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* 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 (Immelgluck et al. 1990; Reuter et al. 1990; Graba et al. 1992; Hinz et al. 1992; Vachon et al. 1992; Hrusak 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*), abdominial-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. 1983, 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 (ANTPC), is expressed in PS4–PS13 (Carroll et al. 1986,
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 effect 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: w1185/w1185, HS:Ubx-ia/ TM6B [the (+) stock] and w1185/w1185, HS:Ubx-iaFS Df(3R)Ubx109/TM6B [the (−) stock]. The two heat shock-Ubx loci have been previously described (Mann and Hogeness 1990). The HS:Ubx-iaFS gene was recombined onto a chromosome carrying Df(3R)Ubx109/TM6B [the (−) stock]. The two heat shock-Ubx loci have been previously described (Mann and Hagen 1990). Here, HS:Ubx refers to the HS:Ubx-ia gene and HS:Ubx-iaFS refers to the HS:Ubx-iaFS gene, which are both homozygous lethal third chromosome insertions. The “wild-type” stock (used for polytene chromosome analyses and in situ hybridizations) was the w1185/w1185 derivative of Canton-S obtained from G. Rubin. Ser’ Antp’ Ubx’ Abd-A’ Abd-B’ refers to embryos of the genotype Scf1/ AntpR (R) Scf1/ AntpR (R) Scf1/ AntpR (R) Ubx*IX2 abd-A M Abd-B48S and has been previously described (Chan and Mann 1993). The Scf1/ Ubx*IX2 abd-A M Abd-B48S Scf1/ AntpR (R) abd-A M Abd-B48S and Scf1/ AntpR (R) Ubx*IX2 Abd-B48S 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 Nüsslein-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 flp-lacZ gene (made by S. Crews and kindly provided to us by Y. Hiromi) or TM6B,22UZ which contains a Ubx-lacZ gene (Irving 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; 5×SSC; 10 mM NaPO4, pH 7.0; 0.1% Tween 20; 1× Denhardt’s; 1 mg/ml tRNA. The length of protease K treatment (3–5 min) was optimized for each probe.

A ’ Ubx probe (used in Figure 6B) was generated by digoxy-
genin labeling the 1.2-kb Xhol to EcoRI fragment of the Ubxa-
la cDNA (KORNFELD et al. 1989). This fragment is entirely
derived from the 5'-most Ubxa exon. As the Ubxa 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' Ubxa probe (used in
Figure 6C) was generated by digoxigenin labeling the 5' EcoRI to
Xhol fragment of the Ubxa-la cDNA and is primarily derived
from the 5'-most Ubxa exon.

To prepare probes for the putative target genes isolated here,
dividual phage DNAs were simultaneously restricted with Sad and Xhol endonucleases (which do not digest the
phage arms), electrophoresed on an agarose gels, blotted to
nitrocellulose, and probed with the original radiolabeled sub-
tracted cDNA (data not shown). Hybridizing fragments were
gel-purified and labeled individually with digoxigenin nucleo-
tides. The labeled fragments were pooled and used as the in situ
probe. In all cases, 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 nervous 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 trip 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 iso-
lated and were used as probes in in situ hybridization experi-
ments (P. G. FERNSTEIN 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'-GGC-
AATTCTGTAAATGTGCCTATG) and PF10 (5'-GGCGA-
TCCTGTAAATGAACTCCA) in a PCR reaction with phage
DNA was isolated from most of the 89 plaque-purified
phage. DNA was isolated from most of the 89 plaque-purified
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
Ubxa 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 se-
quences indicated that the expression of 412 may be
Ubxa-inducible. This prediction has been confirmed by
Northern hybridization of mRNA isolated from heat
shocked HS:Ubxa and HS:Ubxa-FS embryos (data not shown).
Moreover, in situ hybridization experiments demonstrate that 412 is expressed in a highly Ubxa-
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

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

RESULTS

Isolating Ubxa activated target genes by subtractive
hybridization: When Ubxa proteins are ubiquitously ex-
pressed 3–6 hr after egg laying (AEL) by the heat-in-
ducible promoter from the hsp70 gene (a HS:Ubxa gene
PS0 to PS5 are transformed into PS6-like metameeres
(GONZALEZ-REYES and MORATA 1990; MANN and HOG-
NESS 1990). For one of the Ubxa isoforms (UBX-la) (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 Ubxa protein
containing a frameshift mutation within the homeodomain
(UBX-FS) is similarly expressed no transformations are
observed. Because both the cuticle and peripheral neu-
ron phenotypes closely mimic a wild-type PS6, we rea-
soned that Ubxa target genes that are normally up-regu-
lated by Ubxa in PS6 should be more abundant in heat
shocked HS:Ubxa embryos than in heat shocked HS:Ubxa-
FS embryos. These Ubxa-inducible sequences were par-
tially purified using a subtractive hybridization proce-
dure (see MATERIALS AND METHODS). The single-
stranded cDNA resulting from the subtractive hybridiza-
tion 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 radiola-
beled 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. DNA was isolated from most of the 89 plaque-purified
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
Ubxa activated clones (Uacs) isolated using a similar screen
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TABLE 1

Summary of isolated phage

<table>
<thead>
<tr>
<th>Phage</th>
<th>Name</th>
<th>Number</th>
<th>Location</th>
<th>UBX inducible</th>
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<tr>
<td>1</td>
<td>nerry</td>
<td>1</td>
<td>99F</td>
<td>+</td>
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<tr>
<td>2</td>
<td>xerox</td>
<td>3</td>
<td>15–20 sites</td>
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<tr>
<td>3</td>
<td>—</td>
<td>1</td>
<td>61A</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>furrow</td>
<td>1</td>
<td>48B/C</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>412^</td>
<td>68</td>
<td>~25 sites</td>
<td>+++</td>
</tr>
<tr>
<td>8</td>
<td>belt</td>
<td>1</td>
<td>28E/F</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>—</td>
<td>1</td>
<td>13C</td>
<td>–</td>
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<tr>
<td>10</td>
<td>gang of three</td>
<td>2</td>
<td>ND</td>
<td>+</td>
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<tr>
<td>13</td>
<td>—</td>
<td>1</td>
<td>71B/C</td>
<td>–</td>
</tr>
<tr>
<td>18</td>
<td>lips</td>
<td>1</td>
<td>82E/F</td>
<td>+</td>
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^ 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.

^ The number of cross-hybridizing phage in each group is shown.

^ Cytological position as determined by in situ hybridization to polytene chromosomes. ND, not determined.

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

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

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...
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 ANT (in HS:UBX or HS:ANT 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 effect 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 ANT may both activate lips expression in similar patterns. Thus, the lips expression pattern would appear wild type in UBX embryos because ANT, 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 (prososcopedia (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), ANT, UBX, abd-A, and Abd-B). Thus, nearly all homeotic gene activity is eliminated in the thorax and abdomen (trunk) in Scr ANT UBX abd-A Abd-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 ANT, UBX, or abd-A but still mutant for the four remaining trunk homeotic genes were also constructed (Figure 2A). Cuticle preparations of first instar larvae having these genotypes illustrate the Scr ANT UBX abd-A Abd-B (ground state) identity and the identities generated by ANT, UBX, or abd-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 ANT 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-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 ANT and UBX can independently generate the T2 and A1 nervy patterns, respectively. In contrast, when only abd-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 ANT UBX abd-A Abd-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 ANT UBX abd-A Abd-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-A Abd-B (“ground state”), Scr Antp abd-A Abd-B (ANTP*), Scr Antp abd-A Abd-B (UBX*), and Scr Antp Ubx Abd-B (ABD-A*) were generated. Md, mandibular; 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 (approximate 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-A Abd-B embryos (C) all thoracic and abdominal segments have acquired the ground state identity (GS, only one segment is labeled). In Scr Ubx abd-A Abd-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 Ubx abd-A Abd-B embryos (E) T1 and T2 are transformed to the ground state (GS); T3 partially transformed to the ground state, and all abdominal segments appear A1-like (A1, only one segment is labeled). In Scr Antp Ubx Abd-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).
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 Ser– Anitp– Ubx– Abd–A– Abd–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 Ser– Anitp– Ubx– Abd–A– Abd–B– embryos, suggests that this aspect of the early lips expression pattern is regulated by homeotic genes.

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

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

(Figure 7A). When compared to the sequence database, the human gene \textit{ETO} (also called \textit{MTG8} and \textit{CDR}) had the highest degree of similarity (Erickson et al. 1992, 1994; Nisson et al. 1992; Koizumi et al. 1993; Miyoshi et al. 1993) (Figure 7B). Throughout their ORFs, these two predicted proteins are \textasciitilde30\% identical, with three subregions exhibiting 45–55\% identity (Figure 7B). The high degree of similarity throughout their ORFs suggests that \textit{nervy} is a Drosophila homologue of the \textit{MTG8(ETO)} gene. \textit{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 \textit{AML1}, which contains a highly conserved domain present in the Drosophila gene \textit{runt} (the \textit{runt} domain). Thus, gene fusions between the human homologues of two Drosophila genes, \textit{nervy} and \textit{runt}, are associated with myeloid leukemias.

In addition to the overall similarity between \textit{nervy} and \textit{MTG8(ETO)}, several other features of the predicted protein are noteworthy. First, database searches also identified a region of \textit{nervy} that is similar to the Drosophila coactivator TAF110 (Hoeỳ et al. 1993) (Figure 7C). This region is also conserved in \textit{MTG8(ETO)} (Erickson et al. 1994). Second, \textit{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 \textit{nervy} and \textit{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 \textit{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.
While the subtractive hybridization approach is more activated target genes? Using a subtractive hybridization screen, we have identified genes whose expression is on the ability of homeotic proteins to bind DNA. Thus, why expression, it may not always identify directly regulated likely to identify homeotic-induced differences in gene expression that are induced by ectopic homeotic gene approach depends upon isolating differences in gene expression in wild type embryos. Moreover, the regulation of these target genes in ectopic positions, they are regulated by other homeotic genes can activate 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 nery map close to UBX binding sites on salivary gland polytene chromosomes (J. BOTAS and D. S. HOGENESS, unpublished observations). In addition, UBX binding sites have been identified within a large nery intron and within the 412 LTR (P. G. FEINSTEIN, S.-K. Chan, and R. S. MANN, unpublished observations).

**lips, belt, and nery 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
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 Ser′ Antp− Ubx− abd-A′ Abd-B′ have a different pattern of target gene expression than wild-type embryos. Ser′ 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 *nerve* could be altered by *Antp*, *Ubx*, and *abd-A*. *nerve*, 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 *nerve* pattern. We therefore infer that *Ubx*, not *abd-A*, is more likely to be the relevant regulator of the late *nerve* expression pattern in the wild-type abdomen.

In *Scr* *Antp* *Ubx* *abd-A* *Abd-B* - stage 15 nerve cords, *nerve* 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 *nerve* expression. This is an apparent contradiction to the goal of the subtractive screen, which aimed at isolating *Ubx*-activated genes. However, earlier in embryogenesis, *nerve* expression was activated by *Ubx* (Figure 1). It therefore appears that *Ubx* can be a repressor or an activator of *nerve* 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 *nerve* 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 *nerve* 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 *nerve* expression pattern in stage 11 embryos. Thus, at least in *nerve*-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; Sermoen 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 (Sermoen 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 *nerve*. Using deficiencies, *lips* has been mapped to a small interval within Df(3R)H6–7 (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 *1126*, 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; Ferrer 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 (Jørgens 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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