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Genetic analysis of an *intersex* allele (ix^5) that regulates sexual phenotype of both female and male *Drosophila melanogaster*

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Summary

An allele of *intersex* (ix^5) of *Drosophila melanogaster* has been characterized. The genetic analysis of the allele demonstrated that like other point mutations of *ix*, the ix^5 allele also transformed diplo-X individuals into intersexes. The ix^5 mutation also affects the arrangement of sex comb bristles on the forelegs of males, although they had morphologically nearly normal male genitalia. They often fail to display a sustained pattern of courtship activity when tested. Orcein-stained squash preparations of testes from ix^5 males revealed a defect in spermatogenesis. Our results, taken together with those of McRobert & Tompkins (1985), indicate that the ix^+ gene also functions in male sex determination.

1. Introduction

In *D. melanogaster* sex determination is controlled by a set of genes that act together within a regulatory hierarchy to control sexual phenotype. At the bottom of the somatic sex determination hierarchy lie the *double sex* (*dsx*), *hermaphrodite* (*her*) and *intersex* (*ix*) genes that are responsible for implementing sexual differentiation.

Prior analyses of four alleles of *ix* (ix^1, ix^2, ix^3 and ix^4) by Baker and his co-workers (Baker & Ridge, 1980; Chase & Baker, 1995) had suggested that the *ix* gene is required to function with the female-specific product of the *dsx* gene to implement female sexual differentiation in diplo-X animals. However, a growing number of workers argued that *ix* may function not only with *dsx* to control many aspects of somatic sex, but also independently of *dsx* to regulate other aspects of somatic sex. McRobert & Tompkins (1985), for instance, noted that haplo-X ix^1 homozygotes exhibit decreased levels of male courtship behaviour. Chase & Baker (1995) observed that, while DSX proteins are capable of binding to the sex-specific enhancer site of the *yolk protein* genes, the product of ix^+ may not be required to achieve high levels of YP transcription. They therefore suggested the need to identify the spectrum of targets of *dsx* and *ix* genes to know precisely the role of these genes in sex determination in *Drosophila*. Recently, Keisman *et al.* (2001) noted that *dsx* regulates the anterior-posterior (A/P) organizer to control sex-specific patterns of growth in the genital imaginal disc of *Drosophila*. However, so far as our knowledge is concerned, the spectrum of targets of the ix^+ gene has never been critically analysed.

Earlier, Chatterjee (1994) isolated an allele of *ix*, ix^4 [described as ix^5 in this paper as Chase & Baker (1995) have already referred to one temperature-sensitive allele of *ix* as ix^4], and noted that the new mutant allele not only transforms diplo-X individuals into intersexes, but also alters the pattern of arrangement of the sex comb bristles of the haplo-X males. However, it was not clear whether the phenotype observed in ix^5 mutants was the consequence of partial loss-of-function of the allele, or the allele not reflecting the full spectrum of *ix* function. Alternatively, ix^5 might be a female-specific allele at the *dsx* locus, where null mutation transforms both diplo-X and haplo-X individuals into phenotypic intersexes. In view of these considerations, mapping and characterization of the allele are necessary.

Prior analysis of four different *ix* alleles by Baker and his co-workers (Baker & Ridge, 1980; Chase & Baker, 1995) had suggested that intersexual phenotypes of *ix* mutants are variable. Since a precise map of growth specificity of the genital disc derivatives of different *ix* mutants would help an understanding of the target tissues of the ix^+ gene in *Drosophila*, an

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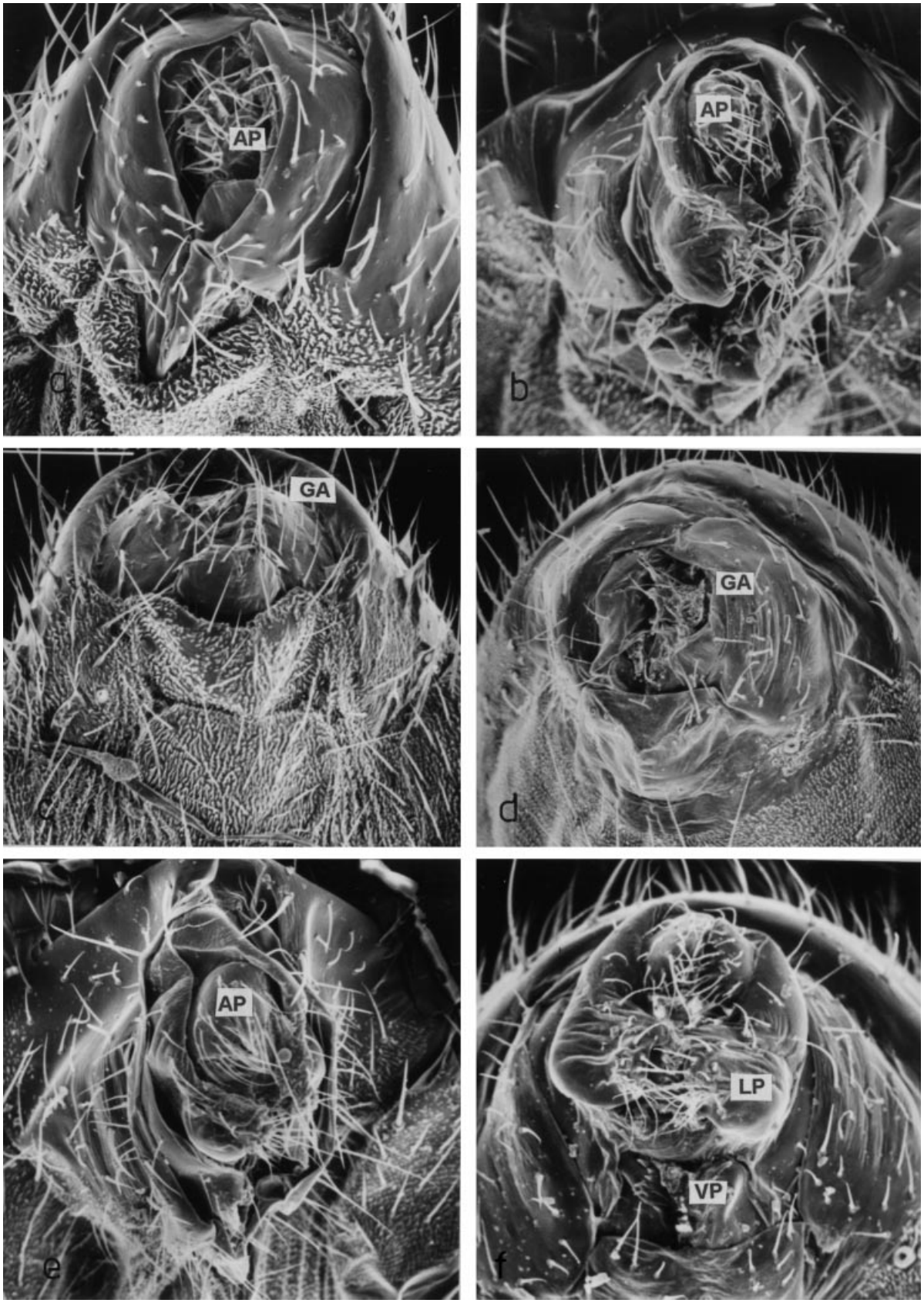

Fig. 1. Scanning electron micrographs showing the terminalia of: (*a*) an XX, ix^1/ix^1 individual; (*b*) an XX, ix^2/ix^2 individual; (*c*) an XX, ix^5/ix^5 individual, reared at 18 °C; (*d*), (*e*) two XX, ix^5/ix^5 individuals reared at 25 °C; and (*f*) an XX, ix^5/ix^5 individual reared at 29 °C. Note the robustness of the intersexual characters of the XX, ix^5/ix^5 individual reared at 29 °C. AP, anal plate; GA, genital arch; LP, lateral plate; VP, vaginal plate.

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attempt has also been made here to analyse the morphological phenotypes of adults with different *ix* alleles using scanning electron microscopy (SEM). SEM provides high resolution, large depth of focus and apparent oblique illumination which gives the impression of three-dimensional structure.

In this paper we have therefore made an attempt to map and characterize the ix^5 allele and to reassess the function of ix^+ in the sex determination pathway of *Drosophila*. Our results reveal that ix^5 is allelic to ix^1 and ix^+ also functions in the male sex determination pathway.

2. Materials and methods

(1) *Fly stocks*

(a) *The* ix *alleles*. ix^1 is a spontaneous *ix* allele first reported by Morgan *et al*. (1943) and employed by Kroeger (1959). We have used the following ix^1 stocks for the present investigation: (a) B^sY, *pr cn* $ix^1/SM5$; (b) B^sY, *pr cn* ix^1 *bw*/*SM5*; and (c) *y* sn^3, *pr cn* $ix^1/SM5$. ix^2 is a UV-induced *ix* allele first reported by Meyer (1958). Description of the two *ix* alleles can be found in Baker & Ridge (1980) and Chase & Baker (1995).

(b) *New allele of* ix. ix^5 is a spontaneous *ix* allele (Chatterjee, 1994) maintained in this laboratory as B^sY, *pr cn* $ix^5/SM5$. Salivary gland chromosome analyses indicated that the mutant flies are chromosomally normal.

(c) *Other fly stocks*. Other fly stocks include (a) *en lacZ*/*CyO*; (b) *Df* (*2R*) en^B [47E3-6; 48A4] which was obtained from Dr P. Sinha, Indore and (c) In (1) dl-49, *Sxl* $^{f\#1}$ v^{of} g^4, Dp (1;3) sn^{13al} which was obtained from Dr R. Nothiger, Switzerland.

The Oregon R strain, isogenic for the second and third chromosomes, obtained from our laboratory, was used as a wild-type strain.

Flies were raised on standard *Drosophila* food medium containing cornmeal, molasses, yeast and agar-agar. Propionic acid was added as mould inhibitor. Adults and all developmental stages were reared at 24 ± 1 °C. Descriptions of mutations and chromosomes can be found in Lindsley & Zimm (1992).

(ii) *Preparation of the specimen for microscopy*

Specimen were prepared for light microscopy as described by Szabad (1978) and mounted in Euparol. External genitalia and sex combs were examined under a compound microscope (Zeiss) using $\times 10$ ocular and $\times 40$ objective lenses. For SEM, the flies were sputter-coated with gold–palladium and viewed at 25 kV with a Hitachi S 530 microscope. Photomicrographs were taken whenever necessary.

(iii) *Preparation of testes squash*

Orcein-stained testes were prepared for light microscopy following the methods of Lifschytz & Harven (1977). Slides were examined under a Zeiss phase-contrast microscope.

(iv) *Behavioural assay*

Male courtship behaviour was assayed and the courtship indices (C.I.) were measured as described by McRobert & Tompkins (1985), except that very light anaesthesia with ether was used during initial collection of some adults.

3. Results

(i) *Localization*

Chase & Baker (1995) have localized the *ix* gene by examining the patterns of complementation of *ix* point mutations with a set of overlapping deletions. They noted that the *ix* gene is located to the cytogenetic interval between 47E3–6 and 47F11–18 of the salivary gland chromosome. Our results also reveal that ix^5 is complemented by *en lacZ* (48A), but not complemented by *Df* (*2R*) en^B (data not included). It seems, therefore, that ix^5 is allelic to ix^1. A similar range of intersexual morphological characteristics was observed for each of the *ix* alleles at 18 °C and/or 29 °C and independent of whether the mutation was homozygous or hemizygous (see below). These results clearly indicate that the ix^5 mutant does lie within the 47E3–6 to 47F11–18 region of the second chromosome.

(ii) *Phenotypes*

The morphological phenotypes of XX, ix^1/ix^1 and XX, ix^2/ix^2 are shown in Fig. 1*a*, *b*. As expected, these flies were phenotypically intersexes with simultaneous realization of both male and female developmental pathways. Furthermore, to assess whether the range of intersexual characteristics of XX, ix^5/ix^5 corresponds to the phenotype associated with those previously known *ix* mutations, morphology of terminal abdominal segments of the animals homozygous for the ix^5 mutant, raised at different temperatures, was examined using SEM.

Our results reveal that the increased robustness of the intersexual phenotype was largely, but not entirely,

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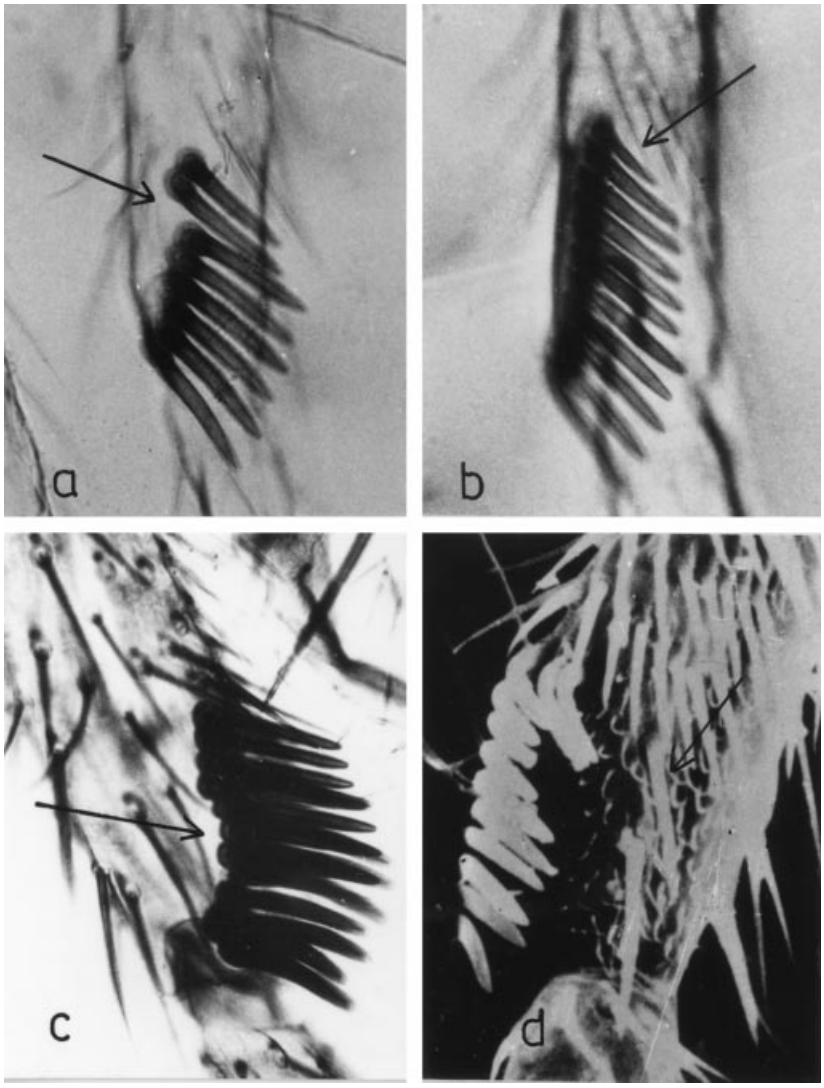

Fig. 2. Morphology of the foreleg basitarsus of XY, ix^5/ix^5 individuals. Note the arrangements of the sex comb teeth of the flies. (*a*) An example of the sex comb of foreleg where teeth were arranged in more than one row (arrow). (*b*) An example of sex comb of foreleg where some teeth were slender and pointed in appearance (arrow). (*c*) An example of irregular and clustered arrangement of the sex comb teeth (arrow). (*d*) An example of the arrangement of sex comb teeth and mechanosensory bristles (arrow) in XY, ix^5/ix^5 males, using SEM.

attributable to the effect of temperature (see below). As it appears from Fig. 1*c*, when XX, ix^5/ix^5 flies were reared at 18 °C, the sixth and seventh sternite bristles of some animals were female-like. The male derivatives of the genital disc, genital arch and phallus apparatus were poorly formed in the flies. The anal plates were laterally positioned as in males, but they remain fused (Fig. 1*c*). When the ix^5 flies were reared at 25 °C, intersexual characters were seen with variable phenotypes. For example, in some flies the terminal segments were not clearly differentiated (Fig. 1*d*). In these intersex flies, none of the male or female genital organs of the flies were developed and the anal plate remained undifferentiated. However, their segmentation pattern followed the male pathway although a genital-arch-like structure was developed (Fig. 1*d*). In some flies, simultaneous realization of both male and female developmental pathways was noted (Fig. 1*e*). When the flies were reared at 29 °C, both male and female genital primordia developed simultaneously and differentiated distinctive intersexual characters of the terminal abdominal segment of the body (Fig. 1*f*). In these individuals a rudimentary penis-like structure was noted. Curiously, it may be noted here that, as seen from the photomicrographs (Fig. 1*a–f*), the morphology of the bristles of the terminal abdominal segments of all intersex flies was abnormal, although the penetrance and expressivity were not the same. Together these data clearly suggest that the ix^5 allele represents a substantial, if not complete, loss-of-function mutation.

In XY, ix^5/ix^5 individuals sexual phenotypes are male except for the morphology of the bristles of the terminal abdominal segments.

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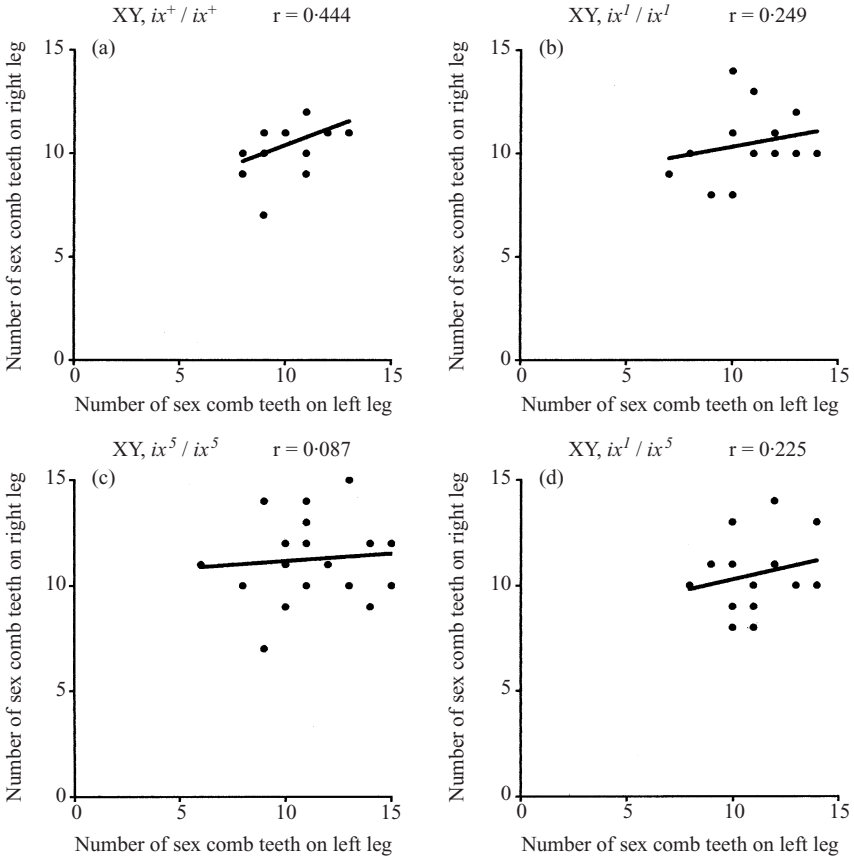

Fig. 3. Regression analysis of the number of right and left sex comb teeth of the forelegs of males with genotype (*a*) ix^+/ix^+, (*b*) ix^1/ix^1, (*c*) ix^5/ix^5 and (*d*) ix^1/ix^5.

(iii) *The genital disc*

When genital discs of XX, ix^1/ix^1 and XX, ix^5/ix^5 larvae were examined critically, it was noted that female and male genital primordia of the disc developed simultaneously in both types of flies. This observation further established the fact that the pattern of organization of the genital disc of XX, ix^1/ix^1 and XX, ix^5/ix^5 individuals is comparable. In this context, it may be noted here that in some larval genital discs of ix^5 individuals there was a loss of monolayer organization. Analysis of terminal abdominal segments of some (5–10%) XX, ix^5/ix^5 flies also showed that a large mass of chitinous outgrowth protrudes from the vaginal opening. The structure has also been noted earlier in XX, ix^1/ix^1 flies and referred to as a 'genital knob' (see Lindsley & Zimm, 1992).

(iv) *Male sex comb*

The effects of the ix^5 mutation on the arrangement of the sex comb on the foreleg of XY, ix^5/ix^5 males are shown in Fig. 2*a–d*. As it appears from the photomicrographs, in some males the sex comb teeth were arranged in more than one row (Fig. 2*a*). In some flies, sex comb teeth were slender and pointed in appearance (Fig. 2*b*). An irregular and clustered arrangement of the sex comb teeth was also noted in some flies (Fig. 2*c*). Closer examination showed that the structure and arrangement of small bristles (mechano-sensory bristles) near the sex comb areas were abnormal (Fig. 2*d*). It therefore appears that the ix^5 mutation causes disruption of cell lineages, at least in the sex comb area of males.

An analysis of the number of sex comb teeth of *ix* (ix^1 and ix^5) males further indicates that there was a positive correlation between the right and left side measurements. The left–right correlations of the comb tooth number in the ix^1/ix^1, ix^5/ix^5 and ix^1/ix^5 males were 0·249, 0·087 and 0·225 respectively (Fig. 3). The low correlation between left and right combs, coupled with the high variation for the trait (see Fig. 2*a–d*) in ix^5 males, clearly suggests that the product of the *ix* locus functions in a cell-autonomous manner in the foreleg, and is required in the leg disc after the resumption of cell division at pupariation for sexually dimorphic differentiation of the foreleg.

(v) *The internal reproductive organs, gonadal morphology and fertility*

As reported earlier in other *intersex* individuals, the morphology of the internal reproductive organs of

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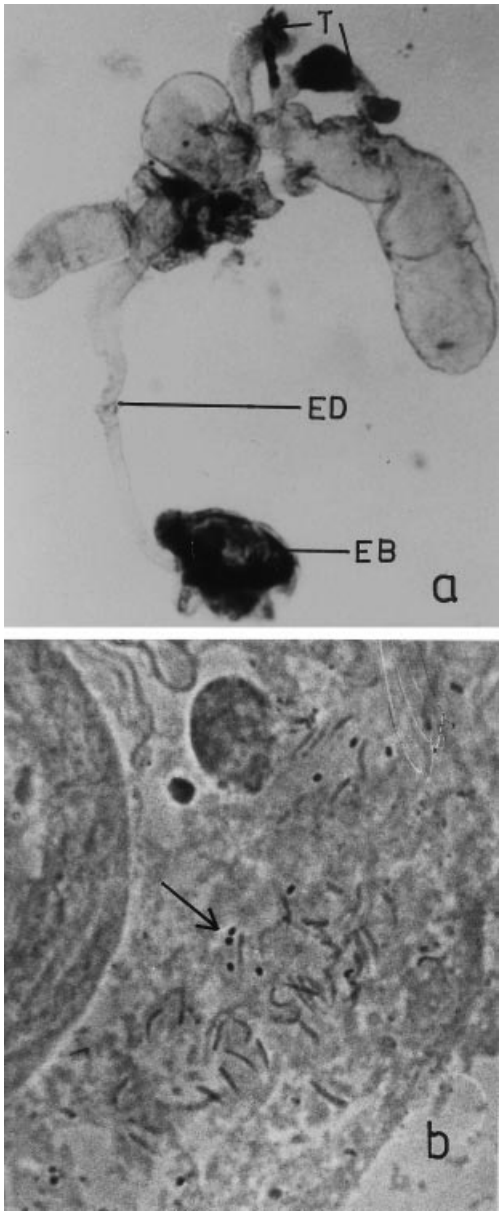

Fig. 4. Effects of ix^5 mutation on the internal reproductive system of XY, ix^5/ix^5 individuals at 29 °C. (*a*) An example of a rudimentary testis that was developed due to shifting the flies at restrictive temperature (29 °C). (*b*) Squash preparation of the testes shows spermatid heads of ix^5 males at restrictive temperature 29 °C. Note the abnormal round sperm head morphology (arrow) in XY, ix^5/ix^5 flies reared at 29 °C. EB, ejaculatory bulb; ED, ejaculatory duct; T, testis.

XX, ix^5/ix^5 individuals was also intermediate between male and female. However, the internal reproductive organs of the haplo-X, ix^5 individuals were comparable to those of the normal males. However, when ix^5 males were reared at restrictive temperature (29 °C) the size of the gonads was reduced considerably (Fig. 4*a*). These males were partially sterile in comparison with their control sibs. Orcein-stained squash preparations of testes from ix^5 males further showed that at restrictive temperature there was an increased frequency of dense, round sperm heads (Fig. 4*b*) instead of the long, narrow, condensed sperm heads of the wild-type males. It is possible that during spermatogenesis there may be a temperature-sensitive process that ix^+ regulates. Put another way, our results indicate that the ix^+ gene product is required at a well-defined stage of spermatogenesis, corresponding to the late primary spermatocyte–meiosis early spermatid interval.

(vi) *Sexual behaviour*

Previous behavioural analysis of haplo-X ix^1 homozygotes indicated that although these individuals are morphologically normal males they exhibit decreased levels of courtship behaviour (McRobert & Tompkins, 1985; Tompkins, 1998). We have also noted here that XY, ix^5/ix^5 males elicit much less courtship than their control sibs (data not included). On the other hand, the sexual behaviour of the diplo-X flies homozygous for the ix^5 mutation was almost female-like.

(vii) *Interaction with Sxl*

To see whether there is any interaction with *ix* and *Sxl* mutant alleles, female flies heterozygous for $Sxl^{f\#1}/Sxl^+$, ix^5/ix^5 were generated and examined under the microscope as appropriate. Our results reveal that, like other *ix* alleles, there was weak genetic interaction between the *Sxl* and ix^5 mutant. In fact $Sxl^{f\#1}$ has no great effect on the differentiation of sexually dimorphic tissues of haplo-X and diplo-X individuals carrying the ix^5 mutant.

4. Discussion

The genetic analysis of the ix^5 allele, as presented in this paper, demonstrates that, like other point mutations of *ix*, the ix^5 allele also transformed diplo-X individuals into intersexes. A comparison of morphological and behavioural phenotypes of homozygotes of ix^1, ix^2 and ix^5 indicates that these alleles of *ix* transform diplo-X individuals into intersexes. In addition, our data reveal that in ix^5 males, sex comb teeth were frequently arranged in more than one row. Morphologically, the sex comb bristles were less masculinized. The ix^5 mutant also affects the morphology of the mechanosensory bristles of the terminal abdominal segment of both male and female. These observations together lead us to believe that the *ix* mutant can also affect secondary sexual characters of haplo-X animals.

McRobert & Tompkins (1985) noted that haplo-X ix^1 homozygotes exhibit decreased levels of male

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courtship behaviour and that diplo-X ix^1 homozygotes behave as normal females. The data presented in this paper further provide evidence that, like ix^1 males, ix^5 males also exhibit decreased levels of courtship behaviour. We know that, like other aspects of sexual differentiation, sexual behaviour is governed by a hierarchy of sex regulatory genes (Burtis *et al*., 1991; see Tompkins, 1998). Mutations at these loci affect flies' courtship behaviour (Hall, 1994). However, we cannot rule out the possibility that the decreased level of courtship index relative to the Oregon R control is due to the abnormal arrangement of the sex comb teeth of ix^5 males, since both Cook (1977) and Coyne (1985) noted that males lacking the foreleg tarsal segment holding the sex comb have great difficulty in grasping female genitalia and subsequently have reduced male mating ability. This view was greatly strengthened when we noted that the row of bristles near the tip of the female ovipositor is anchored by the male sex comb bristles during copulation (Coyne, 1985). Thus, having evidence in hand that there are abnormal arrangements of the sex comb teeth in ix^5 males, it is not unreasonable to infer an indirect role of *ix* in regulating the sexual behaviour of males. Abnormal bristle morphology of the terminal abdominal segments of the ix^5 homozygotes (Fig. 1 *a–f*) supports this view.

Results of our study further reveal that in ix^5 males fertility is reduced significantly. At restrictive temperature (29 °C), ix^5 males developed as almost sterile males. However, when raised at permissive temperature (18 °C) they developed as sexually normal and fertile males. These data together provide evidence that normal function of ix^+ not only acts in the pathway with *dsx* to control morphology of diplo-X animals, but also acts in a separate pathway for sexual differentiation.

When the above analyses of the ix^5 allele are considered together with the results of previous epistatic analyses of ix^1 and ix^2 with *dsx*, *tra* and *tra-2* (Baker & Ridge, 1980; Chase & Baker, 1995) and the observation that the transcriptional profile of *dsx* is unaltered in heterozygous ix^1/ix^2, ix^1/ix^3 and ix^2/ix^3 genetic backgrounds (Nagoshi *et al*., 1988), ix^+ would appear to be positioned within the sex determination regulatory hierarchy at the same level as, or subsequent to, *dsx*. Data presented here further indicate that the ix^+ product is required not only to repress male differentiation function in diplo-X animals but also to function in haplo-X individuals in some aspects. The multiple roles of ix^+ represent the functioning of IX protein. It is possible that *ix*'s different functions are due to spatial and/or temporal control of either *ix*'s activity or the activities of factors that interact with other sex regulatory genes, including *her*, or its targets (Li & Baker, 1998). This is not a unique property of ix^+, since, for example, the *sisterless-b* (*sis-b*) gene also encodes a single protein which is necessary for the activation of the *Sxl* early promoter (Sxl_{pe}) and for the development of the peripheral nervous system, depending on when *sis-b* is expressed and what factors it interacts with (reviewed by Cline & Meyer, 1996; Villares & Cabrera, 1987). Investigations at the molecular level should lead to finer understanding of the role of the ix^+ product in the regulation of the sex determination process.

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