Gene regulation in *Drosophila mulleri*, *D. arizonensis*, and their hybrids: The nucleolar organizer*

(genetic interaction/interspecific hybridization/genetic divergence/suppressed loci)

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ABSTRACT Typically, *Drosophila* have nucleolar organizer regions (NORs) confined to the sex chromosomes. Salivary gland cells of hybrids between *Drosophila mulleri* females and *D. arizonensis* males exhibit features in nucleolar organizer regulation that differentiate the species on one hand, and which show an interplay between the X and the microchromosome on the other hand.

In the hybrid females only the X chromosome from *D. arizonensis* appears to be attached to the nucleolus. In the hybrid males the X chromosome, from *D. mulleri*, also does not seem to contain a functional NOR. However, in hybrid males the microchromosome from *D. arizonensis* increases greatly in size and appears to be associated with the nucleolus. The increase in size of the microchromosome involves a 4-fold increase in DNA content. In *D. arizonensis* and in hybrid females the NOR of the microchromosome appears to be suppressed. In the absence of an *arizonensis* X chromosome, the NOR of the microchromosome typically is active, while the NOR on the *mulleri* X chromosome remains suppressed. Therefore, the ribosomal cistrons and interchromosomal regulator element appears to be duplicated on both the X chromosome and microchromosome of *D. arizonensis*, but with epistatic suppression of the microchromosomal NOR by the *arizonensis* X-linked NOR. Either *arizonensis* NOR, X linked or microchromosomal, suppresses the *mulleri* NOR.

The vital role played by ribosomes in the physiological economy of a cell is clearly illustrated in the systems that have evolved to ensure an adequate supply of 18S and 28S rRNA. Not only are the ribosomal cistrons amplified and transcribed in a special organelle, the nucleolus, but a variety of "backup" strategies have been observed in a range of eukaryotes. These alternative or compensatory abilities are activated when unusual conditions arise. For example, oocytes of vertebrates and invertebrates synthesize prodigious amounts of polypeptides, and some species have high degrees of nuclear extra-chromosomal replication of DNA (1-3). Compensatory replication of the rRNA genes of *Drosophila melanogaster* also occurs when one nucleolar organizer chromosome is completely or partially deficient in its nucleolar organizer region (NOR). In some of these cases the additional copies of rRNA genes (rDNA) are structurally integrated into the genome (4), while in other cases they are not (4, 5). In maize, there appears to be a reserve of ribosomal cistrons that may be activated (6). Also, when NORs are totally absent, nucleolar material (which probably contains some rRNA) is synthesized by alternative loci (7).

These examples illustrate two alternative strategies of achieving rDNA synthesis, extra-chromosomal and chromosomal, as well as the possibility of latent cistrons being available for emergency use. The experiments reported here suggest that

the various ways for having additional rDNA when needed in abnormal circumstances that have been observed in several species may be options available for a single species. Which particular option is activated depends upon the response of a regulation system to the circumstances.

MATERIALS AND METHODS

The stock of *D. mulleri* is a pool of twenty isofemale lines from Guayalejo, Mexico, and the stock of *D. arizonensis* is a pool of 25 isofemale lines from the same area. The hybrids were obtained in crosses between *D. mulleri* females and *D. arizonensis* males. This cross yields fertile females and sterile males, while the reciprocal cross is incompatible (22).

The squashes of salivary glands and brains were prepared using two stain techniques: lacto aceto-orcin (8) and Feulgen (9). About 60 hybrid larvae of both sexes and more than 200 larvae of each parental species were analyzed.

The relative width in a salivary gland preparations of chromosome 6 (microchromosome) was measured in photographic prints, taking the ratio of the major width of this chromosome to the width of a dark condensed band considered to be representative of the general width of another autosome. Synapsed chromosomes were measured in the parental species and asynapsed single chromosomes were measured in the hybrids. In the hybrids, the same autosomal band was used for comparison of both microchromosomes in a cell.

For DNA content estimates, the area of chromosome 6 was measured in black-and-white transparency enlargements of the same magnification, and the average percent absorbance of Feulgen-stained chromosomes was obtained from scans with a Gilford 250 spectrophotometer (narrow slit, 500 nm). Care was taken to make all exposures in the linear range of the emulsion and transparencies were developed together. Measurements taken from several chromosomes were averaged. Using a parental species as a reference, the relative amount of DNA per chromosome was calculated from the product of area and average absorbance. In the parental species, measurements were of synapsed chromosomes, so the DNA values were divided by two.

RESULTS

The *D. mulleri* and *D. arizonensis* X chromosomes are homeosequential. However, they can be recognized in salivary gland cells by some consistent morphological differences. For example, there is a distinct difference in the thickness of a single

Abbreviations: NOR, nucleolar organizer region; rDNA, genes for rRNA.

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FIG. 1. Proximal part of the X chromosomes in hybrid females showing the band (arrows) used as a morphological marker for identification (M = *D. mulleri*; A = *D. arizonensis*). The scale in this and following figures corresponds to 10 μ m.

band (Fig. 1) located in section F-2 (map in ref. 10), and a difference in the shape of the proximal end. The banding pattern of the proximal 20% (map sections F-5 to F-11) of the X chromosome of both species does not show homology and this region was never found synapsed in the hybrids. The homology of the distal 80% of this chromosome is apparent. Synapsis for some portions of this region may be observed.

In the salivary gland cells of *D. mulleri* and *D. arizonensis*, the proximal end of the X chromosome frequently is attached to the nucleolus, indicating a common location for the NOR in both species (Fig. 2). In the hybrid female cells that displayed the X chromosome attached to the nucleolus after squashing, the *D. arizonensis* alone was involved (Fig. 3) in about 90% of the nuclei. The nucleolus appeared normal.

Most of the *D. mulleri* X chromosomes of the male hybrids are unusually wider than the autosomes of the same cells or than the X chromosomes of the males in the parental species (Fig. 4 A, B, and C). They stain lighter, however, so the increase in width does not necessarily imply additional polyteny. In these hybrids, the X chromosome sometimes is positioned adjacent

FIG. 2. Association between the X chromosome and the nucleolus (N) in *D. mulleri* and *D. arizonensis*. (A) *D. arizonensis* male; (B) *D. arizonensis* female; (C) *D. mulleri* male; (D) *D. mulleri* female. Note strands which connect nucleolus to separate X chromosomes in the females and males. The magnifications in A-D are the same.

to the nucleolus. Although no clear connection between the X and the nucleolus was observed, the possibility of a connection could not be excluded in some cases (Fig. 4D).

The banding pattern of chromosome 6 is not as distinct as in the other chromosomes. It shows variable degrees of banding disorganization not associated with the larval developmental stages. These phenomena are observed in *D. mulleri* and *D. arizonensis*, but are more striking in *D. mulleri*. In both species, one to three heterochromatic dark granules are visible at the proximal end of the polytene chromosome 6 (Fig. 5 A and B).

The morphology of the polytene chromosome 6 found in the parental species is preserved in the hybrid females. They are asynapsed, as are the other chromosomes. However, the microchromosomes frequently appear joined to each other by the proximal regions, directly or through strands (Fig. 5 C and D). In the hybrid males, one of the microchromosomes increases

Table 1. Distribution of average width of the microchromosomes relative to autosomes in males and females of *D. mulleri*, *D. arizonensis*, and the interspecific hybrids

Source	Sex	Microchromosome		No. of chromosomes with the indicated ratio to reference band [†]										Average ratio
		Type*	No. measured	1.0-1.5	1.6-2.0	2.1-2.5	2.6-3.0	... 3.6-4.0	4.1-4.5	4.6-5.0	5.1-5.5	... 6.6-7.0	... 7.5-8.0	
<i>D. arizonensis</i>	♀	A	22	13	9	—	—	—	—	—	—	—	—	1.47
	♂	A	22	10	9	2	1	—	—	—	—	—	—	1.68
<i>D. mulleri</i>	♀	M	20	8	10	2	—	—	—	—	—	—	—	1.67
	♂	M	22	7	11	4	—	—	—	—	—	—	—	1.70
Hybrid	♀	A	20	7	9	3	1	—	—	—	—	—	—	1.76
		M	20	3	11	4	2	—	—	—	—	—	—	2.04
	♂	A	19	—	—	1	3	7	2	2	2	1	1	4.25
		M	21	1	7	9	4	—	—	—	—	—	—	2.26

* A = *D. arizonensis*; M = *D. mulleri*.

[†] See text for procedure.

FIG. 3. Hybrid female cells showing only the *D. arizonensis* X attached to the nucleolus. The arrow points to the morphological marker of the *D. arizonensis* X. A = *D. arizonensis* proximal end; M = *D. mulleri* proximal end; N = nucleolus; X = distal ends of the X chromosomes.

greatly in size without loss of the staining intensity, while it completely loses the banding pattern (Fig. 5 E and F). The morphological differences of the polytene microchromosome pair between hybrid females and males are so clear and constant that they are easy characters for identification of the sex of the larvae from which the salivary glands were dissected.

The relative widths of the polytene microchromosomes in males and females of *D. mulleri* and *D. arizonensis*, and hybrids (Table 1), show that this chromosome is slightly narrower in *D. arizonensis* than in *D. mulleri*, and in both species it is narrower in females than in males. Both microchromosomes in hybrid females or males are wider than those in larvae of the parental species. But in males the large microchromosome is about 2.5 times wider than the normal microchromosome observed in cells of either parental species. The variability of width is greater in the hybrids than in the parental species, and is especially pronounced in the enlarged microchromosome of the male hybrids. The enlarged microchromosome is also highly variable morphologically. Sometimes it partially segregates into two to five parts (Fig. 5 E and F). In extreme cases, it appears completely unravelled.

The relative amount of microchromosomal DNA estimated from Fuelgen-stained cells (Table 2) indicates that there are similar amounts per polytene microchromosome of *D. mulleri*, *D. arizonensis*, the hybrid female, and the small polytene chromosome 6 of the hybrid male. The enlarged microchromosome of the hybrid male, however, had over four times the DNA of the others, suggesting the occurrence of two additional replications of the DNA in the polytenization of this chromosome. Should the disproportionate replication be restricted to the rDNA (11) assumed to be present in this chromosome, then the rDNA amplification may be much greater than 4-fold. The estimation of numbers of cistrons will follow in a subsequent paper.

The large polytene microchromosome in the hybrid males is frequently associated with the nucleolus (Fig. 6A). Sometimes, when this association is not visible, a small nucleolus-like body is seen next to this chromosome, apparently being formed there. Both observations reinforce the indication that this chromosome has an active NOR in the hybrid males.

So far, we cannot identify the origin of the enlarged microchromosome in hybrid males with certainty. However, the

Table 2. Relative amounts of DNA per polytene microchromosome in *D. mulleri*, *D. arizonensis*, and the interspecific hybrids

Source	Chromosome	k (DNA/chromosome)* ± SEM	Comparison	Relative DNA per chromosome ± SEM
<i>D. arizonensis</i> parent	A	13.69 ± 1.98	A:A	1.00
<i>D. mulleri</i> parent	M	15.33 ± 2.17	M:A	1.12 ± 0.35
Hybrid ♂	A _H	63.39 ± 1.46	A _H :A	4.63 ± 0.02
	M _H	18.15 ± 0.85	M _H :M	1.18 ± 0.09

A = *D. arizonensis*; M = *D. mulleri*; H = chromosome in a hybrid.
* See text for estimation procedure.

FIG. 4. (A–C) Cells showing the widths of X chromosome in males relative to autosomes. (A) Hybrid; (B) *D. mulleri*; (C) *D. arizonensis*; (D) possible connection between the X chromosome and the nucleolus in male hybrid. Enlargements in A–C are the same.

normal size microchromosome sometimes retains its banding structure and resembles the *D. mulleri* microchromosome. This fact allows us to presume that the enlarged microchromosome is derived from *D. arizonensis*. We could not detect a differ-

FIG. 5. Microchromosomes. (A) *D. arizonensis*; (B) *D. mulleri*; (C and D) hybrid females; (E and F) hybrid males. A = *D. arizonensis*; M = *D. mulleri*.

FIG. 6. Nucleolar association with microchromosome. (A) The enlarged microchromosome (arrow a) and the nucleolus in a hybrid male cell. Note small microchromosome (arrow b), and X chromosome (X) without nucleolus. (B) The enlarged microchromosome (arrow) and a nucleolus-like body (NL). N = nucleolus. The proximal end of chromosome 3 is connected to the microchromosome.

ence in size between the homologous microchromosomes in metaphases in brain squashes of hybrid males (Fig. 7). Therefore, the enlargement appears to be confined to one of the polytene chromosomes.

DISCUSSION

Different features of the control system for rRNA synthesis have been shown in intraspecific studies of several organisms. From them, we can deduce that this control encompasses basically two separate genetic processes: DNA amplification and gene activation. Variation in ribosomal gene activity in hybrids provides information on the dominance and epistatic relations of NOR regulation. Such studies have involved hybrids of both plants (12–14) and animals (15–17). The dominance for NOR activity ordinarily is the same in reciprocal crosses, although a strong maternal influence often is found (18). In mouse-human somatic cell hybrids, suppression of the rRNA production of one of the species involved was affected by the specific chromosomes that segregated in a cell line (19, 20).

In the present experiments, *Drosophila* hybrids showed si-

FIG. 7. Metaphase brain squashes of hybrid males showing the five telocentric chromosomes (including the X and Y) and the microchromosome. No difference in size between the homologous microchromosomes is apparent. A and B are preparations from different individuals.

multaneously DNA amplification and gene activation in polytene cells apparently under strong dominance of the regulation system of one species over that of the other species. The *Drosophila* female pattern parallels that observed in *Xenopus* (16), but *Drosophila* males apparently introduce a new effect with respect to regulation of ribosomal cistrons.

Consistent phenotypic and cytological characteristics reveal changes in pattern of rRNA synthesis occurring in hybrids between *D. mulleri* and *D. arizonensis*. The males display the bobbed phenotype involving morphological features and rate of development (21). Cytologically, the females show that a single NOR from *D. arizonensis* is active, as inferred from the connection of the proximal region of this X chromosome with the nucleolus. The extent of the rRNA control system is not fully revealed by the hybrid females; the hybrid males indicate a latent NOR contained in or intimately associated with the microchromosomes, which is activated in their cells. The DNA content of the microchromosomes increases more than 4-fold, suggesting there were at least two additional replication cycles of polytenization confined to this chromosome. On the basis of its morphology, the nucleolus associated with the microchromosome appears to be normal, although this NOR is usually repressed. However, the persistence of the bobbed phenotype in some males indicates that the activated NOR is not fully equivalent in these males to the normally active NOR. The bobbed phenotype shows variable expressivity, which might be related to the variability detected in the relative size of the enlarged microchromosome. Presumably, the variable size of this chromosome represents different degrees of DNA amplification, although regulation of transcription may be involved.

In several cases involving other species of *Drosophila*, the microchromosome and X chromosome exhibit tendencies for pairing and have a similar action of lethal genes. It has been suggested that a degree of homology exists between these two chromosomes (22). It is possible that the *D. arizonensis* microchromosome and X chromosome share homologous ribosomal cistrons, but differ in responses to regulator signals. There is no evidence that these chromosomes in *D. mulleri* share these loci. In such a case, it would not be possible to observe a nucleolus produced by the *D. mulleri* microchromosome.

It is surprising that the *D. mulleri* NOR remains nonfunctional in hybrid males. In the absence of the *D. arizonensis* X, one would expect that the *D. mulleri* NOR would assume the

entire functional role of nucleolus formation. Instead, however, a latent Nor was activated. Therefore, the dominance cannot be directly a function of one X-linked NOR relative to the other. The indication that the activated NOR is in, or associated with, the microchromosome from the same genome as the "dominant" NOR in females suggests that the dominance acts at a distance, although the chromocenter connects all of these chromosomes and their associated NORs in polytene cells. The location of the *D. arizonensis* regulation system would appear to be restricted to homologous segments of the X and Y chromosomes and to the autosomes. We postulate that the *D. arizonensis* microchromosome and X chromosome contain (i) ribosomal cistrons, and (ii) the regulatory site suppressing the *D. mulleri* NOR. Furthermore, the NOR on the *arizonensis* microchromosome is regulated by the *arizonensis* X chromosome.

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