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INTRODUCTION

A number of genes are known in Drosophila melanogaster whose alternative alleles determine the differentiation of structures in alternative patterns. In genetic mosaics most of these pattern genes behave autonomously. Even a small patch of tissue of one genotype present on an individual made up mostly of tissue of another genotype differentiates according to its own typical pattern, regardless of the surrounding tissue.

A case in which pattern differentiation in mosaics occurs nonautonomously is that of eyeless-dominant (ey^D) (Stern and Tokunaga, 1967). This genotype is represented by the insertion of a chromosome section of unknown origin into chromosome 4. Apart from its effect on the eye, it leads to the differentiation of multiple instead of single sex combs on the basitarsus of male flies. This abnormal differentiation is correlated with other abnormalities of the tarsi. Frequently, in both sexes, the distal part of the basitarsus is enlarged and fails to develop a normal joint between it and the second tarsal segment. In males the supernumerary sex combs are found on the enlarged epidermis of the basitarsus. In mosaics in which the main part of the body contains the genotype ey^D, but in which part of the sex comb region is not-ey^D (i. e., normal),

the latter tissue differentiates according to the ey^D pattern: more than one row of sex comb teeth instead of a single row is formed in the normal patch.

Three different interpretations were offered to account for the nonautonomy of ey^D differentiation. One of these is based on the assumption that preceding sex comb differentiation, the ey^D genotype leads to an altered (not normal) prepatter of the future sex comb site. The alteration consists in an enlargement of the prepatter area. Subsequently, differentiation of sex comb teeth occurs on the enlarged prepatter, resulting in increased comb production. A second interpretation rests on the possibility that the not-ey^D cells, which originated as a result of somatic crossing over, remained under the influence of their former ey^D constitution, either as a result of persistent ey^D messenger RNA or in consequence of differentiation sequence that had been initiated earlier under the influence of ey^D. A third interpretation is based on the assumption that the formation of multiple sex combs in ey^D is not due to local action of ey^D tissue in the tarsus, but that it depends on an ey^D substance produced elsewhere in the larval body.

The first of the interpretations seemed to be well in agreement with the morphogenetic findings in whole and mosaic ey^D flies, but the facts were also compatible with the third hypothesis--that of a circulating or diffusing ey^D substance. The second hypothesis, that of pre-determination, seems less likely, since earlier work with two recessive genes for extra sex combs had shown autonomy of differentiation in homozygous extra sex comb tissue on a heterozygous background at as late a stage as in larvae 69 to 94 hours old* (Tokunaga, 1961; Tokunaga

and Stern, 1965). Since the mosaic patches in the ey^D experiment were induced in larvae less than 72 hours old the predetermination hypothesis may be regarded as excluded, unless the very unlikely assumption is made that the dominant ey^D behaves in an opposite way from the recessive sex comb genes.

This paper reports the results of transplantation experiments which lead to the exclusion of the hypothesis of an ey^D substance produced outside the leg anlagen.

MATERIAL AND METHODS

Pairs of disks of male first legs from yellow (y, 1) larvae 48 ± 1 hours after hatching at $25 \pm 0.5^\circ \text{C}$ (about 70 hours old reckoned from egg deposition) were carefully dissected out in Drosophila Ringer's solution and transplanted into ey^D/+ larvae of the same age. As controls, transplants were produced in the combinations y \rightarrow y and ey^D \rightarrow y. After eclosion of the host flies the transplants were dissected out under the dissecting microscope and fixed in 70% alcohol and the tarsal segments were mounted between two cover glasses in André medium. These preparations were studied under the compound microscope. The transplants of those host flies which had developed into pupae but had failed to eclose were also examined.

The transplantation procedures essentially followed the original methods of Ephrussi and Beadle (1936), but were modified to facilitate the work with younger larvae. The tip of the transplantation needles was very thin-walled and had a diameter of about 0.07 mm. The elliptic opening of the needle was made smooth by grinding on Arkansas stone moistened with glycerin, and its large diameter formed the base of a rectangular

triangle whose larger side was about 0.15 mm long. No constriction was present in the neck of the needle. Instead, installation of a spring in the syringe and maneuvering with a screw greatly improved the degree of control in sucking up and injecting the transplant.

At the time of transplantation, when the larvae (about 70 hours after egg laying) are kept at $25 \pm 0.5^\circ\text{C}$, each of a pair of first-leg disks is connected to the ventral ganglion and trachea by a long neural process and by a tracheal process, respectively. The two disks are not yet separated from each other at the larval median line, and a common stalk connects the disks to the ventral hypodermis of the first thoracic segment. The stalk is still rather broad and short, requiring careful dissection for separation of the disks from the ventral hypodermis. According to Auerbach (1936), at this stage the wall of the disk is composed of a single layer of cells arranged regularly around the lumen. A dorsal thickening of the wall will later develop into the leg proper. Morphologically, the tarsal segmentation seems to be initiated more than 17 hours after the time of transplantation.

RESULTS

No fully normal legs developed in the transplanted disks. This was the result of the invariable failure of the disks to evaginate, and in addition incomplete development of leg structures occurred independently of the absence of evagination. Fairly well-developed transplants consisted of pairs of legs each of which had formed a folded chitinized single-layered integument sac, the two sacs being connected to each other at a part of the proximal end of each leg. Usually a coxa, trochanter, and femur, with their bristles, were found in the folded integument. At the distal

end of the sac, the leg segment had evaginated inside the sac and developed toward the proximal direction. Identifiable tibiae and tarsal segments were found in this position, although considerable variation was observed in the degree of evagination of the leg segments.

As already described by Bodenstein (1941), most of the bristle patterns in transplants are irregular and difficult to analyze, but sex combs and claws are structures which can be recognized even in poorly developed tarsal segments. Among the developed transplants, those which had at least recognizable tarsal segments were selected and subjected to a detailed study of the sex comb pattern. A few underdeveloped transplants has some well-formed teeth inside a segmented integument that has failed to evaginate. These cases were excluded from the data because of the difficulties of identifying the teeth-forming segment.

A. Development of Sex Comb in Transplants

The development of sex combs in the transplants was very variable. Some transplants formed several transverse bristle rows at the distal part of the tibia, but the basitarsus was very poorly developed and did not have identifiable transverse bristle rows or sex comb. In other transplants the opposite situation prevailed.

The sex comb was usually found on a lobe of the basitarsus which protruded from its distal region. There, the comb structure varied from being a mere chitinous mass to being clearly differentiated as a number of teeth. In fairly well-developed combs from the $y \rightarrow \underline{y}$ and $y \rightarrow \underline{ey}^D$ series the teeth were arranged in a continuous row. Many such rows however, showed one or more gaps between the teeth, suggesting that if the row had developed fully there might have been more that would

have filled the gaps (Fig. 1, d, e). In a few cases one tooth was located outside the row formed by the other teeth (Fig. 1, a). The development of the basitarsal lobe itself was variable, particularly in transplants with underdeveloped combs. Here the arrangement of teeth was distorted and did not conform to a single row (Fig. 1, b). In the $\underline{y} \rightarrow \underline{y}$ series, two combs out of 58, clearly had formed more than one row (Fig. 1, c).

Two unusual transplants were also found in the $\underline{y} \rightarrow \underline{ey}^D$ series. In one case out of 124 the basitarsal lobe had formed a symmetrical double structure. The sex comb teeth had also developed in symmetrical arrangement along the edge of the lobe, thus forming two combs (one with five teeth, another with six teeth and one socket) which were connected at the constricted center of the lobe (Fig. 1, f). It is not clear from the differentiated structures whether the two combs should be regarded as the products of initially abnormal differentiation or as an initially continuous single comb which later became constricted. In the other unusual transplant of the $\underline{y} \rightarrow \underline{ey}^D$ type two separate lobes had clearly developed in a basitarsus and two separate groups of sex comb teeth had formed, one consisting of 21 teeth altogether and the other of 10 teeth (Fig. 1, g).

In the $\underline{ey}^D \rightarrow \underline{y}$ series, 83 combs out of 109 showed more than a single row of teeth either on a normal or a continuously folded lobe at the sex comb site (Fig. 1, i, j, k). The remaining 26 combs, most of them with a low number of teeth, others with gaps between teeth, could not be identified as consisting of a single or of more than one comb entity.

In several ey^D transplants as well as in one y transplant the teeth were formed not only on the typical sex comb site but also on neighboring proximal transverse bristle rows. Apparently in these cases bristles of the transverse rows had differentiated into teeth. One ey^D transplant showed a mass of 11 teeth at the proximal end of the basitarsus, quite separate from a group of 55 teeth at the distal normal site. This basitarsus thus had formed 66 teeth, the highest number in any transplant.

B. Number of Teeth in Transplants

As a control for the number of teeth per comb in the transplants, the number of teeth in the sex combs of the y male hosts of the $y \rightarrow y$ and $ey^D \rightarrow y$ transplant series were determined. Out of a total of 42 host males in which at least one of the transplanted legs had developed a sex comb, 83 forelegs were available for inspection. The number of teeth per comb ranged from 10 to 16, the mean number being 13.16 ± 0.12 . In the same way, 98 forelegs of 49 ey^D host males were checked. The number of teeth per comb ranged from 9 to 42, the mean number being 27.49 ± 0.79 (Fig. 2, A).

In the transplants, several types of underdeveloped teeth were found besides well-developed teeth. Most of the abnormalities encountered concerned the development of trichogen cell, as judged by formation of structures intermediate between bristles and teeth; splitting of the tip of the tooth, often accompanied by large diameter but short length (Fig. 1, b, e); absence or decrease of pigmentation; small size often accompanied by abnormal pigmentation or lack of trichogen cell development although a socket was presented (Fig. 1, f). In some cases the tormogen cell failed to develop so that only the tooth proper was found. Both abnormally developed

teeth and well developed teeth were scored.

As shown in Fig. 2 B, the number of teeth on the 58 transplanted legs of the $y \rightarrow y$ series ranged from 2 to 33, all except one having not more than 16 teeth, which is the highest class in the control series. The comb with 33 teeth was different from the rest not only in the extra number of teeth but also in the arrangement of teeth. It clearly had multiple rows. This comb as well as another multiple comb with 12 teeth (Fig. 1, c) show that the occasional differentiation of extra sex comb structure in transplants can be independent of the ey^D genotype. In the two cases under discussion the multiple combs were formed by not- ey^D disks in not- ey^D hosts.

Among the 124 legs of the $y \rightarrow ey^D$ transplant series, the type of variation in the number of sex comb teeth was similar to that in the $y \rightarrow y$ series (Fig. 2, B). The number of teeth in a sex comb ranged from 1 to 31, all except 3 having not more than 16 teeth per comb. The three exceptions had 31, 19, and 21 teeth. The comb with 31 teeth was one of the malformations mentioned before (Fig. 1, g). The comb with 19 teeth was the one in which all the bristles on the distal part of the basitarsus differentiated into teeth, another abnormally developed basitarsus. The last exceptional case had 19 teeth on the basitarsus, and in addition 2 teeth on the distal part of the second tarsal segment (Fig. 1, i). The 19 teeth were arranged in three groups--4, 7, and 8--with two gaps in between. This may be considered either as an example of a distorted continuous row of teeth with one tooth outside the row next to the middle group, or as a multiple comb.

In both the $\underline{y} \rightarrow \underline{y}$ and $\underline{y} \rightarrow \underline{ey}^D$ series, almost all the transplants, whether in a \underline{y} or in an \underline{ey}^D host, developed no more sex comb teeth than the highest class of the control \underline{y} sex combs. The four exceptionally overgrown cases in both series can be regarded as malformations of injected disks caused by the abnormal condition of transplantation and not as an expression of an \underline{ey}^D genotype. The similarity of the two transplant series in regard to the distribution curves of the numbers of teeth suggests that neither the rare overgrowths nor the more frequent incomplete development of the combs are due to the difference of the host genotypes \underline{y} and \underline{ey}^D . One can conclude from the above data that the \underline{y} transplants developed autonomously, regardless of their host.

In contrast to the above series, the $\underline{ey}^D \rightarrow \underline{y}$ transplants have shown a much wider range of variation in the number of sex comb teeth (Fig. 2, B). Among the total 109 transplanted legs with comb, the number of teeth ranged from 2 to 66. When one compares the data with those from the control \underline{ey}^D hosts, one finds that 90 belong to the normal range of variation (between 9 to 42), 15 belong to a subnormal class (2 to 8), and the remaining 4 belong to a supernormal class (49 to 66). As mentioned earlier, the comb with 66 teeth is an example of malformation. The same holds for the case with 49 teeth, with its 43 teeth on the distal ventral area of the basitarsus and a separate row of six teeth in the middle of the anterior part of the basitarsus. The rest of the four overgrown combs were of typical \underline{ey}^D type (Fig. 1, k).

The data fit well the interpretation that there is autonomous differentiation of the \underline{ey}^D leg disks and that the subnormal and supernormal combs owe their peculiarities to the abnormal situations caused by

transplantation. They correspond thus to the abnormal combs from the $y \rightarrow y$ and $y \rightarrow ey^D$ transplantation series. This conclusion is supported by the study of the arrangement of teeth in combs with abnormal teeth numbers.

DISCUSSION

In all experiments, the great majority of transplanted leg disks behaved autonomously in regard to the differentiation of numbers of sex comb teeth. Earlier work of Bodenstein (1941), who transplanted fragments of third instar male forelegs at a period 12 to 24 hours before pupation, had shown that no regulation occurs with respect to number of teeth and claws. The combined number of teeth from fragments of single leg disks in the wild type Oregon R strain varied between 3 and 13, thus exhibiting no increase over the normal number. This result was confirmed by Nöthiger and Schubiger (1966) in experiments with 96-hour-old disk fragments transplanted into hosts of the same age. On the other hand, they did find increased numbers of various structures, including sex comb teeth, when the transplants were 70 to 80 hours old and the hosts 55 to 58 hours old, thus providing a prolonged period of growth amounting to some 70 hours. Recently, Schubiger (1968) transplanted 72- to 80 hour-old half disks into 55- to 60-hour-old larvae, and compared the differentiation of the transplants with those of 110- to 115-hour-old donor disk halves. In these experiments emphasis was laid on the differentiation of the "edge bristle" of the trochanter, and it was demonstrated that the potency for formation of supernumerary edge bristles disappears during the period of from 72 to 115 hours. Concerning the sex combs it is stated that some have been found with as many as 50 teeth.

It should be remembered that none of the former experiments, conducted with disk fragments, are fully comparable to those with whole disk transplants described in this report. The transplants in this work had a period of some 50 hours available before pupation of the host. In conformity with the results of Nöthiger and Schubiger, and of Schubiger, in all series some transplants had more teeth than the typical range of these structures. In addition some gross malformations were observed, e. g., a leg with two tarsi joined to a common tibia, or disks that developed three partial legs each having a sex comb. Such abnormalities are not unexpected if one considers that at the time of transplantation the leg disks were in a very primitive state of development and that injuries during transplantation might easily result in underdevelopment as well as regulative overdevelopment of leg structures. Regardless of the variability in the manifestation of the developmental capacity in the transplant, the data obtained in the experiments described here have shown clear-cut autonomy in differentiation of sex comb pattern of not-ey^D tissue in ey^D host and of ey^D tissue in not-ey^D host. This result eliminates the third interpretation listed in the introduction, according to which the nonautonomy of not-ey^D tissue in sex comb differentiation observed in genetic mosaics is caused by a circulating or diffusing ey^D substance produced outside the first-leg disks. Therewith the interpretation is strengthened which assigns the differentiation in mosaics to a difference between the prepatterns controlled by the ey^D and not-ey^D genotypes.

SUMMARY

Mosaics consisting of tissue of normal genotype in the sex comb region of otherwise ey^D males had shown nonautonomy of sex comb differentiation, i. e., production of multiple combs by not-ey^D cells. In work reported in this paper, it was investigated whether the nonautonomy depends on local action of ey^D in the first-leg imaginal disk or on a ey^D substance produced elsewhere in the larval body. Disks of male first legs of y larvae seventy hours old were transplanted into ey^D larvae of the same age. As controls, transplants were produced in the combination y to y, and ey^D to y. Regardless of variability in the manifestation of the development capacity of transplants, the data obtained show clear-cut autonomy in differentiation of sex comb patterns of not-ey^D tissue in ey^D host and of ey^D tissue in not-ey^D host.

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Footnotes

Title page: * This work was carried out under auspices of the United States Atomic Energy Commission and supported in part by National Science Foundation grant GB2532.

p. 2 : * The age of larvae given in this paper without explanation is the age after egg deposition.

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Figure 1. Sex comb regions of transplants.

a-c: $\underline{y} \rightarrow \underline{y}$ transplants

d-h: $\underline{y} \rightarrow \underline{ey}^D$ transplants

i-k: $\underline{ey}^D \rightarrow \underline{y}$ transplants (k: 4 teeth on the opposite side are not shown.)

Teeth indicated by dotted outline lie below those with full outline, either on the same plane as the latter or on the opposite plane.

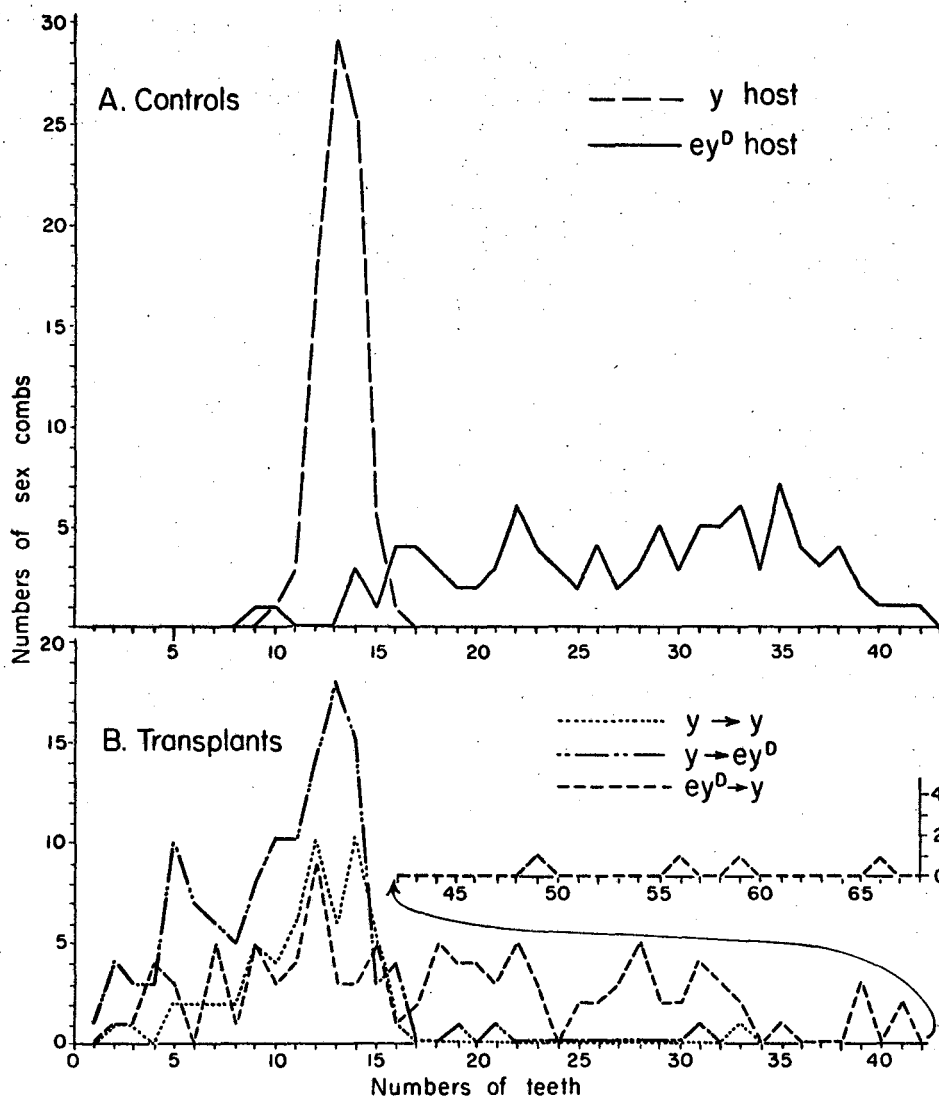
The dotted lines in d, g, h and j correspond to the edges of the transplants on the opposite side.

Figure 2. Frequency distributions of sex combs according to numbers of teeth.



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Fig. 1



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Fig. 2

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