To the Graduate Council:

I am submitting herewith a thesis written by Xiaojia Ren entitled "Mapping the rDNA X-Y Pairing site in *Drosophila melanogaster* Males: Test of rDNA Structural Region." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfilment of the requirements for the degree of Master of Science, with a major in Zoology.

Bruce D. McKee

Bruce D. McKee, Major Professor

We have read this thesis
and recommend its acceptance:

Ranjan Ganguly

Mary Ann Handel

Accepted for the Council:

Lew Minkel

Associate Vice Chancellor
and Dean of The Graduate School

MAPPING THE rDNA X-Y PAIRING SITE IN *Drosophila melanogaster* MALES: TEST OF rDNA STRUCTURAL REGION

A Thesis

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Degree

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ABSTRACT

Homologous chromosome pairing during early prophase I of meiosis is essential for subsequent meiotic events, including chromosome segregation. To understand how homologs pair, one important step is to find out where they pair. Is meiotic chromosome pairing sequence-specific? In *Drosophila* males, there is no crossingover and chiasma formation when chromosomes pair. Those phenomena simplify the analysis of pairing. Sex chromosome pairing in the males is mediated by ribosomal DNAs which are located in the X heterochromatin and near the base of the short arm of the Y. It has been shown that one complete copy of rDNA can stimulate X-Y pairing. Insertions of the 5′ half of a rDNA gene including the promoter and upstream intergenic spacer at ectopic locations on the heterochromatically deficient X can restore X-Y pairing capacity. In this study we introduced a fragment containing most of the rDNA transcription unit but lacking the promoter and spacer sequences into embryos by P element-mediated transformation. Two X-linked transformants were identified by polytene chromosome in situ hybridization and genomic Southern blot analysis. We used two experimental approaches to investigate the function of this piece of rDNA in meiotic X-Y pairing. Both testis squashes and progeny testing to determine the X-Y non-disjunction rate give the same result: this piece

of rDNA can not stimulate X-Y chromosome pairing during meiosis. This result together with previous studies demonstrates that the ribosomal DNA promoter region is the only region that contributes to X-Y chromosome pairing in *D. melanogaster* males and also implies that meiotic pairing may be localized in certain regions within a gene.

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CHAPTER 1

INTRODUCTION

1. The cytological overview of meiotic prophase I

Meiosis is a germ cell nuclear division in which the chromosome complement is precisely halved and was first discovered by Weismann (1887). Hapliod gametes are formed from diploid cells at the end of meiosis. During meiosis, a single round of DNA replication is followed by two rounds of chromosome segregation instead of single round in mitosis. The first cell division is called meiosis I, and the second division is called meiosis II. Meiosis I involves segregation of homologous chromosomes, thus achieving the halving of chromosome number. The process of meiosis II is similar to mitosis: sister chromatids segregate from one another.

The complex and fascinating events which make meiosis I unique occur in meiotic prophase I. Meiotic prophase I is the period between the end of DNA replication and the time of metaphase I spindle formation. Meiotic prophase involves chromosome pairing, synapsis and recombination. Sequences on a maternally-derived chromosome pair and align with homologous sequences on a paternally-derived chromosome. Synapsis continues in a zipper-like manner mediated by the synaptonemal complex (SC). Sc is a long ladderlike structure with two lateral elements on each homologue side and one central

element in the middle (Fig.1). The SC is thought to have some functions in meiotic recombinaton (Von Wettstein et al., 1984). Before and after synapsis is complete, recombination nodules appear at intervals on synaptonemal complex. They are small, dense proteineous structures thought to contain localized meiotic recombination enzymes (Carpenter, 1975; 1988) and to mediate chromosomal exchange and recombination (Rasmussen and Holm, 1978). There are two kinds of nodules, early and late. Early nodules are abundant and may play a role in the initiaton of matching homologous chromosomes (Albini and Jones, 1987; Anderson and Stack, 1988). Late nodules are less abundant and their distribution correlates with chiasmata (Carpenter, 1988; Holm and Rasmussen, 1980). The chromosomal exchanges result in crossovers (or gene conversions) between two non-sister chromatids.

When the SC dissolves, the two homologous chromosomes pull away from each other to a certain extent and are stabilized by chiasmata. Genetic data suggest that chiasmata are necessary to ensure chromosome segregation in most organisms (Jones, 1987; Hawley, 1988). Mutations that disrupt meiotic recombination and therefore inhibit chiasma formation also cause high frequencies of meiosis I-nondisjunction (Roeder 1990; Hawly 1988; Baker et al., 1976). The *ord* mutation may have a defect in chiasma maintenance in *Drosophila melanogaster* (Mason, 1976). In homozygous ord females, the probability of non-disjunction is the same for

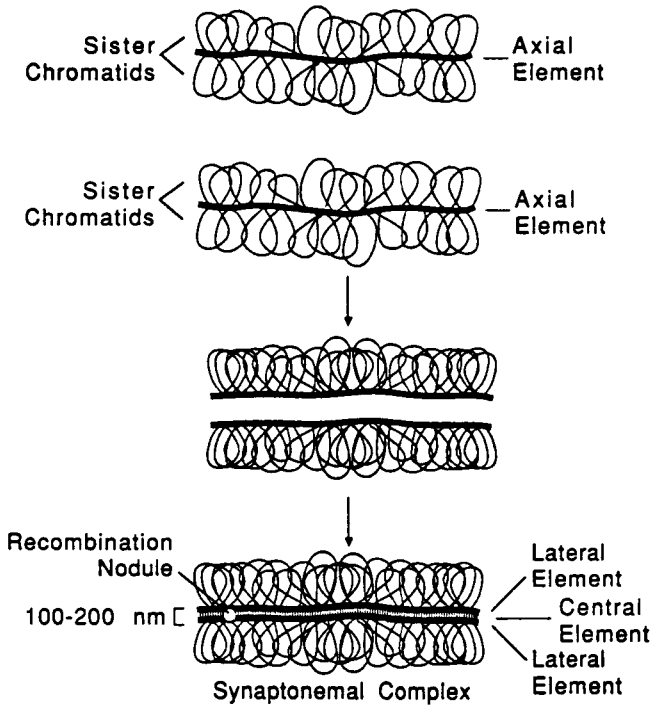


Figure 1. Structure and assembly of the synaptonemal complex (Adapted from Roeder, G. S., 1990).

both exchange and non-exchange bivalents. Thus, *ord* may affect the ability of those residual exchanges that do occur to ensure segregation.

2. Recent understanding of the molecular basis of meiotic pairing

All sexually reproductive organisms must undergo meiosis to reproduce haploid gametes from diploid cells. There are two significant characteristics of that lengthy prophase I of meiosis. One is homologous chromosome synapsis (the appearance of SC tripartite structure) and pairing (the processes that brings homologs together prior to and during synapsis) (Alani et al, 1990); the other is high levels of genetic recombination between homologs. The mechanism of pairing and synapsis and their relationship with recombination are complex and still not well understood.

Several studies support the idea that chromosome synapsis and genetic recombination are related events. First, a number of mutations have been identified that affect both synapsis and recombination (Giroux et al., 1986, 1989; Engebrecht and Roeder, 1990; Hollingsworth and Byers, 1989). The *RAD50* gene of S. cerevisiae is required during meiosis for both recombination and chromosome synapsis (Alani et al, 1990). *RAD50* null mutations block both meiotic recombination and formation of SC at early stages, while non-null mutations

block both processes at intermediate stages. Second, when SC assembly is restricted to specific chromosome segments in some organisms, recombination is similarly limited (von Wettstein et al., 1984). Third, the timing of recombination intermediates (such as double-strand breaks) products formed corresponds to the time of SC formed (Kleckner et al., 1991). The popular current view is that recombination precedes and is necessary for pairing and that recombination and pairing are related processes.

The molecular basis of recombination has been studied in recent years. These studies have found that recombination (and gene conversion) is initiated at specific sites (those initiation sites are called recombination hot spots) (Alani et al., 1989; Nicolas et al.,1989). DNA double strand breaks were found to occur in genes undergoing recombination (Sun et al., 1989; Cao et al., 1990) as well as in whole meiotic chromosomes (Drora et al., 1992). Several double strand break sites were identified and correlated with kown recombination hot spots. Those double strand breaks are thought to be early intermediates in meiotic recombination and they might be generated by a site-specific nuclease, by a topoisomerase, or by a endonuclease in response to changes in chromatin structure (this kind of enzyme is yet to be discovered). The double strand break fragments were found to end in extensive 3′-overhanging, single-stranded tails (Sun et al., 1991). These 3′-overhanging, single-stranded tails are thought to be

generated by single strand-specific exonucleases and they invade its homologous nonsister chromotids by strand-transferase. The identification of this strand-transferase is in process. A yeast gene, called *DMC1*, which is necessary for the completion of meiotic synapsis and recombination, encodes a protein similar to the *E.coli recA* protein (Bishop et al., 1992). *RecA* helps two homologous DNA molecules to pair and catalyzes strand-exchange reactions leading to the formation of heteroduplex DNAs (West, 1992; Kowalczykowski, 1991). Heteroduplex DNA molecules have been detected in Saccaromyces cerevisiae meiotic cells (Lichten et al., 1990).

The above experimental findings support the hypothesis that pairing involves a DNA-level homology search that culminates in heteroduplex DNAs. We will continue to learn more about meiosis with these and similar molecular-level studies.

3. Genetic characteristics of *D.melanogaster* males

Chromosome recombination and meiotic related structures (SC and chiasmata) are absent in *Drosophila* males (Morgan, 1912; Meyer, 1960). Furthermore, males have no distributive pairing (Holm, 1976), a size-based segregation system for non-exchange homologs found in *Drosophila* females (Grell, 1976; Hawley, 1989).

Drosophila male meiosis is of interest in part because of the lack of recombination. Despite this lack, homologs pair

during prophase I and segregate regularly during anaphase I. This raises questions about the association between pairing and recombination suggested by the studies reviewed above. Moreover, as noted above, in most organisms, regular homolog segregation depends upon chiasmata, the products of exchange. However, in *Drosophila* males, no chiasmata are formed yet homolog segregation occurs with high fidelity.

Because of the simplified character of male meiosis, it has been possible to partially localize chromosome segments involved in pairing by cytogenic mapping. The heterochromatin of chromosome 2, which is a large metacentric autosome, lacks meiotic pairing ability in *Drosophila* males, and pairing appears to be restricted to the euchromatic regions. Yamamoto (1979) showed that free duplications for 2R heterochromatin fail to pair with each other or with complete second chromosomes. The compound chromosomes for the left and right arms of chromosome 2 [C(2L) and C(2R)] fail to segregate regularly from each other although they share large amount of heterochromatic homology. C(2L) and C(2R) pair regularly only when they share euchromatic as well as heterochromatic homology (Gethman 1976; Hilliker et al. 1982). The distribution of autosomal pairing ability in Drosophila males was investigated by chracterizing the pairing and segregation patterns of 2-Y transpositions (Mckee, 1993). It was found that all transpositions including euchromatin of chromosome 2, either small or large, have pairing ability with chromosome 2

(quadrivalents involving the second and sex chromosome bivalents were increased). Transposition that involved only heterochromatin had no effect on quadrivalent frequency. One region containing the base of 2L arm was found to be more effective in pairing and segregation. It was concluded that autosomal pairing in males is also dependent on general homology, despite the lack of homologous recombination.

4. rDNA and its function in X-Y pairing in *D. melanogaster* males

Cytogenetic studies suggested that sex chromosome pairing in *Drosophila* males might be limited to the nucleolus organizers (NOs, site of rDNA), which are located in the centric heterochromatin region of the X and near the base of the short arm of the Y (Cooper, 1964; Ritossa, 1976). When chromosomes in male meiotic prophase I appear visible, they have already paired and stay in pairing until anaphase I. Unlike autosomes which show pairing all the way along their lengths, X-Y pairing occurs at discrete, heterochromatic sites known as collochores. Large deletions for XL heterochromatin disrupt X-Y pairing and result in frequent X-Y nondisjunction and skewed progeny recovery ratios (Muller and Painter, 1932; Gershen, 1933; Cooper, 1964; Mckee, 1984). For the largest such deletions, X-Y disjunction is essentially random (Mckee and Lindsley, 1987).

All free X duplications (deletions for most of the X

euchromatin, some of which also include heterochromatin) that fail to disjoin regularly from an attached XY are NO minus (Lindsley and Sandler, 1958). All X heterochromatic deficiencies that disrupt X-Y pairing and disjunction to certain degrees are bobbed-lethal (recessive lethal caused by rDNA insufficiency), and most bobbed-lethal are also pairing-disfunctional (Mckee and Lindley, 1987). Although there are bobbed-lethal Xh deficiencies that retain normal X-Y pairing abilities, such deficiencies may not be completely rDNA minus. One such bobbed-lethal, pairing-positive deficiency ($In(1)w^{m4L}w^{m51bR}$) retains 6-8 rDNA genes by in situ hybridization to mitotic chromosomes (Appels and Hilliker, 1982). These findings have stimulated molecular studies of the role of rDNA in X-Y pairing.

The NO on either X or Y chromosome contains 200-250 tandem copies of rDNA. One complete rDNA copy (Fig.2) contains an 8 kb transcription unit (TU) and 3-4 kb intergenic spacer (IGS). The TU consists of 18S, 5.8S and 28S rRNAs as well as external transcribed spacer (ETS) and internal transcribed spacer (ITS). IGS consists of 1900 bp region (the unique region following the transcription termination) and 240 bp repeats region.

A plasmid called pP(rib, ry)7 (Fig.3E) containing a single, complete rDNA was transformed into the D. melanogaster germline (Karpen et al., 1988). Chromosomal insertions of pP(rib, ry)7 (known as [rib7]) are transcribed in polytene

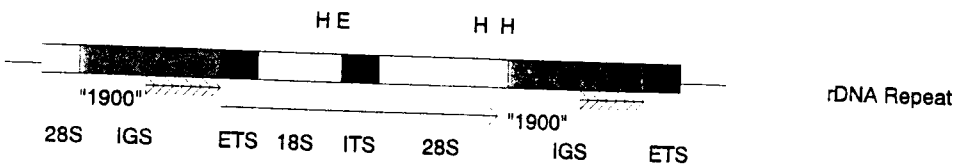


Figure 2. One rDNA repeat. IGS=intergenic spacer, including "1900" bp region and 240 bp repeats. ETS=external transcribed spacer. ITS=internal transcribed spacer(including 5.8S rDNA). 18S and 28S are responsible for 18S and 28S rRNAs. E=EcoRI, H=HindIII.

Figure 3. Plasmid constructions.

A. rDNA repeat (see Figure 2).

B. pP(rib, w^{hs})12 as microinjection vector. It contains *Hind*III fragment (rib12) including part of the 18s region, the ITS and most of the 28S gene.

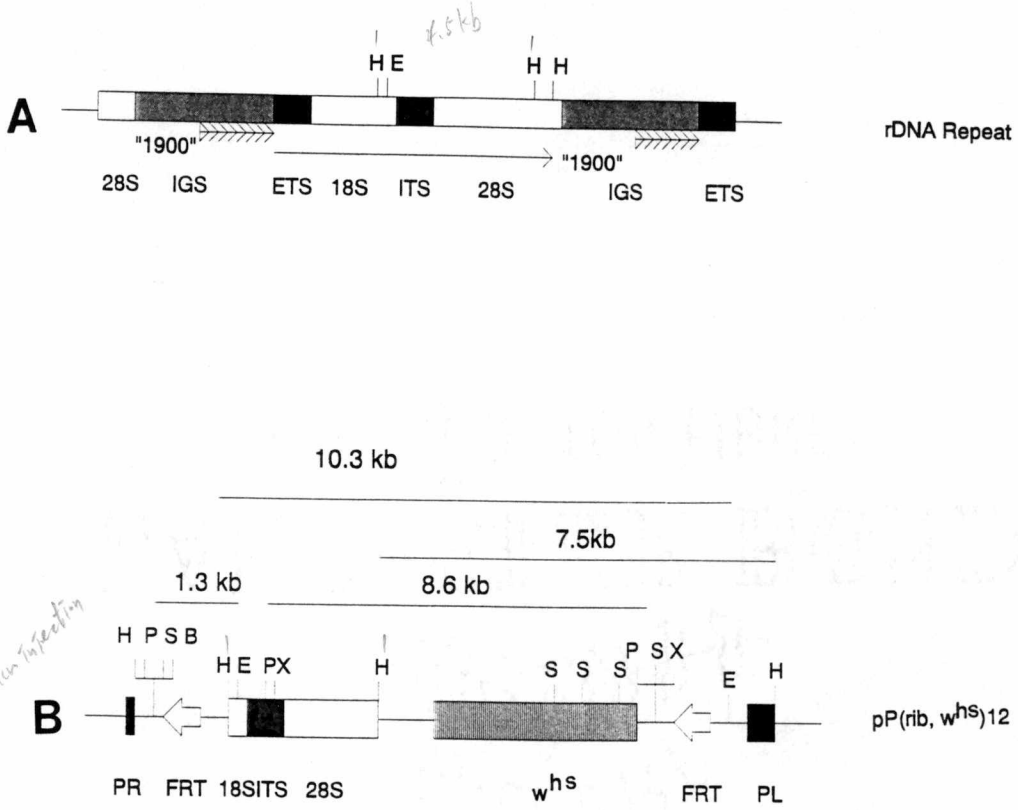
C. The rib12 rDNA cloned into Bluescript.

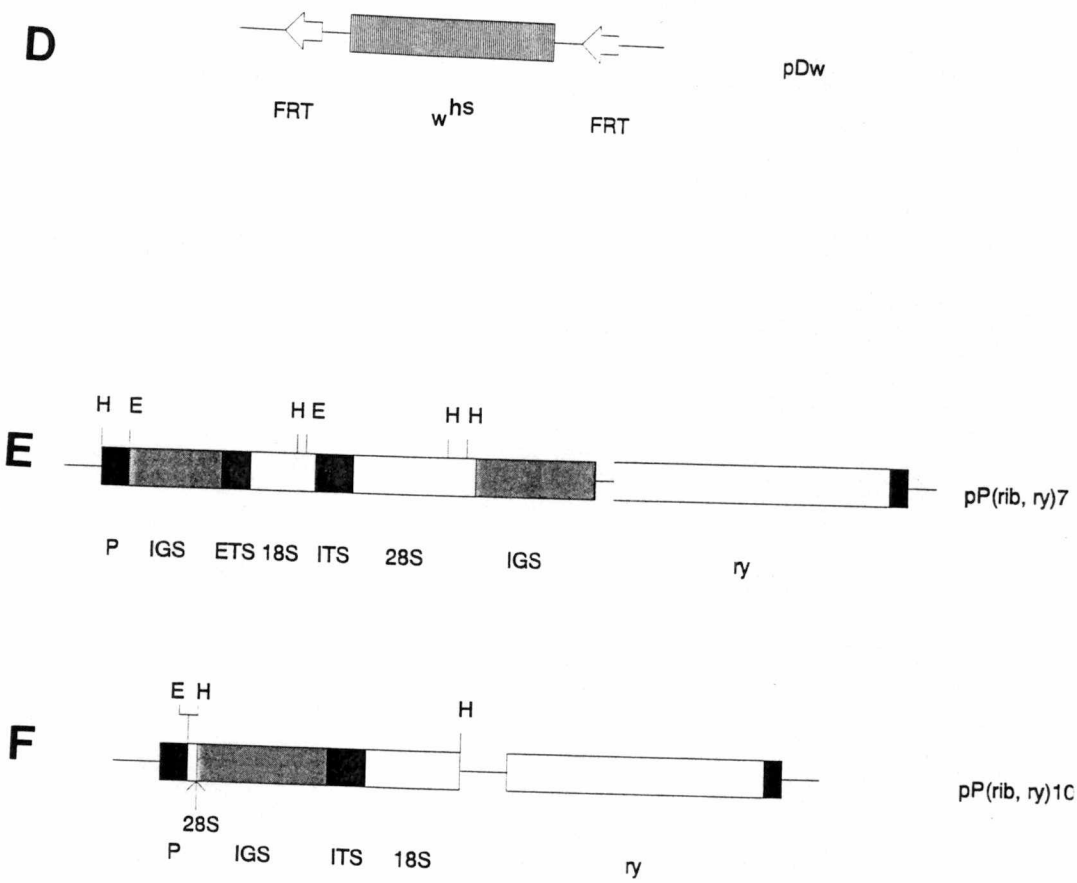
D. w^{hs} was cloned into pMMD2 vector carrying direct repeat FRTs.

E. pP(rib,ry)7 contains single copy of rDNA repeat in Carnegie 20 carrying P and rosy marker.

F. pP(rib, ry)10 contains approximately 5′ half of complete rDNA gene in Carnegie 20.

B=*BamH*I, E=*EcoR*I, H=*Hind*III, P=*Pst*I, S=*Sal*I, X=*Xba*I.


A
H E
H H
"1900"
"1900"
28S IGS ETS 18S ITS 28S IGS ETS
rDNA Repeat
10.3 kb
7.5kb
1.3 kb
8.6 kb
H P S B
H E PX
H
S S S
P S X
E H
B
PR FRT 18SITS 28S
w^hs
FRT PL
pP(rib, w^hs)12
C
H H
pKS
pKS
18S ITS 28S
p(rib12)


D
FRT
w^hs
FRT
pDw
E
H E
H E
H H
P IGS ETS 18S ITS 28S IGS ry
pP(rib, ry)7
F
E H
H
28S
P IGS ITS 18S ry
pP(rib, ry)1C

chromosomes and induce mininucleolus formation at their insertion sites. It was shown that [rib7] could stimulate X-Y pairing and disjuncton and reduce progeny ratio distortion when located on a heterochromatically deficient X but not on a autosome (Mckee and Karpen, 1990). These results implied that rDNAs functioned as X-Y pairing sites and normal sperm development required X-Y pairing.

5. Research purpose

To define the rDNA sequences involved in X-Y pairing, rDNA subfragments encompassing various regions of the rDNA repeat have been similarly tested. A transposon named pP(rib, ry)10 containing the 5′ half of an rDNA repeat including the promoter (ETS region) and upstream IGS region (Fig.3F) was transformed into the germline and its pairing ability was tested. The result showed that this fragment also could stimulate X-Y pairing and rescue progeny ratio distortion (Merrill et al., 1992). Moreover, smaller rDNA fragments consisting of just the IGS or the IGS plus ETS are also capable of stimulating X-Y pairing and rescuing progeny ratio distortion (Merrill et al., 1992). A series of deletions within a [rib7] insertions was generated by P element-mediated destabilization (Mckee et al., 1992). Analyses of these deletions′ pairing abilities showed that rDNA fragments containing only 240 bp IGS repeats could stimulate pairing and

that pairing ability was proportional to the copy number of 240 bp repeats. It was hypothesized that the rDNA transcription unit does not contribute to X-Y pairing and the rDNA promoter/IGS region is the only region to contribute to X-Y pairing in D. melanogaster males. The main purpose of this investigation is to test this hypothesis.

The pP(rib, w^{hs})12 vector (Fig.3B) containing most of the rDNA transcription unit but lacking the promoter and IGS sequence was constructed and injected into embryos. X-linked insertions from this injection were studied. It was found that single copy insertions of this fragment failed to stimulate X-Y pairing, thus confirming the hypothesis.

CHAPTER 2

MATERIALS AND METHODS

1. Plasmid pP(rib,w^{hs})12 Construction

A 4.5kb *Hind*III fragment (Fig. 3A) containing almost all of the 28S gene, all of the internal transcribed spacer (ITS), and the 3′ end of 18S gene was cloned into the pDw vector, carrying w^{hs} (white gene marker) and direct repeat sequence FRTs (site-specific recombination targets from yeast 2 μm plasmid) (Fig. 3D). A fragment containing the rDNA, the w^{hs} and the two FRTs was then removed by digestion with BamHI and EcoRI and cloned into the P vector Carnegie 2, generating pP(rib,w^{hs})12 (Fig.3B). This plasmid was microinjected into the embryos.

2. Plasmid p(rib12) Construction

The same above 4.5kb HindIII fragment was cloned into the HindIII site of bluescript. This plasmid was used as a probe (for the rDNA fragment) in in situ hybrizidation.

3. *Drosophila* Transformation

Germline transformation was carried out as described by Spradling and Rubin (1982, 1986) and Rubin and Sprandling (1982,1983). The strain used for microinjection is *yw*,

TM3/[Δ2,3](99B). This strain was maintained at 18°C on standard cornmeal-agar medium containing yeast and molasses. The flies were allowed to lay eggs for at least one hour. These eggs were discarded. Eggs laid subsequently on grape juice-agar plates were collected every 30 minutes, dechorionated, desiccated and microinjected with pP(rib, w^{hs})12 at a concentration of 100-1000 ng/μl. The injected embryos were incubated at 18°C until they became adults. G_o stubble flies (genotype is *yw, TM3/[Δ2,3]*) were crossed individually to *yw* males or females (Fig.4). G_o non-stubble flies (genotype is yw, [Δ2,3]/[Δ2,3]) were first crossed to yw, TM3/[Δ2,3] males or females individually, then their stubble progeny were crossed to yw males or females individually in order to get rid of Δ2,3 transposase source. Flies with orange eye color (indicative of transformation) were recovered from the G_1. No expression of w^{hs} was seen in the Go, in agreement with previus observations (Klemenz, R. et al. 1987).

4. Southern Blotting

Genomic DNA was isolated from twenty to thirty adult flies by the method of Bender et al. (1983) or Steller (1985) and G. Rubin (1983). The DNA was digested for 6 hours by restriction enzymes and separated on a 0.5% agarose gel by electrophoreis in TBE buffer (Sambrook et al., 1989). The DNA was nicked by soaking the gel for 15 minutes in 0.25 M HCl, then denatured by incubation for 30 minutes in 0.4 M NaOH /

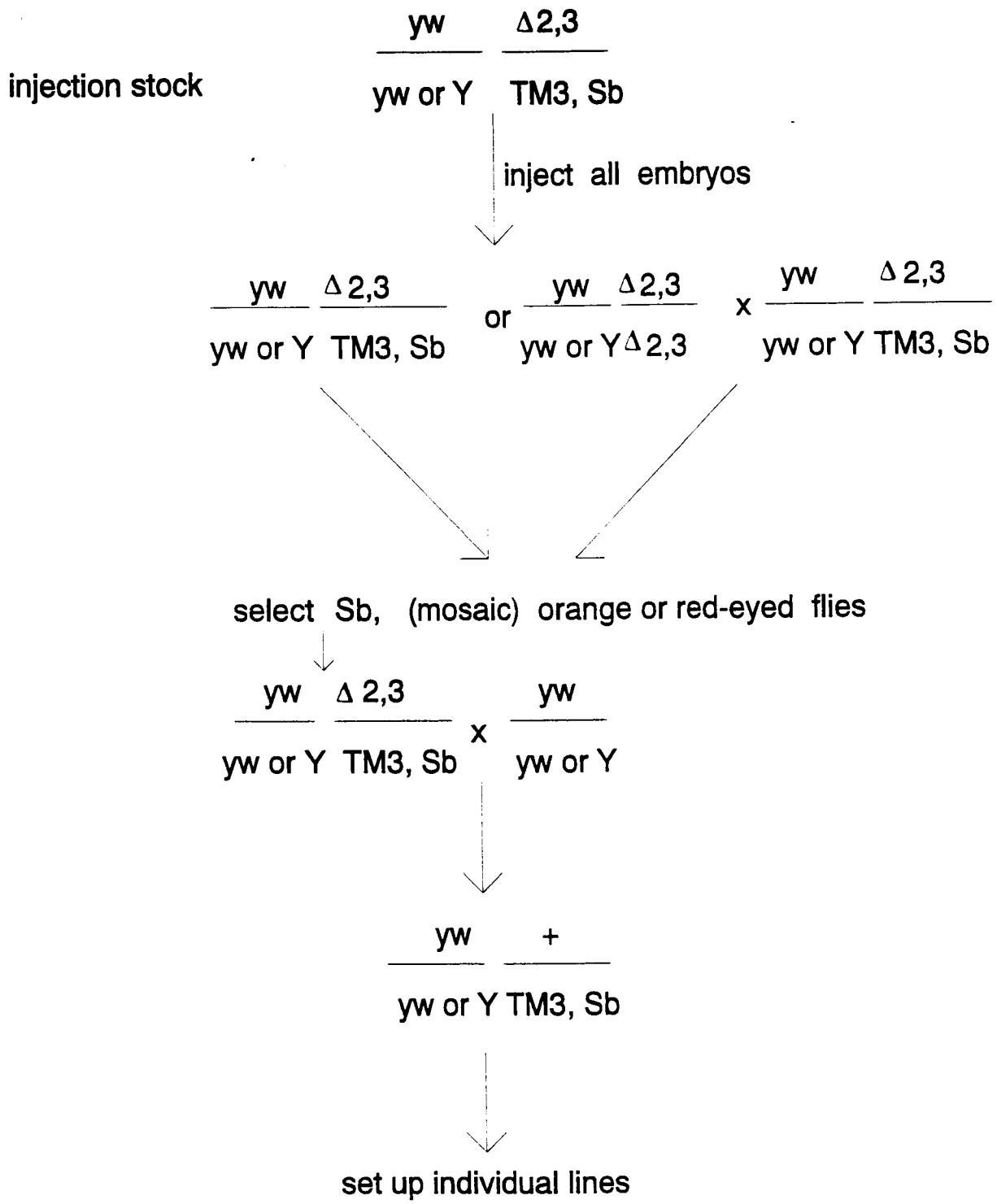


Figure 4. Crossing scheme for microinjection.

0.6 M NaCl and neutralized by incubation for 30 minutes in 0.5 M Tris-HCl (pH 7.5) / 1.5 M NaCl. DNA was transfered to GeneScreen Plus hybridization transfer membranes (Dupont / NEN) by the Southern blot method (Southern, 1975). The membranes were prehybridized in 50% formamide, 1% SDS, and 5X SSPE (Sambrook et al., 1989) for at least 15 minutes at 42 °C. The DNA probes were labeled by using Random Primers DNA Labeling System (BRL). Hybridization was carried out at 42 °C waterbath with constant agitation overnight. Membranes were washed with 2X SSC twice for 5 minutes each at room temperature, 2X SSC / 1% SDS twice for 30 minutes each at 65 °C, 0.1X SSC twice for 30 minutes each at room temperature and exposed to Kodak XAR5 films for various time at -70 °C.

5. Testes Squashes

Male meiotic (Fig.5) chromosomes were prepared by the method of Lifschytz and Hareven (1977). 0-2 days young male adults were dissected in testes buffer (7% NaCI, Ashburner, 1989) and fixed in 45% acetic acid for 30 seconds. After staining in 3% orcein-60% acetic acid for 5 minutes, the testes were squashed in a solution 1:1 mixture of 60% acetic acid and 2% lactic-acetic-orcein. The chromosomes were examined under phase-contrast optics using a Zeiss Universal Axioplan photomicroscope and photographed using Kodak T-MAX 100 film.

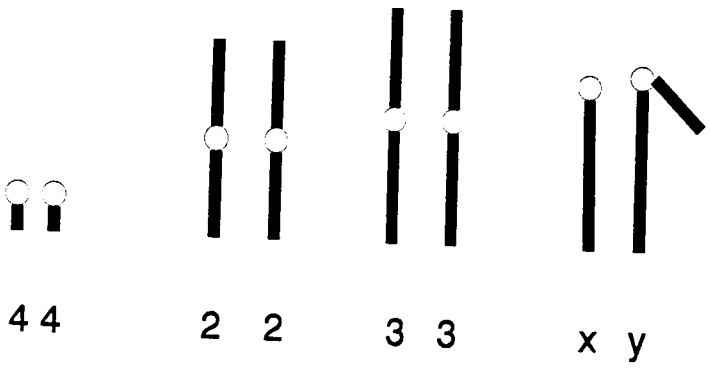


Figure 5. Drosophila male karyotype.

6. In Situ Hybridization

In situ hybridization to salivary gland chromosome using biotin-labeled DNA probes was was carried out as described in Engels et al. (1991). Larvae were cultured in standard cornmeal-molasses-yeast agar at 22-23°C under uncrowded conditions. The salivary glands were dissected and fixed in 45% acetic acid on subbed slides, squashed under siliconized coverslips and kept at 4°C in 24-48 hours for chromosome flatness. The coverslips were removed in liquid nitrogen and chromosomes were fixed again in 3:1 mixture of methanol and acetic acid. After dehydrating in ethanol grades, the slides were air dried. The slides were then washed in 2X SSC at 65°C for 30 minutes and in 2X SSC at room temperature for 2 minutes. The slides were immersed in 2 liters of 100mM TEA and 10 ml acetic anhydride was added while stirring the solution vigorously. Once the acetic anhydride was dissolved, stirring was stopped and the slides were left to sit in the solution for 10 minutes. The slides were washed in 2X SSC twice, 5 minutes each, and dehydrated in 70% ethanol twice (5 minutes each), in 90% ethanol twice (5 minutes each). After denaturing in 70 mM NaOH for 3 minutes at room temperature, the slides were dehydrated, air dried, and used for hybridization.

The DNA probes to be used for hybridization were prepared by using BioNick labeling system (Gibco-BRL) and purified by using column chromatography. The probes were denatured by

boiling 5 minutes before using. Ten microliter probe was placed on the chromosomes for hybrydization. The chromosomes were coverd with siliconized coverslips, sealed with rubber cement, and incubated at 37-58°C in a moisturized chamber overnight. On the following day, the rubber cement was peeled off and the coverslips were removed by soaking the slides in 2X SSC at 53°C. The slides were washed with three changes in 2X SSC at 53°C for 20 minutes each, two changes in PBS solution at room temperature for 10 minutes each, two changes in 2% BSA in TMN1, triton X-100 (first wash: 20 minutes at 42°C, second wash: 10 minutes at room temperature). Fifty microliter SA/AP (2 μl streptavidin/alkaline conjungate in 1 ml TMN1, triton X-100) was pipeted onto each slide where the coverslips were marked. Another 50 μl was added to each slides after 5 minutes. The slides were then left to sit for another 5 minutes. Each slide was rinsed with 3-4 ml TMN1, triton X-100 by holding the slide over a beaker. The slides were washed twice in TMN1, triton X-100 (three minutes each wash) and twice in TMN2 (3 minutes each wash). NBT/BICP solution (30μl) was pipeted onto the chromosomes. A slide with chromosome side facing down was held over a coverslip to make sure the droplet would adhere to the slide. The slide was then flipped over carefully so that the coverslip did not slip off. The slides were then placed on a flat tray and incubated in dark for 2 hours at room temperature. The slides were then rinsed in distilled water and stained with the

orcein solution for about 10 seconds. After being rinsed in water, the slides were air dried. To examine the slides, a drop of sterile water was pipeted on the chromosomes and a siliconized coverslip was placed on it. To photograph, the coverslip edges were sealed with rubber cement to prevent from oil going under the coverslip and ruining the slide.

7. Data Analysis

The X-Y disjunction frequency (P) is defined as the fraction of secondary spermatocytes that include an X or a Y but not both ((X + Y)/(X + Y + XY + O)).

P values were estimated from testis squash data using the formula P = (2a + d)/(2a + 2b + C + d + e) (metaphase I and anaphase I: a = X and Y go to different poles, b = X and Y go to the same pole. meiosis II: c = a cell has no sex chromosomes at all, d = a cell contains either X or Y, e = a cell contains both X and Y.). The variance is V = P(1-P)/N.

P values were also estimated from progeny class frequencies by the method of Mckee (1984; also see Mckee and Lindsly, 1987). The formulae are:

$$P = 1/[1 + (o(XY)o(O)/o(X)o(Y))^{1/2}];$$

$$Rx = [(o(XY)o(X)/(o(Y)o(O))]^{1/2};$$

$$Ry = [(o(XY)o(Y)/(o(X)o(O))]^{1/2}.$$

The variances are:

$$V_j = 1/[\Sigma i((1/e(i))(\partial e(i)/\partial j)^2)N]$$

i presents the progeny classes: X, Y, XY, O. Rx and Ry are chromosome viability parameters defined as the viability of sperm that carry the relevant chromosome divided by the viability of otherwise identical sperm that lack of it. j presents the parameters: Rx, Ry (Kempthorne, 1969).

The pairwise comparisons of parameters obtained from the same genetic crosss involving different lines were analyzed by the z statistic: (The method is available in both assays)

$$z = (j_1 - j_2)/(V(j_1) + V(j_2))^{1/2}.$$

8. Fly strains and Genetic crossses

The *Drosophila* strains mentioned in this thesis are described below: [see Lindsley and Grell (1968) for details]

yw: two recessive genes on X chromosome determine body color (yellow) and eye color (white);

TM3: a third chromosome inversion with a dominant stubble (Sb) marker;

[Δ2,3](99B): a third chromosome carries Δ2,3 transposase gene with deletion at 99B region;

FM6, y^2, w^a, B: an X chromosome balancer carrying multiple inversions, FM6 effectively eliminates recombination in FM6/+ heterozygote;

Df(1)X-1: a large deletion in X chromosome heterochromatin

(rDNAs location) region;

B^sYy^+: Y chromosome that carries a duplicated X tip attached to the end of YS and three proximal X bands attached to the tip of YL.

Genetic crosses for microinjection, testis squashes and progeny counting test are showed in Fig.4 and Fig.6.

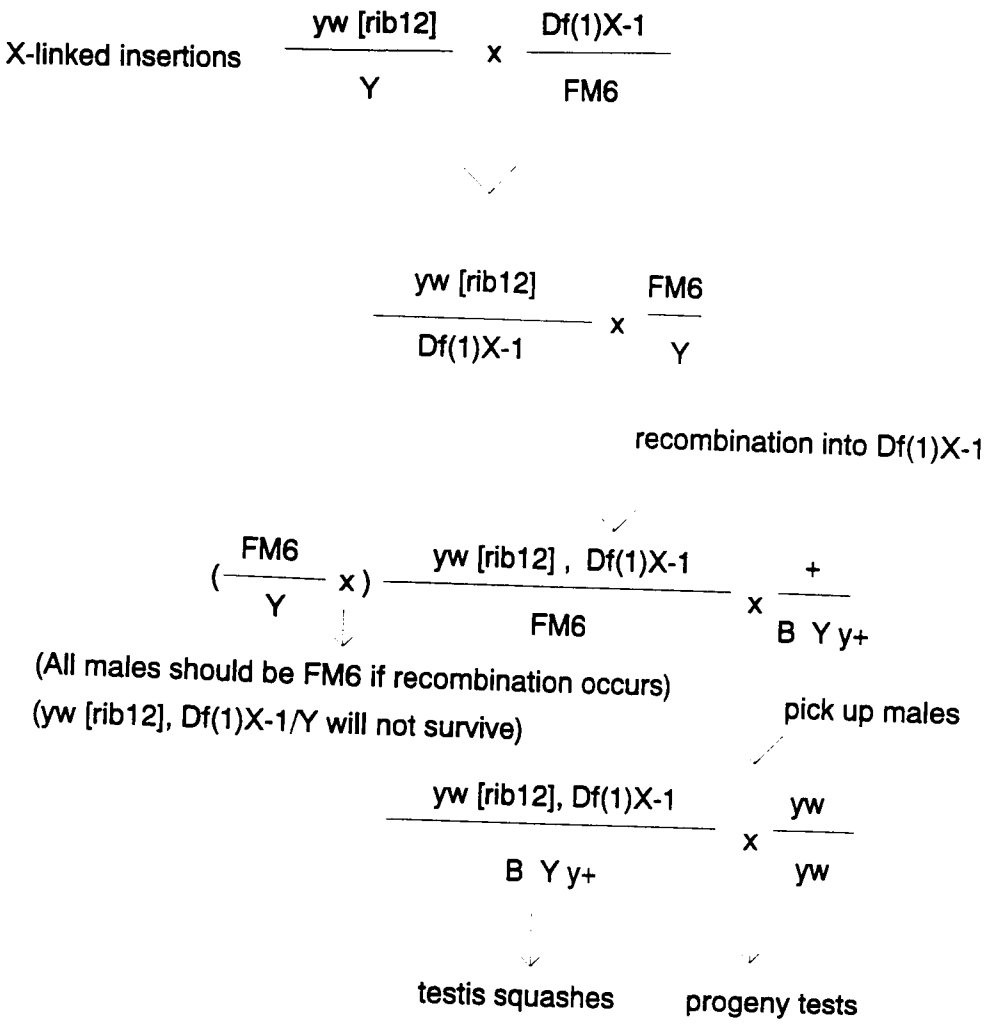


Figure 6. Crossing scheme for testis squash and progeny counting test.

CHAPTER 3

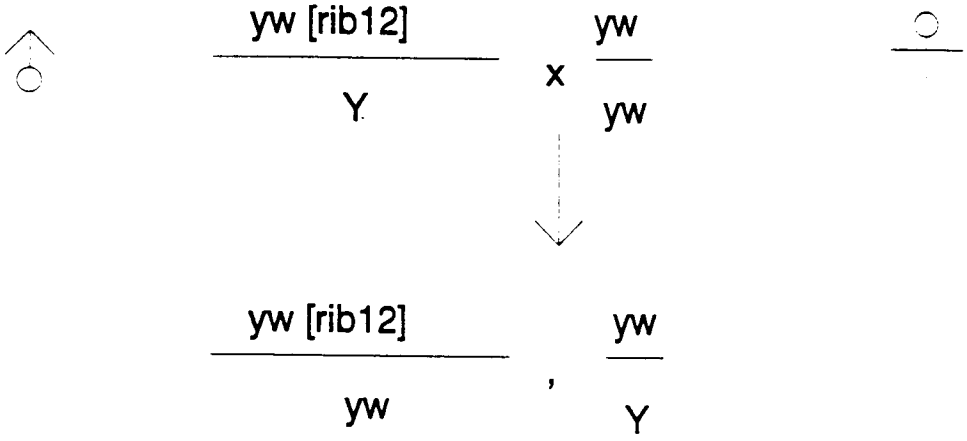
RESULTS

1. 5-1 and 3-21 Were Two X chromosome Transgenic Insertions

The pP(rib12, w^{hs}) was transformed into the germline of white-eyed flies (yw, TM3/Δ2,3) by embryo microinjection. w^{hs} is a mini-white gene constructed by Klemenz et al (1986). It is small relative to the wild-type white gene, easier to manipulate, and transforms more readily. Δ2,3 is an inserted P element construct on chromosome 3 that functions as a stable, genomically integrated transposase source. Transformants carrying an insertion of pP(rib, w^{hs})12 (known as [rib12]) on chromosomes X, 2, and 3 were obtained. The scheme for identifying the chromosomes on which the insertions were located is shown in Fig.7. The number of transformants on different chromosomes is shown in Table 1. Two independent X-linked transformants were identified, one is 5-1 and the other is 3-21. The 5-1 insertion resulted in orange-colored eyes in a single dose (when heterozygous with the original yw X chromosome). When the 5-1 w^{hs} insertion is present in two doses (homozygous), the eye color was bright-red eyes. However, the 3-21 insertion always results in bright red eye color in either the heterozygous or homozygous genotype.

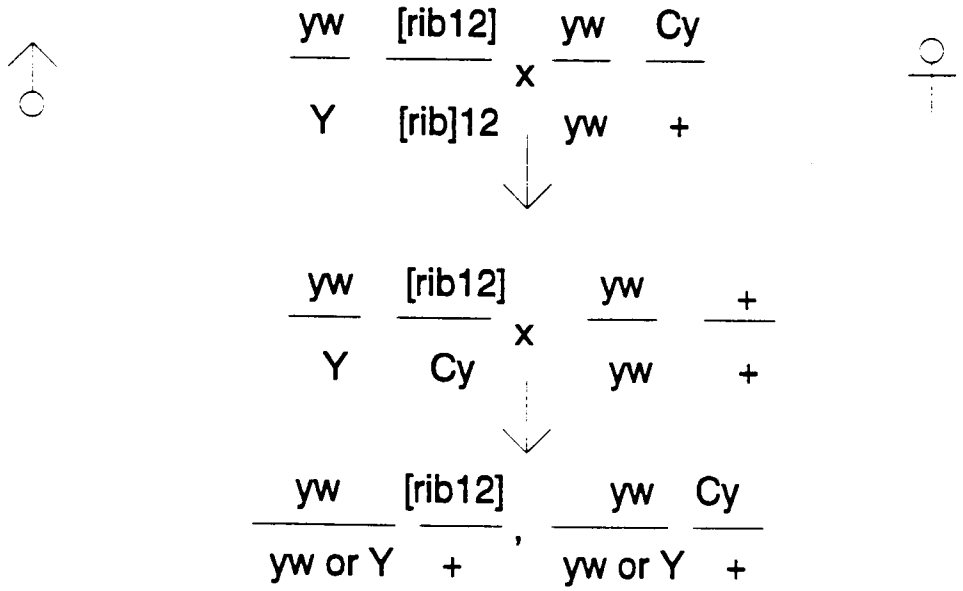
Figure 7. The crossing scheme for identifying insertions on which chromosome.

If insertions on chromosome X



All of females are orange-eyed, all of males are white-eyed.

If insertions on chromosome 2



All non-Cy flies are orange-eyed, all Cy flies are white-eyed.

If insertions on chromosome 3

$$\frac{yw}{Y}\ \frac{\Delta 2,3\ [rib12]}{TM3,\ Sb} \times \frac{yw}{yw}\ \frac{+}{+}$$

$$\downarrow$$

$$\frac{yw}{yw\ or\ Y}\ \frac{\Delta 2,3\ [rib12]}{+}\ ,\ \frac{yw}{yw\ or\ Y}\ \frac{+}{TM3,\ Sb}$$

If insertions on chromosome 3, they were discarded due to homozygous never obtained.

Table 1. Microinjection efficiency for pP(rib, w^{hs})12.

No. embryos injected	No. G_0 flies	No. G_0-laid transformants
about 400	74(4 died, 15 sterile)	8*

* One G_0 fly laid both x-linked and autosomal inserts separately.

No. X inserts	No. autosomal inserts
2	6

2. 5-1 and 3-21 Carry Insertions at 7C and 6F-7A

Chromosomal insertion sites were mapped by in situ hybridization to salivary gland polytene chromosomes. The probe for w^{hs} was pDw-a plasmid which carries w^{hs}. The probe for rDNA insertion was p(rib12) which carries the same rDNA fragment as pP(rib, w^{hs}) (see Materials and Methods). Both probes gave hybridization signals which proved that w^{hs} and the rDNA fragments were present in the insertions. Both probes also gave the same results-that the 5-1 insert was in the 7C region and 3-21 was in the 6F-7A region (Fig.8 and 9). Both insertions appeared to be single insertions.

3. The rib12 rDNA insert was intact in both 5-1 and 3-21

Genomic Southern blot analysis was carried out to check if the rDNA insert was intact. The results are described in three parts according to the different probes used.

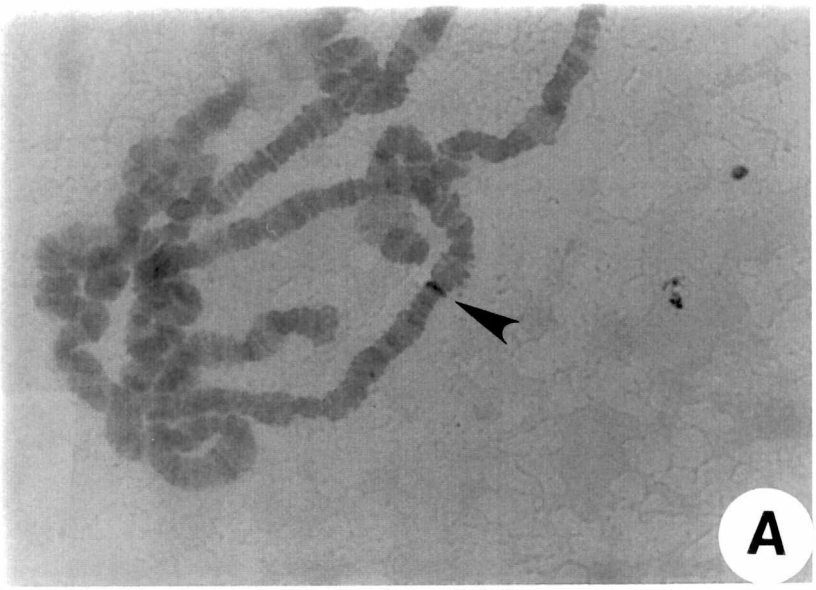
1). P-left Probe

5-1, 3-21 and yw, TM3/Δ2,3 (control line) genomic DNAs were digested with HindIII and probed with P-left (Fig. 10A). Both 5-1 and 3-21 lines gave the expected 7.5kb fragment that includes the PL, FRT, w^{hs} sequences (Fig.3B). There is a 500 bp faint band which is PL in yw, TM3/Δ2,3 control line (Fig.10A). 5-1 and 3-21 genomic DNAs were digested with EcoRI and probed with P-left again (Fig. 10B). There is a EcoRI

Figure 8. In situ hybridization of Biotin-labeled pDw(containing w^{hs}) to 5-1 and 3-21.

A: 5-1 insert at 7C.

B: 3-21 insert at 6F-7A.


A

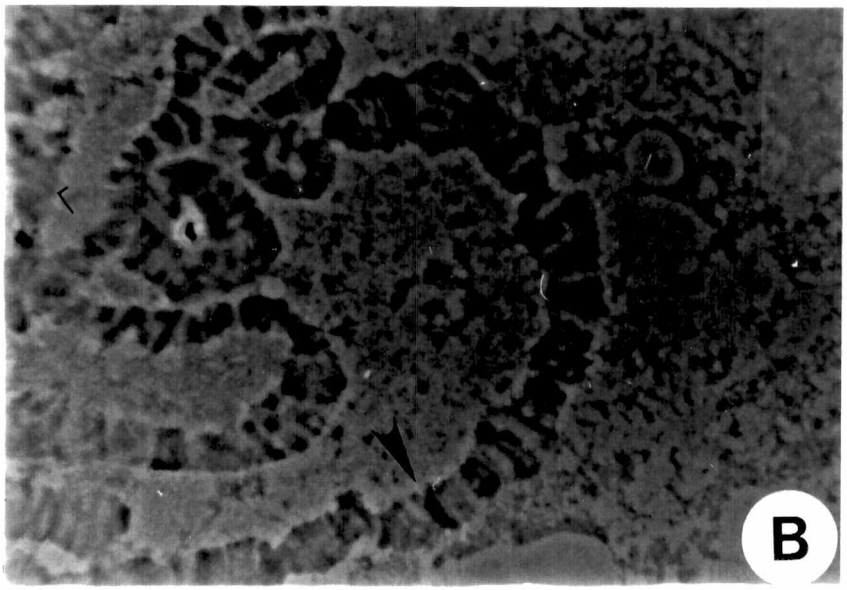
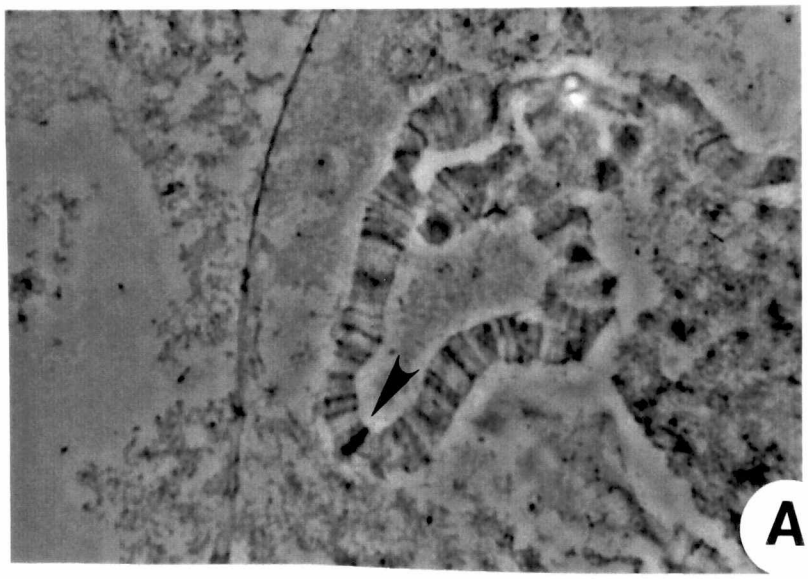

B

Figure 9. In situ hybridization of Biotin-labeled prib12 to 5-1 and 3-21.

A: 5-1 insert at 7C.

B: 3-21 insert at 6F-7A.


A

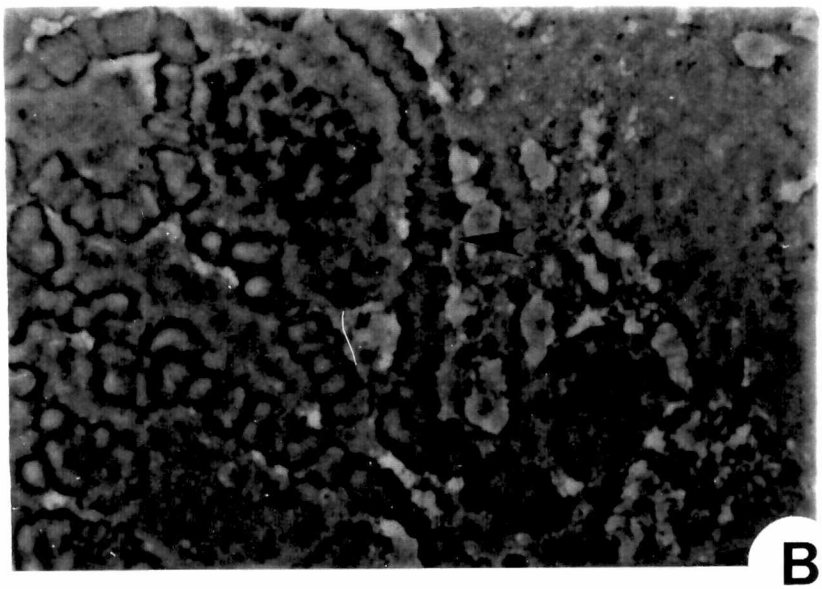
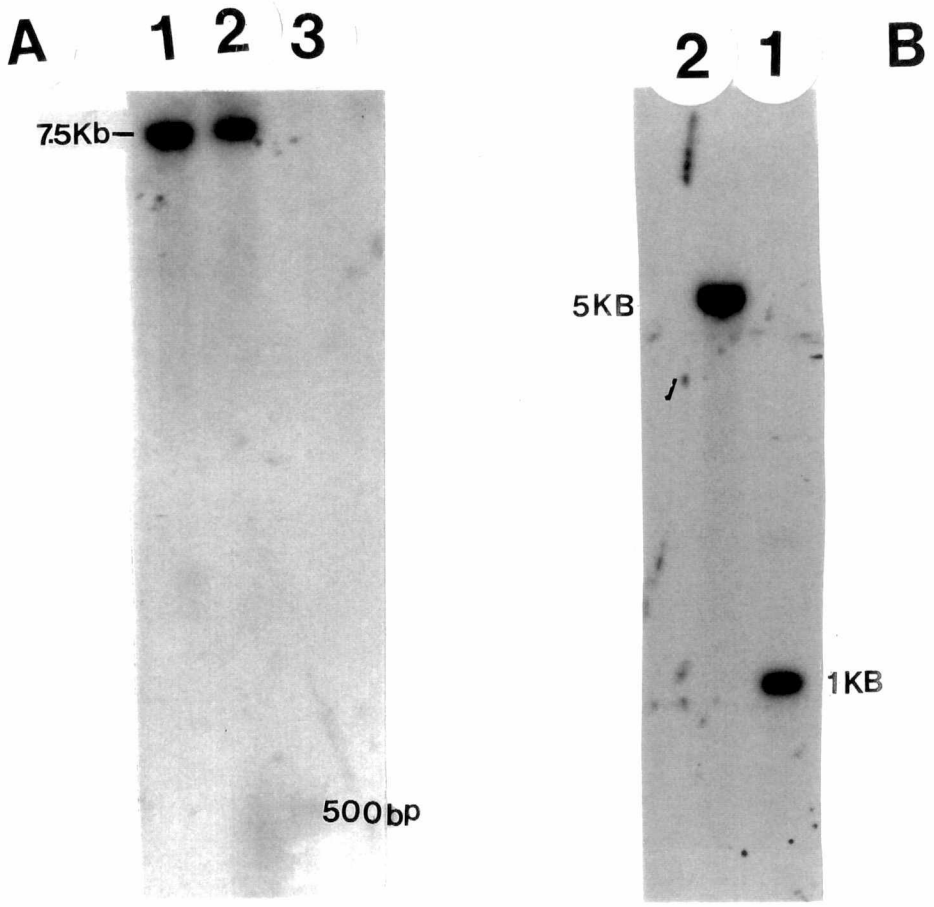

B

Figure 10. Autoradiograms of 5-1 and 3-21 genomic southern blots with P-left probe.

A: HindIII digested genomic DNA. 7.5 kb bands are visible in lanes 1 (5-1) and 2 (3-21). Lane 3 is the stock for microinjection.

B. EcoRI digested genomic DNAs showing (lane 1) and 3-21 (lane 2) inserts at different locations in the X chromosome.

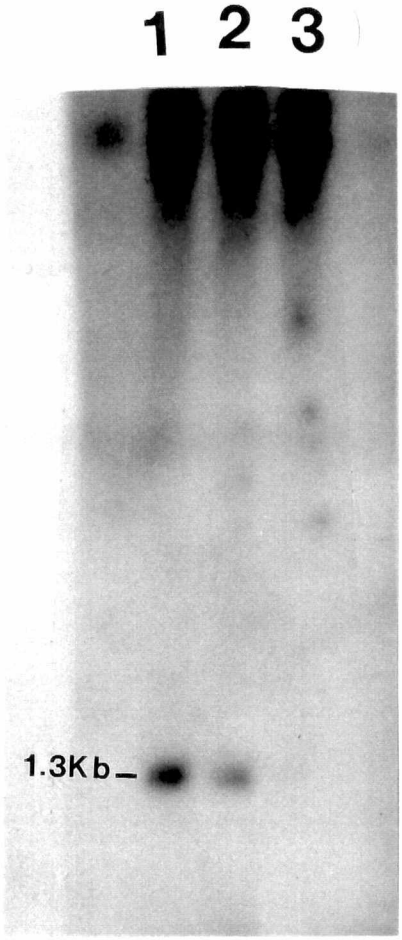

A
1 2 3
7.5Kb
500bp
2 1
B
5KB
1KB

site next to the P-left in the insert (Fig. 3B) and the other EcoRI cutting site must be somewhere on the X chromosome. If the 5-1 and 3-21 insertions were inserted into the same site on X chromosome, the other EcoRI site for both of them would be same and the hybridized bands of two lines in figure 10b would be the same size. However, if the insertions were at different sites, the bands would be expected to be different. From Figure 10b (as well as figs. 8 and 9), these two inserts are obviously in different locations.

2). FRT Probe (see plasmid construct in Materials and Methods for FRT description)

5-1, 3-21 and yw, TM3/Δ2,3 (as control) genomic DNAs were digested with BamHI and EcoRI and probed with the 700bp BamHI/HindIII FRT fragment from pP(rib12, w^{hs}). Both 5-1 and 3-21 gave the expected 1.3 kb bands (Fig. 11). This result means that the HindIII/EcoRI rDNA fragment on the right of [rib12] was in the insert. No 1.3 kb band was observed in the control. Both the control and the insertion lines showed high molecular weight hybridization bands. They are probably due to the FRT homologous sequences from Drosophila, since those kind of bands are obtained every time when FRT was used as a probe.

Figure 11. Autoradiograms of 5-1 and 3-21 genomic southern blots with FRT probe. 1.3 kb bands are visible in both lane 1 (5-1) and lane 2 (3-21) and is not visible in lane 3 (stock for microinjection).


1 2 3
1.3Kb

3). SalI w^{hs} Probe

5-1, 3-21, and yw (as control) genomic DNAs were digested with XbaI, PstI, and EcoRI separately and probed with the SalI fragment from w^{hs} (the left SalI fragment in w^{hs}, see Fig. 3B). The sizes of the expected hybridized bands from each of the three digestions in 5-1 and 3-21 were: 10.3 kb EcoRI band, 8.2 kb XbaI band, 8.6 kb PstI band (Fig. 3B). All 5-1 and 3-21 lanes gave the expected bands (Fig.12). The bands in the 5-1 and 3-21 lanes which were the same size as the bands in the three yw lanes were due to hybridization to white sequence in the yw stock.

The 10.3 kb EcoRI band from the insert in 5-1 and 3-21 was approximately the same size as the band from yw.

4. [rib12] Function in Meiotic X-Y Pairing

[rib12] was introduced on to a rDNA-minus, pairing-deficient X chromosome by recombination (Fig.6). Then, two independent experimental approaches (testis squash and progeny counting) were used to investigate [rib12] function in meiotic X-Y pairing. Both testis squash and progeny counting gave the X-Y non-disjunction rate (see Materials and Methods).

The following X-Y chromosome behaviors can be observed from spermatocytes in orcein-stained testis squash preparations:

Late prophase I: X, Y are paired or unpaired (Fig. 13 A+E)

Metaphase I: X, Y are paired or unpaired, X, Y go to the same pole or opposite poles

Figure 12. Autoradiograms of 5-1, 3-21 and yw (control) genomic southern blots with w^{hs} SalI probe.

A. 8.6 kb bands showing in both 5-1 and 3-21 in PstI digestion.

B. 8.2 kb bands showing in both 5-1 and 3-21 in XbaI digestion.

C. 10.3 kb bands showing in both 5-1 and 3-21 in EcoRI digestion (see Results for explanation of control lane band).

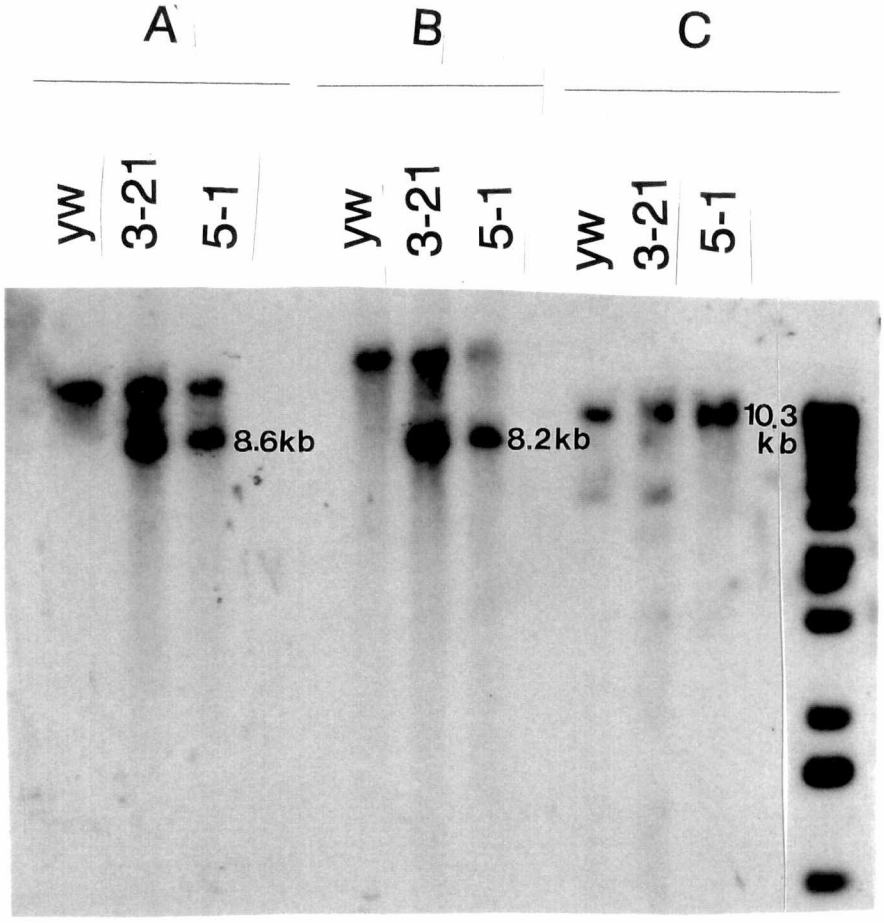

A
B
C
yw
3-21
5-1
yw
3-21
5-1
yw
3-21
5-1
8.6kb
8.2kb
10.3
kb

Figure 13. Primary and secondary spermatocytes from testis squashes with aceto-orcein staining.

A: late prophase I, X, Y are paired.

B: anaphase I, X, Y go to the oposite poles.

C: late prophase II, the cell has an X.

D: late prophase II, the cell has a Y.

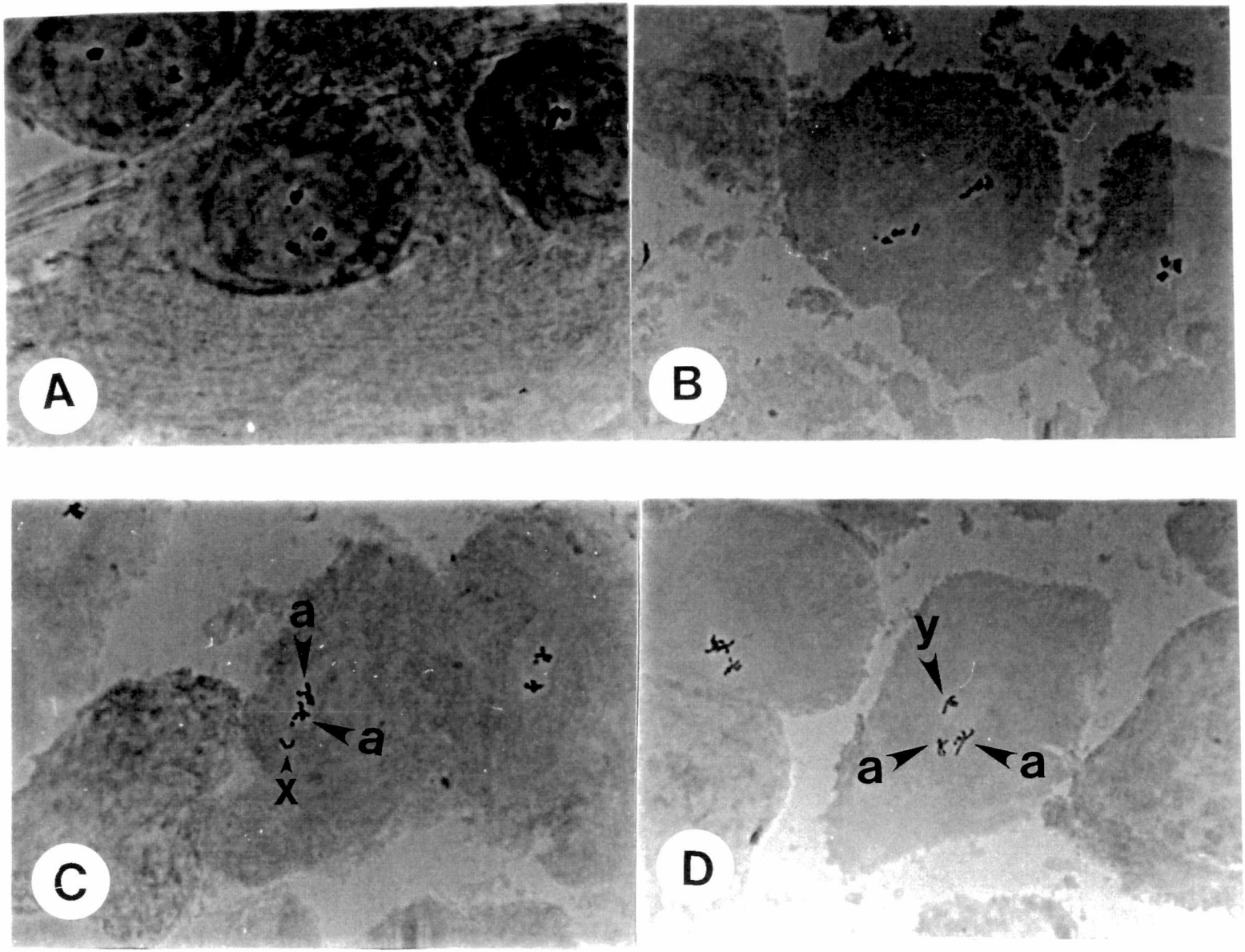
E: late prophase I, X, Y are unpaired.

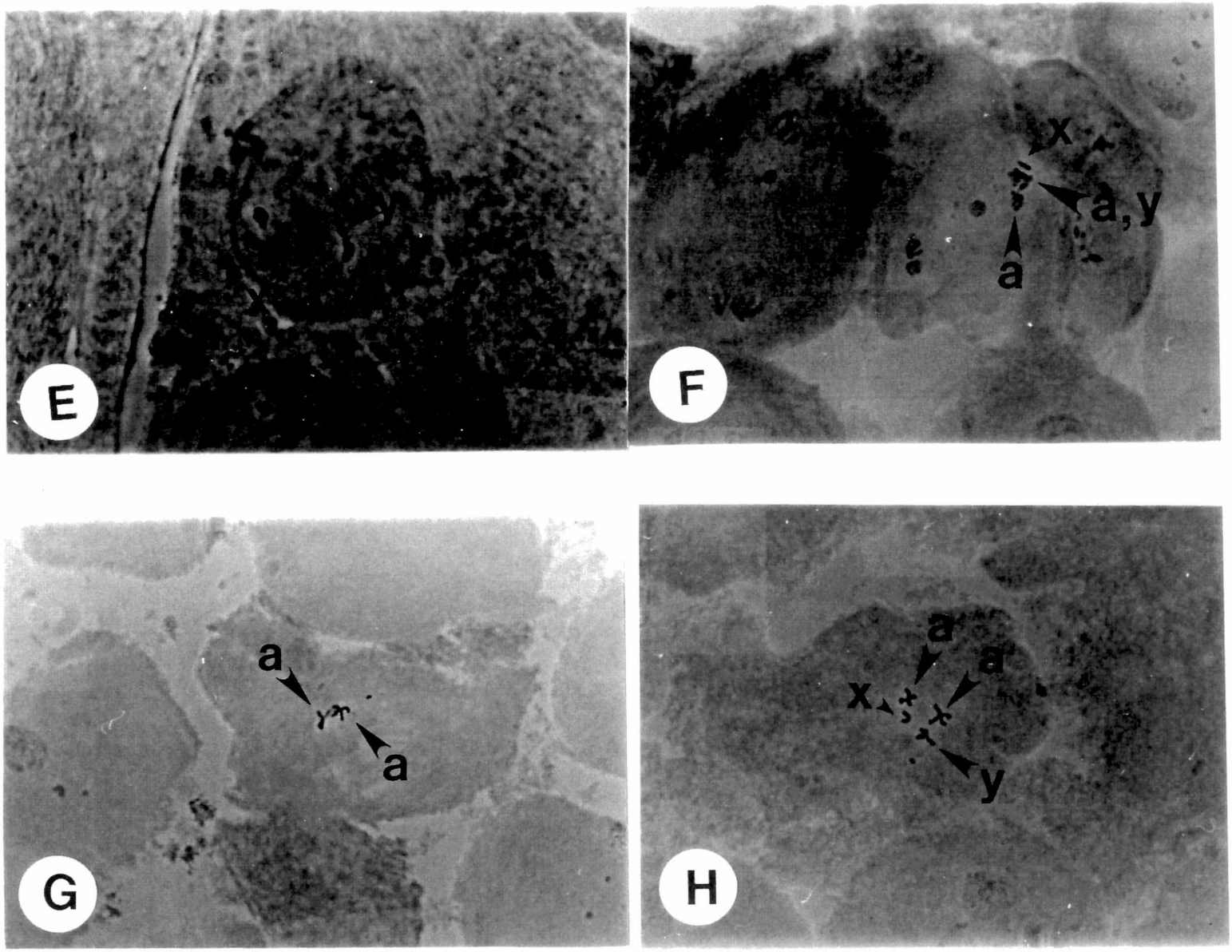
F: anaphase I, X, Y go to the same pole.

G: late prophase II, the cell has no sex chromosomes.

H: late prophase II, X, Y in the same cell.

x=X chromosome, y=Y chromosome, a=Autosome.


A
B
a
a
X
C
y
a
a
D


E
F
x
a, y
a
G
a
a
H
a
a
x
y

Anaphase I or Telophase I: X,Y go to the same pole (4:2) (Fig. 13F) or opposite poles (3:3) (Fig. 13B) 2, 3, 4 stand for the chromosome numbers at each pole (chromosome number 4 is not included due to its smallness)

Metaphase II: X, Y are in different cells-(3) (Fig. 13C,D); X, Y are in same cells-(4) (Fig. 13H); both X, Y are absent in cells-(2) (Fig. 13G); 2, 3, 4 stand for chromosome numbers in each cell (chromosome number 4 is not included due to its smallness)

The disjunction frequency (P) (as calculated from the cytological data) for 5-1 was 0.50 and for 3-21 was 0.55(Table 2A). The P values for both 5-1 and 3-21 strains were not significantly different from the P value (0.51) in Df(1)X-1 males without rDNA insertion (from z test, see Materials and Methods). Thus, the [rib12] insert does not improve X-Y pairing.

The frequency of X-Y disjunction was also calculated by crossing [rib12], Df(1)X-1/BsYy$^+$ males with chromosomally normal yw females to count the four different kinds of progenies:Bar or non-Bar females, Bar or non-Bar males. Those four kinds of progenies result respectively from four different kinds of sperms: X, XY, Y, O.

The P value for 5-1 was 0.60 and for 3-21 was 0.52 (Table 2B). Again, both values were not significantly different from the P value (0.55) in the Df(1)X-1 males (from z test, see

Table 2. The X-Y disjunction frequencies from both testis squashes (A) and progeny counting test (B). Calculations are given in Materials and Methods.

A. Testes Squash Data

Paternal X Chromosome	Ectopic rDNA Dose	Meiosis I X-Y(a)	Meiosis I X-Y(b)	Meiosis II O(c)	Meiosis II X or Y(d)	Meiosis II XY(e)	P* ± (C.I.)
Df(1)X-1	0	23	19	53	108	57	.51 ± .06
(rib7)(1A)	1	18	9	52	166	46	.64 ± .05
(rib12)(5-1)	trpt unit	70	62	35	41	23	.50 ± .08
(rib12)(3-21)	trpt unit	65	41	30	32	20	.55 ± .09

$$P^* = (2a + d) / (2a + 2b + c + d + e)$$

B. Progeny Count Data

Paternal X Chromosome	Sperm Class Totals X	Y	XY	O	P* ± (C.I.)
Df(1)X-1	871	217	54	2042	.55 ± .02
(rib7)(1A)	2572	1693	523	1771	.68 ± .01
(rib12)(5-1)	1115	269	46	2971	.60 ± .01
(rib12)(3-21)	798	203	64	2163	.52 ± .02

P* = frequency of X-Y disjunction

Materials and Methods). Thus the conclusion from the progeny tests is also that the [rib12] insert can not stimulate X-Y pairing.

CHAPTER 4

DISCUSSION

This thesis tested the X-Y pairing ability of an ectopic rDNA fragment containing most of rDNA transcriptional unit (minus promoter and spacer sequences) located on chromosomes of transgenic lines 5-1 and 3-21. The effect of this truncated rDNA fragment on X-Y disjunction was determined by recombining the insertions onto a heterochromatically deficient X and measuring the X-Y disjunction both cytologically and genetically. Although one insertion is in the 7C region and the other is in 6F-7A region, the results from those two insertions tested under the same experimental procedures were the same: this piece of rDNA does not stimulate X-Y paring. These results extend previous studies of rDNA pairing.

A single complete copy of rDNA in p(rib, ry)7 was transformed into the 1A1-4 region of the X chromosome in D. melanogaster (Karpen, 1987; Karpen et al, 1988; Mckee and Karpen,1990). The complete rDNA insertion could stimulate X-Y pairing and its double insertions stimulate pairing more strongly (Mckee and Karpen, 1990).

rDNA pairing was also studied in other (different from above) X-linked, transgenic insertions of three fragments of

a rRNA gene(Merrill et al.,1992). All three fragments (rib10, rib6ΔH, rib10Δ3) retain the promoter and upstream IGS region, including 6-9 tandem copies of a 240 bp repeat, which contains a sequence exactly the same as the rDNA promoter. The constructs of those three fragments differ one from another in the length of downstream sequences: about 2.2 kb (rib10) retains all of the ETS and about 2/3 of 18S region, 1 kb (rib6ΔH) retains all of the ETS and only 150 bp, (rib10Δ3) retains only 140 bp of the ETS. The results from testing those three insertions clearly showed that a complete rDNA was not required for stimulation of X-Y disjunction and that the rDNA promoter and upstream spacer sequences have the ability to stimulate X-Y pairing.

A similar conclusion was reached in another study in which deletions were generated in vivo by exposing [rib7](1A1-4) to a P transposase source [Δ2,3](99B) (see method: Daniels et al., 1985; Dreesen et al., 1991). The deleted products containing partial excisions with breakpoints at various sites within the [rib7] transposon (Mckee et al, 1992) were recovered and tested for pairing ability both cytologicaly and genetically. Complete deletions of the rRNA transcription unit did not affect the ability to stimulate X-Y pairing as long as most of the intergenic spacer (IGS) remained. Within groups of deletions that lacked the entire transcription unit and differ only in length of residual IGS material, pairing ability was proportional to the dose of 240-bp intergenic

spacer repeats. The fact that complete deletions of the rDNA transcription unit did not reduce pairing ability led to the speculation that X-Y pairing ability is limited to the intergenic spacer region of the rDNA. The present results confirm this hypothesis by showing that when the rDNA transcription unit without a promoter/IGS sequences, no pairing stimulation is observed.

Intergenic spacer sequences may also mediate X-Y pairing in Drosophila simulans, a close relative of D.melanogaster. The D. simulans Y chromosome lacks rDNA transcription units (no nucleolus organizer). Those unusual Y chromosomes pair and disjoin regularly from the X chromosomes (which have nucleolus organizers) during male meiosis. It has been shown that Y chromosome in D. simulans carries amplified rDNA spacer (3000 kb tandemly repeated 240-bp sequences) homologous to the IGS repeats in X chromosome (Lohe and Roberts, 1990).

From the experimental evidence presented in this thesis and the findings in other earlier studies, we conclude that rDNA pairing is sequence-specific.

The capacity of [rib12] in X-Y pairing needs to be tested further. The research in this thesis tested the function of one single copy of [rib12] in X-Y pairing. It is possible that [rib12] pairing ability depends on its copy number, i.e., that there is a minimum copy number for [rib12] to involve in pairing (some kind of weak contribution).

Experimental evidence showed that the minimum copy number

of 240 bp IGS repeats which positively stimulate pairing may be five (Mckee et al., 1992). Although there are no rDNA genes in D.simulans Y chromosome, there are amplified spacers. The copy number of the spacer was amplified that could be in order to match the same length of pairing distribution with X chromosome. It is not clear whether these results indicated a requirement for a minimum length of homology or a minimum repeat copy number. If length of homology is the crucial factor, the single [rib12] insertions should be more than adequate as they provide over 4.5 kb of homology (compared to 1.2-3.0 kb of homology for various pairing-positive IGS insertions (Mckee et al., 1992; Merrill et al., 1992). However, if repeats are required, a complete test of pP(rib, w^{hs})12 will require amplification of the insert.

The present experiments have been designed to facilitate testing of duplications of the rDNA in pP(rib, w^{hs})12. pP(rib, w^{hs})12 contains two directed repeat recombination sites-FRT from yeast 2 μm plasmid (Michael, M. C., 1988). [rib12] and w^{hs} were flanked by those two FRTs. The DNA sequence flanked by FRTs can be amplified or reduced due to site-specific FRT recombination mediated by FLP (site-specific recombinase). So we can get amplified [rib12] and test their pairing ability.

The purpose of finding out where chromosomes pair is to find out how they pair. More studies are needed to show what mechanism is involved in pairing (eg. what kind of proteins

bind to the promoter/IGS sites and how those proteins interact with one another). The identification of a small sequence that promotes X-Y pairing should facilitate such studies.

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VITA

Xiaojia Ren was born on September 2, 1963 in Beijing, China. She finished her primary education in Beijing and her high school education in Yantai area, Shandong Province. She decided to study science after reading several scientists' biographies and later chose biology as her undergraduate major because the 21st century is to be the Century of Biology. She graduated from Lanzhou University with a B.S. in Cell Biology in 1986.

After being an instructor of Microbiology in the Department of Biochemistry at Yantai University for two and half years, She came to the United States to join her husband in 1989. In the Spring of 1991, she began her graduate study at Dr. Bruce D. Mckee's laboratory of the Department of Zoology at the University of Tennessee, Knoxville. She received her Master of Science degree in Zoology in December, 1993 and continued to work in the same field.