Enhancer Traps in the Drosophila Bithorax Complex
Mark Parasegmental Domains

Kimberly McCall,* Michael B. O’Connor† and Welcome Bender*

*Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, and †Department of Biochemistry and Molecular Biology, University of California at Irvine, Irvine, California 92717

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ABSTRACT

Eight P elements carrying a β-galactosidase (lacZ) reporter have been mapped to sites within the Drosophila bithorax complex. The bithorax complex contains three homeotic genes, and at least nine regulatory regions which control their expression in successive parasegments of the fly. The enhancer traps inserted at the promoter of one of the genes, Ultrabithorax, express lacZ in patterns which mimic the Ultrabithorax protein pattern. Enhancer traps in the regulatory regions do not mimic the endogenous genes, but express lacZ globally in the relevant parasegments. Some P elements carry large DNA fragments upstream of the lacZ promoter but internal to the P element. In cases where these internal sequences specify a lacZ pattern, that pattern is generally suppressed when the element is inserted in the bithorax complex. In embryos mutant for genes of the Polycomb group, the lacZ expression from the enhancer traps spreads to all segments. Thus, the enhancer traps reveal parasegmental domains that are maintained by Polycomb-mediated repression. Such domains may be realized by parasegmental differences in chromatin structure.

The initial boundaries of homeotic gene expression are likely to be established by the action of gap and pair-rule genes (Qan et al. 1991; Müller and Bienz 1992; Shimell et al. 1994). After these gene products decay in early embryogenesis, the roles of maintenance of homeotic gene repression and expression are taken over by the genes of the Polycomb and trithorax groups, respectively. The Polycomb group (Pc-G) of genes are required for maintaining repression of the homeotic genes. Mutations in these genes result in expression of the homeotic genes beyond their normal boundaries (White and Wilcox 1985; Struhl and Akam 1985; Celniker et al. 1990; Simon et al. 1992). Mutations in the trithorax group result in the loss of expression of homeotic genes in specific cells, tissues or parasegments (Breen and Harte 1993).

P elements containing the lacZ gene have been used as enhancer detectors in Drosophila (O’Kane and Gehring 1987). For several of such “enhancer trap” insertions, their positions relative to the adjacent transcription unit have been mapped, and the pattern of the endogenous transcript has been compared to the pattern of β-galactosidase (β-gal) from the P element. For example, enhancer traps in the elav, fasciclin III, Couch potato and connectin genes have all been mapped to the 5′ end of the endogenous transcript, and the lacZ patterns look very similar to the protein or RNA patterns produced by these genes in wild-type embryos (Bier et al. 1989; Wilson et al. 1989; Belen et al. 1992; Nosz et al. 1992). For other enhancer traps that have been characterized, β-gal is produced in a subset of the tissues.
that express the adjacent gene (e.g., Toll, rhomboid (Bellem et al. 1989; Bier et al. 1990)). Two enhancer
traps have been characterized in the homeotic gene clusters. One is inserted just upstream of the Antennapedia
P1 promoter and mimics the expression pattern of this transcript (Engstrom et al. 1992). Another has been
mapped to the iab-7 regulatory region of AbdB, and it appears to respond only to enhancers from within this
regulatory region (Galloni et al. 1993).

In this report, we describe eight P element enhancer traps that are inserted in the BX-C. Three of these lines
behave like enhancer traps in most other loci and mimic the pattern of the adjacent gene. Five enhancer traps,
however, do not reproduce the patterns of the homeotic genes, but they faithfully mark the parasegmental limits
of the regulatory domains.

MATERIALS AND METHODS

Drosophila strains: Insertions in the BX-C were identified by phenotype or ß-gal pattern. Many carried DNA fragments
from the complex (see Table 1) cloned into the SacI site of pMBO140 (Simon et al. 1990). The transformant line bxex
(3-51) was kindly provided by Christian Klambt and Corey Goodman; the UbxPac(-31) strain was kindly provided by Jeff
Simon. The Jbx(31) insertion (Mazo et al. 1990) was recombined onto the bxPac(3-51) and bxdPac(-13) chromosomes. The following
Pc-G mutations were used: esc, esc(10) Pc and Pc(3) (Lindsey and Zimm 1992). esc embryos were generated by
crossing esc/esc females to esc/esc males that were heterozygous for one of the enhancer traps on the third chromosome. The 02-3 chromosome (Robertson et al. 1988) was used in generating derivative lines contains a stably integrated source of P transposable.

Mapping of P elements: The P element insertions bxPac(-31), UbxPac(-31), UbxPac(-13) and bxdPac(-13) were recovered from bacteriophage λ genomic libraries. The libraries contained genomic DNA fragments generated by partial digestion with Sau3AI; these were inserted into the EMBL3 vector. The P element insertions bxPac(-31) (bxPac(-31) and bxdPac(-13)) were mapped using inverse PCR (Ochman et al. 1988), using primers homologous to the 3' end of the P element. The resulting product was radio labeled using PCR and was hybridized to a Southern blot containing EcoRI-digested phage clones carrying genomic DNA from the BX-C. The orientations of the P elements were determined by Southern blot. The junction fragments from several P element lines were sequenced using the Sequenase protocol (U.S. Biochemical Corp.).

Antibody staining: Embryos were fixed, stained and dissected as described (Karch et al. 1990) using a mouse mono-
clonal against ß-gal (Promega), followed by an HRP-conjugated goat-anti-mouse secondary antibody (Bio-Rad).
The gut and visceral mesoderm are pulled away from the epidermis in these dissections, and are not included in the
photographs here. X-gal staining of larval tissues was performed as described in Glaser et al. (1986). The anti-USB antibody was the FR.3.38 monoclonal (White and Wilcox 1984), generously provided by Ian Duncan. The anti-ABDB was the 1A229 monoclonal (Celinker et al. 1989) generously provided by Sue Celinker.

Detection of RNA in situ: A 4.1 kb EcoRI-HindIII fragment from the rosy gene (coordinates 0.0 to +4.1, (Cote et al. 1986)) was cloned into pGEM (Promega). This plasmid was digested with PstI, which cuts 1.1 kb downstream from the EcoRI site, and a digoxigenin-labeled RNA probe was produced using SP6 polymerase (New England Biolabs) following directions from the Genius kit (Boehringer Mannheim). The resulting probe is labeled from +1.1 to +4.1, which corre-
sponds to a region that is deleted in the ry0b strain (Cote et al. 1986). Anti-sense lacZ was generated using pGEMAlacZ, kindly provided by Francisco Peligri. Subsequent treatment of the RNA probe and hybridization to embryos was performed using the procedure of Gavis and Lehman (1992).

Generation of derivative lines: The UbxPac(-31) insertion was derived from the bxPac(-31) insertion through dysgenesis. The bxPac(-31) was combined with the 02-3 source of transposable for one generation in males, and the bxPac(-31) chromosome was recovered from a group of unselected male progeny.

Derivatives of iab-7Pac(-132) were generated by crossing iab-7Pac(-132)/sb 02-3 males to ry- females and recovering
male ry' progeny that lacked patches of dark tissue on anterior abdominal segments. One derivative line (iab-7Pac(-132/)) had the pattern shown in Figures 1G and 2, E and F. The structure of the P element inserted in the derivative line was determined by Southern blot using probes from the iab-7, bxd/ib-2 and Ubx promoter regions.

Two transformant lines exPac(31)/sb 02-3 males were crossed to ry0b females, and independent lines were established from ry' male progeny. Several derivative lines (iab-8Pac(-132)) had the lacZ pattern shown in Figures 1H and 3B.

RESULTS

Figure 1 shows the embryonic lacZ expression patterns from eight enhancer trap lines, aligned according to their sites of insertion within the bithorax complex. The enhancer trap lines are named according to their sites of insertion within the bithorax complex. The enhancer trap lines are indicated by brackets in parentheses. Two of the bxPac lines are inserted at approximately the same location (3-31); these are indicated by A and B, referring to the order in which the lines were isolated. Deletion derivatives of initial P element lines are indicated with a Δ. Three of the enhancer trap lines have insertions very close to the Ubx promoter (Figure 1, A, B and C). These lines have lacZ expression patterns that are very similar to that of the endogenous Ubx gene. The remaining five P elements, however, are inserted into the regulatory regions of Ubx (Figure 1, D, E and F) or AbdB (Figure 1, G and H). Two transformant lines express ß-gal from the P element promoter, but the remaining lines express ß-gal from the Ubx promoter (Table 1). The lines containing the Ubx promoter also contain various regulatory DNA fragments from the BX-C within the P elements (Table 1). These were recovered from a series of experiments where we were analyzing the regulatory DNA for enhancer properties (M. O’Connor and J. Simon, unpublished results).

The Ubx promoter insertions: The enhancer traps shown in Figure 1, A, B and C, map just upstream of the Ubx promoter. The most proximal enhancer trap, UbxPac(-31) (Figure 1A) is inserted 13 nucleotides upstream of the Ubx transcriptional start site (Saari and Bienz 1987). This insertion is homozygous lethal, and
homogygous first instar larvae have a characteristic Ubx phenotype, i.e., segment A1 is transformed to a thoracic segment and thoracic pits appear on the abdominal segments. The Ubx protein pattern resembles that of a strong bxd mutation (Beachy et al. 1985; White and Wilcox 1985) although it is also reduced in PS5 (data not shown).

The bxdPlac(-31)A insertion is 135 bp upstream of the Ubx start site (Figure 1B). Homozygotes die as pharate adults with a severe bxd transformation. Animals dissected from their pupal cases are missing the first abdominal tergite, have an additional band of postnotal tissue, and have halteres that are strongly transformed toward wing in the posterior compartment. Homozygous embryos have reduced levels of Ubx protein in PS6–12; the pattern in PS6 now resembles that of PS5 (data not shown).

The bxdPlac(-31)A P element (Figure 1C) is inserted 196 bp upstream of the Ubx start site. Heterozygotes over the TM2 (Ubx13) balancer chromosome are viable, but the halteres are enlarged to about five times the size of TM2/+, and the first abdominal tergite is reduced in size. Homozygotes are similarly transformed but they have reduced viability and those that do survive usually have unexpanded wings. We have been unable to separate this wing phenotype from the bxd phenotype by recombination. Furthermore, some ry derivatives of the bxdPlac(-31)A line, induced by hybrid dysgenesis, lack the wing phenotype. Both observations suggest that the wing phenotype is associated with the bxdPlac(-31)A insertion. The Ubx protein pattern in embryos appears normal.
All three of these enhancer traps have a \( \beta \)-gal expression pattern that mimics the \( Ubx \) protein pattern in several respects. They have expression in the epidermis and CNS from PS5-13, with the highest level of expression in PS6. All three lines have expression in the visceral mesoderm in PS7, and the two most proximal lines have expression in the CNS midline cells of PS4. The most distal insertion, \( bxd^{\text{Plac(-31)A}} \), has much less staining in PS5 than the other two lines (Figure 1C).

In the three insertion lines (especially the \( bxd^{\text{Plac(-31)B}} \) line), young embryos express \( \beta \)-gal weakly in the lateral epidermis and head in a pattern similar to the basal pattern produced from the \( Ubx \)-lacZ construct (BIENZ et al. 1988; SIMON et al. 1990). But by 10 hr of development, there is no evidence of the basal pattern in these or any of the lines shown in Figure 1. We stained for \( lacZ \) RNA in embryos from three enhancer trap lines (\( bxd^{\text{Plac(-31)B}}, bxd^{\text{Plac(-13)}}, \) and \( iab^{\text{Plac(159)}} \)) to measure more accurately the time of \( lacZ \) shut off. RNA expression in the head is last observed at about 6 hr of development (data not shown).

The \( bxd^{\text{Plac(-31)A}} \) and \( bxd^{\text{Plac(-31)B}} \) elements contain DNA fragments from the BX-C regulatory regions upstream of the \( Ubx \) promoter (Table 1). Control lines containing these constructs inserted outside the BX-C have \( \beta \)-gal patterns that initiate with a PS6 boundary in early embryos, but at late times \( \beta \)-gal is expressed throughout the embryo (data not shown). The position in the BX-C prevents the anterior expression. This position effect is also observed in the \( bxd^{\text{Plac(+13)}}, \) and \( iab^{\text{Plac(+159)}} \) lines and is discussed in more detail below. Similarly, \( Ubx^{\text{Plac(-31)}} \) contains DNA from the \( bx \) region. At late stages of embryogenesis, insertions outside the BX-C that contain this \( bx \) fragment express \( \beta \)-gal in the anterior thorax and head, but this anterior expression does not occur in \( Ubx^{\text{Plac(-31)}} \) embryos.

**The PS5 insertions:** The \( bx^{\text{Plac(-61)}} \) P element is the PZ construct (MLODZIK and HIROMI 1992), with a P element promoter driving \( \beta \)-gal. The resulting enzyme includes the nuclear localization signal from the P element. The insertion maps at -61 kb on the BX-C map, within the large \( Ubx \) intron. It is oriented with the \( lacZ \) gene transcribed in the same direction as the \( Ubx \) gene. Animals homozygous for the insertion have a very mild \( bx \) phenotype, with slightly enlarged halteres. Heterozygotes over a strong \( Ubx \) allele (such as \( Ubx^{130} \) on the TM2 chromosome) show a slightly stronger transformation, with a few small wing bristles on the capitellum. The pattern of \( Ubx \) protein in homozygous embryos appears normal. There is strong and uniform \( lacZ \) staining in nuclei of PS5 and somewhat weaker cell-specific expression in PS6-12 (Figure 1D). There is also strong expression in the nuclei of the amnioserosa, and in a group of cells in the mandibular lobe of the head. There is no apparent staining of the visceral mesoderm.

The \( bx^{\text{Plac(-61)}} \) insertion was derived from the \( bx^{\text{Plac(-61)}} \) line by hybrid dysgenesis (see MATERIALS AND METHODS). This element is an exact inversion of the \( bx^{\text{Plac(-61)}} \) element. Both junctions between the P element and the BX-C were sequenced, and the target site duplications and flanking sequences were identical in the two insertions. Although only the orientation of the element has been changed, the phenotype and \( lacZ \) patterns are dramatically altered. The \( Ubx^{\text{Plac(-61)}} \) insertion is a strong \( Ubx \) mutation; homozygotes die as larvae, with a strong transformation of the first abdominal segment to a third thoracic type. Furthermore, homozygous embryos lack \( Ubx \) protein as detected with \( Ubx \) antibody. This \( Ubx^{\text{Plac(-61)}} \) phenotype may be caused by premature termination of the \( Ubx \) transcript when the P element is in this orientation. The phenotype of \( Ubx^{\text{Plac(-61)}} \) is probably not due to any second site mutations, since most \( ry \) revertants induced by hybrid dysgenesis are wild type. The \( lacZ \) expression is strong and uniform in PS5-11, and somewhat weaker in PS12 (Figure 1E). In older embryos, the PS5 expression is slightly stronger than that of PS6-11, but there is not the clear difference between parasegments 5 and 6 seen in \( bx^{\text{Plac(-61)}} \), and the epidermal staining in PS6-11 appears to be virtually universal. As in the \( bx^{\text{Plac(-61)}} \) line, there is also expression in the amnioserosa and the mandibular lobes, but not in the visceral mesoderm.

<table>
<thead>
<tr>
<th>Insertion</th>
<th>Isolate name</th>
<th>Promoter(^a)</th>
<th>Regulatory DNA(^b)</th>
</tr>
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<tbody>
<tr>
<td>( bx^{\text{Plac(-61)}} )</td>
<td>3-my128</td>
<td>( P (-) )</td>
<td>None</td>
</tr>
<tr>
<td>( Ubx^{\text{Plac(-61)}} )</td>
<td>3-my18,42-4</td>
<td>( P (+) )</td>
<td>None</td>
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<tr>
<td>( Ubx^{\text{Plac(-31)}} )</td>
<td>bx17J-36</td>
<td>( Ubx (-) )</td>
<td>17 kb bx -63 to -46</td>
</tr>
<tr>
<td>( bxd^{\text{Plac(-31)B}} )</td>
<td>UC31(1401-116A)</td>
<td>( Ubx (-) )</td>
<td>13 kb bxd/iab-2 +36 to +23</td>
</tr>
<tr>
<td>( bxd^{\text{Plac(13)}} )</td>
<td>UC42(1403-96)</td>
<td>( Ubx (-) )</td>
<td>18 kb bxd/iab-2 +30 to +12</td>
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<tr>
<td>( iab^{\text{Plac(132)}} )</td>
<td>UC47(1357-121)</td>
<td>( Ubx (-) )</td>
<td>7 kb bxd/iab-2 +23 to +30</td>
</tr>
<tr>
<td>( iab^{\text{Plac(132)A}} )</td>
<td>UC43(1402-7)</td>
<td>( Ubx (-) )</td>
<td>15 kb bxd/iab-2 +30 to +22 +36 to +30</td>
</tr>
<tr>
<td>( iab^{\text{Plac(159)}} )</td>
<td>UC21-10</td>
<td>( Ubx (-) )</td>
<td>1 kb bxd/iab +31 to +30</td>
</tr>
<tr>
<td>( iab^{\text{Plac(159)A}} )</td>
<td>UC21-10,14d</td>
<td>( Ubx (-) )</td>
<td>11 kb iab-3 +67 to +56; 4 kb Mep +99 to +95</td>
</tr>
</tbody>
</table>

\(^a\) Direction of transcription of \( lacZ \) gene relative to the endogenous \( Ubx \) gene (\( \rightarrow \)).

\(^b\) Safl DNA fragments from the BX-C. Coordinates are from the BX-C walk; the first coordinate is adjacent to the \( Ubx \) promoter in the pMB0140 construct.
The PS6 insertion: The *bxd*\textsuperscript{PLac(+13)} insertion maps in the *bxd* region at +13 on the BX-C map (Figure 1F). Homozygotes are fully viable, and they show only a slight reduction of the A1 tergite. The A1 reduction is enhanced in animals that are heterozygous for this P element and the TM2 (Ubx\textsuperscript{130}) balancer. The pattern of Ubx protein in homozygous embryos appears normal. The *incZ* gene from this insertion is expressed strongly and uniformly in the epidermis and CNS of PS6–12 and these boundaries of expression are maintained throughout embryogenesis. This expression pattern is appropriate for the *bxd* regulatory domain since *bxd* mutations affect PS6 and posterior parasegments. This line shows staining in the visceral mesoderm of PS7, and at later embryonic stages staining becomes stronger in the CNS of PS6.

The PS12 insertion: Our initial insertion in the *iab-7* region, called *iab-7*\textsuperscript{PLac(+132)}, is located at +132. The line programs a *lacZ* pattern with cell-specific expression in PS6–13, with enhanced staining in PS12 (Figure 2, C...
and D). The P element contains DNA from the bxd/iab-2 region (Table 1) which programs the anterior boundary in PS6. In control lines, where the construct is not in the BX-C, this boundary is transient, and there is substantial staining anterior to PS6 at late embryonic stages (Figure 2, A and B). In the iab-7 line, however, the PS6 boundary is much sharper, and is maintained throughout embryogenesis (Figure 2, C and D). Early embryos show some anterior expression in the basal Ubx-lacZ pattern (Figure 2C), but the basal pattern disappears by the time of germ band retraction, as in the Ubx promoter enhancer traps. Thus, the position in the iab-7 region prevents expression of β-gal anterior to PS6.

Adult males that are heterozygous for this insertion have patches of dark pigment on anterior abdominal tergites, similar to the Mcp mutation (Lewis 1978). Homozygous males have more and larger patches of ectopic pigmented tissue, have a sixth tergite that is reduced in size (like Fab7) (Gurkovics et al. 1990), and have a partial seventh tergite (like weak iab-7 mutations). Thus, these flies have a gain-of-function (like Fab7) and a loss-of-function (iab-7) phenotype. The gain-of-function phenotypes may result from ectopic activation of the AbdB gene by the bxd/iab-2 sequences. No ectopic AbdB expression was observed in embryos, but there could be misexpression later in development.

We used hybrid dysgenesis to delete the internal bxd/iab-2 sequences, selecting for ry+ derivative lines that were reverted for the dominant phenotype. One derivative line (iab-7^Plac(+159), Figures 1G and 2, E and F) has a deletion of most of the bxd/iab-2 DNA (see map in Figure 2). Homozygotes of this line have only the iab-7 loss-of-function phenotype; males have a small seventh tergite. The bxd/iab-2 pattern is absent in this line, and lacZ is expressed in the epidermis, mesoderm and CNS of PS12 (Figures 1G and 2, E and F). This is the expected expression domain, since iab-7 mutations primarily affect PS12. The PS12 stripe of the iab-7 insertion is maintained throughout embryogenesis and is especially sharp at late stages in the CNS (Figure 2F). There is anterior epidermal expression observed in some embryos (Figure 1G) that may be due to the remaining bxd/iab-2 DNA on the P element, but this anterior expression is absent at 12 hr. β-gal is also expressed in the visceral mesoderm, the tracheal tubes and the posterior gonad (Figure 2F). This pattern is very similar to the bll insertion at -124.7, reported by Galloni et al. (1993). The principal difference is that the bll insertion expresses β-gal in PS12-14, whereas iab-7^Plac(+159) does not have significant β-gal expression posterior to PS12. We have examined an additional insertion of the "PZ" element in the iab-7 region, discovered by Lynne Schneider and Allan Spradling. Its lacZ expression pattern resembles that of iab-7^Plac(+159) or bll (data not shown).

The PS13 insertion: The initial insertion in the iab-8 region (iab-8^Plac(+159), Figure 3A) was mapped to +159 on the BX-C map. Two distinct bands were generated by inverse PCR from the P element 3' end, and each was cloned and sequenced. One contained the 3' P element sequence and adjacent AbdB sequence, placing the P element 253 bp upstream of the proximal AbdB promoter (AbdB-m or AbdB-I) (Zavortink and Sakonju 1989; Celinker et al. 1990). The other cloned fragment contained the 3' P element sequence adjacent to plasmid vector sequence. Southern blot analysis confirmed that pUC8 sequences were integrated along with two P element constructs at AbdB (map in Figure 3A). This double insertion is homozygous lethal. The homozygous first instar larvae lack posterior spiracles, and the A8 denticle belt is transformed toward a more anterior denticle belt. Some larvae are more severely transformed, with a partial A9 denticle belt and chitinous plates near the anal pads. AbdB protein appears reduced in all para-segments of homozygous embryos, especially PS10–13 (data not shown).

The iab-8 insertion contains DNA from the Mcp and iab-3 regions (Table 1, map in Figure 3). Control lines with this construct outside the BX-C show the basal Ubx-lacZ pattern, with occasional embryos showing a weak PS8 boundary, which is normally programmed by this iab-3 DNA fragment [as in the iab-3-11.5 construct of Simon et al. (1990)]. The double insertion at the iab-8 site (iab-8^Plac(+159)), however, expresses the PS8 pattern strongly, as well as aspects of the Ubx-lacZ basal pattern (Figure 3A). In addition, there is a strong stripe of expression in PS13.

We generated several derivatives of this line that contain only one copy of the P element (see MATERIALS AND METHODS). These lines are still homozygous lethal. The phenotype resembles that of the double insertion, except none of the first instar cuticles have a partial A9 denticle belt or chitinous plates. AbdB protein appears to be expressed at normal levels in PS14 but is reduced in PS10–13 of homozygous embryos (data not shown). The β-gal pattern from a single P element line, iab-8^Plac(+159), is shown in Figures 1H and 3B. This line stains intensely in the epidermis and CNS of PS13, and most of the anterior staining seen in the double insertion is absent. It is striking that this insertion is so close to the AbdB promoter, yet the pattern does not look like the AbdB expression pattern. The transcript from the AbdB-I promoter is in PS10–13 in wild-type embryos (Kuziora and McGinnis 1988; Sanchez-Herrero and Crosby 1988). Some embryos have weak β-gal expression in the epidermis of PS8–12 (Figure 3B) which may be due to the iab-3 fragment on the P element or weak response to iab-5, -6 and -7 enhancers, but, in any case, there is no staining anterior to PS13 in the CNS.

Expression in Pc-G and trx mutants: Mutations in the trithorax locus cause a reduction in the pattern of Ubx
Enhancer Traps in the Bithorax Complex

We have looked at two enhancer traps from the Ubx regulatory regions for changes in their expression patterns in \textit{trp}' mutant embryos (Figure 4, B and E). Both lines show cell-specific reductions in \(\beta\)-gal expression. The most dramatic reduction is observed in PS5 of bx\textsuperscript{\textit{P}}\textsuperscript{\textit{stat}(-61)}, where \(\beta\)-gal is expressed at high levels in \textit{trp}' embryos but at a much lower level in \textit{trp}' embryos (compare Figure 4, A and B). \textit{trp}' bx\textsuperscript{\textit{P}}\textsuperscript{\textit{stat}(+13)} embryos (Figure 4E) have a less uniform \(\beta\)-gal expression pattern than \textit{trp}' embryos (Figure 4D), with the highest \(\beta\)-gal levels at the segment borders.

Mutations in the Polycomb group (Pc-G) cause the homeotic genes to spread beyond their initial segmental boundaries. Figure 4, C and F, shows the expression pattern of two transformant lines in embryos that are mutant for the \textit{extra sex combs} gene (\textit{esc}, a Pc-G member). The embryos shown lack both maternal and zygotic \textit{esc} function (STRUHL and AKAM 1985). The \textit{lacZ} expression in these enhancer trap lines appears in the normal position in early embryos, but it spreads beyond the normal anterior and posterior boundaries during the extended germ band stage (~5 hr). The Ubx, \textit{abdA} and \textit{AbdB} genes spread beyond their normal domains in \textit{esc}' embryos at about the same time in development (STRUHL and AKAM 1985; SIMON et al. 1992). The other insertions shown in Figure 1 have also been examined in \textit{esc} null backgrounds, and all show \textit{lacZ} pattern expansion.
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throughout the body axis. Four insertions \((bx^{Plac(-61)}\), \(bx^{Plac(+13)}\), \(iab-7^{Plac(+132)\Delta}\) and \(iab-8^{Plac(+159)\Delta}\) have been tested in embryos lacking zygotic Polycomb-like, and one \((bx^{Plac(-31)\Delta})\) has been examined in embryos lacking zygotic Polycomb; all five cases show \(lacZ\) anterior to the normal boundaries.

Expression from the rosy promoter: All of the \(P\) elements in this study carry the \(rosy\) gene as a marker for transformation. We examined \(rosy\) expression in three enhancer traps. Two of these \((bx^{Plac(+13)}\) and \(iab-7^{Plac(+132)\Delta}\) carry a deletion at the \(rosy\) locus \((ry^{506})\). Thus, all \(rosy\) expression observed in these lines comes from the copy of \(rosy\) carried on the \(P\) elements. Wild-type embryos express \(rosy\) in a broad ventral domain at the blastoderm stage and in the anterior and posterior midgut invaginations during gastrulation and germ band extension \((Dome et al. 1989; data not shown)\). After germ band retraction, some embryos express \(rosy\) in the gut underlying PS8 and there is very weak staining in the malpighian tubules \((data not shown)\). There is no detectable \(rosy\) mRNA in the epidermis, somatic mesoderm or CNS of wild type embryos after germ band retraction. The three enhancer trap lines express \(rosy\) in the wild-type pattern combined with new domains of expression beginning at the blastoderm stage. In the \(bx^{Plac(+13)}\) line, \(rosy\) mRNA is expressed in a new domain with a PS6 boundary \((Figure 5A)\), although the pattern is not as uniform as the \(\beta\)-gal pattern \((Figure 1F)\). \(iab-7^{Plac(+132)\Delta}\) expresses \(rosy\) in PS12 and more posterior

Figure 4.—\(\beta\)-gal expression of BX-C enhancer traps in \(trc\) and \(esc\) mutant backgrounds. All embryos are at the germ band retracted stage at about twelve hr of development. \((A) bx^{Plac(-61)}\) homozygote, \((B) bx^{Plac(-61)}\) homozygote, \((C)\) embryo from \(esc^{10}/esc^{2}\); \(bx^{Plac(-61)}\)/+ parents, \((D) trc^{811}\) heterozygote, \((E) trc^{811}\) embryo, \((F)\) embryo from \(esc^{10}/esc^{2}\); \(bx^{Plac(+13)}\)/+ parents. Parasegments 5 and 6 are indicated.

Expression from the \(rosy\) gene in \(BX-C\) enhancer traps. \(rosy\) mRNA was detected using a digoxigenin-labeled antisense-\(rosy\) RNA probe. \((A) bx^{Plac(+13)}\); \((B)\) ia\(b7^{Plac(+132)\Delta}\). Parasegments 6 and 12 are indicated.

parasegments \((Figure 5B)\); the \(\beta\)-gal pattern \((Figure 1G)\) is limited to PS12. In this line, \(rosy\) is expressed at much higher levels in the CNS than in other tissues. \(bx^{Plac(-61)}\) embryos express \(rosy\) in PS5–12 at early times, but by germ band retraction \(rosy\) appears only in lateral spots in PS5 and PS6 \((data not shown)\). Although the cell and tissue specificity differs between \(rosy\) and \(Ubx-lacZ\) in these enhancer traps, the anterior boundaries of expression are the same.

\(\beta\)-gal expression in the larva: We examined the \(\beta\)-gal patterns in the third instar larval CNS to determine if the
pattern boundaries are maintained. All the BX-C enhancer traps shown in Figures 1 and 2 were stained; four are shown in Figure 6. Parasegmental boundaries were confirmed by double staining with monoclonal antibody BP101, which labels the segmental ganglia (data not shown). The CNS from a bxd'21' r(+) larva stains in a few cells ahead of the obvious stripe in PS6 (Figure 6B). With the exception of these few cells, the larval CNS patterns in all lines resembled the late embryonic CNS patterns, with strict maintenance of the anterior boundaries.

**DISCUSSION**

**Traps sense local enhancers?** A simple view of enhancer traps is that they respond to regulation from nearby enhancers. The mechanisms of enhancer function are only partly understood. In some cases, transcription factors (such as the GAL4 or GCN4 proteins in *Saccharomyces cerevisiae*) bind to a site near the promoter and interact with basal transcription machinery. These factor binding sites must be within a few kb of a promoter to exert their effect (Sadowski et al. 1988). Some promoters clearly interact with regulatory sequences over much larger distances. Perhaps distant enhancers are brought close by looping (Dunn et al. 1984; Benson and Piroirta 1988). If the long-distance regulation requires special DNA sites at or near the promoter, then an enhancer trap might respond to distant regulatory sequences only if the mobile element inserts near a natural promoter. It is common for P elements in Drosophila to insert very close to endogenous transcription start sites, and such promoter-proximal P enhancer traps often reproduce the expression pattern of the endogenous gene. The three insertions which lie immediately upstream of the Ubx transcription start site are typical of this group; they give patterns similar to the normal pattern of Ubx expression. But the other five insertions do not reproduce the patterns of the endogenous genes, and, with the exception of iab-5Plac(+159), they do not lie near known endogenous promoters. It is difficult to explain their patterns as a response to transcription factors bound near the insertion sites, for several reasons.

**Uniform lacZ expression:** Each enhancer trap in the regulatory regions expresses lacZ in the appropriate parasegment, and the expression is fairly uniform in every case. For the enhancer traps marking PS5, the lacZ patterns are clearly different from the pattern of the endogenous Ubx gene, which is expressed only in a small subset of PS5 cells. Moreover, the regulatory fragments of the bithorax complex that have been tested typically do not drive uniform expression within a parasegment. The "abx" enhancer, for example, gives a graded pattern across the epidermis of PS5, and marks a subset of cells in the central nervous system (Simon et al. 1990; Müller and Bienz 1991). We have examined the lacZ RNA pattern in the UbxPlac(-61) line to determine if the uniform expression is due to the perdurance of β-gal protein from early stages. The lacZ RNA in PS5 remains widespread in the epidermis and virtually universal in the cells of the CNS throughout embryonic development (data not shown). The β-gal expression pattern in the larval CNS suggests that the uniform expression continues through later stages of development (Figure 6A).

**Three different promoters:** The regulatory elements that turn on the enhancer traps in specific parasegments appear not to require a special promoter for their action. In six examples discussed here, the Ubx promoter is directing the expression of β-gal. This is the appropriate promoter for insertions near the natural Ubx RNA.
start site, and for the insertion at +13 in the bxd region. But the iab-7 and iab-8 insertions are responding to information that normally directs an AbdB promoter. The two insertions at -61, in the bx region, have P element promoters driving β-gal. Moreover, the promoter for the rosy transcription unit, in the three cases examined (Figure 5), is also turned on in the appropriate parasegments.

**Action at a distance:** Although we have not mapped the regulatory sequences that drive any of the lacZ patterns described here, it is clear that they must act at a considerable distance. The six elements which contain the Ubx promoter fused to lacZ have various DNA fragments inserted into the P element upstream of the promoter. There is between 3.4 and 20 kb of DNA between the Ubx/lacZ RNA start site and the upstream edge of the P element. The downstream edge of the P element is 12 kb from the Ubx/lacZ RNA start site. In the examples of rosy promoter regulation, the rosy RNA start site (Curtis 1990) lies 15.4 or 9.4 kb (Figure 5, A and B, respectively) from the upstream edge of the P element, and 6.0 kb from the downstream edge.

**Patterns do not reflect flanking enhancers:** The DNA flanking these enhancer traps has been tested for enhancer properties using a lacZ reporter system (Simon et al. 1990; Qian et al. 1991; Müller and Bienz 1991; Busturia and Bienz 1993; M. O'Connor, unpublished results) and the patterns generated from these constructs do not look like the patterns from our enhancer traps. For example, an element only 2 kb to the left of the P element promoter in the UbxPlac(-61) insertion has been analyzed in detail, and has been shown to drive β-gal in a PS6 restricted pattern (Qian et al. 1991), in contrast to the PS5 boundary of the enhancer traps. The closest PS5 element that has been mapped (the "abx" element) is 17 kb to the left of the PS5 enhancer traps (Simon et al. 1990; Müller and Bienz 1991). Thus, the enhancer traps must be responding to parasegmental regulation specified at sites distant from the edge of the P element, but the traps do not recognize nearby or distant enhancers that specify cell types.

**Suppression of internal enhancers:** The "position effects" of the bithorax complex not only turn on new patterns from the enhancer trap P elements, they also turn off patterns that the P elements would normally express. The Ubx promoter/lacZ fusion present on most of our enhancer traps (Table 1) has been shown to produce a "basal" pattern of weak lacZ expression in the lateral epidermis and head when it is inserted at random in the chromosomes (Bienz et al. 1988; Simon et al. 1990). We have noted this basal pattern in insertions outside the bithorax complex of the P elements of the UbxPlac(-31), bxPlac(-13), iab-7Plac(+132), and iab-8Plac(+199a) enhancer traps. The same basal pattern is frequently seen in random insertions of the PZ element, with the P promoter driving lacZ (Mlodzik and Hiromi 1992).

The basal lacZ pattern is repressed in all eight lines shown in Figure 1. This is seen most clearly for the iab-7Plac(+132) element when inserted inside and outside the bithorax complex (Figure 2, A and C). There is a prominent spot of expression in the mandibular lobe of the head in the bxPlac(-61) line and much weaker expression in the same place in the UbxPlac(-61) line. This spot persists through embryonic development, and represents the only apparent exception to the repression of lacZ in the anterior segments.

The normal level of rosy expression in embryos is quite low, and it is not clear whether the rosy gene internal to the enhancer traps is turned off in the more anterior parasegments. However, all enhancer traps recovered within the BX-C carry rosy as a transformation marker; none carry white, although several large screens have been performed using white P elements (Bier et al. 1989; Nosè et al. 1992). rosy is a non-autonomous gene (Hadorn and Schwenk 1956), but white is a cell-autonomous gene in the eye disc, an anteriorly derived structure (Brable and Ephrussi 1996). The white gene inserted into the BX-C might be repressed, and such a transformant would be undetectable.

**Patterns are maintained by Polycomb group, not by feedback from homeotics:** The parasegmental boundaries of the homeotic genes appear to be set by the gap and pair-rule regulators (Qian et al. 1991; Shimell et al. 1994). The lacZ patterns of the enhancer traps are likely specified by the gap and pair-rule genes, not by the homeotics. The enhancer traps express β-gal before Ubx or AbdB proteins are detectable (data not shown), and their cell-specificity differs from that of Ubx or AbdB. The UbxPlac(-61) insertion is an apparent Ubx null mutation, and the iab-8Plac(+159) insertion causes a strong AbdB mutation, but in both cases, embryos homozygous for the insertion (i.e., lacking Ubx or AbdB) give lacZ patterns identical to heterozygotes (although more intense). We have also shown that the bxPlac(-61) pattern is unchanged in a Ubx- background (data not shown).

If the enhancer traps are responding to the gap and pair-rule signals, then some mechanism must maintain the pattern boundaries after the gap and pair-rule stripes disappear, at 3–4 hr of embryogenesis. That mechanism involves the genes of the Polycomb family, since the anterior restriction of β-gal is lost in Pc-G mutants (Figure 4, C and F). The high level of expression of β-gal in PS5 in bxPlac(+61) embryos is dependent on the trithorax gene (Figure 4B), which may play an antagonistic role to the Polycomb family (Ingham 1983).

**Chromosome structure alternative:** It has been suggested that the bithorax complex includes a series of domains whose chromosome structure differs from segment to segment (Peifer et al. 1987). This accessibility model helps to explain the sequential order of regulatory regions, and the phenotypes of some mutations
Figure 7.—Chromosome accessibility model. Each line depicts the bithorax complex in one parasegment for parasegments 4 through 7. The triangle represents the bxdsac(-13) P element. The black ovals represent the Polycomb group of gene products binding to the BX-C, making the DNA inaccessible to transcription factors. In PS5, the bx region is active (shown by the open oval) and the Ubx gene is transcribed, but the bxd region is still repressed by the Pc-G. In PS6 the bxd region is accessible to transcription factors and the lacZ gene is transcribed (indicated by the arrow producing β-gal). More distal regions of the BX-C become progressively accessible in more posterior parasegments.

caused by rearrengements. Figure 7 diagrams consequences of the model for one of the enhancer traps, the bxdsac(+13) trap in the PS6 regulatory domain. In the head and parasegments 1-5, this region of the complex would have proteins of the Polycomb family bound to it, and it might be sterically unable to interact with RNA polymerase and transcription factors (PARO 1990; Eissenberg and Elgin 1991). This view is supported by the protein similarity between Polycomb and the HP1 heterochromatin protein (PARO 1990), as well as by the demonstrated binding of Polycomb protein to the bithorax complex in a segment where the complex is repressed (Zink and Paro 1989). In parasegment 6 (as well as PS7-13), the structure of the chromosome around the +13 enhancer trap is altered so that transcription factors have easy access. In the most simple and extreme view, this access alone is sufficient for constitutive transcription of lacZ promoters. The promoter of the rosy gene would also be regulated in a parasegment-specific way by the chromosome state, although there may be additional cell-specific regulatory constraints due to the rosy regulatory sequences. Galloni et al. (1993) have also proposed a model of chromatin accessibility to explain the lacZ pattern they observe from an enhancer trap in the iab-7 region. The results described here with a more extensive collection of enhancer traps support such a model.

There are examples of eukaryotic enhancers which lie tens of kilobases from their natural promoters, and which can interact with multiple promoters (Kim et al. 1992). It is possible that such enhancers act by looping without the need for a unique target sequence at the promoter. It is also possible that these enhancers affect chromatin accessibility.

Competitive regulatory signals: Several enhancer trap lines carry regulatory DNA fragments from the bithorax complex within the P element, and these sequences direct segment-specific lacZ expression when the P elements are inserted at other places in the genome. When these elements lie within the complex, there is a potential competition of regulatory information between sequences inside and outside the elements. In four cases (Ubx2ac(-31), bxdsac(-31)B, bxdsac(-31)A and bxdsac(+13)), the internal DNA fragments initiate a lacZ pattern beginning in PS6, but the lacZ pattern from the external regulation overlaps the PS6 boundary. Thus, one cannot tell whether the internal sequences are functional.

In the iab-7Puc(+132) line, the initiation element inside the P transposon is activated in PS6-13 (Figure 2A) by gap and pair-rule products, which should block Pc-G repression. But the initiation element(s) outside the P transposon is repressed by gap and pair-rule genes in parasegments anterior to PS12 (Figure 2E), and this repression would normally be fixed by the Pc-G. In this case, both lacZ patterns are superimposed (Figure 2C). Apparently activation prevails over repression, since the final pattern looks like the summation of positive inputs. The prevention of Pc-G repression in PS6-11 might be expected to cause anterior misexpression of the AbdB gene. The Mcp-like and Fab-like phenotypes are indications of such misexpression.

The internal sequences of the iab-7Puc(+132) P element are not able to maintain the PS6 lacZ boundary when this P element is inserted at other chromosome locations (Figure 2B), but in the iab-7 location, adjacent BX-C sequences impose strict maintenance of this boundary (Figure 2D). This context-dependent fixation of the PS6 boundary is similar to the results from Simon et al. (1993), where Polycomb response elements from one DNA fragment can act on pattern initiation elements on an adjacent DNA fragment.

The other enhancer trap with a potential conflict is the iab-8Puc(+159) insertion. This P element contains DNA from the iab-3 and Mcp regions. The iab-3 fragment normally programs a pattern starting in PS8...
(Simon et al. 1990), but that pattern is not detectable in most embryos that contain this particular construct outside of the BX-C. The Mbp DNA fragment, which has been proposed to act as a boundary element (Gayrrovics et al. 1990), may be blocking activation of the Ubx promoter by the iab-3 DNA (see map in Figure 3B). In the double iab-8 insertion (iab-8del(+159)), the iab-3 sequences on the distal P element could affect the Ubx promoter on the proximal P element, without an intervening Mbp element (Figure 3A). The double insertion shows superimposed patterns from internal and external regulation, with PS8 and PS13 boundaries respectively. It also shows the Ubx promoter basal pattern in parasegments 3–7 (Figure 3A), which is not observed in the other enhancer traps within the complex. It is possible that the two Mbp boundaries on the construct act to form an isolated domain shielded from outside repression.

Additional utility of BX-C enhancer traps: These enhancer traps should be useful for further genetic analysis of the bithorax complex. If the P elements are exposed to P transposable, they excise with a high frequency, and imprecise excisions generate deletions of adjacent chromosomal sequences (Daniels et al. 1985; Galloni et al. 1993). The enhancer trap elements also allow the possibility of gene conversion in the neighborhood of the insertion site (Gloor et al. 1991). This should permit a detailed analysis of regulatory sequences in the context of the normal complex, or the insertion of more sophisticated probes of chromosome structure.

The parasegment-specific expression from these enhancer traps show which regulatory domain the traps have marked, and this may prove to be the best way to map the extents of the successive domains. The present mapping, based on the phenotypes of a limited number of mutations, is rather crude, especially for the domains of the abdominal region (PS8–14). It may be possible to generate additional enhancer trap lines in the complex by local jumping (Zhang and Spradling 1993) or homing of P elements (Hama et al. 1990).

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LITERATURE CITED


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