Isolation and characterization of flightless mutants in Drosophila melanogaster

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SUMMARY

Since animal behaviour is executed through neuronal circuits including sensory receptors and muscle, genes vital for their development and differentiation must be found among mutants having behavioural anomaly. After mutagenesis with ethyl methanesulphonate (EMS), we screened for X-linked flightless mutants of Drosophila melanogaster by using a column-type flight tester. Approximately 10⁴ individuals were screened and 21 mutant genes were isolated. Chromosomal mapping and complementation experiments revealed that they belong to 15 cistrons randomly located on X chromosome, three cistrons having more than two alleles. Two of the isolated mutants (fltO² and fltH, which are recessive both behaviourally and morphologically) were analysed with the mosaic fate mapping technique, and both were found to have their primary foci in mesodermal region of blastoderm, suggesting that the genes exert their primary effect in indirect flight muscle.

Electronmicroscopic studies on the muscles from four alleles of the fltO² cistron revealed an abnormality in myofibrillar arrangement. A possible deficit within Z-band components is discussed in relation to wings-up B mutants. The indirect flight muscle of fltH was also examined, and it was found that sarcomere length and diameter of myofibrils were abnormal. It was postulated that a possible factor which controls size of myofibrils is defective in this mutant. These examples indicate the advantage of combining ultrastructural examination with genetic mosaic mapping technique.

INTRODUCTION

Behavioural circuits and their components, including sensory receptors, nervous system and muscle, differentiate during development according to the genetic programme inherited by an organism. Their development may be partly modified by environment, but the plasticity itself is also under genetic control. To reveal how genes govern and construct such a complex system, one approach is to mutate relevant genes one at a time to analyse how they perturb the system. Drosophila is one of the most ideal organisms for such analysis (see Benzer, 1973). However, such an analysis may not be simple. One serious problem is that interactions between organs mean that a local mutant character may often be caused by abnormal gene function in another tissue. Hotta & Benzer (1972)

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realized that the primary 'focus' for each behavioural gene could be identified on a blastoderm fate map made by means of genetic mosaics. The method is to make composite, mosaic individuals, partly normal and partly mutant, and to ask of which tissue the genotype determines the mutant character in question. The primary focus of the gene is regarded as a site where the gene exerts its primary action to cause mutant characters. Its location can be shown as a point (or an area) on a blastoderm fate map by calculating distances (correlation of genotype) between the focus and various external structures. The method has been applied to a number of behavioural mutations (see Hall, Gelbart & Kankel, 1976 for a review). Recently the mosaic technique was also applied to dissect such complex behaviours as male courtship action patterns (Hotta & Benzer, 1976).

For the genetic dissection to work, the behavioural system to be studied must be dispensable in a sense that its malfunction does not lead to lethality of the individual. *Drosophila* flight behaviour is one of the most ideal systems, since it is not essential for survival (at least in the laboratory), and it has been already analysed in detail (Levine, 1973a; Shafiq, 1963). One flightless mutant has been already analysed electrophysiologically and abnormal output pattern of motor-neurons was observed (Levine & Wyman, 1973), although its primary focus is unknown. There are also several other flightless mutations which are accompanied with wing position abnormalities. Among them, *wings-up A* (*wupA*), *wings-up B* (*wupB*) (Hotta & Benzer, 1972, 1973), *indented thorax* (*int*) and *flap-wing* (*flw*) (Deak, 1977) have been shown to have mesodermal foci, most likely in the indirect flight muscle.

In this paper, we report our isolation of 21 sex-linked flightless mutations without wing position abnormality in *Drosophila melanogaster*. They belong to 15 cistrons, two of which were found to have their primary foci in indirect flight muscle. Ultrastructure of the muscle was examined, and we could demonstrate cistron-specific abnormalities of the myofibrillar organization in these mutations.

**MATERIALS AND METHODS**

*Strains used and rearing conditions*

Canton-S strain of *Drosophila melanogaster* was used as a wild type. Flies were raised at 24 ± 1 °C and fed on the usual cornmeal-agar medium on which live yeast suspension was sprayed. Soon after eclosion, flies were collected under light ether anaesthesia and kept in clean bottles. We took care to avoid crowded conditions and to keep food always fresh. This is important for obtaining reproducible results in a behavioural experiment. All experiments were carried out at 24 ± 1°C when flies are between 4 and 7 days after eclosion. We found that the flying ability of normal flies is best under these conditions.
Fig. 1. Left: flight tester used for measuring flying ability. It is a conventional 1000 ml graduated cylinder, inside wall being coated with paraffin oil. When flies are dumped into the cylinder through the funnel at the top, they start flying horizontally until they hit the inside wall and are trapped in oil, so the level at which they are stuck reflects their flying ability. Distance between bottom of the cylinder and lower tip of the funnel was divided into six regions. They are called landing height 1 to 6, counted from the lowest. Right: flying ability curves of wild-type males (solid line) and females (dotted line) measured by the flight tester. Number of flies landed in each region are plotted against the landing height. Flies which fell to the bottom or were stuck above the tip of funnel were plotted at landing height 0 and 7 respectively.

Measurement of flying ability

Flying ability was measured with two different methods.

(1) For measurement as a group, we used a simple flight tester originally devised by Dr S. Benzer (1973), with some modifications (Fig. 1). It is a conventional 1000 ml graduated cylinder, the inside wall of which is coated with paraffin oil. When flies are released at the top, they start to fly horizontally. Therefore, the points at which they hit and were stuck in the oil film reflects their flying ability. The entire length of the cylinder was divided into six regions, and they were called landing height 1 to 6 from the bottom. The number of flies landed was counted for each landing height. Flies which fell to the bottom or were stuck above the landing height 6 were plotted at landing height 0 and 7 respectively.
(2) Flying ability of the isolated mutants was also examined individually. A fly was gently put on the table with a glass suction tube and examined to see if it could take off.

**Mutagenesis and mutant isolation**

We used a chemical mutagen ethyl methanesulphonate (EMS) which is known to induce mostly point mutations. According to the procedure of Lewis & Bacher (1968), young wild-type males of 24–48 h after eclosion were fed for 24 h with 0.025 M EMS dissolved in 1% sucrose solution. The EMS treatment induced approximately one lethal mutation per one X chromosome. The dose can be shown to be optimum for isolation of non-lethal mutants. Higher dosage could increase frequency of hitting flight-specific genes but concomitantly would increase the frequency of lethal mutations on the same chromosome.

For isolation of sex-linked mutants, we crossed EMS-treated males to attached X(yf: = ) virgin females so that the mutagenized X chromosome should be inherited by F₁ males (Benzer, 1967). At the age between 4 and 7 days after eclosion, they were screened for flightlessness by means of the flight tester, of which the bottom was cut off and replaced with a beaker. We collected the flies which fell into the beaker and examined to see if they looked normal. Those which did not have any obvious morphological abnormality were crossed to yf: = virgins individually. The isogenic male progeny of the following generations were examined repeatedly, and those which showed deficit in flight were saved and balanced on FM6 or FM7b. By this procedure, we could identify recessive as well as dominant X chromosomal flightless mutations as long as they do not affect either viability or fertility of the males.

**Chromosomal mapping and complementation test**

For mapping loci of the flightless mutations on the X chromosome, males of a mutant line were crossed to y sc cho cv v f yf y+ virgins (for the details of mutant, see Lindsley & Grell, 1968). y+ is a small fragment of X chromosomal tip containing a normal allele of y and is translocated to the right of centromere. F₂ males were classified by expression of markers, and several from each recombinant class were crossed to yf: = virgins individually. The flying ability of isogenic male progeny of the crosses was then examined with the flight tester. The test could generally reveal the approximate position of each mutant locus relative to the reference marker genes. Among mutant candidates, only those of which loci could be mapped properly were classified as established mutants.

Complementation tests were carried out between all pairwise combinations of the 20 isolated mutant lines. Males from each line were crossed to virgins of another line, and the flying ability of F₁ heterozygous females was compared to that of normal females.
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Focus mapping by means of genetic mosaics

The general procedures of the mosaic analysis are identical to the blastoderm fate mapping technique originally used by Hotta & Benzer (1972). Males, whose X chromosome has a recessive, flightless gene and recessive marker genes for adult cuticular structures, such as y (yellow body and hair colour) and cho (chocolate eye colour), were crossed to In(l)\(w^{vC}\) females. F1 daughters of the cross frequently lose the \(w^{vC}\) chromosome from one of the daughter nuclei during the initial nuclear divisions to make gynandromorphs. In female cells, the recessive behavioural, as well as marker (y and cho) mutations are not expressed because of the presence of their wild-type alleles on the \(w^{vC}\) chromosome, while they are uncovered in the male parts of the gynandromorph. The difference in colour enables us to distinguish normal female area from mutant male parts in the cuticle. Their surface mosaicism patterns thus revealed were recorded individually and were compared with their flight ability. Then a correlation table was made for each landmark separately as is shown below, where \(a_{ij}\) and \(b_{kl}\) represent the numbers of flies classified in each category.

<table>
<thead>
<tr>
<th>Behavioural character</th>
<th>Both sides normal</th>
<th>One side normal one mutant</th>
<th>Both sides mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>(a_{11})</td>
<td>(a_{10})</td>
<td>(a_{00})</td>
</tr>
<tr>
<td>Mutant</td>
<td>(b_{11})</td>
<td>(b_{10})</td>
<td>(b_{00})</td>
</tr>
</tbody>
</table>

Distances on the blastoderm fate map between the flightlessness focus and various surface landmarks were calculated according to the equations of Hotta and Benzer, both with submissive and domineering models.

We also calculated the distances under the assumption that the focus is on the midline of the blastoderm. In this case, the equation for calculating fate map distances between the behavioural focus \(f\) on the midline and each surface landmark \(A\) is simplified as

\[
\bar{Af} = \frac{a_{00} + b_{11} + \frac{1}{2}(a_{10} + b_{10})}{\text{number of total mosaics}}.
\]

When a model employed is incorrect, distance between bilateral foci \((ff')\) would become a negative value, and distance between the focus and any of the surface landmarks would apparently increase. Distances calculated with the submissive or domineering foci model approach those calculated by the midline focus model as the distance between the pair of symmetric foci \((ff')\) tends to zero.
Fig. 2. Loci of mutants. The isolated mutants were mapped to five chromosomal segments separated by the morphological genes shown in this figure. The standard map positions of these reference genes are also shown in the top line of this figure. Mutant males were crossed to \texttt{y sc cho cv y+ vkg m} and their F\textsubscript{2} recombinant sons were crossed to \texttt{yf - } (attached A') virgins individually. The isogenic F\textsubscript{2} males were then examined for their flying ability by the flight tester. Nineteen lines could be mapped unambiguously, while one was found to be a double mutant. Therefore, there are 21 mutations, which were found to belong to 15 cistrons by complementation tests. These cistrons were named \texttt{flt} (flightless) and each cistron was identified by adding an alphabetical letter. In case more than two mutations are found to be alleles of a cistron, they are identified by superfix. The fifteen cistrons are located on an X chromosome without any obvious regularity.

\textit{Histological and ultrastructural observations}

Thoraces of 4- to 7-day-old adult flies were cut bilaterally with a razor blade and fixed in 3\% glutaraldehyde and 1\% osmium tetroxide. The specimens were stained with 1\% uranyl acetate, and then dehydrated and embedded in Epon. Sections for light and electron microscopy were made with a JUM-7 ultramicrotome (JEOL Ltd.). Thin sections were stained with Reynolds solution and examined and photographed with a JEM 100B electronmicroscope (JEOL Ltd.). For histological observations, thick sections were placed on a glass slide, stained with toluidine blue and photographed with a Nikon light microscope.

\textbf{RESULTS}

\textit{Isolated mutants and their chromosomal loci}

By means of the modified flight tester, ca. 10\textsuperscript{4} \textit{F\textsubscript{1}} males were screened for flightlessness. Among them 20 individuals were shown to possess sex-linked, recessive mutations by the criteria described in Methods.

We carried out chromosomal mapping of all 20 lines and also complementation tests between all 190 pairwise combinations. Among them, only one line was found to have double mutations. They were separated by recombination, and both were shown to cause flightlessness. All other lines were judged to be single mutations. The complementation tests classified them into 15 cistrons. They were named \texttt{flt} (flightless), and each cistron was identified by adding an
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Fig. 3. Classification of mutants with flight behaviour. (a) Type 1; flying-ability curve has a single peak at landing height 0. Those genes which belong to this group are, \textit{fltD, fltH, fltK, fltL}, and all four alleles of \textit{fltO}. (b) Type 2; flying curve does not have a single, clear peak. Those which belong to the group are, \textit{fltA, fltB, fltC, fltE, fltF}, all alleles of \textit{fltG, fltI}, all alleles of \textit{fltJ, fltM}, and \textit{fltN}. The curves shown are taken from (a) \textit{fltD} and (b) \textit{fltB}.

The fact that all alleles of \textit{fltO} cistron lack flight ability entirely suggests that the gene is essential in a basic flight mechanism. The same may be true for other

Flight behaviour of the mutants

The mutants were classified into two groups based on the results of the flying ability tests (Fig. 3); Type 1, all individuals fall to the bottom and, therefore, their flying ability curve has a single peak at landing height 0. Those genes which belong to this group are \textit{fltD, fltH, fltK, fltL, fltO, fltO^2, fltO^3} and \textit{fltO^4}. The fact that all alleles of the \textit{fltO} cistron lack flight ability entirely suggests that the gene is essential in a basic flight mechanism. The same may be true for other
genes, but lack of alleles does not permit us to argue that way. None of these mutants were able to take off from a flat table, although most of them can jump as normal flies. Only \( \text{fltK}, \text{fltD} \) and \( \text{fltO} \) were judged to have significantly reduced ability to jump.

There are genes which cause partial flightlessness. They were grouped as Type 2. In general, their flying ability curves do not have a single peak. Repeated trials to select from these strains a subline which has a single peak in this flight test were never successful and, therefore, it is concluded that this is an intrinsic character of the genes and not due to heterogeneity of their genetic background. Those which belong to this group are \( \text{fltA}, \text{fltB}, \text{fltC}, \text{fltE}, \text{fltF}, \text{fltG}, \text{fltG}^2, \text{fltI}, \text{fltJ}, \text{fltJ}^2, \text{fltJ}^3, \text{fltM} \) and \( \text{fltN} \). When they were gently released on a table, \( \text{fltJ}, \text{fltF} \) and \( \text{fltG}^* \) were unable to take off. Among them, most of \( \text{fltF} \) flies could not jump at all, while \( \text{fltJ} \) and \( \text{fltG}^2 \) had normal jumping ability. Flying ability of other Type 2 mutations was generally very variable; some could take off but others did not. Jumping ability of these mutants was mostly normal. Worth mentioning is \( \text{fltC} \), in which there is a clear separation between individuals which can and cannot fly. This is consistent with the fact that the flying ability curve of \( \text{fltC} \) has two distinct peaks. The \( \text{fltC} \) mutation is temperature-sensitive; when these mutants are raised at 29 °C, their flying ability curve becomes that of Type 1.

**Foci of mutants**

Two mutations \( \text{fltO}^2 \) and \( \text{fltH} \) were chosen for the focus mapping, since both lack flying ability with complete penetrance. We collected 125 gynandromorphs for \( \text{fltO}^2 \) and 109 for \( \text{fltH} \), scored them individually for 41 pairs of surface landmarks and examined all the mosaics for their flying ability. Though mutant individuals of these lines are entirely flightless, some mosaics were found to be neither normal nor flightless; they retained some gliding ability when they were released in mid-air. We interpreted this to mean that the focus is actually of finite size through which the mosaic dividing line happened to cut across in these intermediate cases. The correlation between genotypes of symmetrical surface landmarks and the individual's flying behaviour was tabulated in matrix form (Tables 1, 2), in which the intermediate cases were counted half as normal and half as mutant. We calculated the distances between bilateral foci (\( \bar{f}f' \)) and between focus and 41 landmarks (\( \bar{Af} \)). For \( \text{fltO}^2, ff' \), calculated under the assumption that the mutant focus is submissive, is mostly negative, while \( \bar{f}f' \) calculated with domineering model is positive. Distances between surface landmarks and \( \text{fltO}^2 \) focus are smaller if the focus is assumed to be domineering, than if it is assumed to be submissive. On the contrary, the converse is true for \( \text{fltH} \). So we conclude that \( \text{fltO}^2 \) focus is domineering and that \( \text{fltH} \) focus is submissive. From these data, foci of both \( \text{fltO}^2 \) and \( \text{fltH} \) were mapped to anterior ventral region of blastoderm fate map (Fig. 4). This is consistent with the fact that
Table 1. Summary of focus mapping for the flightless mutant fltO^2

Correlation matrices for eight surface landmarks are presented. Meaning of \(a_{ij}\) and \(b_{kl}\) is given in Methods. Distances between landmarks and the ipsilateral behavioural focus (\(A_f\)) were calculated from the correlation matrices. Equations used for submissive (sub.) and domineering (dom.) models were already described in a previous paper (Hotta & Benzer, 1972). An equation for the midline (mid.) focus model is given in Methods. Abbreviations for surface landmarks. IV, inner vertical bristle; PR, proboscis; I-coxa, prothoracic coxa; II-coxa, mesothoracic coxa; III-coxa, metathoracic coxa; ANP, anterior notopleural bristle; Gt, dorsal external genitalia; 2s, second abdominal sternite.

<table>
<thead>
<tr>
<th>Surface landmarks</th>
<th>Correlation matrices</th>
<th>Calculated distances in sturts</th>
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<tbody>
<tr>
<td></td>
<td>(a_{ij})</td>
<td>(b_{kl})</td>
</tr>
<tr>
<td>IV</td>
<td>34.5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>14.5</td>
<td>16</td>
</tr>
<tr>
<td>PR</td>
<td>21.5</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>22.5</td>
</tr>
<tr>
<td>I-coxa</td>
<td>27</td>
<td>17.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>II-coxa</td>
<td>27</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>17.5</td>
</tr>
<tr>
<td>III-coxa</td>
<td>24</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>21</td>
</tr>
<tr>
<td>ANP</td>
<td>24</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>27</td>
</tr>
<tr>
<td>Gt</td>
<td>29</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>25</td>
</tr>
<tr>
<td>2s</td>
<td>27.5</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td>14.5</td>
<td>30</td>
</tr>
</tbody>
</table>

Similar map distances could be obtained by assuming that the focus is on the midline (Tables 1, 2).

This region has been shown by embryological studies to be an area where primordial mesoderm arises (Poulson, 1965), and the two foci are located close to the foci of \(wup-A\) and \(wup-B\) (see Fig. 4), mutants known to have their foci within indirect flight muscle (Hotta & Benzer, 1973).

Histological and ultrastructural observations

The indirect flight muscle of \(Drosophila\) consists of six median pairs of anteroposterior dorsal longitudinal muscle fibres and seven lateral pairs of oblique dorsoventral muscle fibres. These muscle fibres, which produce power for the wing beat, attach to thoracic cuticle at both ends without any direct contact with wings. They are fibrillar-type muscles. Tubular muscles are also involved
in flight as direct wing muscles which attach to the base of a wing directly, regulating its beating angle.

(1) Normal morphology of *Drosophila* indirect flight muscle

In longitudinal sections of a wild-type indirect flight muscle viewed under a light microscope, long straight myofibrils with a diameter of approximately 1.7 μm are seen to run parallel, and Z bands and H lines are recognizable separating 3.5 μm long sarcomeres. They are also visible with a phase-contrast microscope, when the muscle was macerated in physiological saline solution, in which myofibrils of tubular muscles do not maintain structural integrity. With the electronmicroscope straight myofibrils are seen to run parallel to each other, with many mitochondria filling the intermyofibrillar space (Fig. 5). In cross-sections (Fig. 6), the arrangement of both filaments is hexagonal. The Z band also has an internal hexagonal structure which is similar to the Z band structure in honey-bee flight muscle already reported (Saide & Ulrick, 1973). The thick filaments are hollow except at the region of H line and both ends. The sarcoplasmic reticulum and the T-system forming dyads are often seen adjacent to myofibrils, but the internal membrane system is sparse compared with that in tubular-type muscles. Our observations confirmed the *Drosophila* flight muscle morphology already reported by Shafiq (1963).
(2) **Morphology of flightless mutants**

Using electronmicroscopy we examined the indirect flight muscle of *fltH* and four *fltO* alleles whose foci were located in these muscles.

We found three symptoms in the myofibrils of *fltH* indirect flight muscle, of which a typical example is shown in Fig. 7. Their myofibrils are wavy and their diameter is larger than normal. Thick and thin filaments are occasionally disorganized with deficient Z bands. Their sarcomere length is also abnormal. Spacing between adjacent myofibrils is relatively large with a large number of mitochondria in between. In toluidine blue-stained sections, the size of muscle cells themselves was found to be normal. Relative frequency of these symptoms varied even within a single muscle fibre. It was common to find thick myofibrils with abnormal sarcomere length immediately adjacent to almost normal myofibrils. These symptoms are recessive, i.e. myofibrils of *fltH* heterozygote are morphologically normal.

In toluidine blue-stained sections of *fltO* indirect flight muscle, which is an allele of *fltO*², muscle fibres were somewhat wavy with amorphous internal structure. By electronmicroscopy, we found that the arrangement of the myofilaments was disorganized in this mutant (Fig. 8). Z bands were frequently absent, split or distorted, and myofibrils, if any, were tortuous and not parallel to each other. Thick and thin filaments within a sarcomere adjacent to an apparently normal Z band have a nearly normal arrangement. Outside the normal area, thick filaments run freely while thin filaments are often found to aggregate, forming a bundle with electron-dense striations with ca. 130 nm periodicity (Fig. 9). A typical bundle has 10–20 striations with fine fibrous appearance. There is no indication that thick filaments were also involved in the structure. Such bundles have never been observed in the wild-type muscle under normal conditions.

*fltO*², *fltO*³ and *fltO*⁴ belong to the same cistron as *fltO*, and their symptoms are similar but less marked than that of *fltO*. The striated bundles are also seen frequently in *fltO*². The symptom of *fltO*³ is even milder. The myofibrils run almost straight and parallel while their diameter is variable. Many of the thick myofibrils had distorted Z bands where they were torn longitudinally. We found that *fltO*⁴ indirect flight muscle had a normal arrangement in the central part of each myofibril (Fig. 10). However, the diameter of its Z band was somewhat smaller than that of normal muscle, and the most peripheral arrangement of filaments was loose. Local interaction between thick and thin filaments is still maintained at the disorganized periphery of *fltO*⁴ myofibrils.

**DISCUSSION**

In this paper, we reported our attempt to induce and collect a number of flightless mutants systematically. Chemical mutagenesis with EMS is known to
Fig. 4. For legend see opposite.
induce small, possibly point, mutations (Lim & Snyder, 1974). We hunt only for X-linked mutations, since they are the easiest to screen for. By our flight tester, 20 lines of flightless mutations were isolated from 10⁴ F₁ males. On an average, one mutant was isolated from several hundred F₁ males. The efficiency of screening is comparable to that of Shepperd (1974) who developed a sophisticated machine for isolating flightless mutants.

However, we might have overlooked certain specific types of flightless mutants. When a normal fly takes off from the ground, it pulls up both its wings first, and then jumps, before starting wing beat (Boettinger & Furshpan, 1952). When flies are dumped into the flight tester, however, jumping is not a necessary process to initiate flight. Any mutant which could fly but not jump would not be isolated by our method. Furthermore, if a mutation affects direct wing muscles, the F₁ male might not be able to vibrate its wings properly in courtship behaviour, making such males effectively sterile. Such mutations would not have been noticed either.

Fig. 4. Upper figure: a pictorial sketch of an embryonic fate map of *Drosophila melanogaster*. The fate map represents a right hemisphere of a blastoderm seen from inside. Adult surface structures are placed so that a distance between any two landmarks is approximately proportionate to a probability that the two landmarks are of different genotypes among entire mosaic ensemble made with the present method. drd, drop-dead focus; HK-I, II, III, hyperkinetic leg shaking foci for pro-, meso-, and metathoracic legs respectively; wup-A, -B, foci for two wings-up mutants of which mesodermally derived indirect flight muscles are degenerate or malformed. For more details of mapping these foci and structures, see Hotta & Benzer (1972).

In addition, other internal structures and foci are shown. MC, male courtship focus which must be male in order to trigger chain of male courtship towards a female, such as orientation, following and wing vibration (Hotta & Benzer, 1976). AMG, anterior midgut; GON, gonad; MP, Malpighian tubule. These are adapted from Janning (1974a, b). SP, SB and OG, supraoesophageal, suboesophageal and optic ganglia which are adapted from Kankel & Hall (1976). Combining these data with data of histological tracing of cell-lineage during embryonic development, especially of Poulson (1965), regions on a blastoderm which give rise to central nervous system, including brain and thoracic ganglia, and mesodermal structures are inferred and shown with dotted lines.

Middle figure: focus for the *fltO²* flightless mutant. It is mapped under the assumption that the mutant focus is domineering. See Table 1 for the calculations.

Lower figure: focus for the *fltH* flightless mutant. It is mapped under the assumption that the mutant focus is submissive. Distances for the flightless mutant foci from a number of strategic landmarks on the adult surface are given in sturts; one stunt representing a probability of 1 % that, among the entire set of adult mosaics formed by this method, the two structures in question were of different genotype. Data on which the mapping calculations are based is summarized in Table 1. For mutants like *fltO²* and *fltH* which have their foci near the midline, a simpler calculation method under the assumption that a focus is on the midline is given in the text. From the map position of two mutant foci, it is concluded that both of these flightless genes have their primary focus within mesodermal structures, most likely in indirect flight muscles. See Table 2 for the calculations.
Fig. 5. An electronmicrograph of a longitudinal section of a wild-type indirect flight muscle. Straight myofibrils with approximately 1.7 μm diameter run mutually parallel, mitochondria being tightly packed between them. Z bands run perpendicularly to myofibrils. A large portion of a sarcomere is occupied by A band, I bands being very narrow. The sarcoplasmic reticulum and T system are seen to make dyads adjacent to myofibrils, but they are relatively underdeveloped. In these muscles, they are found more frequently in the region of A bands, but rarely near H lines and Z bands. Glycogen particles are seen in the region of H lines and between myofibrils. Scale bar = 2 μm.

By the complementation test and chromosomal mapping, the 21 isolated mutant genes were classified into 15 cistrons. Three among the 15 cistrons have more than two isolated alleles. All of these repeats, however, may not be independent. Since EMS is known to act also premeiotically, two $F_1$ males having an identical mutation can arise as a pair in a same batch. Such a possibility can be raised for $fltI$ and $fltI^2$, and for $fltG$ and $fltG^2$. Excluding the two mutations discussed above, the average number of alleles repeatedly isolated from a cistron is 1.3. The number is still small so that we cannot exclude a possibility that there are more flight behavioural genes on the X chromosome yet to be discovered.

Once many mutants have been isolated and their behavioural syndromes characterized, a next question to ask is how each mutant gene leads to a specific
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Fig. 6. An electronmicrograph of a cross-section of a wild-type indirect flight muscle. Hexagonal arrangement of thick and thin filaments is seen. Z band (Z) also has a hexagonal internal structure. Thick filaments are seen to be hollow except in the region of H line (H) and at both ends. Scale bar = 2 μm.

developmental and behavioural deficit. Although electrophysiological, histological and biochemical techniques help us analyse the link between the gene and behaviour, experimental findings with these methods cannot be interpreted easily. In a multicellular organism, intercellular or organ-to-organ interactions are so common that a local mutant character found might be caused by gene function elsewhere. The mosaic fate mapping technique thus plays a key role in overcoming such a difficulty, since it clarifies the primary focus of each mutant gene where malfunction of the gene causes the behavioural deficit in question.

In this paper, two new flightless mutants were analysed with the fate map method. The calculated position for fltO² and fltH foci were found to be close to each other.

We concluded that these mutations have their foci in the indirect flight muscle because of the following reasons. (i) Their foci are in the region which had been shown by the embryological studies to be primordial mesoderm (Poulson, 1965). If the primary focus of these mutations was in the thoracic nervous system
which innervates the flight muscle, the calculated site of foci should be located further from ventral midline and closer to leg primordia. (ii) Their foci are located close to the foci of \textit{wup-}A and \textit{wup-}B (see Fig. 4) which are known to be within indirect flight muscle (Hotta \& Benzer, 1973). Their foci are also very close to the foci of \textit{int} and \textit{flw} which have severe morphological aberrations in their indirect flight muscles (Deak, 1977). (iii) These mutants have normal ability in walking, jumping, holding up wings before starting flight and vibrating wings in mating behaviour, which are governed by tubular muscles. On the contrary, they lack the ability to beat wings when tethered, suggesting functional defect in indirect flight muscle system. (iv) The indirect flight muscles of \textit{fltO} and \textit{fltH} have an abnormal morphology. On the contrary, we could not find any abnormality in the direct wing muscles of these mutants.

The two mutant maps, however, look slightly different; most distances being longer in \textit{fltH} than in \textit{fltO} (Fig. 4). One possible reason for this lies in the assumption that the focus is a point. If indirect flight muscle is the focus, it
must be an elongated area. Since \( \text{flt}^O \) is domineering while \( \text{flt}^H \) is submissive, the error may be significantly different between the two cases.

Since the focus of \( \text{flt}^O \) has been found to be in indirect flight muscle, it is expected that the other three alleles of the cistron also have their primary defects in indirect flight muscle. Therefore, we performed comparative electron-microscopic studies of indirect flight muscle in these alleles to see if they look similar. They were found to have a deficiency in Z bands and a disorganized arrangement of myofibrils in common, although the symptoms differ quantitatively between the alleles. In \( \text{flt}^O \) and \( \text{flt}^O \), thin filaments are frequently seen to be bunched together with electron-dense periodic striations. The ‘striated bundles’ have also been observed in indirect flight muscle of homozygous \( \text{wup-B} \) mutant (K. Mogami et al., unpublished), an X-linked flightless mutation with vertically held wings, whose primary focus is in indirect flight muscle and the deficit is suspected to be in Z bands (Hotta & Benzer, 1973). The similarity of syndrome between \( \text{flt}^O \) alleles and \( \text{wup-B} \) suggests that the former also have
a deficit in Z bands. However, these two genes are distinct, since the chromosomal locus of \textit{wup-B} is between \textit{vermilion} and \textit{forked} while that of \textit{fltO} is between \textit{forked} and a centromere. Mutant characters of the two genes are also different. For example, all \textit{fltO} alleles are completely recessive with respect to both flightlessness and morphological abnormalities, while \textit{wup-B} is dominant in these respects.

In \textit{fltH}, of which the focus has also been found in indirect flight muscle, there are three symptoms: larger diameter of myofibrils, distortion of filament arrangement and abnormal sarcomere length. Among them, the large myofibrillar diameter is a constant symptom, while others are variable. Peristianis & Gregory (1971) found that a developing myofibril of a blowfly \textit{Calliphora} divides longitudinally and, therefore, a myofibril at 54 h after puparium formation contains twice as many filaments as that 18 h later. Although such division has not been reported in \textit{Drosophila}, the symptom of \textit{fltH} could be explained if it is assumed that the deficit of this mutant is in the mechanism which keeps the size of myofibril constant. The sarcomere length is also abnormal in this mutant. Figure 7 shows that I bands and H bands are clearly discernible even in a short sarcomere.
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of only 2.2 μm length. Although length of the thick and thin filaments cannot be measured reliably in such a sectioned specimen, the presence of H and I bands suggests that the filament length is also abnormal. Shafiq (1963) reported that the myofibrils in Drosophila grow with an increase in both length and diameter of sarcomeres. It is possible that the gene functions in such developmental processes.

In these examples, the foci were indeed found to be the site of most obvious morphological deficits. This fact indicates that the mosaic method is working properly, and that the morphological abnormalities observed must be closely related to the mutant gene dysfunction. However, the focus may not always have apparent abnormality. For example, a preliminary mosaic analysis suggested that fltl focus is also in ventral mesoderm, but we could not find any abnormality in these muscles, neither with light nor electron microscopy. In such cases, subtle biochemical or physiological abnormality should be sought in the focus structure.

In this paper, we could demonstrate the presence of genes which affect
morphology of a specific set of muscles. By extending this approach, we will also be able to genetically dissect specific sets of neural circuits. In analysing such neurogenic mutants, the electrophysiological method developed by Levine & Wyman, (1973) and Levine (1973b) will be especially useful.

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REFERENCES


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Note added at Proof Stage
While this article was in the press, papers by Homyk et al. (Genetics, 87, 95–104, 105–128) appeared, in which they described their isolation of mutations with decreased flight ability. Some of their mutants were also analysed by means of the genetic mosaic technique. Although they did not examine their mutants histologically, some of them are behaviourally similar to ours.