The EMBO Journal vol.15 no.2 pp.334–343, 1996

Functional hierarchy and phenotypic suppression among *Drosophila* homeotic genes: the *labial* and *empty spiracles* genes

Ana Macías and Ginés Morata

Centro de Biología Molecular, Universidad Autónoma de Madrid, 28049 Madrid, Spain

The *Drosophila* homeotic cluster (HOM-C) is made up of eight genes, which specify the identity of cephalic, thoracic and abdominal segments. These genes can be ordered in a hierarchy which correlates with their position along the 5′–3′ transcriptional direction. When they are absent, thoracic and abdominal body segments develop the same 'ground' pattern, which is thoracic-like but also includes cephalic structures (sclerotic plates). We find that these plates are specified by the homeobox gene *empty spiracles* (*ems*) which is not a member of the HOM-C and which is expressed in all body segments. *ems* mutations, however, only produce defects in anterior head structures and the posterior spiracles. The *ems* product has the potential to induce sclerotic plates but this potential is suppressed by any of the HOM-C genes, including the *labial* gene, which we show to be the lowest ranking of the HOM-C hierarchy. This suppression does not occur at the transcriptional or translational level because the *ems* function is suppressed in cells containing the *ems* product. Thus, this appears to be the first case of phenotypic suppression operating in normal development. We propose that *ems* was originally a member of the HOM-C which escaped from the complex and has also acquired new functions during evolution.

Keywords: *ems*/homeotic complex/*lab*/phenotypic suppression

Introduction

One of the most exciting findings in developmental biology in recent years is that homeotic genes—the prime determinants of morphological patterns—form clusters which exhibit similar organization and structure in different animal groups (Duboule and Dolle, 1989; Graham *et al.*, 1989). The implication is that today homeotic complexes, represented in *Drosophila* by the ANT-C genes *labial* (*lab*), *proboscipedia* (*pb*), *Deformed* (*Dfd*), *Sex combs reduced* (*Scr*) and *Antennapedia* (*Antp*) and the BX-C genes *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*) and collectively called the HOM-C (Akam, 1989), and by their homologues in the Hox-C of vertebrates, derive from a common ancestor that already existed some 500 million years ago (Akam *et al.*, 1994). Not only has the overall organization of the homeotic cluster been conserved, but certain properties of the homeotic genes are also common to different animal groups. Most notable is collinearity, a striking correlation between the order of the genes within the cluster and their expression pattern in the body (reviewed in McGinnis and Krumlauf, 1992; Duboule and Morata, 1994). Another aspect of collinearity is the existence of a functional hierarchy among the members of the homeotic clusters so that the more posterior acting genes impose their developmental specificity upon the anterior genes (Struhl, 1983; Busturia and Morata, 1988).

The mechanisms behind functional hierarchy have not been elucidated. In *Drosophila*, the expression patterns of homeotic genes can be manipulated by various methods so that aspects of the hierarchy can be tested experimentally. By linking the homeotic coding sequences to the *hsp70* promoter, the restricted expression patterns of the homeotic genes can be replaced by a generalized and uniform distribution of the product (Gibson and Gehring, 1988; Kuziora and McGinnis, 1988; González-Reyes and Morata, 1990; Mann and Hogness, 1990), and the morphological effects noted. The general observation is that the homeotic gene product will only have an effect in the body region anterior to the normal domain of the gene. This has been seen not only at the morphological level, but also when looking at the expression of homeotic target genes (Reuter *et al.*, 1990; Capovilla *et al.*, 1994). The lack of effect in the posterior regions can only be explained by functional inactivation of the homeotic protein due to the products of the resident homeotic genes; if the suppressing gene is not present, the heat shock-driven product is active (González-Reyes and Morata, 1990). This functional inactivation of one homeotic product by another, which is obviously not at the transcriptional level, has been called 'phenotypic suppression' (González-Reyes and Morata, 1990), and may underlie the functional hierarchy. The rule would be that one homeotic product inactivates others that act anterior to its domain but is inactivated by those that act posterior to its domain. The rule predicts that *lab*, the most anterior acting gene, should be inactivated by all the others. In vertebrates, a similar phenomenon, termed 'posterior prevalence', has also been observed for the *Hox* genes (Duboule, 1991), and recent experiments expressing *Hox* genes in *Drosophila* under heat shock control suggest that the two phenomena are equivalent (Bachiller *et al.*, 1994). However, the functional significance of phenotypic suppression in normal development is uncertain, because the cases reported so far (reviewed in Duboule and Morata, 1994) involve artificial situations which do not occur *in vivo*.

The homeobox gene *empty spiracles* (*ems*) is necessary for normal development of the anterior segments of the larval head (Cohen and Jürgens, 1990), and also has a secondary and later function in the development of posterior spiracles (hence the name), where it acts downstream of *Abd-B* (Jones and McGinnis, 1993). However, even though requirement for the gene is restricted to the anterior and posterior ends, *ems* is expressed in all trunk segments

© Oxford University Press

where it has no apparent function. Two homologues of *ems* have been found in vertebrates (Simeone *et al.*, 1992) and are expressed in the anterior brain in mouse embryos, indicating a function there. It is of interest that the nematode *Hox* complex contains a homeobox gene with some homology to *ems* (Wang *et al.*, 1993), suggesting that the original complex may have included an *ems* homologue (see the review by Manak and Scott, 1994).

In this report we study the hierarchy of homeotic function with reference to the *lab* and *ems* genes. We show that both genes are inactivated by all the other HOM-C genes. However, while *lab* expression does not overlap in wild-type embryos with the other HOM-C genes, *ems* expression does, thus providing the first example of phenotypic suppression operating in normal development. We also show that the cephalic structures specified by *ems* are the result of its metameric expression, which is not regulated by *bicoid* (*bcd*) and *hunchback* (*hb*) and probably represents the primordial *ems* function. The tiers of expression controlled by *bcd–hb* and by *Abd-B* are probably recent evolutionary acquisitions.

Results

Larval segment patterns in the absence of HOM-C function: a mixture of thoracic and cephalic structures

As the pattern along the anteroposterior axis of the body is established by the activity of the eight HOM-C genes, it is of interest to define the 'ground' pattern upon which these genes act. This would be best done by studying the pattern of larvae lacking all the HOM-C genes, but this is impractical because the relevant chromosomal deletions include other genes which compromise the phenotype. However, a good approximation is provided by the quintuple (*Scr⁻ Antp⁻ Ubx⁻ abd-A⁻ Abd-B⁻*) combination (Struhl, 1983) in which all the thoracic and abdominal segments are devoid of HOM-C function. Although larvae of this genotype contain the wild-type *lab*, *pb* and *Dfd* genes, we have observed, by staining these embryos with specific probes, that they are not expressed in the thorax and abdomen, except for a low level of *lab* expression in the posterior compartments, which we believe has minor or no significance (see below). Thus, the thorax and abdomen of these larvae appear to define the ground pattern.

In every segment of the thoracic and abdominal region, larvae mutant for the quintuple combination differentiate a mixed pattern (Figure 1A), including thoracic-like denticle belts and cephalic structures, termed 'sclerotic plates' (sp) by Lewis (1978). It is not clear what piece of the head is made by the sp; they could be part of the lateral plates which derive from the procephalon (Jürgens *et al.*, 1986). The thoracic-like pattern of the ground segment is expected on evolutionary grounds (Lewis, 1978), but the differentiation of the cephalic sp is not. They might indicate the existence of a 'cephalic' gene, which becomes derepressed in the absence of the thoracic and abdominal homeotic genes. We have tested the possible implication of the HOM-C genes in the specification of the sp by examining *Df(3R)Scr Df(3R)P9* larvae which lack all HOM-C genes (Lindsley and Zimm, 1992). We find (Figure 1B) that though these larvae show pair-rule phenotype because of the lack of *fushi tarazu*, they differentiate sp plates in some of the remaining segments, thus demonstrating that the sp are produced by a gene(s) from outside the HOM-C.

Functional hierarchy of the HOM-C genes: the status of labial

Assuming that the sp in the quintuple combination are the markers of an unidentified cephalic gene, we have tested the function of this gene by individually expressing *lab*, *Dfd* and *Ubx* gene products under heat shock control in the quintuple combination. We find that each of these gene products suppresses the differentiation of the sp and yet each specifies its characteristic segment patterns; *Dfd* induces the differentiation of mouth hooks and cirri (Kuziora and McGinnis, 1988), while *Ubx* induces the appearance of abdominal segments (González-Reyes and Morata, 1990; Mann and Hogness, 1990).

We describe in more detail the experiments on *lab*, which specifies cephalic structures and is the most anteriorly expressed and the most 3′ of the ANT-C (Diederich *et al.* 1989; Merrill *et al.*, 1989; Kaufman *et al.*, 1990) and is therefore expected to occupy a low rank in the homeotic hierarchy (Struhl, 1983; Busturia and Morata, 1988; González-Reyes and Morata, 1990). We have used a *hsp70-lab* (Heuer and Kaufman, 1992) transgene (referred to as HSL) which can induce a high and generalized distribution of the *lab* product during the embryonic period. Heat pulses were given to quintuple embryos as well to as other combinations possessing different combinations of HOM-C genes. Staining with an anti-lab antibody shows, following a heat shock, a generalized presence of the product all over the body. Several hours after the heat pulse, there is still a high level of *lab* product in the anterior compartments of the trunk segments, but none in the posterior compartments. This is probably due to autoregulation, as previously described for *lab* and other homeotic genes (Kuziora and McGinnis, 1988; Chouinard and Kaufman, 1991; Tremml and Bienz, 1992).

Heat-shocked HSL *Scr⁻ Antp⁻ Ubx⁻ abd-A⁻ Abd-B⁻* larvae lack sp but differentiate in every thoracic and abdominal segment a stereotyped arch-shaped cephalic structure (Figure 2B), which appears in the ventral region and results from the fusion of material at the left and right sides. As the pattern elements from the labial and maxillary segments are present in the heat-shocked larvae, the *lab*-induced structure probably derives from the anterior head (Merrill *et al.*, 1989). This head piece probably represents the contribution of the *lab* gene to head development and, judging from its aspect and position, it may be part of the ventral arms. As a result of the ubiquitous *lab* expression, the metameric structure of the quintuple embryos tends to disappear (Figure 2B); the groups of transformed primordia frequently fuse, as occurs in normal head development. This observation is consistent with the idea that head development is built upon a ground thoracic-like pattern. In HSL *Scr⁻ Antp⁻ Ubx⁻ abd-A⁻* embryos, the same cephalic element appears in the region corresponding to parasegments 3–10, indicating that the *Abd-B* product suppresses *lab* function, whereas in HSL *Scr⁻ Antp⁻* larvae the cephalic element only appears in the thorax (Figure 2C), again indicating a suppression of *lab* function, in this case by *Ubx* and *abd-A*. Finally, in HSL embryos

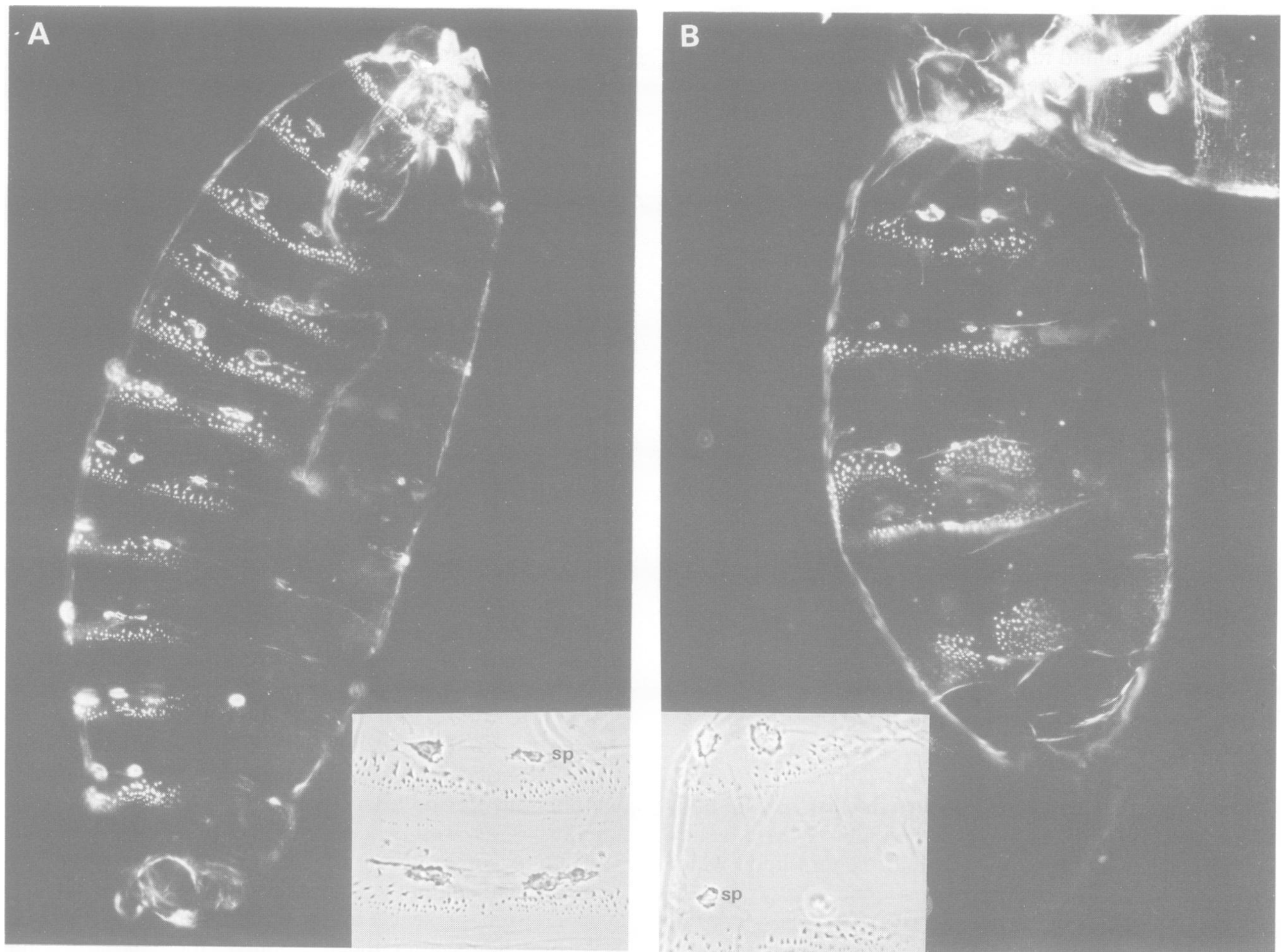

Fig. 1. (**A**) Dark field ventral view of a larvae of genotype *Scr⁻ Antp⁻ Ubx⁻ abd-A⁻ Abd-B⁻* deficient for the activity of the thoracic and abdominal homeotic genes. The sclerotic plates (sp) appear as refringent structures anterior to each denticle belt. Two segments with the corresponding pairs of sp are shown in phase contrast in the inset at the bottom right corner. Note that all body segment develop the same pattern. (**B**) Larvae of the genotype *Df(3R)Scr Df (3R)P9* in which all the HOM-C genes are deleted. It shows pair-rule phenotype because it is also deficient for the *ftz* gene which is located within the ANT-C. Nevertheless, denticle belts and sp similar to those in (A) can be seen in dark field view and in the phase contrast inset.

possessing a normal set of homeotic genes, the generalized expression of *lab* only produces pattern alterations in the head (Figure 2D); the rest of the body remains normal. In these larvae the alterations are more difficult to study because of the interference of other cephalic structures with similar texture and the effect of the heat shock on other phenomena such as the involution of the head (Merrill *et al.*, 1989). However, it is clear that the modifications affect only the most anterior structures, while those that derive from the gnathal segments, like labial sensory organs, maxillary sense organs, cirri or mouth hooks, are unaffected (Figure 2E). Since the latter are specified by the *Scr* and *Dfd* genes (Wakimoto and Kaufman, 1981; Chadwick and McGinnis, 1987; Kaufman *et al.*, 1990), the result indicates that the *lab* product is functionally suppressed by those of *Scr* and *Dfd*.

In summary, our results indicate that the sp are suppressed by any HOM-C gene. The latter can be ordered in a hierarchical rank and among them the *lab* gene occupies the lowest position, corresponding to its location in the 3′ end of the complex and to its expression domain anterior to those of the other HOM-C genes. Although the gene specifying the sp is not in the HOM-C, it follows from the logic dictated by the hierarchy that the sp should derive from an embryonic segment from the most anterior region of the head, the procephalon (see Campos-Ortega and Hartenstain, 1985). Recently, we correlated the suppression of sp in the abdomen of *Abd-B⁻* larvae with a homeotic transformation of an anterior head segment (Bachiller *et al.*, 1994), also suggesting that the gene responsible for sp differentiation is involved in anterior head development.

The ems gene is responsible for differentiation of the sclerotic plates

Since the sp probably derive from an anterior head segment, we looked for genes that specify the procephalon. Several genes, *ems*, *orthodenticle* (*otd*) and *buttonhead* (*btd*), are possible candidates. Their mutations remove distinct portions of the anterior head (Cohen and Jürgens, 1990). Moreover, the *ems* and *otd* genes encode homeodomain proteins (Dalton *et al.*, 1989; Finkelstein *et al.*, 1990; Walldorf and Gehring, 1992).

To check the requirement of *ems* function for the differentiation of sp, we constructed a chromosome *Scr⁻ Antp⁻ ems⁻ Ubx⁻ abd-A⁻ Abd-B⁺* and compared its larval

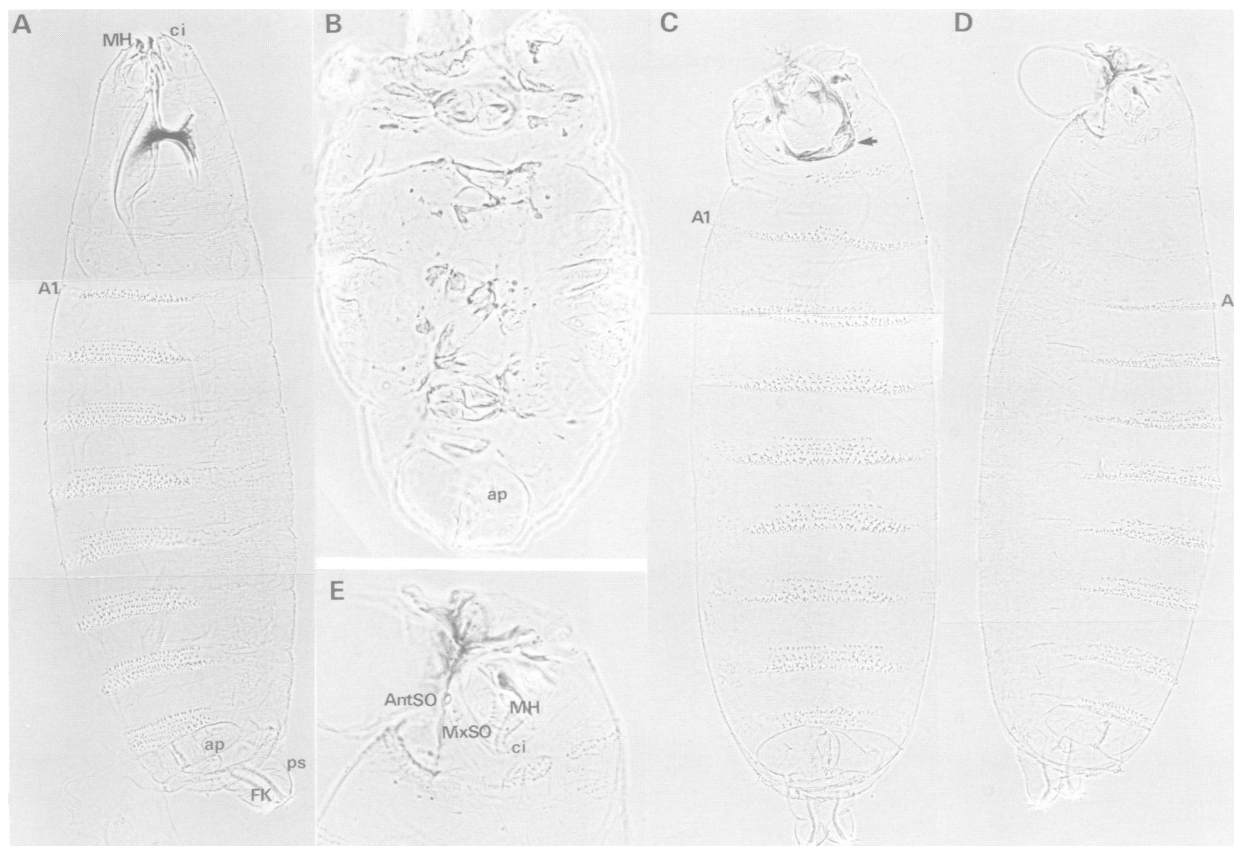

Fig. 2. (**A**) Ventral view of a wild-type larva. Cephalic structures like mouth hooks (MH) and cirri (ci), and posterior structures like anal pads (ap), posterior spiracles (ps) and filzkorpers (FK) are indicated. The position of the first abdominal segment (A1) is also shown. (**B**) HSL *Scr⁻ Antp⁻ Ubx⁻ abd-A Abd-B⁻* larva after heat shock (without heat shock the figure would be identical to Figure 1A). The metameric organization tends to disappear, and all trunk segments (except the terminalia, the ap remain) differentiate cephalic elements which often fuse and are hard to identify. (**C**) HSL *Scr⁻ Antp⁻* larva after heat shock. The morphological effect extends only to the head and thorax because, in the abdomen, the BX-C products inactivate the *lab* product. Note that from the A1 segment down to the posterior end of the body the pattern is entirely normal. The thorax is modified and shows the same cephalic element (arrow) observed in all segments in the larva in (B). (**D**) HSL larva after heat shock. The effect is restricted to the anterior head due to the inactivation of the *lab* product by the other ANT-C and BX-C products; the thorax and abdomen are normal. (**E**) Higher magnification of the larva in (D) to show that the effect of *lab* is restricted to the anterior head. The involution of the head is partially prevented, but the first thoracic segment and the MH, ci and maxillary sense organ (MxSO) derivatives of the maxillary segment are all present, indicating that the effect of *lab* is anterior to this segment.

phenotype with that of *Scr⁻ Antp⁻ ems⁺ Ubx⁻ abd-A⁻ Abd-B⁺*. We have used the null *ems*^{9Q} allele (Dalton *et al.*, 1989). Another test consisted of the comparison between *Scr⁻ Antp⁻ ems⁺* and *Scr⁻ Antp⁻ ems⁻* for the differentiation of sp in T1p or *ems⁺ Abd-B⁻* with *ems⁻ Abd-B⁻* for the differentiation of sp in A8p (Casanova *et al.*, 1986). In all these experiments, the clear result (Figure 3A and B), is that the differentiation of sp depends on *ems⁺* function. While in *Scr⁻ Antp⁻ ems⁺ Ubx⁻ abd-A⁻ Abd-B⁺* larvae all thoracic and the anterior abdominal segments develop sp, in *Scr⁻ Antp⁻ ems⁻ Ubx⁻ abd-A⁻ Abd-B⁺* the sp are absent in the great majority of segments. In a few cases, especially in T1p (and in A8p of *ems⁻ Abd-B⁻*), there are vestigial sp, suggesting that some other gene makes a minor contribution. We have also checked the effect of the elimination of *otd* in similar combinations of HOM-C mutants and found that the medial region is lacking in *otd⁻* larvae. Since this is the region where the sp come from, this result does not allow any firm conclusion about the possible role of *otd*. Mutations in the *btd* gene in similar combinations did not alter the sp.

Embryonic expression of the ems gene

We have studied the embryonic expression of *ems* in order to identify which aspect may be responsible for the sp. The normal expression of *ems* has already been described (Dalton *et al.*, 1989; Walldorf and Gehring, 1992), but we have re-examined it by the whole mount *in situ* method (Tautz and Peifle, 1989) and by using a polyclonal antibody raised in our laboratory. Initially, the *ems* product is restricted to the anterior region of the embryo, in the head-forming region. Later it appears in every trunk segment, around the tracheal pits and in a ventral stripe abutting the anteroposterior compartment border. Most of the *ems* expression is localized in the anterior compartments. By stage 10, a new expression domain, formed by the precursor cells of the filzkorpers, appears in A8. This late domain does not develop in *Abd-B⁻* embryos (Jones and McGinnis, 1993). We also looked at *ems* expression in *bcd⁻* embryos and found no *ems* product in the head in early embryonic stages, but after stage 8 they show the striped trunk expression (Figure 4A) equivalent to that seen in normal embryos (Dalton *et al.*, 1989). Thus, only

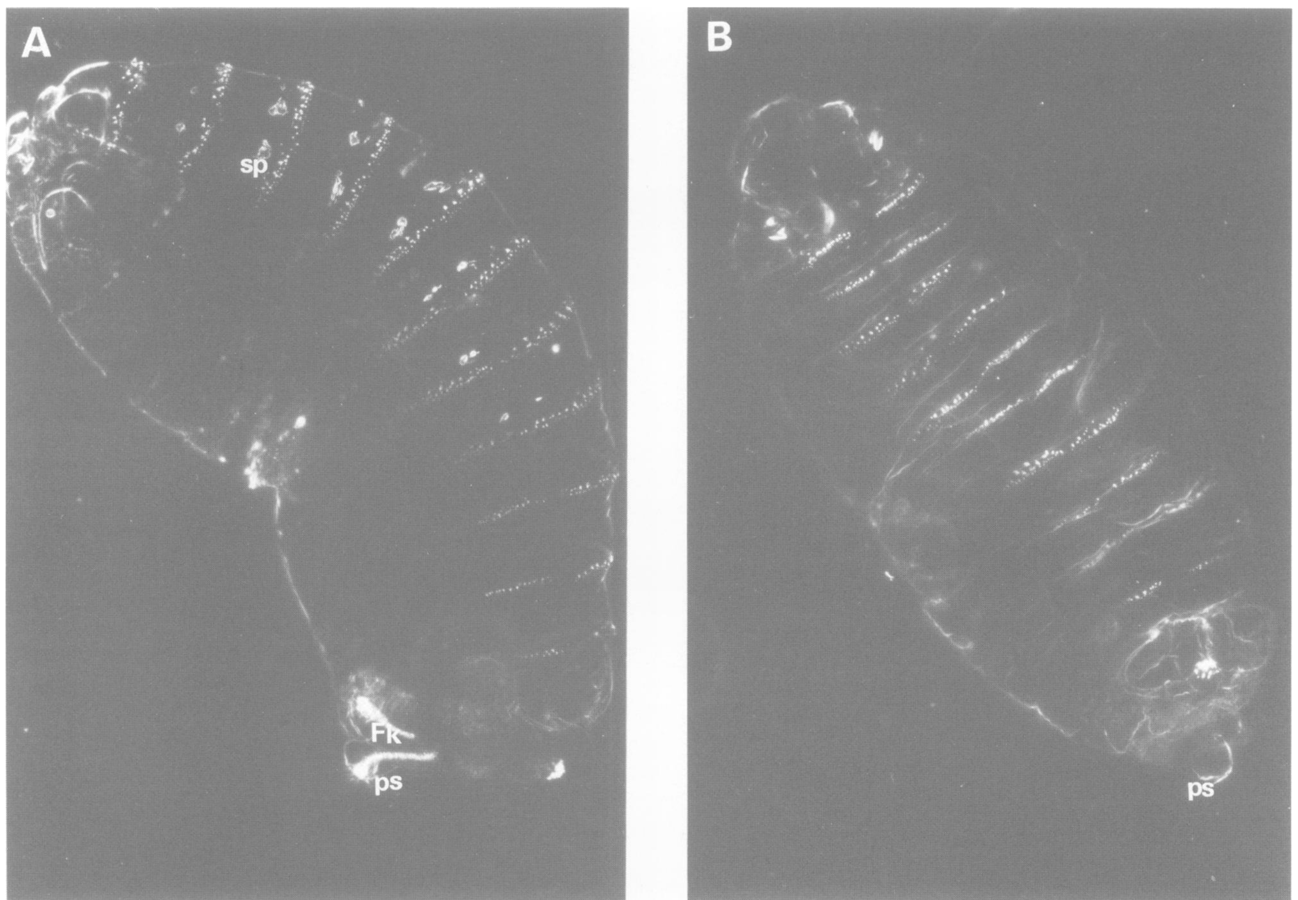

Fig. 3. (**A**) *Scr⁻ Antp⁻ ems*$^+$ *Ubx⁻ abd-A⁻ Abd-B*$^+$ larva show the differentiation of sclerotic plates (sp) in every thoracic segment and abdominal segments A1–A5. All these segments show the same thoracic-like denticle belts. In segments A6–A8, the sp do not differentiate and the denticle belts are also different due to the presence of the *Abd-B* product. These larvae develop normal posterior spiracles (ps) and filzkorpers (FK). (**B**) Segment pattern of a *Scr⁻ Antp⁻ ems⁻ Ubx⁻ abd-A⁻ Abd-B*$^+$ larva. The sp are lacking but the thoracic-like denticle belts are as in (A). The only difference between these two genotypes is that the latter is defective for *ems* function. Note also the lack of FK, but not of ps, a diagnostic criterion for *ems* mutations.

part of the *ems* expression is dependent on the *bcd* (see also Simpson-Brose *et al.*, 1994). A similar expression is seen in embryos in which both the *bcd* and the *hb* functions are inactivated by ectopic *nos* expression using the *nosA* construct (Gavis and Lehmann, 1992).

We next checked which tier of *ems* expression is responsible for sp differentiation. In particular, the metameric *ems* expression was a likely candidate as the sp appear along the body trunk in larvae deficient for the thoracic and abdominal homeotic genes. We constructed larvae lacking the *Scr*, *Antp*, *Ubx*, *abd-A* and *Abd-B* functions and, because they bear the *nosA* construct, they also lack *bcd* and *hb* functions (see Materials and methods for details). These embryos contain only *ems* metameric expression, as reported earlier (Dalton *et al.*, 1989; Simpson-Brose *et al.*, 1994). They develop into larvae with duplicated telson structures at their ends, but the intervening segments develop just as those in the quintuple combination *Scr⁻ Antp⁻ Ubx⁻ abd-A⁻ Abd-B⁻* and exhibit well formed sp (Figure 4B).

Metameric embryonic expression of ems is unaltered in the presence of suppressing genes

Having established that the *ems* striped expression is responsible for sp differentiation in the absence of the homeotic thoracic and abdominal genes, we tested whether these genes suppress transcription or translation of the *ems* product. The comparison between wild-type and quintuple embryos has been done at the level of both RNA and protein expression and shows (Figure 4C and D) one difference only: the late filzkorpers-specific expression known to depend on *Abd-B* (Jones and McGinnis, 1993). All other features are indistinguishable between the two genotypes. This result strongly suggests that the suppression of *ems* does not occur at the level of transcription and appears to be the result of phenotypic suppression.

The possibility of transcriptional regulation of *ems* by the ANT-C and the BX-C genes was further investigated by examining the expression of *Pc⁻* embryos in which the regular homeotic genes are derepressed (Wedeen *et al.*, 1986), and high levels of *abd-A* and *Abd-B* products were found, especially in the trunk segments. *ems* is expressed as in the wild-type.

Lack of ems adult function

Using the specific ems antibody and the RNA probe, we have looked at the expression of *ems* in the imaginal discs, which contain the precursor cells of adult structures, and found little expression. In the wing and haltere discs there are three patches, one in the thorax and two in the

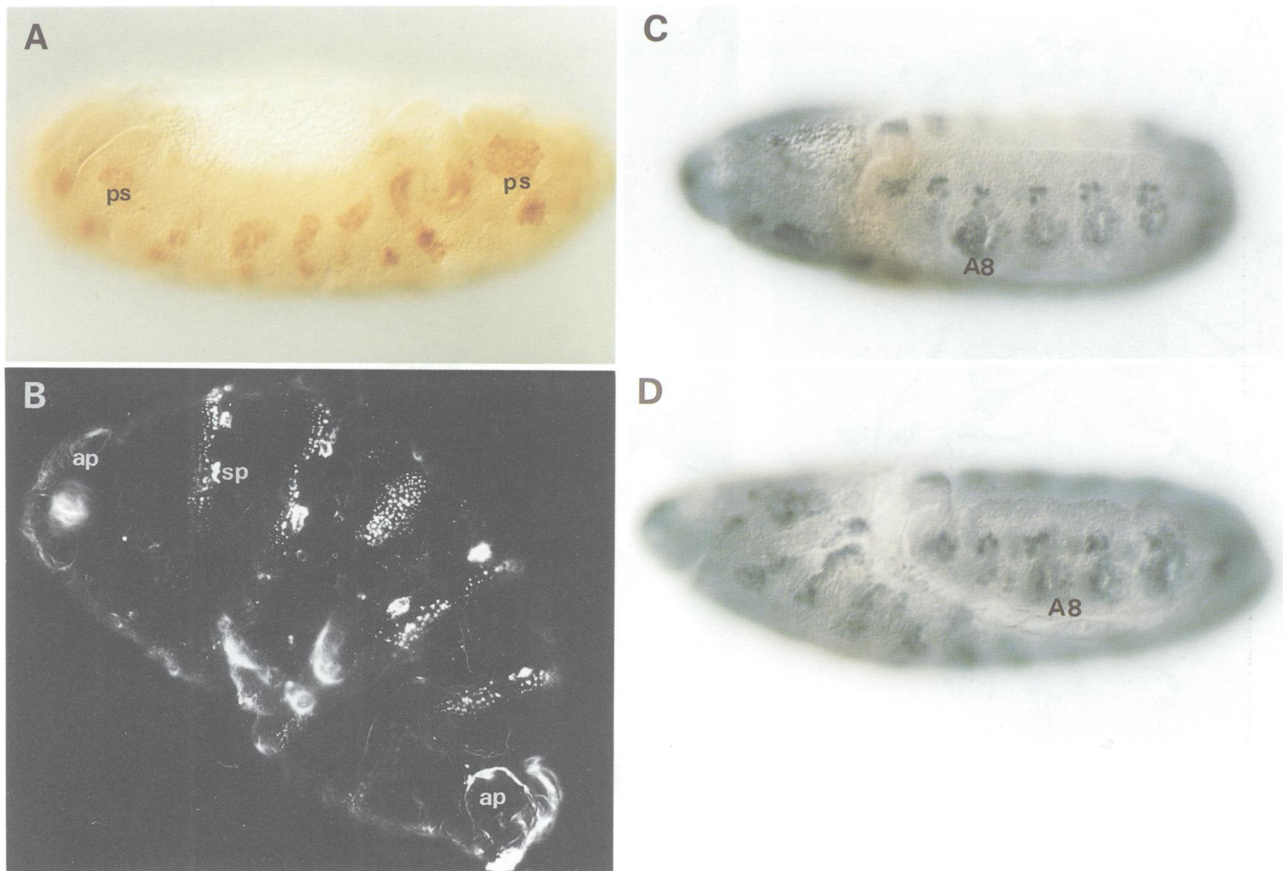

Fig. 4. (**A**) Anti-ems antibody staining of a *nosA* embryo, defective in *bcd* and *hb* activities, showing the metameric *ems* expression. (**B**) Larva of *nosA Scr⁻ Antp⁻ Ubx⁻ abd-A⁻ Abd-B⁻* genotype showing sp in the segments that remain, demonstrating that these cephalic elements do not depend on the *bcd–hb* cascade. (**C**) *ems* expression in the abdominal segments of a wild-type embryo at the extended germ band period. The ems protein accumulates around the tracheal pits and in a group of cells in a more ventral position. In the A8 segment, the precursor cells of the filzkorpers also accumulate the product. (**D**) A *Scr⁻ Antp⁻ Ubx⁻ abd-A⁻ Abd-B⁻* embryo stained with the anti-ems antibody. The only difference that can be observed with respect to (C) is the lack of stain in the filzkorpers precursor cells in A8, which is stained just like the other segments.

proximal region of the appendage. In the leg discs the ems label is restricted to a region in the periphery of the disc, corresponding to the proximal regions of the appendage.

Since a low, and yet functional, level of *ems* product might be undetectable by the labelling methods used, we checked the functional requirements by examining the developmental effects in the adult structures of clones of cells lacking wild-type *ems* function (see Materials and methods for details). Two different experiments were performed. In the first, the mutant clones were deficient for *ems* function only, and different body parts were inspected for marked clones. A large number of clones were found and they all differentiate normally. For example, out of a total of 430 heads irradiated at 48–96 h of development, 33 Ki^+ ems^{9Q} Sb^+ $M(3)w^+$ clones were found that covered all adult head structures and none of them showed any significant pattern alteration (Figure 5A). Out of a sample of 408 legs irradiated at 24–48 h of development, we found 14 large clones (Figure 5B) which differentiate normal leg patterns. Similar numbers of clones were studied in other body regions and found to have normal patterns.

The second experiment was conceived to test the possible suppression of *ems* by *Antp* and *Scr*. It is known that *Scr⁻ Antp⁻* clones in the first and second legs differentiate antenna-like structures (Struhl, 1982) and, since *Antp* and *Scr* suppress *ems* function in the embryo, we reasoned they could also suppress some adult *ems* function. Thus, we generated and compared the phenotype of *Scr⁻ Antp⁻ ems⁺* and *Scr⁻ Antp⁻ ems⁻* clones in the legs. Our triple mutant clones in first and second legs ($n = 7$) differentiate antennal structures (Figure 5C) similar to those reported by Struhl (1982) for *Scr⁻ Antp⁻ ems⁺* clones and which we also observed in our control experiments. Thus, there appears to be no requirement for *ems* function in adult development. This contrasts with the need for *ems* function for the normal development of the larval head and is a intriguing observation, for it suggests that the adult and larval heads follow, at least in part, different developmental strategies.

Discussion

ems is the gene responsible for sclerotic plates and is phenotypically suppressed by the HOM-C genes in normal development

Our results indicate that the cephalic sp, which appear in every segment of larvae lacking the HOM-C genes, depend on the function of a gene responsible for anterior head development, which we identify as the homeobox gene *ems*. It is possible that some other gene might make a

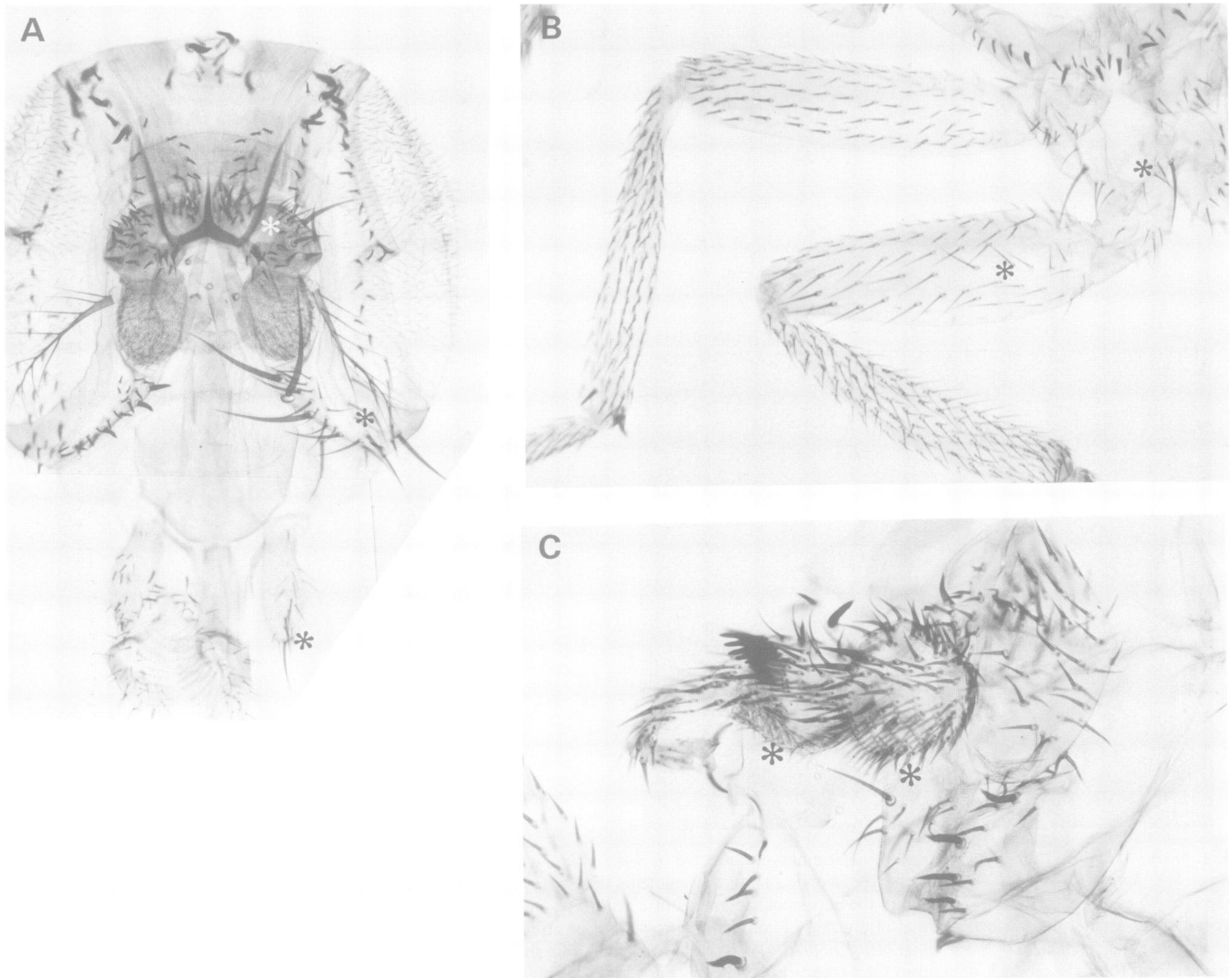

Fig. 5. (**A**) A Ki^+ ems^{9Q} Sb^+ $M(3)w^+$ clone extending to the antenna * to the ventral head * and to the maxillary palp *, and exhibiting a normal pattern. (**B**) Clone of the same genotype as in (A) in the third leg * showing a normal pattern. (**C**) Clone of genotype Ki^+ $Scr^{C1}Antp^{Ns+C3}ems^{9Q}$ Sb^+ $M(3)w^+$ in the first leg differentiating normal distal leg but in the proximal region differentiating antennal structures * corresponding to the third antennal segment. These clones differentiate just like the Ki^+ $Scr^{C1}Antp^{Ns+C3}$ ems^+ Sb^+ $M(3)w^+$ reported by Struhl (1982).

minor contribution. The presence of sp in *Scr*⁻ *Antp*⁻ *Ubx*⁻ *abd-A*⁻ *Abd-B*⁻ *nosA* larvae indicates that sp are produced by the metameric expression of *ems*. The results presented here and in previous work (Lewis, 1978; Struhl, 1983) indicate that the differentiation of the sp can be suppressed by the activity of any homeotic gene, including cephalic genes like *lab*, *Dfd* or *Scr*. This suppression is not due to transcriptional repression, because (with the exception of the filzkorpers domain) the distribution of ems protein is the same in *Scr*⁻ *Antp*⁻ *Ubx*⁻ *abd-A*⁻ *Abd-B*⁻ mutant embryos, where *ems* is fully functional, and in wild-type embryos where it is fully suppressed (Figure 4C and D). Even the excess of homeotic function produced by overexpression of *Ubx* and other homeotic genes fails to have an effect on *ems* expression. Thus, this is an example of phenotypic suppression similar to those in which although different homeotic products are co-expressed only one of the products is functionally dominant (González-Reyes and Morata, 1990; Sánchez-Herrero *et al.*, 1994). However, the significant difference is that, while the heat shock experiments describe an artificial situation, the silencing of *ems* by the HOM-C genes provides the first example of phenotypic suppression operating in normal development, suggesting that it may be functionally significant. The similar phenomenon 'posterior prevalence', described by Duboule (1991) for the mouse *Hox* genes, indicates that it has been conserved in evolution.

ems may have been a member of the original Hox complex; the control by bcd and hb may be a recent acquisition

The involvement of the homeobox *ems* gene in the segment patterning along the anteroposterior axis is of interest because all the other homeobox genes connected with this process, with the possible exception of *otd*, belong to the HOM-C whereas *ems* does not. However, a homeobox gene with some homology to *ems* has been found recently in the nematode *Hox* complex (Wang *et al.*, 1993), raising the possibility that *ems* might have been part of the original homeotic complex. Moreover, *ems* contains an intron in the some position as *lab*, *pb* and *Abd-B*, and the ems protein includes the hexapeptide IYPWMK, characteristic of the homeotic cluster genes (for a review,

see Gehring *et al.*, 1994). Note that in *Drosophila* and perhaps other higher dipterans, the homeotic complex may be falling apart: the ANT and the BX subcomplexes have already separated, whereas in other insects like *Tribolium* (Beeman, 1987) they are linked. In primitive chordates such as *Amphioxus* there is one Hox complex with all genes tightly linked (García-Fernández and Holland, 1994) and, even in mammals where large scale duplications have produced four Hox paralogue copies, the original linkage is conserved (Duboule and Dolle, 1989; Graham *et al.*, 1989). In *Drosophila*, *ems* is located in the same chromosome arm as the ANT-C and BX-C genes; the disintegration of the original complex may have produced two major groups of genes, the ANT-C and the BX-C, leaving a third group, the *ems* gene, in between.

The formation of sp is induced by the *bcd–hb*-independent metamerized expression of *ems*, which is the function suppressed by the thoracic and abdominal homeotic genes. Given the evolutionary conservation of the homeotic genes, it is possible that this metamerized expression represents the primordial function of *ems*, connected with the formation of head structures by modifying an archetypal thoracic-like pattern. The idea that the primordial segment pattern is thoracic-like (suggested by Lewis, 1978) is supported by the phenotype of larvae like the one in Figure 3B in which the absence of the *ems* and the HOM-C genes produces a pattern resembling a thoracic one. It is also supported by recent observations in which the ectopic expression of *Hoxd* genes in the head of *Drosophila* produces an inactivation of the resident homeotic genes which results in a thoracic transformation of the head segments (Bachiller *et al.*, 1994). The implication is that the head develops from a modification of a thoracic-like scaffold and that *ems* was the gene (or one of the few) first involved in this process. The tiers of *ems* expression regulated by *bcd–hb* and by *Abd-B* might be evolutionary novel acquisitions in which the *ems* product is re-used for a different function. This is consistent with the idea that *bcd* is a member of the *Drosophila* ANT-C not conserved in other species, and has a recent origin within the ANT-C (Simpson-Brose *et al.*, 1994). However, we note that the expression of the *emx* genes in vertebrates is restricted to the cephalic region (Simeone *et al.*, 1992), which in principle argues against our interpretation.

The metameric *ems* function was modified by the ANT-C and BX-C products, which altered the *ems*-dictated pattern towards gnathal, thoracic and abdominal patterns, a process that involved the silencing the *ems* product through phenotypic suppression. As has been pointed out (Duboule, 1991; Duboule and Morata, 1994), the phenotypic suppression/posterior prevalence phenomenon might be a mechanism invented by evolution to eliminate a homeotic gene function no longer required in development. Though, in principle, one gene function could be eliminated more directly by switching off transcription, this requires fine tuned *cis*-acting machinery connected with *trans*-acting products which have to be regulated temporally and spatially as, for example, the expression of the gap genes (Hulskamp *et al.*, 1990; see also an overall description in Lawrence, 1992). Evolution may have found it simpler to silence a function just by adding a gene product which competes out the original one.

Materials and methods

Mutant chromosomes and stocks

The mutant alleles *ems*^{9Q64}, *ems*^{9H83}, *btd*^{XG81}, *otd*^{YH13}, have been described (Jürgens *et al.*, 1984; Wieschaus *et al.*, 1984; Dalton *et al.*, 1989). Embryos homozygous for *ems*^{9Q64} do not show detectable label with anti-ems antibody. The ANT-C mutations were *Scr*^{C1} and *Antp*$^{Ns-C3}$ (Struhl, 1983); *bcd*^{1} (Frohnhöfer and Nüsslein-Volhard, 1986) and the *Df(3R)Scr* (Lewis *et al.*, 1980) in which all the ANT-C genes are deleted. The BX-C mutations *Df(3R)P9* (Lewis, 1978) and the triple combination *Ubx*^{MX2} *abd-A*^{M1} *Abd-B*^{M8} (Casanova *et al.*, 1987) are completely deficient in BX-C function, while *Df(3R)109* (Lewis, 1978; Morata *et al.*, 1983) is deficient for *Ubx* and *abd-A* activity, but retains *Abd-B* function. Embryos homozygous for *Df(3R)109* develop normal terminalia, which have been used as a diagnostic feature. *Abd-B*^{M1} is a null mutation (Casanova *et al.*, 1986). *Pc*^{3} is a strong mutation at the *Polycomb* locus (Lewis, 1978). It was balanced with TM3 *hb–lacZ* to allow identification of homozygous embryos. The *nosA* construct (Gavis and Lehmann, 1992) inactivates the *bicoid* and *hunchback* products. The progeny of *nosA* females show null *bcd* and *hb* phenotype. *nosA/Scr*$^-$ *Antp*$^-$ *Ubx*$^-$ *abd-A*$^-$ *Abd-B*$^-$ females were crossed to *Scr*$^-$ *Antp*$^-$ *Ubx*$^-$ *abd-A*$^-$ *Abd-B*$^-$ /TM6B males to generate embryos lacking the function of the five homeotic genes and *bcd* and *hb* activity.

Mitotic recombination experiments

We used X-ray-induced mitotic recombination and the Minute method (Morata and Ripoll, 1975) to generate cells homozygous for the *ems*^{9Q} allele which at the same time lose the dominant marker mutants *Ki* and *Sb*^{63}. The chromosome *Ki Sb*^{63} *M(3)w*^{124} has been used to mark *ems*^{9Q} mutant cell clones induced in the right arm of the third chromosome (Struhl, 1982). Since *ems* is located in between *Ki* and *Sb*, any event of mitotic recombination proximal to *Ki* (and many but not all proximal to *Sb*) will result in a cell mutant for *ems*^{9Q} which at the same time loses the dominant *Ki*, *Sb*^{63} and *M(3)w*^{124} alleles, thus showing wild-type bristle phenotype. Because of the loss of the retarding *Minute* condition, it will also proliferate faster than surrounding cells. The same marking method has been used to generate clones deficient for *Scr*, *Antp* and *ems* functions. The chromosome *Scr*^{C1}*Antp*$^{Ns-C3}$*ems*^{9Q} was obtained by recombining the *ems* mutation with the double mutant *Scr*^{C1}*Antp*$^{Ns-C3}$ chromosome (a gift from G.Struhl).

Heat shock treatments and cuticular preparation of larvae and adults

The following heat shock genes were used: *hsp70-lab* (HSL; Heur and Kaufman, 1994), *hsp70-Dfd* (HSD; Kuziora and McGinnis, 1988) and *hsp70-Ubx* (HSU; González-Reyes *et al.*, 1990). Embryos were collected and treated as described in González-Reyes and Morata (1990) and González-Reyes *et al.* (1992) and given two heat pulses (37°C, except for HSL, 35°C) of 45 min, one at 3–5 h and the second at 5–7 h of development.

First instar larvae were dechorionated in commercial bleach and the vitelline membrane removed using heptano-methanol 1:1. Then, after washing with methanol and 0.1% Triton X-100, larvae were mounted in Hoyer's lactic acid 1:1 and allow to clear at 60°C for at least 2 h. Adult flies of the appropriate genotype were dissected in alcohol and cut in pieces. These were subsequently treated with 10% KOH to digest internal tissues, washed with propanol and mounted in Euparal.

Antibody production

For general procedures in antibody production, we followed the protocols as in Harlow and Lane (1988). Bacterial ems protein was prepared as a fusion with GST using the pGEX expression plasmid and *Escherichia coli* strain HB101 as described (Smith and Johnson, 1988; Guan and Dixon, 1991). A *Bcl*I–*Bam*HI fragment extending from nucleotide 957 of the *ems* cDNA to the *Bam*HI site in the pGEM 4 polylinker (Dalton *et al.*, 1989) was cloned into the *Bam*HI site of pGEX 3X expression vector. The GST–ems fusion protein contains the C-terminal 272 amino acid residues of ems, which includes the homeodomain. A polyclonal antiserum against this GST–ems fusion protein was raised in rats. We performed four injections with 150 μg of protein in each dose. The second injection was 30 days after the first, and the other boosters were given with a 2-week interval.

Antibody staining and in situ hybridization

The protocol for antibody staining used is described in Macías *et al.* (1990). To reduce background, the embryos were incubated for 5 min

in glutaraldehyde solution 1% in phosphate-buffered saline 0.1% Tween (PBT) before adding the DAB staining solution. *In situ* hybridization was performed as originally described by Tautz and Pfeifle (1989), with the modifications introduced by M.Klingler (Simpson-Brose *et al.*, 1994). In many of the experiments, we performed a double staining procedure with the set of antibodies available in the laboratory (anti-Scr, Ubx, abd-A, Abd-B, ems, etc.) to identify the appropriate embryos in crosses.

Acknowledgements

We thank Ernesto Sánchez-Herrero and Sergio González for their help and suggestions and Ernesto Sánchez-Herrero, Isabel Guerrero and Peter Lawrence for critical reading of the manuscript. We also thank the following people who sent us various materials: Ernest Wimmer for the *btd*^{XG81} allele, Robert Finkelstein for the *otd* mutations and *otd* cDNA, Bill McGinnis for the *ems* alleles and cDNA, Gary Struhl for the *Df(3R) Scr Df(3R)P9* chromosome, Mariann Bienz for the *hsp70-lab* minigene, T.Kaufman for the lab antibody and Claude Desplan for the *nosA* construct. Rosa Gonzalez gave excellent technical assistance. This work has been supported by grants from the Dirección General de Investigación y Ciencia and the Human Frontier Science Program Organization (RG 372/94). We also received an institutional grant from the Ramon Areces Foundation.

References

Akam,M. (1989) Hox and HOM: homologous gene clusters in insects and vertebrates. *Cell*, **57**, 347–349.

Akam,M., Auerof,M., Castelli-Gair,J., Dawes,R., Falciani,F. and Ferrier,D. (1994) The evolving role of Hox genes in arthropods. *Development* (Suppl.), 209–215.

Bachiller,D., Macías,A., Duboule,D. and Morata,G. (1994) Conservation of a functional hierachy between mammalian and insect Hox/HOM genes. *EMBO J.*, **13**, 1930–1941.

Beeman,R. (1987) A homeotic gene cluster in the red flour beetle. *Nature*, **327**, 247–249.

Busturia,A. and Morata,G. (1988) Ectopic expression of homeotic genes caused by the elimination of the *Polycomb* gene in *Drosophila* imaginal epidermis. *Development*, **104**, 713–720.

Campos-Ortega,J. and Hartenstein,V. (1985) *The Embryonic Development of Drosophila melanogaster.* Springer-Verlag, Heidelberg.

Capovilla,M., Brant,M. and Botas,J. (1994) Direct regulation of dpp by *Ultrabithorax* and its role in *Drosophila* midgut morphogenesis. *Cell*, **76**, 461–475.

Casanova,J., Sánchez-Herrero,E. and Morata,G. (1986) Identification and characterization of a parasegment specific regulatory element of the *Abdominal* gene of *Drosophila. Cell*, **47**, 627–636.

Casanova,J., Sánchez-Herrero,E., Busturia,A. and Morata,G. (1987) Double and triple mutant combinations of the *bithorax* complex of *Drosophila. EMBO J.*, **6**, 3103–3109.

Chadwick,R. and McGinnis,W. (1987) Temporal and spatial distribution of transcripts from the *Deformed* gene of *Drosophila. EMBO J.*, **6**, 779–789.

Chouinard,S. and Kaufman,T.C. (1991) Control of expression of the homeotic *labial* (*lab*) locus of *Drosophila melanogaster*: evidence for both positive and negative autogenous regulation. *Development*, **113**, 1267–1280.

Cohen,S.M. and Jürgens,G. (1990) Mediation of *Drosophila* head development by gap-like segmentation genes. *Nature*, **346**, 482–485.

Dalton,D., Chadwick,R. and McGinnis,W. (1989) Expression and embryonic function of *empty spiracles*: a *Drosophila* homeo box gene with two patterning functions on the anterior–posterior axis of the embryo. *Genes Dev.*, **3**, 1940–1956.

Diederich,R., Merrill,V., Pultz,M. and Kaufman,T. (1989) Isolation, structure, and expression of *labial*, a homeotic gene of the *Antennapedia* complex involved in *Drosophila* head development. *Genes Dev.*, **3**, 399–414.

Duboule,D. (1991) Patterning in the vertebrate limb. *Curr. Opin. Genet. Dev.*, **1**, 211–216.

Duboule,D. and Dolle,P. (1989) The structural and functional organization of the murine HOX gene family resembles that of *Drosophila* homeotic genes. *EMBO J.*, **8**, 1497–1505.

Duboule,D. and Morata,G. (1994) Colinearity and functional hierarchy among genes of the homeotic complexes. *Trends Genet.*, **10**, 358–364.

Finkelstein,R., Smouse,D., Capaci,T., Spradling,A. and Perrimon,N. (1990) The *orthodenticle* gene encodes a novel homeo domain protein involved in the development of the *Drosophila* nervous system and ocellar visual structures. *Genes Dev.*, **4**, 1516–1527.

Frohnhöfer,H. and Nüsslein-Volhard,C. (1986) Organization of anterior pattern in the *Drosophila* embryo by the maternal gene *bicoid. Nature*, **324**, 120–125.

García-Fernández,J. and Holland,P.W.H. (1994) Archetypal organization of the *Amphioxus* Hox gene cluster. *Nature*, **370**, 363–566.

Gavis,E. and Lehmann,R. (1992) Localization of nanos RNA controls embryonic polarity. *Cell*, **71**, 301–313.

Gehring,W., Affolter,M. and Bürglin,T. (1994) Homeodomain proteins. *Annu. Rev. Biochem.*, **63**, 487–526.

Gibson,G. and Gehring,W. (1988) Head and thoracic transformations caused by ectopic expression of *Antennapedia* during *Drosophila* development. *Development*, **102**, 657–675.

Gónzalez-Reyes,A. and Morata,G. (1990) The developmental effect of overexpressing a Ubx product in *Drosophila* embryos is dependent on its interactions with other homeotic products. *Cell*, **61**, 515–522.

González-Reyes,A., Urquía,N., Gehring,W.J., Struhl,G. and Morata,G. (1990) Are cross-regulatory interactions between homeotic genes functionally significant? *Nature*, **344**, 78–80.

González-Reyes,A., Macías,A. and Morata,G. (1992) Autocatalysis and phenotypic expression of *Drosophila* homeotic gene *Deformed*: its dependence on polarity and homeotic gene function. *Development*, **116**, 1059–1068.

Graham,A., Papalopulu,N. and Krumlauf,R. (1989) The murine and *Drosophila* homeobox gene complexes have common features of organization and expression. *Cell*, **57**, 367–378.

Guan,K. and Dixon,J.E. (1991) Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase. *Anal. Biochem.*, **192**, 262–267.

Harlow,E. and Lane,D. (1988) *Antibodies: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Heuer,J.G. and Kaufman,T.C. (1992) Homeotic genes have specific functional roles in the establishment of the *Drosophila* embryonic peripheral nervous system. *Development*, **115**, 35–47.

Hulskamp,M., Pfeifle,C. and Tautz,D. (1990) A morphogenetic gradient of hunchback protein organizes the expression of the gap genes *Kruppel* and *knirps* in the early *Drosophila* embryo. *Nature*, **346**, 577–580.

Jones,B. and McGinnis,W. (1993) The regulation of *empty spiracles* by *Abdominal-B* mediates an abdominal segment identity function. *Genes Dev.*, **7**, 229–240.

Jürgens,G., Wieschaus,E., Nüsslein-Volhard,C. and Kluding,H. (1984) Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster. Wilhelm Roux's Arch. Dev. Biol.*, **193**, 283–295.

Jürgens,G., Lehman,R., Schardin,M. and Nüsslein-Volhard,C. (1986) Segmental organization of the head and embryo of *Drosophila melanogaster. Roux's Arch. Dev. Biol.*, **195**, 359–377.

Kaufman,T., Seeger,M. and Olsen,G. (1990) Molecular and genetic organization of the *Antennapedia* gene complex of *Drosophila melanogaster. Adv. Genet.*, **27**, 309–361.

Kuziora,M. and McGinnis,W. (1988) Autoregulation of a *Drosophila* homeotic selector gene. *Cell*, **55**, 477–485.

Lawrence,P.A. (1992) *The Making of a Fly.* Blackwell Scientific Publications, Oxford.

Lewis,E. (1978) A gene complex controlling segmentation in *Drosophila. Nature*, **276**, 565–570.

Lewis,R., Wakimoto,B., Denell,R. and Kaufman,T. (1980) Genetic analysis of the *Antennapedia* gene complex (ANT-C) and adjacent chromosomal regions of *Drosophila melanogaster.* II. Polytene chromosomal segments 84A–84B1,2. *Genetics*, **93**, 383–397.

Lindsley,D.L. and Zimm,G. (1992) *The Genome of Drosophila melanogaster.* Academic Press, San Diego, CA.

Macías,A., Casanova,J. and Morata,G. (1990) Expression and regulation of the *abd-A* gene of *Drosophila. Development*, **110**, 1197–1207.

Manak,R.J. and Scott,M.P. (1994) A class act: conservation of homeodomain protein functions. *Development* (Suppl.), 61–71.

Mann,R. and Hogness,D. (1990) Functional dissection of Ultrabithorax proteins in *D.melanogaster. Cell*, **60**, 597–610.

McGinnis,W. and Krumlauf,R. (1992) Homeobox genes and axial patterning. *Cell*, **68**, 283–302.

Merrill,V., Diederich,R., Turner,F. and Kaufman,T. (1989) A genetic and developmental analysis of mutations in *labial*, a gene necessary for proper head formation in *Drosophila melanogaster. Dev. Biol.*, **135**, 376–391.

Morata,G. and Ripoll,P. (1975) Minutes: mutants of *Drosophila* autonomously affecting cell division rate. *Dev. Biol.*, **42**, 211–221.

Morata,G., Botas,J., Kerridge,S. and Struhl,G. (1983) Homeotic transformations of the abdominal segments of *Drosophila* caused by breaking or deleting a central portion of the bithorax complex. *J. Embryol. Exp. Morphol.*, **78**, 319–341.

Reuter,R., Panganiban,R., Hoffmann,F. and Scott,M. (1990) Homeotic genes regulate the spatial expression of putative growth factors in the visceral mesoderm of *Drosophila* embryos. *Development*, **110**, 1031–1040.

Sánchez-Herrero,E., Guerrero,I., Sampedro,J. and Gónzalez-Reyes,A. (1994) Developmental consequences of unrestricted expression of the *abd-A* gene of *Drosophila*. *Mech. Dev.*, **46**, 153–167.

Simeone,A., Gulisano,M., Acampora,D., Stornaiuolo,A., Rambaldi,M. and Boncinelli,E. (1992) Two vertebrate *homeobox* genes related to the *Drosophila empty spiracle* gene are expressed in the embryonic cerebral cortex. *EMBO J.*, **11**, 2541–2550.

Simpson-Brose,M., Treisman,J. and Desplan,C. (1994) Synergy between the hunchback and bicoid morphogens is required for anterior patterning in *Drosophila*. *Cell*, **78**, 855–865.

Smith,D.B. and Johnson,K.S. (1988) Single-step purification of polypeptides expressed in *E.coli* as fusions with glutathione S-transferase. *Gene*, **67**, 31–40.

Struhl,G. (1982) Genes controlling segmental specification in the *Drosophila* thorax. *Proc. Natl Acad. Sci. USA*, **79**, 7380–7384.

Struhl,G. (1983) Role of *esc* product in ensuring the selective expression of segment-specific genes in *Drosophila*. *J. Embryol. Exp. Morphol.*, **76**, 297–331.

Tautz,D. and Pfeifle,C. (1989) A non-radioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma*, **98**, 81–85.

Tremml,G. and Bienz,M. (1992) Induction of *labial* expression in the *Drosophila* endoderm: response elements for dpp signalling and for autoregulation. *Development*, **116**, 447–456.

Wakimoto,B. and Kaufman,T. (1981) Analysis of larval segmentation in lethal phenotypes associated with the *Antennapedia* gene complex in *Drosophila melanogaster*. *Dev. Biol.*, **81**, 51–64.

Walldorf,U. and Gehring,W.J. (1992) *Empty spiracles*, a gap gene containing a homeobox involved in *Drosophila* head development. *EMBO J.*, **11**, 2247–2259.

Wang,B.B., Müller-Immergluck,M.M., Austin,J., Robinson,N.T., Chisholm,A. and Kenyon,C. (1993) A homeotic gene cluster patterns the anteroposterior body axis of *C.elegans*. *Cell*, **74**, 29–42.

Wedeen,C., Harding,K. and Levine,M. (1986) Spatial regulation of *Antennapedia* and *bithorax* gene expression by the *Polycomb* locus in *Drosophila*. *Cell*, **44**, 739–748.

Wieschaus,E., Nüsslein-Volhard,C. and Jürgens,G. (1984) Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. *Wilhelm Roux's Arch. Dev. Biol.*, **193**, 296–307.

Received on August 9, 1995; revised on September 19, 1995