Review TRENDS in Genetics Vol.23 No.7

The stars and stripes of animal bodies: evolution of regulatory elements mediating pigment and bristle patterns in *Drosophila*

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Evolution has generated enormous morphological diversity in animals and one of the genetic processes that might have contributed to this is evolution of the *cis*-regulatory sequences responsible for the temporal and spatial expression of genes regulating embryonic development. This could be particularly relevant to pleiotropic genes with multiple independently acting regulatory modules. Loss or gain of modules enables altered expression without loss of other functions. Here I focus on recent studies correlating differences in morphological traits between related species of *Drosophila* to changes in *cis*-regulatory sequences. They show that ancestral regulatory modules have evolved to mediate different transcriptional outputs and suggest that evolution of *cis*-regulatory sequences might reflect a general mechanism driving evolutionary change.

Mechanisms for generating morphological diversity

Evolution has generated an enormous variety of sizes, shapes and colours of animals. Morphological diversity can be traced back to the activity of genes regulating embryonic development, and several different genetic mechanisms might have contributed to this variation in gene activity. Modification of protein specificity has been shown to underlie some differences in morphology but, because of the need to maintain the triplet code and to retain protein function, coding sequences are subject to considerable constraint. Furthermore, it is now clear that most animals share a similar toolkit of proteins with which to construct their bodies, and evolution of this would be insufficient to account for all of the diversity [1]. Another process implicated in morphological change is gene duplication. Duplication of HOM(Hox) genes, for example, is associated with variation in body plans [2]. Evolution of expression or activity of microRNAs might also contribute [3]. Changes in the spatial or temporal expression of regulatory genes would have important morphological consequences, particularly if their products regulate several downstream targets. Indeed, examination of species-specific allelic expression of 29 genes in hybrids between *Drosophila melanogaster* and *D. simulans* revealed that about half of them had evolved as a result of *trans* effects of this sort [4]. Changes in *cis*, that is, altered gene expression as a result of evolution of *cis*-regulatory sequences, have been invoked recently as one likely mechanism driving morphological change [1,5–8]. Regulatory sequences tolerate considerable variation in number and topology of binding sites, are therefore subject to fewer constraints than coding sequences and might evolve more easily [9]. Furthermore, it is now known that many eukaryote genes are pleiotropic (controlling several distinct and seemingly unrelated phenotypic effects) with modular promoters and independently acting *cis*-regulatory sequences to mediate expression at different times and places [7,9,10]. This important feature enables the loss and gain of expression domains without interfering with other functions of a gene.

Quantitative genetic analysis – the study of the number of genes involved, their relative contributions and the interactions between them – has been applied to divergent morphological traits but is limited to species that interbreed. By contrast, comparison of embryonic development between different species has uncovered numerous examples of changes in gene expression that correlate with different morphologies. However, these mostly concern slowly evolving characters between widely divergent species, such as limb loss in snakes and birds, making investigation of how such changes came about all but impossible. A possible compromise is to examine rapidly evolving traits between related species (or within species) displaying small differences in morphology [5,6]. This requires the choice of a tractable trait whose underlying genetic basis is well understood in a model species. *D. melanogaster* is a well-studied organism and can be compared with other *Drosophila* species with divergent features within a defined phylogenetic grouping. I shall review recent papers describing changes in the responses of the *yellow* and *achaete-scute* genes in different species, to the networks of genes regulating development of the wing and thorax that are conserved between species. Gene expression patterns correlate with changes in pigmentation and bristle positions and suggest that evolution of *cis*-regulation could be one mechanism driving morphological change.

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Available online 11 May 2007.

 0168-9525/$ – see front matter doi:10.1016/j.tig.2007.04.006

Evolution of *cis*-regulatory sequences at the *yellow* locus underlie variation in pigment patterns

The wing bears specific patterns of sensory organs, important for flight, and veins that act as conduits for axons from the sensory organs and also restrict flexibility of the wing. The wing is patterned by two diffusible proteins, Decapentaplegic (DPP) and Wingless (WG), whose expression domains are established in early wing development as a result of inductive interactions between the different compartments of the wing imaginal disc (see Box 1). The gene *engrailed* (*en*) mediates the difference between anterior and posterior compartments, and this role is conserved throughout the arthropods [11]. The positioning of all morphological features, including the veins, can be traced back to the activity of Engrailed (EN), Apterous (AP), DPP, WG and their targets. The prepattern of proteins governing wing development is likely to be well conserved across the Diptera (the true flies) [12]. Expression of the main patterning genes *en*, *ap*, *wg* and *vg* is conserved in the wings of the butterfly *Precis coenia*, suggesting, because Lepidoptera and Diptera diverged ~200 Mya, an ancient origin for this regulatory network [13].

Late in the development of insects, melanin precursors are secreted by epidermal cells into the extracellular matrix, where they oxidize to melanin and are incorporated into the cuticle [14]. Many species of Drosophilidae have intricate species-specific patterns of darkly pigmented and light-coloured wing regions. The wing veins are instrumental in the establishment of the pattern of melanin on the wing because the melanin precursors diffuse from the vein haemolymph (Box 1). Therefore even species with no wing melanization, such as *D. melanogaster*, possess a basic pattern for melanization resulting from the preformed pattern of veins [15]. The pattern of veins is widely conserved throughout the Drosophilidae, and wing melanin patterns are likely to be vein-dependent in all drosophilids [15]. Enzymes producing the melanin precursors are not rate limiting [16]. In *D. melanogaster* the pattern of pigmentation is the result of the restricted transcription of two enzymes encoded by *yellow* (*y*) and *ebony* (*e*) [17,18]. The *yellow* gene is required for black

Box 1. Selector genes and organizers regulating wing development

Patterning of the *Drosophila* wing is initiated through the selector gene *engrailed*, whose expression in the posterior (P) compartment of the wing imaginal disc (pale blue zone in Figure I) is inherited from the embryo (reviewed in Refs [56,57]). The anterior–posterior compartment border is the source of a signal that patterns the *Drosophila* wing along the anterior–posterior (A–P) axis. Engrailed, a homeodomain-containing transcription factor, regulates expression of the secreted protein Hedgehog (HH), whose limited diffusion induces the expression of DPP – a ligand of transforming growth factor-β (TGF-β) type – in a small band of cells of the anterior (A) compartment immediately adjacent to the P cells (maroon band in Figure I). Only A cells are programmed to respond to HH through their expression of Ci, a zinc finger protein required for reception of the HH signal. This restricts production of the DPP signal to the A–P boundary. The concentration of DPP forms a gradient throughout the wing decreasing from the boundary. The DPP morphogen can elicit a precise threshold response by activating target genes at specific concentrations. Its activity is refined by an opposing gradient of Brinker. The *brinker* gene is repressed by DPP but in turn functions to repress DPP target genes. Different target genes are activated at different threshold concentrations of DPP and WG throughout the wing. DPP targets include *vestigial*, a gene promoting wing identity, *optomotor-blind*, a gene required for distal wing development, and *spalt*, a gene required for wing vein patterning (for review see Ref. [58]). The wing is also divided into dorsal (D) and ventral (V) compartments by another selector gene, *apterous* (*ap*), whose expression is restricted to the D compartment (dark blue in Figure I). A second organizer, Wingless, is set up by inductive interactions between the cells of the D and V compartments (yellow in Figure I). Veins are positioned by the gradients of Hedgehog and DPP signalling, which set up a pattern of epidermal growth factor (EGF) signalling at the positions of each vein (pink lines in Figure II), and expression of Blistered (also called serum response factor) in the intervein regions (Figure II). For a review of the genetic control of vein development see Ref. [59].

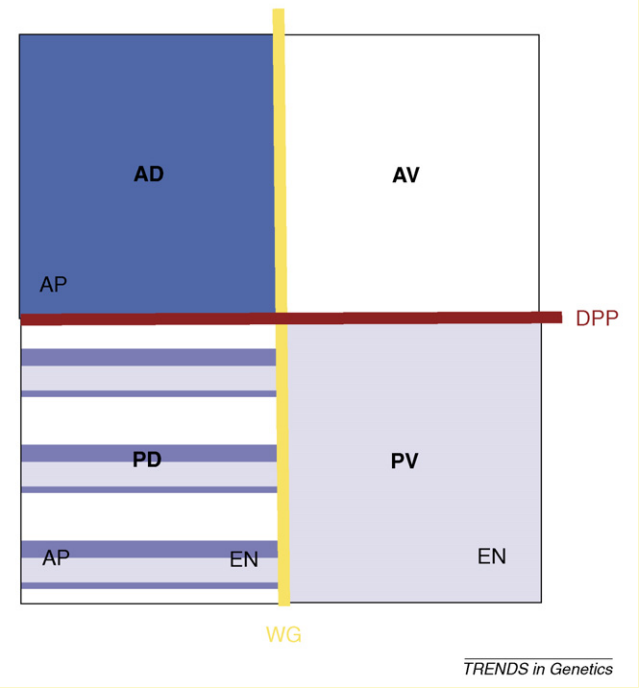

Figure I. Key selector gene products responsible for patterning the *Drosophila* wing. AD, anterior dorsal; AV, anterior ventral; PD, posterior dorsal; PV, posterior ventral.

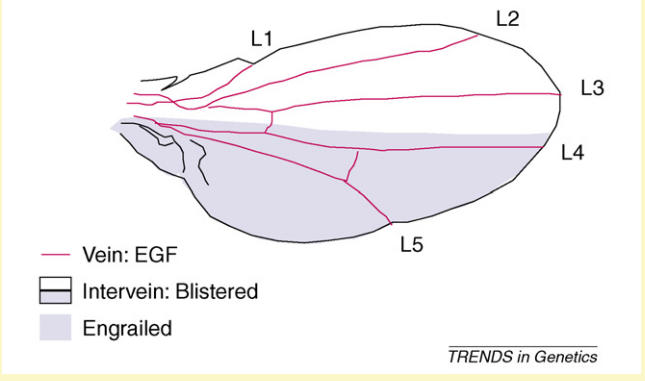

Figure II. Domains of genetic activity and developmental cues involved in positioning of veins (L1–L5) in the *Drosophila* wing. Engrailed is restricted to the P compartment (pale blue), EGF is expressed in the veins (pink) and Blistered is found in the interveins (white).

melanin (dopa-melanin) and its expression correlates with black pigmentation. Ebony converts dopamine to *N*-β-alanyl-dopamine synthetase (NBAD), which is oxidized to produce tan pigment. The *ebony* gene is not spatially regulated but expression levels vary.

Two recent papers show that variation in wing pigmentation between species correlates with changes in the response of the *yellow* (*y*) gene in different species to the conserved gene network [19,20]. The *y* locus contains several independently acting *cis*-regulatory elements that regulate expression in different parts of the body (Figure 1a), reviewed in Ref. [17]. A dark spot of pigment is found on the anterior distal wing of *D. biarmipes* and *D. elegans*, both members of the *melanogaster* group of drosophilids that diverged ~15 Mya [20] (Figure 1b). Phylogenetic analysis indicates that the common ancestor of this group was unspotted, so the spot is a derived character; it was secondarily lost in *D. melanogaster* [20]. The spot is preceded by expression of *y* (Figure 1d). A specific, regulatory 675-bp sequence, the 'spot' element, recapitulates most of the expression of *y* in the spot region: when coupled to a reporter gene encoding green fluorescent protein it drives expression in a similar spot region in transgenic *D. melanogaster* [19,20]. Sequences required for activation of *y* in the spot region of *D. biarmipes* were localized to a small region of ~28 bp [19]. In addition to sequences for activation, the 'spot' element of *D. biarmipes* contains two binding sites for EN. These are not present in the orthologous *cis*-regulatory region of the *D. melanogaster y* gene. Loss of these binding sites causes the expression of *y* to expand into the posterior compartment [19]. The 'spot' element of *D. biarmipes* and *D. elegans* can therefore respond to proteins of the conserved ancestral wing circuitry present in *D. melanogaster* and has evolved to acquire binding sites for these. It also seems that more than one change has occurred during the evolution of this element.

The use of chimeric transgenes bearing sequences of the 'spot' element of *D. elegans* and of the orthologous region of the closely related *D. gunungcola*, a species with no spot (Figure 1c), enabled Prud'homme *et al.* [20] to pinpoint a region of ten divergent nucleotides involved in spot

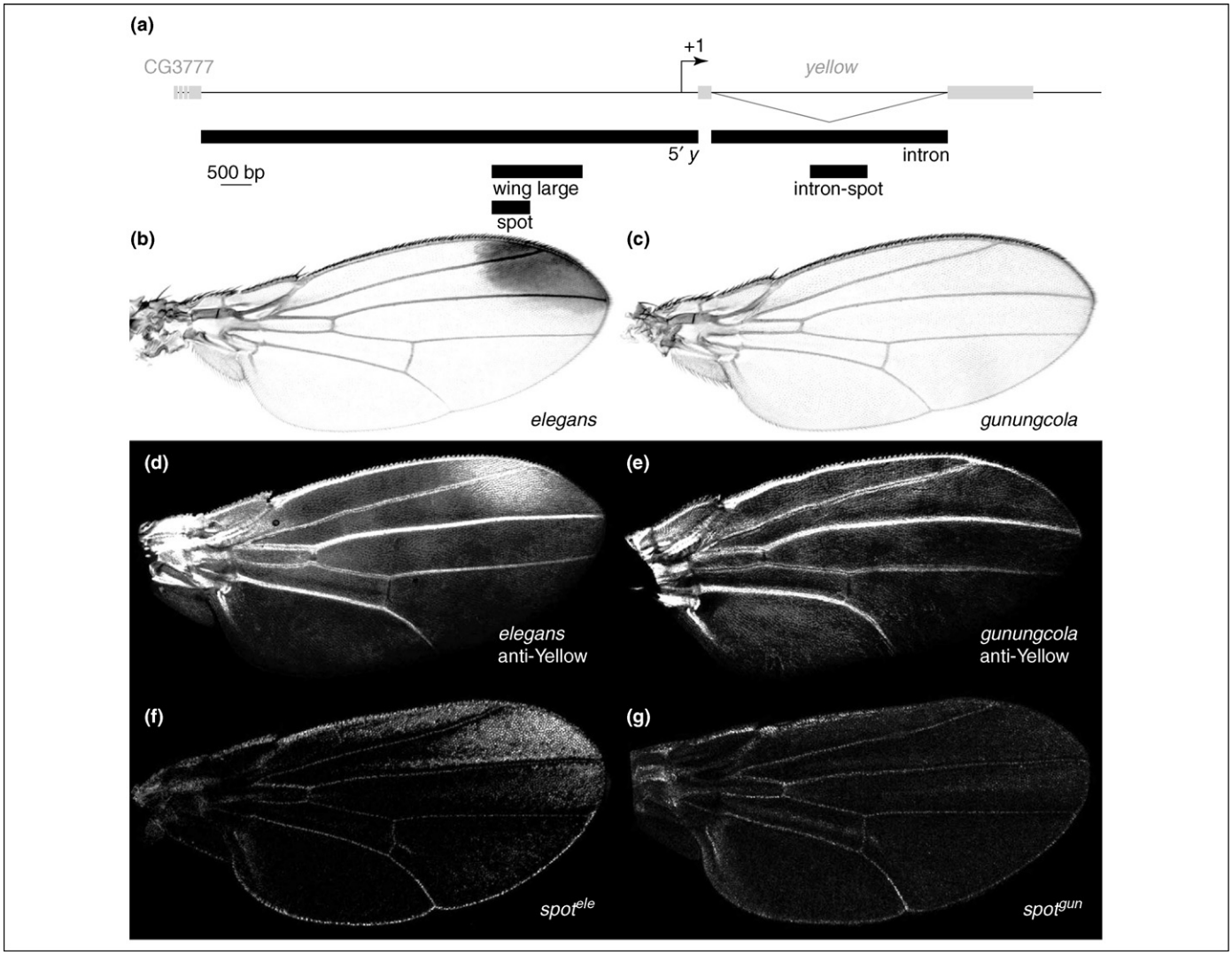

Figure 1. Sequence evolution of the 'spot' regulatory element of the *yellow* gene is responsible for the loss of the pigment spot in *Drosophila gunungcola*. **(a)** Diagram of the *yellow* locus indicating the location of the regulatory elements 'spot' and 'intron-spot'. **(b,c)** Adult male wings of *D. elegans* and *D. gunungcola*. Only *D. elegans* bears a pigment spot. **(d,e)** The pigment spot of *D. elegans* is preceded by the expression of *yellow*, which is expressed in the veins of both species. **(f,g)** The 'spot' element of *D. elegans* drives reporter gene expression in the spot region. The orthologous region from *D. gunungcola* no longer has this activity. Reproduced, with permission, from Ref. [20].

formation. Removal of these nucleotides from the 'spot' element of *D. elegans* renders the sequence inactive, whereas their addition to the orthologous region of *D. gunungcola* confers the ability to direct expression in the region of the pigment spot, when assayed in *D. melanogaster* hosts. This indicates that one of the transcription factors involved in the regulation of *y* in the spot region can activate transcription through binding to this sequence and that variation of only a few nucleotides could cause loss of the spot.

The authors determined that the 'spot' sequence is embedded in a larger region ('wings large', Figure 1) that mediates expression over most of the wing. An orthologous region of the *y* gene of *D. pseudoobscura*, an outgroup species devoid of a spot, drives ubiquitous wing expression [19]. They suggest that the 'spot' sequence arose from a pre-existing element responsible for expression in the wing that, by subfunctionalization (divergence after duplication such that each copy carries out an individual function), gave rise to independent wing and spot elements.

The authors also examined the role of *y* in spot formation in *D. tristis*, a species in the *obscura* group that has independently gained a similar spot of pigment on the wing [20] (Figure 2). The *yellow* gene is also expressed in the spot region in this species, but, remarkably, expression is regulated by a different *cis*-regulatory element, situated in the intron (known as 'intron-spot') (Figure 1a). The lack of any sequence similarity between this element and the 'spot' element of *D. biarmipes* and *D. gunungcola* is consistent with an independent origin of the two. The element driving *y* expression in *D. tristis* is closely associated with sequences that mediate expression in the wing veins. The orthologous region from *D. guanche*, another species from the *obscura* group but devoid of a wing spot, gives expression in the wing veins. This suggests that the 'intron spot' element of *D. tristis* evolved through the co-option of an ancestral element for vein expression. More than one molecular path has thus evolved to mediate expression of *y* in the wing spot but both cases involve an ancestral regulatory element for expression in the wing.

Evolution of *cis*-regulatory sequences at the *sc* locus underlie variation in bristle patterns

Like the wing, development of the thorax of *Drosophila* depends on a conserved gene regulatory network. Decapentaplegic, WG and AP, together with the selector genes *pannier* (*pnr*) and *iroquois* (*iro*) pattern the dorsal thorax of *Drosophila*, the notum (see Box 2). As with the wing, all aspects of the morphology of the notum – tendons, bristles and pigmentation – depend on these genes. The thorax of dipterans is enlarged to house the powerful indirect flight muscles. Flight in dipteran flies is specialized, so some of the genes patterning the thorax might not be conserved in other insects. However, the pattern of flight muscles and their attachment sites on the cuticle is greatly conserved throughout the Diptera [21,22]. Expression of *stripe* preceding tendon development is conserved between *Anopheles gambiae* and *D. melanogaster*, two species with a divergence time of ~200 My. Likewise expression of *pnr* is restricted to the medial thorax of both species [23]. *Calliphora vicina* and *D. melanogaster*, two species with a divergence time of ~100 My, display similar patterns of expression of *pnr*, *iro*, *wg* and *u-shaped* (*ush*) [24]. This suggests that the thoracic gene regulatory network is conserved within the cyclorraphous Diptera (species that pupate inside a modified larval skin: the puparium).

Mechanosensory bristles form as a result of the activity of the proneural genes *achaete* (*ac*) and *scute* (*sc*) whose expression on the notum is activated by PNR and IRO (Box 2). *achaete* and *sc* encode transcription factors of the basic helix–loop–helix family that, together with the protein Daughterless, provide neural potential to cells [25–28]. The large mechanosensory bristles, macrochaetes, are arranged into species-specific patterns on the notum that,

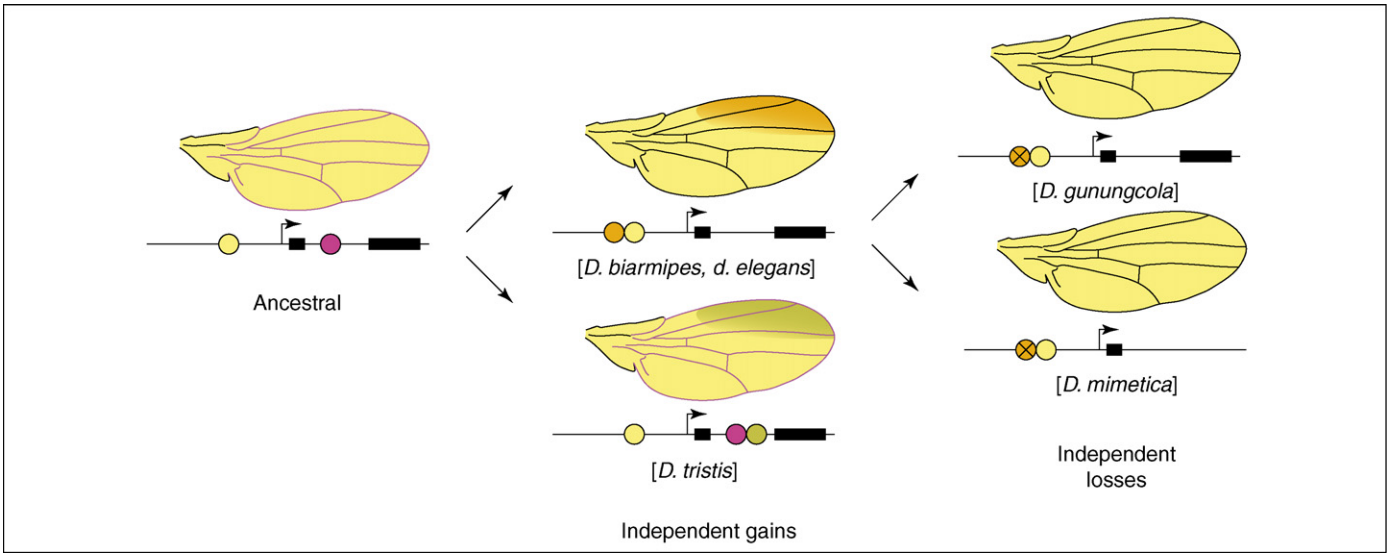

Figure 2. The convergent evolution of the pigment spot in *Drosophila tristis* has involved a *cis*-regulatory element of the *yellow* gene, 'intron spot', which is different from that present in *D. biarmipes* and *D. elegans*, 'spot' (shown in Figure 1). The common ancestor of *D. biarmipes, D. elegans* and *D. tristis* did not have a wing spot. Expression of *yellow* in the spot region evolved twice: once in the *D. biarmipes-D. elegans* lineage and once in the *D. tristis* lineage, through the co-option of two different pre-existing *cis*-regulatory elements (the 'spot' element, symbolized by yellow to orange circles, and the 'intron-spot' element symbolized by pink to green circles). The spot was lost independently in *D. gunungcola* and *D. mimetica* through inactivation of the 'spot' element. Reproduced, with permission, from Ref. [20].

Box 2. Selector genes and organizers regulating thorax development

The thorax develops from part of the wing disc and is also divided into A, P, D and V compartments. The notum, shown in Figure I, is the dorsal, anterior component of the thorax. It is patterned by DPP (maroon zone in Figure I) and the activity of *ap*. Other selector genes are required. Early in development the genes of the *iroquois* (*iro*) complex are expressed over the notum, but later the selector gene *pannier* (*pnr*) is activated by DPP over the medial half of the notum (dark-brown zone in Figure I), restricting *iro* to the lateral half (pale-green zone) [60–63]. The *pannier* gene encodes a transcription factor of the GATA family and the genes of the *iro* complex encode homeodomain-containing proteins [64,65]. Pannier activates target genes when not associated with U-shaped, the product of *ush*, a gene expressed in a smaller domain than that of *pnr* and also regulated by DPP [60,66]. Together *pnr* and *iro* pattern most of the notum. A further genetic subdivision is afforded by *eyegone* (*eyg*), a Pax-homeobox-containing gene whose expression is restricted to the anterior central region of the disc (orange and bright-green zones) through the antagonistic regulatory activities of PNR and IRO [67]. The combined activities of *pnr*, *iro* and *eyg* subdivide the notum into four distinct genetic subdomains. A further prepattern gene, *islet*, is expressed in the posterior notum [42].

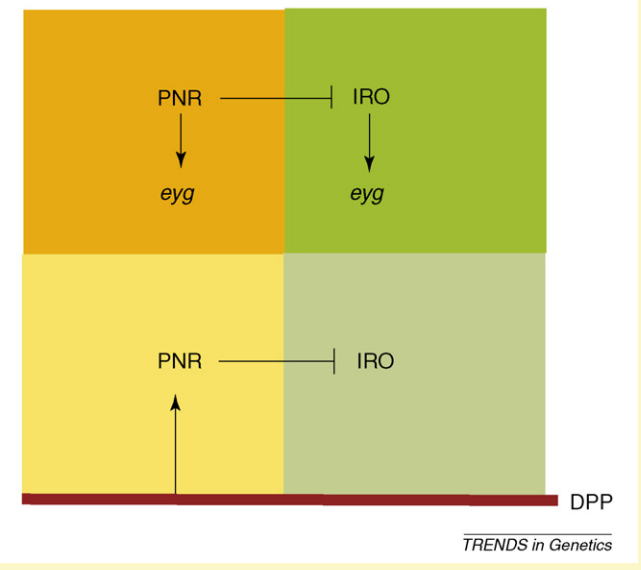

Figure I. Principal selector genes and their products responsible for subdivision of the notum into four domains during development of the thorax in *Drosophila*.

Development of bristle precursors depends on expression of the *achaete-scute* genes [39]. They are activated by Pannier and Iroquois and are also dependent on Wingless for their expression [40,65,68]. The *wingless* gene is activated by Pannier where the levels of U-shaped, a repressor of Pannier, are low (yellow zone in Figure II) [41,66]. The indirect flight muscles that lie just below the surface of the notum attach to the cuticle through tendons whose precursors are selected from the disc epithelium [69–72]. Tendon development depends on expression of *stripe*, a gene encoding a transcription factor with zinc finger motifs [69,71]. The *stripe* gene is activated by Apterous and Pannier and repressed by Wingless (violet zone in Figure II) [73,74]. The formation of bristles is antagonized by the activity of Stripe [22]. The domains of expression of *achaete-scute* and *stripe* do not overlap thus leading to the spatial segregation of tendons and bristles [22,24].

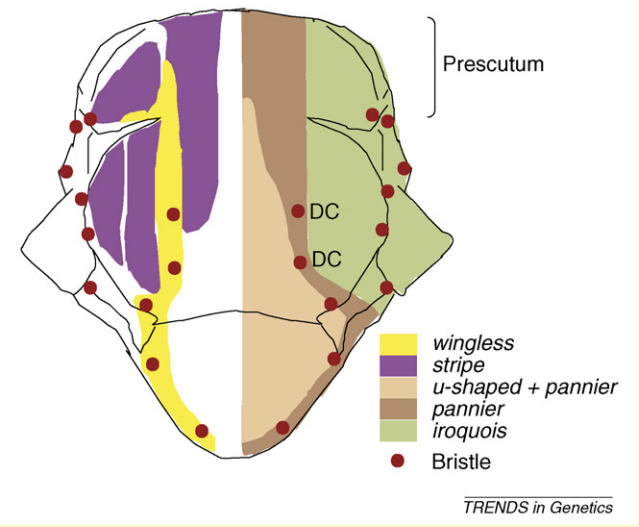

Figure II. Domains of expression of genes regulating the development of bristles in the epithelium of the *Drosophila* thorax. The diagram is of an adult thorax but indicates the domains of expression of genes during development of the thoracic disc. All genes are expressed on both hemithoraces (each from one imaginal disc) but for ease of description some are shown on the left and others on the right. The expression of *stripe* (violet) and *wingless* (yellow) is shown on the left side of the thorax. Tendons arise from the domains of *stripe* expression. The expression of *pannier* (brown and pale brown), *u-shaped* (pale brown) and the genes of the *iroquois* complex (green) are shown on the right side. The prescutum is the anterior part of the scutum, which in *D. melanogaster* does not bear any dorsocentral (DC) bristles.

particularly in cyclorraphous flies, are often present in an invariant pattern [29,30]. Amongst the cyclorraphous flies examined so far there is a correlation between the spatial expression of *sc* and bristle patterns. The *scute* gene is expressed in discrete domains on the thorax, either in stripes or in small clusters of cells called proneural clusters, at the sites of formation of the future bristle precursors (Figure 3a) [31–34].

Bristle patterns on the notum do not evolve as fast as the pigment spot on the wing: the position of some bristles is remarkably constant throughout the Drosophilidae [35–37]. Most of the ~4000 species of the Drosophilidae have two dorsocentral (DC) bristles [36,37] (Box 2). The *ac-sc* genes are part of a gene complex bearing numerous *cis*-regulatory elements for expression in different proneural clusters of cells on the notum (Figure 3c) [38,39]. In *D. melanogaster* the DC bristles arise from the DC cluster of *ac-sc* expression [31,32]. A specific sequence, the DC element (DCE; shown in pink on Figure 3c), mediates expression of *ac-sc* in this cluster [40]. The DCE binds to PNR through specific GATA binding sites [40]. The *pannier* gene is broadly expressed (Box 2). The activity of PNR on this regulatory sequence is restricted dorsally through the repressor activity of USH and posteriorly through the antagonistic activity of Islet [41,42] (Box 2). It is not known what restricts expression anteriorly. A transgene bearing the DCE and *sc* can rescue the two DC bristles in an animal devoid of endogenous *ac-sc* expression [43]. An orthologous DCE from *D. virilis*, a distantly related drosophilid similarly bearing two DC bristles, can also rescue two DC bristles in transgenic *D. melanogaster* hosts when coupled with *sc* [44].

D. quadrilineata, a species belonging to the *immigrans* subgroup, bears four instead of two DC bristles (Figure 4). The four bristles form a row, with the additional bristles located anteriorly. These additional, anterior bristles represent a trait newly acquired in *D. quadrilineata*,

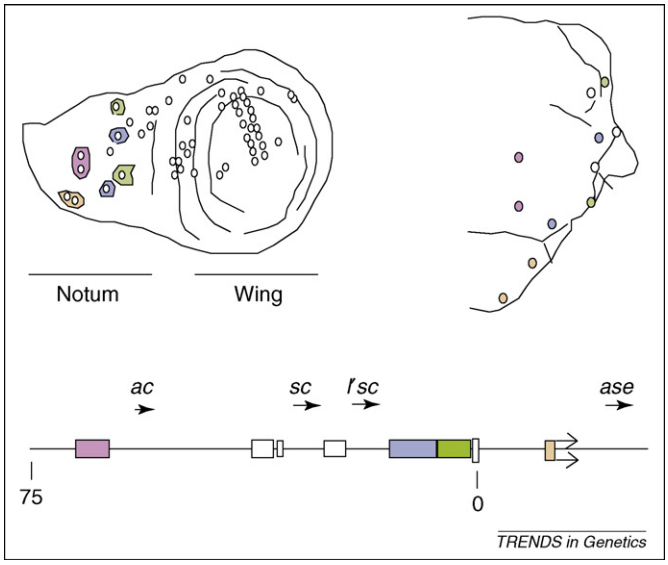

Figure 3. The *achaete-scute* complex bears many *cis*-regulatory elements driving expression in different proneural clusters. **(a)** The positions of the proneural clusters of *achaete-scute* expression in the imaginal disc. **(b)** The positions of the bristles on the notum arising from each cluster. Each proneural cluster gives rise to one or two bristle precursors on the notum. **(c)** The *achaete-scute* complex showing some of the independently acting *cis*-regulatory elements that mediate expression of *achaete* and *scute* in different proneural clusters. Colours for regulatory element, proneural clusters and bristles correspond. The dorsocentral regulatory element, proneural cluster and bristles are shown in pink. ***l'sc, lethal of scute; ase, asense***. Adapted, with permission, from Ref. [38].

Figure 4. The dorsocentral regulatory elements of *Drosophila quadrilineata* and *D. melanogaster* mediate different phenotypic outputs. **(a,b)** The thoraces of *D. melanogaster* and *D. quadrilineata*, respectively. *D. melanogaster* has two and *D. quadrilineata* four dorsocentral bristles (white arrowheads). **(c)** When the dorsocentral regulatory element of *D. melanogaster* is coupled to *scute* it mediates the development of two dorsocentral bristles in transgenic *D. melanogaster* hosts. **(d)** When the dorsocentral regulatory element of *D. quadrilineata* is coupled to *scute* it mediates the development of four dorsocentral bristles in transgenic *D. melanogaster* hosts. Reproduced, with permission, from Ref. [44].

because it is thought that the ancestor to the Drosophilidae had only two, posterior DC bristles [29,36,37]. An orthologous DCE from *D. quadrilineata* coupled with *sc* can mediate the formation of a row of four DC bristles in *D. melanogaster*, instead of the two usually present in this species [44] (Figure 4). The *trans*-regulatory proteins of *D. melanogaster* can recognize the *D. quadrilineata* sequence, which has therefore co-opted pre-existing parts of the conserved regulatory landscape. It is not yet known which region(s) of the DCE is responsible for this difference. *D. melanogaster* and *D. quadrilineata* are distantly related and their DCEs have undergone significant sequence turnover [44]. Analysis of species more closely related to *D. melanogaster* on the one hand, and to *D. quadrilineata* on the other, might display less sequence divergence and enable the determination of the region(s) of the DCE that mediate this difference.

Expression of *sc*, visualized by *in situ* hybridization, at the DC site in *D. quadrilineata* is less like a cluster and more like a streak, reflecting the extended row of bristles seen in this species. Consistent with this, the domain of expression mediated by the *D. quadrilineata* DCE in transgenic *D. melanogaster* also extends in a more anterior direction. The DCE of *D. quadrilineata* has therefore evolved to mediate *sc* expression in a modified domain leading to the formation of additional anterior DC bristles. Unfortunately the factor restricting activity of the DCE to posterior regions in *D. melanogaster* is unknown. However, the row of bristles is situated in a domain free of expression of *stripe*, devoid of tendons and where DC bristles are frequently located in species belonging to other families of cyclorraphous flies.

The *D. quadrilineata* DCE does not perfectly recapitulate the *D. quadrilineata* bristle pattern when expressed in *D. melanogaster*, in that the anterior bristles do not extend as far as the prescutum (Figure 4). Some changes in *trans*-acting factors or other regulatory regions of *sc* might therefore also have taken place.

Morphological evolution through changes in genes responding to a conserved prepattern

There are several features common to the evolution of the pigment and bristle patterns. Both *y* and *sc* respond to a conserved genetic prepattern made earlier in development. The genetic networks controlling these traits also regulate the development of most other morphological features of the wing or thorax. Any modification of the regulatory landscape would interfere simultaneously with all other aspects of the morphology that depend on it. Positions of veins are unchanged throughout the Drosophilidae and those of thoracic tendons throughout the Diptera. Modification of the selector genes and diffusible signals that pattern the wing and notum, or even of expression of later-acting genes required for positioning of veins or tendons, would be likely to impair flight. Modification of the *trans*-acting factors is therefore perhaps less likely in these cases. The *trans*-regulatory proteins of *D. melanogaster* recognize the regulatory sequences from the other *Drosophila* species so the propensity to make a wing spot or anterior DC bristles is present in the prepattern of *D. melanogaster*, which is itself devoid of these traits. Tinkering with the response to a conserved regulatory

landscape enables modification of only a localized aspect of the final pigment or bristle pattern. *Cis*-regulatory sequences of *y* and *sc* have thus evolved to respond differently to the prepattern. It seems that in both cases the changes have involved regulatory elements that were already present. Slight modification of pre-existing regulatory elements would involve fewer steps than the *de novo* generation of new ones [20]. The fact that evolution of different, but nevertheless pre-existing, regulatory elements of the *y* gene underlies a convergent change in gene expression in *D. tristis* and *D. elegans*, enables the hypothesis that this is a common evolutionary route.

Both of the examples discussed here involve traits that are elaborated late in development and both involve change in late-acting genes responding to a conserved prepattern. Does this mean that the number of target genes available for such change is small? The *yellow* and *e* genes both intervene at the last possible moment in the generation of pigmentation, when the veins are already in place, enabling melanin precursors to diffuse into the wing. The *yellow* gene has been the target for convergent evolution of expression in different species. A decrease in expression of *e* accompanies the increase in expression of *y* in wing spots, but it is not known how *e* is regulated. Of course more species need to be investigated but it remains possible that *e* does not have a modular promoter that would readily enable localized changes in gene expression. It is not yet known whether differences in *cis*-regulatory modules at the *ac-sc* gene complex underlie different bristle patterns in species other than *D. quadrilineata* and *D. melanogaster*. The *scute* gene is expressed earlier in development than *y* but there is only a narrow time window, restricted to the latter half of the third larval instar (the last larval stage before pupariation) and the first hours after pupariation, within which the epithelium is competent to respond to SC to produce macrochaete precursors [45,46]. This might also limit the number of target genes available for the modification of bristle patterns. However, at least one documented difference between species has another underlying cause. The bristle patterns of two species of Calliphoridae, *Calliphora vicina* and *Phormia terranovae*, arise from identical spatial expression patterns of *sc* but there is a temporal change, such that late expression of *sc* in *P. terranovae* misses the time window of competence for macrochaetes [47]. The genetic basis for this difference is unknown.

I have discussed two examples of evolution of expression of late-acting genes. Although modification of gene expression early in embryogenesis is expected to have a greater knock-on effect during subsequent development, this does not mean that all morphological variation results from changes in late events. Variation in adult morphology can be generated at diverse stages and through various means. For example, the same prepattern of regulatory proteins underlies development of both the haltere and the wing of *Drosophila*, but the haltere is considerably smaller in size. This difference results from the activity of the selector protein Ultrabithorax (UBX), which acts early in imaginal disc development. UBX causes elevated levels of the DPP receptor Thick-vein in the haltere disc; this traps DPP close to its source along the anterior–posterior boundary (see Box 1). Consequently fewer cells are exposed to the signal and this results in less growth [48]. Shifts in the timing of early developmental events can be important in driving evolutionary change, even in vertebrates [49,50]. For example, the *Hoxc8* early enhancer drives expression earlier in the paraxial mesoderm of the chick than in that of mouse and this results in more posterior expression. Consequently the chick has a shorter thoracic region. When transformed into the mouse the chick early enhancer drives more posterior expression [51].

Concluding remarks

Recent research reflects a growing interest in the evolution of *cis*-regulatory sequences as one means of effecting morphological change [51–55]. Regulatory sequences might be more amenable to evolutionary change than coding sequences. Any change that results in a gain of gene expression at a new location will result in an increase in complexity of the genome and of the morphology of a species. The results suggest that evolution of pre-existing regulatory elements could be a more common feature than the acquisition of new ones. It remains to be shown how the forces of selection act through small changes in regulatory sequences. Further investigation into other cases of morphological diversity between closely related species will hopefully ascertain whether the cases discussed here illustrate a general mechanism driving evolutionary change.

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