

Functional analysis of *eve* stripe 2 enhancer evolution in *Drosophila*: rules governing conservation and change

Michael Z. Ludwig^{1,2,*}, Nipam H. Patel^{2,3} and Martin Kreitman¹

¹Department of Ecology and Evolution, University of Chicago, 1101 E. 57th Street, Chicago, IL 60637, USA

²Department of Organismal Biology and Anatomy and ³Howard Hughes Medical Institute, MC1028, N-101, 5841 S. Maryland Avenue, Chicago, IL 60637, USA

*Author for correspondence (e-mail: mludwig@midway.uchicago.edu)

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SUMMARY

Experimental investigations of eukaryotic enhancers suggest that multiple binding sites and *trans*-acting regulatory factors are often required for wild-type enhancer function. Genetic analysis of the stripe 2 enhancer of *even-skipped* (*eve*), an important developmental gene in *Drosophila*, provides support for this view. Given the importance of *even-skipped* expression in early *Drosophila* development, it might be predicted that many structural features of the stripe 2 enhancer will be evolutionarily conserved, including the DNA sequences of protein binding sites and the spacing between them.

To test this hypothesis, we compared sequences of the stripe 2 enhancer between four species of *Drosophila*: *D. melanogaster*, *D. yakuba*, *D. erecta* and *D. pseudoobscura*. Our analysis revealed a large number of nucleotide substitutions in regulatory protein binding sites for bicoid, hunchback, Kruppel and giant, as well as a systematic change in the size of the enhancer. Some of the binding sites in *D. melanogaster* are either absent or modified in other species. One functionally important bicoid-binding site in *D. melanogaster* appears to be recently evolved.

We, therefore, investigated possible functional consequences of sequence differences among these stripe 2

enhancers by P-element-mediated transformation. This analysis revealed that the *eve* stripe 2 enhancer from each of the four species drove reporter gene expression at the identical time and location in *D. melanogaster* embryos. Double staining of native *eve* protein and transgene mRNA in early embryos showed that the reporter gene mimicked native *eve* expression and, in every case, produced sharply defined stripes at the blastoderm stage that were coincident with *eve* stripe 2 protein.

We argue that stripe 2 *eve* expression in *Drosophila* evolution can be viewed as being under constant stabilizing selection with respect to the location of the anterior and posterior borders of the stripe. We further hypothesize that the stripe 2 enhancer is functionally robust, so that its evolution may be governed by the fixation of both slightly deleterious and adaptive mutations in regulatory protein binding sites as well as in the spacing between binding sites. This view allows for a slow but continual turnover of functionally important changes in the stripe 2 enhancer.

Key words: Evolution, *Drosophila*, Transcription, Enhancer, *even-skipped*

INTRODUCTION

Differential gene expression during development can now be described in terms of the interactions between *cis*-acting elements and *trans*-acting factors (Arnone and Davidson, 1997). Recent success in correlating the expression of key regulatory factors in development with major evolutionary features of arthropod and vertebrate body plans is predicated on the parallel assumption that evolutionary differences in gene expression will also be understood in terms of specific changes the same *cis*- and *trans*-acting factors. Since many of the *trans*-acting regulatory factors are highly conserved proteins, it has been suggested that regulatory evolution is brought about primarily by substitutions in *cis* regulatory sequences rather than in the proteins themselves (Averof et al., 1996).

A detailed experimental dissection of a regulatory sequence has been carried out for the stripe 2 enhancer governing the transcriptional regulation of a pair-rule gene, *even-skipped* (*eve*) in *Drosophila* (Stanojevic et al., 1991; Small et al., 1992; Arnosti et al., 1996). *eve* encodes a homeodomain-containing protein that is expressed during embryonic development in both arthropods and vertebrates (Kenyon, 1994; Patel et al., 1994). The spatial and temporal patterns of embryonic expression of *eve* are tightly regulated in *D. melanogaster*; but are different among diverse insect species, such as the fruitfly, grasshopper and beetle (Patel et al., 1994). In *Schistocerca*, for example, *eve* does not exhibit a pair-rule pattern of expression (Patel et al., 1992), whereas in *Tribolium* it does show pair-rule expression, but with the formation of segments proceeding sequentially rather than simultaneously, as in *Drosophila* (Patel

et al., 1994). In *Schistocerca*, vertebrates and *C. elegans*, *eve* homologs are expressed in the posterior regions of embryos, suggesting a role in the specification of posterior identities rather than defining segmental boundaries throughout the embryo (Patel et al., 1992, 1994; Ruiz i Altaba and Melton, 1989; Bastian and Gruss, 1990; Ahringer, 1996). Indeed, mutations in *C. elegans vab-7* (the *eve* ortholog) lead to the deletion of posterior structures (Ahringer, 1996).

eve plays a key role in the segmentation process of *D. melanogaster* (Nusslein-Volhard et al., 1985). Prior to the completion of cellularization, *eve* is expressed in series of seven transverse stripes in the blastoderm (Frasch and Levine, 1987). The transcriptional regulation of these stripes is complex. The *eve cis*-regulatory region contains a series of separate modular enhancers that control the expression of individual stripes (Goto et al., 1989; Harding et al., 1989; Small et al., 1992, 1996). Stripes 2 and 3 are controlled by nonoverlapping enhancers which are separated by approximately 1.5 kb of 'spacer' sequence (Small et al., 1993, 1996). The stripe 3 enhancer sequence also regulates the expression of stripe 7 (Small et al., 1996). Short-range repression permits these enhancers to direct transcription from a common promoter independently (Small et al., 1993; Gray et al., 1994).

Extensive in vitro mutagenesis of *trans*-acting factor-binding sites in a 480 bp Minimal Stripe 2 Element (MSE) has allowed the refinement of a mechanistic model for stripe 2 activation and repression (Small et al., 1992; Arnosti et al., 1996). The *D. melanogaster* MSE contains 12 transcription factor-binding sites, including six activator and six repressor sites. According to the model, binding of bicoid and hunchback in the MSE is required for the activation of *eve* transcription in the anterior half of the embryo. The stripe borders are determined by the activities of two repressors, giant in anterior region and Kruppel in posterior region (Arnosti et al., 1996). The fact that four of six bicoid and hunchback activator sites directly overlap giant or Kruppel repressor sites led to the early suggestion that these repressors might define the stripe borders through competition for shared binding sites (Stanojevic et al., 1991; Small et al., 1992). However, the overlap of giant-binding sites with bicoid and hunchback sites has recently been shown not to be essential for giant function in vivo (Arnosti et al., 1996). The authors interpreted this result as indicating that giant and Kruppel repress *eve* transcription at stripe boundaries by a mechanism involving short-range quenching.

In an attempt to understand the relationship between sequence evolution and enhancer structure-function, we previously compared the stripe 2 enhancer region in population samples of the sibling species, *D. melanogaster* and *D. simulans* (Ludwig and Kreitman, 1995), in another closely related species, *D. erecta* (Kreitman and Ludwig, 1996), and in a distantly related species, *D. picticornis* (Sackerson, 1995). Interspecific comparisons revealed that most, but not all, of the *D. melanogaster* homologs of the bicoid, hunchback, Kruppel and giant protein-binding sites were present in the other species. One binding site for bicoid, bcd-3, was not present in either *D. erecta* or *D. picticornis*, indicating that it was a recently evolved site in *D. melanogaster* (Kreitman and Ludwig, 1996). Surprisingly, this site has been experimentally shown to be functionally important in the *D. melanogaster* MSE (Small et al., 1992). Nucleotide substitutions were

present in the majority of binding sites; only three binding sites were completely conserved in all of the species. The spacer regions separating adjacent binding sites also differed in sequence as well as in length. Similar mutational changes were found segregating as polymorphisms within species (Ludwig and Kreitman, 1995).

Here we investigate whether the evolutionary changes in the stripe 2 enhancers of four *Drosophila* species have any discernible effects on the timing or spatial localization of stripe 2 expression. A functional analysis of the *eve* stripe 2 enhancers from *D. melanogaster*, *D. yakuba*, *D. erecta* and *D. pseudoobscura* was carried out using a reporter gene in P-element-mediated transformants. We were able to spatially and temporally localize stripe 2-driven *lacZ* in early embryos by using a double staining technique for native *eve* protein and *lacZ* mRNA, and by including the *melanogaster* stripe 3+7 *eve* enhancer as an internal standard in our constructs. The goal of this work is to relate patterns of natural sequence variation in the *eve* stripe 2 enhancer to our experimentally derived understanding of its function.

MATERIALS AND METHODS

Drosophila stocks

D. erecta (stock number 1013) was obtained from the *Drosophila* Species Stock Center in Bowling Green. *D. yakuba*, *D. pseudoobscura* (Est-5^{1.00}) and *D. melanogaster* Oregon RC were obtained from Michael Ashburner, R. Richmond and C. I. Wu, respectively. Transgenic lines of *D. melanogaster* containing a stripe 2,3+7-*lacZ* gene fusion construct was kindly provided by S. Small (line 51 and 55 transformed with construct 2; see Small et al., 1993).

Cloning, amplification and sequencing

Genomic DNA was prepared from single adult male flies as previously described (Ludwig and Kreitman, 1995). The *eve* regulatory region of *D. erecta*, containing part of the coding region, the proximal promoter and part of the stripe 2 regulatory element, was cloned as a PCR fragment of approximately 1600 bp length. Primers for the amplification of this fragment (5'catcttctcggcgctttg3' and 5'ctgccgttcaaggagttatc3') were designed from conserved sequences in *D. melanogaster* and *D. simulans*. The 5' portion of the *D. erecta* stripe 2 element was obtained by inverse PCR (Ochman et al., 1989).

Two sets of universal primers for the amplification of the stripe 2 enhancer region were then designed from aligned sequence comparisons of *D. melanogaster* (Canton-S GenBank reference X78903), *D. picticornis* (Sackerson, 1995), and our sequences of *eve* from different alleles of *D. melanogaster* and *D. simulans* (Ludwig and Kreitman, 1995), and *D. erecta*. One set of primers (containing 5' *EcoRI* sites) –

U1+: 5'aaaagaattcatttctgctgctgagctg3' and

U2–: 5'aaaagaattctgrtctctyccatrttrta3', or

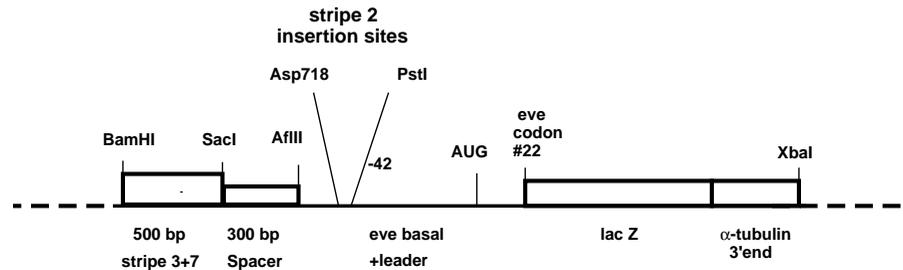
U3–: 5'aaaagaattcmtgccrttcarsgarttrtc3' – was used to amplify a region that spanned the autoregulatory region to the coding region of *eve*. This fragment was then reamplified with nested primers (containing 5' *Bam*HI or *Eco*RI sites) –

U4+: 5'aaaaggatccgagatcggcgctttgtgag3' and

U2–: 5'aaaagaattctgrtgrctyccatrttrta3', or

U3–: 5'aaaagaattcmtgccrttcarsgarttrtc3' – so that it extended from the spacer between the elements for stripes 3+7 and 2 to the *eve* coding region. All PCR fragments were cloned into the Stratagene vector, pBluescript II SK+. Sequencing templates were prepared from amplification of the cloned inserts with the universal M13 primers (–20) and Reverse. All sequences were determined for both strands

Fig. 1. Physical organization of the stripe 3+7 and stripe 2 enhancers in relation to the *eve* proximal promoter and *lacZ* reporter gene in the P-element vector, CaSpeR.



using template-specific primers. To eliminate PCR and cloning errors, each sequence was confirmed by sequencing PCR templates obtained directly from the amplification of genomic DNA. Sequencing was carried out on an Applied Biosystems Model 373a automated sequencer using TAG DyeDeoxytm terminator cycle sequencing chemistry, as described in Ballard and Kreitman (1994).

Stripe 2 elements

The region containing the stripe 2 element for each species was also obtained by PCR. A 2.5 kb fragment containing the stripe 2 element was amplified from DNA prepared from a single fly using primers U4⁺ and U3⁻. Nested primers based on conserved sequences, Kr6⁺: 5'aaaaggtaccaatataaccaataatt3' and U5⁻: 5'aaagaattcaaacatttattatgatataatca3', were then used to obtain the stripe 2 element from the previously amplified fragment. The stripe 2 enhancer sequences from the four species used in the transformation experiments, therefore, are identical in that they begin and end at completely conserved sequences flanking the enhancer, and they contain all the DNA between these conserved sites.

The primers, Kr6⁺ and U5⁻ contained the restriction sites for *Asp718* and *EcoRI*, respectively, at their 5' ends. Following digestion with these enzymes, the PCR fragments were cloned into *Asp718* and *EcoRI* sites of the plasmid. Inserts with correct sequences were identified for further use by sequencing independent clones.

P-element transformation vector

The organization of the transformation vector, CaSpeR *eve* 3, *lacZ*, is shown in Fig. 1. This construct contains a minimal *eve* stripe 3+7 enhancer (~500 bp), a 3' spacer (~300 bp), and the *eve* proximal promoter linked to *lacZ*. The stripe 3+7 enhancer and 3' spacer of *D. melanogaster* was obtained from the plasmid pE5.2 *lacZ* (Small et al., 1993) as an 800 bp *Bam*HI-*Afl*II fragment. pE5.2 *lacZ* contains a 5.2 kb *Pst*I fragment from the *eve* promoter inserted into the *Pst*I site at -42 of pELI (Lawrence et al., 1987). pELI *lacZ* contains the basal *eve* promoter (from -42), the intact 100 bp untranslated leader and the coding sequences for the first 22 amino acids of the *eve* protein fused to amino acid number 5 of the *lacZ* coding sequence. A fragment containing the minimal stripe 3+7 enhancer, the spacer and the *eve* proximal promoter-*lacZ* fusion was cloned into the P-element transformation vector CaSpeR (Thummel et al., 1988) using unique *Bam*HI and *Xba*I sites. A unique restriction site, *Asp718* (*Kpn*I), was inserted near the *Pst*I site located downstream of the spacer region, so that stripe 2 enhancer elements from different species could be cloned into this vector in the proper orientation. To accommodate this cloning strategy, unique restriction sites in the polylinker, *Pst*I and *Eco*RI, were eliminated. The stripe 2 and stripe 3+7 enhancers are separated in the final construct by the native 300 bp spacer to ensure autonomous regulation of the *lacZ* reporter (Small et al., 1993).

P-transformation and whole-mount in situ hybridization

P-element-mediated germline transformation was carried out according to Robertson et al. (1988). For each construct, at least one insertion in each of the three major chromosomes of *D. melanogaster* was generated to control for the influence of position effect on *lacZ*

expression (Ludwig et al., 1993). Between 6 and 10 independent stable transformed lines were generated for each construct and at least three independent lines were examined for *lacZ* expression. Embryos were doubly stained to allow simultaneous detection of *eve* protein and *lacZ* mRNA by in situ hybridization. *eve* protein was detected using an anti-*eve* monoclonal antibody (mAb 2B8; Patel et al., 1994), HRP-conjugated secondary antibody and DAB histochemistry, according to a rapid staining protocol with an addition of RNase inhibitor (Patel, 1994, 1996). *lacZ* mRNA was detected by in situ hybridization using a DIG-labeled antisense *lacZ* RNA probe. The preparation of the probes and the whole-mount in situ hybridization was carried out according to a protocol provided by S. Small (Jiang et al., 1991a).

Analysis of enhancer stripe 2 expression using a reporter gene

We evaluated the position of *lacZ* expression relative to native *eve* protein expression at a fixed timepoint in embryonic development. The expression of *lacZ* mRNA driven by the stripe 3+7 enhancer provided an internal control for the timing of development. The position of the experimental stripe 2-*lacZ* mRNA was evaluated when the *lacZ* mRNA stripes 3+7 precisely coincided with the corresponding stripes of the native *eve* protein. The experimental justification for this procedure is given in the Results section and in Fig. 2. As an additional way to assess the stage of development, we

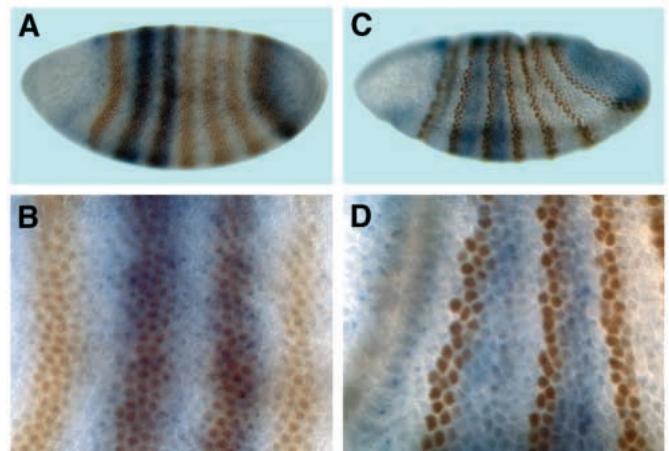


Fig. 2. *eve* protein (brown) and *lacZ* mRNA (purple) simultaneously detected in a transgenic line of *D. melanogaster* (provided by S. Small) transformed with the *D. melanogaster* stripe 2 (MSE), 3+7-*lacZ* gene fusion. (A) Stage 5(2)-5(3) blastoderm; (B) high magnification view of stripes 2 and 3, from A. (C) Early germ band extension (stage 6); (D) high magnification view of stripe 2 and 3, from C. (A,B) Embryos at the mid-cellularization stage, when the native *eve* expression and *lacZ* expression coincide along stripes 2, 3 and 7. (C,D) The discordance of native *eve* stripes relative to persistent *lacZ* mRNA stripes (see text for details).

also analyzed the extent of cellularization in each embryo. In all cases, our comparisons of stripe 2 expression occurred at mid-cellularization.

Alignment of DNA sequences

The *eve* stripe 2 regions from *Drosophila* species were initially aligned with ClustalV with gap penalty = 8 and length gap penalty = 2. Manual adjustment of this alignment was necessary to improve local alignments of some of the binding sites. The GenBank references for the sequences are AF042712 (*D. pseudoobscura*), AF042711 (*D. erecta*), AF042710 (*D. yakuba*) and AF042709 (*D. melanogaster*).

RESULTS

Characterization of eve stripe 2-binding site evolution

Inspection of the aligned sequences, shown in Fig. 3, allowed the identification of homologous sequences corresponding to many, but not all, of the *D. melanogaster* DNA-binding sites (Fig. 4). The spacers between the conserved binding sites vary in length and are not well conserved as a rule. Fig. 4 contains binding site sequences from *D. melanogaster*, which were identified by DNA footprinting (Stanojevic et al., 1991), and the homologous sequences from five other species. Included in this table are the sequences from the three species that are the subject of this study as well as the sequences from two other

species, *D. simulans* (Ludwig and Kreitman, 1995) and *D. picticornis* (Sackerson, 1995).

Two binding sites do not have obvious functional homologs in one or more species (Fig. 4). A hb-1 site is entirely absent in *D. erecta* and no corresponding sequence can be found in *D. pseudoobscura*. Only *D. melanogaster* has a viable bcd-3 site, TATAATCGC, including the required central motif, TAAT. The homologous sequences of the two related species, *D. yakuba* and *D. erecta*, TGCACCTCGG and TATGTATCGC, respectively, probably cannot be functional as bicoid-binding sites. No homologous sequence can be identified in *D. pseudoobscura*. The presence of the bcd-3-binding site only in *D. melanogaster* and *D. simulans* indicates that this is a relatively new site that evolved in the ancestral lineage leading to these two species.

Of the 17 known binding sites in *D. melanogaster* (see Figs 3, 4), only three (kr-6, kr-5 and bcd-5) are completely conserved among all six species. Many of the binding sites, however, differ by only one or two base changes, indicating that these sequences must be functionally constrained. Another indication of functional constraint is the fact that the vast majority of mutational changes in these binding sites occurred only once in the phylogeny of the species. For example, of 17 variable positions in the six Kruppel-binding sites (Fig. 4), only two of them have more than one mutational change. One of them, position 8 in Kr-1, has mutated (at least) twice to three

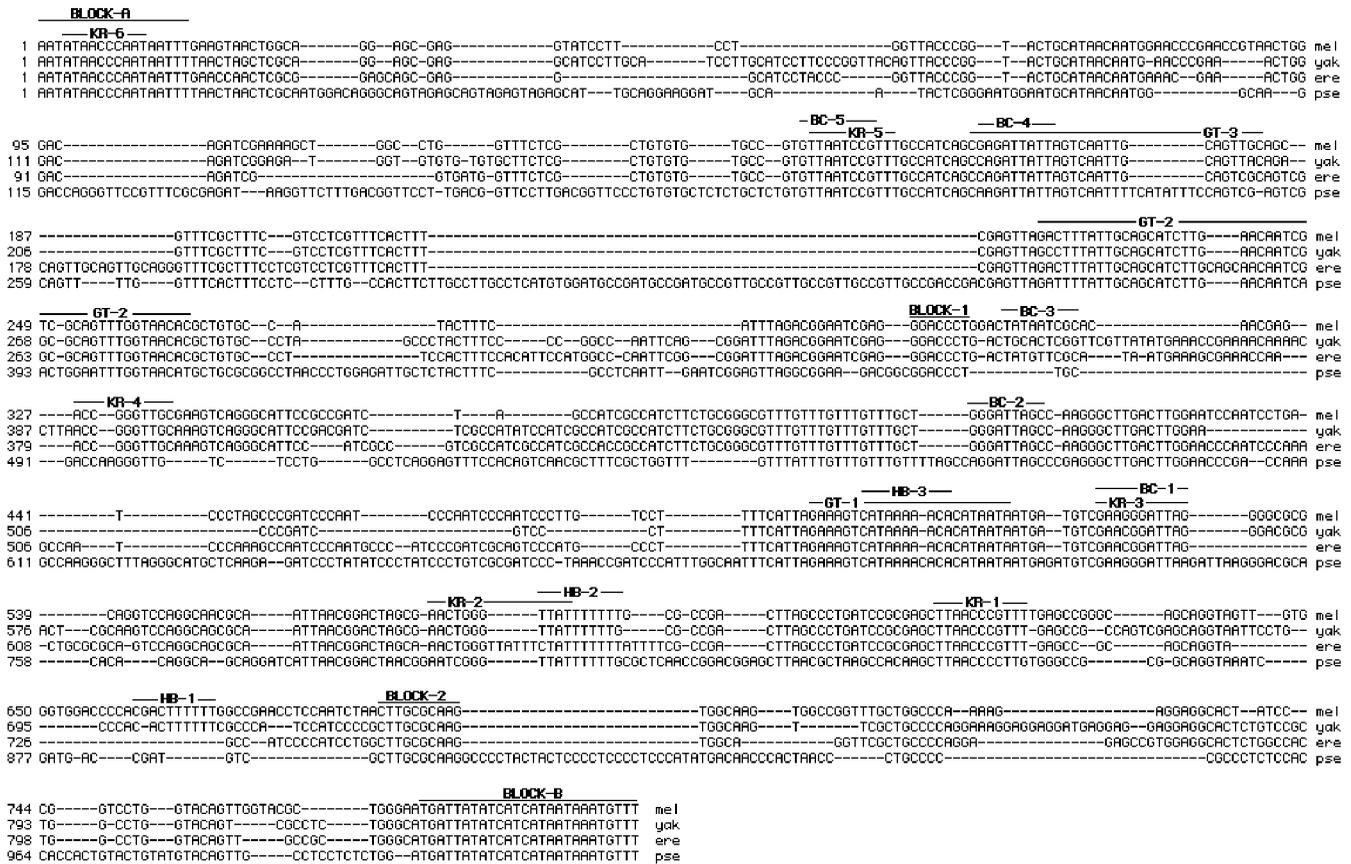


Fig. 3. Alignment of *eve* stripe 2 enhancer regions from four species of *Drosophila*. Gaps in aligned sequences are indicated by dashes. The binding sites in *D. melanogaster* for the *trans*-acting factors, bicoid (BC), hunchback (HB), Kruppel (KR) and giant (GT), are shown above the sequence. Blocks 1 and 2 are conserved sequences (see text for detail). *mel*, *D. melanogaster*; *yak*, *D. yakuba*; *ere*, *D. erecta*; *pse*, *D. pseudoobscura*.

nucleotides (G, C and A). The other doubly mutated site, position 4 in kr-3, is one in which non-sister species share a mutational change, G→C. The best available phylogeny of the *D. melanogaster* species subgroup (Jeffs et al., 1994), places *D. yakuba* as a sister-species of *D. melanogaster* and *D. simulans*, and *D. erecta* as the outgroup of these species. If this phylogeny is correct, then position 4 in kr-3 mutated once from G→C prior to the split of *D. erecta* from the other subgroup species, and mutated again back to G following the split of *D. yakuba* from its sister species. Thus, this site has a convergent mutation, G→C→G.

D. picticornis differs from *D. pseudoobscura* and the four *D. melanogaster* subgroup species at 11 nucleotide positions in Kruppel-binding sites. These sites are well conserved, however, in the five Sophophoran species: only one mutational difference can be found at these positions. Given the cumulative evolutionary time separating the five Sophophoran species, many of these sites must be functionally constrained in the Sophophoran species. It would appear, therefore, that essentially every individual nucleotide position in the six Kruppel sites is functionally constrained. This raises the interesting question, addressed in the discussion, as to whether the observed changes in these binding sites can be selectively neutral ones, given the strong indication that none of the sites at which these changes occur are free to evolve.

Length changes

The stripe 2 enhancer is bordered on the 3' and 5' sides by completely conserved blocks of 18 bp and 26 bp, respectively (marked as blocks A and B in Fig. 3). The *D. pseudoobscura* stripe 2 enhancer region, at 1027 bp, is 28% larger than the corresponding 798 bp region in *D. melanogaster*. The *D. erecta* and *D. yakuba* stripe 2 regions, 849 bp and 844 bp, respectively, are intermediate in length. We investigated the distribution of length changes across the stripe 2 enhancer by identifying all of the intervals separating conserved binding sites. We refer to these intervals as 'spacers'. Since bcd-3 and hb-1 sites could not be identified in all of the species, we substituted conserved blocks located adjacent to these sites (identified as blocks 1 and 2 in Fig. 3). Spacer lengths in the four species differ in 11 of the 12 intervals (Table 1). The one invariant spacer interval is a

conserved 9 bp sequence located between kr-5 and gt-3. Its invariant length, therefore, is likely to be due to specific constraints on that sequence. *D. melanogaster* and *D. pseudoobscura* have the smallest and largest spacer lengths, respectively, differing by an average of 37.7%. Interestingly, the least variable spacer, excluding the invariant kr-5 to gt-3 spacer, as measured by the coefficient of variation, is the one located in the middle of the stripe 2 element between kr-4 and bcd-2. Previously, we showed that the middle of the enhancer, from gt-3 to gt-1, is more variable in terms of length than two clusters of overlapping binding sites that flank this region (Kreitman and Ludwig, 1996). The present data indicate, however, that the length variability in the middle region is not uniformly distributed.

Although 11 of the 12 spacers vary in length among the species, their relative lengths do not change appreciably (Table 1). Thus, for example, the rank order correlation of spacer lengths between *D. melanogaster* and *D. pseudoobscura* is $r=0.79$, and is nearly identical to that between the much more

	kr-6	kr-5	kr-4	kr-3	kr-2	kr-1
<i>mel</i>	ATAACCCAAT	TTAATCCGTT	ACC--GGGTTGC	GAAGGGATTAG	ACTGGGTTAT	TTAACCCGTTT
<i>sim</i>-.....
<i>yak</i>-.....	...C.....
<i>ere</i>-.....	...C.....
<i>pse</i>AA.....-A	.TC.....C..G
<i>pic</i>-.....A.	AGG.....	.T...C....	C...G..AC.G

	bcd-5	bcd-4	bcd-3	bcd-2	bcd-1
<i>mel</i>	GTTAATCCG	GAGATTATT	TATAATCGC	GGGATTAGC	GAAGGGATTAG
<i>sim</i>	C.....	.C.....
<i>yak</i>	C.....	.GC.C...GC.....
<i>ere</i>	C.....	...GT....C.....
<i>pse</i>	A.....	N/A	A.....A
<i>pic</i>C.....	N/A	.A.....G	AGG.....

	hb-3	hb-2	hb-1	gt-3
<i>mel</i>	CATAAAA-ACA	TTATTTTTT	CGATTTTTT	CGAGATTATTAGTCAATTG-----CAGTTGC
<i>sim</i>C.....	.C.....
<i>yak</i>G	..C.....	.C.....
<i>ere</i>	C.....	N/A	.C.....
<i>pse</i>C...	N/A	.C.....TTCATATTC...C.-
<i>pic</i>	..C.C...GC...	N/A	...C.....C..T...TTCC-ATTT-.TC.CTA

	gt-2	gt-1
<i>mel</i>	GACTTTATTGCAGCATCTTG----AACCAATCGTC-GCAGTTTGGTAACAC	GAAAGTCATAAAA-ACACATAATA
<i>sim</i>
<i>yak</i>	.C.....
<i>ere</i>CAGC.....G.....
<i>pse</i>	.T.....
<i>pic</i>	.T.....C.-T..AC.C.--.T.C.C.-.G.....G

Fig. 4. Stripe 2 enhancer binding sites in *D. melanogaster* and homologous sequences from five other *Drosophila* species. N/A, no homologous sequence identified. *mel*, *D. melanogaster*; *sim*, *D. simulans*; *yak*, *D. yakuba*; *ere*, *D. erecta*; *pse*, *D. pseudoobscura*; *pic*, *D. picticornis*.

Table 1. Distances between putative binding sites or conserved motifs

Region bounded by ¹	KR6 - KR5	KR5- GT3	GT3- GT2	GT2- B11	B11- KR4	KR4- BC2	BC2- GT1	GT1- BCD1	BC1- HB2	HB2- KR1	KR1- B12	B12- B1B	Total
<i>D. melanogaster</i>	125	9	37	33	21	66	78	8	40	27	64	72	573
<i>D. yakuba</i>	144	9	37	56	43	78	39	8	45	26	57	84	619
<i>D. erecta</i>	114	9	58	61	31	78	90	8	39	31	31	71	614
<i>D. pseudoobscura</i>	187	9	111	70	4	69	117	10	49	37	34	100	789
Average	142.5	9	60.75	55	24.75	72.75	81	8.5	43.25	30.25	46.5	81.75	648.8
Standard deviation	32.15	0	34.93	15.77	16.5	6.185	32.4	1	4.646	4.992	16.46	13.52	95.74
Coefficient of variation	0.226	0	0.575	0.287	0.667	0.085	0.4	0.118	0.107	0.165	0.354	0.165	0.148
Maximum difference	73	0	74	37	39	12	78	2	9	10	33	29	216
Maximum % difference	0.646	0	2.0	1.121	9.75	0.182	2	0.25	0.231	0.385	1.065	0.408	0.377

¹Spacer lengths were determined from the DNA sequence alignment in Fig. 3. B11 (Block 1) and B12 (Block 2) are conserved motifs; all other spacer boundaries are putative binding sites.

closely related species, *D. melanogaster* and *D. yakuba* ($r=0.81$). This suggests that selection may limit the range of permissible lengths for each spacer.

Pattern of native stripe 2 and transgene expression during embryonic development

We investigated the dynamics of transgene expression relative to native *eve* expression by assembling a developmental series of stained embryos. A representative series is shown in Fig. 5 for the construct containing the *D. erecta* stripe 2 element. In each of the experimental constructs from the four species, the spatial localization of *lacZ* mRNA stripes changes during embryogenesis relative to native *eve* protein expression. As expected, a broad anterior band of *lacZ* transcript is initially present in early embryos, which then resolves into distinct stripes at around the time of cell wall formation in the syncytial blastoderm. At the first appearance of well-resolved native *eve* stripes, the *eve* protein and the *lacZ* transcript coincide on a cell-for-cell basis along the full length of the stripes. Subsequently, however, the native *eve* stripes are positioned slightly forward in the embryo relative to the *lacZ* stripes, and this is apparent for both stripe 2 and for stripes 3+7. We have also observed the same phenomenon in two transgenic lines containing a different construct of *D. melanogaster* *eve* stripe 2 and 3+7 enhancers, provided by S. Small (Fig. 2). After cellularization, native *eve* stripes undergo the process of refinement, in which they narrow from about four cells to about two cells by loss of expression in the posterior region (Warrior and Levine, 1990). The control of this process requires an upstream *cis*-acting element, which is located approximately 5 kb from the transcriptional start site. This element has been shown to respond to early stripe *eve* expression and to regulatory inputs from other pair-rule genes (Goto et al., 1989; Harding et al., 1989; Jiang et al., 1991b; Fujioka et al., 1995). The fact that our constructs and the construct provided by S. Small do not contain the autoregulatory element, probably explains the misalignment of the endogenous *eve* protein stripes and the persistent of *lacZ*-mRNA in the transgenic embryos at stages following the completion of cellularization.

Pattern of stripe 2 *lacZ* expression from different species

We compared the spatial patterns of stripe 2 transgene expression from four different species to ask whether the evolutionary differences in the stripe 2 sequences had

detectable effects on either the anterior-posterior localization or the width of the stripes. To ensure that each stained embryo being compared was at the same timepoint in development, we took advantage of the fact that when both native and transgene stripes are first sharply resolved at the mid-cellularization stage, the 3+7 *lacZ* stripes coincide nearly perfectly with their corresponding native *eve* stripes. Since all of our constructs carried a common 3+7 element, we restricted our analysis to

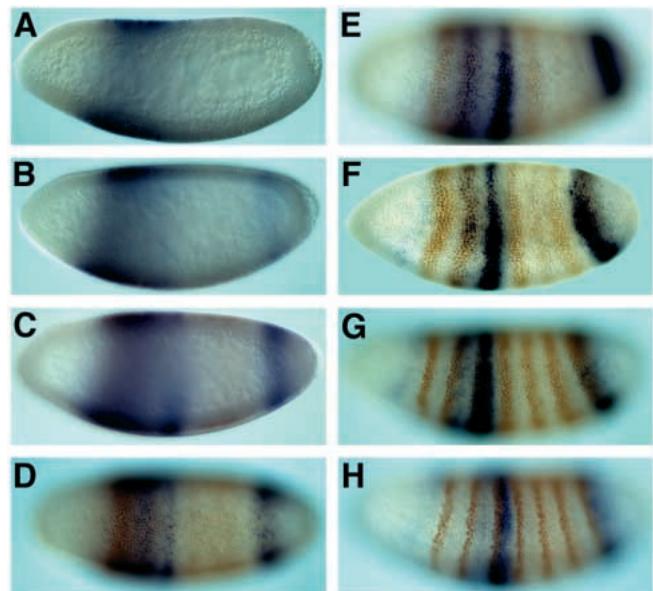


Fig. 5. Developmental series of *D. melanogaster* embryos transformed with the *D. erecta* stripe 2 enhancer and *D. melanogaster* stripe 3+7 enhancer-*lacZ* gene fusion. (A-H) *eve* protein (brown) and *lacZ* mRNA (purple) simultaneously detected in the embryos at precellularization stage to beginning of gastrulation stage. (A-C) Sagittal focus; (D-H) superficial focus. (A-C) The process of the activation of *D. erecta* stripe 2 *lacZ* and *D. melanogaster* stripe 3+7 *lacZ* expression. (D) The beginning of *eve* protein stripe maturation; the anterior border of the *eve* stripe 1 is defined. (D-F) Early *eve* protein and *lacZ* mRNA stripe formation. (F) Mid-cellularization stage, when native *eve* expression and *lacZ* expression coincide along stripes 2, 3 and 7. A decrease in stripe 2 *lacZ* expression relative to stripe 3 *lacZ* expression can be seen at this stage. (G,H) The discordance of native *eve* stripes relative to persisting *lacZ* mRNA stripes is apparent in panels (see text for details).

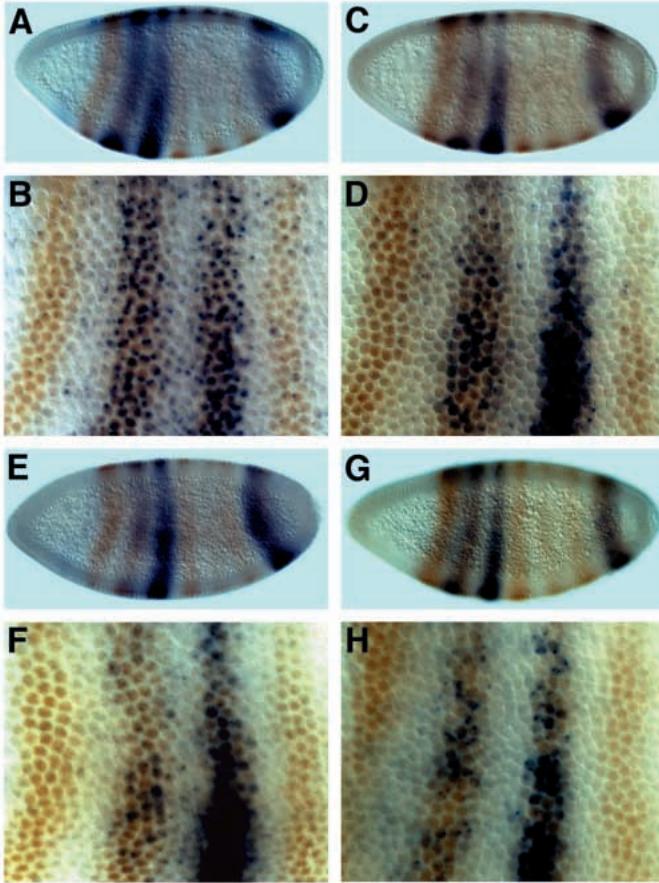


Fig. 6. Comparison of *lacZ* mRNA expression driven by stripe 2 enhancers from *D. melanogaster*, *D. yakuba*, *D. erecta* and *D. pseudoobscura*. *eve* protein (brown) and *lacZ* mRNA (purple) are simultaneously detected in the embryos transformed with the stripe 2 enhancers from each of the four species and *D. melanogaster* stripe 3+7 enhancer-*lacZ* gene fusion. Embryos were selected to be at the same timepoint in development by choosing ones in which the native *eve* protein stripes 3 and 7, and the *lacZ* stripes coincided, and by observing the extent of cellularization. (A,B) Stripe 2 enhancer from *D. melanogaster*; (C,D) stripe 2 enhancer from *D. yakuba*; (E,F) stripe 2 enhancer from *D. erecta*; (G,H) stripe 2 enhancer from *D. pseudoobscura*. (A,C,E,G) Sagittal focus; (B,D,F,H) higher magnification in superficial focus of stripes 2 and 3, from the embryos in A,C,E,G. The *eve* stripe 2 enhancer regions from all four species produce a pattern of *lacZ* expression that is coincident with the *D. melanogaster* native *eve* stripe 2.

those embryos in which the native and transgene 3+7 stripes coincided. Those embryos were assumed to be at a nearly identical timepoint in development, at least with respect to the progression of the morphogenetic gradients affecting the spatiotemporal expression of stripes 3+7.

Representative double-stained embryos of transformed lines bearing stripe 2 enhancers from *D. melanogaster*, *D. yakuba*, *D. erecta* and *D. pseudoobscura* are shown in Fig. 6. The *eve* stripe 2 enhancer regions from all four species produce a pattern of *lacZ* expression that is coincident with the *D. melanogaster* native *eve* stripe 2. After examining double-stained embryos from each of the replicate transformed lines, we did not detect any consistent shift or expansion of stripe 2

transgene expression. We conclude that the evolutionary differences in the *eve* stripe 2 enhancers have little or no effect on the function of the enhancer in terms of the spatial localization of early stripe 2 expression.

Level of stripe 2 *lacZ* expression from different species

The staining technique used in this study did not permit accurate quantification of *lacZ* expression. However, we did notice that the *lacZ* stripes produced by non-*melanogaster* stripe 2 enhancers were noticeably less intense when compared to the *D. melanogaster* stripe 3+7 control, at the time of their coincidence with native *eve* stripe 2. The *lacZ* staining intensity was lowest for the *D. erecta* stripe 2 construct. These qualitative differences in staining intensity could be seen in independent transformants of each construct. We also noticed that these stripes were not as uniform along the dorsal-ventral axis. All of these constructs contained the homologous region of stripe 2 enhancer DNA (see Materials and methods), so any difference in expression must be due to mutational differences in the enhancers. Possible explanations for the reduced *lacZ* expression by the non-*melanogaster* stripe 2 enhancers are presented below.

DISCUSSION

Conservation of the stripe 2 enhancer expression

The experimental results presented here show that the evolutionary divergence of the four *eve* stripe 2 enhancers has no discernible effect on either the timing or spatial localization of stripe 2 expression. Each of the four stripe 2 enhancers directs *lacZ* expression to the same set of cells that are expressing native *eve* protein in *D. melanogaster*, and they do so at identical timepoints in embryonic development (within the time resolution of our analysis). *eve* stripe 2 expression, therefore, is functionally conserved to a remarkable degree in these species. This functional conservation, we hypothesize, must be the consequence of stabilizing selection maintaining a single narrow band of *eve* expression in the early embryo.

The lack of evidence for functional evolution of the stripe 2 enhancer implies, by logical extension, that there has also been no species-specific coevolution of this enhancer with the morphogens to which they are responding. We hypothesize that the spatial and temporal expression of these morphogens must be nearly the same in each of the species in order that the stripe 2 enhancers from each of them respond identically to the regulatory signals of *D. melanogaster*. More specifically, features of the *trans*-acting factors – bicoid, hunchback, Kruppel and giant – responsible for the enhancer's activity must also be functionally conserved in each of the four species. This argument is consistent with the observation that the domains of expression of many segmentation genes are largely conserved within the Diptera (Sommer and Tautz, 1991). The experimental test of this prediction, however, awaits the reciprocal transformation of the stripe 2 reporter constructs into non-*melanogaster* species.

Evolutionary changes in the stripe 2 enhancer

In contrast to the functional conservation of the stripe 2 enhancer expression, we found that two binding sites, *bcd*-3 and the hb-

1, do not have obvious homologs in the other species. *D. pseudoobscura*, *D. erecta* and *D. yakuba* do not have a bcd-3 site, and *D. pseudoobscura* and *D. erecta* do not have a hb-1 site. (It is also possible that *D. yakuba*'s hb-1 site is nonfunctional, even though a mutated version of it can be identified.) Of the two sites not present in these species, one of them, the bcd-3 site, has previously been shown to be required for 'normal' MSE expression (Small et al., 1992). It is possible that the reduced transgene expression observed for the non-*melanogaster* constructs is the result of the smaller number of activator binding sites present in their stripe 2 enhancers. If true, the evolutionary gain of the bcd-3 and hb-1 sites in *D. melanogaster* and *D. simulans* may be an adaptive response to a reduction in the level of bicoid and/or hunchback proteins in these species. At present, there is no empirical evidence bearing on this hypothesis. An alternative evolutionary hypothesis for the gain of these sites in the lineage leading to *D. melanogaster* is presented below.

All of the remaining 14 binding sites identified in *D. melanogaster* are conserved at the sequence level, but only three of them completely so. A striking feature of the large majority of the nucleotide substitutions in the binding site sequences is that each substitution is present only once in the phylogeny. In other words, most of the substitutions in the binding sites occur at otherwise conserved, and presumably functionally important, positions. This suggests that the observed changes at these sites must not be selectively neutral. Rather, we speculate that they are likely to be either adaptive substitutions or slightly deleterious mutations fixed by genetic drift. A site in which a slightly deleterious mutation has been fixed is a good candidate for a subsequent convergent substitution by the adaptively favored mutation. Two convergent mutations can be identified in all of the binding sites, the aforementioned position 4 in kr-3 (also bcd-1) and position 2 in gt-3. The latter site, however, has mutated to three of four possible nucleotides, and may be one of the few exceptional binding site positions that is not functionally constrained.

Nearly all of the spacers that separate adjacent binding sites in the stripe 2 enhancer are variable in length. For example, in *D. melanogaster*, the gt-2-binding site is 53 bp from bcd-4, its closest activator site, but it is 135 bp from the bcd-4 site in *D. pseudoobscura*. A rough proportionality of spacer lengths, however, is maintained in each species, indicating that there may be evolutionary constraints on the magnitude of acceptable changes. Nevertheless, the differences in spacer lengths among species raises the possibility that the dynamics of quenching of specific activators by nearby repressors may vary.

Evidence supporting spacing requirements for transcription activation and repression shows that insertions and deletions in an enhancer, even small ones, have the potential to be subject to natural selection. Previous studies in *Drosophila* and yeast have shown that the spacing between interacting bicoid-binding sites is critical for activation of transcription, although the spacing is different in the two species (Hanes et al., 1994). Mechanisms of short-range transcriptional repression, such as local quenching and dominant repression, require close linkage (<100 bp) of the repressor with upstream activators (Gray et al., 1994; Gray and Levine, 1996).

Evolutionary explanations for functional conservation and structural change

The stripe 2 enhancers of the four *Drosophila* species contain

numerous differences, including the number of binding sites, the sequences of the binding sites and the spacing between them. Despite these differences, they all drive *lacZ* expression at the appropriate time and location in *D. melanogaster* early embryos. We now consider two evolutionary mechanisms to account for this functional conservation of the stripe 2 enhancer in the face of the observed structural differences. First, all of the sequence changes might be selectively neutral. It is reasonable to speculate that base substitutions and small length changes in the spacer regions have no functional effect on eve stripe 2 expression. If so, then these substitutions will have been selectively neutral. However, at least one change – the gain of a bcd-3 site in *D. melanogaster* – has been shown to be functionally important, and this change is not likely to have been selectively neutral. Otherwise, one would have to argue that the results from transgene analysis of the MSE do not apply to the in vivo expression of the native stripe 2 element. We think this is unlikely, due to the inherently greater sensitivity of natural selection in these species to detect mutations of extremely small functional effect, including ones that cannot possibly be measured experimentally. Codon preference in *Drosophila* is a good example of selection acting on synonymous mutations that have extremely subtle effects on the expression level of a gene (Akashi, 1995).

We have previously argued that the evolution of multiple binding sites for bicoid, hunchback, Kruppel and giant in the stripe 2 enhancer can be understood in terms of selection for functionally robust localization of eve expression, possibly owing to selection for the canalization of pair-rule gene expression. A mechanistic explanation for multiple binding sites provides support for this argument. Ma et al. (1996) recently showed that multiple binding sites for bicoid promote cooperative binding of this protein in an enhancer element of the hunchback gene, and that this cooperativity is necessary to achieve a sharp on/off switch of gene expression. We propose that many of the substitutions in the binding sites of the stripe 2 enhancer and some of the length changes in the spacers have functional effects on stripe 2 expression, but that the magnitude of these effects are ameliorated by functional robustness of the enhancer. In addition, flexibility in the structural design of the enhancer allows for rapid compensatory evolutionary changes, leading to overall functional conservation.

This proposition is compatible with the view that stabilizing selection acting on the timing and spatial localization of stripe 2 expression is the main evolutionary force governing this enhancer's evolution. Mutations of small effect, including slightly deleterious ones, can become fixed under stabilizing selection when there is functional 'redundancy' and epistasis, or when a large number of segregating mutations are contributing to a quantitative character (Kimura, 1981). Both are characteristic features of the stripe 2 enhancer architecture. Adaptive compensatory changes would be required in order to re-establish optimal regulatory performance after the fixation of deleterious mutation by genetic drift. The emergence of the bcd-3 site in *D. melanogaster* and *D. simulans*, according to this argument, may be a specific evolutionary response to the flux of weakly functional substitutions occurring at other activator sites.

eve stripe 2 expression is influenced by a number of factors extrinsic to the enhancer itself, each of which can also be subject to stabilizing selection to maintain the optimal

regulation of *eve* stripe 2 expression. These extrinsic factors include the distance between the enhancer and the basal promoter, the basal transcriptional complex through which it acts, and the structure of the *eve* autoregulatory element. However, the fact that the stripe 2 enhancers from the four species produce indistinguishable temporal and spatial patterns of expression in our experiments, indicates that these extrinsic factors are not coevolving with the stripe 2 enhancers to regulate these aspects of gene expression.

Levels of gene expression driven by the stripe 2 enhancers from different species

The constancy seen in the spatial and temporal patterns of the enhancer-driven *lacZ* expression is not observed for levels of stripe 2 *lacZ* expression, which appear to be lower when driven by non-*melanogaster* sequences, especially that of *D. erecta*. One possible explanation for this observation is that non-*melanogaster* sequences lack the *bcd-3* and *hb-1* activator binding sites. As has previously been shown by Small et al. (1992), mutations in the low-affinity *bcd-3* activator site caused a reduction, but not loss, of stripe 2 expression. The significance of the *hb-1* activator binding site for stripe 2 expression has not been determined experimentally, but its absence will decrease the number of activator molecules that can be bound to the enhancer. This decrease might weaken the overall activation ability of the enhancer (Arnosti et al., 1996). Alternatively, it is possible that the absence of *hb-1* in *D. erecta* and *D. pseudoobscura*, and its possible absence in *D. yakuba*, allows Kruppel protein bound to the *kr-1* site to repress transcription by directly interacting with the proximal promoter in our construct. Similarly, if there are direct interactions between any of the activators and the proximal promoter in our construct, then length differences in the enhancers may be influencing the strength of those interactions. Finally, it is possible to hypothesize the presence of unidentified repressor binding site(s) in these species' enhancers that are not present in the *D. melanogaster* stripe 2 enhancer.

Rules of enhancer evolution

The substitution pattern seen in this 5' regulatory region of *eve* is similar to that found in other regulatory regions containing enhancer elements. Regulatory control regions of *fushi tarazu* (*D. melanogaster* vs. *D. hydei* [Maier et al., 1990]), *hairy* (*D. melanogaster* vs. *D. virilis* [Langland and Carroll, 1993]), and *vestigial* (*D. melanogaster* vs. *D. virilis* [Williams et al., 1994]) all exhibit nearly identical patterns of substitution: small blocks of strongly conserved sequences interspersed among stretches containing many base substitutions and length changes. All three genes play essential roles in developmental processes and, like *eve*, the regulatory control of expression in these genes is expected to be functionally conserved. Not surprisingly, in each of the three cases, P-element-mediated transformation of a non-*melanogaster* regulatory sequence directed the expression of a reporter gene in *D. melanogaster* in a spatially and temporally conserved manner. Given that these species diverged from their most recent common ancestor with *D. melanogaster* approximately 60 million years ago, the blocks of conserved sequences are almost certainly the result of natural selection (Hartl and Lozovskaya, 1994).

Only bicoid, hunchback, Kruppel and giant are known to be

required for *eve* stripe 2 expression, and many of the conserved blocks in the stripe 2 enhancer are binding sites for these proteins (Fig. 3). Thus, factor binding appears to be the major selective constraint acting on the stripe 2 enhancer. There are conserved blocks in the stripe 2 enhancer, however, that are not known factor binding sites. These sites are not likely to be cryptic bicoid, hunchback or Kruppel sites since none of them have the required sequence motifs for these proteins. (The presence of cryptic giant-binding sites cannot be ruled out because no consensus is available for this protein.) It will be interesting to know whether these conserved blocks are binding sites for proteins, or whether they play other structural roles in enhancer function.

Almost all enhancers have a modular design and are characterized by having multiple binding sites for each of a small number of positive and negative regulators (Arnone and Davidson, 1997). The multiplicity of binding sites may be important in cooperative binding and in assuring robust performance. Quenching is also a general mechanism for negative regulation in enhancers. Thus, we expect that many enhancers will evolve in a manner similar to that of the *eve* stripe 2 enhancer. It should be of general interest, therefore, to determine how functional conservation of the stripe 2 expression pattern is achieved in evolution, given evidence that some changes are likely to have been selected.

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