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Regulatory elements of the bithorax complex that control expression along the anterior – posterior axis

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The *Drosophila* bithorax complex (BX-C) controls segmental development by selectively deploying three protein products, *Ubx, abd-A* and *Abd-B*, within specific segments along the body axis. Expression of these products within any one segment (or, more accurately, parasegment) is affected by mutations clustered in a particular region of the BX-C. The regulatory regions defined by this genetic analysis span 20 – 50 kb and there is one region for each segmental unit. Here we describe regulatory elements from several of these regions, identified by fusion to a *Ubx – lacZ* gene and analysis in germline transformants. A small DNA fragment from the *abx* region programs expression with an anterior boundary in the second thoracic segment (parasegment 5). This anterior limit is appropriate, since the *abx* region normally controls *Ubx* in parasegment 5. Other regulatory regions of the BX-C that control development of parasegments 6, 7 or 8 contain similar regulatory elements that program expression with anterior limits in parasegments 6, 7 or 8, respectively. These experiments define a class of BX-C regulatory elements that control expression along the anterior – posterior axis. The early appearance of the *lacZ* patterns in embryos suggests a role for these elements in the initial activation of expression from the BX-C.

Key words: anterior – posterior axis/bithorax complex/*Drosophila*/homeotic/*Ultrabithorax*

Introduction

The early *Drosophila* embryo is initially divided into segments and then the segments are differentiated one from another. The differentiation of the segments in the posterior half of the thorax and in the abdomen is controlled by genes of the bithorax complex (Lewis, 1978; Bender *et al.*, 1983; Karch *et al.*, 1985). The functions of the bithorax complex are executed by three protein-coding transcription units, *Ubx, abd-A* and *Abd-B* (Regulski *et al.*, 1985; Sanchez-Herrero *et al.*, 1985; Tiong *et al.*, 1985; Casanova *et al.*, 1987). Null mutations in any of these transcription units cause morphological transformation of groups of segments into more anterior segments resulting in embryonic lethality.

The regions affected by *Ubx* mutations are slightly offset relative to the edges of the segments; these units have been called parasegments (Martinez-Arias and Lawrence, 1985). Thus, in embryos, parasegment 5 consists of the posterior quarter of the second thoracic segment and the anterior three-quarters of the third thoracic segment. *Ubx* null mutations transform parasegments 5 and 6 into parasegment 4, and, in agreement with the mutant phenotype, parasegments 5 and 6 are major sites of *Ubx* expression in embryos (Beachy *et al.*, 1985; White and Wilcox, 1985a and see Figure 3A). In early embryos, the most anterior activation of *Ubx* occurs in parasegment 5 in a mosaic pattern in a modest number of cells. In parasegment 6, *Ubx* appears more uniform with expression in all or nearly all cells. *Ubx* expression decreases in the more posterior parasegments, in response to *trans*-repression by the *abd-A* and *Abd-B* products (Struhl and White, 1985) which predominate in these more posterior regions (Celniker *et al.*, 1989; Karch *et al.*, 1990). *abd-A* is expressed with a sharp anterior boundary in parasegment 7. The confinement of *Ubx* and *abd-A* expression to particular parasegments is critical since abnormal development occurs in mutants that express either product ectopically in inappropriate parasegments (White and Akam, 1985; Karch *et al.*, 1990).

A molecular map of the region of the bithorax complex (BX-C) that encompasses the *Ubx* transcription unit is depicted in Figure 1A. Four *Ubx* exons are distributed over 75 kb, with alternative splicing of the internal microexons to the common 5′ and 3′ exons generating a family of *Ubx* proteins (O'Connor *et al.*, 1988; Kornfeld *et al.*, 1989). A number of the DNA lesions that disrupt the *Ubx* transcription unit have been mapped and these cause embryonic lethality (Bender *et al.*, 1983).

A large number of mutations in this portion of the BX-C that fail to disrupt the *Ubx* transcription unit have also been mapped (Bender *et al.*, 1983, 1985; Peifer and Bender, 1986) and some of these are depicted above the DNA line (Figure 1A). These mutations tend to cause less severe phenotypes and can be divided into two classes according to the affected anatomical domain of the fly. The first class includes the *abx/bx* mutations which affect specifically the development of parasegment 5 and are spread out within the large third *Ubx* intron between map positions −80 and −55. The second class includes the *bxd/pbx* mutations which affect the development of parasegment 6 and are spread upstream of the *Ubx* transcription unit between map positions −30 and +20. These parasegment-specific mutations interfere with regulatory information necessary for the proper spatial distribution of *Ubx*. Thus, the *abx/bx* mutations define a regulatory region that programs the spatial distribution of *Ubx* protein in parasegment 5, and the *bxd/pbx* mutations define a regulatory region that programs the spatial distribution of *Ubx* protein in parasegment 6 (White and Wilcox, 1985b; Cabrera *et al.*, 1985). In this way, the *Ubx* promoter is capable of interacting with *cis*-regulatory regions in positions 50 kb away in either upstream or downstream locations.

The control regions for *abd-A* and *Abd-B* can also be

divided into a series of large parasegment-specific regulatory regions (Karch *et al.*, 1985, 1990; reviewed in Peifer *et al.*, 1987). The *iab-2, iab-3* and *iab-4* regulatory regions map to the right of the *bxd/pbx* region and control the distribution of *abd-A* in parasegments 7, 8 and 9, respectively. Parasegment-specific regulatory regions for *Abd-B* map even further to the right. Strikingly, these BX-C regulatory regions are arranged along the chromosome in the same order as the parasegments they affect are arranged along the anterior−posterior (A−P) axis of the fly.

We wish to understand how *Ubx* and *abd-A* are activated in the appropriate parasegments. The linear order of the regulatory regions, and their large sizes, suggested to us that the assignment of *Ubx* or *abd-A* to a particular parasegment is a property of a large chromosomal domain (Peifer *et al.*, 1987). Such large regulatory regions are difficult, at present, to transform into *Drosophila*. Instead, we have begun to explore the large regulatory regions by testing relatively small DNA segments in germline transformation experiments. Surprisingly, we find that a small DNA fragment from the *abx* region programs expression with a proper anterior limit in parasegment 5. We also identify regulatory elements from the *bxd, iab-2* and *iab-3* regions that program appropriate anterior limits in parasegments 6, 7 and 8, respectively. We will refer to these elements, which share the ability to control expression along the A-P axis, as parasegmental control elements.

Results

Strategy and transformation vectors

The strategy for identification of regulatory elements in the BX-C was to clone various DNA restriction fragments, whose regulatory properties were to be tested, upstream of

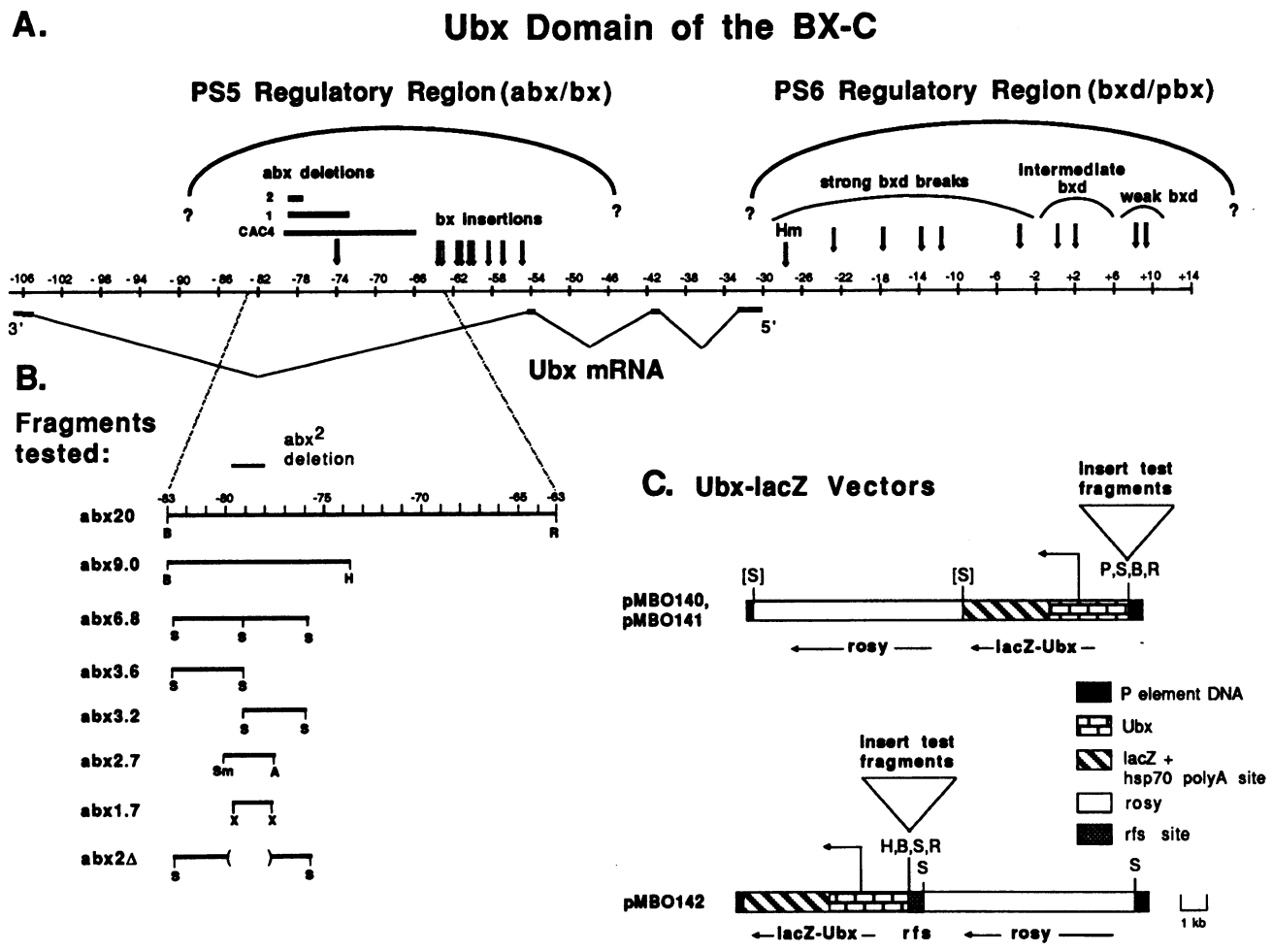

Fig. 1. *Ubx* regulatory regions and germline transformation constructs. **A.** Map of the *Ubx* transcription unit and associated regulatory regions. Map coordinates in kb are as in Bender *et al.*, (1983). The *Ubx* transcription unit is shown below the DNA line. Mutant DNA lesions with primary effects in parasegment 5 development (*abx* deletions, *bx* insertions) and in parasegment 6 development (*bxd* breaks) are shown above the DNA line. The *Hm* (*Haltere-mimic*) *bxd* break (Bender *et al.*, 1985) occurs ~3 kb upstream of the *Ubx* transcription unit (Bienz *et al.*, 1988). The question marks reflect uncertainty in the extents of the parasegment-specific regulatory regions as defined by the genetics. **B.** *abx* fragments tested in germline transformation experiments. All fragments are subfragments of abx20 and are aligned vertically. abx2Δ is a 1.5 kb deletion, that corresponds to the abx^2 mutant lesion, within the context of the abx6.8 fragment. Restriction sites: B, *Bam*HI; R, *Eco*RI; H, *Hin*dIII; S, *Sal*I: Sm, *Sma*I: A, *Apa*I; X, *Xho*I. **C.** *Ubx−lacZ* P element transformation vectors. pMBO140 and pMBO141 are identical except pMBO140 contains *rosy* on a 7 kb *Hin*dIII fragment and lacks the bracketed *Sal*I sites whereas pMBO141 contains *rosy* on an 8 kb *Sal*I fragment and has the bracketed *Sal*I sites. Perpendicular arrow indicates the *Ubx* transcription start site. Arrows below the maps indicate the directions of transcription. pMBO140 and pMBO141 are derivatives of Carnegie 3 (Rubin and Spradling, 1983). pMBO142 is a derivative of the F factor plasmid, pMBO132 (O'Connor *et al.*, 1989). The vector backbones are not shown. *rfs* is a site for the F factor site-specific recombinase used for adding DNA by recombination (see O'Connor *et al.*, 1989). All sites are shown only for *Sal*I.

a *Ubx−lacZ* reporter gene in a P element transformation vector. Several transformant lines for each construct were generated and analyzed for reproducible patterns of β-galactosidase expression. Expression was examined in embryos using an antibody directed against the *lacZ* portion of the fusion protein and, in larvae, by using the histochemical stain Xgal. A list of transformant lines described in this work is presented in Table I.

The structures of the transformation vectors used in this study are illustrated in Figure 1C. Each vector contains a hybrid gene possessing an in-frame protein fusion of *Ubx* 5′ exon sequences to *Escherichia coli lacZ* sequences. Each vector also contains the entire non-coding portion of the *Ubx* 5′ exon, the transcription start site and 1.7 kb of 5′ flanking DNA, which together, we assume, contain basal promoter elements. The hybrid gene was placed within a P element vector (Rubin and Spradling, 1983) containing *rosy* as a phenotypic marker for transformation. pMBO140 contains a unique *SalI* site upstream of the fusion gene and served as the recipient for most test fragments. pMBO142 contains the hybrid gene within a special 'building' vector so that once a test fragment has been inserted, additional adjacent sequences can be added by recombination (O'Connor *et al.*, 1989).

Table I. Summary of transformant lines and expression results

Construct	No. of lines with consensus pattern	No. of lines stained	Expression patterns 12 h	6 h	discs
abx20	4	4	PS5, uniform	ep, CNS, m, fb	+
abx9.0	3	3	PS5, pair-rule	ep, CNS	+
abx6.8a[a]	5	7	PS5, pair-rule	ND	+
abx6.8b	17	19	PS5, pair-rule	ep, CNS	+
abx6.8c	2	2	PS5, pair-rule	ep, CNS	+
abx6.8d	4	4	PS5, pair-rule	ND	+
abx6.8e[b]	4	8	PS5, pair-rule weak	−	+
abx6.8HS	5	5	PS5, pair-rule	ep, CNS, modified[c]	+
abx3.6	10	12	basal	−	−
abx3.2[b]	4	9	PS5, pair-rule, weak	−	+
abx2.7	13	16	PS5, pair-rule	ep, CNS, weak	+
abx1.7	7	9	PS5, pair-rule	ep, CNS, weak	+
abx2Δ	6	6	basal	−	+
pMBO140	5	5	basal	−	−
pMBO142	2	2	basal	−	−
bxd14.5	4	5	PS6, uniform	ep, CNS, m	ND
iab-2−11.0	11	12	PS7, pair-rule	ep, CNS	ND
iab-3−11.5	6	7	PS8, pair-rule[d]	ep, CNS	ND

The constructs listed are depicted in Figures 1 and 5A. The consensus pattern is the 6 h expression pattern common to the majority of distinct transformant lines with the same construct. Occasionally, transformant lines exhibited exceptional expression patterns, presumably due to the chromosomal location of the insert. Such 'position-effect' lines with patterns that obscured the consensus pattern were scored as non-consensus.

Staining in tissues at 6 h: PS5, expression restricted to parasegment 5 and more posterior parasegments; pair-rule, strong expression in odd-numbered parasegments; uniform, strong expression in odd and even parasegments; weak, overall reduction in staining intensity relative to abx6.8b; basal, depicted in Figure 2.

Staining in tissues at 12 h: ep, epidermis; CNS, central nervous system; m, muscle; fb, fat body; −, very weak or no staining; ND, not determined. Staining in third instar larval imaginal discs: +, staining in all discs; −, no staining in discs.

[a]abx6.8 construct designations: a, abx6.8 fragment inserted upstream of *Ubx−lacZ* in same orientation relative to *Ubx* promoter as in wild-type; b, upstream of *Ubx−lacZ* in opposite orientation; c, downstream of *Ubx−lacZ* in same orientation as in wild-type; d, downstream of *Ubx−lacZ* in opposite orientation; e, downstream of *rosy*.

[b]For these constructs with weak expression, non-consensus lines include those showing the basal pattern.

[c]The differences between the abx6.8HS and abx6.8 patterns at 12 h are shown in Figure 3.

[d]The iab-3−11.5 pattern has slightly higher expression in the even-numbered parasegments.

Expression from the basal construct

As a control for transformation of constructs with putative regulatory DNA fragments, we examined expression from the basal constructs (pMBO140, pMBO142, Figure 1C) in transformants. Figure 2 shows a typical basal transformant line at two developmental stages. At germ band extension (6 h of embryogenesis), there is weak β-galactosidase expression in lateral epidermal patches in the anterior portions of each parasegment. There is also significant staining in the head region. By 12 h of embryogenesis, the staining becomes even weaker. This pattern appears identical to that described previously for *Ubx−lacZ* fusion genes containing < 1.7 kb of 5′ flanking *Ubx* DNA (Bienz *et al.*, 1988).

This basal pattern is very different from the wild-type *Ubx* pattern (Figure 3A). One obvious difference is in the number and locations of expressing cells per parasegment. Another major distinction is the lack of a proper anterior boundary of expression. Indeed, this basal pattern is likely independent of the *Ubx* sequences on the construct since *lacZ* genes fused to other promoters program the same embryonic pattern (Boulet and Scott, 1988; Bellen *et al.*, 1989; Ghysen and O'Kane, 1989).

Identification of an early-acting parasegmental control element within the abx/bx region

Our search for *Ubx* regulatory elements has been guided by the large number of mapped and characterized mutant lesions

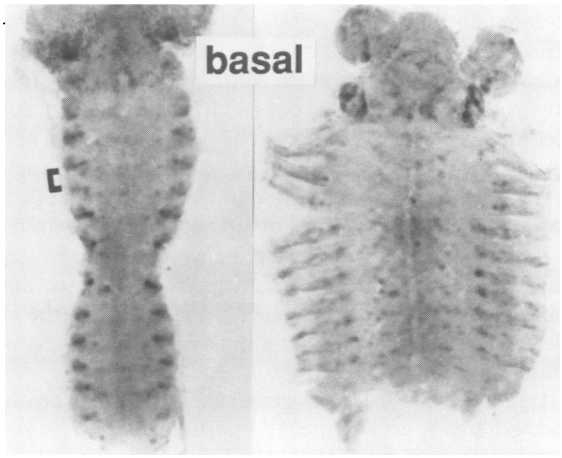

Fig. 2. Expression from the basal construct. Embryos are transformed with the basal construct, pMBO142, and were stained with antibody to β-galactosidase. Embryos here and in subsequent figures are oriented with anterior towards the top and were dissected as described in Materials and methods. Bracket indicates parasegment 5. An ~6 h embryo is on the left and an ~12 h embryo is on the right.

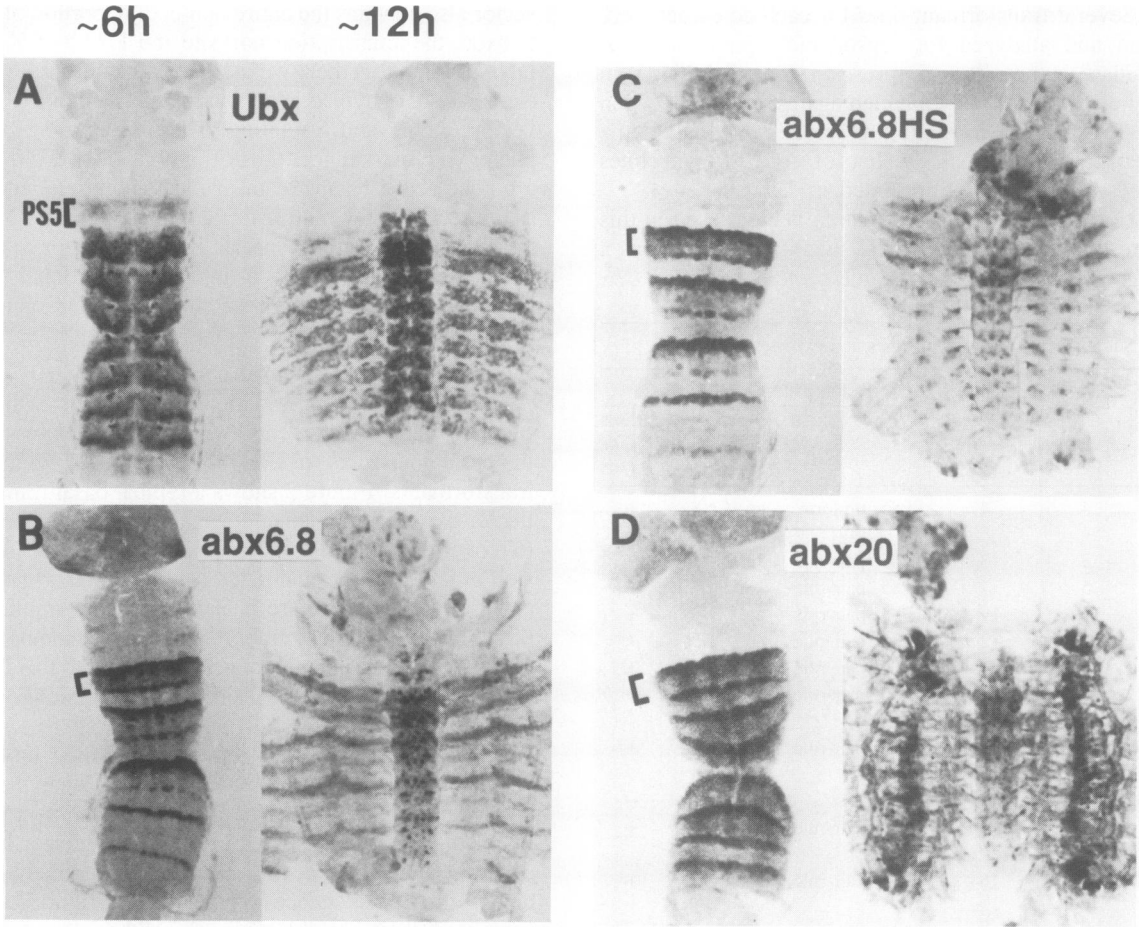

Fig. 3. Expression from *abx* constructs in embryos. Embryos in (**A**) were stained with antibody to Ubx. All other embryos were stained with antibody to β-galactosidase. **A**, Wild-type; **B**, abx6.8 transformant; **C**, abx6.8HS transformant; **D**, abx20 transformant. Brackets indicate parasegment 5. Approximately 6 h old embryos are on the left and ~12 h old embryos are on the right. In 12 h embryos, the CNS is midventral. 12 h abx6.8 embryos show primarily CNS and epidermal expression. 12 h abx20 embryos show expression in the CNS, epidermis, muscles, fat body and tracheae.

in the *Ubx* domain (Bender *et al.*, 1983, 1985; Peifer and Bender, 1986). The lesions that are easiest to interpret are the deletions since they remove DNA regulatory elements without introducing extraneous elements. Therefore, our initial studies have concentrated on the *abx* region located between −80 and −66, as defined by the three overlapping deletions abx^1, abx^2 and abx^{CAC4} (Figure 1A). The *abx* mutations cause transformation of parasegment 5 structures into those characteristic of parasegment 4 (Peifer and Bender, 1986). In the adult, the most obvious phenotype is transformation of anterior haltere to anterior wing. These phenotypes and the examination of *Ubx* expression in *abx* mutant embryos (White and Wilcox, 1985b) indicate that the DNA deleted in *abx* mutants is important for *Ubx* expression in parasegment 5.

The abx6.8 construct was generated by inserting *abx* DNA from −82 to −75 (Figure 1B) upstream of *Ubx−lacZ* in the basal construct, pMBO141. Examples of the staining observed at several developmental stages with abx6.8 transformant lines are shown in Figure 4. Expression is detectable during gastrulation at 3 h of embryogenesis in the pattern shown in Figure 4A. Three or four transverse bands of staining are seen with the anterior and strongest band at ~50% egg length. At this stage, expression is observed in ectodermal cells but it is absent in mesodermal precursor cells. This distinction is evident in Figure 4A as an interruption in the transverse stripes along the ventral midline. This developmental stage precedes the detection of *Ubx* protein in embryos, but it closely follows the initial time of expression of *Ubx* RNA (White and Wilcox, 1985a; Akam and Martinez-Arias, 1985).

As the germ band extends, this expression pattern intensifies (Figure 4B). By the time of complete germ band extension, a metameric epidermal staining pattern is seen. Embryos at this stage were double-stained with antibodies to both β-galactosidase (blue signal) and *engrailed* (brown signal) (Figures 4C and 5). Engrailed protein marks the anterior portion of each parasegment. The most striking aspect of the β-galactosidase pattern is an anterior boundary that coincides with the anterior edge of parasegment 5. In some transformants, features of the basal pattern, such as the lateral epidermal patches and weak staining in the head region, are also seen.

There are other notable aspects of the β-galactosidase expression pattern. First, expression is modulated along the A−P axis in a pair-rule fashion. Staining is more intense in parasegments 5, 7, 9, 11 and 13 and is weaker in the intervening parasegments. Another prominent feature of the

pattern is the gradient in the staining intensity along the A−P axis. The strongest expression is seen in parasegment 5, with progressively weaker staining in fewer cells in parasegments 7, 9 and 11 and barely detectable staining in parasegment 13. This feature is apparent even in the earliest embryos when staining is first detected (Figure 4A). Finally, the anterior portions of the individual parasegments are more intensely stained than the posterior portions. This anterior bias in expression is illustrated in Figure 4C where the strongest β-galactosidase expression within the odd-numbered parasegments coincides with the *engrailed* expression at the anterior margins of these parasegments.

As embryogenesis proceeds further, expression begins to spread forward of parasegment 5. This is first seen at ~9 h of embryogenesis when β-galactosidase appears in parasegment 4 in the developing central nervous system (CNS) (Figure 4D, arrow). By the time dorsal closure is complete at ~12 h (Figure 4E), the most pronounced staining is in the CNS, although pair-rule modulated expression remains in the epidermis. By this time, there is significant staining in the anterior CNS, most noticeably in parasegments 3 and 4. This breakdown in the parasegment 5 boundary is also apparent at later stages of embryonic development (Figure 4F) and during larval stages as assayed in imaginal discs. Wild-type *Ubx* expression is limited to the disc derivatives of parasegments 5 and 6: the second and third leg discs, the wing disc and the haltere disc (White and Wilcox, 1984; Beachy *et al.*, 1985). The abx6.8 transformants, however, express *Ubx−lacZ* in all of the imaginal discs. For example, Figure 4G shows appropriate expression in the haltere disc, as well as inappropriate anterior expression in the eye−antennal disc. Thus, the abx6.8 construct contains regulatory elements that provide proper parasegmental control during initiation of expression, but lacks the information needed for the maintenance of the parasegmental pattern through later development.

Tissue and cell specificity in abx6.8 transformants

The abx6.8 construct appears to contain partial information for the spatial distribution of expression within specific tissues and cells. At germ band extension, the most prominently staining tissue in the transformants is the epidermis, by mid-embryogenesis most of the expression is concentrated in the CNS (Figure 3B), and third instar larval expression is primarily in imaginal discs. These tissues are major sites of *Ubx* expression at these respective times (White and Wilcox, 1985a; Brower, 1987). There are, however, incorrect aspects of the tissue and cellular distributions as well. For example, in 6 h old abx6.8 transformants, the entire epidermal layer of cells within parasegment 5 expresses *Ubx−lacZ* (Figure 3B). In contrast, *Ubx* is expressed in parasegment 5 in a small number of epidermal cells along the anterior edge of the parasegment and in lateral areas surrounding the tracheal pits (Figure 3A). Much of the posterior portion of parasegment 5 lacks *Ubx*. Thus, the *Ubx* and *Ubx−lacZ* patterns are both strongest in the anterior portion of parasegment 5 but the cell-specific patterns do not precisely match.

The 6.8 kb abx fragment has enhancer-like properties

In its normal context, the abx6.8 fragment is located 40 kb downstream of the *Ubx* promoter. If this DNA region controls normal *Ubx* transcription, it clearly must act at a distance as an 'enhancer'. To test this directly, the abx6.8 fragment was inserted into each of three locations in the vector pMBO141: immediately upstream of *Ubx−lacZ*, immediately downstream of *Ubx−lacZ* and immediately downstream of the *rosy* gene. Both orientations of the fragment located upstream and downstream of *Ubx−lacZ* were tested and one orientation of the fragment inserted at the site distal to *rosy* was examined. All five constructs generated similar patterns, with an anterior limit in parasegment 5, when introduced into flies (examples in Figure 6A−C).

Another property of an enhancer element is its ability to act upon a heterologous promoter. The abx6.8 fragment was tested upstream of an *hsp70−lacZ* fusion gene (Lis *et al.*, 1983) in the construct abx6.8HS. As illustrated in Figure 3C, at 6 h of embryogenesis, the pattern of β-galactosidase expression directed by the abx6.8 fragment fused to the *hsp70* promoter appears identical to that for the abx6.8 fragment fused to the *Ubx* promoter. As shown in Figure 7A, expression from the abx6.8HS construct is detected as early as the blastoderm stage, just after the first detection of endogenous *Ubx* transcription (Akam and Martinez-Arias, 1985). Expression first appears in two stripes in the blastoderm with additional, weaker staining posterior stripes becoming detectable during gastrulation. In late embryos, the abx6.8HS construct programs a slightly different pattern of CNS expression, involving fewer cells per parasegment, and much reduced staining in the epidermis (compare Figure 3B and C). These epidermal and CNS staining differences could be due to a shorter half-life for the *hsp70−lacZ* product versus the *Ubx−lacZ* product. We also note that *Ubx−lacZ* protein is distributed uniformly within cells but *hsp70−lacZ* appears concentrated at the cell periphery.

Test for autoregulation

Several segmentation and homeotic genes have been shown to autoregulate their own expression at certain times and in certain tissues during development (Hiromi and Gehring, 1987; Kuziora and McGinnis, 1988; Goto *et al.*, 1989; Harding *et al.*, 1989). In particular, the expression of *Ubx* in the visceral mesoderm requires an active *Ubx* product (Bienz and Tremml, 1988). It was conceivable that endogenous *Ubx* product could mediate the initial activation of the *abx* constructs. In order to address this, abx6.8 transformants were crossed into a background deficient for the entire BX-C (*DfP9/DfP115*). The resulting stained embryos showed no changes in the early patterns of β-galactosidase. Thus, the early anterior boundary of *Ubx−lacZ* expression in parasegment 5 is set independently of the BX-C. The expression pattern was somewhat altered, however, in later stage (12−16 h) abx6.8 embryos lacking the BX-C. *Ubx−lacZ* expression was increased in the CNS in parasegments 7−13 (not shown), consistent with *trans*-repression of the construct by *abd-A* and *Abd-B* in the wild-type embryos (Struhl and White, 1985).

Dissection of the abx6.8 construct

In order to localize further the parasegmental control element, deletion derivatives of the abx6.8 construct (Figure 1B) were tested. Expression patterns from this series of constructs in 6 h and 12 h embryos and in larval imaginal discs are summarized in Table I.

The abx6.8 fragment was divided approximately in half

to generate the abx3.6 and abx3.2 constructs (Figure 1B). Transformants containing the abx3.6 construct failed to yield β-galactosidase expression above the basal pattern. Transformants containing the abx3.2 construct occasionally showed (four lines out of nine) a parasegment 5-restricted expression pattern in early embryos albeit at reduced levels (Figure 6D). Thus, the abx3.2 fragment is sufficient to program the parasegment 5-restricted pattern but DNA from

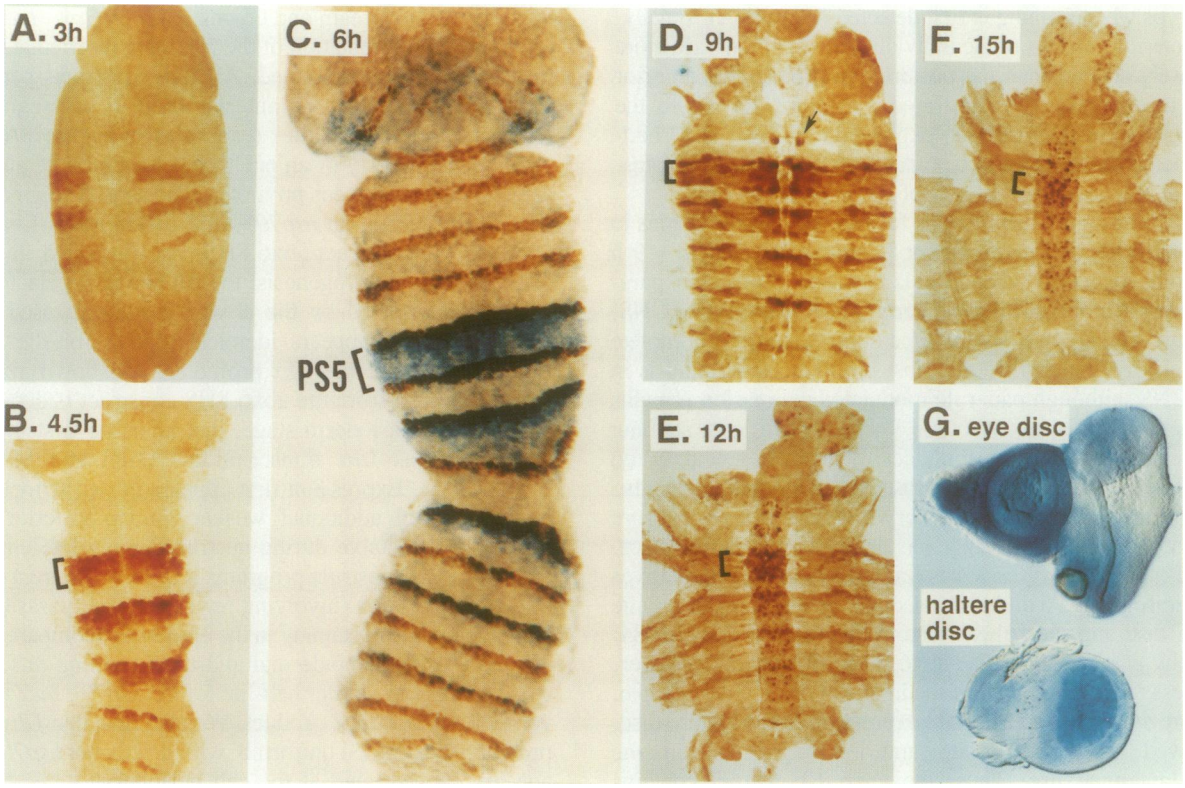

Fig. 4.

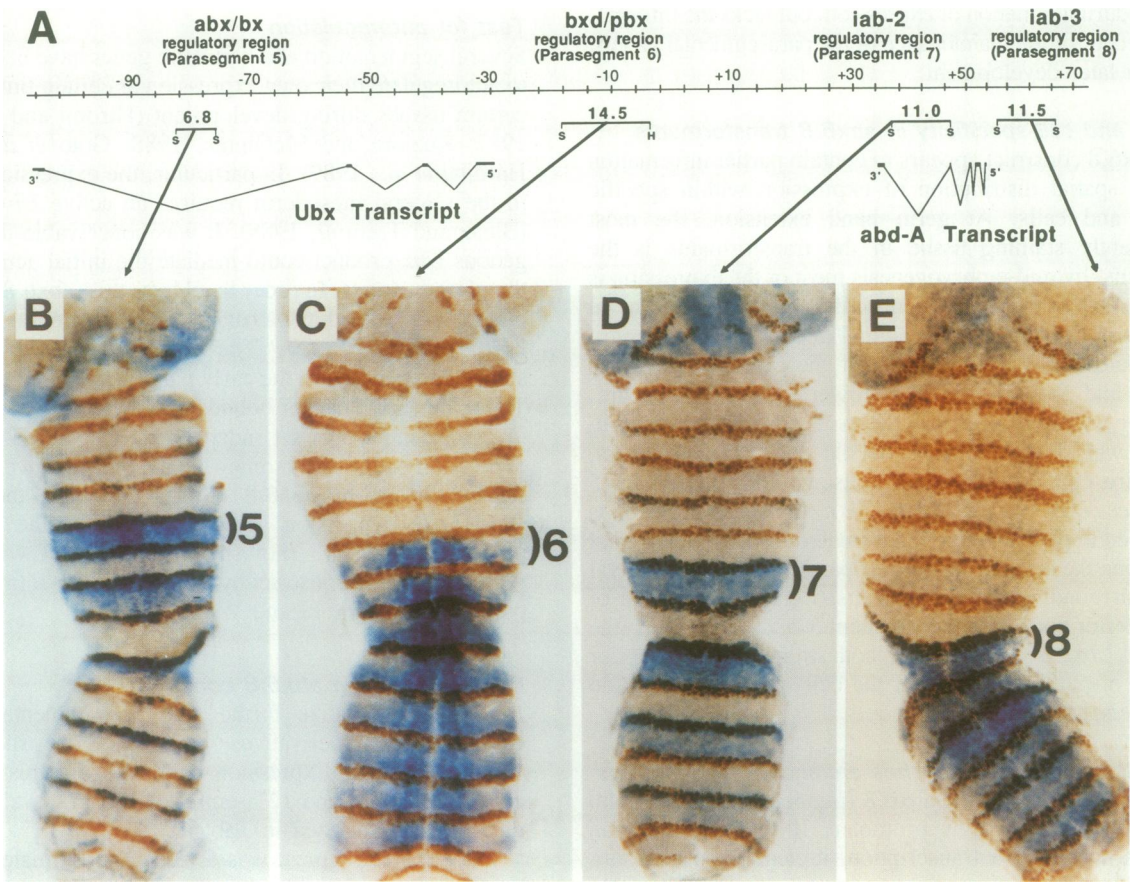

Fig. 5.

the abx3.6 fragment may contribute to the level of expression.

Further localization of regulatory elements was obtained by analyzing expression from the abx2.7 and abx1.7 constructs (Figure 1B). Transformants containing either of these constructs produced the early expression pattern characteristic of abx6.8 transformants at comparable levels (compare Figures 6A and E). The only difference is that some β-galactosidase begins to appear anterior to the parasegment 5 boundary in the abx2.7 and abx1.7 transformants at 6 h (arrows, Figure 6E). Thus, the abx1.7 fragment defines the smallest amount of DNA, identified so far, that is sufficient to contain the parasegmental control activity.

The DNA lesion in abx^2 mutants (a 1.5 kb deletion) is completely contained within the abx6.8 fragment (see Figure 1B). The equivalent *SalI* fragment from abx^2 genomic DNA was isolated and inserted into the pMBO140 vector to generate the abx2Δ construct (Figure 1B). Germline transformants containing this construct lacked embryonic expression above the basal pattern (Figure 6F). Since this shows that at least part of the parasegmental control element localizes to the 1.5 kb removed in abx^2 mutants, it is important to examine how *Ubx* expression is altered in such mutants. We have repeated the observations of White and Wilcox (1985) who reported that *Ubx* expression in abx^2 mutants is reduced in certain CNS cells but epidermal staining appears unaffected. This staining difference is first seen at ~ 9 h when the central nerve cord begins to condense. We have also examined *Ubx* expression in embryos homozygous for the larger deletion, abx^{CAC4} (see map, Figure 1A) and it appears indistinguishable from that in abx^2 embryos (not shown). Thus, certain parasegment 5 cells can activate *Ubx* expression correctly even in the absence of the parasegmental control element defined here. We suggest that this reflects the presence of multiple parasegmental control elements within the *abx/bx* region (see Discussion).

Exploration for additional regulatory elements in flanking sequences within the abx region

Although certain features of the abx6.8 expression pattern are reminiscent of wild-type *Ubx* expression, a number of features, such as the cell specificity in the epidermis, are inappropriate. It is likely that necessary regulatory elements are located elsewhere in the *abx/bx* region in DNA absent from the abx6.8 construct. To address this, larger constructs that contain the abx6.8 fragment and additional flanking DNA were tested. The abx9.0 fragment adds ~ 1 kb to both ends of the abx6.8 fragment (Figure 1B). abx9.0 transformants, however, displayed expression identical to that in abx6.8 transformants at all stages.

A recombination strategy (O'Connor *et al.*, 1989) was then used to insert 11 kb of flanking DNA into the abx9.0 construct to generate the abx20 construct (Figure 1B). The abx20 construct contains DNA from −83 to −63 and covers all three *abx* deletions as well as the insertion points of transposable elements associated with the *bx* alleles bx^{F31}, bx^G, bx^{AV} and bx^9 (Peifer and Bender, 1986). abx20 transformants show the same anterior border of expression at the front edge of parasegment 5 as seen in abx6.8 transformants (Figure 3D). However, there are several differences in the abx20 embryonic staining pattern when compared with the shorter constructs. First, expression in gastrulation stage embryos now includes the mesodermal precursor cells on the ventral surface (compare Figures 7B, 7A and 4A). The mesodermal derivatives continue to stain through later stages such that by 12 h of embryogenesis there is significant labeling of muscles, fat body and tracheae (Figure 3D). Another difference between the abx20 and abx6.8 patterns is increased expression in the even-numbered parasegments such that the pair-rule aspect of the abx20 pattern is greatly reduced (Figure 3D). This more uniform expression along the anterior−posterior axis now more closely resembles wild-type *Ubx* expression (Figure 3A).

A number of inappropriate aspects of expression are still observed in the abx20 transformants, however. The cell-specific pattern of expression in parasegment 5 of early embryos resembles the incorrect pattern in the abx6.8 transformants. Also, by 12 h of embryogenesis, the sharp parasegment 5 boundary is not maintained in either the ectoderm or the mesoderm. Examination of expression in larval tissues similarly revealed that all imaginal discs accumulate β-galactosidase. We suspect that even larger segments of *abx/bx* DNA are necessary in order to program the wild-type *Ubx* pattern in parasegment 5.

An ordered array of parasegmental control elements in the BX-C

The BX-C consists of an ordered series of regulatory regions, one for each parasegment from 5 through 13 (Lewis, 1978; Karch *et al.*, 1985, 1990). We wondered whether each of these regulatory regions might contain elements similar to the *abx* parasegmental control element. In particular, we searched the *bxd* regulatory region, which controls *Ubx* expression in parasegment 6 and the *iab-2* and *iab-3* regions, which control *abd-A* in parasegments 7 and 8, respectively.

Figure 5 presents the results for three fragments, one from each of these regulatory regions. The map in Figure 5A shows the portion of the BX-C that encompasses the *Ubx* and *abd-A* transcription units. The transformant embryos in Figures 5B−E have been double-stained with antibodies to β-galactosidase (blue signal) and *engrailed* (brown signal).

Fig. 4. Time course of expression from the abx6.8 construct. Embryos were stained with antibody to β-galactosidase. Embryo in (**C**) was double-stained with antibodies to β-galactosidase (blue signal) and to *engrailed* (brown signal). The anterior margins of the *engrailed* stripes demarcate the anterior boundaries of the parasegments. Imaginal discs in (**G**) were stained for β-galactosidase activity with Xgal. Brackets indicate parasegment 5. The arrow in panel (**D**) points to expressing cells in parasegment 4. Approximate ages of embryos are indicated in hours.

Fig. 5. Anterior expression boundaries in four successive parasegments. **A.** Map of the *Ubx* and *abd-A* transcription units and four of their associated regulatory regions. Map coordinates are in kb as in Figure 1. Below the map are four DNA fragments tested in germline transformants after insertion into pMBO140 or pMBO141 (Figure 1C). Restriction sites: S, *SalI*; H, *Hin*dIII. The corresponding expression patterns from these four constructs in ~ 6 h embryos are shown in **B**−**E**. These embryos were double-stained with antibodies to β-galactosidase (blue signal) and to *engrailed* (brown signal). The anterior margins of the *engrailed* stripes demarcate the anterior boundaries of the parasegments. Numbers indicate individual parasegments. **B**, abx6.8 transformant; **C**, bxd14.5 transformant; **D**, iab-2−11.0 transformant; **E**, iab-3−11.5 transformant.

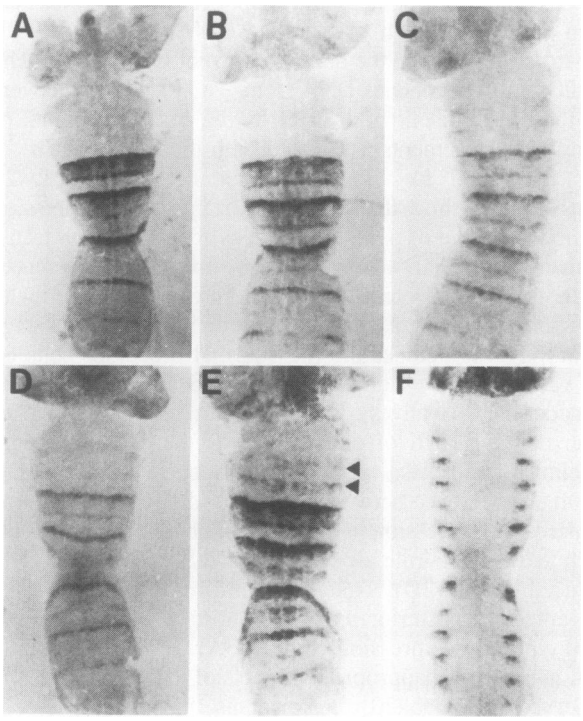

Fig. 6. Tests for enhancer properties and dissection of the abx6.8 fragment. Embryos were stained with antibody to β-galactosidase. **A.** Transformant with abx6.8 fragment upstream of *Ubx−lacZ* in opposite orientation (abx6.8a, see Table I). **B.** Transformant with abx6.8 fragment downstream of *Ubx−lacZ*. **C.** Transformant with abx6.8 fragment downstream of *rosy*. **D.** abx3.2 transformant. **E.** abx1.7 transformant. Arrows indicate expression detectable at this stage in parasegments 3 and 4. **F.** abx2Δ transformant.

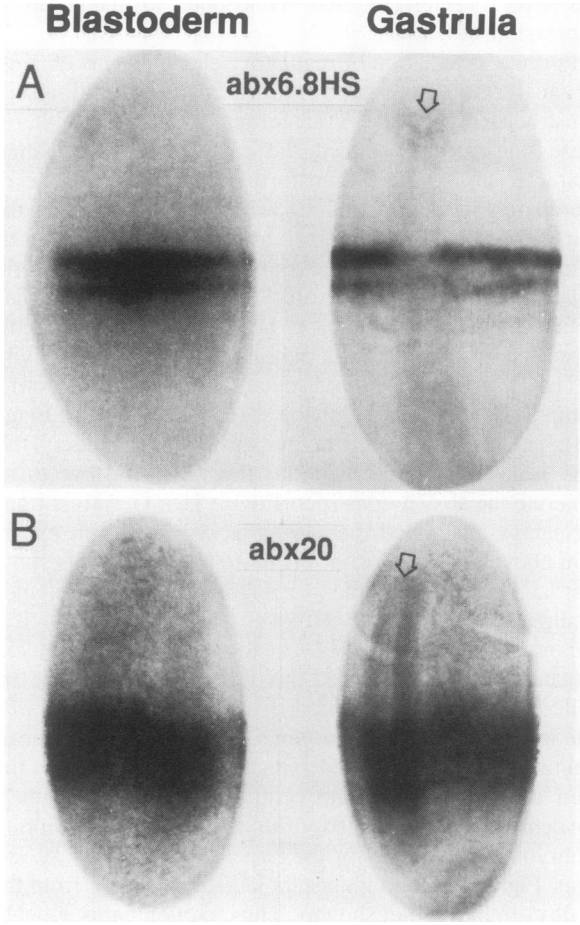

Fig. 7. Early expression from abx6.8HS and abx20 constructs. Embryos were stained with antibody to β-galactosidase. **A**, abx6.8HS transformant embryos; **B**, abx20 transformant embryos. Embryos on the left are at late blastoderm stage and embryos on the right are at early gastrulation. Open arrows indicate the ventral furrow. Expression from these two constructs is detectable at earlier times than from the abx6.8 construct.

The bxd14.5 construct contains *bxd* DNA, from map coordinates −18.5 to −4, inserted upstream of *Ubx−lacZ* in the vector pMBO140 (Figure 1C). As shown in Figure 5C, this construct programs β-galactosidase expression in a 6 h embryo with an anterior boundary in parasegment 6. The iab-2−11.0 construct contains DNA from the large *abd-A* intron (coordinates +36.5 to +47.5) and it programs β-galactosidase expression with a sharp anterior boundary in parasegment 7 (Figure 5D). Similarly, the iab-3−11.5 construct contains *iab-3* DNA upstream of the *abd-A* promoter (coordinates +56 to +67.5) and it directs β-galactosidase with an anterior limit in parasegment 8 (Fig. 5E).

Figure 5B depicts an abx6.8 transformant embryo for the purpose of comparison with the other three transformant patterns. The striking similarity among all four constructs is that they program anterior limits that are appropriate, given the genetically defined roles of the four regulatory regions. The abx6.8 and iab-2−11.0 cell-specific patterns are also remarkably similar at this stage. Both patterns are pair-rule modulated with expression predominating in the odd-numbered parasegments. The iab-3−11.5 construct shows weaker pair-rule modulation whereas the bxd14.5 construct is not pair-rule modulated at all. The more uniform bxd14.5 expression pattern is reminiscent of the abx20 pattern. The bxd14.5 pattern is also distinct in that it is stronger in the posterior portions of each parasegment, whereas the other three patterns are stronger in the *engrailed*-expressing anterior portions of the parasegments. The differences in pattern may be imposed by cell-specific regulatory elements also present on these fragments.

The four constructs in Figure 5 are all expressed in gastrulae prior to the detection of *Ubx* or *abd-A* protein (not shown). In addition, the anterior expression boundaries for all four constructs are unchanged in mutants lacking *Ubx* and *abd-A*, confirming that none of these parasegmental control elements are simply activated by endogenous BX-C products.

Discussion

Expression domains with parasegment-specific anterior limits

The bithorax complex controls pattern formation along the A−P axis by selectively expressing the homeotic products, *Ubx, abd-A* and *Abd-B,* along that axis. This selective expression does not involve one homeotic product per parasegment. Rather, once a homeotic product is activated in a particular parasegment, its expression is repeated in the more posterior parasegments (Beachy *et al.*, 1985; White and Wilcox, 1985a; Celniker *et al.*, 1989; Karch *et al.*, 1990; reviewed in Peifer *et al.*, 1987). For example, although *Ubx* is primarily required for the proper development of para-

segments 5 and 6, *Ubx* protein is also expressed in parasegments 7–13 (Beachy *et al.*, 1985; White and Wilcox, 1985a and see Figure 3A). Single BX-C regulatory regions are sufficient to program such expression in an array of parasegments with an anterior limit. For example, embryos that express *Ubx* primarily under the control of the *abx* region can be generated by cutting away all DNA to the right of the *Ubx* promoter region at the translocation *Hm* break (see Figure 1A). Such embryos still express *Ubx* in parasegments 5–12 (Bienz *et al.*, 1988; W.Bender, unpublished). Similarly, embryos containing a translocation break just upstream of the *abd-A* promoter, leaving *abd-A* under the control of the *iab-2* region, still express *abd-A* in parasegments 7–13 (*iab-3*^{277} in Karch *et al.*, 1990). Thus, a fundamental task of the BX-C regulatory regions is to specify a broad expression domain with an anterior limit in a particular parasegment.

Here, we describe regulatory elements from the *abx, bxd, iab-2* and *iab-3* regions that specify appropriate anterior boundaries in parasegments 5, 6, 7 and 8, respectively. Like the BX-C regions in their wild-type contexts, these elements program expression in a broad array of parasegments posterior to the boundary.

Setting the anterior limit in early embryos

All of our constructs that program parasegmentally restricted expression are activated in early embryos. Depending upon the particular construct, expression is first detected between 2 and 4 h of embryogenesis, either in the cellular blastoderm or during early gastrulation. This appearance of β-galactosidase precedes the appearance of *Ubx* and *abd-A* proteins (during germ band extension at ~4 h) but follows the time of appearance of *Ubx* RNA (Akam and Martinez-Arias, 1985). The appearance of *Ubx–lacZ* protein prior to endogenous *Ubx* in the transformants could be due to the much smaller *Ubx-lacZ* transcription unit versus that for *Ubx*, or to post-transcriptional controls upon *Ubx* that fail to operate upon the constructs. In any case, these times of appearance suggest that the parasegmental control elements on these constructs respond to regulatory machinery involved in the initiation of BX-C expression.

As expected from the time of appearance, the early β-galactosidase anterior boundaries are unchanged in abx6.8, bxd14.5, iab-2–11.0 or iab-3–11.5 transformant embryos that lack *Ubx* and *abd-A*. In contrast, an element located just upstream of the *Ubx* promoter that programs *Ubx–lacZ* expression in a single parasegment of the visceral mesoderm is completely dependent upon endogenous *Ubx* protein (Bienz and Tremml, 1988; Bienz *et al.*, 1988). Thus, the visceral mesoderm element reflects an autoregulatory function whereas the parasegmental control elements described here likely reflect early-acting initiation functions.

Several *trans*-acting factors that specify anterior–posterior position in early embryos could play a role in determining the anterior expression boundaries in the transformants. The gap gene *hunchback* (*hb*) is expressed in an anterior domain in blastoderm embryos with a posterior limit at ~50% egg length (Tautz, 1988). This posterior limit is specified as a concentration dependent response to the *bicoid* gene product (Struhl *et al.*, 1989; Driever and Nusslein-Volhard, 1989), which is expressed in an A–P gradient, highest at the anterior end (Driever and Nusslein-Volhard, 1988). Gradient molecules expressed with the opposite gradient polarity, high at the posterior end, have also been described (MacDonald and Struhl, 1986; Mlodzik and Gehring, 1987).

Since gap gene products are expressed in discrete anterior–posterior domains in early embryos, they could also specify the anterior expression boundaries in our transformants. Repression by the gap gene product, *Kruppel*, defines the anterior boundary of the sixth stripe of expression of the pair-rule gene, *hairy* (Pankratz *et al.*, 1990). In a similar way, repression by *hunchback* could define the parasegment 5 anterior limit in the *abx* transformants, as well as for wild-type *Ubx*. The observation that *Ubx* expression spreads anteriorly in *hb* mutants is consistent with this possible role (White and Lehmann, 1986; Irish *et al.*, 1989).

Expression from the abx6.8 and iab-2–11.0 constructs is modulated in pair-rule fashion, predominating in the odd-numbered parasegments. Indeed, the earliest expression in *abx* transformants is in blastoderm embryos in a pattern of discrete stripes (Figure 7A). A double-staining experiment using *engrailed* antibody shows that the edges of these stripes match the parasegmental boundaries cell for cell (Figures 4C and 5D). This modulation of the pattern immediately suggests involvement of at least one *trans*-acting factor with a pair-rule distribution. Indeed, the pair-rule gene products *fushi tarazu* (*ftz*), which is required for the correct activation of *Ubx* in parasegment 6, and *even-skipped*, have been considered as possible direct regulators of *Ubx* (Duncan, 1986; Ingham and Martinez-Arias, 1986; Martinez-Arias and White, 1988). In addition, although *Ubx* protein is not expressed with pair-rule modulation, *Ubx* RNA is expressed in a pair-rule fashion (in PS 6, 8, 10 and 12) during gastrulation (Akam and Martinez-Arias, 1985), indicating that at least transient pair-rule regulation of *Ubx* does occur.

We have attempted to assess the roles of particular gap and pair-rule products in regulating our constructs by examining the changes in *lacZ* expression in *hunchback, Kruppel* and *ftz* mutant embryos. For example, expression from any of the abx6.8, abx20, iab-2–11.0 and iab-3–11.5 constructs in a *hb* mutant background appears to spread anteriorly to a position closer to the cephalic furrow. It is unclear whether this is due to loss of direct *hb* repression of the constructs or an indirect result of changes in segmentation that *hb* causes through its effects upon many other genes. Complex cross-regulatory interactions among gap and pair-rule products occur in the precellular blastoderm (Jackle *et al.*, 1986, reviewed by Carroll, 1990). Since the transformant patterns are first detected later, in embryos at late cellular blastoderm or at gastrulation, there is sufficient time for indirect effects. We hope to assess direct interactions by identifying DNA binding sites for gap or pair-rule products *in vitro*, mutating them and then testing them for regulation *in vivo*. Since our *abx* constructs program the parasegment 5 anterior limit regardless of the promoter used, the potential regulatory sites are limited to the *abx* DNA. Localization of the parasegmental control element to a 1.7 kb *abx* fragment presents a manageable target for such binding studies.

Maintenance of the anterior expression boundary

Once the anterior limit in *Ubx* expression is set during early embryogenesis, it is preserved throughout the remainder of development (White and Wilcox, 1985a; Brower, 1987). Thus, segmentation gene products cannot be the only regulators of this limit since their early expression patterns

decay after 3–4 h of embryogenesis (Driever and Nusslein-Volhard, 1988; Tautz, 1988). There is genetic evidence for another set of regulators, genes like *Polycomb* (*Pc*) and *extra sex combs* (*esc*) (Struhl, 1981; Duncan and Lewis, 1982; Duncan, 1982; Jurgens, 1985), that maintain the early limits of homeotic expression. For example, in *esc* mutant embryos, *Ubx* RNA first appears in the proper parasegments, but by 6 h of embryogenesis, it spreads indiscriminately to all parasegments (Struhl and Akam, 1985).

Our constructs maintain their anterior boundaries of expression in embryos to varying degrees. Expression in abx1.7 transformants begins to spread anterior of parasegment 5 at 6 h (Figure 6E), expression in abx6.8 transformants spreads forward at 9 h (Figure 4) and the *bxd, iab-2* and *iab-3* transformants show restricted expression through 12 h (not shown). We are currently testing whether these differences reflect regulation by genes like *Pc* and *esc*.

Requirement for large and complex regulatory regions

The homeotic genes of the BX-C are exceptional in their requirement for distant and extensive regulatory regions. The magnitude of these large regulatory regions does not merely reflect large stretches of spacer DNA since at least four distinct intervals in the *bxd* region are required to specify different aspects of the pattern (Bender *et al.*, 1985; W.Bender, unpublished; K.Irvine, S.Helfand and D.Hogness, personal communication). Our analysis of a portion of the *abx/bx* region is consistent with multiple, separable *cis*-acting elements that confer tissue and cell specificity. As summarized in Table I, different DNA regions appear to be involved in epidermal, mesodermal, nervous system and imaginal disc expression. Expression from our largest construct, abx20, shows the greatest overall resemblance to wild-type *Ubx* expression (compare Figures 3A and D). But these identified regions are not sufficient for proper regulation since there are incorrect features in the tissue and cell specificity of expression. Thus, *Ubx* regulation within parasegment 5 likely requires a greater expanse of DNA than we have examined here. Additional regulatory elements are possible in the DNA between −63 and −55, which is the location of several *bx* mutant lesions (Figure 1A) that cause parasegment 5-specific phenotypes. It is also possible that DNA elsewhere within the *Ubx* transcription unit contains regulatory information that has not been revealed by the available mutations. Similarly, the extents of the bxd14.5, iab-2−11.0 and iab-3−11.5 constructs are not sufficient for correct cell-specific patterns.

In an analysis of another homeotic gene, *Antennapedia* (*Antp*), 10 kb of DNA upstream of the P2 promoter was sufficient for many aspects of normal *Antp* P2 expression (Boulet and Scott, 1988). However, this 10 kb *Antp* construct, like our abx20 construct, fails to provide precise cell-specific control. Another similarity to our *abx* constructs is that, at 12 h of development, there is incorrect expression along the anterior–posterior axis. (Unfortunately, the *Antp–lacZ* pattern is not detectable early enough to compare it with our *abx* pattern at times when the sharp anterior boundary is seen.) Taken together, these analyses indicate that 10–20 kb portions of homeotic gene regulatory regions can confer aspects of proper expression, but more DNA is required for wild-type patterns.

Common elements in the BX-C for reading the anterior–posterior axis

Given the intricate patterns of homeotic gene expression, it is not surprising to find multiple tissue and cell-type regulatory elements. The novel result is the discovery of a distinct class of regulatory element that programs restricted expression along the A-P axis, even when removed from its context within complex regulatory regions. We show that the *abx, bxd, iab-2* and *iab-3* regulatory regions each contain at least one such parasegmental control element. Recently, we have located a similar DNA element in the *iab-4* region that programs an anterior boundary in parasegment 9 (M.O'Connor, unpublished results.)

Like their *Drosophila* counterparts, the mammalian homeotic gene complexes contain a set of genes arranged along the chromosome in the same order as their expression domains along the A–P axis (Duboule and Dolle, 1989; Graham *et al.*, 1989). Recently, enhancer elements from the mouse *Hox* loci have been described that, like the elements reported here, restrict expression along the A–P axis in early embryos (Tuggle *et al.*, 1990). It will be interesting to compare the mechanisms by which these elements control restricted expression, especially since mammals determine their segments in a cellular embryo whereas segment determination in flies occurs in a multinucleate syncytium.

It is not yet clear how many parasegmental control elements reside in each BX-C regulatory region. We are currently testing successive pieces of DNA across 200 kb of the BX-C, from the 3′ end of *Ubx* through the *iab-4* regulatory region. Although we have found only one such element in the *abx/bx* region so far, we suspect there is at least one other since epidermal *Ubx* expression in parasegment 5 is normal in abx^2 mutant embryos (White and Wilcox, 1985). The *bxd* region clearly contains multiple parasegmental control elements since at least two other *bxd* fragments program a parasegment 6 anterior boundary (M.O'Connor, J.Simon and W.Bender, unpublished) in addition to the one described here (Figure 5C). Comparison of the properties and DNA sequences of these parasegmental control elements should provide further understanding of BX-C regulation along the A–P axis.

Wild-type homeotic gene expression, which displays cell specific as well as parasegmental patterning, likely requires interactions between the two types of elements. For example, *Ubx* expression may require input from one set of regulatory elements that controls the three-dimensional array of expressing cells and a second set of parasegmental control elements that assigns this pattern to the appropriate parasegments. In developmental terms, the cell specific controls would program formation of a part of the fly anatomy and the parasegmental controls would determine where the part appears.

Materials and methods

Drosophila strains and crosses

A homozygous ry^{502} stock was used as the recipient for all transformation experiments. The balancer stock T(2:3) Ap^{Xa}/CyO; TM2 (ry^-) was used to assess the chromosomal linkage of inserts. The same balancer stock was used to make second and third chromosome inserts homozygous. X-linked inserts were made homozygous by inbreeding. Lethal inserts were maintained in stock using the FM6, CyO or TM2 balancer chromosomes.

abx6.8 transformants deficient for the entire BX-C were generated using

Df(3R)P115 and *Df(3R)P9*, large deletions that remove the entire BX-C. The bxd14.5, iab-2−11.0 and iab-3−11.5 transformants were examined in a background lacking *Ubx* and *abd-A* using *Df(3R)P2*. In all cases, mutant embryos were identified independently by lack of simultaneous staining with antibody to Ubx or abd-A protein.

Ubx−lacZ constructions

A *Ubx−lacZ* fusion gene was generated by joining the 2.9 kb *Pst*I fragment containing the 5′ end of the *Ubx* gene to the *lacZ* gene contained in the vector pSKS107 (Casadaban *et al.*, 1983). The 2.9 kb of *Ubx* DNA includes 1.7 kb of 5′-flanking DNA and 1.2 kb of the first exon. The in-frame protein fusion occurs between the 56th amino acid of *Ubx* and the 8th amino acid of *lacZ*. A 6 kb *Bgl*II-*Eco*RI fragment containing this *Ubx−lacZ* gene and the poly(A) addition signal from the *Drosophila hsp70* gene was inserted between the *Bam*HI and *Eco*RI sites of the P element vector Carnegie 3 (Rubin and Spradling, 1983) to generate the plasmid pMP1204. A 7 kb *Hin*dIII fragment containing the *rosy* gene was inserted downstream of the *Ubx−lacZ* gene in pMP1204 to generate the transformation vector pMBO140. Transcription of *Ubx−lacZ* and *rosy* are in the same direction as shown in Figure 1C. pMBO140 contains a unique *Sal*I site in the polylinker upstream of *Ubx−lacZ*. pMBO141 is identical to pMBO140 except the *rosy* gene was inserted on an 8 kb *Sal*I fragment and it contains a total of three *Sal*I sites (see Figure 1 C). Plasmid pMBO142 contains the same basic fusion gene as pMBO140 except the fusion was at the 8th codon of *Ubx* at a *Stu*I site. The remainder of pMBO142 consists of the P element 'building' vector, pMBO132, which allows addition of DNA upstream of 5′ *Ubx* by recombination (O'Connor *et al.*, 1989). pMBO142 contains *rosy* on the 8 kb *Sal*I fragment inserted upstream of *Ubx−lacZ* as shown in Figure 1C. abx20 and abx9.0 are derivatives of pMBO142. All other *abx/Ubx−lacZ* constructs are derivatives of pMBO140 or pMBO141. abx6.8HS was constructed by inserting the 6.8 kb *Sal*I *abx* fragment upstream of an *hsp70−lacZ* fusion gene (Lis *et al.*, 1983) in a P element vector provided by R.Padgett. The bxd14.5, iab-2−11.0 and iab-3−11.5 constructs were made by inserting the fragments depicted in Figure 5A into the *Sal*I site of pMBO140. Additional details of constructions are available upon request.

P element mediated transformation

Germline transformation was performed essentially as described (Rubin and Spradling, 1982). Host embryos were injected with DNA solutions containing 50 μg/ml each of the two helper plasmids pπ25.7 wc (Karess and Rubin, 1984) and pUChsπΔ2-3 (Mullins *et al.*, 1989) and 600 μg/ml of the various *Ubx−lacZ* constructs. Transformant flies were identified by *rosy*$^+$ eye color.

Immunohistochemical and enzymatic staining

Embryos were dechorionated, fixed and devitellinized as described (Mitchison and Sedat, 1983; G.Struhl, personal communication). The fixed embryos were stained as described (Karch *et al.*, 1990) using either a mouse monoclonal against β-galactosidase (Promega) or a monoclonal against Ubx (FP3.38, White and Wilcox, 1984, gift from G.Struhl).

Embryos were double-stained with antibodies against β-galactosidase and *engrailed* as follows. The primary antibodies were a mouse monoclonal against *engrailed* (Patel *et al.*, 1989, gifts from D.Smouse and S.Bray) and a rabbit polyclonal against β-galactosidase (Cappel) and were incubated simultaneously with the embryos. Secondary antibodies were goat anti-mouse coupled to horseradish peroxidase and goat anti-rabbit coupled to alkaline phosphatase (Biorad). The peroxidase reaction was performed first, as above, followed by the alkaline phosphatase reaction using the substrate kit III (blue) from Vector Laboratories.

β-galactosidase activity in larvae was detected with Xgal as described (Glaser *et al.*, 1986). Color development was allowed to proceed at 25°C until significant blue product accumulated, which usually occurred within 1 h for imaginal disc staining.

Embryo dissection and mounting

Embryos were dissected with sharp tungsten needles using a Zeiss dissecting microscope at 40× magnification. The embryos were dissected and mounted in Immumount (Shandon), an aqueous mounting medium. Germ band extended (6 h) embryos were dissected by detaching the most posterior portion of the embryo from its attachment to the dorsal part of the head, and then flipping the germ band out to display the entire ectodermal surface in two dimensions. Germ band retracted and later stage embryos were dissected by making an incision along the dorsal midline and teasing out the gut tube and associated visceral mesoderm. The remaining tissues, consisting of the midventral CNS, more lateral epidermis and underlying mesodermal derivatives were then flattened in two dimensions. Embryos were flattened under a cover slip oriented with the ectodermal surface up. Permanent slides were obtained after hardening of the Immumount.

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References

Akam,M.E. and Martinez-Arias,A. (1985) *EMBO J.*, **4**, 1689−1700.
Beachy,P.A., Helfand, S.L. and Hogness,D.S. (1985) *Nature*, **313**, 545−551.
Bellen,H.J., O'Kane,C.J., Wilson,C., Grossniklaus,U., Pearson,R.K. and Gehring,W.J. (1989) *Genes Dev.*, **3**, 1288−1300.
Bender,W., Akam,M., Karch,F., Beachy,P.A., Peifer,M., Spierer,P., Lewis,E.B. and Hogness,D.S. (1983) *Science*, **221**, 23−29.
Bender,W., Weiffenbach,B., Karch,F. and Peifer,M. (1985) *Cold Spring Harbor Symp. Quant. Biol.*, **50**, 173−180.
Bienz,M. and Tremml,G. (1988) *Nature*, **333**, 576−578.
Bienz,M., Saari,G., Tremml,G., Muller,J., Zust,B. and Lawrence,P.A. (1988) *Cell*, **53**, 567−576.
Boulet,A.M. and Scott,M.P. (1988) *Genes Dev.*, **2**, 1600−1614.
Brower,D. (1987) *Development*, **101**, 83−92.
Cabrera,C.V., Botas,J. and Garcia-Bellido,A. (1985) *Nature*, **318**, 569−571.
Carroll,S.B. (1990) *Cell*, **60**, 9−16.
Casadaban,M.J., Martinez-Arias,A., Shapira,S.K. and Chou,J. (1983) *Methods Enzymol.*, **100**, 293−308.
Casanova,J., Sanchez-Herrero,E., Busturia,A. and Morata,G. (1987) *EMBO J.*, **6**, 3103−3109.
Celniker,S.E., Keelan,D.J. and Lewis,E.B. (1989) *Genes Dev.*, **3**, 1424−1436.
Driever,W. and Nusslein-Volhard,C. (1988) *Cell*, **54**, 83−93.
Driever,W. and Nusslein-Volhard,C. (1989) *Nature*, **337**, 138−143.
Duboule,D. and Dolle,P. (1989) *EMBO J.*, **8**, 1497−1505.
Duncan,I. (1982) *Genetics*, **102**, 49−70.
Duncan,I. (1986) *Cell*, **47**, 297−309.
Duncan,I. and Lewis,E.B. (1982) In *Developmental Order: Its Origin and Regulation.* Alan R.Liss, Inc., New York, pp. 533−554.
Ghysen,A. and O'Kane,C. (1989) *Development*, **105**, 35−52.
Glaser,R.G., Wolfner,M.F. and Lis,J.T. (1986) *EMBO J.*, **5**, 747−754.
Goto,T., MacDonald,P. and Maniatis,T. (1989) *Cell*, **57**, 413−422.
Graham,A., Papalopulu,N. and Krumlauf,R. (1989) *Cell*, **57**, 367−378.
Harding,K., Hoey,T., Warrior,R. and Levine,M. (1989) *EMBO J.*, **8**, 1205−1212.
Hiromi,Y. and Gehring,W.J. (1987) *Cell*, **50**, 963−974.
Ingham,P. and Martinez-Arias,A. (1986) *Nature*, **324**, 592−597.
Irish,V.F., Martinez-Arias,A. and Akam,M. (1989) *EMBO J.*, **8**, 1527−1537.
Jackle,H., Tautz,D., Schuh,R., Seifert,E. and Lehmann,R. (1986) *Nature*, **324**, 668−670.
Jurgens,G. (1985) *Nature*, **316**, 153−155.
Karch,F., Weiffenbach,B., Peifer,M., Bender,W., Duncan,I., Celniker,S., Crosby,M. and Lewis,E.B. (1985) *Cell*, **43**, 81−96.
Karch,F., Bender,W. and Weiffenbach,B. (1990) *Genes. Dev.*, **4**, 1573−1587.
Karess,R.E. and Rubin,G.M. (1984) *Cell*, **38**, 135−146.
Kornfeld,K., Saint,R.B., Beachy,P.A., Harte,P.J., Peattie,D.A. and Hogness,D.S. (1989) *Genes Dev.*, **3**, 243−258.
Kuziora,M.A. and McGinnis,W. (1988) *Cell*, **55**, 477−485.
Lewis,E.B. (1978) *Nature*, **276**, 565−570.
Lis,J.T., Simon,J.A. and Sutton,C.A. (1983) *Cell*, **35**, 403−410.
MacDonald,P.M. and Struhl,G. (1986) *Nature*, **324**, 537−545.
Martinez-Arias,A. and Lawrence,P.A. (1985) *Nature*, **313**, 639−642.

Martinez-Arias,A. and White,R.A.H. (1988) *Development*, **102**, 325–338.
Mitchison,T.J. and Sedat,J. (1983) *Dev. Biol.*, **99**, 261–264.
Mlodzik,M. and Gehring,W.J. (1987) *Cell*, **48**, 465–478.
Mullins,M.C., Rio,D.C. and Rubin,G.M. (1989) *Genes Dev.*, **3**, 729–738.
O'Connor,M.B., Binari,R., Perkins,L.A. and Bender,W. (1988) *EMBO J.*, **7**, 435–445.
O'Connor,M.B., Peifer,M. and Bender,W. (1989) *Science*, **244**, 1307–1312.
Pankratz,M.J., Seifert,E., Gerwin,N., Billi,B., Nauber,U. and Jackle,H. (1990) *Cell*, **61**, 309–317.
Patel,N.H., Martin-Blanco,E., Coleman,K.G., Poole,S.J., Ellis,M.C., Kornberg,T.B. and Goodman,C.S. (1989) *Cell*, **58**, 955–968.
Peifer,M. and Bender,W. (1986) *EMBO J.*, **5**, 2293–2303.
Peifer,M., Karch,F. and Bender,W. (1987) *Genes Dev.*, **1**, 891–898.
Regulski,M., Harding,K., Kostriken,R., Karch,F., Levine,M. and McGinnis,W. (1985) *Cell*, **43**, 71–80.
Rubin,G.M. and Spradling,A.C. (1982) *Science*, **218**, 348–353.
Rubin,G.M. and Spradling,A.C. (1983) *Nucleic Acids Res.*, **11**, 6341–6351.
Sanchez-Herrero,E., Vernos,I., Marco,R. and Morata,G. (1985) *Nature*, **313**, 108–113.
Struhl,G. (1981) *Nature*, **293**, 36–41.
Struhl,G. and Akam,M.E. (1985) *EMBO J.*, **4**, 3259–3264.
Struhl,G. and White,R.A.H. (1985) *Cell*, **43**, 507–519.
Struhl,G., Struhl,K. and MacDonald,P.M. (1989) *Cell*, **57**, 1259–1273.
Tautz,D. (1988) *Nature*, **332**, 281–284.
Tiong,S., Bone,L.M. and Whittle,R.S. (1985) *Mol. Gen. Genet.*, **200**, 335–342.
Tuggle,C.K., Zakany,J., Cianetti,L., Peschle,C. and Nguyen-Huu,M.C. (1990) *Genes Dev.*, **4**, 180–189.
White,R.A.H. and Akam,M.E. (1985) *Nature*, **318**, 567–569.
White,R.A.H. and Lehmann,R. (1986) *Cell*, **47**, 311–321.
White,R.A.H. and Wilcox,M. (1984) *Cell*, **39**, 163–171.
White,R.A.H. and Wilcox,M. (1985a) *EMBO J.*, **4**, 2035–2043.
White,R.A.H. and Wilcox,M. (1985b) *Nature*, **318**, 563–567.

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