed by Loidl, 1993). However, the connection that keeps the two homologs joined under spindle fiber tension prior to the first division is provided by the chiasmata, results of reciprocal recombination events, exchanges between one sister chromatid of each homolog. The chiasmata can be seen until the initiation of the segregation of homologs. The second meiotic division, the equational division, is similar to that in mitosis: sister chromatids segregate from one another.

Almost one-third of the time required for spermatogenesis in the mouse is devoted to the various phases of meiosis, principally to prophase of meiosis I (Kofman-Alfaro and Chandley, 1970). Prophase I stage is typically divided into the leptotene, zygotene, pachytene, diplotene, and diakinesis substages based primarily on cytological observations (Solari, 1970; Moses, 1980; Goetz *et al.*, 1984). Leptotene spermatocytes are characterized by diffuse chromatin with little indication of chromosome pairing. It is not clear when homologs begin to pair; however, zygotene is characterized by the formation and beginning of synapsis of the SC, which is assumed to be an indicator of the completion of homolog pairing. The SC is a prominent and unique nuclear structure of meiotic cells and a number of its structural components have been isolated from pachytene spermatocyte nuclei (Heyting *et al.*, 1985, 1988; Behal, *et al.*, 1988). The assembly and disassembly of

SC's are important markers for staging meiotic prophase. During the lengthy pachytene stage cells increase considerably in size. They have a thin rim of cytoplasm, a large nucleus with thick strands of bivalent chromosomes, and a prominent heterochromatic region adjacent to the nuclear envelope called the sex body, which is comprised of the X and Y chromosomes. SC's are prominent and fully formed during the pachytene stage. The transition from pachytene to diplotene is characterized by a gradual desynapsis of the SC's, starting with the X-Y pair, and then autosomal desynapsis. During diplotene and diakinesis the recombination events (crossovers) that occurred are visible as chiasmata, and further chromosomal condensation becomes apparent. Toward the end of diakinesis the sex body, nucleolus and nuclear membrane have disappeared and a spindle apparatus becomes apparent in conjunction with fully condensed metaphase chromosomes. The first meiotic division follows rapidly, and is the time when the homologs segregate.

Analysis and dissection of the events of prophase I of meiosis has been difficult in mammals; investigation and definition of meiotic prophase I events has relied mostly on other model organisms, particularly *Saccharomyces cerevisiae* (recently reviewed by Roeder, 1995) and *Drosophila.* (recently reviewed by Orr-Weaver, 1995).

A defining feature of meiosis is the pairing and recombination of homologs (recently reviewed by Loidl, 1994) and recent evidence suggests that much, and possibly all, meiotic recombination, at least in *Saccharomyces cerevisiae,* is initiated via the formation and subsequent repair of double-strand DNA breaks (DSBs). In yeast the first chemical change in DNA during meiosis is the occurrence of DSB's in early prophase (Game *et al.*, 1989; Zenvirth *et al.*, 1992). At least some DSBs are then converted to recombination intermediates (Schwacha and Kleckner, 1994, 1995), with DSB distributions mirroring those of meiotic crossovers (Game, 1992; Wu and Lichten, 1994). Mutations in several yeast genes result in a complete loss of DSBs and an absolute defect in

meiotic recombination (Cao *et al.*, 1990; Rockmill *et al.*, 1995). Although not in a meiotic context, it was also recently reported that DSBs created by the use of restriction enzymes that produce blunt ended DSBs induced illegitimate (non-homologous) recombination of mammalian chromosomes in CHO cells (Phillips and Morgan, 1994).

Several observations indicate that local DNA sequence is not the primary determinant of DSB positioning. All DSB sites examined to date have been found to lie in regions of chromatin that are DNase I hypersensitive (Wu and Lichten, 1995), and mutations that either open or constrict chromatin at a locus cause parallel changes in the frequency of DSBs (Fan *et al.*, 1995).

The critical timing and mechanisms of recombination events clearly have not been elucidated as yet, and it is not even certain that DSBs are the initiators of recombination. Additional observations have demonstrated that homologs interact long before DSBs are formed (Weiner and Kleckner, 1994), and it is not clear when the resolution of recombinational events is complete. Mature recombinants can be detected at, or immediately after, the end of pachytene (Padmore *et al.*, 1991).

Aside from the many unresolved mechanistic issues, significant progress is being made regarding identifying genes involved in meiosis. There is a growing list of meiotic-function genes, mostly from studies in yeast. It is hoped that this will initiate the identification of mammalian homologs, but few have been identified so far.

Yeast Rad51 protein is a molecular homolog of RecA protein, and has been shown to be involved in the repair of double-strand breaks in meiosis (Shinohara *et al.*, 1992). The mouse Rad 51 homolog exhibits high levels of expression in testis, ovary, thymus and spleen suggesting it may be involved in recombination events of meiosis, and antibody formation and class switching in somatic cells (Shinohara *et al.*, 1993). Immunolocalization studies in spermatocyte and oocyte preparations suggest that Rad51

protein is functionally involved in multiple steps in meiosis from before synapsis through metaphase I (Ashley *et al.*, 1995).

PMS2, another of the mouse meiotic genes that has recently been identified has greatest homology to the yeast *PMS1* gene which is a *mutL* homolog, a gene important in the correction of replication errors in *E.coli*. In yeast, mismatch repair defects result in failure to correct mispairs in heteroduplex DNA, which affects genetic recombination processes (Petes *et al.*, 1991), and is associated with low meiotic spore viability (Williamson *et al.*, 1985). Using gene targeting in embryonic stem cells Baker *et al.*, (1995) created *PMS2*-deficient animals. These animals were more prone to sarcomas and lymphomas, and in addition, males were infertile, producing only abnormal spermatozoa. The analysis of SC formation during prophase I of meiosis indicates abnormalities in chromosome synapsis. These observations suggest strong links between mismatch repair, genetic recombination and chromosome synapsis in meiosis.

The human *XRCC1* gene has recently been shown to be involved in DNA strand-break repair, and a study was recently undertaken to characterize the expression of *Xrcc-1*, the mouse homolog. Enriched populations of pachytene spermatocytes and round spermatids displayed significantly higher levels of *Xrcc-1* expression than other mouse tissues. The relatively high levels of expression identified in male germ cells indicate Xrcc-I may have an important role in male germ cell physiology, and may have specific meiotic function (Walter *et al.*, 1994).

Investigating the Events of Meiotic Prophase I

Spermatogenesis as a System to Study Meiosis

Spermatogenesis provides an excellent model system for the study of meiosis since

in mammals meiosis I is more easily studied in males than in females for several reasons. Spermatogenesis occurs repeatedly during the adult life of males. In contrast, oogenic meiosis is initiated synchronously in all germ cells during fetal development and is discontinuous; oocytes arrest at diplotene, until released from this stage by ovulatory hormonal controls at puberty when they resume meiosis. The developmental biology of testicular development through puberty is well established in the mouse, and this facilitates the isolation of developmentally staged testes with only certain stages of germ cell development represented. In addition the isolation from both adult and juvenile mice of large numbers of highly enriched populations of meiotic cells is possible. This has enabled investigations of short term cultures of spermatocytes and inquiry into the controls and timing of spermatogenic progression. Another important feature of spermatogenesis in the mouse is that, compared to many other organisms, the quality of cytogenetic preparations for visualization of the meiotic events of SC formation and chromosome and chromatin behavior is general excellent .

An additional advantage exploited here is that mammals exhibit a sexual dimorphism in meiotic chromosome behavior; in oocytes the sex chromosomes have a euchromatic conformation and are transcriptionally active while in spermatocytes the sex chromosomes are heterochromatic and transcriptionally inactive.

The Behavior of the X-Y Chromosome Pair in Meiotic Prophase

An interesting feature of meiotic prophase in male mammals is the behavior of the sex chromosomes during this stage. By the pachytene sub-stage, the X and Y chromosomes assume a chromatin configuration strikingly different from that of the autosomes, a conspicuous structure termed the sex vesicle or X-Y body (Solari, 1989; Handel and Hunt, 1992). Although the biochemical nature of this chromatin configuration

of the X-Y body is not known, it appears morphologically different from that of the autosomal chromosomes in all histological preparative techniques. This suggests fundamental differences in the packaging of the sex and autosomal chromosomes during this stage. There are also differences in the behavior of the sex chromosomes and the autosomes. During pachytene the autosomes are fully paired whereas the X and Y chromosomes are paired only in a short homologous terminal region, the pseudoautosomal region. Genetic recombination is restricted to this region. In addition, the autosomal chromosomes are transcriptionally active while the X-Y pair become transcriptionally inactive during this time.

Although cytological and autoradiographic observations suggest that the sex-chromosomes are inactive in the pachytene stage, the time of onset of inactivation is not known. It is not known for certain whether all genes on the sex chromosomes are inactivated, and how, or why they should be inactivated in the first place. It has been shown, however, that the sex body is first evident late in the zygotene stage of prophase (Monesi, 1965).

The reason for these structural and functional differences between the sex chromosomes and the autosomal chromosomes is not known. McKee and Handel (1993) have suggested that the special behavior of the sex chromosomes during male meiosis is related to the recombinational status of these chromosomes. The differential condensation is proposed to be a meiotic adaptation to prevent the initiation of potentially damaging recombination events in the differentiated X and Y chromosomes. The transcriptional inactivity of the X-Y body is viewed then as a consequence of chromosome condensation rather than as a requirement for proper progression of spermatogenesis as previously postulated (Lifschytz and Lindsley, 1972).

The consequences of temporary X-chromosome inactivation are far-reaching for

spermatocytes, and would seem to be of considerable "inconvenience" for spermatocytes as the X-chromosome encodes several proteins with housekeeping functions that are presumably required by all metabolically active cells. Several strategies have been suggested and investigated for maintenance of important proteins, such as the stabilization of protein product, or the specific use of an autosomally encoded back-up gene. It may also be possible that some X-linked genes escape X-chromosome inactivation during spermatogenesis, although this has not been demonstrated, as it has for X-chromosome inactivation in somatic cells (Goodfellow *et al.*, 1984). Genetic investigations have suggested that X-Y body formation is not an intrinsic property of the pairing of sex chromosomes as absence of a pairing partner or interruption of the sex chromosome structure do not affect sex body formation. Its formation may be controlled by factors originating from the autosomes, or from a function of the environment that surrounds the meiotic cell (Handel *et al.*, 1994).

Little is known about the mechanism of sex-chromosome inactivation during spermatogenesis but the mechanisms involved in X-Y body formation appear to be different than those controlling X-chromosome inactivation in somatic cells of female mammals. Mammals compensate for different doses of X-chromosome linked genes in male (XY) and female (XX) somatic cells by terminally inactivating all but one X chromosome in each cell. A transiently inactive X chromosome is also found in germ cells, specifically in premeiotic oogenic cells and in meiotic and postmeiotic spermatogenic cells. The *Xist* (X-inactive specific transcript) gene which is involved in X-chromosome inactivation is expressed from the inactive X-chromosome in female somatic cells. It has also been shown to be expressed in germ cells of both sexes, but only at those stages when an inactive X chromosome is present (McCarrey and Dilworth, 1992). It had been suggested that, *Xist* as a regulator of X-chromosome inactivation, may also function as the

mechanism by which X inactivation is initiated or maintained in germ cells as well. Although McCarrey and Dilworth (1992) report the expression of *Xist* in the mouse testis around the time of X-Y chromosome condensation, others have since reported that *Xist* expression in mouse testis is very low, and does not fit temporally with X-Y body formation (Kay *et al.*, 1993). Also, in contrast to the situation in somatic cells where DNA modification in the form of DNA methylation is involved in transcriptional inactivity, methylation events do not appear to be involved in sex chromosome inactivation in male meiotic cells (Driscoll and Migeon, 1990). However, recently a 51kD protein was identified in spermatogenic cells that was shown by antibody localization to be localized to the whole X-Y body and the centromeric heterochromatin of autosomes (Smith and Benavente, 1995). They speculate that it may be involved in a mechanism rendering chromosomal regions inaccessible for transcription and/or recombination events.

Chromatin Configuration During Meiosis

The size and complexity of eukaryotic DNA has demanded stringent structural organization of the genetic material, which has to meet two basic requirements: 1) The DNA must be able to be packaged, at times, into a very compact form, as in metaphase chromosomes. 2) The DNA must be accessible to various proteins and enzymatic machinery to enable replication, transcription, and recombination to take place and at times for the DNA to be repaired.

A phenomenon which is poorly understood is the mechanism by which chromatin is packaged and maintained in different conformations often broadly described as "inactive" heterochromatic regions, as seen in the X-Y pair of the sex body, or "active" euchromatic regions. It has been well established that active chromatin, or potentially active chromatin is preferentially sensitive to digestion by nucleases (Hutchison and Weintraub, 1985). In

addition to this general nuclease sensitivity in the transcribed regions, there are chromatin regions that are up to 10 times more nuclease-sensitive, usually defined as DNase hypersensitive regions (Kuo and Plunkett, 1985; Gross and Garrard, 1988). A number of studies have investigated the gross chromatin configuration of interphase and condensed metaphase chromosomes, as well as the chromatin configuration of meiotic chromatin, using the method of DNase sensitivity.

The gross structural features of pachytene chromatin have shown that the nucleosomal repeat lengths of 196 bp are identical with those of interphase chromatin, but interestingly, under identical conditions of digestion, pachytene chromatin was 25% more accessible to DNase I than interphase chromatin, suggesting a loosened higher order structure for pachytene chromatin (Rao *et al.*, 1983; Rao and Rao, 1987). In another study investigating the DNase hypersensitivity of spermatogenic cells, McPherson and Longo (1992) used in-situ nick translation techniques to determine the localization and degree of nuclease sensitive regions. They find that the incorporation of radioactive nucleotides at nuclease nick sites was related to nuclear area and DNA content, suggesting that functional and structural modifications in chromatin were greatest in pachytene spermatocytes. The transcriptionally inactive mature spermatozoan nuclei was DNase insensitive under any of the conditions tested, although the chromatin of mature sperm lacks nucleosomes. This lack of hypersensitivity undoubtedly reflects the condensed packaging of the chromatin distinctive of this particular cell.

Another interesting finding is that a significant fraction of pachytene chromatin is associated with poly-ADP-ribosylated nuclear proteins, and this fraction of DNA has a high number of internal strand breaks (Satyanarayana and Rao, 1989). Poly-ADP-ribosylation of nuclear proteins is thought to be directly related to DNA repair (Smerdon 1991). It is known that a substantial fraction of the genome is subject to repair synthesis in pachytene-

stage spermatocytes; this fraction of the genome is called P-DNA (for review, Stern, 1990). The function of P-DNA repair synthesis is not clear, but it also is thought to be related to recombination. The fraction of DNA in the mouse undergoing pachytene repair, has been shown to be highly susceptible to DNase digestion (Stubbs and Stern, 1986), and has a high frequency of internal strand breaks.

Chromatin Configuration and Recombination

A key question of interest then in this study is to investigate the possible links between chromatin configuration and meiotic recombination. Homologous recombination resulting in the exchange of genetic information between homologous chromosomes, serves three principal functions: 1) Recombination generates new combinations of alleles, which may have an evolutionary advantage. 2) In most organisms recombination is required for the proper segregation of chromosomes at meiosis I (Hawley, 1989). 3) Recombination is used to repair certain types of DNA damage (Szostak *et al.*, 1983). Although the frequency of recombination between any two genetic markers is approximately proportional to the physical distance between them, some regions of chromosomes - recombination hot spots - undergo higher rates of recombination than others (reviewed by Lichten and Goldman, 1995). In eukaryotes, hot spots of recombination have been identified from yeast (Gutz, 1971) to humans (Steinmetz *et al.*, 1987). Because recombination hot spots presumably circumvent some rate-limiting steps of recombination, elucidating how hot spots function has become a major target for understanding how, and where recombination occurs.

It is a crucial and still poorly understood issue how and where recombination sites are determined in meiotic cells, but the evidence seems to point more toward the modification of the chromosomal substrate in some way. This does not exclude the

possibility at all, that some recombinational sites are modified by binding of proteins to specific DNA sequences, and Wahls and Smith (1994) describe the purification of just such a protein that binds to the *M26* allele of the *ade6* gene in *S. pombe*, and identification of its specific DNA recognition sequence. The state or conformation of chromatin at a "higher than local sequence level" is considered to be the most likely controlling factor in determining the accessibility of recombinational machinery to initiation sites. The state of chromatin is known to play a role in the target site accessibility in yeast mating-type switching (Nasmyth, 1982) and is invoked in the initiation of recombination at the mouse Eß meiotic recombination hot spot (Shenkar *et al.*, 1991) as well as in switch-site activation in immunoglobulin gene rearrangement (Burton and Vitetta, 1990). In yeast it is suggested that the level of recombination at a certain position is governed by local chromatin conformation (Nicolas and Petes, 1994). Furthermore recent studies by Wu and Lichten (1994) and Ohta *et al.* (1994) indicate that the features of chromatin structure that are established before meiosis play a role in determining where meiotic recombination events are initiated.

In mammalian meiotic cells, apart from a few known recombinational hot spot regions, it has not been possible yet to identify DSB sites and recombination sites. At a higher order level though, it is possible to investigate the positioning of chiasmata, the resultant structure of a recombinational event, and to determine the DNase sensitivity of specific chromosomal regions. The distribution of crossovers/chiasmata has been demonstrated by both genetic and cytological experiments to be position dependent rather than DNA-sequence dependent.

Heterochromatic regions of the genome are usually largely devoid of chiasmata (Loidl, 1994), and the exclusion of recombinatory events from heterochromatin or other specific chromatin regions is most likely attributed to a particular chromatin conformation

which prevents the access of recombinatory enzymes. The extraordinary heterochromatic chromatin conformation of the X-Y pair during male spermatogenesis is proposed as a mechanism to avoid ectopic recombination (McKee and Handel, 1993).

The important meiotic events of genetic exchange and chromosome behavior already described here lead on into the the first meiotic division and resultant segregation of homologs. Many of the manipulations required for the investigation of these events, and in particular, the control and timing of these events require that we isolate meiotic cells and culture them. The primary culture of spermatocytes has been important in development of this work.

The Culture of Spermatocytes

It is clear that Sertoli cells play a vital role in the development of germ cells, and numerous studies using isolated populations of cells have investigated the interactions between Sertoli cells and germ cells. Evidence points clearly to the fact that Sertoli cells provide the right environment for spermatogenic development, but may also provide a specific architecture within which germ cells develop.

It is not surprising that spermatocytes lose viability without the presence of the "nurse" Sertoli cells, or that purified spermatocyte cultures are defined as short-term events. Attempts at *in vitro* culture have involved a number of strategies. Parvinen *et al.*, (1983) reported the completion of meiosis and initiation of spermatogenesis in incubated seminiferous tubular segments, and approaches similar to this in other organisms have also been successful. Another method that has had moderate success has been to co-culture germ cells with Sertoli cells. Early reports using mixed populations of germ cells with Sertoli cells reported meiotic progression for periods of 7-10 days (Dietrich, 1983). Spermatocyte-Sertoli co-culture systems have many variations (Tres and Kierszenbaum,

1983; and reviewed in Kierszenbaum, 1994) including the use of an immortalized Sertoli-like cell line 15P-1 (Rassoulzadegan *et al.*, 1993). None of these methods supports full spermatogenesis although transmeiotic differentiation is reported in some, but all of these methods suffer from the same problem: analysis of spermatogenesis and meiosis is complicated by the presence of a "contaminating" somatic cell type - the Sertoli cell.

Potentially one of the most interesting advancements for spermatogenic meiosis *in vitro* was the development of transformed germ cell lines reported by Hofmann *et al.* (1994). They established two germ cell lines by cotransfecting primary mouse testicular germ cells, preleptotene spermatocytes, with the simian virus 40 large tumor antigen gene and the gene coding for a temperature-sensitive mutant of p53. They reported that both cell lines expressed germ cell-specific enzymes, and gave rise to cells with a haploid DNA content and the morphological and biochemical features of round spermatids, although no studies were done on the critical cytological features of SC formation and pairing and segregation. It is not clear that these cells were undergoing meiotic differentiation, and other groups using these cell lines have reported abnormalities not consistent with *in vitro* meiosis, the lack of expression of appropriate marker genes, and failure to detect haploid cells. (Wolkowicz and Herr, 1995). As yet no one has identified a satisfactory *in vitro* culture system for developing spermatogenic cells, it is a somewhat intractable problem without the associated Sertoli cells being present, as in the testis. Previous analyses of purified or enriched pachytene, or earlier meiotic prophase spermatocytes in culture have not focused on the unique cytogenetic events of meiosis, as in this study.

Significance and Specific Aims

Many of the complex processes that occur during meiosis, particularly mammalian meiosis, are not well understood. Little is known about the control, regulation and timing of the processes of homology recognition, chromosome pairing and recombination, and although the chromatin structure changes dramatically during prophase I, it is not understood how this relates to meiotic function. Research into the events of meiosis at the molecular level rather than the genetic level has grown enormously in recent years, mainly in yeast where the availability of large numbers of meiotic mutants has allowed the ordering and characterization of some of the events of meiosis. In mammals these important events are most easily studied in male gametogenic cells, the spermatocytes. Some of these events have been investigated within the aims of this research, by the following goals:

- The investigation of chromatin configuration and chromosome behavior during the pachytene stage of spermatogenesis, and how this relates to meiotic function (Section II).
- The development of primary cultures of spermatocytes which are specifically used to monitor and examine meiotic events (Section III).
- An investigation of the cell cycle controls involved in the G2/M meiotic progression (Section IV).

The unique behavior of the sex chromosomes during this transition is the focus for section II. The hypothesis proposed by McKee and Handel (1993) that this behavior of the sex chromosomes is a mechanism to prevent recombination between non-homologous chromosomes and that acquisition of a different chromatin structure is the means by which this occurs. The analysis of the conformation of chromatin in both autosomes, and sex chromosomes was undertaken using DNase sensitivity (DS) assays. Examining DS in

pachytene cells has allowed a comparison of chromatin that is recombinationally active in the autosomes with chromatin that is recombinationally inactive in the sex body. The results of these experiments demonstrate that sequences located on the X-chromosome are indeed generally more nuclease resistant,

Techniques isolating nearly pure populations of germ cells at relevant stages of meiosis have already been well developed, but few have used the resulting cells for the purpose of the study of meiotic events. This study describes short-term culture methods and the specific assessment of chromosome pairing and synapsis as a measure of the continuity of meiotic processes during culture. It is also shown that these cultures can be used for the investigation of agents that might control chromosome structure and function during meiotic prophase, as well as for agents that are suspected mutagens, particularly meiotically acting aneuploidogens or clastogens. The results of this work (section III) have led to the continued use of these primary cultures to investigate the timing and biochemical events of the G2/ M transition (section IV).

One of the agents used in cultured cells was okadaic acid (OA), a protein phosphatase inhibitor. Treatment of spermatocytes with OA leads to a premature and rapid onset of metaphase I. Biochemical analyses have shown that during this transition, at least some of the universal cell cycle signals are involved. Treatment with OA seemingly abrogates a checkpoint that normally holds spermatocytes in prophase for a few more days. Since OA inhibits several protein phosphatases and perturbs protein phosphorylation, the phosphorylation status of critical proteins may be an important element of spermatogenic meiosis cell cycle control. In addition to these findings, this procedure of induced metaphase transition is now being used to investigate the timing of when spermatocytes become competent to progress with this transition. That is, what are the determining factors for progression to the first metaphase. Are they primarily genetic - the completion

of recombination events, or are the presence, or status, of cell cycle proteins the primary determinants of this prophase-metaphase transition?

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PART II

An assessment of meiotic chromatin configuration and implications for meiotic function in mammalian spermatocytes*

* This chapter is intended to be submitted for publication as Wiltshire, T., Park, C., and Handel, M.A. (1996), however, additional experimental results will be included in the final manuscript.

ABSTRACT

During the pachytene stage of meiotic prophase in male mammals, the X and Y chromosomes become transcriptionally inactive and establish a chromatin domain, the sex body, that is visually distinct from the transcriptionally active autosomes. We sought objective criteria to describe chromatin differences by assessing DNase-I sensitivity (DS) of sex chromosome and autosomal sequences. Nuclei were prepared from pachytene spermatocytes and treated with various concentrations of DNase and extracted DNA was then digested with a restriction enzyme prior to Southern blotting. Blots were hybridized with probes for X-linked and autosomal sequences. Among these were some gene sequences expressed, and some not expressed, in pachytene spermatocytes. There was a wide range of DS in different genomic sequences; however, the X-linked sequences generally show significantly less DS than do autosomal sequences. Interestingly, a hot spot of recombination (Eb) shows a high level of DS while a cold spot of recombination (centromeric satellite region) showed low DS similar to X-linked sequences. We also examined the DS of two transgenic lines of mice, that have the same tyrosinase transgene insert, *TyBs* . In one line, the*TyBs* insert is X-linked, and in the other line it is autosomal. In these mice the same insert sequence shows less DS when X-linked than when autosomal. These results support the hypothesis that in pachytene spermatocytes the chromatin configuration of the X-Y pair in the sex body is more condensed and inaccessible to enzymatic digest, while the autosomal chromatin is in a more open configuration. This may well be related to recombination status. In addition we examined the DS of some of the same genes in the earlier leptotene/zygotene prophase stage, and find that while autosomal-gene nuclease sensitivity remains essentially the same, X-linked sequences are more sensitive, reflecting the obvious cytological changes that occur during this transition through meiotic prophase, when the X-Y pair become condensed.

CHAPTER I

INTRODUCTION

Mammalian sex chromosomes exhibit marked sexual dimorphism in behavior during gametogenesis. The transcriptional, recombinational and pairing behavior, as well as the morphology of the sex chromosomes during meiotic prophase in mammalian males differs dramatically from that of the autosomes and from that of the sex chromosomes during female meiosis. In females the homomorphic X chromosomes pair and participate in recombination over their entire length and both are transcriptionally active with a chromatin conformation indistinguishable from the autosomes. In marked contrast, during male meiosis, pairing and recombination of the heteromorphic X and Y chromosomes is limited to a specific domain, the pseudoautosomal region (PAR). The X-Y pair is transcriptionally inactive (reviewed by Handel, 1987) and assumes a chromatin configuration distinct from that of the autosomes, hence the terminology sex vesicle, sex body or X-Y body (Solari, 1989). The reasons for these structural and functional differences between the sex chromosomes and the autosomal chromosomes during male meiotic prophase is not known. McKee and Handel (1993) suggested that the special behavior of the sex chromosomes during male meiosis could be related to the recombinational status of these chromosomes. The differential condensation is proposed as a meiotic adaptation to prevent the initiation of potentially damaging recombination events in the differentiated X and Y chromosomes. The transcriptional inactivity of the X-Y body is then viewed as a consequence of chromosome condensation rather than as a requirement for proper progression of spermatogenesis as previously postulated (Lifschytz and Lindsley, 1972), and in this view there is no need to postulate that products of the X

chromosome are inhibitory to spermatogenesis (Forejt, 1982).

Little is known about how chromatin conformation relates to meiotic function, although recent studies in yeast have indicated that the state or conformation of chromatin at a "higher than local sequence level" is considered to be the most likely controlling factor in determining the accessibility of recombinational machinery to initiation sites (Nicolas and Petes, 1994). In yeast all DSB sites examined to date have been found to lie in regions of chromatin that are DNase I sensitive (Wu and Lichten, 1995). In contrast, in mammalian meiotic cells so far it has not been possible to identify DSB sites and potential recombination sites, although a limited number of recombinational hotspots have been identified (reviewed by Lichten and Goldman, 1995).

Here we used DNase I sensitivity assays to assess the chromatin conformation both of spatially separated regions of chromatin and of temporally distinct prophase stages. We determined the differences between sex and autosomal chromatin, in both pachytene (P) and leptotene/zygotene (L/Z) substages. Differences are consistent with the potential recombinational status of the chromatin. We also used transgenic lines of mice to examine DS of the same transgene that has been inserted in either autosomal or sex chromatin. Our findings support the idea that during the pachytene stage the recombining autosomal chromatin is generally more nuclease sensitive than is recombinationally inactive sex chromatin, confirming the cytological differences seen in chromatin conformation. At the leptotene/zygotene stage, X-chromatin is more DS than at pachytene, although there is little change in the DS of autosomal chromatin. The possible relationship of these differences in chromatin configuration during meiosis to recombination raises intriguing possibilities about both chromatin function and timing of meiotic events.

CHAPTER II

MATERIALS AND METHODS

Animals

The BALB/c mice used in this study were purchased from Harlan-Sprague-Dawley. XX*Sxr* mice were produced in our breeding colony by matings of XY*Sxr* males to XX or XO females. In a typical breeding scheme, XY*Sxr* males were hemizygous for one of two X-linked markers, Tabby (*Ta*) or Greasy (*Gs*), both which affect coat characteristics, while females were genetically unmarked. XX*Sxr* reversed males were identified by a variegated coat pattern for the appropriate X-linked marker and by smaller than normal testes. Transgenic mice carrying low-copy number inserts of the tyrosinase minigene *TyBS* were obtained from Dr. R Woychik (Oak Ridge National Laboratory). Two lines of transgenic mice were used: Tg3409B, carrying an autosomal insert, and Tg3142A, carrying the X-linked transgene. Transgenic mice have a characteristic mottled grey fur coloration indicating a functional tyrosinase gene. All transgenic mice were confirmed as such by genotyping on Southern blots of tail DNA.

Enrichment of cells

Testes of adult Harlan-Sprague-Dawley mice were used as a source of germ cells. Testis tubules were suspended in EKRB[120.1 mM NaCl, 4.8 mM KCl, 25.2 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM MgSO4.7H_2O, 1.3 mM $CaCl_2$, 11 mM glucose, 1X essential amino acids (Sigma), 1X nonessential amino acids (Sigma)] and digested with collagenase and trypsin as described (Romrell et al., 1976; Bellvé et al., 1977). After tubule digestion, trypsin was removed by washing in EKRB containing 0.5% bovine

serum albumin (BSA), and the single-cell suspension of germ cells was sedimented at unit gravity through a 2-4% BSA gradient generated in a medium-sized STAPUT chamber (Johns Scientific, Ontario). Gradients were collected after 2.5 hours in 10 ml fractions at a rate of 10 ml/45 seconds. Fractions were assessed for morphology and purity by light microscopy using Nomarski optics. Pachytene spermatocytes were isolated from the testes of adult mice (> 8 weeks old) and the average purity of the fractions used was greater than 85%; the principle contaminants were Sertoli cells and symplasts composed of round spermatids. Leptotene/zygotene spermatocytes were isolated from testes of 17 day old mice and the average purity of fractions used was 85-90%; the principle contaminants were spermatogonia and Sertoli cells. Spermatogonia were isolated from testes of 8 day old mice, with purities of ~90%, and contaminants of preleptotene spermatocytes and Sertoli cells. Pooled germ cells were washed once and resuspended in the appropriate buffer for isolation of nuclei and DNase I digestion, or RNA extraction.

Isolation of nuclei, DNase I digestion and Southern blotting

Pachytene nuclei were isolated according to a modification of the method of Wu (1989). Enriched fractions of STAPUT isolated cells were resuspended in Nuclei Buffer 1 (NB1: 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 15 mM Tris-Cl, pH 7.5, 300 mM sucrose, 0.5 mM DTT, 0.1 mM PMSF, 3.6 µg/ml aprotinin), at approximately 2 x 10^6 cells/ml and homogenized (20 strokes in a Dounce homogenizer) on ice. The homogenate was layered over a cushion of Nuclei Buffer 2 (NB2: 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 15 mM Tris-Cl, pH 7.5, 1200 mM sucrose, 0.5 mM DTT, 0.1 mM PMSF, 3.6 µg/ml aprotinin) in Corex tubes, and centrifuged at 8000 x g for 20 minutes, in a swinging bucket rotor. Nuclei pellets were resuspended in DNase I digestion buffer (85 mM KCl, 1 mM $CaCl_2$, 5 mM PIPES buffer pH 7.5, 5% sucrose, 5%

glycerol) at a concentration of 7.5 x 10^5 nuclei/100 µl of buffer. Nuclei were stored at -80°C for periods of up to 5 days. Stored aliquots were thawed and combined, and nuclei concentrations reassessed by microscopy prior to use in the DNase assay.

DNase I (Promega RQ-1 DNase, 1U/ µl) was added directly to aliquots of nuclei held on ice in DNase I digestion buffer at appropriate concentrations, and digestion performed for 15 minutes at 20° C according to the methods of Bellard *et al* (1989). Stop buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 15 mM EDTA, 0.3% SDS) was added, and the lysed nuclei were treated with DNase free 50 µg/ml RNase(Promega) for 1 hour at 37° C, and then with 50 µg/ml Proteinase K (Promega) overnight at 37° C. Purified DNA was prepared using standard methods of phenol/chloroform extraction and ethanol precipitation (Sambrook *et al.* 1989). DNA was quantitated using a Beckman DU650 spectrophotometer. Undigested genomic DNA was also prepared by the same methods from mouse liver.

DNA samples were electrophoresed on 1% agarose gels, using TBE buffer overnight at 25-30 volts. Gels were stained with ethidium bromide, photographed, and then DNA was transferred to nylon membrane (Hybond+, Amersham) using a vacuum blotter (Pharmacia, Piscataway, New Jersey) and crosslinked to the nylon membrane with a UV Stratalinker (Stratagene).

Isolation of RNA and Northern blotting and hybridization

RNA was isolated by the method of Cathala *et al.* (1983). Enriched fractions of STAPUT cells were homogenized in lysis buffer (5 M guanidinium isothiocyanate, 10mM EDTA, and 50 mM Tris-Cl, pH 7.5) by passing the sample through a 21-gauge needle 20 times. The RNA was precipitated overnight in 7 volumes 4 M lithium chloride at 4° C. Samples were centrifuged for 90 minutes at 10,000 x g and the pellets resuspended in RNA

solubilization buffer (10 mM Tris-Cl, pH 7.5, 1mM EDTA, 0.1% SDS) and treated with 40 μg/ml proteinase K for 30 minutes at 37° C. The samples were then extracted with phenol:chloroform (1:1), followed by chloroform. Following precipitation with 0.2 M sodium acetate, pH 5.2 and 2 volumes 95% ethanol, RNA was pelleted by centrifugation and quantitated spectrophotometrically.

Gels for Northern blotting were run using standard methods (Sambrook *et al.*, 1989) in order to verify RNA integrity. RNA was denatured at 68° C in 50% formamide for 15 minutes prior to electrophoresis. The 1% agarose gels contained 2.2 M formaldehyde and 1X MOPS buffer [3-(N-morpholino)propanesulfonic acid] and were run at 5 volts/cm^2 for 2.5 hours. Following electrophoresis gels were stained with ethidium bromide and RNA integrity was evaluated by the presence of the 28S and 18S ribosomal RNA bands. RNA was transferred to nylon membrane (Hybond+, Amersham) using a vacuum blotter (Pharmacia, Piscataway, New Jersey) and then crosslinked to the nylon membrane with a UV stratalinker (Stratagene). Prehybridization of blots was carried out at 42°C for 1 hour with 50% formamide, 5X SSC, 5X Denhardt's solution, 50 mM potassium phosphate buffer, pH 7.4, and 250 μg/ml denatured fish sperm DNA. Hybridization of Northern blots was performed in prehybridization solution with 10% dextran sulfate and 1-2 x 10^6 cpm/ml of denatured radioactive DNA probe at 42°C overnight. Washing consisted of two 42°C washes with 1X SSC, 0.1% SDS for 15 minutes, and two washes at 65°C in 0.1X SSC for up to 15 minutes each. Blots were exposed to X-ray film (XR-100, Sterling X-ray film) at -80°C, for up to 72 hours.

Probe preparation and Southern hybridization

Although the probes were in a variety of plasmid vectors, standard molecular biology techniques of plasmid isolation, and purification using Nucleobond AX 100

columns (Macherey-Nagel) were used in all cases. One exception was Zfy-2 where the probe was prepared by PCR from the 5' end of the cDNA sequence using primers designed using the Primer 2.2 program (Whitehead Institute), and supplied by Bioserve Biotechnologies (Laurel MD.). All probes used in both Southern and Northern hybridizations were radiolabeled with deoxycytidine 5'-[α-^{32}P]triphosphate (New England Nuclear, dCTP[^{32}P]) by random primer labeling (Feinberg and Vogelstein, 1983). The unincorporated nucleotides were separated from the labeled probes by gel filtration chromatography on Sephadex G-50 using 0.45 μm nylon microspin columns from PGC Scientifics (Gaithersberg, Maryland). The average specific activity for probe labeling by this method was 1 x 10^9 cpm/μg DNA. Blots were prehybridized with: 6X SSC, 0.5X Denhardt's solution, 0.5% SDS, and 100 mg/ml denatured fish sperm DNA, for 1 hour at 68°C. Radiolabeled probe was added at a concentration of 10 ng per ml of hybridization solution. Following an overnight hybridization, excess radioactivity was washed from the blots with two low stringency washes at room temperature; the first consisted of 2X SSC, 0.5% SDS, for 5 minutes, and the second of 2X SSC, 0.1% SDS for 15 minutes with shaking. Two higher stringency washes were performed with 0.1X SSC, 0.5% SDS; the first was at 37°C for 30 minutes, and the second was at 68°C for 15-30 minutes. Blots were placed on X-ray film (XR-100, Sterling X-ray film) for 24-96 hours at -80°C with intensifying screens.

Densitometry

Laser densitometry was performed with a Molecular Dynamics 300 series computing densitometer and data analyzed with Imagequant software. The data obtained

from this analysis were expressed as densitometric intensity units, determined from the area of the integrated curve as measured for each band and the resultant DS was expressed as the percentage loss of intensity, i.e., the percentage difference between the most intense band (no DNase I) and least intense band (most DNase I).

CHAPTER III

RESULTS

DNase sensitivity of pachytene chromatin

One of the critical, but difficult factors in comparing DS of different sequences is to ensure that they are compared under similar conditions. For assessment of all pachytene sequences enough nuclei were prepared such that the nuclease digest was undertaken with relatively large aliquots of nuclei. To accomplish this, nuclei were frozen, stored, and then pooled with additional fractions of pachytene spermatocytes. In this manner, aliquots of extracted DNA from a single DNase I digest could be treated with different restriction enzymes, electrophoresed, and Southern blotted. All probes were hybridized to either one of two Southern blots prepared under identical conditions of DS treatment, one blot of DNA treated with EcoR1 and the other of DNA treated with BamH1. Probes were previously hybridized to genomic Southern blots digested with a range of restriction enzymes (data not shown). EcoR1 and BamH1 digests were selected because all probe sequences used in these experiments exhibited distinct patterns of one or two bands with one or both of these enzymes.

A number of different probes were used to assess DS of sex chromosomes and autosomal chromosomes from pachytene spermatocytes. Typical hybridization patterns on Southern blots are shown in Figure 1 for the autosomal sequences *Topo 2* and *Ldh-c*, and in Figure 2 for the X-linked sequences *Hprt* and *Timp*. The bands were scanned by densitometry and individual band intensity expressed as a percentage of the maximum intensity of hybridization when no nuclease was present (Lane 1 in each case). Where two

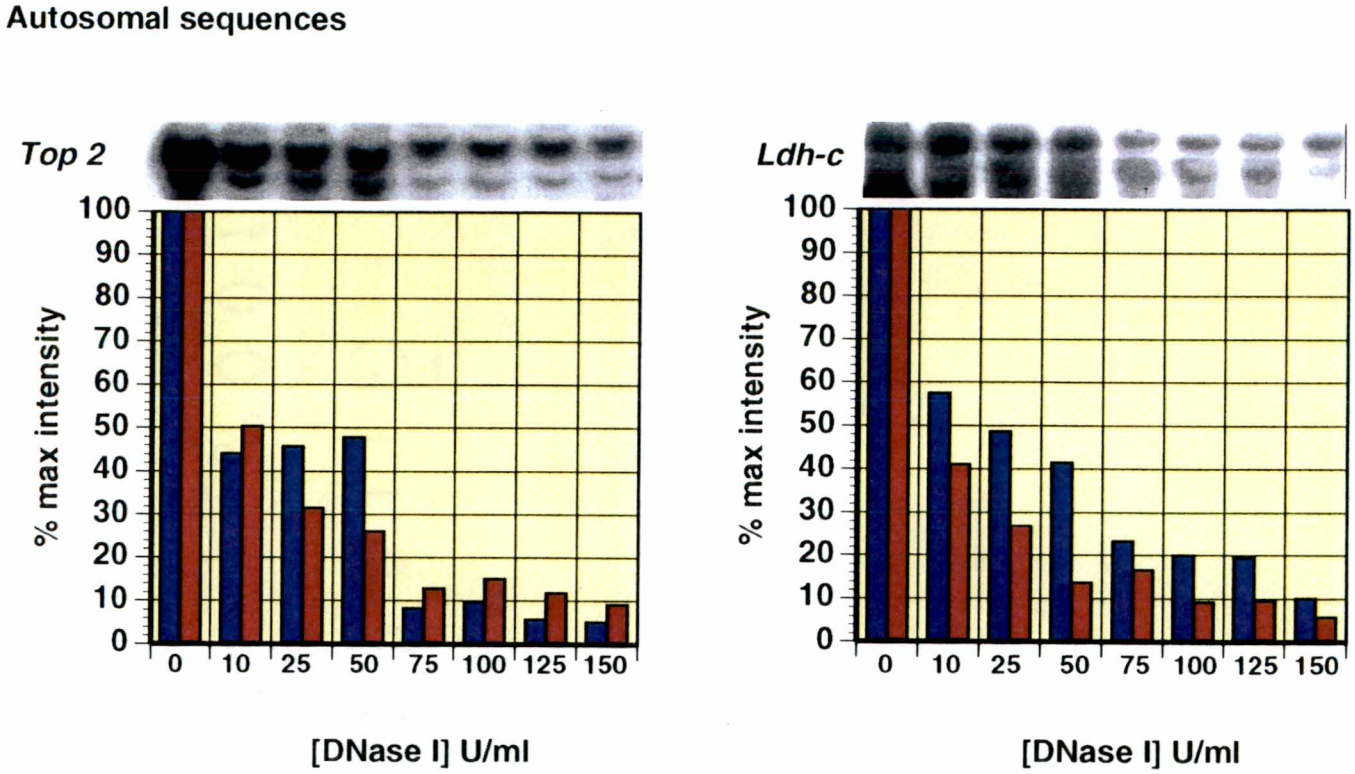


Fig 1. DNase sensitivity of two representative autosomal sequences *Top 2* and *Ldh-c*. *Top 2* and *Ldh-c* probes were hybridized to a Southern blot of BamH1 digested pachytene genomic DNA. Nuclei had previously been digested with 0-150 U/ml of DNase I, the DNA extracted, electrophoresed on a 1% agarose gel and transferred to nylon membrane. The bands in the autoradiogram were scanned by densitometry and expressed in the corresponding graph as a percentage of the maximum intensity. Two sets of bands, ■ (top band) and ■ (bottom band) were scanned for each sequence.

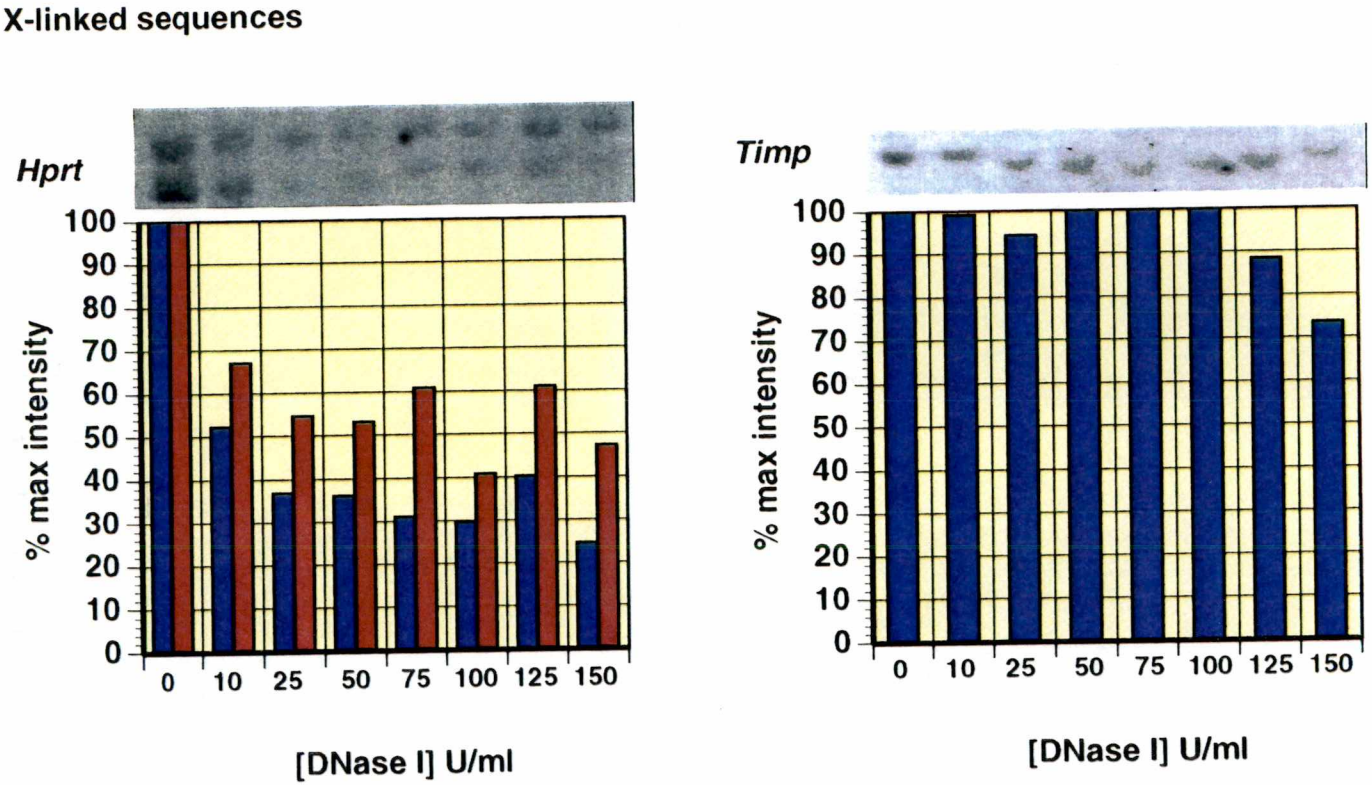


Fig 2. DNase sensitivity of two representative X-linked sequences *Hprt* and *Timp*. *Hprt* and *Timp* probes were hybridized to a Southern blot of BamH1 digested pachytene genomic DNA. Nuclei had previously been digested with 0-150 U/ml of DNase I, the DNA extracted, electrophoresed on a 1% agarose gel and transferred to nylon membrane. The bands in the autoradiogram were scanned by densitometry and expressed in the corresponding graph as a percentage of the maximum intensity. Two sets of bands, ■ (top band) and ■ (bottom band) were scanned for *Hprt* and one set for *Timp*.

bands were present, both were scanned as shown for *Topo II* , *Ldh-c* and *Hprt.* Table I summarizes the DS data for all probes used in pachytene chromatin analysis. The differential DS is expressed as the percentage difference in band intensity between the bands where no DNase was present, and the bands at the maximal concentration of DNase.

There is a wide range of DS for the autosomal sequences tested, and some variability in X-linked sequences as well. However, in general, the X-linked sequences were consistently more resistant to nuclease digestion. The average percent decrease in band intensity for autosomal gene sequences tested was 77%, as compared to an average of 39% decrease in band intensity for gene sequences on the X and Y chromosomes. Tcp-10 appears to be an exception to the generally higher DS of autosomal sequences, and while there is a band which shows greater resistance to nuclease digestion, this sequence also has a DNase hypersensitive site (DHS), that is, a band digested at the lowest DNase concentration. This feature of DHS was only observed for autosomal genes, and not X-linked genes.

Since we were interested in the possibility that our DNase I assay was defining chromatin regions that are simply transcriptionally active, the expression pattern for all genes studied was determined by hybridization to meiosis-cell specific Northern blots as in Figure 3. Those probes representing non-coding regions were also hybridized to the Northern blots. The expression patterns of all sequences used are summarized in Table 1. While most are not expressed in the P or L/Z stage, *Ldh-c* and *Topo 2* are expressed. *Ldh-c* is expressed in P but not in L/Z, and *Topo 2* is expressed in both. Similar analyses of *Igf-2* (Figure 4), which is not expressed in prophase, and other non-expressed genes (Table 1) clearly indicate that the DS we have measured is not determined solely by transcriptional status.

We also tested DS for sequences known to recombine and not recombine. Little is

Table 1. Meiotic Cell DNase Sensitivity for Selected Sequences

Gene /sequence	Location	Expressed in P[4]	Expressed in LZ[4]	Hypersensi -tive region[5]	DS - % [6] decrease in P band intensity	DS - % [6] decrease in LZ band intensity
H19	autosomal	-	+	+	65	
Top 2	autosomal	+	+	-	93	85
Igf-2[1]	autosomal	-	-	+	86	74
Igf-2[2]	autosomal	-	-	+	93	
Igf-2[3]	autosomal	-	-	-	84	
Prm-1	autosomal	-	-	+	84	
Ldhc	autosomal	+	-	+	92	
Tcp-10	autosomal	+	-	+	17	
Eb (MHC)	autosomal	-	-	+	85	
Hprt	X-linked	-	+	-	65	83
Timp	X-linked	-	-	-	26	74
Otc	X-linked	-	-	-	56	
Zfy-2	Y-linked	-	-	-	10	
pMCK104	mouse minor satellite sequence	-	-	-	86	
pBSAT-1	mouse major centromeric satellite sequence	-	-	-	47	
70-38	X-linked satellite sequence	-	-	-	62	

[1,2,3] Three regions of *Igf-2* were assessed for DNase sensitivity; [1] a region 5 kb upstream from the promoter, [2] the promoter region, and 5' end of the gene, and [3] a 3' region of the gene.

[4] All sequences were assessed for transcript expression by Northern blotting procedures

[5] Hypersensitive regions represent those hybridized bands which disappear in the presence of low concentrations of DNase.

[6] The % decrease in band intensity is the percentage loss of band intensity between the zero DNase band and the 150 U/ml DNase band. Where more than one band was scanned an averaged figure is shown.

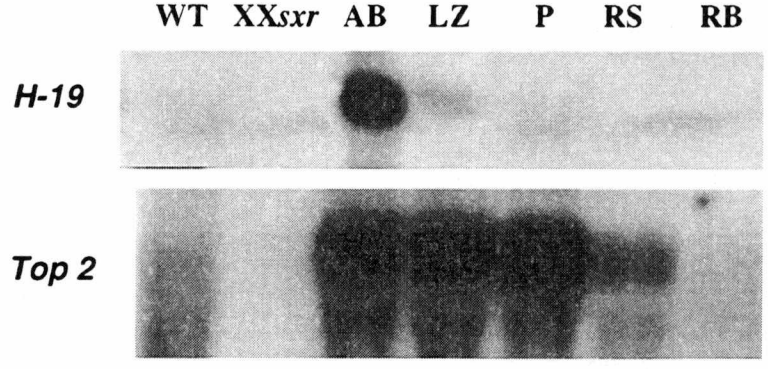


Fig 3. Northern expression patterns for two gene sequences, *H-19* and *Top 2*, expressed during meiotic prophase I. The cell-specific Northern blot has total cellular RNA (15 µg/lane) from, WT - whole testis, XX*sxr* testes, A/B - type A and B spermatogonia, L/Z - leptotene and zygotene spermatocytes, P - pachytene spermatocytes, RS - round spermatids, RB - residual bodies. RNA from these sources was denatured, electrophoresed in a 1% agarose gel and transferred to nylon membrane. Northern blots were probed with all sequences used in this study. Most were not expressed during meiotic prophase.

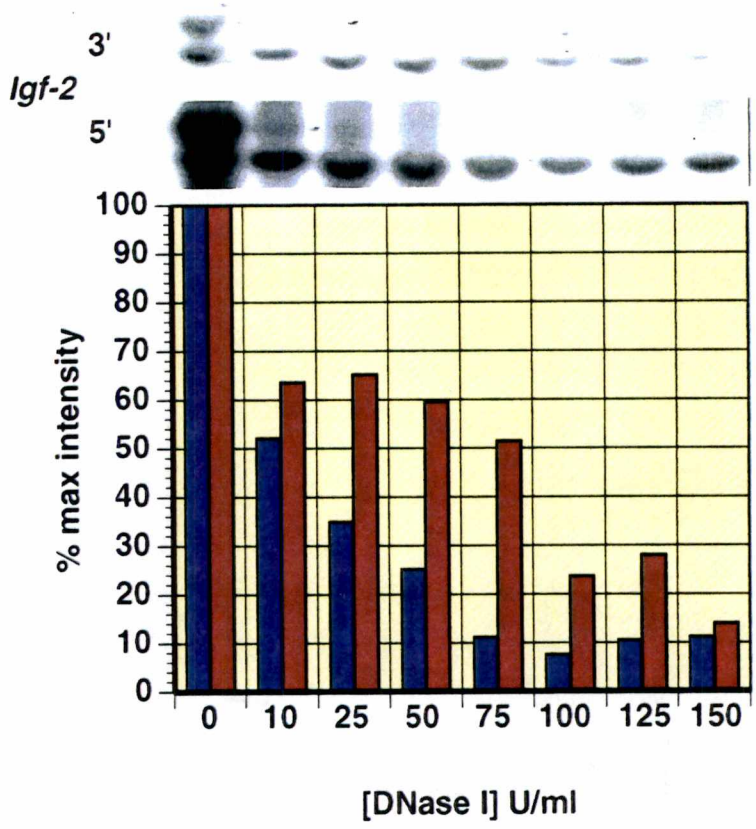


Fig 4. DNase sensitivity of regions of the autosomal sequence *Igf-2*. DS of the 5' region ■, which includes the promoter region of *Igf-2* , is more sensitive to nuclease attack than the 3' region of the gene ■. An *Igf-2* probe was hybridized to a Southern blot of BamH1 digested pachytene genomic DNA. Nuclei had previously been digested with 0-150 U/ml of DNase I, the DNA extracted, electrophoresed on a 1% agarose gel and transferred to nylon membrane. The bands in the autoradiogram were scanned by densitometry and expressed in the corresponding graph as a percentage of the maximum intensity.

known about specific sites of mammalian recombination, but the *Eb* region of MHC is recognized as a hot spot of recombination, at least in some strains of mice (Shenkar *et al.* 1989). Although it has not been shown as a recombination hotspot in BALB/c mice and is not expressed in spermatocytes, this region expresses a high degreee of DS as shown in Figure 5. Two regions of predicted recombinational inactivity, the mouse major centromeric satellite sequence and an X-linked satellite sequence, show low DS, while a mouse minor satellite sequence which has been implicated in recombination (Chandley and Mitchell, 1988) is more DS (Table 1).

Transgenic mice

As an additional test of DS between the two distinct chromatin regions in the pachytene nucleus, the X-Y pair and the autosomes, we examined the DS of the same gene sequence when it is present in one or the other of those regions. The Tg 3142A line of mice have an X-linked tyrosinase transgene insertion and Tg 3049B an autosomal insertion of the same transgene. The same analysis was done (data not shown) using the *TyBs* minigene to probe DNase I digested DNA from these mice. Currently these data show that the X-linked transgene exhibits an equivalent resistance to nuclease digestion as the autosomal transgene, although this result has been complicated by the fact that DNase digests of the autosomal transgene line were not digested to the equivalent level of the X-linked transgene line. These experiments will be repeated when more transgenic mice are available.

DNase sensitivity of L/Z chromatin

The data obtained from pachytene nuclei corroborate the observed chromatin conformation from cytological studies of pachytene spermatocytes in that the X-Y

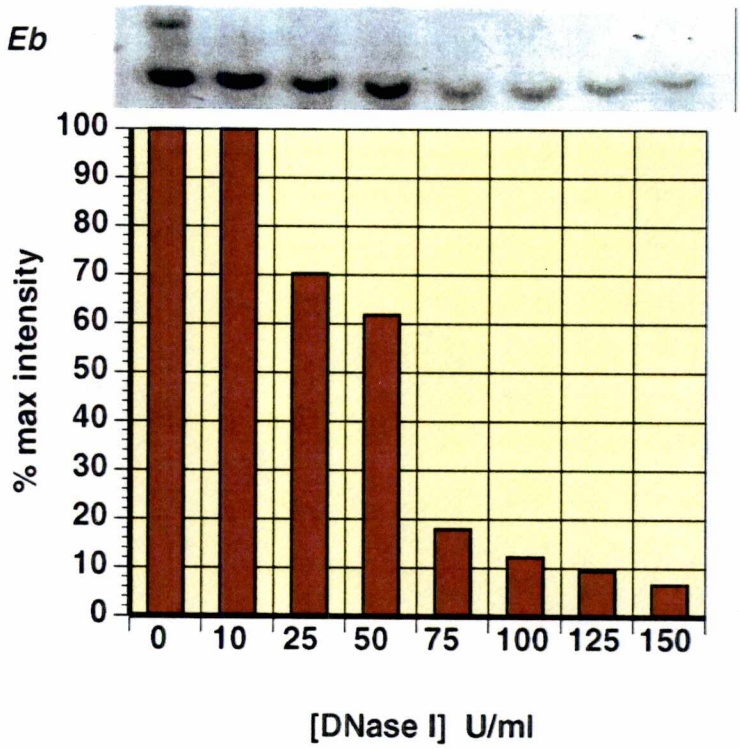


Fig 5. DNase sensitivity of the *Eb* locus, an autosomal sequence. An *Eb* probe was hybridized to a Southern blot of BamH1 digested pachytene genomic DNA. Nuclei had previously been digested with 0-150 U/ml of DNase I, the DNA extracted, electrophoresed on a 1% agarose gel and transferred to nylon membrane.The bands in the autoradiogram were scanned by densitometry and expressed in the corresponding graph as a percentage of the maximum intensity.

chromatin is generally less DS than autosomal chromatin. However, to address the issue of meiotic timing of X-Y chromatin condensation, similar, but more limited studies were undertaken on L/Z spermatocytes. L/Z nuclei were prepared in the same manner as for pachytene nuclei, with the same conditions of nuclease digestion. Typical Southern blots and densitometric scan data are shown for *Hprt* in Figure 6, and other results are shown in Table 1. These data indicate that the DS of autosomal sequences examined does not change much in the transition from L/Z to P but that X-linked sequences may be more nuclease sensitive in L/Z than P. This has been demonstrated in only two cases so far (*Hprt* and *Timp*) and more data is required to confirm this finding.

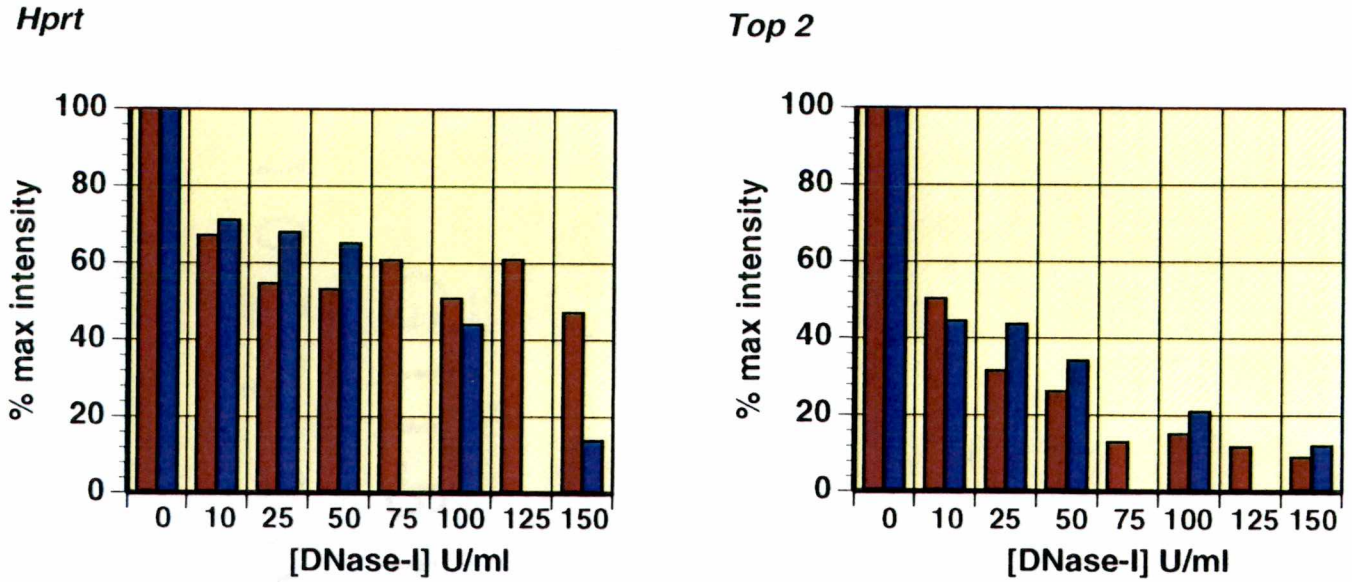


Fig 6. DNase sensitivity of the X-linked sequence *Hprt* and the autosomal *Top 2* sequence determined in spermatocytes in the pachytene stage ■, and during the leptotene/zygotene stage ■. *Hprt* and *Top 2* probes were hybridized to a Southern blot of BamH1 digested pachytene genomic DNA. Nuclei had previously been digested with 0-150 U/ml of DNase I, the DNA extracted, electrophoresed on a 1% agarose gel and transferred to nylon membrane.

CHAPTER IV

DISCUSSION

The sex chromosomes at the pachytene stage of prophase I in male spermatocytes become transcriptionally inactive and appear condensed and dark staining in the sex body. It is not clear why this happens, but inactivation of X-Y chromatin may be a mechanism by which spermatocytes prevent the initiation of potentially damaging recombination events in these non-homologous chromosomes (McKee and Handel 1993). This hypothesis proposes that the heterochromatic conformation of the X and Y chromosomes in spermatocytes prevents promiscuous recombination, and that the diminished transcriptional activity of sex chromosomes in pachytene spermatocytes is a resultant, but secondary, consequence. Further evidence is needed to support or reject this hypothesis.

It is well established that active chromatin, or potentially active chromatin, is preferentially sensitive to digestion by nucleases, such as DNase I in the most widely accepted and utilized assay (Weintraub and Groudine, 1976). Although most studies using DNase I-sensitivity have been directed toward transcriptional activity, the accessibility of DNA to the transcriptional machinery, and the enzymes involved in recombination almost certainly depend on similar features of chromatin conformation. The idea that recombination events are regulated by chromatin conformation is also consistent with molecular studies of recombination hotspots. In both yeast (Stapleton and Petes, 1991) and mice (Shenkar et al., 1991) hot spots for meiotic recombination have been mapped to sites coincident with gene promoters or enhancers. Recent studies in yeast have shown that an important feature of a recombination event is the appearance of a DNA double strand break (DSB), and all DSB sites examined to date have been found to lie in regions of

chromatin that are DNase I hypersensitive (Wu and Lichten, 1995). Mutations that either open or constrict chromatin at a locus cause parallel changes in the frequency of DSB's (Fan et al., 1995).

In this study analysis of the DS of both autosomal and sex-linked sequences revealed that sequences on the X-Y pair were not insensitive to DNase I digestion but were, in general, more resistant than the autosomal sequences examined. This observation raised two questions: first, is DS in pachytene spermatocytes predicted solely by transcriptional activity of sequences tested? Second, is the differential condensation of the sex and autosomal chromosomes related to timing of meiotic events?

The observations reveal that gene sequences that are transcribed in pachytene spermatocytes have a high level of DS. However, they are not the only ones that do so, for some non-transcribed genes in pachytene spermatocytes, for example *H-19* and *Igf-2*, also show quite high DS. Although the *Igf-2* gene is not transcribed in pachytene spermatocytes, a detailed analysis of three regions of the gene showed that the promoter region had a high level of DS, suggesting that it was open to nuclease attack. Thus the assay we used did not simply assess transcriptional activity. It quite possibly reveals transcriptional potential; but, based on yeast studies it also may reveal recombinational potential. That this may be the case is supported by our observations that the *Eb* region, a hotspot of recombination, is nuclease sensitive, while the mouse major centromeric satellite sequence, a region of low recombination rate, is not sensitive. Few regions of the mammalian genome have been designated either as hotspots or as regions with low levels of recombination, and generally little is known about rates of recombination in specific regions. Our observations of nuclease sensitivity of these regions do not yet prove a link to recombination, but the emerging patterns parallel those in yeast. Thus, sites of recombination may be determined by local chromatin structure.

The second question regarding the differential condensation of the X-Y chromatin raises some important issues about the timing of events of meiosis. Information about the timing of the homology search, homolog pairing and the initiation of recombination events comes almost entirely from studies in yeast which indicate that the initiation of recombination events and formation of DSBs occurs very early in meiotic prophase, prior to, or coincident with synapsis (Padmore et al, 1991). If the mouse also follows the same pattern of events, we would predict that the heterochromatin formation of the X-Y pair would occur prior to the initiation of recombination, if condensation exists to prevent non-homologous recombination. However, several lines of evidence suggest that condensation of X-Y chromatin has not yet occurred in mouse zygotene spermatocytes. Cytologically the X-Y pair are not typically seen as a condensed region of the nucleus until pachytene, although it has been demonstrated by some that the sex-body is first evident late in zygotene (Monesi 1965). Transcription is also consistent with this view. Transcript for *Hprt* , an X-linked gene, is present in P (Shannon and Handel, 1992), indicating that the chromatin may still be available (open) for transcription during L/Z. Thus, it is interesting that the limited results we have so far obtained in this study indicate that the sex pair may be more sensitive to nuclease digestion during L/Z than during P. These data would not support the hypothesis that the reason for condensation of the X-Y pair is to prevent recombination if we assume that in mice, as in yeast, recombination is initiated early in prophase, in L or Z cells. However, two factors complicate this conclusion. First, all the analyses that have been completed so far have been done using mixed populations of L and Z cells representing the two meiotic substages where initiation of recombination might occur. Thus, it may well be that the increased nuclease sensitivity that we detect in L/Z X-linked sequences is due not to Z cells but to leptotene chromatin, which might be expected to be more DS, than Z chromatin.

Second, there is no evidence available as yet that events of recombination in mammals do conform to the same time scale of the meiotic events of DSB formation as defined from yeast. In fact, with the considerably larger and more complex genome, and the presence of many repetitive element sequences in the mouse, it may be reasonable to speculate that homologs should be fully paired prior to the initiation of recombination, rather than concurrently, as in the case of yeast, which have simpler genomes without dispersed repeat sequences. Further investigations in this area should allow us to ascertain the validity of the original hypothesis, or may in fact determine that there are some significant differences in the timing of yeast and mammalian meiotic events.

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PART III

Culture of Pachytene Spermatocyes for Analysis of Meiosis*

* This chapter was published in its entirety as Handel, M.A., Caldwell, K.A., and Wiltshire, T. (1995). Culture of Pachytene Spermatocytes for Analysis of Meiosis. Dev Genet. 16: 128-139. This chapter also appeared in the doctoral dissertation of Kim Caldwell as both Tim Wiltshire and Kim Caldwell contributed equally to this research. Inclusion of this chapter in both dissertations was approved by members of both doctoral dissertation committees.

CHAPTER I

INTRODUCTION

Spermatogenesis is comprised of three major phases: mitotic proliferation of the spermatogenic stem cells and spermatogonia, meiosis, and post-meiotic haploid differentiation, or spermiogenesis. Of these three, meiosis most closely defines gametogenesis, for no mammalian cells other than the germ cells undergo meiosis. The unique events of meiosis include the pairing and recombination of chromosomes during prophase of meiosis I, and the segregation of homologous chromosomes during anaphase of meiosis I. In mammals, these important events are more easily studied in male gametogenic cells (the meiotic spermatocytes) since meiosis occurs during adult life. In female gametocytes these events occur during fetal life. However, little is known about the basic mechanisms governing the structure and behavior of chromosomes during mammalian meiotic prophase. Transitions in chromosome structure and function during meiosis are even more dramatic than those that occur in mitosis. In meiotic prophase, chromosomes must engage in a homology search to find their homologs, they must both establish and maintain pairing and synapsis, and they must carry out the steps of recombination. Late in meiotic prophase, chromosomes undergo condensation, and recombination events are resolved as crossovers, cytologically visible as chiasmata, at the end of meiotic prophase. With the end of meiotic prophase comes the transition to meiotic metaphase, with maximal condensation of the chromosomes; at this stage, the chiasmata stabilize the alignment of homologs at the spindle equator, thus ensuring regular segregation of homologous chromosomes from one another during the ensuing anaphase (Hawley, 1988).

The events of meiosis I are all essential for production of chromosomally balanced

gametes; errors in pairing or recombination in meiotic prophase lead to errors in anaphase I segregation, giving rise to aneuploid offspring by the production of chromosomally unbalanced gametes. Mutagens, in particular aneuploidogens and clastogens, can induce such error (for recent review, see Dellarco, 1993). To understand the etiology of both sporadic and induced chromosomally abnormal gametes, better understanding of the normal mechanisms of chromosome behavior during meiosis I is required. Acquisition of such information would be facilitated by experimental analysis of the events of meiosis I. Much of what we now know about the behavior of chromosomes during mammalian meiotic prophase derives from essentially descriptive analyses of meiotic germ cells in situ. These studies have been extremely useful and have informed us about, for instance, the molecular structure of the synaptonemal complex (Heyting et al., 1988) and the cytogenetics of pairing failure (de Boer and de Jong, 1989). More direct experimental analyses of chromosome structure and function during the critical events of meiotic prophase have been limited primarily by the inability to manipulate in vitro gametocytes of the appropriate stage.

While it would be advantageous to have all the events of meiosis carried out by purified spermatocytes in culture, this goal is not yet possible. Previous analyses of purified or enriched pachytene, or earlier meiotic prophase spermatocytes in culture have not focused on the unique cytogenetic events of meiotic prophase. Instead, these studies were conducted to investigate stage-specific protein synthesis and modification during spermatogenesis (Gerton and Millette, 1986; O'Brien, 1987; Smith et al., 1992) or the effect of these germ cells on the metabolism and secretions of cocultured Sertoli cells (Djakiew and Dym, 1988). A very promising recent study (Rassoulzadegan et al., 1993) suggests that early pre-meiotic-prophase spermatogenic cells can indeed undergo events of meiosis in coculture with a transformed Sertoli cell line; however, normal chromosome

dynamics, such as pairing and formation of the synaptonemal complex, were not demonstrated in this study. Thus, although successful short-term cultures of pachytene spermatocytes have been reported, these systems have not yet been exploited for investigation of the defining events of the pachytene substage of meiotic prophase, namely maintenance of chromosome pairing and recombination.

Our aims in the present work were to utilize the short-term culture methods previously reported (O'Brien, 1987) for pachytene spermatocytes to establish whether chromosome pairing and synapsis are maintained in cultured cells. We also show that these cultures can be used for investigation of effects of agents that might control chromosome structure and function during meiotic prophase, as well as for agents that are suspected mutagens, particularly meiotically acting aneuploidogens or clastogens. We have used primarily cytogenetic criteria to assess the cultured cells in order to determine how accurately they maintain the typical pachytene state. In particular, we have concentrated on the presence of the synaptonemal complex as being indicative of the maintenance of chromosome pairing, and also on the unique morphology and metabolism of the sex chromosomes, which form a structurally and functionally unique domain in the pachytene nucleus (Handel et al., 1993). We find that in such cultures, pairing and synapsis are in fact maintained and that cells make progress through meiotic prophase. Analysis of experimental agents affecting chromosomes provides interesting insights into the structure and function of chromosomes and unique configuration of the sex chromosomes during critical periods of chromosome pairing and recombination. These studies thus suggest that short-term cultures can be useful not only for screening potential mutagens, but more importantly, for experimental analysis of the structural and functional transitions in meiotic chromosomes in spermatocytes. Continuing studies in these areas are likely to provide insights into factors controlling chromosome behavior and transition through the cell cycle

to metaphase and anaphase.

CHAPTER II

MATERIALS AND METHODS

Enrichment of cells and culture

Testes of adult Harlan-Sprague-Dawley mice were used as a source of germ cells. Testis tubules were suspended in EKRB[120.1 mM NaCl, 4.8 mM KCl, 25.2 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM MgSO4.$7H_2O$, 1.3 mM $CaCl_2$, 11 mM glucose, 1X essential amino acids (Sigma), 1X nonessential amino acids (Sigma)] and digested with collagenase and trypsin as described (Romrell et al., 1976; Bellvé et al., 1977). After tubule digestion, trypsin was removed by washing in EKRB containing 0.5% bovine serum albumin (BSA), and the single-cell suspension of germ cells was sedimented at unit gravity through a 2-4% BSA gradient generated in a medium-sized STAPUT chamber (Johns Scientific, Ontario). Gradients were collected after 2.5 hours in 10 ml fractions at a rate of 10 ml/45 seconds. Fractions were assessed for morphology and purity by light microscopy using Nomarski optics. The average purity of the pachytene spermatocyte fractions used was greater than 80%; the principle contaminants were Sertoli cells and symplasts composed of round spermatids. Pooled pachytene spermatocytes were washed once and resuspended in HEPES (25 mM)-buffered MEMα (Sigma) culture medium supplemented with 25 mM $NaHCO_3$, 5% w/v fetal bovine serum (Hyclone), 10 mM sodium lactate, 59 µg/ml penicillin, 100 µg/ml streptomycin (modified from O'Brien, 1987). Cells were plated at a concentration of 2.5 x 10^6 cells/ml in 4-well Nunclon dishes and cultured at 32°C in a gaseous environment of 5% CO_2 in air. Cell viability was determined using trypan blue dye exclusion; the typical viability at the onset of culturing was 96% and 90% after 24 hours in culture.

Experimental treatments

Actinomycin D (Sigma) was added to the cultured cells at 5 μg/ml for one hour. Camptothecin (Sigma) was added at 50 μM for 18 hours after a one-hour incubation. Okadaic acid (ICN Biomedicals) was added to the cells at varying concentrations for up to 6 hours after the cells were incubated overnight. All incubations were in standard conditions as described.

Cytological methods

Cell pellets were fixed for light and electron microscopy in 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ for 30 minutes, rinsed, postfixed in 1% osmium tetroxide in the same buffer, dehydrated through graded ethanols, infiltrated with propylene oxide, and embedded in Epon. Thick sections for light microscopy were stained with toluidine blue and sections for electron microscopy were stained with uranyl acetate and lead citrate.

In order to visualize synaptonemal complexes, the methods of Dresser and Moses (1980) were used for microspreading of cell nuclei, and complexes were stained with silver (Howell and Black, 1980). Distribution of cells in various substages of meiotic prophase was determined using the criteria of Moses (1980).

A modification of the Evans procedure (Evans et al., 1964) was also used to analyze the meiotic substages. Briefly, 2.5 x 10^6 cultured cells were pooled and pelleted by centrifugation. After washing twice, the cells were processed by the method of Evans (Evans et al., 1964). These air-dried meiotic cell preparations were stained with Gurr Giemsa in phosphate buffer.

Metabolic labeling

Incorporation of nucleotide precursor into RNA was monitored by following the uptake of ^{3}H-uridine, administered in pulses. Incorporation of uridine was measured both by determining acid-precipitable counts and by autoradiography to visualize nuclear sites of incorporation. Cells were pulsed with ^{3}H-uridine (ICN, 60 Ci/mmol) at a final concentration of 20 mCi/ml. At various time intervals, samples were removed and acid precipitated prior to liquid scintillation counting. Autoradiographic studies were performed on cells prepared by the Evans method and by microspreading as described above. The slides were dehydrated through graded ethanols and then coated with Kodak NTB-2 photographic emulsion diluted 1:1 with distilled water. Exposure time was 5 days at 4°C. Slides were developed with D19 developer (Kodak) and fixed with Kodafix (diluted 1:3 with distilled water). Cells prepared by the Evans method were stained with Gurr Giemsa after autoradiography.

CHAPTER III

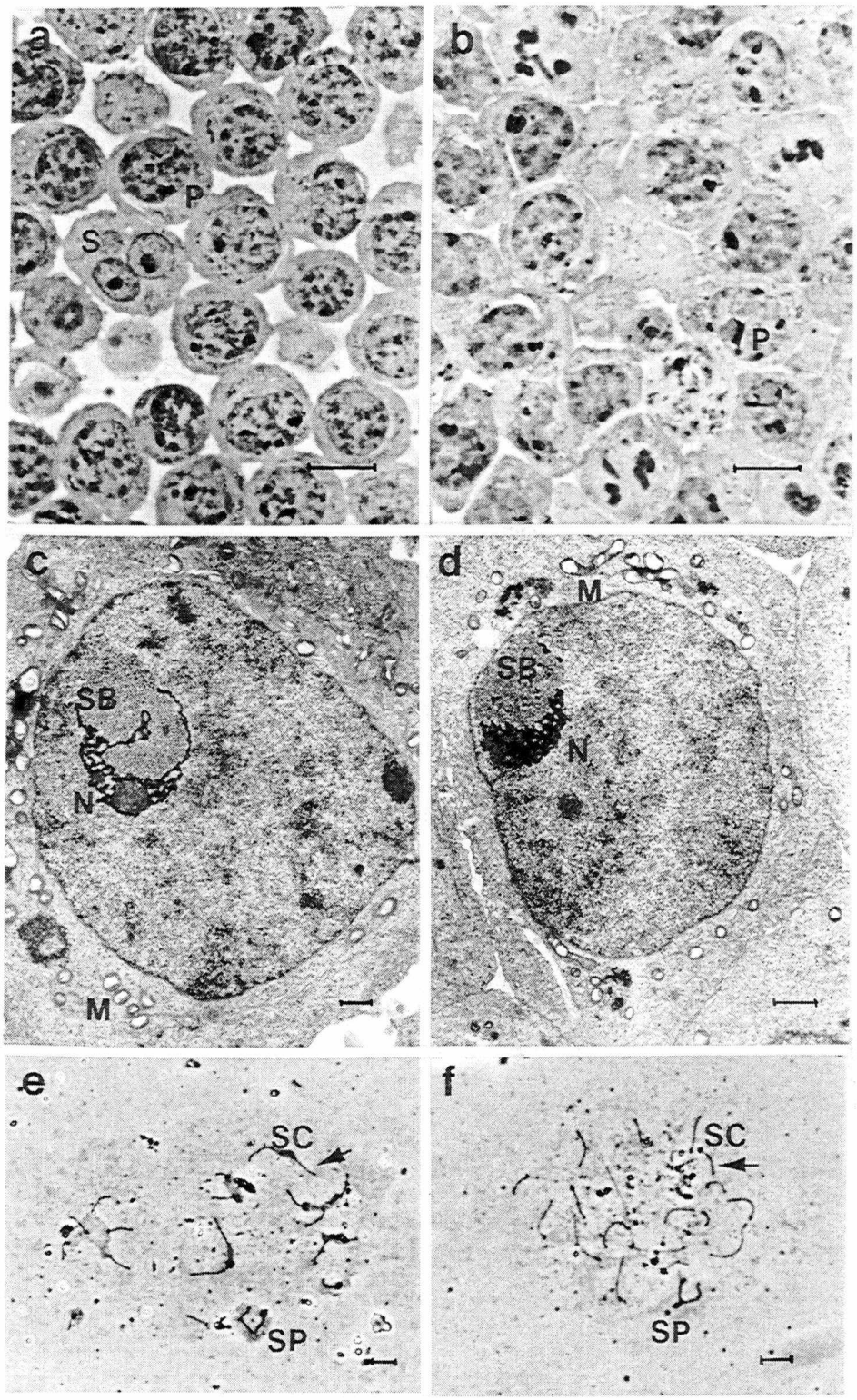
RESULTS

Pachytene spermatocytes show typical morphological features in culture

The defining characteristics of mammalian pachytene spermatocytes are primarily morphological. The autosomal chromatin is clearly less condensed than the sex chromatin and the chromosomes are paired around the typical tripartite array of the synaptonemal complex (SC). The sex chromosomes are largely unpaired, and form the unique chromatin domain of the sex body. Thus a primary objective of this work was to demonstrate these typical features for pachytene spermatocytes cultured in vitro. Cultured cells were prepared by several techniques and analyzed by both light and electron microscopy (Fig. 1b, d, and f) to assess if their morphological features, particularly those indicative of chromosome behavior, were consistent with those observed in pachytene spermatocytes in situ and recovered directly from unit gravity enrichment (Fig. 1a, c, and e). Figure 1b illustrates a cross section of cultured pachytene spermatocytes, demonstrating that the typical patterns of chromatin staining are maintained in these cultured cells when compared to pachytene spermatocytes that have been enriched through the STAPUT procedure, but not cultured (Fig. 1a). The sex body and associated darkly stained nucleolus are evident in many cells. These same features are revealed in ultrastructural analysis (Fig. 1c and d), where the characteristic morphology of the sex body is seen. Mitochondria are in their typical pachytene germ-cell configuration, clustered around the nucleus and with contracted cristae and a highly dilated intermembrane space.

When nuclei are prepared by the microspreading method, the morphology of the SCs are demonstrated (Fig. 1e and f). At the level of ultrastructure, the SCs are seen to be

Fig. 1. Micrographs showing features of morphology of pachytene spermatocytes at the outset of culture (**a,c,e**) and after 24 hr of culture (**b,d,f**). **a,b:** Light micrographs of cells showing nuclei of pachytene spermatocytes (P), and a few containing symplasts of round spermatids (S). Bar = 10 μm. **c,d:** Electron micrographs of cells showing the typical features of pachytene cells, including the sex body (SB), nucleolus organizer (N), and mitochondria (M). Bar = 1 μm. **e,f:** Phase-contrast micrographs of nuclei prepared by the microspreading procedure and silver stained to demonstrate the synaptonemal complexes (SC) between paired homologs, as well as the sex pair (SP). Bar = 10 μm.


a
P
S
b
P
c
SB
N
M
d
M
SB
N
e
SC
SP
f
SC
SP

normal (Fig. 2a), with only occasional breaks due to spreading artifacts, and the typical thickened appearance of the unpaired axes of the sex chromosomes is seen (Fig. 2b). Thus pachytene spermatocytes in conditions of short-term cell culture show typical morphological features of pachytene spermatocytes and chromosome synapsis is maintained in an apparently normal fashion.

Pachytene spermatocytes are viable and progress through meiotic prophase in culture

If culture conditions are satisfactory, cells should show some developmental progress; in this case, through meiotic prophase. At specific time intervals in culture, we determined cell viability and distribution of cells in the various substages of meiotic prophase. During the first 24 hours of culture viability is high (96% after enrichment of cells, 92% at 12 hours of culture, and 90% at 24 hours); after the first 24 hours viability drops more rapidily (70% - 80% at 48 hours, and 10% - 30% by 90 hours). For this reason, we limited our experimental analyses to the first 24 hours of culture, which is sufficient for determination of effect of experimental manipulation of the maintenance and morphology of chromosome synapsis.

Nuclei were prepared for visualization of SCs by the microspreading technique at various time intervals and progress through meiotic prophase assessed by analysis of SC morphology. Criteria have been established (Moses, 1980) for assessing stages of meiotic prophase at the cytological level; these criteria are based on the morphology of SCs as visualized in microspread nuclei, their terminal knobs, their degree of synapsis, and staining density and position of the sex body.

This technique, by virtue of assessing SC morphology, permits scoring of cells

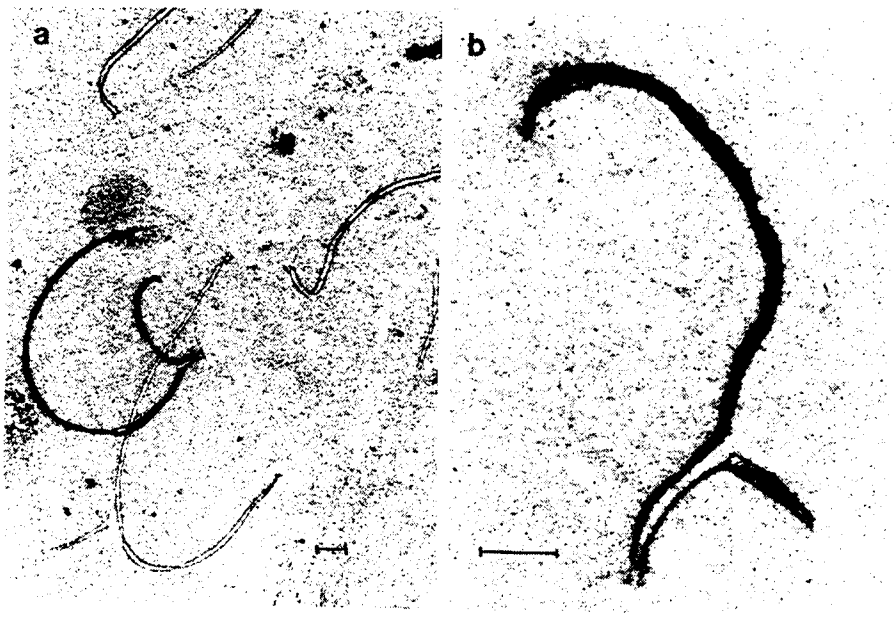


Fig. 2. Electron micrographs of synaptonemal complexes in cells cultured for 24 hours and prepared by the microspreading procedure. a: Seen here are profiles of the paired axes of the synaptonemal complex of autosomal chromosomes as well as the largely unpaired sex chromosomes with thickened lateral elements. Bar = 1 μm. b: Higher magnification view of a sex pair, showing the terminal paired region as well as the thickened lateral elements of the unpaired region. Bar = 1 μm.

through the substages of meiotic prophase. When compared to the distribution data obtained by Moses (1980) on cells released from macerated testes, the distribution of cells in the enriched pachytene fraction (Fig. 3, t=0) shows, as expected, an enrichment for the larger, mid-to-late pachytene cells. Furthermore, as can be seen from Figure 3, the cultured cells exhibit progress through meiotic prophase. Significant progress ($P < 0.05$) through prophase is made over 48 hours but no significant progress would be expected during a 24 hour culture period as this represents a small percentage of the total time for the pachytene stage of meiotic prophase. Thus cultured pachytene spermatocytes are viable and progress toward late stages of meiotic prophase.

Transcription occurs in a morphologically normal pattern in cultured pachytenes

One of the hallmarks of metabolism of pachytene spermatocytes is that the autosomal chromosomes are actively engaged in transcription while the sex chromosomes are transcriptionally inactive (evidence reviewed, Handel, 1987). Thus we assessed the nuclear distribution of transcriptional activity in cultured pachytene spermatocytes. Figure 4 illustrates the observations for nuclei prepared by the Evans air-drying procedure for visualization of pachytene chromatin. The autosomal domain is characterized by abundant incorporation of uridine, while the sex chromosomes, in the sex body, are transcriptionally inactive. Cultured pachytene spermatocytes thus reflect the same intranuclear distribution of transcription as do their counterparts in situ.

Cultured pachytene spermatocytes are affected by agents that alter chromatin structure

Since we are interested primarily in the behavior of chromosomes during meiotic

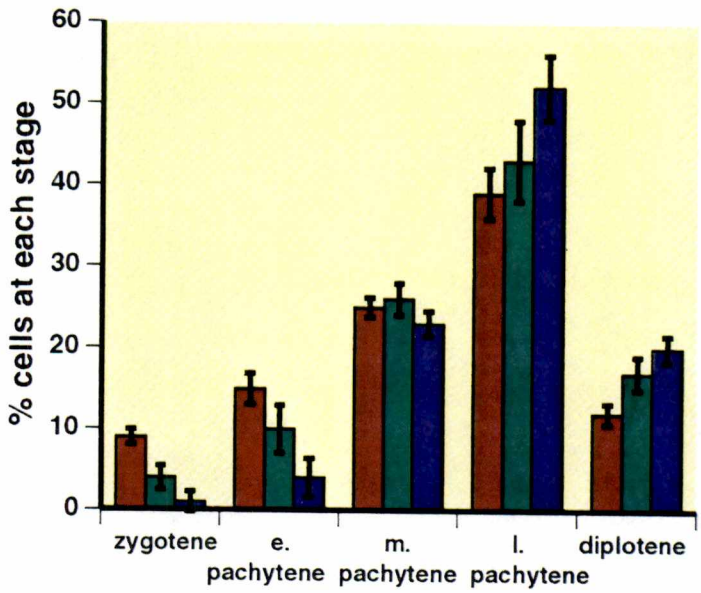


Fig 3. Pachytene spermatocytes show progress through meiotic prophase in culture. Cells prepared by microspreading were scored according to the criteria of Moses (1980). Samples were taken at the outset of culture ■ , at 24 hours ■ , and at 48 hours ■ . These data represent the combined results of three repeat experiments; a total of 1600 cells were scored.

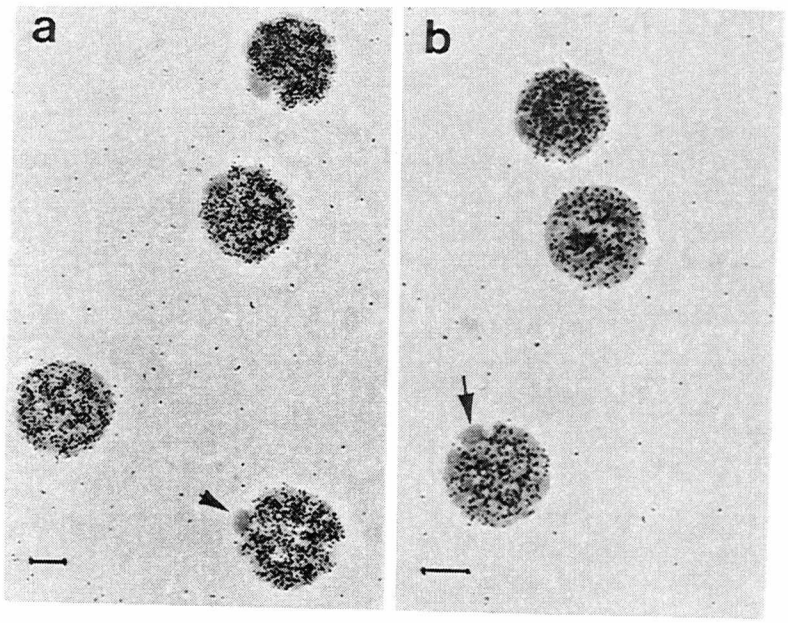


Fig. 4. Autoradiographs of air-dried preparations of pachytene cells indicate spatial distribution of incorporation of uridine. a: Cells were labeled immediately after removal from testes. b: Cells labeled after overnight culture. Note absence of label over the sex body (arrow). Bar = 10μm.

prophase, we have used cultured primary spermatocytes to investigate aspects of chromosome structure and its transitions by using agents known to affect, directly or indirectly, chromosome structure and behavior. The agents we used are: actinomycin D (a DNA intercalator that inhibits transcription), camptothecin (an inhibitor of topoisomerase I), and okadaic acid (a phosphatase inhibitor known to induce the G2-metaphase [G2-MI] cell cycle transitions in chromatin).

Actinomycin D: Actinomycin D (AMD) intercalates into DNA and has previously been used for cytological assessment of chromatin configuration (Darzynkiewicz et al., 1969; Barcellona et al., 1974). Because of both its intercalatory activity and resultant inhibition of transcription we were interested in the effect of AMD on meiotic prophase chromosome structure. When used in concentrations sufficient to inhibit the incorporation of uridine, AMD had a dramatic effect on prophase chromatin structure. Within one hour after addition of AMD, chromatin became highly condensed (Fig. 5); SCs were largely obscured by the highly condensed chromatin, but apparently were not disassembled. Interestingly, AMD had a differential effect on autosomal and sex chromatin. Early in the treatment, sex chromatin was affected to a lesser degree (Fig. 5); with longer treatment and/or higher concentrations of AMD, the sex chromatin also became condensed. Thus although the sex chromatin appears to be less accessible to AMD than does the autosomal chromatin, it too is ultimately affected.

Camptothecin: A major role of topoisomerases is in unwinding chromatin and they are believed to affect the processes of transcription, replication and recombination (Liu, 1989). Thus, inhibitors of topoisomerases might affect meiotic chromatin structure and/or progress through meiotic prophase. Pachytene spermatocytes were cultured in media supplemented with camptothecin, a topoisomerase-I inhibitor. Spermatocytes thus treated did not differ in viability from controls during culture, and furthermore, there were

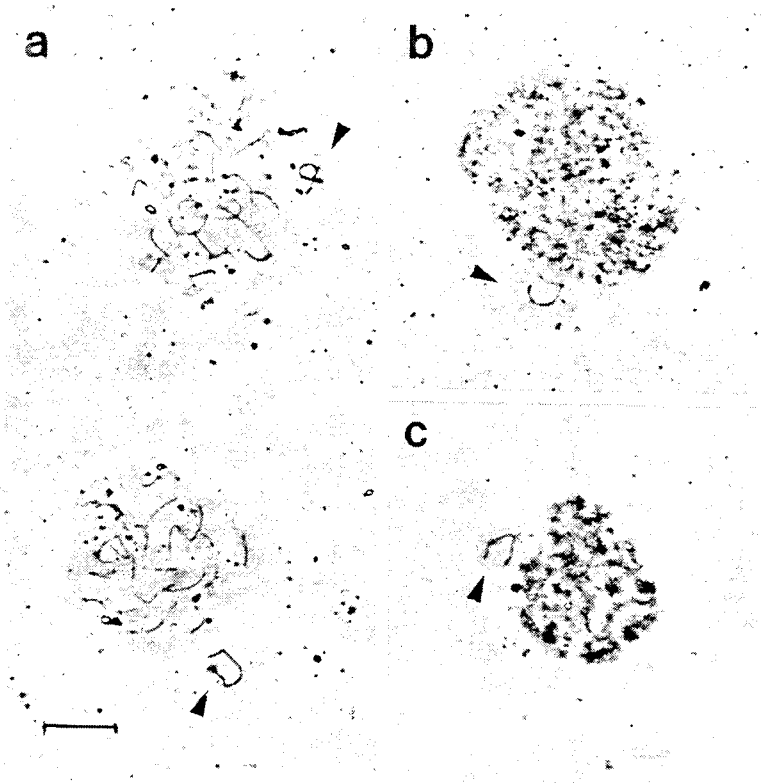


Fig. 5. Phase contrast micrographs of microspread preparations of cells demonstrate the chromatin condensation effect of actinomycin D treatment. a: Untreated cultured cells. b,c: Cells incubated for 1 hour in the presence of 5 μg/ml actinomycin D. Note that the sex body (arrow) does not show dramatic condensation. Bar = 20 μm.

no obvious differences in chromatin structure as assessed from air dried meiotic preparations and DAPI-stained cells (data not shown). Interestingly, however, scoring of distribution of cells in meiotic prophase substages revealed a change in the apparent rate of progression through meiotic prophase I by the camptothecin-treated cells. Among the treated cells, there were more cells with the characteristics of the diplotene stage as assessed from morphology of the SC. Figure 6 shows camptothecin-treated cells demonstrating the desynapsed (diplotene-like) appearance of the autosomal and sex chromosome axes. Figure 7 shows that the proportion of cells with these characteristics increases over time with exposure to camptothecin. However, although there was an increased proportion of diplotene-like cells, we did not observe the expected concurrent increase in the proportion of metaphase I (MI) scored. Thus, the increased frequency of diplotene-like cells may represent premature desynapsis induced by the topoisomerase inhibitor rather than true meiotic progression to MI.

Okadaic Acid: In the analysis of oocyte meiotic maturation, the phosphatase inhibitor okadaic acid (OA) has proved to be a useful reagent in elucidating transition to meiotic metaphase and associated cell-cycle control points (reviewed by Eppig, 1993). However, oocytes that have been experimentally treated with OA have been in the dictyate stage, post-pachytene, after disassembly of SCs, and presumably after the time of recombination. It is not known if cells in mid-pachytene, with paired and synapsed chromosomes, presumably undergoing recombination, are able to rapidly undergo the changes necessary to form condensed metaphase chromosomes. To learn more about the maintenance of pachytene and the regulation of G2-MI transition, we cultured pachytene spermatocytes in the presence of OA. As shown in Figure 8a, control cultures not treated with OA typically consist of less than 4% MI figures. However, cultures treated with OA show a dramatic increase in the proportion of MI figures (Fig. 8b). The number of MI

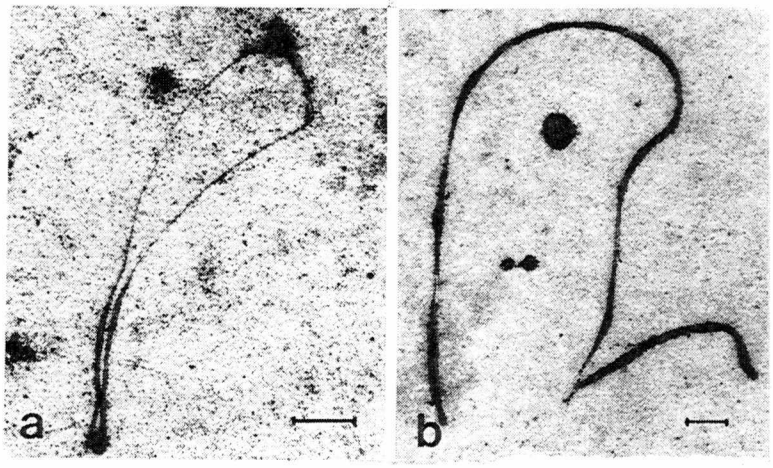


Fig. 6. Electron micrographs illustrating desynapsis frequently found in chromosomes of cells cultured for 18 hours in the presence of 50 μM camptothecin. a: An autosomal homolog is undergoing desynapsis. b: The X-Y pair in the same cell is almost completely desynapsed. Bar = 1 μm.

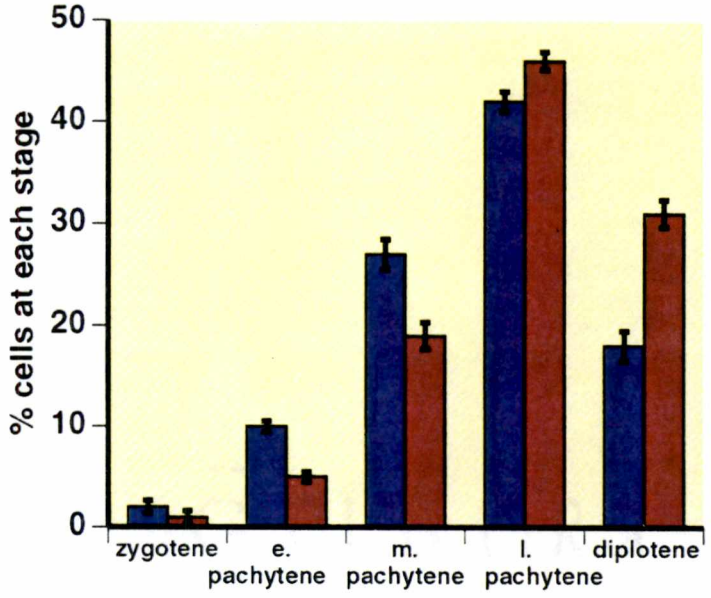


Fig. 7. Distribution of cells in meiotic prophase substages, scored according to the criteria of Moses (1980). Samples were removed for scoring after 18 hr of culture for both control, untreated, n = 800 ■ and cells incubated in 50 μM camptothecin, n = 1000, ■. Cells with desynapsis were scored as "diplotene" although as discussed in text, these cells may not be true diplotene.

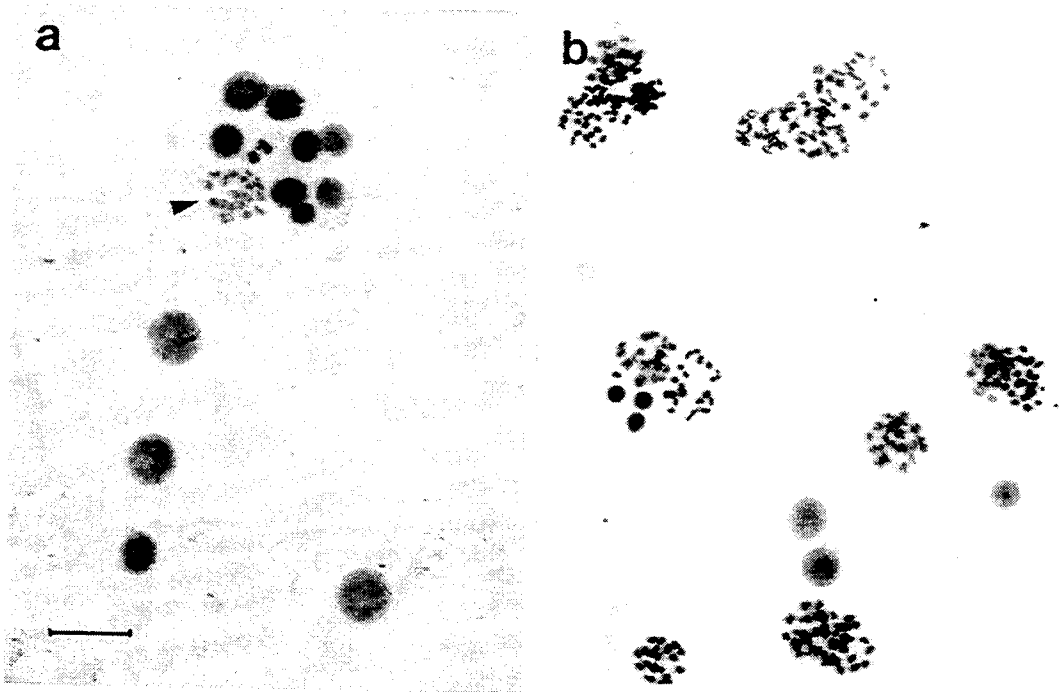


Fig. 8. Air-dried meiotic chromosome preparations of cultured cells before (a), and after (b) treatment with 5 μM okadaic acid. Note the prevalence of pachytene cells among untreated cells; this photograph was selected to demonstrate a meiotic cell, (arrow) which represent less than 4% of the total. In contrast, after treatment, some late meiotic prophase (diplotene) and many metaphase I cells are common. Bar = 50 μm.

figures scored increases with increasing concentrations of OA and length of exposure (Table 1). A concentration of 5 μM OA appears to maximally induce MI, as cultures treated with higher concentrations do not show significant differences in proportion of MI figures. Thus, these results suggest that the experimental induction of MI chromosome condensation in pachytene spermatocytes proceeds via cell-cycle controls similar to those operating in mitotic cells and during meiotic maturation of oocytes.

Table 1. Effects of varying concentrations and length of treatment of okadaic acid on cultured pachytene spermatocytes

	Hours of treatment			
O.A. Conc. (μM)	1	2	3	6
0	4[a]	ND	2	4
1	9	7	15	38
2	7	12	18	58
5	14	20	34	86
10	24	26	ND	83

[a]these data indicate the percentage of cells in metaphase I. Cells scored, n=2200, represent the pooled data from two experiments.
ND - Not determined

CHAPTER IV

DISCUSSION

Experimental analysis of the molecular events of meiotic prophase and regulation of the transition to metaphase during mammalian spermatogenesis has been seriously hampered by the lack of a system for culture in vitro. Some greater success in achieving meiosis in vitro has been obtained in culturing spermatogenic cells of lower vertebrates where germ cells can be cultured in cysts with their associated somatic cells (Abé, 1987 for review, see also Risley, 1983; Risley et al., 1987; Dubois and Callard, 1991). Such cultures have been used with considerable success to monitor effects of potential mutagens on meiotic cells (e.g., Risley et al., 1988; Risley et al., 1991). Additionally, mammalian spermatocytes have been cultured briefly in vitro for analysis of unscheduled DNA synthesis in response to mutagens previously administered in vivo, the "in vivo/in vitro " DNA repair assay (Working and Butterworth, 1984; Working, 1989). Recently, meiosis has been reported to occur in vitro in cultures of immortalized germ cells (Hofmann et al., 1994); or in cultures utilizing immortalized Sertoli cells (Rassoulzadegan et al., 1993), however, the cytogenetic events of meiosis have not been investigated in detail in these studies.

The culture method described here was utilized with the aim of providing a system for experimental analysis of meiotic prophase and the transition to metaphase and anaphase in mammalian spermatocytes. In order to be an appropriate model for meiosis, cells in culture should exhibit the normal morphological features, should maintain chromosome pairing and synapsis, and should carry out the molecular events of genetic recombination that are presumed to occur during the pachytene substage of meiotic prophase.

Unfortunately, we do not know enough of the molecular attributes of some of these processes to be able to assess them either in cultured cells or meiocytes in situ. Nonetheless, we demonstrate here that spermatocytes in short-term cultures retain chromosomal pairing and synapsis and other typical morphological features of meiotic prophase as well as the normal subnuclear distribution of transcriptional activity. Much remains to be done to develop the appropriate conditions for execution of meiotic prophase events in vitro; however, as we show here, cultured spermatocytes can be useful for experimental analyses that provide some insights into the structure and function of chromosomes during meiotic prophase and the transition to metaphase.

Key morphological features of meiotic prophase are maintained in cultured spermatocytes

In the absence of well-defined molecular landmarks for meiotic prophase in mouse spermatocytes, our initial criteria for assessing spermatocytes in culture were primarily morphological. Most importantly, there is maintenance of chromosome pairing and the SC between paired homologs in cultured cells. Thus pairing and maintenance of the SC do not seem to require the presence of Sertoli cells. The SC is also not particularly labile or susceptible to environmental stress, for even at times when there is general decrease in viability of the cultured spermatocytes (after 48 hours of culture), chromosome pairing and synapsis are maintained. Additionally, the morphologically differentiated chromatin domains of the sex chromosomes (forming the sex body) and the autosomal chromosomes are distinct in cultured spermatocytes. Finally, cytoplasmic features typical of pachytene cells are found in the cultured spermatocytes; in particular the mitochondria are in circumferential array around the nucleus and show the contracted cristae and expanded intermembrane space found to be characteristic of mammalian meiotic prophase

spermatocytes (André, 1962). Although these observations on maintenance of typical pachytene morphology are not particularly surprising, they are essential for demonstrating the validity of this culture system for the investigation of factors that might influence the maintenance of the chromosome pairing presumed to be essential for recombination and subsequent normal segregation of homologs.

Additionally, pachytene spermatocytes in culture faithfully retain the subnuclear distribution of transcriptional sites found in their counterparts in situ. That is, the autosomal chromosomes actively incorporate tritiated uridine, while the sex chromosomes are transcriptionally inactive. It is not known if transcriptional inactivation of the sex chromosomes exists to prevent production of products inhibitory to spermatogenesis as proposed by Forejt (1982) or if it is related to the absence of recombination over much of the sex chromosomes as suggested by McKee and Handel (1993). The maintenance of this transcriptional asymmetry in cells in vitro suggests both that it is not under immediate testicular control and that these cultured cells can be useful for examining short-term parameters of pachytene transcription, which may lead to some insights about why the sex chromosomes are inactivated.

Because we wished to establish culture conditions useful for analysis of events, changes in levels of enzyme activity, proteins, and transcript levels in purified populations of pachytene spermatocytes, we deliberately chose to culture spermatocytes without the benefit of coculture with Sertoli cells. Undoubtedly the absence of Sertoli cells is a major factor contributing to the limited viability of spermatocytes in culture. It is unlikely that meiosis in vitro, from the initiation of preleptotene DNA synthesis to the completion of the two meiotic divisions, will be possible without the presence, or at least the products, of Sertoli cells. A number of studies indicate that the initiation and completion of meiosis are hormonally dependent and require FSH and/or androgen (see for example, Sun et al.,

1990); it is the Sertoli cells that possess FSH receptors and thus mediate the hormone response. Although coculture of spermatocytes with Sertoli cells will probably be necessary for the initiation and completion of meiosis in vitro, it will complicate analysis of spermatocyte-specific processes. The system we have chosen, culture of enriched populations of spermatocytes, facilitates monitoring of transcripts and protein as well as synthetic events of the spermatocytes. Furthermore, our observations have demonstrated that certain features of the pachytene substage of meiotic prophase do not require immediate control by Sertoli cells over the short term. The interesting, but presently unanswerable, question is whether Sertoli-cell products are required for spermatocytes to carry out the molecular events of recombination.

Meiotic chromosome structure and behavior can be experimentally altered in vitro

Currently, in the absence of well-defined molecular criteria, the hallmarks of the events and progress of meiotic prophase lie in the structure of chromosomes and their behavior, e.g., the pairing of chromosomes, the chromatin structural differences between the autosomal and the sex chromosome domains, and the desynapsis and condensation of chromosomes as they progress to the first meiotic metaphase. We were interested in determining the usefulness of cultured spermatocytes for analysis of effects of agents that might affect or disrupt meiotic chromosome structure and behavior. Analysis of the effects of such agents ultimately can not only provide useful information about control of chromosome behavior but also can serve to identify and screen for mutagenic agents that might act as germ-cell aneuploidogens or clastogens (as demonstrated for lower vertebrates by Risley et al., 1988; 1991). For this initial test of the utility of our culture system, we chose agents that might be expected to affect meiotic chromosome structure and behavior,

such as pairing and condensation in the transition from meiotic prophase to metaphase. We used a DNA-intercalating agent (actinomycin D) that inhibits transcription and has been used to assay chromatin configuration, a topoisomerase inhibitor (camptothecin) that can induce DNA strand breaks but whose effects on mammalian meiosis are unknown, and a phosphatase inhibitor (okadaic acid) known to induce metaphase transitions in chromosome structure in meiotic oocytes.

Since autosomal chromosomes of pachytene spermatocytes are actively transcribing RNA, and since there is an apparent (although possibly indirect) relationship between ongoing transcription and recombination (reviewed by McKee and Handel, 1993), we were interested in the effects of AMD on meiotic chromosome morphology and behavior. AMD inhibits transcription by intercalating into DNA, thus, presumably hindering the transcription complex. Interestingly, AMD, as a DNA intercalator, has also been used to assess whether chromatin is accessible to AMD; some of these studies have analyzed spermatogenic cells of different stages and species (e.g., Barcellona et al., 1974 and Darzynkiewicz et al., 1969). In the present study, when cultured pachytene cells were treated with AMD in concentrations sufficient to inhibit RNA synthesis, the chromatin was dramatically affected. Within one hour of treatment, the autosomal chromatin became quite condensed, with the underlying SCs being mostly obscured. In contrast, the sex body was relatively unaffected early in treatment, although it too subsequently became condensed. The mechanism by which AMD induces this effect is not known; however, since the sex body is not transcribing RNA, it seems more likely that the effect is the morphological manifestation of AMD intercalation rather than the result of inhibition of transcription. Nonetheless, the observations have interesting implications with respect to the relative effect on autosomal versus sex chromatin. They are consistent with the idea that the sex chromatin is in a functionally and structurally different conformation than the autosomal

chromatin, a relatively inaccessible one that may exclude AMD as well as transcription factors. This kind of experiment demonstrates the utility of cultured pachytene cells for analysis of experimental agents that may provide insights into the structure and function of meiotic prophase chromosomes.

Camptothecin is a cytotoxic alkaloid whose intracellular target is topoisomerase I. The end result of camptothecin-topoisomerase I interaction is induction of double-strand breaks in replicating DNA (Ryan et al., 1991) and these breaks can trigger p53-dependent DNA damage responses (Nelson and Kastan, 1994). In general, topoisomerase inhibitors such as camptothecin interact with DNA in complexes that trap the enzyme, the so-called cleavable complex. In the case of camptothecin, the lethal cell damage occurs as a result of the interaction of the cleavable complex with the enzymes of DNA replication (Ryan et al., 1991). It is not known if lethal cleavage can occur in association with the repair replication characteristic of mammalian pachytenes (Stubbs and Stern, 1986) or if indeed camptothecin affects meiotic chromosome pairing. In fact, the role of topoisomerase in meiosis is not clear. In the yeast Saccharomyces cerevisiae, mutants for topoisomerase I are able to complete meiosis, suggesting topoisomerase I is not essential for meiotic function in this species (Christman et al., 1988). On the other hand, in Drosophila melanogaster, X-Y chromosome pairing sites are characterized by repeats of the consensus binding sequence for topoisomerase I (McKee et al., 1992), suggesting that the enzyme may be important for chromosome pairing in this case. Although a topoisomerase I gene is expressed in the mammalian testis (Reddy and Handel, unpublished), it is not known if it is involved in meiosis. Our results here do not provide evidence for a dramatic effect of the topoisomerase-I inhibitor camptothecin on spermatocyte morphology, viability, or, indeed, on the morphology of the SC present between paired homologs. There is no evidence for breaks in the SC or for non-homologous pairing, as found for some mutagenic agents

(Allen et al., 1987; Allen et al., 1988; Backer et al., 1988). However, we did observe a marked difference between camptothecin-treated and untreated cells in the progress of cells to a desynapsed (apparent diplotene stage) configuration. Since there was no increase, as might be expected, in the number of camptothecin-treated cells reaching metaphase I, it appears that camptothecin treatment induces desynapsis of chromosome homologs. This experiment demonstrates the utility of cultured pachytene spermatocytes for rapid identification of potentially mutagenic agents that might affect chromosome pairing. The interesting observation made here could in fact imply a role for topoisomerase I in the maintenance of chromosome synapsis and should therefore be investigated further with both analysis of topoisomerase I activity in treated cells and determination of whether the treated cells can be stimulated to progress to metaphase.

Although our primary goal in establishing the culture system was analysis of the structure and behavior of meiotic prophase chromosomes, we were curious to see if the cells could be induced to progress to metaphase, thus, demonstrating the utility of the culture system for investigation of meiotic cell-cycle controls. OA has proven to be a useful reagent for investigation of cell-cycle progress. It is a phosphatase inhibitor that can induce meiotic metaphase ("maturation") of mammalian oocytes (reviewed by Eppig, 1993). A priori, it was not known if mid-pachytene spermatocytes, still carrying out the events of meiotic prophase, including recombination, would be able to respond to the stimulus to condense chromatin to form metaphase chromosomes. Our results show that indeed cells treated with OA do condense chromatin with a resultant dramatic increase in the proportion of cells with MI metaphase figures. The OA-treated cells also show other biochemical and physiological features of MI cells (Wiltshire et al., 1995). It is not known how pachytene cells assess that they have completed prophase, but since the transition to MI that we have observed would normally take 2-4 days, it is clear that OA has overridden

a cell-cycle checkpoint that normally holds pachytene cells in prophase. It is not surprising that spermatocytes should utilize the seemingly universal patterns of phosphorylation and dephosphorylation in undergoing the meiotic G2-MI transition. However, it was previously unknown if these same universal cell cycle features might also control the completion of recombination events and disassembly of the SC. Although some common controls are apparently active, there may also be controls unique to meiotic prophase cells. Cultured pachytene cells may well provide a useful resource for investigation of the meiotic cell cycle and its checkpoints.

Apart from demonstrating the utility of culturing pachytene spermatocytes, this work has presented interesting cytological observations that merit further study. What is clearly needed for the analysis of mammalian meiosis are the molecular assays to pursue such findings.

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PART IV

Induced Premature G2/M-Phase Transition in Pachytene Spermatocytes Includes Events Unique to Meiosis*

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ABSTRACT

Little is known about the control of events ending the lengthy prophase of meiosis I and leading to the G2/M-phase transition in mammalian spermatocytes, primarily because the relevant late pachytene, diplotene and MI cells are present in low numbers in the testis and it is not possible to isolate them in significant numbers. We have utilized short-term cultures of pachytene spermatocytes from the mouse to study events of the G2/M cell-cycle transition induced by the protein phosphatase inhibitor okadaic acid (OA). Treatment of cultured pachytene spermatocytes with OA induced a rapid and premature onset of events leading to the M phase, visualized cytologically by nuclear envelope breakdown and chromosome condensation. After OA treatment, condensed chromosomes were seen as bivalents, not as univalents. Treatment with OA induced disassembly of synaptonemal complexes and resolution of crossovers as cytologically visible chiasmata. Chiasmata counts were similar in treated cells and control cells. Thus, surprisingly, even though the treated cells were in the pachytene substage of meiotic prophase, events of recombination were apparently completed to the point of chiasma formation in the majority of these cells. The sex chromosomes, forming the sex body of the pachytene spermatocyte, lagged behind the autosomal chromosomes in their condensation and progression toward the M phase. Treatment with OA induced an increase in histone H1 kinase activity, generally used as an indicator of MPF activity; furthermore, the OA-induced cell-cycle transition does not require new protein synthesis. These results suggest that OA treatment overrides a cell-cycle checkpoint control that normally keeps pachytene spermatocytes in a lengthy prophase, and that this control may be exerted by regulation of protein phosphorylation status.

CHAPTER I

INTRODUCTION

The cell cycle of the first meiotic division of mammalian male germ cells differs in several significant ways from a typical mitotic cell cycle, and even from the mammalian oocyte cell cycle. During the lengthy first prophase homologous chromosomes pair, a process thought to be maintained or mediated by the synaptonemal complex. They also participate in recombination, an exchange of DNA later manifested as chiasmata. During progression from late prophase to the first meiotic metaphase (the G2/MI transition), the synaptonemal complex disappears, the chromosomes condense, the nuclear envelope breaks down, the spindle forms and the paired chromosomes, now held together by chiasmata, orient on the spindle apparatus. Anaphase I, achieving the separation of homologous chromosomes, and the second meiotic division ensue rapidly thereafter. Little is known about the control of the cell-cycle G2/M-phase transition during the first meiotic divisions of spermatocytes, although a regulatory mechanism that assesses the completion and fidelity of recombination prior to dissolution of the synaptonemal complex and progress through the G2/M transition can be inferred. Many of the events of the G2/M transition of a mitotic cell cycle probably also occur in meiosis I spermatocytes, but in addition, there must be processes to ensure that it is the homologous chromosomes, not chromatids, that are correctly separated at the first meiotic anaphase.

Our lack of information about the events comprising the G2/M transition in meiosis I male germ cells is in striking contrast to our considerable knowledge about these events in oocytes. Indeed, the investigation of events of the oocyte G2/M transition provided much of the foundation for the discovery of universal mechanisms of cell-cycle control (Murray

and Hunt, 1993). Oocytes are arrested at the end of prophase I, after the completion of recombination and disappearance of the synaptonemal complex. The initiation of the G2/M transition, or meiotic maturation, is under control of hormones and the surrounding somatic cells of the follicle. This aspect of the biology of female meiotic cell cycle has made the transition to the meiotic M phase particularly amenable to experimental analysis. The basic elements of the signals for the oocyte G2/M transition are now well known. The key molecular complex involved is metaphase-promoting factor, MPF (formerly known as maturation-promoting factor). MPF was first isolated from non-mammalian oocytes, but subsequently shown to be present universally in mitotic cells and oocytes (Murray and Hunt, 1993). MPF consists of a 34kD protein, homologous in all species to the p34 product of the fission yeast *cdc2+* gene, and cyclin. The p34^{cdc2} is a serine-threonine protein kinase whose activity is regulated by its association with cyclin B as well as by its phosphorylation status (Lewin, 1990; Murray and Hunt, 1993; Murray and Kirschner, 1989; Nurse, 1990). MPF phosphorylates a number of proteins, but its H1 kinase activity is most frequently used to monitor MPF activity. Inactivation of MPF at the transition from metaphase to anaphase is coupled with the proteolytic degradation of cyclin and the reactivation of MPF at the end of G2 thus requires new cyclin B. In mammalian oocytes MPF activity increases at the time of germinal vesicle breakdown and peaks at metaphase I (Choi *et al.*, 1991). Although MPF is both necessary and sufficient for spontaneous G2/M transition in mammalian oocytes, the hierarchy of events for its activation, its direct and indirect substrates, and whether there are alternative pathways for the G2/M transition are not clear.

Until now, analysis of cell-cycle events of the G2/M transition of mammalian spermatocytes has been hampered by the difficulty in obtaining sufficient quantities of appropriate cells for experimental analysis. However enriched fractions of pachytene

(mid-prophase) spermatocytes can be obtained (Bellvé *et al.*, 1977) and have been advantageously used to determine the presence of proteins implicated in the G2/M transition. Thus it has been shown that mouse spermatogenic cells express both cyclin B1 and B2, but that cycB1 levels are higher in postmeiotic cells than in pachytene spermatocytes, while cycB2 was most abundantly expressed in pachytene spermatocytes (Chapman and Wolgemuth, 1992; Chapman and Wolgemuth, 1993). In contrast to transcript levels, the highest levels of cyclin B1 protein were found in pachytene cells, as was also $p34^{cdc2}$ protein and immunoreactive H1 kinase activity (Chapman and Wolgemuth, 1994). These interesting and informative studies suggest that the molecules of universal cell-cycle control are present, and perhaps active, in pachytene cells prior to the G2/M transition. However, it was not possible by the means used in these studies to assess presence or activity of these proteins in the relevant diplotene/MI spermatocytes undergoing the G2/M transition; these cells are neither abundant nor possible to enrich from testes.

Recently we have devised a means to circumvent this problem by culturing mid-to late-pachytene cells, which can be obtained by enrichment procedures, and experimentally inducing them to undergo the G2/M-phase transition. We have previously reported (Handel *et al.*, in press) that pachytene spermatocytes in short-term cultures maintain chromosome pairing and typical synaptonemal complex arrays as well as the unique sub-nuclear distribution of transcriptional activity characteristic of spermatocytes in vivo. Here we asked if it is possible for these mid-pachytene cells, presumably carrying out the event of recombination, to be induced to the G2/M transition. For experimental induction of the G2/M transition, we used treatment with okadaic acid (OA). OA is a polyether monocarboxylic acid that is a specific inhibitor of protein phosphatases (PPs), both PP1 (ATP-Mg^{2+}-dependent) and the related PP2A (polycation-stimulated) (Cohen *et al.*, 1990).

Experimental treatment with OA induces hormone-independent meiotic maturation (G2/M transition) of oocytes of several species (Goris *et al.*, 1989; Picard *et al.*, 1989; Picard *et al.*, 1991) including the mouse (Alexandre *et al.*, 1991; Chesnel *et al.*, 1994; Gavin *et al.*, 1991; Gavin *et al.*, 1994; Schwartz and Schultz, 1991). In the studies reported here, we asked if treatment with OA could induce a premature G2/M transition in cultured pachytene spermatocytes, and, if so, if cell-cycle events unique to meiosis, i.e., synaptonemal complex disassembly and resolution of crossovers as chiasmata, not previously analyzed with respect to cell-cycle events, also occur during the induced G2/M transition. We found that OA treatment of pachytene spermatocytes does indeed induce a rapid and premature G2/M transition which was accompanied by disassembly of the synaptonemal complex and resolution of crossovers as chiasmata. An interesting differential behavior of the autosomal chromosomes and the sex chromosomes in response to OA treatment was observed. Additionally, the G2/M transition was accompanied by an increase in histone H1 kinase activity and did not require new protein synthesis. Thus the meiotic cell-cycle transition of spermatocytes can be prematurely induced by an agent affecting the universal cell-cycle regulators, and, furthermore, events unique to meiosis (e.g., synaptonemal complex breakdown) seem to be coordinately controlled. OA treatment appears to abrogate cell-cycle checkpoints that maintain the normally lengthy meiotic first prophase of male germ cells.

CHAPTER II

MATERIALS AND METHODS

Preparation of Cells and Culture Procedures

Testes of adult CD1 mice (Harlan) were used as a source of germ cells. Testis tubules were suspended in EKRB (120.1 mM NaCl, 4.8 mM KCl, 25.2 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.3 mM $CaCl_2$, 11 mM glucose, 1X essential amino acids [Sigma], 1X nonessential amino acids [Sigma]) and digested with collagenase and trypsin as described (Bellvé, *et al.*, 1977; Romrell *et al.*, 1976). After tubule digestion, trypsin was removed by washing in EKRB containing 0.5% bovine serum albumin (BSA), and the single-cell suspension of germ cells was sedimented at unit gravity through a 2-4% BSA gradient in a STA-PUT chamber (Johns Scientific, Ontario). Gradients were collected after 2.5 hours in 10 ml fractions at a rate of 10 ml/45 seconds. Fractions were assessed for morphology and purity by light microscopy using Nomarski optics. The average purity of the pachytene spermatocyte fractions used was greater than 80%; the principle contaminants were Sertoli cells and symplasts composed of round spermatids. Pooled pachytene spermatocytes were washed once and resuspended in a culture medium modified from one previously described (O'Brien, 1987): HEPES (25 mM)-buffered MEM-α (Sigma) culture medium supplemented with 25 mM $NaHCO_3$, 5% w/v fetal bovine serum (Hyclone), 10 mM sodium lactate, 59 μg/ml penicillin, 100 μg/ml streptomycin. Cells were plated at a concentration of 2.5 x 10^6 cells/ml in 4-well Nunclon dishes and cultured at 32°C in a gaseous environment of 5% CO_2 in air. Cell viability was determined using trypan blue dye exclusion; the typical viability at the onset of culturing

was 96% and 90% after 24 hours of culture.

Okadaic Acid Treatments

After 12 hours of culture the culture medium was supplemented with OA (ICN Biomedicals) at concentrations of 2-10 μM,with or without 10 μg/ml cycloheximide (Sigma). Culturing was continued for up to 6 hours with aliquots taken throughout the culture period for analysis of cytology, histone H1 kinase activity, and protein synthesis. OA was prepared as a 250 μM stock solution in 100% ethanol. Since ethanol negatively affects spermatocyte viability, equal volumes of MEM- α were added to aliquots of OA and partially dried to remove ethanol before being added to the culture media.

Cytological Methods

Pelleted cultured cells were fixed for light and electron microscopy in 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ for 30 minutes, rinsed, postfixed in 1% osmium tetroxide in the same buffer, dehydrated through graded ethanols, infiltrated with propylene oxide, and embedded in Epon. Thick sections for light microscopy were stained with toluidine blue and sections for electron microscopy were stained with uranyl acetate and lead citrate.

A modification of the Evans procedure (Evans *et al.*, 1964) was also used to analyze the meiotic substages. Briefly, 2.5 x 10^6 cultured cells were pooled and pelleted by centrifugation. After washing twice in 2.2% sodium citrate, the cells were processed by the method described (Evans *et al.*, 1964) . These air-dried meiotic cell preparations were stained with Gurr Giemsa in phosphate buffer. Stages in the progress of cells from the pachytene substage of meiosis I through to metaphase of meiosis I were scored by light

microscopy at 100X using classical cytogenetic criteria.

Chiasmata were scored in cells that did not exhibit hypercondensation by observation with an oil-immersion 100X objective.

Histone H1 Kinase Assay

To assess the effect of OA on histone H1 kinase activity assays were performed using a modification of the method of Meijer *et al.* (1989). Crude pachytene cell lysates were prepared by centrifuging cells from 500 µl culture, washing, and then resuspending in 150 µl of H1 kinase buffer containing 25 mM MOPS (pH 7.2), 60 mM ß-glycerolphosphate, 30 mM p-nitrophenylphosphate, 15 mM EGTA, 15 mM $MgCl_2$, 100 µM Na_3VO_4, 1 mM DTT, 1 mM PMSF, BSA (1 mg/ml). Lysates were frozen at -70° C. Thawed lysates were vortexed and centrifuged at 12000 rpm for 10 min. at 4°C. Kinase reactions using 5 µl of lysate were carried out for 1 hour at 30°C in a total volume of 25 µl in H1-kinase buffer supplemented with 100 µg/ml histone H1 (type III-S; Sigma), 1 µg/ml cAMP-dependent protein kinase inhibitor peptide and 100 µCi/ml γ-^{32}P ATP. The reactions were terminated by spotting 20 µl of the reaction mix onto phosphocellulose p81 filters (Whatman) followed by 5 washes in 1% phosphoric acid. Incorporation of ^{32}P into histone H1 was determined by scintillation counting. Protein in the lysates was estimated by the method of Bradford (1976).

Metabolic Labeling

For assessment of protein synthesis by uptake of ^{35}S-methionine, spermatocytes were either resuspended in a methionine/cysteine-deficient MEM-α (ICN Biomedicals) medium, or after a period of culture in the standard media, centrifuged and resuspended in the

deficient media before addition of 0.5 μCi/μl Trans ^{35}S-label (ICN Biomedicals, >1000Ci/mmol). The amount of ^{35}S-labeled compounds incorporated into protein was determined by precipitation of 5 μl of lysate with 10% TCA collected on glass fiber filters (GF/A;Whatman) followed by scintillation counting.

CHAPTER III

RESULTS

OA induces rapid and premature transition of pachytene spermatocytes from prophase toward M phase

In these experiments, we investigated the cytology of the effect of OA on the progression of pachytene spermatocytes through the G2/M-phase boundary. Previously, we reported (Handel et al., in press) that OA, in concentrations of 1 μM - 10 μM, initiates rapid chromosome condensation in a dose-dependent manner. Here, cytological methods were used to determine the time course for this process. Cell stages were scored from air-dried meiotic preparations, according to their levels of chromatin condensation and morphology of chromosomes. Fig. 1 illustrates the cytological appearance of the specific cell stages that were scored from late prophase to metaphase I. The presence of significant numbers of cells in intermediate stages between pachytene and MI, namely in the morphologically recognizable substages of late pachytene and diplotene, implies that meiotic progression has occurred, rather than just a premature, rapid chromatin condensation, as seen in some instances of OA response (Dyban *et al.*, 1993). Other cytological changes (see below) also indicate true progression through the G2/M border rather than merely condensation of chromatin. Fig. 2 illustrates the time course of the G2/M transition. Very few (2-4%) control spermatocytes, not treated with OA, progressed to the M phase; these metaphases were the ones scored as controls for studies described below. This is consistent with the known timing for spermatogenesis in vivo; the development of spermatocytes from mid-pachytene through to MI normally takes several days. In contrast, 2 μM OA induced a significant change in the numbers of cells in each of

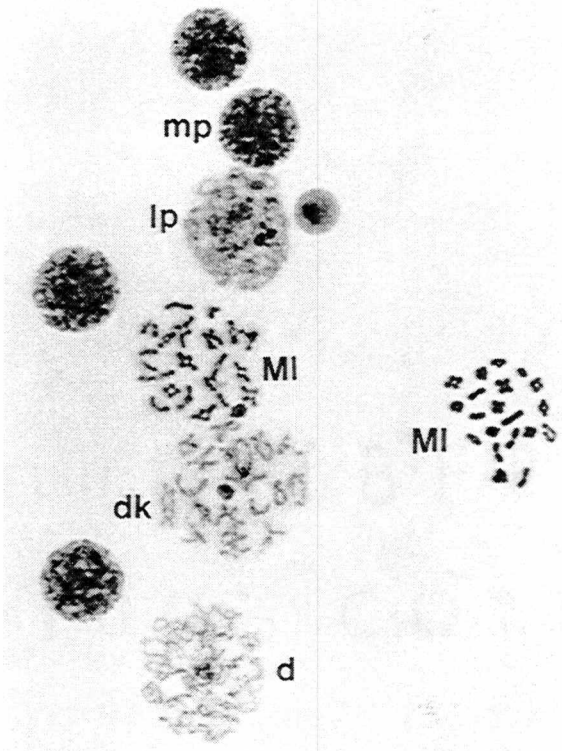


Fig. 1. Bright field micrograph of air-dried pachytene spermatocytes cultured for 2 hours in 5μM OA-supplemented medium. The OA-induced rapid progress of pachytene cells through to MI can be visualized in a number of distinct prophase substages shown here, mid-pachytene (**mp**), late-pachytene (**lp**), diplotene (**d**), diakinesis/M1 (**dk**), and fully condensed MI (**MI**). The round spermatid seen here is not a result of meiotic progression, but is a contaminant of the cultures. Note features of increasing chromatin condensation, and the presence of the sex body (arrows). Bar = 10 μm.

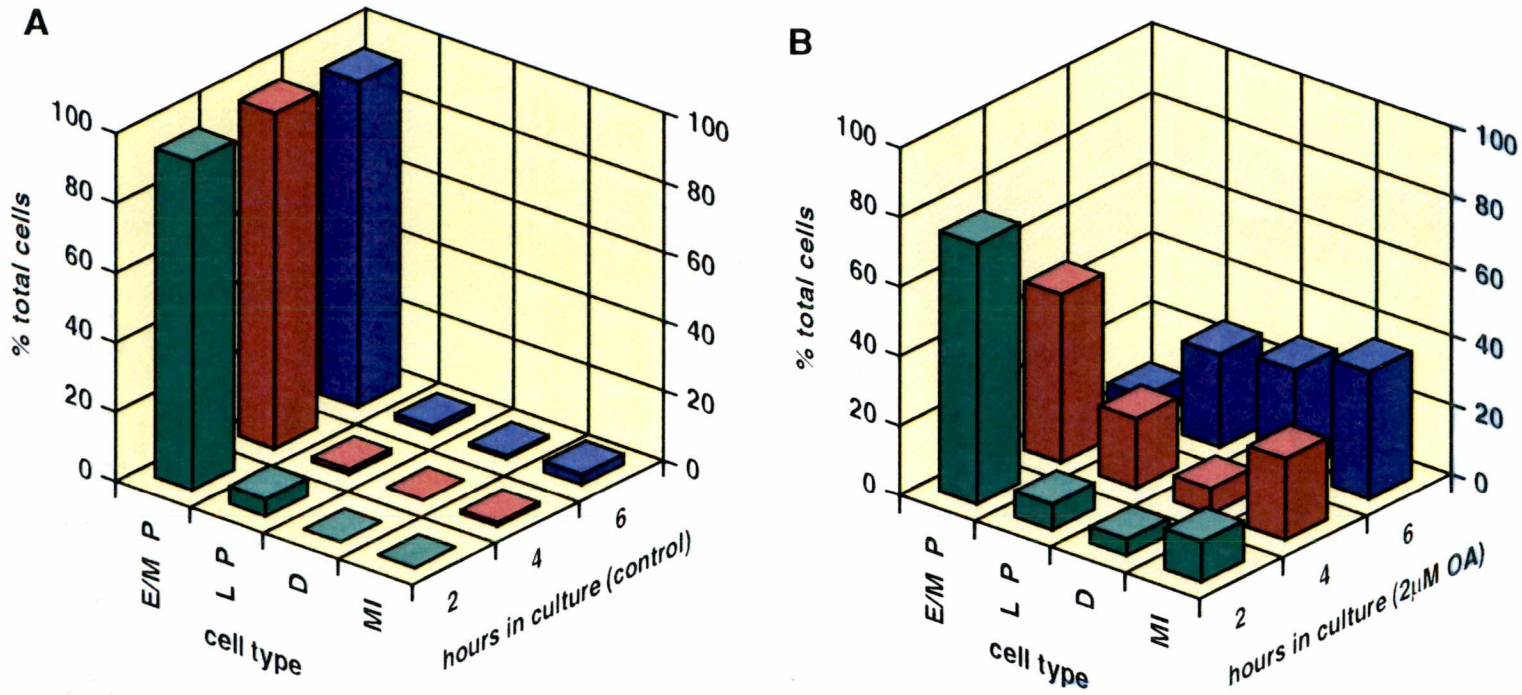


Fig. 2. Time course of OA-induced meiotic progression, from mid-pachytene spermatocytes at the outset of culture through to M-phase at 2 hours ■ , 4 hours ■ , and 6 hours ■. Cells were scored according to the categories shown in Fig 1. **A**) For untreated cultured cells there is little change observed in the distribution of stages over 6 hours. **B**) Cells treated with 2μM OA show significant changes in stage distribution, with a rapid increase in the number of metaphase figures, as well as in the intermediate stages.

the developmental stages of late prophase/metaphase. After 6 hours of culture over 60% of cells progressed to diplotene/MI stages. After treatment with higher concentrations of OA (5 μM - 10 μM), over 80% of the cells progressed to M phase within 6 hours (Handel *et al.*, in press).

The G2/M transition induced by OA treatment involves events unique to meiosis as well as events common to mitosis

The cytological hallmarks of the typical mitotic cell-cycle G2/M transition include nuclear envelope breakdown and chromatin condensation. The cell-cycle transition of spermatocytes also involves several events not occurring in a mitotic cell cycle. These include disassembly of the synaptonemal complex as well as resolution of sites of recombination as chiasmata. It is particularly important to assess whether these events occur in pachytene spermatocytes induced to the G2/M transition. At the time of OA treatment, these cells were in mid-prophase, a time when recombination is generally thought to be occurring; it was not known if recombination events could be completed to the stage of chiasma formation when a premature cell-cycle transition was induced. We wished to determine if chromosomes would condense as bivalents or as univalents, if the synaptonemal complex would disassemble, and if chiasmata would be formed in a premature induction of the M phase.

We assessed the ultrastructure of cells in the transition from the pachytene stage of meiotic prophase to M phase. Untreated spermatocytes exhibited the characteristic morphology of a pachytene nucleus, including the tripartite synaptonemal complex and sex chromosomes condensed to form the sex body (Fig. 3a). After treatment for 2 hours with 2 μM OA, most cells exhibited some condensed chromatin but profiles of synaptonemal complex were still visible (Fig. 3b). After 4-6 hours of treatment, the chromatin became

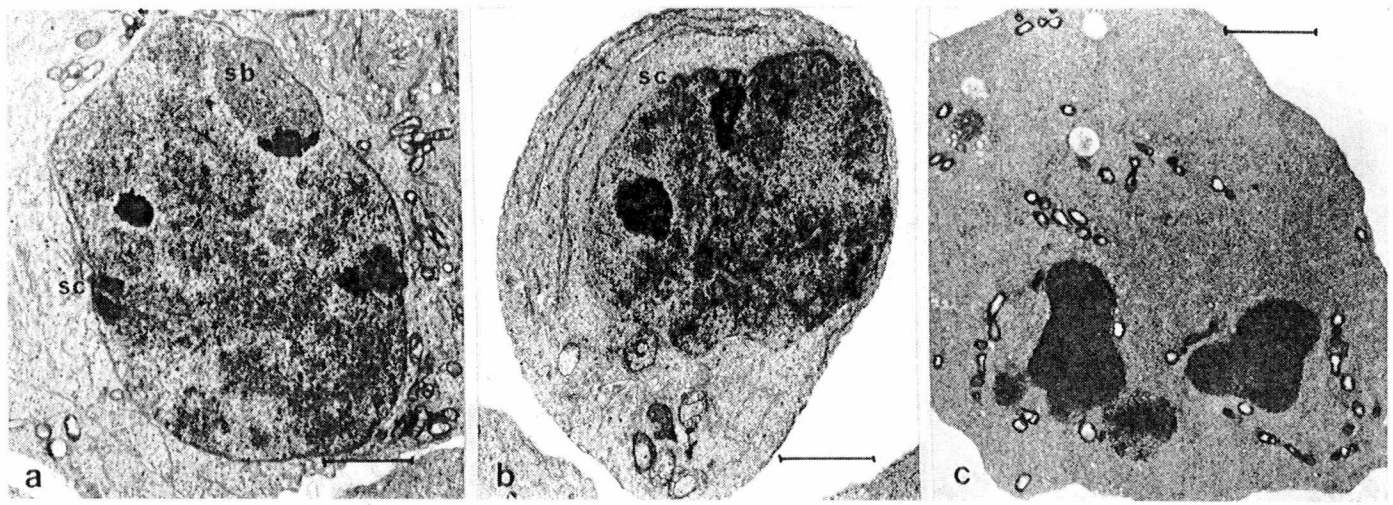


Fig. 3. Electron micrographs of pachytene spermatocytes cultured without OA (**a**) or with 2μM OA (**b**,**c**). **a**) Typical untreated spermatocyte nucleus showing a sex body (**sb**) and synaptonemal complex (**sc**). **b**) Nucleus of spermatocyte cultured for 2 hours in the presence of 2μM OA, showing condensed chromatin and a few profiles of synaptonemal complex (**sc**). **c**) Spermatocyte cultured for 6 hours in the presence of 2μM OA. At this time, chromatin is highly condensed and no synaptonemal complex is seen. Bars = 2μm.

quite condensed and synaptonemal complex was no longer visible (Fig. 3c). These observations suggest that disassembly of synaptonemal complex occurred during the induced and premature G2/M transition, just as it normally does at the end of meiotic prophase.

From Fig. 3 it can also be seen that the cell-cycle transition was accompanied by breakdown of the nuclear envelope, which was expected since it had been observed in other kinds of cells treated with OA. Additionally, cells treated with OA were characterized by the presence of many membranous vesicular structures (Fig. 4).

OA may have an effect on cellular membranes other than the nuclear envelope, since such a proliferation of membranous profiles was not seen in testicular or untreated cultured spermatocytes in the late meiotic prophase - early metaphase stages. Additionally, although the mitochondria appeared normal in morphology, they regularly were found clustered at one side of the cell with the nucleus likewise eccentrically situated, apparently as a result of the centrifugation used to collect these cell pellets. These internal rearrangements were not seen in similarly centrifuged untreated cells, and may therefore indicate disruption of cytoskeletal elements.

Since we induced a G2/M transition in meiotic prophase cells at a stage when it is thought that events of recombination are occurring, we particularly wanted to know if recombination events (crossovers) could be resolved as typical chiasmata during the rapid and premature cell-cycle progress. Fig. 5a illustrates that condensed chromosomes are present as bivalents, not as univalents, and that chiasmata were seen in the prematurely condensed chromosomes of OA-treated cells. To determine the mean chiasmata count in OA-treated cells, we scored chiasmata number from 20 diakinesis/metaphase figures. The controls for this analysis were from among the low percent of cells that undergo the G2/M transition in cultures not treated with OA; here too we counted chiasmata in 20

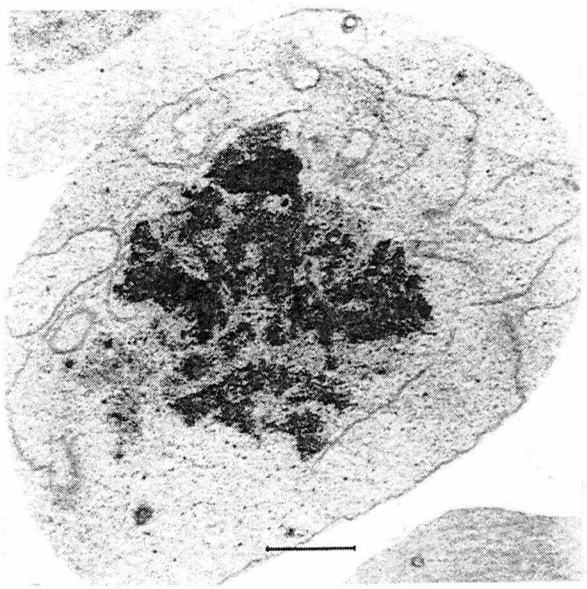


Fig. 4. Spermatocyte cultured for 4 hours with 2μM OA; note the proliferation of membrane profiles. Bar = 2μm.

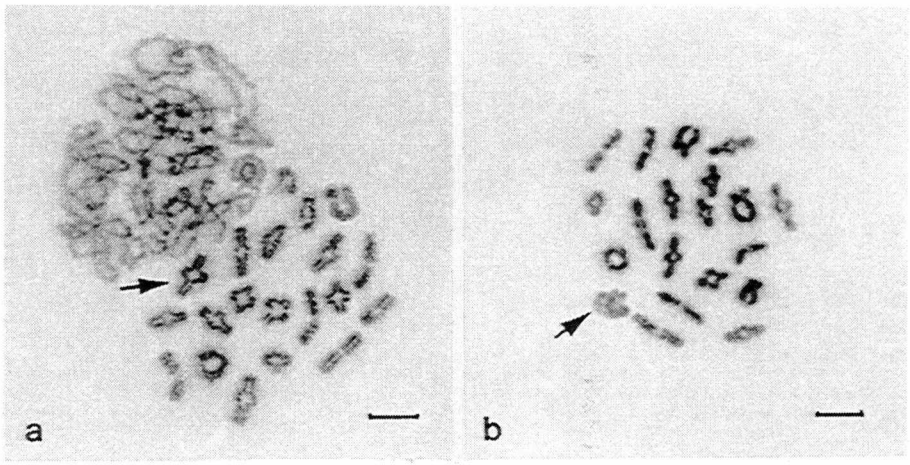


Fig. 5. Air-dried preparations of OA-induced condensed metaphase figures reveal typical patterns of chiasmata, and chromosomes are present as bivalents (not as univalents). Spermatocytes were treated with 5μM OA for 2 hours. **a**) This figure illustrates chiasmata (arrow) seen in condensed chromosomes after OA treatment. **b**) In this metaphase the sex chromosomes are still in the sex-body configuration (arrow) that is characteristic of pachytene and diplotene substages but not characteristic of normal metaphase. Bars = 10μm.

diakinesis/metaphase figures. We found no significant differences in the chiasmata counts in these two populations, with an average of 1.09 chiasma per bivalent in control cells and 1.08 per bivalent among the metaphases from treated cells. Thus, chiasmata were formed in normal numbers in the OA-treated cells; however, even though the chiasmata were functional to maintain homologous chromosomes in bivalents, it was not possible to determine if the chiasmata were functional at the metaphase/anaphase transition or if they represented normally completed recombination events.

Observation of air-dried meiotic chromosome preparations revealed an asynchrony in the behavior of the autosomal and sex chromosomes in response to OA treatment. Autosomal chromosomes exhibited rapid condensation induced by OA, while frequently, the condensation of the sex chromosomes lagged or did not occur (Fig. 5b).

Table 1 shows that a significant number of metaphases from treated cells also have a clearly defined sex body, and the frequency of these cells increases with increased OA concentration. Among the infrequent metaphase figures from cells not treated with OA, various degrees of condensation of the X and Y chromosomes with respect to the autosomes were seen; however, fully formed sex bodies, as seen in the OA-treated cells, were not observed. This observation correlates with others that many aspects of the meiotic behavior of the sex chromosomes of spermatocytes is at variance or temporally asynchronous with respect to the behavior of the autosomes (Handel and Hunt, 1992).

Spermatocyte progress through the G2/M transition is accompanied by an increase in histone H1 kinase activity

In mitotic cells as well as meiotic oocytes, progress into the M phase is accompanied by an increase in histone H1 kinase activity, and this activity is generally considered to be attributable to MPF. We found that OA treatment of spermatocytes induced a 2.5-fold

TABLE 1. Effects of Concentration and Time of Treatment of Okadaic Acid on Autosomal and X-Y Pair Chromosome Condensation

O.A. Conc (μM)	Hours of Treatment		
	2	4	6
0	0[1]	0	0
2	20	27	29
5	52	50	45

[1] These data indicate the percentage of metaphase I figures that also show a clearly defined sex body.

increase in histone H1 kinase activity over levels present in untreated pachytene spermatocytes (Fig. 6). It would be interesting to know whether this level of H1 kinase activity reflects that in spermatocytes spontaneously undergoing the G2/M transition; however, it is not possible to isolate these cells for such an analysis.

The OA-induced G2/M transition is independent of protein synthesis

Pachytene spermatocytes cultured with OA rapidly underwent the premature G2/M transition even in the presence of the protein synthesis inhibitor cycloheximide. Fig. 7 shows that 10 μg/ml cycloheximide did not significantly affect the rate at which cells progressed to M phase during culture with 5 μM OA. The fact that 75% of cells progressed to M phase after 6 hours of culture in the presence of both cycloheximide and OA implies that protein synthesis is not required for this progression. Cycloheximide alone added to culture media had no effect on spontaneous progression to M phase as both control cultures and cycloheximide-treated cultures (without OA) had <4% MI figures.

Previous studies have shown that OA can inhibit protein synthesis (Cohen *et al.*, 1990; Schwartz and Schultz, 1991). Biosynthetic labeling was performed to evaluate the effect of OA on spermatocyte protein synthesis, and the incorporation of ^{35}S-methionine was assessed following OA and cycloheximide treatments. Treatments with cycloheximide and OA both at the time of addition and 2 hours prior to the addition of ^{35} S-methionine were made to assess how rapidly these agents could affect the spermatocytes. Results (Fig. 8) indicate that OA treatment did decrease overall protein synthesis as compared to untreated cells; however, treatment with cycloheximide had a far more profound effect in decreasing protein synthesis. Taken together, these data (Figs. 7 and 8) suggest that while protein synthesis was not required for the spermatocyte G2/M transition, inhibition of protein synthesis did not itself induce the cell-cycle transition.

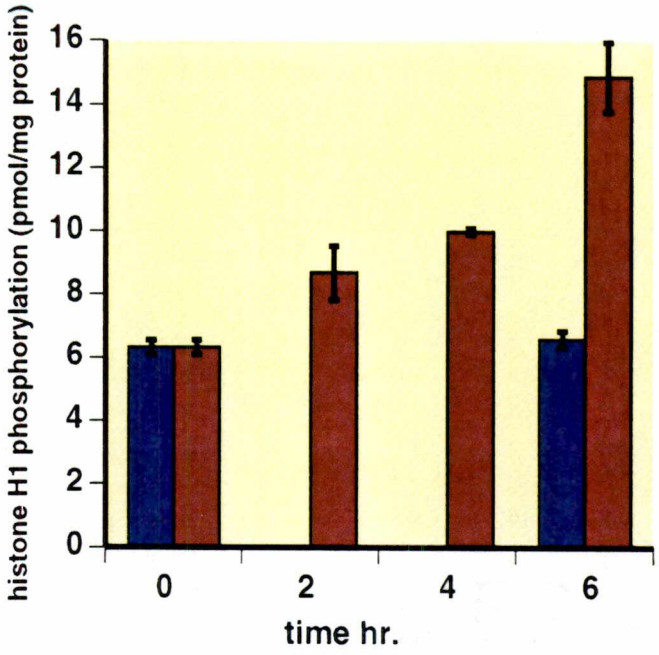


Fig 6. The effect of treatment with OA on histone H1 kinase activity. Pachytene spermatocytes were cultured in the presence of 10μM OA ■, for up to 6 hours and histone H1 kinase activity assessed in cell lysates. OA treatment induced a 2.5-fold increase in histone H1 kinase activity . At 6 hours over 80% of treated cells have progressed to M phase. Controls ■, which have no added OA, maintain a constant level of histone H1 kinase activity.

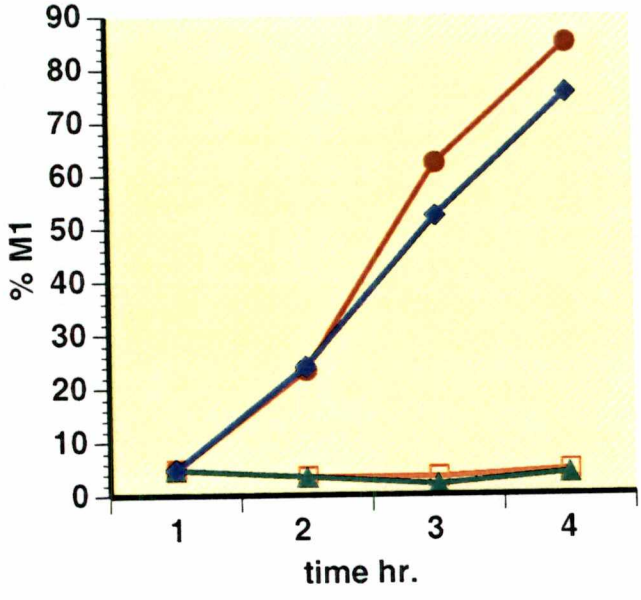


Fig. 7. Time course of progression to M phase by pachytene spermatocytes incubated in medium containing either OA and/or cycloheximide. Spermatocytes were incubated in medium containing 5 μM OA (●), or 5 μM OA and 10μg/ml cycloheximide (◆), or 10μg/ml cycloheximide (▲), or no additives (□). Data were pooled from repeat experiments and expressed as the mean.

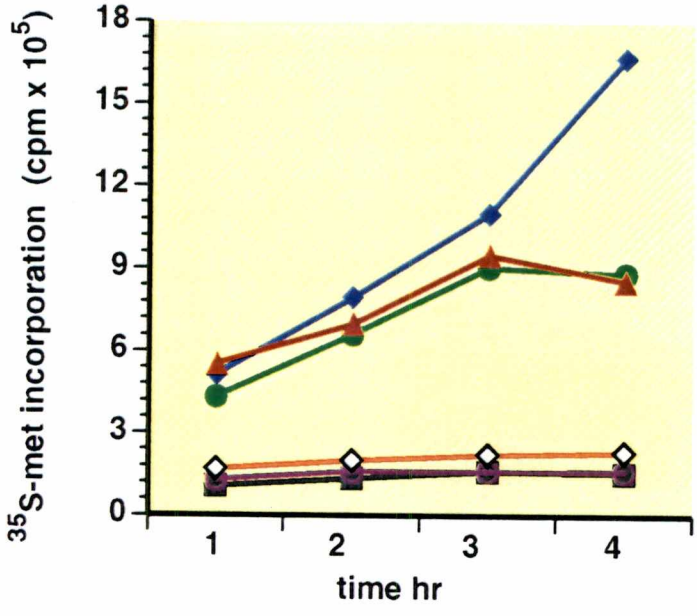


Fig. 8. The effects of OA and cycloheximide on spermatocyte protein synthesis were evaluated by ^{35}S-radiolabelling of cell proteins. Spermatocytes were incubated in media containing 5 µM OA (●,▲) or 10 µg/ml cycloheximide (○,■), or 5 µM OA and 10 µg/ml cycloheximide (◇), or in controls with no additives (◆). Cultures were carried out with cycloheximide and OA added at the time of ^{35}S-methionine addition (◆,●,◇,■), or added 2 hours prior to the addition of ^{35}S-methionine (▲,○). Data were pooled from repeat experiments and expressed as the mean.

CHAPTER IV

DISCUSSION

OA treatment of cultured pachytene spermatocytes induces within several hours a rapid and premature transition to M phase, a process that normally takes several days. This transition involves events unique to meiosis including disassembly of synaptonemal complexes and resolution of recombinations as cytologically visible chiasmata. The progress of pachytene cells to M phase is dependent on the concentration of OA, and at levels of 5 μM OA or greater, virtually all of the cells, representing stages of early to mid and late pachytene, undergo the cell cycle transition. The induced G2/M transition is accompanied by a nearly three-fold increase in histone H1 kinase activity and does not require protein synthesis. Since OA perturbs the phosphorylation status of common cell-cycle regulators, these results, coupled with other studies, suggest that these regulators may also be involved in the spontaneous G2/M transition of mouse spermatocytes. Events unique to meiosis may be directly or indirectly tied to cell-cycle regulation and may involve the phosphorylation status of the relevant proteins.

Cell-cycle regulators and OA effects on G2/M transition in pachytene spermatocytes

These results confirm previous observations reported for oocytes of a variety of species demonstrating that okadaic acid induces a G2/M transition that can be associated with an increase in MPF activity (Alexandre *et al.*, 1991; Gavin *et al.*, 1991; Gavin *et al.*, 1994; Goris *et al.*, 1989; Hampl and Eppig, 1994; Jessus *et al.*, 1991; Picard *et al.*, 1989; Picard *et al.*, 1991; Rime *et al.*, 1990; Rime and Ozon, 1990; Schwartz and Schultz, 1991).

The effects of okadaic acid on the cell cycle are thought to occur by a pathway that involves inhibition of a phosphatase that in turn inhibits the action of the cdc25 phosphatase essential for activation of MPF, although other pathways are not excluded (Cohen *et al.*, 1990).

The fact that okadaic acid induces the G2/M transition and a concurrent increase in histone H1 kinase activity in spermatocytes has several implications. First, these observations suggest that both the spontaneous and the induced G2/M transitions could be regulated by MPF activation. Further support for this idea comes from the finding of relevant transcripts and protein components of MPF (Chapman and Wolgemuth, 1992; Chapman and Wolgemuth, 1993; Chapman and Wolgemuth, 1994) in spermatocytes. Additionally, both this study and that of Chapman and Wolgemuth (1994) report H1 kinase activity, generally assumed to represent MPF activity, in pachytene spermatocytes. Thus, it is anticipated that the in situ G2/M transition of mouse spermatocytes involves cyclins and the p34^{cdc2} components of MPF. However, this hypothesis is not proven; both MPF and histone H1 kinase activity are present in the right cells at approximately the right time, but it has not been demonstrated that inhibition of MPF activity inhibits the G2/M transition in these cells either in vivo or in vitro, and thus alternative pathways for the G2/M transition are not excluded. In this regard it should be noted that the increases in histone H1 kinase that we have documented are modest, about 3-fold. Interestingly, it has been found for mouse oocytes that a small stimulation of histone H1 kinase may account for germinal vesicle breakdown, but that a greater (8-fold) increase in activity occurs at the time of metaphase (Gavin *et al.*, 1994; Hampl and Eppig, 1994). There is no further increase in histone H1 activity following okadaic-acid stimulation of oocytes, either at germinal vesicle breakdown or during the following hours of culture, and therefore, okadaic acid may activate an alternative pathway leading to germinal vesicle breakdown that is MPF-independent and may involve p42^{MAPK} activation (Gavin *et al.*, 1994). It is possible

that meiotic progress in OA-treated cells is mediated in part or in full by other kinases.

An additional implication of these observations is that normally the transition from pachytene through diplotene to the meiotic divisions must be stringently controlled. Our observations and those of Chapman and Wolgemuth (Chapman and Wolgemuth, 1992; Chapman and Wolgemuth, 1993; Chapman and Wolgemuth, 1994) suggest that pachytene cells contain both active MPF (assayed as H1 kinase) and pre-MPF, capable of being activated, since an increase in histone H1 kinase activity was found after treatment with OA. Yet, in spite of the fact that pachytene spermatocytes may have sufficient precursors to drive meiosis, meiosis does not occur and, in fact, the transition toward the division phase occurs very slowly (over days). Regulation of MPF (and/or other kinases) must therefore be a key element in controlling the timing of the meiotic divisions. How might kinase activity be regulated in spermatocytes? Clearly, cyclins that can form active MPF are present in pachytene spermatocytes (Chapman and Wolgemuth, 1992; Chapman and Wolgemuth, 1993; Chapman and Wolgemuth, 1994); however, other activating elements might act very slowly. The normally slow transition may involve translational regulation of the MPF components or regulators, as has been found to control the gradual increase in histone H1 kinase activity observed over the several hours of mouse oocyte meiotic maturation (Hampl and Eppig, 1994) and during oocyte growth (Chesnel and Eppig, 1994). Alternatively, it may be that the necessary components for the G2/M transition are present and potentially active in pachytene spermatocytes but are sequestered in cytologically inactivated configurations, as has been proposed for mouse oocytes (Albertini, 1992).

Although the effects of okadaic acid on spermatocytes are similar to those observed in oocytes, there are important differences. First, pachytene spermatocytes are in an earlier stage of meiotic prophase than are grown oocytes; thus more and different events

(associated with the completion of meiotic prophase) are involved than in OA-induced meiotic maturation of oocytes. Second, there are regulatory differences in cell cycle control. Spermatocytes have a continuous cell cycle in that they are not arrested at the diplotene (dictyate) stage, and additionally, it is known that the c-mos protein is required for oocyte maturation, but not for normal spermatogenesis (Colledge *et al.*, 1994; Hashimoto *et al.*, 1994). How these differences might affect the details of cell-cycle regulation is not known.

An important and unresolved question is the extent to which the treated spermatocytes are impaired by okadaic acid and how this might affect their cell-cycle progress. Clearly, okadaic acid has many effects on cells resulting from general inhibition of protein phosphatases types 1 and 2A, including effects on metabolism, glucose transport, and ion flux (Cohen *et al.*, 1990). These effects may in fact account for abnormalities such as the observed repositioning of organelles after centrifugation as well as the proliferation of internal cellular membranes that we and others have observed (Alexandre *et al.*, 1991). Additionally, and importantly for investigations of cell-cycle transition, continuous treatment with okadaic acid inhibits formation of the spindle apparatus (Alexandre *et al.*, 1991; Kalous *et al.*, 1993; Rime and Ozon, 1990). Our ultrastructural analysis confirmed the lack of a normal metaphase-I spindle, and the highly condensed bivalents observed remain scattered rather than associated in an orderly manner with a spindle. This was also found by Kalous (1993). The ultimate test of success and normalcy of the induced G2/M transition would be to monitor progress through the two meiotic metaphases and determine if the resulting haploid cells were competent to undergo spermiogenesis; this has not been possible with these methods.

Cytogenetic events of the meiotic G2/M transition

These studies provided an unprecedented opportunity to examine the relationship of events unique to meiosis, such as resolution of genetic recombination events as chiasmata and disassembly of the synaptonemal complex, to cell-cycle control. Chiasmata, the visible outcomes of genetic exchange events, are important to ensure the precise and regular segregation of homologous chromosomes at the meiosis I anaphase (Carpenter, 1994) and aneuploidy is associated with reduced recombination of the relevant chromosome (Hassold *et al.*, 1993). Thus, production of euploid gametes requires that recombination occurs and is completed prior to segregation of chromosomes.

In this study we were interested in events associated with recombination, since the cells were treated with OA during the stage where recombination is thought to be occurring. Disassembly of synaptonemal complex occurred as part of the OA-induced G2/M transition. Chromosomes in OA-treated cells were present as bivalents; univalents would be expected if recombination had not occurred or had been disrupted. Chiasmata were present in the condensed chromosomes of OA-treated cells and the chiasmata frequency in the OA-treated cells was similar to that in cells that entered M phase spontaneously. Thus, resolution of recombination events as chiasmata that maintain homologs in bivalent configuration seemed to occur, although further functional competence of these chiasmata could not be assessed in the absence of a spindle. Finding the intermediate substages of late prophase (late pachytene and diplotene), suggested true cell-cycle progress in OA-treated spermatocytes rather than chromatin condensation only, such as has been found for mouse zygotes treated with OA early in the cell cycle (Dyban *et al.*, 1993). Taken together, these observations indicate that meiotic events, involving formation of chiasmata and the condensed chromosome morphology that ensure successful anaphase segregation, occur during the premature OA-induced transition to M phase. Thus, events unique to meiosis

might indeed be controlled, directly or indirectly, by universal cell-cycle regulators.

An intriguing observation was that the disappearence of the sex body and individualization of the sex chromosomes was temporally asynchronous, lagging with respect to autosomal chromosome condensation. During pachytene the nonhomologous sex chromosomes of the spermatocyte form a unique domain known as the sex body, where chromatin is more condensed and transcriptionally inactive, in contrast to the autosomes (Handel and Hunt, 1992). This behavior may be related to the general lack of recombination in the sex chromosomes (McKee and Handel, 1993). Chromatin of the sex body may be more resistant to experimental disruption by agents binding to DNA, such as actinomycin D (Handel *et al.*, in press). Therefore, the sex body chromatin may lag in the induced G2/M transition because the relevant effectors (such as MPF, other kinases, or phosphatases) cannot gain access to the chromatin, or that the chromatin of the sex body may be sufficiently different in protein composition from the autosomal chromatin that its response to OA differs.

What happens during pachytene and why is it so lengthy?

It was an unexpected observation that OA induced such seemingly normal morphological aspects of the meiotic G2/M transition in pachytene spermatocytes. Even if recombination is initiated prior to pachytene (Hawley and Arbel, 1993; Padmore *et al.*, 1991), it has previously been assumed that the main events of recombination and conversion of recombination events to chiasmata occur during pachytene (Padmore *et al.*, 1991). The time interval from mid-pachytene to MI metaphase in mouse spermatocytes is normally days, not hours, as is the case in the OA-induced M-phase transition. The supposition might previously have been that the process takes days because this much time is needed to carry out the genetic events of recombination. However, our results suggest

that the recombination events have reached the point where chiasmata can be formed, consistent with recent data suggesting that important events in the recombination pathway have occurred by early to mid pachytene (Collins and Newlon, 1994; Schwacha and Kleckner, 1994). This is interesting since previously there was no information about when competence to form chiasmata might arise relative to the time when they are visible, at the end of meiotic prophase.

Thus, surprisingly, it may not be the genetic events of pachytene that account for the length of time that the spermatocyte spends in this phase of its differentiation. Instead, as indicated by the dramatic increase in size of spermatocytes during pachytene, the length of pachytene may be determined by the need to synthesize precursors that will be used for postmeiotic spermiogenesis. In other words, pachytene may be the spermatogenic equivalent of the arrested diplotene (dictyate) period of oocyte growth (Eppig, 1993).

Cell-cycle checkpoints describe the mechanism by which cells assess the completion of events required for successful progress through the cell cycle and production of euploid cell products (Hartwell and Weinert, 1989). There may be a number of spermatogenic meiosis cell-cycle checkpoints and these may assess both completion of recombination and accumulation of precursors for spermiogenesis. Treatment with OA seemingly abrogates a checkpoint that normally holds mid-pachytene spermatocytes in prophase for several more days. Since OA inhibits several protein phosphatases and perturbs protein phosphorylation, the phosphorylation status of critical proteins may be an important element of spermatogenic meiosis cell-cycle control.

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PART V

SUMMARY AND FUTURE DIRECTIONS

CHAPTER I

Investigations in mammalian meiotic prophase

The unique events of meiosis include the pairing and recombination of homologous chromosomes during prophase of meiosis I. Completion of these events is essential for the production of chromosomally balanced gametes. The sequence of events of meiosis I, the mechanisms, and genes involved are slowly being unravelled, mostly from studies in yeast; still little is known about these events in mammalian meiosis. These events are most easily studied in male gametogenic cells (meiotic spermatocytes), as meiosis occurs during adult life (while in the female these events occur during fetal life). In this study some of the basic mechanisms governing the structure and behavior of chromosomes, and the timing of events during male mammalian meiotic prophase are investigated.

The focus of this research has been on analyses of specific events of meiosis as they occur in mouse spermatogenic cells. This has developed as three main areas of work: defining chromatin structure and function during prophase I, cell culture to establish a system for experimental analysis of meiosis, and cell cycle analysis. This summary describes the continuation of these areas of work and some suggestions for future research. The possible directions are exciting, and have the potential to open up new areas of investigation in mammalian meiosis.

Chromatin structure and behavior during prophase I

Although a great deal is known about the relationship between chromatin configuration and gene transcription, remarkably little is known about the structure of

chromatin involved in the meiotic process of homology recognition, chromosome pairing and recombination. As previously reviewed, a number of observations has led to the conclusions that the chromatin domain of the sex chromosomes is structurally different from that of the autosomes in pachytene spermatocytes. The sex chromatin is also functionally different from the autosomes in that it is transcriptionally inactive, is not fully paired, and is involved in recombination, whereas autosomal chromatin is paired and recombining. The inactivation does not appear to be a requirement for the metabolic needs of the germ cell, and the control of this sex-chromosome inactivation in spermatogenic cells does not appear to involve the same stabilization events as for X-inactivation in females. In addition, the formation of this X-Y body does not require an intact Y chromosome, or the Y pairing region, and does not depend on successful X-Y pairing (Handel *et al.,* 1994).

The reasons for this behavior of the X-Y pair during meiotic prophase are not known. As previously mentioned, the work described in Part II was developed to test a hypothesis that this heterochromatic conformation may be a means of preventing potentially damaging recombination events in non-homologous chromosomes. The investigation had two key approaches: first, to demonstrate that differences in chromatin structure do exist between the autosomal and sex chromatin domains, and second, to correlate differences with meiotic events occurring during prophase I.

This study showed that differences in nuclease sensitivity do exist between the X-Y pair and autosomes during the pachytene stage of prophase. Most studies have related DNase-I sensitivity to transcriptional activity; however, the accessibility of DNA to the transcriptional machinery, and to enzymes of recombination, almost certainly depend on similar features of chromatin conformation. The idea that recombination events are regulated by chromatin conformation is also consistent with molecular studies of recombination hotspots (reviewed in Lichten and Goldman, 1995). In both yeast

(Stapleton and Petes, 1991) and mice (Shenkar *et al.*, 1991) hot spots for meiotic recombination have been mapped to sites coincident with gene promoters or enhancers. Recent studies in yeast have shown that an important feature of recombination events is the appearance of DNA double strand breaks (DSB), and all DSB sites examined to date have been found to lie in regions of chromatin that are DNase I hypersensitive (Wu and Lichten, 1995).

At the cytological level, in another study, we used the methods of DNase nicking and nick-translation to assess the overall differences in chromatin configuration between the non-recombining X-Y pair and recombining autosomes during pachytene stages. The X-Y pair was more resistant to nuclease attack. These cytological observations are bolstered by data reported here on DS of specific gene sequences, which allows a more critical and definitive assessment of differences in sex chromatin from that of autosomal chromatin.

Genomic regions were scanned with complete cDNA probes to assess generalized DNase I sensitivity rather than DNase I hypersensitivity (DHS) associated specifically with transcriptional activity. Nonetheless, the method detected hypersensitive regions as well. The probes used varied from cDNAs that recognize quite large genomic regions, to more specific partial cDNAs, and some probes that recognize non-coding sequences as well (see Appendix 1 for probe data, and Appendix 2 for genomic data of probe recognition site).

It was originally proposed that this study should make specific comparisons between DS of gene sequences in pachytene spermatocytes and the same sequences in somatic cells. This comparison would have given information regarding changes that had occurred, if any, in meiotic chromatin conformation, specifically regarding the X and Y chromosomes. It proved to be difficult, and not necessarily valid, to make those comparisons for several reasons. The isolation of nuclei is usually undertaken utilizing one of two methods; non-ionic detergents to lyse cells or homogenization of cells. Pachytene

spermatocyte nuclei could not easily be isolated using the generally preferred former method. The numbers of nuclei available are strictly limited, and even very low levels of detergent made these nuclei sticky and very difficult to recover as a useable nuclear substrate. Homogenization methods worked well with spermatocytes; perhaps because there are relatively very low levels of endogenous DNase, which can become active during homogenization. The somatic tissues tested, liver and spleen, exhibited high levels of endogenous DNase. Thus, it was not possible to utilize the same isolation method for nuclei from germ cells and somatic tissue. Furthermore, since different tissues have varying amounts of endogenous nucleases, comparisons of exogenous DNase concentrations are difficult to evaluate.

Despite these difficulties some further controls are needed for this study prior to publication. Now that an array of data has been compiled for DS sensitivity of X-linked and autosomal sequences within pachytene spermatocytes, it should be possible to examine the same sequences in a somatic cell type. The comparison to be made is not of the DS of sequences between different cell types, but of the relative difference in DS of X-linked and autosomal sequences between somatic and meiotic cells. This work is now in progress.

Analysis of the DS of both autosomal and sex-linked sequences clearly confirms that sequences on the X-Y pair, although not totally resistant to DNase I digestion, were generally more resistant than the autosomal sequences examined. This is in keeping with the cytology of the sex body, where the X-Y pair certainly appear in a more condensed chromatin configuration than autosomes during the pachytene stage. Our observations on DS raise two questions: first are we simply assaying a consequence of transcriptional activity of gene sequences by these DS assays? Second, is the differential condensation of sex chromosomes a result of a requirement to avoid promiscuous recombination? Neither of these questions can be answered directly with these data, although we have made some

progress that yields tantalizing clues.

With respect to transcription, these data showed that those gene sequences that are transcribed during the pachytene stage generally are in the upper range of DNase sensitivity; however, some genes that are not transcribed during pachytene, (e.g., *H-19*, and *Igf-2*) also showed quite high DS. A more detailed analysis of three regions of the *Igf-2* gene revealed that the promoter region had higher DNase sensitivity, suggesting that, although it is not transcribed, this region of chromatin is more open to nuclease attack. This is not surprising as many studies have shown DS and DHS to be linked to promoter regions. The reverse is true for *Tcp-10* which is transcribed in pachytene but shows low DS in the main band although it also has a strong hypersensitive site The *Tcp-10* probe, however, covers a smaller genomic region than do most of the other probes used.

These observations suggest that our assay is not assessing chromatin configuration resulting solely from transcriptional activity, but may indicate transcriptional potential. In the light of recent evidence regarding DS of recombinational sites, this may be equivalent to recombinational potential; thus, an open or accessible chromatin conformation is a common feature of both activities. In contrast is the behavior of heterochromatin. Centromeres are heterochromatic, highly compact, higher-order chromatin structures (Sumner, 1991). They are considered "cold spots" of recombination, and interestingly we found that pBSAT-1, the mouse major centromeric satellite consensus sequence dimer, was not DS. Finally a region of the *Eb* gene known to be a meiotic hot spot of recombination is highly DS although this region is not transcribed. Thus, these data are consistent with a link between DS and recombinational status of chromatin although they do not prove such a link.

The relationship between our observations and the hypothesis that formation of the sex body is a means to avoid illegitimate recombination between the X and the Y

chromosomes raises some important issues about the timing of events of meiosis. Information about the timing of the homology search, homolog pairing and the initiation of recombination events comes almost entirely from studies in yeast. Here the initiation of recombination events and formation of DSBs occurs very early in meiotic prophase prior to, or coincident with synapsis (Padmore *et al.*, 1991). If the same timing occurs in mouse we would predict the heterochromatin formation of the X-Y pair would occur early in meiotic prophase. However, evidence suggest that this is not the case. Cytologically the X-Y pair are not typically seen as a condensed region of the nucleus in leptotene or zygotene, but are in pachytene; although, some find that the sex-body is first evident late in zygotene (Monesi 1965). Additionally, evidence from several molecular studies indicates that X-linked genes are active in leptotene and zygotene prior to when they become transcriptionally inactive in the pachytene stage. The transcript for *Hprt*, for example, has been detected in L/Z cells but is not present in P cells (Shannon and Handel, 1992), indicating that the chromatin may still be open for transcription during L/Z. Thus, it is relevant that we have preliminary evidence that the sex pair is more sensitive to nuclease digestion during L/Z than during pachytene. These data would not support the hypothesis that the X-Y pair is condensed to prevent recombination if we assume that in the mouse, as in yeast, recombination is initiated at L/Z. Two factors complicate this conclusion. First, all our analyses have been with mixed populations of L and Z cells. Thus, cells representing the two stages that adjoin the proposed timing of the initiation of recombination are not resolved. We are addressing this by working with more highly enriched cell specific stages.

Second, and most importantly, there is as yet no evidence that mouse conforms to the same time scale as yet for the meiotic events of DSB formation. Perhaps in mammals, which have considerably larger and more complex genomes than yeast, homologs must be

fully paired (the end of zygotene) before recombination events can safely be initiated. In mouse (mammals) there are a lot of repetitive elements throughout the genome and pairing these regions between chromosomal homologs before any DSBs occurred would ensure recombination occurred only between homologs. In this view, the DSBs that initiate recombination might occur in pachytene, after full pairing is established, at the time we observe maximal DNase resistance of the sex chromosomes.

It is essential therefore to clarify issues of timing of meiotic prophase events. We plan experiments to detect DSBs in mice. Success is dependent both on the ability to obtain quite specific stages of prophase spermatocytes, and on sensitive detection methods. We are making progress on the former goal, and sensitive PCR methods that have been developed to detect DSBs in yeast (Xu, *et al.,* 1996) can be applied to mammalian spermatocytes. These studies may well allow us to determine the timing of DSB formation and the resultant recombination events. Results from this type of investigation will then allow us to further address the question of why the X-Y pair forms a heterochromatic region during pachytene.

Primary culture of spermatocytes

An impediment to the experimental investigation of prophase I in meiosis has been the lack of an appropriate system for experimental manipulation. In Part III we described a simple system for the short term primary culture of pachytene spermatocytes. As yet no one has defined a satisfactory culture system for differentiation of spermatogenic cells. It has been a somewhat intractable problem to establish a substitute for the Sertoli cell role in testis function. Our culture procedures are simple but allowed us to advance in the investigation of the prophase sub-stages of meiosis. Part III describes cytological and biochemical analyses of spermatocytes in culture to determine viability and the continuance

of normal cellular characteristics. In particular, we assayed parameters of cell function pertinent to meiotic prophase, namely chromosome pairing and synapsis. A number of experiments, not reported in our publication were carried out to ascertain the optimal short term culture conditions. Different combinations of various additives were tested to determine effects on cell viability, particularly in serum-free media. All reagents tested had previously been reported to improve spermatocyte viability; they included insulin, transferrin, EGF, retinol, epinephrine, and norepinephrine. While some of these reagents may be required for long term survival of spermatocytes, none of them produced significant improvements in viability, and instead, a relatively simple media that included fetal bovine serum was used for all culture studies.

In this system conditions are still inadequate for long-term progress as spermatocytes begin to lose viability after about 48 hours in culture. However, after 90-120 hours 25 -30% of cells still survive and evidence can be seen of progression through meiotic prophase. The future of cell culture systems for spermatocytes lies in more carefully ascertaining the intricate interactive signalling between spermatocytes and Sertoli cells and defining the delicate balance required to maintain the correct environment for meiotic germ cell transition. Certainly the best spermatocyte culture systems reported to date rely on co-culture of Sertoli cells and germ cells. Even then, complete spermatocyte development may depend unconditionally on the specialized architecture of the Sertoli cell, and cell-cell contacts. The major difficulty with co-culture systems at least in the study of meiotic prophase events are the problems associated dealing with, and analysis of, mixed populations of cells.

Events of the G2/M transition

The culture system described has facilitated analysis of meiotic cell-cycle events in spermatogenesis. Most investigations of mammalian meiotic cell-cycle have been done using oocytes; however, arrested oocytes have already progressed through the earlier prophase I stages. Only in fetal oocytes would it be possible, although technically very difficult, to investigate the meiotic stages up to the metaphase I arrest. Thus, isolated spermatocytes have been used to study the links between the genetic events of prophase I and the cell cycle transition to metaphase I. It has been assumed, perhaps correctly, that many of the principal molecules involved in the mitotic cell cycle would also be key players in the meiotic cell cycle. Recent studies have shown that both cyclin B1 and cyclin B2, regulatory cell cycle proteins, are both expressed in germ cells (Chapman and Wolgemuth, 1993). Also recently reported was the developmentally regulated pattern of expression of Cdc25 genes which encode protein threonine/tyrosine phosphatases that activate cyclin-dependent kinases in the cell cycle (Wu and Wolgemuth, 1995). These studies, however, did not assess developmental regulation during the G2/M transition. Some of the G2/M transition events that occur as spermatocytes progress through prophase I, are described in Part IV. Treatment of spermatocytes with OA led to a rapid and premature onset of metaphase I. These results were particularly surprising as it was expected that mid-pachytene spermatocytes in the midst of recombination could not proceed to metaphase so quickly. Mid-pachytene cells normally take at least two to three days to develop to the metaphase I (MI) division. Biochemical analysis of histone phosphorylation suggests that OA induces an increase in histone HI kinase activity. Histone HI kinase activity is assumed to represent MPF activity (although histone HI kinase may be only one of many kinases affected) and MPF is known to be a key molecular complex involved in cell cycle regulation. Protein synthesis assays using ^{35}S-methionine uptake demonstrated that

protein synthesis was not required for the pachytene to MI transition. These data have important implications for our understanding of the timing of events of meiotic prophase and cell cycle controls involved in the G2/M cell cycle transition, and raise new questions and directions.

An important and unresolved question is the extent to which the treated spermatocytes are impaired by okadaic acid and how this might affect their cell-cycle progress. Unfortunately for investigations of cell-cycle transition, continuous treatment with okadaic acid inhibits formation of the spindle apparatus (Alexandre *et al.*, 1991; Kalous *et al.*, 1993; Rime and Ozon, 1990). Our ultrastructural analysis confirmed the lack of a metaphase-I spindle in OA-treated spermatocytes, and the highly condensed bivalents remained scattered, rather than associated with a spindle. The ultimate test of success and normalcy of the induced G2/M transition would be to monitor progress through the two meiotic metaphases and determine if the resulting haploid cells were competent to undergo spermiogenesis; this has not been possible with these methods. We have tried several strategies to develop conditions permissible for anaphase and completion of meiosis. Attempts to wash out OA from treated cells was not successful; interestingly, spermatocytes treated in this manner appeared to progress to an interphase like state, rather than to any expected meiotic stage. It has been confirmed that this also occurs in Xenopus oocytes similarly treated (Risley. M., Pers. commun). Pulse treatments with OA at levels sufficient to initiate the G2/M transition, but not high enough to inhibit spindle formation have also been attempted. Results were not conclusive. Although it did seem that more round spermatids result from this treatment, this has not as yet been rigorously tested; it is possible, for example, that contaminating round spermatids simply have a higher survival rate in this treatment.

Important questions that have arisen from our studies are: when does a

spermatocyte become competent to undergo the G2/M transition? and, what factors determine this competency? There are most likely two contributing influences here. One is the complex events of genetic recombination that occur in meiotic prophase; it is reasonable to propose that these must reach a certain critical stage in order for the spermatocyte to proceed to metaphase. It has been well documented in yeast meiosis that genetic factors act as cell-cycle checkpoint controls; furthermore, it is known that there are continuous checkpoints in the mitotic cell cycle (for example DNA strand breaks must be repaired) (reviewed by Hunter and Pines, 1994). Failure to pass such checkpoints may have disastrous effects for the cell or the organism. A second factor contributing to spermatocyte competence is that the critical subcellular regulators must be present and active for progression through this transition.

We used primary spermatocyte cultures to investigate these issues. We find that spermatocytes in the zygotene stage, when OA-stimulated, are not competent to progress to MI. Results indicate that while these zygotene cells cannot progress, the earliest pachytene cells (those from 17 day old mice) are competent to undergo the G2/M transition (Figure 1). Interestingly, OA treatment does affect zygotene cells in that chromatin becomes quite fragmented and condensed (Fig 2) rather than condensing into chiasmate chromosomes. Thus, these studies reveal that competence for the G2/M transition is likely to be acquired early in the pachytene stage.

Studies of the biochemical requirements for the OA-induced G2/M transition are also continuing in our laboratory. Recently it was shown that Topo II plays a critical role in the G2/M transition. Blocking the activity of Topo II by use of specific inhibitors blocked the OA-induced G2/M transition. Additionally, although MPF activity increases in the OA-induced G2/M transition in pachytene spermatocytes (Wiltshire *et al.*, 1995), it is not known yet whether there is functional MPF in L/Z spermatocytes. This may well be

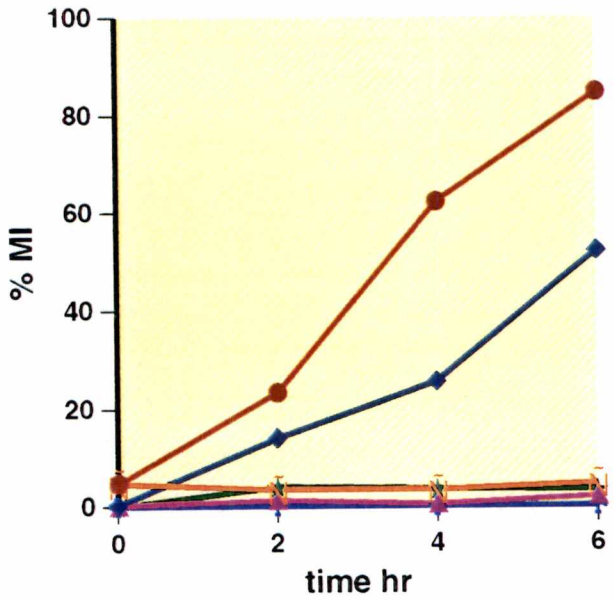


Fig 1. Time course of OA-induced meiotic progression shown as the percentage of cells that proceed through to an MI chromosome configuration. Spermatocytes were treated with 5 μM OA, and assessed at 2 hour intervals: ★ leptotene/zygotene spermatocytes, ◆ juvenile pachytenes, ● adult pachytenes. Controls are untreated and assessed at the same 2 hour intervals: ✣ leptotene/zygotenes, ▲ juvenile pachytenes, □ adult pachytene cells.

	Treatments								
	Control			2μM OA			5μM OA		
hours Cell Types	2	4	6	2	4	6	2	4	6
Normal LZ	76*	73	71	71	60	48	61	55	36
Condensed	1	11	9	13	20	21	20	17	25
Punctate	4	4	3	2	10	24	8	15	28
Other	19	12	17	14	10	7	11	12	11

* % of cells in each category

Fig 2. After OA treatment, chromatin of L/Z cells condenses but does not achieve the characteristic MI configuration of individual chiasmate bivalents, as is the case for OA-treated pachytene spermatocytes. Chromatin characteristics were scored as: normal L/Z, condensed (c), highly condensed and punctate (p), and other patterns.

one of the factors regulating competency for G2/M progression.

A final and intriguing observation in the OA treatment of spermatocytes was that the disappearance of the sex body and individualization of the sex chromosomes was temporally asynchronous, lagging with respect to autosomal chromosome condensation. The sex body chromatin may lag in the induced G2/M transition because the relevant effectors (such as MPF, other kinases, or phosphatases) cannot gain access to the chromatin, or that the chromatin of the sex body may be sufficiently different in protein composition from the autosomal chromatin that its response to OA differs. These observations are thus consistent with those we have made (Part II) demonstrating that pachytene sex chromosomes are relatively DNase resistant by comparison to the autosomes.

Conclusion

The studies described in this dissertation are in some ways diverse, yet all focus on events of mammalian meiotic prophase I. The different aspects of this study, while answering some questions about meiosis, have really come face to face with some of the central mechanistic questions of meiosis: what are the key elements in control of meiotic cell cycle events, and how and when does a meiotic cell process the complex exchange of genetic information? These questions are starting to be addressed in mammalian systems and these studies contribute to this research.

The establishment and characterization of a cell culture system for pachytene spermatocytes provides a useful tool to study reagents and possible effectors of meiotic processes. The study of cell cycle controls proved to be an important entry into investigation of the complex biochemical mechanisms involved in the progress through prophase I. Our studies assessing chromatin configuration of the X-Y pair and reasons for

the special behavior of the sex chromosomes during the pachytene stage are leading us into a new and exciting area: the events of recombination and the mechanism by which those events occur.

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APPENDIX 1

Probe Data

Gene /sequence	Probe size (kb)	Probe structure
H-19	1.8	full length cDNA
Top 2	2.3	full length cDNA
Igf-2[1]	2.2	non-coding region 5kb upstream of *Igf-2* start site
Igf-2[2]	2.6	5' fragment - genomic region including promoter and 5' end of the gene
Igf-2[3]	1.6	3' fragment - partial cDNA of 3'end of the gene
Prm-1	0.4	full length cDNA
Ldh c	0.45	partial cDNA 3'end
Tcp-10	0.5	partial cDNA
Eb (MHC)	1.8	partial cDNA
Hprt	1.3	full length cDNA
Timp	0.35	partial cDNA (mid-portion)
Otc	1.4	full length cDNA
Zfy-2	0.8	partial cDNA 5'end
pMCK104	0.273	repeat sequence
pBSAT-1	0.471	repeat sequence
70-38	9.5	repeat sequence
TyBS minigene	4	full length cDNA and attached promoter region

APPENDIX 2

Genomic data

Gene /sequence	Location	Size of genomic region of probe recognition (kb)
H-19	autosomal	~35
Top 2	autosomal	~30
Igf-2[1]	autosomal	2.2
Igf-2[2]	autosomal	2.6
Igf-2[3]	autosomal	1.6
Prm-1	autosomal	~0.5
Ldh c	autosomal	-
Tcp-10	autosomal	
Eb (MHC)	autosomal	
Hprt	X-linked	33
Timp	X-linked	~6.5
Otc	X-linked	
Zfy-2	Y-linked	~18
pMCK104	mouse minor satellite sequence	different regions in genome of variable size
pBSAT-1	mouse major centromeric satellite sequence	different regions in genome of variable size
70-38	X-linked satellite sequence	9
TyBS minigene	X-linked (Tg 3142A)	4.5[1]
	autosomal (Tg 3409B)	4.5[1]

[1] the insert size of TyBS is 4.5kb, but it is not known how many tandem repeats are present in the insertion sites

VITA

Tim Wiltshire grew up and was educated in Christchurch, New Zealand. Tim attended the University of Canterbury and graduated with a Bachelor of Science degree with a major in Organic Chemistry. He attended Christchurch Teachers College, and graduated with a Diploma in Teaching, and taught High School classes, in Chemistry, Biology, and General Sciences, in New Zealand for a number of years.

In the 1990 he started to further his education by completing a post-Graduate Diploma in Biotechnology at the University of Otago, New Zealand, and subsequently enrolled at the University of Tennessee, Knoxville in 1991. Tim received his Ph.D in 1996, conducting his dissertation research in the laboratory of Dr. Mary Ann Handel, in the Biochemistry, Cellular, and Molecular Biology Department.

Tim has accepted a post-Doctoral position in the laboratory of Dr. Roger Reeves at The Johns Hopkins University in Baltimore.