Absence of resistance genes against male-killing action of the SRO in *Drosophila melanogaster*

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(Received December 12, 1984)

ABSTRACT

Males of *Drosophila melanogaster* bearing various X chromosome segmental aneuploidies were tested for sensitivity/resistance to the male killing action of the Sex Ratio Organism (SRO). It was found that there is no region on the X chromosome which makes, when duplicated, males resistant to the SRO. A region which makes hetero-deficient females sensitive to the SRO also was not found.

Searches for a gene(s) that can give males resistance to the SRO action were made using the X chromosomes continuously mutagenized for seven successive generations, the second and third chromosomes obtained from a natural population (100 lines), and the cytoplasm of 100 isofemale lines and twenty-two mass lines from various natural populations. No such genes were recovered. A possible reason for the failure of recovering such a gene is discussed.

1. INTRODUCTION

The sex-ratio organisms (SRO) in *Drosophila* are transovarially transmitted spiroplasmas (Class Molicutes, Order Mycoplasmales) that cause a sex-specific lethality in early developmental stages (review, Williamson and Poulson 1979). Several SRO strains are known each derived from a single wild-caught female of four neotropical *Drosophila* species: *D. nebulosa* (NSRO), *D. willistoni* (WSRO), *D. equinoxialis* (ESRO), and *D. paulistorum* (PSRO). The SRO is found most abundantly in adult female hemolymph, and the injection of the SRO-laden hemolymph into normal females of the same species or different species of *Drosophila* generally establishes a permanent infection and results in male lethality. Thus most studies have employed *D. melanogaster* as an experimental host animal where extensive genetic manipulation is possible. The mechanism of male-specific lethality has been a center of the work on SRO, but it still remains largely unknown except for the facts that

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the SRO kills single-X individuals regardless of their phenotypic sex (Sakaguchi and Poulson 1963; Miyamoto and Oishi 1975; Watanabe 1975; Fujihara, Kawabe and Oishi 1978), that XO cells in gynandromorphs can survive only in small patches and the lethal focus is in the primordial mesoderm and/or nervous system (Tsuchiyama, Sakaguchi and Oishi 1978), and that the SRO affects diploid cells but not polytene or polyploid cells among identifiable cells in the early stage-derived embryo primary culture (Koana and Miyake 1983).

It would be most useful for such studies to obtain non-male-killing variants of the SRO and also mutants in Drosophila that resist the male-killing action. A non-male-killing spiroplasma strain is known in D. hydei (Ota et al. 1979), and a variant of NSRO, NSRO-A, is recently reported which has lost the male-killing action but otherwise retains the characteristics of NSRO (Yamada, Nawa and Watanabe 1982). Attempts have been made earlier to identify Drosophila species where the newly-introduced SRO establishes infection but without male-specific lethality. In all species examined successful infection is always accompanied with the male-killing (Williamson and Poulson 1979). A systematic search for genetic variants that allow the SRO infection but resist the male-killing action in D. melanogaster, on the other hand, has never been conducted.

In the present study, we have examined (1) whether there is a region on the X chromosome which when duplicated in the male or deficient (hemizygous) in the female can give resistance or sensitivity, respectively, to the action of SRO using segmental aneuploids, and (2) whether there is a gene(s) in the genome or in the cytoplasm that makes males resistant to the SRO's male-killing action using mutagenized chromosomes and stocks established from natural populations.

2. RESULTS AND DISCUSSION

(1) The X chromosome segmental aneuploids.

Miyamoto and Oishi (1975) earlier proposed, following their own observation combined with others' that only single-X individuals are affected by the SRO regardless of their phenotypic sex, that there would be a gene(s) on the X chromosome which is not dosage compensated and whose product, reacting with the SRO, is critical to the normal early development of a fly. According to their proposal, males with a certain segment of the X chromosome duplicated would survive and females heterozygous for a deficiency of that particular segment would be killed.

Duplications and deficiencies of X chromosome segments, each for a small region but together covering the entire X chromosome, were generated by using X;Y translocation stocks with different X chromosome breakpoints (Table 1). Experimental details are the same as those described by Stewart
and Merriam (1975, see their Figure 2), except that these stocks were infected by the SRO prior to the experiments and the SRO-infected females were used for crosses.

The results are shown in Table 2. For all of these segments, males with duplications appeared in the control but not in the SRO-infected experimental. Viabilities of females carrying deficiencies or duplications were generally in agreement with the results of Stewart and Merriam (1973). In about half the cases females heterozygous for deficiencies did not appear even in the control. However, whenever such females appeared they also appeared in the SRO-infected flies.

The region 11F2–6–12A1–12C was unique in that males with this region duplicated appeared at a low frequency. Thus this region was examined by a large-scale experiment (Table 2). Females heterozygous for a deficiency for this region also appeared at a low frequency, and the region was further examined by using five independent deficiencies: 10E1–11A7 by Df(1)KA6, 11D1–2–11F1–2 by Df(1)N12, 11D–12A1–2 by Df(1)c246, 12A6–7–12D3 by Df(1)HA92, and 12E1–13A5 by Df(1)KA9 (for these deficiencies see Craymer and Roy 1980). In all cases females heterozygous for the deficiency were viable in the control as well as in the SRO-infected flies (data not shown).

Among these deficiency-bearing stocks, Df(1)c246 is somewhat peculiar: c246/FM6 females infected with SRO-containing hemolymph rarely trans-
mitted SRO to their G, and hence it was rather difficult to obtain a c246 strain with the complete sex ratio condition. A complete SR strain also tended to "revert" to normal one-to-one sex ratio. It is probable that c246/FM6 females have a low viability and that a high concentration of SRO is toxic to them. Then, surviving females may usually have low SRO concentration which is insufficient to kill their sons. However, it is still possible that the chromosomal region 11D—12A1—2 has a special relationship to the SRO multiplication.

The region 1—3 was also examined in three subdivisions; females heterozygous for a deficiency for 2C1—3C4, generated by the use of T(1;2)wvco (Lindsley and Grell 1968), were quite viable in the SRO-infected flies as well as in the control, and so were the females heterozygous for a deficiency for 3C2—3E8, generated by T(1;2)w64d (Craymer and Roy 1980). Duplication males for these regions were viable in the control but lethal in the SRO-infected flies. No heterozygous deficient females were obtained for the region 1—3A.

Table 2. Effect of SRO on viability of flies with various X chromosome segmental duplications or deficiencies

<table>
<thead>
<tr>
<th>X chromosome segment</th>
<th>Number of flies*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Dp ♀</td>
</tr>
<tr>
<td>1—3D5—E1</td>
<td>S R</td>
</tr>
<tr>
<td></td>
<td>Cont.</td>
</tr>
<tr>
<td>3D5—E1—4B**</td>
<td>S R</td>
</tr>
<tr>
<td></td>
<td>Cont.</td>
</tr>
<tr>
<td>4B—5C</td>
<td>S R</td>
</tr>
<tr>
<td></td>
<td>Cont.</td>
</tr>
<tr>
<td>5C—8C</td>
<td>S R</td>
</tr>
<tr>
<td></td>
<td>Cont.</td>
</tr>
<tr>
<td>8C—9B</td>
<td>S R</td>
</tr>
<tr>
<td></td>
<td>Cont.</td>
</tr>
<tr>
<td>9B—11A6—7</td>
<td>S R</td>
</tr>
<tr>
<td></td>
<td>Cont.</td>
</tr>
<tr>
<td>11A6—7—11F2—6—12A1</td>
<td>S R</td>
</tr>
<tr>
<td></td>
<td>Cont.</td>
</tr>
<tr>
<td>11F2—6—12A1—12C</td>
<td>S R</td>
</tr>
<tr>
<td></td>
<td>Cont.</td>
</tr>
<tr>
<td>12B9—C1—12C***</td>
<td>S R</td>
</tr>
<tr>
<td></td>
<td>Cont.</td>
</tr>
<tr>
<td>12B9—C1—15A</td>
<td>S R</td>
</tr>
<tr>
<td></td>
<td>Cont.</td>
</tr>
<tr>
<td>15A—16C</td>
<td>S R</td>
</tr>
<tr>
<td></td>
<td>Cont.</td>
</tr>
<tr>
<td>16C—20</td>
<td>S R</td>
</tr>
<tr>
<td></td>
<td>Cont.</td>
</tr>
</tbody>
</table>

** : Results at 18°C. At 25°C no Dp ♀ were obtained even in the control.
***: Only for this segment Df ♀ were obtained (363 in the control and 0 in SR).
(generated by \(Dp(1; f)R\), Lindsley and Grell 1968) in the control as well as in the experimental. However, duplication males were viable in the control but not in the experimental (data not shown).

It is clear then that there is not a single locus or loci closely linked together and occupying a single region on the X as proposed by Miyamoto and Oishi (1975) which provides resistance to SRO. It is still possible, if not probable, that the genes as proposed are present on the X occupying widely separated sites.

(2a) **EMS treatment of Oregon-R stock.**

There may be multiple loci in the genome that respond to the SRO's male-killing action. If such loci were all on the X chromosome, we might be able to obtain by simple mutagenesis experiments an X chromosome that gives resistance to the male-killing action. An Oregon-R stock was treated with EMS according to the procedure of Lewis and Bacher (1968) using 30 males each for 7 successive generations as shown in Fig. 1. Lethal and sterile muta-

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![Diagram](image_url)

Fig. 1. Results of a continuous mutagenesis experiment in the search for X chromosome resistance gene against the male-killing action of SRO.
tions on the X chromosome would be automatically eliminated but other mutations would accumulate. Starting from the fourth generation, 20 males in each generation were taken and crossed with 30 females of ORNSR (an Oregon-R stock carrying NSRO, which had been established previously and showing complete absence of males in each generation). Female progeny were crossed with Oregon-R males to test for the appearance of males in the following generation. Numbers of progeny females and males thus obtained are shown in Fig. 1 in parentheses. No males appeared in G1, G2, G4, and G5, while about 3,000 females emerged in each generation. A single male was obtained in G6, but it proved to be non-resistant upon subsequent examination.

(2b) Survey of autosomes from a natural population.

We next made a search for a resistance gene against the action of SRO in the second and the third chromosomes. Males of D. melanogaster collected from a natural population at Katsunuma were individually crossed with females from a balanced stock, SM1(Cy)/Pm; TM3(Sb)/Pr (SM1 and TM3 are multiply inverted balanced lethal chromosomes of the second and third chromosomes, respectively, and Pm and Pr are dominant marker genes of the second and third chromosomes, respectively; see Lindsley and Grell 1968), and, using the routine methods for the isolation of chromosomes, the second and third chromosome sets were obtained in the F3 generation. One hundred sets of such II-III combinations were chosen for examination.

Three SM1; TM3 males from each of these sets were crossed with three 4-day-old females of ORNSR (Fig. 2a). The F1 progeny were separated according to their dominant markers into four classes and sexed (A, B, C and D in Fig. 2a). If a dominant gene giving resistance to the NSRO’s male-killing action was present, B and D males should appear when it was on the third chromosome and C and D males when it was on the second chromosome. The results are presented in the histogram in Fig. 2a and show that no such genes existed among the tested chromosomes, that is, all the tested lines produced no or almost no males.

Three wildtype F1 females (D in Fig. 2a) were taken from each line, aged for four days, and were backcrossed to the males of the balanced stock line (Fig. 2b). The F2 progeny, which gave four phenotypically different classes (E, F, G and H in Fig. 2b), were sexed. If a recessive gene resistant to the NSRO’s male-killing action was present, F and H males should appear when it was located on the third chromosome and G and H males when it was on the second chromosome. The results (histogram in Fig. 2b) show that 42 lines had at least some males in the F2 progeny. Among these male-appearing lines, 7 had males of only F and H classes, thus giving a possibility of the existence of a recessive resistant gene on the third chromosome. Others had
males of various classes but they always included $SM1; TM3$ (E) males. The results presented in the histogram were based on the pooled data. Parental flies had been transferred into new culture bottles at 2-day intervals for 4 times and the progeny had been counted in four successive broods. When the sex ratios (number of female progeny/total number of progeny) were examined according to the brood, five out of the 7 putative resistant lines showed complete absence of males by the fourth brood and thus they were not the ones with a resistance gene. Two other lines, K32 and K54, did not show complete sex ratio in this generation but they also did not have the resistance gene (see below).

We have chosen NSRO as the experimental material since it has the strongest known male-killing action. The established Oregon-R stocks carrying NSRO usually produce no male progeny even in the first brood (Oishi 1971).
Disturbances in the genotypes of flies by introducing alien genomes might have caused a delay in the rate of NSRO multiplication, and thus the delayed expression of male-killing. All 17 lines including K32 and K54, which showed less than 0.76 sex ratios in crosses shown in Fig. 2b, were reexamined. The F₁ females (D in Fig. 2a) were aged for 7 days before mating. None produced male progeny. It is clear that no genes were present which give resistance to the SRO’s male-killing action among 100 each of the second and the third chromosomes we examined, and in 100 sets of such II-III combinations. It is possible that these lines require the presence of a higher concentration of SRO to effect the male-killing. Alternatively they may be carrying genes that delay the multiplication of SRO so that the saturation level of SRO can be reached a few days later than normal.

(2c) Survey of cytoplasm.

The SRO-infected males are affected at early developmental stages though the actual lethal stage may be later (Williamson and Poulson 1979). That different sources of cytoplasm may carry some genetic factors against the male-killing action of SRO was next examined. One hundred isofemale stocks of *D. melanogaster* were established from the Katsunuma population, each derived from a single female inseminated in nature. Twenty-two mass stocks of *D. melanogaster* were also prepared each derived from several males and females in different populations: eleven stocks were from Japanese populations (Kitami, Tsuruga, Akayu, Okayama, Nagasaki, Shingu, Kochi, Hachijo, Ogasawara, Yakushima, and Iriomote) and eleven others were from foreign countries (Singapore, Hong Kong, Philippines, Taiwan, Yugoslavia, Nepal, Tahiti, Kenya, New Guinea 1, New Guinea 2, and Madagascar). About 0.1 μl each of hemolymph from ORNSR females was injected into test females, 10 for each stock. The injected females were individually placed in vials, mated, and they were transferred into new culture vials every two days until the 6th vials. In the next generation, flies were sexed for each vial to obtain the sex ratios in the 6 successive broods. Ninety-two isofemale stocks and 19 mass stocks were successfully examined. The sex ratio increased with the brood number in all cases and eventually became 1.0 (Figure 3). Stocks varied, however, with respect to the period needed to attain the complete sex ratio, which ordinarily is in the range of 3–4 days (Williamson 1969; Watanabe and Yamada 1977). They were from Rank 1 (R1) of rapid establishment (2 days) to Rank 5 (R5) of slow establishment (10 days) (average R3, 6 days). Numbers of isofemale stocks and mass stocks belonging to each Rank are shown in Fig. 3. Oregon-R was treated similarly as a control and was categorized as R2. Thus no cytoplasmic factors were detected modifying the action of SRO. Possibilities as mentioned above in (2b) remain here also. It should be noted that these examinations served at the same time as a search
That we were not able to recover any resistance genes in all these attempts does not of course prove that such genes do not exist. It may be that such a gene is present on the fourth chromosome or that several genes are responsible for the establishment of resistance and they are widely dispersed on one or more chromosomes.

Other possibilities, however, may be worth considering. We have been concerned with the male-specific killing effect of the SRO, and as such have been looking for a mutant that rescues males. There may be a gene(s) which is essential for the survival of males, and the action of SRO may be to inactivate the gene. Most mutations would act to inactivate the gene and such mutations would result in male lethality even in the absence of SRO. Male-specific lethals in *D. melanogaster* which are known to affect dosage compensation in males (Belote and Lucchesi 1980a; Okuno, Satou and Oishi 1984) may provide one such possibility, though they are all late-lethals contrary to the situation in the SRO-infected males. At least three male-specific lethals are known on the second chromosome and one on the third (Belote and Lucchesi 1980b; Uchida, Uenoyama and Oishi 1981) and the wildtype products of these loci are essential for males but not for females (Belote 1983). Consequently we may have to expect mutations in all of these loci such as to make them insensitive to the repressing action of the SRO.

We are grateful to Dr. K. Oishi of Kobe University for much help in the preparation of the manuscript. We thank Dr. J. R. Merriam of UCLA for providing us with the X–Y translocation stocks and unpublished information on cytological breakpoints and the Mid-America Drosophila Stock Center for supplying some X–Y translocation stocks. We also thank Y. Onuma and M. O. Okada for their skillful technical assistance.
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