To the Graduate Council:

I am submitting herewith a thesis written by Lynn Victoria Eisenhour entitled "Molecular Genetic Analysis of rDNA Insertions in *Drosophila melanogaster*." 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 Master of Science, with a major in Zoology.

Bruce D. McKee

Bruce D. McKee, Major Professor

We have read this thesis
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and Dean of The Graduate School

Molecular Genetic Analysis of rDNA Insertions In *Drosophila melanogaster*

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Lynn Victoria Eisenhour

May 1994

DEDICATION

To all my family and friends who have encouraged, supported and prayed for me during this study. May the Lord richly bless all of you.

ACKNOWLEDGEMENTS

I would like to express sincere thanks to my major professor, Dr. Bruce D. McKee, for his patience, guidance, encouragement, and for believing in me throughout this study. I would also like to thank the members of my committee, Dr. Mary Ann Handel and Dr. Ranjan Ganguly, for their comments and assistance during this study. I would also like to thank Ledare Habera for her friendship and patience while teaching me molecular biology techniques. To all those who work in Dr. McKee's lab, I thank them for the laughter and assistance to help me get through the tough days.

Words will never express my deepest and warmest appreciation to my husband, David, who has been my constant source of strength and encouragement during this study. Most of all, I appreciate him for his patience and love even though we've been far away from one another.

ABSTRACT

Homologous chromosome pairing is essential for the subsequent events in meiosis. Pairing, in most organisms, leads to recombination, in which chiasmata are formed and hold the chromosomes together until segregation at Anaphase I. This is not so in *Drosophila melanogaster* males, where there is no recombination or chiasma formation, but there is pairing of the homologs. Therefore, it is important to locate where pairing occurs in order to understand how homologous chromosomes pair. It is known that in *Drosophila* the autosomes pair in the euchromatin and that the sex chromosomes pair in the heterochromatin. The pairing of the X and Y is mediated by ribosomal DNAs (rDNAs) which are located near the base of the short arm of the Y and in the centric heterochromatic region of the X. A single, complete copy of an rDNA repeat has been shown to stimulate pairing of the sex chromosomes when located on a heterochromatically deficient X chromosome. Also, insertions of the 5′ half of the rDNA which includes all of the external transcribed spacer (ETS) region, part of 18s gene, and a part or all of the promoter region, have been shown to restore pairing. Three X-linked transformation stocks have been found, each transformed with the same transformation vector, which includes only 180bp of the promoter region and the entire intergenic spacer. These three transformed stocks have different X-Y pairing abilities.

The present study was undertaken to ascertain the basis for the differences in their pairing abilities. *In situ* hybridization to polytene chromosomes in the salivary glands of the larvae in each of the stocks was done to locate the insertion site. Two of the stocks have an insertion site at the tip of the X and one stock has the insertion site at 14D of the X. Also, genomic Southern blot anaylsis and cloning of the rDNA fragment were done to determine the structure of the rDNA insert to understand why there is such a difference in the pairing ability of each of these stocks. Southern blot analysis revealed that there was a size difference among the three stocks. From cloning, it was shown that the difference lies within a repetitive region of the intergenic spacer.

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Chapter I

INTRODUCTION

Meiosis is a process during which cells undergo two successive nuclear divisions to form cells that contain half the number of chromosomes. The first division, Meiosis I, is characterized by the pairing of homologous chromosomes and their separation. Meiosis II, the second division, is much like mitosis with the exception that there are only half as many chromosomes. The most striking and complex phase in meiosis is Prophase I. In this phase, homologous chromosomes pair and undergo, in most organisms, genetic recombination before segregating to opposite poles.

Homologous chromosome pairing is essential for subsequent events in meiosis. Pairing is, in most organisms, accompanied by synapsis, formation of a three part structure called the synaptonemal complex (SC). The precise role of the SC is yet unclear, but is thought to play some role in the meiotic recombination event between the homologous chromosomes (Von Wettstein et al., 1984). With the appearance of the SC, there is also the appearance of small, dense proteinaceous structures, called recombination nodules, at intervals along the length of the SC. These nodules are thought to contain localized meiotic recombination enzymes (Carpenter, 1975) and aide in the exchange of genetic information between non-sister chromatids (Rasmussen and Holm, 1978). With the disappearance

of the SC and recombination nodules at the completion of synapsis, the chromosomes begin to pull apart, but are held together by chiasmata, which stabilize the structure until segregation at Anaphase I. The number of the chiasmata along the chromosomes correlates with the number and distribution of the recombination nodules before they disappear (Carpenter, 1988; Holm and Rasmussen, 1980). The chiasmata are believed to help the bivalents "line up" at the metaphase plate with the proper orientation of the centromeres to ensure proper segration of the chromosomes to opposite poles (Hawley, 1988). Chromosomes that have problems pairing also have difficulty achieving bipolar stability at the metaphase plate. The failure of the bivalents to pair and achieve the proper orientation in metaphase leads to nondisjunction, loss of chromosomes, and failure of the cells to develop normally (Baker et al., 1976).

Recombination of homologous chromosomes is known to occur in most, but not all, sexually reproducing organisms. However, in the case of *Drosophila melanogaster* males, there is no exchange of genetic information (Morgan, 1912), formation of a synaptonemal complex (Meyer, 1960), or chiasmata (Cooper, 1950). Despite the lack of recombination, the homologous chromosomes pair and segregate from one another regularly (Morgan, 1912; White, 1973). This raises the question about how homologous chromosomes can pair and segregate from one another without any meiotic recombination

as suggested in the studies reviewed above.

Because meiosis is simple in *Drosophila* males, due to the lack of recombination or formation of chiasmata, it has made the study of homologous chromosome pairing and segregation more straightforward than in chiasmate organisms. It is known that chromosome 2, large metacentric chromosomes, appears to pair only in the euchromatic regions of the chromosomes. Yamamoto (1979) showed that a mini-chromosome II, comprised of mostly heterochromatin, failed to pair regularly with a normal second chromosome. Furthermore, compound chromosomes for the left and right arms of chromosome 2 [C(2L) and C(2R)] failed to segregate from one another regularly even though they share a large portion of heterochromatic homology. Only when C(2L) and C(2R) shared euchromatic homology along with heterochromatic homology did these chromosomes segregate regularly (Gethmann, 1976; Hilliker et al., 1982). Further studies using the second chromosome showed that autosomal pairing in *Drosophila* males is dependent on general homology (McKee et al., 1993).

In contrast to autosomal pairing, sex chromosomes are known to pair at heterochromatic sites. Cytogenetic studies have localized these sites to nuceolus organizers, which is the site of rDNA and located near the base of the short arm of the Y and in the centric heterochromatic region of the X (Cooper, 1964). Large deletions in the XL heterochromatin have been shown to disrupt X-Y nondisjunction (Muller and

Painter, 1932; Gershenson, 1933; Cooper, 1964).

There are two nucleolus organizers in *Drosophila*, one in the X and one in the Y, each consisting of 200-250 tandem copies of rDNA. One complete rDNA repeat (Figure 1) contains an 8Kb transcriptional unit and an 3-4Kb intergenic spacer (IGS). The IGS region contains a "1900" region, which is defined by two AluI sites on each end, and a series of 240bp repeats, which are also defined by two AluI sites on either side of them. The transcriptional unit of the rDNA contains an external transcribed spacer (ETS), an 18s gene, an internal transcribed spacer (ITS), and a 28s gene.

McKee and Karpen (1990) used P-element transformation to show that a single, complete rDNA repeat stimulates X-Y pairing when located on a heterochromatically deficient X [Df(1) X-1] (Figure 2). This raised the question of what part of the rDNA was responsible for the meiotic pairing of the X and Y chromosomes.

An approach to this question involved the construction of P10, a P-element transformation vector. P10 (Figure 1B) contains the sequences from the Hind III site in the 28s gene (Figure 1A) at the left of the IGS to the first Hind III site in the 18s gene of the rDNA. Merrill (1990) found that this fragment of the rDNA stimulated X-Y pairing when located on the Df(1)X-1 chromosome. Because this study of a truncated rDNA fragment gave positive results in pairing (Merrill et al., 1992), the P10Δ3 transformation vector was constructed to

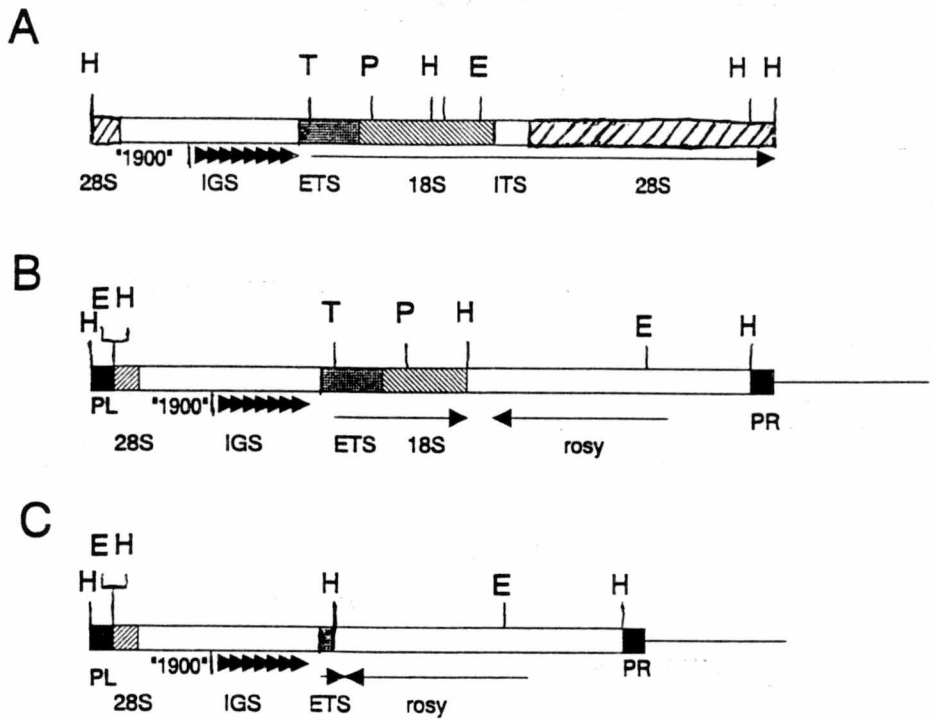

Figure 1. Restriction maps of rDNA and P-element constructs. A. Single rDNA repeat. B. P10 transformation vector C. P10Δ3 transformation vector.

PL and PR=left and right ends of the P-element
--►=transcription unit IGS=intergenic spacer
◄--=rosy transcription unit "1900"=1900 region
ETS=external transcribed spacer ITS=internal transcribed spacer

A=SalI H=HindIII P=HpaI T=SstI ►=240bp repeats

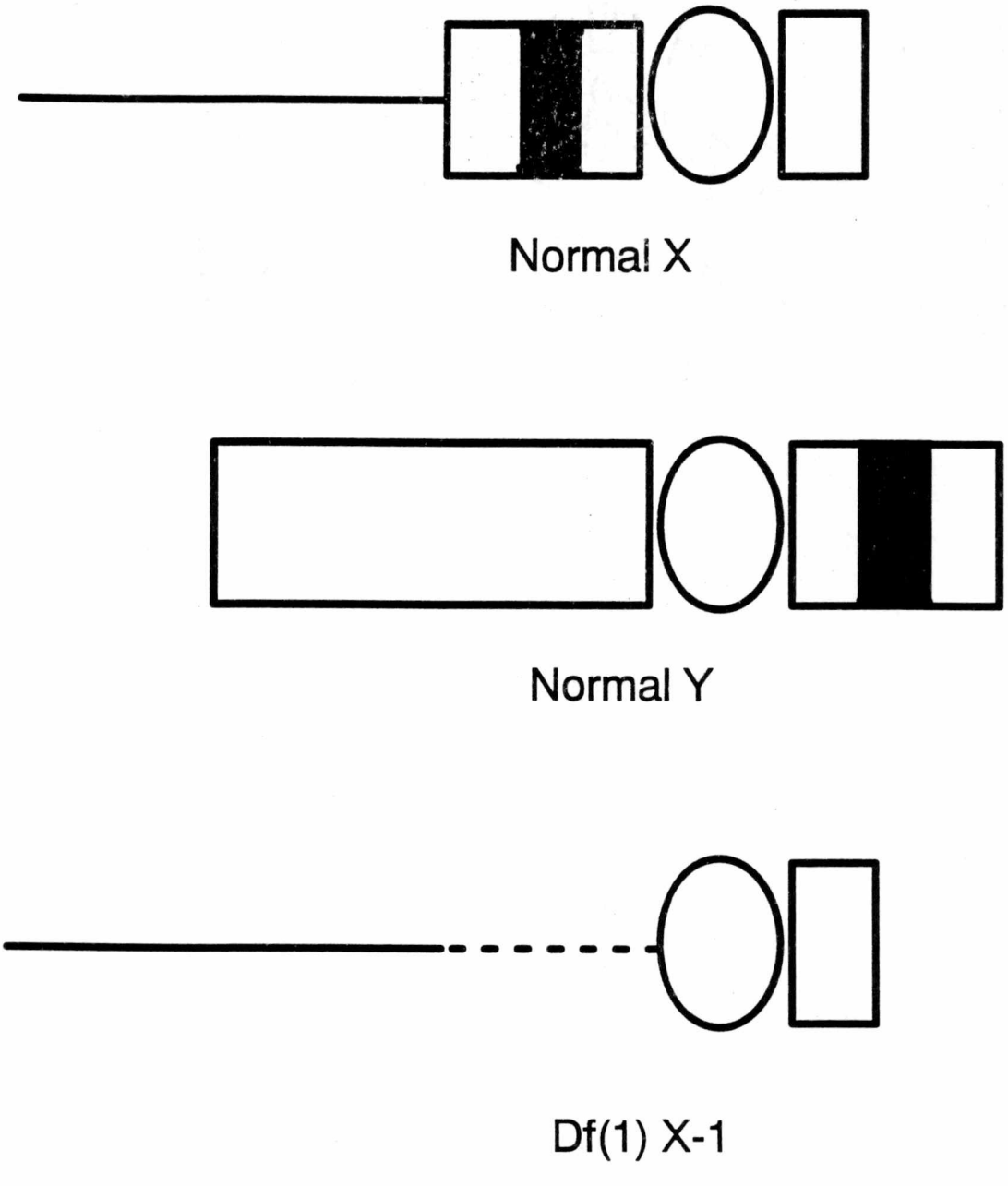

Figure 2. Comparison of normal X and Y chromosomes to Df(1) X-1 chromosome.
□=heterochromatin —=euchromatin ■=rDNA
○=centromere ---=deleted heterochromatin

see how much of the 5′ end of the rDNA was actually needed to stimulate pairing of the sex chromosomes.

The P10Δ3 construct (Figure 1C) contains the sequences in the P10 vector, but does not have the 18s gene and only 140bp of the ETS region. Three different *Drosophila* stocks were obtained that carried P10Δ3 inserted on the X chromosome. These stocks were tested for meiotic pairing ability and it was found that they each had a different pairing ability: P10Δ3 X-2 showed the weakest ability to pair, P10Δ3 X-1 showed a moderate ability, and P10Δ3 X-3 showed the strongest ability, which proved to be a stronger pairing ability than a stock that contained a single, complete rDNA unit (Merrill et al 1992). This led to the question of what causes pairing differences among these three X-linked rDNA insertions when they all three presumably carry the same P10Δ3 construct.

There are two different hypotheses as to what could be causing the difference in pairing ability. One hypothesis is that chromosomal position of the rDNA insertion may be having an effect on pairing (Rubin and Spradling, 1983). The other hypothesis is that the molecular structure of the rDNA has somehow been altered among the three inserts. The main purpose of this research was to test both of these hypotheses.

In situ hybridization of salivary glands from larvae in each of the stocks was used to located the insertions of the rDNA on the X chromosome. Also, genomic Southern analysis and cloning of the rDNA fragments was done to determine the

structure of the rDNA insert in each of the stocks.

Chapter II

METHODS AND MATERIALS

In situ Hybridization

Larvae were cultured in uncrowded conditions in standard cornmeal-molasses-yeast agar at 22-23°C (Ashburner 1989). Salivary glands from third instar male larvae were dissected in 45% acetic acid on subbed slides and gently squashed under a siliconized coverslip. These slides were then stored at 4°C overnight, to ensure flatness of the chromosomes. The coverslip position was marked with a glass etcher and removed by placing the slide in liquid nitrogen. The slides were dehydrated in 3:1 methanol:acetic acid, 50% ethanol, 75% ethanol, and 95% ethanol for 10 minutes each at room temperature and air dried. The slides are washed in 2X SSC at 65°C for 30 minutes, 2X SSC at room temperature for 2 minutes, then acetylated while hanging in 2L of 100mM TEA while 10ml of Acetic Anhydride was added to the stirring solution. Stirring was stopped once the Acetic Anhydride was dissolved and the slides were incubated in this same solution for 10 minutes. The slides were washed twice in 2X SSC, 5 minutes each, dehydrated twice in 70% ethanol and twice in 95% ethanol at room temperature for 5 minutes each, denatured in 70mM NaOH for 3 minutes, washed in 2X SSC for 5 minutes, then dehydrated as described above and air dried. The biotin-labeled DNA probe was prepared by using the BioNick Labeling System (Gibco-BRL) and purified using a G-50 Sephadex column. The

biotinylated probe was then precipitated with 20μg salmon sperm DNA, 10% total volume 3M NaOAc, and 2X volume 100% ethanol at -80°C for 20 minutes, washed with 70% ethanol, dried, and rehydrated in 150μl hybridization buffer (Ashburner 1989), then stored at -20°C until ready to use. Probes were denatured by boiling for 5 minutes before pipetting 10μl of the probe onto the slide where the coverslips were marked. The slides were covered with siliconized coverslips and sealed with rubber cement. Hybridization was carried out in a moist chamber in a 58°C waterbath for 12-18 hours. The next day, the rubber cement was removed without moving the coverslip and the slides were washed three times in 2X SSC at 53°C for 20 minutes each, twice in 1X PBS at room temperature for 10 minutes each, twice in 2% BSA in TMN1, triton X-100 (first wash: 42°C for 20 minutes, second wash: room temperature for 10 minutes). 50μl SA/AP (2μl streptavidin/alkaline conjugate in 1ml TMN1, Triton X-100) was pipetted on to the area where the coverslips were marked. After 5 minutes, another 50μl SA/AP was added to the slides, then let to stand for another 5 minutes. Each slide was rinsed with 3-4ml TMN1, triton X-100 by holding the slide over a beaker. The slides were washed twice in TMN1, Triton X-100 for 3 minutes each and twice in TMN2 for 3 minutes each. Next, 30μl NTB/BICP was pipetted onto the center of a plastic coverslip. Each slide, with the chromosome side facing downward, was held over the coverslip to ensure the droplet would adhere to the slide.

The slides were carefully flipped so that the coverslip would not slip off and then placed in a flat tray in the dark for 2 hours. The coverslips were rinsed off by swishing the slide in a dish of distilled water. Each slide was stained with a few drops of 2% Aceto-Orcein solution for approximately 10 seconds, rinsed with distilled water, and air dried. To view and photograph the slides, 25μl of sterile water was pipetted onto the slide, a siliconized coverslip was placed on the slide, and sealed around the edges with rubber cement to prevent the microscopic oil from getting under the coverslip and ruining the chromosomes.

Preparing CsCl Purified Genomic DNA

After collecting 1000 fruit flies, approximately 250 flies were ground in 15ml of cold NIB solution until the liquid had a "strawberry mush" appearance to it. The liquid was filtered through a nylon mesh into a 50ml Oak Ridge centrifuge tube to remove the majority of the fly debris. This procedure was repeated with the rest of the flies, putting a total of 30ml (approximately 500 flies worth of solution) of the solution in each Oak Ridge tube. The tubes were centrifuged at 4°C for 7.5 minutes at 7,000 rpm. The supernatant was discarded and 4.8ml NIB was added to one Oak Ridge tube, mixed using a glass pipette to make sure to pellet was dissolved into the solution, and combined with the other tube of the same sample. To each sample, 1.2ml 10% Sarkosyl

was added and mixed by inversion until the solution became viscous. In a 15ml graduated centrifuge tube, 12.4g of dry CsCl was added along with 2ml of 2% Sarkosyl in NIB to wet the CsCl. The sample solution was poured into the tube containing the CsCl, adding 2% Sarkosyl in NIB to bring the total volume to 13ml, and mixed gently to dissolve the CsCl into the solution. The tube was centrifuged for 5 minutes in a clinical centrifuge at the top speed to remove any extra fly debris. Any fly debris at the top of the tube was removed and the solution was transferred to a 13ml ultracentrifuge tube, sealed, and centrifuged at 15°C for 2 days at 55,000 rpm. DNA was collected from the ultracentrifuge tube in an 1.5ml Eppendorf tube by dripping the liquid from the tube through a small hole just above the RNA line at the bottom of the tube. The DNA was very viscous as it dripped out of the tube and was dialyzed in 2L TE pH 8 for 3 hours, changing the TE every 1.5 hours. The DNA was extracted once in equal volumes of phenol:chloroform, chloroform:isoamyl alcohol and precipitated with 10% volume 3M NaOAc and 2X volume 100% cold ethanol at -20°C for at least 3 hours. The DNA was pelleted by centrifugation at room temperature for 15 min at 14,000 rpm, washed with 70% cold ethanol, air dried, and rehydrated in a total volume of 250ml TE pH 8. The sample was stored at 4°C until ready for use.

Southern Blotting

CsCl purified DNA and plasmid/phage DNA was digested with the appropriate restriction enzymes for 6-8 hours and separated on a 0.7% agarose gel by electrophoresis in a 1X TBE buffer (Maniatis et al., 1982). The DNA in the gel was nicked by soaking in 0.25M HCl for 10 minutes, denatured in 0.4M NaOH/0.6M NaCl for 30 minutes, and neutralized in 0.5M Tris-HCl pH 7.5/1.5M NaCl for 30 minutes. The DNA was transferred to GeneScreen Plus hybridization transfer membranes (Dupont/NEN) by the Southern blotting method (Southern, 1975). The membranes were prehybridized in 50% formamide, 1% SDS, 5X SSPE (Maniatis et al., 1982) and salmon sperm DNA for at least 15 minutes at 42°C. The DNA probes were labeled by using the Nick Translation System (Gibco BRL). Hybridization was carried out in a 42°C waterbath with constant agitation overnight. The membranes were washed twice with 2X SSC at room temperature for 5 minutes, twice with 2X SSC/1% SDS at 65°C for 30 minutes, twice with 0.1X SSC at room temperature for 30 minutes and exposed to Kodak XAR5 films at -70°C for varying times.

Insert Purification

For each of the P10Δ3 stocks, 40μg of CsCl purified DNA was digested with EcoRI for 6-8 hours and separated on a 0.7-1.0% low melting-point agarose gel by electrophoresis (Maniatis et al 1982) in a 1X TAE buffer. The DNA was cut out

of the gel at the appropriate size using a long wave UV lamp to remove excess gel and weighed. DNA was extracted from the gel with Gelase enzyme (Epicentre Technologies) for 1 hour at 45°C, once with equal volumes of phenol:chloroform and chloroform:isoamyl alcohol, and precipitated with 2X volumes 100% cold ethanol at -20°C for at least 3 hours. The DNA was pelleted by centrifugation at room temperature for 15 minutes at 14,000 rpm, washed in 70% cold ethanol, air dried, and rehydrated in 5 μl TE pH 8. The insert sample was stored at 4°C until ready for use.

Library Construction

Two different vectors were used to clone the different size EcoRI fragments. EMBL4 was used for P10Δ3 X-2 and X-3 and λZAP II was used for P10Δ3 X-1. Each purified DNA fragment was ligated into their respective EcoRI prepared vectors at 4°C overnight (Maniatis et al., 1982). The DNA was packaged into phage heads using the Gigapack Packaging System (Statagene). To the Freeze/Thaw tube in the kit, 5μg of the ligation and 15μl sonic extract was added. The mixture was allowed to incubate at room temperature for 2 hours. After the incubation time was complete, 500μl SM (phage dilution buffer) and 20μl chloroform was added to the tube and mixed gently. The tube was spun briefly to sediment the debris. The library was stored at 4°C.

Library Screening

After titering the library to determine the concentration of phage, the library and the appropriate bacterial host was plated onto NZYM plates in a NZYM top agar overlay and grown at 37°C overnight (Maniatis et al., 1982). The plates were chilled at 4°C for at least an hour (Benton and Davis, 1977). Nitrocellulose transfer and immobilization membranes (Schliecher and Schuell) were placed on top of the chilled plates for 2-3 minutes to lift the plaques and ink spots were made so the first and second membrane lifts could be matched up later for picking the proper plaques. The membranes were soaked in a lysis solution (1.5M NaCl/0.5M NaOH) for 1 minute, a neutralizing solution (1.5M NaCl/0.5M Tris-HCL pH 8) for 5 minutes, a rinsing solution (2X SSPE pH 7.2) for 2-5 minutes, and allowed to air dry. The membranes were baked in a vacuum oven at 80°C for 2 hours. After the baking process, the membranes were stored in "Seal-a-Meal" baggies at 4°C until ready to use. The membranes were prehybridized in 50% formamide, 0.01% SDS, 5x SSPE, 5X Denhardt's solution (Benton and Davis, 1977), and salmon sperm DNA at 42°C for 2 hours. The DNA probes were labeled by using the Nick Translation System (Gibco BRL). Hybridization was carried out in a 42°C waterbath with constant agitation overnight. The membranes were washed once with 2X SSC/0.5% SDS at room temperature for 5 minutes, once with 1X SSC/0.1% SDS at 68°C for 30 minutes to 1 hour and exposed to Kodax XAR5 films at -70°C for varying

times.

***In vivo* Excision of Plasmid From λZAP II Vector**

In a 50ml conical tube, 200μl bacterial host cells (OD_{600}=1.0) and 200μl λZAP II phage pick stock were combined and incubated at 37°C for 15 minutes according to the manufacturer's instructions (Stratagene). To the bacteria/phage mixture, 5ml 2X YT media was added and the mixture was incubated at 37°C with shaking for 3 hours. After the incubation time was complete, the tubes were placed in a 70°C waterbath for 20 minutes, then spun in a clinical centrifuge for 5 minutes at 4,000 g. The supernatant, which contains the packaged pBluescript phagemid, was transferred into a sterile tube and was stored at 4°C until ready for use. To recover the plasmid, 10μl phagemid stock and 200μl bacterial host cells were combined, incubated at 37°C for 15 minutes, and plated 1-100μl on LB-Amp plates, then incubated at 37°C overnight. The next day, bacterial colonies were picked and grown in 2ml LB-Amp liquid at 37°C with shaking for 8 hours (Maniatis et al., 1982). After the incubation time was complete, 1ml of the liquid was transferred to a 1.5ml Eppendorf tube, centrifuged for 3 minutes at 14,000 rpm, and the supernatant discarded. To resuspend the cells, 100μl 10mg/ml lysozyme/Solution I (50mM glucose/25mM Tris-HCl pH 8/10mM EDTA) was added and incubated at room temperature for 5 minutes (Birnboim and Doly, 1979). To the tube, 200μl

Solution II (0.2N NaOH/1% SDS) was added, mixed by inversion, and kept on ice for 5 minutes. Next, 200μl Solution III (5M KAc) was added, mixed by inversion, and kept on ice for 5 minutes. After the incubation on ice was complete, the tube was centrifuged for 8 minutes at 14,000 rpm and the supernatant was transferred to a new tube. To the supernatant, 5μl 1mg/ml RNase was added and incubated at 37°C for 20 minutes. The DNA was extracted twice with phenol:chloroform, once with chloroform:isoamyl alcohol, and precipitated with 10% volume 3M NaOAc and 2X volume 100% cold ethanol. The DNA was pelletted at room temperature at 14,000 rpm, washed with 70% cold ethanol, air dried, and rehydrated with 40μl of TE pH 8.

Isolation of Phage DNA

After plaque picks were isolated and stored in 1ml SM containing a drop of chloroform at 4°C for 4-6 hours, 50-100μl of the bacteriophage suspension and 200μl of the appropriate bacterial host cells (OD_{600}=1.0) were incubated at 37°C for 20 minutes, plated on NZYM plates in an NZYM top agarose, and incubated at 37°C overnight (Maniatis et al., 1982). The next day, 5ml SM was directly poured on top of the plate and gently shaken constantly at room temperature for 2 hours. The liquid was transferred to a 15ml graduated centrifuge tube and centrifuged at 4°C for 10 minutes at 8000 g to remove the bacterial debris. The supernatant was

transferred to a new tube, 1μg/ml of RNase A and DNase I were added and incubated at 37°C for 30 minutes. After the incubation time was complete, an equal volume of a solution containing 20% PEG/2M NaCl in SM was added to the tube and incubated at 0°C for 1 hour. The bacteriophage particles were recovered by centrifugation at 4°C for 20 minutes at 10,000 g. The supernatant was removed and the tube was inverted to drain away any excess liquid for 10-15 minutes. To the pellet, 500μl SM was added and resuspended by vortexing. The solution was centrifuged at 4°C for 2 minutes at 8,000 g to remove any excess debris. The supernatant was transferred to a 1.5ml Eppendorf tube, 5μl 10% SDS and 0.5M EDTA pH 8 were added and incubated at 68°C for 15 minutes. The DNA was extracted once with phenol:chloroform and chloroform:isoamyl alcohol, precipitated with an equal volume Isopropanol at -70°C for 20 minutes. The DNA was pelletted at room temperature for 15 minutes at 14,000 rpm, washed with 70% cold ethanol, air dried, and rehydrated with 50μl TE pH 8. The phage DNA was stored at 4°C until ready for use.

Chapter III

RESULTS

In situ Hybridization

Insertions of the rDNA on the X chromosome were mapped by *in situ* hybridization to salivary gland polytene chromosomes. The probe used for the hybridization was Carnegie 20 (C20). This probe was used because it contains the *rosy* gene, which is also present within the P-element construct (P10Δ3) used to make the rDNA inserts. The probe gave a single hybridization signal on the X chromosome for each of the three P10Δ3 stocks. P10Δ3 X-1 was located at 14D (Figure 3) and both P10Δ3 X-2 and X-3 were located at 1A, the tip of the X (Figure 4).

Restriction Analysis

A summary table (Table 1) containing the expected rDNA insert bands in the P10Δ3 transformation vector and rDNA insert bands that were found in each of the transformation stocks and clones used has been included to help in the understanding of the restriction digests.

Genomic Southern Blot Analysis

Genomic DNA Southern blot analysis was carried out to see if there was any difference in the size of the rDNA insert in each of the P10Δ3 insertion lines and to attempt to localize where the difference was within the rDNA. A detailed restriction map of the P10Δ3 transformation vector (Figure 5)

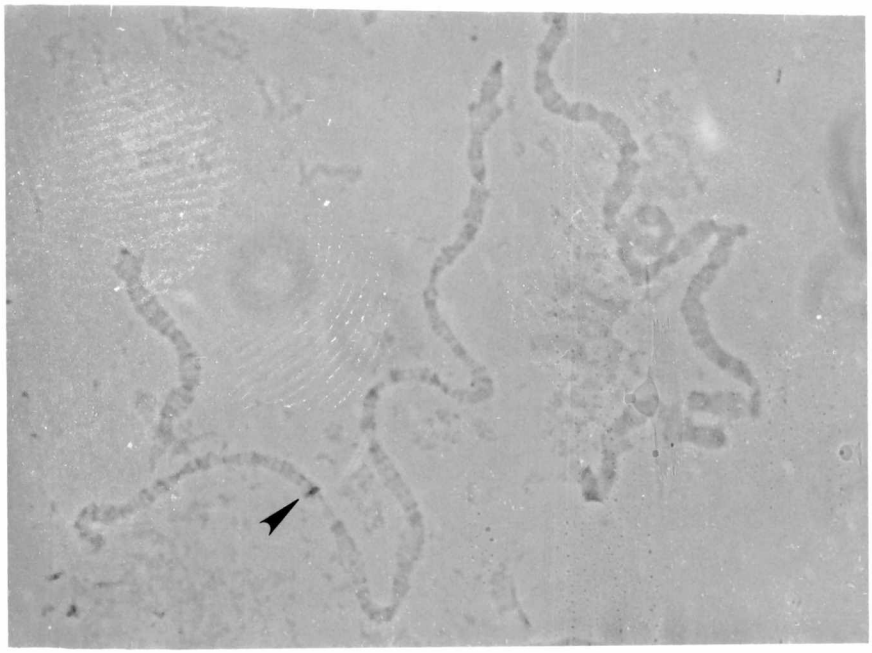

Figure 3. *In situ* hybridization of biotin-labelled C20 to P10Δ3 X-1. Arrow indicates the hybridization band.

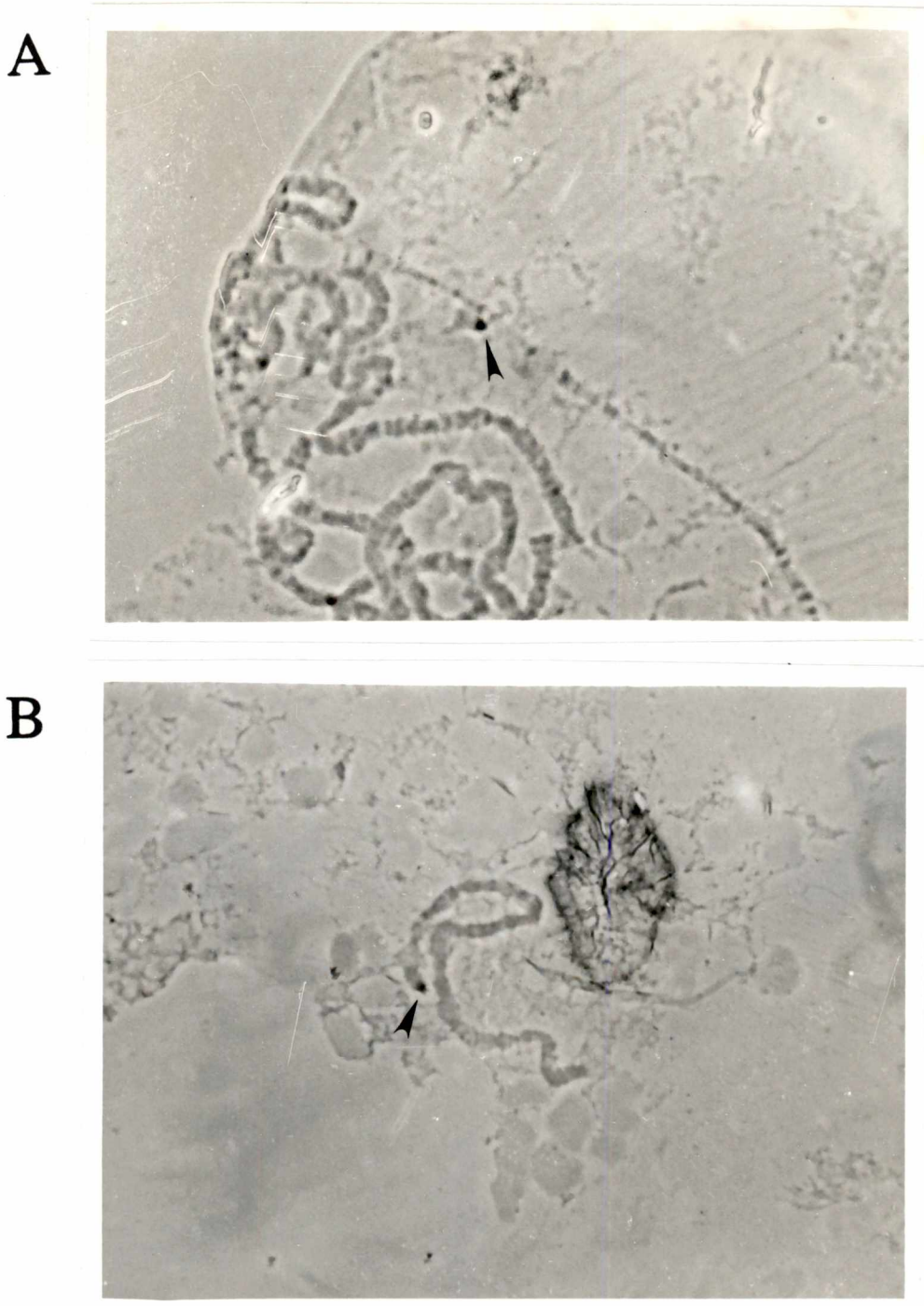

Figure 4. *In situ* hybridization of biotin-labelled C20 to A) P10Δ3 X-2 and B) P10Δ3 X-3. Arrows indicate hybridization bands.

Probe/Type of Digest	Expected bands P10Δ3	Bands that were found P10Δ3 X-1	P10Δ3 X-2	P10Δ3 X-3	Reference figures
Rosy 4.1 probe Genomic Digest					
EcoRI	8.2Kb	8.0Kb	6.3Kb	11.5Kb	6,7
XhoI/NdeI	2.4Kb	2.4Kb	2.4Kb	3.0Kb	8
C20 vector with 21x240bp repeats probe Clone Digest					
NheI	240bp >12Kb	240bp 9.5Kb	none >12Kb	NA	9A
HindIII	4.1Kb	3.9Kb	2.3Kb	NA	9B
NheI/HindIII	240bp 480bp 1.9Kb	240bp 480bp 1.9Kb	2.3Kb none none	NA	9C
ScaI/BstBI	500bp 800bp 1.1Kb	500bp 800bp 1.1Kb	none 800bp none 7.0Kb	NA	10A
ScaI/HindIII	500bp 1.2Kb 2.5Kb	500bp 1.2Kb 2.3Kb	none none 2.3Kb	NA	10B

Table 1. Summary table of expected and found rDNA insert bands with appropriate restriction digestions and probes.

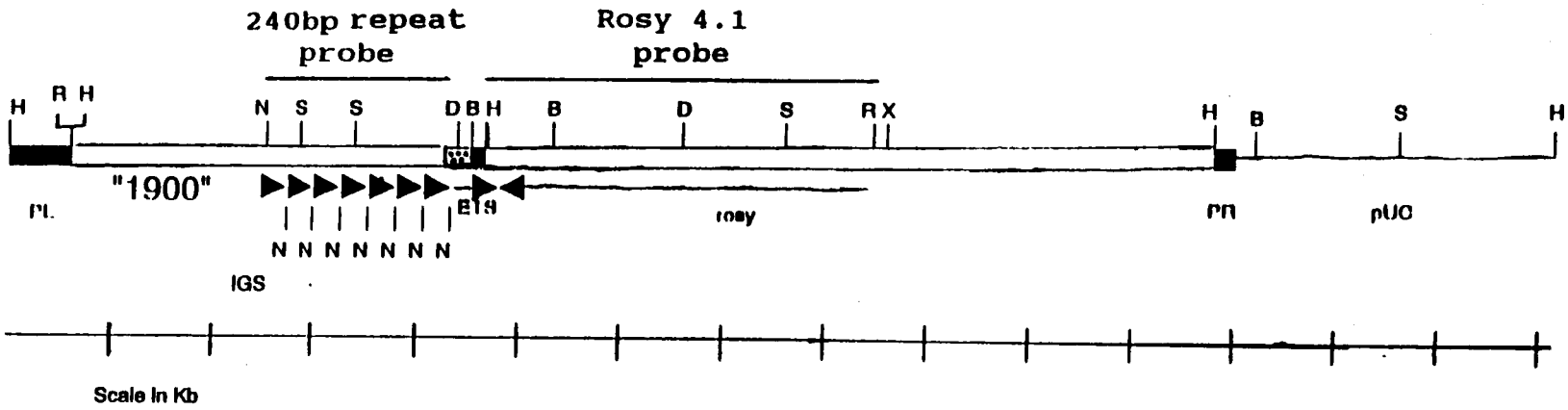

Figure 5. Detailed restriction map of the P10Δ3 transformation vector.

"1900"=1900 region -▸=rDNA transcriptional unit ▸=240bp repeat
◂-=rosy transcriptional unit PL and PR=left and right ends of P-element
-=vector sequences

B=BstBI D=NdeI H=HindIII N=NheI R=EcoRI S=ScaI X=XhoI

was used as a comparison in the analysis of the rDNA insertions in each of the stocks. The intensity of some bands in these digests appears to be less in some lanes than in others due to the copy number differences, males having just one copy of X-linked insertions but two copies of the native *rosy* locus, and the length of homology between the probe and target DNA.

Rosy 4.1 Probe

Nuclear DNA extracted from males in each of the P10Δ3 stocks was digested with EcoRI and probed with Rosy 4.1 (Figure 6). Probes within the rDNA itself could not be used because there would be hybridization to the multiple bands in the nucleolus organizers which would cause problems in the identification of the rDNA insert in each of the stocks. Each of the P10Δ3 stocks was set up as an attached-X stock, meaning that the females carry an attached-X chromosome and males carry the rDNA insertion on the X chromosome. Nuclear DNA extracted from females was used as a control for the digestion so that the native *rosy* locus could be identified. The expected size of the EcoRI band containing the rDNA insert and part of the *rosy* gene was 8.2Kb and the native *rosy* locus band was 12 Kb. This expected EcoRI insert size was not found in any of the P10Δ3 transformation lines. However, the P10Δ3 X-1 line has a band that is slightly smaller, approximately 200-300bp, than the original vector (Figure 7). This difference

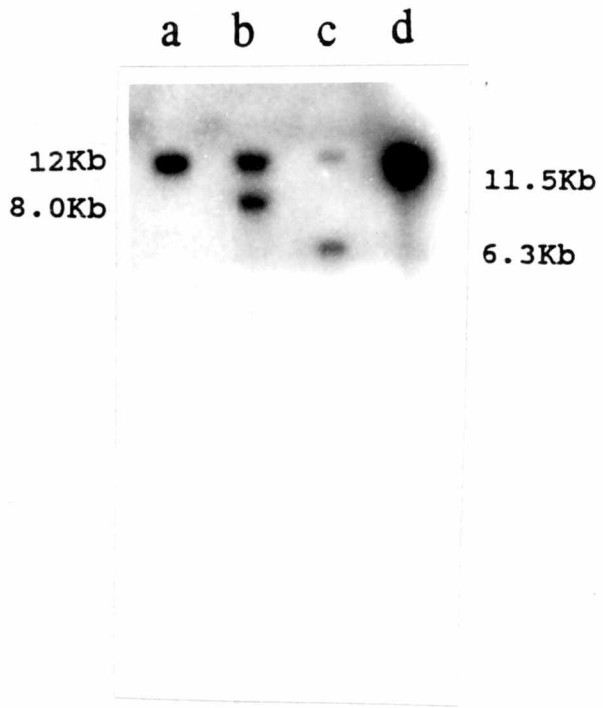

Figure 6. Genomic DNA digestion with EcoRI probed with Rosy 4.1. a) female control, b) P10Δ3 X-1, c) P10Δ3 X-2, and d) P10Δ3 X-3.

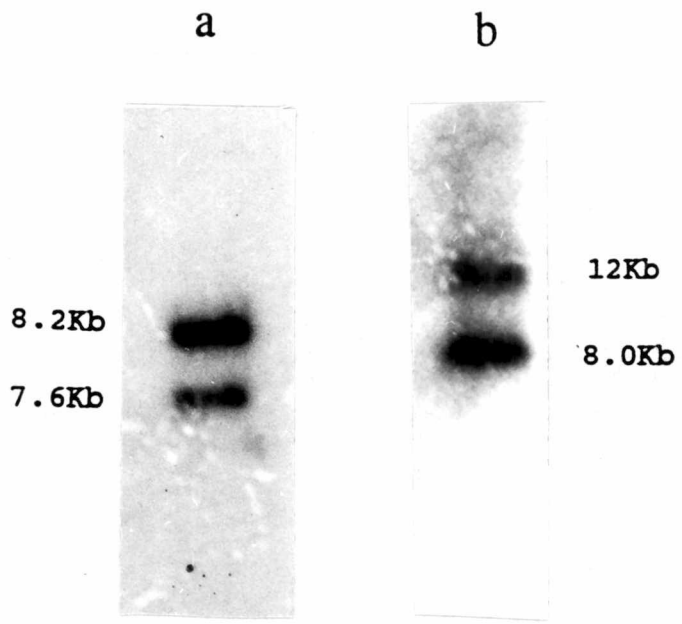

Figure 7. EcoRI digestion comparison between a) P10Δ3 transformation vector and b) P10Δ3 X-1 genomic DNA probed with Rosy 4.1.

may be due to the instability of the 240bp repeat region, in which a 240bp repeat was lost during the insertion process. In the P10Δ3 lane, a band appears at 7.6Kb which contains the rest of the *rosy* gene and the vector sequences. P10Δ3 X-2 and X-3 were not the expected insert sizes. P10Δ3 X-2 was approximately 2Kb smaller than the original vector. The P10Δ3 X-3 EcoRI fragment was approximately 3Kb larger than the original vector. This can not be seen clearly in the photograph (Figure 6), but the autoradiogram shows that this band is a doublet with the native *rosy* locus. This difference indicated that P10Δ3 X-2 had undergone some type of deletion event and P10Δ3 X-3 had undergone some type of insertion event.

Each of the P10Δ3 male and female genomic DNAs were digested with XhoI/NdeI and probed with Rosy 4.1 (Figure 8). This digest was to determine if the differences among the lines was in the *rosy* gene, the promoter region of the rDNA, or the region upstream of the promoter (Figure 5). Both P10Δ3 X-1 (not shown) and X-2 showed bands at the expected band size of 2.4Kb from the NdeI site in the upstream region of the promoter to the NdeI site within the *rosy* gene. The band at 2.3Kb is from the NdeI site to the XhoI site in the *rosy* gene. The P10Δ3 X-3 showed a larger band at 3Kb indicating that there was an insertion of some type that took place within this region. This difference of 0.6Kb does not fully account for the increased size of P10Δ3 X-3.

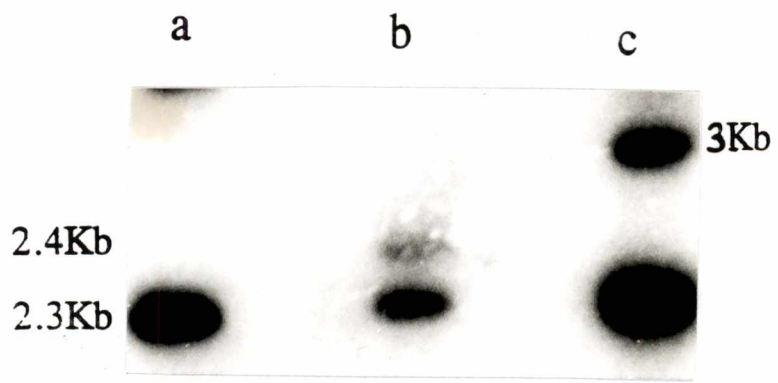

Figure 8. Genomic DNA digestion with XhoI/NdeI probed with Rosy 4.1. a) female control, b) P10Δ3 X-2, c) P10Δ3 X-3.

Clone Southern Blot Analysis

Plasmid and phage DNA Southern blot analysis was carried out to further localize the differences detected by genomic Southern blot analysis.

Purified EcoRI fragments containing the rDNA insert were ligated into cloning vectors to further characterize the rDNA. The P10Δ3 X-1 was isolated, dephosphorylated, and cloned into λZAPII. After the plaques were screened with a gel purified Rosy 4.1 insert probe, 16 positive plaques were found that may have contained the rDNA insertion. Due to the nature of the cloning vector, the rDNA was inserted into a pBluescript plasmids which was evicted from the bacteria and digested with EcoRI to see which of the plasmids had the correct size fragment. One plasmid was found that contained the correct size fragment and a large preparation of this plasmid was made for the Southern blot analysis. P10Δ3 X-2 and X-3 were cloned into EMBL4. The P10Δ3 X-2 insert was not dephosphorylated because the size of the insert was smaller than the vector would take for proper cloning, so random EcoRI fragments that were cut out of the gel along with the rDNA insert were present when the ligation took place. After screening phage plaques with Rosy 4.1 probe, 8 positive plaques were found that may have contained the rDNA insert. Phage DNA was extraced from the plaques and digested with EcoRI to see which of the plaques contained the correct size fragment. Two phage

DNAs were found to contain the correct size fragment and one was used for the Southern blot analysis. The P10Δ3 X-3 insert cut out of the gel was dephosphorylated and ligated into the cloning vector. After screening with Rosy 4.1 probe, 5 positive plaques were found that may have contained the rDNA insert. Phage DNA was extracted from the plaques and digested with EcoRI to see which of the plaques contained the correct size fragment. None of the phage DNAs contained the rDNA fragment. As seen in Figure 6, the rDNA insert size runs at the same or nearly the same size as the native *rosy* locus. The band cut out of the gel likely contained not only the insert but also the native *rosy* locus in equal proportions, so each positive plaque had a 50% chance of containing the rDNA insert. The probe, Rosy 4.1, that was used to determine which plaque contained the EcoRI fragment in question hybridizes to the *rosy* locus. This problem was not discovered until Southern blot analysis was done using the 240bp repeat probe. Therefore, no P10Δ3 X-3 clone was available for the rDNA analysis in this study.

240bp Probe

Plasmid and phage DNAs were digested with NheI, NheI/HindIII, and HindIII and probed with a series of 240bp repeats in the C20 vector (Figure 9). The P10Δ3 transformation vector was used as a control for the experiments.

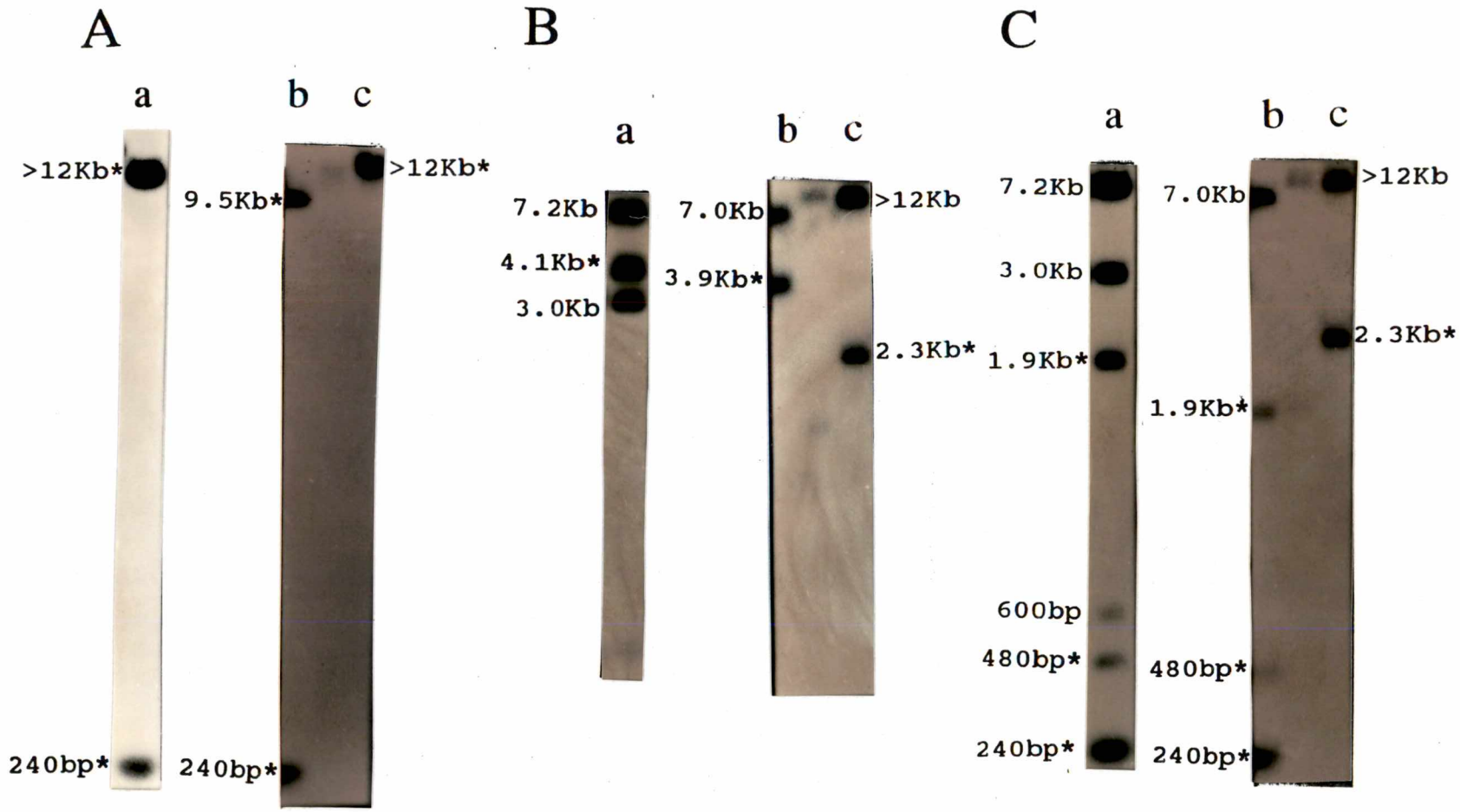

Figure 9. Clone digestion with A) NheI, B) HindIII, and C) NheI/HindIII probed with a series of 240bp repeats in C20. a) P10Δ3 control, b) P10Δ3 X-1 clone, and c) P10Δ3 X-2 clone. Asterisks indicate bands containing rDNA.

The NheI digest was used to determine if there were any 240bp repeats present in the rDNA insert clone of each of the clones. The P10Δ3 X-1 clone showed a strong band at 240bp indicative of a series of 240bp repeats, whereas, P10Δ3 X-2 showed no hybridization at 240bp, meaning that there were no 240bp repeats in this clone (Figure 9A). The bands at 9.5Kb and >12Kb in all the lanes represent the rest of the rDNA, *rosy* gene and vector sequences.

The HindIII digest was used to check the size of the rDNA insert itself (Figure 9B). The expected size band of the rDNA insert was 4.1Kb (Figure 5). P10Δ3 X-1 shows a slightly smaller band (3.9Kb), approximately 200-300bp, than the expected size band. This was expected to be smaller, because when looking at the comparison of the EcoRI digestion P10Δ3 transformation vector and P10Δ3 X-1 (Figure 7), it was shown that P10Δ3 X-1 was slightly smaller than the vector possibly due to the instability of the 240bp region during transformation. The other bands that appear in the P10Δ3 lane are a 7.2Kb band, which is from the *rosy* gene, and a 3.0Kb band, which contains vector sequences. On the autoradiogram, there is a band at 600bp in the P10Δ3 lane which is not seen in this photo. The 7.0Kb band that appears in the P10Δ3 X-1 lane contains the *rosy* gene and vector sequences. P10Δ3 X-2 shows an expected 2.3Kb band, containing the "1900" region and the promoter region along with the upstream sequences, that would be found due to the lack of 240bp repeats. There is

also a higher molecular weight band (>12Kb) that contains the *rosy* gene and the vector sequences.

The NheI/HindIII digest was used to determine if the "1900" region was present in each of the clones (Figure 9C). There are two bands (7.2Kb and 3.0Kb) in the P10Δ3 lane which are the same bands seen in the HindIII digest (Figure 9B). The 600bp band, which is P-Left (Figure 5), that appears in this photo is the same one that should appear in the HindIII digest (Figure 9B). Both the P10Δ3 and P10Δ3 X-1 lanes show a band at 1.9Kb indicating that the "1900" region is intact. The 240bp band is seen as it was in the NheI digest (Figure 9A). There is also another expected band at 480bp from the NheI site at the edge of the upsteam sequences next to a 240bp repeat to the HindIII site at the 5′ end of the promoter (Figure 5). P10Δ3 X-2 shows the same band pattern that was seen in the HindIII digest (Figure 9B), which further establishes that this clone lacks the region of 240bp repeats.

Plasmid and phage DNAs were digested with ScaI/BstBI and ScaI/HindIII and probed with a series of 240bp repeats in C20 vector (Figure 10). Both of the digests were used to determine if there was any change within the promoter region of the rDNA (Figure 5).

The ScaI/BstBI digest shows the expected band of 1.1Kb from the BstBI site at the edge of the promoter to the first ScaI site within the 240bp repeat region of the IGS near the promoter in the P10Δ3 vector and P10Δ3 X-1 (Figure 10A). In

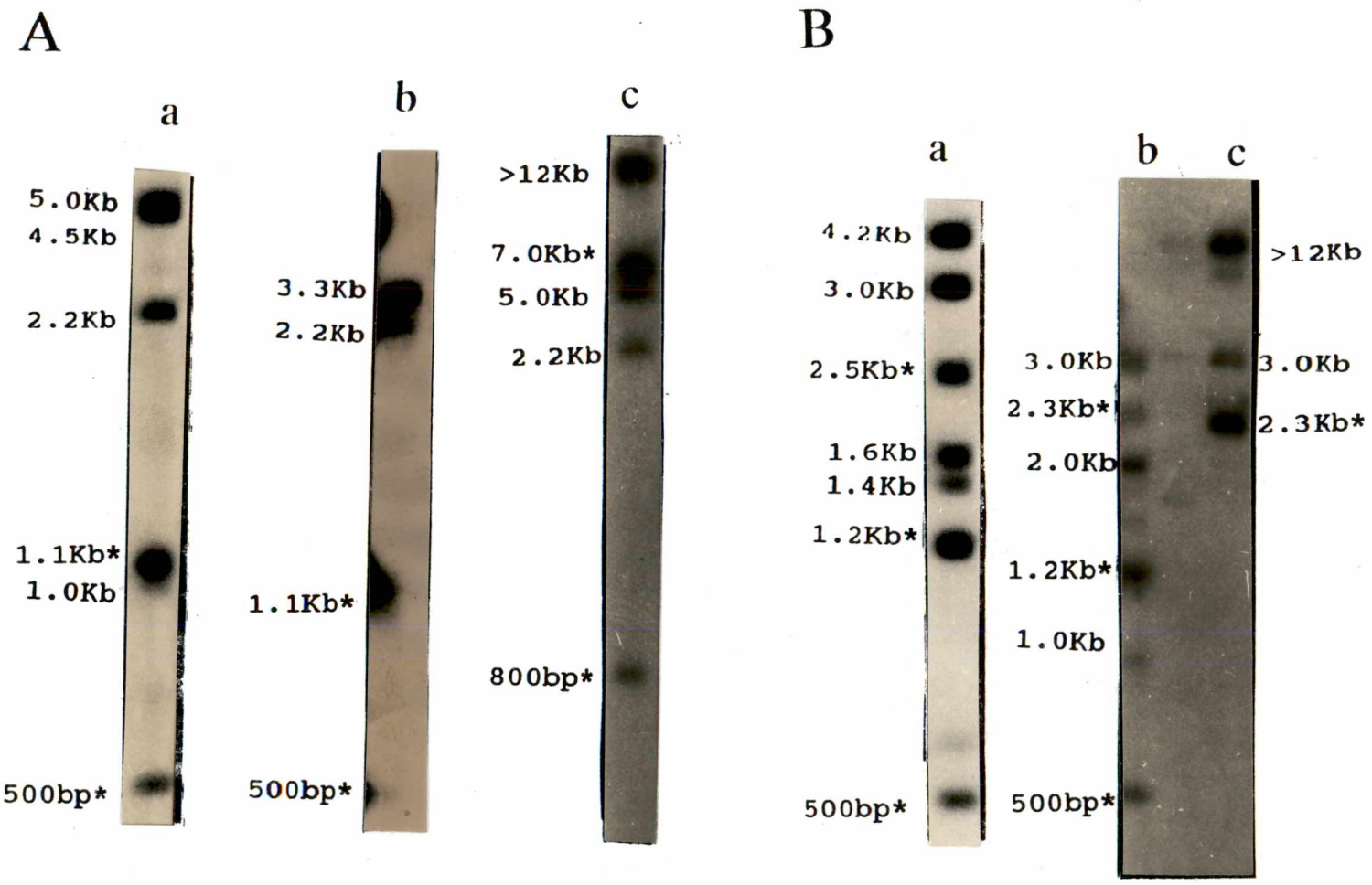

Figure 10. Clone digestion with A) ScaI/BstBI and B) ScaI/HindIII probed with a series of 240bp repeats in C20. a) P10Δ3 control, b) P10Δ3 X-1 clone, and c) P10Δ3 X-2 clone. Asterisks indicate bands containing rDNA.

the P10Δ3 lane, there is another band at 1.0Kb, which appears as a doublet in the photo, that is from the BstBI and ScaI sites that are found in the vector. An 800bp band from the BstBI site at the edge of the promoter to the BstBI site in the *rosy* gene does appear in the P10Δ3 X-2 lane to show that the promoter region is intact. This band does appear on the autoradiogram in the P10Δ3 and P10Δ3 X-1 lanes, but cannot be clearly shown in the photos without overexposing the picture and blurring the other bands. The expected 500bp band from the ScaI fragment within the 240bp repeat region of the rDNA appears in the P10Δ3 and P10Δ3 X-1 lanes, but not in the P10Δ3 X-2 lane due to the lack of the 240bp repeat region. In the P10Δ3 lane, there is a 5.0Kb band, which contains the "1900" region, the rest of the 240bp repeats on the left side of the ScaI fragment, and vector sequences and a 4.5Kb band, which contains sequences on the right side of the ScaI site in the *rosy* gene. There is also a 2.2Kb band from the BstBI site to the ScaI site in the *rosy* gene that appears in all the lanes. In the P10Δ3 X-1 lane, a band appears at 3.3Kb which contains the other side of the rDNA to the left of the ScaI site to the ScaI site in the vector. A band at 7.0Kb from the BstBI site at the edge of the promoter to a BstBI site within the left arm of the vector (location unknown) in the P10Δ3 X-2 lane contains the rDNA and vector sequences. Another band at 5.0Kb is from the right side of the ScaI site in the *rosy* gene to a ScaI or BstBI site within the random EcoRI fragments. The

higher molecular weight band (>12Kb) seen is uncut phage DNA.

The ScaI/HindIII digest (Figure 10B) also shows that the promoter region at 1.2Kb in P10Δ3 X-1 is unchanged from the original P10Δ3 transformation vector. The expected 500bp ScaI fragment, which is within the 240bp reion in the IGS, is present in the P10Δ3 and P10Δ3 X-1 lanes. The band containing the 240bp repeat region to the left of the ScaI fragment and the "1900" region of the rDNA is present in both of the P10Δ3 (2.5Kb) and P10Δ3 X-1 (2.3Kb) lanes. The 600bp band, which is P-Left (Figure 5), that appears in this photo is the same one that should appear in the HindIII digest (Figure 9B). In the P10Δ3 lane there are bands that appear at 1.4Kb and 1.6Kb which are from the vector that has a ScaI in the middle and 3.0Kb and 4.2Kb which are from the *rosy* gene. In the P10Δ3 X-1 lane, there are bands that appear at 1.0Kb which is from the edge of the rDNA to the ScaI site in the vector, 2.0Kb which is from the ScaI site in the *rosy* gene to the ScaI site in the vector, and a 3.0Kb band which is from the *rosy* gene. Neither the 500bp ScaI fragment or the 1.2Kb fragment is present in P10Δ3 X-2, which confirms that there are no 240bp repeats within this stock. However, there is a 2.3Kb band which is the same size as the band found in the HindIII digest (Figure 9B) that contains the "1900" region, the sequences upstream of the promoter, and the promoter itself. The same 3.0Kb band that appears in all the other lanes from the *rosy* gene also is present in the P10Δ3 X-2 lane. The high

molecular weight band (>12Kb) contains uncut phage DNA.

Restriction Mapping of the rDNA Fragments

Using the data obtained from the genomic and clone Southern blot analysis along with the restriction sites already known in the P10Δ3 transformation vector, detailed maps of the P10Δ3 X-1 and X-2 rDNA inserts in their cloning vectors were made (Figure 11).

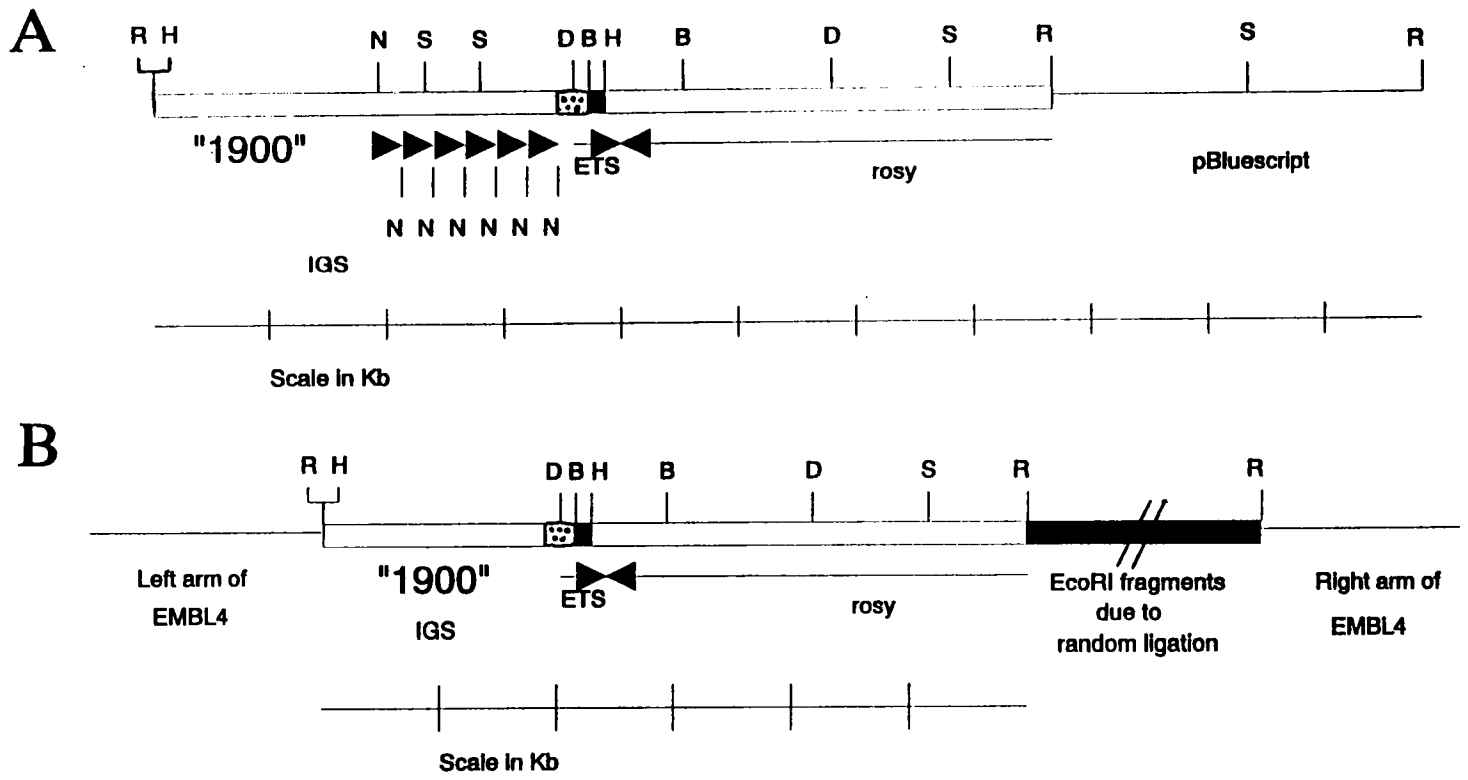

Figure 11. Detailed restriction maps of the A) P10Δ3 X-1 and B) P10Δ3 X-2 clones.

"1900"=1900 region -▸=rDNA transcriptional unit ▸=240bp repeat
◂-=rosy transcriptional unit -=vector sequences

B=BstBI D=NdeI H=HindII N=NheI R=EcoRI S=ScaI

Chapter IV

DISCUSSION

This study was designed to test two hypotheses to explain differences among the three X-linked insertions of P10Δ3 in ability to stimulate X-Y meiotic pairing. It was shown, in a previous study, that P10Δ3 X-2 had the weakest pairing ability, P10Δ3 X-1 had moderate pairing, and P10Δ3 X-3 had the strongest pairing ability and was stronger than a single, complete rDNA repeat (Merrill et al., 1992). One hypothesis is that the position of the rDNA inserts on the X chromosome affected their ability to stimulate the pairing of the X and Y chromosomes. The second hypothesis is that there are differences in the structures of the three rDNA inserts that affected their ability to stimulate X-Y pairing.

The position effect hypothesis was tested by *in situ* hybridization to polytene chromosomes from P10Δ3 transformation stocks to locate the position of the rDNA insertions on the X chromosome. It was found that one of the stocks had an insertion at 14D (P10Δ3 X-1) and the other two had insertion sites at 1A (P10Δ3 X-2 and X-3). These data show that the hypothesis of position effect was incorrect, because the two inserts, P10Δ3 X-2 and X-3, found at 1A have radically different pairing abilities (Merrill et al., 1992).

This investigation supports the second hypothesis that there was a change in the molecular structure of the rDNA

inserts. Genomic Southern blot analysis revealed that each of the transformation stocks had a different size EcoRI fragment that contains the rDNA insert and part of the rosy gene. P10Δ3 X-2 was the smallest (6.3Kb), P10Δ3 X-1 was close to the same size as the original transformation vector (8.0Kb), and P10Δ3 X-3 was the largest (11.5Kb). These data indicate that there was a deletion in P10Δ3 X-2 and an insertion in P10Δ3 X-3. Other data presented in this study show that the deletion in P10Δ3 X-2 occurred between the 1900 region in the IGS and the promoter region, deleting all of the 240bp repeats. The specific region that was affected in P10Δ3 X-3 is still unclear. Southern blot analysis suggests that there may have been an insertion event within the promoter region, but the location and extent is not determined.

There are three hypotheses about the cause of the changes in the rDNA inserts in P10Δ3 X-2 and X-3. One is that there was a sister chromatid crossover event, coupled with a misalignment of the 240bp repeats on the X chromosome in the germ line of the G_0 parent of these two stocks causing one of the chromatids to gain extra 240bp repeats and the other to lose them (Figure 12). When the chromatids segregated, two different X chromosomes were present (Hipeau-Jacquotte et al., 1989; Coen and Dover, 1983). This first hypothesis may be correct, but the question remains that if the crossover event took place, why is there a change in the promoter region in P10Δ3 X-3? The second hypothesis is that there may have been

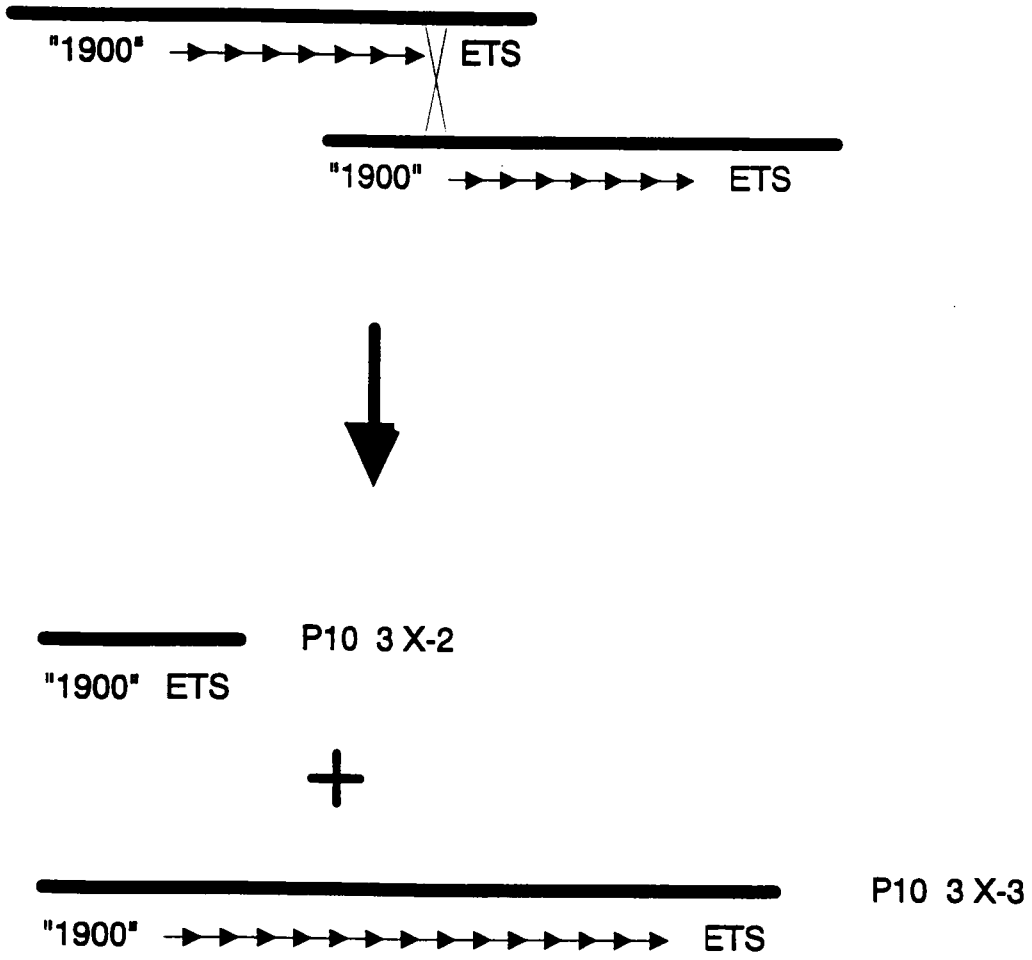

Figure 12. Proposed sister chromatid crossover event.

"1900"=1900 region -▸=240bp repeat ETS=external transcribed spacer Thick black lines=chromatids

a deletion of DNA within the rDNA region of P10Δ3 X-2 and an insertion of foreign DNA from another place in the genome into the rDNA of P10Δ3 X-3 that is causing the difference. One observation made by Engels (1989) to support this hypothesis showed that excision breakpoints occur more often between copies of short repeats, such as 240bp repeats, deleting the repeated region. This type of deletion could have occurred within the rDNA of P10Δ3 X-2 causing the change in the molecular structure of the rDNA. Another observation Engels (1989) made to support this hypothesis was that chromosome breakage brought about by P element mobilization can lead to the random rejoining of DNA fragments causing a rearrangement of the DNA. A rearrangement of the rDNA of P10Δ3 X-3 may have occurred in which one breakpoint was within the promoter of the rDNA and a DNA fragment from another place in the genome randomly inserted at the breakpoint in the promoter when the fragments rejoined. The third hypothesis is that there may have been a crossover event between sister chromatids in the germ line of the G_0 parent of P10Δ3 X-2 and X-3 causing a change in the 240bp repeats and that after the chromatids segregated, a random insertion of a foreign DNA fragment at a breakpoint within the promoter of P10Δ3 X-3 caused the difference in the molecular strucure of the rDNA in these two P10Δ3 transformation stocks.

Although it is uncertain which of these hypotheses explains the origin of the difference shown between P10Δ3 X-2

and X-3, it is clear from this study that changes within the IGS/promoter region, particularly in the copy number of the 240bp repeats, can lead to changes in the ability to stimulate X-Y pairing. This conclusion is consistent with the results of other studies involving pairing ability of rDNA fragments.

Results from testing several X-linked insertion lines showed that a single, complete rDNA was not required for stimulating pairing of the X and Y chromosomes and that insertions of the IGS/promoter region and IGS/promoter/ETS region alone proved capable of stimulating pairing (Merrill et al., 1992). Similar results were found in another study in which deletions within a complete rDNA repeat were generated *in vivo* by exposing an inserted rDNA repeat to a transposase source that would cause breakpoints at various sites in the rDNA (McKee et al., 1992). Progeny were recovered and tested for pairing ability both cytologically and genetically. Deletions that encompassed the rDNA transcriptional unit were found not to have an affect on the pairing ability of the X and Y chromosomes as long as some of the IGS region was present. Among the deletions that only had a difference in the size of the IGS region, pairing ability was proportional to the number of 240bp repeats. The IGS region was speculated to be the location where the X and Y chromosomes paired. This hypothesis was tested in another study in which only the transcriptional unit of the rDNA was inserted onto the heterochromatically deficient X chromosome (Ren, 1993). It

was found that the transcriptional unit was not responsible for the pairing of the sex chromosomes.

This study has shown that the number of 240bp IGS repeats is correlated with the pairing ability of the sex chromosomes. Further analysis of the P10Δ3 X-3 rDNA insert will determine if there is an increase in the number of 240bp repeats as compared to the results shown in this study of P10Δ3 X-1 and X-2 that is responsible for the strong pairing ability. Further investigations to narrow down the sites of pairing in the X and Y chromosomes will help to determine what mechanism is involved in pairing.

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VITA

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