Dissecting the temporal requirements for homeotic gene function

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SUMMARY

Homeotic genes confer identity to the different segments of Drosophila. These genes are expressed in many cell types over long periods of time. To determine when the homeotic genes are required for specific developmental events we have expressed the Ultrabithorax, abdominal-A and Abdominal-Bm proteins at different times during development using the GAL4 targeting technique. We find that early transient homeotic gene expression has no lasting effects on the differentiation of the larval epidermis, but it switches the fate of other cell types irreversibly (e.g. the spiracle primordia). We describe one cell type in the peripheral nervous system that makes sequential, independent responses to homeotic gene expression. We also provide evidence that supports the hypothesis of in vivo competition between the bithorax complex proteins for the regulation of their down-stream targets.

Key words: Ultrabithorax, abdominal-A, Abdominal-B, bithorax complex, GAL4, embryogenesis, Drosophila

INTRODUCTION

Segments of the Drosophila embryo are first defined at around the time of gastrulation. At this stage molecular probes show that the segments are all very similar, but as development proceeds, differences between them become increasingly apparent. These differences depend on the expression of the homeodomain proteins encoded by the homeotic genes of the Antennapedia and bithorax complexes (Lewis, 1978; Kaufman et al., 1980).

The homeotic genes are first transcribed in the blastoderm, primarily under the regulation of segmentation gene products of the gap and pair-rule classes (Dessain and McGinnis, 1991). Their early transcription patterns evolve rapidly, reflecting the dynamic generation of pattern in the blastoderm, but by the end of gastrulation, each of the homeotic genes is expressed in a defined spatial domain encompassing one or more segment primordia (Harding et al., 1985; White and Wilcox, 1985a; Carroll et al., 1986; Mahaffey and Kaufman, 1987; LeMotte et al., 1989; DeLorenzi and Bienz, 1990; Karch et al., 1990; Macias et al., 1990). These expression domains are maintained throughout subsequent development in the absence of the segmentation gene products, by a memory mechanism that involves genes of the Polycomb and trithorax groups (Paro, 1990; Kennison, 1993). Later in development the levels of individual homeotic proteins within each segment vary in different cell types and at different developmental stages. This reflects interactions between the homeotic genes, and their regulation by other patterning genes (e.g. the segment polarity genes) (White and Wilcox, 1985b; Martínez Arias and White, 1988; Irvine et al., 1993).

This evolving pattern of homeotic gene expression may have phenotypic effects on all stages of development. For example, Ultrabithorax (Ubx) is required to establish the difference between second and third thoracic segments. In the embryo, this difference is reflected in the number of cells forming the imaginal (i.e. adult) primordium of each segment (Bate and Martínez Arias, 1991). In the larva, the difference is apparent in the form of the imaginal discs, and in the pupa, in the production of wing (T2) instead of haltere (T3) cuticle.

In principle, only the very earliest of these differences need depend directly on the homeotic gene products: subsequent development may be affected indirectly, by the downstream consequences of early homeotic gene action. However, experiments that remove the products of homeotic genes from epidermal cells at different stages of development have shown that adult epidermal cells require the homeotic proteins until late stages of development to maintain their normal state of differentiation - a clone of cells lacking the homeotic gene Ubx in the haltere will develop as wing tissue, even though the ancestors of these cells expressed UBX protein until just prior to the last epidermal decision (Morata and García-Bellido, 1976; Kerridge and Morata, 1982). Therefore these cells must be checking their 'homeotic address' continuously.

This, and similar experiments, have led to the generalisation that homeotic gene products are required continuously for the normal development of segment-specific structures. However, this hypothesis has been tested adequately only for adult...
cuticular structures. The technique used to produce clonal patches of mutant cells (somatic recombination) cannot be used effectively to study larval differentiation, and it has not been used for internal tissues. Moreover, it cannot be reversed, allowing normal homeotic gene expression to be restored at subsequent stages.

In this paper we examine whether, in other situations, homeotic gene products may be required only transiently to elicit particular developmental decisions, and whether the competence of a cell to make such a choice may be restricted to a particular ‘window of opportunity’. This situation would be more akin to that frequently encountered in other developmental systems, where competence to respond to a cell extrinsic signal is frequently restricted in time and space.

To test this hypothesis, we have used GAL4 mediated induction to modify the pattern of homeotic gene expression at defined times during development. The essence of this technique (Brand and Perrimon, 1993) is to cross two parental strains that carry different transgenic constructs. One carries GAL4 coding sequences under the regulation of a promoter with the desired spatial or temporal specificity; the other carries coding sequences for the protein of interest under the regulation of the GAL4 upstream activating sequences (UAS). Neither of these constructs alone affects the viability of the fly. Crossing the two strains allows the generation of numerous embryos that show the same pattern of ectopic protein expression.

To direct ectopic homeoprotein expression we have used three different GAL4 lines that are expressed at different times during development. These three lines have been used to induce expression of homeotic genes during the three different GAL4 lines that are expressed at different times during development. These three lines have been used to induce expression of proteins encoded by the homeotic genes Ultrabithorax, abdominal-A and Abdominal-B (UBX1A, ABDA or ABDB in, for details see Materials and Methods). The promoters driving GAL4 differ both in their spatial and temporal specificity, but by focusing on regions where all three lines are active, we analyse specifically the effects of expressing homeotic genes at different times.

We find that continuous homeotic gene expression is required for some functions, while transient expression is sufficient for other developmental effects.

**MATERIALS AND METHODS**

**GAL4 lines and fly stocks**

The **Kr-GAL4** line carries a fusion of GAL4 coding sequences to a promoter fragment taken from the *Krüppel* gene (see below). This line is not expressed in the central nervous system. The other lines used carry enhancer traps in which GAL4 is activated by genomic lines and fly stocks

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All the GAL4 and UAS stocks used in this study are homozygous viable (Table 1). Crossing the driving GAL4 lines with the UAS expression lines renders 100% embryos of the same genotype, heterozygous for both the inducer and responder constructs.

All lines inserted on the third chromosome were recombined to the TM6B balancer chromosome. Crosses between these lines rendered 25% **Df(2R)Ubx109** homozygous embryos simultaneously ectopically expressing UBX and ABDB proteins. All of them are unequivocally recognisable by their phenotypes. A **UAS-abd-A 21.6**; **Df(2R)Ubx109/4** mothers were used for ABDA ectopic expression in **Ubx**-**abd-A** embryos.

**Constructs**

The three expression constructs used carry coding sequences derived from cDNAs cloned down-stream of the GAL4 response element **UAS** (Fischer et al., 1988).

Construction of **p[UAS-Ubx]**: A 1.489 bp **DraI** fragment encompassing the **Ubx** coding sequence, 229 bp of 5′ untranslated sequence and 127 bp of 3′ untranslated sequence was taken from a **Ubx** cDNA encoding protein form 1A (pET3a, Rob White, personal communication), and blunt end ligated into the **SmaI** site of pBluescript. The Ubx cDNA was then removed from this construct as a **KpnI-NotI** (partial) fragment and inserted into these sites of p[**UAS**] (Brand and Perrimon, 1993).

Construction of **p[UAS-Abd-Bm]**: A 1.888 bp **SspI-BglII** fragment encompassing the **Abd-Bm** coding sequence, 475 bp of 5′ untranslated sequence and 225 bp of 3′ untranslated sequence was taken from the **Abd-Bm** cDNA, c23 (Kelsh, 1991), and subcloned into pBluescript opened with **HincII** and **BamHI**. The Abd-Bm cDNA was then removed from pBluescript as a **Xhol-XbaI** fragment and inserted into these sites of p[**UAS**] (Brand and Perrimon, 1993).

Construction of **p[UAS-Abd-A]**: The **p[UAS-Abd-A]** construct has already been described (Greig and Akam, 1993).

Construction of **p[Kr-GAL4]**: Sequences encoding a truncated form of GAL4 protein with 75% of the full transcriptional activity [pMA242 (Ma and Ptashne, 1987)] were inserted downstream of a 5.1kb **Krippel** control region/hsp fusion. This fusion contains the 5kb **PstI-SalI** fragment of the **Kr** control region (Fig. 1 in Hoch et al., 1990). The transcript was terminated by a **SV40** poly(A) site and everything inserted into pCaSpeR. Further details of the construct can be obtained from G. M. or Maria Leptin on request.

**Preparation of the larval cuticle**

24-hour old embryos were dechorionated in bleach. The vitelline membrane was removed by treatment with heptane and methanol. After washing the embryos in 0.1% **Tween** they were mounted in **Hoyer’s mountant** and kept at 60°C for 3 days.

For scanning electron microscopy, the cuticles where fixed following a standard protocol (Ashburner, 1989).

**Antibody stainings**

The following antibodies were used: a polyclonal anti-CUT (Blochinger et al., 1990), a polyclonal anti-POXN (Dambly-Chaudière et al., 1992), the monoclonal F9.38 anti-UBX (White and Wilcox, 1984), the polyclonal anti-ABD-A (Macías et al., 1990) provided by Jordi Casanova and the monoclonal 1A2E9 anti-ABD-B (Celniker et al., 1989). All stainings were carried out under standard conditions and developed with the Vectastain Elite ABC kit.

**Table 1. GAL4-driving lines and UAS-expression lines used in this study.**

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RESULTS

Efficient induction of homeotic genes mediated by GAL4 expression

Fig. 1 illustrates the patterns of ectopic protein expression driven by the three GAL4 lines that we have used.

The Krüppel-GAL4 (Kr-GAL4) line drives expression of homeotic proteins from the onset of gastrulation until the extended germ band stage (3-5.20 hours). The promoter is active in the middle of the embryo, in a region encompassing parasegments 5 to 9. Induced protein levels are highest in the middle of this region, and lower at the edges (Fig. 1). This pattern of expression resembles that of the Krüppel gene (Hoch et al., 1990) but the appearance of induced proteins is delayed by 0.5-1 hour, presumably the extra time required for the GAL4 intermediate to be transcribed and translated. Even so, the ectopic BX-C proteins are expressed slightly earlier than the endogenous homeotic proteins, which are first detectable (under the same staining conditions) at stage 9, about 40 minutes later (data not shown and DeLorenzi and Bienz, 1990; Macías et al., 1990; Irvine et al., 1991). Levels of ectopic protein fall during stage 11 (5-7 hours). By the time the germ

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Fig. 1. The pattern and timing of homeotic gene expression induced by the Krüppel, hairy and 69B-GAL4 constructs. The patterns of ABD-A protein induced from a UAS-abd-A transgene when GAL4 is expressed from the Krüppel, hairy or 69B promoters. Rows A to E show progressively older stages: (A) stage 9, 3.40-4.20 hours; (B) stage 10, 4.20-5.20 hours; (C) stage 11, 5.20-7.20 hours; (D) stage 14, 10.20-11.20 hours; (E) stages 16-17, after 13 hours. All embryos are stained with anti-ABD-A antibody. These embryos are deficient for the endogenous Ubx and abd-A genes. Therefore the only protein detected by the antibody derives from the induced abd-A transgene. Note the difference in the time of appearance and disappearance of ABD-A expression in the three lines. Expression of the Kr line disappears from the trunk after the extended germ band stage; high levels of protein only persist in the amnioserosa after germ band retraction. After stage 15, most of the expression driven by the Krüppel and hairy lines disappears; expression is only detectable in some internal organs.
band begins to retract, only faint traces of ectopic protein are detectable in the trunk segments, and these are in the cytoplasm.

The activity of the hairy-GAL4 (h-GAL4) line has been described previously (Brand and Perrimon, 1993). Homeotic proteins induced by this driver are first detectable at stage 10, (4.20-5.20 hours) in seven stripes throughout the ectoderm (Fig. 1). Levels of expression increase to a maximum during stage 11 (5.20-7.20 hours) and thereafter slowly decrease. During germ band retraction ectopic protein is still detectable in nuclei, but it disappears shortly thereafter.

Expression driven by the 69B line (Brand and Perrimon, 1993) (hereafter called 69B-GAL4) first appears during early stage 11 (5.20-6.00 hours) (Fig. 1), well after endogenous homeotic proteins are expressed at high levels. Initially, the levels of ectopic homeotic proteins are higher in even numbered parasegments. By late stage 11, ubiquitous expression appears throughout the epidermis, with higher levels in the anterior row of cells in each segment. General expression is maintained throughout all stages studied, though induced protein levels do decrease slightly during stage 17. From the results described below, we conclude that functional levels of homeotic protein are present throughout the segment in 69B embryos.

**Continuous homeotic gene expression is required for the down-regulation of the BX-C genes**

Homeotic genes expressed in more posterior regions of the embryo generally down-regulate those expressed more anteriorly (Hafen et al., 1984; Struhl and White, 1985). This effect is
probably direct (Krasnow et al., 1989; Appel and Sakonju, 1993). We have used it both to monitor the effectiveness of our induced proteins, and to ask whether the repression of a homeotic gene has lasting effects on its regulation. Transient expression of ABD-A under the control of Kr-GAL4 (Fig. 2C) or h-GAL4 (not shown) down-regulates the endogenous Ubx gene, but this effect is observed only while ABD-A protein is present at high levels. When ABD-A protein levels fall, normal Ubx expression is restored (Fig. 2D). Because the genes that originally specified this normal pattern are no longer active, the ability to restore normal expression shows that Ubx ‘memory’ mechanisms (e.g. those controlled by proteins of the Polycomb and trithorax groups) are not disturbed by this transient repression.

Expression of Ubx does not recover if the 69B-GAL4 line is used to drive ABD-A expression, presumably because ABD-A persists at high levels throughout the stages studied (not shown).

ABD-Bm protein also represses Ubx and abd-A (Fig. 2G) when expressed under GAL4 regulation. However, when the levels of ectopic ABD-Bm protein fall, normal expression of Ubx and abd-A is not fully restored. Repression is maintained for a longer period, even in the absence of detectable ectopic protein. This could indicate that ABD-Bm binds more stably than ABD-A to its down-stream targets, or it may simply reflect the relative sensitivities of the antibodies used to detect the proteins. More significantly, transient expression of ectopic ABD-Bm protein de-stabilises the normal repression of Ubx and abd-A in anterior segments. After levels of ABD-Bm protein decrease, scattered cells express UBX and ABD-A in anterior segments, where they would not normally be expressed (Fig. 2H). The proportion of embryos with cells expressing Ubx or abd-A ectopically ranged from 40-80% in different experiments; the specific cells affected varied from embryo to embryo. These observations suggest that ectopic ABD-Bm disturbs the memory mechanisms that maintain Ubx and abd-A expression. Lamka et al. (1992) infer the existence of a similar phenomenon on the basis of cuticular phenotypes following heat shock induction of ABD-Bm protein.

Fig. 3. Cuticle in embryos expressing UBX transiently at different times during development. Cuticle preparations of late embryos [arrowheads point to the denticle belt of PS6 (A1 in the wild-type)]. (A) An embryo deficient for the Ubx and abd-A genes (homozygous for the deficiency Ubx109). The denticle belts of T3-A7 (parasegments 5-12) all resemble those of T2 (PS4). (B-D) Similar embryos expressing UBX under the regulation of the Krüppel-, hairy- and 69B- GAL4 constructs. Early induction by the Kr-GAL4 line has little effect on cuticle development (B). Later expression with the h-GAL4 line (C) slightly transforms alternate segments to resemble the wild-type A1. The transformation is complete when UBX is induced with the 69B line (D). In this case, the orientation of the denticles and the shape of the belts are like that of A1, however the denticles are slightly bigger, and are spread more widely. (E) Wild-type cuticle for comparison. The two rows of ventral pits (or kölbchen) can be seen in A-C. These are not present in D due to the ectopic UBX expression. In C and D, UBX expression in the cephalic segments has blocked head involution and eliminated elements of the mouth parts. Similar phenotypes have been reported following heat shock induction of UBX.
UBX proteins are not known to repress more posterior homeotic genes during normal development. We were therefore surprised to see that ectopically expressed UBX protein is able to repress the *abd-A* gene (Fig. 2I). This repression was observed in embryos from stage 9 to 10 following *Kr-GAL4* induction, from stages 11 to 14 following *h-GAL4* induction and from stage 11 to 14 following *69B-GAL4* induction. In the last case repression was obvious only in the cells of the anterior compartment, where highest levels of UBX protein are produced. Thus ‘anterior’ genes are able to repress ‘posterior’ ones under these conditions. As for the case of repression by ABD-A protein, this down regulation is not maintained when levels of UBX decay (Fig. 2J).

**Transient early expression of homeotic genes has no lasting effect on the cells that will secrete the larval cuticle**

The experiments above demonstrate that functional levels of BX-C proteins are expressed following induction with the GAL4 lines. We next examined how this transient expression affects subsequent development.

For the epidermal cells that will give rise to the larval cuticle the result is clear: early expression mediated by the *Kr-GAL4* line has virtually no effect on the shape or morphology of the denticle belts that serve as markers of segment identity (Figs 3B, 4B), although it does affect certain elements of the peripheral nervous system (see below). The slightly later expression mediated by the *h-GAL4* line has a moderate effect on cuticle morphology (Figs 3C, 4C), whereas the *69B-GAL4* line, expressed only after 5.20 hours, elicits a complete (and in some respects excessive) transformation towards the phenotype specified by each homeotic gene (Figs 3D, 4D). Thus the response of the cells secreting the denticle belts of the cuticle parallels the observed effects on cross-regulation: homeotic proteins must be present at the time when the cells are specifying cuticular patterns to affect their behaviour.

**Transient expression of UBX and ABD-A suppresses the formation of anterior spiracles irreversibly**

The results above show that transient homeotic gene expression during development has only transient effects. To see if this is generally true, we have utilised markers that reveal other aspects of segment differentiation and allow us to assess

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**Fig. 4.** Cuticle transformations produced by expression of ABD-Bm protein in wild-type embryos. (A) wild-type embryo. (B–D) Ectopic expression of ABD-Bm with *Krüppel* (B), *hairy* (C) and *69B* (D) -GAL4 driving constructs. Ectopic filzkörper (some marked with arrowheads), form after induction with each of the three driving constructs, but the three constructs differ markedly in their effects on the denticle belts. Ectopic expression with the *Kr-GAL4* line has only a very slight effect on denticle belts (strongest in A2 and A3); with the *h-GAL4* line the denticle belts are more severely reduced (in alternate segments). Ectopic expression with the *69B* line deletes the ventral denticle belts almost completely. Similar but milder effects on the denticle belts were observed by Lamka et al. (1992) after heat shock induction of ABD-Bm proteins.
the behaviour of the cells long before cuticle formation. One such marker is the antibody to the CUT homeodomain protein (Blochinger et al., 1990). Cut is required for a number of cell specification events during embryogenesis, particularly in the peripheral nervous system (PNS) (Bodmer et al., 1987). However, among the earliest and most prominent sites of CUT expression are the placodes that give rise to the anterior and posterior spiracles (Blochinger et al., 1990). These structures are unique to single segments. The anterior spiracle arises in the first thoracic segment (T1p, parasegment 4), the posterior spiracle in the anterior of A8.

In wild-type embryos, CUT staining of the anterior spiracle is first visible at stage 11, in the dorsolateral part of T1p. Later, these labelled cells form a characteristic doughnut-shaped structure (Fig. 5A). In Ubx− embryos, anterior spiracles form in T2 and T3 as well as in T1. These ectopic spiracles stain for CUT at the same time as the T1 spiracle (data not shown), and differentiate fully in mutant third instar larvae (Lewis, 1978). Ubx− abd-A− embryos die before hatching, so the anterior spiracles never differentiate. However, CUT staining reveals an anterior spiracle at the appropriate location in each thoracic and abdominal segment from T1 to A6 (Fig. 5B). Thus anterior spiracles appear to be suppressed, in normal development, by the expression of either UBX or ABD-A proteins.

We have tested the effect of transient ectopic expression of both UBX and ABD-A proteins on these structures. In Ubx− abd-A− mutant embryos the early transient expression of UBX or ABD-A is sufficient to repress spiracle formation irreversibly, wherever the ectopic protein is produced. Thus with the Kr-GAL4 driver line, anterior spiracles are suppressed completely in PS 7 and 8, incompletely in 5, 6 and 9 where protein levels are lower (Fig. 5C). This contrasts with the lack of lasting effect that the same regime of induction has in the epidermis.

With the h-GAL4 driver line, anterior spiracles are suppressed in alternate parasegments (Fig. 5D), mirroring the activity of this promoter. With the 69B-GAL4 line they are suppressed in all segments, showing that their development is still sensitive to repression at late stage 11.

**Formation of posterior spiracles by transient expression of ABD–Bm**

While the anterior spiracle is repressed by UBX or ABD-A, the posterior spiracle is induced by ABD-B (Sánchez-Herrero et al., 1985; Lamka et al., 1992; Kuziora, 1993). The posterior spiracle therefore provides a positive assay for the effects of transient homeotic gene expression.

The wild-type posterior spiracle is first detectable with anti-CUT antibody at stage 10, as a patch of dorsolateral cells in the anterior compartment of A8 (Fig. 6). ABD-Bm protein driven with any of the three GAL4 lines causes ectopic patches of CUT staining in the anterior compartments of more anterior segments. We interpret these as the primordia for ectopic posterior spiracles. These ectopic primordia will go on to develop filzkörper and spiracular hairs, terminally differentiated structures that are unique to the posterior spiracle (Fig. 4). Thus this effect of transient ABD-Bm expression persists throughout embryogenesis, even when the expression was induced from the early acting Kr-GAL4 line.

Because the three GAL4 lines are active at different times during development, we can test whether the onset of spiracle development reflects the timing of ABD-B expression. The Kr-GAL4 driver line expresses ABD-Bm at stage 5 (early gastrula), slightly earlier than the endogenous ABD-B protein. Even so, CUT protein first appears in the ectopic posterior spiracles at the same time as in the normal posterior spiracle in A8. This observation suggests that for ABD-Bm protein to have any effect, cells must first become competent to activate cut at the appropriate position in the segment. This competence is achieved independently of posterior determining genes, as whatever factor is required for activation of CUT in the
posterior spiracle is also located in the mid segments of the embryo.

The 69B-GAL4 driver line induces detectable ABD-Bm protein for the first time at stage 11 (6 hours), after CUT expression reveals the normal posterior spiracle. By late stage 11, patches of CUT expression show that the ectopic posterior spiracles have been specified in these embryos. Thus ectopic spiracles can be induced 1-2 hours later than the normal ones: the competence to enter this developmental pathway persists from stage 10 until at least stage 11.

Some peripheral sensory organs make sequential independent, responses to homeotic gene expression

To investigate effects on PNS elements during early developmental stages we have stained neural precursor cells with anti-POXN (Dambly-Chaudière et al., 1992) and anti-CUT antibodies (Blochinger et al., 1990).

Anti-POXN stains cells of the PNS that will give rise to poly-innervated sensory organs (Dambly-Chaudière et al., 1992). Some of these cells give rise to homologous but different structures in the thorax and in the abdomen (Dambly-Chaudière and Ghysen, 1987). A dorsal row of POXN-positive cells (Fig. 7) become kölbchen in the thorax (the dorsal pits), but small sensory hairs in the abdomen (compare Fig. 8A and B with C). These sense organs differ, not only in their terminal differentiation, but also in their position. In thoracic segments T2 and T3, the dorsal POXN-positive precursor cells migrate to a more ventral position than those in the abdomen (Fig 7A, arrowheads). Both the differential migration and the terminal differentiation of these precursors are determined by the bithorax complex genes. In Ubx-"abd-A" embryos all the dorsal POXN-positive cells in the abdomen now migrate ventrally (Fig 7B), and differentiate as kölbchen, as in the thorax.

Transient early expression of UBX or ABD-A using the Kr or h-GAL4 lines prevents the ventral migration of these cells (Fig. 7C,D). They differentiate in the position of the normal abdominal cells, but instead of making dorsal hairs, they make misplaced kölbchen (Fig. 8D). Thus early expression is sufficient to transform their early behaviour, but not their late. Later expression, with 69B, affects both their migration (Fig. 7E) and their differentiation (Fig. 8F).

The ventral POXN-positive cells do not show this differential migration, but they do make different structures in the thorax and abdomen. In the thorax, they make the kölbchen that appear in cuticle preparations as the ventral pits. In the abdomen, they make distinct ventral papillae. The differentiation of these ventral cells responds to ectopic homeoprotein expression at the same time as that of their dorsal partners: the ventral pits are largely unaffected by early ectopic expression of UBX or ABD-A, but their appearance is suppressed by later expression driven by the 69B line (Fig. 3B,C).

The PNS cells that will form the Keilin’s organs show a different temporal sensitivity to homeotic proteins. Keilin’s organs form in the thoracic segments, but not in the abdomen. They can be recognised as a cluster of cells expressing the CUT protein. The fate of their abdominal homologues is unknown. Anti-CUT staining and cuticle preparations show that formation of the Keilin’s organs is suppressed efficiently by transient UBX or ABD-A expression, from either the Krüppel

![Fig. 6. Ectopic posterior spiracles can be induced at different developmental stages. Embryos stained with anti-CUT antibody at stage 9 (A), 10 (B) or 11 (C). Left column: wild-type embryos. The posterior spiracle does not stain at stage 9; the only stained cells are those in the future Malpighian tubules (MT). Cells of the posterior spiracle first stain at stage 10 (B, arrowhead). Staining is maintained at stage 11 and later. Centre column: embryos expressing ABD-Bm with the Kr-GAL4 line. At stage 10 staining appears simultaneously in both ectopic and endogenous spiracles (arrowheads). Right column: embryos expressing ABD-Bm with the 69B-GAL4 line. At stage 10 only the endogenous spiracle stains (arrowhead). By stage 11 ectopic spiracles are detectable in most segments (arrowheads).](image-url)
Temporal requirements for homeotic genes

or hairy promoters (not shown). Their formation is also suppressed by later expression driven by the 69B-GAL4 line.

Competitive interactions between homeotic proteins

In the course of these experiments, we noted certain effects of ectopic homeoprotein expression that differ from those previously published. Most notably, we observe that in otherwise wild-type embryos, the ectopic expression of UBX and ABD-A is able to modify the development of structures normally controlled by ABD-B. When the 69B-GAL4 construct drives UBX or ABD-A expression, the posterior spiracles are always reduced to half their wild-type size (compare Fig. 9A and C). In most of the embryos (75%) a small A9 denticle belt is formed anterior to the anal plates, and the anal tuft is often reduced (compare Fig. 9B and D). Induction with h-GAL4 cause similar but weaker effects on the posterior spiracles. All these effects suggest that ectopic UBX and ABD-A are able to compete with ABD-B for common binding sites.

The down regulation of abd-A by ectopic UBX protein might be expected to produce an analogous transformation of abdominal segments A2-A7 towards the phenotype of A1. The denticle belts of these abdominal segments were not affected by ectopic Ubx expression under our experimental conditions. However, Lamka et al. (1992) observed a transformation of this type following heat shock induction of Ubx. Our constructs may not be driving sufficient levels of UBX protein in the relevant cells at the right time.

DISCUSSION

The homeotic genes specify segment identity in many different tissues of both insects and vertebrates (McGinnis and Krumlauf, 1992). Expression of these genes is turned on during early stages of embryogenesis and maintained throughout development. Thus, the homeotic genes are expressed in cells that have very different histories, and differentiated states. It is clear that the response a cell makes to the expression of a particular homeotic gene is conditioned by this past history and may differ at different points along a single cell lineage. Here we have begun a temporal dissection of the requirement for the BX-C genes in different cell types. We have chosen to use GAL4 mediated induction (Brand and Perrimon, 1993) to induce ectopic expression as this allows large populations of embryos to be subjected to identical temporal profiles of ectopic protein expression. To examine the immediate consequences of ectopic expression we have used early marker genes for segment-specific structures.

Cell 'identity' responds to homeoprotein expression in distinct ways

One striking feature of our results is that transient early expression, as induced by the Krüppel promoter, may have profound effects on the differentiation of some structures (e.g. the spiracle primordia, Keilin’s organs), while similar levels of
the same proteins have little or no effect on the differentiation of the surrounding larval epidermis, even though at a later stage of development the terminal differentiation of this tissue will be sensitive to the same homeoproteins. It seems that these two groups of cells respond to homeotic gene expression in quite different ways. In the case of the spiracle primordia, there is an immediate and lasting effect: when the ectopic homeotic gene product decays, these cells will not revert to the pathway normally followed in its absence. In contrast, the terminal differentiation of the epidermis is virtually unaffected, even though we know that the ectopic homeoprotein was effective in modifying the behaviour of the cells, as reflected in the transient down-regulation of other homeotic genes.

We guess that the irreversible induction of the posterior spiracle with ABD-Bm reflects the activation of an autonomous ‘subroutine’ that becomes independent of the homeotic gene that induced it, perhaps by the activation of down-stream transcription factors that maintain their own
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expression. Similarly, the repression of the anterior spiracle by UBX/ABD-A may reflect a block to the induction of a similar subroutine, the competence for which passes when the transient expression of other necessary signals (e.g. segment polarity gene products) fades. Many other aspects of homeotic gene function may behave similarly - for example, the induction of the salivary gland by SCR protein (Panzer et al., 1992) and the suppression of leg primordia in the abdomen by UBX or ABD-A proteins (Vachon et al., 1992).

An interesting corollary of this model is that, once a downstream subroutine is established, re-expression of the same homeotic gene in cells committed to a certain pathway may have a developmental specificity quite different from that which it has in the homologous cells of other segments, where the same earlier decision was not taken. For example, once a cell is specified as leg, $UBX$ may be used to modulate the structure of the leg, rather than to suppress the formation of the leg primordium.

In other cells, by contrast, the transient expression of the homeotic genes results in no irreversible change in cell fate. Specific characteristics of the cells behaviour are altered at particular times in development - for example, the decision whether or not to make a denticle, or what sort of denticle to make, in response to segment patterning. However, subsequent responses to the homeotic genes are unaffected by earlier decisions. Thus a cell can adopt a hybrid state. This is seen clearly in the dorsal POXN-positive cells of the PNS. These cells make at least two distinct responses to the presence of UBX or ABD-A protein. One is the decision whether to migrate, the other defines the pathway of terminal differentiation followed by this particular PNS precursor cell in each segment. Our results indicate that the two decisions are independent: a cell that stays in the dorsal position does not have to form the sensory organ typical of that position, but retains the option, depending on what homeoprotein is expressed at a later stage, to differentiate along either pathway. The expression of the homeoprotein in these cells is not switching the cell between alternative fates in different segments, but repeatedly modulating the response that a single cell type makes to other developmental stimuli.

Homeotic gene expression and the timing of developmental events

Using cut expression as a marker, we have asked whether altering the timing of Abd-B expression concomitantly alters the time at which appropriate cells of the ectoderm become specified to form posterior spiracle. Precocious expression of Abd-B is not able to initiate cut expression any earlier than is endogenous Abd-B expression, suggesting that the rate-limiting step is the cells acquisition of the specific competence to respond. Cells in all trunk segments acquire this competence at about the same time, as judged by the simultaneous appear-

Fig. 9. Tail structures are modified by ectopic expression of UBX and ABD-A proteins. (A,B) Posterior segments of a wild-type embryo in lateral (A) or ventral view (B). The posterior spiracle bulges out at the end of the wild-type embryo. Note the characteristic denticles on the base of the spiracle (arrow). The filzkörper (fk) are entirely located in this everted structure. The denticle belt of A8 is labelled. Posterior to this are the anal pads and the anal tuft (t). (C) Lateral view of a 69B-GAL4 embryo expressing ABD-A ectopically in an otherwise wild type background. The posterior spiracles are reduced in size and the filzkörper are located inside the body of the embryo. The characteristic denticles on the base of the spiracle are missing (arrow). The anal tuft is reduced, and a small A9 denticle belt appears between the A8 belt and the anal pads. (D) Ventral view of a 69B-GAL4 embryo expressing UBX ectopically in a wild-type background. The anal tuft is reduced, and a small A9 segment forms (arrowhead). The posterior spiracles are out of focus. The denticle belts of abdominal segments A2-A7 appear normal.
ance of normal and ectopic spiracles in embryos expressing ABD-Bm under the influence of the Kr-GAL4 line. However, this window of competence extends beyond the timing of normal specification. Ectopic ABD-Bm protein, expressed later in development, induces ectopic patches of CUT expression in the appropriate position in the larval ectoderm as late as stage 11, at least 1 hour after the normal posterior spiracle primordia are visible. These patches presumably correspond to ectopic spiracles, for corresponding filzkörper differentiate beneath the cuticle of these embryos by the end of embryogenesis. We do not know whether the corresponding ectodermal cells remain competent to initiate this developmental pathway even much later - for example during each moult cycle of larval life.

Cross-regulatory interactions between the homeotic genes and their products

Previous experiments using heat shock to induce ectopic gene expression have reported that ‘anterior’ homeoproteins are generally unable to modify the phenotype induced by those homeoproteins that are normally expressed more posteriorly (González-Reyes and Morata, 1990; González-Reyes et al., 1990; Mann and Hogness, 1990; but compare Kuziora and McGinnis, 1988). This has been taken to imply a functional hierarchy among these proteins, perhaps in binding affinities to a common set of target genes (González-Reyes et al., 1990).

This conclusion has been challenged by Lamika et al. (1992) who found that when two homeoproteins are co-expressed by heat shock induction, the resulting phenotype depends on the ratio of the two proteins, and not simply on a hierarchy of interaction. This suggests that the interactions between the two proteins are competitive, not hierarchical. A similar conclusion is supported by the observation that the ectopic expression of anterior homeoproteins does modify the development of cells in the PNS, even in the presence of more posteriorly acting genes (Heuer and Kaufman, 1992).

Our experiments support a competitive model. We show here that UBX protein can affect the phenotypic consequences of endogenous Abd-B expression in the posterior of the embryo, not just the effects of ectopic expression. The size of the posterior spiracle is significantly reduced when UBX or ABD-A is expressed under the control of the 69B-GAL4 line, and an extra A9 segment denticle belt is formed.

Our observations differ from those of some previous experiments that used heat shock to induce homeotic gene expression. This is probably not due to higher levels of ectopic protein expression in our experiments. It is unlikely that induced protein levels with the GAL4 technique are significantly higher than those at the peak of ectopic heat shock induction. Moreover, a multiple heat shock regime can maintain high levels of induced proteins throughout much of embryogenesis (González-Reyes and Morata, 1990). However, our experiments do not involve the disruption of endogenous gene expression by heat shock (Maldonado-Codina et al., 1993). It may be that the effects of heat shock not only stop endogenous gene expression, but also alter the response of genes to new transcription factors in the period immediately following the heat shock.

Reciprocal inhibition of homeotic genes

We observe that UBX protein is capable of down-regulating the activity of the endogenous Abd-A gene - another effect that has not been seen following heat shock induction. This is the converse of the well documented transcriptional cross-regulation of more anterior homeotic genes by more posterior ones (Hafen et al., 1984; Struhl and White, 1985).

If UBX protein can suppress the transcription of the Abd-A gene, as well as vice versa, then the normal establishment of the overlapping Ubx and abd-A expression patterns may involve an element of competition: whichever protein first accumulates to high levels within a given cell will be more likely to achieve stable expression by down-regulating its competitor.

Repression of Abd-A by UBX is not widespread in normal development. This is shown by the fact that Ubx mutations do not obviously alter the patterns of Abd-A protein expression (Karch et al., 1990). However, no careful study has examined whether Ubx mutants affect the cellular specificity of Abd-A expression within the abdominal segments. For example, down-regulation by UBX may affect the relative levels of Abd-A and Ubx in the mesoderm or in specific neurones of the CNS of segments A2-A7.

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