Evolution of Developmental Genes: Molecular Microevolution of Enhancer Sequences at the Ubx Locus in Drosophila and Its Impact on Developmental Phenotypes

Jaros Phinchongsakuldit, Stewart MacArthur, and John F. Y. Brookfield

Institute of Genetics, University of Nottingham, Queens Medical Centre, Nottingham, United Kingdom

Homeotic genes, which function to specify segment identity along the anterior-posterior axis of embryos, are controlled by extensive batteries of enhancer sequences. We have investigated patterns of interspecific and intraspecific molecular variation in three enhancers of the Ultrabithorax (Ubx) locus, which are bx-32.8, pbx-32.7, and bxd-4.1, from the Drosophila melanogaster species group. These enhancer sequences control Ubx expression by binding to multiple transcription factors encoded by gap, pair-rule, and dorsoventrally expressed genes. Sequence comparisons reveal purifying selection acting on all three enhancers, both in bases binding transcription factors and in bases whose functions are as yet unknown. Neutrality tests largely fail to reject a neutral evolution model. However, using a matrix similarity value to reflect the binding affinity of the protein-binding sites, interspecific and intraspecific variation that may have potential to affect the binding affinity of the sequences homologous to those binding transcription factors in D. melanogaster are discovered, suggesting evolutionary flexibility in the way in which these sequences function in the control of development. As a means of measuring the impact of intraspecific variation on observable phenotypes, we have induced Ubx mutant phenocopies with embryonic ether treatment, and find strong and highly significant variation between D. melanogaster strains in their phenocopy frequencies. This variation shows no significant correlation with the strengths of the mutant phenotypes when the strains are heterozygous with a Ubx null mutation. Estimated phylogenetic trees have been constructed for the three enhancer regions investigated. Neither of the two phenotypic traits investigated shows any significant associations with the phylogeny of any of the three enhancers.

Introduction

Animal development is controlled by the creation of regular and repeatable patterns of gene expression, which varies between tissues and between stages of development. The process of differential gene expression is brought about in large part by the interaction of transcription factors with their target sequences, which make up enhancers, creating cis-acting control of transcription (Arnone and Davidson 1997). The evolution of development therefore must involve changes in transcription factors, in their cis-acting targets, or in both (Tautz 2000). However, a number of studies have shown that the amino acid sequences of many developmentally important transcription factors have been highly conserved among moderately diverged species (Kassis et al. 1986; Treier, Pfeifle, and Tautz 1989; Pan, Valentine, and Courey 1994; Sackerson 1995; Terol, Perez-Alonso, and de Frutos 1995). This is in accord with what one might theoretically expect, because a change in the functional properties of a transcription factor would be expected to have an impact in its interactions with multiple target genes, and the overall effect would be very unlikely to be advantageous. Changes in cis-acting control sequences, however, are likely to create a more specific and restricted effect on gene expression, which would have a higher probability of being advantageous to the organism. The control of development is likely to evolve over the short term mainly by changes in cis-acting enhancer sequences (Warren et al. 1994; Averof and Patel 1997; Weatherbee et al. 1999; Stern 2000).

Typically, regulatory sequences can evolve unexpectedly rapidly between diverged species so that only sequences from closely related species can be reliably aligned. The comparison of the regulatory sequences between moderately diverged species often shows an inter- dispersion pattern of small conserved sequences, most of which are identified as binding sites for transcription factors (Ludwig, Patel, and Kreitman 1998; Wilde and Akam 1987; Kassis et al. 1989; Pfeifle, and Tautz 1989; Maier, Preiss, and Powell 1990; Langeland and Carroll 1993; Lukowicz et al. 1994; Pan, Valentine, and Courey 1994; Sackerson 1995; Kreitman 1998; Wolff et al. 1999; Kim 2001; Dellino, Tatout, and Pirrotta 2002). It is interesting that, while the sequences of regulatory elements have rapidly evolved, their functions in controlling restricted gene expression have been found to be highly conserved between species. In transgenic experiments, the regulatory sequences from one species can drive the expression of a reporter gene in the same way as do the native regulatory sequences (Langeland and Carroll 1993; Lukowicz et al. 1994; Pan, Valentine, and Courey 1994; Ludwig, Patel, and Kreitman 1998; Wolff et al. 1999). Furthermore, a transformation of the coding and regulatory sequences from one species into another can often rescue the mutant phenotype in the same way as can the native gene (Maier, Preiss, and Powell 1990; Lukowicz et al. 1994). The fact that nucleotide changes are sometimes observed in protein-binding sites that have been shown to be functionally important in one species suggests that regulatory sequences may evolve under stabilizing selection (Ludwig et al. 2000). The functional redundancy of regulatory sequences may allow slightly deleterious mutations to be fixed by genetic drift, and then adaptive changes would be selected to compensate for these slightly deleterious mutations. Evidence for stabilizing selection acting on regulatory sequences has been shown in the case...
of the *Drosophila even-skipped* stripe 2 enhancer (Ludwig et al. 2000). The *even-skipped* stripe 2 enhancer of *D. melanogaster* and *D. pseudoobscura* can individually control the correct expression of *even-skipped* in transgenic *D. melanogaster* embryos. However, the chimeras constructed by combining halves of the enhancer from the two species no longer drive the correct *even-skipped* expression. Based on this finding, the authors suggested that stabilizing selection has maintained the overall function of the *even-skipped* stripe 2 enhancer by selecting for compensatory mutations, and this may be a general evolutionary mode for many other regulatory sequences. Such a pattern of compensatory changes regulated by stabilizing selection has allowed enhancer sequences to become remarkably diverged between moderately diverged species and yet still be able to create the same gene expression pattern.

That enhancer sequences can evolve unexpectedly quickly between species restricts the phylogenetic domain of inference about developmental control that can be derived from model systems such as *Drosophila*. However, while this reduces the capacity of *Drosophila* to act as a model system for phylogenetically ancient patterns of expression, our great understanding of the evolutionary process in this genus suggests that it will be a useful tool to study the microevolutionary processes that operate on this kind of sequence. Investigating the ways in which the functionally important parts of enhancer sequences evolve within the genus will give us some insight into the ways in which an evolving array of transcription factor binding sites can nevertheless maintain a gene expression pattern. Evolutionary change can potentially be brought about in two different ways—either by neutral or nearly-neutral changes fixed by genetic drift or by changes driven by natural selection. In determining the relative role of these two processes in sequence change, some insight can sometimes be gained from knowledge of the function of the sequences, but another very powerful set of investigative tools comes from population genetics. Interspecific variation may not, in itself, reveal whether changes observed are neutral or selected, but these two models have differing predictions for the patterns of variability within populations, and so the simultaneous consideration of within-species and between-species divergence can often detect evidence of natural selection.

Ultrabithorax (*Ubx*) is one of the homeotic genes in *Drosophila* (Lewis 1978). It functions to control segment identity of the parasegments 5 and 6. Temporal and spatial expression of *Ubx* is regulated by interaction between transcription factors and regulatory elements located in the intron and the 5′ flanking region. These regulatory elements are classified into four clusters, which are *anterobithorax* (*abx*), *bithorax* (*bx*), *bithoraxoid* (*bxd*), and *postbithorax* (*pbx*). Mutations in these regulatory elements cause transformation of the third thoracic segment toward the corresponding part of the second thoracic segment. The amino acid sequences of *Ubx* are highly conserved, showing 94% overall amino acid identity between *D. melanogaster* and *D. virilis* (Bomze and López 1994), and 78% identity between *D. melanogaster* and *Musca* (Wilde and Akam 1987). In addition, the process of *Ubx* alternative splicing and the time-specific and tissue-specific expression of each UBX isoform have been highly conserved among *Drosophila* species (Bonze and López 1994), suggesting that the mechanisms of *Ubx* regulation are also highly conserved. However, it is found that the subtle morphological differences in the trichome patterns on the posterior femur of the second leg between *D. melanogaster* and its sibling species *D. simulans* are due to the differences in the levels of *Ubx* expression in the posterior femur during late pupal development (Stern 1998). These differences have been shown to be driven by the divergence of cis-regulatory sequences at the *Ubx* locus between the two species, because the rescue of a *Ubx* null allele by its own wild-type allele cannot be duplicated by the wild-type allele from another sibling species, even a species showing an identical *Ubx* amino acid sequence. In addition, it is evident that polymorphisms at the *Ubx* locus partly play a role in controlling the frequency of bithorax phenocopies induced by ether vapor (Gibson and Hogness 1996).

Here, we study the process of enhancer sequence evolution by investigating the patterns of microevolutionary change in three *Ubx* enhancer sequences, which are named *bx*-32.8, *bxd*-4.1, and *pbx*-32.7, both within and between species. The locations of these three enhancer sequences in the *Ubx* gene map are shown in figure 1. The *bx*-32.8 enhancer is 730 bp in length and is located approximately 32.8 kb downstream from the transcription start site. This enhancer is equivalent to the BRE fragment, which functions to regulate *Ubx* expression in epidermal cells in the even parasegments from PS6 through PS12 (Qian, Capovilla, and Pirodda 1991). The *bxd*-4.1 enhancer is 549 bp in length and is located 4.1 kb upstream from the transcription start site. This enhancer contains no HUNCHBACK binding sites, and does not regulate *Ubx* expression in a parasegmental pattern like the *bx*-32.8 enhancer. Its specific function has not been investigated. However, it is located adjacent to the regulatory sequence controlling *Ubx* expression in visceral mesoderm (the sequence between 1.7 to 3.1 kb upstream of the *Ubx* transcription start site) (Bienz et al. 1988). The *pbx*-32.7 enhancer is 1,036 bp in length and is aligned with the U31961 sequence at base positions 209118–210153. It is equivalent to the PBX (or 2206Hf) fragment, and it functions to regulate *Ubx* expression in epidermis and mesoderm in the even parasegments from PS6 through PS12 (Müller and Bienz 1992; Pirodda et al. 1995). The initial expression of *Ubx* is regulated by products of gap and pair-rule genes. The gap gene product HUNCHBACK binds to the *bx* and the *pbx* elements and represses expression in the anterior half of the embryo, whereas repression by TAILLESS defines the posterior limit of the *Ubx* expression pattern (Qian, Capovilla, and Pirodda 1991, 1993; Muller and Bienz 1992). FTZ binding to sites in both *bx* and *pbx* stimulates expression in the even-numbered parasegments posteriorly from PS6, whereas the subsequent strengthening of expression in the anterior of each parasegment is thought to result from EN activation. TWIST stimulates expression from *bx* in the mesoderm (Qian, Capovilla, and Pirodda 1993).
In this study, a matrix similarity value, based on Quandt et al. (1995), was used to reflect the binding affinity of the protein-binding sites and thus to assess dynamic changes in the protein-binding sites over the microevolutionary scale. We also investigate the evidence that wild-type strains of *D. melanogaster* vary genetically in Ubx function as measured by the phenocopy frequency after ether treatment and by the ability of the strains to complement a null allele. The variation in the two phenotypes among the strains was compared to *Ubx* phylogenetic trees.

**Materials and Methods**

**Fly Species and Strains**

The *Drosophila* species studied were the eight closely related species of the melanogaster species subgroup. The *D. melanogaster* strains were collected from natural locations and kindly provided as follows. Canton S, Draveil, Kishinev, Loua, and Petit Bourg were collected from USA, France, Moldova, Zaire, and Guadeloupe (West Indies), respectively. These strains were provided by P. Capy (Gif). Harwich was collected from USA and provided by M. Ashburner (Cambridge). NW12B was collected from North Wootton (UK) and provided by P. Corish (Leicester). Samarkand, Tabilk Cellar, Tahiti, and Tex were collected from Uzbekistan, Australia, South Pacific, and Texas (USA), respectively. These strains were provided by B. Clarke (Nottingham). The Monty strains were collected from Montpellier (France), and the RD3F55 and RD8F55 strains were collected from Nottingham (UK). Each *D. melanogaster* strain was inbred in the laboratory in a long history of single brother-sister mating.

The extraction of chromosomes II and III was carried out in some *D. melanogaster* strains by crossing an individual fly from a *D. melanogaster* wild-type stock to C23a [In(2R) Cy spβ In(2LR) Pm(bwV1) ds3k b ap; In(3LR) D cxF ru h/Sb], then crossing an individual F1 female or male carrying the Pm and D mutations to C23a again, and collecting F2 progeny carrying the Pm and D mutations and letting them cross to each other, finally collecting wild-type F3 progeny, which are now homozygous for chromosomes II and III isolated from their original stocks. The chromosome extraction stocks are given new names by adding a number after their original strain’s name, as follows: Draveil(2), Draveil(3), Kishinev(7), Loua(4), Monty3(2), Monty5(6), Monty8(1), Monty9(3), Monty9(15), Monty11(4), Monty20(2), Petit Bourg(3), Tahiti (7), Tex(10).

Other isofemale *Drosophila* species and strains were obtained from the Species Resource Centre. The seven *D. simulans* strains are Sim161 (14021-0251.161), Sim162 (14021-0251.162), Sim163 (14021-0251.163), Sim165 (14021-0251.165), Sim166 (14021-0251.166), Sim167 (14021-0251.167), and Sim175 (14021-0251.175). *D. sechellia* is Fly base stock number 14021-0248.2. The two *D. mauritiana* strains are line 4 (14021-0241.4) and line 5 (14021-0241.5). Single brother-sister inbreeding from the original *D. teissieri* (14021-0257.0) stock was carried out for five generations in the laboratory, deriving...
two D. teissieri strains, 1 and 5. D. yakuba, D. orena, and D. erecta are Fly base stock numbers 14021-0261.0, 14021-0245.0, and 14021-0224.0, respectively.

DNA Extraction

Genomic DNA extraction used commercial DNA extraction kits from QiaGen (QIAamp Tissue Kit, QIA-GEN DNA Mini Kit, and QIAGEN DNA Blood Mini Kit) and Gentra system, Flowgen (Puregene DNA Isolation Kit). The methods of DNA extraction followed the protocols given with these commercial kits. However, in the last step of the extraction, the DNA was eluted with sterile distilled water preheated to 70°C. The DNA solution was stored at −20°C. All these commercial kits provided suitable DNA for polymerase chain reactions (PCR).

PCR and DNA Sequencing

The following primers were used to amplify the bx-32.8, bx4.1, and pbx32.7 enhancer sequences. Two pairs of primers were used to amplify two overlapped sequences of the pbx32.7 enhancer, giving a total length of 1,036 bp (table 1).

<table>
<thead>
<tr>
<th>Amplified Sequence</th>
<th>Primers</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>bx-32.8 (730 bp)</td>
<td>5′-CAT CAG GTG AAC CTC CTG CG-3′</td>
<td>58°C</td>
</tr>
<tr>
<td></td>
<td>5′-ATC CGA TCG TGT GGC TCC G-3′</td>
<td></td>
</tr>
<tr>
<td>bx4.1 (549 bp)</td>
<td>5′-TTC CGT TGC TGC TGC AGC G-3′</td>
<td>55°C</td>
</tr>
<tr>
<td></td>
<td>5′-GAG CAC AAA CAG AGG TCC G-3′</td>
<td></td>
</tr>
<tr>
<td>pbx32.7 (1036 bp)</td>
<td>5′-TGC GGC GTC GCT CTA GAT C-3′</td>
<td>55°C</td>
</tr>
<tr>
<td></td>
<td>5′-AAC CTC CCC TAT GTC GTC C-3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5′-TCG GAA TGG CAG ACA TCC C-3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5′-GAT GTG GGC ATT CGG ACG G-3′</td>
<td>58°C</td>
</tr>
</tbody>
</table>

The PCR was conducted in a 50 μl reaction volume (5 μl 10 × PCR buffer, 4 μl 25 mM MgCl₂, 1 μl 10 mM dNTPs, 2 μl of each 10 pmol Primer, 0.4 μl Red Hot DNA Polymerase/Advanced Biotechnologies, 2 μl 40 ng template DNA, and 33.6 μl sterile distilled water). Reactions were carried out for 1 cycle of 2 min at 94°C, followed by 30 cycles of 1 min at 94°C for denaturation, 30 s at the annealing temperature (depending on which primers were being used), and 1 min at 72°C for extension, then followed by one cycle of 10 min at 72°C and 2 min at 4°C.

The PCR product was purified using the low melting point (LMP) agarose DNA purification/freeze-and-thaw method. The PCR product was separated on a 1% LMP agarose gel, and then the band was cut out. The gel slice containing PCR product was melted at 65°C in 400 μl TE buffer for 10 min, and snap frozen in liquid Nitrogen for 2 min. The solution was thawed at 37°C for 30 min. The LMP gel was separated from the solution by spinning at 13,000 rpm in a bench centrifuge for 5 min. Supernatant containing DNA was removed and saved in a new tube. DNA was precipitated with 100 μl 0.3 M sodium acetate and 900 μl 100% ethanol at −20°C overnight, and then spun down at 13,000 rpm for 15 min. The pellet was washed with 1 ml 70% ethanol, dried, and dissolved in 30 μl sterile distilled water. The purified PCR products were kept at −20°C until used.

The purified PCR products were sequenced using the same primers that were used to perform the PCR reactions. DNA sequencing was performed by using ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) and run on an ABI 377 automated DNA sequencer. All sequences were investigated for both strands.

Sequence Analysis

The bx-32.8 sequence aligned to the complementary strand of the GenBank DNA sequence, accession number U31961, at base positions 275750–276480. (The G at base 275754 in this sequence is absent in all other sequences and is probably an error.) The bx4.1 and pbx32.7 sequences aligned to the U31961 sequence at base positions 238196–238744 and 209118–210153, respectively. Pairwise alignments using the “align” program (Myers and Miller 1988) were performed to examine the sequencing accuracy, to edit, and to complete the sequences. Multiple alignments of the completed sequences from all species and strains were produced using the ClustalW package (Thompson, Higgins, and Gibson 1994; Higgins and Sharp 1998), and manually edited. Neighbor-Joining trees with 1,000 bootstrap replications were constructed using the method of Saitou and Nei (1987) and plotted with the TreeView program (Page 1996).

From the multiple alignments, the numbers of fixed changes between the species and the numbers of polymorphisms were counted. The minimum number of mutational steps separating the sequences observed was estimated from the branches in the estimated phylogenetic trees and from the true species phylogeny (Ashburner 1989). Deletion or insertion of any number of contiguous bases is counted as one change. However, if changes, created either by slippages or by nucleotide substitutions, were seen in the sequence included in any of the indels, then the number of such changes was also counted and included in the analysis. To calculate the numbers of fixed changes per nucleotide, the length of the D. melanogaster sequence from GenBank, accession number U31961, was used as the standard length. The DnaSP version 3.51 program was used to calculate intraspecific variation (π and θ) and to perform neutrality tests (Tajima’s D, and Fu and Li’s D, and the HKA test) (Rozas and Rozas 1999).
this program, gaps in the alignment were excluded from the analyses.

Among the three enhancers studied, only the bx-32.8 and pbx32.7 enhancers have been previously investigated for their protein-binding sites in *D. melanogaster* using a DNA footprinting technique (Pirrotta et al. 1995; Qian, Capovilla, and Pirrotta 1993). Sequence analyses comparing protein-binding and non-protein-binding sites were therefore carried out on these two enhancers. The numbers of fixed changes and polymorphisms were used for a neutrality test equivalent to the McDonald-Kreitman test (McDonald and Kreitman 1991) when applied to these non-coding sequences (Jenkins, Ortori, and Brookfield 1995).

Estimation of Matrix Similarity of Protein Binding Site

The calculation of a matrix similarity value is based on Quandt et al. (1995). This is involved in the construction of a binding consensus matrix for a transcription factor and the calculation of a consensus index value ($C_i$) for each base position within the consensus matrix. The nucleotide distribution matrices for the binding consensus of the FUSHI TARAZU (Ftz), HUNCHBACK (Hb), ENGRAILED (En), TAILLESS (Tll), and TWIST (Twi) proteins were created by the multiple alignments of 52 Ftz, 67 Hb, 13 En, 27 Tll, and 13 Twi binding sites, respectively. The references for these protein-binding sites are available on the Brookfield Laboratory Web site (http://evol.nolt.ac.uk/~jb_lab/index.htm); and the references for the Hb binding sites were kindly provided by C. Bergman. Note that the binding sites in the bx-32.8 and pbx32.7 enhancers are not included in the alignments. The consensus index value ($C_i$) represents conservation of each base position within the consensus matrix. It is calculated as follows:

$$C_i(i) = \frac{100}{\ln 5} \times \left[ \sum_{b, A, C, G, T, \text{gap}} P(i, b) \times (\ln P(i, b) + \ln 5) \right],$$

where $P(i, b)$ is the frequency of nucleotide $b$ at position $i$.

The maximum value of $C_i$ is 100. This happens when the base position in the consensus shows total conservation of base identity. The minimum value of $C_i$ is 0, which happens when the position shows an equal distribution of all four nucleotides and gaps. A matrix similarity value was calculated as follows:

$$\text{Matrix similarity} = \frac{\sum_{j=1}^{n} C_j(j) \times \text{score}(b, j)}{\sum_{j=1}^{n} \max \text{. score}(j)};$$

where $C_j(j)$ is the consensus index value of position $j$, $n$ is the length of the consensus matrix, $\text{score}(b, j)$ is the matrix value for base $b$ at position $j$, and $\max \text{. score}(j)$ is $\max \{\text{score}(b, j); b \in A, C, G, T, \text{gap}\}$. The maximum value of a matrix similarity is 1. This happens in the case that the candidate sequence is identical to the most conserved nucleotide at every position of the consensus matrix. Because the matrix value (score) is multiplied by the $C_i$ value, mismatches at highly conserved positions will affect the matrix similarity value more than do mismatches at less conserved positions.

The core sequence within each protein-binding site of *D. melanogaster* was identified by its having the highest matrix similarity value. To achieve this, the matrix similarity value was calculated for every candidate sequence by successively shifting one base along the protein-binding sequence, as well as its complementary strand. This was performed by our program written in the Perl language. To evaluate the potential effects of evolutionary changes on the function of these protein-binding sites in other *Drosophila* species, the matrix similarity values were calculated for the sequences homologous to the *D. melanogaster* core sequences. Changes in the matrix similarity values compared to those of *D. melanogaster* suggest what might be the functional effect of nucleotide changes on the binding affinity of the sites.

Measurement of Interstrain Variation in Bithorax Phenocopy Frequency

The bithorax phenocopies are first observed in embryos treated with ether at syncytial blastoderm (1:00–2:00 h), are at the highest frequency in embryos treated at cellular blastoderm to gastrulation (2:30–3:30 h), and are not observed with treatments after germ band extension (5:30 h) (Bownes and Seiler 1977). In this study, a 10-min ether treatment was conducted on 3.0 ± 0.5-h-old embryos from different strains of *D. melanogaster*. Eggs were collected over a 1-h period, and placed on a 1.25% agar slice. After 2.5 h, the agar slice was transferred into a glass vial containing ether-saturated cotton wool. The eggs were exposed to ether vapor for 10 min and left in the fume cupboard for 30 min. After that, they were transferred to a new vial of commale medium containing 20–40 eggs per vial. Two weeks later, the emerging adults were scored. The bithorax phenocopies show a transformation of metathoracic to mesothoracic segments, imitating the Ultrabithorax (Ubx) mutant phenotype (fig. 2). The phenocopies were transferred in 70% ethanol, dehydrated with 100% ethanol, and kept in 90% glycerol. The detailed characteristics of phenocopies were observed under a microscope.

Measurement of *Ubx* Complementation Ability

Single-pair crosses between a virgin female from different chromosome extraction strains of *D. melanogaster* and a male from the y(1) strain were carried out. The y(1) strain [(1)y; TM1: In(3LR)Mec $^{kn1}$-1 $\text{sbd}^1$/TM2: In(3LR) $\text{emc}^c$ $\text{Ubx}^{130}$ $c^1$] was from Bloomington Stock Center (Indiana, USA), stock number 1628. The $\text{Ubx}^{130}$ heterozygous progenies were identified by the presence of atypical bristles on the capitellums of the halteres (fig. 3). Halteres of the heterozygotes were removed with small needles, transferred to a drop of water on a glass microscope slide, and covered with a coverslip. The numbers of atypical bristles on the halteres on each side were scored under a light microscope.
Results
Evolutionary Changes in the Ubx Enhancer Sequences

Neighbor-Joining trees based on the sequences of the bx-32.8, bxd4.1, and pbx32.7 enhancers are shown in figure 4(a–c). The overall picture shows that the topology of the trees confirms the widely accepted phylogeny of the melanogaster species subgroup, with D. erecta as the outgroup species (Ashburner 1989). The exception is the bxd4.1 tree, in which D. melanogaster is found on the same branch as D. simulans. However, the sequences of D. melanogaster are still very well grouped together: D. mauritiana and D. sechellia are sometimes found within the D. simulans branch, for example, as is seen in the bx-32.8 and bxd4.1 trees. This phylogenetic pattern is a result of these three species being closely related species that share recent coancestry. The variation in the positions of the D. melanogaster strains among the three trees shows evidence of recombination within the Ubx locus during evolutionary time. This variation is also confirmed by assessment of linkage disequilibrium. In the D. melanogaster sequences, there are four polymorphic sites in the bx-32.8 enhancer, seven in the pbx32.7 enhancer, and nine in the bxd4.1 enhancer, where the rarer variant is found more than once in the sample. Six pairwise linkage disequilibrium tests in bx-32.8 revealed one linkage disequilibrium that was significant at the 1% level and three others that were significant at the 5% level. For pbx32.7, all 21 pairwise linkage disequilibria were significant at the 1% level. Of 36 tests in bxd4.1, nine were significant at the 1% level and five others were significant at the 5% level. However, we found no significant evidence of linkage disequilibrium between variants in different enhancers.

Total numbers of fixed changes and of polymorphisms in the three enhancers are shown in Table 2. The numbers of fixed changes per nucleotide between D. melanogaster and D. simulans for the three enhancers (0.015 for bx-32.8, 0.009 for bxd4.1, and 0.032 for pbx32.7) are smaller than the average divergence rate of synonymous sites between the two species (0.072) (Sharp and Li 1989). This reveals that sequences of the three enhancers have evolved under purifying selection. The total numbers of fixed changes per nucleotide between all eight Drosophila species, for the three enhancers, are found to be significantly different among the enhancers ($\chi^2 = 115.30$, 2 df, $P < 0.001$). This suggests that the enhancers have evolved at different rates, with bx-32.8 the most conserved enhancer of the three, followed by bxd4.1 and pbx32.7.
Nucleotide Polymorphisms and Neutrality Tests

Table 3 shows a summary of nucleotide diversities within the *D. melanogaster* and the *D. simulans* samples, as well as the results from the neutrality tests. The *D. simulans* samples reveal higher nucleotide variation measured by η and θ than do those from *D. melanogaster*, for all three enhancers. This is consistent with the study of Moriyama and Powell (1996), which showed that, on average, *D. melanogaster* has a lower degree of DNA polymorphism than *D. simulans*. In addition, the levels of nucleotide diversity among the three enhancers in the *D. melanogaster* samples are similar to the average level of nucleotide diversity relative to their local recombination rate (Begun and Aquadro 1992; also see Aquadro and Begun 1993).

Reflecting the numbers of fixed changes per nucleotide, *bx*-32.8 shows the lowest nucleotide diversity, followed by *bxd*4.1 and *pbx*32.7. Hudson-Kreitman-Aguade (HKA) tests for pairwise comparison between the three enhancers fail to reject the null hypothesis of neutral evolution (data not shown). The neutrality tests, Tajima’s D and Fu and Li’s D with an outgroup species, were performed to test for a non-neutral distribution of observed allele frequencies within the population (Fu and Li 1993; Tajima 1989). A significantly negative Tajima’s D value is found for the *bx*-32.8 enhancer in the *D. melanogaster* samples. Correspondingly, significantly negative Fu and Li’s D values are found for the *bx*-32.8 enhancer both for the *D. melanogaster* and the *D. simulans* samples. This suggests that there may have been a selective sweep operating on the *bx*-32.8 enhancer in *D. melanogaster*, and possibly a second in *D. simulans*. However, negative D values can be created by population expansion, which, without selection, results in high numbers of variable sites relative to the gene diversity. *D. melanogaster* and *D. simulans* are fruit fly species which have been commensal with humans for many thousands of years. As the human population has increased, populations of *D. melanogaster* and *D. simulans* have also expanded, which could have created the significantly negative D values of the *bx*-32.8 enhancer in the *D. melanogaster* and the *D. simulans* samples.

Evolutionary Comparison of Protein-Binding and Non-Protein-Binding Sites

Sequences of around 499 bp of the *bx*-32.8 enhancer and around 579 bp of the *pbx*32.7 enhancer have been investigated with a DNA footprinting technique to identify the DNA binding sites for some of the transcription factors encoded by gap, pair-rule, and dorsoventrally expressed genes (Pirrotta et al. 1995; Qian, Capovilla, and Pirrotta 1993). The variability within these two sequences is shown...
Based on the results from the DNA footprinting, bases in the bx-32.8 and pbx32.7 enhancers can be classified into two types; protein-binding and non-protein-binding sites. Table 4 shows a summary of numbers of fixed changes between species and of polymorphisms in the protein-binding and non-protein-binding sites. The question is whether sequences of the protein-binding sites are more conserved than those of the non-protein-binding sites, because one might expect sequences of the protein-binding sites to evolve under a functional constraint. Interestingly, the results from table 4 show that numbers of fixed changes per nucleotide of the two base types are not significantly different from each other for either the bx-32.8 or pbx32.7 enhancers (\(\chi^2 = 0.852, 1\) df, \(P = 0.356\) and \(\chi^2 = 0.004, 1\) df, \(P = 0.949\), respectively). This suggests that the sequences of both protein-binding and non-protein-binding sites in the bx-32.8 and pbx32.7 enhancers have evolved at the same rate.

Using data from intraspecific and interspecific variation, a neutrality test equivalent to the McDonald-Kreitman test can be applied to these noncoding sequences (Jenkins, Ortori, and Brookfield 1995). Ratios of numbers of fixed changes to polymorphisms between protein-binding and non-protein-binding sites are compared. The results show a significant difference for the bx-32.8 enhancer (\(P = 0.027\)) but no significant difference for the pbx32.7 enhancer (\(P = 0.093\)). This suggests that there might be some forms of positive selection operating on the bx-32.8 enhancer. It is noteworthy that this significant result in the bx-32.8 enhancer is due to the higher than expected numbers of polymorphisms found in the protein-binding sites and fixed changes seen in the non-protein-binding sites. It is therefore possible that either there is selection to maintain polymorphisms in the protein-binding sites or the sequences of the non-protein-binding sites have evolved adaptively. This observation is hard to explain, however, because little is known about the functions of the sequences of the non-protein-binding sites. It is possible that some of the “non-protein-binding” sites, in reality, bind unknown proteins. If as few as three of the 31 non-protein-binding sites in the bx-32.8 sequence that show fixed changes between species were reclassified as protein-binding, the result for bx-32.8 would no longer be significant.

Table 2
Total Numbers of Nucleotide Variations in Ubx Enhancers

<table>
<thead>
<tr>
<th>Enhancer</th>
<th>Length (bp)</th>
<th>F</th>
<th>P</th>
<th>Changes per Nucleotide</th>
<th>Between eight species</th>
<th>F</th>
<th>P</th>
<th>Changes per Nucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>bx-32.8</td>
<td>730</td>
<td>11</td>
<td>26</td>
<td>0.015</td>
<td>64</td>
<td>40</td>
<td>0.088</td>
<td></td>
</tr>
<tr>
<td>bx44.1</td>
<td>549</td>
<td>5</td>
<td>30</td>
<td>0.009</td>
<td>76</td>
<td>44</td>
<td>0.138</td>
<td></td>
</tr>
<tr>
<td>pbx32.7</td>
<td>1036</td>
<td>33</td>
<td>55</td>
<td>0.032</td>
<td>290</td>
<td>101</td>
<td>0.280</td>
<td></td>
</tr>
</tbody>
</table>

a Polymorphisms are observed from a comparison of 23 D. melanogaster, and seven D. simulans sequences.
c Polymorphisms are observed from a comparison of 23 D. melanogaster, and seven D. simulans, two D. mauritiana, and two D. teissieri sequences.

Note.—Numbers of changes per nucleotide, comparing among the eight species of the bx-32.8, bx44.1, and pbx32.7 enhancers, are significantly different from one another (\(\chi^2 = 115.30, 2\) df, \(P < 0.001\)). F = fixed changes between species; P = polymorphisms.
Changes in Protein-Binding Sites

Both bx-32.8 and pbx32.7 enhancers contain DNA binding sites for the same transcription factors, which are Ftz, Hb, En, Tll, and Twi. Nucleotide variability in each type of transcription factor binding site in the bx-32.8 and pbx32.7 enhancers is shown in Figure 6a and 6b. The numbers of nucleotide changes of each protein-binding type, derived from the total numbers of changes in the bx-32.8 and pbx32.7 enhancers, are shown in Table 5. One can ask whether the DNA binding sites of each transcription factor have evolved at the same rate and whether there is positive selection operating on any particular types of protein-binding sites. It is found that the numbers of fixed changes per nucleotide are not significantly different between the different types of proteins ($\chi^2 = 1.518$, 4 df, $P = 0.823$). This suggests that the sequences binding each type of protein have evolved at the same rate. Furthermore, the ratios of number of fixed changes to polymorphisms are also not significantly different between the types of protein bound ($\chi^2 = 0.437$, 4 df, $P = 0.979$), suggesting that there is no positive selection on any particular protein-binding type.

The core sequence within each protein-binding site was identified (fig. 6a and 6b). It is found that some of the large protein-binding sites contain more than one core sequence—for example, the Ftz3, Ftz4, Hb3, and Tll3 sites of the bx-32.8 enhancer. It is possible that those core sequences are individual protein-binding sites that are located close to each other. The matrix similarity value calculated for each core sequence seems to correlate with the binding affinity of the binding site investigated by a DNA footprinting assay. This can be seen from the comparison of the matrix similarity values of the binding sites in the bx-32.8 enhancer with the footprinting results of Qian, Capovilla, and Pirrotta (1993). For example, the weak Ftz1 and Ftz2 binding sites show low matrix similarity values (0.33 and 0.52, respectively) whereas the strong Tll1, Tll2, and Twi1 binding sites show high matrix similarity values of 0.95, 0.95 and 0.99. However, exceptions can be seen for some of the binding sites. For instance, the Twi3, Twi4, and En2 sites were shown to be weak binding sites, and their matrix similarity values are as high as 0.76, 0.76, and 0.73. This may be because the binding affinity of the site also depends on the structure of the DNA strand. In general, however, the matrix similarity can be used to reflect the binding affinity of the DNA target site to the transcription factors. A high matrix similarity value reflects a strong protein-binding site, whereas a low matrix similarity reflects a weak protein-binding site. It is interesting that not all of the conserved binding sites show high matrix similarity values. This implies that some of the weak binding sites are functionally important and have therefore been conserved among Drosophila species.

Table 3

| Enhancer | $\pi$/site | $\theta$/site | $D_{Tajima}$ | $D_{Fu & Li}$
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>bx-32.8</td>
<td>0.214</td>
<td>0.450</td>
<td>-1.814*</td>
<td>-2.335*</td>
</tr>
<tr>
<td>bx-32.1</td>
<td>0.649</td>
<td>0.586</td>
<td>0.372</td>
<td>0.339</td>
</tr>
<tr>
<td>pbx32.7</td>
<td>0.685</td>
<td>0.770</td>
<td>-0.418</td>
<td>-0.610</td>
</tr>
</tbody>
</table>

| Enhancer | $\pi$/site | $\theta$/site | $D_{Tajima}$ | $D_{Fu & Li}$
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>bx-32.8</td>
<td>0.655</td>
<td>0.842</td>
<td>-1.228</td>
<td>-2.023*</td>
</tr>
<tr>
<td>bx-32.1</td>
<td>1.374</td>
<td>1.273</td>
<td>0.439</td>
<td>0.644</td>
</tr>
<tr>
<td>pbx32.7</td>
<td>0.981</td>
<td>1.184</td>
<td>-0.979</td>
<td>-0.972</td>
</tr>
</tbody>
</table>

* Significant at $P \leq 0.05$. 

Table 3 shows some examples of specific changes in the protein-binding sites which might disrupt the ability of the binding sites to interact with transcription factors. This disruption is predicted by the nucleotide changes being in the core sequence of the binding sites and causing a great change in the matrix similarity values. For example, the fixed substitutions in Twi2 binding sites of the bx-32.8 enhancer in D. teissieri, D. yakuba, D. orena, and D. erecta cause a reduction of the matrix similarity value of the sequences lower than that of D. melanogaster (fig. 6a). It is possible that this bx-32.8 Twi2 binding site is no longer a protein-binding site in those Drosophila species, or it may be only a weak binding site. Another example is in the Hb2 and Twi1 binding sites of the pbx32.7 enhancer (fig. 6b). The fixed changes in the pbx32.7 Hb2 of D. yakuba, D. orena, and D. erecta, and those in the pbx32.7 Twi1 of D. teissieri and D. yakuba, also cause a great reduction of the matrix similarity value of the sequences. Many of the polymorphisms are also found to greatly affect the matrix similarity values, as can be seen in the bx-32.8 Ftz1, Ftz2, Twi2, Twi4 binding sites and the pbx32.7 Ftz4, and Hb2 binding sites. Interestingly, some of those polymorphisms are found within the species while the binding sites are highly conserved among Drosophila species. For instance, the ‘‘T’’ polymorphism in the Twi4 binding site of the bx-32.8 enhancer in D. melanogaster causes a reduction of the matrix similarity value from 0.76 to 0.53, and the ‘‘G’’ polymorphism in the bx-32.8 Ftz2 binding site of the D. simulans species complex causes a reduction of the matrix similarity value from 0.52 to 0.27.

Numbers of fixed changes per nucleotide between bases in the core sequences and those outside the core sequences, including the non-protein-binding sites, have been compared (data not shown). Interestingly, although in this case only small regions of the protein-binding sites are considered, the numbers of fixed changes per nucleotide in the core sequences and those outside the core sequences are not significantly different from each other for either the
FIG. 5.—Nucleotide variations in 499 bp of the bx-32.8 enhancer (a) and 579 bp of the pbx32.7 enhancer (b). A dot and dash indicate identity and a gap, respectively, in the aligned sequences. Above the sequences are shown the nucleotide positions. Numbers of fixed changes and polymorphisms at each base position are shown below the sequences. An insertion or a deletion of contiguous bases is counted as one change. Shaded boxes indicate bases in the protein-binding sites, which are referred from Qian, Capovilla, and Pirrotta (1993) and Pirrotta et al. (1995). Dark-shaded boxes indicate bases in the core sequences of the protein-binding sites. Changes in the core sequences which affect the matrix similarity values are highlighted in white.
Table 4
Evolutionary Comparisons Between Protein-Binding and Non-Protein-Binding Sites

<table>
<thead>
<tr>
<th>Enhancer</th>
<th>Base Types</th>
<th>Fixed Changes</th>
<th>Polymorphisms</th>
<th>Length (bp)</th>
<th>Fixed Changes per Nucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>bx-32.8</td>
<td>Protein-binding</td>
<td>22</td>
<td>23</td>
<td>237</td>
<td>0.093</td>
</tr>
<tr>
<td></td>
<td>Non-protein-binding</td>
<td>31</td>
<td>11</td>
<td>262</td>
<td>0.118</td>
</tr>
<tr>
<td>pbx32.7</td>
<td>Protein-binding</td>
<td>43</td>
<td>11</td>
<td>241</td>
<td>0.178</td>
</tr>
<tr>
<td></td>
<td>Non-protein-binding</td>
<td>61</td>
<td>31</td>
<td>338</td>
<td>0.180</td>
</tr>
</tbody>
</table>

Note.—Numbers of fixed changes per nucleotide of the protein-binding and non-protein-binding sites are not significantly different in either the bx-32.8 or pbx32.7 enhancers, with $\chi^2 = 0.852$, 1 df, $P = 0.356$ and $\chi^2 = 0.004$, 1 df, $P = 0.949$, respectively. In the comparison of the number of fixed changes to polymorphisms ratios between the protein-binding and non-protein-binding sites, two-tailed Fisher’s exact tests show a significant result in the bx-32.8 enhancer ($P = 0.027$) but an insignificant result in the pbx32.7 enhancer ($P = 0.093$).

1 Numbers of fixed changes were obtained from a comparison of the sequences from eight Drosophila species, namely D. melanogaster, D. simulans, D. mauritiana, D. sechellia, D. teissieri, D. yakuba, D. orena, and D. erecta.

2 Numbers of polymorphisms were obtained from a comparison of 23 D. melanogaster, 7 D. simulans, 2 D. mauritiana, and 2 D. teissieri sequences.

bx-32.8 or pbx32.7 enhancers ($\chi^2 = 1.540$, 1 df, $P = 0.215$ and $\chi^2 = 0.809$, 1 df, $P = 0.368$, respectively). This finding shows that the sequences outside the core sequences have evolved at the same rate as those in the core sequences. In addition, comparison of the fixed changes to polymorphism ratios between nucleotide changes which affect and do not affect the matrix similarity values yields an insignificant result ($P = 0.537$), suggesting that the pattern of nucleotide variation in the core sequences is consistent with a neutral model (data not shown).

Variation in Ubx Homeotic Stability

Table 6 shows the rates of induction of bithorax phenocopies for each of the D. melanogaster strains, along with the number of atypical bristles on halteres of strains heterozygous for Ubx. The strains show remarkable variation in the phenocopy frequencies, with Monty8(1) showing the highest frequency of phenocopies. The transformation of the metathoracic to the mesothoracic segment in phenocopies varies in size (fig. 2). We found that the higher the phenocopy frequency, the more severe the transformation appears to be. The expressivity of bithorax phenocopies observed is consistent with the expressivity reported in previous studies (Bownes and Seiler 1977; Capdevila and García-Bellido 1978; Ho, Bolton, and Saunders 1983) in many respects, such that

1. The numbers of individuals showing transformations on both sides, and the numbers of individuals showing transformations on both haltere and leg imaginal discs of the same side, are higher than the expected numbers under an assumption of independence.
2. The effects of ether vapor are seen more in the haltere imaginal disc than in the leg imaginal disc.
3. Phenocopies show transformation of the anterior compartment more frequently than the posterior compartment.

As with the phenocopy induction rates, the numbers of atypical bristles on the halteres of flies heterozygous for Ubx also vary significantly among the strains. The mean number of bristles varies between 1–9 bristle(s) per haltere. Heterozygous Loua(4) shows the highest number of atypical bristles, suggesting that this strain has the lowest ability to rescue the Ubx phenotype. We even found that a few heterozygous Loua(4) flies showed transformation of the thoracic compartment, or parts of the haltere which had a wing-like appearance (data not shown). We also found, based on information from the Monty5(6), Draveil(3), Petit Bourg(3), Draveil(2), and Monty8(1) strains, that the frequency of phenocopies is not significantly correlated across strains with the number of atypical bristles ($r = -0.271, P = 0.660$). The Monty8(1) strain, which has a very high frequency of phenocopies, shows a low number of atypical bristles when heterozygous with Ubx.

Discussion

Conservation and Divergence of Ubx Enhancer Sequences

The sequences of the bx-32.8, bxd4.1, and pbx32.7 enhancers are found to be highly conserved. This reveals a strong functional constraint of the enhancers in the Drosophila melanogaster subgroup species. The differences in conservation levels among the three enhancers suggest that they have evolved subject to different degrees of constraint. The bx-32.8 enhancer shows the highest degree of constraint among the three enhancers studied. Unlike the others, the bx-32.8 enhancer is located in an intron. The requirement for transcription through this sequence, and the requirement for accurate splicing of this sequence from the transcript, might potentially impose further selective constraint on an enhancer sequence in an intron. Such constraint would add to the selection arising from its impact on the initiation of transcription. Using information about the protein-binding sites in the bx-32.8 and pbx32.7 enhancers, we have found that the sequences of the target sites of each transcription factor in the enhancers have evolved at the same rate. The sequences of both protein-binding and non-protein-binding sites reveal the same rate of divergence for either the bx-32.8 or the pbx32.7 enhancers. This observation can be seen, even in the comparison of the core sequences with the others, suggesting an equal degree of functional constraint for the two base types. It is possible that the sequences of the non-protein-binding sites function as binding sites for other transcription factors, or they may function to maintain the
Fig. 6.—Changes to the protein-binding sites of the bx-32.8 (a) and pbx32.7 (b) enhancers. Shaded boxes indicate the core sequences, which give the highest matrix similarity value. The matrix similarity value of each interacting site is shown beside the sequence alignment. Nucleotide changes which cause changes in the matrix similarity values are indicated by dark boxes. The binding affinity of each protein-binding site in the bx-32.8 enhancer previously investigated by the DNA footprinting assay (Qian, Capovilla, and Pirrotta 1993) is described, but there is no information for the protein-binding sites in the pbx32.7 enhancer.
secondary structure of the enhancers. Both bx-32.8 and pbx32.7 enhancers drive Ubx expression during early embryogenesis (Qian, Capovilla, and Pirrotta 1991; Pirrotta et al. 1995). It is possible that other gap and pair-rule genes or dorsoventrally expressed genes might bind to the bx-32.8 and pbx32.7 enhancers, but they have not been experimentally investigated.

Neutrality tests largely fail to reject a neutral evolution model. Although significantly negative Tajima’s D, and Fu and Li’s D for D. melanogaster and D. simulans are found for the bx-32.8 enhancer, this is probably as a result of population expansion rather than an effect of selection. The evolutionary comparisons of nucleotide variation between types of transcription-factor-bound protein-binding, and non-protein-binding sites, and the changes affecting and not affecting the matrix similarity values of the core sequences, show that the pattern of nucleotide variation in the bx-32.8 and pbx32.7 enhancers is consistent with a neutral evolution model. One exception is in the comparison of nucleotide variation in the protein-binding and non-protein-binding sites of the bx-32.8 enhancer, which shows a significant deviation from neutrality. The data show a significant excess of protein-binding polymorphisms and/or non-protein-binding fixed changes in the bx-32.8 enhancer (table 4). In principle, if this significance is not simply the outcome of multiple testing without Bonferroni correction, two types of explanation are possible: either there is selection to maintain diversity in the protein-binding sites in this bx region, or there has been adaptive change in the non-protein-binding sites.

Sequences of the protein-binding sites vary among the Drosophila species. The fact that the nucleotide changes are not shared by all the protein-binding sites for a given transcription factor implies that the changes observed do not result from any change in the binding site that is the target for binding by an altered transcription

### Table 5

<table>
<thead>
<tr>
<th>Strains</th>
<th>Length (bp)</th>
<th>Hb</th>
<th>En</th>
<th>Tll</th>
<th>Twi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monty5(6)</td>
<td>218</td>
<td>213</td>
<td>73</td>
<td>102</td>
<td>121</td>
</tr>
<tr>
<td>Fixed changes</td>
<td>32</td>
<td>25</td>
<td>8</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>Polymorphisms</td>
<td>17</td>
<td>17</td>
<td>4</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Changes per nucleotide</td>
<td>0.147</td>
<td>0.117</td>
<td>0.110</td>
<td>0.108</td>
<td>0.132</td>
</tr>
</tbody>
</table>

**NOTE.**—Numbers of fixed changes per nucleotide in the protein-binding sites of each transcription factor type are not significantly different ($\chi^2 = 1.518$, 4 df; $P = 0.823$). There is no significant difference among the ratios of number of fixed changes to polymorphisms in the protein-binding sequences of each transcription factor type ($\chi^2 = 0.437$, 4 df; $P = 0.979$).

### Table 6

<table>
<thead>
<tr>
<th>Strain</th>
<th>N.T.</th>
<th>n*</th>
<th>Frequency of Phenocopies (%)</th>
<th>Measurement of Ubx Complementation Ability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Measurement of Ubx Complementation Ability</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Number of Atypical Bristles</td>
</tr>
<tr>
<td>Tex</td>
<td>26</td>
<td>857</td>
<td>0.00a</td>
<td>—</td>
</tr>
<tr>
<td>Monty9</td>
<td>15</td>
<td>711</td>
<td>0.00a</td>
<td>—</td>
</tr>
<tr>
<td>Monty3</td>
<td>21</td>
<td>751</td>
<td>0.19a</td>
<td>—</td>
</tr>
<tr>
<td>Loua</td>
<td>44</td>
<td>1500</td>
<td>0.75 ± 0.26b</td>
<td>—</td>
</tr>
<tr>
<td>Tahiti</td>
<td>15</td>
<td>715</td>
<td>3.58 ± 0.09e</td>
<td>—</td>
</tr>
<tr>
<td>Kishinev</td>
<td>29</td>
<td>945</td>
<td>3.63 ± 0.71c,d</td>
<td>—</td>
</tr>
<tr>
<td>Monty5(6)</td>
<td>14</td>
<td>776</td>
<td>6.34 ± 1.28d,e</td>
<td>116</td>
</tr>
<tr>
<td>Draveil(3)</td>
<td>11</td>
<td>665</td>
<td>7.57 ± 1.52e</td>
<td>112</td>
</tr>
<tr>
<td>Petit Bourg(3)</td>
<td>12</td>
<td>762</td>
<td>8.00 ± 1.32e</td>
<td>102</td>
</tr>
<tr>
<td>Draveil(2)</td>
<td>13</td>
<td>705</td>
<td>17.34 ± 2.11f</td>
<td>138</td>
</tr>
<tr>
<td>Monty8(1)</td>
<td>11</td>
<td>635</td>
<td>44.57 ± 2.59g</td>
<td>118</td>
</tr>
<tr>
<td>Tex(10)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>190</td>
</tr>
<tr>
<td>Tahiti(7)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>86</td>
</tr>
<tr>
<td>Monty9(3)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>126</td>
</tr>
<tr>
<td>Kishinev(7)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>192</td>
</tr>
<tr>
<td>Monty9(15)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>132</td>
</tr>
<tr>
<td>Loua(4)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>158</td>
</tr>
</tbody>
</table>

**NOTE.**—Dash, not observed; N.T., number of treatments; n*, number of eggs treated; n**, number of halteres examined. The frequencies of phenocopies shown are calculated using total numbers pooled from N.T. Numbers of atypical bristles shown are the average atypical bristles on a haltere. There is a significant correlation between the atypical bristle numbers in the left and the right haltere, with $P < 0.001$ for all but three strains, and with $P = 0.017$ for Monty8(1), although Monty5(6) and Loua(4) show $P = 0.563$ and 0.999, respectively.

* A $\chi^2$ test shows a significant difference between the frequencies of bithorax phenocopies from each D. melanogaster strain ($P < 0.001$). Homologous subsets of the phenocopy frequencies significantly different at $P < 0.05$, classified using pairwise $\chi^2$ tests, are shown by different lettered superscripts.

** An ANOVA shows a significant difference among average numbers of atypical bristles from each of the strains ($P < 0.001$). Homologous subsets of the means significant at $P < 0.05$ are performed by Scheffé method and shown by different lettered superscripts.

Pearson correlation between the phenocopies frequencies and the numbers of atypical bristles of Monty5(6), Draveil(3), Petit Bourg(3), Draveil(2), and Monty8(1), is not significant ($r = -0.271$, $P = 0.660$).
factor in another species. In this study, the matrix similarity value was used to reveal dynamic changes of the protein-binding sites between species or strains. The change in matrix similarity value resulting from a substitution depends on the \( C_l \) value of its position. Changes in a highly conserved position (with a high \( C_l \) value) may greatly change the matrix similarity values. Because the only way a base change can fail to change the matrix similarity value is if the scores of the two bases at the position are the same, most changes will affect the matrix similarity values. Although there is no evidence to measure quantitatively the degree to which a change in the matrix similarity value causes a difference in the biochemical function of a site, the matrix similarity value correlates with the binding affinity of the binding site investigated by DNA footprinting to the extent that a strong or a weak protein-binding site can be revealed by its having a high or a low matrix similarity value (see Results). Important changes in the protein-binding sites are thus predicted if the binding site homologs show a great difference in their matrix similarity values. In this case, we found 4 of the 17 binding sites of the bx-32.8 enhancer (Ftz1, Ftz2, Twi2, and Twi4) and 3 of the 22 binding sites in the pbx32.7 enhancer showing a great difference in the matrix similarity values.

Although these data suggest dynamic changes in some protein-binding sites in the bx-32.8 and pbx32.7 enhancers over the microevolutionary scale of Drosophila evolution, it is possible that these dynamic changes in the protein-binding sites are, at most, only slightly deleterious and do not affect the functions of the enhancers. It is speculated that stabilizing selection may also play a role in the evolution of Ubx regulatory sequences, in the same way seen in the even-skipped stripe 2 enhancer (Ludwig et al. 2000), where selection has operated to maintain the overall functions of the Ubx enhancers during evolution, while allowing a rapid turnover of their sequences to occur. This may happen through the fixation of slightly deleterious mutations being compensated by adaptive changes at other sites. In this case, failure to achieve functional compensation will result in changes in the gene-expression pattern. Alternatively, it can also be imagined that sequence turnover may occur in the way in which new protein-binding sites are created by the fixation of neutral or selected mutations, and thus, some of the former protein-binding sites that are no longer necessary can be lost. These two models of enhancer evolution also explain the outcome of the gain and loss of protein-binding sites in regulatory sequences over the macroevolutionary scale, which is observed in regulatory sequences of many regulatory genes including Ubx (Dellino, Tatout, and Pirrotta 2002; Wilde and Akam 1987).

Impact of Intraspecific Variation at the Ubx Locus

The failure to detect a sign of positive selection does not necessarily mean that all of the variability observed in Ubx sequences is neutral. It is possible that the neutrality tests are not sensitive enough to detect any sign of selection. For example, in the McDonald and Kreitman test, and other tests for adaptive change, if only a few adaptive changes occur, these will be insufficient to make the results of the tests significant (Brookfield and Sharp 1994). In this study, to measure functional variation of the Ubx alleles among D. melanogaster strains, we used two phenotypic tests, the induction of bithorax phenocopy and the Ubx\(^{130}\)/Ubx\(^{+}\) complementation test. The results show that the D. melanogaster strains differ greatly in their frequencies of phenocopy induction, as well as in the numbers of atypical bristles on their haltere when they are heterozygous with Ubx\(^{130}\). However, variation in the phenocopy frequency and the atypical bristle numbers is not correlated with the positions of the strains on the Ubx trees in figure 4. Strains that show significantly different phenocopy frequencies, or that have significantly different numbers of atypical haltere bristles (when heterozygous with Ubx\(^{130}\)), can be found to have identical Ubx regulatory sequences, whereas strains that show the same levels of phenocopy frequencies and numbers of haltere atypical bristles can be found on different branches in the trees. It is possible that the variation of these two phenotypes is caused by variation in the other regulatory regions of Ubx, in addition to bx-32.8, bxd4.1 and pbx32.7, or it may be that the variation of the phenotypes is a result of the interaction of variations in several regions of the genome in addition to Ubx.

The differences in the phenocopy frequencies among the strains cannot be explained by their Ubx alleles having differing levels of complementation ability because there is no significant correlation between the phenocopy frequency and the strength of the Ubx\(^{130}\)/Ubx\(^{+}\) mutant phenotype (\( P = 0.660 \)). An obvious example is that Monty8(1) shows a very high phenocopy frequency but has a low number of atypical bristles on the haltere when the strain is heterozygous with Ubx\(^{130}\). It is possible that these two phenotypes are the result of different mechanisms. The differences in the numbers of atypical bristles on the haltere among Ubx\(^{130}\) heterozygous strains may be caused by differences in the Ubx alleles of each strain, or it may be the result of differences in the response of downstream genes to one dose of UBX protein. However, the differences in the other susceptibility between the strains may be caused by differences in the temporal and spatial regulation of Ubx expression, which may include the regulation of both the initial activation/repression and the maintenance of activating/repressing states. Because the sensitive period for ether induction of phenocopy is very specific, if the different strains were to be laying eggs at differing stages of development, they would differ systematically in the stage of development at which their eggs were being exposed to ether. If so, this could potentially create consistent differences between strains in their frequencies of phenocopies. However, other data (Phinchongsakuldit and Brookfield, in preparation) imply that at least some of the variation in phenocopy frequency maps to Ubx, which would not be expected under this model.

A QTL (quantitative trait loci) study for haltere/wing margin transformation in Ubx\(^{130}\) heterozygotes showed that the variation in genes mapping to the left arm of chromosome III, at bands 62–65, is responsible for the variation in the atypical bristle numbers on the Ubx\(^{130}\)/Ubx\(^{+}\)
halteres (Gibson, Wemple, and van Helden 1999). This result gives an example of the genetic variation outside the \textit{Ubx} locus that affects the variation of \textit{Ubx} homeotic stability. Both \textit{Ubx}\textsuperscript{130} and \textit{Ubx}\textsuperscript{1} are null mutations, but, whereas \textit{Ubx}\textsuperscript{130} is associated with many inversions on the third chromosome, \textit{Ubx}\textsuperscript{1} is a point mutation caused by a \textit{doc} element insertion in the first exon. However, the two mutations have the same phenotypic effects of creating an enlarged haltere with atypical bristles in heterozygotes. It is possible that the locus discovered by Gibson, Wemple, and van Helden may also affect the phenotype of individuals heterozygous for the \textit{Ubx}\textsuperscript{130} allele. It was shown that the variation in the ether susceptibility is at least partly caused by polymorphism in the \textit{Ubx} regulatory sequences (Gibson and Hogness 1996). Other genetic backgrounds contributing to the variation in ether susceptibility have not been identified.

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Richard Thomas, Associate Editor

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