Two or Four Bristles: Functional Evolution of an Enhancer of scute in Drosophilidae

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Changes in cis-regulatory sequences are proposed to underlie much of morphological evolution. Yet, little is known about how such modifications translate into phenotypic differences. To address this problem, we focus on the dorsocentral bristles of Drosophilidae. In Drosophila melanogaster, development of these bristles depends on a cis-regulatory element, the dorsocentral enhancer, to activate scute in a cluster of cells from which two bristles on the posterior scutum arise. A few species however, such as D. quadrilineata, bear anterior dorsocentral bristles as well as posterior ones, a derived feature. This correlates with an anterior expansion of the scute expression domain. Here, we show that the D. quadrilineata enhancer has evolved, and is now active in more anterior regions. When used to rescue scute expression in transgenic D. melanogaster, the D. quadrilineata enhancer is able to induce anterior bristles. Importantly, these properties are not displayed by homologous enhancers from control species bearing only two posterior bristles. We also provide evidence that upstream regulation of the enhancer, by the GATA transcription factor Pannier, has been evolutionarily conserved. This work illustrates how, in the context of a conserved regulatory landscape, evolutionary tinkering of pre-existing enhancers can modify gene expression patterns and contribute to morphological diversification.

Introduction

Development is a complex process during which a plethora of regulatory mechanisms progressively unfold to ensure correct spatio-temporal expression of the genome. Morphological evolution occurs when mutations modifying these mechanisms produce a new phenotype, are tolerated, and fixed in a population [1,2]. Although phenotypic evolution could result from changes at many regulatory levels [3], there has been an ever-growing emphasis that modular cis-regulatory enhancers might be the major mutational targets [4–12]. A few studies correlate evolutionary changes in cis-regulatory regions to anatomical traits that differentiate species. Examples include linkage of the evolution of a Hoxc8 enhancer with changes in vertebrate axial identity, of a lin-48 enhancer with modifications of the nematode excretory duct, and of yellow enhancers with diversification of pigment patterns in Drosophilidae [13–17]. Functional tests of other cases would help decipher the complex relationship existing between evolution of cis-regulatory sequences and morphological evolution.

Within dipteran flies, bristle patterns are variable, but often stereotyped and species specific [18]. Indeed within the Schizophora, a monophyletic group of the Diptera, the large bristles, macrochaetes, can be homologised. The genetic basis of bristle development in Drosophila melanogaster has been intensively investigated over several decades [19,20]. The positions of bristles on the thorax depend on the precise spatial expression of the achaete-scute (ac-sc) genes, mediated by numerous independently acting enhancers [21,22]. Bristle patterns therefore offer an ideal paradigm to study evolutionary changes in gene regulation. Within the Schizophora, different patterns correlate with changes in sc expression [23–26]. Such changes could result from alterations in trans-acting factors or to cis-regulatory changes at the sc locus itself. The expression domains of the trans-acting factors are unchanged between Calliphora vicina and D. melanogaster suggesting conservation of a trans-regulatory gene network throughout the 100 million years (Myr) of evolution separating these two species [27]. This prompts investigation of cis-regulatory sequences.

The proneural ac-sc genes are expressed in small clusters of cells on the notum, proneural clusters, at the sites of formation of bristle precursors [28,29]. Expression is mediated by a number of enhancer modules of which one, the dorsocentral enhancer (DCE), has been characterized in some detail. It interacts with the GATA transcription factor Pannier (Pnr). Pnr binds this element and loss of function of pannier (pnr) abolishes sc expression at the dorsocentral (DC) site [21,30–32]. Furthermore, mutation of GATA sequences shown to bind Pnr causes a loss of enhancer activity when assayed in reporter gene constructs [30]. Here we have analyzed the activity of this enhancer from other...
species of Drosophilidae with variable numbers of dorsocentral bristles to examine its possible evolution. First, we show that, despite significant sequence turnover, its function has been retained between species with a divergence time of up to 60 Myr, that, like D. melanogaster, bear two DC bristles. Second, we demonstrate functional evolution of the enhancer in a species with four to five DC bristles.

**Results**

**A Secondary Gain of Anterior DC Bristles in D. quadrilineata**

The last common ancestor of the Schizophora is thought to have possessed four longitudinal rows of bristles extending from anterior to posterior on the scutum [18,33,34]. The Schizophora comprises the calyptrate and acalyptrate lineages, and many species of Calyptrata retain four complete bristle rows (Figure 1A). The Acalyptrata display reduced, derived patterns due to partial or complete loss of rows [18,33]. Bristle loss is most frequent on the anterior notum [35]. Absence of the anterior bristles of the DC row, particularly those situated in the prescutum anterior to the transverse suture is an apomorphic character found in many Acalyptrata [18,35]. Indeed the presence of only two, posteriorly situated DC bristles is a plesiomorphic feature of the family Drosophilidae [36]. Thus many extant and extinct Drosophila species display two posterior DC bristles at stereotyped locations (Figure 1) [35–37]. This is the case for D. virilis and D. melanogaster, for example, separated by 60 Myr of independent evolution (Figure 1A, 1B, and 1D). A few Drosophila species, like D. quadrilineata, display anterior DC bristles on the scutum as well as on the prescutum, thereby mimicking the ancestral situation of the Schizophora (Figure 1A and 1C). These are thought to have arisen by secondary gain. D. quadrilineata belongs to the immigrans subgroup [38,39], implying that it is more closely related to D. virilis than it is to D. melanogaster [40].

The DCE from D. quadrilineata Allows Development of Four DC Bristles in D. melanogaster

To examine whether the mutation(s) responsible for the re-emergence of anterior DC bristles reside in the sequence of the DCE of D. quadrilineata (Dq-DCE), we constructed sc minigenes. It has previously been demonstrated that, when used to drive the expression of a sc minigene in an ac-sc null mutant background, the DCE of D. melanogaster (Dm-DCE) is sufficient to rescue the formation of the two posterior DC bristles [41]. Following a similar strategy, we compared orthologous DCEs from species with two DC bristles, D. melanogaster and D. virilis (Dv-DCE), or four DC bristles, D. quadrilineata. We subcloned the enhancers upstream of a sc minigene, and generated four independent insertion lines for each transgene in D. melanogaster hosts. When assayed in a wild-type background, all lines bearing Dm-DCE-sc and Dv-DCE-sc display an unchanged pattern of two posterior DC bristles (Figure 1E and 1G). Remarkably, one third of the flies bearing Dq-DCE-sc (four independent lines were examined) exhibit a row of four DC bristles that include bristles in a more anterior location (Figure 1F), thereby mimicking the phenotype of D. quadrilineata itself (Figure 1C).

To rigorously compare the ability of homologous DCEs to rescue DC bristle formation in D. melanogaster hosts, we took advantage of Df(1)91B, a 45-kilobase (kb) deletion that removes ac and the DCE, but leaves sc and most other regulatory elements intact [42]. This viable recessive mutant, hereafter referred to as ac wind the gap (ac wtg), exhibits a dramatic decrease in DC bristles: 80% of the scored heminotures are devoid of them entirely (Figure 2A). A single copy of the Dm-DCE-sc or Dv-DCE-sc minigenes rescues the two posterior DC...
bristles characteristic of both *D. melanogaster* and *D. virilis* in nearly all flies (Figure 2A). A single copy of *Dq*-DCE-sc also rescues DC bristles: in a little less than half the cases, there are two bristles, but in the others, there are three to five bristles (Figures 2A and S1). Note that some *D. quadrilineata* flies bear five DC bristles. Despite the expected variability between different insertion lines, the weakest *Dq*-DCE-sc strain still rescues more bristles than the *Dm*-DCE-sc or *Dv*-DCE-sc controls (Figure S1). Interestingly, the bristles are usually aligned, and extra bristles are mainly situated at more anterior locations on the scutum, sometimes at the level of the transverse suture (white arrowheads in Figure 2A). Bristles anterior to the transverse suture were not observed. Hence, the *Dq*-DCE-sc minigene is sufficient to confer on *D. melanogaster*, at least partially, a phenotype characteristic of *D. quadrilineata*.

The *Dq*-DCE Drives Expression in a Longitudinal Stripe Extending Anteriorly

To understand how homologous DCEs promote the emergence of different bristle patterns, we compared the expression of the proneural gene *sc* by in situ hybridisation in third larval instar wing discs of wild-type flies and transgenic *acmtg* mutants (Figure 2B–2G). In *D. melanogaster*, the proneural cluster that gives rise to the DC bristles is oval in shape with its long axis orientated roughly parallel to the antero-posterior axis (Figure 2B). By contrast, in *D. quadrilineata*, *sc* is expressed in the region corresponding to the site of origin of the DC bristles in a streak of cells that is elongated anteriorly (Figure 2E). This elongated proneural cluster does not extend in a straight line, but makes a sharp turn to become parallel to the midline (Figure 2E). As expected, *sc* expression is undetectable in the DC cluster of hemizygous *acmtg* wing discs (Figure 2C). It is expressed in the other proneural clusters, such as the SC and PA, although at weaker levels than the wild type (Figure 2B and 2C). One copy of the *Dm*-DCE-sc or *Dv*-DCE-sc minigene restores the expression of *sc* in an oval-shaped cluster in *acmtg* mutants (Figure 2D and 2G). The cluster rescued by the *Dq*-DCE-sc transgene, however, displays *sc* expression in an elongated streak, extending anteriorly and following the midline of the disc. This is similar to endogenous *sc* expression in *D. quadrilineata* wing discs (compare Figure 2E with 2F). Our results reveal that functional changes within the *Dq*-DCE are sufficient to confer upon *D. melanogaster*, a bristle pattern typical of *D. quadrilineata* resulting from a proneural cluster of elongated shape. Importantly, these properties are unique to the *Dq*-DCE because they are not displayed by the controls *Dm*-DCE and *Dv*-DCE.

Pairwise Comparison of the Activity of Orthologous DCEs

Although the in situ hybridisation for *sc* revealed functional divergence between homologous DCEs (Figure 2), only double stainings performed at cellular resolution can provide an accurate comparison of enhancer activity. It has previously been shown that the *Dm*-DCE drives the expression of a cytoplasmic form of β-Gal in cells of the DC proneural cluster [30]. We have used this *lauZ* reporter line as an internal reference, and compared it to other DCEs driving the expression of a nuclear form of GFP. In addition to *Dq*-DCE and *Dv*-DCE, we have included the DCE from *D. eugnathi* (*De*-DCE), whose phylogenetic position is closer to *D. melanogaster* (Figure 1) [40,43].

We first verified that two independent transgenes of the *Dm*-DCE, driving expression of β-Gal or GFP, are indeed active in precisely the same cells of the disc (Figure 3A). Expression driven by the orthologous DCEs overlaps in each case with the endogenous DC cluster, but the expression domains differ in detail. The pattern of GFP driven by the *Dq*-DCE extends significantly farther anteriorly than that of the *Dm*-DCE (arrow in Figure 3B). The *Dv*-DCE drives expression in a larger cluster of cells that overlaps only partly with the endogenous one: it is moderately displaced dorsally (arrowhead in Figure 3C) and anteriorly (arrow in Figure 3C). The *Dq*-DCE drives expression in a completely overlapping domain that is a little broader than the endogenous one and extends slightly anteriorly (arrow in Figure 3D).

Although the *Dv*-DCE drives the expression of a reporter gene in cells located more anteriorly than the endogenous DC cluster (Figure 3C), it is unable to induce the formation of anterior DC bristles (Figure 2A). To examine this in more detail, we compared the anterior limit of expression of the reporter genes with respect to an independent spatial reference. We focused on *stripe* (*sr*), a gene expressed in four distinct domains in third larval instar wing discs from which tendon precursor cells are selected [44]. These domains are adjacent to the proneural clusters of *sc* expression [34]. The dorsoventral muscles 2 and 3 (DVM) tendons extend along the anterior–posterior axis, stopping just below the transverse suture (Figure 3E). The anterior limit of the expression domain of *sr* corresponding to the DVM precursors thus provides a sharp, reliable spatial landmark (arrows in Figure 3E–3H). We simultaneously compared *sr* expression with the activity of the *Dm*-DCE and *Dq*-DCE (Figure 3F) or of the *Dv*-DCE and *Dv*-DCE (Figure 3G). All three enhancers mediate expression in a cluster of cells abutting the dorsal aspect of the DVM st expression domain (Figure 3F and 3G). Activity of the *Dv*-DCE spreads diffusely in a dorso-anterior direction (Figure 3G), whereas the *Dq*-DCE is active in an elongated cluster of cells that bends parallel to the DVM st expression domain and to the dorsal midline of the disc (Figure 3F). The anterior limit of expression mediated by the *Dm*-DCE and the *Dv*-DCE in most of the cases analyzed, ends at a position posterior to the anterior limit of *sr* expression (Figure 3H and 3I). The *Dq*-DCE, on the other hand, reproducibly mediates GFP expression anterior to the anterior-most limit of *sr* expression (Figure 3H and 3I). Thus, the *Dq*-DCE drives expression more anteriorly than the *Dv*-DCE, and it is this feature that allows the *Dq*-DCE to induce the formation of anterior DC bristles up to the level of the suture.

The Response of Orthologous Enhancers to Variations in *pnr* Activity

Unfortunately, to date, the mechanism responsible for restricting the activity of the DC enhancer in the anterior direction has not been discovered. However, the direct input of Pnr and U-shaped (Ush), essential for the correct activity of the *Dm*-DCE along the dorso-lateral axis, has been extensively analyzed. In order to shed light on the ancestry and the functional conservation of the regulation by Pnr and Ush, we compared the sequences of orthologous DCEs, as well as their relative activities in various mutant backgrounds.

Sequence alignments reveal that the DCEs are greatly
variable in size and have undergone considerable turnover. Only the extremities display significant levels of similarity between all species examined (shown in blue in Figure 4A). The central region is poorly conserved. The elements from D. melanogaster (1.5 kb) and D. eugracilis (2 kb) are more similar to each other than to the others, in accordance with their closer phylogenetic relationship (Figure 4A). The enhancers from D. virilis and D. quadrilineata share a relatively large size (4.1 and 3.3 kb, respectively) and a conserved stretch of about 300 nucleotides that is absent from the D. melanogaster and D. eugracilis sequences (labelled Dq-Dv in Figures 4A and S2). Putative binding sites for Pnr are present in all species (rectangles in Figure 4A). Mutation of a specific Pnr binding site severely reduces activity of the Dm-DCE [30]. This site is embedded within a stretch of 16 nucleotides perfectly conserved between the four species (asterisks in Figure 4A). Interestingly, two other neighbouring GATA sequences can be recognised as homologous between all species (red rectangles in Figure 4A). Conservation overall, however, is low, and the number, spacing, and orientation of the remaining putative Pnr binding sites are extremely variable (Figures 4A and S2).

In D. melanogaster, pnr is expressed in a broad medial domain, but activates sc in discrete proneural clusters [31]. Expression of sc mediated by the Dm-DCE is a direct consequence of Pnr binding [30]. DCE function is restricted dorsally through the repressor activity of Ush, which forms heterodimers with Pnr and prevents activation of sc [32,45]. We found that the activity of the Dv-DCE and the Dq-DCE in D. melanogaster is restricted to a lateral cluster of cells completely included within the expression domain of pnr (Figure 4B and 4C). This suggests that, despite significant sequence turnover, the divergent DCEs require Pnr and are efficiently repressed dorsally by Ush. We examined behaviour

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**Figure 2. Rescuing Activity of scut e Minigenes**

Minigenes comprising the enhancers of D. melanogaster, D. quadrilineata, and D. virilis and sc (Dm-DCE-sc, Dv-DCE-sc and Dq-DCE-sc) were assayed in ac<sup>mtg</sup> mutants.

(A) Pictures of typical adult hemithoraces characterised by the total number of bristles (indicated at the top), the position of the anterior-most bristle (white arrowheads), and of the smaller intermediate bristles (black arrowheads). Each hemithorax category is given a colour code below. The associated histograms show the percentage of hemithoraces falling in the above categories for the genotypes examined. This summarises the results from four independent insertion lines of each transgene, with at least 100 hemithoraces scored for each line.

(B–G) In situ hybridisation for sc performed on third instar larval wing discs of the genotypes indicated. In D. quadrilineata, we observed expression of sc at the site of origin of the DC, scutellar (SC), and postalar (PSA) bristles, but noted its absence at the presutural position (see asterisk [*] in [E]). D. quadrilineata flies lack the presutural bristle (arrows in Figure 1B–1D). Expression corresponding to the presutural bristles of D. melanogaster is labelled “sut” in (B). The dotted lines in (B) and (E) indicate the midline (m). Anterior is up, posterior down, dorsal to the left, and lateral to the right.

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of the DCEs in the context of various mutant alleles of \textit{pnr}.

We used \textit{pnr}^{VX4}, a strong loss of function allele, \textit{pnr}^{V1}, a hypomorphic allele and \textit{pnr}^{D1}, a gain of function allele with a missense mutation that disrupts the interaction of Pnr with Ush [31,32,45]. Activity of the \textit{Dm-DCE} was compared with that of the \textit{Dv-DCE} (Figure 4D) and that of the \textit{Dq-DCE} (Figure 4E). We observed that the enhancers react in a similar fashion to four different mutant backgrounds. The expression domains are reduced in loss of function genotypes and expanded in gain of function genotypes.

**Discussion**

**A Conserved \textit{trans}-Regulatory Landscape**

To date, a single \textit{trans}-regulator of the DCE, the GATA factor Pnr, has been identified in \textit{D. melanogaster} [30,31]. We present evidence that the activity of Pnr is conserved and positively regulates the DCE enhancers from distantly related Drosophilidae. When assayed in \textit{D. melanogaster}, the \textit{Dv-DCE} and \textit{Dq-DCE} are active in groups of cells completely included within the expression domain of \textit{Dm-pnr}. It is significant that an essential, high-affinity Pnr binding site in the \textit{Dm-DCE} is

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**Figure 3.** Detailed Comparison of the Activity of Orthologous DCEs

(A–D) \textit{D. melanogaster} wing discs expressing a cytoplasmic form of \textbeta-Gal (red) under the control of the \textit{Dm-DCE}, and a nuclear form of GFP (green) under the control of the DCE from \textit{D. melanogaster} (A), \textit{D. quadrilineata} (B), \textit{D. virilis} (C), or \textit{D. eugracilis} (D). A phalloidin counter-stain reveals actin in blue. In (B–D), the arrows point to anterior cells expressing GFP but not \textbeta-Gal. In (C), a few posterior cells strongly express \textbeta-Gal but weakly GFP (arrowhead).

(E) Adult hemithorax of an \textit{ebony} mutant, the muscle attachment sites appear as unpigmented cuticle. The schematic drawing shows the sites of muscle attachment (red) and the DC bristles (black dots).

(F) and (G) \textbeta-Gal antibody staining (red) revealing the expression of both \textit{sr-lacZ} (enhancer trap line, nuclear signal) and of the \textit{Dm-DCE-lacZ}. The expression of GFP driven by the DCE enhancers of \textit{D. quadrilineata} (F) or \textit{D. virilis} (G) is shown in green. The regions indicated by the dashed boxes are shown in (H). In (E–G), the site of DVM 2–3 tendons is indicated (DVM). (H) Comparison of \textit{sr} expression in the DVM (red) with the activity of the DCEs of \textit{D. melanogaster}, \textit{D. quadrilineata}, or \textit{D. virilis} driving GFP (green). The dotted lines demarcate the anterior limit of activity of the DCEs. The samples shown are in a similar position to the dashed boxes in (F) and (G). In (E–H), the arrows point to the anterior limit of the DVM. The samples in (H) correspond to three categories depending on whether GFP signal was observed posterior to, at the same level as, or anterior to the anterior limit of DVM \textit{sr} expression. The percentages of discs falling into each category are indicated.

(I) The histograms detail the frequency of each category, for the DCE from \textit{D. melanogaster} (one transgenic line), \textit{D. quadrilineata} (six lines), or \textit{D. virilis} (six lines). The numbers of discs examined per line are indicated (n).
conserved in the DCEs of the other species (Figure S2). Note that the three conserved Pnr binding sites are clustered in a region of the DCE that is required for activity and is sufficient in D. melanogaster to direct weak expression by itself [30]. Expression of sc mediated by the Dm-DCE is restricted dorsally through the repressor activity of Ush that associates with Pnr to prevent activation [32,45]. In gain-of-function pnr alleles that are insensitive to Ush, activity of the Dv-DCE and the Dq-DCE, like the Dm-DCE, expands dorsally. We have cloned most of the open reading frame of pnr from D. quadrilineata and found that, in D. virilis, the two zinc fingers are perfectly conserved (Figure S3), suggesting that Dq-Pnr and Dv-Pnr may also bind Ush within their respective species [31,32,45]. Hence, it is most likely that Pnr and Ush are direct, evolutionarily conserved regulators of the DCE within Drosophilidae. Indeed the expression domain of pnr, as well as other upstream regulators, has been found to be conserved in other families of flies [23,27]. Even Pnr from the mosquito Anopheles gambiae is able to regulate ac-sc in transgenic D. melanogaster, suggesting conservation of pnr function throughout the Diptera [26].

Morphological Diversification through cis-Regulatory Evolution

D. quadrilineata is phylogenetically distant from D. melanogaster and displays four instead of two DC bristles. Our results demonstrate that this secondary gain is partly due to evolution of the cis-regulatory sequence that drives sc expression at the DC site. A Dq-DCE-sc minigene, present in transgenic mutant D. melanogaster devoid of the endogenous DC proneural cluster of ac-sc expression, is not only able to rescue posterior bristles, but also allows development of more anterior bristles. It thus mimics the DC phenotype of D. quadrilineata itself. Expression driven by the Dq-DCE in D. melanogaster extends anteriorly in a domain that is longer and thinner. Although we have been unable to test the Dq-DCE in D. quadrilineata itself, it is active in D. melanogaster in a domain that is similar to the DC domain of sc expression in D. quadrilineata visualized by in situ hybridisation. This suggests that the Dq-DCE autonomously reproduces an expression pattern similar to the endogenous one in D. quadrilineata. Expression of sc mediated by the Dm-DCE is restricted laterally through lack of Pnr, dorsally through the repressor activity of Ush and posteriorly through the antagonistic activity of Islet [32,45,46], but it is not yet known what restricts expression in an anterior direction. The anterior expansion seen with the Dq-DCE indicates that this sequence may be at least partially insensitive to whatever factors limit anterior expression driven by the Dm-DCE. Alternatively it may contain new information not present in the other species.

Our observations demonstrate an altered response of the D. quadrilineata sequence to the upstream regulators of D. melanogaster. This response should reside in the sequence of the Dq-DCE itself that is sufficient to modify the phenotype of D. melanogaster when used to drive sc. Thus the exchange of a single, well-defined enhancer is sufficient, not only to reproduce an expression pattern, but also to partially transform a morphological trait of one species into that of another. We propose that a change in cis, within a pre-existing regulatory element of sc, contributed to the evolution of the bristle pattern observed in D. quadrilineata by altering the region where it is expressed.

The Dv-DCE, in D. melanogaster, drives expression in a larger cluster that expands predominantly in a dorsal direction. A Dv-DCE-sc minigene, however, allows the development of only two bristles positioned at the correct locations. The most likely explanation for the fact that the expanded expression driven by Dq-DCE-sc leads to additional bristles, whereas that of the Dv-DCE-sc does not, is probably linked to the different locations of the cells expressing sc. It seems that, in D. melanogaster, the region anterior to the two DC bristles is competent to produce bristles. This region is situated between the domains of expression of sr, a repressor of macrochaete development, and overlaps a band of expression of wingless (wg), a gene encoding a secreted factor that is required to maintain sc expression and to repress sr [47,48]. It is possible to select for additional anterior DC bristles, but not for macrochaetes on either side of the DC row where sr is expressed but wg is not [34]. Notably, anterior DC bristles were present in the ancestor common to D. melanogaster and D. virilis [18,33]. The curved shape of the Dq-DCE-driven expression domain means that it avoids overlap with the domains of expression of sr and shows significant overlap with that of wg. Therefore only the Dq-DCE drives expression in an anterior location that is competent to produce bristles.

Nevertheless transgenic D. melanogaster expressing Dq-DCE-sc do not perfectly reproduce the bristle pattern of D. quadrilineata. The anterior-most DC bristle, the scapular bristle, is absent. This bristle is situated in the prescutum, anterior to the transverse suture. It may be that this difference is attributable to changes in factors that negatively or positively regulate the enhancer in trans. It is also possible that full enhancer activity requires sequences on either side of the fragment tested. Additionally, the modification of cis-regulatory elements lying elsewhere within the D. quadrilineata ac-sc complex could also have contributed to the emergence of the additional bristles. However, it is equally possible that other extraneous factors are responsible that cannot be controlled for in these experiments. For instance, it has been shown that differences in the timing of bristle precursor formation between species can influence the development of macrochaetes [24].

Phenotypic Stability and Enhancer Evolution

The two DC bristles resulting from the activity of Dv-DCE-sc are situated at exactly the correct positions despite the fact that the Dv-DCE drives expression in a cluster of cells that is larger and displaced dorsally when compared with that of D. melanogaster. Thus the fly can compensate for this degree of imprecision in sc expression at the DC site. The explanation for this probably lies in the manner in which the bristle precursors are selected from the proneural cluster. Notch-mediated lateral signalling allows the selection of only two cells destined to become precursors with the appropriate spacing [49]. However, the choice of these cells is not random, but biased by external factors such as the repressors emc and sr, whose activity causes the precursors to arise at similar positions within the DC cluster of all individuals [34,50,51]. Their site of origin is in fact located within the region of overlap of expression driven by the Dm-DCE and the Dv-DCE. Positioning of bristle precursors thus results from restricted expression of sc in the proneural clusters as well as other cues.
that constrain the choice of precursor cell. Together, these two inputs lead to a robust patterning mechanism that is resistant to mild perturbations such as the shifting of the proneural cluster observed for Dv-DCE activity.

The ability of poorly conserved enhancers to drive expression of reporter genes in homologous tissues when transferred between species of similar morphology has been widely documented in the literature [52–57]. Where a detailed comparison of enhancer activity allowed a rigorous assessment of the degree of conservation, two different outcomes have been observed. On the one hand, transferring enhancers between related species of Drosophila (e.g., even-skipped), or of nematodes (e.g., lin-48) revealed a perfect conservation of activity [58,59], a phenomenon attributed to stabilizing selection [58]. On the other hand, the regulatory regions exchanged between species of sea urchins (e.g., endo-16) or ascidians (e.g., Otx) did not perfectly recapitulate the endogenous expression pattern [60,61]. The DCEs from D. eugracilis and D. virilis behave like the latter: they drive reporter gene expression in a cluster of cells that is not perfectly co-incident with that of the endogenous DC cluster. The slightly different expression patterns could be due to the divergent sequences, or could result from co-evolution between the enhancer and its regulatory environment [62–65]. Indeed earlier experiments have hinted that co-evolution between Pnr and its target sequences may be occurring [66].

Role of Selection in Shaping Bristle Patterns

The role of the sensory macrochaetes in behaviour is not known. Many species of Acalyptrata have ancient stereotyped patterns in which the number and precise position of each bristle is invariant [18]. The bristle patterns of the Drosophilidae are remarkably conserved, and the majority of the nearly 4,000 species have two DC bristles [67,68]. The evolutionary stability of the many bristle patterns suggests a role for selective forces to maintain them. D. quadrilineata is unusual among Drosophilidae in having four or five DC bristles. The anterior-most DC bristles would allow additional

Figure 4. Divergent Enhancers Display a Similar Response to Pnr and Ush

(A) Diagram representing the DCE sequences of D. eugracilis, D. melanogaster, D. virilis, and D. quadrilineata. The scale is shown at the top left. Only the blue regions connecting adjacent enhancers are alignable. A 300-bp region shared exclusively between D. quadrilineata and D. virilis is indicated (Dq-Dv). Small vertical rectangles symbolise all the putative GATA sites found in the forward (top) or reverse (bottom) strand. They are white when they exist only in one species, red when found in all species, and half-black when shared by two or three species. The asterisks (*) mark a conserved Pnr binding site essential for normal activity of the D. melanogaster enhancer.

(B) and (C) The D. virilis (B) and the D. quadrilineata (C) enhancers drive GFP expression (green) within the pnr expression domain, which is visualized by β-Gal antibody staining of pnr-Gal4/UAS-lacZ wing discs (red). A phalloidin counter-stain reveals actin in blue.

(D) and (E) Activity of the D. virilis (D) or D. quadrilineata (E) DCEs driving the expression of GFP (green) in the five genotypes indicated. In all cases, the activity of the D. melanogaster DCE driving expression of a cytoplasmic form of β-Gal (red) was used as an internal reference. White double-headed arrows delimitate the dorso-lateral width of the clusters of cells expressing GFP.

D1, pnrD1; V1, pnrV1; VX4, pnrVX4; WT, wild-type.

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positioned sensory input, and it is possible that they confer a selective advantage. However, it is important to note that not all morphological change needs be driven by selection. Kimura proposed a neutral theory of molecular evolution in which mutations with null or negligible effect can become passively fixed in populations [69]. Similarly, natural selection alone may not explain the infinite number of subtle morphological variations displayed by the many species of *Drosophila* described [70]. Exploratory behaviour is an intrinsic property of biological systems [2], and one may therefore also speculate that evolution can proceed through a series of viable, seemingly useless, phenotypes.

**Materials and Methods**

**Cloning of sc and pnr from D. quadrilineata.** For *sc*, a primer pair was designed against the regions coding for the conserved Sc N-terminal (GQYHIMP) and C-terminal (EELDYS) motifs (5′-forward 5′-CGC TAY CAG CAC ATH TTG CC-3′ and reverse 5′-DAT ATA GTC GAG DAT YTC CTC-3′). Using genomic DNA as a template, these primers amplified a 1,010–base pair (bp) PCR product. For *pnr*, two primer pairs were designed in conserved regions to amplify small fragments of exon 2 or exon 4 (Exon2-forward 5′-GGC ACT ACC ACA ACG T-3′ and Exon2-reverse 5′-GGG CGA TTC ATG CCG TTC AT-3′; and Exon4-forward 5′-GGA GCC GGC TGC CAC CAA-3′ and Exon4-reverse 5′-GAC ATT GTG CTT ATG ATG GTA-3′). Next, the DCE sequences obtained were used to design a specific reverse primer in exon 2 (5′-TAT GCA GTT CCA GTT TGG GGA-3′) and a specific forward primer in exon 4 (5′-GTA GCA GTT CAT CCA GTC-3′), amplifying a 1,033-bp *pnr* cDNA by RT-PCR.

**Cloning of the DC enhancers and transgenesis.** Specific PCR primers used to amplify the D. melanogaster and D. viridis enhancers were designed according to previously published sequences (melano-forward 5′-GAA GCC CAT TAT ACC GAC AAA AGT G-3′ and melano-reverse 5′-GAC AAT GAA ATT TTT TGG CAA-3′; and viridis-forward 5′-ACG GCC GGC ATT ATT TTT TGA CCA-3′ and viridis-reverse 5′-GAA GCA CTT AAC GCC AAA AGT G-3′). The forward primer was designed in the region coding for the conserved NARQGSQWW C-terminal motif of the neighbouring gene *yellow* (5′-ATG CCC GCC AAT CTG GTT GGT G-3′) and the reverse primer in a conserved region downstream of the DCE (5′-GAA AAT TTT TGA CCA CCT TGT GTC-3′). For *D. eugracilis*, the primer sequences were also chosen to amplify the D. melanogaster and D. viridis enhancers to the specific reverse primer in exon 2 (5′-GAA GCA CTT AAC GCC AAA AGT G-3′) and a specific reverse primer in exon 4 (5′-GTA GCA GTT CAT CCA GTC-3′), amplifying a 1,033-bp *pnr* cDNA by RT-PCR.

**Supporting Information**

**Figure S1. Detailed Phenotype of the scute Minigene Experiment**

The legend of the top pictures is as in Figure 2. The table shows the number of hemithoraces in each phenotypic category for four independent insertion lines of each genotype indicated.

Found at DOI: 10.1371/journal.pbio.0040386.s001 (200 KB DOC).

**Figure S2. Conservation and Variation between Distantly Related DCE Sequences**

(A) The legend of the diagram is as in Figure 4. The alignable regions are named R1, R2, R3, and R'Dq-Dv". The corresponding alignments were performed with ClustalW [73].

(B) Sequence alignment of the DCEs from *D. melanogaster* and *D. eugracilis*. The poorly conserved central region is shown in green. In (A) and (B) the putative Pnr binding sites are highlighted in grey when they are found in one sequence only; in black when they are found in two sequences; and in red when they are conserved between all four species.

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**Figure S3. Sequence Comparison of Scute and Pnr Proteins**

The alignments of the *viridis*, *D. quadrilineata*, and *D. melanogaster* protein sequences were performed with ClustalW [73]. The bHLH domain of Scute and the two zinc fingers of Pnr are underlined. Residues known to be crucial for the interaction with Ush are highlighted in red in the *D. melanogaster* Pnr sequence.

Found at DOI: 10.1371/journal.pbio.0040386.s003 (33 KB DOC).

**Accession Numbers**

The GenBank ([http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)) accession numbers for the *D. quadrilineata* sequences are DQ992393 (DCE), DQ992392 (scute), and DQ992395 (*pnr*). The accession number for the *D. eugracilis* DCE is DQ992394.

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**Author contributions.** SM conceived and designed the experiments. SM and PS analyzed the data. SM and PS wrote the paper.

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