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Genetics

A *Bam*HI repeat element is predominantly associated with the degenerating neo-Y chromosome of *Drosophila miranda* but absent in the *Drosophila melanogaster* genome

(repetitive DNA/*in situ* hybridization/chromosome degeneration/Y chromosome evolution)

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ABSTRACT **In *Drosophila miranda*, females have two X1 and two evolving X2 chromosomes, and males have one of each of these two X chromosomes and a Y chromosome. In males, the homologue of the X2 chromosome, the neo-Y chromosome, is attached to the Y chromosome and is under the process of degenerative evolution. We have examined a developmentally regulated X2/neo-Y chromosome-linked gene, *549mr*, of *D. miranda* and found that the neo-Y chromosome-linked copy of this gene (*549mr-NY*) contains an insertional DNA. We discovered that sequences similar to those in the insertional DNA are present in multiple copies in the genome of both sexes of *D. miranda* but are more abundant in the males. The insertional DNA also identified a 1.1-kilobase *Bam*HI repeat that is present in at least 6-fold excess in the male genome as compared to the female. This *Bam*HI repeat and similar DNA sequences are predominantly concentrated on the evolving neo-Y chromosome, but very few are found on the homologous X2 and other chromosomes. The *Bam*HI repeat also hybridizes with 2.0- and 1.8-kb RNAs and many other RNA species, which together are also ≈6-fold greater in males. No sequences similar to the *Bam*HI repeat are found in *Drosophila melanogaster*. Moreover, the *Bam*HI repeat is not homologous to P, *copia*, or other *D. melanogaster* transposable elements. This repeat, named the *NY* element, may be involved in gene disruption and the process of degenerative evolution of the neo-Y chromosome.**

Except for the rRNA genes and the *Stellate* locus, the Y chromosome of *Drosophila* does not carry the alleles of any other X chromosome-linked genes. The loss of alleles of these genes on the proto-Y chromosome is thought to be the result of degenerative evolution (1–3). Consequently, the males have one and females have two doses of X chromosome-linked genes because they have one and two doses of the X chromosome, respectively. To equalize the X chromosome-linked gene products between males and females, a gene regulatory mechanism called dosage compensation has evolved (4). In *Drosophila*, dosage compensation is manifested by transcriptional hyperactivation of the X chromosome in males (5, 6).

To explain the evolution of the Y chromosome, it has been hypothesized that the functional degeneration of this chromosome is a result of the loss of recombination with its homologue and progressive accumulation of mutations (1–3). Clearly identification of a system in which the degenerative process is in progress will enhance our understanding of the mechanism of Y chromosome evolution. *Drosophila miranda* has a karyotype that makes it an ideal material for this purpose. In this species, the males have one and the females have two X1 chromosomes (7, 8). However, during the course of evolution, the ancestral chromosome element C in this species has fused with the Y chromosome and become restricted to the males as a neo-Y chromosome (7, 8). The free homologue of the neo-Y on the other hand has become a second X chromosome (X2), which exists in one copy in males but in two copies in females (8). In polytene nuclei the neo-Y looks highly coiled and diffusely stained, while the X2 chromosome shows the typical banding pattern of polytene chromosomes (8). Transcription autoradiographic studies have demonstrated that different areas of the X2 chromosome show full to partial dosage compensation (9, 10), implying that the corresponding regions on the neo-Y chromosome are either totally inactive or have reduced activity.

Although it has been proposed that invasion of repetitive DNA is the cause of degenerative evolution of the neo-Y chromosome (8), there has not been any molecular evidence for invasion of the neo-Y chromosome or a neo-Y chromosome-linked gene with a discrete repeat element. To understand the molecular basis of neo-Y degeneration, we have used the developmentally regulated neo-Y/X2 chromosome-linked gene *549mr* of *D. miranda*. We show that the neo-Y chromosome-linked copy of this gene has an insertional DNA that has strong sequence similarity with a 1.1-kilobase (kb) *Bam*HI repeat. We demonstrate that this repeat, called the *NY* element, is present in multiple copies in the genome and is predominantly associated with the neo-Y chromosome of *D. miranda*. The number of *NY* elements is ≈6-fold greater in male than in female *D. miranda*. The *NY* element also hybridizes with many RNAs that are more abundant in males than in females. Sequences similar to the *NY* element are not detectable in male and female *Drosophila melanogaster* and in the *P* transposable element.

MATERIALS AND METHODS

Stocks. Wild-type *Drosophila melanogaster* strain Oregon-R and *D. miranda* strain S204 were used. These flies were raised at 23°C and 18°C, respectively, on standard cornmeal/agar/molasses medium under a 12-hr dark/light cycle.

Library Construction, Cloning, and Blot Hybridizations. Construction of the genomic library of mixed male and female *D. miranda* has been described (11). The same procedure was used to construct a genomic library of male *D. miranda* at the *Bam*HI site of λEMBL3 phage. The *D. melanogaster* head cDNA library was a gift from Seymour Benzer and Elliot Meyerowitz (Caltech). Screening of the libraries was done as described (11). Subcloning of DNA fragments was done in the pBluescript plasmid (Stratagene). Genomic DNA and RNA were prepared as described (11). DNA and RNA blots were prepared on Hybond nylon membranes (Amersham) according to standard procedures. All Southern blots were hybridized at 68°C in 6× standard saline citrate (SSC)/5× Den-

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hardt's solution/sheared salmon sperm DNA (0.1 mg/ml) and washed at 58°C in 0.1× SSC/0.1% SDS. The hybridization solution for Northern blots was the same as that for the Southern blots except that the solution contained 50% formamide and hybridization was done at 42°C. Subsequently the blots were washed at 58°C as described (11). Radiolabeled DNA probes were prepared by the random hexamer-priming method (12) with ^{32}P-labeled dCTP or dATP. The specific activity of the probes ranged between 1×10^9 and 2×10^9 cpm/μg.

***In Situ* Hybridization to Polytene Chromosomes.** Polytene chromosomes from larval salivary glands were prepared, processed, and hybridized with ^{3}H-labeled probes as described (11). DNA fragments were labeled with [^{3}H]TTP by random priming to a specific activity of 2–3 $\times 10^7$ cpm/μg. For each slide $\approx 1.5 \times 10^5$ cpm was used.

Measurement of Chromosome Length. To measure the distance of the site of *in situ* hybridization of the *549mr* probe from the tip of the X2 and neo-Y chromosomes, photographs of the chromosome were first digitized. Measurements on the digitized images were done by using a set of image-processing programs (IMAGE) developed by Wayne Rasband (National Institutes of Health). The distance was expressed in arbitrary units.

RESULTS

Gene *549* Is Complex and Developmentally Regulated. Gene *549* is located on chromosome 2R of *D. melanogaster* (13). Since the 2R chromosome of *D. melanogaster* and the X2 and neo-Y chromosomes of *D. miranda* are thought to have originated from the ancestral chromosome element C (7), it is expected that, in *D. miranda*, gene *549* will be located on the X2 and neo-Y chromosomes. For this reason, we chose to use gene *549* to examine the neo-Y chromosome. It has been shown that clone *549* of *D. melanogaster* hybridized with 2.9-, 1.9-, and 1.0-kb RNA species (13, 14). For further characterization, a cDNA clone of this gene was isolated from a head cDNA library and hybridized with RNA isolated from adult heads and bodies of *D. melanogaster*. The results (Fig. 1) showed that the cDNA probe hybridized with all three RNA species. The 2.9-kb RNA is head-specific because it is found only in the head. The cDNA probe also hybridized with three RNAs of *D. miranda* (Fig. 1, lane c). These results indicate that gene *549* is active in the adult flies of both species and that it probably transcribes three RNA species. Sequence analysis of a cDNA clone of gene *549* of *D. melanogaster* (K.D.S. and R.G., unpublished observations) revealed that gene *549* is similar to a family of genes coding for protein kinase-dependent activator proteins of tyrosine and tryptophan hydroxylases, key enzymes in the regulation

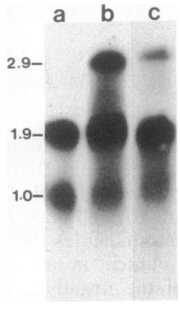

FIG. 1. *549* RNA in adult *D. melanogaster* and *D. miranda*. Northern blots containing poly(A)$^+$ RNA of body (lane a) and head (lane b) of *D. melanogaster* and head of *D. miranda* (lane c) were hybridized with a cloned cDNA of *549ml* gene of *D. melanogaster*. Sizes of bands (in kb) are indicated at left.

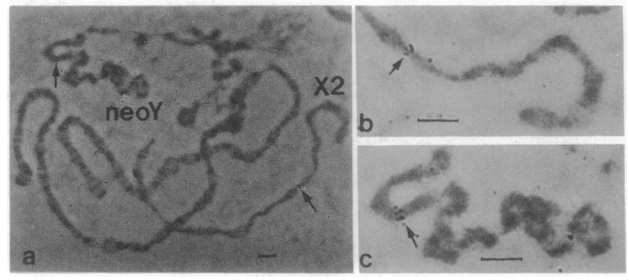

FIG. 2. (*a*) *In situ* hybridization of ^{3}H-labeled *549mr-2* DNA with the polytene chromosomes of a male *D. miranda* nucleus. (*b* and *c*) Photographs of X2 and neo-Y chromosomes from *a* under higher magnification. Arrows indicate the sites of hybridization. (Bars = 10 μm.)

of catecholamine and serotonin levels in neurons (15). These results will be published elsewhere.

The neo-Y Chromosome-Linked Copy of Gene *549mr* Is Different from the X2 Chromosome-Linked Allele. Using a 3.3-kb *Hin*dIII fragment containing the transcribed region of gene *549ml* of *D. melanogaster* as a probe, we isolated the homologous 549mr-2 clone of *D. miranda*. This clone hybridized with only X2 chromosomes at the 25CD region as well as with the neo-Y chromosomes of *D. miranda* (Fig. 2 *a–c*). Curiously, hybridizations on the X2 and neo-Y chromosomes were not at corresponding positions (Fig. 2 *b* and *c*). On the X2 chromosome, the site of hybridization was 23.41 units, whereas in the case of neo-Y it was 30.62 units, from the tip of the chromosome. Thus, neo-Y and/or X2 chromosomes may have undergone chromosomal rearrangement. This prompted us to investigate whether the X2 and neo-Y chromosome-linked copies of gene *549mr* had acquired any other polymorphisms at the molecular level.

To test this, Southern blots containing *Eco*RI-digested genomic DNA of male and female *D. miranda* were probed with a mixture of ^{32}P-labeled fragment 1 (1.6×10^7 cpm) and fragment 2 (0.3×10^7 cpm) of clone *549mr-2* of *D. miranda* (Fig. 3, map a). These two fragments are located within a 2.9-kb *Sal* I fragment (Fig. 3, map a, between the arrows) that hybridized with all three RNA species of *D. miranda* (data not shown). Strong hybridization with a 3.0-kb *Eco*RI DNA fragment from female *D. miranda* was observed (Fig. 4, lane b). It is evident that fragment 1 has hybridized with the 3.0-kb fragment because fragment 1 is located within a 3.0-kb *Eco*RI genomic DNA (Fig. 3, map a). A 1.5-kb *Eco*RI fragment from female *D. miranda* also hybridized (Fig. 4, lane b), but the signal intensity was much lower than that seen with the 3.0-kb fragment. Presumably, the 1.5-kb fragment hybridized with fragment 2, which had a lower level of radioactivity. This implied that the 1.5-kb fragment is partially present in clone

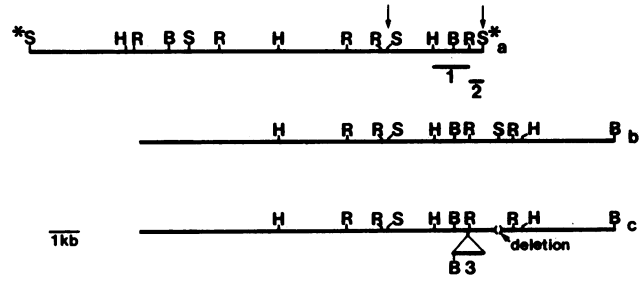

FIG. 3. Restriction maps of 549mr-2 (map a), 549mr-X2 (map b), and 549mr-NY (map c) clones. The boundaries of 549mr-X2 and 549mr-NY clones have not been determined. The *Sal* I fragment between arrows hybridizes with three RNA species. The *Sal* I sites with asterisks are the restriction enzyme sites in the polylinker of the λEMBL3 cloning vector. R, *Eco*RI; B, *Bam*HI; S, *Sal* I; H, *Hin*dIII.

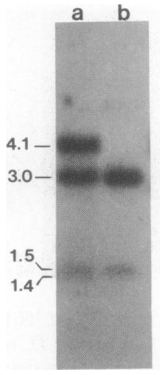

FIG. 4. Blot hybridization of *Eco*RI-digested genomic DNA of male (lane a) and female (lane b) *D. miranda*. A mixture of ^{32}P-labeled fragment 1 (1.6×10^7 cpm) and fragment 2 (0.3×10^7 cpm) (Fig. 3, map a) was used as probes.

549mr-2 (Fig. 3, map a). Analysis of more genomic clones confirmed this to be the case (see below). Since the females have two X2 chromosomes but no neo-Y, we conclude that both the 3.0- and 1.5-kb *Eco*RI fragments were X2 in origin.

Hybridization with genomic DNA from males was found to be different (Fig. 4, lane a). Besides the X2 chromosome-specific 3.0- and 1.5-kb fragments, 4.1- and 1.4-kb *Eco*RI DNA fragments from males also showed hybridization (Fig. 4, lane a). The X2 chromosome-specific fragments were expected to be present in the males because the males have one X2 chromosome. Therefore, the male-limited 4.1- and 1.4-kb fragments were of neo-Y origin because the neo-Y chromosome is male-limited. A stronger hybridization signal on the 4.1-kb DNA suggested that this DNA had hybridized with fragment 1, which had a higher level of radioactivity than fragment 2 (see above). On the basis of the same logic, we presumed that the 1.4-kb DNA, which had a faint hybridization signal, must have hybridized with fragment 2. This implies that 4.1-kb and 1.4-kb male-specific DNA correspond to the 3.0- and 1.5-kb X2 chromosome-specific DNA, respectively. If all these interpretations are correct, two types of genomic clones should be present in the male genomic library, one carrying the X2 chromosome-specific and the other carrying the male—i.e., neo-Y chromosome-specific—*Eco*RI fragments.

Thus, a male genomic library, constructed in EMBL3 λ phage, was screened with fragment 1 of the 549mr-2 clone (Fig. 3, map a), and 11 clones were isolated. Restriction mapping and Southern blot hybridization revealed that two clones were incomplete and only had a portion of fragment 1 shown in Fig. 3, map a. Six clones (549mr-X2) had both the X2 chromosome-specific 3.0- and 1.5-kb *Eco*RI fragments (Fig. 3, map b) which hybridized with fragments 1 and 2, respectively. On the other hand, the remaining three clones (549mr-NY) had the male-specific 4.1- and 1.4-kb *Eco*RI fragments (Fig. 3, map c), and these fragments hybridized with fragments 1 and 2, respectively. That these and other restriction fragments of two clones are colinearly arranged (Fig. 3) and that the corresponding fragments of two clones have sequence similarity were determined by hybridizing DNA fragments of one clone to those of the other (data not shown).

It is clear from the restriction maps that the 549mr-X2 and 549mr-NY clones are identical except that in 549mr-NY clones a 1.1-kb *Bam*HI–*Eco*RI DNA insertion (Fig. 3, map c, fragment 3) and a 0.1-kb deletion (which removed a *Sal* I site) are present within two *Eco*RI fragments corresponding to the 3.0- and 1.5-kb *Eco*RI fragments of the 549mr-X2 clone, respectively (Fig. 3, maps b and c). As a result, the sizes of these two *Eco*RI fragments on the neo-Y are 4.1 and 1.4 kb, respectively. Thus, the X2 and neo-Y chromosome-linked 549mr clones show restriction pattern polymorphism. Although the 3.0-kb *Eco*RI fragment of 549mr-X2 hybridizes with three RNA species, we do not know whether the insertion or deletion has disrupted the coding region of the *549mr* gene.

Sequences Similar to the Insertional DNA Element Are Predominantly Present in Multiple Copies in the Genome of Male *D. miranda*. To determine whether the 1.1-kb insertional DNA found in clone 549mr-NY (Fig. 3, map c) is present in single or multiple copies in the genome, Southern blots containing *Bam*HI-digested genomic DNA of *D. miranda* as well as of *D. melanogaster* were probed with ^{32}P-labeled 1.1-kb *Bam*HI–*Eco*RI insertional DNA of the 549mr-NY clone (Fig. 3, map c, fragment 3). Several *Bam*HI fragments of DNA from male and female *D. miranda* hybridized with the probe (Fig. 5 *Lower*, lanes a, b, e), but the highest level of hybridization was observed with a 1.1-kb *Bam*HI fragment of DNA from both male and female *D. miranda*. However, the intensity of hybridization of the 1.1-kb *Bam*HI fragment per unit amount of DNA loaded (as determined by densitometric scanning of Fig. 5) was found to be ≈6-fold greater in the DNA from males. This suggests that the copy number of the 1.1-kb *Bam*HI fragment is at least 6-fold greater in the male. In fact the 1.1-kb *Bam*HI DNA was clearly visible in the male but not in the female DNA in the ethidium bromide-stained gel (arrow in Fig. 5 *Upper*), indicating its repeated nature in the genome. No hybridization of fragment 3 was observed with DNA from male or female *D. melanogaster* (Fig. 5 *Lower*, lanes c and d), although these DNA were present in 5- to 7-fold excess of the *D. miranda* DNA loaded in lane b (see Fig. 5 *Upper*). Since the insertional DNA (fragment 3 in Fig. 3, map c) in clone 549mr-NY does not have two *Bam*HI sites like the *Bam*HI repeat, we believe that

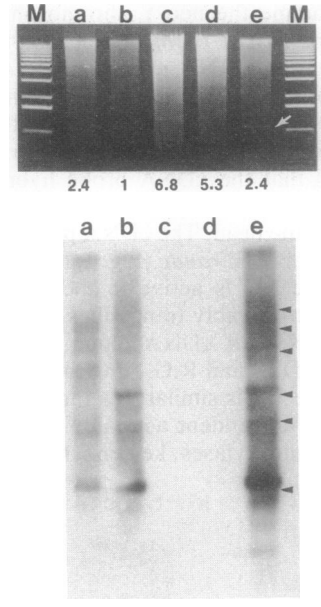

FIG. 5. (*Upper*) Photograph of an ethidium bromide-stained gel containing *Bam*HI-digested genomic DNA of female *D. miranda* (lane a), male *D. miranda* (lane b), female *D. melanogaster* (lane c), male *D. melanogaster* (lane d), and male *D. miranda* (lane e). The value given below each lane indicates the quantity of DNA in that lane relative to the DNA content in lane b as determined by laser densitometric scanning of the negative. The arrow indicates the 1.1-kb *Bam*HI repeat in male DNA. Lanes M, 1-kb DNA ladder (BRL) used as size markers. (*Lower*) Autoradiogram of the Southern blot of the gel shown in *Upper*. ^{32}P-labeled fragment 3 of the 549mr-NY clone (Fig. 3, map c) was used as a probe. Arrowheads indicate (from top to bottom) 5.0-, 4.0-, 3.0-, 2.0-, 1.6-, and 1.0-kb marker DNA.

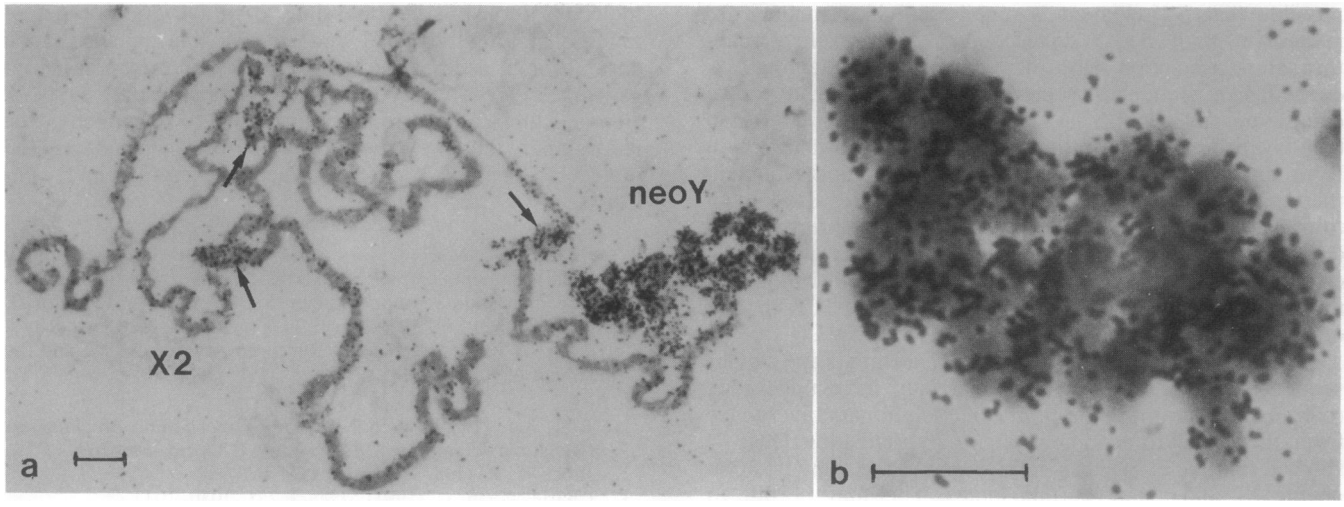

FIG. 6. (*a*) *In situ* hybridization of the polytene chromosomes of male *D. miranda* with ^{3}H-labeled *NY* element. Arrows indicate hybridization with the proximal heterochromatin. (*b*) neo-Y chromosome showing hybridization with the probe. The autoradiographic exposure was 5 days. (Bars = 10 μm.)

either part of the repeat is present in fragment 3 or one *Bam*HI site of the repeat has mutated.

The 1.1-kb *Bam*HI Repeat Is Predominantly Associated with the neo-Y Chromosome. The 1.1-kb *Bam*HI repeat, clearly visible in the male genomic DNA (arrow in Fig. 5), was isolated from an agarose gel and subcloned in pBluescript plasmid. Restriction mapping showed that the *Bam*HI repeat does not have sites for *Eco*RI, *Sal* I, *Xho* I, *Pst* I, *Xba* I, *Kpn* I, and *Hin*dIII. Subsequently, purified *Bam*HI repeat was hybridized with the polytene chromosomes of male *D. miranda*. Heavy hybridization was observed over the neo-Y chromosome (Fig. 6). Hybridization was also seen over the proximal heterochromatin, X2 chromosome, and other chromosomes, but it was much lighter than that seen on the neo-Y chromosome. In female nuclei, a low level of hybridization was seen over the chromocenter (data not shown). Thus, the 1.1-kb *Bam*HI repeat is predominantly associated with the neo-Y chromosome and, therefore, we named it the *NY* element. The same pattern of hybridization was seen when fragment 3 (Fig. 3, map c) was used as a probe.

The *NY* Element Hybridizes with RNA of Different Sizes. The 1.1-kb *NY* element DNA was also used as a probe for blots containing total RNA of *D. miranda* and *D. melanogaster*. In the case of *D. miranda*, the *NY* element hybridized with many RNAs, forming a smear. However, in the background of the smear, faint hybridization with 2.0- and 1.8-kb RNA was visible (Fig. 7 *Right*, lanes a and b). On the basis of a laser densitometric scan of the negative of the stained gel (Fig. 7 *Left*) and the autoradiogram (whole lane), it was estimated that the hybridization signal per unit amount of RNA was ≈6-fold higher in males, suggesting that the males have a 6-fold higher level of *NY* element sequence-containing RNA. No hybridization was detected with the total RNA of male and female *D. melanogaster*, even after a longer exposure (Fig. 7 *Right*, lanes c and d).

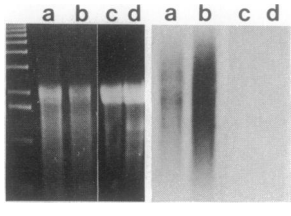

FIG. 7. (*Left*) Photograph of ethidium bromide-stained gels containing 2.0 μg of female *D. miranda* RNA (lane a), 1.0 μg of male *D. miranda* RNA (lane b), 4.0 μg of female *D. melanogaster* RNA (lane c), and 4.0 μg of male *D. melanogaster* RNA (lane d). The 1.0-kb ladder (BRL) was used as markers. The gels were blotted and hybridized with ^{32}P-labeled *NY* element. (*Right*) Autoradiograms of the gel blots in *Left*. Lanes a and b were exposed overnight, whereas lanes c and d were exposed for 72 hr.

DISCUSSION

On the basis of the results presented, several conclusions can be drawn. First, multiple copies of the *NY* element and similar sequences are found in the genome of *D. miranda*. Second, compared to the female genome the *NY* element is present in at least 6-fold greater abundance in the male genome. Third, *NY* element-like sequences are found in 2.0-kb, 1.8-kb, and many other RNA species, which are also ≈6-fold greater in male than in female *D. miranda*. Fourth, and most importantly, the *NY* element and similar sequences are predominantly associated with the neo-Y chromosome.

Although we cannot conclude whether the *NY* element and similar sequences are found only in *D. miranda* or *Drosophila obscura* species group, it is clear from the hybridization studies that *NY*-element-like sequences are not detectable in the genome of the Oregon-R strain of *D. melanogaster*. These results suggest that the *NY* element is different from *copia*, *412*, *249*, and other transposable elements that are found in the Oregon-R strain (16). We were also unable to find hybridization of the *NY* element with the cloned *P* element of *D. melanogaster*, even under low stringency washing conditions (data not shown). Therefore, the *NY* element is not any one of the above transposable elements; it is probably a previously unknown transposable element that is active in the genome of *D. miranda*. Conservation of this element as a *Bam*HI fragment in the genome makes this possibility stronger.

Results of Northern hybridization showed that sequences similar to the *NY* element are found in 2.0-kb, 1.8-kb, and other RNA species of male and female *D. miranda* but not in *D. melanogaster*. Although the 2.0- and 1.8-kb RNA are similar in size to *Drosophila* ribosomal RNA, it is unlikely that they are rRNA. If they were, hybridization of the *NY* element with *D. melanogaster* RNA would have also been observed unless only the rRNA of *D. miranda* contains *NY*-element-like sequences. If this is the case, it must be assumed that *D. miranda* males produce 6-fold greater amounts of rRNA, since the level of hybridization in males was 6-fold greater than in the females. Alternatively it is

possible that *NY* element is mobile and its remnants are present in many neo-Y linked genes. As a result of read-through transcription, RNA of various sizes containing *NY*-element-like sequences is produced. In addition, it is also equally possible that the *NY* element is actually larger than the 1.1-kb *Bam*HI repeat and transcribes a 2.0- and 1.8-kb RNA autonomously. Various transposable elements found in the *Drosophila* genome (e.g., *copia* and *412*) are also known to transcribe poly(A)$^+$ and poly(A)$^-$ RNA of different sizes (17, 18). In this respect, the *NY* element is like a transposable element. However, further investigation is needed to clarify these speculations as well as the origin of 2.0-kb, 1.8-kb, and other RNA hybridizing with the *NY* element.

In addition to accumulating many *NY* elements and similar sequences, the neo-Y chromosome may have undergone rearrangements during the course of evolutionary divergence from the X2 chromosome. *In situ* hybridization (Fig. 2) showed that locus *549mr* is not located at the same position on the X2 and neo-Y chromosomes. This may be due to inversion of the locus *549*-containing segment of the neo-Y relative to the X2 chromosome or to accumulation of *NY*-element-like sequences or other sequences between the locus *549mr* and the tip of the neo-Y chromosome. We have also found a small deletion in the *NY* copy of the *549mr* gene (Fig. 3, map c). Similarly, duplication has been detected in the neo-Y chromosome-linked copy of the *LCP* gene of *D. miranda* (19). The same study also detected a 3.1- and 2.3-kb insertional DNA in the *LCP* gene (18). However, it is not known whether, like the *NY* element, these insertional DNA sequences in the *LCP* gene are also repeated and predominantly associated with the neo-Y chromosome. Nevertheless, it is clear from the present and previous (18) studies that the neo-Y chromosome is a target for various aberrations, including accumulation of the *NY* element.

It is believed that a chromosome becomes susceptible to the accumulation of various types of mutations along with gradual loss of genetic activity when it becomes permanently heterozygous in one sex. The process of loss of genetic activity may be enhanced if the homologue of this chromosome concomitantly acquires a mechanism to compensate for the lost genetic activity of the permanently heterozygous homologue (i.e., dosage compensation). Scenarios like these have been discussed by various investigators in relation to the degenerative evolution of the Y chromosome (1–4). Sequences like *NY* elements that are heavily concentrated on the neo-Y chromosome may function as mutagens and disrupt function of many genes. By using the *NY* element as a molecular tool, it is now possible to study various types of mutations, repetitive DNA, and transposable elements that a chromosome, such as the neo-Y, may acquire when it presumably evolves under lower selection pressure. The *NY* element can also be used to isolate many neo-Y linked genes and compare them with their X2 alleles to gain insight into the molecular basis of Y chromosome degeneration in *D. miranda*.

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