A test of reciprocal X–Y interactions as a cause of hybrid sterility in *Drosophila*

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ELUCIDATION of the nature of the gene interactions that underly the sterility of interspecific hybrids is important in evolutionary biology[1,2]. The interactions between the heterospecific X and Y (or Z and W) chromosomes are often used as an explanation for two reasons. First, the fertility of the hybrids of the heterogametic sex is much more often affected than that of the homogametic sex (Haldane's rule[3]) and X–Y interactions are specific to the heterogametic sex. Second, sex chromosomes, especially the X chromosome, are often considered to be of special importance in determining the fertility of hybrids[1,2,4]. X–Y interactions have been addressed in studies of males with a heterospecific Y chromosome in a mixed genetic background[5-8]. A more stringent test of the X–Y interaction model requires each X chromosome sterility factor to be tested separately for its interaction with the Y chromosome in a homogeneous background of the pure species. Here we report such a test of the X–Y interaction model and conclude that X–Y interactions should not be assumed to be the only or even the most common cause of hybrid sterility.

Crosses between *Drosophila simulans* and its sibling species *D. sechellia* result in fertile F_1 females and sterile F_1 males, and thus follow Haldane's rule. At least three X-linked factors that cause male sterility when introgressed from *D. sechellia* into *D. simulans* have been mapped by linkage analysis[9]. We used an approach that relies on interspecific DNA variations as markers to construct a fine-scale physical map of X-linked sterility factors between *D. simulans* and either of its two sibling species, *D. mauritiana* or *D. sechellia*[10]. We constructed a stock with a Y chromosome introgressed from *D. sechellia* into a nearly pure *D. simulans* background (type A males in Fig. 1; for details of the construction of type A males, see ref. 11). The identity of the Y chromosome was further confirmed by probing genomic DNA from both sexes with the Stellate sequence[12], which hybridizes predominantly to the Y chromosome (Fig. 2). The fertility of type A males indicates the absence of sterility interactions between Y_{sec} (the Y chromosome of *D. sechellia*) and the genome of *D. simulans*. In fact, only after repeated mating to several females do type A males produce fewer offspring than the pure *D. simulans* males (N.A.J., unpublished results). The fertility rules out interactions between Y_{sec} and genes on X_{sim} (the X chromosome of *D. simulans*) but provides no information on the reciprocal interactions betwen X_{sec} and Y_{sim} because such gene interactions are often asymmetric for hybrid sterility[13,14].

In a parallel experiment, we attempted to introgress the Y chromosome of *D. simulans* into *D. sechellia* but were unsuccessful in producing fertile males with Y_{sim}. The lack of success suggests sterility interactions between Y_{sim} and *D. sechellia* genes ($P<0.01$ if Y_{sim} has no sterility effect; see the legend to Fig. 2). In other words, the reciprocal genotype of type A (with blank and solid bars reversed) appears sterile. In an independent study, Zeng and Singh (personal communication) succeeded in introgressing Y_{sec} into *D. simulans* by a different method, which also failed to yield *D. sechellia* males with an introgressed Y_{sim}. Because X_{sec} interacts with the *D. simulans* background (the sterile type B males of Fig. 1) and Y_{sim} apparently interacts with *D. sechellia* genes because it was non-introgressable, it is natural to formulate the null hypothesis that X_{sec} (or some segments of it) interact with Y_{sim}. An alternative hypothesis is that X_{sec} interacts with the autosomes of *D. simulans*[1] and Y_{sim} with the autosomes of *D. sechellia*[15] but X_{sec} and Y_{sim} do not interact with each other.

Our test of the null hypothesis of X_{sec}–Y_{sim} interactions consists of two steps. (1) Three different X-linked regions were individually introgressed from *D. sechellia* into *D. simulans* by repeatedly backcrossing hybrid females to *D. simulans* males for more than 15 generations[10]. Each introgression contains a factor(s) that completely sterilizes the males. Sterility must be the result of interactions between the small introgressed segment and the *D. simulans* genome because the introgressed segment itself does not cause sterility in its own *D. sechellia* background. An example of such an introgression genotype is shown in Fig. 1 (type B). (2) We then took advantage of the fact that the Y_{sec} chromosome is fully introgressable into *D. simulans* and replaced the Y_{sim} chromosome of the type B sterile males with the Y chromosome from *D. sechellia* (type C of Fig. 1). If sterility is caused by X-Y interactions, co-introgression of Y_{sec} with each X_{sec} sterility factor should lead to fertility rescue, or at least to a more advanced spermatogenic development.

The results are summarized in Table 1 for each of the three regions of the X chromosome, marked with the visible markers, *y* (*yellow*, 1B on the polytene chromosome map), *v* (*vermilion*, 10A) or *f* (*forked*, 15F), respectively. Co-introgression of Y_{sec} has no effect on the sterility caused by any of the three introgressions. The criteria for sterility are that no progeny are produced in test crosses and no motile sperm can be detected in the male's seminal vesicle. (Note that Y_{sec} alone does not

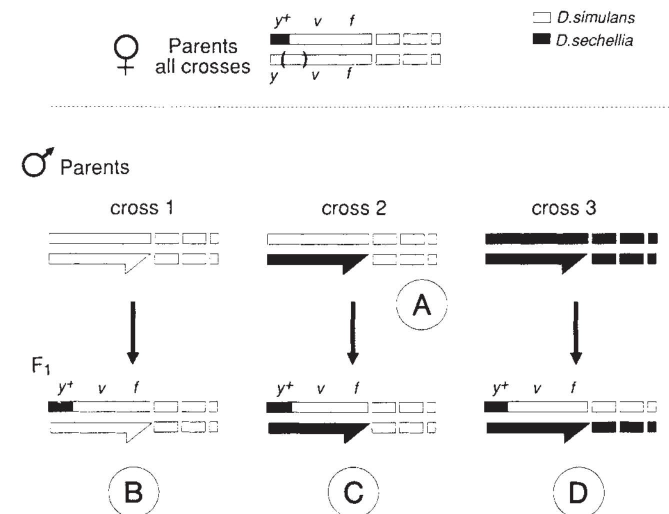

FIG. 1 Crosses generating males of genotypes B, C and D. These males are all sterile (their spermatogenic phenotypes given in Table 1), whereas type A males have normal fertility. The genotype of the female parent is the same for all three crosses and is given at the top. These females have been backcrossed to *D. simulans* males for at least 15 generations[10]. The X chromosome, marked with *y*, *v* and *f*, is drawn to a larger scale. The parentheses represent the breakpoints of an inversion at 2B and 8B. This inversion ensures that almost all y^+-bearing chromosomes in F_1 males carry the same introgression without undergoing crossing-over in that region[10]. Type A males, the male parent in cross 2, were constructed as described in ref. 11, crosses 1 and 2 were repeated for two other introgressions marked with v^+ and f^+, respectively. There are no inversions to prevent crossover in these two regions. For the *f* region, the *D. simulans* chromosome is marked with *g* (12B), *f* (15F) and *Bx* (17A) markers, thus ensuring the introgression spanning 12B and 17A would not be eroded by recombination. For the *v* region, we relied only on male sterility itself to ensure that the sterility factor is still present. Periodically, we used a single female parent to continue the cross in order to purge the stocks of heterogeneity in the size of introgression due to recombinations.

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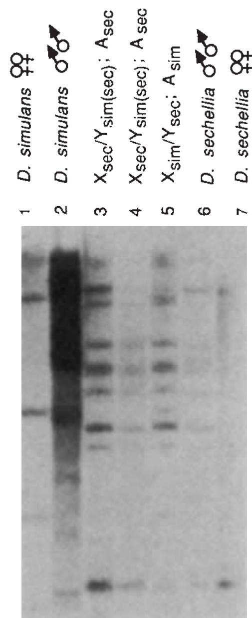

FIG. 2 Molecular identification of the introgressed Y chromosome. We used the Stellate sequence (plasmid pSX1.3 of ref. 12) to probe genomic DNA of *D. simulans* females (lane 1), *D. simulans* males (lane 2), *D. sechellia* males (lane 6), *D. sechellia* females (lane 7) and the three introgression lines described below. (The signals shown are largely Y-specific but some X-specific bands are also identifiable. These Y-specific signals are species-specific.) The X_{sim}/Y_{sec} males (lane 5) are *D. simulans* flies with an introgressed Y_{sec}, as described in ref. 11. The identity of the Y chromosome is confirmed to be of the *D. sechellia* origin. The $X_{sec}/Y_{sim(sec)}$ males (lanes 3 and 4; two independent lines) were expected to carry Y_{sim} but in fact carry Y_{sec}. These males were obtained by crossing $C(1)\,y\,w/Y_{sim}$; $(1/2A_{sim}, 1/2A_{sec})/A_{sim}$ females to *D. sechellia* males. $C(1)\,y\,w$ is the attached-X chromosomes of *D. simulans* and *A* denotes autosomes. These $C(1)\,y\,w$ females were from the F_2 generation of the cross of ref. 11. The expected genotype of their male progeny (F_3) is X_{sec}/Y_{sim}; $(3/4A_{sim}, 1/4A_{sec})/A_{sec}$. But because of possible nondisjunction between $C(1)\,y\,w$ and the Y chromosome in previous generations, a small fraction of the F_2 females may be $C(1)\,y\,w/Y_{sim}/Y_{sec}$ and so half of their F_3 sons could be X_{sec}/Y_{sec}; $(3/4A_{sim}, 1/4A_{sec})/A_{sec}$. Among 3,800 F_3 male progeny, possibly from such a mixture of F_2 females, only two were fertile. (The high incidence of sterility is due to interactions between the autosomes of *D. simulans* and either the X or the autosomes of *D. sechellia.*) Each of these two males were backcrossed to *D. sechellia* virgin females for seven generations to establish a pure line and then their Y chromosomes were examined for the Stellate genotype. The fact that both fertile males were X_{sec}/Y_{sec}, instead of the expected X_{sec}/Y_{sim} genotype, suggests that Y_{sim} causes male sterility when introgressed into the genome of *D. sechellia*. A statistical argument can be made as follows. (1) In F_3, X_{sec}/Y_{sim} males are estimated to outnumber X_{sec}/Y_{sec} males by 25-fold. (We obtained this estimate by counting the metaphase Y chromosomes in the neuroblasts of $C(1)\,y\,w$ females and found one out of the 25 females examined to have two Y chromosomes. The rate of *de novo* nondisjunction in these females is negligible because wild-type male progeny of $C(1)\,y\,w$ females are often 100% fertile.) (2) Because the background sterility interactions in F_3 males (that is between X and autosomes or between autosomes) are the same for both types, the probability of recovering a X_{sec}/Y_{sec} male should only be half of 1/25(=0.02), if the introgression of Y_{sim} does not cause sterility. Under that assumption, the probability that the only two fertile males recovered are both X_{sec}/Y_{sec} should be much less than $0.01(\gg 0.02^2)$. The results thus invalidate an earlier statement of ours in ref. 11 claiming the fertility of X_{sec}/Y_{sim}; A_{sec}/A_{sec} males. The observations provide a strong argument for molecular identification of introgressed materials.

METHODS. Genomic DNA was digested with *Xba*I, electrophoresed in a 0.8% agarose gel and blotted onto a Hybond-N membrane. Hybridization was done at 60 °C in 6×SSPE, Blotto and 1% SDS overnight. The membrane was washed for 15 min in 4×SSC and 0.2% SDS at room temperature, then 4×SSC and 0.1% SDS for 30 min at 37 °C and finally in 2×SSC and 0.1% SDS for 30 min at 37 °C.

TABLE 1 Fertility and spermatogenic development in males with genotypes shown in Fig. 1

	Genotype B	Genotype C	Genotype D
y region	Sterile Small deformed testes 3.40±1.43* (10) Much reduced number of spermatocytes	Sterile Small deformed testes 3.93±0.96* (36) Much reduced number of spermatocytes	Sterile Normally shaped testes 5.85±0.92 (22) Number and size of spermatocytes and spermatids normal†
f region	Sterile Normally shaped testes 3.93±0.73 (18) Smaller spermatocytes, number reduced	Sterile Normally shaped testes 4.90±1.24 (10) Smaller spermatocytes, number reduced	No data
v region	Sterile	Sterile	No data
Control (fertile males) 7.38±0.58 (20)			

These are the results of crosses in Fig. 1 repeated for three different regions of the X chromosome (see text). The numbers are the length of the testes±s.e., representing the maximal dimension of the testes in their normal coiled state. The units are in 0.1 mm. The numbers in parentheses are the number of testes, measured and observed cytologically. The control value is from the males of the *y, v, f* stock of *D. simulans*. The term spermatocyte refers to mature primary spermatocytes ready to enter meiosis. All descriptions are relative to the fertile males.

* The length of the deformed testes are overestimates of their actual sizes because these testes are not coiled and are much thinner (Fig. 3); hence, in comparison with normally shaped testes of equal length, their areas seen under a microscope are less than half of the normal ones.

† Occasionally, low numbers of motile sperm were observed in these males.

cause any spermatogenic defect in the *D. simulans* genome.) Because these criteria do not take into account the possibility of partial rescue of spermatogenic development in type C males, which remain sterile, we also compared the testes and spermatocytes in males of various genotypes (Table 1).

With the introgressed region marked with y^+ (wild type of *yellow*), sterility is characterized by very small testes as shown in Fig. 3*b*. The total volume is on average less than 20% of the normal ones. These testes do not have the normal coiled appearance (thus making its length measurement an underestimate of the severity of the defects). The mature primary spermatocytes, which are ready for meiosis, in the normal and type B males are shown in Fig. 3*c* and *d*, respectively. In general, there are very few mature primary spermatocytes in the testes of the sterile type B males. To show the difference, we selected a sterile sample that did have some spermatocytes (Fig. 3*d*), in contrast to one from a typical fertile male (Fig. 3*c*). The defect is clearly premeiotic and is much more severe than that in sterile F_1 males, which have normal-looking meiocytes (D.E.P., unpublished results). The important point is that, in type C males, neither the testicular morphology nor the spermatogenic development seems different from those of type B males. The testes of type C males are on average slightly larger, but the difference is not statistically significant.

Spermatogenic defects associated with the introgression of the *forked* region are less severe than those of the *yellow* region. The testes in type B males are shorter than normal but have the normal appearance and, hence, a volume closer to that of normal males. Mature spermatocytes are abundant, although smaller and not as numerous as normal males. As in the case of the *yellow* region, cytological observations on type C males are indistinguishable from those on type B males; thus the Y replacement has no observable effect on the spermatogenic development arrested by the f^+-linked factor.

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Ideally, the introgression in type B males should contain only one sterility factor. Preliminary results from molecular mapping are indeed suggestive, although not conclusive, of only one major factor in the *y* and *f* regions from *D. sechellia*[10]. If there are two independent sterility factors on the introgressed segment, our sterility test can only rule out that both factors interact with Y_{sim}. (For this reason, previous tests using whole X introgression, for example ref. 8, only ruled out that all X-linked factors interact with Y.) But the absence of rescue at the cytological level enables us to make a stronger inference: if there are two factors on the introgressed segment and one of them interacts with Y_{sim}, the replacement of Y_{sec} might, in some instances, improve the spermatogenic development. Because such a phenomenon was not observed in either of the two regions analysed, the possiblity for undetected X–Y interactions is reduced. A more direct line of evidence will be the confirmation of a single factor by molecular means, which is still in progress.

The question is: with what do the introgressed X-linked factors interact to cause male sterility? Because X-autosome interactions seem to be the most likely candidate, we constructed type D males as in Fig. 1. This is not a direct test as the autosomes introduced from *D. sechellia* for rescue create sterility on their own (by interacting with X or autosomes of *D. simulans*). Nevertheless, type D males with the y^+-region introgression have a closer to normal spermatogenic development than the corresponding type C males (Table 1). Although the mature spermatocytes and the spermatids (that is, the immediate products of meiosis) are usually not observed in type B or C males, they are readily observable in type D males. The difference between type C and D males is that type D males carry a copy of autosomes from *D. sechellia* (A_{sec}), which may provide the missing X_{sec}–A_{sec} interactions required for testicular development. The observation is consistent with the X-autosome interaction model proposed 50 years ago[1,2].

In the cross between *D. simulans* females and *D. sechellia* males, sterility does not result from the interaction between Y_{sec} and *D. simulans* genes, including X_{sim}, because it is possible to introgress Y_{sec} into an otherwise pure *D. simulans* background. In the reciprocal cross, several X_{sec} genes (the three sterility factors marked by *y*, *v* and *f*) interact with the *D. simulans* background, whereas Y_{sim} apparently interacts with *D. sechellia* genes (hence the non-introgressability of Y_{sim} into the *D. sechellia* background). Replacing Y_{sim} with Y_{sec} does not lessen the sterility effect of any of the three X_{sec} genes tested, implying that X-autosome and Y-autosome interactions are causing sterility rather than X-Y interactions. In *Drosophila*, the Y chromosome either has no effect on hybrid sterility[7,8] or, when it does, has not been shown to interact with the heterospecific X in a homogeneous genetic background. Inferences on the genic interactions of hybrid sterility from heterogeneous complex genotypes, such as the backcross F_2 hybrids, can be divergent even from essentially the same results; for example, between those in refs 1 and 6. In another study where Y causes sterility in a homogeneous background (A. C. Pantazidis, V. K. Galanopoules and E. Zouros, manuscript in preparation), its interaction was clearly demonstrated with an autosomal factor by means of fertility rescue. Although it has often been accepted[16,17], evidence for the X–Y interaction model of hybrid sterility has remained inconclusive. It is thus prudent not to emphasize X–Y interactions at the expense of other plausible models such as sex chromosome–autosome interactions. □

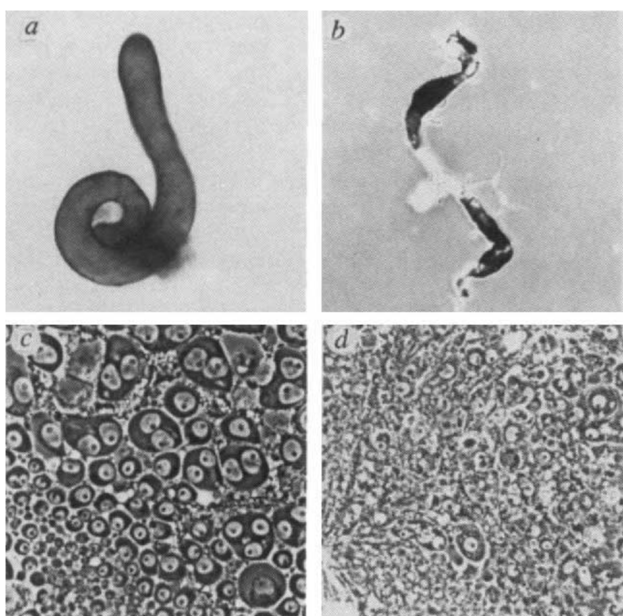

FIG. 3 A severe phenotype of male sterility resulting from the introgression of an X-linked factor. *a*, *c*, Whole testis and primary spermatocytes in pure *D. simulans* males. *b*, *d*, A pair of testes and primary spermatocytes in *D. simulans* males whose *yellow*-locus region is introgressed from *D. sechellia*.

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Cytosolic free calcium mediates red light-induced photomorphogenesis

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LIGHT is a primary environmental signal regulating plant growth and form[1]. The receptor phytochrome responds to red light and induces changes in membrane properties and gene expression[2,3] through an unknown transduction pathway which is the subject of intense study. Etiolated wheat leaf protoplasts swell in response to red light[4], by a mechanism believed to be similar to that involved in phytochrome-regulated leaf growth and unrolling[5–7]. Here we report that this physiological response is preceded by a transient increase in free calcium in the cytosol, followed by a decrease to below resting level. The timing of this response varied between protoplasts. Cytosolic calcium transients induced by photolytic release of calcium or inositol 1,4,5-trisphosphate from chemically caged forms[8–13] inside these protoplasts resulted in red light-induced increases in protoplast volume being mimicked. Our results support the hypothesis that phytochrome-mediated signals

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