The EMBO Journal vol.4 no.7 pp.1689–1700, 1985

The distribution of *Ultrabithorax* transcripts in *Drosophila* embryos

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Communicated by P.Lawrence

We have used *in situ* hybridization to monitor the distribution of *Ultrabithorax* (*Ubx*) transcripts during the early stages of *Drosophila* embryogenesis. When first detectable, in the late syncytial blastoderm, *Ubx* transcripts are broadly distributed from 20 to 50% egg length (measured from the posterior pole). By the completion of cellular blastoderm formation, a precisely bounded zone one segment wide is defined by the accumulation of high levels of *Ubx* transcripts. This zone is probably the primordium for parasegment 6, that is, the posterior compartment of the third thoracic segment (T3) and the anterior compartment of the first abdominal segment (A1). Following gastrulation, seven metameric units of the germ band express *Ubx* transcripts in most or all cells of both the ectoderm and the mesoderm. This principal domain of *Ubx* expression is sharply bounded at superficial grooves in the embryo which probably mark the A/P compartment boundaries in T3 and A7, and so consists of parasegments 6–12. Outside this region, *Ubx* transcripts can be detected in only a few cells of the ectoderm of parasegments 5 and 13, and in the amnioserosa. This distribution suggests that the major role of *Ubx* in the early embryo lies in the morphological domain generally ascribed to the control of the *bithoraxoid* gene. Later in embryogenesis, the pattern of *Ubx* transcript accumulation is different in each of the major germ layers, suggesting that different controls regulate *Ubx* in each. In the ectoderm, but not in the mesoderm, *Ubx* transcripts accumulate differentially in anterior and posterior compartments. Probes prepared from different regions of the *Ubx* transcription unit show related but different patterns of hybridization. These patterns suggest that the 3.2-kb *Ubx* transcript is distributed throughout the wider domain of *Ubx* expression, but that the 4.7-kb transcript accumulates preferentially in parasegment 6.

Key words: in situ hybridization/bithorax complex/embryology/spatial regulation/homoeotic

Introduction

Ultrabithorax (UBX) is one of the principal functional domains of the bithorax complex (BX-C) in *Drosophila* (Lewis, 1978; Sanchez-Herrero *et al.*, 1985; reviewed in Lawrence and Morata, 1983). It serves as a major determinant of segment identity in a morphological domain of the fly which extends from the antero/posterior (A/P) compartment boundary in the second thoracic segment (T2) to the equivalent boundary in the first abdominal segment (A1) (Lewis, 1981; Morata and Kerridge, 1981; Hayes *et al.*, 1984; Struhl, 1984).

The organization of the Ultrabithorax domain is complex. It contains at least two transcription units but only one of these is

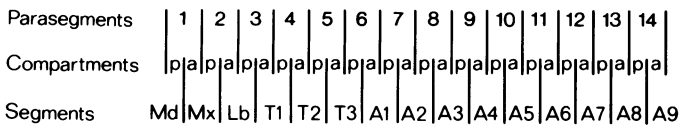


Fig. 1. The relationship between parasegments, compartments and segments.

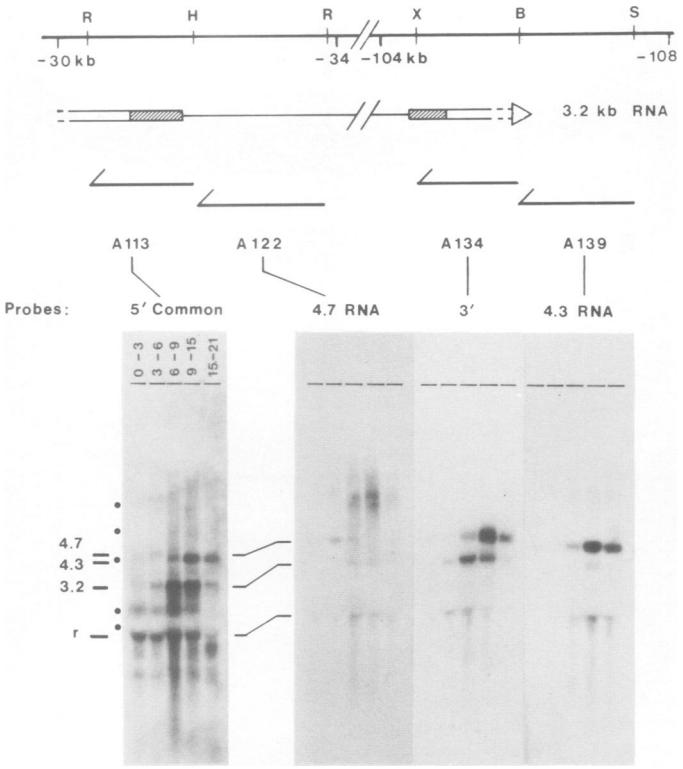


Fig. 2. Transcripts homologous to the *Ubx* probes used for *in situ* hybridization. The structure of the *Ubx* 3.2-kb RNA is shown in relation to genomic sequences at the 5′ and 3′ extremes of the *Ubx* unit; sequences encoding two microexons lie between the regions shown on this diagram (Beachy *et al.*, 1985). Single-stranded probes derived from the indicated restriction fragments were hybridized to total RNA from embryos of the indicated ages (h at 25°C after egg laying). The probes were prepared from subclones in M13 isolated from BX-C genomic clones (Bender *et al.*, 1983), we refer to these fragments as the *Ubx* 5′ common probe (clone A113; Akam, 1983); the 4.7 RNA probe (clone A122), the *Ubx* 3′ probe (clone A134, truncated with *Xho*I) and the 4.3 RNA probe (clone A139). In each case the orientation of the M13 clone ensures that only transcripts from the same strand as the 3.2-kb RNA are detected. The three major *Ubx* transcripts have been assigned as 4.7, 4.3 and 3.2 kb following Beachy *et al.* (1985) and Saint and Hogness (unpublished results), and are consistent with the mobility of denatured DNA standards. Apparent bands located immediately below the rRNA probably result from hybridization to heterogeneous RNA fragments which have been compressed into this region of the gel. Numbers on the DNA map refer to the coordinates of the BX-C as defined by Bender *et al.* (1983). R = *Eco*RI site; H = *Hind*III site; X = *Xho*I site; B = *Bam*HI site; S = *Sal*I site. Dots adjacent to the A113 autoradiogram indicate the location of denatured DNA fragments of 9.46, 6.56, 4.37, 2.32 and 2.03 kb. The filter probed with A113 carries 5 μg total RNA/track. The 6–9 h RNA sample on this filter is partially degraded. The remaining filters carry 10 μg of RNA per track. Autoradiographs for the 4.3 RNA and 4.7 RNA probes derive from consecutive hybridizations to the same filter.

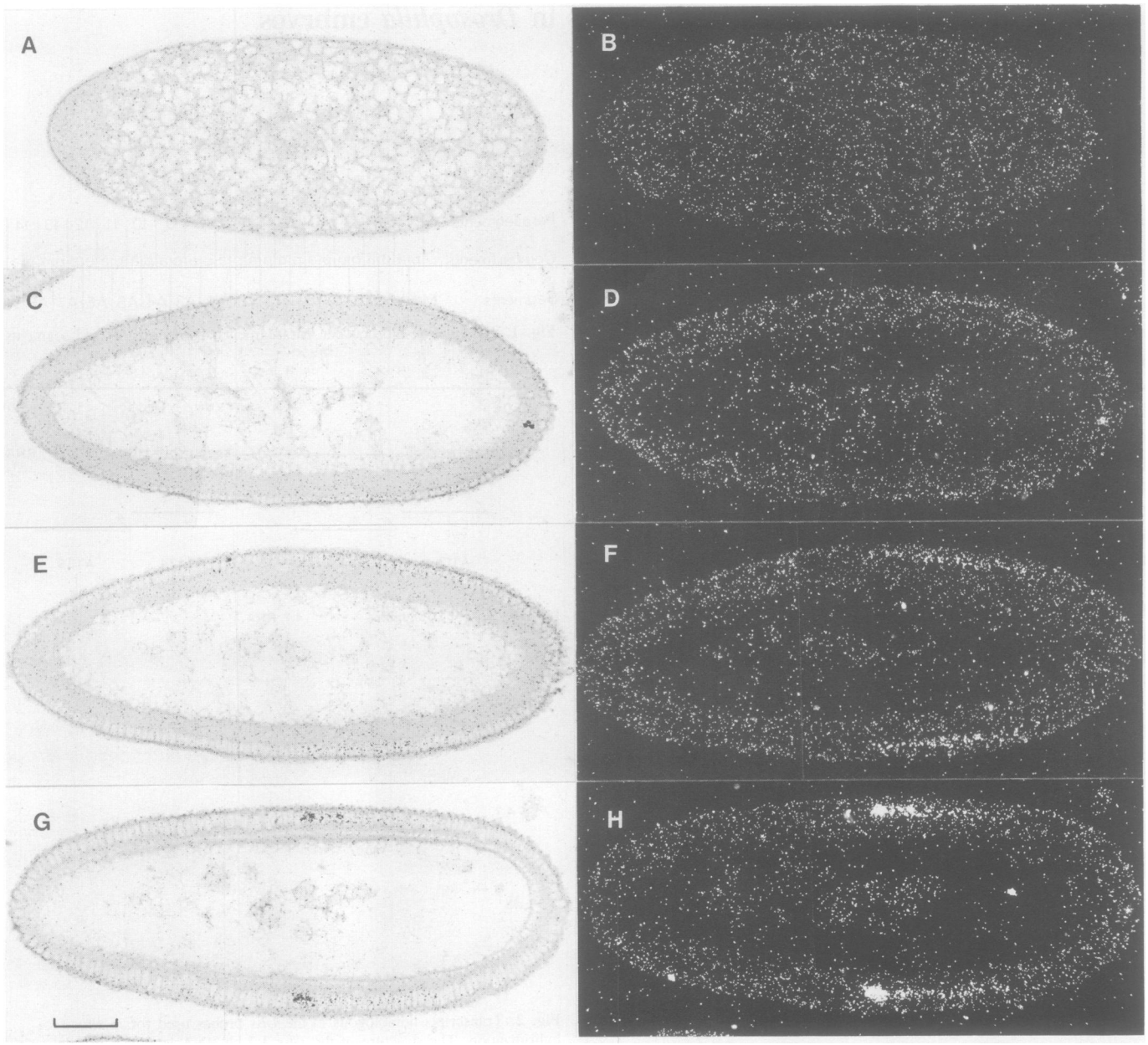


Fig. 3. Hybridization of the *Ubx* 5′ common probe to the developing blastoderm. This series of four sections seen in bright field (**A,C,E,G**) and dark field illumination (**B,D,F,H**) are taken from a single slide probed with the *Ubx* 5′ common probe. **Panels A** and **B**, unfertilized egg. The yolk is not segregated from the cytoplasm; background labelling is uniform throughout the egg. **Panels C** and **D**, late syncytial blastoderm after 13th nuclear cleavage. Yolk (unstained) is now segregated into the centre of the egg. Nuclei form a monolayer around the periphery of the egg, but cell membranes have not yet formed. At this stage, the first traces of localized labelling can barely be detected above background levels (see text). **Panels E** and **F**, early stage of cell formation. The nuclei have begun to elongate as membranes pull down around them. *Ubx* labelling is clearly visible over nuclei in the posterior half of the egg. **Panels G** and **H**, late cellular blastoderm. A prominent zone of labelling has been established in the centre of the egg. Here and in the earlier stages the specific labelling is detected only over the nuclei. The plane of section is near to the horizontal midline in each of these sections. In these and subsequent figures we follow the convention that anterior is to the left and, in vertical sections, dorsal is at the top. (Probe A113 at 0.13 μg/ml. Hybridization with dextran sulphate at 25°C for 40 h. Autoradiographic exposure = 20 days, scale = 50 μm).

known to encode proteins. This, the *Ultrabithorax* (*Ubx*) transcription unit, gives rise to a family of processed RNA products, at least one of which encodes a protein in exons which span 70 kb of the genome (Beachy *et al.*, 1985; Akam *et al.*, 1984; White and Wilcox, 1984). Many mutations which inactivate this protein coding frame abolish all functions of the UBX domain; other mutations, within the *Ubx* transcription unit (*bx, abx*), or within the adjacent 'upstream' transcription unit (*bxd, pbx*), abolish only some functions of the UBX domain (Lewis, 1978; Bender *et al.*, 1983).

We have previously described the distribution of transcripts derived from the *Ubx* unit (Akam, 1983). Here we describe the distribution of *Ubx* transcripts in the embryo, with particular emphasis on the early embryonic stages.

It has been suggested that elements of the bithorax complex are deployed out-of-frame with normal segments (Struhl, 1984; Hayes *et al.*, 1984). We find that the expression of the *Ubx* transcription unit in embryos can best be described with reference to units which extend from the A/P compartment boundary in one segment to the equivalent boundary in the next segment. Martinez-Arias and Lawrence (1985) propose the term parasegment for this unit, and we shall use this term here. The registra-

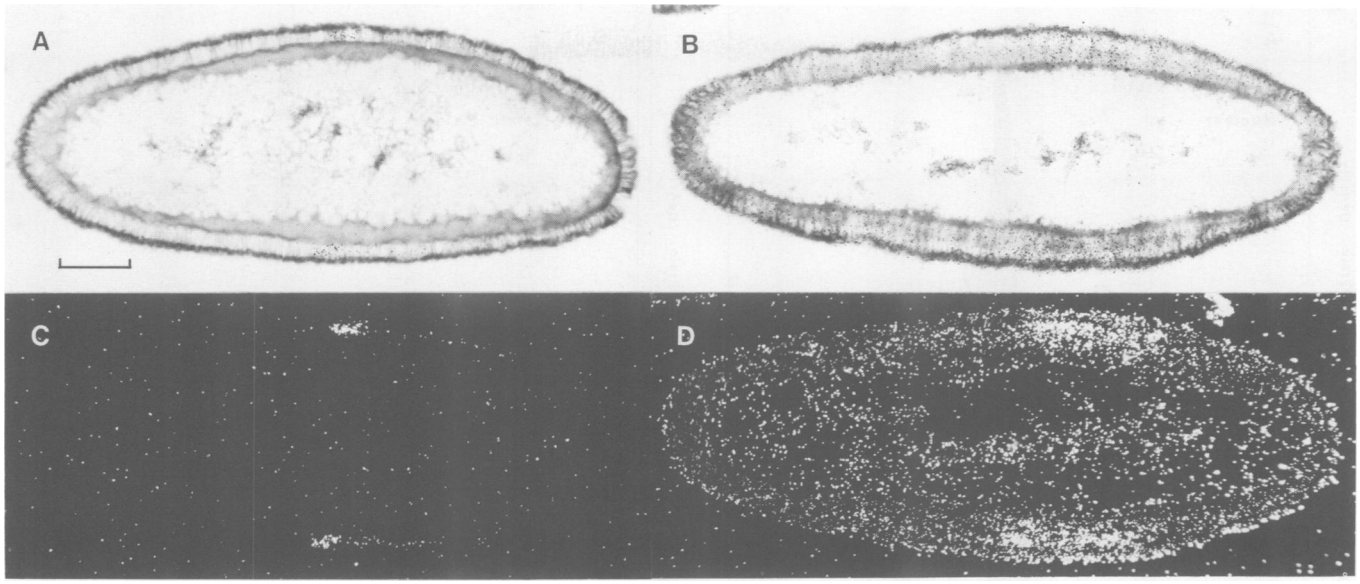


Fig. 4. Hybridization of other *Ubx* probes to the cellular blastoderm. **Panels A** (bright field) and **C** (dark field illumination), early cellular blastoderm hybridized with the 4.7 RNA probe. Prominent labelling at the centre of the egg is already clearly detectable, exclusively over the nuclei. **Panels B** and **D**, late cellular blastoderm hybridized with the *Ubx* 3′ probe. Localized labelling is detected over both nuclei and cytoplasm of an area within the posterior half of the egg. The marked differences in the levels of background labelling shown here and in Figure 3 are characteristic of each probe. They do not result from hybridization to BX-C transcripts. (Details of control experiments are given in Material and methods.) (Probes: **A,C**: A122 at 0.16 μg/ml. Hybridization with dextran sulphate at 32°C for 23 h. Autoradiographic exposure = 13 days. **B,D**: A134 at 0.36 μg/ml. Hybridization with dextran sulphate at 32°C for 16 h.) Autoradiographic exposure = 20 days, scale = 50 μm.

tion of segments and parasegments is illustrated in Figure 1.

Results

Three major size classes of *Ubx* RNA appear during the first 8 h of embryogenesis. The structure of one of these, a 3.2-kb polyadenylated transcript, has been characterized in some detail (Beachy *et al.*, 1985, see Figure 2).

We discuss here results obtained with probes prepared from two genomic fragments which include the major parts of the 5′ and 3′ exons of this 3.2-kb RNA species. The 5′ probe, which we refer to as the *Ubx* common probe, hybridizes additionally to both of the other major embryonic RNAs (species of 4.3 and 4.7 kb) and also to higher mol. wt. RNA, presumably nascent or partially processed transcripts of the *Ubx* unit. The 3′ probe also hybridizes to the 4.3-kb RNA species, but does not efficiently detect sequences of either the 4.7-kb RNA, or nascent transcripts (Figure 2).

We have also used a number of probes to sequences which are not present in the processed 3.2-kb transcript. One of these is a genomic fragment located immediately 3′ to the common probe, in sequences spliced out of the 3.2-kb transcript (Beachy *et al.*, 1985). This probe does not hybridize with either the 3.2- or 4.3-kb *Ubx* RNA, but detects principally the processed 4.7-kb species in 3−6 h embryos. We refer to this as the '4.7 RNA' probe. In older embryos it detects higher mol. wt. nascent or partially processed transcripts. Another fragment derived from the extreme 3′ end of the *Ubx* unit hybridizes efficiently only to the 4.3-kb RNA species (Figure 2). We refer to this as the '4.3 RNA' probe.

The pattern of Ubx transcript accumulation in the blastoderm and early gastrula (2−4 h)

We detect no specific hybridization with *Ubx* probes to embryos before the final nuclear cleavage in the blastoderm. After this last cleavage, specific hybridization is first detected with the *Ubx* common probe, in a zone extending from ~20% to 50% egg length. (Figures 3, 5; all egg lengths are measured from the posterior pole.) As cell membranes form, *Ubx* transcripts become more abundant and by the start of gastrulation a complex distribution is already apparent. This pattern can be simply described as the accumulation of *Ubx* transcripts throughout a region of the egg which includes the primordia for the third thoracic and first seven abdominal segments. Within this region there is differential expression along both the antero/posterior (A/P) and the dorso/ventral (D/V) axes of the egg.

The antero-posterior axis. The primordia for the ectoderm of the post-oral segments occupy the lateral regions of the cellular blastoderm from 10 to 60% egg length (Poulson, 1950; Lohs-Schardin *et al.*, 1979; Underwood *et al.*, 1980; Technau and Campos-Ortega, 1985; Hartenstein *et al.*, 1985). The average width of primordia for thoracic and abdominal segments has been estimated rather precisely as 4% of the egg length, but the location of cells which will give rise to each segment can at best be assigned within 2% egg length. The boundary between T3 and A1, for example, has been placed at 52% (Hartenstein *et al.*), 48% (Lohs-Schardin *et al.*), or 45−50% egg length (Underwood *et al.*, *op. cit.*).

When the *Ubx* common probe is hybridized to embryos which have completed cellular blastoderm formation, a single narrow zone within the presumptive segmental ectoderm is labelled 5- to 10-fold more strongly than any other (Figures 3, 5, 6). This zone, three to four cells wide, has the expected width of a single segment primordium, and lies between 45 and 50% egg length. From its position we identify it as either the primordium for abdominal segment 1, or for a metameric unit which includes the posterior part of T3 and the anterior part of A1. This labelled zone overlaps but lies just posterior to the third stripe of cells which accumulate the *fushi-tarazu* transcript (data not shown). As this *fushi-tarazu* stripe almost certainly spans the T3/A1 border (Hafen *et al.*, 1984), we are confident that the *Ubx* zone lies posterior to the primordium for T3. We believe that it identifies

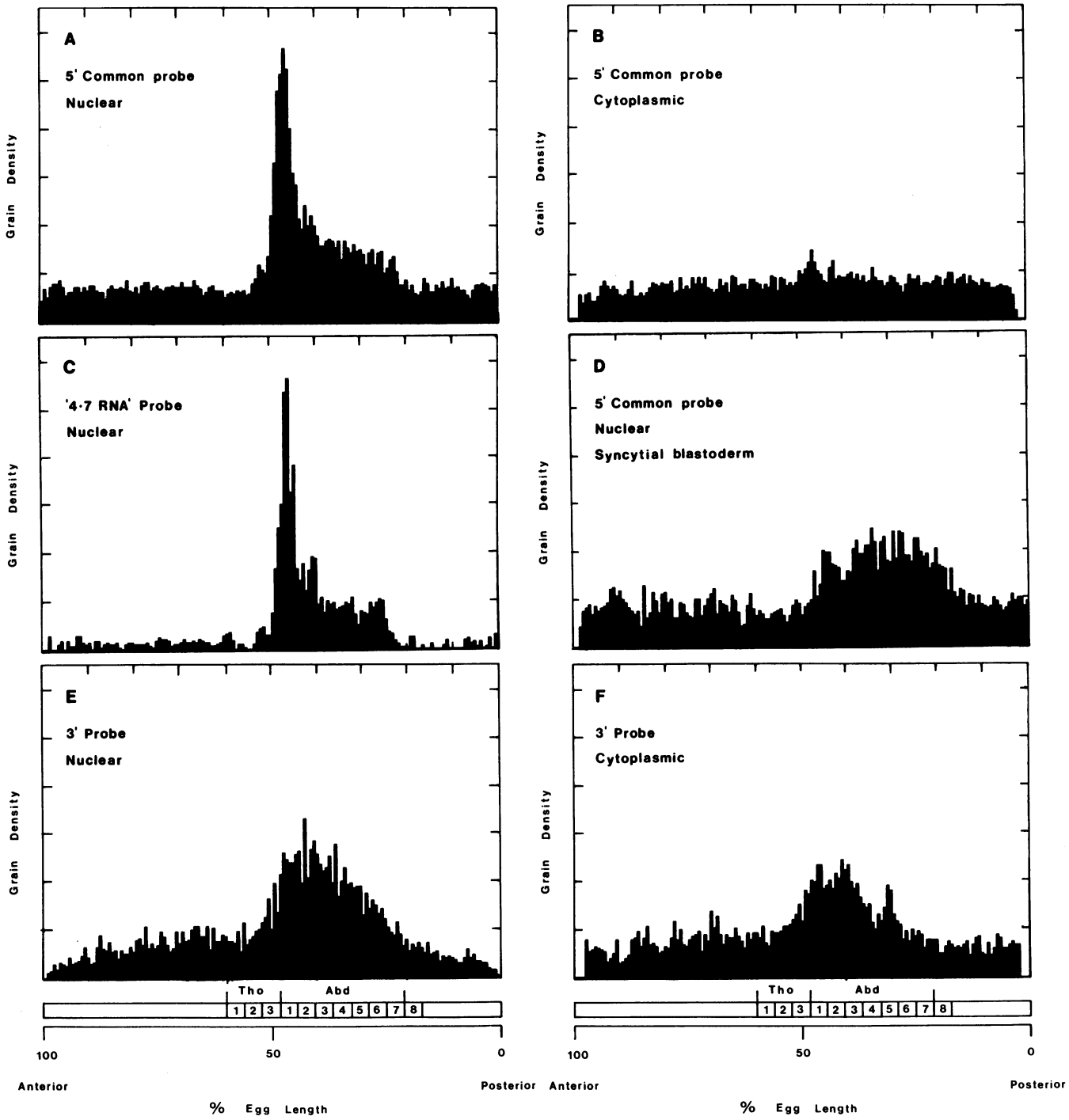


Fig. 5. Histograms showing the distribution of grains along the A/P axis of blastoderm stage embryos hybridized with *Ubx* probes. **A** and **B**: grains over the nuclei (**A**) and cytoplasm (**B**) of late cellular blastoderm embryos hybridized with the *Ubx* 5′ common probe A113. **C**: distribution over the nuclei of a late cellular blastoderm embryo hybridized with the '4.7 RNA' probe. No cytoplasmic label is detectable with this probe. **D**: distribution over the nuclei of the syncytial blastoderm stage embryo shown in panel E of Figure 3, hybridized with the *Ubx* common probe. **E** and **F**: grains over the nuclei (**E**) and cytoplasm (**F**) of late cellular blastoderm embryos hybridized with the *Ubx* 3′ probe A134. The data in these histograms are taken from the lateral region of the embryo, between 25% and 75% along the dorso-ventral axis. Somewhat different distributions are observed along the dorsal and ventral midlines (see text). The scale of segment primordia is taken from the laser ablation studies of Lohs-Schardin *et al.* (1979). **A,B**: four sections from each of two embryos including the embryo shown in panel G of Figure 3. 7830 nuclear and 4850 cytoplasmic grains entered; **C**: seven sections from a single embryo, 1745 grains entered; **D**: four sections from a single embryo, 3920 grains entered; **E**: four sections from each of two embryos including the embryo shown in Figure 4B, 11 000 grains entered; **F**: four sections from the embryo shown in Figure 4B, 2700 grains entered.

the primordium for parasegment 6 (see Discussion).

By the time that gastrulation has started, there is a sharp boundary in the lateral regions of the embryo at the anterior margin of the labelled band; maximally labelled cells in parasegment 6 and unlabelled cells in parasegment 5 are adjacent (Figure 6). Posterior to parasegment 6 a more complex pattern is evident (Figures 3, 5 and 6). Significant levels of hybridization extend back to 20% egg length, through the primordia for abdominal segments 2–7. For a short period following the onset of gastrulation, the distribution of *Ubx* transcripts defines 4 'pair-rule' stripes

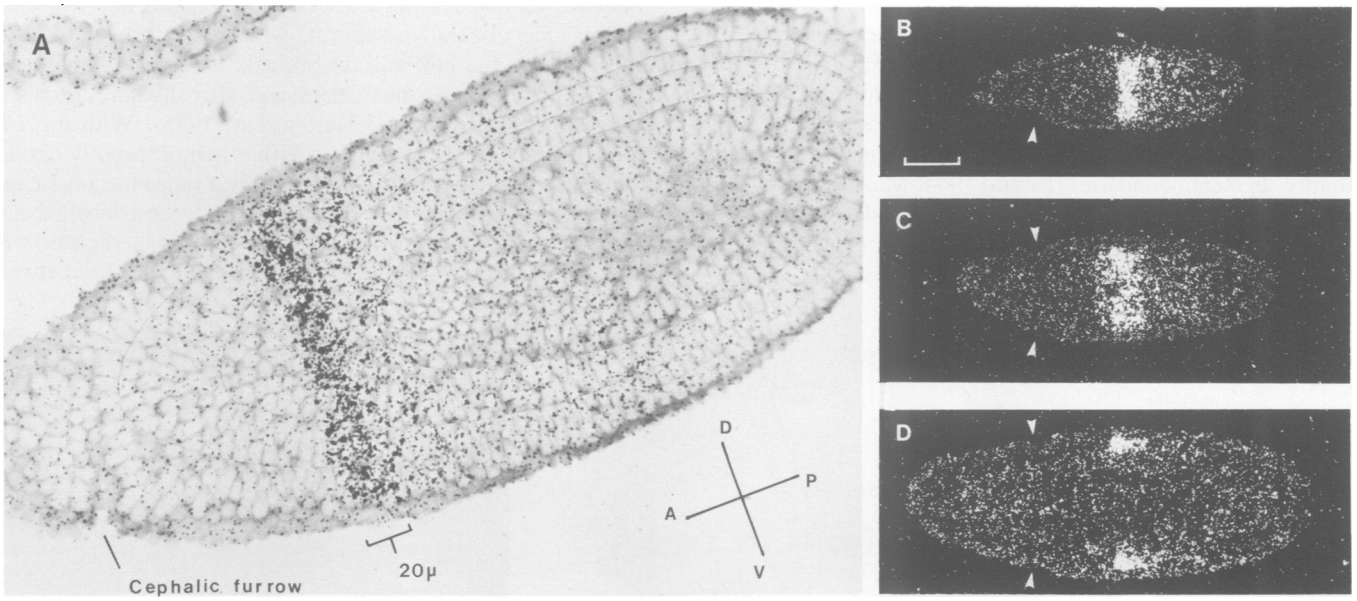


Fig. 6. *Ubx* expression in the early gastrula. **Panel A**: lateral view of an early gastrula hybridized with the *Ubx* 5′ common probe. This photographic montage was constructed by superimposing the nuclear zones from a series of sections through a gastrulating embryo. The embryo was hybridized on the same slide as the embryos shown in Figure 3. **Panels B–D**: comparison of *Ubx* expression in dorsal and lateral regions of the early gastrula. Embryos hybridized with the *Ubx* 5′ common probe, shown in dark field illumination. **B** is a lateral section comparable with that shown in **A**. **C** and **D** are horizontal sections through a similar aged embryo on the same slide. The abrupt anterior boundary of the labelled zone in lateral regions is clearly visible in **A, B** and **D**, but in **C**, where the section grazes the dorsal surface, the labelled zone is broader and extends more anteriorly. Arrows indicate the location of the cephalic furrow in each section. **B–D**: probe A113 at 0.12 μg/ml. Hybridization without dextran sulphate at 25°C for 40 h. Autoradiographic exposure = 26 days, scale = 50 μm.

(parasegments 6, 8, 10, 12; see Akam, 1985).

No *Ubx* labelling is detected in regions of the blastoderm which will give rise to the head, the endoderm, the foregut or hindgut. The pole cells are not labelled.

Variations on this pattern are observed when other *Ubx* probes are used. With the '4.7 RNA' probe, the labelling of parasegment 6 in the blastoderm is particularly prominent. With the 3′ probe, the same primordia are labelled, but hybridization is very weak in the blastoderm, and the specific labelling of parasegment 6 is not observed (Figures 4 and 5). At this stage no specific hybridization is observed with the '4.3 RNA' probe.

The dorso-ventral axis. In thoracic and abdominal regions, the circumference of the egg is subdivided into at least three primordia. Cells of the dorsal surface will give rise to the amnioserosa, the lateral regions will give rise to the ectoderm and the most ventral cells will form the mesoderm. *Ubx* is expressed in all three of these regions, although differences exist between them. In the blastoderm, the *Ubx* probes label the midventral regions (presumptive mesoderm) more weakly than the dorsolateral regions. Hybridization to the presumptive mesoderm becomes much stronger at the time of ventral furrow formation.

Cells along the dorsal surface of the embryo clearly show localized accumulation of *Ubx* transcripts, but no single labelled metameric primordium can be defined. The labelled zone extends somewhat more anteriorly than it does in the lateral regions, and the prominently labelled area covers ~10% of egg length, rather than the 4% observed laterally (Figure 6).

Cytological location of Ubx transcripts. Cells of the blastoderm are sufficiently large to allow the clear distinction of nuclear and cytoplasmic RNA. With both the *Ubx* common probe and '4.7 RNA' probe, specific hybridization is essentially confined to the nucleus; <5% of the label appears over the cytoplasm (Figures 3, 5). With the 3′ probe, the weak hybridization observed in the blastoderm is clearly detectable over both nucleus and cytoplasm (Figures 4, 5).

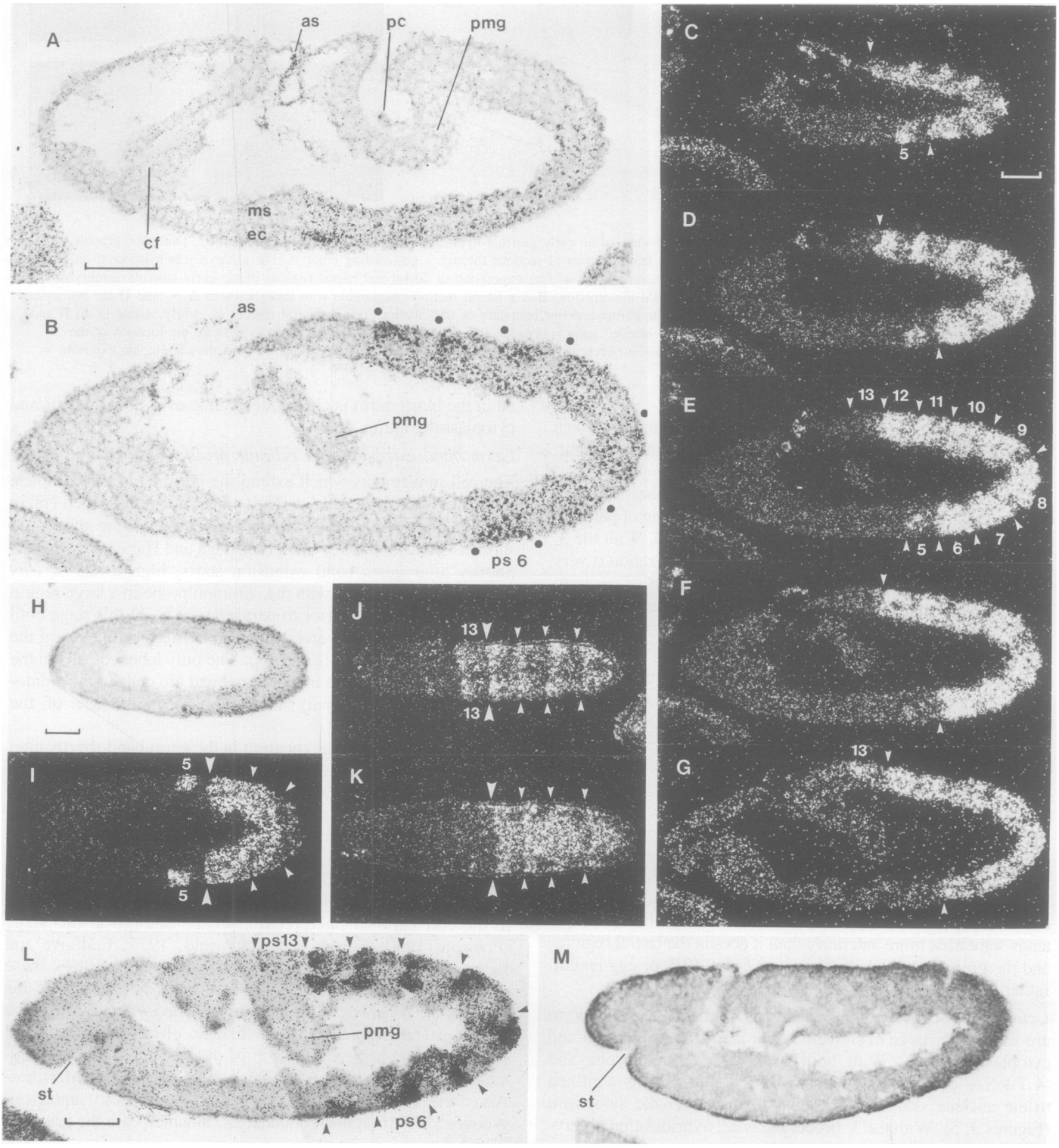
Germ band extension and cellular proliferation (4–10 h)

The cell movements which extend the germ band are complete within 1 h of the onset of gastrulation. The embryo then enters a period of cell proliferation which will end when the germ band shortens (Poulson, 1950; Campos-Ortega and Hartenstein, 1985). Shortly after germ band extension starts, high levels of *Ubx* transcripts are detected with the common probe in a large region of the germ band posterior to parasegment 6. At this stage both the inner (mesodermal) and outer (ectodermal) cell layers of the germ band are labelled (Figure 7). The only labelled cells in the embryo other than those in the germ band are cells of the amnioserosa, derived originally from the dorsal surface of the blastoderm.

Segmentation becomes apparent in the germ band shortly after elongation is complete, as regularly spaced bulges in the mesoderm (Poulson, 1950). By 6 h, segmental repeats are visible in the ectoderm as a series of grooves on the surface of the embryo. Seven of these metameric units are extensively labelled when the *Ubx* common probe is hybridized to 5–7 h embryos. In medial sections the boundaries of the heavily labelled region clearly lie at the superficial grooves (Figures 7,8). According to the conventional interpretation of the embryo at this stage (Poulson, 1950; Turner and Mahowald, 1977; Fullilove and Jacobson, 1978; Hartenstein and Campos-Ortega, 1984), these superficial grooves are presumptive segment boundaries. If this were so, the zone which is extensively labelled with the *Ubx* probe would extend from the anterior margin of the first abdominal segment to the posterior margin of the seventh abdominal segment. For reasons discussed below, and elsewhere (Martinez-Arias and Lawrence, 1985) we believe that these superficial grooves are parasegment boundaries, in which case the principal

labelled zone includes parasegments 6 – 12, from the presumed A/P compartment boundary in the third thoracic segment to the equivalent boundary in the seventh abdominal segment (cf. Figure 1). In this region, the ectoderm shows clear modulation of labelling intensity within each metamere. If the visible repeats are parasegments, and the position of the tracheal pits delimits the position of future segment boundaries (Keilin, 1944; Martinez-Arias and Lawrence, 1985), it appears that, in the ectoderm of the abdominal segments, the anterior compartments are labelled much more strongly than the posterior ones (Figures 7,8).

Between 5 and 7 h, the mesoderm segregates into two components, the visceral mesoderm, which will form the muscle layers around the gut, and the somatic mesoderm, which will give rise to the somatic musculature and other structures (Poulson, 1950; Campos-Ortega and Hartenstein, 1985). With the *Ubx* common probe, the somatic mesoderm is homogeneously labelled throughout parasegments 6 – 12, but only a single metameric unit of the visceral mesoderm is labelled. By 7 h, these labelled cells of the visceral mesoderm are located almost in register with parasegment 7, but when first distinguishable they are between



parasegments 6 and 7, and probably originate in parasegment 6. The mesoderm is not labelled in parasegments 5 or 13. In these units, only a few ectodermal cells are labelled (Figures 7,8). In parasegment 5, the cells are located laterally around the tracheal pit (Figure 8); in parasegment 13, they are located superficially, in the anterior compartment of A8 (Figure 7).

The '4.7 RNA' probe labels the same structures as the *Ubx* common probe. The preferential labelling of parasegment 6 remains prominent in the early stages of germ band elongation, but labelling of all segments in the later stages is weak (Figure 7). It seems likely that this probe is detecting the distribution of both the 4.7-kb RNA species, and also of those unprocessed transcripts from the *Ubx* unit which include sequences complementary to the probe as part of their introns (see Discussion).

The *Ubx* 3′ probe also labels the same structures as the 5′ common probe in the extended germ band (Figure 7), but the preferential labelling of parasegment 6 seen in the early stages of germ band extension with the common probe is not seen with the 3′ probe. The '4.3 RNA' probe shows no detectable signal at this stage.

Germ band shortening and organogenesis (10–20 h)

By 10 h the germ band of the embryo has retracted, and the thoracic and abdominal segments lie close to their final locations. Hybridization of the *Ubx* common probe to the ectoderm in the shortened germ band corresponds to that in the extended germ band. Along the ventral midline, the relationship of the labelled ectodermal structures to the visible grooves on the surface of the embryo is essentially unchanged. Laterally, the grooves, which will finally demarcate the segment boundaries are already forming, shifting the apparent position of the label (see legend to Figure 9).

Shortly after germ band shortening, a large region of the nervous system still contains high levels of *Ubx* transcripts (Figure 9). By 12 h, when the ladder of commissures can be used to identify segmental repeats within the nervous system (Thomas *et al.*, 1984), the *Ubx* common probe detects transcripts in all ganglia from T2 to A7 (Akam, 1983; Figure 9). As condensation of the nerve cord progresses the relative abundance of *Ubx* transcripts in the posterior ganglia declines. Maximal labelling remains in a single metamere, which overlaps cells in T3 and A1. We believe this to be the neural derivative of parasegment 6 (Figures 9 and 10). In these later stages both the *Ubx* 3′ probe and the '4.3 RNA' probe detect a pattern in the nervous system similar to that seen with the 5′ probe (data not shown).

Labelling in the somatic mesoderm after germ band shortening resembles the pattern in the earlier embryo, in that a contiguous block of cells is labelled, but the anterior margin of this labelled zone appears to have moved, and is now located between parasegments 6 and 7 of the ectoderm (Figure 9). In the visceral mesoderm, which by 10 h has attached to the developing midgut, one small region labels very strongly with the *Ubx* common probe. As the anterior and posterior midgut rudiments fuse and engulf the yolk, these labelled cells come to lie at the first major constriction of the developing midgut (Figure 9). In the hatching embryo a region of the midgut is still encircled with labelled muscle cells. Cells around the hindgut are labelled with the *Ubx* common probe after 14 h. From our preparations it is not clear whether these are gut cells or visceral mesoderm (data not shown).

After the shortened germ band stage, the 4.7 RNA probe shows only weak labelling of those structures which label more strongly with the *Ubx* common probe. A probe derived from slightly more 3′ sequences of the *Ubx* intron shows a similar pattern of labelling, suggesting that this hybridization is principally to unprocessed transcripts (data not shown).

Discussion

Lewis (1978, 1981) suggested that each gene of the BX-C is expressed in a particular segment along the A/P axis of the *Drosophila* embryo, within which it plays a major role in the control of segment identity, and in more posterior segments, where it is required in concert with other BX-C functions to evoke a completely wild-type phenotype. The pattern of *Ubx* transcript accumulation which we observe in the early embryo reflects both these aspects of BX-C function. In the blastoderm a single metameric unit is distinguished by the accumulation of high levels of *Ubx* transcripts. Slightly later in development, *Ubx* is expressed at high levels in this and in the six more posterior metameres of the germ band.

However, the precise pattern of *Ubx* expression which we observe could not easily have been predicted from previous genetic analysis. Lewis (1978) attributed to *Ubx* a primary role

Fig. 7. Expression of *Ubx* in embryos 4–7 h old. **Panels A–K** were hybridized with the *Ubx* 5′ common probe, **panel L** with the 3′ probe and **panel M** with the 4.7 RNA probe. **Panels A–F** are sections from the same slide as those illustrated in Figures 3 and 6A, and so relative labelling is directly comparable. **Panel A**: sagittal section of 4.5 h old embryo. The germ band is extending. The probe hybridizes to a wide zone of the germ band, but prominent labelling of the most anterior labelled metamere is still visible. **Panel B**: slightly oblique sagittal section through a 5–6 h embryo. The germ band is almost fully extended. A sharply bounded zone of seven metameric units is strongly labelled. We believe these are parasegments 6–12 (dots). **Panels C–G**: a series of sections through the same embryo as **B**, shown in dark field illumination; **C** is lateral, **G** is medial. **F** is the dark field image of **B**. Presumed parasegment boundaries are indicated (arrowheads). In the ectoderm, the posterior and anterior compartments of each parasegment are differentially labelled (see also Figures 8 and 9). **E** and **F** show that at this stage the bounds of *Ubx* expression in the mesoderm of parasegments 6–12 are in register with those in the ectoderm. In the lateral sections (**C–E**), some ectodermal cells are clearly labelled in parasegment 5. Medially (**G**), labelling of ectodermal cells can be seen in parasegment 13. **Panels H** and **I**: horizontal sections through the ventral germ band of a 6 h embryo (**H**, bright field; **I**, dark field). The primordia for parasegments 6, 7 and part of 8 are labelled in both the ectoderm and the mesoderm. Labelled ectodermal cells in parasegment 5 are visible on both sides of the embryo. Compare the location of these cells with those visible in the sagittal sections of **C, D** and **E**. Large arrowheads indicate the anterior margins of parasegment 6. **Panels J** and **K**: dark field micrographs of horizontal sections through the dorsal germ band of a 7 h old embryo. **J** is a section grazing through the ectoderm. **K** cuts the same embryo at a deeper level, passing through the mesoderm. Large arrowheads indicate the posterior margin of parasegment 12. Both ectoderm and mesoderm are labelled in parasegments 9–12, but only ectodermal cells are detectably labelled in parasegment 13. The differential labelling of posterior and anterior compartments is not apparent in the mesoderm. Compare with the corresponding regions in E–J. **Panel L**: sagittal section through a 7–8 h old embryo hybridized with the *Ubx* 3′ probe. The pattern of labelling is comparable with that seen with the *Ubx* 5′ common probe. Notice, however, a difference in labelling intensity within parasegment 6. **Panel M**: sagittal section through a 5–6 h old embryo hybridized with the '4.7 RNA' probe. Only weak hybridization is observed to the same region of the germ band that is labelled with the *Ubx* 5′ common probe. as, amnioserosa; cf, cephalic furrow; ec, ectoderm; ms, mesoderm; pc, pole cells; pmg, posterior mid-gut; ps, parasegment; st, stomodaeum. Hybridization: for **A–F**, see the legend to Figure 3. The section in **G** was treated identically to the above except that the probe did not contain dextran sulphate. **H,I**: probe A113 at 0.12 μg/ml. Hybridization with dextran sulphate at 32°C for 23 h. Autoradiographic exposure = 20 days. **J,K**: probe A113 at 0.2 μg/ml. Hybridization without dextran sulphate at 37°C for 40 h. Autoradiographic exposure = 38 days. **L**: probe A134 at 0.15 μg/ml. Hybridization with dextran sulphate at 32°C for 23 h. Autoradiographic exposure = 13 days. **M**: probe A122 at 0.1 μg/ml. Hybridization with dextran sulphate at 32°C for 23 h. Autoradiographic exposure = 20 days. Scales: **A,B** = 50 μm; **C–G** = 50 μm; **H–K** = 50 μm; **L,M** = 50 μm.

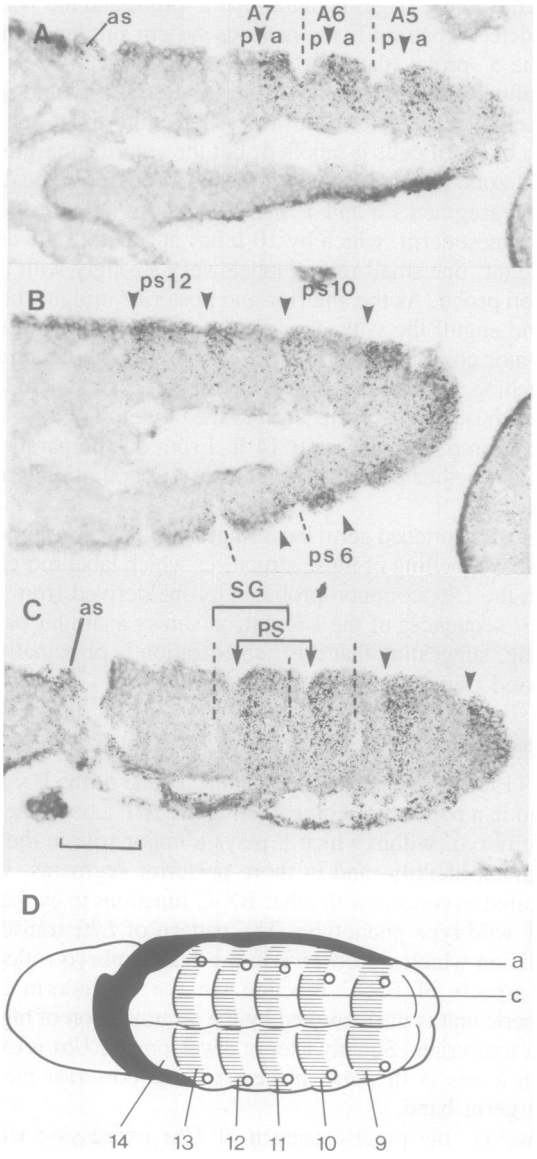


Fig. 8. Metameric boundaries of *Ubx* expression. **Panels A** and **B**: oblique sagittal sections through the germ band of a 7–8 h old embryo, showing the relation of *Ubx* expression to tracheal pits (indicated by dotted lines) and superficial grooves (indicated by arrowheads). Note that on the dorsal surface of the extended germ band embryo, the posterior part of each segment lies towards the head. In **A** the section is lateral, passing through the tracheal pit dorsally (at the level of line a on **D**). Strongest labelling with the *Ubx* probe lies immediately to the left (posterior) of these openings, in the presumed anterior compartments (a), Superficial grooves are not visible in this lateral section. In **B** the section is more medial, passing through the superficial grooves dorsally and the tracheal pits ventrally. Dorsally, the label is located to the right of the superficial grooves, still in the anterior compartments. On the ventral surface, arrowheads bound parasegment 6. Labelled cells in parasegment 5 cluster around the tracheal pit. **Panel C**: the section is very oblique. At the top it is close to medial (indicated by line c on **D**), and the superficial grooves are clearly visible (arrows). The tracheal pits can be seen as holes in the section, beneath the dotted lines. Segment (SG) and parasegment (PS) repeats are indicated (see text for details). The P compartments appear to be smaller than the A compartments. Maximal labelling of the ectodermal cells is observed in the anterior compartment (a) adjacent to the parasegment boundary. **Panel D** is a representation of the dorsal surface of an extended germ band embryo. Regions of the ectoderm which label prominently with the *Ubx* probe are indicated by hatching. Parasegments (ps) are numbered; tracheal pits are shown as circles and the superficial grooves are indicated. The amnioserosa (as) is shaded. (Probe A113 at 0.2 μg/ml. Hybridization without dextran sulphate at 37°C for 40 h. Autoradiographic exposure = 38 days, scale = 50 μm).

in defining the identity of the third thoracic segment. The phenotype of *Ubx* mutations in clones, and the epidermal phenotype of Ubx^- embryos defines a larger domain within which *Ubx* functions play a major role (Morata and Kerridge, 1981; Sanchez-Herrero *et al.*, 1985). This domain extends from the A/P compartment boundary in T2 to the A/P boundary in A1, spanning parasegments 5 and 6. Our results, summarized in Figure 11, suggest that the domains of *Ubx* expression in the early embryo are indeed parasegments, but show that *Ubx* is expressed very differently in parasegments 5 and 6. *Ubx* is active in all cells of the mesoderm and ectoderm of parasegment 6, but only in a subset of the ectodermal cells of parasegment 5.

We suggest that the primary role of *Ubx* in early development is to specify the characteristics of parasegment 6, and in conjunction with other BX-C functions, of more posterior regions of the abdomen. This is the morphological domain previously ascribed to the control of the *bithoraxoid* gene. According to our view, mutations in the *bxd* region would exert their effect by regulating aspects of *Ubx* expression, as has been suggested previously (Ingham, 1984; Beachy *et al.*, 1985), rather than the reverse (Lewis, 1978).

What then, is the role of *Ubx* in parasegment 5, and where are the transcripts which mediate these effects? Genetic analysis suggests at least two different roles. One function of *Ubx* is required early in the development in both posterior T2 and (probably) T3 (Morata and Kerridge, 1981). Later, other functions of *Ubx* are essential in the anterior compartment of T3, but no longer in T2. Struhl (1982) has suggested that the early function acts only to suppress *Scr* and perhaps other homoeotic genes. It is possible that very low levels of *Ubx* expression, undetectable in our experiments, may mediate this effect. An attractive hypothesis is that, in early blastoderm stages, *Ubx* may be transcribed at low levels in all the cells which will form parasegment 5, even though, in the late cellular blastoderm, only a small group of these cells contain detectable *Ubx* RNA.

There is good correlation in later stages between the cells in which we observe *Ubx* transcripts and the structures in T3 which are affected by *Ubx* mutations. These include the epidermis and tracheal system, the imaginal discs (Akam, 1983) and the nervous system. Moreover the muscles of T3, in which no *Ubx* transcripts are detectable, are not transformed by *Ubx* mutations (Lawrence, 1983; J.Hooper, unpublished observations). Therefore it is likely that the transcripts which mediate *Ubx* functions in T3 are among those which we detect.

These trancripts may be under different genetic controls from those which activate *Ubx* in parasegment 6 and more posterior segments of the early embryos. This is suggested by the exceptional distribution of transcripts in parasegment 5 of the embryo, and is strongly indicated by the functional organization of the *Ubx* domain itself. Sequences of the *Ubx* transcription unit alone can direct the normal development of parasegment 5 (Morata and Kerridge, 1981). However, the *Ubx* functions which are required in and posterior to parasegment 6 can only be expressed when sequences spanning both the *Ubx* unit and the adjacent *bxd/pbx* region are contiguous (Lewis, 1978; Bender *et al.*, 1983). Yet other controls may act on *Ubx* in the post-embryonic lineages, for the mutations *bithorax* and *postbithorax* eliminate *Ubx* functions in single compartments of the adult epidermis but have little effect on the development of the larva.

Generation of the blastoderm pattern

The mechanisms which regulate *Ubx* expression define a band of cells in the celullar blastoderm 20–25 μm wide, within which a unique pattern of *Ubx* expression is maintained. The anterior

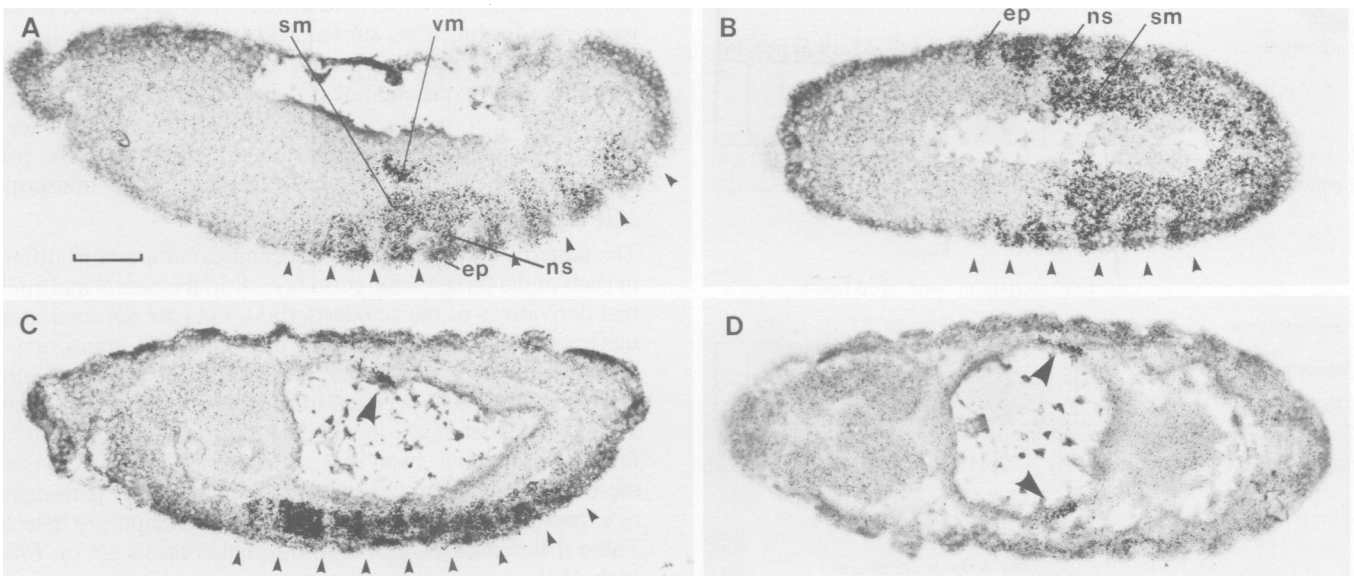


Fig. 9. *Ubx* expression in 10 – 15 h old embryos. The four embryos shown were all hybridized with the *Ubx* 5′ common probe A113. Autoradiographic exposures are comparable. **Panel A**: sagittal section of a 9 – 10 h old embryo. The germ band is shortening. At this stage the superficial grooves deepen and drag in the external remains of the tracheal pits. It is not clear whether at this stage the grooves can be defined unambigously as segmental or parasegmental (Martinez-Arias and Lawrence, 1985). Arrowheads indicate parasegments 5 – 12 or segments T3 to A7. By comparing this section with resin-embedded sections of the same age, we assign the histological nature of the labelled regions as indicated. The developing ganglia of the nervous system (ns) and the external epidermis (ep) of parasegments 6 – 12 are strongly labelled; some labelled cells are also visible in parasegment 5. A block of the somatic mesoderm (sm) is uniformly labelled. This extends back from the level of parasegment 7 to, in adjacent sections, at least the level of parasegment 11. (This section passes through no somatic mesoderm posterior to parasegment 9.) A few cells in the mesodermal region of parasegment 6 are also labelled (see also **B**). The isolated group of strongly labelled cells at the level of parasegment 7 is a small region of the visceral mesoderm (vm). Adjacent sections show that this is the only region of the visceral mesoderm which is labelled. The midgut and anterior structures are not labelled. **Panel B**: horizontal section through an embryo 9 – 10 h old. Symbols are as in **A**. Prominent labelling of the somatic mesoderm starts at the level of parasegment 7. **Panel C**: near sagittal section of a 12 – 14 h old embryo. Most *Ubx* transcripts at this stage are detected in the nervous system. One metamere is uniformly and strongly labelled. By comparison with the pattern of expression of *Ubx* transcripts in embryos at a slightly later stage (Akam, 1983 and Figure 10), and of *Ubx* proteins at similar stages (White and Wilcox, 1984; Beachy *et al.*, 1985), we believe this metamere to be parasegment 6. *Ubx* transcripts are also detected in parasegment 5, and in parasegments 7 – 12 of the nervous system. The only other cells prominently labelled at this stage are the visceral mesoderm at the first principal constriction of the midgut (large arrowhead). Myoblasts and epidermal cells in the abdominal region are also labelled, but these tissues are hard to analyse in frozen sections of these stages. **Panel D**: horizontal section of a 14 – 15 h old embryo. Labelling of the visceral mesoderm is evident (arrows). Probe = A113 at 0.1 μg/ml. Hybridization without dextran sulphate at 37°C for 40 h. Autoradiographic exposure = 22 days. Scale = 50 μm.

margin of this band is defined by an abrupt transition between cells which do not detectably express *Ubx*, and those which express it at high levels. Precisely defined zones of expression have also been observed in the blastoderm with probes for other homoeotic genes (Levine *et al.*, 1983; McGinnis *et al.*, 1984). These zones are presumably the molecular correlates of the mosaic of determined states in the blastoderm (Lohs-Schardin *et al.*, 1979; Underwood *et al.*, 1980; Simcox and Sang, 1983).

This precisely bounded zone is not apparent when transcripts are first detectable, but is generated during the early period of transcript accumulation (Figures 3, 5). This suggests that two processes may be involved in the generation of the mature blastoderm pattern; an early process, effective when transcription first starts in the syncytial blastoderm, which must be dependent on the position of a given nucleus within the egg, and a subsequent process, perhaps dependent on the interaction between neighbouring nuclei, which makes the final mosaic of states more precise. There is genetic evidence for maternally controlled positional cues underlying the first step (Lewis, 1978; Nüsslein-Volhard *et al.*, 1982). Early interactions between the functions of homoeotic genes (Struhl, 1982) and probably also segmentation functions, might mediate the second step (see Akam, 1985).

The precise boundaries of *Ubx* expression are different in the dorsal and lateral regions of the blastoderm; a single strongly labelled parasegment primordium cannot be identified along the dorsal midline. This suggests that the first step in the regulation of *Ubx* may be dependent only on position along the antero-posterior axis of the egg, but that the postulated second step may be sensitive also to position along the dorsal/ventral axis.

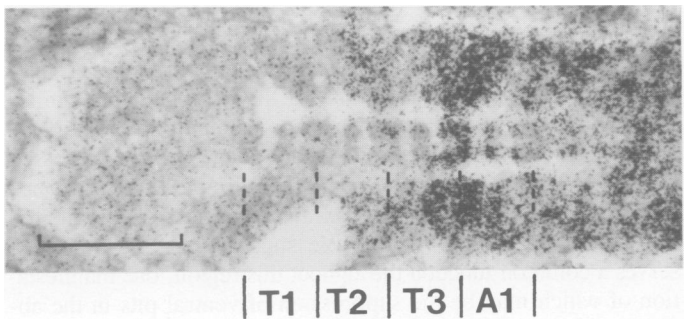


Fig. 10. *Ubx* RNA accumulation in neuromeres at 14 – 15 h of development. The section passes horizontally through the ventral ganglion. Lateral commissures are apparent as unstained gaps between the medial cell bodies. The segment boundaries have been indicated at the approximate position of the intersegmental nerve (Thomas *et al.*, 1984). T1, T2, T3: thoracic neuromeres; A1: neuromere of first abdominal segment. Hybridization conditions are as for Figure 9.

Segmental regulation of transcription and processing

Our observations show that the excess of transcripts which ac-

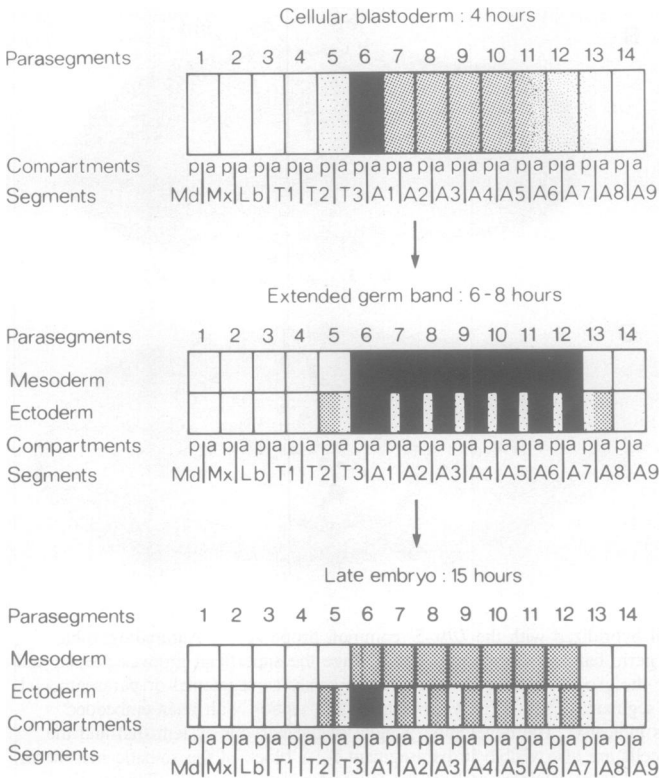


Fig. 11. A summary of the pattern of hybridization of the *Ubx 5′* common probe to developing embryos. A qualitative attempt has been made to distinguish tissues in which most or all cells accumulate high levels of *Ubx* transcripts (dark shading), from tissues which show lower levels of labelling or in which only a small proportion of cells are labelled (hatching or stipple). The details of the pattern are described in the text.

cumulate at blastoderm in parasegment 6 do not include the 3′ *Ubx* exon. This could result from the accumulation in parasegment 6 of specific RNA species which do not include 3′ sequences, and/or from an increase in the rate of transcript initiation occurring at this time only in parasegment 6.

At least two processed *Ubx* RNAs accumulate in embryos between 3 and 6 h. In the latter half of this period, when similar levels of *Ubx* transcripts have accumulated in parasegments 6–12, the 3.2-kb species is predominant. Since processed transcripts account for most of the homology detected, at least with the 3′ probes, the 3.2-kb transcript must be distributed throughout this entire region. We suggest that this transcript serves a common function throughout this region, one manifestation of which may be the suppression of ventral pits in the abdominal segments (Lewis, 1978).

A second RNA species of 4.7 kb is most abundant in the earlier part of this period, and is virtually undetectable after 6 h (R.Saint and D.Hogness, personal communication). At the blastoderm stage, the 4.7 RNA probe hybridizes on filters principally to this 4.7-kb species; *in situ* it hybridizes principally to parasegment 6, suggesting that the 4.7-kb species accumulates in this parasegment. However, all *Ubx* 5′ probes which we have used, including a probe from a region which is not detectably present in the 4.7-kb species, also show specific accumulation in this parasegment of the blastoderm (unpublished results). Therefore, it seems that both enhanced transcription of the *Ubx* unit and the accumulation of the 4.7-kb RNA contribute to the strong labelling of parasegment 6. It is possible that the 4.7-kb transcript, expressed transiently in the blastoderm, serves to define the identity of parasegment 6, and to determine the expression of parasegment specific functions subsequently.

Later in development, when the 4.3-kb species is predominant, the differential expression of *Ubx* in parasegment 6 is again apparent in the nervous system. The 4.7-kb RNA accounts for little or none of this later expression, for the '4.7 RNA' probe hybridizes only weakly, probably to unprocessed transcripts.

Ubx expression in different germ layers

The patterns of *Ubx* transcript accumulation are very different in each of the three major germ layers. In the neural and epidermal derivatives of the ectoderm transcripts are detected in nine metameres, but as development proceeds, *Ubx* transcripts accumulate differentially both between cells within a single metameric unit, and between metameres. In the somatic mesoderm a block of seven metameres accumulate transcripts uniformly throughout most of embryogenesis. In the visceral mesoderm only a single metameric unit, probably parasegment 6, expresses *Ubx*. In the endoderm, no transcripts are detected. These differences suggest that different controls act on *Ubx* in each of the germ layers.

The registration of segments, parasegments and Ubx expression

Many mutations of the UBX domain transform regions of the body which respect either segment or compartment boundaries (Lewis, 1981; Garcia-Bellido *et al.*, 1973; Morata and Kerridge, 1981). Therefore, it is likely that the abrupt boundaries of *Ubx* expression which we observe are also segment or compartment boundaries. In the extended germ band, these boundaries coincide with the ectodermal grooves on the surface of the embryo which have been interpreted in the past as the segment boundaries. Later, in the nervous system, the unit which is principally labelled with *Ubx* probes is not a single segmental ganglion, but approximates more closely a parasegmental unit. This is evident both from *in situ* hybridization to RNA (Akam, 1983 and Figure 10) and from antibody binding to *Ubx* protein in whole nervous system preparations (Beachy *et al.*, 1985; White and Wilcox, 1984).

If we were to accept the conventional interpretation of the ectodermal grooves on the embryo, then we would conclude that the domains of *Ubx* expresssion shift from segments in the extended germ band to parasegments in the nervous system. In view of mounting genetic evidence (Morata and Kerridge, 1981; Struhl, 1984; Sanchez-Herrero *et al.*, 1985), we prefer the view that the principal units of *Ubx* expression are parasegments throughout development. We conclude that, in the extended germ band, the principal labelled zone extends through parasegments 6–12, although some ectodermal cells are labelled in parasegments 5 and 13. This interpretation implies that the visible grooves in the early embryo are parasegment, not segment, boundaries. Martinez-Arias and Lawrence (1985) adduce additional morphological evidence for this point of view.

A single metamere is prominently labelled in the blastoderm. The prominent accumulation of *Ubx* transcripts in this metamere persists until parasegments are visible, during and for a short time after germ band extension (Figure 7). The labelled unit is parasegment 6. On the assumption that this label defines the same cells as those demarcated in the blastoderm, we conclude that the single primordium defined in the blastoderm is parasegment 6.

In the early gastrula, labelled cells of the mesoderm are precisely in register with those of the ectoderm. The anterior boundary of *Ubx* expression in the mesoderm is therefore presumably at the level of the A/P compartment boundary in the third thoracic segment. Shortly thereafter, the mesoderm appears to be

segmented in register with the ectodermal grooves, and is therefore presumably divided into parasegmental units. After germ band shortening the anterior boundary of expression in the mesoderm has shifted back to between parasegments 6 and 7. Subsequently, in the third larval instar, the muscles of abdominal segments 1 – 7 accumulate *Ubx* transcripts, whereas the major muscles of the third thoracic segment do not (Akam, 1983). It seems likely that the intersegmental muscles of the first (and by extension, each) abdominal segment derive from a parasegment primordium, and shift back one half segment relative to the ectoderm to attach at the anterior and posterior boundaries of the abdominal segments (Lawrence and Johnston, 1984a, 1984b; Martinez-Arias and Lawrence, 1985).

Compartments and Ubx expression

In the ectoderm of the post-blastoderm embryo, *Ubx* transcripts accumulate differentially in regions within a single parasegmental unit. In each abdominal primordium of the extended germ band the grain density increases posteriorly from one parasegment boundary to another, across the tracheal pit. At the parasegment boundary, there is a sharp discontinuity (Figures 7 – 9). We surmise that this discontinuity at the parasegment boundary corresponds precisely to the A/P compartment boundary as defined by lineage studies. Using the tracheal pits as landmarks for the future segment boundaries (Keilin, 1944; Martinez-Arias and Lawrence, 1985), our data indicate that the A compartments accumulate more *Ubx* transcripts than the P compartments. A similar distribution is visible later in the ventral nerve cord (Figures 9, 10; Akam, 1983; Beachy *et al.*, 1985; White and Wilcox, 1984). In the mesoderm, we have not observed differential accumulation of *Ubx* transcripts within single parasegmental units. Martinez-Arias and Lawrence (1984) suggest that the mesoderm is built exclusively from parasegmental units, but that in the ectoderm compartments are defined within these. Our results suggest that the expression of *Ubx* is modulated by the establishment of these compartmental units.

Materials and methods

Filter hybridization to RNA

RNA was prepared according to Fyrberg *et al.* (1980), with minor modifications (K.Howard, personal communication). Electrophoretic separation and transfer to nitrocellullose filters were carried out as described by Maniatis *et al.* (1982), using 5 or 10 μg of total embryonic RNA for each gel track. Single-stranded ^{32}P-labelled probes were prepared from M13 subclones under conditions similar to those used for *in situ* hybridization probes, except that labelled triphosphate concentrations limited the extent of transcription to 1 – 2 kb. Reactions were denatured immediately after synthesis and newly synthesized DNA was separated from template by electrophoresis on agarose. Gel fractions containing DNA between 50 and 2000 bases long were melted and dispersed directly in hybridization buffer. Filters were hybridized in 5 x SSPE, 50% formamide at 45°C for 16 h, and washed in 0.2 x SSPE, 0.1% SDS at 45°C.

Preparation of in situ hybridization probes

Single-stranded probes prepared from *Ubx* subclones were labelled with ^{3}H to a specific activity of ~ 4 x 10^{8} d.p.m./μg, using minor modifications of a protocol described previously (Akam, 1983). After second strand synthesis on M13 templates, newly synthesized material was isolated (by denaturation in alkali or formamide and electrophoresis on agarose) either as a discrete restriction fragment or as heterogeneous low mol. wt. material (500 – 2000 bases) released from the template without truncation. This material was reduced to a mean size of 50 – 200 bases as described previously, and resuspended in hybridization buffer (Akam, 1983), generally at a concentration of 0.1 – 0.2 μg/ml. In some experiments this hybridization buffer was made to a final concentration of 10% w/v with sodium dextran sulphate (Pharmacia).

Preparation of sections

Embryos of the wild-type strains Canton-S, Oregon R or Barton were collected from cage populations and reared at 25°C. Embryos harvested at the desired age were pre-fixed and demembranated to facilitate sectioning, using a modification of the procedure of Sedat and Mitchison (1983). They were dechorionated in commercial bleach, washed thoroughly, and then shaken vigorously for 10 min at room temperature in a two-phase mixture (1:1) of heptane and 4% formaldehyde in phosphate buffered saline (Form-PBS). (PBS is 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4. Formaldehyde was freshly prepared from paraformaldehyde before use.) The permeabilized and pre-fixed embryos were transferred to a two-phase mixture (1:1) of heptane:buffer ME which was pre-cooled and maintained in a dry-ice ethanol bath. (Buffer ME is 90% methanol, 10% water, 0.5 M EGTA, pH 8.0.) After further vigorous shaking for 10 min, the vitelline membranes were ruptured by rapidly warming the embryos to room temperature. Demembranated embryos, which now sink in the methanol phase, were transferred to fresh buffer ME, rehydrated by settling through mixtures of Form-PBS and buffer ME (1:9, 7:3, 1:1, 3:1; 2 x 2 min each), then settled through Form-PBS and PBS (twice each) before mounting in OCT compound to section at −18°C.

Sections were picked up on subbed or polylysine-coated slides, post-fixed in Form-PBS and dehydrated as described by Hafen *et al.* (1983). Polylysine-coated slides retain sections much more efficiently than subbed slides during subsequent procedures, but acetylation prior to hybridization is required to block fully the binding of probe to the polylysine coating (see below).

Immediately prior to hybridization the sections were pre-treated as described by Hafen *et al.* (1983), except that the acid step was omitted, and immediately after post-fixation, sections on lysine-coated slides were acetylated (Hayashi *et al.*, 1978) by incubation for 10 min in 0.25% acetic anhydride freshly dissolved in triethanolamine buffer, 0.1 M, pH 8.0.

Hybridization, washing and autoradiography were as described previously (Akam 1983), except that hybridization temperatures between 25 and 37°C were used [the lower temperatures for probe preparations containing short (~50 base) fragments], and hybridization time was reduced to 16 h for probes containing dextran sulphate. Sections were stained with toluidine blue (0.02% for 2 min) dehydrated through ethanol and xylene and mounted in permount.

The age of sectioned embryos was estimated from their morphology by reference to Poulson (1950) and Campos-Ortega and Hartenstein (1985), and is expressed as hours at 25°C after egg laying. To identify particular structures and germ layers in late embryos, the hybridized sections were compared with resin-embedded embryos sectioned at similar stages (AMA, unpublished results).

Control experiments

We have hybridized *Ubx* probes to embryos deficient for all of the BX-C (*Df(3R)P9*), and to embryos homozygous for a deletion which removes the entire *Ubx* transcription unit (*Df(3R)bxd*^{100}, see Lewis, 1978). These embryos lack the specific labelling which we attribute to the *Ubx* RNA. The background levels of binding of probe to endodermal structures, and to the anterior segments of the embryo are as in the wild-type, indicating that this background is not due to low levels of *Ubx* transcripts.

The pattern of labelling with the 3′ probe is not changed when the probe is prepared by truncation at a *Bgl*II site which removes all but 35 bases of the homeobox (McGinnis *et al.*, 1984). This indicates that mismatch hybridization to homeobox homologues contributes no significant signal in these experiments.

We have also hybridized embryo sections with M13 probes containing sequences complementary to those of the *Ubx* 5′ and 3′ probes, and the '4.7 RNA' probe. None of these show localized labelling of embryo sections. The background levels of binding observed with comparable preparations of these six probes vary by a factor of at least 5. The *Ubx* common probe is particularly 'noisy'.

Analysis of autoradiographs

A semi-automated procedure was used to quantitate autoradiographic labelling along the antero-posterior axis of blastoderm embryos, using a computer program developed in collaboration with Michael Farthing. Serial sections of a chosen embryo were photographed using a x 25 planapo objective, and printed at ~40 cm length. To establish a common coordinate system for the series, sections were aligned by superimposing their outlines, generally with an accuracy estimated to be better than ± 1.5% of egg length. The outline of the nuclear and cytoplasmic regions and the location of grains in each section were recorded using a digitizer (Graf-Bar GP7) and grain counts and areas over each tissue were calculated for 140 – 160 columns along the axis of the embryo. Summed values for each column in the series of sections were computed, and the distribution adjusted to fit a plot of 150 columns along the egg axis.

Acknowledgements

We thank Helen Moore for assistance with many aspects of this work, most notably her skilled hours at the microtome, and Michael Farthing for writing the autoradiograph analysis program. Michael Bate and Jose Campos-Ortega answered many questions of embryology; on some matters we agree to differ. Criticisms and comments from Michael Ashburner, Antonio Gracia-Bellido, David Hogness and Philip Ingham helped us with particular points of interpretation. Our

parasegmental view of the embryo evolved in discussion with Peter Lawrence, and his goading greatly improved the manuscript. This work was supported by an EMBO fellowship to AMA, and by the Medical Research Council of Great Britain.

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Received on 29 March 1985; revised on 10 May 1985