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Identification of Homeotic Target Genes in *Drosophila melanogaster* Including *nervy*, a Proto-Oncogene Homologue

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ABSTRACT

In Drosophila, the specific morphological characteristics of each segment are determined by the homeotic genes that regulate the expression of downstream target genes. We used a subtractive hybridization procedure to isolate activated target genes of the homeotic gene *Ultrabithorax* (*Ubx*). In addition, we constructed a set of mutant genotypes that measures the regulatory contribution of individual homeotic genes to a complex target gene expression pattern. Using these mutants, we demonstrate that homeotic genes can regulate target gene expression at the start of gastrulation, suggesting a previously unknown role for the homeotic genes at this early stage. We also show that, in abdominal segments, the levels of expression for two target genes increase in response to high levels of *Ubx*, demonstrating that the normal down-regulation of *Ubx* in these segments is functional. Finally, the DNA sequence of cDNAs for one of these genes predicts a protein that is similar to a human proto-oncogene involved in acute myeloid leukemias. These results illustrate potentially general rules about the homeotic control of target gene expression and suggest that subtractive hybridization can be used to isolate interesting homeotic target genes.

MORPHOLOGICAL differences along the anterior to posterior (a/p) axis of *Drosophila melanogaster* are determined by the homeotic selector genes, which are clustered in the genome in either the bithorax or Antennapedia complexes. Altering the expression of these homeotic genes produces homeotic transformations, which are the conversions of one body structure into another (LEWIS 1978; WAKIMOTO and KAUFMAN 1981). Homeotic genes, therefore, behave as master regulators that control the activity of subordinate downstream target genes (GARCIA-BELLIDO 1975). Consistent with this suggestion, homeotic genes all encode proteins that contain a homeodomain, which is a sequence-specific DNA-binding motif present in many eukaryotic transcription factors (SCOTT *et al.* 1989). Homeotic genes are present in all animals suggesting that this evolutionarily conserved subset of homeodomain proteins is important for differentiating a/p morphologies throughout the animal kingdom (MCGINNIS and KRUMLAUF 1992).

To understand how homeotic genes generate different morphologies along the a/p axis, it is important to identify and characterize their downstream target genes. However, to date only a handful of target genes have been identified (reviewed in ANDREW and SCOTT 1992; BOTAS 1993). Among these examples are genes that encode very different types of proteins, including the cytoskeletal protein β-tubulin, a MyoD homologue encoded by *nautilus*, a homeodomain protein encoded by *Distal-less*, and secreted signaling molecules (IMMERGLUCK *et al.* 1990; REUTER *et al.* 1990; GRABA *et al.* 1992; HINZ *et al.* 1992; VACHON *et al.* 1992; HURSH *et al.* 1993; O'HARA *et al.* 1993; MICHELSON 1994). These examples suggest that homeotic target genes have very diverse functions in development.

Because homeotic genes dictate morphology by controlling target gene expression, it is also important to understand the rules that govern this regulation. However, several features of the homeotic genes complicate this analysis. First, both the expression and function of different homeotic genes extensively overlap along the a/p axis. For example, the three homeotic genes in the bithorax complex (BX-C), *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*), and *Abdominal-B* (*Abd-B*) are expressed in overlapping domains and function in many of the same parasegments (LEWIS 1978; BEACHY *et al.* 1985; KARCH *et al.* 1985, 1990; SÁNCHEZ-HERRERO *et al.* 1985; WHITE and WILCOX 1985; CARROLL *et al.* 1988; CELNIKER *et al.* 1989; MACIAS *et al.* 1990). Specifically, *Ubx* is expressed in parasegment (PS) 5–PS13, *abd-A* is expressed in PS7–PS13, and *Abd-B* is expressed in PS10–PS14 (see Figure 2A). In addition, *Antennapedia* (*Antp*), a homeotic gene located in the Antennapedia complex (ANTP-C), is expressed in PS4–PS13 (CARROLL *et al.* 1986,

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1988; KAUFMAN *et al.* 1990). These expression patterns, together with the phenotypes generated by loss-of-function homeotic mutations (reviewed in MCGINNIS and KRUMLAUF 1992), suggest that the specific morphologies of many parasegments require the action of multiple homeotic genes.

A second hallmark of the homeotic genes is that they cross-regulate each other's expression. These cross-regulatory interactions are important because they account for the homeotic transformations that are produced in loss-of-function homeotic mutations. For example, *Ubx* mutations result in a higher level of *Antp* expression within PS5 and PS6, transforming these parasegments into copies of PS4 (HAFEN *et al.* 1984; CARROLL *et al.* 1986). Similarly, mutations in *abd-A* and *Abd-B* result in the derepression of *Ubx* in PS7–PS13, transforming them into copies of PS6 (STRUHL and WHITE 1985). These cross-regulatory interactions are important to take into consideration when analyzing how homeotic genes regulate their targets. For example, if a particular target gene is regulated similarly by *Ubx* and *abd-A*, removing *abd-A* function would not be sufficient to see an affect on target gene expression.

In the work described here, a subtractive hybridization protocol was used to isolate *Ubx*-activated target genes. The expression patterns of three of these target genes indicated that they were regulated by multiple homeotic genes in addition to *Ubx*. Therefore, a set of genotypes were generated that measures the regulatory contribution of individual homeotic genes to a complex target gene expression pattern. In this way, both the complications of overlapping homeotic gene expression and cross-regulatory interactions were avoided. This study provides some general insights about how homeotic genes control the expression of their targets. Moreover, one of these genes appears to be the homologue of a proto-oncogene, suggesting that this approach has identified novel and potentially interesting homeotic target genes.

MATERIALS AND METHODS

Preparation of the subtracted cDNA probe: The genotypes of the two fly stocks used for the subtraction were: w^{1118}/w^{1118}; *HS:Ubx-Ia/TM6B* [the (+) stock] and w^{1118}/w^{1118}; *HS:Ubx-IVaFS Df(3R)Ubx*^{109}*/TM6B* [the (−) stock]. The two heat shock-*Ubx* loci have been previously described (MANN and HOGNESS 1990). The *HS:Ubx-IVaFS* gene was recombined onto a chromosome carrying *Df(3R)Ubx*^{109} to reduce the amount of biologically active *Ubx* gene product in the (−) stock. *Df(3R)Ubx*^{109} is also deficient for the *abd-A* gene. The two stocks were each expanded to two large houses. Three-hour embryo collections were harvested on yeasted grape plates, aged at 25° for 3 hr, washed into nylon mesh chambers, and heat shocked at 37° for 50 min as described (MANN and HOGNESS 1990). After the heat shock, the embryos were returned to 25° in the nylon mesh containers and incubated for a further 3 hr. The embryos were then dechorionated in 60% bleach, rinsed with distilled water, transferred to microfuge tubes, and quickly frozen by immersing the tubes in liquid N_2. After 10 g of embryos for each genotype were harvested, total RNA was isolated and doubly poly(A)$^+$ selected as described (KORNFELD *et al.* 1989).

The subtractive hybridizations were carried out as described (HEDRICK *et al.* 1984; DAVIS 1986) with the following modifications. The doubly poly(A)$^+$ selected mRNA (20 μg) obtained from the (+) stock was used as a template for cDNA synthesis using both random hexamer primers (at 1.5 mg/ml) and oligo d(T) primers (at 100 μg/ml). Trace amounts of (^{32}P)deoxycytosine triphosphate were included during the cDNA synthesis to allow the detection of newly synthesized cDNA. To reduce RNA secondary structure Na pyrophosphate was included at a concentration of 4 mM. The resulting (+) cDNA (~13 μg) was hybridized with a twofold excess of (−) poly(A)$^+$ mRNA and single-stranded nucleic acid was purified using hydroxyapatite chromatography. The resulting subtracted cDNA was rehybridized with the same amount of (−) mRNA used in the first subtraction (now approximately a 10-fold excess) and single-stranded material was purified as before. After the second subtraction, ~1.7% of the original cDNA remained single stranded.

Approximately half of the subtracted cDNA was radiolabeled by multiple rounds of random hexamer primed DNA synthesis (FEINBERG and VOGELSTEIN 1983). This probe was used to screen ~30,000 plaques of a Drosophila genomic DNA library constructed in the phage vector FIX (Stratagene) (MOSES *et al.* 1989). Only plaques hybridizing on duplicate filters were picked and purified using standard procedures (SAMBROOK *et al.* 1989).

***D. melanogaster* strains:** The w^{1118}/w^{1118}; *HS:Ubx-Ia/TM6B* [the (+) stock] and w^{1118}/w^{1118}; *HS:Ubx-IVaFS Df(3R)Ubx*^{109}*/TM6B* [the (−) stock] were previously described (MANN and HOGNESS 1990). Here, *HS:Ubx* refers to the *HS:Ubx-Ia* gene and *HS:Ubx-FS* refers to the *HS:Ubx-IVaFS* gene, which are both homozygous lethal third chromosome insertions. The "wild-type" stock (used for polytene chromosome analyses and *in situ* hybridizations) was the w^{1118}/w^{1118} derivative of Canton-S obtained from G. RUBIN. *Scr*$^-$ *Antp*$^-$ *Ubx*$^-$ *abd-A*$^-$ *Abd-B*$^-$ refers to embryos of the genotype *Scr*^{C1} *Antp*$^{Ns+RC3}$ *Ubx*^{MX12} *abd-A*^{M1} *Abd-B*^{M8} and has been previously described (CHAN and MANN 1993). The *Scr*^{C1} *Ubx*^{MX12} *abd-A*^{M1} *Abd-B*^{M8}, *Scr*^{C1} *Antp*$^{Ns+RC3}$ *abd-A*^{M1} *Abd-B*^{M8}, and *Scr*^{C1} *Antp*$^{Ns+RC3}$ *Ubx*^{MX12} *Abd-B*^{M8} stocks were generated by recombining previously described chromosomes (STRUHL 1983; CASANOVA *et al.* 1987). For the *in situ* hybridizations of *HS:Ubx* embryos, embryos homozygous for the *HS:Ubx-Ia P* element were examined. The *HS:Antp* stock (P2–3) contains a homozygous viable *P* element insertion and was kindly provided by M. SCOTT. For all of the above stocks, first instar larval cuticle preparations were examined to confirm the expected phenotypes (WIESCHAUS and NUSSLEIN-VOLHARD 1986).

For unambiguously determining which embryos were homozygotes of the *HS:Ubx-Ia* gene or homozygotes of any of the mutant homeotic chromosomes, one of two marked balancer chromosomes were used: either *TM3B*, containing a *ftz-lacZ* gene (made by S. CREWS and kindly provided to us by Y. HIROMI) or *TM6B,22UZ* which contains a *Ubx-lacZ* gene (IRVINE *et al.* 1991). In experiments using these balancer chromosomes, a *lacZ* probe was included in the *in situ* hybridizations and the non-*lacZ*-staining embryos were studied.

RNA *in situ* hybridization to whole embryos: RNA *in situ* hybridization to whole embryos was performed as described (TAUTZ and PFEIFLE 1989) using the following hybridization conditions: 50% formamide; 5XSSC; 10 mM $NaPO_4$, pH 7.0; 0.1% Tween 20; 1× Denhardt's; 1 mg/ml tRNA. The length of proteinase K treatment (3–5 min) was optimized for each probe.

A 3′ *Ubx* probe (used in Figure 6B) was generated by digoxy-

genin labeling the 1.2-kb *Xho*I to *Eco*RI fragment of the *Ubx-Ia* cDNA (KORNFELD *et al.* 1989). This fragment is entirely derived from the 3′-most *Ubx* exon. As the *Ubx* transcription unit is >70 kb, this probe will only identify mature or nearly mature transcripts (see also AKAM and MARTINEZ-ARIAS 1985; SHERMOEN and O'FARRELL 1991). A 5′ *Ubx* probe (used in Figure 6C) was generated by digoxygenin labeling the 5′ *Eco*RI to *Xho*I fragment of the *Ubx-Ia* cDNA and is primarily derived from the 5′-most *Ubx* exon.

To prepare probes for the putative target genes isolated here, individual phage DNAs were simultaneously restricted with *Sac*I and *Xba*I endonucleases (which do not digest the phage arms), electrophoresed on an agarose gels, blotted to nitrocellulose, and probed with the original radiolabeled subtracted cDNA (data not shown). Hybridizing fragments were gel-purified and labeled individually with digoxygenin nucleotides. The labeled fragments were pooled and used as the *in situ* probe. In all cases tested, individual fragments produced the same pattern as the pooled fragments, suggesting that the expression pattern of a single gene was visualized (data not shown). The original *nervy* phage insert is 18.5 kb and the hybridizing fragments are 6.0 and 1.8 kb. The original *belt* phage insert is 16 kb and the hybridizing fragments are 6.5, 3.0, 2.6, 1.4, and 1.2 kb. The original *lips* phage insert is 17.5 kb and the hybridizing fragments are 5.0, 4.5, 3.0, 1.8, and 1.4 kb. cDNA clones representing these genes have been isolated and were used as probes in *in situ* hybridization experiments (P. G. FEINSTEIN and R. S. MANN, unpublished data). In all cases, hybridization with cDNA probes was qualitatively the same but quantitatively stronger than with genomic probes. The 412 probe was generated by amplifying long terminal repeat sequences using oligonucleotides PF8 (5′-GCGAATTCTGTAAGTAATGTGCCTATG) and PF10 (5′-GCGGATCCTGTAATGATGAACTCCA) in a PCR reaction with phage 5 DNA as the template. For each probe in Figure 1, the hybridizations were done in parallel with the same probe, therefore, the signal intensities are directly comparable.

***In situ* hybridization to polytene chromosomes:** The identical digoxygenin-labeled genomic DNA probes described above were used for hybridization to polytene chromosomes. These hybridizations were done as described (ASHBURNER 1989) except that a horseradish peroxidase-conjugated antidigoxygenin antibody (Promega) was used.

cDNA isolation and sequencing: The 1.0-kb *Eco*RI to *Sma*I fragment of the *nervy* genomic phage was radiolabeled and used to probe a λgt11 cDNA library (ZINN *et al.* 1988). Screening 800,000 plaques yielded three different phage. No evidence for alternative splicing was apparent. The longest phage insert was 3.0 kb and, when used as a probe in *in situ* hybridization experiments, generated the same expression pattern as the *nervy* genomic probe. Both strands of this phage were completely sequenced using standard procedures and the nucleotide sequence has been submitted to GenBank (no. U21717). Two potential initiator methionines (codons 1 and 2) were the only ATG codons in frame with the remainder of the ORF. In addition, stop codons were present 5′ to these ATGs in all reading frames. A putative polyadenylation signal was identified near the 3′ end of this cDNA.

RESULTS

Isolating Ubx activated target genes by subtractive hybridization: When UBX proteins are ubiquitously expressed 3–6 hr after egg laying (AEL) by the heat-inducible promoter from the *hsp70* gene (a *HS:Ubx* gene) PS0 to PS5 are transformed into PS6-like metameres (GONZALEZ-REYES and MORATA 1990; MANN and HOGNESS 1990). For one of the UBX isoforms (UBX-Ia) (KORNFELD *et al.* 1989) this transformation phenotype can be identified by analyzing either the first instar larval cuticle that is secreted during the second half of embryogenesis or by observing the pattern of segmentally repeated peripheral neurons (MANN and HOGNESS 1990). In contrast, when a mutant UBX protein containing a frameshift mutation within the homeodomain (UBX-FS) is similarly expressed no transformations are observed. Because both the cuticle and peripheral neuron phenotypes closely mimic a wild-type PS6, we reasoned that *Ubx* target genes that are normally up-regulated by UBX in PS6 should be more abundant in heat shocked *HS:Ubx* embryos than in heat shocked *HS:Ubx-FS* embryos. These UBX-inducible sequences were partially purified using a subtractive hybridization procedure (see MATERIALS AND METHODS). The single-stranded cDNA resulting from the subtractive hybridization was radiolabeled and used to probe a Drosophila genomic DNA library (MOSES *et al.* 1989).

Classification of the genomic phage identified by the subtracted cDNA: Of the 30,000 plaques screened, ~250 recombinant phage hybridized to the radiolabeled subtracted cDNA. Of these, 100 were picked and 89 retested positive after the third consecutive plating. DNA was isolated from most of the 89 plaque-purified phage and the Drosophila DNA inserts were radiolabeled and used as probes on nitrocellulose filters where all 89 phage were represented in a grid. In addition to these 89 phage, eight additional phage representing *Ubx activated clones* (*Uacs*) isolated using a similar screen from cultured Drosophila cells were also included on the filters (K. KORNFELD, J. CHUNG, and S. MUNROE, unpublished data). None of the 89 phage inserts hybridized to the eight *Uac* phage (data not shown). The 89 phage were divisible into 20 nonoverlapping groups, which were numbered 1 to 20 (Table 1).

One group, represented by phage 5, was remarkable because it contained a sequence that was present in 65 of the 89 phage (Table 1). Partial DNA sequencing of the phage 5 insert demonstrated that the relevant cross-hybridizing sequence was a long terminal repeat (LTR) from the Drosophila retrotransposon 412 (FINNEGAN *et al.* 1978; WILL *et al.* 1981; MARTIN *et al.* 1983; BINGHAM and ZACHAR 1989). The isolation of 412-containing sequences indicated that the expression of 412 may be *Ubx*-inducible. This prediction has been confirmed by Northern hybridization of mRNA isolated from heat shocked *HS:Ubx* and *HS:Ubx-FS* embryos (data not shown). Moreover, *in situ* hybridization experiments demonstrate that 412 is expressed in a highly UBX-inducible pattern in the mesoderm during embryogenesis (Figure 1, A and B). Interestingly, 412 has also been shown to be a downstream target of the homeotic genes *abd-A* and *Abd-B* (BROOKMAN *et al.* 1992).

A group of three phage, represented by phage 2, also contained a middle repetitive sequence because both

TABLE 1

Summary of isolated phage

Phage[a]	Name	Number[b]	Location[c]	UBX inducible[d]
1	nervy	1	99F	+
2	xerox	3	15–20 sites	+++
3	—	1	61A	−
4	furrow	1	48B/C	−
5	412[e]	68	~25 sites	++++
8	belt	1	28E/F	++
9	—	1	13C	−
10	gang of three	2	ND	++
13	—	1	71B/C	−
18	lips	1	82E/F	+

[a] Each phage listed represents a group of cross-hybridizing phage. Probes for phage −6, −7, −11, −12, −14, −15, −16, −17, and −19 generated little or no *in situ* hybridization signal in wild-type embryos and have not been pursued further.

[b] The number of cross-hybridizing phage in each group is shown.

[c] Cytological position as determined by *in situ* hybridization to polytene chromosomes. ND, not determined.

[d] UBX inducibility was judged by either whole mount *in situ* hybridization of embryos or Northern hybridization analysis; see text for details.

[e] Sixty-five of the phage in this group had at least one long terminal repeat (LTR) of the Drosophila retrotransposon 412. The remaining three phage included in this group contained sequences that were located next to a solo-LTR in the genome.

phage 2 insert DNA and related cDNA clones hybridized to ~15 bands on polytene chromosomes (data not shown). Partial sequencing of phage 2-related cDNAs revealed no homology to any sequence in the GenBank data base suggesting that this sequence may be a novel repeated element, which we preliminarily name *xerox* (Table 1). Expression of phage 2-related sequences was highly UBX-inducible during embryogenesis (data not shown).

The 16 remaining phage were represented only once among the 89 phage. Hybridizations to salivary gland polytene chromosomes using probes for seven of these 16 phage demonstrated that they represent unique sequences in the Drosophila genome (Table 1). We focused on three genes represented by phage 1, 8, and 18 because their expression patterns suggested that they were regulated by homeotic genes during embryogenesis (see below). Based on their expression patterns, these putative target genes have been named *nervy, belt,* and *lips,* respectively (Table 1).

***HS:Ubx* embryos express elevated levels of the putative target genes:** Putative target genes isolated by this subtractive hybridization procedure should be expressed at higher levels in response to ubiquitous UBX expression. This was confirmed for the genes isolated here by comparing their expression patterns following ubiquitous UBX or UBX-FS expression (in *HS:Ubx* or *HS:Ubx-FS* embryos, respectively). *HS:Ubx* and *HS:Ubx-FS* embryos were collected in parallel for 3 hr, aged for 3 hr at 25°, heat shocked at 37° for 1 hr, and allowed to recover at 25° for 3 hr before fixation. For most of the genes isolated here, activation of the putative target gene was observed (Figure 1 and data not shown). Specifically, *lips,* which was primarily expressed in two

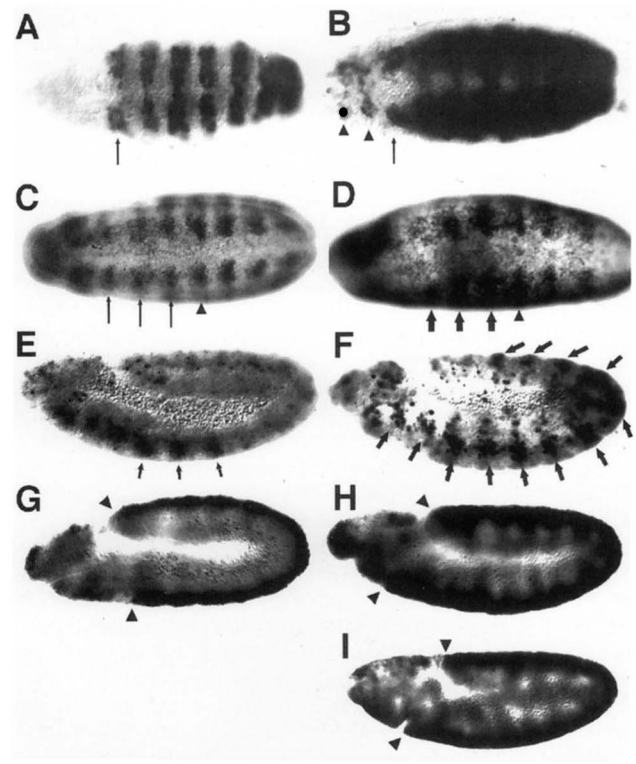


FIGURE 1.—*lips,* 412, *belt,* and *nervy* are induced by ubiquitous UBX expression during embryogenesis. *HS:Ubx-FS* (A, C, E, and G), *HS:Ubx* (B, D, F, and H), and *HS:Antp* (I) stage 11 embryos stained for 412 (A,B); *lips* (C and D); *nervy* (E and F); and *belt* (G–I) RNA by *in situ* hybridization. The embryos in A–D are ventral views of germband elongated embryos, therefore only anterior segments are visible; the remaining panels are lateral views. At this stage, 412 RNA is present in the mesoderm of PS2 to PS14 (the arrows in A and B point to PS2 expression). After ubiquitous UBX expression (B), 412 expression is highly induced in PS2 to PS14. In addition, two additional anterior parasegments PS0 and PS1 express 412 (B, arrowheads). At stage 11, *lips* was expressed in two patches of ectodermal cells per segment and the three thoracic segments expressed lower levels (C, arrows). After ubiquitous UBX expression, the level of *lips* RNA was elevated in the three thoracic segments (D, arrows). The arrowheads in A and B point to *lips* expression in the first abdominal segment (A1), which appeared the same. *nervy* was expressed in nervous system precursor cells in head, thoracic, and abdominal segments and the three thoracic segments expressed higher levels (E, arrows). Elevated levels of *nervy* RNA were observed in all segments in response to ubiquitous UBX expression (F, arrows). *belt* was expressed at uniform levels throughout the germ band from T1 through A8 (the arrowheads in G mark the extent of this expression). Additional *belt* expression in the head was weaker and out of the plane of focus. Either ubiquitous UBX (H) or ubiquitous ANTP (I) expression induced ectopic *belt* expression anterior to T1 (the arrowheads in H and I mark the extended *belt* expression pattern).

patches of ectodermal cells in each parasegment, was induced to higher levels in head and thoracic segments (Figure 1, C and D). Expression of *nervy,* which was limited to the nervous system, was induced to higher levels in head and abdominal segments (Figure 1, E and F). Expression of *belt,* which was uniformly expressed throughout the thorax and abdomen, was induced in head segments by either UBX or ANTP (in *HS:Ubx* or *HS:Antp* embryos, respectively; Figure 1, G–I).

A set of genotypes to analyze the regulation of target gene expression by homeotic genes: The three putative target genes described here were expressed in many segments throughout the embryo. Moreover, removing only a single homeotic gene often had no affect on the expression pattern. For example, *lips* expression in Ubx^- embryos appeared wild type (data not shown). One explanation for this result is that *Ubx* is not a regulator of *lips* expression in wild-type embryos. Alternatively, *Ubx* and *Antp* may both activate *lips* expression in similar patterns. Thus, the *lips* expression pattern would appear wild type in Ubx^- embryos because *Antp,* in the absence of *Ubx,* would be derepressed in the *Ubx* domain, resulting in an equivalent pattern of *lips* expression.

To distinguish between these possibilities, a set of genotypes was constructed that measures the contribution of a single homeotic gene to a complex expression pattern (Figure 2A). This approach depends on the fact that the homeotic genes that are expressed in the head [*proboscopedia* (*pb*), *labial* (*lab*), and *Deformed* (*Dfd*)] are only weakly derepressed in the absence of the five homeotic genes of the thorax and abdomen [*Sex combs reduced* (*Scr*), *Antp, Ubx, abd-A,* and *Abd-B*]. Thus, nearly all homeotic gene activity is eliminated in the thorax and abdomen (trunk) in Scr^- $Antp^-$ Ubx^- $abd\text{-}A^-$ $Abd\text{-}B^-$ embryos. In animals of this genotype all trunk segments have an identity that approximates the "ground state," which is defined as the identity obtained in the absence of all homeotic gene activity (LEWIS 1978; STRUHL 1982). In addition to this quintuple mutant, mutant stocks that were wild type for either $Antp^+$, Ubx^+, or $abd\text{-}A^+$ but still mutant for the four remaining trunk homeotic genes were also constructed (Figure 2A). Cuticle preparations of first instar larva having these genotypes illustrate the Scr^- $Antp^-$ Ubx^- $abd\text{-}A^-$ $Abd\text{-}B^-$ (ground state) identity and the identities generated by $Antp^+$, Ubx^+, or $abd\text{-}A^+$ in isolation (Figure 2, B–E).

Homeotic-specific control of the late *nervy* expression pattern: The *nervy* expression pattern was very complex and dynamic (Figure 3). *nervy* expression was first detected in a large fraction of central nervous system (CNS) neuroblasts during the first stage of neuroblast formation (during early stage 9 of embryogenesis; Figure 3A) (DOE 1992). During embryogenesis, *nervy* expression apparently remained restricted to precursors of the central and peripheral nervous systems (Figure 3, B–F). At three different stages during embryogenesis *nervy* was expressed in more cells and/or at higher levels in thoracic segments than in abdominal segments (Figure 3, C, D, and F). Interestingly, these stages are preceded by and separated by stages when the *nervy* expression pattern appeared identical in all segments (Figure 3, A, B, and E).

When CNS development is nearly complete (by stage 15), clear differences in *nervy* expression were observed between the thoracic and abdominal segments (Figure 3, F and G). Specifically, each of the three thoracic segments had more *nervy*-expressing cells than the abdominal segments. In nerve cords doubly stained for *nervy* RNA and *engrailed* (*en*) protein, most of the thoracic-specific *nervy* expression colocalized with *en,* indicating that these cells are within the posterior compartments of these segments (Figure 3G).

The segment-specific *nervy* expression pattern seen in dissected nerve cords of wild-type stage 15 embryos suggests that the homeotic genes differentially regulate its expression. This prediction was tested by analyzing *nervy* expression in embryos that express none or only a single wild-type trunk homeotic gene (see Figure 2A). In the ground state, *nervy* expression was derepressed throughout but still limited to the CNS (Fig. 4, B and C). When only $Antp^+$ is present, the pattern normally present in the second thoracic (T2) segment was reiterated throughout the CNS (Figure 4D). A similar result was also observed in Ubx^- $abd\text{-}A^-$ embryos (data not shown). When only Ubx^+ was present, the pattern normally present in the first abdominal segment (A1) was reiterated throughout the abdomen (Figure 4E). The thoracic segments of these embryos express *nervy* as it was expressed in the ground state, consistent with a lack of homeotic gene activity. *Ubx* can also generate the A1 *nervy* expression pattern when ectopically expressed: in *HS:Ubx* embryos the A1 *nervy* pattern was observed in all thoracic and abdominal segments and in at least two head segments (Figure 4A). Thus, it appears that *Antp* and *Ubx* can independently generate the T2 and A1 *nervy* patterns, respectively. In contrast, when only $abd\text{-}A^+$ was present (Figure 4F), the *nervy* expression pattern in abdominal segments was unlike the ground state pattern or any pattern present in wild-type nerve cords. Therefore, while *abd-A* can regulate *nervy* expression, it cannot, on its own, generate a normal abdominal pattern. These results suggest that *Ubx,* not *abd-A,* is responsible for generating the *nervy* expression pattern in the abdominal segments of wild-type stage 15 embryos.

The late *lips* expression pattern can be specified by *Ubx*: As with *nervy,* the *lips* staining pattern in wild-type embryos was easily distinguished from the pattern in Scr^- $Antp^-$ Ubx^- $abd\text{-}A^-$ $Abd\text{-}B^-$ embryos. In wild-type germ band shortened embryos, *lips* expression is primarily observed in a dorso-ventral oriented row of cells in the posterior portion of each thoracic and abdominal segment (Figure 5A). In contrast, in Scr^- $Antp^-$ Ubx^- $abd\text{-}A^-$ $Abd\text{-}B^-$ embryos this row of staining was replaced by a single cluster of *lips*-positive cells in each segment

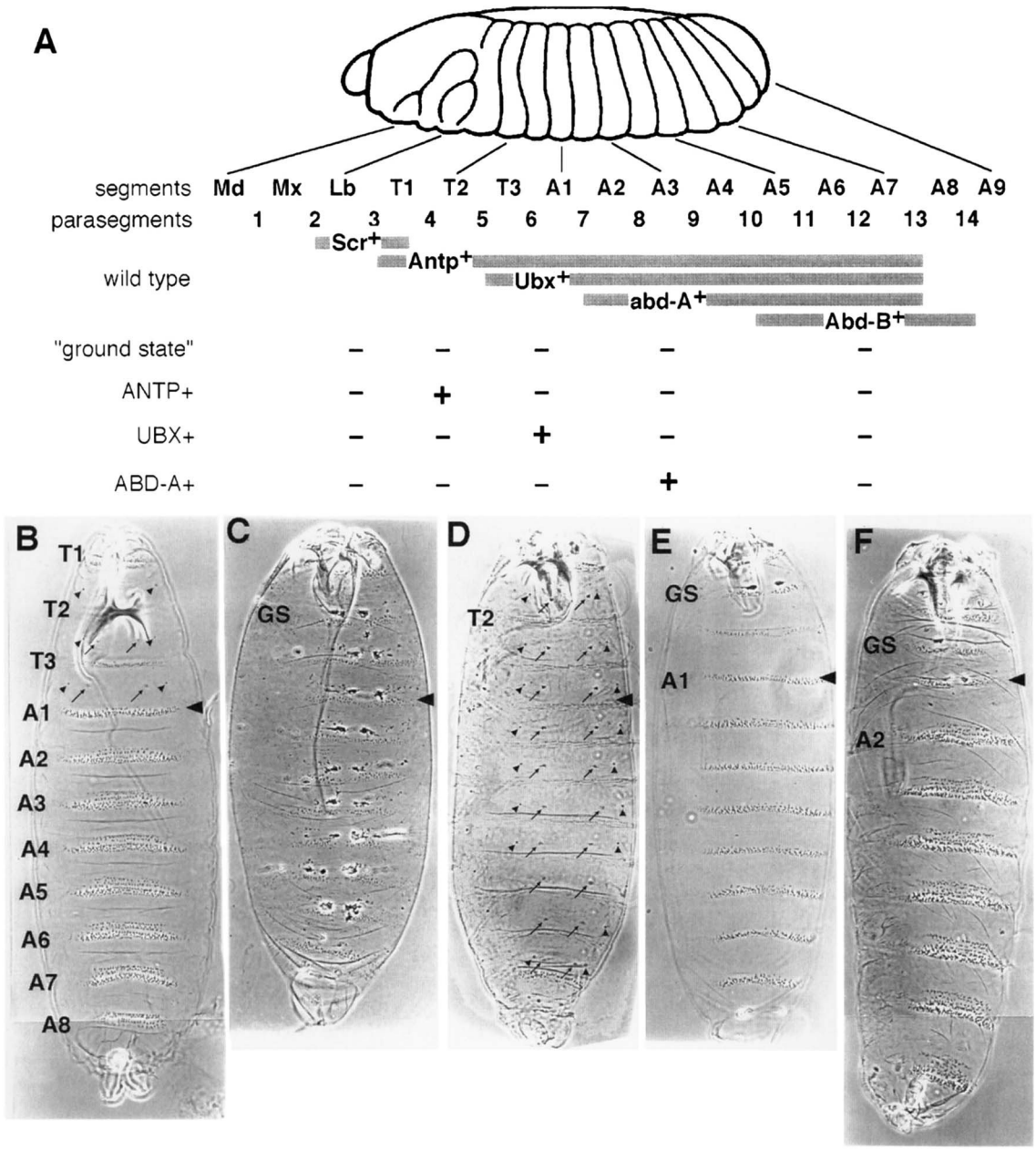


FIGURE 2.—A set of genotypes for analyzing homeotic regulation of target gene expression. (A) Schematic drawing of a Drosophila embryo close to the end of embryogenesis with the segments and parasegments indicated. The wild-type expression domains of *Scr, Antp, Ubx, abd-A,* and *Abd-B* (illustrated with gray bars) overlap during most of embryogenesis and are refined by cross-regulatory interactions. For example, *Ubx* is expressed in PS7 to PS13 at lower levels than in PS6 because of repression by *abd-A* and *Abd-B* (STRUHL 1982; HAFEN *et al.* 1984; STRUHL and WHITE 1985; RILEY *et al.* 1987; SCOTT and CARROLL 1987). Because of this complexity, embryos having the genotypes Scr^- $Antp^-$ Ubx^- $abd\text{-}A^-$ $Abd\text{-}B^-$ ("ground state"), Scr^- Ubx^- $abd\text{-}A^-$ $Abd\text{-}B^-$ ($ANTP^+$), Scr^- $Antp^-$ $abd\text{-}A^-$ $Abd\text{-}B^-$ (UBX^+), and Scr^- $Antp^-$ Ubx^- $Abd\text{-}B^-$ ($ABD\text{-}A^+$) were generated. Md, mandibullar; Mx, maxillary; Lb, labial; T1–T3, the first, second and third thoracic segments; A1–A9, abdominal segments 1–9). (B–F) First instar larval cuticle preparations of the genotypes described in (A). The large arrowhead in each panel points to the equivalent position along the anterior-posterior axis (approximately the T3/A1 boundary). When visible, the thoracic specific Keilin's organs (small arrows) and ventral pits (small arrowheads) are indicated. In wild-type embryos (B) three thoracic (T1–T3) and eight abdominal segments (A1–A8) were visible. In Scr^- $Antp^-$ Ubx^- $abd\text{-}A^-$ $Abd\text{-}B^-$ embryos (C) all thoracic and abdominal segments have acquired the ground state identity (GS, only one segment is labeled). In Scr^- Ubx^- $abd\text{-}A^-$ $Abd\text{-}B^-$ embryos (D) T1 is transformed towards a more anterior identity and the remaining thoracic and abdominal segments appear T2-like (T2, only one segment is labeled). In Scr^- $Antp^-$ $abd\text{-}A^-$ $Abd\text{-}B^-$ embryos (E) T1 and T2 are transformed to the ground state (GS), T3 is partially transformed to the ground state, and all abdominal segments appear A1-like (A1, only one segment is labeled). In Scr^- $Antp^-$ Ubx^- $Abd\text{-}B^-$ embryos (F) T1–T3 and A1 are transformed to the ground state (GS) and the remaining segments appear A2- or A3-like (A2, only one segment is labeled).

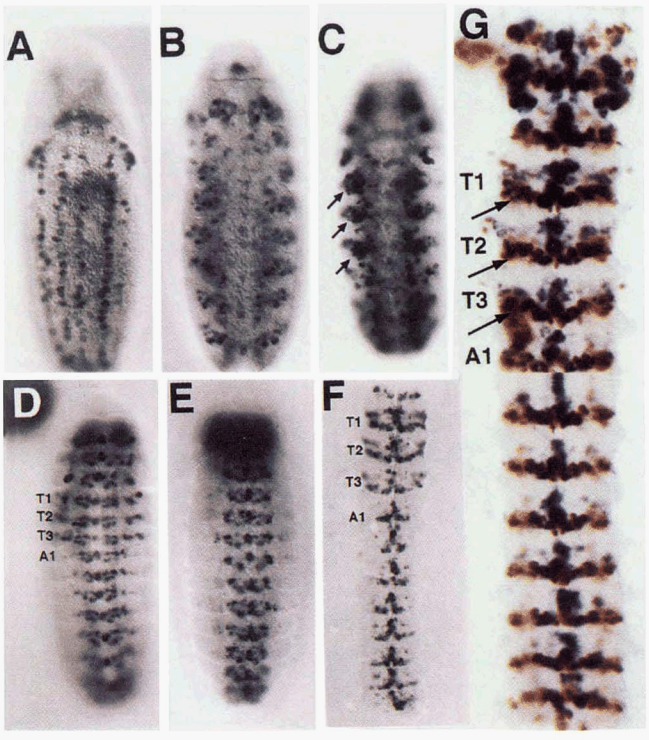


FIGURE 3.—Expression of *nervy* during embryogenesis. All panels are photomicrographs of embryos (A–E) or dissected nerve cords (F and G) stained for *nervy* RNA; anterior is up. (A) *nervy* expression began in a subset of neuroblasts in stage 9 embryos. (B) In stage 10 embryos, *nervy* expression appeared to be excluded from the CNS and may be limited to the peripheral nervous system (PNS). No segmental differences in *nervy* expression were apparent at these early stages. (C) In stage 11 embryos, *nervy* expression in thoracic segments (arrows) was elevated relative to its expression levels in abdominal segments. (D) In late stage 12 embryos (close to the end of germ band shortening) *nervy* expression close to the ventral midline was observed. At this stage, the thoracic segments (T1–T3) express *nervy* in additional, more laterally positioned cells than abdominal segments (*e.g.*, A1). (E) After germ band shortening is complete (stage 14) *nervy* expression again appeared identical in all thoracic and abdominal segments. (F) In a dissected nerve cord from a stage 15 embryo clear differences in the *nervy* expression pattern were again visible in thoracic and abdominal segments. (G) A dissected stage 15 nerve cord stained for *nervy* RNA and en protein (brown). The additional thoracic-specific *nervy*-positive cells also expressed en (arrows).

(Figure 5B). This "ground state" *lips* pattern was transformed to a wild-type-like pattern by Ubx^+ in posterior segments (Figure 5C). However, $abd\text{-}A^+$ did not generate a wild-type *lips* expression pattern (Figure 5D). The *lips* expression pattern in abdominal segments is more likely controlled by *Ubx* rather than by *abd-A*. However, *abd-A* is a regulator of *lips* expression because the pattern observed in Scr^- $Antp^-$ Ubx^- $Abd\text{-}B^-$ embryos was different than the ground state (Scr^- $Antp^-$ Ubx^- $abd\text{-}A^-$ $Abd\text{-}B^-$) pattern.

Expression of *lips* is regulated by homeotic genes early in embryogenesis: In wild-type embryos that have just completed cellularization (stage 5–6) *lips* transcripts were observed along the dorsal surface [most intensely from 19 to 66% egg length (EL; 0% is the posterior pole)], in two intense transverse stripes (at 50 and 57% EL), and in a weaker transverse stripe at 43% EL (Figure 6A). Strong expression was also observed in a wide band near both poles (at ~14 and 84% EL). These bands are more intense dorsally. In addition to this expression, four weak stripes were usually observed (two anterior to 57% and two posterior to 43%). *In situ* hybridization experiments using probes for both *lips* and *Ubx* at this stage demonstrated that the *lips* stripe at 50% EL approximately coincides with *Ubx* expression at this stage in PS6 (Figure 6, B and C).

This expression pattern suggested that homeotic genes may play a role in regulating *lips* expression at this stage of embryogenesis. Because of the potential for cross-regulation by the homeotic genes, this possibility was initially tested by removing all homeotic genes that are normally active in the thoracic and abdominal segments. Thus, the expression of *lips* was analyzed at stage 6 in Scr^- $Antp^-$ Ubx^- $abd\text{-}A^-$ $Abd\text{-}B^-$ embryos (Figure 6D). No stripes of *lips* expression were observed in embryos of this genotype. *lips* expression along the dorsal surface and at the two poles appeared unchanged in these embryos. These results suggest that the five trunk homeotic genes differentially activate *lips* expression to generate the early striped pattern.

To further investigate a regulatory role for *Ubx* at this stage, we examined *lips* expression in *HS:Ubx* and *HS:Ubx-FS* embryos. *HS:Ubx* and *HS:Ubx-FS* embryos were collected in parallel for 3 hr, heat shocked at 37° for 35 min, and allowed to recover at 25° for 1 hr before fixation. Interestingly, four strong stripes of *lips* expression were observed in many of the *HS:Ubx* embryos (Figure 6F). The additional two strong stripes of expression apparently coincide in position with stripes that are present but much weaker in wild-type embryos (compare with Figure 6A). *lips* expression along the dorsal surface and at the two poles appeared unchanged in these embryos. The four-striped *lips* pattern was never observed in wild-type embryos, nor was this pattern seen in *HS:Ubx-FS* embryos (Figure 6E). The inducibility of these stripes by UBX, together with the lack of stripes observed in Scr^- $Antp^-$ Ubx^- $abd\text{-}A^-$ $Abd\text{-}B^-$ embryos, suggests that this aspect of the early *lips* expression pattern is regulated by homeotic genes.

The *nervy* protein is similar to that encoded by a proto-oncogene: To further characterize these three putative target genes, cDNA clones representing *lips*, *belt*, and *nervy*, were isolated and sequenced. The characterization of the *nervy* cDNAs is presented here, while that for *lips* and *belt* will be given elsewhere.

Three *nervy* cDNAs, that appeared to differ only at their 5′ and 3′ ends, were isolated. The DNA sequence of the longest cDNA identified one large open reading frame (ORF) encoding a predicted protein of 76 kDa

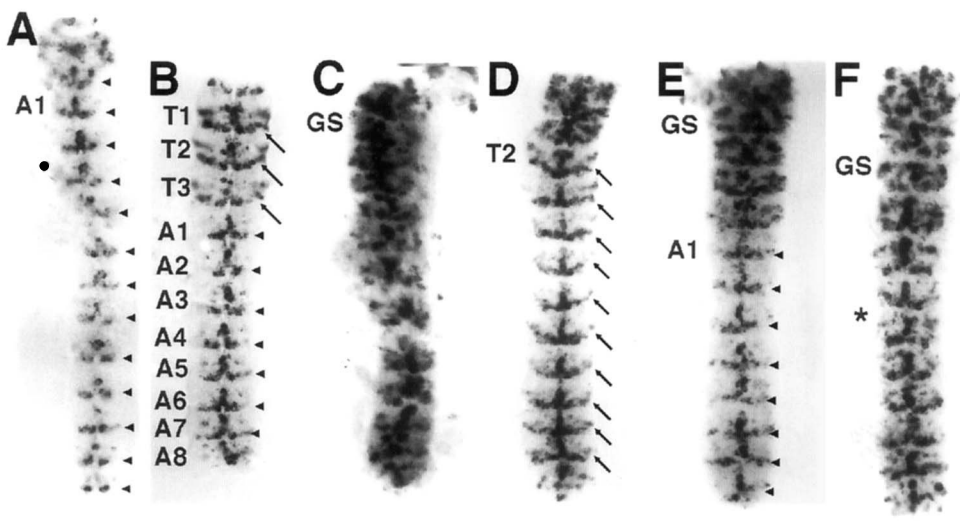


FIGURE 4.—Homeotic control of the late *nervy* expression pattern. All panels show dissected nerve cords from stage 15 embryos that were stained for *nervy* RNA expression; anterior is up. (A) *HS:Ubx*; (B) wild type; (C) *Scr*$^-$ *Antp*$^-$ *Ubx*$^-$ *abd-A*$^-$ *Abd-B*$^-$; (D) *Scr*$^-$ *Ubx*$^-$ *abd-A*$^-$ *Abd-B*$^-$; (E) *Scr*$^-$ *Antp*$^-$ *abd-A*$^-$ *Abd-B*$^-$; (F) *Scr*$^-$ *Antp*$^-$ *Ubx*$^-$ *Abd-B*$^-$. The ground state (GS, only one segment is labeled in C, E, and F), thoracic (T1–T3), abdominal (A1), and novel (*, only one segment is labeled in F) segment identities are indicated. The T2 pattern (B and D) is indicated with arrows and the abdominal pattern (A, B, and E) is indicated with arrowheads.

(Figure 7A). When compared to the sequence database, the human gene *ETO* (also called *MTG8* and *CDR*) had the highest degree of similarity (ERICKSON *et al.* 1992, 1994; NISSON *et al.* 1992; KOZU *et al.* 1993; MIYOSHI *et al.* 1993) (Figure 7B). Throughout their ORFs, these two predicted proteins are ~30% identical, with three subregions exhibiting 45–55% identity (Figure 7B). The high degree of similarity throughout their ORFs suggests that *nervy* is a Drosophila homologue of the *MTG8*(*ETO*) gene. *MTG8*(*ETO*) is a proto-oncogene because it composes most of the fusion transcript that is consistently present in acute myeloid leukemias containing the t(8:21) translocation (MIYOSHI *et al.* 1993). Interestingly, the translocation partner in these leukemias is the gene *AML1,* which contains a highly conserved domain present in the Drosophila gene *runt* (the runt domain). Thus, gene fusions between the human homologues of two Drosophila genes, *nervy* and *runt,* are associated with myeloid leukemias.

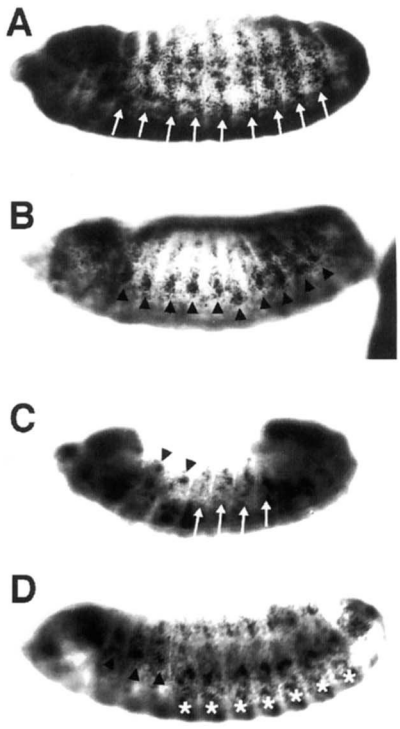


FIGURE 5.—The late expression pattern of *lips* can be generated by *Ubx*. All panels are photomicrographs of stage 14 embryos stained for *lips* RNA; anterior is to the left. In wild-type embryos (A) *lips* was expressed in a similar row of cells in all thoracic and abdominal segments (arrows). In *Scr*$^-$ *Antp*$^-$ *Ubx*$^-$ *abd-A*$^-$ *Abd-B*$^-$ embryos (B) *lips* was expressed predominantly in a single cluster of cells in each thoracic and abdominal segment (the ground state pattern, arrowheads). In *Scr*$^-$ *Antp*$^-$ *abd-A*$^-$ *Abd-B*$^-$ embryos (C) T1 and T2 exhibited the ground state pattern (arrowheads) and more posterior segments exhibited the wild-type pattern (arrows). In *Scr*$^-$ *Antp*$^-$ *Ubx*$^-$ *Abd-B*$^-$ embryos (D) T1–T3 expressed *lips* in the ground state (arrowheads) and all abdominal segments expressed *lips* in a novel pattern (*).

In addition to the overall similarity between *nervy* and *MTG8*(*ETO*), several other features of the predicted protein are noteworthy. First, database searches also identified a region of *nervy* that is similar to the Drosophila coactivator TAF110 (HOEY *et al.* 1993) (Figure 7C). This region is also conserved in *MTG8*(*ETO*) (ERICKSON *et al.* 1994). Second, *nervy* also contains two putative zinc-fingers (Figure 7D). This Cys-His region does not match the sequence of previously defined DNA-binding zinc-fingers and therefore may not represent a DNA-binding domain (HARRISON 1991). However, this region was very highly conserved between *nervy* and *MTG8*(*ETO*) (28 of 40 amino acids were identical, including all Cys and His residues), suggesting that it has an important function. Moreover, a similar pattern of Cys and His residues is also present in the programmed cell death-induced rat gene RP-8 (OWENS *et al.* 1991). This Cys-His region is conserved in RP-8 homologues isolated from mouse and *Caenorhabditis elegans* (WILSON *et al.* 1994; D. L. VAUX, unpublished GenBank submission). The evolutionary conservation of this sequence suggests that it may represent a novel Cys-His protein motif.

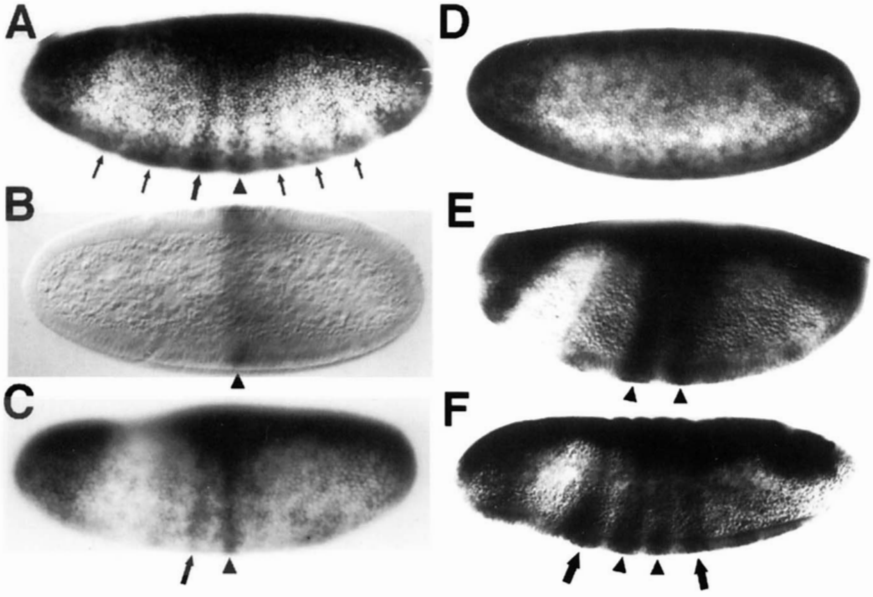


FIGURE 6.—*lips* is regulated by homeotic genes very early in embryogenesis. (A) The wild-type *lips* expression pattern during cellularization (stage 5). Expression was strongest along the dorsal surface and in two transverse stripes at 50% EL (arrowhead) and at 57% EL (thick arrow). In addition, weaker stripes are also visible (thin arrows). (B and C) Similarly aged embryos as in (A) but probed for *Ubx* expression (B) or both *Ubx* and *lips* (C). One of the two strong stripes of *lips* expression appeared to coincide with *Ubx* expression at this stage (arrowheads in B and C). The *Ubx* probe used in (B) was entirely derived from the 3′-most *Ubx* exon and therefore only identified mature or nearly mature transcripts. In *Scr*$^-$ *Antp*$^-$ *Ubx*$^-$ *abd-A*$^-$ *Abd-B*$^-$ stage 5 embryos (D) the stripes of *lips* expression were not visible whereas the dorsal expression was unchanged. In wild-type stage 6–7 embryos, *lips* expression was strongest dorsally and in two of the central *lips* stripes; ubiquitous UBX-FS expression (E) did not alter this wild-type pattern. In contrast, ubiquitous UBX expression resulted in four strong stripes of *lips* expression [F; arrowheads point to *lips* stripes at 50 and 57% EL (also in E) and arrows point to an increase in *lips* expression].

DISCUSSION

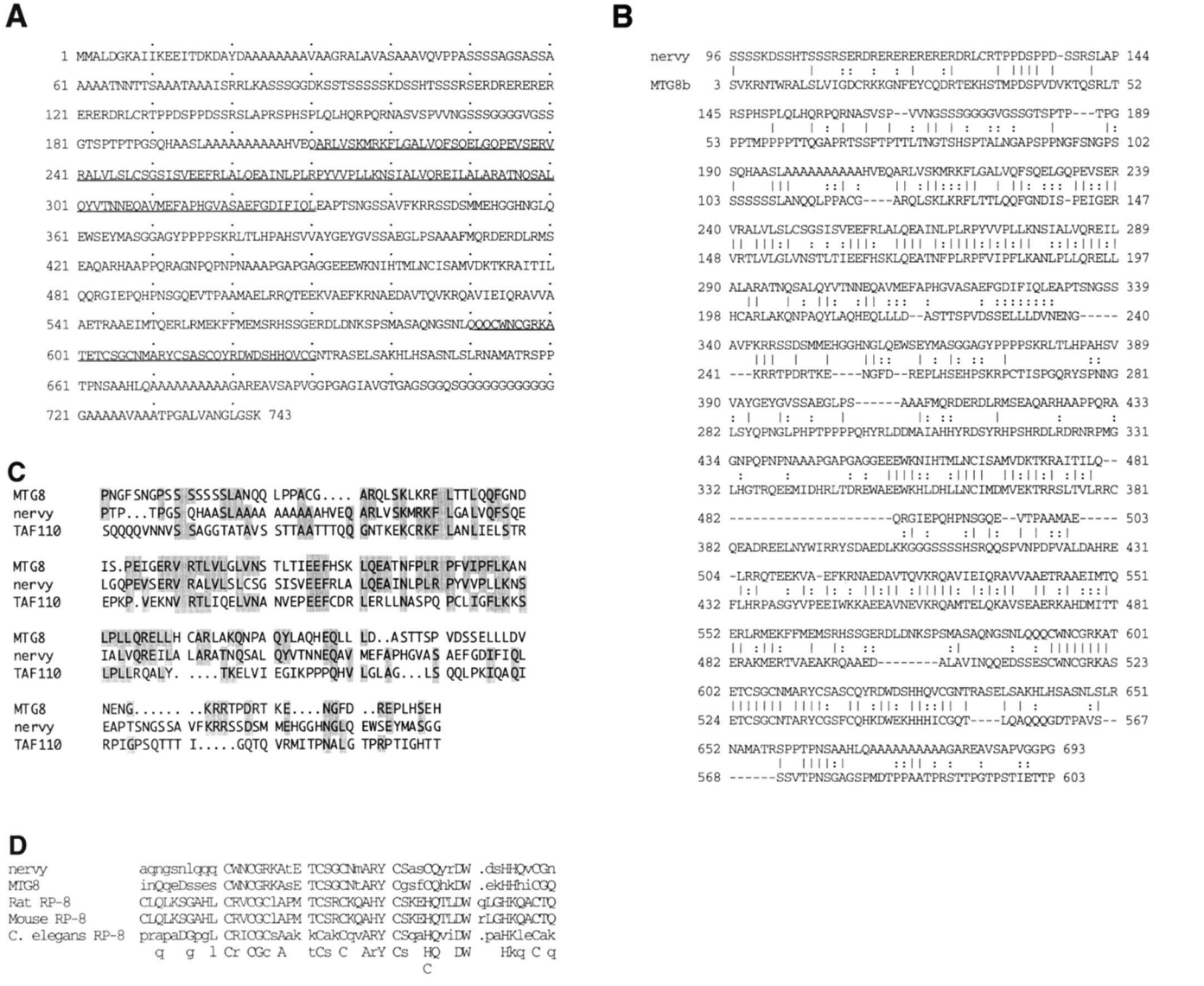
Did the subtractive hybridization enrich for UBX-activated target genes? Using a subtractive hybridization screen, we have identified genes whose expression is regulated by homeotic proteins in Drosophila. This approach depends upon isolating differences in gene expression that are induced by ectopic homeotic gene expression *in vivo*. In the screen described here, the most highly represented sequence in the subtractive probe was 412, consistent with its strong UBX-inducibility (Figure 1). In addition, 412 sequences are present ~25 times per genome. These two facts account for why 412 related clones represented ~76% of the phage isolated in this screen. However, many of the remaining clones also contained UBX-inducible sequences (Table 1; Figure 1 and data not shown). These data suggest that subtractive hybridization can be used to isolate the downstream targets of homeotic proteins. By avoiding the repeated isolation of 412, future screens should be successful at isolating additional single copy homeotic targets.

In contrast to previously described methods (GOULD *et al.* 1990; GRABA *et al.* 1992), this approach relies on the induction of target gene expression and not only on the ability of homeotic proteins to bind DNA. Thus, while the subtractive hybridization approach is more likely to identify homeotic-induced differences in gene expression, it may not always identify directly regulated target genes. An alternative approach to isolating homeotic target genes that depends on identifying Ubx-responsive binding sites in yeast has also been described (MASTICK *et al.* 1995).

Presently, there is no strong evidence that the regulation of the target genes described here is direct. However, consistent with their direct regulation, *lips, belt,* and *nervy* map close to UBX binding sites on salivary gland polytene chromosomes (J. BOTAS and D. S. HOGNESS, unpublished observations). In addition, UBX binding sites have been identified within a large *nervy* intron and within the 412 LTR (P. G. FEINSTEIN, S.-K. Chan, and R. S. MANN, unpublished observations).

***lips, belt,* and *nervy* are regulated by more than one homeotic gene:** Although this screen was designed to isolate target genes activated by *Ubx,* in all cases the wild-type expression patterns for the genes characterized here imply that they are regulated by other homeotic genes in addition to *Ubx.* Further evidence that these genes are regulated by multiple homeotic proteins comes from studying animals in which homeotic gene expression was altered. For example, the expression of *belt* in germ band extended embryos was induced in anterior parasegments by either ubiquitous *Ubx* or *Antp* expression. These results suggest that both of these homeotic genes can activate *belt* expression equivalently in these parasegments.

While experiments that use the ubiquitous expression of homeotic gene products demonstrate the inducibility of these target genes in ectopic positions, they do not address how homeotic genes regulate their expression in wild type embryos. Moreover, the regulation



C

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MTG8      PNGFSNGPSS SSSSSLANQQ LPPACG.... ARQLSKLKRF LTTLQQFGND
nervy     PTP...TPGS QHAASLAAAA AAAAAAHVEQ ARLVSKMRKF LGALVQFSQE
TAF110    SQQQQVNNVS SAGGTATAVS STTAATTTQQ GNTKEKCRKF LANLIELSTR

MTG8      IS.PEIGERV RTLVLGLVNS TLTIEEFHSK LQEATNFPLR PFVIPFLKAN
nervy     LGQPEVSERV RALVLSLCSG SISVEEFRLA LQEAINLPLR PYVVPLLKNS
TAF110    EPKP.VEKNV RTLIQELVNA NVEPEEFCDR LERLLNASPQ PCLIGFLKKS

MTG8      LPLLQRELLH CARLAKQNPA QYLAQHEQLL LD..ASTTSP VDSSELLLDV
nervy     IALVQREILA LARATNQSAL QYVTNNEQAV MEFAPHGVAS AEFGDIFIQL
TAF110    LPLLRQALY. ....TKELVI EGIKPPPQHV LGLAG...LS QQLPKIQAQI

MTG8      NENG..... ..KRRTPDRT KE....NGFD ..REPLHSEH
nervy     EAPTSNGSSA VFKRRSSDSM MEHGGHNGLQ EWSEYMASGG
TAF110    RPIGPSQTTT I.....GQTQ VRMITPNALG TPRPTIGHTT
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D

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nervy             aqngsnlqgq CWNCGRKAtE TCSGCNmARY CSasCQyrDW .dsHHQvCGn
MTG8              inQqeDsses CWNCGRKAsE TCSGCNtARY CgsfCQhkDW .ekHHhiCGQ
Rat RP-8          CLQLKSGAHL CRVCGClAPM TCSRCKQAHY CSKEHQTLDW qLGHKQACTQ
Mouse RP-8        CLQLKSGAHL CRVCGClAPM TCSRCKQAHY CSKEHQTLDW rLGHKQACTQ
C. elegans RP-8   prapaDGpgL CRICGCsAak kCakCqvARY CSqaHQviDW .paHKleCak
                    q   g  l Cr CGc A   tCs C  ArY Cs  HQ  DW    Hkq C q
                                                    C
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FIGURE 7.— *nervy* encodes a homologue of a proto-oncogene. (A) The sequence of the predicted nervy protein. The underlined region from residues 211–340 corresponds to a region with similarity to TAF110 (C) and the underlined region from residues 591–630 corresponds to a region with an evolutionarily conserved pattern of Cys and His residues (D). (B) A bestfit comparison of the predicted proteins encoded by the *nervy* (top line) and *MTG8b* (bottom line, also called *ETO*) cDNAs. The two ORFs are ~30% identical from nervy residues 96–693. Three regions are more highly conserved than the average: nervy residues 211–308 (48%), residues 454–480 (55%), and residues 519–632 (46%). Vertical lines indicate identical amino acids and colons indicate similar amino acids. Gaps in the sequence are indicated by dashes. (C) Sequence similarities between TAF110, nervy, and MTG8/ETO. The regions shown in this figure are: TAF110, residues 261–436; nervy, residues 184–370; and MTG8b, residues 94–261. Gaps in the sequence are indicated by periods. (D) A pattern of Cys and His residues is conserved in nervy, MTG8, and RP-8 homologues isolated from three species. Rat RP-8 was originally identified as a gene that is induced in neurons upon programmed cell death (OWENS *et al.* 1991). The bottom line summarizes the conserved residues in these five sequences. All conserved Cys or His residues are indicated with capital letters. Additional conserved residues are indicated with capital letters if present in all five sequences and small letters if present in at least three of the five sequences. Nervy residues shown here are 583–631. All five of these sequences share the following consensus: $\underline{\mathbf{C}x_2\mathbf{C}}$ – $\mathbf{G}x_2\mathbf{A}x_3$ – $\underline{\mathbf{C}x_2\mathbf{C}}$ – $x_2\mathbf{A}x\mathbf{Y}$ – $\underline{\mathbf{C}x_3\mathbf{C}/\mathbf{H}}$ – $\mathbf{Q}x_2\mathbf{DW}x_{2-3}$ – $\underline{\mathbf{H}x_3\mathbf{C}}$, where x is any residue and potential zinc-chelating residues are underlined. The two sets of bolded residues indicate the two putative fingers.

of target gene expression is complicated by two facts: first, target genes are often expressed in many segments and are, therefore, expressed within the domains of several different homeotic genes. Second, homeotic genes often cross-regulate each other. Thus, removing the function of a single homeotic gene may not adequately address its regulatory role.

To better characterize target gene regulation, a set of genotypes was constructed and used to assess the regulatory contribution of individual homeotic genes to these complex expression patterns. The method requires that embryos of the genotype *Scr*$^-$ *Antp*$^-$ *Ubx*$^-$ *abd-A*$^-$ *Abd-B*$^-$ have a different pattern of target gene expression than wild-type embryos. *Scr*$^-$ *Antp*$^-$ *Ubx*$^-$ *abd-*

A$^-$ *Abd-B*$^-$ animals have no wild-type homeotic gene function in thoracic and abdominal (trunk) segments and therefore have a segment morphology that approximates the ground state (LEWIS 1978). The pattern of target gene expression in animals of this genotype was compared with the pattern observed in embryos that have only a single functional trunk homeotic gene.

This approach was used to demonstrate that the ground state pattern of *lips* could be altered by *Ubx* and *abd-A* and that the ground state pattern of *nervy* could be altered by *Antp, Ubx,* and *abd-A*. *nervy,* which was expressed in different patterns in thoracic and abdominal segments, was shown to be differentially regulated by *Antp, Ubx,* and *abd-A*. Interestingly, while *Antp* and *Ubx* generated patterns that appeared similar to the wild-type T2 and abdominal patterns, respectively, *abd-A* did not produce a recognizable *nervy* pattern. We therefore infer that *Ubx,* not *abd-A,* is more likely to be the relevant regulator of the late *nervy* expression pattern in the wild-type abdomen.

In *Scr*$^-$ *Antp*$^-$ *Ubx*$^-$ *abd-A*$^-$ *Abd-B*$^-$ stage 15 nerve cords, *nervy* was generally derepressed relative to its expression in embryos containing a functional homeotic gene. Thus, at this late stage of embryogenesis, the homeotic genes appear to be repressors of *nervy* expression. This is an apparent contradiction to the goal of the subtractive screen, which aimed at isolating *Ubx*-activated genes. However, earlier in embryogenesis, *nervy* expression was activated by *Ubx* (Figure 1). It therefore appears that *Ubx* can be a repressor or an activator of *nervy* expression at different developmental stages. Assuming this regulation is direct, these data suggest that different cofactors, present at these different stages, modify the regulatory activity of the homeotic genes.

The down-regulation of *Ubx* in the abdomen is functional: During wild-type embryogenesis, *Ubx* expression is down-regulated in parasegments posterior to PS6 by the products of the *abd-A* and *Abd-B* genes (BEACHY *et al.* 1985; STRUHL and WHITE 1985; WHITE and WILCOX 1985). While eliminating *Ubx* expression in these segments leads to their partial transformation to a thoracic-like segment, increasing *Ubx* expression leads to only minor phenotypic alterations in the abdominal cuticle (LEWIS 1978; GONZALEZ-REYES and MORATA 1990; MANN and HOGNESS 1990; LAMKA *et al.* 1992). The lack of a strong effect resulting from the overexpression of UBX in abdominal segments (called phenotypic suppression) suggests that the normal down-regulation of *Ubx* in these segments may be functionally irrelevant (GONZALEZ-REYES *et al.* 1990). One limitation to these studies is that the identities of abdominal segments were determined only for the first instar larval cuticles.

Using *nervy* expression as a marker, we observed a strong phenotypic effect resulting from the overexpression of UBX in abdominal segments of stage 11 embryos. Specifically, ubiquitous expression of UBX caused *nervy* to be highly expressed in all segments (Figure 1). This result suggests that the down-regulation of *Ubx* in the abdomen is important for generating the wild-type *nervy* expression pattern in stage 11 embryos. Thus, at least in *nervy*-expressing cells, the down-regulation of *Ubx* in the abdomen appears to have a function. In addition, we also observed that the expression of the 412 retrotransposon is highly induced in abdominal segments in response to UBX overexpression (Figure 1). These data suggest that, for some target genes, high levels of UBX can dominate wild-type regulation by *abd-A*.

***lips* may be a very early homeotic target gene:** One of the most striking expression patterns exhibited by the target genes isolated in this screen was the early (stage 6) striped pattern of *lips*. Its pattern of seven stripes of different intensities is unusual for genes expressed at this stage. Although earlier acting segmentation genes could be responsible for this differential expression of *lips,* three observations argue that homeotic proteins regulate *lips* expression at this stage. First, one of the stronger stripes of *lips* expression approximately coincided with early *Ubx* expression in PS6. Second, *in situ* hybridization to whole embryos showed a reproducible induction of *lips* expression in response to ectopic UBX expression at this stage. Third, *Scr*$^-$ *Antp*$^-$ *Ubx*$^-$ *abd-A*$^-$ *Abd-B*$^-$ embryos did not exhibit the *lips* stripes in stage 6 embryos but retained the other aspects of the *lips* pattern.

These data suggest that homeotic gene products may act earlier in development than was previously known. The earliest time that UBX proteins have been visualized during embryogenesis is early stage 9 (WHITE and WILCOX 1985; IRVINE *et al.* 1991). However, *in situ* hybridization experiments using probes directed against both the 3′ and 5′ ends of the *Ubx* transcript suggest that mature *Ubx* mRNAs are present when gastrulation begins (AKAM and MARTINEZ-ARIAS 1985; SHERMOEN and O'FARRELL 1991; this work). Furthermore, measurements of the time required to transcribe the *Ubx* transcription unit suggest that mitotic cycle 14 is long enough to produce mature transcripts (SHERMOEN and O'FARRELL 1991; O'FARRELL 1992). These results imply that homeotic proteins are present and may be regulating the expression of downstream target genes at this time in development.

Homeotic target gene function: To understand the control of segment morphologies by homeotic genes, it is important to characterize the function as well as the regulation of their downstream target genes. At this time, point mutations are not known to exist for *lips* or *nervy*. Using deficiencies, *lips* has been mapped to a small interval within *Df(3R)6–7* (generously provided by S. WASSERMAN) (P. G. FEINSTEIN and R. S. MANN, unpublished results). This interval includes the genes *canoe,* in which mutations produce a dorsal closure phenotype, and *l126,* in which mutations produce a pair-rule phenotype (JÜRGENS *et al.* 1984; P. G. FEINSTEIN

and R. S. MANN, unpublished results). Experiments are in progress to determine if either of these genes correspond to *lips*. Interestingly, heat shock-induced misexpression of *lips* produces highly aberrant first instar cuticles, consistent with it playing a role in segment identity determination (P. G. FEINSTEIN and R. S. MANN, unpublished results).

There are no simple deficiencies for *nervy*. In addition, because its expression is limited to the nervous system, an affect on the first instar cuticle morphology would not be expected. Instead, it is more likely that *nervy* mediates a segment-specific identity function of the homeotic genes in the central nervous system. The similarity between *nervy* and a proto-oncogene suggests that this will be an interesting gene to characterize further. Specifically, throughout their ORFs, *nervy* shares a high degree of sequence similarity with the human gene *MTG8(ETO)*. Interestingly, *MTG8(ETO)* is highly expressed in the central nervous system of humans, suggesting that its function in this tissue may be evolutionarily conserved (ERICKSON *et al.* 1994).

MTG8(ETO) is often translocated to the *AML1* gene in acute myeloid leukemias [t(8:21)] (ERICKSON *et al.* 1992, 1994; NISSON *et al.* 1992; KOZU *et al.* 1993; MIYOSHI *et al.* 1993). *AML1* contains a runt domain that is named for the Drosophila segmentation gene, *runt*, that shares this region (DAGA *et al.* 1992). The runt domain has DNA-binding and protein-protein interaction activities and a consensus ATP binding site (KAGOSHIMA *et al.* 1993; MEYERS *et al.* 1993; OGAWA *et al.* 1993). The fusion transcript present in the t(8:21)-containing leukemias has the runt domain of *AML1* fused in frame to nearly the entire *MTG8(ETO)* coding sequence (MIYOSHI *et al.* 1993). The consistent structure of this fusion transcript in independent leukemias suggests that both the *AML1* and *MTG8(ETO)* portions are important for oncogenesis. Our finding that much of the *MTG8(ETO)* coding sequence is conserved in Drosophila suggests an evolutionarily conserved function for this gene. Furthermore, expression of *nervy* in segregating neuroblasts suggests an early regulatory role for this gene in the developing nervous system. Interestingly, *runt*, like *nervy*, is also expressed in the developing central and peripheral nervous systems (DUFFY *et al.* 1991).

The predicted *nervy* protein contains two putative zinc-fingers and a region of similarity with the TATA binding protein-associated factor TAF110. Both of these features suggest that *nervy* encodes a transcription factor. TAF110 has properties of a coactivator because it can mediate an interaction between the basal transcription machinery and the transactivators SP1 and CREB (HOEY *et al.* 1993; FERRERI *et al.* 1994). Interestingly, the region of similarity between *nervy* and TAF110 partially overlaps with the SP1 and CREB interaction domain. These data suggest that the nervy protein interacts with these or other transcription factors. In future experiments, it will be interesting to explore the functional relationship between *nervy* and *runt* in the nervous system and to investigate the significance of the homeotic control of *nervy* expression.

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Note added in proof: The gene identified here as *lips* is likely to be identical to *canoe* (JÜRGENS *et al.* 1984) for the following reasons. By sequencing genomic DNA derived from a strong *canoe* allele we have identified a point mutation that results in a stop codon near the start of the *lips* ORF (P. FEINSTEIN and R. MANN, unpublished results). In addition, the sequence reported by MIYAMOTO *et al.* (1995), which they suggest is *canoe*, is nearly identical to our sequence of the *lips* ORF (P. FEINSTEIN and R. MANN, unpublished results).

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