RESEARCH ARTICLE

The costae presenting in high-temperature-induced *vestigial* wings of *Drosophila*: implications for anterior wing margin formation

DAXIANG YANG*

Department of Zoology and Animal Physiology, College of Biological Sciences, China Agricultural University, Beijing, 100094, China

Abstract

It has long been noted that high temperature produces great variation in wing forms of the *vestigial* mutant of *Drosophila*. Most of the wings have defects in the wing blade and partially formed wing margin, which are the result of autonomous cell death in the presumptive wing blade or costal region of the wing disc. The *vestigial* gene (*vg*) and the interaction of Vg protein with other gene products are well understood. With this biochemical knowledge, reinvestigations of the high-temperature-induced vestigial wings and the elucidation of the molecular mechanism underlying the large-scale variation of the wing forms may provide insight into further understanding of development of the wing of *Drosophila*. As a first step of such explorations, I examined high-temperature-induced (29°C) vestigial wings. In the first part of this paper, I provide evidences to show that the proximal and distal costae in these wings exhibit regular and continuous variation, which suggests different developmental processes for the proximal and distal costal sections. Judging by the costae presenting in the anterior wing margin, I propose that the proximal and distal costal sections are independent growth units. The genes that regulate formation of the distal costal section also strongly affect proliferation of cells nearby; however, the same phenomenon has not been found in the proximal costal section. The distal costal section seems to be an extension of the radius vein. *vestigial*, one of the most intensely researched temperature-sensitive mutations, is a good candidate for the study of marginal vein formation. In the second part of the paper, I regroup the wing forms of these wings, chiefly by comparison of venation among these wings, and try to elucidate the variation of the wing forms according to the results of previous work and the conclusions reached in the first part of this paper, and provide clues for further researches.

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Introduction

It has long been noticed that high temperature increases the size of the wing of the *vestigial* mutant of *Drosophila* and produces great variation in wing forms, which vary from normal vestigial to wild-type-like wing. Most of the wings are characteristic of deleted structures in wing blade and partially formed wing margin (Roberts 1918; Harnly 1930; Li and Tsui 1936). Researchers in the 1930s were attracted by the phenomenon, but the limited methods available at that time handicapped them and further research could not be done.

It was not until the late 1960s that Fristrom provided cytological evidences to show that the defect in vestigial wings is due to extensive cell death in the presumptive wing blade and margin region in wing disc (Fristrom 1968). Bownes and Roberts (1981) further demonstrated that the cell death is autonomous.

After the seminal work of Waddington (1940), which attempted to understand the genetic basis of wing vein formation, development of the wing vein of *Drosophila* has been used as a model system for studying epithelial morphogenesis and understanding how genes regulate development. Taking advantage of the large number of viable wing vein mutations and relatively simple structure of the wing and imaginal disc, numerous studies at cellular and molecular level have been carried out (for a review, see Garcia-Bellido and de Celis 1992; de Celis 1998, 2003; Bier 2000). The *vestigial* gene (*vg*) and interaction of Vg protein with other gene products in wing vein formation are well understood. In *Drosophila vg* encodes a novel nuclear protein that is re-

*E-mail: dxyoung@163.com.

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quired for formation of the entire wing. *vg* is expressed in the presumptive wing margin and dorsal/ventral wing blade in a graded fashion (Williams *et al*. 1991, 1993, 1994). During growth of the *Drosophila* wing, short-range or long-range signals from the dorsal/ventral boundary and wing blade activate different enhancers to regulate *vg* expression (Cohen 1996; Kim *et al*. 1996). Vg protein combines with Sd, the product of the gene *scalloped (sd)*, forming a transcriptional activation complex, which regulates downstream genes involved in development of the wing (Campbell *et al*. 1992; Halder *et al*. 1998).

With this biochemical knowledge about *vg* and current tools, one can resume the suspended research mentioned earlier and unravel the complex molecular mechanism underlying the large-scale variation in high-temperature-induced vestigial wings. As a beginning, I reinvestigate the high-temperature-induced (29°C) vestigial wings morphologically. In the first part of this paper, I provide evidences to show that the proximal and distal costal sections (see figure 1) present in these wings exhibit regular and continuous variation. Judging by the costae presenting in the anterior wing margin, I propose that the proximal and distal costal regions are independent developmental units. The gene(s) that control marginal vein formation also significantly affect proliferation of cells nearby. Development of the marginal vein is intriguing but less understood, one of the reasons being viable mutants that show desired phenotypes are not available (Garcia-Bellido and de Celis 1992). It is possible to find such phenotypes in viable spontaneous or induced temperature-sensitive mutants (Driver 1931; Suzuki 1970). *vestigial* seems to be a good candidate. In the second part of the paper, I regroup the wing forms of these vestigial wings, chiefly by comparison of venation among wings. This work aims at elucidating some of the variation of the wing forms according to the results of earlier work and the conclusions reached in the first part of this paper, and at providing clues for further research.

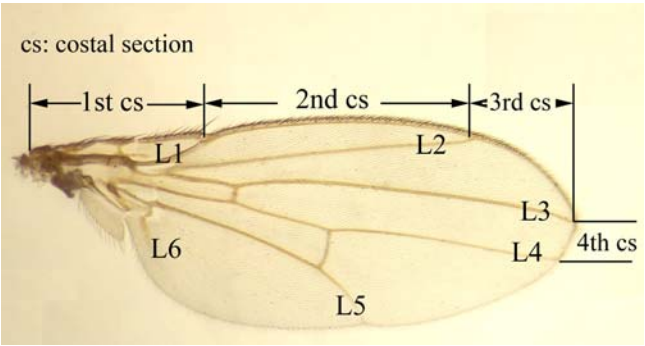

Figure 1. Wild-type wing to show nomenclature used in this paper. The first costal section (cs) is referred to as proximal costal section, and the second to fourth costal sections comprise distal costal section.

Materials and methods

A pure stock of *vestigial* mutant was used in the experiments. Food was the common cornmeal medium, and flies were maintained in 29°C incubators. A bottle of wild-type flies was also set in the same incubator as control.

Emerging flies were etherized and fixed with 95% alcohol. Both wings of all fixed *vestigial* flies, and right wings of 60 wild-type flies (30 male and 30 female) randomly drawn from culture bottles were removed in isopropanol with a pair of dissecting needles under a binocular stereomicroscope. Images of wings were captured using a digital camera, which was connected to an ocular of a light microscope with an adapter.

The typical wing types in the *vestigial* population were grouped mainly by similarity of wing vein pattern. Wing shape was also considered, and was roughly estimated by the remaining wing blade. Wings with similar wing vein pattern and wing shape are considered to belong to the same group. Assessments of resemblances were performed using Adobe Photoshop software. The typical vestigial wing vein patterns were determined and compared with the typical wild-type wing venation. The typical wild-type wing venation was established by the following steps: Superpose 30 right wings of flies of the same sex; compare the wing vein pattern in the background image with those in the upper layers; make the image in the second layer semitransparent and those in the third to thirtieth layers invisible; align wings in the background and the second layer along the L3 and anterior cross vein (ACV). If the two wings match perfectly, then drop the second layer; if not, make the second layer invisible and compare wings in the background and the third layer; and so on. Finally make all remaining wings opaque and visible, erase intervein regions in each wing, then merge down all layers. Change the colour of the resulting image and replace the solid lines of the veins with dotted lines. Clearly, the 'typical' wild-type wing demonstrates the positional ranges of longitudinal and cross veins in a population. A wing is considered to be wild type if its longitudinal veins fall in the longitudinal vein range and at the same time its cross veins fall in the cross vein range of the 'typical' wild-type wing.

Results

Some partially formed distal costal sections are parts of the intact distal costal region, and show regular and continuous variation

A total of 1993 individuals were examined. In most of the wings, the 4th costal section and the tissue in the vicinity were usually deleted (figure 2,A–C). This made me limit my observations to the 1st to 3rd costal sections. My observations showed that in some wings, the partially formed distal costal sections were in fact connected by prevein or poorly developed vein(s), and these vein structures formed a continuous 2nd and 3rd costal margin (figure 2,D–F). This indicates that in these wings the 2nd and 3rd costal regions had not suffered cell degeneration. The partially developed vein(s) are

in fact the intact end product of a developmental phase. My analyses mainly focus on these wings.

An intriguing observation was that the tips of these wings invariantly had a segment of fully developed vein which bore bristles (figure 2,A–C). I did not find a segment of vein without bristles in this region. The vein usually started at the tip of the L3, and sometimes extended to the middle of the 2nd costal section. The length of the segment varied with wing. In some wings, I could find another segment of vein extending from L1 and running down the edge of the marginal cell (figure 2,F); this segment of vein obviously developed from the prevein (figure 2,D&E), which usually does not bear bristles. This seems to be consistent with the observation that the sensory organs develop independently of the veins (Sturtevant and Bier 1995). At the middle of the 2nd costal region, I usually found one or several pieces of dot-like vein, carrying one or two bristles (figure 2,H), interspersed in a relatively long segment of poorly developed vein. However, I did not find a segment of fully developed vein, connected by such poorly developed vein at both sides, presenting at the middle of the 2nd costal region.

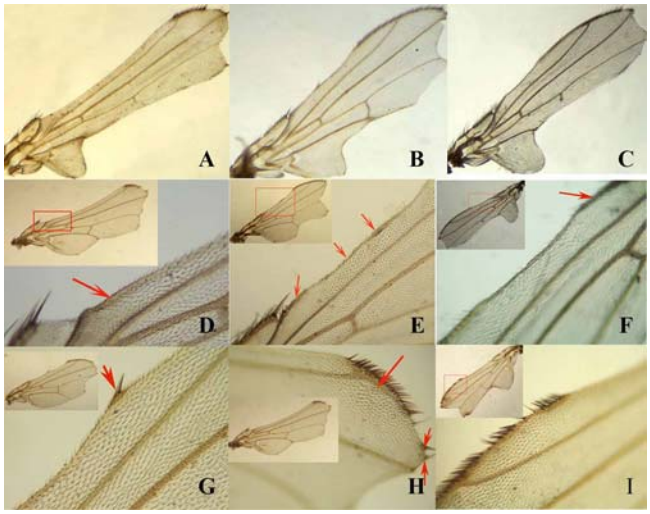

Figure 2. Partially developed distal costal section found in high-temperature-induced vestigal wings. See text for explanation.

I did not find some types of partially formed vein I would have expected in the distal costal region (figure 3,A–D) in this population. For example, I never found a wing carrying only the proximal part of 2nd costal section, or several independent segments of veins randomly distributed along the distal costal region.

All of these observations suggest a developmental model for the distal costal section. The relevant gene(s) may be expressed near the tip of L3 and initiate marginal vein development, and the costa protracts to meet another part which runs from L1. The distal costal section seems not to be formed continuously, just as Waddington (1940) described, but formed segment by segment. The veins shown in figure 2,H&I may be the very product of such a process.

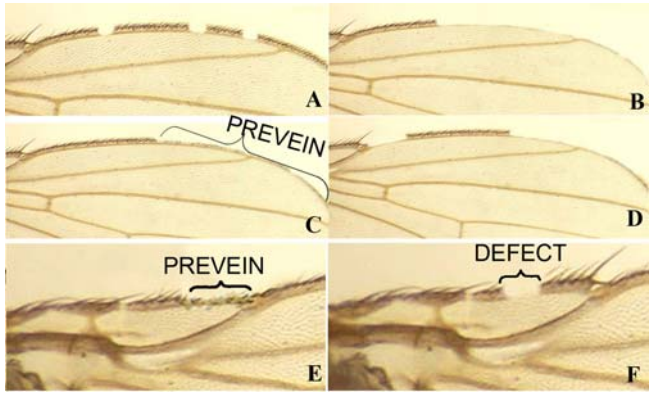

Figure 3. Some partially developed distal (A–D) and proximal (E–F) costal sections not found in the population examined.

Note the marginal cell edge directly under the prevein or poorly developed vein. The region was usually bowed and sunken more noticeably (figure 2,A–F) than that covered by fully developed vein. The position of L2 was usually lower than in the wild-type wing. These observations suggest that the gene(s) that regulate development of the distal costal section may also significantly promote local cell differentiation. The example shown in figure 2,G may support this suggestion. The 'hill' is proposed to be created by the gene(s) relevant to the formation of the apex—the dot-like vein.

The distal costal section seems to be the extension of the radius vein

Note the joint where L1 meets the 2nd costal section in figure 4, and see also figure 2,E. In some wings, I could find a trace of vein structure in the 2nd costal region (figure 4,A).

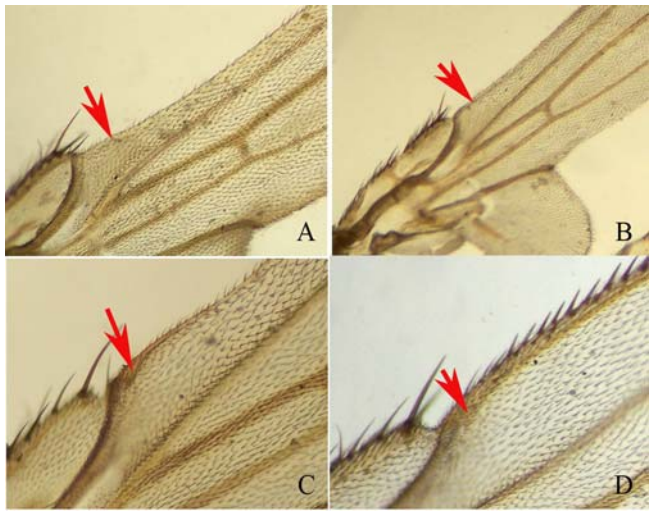

Figure 4. The junction where L1 meets the 2nd costal section.

The tiny costal vein became distinct and connected with L1 (figure 4,B). The joint where L1 met the 2nd costal section was sometimes bigger in size (figure 4,C). The information in figure 4 suggests that the growing vein runs from the joint towards the base of the wing, and then other structures, such as sensory organs, develop. It seems that the final step in development of the distal costal section is production of certain substances that wrap the newly formed marginal vein (figure 4,D).

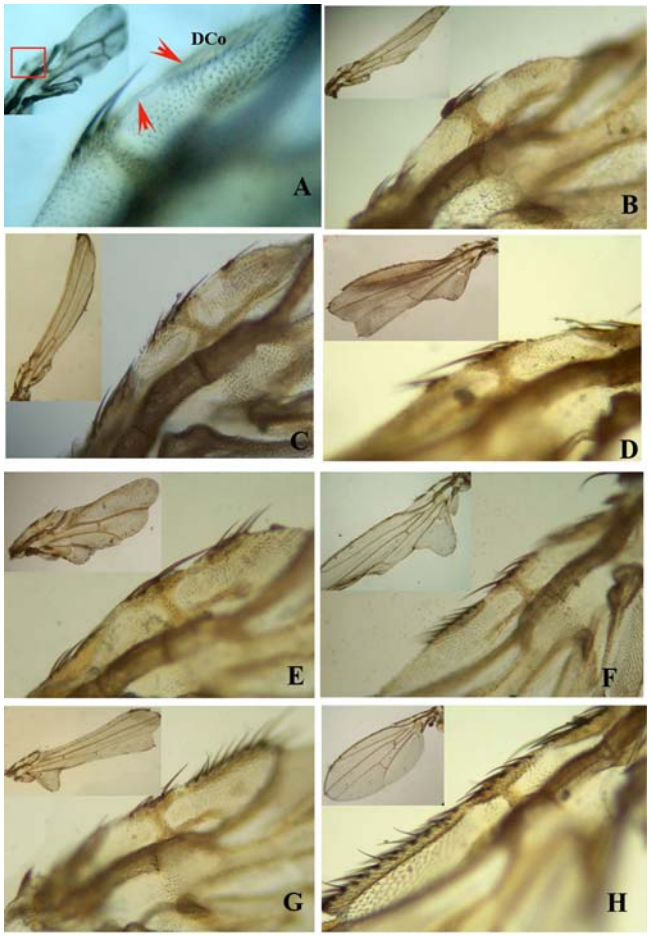

Figure 5. Typical proximal costal sections found in high-temperature-induced vestigal wing (A–G), and proximal costal section of wild-type wing (H).

The variation of proximal costal section ranges from 'normal vestigial' to 'wild type'

Typical proximal costal sections in these vestigial wings are shown in figure 5. The variations range from 'normal vestigial' (figure 5,A) to 'wild type' (figure 5,G). In normal vestigial wing, the costal cell edge was round. A small piece of medial costa (MCo) presented at the top of the humeral cross vein, with or without bristles. In some individuals, I found both sides of this segment of vein connected by tiny vein structures (figure 5,A), which suggests that the piece of MCo is not a remnant of degeneration of neighbouring tissue. This circumstance was also found in some elongated wings (figure 5,B). The connection between proximal costa (PCo) and MCo gradually grew to normal size (figure 5,C–F). The combined PCo–MCo joined the short segment of distal costa (DCo), and the edge of the costal cell gradually became level (figure 5,E&F).

I would have expected that a tiny prevein or similar structure would follow the short segment of distal costa, and cover the remaining region that should be covered by DCo (figure 4,E). It is interesting, however, that in the 1993 vestigial flies examined I did not find such a partially formed proximal costal section, and I did not find a split DCo as shown in figure 4,F as well. I also noticed that the edge of the proximal costa cell not covered by vein did not become concave, and no nick or other deletion of structure was found at the edge either (figure 5,E&F). These observations suggest that this vein-deleted region does not suffer cell death, and during development of the proximal costal section DCo extends gradually in one direction to its full length.

Wing forms are abundant, but wing types are rather limited

Li and Tsui (1936) had tried to group the high-temperature-induced vestigial wings. However, their methods of grouping, chiefly based on wing shape, have inherent disadvantages. Most of the wings have deleted structures in the wing blade (Fristrom 1968; Bownes and Roberts 1981), the region of cell death is not constant, and the pair of wings carried by the same fly are not even symmetric (figure 6,A&B). Figure 6,A shows the pair of quite different wings carried by one fly in this population. However, when the left wing was superposed on the right, the wing vein patterns in the remaining wing blade, and the edges of basal parts of the two wings matched perfectly. Figure 6,B is another example. The positions of L3, L4 and ACV, and the region of underdeveloped 3rd anterior cell of the left wing matched quite well with the right. There is no evidence so far that a *vestigial* fly may bear different-type wings. These may explain the phenomenon that puzzled Li and Tsui (1936). As a classification base, wing shape may be rather misleading; the wing vein pattern, together with the wing shape, which is judged by the remaining anterior or posterior marginal edge when the wing has defects, are thought to be more appropriate. Wings with similar wing vein patterns and shapes are supposed to be of the same type. The similarity assessments of wing vein pattern and shape can be performed with Adobe Photoshop software conveniently. When typical vestigial wing types are determined, they are compared with the typical wild-type wing venation (figure 6,C). Among wild-type wings, there exist many minor variations, such as in wing size, and longitudinal and cross vein position. The wing vein pattern established as described in Materials and methods helps to adjust for the possible random error in venation comparison.

The population studied here duplicates all the wing forms observed by Roberts (1918), Harnly and Harnly (1935) and

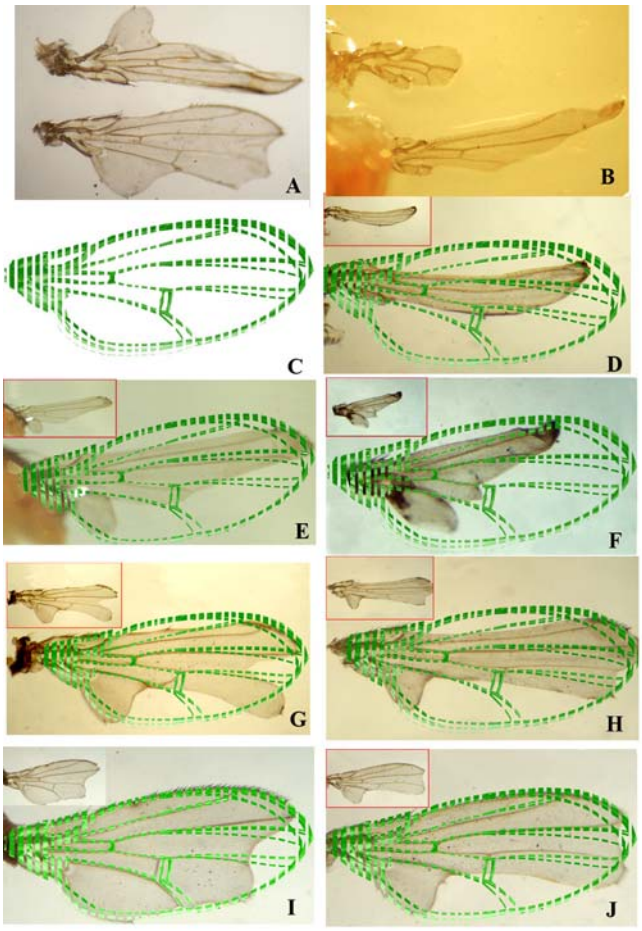

Figure 6. Typical wing forms found in male flies raised under 29°C.

Li and Tsui (1936). On the basis of the results of venation comparison, wings in this population can be divided into four types, with the normal vestigial wing and wild-type-like wing (images not shown) at the ends of the range. Figure 6 shows typical wing forms of male flies in the population; the wing types of female flies are the same as those of the male.

Type I: Normal vestigial wing.

Type II (figure 6,B&D): The wing veins become more distinct than in the normal vestigial wing. L2 appears, and the tissues in marginal cell, submarginal cell, 1st posterior cell, 2nd posterior cell begin to grow. However, 3rd posterior cell is underdeveloped. The reason I include the wing shown at top of figure 6,B in this type has been stated previously. Sometimes, at the tip of these wings, a quite short segment of 3rd costal section can be found, but one can't find prevein or poorly developed vein in 2nd or 3rd costal region.

Type III (figure 6,E–H): The 3rd posterior cell is fully developed. Although in different wings of this type the wing blade may suffer cell degeneration to various degrees, the basal part of the wings, judged by the remaining tissues, is well matched to that of a wild-type wing, and the central veins L3 and L4 match those of wild-type wing as well. But the size of the marginal and submarginal cells is smaller, and the position of L2 is usually lower than in wild-type wing. In 3rd costal region, one can usually find a segment of fully developed vein, and remaining parts of 2nd and 3rd costal regions are usually found to be covered by prevein or poorly developed veins.

Type IV (figure 6,I&J) Both wing venation and wing shape are perfectly matched to those of wild-type wing. The distal costal section is complete or has little break. This type of wing is considered to be fully developed.

The observations show that, although the wing shapes in this population are abundant, the wing vein patterns are rather limited. Unfortunately, I did not compare my results with those of Li and Tsui (1936), as they did not provide photographs for each wing. Except in the normal vestigial wings, the 1st costal section varies from incomplete to complete. Normal vestigial type 1st costal section is seldom found in wings of type III and type IV. The difference between type III and type IV is that the 2nd costal section of type III wings is not fully developed. I also found scalloped wings (data not shown) in this population, as described by Li and Tsui (1936), but this type of wing accounts for a rather small proportion and would not be considered as a typical wing type.

Discussion

The regular and continuous variation in costae may provide insight into understanding marginal vein development

Although the molecular mechanisms that control *Drosophila* wing vein pattern are well understood, relatively little is known about formation of the marginal vein. As regards development of the proximal costal region, we know little. One approach to improve understanding is to find some viable mutants that show the desired phenotype. Some temperature-sensitive mutants, such as *vestigial*, may be good candidates.

In this paper, I have shown that in high-temperature-induced *vestigial* wings the variation present in wing margin is regular and continuous. These variations suggest different developmental processes for the proximal and distal costal sections and imply that the two parts of the costal region are regulated by different genes.

Silber *et al*. (1997) have demonstrated that under high temperature *vg* transcription increases in wing disc of *vestigial* fly, and suggest that a *vg*-wing-specific enhancer may be involved in the thermoregulation. The increase of Vg protein explains development of more wing structures, as Vg is required for cell proliferation (Williams *et al*. 1991). However, some more questions remain for further research: (i) When temperature increases, does Vg increase at the same rate as the presumptive wing margin and wing blade? (ii) We know *vg* expression is regulated by different enhancers in dorsal/ventral boundary and wing blade (Kim *et al*. 1996). What specific enhancers are involved in the thermoregulation? (iii) Is the increase of Vg the only reason that explains the continuous variation shown in proximal and distal costal sections?

The costa of Drosophila wing: one longitudinal vein or two independent developmental units?

The proximal and distal costal sections, which are both referred to as costa by entomologists (Stark *et al*. 1999), are in fact innervated by different marginal nerves (Palka *et al*. 1979; Murray 1984) and are quite different morphologically. Are they regulated by the same set of genes during formation of the anterior margin? The answer seems to be negative. The present observations suggest that the proximal and distal costal sections develop independently. A fully developed proximal costal section was usually found to be followed by a partially developed distal costal section and vice versa. As seen in figure 2,A, the proximal costal section is complete; however, a long segment of the 2nd costal section has not formed.

The whole distal costal section seems to be formed segment by segment, one initiates near the tip of L3, another extends from L1. The genes that regulate development of the distal costal section also significantly affect proliferation of cells directly under the vein. The *wingless* (*wg*) gene is supposed to be involved in regulation of this process (Díaz-Benjumea and Cohen 1993). The proximal costal section has a unique process of formation. It seems that MCo, DCo and PCo develop independently first, then join together, and then DCo protracts along the costa cell edge to its full length. The characteristic concave, which appears under the prevein or poorly developed vein in the distal costal section, does not show up in the region covered by prevein structure in the proximal costal region. These observations suggest that the proximal and distal costal sections are independent growth units, and are regulated by different genes during their formation. Díaz-Benjumea and Cohen (1993) demonstrated how the wing margin is formed at the dorsal/ventral boundary. They showed how juxtaposition of different lineages of cells induces formation of the distal costal section. The molecular basis underlying formation of the proximal costal section is not understood.

Which is L1?

From the first branch of radius vein to the base of 4th costal section there exists a continuous conduit; this is clear from the fact that a bundle of axons passes through it (Murray 1984). However, there is still considerable debate over a simple question: Should this conduit be considered as one vein or two veins? Traditional *Drosophila* geneticists tend to take the distal costal section as part of L1 (Garcia-Bellido and de Celis 1992). However, entomologists suggest that this is a mistake (Stark *et al*. 1999), In the opinion of entomologists, L1 should be the vein running from the origin of the radius to the very beginning of 2nd costal section, and 2nd costal section should be taken as part of the costa. de Celis (2003) obviously took this opinion, and redefined L1 as an incomplete vein which does not reach the wing margin. My present observations seem to favour the viewpoint of the traditional *Drosophila* geneticists. It seems that at least part of the distal costal section is formed by such a two-step process: first, the radius vein extends along the edge of marginal cell, and then the other structures, such as bristles and other sensory organs, develop independently.

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