

SILVER STAINING AND THE NUCLEOLAR ORGANIZING ACTIVITY IN *Drosophila* SPECIES OF THE MULLERI COMPLEX AND THEIR HYBRIDS

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ABSTRACT

Silver techniques used in salivary gland preparations of 6 species in the mulleri complex and their hybrids differentially stained the nucleoli and the threads which attach these organoids to the NOR-bearing chromosomes. This ability to discriminate the association between nucleoli and chromosomes confirmed previous observations made on preparations stained by lacto-acetic orcein as to the involvement of X chromosomes and microchromosomes in the nucleolar organizing activity of the species studied, and as to the nucleolar dominance in hybrids. Furthermore, silver staining techniques revealed changes in the size, shape and number of nucleoli of hybrids, supporting and extending previous observations that suggest that the hybridization of these species disturbs nucleolar synthesis in a manifold way.

INTRODUCTION

In several organisms the nucleolar organizing regions (NORs) may be clearly located on account of associated nucleolar material for whose synthesis these regions are responsible (McClintock, 1934). Following the demonstration that ribosomal cistrons exist in multiple copies in the NORs (Ritossa and Spiegelman, 1965) the technique of *in situ* hybridization with labelled

ribosomal RNA and subsequent autoradiography (Gall and Pardue, 1969) became extensively used for identifying these chromosomal regions. Recently, silver methods which stain differentially NORs (Goodpasture and Bloom, 1975; Howell *et al.*, 1975; Bloom and Goodpasture, 1976; Howell, 1977) have been successfully applied to a variety of animals including mammals, frogs and insects (e.g. Goodpasture and Bloom, 1975; Ragghianti *et al.*, 1977; Varley, 1977; Howell, 1977; Ferreira *et al.*, 1978; Ruiz *et al.*, 1978). It has been demonstrated that silver methods only stain NORs which contain functionally active ribosomal cistrons (Miller, D.A. *et al.*, 1976; Miller, O.J. *et al.*, 1976; Howell, 1977). Silver apparently binds to proteins which are associated with rRNA accumulating around active NORs (Howell, 1977; Schwarzacher *et al.*, 1978). These are acidic proteins (Schwarzacher *et al.*, 1978) exhibiting reactive sites (Lischwe *et al.*, 1979; Mamrack *et al.*, 1979).

The specificity for active NORs makes silver techniques very valuable for studies of nucleolar organizing activity in interspecific hybrids, since it has been shown in several of them that ribosomal RNA of one species is exclusively expressed. This seems to be the case for hybrids involving *Drosophila* species in the mulleri complex in which nucleolar dominance is apparently a general feature (Bicudo and Richardson, 1977; Bicudo, 1979). This nucleolar dominance was inferred from the observation in hybrid females of a single X chromosome (which is NOR-bearing in the parental species) attached to the nucleolus in most of their salivary gland cells. In hybrid females for which morphological chromosome markers are available, the specific origin of the X-linked dominant NOR could be determined. Thus, in the female hybrids of *D. mulleri* females with *D. arizonensis*, *D. navojoa*, *D. mojavensis*, *D. wheeleri* and *D. aldrichi* males, the dominant NORs are respectively from *D. arizonensis*, *D. navojoa*, *D. mojavensis*, and from *D. mulleri* for the remaining hybrids. Besides, the observations indicated that the microchromosome pair is also involved in the nucleolar activity of these species, operating intraspecifically as a secondary nucleolar organizer, but able to assume the role of main nucleolar organizer in male hybrids of some of the species studied. In these cases one element of the microchromosome pair is submitted to a DNA amplification apparently by additional polyteny, and is activated. The activation of the microchromosome was observed in hybrid males in which, as a consequence of the direction of the interspecific crosses, the X-linked NOR dominant in the females is absent. The activation of the microchromosome was considered indicative of dominance at the regulatory level because

in these males the recessive X-linked NOR does not operate as the main NOR in spite of the absence of the dominant X-linked NOR; instead, one microchromosome apparently of the same specific origin as the dominant X-linked NOR is activated.

This paper reports observations on salivary gland preparations submitted to silver staining techniques, using the same species in the *mulleri* complex and hybrids preliminarily studied in preparations stained with lacto-acetic orcein. The results reinforced some of the findings described above and indicated other aspects of the nucleolar organizing activity affected by hybridization.

MATERIALS AND METHODS

Drosophila mulleri (Guayalejo, Mexico), *D. arizonensis* (Guayalejo, Mexico), *D. mojavensis* (Baja California, Mexico), *D. aldrichi* (Austin, USA), *D. navojoa* (Navojoa, Mexico) and *D. wheeleri* (Baja California, Mexico) were used. All of them have 6 pairs of chromosomes, 5 acrocentric and one pair of microchromosomes (Wharton, 1943; Bicudo, 1979).

Hybrids were obtained from mass crosses prepared in vials (using 10 to 30 pairs of flies) or in population cages (using up to 400 pairs of flies), depending on the fecundity of each type of interspecific cross. Hybridizations involved both directions of crosses between *D. arizonensis* and *D. mojavensis*, and crosses between *D. mulleri* females and males of every other species.

Late third instar larvae from the stocks or those obtained in the interspecific mass crosses were used for salivary gland preparations. They were dissected in Shen's or Demerec's solutions, fixed for 2 minutes in absolute ethanol: glacial acetic acid (3:1), transferred to 50% lactic acid for 3 minutes and then squashed in 45% acetic acid on gelatinized slides. The preparations were immersed in liquid nitrogen and the cover glass flipped off with a razor blade. The slides were then air dried or immersed in 70%, 95% and absolute ethanol before air drying.

For mitotic preparations larvae from the early third instar were used. The ganglia were dissected in Shen's or Demerec's solution, transferred to a drop of Colchicine solution (10 μ g/ml) for 10 minutes, placed in a drop of 0.4% KCl for 10 minutes, fixed for 3 minutes in 3:1 absolute ethanol: glacial acetic acid and squashed in 45% acetic acid. Coverlips were removed as described for salivary gland preparations.

Two staining techniques were used: the Ag-AS (Goodpasture and Bloom, 1975), which is basically the same as AS-SAT (Howell *et al.*, 1975), and Ag-I (Bloom and Goodpasture, 1976). Both procedures were used for mitotic and salivary gland chromosomes. The Ag-I technique has 2 advantages over Ag-AS: (1) it is simpler and (2) it stains preparations slowly, allowing a better control of the intensity of staining.

Nucleolar areas were measured in silver stained salivary gland cells of parental species and of hybrids using the following process: photomicrographic negatives were projected by the enlarger onto a sheet of graph paper (10 x 10 to the cm) where the nucleolar contour was traced. A constant magnification was used. Nucleolar areas scored in the paper were converted to μm^2 according to the magnification used.

RESULTS

None of the silver staining techniques used, even with some modifications, gave differential staining of NORs in mitotic preparations of parental species and hybrids. In salivary gland cells the nucleoli and the threads which attach them to the NOR-bearing chromosomes exhibited the dark yellow or brown color which characterizes NORs in mitotic chromosomes of other organisms treated by these techniques, while the chromosomes remained pale yellow.

The pattern of association of nucleoli with chromosomes shown by differential silver staining was the same as previously observed, with some difficulty, in salivary gland preparations of these species and hybrids stained with lacto-acetic orcein (Bicudo and Richardson, 1977; Bicudo, 1979). Intraspecifically this association involves in most cases the X chromosome, whereas in hybrids both the X chromosome and the microchromosome may be involved. For example, in Figure 1 the association of the X chromosome with the nucleolus is shown in a *D. wheeleri* male cell (Figure 1A) and in a cell of a female hybrid between *D. mulleri* and *D. aldrichi* (Figure 1B). In the hybrid female cell this association involves a single X chromosome, the one derived from *D. mulleri*. The unichromosomal attachment to the nucleolus also characterizes hybrid females of the other species studied.

The distinct staining of the nucleoli also made it possible to analyze the shape, number and size of this organoid in the parental species and hybrids. Typically any one of the species analyzed has one single round or oblong nucleolus in the salivary gland cells. However, in males and females of all the

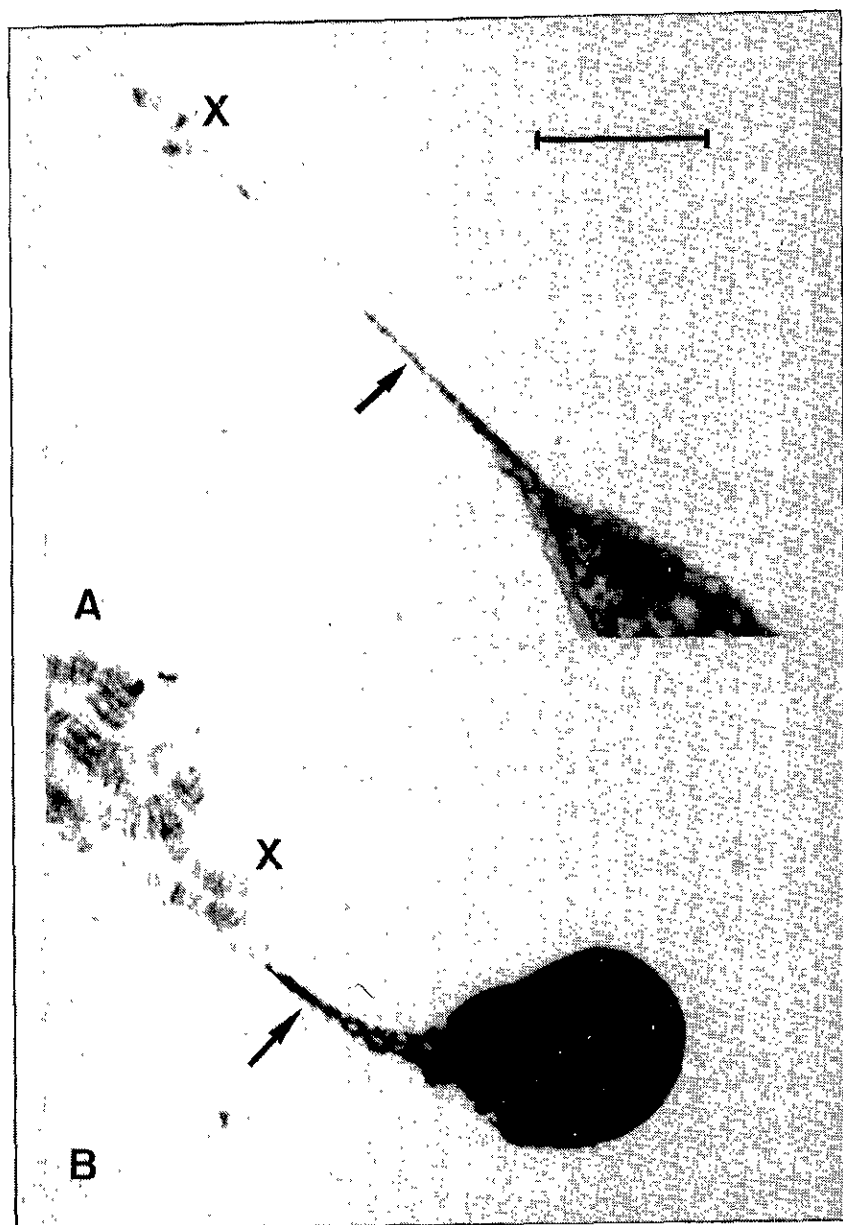


Figure 1 - A. *D. mulleri* male cell: Ag-stained nucleolus and threads (arrow) attaching the X chromosome to the nucleolus. B. *D. mulleri*/*D. aldrichi* hybrid female: Ag-stained nucleolus and threads (arrow) attaching a single X chromosome (from *D. mulleri*) to the nucleolus. Bar in each figure represents 10 μ m.

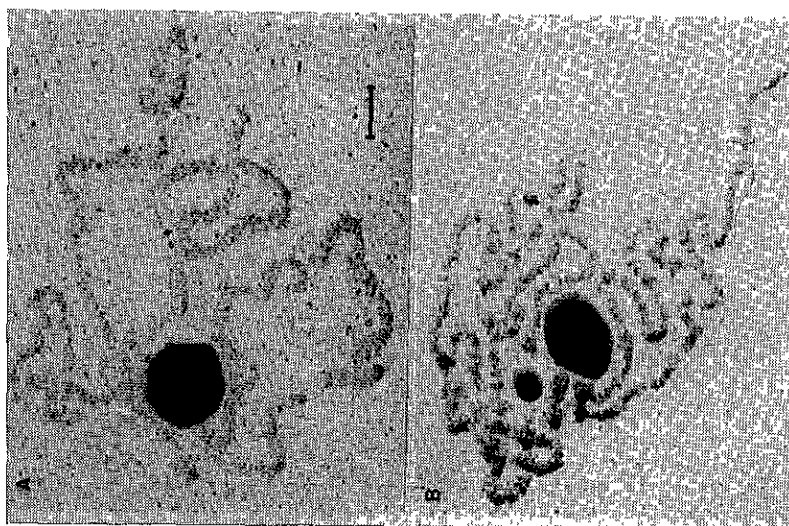


Figure 2 - A and B. *D. mulleri* female: salivary gland cells exhibiting one single nucleolus (2A) and one additional nucleolus (2B).

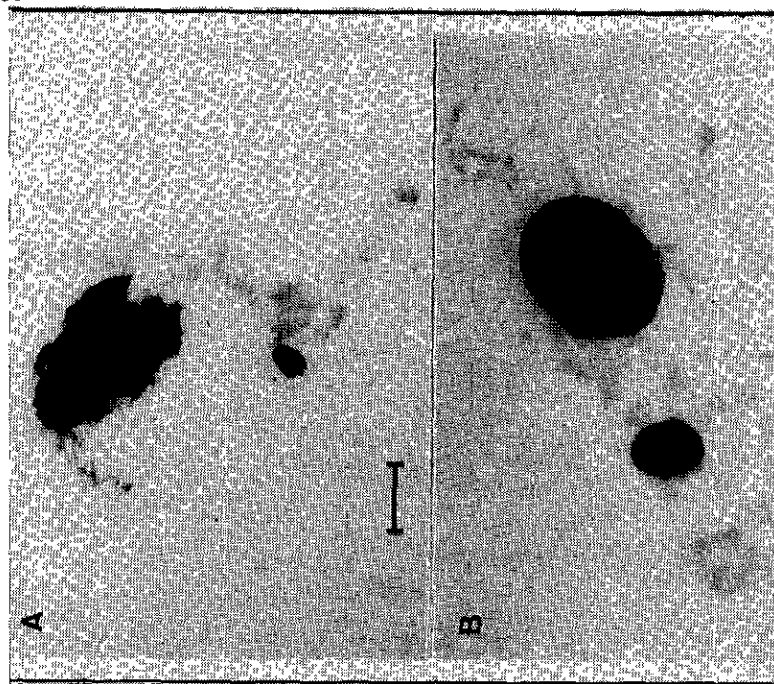


Figure 3 - A and B. *D. aldrichi* male: two salivary gland cells showing one additional nucleolus of different size.

species analyzed some slides showed one or two cells in which, besides one apparently normal nucleolus, another (rarely two) of small size was present (Figure 2). Intraspecifically the additional nucleolus shape followed that of normal nucleoli, being round or oblong. Among the 6 species analyzed, *D. aldrichi* exhibited the highest frequency of cells per larva containing the additional nucleolus: 5 or 6 salivary gland cells in several larvae (Figure 3).

In hybrids the frequency of larvae and cells per larva containing an additional nucleolus increased in comparison with the parental species. In some cells two of these extra organoids were present. Hybrids between *D. mulleri* and *D. aldrichi* showed the highest percentages of cells with one or two extra nucleoli; in some individuals this percentage attained 50% of the salivary gland cells. In these hybrids the large and the small nucleoli (mainly the small ones) were very variable in size and shape (Figure 4). Next in decreasing order of frequency of cells bearing additional nucleoli were the hybrids between *D. mulleri* and *D. wheeleri* with about 5 cells exhibiting one or two additional nucleoli in some larvae. In the remaining hybrids the frequency was lower: one or two cells in few larvae; in every case one single additional nucleolus per cell was found.

In hybrids between *D. mulleri* and *D. mojavenensis* the nucleolus frequently had a very irregular shape (Figure 5).

Recognition of the chromosome to which the additional nucleoli were attached was not possible in many cases mainly because Ag-staining techniques markedly affect the chromosomal structure. When recognition was possible, the small additional nucleoli were seen in association with the X chromosome or with the microchromosome. For example, in some cells of hybrid females from both directions of crosses between *D. mojavenensis* and *D. arizonensis* the additional nucleolus was observed in association with the microchromosome (Figure 6); in hybrid males of crosses between *D. mulleri* and *D. mojavenensis* it was found attached to the X chromosome (Figure 7); in a cell of a female hybrid between *D. mulleri* and *D. wheeleri* it was apparently associated with the *D. wheeleri* X chromosome whereas the great nucleolus was attached to the *D. mulleri* X chromosome (Figure 8).

Another aspect observed was a variation in the degree of compactness of the nucleolar structure. Compact and sponge-like nucleoli were found in the same gland, sometimes in neighboring cells (Figure 9).

Our observations suggested that besides the degree of chromosome polyteny (and consequently the nucleus and cell sizes), the degree of compactness of the nucleolus structure is involved in the variation of nucleolus size

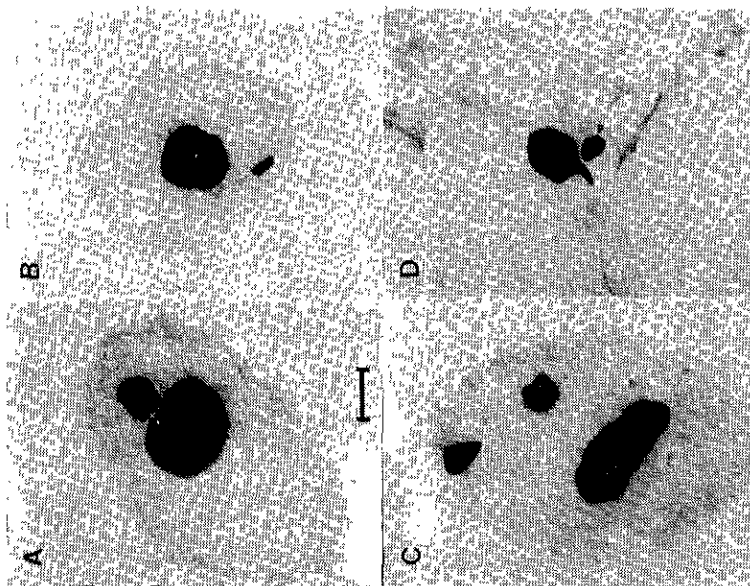


Figure 4 - A-D. *D. mulleri*/*D. aldrichi* hybrid female: Cells varying as to the number, shape and size of the additional nucleoli. The large nucleolus also varies in size and shape.

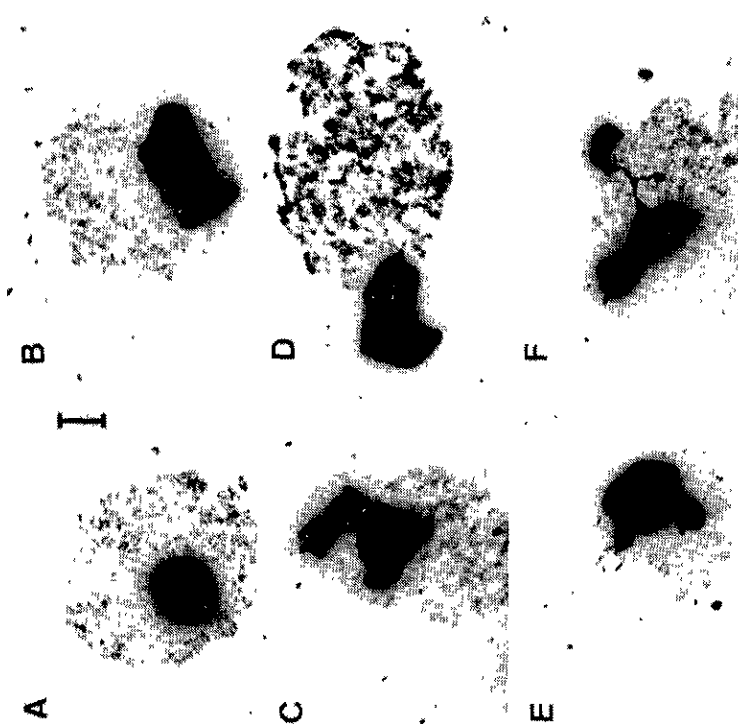


Figure 5 - A-F. *D. mulleri*/*D. mojavensis* hybrid male. Cells in the same salivary gland exhibiting a nucleolus of normal shape (5 A) and a nucleolus of very irregular shape.

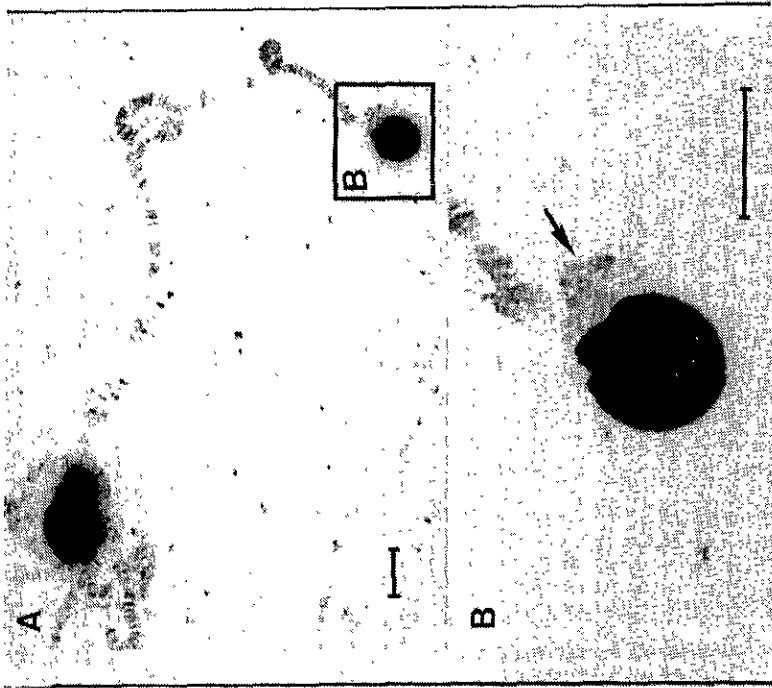


Figure 6 - A-B. *D. mojavensis* hybrid female. A. Chromosomal set showing the additional nucleoli attached to the microchromosome; B. Detail of A.

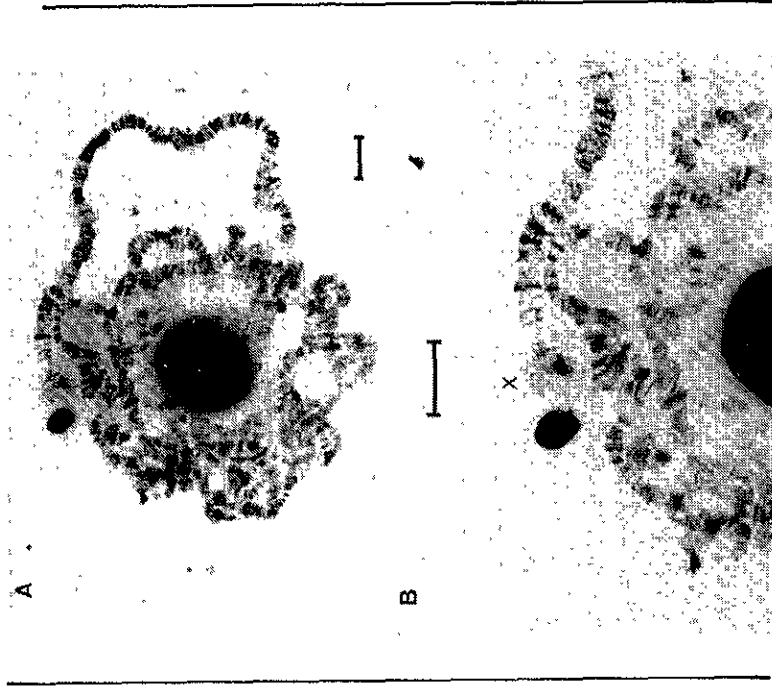


Figure 7 - A and B. *D. mulleri*/*D. mojavensis* hybrid male. Chromosomal set showing the additional nucleoli associated with the X chromosome. B. Detail of A.



Figure 8 - A and B. *D. mulleri*/*D. wheeleri* hybrid female. A. Unichromosomal association of the single nucleolus with the *D. mulleri* X chromosome. B. The large nucleolus is associated with the *D. mulleri* X chromosome and the small nucleolus (arrow) with the *D. wheeleri* X chromosome.

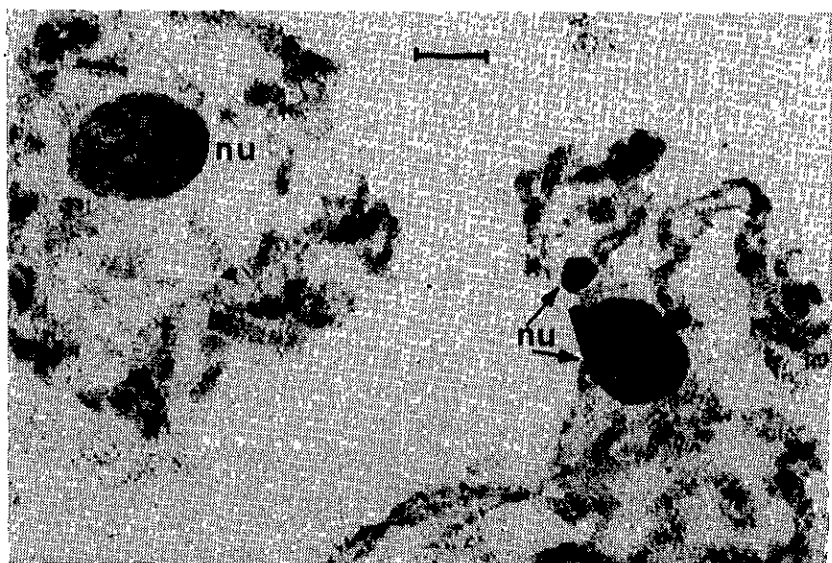


Figure 9 - *D. mulleri*/*D. mojavensis* hybrid female. Two neighbouring cells exhibiting a sponge-like nucleolus (at left) and a compact nucleolus (at right). Cell at right has an additional nucleolus.

in the parental species and hybrids, the sponge-like nucleoli being in general larger than the compact ones. Our observations also suggested that in general hybrids have larger nucleoli than the parental species. In order to test these observations, nucleolar areas were measured in late third instar larvae, in salivary gland cells bearing chromosomes with apparently similar degrees of polyteny. In Table I the mean nucleolar areas of cells with one single nucleolus are shown separately for (a) parental species and hybrids, respectively numbered from 1 to 11 and from 12 to 22, and (b) structurally compact and sponge-like nucleoli.

In cells carrying a single compact nucleolus, the mean nucleolar areas for flies from the same species and same hybrid or from different species and different hybrids were very variable. The analysis of variance calculated for species and hybrids gave X values of respectively $F_{9, 129} = 33.70$ and $F_{9, 139} = 24.97$ with $P < 0.005$. The high standard deviation values indicate that the nucleolar areas are also very different for salivary gland cells from the same fly.

These data also showed that the nucleolar areas from hybrids are in general larger than those from the parental species. When the mean of the 10 means for species and hybrids (respectively $192.68 \mu\text{m}^2$ and $305.94 \mu\text{m}^2$) were compared, the Student "t" value was significant at the 0.01 level ($t_{18} = 3.10$).

In the various species, sponge-like nucleoli were detected in a *D. mulleri* male, in two *D. mulleri* females, and in a *D. arizonensis* female. In all of the females the sponge-like nucleoli occurred side by side with compact nucleoli, the mean area of the first kind of nucleoli being between 1.5 and 2-fold the mean area of the nucleoli of the second kind. In the *D. mulleri* male all cells had sponge-like nucleoli and their mean was about 2.7-fold the mean of the means for compact nucleolus of *D. mulleri* flies ($208.03 \mu\text{m}^2$).

In hybrids, sponge-like nucleoli were detected in 3 females: a *D. mulleri*/*D. aldrichi*, a *D. mulleri*/*D. mojavensis* and a *D. arizonensis*/*D. mojavensis*. In the first fly the single sponge-like nucleolus had an area about 1.7-fold the mean area of the compact nucleoli in the same larva. In the second hybrid also a single sponge-like nucleolus was detected, but it was very large: its area was more than 7-fold the mean area of the compact nucleoli in the same larva. In the third hybrid only sponge-like nucleoli were detected and their mean area was about 2.8-fold the mean of the means for compact nucleoli of the other hybrids of the same species. Thus, although the number of sponge-like nucleoli found was low, the observations suggested that nucleoli with this structure also have larger areas in hybrids than in parental species.

Table I - Means and standard deviations for nucleolus area (in μm^2) in cells of 22 parental species and hybrid flies carrying one compact or one sponge-like nucleolus. In parentheses, number of scored cells. Asterisks indicate absolute values for single cells.

Species or Hybrids		Sex	One compact nucleolus		One sponge-like nucleolus	
1.	MU	M	142.43 $\pm$ 34.30	(15)		
2.	MU	M			553.84 $\pm$ 88.33	(15)
3.	MU	F	181.78 $\pm$ 32.80	(16)		
4.	MU	F	355.94 $\pm$ 60.96	(7)	561.55 $\pm$ 161.02	(6)
5.	MU	F	151.97 $\pm$ 42.86	(15)	305.87 $\pm$ 87.68	(5)
6.	WH	F	73.04 $\pm$ 14.85	(15)		
7.	WH	F	160.06 $\pm$ 39.51	(15)		
8.	AL	M	218.50 $\pm$ 52.83	(16)		
9.	AR	F	189.28 $\pm$ 47.12	(16)		
10.	AR	F	285.68 $\pm$ 66.10	(9)	464.52 $\pm$ 118.82	(6)
11.	NA	F	168.16 $\pm$ 38.06	(15)		
12.	MU x AL	F	209.01 $\pm$ 41.12	(17)		
13.	MU x AL	F	231.27 $\pm$ 68.16	(11)	*406.22	— (1)
14.	MU x NA	F	424.03 $\pm$ 65.94	(15)		
15.	MU x WH	F	340.30 $\pm$ 83.43	(16)		
16.	MU x WH	F	298.66 $\pm$ 58.18	(15)		
17.	MU x MO	M	250.80 $\pm$ 44.34	(16)		
18.	MU x MO	M	442.19 $\pm$ 71.35	(14)		
19.	MU x MO	F	366.12 $\pm$ 85.14	(15)	*2361.13	— (1)
20.	AR x MO	M	205.28 $\pm$ 35.88	(16)		
21.	AR x MO	F			720.48 $\pm$ 41.94	(13)
22.	MO x AR	F	291.74 $\pm$ 88.56	(14)		

The additional nucleoli showed a compact structure in every case.

Mean nucleolar areas for cells carrying one or two additional nucleoli are presented in Table II. Considering cells with compact nucleoli, nucleolar

Table II - Mean or absolute nucleolus area for cells bearing one or two additional nucleoli, given separately for cells with compact and with sponge-like nucleoli. $>$ = area of the large nucleolus; $<$ = area of the small nucleolus or nucleoli, t = sum of the large and small nucleolus areas. N = number of cells scored. Numbers for the parental species and hybrid flies correspond to those in Table I.

Species or Hybrids	Sex	Number of additional nucleoli	Compact nucleoli			Sponge-like nucleoli		
			t	$>$	$<$	t	$>$	$<$
1. MU	M	2	156.94	113.13	44.00	(2)		
5. MU	F	1				410.66	382.22	28.44 (1)
8. AL	M	1	212.21	177.11	35.10	(2)	461.43	414.44 (2)
10. AR	F	1	256.89	225.33	31.56	(1)		
11. NA	F	1	175.83	156.22	19.61	(2)		
13. MU x AL	F	1	308.98	261.42	47.56	(10)	548.44	463.56 (1)
14. MU x NA	F	1	215.56	162.67	52.89	(1)		
18. MU x MO	M	1	360.44	333.78	26.67	(1)		
22. MO x AR	F	1	520.15	420.29	41.57	(3)		

areas suggested that intraspecifically the total area (sum of the areas of nucleoli present in a cell) follows approximately the area of the single compact nucleolus in the same fly. For example, the mean total nucleolar area calculated for the *D. aldrichi* male (number 8 in Tables I and II) was $212.21 \mu\text{m}^2$, close to $218.50 \mu\text{m}^2$ obtained for the single compact nucleolus in the same larva. The results were similar for the *D. arizonensis* female (number 10 in the same Tables), for the *D. navojoa* female (number 11) and also for the *D. mulleri* male (number 1) whose cells were carrying two additional nucleoli.

In hybrids, however, the total nucleolar area of cells with additional nucleoli did not follow the mean areas of the single nucleolus. The same was true for sponge-like nucleoli, intraspecifically or in hybrids.

For cells carrying compact nucleoli the mean areas of the additional nucleoli in percentages of the total nucleolar areas were as follows: 14.00% (fly 1), 16.54% (fly 5), 12.28% (fly 10), 11.15% (fly 11), 15.39% (fly 13), 24.53% (fly 14), 7.40% (fly 18) and 7.99% (fly 22). Variation was thus greater among hybrids (flies 13 to 22). For cells with sponge-like nucleoli, the percentages were: 6.92% (fly 5), 11.27% (fly 8) and 15.48% (fly 13).

DISCUSSION

The staining pattern described here for most of the salivary gland preparations was expected on the basis of previous studies which indicated that silver in staining techniques for NORs does not bind to rDNA, but to proteins associated with rRNA: only the nucleolar material and the threads attaching it to the NOR-bearing chromosomes (presumably the nucleolar material around the threads) exhibited the differential staining. Besides, it has been known since Ruzika (1891, in Hubbell *et al.*, 1979) that nucleoli are stained in cytological preparations with silver nitrate.

The clear discrimination of the nucleolar material and the threads which associate it with the chromosomes propitiated in the present study a detailed analysis which reinforced previous observations made on preparations stained with lacto-acetic orcein, indicating that the X chromosome and the microchromosomes are involved in the nucleolar organizing activity of the species studied (Bicudo and Richardson, 1977; Bicudo, 1979). Besides, the staining of threads attaching one single X chromosome to the nucleolus in hybrid females reinforced the idea (also indicated by the above mentioned investigations) that a nucleolar dominance occurs in these hybrids.

The easy recognition of the Ag-staining nucleolar material also made it possible to analyze the number, shape and size of nucleolar bodies. Small bodies Ag-stained as the nucleolus confirmed the existence of additional nucleoli in some salivary gland cells of the species and hybrids studied. They were two at the most and were previously observed in studies in which preparations stained with lacto-acetic orcein were used (Bicudo and Richardson, 1977; Bicudo, 1979). In these studies they were termed nucleolus-like bodies because there was no certainty as to their nature.

Why are these additional nucleoli present in some cells and why do they have a smaller size? It is already known that NORs in a cell may associate to form a single large nucleolus or every NOR may work separately to form so many smaller than the single but equal-sized nucleoli as the NORs (e.g. Ferguson-Smith and Handmaker, 1961; Henderson *et al.* 1973; Macera and Bloom, 1980). In the present study some observations support the hypothesis that additional nucleoli are independently produced by activated secondary and/or recessive NORs which are supposed to be normally repressed. For example, in hybrid females between *D. mulleri* and *D. wheeleri* the small silver-stained additional nucleolus was associated with the *D. wheeleri* X chromosome which has the recessive NOR according to previous data (Bicudo, 1979), whereas in female hybrids between *D. mulleri* and *D. mojavensis* it was found in association with the microchromosome, considered a secondary NOR in the species studied (Bicudo and Richardson, 1977; Bicudo, 1979). Both kinds of associations were also previously observed in salivary gland preparations of the same species and hybrids, stained with lacto-acetic orcein, and also in female hybrids between *D. mulleri* and *D. aldrichi* in which the additional nucleolus (nucleolus-like) was attached to the *D. aldrichi* X chromosome, bearer of the recessive NOR.

Some results in the literature reinforce this hypothesis: the association of a small nucleolus with the recessive NOR while a normal size nucleolus was attached to the dominant NOR was previously described for cells of hybrids *Ribes nigrum*/*R. grossularia* (Keep, 1960, 1962).

One observation in this study does not seem to be in accordance with the hypothesis that additional nucleoli are due to activation of secondary and/or recessive NORs. In hybrid males obtained in crosses between *D. mulleri* females and *D. mojavensis* males, in which the *D. mulleri* X chromosome (in the absence of the *D. mojavensis* X) apparently operates as the main NOR (Bicudo, 1979), the additional nucleolus was attached to it. Another observation made on preparations stained with lacto-acetic orcein

which apparently disagrees with the hypothesis under discussion, was the finding of "nucleolus-like" bodies in association with the amplified microchromosome in male hybrids between *D. mulleri* and *D. arizonensis* (Bicudo, 1977). The amplified microchromosome apparently operates as the main NOR in these male hybrids. However, these cases could be explained by the existence of some cell autonomy in the nucleolar organizing activity which was previously indicated in the same hybrids: although for every hybrid there is a predominant cell behavior as to the dominant NOR, some variation was observed among cells (Bicudo, 1979). Cell variation in nucleolar dominance was also described for hybrids between *D. melanogaster* and *D. simulans* (Durica and Krider, 1977).

In turn, the small size of the additional nucleolus could be indicative of (1) a poor activity of the rDNA cistrons or (2) a smaller number of rDNA cistrons present in the NORs which produce them. These hypotheses reflect two possibilities for control of NOR activity in the species and hybrids studied: respectively the "silencing" of recessive or secondary NORs at the transcriptional level, turning off rDNA cistrons; or their "silencing" as a consequence of control of rDNA replication in the polytenization process. According to the first hypothesis, the "silent" recessive or secondary rDNA could partially escape from the regulatory mechanism which turns it off at the transcriptional level, and could operate at a lower degree producing the small additional nucleoli. In the second hypothesis, additional nucleoli would be formed in cells in which some replication of rDNA in recessive or secondary NORs had occurred.

Evidence in support of the first hypothesis for recessive NORs is derived from studies on *D. melanogaster*/*D. simulans* hybrids, in which the nucleolar organizer of the first species is dominant over that of the second (Durica and Krider, 1977) and also on hybrids between *Crepis capillaris* and *C. dioscorides*, in which the first species is dominant over the second (Doerschug *et al.*, 1976). In both cases measures of the rDNA content in the parental species and hybrids indicated that suppression of the recessive NORs occurs at the transcriptional level. On the other hand, there is also some evidence for differential replication, in the polytenization process, of the rDNA cistrons from different NORs in *D. melanogaster* (Endow and Glover, 1979), which could support the second hypothesis. In addition, in several other instances a correlation was described between the number of rRNA genes and the size of the nucleolus (e.g. Miller and Brown, 1969; Miller and Knowland, 1970; Sinclair *et al.*, 1974; and Flavell and O'Dell, 1979) as well as between transcriptional level and nucleolus size (Sinclair *et al.*, 1974).

The available evidence is not sufficient to choose between these hypotheses. Anyway, the present study indicates that the nucleolus size is regulated in the species studied. In spite of the scarce number of cells bearing additional nucleoli scored, the values for total nucleolar areas were close to the means for cells from the same fly, bearing a single nucleolus. This intraspecific control of nucleolar size was expected in view of the fact that nucleolar size has been correlated with the rate of rRNA synthesis (e.g. Kurata *et al.*, 1978).

Although devoid of interpretation at the present level of our observations, nucleolus structure was found to be related to nucleolar size. Sponge-like nucleoli were considerably larger than compact ones, regardless of their occurring in the same or in different flies. However, even considering cells with apparently similar degrees of polyteny and nucleolar compactness, some variation in mean nucleolar areas was detected among flies from the same or different species and from the same or different hybrids. Nucleolar areas also varied among different salivary gland cells from the same fly.

Additional nucleoli were found in salivary gland cells of the species and hybrids, but the frequency of larvae and cells exhibiting them was greater in hybrids. Similarly, changes in the normal round and oblong shape of nucleoli were more frequent in hybrids. Also in hybrids, the mean nucleolar areas were predominantly greater than in the parental species and the similarity of the nucleolar areas between cells with one or more nucleoli, observed intraspecifically, was not detected. These observations show that the inter-specific chromosome interactions related to nucleolar formation are inadequate. Such inadequacy had already been revealed for the same hybrids by the switching on of regulatory mechanisms including activation paralleled by DNA amplification of a secondary nucleolar organizer (the microchromosome) to operate as the main NOR in some hybrid males (Bicudo and Richardson, 1977; Bicudo, 1979). All of these findings indicate that the species studied diverged in the evolutionary process as to the elements involved in the chromosome interactions for nucleolar organizing activity.

ACKNOWLEDGMENTS

I thank Dr. Iris Ferrari for reading the manuscript, Dr. Celso A. Mourão for helpful discussion, and Dr. Carlos Daghljan for checking the wording. This research was partially sponsored by Fundação de Amparo à Pesquisa – FAPESP (Grants 70/562 and 76/1227) and Conselho Nacional do Desenvolvimento Científico e Tecnológico – CNPq (Grant 2222.1422/77).

RESUMO

Técnicas de coloração pela prata aplicadas a preparações salivares de 6 espécies de *Drosophila* do complexo *mulleri* coraram diferencialmente os nucléolos e os filamentos que se associam aos cromossomos nucleolares. A associação entre cromossomos e nucléolos, *tornada assim facilmente visível, confirmou observações anteriores feitas em preparações coradas pelaorceína lacto-acética quanto ao envolvimento dos cromossomos X e dos microcromossomos na atividade organizadora nucleolar das espécies estudadas. Além disso, essas técnicas de coloração revelaram modificações em tamanho, forma e número dos nucléolos dos híbridos, reforçando e ampliando observações anteriores segundo as quais a hibridação das espécies em estudo perturba a síntese nucleolar de várias maneiras.*

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(Received March 13, 1981)