THE DIFFERENTIATION OF A SECONDARY SEX COMB UNDER THE INFLUENCE OF THE GENE ENGRAILED IN DROSOPHILA MELANOGASTER[1]

CHIYOKO TOKUNAGA[2]

Department of Zoology, University of California, Berkeley

Received August 16, 1960

THE pattern of distribution of bristles in *Drosophila melanogaster* is under genic control. Thus, the first tarsal segment of a foreleg of female genotype does not differentiate the row of heavy bristles which constitutes a sex comb while this structure is formed on a foreleg of male genotype. The determination of the sex comb was studied by STERN and HANNAH (1950) by means of analysis of gynandric mosaic forelegs. It was found that the differentiation of sex comb teeth occurred whenever cells of male genotype were present in the region of the forelegs where sex combs normally develop and that the occurrence of this differentiation was independent of the relative sizes of the female and male areas of the mosaic tarsal segment. It was concluded that during development of a foreleg a certain region is singled out from the rest, whose property it is to evoke differentiation of a sex comb if the cells of the region are male but which is not able to cause such differentiation if the cells are female. STERN (1954a, 1957) called the invisible pattern of regional differentiation which precedes that of morphogenetic differentiation a prepattern. Female cells, then, do not respond to the prepattern of a sex comb, while male cells do so fully. Cells of triploid intersexual genotype seem to be intermediate in their sensitivity so that fewer cells respond by formation of sex comb teeth (STERN 1956a, HANNAH-ALAVA and STERN 1957).

In a series of papers the presence of invariant prepatterns, but of differential response of different genotypes, was also demonstrated for the differences between the bristle patterns of achaete and nonachaete, hairy and nonhairy, Theta and non-Theta, scute and nonscute (STERN 1954a,b, 1956b; STERN and SWANSON 1957). KROEGER (1959a,b) has applied and extended the concept of prepattern to the interpretation of his interesting experiments on mosaics between the fore and hind wings of the moth Ephestia, and between the male and female genital discs of Drosophila. In both cases invariant prepatterns seem to be involved. Recently, HANNAH-ALAVA (1958a) has described a case which may constitute genic determination of a prepattern itself.

The present study concerns the action of the recessive autosomal gene engrailed (*en*) which leads to the appearance of a secondary sex comb on male forelegs which forms approximately a mirror image of the normal, primary sex comb. By the use of gynandric mosaics it had been shown that the foreleg of a homozy-

[1] Supported by a grant from the National Science Foundation to CURT STERN.

[2] Present address: Kobe College, Nishinomiya, Japan.

gous *en/en* female possesses a prepattern for the secondary sex comb like that of the homozygous *en/en* male (STERN 1954b). The question remained whether a not-engrailed heterozygous *en/+* male foreleg also possesses such a prepattern. If so, the difference between *en/en* and *en/+* males would not consist of a difference in prepattern organization but solely in response of the two genotypes to an invariant prepattern: *en/en* responding by differentiation of a secondary sex comb and *en/+* failing to respond. This problem was attacked by means of analysis of forelegs mosaic for *en/en* and *en/+* tissue. Such mosaicism resulted from somatic crossing over induced by irradiation (BECKER 1957).

METHODS

In all experiments, larvae heterozygous for engrailed, *en/+*, were irradiated with X-rays and the forelegs of the resulting adult male flies examined for evidence of somatic crossing over between the kinetochore of chromosome 2, located at 55.0, and the locus of *en*, located in the right arm at 62.0. As a criterion for determining whether a particular tissue area was *en/en* or *en/+*, the coloration of bristles and teeth of sex combs was used. This was made possible by introducing a recessive marker gene for coloration in heterozygous state which in the majority of cases of somatic crossing over was to become homozygous together with *en*. The forelegs of treated individuals were examined for mosaicism under a 60-fold magnification of a dissecting microscope, and flies with variegation on a foreleg or with a secondary sex comb were subjected to detailed study under a 680-fold magnification under a binocular compound microscope.

Three separate experiments were conducted as described below. The effective X-ray dosage in the first, preliminary experiment varied from approximately 1000r to 2200r and the age of the larvae varied from 24 hours to 72 hours after hatching. In the definitive experiments I and II, the dosages were 1500r and 1800r, and the larval age was again varied. Most cultures were kept in an incubator at 25° ± 1°C, but some of the larvae were kept at 17°C until they were irradiated.

For the preliminary experiment, straw^3 (*stw^3*, 55.1, bristles and hairs strawish yellow) was selected as the marker. Twelve males with one foreleg each showing a secondary sex comb in the normal position and many forelegs with variegation for the primary sex comb were obtained. Each of the secondary sex combs consisted of one or more teeth, all straw^3 in color and distinct from the normal dark tooth color of the primary sex comb on the same tarsal segment. The experiment was successful as far as induction of appropriate somatic variegation was concerned. However, detailed examination of the tarsi, which were mounted in euparal between two cover glasses, met with difficulties. While the color of the teeth of sex combs could be clearly classified as either straw or not straw, the difference in color often was not clear in the more slender bristles. Thus, it was not possible to determine exactly how large a region was occupied by homozygous *stw^{3}en* tissue.

Accordingly, experiments were devised in which the marker gene straw^3, was

replaced by the gene yellow (y,1,0.0) which is known to be a good marker (STERN and HANNAH 1950). Since yellow normally is located on the X chromosome, the use of a translocation was indicated which would link this locus with engrailed. Two different procedures were employed in experiments referred to as I and II, respectively.

Experiment I made use of translocation T(1;2) sc^{S2} in which the tip of the X chromosome which contains the normal allele of yellow and the tip of the right arm of the second chromosome are interchanged (Figure 1A). Larvae were obtained in which one each of the first and second chromosomes carried the translocation and the wild-type alleles of yellow and engrailed, and the other first and

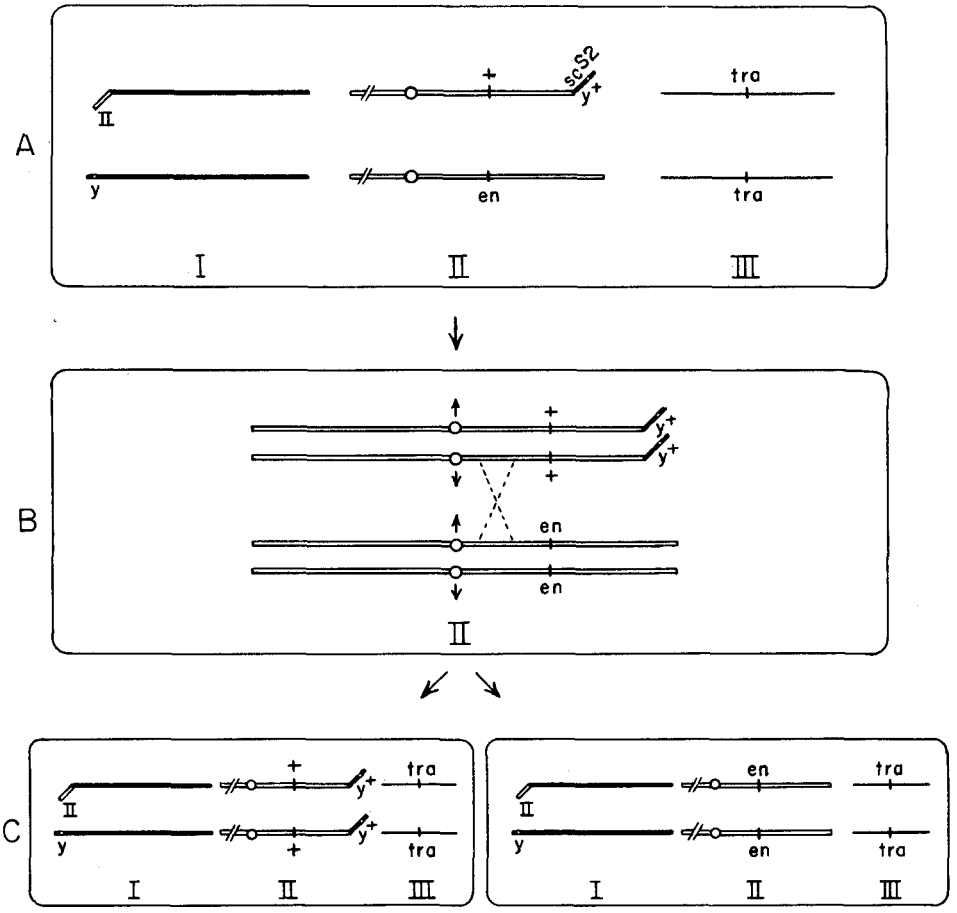


FIGURE 1.—Experiment I. A. Genetic constitutions of the first (I), second (II) and third (III) chromosome in heterozygous *en* larva before X-ray irradiation. B. Somatic crossing over between the centromere (o mark) and the *en* locus on the second chromosome. C. Resulting two daughter cells; left, cell with y^+, en^+ phenotype; right, cell with *y*, *en* phenotype. Black heavy line = X chromosome; double line = second chromosome; black thin line = third chromosome. *y*, yellow; sc^{S2}, scute bristle effect; *en*, engrailed; *tra*, transformer.

second chromosomes carried the recessives yellow and engrailed. Somatic crossing over between the kinetochore and the locus of engrailed (Figure 1B) would lead to the formation of two new genotypes (Figure 1C) one of which is homozygous for engrailed and hemizygous for yellow. Cells with this genotype, like the rest of the fly, would normally be female and therefore be unsuitable for an analysis of sex comb differentiation. However, the experiment was so designed as to make the flies homozygous for the third chromosome gene *tra* which transforms XX individuals into phenotypic males (STURTEVANT 1945). The experiment succeeded in furnishing a number of XX males with secondary yellow-toothed sex combs and with variegation in the primary sex comb area. However, one of the foundation stocks was highly infertile so that it was difficult to obtain a sufficient number of desired flies.

This led to the planning of experiment II which circumvented the use of the *tra* gene. Male larvae were obtained whose first and one second chromosomes were of the T(1;2)sc^{s2} type and whose other second chromosome carried engrailed. In addition they carried a special Y chromosome [Y(II-15i)], obtained by E. NOVITSKI, which possesses a short insertion of the left end of an X chromosome with the recessive yellow allele and the wild type allele of scute (Figure 2). Such males are not yellow since they carry y^+ in the translocation. However, crossing over in the second chromosome between the kinetochore and the locus of engrailed results in cells with two normal second chromosomes homozygous for *en*, a first chromosome deficient for *y* (and carrying a duplication for the tip of chromosome 2), and a Y chromosome compensating for the deficiency in the X and carrying *y*. Such cells, then, are *y en/en*. This experiment provided numerous clearly marked mosaic tarsi.

It may be added that the *en* carrying second chromosome also had straw[3] so

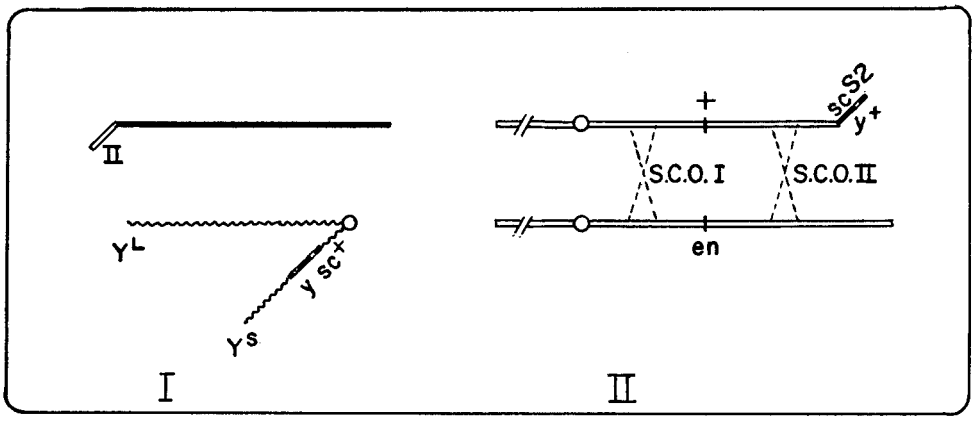


FIGURE 2.—Experiment II. Genetic constitutions of the first (I) and second (II) chromosomes. Black solid line = X chromosome; double line = second chromosome; wavy line = Y chromosome; Y^L = long arm of the Y chromosome; Y^S = short arm of the Y chromosome; S.C.O.I. and II = two possible positions of somatic crossovers; *y* = yellow; sc^+ = scute$^+$; sc^{s2} = scute bristle effect; *en* = engrailed.

that homozygous *en/en* cells were not only hemizygous for *y* but also homozygous stw^3/stw^3.

Sex combs and chaetotaxy in the tarsus of homozygous engrailed males: Although BRASTED (1941) had dealt with the morphology of the first tarsal segment of *en/en* males, it was necessary to establish specific control data for the present experiments since both genetic and environmental variations influence the patterns. Such data were obtained for homozygous wild type and heterozygous *en* males and for homozygous *en/en* males of three genotypes corresponding to those used in the three experimental series (Figure 3; Table 1). In contrast to the limited variability in appearance of the primary sex comb there is high variability of the secondary comb in number and position and, occasionally, in shape of the teeth. The number of teeth in the secondary comb varied from 0 to 12, and their mean number in one genotype was significantly larger than in the two other genotypes. The shape and size of teeth in the secondary comb were usually like those of typical primary teeth, but some were intermediate between the blunt typical teeth and the pointed macrochaetae (e.g., the proximal tooth in Figures 4a, b). Also, at times there were rather distinct variations in length of teeth of the same secondary comb. Most variable was the arrangement of the teeth. When the number of teeth was small, e.g. two or three, they were usually arranged in a line parallel to the length of the primary comb so that the two combs formed approximate mirror images. Larger secondary combs were often more irregular,

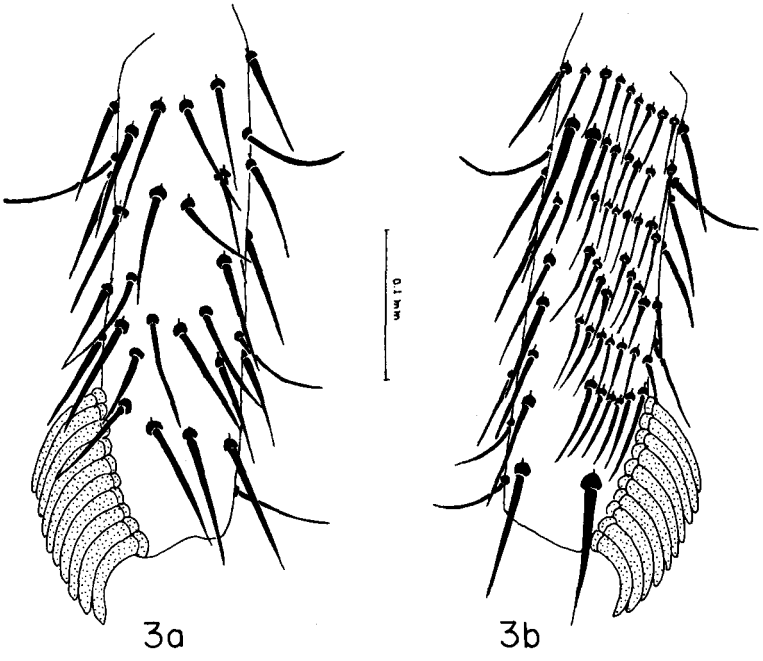


FIGURE 3.—First tarsal segment of a wild type male foreleg (Canton-S). a, dorsal side; b, ventral side; black bristles = + phenotype; dotted bristles = + phenotype sex comb teeth; (Camera lucida drawing).

TABLE 1

*Number of teeth in the primary (a) and secondary (b) sex combs in wild type, homozygous engrailed and heterozygous engrailed males**

(a) Genotype†	Primary sex comb: Distribution of the sample according to teeth number 7	8	9	10	11	12	13	Mean number
(1) Canton-S (wild type)	0	1	13	24	11	1	0	9.96 ± 0.11
(2) *stw³ en* / +	0	0	8	22	17	3	0	10.30 ± 0.11
(3) *stw³ en*	0	5	6	18	13	7	1	10.28 ± 0.17
(4) *y ac sn³; stw³ en; tra/Ubx*	0	2	9	11	23	4	1	10.42 ± 0.15
(5) *y ac sn³*/Y(II-15i)*y sc⁺; stw³ en*	2	8	14	12	13	1	0	9.58 ± 0.17

(b) Genotype cf part (a)	Secondary sex comb: Distribution of the sample according to teeth number 0	1	2	3	4	5	6	7	8	9	10	11	12	Mean number
(3)	1	5	10	12	12	6	2	0	1	1	0	0	0	3.36 ± 0.24
(4)	1	6	6	12	11	10	1	2	0	0	1	0	0	3.56 ± 0.25
(5)	0	2	2	4	5	8	5	11	5	4	2	1	1	6.08 ± 0.35

* For each sample, 50 tarsals from the left forelegs of 50 flies were studied.
† Symbols explained in text except *Ubx* (=ultrabithorax).

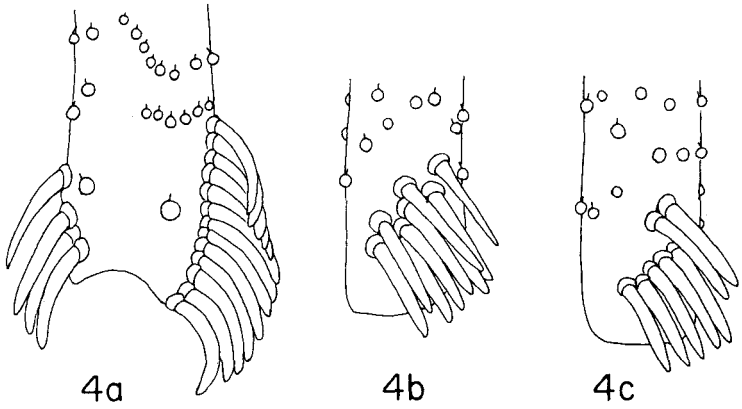


FIGURE 4.—Part of first tarsal segments of homozygous *en* male forelegs. a. Genotype: *stw³ en*. The secondary sex comb has four teeth. b, c. Genotype: *y ac sn³* Y(II-15i) *y sc⁺; stw³ en*. Only the secondary sex comb site is shown (*ac*=achaete, *sn³*=singed³). White circles indicate the positions of the *stw³* bristles (a), or yellow bristles (b, c).

being arranged in a discontinuous row or distributed irregularly over a wider area (Figure 4a-c).

In order to define the location of this area it is necessary to discuss the standard pattern of chaetae on the first tarsal segment of the male foreleg. A map of this pattern was first made by HANNAH-ALAVA (1958a,b). It formed the basis of a slightly changed version which was used for the present work. This version is

shown in Figure 5. A series of longitudinal and transverse rows of bristles exists which will be discussed individually. In each row considerable variation exists from fly to fly. This variability is smallest in rows 4, 5 and 6 including the bractless bristles between them.

Row 1 is easily located on a leg except that its bristles sometimes are arranged in a zig-zag manner which may make it difficult to distinguish them from those of row 2. Similarly, row 3 is strongly variable. The high variability of bristles centered in the area which includes rows 1 to 3 made it difficult to define a standard pattern for row 2. The same is true for the area between row 6 and the adjacent transversal rows, which includes row 7 in whose neighborhood the primary sex comb is located. In the diagram, Figure 5, the most frequent numbers of bristles, including bractless ones, found in 50 forelegs, have been represented in rows 1, 1′, 3, 4, 5 and 6, as well as that of the teeth in the primary comb. Rows 2 and 7 are represented somewhat more arbitrarily. The same is true for the transverse rows, six such rows being most frequent but by no means found exclusively.

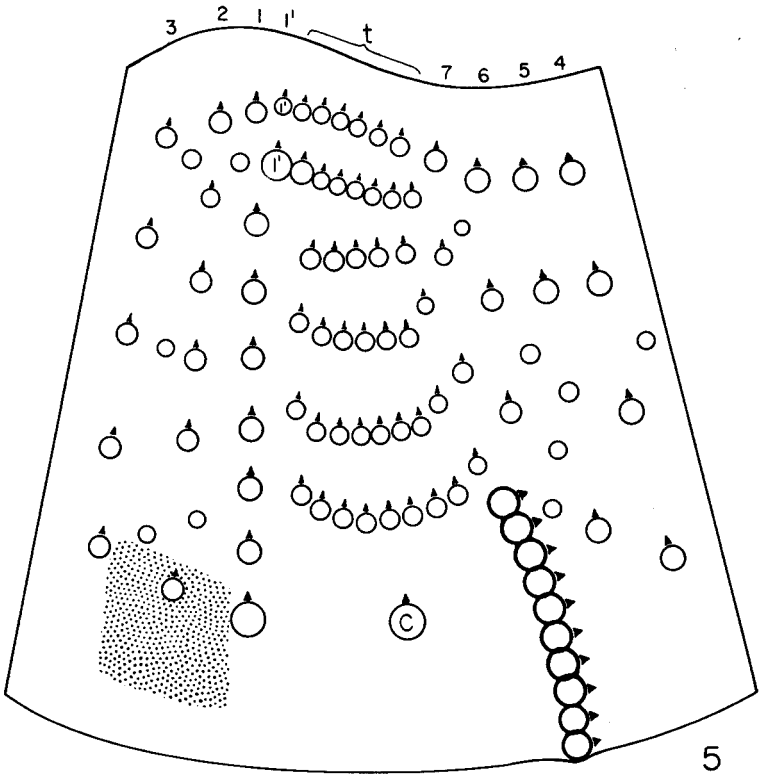


FIGURE 5.—Schematic representation of the standard bristle pattern on the first tarsus of the male foreleg in *Drosophila melanogaster*. Light circles with triangles = normal bristles with bracts; heavy circles = primary sex comb teeth; light circles = bractless bristles; dotted area = the secondary sex comb area in homozygous *en* tarsi; 1, 1′, 2,3,4,5,6,7 = numbers assigned to the longitudinal bristle rows; t = transverse rows on the ventral side of the tarsus; C = central bristle.

A more definite map of the bristles will have to be drawn after a detailed study of cell lineage.

In contrast to the highly localized, narrow zone occupied by the primary sex comb, teeth of the secondary sex comb may be found over a distal tarsal area which is about as wide as it is long. This is indicated by stippling in Figure 5. Generally, the proximal end of the secondary comb lies between the terminal bristles of rows 2 and 3, but it may be shifted to the area between rows 1 and 2. The distal end of the comb never reaches the edge of the segment, and, in the nonmosaic legs studied, no bristles were found between the distal end of the comb and that of the segment.

Mosaic forelegs

Variegation including the area of the secondary sex comb: In the three experimental series a total of 81 first forelegs was found in which a secondary sex comb had differentiated on initially heterozygous *en*/+ males. Sixty-one cases came from experiment II. Table 2 gives data on the frequency of these mosaic segments after irradiation of larvae of different ages. The mean frequency was close to one mosaic in 174 tarsi, and there were no striking differences between the frequencies obtained in the different subgroups.

Among the 81 tarsal segments mentioned, 69 came from experiments I and II. Sixty-seven of these were available for the determination of the area occupied by *en/en* tissue since it was marked by bristles of yellow coloration (Table 3). Apparently, single somatic crossovers had occurred between the kinetochore and the locus of *en* thus giving rise to *y en/en* genotypes.

1. In 20 tarsi only the secondary sex comb itself showed the yellow phenotype. Twelve of these "sex combs" consisted of a single tooth only. These mosaic tarsi

TABLE 2

*The frequency of the occurrence of the variegated tarsals which differentiated a secondary sex comb after irradiation at various larval ages (Exp. II)**

Larval age at irradiation (hour unit after hatching)		Total number of wild-type males among treated flies	Number of variegated tarsi with secondary sex comb	Frequency percent
A	12–29	279	2	0.35
	19–37	793	5	0.31
	27–48	1651	20	0.60
	36–56	668	16	1.19
	47–72	906	4	0.22
	Total	4297	47	0.54
B	27–72	805	11	0.68
	72–127	193	3	0.77
	Total	998	14	0.70
	Grand total	5295	61	0.57

* X-ray dosages were 1500r and 1800r. Incubator temperature was 25°C±1°C. in A, but in B the larvae were kept in 17°C until they were irradiated.

indicate that even small areas of *en/en* genotype differentiate a secondary sex comb if located within the prospective area.

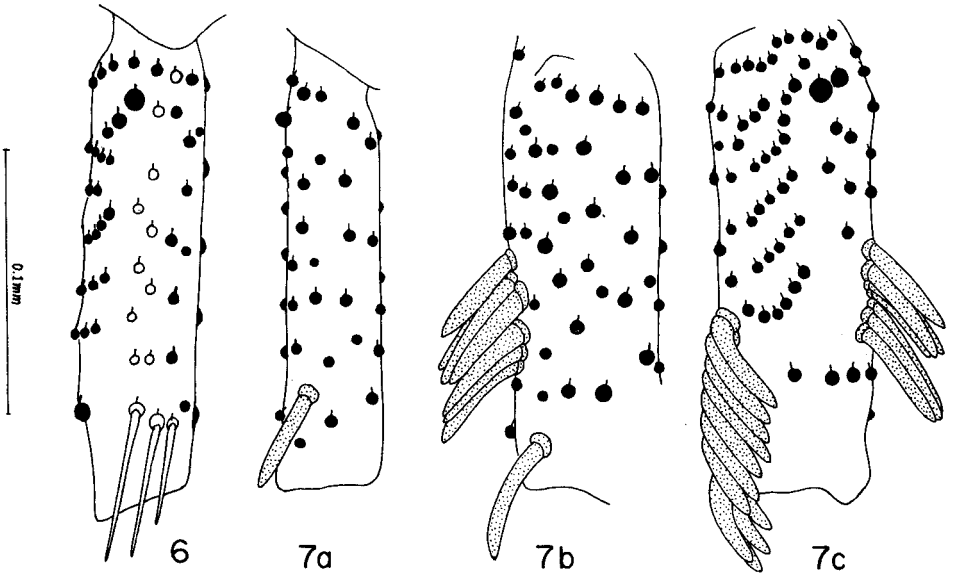
2–4. In 47 tarsi, apart from the teeth of sex combs, bristles in the surrounding areas were yellow. These bristles belonged either to row 1 or 2 or to both rows, and sometimes included, in addition, bristles in rows 1′ and 3. There were three tarsi in which bristles of rows 2 and 3 were yellow but no tarsus with a secondary sex comb in which yellow bristles in row 3 only were found. Likewise, yellow bristles in row 1′ were accompanied by a secondary sex comb only when row 1 also had yellow bristles. These data suggest a close developmental relation between the area of the secondary sex comb and the areas of rows 1 and 2 and occasional shifts of the border lines between the two rows 1 and 1′, and 2 and 3, respectively.

There were two exceptional tarsi in experiment II, not listed in Table 3, on which bristles near to or within the area of potential secondary sex comb differentiation were yellow but on which no sex comb teeth had formed. One of these segments is illustrated in Figure 6. All bristles of row 1 and some of row 2 were yellow. The other segment had only one yellow bristle which was the most distal one in row 2. These two mosaic segments may possibly correspond to cases in which a secondary sex comb fails to differentiate even in nonmosaic *en/en* males (see, for instance, the two forelegs referred to in Table 1, rows 2 and 3 from below, which had no secondary comb). This would appear unlikely, however, since in the stock used in the experiment (Table 1, last row) not a single leg without a secondary comb was found. A more likely explanation of the mosaic shown in Figure 6 is that it was a product of somatic crossing over to the right of the *en* locus (see Figure 2, II) which would result in a *y en*/+ genotype. The same explanation

TABLE 3

Part 1: The relation of the secondary sex comb to the phenotypes of the neighboring bristles in mosaic tarsi (Exp. I and II). Part 2: The relation between the number of teeth in the secondary sex comb and the phenotypes of the neighboring bristles in mosaic tarsi (Exp. II)

Part 1 (Exp. I and II)		Part 2 (Exp. II)								
			Distribution of the secondary sex comb according to their teeth number							
Mosaic area	Number of tarsi	Number of tarsi	1	2	3	4	5	6	7	Mean teeth number
A Secondary sex comb yellow:	67	59	34	11	7	4	1	0	2	1.8
1. Only secondary sex comb yellow: rest of bristles +	20	14	12	2	0	0	0	0	0	1.1
2. Some bristles belonging to row 1 or 2 yellow:	31	29	15	7	3	3	0	0	1	1.9
3. Some bristles belonging to row 1 and 2 yellow:	13	13	4	2	4	1	1	0	1	2.7
4. Some bristles belonging to row 2 and 3 yellow:	3	3	3	0	0	0	0	0	0	1.0
B Secondary sex comb wild phenotype	2	2	2	0	0	0	0	0	0	1.0



FIGURES 6–13.—Mosaic tarsals on heterozygous *en*/+ male forelegs, induced by X-ray irradiation. (Camera lucida drawings). The secondary sex comb is shown in each case. The primary sex comb is shown in some cases only, either in detail or in outline. The positions of the bristles on the tarsus are indicated as in Figure 5, except in Figure 6 where three bristles at the end of rows 1 and 2 are shown completely. Open symbols = yellow bristles or sex comb teeth; solid symbols = wild type bristle; dotted symbols = wild type sex comb teeth. All from Experiment II except No. 4-a which is from Experiment I.

FIGURE 6.—No. 67. FIGURE 7.—a. No. 27; b, c. No. 49 dorsal and ventral side.

may be valid for the other mosaic, but it is also possible that the single yellow distal bristle in row 2 which was located at the shifting critical borderline of the potential secondary sex comb area represented a very small number of cells which did not lie in the sex comb determining region of this specific segment.

Two tarsal segments were found which had formed secondary sex combs but whose teeth were not yellow (Table 3, Part 1B). These segments were not variegated for yellow—their whole surface being non yellow. One of the segments had only a single secondary comb tooth and, distal to it, a bractless bristle (Figure 7a). The other had one tooth located within the area typical for the secondary comb and had in addition, in the abnormally broadened area between rows 1 and 3, an irregularly arranged group of seven or more teeth (Figure 7b,c). The location of these teeth was near the typical area for a secondary sex comb, and the abnormal width of the tarsal region between rows 1 and 3 which was accompanied by an increase in the number of bristle rows in this region is evidence of some disturbance in the differentiation of the leg bud, perhaps caused by the irradiation (larvae kept at 17°C until exposure to X-rays).

The non-yellow, engrailed phenotype of these two segments may be accounted for by assuming the occurrence of somatic double crossovers, one between the

kinetochore and the locus of *en* and another between the latter and the distal translocated part of the second chromosome (Figure 2, II, s.c.o.I and II).

In Part 2 of Table 3, data are presented concerning the numbers of teeth in the secondary sex combs as related to the presence or absence of yellow bristles in rows 1, 2 and 3. The mean number of teeth increases from 1.1 in group 1 where no yellow bristles were present to 2.7 in group 3 where yellow bristles occurred in both rows 1 and 2, the number being intermediate, namely 1.9, in group 2 where yellow bristles were restricted to either row 1 or 2. The differences between the three distributions of numbers of teeth are significant at the five percent level. There is thus a correlation between the size of the yellow area adjacent to that of the secondary sex comb and the number of teeth differentiated. Such a correlation is not unexpected since the chance that a yellow area will include that of the prospective secondary sex comb should increase with the size of the yellow area. This correlation, however, does not signify that the size of the secondary sex comb depends on that of the surrounding tissue. This may be shown by a discussion of some selected cases:

(a) In Figure 8a-c, two segments are illustrated, both of which had seven secondary sex comb teeth. On the first segment all bristles of row 1, eight in number, were yellow; in the second segment all those of both rows 1 and 2 were yellow also. In contrast to these two segments, a third (Figure 8d) had only a single secondary tooth. Nevertheless, again all bristles on rows 1 and 2, except the bractless ones, were yellow.

(b) In Figure 9a and b segments are illustrated both of which had four secondary

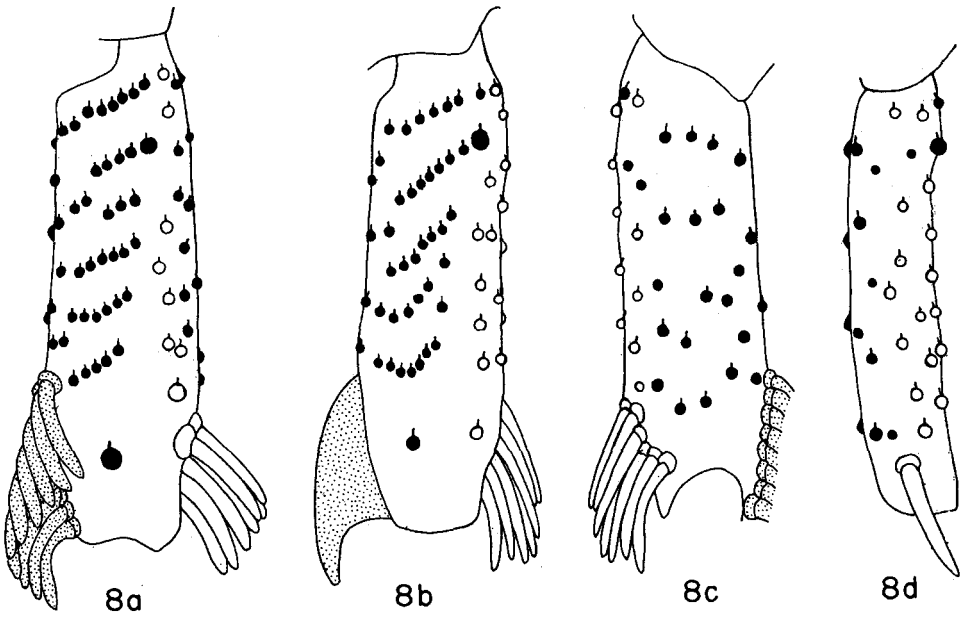


FIGURE 8.—a. No. 48; b, c. No. 24, ventral and dorsal side; d. No. 66.

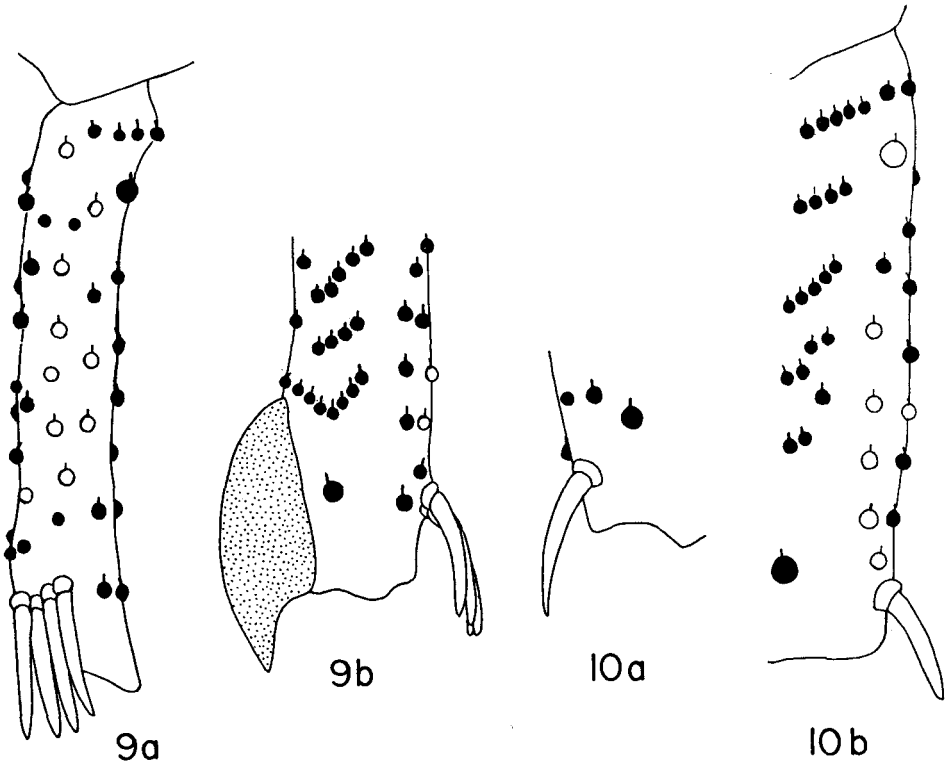


FIGURE 9.—a. No. 61; b. No. 44. FIGURE 10.—a. No. 4-a; b. No. 20.

sex comb teeth. In one of these, ten bristles of row 2 were yellow, in the other only one in row 1 and one in row 2.

(c) A certain tarsal segment had only one secondary tooth though adjacent to three yellow bristles in row 1, in contrast to another segment which had three teeth and no yellow bristles at all.

It is thus clear that the size of secondary combs depends on the size of the *en/en* area within the potential comb region independently of the total size of the yellow engrailed patch of tissue.

There remains a brief discussion of certain peculiarities concerning the secondary sex comb in variegated tarsi. In general no differences between these combs and those on homozygous engrailed males were noticed either in shape, size, position or arrangement of teeth. Occasionally variants were found of types also seen in the controls. Thus, among the secondary sex combs consisting of a single tooth, that of one was intermediate in shape between a typical macrochaeta and a sex comb tooth (Figure 10a), those of three were unusually short (Figure 10b), and that of another unusually thin. In still other instances, the tooth was straight instead of being slightly curved. Since, as stated, these variants occurred also in nonmosaic tarsal segments, it is not necessary in the mosaic segments to invoke an influence of the neighboring *en/+* tissue. However, a few variants were ob-

served which were not encountered in the controls. On one mosaic tarsus the single tooth of the secondary comb had differentiated at the position of the most distal end bristle of row 1, i.e., laterally of the area of normal differentiation. Only one bristle, that adjacent to the tooth, in row 1 was yellow, all others being non yellow. The tooth itself was straight and pointed in the same direction as the normal end bristle of row 1 which it replaced (Figure 11). In three other cases one of the normal, nonyellow bristles of row 2 had differentiated between the secondary sex comb and the distal end of the tarsal segment (Figure 7a, 12a-c). These may have been mosaic tarsi in which the homozygous *en* tissue occupied only a small part of the proximal secondary sex comb area while the distal part contained heterozygous *en*/+ tissue in that area in which the distal bristle of row 2 had formed.

Variegation including the area of the primary sex comb: In experiments reported in this paper 85 mosaic tarsal segments possessed areas occupied by new genotypes obtained from somatic segregation which included the area of the primary sex comb. (Of these, 36 were found in the preliminary experiment, 3 in Experiment I, and 46 in Experiment II.) Among these tarsi, 49 could be analyzed in detail as to the extent of the homozygous engrailed area (Table 4). It is seen that the extent of the area varied from such small size as to include only a single

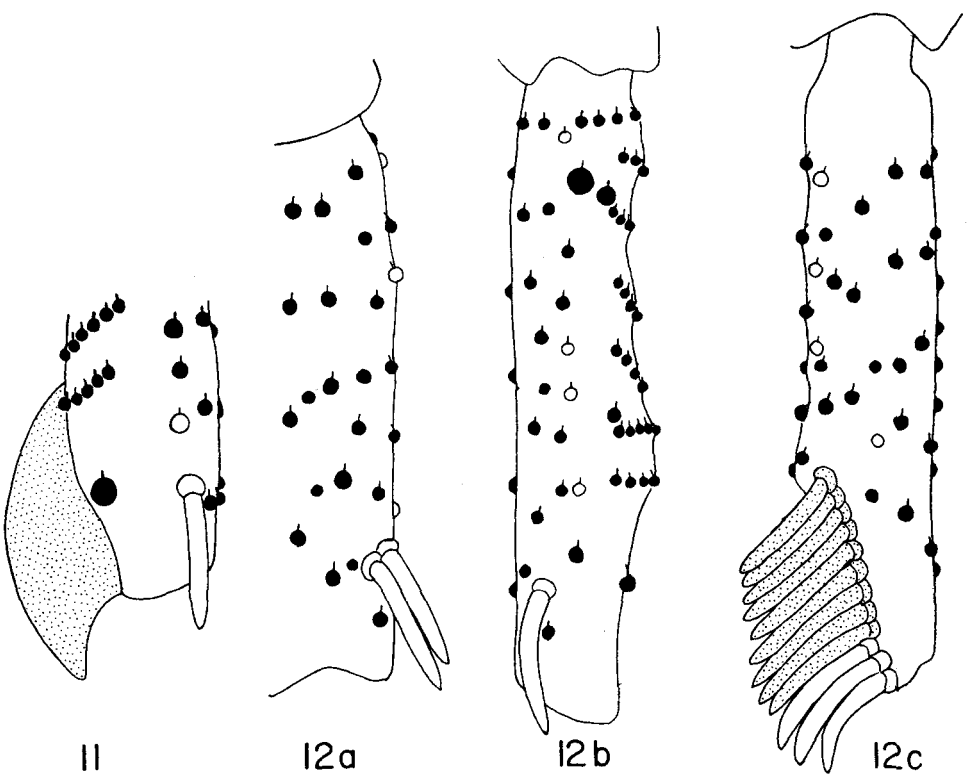


FIGURE 11.—No. 17. FIGURE 12.—a. No. 54; b, c. No. 65 (outer and inner side).

TABLE 4

Classification of the mosaics in the primary sex comb area according to the mosaic part

	Primary sex comb teeth	Other bristles	Yellow bristles not including primary sex comb: Number of bristles	Yellow bristles not including primary sex comb: Number of rows to which the yellow bristles belong
A	mosaic (48)*	+ (28)	0	
		mosaic (20)	1 (5)	No.3.5† (1), 5.5 (1), 6 (1), No.7 (1), transverse row (1).
			More than 2 (15)	No.5 (1) transverse rows (6) transverse rows + No.1′ (3) transverse rows + No.7 (2) transverse rows + No.5.5 + No.6 + No.7 (1) No.7 + No.5.5 + secondary sex comb tooth + No. 1 (2)
B	yellow (1)	mosaic (1)	Many (1)	transverse rows + No.1′ + No.4 + No.5 + No.6 + No.7 (1)

* Number of cases is shown in ().
† No.3.5 means the bractess bristle between row 3 and row 4; the same for No.5.5 (between row 5 and 6).

yellow tooth on an otherwise not yellow segment to one segment in which the yellow area included rows 4, 5, 6, 7, the whole primary sex comb, all transverse bristle rows and row 1′ (Figure 13a,b). Indeed, this last named segment had the largest homozygous engrailed area of any mosaic tarsus obtained. More such extensive segregated areas could be expected in the future if the larvae were irradiated at younger stages and less heavily.

In general, the yellow bristles or segments with variegation involving the primary sex comb were confined to rows 4, 5, 6, the transverse rows and row 1′. Only two exceptions were encountered, in experiment II. These two tarsal segments had each two separate areas of yellow tissue, one involving the primary, the other the secondary sex comb area. They have already been partly discussed under variegation in the area of the secondary comb. Here, they are once more considered in regard to the primary comb area. One of these segments had ten primary teeth, the most distal one of which was yellow as were also some bristles between row 6 and the transverse rows as well as two out of the three bractless bristles between rows 5 and 6 or row 5.5. The secondary teeth were two in number, yellow and accompanied by six yellow bristles in row 1. The other segment (Figure 12b,c) had 12 primary teeth of which the three most distal ones were yellow as were also three bristles between row 6 and the transverse rows, as well as one of the three bractless bristles of row 5.5 (Figure 12b,c). The single secondary tooth was yellow and accompanied by four yellow bristles in row 1. It seems most likely that each of these two tarsal segments showed the results of two independent occurrences of somatic crossing over in the imaginal leg disc of the irradiated larvae.

The border lines between the yellow and not yellow areas in this group of variegated segments usually fell between the various bristle rows. Some exceptions were seen. Thus, occasionally rows 1′ and 1 were yellow, while the transverse rows were not yellow or *vice versa;* or, a bristle in row 3 or 4 corresponded in phenotype to that of its adjacent row rather than its own. An example is shown in Figure 13a,b where the distal bristle of row 4 is not yellow as were those of rows 3, 2, and 1, while the other three bristles of row 4 together with all bristles in the rest of the segment were yellow. It is thus clear that the cell lineage concerning the origin of bristle rows in the tarsal segment is not absolutely fixed but shows occasional shifting.

Some primary sex combs had teeth which had either no or only a small amount of pigment, and the same was true for some bristles. Such unpigmented teeth or setae occurred in otherwise not variegated segments and, in a few cases occurred together with a typically yellow tooth or teeth of a variegated primary comb.

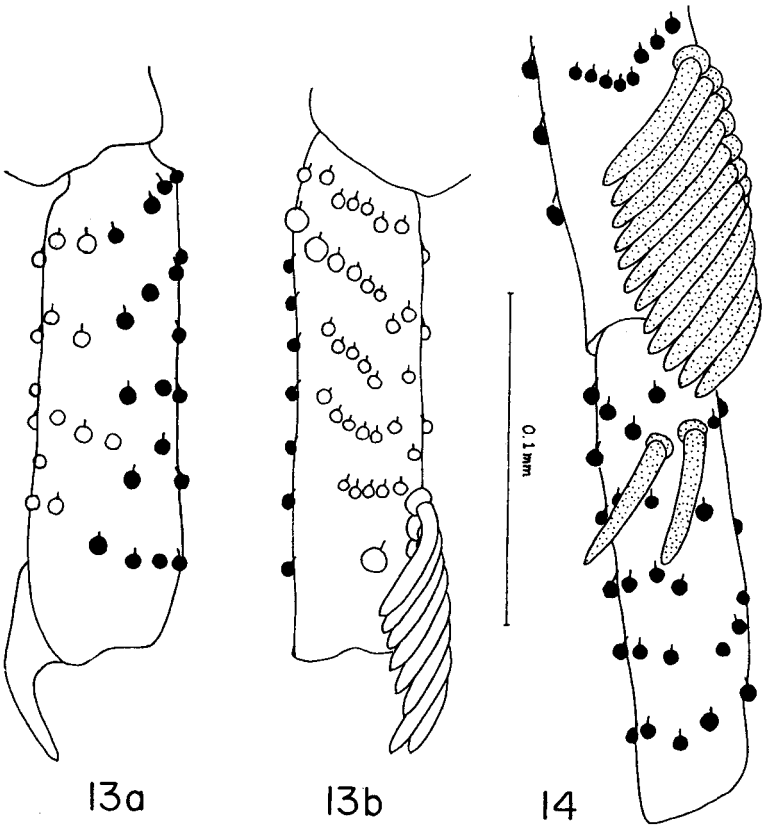


FIGURE 13.—a, b. No. 51 (dorsal and ventral side).

FIGURE 14.—Extra sex comb differentiation on the second tarsal segment of a foreleg, induced by X-ray irradiation of a heterozygous *en*/+ male larva. No. 12 from Experiment II.

The nature of this absence of pigment is not understood. The cases of this type of variegation were not included in Table 4, but they are summarized in Table 5.

The data reported both in the present and preceding sections show that there is only a remote developmental relation between the area of the secondary sex comb and the area covering rows 4, 5, 6, 7 and the transverse rows in contrast to the close relation to rows 1, 2 and 3. This follows from the fact that most of the variegated segments showed either variegation within rows 1 to 3 or outside of this area.

Abnormalities in the forelegs of treated males: Apart from genetic mosaicism, a number of malformations were induced by the larval irradiations. Sometimes, though rarely, general malformation of the tarsal structure was observed accompanied by disturbance of the entire arrangement of bristles including the primary sex comb. Most frequent were irregular arrangements of the primary teeth and the primary comb. At times sex comb teeth had differentiated on the second tarsal segment (Figure 14), or on the third or fifth segment.

It was decided to study whether the induction of abnormalities of sex comb differentiation was connected with the fact that the treated larvae were all heterozygous for engrailed, or whether this was a general property of various constitutions. Accordingly, larvae of varying ages of both homozygous nonengrailed (Canton-S and stw^3/T(Y;2)*B*) and engrailed (*pr en* and stw^3 *en*) genotypes (Table 6) were irradiated. Of the four stocks treated stw^3/T(Y;2)*B*, which is free from *en*, was the most sensitive one in terms of induced extra sex comb teeth. As in the cases of extra teeth in *en*/+ males, most extra teeth appeared close to the primary sex comb on the first tarsal segment. Where the extra teeth formed on a more distal tarsal segment it was always located on the inner or ventral side of the tarsus, *i.e.* the same side where the primary comb is differentiated, but without preference for a proximal, intermediate or distal position on the segment.

These observations suggest that the differentiation of extra teeth is related to the area of the primary sex comb and its developmentally correlated regions on the more distal tarsal segments. Such a developmental relation is demonstrated by the cell lineage in genetic mosaics along the tarsus—in those instances where the variegation of the first segment extended to other segments a given genotypic area occurred on the different segments along the same longitudinal line, that is, on the same side of the tarsus.

TABLE 5

Classification of tarsi mosaic in the primary sex comb area according to variegated teeth pigmentation

	Pigmentation of the not wild type teeth of the primary sex comb			
	No pigment	No pigment plus yellow teeth*	Yellow*	Total
Preliminary exp.	10	3	33	46
Exp. I	0	0	3	3
Exp. II	6	3	43	52

* In the preliminary experiment straw^3 instead of yellow was present.

TABLE 6

The differentiation of extra sex comb teeth on male tarsal segments after X-ray irradiation

Genotypes*	Age at irradiation† (hour unit after hatching)	Number of irradiated larvae	Percent of emerged adult flies	Adult males				
				Total	Irregular primary sex comb	Cases of extra sex comb teeth differentiated		
						On first tarsus	On second tarsus	Percent per total male tarsals
Canton-S	31–78	4567	81.2	1776	113	2	0	0.05
stw^3/T(Y;2)*B*	30–78	1999	55.3	1107	160	33	2	3.29
pr en	30–78	1763	23.87	173	9	4	0	1.15
stw^3 *en*	50–75	2133	72.85	776	26	8	0	0.51

* *pr*=purple eye color; T(Y;2)*B*=a Y-2 translocation. The *stw*^{3} *en* stock was obtained from crosses of the *pr en* and *stw*^{3}/T(Y;2)*B* stocks.
† The X-ray dosage was about 1450r, effective age of irradiation for extra teeth differentiation was 45 to 78 hours after hatching.

The extra teeth induced by X-rays then seem to be due to disturbances caused in the prepattern area of the primary sex comb. If there is a prepattern for a secondary sex comb, as suggested by the results of the study of mosaics, it remains to be determined why no disturbances resulting in extra teeth were induced in the prepattern area of the secondary comb.

DISCUSSION

The engrailed prepattern of a secondary sex comb: The results of the study of first tarsal segments mosaic for heterozygous and homozygous engrailed tissues show clearly that the heterozygous *en*/+ segment possesses a singular region which is able to evoke the differentiation of a secondary sex comb provided it contains cells competent to respond to this prepattern. Heterozygous cells are not competent to do so, but homozygous *en/en* cells, even if present in a small patch only, react by formation of sex comb teeth. It may be assumed that homozygous normal +/+ segments also possess the prepattern for secondary sex comb formation since the general chaetotaxy of +/+ and *en*/+ segments is alike.

The appearance of a secondary sex comb caused by a single-locus mutation is dependent on a prepattern which remains normally unknown but whose existence is made explicit by the response of the mutant genotype. It has been pointed out before by STERN (1954a,b) that the absence of a primary sex comb in various species of Drosophila and its presence in other species is not necessarily to be regarded as a difference in fundamental organization of the developing forelegs, but possibly only as a difference in response to an invariant prepattern. The first appearance of a sex comb in evolution could then be due to a single mutation which on account of its production of a strikingly new structure could be called a macromutation. This interpretation of the origin of a primary sex comb could be countered by the question whether the species without comb are not perhaps derived from species with a comb. In this case the prepattern of the latter species and therewith the origin of the primary comb could have been the result of an

accumulation of a polygenic system, in response to selective forces favoring a sex comb, and the absence of a comb in spite of the presence of a prepattern in comb-less species only the result of mutational loss of response.

This argument would not apply to the secondary sex comb. There are no Drosophila species which possess such a structure normally so that it cannot be assumed that the presence of a prepattern for this comb is the result of former selective agents specifically involved in the establishment of this structure. Rather, this developmental prepattern is a potential evolutionary prepattern ready to become manifest if selection should favor the formation and establishment of a secondary sex comb. Since, in a sense, the secondary comb is a mirror image of the primary, the formation of the two prepatterns in development may be the consequence of a single process of determination which results in two symmetrically placed singular regions.

The analysis of the distribution of secondary sex comb teeth on the tarsal segment indicates the existence of a relatively large prepattern area with a gradient of effectiveness in evocating tooth formation—strong at the normal site of the secondary comb and weaker toward the periphery. Thus, in mosaics an *en/en* cell which happens to occupy only a peripheral area will differentiate a tooth even though normally in this area the typical end bristle of row 1 would have been formed. Such differentiations are similar to those found in achaete–non-achaete mosaics. For these STERN (1954a,b) found some specimens in which ac^+ tissue which is normally responsible for the differentiation of the thoracic dorsocentral bristles did not cover the site of these bristles but only an area close to it. Had the whole individual been ac^+ no dorsocentral bristle would have been formed in this area. In these mosaics, however, where no dorsocentral bristles could differentiate at the typical sites, the neighboring area did evoke this differentiation. An interpretation of this situation assumed a prepattern area with a gradiant of effectiveness: a peak at the site typical for the differentiation but potential evocation at lower levels away from the typical site (STERN 1956a). Normally, differentiation of a structure in the peak area acts as an inhibitor of differentiation outside of it, but failure of differentiation in the peak area permits differentiation in a peripheral region of the prepattern area. A similar interpretation may apply to the prepattern of the secondary sex comb. On the other hand, it is possible that the location of the peak of its prepattern may vary from one tarsus to another. This is suggested by the variations in the position of the secondary comb in different homozygous engrailed male individuals. An alternative interpretation to the gradient and inhibition concept of the formation of a secondary tooth at the position of a normal terminal bristle of row 1 is, therefore, the assumption that in these cases the peak of the prepattern happened to be at or near the end of row 1.

The developmental relations between the prepatterns of the primary and secondary sex combs: A study of cell lineage in the mosaic first tarsal segments of males leads to the conclusion that this segment can be divided developmentally into two parts. One of these includes the primary sex comb area, the transverse rows on the ventral side and the adjacent rows on the inner side of the segment;

the other includes the secondary sex comb area and the rows on the outer side. The close association between each sex comb and the bristle rows listed and the very rare associations between the two combs themselves suggest that the separate differentiation of the two prepatterns occurs at a rather early developmental stage.

There is a striking difference in the variability of the primary and secondary sex combs in homozygous engrailed males, the secondary comb being much more variable in extent and position than the primary. Another difference between the two is the fact that larval irradiation causes differentiation of extra teeth in areas related to the primary comb but not in those related to the secondary comb. The normal genotype seems to be specifically sensitive to evocation of teeth in the area of the primary comb and insensitive to evocation in the area of the secondary comb, while the engrailed genotype is specifically sensitive to respond to evocation in the area of the secondary comb and does not increase response in that of the primary. It is not clear whether these differences are exclusively related to response or whether there are also intrinsic differences in the two prepatterns.

SUMMARY

1. In *Drosophila melanogaster*, the recessive autosomal allele engrailed (*en*) leads to the formation of a secondary sex comb on the first tarsal segment of the male foreleg. The developmental action of *en* was studied by means of patches of *en/en* tissue on *en/+* forelegs. These mosaics were obtained by induction of somatic crossing over as a consequence of irradiation by X-rays of *en/+* larvae.

2. In order to distinguish *en/en* and *en/+* tissues pigment markers were introduced. The autosomal recessive straw[3] failed to give clear separation of the two tissues, but the X-linked recessive yellow, translocated to the *en*-carrying autosome was made to serve as a successful marker.

3. Most mosaic genotypes can be explained as resulting from somatic crossing over between the kinetochore and the locus of *en*, and a few by crossing over between *en* and the distal end of the chromosome or by double crossing over on both sides of *en*.

4. The standard bristle pattern of the first tarsal segment of the male foreleg in wild-type flies and the characteristics of the secondary sex comb in *en/en* males were investigated.

5. When *en/en* tissue occupies the area of the secondary sex comb on *en/+* tarsi differentiation of teeth occurs even when the homozygous area is small. This indicates that nonengrailed as well as engrailed forelegs possess a prepattern for a secondary sex comb and that the difference in final phenotype depends on the response of the genotypes.

6. In a mosaic tarsal segment, a small area of *en/en* tissue located adjacent to the area occupied by a secondary comb in nonmosaic *en/en* segments, may form a sex comb tooth. This suggests either the existence of a gradient within the prepattern region of the secondary comb and inhibition of differentiation outside the peak area by formation of a sex comb within it, or possibly individual variations in the location of the peak of the prepattern.

7. Irradiation by X-rays of wild type or *en/en* male larvae leads to the formation of extra sex comb teeth on the first and more distal tarsal segments in regions associated with the primary sex comb only.

8. Cell lineage studies in mosaics suggest an early developmental separation in the leg discs of the regions determining the primary and secondary sex combs.

ACKNOWLEDGMENTS

The author wishes to express her deep appreciation to DR. C. STERN for his invaluable suggestions and guidance during the experiment, and also to DR. E. NOVITSKI for his helpful suggestions and kindness in supplying his special stocks. Acknowledgment must also be made to the United States Educational Commission for its generous support which made it possible for the author to work at the University of California.

LITERATURE CITED

BECKER, H. J., 1957 Über Röntgenmosaikflecken und Defektmutationen am Auge von Drosophila und die Entwicklungsphysiologie des Auges. Z. Ind. Abst. Vererb. **88**: 333–373.

BRASTED, A., 1941 An analysis of the expression of the mutant "engrailed" in *Drosophila melanogaster*. Genetics **26**: 347–373.

HANNAH-ALAVA, A., 1958a Developmental genetics of the posterior legs in *Drosophila melanogaster*. Genetics **43**: 878–905.

1958b Morphology and chaetotaxy of the legs of *Drosophila melanogaster*. J. Morphol. **103**: 281–310.

HANNAH-ALAVA, A., and C. STERN, 1957 The sex combs in males and intersexes of *Drosophila melanogaster*. J. Exptl. Zool. **134**: 533–556.

KROEGER, H., 1959a Determinationsmosaike aus kombiniert implantierten Imaginalscheiben von *Ephestia kühniella* Zeller. Wilhelm Roux' Arch. Entwicklungsmech. **151**: 113–135.

1959b The genetic control of genital morphology in Drosophila. A study of the external genitalia of sex mosaics. Wilhelm Roux' Arch. Entwicklungsmech. **151**: 301–322.

STERN, C., 1954a Two or three bristles. Am. Scientist **42**: 213–247.

1954b Genes and developmental patterns. Caryologia (Suppl. Proc. 9th Intern. Congr. Genet.) **6**: 355–369.

1956a Genetic mechanisms in the localized initiation of differentiation. Cold Spring Harbor Symposia Quant. **21**: 375–382.

1956b The genetic control of developmental competence and morphogenetic tissue interactions in genetic mosaics. Wilhelm Roux' Arch. Entwicklungsmech. **149**: 1–25.

1957 The role of genes in differentiation. Proc. Intern. Genet. Symp. Suppl. Vol. Cytologia 1956: 70–72.

STERN, C., and A. M. HANNAH, 1950 The sex combs in gynanders of *Drosophila melanogaster*. Portugaliae Acta Biol. Ser. A. (R. B. Goldschmidt Volume): 798–812.

STERN, C., and D. L. SWANSON, 1957 The control of the ocellar bristle by the scute locus in *Drosophila melanogaster*. J. Fac. Sci. Hokkaido Univ. Ser. VI. **13**: 303–307.

STURTEVANT, A. H., 1945 A gene in *Drosophila melanogaster* that transforms females into males. Genetics **30**: 297–299.