Conserved regulatory architecture underlies parallel genetic changes and convergent phenotypic evolution

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Similar morphological, physiological, and behavioral features have evolved independently in different species, a pattern known as convergence. It is known that morphological convergence can occur through changes in orthologous genes. In some cases of convergence, *cis*-regulatory changes generate parallel modifications in the expression patterns of orthologous genes. Our understanding of how changes in *cis*-regulatory regions contribute to convergence is hampered, usually, by a limited understanding of the global *cis*-regulatory structure of the evolving genes. Here we examine the genetic causes of a case of precise phenotypic convergence between *Drosophila sechellia* and *Drosophila ezoana*, species that diverged ∼40 Mya. Previous studies revealed that changes in multiple transcriptional enhancers of *shavenbaby* (*svb*, a transcript of the *ovo* locus) caused phenotypic evolution in the *D. sechellia* lineage. It has also been shown that the convergent phenotype of *D. ezoana* was likely caused by *cis*-regulatory evolution of *svb*. Here we show that the large-scale *cis*-regulatory architecture of *svb* is conserved between these *Drosophila* species. Furthermore, we show that the *D. ezoana* orthologs of the evolved *D. sechellia* enhancers have also evolved expression patterns that correlate precisely with the changes in the phenotype. Our results suggest that phenotypic convergence resulted from multiple noncoding changes that occurred in parallel in the *D. sechellia* and *D. ezoana* lineages.

parallel developmental evolution | evolutionary developmental biology | enhancer function

The repeated occurrence of similar, or sometimes identical, evolutionary changes in independent lineages has occurred commonly and, at the phenotypic level, is called convergence. Phenotypic convergence is correlated often with transitions to similar environments, which provides compelling evidence that convergence resulted from response to similar patterns of natural selection. Examples include flippers and fins in cetaceans and fish, wings in birds and insects, and the eyes of mammals and octopi.

Developmental mechanisms can evolve in similar ways in independent lineages and can cause convergence, which has been called parallel developmental evolution (1). Multiple examples of parallel developmental evolution (2–13) have been reported in recent years. These data provide evidence for the contribution of similar changes in developmental mechanisms to phenotypic convergence. In particular, changes in *cis*-regulatory regions have contributed extensively to parallel developmental evolution. However, we do not yet have a detailed understanding of how these *cis*-regulatory changes contribute to convergence, partly because we currently have a limited understanding of the global *cis*-regulatory architecture of evolving genes.

Here we focus on a case of morphological convergence, the loss of larval dorsal and lateral cuticular extensions—michrotrichiae (hereafter called trichomes)—that occurred in evolutionary lineages that last shared a common ancestor at least 40 Mya (11). Previous studies provided preliminary evidence that similar changes in the same developmental mechanism caused this case of convergence. Given the deep evolutionary divergence between these lineages, together with the phylogenetic evidence that the loss of these trichomes is evolutionarily derived in both lineages, this appears to be a case of parallel developmental evolution. Here we provide functional evidence that supports this hypothesis.

Study System. The pattern of trichomes has evolved multiple times in larvae of the genus *Drosophila* (14, 15), possibly in response to natural selection (16). In the *Drosophila melanogaster* and *Drosophila virilis* species groups, most species produce trichomes over much of the dorsal and lateral surface of the first-instar larva (Fig. 1*A*). Within the *D. melanogaster* species group, *Drosophila sechellia* has evolved first-instar larvae in which the so-called quaternary cells differentiate naked cuticle (15–18) (Fig. 1*D*). Likewise, several species of the *D. virilis* group produce first-instar larvae with different degrees of naked cuticle (11, 14). In this work we focus on the larvae of *Drosophila ezoana*, which differentiate quaternary cells with naked cuticle on all body segments, resembling the phenotype of *D. sechellia* (Fig. 1 *D* and *F*).

In *Drosophila* larvae, differentiation of cells with trichomes, as opposed to smooth cuticle, is controlled by the transcription factor *Shavenbaby* (*svb*) (19), whose activity is both necessary and sufficient to produce trichomes (20). The complex embryonic expression pattern of *svb* in *D. melanogaster* is determined by the activity of seven enhancers that are distributed throughout a region ∼90 kb upstream of the *svb* first exon (17, 18) (Fig. 2). Five of these enhancers drive expression in quaternary cells (Fig. 2). The evolution of naked cuticle in *D. sechellia* resulted entirely from changes in the *D. sechellia* orthologs of these five *D. melanogaster* enhancers (17, 18). All of these evolutionary changes on the *D. sechellia* lineage cause reduced levels of enhancer activity, leading to the absence of *svb* mRNA in quaternary cells.

Three pieces of evidence reported previously suggested, but did not prove, that *cis*-regulatory changes in *svb* caused the convergent evolution of naked cuticle in species of the *D. virilis* group (11). First, six genes that regulate *svb* expression are expressed similarly in species with divergent trichome patterns (14), suggesting that the regulatory cascades upstream of *svb* have been conserved in species with divergent trichome patterns. Second, interspecific crosses demonstrated that the difference in trichome patterns

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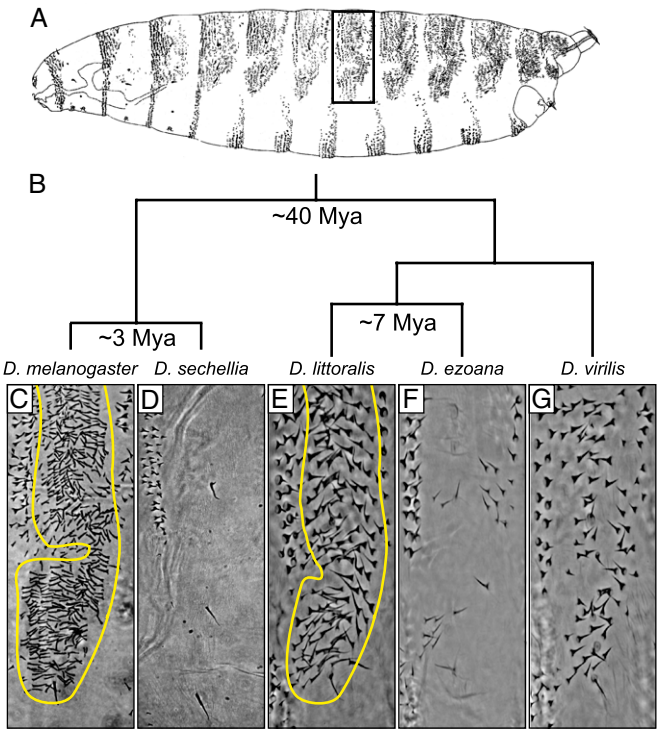

Fig. 1. Convergent evolution of a naked dorso-lateral cuticle phenotype in first-instar larvae of *D. sechellia* and *D. ezoana*. (*A*) Drawing from the lateral perspective of a *D. melanogaster* first-instar larva. The dark rectangle indicates the cuticle region shown in *C*–*G*. (*B*) Phylogenetic relationships and estimated divergence dates between species discussed in this article. Dorso-lateral cuticle of the fourth abdominal segment for five *Drosophila* species. The "hairy" phenotype present in *D. melanogaster* (*C*), *D. littoralis* (*E*), and *D. virilis* (*G*) is the ancestral state in the genus *Drosophila*. Quaternary trichomes are outlined in *D. melanogaster* (*C*) and *D. littoralis* (*E*). Quaternary trichomes were lost independently in *D. sechellia* (*D*) and *D. ezoana* (*F*).

between two species of the *D. virilis* group was caused by a locus on the X chromosome—the location of *svb*—and that the "naked cuticle" allele is recessive to the "hairy" allele, just as *svb* is in the *D. melanogaster* species group (11). Third, analysis of *svb* expression in these species revealed a precise correlation between the presence of mRNA and the pattern of trichomes (11).

In this work, we performed a series of experiments revealing that the position and function of enhancers in the *cis*-regulatory region (what we call the functional architecture) of the *svb* gene has been conserved between species that diverged ~40 Mya. We also demonstrate that orthologous enhancers from the *svb cis*-regulatory region have evolved independently in *D. ezoana* and *D. sechellia* and it is likely that these *cis*-regulatory changes have caused the precise morphological convergence between these species.

Results

Architecture of the *Cis*-Regulatory Region of *svb* in *Drosophila virilis*. To test the hypothesis that *svb* enhancers evolved to generate diversity of the trichome pattern in the *D. virilis* group of species, we first performed a functional analysis of the *svb cis*-regulatory region in *D. virilis*. We were concerned that a search guided only by sequence conservation might generate an incomplete picture of the *D. virilis svb cis*-regulatory architecture (21). For example, new enhancers may have evolved in the *D. virilis* lineage. Therefore, we performed a comprehensive and unbiased survey of the entire *cis*-regulatory region of the *D. virilis svb* locus (a ~132 Kb region between *SIP3* and the *svb* first exon), by assaying 35 ~5 Kb reporter constructs. This region corresponds to the ~90 Kb region containing all seven *svb* embryonic enhancers in *D. melanogaster* (Fig. 2). In addition, we were concerned that some of the *trans*-regulatory factors that regulate *svb* expression might have evolved new functions between *D. melanogaster* and *D. virilis*. Therefore, instead of analyzing the constructs in *D. melanogaster*, we tested all constructs in transgenic *D. virilis* embryos. This effort uncovered six regions that drive reporter gene expression in patterns that resemble parts of the *svb* expression pattern (11) in epidermal cells of the embryo (Fig. 2).

Orthologous Enhancers Generate the Embryonic Expression Pattern of *svb* in Phylogenetically Distant *Drosophila* Species. To determine whether these *D. virilis* enhancers represented orthologs of the *D. melanogaster svb* enhancers, we tested for positional conservation, functional similarity, and sequence similarity between the two species. First, we tested for positional conservation between possible orthologs by identifying conserved "anchors" of 30 bp across the locus (22) (Fig. 2). The collinear synteny of these anchors indicates that the entire *cis*-regulatory region of *svb* has not experienced rearrangements between these two species on the scale of tens of kilobases. Moreover, the locations of the conserved anchors reveal that six *svb* enhancers are positioned in the same relative sites within the *D. melanogaster* and *D. virilis svb* loci (Fig. 2). We did not find a *D. virilis* enhancer in the region orthologous to *D. melanogaster* enhancer *A*.

Second, we tested for functional similarity between the *D. virilis* and *D. melanogaster* enhancers by examining their detailed expression patterns (Fig. 2). We found that the expression patterns of all six *D. virilis* enhancers are similar to the patterns driven by their positional homologs (Fig. 2). Overall, putatively orthologous enhancers are active mainly in the same segmental and dorsal-ventral spatial domains. For example, the *D. melanogaster* putative orthologs of the three *D. virilis* enhancers that drive expression in cells giving rise to the ventral denticle belts also drive expression in cells giving rise to ventral denticle belts in *D. melanogaster*. We also detected several differences in expression between putative orthologs. First, *D. melanogaster 7* drove expression in dorsal primary and tertiary cells and weakly in dorsal quaternary cells, whereas *D. virilis 3* does not drive any detectable expression in dorsal cells. In contrast, *D. virilis 24* drives expression in dorsal primary and tertiary cells, and *D. melanogaster DG3* does not. In both species, only a single enhancer drives in primary and tertiary dorsal cells. It therefore appears that this function has shifted between enhancers during the divergence of *D. melanogaster* and *D. virilis*. We also detected apparently weaker expression in dorsal quaternary cells driven by *D. virilis 19* than by *D. melanogaster Z*.

Third, to test whether sequence similarity supported orthology between functionally similar enhancer regions, we used *D. virilis* and *D. melanogaster* enhancer sequences as queries in reciprocal BLAST (23) searches. Despite extensive divergence, we detected significant reciprocal sequence similarity in or near each of the six enhancer pairs (Figs. S1 and S2). Thus, comparisons of position, function, and sequence suggest that six pairs of *svb* enhancers are true orthologs between *D. melanogaster* and *D. virilis*.

Parallel Genetic Changes in Enhancers *8* and *19* of *D. ezoana* and the Evolution of a Convergent Cuticular Morphology. Three of the *D. virilis* enhancers—*8*, *19*, and *26*—drive expression in the dorsal and lateral domains that show evolving trichome patterns in the *D. virilis* species group. Most of the dorso-lateral expression is driven by enhancers *8* and *19*. Similarly, *D. melanogaster E6* and *Z*, which are the orthologs of *D. virilis 8* and *19*, generate most, but not all, of the dorsal and lateral expression in *D. melanogaster*. These two enhancers lost their activity in the *D. sechellia* lineage, which caused the naked cuticle phenotype of this species (17, 18). To test whether these enhancers have evolved also in the *D. virilis* species group, we cloned the orthologous regions from two species of the *D. virilis* group with contrasting dorso-lateral trichome patterns (see *Materials and Methods* for details); *Drosophila littoralis*

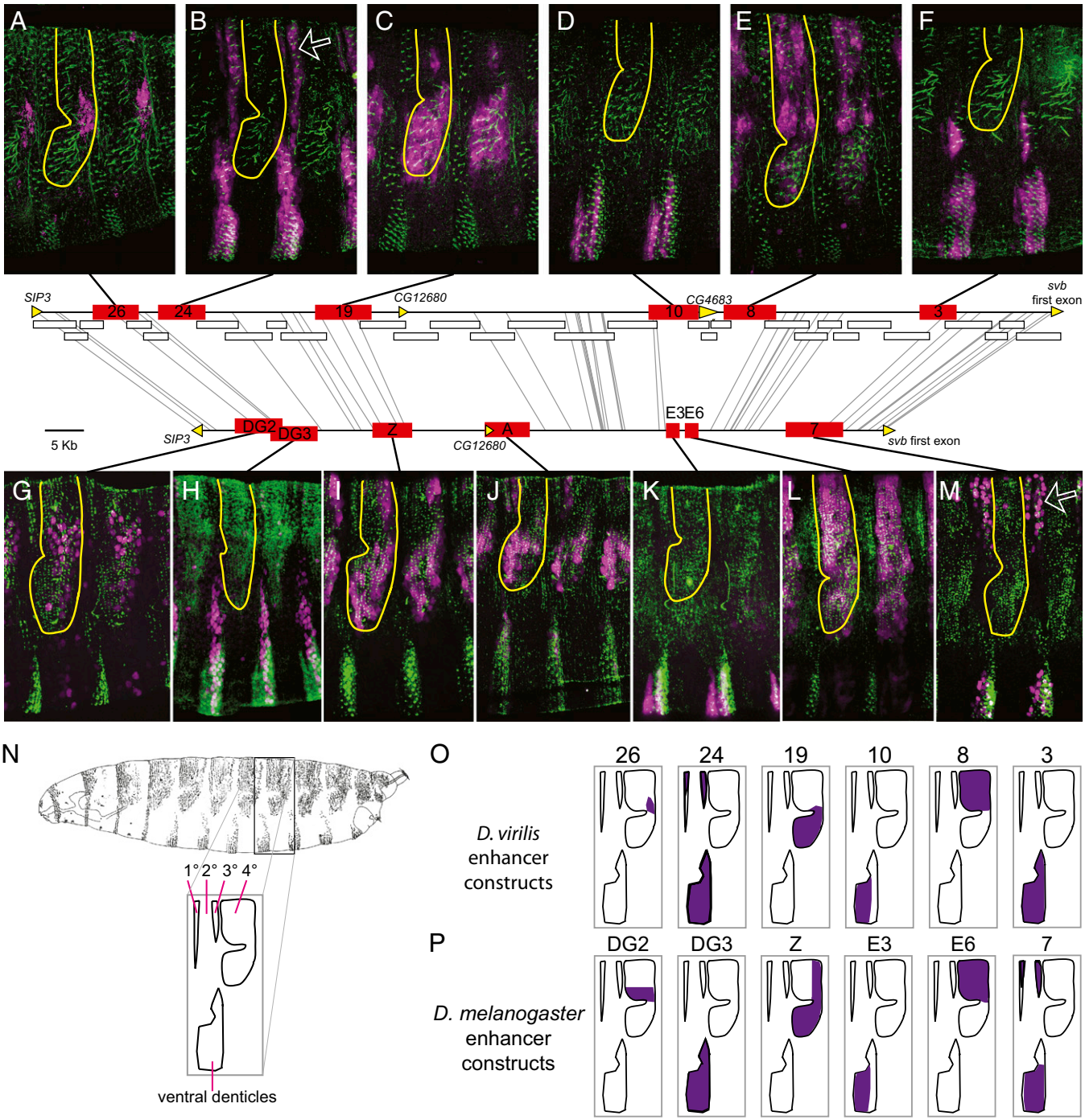

Fig. 2. Positional and functional conservation between *svb* embryonic enhancers of *D. melanogaster* and *D. virilis*. Horizontal lines schematize the *svb cis*-regulatory region in *D. virilis* (*Upper*) and *D. melanogaster* (*Lower*). Thin lines connect identical 30 bp sequences ("anchors") between the orthologous regions. The fact that none of these lines cross implies that the orthologous *svb* regions are largely collinear. White rectangles correspond to *D. virilis* DNA fragments tested for enhancer activity in transgenic *D. virilis* embryos that did not drive expression in embryonic epidermis. Yellow arrows specify coding regions. Red rectangles indicate the position of embryonic enhancers for the two species. Positional conservation is evident for six enhancer pairs. Expression patterns of *D. virilis* enhancers (*A*–*F*) and of *D. melanogaster* enhancers (*G*–*M*) are shown. Quaternary trichomes are outlined in all panels. Similar expression patterns are driven by the orthologous enhancers *26* (*A*) and *DG2* (*G*), *24* (*B*) and *DG3* (*H*), *19* (*C*) and *Z* (*I*), *10* (*D*) and *E3* (*K*), *8* (*E*) and *E6* (*L*), and *3* (*F*) and *7* (*M*). White arrows highlight the dorsal expression pattern encoded by different enhancers in the two species. Pictures were taken from stage 15–16 embryos. Trichomes are stained in green; *lacZ* reporter expression is purple. Different embryos display slightly different rotations along the dorso-ventral axis. (*N*) Drawing of a lateral view of the *D. melanogaster* first-instar larval cuticle and, below, a diagram of the major cuticular domains is shown. On the dorsal surface, the primary (1°), tertiary (3°), and quaternary (4°) cells differentiate trichomes, and the secondary (2°) cells differentiate naked cuticle (38). The ventral denticle belts are also labeled. (*O*) The epidermal domains in which the six *D. virilis* enhancers drive expression is illustrated with purple shading. (*P*) The epidermal domains in which the six *D. melanogaster* enhancers that appear to be orthologous to the six *D. virilis* enhancers, based on shared chromosomal synteny and sequence similarity are illustrated with purple shading. The expression patterns of the *D. virilis* enhancers are placed directly above the expression patterns of their putative orthologs in *D. melanogaster*.

produces trichomes where enhancers *8* and *19* are active (Figs. 1*E* and 2), while *D. ezoana* displays a *D. sechellia*–like phenotype (Fig. 1*F*). As a control, we cloned the homologous regions of enhancer *3* from both species. Enhancer *3* is expressed primarily in ventral trichome-producing cells, which have not evolved between *D. littoralis* and *D. ezoana*.

We observed that all three enhancers isolated from *D. littoralis* drove expression in patterns that were extremely similar to the patterns driven by the homologous *D. virilis* enhancers (Fig. 3). This implies that the common ancestor of *D. littoralis* and *D. ezoana* also possessed all three active enhancers. Enhancer *3* is still active in *D. ezoana* and drives expression in the same domains as the *D. littoralis* and *D. virilis* orthologs. In contrast, *D. ezoana* enhancers *8* and *19* produce no detectable expression. These results are consistent with the pattern of *svb* mRNA in *D. ezoana* embryos that was reported previously (11). Because all of these assays were performed in the common *trans*-regulatory landscape of *D. virilis*, which is competent to drive dorsal and lateral expression from the *D. virilis* and *D. littoralis* enhancers, the changes in *D. ezoana* enhancers *8* and *19* are likely to have caused the previously observed changes in *svb* expression in *D. ezoana*.

svb is required for trichome development in *D. melanogaster* (20). We therefore tested whether *svb* is also required for trichome development in *D. virilis* by performing a knockdown of *svb* mRNA. Injection of two independent siRNAs in *D. virilis* embryos caused loss of trichomes in larvae (Figs. S3 and S4), confirming that *svb* is also required for trichome development in *D. virilis*. Taken together with previous findings of conserved expression patterns of *svb* regulators and genetic linkage of trichome patterns with *svb* in the *D. virilis* species group (11, 14), our current observations suggest that genetic changes in *svb* enhancers contributed to the evolved trichome pattern in *D. ezoana*. We are not able to rule out, however, additional contributions from loci closely linked to *svb*.

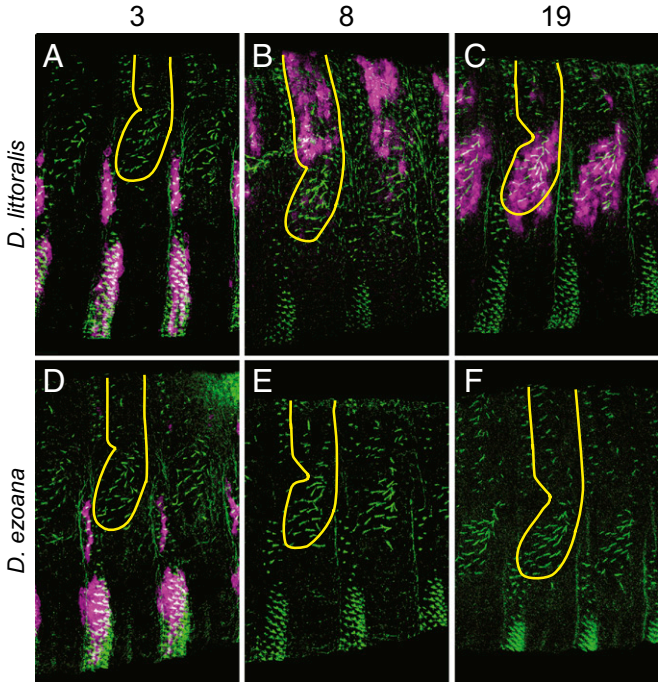

Fig. 3. *Cis*-regulatory changes in evolutionarily conserved *svb* enhancers underlie the convergent loss of quaternary trichomes in *D. ezoana*. *D. littoralis* enhancers *3* (*A*), *8* (*B*), and *19* (*C*) drive expression patterns that cannot be distinguished from those produced by the *D. virilis* orthologous enhancers (Fig. 2). (*D*) The expression pattern of the *D. ezoana* enhancer *3* also is conserved. In contrast, *D. ezoana* enhancers *8* (*E*) and *19* (*F*) do not produce detectable expression. This explains, at least in part, the absence of quaternary trichomes in *D. ezoana*. In *D. sechellia*, the parallel inactivation of *E6* and *Z* (the orthologs of *D. virilis 8* and *19*) likely caused the convergent loss of quaternary trichomes. The activity of all *D. ezoana* and *D. littoralis* constructs were tested in transgenic *D. virilis* embryos. Differences in the activity of these enhancers therefore represent differences in the enhancer sequences from each species. Colors and symbols as in Fig. 2.

Discussion

We have found that the functional architecture of the *svb cis*-regulatory region was conserved between *D. melanogaster* and *D. virilis*. Similarly, in insects, some enhancers of "even-skipped" (24) and of some dorso-ventral patterning genes (25) have maintained their ancestral positions. Within single *svb* enhancers, DNA sequences have diverged substantially without causing major changes in enhancer function, a feature that has been observed previously (26). However, at a larger scale, the functional organization of the whole *svb* regulatory region has been largely conserved. This higher order conservation is reminiscent of the structural conservation of *Hox* gene clusters (27), suggesting that long-range molecular interactions, such as enhancer–enhancer or enhancer–promoter interactions (28), constrain the evolution of large *cis*-regulatory regions, such as those found in *svb* and in *Hox* genes. In other words, the spacing and order of enhancers in the *cis*-regulatory region of *svb* might be crucial for the occurrence of precise physical contacts between different regulatory elements or between regulatory elements and the core promoter.

Parallel evolution underlying convergence of trichome patterns had been inferred previously from genetic studies coupled with expression assays (11). Our current functional study supports the view that not only the same gene, *svb*, underlies this convergence, but that two orthologous enhancers have changed in similar ways in *D. sechellia* and *D. ezoana*.

There are multiple reasons why *svb* may be a favored locus of evolutionary change, and additional reasons why specific enhancers may be particularly favored. First, *svb* acts as a single master regulator of larval trichome development. *svb* function is both required for development of these trichomes and sufficient to induce expression of a large set of downstream genes that regulate and contribute to trichome development (20, 29). Thus, manipulation of the *svb* expression pattern can, on its own, generate diversity of trichome patterns. It is not clear that any other single gene can cause such a specific morphological change without disrupting other aspects of larval development (29). *svb* expression is regulated by a large number of signaling pathways and transcription factors, and manipulations of these factors can alter trichome patterns (30). However, these manipulations are likely to have pleiotropic deleterious effects, in addition to altering trichome patterns. Thus, the *svb* locus may be a favored target for evolutionary change underlying trichome patterns both because these changes minimize pleiotropic effects and because *svb* can instruct the entire module of trichome morphogenesis (31, 32).

The parallel evolution of individual enhancers of *svb* comes as more of a surprise. At the initiation of this work, given the extreme sequence divergence of *D. melanogaster* and *D. virilis*, it was not clear that the *svb* enhancer region would be both spatially and functionally conserved. The conserved *svb* enhancer functions between *D. melanogaster* and *D. virilis*, and the observation that orthologous enhancers seem to have evolved in similar ways to generate convergent evolution, implies that our ability to "predict" patterns of genomic evolution may improve as our understanding of genome function improves.

Although we have found some sequence similarity between *D. melanogaster* and *D. virilis svb* enhancers, the sequences align poorly. Therefore, at this stage, we cannot determine if the nucleotide changes that inactivated *D. ezoana 8* are similar to those described recently for *D. sechellia E6* (16). It will be interesting to determine whether, despite this sequence divergence, orthologous transcription factors regulate orthologous enhancers and whether,

at an even more detailed level of analysis, similar patterns of transcription factor binding site gain or loss have generated developmental parallelism in *D. sechellia* and *D. ezoana*.

Materials and Methods

Design of piggyBac Reporter Vectors. The polylinker-hs43-lacZ region from pCaSpeR-hs43lacZ (33) was PCR-amplified using primers BglII-FW (AGATCTAGATCTTACTAGAATTCGGT) and BglII-RV (AGATCTAGATCTAATTTGCGAGTACG). This 4.6 Kb fragment was cloned into pGEMT (Promega), released by cutting with BglII, and subcloned into the unique BglII site of both piggyBac PB (34) and piggyBac-enhanced yellow fluorescent protein (eYFP) (35), yielding piggyBac-hs43lacZ and piggyBac-eYFP-hs43lacZ, respectively. The difference between these two reporter vectors is the transformation marker; piggyBac-hs43lacZ carries *miniwhite*, whereas piggyBac-eYFP-hs43lacZ contains *Pax6*::eYFP.

Reporter Constructs. The *svb* gene is located in scaffold 13042 of the *D. virilis* sequenced genome. The dissected region corresponds to bases 298789–434870 of this scaffold. PCR fragments were amplified from genomic DNA of *D. virilis white* (*Drosophila* Species Stock Center strain 15010–1051.53) using the primers listed in Table S1. These fragments were cloned into pGEMT (Promega), excised using NotI, and subcloned into the NotI site of the piggyBac-hs43lacZ polylinker. Fragments that contained internal NotI sites were subcloned using the CloneEZ kit (Genscript). Recombinant plasmids were coinjected with the helper vector pHSPpBac (36) into *D. virilis white* embryos. At least three independent transgenic lines were established for each construct.

Fragments *3*, *8*, and *19* from *D. ezoana* (*Drosophila* Species Stock Center strain 15010–0971.00) and *D. littoralis* (*Drosophila* Species Stock Center strain 15010–1001.00) were amplified using the primers listed in Table S2. Primers were designed based on *D. virilis* sequence. *D. littoralis* and *D. ezoana* fragments *8* and *19* are slightly larger than their *D. virilis* ortholog; these fragments had to be amplified with primers that flank the enhancers originally defined in *D. virilis*. Fragments *3*, *8*, and *19* from *D. ezoana* and *D. littoralis* were fully sequenced (GenBank accessions JN792404–JN792409). Primers used to amplify fragments *3*, *8*, and *19* from *D. littoralis* and fragments *3* and *8* from *D. ezoana* had SacII (forward) and SalI (reverse) sites added. These fragments were cloned into pGEMT (Promega), excised with SacII and SalI, and subcloned into piggyBac-eYFP-hs43lacZ (the polylinker was cut with SacII and XhoI). Both primers used to amplify fragment *19* from *D. ezoana* had XhoI sites added. These fragments were cloned into pGEMT (Promega), excised with XhoI, and subcloned into piggyBac-eYFP-hs43lacZ (the polylinker was cut XhoI).

The *D. melanogaster* enhancer constructs *DG2*, *DG3*, *Z*, *A*, *E3*, *E6*, and *E7* were reported in previous publications (16–18). The image of enhancer *7* in Fig. 2 is from a new *7*::luciferase construct that was made by Ella Preger (Janelia Farm Research Campus, Howard Hughes Medical Institute, Ashburn, VA), who kindly provided fixed embryos for our use.

The precise expression domains of the enhancer constructs were determined by double-staining embryos with a mouse anti-*βGal* antibody (Promega) and a rabbit anti-*Dusky-like* antibody (37). Fluorescent secondary antibodies were used. Stained embryos were examined with a confocal microscope.

Preparation of Cuticles. We made overnight embryo collections and transferred embryos to plastic Petri dishes containing distilled water and maintained them in a 25 °C incubator. Two days later, we collected first-instar larvae and incubated them at 60 °C for 4 h. Subsequently, larvae were mounted on a microscope slide in a drop of 1:1 Hoyer's solution–lactic acid mixture. After overnight drying, the cuticles were imaged with phase-contrast microscopy.

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