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Genetics

Incomplete dosage compensation in an evolving *Drosophila* sex chromosome

(RNA synthesis/regulatory evolution/*Drosophila miranda*/autoradiography)

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ABSTRACT **Cellular autoradiography was used to measure relative rates of chromosomal RNA synthesis and to examine the regulatory phenomenon of X-linked dosage compensation in *Drosophila miranda*, a species containing two distinct, nonhomologous X chromosomes (X^1 and X^2). The X^1 chromosome was found to be dosage-compensated, since the rate of RNA synthesis along the single X^1 chromosome in males equaled that of both X^1 chromosomes in females. Unlike other sex chromosomes that have been studied, the more recently evolved X^2 heterochromosome exhibited regional differences in transcriptional activity when males and females were compared. The distal 10% of the X^2 was not dosage-compensated, whereas the majority of an interior segment, representing 30% of the X^2 chromosome's length, was found to be dosage-compensated. Our data are consistent with the idea that the evolution of X^2 dosage compensation has paralleled the differentiation of the X^2 sex chromosome. In addition, gene rearrangement seems to have accompanied the acquisition of a dosage-compensatory mechanism in the X^2.**

The phenomenon of dosage compensation in *Drosophila* has been examined in a number of species within the genus. In all cases it appears as if a regulatory system modulates the amount of X chromosome gene products formed in such a way that males with one X chromosome and females with two X chromosomes have equal amounts of product. An X-linked gene is therefore twice as active in males as in females (1). The mechanism occurs at the level of transcription (2) and is dependent upon the activity of one or more autosomal genes (3–6). X-linked genes need not be located on the X chromosome to be dosage-compensated, since (*a*) X chromosome genes translocated to autosomes still exhibit compensation (7–11) and (*b*) autosomal regions inserted into the X do not exhibit compensation (12, 13). Therefore, the regulatory elements responsible for dosage compensation are probably interspersed along the X chromosomes.

As an approach toward understanding the evolution of dosage-compensatory mechanisms, we have examined a *Drosophila* species that has recently undergone evolutionary differentiation of its sex chromosomes. *Drosophila miranda*, which exhibits a unique sex-chromosome constitution, is a morphologically similar, reproductively compatible sibling species of *D. pseudoobscura* (14). *D. miranda* has two chromosomes (X^1 and X^2), each of which exists in a single copy in males and in two copies in females (15). Both arms of the X^1 chromosome are homologous to the X chromosome arms of *D. pseudoobscura*, whereas the X^2 is largely homologous to the autosomal third chromosome of *D. pseudoobscura* (15). The X^2 evolved as a consequence of a Y-3 chromosomal fusion in the *D. miranda* lineage (16). Ancestral males are presumed to have had one copy of the new Y-3 fusion chromosome and one copy of the "free" third chromosome, whereas females had two copies of the latter. Eventually, most third chromosome genes on the new Y degenerated, and the "free" third chromosome gradually differentiated into a sex chromosome (the X^2). Because limited genetic homology still exists between the X^2 and Y (16), the evolution of the once autosomal X^2 into a sex chromosome appears to be incomplete.

Unless a system of dosage compensation had evolved during the differentiation of the X^2 chromosome, haploidy for large regions of the X^2 would drastically alter genic balance in the males (17). Selective pressures should therefore have favored the evolution of a dosage-compensatory mechanism for those X^2 regions originally autosomal and hence lacking homologous loci on the Y chromosome. By means of quantitative cellular autoradiography we have examined the relative rates of transcription along the male and female *D. miranda* polytene X^1 and X^2 chromosomes.

MATERIALS AND METHODS

Maintenance of *D. miranda*. The wild-type stock of *D. miranda* used for the present study was obtained from M. Steinemann of the Max-Planck-Institut für Biologie, Tübingen, W. Germany. All cultures contained ample quantities of yeast and were maintained at 18°. For chromosome preparations and autoradiography, salivary glands were collected from late third instar larvae, that had everted their spiracles.

Preparation of Salivary Gland Chromosomes. Larvae were dissected in 18° Ephrussi–Beadle saline solution at pH 7.2. Excised salivary glands were fixed in acetic acid/ethanol (1:3) for 3–5 min and squashed in 50% acetic acid. Chromosome spreading was monitored under the phase contrast microscope before the slides were placed on dry ice. The glands were postfixed in absolute ethanol and stained in 1% Giemsa solution. The chromosome arms were identified by means of the chromosome map of Dobzhansky and Tan (15). Photomicrographs of *D. miranda* salivary gland chromosomes were taken by F. Arcos-Teran.

Autoradiographic Procedure. Individual salivary glands excised as above were transferred to 20 μl of 18° Ephrussi–Beadle saline containing 50 μCi of [^{3}H]uridine per ml ([^{3}H]-uridine specific activity = 25–30 Ci/mmol). The glands were incubated in this solution for 5 or 10 min, then fixed and squashed. After the tissue was postfixed in ethanol, the slides were placed in a formaldehyde/ethanol (3:1) solution for 2 hr. The slides were exhaustively rinsed with distilled H_2O and covered with Kodak AR-10 autoradiographic film. After exposure for 10 days at 4°, the autoradiographs were developed with Kodak D-19B developing solution, rinsed, and Giemsa-

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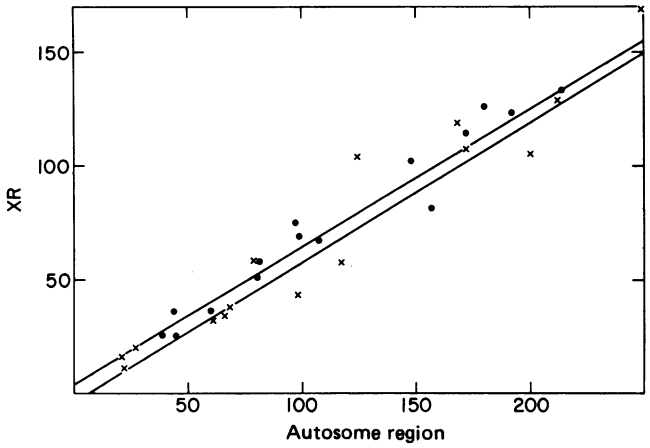

FIG. 1. Scatter diagram and best-fit regression line of chromosome arm XR (distal 20%) against 4th chromosome incorporation of tritium in *D. miranda* male and female salivary gland nuclei. The ordinate represents the number of silver grains located over the distal 20% of the XR chromosome; the abscissa is the number of grains over the distal 30% of the 4th chromosome. Each point represents XR and 4th chromosome values from a single nucleus. ●, Male nuclei; ×, female nuclei.

stained. Grain counting was done at a magnification of ×2000 under bright-field illumination. RNase-treated slides were prepared by the procedure of Sederoff *et al.* (18).

Analysis of Data. Since the number of silver grains over a chromosomal region varied greatly within and between salivary glands, it was necessary to establish a rate of RNA synthesis relative to a standard autosomal rate for each of the X^1 and X^2 regions under study (5). This was accomplished for each nucleus by measuring the number of grains over the X^1 (or X^2) segment in question relative to the number of grains over a section of the autosomal 4th chromosome. For any particular X chromosome region, a minimum of 15 nuclei from at least six individuals were measured for each sex.

Technical problems made it difficult for us to collect enough data to allow separate comparisons within and among glands, as Maroni and Plaut have recommended (5). Although our statistical treatment involves the analysis of the whole population of nuclei, the fact that we studied three distinct X-chromosome regions independently in relation to the same autosomal standard provides us with some internal control.

The data for male and female nuclei were compared by regression analysis of X compared to autosomal grain counts. The regression coefficient b is a measure of transcriptional activity within the sex chromosome segment relative to the activity of the autosomal section. The significance of differences in b between males and females was evaluated by a t-test. Our method of comparison depends upon the reliability with which sex chromosome transcriptional activity can be determined when expressed relative to the selected 4th chromosome segment. In all cases the correlation coefficient r was greater than 0.90, indicating that changes in the absolute number of silver grains involve the same relative change in the number of grains over the X^1 or X^2 segment and the 4th chromosome region used as a standard. Since the X^1 (or X^2) chromosome activity is expressed in amounts relative to the autosomal region, it is important to establish that incorporation over the autosomal segment is equal in males and females. At the 5% level of significance, t-tests indicate that, for all X^1 (or X^2) regions examined, there were no significant differences between males and females in 4th chromosome labeling.

Identification of Chromosome Regions. No detailed study yet exists of the banding pattern in salivary gland chromosomes of *D. miranda*. Dobzhansky and Tan made a comparison of gene arrangement in *D. pseudoobscura–D. miranda* hybrids. However, they made no attempt to detect all the bands existing in *D. miranda* (15). In the present investigation segments were selected for either ease of identification or homology to regions of the *D. pseudoobscura* genome. The number of silver grains was examined over four chromosomal sections of *D. miranda*. The autosomal region corresponds to the distal 30% of the 4th chromosome and represents a segment from within division 96 to the tip of the Dobzhansky–Tan map. A section of the right arm of the X chromosome from a point within division 38 to the tip was chosen; this is approximately equivalent to the distal 20% of XR. Two regions of X^2 were investigated, together amounting to about 40% of this chromosome. The first segment represents the distal 10% of the X^2 and is entirely homologous to division 80–81 of the *D. pseudoobscura* third chromosome. The other section of the X^2, from a point within division 71 to the end of division 80, is equivalent to nearly 30% of the chromosome. Most of this region has little banding pattern homology to *D. pseudoobscura*.

Table 1. Regression analysis of sex chromosome transcriptional rates relative to the autosomal segment

	$\overline{X}$	$\overline{\text{Autosome}}$	r	$b\pm$	t	P
Region: XR						
Males	74.8	114.4	0.97	0.62 ± 0.09	0.38	>0.70
Females	70.0	112.3	0.97	0.65 ± 0.12		
Region: X^2, tip						
Males	37.0	88.4	0.95	0.40 ± 0.06	7.51	<0.001
Females	73.1	91.9	0.97	0.78 ± 0.08		
Region: X^2, middle						
Males	89.9	112.3	0.96	0.70 ± 0.11	1.92	0.05
Females	75.3	92.2	0.97	0.85 ± 0.12		

$\overline{X}$ and $\overline{\text{Autosome}}$ represent the average number of silver grains found in each region, r is the correlation coefficient for the X-autosome comparison, b is the regression coefficient, and ± the 95% confidence interval. Correction of the grain counts from female nuclei for β self-absorption (5) alters the male to female regression coefficient ratio by only 13% and has no effect on the interpretation of the data.

RESULTS

Significant labeling of all chromosomes occurred when glands were incubated in [^{3}H]uridine. Silver grains represent [^{3}H]RNA, since chromosomes treated with RNase after the [^{3}H]uridine pulse showed no appreciable incorporation. We were assured that the number of silver grains over a chromosome region actually represents new RNA transcription because with increasing incubation proportionately more grains appeared over the chromosomes. Transport of RNA from the chromosomes was negligible under our conditions, for there was an insignificant number of grains over the cytoplasm. Since RNA synthesis was measured along an X-chromosome region relative to an autosomal segment from the same nucleus, it was deemed unnecessary to establish that salivary gland [^{3}H]UTP pools reached equilibrium during the incubation used.

D. miranda XR

Relative rates of chromosomal RNA synthesis were measured for the distal 20% of XR in *D. miranda*. A scatter diagram and best-fit regression line of XR against 4th chromosome incorporation is shown for each sex in Fig. 1. Statistical analysis of the data is presented in Table 1. The slope of the regression line

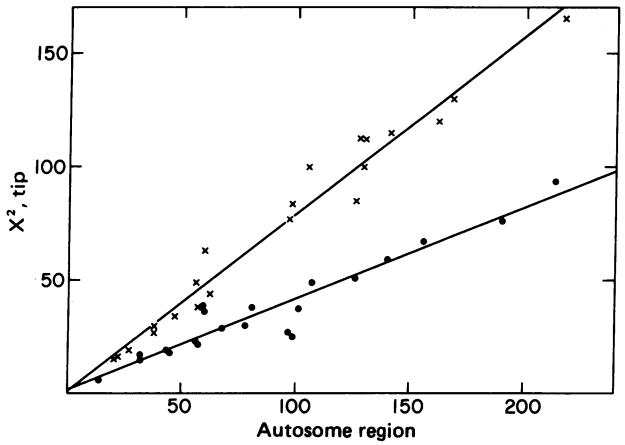

FIG. 2. Scatter diagram and best-fit regression line of chromosome X^2 (distal 10%) against 4th chromosome incorporation of tritium in *D. miranda* male and female salivary gland nuclei. The ordinate represents the number of silver grains located over the distal 10% of the X^2 chromosome, and the abscissa is the number of grains over the distal 30% of the 4th chromosome. Each point represents X^2 tip and 4th chromosome values from a single nucleus. ●, Male nuclei; ×, female nuclei.

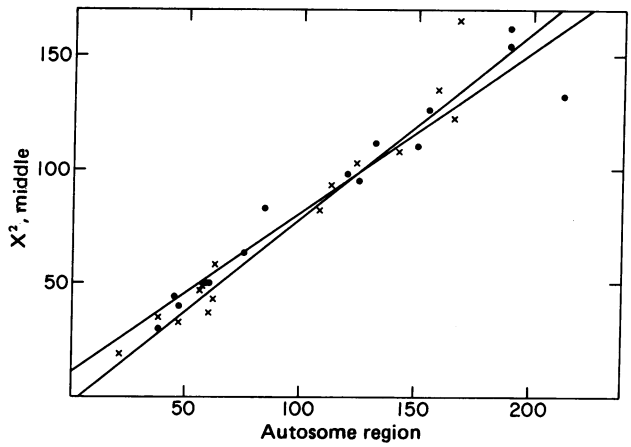

FIG. 3. Scatter diagram and best-fit regression line of chromosome X^2 (middle region) against 4th chromosome incorporation of tritium in *D. miranda* male and female salivary gland nuclei. The ordinate represents the number of silver grains located over 30% of the X^2 adjacent to the tip region; the abscissa is the number of grains over the distal 30% of the 4th chromosome. Each point represents X^2 middle and 4th chromosome values from a single nucleus. ●, Male nuclei; ×, female nuclei.

(*b*) is nearly identical in males and females ($P > 0.7$). We conclude that the distal 20% of the right arm of the X chromosome is therefore dosage-compensated in *D. miranda*, as it is in the corresponding section of XR in *D. pseudoobscura* (19).

D. miranda X^2

The regions of the X^2 chromosomes that were studied together comprise about 40% of its length. One of the two regions corresponds to the distal 10% of the X^2. Scatter diagrams and statistical analysis of this section are shown in Fig. 2 and Table 1. There is great disparity between the males and the females in the rate of RNA synthesis within this tip region. Slopes of the regression lines are 0.40 for males and 0.78 for females, and indicate that the rate of RNA synthesis is almost twice as great in females as in males. A *t*-test confirms that the observed differences are significant ($P < 0.001$). We conclude that the X^2 tip region is uncompensated in *D. miranda* since the rate of RNA synthesis is proportional to the number of copies of the X^2 tip region present, two in females, one in males. No compensation mechanism for the males has evolved for these X^2-linked genes.

The second X^2 segment we examined extends from the proximal boundary of the X^2 tip region to approximately the middle of the chromosome. Scatter diagrams and statistical analysis of this X^2 middle section are shown in Fig. 3 and Table 1. Although points from male and female nuclei are mingled, the best-fit regression lines are somewhat different in males and females. The male regression coefficient is 20% lower than the female value. A *t*-test indicates that the observed disparity is just on the borderline of statistical significance ($P \sim 0.05$). It is therefore possible that the rates of RNA synthesis are in fact equal in this region in males and females, the observed difference being attributable to random error. Even if the regression coefficients are significantly different, the actual disparity between males and females cannot be great. We conclude that either the entire middle section of the X^2 chromosome is dosage-compensated, or almost so, perhaps varying from subsegment to subsegment or gene to gene.

We measured the relative rate of RNA synthesis in just the distal 10% of the middle section of the X^2 chromosome. This subsection contains approximately 10 chromomeric bands and extends from within division 94 to the proximal boundary of the X^2 tip region. When the number of silver grains over this subsection was plotted against the number of grains over the 4th chromosome region used as a control, we found that the males and females have parallel regression lines and equal slopes for this small subsegment (Fig. 4). There was no statistically significant difference between the slopes, but the rate of incorporation over the 10-chromomere subsection was quite low, with an average of eight grains per nucleus. It is possible that small differences may exist; however, we feel the observed equality indicates that some degree of dosage compensation exists here. It is interesting that this dosage-compensated region adjoins the dosage-uncompensated X^2 tip region.

DISCUSSION

In the genus *Drosophila*, the evolution of the sex chromosome is generally supposed to involve the differentiation of originally

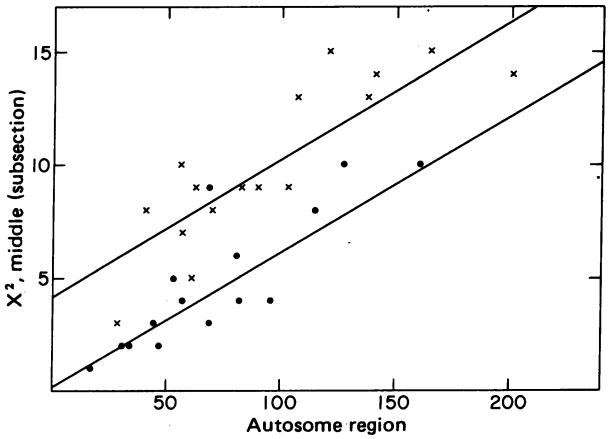

FIG. 4. Scatter diagram and best-fit regression line of chromosome X^2 (middle subsection) against 4th chromosome incorporation of tritium in *D. miranda* male and female salivary gland nuclei. The ordinate represents the number of silver grains located over a 10-chromomere subsection of the X^2 middle region; the abscissa is the number of grains over the distal 30% of the 4th chromosome. Each point represents X^2 (middle subsection) and 4th chromosome values from a single nucleus. ●, Male nuclei; ×, female nuclei.

homologous chromosomes into two distinct genetic entities. One of the chromosomes thereafter retains most of the original genetic material and becomes a heterochromosome (the X), existing in one copy in males and in two copies in females. The other element almost invariably degenerates and appears largely genetically empty (the Y) (20). Finally, the males are haploid for most heterochromosome genes, and that is an alteration of genic balance so extreme as normally to be lethal. In order to restore proper genic balance, the sex chromosomes must also evolve a regulatory mechanism involving the heterochromosome, so that single gene activity in males is increased to equal that of the two alleles present in females. Seven species from two *Drosophila* subgenera have been examined to date, and all of them exhibit dosage compensation for X-linked genes (19, 21–23). The most extensive study of the evolution of dosage compensation has been made in *D. pseudoobscura*, the X chromosome of which consists of two arms. It is believed that this metacentric X chromosome resulted from a Robertsonian fusion between the original ancestral rod-X chromosome (homologous to the X in *D. melanogaster*) and an autosome (24, 25). Therefore, one of the X-chromosome arms has a more recently evolved status as a sex-chromosome element. Measurements of relative rates of transcription and levels of X-linked enzyme activity indicate that both arms are dosage-compensated in *D. pseudoobscura* (19, 21). In *D. willistoni*, another species containing a metacentric X chromosome, X-linked enzyme activities on both arms are also equal in males and in females (21).

A study of dosage compensation in *D. miranda*, the X^2 chromosome of which appears to be in the process of sex chromosome differentiation, should therefore be illuminating. The *D. miranda* X^2 chromosome has resulted from a fusion of the Y chromosome with an autosome. The translocated autosome element has become genetically degenerate and heterochromatic, like a typical Y chromosome, and the free autosome has differentiated into the X^2 heterochromosome. Unlike the heterochromatic Y chromosome of other *Drosophila* species, the Y chromosome of *D. miranda* still contains some regions of euchromatic material interspersed within the heterochromatin (16), so perhaps not all of the translocated genes have degenerated. In fact, in *D. miranda* spermatogenesis the X^1 and X^2 chromosomes pair noncompetitively with the Y to form a trivalent association during the first meiotic metaphase (26, 27). Such pairing constitutes further evidence for a remaining euchromatic homology between the X^2 and Y chromosomes. Moreover, by repeated backcrossing of partially fertile *D. miranda–D. pseudoobscura* hybrid females, MacKnight demonstrated that some genetic homology exists between the *D. miranda* X^2 and Y chromosomes. It seems clear that X^2 sex chromosome differentiation is not complete in *D. miranda*.

Dosage compensation appears to be a necessary regulatory adaptation to the X-chromosome haploidy characteristic of the evolution of the *Drosophila* sex chromosomes. Our results indicate that the X^2 chromosome is incompletely dosage-compensated. Along the distal 10% of the X^2 chromosome, the rate of chromosomal RNA synthesis was almost twice as great in females (containing two copies of the X^2 chromosome) as in males (with one X^2 chromosome). The X^2 tip region therefore lacks dosage compensation. In contrast to the X^2 tip region, an interior segment representing 30% of the X^2 length was found to be dosage-compensated. Since the ancestors of *D. miranda* diverged from those of its sibling species *D. pseudoobscura*, a chromosome that still exists as an autosome in *D. pseudoobscura* has in part differentiated into a dosage-compensated sex chromosome in *D. miranda*. The incomplete degeneration of homologous X^2 loci on the Y chromosome of *D. miranda* is thus correlated with an incomplete evolution of dosage compensation along the X^2 chromosome. Genes within the uncompensated X^2 tip region almost certainly have homologous loci on the Y chromosome, for it is difficult to believe that such a large euchromatic region of the genome could remain haploid in males without dosage compensation (14, 28). The evolution of X^2 dosage compensation in general parallels differentiation of the X^2 chromosome. Such a regulatory mechanism seems indispensable in the evolution of translocated autosomal segments that become a part of the sex chromosomes in *Drosophila*.

The transcriptional activity within a 10-chromomere subsection of the X^2 middle section, just adjacent to the dosage-uncompensated distal segment, appeared to be equal in males and females. The fact that one interchromomeric interval may separate a dosage-compensated subsection from an uncompensated one (the X^2 tip region) suggests the existence of precise, localized compensatory evolution and regulation. We observe no alteration of gene activity, like a position effect, that spreads in a polarized manner from dosage-compensated loci into adjacent regions or the reverse (29, 30).

It is interesting to note that the pattern of chromomeres within the uncompensated X^2 tip region is identical cytologically to the tip region of the third chromosome of *D. pseudoobscura*. Although mapping studies have shown that the X^2 middle section also contains loci that are homologous to those on the third chromosome of *D. pseudoobscura*, its pattern of chromomere bands has been modified extensively. Thus, a striking correlation exists between the extent of chromosomal rearrangement and the establishment of dosage compensation. Both the X^2 middle region in *D. miranda* and a section of the third chromosome in *D. pseudoobscura* are known to be highly polymorphic in gene arrangement. Sixteen naturally occurring inversions within this *D. pseudoobscura* third chromosome region have been found (31). Furthermore, the Whitney race of *D. miranda* exhibits inversion polymorphism within the X^2 midsection (32). It is evident that this region undergoes changes in structural organization at a much greater frequency than most other regions of the *D. miranda* genome. One is tempted to speculate that such changes in chromosome organization have somehow facilitated or accelerated the evolution of a mechanism of dosage compensation within the midsection of the X^2 chromosome.

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