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Loss of Notum Macrochaetae as an Interspecific Hybrid Anomaly Between *Drosophila melanogaster* and *D. simulans*

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ABSTRACT

With the aim of revealing genetic variation accumulated among closely related species during the course of evolution, this study focuses on loss of macrochaetae on the notum as one of the developmental anomalies seen in interspecific hybrids between *Drosophila melanogaster* and its closely related species. Interspecific hybrids between a line of *D. melanogaster* and *D. simulans* isofemale lines exhibited a wide range in the number of missing bristles. By contrast, *D. mauritiana* and *D. sechellia* lines showed almost no reduction in bristle number in hybrids with *D. melanogaster.* Genetic analysis showed that the *D. simulans X* chromosome confers a large effect on hybrid bristle loss, although X-autosome interaction may be involved. This suggests that at least one genetic factor contributing to hybrid anomalies arose recently on a *D. simulans X* chromosome. Moreover, the results indicate sex dependency: the male hybrids were more susceptible to bristle loss than the female hybrids were. Use of cell type markers suggests that the defect does not lie in cell fate decisions during bristle development, but in the maintenance of neural fate and/or differentiation of the descendants of sensory mother cells.

ALTHOUGH one of the most important factors determining rates of DNA sequence evolution is the degree of selective constraint (Kimura 1983), "developmental constraints" (Alberch 1982) may shape the future evolution of morphology and developmental mechanisms of organisms. To some extent, the constraints come from the evolutionary history of a species. Indeed, Alberch and Gale (1985) showed that the different patterns of digital loss in the salamander and frog hind limbs are consistent with the sequence of digital differentiation: the most frequently affected digits tend to be the last ones to be formed—the fourth and fifth digits in salamanders and the first digit in frogs. On the other hand, highly complicated genetic systems connected with interactive networks probably define a very rugged multidimensional fitness landscape, showing the presence of many peaks, each separated by valleys, as represented by Wright's shifting balance theory (1931). Knowledge of genetic differences and evolutionary paths among closely related species, as well as distantly related species having distinct developmental mechanisms, is an important clue for understanding evolution at organismal and population levels.

Species differences can be revealed through inviability, sterility, and morphological anomalies of interspecific hybrids, even if there is remarkable morphological similarity between species. The genetic and molecular bases of these hybrid anomalies have been a long-standing topic in evolutionary biology. Fixation of recessive advantageous mutations may be involved in hybrid sterility and inviability (Charlesworth *et al.* 1987). Because hybrid anomalies most likely involve two or more genes, the interspecific variation responsible for anomalies may also be useful as a source to study interactions among genes. Provided with the existing knowledge of the genetics and the genetic tools of a number of mutants, deficiency and duplication chromosomes, and cell markers, *Drosophila melanogaster* is one of the most favorable organisms for detailed analysis of hybrid anomaly. Related Drosophila studies, however, have focused on species other than *D. melanogaster* (*e.g.*, Coyne 1984; Orr 1987; Cabot *et al.* 1994), and there are relatively few studies of *D. melanogaster*, such as partial hybrids produced from crosses between triploid *D. melanogaster* females and irradiated males of *D. simulans*, and rescue mutations of hybrid viability (*e.g.*, Muller and Pontecorvo 1940; Watanabe 1979). This is simply because all the progeny of interspecific crosses between pairs of *D. melanogaster* and its three most closely related species, *D. simulans*, *D. mauritiana*, and *D. sechellia*, are sterile. Thus, no second-generation hybrids can be produced, although recently a rescue mutant of hybrid female sterility between *D. melanogaster* and *D. simulans* was found in *D. simulans* (Davis *et al.* 1996). Use of the suitable genetic tools in *D. melanogaster*, however, would allow us to analyze the genetic basis of species differences using first-generation hybrids if effects of the *D. simulans*, *D. mauritiana*, or *D. sechellia* genomes are not completely dominant over the *D. melanogaster* genes.

One of the developmental anomalies in hybrids between *D. melanogaster* and *D. simulans* is loss of notum

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bristles (Figure 1A; Sturtevant 1920; Biddle 1932), the pattern of which is fixed within each species and identical between species (see Figure 1, B and C for the pattern of the wild-type *D. melanogaster*). Bristle formation has long been studied as a model system of pattern formation and its evolution (*e.g.*, Sondhi 1962). Analyses of expression and detailed mutant phenotypes of genes involved in various aspects of bristle development have led to the proposal of a progressive determination model for the formation of sensory organs (Ghysen and Dambly-Chaudiere 1989; Jan and Jan 1993). Several key points in bristle development include the singling out of precursors from proneural clusters, specification of neuronal identity and neural types, and asymmetric cell divisions producing four different cells: shaft, socket, neuron, and sheath cells. These accumulated findings on the developmental mechanisms serve as guides to understanding the genetic basis of species differences and their evolutionary history.

This article presents evidence that bristle loss in interspecific hybrids is found between *D. melanogaster* and *D. simulans*, but not between pairs of *D. melanogaster* on one hand, and *D. mauritiana* and *D. sechellia* on the other. This suggests that at least one genetic factor contributing to hybrid bristle anomaly arose recently in the *D. simulans* lineage. No clear anomaly was found in the emergence and divisions of sensory mother cells (SMCs) revealed by a transformant line, A101, and a rabbit anti-ASENSE (ASE) antibody. Hybrid pupae of 15 hr APF (after puparium formation), however, had no, or very reduced, levels of staining with the anti-CUT antibody at a large number of sites. Immunostaining using a nerve-specific antibody detected no neurons at many sites in the hybrid pupae as well. These results suggest that the defect does not lie in the cell fate decisions during the development of bristles, but in the maintenance of neural fate and/or differentiation of the descendants of SMCs. We provide evidence for a large effect of the *D. simulans X* chromosome and sex-dependent action on the bristle loss of hybrids.

MATERIALS AND METHODS

Population survey of inter- and intraspecific variation in the number of missing bristles on the notum in hybrids with *D. melanogaster*: In order to study the degree of hybrid anomaly as the number of missing bristles, crosses were made between *C(1)RM, y w^a* females of *D. melanogaster* [*Basc/C(1)RM, y w^a* was provided by the Mid-America Drosophila Stock Center (Bowling Green, OH), TT-35 in this article] and males from isofemale lines of four species: 100 lines of *D. simulans*, 34 of *D. mauritiana*, nine of *D. sechellia*, and eight of *D. melanogaster*. These are listed below:

D. simulans: S-2, S-11, S-19, and S-46 (B. Congo, 1983), SF2 and SF20 (South France, 1983), S-5 (Raleigh, 1984), Tananarive (1984), SA-10 (South Africa, 1983), T-6 (Tunisia, 1983), A-1 (Australia, 1986), and *Lhr* (K18) provided by C. C. Laurie; *y^2 w^{am} m^{65}* provided by the Bloomington Drosophila Stock Center; S-23 (Ethiopia 225.1) and S-24 (Tsimbazaza, Madagascar, 1980) lines provided by M. Ashburner; 21 lines from Zimbabwe, eight from Reunion (1979), 11 from Tananarive (1979), and 12 from Nairobi (1979) provided by the Genetic Strain Research Center, National Institute of Genetics (Mishima, Japan); 10 from St. Denis, Reunion (1987), five from Seychelles (1987), 10 from Antananarivo, Madagascar (1993), and eight from Ogasawara, Japan (1993) provided by S. C. Ishiwa.

D. mauritiana: Robertson (1979), 75 (1981), 152 (1981), Petite Reviere (1985), Les Galets (1985), and lig.21 provided by C. C. Laurie; *sn^1*; *j^1*; *ir^1*, and *y^1 pm^1* stocks provided by the Bloomington Drosophila Stock Center; 16 lines (1979) provided by the Genetic Strain Research Center, National Institute of Genetics; 10 lines (1987) provided by S. C. Ishiwa.

D. sechellia: Robertson (1980), 228 (1981), SS78 (1987), MAT iso6 (1989), and MBT iso7 (1989) provided by C. C. Laurie; four lines (1987) provided by S. C. Ishiwa.

D. melanogaster: Raleigh 84 (1982), Netherlands 218 (1982), Kochi 27, Japan (1982), F. Australia 7 (1980), V. France 7-2 (1978), B. W. Africa 7CA, 9C, and 27 (1978) provided by C. C. Laurie.

A survey of the above 151 lines of the four species was carried out in six separate sets of experiments. The crosses designed to examine the loss of bristles, basically one cross for each line, were made between ~20 pairs of TT-35 females and males of the above lines. Every three days, all the parental flies were transferred to new vials. This was done two or three times. Five male progeny were sampled from each of three vials, making a total sample size of 15 males (5 males × 3 vials) per cross, with a few exceptions. Some crosses, particularly involving *D. sechellia*, yielded only a few progeny. Less than 15 male hybrids were examined for two lines of *D. simulans* (10 hybrids for each line) and two *D. sechellia* lines (four and 14 hybrids). In addition, the data were pooled from two or three crosses for one line of *D. simulans* (a total sample size of 30 males) and four lines of *D. sechellia* (18–31 males sampled per line). For each sampled male, the number of missing bristles was examined for 13 pairs of macrochaetae on the notum and humeri (see Figure 1C).

In addition to the above stocks, adult male flies of *D. simulans* and *D. melanogaster* were collected in Kofu, Japan, in September 1995. Thirty-eight males of *D. simulans* and 20 of *D. melanogaster* were individually mated to *C(1)RM* females of *D. melanogaster.* As mentioned above, 15 male hybrids from three vials for each line were examined for bristles. However, in the case of six crosses, the sample sizes ranged from five to 14 males. Isofemale lines of *D. simulans* were also established from the females collected in Kofu at the same time. Two years later in September 1997, one male from each of five isofemale lines was examined for loss of bristles in hybrids with *C(1)RM* females of *D. melanogaster* in the same manner as the field-collected males. Fifteen hybrids for each cross except one cross (13 hybrids in this case) were studied for bristles.

Interpopulation differentiation in *D. simulans* was examined by an analysis of variance. The analysis was done only on the data of the four populations from the above population survey [St. Denis, Reunion (1987), Seychelles (1987), Antananarivo, Madagascar (1993), and Ogasawara, Japan (1993)] because measurements from these populations were contemporary. The mean number of missing bristles on the notum were obtained from 15 hybrid males for each line except for one, where 10 hybrids were employed in the calculation. The one-way analysis of variance was done using these line means. The model for the analysis is $Y_{ij} = \mu + P_i + \varepsilon_{j(i)}$, where P_i is the effects of the ith population (i = 1, 2, 3, 4) and $\varepsilon_{j(i)}$ is the residual.

Study of bristle anomaly in *D. simulans*-*D. mauritiana* hybrids and intraspecific heterozygotes of *D. simulans* strains: Bristle

anomaly was studied in *D. simulans-D. mauritiana* hybrids and in progeny from the crosses between pairs of the *D. simulans* stocks as well as *D. simulans-D. melanogaster* hybrids (see Table 2 for results). The S-11 (B. Congo, 1983, renamed as Sim-5 in this article) strain of *D. simulans* was mainly used in the following experiments, because this showed the greatest number of missing bristles in the interspecific hybrids with the *C(1)RM, y* w^a females of *D. melanogaster* (the mean ± SEM was 13.9 ± 0.9 using the original isofemale line). Inbred lines of *D. simulans*, *D. mauritiana*, and *D. sechellia* were made from some of the isofemale lines that were studied in the population survey of intra- and interspecific variations described above. These inbred lines and three isofemale lines of *D. melanogaster* were employed in this experiment, and a list of them is given below. The number following the letter G in parentheses indicates the number of generations of half sib-matings.

D. simulans: Sim-5 (G12), Congo S-2 (G10), Raleigh S-5 (G11), Ethiopia 225.1 (G12), Tsimbazaza, Madagascar (G12), Zimbabwe (G10), Southern France SF2 (G7), Tananarive (G12), South Africa SA-10 (G12), Tunisia T-6 (G12), and Australia A-1 (G12).

D. mauritiana: Petite Reviere (G5), Les Galets (G5), 75 (G5), and 152 (G5).

D. melanogaster: Raleigh 84, F. Australia 7 (renamed as Mel-4 in this article), and B. W. Africa 7CA (Mel-6 in this article).

The original isofemale lines of these *D. simulans* inbred lines showed a large variation in the number of missing bristles in hybrids with the *C(1)RM D. melanogaster* females. Excluding Sim-5, the number of missing bristles ranged from 0.1 ± 0.1 in Zimbabwe to 7.0 ± 1.0 in Australia A-1.

Crosses were made between 10 pairs of females and males for the homozygous and heterozygous crosses of the *D. simulans* lines, 20 pairs for the *D. simulans-D. mauritiana* hybrids, and between 15 females of Sim-5 and 25 males of each of three *D. melanogaster* isofemale lines with a slight variation in number. The experiments were carried out simultaneously, except for the Sim-5-*D. melanogaster* crosses that were made eight days later. A transfer of the parental flies were done once or twice every three days, and up to five male and female progeny from each vial were examined for the bristle number. The sample sizes averaged 10.2 for the intraspecific crosses of *D. simulans*, 5.3 for the hybrids between the *D. mauritiana* females and Sim-5 males, 15 for the hybrids of the Sim-5 females and *D. mauritiana* males, and 14.3 for the Sim-5-*D. melanogaster* male hybrids. The small sample sizes for the *D. mauritiana*-female/Sim-5-male hybrids was due to the low fecundity of this cross.

Bristle position specificity and stochastic effects on hybrid bristle anomalies: The following five inbred lines of *D. simulans* were employed in the experiment: Sim-5 (G20), Tunisia T6 (G20), Australia A-1 (G20), Ethiopia 225.1 (G20), and South Africa SA10 (G20). Just as in the other experiments, 20 males of each of the above five lines were crossed to 20 TT-35 females with two replicate crosses. Transfer of parental flies was done twice every three days. Five male progeny were sampled from each of three vials, making a total sample size of 30 males (2 crosses × 3 vials × 5 males) per line. All the crosses were made simultaneously.

Before pooling the data from different vials, a two-way analysis of variance for each line was conducted for the number of missing bristles on the left and right heminotum in a fixed model. The model for analysis of variance is

$$Y_{ijk} = \mu + C_i + V_j + (CV)_{ij} + \varepsilon_{k(ij)},$$

where C_i is the effect of the ith cross ($i = 1, 2$), V_j is the effect of jth vial ($j = 1, 2, 3$), $(CV)_{ij}$ is the cross-by-vial interaction, and $\varepsilon_{k(ij)}$ ($k = 1, 2, 3, 4, 5$) is the residual. Only 1 of the 30 F tests (5 lines × 2 heminota × 3 tests) was significant, where the cross-by-vial interaction effect for right heminotum of A1 (G20) hybrids was significant at the 5% level (data not shown). Provided that only small effects of separate crosses and different vials, if any, existed, the data from six vials in two replicate crosses were pooled and analyzed separately for each line (see Figures 4 and 5 for results).

Studies on effects of the *D. simulans X* chromosome and sex-dependent action: Effects of the sex, sex chromosomes, and the maternal factors on the number of bristles were studied in interspecific hybrids between *D. melanogaster* and *D. simulans.* TT-35 (*Basc/C(1)RM, y* w^a*/Y*), Sim-5 (G20), and Mel-6 are already mentioned above. The other stocks employed in this analysis are listed below:

- *Lhr* (K18) stock of *D. simulans*, provided by C. C. Laurie, rescues the inviability of hybrid males from the cross of *D. melanogaster* females to *D. simulans* males (Watanabe 1979).
- *C(1)RM, y w/Y* stock of *D. simulans* was given by J. A. Coyne.
- *D. simulans y w* stock homozygous for the detached-*X* chromosome of the above *C(1)RM, y w* was also provided by J. A. Coyne.
- *Zhr* stock of *D. melanogaster* provided by the laboratory of M. Ashburner rescues the inviability of hybrid females from the cross of *D. simulans* females to *D. melanogaster* males (Sawamura *et al.* 1993).
- *In(1)*w^{m4} + *In(1)AB*, y^2 w^{m4} was provided by the laboratory of M. Ashburner. This rescues the lethality of hybrid males from the cross of *D. melanogaster* females to *D. simulans* males (Hutter *et al.* 1990). This is renamed as TT-25 in this article.
- *D. melanogaster* isofemale line, Mel-4 (F. Australia 7, 1980), provided by C. C. Laurie. It was found that this line also rescues the lethality of hybrid female progeny from the cross of *D. simulans* females to *D. melanogaster* males.
- Six *D. melanogaster* isofemale lines employed in cross (9) in Table 3: Netherlands 218 (1982), Kochi 27, Japan (1982), V. France 7-2 (1978), B. W. Africa 9C and 27 (1978), and Mel-4.

Eleven different kinds of crosses were done as shown in Table 3, crosses (1) through (11). An effort was made to cross 20 pairs of females and males for all the cases. Forty parental flies were transferred to new vials twice, making a total of three vials from one cross just as in the other experiments. When possible, up to five male and five female progeny were sampled from each vial. The number of replicate crosses varied: only one for crosses (2), (6), (7), and (8); two replicates for crosses (3), (4), (5), (10), and (11); and three replicates for cross (1). In total, 10 crosses were done to produce the result of cross (9) using six *D. melanogaster* isofemale lines. Two replicate crosses were made for four out of six lines, and one for the remaining two lines. Sample sizes ranged from 15 to 45, but only two female hybrids from cross (3) were examined. The mean number of missing bristles and its standard error were calculated after pooling the data from replicate crosses, except for crosses (9) through (11). In cross (9), six *D. melanogaster* isofemale lines were separately crossed to the *Lhr* stock of *D. simulans.* The mean number of missing bristles was calculated for each *D. melanogaster* line, then the mean and variance of these six values were computed. In addition, because there was a significant difference in the bristle number of female hybrids from cross (11) between two replicate crosses (1.7 ± 0.4 *vs.* 0.5 ± 0.2, $P < 0.05$), the same calculation procedure as used in cross (9) was used for crosses (10) and (11), employing the mean from each of two replicate crosses as an estimate.

Phase assays of bristle development defects in hybrids: Bris-

tle development in interspecific hybrids was studied with the aim of determining the critical stage in bristle anomaly, using cell markers and mutants in *D. melanogaster.* The *neuralized* (*neu*), A101.1F3/*TM3, Sb* (Boulianne *et al.* 1991), and *Delta* (*Dl*), P[lwB]#850, enhancer trap transposon insertion lines were provided by J. Modolell and the Genetic Strain Research Center, National Institute of Genetics, respectively. The A101.1F3 is a recessive embryonic lethal mutant of *neu* (Boulianne *et al.* 1991), whereas the *Delta* enhancer trap line is homozygous viable without obvious notum bristle abnormality in the homozygous condition. emc^{E6} and $Df(3L)emc5, red^{1}/TM2, emc^{2}\ p^{p}\ Ubx^{130}\ e^{s}$ were provided by the Mid-America Drosophila Stock Center and the Bloomington Drosophila Stock Center, respectively. A *D. simulans* inbred line, Sim-5 (G20), was derived from a Sim-5 stock by 20 generations of half sib-mating. Sim-5 and an isofemale line of *D. melanogaster*, Mel-6 (B. W. Africa 7CA 1978), are already described above. Sim-8 is an isofemale line established from a *D. simulans* female collected in Kofu, Japan, in 1995.

Emergence of sensory mother cells (SMCs) in imaginal wing discs was studied using the β-galactosidase reporter gene expression in the P-transposons of the *neuralized* and *Delta* enhancer trap lines as the markers. ASE and CUT expressions were examined for activation of pan-neuronal genes and neuron-type specification genes, respectively. The neuron-specific mouse antibody 22C10 was employed to observe bristle neurons. Crosses were made between 20 pairs of $C(1)RM,\ y\ w^{a}/Y$; $TM3,\ y^{+}\ Ser$/A101.1F3 or $C(1)RM,\ y\ w^{a}/Y$; P[lwB]#850 females and Sim-5 (G20), Sim-8, or Mel-6 males for the analyses of SMC emergence and 22C10 antibody staining. Progeny from these crosses were examined for the number of missing bristles in adults as well. The CUT and ASE stainings were done for imaginal wing discs of the hybrids between TT-35 females of *D. melanogaster* and Sim-5 (G20) or Mel-6 males.

Effects of *emc* mutants of *D. melanogaster* were also examined in hybrids with *D. simulans.* Crosses were made between 20 pairs of $C(1)RM,\ y\ w^{a}/Y$; $TM3,\ y^{+}\ Ser/Df(3L)emc5,\ red$ females and Sim-5 (G20) males and between 20 pairs of Sim-5 (G20) females and emc^{E6} males. In the former cross, male hybrids carrying the *emc* mutant and the balancer chromosome were compared to evaluate the effects of the mutant.

Hybrids between TT-35 females of *D. melanogaster* and Sim-5 (G20) males were examined for the presence of a bristle socket as well as a shaft for 13 pairs of macrochaetae. Crosses were made between 20 pairs of females and males with six replicates, and transfers of parental flies were done twice every three days. Five male progeny were sampled from each vial, making a total sample size of 90 hybrids (6 crosses × 3 vials × 5 males).

β-Galactosidase activity staining: Imaginal wing discs were dissected in PBS and fixed with 0.75% glutaraldehyde in PBS. Histochemical staining for β-galactosidase activity was carried out as described in Bellen *et al.* (1989).

Antibody staining: Staged larvae and pupae were dissected in PBS and fixed for 20 min in 4% paraformaldehyde in PBS. After being washed in phosphate-buffered saline (PBS), the dissected wing discs and nota were incubated in 10% goat serum in blocking solution (20 mm Tris pH 7.5, 130 mm NaCl, 1 mm EDTA, 0.1% Triton-X, 0.2% bovine serum albumin [BSA)] for a few hours. The primary antibodies were diluted as follows: 1:30 for the mouse monoclonal antibody 22C10; 1:1000 for rabbit anti-β-galactosidase (Cappel); 1:3000 for the rabbit anti-ASE (Brand *et al.* 1993); and 1:20 for the anti-CUT (Blochlinger *et al.* 1990). The anti-ASE antibodies were preabsorbed with embryos aged 0–6 hr before use. The biotinylated anti-rabbit IgG (Vector, Burlingame, CA) and biotinylated anti-mouse IgG (Vector) as secondary antibodies and Vectastain Elite ABC kit (Vector) were used for the ASE and CUT stains. The preparations were stained for horseradish peroxidase (HRP) activity by incubation in diaminobenzidine (DAB). For 22C10/β-galactosidase double-labeling, Cy3-conjugated anti-mouse and fluorescein-5-isothiocyanate (FITC)-conjugated anti-rabbit IgGs were used to label sensory neurons and β-galactosidase, respectively. The anti-β-galactosidase antibody staining of wing discs of 1 hr after puparium formation (APF) was done as described in Usui and Kimura (1993) using mouse anti-β-galactosidase (Promega, Madison, WI) and sheep HRP-conjugated anti-mouse IgG (Amersham, Buckinghamshire, England).

RESULTS

Population survey of inter- and intraspecific variation in the number of missing bristles on the notum in hybrids with *D. melanogaster*: The three species most closely related to *D. melanogaster*, *D. simulans*, *D. mauritiana*, and *D. sechellia* have 26 macrochaetae on their notum including humeri, which is exactly the same as for *D. melanogaster* (Figure 1, B and C). The number of missing bristles per fly was surveyed in interspecific hybrids between *D. melanogaster* females and males of the above three species. The compound-*X* chromosome, *C(1)RM*, stock of *D. melanogaster* (TT-35) was used to produce hybrids. This type of cross usually only produces male hybrids carrying the *X* chromosome of the male parents (Takamura and Watanabe 1980). This mating scheme was chosen because it could detect possible hemizygous effects of the *X* chromosomes of *D. simulans*, *D. mauritiana*, and *D. sechellia.*

An example of the hybrids between *D. melanogaster* and *D. simulans* is shown in Figure 1A, where a great deficiency of macrochaetae and microchaetae can be seen compared with the wild type of both species (Figure 1B). Figure 1C illustrates bristle positions and their names on the notum of *D. melanogaster.* The distribution of the number of missing bristles per fly in interspecific hybrid males is shown in Figure 2A. There were clear-cut genetic differences in the reduction of the bristle number between the *D. melanogaster-D. simulans* hybrids and the hybrids of *D. melanogaster* with *D. mauritiana* or *D. sechellia.* Interspecific hybrids between the compound-*X* chromosome stock of *D. melanogaster* and *D. simulans* isofemale lines exhibited a wide range in the number of missing bristles on the thorax. By contrast, *D. mauritiana* and *D. sechellia* lines showed almost no reduction in bristle number in hybrids with *D. melanogaster.*

The isofemale lines employed in this survey were very heterogeneous in terms of collection year and locations sampled. They were maintained in various laboratories for many years. Thus, the degree of anomaly in hybrids may partly be due to mutations that occurred during maintenance, although there was not any systematic difference in collection dates among the three species. Field-collected males of *D. simulans* (Kofu, Japan) were used in the same survey in order to evaluate genetic

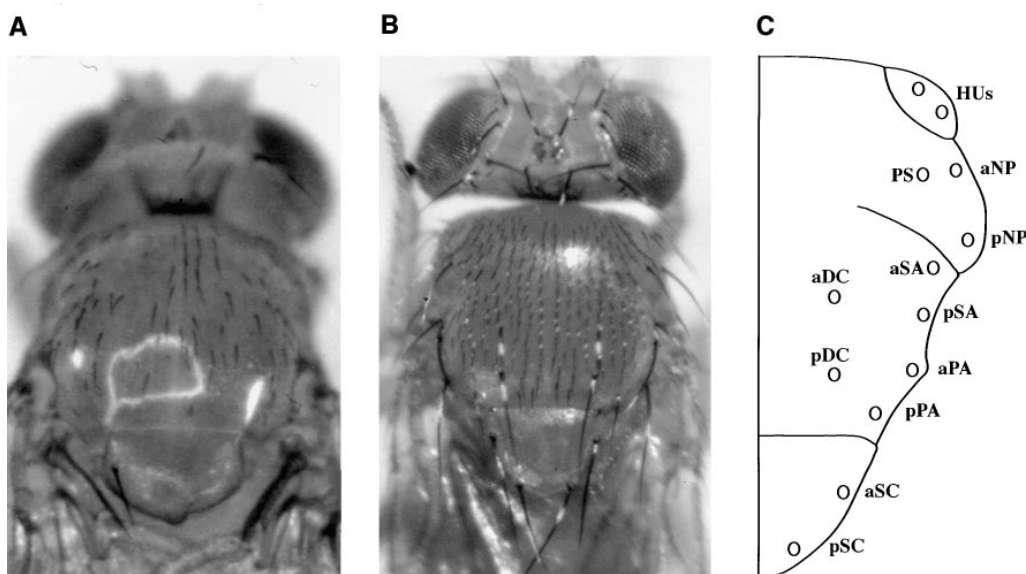

Figure 1.—Defects in bristle formation in interspecific hybrids between *D. melanogaster* and *D. simulans.* (A) Hybrid male from a cross of *C(1)RM/Y* females of *D. melanogaster* and males of *D. simulans* (Sim-5), in which many macrochaetae as well as microchaetae on the notum are lost as compared with a pure *D. melanogaster* male (B). C shows the macrochaete positions on a heminotum and humerus with their nomenclature. PS, presutural; uHU and lHU, humerals; aNP and pNP, notopleurals; aSA and pSA, supraalars; aPA and pPA, post-alars; aDC and pDC, dorsocentrals; aSC and pSC, scutellars.

variation in natural populations. The result is depicted in Figure 2B along with that of a control experiment using *D. melanogaster* males collected in the same locations. These show a great number of missing bristles in the *D. melanogaster-D. simulans* hybrids. Isofemale lines of *D. simulans* originating from females collected at the same time in Kofu were maintained in the laboratory for 24 mon. One male from each of five lines was crossed with TT-35 females of *D. melanogaster*, and then 15 hybrid progeny were examined for bristles. The average number of missing bristles was 6.45 ± 0.66, which is almost identical to that for 38 field-collected males, 6.87 ± 0.37 (Table 1). Thus, maintenance in the laboratory for 24 mon had no effect on the bristle-loss phenotype. Taken together, it can be concluded that the genetic factors responsible for bristle anomalies in *D. melanogaster* and *D. simulans* hybrids are present in both laboratory strains and in natural populations.

It should also be noted that there was a great difference in distribution between males from the stocks of *D. simulans* maintained in the laboratory and those caught in the wild (Figures 2A and 2B). As mentioned above, the population survey shown in Figure 2A was made using heterogeneous groups of lines. Thus, the

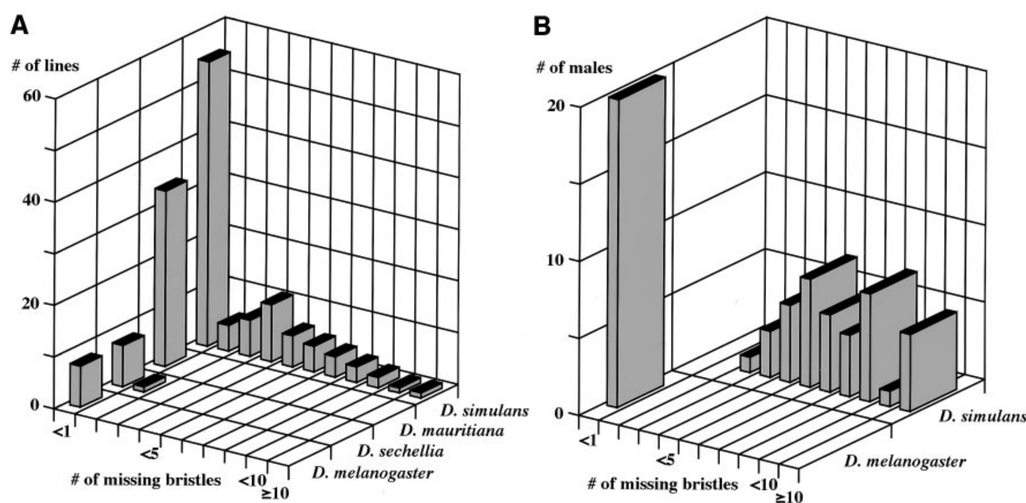

Figure 2.—Distributions of the number of missing bristles in hybrids of the three species with *C(1) RM/Y D. melanogaster* females. Loss of bristles in hybrids with *D. melanogaster* was observed in *D. simulans*, but not in *D. mauritiana* nor in *D. sechellia.* (A) Histogram showing distributions of the number of missing bristles in hybrids of eight lines of *D. melanogaster*, nine of *D. sechellia*, 34 of *D. mauritiana*, and 100 of *D. simulans* with *C(1)RM/Y* females of *D. melanogaster.* The number of missing bristles per fly was calculated as an average of 15 males from three vials for each isofemale line. The mean number of missing bristles was 0.03 ± 0.02 for *D. melanogaster*, 0.48 ± 0.13 for *D. sechellia*, 0.15 ± 0.03 for *D. mauritiana*, and 2.17 ± 0.27 for *D. simulans.* (B) Distributions of the number of missing bristles in hybrids of adult males collected in a natural population. Thirty-eight males of *D. simulans* and 20 of *D. melanogaster* were collected and crossed to *C(1)RM/Y* females of *D. melanogaster.* The mean number of missing bristles per fly was 6.87 ± 0.37 for *D. simulans* and 0.03 ± 0.01 for *D. melanogaster.*

TABLE 1

Interpopulation variation in bristle defects in hybrids with *D. melanogaster*

D. simulans population	No. of lines	Mean number of missing bristles (± standard error of mean)
Zimbabwe	21	4.40 ± 0.57
Congo (1983)	4	5.20 ± 2.92
Nairobi, Kenya (1979)	12	0.29 ± 0.07
St. Denis, Reunion (1979)	8	2.06 ± 0.60
St. Denis, Reunion (1987)	10	0.67 ± 0.36
Seychelles (1987)	5	0.22 ± 0.06
Tananarive, Madagascar (1979)	11	0.45 ± 0.16
Antananarivo, Madagascar (1993)	10	0.38 ± 0.19
Ogasawara, Japan (1993)	8	2.68 ± 0.43
Kofu, Japan (1995)[a]	38	6.87 ± 0.37
Kofu, Japan (1995)[b]	5	6.45 ± 0.66

[a] Thirty-eight field-collected males were individually examined for the bristle in hybrids with *D. melanogaster* (see Figure 2B).

[b] Males from five isofemale lines originated from the females collected in Kofu, 1995, were studied in the same manner as the field-collected males after 24-mon maintenance in the laboratory.

All the other data come from Figure 2A.

data of the *D. simulans* lines in Figures 2A and 2B were classified by population and collection year (Table 1). There was a significant difference in the degree of hybrid anomaly among the populations of *D. simulans* [F of the ANOVA with 3 and 29 degrees of freedom (d.f.) = 11.7, $P < 0.001$, see also materials and methods], although a considerable difference in the mean number was found for the two samples (1979 and 1987) from St. Denis, Reunion (Table 1). In general, the flies collected in Madagascar and Seychelles tended to show much less anomaly, and the strains from the other locations exhibited a wide range of degree of bristle defects. This suggests that at least one genetic factor causing hybrid bristle loss arose recently in one of the *D. simulans* lineages and that it has increased to a considerable frequency in some populations. Interestingly, all males from nature and from isofemale lines of Kofu showed more than three missing bristles per fly in hybrids with *D. melanogaster.* The number of missing bristles of hybrids for eight lines of the Ogasawara population also ranged from 1.2 to 4.1. This may be an indication of the fixation of the anomalous genotype in the Japanese populations.

Study of bristle anomaly in *D. simulans-D. mauritiana* hybrids and intraspecific heterozygotes of *D. simulans* strains: As shown in Table 2, notum bristle loss was not observed in interspecific hybrids between pairs of the *D. simulans* and *D. mauritiana* stocks, nor in heterozygotes between pairs of the *D. simulans* stocks. This suggests that one or more genetic factors arose in the *D. melanogaster* lineage that contributed to hybrid bristle anomalies specifically with *D. simulans* but not in the hybrids with *D. mauritiana.* An alternative explanation may be that the genetic factor(s) responsible for the bristle anomalies arose first in the internal branch from the common ancestor of the four species involved in this study through the common ancestor of *D. simulans* and *D. mauritiana* (and probably *D. sechellia*) ["*a*" to "*A*" substitution in model (2) in Figure 3]. Then another genetic factor(s) occurred in the *D. simulans* lineage ("*b*" to "*B*" substitution) that was compatible with the first one but incompatible with the ancestral allele in *D. melanogaster.* This is a derived-ancestral incompatibility following Orr's (1995) classification. These two possible evolutionary paths of hybrid-anomaly development are presented graphically in Figure 3.

A *D. simulans* strain, Sim-5: The Sim-5 stock was used primarily in the following experiments because it exhibited the greatest number of missing bristles in the compound-*X* survey for the isofemale lines. It should also be mentioned here that a large number of missing bristles appeared in the inbred Sim-5 stock (Table 2). Although we do not know, at this moment, the genetic bases for the bristle loss, the following observations suggest uncoupling of the great loss of bristles in the interspecific hybrids from the bristle reduction in the pure *D. simulans* background. A difference in the sex dependency of the bristle defects was found between the pure *simulans* and hybrid backgrounds. Greater bristle loss was observed in females in the pure *simulans* background (Table 2), whereas only interspecific hybrid males showed a high number of missing bristles, as described later (Table 3). To further test this, females of the inbred Sim-5 (G20) stock were crossed to males of an inbred Tananarive (G20) stock of *D. simulans* that showed no bristle anomaly in the hybrids with *D. melanogaster.* When these F_1 males were crossed with the compound-*X* females of *D. melanogaster*, the interspecific hybrid male progeny showed high numbers of missing bristles. The average number of missing bristles of 90

TABLE 2

Absence of bristle defects in hybrids between *D. simulans* and *D. mauritiana*

Female parent	Male parent	Number of missing bristles (± standard error of mean)	
		Female	Male
Sim-5 (G12)	Three isofemale lines of *D. melanogaster*	Lethal	17.6 ± 0.9
Sim-5 (G12)	Three inbred lines of *D. mauritiana*	0.1 ± 0.1	0.8 ± 0.2
Two inbred lines of *D. mauritiana*	Sim-5 (G12)	0.0 ± 0.0	0.3 ± 0.3
Sim-5 (G12)	Ten inbred lines of *D. simulans*	0.1 ± 0.0	0.4 ± 0.1
Eight inbred lines of *D. simulans*	Sim-5 (G12)	0.0 ± 0.0	0.0 ± 0.0
Sim-5 (G12)	Sim-5 (G12)	4.1 ± 1.2	1.3 ± 0.5

Numbers are unweighted means of multiple lines. There was no significant variation among lines used for each type of cross.

hybrids ± SEM was 11.2 ± 0.5, whereas those in interspecific hybrids of the parental Sim-5 (G20) and Tananarive (G20) strains were 12.8 ± 0.4 and 0.2 ± 0.1, respectively

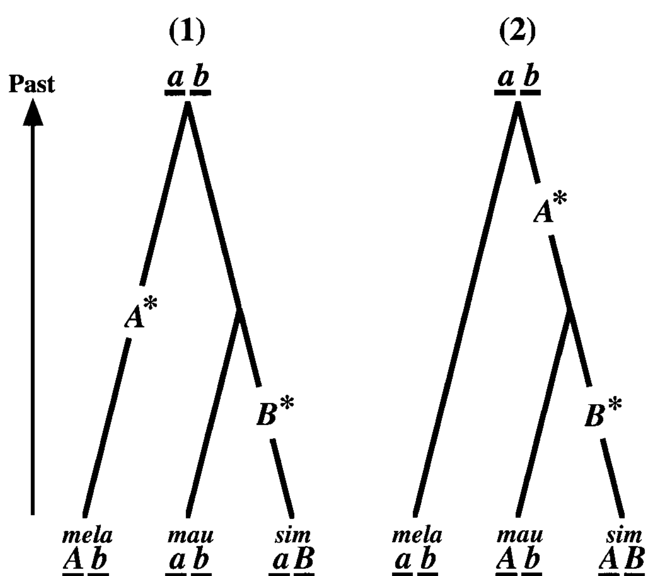

Figure 3.—Possible evolutionary paths leading to the condition that a hybrid incompatibility occurs between *D. melanogaster* and *D. simulans*, but not between *D. melanogaster* and *D. mauritiana* nor between *D. simulans* and *D. mauritiana.* Because *D. sechellia* is in the same situation as *D. mauritiana*, it is not included in this figure. It is assumed here that only two genetic factors are involved in a hybrid incompatibility and that the ancestral alleles are "*a*" and "*b*." It is further assumed, for the sake of simplicity, that all species differences are fixed. "*" represents occurrence of substitutions, "*a*" to "*A*" (*A**) or "*b*" to "*B*" (*B**). In (1), hybrid incompatibility is due to interaction between "*A*" and "*B*" alleles [a derived-derived incompatibility following Orr's (1995) classification], and a derived-ancestral incompatibility (between "*a*" and "*B*") is assumed in (2).

(data not shown). In contrast, the male progeny, as well as females from the crosses of Sim-5 females to 10 inbred lines of *D. simulans*, showed almost no bristle loss (Table 2). The results of these crosses provide a good reference for the hybrid effects and may suggest different causes for bristle loss in the interspecific hybrids and the pure *D. simulans* background.

Sim-5 showed a great number of missing bristles in the hybrids with *D. melanogaster*, but this is not exceptional. Some other African lines showed, on average, more than eight missing bristles per fly in the interspecific hybrids. In addition, many Japanese male flies collected from the wild showed a number of missing bristles in hybrids with *D. melanogaster*, which is comparable to that for the Sim-5 stock. Indeed, five out of 38 males exhibited more than 10 missing bristles per fly in hybrids (Figure 2B). Therefore, because the Sim-5 stock gives a large, but not atypical, degree of bristle loss in hybrids with *D. melanogaster*, this line was chosen for the subsequent analyses.

Bristle position specificity and stochastic effects on hybrid bristle anomalies: It has been found that a certain number of bristle mutants in *D. melanogaster* show strong specificities affecting particular groups of bristles (*e.g.*, García-Bellido 1979). Bristle position specificity in interspecific hybrids was studied using five *D. simulans* inbred lines. The number of missing bristles at each bristle position is given in Figure 4. No strong position specificity was found in general. On the other hand, a remarkable finding is the large amount of variation in the number of missing bristles among flies within each line. The actual number of missing bristles in a sample of 30 flies ranged from nine to 23 for Sim-5 (G20), zero to 14 for T6 (G20), and one to 16 for A1 (G20). This high variation may still be due to segregation of genetic factors responsible for hybrid bristle loss in each line.

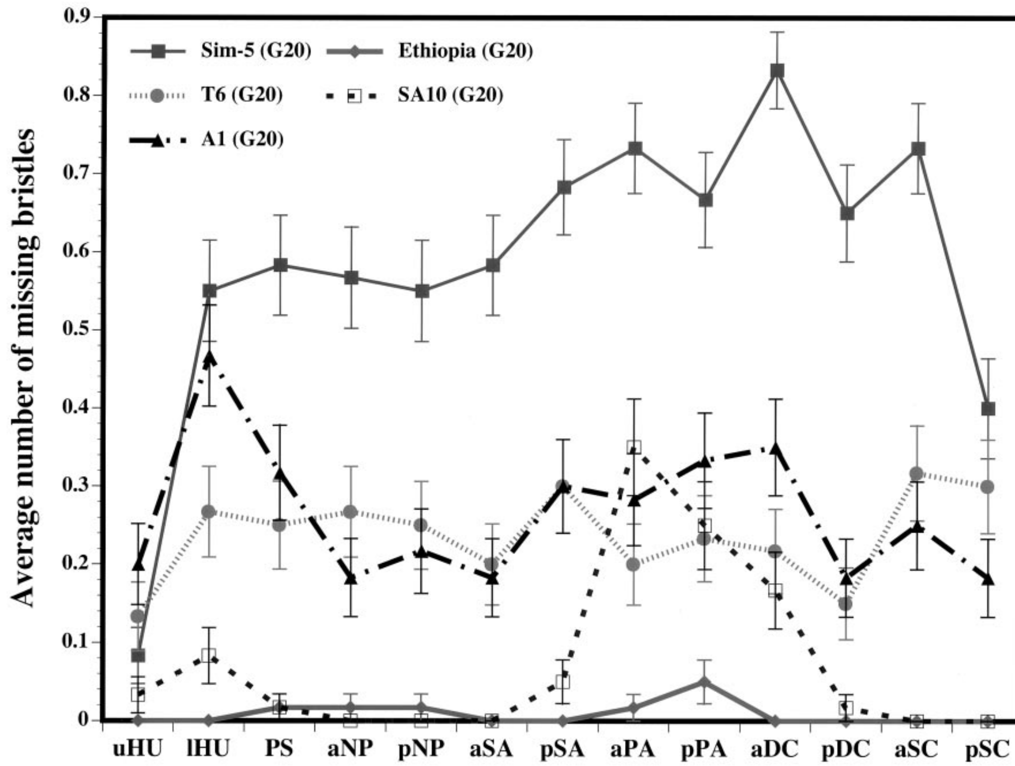

Figure 4.—Low degree of bristle position specificity in bristle loss of hybrids between five *D. simulans* inbred lines and *C(1)RM/Y D. melanogaster* females. The number of missing bristles per heminotum (each can take a value between 0 and 1) is shown for each macrochaete. The number was obtained as the average of 30 hybrid progeny from two replicate crosses for each *D. simulans* line. The error bars represent the standard errors. There is no great position effect in degree of the bristle loss in hybrids, whereas the actual number of missing bristles greatly differed among lines studied. The average number of missing bristles per fly was 15.2 ± 0.7 for Sim-5 (G20), 6.2 ± 0.6 for T6 (G20), 6.9 ± 0.8 for A1 (G20), 0.2 ± 0.1 for Ethiopia (G20), and 1.9 ± 0.3 for SA10 (G20).

To assess the degree of stochastic effects, we analyzed the correlation of the number of missing bristles between left and right heminota in one fly. The results for Sim-5 (G20) and A1 (G20) are presented graphically in Figure 5. Although a considerable variation was found for each heminotum, there is only a very low degree of association between these two numbers. The estimate of the product-moment correlation coefficient was 0.03 for Sim-5 (G20), 0.23 for A1 (G20), and 0.12 for T6 (G20), none of which significantly differ from zero. These results imply that the loss of bristles is, to a large extent, stochastic, although significant between-line differences in the number of missing bristles exist as shown in Figures 2 and 4.

Large effects of the *D. simulans X* chromosome and sex-dependent action: The compound-*X* chromosome stock of *D. melanogaster* was used in the above survey because it allows for examination of the *X* chromosomes

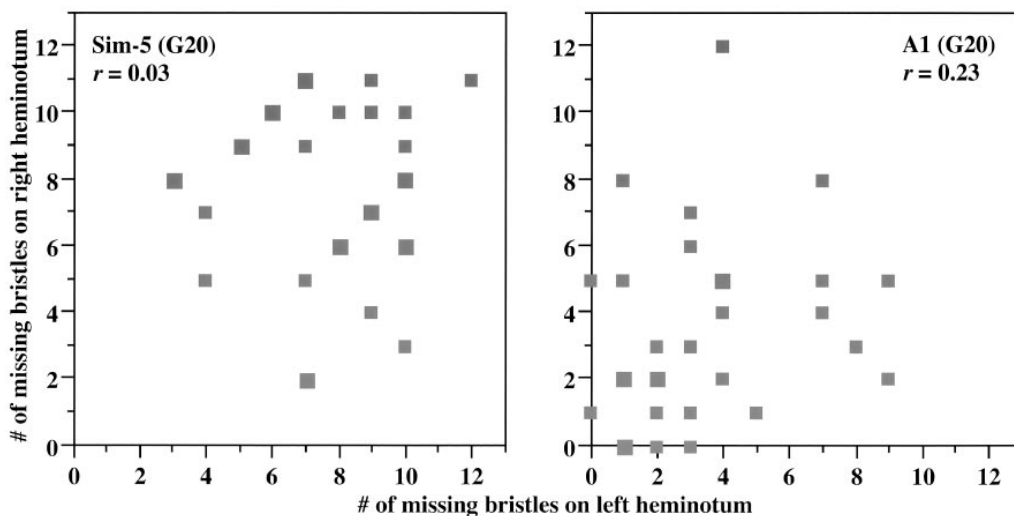

Figure 5.—Low degree of correlation in the number of missing bristles between left and right heminota in one fly. The numbers of missing bristles on left and right heminota were plotted for male hybrids of two *D. simulans* inbred lines, Sim-5 (G20) and A1 (G20), with *C(1)RM/Y D. melanogaster* females. Larger squares represent cases of double occurrences, and *r* stands for estimate of product-moment correlation coefficient. The same result was obtained using T6 (G20)-*D. melanogaster* hybrids, where the correlation coefficient was estimated to be 0.12.

TABLE 3

***X* chromosome and sex effects**

Cross	Female parent	Male parent	Number of missing bristles[h] (± standard error of mean)	
			Female	Male
(1)	TT-35[a]	Sim-5 (G20)	Lethal	14.6 ± 0.6 (*XsYm*)
(2)	Sim-5 (G20)	*Zhr*[b]	5.4 ± 0.7 (*XmXs*)	16.8 ± 0.7 (*XsYm*)
(3)	Sim-5 (G20)	Mel-4[c]	4.0 ± 1.0 (*XmXs*)	17.7 ± 0.6 (*XsYm*)
(4)	Mel-4	Sim-5 (G20)	4.9 ± 0.5 (*XmXs*)	Lethal
(5)	Sim-5 (G20)	Mel-6[c]	Lethal	18.6 ± 0.5 (*XsYm*)
(6)	Mel-6	Sim-5 (G20)	4.5 ± 0.5 (*XmXs*)	Lethal
(7)	TT-25[d]	Sim-5 (G20)	2.8 ± 0.5 (*XmXs*)	0.7 ± 0.3 (*XmYs*)
(8)	TT-35	*Lhr*[e]	0.7 ± 0.2 ($\widehat{XX}$*mYs*)	9.1 ± 1.1 (*XsYm*)
(9)	Six isofemale lines of *D. melanogaster*	*Lhr*[e]	6.3 ± 0.6 (*XmXs*)	0.5 ± 0.2 (*XmYs*)
(10)	TT-35	Sim-detached-X[g]	Lethal	4.7 ± 0.5 (*XsYm*)
(11)	Sim-*C(1)RM*[f]	Mel-4[c]	1.2 ± 0.3 ($\widehat{XX}$*sYm*)	Lethal

[a] A *Basc/C(1)RM, y* w^a stock of *D. melanogaster*.

[b] *Zhr* of *D. melanogaster* rescues the inviability of hybrid females from the cross of *D. simulans* females to *D. melanogaster* males (Sawamura *et al.* 1993).

[c] Mel-4 and Mel-6 are *D. melanogaster* isofemale lines. Mel-4 stock also rescues the lethality of hybrid female progenies from the cross of *D. simulans* females to *D. melanogaster* males.

[d] TT-25 line of *D. melanogaster* carries the *X* chromosome, $In(1)w^{m4}$ + $In(1)AB$, y^2 w^{m4}, and this rescues the lethality of hybrid males from the cross of *D. melanogaster* females to *D. simulans* males (Hutter *et al.* 1990).

[e] *Lhr* of *D. simulans* rescues the inviability of hybrid males from the cross of *D. melanogaster* females to *D. simulans* males (Watanabe 1979).

[f] A *C(1)RM, y w/Y* stock of *D. simulans.*

[g] A *y w* stock of *D. simulans* derived from detachment of the above *C(1)RM, y w* chromosome.

[h] The sex chromosome constitution (*X* and *Y*) is shown for each hybrid progeny, in which "*m*" and "*s*" stand for *D. melanogaster* and *D. simulans* origins, respectively.

Two compound-*X* chromosomes are represented by $\widehat{XX}$.

of *D. simulans* and other species in hemizygous males. In investigating the effect of sex, sex chromosomes, and maternal factors on the number of bristles, interspecific crosses between *D. melanogaster* and *D. simulans* were made using several hybrid rescue stocks. Table 3 summarizes the results, where the *X* and *Y* chromosomes are marked "*m*" and "*s*" for *D. melanogaster* and *D. simulans* origins, respectively. There was a great difference in the number of missing bristles between the two sexes in crosses (2) and (3), in which all the male hybrids carried the *X* chromosome of *D. simulans* and the *Y* chromosome of *D. melanogaster.* The same tendency was also seen in crosses (1), (4), (5), and (6), which produced only one sex. In fact, the numbers of missing bristles in the Sim-5 hybrid females in Table 3 (ranging from 2.8 to 5.4, depending on *D. melanogaster* lines used as female parents) were similar to those of Sim-5 homozygous females (4.1 from Table 2). By contrast, the male progeny from cross (7) showed almost no reduction in bristle number, and they carried the *X* chromosome of *D. melanogaster.* These results suggest that the great reduction of bristles in hybrids is not just a male-specific phenotype, but that much depends on the sex chromosome constitution, the *X* chromosome of *D. simulans* or the *Y* chromosome of *D. melanogaster.*

The functional difference of the *Y* chromosome between *D. melanogaster* and *D. simulans* is well known. Whereas the ribosomal RNA genes are arrayed as tandemly repeated copies on both the *X* and *Y* chromosomes in *D. melanogaster*, the *Y* chromosome of *D. simulans* carries few, if any, rRNA genes (Lohe and Roberts 1990). However, it is difficult to assume that

the presence of the *D. melanogaster Y* chromosome with the functional rRNA genes caused the great reduction of bristles, and that the *D. simulans Y* chromosome did not. Besides the nucleolus organizer, there are only a few known functions of the *Y* chromosome of *D. melanogaster*, including several male fertility factors. Taken together, it is more likely that the *D. simulans X* chromosome is responsible for the loss of bristles and its action is partially recessive. The same result was obtained using the *Lhr* stock that rescues male progeny without the *D. simulans X* chromosome [crosses (8) and (9) in Table 3], although the difference between the *XmXs* females from cross (9) and *XsYm* males from cross (8) was not so great.

A recessive effect of the *D. simulans X* chromosome is not clearly indicated, however, because an effect of sex was also seen. Comparing crosses (10) and (11) revealed that the hemizygous male hybrids showed a statistically greater number of missing bristles than the hybrid females homozygous for the same chromosome. Thus, male hybrids may be more susceptible to bristle loss in hybrids than female hybrids.

Phase assays of bristle development defects in hybrids: The model proposed for the formation of a sensory organ (Ghysen and Dambly-Chaudiere 1989; Jan and Jan 1993) includes the singling out of precursors from proneural clusters, activation of pan-neuronal genes, specification of neuronal types, asymmetric cell divisions producing different cells (shaft, socket, neuron, and sheath cells), and their differentiation in bristle development. Following this model, pan-neuronal precursor genes and selector genes are thought to be involved in neuronal development and differentiation after singling out of precursor cells from the proneural clusters. First, the emergence of SMCs and their cell divisions were studied for late third instar larvae and prepupae up to 1 hr APF by using the enhancer trap line containing an insert in the *neuralized* (A101) locus as a marker (Figure 6). The average number of missing bristles per fly ± SEM was 12.8 ± 1.0 in A101-carrying male hybrids and 12.1 ± 1.1 in TM3-bearing ones in the cross between *C(1)RM, y w^a/ Y; TM3, y$^+$ Ser/* A101.1F3 and Sim-5 (G20). The same number for hybrids from the cross of Sim-5 (G20) with *C(1)RM, y* (TT-35) females was 14.6 ± 0.6 (Table 3). Thus, if there was a *neu* mutant effect of the A101.1F3, it was negligibly small in the interspecific hybrids. The *neuralized* gene is expressed in all SMCs in wing imaginal discs (Boulianne *et al.* 1991). Frequencies of appearance of SMCs for late third instar larvae (data not shown), and those and the number of cells in prepupae up to 1 hr APF at each bristle position were almost the same in *D. melanogaster-D. simulans* hybrids and pure *D. melanogaster* background, as shown in Figure 6. Normal emergence of SMCs in late third instar larvae was confirmed by using the *Delta* enhancer trap line and another *D. simulans* line, Sim-8 (data not shown).

It is known that *extramacrochaetae* (*emc*) acts as an antagonist to the proneural *achaete* and *scute* genes and that there are dosage-sensitive interactions between the *emc* and the *proneural* genes (Moscoso del Prado and García-Bellido 1984; Ellis *et al.* 1990; Garrell and Modolell 1990). The expression levels of these genes may vary among the species, still providing a balanced level between the proneural genes and *emc* in each species. The proneural *achaete* and *scute* genes are on the *X* chromosome, and the *emc* is located on the third chromosome. The present studies, including the expression assays of marker genes, were mainly done in male hybrids carrying the *D. simulans X* chromosome. An imbalance between the proneural genes and the *emc* expression levels could be responsible for a failure of SMC emergence. For instance, if both groups of genes are expressed at higher levels in *D. melanogaster* as compared with *D. simulans*, a lower ratio of the proneural genes to the *emc* is expected in male hybrids carrying the *D. simulans X* chromosome. If this is the cause of the bristle loss, reduction of the *emc* gene product could restore the normal bristle formation. However, *emc* mutants did not rescue the bristle loss (data not shown), which is consistent with the normal emergence of SMCs.

The *ase* gene is one of the pan-neuronal precursor genes and is expressed in most precursor cells (Brand *et al.* 1993). Loss of function mutations of *ase* lead to loss of sense organs (Dambly-Chaudiere and Ghysen 1987; Jarman *et al.* 1993). The *cut* gene is a neuron-type selector gene and is expressed in all external sensory organ precursors and descendants (Blochlinger *et al.* 1993). Loss of its function results in the transformation of an external sensory organ into a chordotonal organ (Bodmer *et al.* 1987). The expression of the ASE and CUT proteins were examined in wing imaginal discs and nota of hybrids, respectively. There was no abnormality in the anti-ASE staining in the wing discs of 1 hr APF as shown in Figure 7, although the fraction of discs having the ASE positive cells at PS differed significantly between the *D. melanogaster-D. simulans* hybrids and the pure *D. melanogaster* background ($P = 0.006$ in Fisher's exact test). This latter finding seems to be, at least partly, due to slower development of the hybrids. It should also be added here that there is no particular position specificity in bristle loss in adult flies as mentioned above (Figure 4). Hybrid pupae of 15 hr APF, however, had no or very reduced levels of staining with the anti-CUT antibody at 26 out of 61 DCs and SCs examined (Figure 8). This fraction is roughly equal to that of bristle loss for these macrochaetae of adults (66 of 120). By contrast, low CUT staining was seen in only one out of 66 DCs and SCs in the pure *D. melanogaster* background, which indicates highly significant heterogeneity (Fisher's exact test for 26/61 *vs.* 1/66, $P < 10^{-8}$).

One possible explanation for missing bristles or loss of shafts is failure of fate choices among four cells comprising an individual bristle: shaft, socket, neuron, and

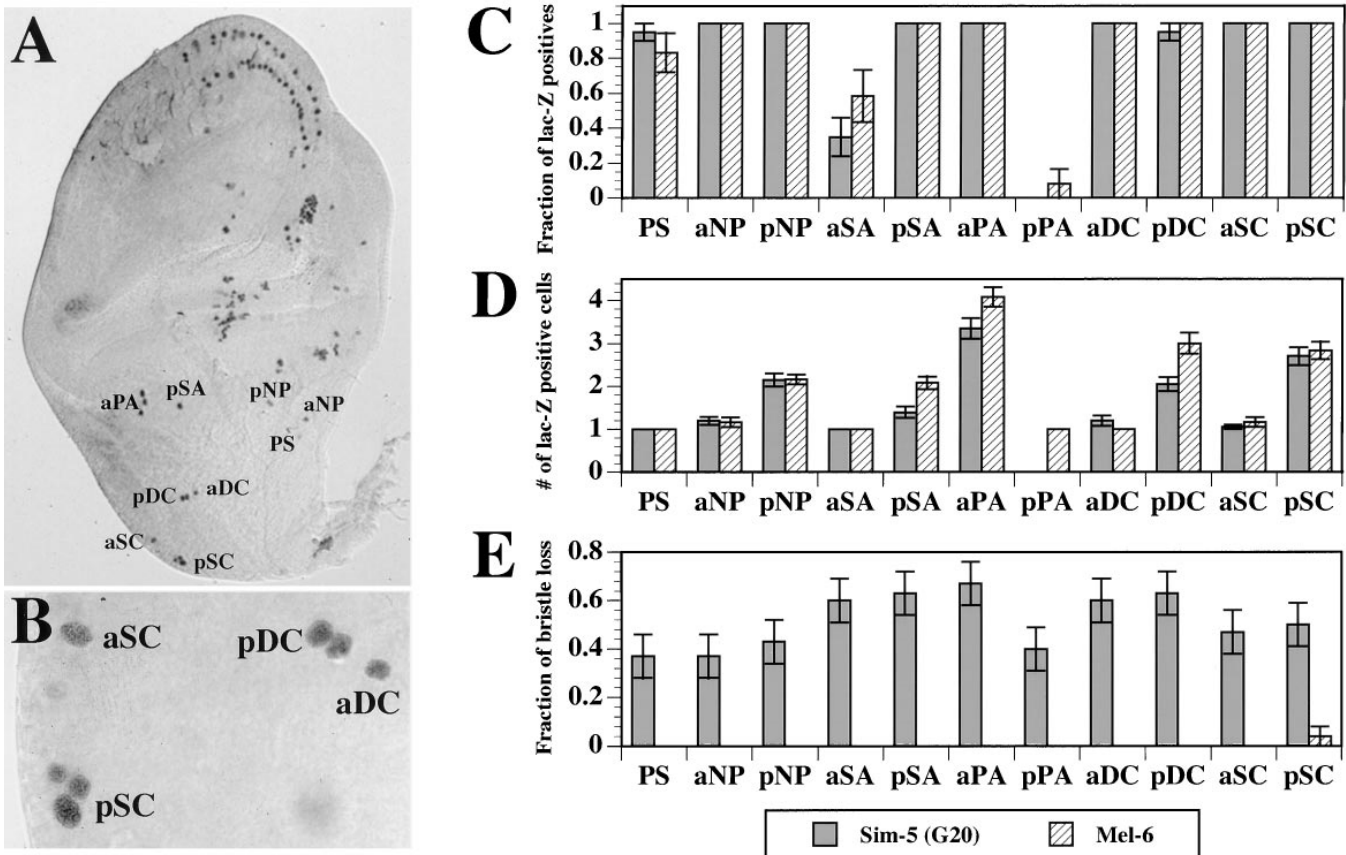

Figure 6.—Normal emergence and cell divisions of the SMCs in prepupae of 1 hr APF. The crosses were made between *C(1)RM/Y*; *TM3/*A101.1F3 *D. melanogaster* females and males of a *D. simulans* line, Sim-5 (G20), or a *D. melanogaster* line, Mel-6. Wing discs from A101.1F3-carrying hybrid prepupae up to 1 hr APF were labeled with anti-β-galactosidase. A typical staining in the Sim-5 hybrid is shown in (A) and magnified in (B). C shows the fraction of presence of stains for each bristle position and D gives the average number of cells in positive stains. Twenty and 12 wing discs were examined for Sim-5 and Mel-6 hybrids, respectively. The number of missing bristles in adults (each takes a value between 0 and 1) is given for each macrochaete position in E, where bristle examination was done on 15 A101-carrying flies for Sim-5 (G20) and 12 for Mel-6. The error bars represent the standard errors. There was no clear difference in C and D between *D. melanogaster-D. simulans* hybrid and pure *D. melanogaster* prepupae in spite of a large number of missing bristles in interspecific hybrids (E).

sheath cells. The *Hairless* mutant, for example, exhibits a double-socket phenotype at the expense of the shaft (Lees and Waddington 1942). On the other hand, the *Delta* mutant can lead to loss of bristles, in which the shaft and socket cells are transformed into a second neuron and sheath cells (Parks and Muskavitch 1993). However, the results of this study indicate that neither of these occurs. The interspecific hybrids lacked both the sockets and shafts at most of the missing bristle positions, indicating no double-socket phenotype. Indeed, for only 36 out of 1148 (3%) missing bristles observed in the hybrids between TT-35 females and Sim-5(G20) males, only a single socket was observed without its shaft. In the other 1112 cases, lack of bristles was accompanied by loss of the sockets. In order to examine the presence of a sensory neuron, staining with a nerve-specific antibody, mAb22C10 (Zipursky *et al.* 1984), was done in the notum of 25-hr APF hybrids of an inbred line, Sim-5 (G20), and *C(1)RM/Y*; A101/*TM3* females of *D. melanogaster*. The average number of missing bristles per fly was 12.8 ± 1.0 in A101-carrying hybrids from this cross. Figure 9 shows that there was a lack of macrochaete neurons, but no double-neuron phenotypes were observed. In sum, loss of bristles in interspecific hybrids was not due to a failure of fate choices among the four cells during bristle development.

Taken together, these results suggest that the defects do not lie in the cell fate decisions during the development of bristles, but in the maintenance of neuronal identity and/or differentiation of the descendants of SMCs.

DISCUSSION

The current study revealed significant effects of the *D. simulans X* chromosomes on the developmental anomaly of bristle formation in the interspecific hybrids,

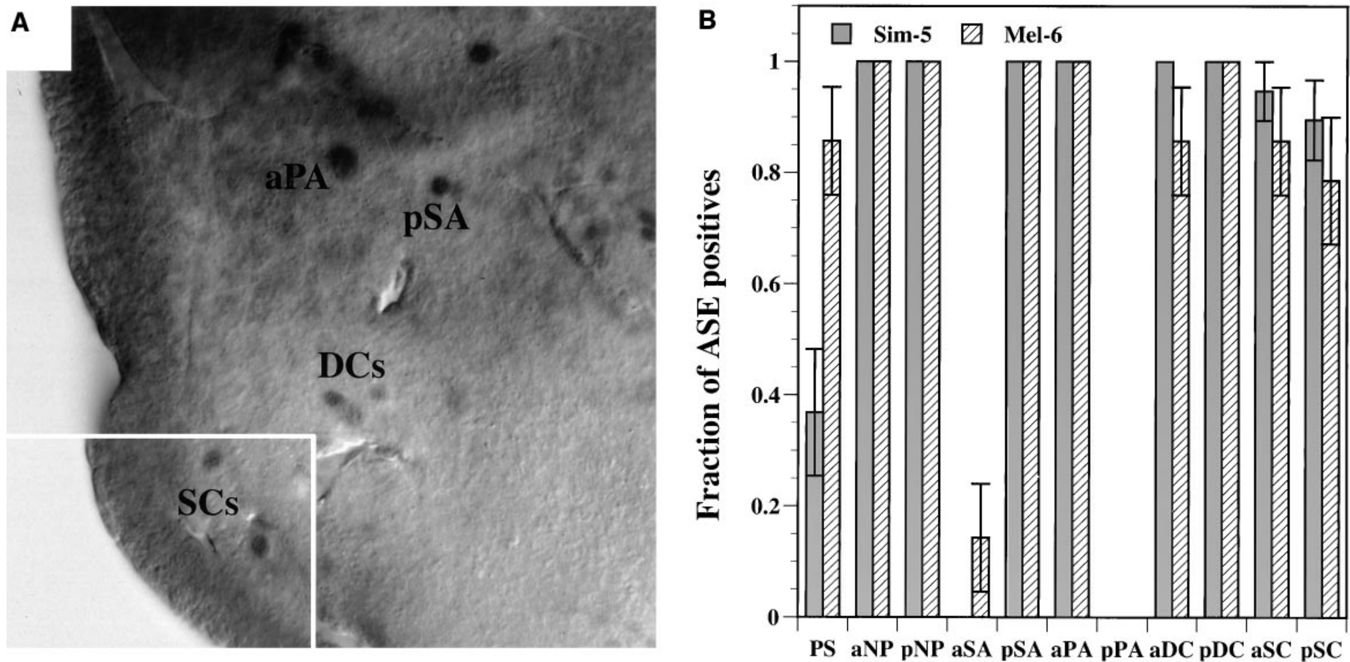

Figure 7.—Normal expression of the *ase* in wing discs of hybrid prepupae of 1 hr APF. A typical staining in the Sim-5 hybrid is shown in A. Appearance of anti-ASE positive cells was not different between *D. melanogaster-D. simulans* hybrids and pure *D. melanogaster* (B), where the vertical axis represents the fraction of discs having the ASE-positive cells. The crosses were done between *C(1)RM/Y D. melanogaster* females and males of Sim-5 (G20) or Mel-6. Ninety and 14 wing discs were examined for Sim-5 and Mel-6 hybrids, respectively. The error bars represent the standard errors. Because identification and assignment of aSC and pSC cells were difficult in a few cases, the actual fraction of discs showing anti-ASE positive cells may be greater than the estimates obtained. This, however, does not affect the conclusion that there was no difference between the *D. melanogaster-D. simulans* hybrid and pure *D. melanogaster* prepupae.

which is consistent with previous studies. Biddle (1932) studied bristle anomaly in hybrids between *D. melanogaster* and *D. simulans* and found that the reduction of bristle numbers is more severe in males than in females. He further showed that the degree of anomaly in the male hybrids varies among the *D. simulans* lines with the largest effects attributable to the *X* chromosome. Muller and Pontecorvo (1940) reported that the bristle reduction and associated abnormality of abdominal banding is due to interaction between gene(s) on the *D. simulans X* chromosome with autosomal gene(s) of *D. melanogaster*, located, at least in part, on the second chromosome. We clearly demonstrated here that the bristle loss was found specifically in *D. melanogaster-D. simulans* hybrids, but not in hybrids of *D. melanogaster* with *D. mauritiana* or *D. sechellia.* Coyne (1985) also found that the *D. simulans-D. mauritiana* hybrids do not show any bristle loss, whereas the *D. melanogaster-D. simulans* hybrids do.

The large effects of the *X* chromosomes detected in this study parallel the findings in the previous backcross studies of hybrid sterility (Coyne and Orr 1989), although the effects of the *X* and the autosomes cannot be compared directly due to the "homozygosity effects" of the *X* chromosomes (Wu and Davis 1993; True *et al.* 1996). The "large *X* chromosome effects" in these genetic analyses do not necessarily mean that the *X* chromosome accumulates hybrid incompatibility factors at a higher rate than the autosomes. Indeed, autosomal introgression of segments of the *D. mauritiana* and *D. sechellia* genomes into *D. simulans* backgrounds shows strong sterility effects in homozygous condition, where the fraction of the autosomal segments showing male sterility is comparable with that of the *X* chromosomes (Hollocher and Wu 1996; True *et al.* 1996). Nevertheless, it is important to notice that the "large *X* chromosome effects" have been found only in hybrid sterility and inviability and not in morphological and behavioral differences between species (*e.g.*, Coyne 1985, 1992; Liu *et al.* 1996). A plausible explanation for this difference is recessive effects in the former (Turelli and Orr 1995), and additive polygenic effects (or lack of directional dominance as a whole) in the latter characters (Charlesworth *et al.* 1987; Liu *et al.* 1996). In this sense, hybrid morphological anomalies, including bristle loss, can be classified into the same class as sterility and inviability, and the genetic bases of hybrid anomalies may be quite distinct from those of between-species morphological differences.

The pronounced defects in hybrid males [see the results in crosses (2) and (3) in Table 3] also parallel the so-called Haldane's rule in postzygotic reproductive isolation (Haldane 1922). In this context, it is intriguing to know whether the genes responsible for the

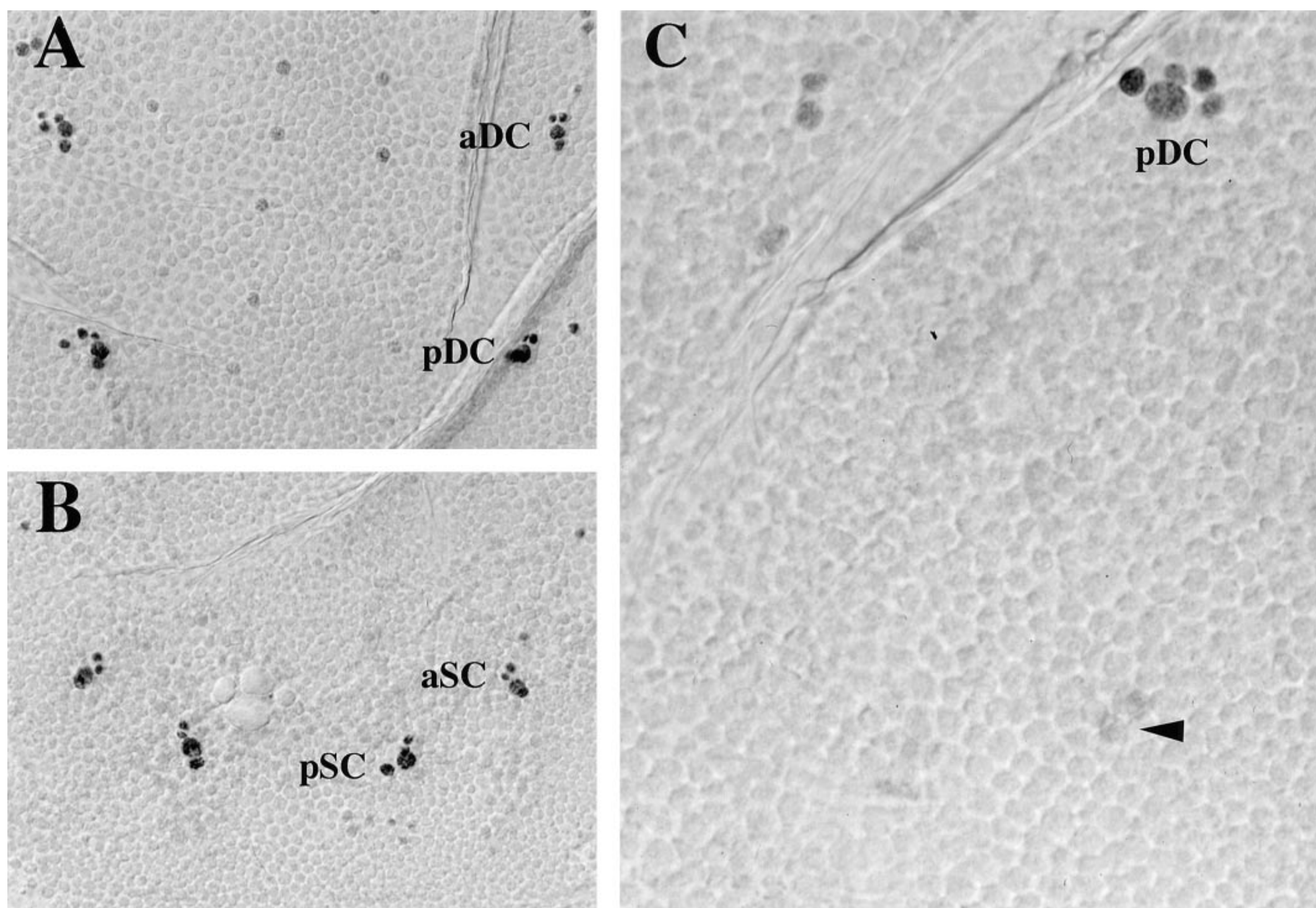

Figure 8.—Failure of the CUT expression in interspecific hybrid pupae of 15 hr APF. A normal staining pattern of DCs (A) and SCs (B) macrochaetae in *D. melanogaster.* C shows a normal staining of pDC macrochaete (clusters of four anti-CUT stained nuclei) but no stain at aSC position in *D. melanogaster-D. simulans* hybrid. The arrowhead refers to the possible position of aSC. Hybrid pupae of 15 hr APF had no or reduced levels of staining with the anti-CUT antibody at a large number of sites (26 out of 61 DCs and SCs), and this fraction was only 1/66 in the pure *D. melanogaster* background.

bristle defects in hybrids also affect male and female fertility in hybrids. Some genes, such as the *Notch* and *Delta*, are known to play roles in oogenesis as well as in neuronal development (Ruohola *et al.* 1991). More recently, it has been suggested that *cut* participates in egg chamber formation (Jackson and Blochlinger 1997). One of the hypotheses to explain Haldane's rule is recessivity of genetic factors causing hybrid sterility and inviability, that is, the dominance theory (Orr 1993; Turelli and Orr 1995). Under this hypothesis, the sex difference is due to a difference in chromosomal genotype, not to sex specificity of genotypic effect. The results of crosses (10) and (11) in Table 3, however, showed a sex difference between comparable genotypes, indicating a certain degree of sex specificity in genotypic effect. From the study of within-species variation of *D. melanogaster*, significant sex-specific effects and epistatic interactions between the mapped QTLs (quantitative trait loci) are observed for abdominal bristle number (Long *et al.* 1995). A strong sex bias has been also found in hybrid sterility between *D. simulans*, on one hand, and *D. mauritiana* and *D. sechellia*, on the other hand, where male sterility factors have evolved much more rapidly than female sterility factors (Hollocher and Wu 1996; True *et al.* 1996). Thus, sex specificity seems to be one of the important factors shaping the evolution of hybrid incompatibility (Wu and Davis 1993; Hollocher and Wu 1996).

A great variability in the degree of the bristle defects was found among the *D. simulans* lines studied. The lines originating from females collected in Madagascar and the nearby small islands did not show any bristle defects, just as in *D. mauritiana* and *D. sechellia*, both of which are endemic on the islands of Mauritius and Seychelles. All the male flies collected from a population in Japan, on the other hand, exhibited a large number of missing bristles. Another example of within-species variation in a hybrid incompatibility study is the rescue mutations of hybrid inviability and sterility found in *D. melanogaster* and *D. simulans* (*e.g.*, Watanabe 1979; Davis *et al.* 1996). These genes themselves could play roles in hybrid inviability (Sawamura *et al.* 1993), al-

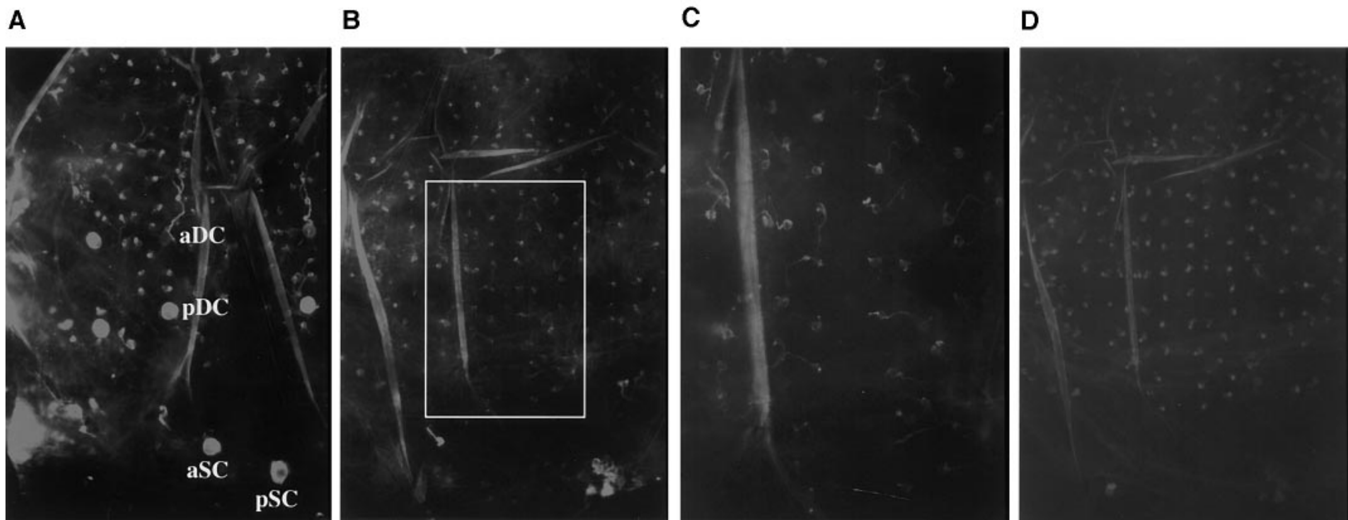

Figure 9.—Absence of a double-neuron phenotype in *D. melanogaster-D. simulans* hybrids. Dissected nota from pupae at the 25 hr APF stage were labeled with the nerve-specific antibody mAb22C10. A shows the normal staining of macrochaetae and microchaetae in *D. melanogaster.* Notum from hybrid pupae of *D. melanogaster* females, *C(1)RM/Y*; *TM3/*A101, and Sim-5 (G20) males was stained simultaneously with mAb22C10 (B and C) and anti-β-galactosidase in A101 (D), in which only microchaetae stains were observed. C shows a higher magnification view of the boxed area of B. The mean number of missing bristles per fly was 12.8 ± 1.0 in A101-carrying hybrids for 13 pairs of macrochaetae on the notum and humeri.

though formally we cannot rule out the possibility that rescue mutations occurred in other genes to circumvent the effects of hybrid incompatibility genes. Thus, the interspecific hybrid analysis should be done with special attention to intraspecific variation. The within- and between-species variation in the bristle defects yields insight into the origin of genetic factors responsible for this anomaly (Figure 3). The results also suggest that a small number of genes on the *D. simulans X* chromosome are involved in the bristle defects.

This study clearly shows that the genetic architecture of bristle formation can change in local populations in the absence of any obvious phenotypic alternation. Hybrid anomaly between species may be developed by successive fixation of incompatibility factors by random genetic drift (*e.g.*, Nei *et al.* 1983) or selective fixation through pleiotropic effects. A correlated response to selection may cause a subtle change in determinants of bristle formation but might be compensated later by another change. Together with the relatively recent origin(s) of some factor(s) causing the hybrid bristle loss, a high degree of within-species variation in *D. simulans* will certainly be useful for studying the process of hybrid-anomaly evolution and the effect of natural selection in this phenomenon.

The data presented here suggest that bristle defects in hybrids lie in maintenance and/or differentiation of precursor cells. We did not detect any cell type transformation (no "double-socket" and no "double-neuron" phenotypes), and *cut* expression was found to be absent or very reduced at many bristle positions, probably resulting in cell death of the precursors. If this is the case, candidate gene(s) responsible for the interspecific hybrid bristle anomaly may play a role in initiating bristle differentiation following *ase* expression in normal condition. Although cell divisions up to 1-hr APF prepupae seems to be normal, loss of bristles in adult flies was accompanied by lack of sockets and neurons at the sites involved. The defects may occur before the cell divisions or in cell-cell communication between the four cells.

Affected bristles in *D. melanogaster-D. simulans* hybrids varied greatly among different flies even from the same cross. This randomly affected pattern is similar to a pattern found in mutants of *D. melanogaster.* The embryos lacking all of the *achaete-scute* complex genes lose 20–25% of their neuroblasts, and their defected patterns are variable as well (Jiménez and Campos-Ortega 1990). Bristle determination in these respects shows some properties of canalized genetic systems (Waddington 1942).

This work was carried out with the purpose of revealing genetic variation accumulated among closely related species during the course of evolution and understanding how differential gene regulation or other mechanisms can produce the same phenotype in different species. The *D. simulans X* chromosome was found to have large effects on the bristle loss of hybrids. Together with a recent origin of at least one genetic factor, this will facilitate isolation of the factor(s) on the *X* chromosome responsible for this hybrid anomaly.

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