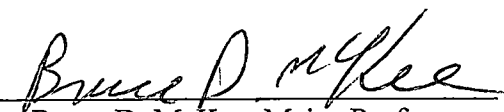
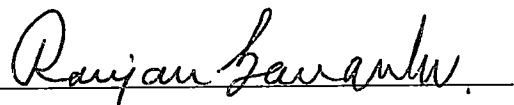


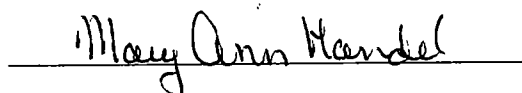
To the Graduate Council:

I am submitting herewith a thesis written by Amy L. Creekmore entitled "Homologous Chromosome Pairing in *Drosophila melanogaster* Male Meiosis." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Masters of Science, with a major in Biochemistry and Molecular and Cellular Biology.


Bruce D. McKee, Major Professor

We have read this thesis and recommend its acceptance:





Accepted for the council:


Interim Vice Provost and
Dean of The Graduate School.

**Homologous Chromosome Pairing in
Drosophila melanogaster Male Meiosis**

A Thesis
Presented for the
Masters of Science Degree
The University of Tennessee, Knoxville

Amy Lynn Creekmore
August 2001

This thesis is dedicated to my mom
Kathy L. Creekmore
and
the loving members of
The First Baptist Church, Oak Ridge
who have taught me how to fly with the wings God gave me.

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ABSTRACT

In the past fifty years many advances in understanding the mechanisms and proteins involved in recombination, the synaptonemal complex, sister chromatid cohesion, and the kinetochore have been made. Nevertheless, the longstanding fundamental question, "What are the physical forces responsible for the attraction and subsequent interactions of homologous chromosomes?," still remains unanswered. (Giroux 1988). How homologous chromosomes in the achiasmatic *Drosophila melanogaster* male pair and are held together was explored in this study by examining a cis-acting factor and a candidate trans-acting factor.

The rDNA in *Drosophila melanogaster* is located on the X and Y chromosomes. Data has shown that the transcription of the cis-acting 240 bp repeats within the rDNA intergenic spacer is essential for X-Y pairing. Transcription being necessary for pairing suggests a direct role of the 240 bp RNA in pairing. The cytological location of the 240 bp repeat RNAs was examined using *in situ* hybridization to the RNAs in testes tissue. Staining, indicating presence of the 240 bp repeat RNAs, was observed in prophase and prometaphase in primary spermatocytes. Direct interactions of the 240bp repeats were examined using *in vitro* and *in vivo* assays. Interactions between *in vitro* transcribed 240bp RNAs were not observed, but peripherally produced 240 bp repeat RNAs (not transcribed from the X or Y chromosomes) did interfere with proper pairing and disjunction *in vitro* in males. This data points to a direct role of 240 bp repeat RNAs in X-Y homolog pairing in male *Drosophila* meiosis.

The cabeza and TLS proteins are 56% identical and share common motifs. Since TLS seems to play a role in mouse meiosis, the *Drosophila* homolog cabeza may play a role in fly meiosis. Two different mutant alleles of the candidate trans-acting factor cabeza were used in this study to characterize its role in meiosis. Meiosis in the cabeza mutants was characterized by looking at live and stained preparations of testis tissue. Meiosis appeared normal in live and stained preparations of testes tissues for both mutants. It appears that the cabeza protein is not essential for *Drosophila* male meiosis.

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LIST OF ABBREVIATIONS

alt pBS	alterered Bluescript plasmid
Df	deficiency
DIG	digoxigenin
DNA	deoxyribonucleic acid
ETS	external transcribed spacer
EWS	Edwing's sarcoma gene
GFP	green fluorescent protein
IGS	intergenic spacer
ITS	internal transcribe spacer
KO	knock out
MI	meiosis I
MII	meiosis II
NDJ	nondisjunction
NO	nucleolus organizer
NOR	nucleolus organizing region
PBS	phosphate buffered saline
pBS	Bluescript plasmid
rDNA	ribosomal DNA
RNA	ribonucleic acid
RNP	RNA nuclear protein
RRM	RNA recognition motif
rRNA	ribosomal RNA
RT	room temperature
SC	synaptonemal complex
SDS	sodium dilaurel sulfate
TBE	Tris, boric acid, EDTA
TLS	translocated in liposacrcoma
TU	transcription unit
UV	ultraviolet

CHAPTER I

INTRODUCTION

In the past fifty years many advances in understanding the mechanisms and proteins involved in recombination, the synaptonemal complex, sister chromatid cohesion, and the kinetochore have been made. Nevertheless, the longstanding fundamental question, "What are the physical forces responsible for the attraction and subsequent interactions of homologous chromosomes?," still remains unanswered. (Giroux 1988). How homologous chromosomes in the achiasmatic *Drosophila melanogaster* male pair and are held together was explored in this study. Very little is known about the trans-acting factors for pairing, but some is known about the cis pairing sites in *Drosophila*. In this study male *Drosophila melanogaster* are used as a model to study a cis factor and a candidate trans-acting factor in homologous chromosome pairing.

Overview of Meiosis

Meiosis: General Aspects

Genetics is the science of heredity and the basis of heredity is meiosis, a special cell division in which the genetic material is reduced to one half in the formation of gametes. Meiosis consists of two divisions that follow one round of DNA synthesis, meiosis I, is a reductional division, and meiosis II, is an equational division (Fig.1). Meiosis I and II are traditionally separated into four stages: prophase, metaphase, anaphase and telophase. Meiosis II is very similar to mitosis, whereas meiosis I is unique because homologous chromosome pair and disjoin.

The unique events that occur during meiosis I that lead to the pairing and segregation of homologous chromosomes occur in prophase. Prophase I is traditionally divided into five stages: leptotema, zygotema, pachynema, diplonema, and diakinesis. Leptotema is characterized by the beginning of chromatin condensation and the formation of the lateral components of the synaptonemal complex (SC). The SC is a tripartite

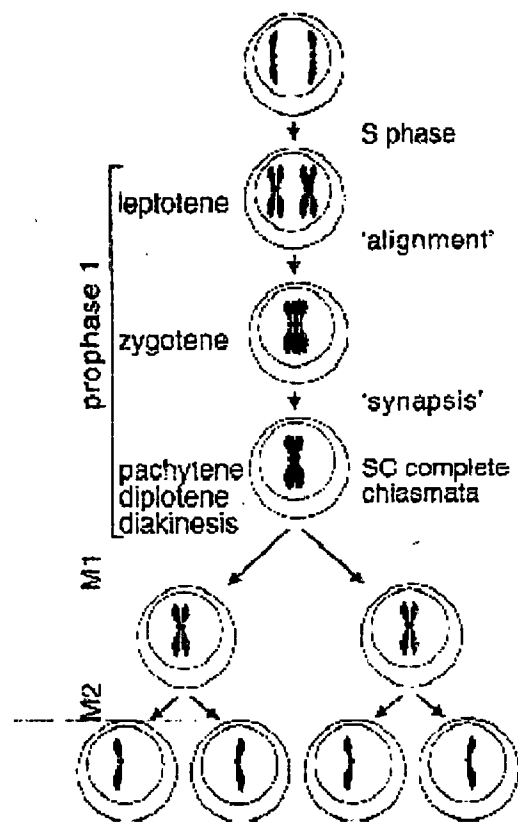


Figure 1. Diagram of Meiosis. Represented are the main events of meiosis. One pair of homologous chromosomes is shown for simplicity. Alignment refers to pairing and synapsis the formation of the synaptonemal complex (SC). M1 and M2 refer to meiosis I and meiosis II, respectively. The diagram was adapted from Cook 1997.

proteinaceous structure containing a central element held by transverse filaments between two lateral elements that interact with the chromatin loops of an univalent chromosome. It is believed that rough alignment or pairing of homologous chromosomes occurs at this stage (von Wettstein et al. 1984).

Through the zygotene stage chromosomes continue to condense and by the beginning of pachytene the SC is fully formed. At this stage, homologous chromosomes are fully aligned or paired and are held together via the SC. Pachynema is characterized by the occurrence of recombination. The SC is thought to facilitate recombination, but not be responsible for recombination. The evidence for this conclusion includes: 1) in *S. pombe* and *Aspergillus nidulans* recombination occurs at high levels despite the failure of SC formation (Roeder 1997), and 2) not all organisms that have SC formation have recombination events such as female moths, male blowflies, and mantids (von Wettstein et al. 1984; Giroux 1988). During the pachytene stage recombination nodules, small dense proteinaceous structures thought to contain localized meiotic recombination enzymes, appear on the SC (Carpenter 1988).

The start of diplotene is marked by the disappearance of the SC. Chiasmata are visible and believed to be holding the homologs together. Chiasmata are commonly accepted to be the result of recombination events since the number and location of recombination events and chiasmata correspond (John 1990; Orr-Weaver 1995). Chiasmata hold homologous chromosomes together to allow interaction with the spindle apparatus, alignment at the metaphase plate, and proper disjunction. Mutations that disrupt meiotic recombination and inhibit chiasmata formation cause high frequencies of meiosis I homolog nondisjunction (Roeder 1990; Hawley 1988). Finally, in diakinesis, chiasmata are resolved and homologous chromosomes line up on the metaphase plate.

Meiosis in Drosophila melanogaster Males

In this study *Drosophila melanogaster* is used as a model organism to study meiosis. Besides the general attributes of *Drosophila* that make them an excellent model system - short generation time, small chromosome number, sequence of the genome

completed, and many mutant alleles available - they also possess attributes that make them ideal for studying meiosis. First, germ line cells undergoing meiosis, in *Drosophila* males, are easily identifiable and are of a size and abundance that makes them well suited for the investigation of the mechanisms of pairing and segregation (Maines and Wassermann 1998). Secondly, the general stages of meiotic cells in male *Drosophila* are well characterized. The third attribute that makes *Drosophila* males ideal for studying meiosis is they do not have SC formation or recombination events. Since it is accepted that recombination and SC formation are most likely mechanisms separate from pairing, this allows for easier characterization of mutants that affect pairing, since secondary effects of improper SC formation and recombination do not have to be considered.

Drosophila male meiosis takes place in two coiled testes tubules (Figure 2). Stages of spermatogenesis are arrayed at fixed intervals progressing along the length of the testis coil. Stem cells are at the apical end (see figure 2) and spermatogenesis takes place as cysts of cells move through the coiled tubules developing from spermatogonia to mature spermatozoa at the basal end that connects to the seminal vesicle. Two types of stem cells divide asymmetrically. Each germ line stem cell divides to produce a spermatogonia and another germ line stem cell and somatic cyst progenitor cells divide producing a cyst cell and another progenitor cell. Two cyst cells encase one spermatogonia cell and then the spermatogonia cell undergoes four mitotic divisions to form a cyst of 16 primary spermatocytes. Incomplete cytokinesis occurs in these divisions and all 16 cells are linked through cytoplasmic bridges called ring canals. (Maines and Wasserman 1998).

Primary spermatocytes then undergo pre-meiotic DNA replication early in a 90 hour extended growth phase. During the extended growth phase transcription is elevated and the volume of the cells increases 25 fold (Cenci 1994). At this stage the cells are called polar spermatocytes because the nucleus is asymmetrically located in the cytoplasm of the cells across from an organelle cluster that contains mostly mitochondria (Cenci 1994) .

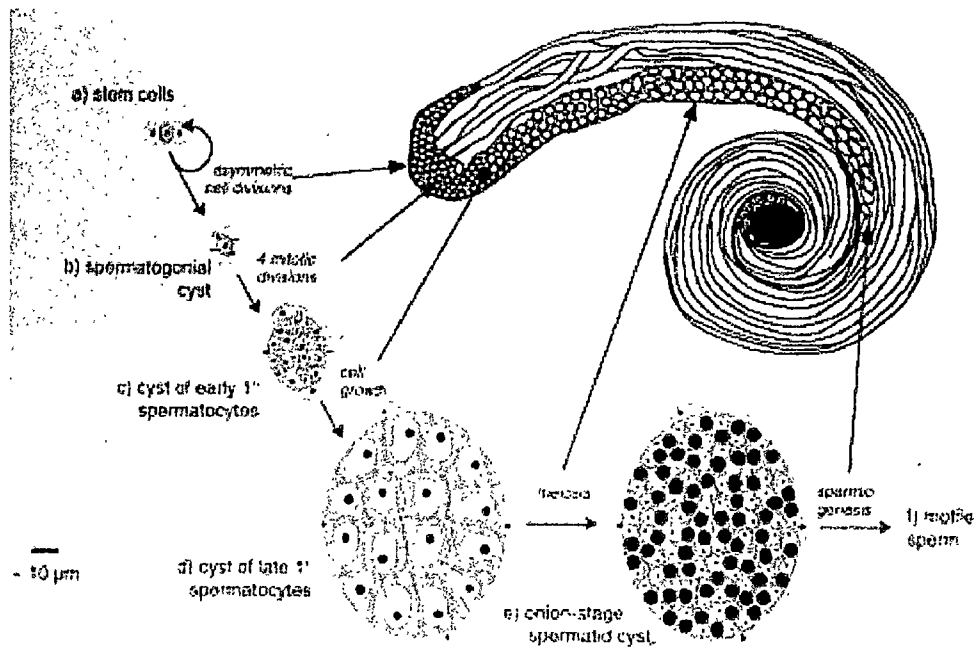


Figure 2. Overview of Spermatogenesis. Represented is one *Drosophila* testis tubule and the approximate locations of cells in landmark stages of spermatogenesis. Illustration adapted from Maines and Wasserman 1998.

In early polar spermatocytes, the chromatin appears as a mass in the center of the nucleus, and as the cell volume increases the chromatin divides into three masses that become situated around the nuclear envelope. The chromosome clumps are believed to correspond to the two large autosomal homolog pairs and the X-Y bivalent, the smallest clump of the three (Cenci 1994). The small fourth bivalent is not visible at this stage. As the chromatin migrates to the nuclear envelope the nucleus assumes a more centric location in the cell, and the mitochondria dissipate. This stage is also characterized by the appearance of Y-loops, lampbrush-like loops associated with the Y chromosome that are visible under phase contrast microscopy.

At the end of the growth phase, the chromatin condenses further and the small fourth homolog pair often becomes visible as the y-loops disintegrate. The chromosomes appear as dense spherical clumps associated with the nuclear envelope (Figure 3). This stage is often referred to as diakinesis although it is accepted that the traditional division of prophase into stages is not applicable in *Drosophila* males since there is no SC formation or recombination (Cenci 1994; McKee 1998). Cells at this stage, in orcein-stained or Hoescht-stained tissue, can be observed for the determination of proper pairing because the bivalents are clearly visible as stained clumps (Figure 3). At this stage, all RNA synthesis has ceased with all RNA required for the rest of spermatogenesis and spermiogenesis present (Gould-Somero and Holland 1974).

As prophase proceeds into what is called prometaphase the nucleus rounds up becoming clearer and the chromosomes start to move toward the metaphase plate. In electron micrographs of sectioned *Drosophila* tissue, prometaphase chromosomes reveal very little structural clues to pairing and cohesion of the chromosomes (Ault et al. 1982). The autosome bivalents appear as two oval-shaped clumps that overlap, and X-Y bivalents appear as clumps that overlap at regions that correspond to the nucleolus organizing regions that contain the rDNA (Ault et al. 1982, 1984).

At metaphase, chromosomes appear as an oblong clump equidistant from the poles at the metaphase plate, the nuclear membrane breaks down, and the chromosomes are

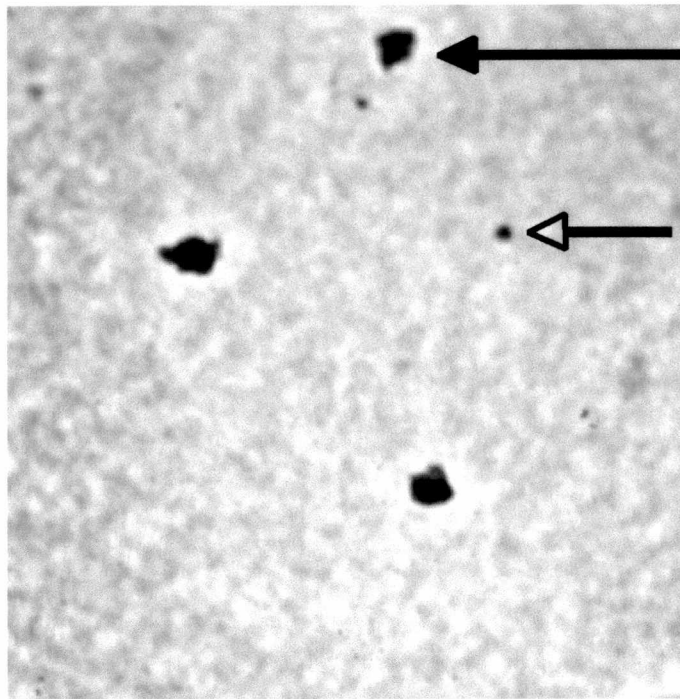


Figure 3. Late Prophase I Chromosomes. Late prophase I chromosomes from orcein stained testes tissue at 100X magnification. Filled arrows indicate the paired sex chromosome homologs and the open arrow the paired fourth chromosomes. The other spots of stained are the two large autosomes. The chromosomes are arrayed along the wall of the disintegrating nuclear envelope. (Photograph provided by Dr. B.D. McKee)

attached to spindle poles via microtubules that form symmetric arrays. At anaphase I, homologs migrate quickly to opposite poles. This is based on the observation that cysts that contain metaphase cells also contain anaphase cells with homologs that have migrated a considerable distance to the poles (Cenci 1994). After the completion of the migration of the homologs to the poles, telophase continues with the microtubule spindle becoming an hourglass shape as two daughter nuclei are formed. The two daughter nuclei become distinct from the cytoplasm and cytokinesis ends with the even distribution of organelles (mainly mitochondria) into the daughter cells.

The transition from meiosis I to meiosis II is characterized by the decondensation of the chromosomes and nuclear enlargement. Prophase II begins with the recondensation of the chromosomes. Two major features distinguish meiosis II and meiosis I. The first is that meiosis II is an equational division in which sister chromatids separate at anaphase. The second feature is the location of the condensing chromosomes in the secondary spermatocytes during prophase. Chromosomes are more centrally located in the nucleus, rather than arrayed along the nuclear membrane like in prophase I. This allows for the discrimination of primary and secondary spermatocytes at prophase in testes tissue stained with orcein or Hoescht dye.

After meiosis I and II, which last approximately 266 minutes total, the post-meiotic stages of spermiogenesis begin with the decondensation of the haploid genetic material (Cenci 1994). As spermiogenesis proceeds, the mitochondria amalgamate and condense in each cell forming a dark, spherical spot known as the mitochondria derivative or nebenkern (Figure 4). Also present in the cell is the nucleus that appears as a milky whit spot. After proper meiosis the nucleus is approximately the same size as the nebenkern. Cells at this stage of spermiogenesis are said to be at the onion stage. The size of the nuclei at the onion stage corresponds to chromosome content (Gonzalez 1989), so proper pairing and disjunction of chromosomes can be extrapolated by observing the size of the onion stage nuclei in live testes preparations. Variation in the size of nuclei in onion cells, whether in relation to the nebenkern or each other, indicates defects in chromosome pairing or disjunction.

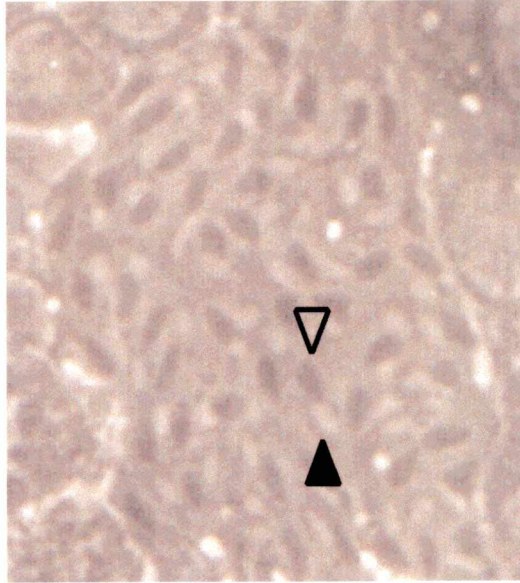


Figure 4. Onion Stage Cells. Live preparation of *Drosophila* testes tissue. Filled arrows indicate the nucleus and the open arrow the nebenkern or mitochondrial derivative.

Chromosome Pairing in *Drosophila melanogaster*: Cis-Acting Factors

While more is beginning to be understood about the molecular mechanisms of homologous chromosome synapsis and recombination, much less is known about the molecular mechanisms of homologous pairing. In homolog pairing little has been discovered about the trans-acting factor(s) that may be involved in pairing (reviewed in Orr-Weaver 1995 and Hawley 1995), but advancements have been made in the cis elements involved in pairing.

Role of Euchromatin in Pairing

Cytological examination of metaphase and prometaphase primary spermatocytes in stained testes tissue in *Drosophila melanogaster*, and the determining of a nondisjunction event through mating crosses, has revealed a lot about the roles of heterochromatin and euchromatin in pairing. Deficiencies of euchromatin or heterochromatin have very different effects on chromosome pairing. Work by Yamamoto (1979) displayed that euchromatin, but not heterochromatin, promotes pairing. Experiments using a mini 2nd chromosome (Dp (2:f)1) deficient for most euchromatin, but containing the heterochromatin, demonstrated pairing with a normal 2nd chromosome cytologically. The minichromosome appeared as an univalent in metaphase I spermatocytes. Also, the 2nd minichromosome showed no pairing with another 2nd minichromosome when present in two copies. Conversely, deletion of heterochromatin from either arm of the 2nd chromosome does not affect pairing.

Rearrangements and translocations of heterochromatin and euchromatin also follow the patterns observed by Yamamoto. Rearrangement of heterochromatin on the 2nd chromosome does not affect pairing, but pairing is disrupted when euchromatin is rearranged. More specifically a 2nd chromosome with duplicated heterochromatin and deleted euchromatin segregates randomly from a normal 2nd chromosome even though they share homology in the heterochromatin (Hilliker et al. 1982). Also, translocation of 2nd chromosome euchromatin to the Y chromosome causes high frequencies of quadrivalent formation between both the 2nd, the X, and the Y chromosomes in metaphase I of spermatocytes. Whereas, translocation of heterochromatin to the Y

chromosome does not affect pairing (McKee et al. 1993). McKee also observed that quadrivalent frequency increased as the size of the euchromatin translocated to the Y chromosome increased, except one spot that seemed to be a "pairing hotspot." This region displayed greater pairing frequencies than expected for its size and was mapped to the histone 2 locus (39DE) which has high rates of transcription (McKee et al. 1993 and personal communication).

Data from translocation experiments deleting heterochromatin or euchromatin of chromosome three, the other large autosome, and the small fourth chromosome also support this idea that euchromatin, but not heterochromatin, promotes pairing (McKee 1998b). All these data suggest that weak pairing sites are widely distributed throughout the euchromatin, with two exceptions. The first is the pairing "hotspot" located in the left arm of the second chromosome corresponding to the histone locus. The other is the X-Y pairing site that is found in heterochromatin and is discussed below.

The X Chromosome Pairing Site

A cytological study by Cooper, in 1964, showed that the X and Y pairing region in *Drosophila* males is confined to the heterochromatin. He observed thin threads that connected the heterochromatin in the long arm of the X chromosome and the short arm of the Y chromosome that he named collochores (Figure 5). Cooper noted that the collochores corresponded to the nucleolar organizing region (NOR), which contains the rDNA, of both chromosomes. Further studies have shown various deletions within the heterochromatin of the X chromosome interfered with pairing, but deletions of euchromatin have no effect. The disruption of X-Y pairing is affected in varying intensities depending upon size and location of the heterochromatin deletion (McKee 1998).

Two chromosomes, Df(1)sc4L-sc8R and Df(1)X-1, are examples that display the varying effects of size and location of deletions. Df(1)sc4L-sc8R is deficient for a large region of heterochromatin that includes the rDNA, and part of the distal heterochromatin is relocated to the tip of the X chromosome (Figure 6). When Df(1)sc4L-sc8R is present with a normal Y (B^sY⁺), X-Y pairing and disjunction vary depending upon temperature

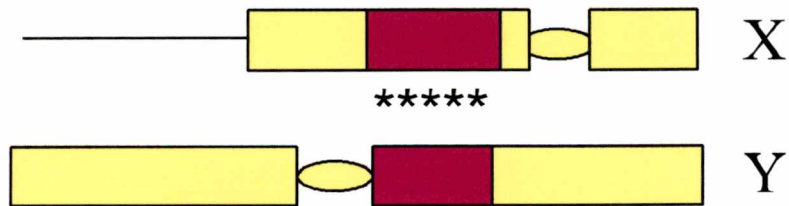


Figure 5. Chromosomal Distribution of Sequences Involved in *Drosophila* Male Meiotic Paring. Yellow boxes represent heterochromatin, magenta boxes represent rDNA, black lines represent euchromatin, and stars represent pairing sites.

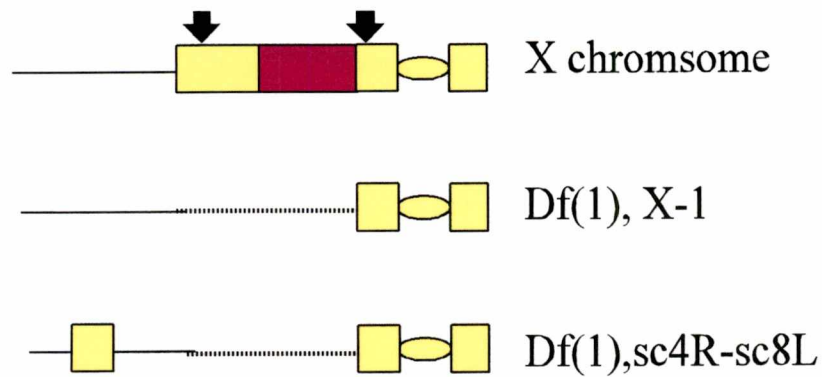


Figure 6. X Chromosome Deficiencies. Yellow boxes represent heterochromatin, black lines euchromatin, magenta boxes the rDNA, and dotted lines the section of the chromosome deleted. Arrows indicate the break points that formed Df(1)sc4-sc8.

and genetic background (McKee 1998). A clearer example of the role of the heterochromatin is Df(1)X-1. This deletion was generated using X-rays in a screen looking for the deletion of the rDNA and the su(f) locus (located in the proximal euchromatin) (Figure 2). When this deficient X chromosome is present with a normal Y there is random disjunction (McKee and Lindsley 1987; McKee and Karpen 1990).

The Role of rDNA in X-Y Pairing

The evidence of X-Y pairing sites being in heterochromatin presented above also implies that rDNA genes are responsible for X-Y pairing. Both the Df(1)sc4L-sc8R and Df(1)X-1 deficiency chromosomes lack rDNA and, furthermore, Cooper's observations point to the nucleolar organizing region, which surrounds the rDNA. The X-Y pairing site being located within heterochromatin is also supported by the experiment in which a deficiency X chromosome that retained a few copies of the rDNA repeats disjoined regularly from a normal Y chromosome (Appels and Hilliker 1982; McKee and Lindsley 1987).

In 1990, work by McKee and Karpen gave decisive evidence for the role of rDNA in X-Y pairing. They found that a P-element insertion, containing a single copy of the rDNA TU inserted into the heterochromatin of the rDNA deficient chromosome Df(1)X-1 (Figure 2), restored pairing with the Y chromosome B^sYy⁺ and B^sY. McKee and Karpen also observed that as more copies of rDNA units were inserted onto Df(1)X-1, the pairing frequency with a Y increased and the magnitude of frequency was proportional to the number of inserted rDNA repeats.

The rDNA repeat units contain 18S, 5.8 S, and 28S rDNA sequences separated by two internal transcribed spacers (ITS) and are flanked at the 5' end by an external transcribed spacer (ETS) (Figure 7). This portion of the rDNA repeat is 8Kb and is known as the transcriptional unit (TU). Another part of the rDNA repeat is the intergenic spacer sequences (IGS) that separates the TUs. The IGS contains repeats of three lengths: 95 bp, 330 bp, and 240 bp (Figure 7) (Hayward and Glover 1989; Simeone et al.

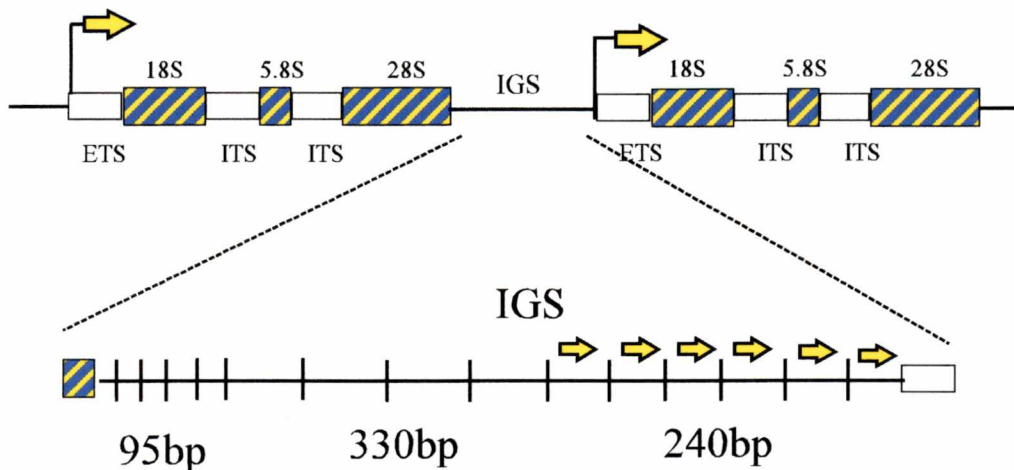


Figure 7. Structure of the rDNA Loci. Shown is one full repeat, which includes the rDNA, ETS, ITS, and IGS spacers, plus part of second repeat. The ETS (external transcribed spacer) and the ITS (internal transcribed spacer) are transcribed with the rDNA (hatched boxes) to make one RNA transcript. The transcript is spliced to give the 18S, 5.8S, and 28S rRNAs (as marked above). The IGS (intergenic spacer), formally known as the NTS (non-transcribed spacer), contains 95bp, 330bp, and 240 bp tandem repeated elements that are 5' to 3' in order (enlarged bottom region). Yellow arrows indicate RNA polymerase I promoter regions. Within the 240 bp repeats of the IGS is a sequence that has homology from -25 to +3 bp of the rDNA promoter start site, which is a minimal polymerase I promoter. (Hayward and Glover, 1989; McKee et al. 1992; Merrill et al. 1992; Ren et al. 1997)

1985). In 1992 Merrill et al. generated a Df(1)X-1 P-element insertions that contained IGS regions with a varying amount of the 5' end of the TU and found that the insertions restored X-Y pairing. It was also observed that pairing frequency increased in an insert-number-dependent manner. McKee et al. (1992) mapped the pairing promoting region of the IGS further using P-elements insertions onto the rDNA/ heterochromatin deficient X chromosome Df(1)X-1 and found that the 240 bp repeats of the IGS increase the X-Y pairing frequency. McKee also observed an increase in X-Y pairing frequencies with the increase of the number of repeats inserted, with the minimum number of 240 bp repeats of six showing significant pairing ability (Table 1). The role of 240bp repeats in pairing is supported by the converse work that showed that the TU does not promote X-Y pairing when copies are inserted on the Df(1)X-1 chromosome via a P-element (Ren et al. 1997).

The 240 bp repeats of the IGS are normally present in 6-12 copies just upstream of the rDNA TU promoter. The 240 bp repeats contain a putative polymerase I promoter (Simeone et al. 1985) (Figure 8) and S1 nuclease protection assays and Northern blot analysis by Murtif and Rae (1985) have shown that they are transcribed. There is evidence that the polymerase I promoters within the repeats, and the transcription of the repeats, allow for the loading and preparation of polymerase I proteins to transcribe the TU of the rDNA in *Drosophila* (Grimaldi et al. 1990; Chooi and Lisby 1984) and in yeast (Liu 1997), although the transcription of the 240 bp repeats seems also to play a role in X-Y pairing. When arrays of 10 or 25 240 bp repeats with mutated pol I promoters were inserted onto the heterochromatin and rDNA deficient Df(1)X-1 chromosome, random disjunction from a B^sYy⁺ chromosome was observed (Table 1) (unpublished data, Sun thesis). Also, work done by Lee demonstrated that an array of 240 pb repeat promoters alone promotes X-Y pairing when inserted onto the Df(1)X-1 chromosome (Lee, thesis).

Table 1. Disjunctional Effects of Mutant or Wild-type 240bp Repeat Promoters

Promoter Type	Repeat Copy Number	X-Y Disjunction Determined Cytologically
None	0	0.50
Mutant	10	0.53
Mutant	25	0.51
Wild-type	2	0.52
Wild-type	5	0.51
Wild-type	6	0.59*
Wild-type	7	0.56*
Wild-type	8	0.62*
Wild-type	10	0.64*
Wild-type	12	0.72*

*Values significantly different from the control sample (no 240bp repeats), $p > 0.05$.
Random disjunction is equal to 0.50 and proper disjunction is equal to one.

AGGTAGGCAG TGGTTGCCGA CCTCTCATAT TGTTCAAAAC
GTATGTGTTC ATATGATTTT GGCAATTATA TGAGTAAATT
AAATCATATA CATATGAAAA TGAATATTTA TTATATGTAT
ATGGAAAAAT GTTGAAATAT TCCCATATTC TCTAAGTATT
ATAGAGAAAA GCCATTTTAG TGAATGGATA ***TAGTAGTGTA***
AGCTAGCTGT TCTACGACAG AGGGTTCAA AACTACTAT

Figure 8. Sequence of the 240 bp Repeat. The putative polymerase I sequence is in bold italics. Primer sequences for 240+ PstI (top) and 240-1 PstI (bottom) are underline and were both preceded with the bases GGG GGC TGC AGA TAG TAG, which contains a PstI restriction site.

Chromosome Pairing in *Drosophila melanogaster*: Trans-Acting Factors

While more is beginning to be understood about the molecular mechanisms of homologous chromosome synapsis and recombination, much less is known about the molecular mechanisms of homologous chromosome pairing. Many mutations have been found to have trans-acting effects on homolog segregation during the first division of meiosis in *Drosophila* females, but not males. These mutations that have been found to affect segregation in females have mostly been mutations in genes that code for proteins involved in recombination though, so the phenotypes observed have been secondary effects. Mutations in the *nod* and *ncd* genes affect non-exchange chromosome segregation in females and code for kinesin motor proteins. The different effects of *nod* and *ncd* mutations on female and male meiosis may be explained by the unique structure of the female meiosis spindle. The spindle forms outward from the chromosomes rather than from the spindle poles to the chromosomes like in male *Drosophila* and most species. This unique spindle formation may require motor proteins that work in different ways, than those required for the more common spindle formation, to segregate chromosomes. When the two genes *ord* and *mei S332* are mutated, both affect female and male meiosis. The *ord* and *mei S332* genes code for proteins involved in sister chromatid cohesion, so the improper segregation observed, again, is a secondary effect.

In homolog pairing little has been discovered about the trans-acting factor(s) that are directly involved in pairing. A couple of mutations having trans-acting effects of homolog pairing have been created in *Drosophila*, but have been lost before they could be fully characterized (Hawley 1995), or seem to affect mitosis as well as meiosis.

Cabeza: A Pairing Protein Candidate

The *cabeza* gene was originally isolated as a partial cDNA in a screen for glycine rich proteins (Haynes et al. 1987). The partial clone displayed homology with RNA recognition motif (RRM) family of proteins specifically the hnRNA A1 gene, a hnRNA binding protein. The overall sequence of *cabeza*, however, was later found to contain some significant differences from the RRM family of proteins, including a divergent RRM

domain and a potential zinc finger motif (Stolow and Haynes 1995).

Cabeza is located on the X chromosome, position 14B, of *Drosophila*. The *cabeza* protein and RNA were shown by Stolow and Haynes (1995) to be expressed in all stages of *Drosophila* development with higher levels present in embryonic and pupal stages and lower levels in adult and larval stages. *Cabeza* protein has been found, in varying levels, in all larval tissues tested including gut, brain, ventral nerve chord, eye-antennal disc, leg disc, and fat bodies. In adults the brain and CNS are enriched with *cabeza* RNA and protein. Alternatively spliced transcripts of *cabeza* have also been observed, using Northern Blot analysis, throughout *Drosophila* development (Stolow and Haynes 1995). The *cabeza* protein, according to Stolow and Haynes, is localized in the nucleus and binds RNA. Using a *cabeza* antibody, Immanuel et al. (1995), has shown co-localization of *cabeza* to chromatin with phosphorylated RNA polymerase II in mammalian cells.

Cabeza shares homology with two genes isolated from human sarcoma cell lines, TLS (POMp75) and EWS (Stolow and Haynes 1995; Immanuel et al. 1995). The TLS and EWS proteins also contain the divergent RRM domain and a potential zinc finger domain and are overall 47% and 55% identical to *cabeza*, respectively, and are 56% identical to on another (Figure 9A). The divergent RRM domains of TLS, EWS, and *cabeza* are very similar with the *cabeza* RRM domain being 50% identical to that of EWS and TLS, and TLS and EWS RRM domains 61% identical to on another. The three proteins diverge from the highly conserved RRM domain within the RNP-1 sub-domain. The RNP-1 sub-domains of the three proteins contain an acid acidic residue at position +3 instead of the highly conserved aromatic residue (Figure 9B); *cabeza* and TLS contain a glutamic acid and EWS an aspartic acid at the +3 position. Also, in loop two of the RRM domain *cabeza*, TLS, and EWS have 12 amino acids instead of the highly conserved two amino acids.

The *cabeza* mammalian homolog, TLS, has been found to bind DNA *in vitro* and RNA *in vitro* and *in vivo* and to promote nucleic acid pairing *in vitro* (Baechtold et al. 1999; Yang 1998; Zinszner 1997; Akhmoedov 1995). When TLS knock out (KO) mice were generated they appeared smaller than their normal siblings (only 70% of the size)

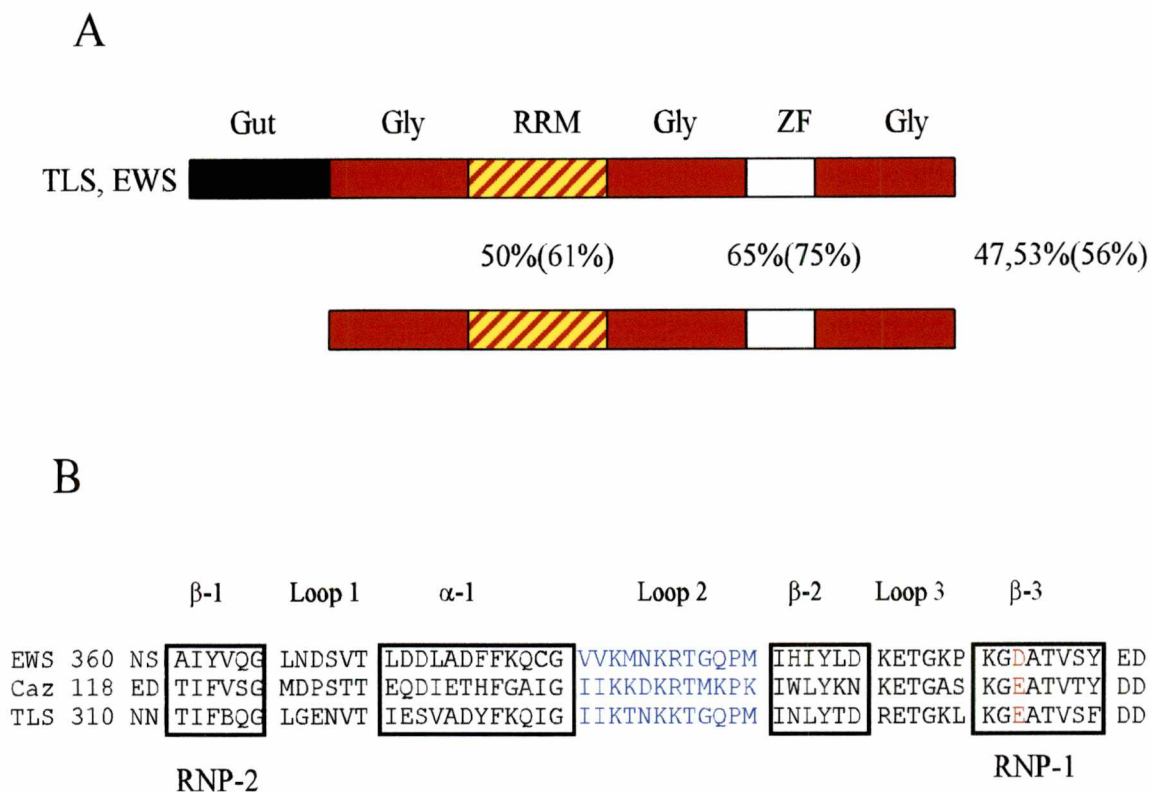


Figure 9. Structural Features of Caz, TLS, and EWS. A) Block boxes represent glutamine rich regions, red boxes glycine rich regions, red and yellow hatched box the RNA recognition motif (RRM) domain, and the unshaded box a zing finger (ZF) domain. Numbers between diagrams indicate percentage of identity between TLS or EWS (in parentheses) and cabeza. B) Comparison of the RRM motifs of EWS, cabeza, and TLS. Amino acids in blue indicate the expanded loop 2 region and the amino acids in red indicate the acid residue that usually is a highly conserved aromatic amino acid. (Figure adapted from Stolow and Haynes 1995.)

and they were sterile (Kuroda et al. 2000). Upon cytological examination of TLS KO mice spermatogenesis, incomplete SC formation, synapsis failure, and non-homologous synapsis of chromosomes was observed. Also, TLS was found to be abundantly expressed in the early stages of spermatogenesis in normal TLS⁺ mice, specifically in primary spermatocytes during the pachytene stage.

Hypotheses to be Tested

A Cis-Acting Factor: Role of 240 bp repeat rRNA in Homolog Pairing

Data has shown that the transcription of 240bp repeats within the rDNA is essential for X-Y pairing. Transcription being necessary for pairing suggests a direct role of the 240 bp RNA in pairing. One of the first logical questions, with this information in mind, is whether 240bp RNA transcripts are at the right place at the right time during spermatogenesis to be involved in pairing. In this study this was addressed using *in situ* hybridization to 240bp RNAs in *Drosophila* testis tissue. Whether 240bp RNAs were present through metaphase I, and the location of the 240 bp RNAs, was observed.

Since transcription being necessary for pairing, it suggests a direct role of the 240 bp RNA in pairing. Direct interactions between 240 bp RNAs were addressed by *in vitro* and *in vivo* experiments. Two sizes of 240 bp repeat RNAs were produced by *in vitro* transcription to perform an *in vitro* transcription interaction assay to observed if 240 bp RNAs directly interact. Computer modeling was used to determine possible direct interactions between 240bp repeat RNAs. The role of 240bp repeat RNAs was tested *in vivo* using a mating cross where a third chromosome served as a peripheral source of 240bp repeat RNA. If 240 bp repeat RNA interacts to induce pairing, whether via direct base pairing promoted by an enzyme or via a protein interacting with strands of 240bp RNA from the X and Y chromosomes, peripheral produced 240 bp RNA would act as a competitive inhibitor and cause nondisjunction.

A Trans-acting Factor: The Pairing Protein Candidate Cabeza

The *cabeza* and TLS proteins are 56% identical and share common motifs. Since TLS seems to play a role in mouse meiosis, the *Drosophila* homolog *cabeza* may play a role in fly meiosis. Two different mutant alleles of *cabeza* have been generated using EMS by Haynes (unpublished). In this study meiosis was characterized in these two mutants to detect if *cabeza* also plays a role in meiosis. Meiosis was characterized by looking at live preparations and stained preparations of testis tissue.

CHAPTER II

MATERIALS AND METHODS

Drosophila Cultures and Chromosomes

Drosophila melanogaster was cultured on standard cornmeal, molasses, agar medium supplemented with yeast at 23-25°C. Crosses were done in vials with two females and one male. Heat shock was administered to larvae on days three and five, after three days of egg laying, for one hour.

The y,w X chromosome contains two recessive genes that determine body color (y being yellow body) and eye color (w being white eyes) and is wild type. The 53B,X-1 chromosome is a Df(1)X-1 chromosome with two [rib 7] P-element insertions (Figure 10A). Df(1)X-1 is an X chromosome deficient for a large portion of the heterochromatin and a small proximal portion of euchromatin. The breakpoints are proximal to the nucleolus organizer (NO) and distal to within l(1)20 Cb (McKee 1992). The [rib7] p-element was constructed as described by Karpen et al. 1988. Briefly, the [rib7] insertion contains a rDNA TU flanked by IGS spacers at the 5' and 3' end that contain eight and three 240bp repeats, respectively, and the rosy gene, which causes a dark red eye color phenotype. Males carrying 53B, X-1 and a normal Y chromosome are not viable, but a duplication present in the B^sYy⁺ Y chromosome can supplement the missing rDNA euchromatic genes, so that 53B,X-1/ B^sYy⁺ males are viable. B^sYy⁺ is a Y chromosome that has a piece of duplicated X chromosome at its tip containing the y⁺ gene and three bands from the proximal X DNA that includes the bar stone (B^s) marker (results in a slit-like eye phenotype) and rDNA (Figure 10B). FM6 and TM3 are balancer chromosomes for the X and 3rd chromosomes, respectively, that contain multiple inversions that eliminate recombination. FM6 contains the white apricot (wa) eye color marker and the bar (B) eye marker that has a restricted to narrow vertical eye phenotypes. TM3 contains the dominant stubble (Sb) marker that displays a short, thick body hair phenotype.

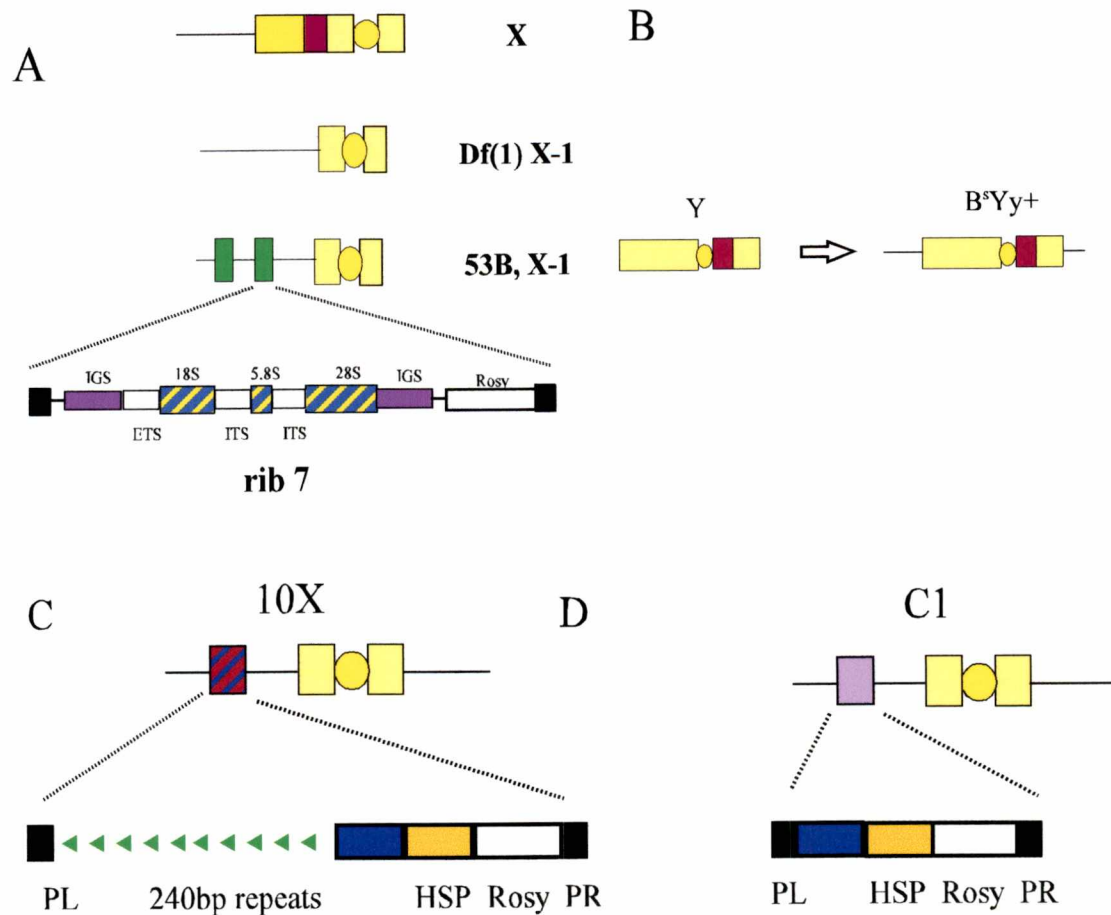


Figure 10. Structure of Drosophila Chromosome Constructs 53B,X-1, B^sYy⁺, 10X, and C1. Yellow boxes = heterochromatin; black lines = euchromatin; magenta boxes = rDNA, black boxes = P-element sequences left (PL) and right (PR). (A) Structure of the 53B,X-1 chromosome. IGS = intergenic spacer; ETS = external transcribed spacer; ITS = internal transcribed spacer. For the 53B, X-1 construct, the Df(1),X-1 chromosome, is a deficiency chromosome lacking a portion of the heterochromatin including the rDNA sequences (compare X and Df(1),X-1 chromosomes) contains two P-element insertions. The P-element insertions contain two IGS each, one IGS containing eight and the other three 240 bp repeats. (B) The B^sYy⁺ chromosome is a Y chromosome marked with the Bar of Stone (severe bar or slit-like eye) on the long arm and y⁺ (wild type body color) on the short arm. These markers are contained in euchromatin translocated from the X.(C) The 10X construct is a P-element insertion into chromosome three. The P-element insertion contains the Rosy gene that causes a bright red eye color (white box) along with the heat shock promoter 26 (HSP; orange box) up stream of ten 240 bp repeats containing mutant pol I minimal promoters (represented by green arrows). Blue boxes represent the lac Z gene. (D) Structure of C1. The C1 chromosome is a 3rd chromosome with a P-element insertion that contains the Rosy gene and the HSP, like the 10X p-element insertion, but no 240bp repeats.

The 10X 3rd chromosome P-element insertion contains an array of ten 240bp repeats with mutant polymerase I promoters (nearly eliminating transcription) downstream of the heatshock 26 promoter and the lac Z gene, which codes for the B-galactosidase protein (Figure 10C). Upstream of the HS promoter is the rosy gene, oriented so that transcription is in the opposite direction. The C1 3rd chromosome has a p-element that does not contain the 240 repeats, but everything else that is present in 10X is present in C1 in the same orientation (Figure 10D).

Drosophila stocks used for cabeza work included two strains that each have a mutant allele for the cabeza (caz) gene, caz¹ and caz², on the X chromosome. Stocks were maintained by utilizing the FM7-GFP balancer chromosome. The FM7-GFP chromosome contains multiple inversion that eliminate recombination in females and also contains a copy of the green fluorescent protein (GFP) that is driven by the actin 5 promoter so that it is constitutively active. Since both caz alleles are late larvae to early pupae lethal recessive lethal, males in the stocks were maintained as FM7-GFP/ Y genotypes. Third instar larvae were sorted based upon fluorescence, since caz¹ or caz²/Y mutants are not fluorescent, so that mutant males could be studied.

Plasmid Constructs

Plasmids containing varying numbers of 240bp repeats were constructed using the Bluescript Plasmid II (pBS)(Gibco) or a Bluescript plasmid with an inverted MCS between the XhoI and BstI restriction sites (alt pBS). The pBS7X, KS⁻ plasmid (Figure 11A) was constructed by inserting an array of seven 240bp repeats into the SmaI and PstI restriction sites of the alt pBS MCS. Then the KS primer sequence was removed by restriction enzyme digestion with SstI and PstI, treated with Klenow polymerase (Gibco, as recommended by the manufacturer), and blunt end self ligation. The pBS5X, KS⁺ plasmid (Figure 11B) was constructed by complete restriction enzyme digestion with BamHI and partial digestion with NheI of the pBS7X, KS⁺ plasmid. A fragment of 1.2 Kb was isolated, containing an array of five 240 bp repeats, and cloned into the BamHI and XbaI restriction sites of the altered pBS MCS, eliminating the SK primer sequence.

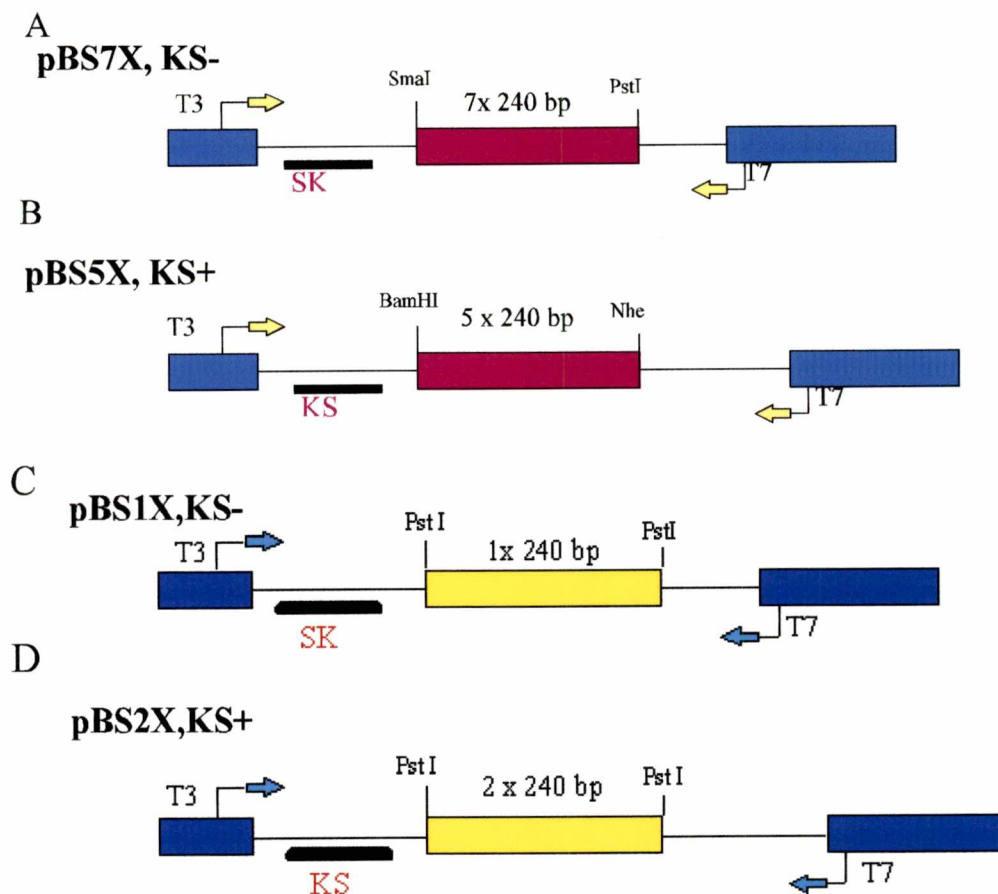


Figure 11. Template Constructs for *In vitro* Transcription. Plasmids were constructed using the Bluescript plasmid (pBS). (A) For the construct containing seven 240 bp repeats (7 X 240 bp) the repeats were inserted into the Sma I and Pst sites (as marked) of an inverted multiple cloning site. The multiple cloning site was inverted between the Xho I and the BstX I sites. The KS sequence, between the Pst I and Sac I site, was cleaved out to remove the sequence between the T7 promoter (T7 indicated by the yellow or blue arrows) and the 240 bp sequences. (B) For the construct containing five 240 bp repeats (5 X 240 bp) the repeats were inserted into the BamH I and Xba I sites of the multiple cloning site of pBS, eliminating the SK primer sequence. (C) and (D) constructs were created by cloning in one or two 240bp repeats, obtained by PCR, into the PstI restriction site of pBS or pBS with an inverted multiple cloning site. KS or SK sequences were then cleaved out with SacI and EcoRV or SstI and SmaI, respectively.

The pBS1X,KS⁻ and pBS2xKS⁺ plasmids (Figure 6, C and D respectively) were constructed by first using PCR with primers 240+1 PstI F (5'- GGG GGC TGC AGA TAG TAG AGG TAG GCA GTG GTT G -3') and 240-1 Pst R (5'- GGG GGC TGC AGA TAG TAG TAT CAT CAA AAA ACT T -3') (Figure 8) to amplify one and two 240 bp arrays with PstI restriction site ends. PCR products were cloned into the PCR II vector as described by the TA Cloning Kit (Stratagene). The pCRII vectors containing either one or two 240bp repeat arrays were then digested with PstI. The two 240bp array (2X) was cloned into the PstI restriction site of the pBS plasmid. SK primer sequence was removed using SstI and SmaI restriction enzymes. Then, the plasmid treated with Klenow polymerase, and then subjected to blunt end self ligation creating pBS2X, KS⁺ (Figure 11D). The 240bp array (1X) was cloned into the alt pBS MCS at the PstI restriction site. The KS primer sequence was removed with SacI and EcoRV restriction digestion. The linearized plasmid was then Klenow polymerase treated and subjected to blunt end self ligation to create pBS1X, KS⁻ (figure 11C). A two 240bp array was also cloned into an alt pBS plasmid in the same way to create alt pBS480, KS⁻.

In vitro Transcription

The RNA Transcription Kit (Stratagene) was used to do assays using *in vitro* transcription. RNA interaction assays were carried out using KpnI digested and Klenow treated plasmids using T7 polymerase as specified by the manufacturer. Either digoxigenin-11-uridine-5'-phosphate (Roche) or α -[³²P]-UTP (NEN) was used as part of *in vitro* transcription reactions to generate labeled riboprobes. To generate anti-sense labeled 240 bp riboprobes, the alt pBS480, KS⁻ plasmid was made linear with EcoRI and Klenow treated and T3 polymerase was used in the *in vitro* transcription reaction along with digoxigenin or [³²P] labeled UTP. For sense labeled 240bp riboprobes, alt pBS480, KS⁻ was made linear with KpnI and Klenow treated and T7 polymerase was used for the *in vitro* transcription reaction.

Slot Blots

Genomic DNA from 20 male or female flies was isolated using the Wizard Genomic DNA Purification Kit (Stratagene). DNA was treated with NaOH at a 1N concentration for one hour at 60°C. The denatured DNA was then neutralized with NH₄OAc. Genomic DNA was applied to Gene Screen Plus hybridization transfer membranes (DuPont/NEN) using the Minifold II slot blot apparatus (Schleicher and Schull) according to the manufacturers specifications. DNA was then UV cross-linked to the membrane and dried under a vacuum at 80°C for 3-4 hours. Membranes were prehybridized for two hours at 42°C in prehybridization solution (50% formamide, 5X Denhardt's, 0.1M Pipes, pH 6.7, 0.8M NaCl, 0.1% SDS, 100 µg/mL sonicated denatured salmon sperm DNA) and hybridized over night at 42°C in prehybridization solution containing 1µg/25mL sense or anti-sense digoxigenin labeled riboprobe. Membranes were washed twice with 2X SSC at room temperature (RT) for five minutes and twice with 0.2X SSC, 0.1% SDS for 30 minutes at 60°C.

Hybridization was then detected using an antibody specific for the hapten-labeled probe. Briefly, membranes were blocked for two hours at RT with blocking solution (5% (w/v) nonfat dried milk in PBS), probed with a mouse anti-digoxigenin primary antibody (Roche) diluted 1:200 in blocking solution, and then probed with a goat anti-mouse alkaline phosphatase conjugated secondary antibody (Sigma) diluted 1:200 in blocking solution. Membranes were washed with PBS three times after each antibody incubation and then once in alkaline phosphatase buffer (150mM NaCl, 5 mM MgCl₂), 100mM Tris-HCl, pH 9.5) after the second PBS washes. Blots were then developed using NBT and BCIP in a 2:1 ratio in alkaline phosphatase buffer and the enzymatic reaction was stopped by washes with PBS.

Northern Blots

Northern blots were prepared with approximately 1µg of *in vitro* transcribed RNA or 5µg of total RNA isolated from yw male *Drosophila* testes using Tri-Reagent (Sigma). (RNA was quantified by 1.2% agarose, 1X MOPS gel electrophoresis using a standard

RNA molecular weight marker to approximate concentrations.) The RNA was fractioned on 1.2% agarose gels containing 8% formaldehyde, 1X MOPS or 1X TBE. RNA was transferred to Gene Screen Plus hybridization transfer membranes (DuPont/NEN) by capillary action over night using 20X SSC. UV cross-linking of the RNA to the membranes was done, followed by drying at 80°C under a vacuum. Membranes were prehybridized for 2-3 hours at 42°C in prehybridization solution (see above) and hybridized in prehybridization solution containing radio-labeled riboprobes or end labeled oligonucleotide probes over night at 42°C. Membranes were washed twice with 2X SSC at room temperature (RT) for five minutes and twice with 0.2X SSC, 0.1% SDS for 30 minutes at 60°C. Blots were exposed over night at RT to Kodak XAR5 films.

In situ Hybridization

Drosophila testis tissue from 1-3 day old yw males were fixed as described by Gonzalez and Glover (1993). Briefly testes were dissected in testes buffer (183 mM KCL, 47 mM NaCl, 10mM Tris-HCl, 1mM EDTA, pH 6.8) and squashed under a siliconized cover slip. Slides were then snap froze and the cover slips removed. Slides were immersed in -20°C methanol for five minutes, -20°C acetone for 1-2 minutes, dried for 30 seconds, immersed in 1% Triton x-100/0.5% acetic acid for 10 minutes at RT, and then air dried for 10 minutes.

For hybridization slides were washed three times after fixation with PBS and then equilibrated in 1:1, PBS: prehybridization solution (50% formamide, 4X SSC, 1X Denhardt's, 10% dextran sulfate, 1mM levamisole). Slides were prehybridized for two hours at 37°C with prehybridization solution containing 100 µg/mL sonicated, denatured salmon sperm DNA and 125 µg/mL tRNA. Hybridization was carried out overnight at 37°C with 1 µg/mL digoxigenin labeled sense or anti-sense 240bp riboprobe in prehybridization solution. Negative controls were incubated with just prehybrization solution and positive controls were sealed with a silicon coated cover slip and rubber cement and heated to 95°C for 10 minutes before the incubation overnight.

All slides were washed twice with 2X SSC for five minutes at RT, twice with 0.2X

SSC for 20 minutes at 37°C, and then three times with PBS for five minutes at RT. Slides were blocked with 10% normal sheep serum (Jackson Laboratories) in PBS for two hours at RT and then incubated with sheep anti-digoxigenin alkaline phosphatase conjugated antibody (Roche) diluted 1:500 in blocking solution for one hour at RT. The slides were then washed three times for 10 minutes with PBS, 0.1% Tween-20, three times for 10 minutes with PBS, and then 10 minutes with alkaline phosphatase buffer (see above). Staining was then visualized by incubating slides with developing solution (alkaline phosphatase buffer with 2:1 NBT:BCIP and 50 ng/mL DAPI) for one hour at RT. Slides were then dehydrated with serial ethanol washes and mounted in glycerol.

Computer Modeling and Data Analysis

The RNA mfold program written by Michael Zuker (University of Washington) was used for computer modeling. The program can be accessed through the internet at the web address <http://mfold.wustl.edu/~folder/cgi-bin/mph-mfold-3.0.cgi>. The 240bp sequence (from Simeone et al. 1985) was entered into the program to observe secondary structure. Also, two repeats were entered into the program to observe predicted conformations.

Nondisjunction frequencies are defined as the fraction of XO and XXY flies of the total counted $(XO+XXY)/(XO+XXY+XY+XX)$. Chi square values were estimated using the formula $\Sigma(o-e)^2/e$, where o is the observed disjunction and nondisjunction values and e is the expected nondisjunction and disjunction values. P values were determined using the chi square (χ^2) contingency test at the 99% confidence interval with one degree of freedom.

Cytological Examination of Spermatogenesis

Testis tissues were prepared for visualization by either orcein staining (Lifshytz and Hareven 1977) or live preparation. Briefly, for orcein staining testes were dissected in testes buffer (183 mM KCL, 47 mM NaCl, 10mM Tris-HCl, 1mM EDTA, pH 6.8) and fixed in 45% acetic acid for 30 seconds. The larval testes were then stained in 3% orcein

stain in 45% acetic acid for five minutes and destained briefly with 60% acetic acid. A drop of 2% orcein stain in 1:1 lactic:acetic acid was then added to the testes and the tissue gently flattened under a cover slip. For live preparations, testes were dissected in testes buffer and flattened under a cover slip. Preparations were examined by phase-contrast microscopy using a Zeiss Universal Axioplan photomicroscope and photographed using Kodak film.

CHAPTER III

RESULTS

A Cis-acting Factor: Role of 240 bp Repeat rRNA in Homolog Chromosome Pairing

Is 240 bp RNA found at the right place at the right time for involvement in pairing?

The digoxigenin labeled riboprobe used for *in situ* hybridization was generated using *in vitro* transcription. The riboprobes were confirmed and the system used was tested by slot blot (Figure 12). Genomic DNA was isolated from yw and 53B,X-1 stocks and blotted onto a membrane using a slot blot apparatus. Membranes were probed with sense or anti-sense 240 bp repeat riboprobes and hybridization was detected using an alkaline phosphatase conjugated antibody system specific for digoxigenin (see Materials and Methods). Bands on the slot blot show a strong staining signal (Figure 7) showing that the riboprobe/ alkaline phosphatase conjugated antibody system functions effectively. The yw and 53B,X-1 flies have significantly different numbers of 240 bp repeats since yw flies have a wild type copy number of rDNA units (1000-2000) and 53B,X-1 flies carry only 22 240 bp repeats on one (or in the case males the only) X chromosome. In the slot blots the 53B,X-1 genomic DNA sample band demonstrates slightly lighter intensity of staining than the band of the yw fly genomic DNA samples (compare rows 1 and 2 of Figure 12A and 12B), suggesting the detection system can be quantitative as well as qualitative.

The specificity of the 240 bp riboprobe was tested using Northern blot analysis. A 1.2% agarose formaldehyde MOPS gel was used to separate total testis RNA and sense and anti-sense 240bp RNA transcribed *in vitro* from the pBS7X, KS- plasmid (Figure 11A). The RNA was then blotted onto a membrane and probed with labeled anti-sense or sense radio-labeled riboprobes (Figure 13). Results using an anti-sense riboprobe revealed an approximately 2Kb band in the total testis RNA sample (Figure 7, lane 1), a 1.4 -1.6 Kb band for the sense 7X RNA (Figure 13, lane 2), and no band for the anti-sense 7X RNA

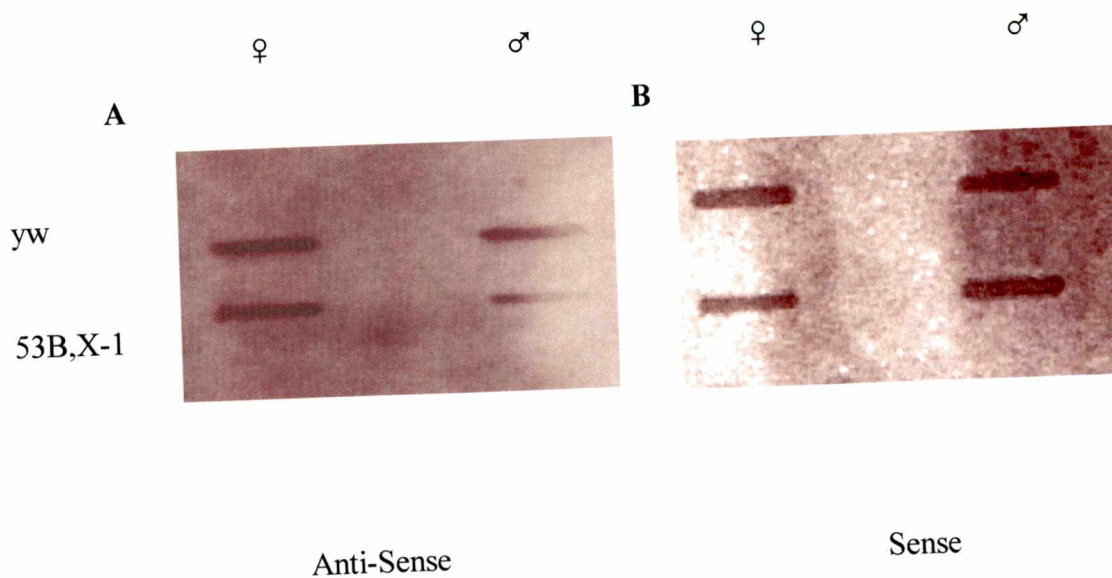


Figure 12. Genomic DNA Slot Blots Probed with Dioxigenin Labeled 240bp Riboprobe. Genomic DNA was isolated from yw females and males and from 53B,X-1/FM6 females and 53B,X-1/BsYy+ male flies. DNA was denatured and blotted onto a membrane and probed with sense or anti-sense dioxigenin labeled 240bp riboprobes generated using *in vitro* transcription. Hybridization was detected using a mouse anti-dioxigenin primary antibody and an anti-mouse alkaline phosphatase conjugated secondary antibody. Bands were visualized by development with NBT/BCIP.

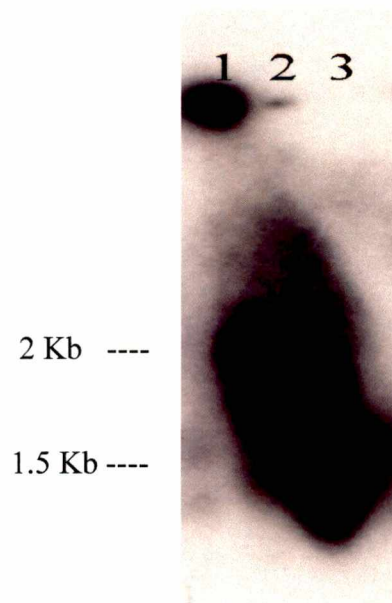


Figure 13. Northern Blot Analysis of Total Testes RNA. Total testes RNA from yw males (lane 1), sense 7X RNA (lane 2) in vitro transcribed, and anti-sense 7X RNA (lane 3) in vitro transcribed, were separated on a 1.2% agarose, 0.8% formaldehyde MOPS gel and blotted onto a membrane. The membrane was hybridized with an anti-sense radio-labeled 240bp riboprobe. Total testes RNA revealed a band of approximately 2Kb, sense 7X RNA a band of approximately 1.6Kb, and anti-sense 7X RNA revealed no signal.

(Figure 13, lane 3). These results were as expected since the 7X RNA should be 1.68Kb (minus as much as 480bp due to early termination of the *in vitro* transcription) and 240bp repeat RNA was found previously by Murtif and Rae (1985) to be approximately 2Kb in size. When the same samples were probed with the sense 240bp riboprobe only one band approximately 1.6kb was observed in the anti-sense 7X RNA lane (data not shown).

After establishing that the digoxigenin labeled 240 bp riboprobe/ anti-digoxigenin alkaline phosphatase conjugated antibody system worked and the specificity of the 240 bp riboprobe was confirmed, *in situ* hybridization to *Drosophila* testes tissue was done. Testes tissue from 1-3 day old yw males were fixed and probed with digoxigenin labeled 240 bp anti-sense or sense riboprobes. Hybridization was visualized using anti-digoxigenin alkaline phosphatase conjugated antibody and developed using NBT/BCIP to create purple/blue staining.

Staining revealed 240 bp RNA is localized to the nucleus. In early prophase, spermatocytes staining is strong and localized to what appears to be the nucleolus (Figure 14A), since positive controls demonstrate the same pattern of staining which indicates the rDNA (Figure 14B). Mid-prophase stage spermatocytes, identified by the presence of Y-loops, are variably stained with some mid-prophase cells stained (Figure 14D) and other mid-prophase cells not stained (Figure 14E). The lacking of staining in some mid-prophase primary spermatocytes may be attributed to 240 bp RNA not being present. However, this is unlikely since transcription is taking place at very high levels during this time, especially at the rDNA loci. Ribosomes are needed for translation of mRNA being produced for later stages of spermatogenesis and spermiogenesis because transcription ceases at late prophase. Another possibility is that rDNA transcripts are not accessible to the probe in the tissue because they are beneath chromatin and RNA. The y-loops at this stage take up a large amount of space in nuclei and may mask the 240bp RNAs.

In late prophase stage spermatocytes all nuclei display staining. In some late stage primary spermatocytes, staining is localized to one spot (Figure 15, A and B) and others two spots (Figure 15, C and D). In cells with two spots of staining in the nucleus, one spot is usually near the nuclear envelope and other near the middle of the nucleus.

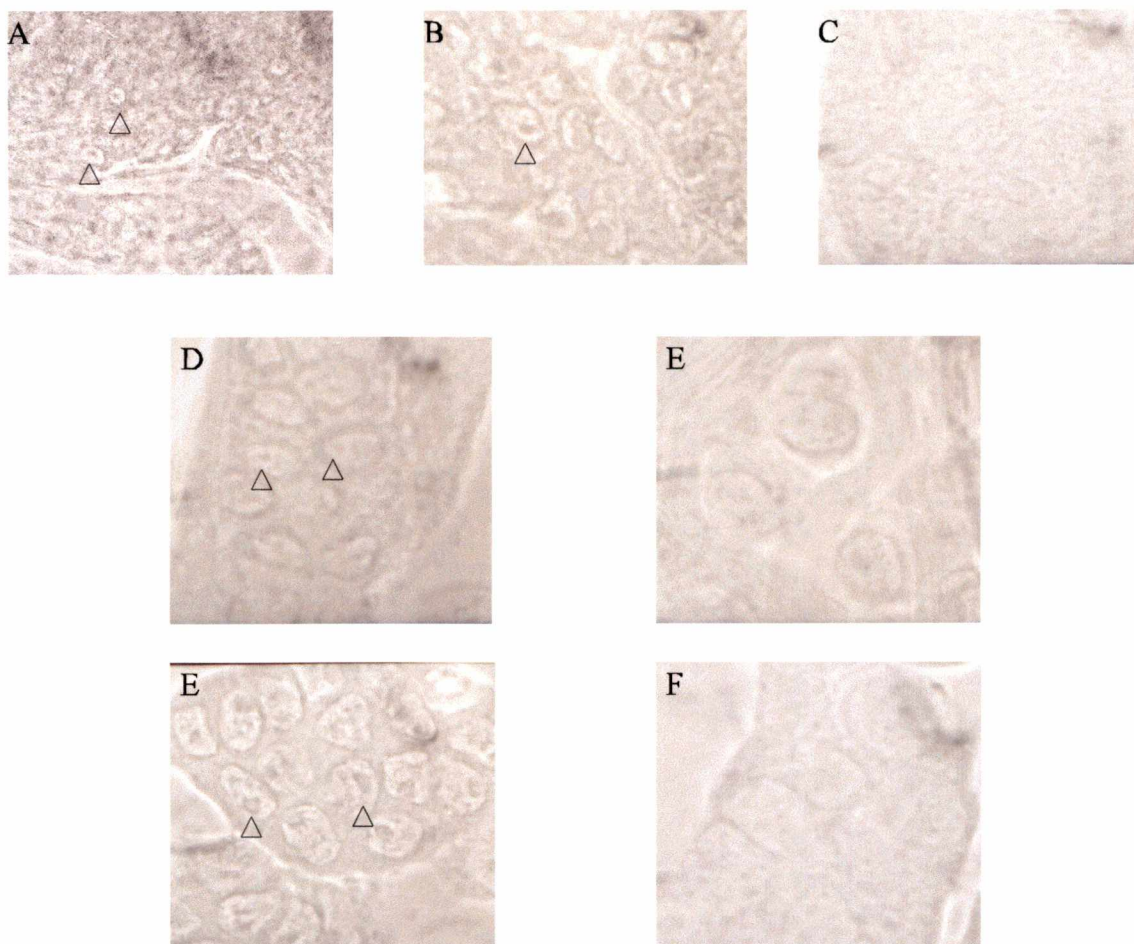


Figure 14. In situ Hybridization to 240bp RNA in Early to Mid-Prophase Primary Spermatocytes. Testes tissues of yw males *Drosophila* were fixed and hybridized with a dioxigenin labeled anti-sense riboprobe. Hybridization was detected via an anti-dioxigenin alkaline phosphatase antibody and visualized with BCIP/NBT development. Experimental samples were hybridized at 37°C, positive control were heated to 95°C before hybridization at 37°C, and negative control were hybridized with prehybridization solution (no probe). All observations at 100X magnification. A) Early prophase I of experimental sample; B) Early prophase I of positive control sample; C) Early prophase I negative control sample; D) Mid-prophase I of experimental sample - staining; E) Mid-prophase I of experimental sample - no staining; F) Mid-prophase I positive control sample; G) Mid-prophase I of negative control sample.

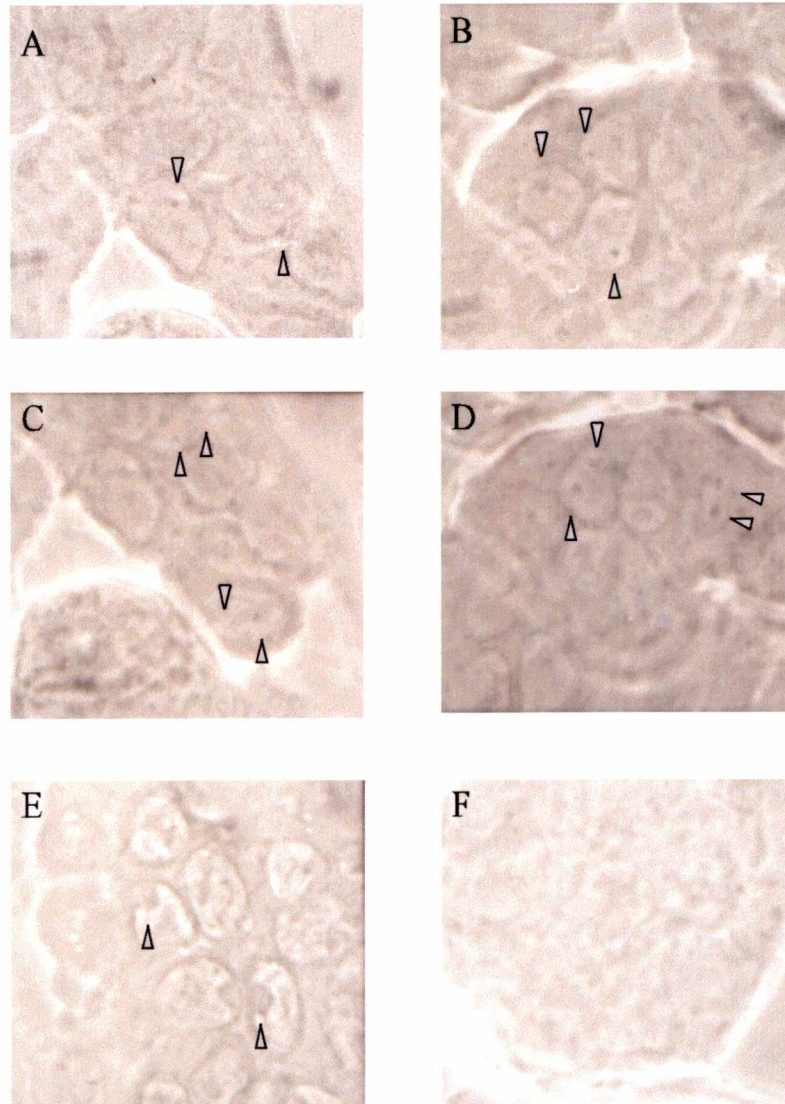


Figure 15. In situ Hybridization to 240bp RNA in Late Prophase Primary Spermatocytes. Testes tissues of yw males *Drosophila* were fixed and hybridized with a dioxigenin labeled anti-sense riboprobe. Hybridization was detected via an anti-digoxigenin alkaline phosphatase antibody and visualized with BCIP/NBT development. Experimental samples were hybridized at 37°C, positive control were heated to 95°C before hybridization at 37°C, and negative control were hybridized with prehybridization solution (no probe). All observations at 100X magnification. All samples late prophase I. A) and B) Experimental - one spot stained; C) and D) Experimental - two spots stained; E) Positive control sample; F) Negative control sample.

stage primary spermatocytes that display just one spot of staining have a spot that is usually near the nuclear envelope. This is the usual location of chromosomes at this stage, on the periphery of the nucleus next to the nuclear envelope, and can be seen clearly in the positive control (Figure 15E). This suggests that the second spot may be 240 bp RNA left over from the nucleolus or X and Y chromosomes that are unpaired, with the later being unlikely.

Staining in very late prophase/ prometaphase is usually found near the nuclear membrane (Figure 16, A and B). This staining is on a condensed chromosome, which appear as a clear spot. DAPI staining shows that the clear spots correspond to condensed chromosomes (Figure 16, E and F). Patchy DAPI stain with a high background was observed for slides that had levamisole in the hybridization buffer. Levamisole decreases background staining by inhibiting non-mammalian alkaline phosphatases; the alkaline phosphatase enzyme conjugated to the anti-digoxigenin antibody used was isolated from calves (Roche product services). The pictures taken in Figure 16 E and F did not have levamisole in the hybridization buffer. This created a high background making stain difficult to interpret. Staining was not obvious in later stages of meiosis I and no staining was observed in meiosis II cells. Scoring cells in metaphase through telophase is difficult do to the events of these phases occurring very quickly, so cells at these stages are observed very often. Also, hybridization was done with sens probes, but no staining was observed.

Staining on at least one chromosome is clearly seen through prometaphse. The location of the staining is most likely the X-Y homolog pair since staining is mostly observed on what seems to be the smallest chromosome clump of the three large visible homolog pairs. This suggests that 240bp repeat RNA may have a role in X-Y pairing since it is at the right place at the right time.

Does 240bp RNA play a direct role in pairing of the X-Y homologs?

Computer modeling was done on the 240bp repeat RNA using the RNA mfold program. Results revealed a structure where 42 bases of two 240bp repeats can base pair

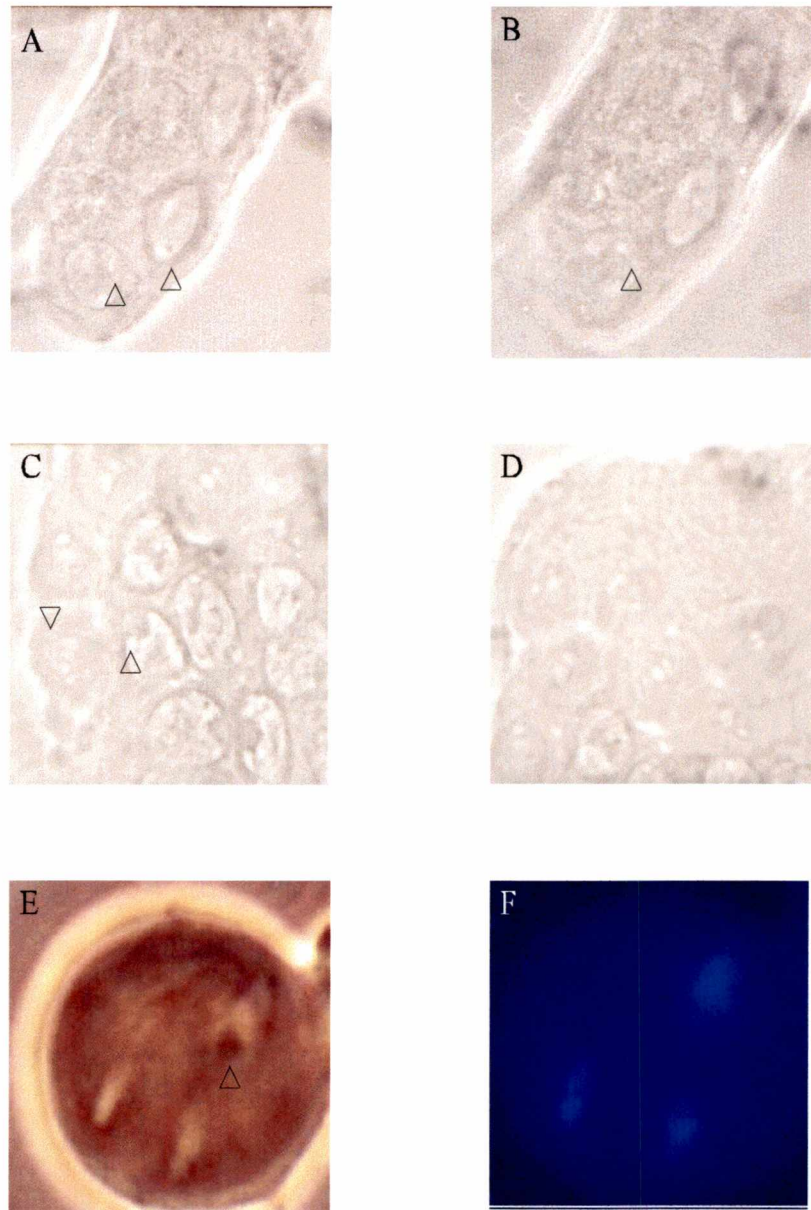


Figure 16. In situ Hybridization to 240bp RNA in Late Prophase/ Prometaphase Primary Spermatocytes. Testes tissues of yw males *Drosophila* were fixed and hybridized with a dioxigenin labeled anti-sense riboprobe. Hybridization was detected via an anti-dioxigenin alkaline phosphatase antibody and visualized with BCIP/NBT development. All observation at 100X magnification. Experimental samples were hybridized at 37°C, positive control were heated to 95 °C before hybridization at 37°C, and negative control were hybridized with prehybridization solution (no probe). A) and B) Experimental sample - two focal planes; C) Positive control sample; D) Negative control sample; E) Experimental, without levamisole; F) DAPI staining of (E).

in an antiparallel fashion (Figure 17). This suggests 240bp repeat RNA may interact via base pairing to pair or hold together the X and Y chromosomes. To test this hypothesis, an *in vitro* transcription interaction assay was utilized.

In the interaction assay linearized pBS5X, KS⁺ (containing an array of five 240bp repeats) and pBS7X, KS⁻ (containing an array of seven 240bp repeats) (Figure 11, A and B) were used for *in vitro* transcription reactions containing either one plasmid or both the plasmids together. *In vitro* transcription of the plasmids create transcripts of two different lengths, and array of and an array of seven repeats, that are also recognized by two different probes. The KS probe is complementary to the 3' end of transcripts from the pBS5X, KS⁺ plasmid, and the SK probe is complimentary to the 3' end of pBS7X, KS⁻ transcripts, because of sequences immediately following the arrays in the plasmids (see Figure 11). Reactions were separated on 1.2% agarose TBE gels, non-denaturing conditions so that transcript interactions, via base pairing, will stay intact; because of the non-denaturing conditions sizes of bands in the gels are relative to secondary structure being present. After reactions were transferred to membranes by capillary action they were probed with SK and KS radio labeled probes. Base pairing of 5X and 7X transcripts would reveal unique bands in the reactions containing both plasmids that would be detected by both the KS and SK probes, but this was not observed (Figure 18). The SK and KS probes did not hybridized to any unique bands (Figure 18, compare lanes 1 and 3 and 4 and 6).

Increasing the template concentration in the *in vitro* transcription reactions, to mimic *in vivo* conditions more closely, did not reveal any 240bp RNA interactions either (data not shown). Heating samples, after transcription but before running then on a gel, to 100°C or 65°C was done to see if this would promote interactions, possibly compensating for a pairing enzyme. Results from this experiment were inconclusive since smears, instead of bands, were seen on the blots (data not shown). This was most likely due to transcripts being denatured and then folding into many different conformations. The interaction assay was repeated using the pBS1x, KS⁻ and pBS2X, KS⁺ plasmids (Figure 11, C and D, respectively). The larger repeat transcripts may have inhibited the

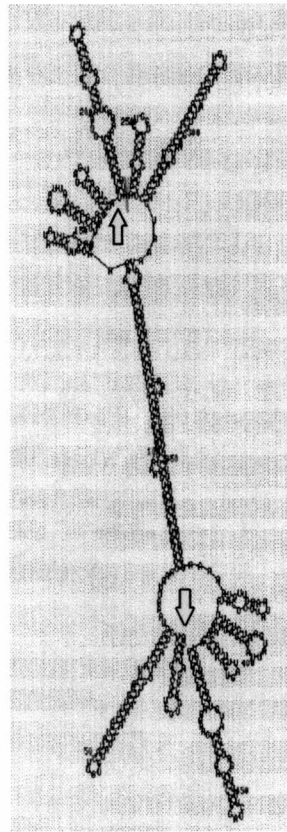
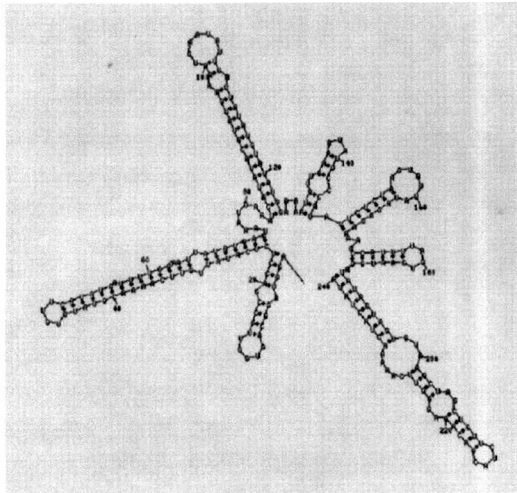


Figure 17. Predicted 240bp RNA Structures. The RNA mfold program was used to predict the secondary structure of the 240 bp RNA sequences. The diagram on the left shows the predicted secondary structure for a single 240 bp repeat. The diagram on the right shows the prediction of how two 240bp repeats may potential interact. Arrow indicate the 5'-end of each sequence.

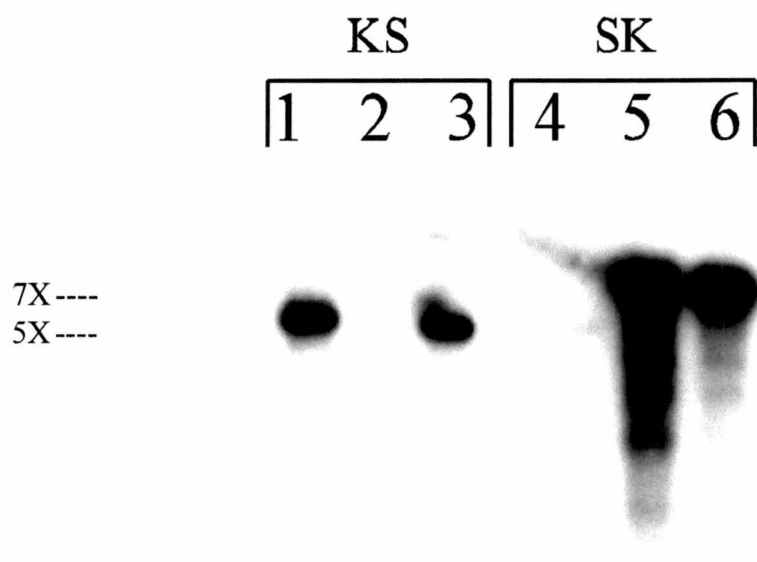


Figure 18. In vitro Transcription Interaction Assay. RNA was transcribed in vitro from the pBS5X, KS+ plasmid (lanes 1 and 4), the pBS7X, KS- plasmid (lanes 2 and 5), or both (lanes 3 and 6) in the same reaction. RNA was separated on a 1.2% agarose TBE gel and blotted onto a membrane. Membranes were probed with radio-labeled KS sequence oligo (lanes 1-3) or SK sequence oligo (lanes 4-6). No unique bands were observed.

interactions *in vitro*, so small size fragments were used. This interaction assay also revealed no unique bands recognized by both the SK and KS probes (Figure 19, compare lanes 1 and 3 and 4 and 6).

The results, from the 240 bp RNA interaction assays, suggest that there is no base pairing between 240bp repeat RNA, but this does not indicate whether 240bp RNA interactions promote pairing *in vivo*. The role of 240bp repeat RNA was tested using a mating cross (Figure 20). In the cross, heat shocked and non-heat shocked 53B,X-1/B^sYy⁺; C1/+ and 53B,X-1/BsYy⁺;10X/+ males were crossed to yw females to detect NDJ (see Figure 20 for crossing scheme). The third chromosome 10X (Figure 10C) contains a p-element insertion with an array of ten 240bp repeats with mutated polymerase I promoters under a HS26 promoter. Upstream of the 240bp repeat array is the lac Z gene that codes for the β -galactosidase protein. A β -galactosidase assay was done previously which determined that there were low levels of transcription of the repeats under normal conditions and that heat shock did induce transcription several fold (personal communication Suchita Das). The 10X chromosome served as a peripheral source of 240bp repeat RNA. If 240bp RNA interacts to induce pairing, whether via direct base pairing promoted by an enzyme or via a protein interacting with strands of 240bp RNA from both chromosomes, peripherally produced 240bp RNA would act as a competitive inhibitor and cause nondisjunction. The third chromosome C1 served as a control, and contains only the heat shock 26 promoter, a small amount of plasmid DNA and the x-gal gene.

Results from the *in vivo* interference crosses revealed 10.5% NDJ in 10X flies with no heat shock, 15.6% NDJ in 10X flies with heat shock, 17.3% NDJ in C1 flies with no heat shock, and 17.4% NDJ in C1 flies with heat shock (Table 2). NDJ was determined by counting females with bar eyes, denoting the genotype yw/53B,X-1/B^sYy⁺ (XXY), and yw males, denoting the genotype yw/0 (X) so only meiosis I NDJ was detected. Upon heat shock, the NDJ of 10X flies increased 5.1%, a significant increase with $P < 0.01$.

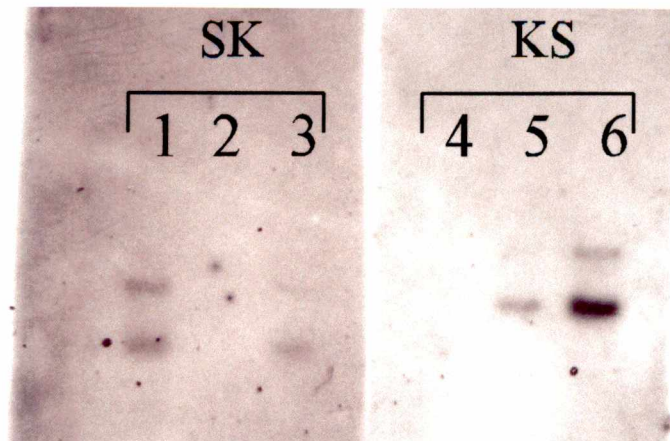


Figure 19. In vitro Transcription Interaction Assay. RNA was transcribed in vitro from the pBS2X, KS+ plasmid (lanes 1 and 4), the pBS1X, KS- plasmid (lanes 2 and 5), or both (lanes 3 and 6) in the same reaction. RNA was separated on a 1.2% agarose TBE gel and blotted onto a membrane. Membranes were probed with radiolabeled KS sequence oligo (lanes 1-3) or SK sequence oligo (lanes 4-6). No unique bands were observed.

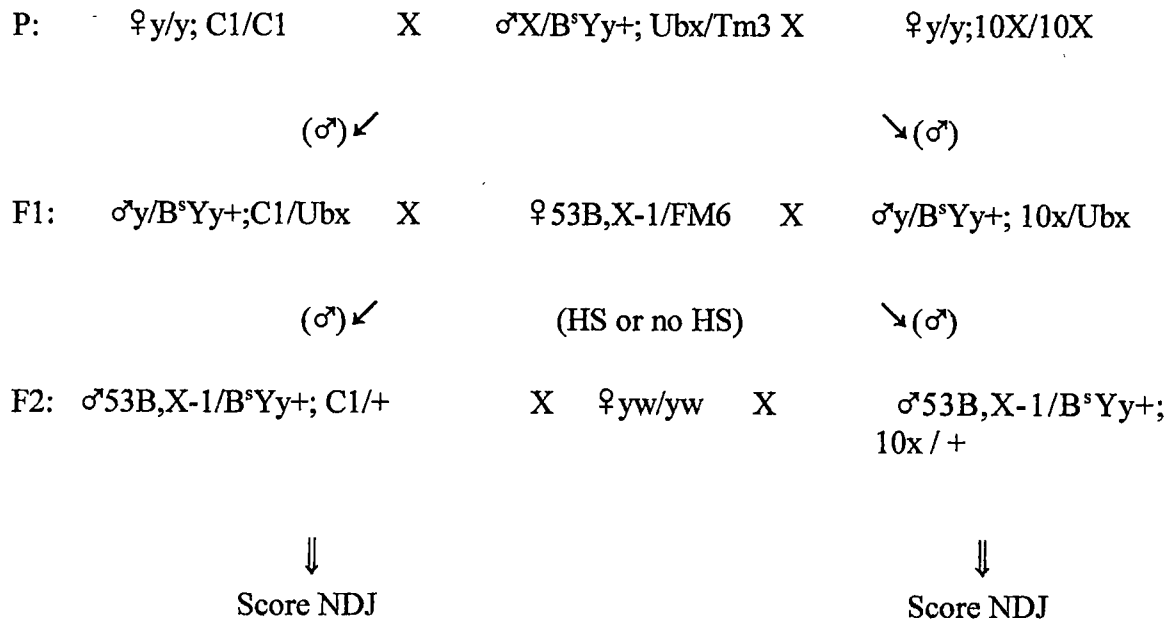


Figure 20. RNA Interference Crossing Schemes. B^sYy⁺ = Y chromosome marked with the Bar of Stone trait, which is slit-like eye phenotype, and wild type body color (y⁺); Ubx = 3rd chromosome marked with ultrabithorax (a large haltere); FM6 = X chromosome balancer; Tm3 = 3rd chromosome balancer; y = X chromosome marked with yellow body color trait; 10X = 3rd chromosome with 10x P-element insertion; C1 = 3rd chromosome with C1 P-element insertion; yw = X chromosome marked with yellow body and white eye traits; ♀ = female *Drosophila*; ♂ = male *Drosophila*, symbol by arrow indicates males were collected from the cross; HS = heat shock, to larvae for 1 hour at days three and five after eggs are laid by females.

Table 2. RNA Interference Cross Results

	XX	XXY	XO	XY	N	Percent NDJ
10X No H.S.	280	15	35	148	478	10.5
10X H.S	1306	70	323	821	2520	15.6*
C1 No H.S.	496	15	150	290	951	17.3
C1 H.S.	759	65	167	508	1332	17.4

* Value significantly different from control value, $p < 0.01$, by χ^2 stringency test.

Heat shock of C1 chromosome flies raised NDJ by only 0.1%, a difference that is not significant. This data suggests that 240bp repeat RNAs play a direct role in X-Y pairing or cohesion, possibly via a protein as suggested above.

A Trans-acting Factor: The Pairing Protein Candidate Cabeza

To confirm the stage of lethality of the *caz*¹ and *caz*² mutants non-fluorescent third instar larvae were collected from each stock and put into vials. All larvae pupated, but none hatched. The specimens inside the pupa cases could be examined under fluorescence light, due to autofluorescence, at 10X magnification. Upon examination of 4-5 day old pupae, the observation was made that the pupa looked as when they first pupated, suggesting no further development and most likely death.

Live preparations of third instar non-fluorescent larvae (*caz*¹ or *caz*²/Y) testes were made to examine onion stage cells. The onion stage of spermiogenesis can be used to detect chromosome content since the size of the nucleus corresponds to chromatin content (Gonzalez 1989). Proper pairing and disjunction of chromosomes can be extrapolated because variation in the size of nuclei, whether in relation to the nebkern or each other, suggests more or fewer chromosomes pointing to defects in chromosome pairing or disjunction. Results of the live preparations revealed nuclei that were very close in size to the nebkern within each cell, and the with each other, for both *caz*¹ and *caz*² mutants, as seen in Figure 21A and 21B, respectively. In Figure 21 filled arrow heads indicate the nucleus and open arrow heads indicate the nebkern. This data suggests proper pairing and disjunction of chromosomes during meiosis in both *caz*¹ and *caz*² mutants.

Data from orcein stained testes tissues from non-fluorescent (*caz*¹ or *caz*²/Y) larvae support the results from the live preparations. Spermatid nuclei were of equal size in the stained tissue from both *caz*¹ and *caz*² mutants (Figure 22A and 22B, respectively). Also, at late prophase I, condensed chromosomes in the *caz* mutants orcein stained testes tissues showed proper pairing (Figure 22C and 22D). Cells demonstrated only three clumps of chromosomes, which are the two large autosomes and the sex chromosome pair, the

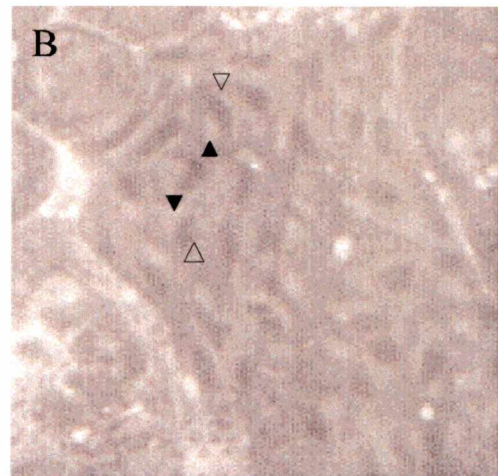
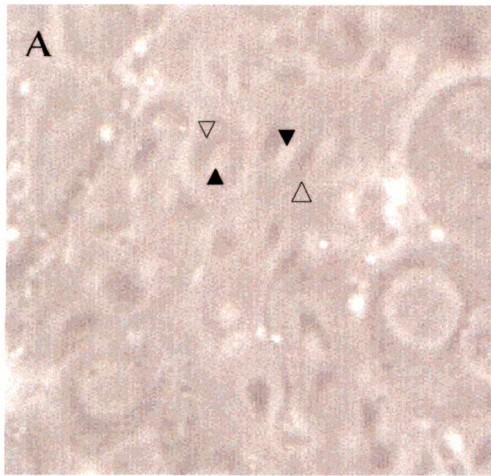


Figure 21. Live Larval Testes Preparations. Representative onion stage cells from live preparations of testes from *caz¹/Y* (A) and *caz²/Y* (B) non-fluorescent larvae. Filled arrows indicate nuclei and open arrows indicate nebenkern.

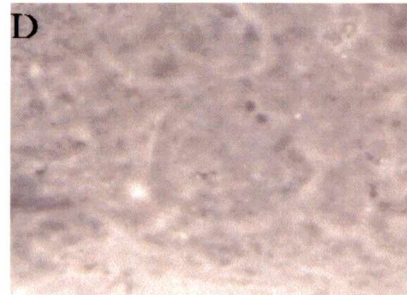
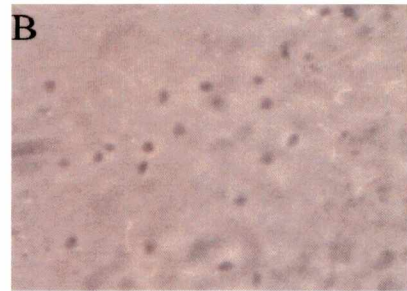
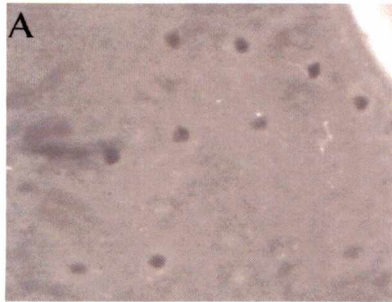


Figure 22. Orcein-Stained Testes Tissues. Testes from *caz*¹/Y (A and C) and *caz*²/Y (B and D) non-fluorescent larvae were fixed with acetic acid and stained with orcein. A and B are mature spermatid nuclei and C and D are late prophase primary spermatocytes.

fourth chromosome is not observed, due to its size, which is common. Mature spermatids and prophase II also appear normal in morphology (Figure 23). Mature spermatids appear normal and of equal size to one another (Figure 23A) and the prophase II sister chromatids appear to have segregated properly from their homolog (Figure 23B, filled arrows).

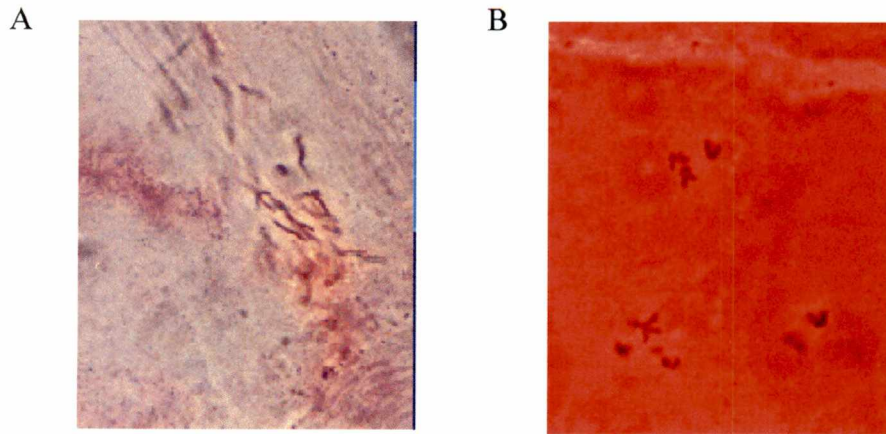


Figure 23. Orcein-Stained Testes Tissues. Testes from *caz/Y* non-fluorescent larvae were fixed with acetic acid and stained with orcein dye. Representative pictures: A) mature spermatids; B) Prophase II.

CHAPTER IV

DISCUSSION

A Cis-Acting Factor: Role of 240 bp repeat rRNA in Homolog Chromosome Pairing

Models of meiotic pairing range from homologous sequences pairing via prion-like proteins (Sybenga 1999) to homologs being brought together through associations with transcription factors transcribing homologous sequences (Cook 1997). Such models change with new findings and new models develop as advances are made. The initial findings about the 240bp sequences promoting pairing, coupled with the fact that they are also transcribed, led to the development of a model with DNA-DNA pairing through strand transfer proteins after transcription opened the DNA (McKee 1992). Additional findings, such as the minimum number of six repeats is required for significant pairing frequencies, that transcription is necessary for pairing, and that the 240bp promoter alone is sufficient for pairing (in an array of 16), does not reduce the plausibility of this model. However, the alternative of RNA-RNA or DNA-RNA interactions can not be ruled out.

The findings in this study provide more compelling evidence for a model of homolog pairing through RNA-RNA or DNA-RNA interactions rather than DNA-DNA interactions. The 240bp RNAs produced from a third chromosome peripheral site (interference assay) showed an increase in NDJ upon induction of transcription of the peripheral RNAs (Table 2). This suggests interference of outside RNAs with pairing, like a competitive inhibitor in an enzyme reaction. A direct role of 240bp RNAs is supported by the finding that 240bp RNAs are detected in stages of spermatocytes in which pairing occurs (the right place at the right time) in *in situ* hybridization experiments (Figures 14-16). A model of RNA-RNA interactions based on the transcription of the 240bp RNAs can include the part of 240 bp RNAs that extrudes from the DNA templates, from the X and Y chromosomes, interacting. The interactions may be by chance base pairing, pairing via a pairing protein, or a protein binding to secondary structure of the RNA repeat from each homolog (Figure 24A). [The plausibility of repeat RNA base pairing is based on

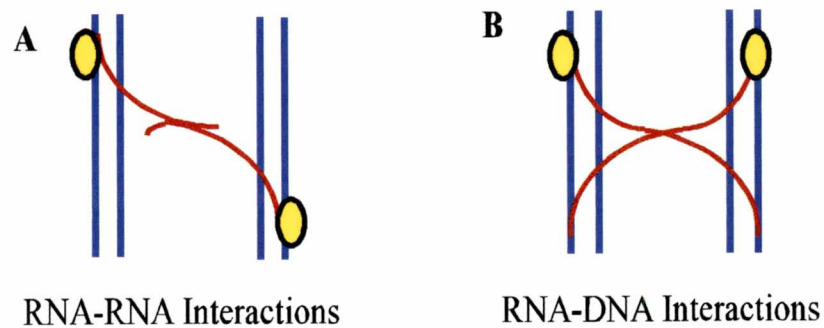


Figure 24. Possible Models for X-Y Chromosome Pairing in *D. melanogaster* males. Blue line = DNA, Red lines = RNA, yellow circles = RNA polymerase I. Shown is general models for pairing of homologous chromosomes. RNA polymerase I is included to account for the need of transcription or at least the binding of pol I to the minimal promoter sequence in the 240 bp repeats.

results obtained from the mfold program (Figure 12) that predicted approximately 42bp can base pairing in an antiparallel fashion.] This would account for the minimum number of six repeats being needed for significant pairing frequencies; the length of the six repeat RNA may be the sufficient length to extrude from the DNA template and induce pairing. Chance base pairing is not likely based on the *in vitro* interaction assay data that demonstrated no interactions, so trans-acting proteins are most likely involved (Figures 18 and 19).

An RNA-DNA pairing model is also supported by the data obtained and is highly plausible. In this model, the part of the repeat RNA transcript that extrudes from the DNA would pair with DNA opened up by transcription (Figure 24B). This model would also account for the minimum number of six 240bp repeats required for significant pairing frequencies and transcription would be doubly important. A mechanism based on this model would also most likely entail proteins that pair nucleic acids instead of just random chance base pairing interactions.

Both of these models would require the 240bp repeat RNA to stay attached to the coding DNA. A possibility is polymerase I (pol I) holding the RNA transcript to the coding strand. This would require pol I to stall during transcription without detaching from the DNA; a downstream pol I promoter, within the 240bp repeats, may cause the stalling without causing the pol I to fall off the DNA strand. A study by Roussel et al. (1996) has shown that pol I transcription complexes localized to chromatin during mitosis of human cells, but without transcription taking place, suggesting stalling or a factor that causes pol I to cease transcription but stay attached to the DNA.

In situ hybridization to detect 240bp repeat RNA in this study detected the presence of 240b repeat RNA as late as prometaphase I. This suggest 240bp RNA has a role in locking the X-Y homologs together until anaphase. This is implied in the RNA-RNA and DNA-RNA interactions pairing models. However, this gives rise to an issue of pairing versus locking role of the 240bp repeat RNAs. It is possible that another factor brings the X and Y chromosomes together and the 240bp repeats are involved in the cohesion or "locking" of the homologs together. The RNAs may be brought into

proximity by being commonly localized to the nucleolus during early prophase I or via shared transcription complexes as suggested by Cook (1997).

If there are RNA-DNA or DNA-DNA interaction via the 240bp repeat RNAs it would explain the string-like structures that Cooper (1964) observed in prophase I holding the X and Y chromosomes together. The next steps will be to test the RNA-DNA and RNA-RNA interaction pairing models. This could include answering questions like where polymerase I is located, and when, in meiosis (immunostaining with a pol I antibody) and whether polymerase I stalling on the DNA is necessary for meiosis. Other questions also need to be addressed such as the role of homology in the interactions. An array of sixteen 240bp putative I promoters when inserted on a Df(1), X-1 rDNA deficient X chromosome restored pairing. This region does not contain the 42 bases predicted to pair, but does share homology with the other homolog (which supports a DNA-RNA interaction model). Insertion of a polymerase II promoter in place of the pol I promoter, or insertion of a 240 bp sequence that contains the polymerase II promoter surrounding by sequence that is not homologous to the homolog, onto a Df(1),X-1 chromosome to observe pairing frequencies with a Y chromosome would address this question. Answers to these questions would shed more light on the role of the RNA in X-Y homolog pairing.

A Trans-acting Factor: The Pairing Protein Candidate Cabeza

The *cabeza* gene does not appear to be involved in meiosis, unlike its mammalian homolog, yet both TLS and *cabeza* both seem to be involved in development. The reduced size of TLS KO mice and the lethality of *cabeza* homozygous mutant flies at the early pupae stage, a time of high rates of cell division and growth, supports this role in development. The lethality of *cabeza* homozygous mutants versus the non-lethality in TLS KO mice can be explained by redundancy in the mouse genome. This is highly likely since EWS is 56% identical to TLS.

EWS and hTAFII68 (transcription activating factor II, 68 Kda), proteins that shares extensive homology with TLS, have been found to co-immunoprecipitate with polymerase II and TFIID (part of the multiprotein transcription preinitiation complex)

(Bertolotti et al. 1996, 1998). Other studies have observed TLS interacting with the nuclear factor κ B (NF- κ B), which is an inducible transcription factor that regulates a wide variety of cellular genes including cytokines and cell adhesion molecules (Uraniski et al. 2001), and polymerase II (Immanuel 1995). Also, evidence shows TLS to have nucleic acid binding and pairing activities (Baechtold et al. 1999; Yang 1998; Zinszner 1997; Akhmoedov 1995). These lines of evidence suggest three different possible functions of TLS or *cabeza*, a transcription or transcription initiation factor, a splicing factor, and a nucleic acid pairing protein.

Cabeza does not seem to be essential for *Drosophila* male meiosis, and TLS very essential in male mouse meiosis. It can be argued that there is different pairing mechanism in the two species. Yet, there has been no direct evidence that pairing occurs by different mechanisms in different organisms, but there has been evidence of different homolog cohesion mechanism. Organisms that form chiasmata seem to rely heavily on the formation of the synaptonemal complex and recombination to hold homologous chromosome together, and achiasmatic organisms do not. Two different pairing mechanisms do not seem likely when taking into consideration the two different effects knocking out TLS has on male and female mice. While male TLS KO mice are completely sterile, female TLS KO mice only have reduced fertility.

Upon cytological examination of TLS KO male mice, spermatocytes showed incomplete SC formation, synapsis failure, and synapsis of non-homologous chromosomes. While this suggests a problem in pairing it also at the same time suggests a possible problem in synaptonemal complex formation or regulation of the SC formation. For these reasons, it is possible that TLS has a role in the mechanism for SC formation, or the SC structure itself, rather than pairing as was tested in this study although two different pairing mechanisms cannot be ruled out. If TLS has a role in the SC formation mechanism, whether regulatory by controlling transcription or a direct role, *cabeza* has well may a role in the SC formation mechanism. So, *cabeza* may affect female *Drosophila*

meiosis, but not male meiosis because there is no evidence of SC formation. Future studies, while technically difficult, should address the role of cabeza in female *Drosophila* meiosis.

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VITA

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After college, Amy worked for over a year at the Oak Ridge National Laboratories in Dr. Griffin's laboratory studying the bioeffects of electromagnetic fields on breast cancer cells. She stayed in east Tennessee and entered The University of Tennessee, Knoxville in August of 1998. Amy officially received the degree of Masters of Science in the department of Biochemistry and Molecular and Cellular Biology in August of 2001. While at UT, Amy studied *Drosophila melanogaster* meiosis in the laboratory of Dr. Bruce D. McKee.

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