INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Genetic, molecular, and cytological characterization

of the paternal effect gene, *paternal loss,*

of *Drosophila melanogaster*

by

Kelly Noel Owens

A dissertation submitted in partial fulfillment
of the requirements for the degree of

Doctor of Philosophy

University of Washington

1996

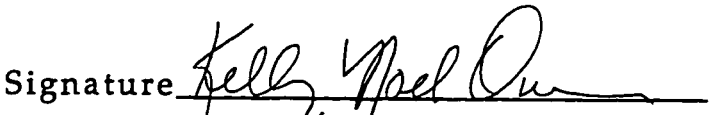
Approved by Barbara T. Wakimoto
Chairperson of Supervisory Committee

Program Authorized to
Offer Degree Department of Genetics

Date 12/13/96

Doctoral Dissertation

In presenting this dissertation in partial fulfillment of the requirements for the Doctoral degree at the University of Washington, I agree that the Library shall make its copies freely available for inspection. I further agree that extensive copying of this dissertation is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for copying or reproduction of this dissertation may be referred to University Microfilms, 1490 Eisenhower Place, P.O. Box 975, Ann Arbor, MI 48106, to whom the author has granted "the right to reproduce and sell (a) copies of the manuscript in microform and/or (b) printed copies of the manuscript made from microforms."

Signature 

Date 12/19/96

University of Washington

Abstract

Genetic, molecular and cytological characterization
of the paternal effect gene, *paternal loss*,
of *Drosophila melanogaster*

by Kelly Noel Owens

Chairperson of the Supervisory Committee
Associate Professor Barbara T. Wakimoto
Department of Zoology

Mutations in the paternal effect gene, *paternal loss (pal)* of *Drosophila melanogaster* cause loss of only the paternally inherited chromosomes during early embryogenesis. In an effort to better understand the role of the paternal contribution to embryogenesis, the genetic and molecular properties of mutations in the *pal* gene were characterized. Comparisons of the original pal^1 allele, a newly isolated dysgenic allele, pal^2, and deficiencies of the locus defined *pal* as a strict paternal effect gene. The weak penetrance of null mutations in the *pal* gene suggested that the *pal* function is partially redundant with other gene functions. The *pal* gene was cloned showing that the gene encodes a small mRNA (0.9 kb) expressed only in the male germline. Expression of the pal mRNA is localized in early primary spermatocytes in a polar perinuclear cap and is homogeneous in later spermatocytes. Sequence analysis of the *pal* cDNAs and genomic clones predicted that the encoded protein is a small highly basic protein. Immunolocalization studies showed that the protein is present in condensing, elongating spermatids and in mature sperm. Preliminary results suggested that the pal protein may enter the embryo in the sperm nucleus. I propose a model in which the pal protein is a sperm-specific chromosomal

protein required for the normal condensation of the sperm nuclei or for the decondensation of the sperm nuclei in the embryo. Loss of the pal protein may disrupt the exchange of sperm-specific chromosomal proteins for maternally provided proteins in the first few minutes of embryogenesis. Defects in this transition may result in late or improperly decondensed paternal chromosomes and their subsequent misbehavior. Progeny of *pal* fathers showed early embryonic defects in mitosis, including chromosomes off the metaphase plate, chromosomes lagging at anaphase and distorted spindles, as well as the presence of micronuclei prior to the first mitosis. These defects may arise in the absence of the pal protein due to an altered interaction of paternal chromosomes with cellular components such as the cytoskeleton or nuclear envelope.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGMENTS

As with any endeavor my work was done with the help and advice of numerous people. I thank my advisor Barbara Wakimoto for her support, advice, and patience throughout my graduate studies. In particular, I want to express my appreciation of the numerous late night conversations we shared about science and teaching and philosophy.

Thanks goes to my committee for their enthusiasm and critical input: Barbara Wakimoto, Breck Byers, Celeste Berg, Steve Hauschka, Lee Hartwell and Bill Moody. Thanks to Breck, Celeste and Barbara for all their great comments on the written part of my dissertation. I thank Steve Hauschka for his ability to listen at times when I needed to talk.

I have been privileged to work in the Wakimoto lab with a superb group of people: John Tomkiel, Glenn Yasuda, Karen Fitch, Kathleen Wilson, Dave Slade, Mark Hamilton, Karen Weiler, Mark Hearn and Mary Howe and numerous others. I've enjoyed our discussions, humor, poker games, and mutual enjoyment of food. Kathleen Wilson was invaluable help with the *pal* project, and I particularly appreciated her cheerful calm attitude. Thanks to Dave Slade for being willing to answer even the most annoying questions and rescuing me on the computer innumerable times. Mary Howe started about the same time as I did in the lab and was a wonderful friend throughout that time as well as a patient bridge teacher.

Much of the work I have done depended on help from a great variety of people in both the genetics and zoology departments. I thank Gerold Schubiger and the members of his lab for a myriad of advice and reagents and friendship. I would especially like to thank Jayne Baker for teaching me about

handling embryos and being willing to persuade me to work out in the mornings. I also thank the Wright lab, particularly Robin Wright and Steve Castillo, who taught me how to run protein gels and generously gave me access to their lab for the protein work I did. I thank the members of the Berg, Braun and Thomas labs for numerous discussions.

I have enjoyed the help and friendship of many of the students and staff in both departments and do not have time to name them all but thanks--particularly my classmates both the first and second groups (Jill Schumacher, Marty Lee, David Pritchard and Eyung Park, and Doreen Gillespie, Theresa Ward, Nick Kosuk, Dave Reiner, Jason, and Bryan Jensen). A special thanks to Jill Schumacher, the other "sperm gal," for words of much needed encouragement and advice starting the antibody experiments. Also, to Dave Pritchard who was always willing to debate philosophical issues at the drop of a hat. I greatly appreciated the camaraderie during the "night shift" and the advice on RNA in situs. I thank Franz Tax for much needed perspective on my work. Thanks to Ann Reynolds for her mentoring as a teacher and for being their to share our various endeavors and books. Also to George Riley and Tracey Lee for their friendship and advice at balancing life with science experiments and small children.

I thank my family my parents, grandparents, and brother, Jimmy, for their unflagging support even if they were not sure why I was still in school. And especially to my mom, Mary, for instilling a love of science and to my dad, Jim, for teaching me how to argue a point (perhaps to some people chagrin). Also to the youngest family member, my son Webb, who's provide my with a long term developmental genetic experiment and for bringing a great deal of joy and humor into my life.

Finally, I thank my husband, John, for being generally wonderful throughout my graduate and undergraduate careers. For understanding when we were living 3000 miles apart so I could be in grad school. For being positive even when I was grumpy. And for being willing to answer questions about protein chemistry at 5am. I could not have possible done this without his love and support.

CHAPTER 1

INTRODUCTION

Embryogenesis requires the fusion and coordination of two vastly different cells: the sperm and the egg. Gametes contribute components to the newly formed zygote, including chromosomes and structural and enzymatic molecules. Failure to properly package these components leads to developmental defects or death. Each gamete supplies different components, packaged in different manners. Inroads have been made in understanding the normal contribution of the egg by analysis of maternal effect mutations. Relatively little is known, however, about the unique contributions of the sperm. In an effort to understand better the role of the paternal contribution to embryogenesis, I have examined the role of a paternal effect gene of *Drosophila*, called *paternal loss (pal)*. I am interested in two aspects of the *pal* mutant phenotype. First, as a paternal effect mutation, *pal* provides a tool for understanding the role of the paternal gamete in embryogenesis. Secondly, as a mutation resulting in loss of paternal chromosomes in the embryo, it provides an opportunity to examine potential differences in the paternal and maternal chromosomes.

In this introduction, I will provide a general background about chromosome loss and parental effects and then focus on paternal effects in particular. Chapter 2 describes the isolation and characterization of new *paternal loss* alleles. Chapter 3 details the molecular characterization of this gene. Chapter 4 discusses production and use of antibodies to localize the *pal* protein. Suggestions for future research comprise Chapter 5.

The accurate transmission of chromosomes is essential for the survival of a cell. A failure to accurately transmit chromosomes creates aneuploidy (reviewed in Baker et al., 1987 and Smith, 1995). Headway has been made in describing components needed for chromosome transmission in yeast with the isolation of conditional mutations in genes involved in cell cycle progression and spindle maintenance (reviewed in Murray, 1995; Hyman and Sorger, 1995) Additionally, the definition of centromere sequences has allowed isolation of centromere binding proteins (Jiang et al., 1993; Strunnikov et al., 1995; Meluh and Koshland, 1995, Sorger et al., 1995). Some centromere binding proteins are likely to affect kinetochore function and therefore accuracy of chromosome transmission. Indeed, mutations of the *CBF1* centromere binding protein gene can cause chromosome instability (Cai and Davis, 1990). These findings may sketch out the general features of mitosis, but the simple mitotic divisions of the single-celled yeast do not reflect the diverse characteristics of mitotic divisions that are observed during development in multicellular eukaryotes.

Variation from the canonical mitotic division is readily apparent in *Drosophila* where divisions are differentially regulated during development. For example, early embryonic divisions occur rapidly without G phases and in a synchronous manner. Later in the larva, divisions are asynchronous and show different features. Some larval cells undergo endoreduplication to form polyploid or polytene tissue, while other larval tissue remains diploid undergoing a more classical mitosis (reviewed in Foe et al. 1993, Edgar, 1995; Reed, 1995). Geneticists have identified molecules important for regulation of the larval mitoses through the study of a class of mutants that die as late larval lethals (reviewed in Gatti and Goldberg, 1991; Edgar, 1995).

What are the molecules regulating or regulated in different types of division? One predicts that this regulation may involve the differential use of components such as actin or tubulin that are required for all divisions, or specialized molecules used only for a particular subtype of mitoses. Understanding the role of molecules differentially used in different division types or during transitions between types of division may lead to a better understanding of the regulation of cell division.

There are several indications that the initial embryonic mitoses may be unique. The transition in the type of division from meiotic division, occurring in the parent, to the first mitotic divisions in the embryo is enormous. In many sexual organisms, the gametes created during spermatogenesis and oogenesis differ vastly from one another in terms of morphology and packaging of organelles. In the embryo, however, one cell must form fusing and coordinating the cellular regulation of these gametes which had existed as separate cells bearing different material in different states. The initial differences in the cellular components contributed by each parent (particularly the nuclear material) suggests that distinct roles may exist for the maternal and paternal contributions within the ensuing embryo. A mutation affecting one parent's role in mitosis would be observed by a parental effect phenotype affecting division.

While traditional Mendelian genetics describes the equivalent transmission of genetic information regardless of its parental origin, some studies reveal this is not always the case. Distinctions between how paternal and maternal chromosomes are handled have been revealed in a variety of organisms. Differential control of paternal and maternal chromosomes or differential expression of alleles of different parental origin are phenomena

often termed imprinting. Though defined in many ways, one of the most historically accurate and inclusive definitions has been provided by Denise Barlow, who designates imprinting as "a reversible process whereby a gametic-specific modification in the parental generation can sometimes lead to functional differences between maternal and paternal genomes in diploid cells of offspring" (Barlow, 1994). These phenomena are of interest because they directly pertain to the transmission of information between generations. The process of chromosome elimination or inactivation observed in *Sciara* and the coccid insects demonstrates that chromosome loss is a normal part of development for some organisms. Moreover, these examples and that of X-inactivation in marsupials and mammals illustrate that differential control of chromosomes dependent on parental origin also occurs as a part of normal development.

Studies by Metz on *Sciara* in the 1930's (reviewed in Brown and Chandra, 1977) provide the first evidence for chromosomal imprinting. Metz and subsequent researchers found that *Sciara* males transmit only their maternal chromosomes to progeny (Metz, 1938, Hughes-Schrader, 1948). It was shown that the entire paternal set is eliminated during male meiosis. This results from the formation at meiosis I of a spindle that appears monopolar. Maternally derived chromosomes move precociously to the single pole to form the nucleus of a gamete containing two sister X chromatids and a set of autosomes, all maternal in origin.

There is also chromosome elimination somatically in cleavage stage *Sciara* embryos. All progeny begin development with a set of autosomes from each parent, one maternal X chromosome and two paternal X chromosomes. During early embryogenesis one paternal X chromosome is

eliminated from the germline of both sexes and the soma of females. In the soma of males both paternal X chromosomes are eliminated. This process results somatically in XO males and XX females. Elimination evidently results from a process in which the paternal X chromosome(s) lag at anaphase during cleavage divisions and is eliminated by extrusion through the nuclear membrane (Berry, 1939,1941). The elimination of paternal chromosomes in the same cell in which maternal chromosomes persist indicates the presence of a mechanism for distinguishing maternal and paternal genomes. Crouse (1960) coined the term imprinting to describe this process by which a molecular event or set of events determine if a chromosome will later behave differently from a homologous chromosome in the same nucleus, such that the two are no longer functionally homologous.

A similar phenomena is observed in coccid insects, which are sedentary plant parasites (reviewed in Brown and Chandra, 1977). Some coccid species eliminate chromosomes in early embryogenesis, much like *Sciara* (Brown and Bennett, 1957; Brown and Wiegmann, 1969). However, other species do not eliminate chromosomes; instead, the paternal set of chromosomes becomes heterochromatic and is rendered genetically inactive (Schrader, 1929; Brown and Nelson-Rees, 1961). During spermatogenesis in these species, the heterochromatic paternal chromosomes do not contribute to sperm, paternal chromosomes being eliminated either in preprophase of meiosis or after the completion of meiosis. Thus meiosis in these coccids produces just two gametes; both of which are derived from euchromatic maternal chromosomes (Brown, 1957 and 1963).

Chromosome imprinting has been observed in situations in which nature has, in essence, created a perturbation in one organism with a second

organism. In *Nasonia vitripennis*, a parasitic wasp, either the presence of the supernumerary chromosome called *paternal sex ratio*, or *psr*, or infection by the bacterium *Wolbachia* (in certain incompatible strains) can disrupt chromosome condensation and cause loss of all paternal chromosomes at the first mitosis (Breeumer and Werren, 1993; Reed and Werren, 1995). Clearly paternal chromosomes must be different from the unaffected maternal chromosomes in the same cytoplasm.

Differential control of chromosomes based on parental origin is not restricted to insects. Mammalian X-inactivation reveals the effect of parental origin (reviewed in Monk and Grant, 1990). Somatic X chromosome inactivation is random in placental mammals, but the paternal X chromosome is preferentially inactivated in extraembryonic membranes (specifically the yolk sac, trophectoderm, and chorion, Takagi and Sasaki, 1975, 1976; West et al., 1977; Harper et al., 1982). The inactivation of the paternal X occurs in approximately the 3.5 day mouse embryo, demonstrating that there is some method of distinguishing or imprinting the paternal versus maternal chromosomes after multiple rounds of division. In marsupials, the paternal X chromosome is inactivated in somatic tissue of the embryo as well as extraembryonic tissue (Cooper et al., 1971; Richardson et al., 1971;Vandeberg et al., 1987). From these observations, researchers (Vandeberg et al., 1987) have suggested that paternal X inactivation prevailed prior to random X inactivation in mammalian evolution. Studies by Amar Klar (1987, 1993) in the fission yeast, *S. pombe*, show that switching of the MAT locus is similarly affected by parental chromosome origin (a or α). This may indicate that the ability to distinguish parental chromosome origin occurred quite early in evolution.

In mammals, mounting data supports the idea that imprinting distinguishes not just the origin of the X-chromosome but also portions of many or all other maternal and paternal chromosomes (reviewed in Peterson and Sapienza, 1993; Barlow, 1994). Pronuclear transplantation experiments demonstrated the requirement in mouse of paternally and maternally derived genomes for normal development (Barton et al., 1984; McGrath and Solter, 1984, 1985; Surani et al., 1984, 1986). Production of Robertsonian translocation or reciprocal translocation to produce uniparental disomic individuals has allowed mapping of particular regions required paternally or maternally (Cattanach and Kirk, 1985; Searle and Beechey, 1990). For some regions in the mouse, both paternal and maternal versions are required, and reciprocal phenotypes can be observed. Parent-of-origin effects are observed in the manifestation of several human diseases including Huntington's chorea (Ridley et al., 1991) Prader-Willi and Angelman syndromes (Nicholls et al., 1989), and some carcinomas (reviewed in Hall, 1990; Clarke, 1990). At least nine genes exhibiting imprinting have been identified: H19 (Bartolomei et al., 1991), Igf2 (DeChiara et al., 1991), Ins1 and Ins2 (Giddings et al., 1994), SnRPN (Leff et al., 1992), Xist (Barlow et al., 1991), Wt1 (Jinno et al., 1994), SP2 (Hatada et al., 1993), Igf2r (Barlow et al., 1992). Correlation of gene expression and methylation state have been made for these loci, though particular methylation patterns vary greatly. Methylation in the mouse arises after fertilization and nuclear fusion and, in some cases, methylation is not observed until after the blastula stage (Brandeis et al., 1993). Clearly, while methylation may be important for determining gene expression, the primary imprint seems likely to precede this secondary effect. Although particular genes have been found to be imprinted on the same chromosome amid

others which are not imprinted, it is not necessary to consider this a gene specific phenomena. Distinguishable differences between maternal and paternal chromosomes must be maintained in order for embryos to retain their viability. Therefore, while chromosomes may be distinguished according to parental origin, only those genes capable of responding to such imprinting would show expression dependent on parental origin.

There is evidence for imprinting in *Drosophila*, despite the lack of methylation (Smith and Thomas, 1981), thereby providing additional evidence that methylation might not be the primary imprint in mammals. Parental effects have been observed in connection with position effect variegation (PEV) (Spofford, 1959, 1961; Hessler, 1961; Dorn, 1985). Expression of variegating alleles of the *white* gene vary depending on whether the rearrangement carrying the gene is transmitted maternally or paternally. Since the *white* gene is not expressed until eye disc differentiation in the late pupal stage, these parental effects are believed to reflect a stable chromosomal imprint that can be transmitted over multiple cell divisions. Similar effects on variegated gene expression are observed with certain rearrangements of the Bithorax-Complex (Kuhn and Packert, 1988), with insertions of *rosy*$^+$ on the Y chromosome (Berg and Spradling, 1991), and with chromosomes affected by the mutation *E(var)3-93D* (Reuter et al., 1993).

Although different organisms may use different mechanisms to maintain a parental imprint, the initial distinction between parental genomes may be determined in a similar manner. In other words, outcomes of imprinting differ (differential gene expression, chromosome elimination or heterochromatinization) but all imprinting may well reflect a common mechanism for distinguishing between parental genomes.

In order to learn more about how parental genomes are distinguished, it seems fruitful to focus on this process in one organism. As noted previously, studies of *Drosophila* show evidence of distinctions between parental genomes. At this point, I will focus on certain mutations in *Drosophila melanogaster* in which chromosome maintenance is affected in a parentally specific manner. Because there is no zygotic transcription initially in *Drosophila*, the process of early embryogenesis is dependent on parental stores. Because of this feature it has been possible to isolate numerous parental effect mutations perturbing development in the early embryo. For this discussion, I will limit descriptions to those parental effect mutations involved in embryonic loss of chromosomes, although some have additional defects in meiosis. As described previously, a parental effect mutation produces defects in progeny of affected parents and often no observable defect in the parents. For example, *nonclaret disjunctional (ncd), mitotic loss inducer (mit)* and *paternal loss (pal)* are all parental effect mutations leading to chromosome loss among progeny of either homozygous mutant female or male parents (i.e. not both sets of parents). *ncd* and *mit* have effects solely when mothers are mutant. In contrast *Horka* is both a paternal effect and maternal effect mutation. All three can affect both maternal and paternal chromosomes, though to markedly different extents. Two points are revealed by the phenotype of these mutations: 1) paternal and maternal chromosomes are distinct from one another in the early embryo and 2) there are sequential changes in the initial embryonic mitoses. These points are discussed below.

The maternal effect mutation *nonclaret disjunctional*, or *ncd*, causes nondisjunction in female meiosis and chromosome loss in early embryogenesis (Davis, 1969). Although both maternal and paternal

chromosomes can be lost, maternal chromosomes are lost ten times more frequently. The majority of maternal chromosomes are lost during the first mitotic division of embryogenesis (Sequeria et al. 1989). Paternal chromosomes are lost after the division with the majority of losses occurring in the second and third divisions (Portin, 1978; Nelson and Szauter, 1992). This distinction between time of loss of maternal and paternal chromosomes suggests a functional distinction between the first and second division, which is reinforced by the observation that only the first division utilizes a gonomeric spindle (a single spindle in which paternally and maternally derived chromosomes do not intermingle).

Sequence analysis of the *ncd* gene revealed that it encodes a kinesin-like motor protein (Endow, 1990; McDonald and Goldstein, 1990). Subsequent *in vitro* assays demonstrated that the NCD protein displays a minus-end directed motor activity as well as a microtubule bundling activity (Walker et al., 1990; McDonald et al., 1990). Matthies et al. (1996) observed the NCD protein on meiotic spindle fibers in wildtype oocytes. In *ncd* mutants, multipolar, frayed spindles were observed, leading the authors to propose that the function of NCD is to bundle microtubules into a bipolar spindle, as had been suggested by *in vitro* experiments (McDonald et al., 1990). The question of what NCD is doing in early cleavage divisions remains open. Matthies et al. (1996) note that meiotic defects are much more severe than mitotic defects in progeny derived from *ncd* mothers and propose that in mitosis is NCD is assisted by another redundant function, that also acts in the microtubule organizing system. While this may explain the differing severity of the meiotic and mitotic defects, it fails to address Szauter and Nelson's observation that paternal chromosomes are not lost in the first mitotic

division but can be lost in second and later divisions. If NCD acts only to bundle the mitotic spindle, why aren't all chromosomes equally affected? Perhaps the later loss of paternal chromosomes in *ncd* mutants reflects a difference between maternal and paternal chromosomes in their ability to bind microtubules during the first mitosis, rather than a difference in NCD function.

Mutations in the kinesin-like motor protein gene *nod* (*no distributive disjunction*) shows meiotic defects similar to those exhibited by mutations in the *ncd* gene, except that only non-exchange chromosomes are affected. Though null mutations have no effect in early embryos, *nod* transcript is present throughout development. Furthermore a dominant mutation of *nod*, nod^{DTW}, causes severe defects in mitotic chromosome segregation, apparently from formation of anaphase bridges between homologues (Rasooly et al., 1991; Gatti and Baker, 1989) in both mutant females and males. Rasooly et al. (1991) suggest that nod^{DTW} acts as a poison product that disrupts other kinesin-like protein functions. The function of *nod* itself, as defined by the null mutations, seems to be restricted to female meiosis. These observations support the idea that different but related molecules may be used in different divisions (meiosis or mitosis at different developmental stages).

The *Horka* gene is defined genetically by a single, dominant, gain of function allele that produces meiotic and mitotic defects when carried by either parent (Erdelyi and Szabad, 1989). *Horka* mutant males show equational nondisjunction during meiosis. Cytological analysis reveals abnormal condensation and arrangement of chromosomes on the metaphase plate, chromosomes lagging at anaphase, and an uneven distribution of

chromatin at telophase (Szabad, 1995). No micronuclei (often indicative of chromosome loss, Wald, 1936; Zalokar et al., 1980) are observed, consistent with the genetic interpretation that only nondisjunction, but not chromosome loss, occurs during meiosis of *Horka* males.

As a paternal effect mutation, *Horka* affects only paternal chromosomes in mitosis, whereas *ncd*, a maternal effect mutation, influences predominantly maternal chromosomes in mitosis. Paternal chromosomes are lost, with the exception of the Y chromosome in the progeny of *Horka* mutant males. Cytological studies of the *Horka* embryonic defect reveal chromosomes lagging at anaphase. The failure to observe loss of maternal chromosomes further supports the idea that paternal and maternal chromosomes are distinct. One could argue that maternal chromosomes are less sensitive to loss than paternal chromosomes. When *Horka* is present in mothers, however, sterility results due to meiotic defects and arrest of progeny at cleavage stage. This observation suggests that maternal chromosomes are as sensitive, if not more sensitive, to this neomorphic product. (It is unreported whether both paternal and maternal chromosomes are affected in arrested embryos or only maternal chromosomes.) Szabad et al. (1995) propose that *Horka* functions at or near the centromere of chromosomes in a parentally specific fashion.

In contrast to *Horka* and *ncd*, the maternal effect mutation, *mitotic loss inducer* , causes loss of paternal and maternal chromosomes at equal rates. No meiotic defects have been observed with this mutation. Time of chromosome loss is estimated to occur in the third or fourth embryonic division by measurements of gynandromorph patch size (Gelbart, 1974). The observation that defects are seen primarily after the first two divisions

suggests that the first and second mitotic divisions are in some way different from the subsequent divisions. (Alternatively, if the mutation is hypomorphic, the protein may be diluted as divisions occur until a sub-threshold level is reached at the third or fourth division.)

While numerous maternal effect mutations exist, there are only a few reported cases of mutations exhibiting a strict paternal effect. Presumably the discrepancy in number of paternal versus maternal effect mutations reflects both the relative contribution of each parent to the embryo and the biased attention paid by researchers. While screens for male sterile mutations have identified paternal effect mutants, those producing motile sperm able to fertilize embryos have been largely ignored in favor of analysis of mutants affecting spermatogenesis *per se* (Lifshytz and Meyer, 1977, Lindsley and Tokuyasu, 1980, and Hackstein, 1991).

In *Drosophila*, three paternal effect mutations have been isolated: *ms(3)K81* (Fuyama, 1984, 1986a,b), sneaky (*snky*, personal communication, K. Fitch and B. Wakimoto), and *paternal loss* (*pal*, Sandler, 1974; Baker, 1975). *pal* and *ms(3)K81* were identified fortuitously in screens of meiotic mutants and natural populations, respectively. On the other hand, *snky* was identified in a screen for paternal effect mutations among P-element induced male sterile mutations. Outside of *Drosophila*, the only isolated paternal effect mutation is *spe-11* in *Caenorhabditis elegans* (Browning and Strome, 1996).

spe-11 was identified in a screen for male sterile mutations in *C. elegans* (Hill et al., 1989). The *spe-11* defects are first visible at fertilization. The pleiotropic effects of *spe-11* include a failure to form a normal eggshell or to complete meiosis, as well as failure to orient mitotic spindles or complete cytokinesis (Strome and Browning, 1996). These are features characteristic of

an unfertilized egg. The ability of maternally expressed *spe-11* to rescue the embryonic defects places the time of function in the early embryo (as opposed to during spermatogenesis). Browning and Strome (1996) favor the role of *spe-11* as an activation signal indicating the beginning of development.

Of the paternal effect mutations in *Drosophila*, the *snky* mutation seems to cause the earliest defects. Progeny of *snky* fathers show no apparent development after sperm entry into the egg. Egg activation, which is normally triggered by ovulation in *Drosophila*, is coincident with fertilization when fertilization occurs. Except for sperm entry, *snky*-derived embryos behave as if they were unfertilized eggs.

The earliest defects observed with the other two identified paternal effect mutations of *Drosophila*, *pal* and *ms(3)K81*, occur later than the *snky* defect. In *ms(3)K81* derived progeny, defects include incomplete pronuclear migration and anaphase bridging as early as the first mitotic division. Two classes of progeny are observed: 90% of animals arrest in early nuclear cycles (prior to cycle 6), and 10% of animals develop as haploids, forming cuticle but failing to hatch as determined by DNA staining and cuticular phenotypes (Fuyama, 1984; Yasuda et al., 1995). Genetic analysis of multiple alleles demonstrate genetically that *ms(3)81* is a null mutation resulting in a strict paternal effect (Yasuda et al., 1995). Yasuda et al. conclude that *ms(3)K81* is essential for participation of the male pronucleus in embryonic development. Furthermore, they suggest that the haploid phenotype represents a more normal course of development when the male pronucleus fails to participate in division, perhaps by failing to migrate properly. When the male pronucleus does participate in the remaining 90% of embryos, the authors suggest it acts as a poison leading to aberrant early nuclear cycles.

Like *ms(3) K81*, the initial *pal* allele, *pal*^{1}, causes only a paternal effect (Baker, 1975); no defects occur in progeny of *pal*^{1} mothers. Embryos produced from *pal*^{1} fathers can lose paternal chromosomes, but no maternal chromosome loss occurs. Unlike *ms(3)K81* or *snky*, *pal*^{1} exhibits incomplete penetrance; many progeny develop normally. Baker (1975) estimated the time of chromosome loss as beginning between the first and second mitotic divisions. (A more extensive discussion of the *pal*^{1} phenotype follows in Chapter 2.)

Understanding the role of parental effect genes may shed light on the requirements for chromosome maintenance and, in particular, the differences required to coordinate the parental contributions in early divisions in *Drosophila*. The differential effect of these parental effect mutations on paternal and maternal chromosomes and their action within a specific window of time provides evidence for a sequential change in chromosome maintenance in the initial divisions. This difference may reflect a continuing refinement of the cell cycle in these initial divisions. During the first division, *snky*, *pal* and *ms(3)K81* affect only paternal chromosomes while *ncd* affects only maternal chromosomes. By the second division, *ncd* can cause loss of paternal chromosomes as well as maternal chromosomes. *Horka* is capable of affecting either maternal or paternal chromosomes but only affects chromosomes derived from the parent bearing the mutation. By the third and fourth divisions, *mit* can affect both paternal and maternal chromosomes (as could *ncd* by the second division). Although it is not clear how late these mutations may act, estimates of time of loss suggest that by the third and fourth divisions *pal*, *Horka* and *ncd* are having progressively decreased effects on chromosome maintenance. Furthermore,

these decreases in sensitivity may reflect a remodeling of paternal and maternal chromosomes to more closely resemble one another. The position effect variegation studies mentioned earlier, however, require that some distinction between parentally-derived chromosomes is maintained until late in development to account for the parental effects observed with variegating rearrangements.

The purpose of my dissertation work is to define better the role of the *pal* gene product. More specifically, I have sought to answer the following questions: What is the function of the *pal* gene? When and where is the product needed? With what components of the cell does the product interact? Why are only paternal chromosomes affected?

I have isolated a new *pal* allele in order to understand the range of phenotypes possible, to provide tools for cloning the gene, and to provide further information about the function of the gene product. Here I report genetic and molecular studies of a new mutant allele, pal^2 and deficiencies of the locus. This allele, designated pal^2 , and pal^- deficiencies have been useful for genetic (Chapter 2) and molecular analyses (Chapter 3) of the gene and for interpreting the nature of the original EMS-induced allele. I have examined the localization of the *pal* protein by immunocytochemistry (Chapter 4) and discuss models of how *pal* may function.

CHAPTER 2

Genetic Characterization of the *pal* gene

The *paternal loss*^{1}*(pal*$^{1)}$ allele was originally isolated by Larry Sandler (1971) in a screen for EMS-induced meiotic mutations affecting disjunction or recombination. The designation of *pal*^{1} (originally *mei-W5*) as a meiotic mutation was based upon the recovery of nullosomic progeny lacking a paternal chromosome. Characterization of the *pal*^{1} allele by B. Baker in 1972 determined that exceptional progeny arise from loss of entire chromosomes, not portions of chromosomes, by several assays for chromosome breakage.

Baker (1975) further characterized the phenotype of this mutation in a series of elegant analyses. He demonstrated that paternal chromosome loss is not caused by nondisjunction, since diplo-exceptional and nullo-exceptional progeny were not recovered with equal frequency. Instead, nullo-exceptional progeny were recovered at much higher rates (19.8% versus 2.4%).

Because homozygous mutant fathers produce nullosomic and mosaic progeny which have lost only paternal chromosomes, Baker designated the gene *paternal loss* or *pal*. Nullosomic progeny presumably arise from loss at or before the first division while mosaic progeny arise from loss during later divisions. The time of chromosome loss, based on patch size in mosaics, was estimated to be predominantly at or before the first division for the X-chromosome and at first or second division for the Y-chromosome. Baker (1975) interpreted the observation of nullosomic and mosaic progeny as resulting from a combination of meiotic and mitotic loss, due to disruption of the normal meiotic process. Thus, he considered *pal* to be a meiotic mutation. However, this designation may be a misnomer. If a chromosome

was lost prior to the completion of meiosis, it could be incorporated into a gamete with its homologue or sister chromatid to produce a diplo-exceptional gamete. Since nondisjunction is not observed above background levels in progeny of *pal* fathers (as evidenced by the low occurrence of diplo-exceptional progeny of *pal*^{1} males), any spermatogenic loss must occur after separation of chromosomes and the completion of meiosis. Hence, the nullosomic and mosaic progeny could arise solely by mitotic loss at the first or subsequent divisions. Therefore, it seems more appropriate to call *pal* a mitotic mutation.

Baker did not observe loss events in the soma of homozygous mutant individuals or in homozygous mutant progeny of heterozygous parents, suggesting that *pal*$^{+}$ activity is not required zygotically. Consistent with this observation, both *pal*$^{+}$ and *pal* embryos derived from *pal* homozygous fathers demonstrate paternal chromosome loss. The *pal* mutation has no observable maternal effect. In summary, the function of the *pal* gene is affected in male parents, altering the normal progression of early embryogenesis in their offspring.

Baker noted that chromosomes differ in susceptibility to *pal*-induced chromosome loss. Loss of the small chromosome 4 occurs most frequently, with lower rates observed for the X chromosome and Y chromosome. Additionally, the rate of loss of a particular chromosome can vary with different genetic backgrounds. Baker used the different susceptibilities of two X chromosomes to map the sensitive region of the X chromosome to the base of the X, proximal to the *carnation* locus. Although Baker favored the idea that the sensitive region was the kinetochore itself , his data did not allow him to map the region more closely than the proximal 50% of the X

chromosome, which includes proximal euchromatin and heterochromatin. Tomkiel (1991) examined the effect of heterochromatic deficiencies on *pal*-induced chromosome loss to determine if the amount of heterochromatin altered the sensitivity to the *pal* function. Deficiencies of heterochromatin correspond with increases in the rate of chromosome loss in *cis*, suggesting that *pal* sensitivity maps throughout the Y chromosome.

All previous work on the *pal* gene used the single EMS- induced mutation pal^1 isolated by Sandler, for which no parental control chromosome is available. Thus, it was not clear if pal^1 is a null allele. In Baker's studies approximately 75% of progeny show no evidence of paternal chromosome loss based on cuticular phenotypes. The weak penetrance of pal^1 suggested that it could be a hypomorphic allele. In the experiments described below, I sought to clarify the null phenotype and the nature of the original *pal* allele and to obtain material useful for cloning the *pal* gene.

Baker mapped pal^1 (1975) to cytogenetic regions 28C-D or 29F-30F of chromosome 2 based on segmental aneuploidy data. To localize the gene more precisely, I tested newly generated deficiencies in the 30A-C region (Lane and Kalderon, 1993) for their ability to complement pal^1. In addition, a new *pal* mutation was generated by *P* transposition mutagenesis. Genetic characterization of the new allele, pal^2, and pal^- deficiencies, indicate that both pal^1 and pal^2 are likely to be null alleles. Thus, the low penetrance of the chromosome loss phenotype may reflect the existence of other factors that can partially compensate for the lack of *pal* function. Additionally, the lack of effect in *pal* mothers indicates that there is no requirement maternally; *pal* function is only required paternally. I will discuss a model to incorporate these observations with previous data.

Materials and Methods

Drosophila stocks

Cultures were raised on standard cornmeal-molasses-brewer's yeast media at 25°C. Except when noted otherwise, mutations are described by Lindsley and Zimm (1992) or Lane and Kalderon (1993). The wildtype strain used was a CantonS isochromosome 2 strain.

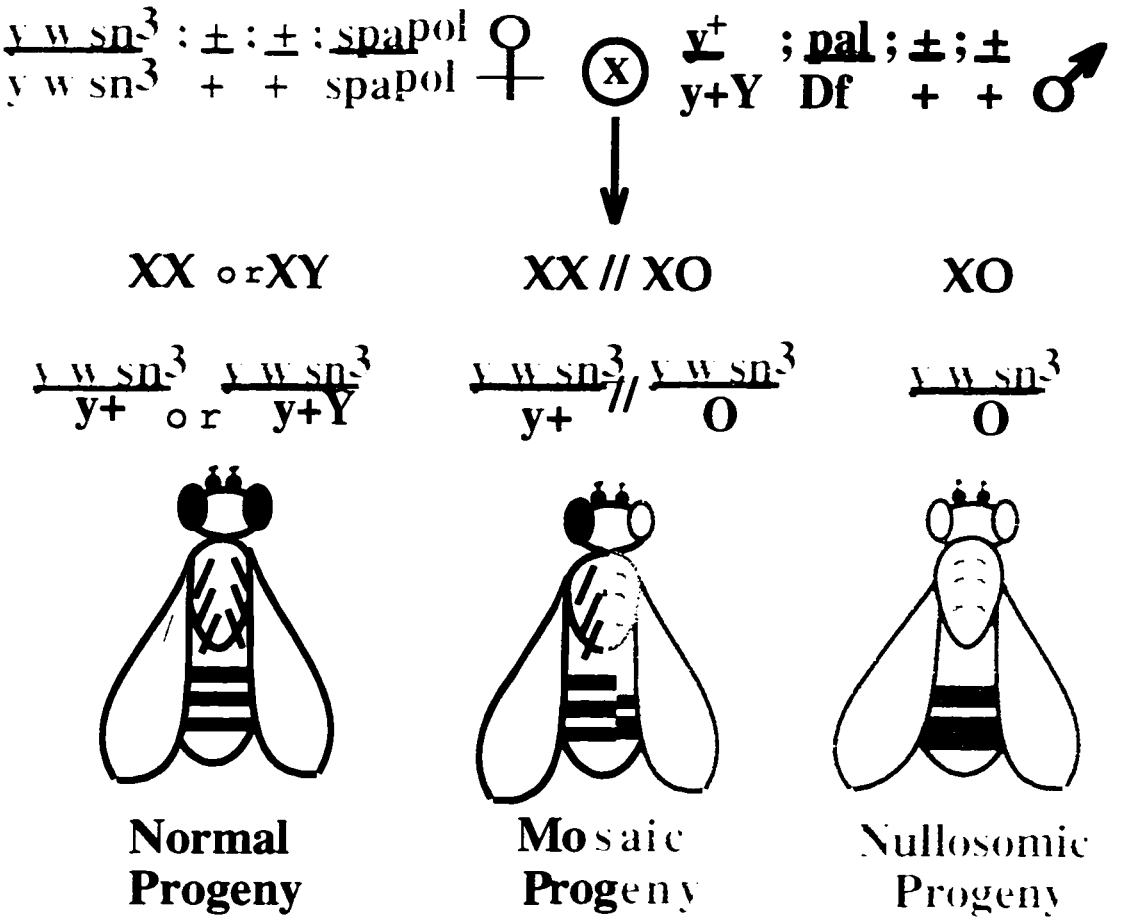
Complementation assay for paternal chromosome loss

To test deficiency chromosomes for the ability to complement the pal^1 mutation, ten to thirty pairs of $y^+Y;Df(2L)/pal^1$ males and $y\ w\ sn^3;\ spa^{pol}$ females were mated in half-pint milk bottles, subcultured after 5 days and parents were discarded after 5 more days. Progeny were scored until day 18 for paternal chromosome loss events. (Fig. 2.1). Loss of the paternal X chromosome is indicated by the presence of tissue expressing the recessive *yellow* and *singed* maternal markers. Loss of the paternal chromosome 4 results in expression of the *sparkling* eye marker or the *Minute* bristle phenotype. The rate of paternal chromosome loss is expressed as a percentage of exceptional progeny among total progeny scored.

Screen for new pal *alleles*

To induce new *pal* alleles, the $P[(w^+, ry^+)A]$ N22 chromosome (Hazelrigg and Levis, 1985), which carries a P element in 30C marked with $white^+$ and $rosy^+$ genes, was exposed to $P[ry^+, \Delta 2\text{-}3]$, an element that encodes P element transposase (Robertson et al. 1988). $w^{1118};\ P[(w^+, ry^+)A]$ N22; ry^{506} females were crossed to $w^{1118};\ Sp/CyO;ry^{506}\ Sb\ P[ry^+, \Delta 2\text{-}3]/TM6,\ Ubx$ males to generate $w^{1118};\ P[(w^+, ry^+)A]$ N22/*Sp* or *CyO*; +/ $ry^{506}Sb\ P[ry^+, \Delta 2\text{-}3]$ sons. Random N22 chromosomes were recovered from these males and established in balanced stocks. Males from each stock of the mutagenized chromosomes

Figure 2.1. Schematic representation of assay for paternal chromosome loss. Deficiencies were tested for the ability to complement the *pal* ^{1} mutation in fathers by crossing such males to females bearing recessively marked X and 4th chromosomes and scoring their progeny for the appearance of tissue showing the recessive phenotypes (yellow pigmentation, white sparkling eyes). The classes of progeny observed when determining the rate loss of the X chromosome are illustrated.


y w sn3 : ± : ± : spapol
y w sn3 + + spapol
y+ ; pal ; ± ; ±
y+Y Df + +
XX or XY
XX // XO
XO
y w sn3 / y+
or
y w sn3 / y+Y
y w sn3 / y+
//
y w sn3 / O
y w sn3 / O
Normal Progeny
Mosaic Progeny
Nullosomic Progeny

(N22*) were mated to *pal*^{1}/*SM5, Cy* females to obtain N22*/*pal*^{1} males. These males were mated to *y w sn*^{3}*; spa* ^{pol} females and at least 200 of their progeny were scored for paternal chromosome loss.

To determine if the *pal*^{2} allele was revertible by P element mobilization, the *pal*^{2} chromosome was exposed to P element transposase for mobilization. *w*^{1118}*; pal*^{2} *P[(w*$^{+}$*, ry* $^{-}$*)]/Sp or CyO; +/ry* ^{506}*Sb P[ry*$^{+}$*, Δ2-3]* males were generated and then crossed individually to *w*^{1118}*; Sco/SM1, Cy lt* females. From this cross, *w*^{1118}*; pal*^{2} *P[(w*$^{-}$*, ry* $^{-}$*)]/Sco or SM1, Cy lt* sons were selected and backcrossed to establish balanced stocks. Males from each stock were tested for paternal chromosome loss among their progeny as described previously.

Lethal phase determination

To assess lethality during development, survival of progeny derived from various *pal* alleles was determined. For each cross, forty mutant or wildtype males were crossed to forty *y w sn*^{3}*; spa*^{pol} females in half-pint bottles at 25°C with daily changes of standard food supplemented with yeast paste. For egg collections, flies were placed in empty half-pint bottles with 1.0% agar plates sprinkled with dry yeast and 45% acetic acid. Eggs were collected for 5-12 hour intervals, counted, and transferred to standard fly food in split bottles. After 36 hours from the mid-point of the collection, egg hatching rates were determined. Surviving progeny were allowed to develop to adulthood and counted until day 18. Paternal chromosome loss was scored as previously described.

Results

The *paternal loss* (pal^1) mutation was mapped by Baker (1975) to Chromosome 2, within the 28C-D or 29F-30F interval on the polytene chromosome map. To localize the *pal* gene more precisely, two approaches were used. In one approach, newly generated deficiencies in the 30A-C region were tested for their ability to complement pal^1. In the other, a new *pal* mutation was generated by *P* transposase mutagenesis. Using the pal^- deficiencies and the new allele, designated pal^2 , it was possible to genetically and molecularly map the gene, as well as to interpret the nature of the original EMS-induced allele for which no parental chromosome was available.

A set of deficiencies for the 30A-C region was generated by Lane and Kalderon (1993) in their study of *DCO*, a gene encoding the catalytic subunit of the Drosophila cAMP-dependent protein kinase. To assay for paternal chromosome loss, y^+ Y; pal^1/Df males were crossed to females carrying recessive markers, and their progeny were monitored for expression of maternal markers (Fig. 2.1). Two types of exceptional progeny could be identified: nullo exceptional progeny in which all scoreable tissue showed loss of a paternal chromosome and mosaic exceptional progeny in which animals have patches of tissue with paternal chromosome loss. Nullo exceptional progeny could also result from meiotic nondisjunction, which occurred at a rate of less than 0.75% for the sex chromosomes and 4th chromosome in control pal^1/+ males (Baker, 1975). A chromosomal deficiency was considered to fail to complement if pal^1/Df males produced nullo exceptional progeny at a rate above the 0.75% background level seen with the pal^1 chromosome.

Complementation results showed that the deficiency, *Df(2L) 30A-C*, fails to complement *pal*^{1} (Table 2.1). I also tested overlapping deficiencies generated by Lane and Kalderon (1993) by gamma irradiation of TE16 [*w*^{a} *rst*$^+$], a chromosome that carries a large transposable element of X chromosomal material inserted into 30C. TE16 and its derivatives, *Df(2L)γ4*, *Df(2L)γ29* and *Df(2L)γ31* and the deficiencies *Df(2L) 50*, *Df(2L) 200* and *Df (2L)A4* complement *pal*^{1} (Table 2.1). Since these deficiencies removed material distal to the transposable element (Lane and Kalderon, 1993, and Fig. 2.2), their ability to complement the *pal* gene set the TE16 insertion site as the distal limit of the *pal* gene.

The TE16 derivatives *Df(2L)γ1*, *Df(2L)γ15*, *Df(2L)γ25*, and *Df(2L)γ27* and the deficiency *Df(2L)232* failed to complement *pal*^{1}. When *Df(2L)γ15/SM5, Cy* males were crossed to *Df(2L)γ25/SM5, Cy* females, the resulting *Df(2L)γ15/Df(2L)γ25* animals were phenotypically normal and fully viable relative to their *Df/SM5, Cy* sibs. The progeny of *Df(2L)γ15/Df(2L)γ25* males, however, exhibited paternal chromosome loss. This result showed that the paternal loss phenotype was not specific to the original EMS-induced *pal*^{1} allele and that at least a portion of the *pal* gene was in the region deleted in both *Df(2L)γ15* and *Df(2L)γ25* (Fig. 2.2).

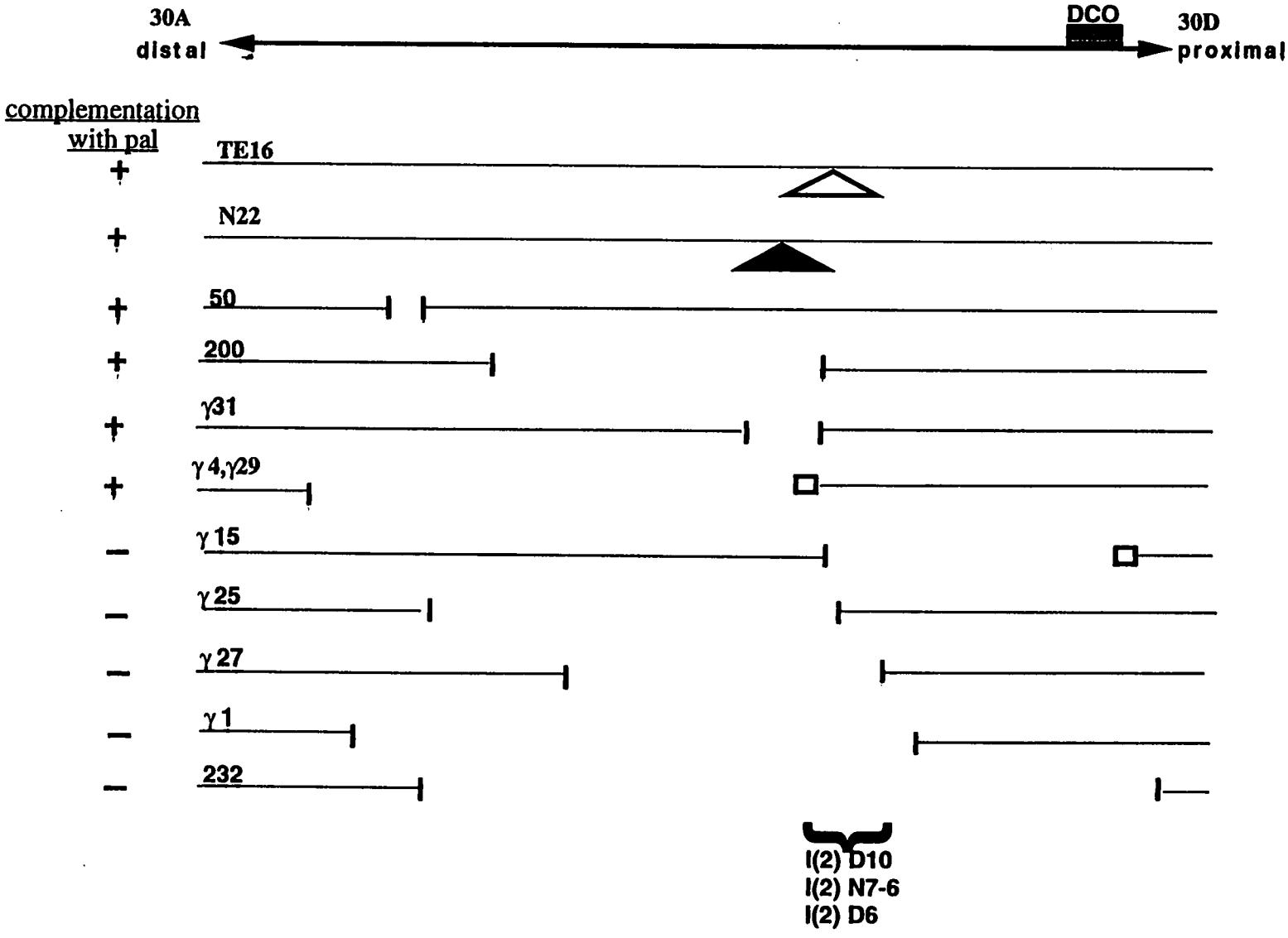
Two known lethal complementation groups fall between the *Df(2L)γ27* and *Df(2L)γ15* breakpoints as defined by complementation analysis (Lane and Kalderon, 1993). Two alleles of one gene, *l(2L)D6* and *l(2L)N7-6*, and the sole allele of the other, *l(2L)D10* , were tested for viability over *pal*^{1} and complementation of the paternal loss phenotype among their progeny. All are fully viable and complement *pal*^{1}.

Table 2.1. Frequency of Paternal Chromosome Loss Caused by Chromsome 2 Deficiencies and Lethal Mutations

Paternal Second Chromosome Genotype	Number of progeny							Percentage of progeny					
	total	XO	XX// XO	XY// XO	4O	44// 4O	total loss events	XO	XX// XO	XY// XO	4O	44// 4O	total loss events
Df(2L)30A-C/pal^1	1701	7	2	1	28	23	61	0.41	0.12	0.06	1.65	1.35	3.59
TE16[w^a*,rst]/pal*^{1}	2676	12	0	0	0	6	18	0.45	0	0	0	0.22	0.67
Df(2L)γ 1/pal^1	512	4	1	3	12	24	44	0.78	0.19	0.59	2.34	4.69	8.59
Df(2L)γ 4/pal^1	953	1	0	0	0	0	1	0.10	0	0	0	0	0.10
Df(2L)γ 15/pal^1	618	2	3	0	13	12	30	0.32	0.48	0	2.10	1.94	4.85
Df(2L)γ 25/pal^1	1257	8	3	0	3	8	22	0.64	0.24	0	0.24	0.64	1.75
Df(2L)γ 27/pal^1	971	5	3	2	20	13	43	0.52	0.31	0.21	2.06	1.34	4.43
Df(2L)γ 29/pal^1	2097	12	0	0	0	3	15	0.57	0	0	0	0.14	0.71
Df(2L)γ 31/pal^1	1020	0	1	1	0	1	3	0	0.10	0.10	0	0.10	0.30
Df(2L)50/pal^1	2496	2	0	0	0	0	2	0.08	0	0	0	0	0.08
Df(2L)200/pal^1	785	0	0	0	0	0	0	0	0	0	0	0	≤0.10
Df(2L)232/pal^1	798	3	0	0	12	15	30	0.37	0	0	0	7.88	3.76
Df(2L)A4/pal^1	2243	0	2	0	1	2	5	0	0.09	0	0.04	0.09	0.23
l(2)D10/pal^1 [a]	1166	1	1	0	1	1	4	0.08	0.08	0	0.08	0.08	0.34
l(2)D10/pal^1 [b]	483	0	0	0	1	0	1	0	0	0	0.21	0	0.21
l(2)D6/pal^1	991	1	0	0	0	1	2	0.10	0	0	0	0.10	0.20
l(2)N7-6 pr cn/pal^1	828	0	0	0	1	0	1	0	0	0	0.12	0	0.12
pal^1*/pal*^{1} trial 1	1940	38	8	0	22	22	90	1.96	0.41	0	1.13	1.13	4.64
pal^1*/pal*^{1} trial 2	614	18	4	2	4	12	40	2.93	0.65	0.32	0.65	1.95	6.51

The progeny of crosses between males of the indicated genotypes and *y w sn*^{3}*; spa*^{pol} females were scored for paternal chromosome loss. Fathers are *w/y*$^+$Y, with the exception of *pal*^{1}*/pal*^{1} males which are *y/y*$^+$*Y* for trial 1 and *+/y*$^+$Y for trial 2 and 3. XO indicates nullosomics with loss of the paternal X chromosome. 40 indicates nullosomics with loss of the paternal chromosome 4. [a] *w/y*$^+$Y*; l(2)D10/pal*^{1} . [b] *+/y*$^+$Y*;l(2)D10/pal*^{1}*; +/ry*^{506}

Figure 2.2. Schematic representation of deficiencies in the 30A-30C region of chromosome 2L. Horizonal lines indicate regions present in the chromosomes, triangles represent insertions, and gaps indicate the relative sizes of the regions deleted based on the complementation data (Lane and Kalderone 1993) and cytogenetic analyses (B. Wakimoto, personal communication). Chromosomes that complement the *pal*^{1} mutation are denoted with the (+) sign; those that fail to complement *pal* are denoted with the (-) sign. Three lethal mutations were mapped to the bracketed area by Lane and Kalderon. The open box symbols indicate uncertainty in the deficiency breakpoints. The grey box represents the *DCO* gene.


30A
distal
DCO
30D
proximal
complementation
with pal
TE16
N22
50
200
γ31
γ4,γ29
γ15
γ25
γ27
γ1
232
I(2) D10
I(2) N7-6
I(2) D6

Isolation of a new **pal** *allele*

I screened for new alleles of *pal* using the strategy described by Tower et al. (1993). The aim was to obtain a P element insertion into the gene that would be useful for genetic studies and molecular mapping. The starting chromosome for mutagenesis was *P*[(*w*$^+$, *ry*$^+$)A] N22, a chromosome that fully complements *pal*^{1} and carries a *P* element marked with the *white*$^+$ and *rosy*$^+$ eye color genes in 30C1-6 (Hazelrigg et al., 1984; Lane and Kalderon 1993). Of the 227 transposon-mutagenized chromosomes screened (representing at least 115 independent events), one chromosome carried a mutation that failed to complement *pal*^{1} . The chromosome carrying this allele, designated *pal*^{2} , retained expression of the *w*$^+$ transgene but lost the ability to confer a *ry*$^+$ phenotype.

Mobilization of the *P*(*white*$^+$, *rosy*$^-$) element in the *pal*^{2} chromosome was induced by providing a transposase source and selecting white$^-$ animals in the next generation. Among these eye color mutants, a portion are expected to carry chromosomes with simple *P* element excisions. None of the 89 independently isolated chromosomes, however, reverted the *pal* phenotype. As shown below, the *pal*^{2} allele appears to be a null allele. Failure to revert this allele suggests that the lesion is not simply an insertion of the *P* element.

Genetic characterization of **pal**^{1} *versus* **pal**^{2}

The *pal*^{2} allele was tested originally in *trans* to the *pal*^{1} allele. To determine if the *pal*^{2} mutation and the *pal*$^-$deficiencies, *Df(2L)γ15* and *Df(2L)γ25* , revealed new properties of the *pal* gene, I compared the effects of various combinations of the *pal* lesions. Homozygous *pal*^{2} males and females are fully viable and fertile when compared to their heterozygous sibs

(data not shown). I did not detect maternal chromosome loss above the background level among progeny of *pal*^{2}/*pal*^{2} mothers and *pal*$^{+}$ fathers (Table 2.2, crosses 1-3). When *pal*^{2} /*pal*^{2} males were crossed to tester females, however, their progeny showed loss of X and 4th chromosomes, as evidenced by nullo and mosaic exceptions (Table 2.2, crosses 6-7). Hence, *pal*^{2} was similar to *pal*^{1} in exhibiting an effect on paternal chromosomes in the early embryo.

The rates of paternal chromosome loss observed in a particular cross with *pal* mutant males can vary between trials, presumably due to variations in culture conditions or sample size. For *pal*^{2}/*pal*^{2} homozygous fathers the rate of chromosome loss among progeny varied from 0.98% to 7.69% in eight trials (Table 2.3). The rates of paternal chromosome loss induced by fathers homozygous or hemizygous for either *pal*^{1} or *pal*^{2} are similar to each other (Table 2.2, crosses 4-9) and to that observed for *Df(2)γ15/Df(2)γ25* males (Table 2.2, cross 10). We conclude that the *pal*^{1} and *pal*^{2} are strong hypomorphic or amorphic mutations. The total rate of paternal chromosome loss is undoubtedly higher since the values in Table 2.2 include only the frequencies of X chromosome mosaics (XX//X0), Chromosome 4 mosaics (44//40), and haplo 4 individuals (4/0). When Baker (1975) monitored X, Y and Chromosome 4 losses induced by *pal*^{1}, he found the rates did not exceed 24%. Taken together, these results suggest that loss of function of the *pal*$^{+}$ product results in low levels of paternal chromosome loss; greater than 75% of the offspring show no evidence of paternal chromosome loss as monitored by adult cuticular phenotypes.

All combinations of *pal* mutant alleles result in similar percentages of mosaic progeny (5 -10%), as monitored in adults (Table 2.2). This relatively

Table 2.2. Rates of Paternal Chromosome Loss and Lethality Induced by the *pal* Mutation

	Paternal Genotype	Maternal Genotype	Total # of progeny	% lethality	% exceptional progeny[b]				% total exceptional progeny
					XO[a]	XX//XO	4O	44//4O	
1.	*y w sn*^{3}; *spa pol*	*pal*^{1}/*pal*^{1}	4234	n.d.					0.07[c]
2.	*y w sn*^{3}; *spa pol*	*pal*^{2}/*pal*^{2}	1565	n.d.	0.12	0.12	0	0	0.12
3.	*y w sn*^{3}; *spa pol*	*N22/N22*	1940	n.d.	0.10	0	0.36	0.15	0.61
4.	*pal*^{1}/*pal*^{2}	*y w sn*^{3}; *spa pol*	404	n.d.	(1.8)	0.5	6.2	3.2	9.6
5.	*pal*^{1}/*pal*^{1}	*y w sn*^{3}; *spa pol*	874	50.3[d]	(1.0)	1.0	4.1	1.7	6.8
6.	*pal*^{2}/*pal*^{2}	*y w sn*^{3}; *spa pol*	1510	21.6	n.d.	0.1	3.6	1.6	5.3
7.	*N22/N22*	*y w sn*^{3}; *spa pol*	1487	19.8	n.d.	0	0.07	0	0.07
8.	*pal*^{1}/*Df(2)γ15*	*y w sn*^{3}; *spa pol*	749	26.9	n.d.	0.9	4.5	2.0	5.6
9.	*pal*^{2}/*Df(2)γ15*	*y w sn*^{3}; *spa pol*	926	18.6	n.d.	0.2	3.8	2.3	6.5
10.	*Df(2)γ25/Df(2)γ15*	*y w sn*^{3}; *spa pol*	750	25.0	n.d.	0.6	3.9	1.0	5.6
11.	*N22/Df(2)γ15*	*y w sn*^{3}; *spa pol*	694	9.8	n.d.	0	0.1	0	0.1

n.d. indicates results not determined.

a The nullo X class was not distinguisable from regular males in crosses 6-11. For crosses 4 and 5, the percentage of X0 progeny is listed but is not included in the total % exceptional progeny to allow direct comparison of crosses 4-11.

b Percent exceptional class is the percentage of exceptional progeny for a particular class among the total progeny.

c Taken from Baker (1975), Table 4, due to a female sterile mutation on the *pal*^{1} chromosome, not present originally.

d Rate of lethality and rate of chromosome loss were determined in separate experiments.

Table 2.3. Variability in the Frequency of Paternal Chromosome Loss

Genotype of paternal second chromosome	trial	Number of Progeny								Percentage of Progeny						
		total	XO	XX//XO	XY//XO	4O	44//4O	total loss events	total loss less XO and XY//XO	XO	XX//XO	XY//XO	4O	44//4O	total loss events	total loss events less XO and XY//XO
pal^{1}/*pal*^{1}	Trial 1	1940	38	8	0	22	22	90	52	1.96	0.41	0	1.13	1.13	4.64	2.68
	Trial 2	614	18	4	2	4	12	40	20	2.93	0.65	0.32	0.65	1.95	6.51	3.26
	Trial 3	332	2	7	0	22[a]	7	38	36	0.60	2.1	0	6.62	2.41	11.44	10.84
	Trial 4	288	4	0	0	15[b]	5	24	20	1.39	0	0	5.21	1.74	8.33	6.94
	Trial 5	254	7	0	1	0	4	16	8	2.76	0	0.39	0	1.57	4.72	3.15
	Trial 6	199	2	0	0	10	2	14	12	1.00	0	0	5.02	1.00	7.03	6.03
	Trial 7	82	3	0	0	4	0	7	4	3.66	0	0	4.88	0	8.54	4.88
	Trial 8	140	2	0	0	10	4	16	14	1.43	0	0	7.14	2.86	11.43	10.00
	Trial 9	131	5	2	1	9	3	20	14	3.82	1.53	0.76	6.87	2.29	15.27	10.69
	Trial 10	229	3	0	0	1	3	7	4	1.31	0	0	0.44	1.31	3.06	1.75
	all trials	**4209**	**84**	**21**	**4**	**97**	**62**	**268**	**180**	**2.00**	**0.50**	**.10**	**2.30**	**1.47**	**6.37**	**4.28**
pal ^{2}/*pal*^{1}	Trial 1	167	**	2	*	4	1		7		1.20	*	2.40	0.60		4.19
	Trial 2	302	2	0	0	14	5	21	19	0.66	0	0	4.64	1.66	6.95	6.29
	Trial 3	238	0	3	0	10	4	17	17	0	1.26	0	4.20	1.68	7.14	7.14
	Trial 4	108	1	0	0	5	1	7	6	0.92	0	0	4.63	0.92	6.48	5.55
	Trial 5	241	1	1	1	19	8	31	29	0.41	0.41	0.41	7.88	3.32	12.86	12.03
	Trial 6	160	2	0	0	5	4	11	9	1.25	0	0	3.12	2.50	6.88	5.62
	all trials	**1216**	**6**	**6**	**1**	**57**	**23**	**94**	**87**	**0.49**	**0.49**	**0.08**	**4.69**	**1.89**	**7.73**	**7.15**
pal^{2}/*pal*^{2}	Trial 1	187	*	0	*	5	2	7	7	*	0	*	2.67	1.07		3.74
	Trial 2	195	*	0	*	11	4	15	15	*	0	*	5.64	2.05		7.69
	Trial 3	595	*	1	*	5	1	7	7	*	0.17	*	0.84	0.17		1.18
	Trial 4	204	*	0	*	2	0	2	2	*	0	*	0.98	0		0.98
	Trial 5	191	*	0	*	2	0	2	2	*	0	*	1.05	0		1.05
	Trial 6	141	*	0	*	4	1	5	5	*	0	*	2.84	0.71		3.55
	Trial 7	103	*	0	*	1	2	3	3	*	0	*	0.97	1.94		2.91
	Trial 8	80	*	0	*	1	1	2	2	*	0	*	1.25	1.25		2.50
	all trials-screen	**1696**	*	**1**	*	**31**	**11**	**43**	**43**	*	**0.06**	*	**1.83**	**0.65**		**2.54**

a One of indicated progeny showed coincident XO and 4O loss events.
b Two of indicated progeny showed coincident XO and 4O loss events. One progeny showed coincident 4O loss and XX//XO mosaicism.

low level of apparent chromosome loss may represent an underestimate since embryos that suffer from the loss of a major autosome or multiple chromosome losses (Baker, 1975) are expected to die before adulthood and are therefore not included among scored adults. To examine this possibility, I determined the percentage of progeny produced by *pal* mutant fathers that survive to hatching and adulthood. The data, summarized in Table 2.4, show that when females are mated to fathers that are hemizygous for pal^1 or pal^2 or trans-heterozygous for the *Df(2L)γ15/Df(2L)γ25* deficiencies, approximately 19 to 29% of their eggs fail to hatch. This decrease in viability relative to wildtype strains is not due to a failure in sperm penetration; approximately equal frequencies of fertilized eggs are produced when fathers are homozygous or wildtype as monitored by DAPI staining of sperm nuclei within the cytoplasm of eggs (data not shown).

Discussion

Both pal^1 and pal^2 are null alleles by genetic criteria; the rates of chromosome loss are equivalent in the progeny of homozygotes as compared to hemizygotes (with *Df(2L) γ15*). No distinctions in phenotype are observed with *Df (2L)γ25/Df(2L)γ15* animals. The phenotype of pal^2 is the same as pal^1 by all genetic tests performed. As null alleles, only a paternal effect phenotype is observed which I interpret to mean that the function of the *pal* gene is required only paternally.

Despite the presumed absence of this gene product, the maximal rate of chromosome loss observed is 21% (Baker, 1975) or 15% in our hands. That is, many animals progress through development with no detectable abnormality in the absence of the *pal* product (greater than 70% in most cases, Table 2.4).

Table 2.4. Lethality associated with *pal* alleles

Genotype	# of eggs	% eggs hatched	% egg to adult survival	% loss among surviving adults
Df(2)γ25/Df(2)γ15	1000	78.8	75.0	5.6
pal^1/Df(2)γ15	1024	85.4	73.1	7.6
pal^2/Df(2)γ15	1137	89.6	81.4	6.5
N22/Df(2)γ15	769	94.1	90.2	0.1

Males were crossed to *y w* sn^3; spa^{pol} females. % loss among surviving adults includes all observed 4th chromosome loss and XX//X0 mosaics. (XO males are not distinguishable in these crosses.)

One explanation for this weak penetrance is that the function of the *pal*+ protein may be partially fulfilled by the product(s) of other gene(s). This may account in part for background variations in the *pal*-induced rate of chromosome loss. Strains may differ in the quantity of particular factors which can compensate for *pal*+ function or in the ability of different chromosomes to respond to these factors.

Any explanation of the *pal* phenotype must account for the fact that only paternal chromosomes are affected among progeny of *pal* fathers. Models invoking a defect in general cellular processes occurring during embryogenesis (for example a defect in the centrosomes thought to be supplied paternally) do not easily explain why maternal chromosomes are not affected. This paternal chromosome specificity may be due to the *pal* product interacting specifically with the paternal chromosome or indirectly by recognizing or affecting some other molecule which interacts with paternal chromosomes.

Perhaps the simplest manner in which to distinguish maternal from paternal chromosomes is to affect a process that only occurs in one sex. The chromosomal changes occurring during spermatogenesis involved in packaging the sperm chromatin are unique to the male germline. The exchange of chromosomal histones for sperm-specific chromosomal proteins is a process termed the histone transition. I propose that the *pal* function is involved in this spermatogenesis specific process. Within this context *pal* protein may directly bind the paternal chromatin. (Alternatively, *pal* protein may not be bound to the chromosomes. If this is the case, it implies that the *pal* protein be must able to recognize paternal chromosomes in some manner, perhaps by virtue of the spermatogenesis specific chromosomal

proteins in order to have an effect on paternal chromosomes but not maternal chromosomes. This explanation seems less parsimonious.)

Why are chromosomes lost in subsequent mitotic divisions? Why does the phenotype persist after the first division if it is due to a defect occurring during spermatogenesis? One possibility is that the defect persists but only the chromosomes bearing strands of DNA formed and packaged during spermatogenesis are lost. Or in other words, the chromosomes are imprinted but cannot pass that imprint to newly replicated strands. Alternatively, there may be some mechanism for passing an imprint to new strands and thus defective paternal chromosomes would give rise to newly formed defective chromosomes. I suggest that the persistent phenotype reflects aberrant chromatin structure distinguishing paternal from maternal chromosomes. For example, if lack of *pal* function results in sperm-specific chromosomal proteins remaining bound to chromosomes beyond the normal reversal of the histone transition. It is just as likely that they cannot be removed during subsequent mitoses. Additionally, the mechanism for histone replacement may operate only during the first division. Therefore, paternal chromosomes which are defective are likely to remain defective. Thus, any chromosome which is defective but was not lost in the first division may be lost in the next, and so on.

In conclusion, *pal* represents a strictly paternal effect gene. The functional redundancy evident from genetic analysis suggests it may be one of a number of products required for the proper maintenance of paternal chromosome structure. Further studies focus on determining the expression of the *pal* RNA and protein to understand better the role of the *pal* product during development.

CHAPTER 3

Molecular characterization of the *pal* gene

Genetic analysis narrowed the cytological localization of the *pal* gene and provided new tools for molecular mapping and cloning of the gene. The pal^2 allele was recovered after transposon mutagenesis of a Chromosome 2 bearing a marked *P* element located in the 30C region. To determine if the associated transposon moved into or near the *pal* gene, Southern analysis was used to molecularly compare the region surrounding the *P* element in the pal^2 and parental chromosomes. Breakpoints of chromosomal deficiencies were mapped at the molecular level to refine the location of the *pal* gene.

Genetic analysis demonstrated a requirement for the *pal* product paternally and also predicted that pal^1 and pal^2 are functionally null mutations. Cloning the gene allowed analysis of the *pal* transcript(s), enabling me to ask when and where the *pal* message is expressed. These studies addressed whether the *pal* is expressed only in the testis and delimited its probable time of function. Furthermore, analysis of the pal gene provided a means to assess the nature of its protein product and function, by homology to known sequences. The cDNA cloning and sequence analysis described was performed in collaboration with Kathleen Wilson.

Materials and Methods

Southern and northern blot analysis

Radio-labeled probes for blot hybridization were prepared by random priming as described by Feinberg and Vogelstein (1984). Southern blots were prepared and processed using standard protocols (Sambrook, Fritsch and Maniatis, 1989). Genomic clones from the 30C region were isolated from a lambda library described by Lane and Kalderon (1993) and were generously

provided by D. Kalderon. Selected fragments from these lambda clones were subcloned into Bluescript II KS- (Stratagene, La Jolla, CA) and used to probe a *Drosophila* cosmid genomic library kindly provided by J. Tamkun (Tamkun et al. 1992). Overlapping cosmid clones spanning the region from the *Df(2L)γ15* breakpoint to a point past the insertion site of the P[w^+,ry^+]A element in N22 were mapped and used to probe Southern blots of *pal* mutant strains. A plasmid bearing the P[w^+,ry^+]A transposon was kindly provided by R. Levis for analyses of the pal^2 chromosome.

For Northern analyses, flies were frozen in liquid nitrogen and RNA was extracted with a guanidium salt/phenol buffer according to manufacturer's specifications (Ultraspec RNA, Biotecx, Houston, TX). RNA was separated on formaldehyde-agarose gels, transferred onto Hybond-N membranes (Amersham, Arlington, IL) and processed following the procedures of Farrell (1993). For the developmental northern blot, embryos were collected from rapidly laying females, aged, homogenized, and processed for RNA extractions. Males lacking a germline were obtained as the progeny of osk^{301}/osk^{301} mothers (Lehmann and Nusslein-Volhard, 1986) mated to Canton S or *osk^{301}/TM3, Sb* males.

Isolation of pal *cDNAs*

A testis cDNA library was screened with a 9 kb genomic fragment to which the pal^2 lesion and the *Df(2L)γ25* breakpoint mapped. The library, kindly provided by T. Hazelrigg, was constructed by Stratagene (La Jolla, CA) by priming testis polyA$^+$ RNA with random primers and oligo-dT. Plating and screening of the lambda library was performed as described by Sambrook, Fritsch and Maniatis (1989). Six putative *pal* cDNA lambda clones were selected based on preliminary restriction digestion patterns for further study.

Inserts were subcloned into the Bluescript plasmid vector (Stratagene, La Jolla, CA). The cDNAs and corresponding genomic regions were sequenced by Kathleen Wilson using dideoxy nucleotides, primers that hybridized to vector or insert sequences and Sequenase according to the manufacturer's recommendations (US Biochemicals, Cleveland, OH). Primers for *Drosophila* sequences were synthesized by DNA International (Lake Oswego, OR).

In situ *hybridization*

Testes were dissected from adult flies in 0.7% sodium chloride, fixed and hybridized with digoxygenin-labeled probes according to the protocols described by Tautz and Pfeifle (1989) and S. DiNardo (personal comm.). To visualize nuclei, testes were stained with 10 μg/ml DAPI (4,6-diamidino-2-phenylindole, Sigma, St. Louis, MO) just prior to mounting in 90% glycerol. Preparations were examined with brightfield optics on a Nikon Microphot microscope to visualize digoxygenin-labeled RNA and epifluorescence optics to visualize DAPI staining.

Sequence analysis

Genomic and cDNA sequence analyses were performed using DNAsis software (Hitachi, San Bruno, CA). Searches for sequence homology were performed using the BLAST program (Altschul et al., 1990) or FASTA program (Pearson and Lipman, 1988) or by searching for protein motifs with BLOCKS program (Henikoff and Henikoff, 1991). Homologies were judged by comparison to random sequence matches generated by FASTA program.

Results

Molecular mapping of the pal *gene*

Southern blot analysis of DNA from the *N22* and pal^2 strains revealed that the pal^2 chromosome carried a deletion within the ry^+ marker gene of

the transposon (Fig 3.1). The *P* element was otherwise intact; the genomic fragments immediately flanking the *P* element were not changed in size, as determined by Southern blot analysis. The *pal*^{2} chromosome, nevertheless, had a 4 kb deficiency noncontiguous with the P element. This deficiency fell within the region deleted in *Df(2L)γ15/Df(2L)γ25* animals and was therefore likely to be the lesion responsible for the *pal* mutant phenotype.

The *Df(2L)γ15* chromosome contains an 80 kb deficiency extending from within the *DCO* gene to the TE16 insertion site (Lane and Kalderon, 1993). The corresponding region was isolated from a genomic lambda library by Lane and Kalderon (1993) as part of a 180 kb chromosome walk to clone the *DCO* gene. These lambda clones and newly isolated genomic clones from a cosmid library were used to localize the proximal breakpoint of *Df(2L)γ25* on the molecular map (Fig 3.1). The results defined a 9.5 kb genomic region of overlap between the *Df(2L)γ15* and *Df(2L)γ25* deficiencies.

Expression of the pal *RNA*

The observation that *pal* mutants show only paternal effect defects suggested that *pal*$^+$ function may be limited to male gametogenesis or early embryogenesis. To identify the *pal* transcript, a 9 kb EcoRI genomic fragment to which the pal^2 lesion and the *Df(2L)γ25* breakpoint map was used to screen a testis cDNA library (Hazelrigg et al. 1984). Four cDNAs with inserts of approximately 0.8 kb were recovered. Northern blots containing 10 ug of polyA$^+$ RNA from adult males and females were hybridized with the 9 kb EcoRI genomic probe or cDNA probes. These probes recognized identical sets of male-specific transcripts: one predominant transcript of approximately 0.9 kb and larger heterogeneous transcripts ranging from the 0.9 kb to 1.35 kb in size (Fig. 3.2A). I have deduced that the larger heterogeneous array of

Figure 3.1. Physical map of the genomic region surrounding the *pal* gene and complementation of deficiencies with *pal* ^{1}. The map illustrates the EcoR1 (R) restriction sites present in the genomic DNA and the relative position of deletions (gaps in lines) in the *Df(2l)γ* and *pal* ^{2} chromosomes. The shaded triangle represents a large TE transposon in the parental chromosome of the *Df(2L)γ* derivative chromosomes (not drawn to scale). The open triangles illustrate a P element insertion marked with the *white* gene (open rectangle) and the *rosy* gene (hatched rectangle). The black rectangle illustrates the position of the sequenced portion of the *pal* gene . The ability of each chromosome to complement the *pal* ^{1} mutation is noted to the left of the map, + indicates loss rates do not exceed loss rates of a control cross, - indicates loss rates of 0.75% or greater.

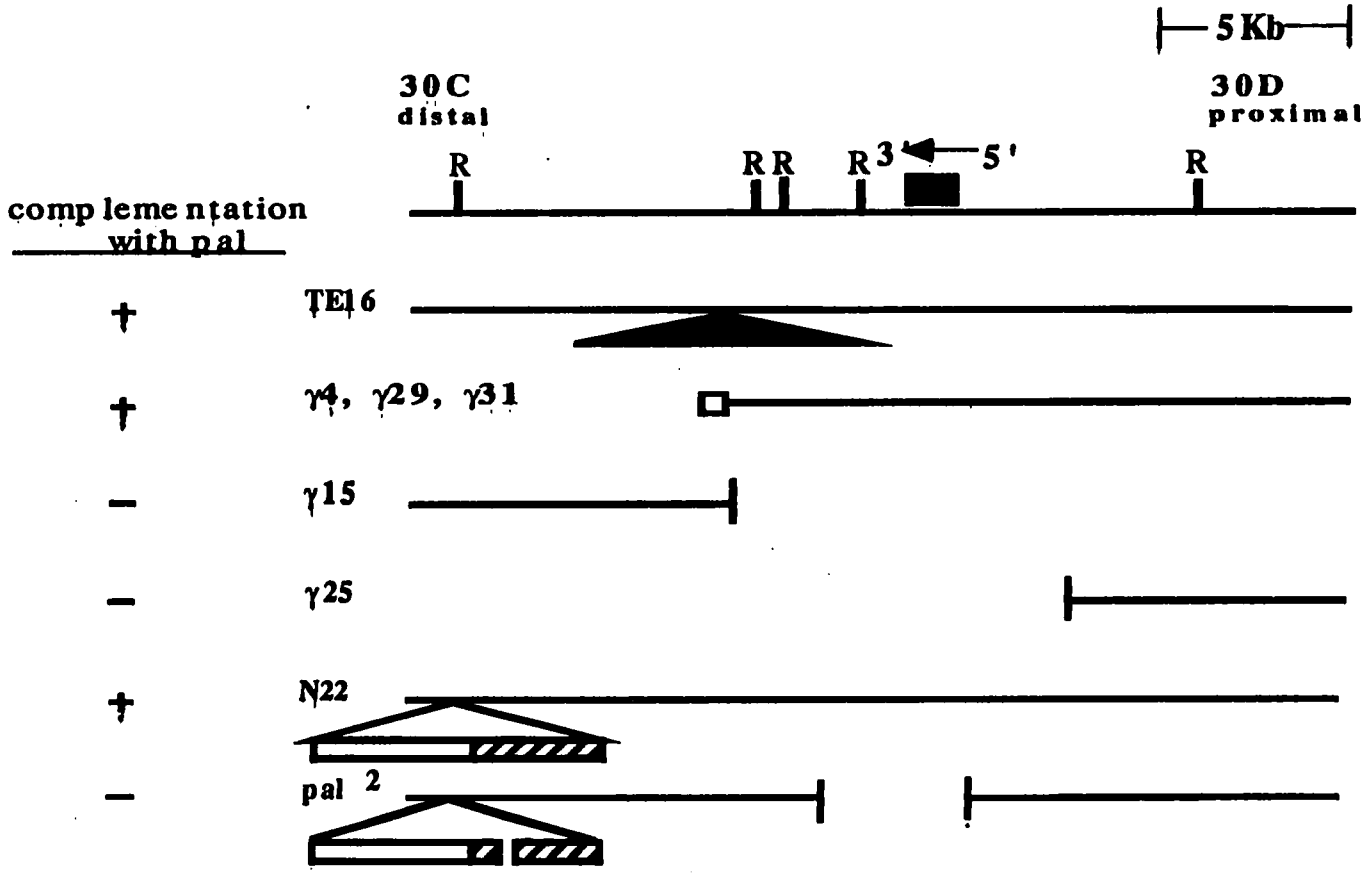
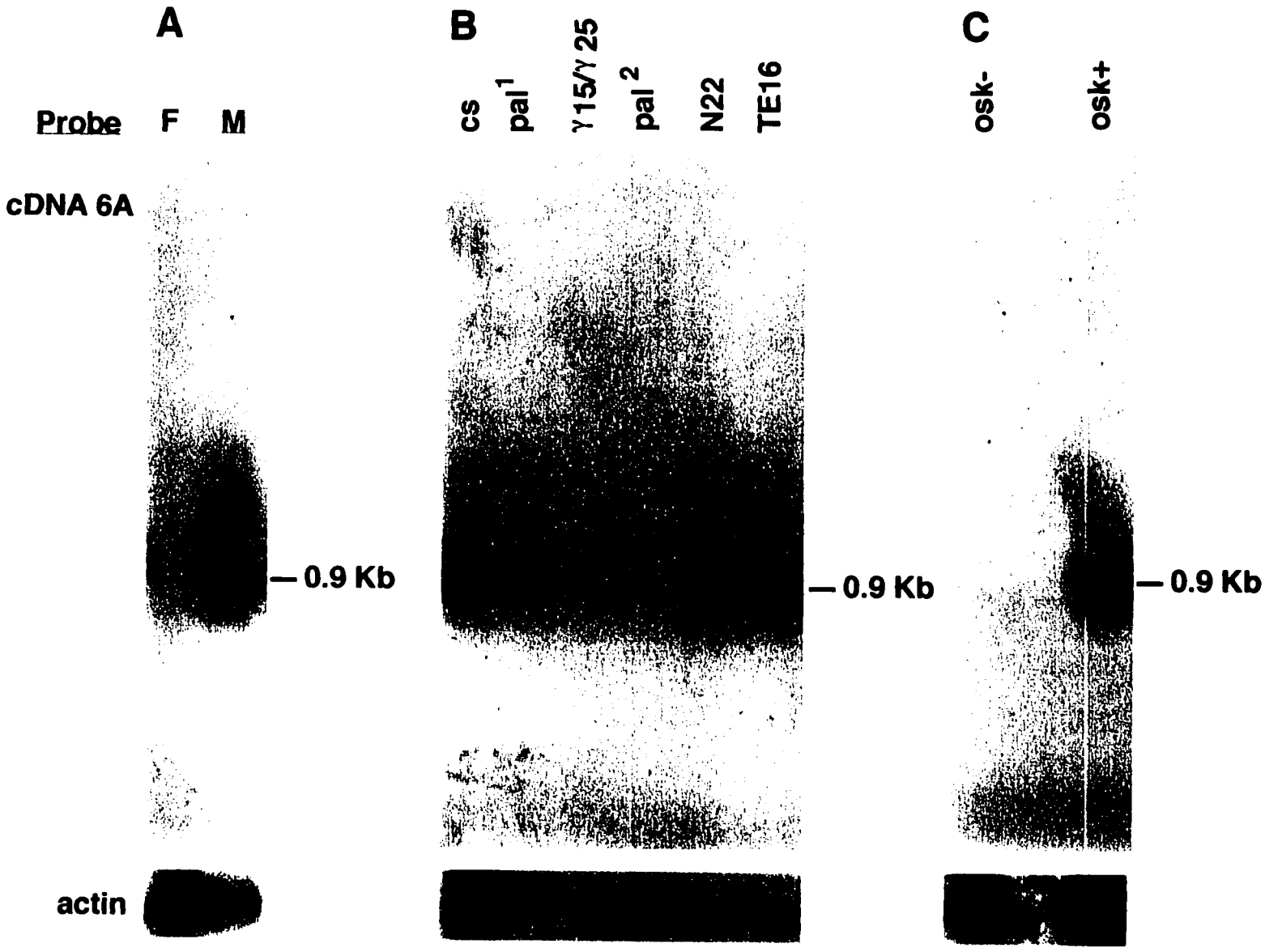

5 Kb
30C
distal
30D
proximal
R
R R
R
3'
5'
R
complemen tation
with pal
+
TE16
+
γ4, γ29, γ31
–
γ15
–
γ25
+
N22
–
pal 2

Figure 3.2. Northern blot analyses of the *pal* transcript. Five (panels B and C) or ten (panel A) micrograms of oligo-dT-selected RNA from adult flies were loaded in each lane, electrophoresed and blotted. Each blot was hybridized with a cDNA6A double-stranded DNA probe, stripped, and reprobed with actin to control for loading differences. Panel **A** shows an autoradiograph of a blot of RNA from wildtype females (F) and males (M). Panel **B** shows an autoradiograph of a blot of RNA from Canton S (cs), pal^1 homozygotes, *Df(2L)γ15/Df(2L)γ25* trans-heterozygotes, and pal^2, *N22* and *TE16* homozygotes. Panel **C** shows an autoradiograph of a blot of RNA from sons of osk^{301} homozygous mothers (osk^-) or $osk^{301}/+$ mothers (osk^+).


A
B
C
Probe
F
M
cDNA 6A
0.9 Kb
actin
cs
pal1
γ15/γ25
pal2
N22
TE16
0.9 Kb
osk-
osk+
0.9 Kb

transcripts is not artifactual and does not result from RNA degradation during extraction because I have detected these transcripts on multiple northern blots that do not show signs of RNA degradation when probed for actin mRNA.

To determine if these hybridizing RNAs were products of the *pal* gene, northern blots containing polyA$^+$ RNA from *pal* mutant males were probed with the genomic and cDNA clones (Fig. 3.2B). No hybridizing bands were detected in the RNA from *pal*^{2} or *Df(2L)γ15/Df(2L)γ25* males. By contrast, transcripts of the expected size are detected in the RNA extracted from *pal*^{1} males, but the most abundant species is reduced in level to 63% of wildtype level when normalized to an actin mRNA control. These data provide strong support that the male-specific transcripts correspond to the *pal* message. The genetic analyses suggests that *pal*^{1}, *pal*^{2} and *Df(2L)γ15/Df(2L)γ25* strains are phenotypically similar. Thus, while *pal*^{1} males retain 63% of wildtype levels of RNA, they probably make little or no functional product.

The tissue- and cell type- specificity of the *pal* transcript was addressed in two ways. First, a northern blot containing mRNA from males that lack germ cells was probed for the *pal* transcript. These males were the progeny of females homozygous for the *oskar*^{301} allele, a maternal effect mutation that fails to form germ cells in the embryo (Lehmann and Nusslein-Volhard, 1986). No hybridization to a *pal* cDNA probe was seen in these mutant males whereas the wildtype pattern seen in sons of *osk*$^+$ mothers (Fig. 3.2 C). We conclude that the *pal* transcripts are germline specific.

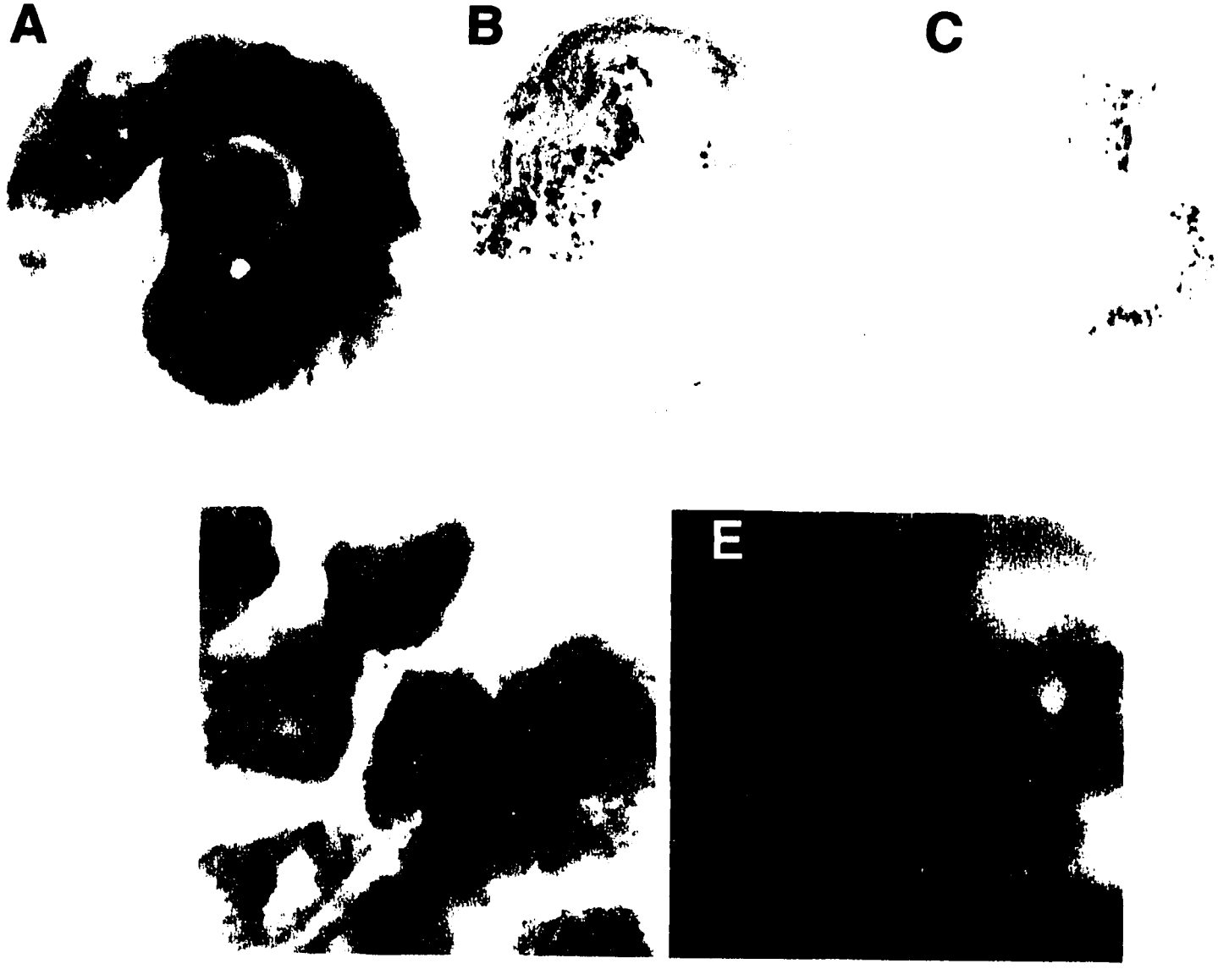
To assess the time when the *pal* transcript is expressed, a developmental northern blot was probed for the *pal* transcript using a cDNA probe. This blot contained 5 μg of polyA$^+$ RNA isolated from the following

stages: 0-12 hr embryos, 12-24 hr embryos, first instar larvae, second instar larvae, third instar larvae, pupae, and adults 0-1 or 2-4 days post-eclosion. Separate RNA extractions were performed for males and females at the third instar larval, pupal and adult stages. The earliest stages of development and females at all stages lack RNAs that hybridize to the *pal* genomic and cDNA probes. Hybridization to a 0.9 kb band is observed, however, in mRNA extracted from males from third instar larval stage through adult stages (data not shown).

Localization of the pal *RNA in testis*

I also used *in situ* hybridization of digoxygenin-labeled cDNA probes to RNA in testes of adult males to examine when and where the *pal* transcripts were detectable during spermatogenesis. Since spermatogenesis is a continuous process, with all stages of gametogenesis present in the adult testes, this technique provides both temporal and spatial information about the *pal* transcript. As shown in Fig. 3.3A for wildtype testes, hybridization is detected in clusters of primary spermatocytes that are located throughout most of the length of the testis. There is no detectable signal in the mitotically dividing spermatogonia located at the tip of the testis, in elongating spermatids, or in maturing sperm bundles. These results are consistent with the northern analysis since the formation of primary spermatocytes begins at the third instar larval stage and continues throughout later development. Interestingly, in the adult testes, the staining in primary spermatocytes shows a striking polar perinuclear localization. The signal appears to be closely apposed to, but outside, the nuclear envelope, as judged by costaining spermatocyte nuclei with the DNA dye, DAPI (Fig. 3.3, D and E). As primary spermatocytes mature, the staining shifts to a cytoplasmic staining and

Figure 3.3. In situ hybridization of the *pal* transcript in testes. Testes were hybridized with a digoxygenin-labeled cDNA probe. Whole mounts of testes from wildtype (A), *pal* ^{2} */pal* ^{2} (B) and *Df(2L)γ15/Df(2L)γ25* (C) adults are shown. A higher magnification of a wildtype testis probed with digoxygenin-labeled cDNA (brightfield, D) and costained with the DNA dye DAPI (fluorescence, E) shows the localization of *pal* transcript within primary spermatocytes in a polar perinuclear cap.


A
B
C
E

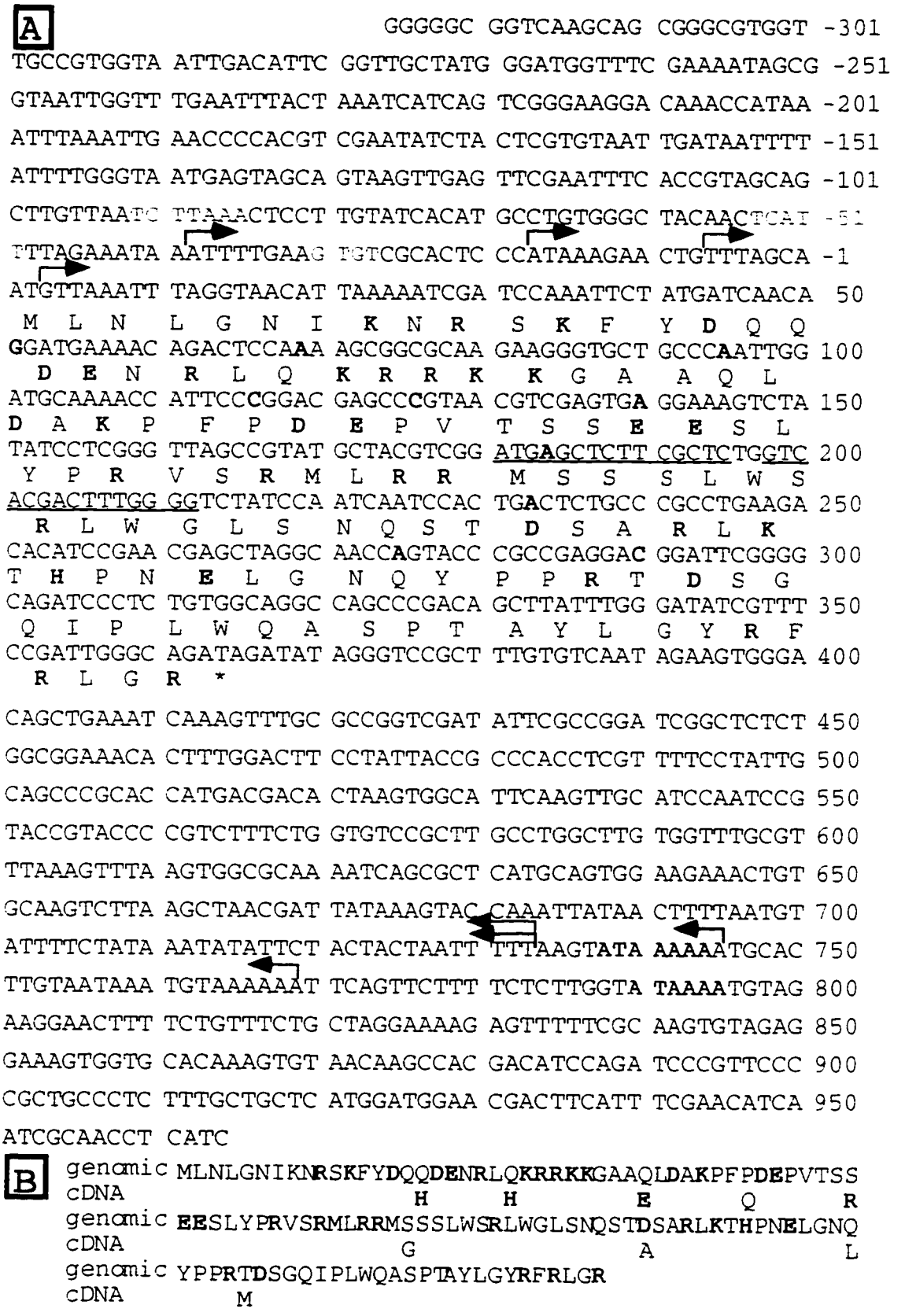
increases in intensity. Because staining was so intense, I could not determine whether or not the polar perinuclear staining is present in addition to more homogenous cytoplasmic staining.

In situ hybridizations to *pal*^{1} testes revealed *pal* gene expression similar to testes of wildtype males. Testes preparations from *pal*^{2} and *Df(2L)γ15/Df(2L)γ25* males, however, exhibited differences. The high intensity of staining throughout the testis was absent and only a low level of hybridization occurred in a small population of primary spermatocytes. Staining from these mutants was diffuse and appeared nuclear. Molecular mapping of the *pal*^{2} and *Df(2L)γ15/Df(2L)γ25* lesions by Southern blot analysis, using high stringency hybridization conditions showed that these deficiencies removed the genomic sequences that hybridize to the cDNA probes used in the *in situ* and northern blot analyses of RNA. I therefore conclude that the low level hybridization observed in the testes of *pal*^{2} and *Df(2L)γ15/Df(2L)γ25* males is due to cross-reacting RNA from a gene outside the *pal* region.

Sequencing of pal *cDNA and genomic clones*

The DNA sequence of four of the *pal* cDNAs and the corresponding genomic region was determined as shown in Fig. 3.4. The cDNAs differ only in length, ranging from 740 to 790 bp in size. (The extent of the cDNAs is indicated by arrows of matching color above the sequence in Fig. 3.4.) None of the cDNAs appear to be full length. The most abundant mRNA detected on northern blots was 0.9 kb (as determined by comparison to RNA molecular weight standards) and all of the cDNAs terminate in short stretches of A's that are present in the genomic sequence surrounding the predicted polyadenylation signal(s) indicated in pink in Fig. 3.4. The cDNAs are identical to each other and to the genomic sequence except for ten

Figure 3.4. Genomic and cDNA sequences of the *pal* gene. A) Nucleotide sequence and derived amino acid sequence of the *pal* gene obtained from genomic clones. The predicted termination codon is indicated with an asterisk. The putative polyadenylation signal(s) are shown in pink. Basic residues are shown in red. Acidic residues are shown in green. Nucleotides differing between the genomic and cDNA sequences are highlighted in blue. Possible promoter region elements (Arkhipova, 1995) are highlighted in gold. The putative sites for RNA-binding proteins, the Y and H box elements (Han et al., 1995) are underlined (also see Fig 3.5). The 5' and 3' ends of the four sequenced cDNAs are indicated by arrows of matching colors above the sequence. B) Comparision of the derived amino acid sequence from genomic and cDNA clones.


A
GGGGGC GGTCAAGCAG CGGGCGTGGT -301
TGCCGTGGTA ATTGACATTC GGTTGCTATG GGATGGTTTC GAAAATAGCG -251
GTAATTGGTT TGAATTTACT AAATCATCAG TCGGGAAGGA CAAACCATAA -201
ATTTAAATTG AACCCCACGT CGAATATCTA CTCGTGTAAT TGATAATTTT -151
ATTTTGGGTA ATGAGTAGCA GTAAGTTGAG TTCGAATTTC ACCGTAGCAG -101
CTTGTTAATC TTAAACTCCT TGTATCACAT GCCTGTGGGC TACAACTCAT -51
TTTAGAAATA AATTTTGAAG TGTCGCACTC CCATAAAGAA CTGTTTAGCA -1
ATGTTAAATT TAGGTAACAT TAAAAATCGA TCCAAATTCT ATGATCAACA 50
M L N L G N I K N R S K F Y D Q Q
GGATGAAAAC AGACTCCAAA AGCGGCGCAA GAAGGGTGCT GCCCAATTGG 100
D E N R L Q K R R R K K G A A Q L
ATGCAAAACC ATTCCCGGAC GAGCCCGTAA CGTCGAGTGA GGAAAGTCTA 150
D A K P F P D E P V T S S E E S L
TATCCTCGGG TTAGCCGTAT GCTACGTCGG ATGAGCTCTT CGCTCTGGTC 200
Y P R V S R M L R R M S S S L W S
ACGACTTTGG GGTCTATCCA ATCAATCCAC TGACTCTGCC CGCCTGAAGA 250
R L W G L S N Q S T D S A R L K
CACATCCGAA CGAGCTAGGC AACCAGTACC CGCCGAGGAC GGATTCGGGG 300
T H P N E L G N Q Y P P R T D S G
CAGATCCCTC TGTGGCAGGC CAGCCCGACA GCTTATTTGG GATATCGTTT 350
Q I P L W Q A S P T A Y L G Y R F
CCGATTGGGC AGATAGATAT AGGGTCCGCT TTGTGTCAAT AGAAGTGGGA 400
R L G R *
CAGCTGAAAT CAAAGTTTGC GCCGGTCGAT ATTCGCCGGA TCGGCTCTCT 450
GGCGGAAACA CTTTGGACTT CCTATTACCG CCCACCTCGT TTTCCTATTG 500
CAGCCCGCAC CATGACGACA CTAAGTGGCA TTCAAGTTGC ATCCAATCCG 550
TACCGTACCC CGTCTTTCTG GTGTCCGCTT GCCTGGCTTG TGGTTTGCGT 600
TTAAAGTTTA AGTGGCGCAA AATCAGCGCT CATGCAGTGG AAGAAACTGT 650
GCAAGTCTTA AGCTAACGAT TATAAAGTAC CAAATTATAA CTTTTAATGT 700
ATTTTCTATA AATATATTCT ACTACTAATT TTTAAGTATA AAAAATGCAC 750
TTGTAATAAA TGTAAAAAAT TCAGTTCTTT TCTCTTGGTA TAAAATGTAG 800
AAGGAACTTT TCTGTTTCTG CTAGGAAAAG AGTTTTTCGC AAGTGTAGAG 850
GAAAGTGGTG CACAAAGTGT AACAAGCCAC GACATCCAGA TCCCGTTCCC 900
CGCTGCCCTC TTTGCTGCTC ATGGATGGAA CGACTTCATT TCGAACATCA 950
ATCGCAACCT CATC
B
genomic MLNLGNIKNRSKFYDQQDENRLQKRRKKGAAQLDAKPFPDEPVTSS
cDNA H H E Q R
genomic EESLYPRVSRMLRRMSSSLWSRLWGLSNQSTDSARLKTHPNELGNQ
cDNA G A L
genomic YPPRTDSGQIPLWQASPTAYLGYRFRLGR
cDNA M

polymorphisms shared by three cDNAs, as indicated in Fig. 3.4. One cDNA contains only nine of these polymorphisms.

The *pal* transcript that corresponds to the sequenced cDNAs lacks introns. The largest open reading frame extends 121 amino acids from initiating methionine to stop codon, with start and stop codons fitting the *Drosophila* consensus reasonably well (Cavener, 1987). However no clear TATAAT box is evident in the 5′ region. The lack of a TATAAT box is not unprecedented. Arkhipova (1995) notes that half of all *Drosophila* promoters with known transcription start regions are driven by TATAAT-less promoters. Other common features in *Drosophila* promoters are observed including a potential initiator element (often at -5/+5) and downstream element (often at +20/-35). There is a sequence loosely fitting the TATAAT consensus in the *pal* sequence. Perhaps with the aid of downstream elements, this region may be able to serve as a promoter region (sequence in gold highlight, Fig. 3.4). As indicated in Fig. 3.4, the location of the 5' ends of the cDNAs sequenced are consistent with this hypothesis.

No significant homology was detected when either the nucleic acid or deduced amino acid sequence was used to search available sequence databanks by FASTA or BLAST programs, or by searching for motifs with BLOCKS program. The predicted protein is basic, with a predicted pI of 10.96 (based on cDNA sequence), due to numerous lysine and arginine residues. Overall the predicted protein is highly charged; hydrophobicity assessment predicts an average hydrophobicity of -1.00 indicating that the predicted protein is hydrophilic (DNasis, using Kyle and Doolittle tables). There is a consensus sequence in the 5′ region for putative Y and H box elements

commonly found in genes expressed during spermatogenesis and whose transcripts are under translational regulation (Fig. 3.5, Han et al., 1995).

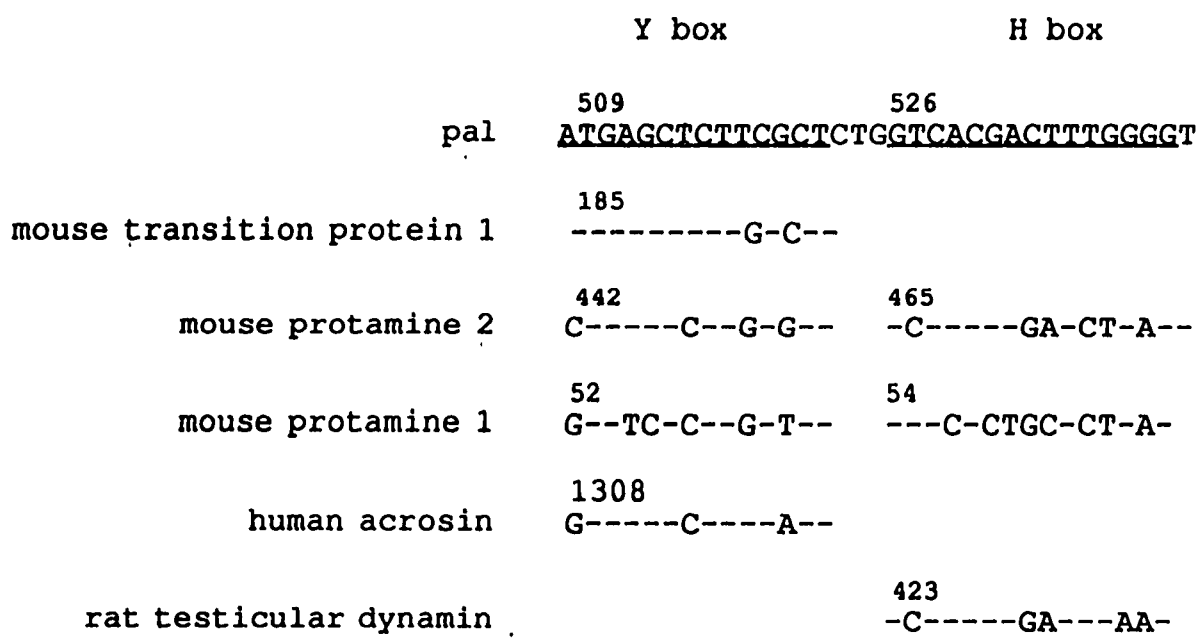
Discussion

Southern and northern analyses revealed a male-specific, germline-specific transcript which is absent or altered in quantity in *pal* mutants (*pal*^{1}, *pal*^{2} and *Df(2L)γ15/Df(2L)γ25*). These observations agree with the genetic predictions for a paternal effect gene. This information suggests that *pal* function is restricted to males because the expression of the gene occurs only in males. It is possible that a similar function is provided by another locus in females for maternal chromosome maintenance. However, this observation also suggests that the functional role of *pal* is a reflection of a distinctly male process, spermatogenesis, requiring a difference in the handling of paternal chromosomes.

The origin of the variation in the transcript size for the *pal* gene is unclear. Because all RNA species are absent in *pal*^{2} and *Df(2L)γ15/Df(2L)γ25*, the larger less abundant RNA species seem to originate from the *pal* gene. No significant variation was observed among the cDNAs isolated. They may be derived from the most abundant 0.9 kb transcript.

Variation could be due to different polyadenylation sites or transcription start sites as seen with some genes (Ayoubi and VanDeVen, 1996; Lewis et al., 1995; Wahle, 1995). Alternatively, many testis-specific messages exhibit variation in the length of poly A$^+$ tails due to translational regulation (Jackson and Standart, 1990, 1993; Kleene et al. 1984; Hecht, 1988). For example, the *protamine-1* transcript varies in length from 580 nucleotides when first transcribed to 430 nucleotides when translated. The *protamine-2* gene is similarly shortened from 830 to 700 nucleotides (reviewed in Hecht,

Figure 3.5. Comparison of putative Y and H box elements. Nucleotide sequence comparison of the putative Y and H boxes of *pal* and several genes with motifs for RNA-binding proteins. Dashes indicate indentical nucleotides. Numbers above each sequence indicate location in the nucleotide sequence (GenBank references, except for *pal*).


Y box
H box
509
526
pal
ATGAGCTCTTCGCTCTGGTCACGACTTTGGGGT
185
mouse transition protein 1
---------G-C--
442
465
mouse protamine 2
C-----C--G-G--
-C-----GA-CT-A--
52
54
mouse protamine 1
G--TC-C--G-T--
---C-CTGC-CT-A-
1308
human acrosin
G-----C----A--
423
rat testicular dynamin
-C-----GA---AA-

1988). Conversely, the Drosophila transcript from the *mst(3)87F* gene lengthens its polyA+ tail from 140 to 380 nucleotides upon translation (Schafer, 1990). For *pal* transcripts, no temporal difference in transcript length was apparent by developmental northern analysis. This experiment, however, does not separate various spermatogenic stages, as required to truly assess whether temporal regulation occurred. Assays could be performed using RNase H digestion to determine if variation in polyA+ tail length accounts for all or part of the variation in the RNA species observed.

In situ hybridization showed expression of the *pal* transcript in primary spermatocytes, which shifted from a polar perinuclear localization to a diffuse cytoplasmic pattern. This observation raises two questions: Is the polar perinuclear localization biologically important? And, is the shift in expression from localized to diffuse reflective of regulation of the transcript? It is possible that this pattern of localization reflects translational control of the *pal* protein. Since there is no transcription post-meiotically in *Drosophila* detectable by bulk labelling studies (Oliveri and Oliveri, 1965; Curgy and Anderson, 1972; Gould et al., 1974) transcripts required meiotically or for the extensive post-meiotic morphogenic events of spermiogenesis must be produced pre-meiotically. (Though not detected by bulk-labelling studies, a few exceptions are known to exist which are transcribed post-meiotically, such as *Hsp70* and *hsr-omega*, Bendena, 1991). Many organisms store transcripts or proteins until required (Iatrou, 1978; Braun, 1989a,b; Garner, 1988; Jeffrey, 1989). Although the *pal* transcript may be translated pre-meiotically, there is no evidence that *pal+* function is required pre-meiotically or during meiosis; thus, it is equally plausible that the *pal* mRNA is translationally regulated. Presence of Y box and H box homologies in the *pal* gene supports the idea that

the *pal* transcripts may be translationally regulated (Spirin, 1994, Sommerville and Ladomery, 1996a,b; Schafer, 1995). Han et al. (1995) suggest that such Y and H box sites (or related sequences) may act as a binding site for the testes/brain RNA-binding protein in the mouse in protamine 2, tau and myelin basic proteins. By analogy, it is possible that these sequences denote a binding site for a regulatory protein involved in translational regulation in Drosophila. One of the better studied examples of translational regulation during spermatogenesis is the protamine transcript in mouse which is produced several days before it is translated. The protamine transcript is localized to snRNPs present throughout the cytoplasm (Balhorn, 1984; Kleene, 1984). In *Drosophila*, several spermatogenic proteins are under translational control (Kuhn, et. al, 1988; Kuhn et al., 1991, Yanicostas and Lepesant, 1990; Schafer et al. 1990, 1993). However, little has been reported about the spatial distribution of these transcripts. Does spatial distribution reflect translational control? A link between localization of transcripts and translational control has been documented for the maternal transcripts encoded by the *Drosophila* pattern formation gene *nanos* (Gavis and Lehmann, 1994; Kim-Ha et al., 1995). The localization of the *nanos* transcript posteriorly appears to be necessary for translation of its transcript. By analogy, the localization of the *pal* transcript may bring it in proximity to factors required either to translate the transcript or, conversely, to inhibit translation. For example, the shift from localized to diffuse expression as the cysts of spermatocytes age may reflect a shift from transcripts that are stored untranslated to those that are actively translated, or vice versa. Lastly, the polarity of the localized *pal* transcript may be due to association with one of the many subcellular structures which are asymmetrically distributed in these

cells, including ER, Golgi, mitochondria, and centrioles (Tates, 1971; Fuller, 1993).

Sequence analyses of the *pal* cDNAs and surrounding genomic region reveal a small open reading frame. No detectable homology was found to this sequence in the databases searched, indicating *pal* is a novel gene. The small size and lack of homology to known proteins eliminates some models for the function of the *pal* protein. Endow et al. (1991) suggest that *pal* might encode a kinesin-like motor protein based upon some similarities in the phenotype with *non-claret disjunctional, ncd.* The *ncd* mutation leads to maternal chromosome loss in embryos of *ncd* homozygous mothers, prompting Endow et al. to posit that *ncd* and *pal* were maternal and paternal analogs, respectively. However, *ncd* additionally results in meiotic nondisjunction and some loss of paternal chromosomes embryonically, differing from the *pal* phenotype. While it is still possible that *pal* is a protein which interacts with a paternally specific motor protein, it is unlikely to function itself as a motor.

Although a protein of 121 amino acid is perhaps smaller than average, proteins of this size have been identified previously. Among them are peptide hormones, splicing factors like *Sxl*, and chromosomal proteins like protamines.

What is most striking about the predicted peptide sequence is the basicity and hydrophilicity. The pI of 10.96 is unusual and places *pal* among a select group of proteins. Proteins with similar features include the histones (pI ranging from 11 to 12, Righetti et al. 1996), and lysozyme (pI=11). (A literature search for basic proteins on Medline showed that 9 of 17 proteins with pI>10 were reported to be DNA or RNA binding.) Presumably the

basicity of the histones is important for the function of these proteins in binding the acidic DNA molecule. Perhaps this characteristic indicates a similar role for the *pal* protein. If the basicity is important for *pal* protein function, it may explain the surprising number of changes between genomic and cDNA sequences. Nine of ten differences lead to amino acid changes which are not conservative. (It is possible that these differences are cloning artifacts. Additionally, cDNA and genomic clones were obtained from different wildtype strains of flies.) These polymorphisms may indicate that the amino acid sequence is less important than a general feature such as basicity and hydrophobicity, which differs little between cDNA and genomic clones.

CHAPTER 4

Cytological characterization of the *pal* gene

When does the *pal* protein act and what is its function? In order to delimit better the time of function of the *pal* protein, cytological analyses were performed in two ways. To examine the expression of the *pal* protein I generated an antibody against a *pal*-fusion protein and used this antibody for immunolocalization. Additionally, in collaboration with Kathleen Wilson, we characterized the defects during early embryogenesis in the progeny of *pal* fathers.

Determination of the expression pattern of the *pal* protein is used to delimit the time period within which the protein could function. In previous studies using northern blot analysis, I demonstrated that the *pal* mRNA was produced only in the testes. *In situ* RNA hybridization indicated that the *pal* mRNA was first expressed in primary spermatocytes. These experiments also revealed an interesting subcellular localization of the *pal* message. The mRNA was initially localized near the nucleus of early primary spermatocytes and later was found throughout the cytoplasm. Does the expression profile of the *pal* protein parallel the *pal* mRNA expression? A delay in the appearance of the *pal* protein would indicate that the message, like many other messages transcribed during spermatogenesis, is under translational control. This result would be interesting in light of the finding that *pal* message shares a sequence motif with other translationally-regulated messages (Chapter 3). Information on the time of protein expression also defines the earliest possible time of *pal* function during development. Similarly, the latest appearance of *pal* protein marks the last possible time of

function. Knowledge about the subcellular distribution of the *pal* protein may provide clues about its function and potential interactions.

Previous genetic analysis inferred events in the embryo from defects observed in adult progeny of *pal* fathers. Two types of adult progeny are observed: nullosomic progeny which show no genetic evidence of a particular paternal chromosome, and mosaic progeny which show loss of a paternal chromosome in only some tissues. Potentially, nullosomic animals could arise from loss events during spermatogenesis, but mosaic progeny must result from zygotic chromosome loss. Based on the size of mutant patches in mosaics, Baker proposed that the *pal* mutation induces paternal chromosome loss during the first few zygotic divisions (1975). Analysis of the phenotype in embryos was used to define more precisely the time of the earliest defects and to characterize the types of abnormalities associated with the paternal chromosome loss.

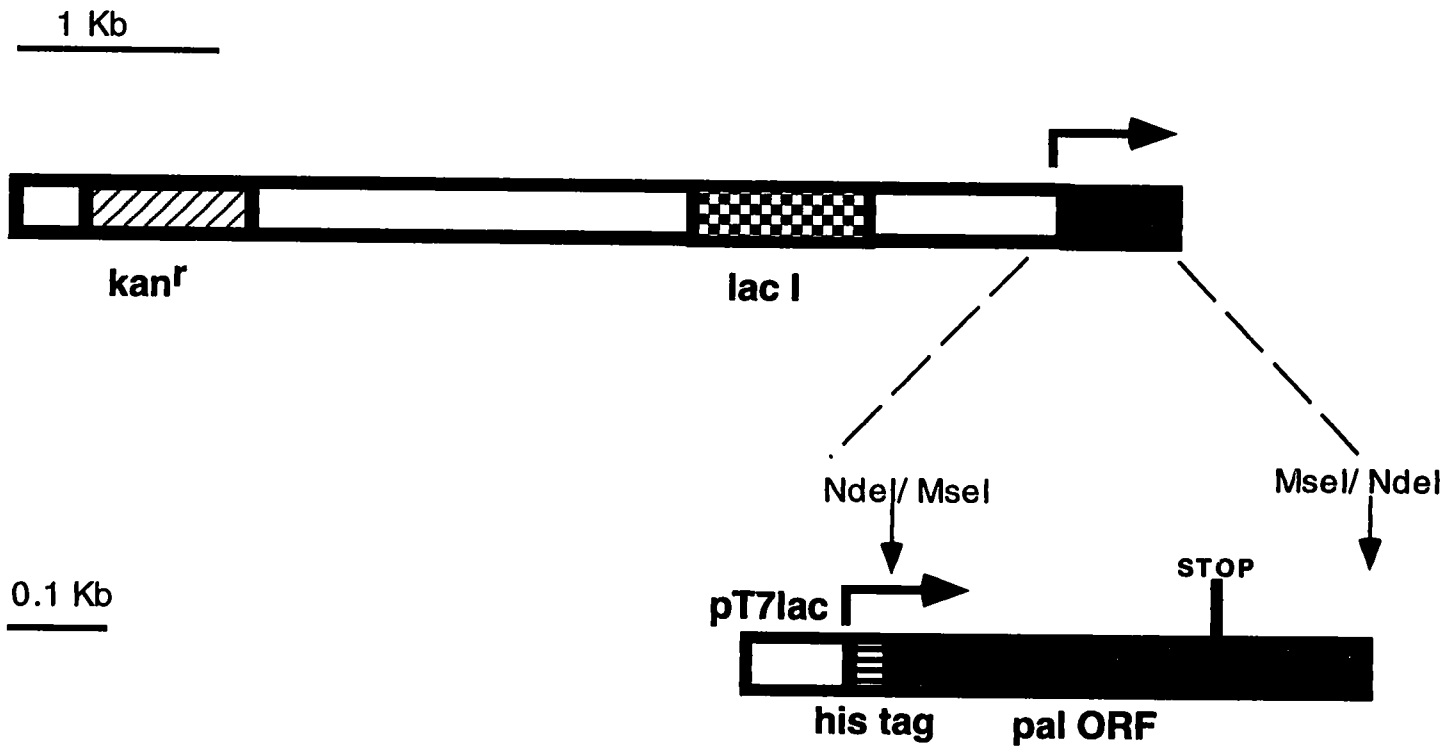
I conclude this chapter with models of *pal* function incorporating data from the genetic, molecular and cytological experiments.

Materials and Methods

Overexpression of the pal *protein in bacteria*

To express the *pal* protein in bacteria at high levels, an expression construct was made. The *pal* cDNA 6A was digested with the restriction enzyme MseI. The resulting 581 bp fragment containing the *pal* open reading frame was isolated and cloned into the NdeI site in the plasmid pET28c (Novagen, Madison, WI) (Fig. 4.1). This construct fuses a polyhistidine tag to the N-terminus of the *pal* protein, hereafter called his-*pal* .

Figure 4.1. Schematic representation of the his-*pal* expression plasmid. The white boxes represent pET28c plasmid sequences, with diagonally striped and checked boxes representing the kanamycin resistance gene and the lacI gene, respectively. The grey box represents the MseI fragment of the *pal* gene cloned into the NdeI restriction site of the pET28c expression vector. The predicted fusion protein is indicated by the arrow and stop. The plasmid sequences provide the T7*lac* promoter, the starting methionine for translation and a polyhistidine tag at the N-terminus of the *pal* sequences.


1 Kb
kan^r
lac I
NdeI/ MseI
MseI/ NdeI
0.1 Kb
pT7lac
STOP
his tag
pal ORF

Initially, the construct was transformed into *E. coli* DH5α to allow isolation and amplification of the plasmid. Subsequently, *E. coli* DE3 cells were transformed with the construct for protein induction. The DE3 strain contains an IPTG-inducible T7 *lac* polymerase gene. For induction of protein, cultures were grown to mid log phase (OD_{600} of approximately 0.6) when IPTG (isopropyl-β-D-thiogalactopyranoside, Sigma, St. Louis, MO) was added to a final concentration of 1 mM. Cells were incubated for an additional 1-3 hours at 37°C.

After transformation of inducible cells with the fusion construct, plasmid stability and potential lethality were monitored by plating cells on selective and non-selective media (as per Novagen, 1995). Colonies plated on IPTG generally do not grow due to overexpression of the fusion protein; only those cells lacking the fusion construct or mutated to eliminate expression should grow on IPTG. Plating tests indicated that there was no or little plasmid instability, as evidenced by the low frequency of colony formation on IPTG or kanamycin/IPTG plates.

To monitor protein production one milliliter samples of cultures were removed, and cells were pelleted for one minute in a microfuge and resuspended in 100-300 microliters of standard protein loading buffer (Ausubel et al., 1995). Samples were frozen at -20°C until use. Time course of protein induction was monitored in 30 minute intervals for three hours to assess the optimal induction time.

Western blots

Protein samples were boiled 5-10 minutes, loaded onto 13% SDS-polyacrylamide minigels and electrophoresed under standard conditions

(Ausubel et al., 1995). Total protein was visualized by staining with Coomassie Blue. For western blots, proteins were transferred with a semi-dry blotting apparatus (Biorad, Hercules, CA) onto NitroBond nitrocellulose membranes (Micro Separations, Inc., Westboro, MA), preincubated 15 minutes or more in transfer buffer (48 mM Tris, pH 9.25, 39 mM glycine, 0.037% SDS, 20% methanol). To verify the identity of the induced his-*pal* protein, a western blot of induced and uninduced cells was probed with anti-polyhistidine antibodies (courtesy of the Katze lab). Immunoreactivity was visualized by secondary incubation with a goat anti-mouse IgG antibody conjugated to alkaline phosphatase and detected with the ECL chemiluminescence system (Amersham, Arlington Heights, IL).

Strip blots were prepared bearing purified his-*pal* protein and total protein from the DE3 bacterial strain. These blots were probed with normalized volumes of sera (preimmune, whole immune, acetone powder subtracted, affinity purified) to assess reactivity and relative purity of affinity purified sera. A secondary goat anti-rabbit IgG antibody conjugated to alkaline phosphatase was used to detect hybridizing rabbit primary antibodies.

*Purification of the his-*pal *fusion protein*

To isolate inclusion bodies from bacterial cells, cultures were induced and harvested by centrifugation (5000 g) and resuspended in 50 mM Tris pH 8. Cells were lysed by incubation for 15 minutes at 30°C with Triton X-100 (0.1% v/v final concentration) and lysozyme (100 µg/ml final concentration). Cells were subsequently sonicated three times for 10 seconds. The resulting suspension was centrifuged at 20,000 g to isolate inclusion bodies and remove soluble proteins. Subsequently inclusion bodies were washed in binding

buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and sonicated. Inclusion bodies were collected by centrifugation at 20,000 g. Proteins were extracted from inclusion bodies by solubilization in binding buffer plus 6M urea for one hour on ice, with sonication every 20 minutes. Preparations were clarified by centrifugation at 39,000 g. Columns composed of a metal-chelating resin (Novagen, Madison, WI) were preequilibrated with 50 mM $NiSO_4$ solution followed by binding buffer containing 6M urea. Supernatants containing the fusion protein were loaded onto the nickel-bound columns. Columns were rinsed with binding buffer with 6M urea, and wash buffer (20 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9, 6 M urea). Bound protein was eluted in a solution of 1M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9, 6M urea. Columns were stripped with 100 mM EDTA, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9 and stored in 20% ethanol at 4°C. All fractions were tested for presence of his-*pal* protein by loading samples normalized for volume onto 13% SDS-PAGE minigels and staining with Coomassie Blue. The fusion protein is highly insoluble in the absence of 6M urea. This feature was used to concentrate the protein by dialysis against PBS (100 mM $NaPO_4$, 130 mM NaCl). The resulting precipitated protein was collected in a small volume of PBS and resuspended in protein gel loading buffer. For immunizations, the his-*pal* fusion protein was further purified by SDS-PAGE and gel slices were isolated and homogenized with PBS.

Immunizations

In an effort to reduce background levels prior to immunization, preimmune sera from eight rabbits were tested for antigenicity to *Drosophila melanogaster* proteins. Sera from each rabbit was used to probe western blots

of *Drosophila melanogaster* proteins extracted from whole adult males, females, hand-dissected testes, and wildtype embryos. For initial extractions, tissues were frozen in liquid nitrogen and homogenized with a Teflon homogenizer in eppendorf tubes with protein loading buffer, prior to boiling 5-10 minutes. Sera were also analyzed by immunocytochemistry against squashes of wildtype testes, following the procedure of Cenci et al. (1992). Three rabbits with the lowest background by these assays were selected for immunization. R&R Rabbitry (Stanwood, WA) performed all care, immunization, and bleeding of rabbits for this study. Rabbits were immunized initially with 200 μg of purified his-*pal* fusion protein and boosted every 21-28 days with 100 μg of purified his-*pal* fusion protein for 6 months. Rabbits were bled approximately 11 days after each immunization. After the fifth boost, all rabbits were terminally sacrificed and serum from each rabbit was collected.

Affinity purification of immune sera

Bacterially-expressed his-*pal* protein was purified as previously described. Protein in elution buffer was dialyzed against PBS, precipitated and resuspended in 0.2% SDS, 0.1M sodium phosphate buffer, pH 6.8. The purified his-*pal* antigen was covalently linked to an AminoLink column (Pierce, Rockford, IL) following manufacturer's specifications. Acetone powders of the DE3 bacterial strain were prepared according to standard methods (Harlowe and Lane, 1993). Aliquots of whole immune sera were incubated with DE3 acetone powders, 1% w/v, for 30 minutes at 4°C, prior to affinity purification to remove antibodies against bacterial proteins. Precipitates were removed by centrifugation for ten minutes at 10,000 g.

Immune serum from each rabbit was loaded onto separate affinity purification columns bearing the his-*pal* antigen and incubated at room temperature for one hour. The columns were washed successively with PBS, PBS plus 0.5M sodium chloride, and finally 0.1M glycine, pH 2.8, to elute bound antibodies. The eluate was collected in one milliliter fractions in eppendorf tubes containing 50 microliters of 1M Tris, pH 9.5, to immediately neutralize the solution. Protein content of each void, wash and elution fraction was assessed by following absorbance readings at OD_{280}. Positive elution fractions were pooled. For subtracted serum, affinity purified antibodies were loaded onto affinity purification columns as described above and the void volume was collected. This void volume is called the subtracted serum, since it should be depleted of antibodies recognizing the his-*pal* fusion protein.

Immunocytochemistry

Initial immunocytochemistry was performed on squashes of adult testes prepared according to Cenci et al. (1992). Whole mount preparations were used for final analyses. Testes or female sperm storage organs were dissected from young adults (0-5 day) in testis dissection buffer (183 mM KCl, 47 mM NaCl, 10 mM Tris-HCl, pH 6.8, 1 mM EDTA, 1:50 dilution of protease inhibitor cocktail). The protease inhibitor cocktail was composed of Complete protease inhibitor mix (Boehringer Mannheim, Indianapolis, IN) made according to manufacturer's specifications and supplemented with 1 μg/ml leupeptin and 1 μg/ml pepstatin). Dissection buffer was replaced with 3.7% paraformaldehyde (EM grade, Electron Microscopy Sciences, Ft. Washington, PA) in PBS (freshly diluted) and tissues were fixed for fifteen minutes at room

temperature. All fixations and washes were performed for fifteen minutes at room temperature unless otherwise noted. Tissue preparations were washed successively with PBS, 0.2% Triton X- 100 in PBS, PBS, fixed in 3.7% paraformaldehyde in PBS, PBS, 0.2% Triton X- 100 in PBS, and washed twice in PBS, then blocked with 4% normal goat serum for 30 minutes. Preparations were incubated with primary antibodies diluted in PBS plus 1% normal goat serum for two hours to overnight. Tissues were rinsed twice in PBS for 30 minutes then incubated with secondary antibody in 1% normal goat serum for two hours. The secondary antibody, BODIPY goat anti-rabbit IgG (Molecular Probes, Eugene, OR), was preabsorbed overnight against fixed wildtype testes. Following incubation with the secondary antibody, tissues were rinsed twice in PBS, stained with 0.5 μg/ml DAPI (4,6-diamidino-2-phenylindole, Sigma, St. Louis, MO), rinsed in PBS twice, then rinsed in 50% and 80% glycerol. Preparations were mounted in 90% glycerol for examination and photography. Embryos (courtesy of K. Fitch) were collected from wildtype Sevelin females crossed to *snky/snky* fathers and fixed in 37% formaldehyde for 5 minutes, briefly devitellinized in methanol and then dehydrated for storage. For antibody staining, embryos were treated as outlined above.

Confocal analysis of pal*-derived embryos*

Embryos of *pal* mutant fathers and wildtype Sevelin mothers were collected at 15 minute intervals on agar plates and aged 0 to 75 minutes before fixation. Chorions were removed by rolling eggs on double stick tape. Vitelline membranes were removed by hand dissection after fixation for 15 minutes in a 1:1 solution of N-heptane and 37% formaldehyde (Theurkauf,

1992). To capture developmental stages before the completion of the first cycle, fixation was completed within 10 to 25 minutes after egg deposition. Fixed embryos were prepared for immunocytochemistry following the methods of Theurkauf (1992) and Baker, Theurkauf and Schubiger (1993). Mouse anti-tubulin antibodies (Amersham, Arlington Heights, IL) and BODIPY-conjugated anti-mouse secondary antibodies (Molecular Probes, Eugene, OR) were used to stain microtubules. Rhodamine-conjugated mouse anti-histone antibody (Chemicon, Temecula, CA) was kindly provided by J. Baker and G. Schubiger and used to stain nuclei. Embryos were viewed with a Biorad 600 Confocal Lasar Microscope.

Results

Determination of when and where the *pal* protein is expressed may delimit somewhat where the *pal* protein may function as well as suggesting with what structure (e.g. the chromosomes, nuclear envelope) the protein interacts. Antibodies directed against the *pal* protein were isolated to use as tools in analyzing the location of the *pal* protein during spermatogenesis and embryogenesis.

To isolate such antibodies, it was necessary first to produce large quantities of the target antigen, the *pal* protein. Such an antigen was produced by creating a vector fusing the predicted *pal* protein to an amino-terminal histidine tag under the control of a inducible bacterial promoter; a fragment from the *pal* cDNA was cloned into a pET expression vector (Novagen, Madison, WI) producing recombinant protein hereafter called his-*pal* (Fig. 4.1). This fusion construct includes all but the first seven amino acids of the predicted *pal* protein. Induction with IPTG allows high level

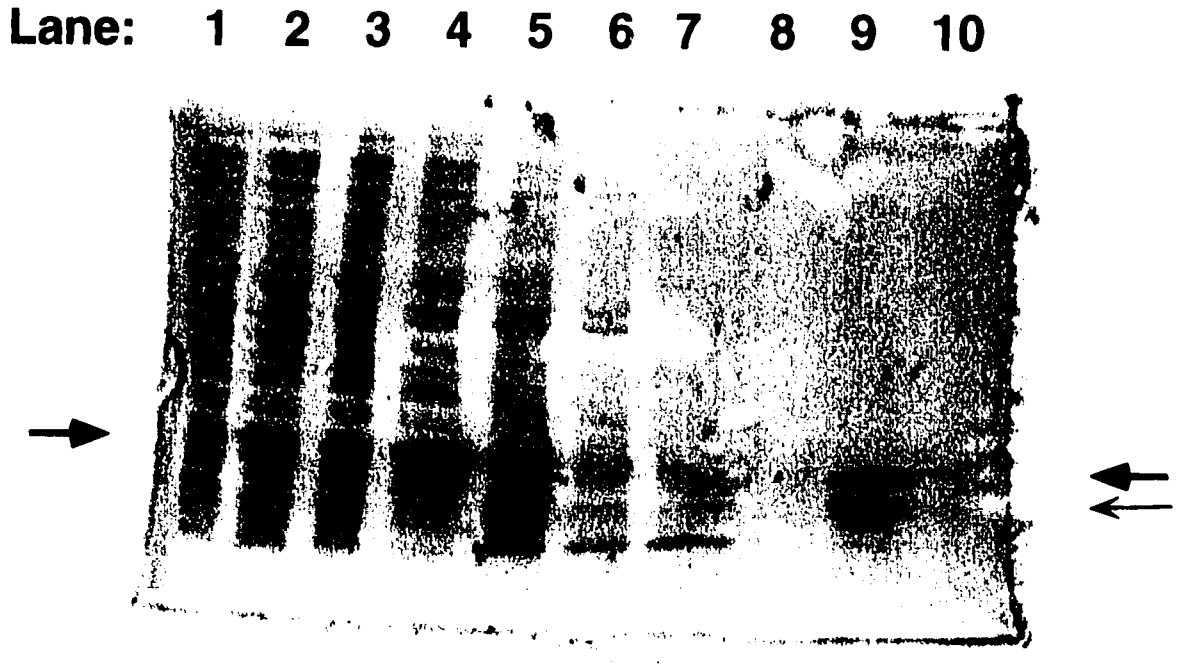
expression of the T7 polymerase and subsequent induction of the T7 promoter of the his-*pal* fusion. The presence of the histidine tag facilitated protein purification.

When IPTG was used to induce the cells to produce high levels of the fusion protein, an obvious band was observed on Coomassie stained protein gels of the induced strain (Fig. 4.2, lanes 2 and 3 versus lane 1). The deduced amino acid sequence was used to predict that the molecular weight of the gene product would be 15.4 kDa and that the pI would be 11.58. The apparent molecular weight was 23 kDa, showing a slower mobility than expected (data not shown). Panyim and Chalkey (1971) have observed that highly basic proteins often migrate more slowly on SDS-PAGE than predicted based on their known molecular weights. Based on their empirically determined migration curves, a 15 kDa highly basic protein would migrate as if it were 22.5 kDa. This prediction corresponds well with the observed size of the induced protein.

To verify that the protein band observed is the his-*pal* protein, western blots of proteins from induced and uninduced cells were made and probed with an anti-histidine antibody (courtesy of Katze laboratory). The 23 kDa protein induced does have an epitope recognized by the anti-histidine antibody suggesting that it is the appropriate protein. Additionally, no other induced proteins were detected in this bacterial strain.

Fractionation of bacterial cells revealed that the his-*pal* fusion protein was recovered in the insoluble fraction. Preparation of inclusion bodies and extraction with 6M urea was performed to isolate the fusion protein. (Fig. 4.2, lanes 4 and 5). Column chromatography was used to further purify the his-*pal* protein away from bacterial proteins. The column is composed of a metal-

Figure 4.2. Protein gel showing purification of his-*pal* antigen. Isolated protein was loaded on 13% SDS polyacrylamide gels, electrophoresed and stained with Coomassie Blue. Protein loaded in each lane was sampled from the same preparation at different steps in the isolation procedure. Lane 1 shows total protein from whole cell preparations of uninduced DE3 cells bearing the his-*pal* plasmid. Lanes 2 and 3 show total protein from induced DE3 cells bearing the his-*pal* plasmid. Lane 4 shows protein isolated by purification and solubilization of inclusion body proteins. Lane 5 shows protein treated as in lane 4 after filtration to remove particulate debris. Lane 6 shows protein obtained from flow through of the Ni^{++} purification column. Lane 7 shows effluent from treatment with binding buffer. Lane 8 shows effluent with wash buffer. Land 9 shows effluent with elution buffer containing the his-*pal* protein. Lane 10 shows effluent when column is stripped with EDTA. The large arrow indicates the induced protein. The thin arrow indicates the degradation product after filtration procedure.


Lane: 1 2 3 4 5 6 7 8 9 10

chelating resin which binds nickel cations, and in turn the nickel cations bind histidine residues. Thus the presence of a polyhistidine tag in the fusion protein allowed selective retention of the fusion protein on the column (Fig. 4.2, lanes 6-10). As seen in Fig. 4.2, lanes 6-8, most bacterial proteins are removed with initial washes. Some degradation of the fusion protein occurred during pretreatment of the protein extract (compare lanes 4 and 5, samples before and after filtering, prior to loading on the column). Observation of smaller degraded form in the elution sample suggest that this degradation occurs at the C-terminus since the his-tag must be present for its retention on the column.

For immunization, the his-*pal* protein was purified additionally by SDS-PAGE. This electrophoresis allowed the elimination of any minor bacterial proteins present of a different molecular weight as well as allowing concentration of the protein. Because the fusion protein was insoluble without the presence of urea or SDS, it was not possible to immunize with a native form of the protein. Instead, gel slices were homogenized for immunization in rabbits.

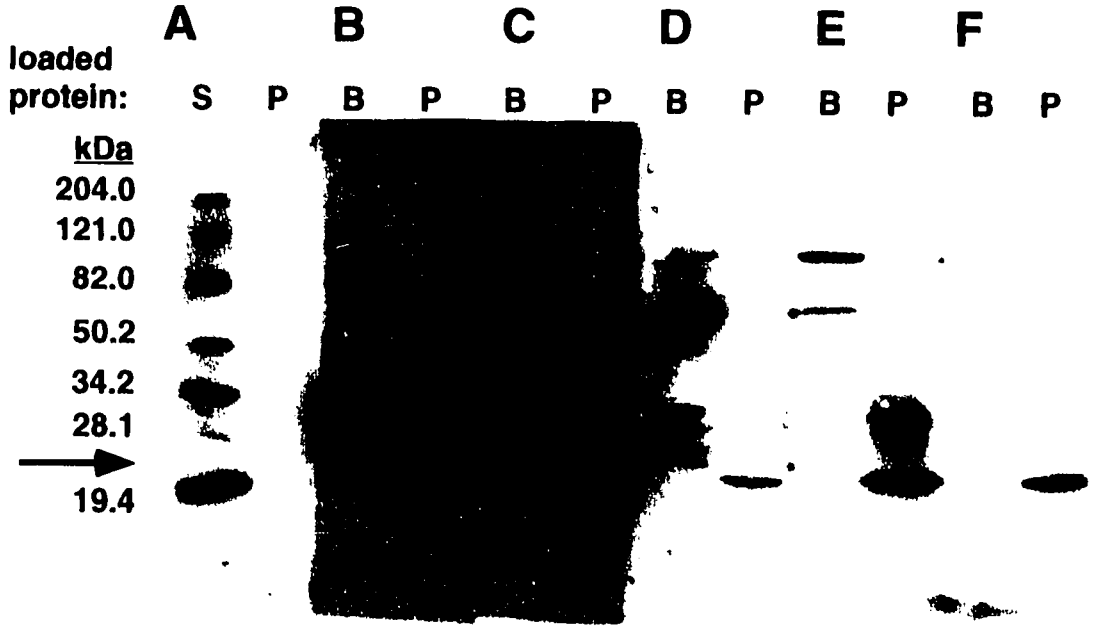
Antisera from each boost of three rabbits were tested against the bacterially expressed antigen to determine presence and titer of antibodies against the antigen as compared to preimmune sera. Western blot analysis revealed weak reactivity of antibodies after the first boost. Increasing titers of anti-his-*pal* antibodies were observed following subsequent boosts. After the fifth boost all animals were sacrificed and the final antiserum was collected. High dilutions (1:5000) of final antiserum easily recognized low levels of the his-*pal* antigen (approx. 30 ng). For most western blot analyses, antibody dilutions of 1:100 or 1:500 were used. (Much less reactivity is observed with

other his-tagged proteins, suggesting that most of the reactive antibodies recognize the *pal* portion of the antigen).

Western blot analysis of *Drosophila melanogaster* proteins revealed numerous bands using both preimmune sera and immune sera raised against the his-*pal* antigen, despite the selection of rabbits for lower preimmune reactivity to *Drosophila*. No new bands were observed among proteins extracted from adult males, females, embryos or hand-dissected testes using immune versus preimmune serum (data not shown). Because the preimmune serum recognized numerous bands, it is quite possible that a *pal* specific band would be masked by comigrating background bands. Similarly, in the initial immunocytochemical analysis of testes squashes, the immune serum did not give a definitive signal above the preimmune background levels.

To eliminate background signal, affinity purification of the total his-*pal* antiserum was performed. Purified his-*pal* antigen was bound covalently to a column. Anti-his-*pal* antibodies were selectively bound and eluted with changes in pH and salt concentration. Fractions of antibodies were tested for reactivity to bacterially expressed his-*pal* antigen by incubation of strip blots bearing purified his-*pal* protein and total bacterial protein (Fig.4.3D-F). Comparison of total immune serum to affinity purified serum showed a decrease in reactivity to bacterial background. Some simultaneous loss of reactivity to his-*pal* antigen was observed. Clearly, some antibodies of weak affinity fail to bind to the affinity purification column as evidenced by the reactivity in the void volume fractions, Fig. 4.3D. It is also possible that strongly bound antibodies may not have eluted from the column. Nevertheless, the affinity purified serum meets the desired requirements in

Figure 4.3. Western blot analyses of antiserum to the his-*pal* protein. Proteins were expressed in bacteria, isolated, electrophoresed on 13% SDS-polyacrylamide gels, and blotted. Each blot was probed with serum, then a secondary goat anti-rabbit IgG antibody conjugated to alkaline phosphatase and signal was detected by autoradiography using the ECL chemiluminescence system. Equal amounts of protein were loaded on each blot of either the purified his-*pal* protein (**P**), total protein from the DE3 cells alone (**B**), or protein standards (**S**). An autoradiograph of blots is shown after incubation with preimmune serum (panel **A**), whole immune serum (panel **B**), immune serum subtracted with bacterial acetone powder (panel **C**), serum derived from flow through of affinity purification column (panel **D**), and affinity purified serum from two fractions (panels **E** and **F**). The arrow indicates the size of the his-*pal* protein, observed by staining with Ponseau S. (A larger band is also observed which seems to be an aggregate of his-*pal* protein forming in older or less denatured preparations.) The molecular weights of the protein size standards are indicated to the left of the figure.


A
B
C
D
E
F
loaded
protein:
S
P
B
P
B
P
B
P
B
P
B
P
kDa
204.0
121.0
82.0
50.2
34.2
28.1
19.4

that the background is reduced and there is reasonable reactivity to his-*pal* for further experiments.

Initial assessment of the affinity purified antibodies by western blot analysis of *Drosophila melanogaster* proteins isolated from whole males or testes revealed a decrease in background levels but no immune-specific reactivity. Further experiments were performed using a variety of extraction conditions, including preparations of histone and non-histone chromosomal proteins, RNA binding protein extractions and membrane protein extractions (Joffe et al., 1977, Kellum et al., 1995, Teng et al., 1994, Matunis et al., 1994, Hortsch et al. 1994). None of the tested conditions permitted detection of a *pal* specific protein. It is possible that the protein is not extracted from insoluble material, or that it is particularly unstable or rare.

Affinity purified antibodies were used for immunocytochemical analysis of whole mount testes preparations. Wildtype testes showed immunoreactivity to nuclei of elongating spermatids most predominantly (Fig. 4.4A and Fig. 4.5E). Costaining with the DNA-binding dye DAPI was used to verify the location of nuclei (Fig 4.4B and Fig. 4.5F). No differential fluorescence within the nuclei was apparent; antibody immunoreactivity was coincident with DAPI staining. Additionally occasional fluorescence of sperm tails in a bundle was observed. Examination of whole mount testes from pal^2 males showed no fluorescence of sperm nuclei in bundles. This observation suggests that the antibody fluorescence is indeed specifically due to the *pal* protein (Fig. 4.4C and D). Furthermore, affinity purified serum was subtracted of antibodies recognizing the his-*pal* antigen (using affinity purification columns). The subtracted serum was used on preparations of wildtype testes and failed to bind specifically (Fig. 4.4E and F). Both in preparations treated

Figure 4.4. Immunocytochemical analysis of testes. Testes from Canton S wildtype (A,B,E and F), and *pal*^{2} males (C and D) were incubated with affinity purified serum raised against bacterially expressed *pal* protein (A,C) or subtracted serum (E), and with the DNA stain, DAPI (B,D,F). Nuclei of bundled elongating spermatids exhibit immunoreactivity in wildtype males (panels A and B). No or little fluorescence is observed in *pal*^{2} males (panels C and D) or in wildtype males incubated with subtracted serum (panels E and F).

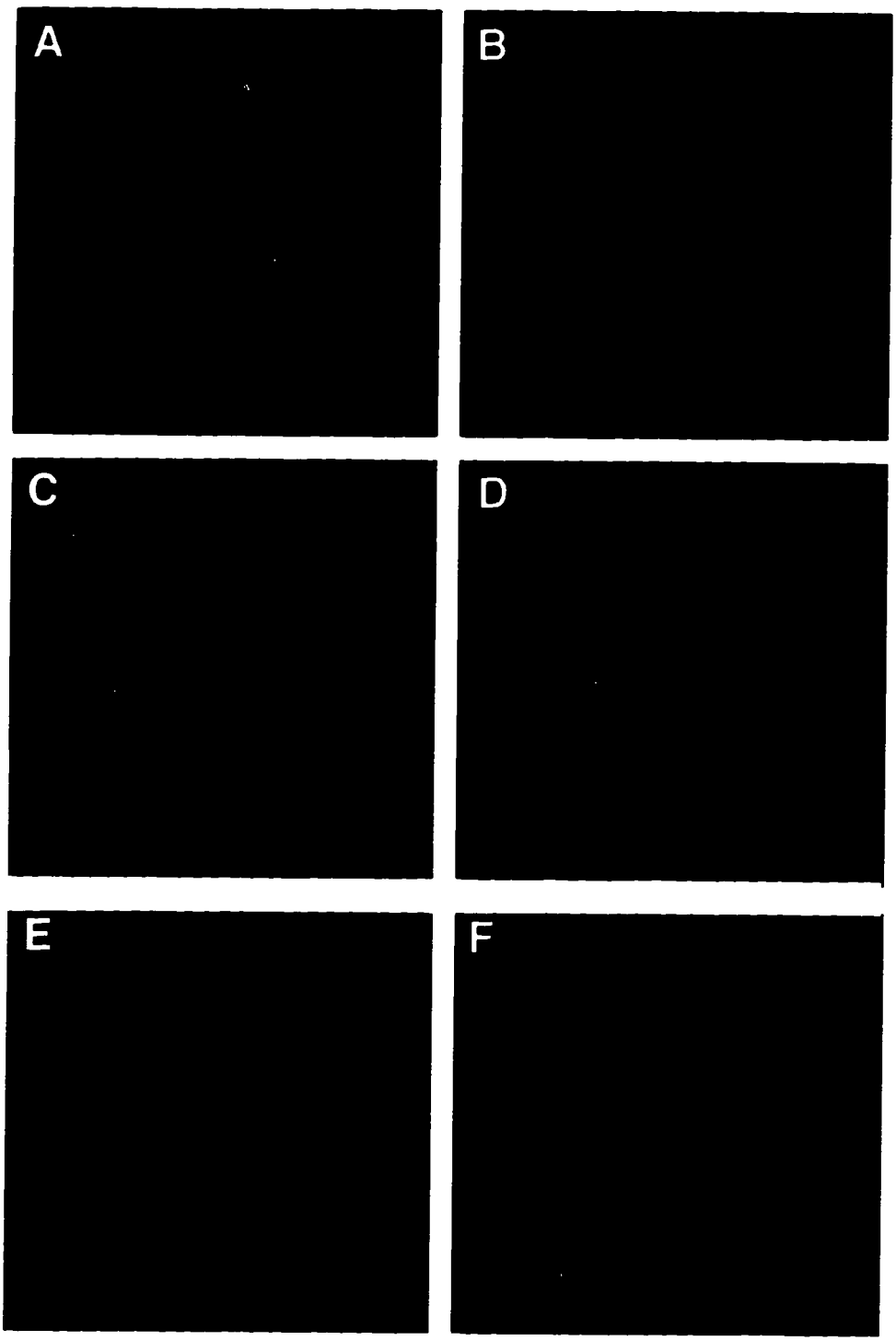
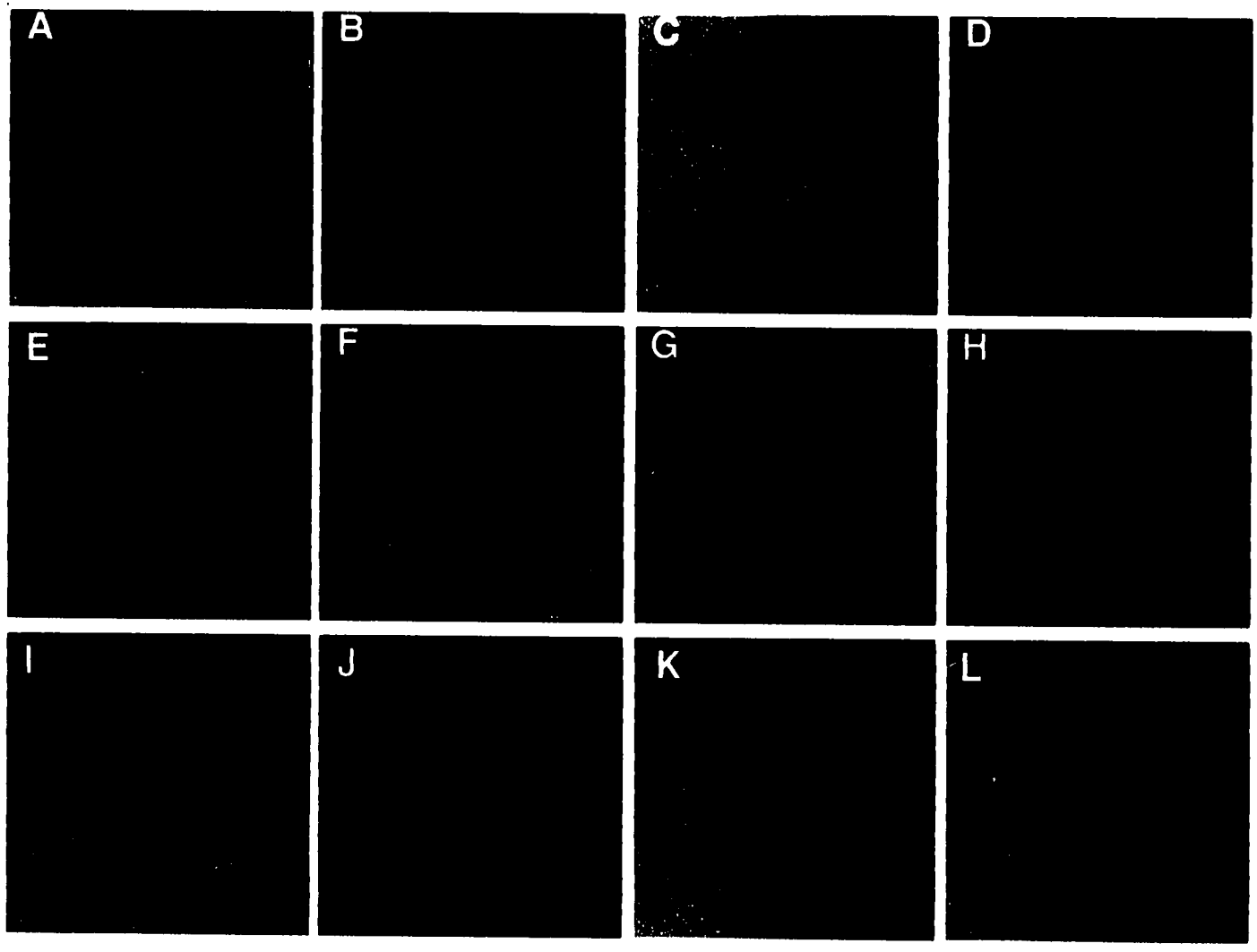

A
B
C
D
E
F

Figure 4.5. Immunocytochemical analysis of dissected testes with affinity purified serum against *pal* protein. Testes from Canton S wildtype males (A-F,I and J) or *pal*^{2} males (G and H) or embryos from *snky* fathers crossed to Sevelin wildtype mothers (K and L) were incubated with affinity purified serum against the *pal* protein (A,C,E,G,I,K) or the DNA stain DAPI (B,D,F,H,J,L). Testes were dissected prior to mounting but after fixation and staining to allow better visualization. Panels A and B show fluorescence in primary spermatocytes. Panels C and D show fluorescence in early round spermatids. Panels E and F show fluorescence in bundled elongation spermatids in wildtype males, while panels G and H show decreased fluorescence in *pal*^{2} males. Panels I and J show fluorescence of individualized sperm. Panels K and L show fluorescence of sperm in embryos derived from *snky* fathers which arrest development after sperm entry.


A
B
C
D
E
F
G
H
I
J
K
L

with subtracted serum and preparations of testes from *pal*^{2} males, immunoreactivity was still occasionally observed to sperm tails in bundles, suggesting that this binding is non-specific.

Other stages of cells in the testes show low levels of fluorescence with affinity purified antibodies. In order to visualize better particular cell types, testes which had been fixed were dissected prior to examination. In primary spermatocytes, fluorescence appears in the cytoplasm, but little or no immunoreactivity is apparent in primary spermatocyte nuclei (Fig. 4.5A and B). In contrast, early round spermatids exhibit very faint fluorescence primarily in the nucleus, and a less detectable signal is observed in the cytoplasm (Fig. 4.5 C and D). It is possible that low levels of protein are present in the cytoplasm which remain indistinguishable from background. Nevertheless, by the elongating spermatid stage bright nuclear fluorescence is observed. Mature individualized sperm appear to bind antibodies though the immunoreactivity is weaker than the signal present in sperm bundles (Fig. 4.5 I and J). It is unclear whether the difference in intensity of the antibody signal is due to a decrease in protein levels, accessibility of the antibody to its epitopes or (perhaps more likely) difficulty in detecting signal from a single sperm in contrast to a bundle of 64 spermatids. The last explanation is supported by a similar observation that DAPI staining of individual sperm is more difficult to detect. It is also plausible that accessibility is limited both for DAPI and antibody staining.

Presence of signal in individualized sperm suggested that the *pal* protein may still be present in sperm nuclei after transfer to females and fertilization of eggs. Additionally, if the *pal* protein is transferred to the embryo, is the *pal* protein removed prior to mitosis or does it persist on

paternal chromosomes? This question was addressed by analyzing embryos derived from *snky/snky* males crossed to Sevelin wildtype females. *snky* is a paternal effect mutation which arrests development shortly after sperm entry with no apparent conversion of the sperm nucleus to a male pronucleus. Thus, by analyzing *snky*--derived embryos, it is possible to view the sperm nucleus in the absence of alterations in the nucleus occurring normally after sperm entry. When *snky*-derived embryos were incubated with *pal* antibodies, fluorescence of the sperm head was observed (Fig. 4.5 K and L). Additionally, fluorescence of the female meiotic products (polar bodies and female pronucleus) was also observed (data not shown). Similarly, when female sperm storage organs were incubated with the affinity purified antibodies, sperm nuclei exhibited fluorescence (data not shown). Nuclei of the storage organ epithelia, however, were also fluorescent. This result suggests that there is a cross-reactive staining of nuclei in storage organ epithelia and female meiotic products. While it is formally possible that the *pal* product is transferred from the sperm nucleus to the female nuclei, this possibility seems unlikely since there is no development of the male pronucleus in *snky*-derived embryos (K. Fitch, personal communication). It seems more plausible that an epitope common to both the *pal* protein and another nuclear protein is identified by antibodies used. This cross-reactivity renders tests of whether staining of later stage embryos is due to *pal* or another protein inconclusive. Similarly, in the testes of pal^2 animals, background signal is observed in most cell stages. Therefore, it is not clear whether fluorescence can decisively be attributed to the *pal* antigen in stages other than the bundled elongating spermatids as opposed to another cross-

reacting species. In the future, perhaps it will be possible to remove such cross-reactive antibodies, allowing this question to be addressed.

Despite the absence of the *pal* protein, *pal* mutants exhibit no apparent abnormalities during spermatogenesis when examined by light microscopy (data not shown). From genetic analysis, the observation of mosaic progeny of *pal* fathers imply that defects do occur in early embryogenesis. To determine the nature of these defects and when they occur, the phenotype of *pal*-derived embryos was compared to wildtype embryos during the earliest stages of development (in collaboration with K. Wilson). In these studies, embryos were collected and fixed in batches at intervals from 15' to 90' after egg deposition. Nuclear and chromosomal morphologies were examined using fluorescent microscopy after staining embryos with DAPI or with an anti-histone antibody. Microtubules and spindle morphology were examined with anti-α-tubulin antibody.

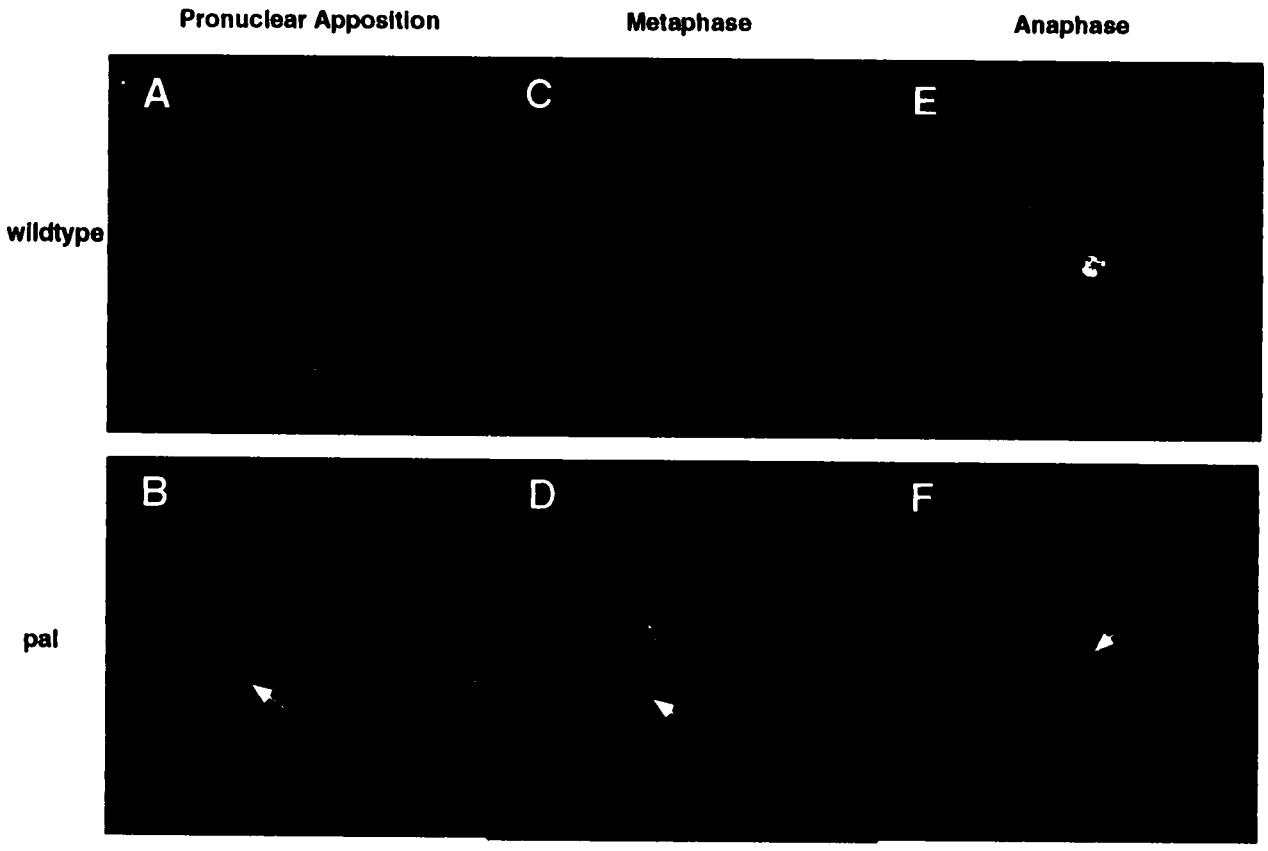
In embryos of wildtype fathers, the sperm nucleus appears as a tightly condensed DAPI-positive body in embryos fixed within 15 minutes of egg deposition. As the sperm traverses the distance from the point of entry at the micropyle to a more central position in the egg, it decondenses into an interphase state. After completion of meiosis, the female pronucleus is formed and migrates to a position about 75% egg length. Once formed, the male pronucleus stains with anti-histone antibody and occupies a position closely apposed to the female pronucleus. The maternal and paternal chromosomes condense into metaphase chromosomes and align in separate clusters on the metaphase plate forming the gonomeric spindle (Huettner, 1924). After the completion of cycle 1, twelve rapid, synchronous nuclear divisions occur before cellularization of the blastoderm.

Embryos of wildtype fathers ranging in age from precycle 1 to cycle 10 were characterized by confocal microscopy (n=107). Of these, only 3.6% showed abnormalities. In contrast, 27% of the 167 embryos derived from *pal*^{1}/*pal*^{1} (n= 65) or *pal*^{1}/*Df(2L)γ15* (n=102) fathers were abnormal. Categories of abnormalities were: the presence of micronuclei adjacent to prophase or interphase nuclei (Figure 4.6,D), mitotic spindles with a chromosome displaced from the spindle (Figure 4.6,E), anaphase configurations with a lagging chromosome (Figure 4.6,F), asynchrony among nuclei of an embryo, and abnormal tubulin arrays, some of which were not associated with nuclei or chromosomes.

Many of these defects are consistent with the idea that the absence of the *pal*$^{+}$ product causes a mitotic chromosome loss that is manifested as a failure of kinetochore function during metaphase or anaphase (Baker 1975). According to this model, the presence of micronuclei at interphase of cycle 2 and later stages may result from chromosome loss at the preceding mitosis, followed by nuclear envelope formation around the isolated chromosome. Observations of cycle 1 embryos, however, provide evidence that chromosome loss events can occur prior to mitosis. Micronuclei adjacent to the male pronucleus were observed prior to or at the stage of apposition with the female pronucleus.

Based on an analysis of the proportion of tissue showing chromosome loss in the progeny of *pal*^{1} fathers, Baker (1975) suggested that most of the loss events occur within the first three or four embryonic cycles. However, in this work, a *pal*$^{-}$associated defect in embryogenesis was observed as early as formation of the male pronucleus.

Figure 4.6. Cytological analysis of embryos from *pal* fathers reveals several aberrations. Embryos from wildtype(A, C, E) and *pal*$^-$ (B, D, F) fathers were incubated with anti-histone antibodies (A-F) and anti-tubulin antibodies (C-F) followed by incubation with rhodamine- and BODIPY-conjugated secondary antibodies, respectively. Panels A and B show pronuclear apposition in cycle 1 embryos. An arrow in panel B indicates micronucleus apposed to the paternal pronucleus. Panels C and D show metaphase stage nuclei in cycle 2 (C) and cycle 5 (D) embryos. An arrow indicates a chromosome off the metaphase plate in the *pal*-derived embryo. Panels E and F show anaphase stage nuclei in cycle 7 embryos. An arrow indicates a chromosome lagging in a *pal*-derived embryo.


Pronuclear Apposition
Metaphase
Anaphase
A
C
E
wildtype
B
D
F
pal

Discussion

Examination of the *pal* protein expression by antibody immunoreactivity revealed that the protein is present in the nuclei of maturing spermatids. Decrease of immunoreactivity with subtracted serum (following preabsorption with the his-*pal* antigen) and decrease or absence in testes of pal^2 males argue that this signal is specific to the *pal* protein. In wildtype animals, fluorescence of spermatids is strongest in late bundled sperm. Although *pal* RNA is readily detected in primary spermatocytes, only low levels of *pal* protein can be detected in the cytoplasm of primary spermatocytes and nuclei of round spermatids. Little or no nuclear fluorescence was observed in meiotic stages of larval testes (data not shown). While low levels of the *pal* protein may be present during meiotic stages, it seems more likely that the *pal* protein is involved in post-meiotic processes for several reasons. Nondisjunction is not observed above background in progeny of *pal* fathers, supporting the idea that spermatogenic loss seems to occur after separation of chromosomes and the completion of meiosis. Perhaps more simply, all the phenotypes observed in *pal* progeny can be explained without invoking a role in meiosis, as discussed later in this chapter.

Observation of fluorescence in bundles of elongating sperm and possible later stages suggest that translational regulation may be important for *pal* expression. The expression of the *pal* RNA is most pronounced in primary spermatocytes and may persist to the round spermatid stage. There is little antibody signal in primary spermatocytes when high levels of *pal* RNA are present. Furthermore, by the time that the protein is the most intense there is no apparent RNA staining by *in situ* hybridization. These data argue

that *pal* RNA is not translated as soon as it is available, rather its translation is regulated in some manner. Perhaps, for instance, the shift of *pal* RNA localization from a tight perinuclear cap to a more homogeneous distribution in the cytoplasm is required for the onset of translation.

Staining of nuclei is consistent with the hypothesis that *pal* encodes a chromosomal protein, as was suggested earlier by the phenotypes associated with the *pal* mutation and by the predicted basicity of this small protein. Nuclear fluorescence appears to coincident with all the DNA in the nucleus, suggesting that the *pal* protein may bind the entire chromosome. However, it is possible that regional differences exist along the length of chromosomes that are not detectable in the small condensing sperm head.

Is the *pal* protein retained on the sperm nucleus of fully mature sperm upon transfer to the egg? Fluorescence of individualized sperm suggest that the *pal* protein may be brought into the embryo, but this remains to be convincingly demonstrated due to an apparent cross-reactive species in embryos. For this reason, it is unclear when the *pal* protein is last present and therefore when the last possible time of function is. If the *pal* protein does not enter the egg, then effects on the embryos of *pal* fathers must be due indirectly to earlier defects caused by the absence of the *pal* protein. Alternatively, the *pal* protein may remain on paternal chromosomes entering the embryo as part of the sperm nucleus at fertilization. If this is the case, it is possible that the *pal* protein functions in the early embryo. The protein, however, could be brought into the embryo, but only function earlier in the testes.

Examination of testes of *pal* mutants by light microscopy failed to detect any abnormalities during spermatogenesis. Analysis of embryos from *pal*

fathers showed that the earliest defect observed was micronuclei present prior to the first division. Additional defects include chromosomes off the metaphase plate, chromosomes lagging at anaphase, distorted spindles, and micronuclei at later divisions. All but the earliest phenotype are consistent with Baker's hypothesis that the *pal* product is a chromosomal protein acting during division to mediate interactions between the kinetochore and the spindle. Observation of micronuclei prior to mitotic division, however, suggest either that the function of the *pal* product is not restricted to mitotic division or that there is a second role of the *pal* product in addition to a mitotic division role. Furthermore, studies by Hardy (1975) demonstrate that micronuclei formed in spermatogenesis are lost prior to the formation of the mature sperm. This observation implies that the micronuclei found prior to cycle 1 in the progeny of *pal* fathers have arisen during the first events of embryonic development.

A model of *pal* function

One way to account for the effect on paternal chromosomes is to postulate that the *pal* protein is a component of the sperm-specific chromatin. My working hypothesis is that the *pal* product may directly affect the condensation or decondensation of chromatin. In particular, *pal* may be involved in the histone transition. Absence of *pal* protein may lead to problems condensing the paternal chromosomes during spermiogenesis and decondensation and/or recondensation of these chromosomes during early embryogenesis. At this time it is unclear whether the *pal* protein functions during spermiogenesis or early embryogenesis.

When the *pal* protein is absent, a defect may occur in the ability of chromosomes to interact with other cellular structures due to the state of

chromosome condensation. The *pal* protein may be important for keeping individual chromosomes grouped together, perhaps by influencing interactions with the nuclear envelope or the cytoskeleton. Difficulty condensing chromosomes during spermiogenesis may result in asynchronous packaging; thus one chromosome may be surrounded in a separate nuclear membrane from the remaining chromosomes. Effects on the condensation of the nucleus during spermiogenesis might be expected to lead to arrest of sperm development as appears to be the case with affected sperm in the Segregation Distorter phenomena (Tokuyasu, 1977 and reviewed in Sandler and Golic, 1985). While there is no problem with condensation of *pal* sperm nuclei or with decondensation in embryos of *pal* fathers that was detectable by light microscopy, effects too subtle for detection may be sufficiently to cause the levels of chromosome loss observed. Similarly, a chromosome which is decondensed late or improperly during embryogenesis could cause separate packaging of this chromosome, and result in micronuclei seen prior to the first mitotic division. During mitosis, improper compaction of the chromosome may prevent or destabilize interactions with the spindle microtubules or related cellular machinery and may cause defects in mitosis. Clearly, from genetic data shown earlier, many progeny of *pal* fathers develop normally. If there are defects in condensation they are not expected to be large effects or else more abnormal animals would be observed.

This hypothesis is appealing because it describes a spermatogenesis specific process and could explain both the observation that only progeny of *pal* fathers (not mothers) are affected and that only paternal chromosomes (not maternal chromosomes) in the embryo are affected. This model is

consistent with the observation that the *pal* protein appears to be bound to the paternal chromosome during spermiogenesis and perhaps persists into embryogenesis.

The histone transition, which is best documented in the mouse, defines the process in which histones are replaced by transition proteins which are replaced in turn by protamines during spermiogenesis (Risley, 1982; Oliva and Dixon, 1991). It is thought that the histone transition is responsible for changes in chromatin architecture resulting in the compaction/condensation of the chromatin to a fraction of its original volume. Cytochemical studies of Das, Kaufmann and Gay (1962 and 1964a) demonstrate that a process analogous to the histone transition of the mouse also occurs in *D. melanogaster*, but indicate that it is a one-step process presumably replacing lysine-rich histones with arginine-rich histone variants, without a final replacement by protamine. Russell and Kaiser (1993) have isolated one possible component of the sperm specific chromatin - a gene with homology to H5-type histones and cysteine-rich protamines. As noted previously, the small size and highly basic nature of the predicted *pal* protein lend credence to the idea that it may act as a chromosomal protein; furthermore, the location of the *pal* protein in the nuclei of the maturing spermatid is appropriate for a role in the condensation of the sperm nucleus. In embryos, the spermatogenesis specific chromosomal proteins are replaced by juvenile somatic histones until cellular blastoderm, when they are replaced by the mature somatic histones (Das, Kaufmann and Gay, 1964b and Das and Gay, 1966). It should be noted that these cytochemical studies provide only a general view of what may be a complex and dynamic process. The molecular components involved in chromosome condensation and

decondensation are just beginning to be isolated in *Drosophila* (dNAP-1, Ito et al., 1996, DF 31; Crevel and Cotterill, 1995). The DF 31 protein is particularly interesting because it causes decondensation of *Xenopus* sperm *in vitro* and thus could be involved in sperm decondensation *in vivo* in *Drosophila* (Crevel and Cotterill, 1995).

Why are chromosomes lost in subsequent mitotic divisions?

If the *pal* protein is a sperm specific chromatin protein and sperm specific chromosome proteins are removed in the formation of the male pronucleus, then why does the phenotype persist after the first division? I suggest that the persistent phenotype reflects aberrant chromatin structure distinguishing paternal from maternal chromosomes and propose two models. In the first model, there may be some mechanism for passing an imprint to new strands and thus defective paternal chromosomes would give rise to newly formed but defective chromosomes. Alternatively, a second model is that perhaps only the chromosomes bearing strands of DNA formed and packaged during spermatogenesis are lost. Or in other words, the chromosomes are imprinted but cannot pass that imprint to newly replicated strands. Other sperm specific proteins may remain improperly bound to chromosomes beyond the normal time in development due to an absence of the *pal* protein. It is likely that the improperly bound proteins will not be removed during subsequent mitoses any more readily than in the first division (particularly since it seems unlikely that the mechanism for the transition to a male pronucleus operates beyond the first division). Thus, any chromosome which is defective but was not lost in the first division may be lost in each subsequent division. Large patch sizes in mosaic embryos of *pal* fathers indicate that most loss events occur early in development. It should

be noted that there is another major remodeling of chromatin at cellular blastoderm (Das, Kaufmann and Gay, 1964b and 1966). This event may represent a second opportunity to rectify defects in *pal*-derived chromosomes. There may also be an earlier remodeling of chromatin after cycle six. Ners and Trever (1994) have observed that HMG-D is present in the nuclei of embryos for the first six divisions in place of histone H1.

How can paternal chromosomes be distinguished from maternal chromosomes? There is some evidence in *Drosophila* for the ability to distinguish chromosomes using chromosomal proteins. An acetylated form of histone H4, a well as the MSL-1 and MLE proteins, are bound preferentially to the more highly condensed X chromosome in *Drosophila* males and are implicated in distinguishing the X chromosome for dosage compensation (Bone et al., 1994; Lavender et al., 1994). Perhaps other chromosomal proteins or modified forms of chromosomal proteins are similarly used to distinguish maternal and paternal chromosomes. Later in development, increased phosphorylation of the heterochromatin binding protein HP-1 coincides with the appearance of heterochromatin (Eissenberg, 1994). It is not clear if the phosphorylation of this protein is causally linked to the formation of heterochromatin. Acetylation of histones has been implicated in cell cycle regulation (reviewed in Turner, 1995; Turner and O'Neill, 1995; Loidl, 1994). The predicted *pal* protein has numerous lysine and serine residues which could be acetylated or phosphorylated, respectively. It remains to be seen whether such modifications of the *pal* protein occur and if so whether they affect function of this protein. Such modifications of chromosomal proteins may affect protein function and may indirectly regulate chromosome function, perhaps by maintaining heritable differences among chromosomes.

In summary, the presence of *pal* protein in nuclei of maturing spermatids suggests a role for *pal* in the process of condensation or decondensation of chromosomes. I will address remaining questions and suggest future studies in Chapter 5.

CHAPTER 5

CONCLUDING REMARKS

Analysis of the paternal contributions allows a more complete understanding of the processes of early embryogenesis. In my dissertation, I have sought to understand better the role of one such paternal effect gene, *paternal loss*. Below I have summarized briefly what is now known about the *pal* gene and its product.

- The *pal* gene is a strict paternal effect gene, expressed only in males. Absence of this function leads to loss of paternal chromosomes in progeny of *pal* fathers.
- Only a proportion of progeny of males homozygous for null alleles exhibit chromosome loss events, suggesting that the function of the *pal* gene is partially redundant with that of other genes.
- The *pal* transcript is testes-specific and is predicted to encode a novel small basic protein of only 121 amino acids. The transcript is expressed in primary spermatocytes and is initially localized as a polar, perinuclear cap, later becoming more diffuse in the cytoplasm.
- The *pal* protein is predominantly detected in the nuclei of elongating spermatids, suggesting that *pal* is a sperm-specific chromosomal protein.

These data suggest that mutations in the *pal* gene affect only paternal chromosomes because the *pal* function is specific to the process of spermatogenesis. I propose that the *pal* protein functions by affecting the condensation state of the sperm chromatin. Absence of this product would result in improper packaging of chromosomes and their failure to interact

appropriately with other cellular components, such as the spindle and nuclear envelope. Many questions remain to be answered. Below, I present some of these questions and a few suggestions about future experiments to address them. Most are encompassed by one of two overriding questions: What is the function of the *pal* protein? How is the expression of *pal* regulated?

What is the function of the *pal* protein? Observation of *pal* protein in the nucleus of elongating spermatids suggests that this small basic protein binds chromatin. If this is the case, does *pal* protein bind DNA directly or indirectly? One could address this question by determining whether *pal* protein can bind DNA as such by performing gel shift assays. This would require the preparation of abundant *pal*+ protein either by bacterially expressing the protein or by purification directly from *Drosophila* tissue. Alternatively, one could determine if the *pal* protein copurifies with DNA in *Drosophila*. This would be facilitated by developing methods to identify *pal* protein on Western blots.

Why are chromosomes lost? What in particular happens during chromosome loss? Is chromosome condensation or decondensation actually affected? I have hypothesized that the *pal* protein functions either in the process of chromosome condensation or decondensation. An assay for condensation is required to test this hypothesis. There are several cytochemical methods for assessing chromatin states (e.g. Das et al., 1964), but these methods do not provide much information about regional differences in condensation state along the length of a chromosome. Ideally, one would like to have more refined methods for examining the state of chromatin in sperm or early embryonic divisions. As an alternative, one could express *pal*

protein ectopically in the salivary glands, where chromosome condensation of the large endoreplicated chromosomes can be more readily monitored. More subtle effects on condensation might be detectable using this approach. While such ectopic experiments would not prove endogenous activity, an observed effect would indicate the potential to affect condensation, justifying further development of more sensitive techniques in the early embryo.

It is unclear whether *pal* functions during spermiogenesis or in early embryogenesis. When is the latest time *pal* protein is present during development? If *pal* protein is present in the embryo, does chromosome loss in progeny of *pal* fathers result directly from absence of a function normally occurring in embryos? Effects in *pal* mutants may be indirect due to an absence of the *pal* protein in the testis. Knowing definitively whether the *pal* protein is present on the sperm as it enters the egg would help address this question. Unfortunately, cross-reactivity of the anti-*pal* antibodies with embryonic nuclei prevents answering this question at this time. One way around this difficulty might be to sort out which antibodies among the polyclonal mix are cross-reactive with other proteins and which are specific to *pal* protein. The polyclonal sera were raised against a *pal* fusion protein containing all but the N-terminal six amino acids. It is possible that the cross-reactive antibodies map to a particular region of the *pal* protein, which could be determined by peptide mapping. If this is the case, small fragments of the *pal* gene could be cloned into an expression vector in order to overexpress smaller regions of the *pal* protein. One could then try to use different portions of the *pal* protein to affinity purify a subset of the antibodies from the polyclonal sera. Hopefully one might identify fractions of antibodies which bind only *pal* protein (i.e. no staining at all in *pal* mutants). One could

then ask when the pal protein is normally removed from the paternal chromosomes.

Are the entering strands of DNA the only ones which are lost or can the defect be passed heritably to DNA synthesized in the embryo? In other words, can we demonstrate the maintenance of an imprint? In order to address this question, one needs a means of distinguishing the paternal chromosomes brought in by the sperm from the newly made copies produced in the embryo. (Experiments are underway by Barbara Wakimoto to use Brd-U labeling of sperm produced by *pal* fathers to follow paternal chromosomes during loss events.) If *pal* is observed on the paternal chromosomes beyond the first division, are all paternal chromosomes labelled, indicative of a persistent imprint, or are only a subset of chromosomes labelled? If only a subset are labelled, does the number correspond to the number of paternal chromosome strands at fertilization? This would indicate failure to maintain a paternal imprint beyond the first division.

Why is the penetrance of the *pal* mutation weak? Are there other factors partially redundant to the *pal* function? If so, what are they? One could try to identify such factors genetically by screen for second site enhancers of the *pal* phenotype. At an extreme, severe chromosome loss is predicted to cause male sterility due to loss of the large autosomes bearing essential functions. The low rates of *pal* loss observed with *pal* null alleles, however, provides ample range for identifying enhancers of *pal*. A laborious but rather straightforward approach would be to mutagenize *pal* strain and test mutagenized *pal/pal* homozygous males for increased rates of paternal chromosome loss (>15%) or male sterility.

Additionally, one could test known mutations directly for interactions with *pal*. If *pal* is indeed a chromosomal protein affecting the condensation state of the paternal chromosomes, one might expect other chromosomal proteins found in sperm or early embryos to affect the severity of the *pal* phenotype. Tomkiel (1991) has suggested that *pal*-induced chromosome loss is affected by deletions of various regions of heterochromatin. Suppressors and enhancers of position effect variegation affect the expression of genes placed in different chromatin context (euchromatin or heterochromatin). Mutations in these genes make reasonable candidates for affecting the rate of chromosome loss induced by pal mutations. Similarly it would be interesting to test mutations in genes encoding chromosomal proteins used in the first divisions, such as the HMG-D (Ners and Travers, 1994) gene, as they become available.

How is the *pal* protein regulated? Is the RNA under translational regulation? Is the localization of the RNA important for regulating translation or protein function? What are the heterogeneous minor forms of *pal* RNA? Are they as suggested due to changes in polyA tail length? If so, does tail length represent a regulated or stochastic event? RNase protection assays to define 5' and 3' ends of the transcript may determine how this variability in transcript size arises, and in particular whether it is due to variation in polyA tail length. With which side of the nucleus is mRNA associated in primary spermatocytes? Anti-centrosomal antibodies could be used to stain centrosomes; alternatively rRNA probes could be used to identify the nucleolus. Both of these organelles are located asymmetrically in primary spermatocytes. Either could provide an orienting landmark to

distinguish where the *pal* transcript resides in the cell in concert with *in situ* localization of the *pal* RNA.

Is the *pal* protein modified covalently (phosphorylated, acetylated) in a way that affects its function? Acetylation of lysine residues appears to important for regulating the function of histone H4 (Turner and O'Neill, 1995). In this case, however, antibodies were raised against various acetylated isoforms of histone H4, enabling the authors to examine the differential use of these isoforms. It would be fortuitous to find antibodies among the isolated polyclonal sera with parallel specificity. Nevertheless, one could test whether particular residues are important for functions, for instance, by performing site-directed mutagenesis to replace lysine residues with non-acetylated residue, transforming the mutated gene into *pal*$^-$background, and assessing if chromosome maintenance is impaired.

By defining the function of the *pal* protein and by identifying and analyzing interacting components, one may gain a better understanding of normal events in the early embryo. The processes occurring in the first embryonic divisions will certainly have features unique to those divisions as well as features shared with many types of division. By better understanding the components that are regulated in different ways, perhaps we will gain a more complete understanding of the dynamic changes occurring during development.

BIBLIOGRAPHY

Arkhipova, I.R., 1995 Promoter elements in *Drosophila melanogaster* revealed by sequence analysis. Genetics **139**:1359-1369.

Ausubel, F.M., Brent, R., Kingston, R.E. et al. *Current Protocols in Molecular Biology*, New York: John Wiley and Sons, 1995.

Ayoubi, T.A. and W.J. VanDeVen, 1996 Regulation of gene expression by alternative promoters. FASEB Journal **10**:453-460.

Baker, B.S., 1972 Tests for chromosome breakage in the meiotic mutant *paternal loss*. Dros Inform Serv **49**:55.

Baker, B.S., 1975 *paternal loss (pal)*: a meiotic mutant in *Drosophila melanogaster* causing paternal chromosome loss. Genetics **80**:267-296.

Baker, B.S., Carpenter, A.T.C. and Gatti, M. On the biological effect of mutants producing aneuploidy in Drosophila. In: *Aneuploidy, Part A: Incidence and Etiology*, edited by Sandberg, A. and Vig, B. New York: Alan R. Liss, Inc., 1987, p. 273-296.

Baker, J., W.E. Theurkauf and G. Schubiger, 1993 Dynamic changes in microtubule configuration correlate with nuclear migration in the preblastoderm *Drosophila* embryo. Journal of Cell Biology **122(1)**:113-121.

Balhorn, R.S., C.T. Weston and A.J. Wyrobek, 1984 DNA packaging in mouse spermatids: Synthesis of protamine variants and four transition proteins. Experimental Cell Research **150**:298-308.

Barlow, D.P., 1994 Imprinting: a gamete's point of view. Trends in Genetics **10**:194-199.

Barlow, D.P., R. Stoger, B.G. Herrmann, K. Saito and N. Schweifer, 1991 The mouse *insulin-like growth factor type-2 receptor* is imprinted and closely linked to the *Tme* locus. Nature **349**:84-87.

Bartolomei, M.S., S. Zemel and S.M. Tilghman, 1991 Parental imprinting of the mouse *H19* gene. Nature **351**:153-155.

Barton, S.C., M.A.H. Surani and M.L. Norris, 1984 Role of paternal and maternal genomes in mouse development. Nature **311**:374-376.

Bendena, W.G., A. Ayme-Southgate, J.C. Garbe and M.L. Pardue, 1991 Expression of heat-shock locus *hsr-omega* in nonstressed cells during development in *Drosophila melanogaster*. Developmental Biology **144**:65-77.

Berg, C.A., and A.C. Spradling, 1991 Studies on the rate and site specificity of P element transposition. Genetics **127**:515-525.

Berry, R.O., 1939 Observations on chromosome elimination in the germ cells of *Sciara ocellaris*. Proceedings of the National Academy of Sciences **25**:125-127.

Berry, R.O., 1941 Chromosome behavior in the germ cells and development of the gonads in *Sciara ocellaris*. Journal of Morphology **68**:547-583.

Bone, J.R. and J. Lavender, 1994 Acetylated histone H4 on the male X chromosome is associated with dosage compensation in *Drosophila*. Genes and Development **8**:96-104.

Brandeis, M., M. Ariel and H. Cedar, 1993 Dynamics of DNA methylation during development. BioEssays **15**:709-713.

Brandeis, M., T. Kafri, M. Ariel, J.R. Chaillel, J. McCarrey, A. Razin and H. Cedar, 1993 The ontogeny of allele-specific methylation associated with imprinted genes in the mouse. EMBO Journal **12**:3669-3677.

Braun, R.E., R.R. Behringer, J.J. Peschon, R.L. Brinster and R.D. Palmiter, 1989a Genetically haploid spermatids are phenotypically diploid. Nature **337**:373-376.

Braun, R.E., J.J Peschon, R.R Behringer, R.L. Brinster and R.D. Plamiter, 1989b Protamine 3'-untranslated sequences regulate temporal translational control and subcellular localization of growth hormone in spermatids of transgenic mice. Genes and Development **3**:793-802.

Breeuwer, A.J. and J.H. Werren, 1993 Cytoplasmic incompatibility and bacterial density in *Nasonia vitripennis*. Genetics **135**:565-574.

Brockdorff, N., A. Ashworth, G.F. Kay, P. Cooper, S. Smith, V.M. McCabe, D.P. Norris, G.D. Penny, D. Patel and S. Rastan, 1991 Conservation of position and exclusive expression of mouse *Xist* from the incative X chromosome. Nature **351**:329-331.

Brown, K.W., A. Gardner, J.C. Williams, M.G. Mott, A. McDermott and N.J. Maitland, 1992 Paternal origin of 11p15 duplications in the Beckwith-Wiedemann syndrome. Cancer Genet Cytogenet **58**:66-70.

Brown, S.W., 1957 Chromosome behavior in *Comstockiella sabalis* (Comstk.)(Coccoidea-Diaspididae). Genetics **42**:362-363.

Brown, S.W., 1963 The Comstockiella system of chromosome behavior in the armored scale insects (Coccoidae: Diasspididae). Chromosoma **14**:360-406.

Brown, S.W. and F.D. Bennett, 1957 On sex determination in the diaspine scale *Pseudaulacaspis pentagona* (Targ.) (Coccoidea). Genetics **42**:510-523.

Brown, S.W. and Chandra, H.S. Chromosome imprinting and the differential regulation of homologous chromosomes. In: *Cell Biology: A Comprehensive Treatise*, edited by Goldstein, L. and Prescott, D.M. 1977, p. 109-189.

Brown, S.W. and W.A. Nelson-Rees, 1961 Radiation analysis of the lecanoid genetic system. Genetics **46**:983-1007.

Brown, S.W. and L.I. Wiegmann, 1969 Cytogenetics of the mealybug *Planococcus citri* (Risso) (Homoptera: Coccoidea): Genetic markers, lethals, and chromosome rearrangements. Chromosoma **28**:255-279.

Browning, H. and S. Strome, 1996 A sperm-supplied factor required for embryogenesis in C. elegans. Development **122**:391-404.

Cai, M. and R. Davis, 1990 Yeast centromere binding protein *CBF1*, of the Helix-Loop-Helix protein family, is required for chromosome stability and methionine prototrophy. Cell **61**:437-446.

Cattanach, B.M. and M. Kirk, 1985 Differential activity of maternally and paternally derived chromosome regions in mice. Nature **315**:496-498.

Cavener, D.R., 1987 Comparison of the consensus sequence flanking translational start sites in *Drosophila* and vertebrates. Nucleic Acids Research **15**:1353-1361.

Cavener, D.R. and S.C. Ray, 1993 Eukaryotic start and stop translation sites. Nucleic Acids Research **19**:3185-3192.

Cenci, G., S. Bonaccorsi, C. Pisano, F. Verni and M. Gatti, 1994 Chromatin and microtubule orgainzation during premeiotic, meiotic and early post-meiotic

stages of *Drosophila melanogaster* spermatogenesis. Journal of Cell Science **107**:3521-3534.

Clarke, A., 1990 Genetic imprinting in clinical genetics. Development Suppl.:131-139.

Cooper, D.W., J.J. VandeBurg, G.B. Sharman and W.F. Poole, 1972 Phosphoglycerate kinase polymorphism in kangaroo provides further evidence for paternal X inactivation. Nature, New Biology **230**:154-155.

Crevel, G. and S. Cotterill, 1991 DNA replication in cell-free extracts from *Drosophila melanogaster*. European Journal of Molecular Biology **10(13)**:4361-4369.

Crevel, G. and S. Cotterill, 1995 DF31, a sperm decondensation factor from *Drosophila melanogaster*: purification and characterization. European Journal of Molecular Biology **14**:1711-1717.

Crouse, H.V., 1960 The controlling element in sex chromosome behavior in *Sciara*. Genetics **45**:1429-1443.

Curgy, W. and W.A. Anderson, 1972 Autonomous mitochondrial RNA synthesis during spermiogenesis in *Drosophila*. Zeitschrift fur Zellforschung und Mikroskopische Anatomie **125**:31-44.

Das, C.C., 1962 Analysis of the progress of spermatogenesis in *Drosophila* Histone transition during *Drosophila* spermiogenesis. Carnegie Institution of Washington Yearbook **61**:504-507.

Das, C.C., B.P. Kaufman and H. Gay, 1964 Histone-protein transition in *Drosophila melanogaster* I. Changes during spermatogenesis. Experimental Cell Research **35**:507-514.

Das, C.C., B.P. Kaufmann and H. Gay, 1964 Histone protein transition in *Drosophila melanogaster* II. Changes during early embryonic development. Journal of Cell Biology **23**:423-430.

Das, C.C., B.P. Kaufmann and H. Gay, 1966 Cytoplasmic basic proteins during embryogenesis in *Drosophila melanogaster*. Journal of Cytology and Genetics **1**:1-5.

Davie, J.R. and M.J. Hendzel, 1994 Multiple functions of dynamic histone acetylation. Journal of Cellular Biochemistry **55**:98-105.

Davis, D.G., 1969 Chromosome behavior under the influence of *claret-nondisjunctional* in *Drosophila melanogaster*. Genetics **61**:577-594.

DeChiara, T.M., E.J. Robertson and A. Efstatiadis, 1991 Parental imprinting of the *mouse insulin-like growth factor II* gene. Cell **64**:849-859.

Dorn, R., V. Krauss, G. Reuter and H. Saumweber, 1993 The enhancerof position-effect variegation of Drosophila, *E(var)3-93D*, codes for chromatin protein containing a conserved domain common to several transcriptional regulators. Proceedings of the National Academy of Sciences **90**:11376-11380.

Edgar, B., 1995 Diversification of cell cycle controls in developing embryos. Current Opinions in Cell Biology **7**:815-824.

Eissenberg, J.C. and T. Hartnett, 1993 A heat shock-derived cDNA rescues the recessive lethalility of mutations in the heterochromatin-associated protein HP1 of *Drosophila melanogaster*. Molecular and General Genetics **240**:333-338.

Eissenberg, J.C., Y.W. Ge and T. Hartnett, 1994 Increased phosphorylation of HP1, a heterochromatin-associated protein of Drosophila, is correlated with heterochromatin assembly. Journal of Biological Chemistry **269**:21315-21321.

Endow, S.A. and M. Hatsumi, 1991 A multimember kinesin gene family in Drosophila. Proceedings of the National Academy of Sciences **88**:4424-4427.

Endow, S.A., S. Henikoff and L. Soler-Niedziela, 1990 Mediation of meiotic and early mitotic chromosome segregation in *Drosophila* by a protein related to kinesin. Nature **345**:81-83.

Erdelyi, M. and J. Szabad, 1989 Isolation and characterization of dominant female sterile mutations of *Drosophila melanogaster*. I. Mutations on the third chromosome. Genetics **122**:111-127.

Farrell, R.E.Jr. *RNA Methodologies*, San Diego:Academic Press, 1993.

Feinberg, A.P. and B. Vogelstein, 1984 A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Analytical Biochemistry **137**:266-267.

Foe, V.E., Odell, G.M. and Edgar, B.A. Mitosis and Morphogenesis in the *Drosophila* embryo: point and counterpoint. In: *The Development of Drosophila melanogaster*, Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1993.

Forejt, J. and S. Gregorova, 1992 Genetic analysis of genomic imprinting: An *Imprintor-1* gene controls inactivation of the paternal copy of the mouse *Tme* Locus. Cell **70**:443-450.

Fuller, M.T. Spermatogenesis. In: *The Development of Drosophila melanogaster*, edited by Bate, M. and Martinez-Arias, A. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1993,

Fuyama, Y., 1984 Gynogenesis in *Drosophila melanogaster*. Japanese Journal of Genetics **59**:91-96.

Fuyama, Y., 1986 Genetics of parthenogenesis in *Drosophila melanogaster*. II. Characterization of a gynogenetically reproducing strain. Genetics **114**:4995-509.

Fuyama, Y., 1986 Genetics of parthenogenesis in *Drosophila melanogaster*. I. The modes of diploidization in the gynogenesis induced by male-sterile mutant, *ms(3)K81*. Genetics **112**:237-248.

Garner, C.C., R.P. Tucker and A. Matus, 1988 Selective localization of messenger RNA for cytoskeletal protein MAP2 in dendrites. Nature **336**:674-677.

Gatti, M. and B.S. Baker, 1989 Genes controlling essential cell cycle functions in *Drosophila melanogaster*. Genes and Development **3**:438-453.

Gatti, M. and Goldberg, M. Mutations affecting cell division in Drosophila. In: Methods in Cell Biology, vol. 35, San Diego: Academic Press, 1991, p. 544-582.

Gavis, E.R. and R. Lehmann, 1994 Translational refulation of *nanos* by RNA localization. Nature **369**:315-318.

Gelbart, W.M., 1974 A new mutant controlling mitotic chromosome disjunction in *Drosophila melanogaster*. Genetics **76**:51-63.

Giddings, S.J., C.D. King, K.W. Harman, J.F. Flood and L.R. Carnaghi, 1994 Allele specific inactivation of *insulin 1 and 2* in the mouse yolk sac indicates imprinting. Nature Genetics **6**:310-313.

Gould-Somero, M. and L. Holland, 1974 The timing of RNA synthisis for spermiogenesis in organ cultures of *Drosophila* testis. Wilhelm Roux's Arch **174**:133-148.

Hackstein, J.H.P., 1991 Spermatogenesis in Drosophila. A genetic approach to cellular and subcellular differentiation. European Journal of Cell Biology **56**:151-169.

Hall, J.G., 1990 How imprinting is relevant to human disease. Development Suppl.:141-148.

Han, J.R., W. Gu and N.B. Hecht, 1995a Testis-brain RNA-binding protein, a testicular translational regulatory RNA binding protein, is present in the brain and binds to the 3' untranslated regions of transported brain mRNAs. Biology of Reproduction **53**:707-717.

Han, J.R., G.K. Yiu and N.B. Hecht, 1995b Testis/brain RNA-binding protein attaches translationally repressed and transported mRNAs to microtubules. Proceedings of the National Academy of Sciences **92**:9550-9554.

Harlowe, E. and Lane, D., *Antibodies: A Laboratory Manual*, New York :Cold Spring Harbor Laboratory, 1988.

Harper, M.I., M. Fosten and M. Monk, 1982 Preferential paternal X-inactivation in extraembryonic tissue of early mouse embryos. Journal of Embryology and Experimental Morphology **67**:127-135.

Hatada, I., T. Sugama and T. Mukai, 1993 A new imprinted gene cloned by a methylation-sensitive genome scanning method. Nucleic Acids Research **21**:5577-5582.

Hardy, R.W., 1975 The influence of chromosome content on the size and shape of sperm heads in *Drosophila melanogaster* and the demonstration of chromsome loss during spermiogenesis. Genetics **79**:231-264.

Hauschteck-Jungen, E. and D.L. Hartl, 1982 Defective histone transition during spermiogenesis in heterozygous *Segregation Distorter* males of *Drosophila melanogaster*. Genetics **101**:57-69.

Hazelrigg, T., R. Levis and G.M. Rubin, 1984 Transformation of the *white* locus DNA in drosophila: dosage compensation, *zeste* interaction, and position effects. Cell **36**:469-481.

Hecht, N.B. Post-meiotic gene expression during spermatogenesis. In: *Meiotic Inhibition: Molecular Control of Meiosis*, Alan R. Liss, Inc., 1988, p. 291-313.

Henikoff, S. and J.G. Henikoff, 1991 Automated assembly of protein blocks for database searching. Nucleic Acids Research **19**:6565-6572.

Hessler, A.Y., 1961 A study of parental modification of variegated position effects. Genetics **46**:463-484.

Hill, D.P., D.C. Shakes, S. Ward and S. Strome, 1989 A sperm-supplied product essential for initiation of normal embryogenesis in *C. elegans* is encoded by the paternal effect lethal gene, *spe11*. Developmental Biology **136**:154-166.

Hortsch, M., Preparation and analysis of membranes and membrane proteins from *Drosophila*. In: *Drosophila melanogaster*: Practical uses in cell and molecular biology, San Diego: Acacemic Press, 1994.

Huettner, A.F., 1924 Maturation and fertilization in *Drosophila melanogaster*. Journal of Morphology **39**:249-265.

Hughes-Schrader, S., 1948 Cytology of coccids (Coccoidea-Homoptera). Advances in Genetics **2**:127-203.

Hyman, A.A. and P.K. Sorger, 1995 The structure and function of kintochores in budding yeast. Annual Review of Cellular and Developmental Biology **11**:471-495.

Iatrou, K. and G.H. Dixon, 1978 Protamine messenger RNA: Its life history during spermatogenesis in rainbow trout. Fed Proc **37**:2526-2533.

Ito, T., M. Bulger, R. Kobayashi and J.T. Kadonaga, 1996 *Drosophila* NAP-1 is a core histone chaperone that functions in ATP-facilitated assembly of refularly spaced nucleosomal arrays. Molecular and Cellular Biology **16**:3112-3124.

Jackson, R.J. and N. Standart, 1990 Do the poly(A) tail and 3' untranslated region control mRNA translation? Cell **62**:15-24.

Jackson, R.J. and N. Standart, 1994 Regulation of translation by specific protein/mRNA interactions. Biochimie **76**:867-879.

Jeffery, W.R., 1989 Localized mRNA and the egg cytoskeleton. International Review of Cytology **119**:151-195.

Jiang, W., J. Lechner and J. Carbon, 1993 Isolation and characterization of a gene *(CPF2)* specifiying a protein component of the budding yeast kinetochore. Journal of Cell Biology **3**:513-519.

Jiang, W., K. Middleton, H.J. Yoon, C. Fouquet and J. Carbon, 1993 An essential yeast protein, CBF5p, binds in vitro to centromeres and microtubules. Molecular and Cellular Biology **13**:4484-4493.

Jinno, Y., K. Yun, E. Nishiwaki, T. Kubota, O. Ogawa, A.E. Reeve and N. Niikawa, 1994 Mosaic and polymorphic imprinting of the WT1 gene in humans. Nature Genetics **6**:305-319.

Joffe, J., M. Keene, and H. Weintraub, 1977 Histones H2a, H2b, H3, and H4 are present in equimolar amounts in chick erythroblasts. Biochemistry 16:1236-1238.

Kawasaki, K., A. Philpott, A.A. Avilion, M. Berrios and P.A. Fisher, 1994 Chromatin decondensation in *Drosophila* embryo extracts. Journal of Biological Chemistry **269**:10169-10176.

Kellum, R., J.W. Raff and B.M. Alberts, 1995 Heterochromatin protein 1 distribution during development and during the cell cycle in *Drosophila* embryos. Journal of Cell Science **108**:1407-1418.

Kimble, M. and K. Church, 1983 Meiosis and early cleavage in *Drosophila melanogaster* eggs: effects of the *claret-non-disjunctional* mutation. Journal of Cell Science **62**:301-318.

Kim-Ha, J., K. Kerr, and P.M. Macdonald, 1995 Translational regulation of *oskar* mRNA by Bruno, an ovarian RNA-binding protein, is essential. Cell **81**:403-412.

Klar, A.J., 1987 Determination of the yeast cell lineage. Cell **49**:433-435.

Klar, A.J. and M.J. Bonaduce, 1993 The mechanism of fission yeast mating-type interconversion: evidence for two types of epigenetically inherited chromosomal imprinted events. Cold Spring Harbor Symposium of Quantitative Biology **58**:457-465.

Kleene, K.C., 1989 Poly(A) shortening accompanies the activation of translation of five mRNAs during spermiogenesis in the mouse. Development **106**:367-373.

Kleene, S.S, W. Connor and G.H. Dixon, 1984 Translational regulation and deadenylation of a protamine mRNA during spermiogenesis in the mouse. Developmental Biology **105**:71-79.

Komma, D.J., S.J. Glass and S.A. Endow, 1993 Constitutive magnification by the *Ybb*$^-$ chromosome of *Drosophila melanogaster*. Genetical Research **62**:205-212.

Kuhn, D.T. and G. Packert, 1988 Tumerous-head-type mutants of the distal bithorax complex cause dominant gain and recessive loss of function in *Drosophila melanogaster*. Developmental Biology **125**:8-18.

Kuhn, R., C. Kuhn, D. Borsch, K.H. Glatzer, U. Schafer and M. Schafer, 1991 A cluster of four genes selectively expressed in the male germ line of *Drosophila melanogaster*. Mechanisms of Development **35**:143-151.

Kuhn, R., U. Schafer and M. Schafer, 1988 Cis-acting regions sufficient for spermatocyte-specific transcriptional and spermatid-specific translational control of the *Drosophila melanogaster* gene *mst(3)gl-9*. European Journal of Molecular Biology **7(2)**:447-454.

Lane, M.E. and D. Kalderon, 1993 Genetic investigation of cAMP-dependent protein kinase function in *Drosophila* development. Genes and Development **7**:1229-1243.

Lavender, J.S., A.J. Birley, J.J. Palmer, M.I. Kuroda and B.M. Turner, 1994 Histone H4 acetylated at lysine 16 and other components of the *Drosophila* dosage compensation pathway colocalize on the male X chromosome through mitosis. Chromosome Research **2**:398-404.

Leff, S.E., C.L. Brannan, M.L. Reed, T. Oacelik, U. Franke, N.G. Copeland and N.A. Jenkins, 1992 Maternal imprinting of the mouse SnRNP gene and conserved linkage homology with the human Prader-Will syndrome region. Nature Genetics **2**:259-264.

Lehmann, R. and C. Nusslein-Volhard, 1986 Abdominal segmentation, pole cell formation, and embryonic polarity require the localized activity of *oskar*, a maternal gene in *Drosophila*. Cell **47**:141-152.

Lewis, J.D., S.I. Gunderson and I.W. Mattaj, 1995 The influence of 5' and 3' end structures on pre-mRNA metabolism. Journal of Cell Science Suppl. **19**:13-19.

Lifschytz, E. and G.F. Meyer, 1977 Characterization of male meiotic-sterile mutations in *Drosophila melanogaster*. Chromosoma **64**:371-392.

Lindsley, D.L. and Tokuyasu, K.T. Spermatogenesis. In: *The Genetics and Biology of Drosophila, Vol. 2*, edited by Ashburner, M. and Wright, T.R.F. New York: Academic Press, 1980, p. 225-204.

Lindsley, D.L. and Zimm, G.G. *The Genome of Drosophila melanogaster*, San Diego:Academic Press, 1992.

Loidl, P., 1994 Histone acetylation: facts and questions. Chromosoma **103**:441-449.

Longo, F.J., L. Mathews and R.E. Palazzo, 1994 Sperm nuclear transformation in cytoplasmic extracts from surf clam *(Spisula solidissima)* oocytes. Developmental Biology **162**:245-258.

Matunis, M.J., E.L. Matunis and G. Dreyfuss, Isolation and characterization of RNA-binding proteins from *Drosophila melanogaster*. In: *Drosophila melanogaster*: Practical uses in cell and molecular biology, San Diego: Acacemic Press, 1994.

Matthies, H.J.G., H.B. McDonald, L.S.B. Goldstein and W.E. Theurkauf, 1996 Anastral meiotic spindle morphogenesis: role of the non-claret disjunctional kinesin-like protein. Journal of Cell Biology **134**:455-464.

McDonald, H.B. and L.S.B. Goldstein, 1990 Identificaiton and characterization of a gene encoding a kinesin-like protein in *Drosophila*. Cell **61**:991-1000.

McDonald, H.B., R.J. Stewart and L.S.B. Goldstein, 1990 The kinesin-like *ncd* protein of *Drosophila* is a minus end-directed microtubule motor. Cell **63**:1159-1165.

McGrath, J. and D. Solter, 1984 Completion of mouse embryogenesis requires both the maternal and paternal genomes. Cell **37**:179-183.

Meluh, P.B. and M.D. Rose, 1990 *KAR3*, a kinesin-related gene required for yeast nuclear fusion. Cell **60**:1029-1041.

Meluh, P.B. and D. Koshland, 1995 Evidence that the *MIF2* gene of *Saccharomyces cerevisiae* encodes a contromere protein with homology to the mammalian centromere protein CENP-C. Molecular Biology of the Cell **6**:793-807.

Metz, C.W., 1938 Chromosome behavior, inheritance and sex determination in *Sciara*. American Naturalist **72**:485-520.

Monk, M. and M. Grant, 1990 Preferential X-chromosome inactivation, DNA methylation and imprinting. Development **Suppl.**:55-62.

Murray, A.W., 1995 The genetics of cell cycle checkpoints. Development **5**:5-11.

Neeson, J., H. Bunemann and V.A. Heinlein, 1994 The *Drosophila hydei* gene *Dhmst101(1)* encodes a testis-specific,repetitive axoneme-associated protein with differential abundance in Y chromosomal deletion mutant flies. Developmental Biology **162**:414-425.

Nelson, C.R. and P. Szauter, 1992 Timing of mitotic chromosome loss caused by the *ncd* mutation of *Drosophila melanogaster*. Cell Motility and Cytoskeleton **23**:34-44.

Ners, S.S. and A.A. Travers, 1994 *HMG-D*, the *Drosophila melanogaster* homologue of HMG 1 protein, is associated with early embryonic chromatin in the absence of H1. European Journal of Molecular Biology **13**:1817-1822.

Nicholls, R.D., J.H.M. Knoll, M.G. Butler, S. Karam and M. Lalande, 1989 Genetic imprinting suggested by maternal heterodisomy in non-deletion Prader-Willi syndrome. Nature **342**:281-285.

O'Neill, LP. and B.M. Turner, 1195 Histone H4 acetylation distinguishes coding regions of the human genome form heterochromatin in a differentiation-dependent but transcription-independent manner. European Journal of Molecular Biology **14**:3946-3957.

Oliva, X. and X. Dixon, 1991 Vertebrate protamine genes and the histone-to-protamine replacement reaction. Progress in Nucleic Acid Research and Molecular Biology **40**:25-94.

Olivieri, G. and A. Olivieri, 1965 Autoradiographic study of nucleic acid synthesis during spermatogenesis in *Drosophila melanogaster*. Mutation Research **2**:366-380.

Panyim, S. and R. Chalkey, 1971 The molecular weight of vertebrate histones exploited by a modified sodium dodecyl sulfate electrophorectic method. Journal of Biological Chemistry **246**:7557-7560.

Patel, C.V. and K.P. Gopinathan, 1987 Determination of trace amounts of 5-methylcytosine in DNA by reverse-phase high performance liquid chromatography. Analytical Biochemistry **164**:164-169.

Pearson, W.R. and D.J. Lipman, 1988 Improved tools for biological sequence comparison. Proceedings of the National Academy of Sciences **85**:2444-2448.

Peterson, K. and C. Sapienza, 1993 Imprinting the genome: imprinted genes, imprinting genes and a hypothesis for their interaction. Annual Review of Genetics **27**:7-31.

Poccia, D. and P. Collas, 1996 Transforming sperm nuclei into male pronuclei *in vivo* and *in vitro*. Current Topics in Developmental Biology **34**:25-88.

Pokrywka, N.J. and E.C. Stephenson, 1991 Microtubules mediate the localization of *bicoid* RNA during *Drosophila* oogenesis. Development **113**:55-66.

Portin, P., 1978 Studies on gyandromorphs induced with the *claret-nondisjunctional* mutations of *Drosophila melanogaster*. An approach to the timing of chromosome loss in cleavage mitosis. Heredity **41**:193-203.

Rasooly, R.S., C.M. New, P. Zhang, R.S. Hawley and B.S. Baker, 1991 The *lethal(1)TW-6*^{cs} mutation of *Drosophila melanogaster* is a dominant antimorphic allele of *nod* and is associated with a single base change in the putative ATP-binding domain. Genetics **129**:409-422.

Rastelli, L., R. Richman and M.I. Kuroda, 1995 The dosage compensation regulators MLE, MSL-1 and MSL-2 are interdependent since early embryogenesis in *Drosophila*. Mechanisms of Development **53**:223-233.

Reed, B.H., 1995 *Drosophila* development pulls the strings of the cell cycle. BioEssays **17**:553-556.

Reed, K.M. and J.H. Werren, 1995 Induction of paternal genome loss by the *paternal-sex-ratio* chromosome and cytoplasmic incompatibility bacteria *(Wolbachia)*: a comparative study of early embryonic events. Molecular and Reproductive Development **40**:408-418.

Reuter, G., I. Wolff and B. Friede, 1985 Functional properties of the heterochromatic sequences inducing w^{m4} position effect variegation in *Drosophila melanogaster*. Chromosoma **93**:132-139.

Richardson, B.J., A.B. Czuppon and G.B. Sharman, 1971 Inheritance of glucose-6-phosphate dehydrogenase variation in kangaroos. Nature, New Biology **230**:154-155.

Ridley, R.M., C.D. Frith, L.A. Farrer and P.M. Conneally, 1991 Patterns of inheritance of the symptoms of Huntington's disease suggestive of an effect of genomic imprinting. Journal of Medical Genetics **28**:224-231.

Righetti, R.G., A. Bossi, A. Gorg, C. Obermaier and G. Boguth, 1996 Steady-state two-dimensional maps of very alkaline proteins in an immobilized pH 10-12 gradient, as exemplified by histone types. Journal of Biochemical and Biophysical Methods **31**:81-91.

Risley, M.S. Chromatin organization in sperm. In: *Chromosomes: Eukaryotic, Prokaryotic and Viral II*, edited by Adolph, S. New York: CRC Press, 1990, p. 61-78.

Robertson, H.M., C.R. Preston, R.W. Philis, D.M. Johnson-Schlitz, W.K. Benz and W.R. Engels, 1988 A stable source of P element transposase in *Drosophila melanogaster*. Genetics **118**:461-470.

Russell, S.H. and K. Kaiser, 1993 *Drosophila melanogaster* male germ line specific transcripts with autosomal and Y-linked genes. Genetics **134**:293-308.

Russell, S.R.H. and K. Kaiser, 1993 *Drosophila melanogaster* male germ line-specific transcripts with autosomal and Y-linked genes. Genetics **134**:293-308.

Sambrook, J., Fritsch, E.F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*, New York:Cold Spring Harbor Laboratory, 1989. Ed. 2

Sandler, L., 1971 Induction of autosomal meiotic mutants by EMS in *D. melanogaster*. Dros Inform Serv **47**:68.

Sandler, L. and K. Golic, 1985 Segregation distortion in *Drosophila*. Trends in Genetics **1**:181-185.

Schafer, M., D. Borsch, A. Hulster and U. Schafer, 1993 Expression of a gene duplication encoding conserved sperm tail proteins is translationally regulated in *Drosophila melanogaster*. Molecular and Cellular Biology **13(3)**:1708-1718.

Schafer, M., R. Kuhn, F. Bosse and U. Schafer, 1990 A conserve element in the leader mediates post-meiotic translation as well as cytoplasmic polyadenylation of a *Drosophila* spermatocyte mRNA. European Journal of Molecular Biology **9**:4519-4525.

Schatten, G. and H. Schatten, 1987 Fertilization: Motility, the cytoskeleton and the nuclear architecture. Oxford Reviews of Reproductive Biology **9**:322-378.

Schrader, F., 1929 Experimental and cytological investigations of the life cycle of *Gossyparia spuria* (Coccidae) and their bearing on the problem of haploidy in males. Z Wiss Zool **134**:149-179.

Searle, A.G. and C.V. Beechey, 1990 Genome imprinting phenomena on mouse chromosome 7. Genetical Research **56**:237-244.

Sequeira, W., C.R. Nelson and P. Szauter, 1989 Genetic analysis of the *claret* locus of *Drosophila melanogaster* . Genetics **123**:511-524.

Smith, M.L. and A.J. Fornace,Jr., 1995 Genomic instability and the role of p53 in cancer cells. Current Opinions in Oncology **7**:69-75.

Smith, S.S. and C.A. Thomas,Jr., 1981 The two-dimensional restriction analysis of *Drosophila* DNAs: males and females. Gene **13**:395-408.

Somerville, J. and M. Ladomery, 1996 Transcription and masking of mRNA in germ cells: involvement of Y box proteins. Chromosoma **104**:469-478.

Somerville, J. and M. Ladomery, 1996 Masking of mRNA by Y box proteins. FASEB Journal **10**:435-443.

Sorger, P.K., K.F. Doheny, P. Hieter, K.M. Kopski, T.C. Huffaker and A.A Hyman, 1995 Two genes required for the binding of an essential *Saccharomyces cerevisiae* kinetochore complex to DNA. Proceedings of the National Academy of Sciences **92**:12026-12030.

Spirin, A.S., 1994 Storage of messenger mRNA in eukaryotes: envelopment with protein, translational barrer at 5' side or conformational masking by 3' side? Molecular and Reproductive Development **38**:107-117.

Spofford, J.B., 1959 Parental control of position-effect variegation: I. Parental heterochromatin and expression of the white locus in compound-X *Drosophila melanogaster*. Proceedings of the National Academy of Sciences **45**:1003-1007.

Spofford, J.B., 1961 Parental control of position-effect variegation. II. Effect of sex of parent contributing *white-mottled* rearrangement in *Drosophila melanogaster*. Genetics **46**:1151-1167.

Strunnikov, A.V., V.L. Larionov and D. Koshland, 1993 *SMC1*: an essential yeast gene encoding a putative head-rod-tail protein is required for nuclear

division and defines a new ubiquitous protein family. Journal of Cell Biology **123**:1635-1648.

Surani, M.A.H., S.C. Barton and M.L. Norris, 1984 Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. Nature **308**:548-550.

Surani, M.A.H., S.C. Barton and M.L. Norris, 1986 Nuclear transplantation in the mouse: Heritable differences between parental genomes after activation of the embryonic genome. Cell **45**:127-136.

Szabad, J., M. Erdelyi, G. Hoffman, J. Szidonya and T.R.F. Wright, 1989 Isolation and characterization of dominant female sterile mutations of *Drosophila melanogaster*. II. Mutations of the second chromosome. Genetics **122**:823-835.

Szabad, J., E. Mathe and J. Puro, 1995 *Horka*, a dominant mutation of *Drosophila*, induces nondisjunction and, through paternal-effect, chromosome loss and genetic mosaics. Genetics **139**:1585-1599.

Takagi, N. and M. Sasaki, 1975 Preferential inactivation of the paternally derived X-chromosome in the extraembryonic membranes of the mouse. Nature **258**:640-641.

Tamkun, J.W., R. Deuring, M. Scott, M. Kissinger, A.M. Pattatucci, T.C. Kaufman and J.A. Kennison, 1992 *brahma*: A regulator of *Drosophila* homeotic genes structurally related to the yeast transcriptional activator *SNF2/SWI2*. Cell **68**:561-572.

Tates, A.D., 1971 Cytodifferentiation during spermatogenesis in *Drosophila melanogaster*: An electron microscopic study. 'S-Gravenhage:Drukkerij J H Pasmans

Tautz, D. and C. Pfeifle, 1989 A nonradioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. Chromosoma **98**:81-85.

Theurkauf, W.E. and R.S. Hawley, 1992 Meiotic spindle assembly in *Drosophila* females: behavior of nonexchange chromosomes and the effects of mutations in the *nod* kinesin-like protein. Journal of Cell Biology **116**:1167-1180.

Tokuyasu, K.T. and W.J. Peacock, 1977 Dynamics of spermiogenesis in *Drosophila melanogaster*. VII. Effects of *segregation distorter (SD)* chromosome. Journal of Ultrastructure Research **58**:96-107.

Tomkiel, J.T., 1991 Rescue of the *abnormal oocyte* maternal effect lethality by *ABO* heterochromoatin in *Drosophila melanogaster*. Genetics **128**:583-594.

Tower, J., G.H. Karpen, N. Craig and A. Spradling, 1993 Preferential transposition of *Drosophila P* elements to nearby chromosomal sites. Genetics **133**:347-359.

Turner, B.M., 1995 Histone H4, the cell cycle and a question of integrity. BioEssays **17**:1013-1015.

Turner, B.M., A.J. Birley and J. Lavender, 1992 Histone H4 isoforms acetylated at specific lysine residues define individual chromosomes and chromatin domains in *Drosophila* polytene nuclei. Cell **69**:375-384.

Turner, B.M. and L.P. O'Neill, 1995 Histone acetylation in chromatin and chromosomes. Seminars in Cell Biology **6**:229-236.

van Daal, A. and S.C.R. Elgin, 1992 A histone variant, H2AvD, is essential in *Drosophila melanogaster*. Molecular Biology of the Cell **3**:593-602.

VandeBerg, T.L., Robinson, E.S., Samallow, P.B. and Johnston, P.G. X-linked gene expression and X-chromosome inactivation: Marsupials, mouse and man compared. In: *Isozymes: Current Topics in Biological and Medical Research*, edited by Markert, C.L. New York: Alan R. Liss, Inc., 1987, p. 225-253.

Wahle, E., 1995 3' end cleavage and polyadenylation of mRNA precursors. Biochimica and Biophysica Acta **1261**:183-194.

Wake, N., N. Takagi and M. Sasaki, 1976 Non-random inactivation of X chromosome in the rat yolk sac. Nature **262**:580-581.

Wald, H., 1936 Cytologic studies on the abnormal development of the eggs of the *claret* mutant type of *Drosophila* simulans. Genetics **21**:264-281.

Walker, R.A., E.D. Salmon and S.A. Endow, 1990 The Dropsophila *claret* segregation protein is a minus-end directed motor molecule. Nature **347**:780-782.

West, J.D., W.I. Freis, V.M. Chapman and V.E. Papaioannou, 1977 Preferential expression of the maternally derived X-chromosome in the mouse yolk sac. Cell **12**:873-882.

Wilhelm, J.E. and R.D. Vale, 1993 RNA on the Move: The mRNA localization pathway. Journal of Cell Biology **123(2)**:269-274.

Xu, D.F., M.A. Brown, J.A. Chambers, B. Griffiths, H. Nicolai and E. Solomon, 1995 Distinct transcription start sites generate two forms of *BRCA1* mRNA. Hum Mol Genet **4**:2259-2264.

Yang, J., L. Porter and J. Rawls, 1995 Expression of the *dihydroorotate dehydrogenase* gene, *dhod*, during spermatogenesis in *Drosophila melanogaster*. Molecular and General Genetics **246**:334-341.

Yanicostas, C. and J-A. Lepesant, 1990 Transcriptional and translational cis-regulatory sequences of the spermatocyte-specific *Drosophila janusB* gene are located in the 3' exonic region of the overlapping *janusA* gene. Molecular and General Genetics **224**:450-458.

Yanicostas, C. and A. Vincent, 1989 Transcriptional and postranscriptional regulation contributes to the sex-regulated expression of two sequence-related genes at the *janus* locus of *Drosophila melanogaster*. Molecular and Cellular Biology **9**:2526-2535.

Yasuda, G.K., G. Schubiger and B.T. Wakimoto, 1995 Genetic characterization of *ms(3)K81*, a paternal effect gene of *Drosophila melanogaster*. Genetics **140**:219-229.

Zalokar, M., I. Erk and P. Santamaria, 1980 Distribution of Ring- X chromsosomes in the blastoderm of gynandromorphic *D. melanogaster*. Cell **19**:133-141.

Zhang, P. and R.S. Hawley, 1990 The genetic analysis of distributive segregation in *Drosophila melanogaster*. II. Further genetic analysis of the *nod* locus. Genetics **125**:115-127.

KELLY NOEL OWENS
408 NE 44th St.
Seattle, WA 98105
(206) 543-6719
Home: (206)634-2869

EDUCATION

Doctor of Philosophy, Dept. of Genetics and Molecular and Cell Biology Program, University of Washington, in the laboratory of Dr. Barbara Wakimoto.
Genetic, molecular and cytological studies of the paternal effect gene, *paternal loss*, were done to understand its role in the early development of *Drosophila melanogaster*, 1988-1989 and 1990-1996.

Research technician, with Dr. Peter Wolk, Michigan State University. Evaluated antibiotic resistance and performed pulse-field mapping in cyanobacteria. Supervised undergraduate student helpers. 1989-1990.

Bachelor of Science, with honors, in **Zoology**, Michigan State University, 1988.

Bachelor of Science, with honors, in **Biochemistry**, Michigan State University, 1988.
Independent undergraduate research involved classical genetics, cytogenetic and molecular analyses in the lab of Dr. Tom Friedman, MSU, 1986-1988.

Diploma, Henry Clay High School, Lexington, KY 40502. Valedictorian.

TEACHING EXPERIENCE

Lab oordinator BIO 202 developmental biology. Supervised TAs, taught laboratory section, coordinated with lecturer Dr. Aimee Bakken, Summer 1996.

Participant in Pugh Foundation Zoology Graduate Seminar on teaching, Fall 1995.

Lecturer, minority youth program, Summer 1995.

Graduate student coordinator for science education partnership program for high school teachers, Summer 1993.

Teaching assistant GEN 356 (genetics for majors) with Dr. Jim Thomas, Spring 1992

Teaching assistant GEN 360 (genetics for non-majors) with Dr. Ann Reynolds, Summer 1991.

Tutor for genetics, 1987-present.

OTHER WORK EXPERIENCE

Secretarial Asst. Genetics Program, MSU, E. Lansing, 1988.
Temporary clerical worker. TEMPS, Inc., Lexington, KY. Summer, 1986.
Asst. Director and Stage Manager. MSU Theater Dept., 1984-1987.
Veterinary assistant. AA Small Animal Emergency Clinic, and Lansdowne Vet. Clinic, Lexington, KY 40502. Weekends and school interim, 1981-1985.

ASSOCIATIONS AND HONORS

Member, **Genetics Society of America,** 1989-present.
Member, **Association for Women in Science,** 1989-present. Chair public relations 1990-92.

Phi Beta Kappa, National Academic Honor Society, 1988.
Mortar Board, National Academic Honor Society, 1987.
Phi Kappa Phi, National Academic Honor Society, 1988.
MSU Distinguished Freshman Scholarship, 4 yr. full tuition, 1984-1988.
IBM Watson Scholar, 1984-1988.
National Merit Scholar, 1984.
Bausch and Lomb Science Award, 1984.
Governor's Scholar of Kentucky, 1983.

PUBLICATIONS AND MEETINGS

Kelly N. Owens, Kathleen L. Wilson, John E. Tomkiel, Daniel Kalderon, and Barbara T. Wakimoto, "Characterization of the structure and expression of *paternal loss (pal)*, a paternal effect gene required for chromosome maintenance in *Drosophila melanogaster*", (in progress).

National Drosophila Conference 1994,
Poster presentation.
Workshop presentation: "Expression of a paternal effect gene, *paternal loss (pal)*" K. N. Owens, L. Wilson, and B. Wakimoto

National Drosophila Conference 1993, Poster presentation: "Genetic and molecular characterization of the paternal-effect genes *paternal loss* and *ms(3)K81*" K. N. Owens, G. K. Yasuda, K. Wilson, E. Cates, G. Schubiger, B. T. Wakimoto.

Zoology Graduate Student Symposium 1992. "Characterization of the paternal effect gene, *pal*."

Friedman, T. B., Owens, K. N., Burnett, J. B., Saura, A. O., and Wallrath, L. L., 1991 " The faint band/interband region 28C2 to 28C4-4(-) of *Drosophila melanogaster* salivary gland polytene chromosomes is rich in transcripts," Molecular and General Genetics 226(1-2): 81-87.

Zoology Graduate Student Symposium 1990, "Genetic Analysis of the paternal contribution of embryogenesis in *Drosophila melanogaster*. "