To the Graduate Council:

I am submitting herewith a dissertation written by Kimberlee Anne Caldwell entitled "Strategies for the Isolation of Genes Expressed Meiotically during Mouse 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 de Doctor of Philosophy, with a major in Life Sciences.

Mary Ann Handel

Dr. Mary Ann Handel, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor
and Dean of the Graduate School

STRATEGIES FOR THE ISOLATION OF GENES EXPRESSED MEIOTICALLY DURING MOUSE SPERMATOGENESIS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Kimberlee Anne Caldwell

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DEDICATION

This doctoral dissertation is dedicated to my sister, Karen Marie Neifer, with all my love and pride and to Dr. Guy Alexander Caldwell, my husband and best friend, who fills my life with joy, sunshine, and love, forever....

I would also like to present a special dedication to children who live with the ragged experience of child abuse. I understand your terror, despair, and the seemingly unending darkness. I know, because I was you, once, a long time ago. Please believe me when I tell you this, it doesn't always have to be this way. Your life can encompass so much more than shame and sadness; there is hope, light, and love, and it is within yourself.

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ABSTRACT

The process of gametogenesis is of paramount importance for reproductive success in mammals. Gamete production includes a specialized form of cell division termed meiosis whereby chromosomally diploid cells become haploid. Although meiosis is genetically essential for the formation of a normal gamete, very little is known of the molecular and cellular events constituting this phase of gametogenesis. An initial and reasonable approach to elucidating factors related to the meiotic process is to isolate genes expressed during meiosis. An overview of the literature, as it is related to this work, is presented in Part I of this dissertation.

Part II of this dissertation outlines the construction of spermatogenic cell-specific cDNA libraries in order to establish a system for the molecular analysis of meiosis in male mammals. Cytological and molecular characterization of these libraries are also described. These libraries represent an important tool for the isolation of genes that are expressed meiotically.

Part III of this dissertation describes the use of the meiotic cDNA libraries in a novel screen for meiotically-enriched cDNA clones. This screen utilizes RNA from meiotically-arrested male-sterile mutant mice for the successful identification of spermatocyte-enriched cDNA clones. The isolation of these clones serves as a foundation for further investigations into the molecular basis of meiosis.

Part IV of this dissertation describes the use of homology screening by PCR to identify a meiotically-expressed cDNA. The isolation and characterization of a novel meiotically-expressed cDNA is outlined in this section. This cDNA is expressed most abundantly in pachytene spermatocytes and in the brain and thymus of mice and contains a putative leucine zipper motif.

Additionally, although a molecular analysis of meiosis will potentially reveal new information, cytological approaches for understanding meiosis should lead to the discovery of different information concerning this process. In this regard, Part V reports the development of a spermatocyte *in vitro* culture system for the analysis of meiosis and interesting observations that have come from *in vitro* manipulation of spermatocytes.

Finally, Part VI serves to summarize the various conclusions and significance of this work and postulates future directions for continued investigation into the molecular basis of meiosis.

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PART I

GENERAL INTRODUCTION

CHAPTER 1

SPERMATOGENESIS AS A SYSTEM TO STUDY MEIOSIS

The process of gametogenesis is of paramount importance for reproductive success in mammals. Gamete production includes a specialized form of cell division termed meiosis whereby chromosomally diploid cells become haploid. Although meiosis is genetically essential for the formation of a normal gamete, very little is known of the molecular and cellular events constituting this phase of gametogenesis. Briefly, meiosis consists of several distinct phases, most notably the lengthy prophase I of meiosis, which includes five substages: leptotene, zygotene, pachytene, diplotene, and diakinesis. Following prophase I, the meiocytes undergo a reductional chromosomal division, or meiosis I (MI). The cells produced from this meiotic division do not undergo chromosome replication, rather a second meiotic division (MII) occurs that subsequently yields haploid gametes.

Understanding the regulation of meiosis is important for several reasons. For example, progress through meiotic prophase is one determinant of gametic success, as meiotic failure can lead to infertility. In addition, errors in meiotic segregation of chromosomes lead to chromosomally unbalanced (aneuploid) gametes, ultimately leading to aborted fetuses or aneuploid offspring. Finally, meiosis is the underlying and most fundamental process governing the inheritance of traits which produces genetic diversity within a population. Most of our current understanding about meiosis in mammals has come from observation. We know that recombination and segregation of chromosomes occurs because recombinant, chromosomally normal offspring are produced. However, the mechanisms governing these meiotic processes and how they are regulated have yet to be elucidated.

In mammals, meiosis is more easily studied in males than in females for several reasons. Spermatogenesis occurs repeatedly during adult life of males. In contrast, oogenesis occurs during fetal development and is discontinuous; oocytes arrest at diplotene until puberty, resuming meiosis when ovulation takes place. Additionally, the cytogenetics of meiotic prophase has been studied more extensively in male mammals, where these events are more accessible and where more gametes are produced. These attributes of spermatogenesis render it an advantageous model system for investigation of the molecular basis of meiotic cell events.

Spermatogenesis in the mouse

Spermatogenesis is a process which involves the production of haploid gametes. This is accomplished through a series of cell divisions, both mitotic and meiotic, and a postmeiotic differentiation phase (Figure 1). Mitotic divisions provide a continual supply of stem cells for the testis and increase the number of germ cells that will proceed through meiosis and become haploid gametes. Meiotic reduction in chromosome number involves the pairing of homologous chromosomes and recombination of genetic material between chromosomes followed by segregation. Upon completion of meiosis, spermiogenesis takes place; this is a process whereby the haploid round spermatids become highly differentiated, eventually leading to the formation of mature sperm. This represents only a superficial overview of spermatogenesis; the following paragraphs will provide more details of this process, including details from analysis of the process in mice.

Primitive stem cells, located at the basal membrane of the seminiferous tubule, divide mitotically to produce spermatogonia. Primitive stem cell division provides a continuing supply of stem cells for the testis and leads to the production of spermatogonia. Spermatogonia undergo four mitotic divisions, ultimately producing preleptotene

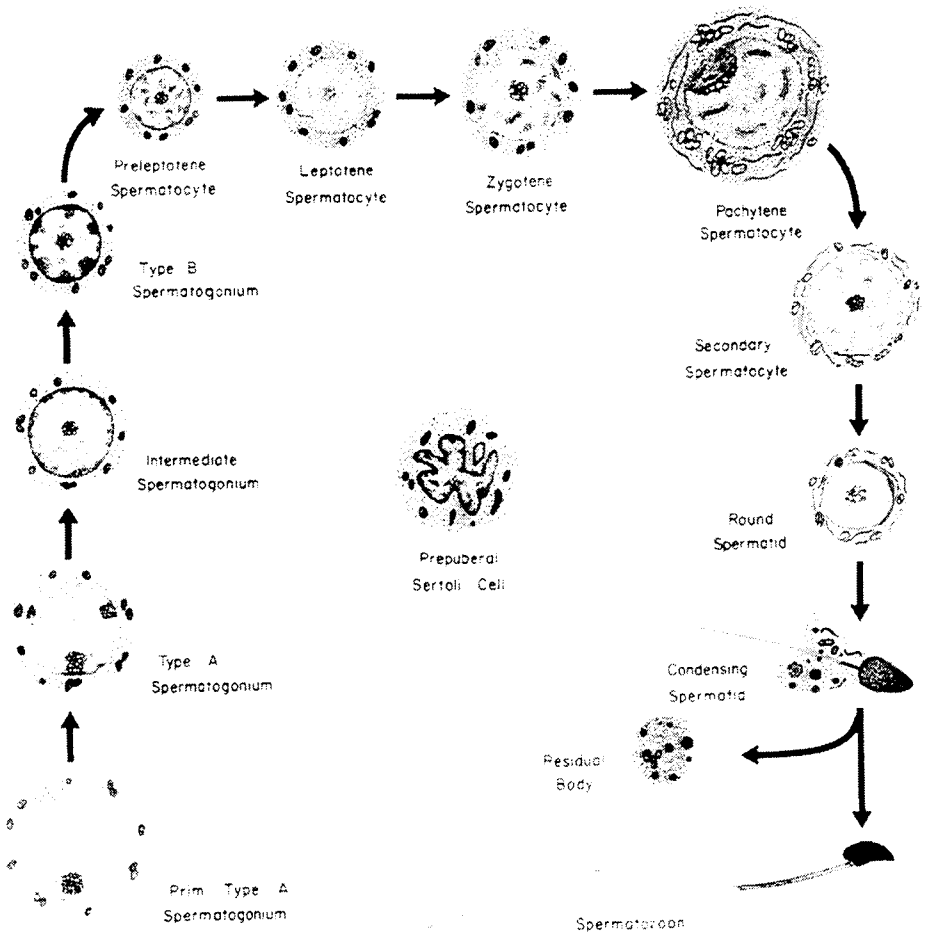

Figure 1. Diagram of spermatogenic lineage in the mouse. Taken from Bellve, AR, JC Cavicchia, CF Millette, DA O'Brien, YM Bhatnagar, and M Dym. 1977. Spermatogenic cells of the prepuberal mouse. J Cell Biol 74:68-85.

spermatocytes (Oakberg, 1956). These early spermatocytes undergo a final round of DNA synthesis prior to progression through meiosis.

Almost one-third of the entire 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). Several important events happen in meiosis I: homologous chromosomes must be paired and recombined; chromosomes must be segregated at MI; and the stage must be set for the ensuing spermiogenic program of differentiation. As mentioned previously, prophase of meiosis I is typically divided into substages. Based primarily on cytological observations (Solari, 1970; Moses, 1980; Goetz *et al.*, 1984), these stages are referred to as leptotene, zygotene, pachytene, diplotene and diakinesis. Leptotene spermatocytes are characterized by diffuse chromatin, with little indication of chromosome pairing. Subsequent evidence of chromosome pairing delineates the start of zygotene. Specifically, the lateral elements of the synaptonemal complex can be visualized after zygotene nuclei are spread on a hypophase and stained with silver. However, the synaptonemal complex is fully formed during the pachytene stage when lateral elements pair to form the tripartite structure. Another hallmark of the pachytene spermatocyte stage is the appearance of the sex body, a distinct chromatin domain within the nucleus where the X and Y chromosomes are sequestered (Solari, 1974). During pachytene, the autosomal homologs become fully paired; and subsequently the sex chromosomes pair in the pseudoautosomal region, a small region of homology between the mostly nonhomologous sex chromosomes. It is in this pseudoautosomal region that recombination occurs between the X and Y chromosomes. In contrast, autosomal recombination is not limited, as the autosomes are homologous over their entire length. By mid to late pachytene the sex chromosomes begin to desynapse, although the autosomes remain fully paired. Diplotene is characterized by complete desynapsis of the sex chromosomes and the onset of

autosomal desynapsis. During late diplotene and early diakinesis, crossovers are visible as chiasmata and further chromosomal condensation is apparent. Toward the end of diakinesis the sex body, nucleolus, nuclear membrane, and chiasmata have disappeared, and a spindle apparatus has appeared. The first meiotic division follows, during which homologous chromosomes segregate. Secondary spermatocytes, the products of this reductional division, proceed fairly rapidly through a second meiotic division, equationally separating chromatids to produce haploid spermatids.

Haploid round spermatids undergo spermiogenesis, a complex process where they become highly differentiated and elongated. The nucleus of a spermatid is initially round but undergoes elongation, coupled with chromatin condensation. Histones are replaced by protamines (reviewed by Balhorn, 1989), which pack chromatin tighter than histones. During spermiogenesis, the nucleus becomes covered by the acrosome, a derivative of the Golgi apparatus. The acrosome contains enzymes that are involved in zona pellucida degradation during fertilization of oocytes. Additionally, although the round spermatid is initially unflagellated, a complex tail is formed and mitochondria form around the base of the tail. Mature sperm are shed into the luminal compartment of the seminiferous tubule and further modifications occur in the epidydimis.

The mouse as a model for mammalian meiosis

The mouse is an experimentally malleable and advantageous organism for learning about the expression and function of genes important for meiosis in mammals. The developmental biology of testicular development and spermatogenesis is well established in the mouse and thereby facilitates the isolation of developmentally staged testes with only certain categories of germ cells present. Cell separation procedures have been developed (Romrell *et al.*, 1976; Bellvé *et al.*, 1977) that permit the purification of meiotic cells, specifically spermatocytes, which can be used as sources of RNA for analysis of

transcription. Techniques for *in vitro* culturing of pachytene spermatocytes (Gerton and Millettte, 1986; O'Brien, 1987; Handel *et al.*, 1995) also permit experimental analyses of gene expression and function. Furthermore, the rich background of classical genetics and availability of a variety of molecular tools facilitate cloning, mapping, and functional studies of meiotically expressed genes in mice. Interspecific crosses and somatic cell hybrid panels are available for mapping purposes as well. Likewise, a number of genetic aberrations that affect meiotic stages of spermatogenesis in the mouse (reviewed by Handel, 1990) have been reported. Such mutations are not only valuable in genetic screens but also in eventually determining putative temporal and genetic hierarchies of gene expression and function during meiosis.

Genetic analysis of meiosis in other organisms

It is assumed from genetic and cytological studies of chromosome pairing and segregation that meiosis is a comparable process in many organisms. Thus, as fundamental as meiosis appears to be, mechanisms and gene products should be highly conserved. Chromosome pairing can easily be visualized in mammalian spermatocytes (Dresser, 1987) following the assembly of the synaptonemal complex (SC), which is a proteinaceous structure that spans the region between two synapsed chromosomes. Two major components of the mammalian SC have been identified immunocytologically that are found only in meiotic prophase nuclei (Heyting *et al.*, 1988). Additionally, genes encoding two synaptonemal complex proteins have been identified (Meuwissen *et al.*, 1992; Lammers *et al.*, 1994). However, virtually nothing is known about the mechanism of homologous chromosome pairing in mammals. It is presumed that the SC participates in homologous chromosome pairing because of its appearance at the time of pairing. Evidence from other species suggests a close relationship between the formation of the SC and genetic recombination, although it is not known if the SC is required for recombination

or if recombination is required for SC formation (reviewed by Roeder, 1990). Additionally, little is known about molecular aspects of genetic recombination and chromosome exchange in mammals.

Recent molecular genetic studies in the yeast *Saccharomyces cerevisiae* have provided exciting and informative insights into recombination and chromosome exchange during meiotic prophase. The apparently intimate and not necessarily hierarchical relationship between SC formation and chromosome exchange is revealed by the fact that most meiotic mutations affect both processes. At least ten genes have been cloned from yeast that identify a subset of meiotic functions involved in chromosome pairing, SC formation, and recombination (Table 1). Since these processes exhibit significant evolutionary conservation, the genes controlling them may also be conserved. A mouse homolog of the yeast DNA repair gene, RAD51, has been identified using a homology-based screening procedure (Morita *et al.*, 1993). The cloning of mouse *rad51* has demonstrated that sequence similarity does exist for this gene between lower eukaryotes and mammals. This suggests that homology in function and gene sequence may exist for other genes between lower eukaryotes and mammals and that homology-based approaches might be an effective method for isolating meiotically expressed genes in the mouse.

Reductional chromosomal segregation at anaphase of MI must be accurate since the developmental consequences of nondisjunction and ensuing aneuploidy usually include lethality or extreme abnormality. The rules governing the normal disjunction and proper exchange of homologous chromosomes have yet to be elucidated. In most species, at least one recombinational exchange (chiasma) per chromosome is essential to ensure proper segregation (reviewed by Hawley, 1988). Correct chromosome segregation is facilitated by preventing centromere separation until after the first meiotic division (Goldstein, 1981).

Table 1. Meiotically expressed genes isolated from yeast that are involved in chromosome pairing and/or recombination

Gene	Reference
HOP1	Hollingsworth *et al.*, 1990
MER1	Engebrecht and Roeder, 1990
RED1	Thompson and Roeder, 1989
SPO11	Atcheson *et al.*, 1987
MEI4	Menees *et al.*, 1992
MEK1	Rockmill and Roeder, 1991
MRE4	Leem and Ogawa, 1992
RAD51	Shinohara *et al.*, 1992
DMC1	Bishop *et al.*, 1992
SEP1[a]	Tishkoff *et al.*, 1995

[a]The *SEP1* gene was not isolated from the laboratory listed in the table, rather these authors identified a meiotic function for *SEP1*. The gene was cloned from several laboratories studying different yeast metabolic functions and has also been refered to as *KEM1* (Kim *et al.*, 1990), *RAR5* (Kipling *et al.*, 1991), and *XRN1* (Larimer and Stevens, 1990).

Likewise sister chromatid cohesiveness, the holding of sister chromatids together throughout the first meiotic division ensures proper disjunction during MI (Nicklas, 1974; Hawley, 1988). These processes may also involve special meiotic adaptations of chromatin and unique spindle motors to facilitate disjunction. However, thus far, the molecular mechanisms governing these processes are not known.

In yeast, mutations that affect chromosome pairing and recombination also affect segregation and lead to spore inviability (the phenotype used to screen for such mutations). Genes that specifically control chromosome disjunction include *SPO13*, a gene required for the separation of homologous chromosomes at MI (Wang *et al.*, 1987) and *TOP2*, the gene encoding topoisomerase II, which is required for the segregation of recombined chromosomes (Rose *et al.*, 1990). Several genes in *Drosophila* that control the meiotic segregation of chromosomes have been cloned as well, including *ncd* (non-claret disjunctional), and *nod*. The product of the *ncd* locus is required in females for normal chromosome segregation during meiosis and shares sequence homology with the *Drosophila* kinesin heavy chain (Endow *et al.*, 1990; McDonald and Goldstein, 1990). Kinesins are microtubule motors, some of which have been implicated in chromosome movement. Likewise, the product of the *nod* locus, required for normal disjunction of non-exchange (distributively paired) chromosomes of *Drosophila* during female meiosis, is also a putative kinesin-like microtubule motor protein (Zhang *et al.*, 1990). These discoveries are significant in regard to the ultimate understanding of meiotic mechanisms. Most theories of proper disjunctional chromosome segregation involve the behavior of chromosomes and their orientation on the spindle apparatus prior to their segregation to the spindle poles. Thus it is likely that both plus-end directed motor proteins (accomplishing proper orientation by pushing chromosomes to the microtubule ends at the spindle equator) and minus-end directed motors (pulling chromosomes to the spindle poles) are important

for meiotic chromosome segregation. As we learn more of the mechanics and components influencing meiotic segregation and associated processes in lower organisms, such information will likely contribute to our ability to identify analogous genes and gene products in mammals.

Theoretical approaches for isolating meiotically expressed genes from spermatocytes

There are several possible approaches for identifying meiotically expressed genes in the mouse. These methods differ with respect to extent of technical difficulty and probable success rate. The various methodologies include homology searches, differential screening of cDNA libraries, and cloning strategies based on expression patterns.

Homology Fundamental mechanisms of meiosis appear to be highly conserved evolutionarily. For example, the SC mediates chromosome pairing in species as diverse as yeast, *Drosophila*, and humans. Such a high level of evolutionary conservation of mechanism suggests that the proteins involved are also likely to be conserved. The underlying hypothesis of homology screening for mouse meiotic genes is that genes unique to meiosis in other species will share high sequence identity and also be specifically expressed in the mouse.

One approach to achieve this goal is direct homology screening in which a meiotically important gene from one organism is used as a probe to identify a homolog in mouse. Initially, this would constitute use of the cross-species probe to screen genomic Southern blots for the presence of a putative homolog. If a potential homolog is found, Northern blot analysis with spermatocyte RNA would reveal whether it is expressed in meiotic cells. This method was initially utilized as a primary approach for the identification of meiotically expressed mouse genes during the course of this dissertation. Unfortunately, mouse homologs of yeast and *Drosophila* genes were not identified by this

method. The results of this analysis are discussed in more detail in the Summary section of this dissertation (Part VI).

A derivative of this homology approach is to utilize the polymerase chain reaction (PCR) in conjunction with degenerate primers, corresponding to consensus regions shared among genes that have been previously identified to be important for meiosis in other species. This approach is limited as it is useful only when the gene being identified is a member of a well-characterized gene family. If a PCR-derived fragment is identified using this technique, full length sequences can be subsequently isolated from a cDNA library. Six new kinesin-like proteins have been identified from *Drosophila* genomic DNA using PCR with degenerate primers to the kinesin heavy chain (KHC) motor domain (Stewart *et al.*, 1991). KHC primers were constructed based on a consensus site found in several kinesin family members. The PCR-based homology approach was utilized to find mammalian and *Xenopus* homologs for the mismatch repair gene *mutS*, originally isolated from *E. coli* (Varlet *et al.*, 1994). In this case, PCR primers were constructed from a *mutS* consensus region, within *mutS* homologs of *S. typhimurium*, *S. cerevisiae*, and humans. Additionally, primers were designed from conserved regions of two *RAD51* genes in *S. cerevisiae* and *S. pombe* to isolate a mouse *rad51* homolog (Morita *et al.*, 1993). This homolog encodes a product involved in mitotic and meiotic recombination, in addition to DNA repair, and has been shown to complement the *rad51* mutation of *S. cerevisiae*. These reports successfully illustrate the utility of the PCR-based approach for identifying meiotically important genes in the mouse.

Differential screening of cDNA libraries Differential library screening has recently provided useful information regarding gene expression in various spermatogenic cell types. Gene sequences primarily expressed in specific cell types of the testis have been identified using this methodology. Thomas *et al.* (1989) screened, by differential hybridization,

adult testis and pachytene spermatocyte cDNA libraries with ^{32}P-labeled cDNA probes from poly A$^+$ RNA of liver, pachytene spermatocytes, and round spermatids. Stage-specific cDNA clones were isolated on the basis of their hybridization to different mixed cDNA probes representing various stages of spermatogenesis. These clones were then classified into categories according to cell-specific expression patterns following Northern blot analysis with purified germ cells. Categories included expression in round spermatids, pachytene spermatocytes leptotene/zygotene spermatocytes, and a category of sequences expressed in both spermatogonia and spermatocytes. Clones specifically expressed in spermatogonia were not isolated in this screen, probably because spermatogonial RNA was not used in the differential screening procedure. Nevertheless, clones of sequences demonstrating differential expression among a variety of spermatogenic cell types were isolated by this method. These cDNA clones will undoubtedly be useful as developmental markers and, perhaps, interesting meiotic factors will be identified from the resultant cDNAs obtained by this cloning strategy.

Differential screening was also used to isolate cDNAs of transcripts expressed during prepuberal germ cell development (Hoog, 1991; Starborg *et al.*, 1992; Kerr *et al.*, 1994). In these recently reported differential screening studies, new sequencing technologies have been utilized to analyze hundreds of differentially expressed cDNAs during spermatogenesis. Poly A$^+$ RNA from mouse liver, kidney, and 6-12 day testis was labeled with ^{32}P using reverse transcriptase and was hybridized to plaques from a 6-12 day testis cDNA library. Clones that did not hybridize with the probes used in this screen were isolated. Negative hybridization with the liver and kidney probes suggested that clones did not represent housekeeping genes and negative hybridization with the prepuberal testis probe indicated that the clones were probably not housekeeping genes expressed in the immature testis, either. In this manner, many clones were identified with preferential expression in the testis. Results from characterization studies of these clones may yield

interesting information regarding meiosis although it is unlikely that many of these clones are meiotic since they were identified from whole testes of mice 6 -12 days old. Spermatogenic cells in the testes of mice between ages 6 - 9 days are undergoing a proliferative mitotic stage and do not begin meiosis until about age 9 or 10 days when the first early spermatocytes appear. Nonetheless, differential screening procedures could be an effective method for the isolation of meiotically expressed genes if appropriate cell populations were compared, such as spermatogonia and leptotene/zygotene spermatocytes, or leptotene/zygotene and pachytene spermatocytes.

Cloning strategies based on expression patterns Several methodologies are now available for identifying differences in gene expression between two or more cell types. These include the procedures of subtractive hybridization (Bowes *et al.*, 1989; Duguid *et al.*, 1988; Travis and Sutcliffe, 1988), differential display (Liang and Pardee, 1992; Liang *et al.*, 1993), and representational difference analysis (Hubank and Schatz, 1994). Currently, I am not aware of any meiotically expressed genes that have been successfully identified using any of these procedures. However, the potential for employing these methodologies to identify meiotically expressed genes exists. The underlying premise behind this perception is that meiosis represents a unique event in germ cells and, as such, specialized, meiosis-specific transcripts are most likely expressed during the process. As these procedures represent alternate and in some cases, novel approaches to those presented in this work, a discussion of their potential utility is warranted and will be expanded upon in the Summary section of this dissertation.

Genes expressed during spermatogenesis

As discussed previously, spermatogenesis is a complex process through which mitotically dividing spermatogonia ultimately become highly differentiated spermatozoa.

This process involves major changes to the spermatogenic cell as it passes through meiosis and undergoes genetic recombination, ultimately becoming haploid and proceeding through spermiogenesis. In this regard, many genes have been identified that are expressed in specific temporal patterns during spermatogenesis. Spermatogenically expressed genes have been found to encode a variety of different proteins, including isozyme variants, structural proteins, and proto-oncogene products. However, many of these genes are not expressed exclusively in the testis. Genes expressed in a temporal manner during spermatogenesis have been divided into categories based on premeiotic (Table 2), meiotic (Table 3), postmeiotic (Table 4), Sertoli cell (Table 5), or Leydig cell (Table 6) expression. A brief description follows which highlights specific genes expressed in the various spermatogenic cell types and emphasizes meiotic gene expression.

Spermatogonial expression of many proto-oncogenes has been described by Wolfes *et al.* (1989) (Table 2). Additionally, two isozymic variants of lactate dehydrogenase are expressed in spermatogonia (Li *et al.*, 1989; Alcivar *et al.*, 1990). Although spermatogonia undergo several rounds of mitosis, genes exhibiting mitotic cell cycle expression have not yet been found to be expressed uniquely at this stage of spermatogenesis.

Several genes have been isolated that are expressed during mammalian meiosis (Table 3). Meiotically expressed genes typically have one of two expression patterns during spermatogenesis. The first expression pattern consists of genes meiotically transcribed but probably utilized postmeiotically. Many of these genes have additional postmeiotic transcription of the gene or postmeiotic translation of the gene product. Several examples of genes which show this expression pattern have been reported. *Pgk-2*, encoding phosphoglycerate kinase-2 is an autosomal variant of *Pgk-1*, which is located on the X chromosome of mice. *Pgk-2* becomes active during pachytene when the X-chromosome is inactivated and is translated postmeiotically (McCarrey and Thomas,

Table 2. Genes expressed premeiotically during spermatogenesis

Gene	Reference
c-*myc*	Wolfes *et al.*, 1989
c-*fos*	Wolfes *et al.*, 1989
c-*jun*	Wolfes *et al.*, 1989
jun B	Alcivar *et al.*, 1990
c-*kit*	Manova *et al.*, 1990
histones	reviewed in Meistrich, 1989
lactate dehydrogenase A	Li *et al.*, 1989
lactate dehydrogenase B	Alcivar *et al.*, 1990
cytochrome c_s	Hake *et al.*, 1990
TH3	Meistrich and Brock, 1987

Table 3. Genes expressed meiotically during spermatogenesis

Gene	Reference
Lactate dehydrogenase C	reviewed in Goldberg, 1977; Weiben, 1981; Fujimoto *et al.*, 1899; Jen *et al.*, 1989
Phosphoglycerate kinase 2	McCarrey and Thomas, 1987
Cytochrome c_t	Hake *et al.*, 1990
Histone varients	reviewed in Meistrich, 1989
Heat shock proteins	reviewed in Wolgemuth *et al.*, 1988; Allen *et al.*, 1988
Mak	Matsushime *et al.*, 1990
meg1	Don *et al.*, 1992
Nek1	Letwin *et al.*, 1992
ferT	Fischman *et al.*, 1990; Keshet *et al.*, 1990
cycB2	Chapman and Wolgemuth, 1992, 1993
zfp-35	Cunliffe *et al.*, 1990
NASP	Welch *et al.*, 1990
Calmodulin	Slaughter and Means, 1989
Calspermin	Ohno *et al.*, 1990
Acrosin	Klemm *et al.*, 1990
Hox-1.4	Rubin *et al.*, 1986; Wolgemuth *et al.*, 1987
DIPas1	Leroy *et al.*, 1989
pyruvate dehydrogenase E1α	Fitzgerald *et al.*, 1994
acrogranin	Anakwe and Gerton, 1990
MSY1	Tafuri *et al.*, 1993

Table 4. Genes expressed postmeiotically during spermatogenesis

Gene	Reference
pim-1	Sorrentino *et al.*, 1988
Protamine 1	Kleene *et al.*, 1985; Klemm *et al.*, 1989
Protamine 2	Yelick *et al.*, 1987
Regulatory subunit type II protein kinase	Oyen *et al.*, 1988
Smooth muscle γ actin	Kim *et al.*, 1989
Tubulin varients	Distel *et al.*, 1984; Villasante *et al.*, 1986; Hecht *et al.*, 1988
t complex polypeptide 1 (TCP1)	Silver, 1985; Wilson *et al.*, 1986, 1989
Transition protein 1	Heidaran and Kistler 1987a,b; Kleene *et al.*, 1988
Transition protein 2	Kleene and Flynn, 1987; Leuerssen *et al.*, 1989
c-abl (mRNA size variant only)	Ponzetto and Wolgemuth, 1985
MEA	Lau *et al.*, 1989
SP-10	Wright *et al.*, 1990
G3PD	Schatte *et al.*, 1990
PH-20	Lathrop *et al.*, 1989
β1,4 gal Tase	Shaper *et al.*, 1990
cAMP PDE	Swinnen *et al.*, 1989
calmodulin-dependent phosphatase	Muramatsu *et al.*, 1992
cycB1	Chapman and Wolgemuth, 1992
Zfp-37	Burke and Wolgemuth, 1992
c-*mos*	Goldman et al., 1987; Mutter and Wolgemuth, 1987; Propst *et al.*, 1987, 1988
int-1	Shackleford and Varmus, 1987

Table 5. Genes expressed in Sertoli cells

Gene	Reference
Follicle-stimulating hormone receptor	Griswold *et al.*, 1986
Androgen-binding protein	Joseph *et al.*, 1985
Ceruloplasmin	Griswold *et al.*, 1986
Transferrin	Griswold *et al.*, 1986
Sulfated glycoproteins	Griswold *et al.*, 1986
Prodynorphin (size varient only)	Garrett *et al.*, 1989

Table 6. Genes expressed in Leydig cells

Gene	Reference
3β-hydroxysteroid dehydrogenase	Bain *et al.*, 1991
Pro-opiomelanocortin	Gizang-Ginsberg and Wolgemuth, 1985
Renin	Deschepper *et al.*, 1986
Angiotensin II receptors	Millan and Aguilera, 1988

1987). Another gene, *D1Pas1*, encoding a member of an ATP-dependent RNA helicase family, is transcribed in pachytene spermatocytes and, to a lesser extent, in round spermatids (Leroy *et al.*, 1989). Another example in this expression category is the mouse homeobox-containing gene, *Hox-1.4*. This gene is transcribed during pachytene and at the same level in round and elongating spermatids (Rubin *et al.*, 1986; Wolgemuth *et al.*, 1987). Finally, *Ldh-3* (encoding Ldh-c) is an example of a gene that is transcribed in both pachytene spermatocytes and in round spermatids, where it is expressed in decreasing levels (Jen *et al.*, 1990). Although the transcript is first translated in pachytenes the amount of protein product increases postmeiotically. It is thought that many gene products translated postmeiotically are initially transcribed during pachytene.

The second pattern of meiotic gene expression involves little postmeiotic transcription. Several genes have been identified with this type of expression and some of their gene products may actually be needed for the meiotic process. The transcript of *meg1* is specifically expressed in spermatocytes with low levels detected in leptotene and increasing levels detected as cells progress through zygotene and pachytene (Don and Wolgemuth, 1992; Don *et al.*, 1994). *Zfp-35* encodes a protein that belongs to the zinc finger family and could possibly be involved in meiotic germ cell development since this motif generally is found in proteins that regulate transcription (Cunliffe *et al.*, 1990). The *cycB2* gene (Chapman and Wolgemuth, 1993) is a member of the cyclin B family. Cyclin B synthesis has been determined to drive meiotic cells into M phase (Westendorf *et al.*, 1989). Since high levels of *cycB2* have been identified in pachytene spermatocytes, this transcript may be involved in the meiotic divisions. The tyrosine kinase-encoding gene *ferT* (Fischman *et al.*, 1990; Keshet *et al.*, 1990) was also identified as having a pachytene-specific transcript. Many of these genes may play a role in meiosis since they are primarily expressed in pachytene spermatocytes. It should be noted, however, that

meiotic function has not been demonstrated for any of these genes. In contrast, genes with meiotic and postmeiotic expression are most likely to be involved in spermiogenic development.

Most of the genes described with meiotic expression in the testis are not unique to the testis but also exhibit expression in some somatic tissues or cell populations. However, a few meiotically-expressed genes are expressed only in the testis and not in any other cell types. Examples of novel testicular genes expressed meiotically are *D1Pas1*, acrosin, *Ldh-c*, *Pgk-2*. Interestingly, all of these genes are transcribed meiotically but have gene products that are utilized postmeiotically.

Many genes have been identified that are expressed postmeiotically (Table 4). Products of several of these genes are found only in the testis, such as the transition proteins 1 (Heidaran and Kistler 1987a,b; Kleene *et al.*, 1988) and 2 (Kleene and Flynn, 1987; Leuerssen *et al.*, 1989), and the protamines (Kleene *et al.*, 1984; Yelick *et al.*, 1987). Additionally, isotypes of some structural proteins are expressed postmeiotically and several proto-oncogenes have been identified that demonstrate a postmeiotic expression pattern.

The somatic cells of the testis, Sertoli and Leydig cells, have also been shown to exhibit cell type-specific gene expression . Sertoli cells (Table 5) express gene products that help maintain the testicular environment, including SGP-2 (Griswold *et al.*, 1986), and signalling proteins, such as inhibin (Mason *et al.*, 1985), which is needed for communication between cells of the testis. Genes expressed in Leydig cells (Table 6) include those involved in steroid biosynthesis.

In summary, the various cell populations of the testis have unique patterns of gene sequences (as shown in Tables 2 - 6). The function of many of these gene products has been analyzed in detail. Gene characterization has provided some knowledge regarding cell regulatory mechanisms. For example, the cloning and characterization of the transition

proteins and protamines during spermiogenesis has led to additional knowledge regarding chromatin configuration and packaging during spermiogenesis (reviewed by Hecht, 1993). Furthermore, the identification of an autosomal variant for phosphoglycerate kinase has provided information regarding X chromosome inactivation and gene expression during male meiosis (McCarrey and Thomas, 1987). Several meiotically expressed genes have been identified (listed in Table 3), however, the availability of these sequences has not significantly increased our knowledge of meiotic mechanisms. This may, in part, be a reflection of the inherent difficulties in elucidating a complex process. There are likely to be many genes involved in meiosis and it will probably be quite some time before knowledge regarding the meiotic process will be gained from the cloning of meiotically expressed sequences alone. It should be noted that although identification of meiotically expressed sequences is an important and reasonable approach to learning about meiosis, there are no guarantees that any given meiotically expressed sequence will contribute additional information regarding the meiotic process. Nevertheless, as more spermatocyte-specific gene sequences are identified and characterized, it remains probable that our understanding of the meiotic process in mammals will be further advanced.

Conclusion

Meiosis is a complex process and unfortunately little is known with respect to its molecular mechanism in mammals. An initial and reasonable approach to elucidating factors related to the meiotic process is to isolate gene sequences expressed during meiosis. Part II of this dissertation describes the construction of stage-specific meiotic cDNA libraries which can be used to isolate genes that are expressed meiotically. These cDNA libraries have proven to be a valuable resource for isolating meiotically-enriched gene sequences, as demonstrated in Part III of this work. In Part III of the dissertation, the

meiotic cDNA libraries are utilized in a novel screen for meiotically-enriched cDNA clones. This screen utilizes RNA from meiotically-arrested male mutant mice for the successful identification of spermatocyte-enriched cDNA clones. Part IV of this dissertation represents an attempt at the use of homology screening by PCR to identify a meiotically-expressed cDNA. The isolation and characterization of a novel meiotically-expressed cDNA is outlined in this section. Additionally, although a molecular analysis of meiosis will potentially reveal new information, cytological approaches for understanding meiosis should lead to the discovery of different information concerning this process. In this regard, Part V reports the development of a spermatocyte *in vitro* culture system for the analysis of meiosis and interesting observations that have come from *in vitro* manipulation of spermatocytes. Finally, Part VI serves to summarize the various conclusions and significance of this work and postulates future directions for continued investigation into the molecular basis of meiosis.

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PART II

CONSTRUCTION AND ANALYSIS OF MEIOTIC cDNA LIBRARIES

CHAPTER I

INTRODUCTION

Spermatogenesis is a highly complex process involving cellular interactions, development, and differentiation. Briefly, primitive stem cells, located at the basal membrane of the seminiferous tubule, divide mitotically to produce Type A spermatogonia, which after a series of mitotic divisions form cell types designated Intermediate, then Type B spermatogonia, and finally, primary spermatocytes upon entering meiotic prophase. Meiosis is a lengthy process and almost one-third of the entire time required for spermatogenesis in the mouse is devoted to various phases of meiosis. In mammals meiosis is a unique process to germ cells. Prophase, the longest stage of meiosis I, involves the pairing of homologs, formation of the synaptenemal complex, recombination, and formation of chiasmata. Segregation of homologs occurs during meiosis I. The result of a second meiotic division, in which sister chromatids separate, is the formation of haploid spermatids. Haploid spermatids undergo a complex process of differentiation, spermiogenesis, whereby the round spermatid becomes a streamlined, elongated spermatid. As the process of spermatogenesis involves a defined course of cellular events, including mitosis, meiosis, and haploid differentiation, concurrent gene expression must be required for directing cell activity.

There have been many studies demonstrating both directly and indirectly that there is differential gene expression during the various stages of spermatogenesis. Evidence for stage-specific transcription during spermatogenesis was first described by Monesi (reviewed by Monesi, 1978). Levels of ^{3}H-uridine incorporation in spermatogenic cells were found to differ among various cell populations within the testis. Furthermore, early studies of protein synthesis, utilizing two dimensional gel electrophoresis, demonstrated

that pachytene spermatocytes, round spermatids, and elongating spermatids contained stage-specific polypeptides, in addition to proteins shared in common by these spermatogenic cells (Boitani *et al.*, 1980; Millette and Moulding, 1981; Kramer and Erickson, 1982; Gold *et al.*, 1983; Stern *et al.*, 1983). Taken together, these studies have found that, in addition to variations in RNA synthesis rates, nascent polypeptides are detected at distinct times during spermatogenesis, thus suggesting that stage-specific gene expression occurs during the course of spermatogenesis.

These results served as a foundation for the experimental isolation of genes expressed specifically during spermatogenesis. Whole testis cDNA libraries were constructed and utilized in differential screening procedures that identified sequences expressed uniquely by specific testicular cells. The majority of the cDNA clones isolated were those corresponding to round spermatid gene expression (Kleene *et al.*, 1983; Dudley *et al.*, 1984; Fujimoto *et al.*, 1984). Two post-meiotically expressed mouse protamine variants were identified using this method (Kleene *et al.*, 1984; Yelick *et al.*, 1985). The identification of cell type-specific cDNA clones utilizing these screening procedures provided confirmation that stage-specific gene expression is present in spermatogenic cell populations. Additionally, these studies demonstrated that differential screening procedures are an effective method for the identification of cDNAs corresponding to stage-specific genes. These initial experimental observations, demonstrating the potential for identification of spermatogenic stage-specific genes, provide the impetus for the development of future differential screening strategies.

An additional differential screening strategy utilizing cDNA libraries constructed from both pachytene spermatocytes and whole testis was subsequently reported (Thomas *et al.*, 1989). In this study, stage-specific cDNA clones were isolated from the two libraries based on hybridization with mixed pachytene cDNA probes and lack of

hybridization with mixed liver cDNA probes. Clones isolated from this procedure were analyzed with respect to cell-type specific expression by Northern blotting and were placed in categories based on expression in either spermatids, pachytene spermatocytes, leptotene/zygotene spermatocytes, or expression in both spermatogonia and spermatocytes. The cDNA clones isolated in this study corroborated the existence of stage-specific gene expression in round spermatids and, additionally, provided evidence for stage-specific gene expression in leptotene/zygotene spermatocytes and pachytene spermatocytes.

Recently, there have been reports on testicular gene expression from analyses of new cDNA libraries constructed from whole testis (Hoog, 1991; Starborg *et al.*, 1992; Kerr *et al.*, 1994; Yuan *et al.*, 1995). These whole testis libraries were generated from 6-12 day testis (Hoog, 1991), 12-13 day testis (Kerr *et al.*, 1994), or from adult testis (Yuan *et al.*, 1995). These studies all utilized new sequencing technologies to analyze hundreds of differentially expressed cDNAs during spermatogenesis. Kerr *et al.* (1994) sequenced the 5' ends of 306 cDNAs isolated from a 12-13 day testis cDNA library. Clones were selected using the criteria of negative hybridization with both brain and 14 day testis cDNA in order to eliminate abundantly expressed clones. Of the clones isolated, 56% were novel and testis-enriched. Yuan *et al.* (1995) partially sequenced 420 mouse testicular expressed sequence tags and identified 42 to be exclusively expressed in the testis; of these, 29 were expressed in round spermatids only, and 13 were expressed in both spermatocytes and round spermatids. In a similar study, more than 100 cDNAs were isolated and partially sequenced from a 6-12 day mouse testis cDNA library (Hoog, 1991; Starborg *et al.*, 1992). Approximately one-third of these cDNAs were found to be predominantly expressed in the testis. Combined results of these studies demonstrated that many genes exist that exhibit expression primarily in the testis. Furthermore, some of the cDNA clones from these genes were found by virtue of the expression of their corresponding RNAs in specific populations of spermatogenic cells. The cDNA clones isolated from

these studies represent valuable reagents for future examination of gene expression and its molecular control during spermatogenesis.

Since most of the cDNA libraries utilized in the studies outlined above were constructed from whole testis, they contain gene sequences expressed in all testicular cells, including spermatogenic, as well as somatic, cells of the testis. However, whole testis cDNA libraries do not adequately represent specific spermatogenic cell populations. The construction of one pachytene spermatocyte cDNA library was reported several years ago; the methodology utilized yielded only 80% of the clones recombinant (i.e., 80% of the vectors contained inserts, 20% did not have inserts) (Thomas *et al.*, 1989). Except for this pachytene spermatocyte cDNA library, there have not been any reports in the literature of the availability or use of germ cell-specific cDNA libraries. cDNA libraries enriched for sequences expressed in specific spermatogenic cell populations could be a valuable resource for the identification of stage-specific cDNA sequences. Differential screening procedures utilizing such stage-specific cDNA libraries have the potential for identification of unique cDNA sequences represented by low level expression. Additionally, such cDNA libraries could be utilized in techniques such as subtractive hybridization (Bowes *et al.*, 1989; Duguid *et al.*, 1988; Travis and Sutcliffe, 1988), differential display (Liang and Pardee, 1992; Liang *et al.*, 1993) and representational difference analysis (Hubank and Schatz, 1994) to isolate stage-specific gene sequences. Furthermore, cDNA libraries constructed from spermatocytes could be useful for elucidating the molecular events of meiotic prophase I through the identification of cDNA clones representing genes important for the meiotic process.

The stage-specific cDNA libraries reported here were constructed from meiotic cells of the testis, specifically, leptotene/zygotene spermatocytes, pachytene spermatocytes and postmeiotic round spermatids. Spermatogenic cells used for cDNA library construction

were cytologically analyzed by both light and electron microscopy in order to verify cell composition. Molecular representation of various gene sequences in the cDNA libraries was studied by systematically hybridizing the cDNA libraries with several different gene sequences, representative of those expected to be expressed in the libraries, and those not expected to be present in the cDNA libraries. The size of all three cDNA libraries was determined to be $> 2 \times 10^6$ clones, of which > 99% are recombinants; the average insert size of the cDNA libraries is 1.4 kb.

The pachytene spermatocyte cDNA library created in this study was utilized in a differential screening procedure to identify cDNA sequences representing genes expressed predominantly during the pachytene stage of meiosis I. This screening, which we refer to as "nearest neighbor" screening, was performed by hybridizing the pachytene library to mixed cDNA probes derived from RNA of leptotene/zygotene and round spermatids. These cells represent the stages immediately preceding and just after pachytene. Clones identified as being negative for hybridization were selected. This differential screening procedure also represented an attempt to elucidate the level of gene expression overlap between pachytene spermatocytes and the two cell stages developmentally flanking the pachytene stage, leptotene/zygotene spermatocytes and round spermatids. The results of this analysis indicated that the pachytene spermatocyte cDNA library contained a minimum of 20% overlap in gene expression with round spermatids and a minimum of 2% overlap with leptotene/zygotene spermatocytes. The construction of stage-specific mouse meiotic cDNA libraries will facilitate subsequent molecular analyses aimed at delineating factors involved in the complex process of meiosis.

CHAPTER II

MATERIALS AND METHODS

Animals

Adult and 17 day old BALB/c mice were purchased from Harlan-Sprague-Dawley (Indianapolis, Indiana). The mice were provided with food and water *ad libitum* and maintained on a 14 hr light/10 hr dark schedule. Mice were killed by cervical dislocation and testes removed immediately.

Isolation of spermatogenic cells

Testes of adult or 17 day old BALB/c 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 $MgSO_4 \cdot 7H_2O$, 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 (Romrell *et al.*, 1976; Bellvé *et al.*, 1977). After tubule digestion, trypsin was removed by washing cells 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). After 2.5 hours for sedimentation, gradients were collected 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. Leptotene/zygotene spermatocytes were obtained from the testes of 17 day old mice. For the isolation of leptotene/zygotene spermatocytes the STAPUT procedure was performed four times, with 65 mice used each time; the average number of cells loaded on a gradient was 3×10^8 with 5×10^6 cells collected for the isolation of RNA. The average enrichment of leptotene/zygotene

spermatocytes used in library construction and probe preparation was greater than 80% as assessed by Nomarski optics; the principle contaminants were spermatogonia and Sertoli cells. Pachytene spermatocytes and round spermatids were isolated from the testes of adult mice (>8 weeks old). One STAPUT isolation with 11 mice was required, with approximately 9 x 10^8 cells loaded on to the gradient. The average enrichment of the pachytene spermatocyte fractions used determined by Nomarski optics was greater than 88% and 7 x 10^6 cells were recovered; the principle contaminants were Sertoli cells and symplasts composed of round spermatids. The average enrichment of the round spermatids determined by Nomarski optics was greater than 79% with 1.4 x 10^7 cells isolated; the principle contaminants were leptotene/zygotene spermatocytes and Sertoli cells. Following cell fixation for electron microscopy (see below), thick sections representing cells isolated from the STAPUT procedure were independently scored for cell composition. From this analysis leptotene/zygotene spermatocytes were enriched 77%, with 6% spermatogonia, 11% Sertoli cells, and 6% unidentified. Pachytene spermatocytes were found to be enriched 89%, with 4% round spermatids and 7% unidentified. Round spermatids used in library construction were found to be enriched 87%, with 7% leptotene/zygotenes, 1% Leydig cells, 5% unidentified.

Cytological methods

Cell pellets from pachytene spermatocytes, round spermatids, and leptotene/zygotene spermatocytes 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.

Isolation of RNA and mRNA

RNA was isolated by the method of Cathala *et al.* (1983); diethylpyrocarbonate-treated glassware and solutions were used in all steps. RNA derived from STAPUT isolated cells was homogenized in lysis buffer (5 M guanidium isothiocyanate, 10 mM 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 4M 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, 1 mM 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. Northern gels were performed following standard methods (Sambrook *et al.*, 1989) in order to verify RNA integrity. Briefly, 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 in these gels.

Poly A$^+$ RNA was isolated with the Dynal magnetic bead system following manufacturers instructions and quantitated as above. By this process, 95 μg of total leptotene/zygotene RNA produced 2 μg of mRNA; 111 μg of total pachytene RNA yielded 3.3 μg of mRNA; and 76 μg of total round spermatid RNA yielded 2.1 μg of mRNA.

Construction of cDNA libraries

cDNA libraries were constructed utilizing the Stratagene ZAP-cDNA synthesis kit (Figure 1). The initial quantities of poly A^+ mRNA used in the first strand cDNA synthesis reaction ranged from 2 to 3.3 μg. The first strand synthesis from an *Xho* I-oligo (dT) primer involved the incorporation of 5-methyl dCTP in order to protect the cDNA during subsequent enzymatic digestion with *Xho* I in the library construction. Second strand synthesis included ^{32}P-dATP to facilitate size selection of newly synthesized cDNA. The first and second strands were analyzed by alkaline gel electrophoresis (Sambrook *et al.*, 1989) in order to assess integrity and length. Leptotene/zygotene first and second strand products were found to be in the range of 3 kb to 100 bp, pachytene was in the range of 6 kb to 100 bp, and round spermatid was 5 kb to 100 bp. After blunt-cutting the ends of the cDNA, *Eco*R I adaptors were ligated and phosphorylated with T4 polynucleotide kinase. The cDNA was then digested with *Xho* I and cDNAs were fractionated after separation on a Sephacryl spin column. The size-fractionated cDNAs were analyzed using agarose gel electrophoresis and cDNAs greater than 0.5 kb were pooled. Pooled cDNAs used for ligations were in the following size ranges: leptotene/zygotene cDNA was 3 kb to 1 kb; pachytene cDNA was 6 kb to 0.5 kb; and round spermatid was 4.5 kb to 0.5 kb. Ligation to vector was performed with approximately 100 ng of cDNA/1 μg of Uni-ZAP XR vector DNA at 12°C overnight. The ligation products were inserted into the host phage using the Gigapack II Gold packaging extract (Stratagene) according to manufacturers instructions. The size of the libraries after packaging was as follows: leptotene/zygotene, 2.2 x 10^6 pfu; pachytene, 3.7 x 10^6 pfu; round spermatid 4.1 x 10^6 pfu. Packaged aliquots of the libraries were amplified in order to produce high-titer stocks. The average titer of the libraries after amplification was approximately 6 x 10^{10} plaque forming units (pfu)/ml. In this manner, three libraries

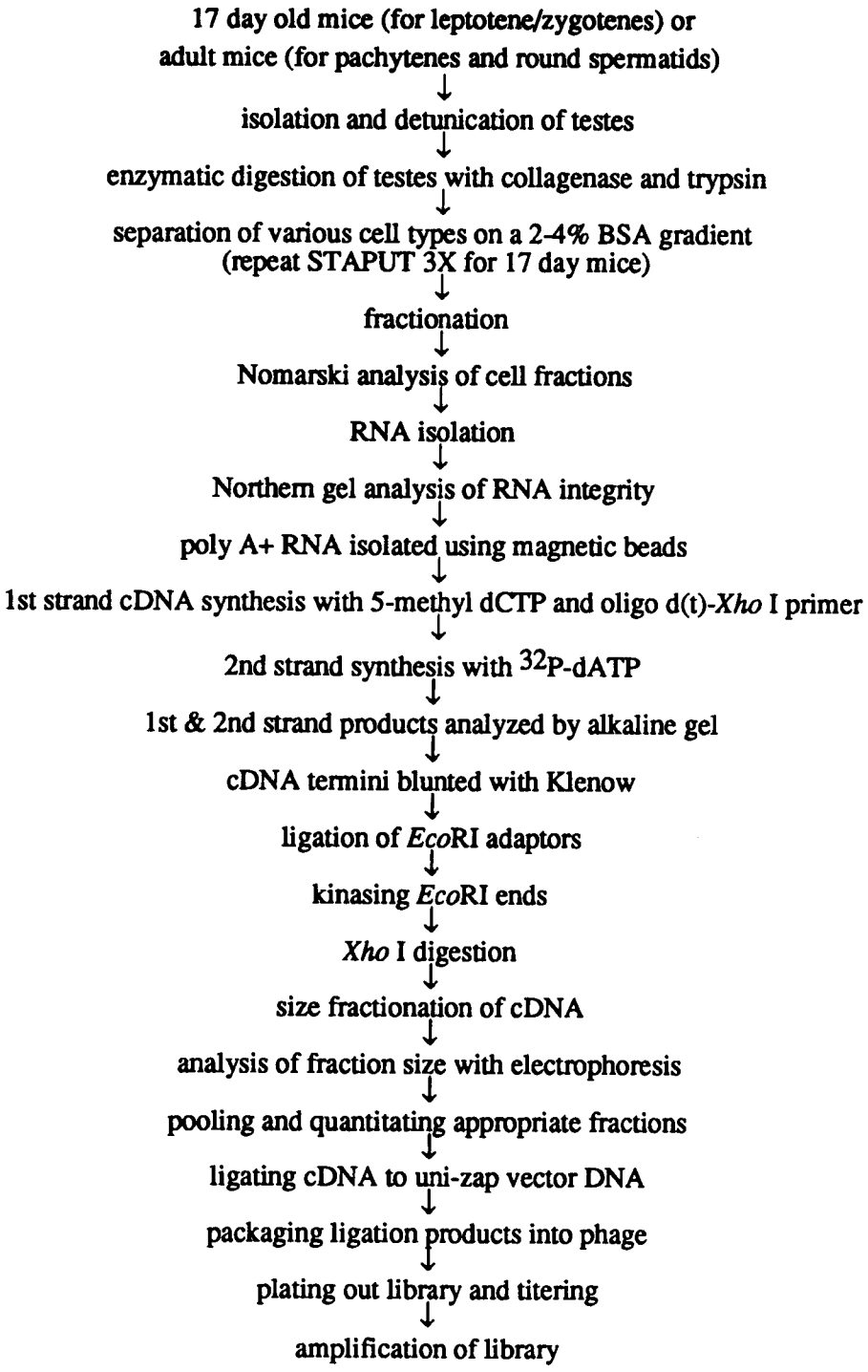


Fig. 1. Flowchart of library construction.

were constructed: one from leptotene/zygotene cell fraction RNA, one from the RNA of the pachytene cell fraction, and one from round spermatid RNA.

Characterization of the average insert sizes of the cDNA libraries

The Uni-ZAP libraries were plated out at approximately 1 x 10^7 pfu/ml and individual plaques were isolated and resuspended in 1 ml of SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-Cl, pH 7.5, 0.01% gelatin). Typically, 2 µl of the diluted phage suspension was lysed by incubation at 70°C for 5 minutes and then placed on ice. This was used as a substrate for a PCR reaction with T3 and T7 primers (100 ng/µl). These primers represent sequences flanking the inserts. Additional constituents of the PCR reactions were 1X Taq DNA polymerase buffer (Promega), 1.5 mM $MgCl_2$, 2.5 units Taq DNA polymerase (Promega), and 0.3 mM dNTPs. Amplification was in a Perkin-Elmer/Cetus DNA thermal cycler using the following profile: one cycle of 94°C for 5 min, 51°C for 3 min, 30 cycles of 72°C for 3 min, 94°C for 1 min 20 sec, 51°C for 2 min, followed by one cycle of 72°C for 7 min. One-tenth of the PCR reaction volume was placed in agarose gels for size analysis with a 1 kb standard marker DNA (GIBCO-BRL). Migration lengths were recorded for both the marker and unknowns. Insert sizes were established by relating to the marker migration standard curve.

Screening of the cDNA libraries for initial characterization

A total of 250,000 pfu of each amplified cDNA library were plated at a concentration of 17,000 pfu/132 mm plate. Two sets of duplicate lifts were taken per plate using nitrocellulose filters (Sambrook *et al.*, 1989). The transferred plaques were denatured for 2 min with 1.5 M NaCl, 0.5 M NaOH, neutralized for 5 min with 1.5 M NaCl, 0.5 M Tris-HCl (pH 8.0) and then crosslinked with 120,000 µJ of UV energy in a UV Stratalinker (Stratagene). The blots were hybridized in duplicate with the various

probes listed in Table 1. The hybridization solution contained 1% BSA, 1 mM EDTA, 0.5 M $NaHPO_4$ (pH7.2), 7% SDS (Ausubel *et al.*, 1987) with ^{32}P-labeled probe at a concentration of 1-2 ng/ml of hybridization solution. Hybridizations were performed overnight at 68°C in a shaking water bath. Low stringency washes contained 0.5% BSA, 1 mM EDTA, 40 mM $NaHPO_4$ (pH7.2), 5% SDS and were performed at room temperature. The high stringency washing solution consisted of 1 mM EDTA, 40 mM $NaHPO_4$ (pH7.2), 1% SDS and was performed in a series of 5-7 short washes and a 20 minute final wash, all at 68°C. Plaques were scored as positive for a probe only if the plaque hybridized on duplicate filters under high stringency conditions.

Probe preparation

All cDNA probes were radiolabeled with deoxycytidine 5'-[α-32P]triphosphate (dCTP[^{32}P]) (New England Nuclear) by the random primer labeling process (Feinberg and Vogelstein, 1983). The unincorporated nucleotides were separated from the labeled probes by gel filtration chromatography using 0.45 μm nylon microspin columns from PGC Scientifics (Gaithersberg, Maryland) with Sephadex G-50. The average specific activity for probe labeling was 1 x 10^9 cpm/μg DNA. The probes used for library analysis are listed in Table 1.

"Nearest neighbor" characterization of the pachytene cDNA library

From the pachytene spermatocyte cDNA library a total of 4,000 pfu were plated. Two sets of duplicate lifts onto nylon membrane (Hybond, Amersham) were taken from the pachytene cDNA library. The transferred plaques were denatured, neutralized, and crosslinked as above. Hybridization and washing conditions were the same as above. For this analysis, hybridization was to mixed cDNA probes derived from RNA of various cell

Table 1. Probes used for screening cDNA libraries

Probe	Source	Size	Type	Reference
18s	mouse	1.9 kb	rDNA	Paynton *et al.*, 1988
28s	mouse	4.8 kb	rDNA	Paynton *et al.*, 1988
3βHSD I	mouse	0.9 kb	cDNA	Bain *et al.*, 1991
β-actin	mouse	1.1 kb	cDNA	Alonso *et al.*, 1986
tubulin	chicken	1.2 kb	cDNA	Woychik, unpublished
Prm-1	mouse	0.5 kb	cDNA	Kleene *et al.*, 1984
c-mos	mouse	0.6 kb	cDNA	Oskarsson et al., 1980
c-fos	mouse	5.4 kb	cDNA	Curran *et al.*, 1983 Miller *et al.*, 1984
c-ras^{K}	mouse	0.5 kb	cDNA	Ellis *et al.*, 1981
Pgk-2:3'	mouse	0.5 kb	cDNA	McCarrey &Thomas, 1988
SGP-2	rat	0.8 kb	cDNA	Collard & Griswold, 1987
Ldh-c	mouse	0.45 kb	cDNA	Wu *et al.*, 1987

fractions. ^{32}P-labeled total cDNA probes complementary to poly A$^+$ RNA were synthesized using a modification of Sambrook *et al.*, 1989, as follows. RNA template at 5 µg was heated to 70°C for 5 min and then chilled immediately in ice water. The denatured RNA was then used in a cDNA synthesis reaction with a ^{32}P-labeled nucleotide and an oligo d(T) primer. The components of the reaction were as follows: 100 units RNasin (Promega), 2.5 µg oligo d(T) 15mer primer (Promega), 1X first strand synthesis buffer (Superscript II buffer from BRL), 0.8 mM dATP, dGTP, dTTP, 4.8 µM dCTP, 500 µCi [α-^{32}P]dCTP, 1mM dTTT, 500 units Superscript II reverse transcriptase (BRL). After 1 hour at 37°C, the reaction was stopped with the addition of 5 µl of 0.5 M EDTA and 5 µl of 10% SDS. The RNA in the reaction was hydrolyzed by incubation with 15 µl of 3N NaOH for 30 minutes at 68°C. Prior to phenol-chloroform extraction, 50 µl of 1 M Tris-Cl (pH 7.4) and 15 µl of 2 N HCl were added. Unincorporated nucleotides were separated by gel filtration chromatography using 0.45 µm nylon microspin columns from PGC Scientifics (Gaithersberg, Maryland) with Sephadex G-50. A total of 5 x 10^7 cpm of radiolabeled cDNA probe was used for each library hybridization. The hybridization solution consisted of 1% BSA, 1 mM EDTA, 0.5 M $NaHPO_4$ (pH7.2), 7% SDS (Ausubel *et al.*, 1987) with ^{32}P-labeled probe at 1-2 ng/ml of hybridization solution. Hybridizations were performed overnight at 68°C in a shaking water bath. Low stringency washes contained 0.5% BSA, 1 mM EDTA, 40 mM $NaHPO_4$ (pH7.2), 5% SDS and were performed at room temperature. The high stringency washing solution contained 1 mM EDTA, 40 mM $NaHPO_4$ (pH7.2), 1% SDS, and this washing was performed in a series of 5-7 short washes with a 20 minute final wash, all at 68°C.

CHAPTER III

RESULTS

Verification of cell populations represented in cDNA libraries

Subsequent to STAPUT sedimentation of spermatogenic cell populations, the cell fractions were analyzed cytologically to determine composition. Each cell fraction isolated contains characteristic contaminants (Romrell *et al.*, 1976; Bellvé *et al.*, 1977). Typical morphological characteristics of the various cells types, such as cell size and degree of granularity, can be visualized using various cytological methods and were important for assessing the enrichment of spermatogenic cells. In the initial assessment, wet mounted cell fractions were scored for purity using Nomarski optics. Subsequently, aliquots of cells representing each cell fraction were fixed, embedded in Epon and sectioned for both light and electron microscopy. The results of these analyses are presented as both numerical data and micrographs depicting typical fields of cells. Tables 2, 3, and 4 contain the results for scoring cell fractions by both Nomarski optics of whole cells and light microscopy of sectioned cells. Figures 2 and 3 contain electron and light micrographs, respectively, for fixed and sectioned pellets of cell fractions.

From this analysis we found the percent enrichment for leptotene/zygotene spermatocytes to be 81% as determined by Nomarski optics and 77% by light microscopy from independent scoring of the sectioned cell pellets (Table 2). Leptotene/zygotene spermatocytes typically represent 13% of the germ cells in the starting preparation used for the STAPUT isolation (Bellvé *et al.*, 1977). Figure 2a illustrates typical morphological features of enriched leptotene/zygotene spermatocytes as visualized by electron microscopy. Nuclei of these cells contain both granular and clumped chromatin. The cell fraction used for creating the leptotene/zygotene spermatocyte cDNA library contained a

Table 2. Cytological and molecular analysis of leptotene/zygotene spermatocyte cDNA library enrichment

Cell Type	% Cells		Probes	Positive Clones[c]
	N[a]	L[b]		
Leptotene/Zygotenes	81	77	c-*ras*^{K}	27 (0.011%)
Spermatogonia	11	6	c-*fos*	22 (0.009%)
Round Spermatids	0	<1	*Prm-1*	0 (0.000%)
Sertoli cells	8	11	SGP-2	12 (0.005%)
Leydig cells	0	0	3βHSD	11 (0.004%)
Unidentified	0	5		

[a]N - Nomarski optics of wet mounted cells from STAPUT-enriched cells

[b]L - light microscopy of pelleted, fixed and sectioned cells

[c]Out of 250,000 independent cDNA clones screened

Table 3. Cytological and molecular analysis of pachytene spermatocyte cDNA library enrichment

Cell Type	% Cells		Probes	Positive Clones[c]
	L[a]	N[b]		
Pachytenes	88	89	*Pgk-2:3'*	32 (0.013%)
			Ldh-c	27 (0.011%)
Round Spermatids	9	4	*Prm-1*	16 (0.006%)
Sertoli cells	3	<1	SGP-2	3 (0.001%)
Leydig cells	0	0	3βHSD	5 (0.002%)
Unidentified	0	6		

[a]N - Nomarski optics of wet mounted cells from STAPUT-enriched cells

[b]L - light microscopy of pelleted, fixed and sectioned cells

[c]Out of 250,000 independent cDNA clones screened

Table 4. Cytological and molecular analysis of round spermatid cDNA library enrichment

Cell Type	% Cells		Probes	Positive Clones[c]
	N[a]	L[b]		
Round Spermatids	79	87	*Prm-1*	43 (0.017%)
			c-*mos*	29 (0.012%)
Leptotene/Zygotenes	15	7	c-*ras*^{K}	10 (0.004%)
Spermatogonia	2	<1	c-*fos*	0 (0.0%)
Sertoli cells	4	0	SGP-2	12 (0.005%)
Leydig cells	0	1	3βHSD	4 (0.002%)
Unidentified	0	4		

[a]N - Nomarski optics of wet mounted cells from STAPUT-enriched cells

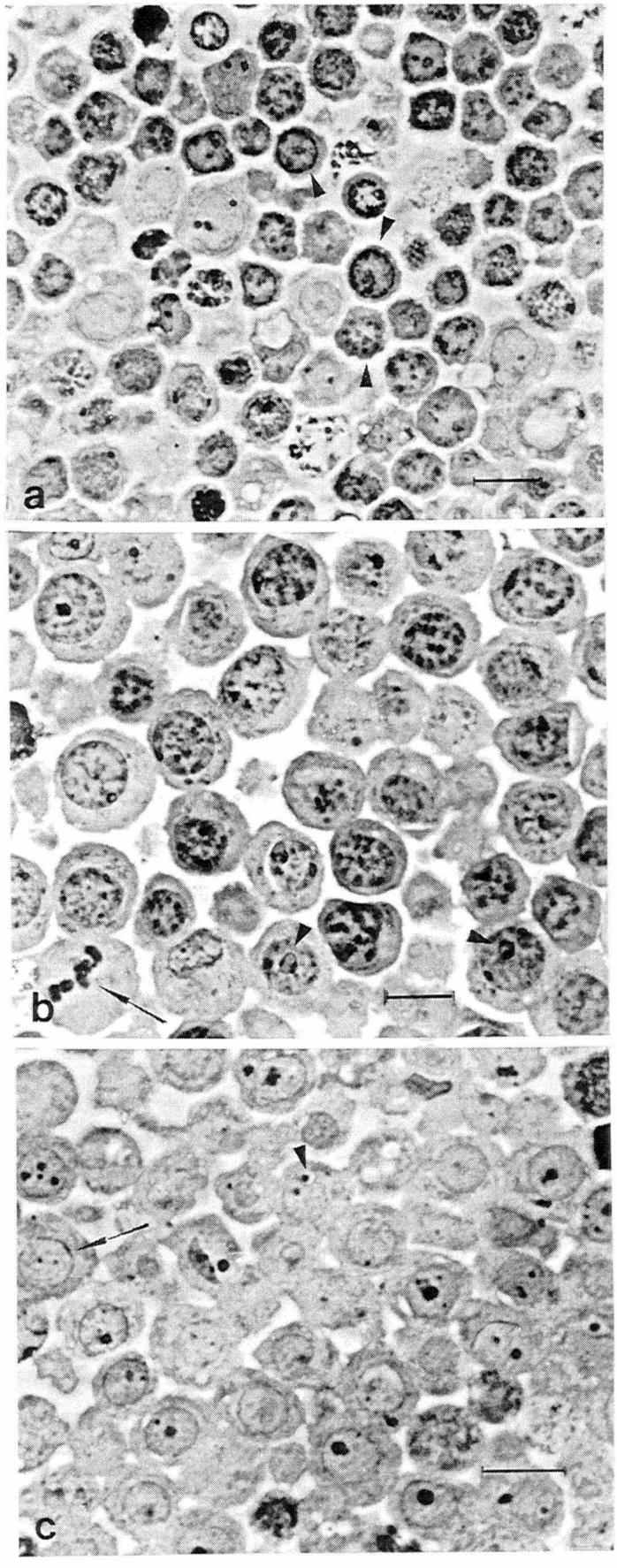
[b]L - light microscopy of pelleted, fixed and sectioned cells

[c]Out of 250,000 independent cDNA clones screened

Figure 2. Electron micrographs showing morphology of cells used in library construction. Cells were fixed, pelleted, sectioned, and stained with uranyl acetate and lead citrate. a: Leptotene/zygotene spermatocytes are shown (arrow). Bar = 2 μM. b: Pachytene spermatocytes with typical features of a sex body (small arrow) and synaptonemal complex (large arrow). Bar = 2 μM. c: Round spermatids are shown with acrosomal granule (large arrow) or acrosomal cap (small arrow). Bar = 2 μM.

a
b
c

Figure 3. Light micrographs showing characteristics of cells used in library construction. Cells were fixed, pelleted, sectioned, and stained with toluidine blue. a: Leptotene/zygotene spermatocytes appear granular and condensed (arrowheads). Bar = 10 μM. b: Pachytene spermatocytes with visible sex bodies (arrowheads) with a contaminating metaphase cell (arrow). Bar = 10 μM. c: Round spermatids showing acrosomal granules (arrowhead) or acrosomal caps (arrow). Bar = 10 μM.


a
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mixture of cell types, primarily leptotene and zygotene spermatocytes. The cellular composition can be visualized most advantageously by light microscopy of sectioned pellets (Figure 3a). The majority of the cells contain the condensed, granular nucleus, typical of leptotene/zygotene spermatocytes (arrowheads).

Pachytene spermatocytes were enriched 88% as determined by Nomarski optics and 89% as determined by light microscopy. Pachytene spermatocytes typically represent 15% of the germ cells in the starting preparation used for the STAPUT isolation (Bellvé *et al.*, 1977). Table 3 provides the cytological analysis for composition of these cell fractions. In addition, Figures 2b and 3b depict pachytene spermatocytes that have been fixed, pelleted, and sectioned. Pachytene spermatocytes have a thin rim of cytoplasm surrounding a large relatively homogeneous nucleus. In Figure 2b, pachytene spermatocytes are shown with a characteristic sex body that is closely associated with a nucleolus (small arrow) and a portion of synaptonemal complex is seen in a cell (large arrow). Contaminants of pachytene spermatocyte fractions are Sertoli cells, which are much larger and more refractile, symplasts of round spermatids and occasional metaphase cells. Figure 3b depicts a lower magnification of pachytene spermatocytes with sex bodies (arrowheads) and a contaminating metaphase cell (arrow).

Round spermatids were also analyzed for enrichment and were found to be enriched 79% by Nomarski optics and 87% by light microscopy (see Table 4). Round spermatids typically represent 31% of the germ cells in the starting preparation used for the STAPUT isolation (Bellvé *et al.*, 1977). Figure 2c is an electron micrograph of a round spermatid cell fraction with cells containing a characteristic large nucleolus-like structure inside the nucleus. Round spermatids are shown in different developmental stages which can be determined by the state of acrosome development from the acrosomal granule (large arrow) to the acrosomal cap (small arrow). Figure 3c is a light micrograph depicting round

spermatids with acrosomal granules (arrowhead) or acrosomal caps (arrow) at lower magnification.

Insert size of spermatogenic cDNA libraries

The leptotene/zygotene spermatocyte cDNA library contained 2.2 x 10^6 clones which were synthesized from 2 μg of mRNA. The pachytene spermatocyte cDNA library was constructed from 3.3 μg of mRNA and contained 3.7 x 10^6 clones. The round spermatid cDNA library contained 4.1 x 10^6 clones which were synthesized from 2.1 μg of mRNA. Since a library of 10^6 clones has a >99% probability of including rare transcripts at a detectable frequency (Sambrook *et al.* 1989) and since these libraries have > 10^6 clones, they probably contain cDNAs representing transcripts in low abundance. Additionally, insert sizes of randomly selected clones were examined for all three cDNA libraries (Table 5). Individual plaques were isolated and insert size analyzed by PCR; similar insert size (an average of 1.4 kb) was found for all three cDNA libraries. The leptotene/zygotene spermatocyte cDNA library contained the smallest average insert size, 1.3 kb, and the pachytene spermatocyte cDNA library contained the largest average insert size, 1.5 kb. The overall range of insert sizes observed for all three cDNA libraries was between 0.4 kb and 3.5 kb.

Gene representation in the cDNA libraries

The cDNA libraries were analyzed with respect to gene representation. Duplicate filter lifts of 250,000 pfu from each library were hybridized with the cDNA probes listed in Table 1. A plaque was considered positive only if it hybridized with a probe on both sets of filters. The results of this molecular analysis are reported below.

Table 5. Insert sizes in cDNA libraries

Library	Plaques Analyzed	Size Range	Average Size
Leptotene/Zygotene	34	2.3 - 0.4 kb	1.3 kb
Pachytene	31	3.1 - 0.7 kb	1.5 kb
Round Spermatid	25	3.5 - 0.5 kb	1.4 kb

Housekeeping genes. Table 6 lists the results of screening the three cDNAlibraries with probes for the "housekeeping" genes actin, tubulin, and ribosomal RNA sequences. The two former genes are expected to be expressed ubiquitously and thus represented in all three libraries while the latter should have been excluded during library construction. Actin cDNA sequences were found in all three libraries. A cDNA probe for tubulin was also used in the initial analysis of the cDNA libraries. Results from screening the three cDNA libraries with a β tubulin cDNA probe show that all three libraries have clones representing tubulin sequences. In contrast to actin and tubulin sequences, ribosomal RNA sequences should not be represented in the libraries. In this regard, the libraries were screened with both 18S and 28S ribosomal DNA sequences (Paynton *et al.*, 1988) and no positive clones were obtained from these hybridizations (Table 6).

Gene representation from somatic cells of the testis. Tables 2, 3, and 4 list the results of hybridizing several probes to the three germ cell cDNA libraries. All three cDNA libraries contained contaminating sequences from the somatic cells of the testis, as determined by the detection of gene sequences expressed exclusively in the Sertoli cells and Leydig cells. Sertoli cells express sulfated glycoprotein-2 [SGP-2] (Collard and Griswold, 1987), and Leydig cell express 3β-hydroxysteroid dehydrogenase type I [3βHSD I] (Bain *et al.*, 1991). The leptotene/zygotene spermatocyte cDNA library contained 0.004% positive clones for the Leydig cell probe, 3βHSD I, and 0.005% clones for the Sertoli cell probe, SGP-2. The pachytene spermatocyte cDNA library contained 0.002% clones for the 3βHSD I and 0.001% clones for SGP-2. The level of hybridization to the round spermatid library was 0.002% for 3βHSD I and 0.005% for SGP-2.

Leptotene/zygotene spermatocyte cDNA library. Table 2 includes both a cytological and molecular analysis of the leptotene/zygotene spermatocyte cDNA library.

Table 6. Analysis of gene representation in cDNA libraries

cDNA Library	18/28S	Actin	Tubulin
Leptotene/Zygotene	0% [a]	0.059%	0.016%
Pachytene	0%	0.049%	0.023%
Round Spermatid	0%	0.034%	0.025%

[a]Percentage of positive plaques of 250,000 independent cDNA clones screened.

The cDNA library was hybridized with a probe for a gene sequence specifically expressed during the leptotene/zygotene spermatocyte substage of meiotic prophase I in order to confirm the presence of gene sequences expected to be present at this stage. The cDNA library was also screened with gene sequences that are not expressed at leptotene/zygotene, but are expressed in contaminating cell types. The leptotene/zygotene spermatocyte cDNA library was screened with a probe for the proto-oncogene c-*ras*^{K}, expressed exclusively in leptotene/zygotene spermatocytes (Wolfes *et al.*, 1989), and 0.011% of the clones hybridized. When a probe for the proto-oncogene c-*fos*, expressed primarily in type B spermatogonia (Wolfes *et al.*, 1989) was used, 0.009% of the clones were positive. Clones, hybridizing to a probe for *Prm-1* (protamine-1), which is expressed solely in round spermatids (Kleene *et al.*, 1984), were not detected in this cDNA library. Round spermatid sequences were not expected to be present in this library as the 17 day old mice used for leptotene/zygotene spermatocyte enrichment have not undergone meiotic divisions (reviewed by Handel, 1987).

Pachytene spermatocyte cDNA library. Table 3 contains the cytological and molecular analysis of pachytene spermatocyte cDNA library enrichment. Pachytene spermatocytes express variants of two housekeeping genes, *Ldh-c* (Thomas *et al.*, 1990) and *Pgk-2:3'* (McCarrey and Thomas, 1988). cDNA clones of both of these genes were used as probes to screen for the presence of pachytene spermatocytes. When the library was probed with *Pgk-2:3'* , 0.013% of the clones hybridized with this sequence and when the library was probed with *Ldh-c*, 0.011% of the clones hybridized with this sequence. When probing with *Prm-1*, which is expressed exclusively in round spermatids (Kleene, *et al.*, 1984), 0.006% of the clones were positive.

Round spermatid cDNA library. Table 4 contains both a cytological and molecular analysis of round spermatid cDNA library enrichment. Round spermatids exclusively

express the proto-oncogene c-*mos* (Wolfes *et al.*, 1989) and *Prm-1* (Kleene *et al.*, 1984). cDNA clones of both of these genes were used as probes to screen for round spermatid gene expression. When the library was probed with c-*mos*, 0.012% of the clones hybridized with this sequence. Likewise, when the library was probed with *Prm-1*, 0.017% of the clones hybridized with it. When probing with c-ras^K, which is expressed exclusively in leptotene/zygotene spermatocytes (Wolfes *et al.*, 1989), only 0.004% of the clones were positive. Hybridization of the round spermatid cDNA library with c-*fos*, which is expressed in spermatogonia, identified no positive clones.

Analysis of unique gene sequences in meiotic libraries

In order to determine minimal values of transcript overlap between two neighboring cell types, a "nearest neighbor" screen was performed. In this procedure 4,000 pfu of the pachytene spermatocyte cDNA library were plated out and duplicate filter lifts made of the plaques. These plaques were then hybridized with ^{32}P-labeled cDNA from the cell types that developmentally border or are "neighbors of" pachytene cells. Thus, leptotene/zygotene spermatocyte RNA and round spermatid, as well as pachytene, RNAs were used as templates for constructing mixed ^{32}P-labeled-cDNA probes, primed from an oligo d(T) primer and hybridized to filters from the pachytene spermatocyte cDNA library. The results of this analysis are shown in Table 7. At least 10% of the pachytene spermatocyte cDNA library inserts contain sequences that are also expressed in round spermatids and, additionally, at least 2% of the pachytene spermatocyte cDNA library inserts are expressed in leptotene/zygotene spermatocytes. The positive control, pachytene ^{32}P-labeled-cDNA hybridized to pachytene cDNA library clones showed that 45% of the plated pachytene library inserts hybridized. Considering the positive control exhibited just 45% hybridization, the values obtained for transcript overlap between neighboring cell types are most likely minimal values and are probably twice the values we obtained. One

Table 7. "Nearest neighbor" analysis of sequence representation in the pachytene spermatocyte cDNA library

Mixed cDNA probe	No. positive plaques (out of 4,800)	% Positive plaques
Leptotene/Zygotene	43	1
Pachytene	2138	45
Round Spermatid	504	11

hundred cDNAs that were shown by this analysis to be expressed in pachytene, but not in the neighboring cell types (leptotene/zygotene spermatocytes and round spermatids), were isolated for further characterization.

CHAPTER IV

DISCUSSION

This report describes the construction of male germ-cell specific cDNA libraries from leptotene/zygotene spermatocytes, pachytene spermatocytes, and round spermatids. Two of these libraries, those from leptotene/zygotene and pachytene spermatocytes, are presumed to represent sequences expressed during meiotic prophase. The cells contributing to these two libraries are the only meiotic cell types that can be isolated from testes with reasonable purity. Thus, they provide a starting point for the investigation of some key elements of meiosis, and cDNA libraries from these cell types could be an important resource. The third library constructed here is composed of postmeiotic, haploid round spermatids and can be a useful reagent for studies of postmeiotic differentiation.

Cytological analysis

Cytological analysis provided information about the cell composition of the libraries. The STAPUT procedure (Romrell *et al.*, 1976; Bellvé *et al.* 1977) used for the isolation of spermatogenic cell populations is based on sedimentation at unit gravity in a BSA gradient and provides enrichment but not purification of various spermatogenic cell populations. It is not possible with current techniques to isolate pure populations of spermatogenic cells. Thus a level of contamination with undesired cDNAs from testicular cell types is to be expected in the cDNA libraries. The cell types that are primary contaminants in a STAPUT-enriched cell population have been previously determined (Romrell *et al.*, 1976; Bellvé *et al.*, 1977). Our cytological analyses of the cell populations isolated and molecular analyses of the resultant libraries show contaminants

consistent with these findings. Cytological assessment of cells is clearly an important step in verifying the composition of enriched fractions. Initially, aliquots of fractions were scored for cell type at the time of enrichment using wet mounts visualized by Nomarski optics. From this assessment it was determined the STAPUT procedure resulted in significant enrichment of leptotene/zygotene spermatocytes (81%), pachytene spermatocytes (88%), and round spermatids (79%). In order to further analyze enrichment of the cells, fixed and embedded STAPUT-enriched cells were prepared from fractions combined for library construction and scored by light microscopy by an independent investigator. From this analysis leptotene/zygotene spermatocytes were enriched 77%, pachytene spermatocytes 89%, and round spermatids 87%. These data from pelleted cells confirm the figures for enrichment gathered from Nomarski optics. Additionally, evidence from electron microscopy confirms levels of cell enrichment derived from Nomarski analysis and adds supporting morphological information (Figures 2 & 3). For example, leptotene/zygotene spermatocytes have a condensed, granular nucleus with an occasional profile of synaptonemal complex lateral element. In contrast, pachytene spermatocytes have a characteristic sex body that is closely associated with a nucleolus and synaptonemal complex elements are commonly seen throughout the nucleus. The round spermatids isolated were identified by various stages of acrosome morphology.

Molecular analysis

The quality of the RNA isolated from each cell type was evaluated by electrophoresis, confirming that high molecular weight, largely undegraded RNA contributed to the cDNA synthesized for the libraries. Molecular methods were used to evaluate the resultant cDNA libraries. Three molecular assessments were made for each library: 1) two housekeeping genes, β-actin and β-tubulin, were analyzed for representation of their cDNAs in the libraries; 2) cell-specific contamination with other

cell types was determined by detection of gene sequences expected to be represented in the libraries when certain cell contaminants were present in the cell fraction utilized as a source of RNA; and 3) the cDNA libraries were analyzed for molecular composition by determining if expected gene sequences were represented in the libraries.

1. Representation of housekeeping gene sequences

There are two cytoplasmic forms of actin, β and γ, and both have been detected in spermatogenic cells (Hecht *et al.*, 1984). The β actin cDNA probe used for this analysis contains the entire coding region of β actin and should hybridize to all actin-encoding mRNAs, as demonstrated previously by Waters *et al.* (1985). In our initial molecular assessment of the cDNA libraries, it was found that clones homologous to the β-actin cDNA probe were represented. The levels of β-actin detected in the three cDNA libraries are comparable to that reported in another study which characterized fetal germ cell libraries for actin sequences and found the level to be 0.04% (Urven *et al.*, 1993).

Both α and β tubulin are present in spermatogenic cells. Our analysis of the three libraries indicates that β-tubulin sequences were represented equally across the three cDNA libraries. The β-tubulin probe used to identify tubulin sequences in these cDNA libraries probably detected numerous tubulin family members, since the cDNA probe for α tubulin contains the coding region for many tubulin family members. Additionally, as expected, no positive clones were detected when the libraries were hybridized with 18S and 28S cDNA sequences, indicating that cDNA synthesis was primed from mRNA and not rRNA.

2. Representation of somatic cell-expressed sequences

In the secondary assessment we performed, all three libraries were hybridized with probes corresponding to cDNA sequences that are known to be expressed in the somatic cells of the testis, Sertoli cells and Leydig cells. As Sertoli cells were observed in the

enriched cell populations, the libraries were expected to contain gene sequences expressed from these contaminating cells, such as SGP-2. All three cDNA libraries were found to have sequences representative of SGP-2.

Very few Leydig cells were observed in our cell populations, as none were detected microscopically in pachytene spermatocytes or leptotene/zygotene spermatocytes. The round spermatid fraction was found to contain 1% Leydig cells, as observed in pelleted round spermatids. Upon hybridizing the Leydig cell-specific probe 3β-hydroxysteroid dehydrogenase I [3βHSD I] to the libraries, positive clones were identified in each library. This was surprising since the percentage of Leydig cells scored cytologically was very low. However, 3βHSD I is expressed abundantly in testes of *XXSxr* mice (Caldwell and Handel, unpublished observation), which are devoid of germ cells. Conclusive molecular analysis of Leydig cell contamination is problematic, as it is not clear that the 'Leydig cell-specific' probe is truly specific, as cross hybridization with other testis cell types may occur (A. Payne, personal communication). Our own Northern analysis of the probe showed it was not expressed in germ cells but we cannot exclude expression in Sertoli cells. It has not been established that better 'specific' probes for Leydig cells are available in this context.

3. Representation of sequences expressed in specific germ cells

The third type of molecular evaluation we undertook with these libraries was to assess the presence of clones representative of gene sequences which are known to be expressed in specific germ cells. Unfortunately for most enriched cell populations, few probes are available which are specific for any given cell type in the context of the contaminating cells present. While it is clear that a more highly expressed gene in a cell type should be represented by greater numbers of clones within that library, comparisons

between the respective libraries for a specific cDNA should be made with caution. Valid comparisons cannot be made between libraries regarding the expression levels and the resultant number of clones in a library for different cDNAs (unless specific quantitative levels of transcript were known for a particular cell type). For instance, in the leptotene/zygotene library, the c-*fos* cDNA probe identified spermatogonial gene expression at a level of 0.009% positive clones, while cytologically 11% of the cells were identified as spermatogonial contaminants. The leptotene/zygotene specific cDNA probe, c-ras^K, identified 0.011% positive clones, although 81% of the cells were leptotene/zygotenes. Superficially, these data might suggest that numbers of spermatogonia were only slightly less than numbers of leptotene/zygotene cells. However, Wolfes *et al.* (1989) studied the levels of various proto-oncogenes during spermatogenesis and found c-*fos* levels to be much higher in type B spermatogonia than were levels of c-ras^K in leptotene/zygotenes (the exposure time for Northern blots hybridized with c-*fos* was 6 hr, as opposed to exposure the exposure time for c-ras^K, which was 2 days). Given this kind of information on relative levels of expression, it is not possible to quantitatively analyze the libraries for cell contamination levels by examining percentages of cDNA clones detected by various probes. It is not likely that a direct link between cell contamination levels and molecular analysis will be possible through examination of gene expression levels of different gene sequences within one specific library unless transcript levels are specifically known for a gene within each cell type.

This molecular analysis did, however, yield some useful information regarding cell contamination levels when comparing a single cell type across different libraries. For instance, the *Prm-1* gene is expressed exclusively during round spermatid development. In the round spermatid library, which is enriched 79% by cytological criteria, 0.017% positive clones were identified. When round spermatids were found as a contaminating cell type in the pachytene library, at 9%, the percentage of positive clones was 0.006%, a value

which does not correlate in linear fashion. If the sequence representation results from the round spermatid library are correct, than we might expect a more proportional value for round spermatid contamination of the pachytene library, with a value closer to 0.002% than to 0.006%. However, each round spermatid scored in the cytological analysis of pachytene spermatocyte enrichment is actually a symplast containing between 2 and 4 round spermatids. We find that some round spermatid symplasts co-sediment with pachytene spermatocytes in our STAPUT procedure. Thus, the level of gene expression in each round spermatid scored is at least 2X the level indicated by the cytological analysis; in this regard, the seemingly high value of 0.006% *Prm-1* expression in the pachytene spermatocytes actually correlates linearly with the results of *Prm-1* expression in the round spermatid library.

The molecular analysis of the cDNA libraries has provided some useful information with respect to confirmation of transcripts that we expect to be present in the cell types represented in the libraries. We can also verify on a molecular level, the contaminants we see cytologically. In all cases the numbers of clones representing genes that are expected to be present in a library are at higher levels than those clones present because of contaminating cell types. Although we find some apparent anomalies with unexpected clone numbers in a library which do not appear to correlate with the known frequency of a particular cell type in a library, these anomalies do not invalidate the characterization of the libraries. No statistical significance can be attributed to the actual number of clones within a library. Likewise, comparative analysis between clone numbers across libraries has little validity. Very little information is available with respect to relative transcript levels for most genes expressed during spermatogenesis. Spermatogenesis is a developmental continuum and gene expression during spermatogenic progression is most likely not entirely distinct between cell types. Additionally, few cDNAs have been

identified with cell-specifc expression patterns during spermatogenesis. Given the limitation that relatively few cell-specific markers have been identified for the cell types used in construction of these libraries, in conjunction with the inevitable problems of contamination with other testicular cell populations, a quantitative molecular analysis is difficult.

Similarities among libraries

These libraries have proven to be useful reagents in the analysis of gene expression during meiosis in several ways. The nearest neighbor screen indicated that many gene sequences represented in the pachytene spermatocyte cDNA library are not represented in either leptotene/zygotene spermatocytes or round spermatid cDNA. A control probe of pachytene cDNA hybridized to 45% of the plaques from the pachytene spermatocyte cDNA library. This unexpectedly low value indicated either that the labeling procedure was either not efficient or that there are many sequences in these cells which are expressed only in low levels. Since the pachytene control hybridization detected only about half of the cDNA clones, this value was used to normalize the figures resulting from hybridization with leptotene/zygotene and round spermatid cDNA probes. With this correction, approximately 20% of the sequences in the pachytene spermatocyte cDNA library were also expressed in round spermatids and 4% of the sequences were expressed in leptotene/zygotene spermatocytes. Since there are quite a few sequences in common between pachytene spermatocytes, and round spermatids, there may be many sequences expressed in pachytenes for subsequent use during spermiogenesis. This observation has support from several studies finding gene sequences first expressed during meiosis and then maintained during spermiogenesis. Genes such as *Pgk-2* and *Ldh-c* exhibit this expression pattern. However, it was surprising that very little transcript overlap was observed between leptotene/zygotene and pachytene spermatocytes, since both represent

meiotic prophase cells. When a mixed leptotene/zygotene cDNA probe is hybridized to plaques from a leptotene/zygotene cDNA library only about 30% of the plaques will hybridize. The efficiency of labeling leptotene/zygotene RNA appears to be low and may account for the surprisingly small amount of overlap demonstrated between the leptotene/zygotene and pachytene spermatocytes. It appears that many of the gene sequences represented in the pachytene cDNA library are not detected in leptotene/zygotene spermatocytes or in round spermatids. Interestingly, there has been some informal discussion at conferences of the possibility that all gene sequences are expressed during meiosis. This theory is derived from the idea that there may be a check point for completion of meiotic prophase requiring "read-through" or the transcription of all gene sequences, perhaps a way of re-setting the genome to a developmental "zero" point. One possible way to examine this hypothesis indirectly would be to try to detect gene sequences in the pachytene library that clearly would not be expected to be expressed in the testis, such as hemoglobin gene sequences.

The leptotene/zygotene and pachytene spermatocyte cDNA libraries have also been utilized in a study designed to identify gene sequences which are enriched during meiosis. This procedure utilized RNA from the testes of meiotically arrested male mice in a screen for meiotically enriched sequences. The clones identified from this procedure are in the process of being analyzed further and are expected to yield information regarding meiosis. This analysis is described in more detail in Part III of this dissertation.

Comparative studies between the two meiotic libraries or between the pachytene and round spermatid library may prove to be reasonable approaches for the isolation of meiotic sequences. For example, in the "nearest neighbor" screen many cDNAs were identified that appeared to represent sequences expressed only during the pachytene stage. Additionally, these libraries could be useful for future studies involved in identification of differentially expressed genes. There are many techniques available for enrichment of

genes expressed in a particular cell type. For example, in order to isolate genes involved in specific meiotic stages, the leptotene/zygotene and pachytene libraries could be used in subtractive hybridization procedures (Bowes *et al.*, 1989; Duguid *et al.*, 1988; Travis and Sutcliffe, 1988). Alternatively, aliquots of the libraries could represent unique sources of selected cDNA pools for use in differential display reactions (Liang and Pardee, 1993; Liang *et al.*, 1993) to identify genes expressed in one meiotic stage but not in another. In addition, these libraries can be a valuable resource for identifying meiotically-expressed gene sequences through straightforward library screening strategies by homology. Recently the pachytene spermatocyte cDNA library constructed in this study was used successfully to identify zinc finger-containing gene sequences (M. Shannon, personal communication).

In all, the three cDNA libraries constructed here are representative of important stages of spermatogenic development and will most likely contain gene sequences of importance for spermatogenic function. Two of the libraries represent gene expression during prophase I of meiosis, leptotene/zygotene spermatocytes and pachytene spermatocytes. During the leptotene/zygotene spermatocyte stages of meiotic prophase I, replicated chromosomes begin their homology search, pairing of chromosomes occurs, and the synaptonemal complex becomes visible; during the pachytene spermatocyte stage of prophase I chromosomes undergo recombination. These libraries should thus contain the essential sequences required for the proper initiation and maintenance of these meiotic events. The third library is composed of post-meiotic round spermatids, a haploid cell population undergoing spermiogenesis, a highly complex process of differentiation. Genes have been isolated that have a role in spermiogenesis, such as the protamines (Kleene *et al.*, 1984) that are expressed exclusively in round spermatids and acrosin (Adham *et al.*, 1989; Klemm *et al.*, 1990), which is transcribed meiotically and utilized postmeiotically; it

is highly likely that other genes with a spermiogenic role will be identified as well. Additionally, the round spermatid library will be useful for comparing gene expression levels between meiotic and postmeiotic cell types with various differential screening methodologies. Therefore, as they become further used and characterized, these spermatogenic cDNA libraries will represent a significant tool for future studies of gene expression during both the meiotic and postmeiotic stages of male germ cell development.

CHAPTER V

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PART III

IDENTIFICATION OF MEIOTICALLY EXPRESSED SEQUENCES THROUGH THE UTILIZATION OF MALE STERILE MOUSE MUTANTS

CHAPTER I

INTRODUCTION

Although meiosis, particularly events of meiotic prophase, is genetically essential for the formation of a normal gamete, very little detail is available concerning the molecular and cellular aspects of this phase of mammalian gametogenesis. From a temporal perspective, meiosis represents a significantly long period of gametogenesis; almost one-third of the entire time required for spermatogenesis in the mouse is devoted to the various phases of meiosis, principally to the relatively long prophase of meiosis I. Prophase I of meiosis is divided into five substages which include: leptotene, zygotene, pachytene, diplotene, and diakinesis. Many events must be accomplished during meiosis, including: homologous chromosome pairing and recombination, faithful segregation of chromosomes at anaphase I, and most probably, preparation for the ensuing spermiogenic program of differentiation. The identification of genes involved in these processes is essential for understanding the mechanisms governing and regulating meiosis.

There are many approaches possible for identification of meiotically expressed genes in the mouse. One of the most common methods reported in the literature for identification of genes expressed during spermatogenesis is differential screening of testis cDNA libraries. Although in many of these studies the investigators were not searching specifically for meiotic gene expression, a few sequences representing meiotically expressed genes were identified (Thomas *et al.*, 1989; Hoog, 1991; Starborg *et al.*, 1992; Yuan *et al.*, 1995). In these studies, whole testis cDNA libraries, from either adult or prepuberal mouse testes, were screened with ^{32}P-labeled cDNA probes from poly A$^+$ RNA of liver and/or kidney, pachytene spermatocytes, and round spermatids. Utilizing these

procedures multiple cDNAs were identified that exhibited a variety of expression patterns during stages of spermatogenesis. For example, Thomas *et al.* (1989) used Northern blot analysis with purified germ cells to classify these clones according to cell-specific expression patterns, including round spermatid expression, pachytene spermatocyte expression, leptotene/zygotene spermatocyte expression, and a category of both spermatogonial and spermatocyte expression. In a comparable study, differential screening was used to isolate cDNAs expressed during prepuberal germ cell development (Hoog, 1991; Starborg *et al.*, 1992). Twenty-six clones were identified as being preferentially expressed in the testis and three of these were expressed mainly in spermatogonia and spermatocytes. In a similar manner, 420 testicular cDNAs were selected as exhibiting differential expression, and partially sequenced. Of these, 13 were found to represent genes expressed in both spermatocytes and spermatids (Yuan *et al.*, 1995). Taken together, these studies report several genes which exhibit meiotic expression, although many of these are expressed also in spermatogenic cells other than the meiotic cells of the testis. These studies were designed to identify spermatogenically expressed genes, and thus the screening procedure did not specifically identify cDNA clones representing meiotically expressed genes. However, differential screening procedures represent a potentially effective method for the isolation of sequences that are expressed during mammalian meiosis.

Several genes showing a meiotic expression profile have been detected in the mouse testis (reviewed by Hecht, 1993). A few of these genes were identified as meiotic variants of genes expressed during other spermatogenic stages, such as *Pgk-2* (McCarrey and Thomas, 1987) and *Ldh-c* (Thomas *et al.*, 1990; Jen *et al.*, 1990), while others were identified as having a common sequence motif, such as the zinc finger motif in *Zpf-35* (Cunliffe *et al.*, 1990). Examination of spermatogenic expression patterns of genes transcribed meiotically has revealed that these genes may or may not be utilized

postmeiotically. Northern blot analysis of spermatogenic expression of these meiotically expressed genes has permitted classification of meiotically transcribed genes into one of two categories - those which exhibit expression in both spermatocytes and spermatids and those with spermatocyte expression only. The products of many meiotically expressed genes, including *Pgk-2* (McCarrey and Thomas, 1987), histone H4 (Meistrich, 1989), *Ldh-c* (Thomas *et al.*, 1990; Jen *et al.*, 1990; Alcivar *et al.*, 1991), and acrogranin (Anakwe and Gerton, 1990) are utilized postmeiotically. Meiotic transcription of these genes may thus facilitate postmeiotic differentiation. It is thought that many gene products translated postmeiotically are initially transcribed during pachytene. In addition to meiotically expressed genes which are utilized postmeiotically, some meiotically expressed genes with limited postmeiotic expression have been described. During the past several years gene sequences have been identified that appear primarily meiotic. Genes that demonstrate this type of expression include *meg1* (Don and Wolgemuth, 1992); *Zfp-35* (Cunliffe *et al.*, 1990), *cycB2* (Chapman and Wolgemuth, 1993), and *ferT* (Fischman *et al.*, 1990; Keshet *et al.*, 1990). Products of these genes may be necessary for the genetic events constituting meiosis since very little postmeiotic expression occurs for these gene products. Further analysis of these clones may prove useful in obtaining new information regarding the meiotic process as it proceeds during spermatogenesis. Clearly, the isolation of additional gene sequences that are expressed during meiosis represents a reasonable approach toward the elucidation of factors necessary for this process. Other strategies that are available for the isolation of gene sequences representative of a particular cell type or function, such as subtractive hybridization (Bowes *et al.*, 1989; Duguid *et al.*, 1988; Travis and Sutcliffe, 1988), differential display (Liang and Pardee, 1992; Liang *et al.*, 1993), or representational difference analysis (Hubank and Schatz, 1994), have not yet been exploited to detect meiotically expressed genes.

A novel alternative approach for the identification of gene sequences expressed during meiosis in the male mouse is reported in this study. This strategy takes advantage of meiotic stage-specific arrest in several male sterile mouse mutants. Most of the information regarding meiotic failure in these mutants is derived from genetic analysis and cytological observations. At least 15 different X-autosome translocations have been described in the literature and spermatogenic failure was always found to occur during meiosis in the testes of mice containing these translocations (reviewed by Russell, 1983). This arrest occurs most frequently prior to metaphase of meiosis I. In addition, time of meiotic arrest varies between different X-autosome translocations (reviewed by Handel, 1987). For example, cytological analysis shows that spermatogenesis is halted during leptotene/zygotene of meiotic prophase I in T(X;11)38H (T38H) translocation heterozygous mice, but during mid-late pachytene in T(X;16)16H (T16H) heterozygous mice. One can infer concurrent differences in gene expression between these two X-autosome translocations as well. This hypothesis provided the rationale for the strategy reported here for enrichment for meiotically expressed gene sequences. Specifically, testicular RNA from T38H mice was utilized to differentially screen cDNA libraries from leptotene/zygotene and pachytene spermatocytes for the isolation of non-hybridizing clones, considered to represent a stage-specific pool of cDNAs. This pool is presumed to consist of those gene sequences expressed after the point of T38H spermatocyte arrest. Thus these sequences may correspond to genes involved in meiotic prophase events after the zygotene stage of prophase I. These clones were then hybridized with heterogeneous cDNA probes from testicular RNA of T16H mice. The rationale behind this screen postulated that positively hybridizing clones might represent gene sequences that are expressed in T16H spermatocytes (since spermatocytes from T16H testes proceed further, to mid to late pachytene, than do spermatocytes from T38H mice). Using this scheme, clones were identified that could thus be representative of a specific "window" of gene

expression during meiotic prophase. In this particular screening strategy the representative meiotic window contains cellular events occurring between the arrest of T38H and T16H spermatocytes, that is between leptotene/zygotene and mid- to late- pachytene of meiotic prophase I.

CHAPTER II

MATERIALS AND METHODS

Animals

Adult BALB/c males were purchased from Harlan-Sprague-Dawley (Indianapolis, Indiana). XX*Sxr* males were bred in our mouse facility by matings of XY*Sxr* males to XX or XO females. Typically, XY*Sxr* males were hemizygous for either Tabby (Ta) or Greasy (Gs), which are X-linked coat markers, while females were genetically unmarked. XX*Sxr* males were identified by variegated coat pattern and by smaller-than-normal testes. T38H and T16H translocation heterozygous male mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). Mice were provided with food and water *ad libitum* and maintained on a 14 hr light/10 hr dark schedule. Mice were killed by cervical dislocation and testes removed immediately.

Cytological methods

Testes from T38H males and T16H heterozygous males 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 1 hour, rinsed, postfixed in 1% osmium tetroxide in the same buffer, dehydrated through graded ethanols, infiltrated with propylene oxide, and embedded in Epon. Sections for electron microscopy were stained with uranyl acetate and lead citrate. Testes from the various mouse strains utilized in this study were also fixed in 2% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium cacodylate and prepared for embedding in Historesin as described by the manufacturer (Jung, Heidelberg). Thick sections for light microscopy were stained with toluidine blue.

Isolation of Spermatogenic Cells

Testes of adult, 17 day old, or 8 day old BALB/c 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 $MgSO_4 \cdot 7H_2O$, 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 (Romrell *et al.*, 1976; Bellvé *et al.*, 1977). After tubule digestion, trypsin was removed by washing cells 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). After 2.5 hours for sedimentation, gradients were collected 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. Leptotene/zygotene spermatocytes were obtained from the testes of 17 day old mice. For the isolation of leptotene/zygotene spermatocytes the STAPUT procedure was performed with 60 mice; the average number of cells loaded on a gradient was 3×10^8 with 5×10^6 cells collected for the isolation of RNA. The average enrichment of leptotene/zygotene spermatocytes used in probe preparation was greater than 80%; the principle contaminants were spermatogonia and Sertoli cells. Pachytene spermatocytes were isolated from the testes of adult mice (>8 weeks old). A STAPUT with 11 mice was required, with approximately 9×10^8 cells loaded on to the gradient. The average enrichment of the pachytene spermatocyte fractions used was greater than 88% and 1×10^7 cells were recovered; the principle contaminants were Sertoli cells and symplasts composed of round spermatids.

Isolation of RNA

RNA was isolated by the method of Cathala *et al.* (1983); diethylpyrocarbonate-treated glassware and solutions were used in all steps. T38H and T16H testes were frozen in liquid nitrogen and pulverized using a mortar and pestle and then homogenized in lysis buffer (5 M guanidium isothiocyanate, 10 mM EDTA, and 50 mM Tris-Cl, pH 7.5) by passing the sample through a 21-gauge needle 20 times. RNA derived from STAPUT isolated cells was treated as above except that the cells were not pulverized in liquid nitrogen, rather they were immediately passed through a 21 gauge needle in the presence of lysis buffer. The RNA was precipitated overnight in 7 volumes 4M 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, 1 mM 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. Northern gels were performed following standard methods (Sambrook *et al.*, 1989) in order to verify RNA integrity. Briefly, 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.

cDNA synthesis from RNA

^{32}P-labeled total cDNA probes complementary to poly A$^+$ RNA from T38H, T16H, pachytene spermatocytes, or leptotene/zygotene spermatocytes were synthesized

using a modification of Sambrook *et al.*, 1989, as follows. RNA template, 5 μg, was heated to 70°C for 5 min and chilled immediately in ice water. The denatured RNA was the template in a cDNA synthesis reaction with a ^{32}P-labeled nucleotide and oligo d(T) primer. The components of this reaction mixture were as follows: 100 units RNasin (Promega), 2.5 μg oligo d(T) 15mer primer (Promega), 1X first strand synthesis buffer (Superscript II buffer from BRL), 0.8 mM dATP, dGTP, dTTP, 4.8 μM dCTP, 500 mCi [α-^{32}P]dCTP,1 mM dTTT, 500 units Superscript II reverse transcriptase (BRL). After 1 hour at 37°C the reaction was stopped by the addition of 5 μl of 0.5 M EDTA and 5 μl of 10% SDS. The RNA in the reaction mixture was hydrolyzed by incubation with 15 μl of 3N NaOH for 68°C for 30 minutes. Prior to phenol/chloroform extraction, 50 μl of of 1 M Tris-Cl (pH 7.4) and 15 μl of 2 N HCl were added. Unincorporated nucleotides were separated by gel filtration chromatography on Sephadex G-50 using 0.45 μm nylon microspin columns from PGC Scientific (Gaithersberg, Maryland).

cDNA library screening

Two cDNA libraries previously constructed (Part II) were utilized in this screening procedure. The strategy is to hybridize with mixed cDNA probes derived from T38H mRNA and to select non-hybridizing plaques. According to the manufacturer (Stratagene) instructions, 6,000 pfu from a leptotene/zygotene spermatocyte cDNA library and 3,000 pfu from a pachytene spermatocyte cDNA library were plated. Plaque lifts were transferred onto nitrocellulose filters (Schleicher and Schuell) and denatured for 2 min with 1.5 M NaCl, 0.5 M NaOH, neutralized for 5 min with 1.5 M NaCl, 0.5 M Tris-HCl (pH 8.0), and crosslinked with 120,000 mJ of UV energy in a UV Stratalinker (Stratagene). Nitrocellulose filters were hybridized with 2-5 x 10^7 cpm radiolabeled cDNA/128-mm filter in 1% BSA, 1 mM EDTA, 0.5 M $NaHPO_4$ (pH7.2), 7% SDS (Ausubel, *et al.*, 1987). Hybridizations were performed overnight at 68°C in a shaking water bath. The

low stringency washes were with 0.5% BSA, 1 mM EDTA, 40 mM $NaHPO_4$ (pH 7.2), 5% SDS, and were performed at room temperature. The high stringency washing solution consisted of 1 mM EDTA, 40 mM $NaHPO_4$ (pH 7.2), 1% SDS; this wash was in a series of 5-7 short washes and a 20 minute final wash, all at 68°C. The filters were placed in cassettes with intensifying screens and X-ray film (Sterling); exposure was for 48 hours at -80°C. Plaques identified on the basis of no hybridization with the T38H cDNA probe were isolated and resuspended in SM buffer (100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-Cl, pH 7.5, 0.01% gelatin). The plaques were rescreened two more times utilizing this process and ultimately plaques which remained negative following three screens with mixed T38H cDNA probes were isolated and resuspended in 1 ml SM buffer.

Southern blot preparation

cDNA library clones that did not hybridize with T38H mixed cDNA probes after 3 rounds of screening were selected for insert isolation by PCR amplification. Individual plaques were isolated and resuspended in 1 ml of SM buffer. Typically, 2 µl of the diluted phage suspension was lysed by incubation at 70°C for 5 minutes and then placed on ice. This was used as substrate for a PCR reaction with T3 and T7 primers (100 ng/µl) complementary to sequences flanking the inserts in the vector. Additional constituents of the PCR reactions were 1X Taq DNA polymerase buffer (Promega), 1.5 mM MgCl2, 2.5 units Taq DNA polymerase (Promega), and 0.3 mM dNTPs. Amplification by a Perkin-Elmer/Cetus DNA thermal cycler used the following profile: one cycle of 94°C for 5 min, 51°C for 3 min, 30 cycles of 72°C for 3 min, 94°C for 1 min 20 sec, 51°C for 2 min, followed by one cycle of 72°C for 7 min. One-fifth of the PCR reaction volume was used for size analysis in agarose gels with a 1 kb standard marker DNA (GIBCO-BRL). Migration lengths were recorded for both the marker and unknowns. After the loading

dye had run two-thirds of the gel distance, gels were photographed and insert sizes were established by relating to the marker migration standard curve. DNA was transferred to nylon membrane (Hybond, Amersham) using a Pharmacia (Piscataway, New Jersey) vacuum blotter. The DNA samples were crosslinked to the nylon membrane by 120,000 μjoules of UV energy in a UV Stratalinker (Stratagene).

Southern hybridization

Southern blots of clones, which were isolated from the cDNA libraries based on negative hybridization with T38H mixed cDNA probes were rescreened with another T38H mixed cDNA probe. Blots were prehybridized with: 6X SSC (1X SSC = 150 mM NaCl, 15 mM NaOAc, pH 7.4), 0.5X Denhardt's solution (50X = 1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA), 0.5% SDS, and 100 μg/ml denatured fish sperm DNA, for 1 hour ar 68°C. Radiolabeled probe was added, at 2-5 x 10^7 cpm/blot for the reverse transcription ^{32}P-labeled cDNA probes generated from total RNA, or at a concentration of 1-2 ng/ml for cDNA probes generated using the random primer labeling process for cDNA inserts. 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. A series of two successive higher stringency washes were performed with 0.1X SSC, 0.5% SDS; the first was at 37°C for 45 minutes, and the second was at 68°C for 45 minutes. A final rinse in 0.1X SSC at room temperature was used to remove excess SDS. Blots were placed on X-ray film (XR-100, Sterling X-ray film) for 24 to 96 hours at -80°C with intensifying screens. The blots were stripped by incubation at 45°C for 30 minutes in 0.4 N NaOH and followed by incubation in 0.1 X SSC, 0.1% SDS, 0.2 M Tris, pH 7.5 at 45°C for 30 minutes. Following removal of probes, blots were rehybridized with mixed

cDNA probes from leptotene/zygotene spermatocyte RNA, pachytene spermatocyte RNA, and T16H testicular RNA following the procedures for hybridization described previously.

Probe preparation

The mouse modifier one cDNA probe (Caldwell and Handel, unpublished; Singh *et al.*, 1991) and the topoisomerase II cDNA probe (Adachi *et al.*, 1992) were radiolabeled with deoxycytidine 5'-[α-^{32}P]triphosphate (dCTP[^{32}P]) (New England Nuclear) by the random primer labeling process (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.

End labeling procedure

The end labeling procedure and hybridization conditions were performed according to Ausubel *et al.* (1987). The oligonucleotide probe representing the zinc finger motif was prepared utilizing the degenerate sequence reported by Hoovers *et al.*, 1992 and was generously supplied by Mark Shannon at ORNL. Approximately 12 pmoles of oligomer was labeled using 20 units of protein kinase K (Promega), 1 X protein kinase K buffer (Promega) and 100 μCi γ^{32}P-ATP (specific activity 4,500 Ci/mmole, ICN) for 1 hour at 37°C and heat inactivated for 10 minutes Following ethanol precipitation of the ^{32}P-labeled oligomer, the entire probe was added to the hybridization mixture. Southern blots were prehybridized using 0.1% SDS, 5 X SSC, 5 X Denhardts, 10% deionized formamide, 100 μg/ml fish sperm DNA, and 50 mM phosphate buffer, pH 7.5 at 37°C. The hybridization solution was the same as the prehybridization solution; hybridization was conducted overnight at 37°C. Blots were washed three times in 6 X SSC, 0.1 % SDS and

exposed to X-ray film (XR-100, Sterling X-ray film) for 24 hours at -80°C with intensifying screens.

CHAPTER III

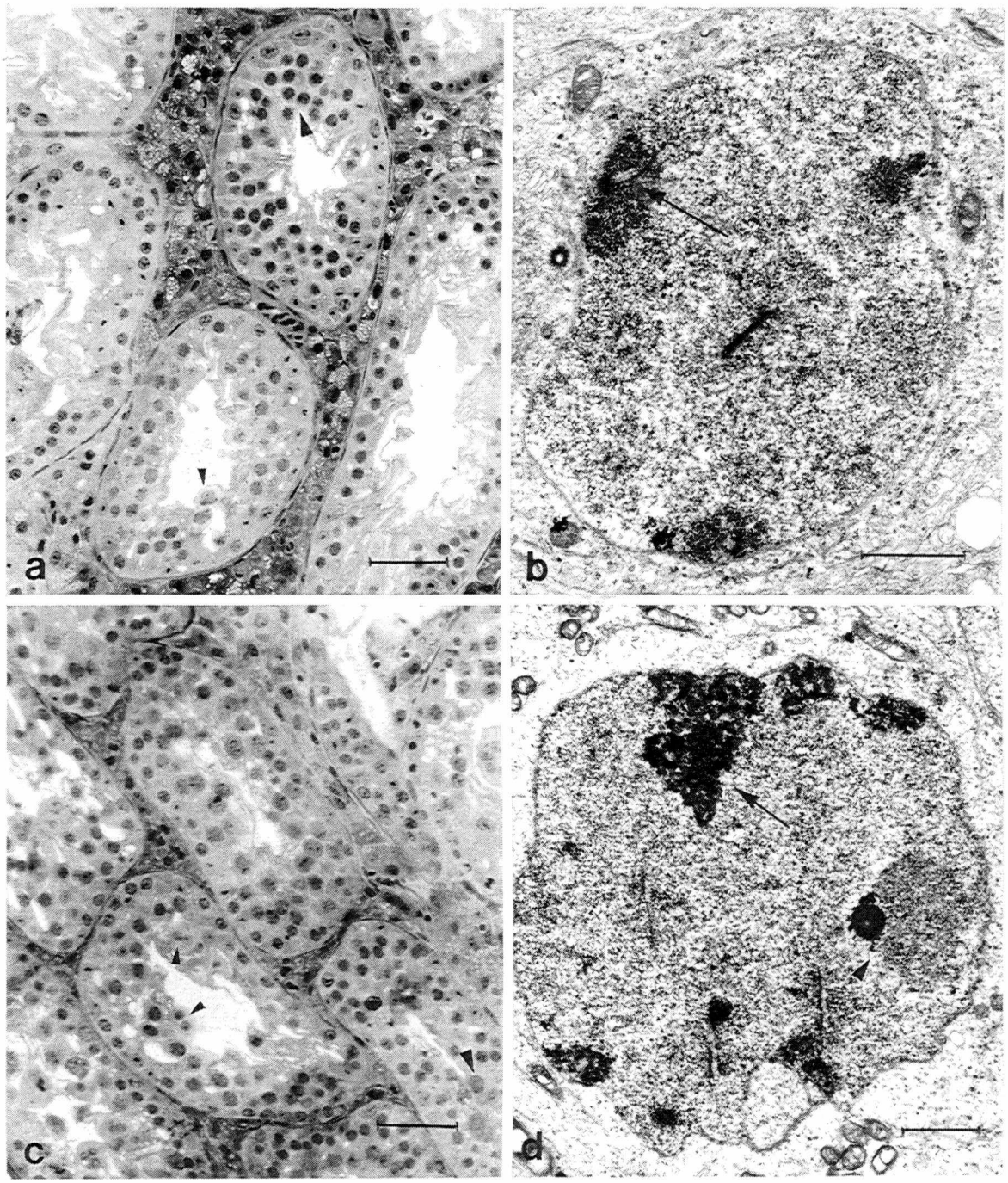
RESULTS

Spermatogenesis in translocation mutant mice is halted during meiosis

X-autosome translocations always cause sterility in male mice, with meiotic arrest typically occurring during meiotic prophase (reviewed by Handel, 1987). Two specific X-autosome translocations causing this form of meiotic arrest are the T38H and T16H translocations. Spermatogenesis is halted during the leptotene/zygotene spermatocyte stage in translocations such as T38H, where the X chromosome breakpoint is in the proximal region, and during the pachytene spermatocyte stage of meiotic prophase I when the X chromosome breakpoint is more medial, as in T16H (Handel *et al.*, 1994; Searle *et al.*, 1983). Figure 1a demonstrates seminiferous tubule cross sections from the testis of a T38H mouse. In the vast majority of tubules, spermatogenic progression was halted at leptotene/zygotene. The spermatocytes, stained with toluidine blue, appeared small and condensed. This photomicrograph was deliberately chosen to demonstrate that early pachytene spermatocytes were also present in a small percentage of the tubules. These pachytene spermatocytes were typically in the early pachytene stage thus not exhibiting an apparent sex body. Other cell types such as spermatogonia and Sertoli cells were also present in the tubules (Fig. 1a). As shown in Fig. 1b, early pachytene spermatocytes in these testes contained normal ultrastructural features. This electron micrograph depicts the nucleus of a leptotene/zygotene spermatocyte with characteristic condensed, granular chromatin and short synaptonemal complex elements visible within the nucleus.

Spermatogenic cells from T16H mice progress further through meiosis than do T38H spermatogenic cells. This is evident from cross-sections of seminiferous tubules from the testis of a T16H mouse (Fig. 1c). As demonstrated by these seminiferous tubule

Figure 1. Micrographs showing meiotic progression of spermatogenic cells in the testes of T38H and T16H mice. a: Light micrograph of seminiferous tubule cross sections from the testis of a T38H mouse showing leptotene/zygotene spermatocytes (large arrowhead) and an early pachytene spermatocyte (small arrowhead). Bar = 50 μM. b: Electron micrograph of the nucleus of a T38H early pachytene spermatocyte with a portion of synaptonemal complex (arrow). Bar = 1 μM. c: Light micrograph of seminiferous tubule cross sections from the testis of a T16H mouse showing mid-late pachytenes (large arrowhead) and postmeiotic round spermatid symplasts (small arrowheads). Bar = 50 μM. d: Electron micrograph of a pachytene spermatocyte from the testis of a T16H mouse showing the nucleus with a sex body and dense body (arrowhead) and a nucleolus (arrow). Bar = 1 μM.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cross-sections, meiosis of spermatocytes was generally found to proceed to mid- or late-pachytene, with occasional metaphase and postmeiotic cells visible. When postmeiotic cells were observed, they were usually in a syncytium, most likely resulting from incomplete cytoplasmic divisions. Other cell types of the testis were also present in the T16H tubules, such as leptotene/zygotene spermatocytes, spermatogonia, and Sertoli cells. The pachytene spermatocytes present contained sex bodies associated with a nucleolus (Fig.1d). Additionally, lateral elements of the synaptonemal complex were also present in the nuclei of these cells which is characteristic of pachytene cellular morphology.

Isolation of cDNA clones not hybridizing to T38H testis cDNA

A mixed cDNA probe from T38H testicular RNA was hybridized to 6,000 pfu of the leptotene/zygotene spermatocyte cDNA library and to 3,000 pfu of the pachytene spermatocyte cDNA library. The cDNA plaques that did not hybridize with the mutant T38H cDNA probe were isolated. Approximately 20% of the plaques from both libraries did not hybridize. However, as this still amounted to a large number of plaques, plaques which exhibited no hybridization were pooled into 12 groups containing roughly 50 plaques each. Clones from these pools were rescreened with another mixed cDNA probe from T38H testicular RNA and once again negative clones were isolated. Most of the clones that were rescreened with the second T38H mixed cDNA probe remained negative for hybridization, however, roughly 20% were positive. Negative clones isolated from this round of screening were individually isolated (not pooled). Ninety-six negative clones were chosen, eight from each pool, and screened one more time with the T38H mixed cDNA probe. None of the 96 clones hybridized with the T38H probe in this third round of screening. Since each pool contributed 8 clones, there was a chance that some of the clones within a pool were duplicates, and this was tested by examining the insert size of

each clone and comparing sizes within each pool. If two or more clones contained the same size insert, from within a pool, it was assumed that they originated from the same cDNA and only one copy of the clone was pursued in future studies. The insert sizes of the cDNAs were identified by amplifying inserts directly from phage utilizing PCR amplification with primers flanking the cloning site. The PCR amplified DNA was electrophoresed on agarose gels and visualized for identification of insert sizes. Sixty-eight independent clones that did not hybridize to the T38H mixed cDNA probe were identified in this manner. Of these clones, 22 were isolated from the pachytene spermatocyte cDNA library and 46 were isolated from the leptotene/zygotene spermatocyte cDNA library. These cDNA clones were then Southern blotted in duplicate onto nylon membrane for further analysis. A final round of screening was performed on one set of blots in which another T38H mixed cDNA probe was hybridized to these immobilized inserts. No clones were identified by positive hybridization.

Sequences homologous to the cDNA clones expressed in meiotic cells from normal mice

In order to establish that the clones represent sequences expressed in germ cells of normal mice the following experiments were performed. cDNA probes were synthesized from normal pachytene and leptotene/zygotene spermatocyte total RNA using an oligo d(T) primer in reverse transcription reactions with ^{32}P dATP. One set of Southern blots containing inserts from the 68 clones was hybridized with a mixed cDNA probe from pachytene spermatocyte RNA and the other set of Southern blots was hybridized with a mixed cDNA probe from leptotene/zygotene spermatocyte RNA. Of the 68 clones isolated, 46 hybridized with the positive control mixed probes, thereby establishing that these 46 clones represented sequences expressed in meiotic germ cells. Of these 46 clones, 19 were from the pachytene cDNA library and 27 were from the leptotene/zygotene cDNA library. The signal intensities from the hybridizations varied as some of the clones

displayed intense hybridization with the mixed cDNA probes and others did not. However, any clone exhibiting hybridization with the control probes, regardless of the level of intensity, was considered positive.

Additional indirect information regarding expression patterns of the clones was obtained by comparing hybridization patterns of these Southern blots. For example, some clones originally isolated from the pachytene library hybridized to both pachytene and leptotene/zygotene cDNA probes, while other clones hybridized only with the leptotene/zygotene cDNA probe, however, the majority of clones isolated from the pachytene library hybridized with only the pachytene mixed cDNA probe. By this analysis the 46 clones were categorized with respect to their inferred expression pattern in pachytene spermatocytes, leptotene/zygotene spermatocytes, or both.

Table 1 provides data on clones isolated from the pachytene spermatocyte cDNA library. The first category in this table represents 53% of the clones originally isolated from the pachytene spermatocyte cDNA library; ten clones hybridized only with the mixed pachytene cDNA probe. Among the remaining isolates, six clones (32%) hybridized to both pachytene and leptotene/zygotene spermatocyte cDNA probes and three clones (16%) did not hybridize with the pachytene cDNA probe but did hybridize with the leptotene/zygotene mixed cDNA positive control probe. The remaining three clones identified from the pachytene spermatocyte cDNA library did not hybridize with either the pachytene or the leptotene/zygotene cDNA probes and were not analyzed further. Table 2 describes clones isolated from the leptotene/zygotene spermatocyte cDNA library. One clone (4%) hybridized only with the pachytene spermatocyte mixed probe and not with the leptotene/zygotene mixed probe. In contrast, three clones (11%) hybridized to both the leptotene/zygotene and the pachytene cDNA probes. The vast majority of the clones identified from the leptotene/zygotene library, however 85% hybridized only with the leptotene/zygotene mixed cDNA probe. The remaining 17 clones did not hybridize with

Table 1. Pachytene spermatocyte cDNA clones that do not hybridize to T38H testis cDNA

Clones hybridizing to	No. clones	% clones isolated	Clones
P probe only	10	53	3, 6, 7, 9, 11, 14, 26, 28, 30, 31
P & LZ probes	6	32	1, 2, 4, 13, 25, 29
L/Z probe only	3	16	5, 17, 20

Table 2. Leptotene/zygotene spermatocyte cDNA clones that do not hybridize to T38H testis cDNA

Clones hybridizing to	No. clones	% clones isolated	Clones
P probe only	1	4	86
P & LZ probes	3	11	56, 88, 91
L/Z probe only	23	85	42, 43, 45, 50, 53, 54, 55, 60, 61, 62, 63, 66, 67, 69, 70, 73, 74, 76, 77, 81, 82, 84, 93

either positive control probes and were no longer examined in this study. It is possible that some of the clones not detected by this positive screening procedure are expressed at low levels and eluded detection by this screen. Mixed cDNA probes reflect the constitution of various mRNAs within a given sample and as such, mRNAs represented at low levels would be labeled at low levels in mixed probes as well. There has been some qualitative assessment of this phenomenon, as studies have found that cDNA probes used in this type of screening procedure can not typically detect sequences that are represented at a level less than 0.05% of the total mRNA (Dworkin and Dawid, 1980; Sambrook *et al.*, 1989).

Analysis of cDNA clones enriched for meiotic expression

Subsequent characterization of the 46 clones established that the average insert size of clones isolated from the pachytene spermatocyte cDNA library was 1.7 kb, whereas clones isolated from the leptotene/zygotene spermatocyte cDNA library were approximately 1.4 kb. The average size of the cDNAs selected by this screening procedure is similar to the average insert size for the libraries: 1.5 kb for the pachytene library and 1.3 kb for the leptotene/zygotene library (Part II of this dissertation).

Since information regarding these cDNA clones was limited to insert size and representation in cDNA, additional hybridizations to these clones were performed with probes for sequences thought to be expressed in meiotic cells. Southern blots containing the 46 clones identified by the screening procedure were hybridized with a degenerate oligomer representing a zinc finger motif. A zinc finger is a consensus sequence found in many transcription factors. The clones were also hybridized with the cDNA for the mouse modifier one protein, which contains a chromo box motif. The chromo box is a sequence believed to have a role in heterochromatin formation (Singh *et al.*, 1991). Additionally, the clones were analyzed for homology with the cDNA for topoisomerase IIα since expression for this gene has been described during mouse meiosis (Reddy and Handel, in

preparation). The end labeled zinc finger oligonucleotide sequence did not hybridize with any of the cDNAs. This result suggested that there may not be a zinc finger-containing transcription factor among the clones identified in this enrichment procedure. Likewise, the cDNA probe for mouse modifier one and topoisomerase IIα did not hybridize with any of the clones, indicating no chromo box-containing cDNAs or putative topoisomerase IIα homology were identified among this cDNA population.

A subset of the enriched sequences are represented in the testes of T16H mice

RNA from testes of T16H mice was isolated and used as a template for the synthesis of ^{32}P-cDNA. One set of Southern blots containing the 46 enriched cDNA inserts were hybridized with this T16H mixed cDNA probe. Nineteen clones were found that hybridized to the T16H mixed cDNA probe, representing 41% of the 46 clones originally isolated as sequences not expressed in T38H testes. Southern blots containing clones that were positive from hybridization were scanned with an imaging densitometer to determine the relative optical density of the bands. This analysis provided a quantitative measure of the amount of hybridization between individual cDNA clones and the mixed cDNA probe from T16H testes since the amount of DNA loaded in the agarose gels was similar as determined by inspection of ethidium bromide stained gels. On the basis of relative optical density values, the clones were organized into classes designated as abundant, moderate, and low level representation in T16H testis cDNA. Clones exhibiting the most intense hybridization signals are listed in Table 3. The abundantly represented class included three clones that clearly contained the highest signal intensities, in the range of 5.7 - 6.7 optical density units. These three clones were originally isolated from either the leptotene/zygotene or pachytene cDNA libraries, but had hybridized to both pachytene and leptotene/zygotene mixed cDNA probes. The seven clones that were moderately

Table 3. Clones hybridizing to sequences with abundant representation in T16H testis cDNA

Clone	Source (LZ or P)	Hybridized to[a]	Size (kb)	Signal intensity
13	P	P & L/Z probes	1.4	6.7
56	LZ	P & L/Z probes	0.5	6.7
88	LZ	P & L/Z probes	1.4	5.7

[a]Refer to Tables 1 and 2

represented in the T16H testis cDNA (signal intensity of 4.2 - 5.3 OD units) are identified in Table 4. Most of these clones were originally isolated from the pachytene spermatocyte cDNA library (5 out of 7). Although the remaining two cDNAs were isolated from the leptotene/zygotene library, they also hybridized with the pachytene mixed cDNA probe (2 out of 7). Thus, all the clones in this category identify sequences represented in pachytene spermatocyte cDNA. Table 5 summarizes the data from the 9 clones that exhibited low levels of representation in T16H testis (signal intensities of 3.8 - 4.0 OD units). Interestingly, all 9 clones in this class represented sequences found only in the leptotene/zygotene spermatocyte cDNA.

The percentage of all 19 clones that were found to be positive for hybridization with the T16H cDNA probe was almost identical between the two spermatocyte cDNA libraries. Of the clones originally isolated from the pachytene spermatocyte cDNA library, 8 out of 19, or 42%, were positive with the T16H cDNA probe, while 11 out of 27, or 41% were found to be positive for the leptotene/zygotene cDNA library. The remaining 27 clones did not hybridize to the cDNA probe from the T16H testes.

In summary, 19 clones have been identified that represent gene sequences which are not represented in the testes of T38H translocation heterozygous mice but are represented in the testes of T16H mice. Since testes from T38H mice demonstrate meiotic arrest during leptotene or zygotene whereas testes from T16H mice undergo meiotic arrest during mid-late pachytene, the 19 cDNA clones identified may represent gene sequences that are expressed between these time points, approximately at the beginning of the pachytene stage.

Table 4. Clones hybridizing to sequences with moderate representation in T16H testis cDNA

Clone	Source (LZ or P)	Hybridized to[a]	Size (kb)	Signal intensity
2	P	P & L/Z probes	3.8	5.3
3	P	P probe	3.1	4.3
17	P	L/Z probe	1.7	4.3
30	P	P probe	1.8	5.3
31	P	P probe	1.4	4.9
86	LZ	L/Z probe	1.0	4.2
91	LZ	P & L/Z probes	1.7	4.5

[a]Refer to Tables 1 and 2

Table 5. Clones hybridizing to sequences with low level representation in T16H testis cDNA

Clone	Source (LZ or P)	Hybridized to[a]	Size (kb)	Signal intensity
5	P	L/Z probe	1.0	3.8
25	P	P & L/Z probes	1.3	3.9
42	LZ	L/Z probe	1.5	3.8
50	LZ	L/Z probe	1.0	4.0
53	LZ	L/Z probe	1.6	3.8
54	LZ	L/Z probe	3.6	3.8
55	LZ	L/Z probe	2.2	3.8
62	LZ	L/Z probe	0.8	3.8
69	LZ	L/Z probe	1.4	3.9

[a]Refer to Tables 1 and 2

CHAPTER IV

DISCUSSION

Spermatocytes from testes of T16H mice proceed further through meiosis than do spermatocytes of T38H mice, to approximately the mid-late pachytene stage of prophase I. Based on this information, a screen was devised that utilized the expressional differences between these mutant mice to isolate cDNA clones that correspond to genes putatively involved in meiosis. In this experimental scheme, testicular RNA from T38H mice was utilized as a mixed cDNA probe to differentially screen meiotic cDNA libraries for clones that would not hybridize. Clones displaying no hybridization with the T38H mixed probe were isolated and were considered to represent gene sequences expressed after the point of T38H spermatocyte arrest. Thus the 46 clones isolated by this procedure reflect genes which are expressed and potentially involved in meiotic events occurring late in the zygotene stage of prophase I. These 46 enriched cDNA clones were then hybridized with a mixed cDNA probe from testicular RNA of T16H mice in order to identify clones that might represent gene sequences that are expressed in T16H spermatocytes. From this screening procedure 19 out of the 46 clones were identified that hybridized to a T16H testicular cDNA probe but not with a T38H testicular cDNA probe. Since T16H spermatocytes proceed farther through meiosis than do T38H spermatocytes, the 19 clones which exhibited negative hybridization with the T38H testicular probe and positive hybridization with the T16H testicular probe would presumably be expressed during a specific time frame in meiosis. In this particular screening strategy, the meiotic window represented includes the cellular events occurring between the arrest of T38H and T16H spermatocytes. Inferring from cytological analysis of the mutant testes from these animals,

the 19 clones identified probably represent sequences expressed between leptotene/zygotene and mid- to late- pachytene of meiotic prophase I.

Cytological analysis of spermatogenic progress in these male sterile mouse mutants has allowed us to confirm previous descriptions of some of the cellular events that are occurring during the window when the 19 meiotically-enriched clones that we have isolated are expressed. As visualized in cross-sections of T38H testes, the cells with the greatest progression have characteristics of leptotene/zygotene spermatocytes. Cells were compact and nuclei stained darkly in a manner similar to that seen in leptotene/zygotene spermatocytes of normal mice. Using electron microscopy, small sections of synaptonemal complex lateral elements were seen, yet sex bodies were not apparent in the majority of the nuclei. These data suggested that the majority of the cells in T38H testes had not progressed past the zygotene stage of meiotic prophase I. As expected, cytological analysis of T16H testes demonstrated that cells from these mice have typically progressed to mid and late pachytene stages of prophase I. This was evident from examination of cross-sections of testes from T16H mice in which many pachytene spermatocytes with discernible sex bodies were observed. Electron microscopy confirmed the presence of many nuclei with sex bodies and associated nucleoli in these spermatocytes as well. Additionally, these cells contain well formed axes of synaptonemal complex in their nuclei, which is often observed in mid-late pachytene of prophase I. Cytological evidence of meiotic failure in these mutants, in conjunction with the cDNA expression patterns of the 19 clones identified in this study, indicates that the clones are expressed between zygotene and mid-late pachytene of meiotic prophase I. Events that may be giving rise to the distinguishing cytological characteristics observed at this stage include an increase in nuclear size as spermatocytes progress through meiosis, which may in part be due to an accumulation of transcripts used postmeiotically, pairing of synaptonemal complex elements to form the mature, tripartite synaptonemal complex, formation of the sex body

and nucleolus association with it. In addition to these differences between the leptotene/zygotene and pachytene spermatocytes, recombination is thought to be well underway during the pachytene stage, the sex chromosomes are inactivated, and the stage must be set for events that occur late in meiotic prophase such as the resolution of crossovers as chiasmata. Therefore, many differences exist between T38H and T16H spermatocytes with respect to progression of meiotic events.

We hypothesize that these meiotic events are accomplished with concurrent changes in gene expression. The use of meiotically arrested testicular RNA in this analysis has proved to be a useful method for the identification of cDNA clones representing genes that may be expressed at a specific stage during meiosis. However, the underlying assumption in these studies is that the cDNA clones identified from the hybridization procedures represent gene sequences that are expressed during specific stages of meiotic prophase. It should be noted that experiments performed in this study have been indirect in providing evidence for gene expression. The preparation of mixed cDNA probes and extraction of the RNA and the construction of cDNA, are inherently imperfect techniques. A more direct approach that can now be pursued is Northern analysis to determine representation of these sequences in RNA.

This screening procedure was performed on clones from leptotene/zygotene and pachytene spermatocyte cDNA libraries. If this screen were to be undertaken again (with another 6,000 plaques from each library), the number of clones generated should be similar since the screening procedure is random. However, false positives and negatives should be considered in any explanation of the outcome of these experiments. It should be noted that the term "false positive" is applied to the negatively-hybridizing clones from the T38H screen or positively-hybridizing from the T16H screen, whereas a "false negative" applies to positively-hybridizing clones from the T38H screen or negatively-hybridizing clones

from the T16H screen. It is difficult to assess which clones identified are either false positives or false negatives in the absence of additional hybridization data. There is reasonable confidence that the isolated clones did not hybridize to mixed cDNA probes from the T38H testicular RNA since this screen was performed a total of 4 times. The first 3 screens involved cDNAs in phage particles, and in the last screen the cDNA inserts were amplified out of the phage and screened on Southern blots. After the initial isolation of the T38H negative clones, all other hybridizations were performed only once. These hybridizations include the screens with leptotene/zygotene spermatocyte, pachytene spermatocyte, and T16H mixed cDNA probes. These screens, which were intended to identify positive clones, may still produce artifacts.

False negatives could also result from this screening methodology and identify clones which hybridize with the mixed cDNA probe from T38H testicular RNA or do not hybridize with the probe from T16H testicular RNA. False negatives from the T38H screen are unlikely since any clone that did hybridize with the mixed T38H probe was excluded from future studies. The second situation, cDNAs that did not hybridize with the mixed T16H probe, is a little easier to assess. It has been shown that typical mixed cDNA probes cannot detect sequences that are represented at a very low levels in the mRNA population (Dworkin and Dawid, 1980; Sambrook *et al.*, 1989). However, this screen was not designed to definitively assess gene expression during meiotic arrest in a quantitative manner but was performed as an initial assessment of the feasibility of such a screening procedure. Ultimately, 19 clones were isolated with meiotic expression patterns that represent sequences with abundant expression in meiotic cells. In the future these clones may be analyzed further with testicular RNA from other meiotically-impaired mice in a similar manner in order to learn more of the temporal pattern of gene expression of the genes represented by the cDNA clones isolated. There are two other male sterile mouse models in which meiosis proceeds to the end of prophase I. These include the murine

mutation, *sks*, skeletal fusions with sterility (Handel et al., 1988; Handel, 1990) and sterile interspecific hybrids between *Mus domesticus* and *Mus spretus* (Matsuda *et al.*, 1992).

Twenty-seven clones failed to hybridize to either T38H or T16H testicular cDNA probes in our screen. These clones could represent sequences that are expressed in low levels in the mutant testes and were therefore not identified by the screening procedure or they could represent sequences not expressed in the testes of these meiotically arrested mice. Alternatively, the possibility exists that translation of the sequences represented by cDNAs had already occurred in the T16H testis, and subsequent message degradation would mean that the messages would no longer be detected by our screening procedure. An approach by which one of these putative scenarios could be delineated would entail hybridization with specific T16H negative clones to a developmental profile blot of T16H testicular RNA. Representation of specific clones in T16H developmentally staged testis RNA would indicate that transcription of the sequence had occurred. Thus, a temporal pattern of expression for a given gene sequence could be established. Likewise, it is also possible that a given gene may not be represented in the T16H testis. In either case, interesting information could be obtained regarding the expression of gene sequences in the meiotic mutants as well as in normal spermatocyte from further analysis of this type.

Alternatively, cDNA clones identified as not hybridizing with mixed cDNA probes from either T38H or T16H testis RNA may be needed for meiotic progression. If these transcripts are not produced, spermatocytes might not be able to progress beyond early meiotic stages. For instance, specific transcripts which encode proteins that play an important role in cell cycle progression, such as cyclins, may not be produced. It was for this reason that an initial assessment of T38H negative clones was performed using sequences for 3 different cDNAs or motifs as probes for hybridization to the clones.

Assessment for the presence of zinc fingers, chromo boxes or for sequences homologous to topoisomerase IIα was performed. None of the cDNAs analyzed had sequences detected by these motifs. These results do not preclude the likelihood that some of the cDNAs identified may actually contain sequences needed for meiotic progression. In this study, we did not perform an exhaustive search for cDNAs representing genes with functions presumed to have a meiotic role. The sequences assessed in this screening procedure were chosen because they were readily available to our laboratory. Although these screenings did not elucidate the function of any of the cDNAs, it did allow for the elimination of possible functions for some of the cDNAs. In the future, it might be worthwhile to obtain probes for consensus regions of kinases and cyclins, as they are both commonly utilized in cell cycle progression.

We know from control hybridizations with mixed cDNA probes from leptotene/zygotene and pachytene spermatocytes that these 27 cDNA clones not hybridizing to T38H or T16H mixed probes must represent genes that are normally transcribed in meiotic cells, yet appear not to be expressed in the testes of these mutant mice. Since these sequences appeared unnecessary for the degree of progress from zygotene to mid-late pachytene represented by T16H spermatocytes, we can hypothesize that these sequences are need for cellular events later than the point of disruption. Alternatively, they may be required during early meiotic events and a lack of expression causes meiotic arrest in these mutants. In order to distinguish between these two possibilities, several experiments could be performed. One experiment which might indicate if some of these sequences are utilized past the point of meiotic disruption would be to hybridize a mixed cDNA probe from round spermatid RNA to Southern blots containing cDNA from a panel of the 27 negative clones. Alternatively, individual clones could be tested for their ability to hybridize to round spermatid RNA by Northern analysis. Whereas this latter method may yield more

definitive results, it is far more labor intensive than use of a mixed probe. If some of the clones displayed hybridization with the round spermatid cDNA probe it would indicate that transcripts represented by these clones are present in round spermatids, thus suggesting that some of these cDNA clones represent genes that might be transcribed in normal spermatocytes for postmeiotic utilization. In this case, we could speculate that genes represented by these sequences undergo transcription well before the RNAs are needed, and are long-lived transcripts not produced in large quantities in the spermatocytes of T38H and T16H mutant mice. If the transcripts are not produced in the spermatocytes of meiotically arrested mice, it might suggest that a control mechanism exists which allows for a spermatocyte to know *a priori* how far it can proceed through the meiotic process. This could be accomplished through a meiotic checkpoint. In this case, normal spermatogenic cells would pass the checkpoint and proceed with transcription of products utilized postmeiotically, whereas with the T38H or T16H spermatocytes, which could not pass this presumed checkpoint, would not be provided with signals for RNA synthesis needed postmeiotically. Alternatively, the clones might represent sequences expressed in normal round spermatids since approximately 10% of the cells which contributed to the pachytene spermatocyte cDNA library were round spermatid contaminants.

Experiments designed to elucidate whether certain genes are required for meiosis may be difficult to perform. However, some of these genes might actually be expressed premeiotically for utilization in meiosis. If this was true, hybridization of the clones with a mixed cDNA probe from spermatogonia might determine if some of the clones are expressed during spermatogonia. Clones with spermatogonial representation would indicate that some gene sequences utilized in meiosis are produced premeiotically and that the meiotically arrested mutants do not express these gene sequences. This would, once again, indicate that there are mechanisms that control gene expression by determination of the potential for cell development.

Finally, although this study demonstrates the utility of male-sterile translocations in screening procedures, it is not clear why X-autosome translocations cause meiotic arrest. Many hypotheses have been tested and eliminated. For example, it was postulated that meiotic arrest was caused by the inability of X and Y chromosomes to pair. However, quantitative analysis of microspread synaptonemal complexes revealed no failure of X and Y chromosome pairing in several X-autosome translocations (Ashley, 1983; Ashley et al., 1982; 1983). It was not the purpose of this study to elucidate the underlying mechanism for meiotic arrest. The meiotic mutants merely served as reagents in this screening strategy for the identification of meiotically expressed sequences. The clones identified simply represent sequences which are not transcribed in the mutant testes and are not necessarily associated with the mutant phenotype of meiotic arrest. However, some of the clones identified in this study may, upon further characterization, provide additional information regarding gene expression in the meiotic cells of the mutants.

The cDNAs identified in our screen represent a unique pool of sequence information which requires further characterization. Individual cDNAs could be analyzed with respect to their differential representation in spermatogenic cells by Northern blot analysis. The elucidation of specific expression patterns will enable subsequent categorization of the individual cDNAs into putative classes. For instance, potential categories may reflect cell-type specific expression. The distribution of clones into various representative classes might provide interesting information regarding meiotic arrest in these cells, or allow for a molecular description regarding the absence of specific gene expression in arrested cells. The cDNAs identified in this study were selected because they were not represented in the testes of meiotically arrested mutants. In order to learn more about gene expression in meiotically arrested cells, we might ask, for example, are many of these clones primarily represented in spermatogonia? If so, what would this indicate about

meiotic arrest? The cells might be arrested because spermatogonial transcripts were not produced. In this regard, these transcripts might not have been produced because the cell was not able to get past a putative cell cycle check point which would allow for continued gene expression. Likewise, are many of these cDNAs representative of gene expression in postmeiotic cells? This might indicate that some transcripts, which are usually produced and stored meiotically, are not being transcribed, suggesting that either meiotically arrested cells do not undergo transcription of sequences required after arrest, or that the lack of specific transcripts caused arrest. A skewing of several clones representative of either category (spermatogonia or round spermatids) might be an interesting observation that could provide an initial basis for investigation of meiotic arrest in these mutants. However, even if nothing is gained through this study with regard to the mechanism of meiotic arrest, the identified clones still might yield interesting information with respect to their expression patterns. These clones, for whatever underlying reason, are not represented in spermatocytes of meiotically arrested cells. This observation, unto itself, is inherently intriguing. Upon delineating the spermatogenic and somatic expression patterns for some of these clones, those exhibiting interesting expression patterns (i.e. primarily meiotic) should probably be sequenced. In addition to expression analysis, a secondary assessment designed to determine which clones should be fully sequenced would potentially involve sequencing of the 5' ends of several clones. These small sequences could be subsequently analyzed by database comparison for the identification of novel genes, which could then be sequenced in their entirety.

In summary, 19 cDNA clones have been identified in this study which represent sequences that are transcribed during a time frame when many meiotic events are occurring. These events include completion of synaptonemal complex lateral elements, formation of the sex body, nucleolus association with the sex body, recombination, and preparation for desynapsis and metaphase I. These clones may all be important for elucidating

mechanisms involved in meiosis and represent an excellent starting point for further dissection of meiotic mechanisms.

CHAPTER V

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PART IV

THE ISOLATION AND CHARACTERIZATION OF A NOVEL cDNA THAT IS EXPRESSED MEIOTICALLY DURING MOUSE SPERMATOGENESIS

CHAPTER I

INTRODUCTION

The process of meiosis is genetically essential for the formation of a normal gamete; however few details are available concerning the molecular aspects of gene expression during this phase of mammalian gametogenesis. The identification of genes expressed during meiosis provides an initial approach to the ultimate goal of understanding molecular mechanisms governing meiosis.

Until recently, most information regarding meiotic function in mouse spermatocytes has come from either cytological observations of meiotic chromosome behavior or from inference via molecular characterization of meiosis in lower eukaryotes. In the past few years, however, some meiotically expressed genes have been isolated from the mouse which show homology to genes identified by genetic studies of lower eukaryotes. An example of this is demonstrated by proteins which contain a conserved structural motif associated with heterochromatin formation termed the chromo box domain. This motif is a region of homology present in *Drosophila* genes which encode Heterochromatin protein 1 and *Polycomb*. Likewise, three genes have been identified from mouse which also contain a region of homology similar to the chromo box domain. These genes have been designated mouse modifiers 1, 2, and 3, respectively (Singh *et al.*, 1991) and their precise functions remain unknown. Interestingly, mouse modifier one contains a testis-specific transcript that is expressed exclusively in pachytene spermatocytes in the mouse testis (Caldwell and Handel, unpublished observation). Furthermore, a mouse homolog for yeast *RAD51* and *E.coli Rec A* has been cloned (Morita *et al.*, 1993). This gene, referred to as mouse *rad51*, has been shown to be involved in mitotic and meiotic recombination, in addition to DNA repair, by complementation analysis of the yeast *rad51* mutant. The

spermatogenic cells that demonstrate mouse *rad51* expression are spermatogonia and pachytene spermatocytes (Morita *et al.*, 1993).

Specific meiotic cell functions, such as recombination, DNA repair, and synaptonemal complex (SC) formation, require specific and specialized protein products. Thus, genes for recombination enzymes, DNA repair enzymes, and SC proteins will most likely have transcripts which are expressed in mouse spermatocytes. In some cases, genes encoding these functions have been identified, particularly when the gene product is also involved in mitotic cell cycle events, such as the topoisomerases. This class of enzymes is most likely involved in DNA repair and transcription since they affect supercoiling of DNA. Antibodies against topoisomerase II recognize meiotic chromosome cores and centromeric regions of paired chromosomes (Moens and Earnshaw, 1989). Additionally, the mouse genes for both topoisomerase I and IIα have been shown to be expressed in spermatocytes (Reddy and Handel, in preparation). It has been suggested that since topoisomerase IIα appears to have a role in transient double stranded breaks, it may possibly be involved in the generation of breaks necessary for successful meiotic recombination (Szostak *et al.*, 1983). Additionally, two major components of the mammalian SC have been identified that are found only in meiotic prophase nuclei (Heyting *et al.*, 1988). After biochemical purification of these SC proteins, genes corresponding to two SC proteins have been cloned by cDNA library screening with antibodies for the SC proteins, these clones are referred to as SCP1(Meuwissen *et al.*, 1992) and SCP3 (Lammers *et al.*, 1994).

As novel genes which contribute to the meiotic process continue to be identified, many will probably be characterized as members of gene families which share similar structural sequence motifs. Some gene families, such as the cyclins, kinases, and kinesins, are obvious choices for identifying meiotically expressed gene members since many

proteins of these types have been implicated in both mitotic and meiotic function in a variety of species. Other classes of genes, such as transcription factors, contain consensus structural motifs which have been shown to be important for directing cell-specific gene expression.

Both cyclins and kinases have been shown to be critical in cell cycle progression. For example, cyclin B synthesis has been determined to drive meiotic cells into M phase (Westendorf *et al.*, 1989). A mouse member of the cyclin B family, the *cycB2* gene, has also been identified which exhibits a meiotic expression pattern (Chapman and Wolgemuth, 1993). High levels of *cycB2* have been detected in pachytene spermatocytes, thus this transcript may represent a protein involved in meiotic cell division. Likewise, the posttranslational regulatory function of protein kinases in complex molecular processes has been well documented. In this regard, the tyrosine kinase-encoding gene *ferT* (Fischman *et al.*, 1990; Keshet *et al.*, 1990) was identified as a pachytene-specific transcript, thereby suggestive of a role for this kinase during the prophase stage of meiosis. Two other novel protein kinase genes, *mak* (Matsushime *et al.*, 1990) and *Nek1* (Letwin *et al.*, 1992) are expressed in mouse meiotic cells as well. The *Nek1* gene has also been shown to be expressed in early round spermatids and may serve in both a meiotic and non-meiotic capacity.

In addition to regulatory proteins that may influence meiotic progression, the existence of structural proteins affecting this process is likely to be revealed. Kinesins, which are microtubule motors, have been shown to be involved in the segregation of chromosomes during meiosis in *Drosophila*. Two examples of meiotically expressed kinesins in *Drosophila* are *ncd* (Endow *et al.*, 1990; McDonald and Goldstein, 1990) and *nod* (Zhang *et al.*, 1990). At present no meiotically expressed mouse kinesins have been reported, however conservation of function suggests this will be forthcoming.

In eukaryotes, transcription factors represent a group of functionally similar proteins directly implicated in mediating cell-specific gene expression (reviewed by Latchman, 1991). Thus, the identification of meiotically expressed gene sequences which possess characteristics of transcription factors will most likely prove important for determining gene regulation during meiosis. Transcription factors possess several distinct structural elements which are characteristic of members of various motif classes. There are three main known structural motifs which a transcription factor may possess: the helix-turn-helix motif (common to homeobox proteins), the zinc finger motif, and the leucine zipper. One zinc finger-containing gene, *Zfp-35,* has been identified in spermatocytes and is expressed during meiosis (Cunliffe *et al.*, 1990). However, to date, there have not been any other putative transcription factors identified from spermatocytes which are primarily expressed during meiosis, although it is likely they will be identified.

In addition to genes which can be easily categorized with respect to function, either from sequence analysis or functional studies, the presence of meiotically expressed genes in mouse which do not fit established parameters is possible. In fact, the *Meg1* gene has been shown to be specifically expressed in spermatocytes, with low levels detected in leptotenes and increasing levels detected as cells progress through zygotene and pachytene (Don and Wolgemuth, 1992; Don *et al.*, 1994). Nevertheless, a putative function for this gene has not been reported, as it does not appear to conform to any known protein families.

In order to identify a putative meiotically expressed gene, this study reports the use of information regarding gene family structure to identify a novel meiotically expressed gene. Surprisingly, a strategy which employed homology screening by PCR with degenerate primers eventually led to the cloning of a nonhomologous cDNA corresponding to a gene expressed during pachytene of meiosis in mouse spermatogenesis. The clone has been designated *Mes*, as it represents a meiotically expressed sequence.

CHAPTER II

MATERIALS AND METHODS

Animals

Adult, 17 day old, and 8 day old BALB/c mice were purchased from Harlan-Sprague-Dawley (Indianapolis, Indiana). XX*Sxr* males were bred in our mouse facility by matings of XY*Sxr* males to XX or XO females. Typically, XY*Sxr* males were hemizygous for either Tabby (Ta) or Greasy (Gs), which are X-linked coat markers, while females were genetically unmarked. XX*Sxr* males were identified by variegated coat pattern and by smaller-than-normal testes. The mice were provided with food and water *ad libitum* and maintained on a 14 hr light/10 hr dark schedule. Mice were killed by cervical dislocation and testes removed immediately.

Isolation of spermatogenic cells

Testes of adult, 17 day old, or 8 day old BALB/c 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 $MgSO_4 \cdot 7H_2O$, 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 (Romrell *et al.*, 1976; Bellvé *et al.*, 1977). After tubule digestion, trypsin was removed by washing cells 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). After 2.5 hours for sedimentation, gradients were collected 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.

Leptotene/zygotene spermatocytes were obtained from the testes of 17 day old mice. For the isolation of leptotene/zygotene spermatocytes the STAPUT procedure was performed two times, with 60 mice used each time; the average number of cells loaded on a gradient was 3×10^8 with 5×10^6 cells collected for the isolation of RNA. The average enrichment of leptotene/zygotene spermatocytes used in library construction and probe preparation was greater than 80%; the principle contaminants were spermatogonia and Sertoli cells. Pachytene spermatocytes and round spermatids were isolated from the testes of adult mice (>8 weeks old). One STAPUT with 11 mice was required, with approximately 9×10^8 cells loaded on to the gradient. The average enrichment of the pachytene spermatocyte fractions used was greater than 88% and 7×10^6 cells cells were recovered; the principle contaminants were Sertoli cells and symplasts composed of round spermatids. The average enrichment of the round spermatids was greater than 79% with 1.4×10^7 cells isolated; the principle contaminants were leptotene/zygotene spermatocytes and Sertoli cells.

Isolation of RNA

The somatic RNA samples utilized in the somatic RNA blot were obtained from Ambion (Austin, Texas). All other RNA samples were isolated as follows, using diethylpyrocarbonate-treated glassware and solutions by the method of Cathala *et al.* (1983). Developmentally staged testes were frozen in liquid nitrogen and pulverized using a mortar and pestle and then homogenized in lysis buffer (5 M guanidium isothiocyanate, 10 mM EDTA, and 50 mM Tris-Cl, pH 7.5) by passing the sample through a 21 gauge needle 20 times. RNA derived from cultured and STAPUT isolated cells was treated as above except that the cells were not pulverized in liquid nitrogen, but were immediately passed through a 21 gauge needle in the presence of lysis buffer. The RNA was then precipitated overnight in 7 volumes 4M lithium chloride at 4°C. Samples were spun for 90 minutes at 10,000 x g and the pellets resuspended in RNA solubilization buffer (10 mM

Tris-Cl, pH 7.5, 1 mM EDTA, 0.1% SDS) and treated with 40μg/ml proteinase K for 30 minutes at 37°C. The samples were then extracted with phenol:chloroform (1:1) and also with 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. Northern gels were performed following standard methods (Sambrook *et al.*, 1989) in order to verify RNA integrity. Briefly, 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 examined for the presence of the 28S and 18S ribosomal RNA bands.

PCR

The degenerate primers for the kinesin heavy chain region were designed according to the specifications of Stewart *et al.*, 1991. The sequence of the primers was as follows: Primer 1, GCGCGAATTC-CT(T/C/G)-(A/T)A(T/C)-CT(T/C/G)-GT(T/C/G)-GA(T/C)-CT(T/C/G)-GCN-GG, 2592-fold degenerate; Primer 2, CGTCTAGA(A/T)-(A/G)T(T/C)-NC(T/G)-(A/G)(A/T)A-NGG-(A/G)AT-(A/G)TG. RNA from pachytene spermatocytes was used in a reverse transcription reaction (Park, 1990) with the following procedure. Pachytene RNA at 2 μg was incubated with 200 units M-MuLV reverse transcriptase (Gibco, BRL), 1 X reverse transcription buffer, 0.5 μg oligo $(dT)_{15}$, 15 units RNasin (Promega), and 1 mM dNTP mix, for 1 hour at 37°C. The reaction was then heated at 75°C for 5 minutes and chilled on ice before the addition of PCR reaction components. The total volume for the PCR reaction was 100 μl, with a final concentration of primers of 15 μM for Primer 1 and 30 μM for Primer 2. Taq polymerase (Promega) was added at 2.5

units and Taq buffer to 1 X final concentration. The following temperature cycle was used, 40 cycles of 94°C for 1 min, 45°C for 2 min, and 72°C for 2 min.

Isolation and sequencing of PCR amplification product

The products from the PCR reaction were electrophoresed on a 2% agarose gel and a band of approximately 200 bp was isolated. This band was eluted from the agarose with the Mermaid kit from Bio101 (LaJolla, CA) and subcloned into pBluescript II SK+ which was digested with *Eco* RI and *Xba* I. The PCR-derived fragments had *Eco* RI and *Xba* I sites incorporated on the ends of the primers. Ligation products were transformed into DH5α cells and plated on media containing X-gal. White colonies were chosen and analyzed for the presence of inserts. Several clones with inserts were then sequenced. Sequencing reactions were performed using the Sequenase 2.0 sequencing system (USB) and reaction products run on 6% polyacrylamide gels. Sequencing data was analyzed on Genbank using a TFASTA search. A clone with 65.5% identity to *Drosophila* kinesin was identified.

Probe preparation

cDNA probes were radiolabeled with deoxycytidine 5'-[α-^{32}P]triphosphate (dCTP[^{32}P]) (New England Nuclear) by the random primer labeling process (Feinberg and Vogelstein, 1983). The unincorporated nucleotides were separated from the labeled probes by gel filtration chromatography using 0.45 μm nylon microspin columns from PGC Scientific (Gaithersberg, Maryland) using Sephadex G-50. The average specific activity for probe labeling was 1 x 10^9 cpm/μg DNA.

Screening of the cDNA libraries for initial characterization

A total of 250,000 pfu from a λgt11 pachytene spermatocyte (gift from M. Eddy and D. Joseph) and 250,000 pfu from a pachytene spermatocyte cDNA libraries (Part II) were plated at a concentration of 15,000 pfu/132 mm plate. The transferred plaques were denatured for 2 min with 1.5 M NaCl, 0.5 M NaOH, neutralized for 5 min with 1.5 M NaCl, 0.5 M Tris-HCl (pH 8.0) and then crosslinked with 120,000 μJ of UV energy in a UV Stratalinker (Stratagene). The hybridization solution contained 1% BSA, 1 mM EDTA, 0.5 M $NaHPO_4$ (pH7.2), 7% SDS (Ausubel *et al.*, 1987) with ^{32}P-labeled probe at a concentration of 1-2 ng/ml of hybridization solution. Hybridizations were performed overnight at 68°C in a shaking waterbath. Low stringency washes contained 0.5% BSA, 1 mM EDTA, 40 mM $NaHPO_4$ (pH7.2), 5% SDS and were performed at room temperature. The high stringency washing solution consisted of 1 mM EDTA, 40 mM $NaHPO_4$ (pH7.2), 1% SDS and was performed in a series of 5-7 short washes and a 20 minute final wash, all at 68°C.

Isolation of mouse genomic DNA

Genomic DNA was isolated from the spleen of a Balb/c mouse according to the following procedure. One mouse spleen was placed in a glass homogenizer containing 5 ml nuclei isolation buffer (50 mM Tris-Cl, pH 7.6, 25 mM KCl, 5 mM $MgOAc{\cdot}7H_2O$, 0.35 M sucrose) and homogenized for 10 strokes with a hand held homogenizer. The homogenate was filtered through cheese cloth and centrifuged at 5,000 rpm for 10 minutes at 4°C. The homogenate was washed in nuclei isolation buffer and pelleted once again. The resulting pellet was resuspended in TE (10 mM Tris-Cl, pH 7.8, 1 mM EDTA) and 0.25 ml 5M $NaClO_4$ was added with gentle swirling for 2 min, followed by the addition of 0.28 ml 10% SDS. Two phenol-chloroform extractions and one chloroform extraction

were then performed. Genomic DNA in the resulting aqueous solution was spooled out with a hooked pasteur pipet following the addition of 0.2 M NaOAc and 2 volumes cold ethanol. This DNA was resuspended in TE and quantitated spectrophotometrically.

Southern hybridization

Clones, on Southern blots, which were isolated from the cDNA libraries based on positive hybridization with either the PCR-derived probe or the 1.3 kb probe derived from the first library screening procedure were hybridized as follows. Blots were prehybridized with: 6X SSC (1X SSC = 150 mM NaCl, 15 mM NaOAc, pH 7.4), 0.5X Denhardt's solution (50X = 1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA), 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 1-2 ng/ml of hybridization solution for Southern blots containing plasmid DNA and at a concentration of 10 ng/ml for blots containing genomic DNA. 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. A series of two successive higher stringency washes were performed with 0.1X SSC, 0.5% SDS; the first was at 37°C for 45 minutes, and the second was at 68°C for 45 minutes. A final rinse in 0.1X SSC at room temperature was used to remove excess SDS. Blots were placed on X-ray film (XR-100, Sterling X-ray film) for 24 to 96 hours at -80°C with intensifying screens.

Sequencing of cDNA

The entire 3.5 kb *Mes* insert, cloned into pBluescript SK^+, was sequenced by The Sequetech Corporation (Mountain View, CA). Double stranded sequencing of plasmid DNA was performed on both strands with overlapping primers and all ambiguities were

resolved by redundant sequencing. The sequence was assessed for open reading frames. There were several open reading frames within one reading frame which all ended with the same stop codon but started at different locations. The longest of these open reading frames, which was 1.4 kb, was analyzed and considered to be the ORF for *Mes*. Sequence analysis included protein and nucleic acid searches of Genbank with both the entire 3.5 kb sequence and the 1.4 kb ORF. Sequence motifs were analyzed with the DNA sequence analysis program, DNAStar (DNASTAR, Inc., Madison, WIsconsin).

Chromosome mapping

These experiments were performed by Mark Shannon at ORNL using the *Mus domesticus/Mus spretus* interspecific backcross panel developed by E. Rinchik and L. Stubbs of ORNL. Southern blots containing DNA from 120 mice derived from the backcross breeding procedure were hybridized with a 700 bp *Xho* I probe from *Mes* using standard procedures discussed previously in the methods and materials. Results of the RFLP analysis were examined with the data analysis program Map Manager and the chromosomal location determined within 99% confidence limits.

Northern blot preparation and hybridization

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 transferred to nylon membrane (Hybond, Amersham) using a Pharmacia vacuum blotting system. Transferred RNA was crosslinked onto nylon membrane using a Stratalinker (Stratagene) at 1280μjoules/cm^2. Prehybridization of the blots occurred at 42°C for 1 hour with 50% formamide, 5X SSC (1X SSC = 150 mM NaCl, 15 mM NaOAc, pH 7.4), 5X Denhardt's

solution (50X = 1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA) 50 mM potassium phosphate buffer, pH 7.4, and 250 μg/ml sheared 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 15 minutes each. Blots were subsequently exposed to X-ray film (XR100, Sterling X-ray film) at -80°C. Exposure times were determined empirically and ranged from 24 hours to 72 hours with intensifying screens.

CHAPTER III

RESULTS

Identification and sequencing of the *Mes* cDNA

The original purpose of this cloning strategy was to isolate a kinesin-like cDNA from the meiotic cells of the male mouse. The availability of a consensus sequence corresponding to the kinesin heavy chain region of kinesin-like proteins (Stewart *et al.*, 1991) allowed for the design of degenerate primers for this region and subsequent employment of PCR to amplify a cDNA sequence from mouse pachytene spermatocyte cDNA. A 160 bp sequence was amplified and upon sequencing, the PCR-derived fragment was found to have 65.5% identity at the nucleic acid level with *Drosophila* kinesin in 160 bp of overlap. When this fragment was used as a probe on Northern blots of RNA from spermatogenic cells, a 3.5 kb transcript was found to be expressed in round spermatids and pachytene spermatocytes. Since the PCR-derived fragment appeared to be a member of the kinesin family and hybridized to transcripts expressed during spermatogenesis, it was used to screen a λgt11 pachytene spermatocyte cDNA library to retrieve a complete cDNA sequence. Following three rounds of screening under stringent hybridization conditions, 3 clones were identified as candidate cDNAs. The insert size of these clones was determined by PCR amplification with primers flanking the cloning site and subsequent fragment sizing by agarose gel electrophoresis. All three clones were found to contain the same size insert, 1.3 kb. One cDNA clone was chosen at random for further experimental analysis. When it was hybridized to a Northern blot of RNA from pachytene spermatocytes and round spermatids, a 3.5 kb message was detected that was more abundant in pachytene spermatocyte RNA than in round spermatid RNA.

The 1.3 kb cDNA clone isolated was too small to represent the whole 3.5 kb mRNA identified by Northern analysis. In order to isolate a larger clone, a new cDNA library was constructed from pachytene spermatocyte mRNA utilizing the Stratagene λ-ZAP system (Part II). This new library was shown to contain a range of cDNA inserts of approximately 0.5 -5.0 kb in size, thereby possibly increasing the chance of successfully isolating a larger cDNA clone. The new cDNA library was hybridized with the previously isolated 1.3 kb cDNA fragment. Four rounds of stringent screening were performed, ultimately identifying 9 clones with homology to the 1.3 kb probe. Subsequent analysis of clone insert size was performed, using PCR with primers that recognize sites flanking the multiple cloning site in phage. The largest cDNA insert was determined to be 3.5 kb. This cDNA was isolated and utilized in all subsequent experimental procedures. In order to verify that the 3.5 kb cDNA exhibited homology to the original 160 bp PCR-derived fragment, a Southern blot was performed in which the 3.5 kb fragment was hybridized with a probe representing the 160 bp fragment. Very strong homology was found to exist between the two cDNAs since X-ray film was developed within half an hour of exposure. Since the 160 bp fragment contained 65.5% identity to *Drosophila* kinesin, the results of library screening and Southern blot analysis led us to believe that the 3.5 kb fragment also shared sequence identity with the kinesin family.

DNA sequencing of the 3.5 kb cDNA led to the identification of a large open reading frame (ORF) consisting of 1428 bases beginning with a methionine and ending with a stop codon (Figure 1). This ORF encodes a protein of 475 amino acids with a predicted molecular mass of 53 kDa (Figure 2). The full cDNA contains approximately 1.3 kb of 5' untranslated sequence and 0.7 kb of 3' untranslated sequence ending in a terminal poly (A) region (Figure 3). The predicted amino acid sequence of the ORF was compared to the Genbank protein data base using the FASTA program (Lipman and Pearson, 1988)

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ATGGACCCGTTTACCGAGGTGAGAGCTCGTGCCGAATTCG 40
GCACGAGCACAACCTGCCTCCAGGCTTTAGTGGGTCGCCG 80
GGCCCCTCCTCCTAGAGCAGTCGCTTTTTCCCGCGCGAGC 120
TTACCTCTGTTGCTCGGGCCACCAGCGGGGGGAAACATGG 160
CGTCTGCTCTGGAGCAGTTCGTGAACAGTGTCCGACAGCT 200

CTCAGCTCAAGGGCAAATGACTCAGCTTTGTGAACTCATC 240
AACAAGAGTGGAGAACTCCTTGCGAAGAACTTGTCCCATC 280
TGGACACTGTGCTTGGGGCTTTAGATGTTCAAGAACACTC 320
TTTGGGTGTCCTTGCTGTTCTGTTTGTGAAGTTCTCAATG 360
CCCAGTGTTCCTGACTTCGAAACACTATTCTCACAGGTTC 400

AGCTTTTCATCAGTACATGTAATGGCGAGCACATTCGATA 440
TGCAACAGATACGTTTGCTGGGCTTTGCCATCAGCTAACA 480
AATGCACTTGTGGAAAGAAAACAGCCCCTTCGAGGAATTG 520
GCATCCTTAAGCAAGCCATAGACAAGATGCAGATGAATAC 560
CAACCAACTGACCTCAGTACATGCCGATCTCTGCCAGCTT 600

TGTTTACTAGCGAAGTGCTTTAAACCTGCCCTTCCATATC 640
TTGATGTGGATATGATGGATATCTGTAAAGAGAATGGAGC 680
CTATGATGCAAAACACTTTCTGTGCTACTACTATTATGGA 720
GGAATGATCTATACGGGGCTGAAGAACTTTGAAAGAGCGC 760
TGTACTTTTATGAGCAGGCTATAACTACTCCTGCCATGGC 800

CGTCAGCCATATCATGTTGGAATCATATAAAAAGTATATT 840
CTAGTGTCTTTGATATTACTTGGCAAAGTACAACAGCTGC 880
CTAAATACACCTCTCAAATAGTGGGTAGATTTATTAAGCC 920
TCTCAGCAATGCTTACCATGAGTTAGCACAAGTTTATTCA 960
ACCAACAACCCGTCTGAGCTGCGGAACCTGGTGAGCAAGC 1000

ACAGCGAGACCTTCACTCGAGACAACAACATGGGCCTGGT 1040
GAAGCAGTGCCTGTCGTCTCTTTATAAGAAGAACATTCAG 1080
AGGCTGACAAAGACTTTTTTAACACTATCATTACAAGATA 1120
TGGCAAGCCGTGTACAGTTATCTGGACCACAGGAGGCAGA 1160
AAAATATGTTCTACACATGATAGAAGATGGAGAAATTTTT 1200

GCAAGTATTAACCAGAAGGATGGAATGGTCAGTTTCCATG 1240
ATAACCCTGAGAAGTATAATAACCCAGCCATGCTTCATAA 1280
CATCGACCAAGAGATGCTAAAATGCATAGAGCTGGATGAG 1320
CGACTGAAGGCTATGGACCAGGAGATCACGGTGAACCCCC 1360
AGTTTGTTCAAAAGAGCATGGGCTCACAAGAAGATGACTC 1400

AGGAAACAAGCCCTCCAGCTACTCTTGA 1428
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Figure 1. Nucleotide sequence of the *Mes* ORF. Nucleotides are numbered in the right margin. The sequence encodes 1428 bases. Nucleotide 1 begins the start of transcription, the first three bases encode a methionine residue. The sequence ends with a stop codon.

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MDPFTEVRARAEFGTSTTCLQALVGRRAPPPRAVAFSRAS 40
LPLLLGPPAGGNMASALEQFVNSVRQLSAQGQMTQLCELI 80
NKSGELLAKNLSHLDTVLGALDVQEHSLGVLAVLFVKFSM 120
PSVPDFETLFSQVQLFISTCNGEHIRYATDTFAGLCHQLT 160
NALVERKQPLRGIGILKQAIDKMQMNTNQLTSVHADLCQL 200
CLLAKCFKPALPYLDVDMMDICKENGAYDAKHFLCYYYYG 240
GMIYTGLKNFERALYFYEQAITTPAMAVSHIMLESYKKYI 280
LVSLILLGKVQQLPKYTSQIVGRFIKPLSNAYHELAQVYS 320
TNNPSELRNLVSKHSETFTRDNNMGLVKQCLSSLYKKNIQ 360
RLTKTFLTLSLQDMASRVQLSGPQEAEKYVLHMIEDGEIF 400
ASINQKDGMVSFHDNPEKYNNPAMLHNIDQEMLKCIELDE 440
RLKAMDQEITVNPQFVQKSMGSQEDDSGNKPSSYS.  476
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Figure 2. Amino acid sequence of the *Mes* ORF. Amino acids are numbered in the right margin. The sequence begins with a methionine residue and ends with a stop codon. The sequence consists of 475 amino acids and encodes a protein with a putative molecular weight of 53 kDa.

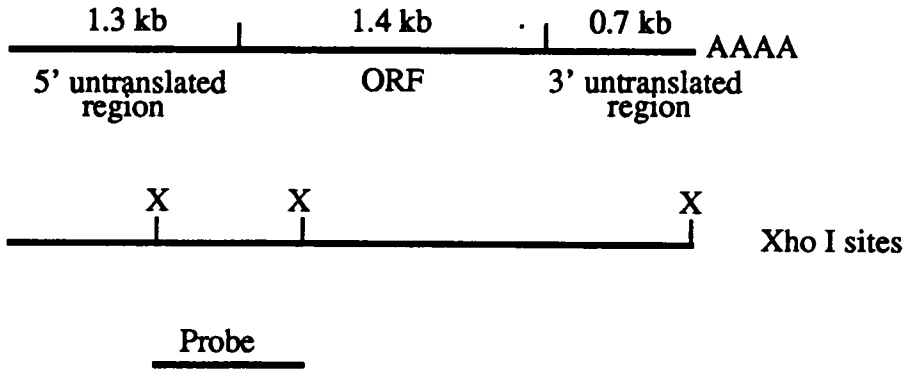

Figure 3. Diagram of the 3.5 kb *Mes* cDNA. Mes contains approximately 1.3 kb of 5' untranslated sequence and 0.7 kb of 3' untranslated sequence. The cDNA contains a terminal poly (A) region. Endonuclease digestion sites for *Xho* I in the cDNA are shown. In addition, the location of the 700 bp *Xho* I fragment used in Southern blot analysis and chromosome mapping is indicated.

and no sequences with significant similarity were identified. However, when the nucleotide sequence of the 1.4 kb ORF was analyzed using a TFASTA nucleic acid search in Genbank, it was found to have 92.8% identity in 404 bp of overlapping sequence to an expressed sequence tag (EST) from human infant brain (Figure 4) (Adams *et al.*, 1993; Polymeropoulus *et al.*, 1993) and at the amino acid level *Mes* and the EST shared 95% identity (data not shown). A high degree of homology between mouse and human genes also exists for homologs of the yeast *Rad51* gene. The mouse and human *Rad51* genes share 91% identity at the nucleic acid level and 99% identity at the amino acid level.

Surprisingly, when both the *Mes* ORF and the entire 3.5 kb sequence were checked for protein or nucleic acid homology in Genbank, no kinesin-like sequences were identified as having homology to the clone isolated. It is not clear why the kinesin-like 160 bp PCR-derived fragment hybridized to the clone isolated in this study. Homology comparison between the PCR-derived fragment and the entire 3.52 kb cDNA clone revealed only a maximum of 86% complementarity to the novel gene over a stretch of 22 bases in the ORF of the cDNA.

Identification of a chromosomal location for *Mes*

In an attempt to determine gene copy number for *Mes* in the mouse genome, a mouse genomic Southern blot was hybridized with the *Mes* cDNA. When the entire 3.5 kb cDNA was used as a probe the resulting signal was a smear with no discernible bands, as can be seen in Figure 5a, indicating the presence of a repetitive element or highly conserved motif in the cDNA sequence. At this time, however, I have not yet analyzed the entire 3.5 kb cDNA for the presence of repetitive elements. In order to circumvent this problem, the 3.5 kb cDNA was digested with *Xho* I, which separated the cDNA into 3 smaller fragments. Three bands were isolated from this digest, including a 700 bp *Xho* I

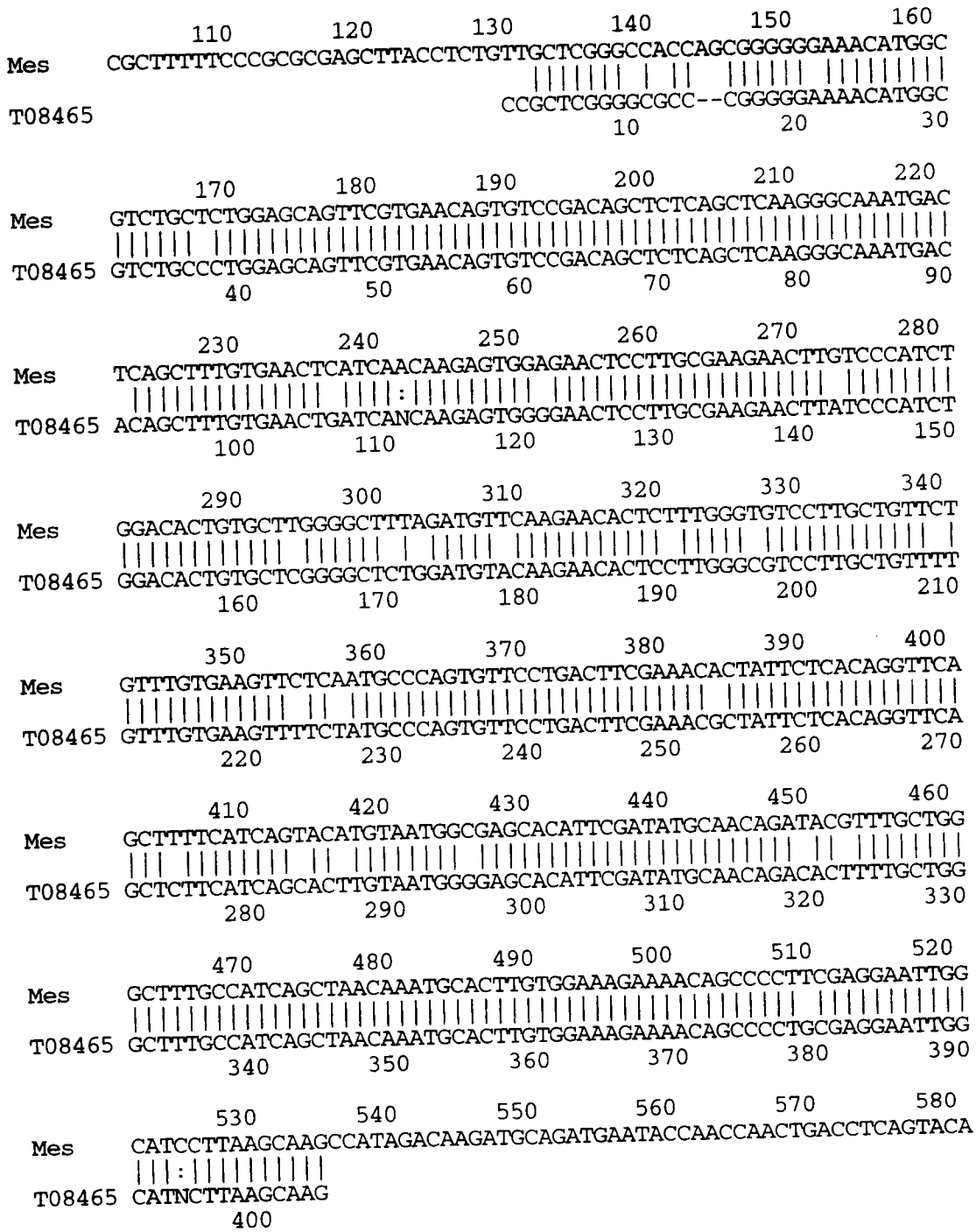

Figure 4. Sequence comparison by a TFASTA search (Genbank) of *Mes* with an expressed sequence tag from infant brain. Human clone=HIBBE73, EST06356 (accession T08465). An identity of 92.8% exists in 404 bp overlap.

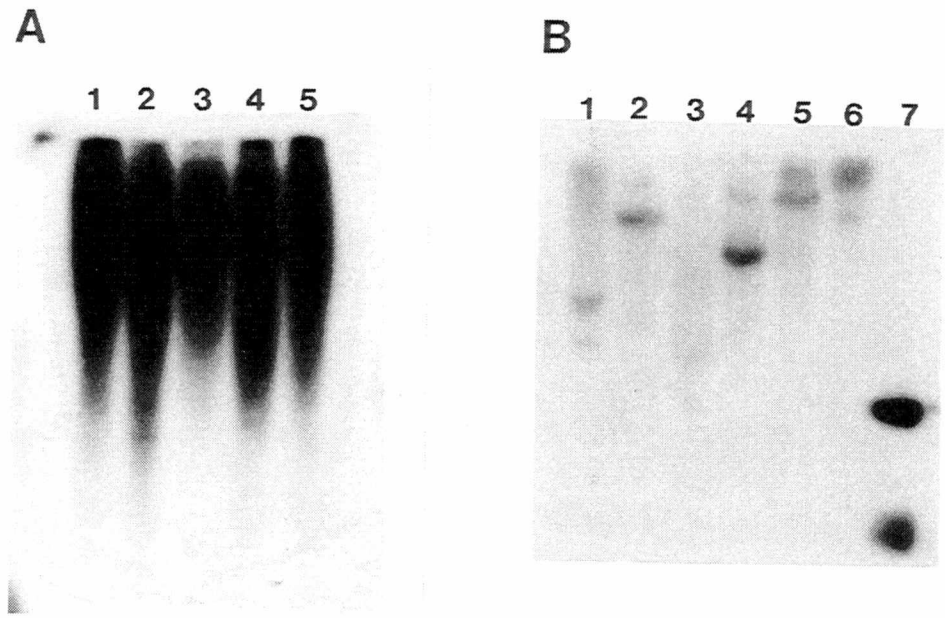

Figure 5. Genomic Southern blots demonstrating the pattern of hybridization when utilizing various *Mes* sequences as probes. A. Mouse genomic Southern blot demonstrating the pattern of hybridization when the 3.5 kb *Mes* cDNA is used as a probe. The lanes are as follows: 1- *Bam* HI, 2- *Eco* RI, 3- *Hind* III, 4- *Pst* I, 5- *Xba* I. B. Genomic Southern blot demonstrating the pattern of hybridization when utilizing the 700 bp *Xho* I fragment as a probe. The lanes are as follows: 1-*Xba* I, 2-*Sac* I, 3-*Pst* I, 4-*Bam* HI, 5-*Kpn* I, and 6-*Xho* I. Lane 7 contains the molecular weight marker.

fragment from Mes, which spanned an area of the cDNA area covering the end of the 5' untranslated region into the beginning of the ORF (Figure 3). This *Xho* I fragment was used to probe a mouse genomic Southern blot from which bands were detected when the genomic DNA was cut as follows: two bands were seen when genomic DNA was digested with *Pst* I, *Sac* I, *Bam* HI, *Kpn* I, *Xba* I and *Xho* I (Figure 5b). These results are suggestive of either a single or two copy gene or a single copy gene with high homology to another gene.

The 700 bp *Xho* I fragment was also used as a probe to identify the chromosomal location of *Mes* in the mouse genome. An interspecific *Mus domesticus/Mus spretus* backcross panel constructed by E. Rinchik and L. Stubbs at the Biology Division of ORNL was used in this analysis. An analysis of the restriction fragment length polymorphism (RFLP) pattern of bands hybridizing with the 700 bp probe in 120 animals determined, within 99% confidence limits, that *Mes* was located on mouse Chromosome 11 in a region approximately 3.4 cM from interleukin 3 and 6.5 cM from acetylcholine receptor beta (Figure 6). This region of mouse chromosome 11 has homology to human chromosomes 17p13.1, 17p11, and 5 (L. Stubbs, personal communication; Lossie *et al.*, 1994).

Analysis of *Mes* expression

In order to determine the expression pattern of *Mes* during spermatogenesis, the 3.5 kb cDNA was used to probe a Northern blot containing RNA from enriched germ cell preparations (Figure 7). The blot contained RNA from various sources including XX*Sxr* mice (which are devoid of germ cells), spermatogonia, leptotene/zygotene spermatocytes, pachytene spermatocytes, round spermatids, residual bodies (the cytoplasmic droplets shed by maturing sperm), and whole testis. The 3.5 kb probe hybridized specifically to a transcript of approximately 1.7 kb, primarily in pachytene spermatocytes. Some

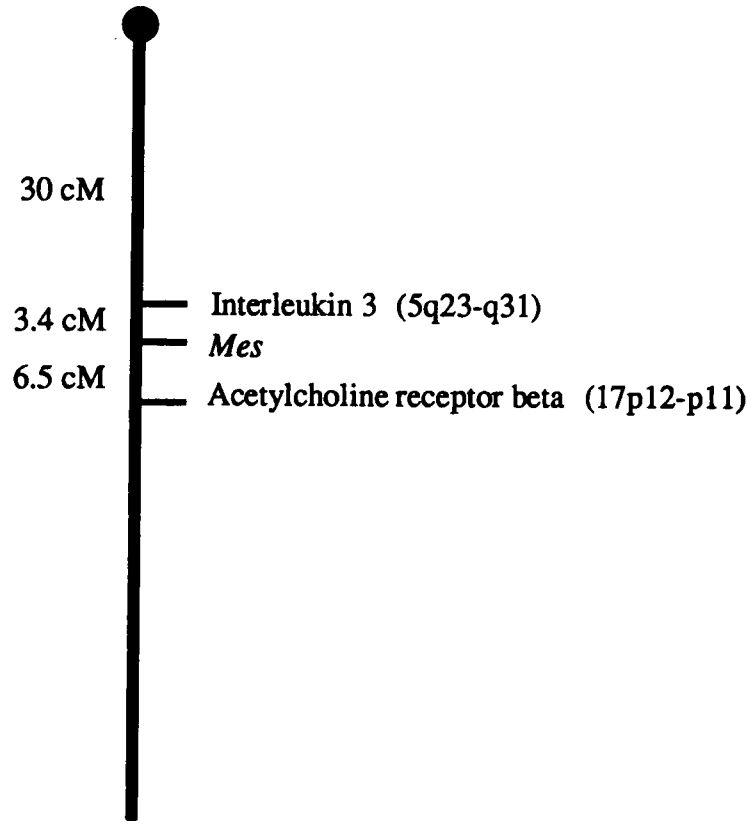

Figure 6. Location of *Mes* on chromosome 11 in the mouse. Distance from the centromere is indicated on the left. Human localizations are shown for interleukin 3 and acetylcholine receptor beta.

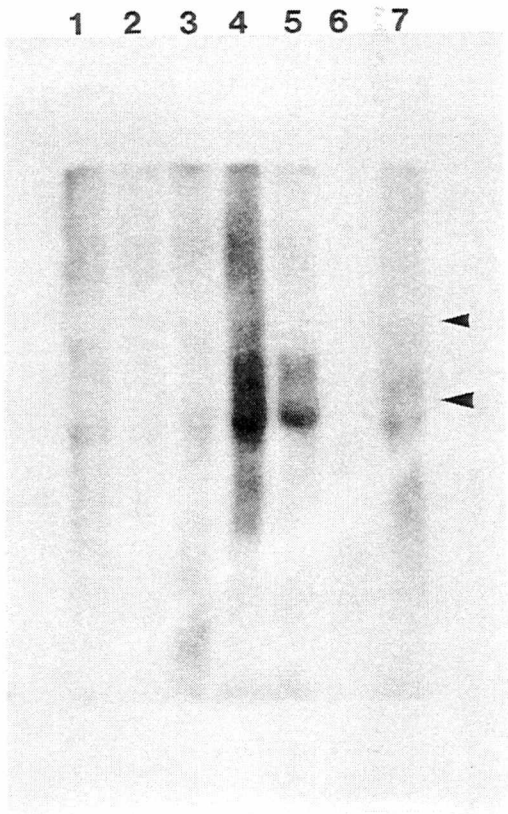

Figure 7. *Mes* transcript levels in testicular cell populations. Total cellular RNA (15 μg) from various cell populations were analyzed as follows: 1-XXSxr testes, 2-spermatogonia, 3-leptotene/zygotene spermatocytes, 4-pachytene spermatocytes, 5-round spermatids, 6-residual bodies, 7-whole testis. RNA from these sources was denatured, electrophoresed in a 1% agarose gel and transferred to nylon membrane. The blot was hybridized with a cDNA probe for the *Mes* gene. Arrowheads indicate positions of 28S and 18S ribosomal RNA bands. The autoradiogram was exposed for 3 days at -80°C.

expression was also detected in round spermatids, although at a lower level, and virtually no expression was observed in the samples from XX*Sxr* testes, spermatogonia, leptotene/zygotene spermatocytes and residual bodies. This expression pattern was observed in 3 additional Northern blots which were hybridized individually with the three *Xho* I fragments of the 3.5 kb *Mes* cDNA (Figure 3).

In order to confirm the meiotic expression pattern of the gene represented by this cDNA, a Northern blot containing RNA representative of developmentally staged testes was hybridized with the 3.5 kb cDNA clone (Figure 8). The seminiferous epithelium of the testis is populated by more advanced spermatogenic cells in a developmental sequence. The major germ cell type present in the testes of 8 day mice are spermatogonia. After day 10 leptotene/zygotene spermatocytes populate the testis. Pachytene spermatocytes appear at day 14, and round spermatids are first present at day 21. The first round of spermatogenesis is completed by day 35 but the mouse is not considered an adult until the testis reaches its maximal size, which is about day 60 (Bellvé *et al.*, 1977). Hybridization of the 3.5 kb clone to a developmentally staged testis blot indicated that *Mes* expression occurs concurrently with specific stages of meiosis. Expression is initially evident in 16 day testes, when pachytene spermatocytes begin to populate the testis and continues in 21 day testes until it then levels off as more of the cells in the testis become round spermatids. Very little expression of *Mes* was detected in XX*Sxr* testes and day 8 testes.

The expression pattern of *Mes* in somatic tissues was also examined (Figure 9). A blot containing mouse RNA from whole testis, adult ovary, brain, embryo, heart, liver, lung, kidney, spleen, and thymus was hybridized with the 3.5 kb *Mes* probe. The results from this hybridization indicated that a 1.7 kb transcript was evident in testis, ovary, and embryo. Moreover, in brain, thymus, and spleen, a 3.5 kb band was present in addition

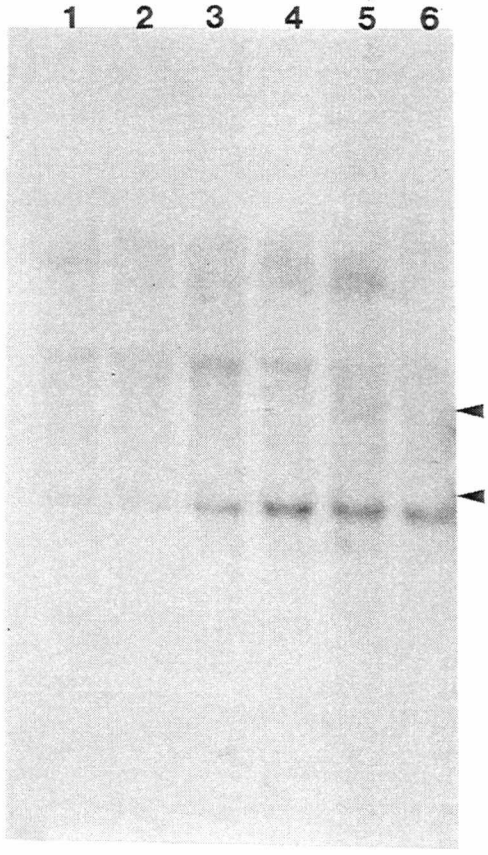

Figure 8. *Mes* transcript levels in testes during postnatal development. Total cellular RNA (12.5 μg) from testes was analyzed as follows: 1- XX*Sxr*, 2- 8 day, 3- 16 day, 4- 21 day, 5-35 day, 6- 55 day. RNA from these sources was denatured, electrophoresed in a 1% agarose gel and transferred to nylon membrane. The blot was hybridized with a cDNA probe for the *Mes* gene. Arrowheads indicate positions of 28S and 18S ribosomal RNA bands. The autoradiogram was exposed for 2 days at -80°C.

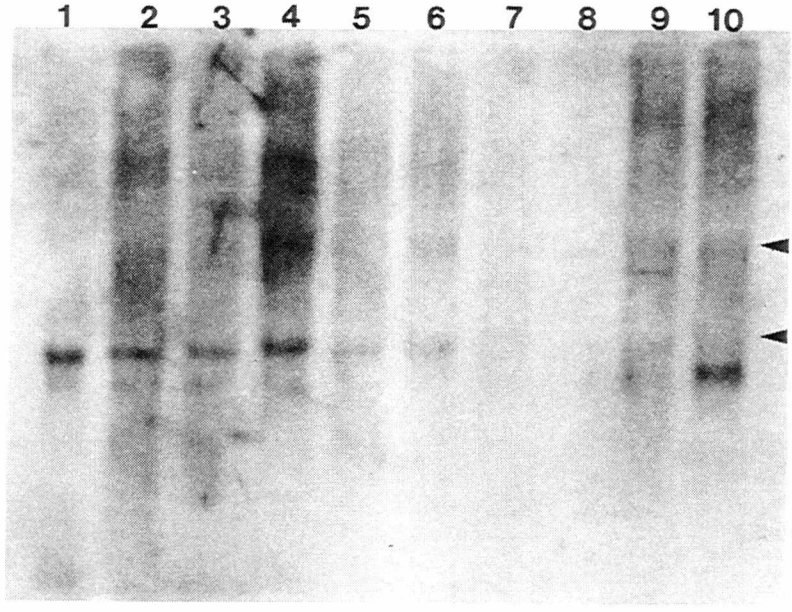

Figure 9. *Mes* transcript levels in mouse somatic tissues. Total cellular RNA (12.5 μg) from various mouse tissues was analyzed as follows: 1-whole testis, 2-ovary, 3-brain, 4-embryo, 5-heart, 6-liver, 7-lung, 8-kidney, 9-spleen, 10-thymus. RNA from these sources was denatured, electrophoresed in a 1% agarose gel and transferred to nylon membrane. The blot was hybridized with a cDNA probe for the *Mes* gene. Arrowheads indicate positions of 28S and 18S ribosomal RNA bands. The autoradiogram was exposed for 3 days at -80°C.

to the 1.7 kb band. There was virtually no hybridization detected with RNA from heart, lung, liver, or kidney.

Putative transcription factor motif identified in the *Mes* cDNA sequence

The 1.4 kb *Mes* ORF was examined for the presence of potential motifs using the DNA sequence analysis program, DNAStar (DNASTAR, Inc., Madison, Wisconsin). This program identified a leucine zipper motif in *Mes*. The leucine zipper structure has been identified in many transcription factors and is characterized by the presence of a leucine residue at every 7th amino acid position over a stretch of 35 amino acids. Typically, 5 leucines aligned in this manner constitute a strict leucine zipper domain. Figure 10 demonstrates the alignment of leucine zipper regions from several leucine zipper-containing transcription factors with the putative leucine zipper region in *Mes*. The three central leucine residues of this motif are conserved in all transcription factors which contain leucine zippers (reviewed by Latchman, 1990).

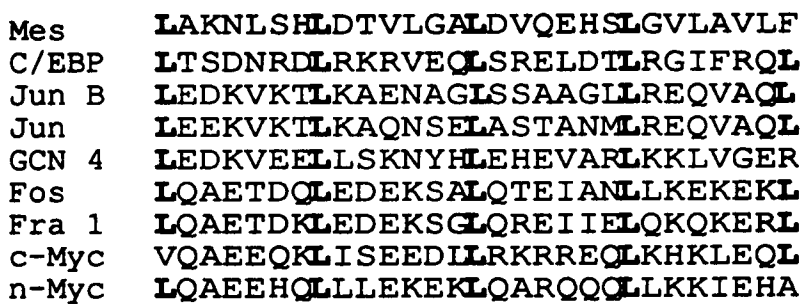

Figure 10. Alignment of the leucine-rich region of *Mes* with several cellular transcription factors. The conserved leucine residues are highlighted. The leucines in this motif occur every 7th amino acid residue over a stretch of 35 amino acids. References for the transcription factors appear in the text.

CHAPTER IV

DISCUSSION

We have isolated a cDNA corresponding to a gene that is expressed meiotically in mouse testis. This gene, which has been designated *Mes* (for Meiotically expressed sequence) is transcribed in pachytene spermatocytes. The cDNA isolated is 3.5 kb in length and includes an ORF of 1.4 kb. Interestingly, this ORF shares significant homology with an expressed sequence tag identified from human infant brain. Additionally, the ORF contains a structural motif representative of a putative leucine zipper, suggesting a possible role for the *Mes* product in the control of transcription.

The screening strategy described in this report was developed for identification of cDNA with sequence homology to the kinesin superfamily. The PCR-based approach initially used to obtain a cDNA fragment encoding a consensus region for the kinesin heavy chain was successful. The PCR-derived fragment was, upon sequencing, homologous to members of the kinesin superfamily. Since this fragment was used in the initial screening procedures to identify larger kinesin-like cDNAs, it was surprising to find that a cDNA without significant sequence homology to kinesin was identified. It is not apparent why the PCR-derived, kinesin-like fragment hybridized with *Mes*. However, following sequence analysis several short regions of homology between the PCR-derived fragment and *Mes* were found to be present. In addition, the PCR-derived fragment was originally cloned into the pBluescript vector (Stratagene) and the pachytene spermatocyte cDNA library used for the isolation of *Mes* was constructed in the λ-ZAP phagemid system (Stratagene), which is a pBluescript-derivative. Small pieces of vector could possibly have hybridized strongly between the probe and library and might have led to chance cloning of

another sequence. Since the 1.3 kb cDNA was used to isolate the 3.5 kb cDNA, the possibility exists that it was also not a kinesin. Several examples in the literature exists whereby homology screening strategies have led to the isolation of seemingly unrelated genes (Narasimhan *et al.*, 1994). It is apparent that the isolation of *Mes* falls into this category as well.

Regardless, a novel gene has been identified that is expressed during meiosis. Transcript levels for *Mes* are most abundant in pachytene spermatocytes, with some postmeiotic transcript detectable, although at a lower level. There is very little transcription of this gene in the mitotic cells of the testis or in early meiotic cells, as expression of *Mes* was barely detectable in spermatogonia or leptotene/zygotene spermatocytes. The meiotic expression pattern was confirmed by results obtained from an experiment in which RNA from developmentally staged testes was probed with the full length *Mes* cDNA. This blot indicated that *Mes* transcript was barely detectable at day 8 of testicular development but increased dramatically in day 16 and day 21 testes. This developmental expression profile corresponds to meiotic progression of spermatocytes. Since the levels of transcript decrease greatly in postmeiotic development, these results are indicative of a possible meiotic function for *Mes*, most likely in the pachytene stage.

Mes is also expressed in some somatic tissues. Interestingly, the expression pattern demonstrated with somatic RNA seems to suggest that a second, larger *Mes*-hybridizing transcript is present in some tissues. In addition to the testis, adult ovary and embryo contained only one primary transcript, the 1.7 kb RNA that is present in pachytene spermatocytes. However, the thymus, spleen, and brain all had a second transcript which appeared to be approximately 3.5 kb in size. This result suggests that perhaps the testis uses alternative transcription initiation or termination sites, or perhaps posttranscriptional processing signals are different for *Mes* transcription. Gene transcripts of alternative sizes are common in the testis. There are several examples of spermatogenically-expressed gene

sequences containing different sized transcripts. For example, it has been reported that there are smaller transcripts in germ cells than in somatic cells for the cAMP-dependent protein kinase subunits RIα, RIIα, RIIβ, and Cα (Oyen *et al.*, 1990). Most of the alternatively sized transcripts identified during spermatogenesis are smaller than the somatic cell transcripts. This has been suggested to be related to changes in transcript stability such that smaller-sized mRNAs are more stable and have longer half-lives (Oyen *et al.*, 1990). Alternatively, the *Mes* cDNA might recognize transcripts of more than one gene, perhaps another gene is homologous to *Mes*. This could be supported from the genomic Southern blot data where more than one band is evident in several of the lanes

A region of 404 nucleotide bases in the *Mes* cDNA was found to have 92.8% identity to an expressed sequence tag from human infant brain. This degree of high identity suggests conservation of function as well. Unfortunately, to date, the human sequence tag does not correspond to a protein or complete sequence with an assigned function (Adams *et al.*, 1993) and in addition, the EST has not yet been assigned a chromosomal location (Polymeropoulos *et al.*, 1993). It is most interesting that *Mes* was found to have two transcript sizes in mouse brain as well, perhaps suggesting there is an additional transcript utilized in the brain but not in the testis.

Sequence analysis of *Mes* led to the discovery of a putative leucine zipper motif within the ORF of this cDNA. A leucine zipper typically consists of a leucine appearing every 7 amino acids over a region of approximately 40 amino acid residues. As shown in Figure 10, the leucine residue alignment of the amino acid sequence for known leucine zipper-containing genes and *Mes* are identical. This structural motif has been identified in a wide variety of transcription factors, including the proto-oncogene products *Myc* (Murre *et al.*, 1989), *Fos* (Curran and Franza, 1988; Kouzarides and Ziff, 1989; Turner and Tjian, 1989), and *Jun* (Curran and Franza, 1988; Kouzarides and Ziff, 1989; Turner and

Tjian, 1989), the CAAT box-binding protein *C/EBP* (Landshulz *et al.*, 1989), and the yeast transcription factor, *GCN4* (Vogt *et al.*, 1987). A leucine zipper alone is not sufficient for a protein to act as a transcription factor. Leucine zipper-containing amino acid sequences form homodimers or heterodimers (reviewed by Johnson and McKnight, 1989) but do not bind DNA, although they are essential for DNA binding since mutations in this region inhibit DNA binding (Landshulz *et al.*, 1989). In some leucine zipper-containing transcription factors, such as C/EBP, Fos, and Jun, it has been shown that there is a basic DNA binding domain adjacent to the leucine zipper which does bind DNA. This evidence indicates that this class of transcription factors contain two separate domains, a leucine zipper region which mediates dimerization and a basic region which binds DNA, and that both are required to regulate transcriptional activation.

In the absence of functional studies it cannot be determined whether *Mes* is a transcription factor. There are many experiments that could be performed in order to elucidate the function of *Mes*. The *Mes* cDNA could be subcloned into a bacterial expression vector in order to produce and purify (by use of gene fusions) large amounts of protein for functional studies. This protein could then be used in DNA binding studies, such as DNAse footprinting or gel mobility shift assays (Kadonga *et al.*, 1987). Likewise, subsequent generation of *Mes* proteins containing specifically altered residues within the leucine zipper motif could also reveal the relative contribution of this region to DNA binding. Additionally, the *Mes* protein could be assayed for its ability to promote transcription of appropriate DNAs in a cell-free transcription system (Mueller *et al.*, 1990). It would be equally interesting to characterize the *Mes* transcript with respect to its localization in the testis and in different stages of spermatogenesis. Moreover, the availability of purified *Mes* fusion protein would facilitate the generation of *Mes*-specific antibodies for use in protein localization studies.

At this time, the function of *Mes* remains uncertain, although the presence of the putative leucine zipper region suggests a possible role for *Mes* as a transcription factor. Nonetheless, it is clear that this gene potentially has a role in meiosis, specifically during the pachytene stage of meiotic prophase I, as suggested by expression analysis. The identification of the *Mes* sequence provides a molecular basis by which its function in the testis, and the putative function of its homolog in human brain, might be ascertained in the future.

CHAPTER V

ACKNOWLEDGEMENTS

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PART V

CULTURE OF PACHYTENE SPERMATOCYTES FOR ANALYSIS OF MEIOSIS*

* This chapter is published in its entirety as Handel, MA, KA Caldwell and T Wiltshire. 1995. Culture of pachytene spermatocytes for analysis of meiosis. Dev Genetics 16: 128-139. This chapter will also appear in the doctoral dissertation of Tim Wiltshire as both Kim Caldwell and Tim Wiltshire 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 $MgSO_4.7H_2O$, 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 of 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, 2.5×10^6 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 μC/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.

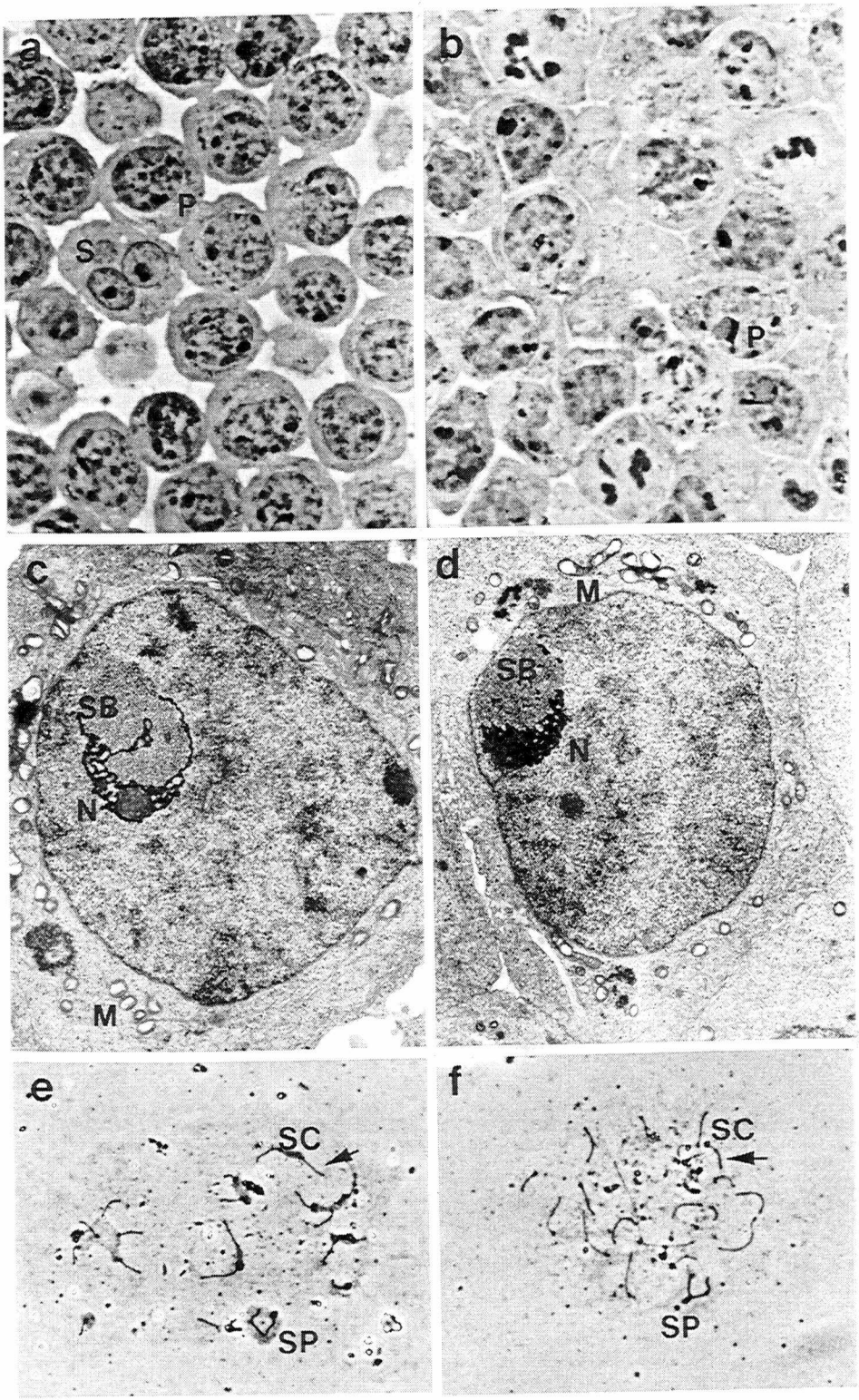
CHAPTER III

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 normal (Fig. 2a), with only occasional breaks due to spreading artifacts, and the typical thickened appearance of the unpaired axes

Fig. 1. Micrographs showing features of morphology of pachytene spermatocytes at the outset of culture (a, c, e) and after 24 hours of culture (b,d,f). a,b: Light micrographs of cells, showing nuclei of pachytene spermatocytes (P), and, a few contaminating 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 photomicrographs 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.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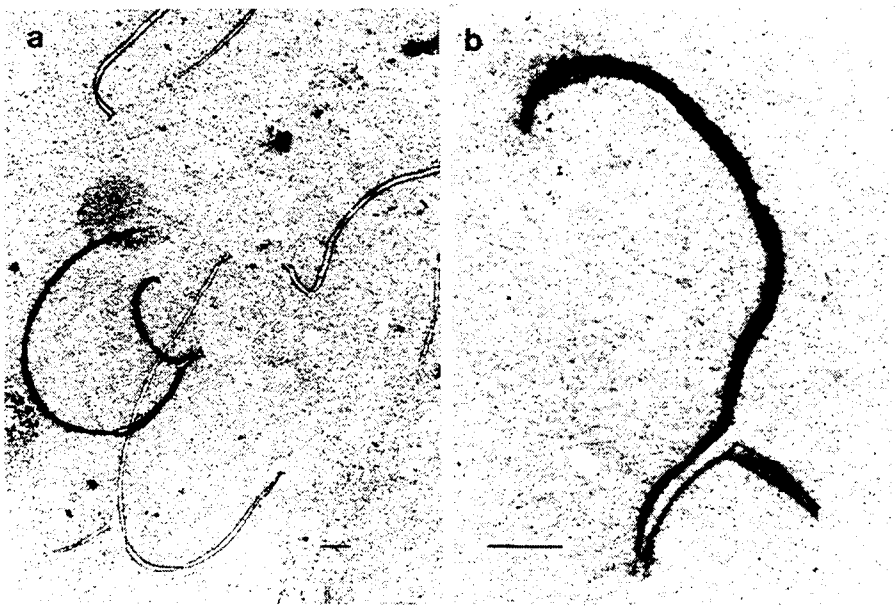

Fig. 2. Electron micrographs of synaptonemal complexes in cells cultured for 24 hours and prepared by the microspreading procedure. a: Seen here are profiles of the paired axes of the synaptonemal complex of autosomal chromosomes as well as the largely unpaired sex chromosomes with thickened lateral 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.

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 rapidly (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 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

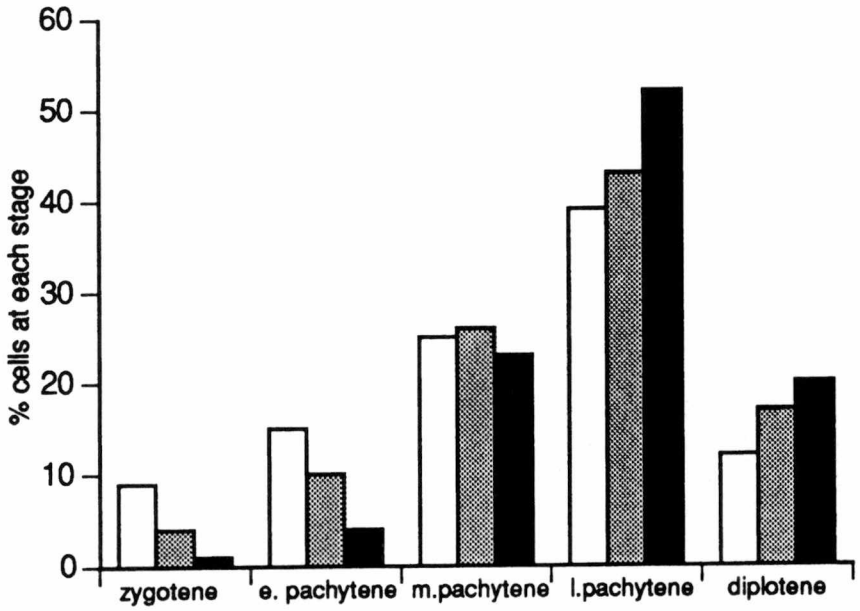

Fig. 3. Pachytene spermatocytes show progress through meiotic prophase in culture. Cells prepared by microspreading were scored according to the criteria of Moses (1980). Samples were taken at the outset of culture (□), at 24 hours (▒), and at 48 hours (■). These data represent the combined results of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

meiotic prophase. Significant progress ($P \leq 0.05$) 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 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).

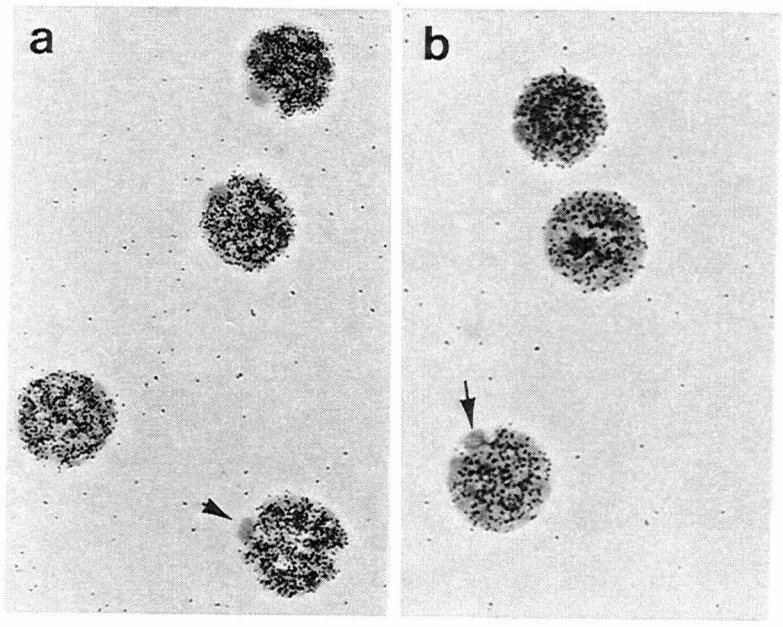

Fig. 4. Autoradiographs of air-dried preparations of pachytene cells indicate spatial distribution of incorporation of uridine. a: Cells were labeled immediately after removal from testes. b: Cells labeled after overnight culture. Note absence of label over the sex body (arrow). Bar = 10μm.

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 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

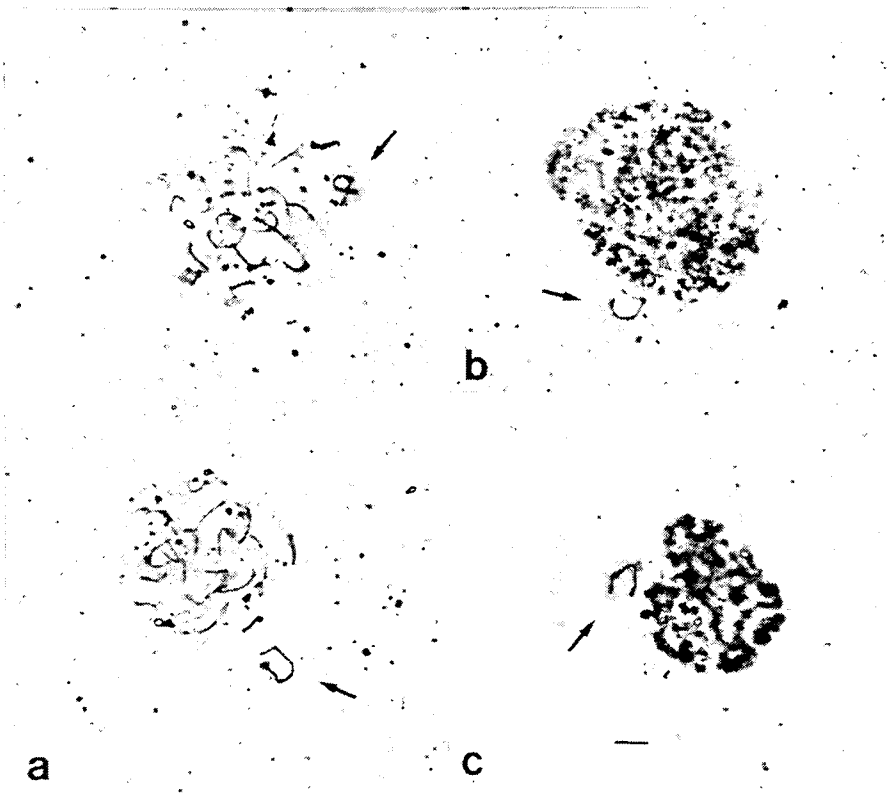

Fig. 5. Phase contrast micrographs of microspread preparations of cells demonstrate the chromatin condensation effect of actinomycin D treatment. a: Untreated cultured cells. b,c: Cells incubated for 1 hour in the presence of 5 μg/ml actinomycin D. Note that the sex body (arrow) does not show dramatic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

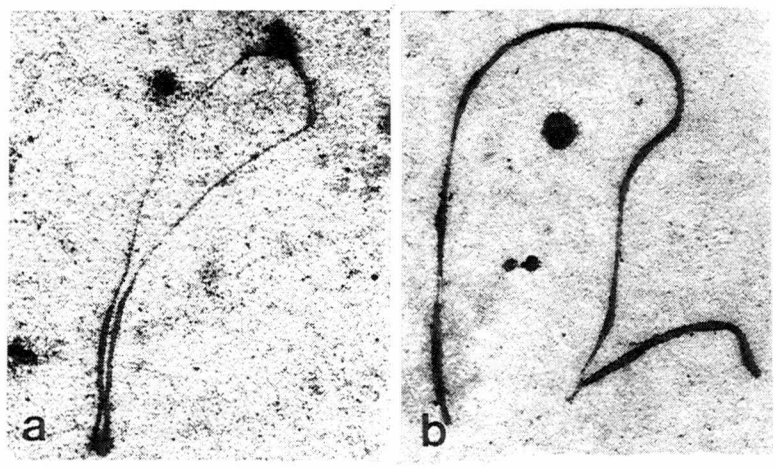

Fig. 6. Electron micrographs illustrating desynapsis frequently found in chromosomes of cells cultured for 18 hours in the presence of 50 μM camptothecin. a: An autosomal homolog is undergoing desynapsis. b: The X-Y pair in the same cell is almost completely desynapsed. Bar = 1 μm.

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 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.

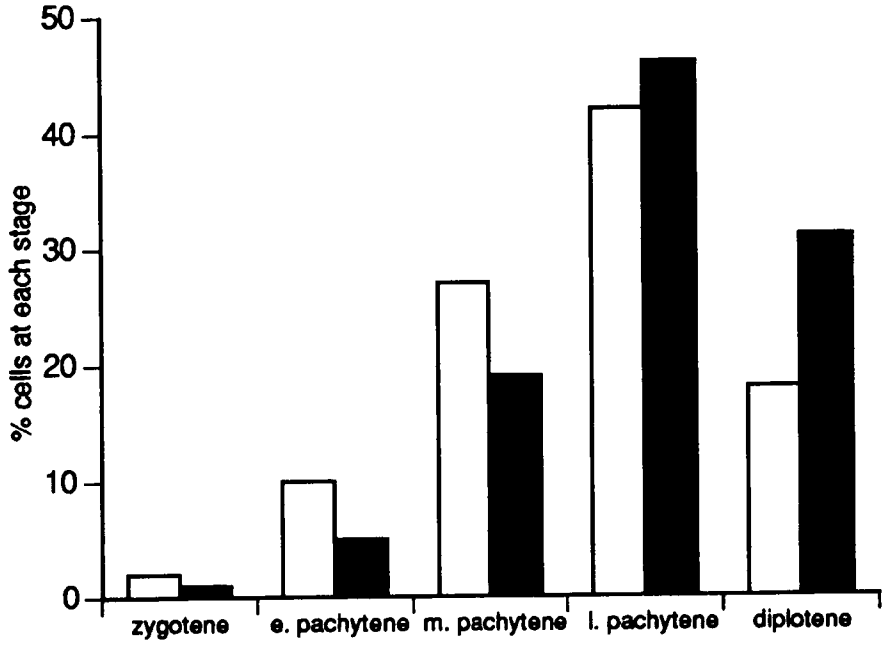

Fig. 7. Distribution of cells in meiotic prophase substages (scored according to the criteria of Moses, 1980). Samples were removed for scoring after 18 hours of culture for both control, untreated, n=800, (□) and cells incubated in 50 μM camptothecin, n=1000, (■). Cells with desynapsis were scored as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

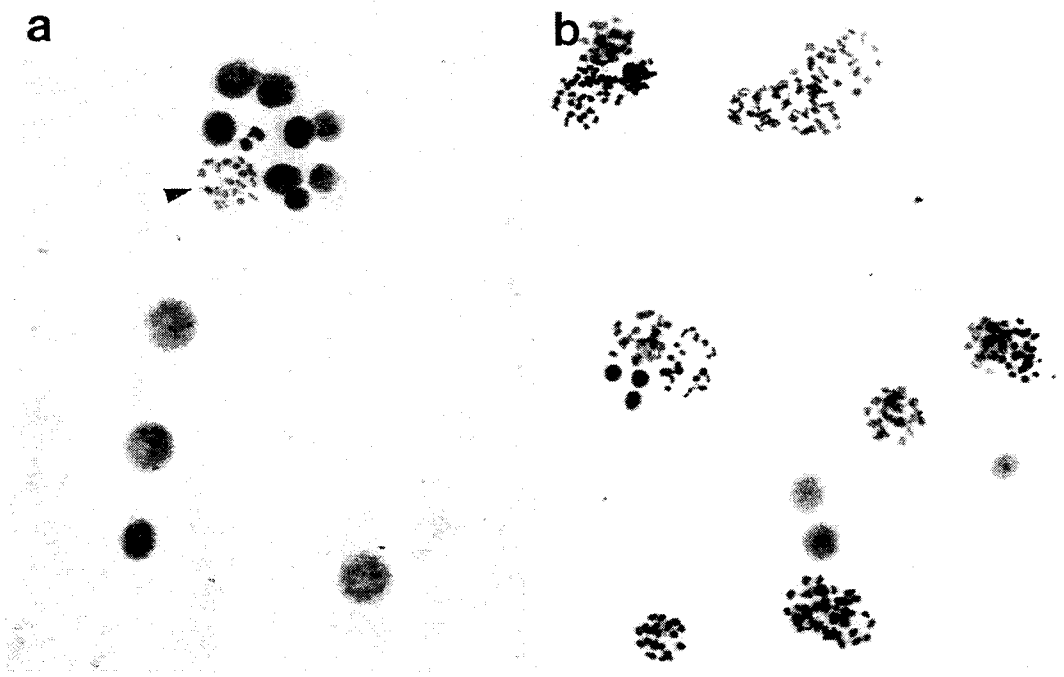

Fig. 8. Air-dried meiotic chromosome preparations of cultured cells before (a), and after (b) treatment with 5 μM okadaic acid. Note the prevalence of pachytene cells among untreated cells; this photograph was selected to demonstrate a meiotic cell, (arrow) which represent less than 4% of the total.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

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.

CHAPTER V

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PART VI

SUMMARY

CHAPTER I

UTILITY OF VARIOUS STRATEGIES FOR THE ISOLATION OF MEIOTICALLY EXPRESSED SEQUENCES

Meiosis is the process governing the genetic inheritance of traits. However, despite continuing advances, the molecular basis of meiosis in mammals remains to be elucidated. The work described in this dissertation was designed to further our understanding of the meiotic process. Clearly, the identification of genes involved in meiosis will ultimately lead to a more complete understanding of the molecular basis of meiosis and the mechanisms regulating this process. Although several genes expressed during meiosis in the mouse have been previously reported (reviewed in Part I) a more comprehensive analysis of this process awaits the identification of additional meiotically expressed genes. In contrast, much more information is available regarding meiotic gene expression in lower eukaryotes where the ease of manipulation of yeast and *Drosophila* has made these organisms particularly amenable to more detailed genetic analyses than can be performed in the mouse. In this regard, many genes have been identified with a role in meiosis in these organisms. Understanding the meiotic process in lower eukaryotes should stimulate further insights into mammalian meiosis. Likewise, information about meiosis stemming from mammalian studies will probably complement and increase the understanding of meiosis in lower eukaryotes. As described in this work, several reasonable approaches exist for analyzing meiosis and isolating meiotically expressed genes in the mouse. Some of these have been utilized in this dissertation with varying degrees of success. In this final section of my dissertation, the pros and cons of these means by which the complex mechanisms of mammalian meiosis can begin to be dissected are discussed.

Implications of this work

This dissertation represents several methodologies all aimed at the goal of identifying meiotically expressed genes. It should be noted that while each approach yields valuable information, the implications of the work as a whole can be difficult to discern. The following represents an attempt to frame these studies within the context of future research goals and objectives.

Direct homology screening Although several distinct approaches have already been described, the initial approach I employed for the identification of meiotically expressed genes has not been previously discussed in this dissertation. This approach was clearly the most straightforward and least labor intensive method to identify mammalian meiotic genes. This technique involved direct homology screening with cDNA probes previously identified from lower eukaryotic meiotically expressed genes. The specific cDNAs utilized in this study were *RED1* (Thompson and Roeder, 1989) and *MEI4* (Menees *et al.*, 1992) which are both well-characterized *Saccharomyces cerevisiae* genes involved in chromosome pairing, and the cDNA for the *ncd*, (Endow *et al.*, 1990; McDonald and Goldstein, 1990) a *Drosophila* gene involved in chromosome segregation. These cDNAs were used to probe mouse genomic DNA on Southern blots. An autoradiograph depicting the results of a typical hybridization with a cross-species probe is shown in Figure 1. This autoradiograph demonstrates the results of hybridizing *MEI4* cDNA to mouse genomic DNA. A lack of hybridization indicates that no homology is present between mouse genomic DNA and the *MEI4* cDNA (lane 2), although hybridization is clearly evident with the control yeast genomic DNA (lane 3). Similar results were obtained when using a cDNA probe for *MER1* or *ncd* (data not shown). Negative results do not necessarily imply there is no homology in gene function since yeast or *Drosophila* gene products may share functional similarities with mouse gene products, but codon usage may be different.

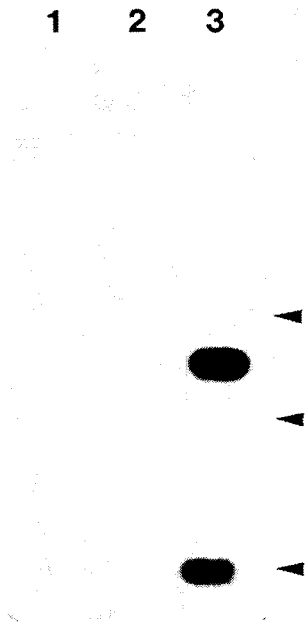

Figure 1. Genomic Southern blot demonstrating the pattern of hybridization of mouse and *S. cerevisiae* DNA when using the yeast *MEI4* gene as a probe. The lanes are as follows: 1- λ Hind III marker DNA, 2- 10 μg mouse genomic DNA digested with *Eco* RI, 3- 1.5 μg *S. cerevisiae* genomic DNA digested with *Eco* RI. Two signals were apparent in the yeast lane, as visualized by the presence of the 3 kb and 0.5 kb bands. Arrowheads indicate the positions of λ Hind III molecular weight markers, 4.4 kb, 2.2 kb, and 0.5 kb. No hybridization was seen with mouse DNA. All methods used in the Southern hybridization procedure were performed as described in other parts of this dissertation.

This is certainly true for yeast, where codon usage is very different from mouse and a positive signal on a mouse genomic Southern blot with a yeast probe might be considered improbable. Although *Drosophila* codon usage is similar to mouse codon usage, some genes may have species specific domains and, therefore, not be entirely homologous to any gene in mouse. In contrast, it might be expected that sequences with highly conserved domains among several organisms would probably be homologous to similar domains in the mouse. Therefore, if only the conserved regions from the various cDNAs were used as a probe in these experiments, hybridization to mouse genomic DNA or Northern blots containing RNA from spermatogenic cells might have been possible. Although not pursued in this analysis, hybridization of mouse genomic DNA with small conserved regions from yeast and *Drosophila* meiotic genes may be an alternative and more successful strategy. Additionally, other gene families may be more amenable to homology screening, depending on the nucleotide organization of genes and organisms involved. The negative results obtained with this primary attempt at direct homology screening subsequently led me to pursue the other strategies discussed in this dissertation.

cDNA library construction A first step toward the experimental goal of identifying novel mammalian meiosis genes involved the construction of cDNA libraries from mRNA of meiotic cells. These libraries provide a resource for studies of meiosis (Part II). Furthermore, there have not been any reports in the literature of cDNA libraries constructed from leptotene/zygotene spermatocytes. Since the STAPUT enrichment procedure for the isolation of early spermatocytes is time consuming and expensive, very often leptotene/zygotene spermatocyte gene expression is studied utilizing cDNA libraries from whole testes of mice between 12 and 17 days old. However, such whole testis cDNA libraries contain, in addition to cDNA sequences representing leptotene/zygotene

spermatocytes, cDNA sequences representing all other cell types present in the testis, including spermatogonia, Sertoli cells, and Leydig cells.

A descriptive molecular analysis of the cDNA libraries is provided in Part II of this dissertation. Unfortunately, the results of this analysis were confusing. I had anticipated that a direct correlation between cytological and molecular analyses of the libraries would be possible. However, upon examination of disparities between cytological scoring data and the characterization of expression of known stage-specific genes, it became apparent that this analysis was not informative. For example, the round spermatid cDNA library was found to contain three times the number of cDNA clones for *Prm-1* as the pachytene spermatocyte cDNA library. The *Prm-1* cDNA sequence represents a gene sequence expressed post-meiotically. On the one hand, finding three-fold greater representation in the round spermatid library fits some expectations. On the other hand, why is the representation in the pachytene library not less than one-third since the percent contamination by round spermatids is far less than 33%? There are several possible explanations for the results I received. My cytological scoring criteria might not have been precise or the existing molecular descriptions of patterns of gene expression during spermatogenesis are not accurate. This is not likely since we used independent means of scoring. More likely is the differences in expression level of those few probes that are cell-specific. Probably we will never be able to control for inherent differences in gene expression levels. Nonetheless, the results do suggest that sequences expected to be in a given library are present in levels higher than sequences that are contaminants. The best we can do with these data is to use the presence of expected sequences as confirmation of the cytological evidence for cell enrichment in the cDNA libraries.

That these cDNA libraries can be an important resource for analysis of meiotically-expressed genes is exemplified by use of both the leptotene/zygotene and pachytene

spermatocyte cDNA libraries for the genetic screening approach described in Part III. Here, the meiotic libraries were utilized in a screen for meiotically-enriched clones based on hybridization patterns with RNA from meiotically arrested testicular cells. In the future, these libraries may well serve as an important reagent for other novel strategies for cloning cDNA sequences represented in the various meiotic stages.

Meiotic cDNA enrichment using meiotic mouse mutants Part III of this dissertation describes the use of the meiotic cDNA libraries for the isolation of cDNA sequences corresponding to genes presumably expressed during mammalian meiosis. This was accomplished by hybridizing plaques from leptotene/zygotene and pachytene cDNA libraries with RNA isolated from testes of T38H translocation heterozygous mice, where germ cells arrest in meiosis I prophase. This analysis was initially undertaken simply to determine if this approach was a reasonable method for the enrichment and isolation of genes expressed during meiosis. Since initial results suggested that the potential existed for identifying gene sequences enriched for meiotic expression, clones were isolated for further characterization. Identification of clones which might represent sequences not expressed in T38H mice suggested that another translocation associated with meiotic germ cell arrest, T16H, might prove useful in further analysis of the cDNAs not hybridizing with the T38H testis RNA. Developing spermatocytes in a T16H testis contain meiotically expressed sequences that are transcribed after the stage of meiosis at which germ cells in the T38H testis remain arrested. Therefore, these two translocations allowed for the differential screening of transcripts. That is, T16H testicular RNA was considered useful for determining if any of the cDNAs identified in the initial T38H screen were present in testes that progress further through meiosis. The results of screening by hybridization with T16H testicular cDNA indicated that several cDNA clones hybridized with T16H, but not with T38H cDNA. Therefore, it became apparent that cytological observations of meiotic arrest might be correlated with differences in gene expression. The results of my study

suggests that this genetic strategy is indeed a feasible option for identifying meiotically-enriched sequences. Mutants meiotically arrested at different times during meiosis appear to exhibit differential expression of certain gene sequences as inferred by identification of sequences from the libraries. In the future, in order to understand the expression patterns of some of these clones in normal spermatocytes, further hybridization analysis could be performed by identifying which clones may be represented in spermatogonia or round spermatids. This type of analysis might yield some information on the longevity of specific transcripts. Additionally, the absence of potentially long-lived transcripts may shed some light on the molecular basis of meiotic arrest in these mutant animals.

The characterization of gene sequences represented in spermatogonia or spermatids, which are not present in meiotically arrested cells but are present in meiotically normal cells, might provide information regarding transcript production potential in cells. For instance, a cell destined for arrest may not receive specific signals for producing transcripts required at subsequent developmental stages. Therefore, it might be interesting to examine the panel of clones identified in this study (Part III) for spermatogonial and round spermatid transcript representation and compare them to an equal number of clones chosen at random from the meiotic cDNA libraries. These experiments would begin to determine whether or not overall gene representation was similar or skewed in the meiotically arrested cells and might provide some suggestive information regarding the meiotic breakdown documented in the mutants.

Additionally, the meiotically identified cDNAs from the screen could be characterized in order to isolate meiotically expressed sequences specifically. In this regard, individual clones could be analyzed with respect to gene expression patterns and by sequence analysis. Alternatively, the clones could be further hybridized with genes assumed to have a role in meiosis, such as cyclins or kinases, in an attempt to narrow

down a possible function for some of the isolates. Although I may appear to be dividing information that might be learned about these clones into two categories (that which can be learned about meiotic failure in the mutants and the isolation of meiotically expressed genes) the line between these two categories is thin. Expression analysis and sequence characterization of some of these clones may assist in developing hypotheses regarding meiotic arrest during the leptotene/zygotene and pachytene stages. For example, if some of the clones encode gene sequences that are essential for meiotic progression, this information may aid in understanding how meiotic failure occurs. In summary, the combined use of the meiotically arrested translocation heterozygous male mice and the cDNA libraries constructed (Part II) provided a novel approach for identifying meiotically enriched cDNA sequences. These cDNAs may contribute useful information toward a more complete understanding of meiotic mechanisms.

Isolation of a meiotically expressed cDNA As discussed in Part IV of this dissertation, a cDNA clone with a leucine zipper-containing sequence was isolated from the pachytene spermatocyte cDNA library. These experiments were originally performed with the intent of isolating a kinesin-like cDNA. The technique utilized for obtaining a kinesin-like cDNA sequence was a derivation of the homology-based approach discussed earlier in this chapter. Degenerate primers derived from conserved regions of sequence within members of the kinesin-like motor domain family were used in a reverse transcription PCR reaction with pachytene spermatocyte mRNA in an attempt to amplify a putative mouse kinesin-like gene fragment. Although a kinesin-like gene sequence was not ultimately cloned from this work, the fragment originally derived from the PCR screen did share significant homology to members of the kinesin superfamily as determined by DNA sequencing. Thus, this procedure for amplifying DNA encoding a conserved domain

within a gene appears to be an effective method for identifying new members of conserved gene families.

It was both interesting and surprising to find that a member of the kinesin superfamily was not identified in subsequent screening since the PCR-derived probe was used in subsequent library screening. However, the PCR-derived probe was used to isolate a 1.3 kb fragment and was not directly used as a probe for the identification of the 3.5 kb fragment. The 1.3 kb fragment served as a probe to identify the 3.5 kb cDNA from the pachytene spermatocyte library. This might account for isolation of a sequence which is not related to the kinesin superfamily. Nevertheless, the novel cDNA isolated from the screening procedure is expressed during meiosis, in fact, primarily in the pachytene stage. This meiotically expressed sequence, which was named *Mes*, shares extensive homology with an EST from human brain cells but does not have homology to any known gene in the database and, therefore, is probably representative of a previously unidentified gene. The cDNA does, however, contain a leucine zipper motif, common to eukaryotic transcription factors, and therefore, potentially encodes a DNA-binding protein. In the future, experiments aimed at determining a putative function for this cDNA are warranted and should be designed to determine if this cDNA encodes a DNA-binding protein. DNA binding studies, such as DNAse footprinting or gel mobility shift assays (Kadonga *et al.*, 1987) would clearly yield information to this end. Likewise, site-directed mutagenesis of the leucine zipper to disrupt DNA binding could also be performed with purified *Mes* protein. Additionally, this putative transcription factor could be immunolocalized, presumably to the nucleus, following antibody production and purification. This cDNA could potentially be interesting with respect to the regulation of gene expression during meiosis and, at least, might serve useful as a molecular marker for the pachytene stage of meiosis.

Culturing of spermatocytes The *in vitro* culture of pachytene spermatocytes was shown to be valuable for experimental evaluation of cytogenetic aspects of meiosis, as described in Part V of this dissertation. Additional experiments designed to study the effects of various reagents on cultured spermatocytes could further our understanding of the meiotic cell cycle. Recently, experiments involving the addition of OA to the pachytene spermatocytes in this culture system have suggested that OA overrides a possible cell cycle check point that usually keeps pachytene spermatocytes in prophase (Wiltshire *et al.*, 1995). Intriguingly, in initial experiments involving the addition of OA to *in vitro* cultured leptotene/zygotene spermatocytes, the chromosomes in these cells do appear to condense but do not progress towards metaphase (Wiltshire and Handel, unpublished observations). This might indicate that the cells have not achieved nuclear competence for metaphase. These observations are exciting with regard to the examination of cellular events that are necessary for successful progression into metaphase, such as chromosome pairing and formation of chiasmata. In the future, it would be interesting to investigate whether the spermatocytes from the T38H and T16H translocation heterozygous males can also bypass this putative cell cycle check point when treated with OA. T16H mice proceed further through meiosis than normal leptotene/zygotene spermatocytes and thus may provide interesting information with respect to cellular competency for meiotic progression. For example, if the T16H spermatocytes can progress towards metaphase, what does this imply regarding meiotic arrest in these cells? It might indicate that a cell cycle check point beyond which leptotene/zygotene spermatocytes cannot proceed beyond was passed by the T16H spermatocytes, but for some other reason (another cell cycle check point, lack of important transcripts?) the cells are not able to finish normal progression through meiosis. If T16H spermatocytes are able to pass this particular check point and the leptotene/zygotene spermatocytes or T38H spermatocytes are not, it might be worthwhile to analyze some of

the clones identified in the mutant screening procedure (Part III) for a putative role in cell cycle progression. This might be accomplished by homology screening with sequences corresponding to genes like cyclins or kinases. The clones which might be candidates for genes of this type would probably be those clones isolated from the pachytene library which hybridize with a pachytene control probe (Table 1, Part III) and were identified as not being expressed in T38H testes but are expressed in T16H testes (Tables 3, 4, and 5, Part III). The previously performed screen identified 3 clones with these characteristics - clones 3, 30, and 31 (Table 4). It is unlikely that one of these clones is intimately involved in a cell cycle check point, as I did not screen to saturation (only 3, 000 pfu from pachytene cDNA library); however, it remains a possibility. A more comprehensive screen could be performed in order to identify sequences, such as these, which might be involved in this particular cell cycle check point, assuming that the T16H spermatocytes progress through prophase and into metaphase upon addition of OA.

Possible applications and novel methods for meiotic analysis

The cDNA libraries constructed in this work will continue to be a valuable resource for the study of molecular aspects of spermatogenesis, and in particular, for molecular analysis of meiosis. These libraries will be instrumental in the isolation of cDNAs and in studies of differential gene expression across the cell types they represent. Additionally, the meiotically expressed cDNA isolated, *Mes*, could also provide information on the meiotic process if it were further characterized and a function were assigned to it. Nevertheless, if given the opportunity to continue this line of investigation, among all available areas of further study, I would choose to pursue a project involving a combination of molecular analyses of the meiotic mouse mutants and cell culture experiments. These experiments, involved in determining if T16H spermatocytes can

proceed through prophase with OA addition, might allow for the identification of cDNAs representative of cell cycle progression. This investigation into the molecular mechanisms of cell cycle check points might represent a line of inquiry which could lead to intriguing insights into the meiotic process. Even if a putative cell cycle control gene was not identified in this study, potential causes, or factors which influence meiotic failure in the translocation mice could be elucidated. Additionally, a worst case scenario would involve the isolation of genes expressed meiotically which do not have a function in cell cycle check points. In this case, these genes would still likely be a welcome contribution to the literature on meiosis.

In addition to utilizing the reagents I have provided in these studies to investigate the molecular basis of meiosis, there are several techniques available for the identification of stage-specific cDNAs for which the cDNA libraries constructed (Part II) may be useful. These techniques include subtractive hybridization, differential display, and representational difference analysis. To date, these techniques have not been utilized to isolate meiotically expressed genes. There are probably several reasons for this, including technical difficulty, expense, and uncertain results. Since these methodologies are similar with respect to the resulting product, following a description of each I will briefly discuss strategies for collectively using these techniques for isolating meiotically expressed sequences.

Subtractive hybridization is a technique that can be utilized for enrichment of RNA sequences expressed in a specific cell population. This technique involves hybridization of single-stranded RNA from two similar cell types and subsequent selection for unhybridized single-stranded RNA representative of a unique nucleic acid pool. The single-stranded fraction usually contains RNA that is differentially expressed between the two cell populations. Enriched RNA of this type can then be used directly as a probe to screen a cDNA library or to create a subtracted library. Both alternatives ultimately allow for

enrichment of sequences expressed more abundantly in one cell type. Many laboratories have utilized this procedure with success. Rothstein *et al.* (1992) successfully employed subtractive hybridization for enrichment of clones expressed during the two-cell stage of mouse embryonic development - 14 novel mammalian genes were identified in this particular study. Furthermore, this technique has been used to isolate other specific transcripts, including scrapie-modulated mRNAs (Duguid *et al.*, 1988) and cortex-specific mRNAs (Travis and Sutcliffe, 1988).

A more recently developed technique, differential display (Liang and Pardee, 1992; Liang *et al.*, 1993) has also been successfully used in several studies to enrich for sequences expressed in specific cell populations. This technique involves hybridizing a series of statistically random primers to mRNA from at least two different cell types. Following subsequent PCR amplification, the PCR products are separated by polyacrylamide gel electrophoresis and examined for different hybridization patterns. Unique bands on gels may indicate differential expression of a specific gene. Furthermore, unique cDNAs can be isolated and utilized in future studies designed to discern the significance of these expression differences. Differential display, DD, has been reported to be successful in several studies, including the identification of a putative tumor suppressor gene in breast cancer cell lines (Sager *et al.*, 1993) and in the isolation of clones that are upregulated by the addition of glucose to cell culture cells (Nishio *et al.*, 1994). This technique might be utilized in an analysis of gene expression differences between leptotene/zygotene and pachytene spermatocytes and could be performed with the previously constructed cDNA libraries. However, differential display may not be appropriate for detecting differential expression of meiotically expressed genes since this technique is usually reported as successful for comparing expression differences between two cell types that are almost identical. An excess of differentially expressed bands, which

may result from an abundance of differential gene expression between leptotene/zygotene and pachytene spermatocytes, may prevent a reasonable analysis by this method.

Representational difference analysis (RDA) is a new technique employed for the isolation of enriched cDNAs. This technique involves both subtraction and PCR amplification (Hubank and Schatz, 1994). Whereas DD (Liang and Pardee, 1992) amplifies fragments from all mRNAs in a specific cell type, RDA eliminates fragments present in two libraries and only differentially expressed sequences remain. RDA is a modification of a procedure which was designed for the isolation of differences between two complex genomes (Lisitsyn *et al.*, 1993). In an initial paper describing the technique, upregulated cDNA clones were identified following the addition of caffeine to cell lines (Hubank and Schatz, 1994). RDA might be more successful than DD for identifying differences in cDNA clones between leptotene/zygotene and pachytene spermatocytes. The technique of RDA would eliminate fragments in common between mRNAs from the leptotene/zygotene and pachytene spermatocytes, thus decreasing the difficulty of interpreting results.

All three of these techniques could be utilized to isolate meiotically expressed sequences. Screening procedures utilizing these techniques could be adapted in an attempt to isolate sequences which may be involved in various stages of meiosis. For instance, in order to isolate sequences representative of the very earliest meiotic signals, one of these differential cloning procedures could be used to discern expression differences which exist between spermatogonia and leptotene/zygotene spermatocytes. Genes transcripts enriched at the stage of zygotene could be isolated by using leptotene/zygotene spermatocytes and prepuberal pachytene spermatocytes (the very earliest spermatocytes, which can be isolated from a STAPUT of 17 day old germ cells). If the identification of sequences from mid pachytene stages is desired, prepuberal and adult pachytene spermatocytes could be used in

such comparative isolations. If sequences from late pachytene are desired, a similar analysis could be performed using pachytene spermatocytes treated with OA and normal, adult pachytene spermatocytes.

Most of the approaches discussed in this dissertation are techniques which will ultimately, I hope, significantly contribute to the elucidation of the molecular basis of mammalian meiosis. At this point, however, all that the scientific community currently possesses are random gene sequences which do not fit together into any evident cascades and many have not even been assigned putative functions. As meiosis is a complex process comprised of numerous events, ultimately many genes will be directly implicated in this process which constitutes an excellent beginning for subsequent inquiry.

Conclusions

The studies described in this dissertation were designed to further our understanding of the molecular basis of meiosis through identification of gene sequences expressed meiotically. In order to determine successful strategies for the isolation of genes expressed meiotically during mouse spermatogenesis various approaches were undertaken. Through successful utilization of several different methodologies, meiotically enriched cDNAs have been identified. Upon further characterization, these cDNA sequences may prove to be a valuable resource for furthering our knowledge of the meiotic process and will be an important reagent for future studies into the molecular basis of meiosis. Likewise, the establishment of a cell culture system for pachytene spermatocytes will provide an important tool to study reagents and possible effectors of the meiotic process *in vitro*. In all, these combined studies represent a foundation from which we can begin to dissect the intricacies of meiotic cell division in mammals.

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