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Cell lineage analysis of kynurenine producing organs in *Drosophila melanogaster*

By MOTI NISSANI

Laboratory of Genetics, University of Wisconsin, Madison, Wisconsin 53706, U.S.A.

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SUMMARY

Six hundred and ten gynandromorphs were produced in which an *X* chromosome loss uncovered the vermilion mutation. The mosaic patterns observed indicate that wild type ocelli are incapable of kynurenine production and that, in addition to the eyes, postembryonic kynurenine producing cells originate from two separate regions of the blastoderm. The positions of these regions on the genetic fate map of *Drosophila melanogaster* correspond to the embryonic precursors which give rise to the kynurenine producing cells of the larval fat body and Malpighian tubes.

1. INTRODUCTION

The first non-autonomous mutation reported in insects was the mutation for vermilion eye colour in *Drosophila melanogaster* (Sturtevant, 1920). Vermilion flies have bright red eyes and colourless ocelli, instead of the wild type dull red eyes and brown ocelli. Sturtevant found that gynandromorphs with genetically vermilion eyes could have vermilion, intermediator wild type eye colours. Vermilion was later discovered in the sibling species, *Drosophila simulans*, and was found to exhibit the same non-autonomous behaviour. Based on the observed gynandromorph patterns, Sturtevant next attempted to determine which internal organs are involved in kynurenine production (Sturtevant, 1932). Since flies with bilateral mosaic heads could have one wild type eye and the other phenotypically vermilion, it could be concluded that genetically wild type eye tissue is sufficient for the appearance of non-vermilion pigmentation. In addition, it was suggested that wild type gonads can bring about a change in genetically vermilion eyes toward wild type phenotype, and that a third internal organ not very closely related (in terms of cell lineage) to the surface areas also produces the same effect (Sturtevant, 1932).

The non-autonomy of vermilion served as the basis for a series of transplantation experiments which resulted in the discovery of the first gene controlled biochemical reaction chain. A *bw v* eye disc implanted in a wild type host did not develop the characteristic colourless eye of the double recessive donor but an intensity of pigmentation approaching the eye colour of *bw* flies (Ephrussi, 1942). Implanted wild type larval gonads in a *bw v* host had no effect on the host eye and ocellar colour, but implanted wild type larval fat body or Malpighian tubes caused a strong modification toward brown colour in the host's eyes and ocelli (Beadle,

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1937). Since the active substance, v^+ or kynurenine, could not be extracted from the fat body, it was concluded that the fat body is capable of kynurenine production. This conclusion was recently corroborated by a more direct approach (Rizki, 1961).

The role of larval Malpighian tubes in kynurenine production could not be as easily interpreted since kynurenine could be extracted from Malpighian tubes of wild type larvae and since these organs are capable of removing kynurenine from the haemolymph. However, a series of ingenious experiments, in which an attempt was made to exhaust wild type Malpighian tubes of kynurenine, made it plausible to conclude that these organs do produce kynurenine (Beadle, 1937). The finding that wild type Malpighian tubes contain the enzyme tryptophan pyrrolase lends additional support to this view (Kaufman, 1962). However, it is still uncertain whether the Malpighian tubes of wild type *D. melanogaster* are capable of kynurenine production (Linzen, 1974).

Advances in Dipteran embryology and the availability of a more accurate and quantitative approach for identification of the anatomical sites at which sex-linked genes exert their primary effects during development (Hotta & Benzer, 1973), suggest that reinvestigation of the behaviour of vermilion in gynandromorphs may provide more definite answers to these problems. In particular, it was hoped that such a study could ascertain the role of the Malpighian tubes in kynurenine production and resolve the apparent discrepancy between gynandromorph and transplantation experiments regarding the role of the gonads in kynurenine production.

2. MATERIALS AND METHODS

A total of 610 gynandromorphs of the constitution *y* sn^3 *v* $un^4/In(1)w^{vc}$; *bw/bw* were obtained. For each gynandromorph all surface landmarks were recorded as either male or female. Intensities of pigmentation of both eyes and of the two lateral ocelli were recorded on an arbitrary 0–5 scale (Beadle, 1937). Each eye, and each of the two lateral ocelli, were recorded separately. In this scale, 0 represents colourless eyes or ocelli not distinguishable from those of *bw v* flies of comparable age and 5 represents an eye or ocellar colour not distinguishable from that of *bw* flies of comparable age. All flies were reared at 25 °C on standard cornmeal/molasses/yeast/agar medium (Strickberger, 1962). Based on this scale gynandromorphs were classified as either having *bw v* eye colour and colourless ocelli, or as having pigmented eyes and ocelli. In a few instances gynandromorphs had barely detectable pigmentation and could not be accurately assigned to either of the above classes.

A list of the genetic variants mentioned in this report, along with a brief characterization, follows. Further information and references can be found in Lindsley & Grell (1968).

bw brown (2–104·5) – brown eyes. Double recessive *bw v* has colourless eyes; the use of *bw* in this experiment increases therefore the resolving power available for determining the intensity of pigmentation of the eyes.

cho chocolate (1–5·4) – Malpighian tubes contain brown pigment.

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cn cinnabar (2–57·5) – phenotypically identical to *v*. Non-autonomous for eye colour.

In(1)w^{vc} unstable ring *X* chromosome, its loss results in the production of gynandromorphs and *XO* males (Hinton, 1955).

sn^3 singed – 3(1–21·0) – singed hairs and bristles.

un^4 uneven – 4(1–54·4) – eye surface rough. Autonomous in gynandromorphs and hence un^4 makes it possible to determine, for a given eye tissue of a gynandromorph, whether it has a *v* or a v/v^+ genetic constitution.

v vermilion (1–33·0) – eye colour bright red. Ocelli colourless.

y yellow (1–0·0) – body and hair colour yellow.

3. RESULTS

The data confirm the earlier observations that the colour of eyes and ocelli in *v* gynandromorphs is non-autonomous and that when kynurenine is not circulated in the haemolymph a v^+ eye tissue is still capable of kynurenine production. However, the v^+; *bw* eyes of such gynandromorphs were lighter (mean grade 4, range 3·5–4·5) than the eyes of *bw* flies of comparable age. This fact suggests that eyes of wild type flies are capable of kynurenine production but are dependent on external kynurenine and/or 3-hydroxy-kynurenine supply in order to produce normal quantities of ommochromes. Such *bw* eyes usually have wine-red or purple colour which appears not only light but also qualitatively different from the light brown colour of genetically *bw v* gynandromorphs with intermediate pigmentation.

Unexpectedly, in all flies, all three ocelli had approximately the same intensity of pigmentation. This effect was most striking in all 22 bilateral head mosaics which had one brown eye and one colourless eye. All these gynandromorphs had three colourless ocelli. It thus seems reasonable to conclude that if no kynurenine is circulating in the haemolymph, wild type ocelli lack pigmentation. This conclusion gains further support from the observations that all gynandromorphs with even a small patch of one colourless eye had three colourless ocelli and that when the ocelli were colourless and one or both eyes were un^{4+}; *bw*, the eyes appeared lighter than the eyes of *bw* flies of comparable age. Indeed, 29 gynandromorphs had complete non-vermilion head, but colourless ocelli. Since ocelli of *cn* mutants are colourless even when 3-hydroxy-kynurenine is available (Beadle, 1937), it is likely that wild type ocelli are capable of producing 3-hydroxy-kynurenine. Hence, it appears that the biochemical steps of the tryptophan-ommochrome pathway preceding 3-hydroxy-kynurenine production do not take place in wild type ocelli and that such ocelli are capable of removing kynurenine from the haemolymph and then catalysing the subsequent steps which lead to production of brown pigments.

The complete non-autonomy of ocellar colour in *v* gynandromorphs facilitated fate mapping analysis of kynurenine producing organs, since it was not necessary to restrict this analysis to gynandromorphs with one or two genetically *v* eyes. In a gynandromorph with two v^+; *bw* eyes the presence or absence of kynurenine producing organs could be determined by the presence or absence of pigmentation in

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its ocelli. The results given below reveal a nearly perfect correlation between the presumptive vaginal plates and one site of kynurenine production and secretion and the existence of an additional separate site which is not closely related, in terms of cell lineage, to any cuticular landmark. Evidence bearing on the genetic fate map location of these two separate sites will be considered now.

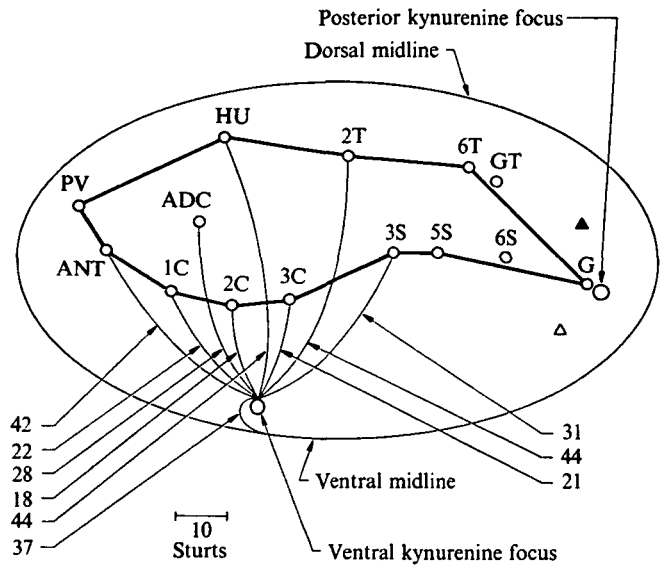

Fig. 1. A genetic fate map of *Drosophila melanogaster* blastoderm, including the ventral and posterior foci of kynurenine production and secretion. This map represents the right half of the embryo, and the closed curve represents the midline. Distances are given in sturts. ADC, anterior dorsocentral bristle; ANT, antenna; G, external genitalia; GT, genital tergite; HU, humeral bristles; PV, post-vertical bristle; 1C, coxa of first leg; 3S, third abdominal sternite; 2T, second abdominal tergite. The open and solid triangles represent fate map positions of the Malpighian tubes reported by Hotta & Benzer (1973) and Janning (1974), respectively.

Based upon the recorded 610 gynandromorphs, a genetic fate map of the cuticular landmarks which appear in Fig. 1 was constructed (Garcia-Bellido & Merriam, 1969). Since the obtained map was essentially identical to the map reported by Hotta & Benzer (1973), the outline depicted in Fig. 1 is taken from their map. The position of the sixth sternite, which was triangulated to a more posterior position than the one reported by Hotta & Benzer, and the position of the external genitalia, were taken from our data.

The position indicated in Fig. 1 for the posterior kynurenine focus was estimated by the following considerations. Firstly, all but two of the 334 gynandromorphs which had one or more well-formed vaginal plates had coloured ocelli. Female composition of no other cuticular landmark showed a comparable correlation with ocellar pigmentation. Secondly, in approximately 10 % of the gynandromorphs scored, only a small segment of their cuticle (8 % or less) was of female composition. Again, the presence of one well-formed vaginal plate was always sufficient for

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non-vermilion pigmentation. In particular, in 10 gynandromorphs the only female cuticular landmark was one well-formed vaginal plate with or without some female anal hairs; all 10 had coloured ocelli. Thirdly, in contrast to the vaginal plates, the genital tergite and the sixth sternite showed no independent correlation with ocellar pigmentation. Among the 102 gynandromorphs (204 sides) which had colourless ocelli, 26 sides had a female genital tergite, 4 sides had a female sixth sternite and in additional 17 sides both structures were of female composition. It can be concluded therefore that the probability for the mosaic dividing line to separate both the posterior kynurenine producing focus and the posterior part of the abdominal cuticle from the presumptive vaginal plates into two regions of unlike constitution is very low and that the vaginal plate embryonic precursors are most likely located between this focus and the posterior histoblasts, i.e. this posterior focus maps to the general area indicated in Fig. 1.

It was noted above that the presence of one well-formed vaginal plate was almost always sufficient for ocellar pigmentation; this relationship indicated that one kynurenine producing focus maps to the posterior-most region of the blastoderm. However, female composition of one vaginal plate was not a necessary condition for ocellar pigmentation. Indeed, 133 flies with well-formed male genitalia had coloured ocelli. This fact suggests that an additional separate region is involved in kynurenine production. The nearly perfect correlation between the vaginal plates and the posterior foci make it possible to map the second site involved in kynurenine production. It is clear that in the great majority or in all flies with complete male external genitalia the posterior foci were also male and that all gynandromorphs with colourless ocelli had no kynurenine producing organs. Analysing therefore the 235 flies belonging to these two groups could yield a close estimate of the map location of the second focus. One problem in such an analysis stems from the fact that these 235 mosaic flies were not chosen at random and hence the results are not strictly comparable to those reported by others. However, a calculation of map distances between various cuticular landmarks based only on these 235 flies indicated that, as expected from the large distance between the position on the fate map of the genitalia and the nearest cuticular structure, the positions of, and distances between, various cuticular structures remain largely unchanged.

Since the bioassay employed is the presence of even a slight change in colour, it may be expected that the model of one pair of bilateral submissive foci may be applicable here, i.e. female composition of only one of the two lateral sites is sufficient to bring about a detectable change in ocellar pigmentation (Hotta & Benzer, 1973). Indeed, since one deals here with a diffusible substance, it is virtually certain that this model is applicable to the present analysis. Also, as will be discussed below, it is almost certain that the focus involved corresponds to the kynurenine producing cells of the fat body. In the case of the fat body there is experimental evidence for the applicability of the bilateral submissive foci model since it is known that transplantation of only a portion of wild type fat body is sufficient to produce a significant change in the *bw v* host, and since there are no regional differences in the effects of various sections of this organ (Beadle, 1937).

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Using the equations developed in order to calculate the distances between such a focus and various surface landmarks (Hotta & Benzer, 1973), the focus was mapped to the ventral position indicated in Fig. 1. As expected, this region corresponds to the general area of the blastoderm which according to embryologists (Sonnenblick, 1950) and geneticists (Falk *et al.* 1973) gives rise to the fat body.

The equations which were used in order to calculate the map position of the ventral kynurenine focus provide only a rough estimate of its accurate position (Merriam & Lange, 1974). It is necessary therefore to outline other considerations which lead to the same conclusion, namely, that this focus is not closely related, in terms of cell lineage, to any cuticular landmark, and that it occupies a midventral position on the genetic fate map of *D. melanogaster*.

Table 1. *Estimated distances between certain cuticular landmarks and the ventral kynurenine producing focus of* Drosophila melanogaster

	ANT	ADC	HU	1C	2C	3C	3S	2T
Estimated distance in sturts	44·6	16·6	27·0	13·2	9·1	9·3	13·8	26·0

Calculations are based upon 102 gynandromorphs which had colourless ocelli. Abbreviations are the same as in Fig. 1.

Firstly, consider the 102 gynandromorphs which had colourless ocelli; it follows from the preceding analysis that all their kynurenine producing organs must have been male, or mutant. Based only upon this class of gynandromorphs, one can estimate the distance in sturts between a given cuticular landmark and its nearest kynurenine producing organ. A similar approach was employed in order to determine cell lineage relationships of certain *X*-linked lethal mutations (Bryant & Zornetzer, 1973). The third coxa, for instance, had female composition in 19 sides, out of a total of 102 gynandromorphs (204 sides), which had colourless ocelli. Hence, an estimate of the distance between the third coxa and its nearest kynurenine producing focus is $19/204 \times 100$ or approximately 9·3 sturts. The results of these calculations are given in Table 1. It is apparent from this Table that although the calculated distances obtained by this procedure tend to be shorter than the map distances which are depicted in Fig. 1, triangulation would yield again a similar, but a more dorsal, map position of the ventral kynurenine producing focus.

Secondly, consider the 38 gynandromorphs which had male external genitalia and only a very small continuous segment of female composition (8 % or less of the entire cuticle was female). If the map position of the ventral kynurenine focus is correct (Fig. 1), it may be possible to predict the nature of the correlation between the locations of these segments and ocellar colour. All 24 segments of gynandromorphs with pigmented ocelli included a cuticular landmark which occupies a ventral position on the genetic fate map of *D. melanogaster* (Fig. 1). Thus, 13 segments included one of the leg coxae, 7 included an antenna, and 4 segments included a second half-sternite. As expected, the observed distribution among the 14 gynandromorphs which had colourless ocelli and only a small segment of female

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composition was different; 4 segments included a female antenna, leg coxa or half a sternite, but, in addition, 10 segments included cuticular landmarks which occupy a dorsal position on the genetic fate map, but did not include a female antenna, leg or any anterior half-sternites. These distributions support the hypothesis that the second kynurenine producing focus has a ventral position on the genetic fate map of *D. melanogaster*.

4. DISCUSSION

The fact that the posterior foci map so close to the vaginal plates raises an interesting embryological possibility. The preceding analysis made it certain that some knyurenine producing cells originate from the posterior-most region of the blastoderm. Other observations suggest the possibility that the Malpighian tubes are the only posterior organs involved in kynurenine production (Beadle, 1937; Kaufman, 1962). Prima facie, it appears therefore that the position of the Malphigian tubes on the genetic fate map corresponds to the position assigned in Fig. 1 to the posterior focus. Two recent attempts were made to place the Malphigian tubes on the adult fate map of *Drosophila melanogaster*. Hotta & Benzer (1973) used the *cho* mutation as a marker of larval Malpighian tubes and reported the position indicated in Fig. 1 by an open triangle. Janning (1974) mapped the adult Malpighian tubes to the position indicated by a solid triangle in Fig. 1; using the lack of the enzyme aldehyde oxidase in the adult as an organ marker. These two positions differ from each other and both are different from the position of the posterior focus reported here. Furthermore, as mentioned earlier, the genital tergite and the sixth sternite had no independent effect on ocellar pigmentation. The positions assigned to the Malpighian tubes by either Janning or Hotta & Benzer imply that the Malpighian tubes are roughly equidistant from the vaginal plate and the genital tergite or sixth sternite, respectively. Assuming that during embryogenesis the posterior kynurenine producing cells do become part of the Malpighian tubes, and that genetic fate mapping is a valid and reliable method, this set of contradictions can be removed by the following hypothesis: the Malpighian tubes originate from a relatively large area in the posterior-most part of the blastoderm, and within this large area, cells with different functions arise from different, more narrowly localized, regions. This hypothesis also resolves the long standing controversy among insect embryologists regarding the exact developmental origin of the Malpighian tubes (e.g. Poulson, 1950; Wigglesworth, 1972), since it implies that, at least for *D. melanogaster*, these disagreements are not the result of inaccurate observations but of an inaccurate assumption, namely that the Malpighian tubes originate from a narrowly localized region of the blastoderm.

However, the only requirements set by the genetic data regarding the position of the posterior focus are that it is very close to the vaginal plate and that the vaginal plate maps between this focus and the posterior abdominal sclerites. In some instances it is difficult to determine accurately the sex of the external genitalia in gynandromorphs and hence no consensus exists in the literature regarding their

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exact location, except that this is in the posterior-most region of the genetic fate map (Garcia Bellido & Merriam, 1969; Bryant & Zornetzer, 1973; Falk *et al.* 1973; Gelbart, 1974; Janning, 1974). It is possible therefore that the posterior kynurenine producing focus and the focus responsible for the brown colour in the Malpighian tubes of *cho* mutants are identical, and that the vaginal plate maps between this focus and the posterior sclerites.

The results reported here resolve the seemingly conflicting evidence regarding the role of the gonads in kynurenine production (Sturtevant, 1932; Beadle, 1937). In 1932 the biochemical aspects of gene function were not understood, and consequently Sturtevant worked under the erroneous assumption that the foci involved produce a substance which suppresses the expression of the gene *vermilion*. Close scrutiny of his results reveals that the only conclusion which may be drawn from them at present is that a correlation in terms of cell lineage exists between the gonads and the posterior kynurenine producing cells. Thus, Sturtevant's data actually support Beadle's observations regarding the effect of transplanted Malpighian tubes on the eye colour of a *bw v* host.

In a recent experiment, *D. melanogaster* gynandromorphs were introduced into a mating chamber containing wild type males. From the observed differential courtship responses of the males it could be concluded that the only important factor which enables a male to distinguish between conspecific males and females and to direct persistent courtship only toward females is a female specific pheromone. Genetic fate mapping indicated that 'the pheromone producing cells of adult *D. melanogaster* are dispersed throughout, and are part of, the female tergites, sternites and thorax' (Nissani, 1975). Since 64 gynandromorphs carried the vermilion mutation on their rod *X* chromosome, it could be also concluded that there is no correlation, in terms of cell lineage, between the composition of these pheromone producing cells and the composition of the kynurenine producing cells. Hence, the prediction was drawn that all kynurenine producing and releasing cells in *D. melanogaster* are unrelated, in terms of cell lineage, to the tergites, sternites or the thoracic integument. The results reported above fully confirmed this prediction.

Throughout this report, reference was made to kynurenine production in *Drosophila*. It is necessary to emphasize, however, that the bioassay employed in both gynandromorph and transplantation studies can detect kynurenine production only when it is accompanied by a subsequent release of kynurenine into the haemolymph. Thus, the analysis above indicated which organs produce *and* release kynurenine. This analysis did not rule out the possibility that some other organ(s) (in addition to the eyes) produce kynurenine, but do not release it into the haemolymph.

The foregoing cell lineage analysis of the vermilion mutation demonstrated that one kynurenine producing focus originates from a mid-ventral position of the blastoderm and thus the analysis provided a further check for the validity of the basic underlying assumptions of genetic fate mapping (Garcia-Bellido & Merriam, 1969), and an *in vivo* demonstration that the larval fat body produces kynurenine. The results also provided much needed evidence that, at least in *D. melanogaster*,

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kynurenine production is not restricted to the fat body (Linzen, 1974), but does occur also in an organ which maps to the posterior-most region of the blastoderm and which most likely corresponds to the Malpighian tubes. Evidence was presented which led to the surprising conclusion that, unlike wild type eyes, wild type ocelli are incapable of kynurenine production and hence appear colourless when kynurenine is absent from the haemolymph. Finally, the analysis raised some interesting questions regarding the embryological origin of the Malpighian tubes in insects.

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