Dosage Compensation in Sciarids Is Achieved by Hypertranscription of the Single *X* Chromosome in Males

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ABSTRACT

Dosage compensation refers to the process whereby females and males with different doses of sex chromosomes have similar amounts of products from sex chromosome-linked genes. We analyzed the process of dosage compensation in *Sciara ocellaris*, Diptera of the suborder Nematocera. By autoradiography and measurements of *X*-linked rRNA in females (*XX*) and males (*XO*), we found that the rate of transcription of the single *X* chromosome in males is similar to that of the two *X* chromosomes in females. This, together with the bloated appearance of the *X* chromosome in males, support the idea that in sciarids dosage compensation is accomplished by hypertranscription of the *X* chromosome in males.

IN organisms in which females and males differ in the number of sex chromosomes, one sex having one and the other sex having two, a process has evolved to eliminate the difference in the doses of the sex chromosome-linked genes in the two sexes. This mechanism is called dosage compensation. This process is accomplished by different mechanisms in the three organisms in which it has been studied so far: *Drosophila melanogaster* (KURODA *et al.* 1993), *Caenorhabditis elegans* (HSU and MEYER 1993) and mammals (BORSANI and BALLABIO 1993). In *D. melanogaster*, the two *X* chromosomes of the females are active and dosage compensation is achieved in males by hypertranscription of its single *X* chromosome. In *C. elegans*, dosage compensation is achieved by hypotranscription of the two active *X* chromosomes in the hermaphrodites. Finally, in mammals, dosage compensation is attained by stable inactivation of one of the two *X* chromosomes in females. In the three organisms, a set of genes have been identified, which are responsible for dosage compensation: the *male-specific-lethal* (*msl*) genes in *D. melanogaster* (BELOTE and LUCCHESI 1980a,b; UCHIDA *et al.*, 1981) the *dumpy* genes in *C. elegans* (HODGKIN 1983; MEYER and CASSON 1986; MENEELY and WOOD 1987) and the *Xist* gene in mammals (BROCKDORFF *et al.* 1991; KAY *et al.* 1993).

In *D. melanogaster*, the study of the *msl* genes has been pursued further. Two of these genes, *mle* (KURODA *et al.* 1991) and *msl-1* (PALMER *et al.* 1993) have been cloned. The gene *mle* encodes a protein containing motifs characteristic of members of a helicase superfamily. The gene *msl-1* encodes a protein that contains an acidic N terminus characteristic of proteins involved in transcription and chromatin modeling. Both MLE and MSL-1 proteins are associated with many sites along the polytenic *X* chromosome in males, but not in females, as is the case also for the histone H4 acetylated at lysine 16 (TURNER *et al.* 1992). These results, together with the fact that the *msl* mutations do not show additive effects (BACHILLER and SÁNCHEZ 1989; GORMAN *et al.* 1993), led to the proposal that the MSL proteins might be components of a heteromultimeric complex that specifically interacts with the male *X* chromosome. Consequently, this chromosome would acquire a chromatin structure, reflected by its pale bloated appearance, that allows a better accessibility to the transcription machinery components. In this context, it is worth mentioning that the inactive *X* chromosome in female mammals is distinguished by a lack of histone H4 acetylation (JEPPESEN and TURNER 1993); thus supporting the idea that the mechanism of dosage compensation is primarily related to chromatin structure.

Little is known on the evolution of the dosage compensation mechanisms. A prerequisite is the elucidation of the genetic complexes governing dosage compensation in different species. Apart from the three organisms mentioned above, dosage compensation has not been thoroughly studied in other organisms. A previous study showed that in *Rhynchosiara americana* uridine incorporation in the single *X* chromosome of male salivary glands was similar to that observed on the double *X* of female larvae (CASARTELLI *et al.* 1969). Here we report on the analysis of dosage compensation in sciarids, Diptera of the suborder Nematocera. We used *Sciara ocellaris*, where females are *XX* and males are *XO*, as an experimental model. We found that in sciarids dosage compensation occurs and is achieved by hypertranscription of the single male *X* chromosome.

MATERIALS AND METHODS

S. ocellaris were raised following the procedure of ROCHA *et al.* (1979).

Autoradiographic analysis: The larval stage was identified by the size and morphology of the eyespot (PERONDINI and

DESSEN 1985), and the sex by the different size of ovaries and testis. The salivary glands were dissected in a sciarid saline solution (TERRA *et al.* 1976) and incubated in this solution containing 10 μCi/ml of [^{3}H]thymidine or [^{3}H]uridine, for 10 or 5 min, respectively, at room temperature. Incorporation was stopped by transferring the glands to 50% acetic acid. Three minutes later, chromosome squashes were prepared for autoradiography by standard procedures (ASHBURNER 1989). Autoradiographs were exposed for 7 days at 4°. The polytene cells came from the anterior region of the salivary glands.

Analysis of the DNA content of polytenic chromosomes: The salivary glands were dissected in sciarid saline solution and transferred to a slide with a drop of 50% acetic acid. After 3 min, squashes were prepared, the slides were incubated for 2 hr in ethanol at −20° and subsequently washed 3 times in 1 × phosphate-buffered saline (PBS) and 0.1% Triton X-100. After hydration, they were incubated for 10 min at room temperature in 1 × PBS containing 0.01 mg/ml of Hoechst 33258. They were washed 3 times for 5 min in 1 × PBS and 0.1% Triton X-100, and subsequently mounted in glycerol-PBS (9:1) containing 1 mg/ml of phenylenedyamine, and studied under a microscope with epifluorescence. Photographs were taken with Plus-X Kodak films. The densitometry was performed with a Molecular Dynamics Computer Densitometer, model 300A.

Analysis of rRNA and histone-RNA: One hundred female and 100 male larvae were homogenized in 1 ml of a guanidine solution (50 ml of 4 M guanidine-thyocyanate, 25 mM sodium citrate, pH 7.0, 0.5% Sarkosyl and 0.36 ml of β-mercaptoethanol). Following the addition of 0.1 ml of 2 M sodium acetate, pH 4.0, 1 ml of phenol and 0.2 ml of a solution of chloroform-isoamyl alcohol (49:1), the mixture was stirred and centrifuged for 20 min at 4°, and 1 ml of isopropanol was added to the aqueous phase to precipitate the RNA. After centrifugation, the pellet of RNA was solubilized in 200 μl of diethyl pyrocarbonate-H_2O. The RNA concentration was measured by spectrophotometry, and a gel was made to ascertain the absence of DNA. The amount of RNA was monitored by hybridization with the rDNA and histone-DNA probes, by means of the slot blot technique. Previously, with the RNA preparation we performed a dilution series in a slot blot that we hybridized, and we measured different exposure times to determine the linear region of the curve corresponding to different RNA concentrations for a given amount of the probe.

For the experiment, the slot contained 15 samples of 6 μg each of female RNA and 15 samples of 6 μg each of male RNA. The prehybridization and hybridization solutions contained 5 × SSC, 5 × Denhardt's solution, 0.25% sodium dodecyl sulfate (SDS), 100 μg/ml of denaturated salmon DNA and 50% formamide. Prehybridization (6 hr) and hybridization (16 hr) were carried out at 42°. After hybridization filters were washed three times (20 min each time) in 2 × SSC and 0.1% SDS at room temperature, in 2 × SSC and 0.1% SDS at 55° and in 2 × SSC and 0.1% SDS at 60°. The filter was first hybridized with the histone probe, and autoradiography was performed. Afterward, the filter was treated to eliminate the histone probe and prehybridized and subsequently hybridized with the rDNA probe. The amount of hybridization was measured by densitometry with a Molecular Dynamics Computer Densitometer, model 300A.

RESULTS AND DISCUSSION

To measure transcription of the *X* chromosome in Sciara females and males, we carried out an autoradiographic analysis. The template activity of the polytenic chromosomes of salivary glands was measured by quantifying the incorporation of [^{3}H]uridine. Since we assayed the template capacity of the *X* chromosome, it was necessary to ascertain that polytenization of the salivary gland chromosomes had already finished; otherwise, any difference in template activity could be attributed to a different degree of polyteny. To determine whether polytenization had already ceased at the "h" phase of the fourth larval instar, the capacity of the chromosomes to incorporate [^{3}H]thymidine was analyzed by autoradiography. As a control, we used salivary gland chromosomes from younger larvae at the "e" phase of the fourth larval instar. Incorporation of [^{3}H]thymidine occurred only in the polytenic chromosomes from "e" but not from "h" phase larvae (data not shown). Thus, polytenization of the salivary gland chromosomes had already finished at the "h" phase.

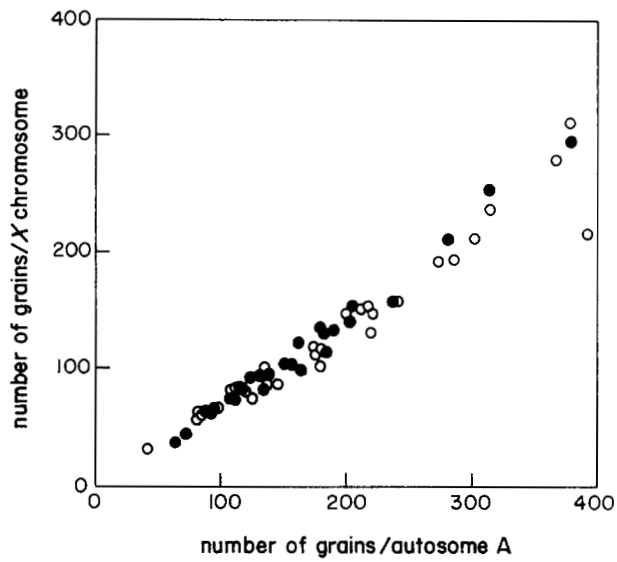

FIGURE 1.—Scatter diagram of the number of grains over the *X* chromosome *vs.* number of grains over the autosome A, corresponding to the autoradiography for measuring [^{3}H]uridine incorporation in female (white dot) and male (black dot) polytenic chromosomes.

Figure 1 shows the autoradiographic analysis of [^{3}H]uridine incorporation into salivary gland chromosomes of female and male larvae at the "h" phase. Each dot represents the number of grains along the *X* chromosome (ordinate) relative to the number of grains along the autosome A (abscissa) within the same nucleus. As a measure of the *X* chromosome transcription activity in relation to the transcription of the autosome A (control), we calculated the slope of the best-fit straight line by regression analysis in both females and males. The statistical analysis comparing both slopes is shown in Table 1. No significant difference ($P > 0.1$) was observed between the sexes. This indicates that the rate of RNA synthesis for the single *X* chromosome in males equals that of the two *X* chromosomes in females; *i.e.*, dosage compensation occurs in *S. ocellaris*.

Despite these results, however, dosage compensation could be the consequence of a higher DNA content (double amount) in the male *X* chromosome with respect to each of the two female *X* chromosomes. One more round of *X* chromosome polytenization could

TABLE 1

Statistical analysis of [^{3}H]UTP incorporation in the *X* and A chromosomes of females and males

Sex	Average number of grains				
	No. of nuclei	Chromosome A	*X* chromosome	b	*t* Student
Females	33	187.1	129.5	0.695	1.444
Males	29	161.4	114.1	0.721	($P > 0.1$)

specifically occur in males, so that the *X* male DNA content would equal the DNA content of both female *X* chromosomes. As a measure of the DNA content, we compared the amount of Hoechst 33258 that binds to the polytenic *X* chromosome relative to the amount of Hoechst that binds to the autosome A within the same nucleus, in both sexes. For this purpose, salivary glands of "h" phase larvae were incubated with Hoechst and the amount incorporated monitored by densitometry. In males the *X*-DNA/A-DNA ratio value was half the value found in females (Figure 2A). This difference was significant ($P < 0.01$), whereas the DNA content of the autosome A was not significantly different between both sexes (data not shown). Therefore, male polytenic nuclei contain half the amount of *X* chromosome DNA than female polytenic nuclei. Consequently, the compensated *X* chromosome RNA synthesis in male relative to female polytenic nuclei cannot be attributed to a similar DNA content of the *X* chromosomes in both sexes.

Although incorporation of [^{3}H]uridine along polytenic chromosomes indicated that dosage compensation exists in sciarids, we performed a more precise study of gene transcription by directly assaying the production of specific *X*-linked RNAs. Initially, we tried the genes *G-6-PDH* and *sgs-4*, known to be located in the *X* chromosome of *D. melanogaster* and showing dosage compensation (LUCCHESI and MANNING 1987), as well as the autosomal genes *Adh* and *sgs-3* as control. Unfortunately, we could not detect the RNA of these four genes in *Sciara* by using the *Drosophila* probes. Then, we chose the rDNA genes, which are localized in the proximal end of the *X* chromosome in Sciara (DESSEN and PERONDINI 1991). The rDNA genes are greatly conserved; therefore, rDNA from *D. melanogaster* was used as a probe (pDm238) (ROIHA *et al.* 1981). Since rDNA genes are expressed in all cells and at all developmental stages, as a control, we analyzed transcripts from an autosomal gene that shows a similar expression pattern. We chose the histone genes, which are also greatly conserved. To quantify the histone RNA we used an heterologous probe (pKG-11) from *Chironomous thummi thummi* (HANKELN and SCHMIDT 1990). As in *S. ocellaris* the localization of histone genes was not known, we performed an "*in situ*" hybridization of *C. t. thummi* histone-DNA on *Sciara ocellaris* polytenic chromosomes, finding that they hybridized to a specific band of the autosome C (data not shown). We measured the amount of rRNA relative to the amount of histone RNA, as described in MATERIAL AND METHODS, in sisters and brothers from the same generation, so it would be unlikely that the rDNA copies would vary between them. No significant differences ($P > 0.05$) were found for the rRNA/histone value between both sexes (Figure 2B), indicating that the rDNA genes in *S. ocellaris* are dosage compensated.

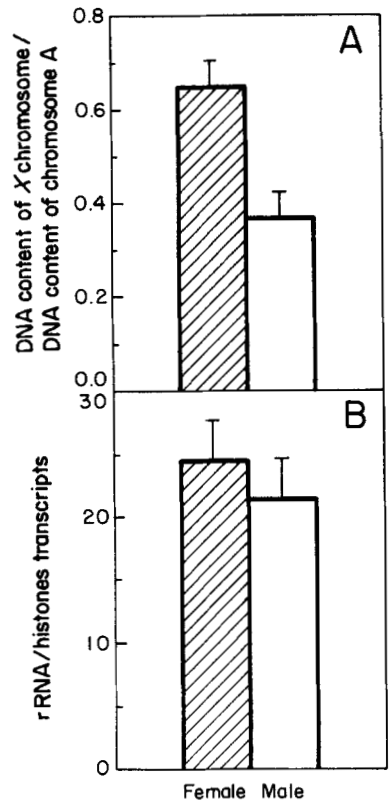

FIGURE 2.—(A) Analysis of the DNA content of the *X* chromosome in relation to the DNA content of the autosome A in salivary glands of "h" phase female and male larvae, corresponding to the measurements of 15 nuclei for each sex. (B) Analysis of the amount of rRNA and histone-RNA in females and males, corresponding to the measurements of 15 samples for each sex. The bars represent the 95% confidence intervals.

All these results demonstrated that the rate of transcription of the single *X* chromosome in males is about twice the rate of transcription of either of the two *X* chromosomes of females. This can be accomplished by either decreasing in females or increasing in males the transcription of the *X*-linked genes. Our results, however, cannot formally distinguish between those two alternative mechanisms. In *D. melanogaster*, the polytenic *X* chromosome of males is as wide as the two paired *X* chromosomes of females, although males contain half the amount of *X* chromosome DNA than females do. This bloated appearance of the male *X* chromosome is lost in males mutant for *msl* mutations, which specifically reduce the transcriptional activity of the male *X*. Those *msl* mutant males show a narrower and more intensely stained *X* chromosome (BELOTE and LUCCHESI

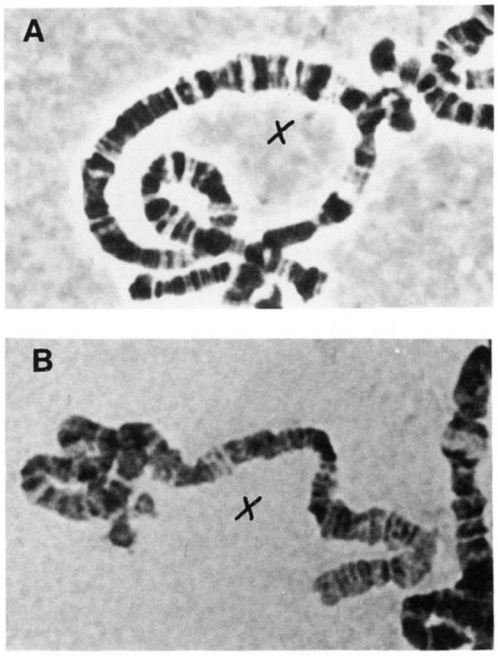

FIGURE 3.—Phase contrast micrograph of polytene chromosomes from female (A) and male (B) *S. ocellaris*. Orcein staining following standard procedures.

1980b; OKUNO *et al.* 1984). Therefore, the bloated appearance of the male *X* chromosome represents a cytologically visible manifestation of its hypertranscriptional activity. The similar bloated appearance of the male polytenic *X* chromosome of *S. ocellaris* (Figure 3) supports the idea of increased transcriptional activity in males rather than decreased transcriptional activity in females.

In conclusion, we have demonstrated that in *S. ocellaris* the rate of transcription of the *X* chromosome in males is greater than the rate of transcription of either of the two *X* chromosomes in females, in spite of the fact that males contain half the amount of *X* chromosome DNA than females do. These results, together with the specific pale bloated appearance of the male polytenic *X* chromosome, argue that in *S. ocellaris*, as in the case of *D. melanogaster*, dosage compensation is achieved by hypertranscription of the single *X* chromosome in males, rather than decreased *X*-linked gene transcription in females. How much the molecular basis of the mechanism of dosage compensation in Diptera has been conserved during evolution is currently under study. We are trying to isolate and characterize in *S. ocellaris* the genes homologous of the *msl* genes of *D. melanogaster*.

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