Distinct Parasegmental and Imaginal Enhancers and the Establishment of the Expression Pattern of the \textit{Ubx} Gene

Vincenzo Pirrotta,* Chi Shing Chan,**1 Donna McCabe* and Su Qian1,2

*Department of Zoology, University of Geneva, CH1211 Geneva, Switzerland, and 1Department of Cell Biology, Baylor College of Medicine, Texas Medical Center, Houston, Texas 77030

Manuscript received May 24, 1995
Accepted for publication September 1, 1995

ABSTRACT

The expression domain of the \textit{Ubx} gene in Drosophila embryos is bounded by the product of the \textit{hb} gene, acting as a repressor. We show that all \textit{Ubx} fragments that bind Hb protein \textit{in vitro} contain parasegmental enhancers active in the embryo in specific parasegmental patterns. We have found three new embryonic enhancer elements in the upstream region, in addition to the two previously identified. Each produces a pattern initially bounded at PS6 by Hb but sooner or later breaks down this boundary and begins to express in the anterior region. These enhancers do not respond to the long-term maintenance mediated by the \textit{Polycomb} group of genes. They also cease functioning after germ band extension. Expression in imaginal tissues is due to a set of entirely separate and independent imaginal disc enhancers. These do not contain Hb binding sites and by themselves have no anterior/posterior positional information, although some distinguish between ventral and dorsal discs. A third kind of element, the \textit{Polycomb} Response Element (PRE), has no enhancer activity but causes long-term maintenance of the expression domain of other enhancers present in the vicinity. The interaction of these elements results in the correct expression of Ubx in imaginal tissues.

THE \textit{Ultrabithorax} (\textit{Ubx}) gene of Drosophila, which specifies the identity of the posterior thoracic and anterior abdominal segments, is probably the best characterized homeotic gene both genetically and molecularly (for review, see Dungan 1987). Its transcription unit is about 75 kb long, and regulatory elements are known to be scattered over a region of over 100 kb, including both upstream and downstream sequences. The principal regulatory mutations have been mapped in four regions, each with a characteristic pattern of defects in specific parasegments, and are arranged from 3' to 5' in roughly the same order as the anterior-posterior order of the segments affected. Thus, the \textit{abx}, \textit{bx}, \textit{bx}d and \textit{pbx} mutations map in regions progressively from 50 kb downstream to 40 kb upstream of the transcription start site. The first two affect principally the posterior T2 and anterior T3 segments (parasegment 5), and the latter two involve posterior T3 and anterior A1 (parasegment 6).

Molecular analysis, using \textit{lacZ} reporter gene constructs, has confirmed the complexity of the regulatory region and has shown that many distinct elements contribute to the overall pattern of expression. Visceral mesoderm expression, for example, is directed by elements located about 3 kb upstream of the promoter and also further upstream in the \textit{bx}d region (Bienz et al. 1988; Irvine et al. 1991). Several enhancer elements are responsible for the parasegmental pattern of expression and correspond roughly to the regulatory regions characterized genetically. Their analysis reveals a distribution of functions that both reflects that expected from the phenotypic effects of the regulatory mutations and differs from expectations in interesting ways. An element from the \textit{abx} region (\textit{ABX}) activates expression in parasegment PS5 but also in PS7, 9 and 11, in stripes progressively decreasing in size and intensity (Simons et al. 1990). An enhancer from the \textit{bx} region (\textit{BX}) activates expression in even-numbered parasegments PS6, 8, 10 and part of 12 (Qian et al. 1991, 1993). A fragment from the \textit{bx}d region (\textit{BXd}) directs expression in all parasegments, including thoracic parasegments where the \textit{Ubx} gene is not normally expressed (Muller and Bienz 1991). An enhancer in the \textit{pbx} region (\textit{PBX}) produces four stripes of expression in the even parasegments, closely resembling the stripes produced by the BX element (Muller and Bienz 1991). Clearly the activity of these enhancers is not limited to the parasegments affected by mutations in the corresponding regions. These enhancers also differ in the details of their expression pattern within the parasegment. In addition, some are active in the somatic mesoderm (BX and PBX), while others direct expression in the central nervous system (ABX and BXd).

The enhancer activities of these elements have been
mapped to short sequences 500–1000 bp in length that,
in some cases, have been shown to contain binding sites
for the products of segmentation genes *hunchback* (*hb*),
*fushi tarazu* (*fz*), *tailless* (*tl*), *engrailed* (*en*) and other
early embryonic regulatory genes. Genetic experiments
indicated that *hb* and *tl* are the principal determinants
of the domain of expression of the *Ubx* gene (Ingham
and Martinez-Arias 1986; White and Lehmann 1986;
Irish et al. 1989; Reinitz and Levine 1990). We have
shown for the BX element that Hb and Tl proteins
act as competitive inhibitors by binding to sequences
overlapping the binding sites of activators (Qian et al.
1991, 1993). A similar mechanism has been proposed
for the regulation of the PBX element by Hb (Zhang
et al. 1991; Müller and Bienz 1992). Hb protein,
therefore, seems to act locally to repress each enhancer
and each activator binding site. In fact, Hb binding sites
were detected in vitro in a number of different frag-
ments from the *Ubx* regulatory region. Since the seg-
mental pattern of *Ubx* as a whole is negatively regulated
by *hb*, we expected that these Hb binding sites would
correspond to parasegmental enhancers. In this article,
we confirmed this by examining the effect of the Hb
binding fragments on *lacZ* reporter gene expression in
the embryo. Of the seven Hb binding fragments, four
correspond to the previously identified ABX, BX, BXD
and PBX elements (Simon et al. 1990; Müller and
Bienz 1991; Qian et al. 1991). Three encode new enh-
ancers. These elements direct different patterns of
expression in the embryo, all initially respecting an
anterior boundary at PS5 or 6. All, however, sooner or
later break down this boundary and express ectopically
in more anterior regions before becoming silent at the
extended germ band stage. This indicates that they lack
ability to respond to two sets of genes that have been
found to be responsible for the maintenance of the
correct expression pattern of the *Ubx* gene (Ingham
and Whittle 1980; Jurgens 1985; see reviews by Paro
1990 and Kennison 1993). The *Polycomb* group of genes
(*Pc-G*) is required to maintain repression in those parts
of the embryo in which the gene is initially repressed
by the gap genes *hb* and *tl*, while the *trithorax* group of
genes (*trx-G*) appears to sustain expression in those
domains in which the gene was initially activated. We
have found that a separate control element, the PRE,
mediates the response to these maintenance systems
(Qian et al. 1994). Our analysis shows also that imagi-
 nal disc expression is not determined by the embryonic
enhancers but by a set of distinct imaginal disc en-
hancers. These are not active in the early embryo and
do not respond directly to the anterior/posterior posi-
tional information provided by the segmentation gap
genes. Alone, they are active in both thoracic and head
imaginal discs. Positional information is conveyed to
them through the *Pc-G* response element, which in turn
depends on the state of activity of the parasegmental
enhancers.

**RESULTS**

**Parasegmental enhancers contain Hb binding sites:**

*In vitro* binding assays with DNA fragments of the
*Ubx* gene reveal a number of Hb binding sites scattered
among the regions genetically identified as containing
regulatory elements (Qian et al. 1991). Two of these
Hb binding sites correspond to the previously identified
ABX and BX elements, about 50 and 30 kb downstream
of the transcription start site, respectively. Several other
Hb binding fragments come from the upstream region.
We began by identifying these fragments in the 48 kb 5’
flanking region represented by genomic clones *X*2218,
2212 and 2206 (Bender et al. 1988). We first subcloned

**MATERIALS AND METHODS**

**Transposon constructs:** All transposon constructs were
assembled in the CaSpeR vector for germ line transforma-
tion (Pirrotta 1988) or its derivatives, where the various DNA
fragments from the *Ubx* regulatory region were placed in front
of the *Ubx* promoter-*lacZ* reporter gene, as described by
Qian et al. (1991). Germ line transformation was performed as
described in that reference.

**Hb binding assays:** Extracts from *Escherichia coli* cells
expressing the Hb protein were prepared as described by
Qian et al. (1991). For Hb binding assays, DNA from lambda or
plasmid clones was cut with suitable restriction enzymes, end-
labeled and incubated with Hb-containing extracts or control
extracts from bacteria not expressing Hb. Precipitation of the
complex was done according to Benson and Pirrotta (1987)
and Stanoevic et al. (1989). For a comparison of the Hb
binding of the different enhancer fragments, a Bluescript
plasmid containing the six *Ubx* enhancer fragments was di-
gested with Hind3, SalI and BamHI to liberate the enhancer
fragments, which were then end-labeled with the Klenow
fragment of DNA polymerase. The labeled DNA was further cut
with Nde1 to further separate the individual enhancers.
Appropriate amounts of uncut, unlabeled DNA of the same plas-
mid were added as competitor, and the mixture was incubated
with Hb-containing bacterial extract in the presence of 30 µg
salmon sperm DNA in 25 µl binding buffer (20 mM Tris pH
7.5, 50 mM NaCl, 0.25 mM EDTA, 1 mM DTT, 10% glycerol).
After 30 min incubation in ice, the mixture was diluted with
300 µl cold binding buffer and centrifuged 5 min, and the
pellet was washed three times with 350 µl cold binding buffer.
The pellet was resuspended in 100 µl extraction buffer (40
µg/ml tRNA, 0.2 mM NaCl, 20 mM EDTA, 1% SDS), phenol
extracted, precipitated and analyzed on a 0.8% agarose gel.

**Histochemical staining:** Embryos were fixed, stained and
mounted by the methods of Lawrence and Johnston (1989).
The rabbit anti-β-galactosidase antibody (Cappel) was preab-
sorbed against 0–14-hr embryos overnight at 1:500 dilution
and used at a further dilution of 1:10 for early stage embryos
or 1:20 for later stage embryos. The anti-β-galactosidase anti-
body was detected as a brown stain using the Vectastain
ABC-HRP kit (Vector Labs). For double staining, the embryos
were washed after anti-β-galactosidase staining and incubated
overnight with monoclonal anti-β-antibody (kindly provided
by T. Kornberg) at 1:1000 dilution. The blue staining was then
developed with the Vectastain ABC-AP kit. Imaginal discs were
dixed with 3% glutaraldehyde in 0.1 M cacodylate buffer, pH
7.5, washed with PBS and stained with X-Gal according to the
protocol of Bellen et al. (1989).

**Footprinting:** Footprinting experiments were carried out
exactly as described by Qian et al. (1993).
EcoR1 or Hind3 fragments containing these binding sites and then mapped them to smaller fragments using Sau3A or Hind1. In all, we found five fragments with significant Hb affinity (see map in Figure 1): 2218S is a 400 bp Sau3A fragment from position −20 in the map of Bender et al. (1983), or about 11 kb upstream of the promoter; 2218R6 is an 800 bp EcoR1 fragment from position −24, containing a weak Hb binding site and corresponding approximately to the enhancer region called BXD by Müller and Bienz (1991); 2212S1 is a 1.3 kb Sau3A fragment from position −11 or 22 kb upstream of the transcription start; 2212S2 is a 1.0 kb Sau3A fragment from position −6 (about 25 kb upstream); finally, 2206H1 is a 1.3 kb Hind1 fragment from position +2 (33 kb upstream) and corresponds to the PBX enhancer of Müller and Bienz (1991). Of these, 2218R6 has the lowest affinity for Hb. We cannot exclude the existence of other, even weaker binding sites that might nevertheless have functional significance. We cloned each of these fragments in a CaSpeR vector for germ line transformation containing an Ubx promoter-lacZ reporter gene (Qian et al. 1991, 1993) and tested transgenic fly lines containing the corresponding transposons for enhancer activity. The results summarized below show that each one of these fragments has an enhancer activity that produces a distinct parasegmental pattern of expression in the early embryo.

2218S: The pattern produced by this enhancer in all six lines studied resembles that of the BX enhancer (Qian et al. 1991, 1993): four broad stripes correspond to parasegments PS6, 8, 10 and 12 (Figure 2, A-C). Like the BX element, 2218S produces stripes that are wider than a parasegment and include both flanking en domains. Like the BX element, it expresses prominently in the mesoderm as well as in the ectoderm but has no detectable activity in the central nervous system. The details of the initial pattern, however, differ from those of the BX element. Expression is first detected by in situ hybridization in the syncytial blastoderm as a weak uniform domain that converts at cellular blastoderm into four thin, sharp stripes, the strongest of which corresponds to PS10. During germ band extension, the pattern consists of four approximately equal stripes, each composed of an even-numbered parasegment domain plus the en domain of the next more posterior PS. Toward the end of germ band extension, a narrow stripe appears in front of PS6, corresponding to the en domain of PS5. Later still, the other thoracic segments begin to stain, at first in narrow stripes and later, during germ band retraction, expanding until the whole embryo stains with greater or lesser intensity. A larger transposon (2218R), including the entire 5 kb EcoR1 fragment of which 2218S is a part, gives a very similar pattern except that the PS6 stripe is much stronger already from the blastoderm stage and is accompanied by a weaker, even more anterior and incomplete stripe that disappears during gastrulation (Figure 2D). From germ band extension on, the 2218R pattern is very similar to that of 2218S except that a narrow but strong PS5 stripe corresponding to the PS5 en domain appears early (Figure 2E). The additional sequences present in 2218R behave as if they strengthened expression specifically in PS6 and thoracic parasegments. In situ hybridization with 2218S shows that the LacZ RNA

![Figure 1](image-url)
FIGURE 2.—Expression patterns of parasegmental enhancers. (A–C) The 2218S enhancer produces a four-stripe pattern by the beginning of gastrulation, seen in ventral view in A. During germ band extension, the four stripes include the even-numbered PS and the en domain (stained in blue) of the next PS (B). By the end of germ band extension, ectopic expression begins in the thoracic PS (C). A larger fragment 2218R enhances expression in the PS5 stripe, and an additional anterior stripe is transiently visible at blastoderm (D). During germ band extension, this anterior stripe reappears as a narrow but strong band in the anterior part of PS5 (E). Expression of the 2218R6 enhancer is not detectable at blastoderm but appears during germ band extension first as at PS6, 10, 11 and 13 (F). By the end of germ band extension, a full stripe pattern is visible and a second, weaker stripe appears just behind the en stripe (blue) in each parasegment, including the thoracic PS (G). During germ band retraction, expression includes all thoracic PS (H). Expression fades in most tissues but appears in the condensing ventral nerve chord (I).

pattern fades after germ band extension and is absent altogether by the end of germ band retraction (not shown).

2218R6: This element is contained in a 0.8 kb EcoR1 fragment, which represents about half of the BXD element of MÜLLER and BIENZ (1991). Their BXD extends for 0.7 kb more to the left up to an EcoR5 site. However, this additional sequence, which is contained in the 2218R fragment mentioned above, by itself produces no expression whatever beyond the basal pattern. Expression driven by the 2218R6 fragment in the 10 lines analyzed becomes detectable only during germ band extension when it appears first as a set of narrow stripes at PS6, 10, 11, 12 and 13 (Figure 2, F–I). The staining is concentrated in the ventral and most posterior part of the parasegment, just in front of the en stripe of the next more posterior parasegment. This pattern is short-lived: stripes appear rapidly in the other abdominal parasegments, and weaker stripes appear also in the thoracic parasegments, resulting in a pattern similar to that reported by MÜLLER and BIENZ (1991) for their BXD element. Before the end of germ band extension, expression is found in all abdominal and thoracic segments in a strong band at the posterior edge of the parasegment and a weaker band just behind the en stripe. The stripes are weaker in the thoracic segments and weakest in PS3 where it lacks the more anterior band (Figure 2G). After germ band retraction, expression fades in the epidermis but staining in the CNS becomes strong when the ventral nerve chord begins to condense (Figure 2H). Because of the differences between our observations and those of MÜLLER and BIENZ, we also examined the pattern produced by the 0.8 kb EcoR1 + 0.7 kb EcoR1-EcoR5 fragment (equivalent to their BXD) but found that, in general, the additional sequence did not significantly alter the expression. The 2218R6 enhancer is unusual in that its activity begins later than the others and has no preference for even- or odd-numbered parasegments. It is likely that it is activated through the action of segment polarity genes.

2212S1 and S2: These two elements produce very similar patterns. Four lines were tested for each construct. In each case, expression begins at the blastoderm as a weak, broad domain, spanning roughly PS6–8. At gastrulation, two stripes corresponding to PS6 and 8 begin to condense and, as the germ band extends, stripes develop also in PS10 and 12 (Figure 3, A–D). These stripes are narrow, predominantly ventral and situated roughly between and not overlapping with the en stripes (Figure 3B). There is little or no expression
FIGURE 3.—Expression patterns of more parasegmental enhancers. The 2212S1 pattern begins at blastoderm as a uniform domain (A), from which four stripes begin to condense. These stripes are in the central and ventral part of PS 6, 8, 10 and 12 (B). The blue stripes are stained by anti-en antibody. The 2212S2 enhancer has a very similar pattern (C). By the end of germ band extension, expression occurs in all PS, including the thoracic and gnathal PS (D). Expression of the 2206Hf enhancer (PBX) begins as a uniform domain at blastoderm (E). During germ band extension, it develops into a four-stripe pattern at PS 6, 8, 10 and 12, but expression is stronger at the anterior edge of each stripe, which soon splits into a stronger and a weaker stripe (F). The four-stripe pattern persists during germ band retraction, but, before it finally fades, expression occurs also in the thoracic PS (G).

in the mesoderm. As germ band extension progresses, expression becomes detectable also in the remaining parasegments, in similar but weaker stripes. By the end of germ band extension, all parasegments, including the thorax, stain equally strongly, but expression is still restricted to the ventrolateral region (Figure 3D). Parts of the head also show strong expression, and the late embryo (end of stage 14) appears uniformly stained. Before staining becomes ubiquitous, obliterating tissue specificity, it is possible to see expression in the condensing ventral nerve cord but it is not clear if it is simply a result of generalized expression rather than specific activation. In situ hybridization shows that expression ceases after germ band extension. The addition of a few hundred nucleotides to the left of 2212S1 (a 1.8 kb Hind 3-Sty 1 fragment) modifies somewhat the pattern obtained with S1 alone by extending expression during germ band extension to the lateral ectoderm in a thin stripe that appears to overlap the next more posterior en band. The pattern produced by these two enhancers resembles that of some pair-rule genes, odd-skipped, runt and even-skipped, which show a similar shift from pair-rule pattern to every parasegment during germ band extension. odd-skipped, in particular, is a good candidate as an activator of these enhancers because its initial expression is in the middle of the even-numbered parasegments, not overlapping with the en stripes (COULTER et al. 1990).

2206Hf: This element corresponds to the PBX element of MÜLLER and BIEZ (1991) and gives a pattern very similar to that produced by the BX element in all six of the lines tested. It initiates at blastoderm as a broad, uniform domain corresponding roughly to PS6–12 (Figure 3E). As with the BX enhancer, expression is more intense along the midline at the cellular blastoderm stage and continues to be strong later in the mesoderm. Four stripes condense at gastrulation, becoming rapidly broader than a parasegment. The anterior edge of each stripe is more intense, and the posterior edge trails off gradually with no sharp boundary. Toward the end of germ band extension, each of these broad stripes splits into two, giving rise to a stronger and broader anterior band and a weaker and narrower posterior band (Figure 3F). The latter eventually fades away but the remaining parasegmental pattern persists until after germ band retraction. Expression in the thoracic and head region appears during this time but remains sporadic rather than uniform and, in consequence, the original domain of expression bounded at PS6 remains distinguishable despite the ectopic expression (Figure 3G). In situ hybridization confirms that weaker stripes appear in the thoracic segments just before the activity
of this enhancer fades during germ band retraction. Therefore, although this element seems to preserve the boundary restriction longer than the other elements described above, it breaks down eventually like the others.

The 2206Hf enhancer activity is localized in a region of about 600 bp which, like the BX enhancer, contains in vitro binding sites for Hb, Tll, Ftz, En and Twi proteins (Figure 4). These binding sites were not tested by in vitro mutagenesis to confirm their in vivo role, but analysis of the activity of this enhancer in mutant embryos shows that it depends on \( hh, \ tll, \ ftz, \ en \) and \( Twi \) proteins (results not shown). The clustering of the binding sites suggests that, as in the BX enhancer (Qian et al. 1993), the activators are competitively regulated by the repressors. Smaller fragments containing only part of these binding sites retain enhancer activity but at a much lower level. A 279 bp Sau3A-BamH1 fragment still produces four stripes, in addition to a strong but nearly continuous expression in the mesoderm. The stripe pattern becomes more distinct in a construct containing two copies of the Sau3A-BamH1 fragment. These results are consistent with our finding that this fragment still contains at least one set of Ftz and En footprinting sites (ftz7 and en3), as well as part of another (ftz5-8 and en2) and that it contains three Twi binding sites overlapping with hb sites 4, 5, 6 and 7. While the twi7 site overlaps with the ftz7 site, consistent with a synergy between the two activators as in the BX enhancer (Qian et al. 1993), the other two are not associated with Ftz and are consistent with the observed mesodermal expression in odd as well as even parasegments (not visible on the focal plane of Figure 5).

To determine if any other active module lacking strong Hb binding sites might be present in the \( bxd-bpx \) region, we surveyed also the activity of additional DNA fragments collectively spanning the region, only some of which are shown in Figure 1. The interval between \(-22 \) and \(+3\), except between \(-20\) and \(-17\), was examined using fragments lacking the five enhancers. We found no other parasegmental enhancer activity other than that associated with the five elements described above. All five enhancer fragments, including the 2218R6 enhancer, are initially active in a restricted domain limited by PS6, but this restriction fails at different times after gastrulation and expression becomes apparent in the anterior of the embryo. The \( in \ situ \) hybridization (not shown) indicates furthermore that all five enhancers cease their activity after germ band extension, except for 2218R6, which continues to be active in the CNS. The activity of the separate enhancers is not appreciably affected by mutations in the \( Pc \) or \( trx \) genes, indicating that they do not respond to the negative nor to the positive maintenance systems mediated by the \( Pc-G \) or \( trx-G \) genes.

**Boundary breakdown and Hb binding:** Although all these elements fail to maintain the boundary restriction, they break down to very different degrees and at very different times during embryonic development, independent of the presence or absence of \( Pc \) function. Both Hb and Tll repressors disappear from their gap gene domains at the early stages of germ band extension. To explain the variable perdurance of the expression boundaries of the different elements, we considered the possibility that it might reflect different abilities to bind and retain Hb protein even when the concentration of free Hb protein plummets rapidly, during germ band extension. Figure 5 shows in fact that the different elements differ widely in their affinity for Hb protein in vitro. 2206Hf is the strongest, followed closely by BX. The 2218S and 2212S1 and S2 enhancers are considerably weaker, and, lastly, 2218R6 is by far the weakest. This ranking of affinity is in excellent agreement with the time at which boundary breakdown occurs: 2218R6 breaks down earliest, followed by 2212S1 and S2, 2218S, then BX and 2206Hf. We suspect, therefore, that even in the absence of a \( Pc \) maintenance mechanism, these different elements are able to retain their bound Hb repressor, in proportion to their affinity for Hb protein. In fact, the difference between the time of breakdown of the 2218R6 enhancer and that of the 2206Hf enhancer is only a matter of a few hours, well within the range of half-life that might be expected in vitro for a protein-DNA complex such as that formed by Hb. We need only to suppose that the
The enhancer fragments and then end labeled. The when it is bound to lanes, labeled In, represent the input pattern concentrations. The other lanes show the fragments precipitated from 0 to 90X, of cold competitor DNA of the same plasmid. The 2212s1 fragment comigrates with a vector fragment that has no binding activity, and the 2218s and 2218R6 fragments also comigrate in this gel.

Hb protein is more resistant to proteolytic degradation when it is bound to DNA. It is also possible that additional mechanisms operate in vivo to stabilize the complex until PcG repression is established.

Imaginal disc enhancers: None of the small embryonic enhancer fragments directs expression in the imaginal discs. Expression in all discs or parts of them is observed in occasional lines but not in most and is attributable to position effects rather than to an intrinsic activity. Consistent expression in imaginal discs was obtained instead with transposons that included sequences from position 0 to -5 of the bsd-phbx region (see Figure 1) and with no other transposon from the upstream region. Transposon 2212B6, which contains no parasegmental enhancer activity, expresses consistently throughout all imaginal discs but much more strongly in the wing, haltere and eye than in the leg discs or antenna in all six lines tested (Figure 6, A–C). The analysis of smaller or overlapping fragments shows that this region contains at least four distinct imaginal disc enhancers, each with a different specificity. The 2212RH1.0 fragment gives LacZ expression in the central or distal part of the leg discs (Figure 6, E and F) and, much more weakly, in the eye disc, in the differentiating part of the retina. This fragment produces only a weak spot of activity in the proximal region of the wing and haltere discs. A complementary pattern is obtained with fragment 2212H2.4, which stains strongly in wing and haltere discs, more weakly in the retinal region of the eye disc and only very weakly in the leg discs. Fragment 2212H1.3 gives staining in the distal part of the leg discs and in an anteroposterior band in the wing and haltere discs (Figure 6, G–J). Fragment 2212B0.3 produces a more variable pattern in all discs: some lines stain the central part of the discs, other lines stain the margins, and some (2 of 14) do not stain any disc. Fragments to the left of the BamHI site at position -5.2 and to the right of the BamHI site at position +1.5 give no imaginal disc expression, nor does the small 2212H0.5 fragment situated between 2212RH1.0 and 2212H1.3. None of these imaginal enhancers is active in the early embryo, although weaker patterns of expression develop after germ band retraction.

We conclude that expression in the imaginal discs is not simply a continuation of embryonic parasegmental expression but is due to a number of distinct imaginal enhancers that are not activated by segmentation genes like the parasegmental enhancers and lack the anteroposterior information conferred by the gap genes and by hh in particular. How then do imaginal disc enhancers receive positional information?

A maintenance element: In addition to the embryonic pattern-forming enhancers and the imaginal disc enhancers, the bsd-phbx region contains another kind of regulatory element, the Polycomb Response Element or PRE. This element, located between the 2212s1 and 2212s2 enhancers, gives no expression pattern by itself but confers maintenance of the expression pattern to other enhancers (Chan et al. 1994). Its activity is illustrated by the 2212H6.5 fragment, which includes both 2212s1 and 2212s2 enhancers, but in contrast to the behavior of these two separate enhancers, it gives an embryonic expression pattern that is maintained throughout embryonic development (Figure 7, A and B). This element responds to the Pc-G genes and is able to establish long-term and long-range repression of enhancers in its general chromosomal vicinity, causing also partial or total repression of the mini-white gene present in the same transposon (Chan et al. 1994). Transposons containing the PRE produce variagated mini-white expression in at least 50% of the lines containing them. In contrast, none of the lines containing the isolated embryonic or imaginal enhancers showed detectable variagation (6–10 lines for each construct). The PRE can be functionally separated from other enhancers and contains no Hb binding sites or enhancer activity of its own. The same PRE fragment also responds to tra function, which stimulates expression either by promoting the activity of other enhancers or, alternatively, by preventing Pc-G repression (L. Rastelli and V. Pirrotta, unpublished results).

Establishment of Pc-G repression depends on early enhancer activity: In the presence of parasegmental enhancers, the PRE can confer long-term maintenance of
repression in the anterior region (Chan et al. 1994). Pirrotta and Rastelli (1994) have argued that the establishment of long-term repression by the Pc-G in the chromatin region surrounding the PRE depends on the state of activity of the enhancers (or promoters) in the region at the time the Pc-G complex is established in the early embryo. The repressive complex is presumably not fully established at the syncytial blastoderm stage, since the enhancers are activated at this time, but must be fully in place before the end of germ band extension, when the first signs of ectopic expression become detectable in Pc-G mutant embryos. The 2218R6 enhancer is not activated at blastoderm like the other enhancers but only later, during germ band extension. If the establishment of the repressive complex at the PRE depends on the state of activity of the enhancer, 2218R6 should be repressed everywhere in the presence of the PRE. About 50% of the transgenic lines carrying a CaSpeR transposon containing the Ubx PRE together with the 2218R6 enhancer driving the Ubx-lacZ gene show various degrees of variegation of the expression of the mini-white gene, indicative of the repressive activity of the PRE. In all the variegating lines, the expression of the 2218R6 enhancer is largely suppressed throughout the embryo, not just in the anterior half as in the case of the 2212H6.5 enhancers (Figure 7, C and D). Little or no repression is seen in the remaining lines, which do not variegate in the eye. The repression, like the eye variegation, is variable and mosaic in character, leaving patches of cells still expressing the 2218R6 pattern. This result suggests that the repressive complex becomes established before or nearly at the same time as the onset of the 2218R6 expression, during germ band extension.

DISCUSSION

Parasegmental enhancers: Our results show that each site in which Hb binding has been detected in vitro contains also a parasegmental enhancer activity. Large transposons representing the entire Ubx gene, including at least 50 kb of upstream sequence, have been tested for enhancer activity (Simon et al. 1990; Müller and Bienz 1991; Qian et al. 1991), and the only pattern-forming embryonic elements found correspond to the seven enhancers ABX, BX, 2218S, 2218R6, 2212S1 and S2 and 2206Hf (PBX), although the existence of some additional and redundant enhancer can-
not yet be rigorously excluded. Together these seven elements most likely determine the initial pattern of expression of the *Ubx* gene in the embryo, directly controlled by and dependent on the segmentation genes. Although they vary widely in their affinity for Hb, these enhancers all initially respect an anterior boundary of expression, confirming the conclusion that the Hb protein acts locally to prevent activation of the enhancer elements by positive factors. We note first that the properties of these enhancers give no indication of a systematic anterior/posterior gradient of activity. Although the most 3' element, ABX, has the most anterior boundary, it is also the only one that has an odd-numbered parasegment specificity. The remaining six elements have an early expression boundary at PS6 and have initially an even-numbered parasegment specificity (or none at all, in the case of the 2218R6 element). It appears therefore that, at least at the level of the separate enhancers, there is no specific mechanism, such as different affinities for Hb, to set different anterior boundaries or to generate specificities of expression in progressively more posterior regions of the *Ubx* expression domain. The more anterior boundary of ABX is probably simply due to its strong activation in PS5, where most of the other enhancers would not be active because of their pair-rule specificity. However, a contribution to expression in PS5 could be made toward the end of germ band elongation by the 2218S, 2218R6, 2212S1 and 2212S2 enhancers.

We note that there is considerable apparent redundancy in the domains of expression of the different elements. Thus, BX, 2218S and PBX (2206Hf) give very similar patterns in the epidermis and in the mesoderm. Similarly, the 2212S1 and S2 enhancers, though very different from the BX class of enhancer, appear to be entirely redundant in their embryonic pattern of expression. It is possible, however, that this apparent similarity in the initial stages masks a greater specificity at the cellular level at later stages. The 2218R6 element is different from the others in its pattern within the parasegment, its later activation and segment polarity-like distribution. To some extent, therefore, different enhancers make different contributions, enriching the overall pattern of *Ubx* expression in the early embryo.

A more difficult question is that of the strength of each contribution. It is difficult to compare the relative enhancer strength of the separate elements because of the effects due to the site of insertion of the transposons. Furthermore, their relative activity in the intact *Ubx* gene may be affected by other factors—for example, their distance from the promoter (MÜLLER and BIEZ 1991; SIMON et al. 1993). In the embryonic pattern of the endogenous *Ubx* gene, early expression in PS5, which according to the activities of the separate enhancers should be largely contributed by ABX, is very weak, suggesting either that the greater distance of this enhancer from the promoter reduces its contribution or that the sum of the other enhancer activities results in much stronger expression in the more posterior parasegments. The pattern of expression of the endogenous *Ubx* gene within a parasegment is different from that of most of the separate enhancers. It is strongest
in the posterior part of the parasegment and weakest in the anterior part, corresponding to the en domain. In fact, it resembles most the pattern of the 2218R6 element, as was noted by Müller and Bienz (1991). This raises the possibility that the 2218R6 may make a proportionally greater contribution than the other elements to the total pattern. However, the fact that bad breakpoints that remove increasing amounts of the upstream region abolish progressively more Ubx pattern elements suggests that all enhancers make a significant contribution to the overall expression of the gene (Peifer et al. 1987).

Expression in other tissues: The BX, PBX (2206Hf) and 2218S elements have prominent expression in the mesoderm. Both BX and PBX contain, in fact, multiple binding sites for the major mesodermal activator Twi, often closely associated with the binding sites of the Hb and TII repressors and of the activator Ftz (Qian et al. 1993). In BX, mesoderm expression occurs only in the even-numbered parasegments, suggesting that Twi action is enhanced by the simultaneous binding of Ftz. In PBX, the Twi binding sites are not always accompanying Ftz binding sites, consistent with the fact that mesoderm expression of PBX persists also in the odd-numbered parasegments.

Besides 2218R6, only ABX activates strong expression in the CNS (Simon et al. 1990; Müller and Bienz 1991), the major site of Ubx expression in the late embryo. We do not know what factors are responsible for this activity. Neural tissue begins to differentiate during germ band extension from neuroblasts that migrate inwardly, but condensation of the ventral nerve chord takes place only at the end of germ band retraction (Campos-Ortega and Hartenstein 1985), when the early enhancers cease functioning. It is possible that neural tissue derived from neuroblasts that initially expressed Ubx continues to express the gene through this process of differentiation. If so, some specific neural factor must be required to stabilize this expression, since only two of the enhancers are active in the CNS. Genes like ftz or eve that activate the Ubx enhancers in the ectoderm are also expressed later in specific sets of cells of the CNS. It is very unlikely, however, that these also function as activators of Ubx in the CNS, since (1) only some enhancers are active in this tissue while others like BX or PBX that are known to be activated by Ftz do not express in the CNS; (2) there is no correspondence between neurons that express Ftz or Eve and those that express Ubx (Doe et al. 1988); and (3) Hb, which acts as a repressor in the ectoderm, is expressed in the differentiating nervous system irrespective of the gap gene territory that originally informs the Ubx domain of expression. Most likely, therefore, CNS expression is activated by some neural-specific factor.

Maintenance: True long-term maintenance of the correct domain of expression is dependent on the PC-G genes and is due to maintenance elements that are distinct and separate from the enhancers. We have found only one PRE in the Ubx gene able to act autonomously to establish repression. The existence of this PRE was also inferred by Simon et al. (1993), who examined the behavior of larger transposons including this region. More recently, however, Chiang et al. (1995) have reported the presence of another, weaker Polycomb protein binding site in the bx region. These results do not exclude the possibility that additional sites interact more weakly with PC-G genes and may, in combination or in certain chromosomal contexts, succeed in establishing PC-G repression (Müller and Bienz 1991). The properties of the PRE are described by Chan et al. (1994). Suffice it to say here that this maintenance element must be able to repress the several Ubx enhancers at a great distance, a type of repression different from that exercised by Hb, which is strictly local (Qian et al. 1991, 1993). In fact, the region containing the PRE, located between 2212S1 and 2212S2 enhancers, possesses no Hb binding sites, yet the maintenance of repression must respond to the positional information specified by Hb. Since the repression is determined by the presence or absence of Hb, the effect of the PC-G complex on a given enhancer must be either mediated by the Hb protein bound at the enhancer (Zhang and Bienz 1992) or a direct consequence of its binding, e.g., the state of activity of the enhancer at the time the Pc-G complex is established. Arguments presented by Chan et al. (1994) support the latter mechanism. This is also consistent with our finding that the 22128R6 enhancer, which becomes active only during germ band extension, is repressed everywhere when combined with the PRE, while enhancers that are active from the blastoderm stage are repressed by the PRE primarily where they were initially repressed by Hb, in the anterior part of the embryo. In further confirmation, we find that imaginal enhancers, which are silent in the early embryo, are also repressed everywhere in the presence of the PRE (S. Poux, C. Kostic and V. Pirrotta, unpublished results).

Imaginal disc expression: The imaginal discs are major sites of postembryonic Ubx expression that must be maintained in accordance with the parasegmental pattern set in the early embryo. Imaginal disc activity is not a continuation of embryonic activity and depends on two separate sets of imaginal disc enhancers, one associated with the ABX region (Simon et al. 1990) and one associated with the PBX region, as reported above. The presence of the latter was inferred by Castelli-Gair et al. (1992) from the properties of a larger PBX enhancer fragment containing our 2212H2.4 enhancer. Christen and Bienz (1994) also reported that the expression of this enhancer in wing and haltere discs of some lines is subject to a parasegmental restriction. We have never observed such a parasegmental restriction and cannot account for this discrepancy at present, but a further account of the imaginal enhancers and their
interaction with the PRE will be given elsewhere (S. Poux, C. Kostic and V. Pierrotta, unpublished results). Neither the abx nor, in our experiments, the pbx set of imaginal enhancers appears to have parasegmental information by themselves or in combination with parasegmental enhancers, and both direct expression in anterior imaginal discs as well as in the PS5 and 6 discs where the Ubx gene is normally expressed. Whence comes then their positional information? We propose that these elements are active or repressed depending on the information derived from the embryonic enhancers and transmitted by the long-range repression due to the Pe-G complex initiated at the PRE. We suppose that, since the imaginal disc enhancers are not active in the embryo at the stage in which the Pe-G repression is established, they are maintained in a repressed state in all cells in which the Ubx gene is not kept in the active conformation by one or more of the parasegmental enhancers. The active state of these parasegmental enhancers prevents the spread of the repressive Pe-G complex and indirectly controls the activity of the imaginal enhancers. It is likely that the same mechanism regulates expression in the CNS and restricts it to its proper parasegmental domain.

The fact that the classical abx, bx, bxd and pbx mutations affect imaginal structures is explained by different arguments in each case. The two pbx mutations known are both deletions that remove both the PBX enhancer and the entire set of imaginal disc enhancers in the upstream region (Bender et al. 1983), leaving only the imaginal enhancers associated with ABX. The observed reduced expression affects more the PS6 compartment that the PS5 compartment. Chiang et al. (1995) propose that this is due to a compartmentalization of the regulatory region such that the ABX imaginal enhancers are active only in PS5. This compartmentalization, if it exists, need not be so strict, allowing the ABX imaginal enhancers to contribute to expression in PS6. bxd mutations are of two kinds. Some are deletions or rearrangements with breakpoints at various places between the Ubx promoter and the pbx region and clearly remove everything that lies upstream, including the imaginal disc enhancers and a variable number of the parasegmental enhancers described here. Several other bxd mutations are caused by the insertion of the gypsy transposable element. It has been shown that gypsy contains a sequence, the target of the su(Hw) product, which when interposed between enhancer and promoter interferes with the communication between them (Holdridge and Dorsett 1991; Jack et al. 1991; Geyer and Cortes 1992; Roseman et al. 1993). gypsy insertions would, therefore, behave not unlike breakpoints, though their effects may vary in importance from one tissue to another depending on the action of the su(Hw) product. The majority of bx insertions are also gypsy insertions (Peifer and Bender 1986) that would be expected to interfere with the BX enhancer as well as with the more distant ABX enhancer and the imaginal disc elements associated with it. The three abx mutations known are deletions that remove from 1.5 to 14 kb from the region containing the ABX enhancer and presumably affect also the associated imaginal enhancers. However, the smallest of these deletions, abx2, does not remove all the imaginal disc enhancers associated with ABX (Simon et al. 1990). White and Wilcox (1985), who examined the expression of the Ubx gene in embryos homozygous for abx2, found that expression is reduced in the anterior part of PS5 in the ventral nerve chord but residual expression is visible in the posterior part. The imaginal discs show a similar change but no effect is seen in the embryonic epidermis. This residual expression in PS5 might be accounted for by a contribution by the 221R86 (BXD) enhancer in the nervous system and by the 2218S enhancer in the epidermis (see Figure 2E), although the existence of other, still undetected enhancers cannot be ruled out. An important difficulty is introduced by the fact that the pbx2 deletion also removes the entire upstream PRE, yet the expression of the Ubx gene is not derepressed in the anterior regions. It is possible that the sum of weaker Pe-G response elements in the rest of the gene, as well as the element found by Chiang et al. (1995) in the BX region is sufficient to maintain a degree of repression even in the absence of the upstream PRE.

We are grateful to Yu Chun He and Jack Guirard for technical assistance, to Sylvain Poux and Corinne Kostic for some of the constructs, and to Tom Kornberg for anti-engrailed antibody. The expert photographic services of Elyre Martinez and Micheline Vautravers are gratefully acknowledged. C.-S.C. was supported by a stipend from the University of Geneva while on leave from the Baylor College of Medicine. The Georges and Antoine Claraz Donation is gratefully acknowledged. This work was supported in part by grants from the National Institutes of Health and from the Swiss National Science Foundation to V.P.

LITERATURE CITED


Jorgens, G., Jack, J., Dorsett, Y. Delotto and S. Liu, 1994 


Communicating editor: R. E. Denell.