

The location of *Ultrabithorax* transcripts in *Drosophila* tissue sections

Michael E. Akam

Department of Genetics, Downing Street, Cambridge CB2 3EH, UK

Communicated by P. Lawrence

Received on 3 August 1983

I have used *in situ* hybridization to map the distribution of transcripts of the *Ultrabithorax* unit in tissue sections of *Drosophila* embryos and larvae. The results confirm the prediction of Lewis that genes of the bithorax complex will show segmentally regulated patterns of expression. Transcripts of the *Ultrabithorax* unit are detected in derivatives of the ectoderm and in some derivatives of the mesoderm, but not in the endoderm. In each of these tissues the transcripts are found in the derivatives of some segments but not of others. In third instar larvae they are most abundant in the imaginal discs of the third thoracic segment, and in a region of the central nervous system that includes parts of the meta-thoracic and first abdominal neuromeres. They are also detected in the nuclei of polytene cells of the larval epidermis, principally in the third thoracic and first abdominal segments, and in the nuclei of larval muscles in the first six abdominal segments. In late stages of embryogenesis the central nervous system is the most prominently labelled tissue; within it transcripts are found only in the neuromeres of the thoracic and abdominal segments. They are most abundant in a region which includes parts of the neuromeres of the metathorax and the first abdominal segment.

Key words: bithorax complex/*Drosophila*/homoeotic genes/*in situ* hybridization/segmentation

Introduction

Mutations in the bithorax complex (BX-C) of *Drosophila* cause segments of the body to develop structures normally characteristic of other segments. In extreme cases they result in the complete replacement of one segment by a duplicate of some other, resulting in spectacular four-winged or eight-legged flies (Lewis, 1963, 1978).

Many features of these homoeotic mutations suggest that they identify genes whose products directly control the state of determination of cells during normal development. It seems that the state of activity of genes of the bithorax complex in each embryonic cell normally depends on the position of that cell. Subsequently this information must be retained through each cell generation, as continued activity of the bithorax complex genes is required at least until cells cease dividing (Morata and Garcia-Bellido, 1976).

Lewis (1978, 1981) has proposed a specific model for the pattern of expression of genes within the bithorax complex. Since deletion of the entire complex has no effect on the segments of the head or prothorax (first thoracic segment) it is assumed not to function in these segments. In such a deletion embryo, the metathorax (third thoracic segment) and all abdominal segments are abnormal; each develops like a mesothorax. Thus, in these segments, bithorax complex functions are assumed to be active, and necessary to establish correct segmental identities.

Molecular clones derived from the bithorax complex have been obtained (Bender *et al.*, 1983). The organization of genes within the complex is not yet clear, but a functional unit corresponding to the *Ultrabithorax* (*Ubx*) gene has been defined (Figure 1, see also Akam, 1983). This unit gives rise to a number of processed transcripts, several of which share common 5' sequences (R.Saint and D.Hogness, unpublished results).

In this paper I describe the use of sequences isolated from the 5' *Ubx* exon to map the pattern of distribution of homologous transcripts in embryos and larvae of *Drosophila* using an *in situ* hybridization technique. This technique allows us to test directly whether the pattern of accumulation of *Ubx* transcripts is segment specific, and to what extent it matches the pattern of *Ubx* function predicted by the Lewis model. I find that transcripts of the *Ubx* gene are detectable *in situ* in derivatives of the ectoderm – both epidermal and neural – and in derivatives of the mesoderm (the larval musculature), but not in tissues derived from the endoderm (midgut). In those tissues where transcripts can be identified, they are found in the derivatives of some, but not all segments. In third instar larvae, they are undetectable in the head and first thoracic segment; there is a complex pattern of labelling in the more posterior segments.

Results

For these experiments I synthesized single-stranded tritium-labelled probes on M13 templates, as outlined in Figure 2. These probes were hybridized to cryostat sections of *Drosophila* embryos or larvae following a procedure based on that of Brahic and Haase (1978). Control experiments considered in more detail in the Materials and methods section (see also Figure 3) confirm that the specific pattern of labelling results from the hybridization of probe sequences to RNA present within the tissue sections.

The DNA fragment from the bithorax complex which I used as a probe is homologous to a number of different processed transcripts. In embryos, the most abundant of these are transcripts with exons which span a 75-kb region of the genome (R.Saint, unpublished results), defined as the *Ubx* functional unit by the distribution of *Ubx* mutations (Figure 1, Bender *et al.*, 1983; Akam, 1983). The precise structure of these RNAs has not yet been established, but the major polyadenylated species contain the sequence of virtually the entire 1.4-kb *HindIII-EcoRI* fragment present in the M13 clones used to prepare single-stranded probes.

These RNA species amount to between 10^{-4} and 10^{-3} % of the total RNA present in eggs during the later stages of embryogenesis. They account for a much smaller proportion of total RNA in larvae, but are relatively enriched in preparations of RNA from isolated imaginal discs (R.Saint, personal communication).

Transcripts have only been detected from one strand of the *Ubx* unit. Single-stranded probe which is homologous to these transcripts is referred to below as the *Ubx* 5' probe. Probe prepared from the complementary strand, which is not

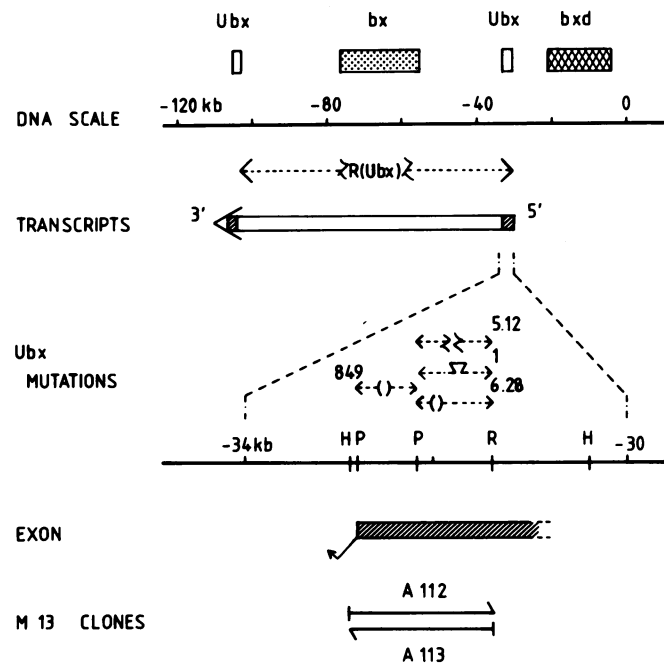


Fig. 1. The *Ultrabithorax* unit. The upper part of the diagram locates the *Ultrabithorax* unit on the molecular map of the proximal bithorax complex (Bender *et al.*, 1983; Akam, 1983). Boxes above the DNA scale show the distribution of DNA lesions in *Ultrabithorax* (*Ubx*), *bithorax* (*bx*) and *bithoraxoid* (*bxd*) pseudopoint mutations. Chromosome rearrangements, $R(Ubx)$, throughout the region indicated below the DNA scale result in extreme *Ultrabithorax* mutations. High mol. wt. transcripts are homologous to this same region. Processed transcripts have homology to exons at the 5' and 3' ends (R.Saint, unpublished results). The region of the 5' *Ubx* exon is enlarged below. The four *Ubx* mutations shown have been located as insertions, deletions or rearrangement breakpoints within the indicated restriction fragments (H = *Hind*III, P = *Pst*I, R = *Eco*RI). *Ubx*^{5.12} is the rightmost of the located chromosome breakpoints associated with a *Ubx* mutation. The approximate locations of exons present in the major *Ubx* transcripts are indicated by cross-hatched boxes. 5' and 3' exons of these transcripts coincide with the locations of *Ubx* pseudopoint mutations, and span the extent of chromosome rearrangements with *Ubx* phenotypes. The 5' exon of the *Ubx* transcripts extends through the *Eco*RI site at -31.5 kb and is terminated by a splice donor site just 5' to the *Hind*III site at -33 kb (R.Saint and M.Goldschmidt-Clermont, unpublished). The promoter used for these transcripts has not been located. Subclone A113 in M13mp8 was used to prepare the *Ubx* 5' probe; clone A112 in M13mp9 to prepare the control probe.

expected to hybridize to *Ubx* transcripts, is referred to as the control probe.

The third instar larvae

The principal structures which are labelled in wild-type (Canton-S) third instar larvae by the *Ubx* 5' probe are the imaginal discs of the third thoracic segment, the haltere and third leg discs. The wing disc, the humeral disc, the first leg disc, the discs of the head and the genital disc all show similar levels of background labelling which are not distinguishable from the non-specific binding of probe to tissue. The second leg disc reproducibly labels at a level 2- to 5-fold above this background level, but very much less strongly than the discs of the third thoracic segment (Figures 3, 4, 5).

Parts of the central nervous system are strongly labelled. The dorsal brain hemispheres, the lateral connectives and the sub-oesophageal ganglion show no labelling; cell bodies within a slice of the ventral ganglion in the region of the first abdominal neuromere are most strongly labelled, and there is also significant labelling of cells both anterior and posterior

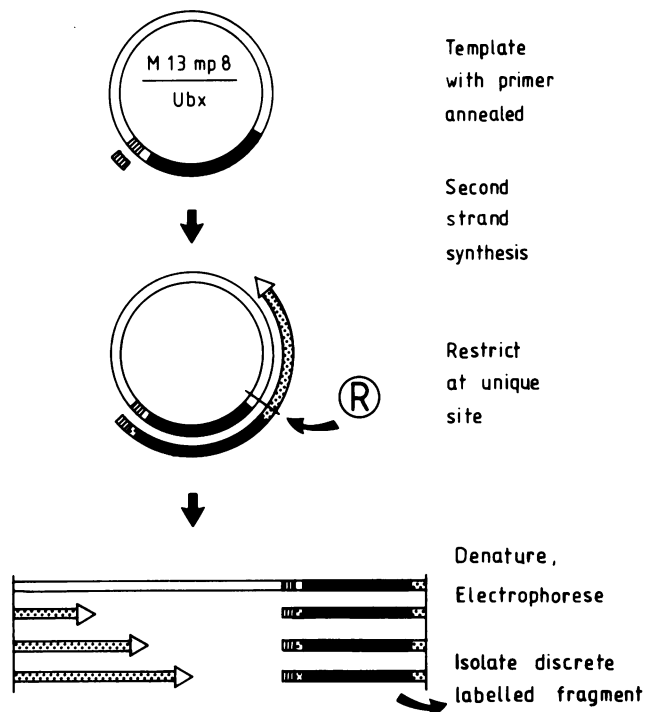


Fig. 2. Isolation of single-stranded hybridization probes from M13 templates. ³H-labelled second strands are synthesized on M13 templates using standard sequencing primers and triphosphate concentrations adjusted such that 30–50% conversion of single to double strand is obtained. The reaction product is restricted with an enzyme which cuts once only, immediately distal to the cloning site. This linearizes the template strand and cleaves the newly synthesized strand to yield a discrete labelled fragment extending from the priming site, through the cloned *Drosophila* insert to the restriction site, and a heterogeneous population of run-on transcripts. The discrete labelled fragment is isolated from the reaction mixture by denaturation and electrophoresis, and then reduced to a mean strand length of 100–200 nucleotides by DNase digestion in the presence of carrier DNA.

to this slice, in the third thoracic and more posterior abdominal neuromeres (Figure 4, and see below).

At first glance no other tissues appear to be significantly labelled. Indeed, most of the polytene tissues of the larva, the midgut, the salivary glands and the malpighian tubules, generally show a lower level of background binding than the diploid epithelia. However, on closer inspection, some polyploid derivatives of the ectoderm and mesoderm are found to be labelled. This is most clearly seen in the musculature of the larva (Figure 6). The larval muscles are syncytial, and carry on their surface large (16 μ m x 8 μ m) polyploid nuclei. Some of these nuclei label very strongly. The label is focussed over a single region of the nucleus <4 μ m in diameter, a region which by ³H autoradiography is not distinguishable from a point source.

Not all muscle nuclei are labelled. There appear to be two reasons for this. First, the point source which is labelled is apparently either present, or accessible to autoradiography, only in certain sections. In cases where the nucleus itself is sectioned, the fragment in one section will frequently label strongly, that in the adjacent section weakly or not at all. Second, and more significantly, muscles in some segments never, or almost never, label (Figure 8). Only the muscles of the first six abdominal segments are frequently labelled. The precise distribution of this labelling is considered in more detail below.

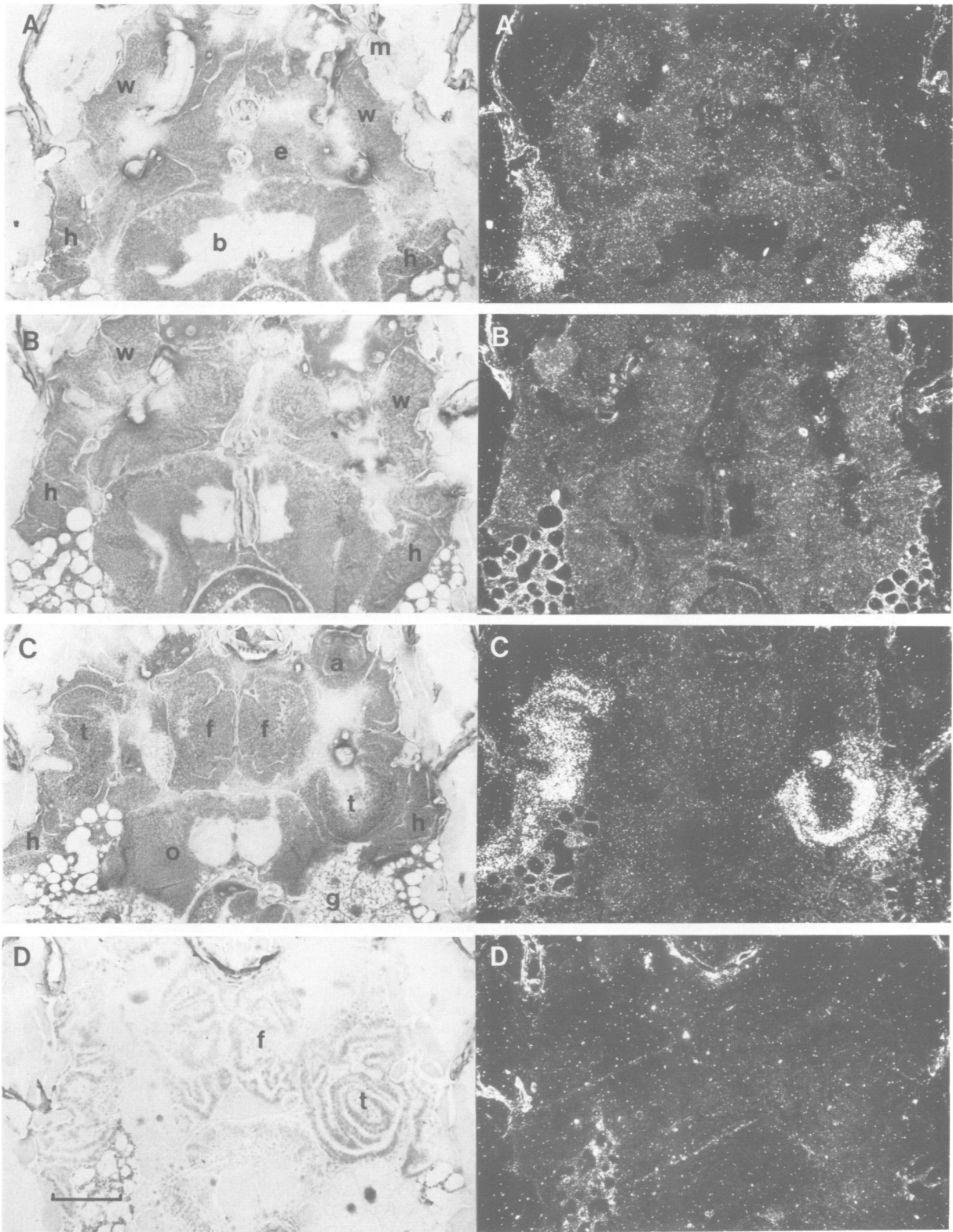


Fig. 3. Hybridization of single-stranded *Ubx* probes to third instar larval sections. **Panel A** and **C**: hybridized with *Ubx* 5' probe. **Panel B**: hybridized with control probe (complementary to message-homologous strand). **Panel D**: hybridized as **panels A** and **C**; section RNase pretreated before hybridization. **Sections A–D** are taken through the anterior region of a single Canton-S third instar larva. The series **A–D** runs dorsal to ventral. Bright and dark field photos of each section are shown. **Section A**: haltere discs (**h**) are labelled with silver grains. Unlabelled structures visible in this section include the dorsal brain hemispheres (**b**), wing discs (**w**), eye-antennal disc (**e**) and muscles (**m**). **Section C**: the principal labelled structures are the third leg discs (**t**), located laterally. The section also grazes the ventral edge of the haltere discs. Note the paired medial first leg discs (**f**) which are unlabelled, as are cells of the suboesophageal ganglion (**o**), and polytene cells of the anterior spiracles (**a**) and the salivary glands (**g**). (**A,C,D**: A113 probe, 0.13 $\mu\text{g}/\text{ml}$, 6×10^4 d.p.m./ μl , 4.9×10^8 d.p.m./ μg . **B**: A112 probe, 0.12 $\mu\text{g}/\text{ml}$, 5×10^4 d.p.m./ μl , 4.4×10^8 d.p.m./ μg . Autoradiographic exposure = 15 days. Scale = 100 μm .)

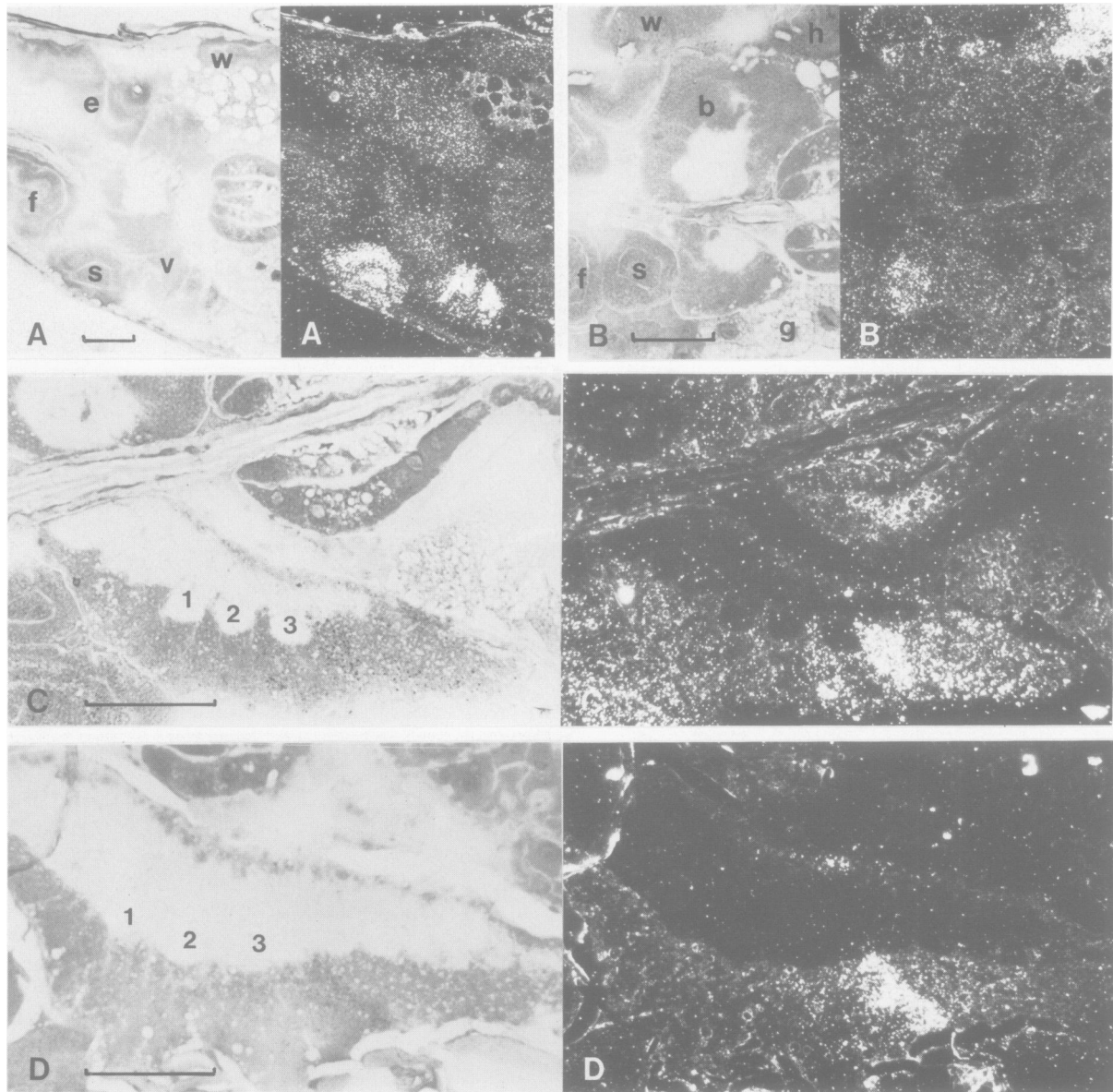


Fig. 4. The ventral ganglion and second leg disc. Vertical or near vertical sections, all probed with *Ubx 5'* probe. **Panel A:** note labelling of a region of the ventral ganglion (v), and more weakly of the second leg disc (s) anterior to it. Background levels of labelling are observed on the first leg disc (f), eye antennal (e) and wing (w) discs. **Panel B:** section includes first leg disc (unlabelled), second leg disc just posterior to it (weakly labelled), unlabelled wing disc at top, and heavily labelled haltere disc (h). **Panels C, D:** Sections through the ventral ganglion. The neuropil of the thoracic nerves are numbered 1–3. The strongly labelled column of cells lie posterior to the metathoracic neuropil; the more weakly labelled cells lie between the meso- and meta-thoracic neuropil and in the more posterior abdominal segments. In **panel C** the weakly labelled second leg disc is visible anterior and ventral to the ganglion. (**A–C:** A113 probe as Figure 3. Exposure 25 days. **D:** A113 probe, 0.2 $\mu\text{g}/\text{ml}$, 5×10^4 d.p.m./ μl , 2.5×10^8 d.p.m./ μg , hybridized 40 h; exposed 23 days. Scale = 100 μm .)

Polytene cells of the epidermis (including the oenocytes) show similar labelling of a point source within their nuclei (Figure 6); again the labelling is confined only to certain segments (see below). The smaller polyploid cells surrounding certain tracheae are also clearly labelled (Figure 6), although in this case it is difficult to distinguish the precise location of the label within the cell.

Some nuclei within the larval fat body show focussed clusters of grains, but the proportion which are labelled is small (< 10% of nuclei). It is unclear whether these nuclei are the nuclei of fat body cells, or nuclei associated with the ramifying tracheae which permeate the fat body.

Polytene cells in part of the hindgut are weakly labelled with the *Ubx 5'* probe. In this case the labelling is distributed

over both nucleus and cytoplasm. It is confined to a region of the hindgut from the point of attachment of the malpighian tubes to the anterior limit of the rectum.

No other specifically labelled tissues have been observed. The male and female gonads have been identified in larval sections and are not detectably labelled. Abdominal histoblasts have not been identified in sections.

The embryonic nervous system

I consider here only the pattern of labelling of the central nervous system of 12–20 h embryos. These embryos show a complex pattern of labelling with the *Ubx 5'* probe, but the nervous system labels more prominently than any other structure.

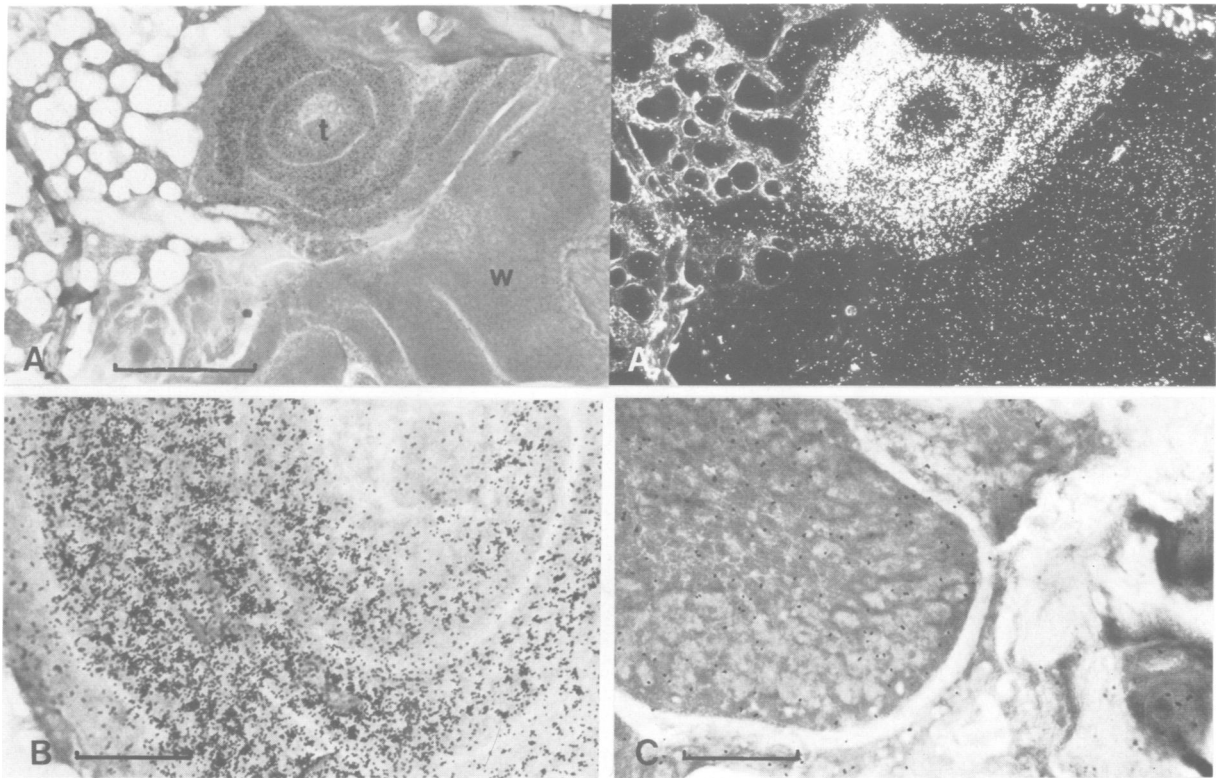


Fig. 5. Third leg, wing and genital discs. **Panel A:** bright and dark-field micrographs of the third leg (t) and wing (w) discs. **Panel B:** enlargement of the same leg disc. The intensity of labelling is modulated by the pattern of folds in the disc, but on average is more intense in the posterior compartment of the disc. **Panel C:** genital disc showing only background labelling. (A–C: A113 probe as Figure 3. Exposure 25 days. Scales: A = 100 μm ; B, C = 20 μm .)

The pattern of labelling is similar but not identical to that of the nervous system in the third instar larva. As in larvae, the dorsal hemispheres and sub-oesophageal ganglion are unlabelled. However, in embryos a large region of the ventral ganglion is strongly labelled, including the neuromeres of eight thoracic and abdominal segments (Figure 7, and see below).

Segment specificity of labelling

Larval muscle and epidermis. Individual polyploid nuclei can clearly be identified in these tissues. I scored series of sections through each of four individuals to establish the distribution of labelled muscle and epidermal nuclei (see the legend to Figure 8 for details).

The results for the muscle nuclei are clear and consistent. Muscles of the pharynx and of the first two thoracic segments are unlabelled. In the third thoracic segment, 4% of muscle nuclei in section are labelled. The proportion rises dramatically to 50–60% in the first four abdominal segments, a proportion which probably corresponds to the presence of a labelled focus in virtually every nucleus. From the fifth to the eighth abdominal segments the proportion of labelled nuclei falls, most markedly between abdominal segments six and seven.

The few muscle nuclei labelled in the third thoracic segment are located close to the posterior boundary of the segment, on oblique muscles. Some of these muscles do not insert on the cuticle at the segment boundary (Szabad *et al.*, 1979); hence their segmental origin is unclear.

Epidermal nuclei show a different and variable pattern of labelling (Figure 8). In each of the four individuals examined in detail, many nuclei of the first abdominal segment were

labelled. In two of these individuals, the epidermal nuclei in other segments were not frequently labelled. In two other individuals labelled nuclei were observed in the posterior part of the second thoracic segment, in the third thoracic segment, and occasionally in abdominal segments 2–6. Inspection of sections from additional individuals confirms this variability, and indicates that the larvae which show label only in the first abdominal segment are the most mature of the wandering larvae – those which have already extruded glue into the lumen of the salivary gland (an event which occurs 3 h before pupariation). This suggests that the abundance of *Ubx* transcripts in the epidermal cells of the larva is declining rapidly during the final stages of the third larval instar.

The tracheal system. Polytene cells surrounding the anterior spiracles are unlabelled by the *Ubx* 5' probe. The slightly more posterior cells surrounding the tracheal trunks in the region of the brain and the wing disc are labelled. The pattern of labelling of the more posterior tracheal cells has not been examined in detail.

The nervous system. During the development of the nervous system the segmental ganglia of the thorax and abdomen first form as separate primordia, clearly separated by non-neural cells. These primordia are juxtaposed, first by germ band shortening, and then by the further contraction of the ventral nervous system to form the single ventral ganglion of the larva (Poulson, 1950). The segmental repeat within this ventral ganglion can be distinguished clearly by the repeating pattern of paired commissures within each neuromere, but the boundaries of individual neuromeres are difficult to identify.

In the nervous system of 12–20 h embryos a region of the ventral ganglion equal in width to one segmental unit is more

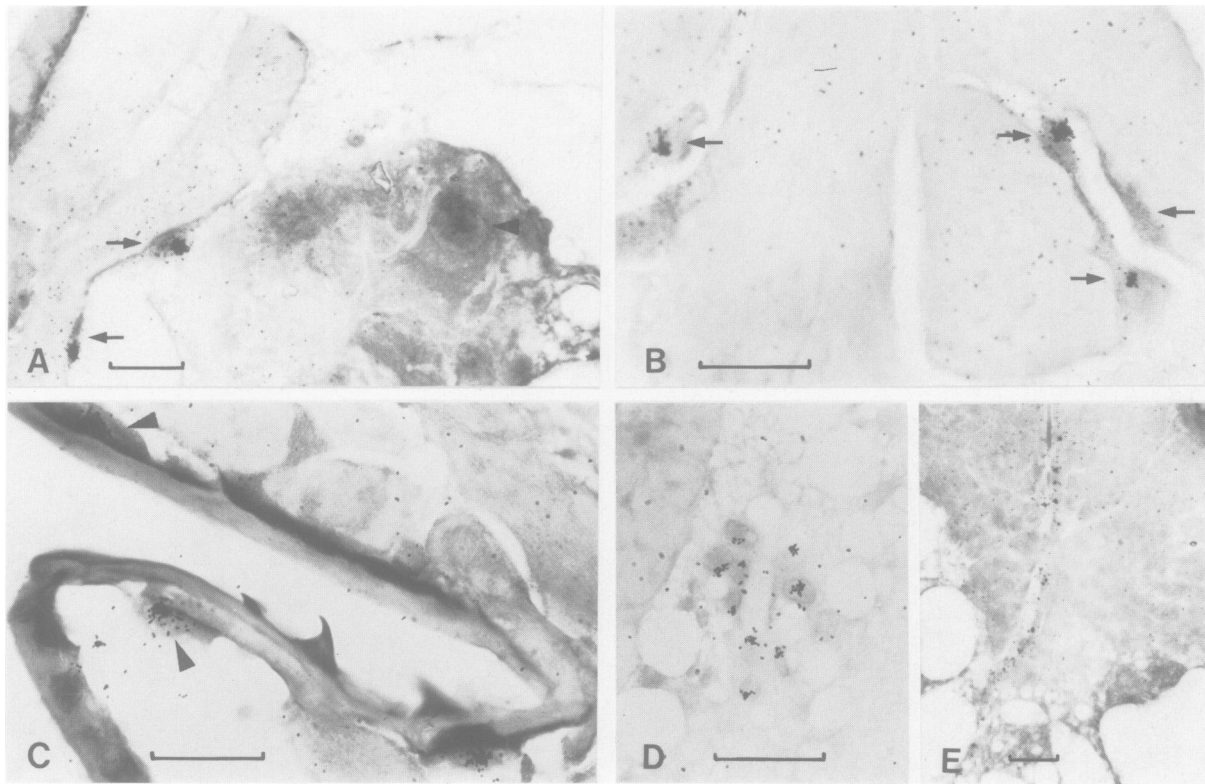


Fig. 6. Nuclear labelling of polytene cells. **Panels A and B:** muscle nuclei (arrows). The polytene nucleus of the midgut cell in **A** (arrowhead) is unlabelled. Not all muscle nuclei in a given section (e.g., **B**) are labelled. In addition to the focussed label over one spot in the muscle nuclei there is a low level of labelling over the rest of the nucleus and over cytoplasmic regions of the muscle fibre. **Panel C:** polyplod epidermal cells underlying cuticle at the boundary of the third thoracic and first abdominal segments. The cytoplasm of the epidermal cells is reduced to a thin layer, with nuclei showing as protuberances from the cuticle surface. The labelled nucleus above is in the third thoracic segment, the two below in the first abdominal. **Panels D, E:** oblique cross-section of tracheal branch in fat body (**D**) and section in plane of a tracheal branch traversing a lymph node (**E**) showing label in the tracheal cells. (**A:** A113 probe as Figure 4D; **B–E:** A113 probe as Figure 3. Exposure 25 days. Scale = 20 μm .)

heavily labelled than its surroundings (Figure 7). This region includes most of the first abdominal neuromere, and probably extends anteriorly into the posterior part of the third thoracic neuromere. A region anterior to this, which labels less heavily, includes most of the third thoracic neuromere and may extend anteriorly into the posterior part of the second thoracic. In abdominal segments 2–7 one part of each neuromere, probably the anterior, is labelled more strongly than the remainder, creating the striped distribution of label which is seen in sections.

One or two sections of a series through each ventral ganglion generally show a small cluster of silver grains located ventrally in the posterior part of the first thoracic neuromere (visible on Figure 7B), suggesting that a few cells at this location may be labelled.

The ventral ganglion of the larva is even more condensed than that of the embryo, but the thoracic neuromeres are marked by prominent bulges in the neuropil associated with each thoracic segmental nerve. The maximally labelled cells lie posterior to the third thoracic segment nerve, in the presumptive first abdominal neuromere. The more weakly labelled cells anterior to this lie between the second and third thoracic nerves, in the posterior second thoracic and/or the anterior third thoracic neuromere. It is not possible to distinguish how many of the more posterior abdominal neuromeres are labelled in larvae (Figure 4).

Discussion

These results strikingly confirm Lewis's prediction that expression of genes of the bithorax complex will be segment specific. I detect *Ubx* transcripts in the epidermis, in the nervous system and in the larval musculature. In each case the derivatives of some segments are labelled; others are not. For any one of these tissues there are few biochemical or ultrastructural differences between the derivatives of the different segments. Thus, the observed pattern of labelling at once provides a powerful internal control for the specificity of *in situ* hybridization, and supports the contention that the products of the bithorax complex are concerned, not with the nuts and bolts of cell differentiation, but with the regulation of segment identity.

Lewis proposed (1978) that the bithorax complex contains at least one gene corresponding to each segment from the metathorax back to the eighth abdominal segment. In the metathorax only the first of these genes, the *Ubx* gene would be active. In each more posterior segment, function of one more gene of the complex would be recruited. The resulting array of bithorax complex products would direct that segment to follow its characteristic path of development. This specific model predicts that the *Ubx* gene will be active in the metathorax, and in each more posterior segment.

My results do not completely support this simple model. They reveal additional complexity; different tissues show dif-

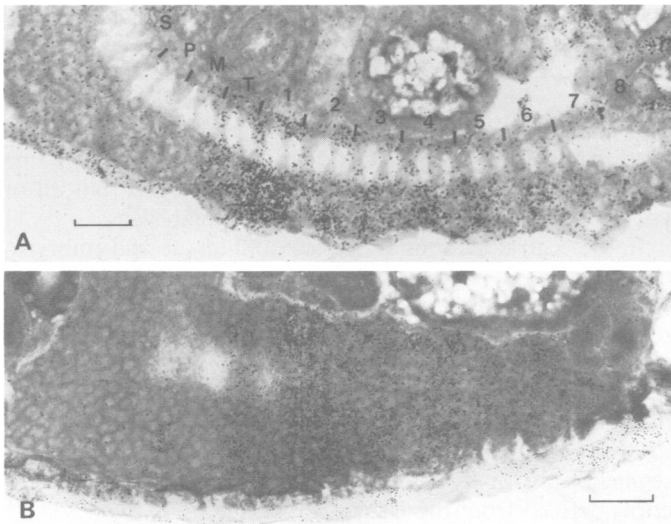


Fig. 7. The embryonic nervous system. Both sections probed with *Ubx* 5' probe. **Panel A:** medial longitudinal section of 14 h embryo. Germ band shortening is complete but the ventral nervous system has not fully condensed. The 'ladder' formed by the commissures in the neuropil is clearly visible. Each neuromere in the thoracic and abdominal region is associated with two lateral commissures separated by cell bodies. The approximate positions of each neuromere are indicated, but the precise boundaries of each segmental derivative cannot be identified. S = sub-oesophageal neuromere. P, M, T, = pro- (first), meso- (second) and meta- (third) thoracic neuromeres. 1–8 = abdominal neuromeres. **Panel B:** lateral sagittal section through the embryonic nervous system after condensation. The section passes through one dorsal brain hemisphere (not shown), the lateral connective and the ventral ganglion. Labelling is limited to the ventral ganglion, and the labelling of 'stripes' within this ganglion is clearly visible. There is a small cluster of silver grains located ventrally at a position one segment anterior to the first labelled 'stripe' (see text). The most posterior segments of the ventral ganglion are not in the plain of this section. (**Panel A:** embryo prefixed in paraformaldehyde before sectioning (see Hafen *et al.*, 1983). Section pretreatment, probe and hybridization conditions as for Figure 3. Exposure 24 days. **Panel B:** A113 probe, 0.2 $\mu\text{g}/\text{ml}$, 5×10^4 d.p.m./ μl . 2.5×10^8 d.p.m./ μg . (Exposure 13 days).

ferent segment-specific patterns of hybridization, and within a single tissue, differential segmental derivatives show different levels of expression.

Tissue specificity of transcription

Lewis's model does not specify which cells require function of the bithorax complex. Epidermal cells must require the autonomous function of the *Ubx* gene. Somatic recombination may be used to make small clones of epidermal cells homozygous for *Ubx* mutations in individuals which are otherwise heterozygous. These clones generally express the same segment transformations as similarly positioned cells in mutant homozygotes (Morata and Garcia-Bellido, 1976; Keridge and Morata, 1982). *Ubx* mutations also cause segment transformations in the nervous system (Jimenez and Campos-Ortega, 1981; Teugels and Ghysen, 1983), but it is not certain that these effects are cell autonomous.

I find that *Ubx* transcripts are clearly detectable in derivatives of the ectoderm (epidermis, nervous system and tracheal cells), and of the mesoderm (larval muscles), but not in the endoderm (midgut), suggesting that all segmentally organized structures express, and may require, bithorax complex functions.

This conclusion relies on a comparison of the amount of RNA detected *in situ* in different tissues. It is less reliable than the observation of a differential distribution for RNA within

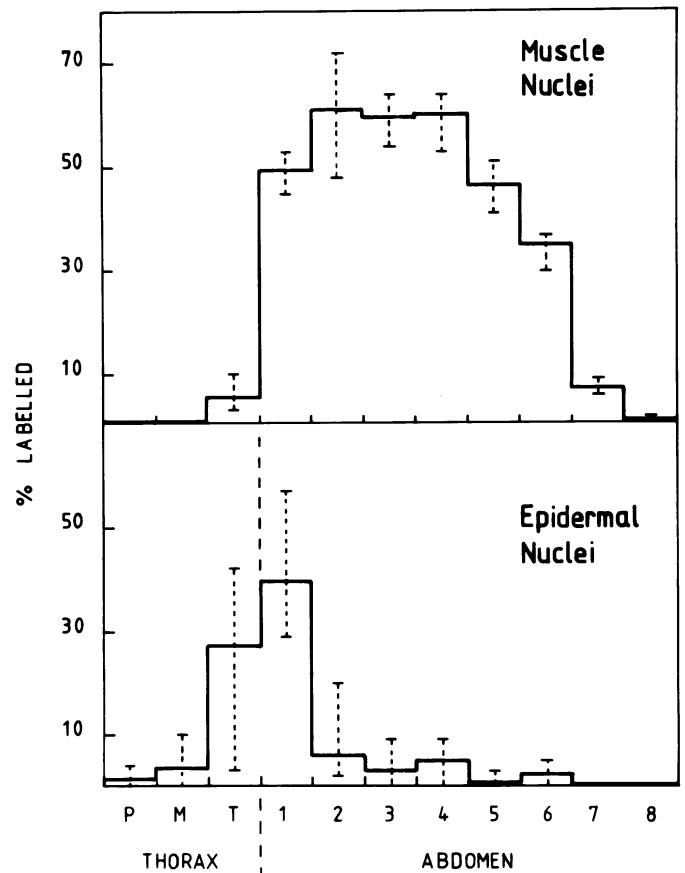


Fig. 8. Segment specificity of nuclear labelling. Longitudinal sections of Canton-S late third instar larvae were hybridized with the *Ubx* 5' probe A113 under the conditions described in Figure 3. Polytene nuclei of the muscles and of the larval epidermis were assigned to segments, taking the segment boundary to be at the major line of muscle attachments at the anterior edge of the ventral denticle belts (Szabad *et al.*, 1979). P, M, T, = first, second and third thoracic segments. 1–8 = abdominal segments. Muscle nuclei located at the segment boundary on muscles which spanned it were attributed to the more anterior segment. Muscles of the head and pharyngeal apparatus are included under the prothorax. Epidermal nuclei: maximally labelled nuclei carried >50 grains; all nuclei with >10 grains were counted as labelled. The average number of grains on unlabelled nuclei in the first thoracic segment was <2. 1710 nuclei scored. (Average 155/segment, range = 80–310.) Muscle nuclei: maximally labelled nuclei carried >100 grains; all nuclei with >15 grains were counted as labelled. The average number of grains on unlabelled nuclei of the pharynx and first thoracic segment was <3. 3280 muscle nuclei scored (average 212/segment, range = 175–404). Between 14 and 20 sections were scored from each of four individuals. Error bars indicate the range of values obtained for the different individuals. Sections from the same individuals were hybridized in parallel either after RNase treatment or with control probe (see Figure 3). In both cases, <1% of muscle or epidermal nuclei were counted as labelled when scored by the same criteria. Ambiguous nuclei: nuclei sandwiched between muscle and cuticle, were scored as a separate class of ambiguous nuclei (15% of all nuclei). Labelling of this class was consistent with that expected for a mixture of the muscle and epidermal nuclei.

an otherwise homogeneous tissue. Detection *in situ* requires that RNA be fixed in section, but remain accessible to probe and capable of hybridizing with it. It is clear that the fixation and pretreatment of sections markedly affects the efficiency of hybridization (Brahic and Haase, 1978; Angerer and Angerer, 1981) and it is likely that the effects of these treatments on a given species of RNA depend on the architecture of the cells, and possibly also on the intracellular location of the RNA.

Nonetheless, I and others have found that cytoplasmic

RNAs in polytene gut and salivary gland cells can readily be detected *in situ* using protocols very similar to that employed here (Hafen *et al.*, 1983; Akam and Carlson, unpublished results). The complete absence of all labelling of midgut, salivary gland and malpighian tubule cells with the *Ubx* probe strongly suggests that *Ubx* transcripts are much less abundant in these tissues than in those ectodermal and mesodermal derivatives which are labelled.

In so far as it has been tested, the tissue distribution which we observe *in situ* is confirmed by the analysis of isolated RNA immobilized on filters. The transcripts are more abundant in preparations of RNA isolated from imaginal discs than in RNA isolated from whole larvae, and can be detected in RNA isolated from haltere discs, but not in RNA similarly prepared from wing discs (R.Saint and M.Goldschmidt-Clermont, personal communication).

Cytological location of transcripts

In polyploid tissues, labelling with the *Ubx* probe is focussed over a point source clearly smaller than the nucleus. In epidermal nuclei this does not appear to be the nucleolus; in muscle nuclei of these preparations nucleoli are rarely visible. Control experiments indicate that the hybridization is strand specific and ribonuclease sensitive. Therefore it is unlikely to result from hybridization to DNA. The simplest explanation for this observation is that a fraction of the RNA which I identify is in the form of nascent transcripts at a site where the bithorax genes of the polyploid (though not obviously polytene) nuclei are clustered. The *Ubx* probe is derived from the 5' end of what appears to be a 70-kb transcription unit. Presumably transcripts must remain close to the gene for at least the time taken to complete transcription (> 40 min).

The nuclear labelling of polytene cells seen in section does not imply that the processed RNA products of the *Ubx* unit are located principally in the nucleus. In both muscle and epidermal cells, cytoplasm is thinly spread, over the muscle fibres or the cuticle, respectively. Muscles whose nuclei are very strongly labelled do show a scatter of grains on the surrounding fibre, particularly when the section 'grazes' its surface. The relative grain densities observed would certainly be consistent with the distribution expected if 50% of hybridization were to a nuclear source of 25 μm^2 , and 50% to a thin layer of cytoplasm scattered over 5000 μm^2 of muscle fibre surface.

In heavily labelled sections of imaginal discs and of the ventral ganglion, grains are clustered, though in this case the size of the foci is comparable with the size of the diploid nuclei, and with the path length of tritium in the emulsion. Again there seems to be a concentration of label in the nucleus, but our results do not rule out the presence of a substantial fraction of the homologous RNA in the cytoplasm.

The spatial pattern of hybridization

I observe a relatively complex pattern of hybridization with the *Ubx* probe. Homologous transcripts are most abundant in the imaginal epidermal cells of the third thoracic segment (haltere and third leg discs). They are not found in the discs of the head or first thoracic segment, are barely detectable in the leg disc of the second thoracic segment, and are not detected in the wing disc of the same segment. I was unable to detect the imaginal histoblasts of abdominal segments 1–8, but the genital disc, a derivative of the most posterior segments, is unlabelled. Transcripts are found in some cells of the larval

epidermis, principally in the third thoracic and first abdominal segments, but the observed differences between individuals suggest that the abundance of *Ubx* transcripts in these cells is changing during the late third instar: these larval cells are destined to die within a few hours.

In contrast to the epidermis, the larval muscles of the first six abdominal segments are consistently labelled, but all or virtually all of the thoracic muscles are unlabelled.

In the central nervous system of both larvae and embryos, transcripts are found only in thoracic and abdominal segments, and are several fold more abundant in a discrete region including most of the first abdominal neuromere and, probably, part of the third thoracic neuromere.

At present I do not know whether this complexity reflects the distribution of the same RNA species in all of those cells to which the *Ubx* probe hybridizes, or whether different segments and/or tissues carry different populations of transcripts derived from the *Ubx* 5' exon.

There is a notable correlation between those epidermal cells which contain detectable *Ubx* transcripts, and those which are phenotypically transformed in *Ubx* mutants. Zygotes homozygous for *Ubx* mutations complete embryogenesis but die as larvae. In these larvae cuticular and tracheal structures of the third thoracic and first abdominal segments are transformed to resemble the homologous second thoracic structures. The more posterior abdominal segments are almost completely unaffected. The effects of *Ubx* mutations on structures of the adult can only be assessed in somatic recombination clones (Kerridge and Morata, 1982) or in mosaics (Miñana and Garcia-Bellido, 1983). When *Ubx* mutations are made homozygous in cells of embryos > 8 h old, only clones in the third thoracic and first abdominal segment show any phenotypic effect. *Ubx* transcripts are most abundant in the epidermal cells of these same segments.

In pre-blastoderm mosaics, or in clones which are induced in embryos younger than 8 h, an additional transformation is observed. In the posterior compartment of the second and third legs, cells homozygous for *Ubx* mutations develop structures characteristic of the first leg, implying a role for the *Ubx* unit in the development of the second thoracic segment. *In situ*, a low level of *Ubx* transcripts is detected in cells of the second leg disc as late as the third larval instar.

There is evidence for some function of the *Ubx* unit in the second abdominal and more posterior segments. Although these segments of the *Ubx* homozygous larva are virtually normal, they do develop ventral pits, which are present only on the thoracic segments of wild type larvae (Lewis, 1981).

The activity of the *Ubx* unit in the abdominal segments is revealed much more clearly in embryos which lack all other functions of the complex. If embryos which lack all bithorax complex functions are compared with the embryos which carry only the *Ubx* unit (carried on the translocated fragment *Dp(3:3)bx^{d100}*), *Ubx* functions are seen to affect the morphology of the tracheal system in all segments from the third thoracic to the seventh abdominal (Lewis, 1981). In a similar test Duncan (1982) observed an effect of *Ubx* functions on epidermal structures in the posterior compartments of all segments from the second thoracic to the sixth abdominal.

It is not clear how these observations relate to the finding of *Ubx* transcripts in the more posterior segments of the embryonic and larval nervous system, and most surprisingly in the muscles of the abdominal segments. One possibility is that *Ubx* functions are expressed in these more posterior segments

of the wild-type, but that they are largely redundant in the presence of products from the *bx*d (*bithoraxoid*) and *iab* (*infra-abdominal*) genes of the complex.

The distribution of transcripts observed in the muscles matches better the pattern expected for products of the *bx*d gene than that for products of the *Ubx* gene; the *bx*d mutations partially transform the first abdominal segment into a thoracic segment. In the Lewis model (1978), products of the *bx*d gene are expected to be absent in the third thoracic segment but present in the first abdominal and more posterior segments. However, the relationship between mutations which affect the proposed *Ubx* function and those which affect the *bx*d function is complex (Lewis, 1981; Bender *et al.*, 1983) and is not simply explained either by Lewis's model or by what is yet known of the molecular architecture of the complex (Akam, 1983). It is therefore possible that some functions attributed to the *bx*d gene may be mediated by transcripts encoded in the *Ubx* unit. The pattern of expression which I observe *in situ* may reflect this.

Materials and methods

Ubx clones

M13 subclones containing the *Ubx* 5' exon in either orientation were constructed by isolating the 1.44-kb *Hind*III-*Eco*RI fragment from the genomic clone pϕDm 3108, and ligating this into vectors mp8 and mp9. This fragment originally derives from the insert of DNA from the *Drosophila melanogaster* strain Canton-S present in clone λCdm 2228 isolated from the Maniatis-Lauer library by Bender *et al.* (1983). The integrity and orientation of the two inserts were confirmed by sequencing the end of each from the same priming site as that used for probe preparation, and by annealing the two complementary templates to yield a 1.44-kb double-stranded fragment which could be excised by *Hind*III-*Eco*RI digestion.

Preparation of probes

Single-stranded probes were prepared using the technique outlined in Figure 2. For a standard reaction, 1 µg of M13 template and 8 ng of pentadecamer primer (BRL or New England Biolabs), were denatured at 90°C for 1 min and then annealed at 60°C for 60 min in 10 µl of 100 mM NaCl, 40 mM Tris-HCl pH 8.3, 40 mM MgCl₂. After annealing, this was diluted to a reaction volume of 40 µl containing 6 units of Klenow polymerase (BRL) in a final reaction buffer containing 10 mM Tris-HCl pH 8.3, 10 mM MgCl₂, 22 mM NaCl, 1 mM dithiothreitol (DTT), 10 µM each labelled dNTP and 50 µM each cold dNTP. Using all four triphosphates tritiated at 10 µM (dTTP at ~100 Ci/mmol; dCTP, dATP at ~60 Ci/mmol, dGTP at ~30 Ci/mmol, New England Nuclear) requires 100 µCi/40 µl reaction.

Second strand synthesis was carried out for 2 h at 25°C, and terminated by heating to 70°C for 5 min. The reaction was made to the appropriate salt concentration; restricted with *Eco*RI (clones in mp8) or *Hind*III (clones in mp9); phenol extracted and the DNA ethanol precipitated. The ethanol pellet was resuspended in 20 µl of 95% formamide/2.5 mM EDTA, pH 8.0, denatured at 90°C for 1 min, and electrophoresed on a neutral low melting temperature agarose gel. Gel bands containing labelled fragments were cut out, melted and digested with a titrated amount of DNase I in the presence of salmon sperm DNA to reduce the mean single strand length of the labelled DNA to 100–200 bases. (500 µl final reaction volume containing 50–100 µl of gel, 50 µg carrier DNA and 10 µg/ml DNase I in 10 mM Tris pH 8.3, 10 mM MgCl₂, incubated 10 min at 37°C.) The reaction was terminated by phenol extraction (twice), ether extraction and ethanol precipitation. Precipitated DNA (90–95% of total counts) was resuspended either in final hybridization buffer or in 10 mM Tris, 1 mM EDTA, pH 8.0, and stored at –20°C.

The calculated specific activity of DNA synthesized using four tritiated triphosphates with the above specific activities is ~4 × 10⁸ d.p.m./µg.

Preparation of sections

Embryos of the wild-type strain Canton-S were collected from cage populations and reared at 25°C. The developmental stage of embryos was estimated after sectioning by reference to Fullilove and Jacobson (1978). Wandering third instar larvae were collected from Canton-S populations raised on yeast-glucose medium.

Live embryos or larvae were mounted directly in OCT compound (Tissue tek II) and frozen by partially immersing the microtome chuck in liquid nitrogen. Frozen sections (~8 µm) were cut at –18°C and picked up on sub-

bed slides (Gall and Pardue, 1971) at room temperature. After drying, sections were fixed in ethanol:acetic acid (3:1, 0°C for 10 min), rinsed in 2 × SSPE to remove the OCT matrix (0°C for 5 min, 2 × SSPE = 360 mM NaCl, 10 mM Na₂HPO₄, 10 mM NaH₂PO₄, 2 mM EDTA, pH 7.0), dehydrated in 70% and 95% alcohol and stored in dry boxes at –20°C for up to 2 months before use.

Immediately before hybridizing, sections were pretreated by incubation in 2 × SSPE at 70°C for 30 min, then in 2 µg/ml Proteinase K (BDH), 10 mM Tris-HCl pH 7.4, 2 mM CaCl₂ at 37°C for 15 min, and dehydrated again.

Hybridization and autoradiography

The final hybridization solution contained tritiated DNA resuspended to a final concentration of 0.1–0.2 µg/ml (~5 × 10⁸ d.p.m./µl) in a solution containing 50% formamide, 600 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.02% Ficoll 400, 0.02% polyvinyl pyrrolidone, 1 mg/ml bovine serum albumin, 1 mg/ml sonicated denatured salmon sperm DNA, 1 mg/ml yeast total RNA and 100 µg/ml poly(A). This was denatured immediately before use (1 min at 90–95°C), chilled on ice, and pipetted directly onto the sections. Slides were incubated for hybridization in humid chambers equilibrated with 50% formamide, 600 mM NaCl, at 25°C for 36–44 h. After hybridization preparations were washed extensively in 50% formamide, 600 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.2, at 25°C (typically three changes in 30 min, then three further changes over 18 h); dehydrated in 70% alcohol, 300 mM NH₄ acetate, in 95% alcohol, and then air dried. Autoradiography was carried out with diluted NTB2 emulsion (Kodak) (Gall and Pardue, 1971). Sections were stained in Giemsa, air dried and mounted in permount.

Sensitivity and specificity of *in situ* hybridization

In preliminary experiments with embryo sections I used probes prepared by nick-translation of a variety of double-stranded DNA fragments, hybridized under conditions which allow the specific detection of abundant RNAs in *Drosophila* tissue sections (Akam and Carlson, in preparation). The *Ubx* probe showed a barely detectable but reproducible pattern of labelling of the ventral nervous system. Other DNA fragments hybridized specifically to other embryonic tissues, but did not label the nervous system.

Single-stranded probes prepared as outlined in Figure 2 proved to be much more efficient hybridization probes. I have made no systematic comparisons of otherwise identical double-stranded and single-stranded probes, but autoradiographic exposures of 5 days using single-stranded probes gave signals comparable with the best 30–50 day exposures obtained with double-stranded probes, and the resulting signal noise ratios were much improved.

With single-stranded probes the complementary sequence provides a control not only for the specificity of binding, but also for the strand specificity of the reaction. Using the pair of M13 vectors mp8 and mp9, which differ only by inverted polylinkers, I have synthesized message-homologous and control probes from exactly the same *Ubx* DNA fragment. M13 sequences contaminating the two probes will be identical in the two cases. The specific activity of the probes derived from the complementary strands is closely similar (e.g., 4.5 × 10⁸ d.p.m./µg for message-homologous, 4.0 × 10⁸ d.p.m./µg for the control strand; calculated from precursor-specific activities and knowing the nucleotide compositions of the two strands), and the similarity in size distribution of the two probes after final DNase digestion has been checked.

Such control strand probes hybridized in parallel with message homologous probes label tissues very weakly, although different tissues do reproducibly label to differing extents (Figure 3). We attribute the majority of this labelling (and similar patterns of background labelling observed with a range of double-stranded DNA probes prepared by nick-translation) to the non-specific binding of labelled DNA to sections. Only a small proportion may result from the hybridization of probe to nuclear DNA. The polytene nuclei of the salivary gland and the midgut contain the highest concentration of DNA in the larval sections; but show no local concentration of label with either message homologous or control probes.

After long autoradiographic exposures it is possible to see that the imaginal discs and nuclei which label most strongly with the *Ubx* message homologous probe do show barely detectable labelling above background with some batches of probe prepared from the complementary template, but not with M13 sequences alone. This may result from trace contamination of the probe preparation with complementary strands generated by hairpin priming during probe synthesis.

As a further control to confirm that the observed labelling is due to hybridization to RNA, sections were pretreated with ribonuclease under conditions identical to those used in the preparation of polytene chromosomes for hybridization to DNA (Gall and Pardue, 1971). In such sections the nuclei remain intact, though much of the Giemsa-staining material in the nuclei and virtually all in the cytoplasm is removed. After this pretreatment the specific labelling of all tissues with the *Ubx* probe is eliminated, including the specific labelling of muscle and epidermal nuclei (Figures 3 and 8).

In addition to the specific patterns of labelling described in the Results section, the surfaces of cuticle lined structures within sections sometimes bind label. This binding is most noticeable to the lining of the pharynx and the tracheae. It is clearly distinguishable from labelling over cells, by its location, its resistance to ribonuclease, and its occurrence with all probes, double- or single-stranded. The intensity of this labelling varies from one probe preparation to another. The causes of this variability have not been determined, but binding is more pronounced with probes of greater single strand length.

Acknowledgements

The conception and early gestation of this project were nurtured in the fertile environment of David Hogness' laboratory at Stanford. Welcome Bender, Robert Saint and Michel Goldschmidt-Clermont freely gave clones and shared unpublished information, for all of which I am most grateful. Julian Burke suggested the technique used to prepare single-stranded probes. Ernst Hafen and Michael Levine were always happy to talk, sometimes about *in situ* hybridization, and provided a gift of embryo sections, including the section illustrated in Figure 7A. José Campos-Ortega and Alain Ghysen helped me to interpret the sections. Helen Moore assisted with many aspects of this work and Peter Lawrence provided critical encouragement throughout. This work was supported by the Medical Research Council of Great Britain.

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