Functional Evolution of a cis-Regulatory Module

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Lack of knowledge about how regulatory regions evolve in relation to their structure–function may limit the utility of comparative sequence analysis in deciphering cis-regulatory sequences. To address this we applied reverse genetics to carry out a functional genetic complementation analysis of a eukaryotic cis-regulatory module—the even-skipped stripe 2 enhancer—from four Drosophila species. The evolution of this enhancer is non-clock-like, with important functional differences between closely related species and functional convergence between distantly related species. Functional divergence is attributable to differences in activation levels rather than spatiotemporal control of gene expression. Our findings have implications for understanding enhancer structure–function, mechanisms of speciation and computational identification of regulatory modules.

Introduction

The annotation of genes from comparative sequence data rests on a fundamental evolutionary dictum, first elaborated by M. Kimura, that the rate of molecular evolution will be inversely related to the level of functional constraint. But the application of this principle would not be interpretable without a corresponding understanding of gene structure and organization (i.e., the genetic code and its degeneracy, the signals for initiation and termination of translation, intron/exon junction sequences, etc). Knowledge of equivalent scope and depth does not exist for cis-regulatory sequences. These sequences often contain docking sites for transcription factors (TFs), but the number of binding sites and the spacing between them vary, and binding-site sequences are often degenerate to the point that they can only be characterized probabilistically. Even more striking is the lack of data relating functional evolution of gene expression to cis-regulatory sequence evolution. There are good reasons to expect the two may be only weakly correlated [1,2]: De novo binding sites can readily evolve [3]; individual TFs often bind at multiple locations and may be exchangeable, and the spacing between binding sites can rapidly evolve. Thus, despite recent progress [4,5], rules have yet to be elucidated for the functional molecular evolution of this critically important component of the genome.

The Drosophila gene even-skipped (eve) produces seven transverse stripes along the anterior–posterior (A–P) axis of a blastoderm embryo (Figure 1). Expression of these early stripes is regulated by five distinct cis-elements (Figure 2A). The best studied of them, the stripe 2 enhancer (S2E), contains multiple binding sites for five TFs, the activators bicoid and hunchback, and the repressors giant, Kruppel, and sloppy-paired [6,7,8]. Maternal deposition of bicoid mRNA in the anterior pole of the egg regulates expression of the other gap genes, which are expressed in broad A–P diffusion gradients. Spatiotemporal control of eve stripe 2 expression is brought about through the integration of these graded signals by the S2E.

We previously used a reporter transgene assay to investigate eve S2E functional evolution in three Drosophila species in addition to D. melanogaster. The sister taxa D. yakuba and D. erecta [9] are separated by approximately 5 million years ago (MYA), while the ancestor they share with D. melanogaster existed approximately 10–12 MYA. In contrast, D. pseudoobscura is a member of a different group and is believed to have split from the melanogaster clade approximately 40–60 MYA. As expected for a trait as ontogenetically important as primary pair-rule stripe formation, the temporal progression of eve stripe expression is nearly identical among the species (see Figure 1A–1D). This functional conservation of gene expression, however, is not reflected in patterns of sequence conservation (see Figures 2B, S1, and S2). Instead, S2E sequences from these species are substantially diverged, including large insertions and deletions in the spacers between known factor-binding sites, single nucleotide substitutions in binding sites, and even gains or losses of binding sites for the activators bicoid and hunchback.

Yet despite these evolved differences, reporter transgene analysis showed that spatiotemporal patterns of gene expression driven by S2Es of all four species are indistinguishable when placed in D. melanogaster [10], indicating that evolved changes in the enhancer have had little or undetectable impact on spatiotemporal control of gene expression. But further experiments with native and chimeric S2Es of D. melanogaster and D. pseudoobscura showed that this functional conservation required coevolved changes in the 5’ and 3’ halves of the enhancer [11], suggesting compensatory (i.e., adaptive) evolution. This functional evidence for adaptive...
substitution, together with indications that levels of gene expression might also differ among the four species' S2Es, raises questions about whether these orthologous enhancers are indeed functionally identical. To overcome limitations inherent in functionally interpreting the overlap of a reporter and native gene expression, here we report results of an in vivo complementation assay to investigate S2E performance. This approach allows us to put the functional equivalency hypothesis to a rigorous test.

Results

Strategy and Proof of Principle

First, we created a fly line, EVEAS2E, in which the native eve S2E was deleted (see Figure 2A). We then attempted to complement, that is, rescue this lethal mutation with the introduction of a transgene, denoted S2E-EVE, containing an eve S2E from one of the four species (D. melanogaster, D. yakuba, D. erecta, and D. pseudoobscura) linked to a functional eve promoter and coding region (Figure 2B). This allowed us to compare both viabilities and developmental consequences among lines differing only in the evolutionary source of their S2E. By genetically manipulating rescue-transgene copy number (Figure 2C), effects of EVE abundance on viability and development could also be investigated.

We created the eve S2E deficiency mutant by removing a 480-bp fragment corresponding to the minimal stripe 2 element (MSE; see Figure S1) from a 15-kb cloned copy of the eve locus [12]. A transgene containing the complete fragment is capable of rescuing eve null mutant flies to fertile adulthood [12]. EVEAS2E is functionally a null allele for stripe 2, as evidenced by the expression of the segment polarity gene, engrailed (en). Establishment of en 14-stripe pattern is a complex process that includes involvement by eve early stripes [13,14]. Eve stripe 2 corresponds to parasegment 3, which is bordered by en stripes 3 and 4. We hypothesized that these en stripes might be developmental indicators of early eve stripe 2 expression. Indeed EVEAS2E embryos lacking a functional S2E (Figure 3A–3F) produce a short parasegment 3 and vestigial en stripe 4 (Figure 3F). This defect alone is almost certainly a lethal condition.

Transgenes containing precisely orthologous S2Es from each of the four species linked to the D. melanogaster eve promoter and coding region were introduced onto the third chromosome. The fragment we chose to investigate is 692 bp in length in D. melanogaster (see Figure S1). It contains the central MSE, and every other previously identified TF-binding site in the S2E region. Notably, this fragment contains completely conserved sequences at its 5' and 3' ends in all four species, thus ensuring that we could compare precisely orthologous fragments. As expected, all four S2E-EVE transgenes express a single early eve stripe in the expected spatial location (see Figure 1E–1H).

Having created the EVEAS2E chromosome line and the S2E-EVE rescue third chromosome lines, we could then produce flies carrying EVEAS2E; S2E-EVE in a doubly balanced configuration (see Figure 2C). Crossing this line with itself or with another line carrying an independent copy of the same S2E allowed us to estimate relative survival to adulthood of offspring carrying one or two copies of the rescue transgene. EVEAS2E homozygotes are embryonic lethal, whereas flies carrying two copies of the D. melanogaster S2Eprobe-EVE transgene in an EVEAS2E genetic background rescue approximately 34% of flies to adulthood (Figure 4). This is approximately the same rescue percentages found for the same genotype (P[EVEG84], R13), which contains the wild-type eve locus (including the native S2E) [12]. This implies that the fragment we used to drive stripe 2 eve expression is complete and that it can function normally when removed from its native context. Importantly, our negative control, S2Eprobe-EVE, does not rescue, indicating that the rescue transgene requires this enhancer to drive eve stripe 2 expression.

Functional Equivalence of the D. melanogaster and D. pseudoobscura S2Es

We evaluated the ability of S2E-EVE rescue constructs to complement the embryonic lethal EVEAS2E deletion by estimating survival to adulthood, based on a genetic design used extensively in Drosophila evolutionary genetics [15]. Viability measurements were made by crossing two independent lines of each rescue transgene to reduce potential recessive fitness effects caused by the site of rescue-transgene insertion. Offspring with two copies of the transgene are doubly hemizygous; few deleterious effects of transgene insertion were observed in these flies (compare, for example, EVEAS2E; R13/CyO; S2E-EVE/S2E-EVE versus EVEAS2E, R13/CyO; S2E-EVE/TM3 survivors in Table S1). Rescue abilities of S2Es from different species can be compared quantitatively because the viability of each S2E-EVE transgene is calculated relative to a standard genotype present in every cross.
Figure 2. Genetic Constructs and Rescue Scheme

(A) Summary map of the eve locus and eve S2E deletion transgene (EVEΔS2E). Adam and Apple are adjacent open reading frames [40]. The late element (Auto) and early stripe enhancers are shown.

(B) S2E-EVE transgenes used to rescue eve function. The rescue EVE locus used is the D. melanogaster eve flanked by 0.9 kb of 5′ and approximately 0.6 kb of 3′ of endogenous sequence. The S2E0-EVE does not have any S2E sequences and is a negative control. The known trans-factor-binding sites in the S2E from D. melanogaster: five bicoid (circles), three hunchback (ovals), six Kruppel (squares), three giant (rectangles), and one sloppy-paired (triangle) binding site. Symbols representing sites 100% conserved compared to D. melanogaster are open, while those diverged are shaded gray. Note the evolutionary gain of novel but functionally necessary [6] activator (bicoid and hunchback) binding sites (red) in D. melanogaster lineage. Full sequences are shown in Figures S1 and S2.

(C) Example of a cross between independent rescue lines and relevant offspring genotypes for the viability assay (see Materials and Methods for details). Genetic notation: b: mutant black; yellow box: native eve; R13 and X’d out yellow box: eveR13 lethal mutant; P(S2EΔEVE) and P(S2EΔEVE) are two independent rescue-transgene inserts with S2E from species A.

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Figure 3. Developmental Series of EVE Abundance

(A–E) Immunofluorescence labeling of time-staged early EVEΔS2E homozygous embryos. This developmental sequence, which corresponds roughly from the initialization of cellularization (A) to its completion (E), takes approximately 45 min at 25°C in wild-type flies [41].

(F) Expression of en in same genotype at stage 10. Arrows mark third and fourth en stripes. Note the short interval between en stripes 3 and 4 (parasegment 3) and the reduced fourth stripe.

(G) EVE expression in stripe 2 during the developmental series around cellularization, where times 1–5 correspond to pictures in A–E. Stage 1 is early cellularization, while the process has been completed for embryos in class 5. The series is comparable to time classes 4–8 on the FlyEx Web site (http://flyex.ams.sunysb.edu/flyex/) [34]. Estimated least square means (±SE) for EVEΔS2E/Cy stock and wild-type line w1118; note the Cy/Cy homozygote is essentially wild-type. Early eve pair-rule expression is not known to be autoregulated (as occurs in postcellularization stages), and we observe a 2-fold difference in early stripe expression, with an additive component (a) of 0.62 and negligible dominance deviation (d/a) = 0.01, for the first two stages. This dosage dependency is lost after the cellularization stage (3), presumably because all embryos carry two copies of the autoregulatory element.

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Evolution of the S2E cis-Element
S2Es from the four species exhibited large differences in rescue abilities that follow neither a phylogenetic trend nor net sequence divergence (Figure 4). The S2E of the most distantly related species, D. pseudoobscura, is completely conserved at only three of 18 TF-binding sites identified in D. melanogaster and is missing two of them entirely (see Figures 2B and S2). It is also nearly 25% longer due to insertions and deletions in the spacers between binding sites. Yet in terms of rescue ability it is indistinguishable from the D. melanogaster S2E.

Functional Divergence of S2Es from Closely Related Species

Given the complete functional conservation of the D. pseudoobscura S2E, we were surprised to discover the failure of the D. erecta transgene to restore viability in EVEAS2E homozygotes (see Figure 4). The inability of the doubly hemizygous S2E<sup>ere</sup>-EVE genotype to rescue cannot be due to deleterious effects of transgene insertion, because the presence of each single transgene has minimal impact on viability (see Table S1). Two additional independent transformants were also investigated, neither of which produced viable adult flies. We conclude, therefore, that the D. erecta sequence, although precisely orthologous to the D. melanogaster and D. pseudoobscura S2E fragments, is nonfunctional when placed in a D. melanogaster embryonic context.

The D. yakuba S2E also exhibits a rescue defect in that two copies of the rescue transgene are required for robust rescue. Flies carrying a single copy of the D. yakuba rescue transgene are less than half as viable as flies carrying one copy of either the D. melanogaster or D. pseudoobscura rescue transgene. A smaller dosage effect on viability of approximately 20% is seen with the S2Es of D. melanogaster and D. pseudoobscura. Since the spatiotemporal expression of eve stripe 2 must be the same for flies carrying one or two copies of a transgene, eve stripe 2 expression level alone must have a measurable influence on fitness.

As expected, embryos carrying one or two copies of either the D. melanogaster or the functionally equivalent D. pseudoobscura S2E rescue transgene exhibit a wild-type en staining pattern, indicating a normal parasegment 3 (Figure 5A–5I). In contrast, the D. erecta S2E exhibits an en pattern defect similar to the one produced in embryos lacking en stripe 2 expression (i.e., EVEAS2E homozygote). The inability to drive normal en expression provides further evidence that the D. erecta S2E is a weak (or nonfunctional) enhancer in the D. melanogaster genetic background.

The D. yakuba S2E also exhibits an en phenotype that correlates with its ability to rescue (Figure 5D and 5E). With two copies of the enhancer present, embryos exhibit a robust en stripe 4, indistinguishable from wild-type. But with only...
one copy present, en stripe 4 expression is shifted anteriorly relative to its neighbors, an indication that parasegment 3 is not forming properly. Some of these embryos survive to adulthood since we do observe one-copy adults in our viability experiment, albeit at a lower than expected percentage. Although adult flies are superficially “normal,” we can observe subtle morphological defects (mouthparts and thoracic structures) in the segments corresponding to parasegment 3.

Differences in eve S2E Expression Levels

To test whether differential gene expression might be the critical functional difference between the S2Es, we quantified eve stripe 2 protein in early embryos. The experimental design allowed us to normalize eve stripe 2 expression in individually stained embryos relative to stripe 3, thus facilitating comparison across embryos and genotypes. We also developed a PCR method to ascertain the genotype of individually stained embryos.

We validated the quantification procedure by comparing eve stripe 2 expression levels in embryos carrying zero, one, or two copies of the S2E in its native position in a wild-type eve locus—that is, EVEAS2E/EVEAS2E, EVEAS2E/Cy, and Cy/Cy embryos, respectively, and a homozygous w1118 line (see Figure 3G). The expected dose dependence is observed in response to EVEAS2E copy number prior to cellularization, followed by a shift to dose independence as control of eve stripe expression is transferred to the late (autoregulatory) element. Unexpectedly, a weak early stripe 2 (estimated to be approximately 20% of the wild-type level) can be detected in EVEAS2E homozygotes; we do not know what drives this stripe.

Normalized stripe 2 expression in early embryos carrying S2Es from D. erecta and D. pseudoobscura is consistent with adult viability (Figure 6). The D. erecta S2E-driven eve expression is too weak to observe statistically significant expression comparing embryos containing zero, one, or two copies of the rescue transgene. Note, however, that this transgene does drive weak eve stripe 2 expression in a fully eve null background (see Figure 1G). Formally, we observe statistically significant effects of gene “dose,” S2E “species” of origin, and most notably a “dose × species” interaction on stripe 2 expression by a mixed-model analysis of variance (ANOVA) (see Tables 1 and S2). Therefore, the major functional evolutionary difference between these enhancers is likely to reside in their activation strengths.

Discussion

Evolution of Enhancer Structure–Function

The D. melanogaster S2E rescue transgene, and its considerably diverged D. pseudoobscura ortholog, each restore complete eve stripe 2 biological activity when placed in a genetic background lacking a native S2E. The DNA fragment we investigated, therefore, entails both the biological and evolutionary units of enhancer function. We chose this fragment based on its extensive prior characterization, including genetic, reverse genetic, and footprinting analyses [6,16,17,18]. In particular, Stanojevic et al.’s [18] TF footprinting data appear to have nicely delineated the functional enhancer.

Our previous experiments with S2Es of these two species demonstrated that both intact enhancers, but not the chimeras between them, drive the correct spatiotemporal pattern of reporter gene expression [11]. The rescue experiments reported here extend this finding by showing that the two orthologs are in fact biologically indistinguishable. These new results reinforce our contention that the phenotypic character—early stripe 2 expression—must be under stabilizing selection. The character itself remains unchanged over evolutionary time despite substitutions in nearly all the TF-binding sites, the gain and loss of some of them, and considerable change in the spacing between sites. This suggests to us that unlike proteins, where functional conservation usually means selective constraint on important amino acids (such as the active site of an enzyme), enhancers have a more flexible architecture that allows modification, and perhaps even turnover, of their “active” sites. Dissimilarities in the structure–function of enhancers and proteins result in different emergent “rules” of molecular evolution.

But the fact that the D. melanogaster and D. pseudoobscura S2Es are biologically indistinguishable does not necessarily imply that enhancer function has been evolutionary static.
Rather, the similar biological activities appear to be the result of convergence. In particular, phylogenetic analysis of S2E sequences indicates that the bcd-3 binding site in *D. melanogaster* was acquired only recently in the lineage leading to *D. melanogaster* (see Figure 2B). (There are also lineage-specific deletions in the spacers flanking both sides of the bcd-3 site in the *D. melanogaster* lineage, which shift the proximal and distal repressors giant and Kruppel binding sites, respectively, closer to this bicoid site. These length changes may have coevolved to enable or increase local repression of this novel activator site.) The bcd-3 site was shown by Small et al. [6] to be required for MSE stripe expression. It seems likely, therefore, that the ancestral S2E lacking this binding site would not properly activate stripe 2 expression in *D. melanogaster*. Perhaps the sensitivity of the enhancer (or more precisely, the fragment investigated) to activator signals has oscillated over evolutionary time, in which case the similarity between the two distantly related species’ S2Es would be an example of functional convergence.

The fact that the S2E fragment from *D. erecta* is essentially unresponsive to the *D. melanogaster* morphogen-gradient environment, but the precisely orthologous segment from *D. melanogaster* (and *D. pseudoobscura*) responds properly, proves that this fragment must contain evolved differences of functional significance between the species. The lack of biological activity of the *D. erecta* transgene in *D. melanogaster* should perhaps come as no surprise, however: Its lower sensitivity to activation may represent the ancestral state of the enhancer. What is surprising is the rapidity with which these functional differences evolve.

Phylogenetic footprinting of distantly related species can readily identify strongly conserved motifs [19] but runs the risk of not detecting enhancers that have retained their function but have evolved structurally. To overcome this, a technique called phylogenetic shadowing—the comparison of noncoding sequences among closely related species—has recently emerged [9]. Our results show that there is no necessary relationship between enhancer phylogenetic (or sequence) relatedness and functional similarity. Closely related species cannot be assumed to be more functionally conserved than distantly related species in enhancer structure-function.

Why Is the *D. erecta* S2E Transgene Not Functional in *D. melanogaster*?

*D. erecta* produces a native early *eve* stripe 2. Why then does the S2E fragment from this species not produce a robust early stripe when placed in *D. melanogaster*? The first possibility is that the fragment we investigated no longer contains a functional enhancer and has been replaced by an equivalent enhancer somewhere else in the *eve* locus. This possibility can easily be ruled out: The overall architecture of the *eve* locus, including all of its 5' and 3' enhancers, is well conserved, and there is no new cluster of the appropriate TF-binding sites that could act as a S2E. Another unlikely possibility is that the locus has been duplicated, and the fragment we investigated has become functionally inert (i.e., equivalent to a pseudogene). There is no indication of a duplicated *eve* locus in the *D. erecta* genome, and all features of the *eve* locus (including its S2E) are intact and do not indicate any degeneration.

This leads us to conclude that the *D. erecta* fragment used in our experiments contains the S2E. We can consider three additional possibilities. The first is that this fragment is no longer the complete biological unit, that is, novel binding sites have evolved in this species distal or proximal to this fragment, which have become assimilated into the active enhancer by a process we call accretion. As Figure 7 shows, *patser*, a binding-site prediction program [20] identifies a single potential bicoid-binding site 135 bp upstream of Block-A (Figure S1), the distal end of the *D. erecta* S2E transgene. This potential site contains an unconventional bicoid-binding motif, TCAATCC. The next closest potential binding site is another 350 bp further upstream and also has an unconventional binding-site sequence (AACAATCGG). So, although we cannot rule out the possibility that these are biologically active sites that contribute to S2E activity, they are relatively distant from the recognized S2E (and other bicoid sites), and their sequences do not have the consensus core motif (TAATC). Future experiments will allow us to formally test whether this enhancer has physically expanded. If so, this would be the first documented case for accretion, the adaptive expansion of an enhancer.

The second possibility is coevolution between the *D. erecta* S2E and its promoter region, such that it is not capable of driving transcription properly from a *D. melanogaster* promoter. We also view this as unlikely for several reasons. First, prior to designing these experiments, we investigated this issue with the core promoters and S2Es of *D. melanogaster* and *D. virilis* (which is an outgroup to the species studied). We could detect no difference in spatial or temporal expression of each S2E with either promoter (Ludwig, unpublished data). Second, the core promoter regions of *D. melanogaster* and *D. erecta* are highly conserved, including complete preservation of both the TATAA and the GAGA site. Indeed there are only four nucleotide differences (and no indels) between the species in a 150-bp stretch containing these sites. Finally, one might expect most functional changes in the core promoter to be pleiotropic, given the presence of more than a dozen other separable enhancers in the *eve* locus, and therefore to be selected against.

The final possibility is that the *D. erecta* S2E fragment does not
contain the entire biological enhancer, but that the trans-acting environment—the morphogen gradients to which the enhancer responds—differ between the species, causing the enhancers to have evolved to accommodate the differences. In other words, the sensitivity, or set point, of the binary (on–off) switch function has coevolved with the trans-acting environment in order for the S2E to maintain the appropriate response to evolved activation inputs. This hypothesis implicates, in particular, evolutionary shifts in the bicoid and/or hunchback activator gradients. As noted above, there has been a lineage-specific addition of the functionally required bcd-3 binding site [6] in D. melanogaster that is not present in any of the other species. Second, there is also a lineage-specific loss of the hunchback-1 (hb-1) site in D. erecta (which may be present in its sister taxon, D. yakuba). We propose that the lack of sites for these activators, and the presence of a species-specific six-base-pair insertion in the overlapping hb-2kr-2 binding sites (Figure S1) reduces the ability of the D. erecta enhancer to respond to the activator gradients of D. melanogaster. This hypothesis predicts stronger activator gradients in D. erecta than in D. melanogaster. Although we have not yet investigated this possibility directly, we note that native eve blastoderm stripes do not reside in the same physical locations in embryos of the two species, but rather are displaced posteriorly in D. erecta compared to D. melanogaster (compare Figure 1A and 1C). A similar effect can be mimicked in D. melanogaster with the addition of extra copies of bicoid gene [21], which shifts the morphogen gradient posteriorly.

The possible independence of spatiotemporal and rheostat activities [22] relates to a long-standing issue in evolutionary genetics—whether developmental constraints are “tunable” [23]. One school holds that features of development are strongly canalized and that deviation from this path will be strongly selected against. An opposing school holds that these developmental constraints can always be overcome by selection. The eve S2E may exhibit elements of both an immutable developmental constraint and a smoothly evolving
trait. Primary pair-rule stripes, such as those laid down by eve in developing blastoderm embryos, establish the positional landmarks that will eventually demarcate segmental identities in the fly. This complex functional network established by gap and pair-rule gene expression imposes strong constraints on spatiotemporal patterns of regulatory gene expression. The S2E must, for example, produce a stripe equidistant from stripes 1 and 3, and at a specific location with respect to other pair-rule genes.

The potential for evolutionary change in the S2E set point or output, on the other hand, may be much less constrained. Genetic variability in the gain and loss of binding sites, polymorphism in binding sites leading to variation in TF binding, and modulation of TF interactions through changes in spacing between binding sites should allow for nearly continuous fine-tuning of these functions. Enhancers should be able to adapt to change in their trans-acting environment. Why might the trans-acting environment for the S2E be evolving? Residing at the head of the hierarchal cascade driving the formation of the A-P axis, perhaps the bicoid morphogen gradient is less constrained than the expression of downstream genes that are more deeply embedded in the interaction network. Or, perhaps there has been natural selection acting on traits such as egg shape, size, or ovariole morphology, leading to changes in the bicoid-diffusion gradient. Egg number and size are, after all, fundamental evolutionary trade-offs. With a properly designed genetic experiment it should be possible to test these hypotheses.

Enhancer Evolution in Relation to Speciation

Postmating isolation is the final step in the speciation process because it involves genetic changes between incumbent species that cause hybrid inviability or infertility. Hybrid breakdown is likely to involve evolutionary changes in at least two interacting genes [24,25]. According to this model, the coevolution of a “speciation” gene with its partner(s) allows it to remain functional in its native background but lethal in the hybrid background. One of the mysteries of this process is the regularity and quickness with which molecular incompatibility arises. Thus, for example, exceedingly closely related species, such as D. simulans, D. sechellia and D. mauritiana, whose common ancestors occurred less than 1 MYA, nevertheless, have evolved postmating isolation involving perhaps hundreds of genes [26]. The question is what components of the genome are involved in this coevolutionary conspiracy?

Cis-regulatory modules and the trans-acting TFs with which they interact provide abundant genetic substrates for coevolution leading to hybrid sterility and inviability [2,5]. If our experiments have captured the entire biological S2Eas of D. erecta and D. yakuba, then the results suggest that this coevolution could involve changes in expression patterns or levels, rather than changes in the protein sequences of the trans-acting factors. The attractiveness of this hypothesis lies in the fact that there must be many more cis-regulatory modules in a eukaryotic genome than there are encoded proteins. Thus there is ample opportunity for the coevolution of enhancers and trans-factors to produce lethal interactions in hybrids, which may explain the abundance of lethal interactions between closely related species.

The regulation of development is often modeled as a logic circuit, with cis-regulatory sequences functioning as switches controlling information flow. The long-term functional preservation of both the spatiotemporal and the activation strengths of the D. melanogaster and D. pseudoobscura S2Es speaks to the general conservation of this genetic network in fruit fly development. Our results also provide an indication that the stoichiometry of the regulatory components could matter critically for normal development, at odds with theoretical predictions [27]. Epistatic changes accompanying interspecific inviability and sterility may therefore arise more readily as a consequence of quantitative shifts in gene expression than as a result of alterations in the topology of the developmental circuits.

Materials and Methods

**Drosophila strains.** Df(2R)eve (D[ eve]) and eve913 (R13): D[ eve] is a deficiency that includes at least three lethal complementation groups [28]. The R13 is null mutation that truncates the protein within the homeodomain [12]. These lethal mutations were balanced over marked balancer chromosome CyO (P{hvb-lacZ}) to allow identification of mutant embryos by immunostaining for β-galactosidase or by PCR analysis for β-galactosidase gene.

**Analysis of embryos.** Histochemical staining with guinea pig polyclonal a-Eve [29] at 1:1,000 dilution, or with a-En monoclonal 4D9 [30] at 1:10 dilution was visualized using HRP-DAB enhanced by nickel [51]. Fluorescent staining of Drosophila embryos with polyclonal a-Eve [29] at 1:1,000 dilution was visualized using Alexa Fluor-594 goat antiguinea pig IgG antibodies (Molecular Probes, Eugene, Oregon, United States) at 1:400 dilution.

**Optical Z-sectioning** (0.8-μm step) of fluorescent embryos was carried out using motorized microscope Axioplan2 (Carl Zeiss, Thornwood, New York, United States), Hamamatsu C4742-95 camera (Hamamatsu City, Japan), and “Openlab” software (Improvision, Emeryville, California, United States).

**Genotyping.** Individual embryos were genotyped after imaging, using a PCR method (three pairs of fluor-labeled PCR primers) and Beckman Coulter CEQ 8000 genetic analysis system for PCR fragment analysis (Allendale, New Jersey, United States; for detailed protocol see Supporting Information). Three sets of fluor-labeled primers (Prodigo, Boulder, Colorado, United States) were used for PCR: P{hvb-lacZ}; (1) marker for SML(CYO) balancer second chromosome (156 bp), +106 adh: 5’TCTGGGAGCCATTTGGTCTGA3’ and –241 lac: 5’TGGGCCCTTCTGGATTAGC3’ and –421 lac: 5’TGGGCCCTTCTGGATTAGC3’; (2) EVE AES2E and native eve locus: 592 bp and 113 bp markers for native S2E and S2E with 380 bp MseI deletion, respectively, +958 F: 5’TAAATCGACAGGCGGATCTC3’ and –115 DF: 5’TCTCCGAGATCGCCTGAGT3’; (3) DMPSRER, 3rd chromosome microsatellite marker for TM3 balancer and rescue transgene-containing chromosome III, DMPROSFR: 5’CGTGATACAAATGTGGTCTC3’ and DMPROSFR: 5’CGTGATACAAATGTGGTCTC3’ and DMPROSFR: 5’CGTGATACAAATGTGGTCTC3’ and DMPROSFR: 5’CGTGATACAAATGTGGTCTC3’ and –421lac: 5’TGGGCCCTTCTGGATTAGC3’; (4) DMPSRER, 3rd chromosome microsatellite marker for TM3 balancer and rescue transgene-containing chromosome III, DMPROSFR: 5’CGTGATACAAATGTGGTCTC3’ and DMPROSFR: 5’CGTGATACAAATGTGGTCTC3’ and –1554 (BstEII) to –1573 kb (BstEII) relative to eve transcription start site (see Figure 2).

**S2E rescue constructs (S2E-EVE).** The S2Es used in this study were cloned previously [10], and their accession numbers are given as supplemental information. The sequences employed in this study are presented in Figure S1; they all begin and end at conserved sequences flanking the S2E (blocks A and B).

The S2E-eve rescue transgenes based on our modification of the Eve pCaSpeR vector provided by M. Fujioka [32] were constructed as follows: 2.76-kb eve pCaSpeR (negative control construct, S2E+/EVE) carries the D. melanogaster wild-type eve genomic DNA fragment from –913 (FspI) to +1.85 kb (BamHI) relative to eve transcription start site, which includes 913 bp intact eve 5’ upstream region, protein-coding sequence, and the polyadenylation site. A unique restriction site Pmel was created instead of the FspI site, so that S2E from different species could be cloned into the 2.76-kb eve pCaSpeR vector by using unique Pmel and NotI sites in the proper orientations. The entire eve region in rescue constructs from D. melanogaster and D. erecta, including both the S2E and eve genomic DNA fragment from –913 to +1.85, were confirmed by sequencing.

**P-transformation.** P-element-mediated germline transformation.
was carried out as described by Rubin and Spradling [33]. A homozygous viable stock with the S2E deficiency transgene P(EVEAS2E) on chromosome II was recombined onto eve mutant chromosomes—either Dif(efe) or eveR13—to create lines that can only drive eve expression from the EVEAS2E transgene. These chromosome combinations are maintained as balanced stocks.

Rescue constructs (S2E-EVE) were chosen for use in the study if they were homozygous viable and were on chromosome III. Between 2 and 4 independent stable transformed lines were generated for each rescue construct and were examined for rescue ability to adulthood and for local eve and en pattern rescue.

Rescue of eve function to adulthood. Each transgenic rescue line was crossed into the evedf17 (R13) P(EVEAS2E) mutant background, generating flies of the genotype w; R13 P(EVEAS2E) or P(S2EAS1-EVE/TM3) Sb. These flies were reciprocally crossed with flies of the genotype w; R13 P(EVEAS2E); P(S2EAS1-EVE/TM3) Sb. A1 and A2 indicate independent transgenic lines with the identical rescue construct (see Figure 2). Adults were scored for both a wild-type wing phenotype (non-CyO) and the black (b) phenotype (indicating R13 homozygotes).

For each reciprocal cross, 100 healthy virgin females from one strain were mated in a standard culture bottle with 100 healthy males from the other. Parents were transferred to fresh culture bottles every 3 d for 24 d. The emerging adult offspring were collected every day from the culture bottles for a period of 10 d for scoring. This approach ensured that mutants with slow development rates were counted. The cultures were kept at 25 °C.

Viability of flies carrying one or two copies of the rescue transgene was measured relative to the number expected based on the count of flies carrying one copy of the dominantly marked second and third chromosome. This genetic design allowed us to estimate viability effects of transgene insertion independent of transgene rescue ability by comparing genotypes carrying one (hemizygous) or two (doubly hemizygous) copies of the rescue transgene in genotypes carrying a wild-type eve locus (i.e., EVEAS2E, R13O; S2E-EVE/TM3 versus EVEAS2E, R13O; S2E-EVE/EVE; see Table S1). Viabilities of rescue-transgene lines were calculated by comparing the number of adult survivors in EVEAS2E homozygotes (EVEAS2E, R13EVE/EVE) relative to the number of survivors in the corresponding genotype with one copy of a functional eve locus (EVEAS2E, R13O; S2E-EVE/ EVE; S2E-EVE).

Localised rescue of eve and en expression patterns. Each transgenic rescue line was crossed into the Dif(efe) P(EVEAS2E) mutant background, generating stock w; Dif(efe) P(EVEAS2E) or P(bb-lacZ); P(S2E-

Embryos of these stocks were stained with anti-Eve and anti-En-Neuroglian antibodies to determine the pattern and level of gene expression. The embryos inspected for eve expression patterns were mated in a standard culture bottle with 100 healthy males from the other. Parents were transferred to fresh culture bottles every 3 d for 24 d. The emerging adult offspring were collected every day from the culture bottles for a period of 10 d for scoring. This approach ensured that mutants with slow development rates were counted. The cultures were kept at 25 °C.

Viability of flies carrying one or two copies of the rescue transgene was measured relative to the number expected based on the count of flies carrying one copy of the dominantly marked second and third chromosome. This genetic design allowed us to estimate viability effects of transgene insertion independent of transgene rescue ability by comparing genotypes carrying one (hemizygous) or two (doubly hemizygous) copies of the rescue transgene in genotypes carrying a wild-type eve locus (i.e., EVEAS2E, R13O; S2E-EVE/TM3 versus EVEAS2E, R13O; S2E-EVE/EVE; see Table S1). Viabilities of rescue-transgene lines were calculated by comparing the number of adult survivors in EVEAS2E homozygotes (EVEAS2E, R13EVE/EVE) relative to the number of survivors in the corresponding genotype with one copy of a functional eve locus (EVEAS2E, R13O; S2E-EVE/ EVE; S2E-EVE).

Localised rescue of eve and en expression patterns. Each transgenic rescue line was crossed into the Dif(efe) P(EVEAS2E) mutant background, generating stock w; Dif(efe) P(EVEAS2E) or P(bb-lacZ); P(S2E-

Image processing and EVE quantification. eve stripes 1, 3, 4, 5, 6, and 7 are always produced from the EVEAS2E locus in our experimental flies, whereas eve stripe 2 comes primarily from the independent S2E-EVE rescue transgene. This allowed us to compare stripe 2 expression levels in different embryos and genotypes by measuring stripe 2 expression relative to an adjacent stripe. We chose the adjacent stripe 3 as a reference, as it has similar temporal and quantitative expression, and we report the stripe 2 expression relative to stripe 3. Image stacks (0.8 μm) were deconvoluted in Huygens Essential (version 2.5.6) from Scientific Volume Imaging (Hilversum, the Netherlands) and 3–5 images in the focal plane collapsed to a single image in Image J (version 1.30, free at http://rsb.info.nih.gov/ij). All were subjected to the same background subtraction and a section corresponding to stripes 1 to 4 in the middle series of the embryo cropped out and saved as a raw pixel file for analysis in Mathematica 5 (www.wolfram.com). The location of each stripe was estimated by fitting second order curves to local maxima/minima of smoothed data, identifying stripes 1 through 4 and the interleaving troughs. The approach worked reasonably, as of 2015 cases analyzed on the rescue transgene for each species, there was agreement between the algorithm and the visual inspection of a detectable trough between stripes 1 and 2 during early stages of embryo development.

The fitted curves were superimposed on the raw data and used as guides for the summarizing of the signal intensity, from the middle 25%, 50%, or 75% of the stripes and the 25% of the troughs. These percentages were derived from the total length between stripes 1 and 4, and therefore represent comparable geometric areas. This is important as we proceed to compare abundances in stripes 2 and 3. Percentages of stripes were used to account for size variation as variables extracted on the basis of absolute pixels were noisier. The measurements were summed from 5 to 11 sections per embryo, each 30-pixel high. The dependence on variables analyzed were ranked from the measured fluorescence in stripe 2 (P2) over stripe 3 (P3) after adjusting the measured background in the separating trough (T2), in general form: Y = (P2 – T2)/(P3 – T2). These variables were derived for the middle 25%, 50%, and 75% of the stripes, but all yielded similar results, and we report on the 75% case. For every embryo we documented a DV index, its degree of rotation along the dorsal–ventral axis (divided into five categories, dorsal, dorsal–middle, middle, middle–ventral, and ventral). In addition we placed the embryos into a series (five classes) around cellularization corresponding to approximately 45 min of development, when the EVE stripes originate and take form (see Figure 3A–3E). The series is based on classes 4–8 for the 14th cleavage cycle available on the FlyEx Web site at http://flyex.ams.sunysb.edu/flyex/ [34].

ANOVA on eve expression. Mixed-model ANOVA was fitted in SAS v8.2 (2002; SAS Institute, Cary, North Carolina, United States) with the main effects of “dose,” that is, how many copies of rescue construct; “species,” designating the origin of the S2E; and a “DV index,” which accounts for orientation, along the dorsal–ventral axis, of measured embryos. Also, “time,” indicating the inferred stage of development, was used as a covariate. Finally, as each embryo was measured several times, we avoided pseudoreplication by nesting the random variable “embryo” within the fixed effects.

The results were identical if the multiple recordings on each embryo were designated as repeated measures within the Proc Mixed statement. We also applied a generalized linear model to the least square mean of the phenotypic values for each embryo. Again results were in agreement (Table 1 and Table S3) as the related model, excluding “species” terms, was used to evaluate the “dose” effects in the EVEAS2E stock. We also investigated the general capacity of the D. pseudoobscura construct to reconstitute the activity of the endogenous gene. The stock with EVEAS2E carried over a Cy balancer was used, and embryos of all three genotypes were collected. The mixed model was constructed in the same way, adding “experiment” as a term. All stocks were constructed to have the same genetic background, and we predicted that individuals homozygous for the EVEAS2E in both datasets would have similar expression. This was corroborated by ANOVA with a reduced mixed model, F(1, 57) = 1.70, p = 0.29, n = 71. Similarly, the EVE expression of the homozygous balancer could not be distinguished from a wild-type strain w1118: F(1, 63) = 1.00, p = 0.32, n = 76 (see Figure 3G).

Using standard quantitative genetic theory [35], we assessed the genotypic effects of the S2E on eve expression in the D. pseudoobscura transgenes and the endogenous gene. The small sample size prohibited formal tests of deviations from additivity.

Sequence analysis. Comparative sequence analysis was conducted with the Avid-Vista suite (http://www-gsd.lbl.gov/vista), using default parameters [36,37]. Binding-site prediction was performed using patser [20] with identical command-line arguments and position-weight matrices used by Berman et al. [38]. Cutoffs for display of predicted binding sites were set to recover all known binding sites in the D. melanogaster S2E–ln(P) = −6.1 for bicon, ln(P) = −8.06 for hunchback, and ln(P) = −6.65 for Kruppel.

Supporting Information

Figure S1. Alignment of eve S2E Regions from Four Species of Drosophila

Gaps in aligned sequences are indicated by dashes. The binding sites in D. melanogaster for the trans-acting factors, bicon (bed), hunchback (hb), Kruppel (kr), sloppy-paired (slp), and giant (gt), are shown above the sequence. The sequences from the four species begin and end at completely conserved sequences, indicated by blocks A and B, flanking the enhancer. mel: D. melanogaster; yak: D. yakuba; eve: D. erecta; pse: D. pseudoobscura.

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Figure S2. Trans-Factor-Binding Sites in D. melanogaster S2E and Homologous Sequences from Three Other Drosophila Species
Binding sites are for five proteins are designated: bicoid (bcd), hunchback (hnb), Kruppel (kr), sloppy-paired (slp), and giant (gt). A: no homologous sequence identified. mel: D. melanogaster; yak: D. yakuba; ere: D. erecta; pse: D. pseudoobscura. The figure is expanded from Ludwig et al. [10] and Ludwig [2].

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Protocol S1. Additional Materials and Methods

Found at DOI: 10.1371/journal.pbio.0030093.sd001 (29 KB DOC).

Table S1. Viability to Adulthood

Adult viability of individuals from reciprocal crosses between two independent transgenic lines with eve 2EVC from the four species and from a negative control (2EVC-EVE); See Figure 2 for constructs and crossing scheme. With Mendelian segregation of the 2nd and 3rd chromosomes, ratios of 4:2:2:1 for the genotypic classes are anticipated. This was used to calculate expected counts and rescue percentages (in brackets) for the classes with one and two copies of rescue constructs. Adjusted rescue percentages, which account for possible detrimental effects of two hemizygous transgenic inserts, are also reported. These data summarized over sexes are represented in Figure 4. Found at DOI: 10.1371/journal.pbio.0030093.s001 (31 KB DOC).

Table S2. ANOVAs on EVE Abundance in Stripe 2

ANOVA on EVE quantity. (A) Analysis on amounts of EVE in stripe 2 D. erecta and D. pseudoobscura rescue data (B) on the rescue data for all four species and (C) the EVEAS2EVC stock. Mixed-model ANOVA (left) fitted the multiple measures per embryo as random. Generalized linear model (right) was fitted on the least square means calculated for each embryo. “Dose” indicates the dose number of rescue transgene per embryo; “Time” indicates a continuous variable of the developmental series; “Species” indicates the origin of the 2EVE; “DV index” indicates orientation of each embryo along the dorsal-ventral axis. VC indicates variance components for “embryos” or residual error, with estimated standard errors and significance according to the z-distribution (VCs can only be estimated with a mixed model). ns, p > 0.05; *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001. Found at DOI: 10.1371/journal.pbio.0030093.s002 (93 KB DOC).

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Competing interests

The authors have declared that no competing interests exist.

Author contributions

MZL and MK conceived and designed the experiments. MZL performed the experiments with EA and JN helping with embryo staining and genotyping. CMB conducted genomic sequence analysis. AP analyzed images. MZL, AP, and MK analyzed the data. MZL and EA contributed reagents/materials/analysis tools. MZL, AP, and MK wrote the paper.

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