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Conservation of a functional hierarchy between mammalian and insect Hox/HOM genes

Daniel Bachiller, Ana Macías[1], Denis Duboule[2] and Ginés Morata[1,3]

European Molecular Biology Laboratory, 6900 Heidelberg, Germany and [1]Centro de Biologia Molecular, UAM-CSIC, 28049 Madrid, Spain

[2]Present address: Department of Zoology, Sciences III, University of Geneva, Quai Ernest-Ansermet, 1204 Geneva, Switzerland

[3]Corresponding author

D.Bachiller and A.Macías have contributed equally to this work

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We have generated several transgenic *Drosophila* strains containing different mouse *Hox* genes under heat shock control and studied how their generalized expression affects *Drosophila* larval patterns. We find that they have spatially restricted effects which correlate with their genetic order and expression pattern in the mouse; as they are expressed more posteriorly in the mouse, they have more extensive effects in *Drosophila*. The generalized expressions of *Hoxd-8* and *d-9* modify *Drosophila* anterior head segment(s), but have no effect in the rest of the body. *Hoxd-10* expression affects head and thorax, but not the abdomen. Finally, *Hoxd-11* alters head, thorax and abdomen. The developmental effect of the *Hox* genes consists of a homeotic transformation of the affected segment(s), which exhibit a 'ground' pattern similar to that obtained in the absence of homeotic information, suggesting that *Hox* genes are able to inactivate *Drosophila* homeotic genes, but do not specify a pattern of their own. A partial exception is *Hoxd-11* which, even though it has a general suppressing effect, can also activate the resident *Abdominal-B* and *empty spiracles* genes in ectopic positions. Our results strongly suggest a general conservation of the functional hierarchy of homeotic genes that correlates with genetic order and expression patterns.

Key words: **homeotic genes/Hox genes/functional hierarchy/phenotypic suppression/posterior prevalence**

Introduction

The homeotic genes occupy a key role in development, for it is through their function that morphological diversity is achieved. In *Drosophila*, where they have been extensively studied, the homeotic complex (the HOM-C, Akam, 1989) consists of eight genes clustered in two groups, the ANT and the BX complexes (Lewis, 1978; Sánchez-Herrero *et al.*, 1985; Kaufman *et al.*, 1990) although the splitting in two groups is not an essential feature; in other insect species (Beeman, 1987) they form a single cluster.

Recently, it has been shown that the homeotic clusters have been conserved in evolution and have a similar organization throughout the animal kingdom; homologs of *Drosophila* homeotic genes have been identified in mammals (the *Hox* genes) and other animal groups, and the relative order of the genes within the homeotic clusters has been found to be conserved (Duboule and Dollé, 1989; Graham *et al.*, 1989). This may turn out to be an important observation, for it suggests that a universal genetic system, the homeotic complex, is responsible for generating the morphological diversity of the animal body. The clustering of the genes and sequence analysis indicate that the *Hox/HOM* complexes arose by successive tandem duplications of an ancestral homeobox-containing gene. An important difference between insects and vertebrates is that in the latter a series of subsequent large scale duplications have generated four *Hox* clusters per haploid genome (the paralog groups), while there is only one homeotic complex in insects (Gaunt *et al.*, 1988; Graham *et al.*, 1989).

A common property of *Hox/HOM* genes in *Drosophila* and vertebrates is that of 'colinearity', a fascinating and mysterious correlation between the order of the genes in the cluster, or 'genetic order', and their expression patterns along the body (Lewis, 1978; Gaunt *et al.*, 1988; Duboule and Dolle, 1989; Graham *et al.*, 1989). The genes located more 5′ are expressed more posteriorly in the body, whether *Drosophila* or the mouse, and moving towards the 3′ end they are expressed more anteriorly. Since the expression patterns are most probably established by *cis*-regulatory elements, the phenomenon of colinearity argues for conservation of regulatory sequences. Indeed, there is recent evidence for conservation of *cis*-regulatory elements of the *Drosophila* gene *Deformed (Dfd)* and its mouse homolog (Awgulewitsch and Jacobs, 1992; Malicki *et al.*, 1992).

In *Drosophila*, the expression patterns (and hence the genetic order) also correlate with a functional hierarchy: the homeotic genes which are expressed in the posterior regions of the body functionally suppress those determining anterior identities (Struhl, 1983; González-Reyes *et al.*, 1990). In several cases it has been shown that this occurs even though the suppressed gene is transcriptionally and translationally active at high levels (González-Reyes and Morata, 1990; Gonzalez-Reyes *et al.*, 1990). This functional silencing of one homeoproduct by another has been called 'phenotypic suppression' (González-Reyes and Morata, 1990).

In vertebrates, the *Hox* genes are expressed in partial overlapping domains and follow a sequential activation that correlates with their position along the cluster (Izpisúa-Belmonte *et al.*, 1991). Genes located at the 3′ end of the complex are expressed earlier than those placed more 5′, and the patterning information appears to be provided by the more posterior acting gene. This phenomenon was termed 'posterior prevalence' (Duboule, 1991), and appears to be similar to the phenotypic suppression by *Drosophila* posterior genes described above. More recently, the idea of posterior genes overriding the effect of anterior ones in the areas in which their expression domains overlap gained

support from the results of the disruption of *Hox* genes in mice. In these mutants, phenotypic alterations are observed in the structures derived from the part of the expression domain of the gene which does not overlap with that of any more posterior gene of the same complex (Chisaka and Capecchi, 1991; Lufkin *et al.*, 1991).

Detailed functional analysis of this phenomenon in vertebrates is hampered by the possibility of functional redundancy and interactions with paralog genes from other complexes, as well as technical difficulties. To overcome some of these problems, we have assayed and compared the morphological effects in *Drosophila* embryos of the *Hoxd-8*, *d-9*, *d-10* and *d-11* genes (see Scott, 1992 for the latest nomenclature of the *Hox* complex). The *Hoxd-9–d-11* genes are homologs of *Drosophila Abdominal-B* (*Abd-B*), and *Hoxd-8* is more related to *abd-A–Ubx–Antp* (Izpisúa-Belmonte *et al.*, 1991). Their expression patterns in the mouse are well known and show a strict correlation with genetic order (Duboule, 1992).

We generated transgenic lines containing gene constructs in which the coding region of each *Hox* gene is under the control of the *hsp70* promoter. In the interpretation of the results we have taken advantage of the previous knowledge of the effects of heat shock driven homeotic products on *Drosophila* embryos (Gibson and Gehring, 1988; González-Reyes and Morata, 1990; González-Reyes *et al.*, 1990; Mann and Hogness, 1990). Our results indicate that there is a correlation between the expression patterns of the *Hox* genes along the antero-posterior body axis of the mouse and the extent of their effect along the antero-posterior body axis of *Drosophila*, suggesting that the functional hierarchy is a universal property of the homeotic complexes.

Results

Developmental effects of Hox genes in Drosophila assayed in different genetic backgrounds

We have used the *hsp70* promoter to drive a high and uniform expression of the *Hox* gene all over the *Drosophila* body. This pattern is superimposed upon the normal expression patterns of *Drosophila* homeotic genes; the effect (or not) on each segment of the Hox product will be indicative of its interactions with the local genes. One problem with this approach is the possibility of cross interactions (Hafen *et al.*, 1984; Struhl and White, 1985; Krasnow *et al.*, 1989) between the mouse and *Drosophila*

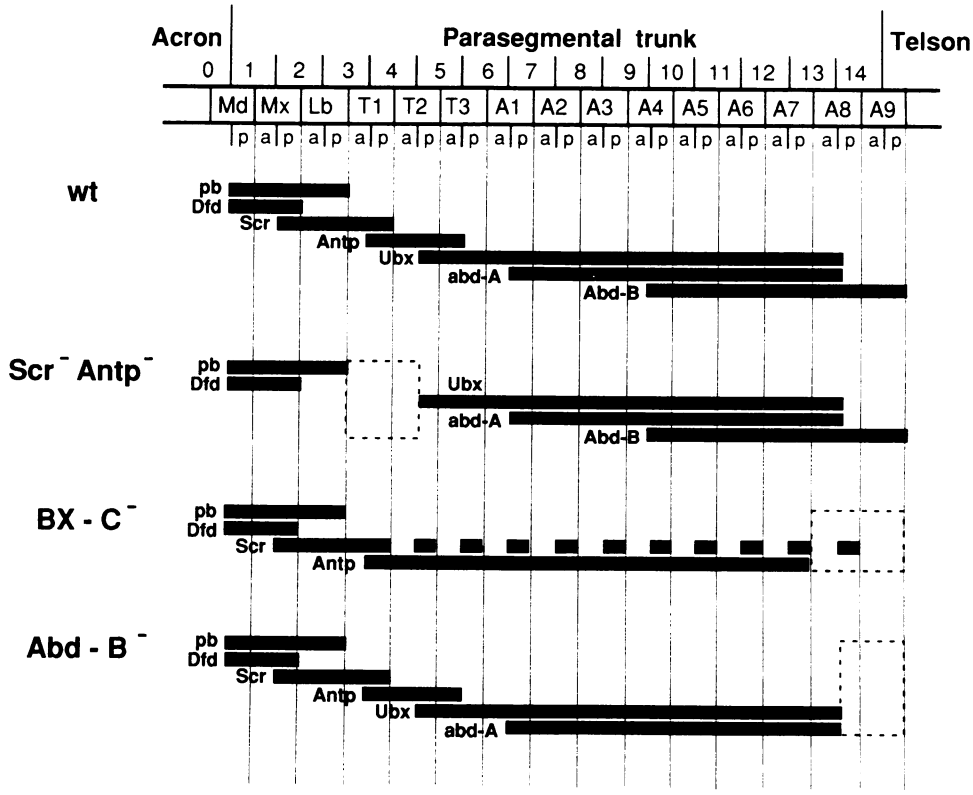

Fig. 1. Expression patterns along the parasegmental trunk of the seven HOM-C genes, *proboscipedia*, (*pb*), *Dfd*, *Scr*, *Antp*, *Ubx*, *abd-A* and *Abd-B* in wild type and mutant genotypes. The more anterior acting gene *labial* has not been considered. The expression patterns are simplified and based on the epidermal expression. In some cases, e.g. *Antp*, the expression in the CNS differs from that in the epidermis, but these complications are not relevant here. Local differences in the levels of expression are also not shown. The parasegments 1, 2 and 3 form most of the posterior part of the head (gnathocephalon) which includes the mandibular (Md), maxillary (Mx) and labial (La) segments. Notice that parasegment 3 also forms the anterior compartment of the first thoracic segment (T1). (1) WT. The entire region from parasegment 1 to 14 is covered at least by one of the seven homeotic genes. The most posterior limit of homeotic expression appears to be the 15th engrailed stripe, marking parasegment 15. (2) *Scr⁻Antp⁻* genotype. The elimination of these two genes results in the T1a–T1p–T2a region, the dashed box, devoid of any known HOM-C information. More anteriorly acting genes like *Dfd* or *pb* are not affected by the loss of *Scr* and *Antp* functions. (3) BX-C⁻ genotype. The loss of the entire BX-C results in derepression of the *Antp* gene down to parasegment 12 (Carroll *et al.*, 1986; Wirz *et al.*, 1986) and of *Scr* in the posterior compartments down to A8p (Pelaz *et al.*, 1993). This defines the region A8a–A9p as without homeotic function, as indicated by the dashed box, except for A8p where there is *Scr* expression (Pelaz *et al.*, 1993), but appears too late to affect the morphology of the compartment. (4) *Abd-B⁻* genotype. The lack of *Abd-B* leaves the region A8p–A9a–A9p, dashed box, without HOM-C activity.

homeoproducts, which may result in a cascade of activation and/or repression of resident homeotic genes. As a consequence, the results observed may be due to a combination of mouse and fly genes. To check on this problem we have studied the effects of the different *Hox* genes, not only in embryos containing a normal set of *Drosophila* homeotic genes, but also in mutant embryos in which at least some segments have no known homeotic activity. In this way we can assay the morphogenetic effects of the different *Hox* genes in isolation. We have used three classes of mutant embryos (Figure 1).

(i) *Scr*$^-$*Antp*$^-$ embryos: these lack the functions of the thorax specifying genes *Sex combs reduced* (*Scr*) and *Antennapedia* (*Antp*). Since these are the only genes of the homeotic cluster active (Kaufman *et al.*, 1990) in the prothorax (T1) and the anterior compartment of the mesothorax (T2), *Scr*$^-$*Antp*$^-$ embryos should in principle contain no homeotic information in this region. The details of the morphology of the *Scr*$^-$*Antp*$^-$ embryos in the thorax are described in Figure 2. The two anterior compartments (T1a and T2a) develop similar thoracic-like denticle belts which, although retaining general thoracic features, are unlike any of the regular thoracic segments. The T1p compartment differentiates some cephalic structures ('sclerotic plates') produced by the inappropriate activation of an as yet unidentified cephalic gene (Lewis, 1978; Struhl, 1983). The fact that there is a thoracic-like pattern (except for the sclerotic plates) in the absence of the thorax determining genes strongly suggests that this may be the 'ground' pattern on which the regular thoracic, abdominal and perhaps cephalic patterns are built.

(ii) *BX-C*$^-$ embryos. In these embryos the lack of BX-C activity produces a derepression of *Antp* in part of the thorax and the abdominal segments A1–A7 (Carroll *et al.*, 1986; Wirz *et al.*, 1986). As a consequence, the anterior compartments of all these metameres develop a T2a pattern. However, this derepression does not reach the A8a compartment (Figure 1), which contains no known homeotic information and differentiates a distinct thoracic-like pattern which closely resembles that found in T1a and T2a of *Scr*$^-$*Antp*$^-$ embryos and must be close to the ground pattern (see Figure 9a). Although there is also ectopic activation of *Scr* in the posterior compartments at the retracting germ band stage (Pelaz *et al.*, 1993) of these embryos (Figure 1), it occurs too late to influence the embryonic pattern in A8p. This compartment differentiates a pair of sclerotic plates, thought to be the result of inappropriate activation of a head specific gene (Lewis, 1978; Struhl, 1983).

(iii) *Abd-B*$^-$ embryos: these contain *Ultrabithorax* (*Ubx*) and *abdominal-A* (*abd-A*) activity in A8a, but not in A8p (White and Wilcox, 1985; Karch *et al.*, 1990; Macías *et al.*, 1990) which develops the same sclerotic plates (Figure 3) as in *BX-C*$^-$.

Unrestricted expression of Hoxd-8 and of d-9 affects only some Drosophila head structures

After a standard heat shock treatment of *Hoxd-8* embryos containing a normal set of homeotic genes, we observe high and uniform expression levels of Hox product (see Material and methods), but a slight, though clear and consistent, morphological effect. A high proportion of embryos, ranging from 60 to 80% in different experiments, show a partial failure of the head invagination. The head is abnormal and some pieces of the head skeleton are consistently missing (Figure 2). Structures which derive from the labial, maxillary and mandibular segments (labial sensory organs, cirri, maxillary sense organs, mouth hooks, T-ribs, ventral arms)

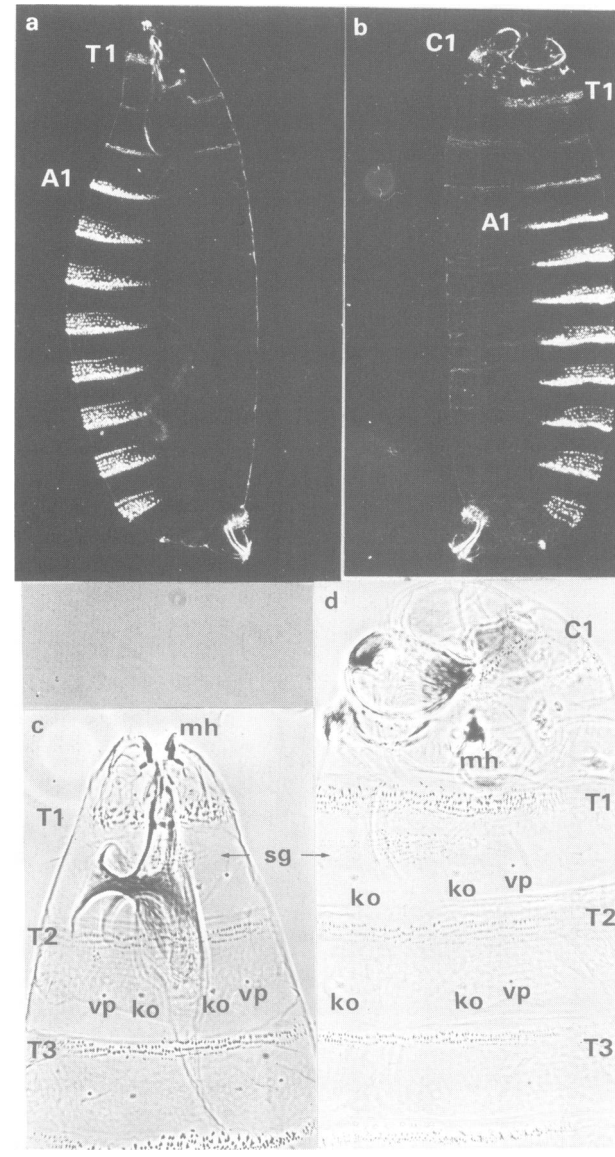

Fig. 2. Developmental effects of *Hoxd-8* and *d-9*. (**a**) Dark field photograph of a wild type larva. Notice the anterior position of the thoracic segments, as indicated by the prothorax (T1). Much of the invaginated head skeleton is situated posterior to T1. (**b**) A strong case of heat shocked *Hoxd-9* larva. Thoracic and abdominal segments are normal but head involution is partially prevented and head skeleton is modified. Note the presence of an extra denticle belt (C1) in dorsolateral position. (**c**) Phase contrast photograph of the head and thorax of a wild type larva in ventral view. The three thoracic (T1, T2 and T3) denticle belts are indicated. Notice in T1 the presence of a centrally located second group of denticles (sg). The three thoracic belts show ventral pits (vp) and Keilin's organs (ko). The mouth hooks (mh) are located in the most anterior position. (**d**) Ventral head and thorax of a *Hoxd-9* larva similar to that portrayed in (b). The head skeleton is altered although some structures like mh and others can be recognized. As a result of the failure of the head involution, the mh are now located in a relatively posterior position in the head. Notice also the presence of a partial denticle belt (C1) in the dorsolateral part of the head (arrow). The three thoracic segments are not modified; the characteristic second group of T1 denticles are present, and T1, T2 and T3 denticle belts are normal. Typical thoracic markers like vp and ko are present.

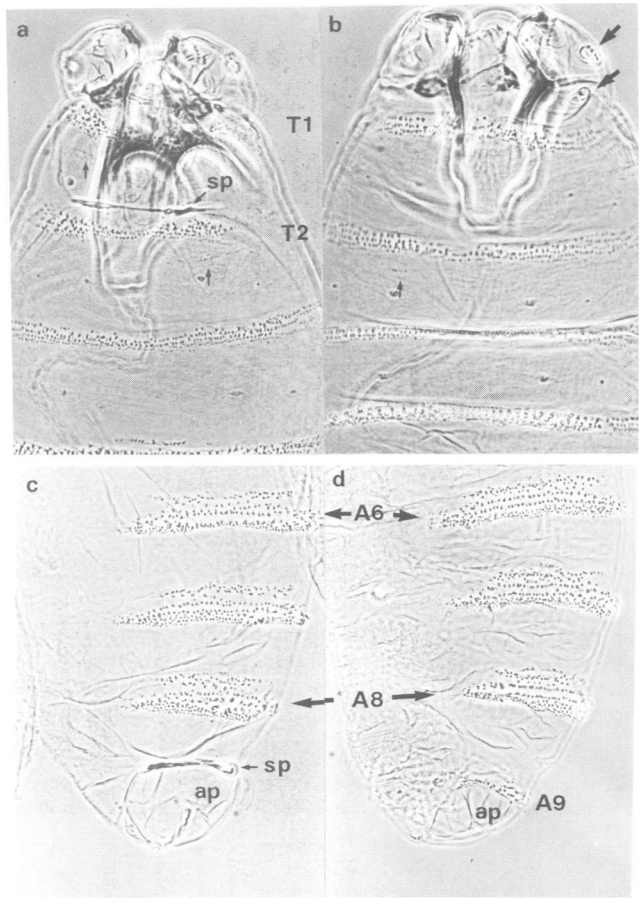

Fig. 3. (**a**) *Scr*$^{-}$*Antp*$^{-}$ larva. The head and thorax of these larvae is quite different from wild type (compare with Figure 2). This is because the *Scr* gene is required to specify the normal T1 segment (Kaufman *et al.*, 1990), while *Antp* is required to specify T2. The T1 and T2 belts are similar and in between, but closer to the T2 belt, there is a pair of refringent cephalic structures, called sclerotic plates (sp), probably produced by the derepression of a head specific gene (Lewis, 1978; Struhl, 1983). The size of denticles is like in T1 but the shape of the denticle belt is characteristic, with a narrowing in the center. Frequently, there are small groups of denticles separate from the main belt (thin arrows). (**b**) *Hoxd-8 Scr*$^{-}$*Antp*$^{-}$ larva after heat shock. It resembles in every aspect a *Scr*$^{-}$*Antp*$^{-}$ larva, but the sclerotic plates are missing. Notice the duplicated maxillary sense organs (thick arrows), a distinctive feature of *Scr* mutations. (**c**) Posterior segments of an *Abd-B*^{M1} larva. The A6, A7 and A8 denticle belts look similar because of the lack of *Abd-B*, responsible for the morphological diversity in the A5–A8 region (Sánchez-Herrero *et al.*, 1985). There is a characteristic region of naked cuticle between the anal pads (ap) and the A8 belt. Just anterior to the anal pads there is a pair of sclerotic plates very similar to those of T1p in *Scr*$^{-}$*Antp*$^{-}$ larvae, which are probably the result of the derepression of the same cephalic gene in the absence of *Abd-B*. These plates appear in the posterior compartment of A8 (Casanova *et al.*, 1986). (**d**) *Hoxd-9 Abd-B*^{M1} larva. The critical difference is the elimination of the sclerotic plates and the presence of an extra denticle belt in A9.

are present, but those deriving from more anterior head segments (vertical plate, dorsal arm, labrum) are often missing. A variable proportion of embryos, from 20 to 60%, show groups of thoracic denticles inside the head, suggesting a partial thoracic transformation of a head primordium. The situation of the thoracic denticles in the transformed head is characteristic, they appear in the anterior dorsolateral part of the head, just the position where ectopic expression of *Drosophila* homeotic genes like *Ubx* or *Antp* produce homeotic transformation of the head C1 segment (González-Reyes and Morata, 1991) which derives from the procephalon, the most anterior region of the head. The thoracic and abdominal segments remain like the wild type.

The morphological effects of *Hoxd-9* in embryos with a normal set of homeotic genes are in general indistinguishable from those found with *Hoxd-8*, although the observed transformations are often stronger; a thoracic belt appears more frequently in the head and is more completely formed. The position of this belt, as in the previous case, corresponds to that of the C1 primordium. These observations suggested that *Hoxd-8* and *d-9* can suppress some cephalic *Drosophila* gene(s), but are suppressed by the *Drosophila* thoracic and abdominal genes. However, this could also reflect some more trivial factor such as differential perdurance of the Hox product in the anterior region. We checked this by overexpressing *Hoxd-8* and *d-9* genes in embryos mutant for *Abd-B*. We reasoned that if they suppress cephalic structures *in situ*, they might suppress the cephalic structures (sclerotic plates) present in A8p, at the end of the abdomen, of *Abd-B*$^{-}$ embryos. The results for the two *Hox* genes point to the same conclusion (Figure 3c and d): out of 16 *Hoxd-8 Abd-B*$^{-}$ heat shocked embryos analyzed, sclerotic plates in A8 were present only in four and these are reduced in size. In addition, in 12 embryos there is an extra thoracic-like denticle belt in A9a. Of a sample of 32 *Hoxd-9 Abd-B*$^{-}$ embryos, only 12 exhibit sclerotic plates after heat shock, and these are underdeveloped or vestigial. In the 32 embryos there is an extra partial or complete thoracic-like denticle belt in A9 (Figure 3d). Control (non-heat shocked embryos of either stock) always show sclerotic plates and never the A9 extra belt.

We have also tested the suppression of head structures by overexpressing *Hoxd-8* and *d-9* in a *Scr*$^{-}$*Antp*$^{-}$ background. Since the results are again essentially identical, we will describe only those of *Hoxd-9*. In the control (non-heated) *Hoxd-9 Scr*$^{-}$*Antp*$^{-}$ embryos, T1 and T2 segments differentiate the thoracic-like (ground) denticle belts described above, and T1p always shows the pair of sclerotic plates (Figure 3a). But the expression of the *Hoxd-9* gene drastically reduces or eliminates these plates without affecting the denticle belts (Figure 3b). After heat shock, only 10% of the *Hoxd-9 Scr*$^{-}$*Antp*$^{-}$ embryos showing transformation in the head still possess the sclerotic plates, and most of these are vestigial.

These experiments indicate that *Hoxd-8* and *d-9* suppress the function of a gene(s) responsible for some specific head structures, which becomes inappropriately active in the T1 segment of *Scr*$^{-}$*Antp*$^{-}$ embryos and in the A8 segment of *Abd-B*$^{-}$ embryos. They also suppress a gene (which may be the same) preventing thoracic development of the A9a primordium of *Abd-B*$^{-}$ embryos. Our results also show that the spatially restricted effect of the ubiquitous expression of *Hoxd-8* and *d-9* is due to phenotypic suppression by the *Drosophila* resident genes; it is the presence of Antp and Abd-B products that prevents the Hoxd-8 and d-9 gene products from having a morphological effect in T1p and in A8p and A9, respectively.

Unrestricted Hoxd-10 gene expression affects Drosophila head and thorax

A standard heat shock treatment given to *Hoxd-10* embryos possessing a normal set of homeotic genes results in an effect extending not only to the cephalic, but also to the thoracic

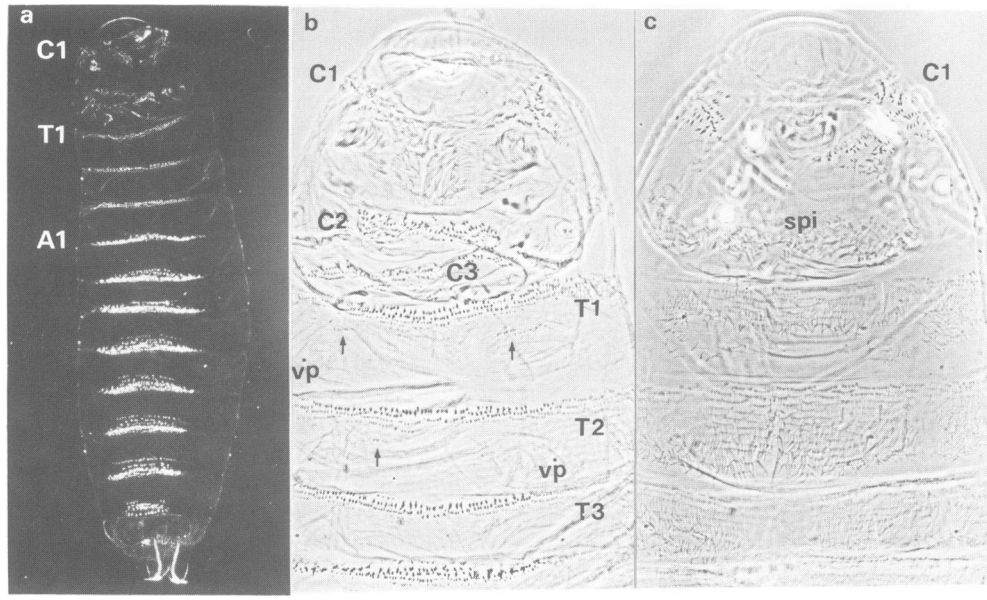

Fig. 4. Developmental effects of *Hoxd-10*. (**a**) Ventral view under dark field of a heat shocked *Hoxd-10* larva showing complete suppression of head involution and modification of head and thoracic segments. The abdominal segments remain unaltered. Abbreviations as in Figure 2. (**b**) Higher magnification of the ventral side of the head and thorax of another *Hoxd-10* larva showing a strong transformation. The C1, C2 and C3 belts differentiate thoracic denticles similar to those in the thorax. The T1 belt is modified (compare with the wild type of Figure 2). The characteristic second group of denticles is eliminated, but there are two small groups of lateral denticles (arrows). Some thoracic markers (vp) are present, but others like the ko are usually missing. (**c**) Dorsal view of another larva to show the characteristic position of the C1 belt and also the presence of the dorsal spinules (spi) of the same type as those found in thoracic and abdominal segments, but which never appear in the head. Notice that practically all head structures have disappeared. On the dorsal side of the T1 segment the spinules are more numerous than in the wild type, another indication of the modification of this segment.

segments. The effect on the head is stronger than in the previous cases; more thoracic-type denticle belts develop at the expense of cephalic structures. While the head of *Hoxd-9* embryos developed at most one (C1) thoracic belt, *Hoxd-10* embryos frequently developed two and sometimes three (Figure 4), indicating that more head primordia are transformed. The latter correspond to the C2 and C3 belts (Gonzalez-Reyes and Morata, 1991) which result from the transformation of the maxillary and labial segments, the posterior part of the head. The size of the denticles is similar to those of the ground pattern described above. On the dorsal side, the transformed head differentiates the characteristic dorsal spinules (Figure 4c) of trunk segments. The aspect and disposition of the remaining cephalic structures resemble those described after overexpression of *Drosophila* homeotic genes like *Ubx* or *Antp* (Gibson and Gehring, 1988; González-Reyes and Morata, 1990, 1991; Mann and Hogness, 1990), indicating that the *Hoxd-10* gene induces a similar cephalic gene suppression. Using a specific antibody for the cephalic gene *Dfd* we find a clear reduction in the Dfd protein levels in *Hoxd-10* embryos after heat shock. However, virtually all embryos still exhibit Dfd protein after several hours of high levels of Hox product, suggesting that a potential trancriptional effect may be of minor significance (see below).

We also tested the possibility that the thoracic-like belts in the head might develop due to a *Hoxd-10*-induced ectopic expression of thoracic genes like *Scr* or *Antp*. However, heat shocked *Hoxd-10* embryos stained with specific anti-*Antp* and anti-*Scr* antibodies show no ectopic activation in the head (Figure 5). Furthermore, we observe that the normal expression domains of these genes (Riley *et al.*, 1987; LeMotte *et al.*, 1989) are frequently altered, showing a reduction in the amount of product. Thus *Hoxd-10* acts as a repressor of *Antp* and *Scr* transcription. However, as in the case of *Dfd*, this repression in the normal domain is partial, ~50% of the embryos show protein levels indistinguishable from the wild type and the rest still retain Scr or Antp product in part of the domain.

Since *Hoxd-10* induces the formation of thoracic-like segments in the head, though not through the function of *Scr* or *Antp*, we checked whether it is able to induce ectopic activation of *teashirt (tsh)*, a gene specific of the trunk segments and known to be inducible by ectopic expression of *Antp* and of *Ubx* in the head (Roder *et al.*, 1992). The result is illustrated in Figure 5. In heat shocked *Hoxd-10* embryos, the normal *tsh* domain is unaltered, but in addition they frequently exhibit one or two ectopic *tsh* stripes in the head. When there is only one stripe, it appears in very anterior position, in the vicinity of the stomodeum, and if there are two, the second appears around the cephalic furrow. These two positions are consistent with those of the C1 and C2 cephalic primordia (González-Reyes and Morata, 1991).

The thoracic segments are also transformed (Figure 4a and b): the distinctive second group of denticles of T1 is eliminated or reduced to two lateral vestiges. The dorsal side is also modified; there is an increase in the number of spinules with respect to the wild type pattern. The T2 segment is also altered: the ventral denticles are thicker than in the normal segment and many embryos (44%, 24/54) show two lateral groups of denticles separated from the main belt and similar to those found in the transformed prothorax. There is little detectable transformation in the belt of T3, although a small percentage (11%, 6/57) of embryos show small groups of separate denticles. The three segments often lose distinctive thoracic markers like Keilin's organs and

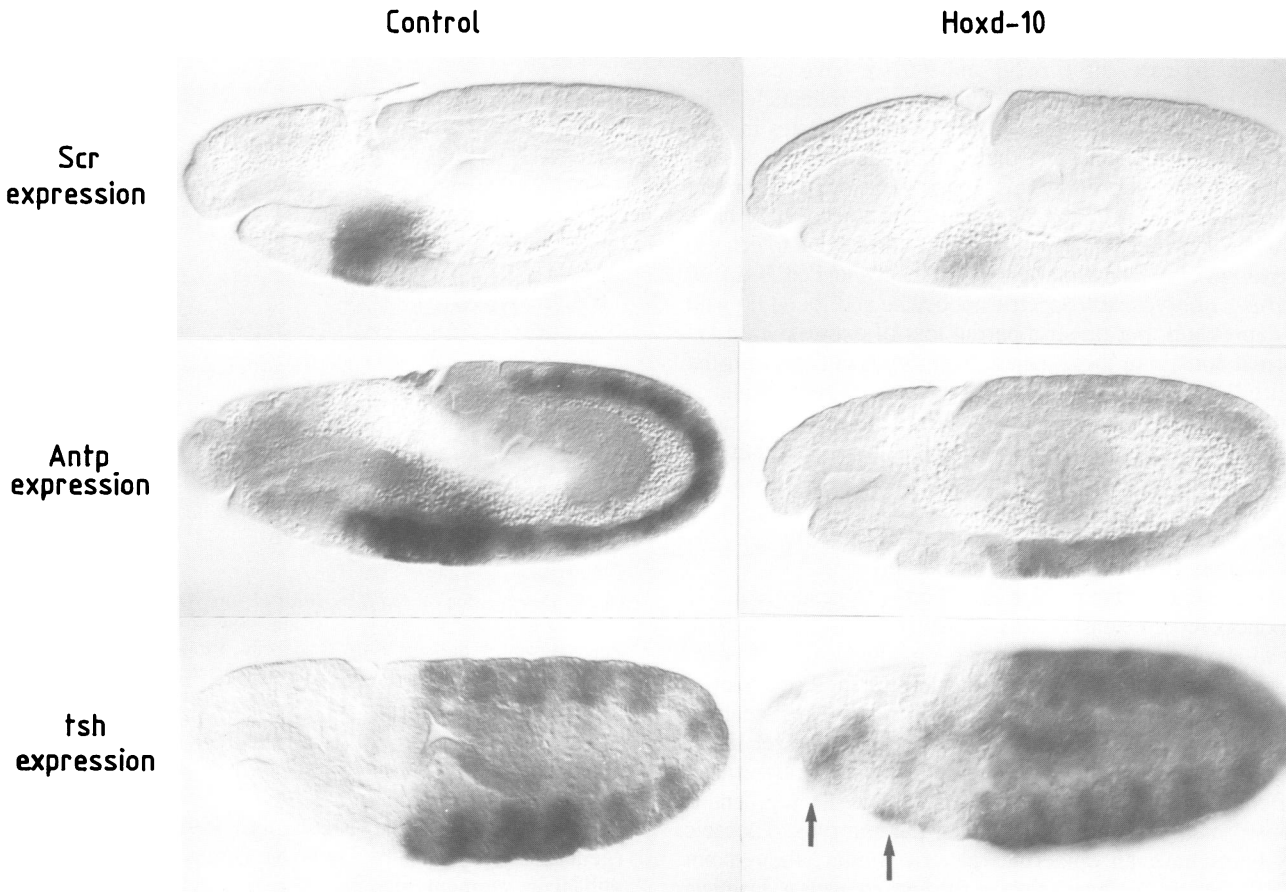

Fig. 5. Effect of *Hoxd-10* on the embryonic (stages 10–11) expression of the *Drosophila* genes *Scr*, *Antp* and *tsh*. The wild type expression of the genes is shown on the left (control) column, while the right column shows the expression of the corresponding genes after heat activation of the *Hoxd-10* gene. The head is to the left. The amounts of Scr and Antp products in the normal domains are reduced, compare left and right embryos, and there is no sign of ectopic expression for either of these genes. In contrast, the normal *tsh* domain is not affected and, in addition, there are two stripes (arrows) of ectopic expression in the head.

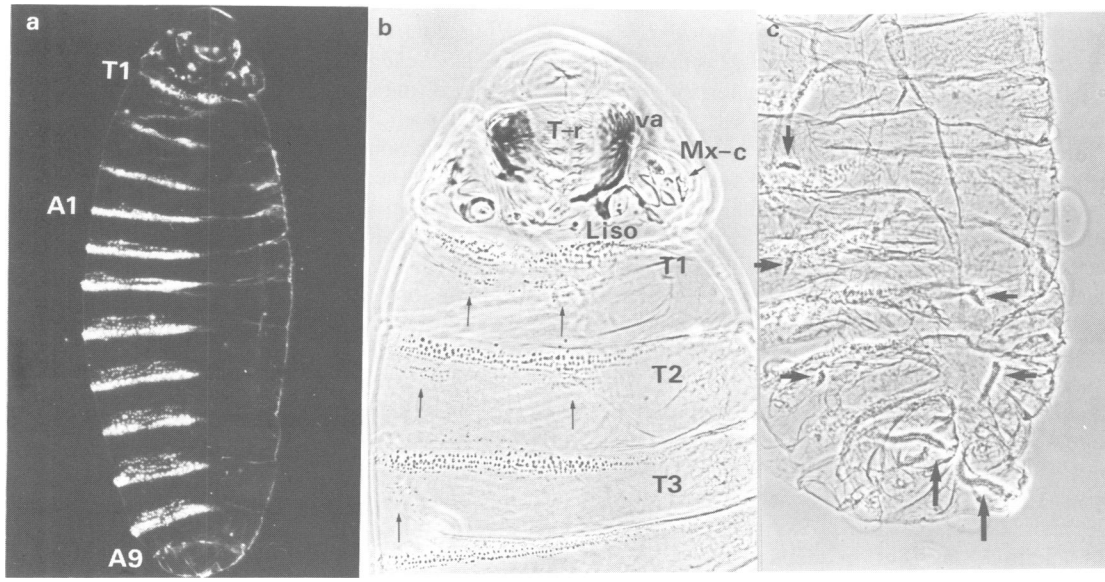

Fig. 6. (**a**) Dark field picture of a *Hoxd-11 Abd-B*^{M1} larva after heat shock showing the effect on head and thorax. Note the presence of a rudimentary A9 belt. (**b**) Ventral view at higher magnification of the head and thorax of a *Hoxd-11 Abd-B*^{M1} larva after heat shock showing the failure of head invagination and the modification of the normal patterns of the three thoracic segments. Some cephalic structures like the maxillary complex (Mx-c), labial sensory organs (Liso), ventral arms (va) and T-ribs (T-r) remain. Notice that the denticle pattern in T1, T2 and T3 is similar to that of *Scr*$^{-}$*Antp*$^{-}$ embryos (Figure 2). The arrows point to the small lateral groups of denticles characteristic of the ground pattern. (**c**) Ventral view of the abdominal region of a *Hoxd-11* larva showing extra filzkorpers. The bigger arrows point to the normal filzkorpers of A8 and the smaller ones to the ectopic filzkorpers in A7, A6 and A5.

ventral pits. In contrast to the head and thorax, we fail to find any consistent alteration in the abdominal segments.

As in the cases of *Hoxd-8* and *d-9* we have tested the effects of *Hoxd-10* in *Abd-B*$^{-}$ embryos. The denticle belts of A1−A8 are not affected, but the cephalic plates in A8p are always eliminated and there is an additional thoracic-like segment in A9a. This extra belt develops more completely than in *Hoxd-8* and *Hoxd-9* embryos. We have also checked whether this extra belt could be due to ectopic activation of *Scr* or *Antp* by staining heat shocked *Hoxd-10 Abd-B*$^{-}$ embryos with specific antibodies and found no gain of expression, but rather a partial loss of expression in the normal domain of these genes. In embryos deficient for the entire BX-C, heat activation of the *Hoxd-10* gene produces a transformation of all cephalic, thoracic and abdominal segments, which resemble the ground pattern. Additionally, there is the extra thoracic-like belt in A9.

Unrestricted Hoxd-11 gene expression affects Drosophila head, thorax and abdomen

In the presence of the regular set of homeotic genes, *Hoxd-11* produces more extensive effects than *Hoxd-8*, *d-9* and *d-10*. Head involution is prevented and some head structures are missing, while others often appear duplicated. The presence of thoracic-like belts in the head is much rarer than in *Hoxd-10* embryos, a phenomenon which may be related to the head duplications (see below). Another significant difference is that, unlike in *Hoxd-10*, the T3 segment is transformed here in most (80%) of the embryos. Thoracic segments have an epidermal pattern similar, though not exactly like that defined above as the ground pattern. Denticle belts are more abdominal-like and thoracic markers like Keilin's organs and ventral pits are absent. The abdominal segments are also affected: denticle belts are frequently modified, although it is difficult to define these effects in terms of a clean homeotic transformation. This is accompanied by a reduction in the levels of expression of *Drosophila* abdominal homeotics like *Ubx* and *abd-A*.

However, the most striking effect of *Hoxd-11* is the ectopic presence of filzkorpers (Figure 6c), characteristic pattern elements corresponding to the eighth (A8) abdominal segment. Since the latter can be ectopically induced by high expression levels of the *Abd-B* gene (Lamka *et al.*, 1992), and there is at least one case reported (McGinnis *et al.*, 1990) of induction of a *Drosophila* homeotic gene by a mammalian cognate, there was the possibility that the effect of *Hoxd-11* was mediated by ectopic activation of the endogenous *Abd-B*. This was tested by examining *Abd-B* expression after *Hoxd-11* heat induction, and also by overexpressing *Hoxd-11* in *Abd-B*$^{-}$ and in *BX-C*$^{-}$ embryos.

Using specific anti-*Abd-B* antibodies, we find after heat shock an alteration of the normal *Abd-B* expression pattern (Celniker *et al.*, 1989; DeLorenzi and Bienz, 1990): in parasegments 10, 11 and 12, some cells which do not normally contain detectable levels of Abd-B protein are labeled. In addition, there is frequently ectopic Abd-B product outside the *Abd-B* domain, in anterior abdominal parasegments and occasionally in thoracic ones, although this expression is restricted to some few cells per metamere (Figure 7).

We also checked the possibility of ectopic activation of *empty spiracles* (*ems*), a gene required to differentiate filzkorpers (Dalton *et al.*, 1989) and known to be regulated

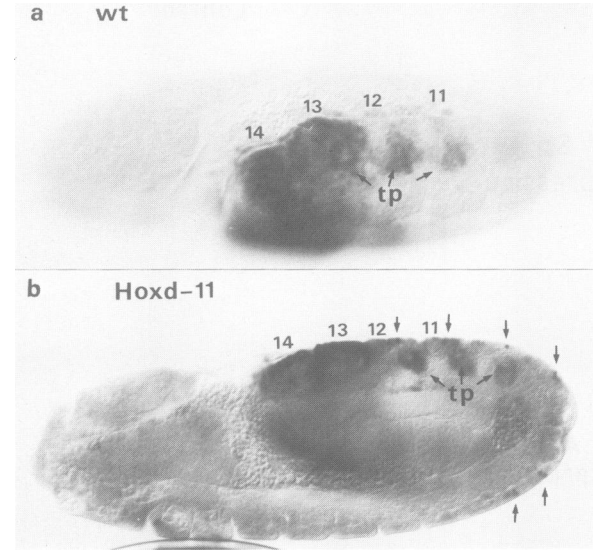

Fig. 7. Derepression of *Abd-B* by *Hoxd-11*. (**a**) Wild type expression of *Abd-B* in a stage 11 embryo in ventral view as revealed by inmunostaining with the anti-*Abd-B* antibody. Parasegments 14 and 13 are strongly labeled but in 12 and 11 (parasegment 10 is out of focus) most of the label is around the tracheal pits (tp). (**b**) After heat activation of *Hoxd-11* there is an increase of *Abd-B* label on the lateral region of parasegments 12, 11 and 10, and also ectopic expression outside the domain (arrows).

by *Abd-B* (Jones and McGinnis, 1993). A *lacZ* line (generously provided by Dr W.McGinnis) carrying an *ems* enhancer element driving β-gal activity in filzkorper precursor cells (Jones and McGinnis, 1993) was used. In this experiment, flies of the homozygous *ems*-β-gal line were crossed to those of the heterozygous *Hoxd-11* stock, so that only 50% of the embryos carry both the *ems*-β-gal and the *Hoxd-11* genes. After heat shock induction of *Hoxd-11*, embryos were doubly labeled for X-gal and Abd-B protein. While control (unheated) embryos of the same cross show *ems* label exclusively in the A8 segment, 40% of the treated embryos (in one particular experiment, 30 out of 74 embryos labeled with X-gal) show ectopic activation of *ems*, which appears in homologous positions in abdominal, thoracic and sometimes head segments (Figure 8). The amount of *ems* derepression is much greater than that detected for the endogenous *Abd-B* gene, indicating that the gain of function of *ems* is not a consequence of that of *Abd-B*. In fact, double staining for β-gal and *Abd-B* frequently shows ectopic *ems* expression in embryos in which *Abd-B* appears to be expressed normally (Figure 8). Thus, the Hoxd-11 product is able to activate directly at least one gene downstream of *Abd-B*. We have explored further this by examining *ems* expression in *Abd-B*$^{-}$ embryos. Double labeling using X-gal and anti-*Abd-B* antibody permits identification of *Abd-B*^{M1} embryos (although these still produce a protein which is recognized by the antibody and yield a low *Abd-B* label). The *Abd-B*^{M1} embryos frequently show ectopic *ems* expression.

Since the *Hoxd-10* gene can ectopically activate *tsh* in the head (Figure 5), we looked for a similar effect of *Hoxd-11* and failed to detect any *tsh* gain of function in the head or alteration of the normal *tsh* pattern. This result is consistent with the observation that *Hoxd-11*, unlike *Hoxd-10*, rarely produces thoracic-like segments in the head.

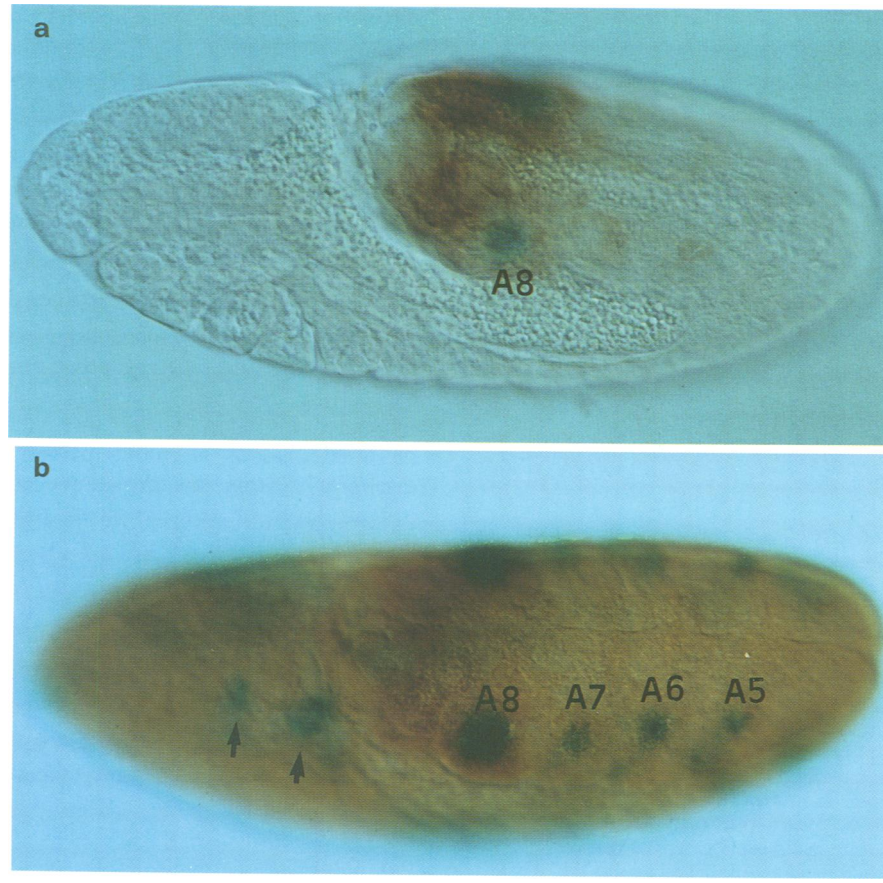

Fig. 8. Derepression of *ems* by *Hoxd-11*. (**a**) Double staining for *Abd-B* antibody and X-gal in a normal embryo (except for the presence of the *ems*-β-gal transgene) to compare the normal product distribution of *Abd-B* (brown), and β-galactosidase (blue-green) driven by the filzkorper-specific *ems* enhancer (Jones and McGinnis, 1993). The X-gal label is restricted to the A8 segment where it marks the filzkorpers' precursor cells. (**b**) Double staining in a *Hoxd-11/ems*−β-gal embryo to show the gain of *ems* activity in homologous positions in abdominal, thoracic (out of focus) and even head segments. Notice that there is no similar gain of *Abd-B* activity. The high overall background is an unwanted consequence of the heat shock.

In *Hoxd-11 Abd-B*$^-$ and *Hoxd-11 BX-C*$^-$ embryos, the head, thoracic and abdominal segments are affected, although in the abdomen the transformations are less extreme than those described in the presence of the endogenous *Abd-B* gene. This, together with the previous observation that endogenous *Abd-B* expression is enhanced, indicates that part of the effect of *Hoxd-11* in the presence of a normal set of homeotic genes is mediated by the endogenous *Abd-B*. However, *Hoxd-11 Abd-B*$^-$ larvae show strong transformations in the head and in the thorax. The head shows on the dorsal side duplications of head structures resembling the dorsal bridge and the epistomal sclerite, but which are difficult to identify in isolation. These duplications are not observed in *Hoxd-10* embryos, and might be related to the ectopic *ems* expression. Since the *ems* gene is involved in the specification of head structures (Cohen and Jurgens, 1990) it is possible that its inappropriate expression in the head may cause the defects observed.

In contrast with the head, the thoracic segments show similar transformations to those described for *Hoxd-10*, although the T2, and especially the T3 belt, exhibit a more complete transformation towards the ground pattern (Figure 6b). The transformation of T3 in *Hoxd-11 Abd-B*$^-$ is significant, for this segment is specified by *Ubx* (Lewis, 1978; Sanchez-Herrero *et al.*, 1985; Kaufman *et al.*, 1990), indicating that *Hoxd-11* is able to suppress *Ubx* function, at least at the characteristic low levels of T3 (White and Wilcox, 1985). The best description of the direct effect of *Hoxd-11* is illustrated in Figure 9; in *Hoxd-11 BX-C*$^-$ embryos all thoracic and abdominal denticle belts are very similar or identical to those of A8 in control *BX-C*$^-$ embryos, clearly indicating a transformation towards the ground pattern. This transformation is stronger than that observed in *Hoxd-10 BX-C*$^-$ embryos. However, and although with low frequency, *Hoxd-11 BX-C*$^-$ embryos can also differentiate filzkorpers. It indicates that the *Hoxd-11* gene is able by itself to specify a characteristic *Drosophila* pattern element. It, however, does not rescue any other aspect of the *Abd-B*$^-$ phenotype.

The interaction of the mouse Hoxd-11 and the Drosophila Antp gene products: a case of genuine phenotypic suppression

The experiments described above indicate that the *Hoxd-8*−*d-11* genes interact with *Drosophila* homeotic genes and either suppress or are suppressed by them. The fact that, for example, the generalized expression of *Hoxd-8*, *d-9* and *d-10* has no effect in parasegment 14 except if *Abd-B* is removed, indicates that *Abd-B*$^+$ phenotypically suppresses these *Hox* functions, and that this cannot be due to down-regulation, for the products are under heat shock control. However, we noticed that *Hoxd-10* reduces transcription of

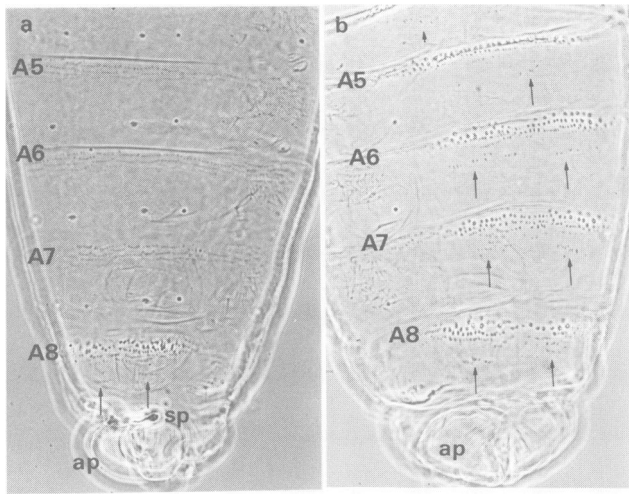

Fig. 9. (**a**) Ventral view of a *Df P9* larva deficient in all the BX-C genes. All the abdominal denticle belts, except A8, look alike and identical to the T2 belt (Lewis, 1978), although only A5, A6, A7 and A8 appear in the photograph. This transformation results from the derepression of *Antp* in thorax and abdomen down to A7 (Hafen *et al.*, 1984; Carroll *et al.*, 1986; Wirz *et al.*, 1986). However, in A8a there is no *Antp* activity and this particular primordium develops without homeotic information with a pattern resembling that seen in T1 and T2 of $Scr^{-}Antp^{-}$ mutants (Figure 2). Note the thicker denticles and the small lateral groups of denticles (arrows) separated from the main belt. Just anterior to the anal pads (ap) there also is the pair of sclerotic plates (sp), the same as in *Abd-B* mutants. (**b**) *Hoxd-11 Df P9* larva after heat shock. All the denticle belts (only A5, A6, A7 and A8 are shown, notice denticle size and satellite groups of denticles indicated by arrows) look alike and as A8, indicating that the expression of the *Hox* gene has repressed *Antp* function. The fact that all the belts look like that of A8 of *Df P9* larvae, where there is no homeotic information, indicates that the *Hoxd-11* does not specify a pattern of its own in the denticle belt, but rather inactivates the resident homeotic products. Note the sclerotic plates are missing.

the endogenous *Antp*, *Scr* and *Dfd* genes, and *Hoxd-11* in addition, that of *Ubx*. This may suggest the suppressing effect of the *Hox* genes may be, at least in part, mediated by their effect on transcription.

We wanted to assess the significance of this transcriptional effect, since in cases reported in *Drosophila* (González-Reyes and Morata, 1990; González-Reyes *et al.*, 1992) transcriptional down-regulation appears to be a secondary consequence of phenotypic suppression. We have tested whether the transformation of the thoracic segments by *Hoxd-11* can also occur in the presence of high, irrepressible levels of Antp product, which normally specifies T2 development and which is transcriptionally down-regulated by *Hoxd-11*. To this end we carried out an experiment co-overexpressing the mouse *Hoxd-11* and the *Drosophila Antp*. Under these conditions cross regulatory interactions at the transcriptional level are barred (González-Reyes *et al.*, 1990). The patterns these two genes specify are readily distinguishable. The activation of the *hsp70-Antp* gene partially or completely transforms the T1 segment towards T2 (Gibson and Gehring, 1988; González-Reyes *et al.*, 1990). The denticles of T1 become thinner and the characteristic second group of denticles is reduced or eliminated. The T2 and T3 segments are not affected and exhibit, like T1, all the distinctive thoracic markers like Keilins organs and ventral pits. The *Hoxd-11* gene induces in the three thoracic segments a ground pattern with thick denticles in the main belt and lateral groups of denticles as distinctive features. Some thoracic markers like Keilins organs disappear.

The *hsp70–Hoxd-11/hsp70–Antp* embryos were obtained crossing flies from a homozygous viable *hsp70–Antp* line to flies of a heterozygous *Hoxd-11* line, (this line is homozygous lethal). Therefore all the F1 embryos contain a *hsp70–Antp* gene known to produce a strong transformation even in one dose (González-Reyes *et al.*, 1990), and half of them bear the *Hoxd-11* gene. After the standard heat shock, we find two kinds of transformed embryos. About half of them show the typical *hsp70–Antp* transformation (Gibson and Gehring, 1988; González-Reyes *et al.*, 1990). The other group of embryos look in every aspect like the *Hoxd-11* embryos described above and illustrated in Figure 6; the three thoracic segments are modified towards a ground pattern and Keilins organs disappear. As this transformation occurs in the presence of a high level of Antp protein, the experiment clearly demonstrates that the Hoxd-11 product functionally inactivates the *Drosophila* Antp product e.g. *Hoxd-11* phenotypically suppresses *Antp*.

Discussion

Using *Drosophila* larval patterns as an assay system we have examined and compared some functional properties of the *Hoxd-8*, *d-9*, *d-10* and *d-11* genes. Our first finding is that the expression of these genes alter *Drosophila* embryonic development but, with the partial exception of *Hoxd-11*, their effect can simply be described as suppression of some *Drosophila* homeotic genes. As a result, the affected segments in each case acquire a thoracic-like pattern which we interpret as the *Drosophila* ground pattern that develops in the absence of homeotic information. For the *Antp* gene we have shown by a double heat shock experiment that its suppression by the *Hoxd-11* gene occurs even if high amounts of Antp products are present, indicating that it occurs at the level of phenotypic suppression. We believe that this is also the case for the suppression of other genes like *Dfd* or *Scr* by *Hoxd-10* and *d-11* in which double heat shocks were not performed due to the lack of appropriate morphological markers. In interactions involving *Drosophila* genes, it has been shown that transcriptional down-regulation is often a consequence of phenotypic suppression (González-Reyes *et al.*, 1992), probably due to the autocatalytic phenomena associated with maintenance of homeotic gene activity.

Our second finding is that there appears to be a correlation between the patterns of expression of the *Hox* genes in the mouse and the extent of their effect along the cephalic, thoracic and abdominal segments of *Drosophila*, thus indicating the existence of a functional hierarchy among the *Hox* complex. The *Hoxd-8* and *d-9* genes have an effect restricted to the C1 cephalic primordium, which is transformed toward a thoracic-like segment. They probably inactivate a head gene(s), and, as shown by the experiments expressing these genes in the absence of *Abd-B*, they can do it even if the head gene(s) becomes active at the posterior end of the body. But in spite of the high and ubiquitous expression levels, *Hoxd-8* and *d-9* fail to produce any segmental transformation in the thorax and abdomen indicating that they are phenotypically suppressed by the

corresponding *Drosophila* genes. This is also shown by the *Abd-B*^{M1} experiments, in which the lack of *Drosophila* thoracic or abdominal gene products in parasegment 14 (A8p−A9a) allows a morphological effect of *Hoxd-8* or *d-9*.

The effect of *Hoxd-10* is stronger than in the previous cases and affects segments located more posterior in the body. In the head, the C1, C2 and sometimes C3 primordia are transformed into thoracic-like segments (Figure 4). This transformation is not due to ectopic activity of *Drosophila* thoracic genes, but it is probably a default state due to the inactivation of head specific genes. One interesting aspect of this transformation is the *Hoxd-10* induced activation of *tsh* (Figure 5), a gene necessary for trunk development (Fasano *et al.*, 1991; Roder *et al.*, 1992). Since there is *tsh* expression in the trunk of embryos deficient for *Scr*, *Antp* and the three BX-C genes (Roder *et al.*, 1992), a genetic condition equivalent to the ground state for the trunk segments, our finding of *tsh* activity in the head of *Hoxd-10* embryos is entirely consistent with the transformation of head segments towards a ground pattern. *tsh* might be directly activated by *Hoxd-10* or else it might result from a default state produced by the suppression of head specific genes.

In the thorax of *Hoxd-10* embryos, T1, T2 and, to a lesser extent, the T3 segments are also modified towards the ground pattern described above. In contrast, the abdomen remains normal. This indicates that in addition to head specific genes, the *Drosophila* genes responsible for thoracic development, *Scr* and *Antp*, are being blocked by *Hoxd-10*. Indeed, we find a reduction in their transcription levels (Figure 5), although the co-overexpression experiment of *Antp* and *Hoxd-11* strongly suggests that this down-regulation is not significant. The lack of effect of the Hoxd-10 product in the abdomen indicates that it is inactivated by the corresponding *Drosophila* genes, *Ubx*, *abd-A* and *Abd-B*, because when the latter are absent, as in the *Hoxd-10 BX-C*$^{-}$ experiment, all segments of the head, thorax and abdomen are equally transformed.

The case of *Hoxd-11* is more complicated because there are two sources of effects; the Hoxd-11 product itself and the ectopic activation of the endogenous *Abd-B* gene. However, the latter is not extensive (Figure 7), and the thoracic segments of *Hoxd-11 Abd-B*$^{-}$ embryos show a transformation stronger than that seen in *Hoxd-10* (Figure 6b). Moreover, in *Hoxd-11 BX-C*$^{-}$ embryos (Figure 9) the transformation of thoracic and abdominal segments into ground pattern is also more complete than in *Hoxd-10 BX-C*$^{-}$, which in turn indicates a stronger suppression of *Scr* and *Antp*. Nevertheless, in addition to the suppressing effect, the *Hoxd-11* gene is able, in the absence of the resident *Abd-B* product, to induce the formation of filzkorpers (Figure 6c) as well as ectopic activation of the *Abd-B* target gene *ems* (Figure 8). As the differentiation of filzkorpers requires both the presence of the ems and the Abd-B products (Jones and McGinnis, 1993), this indicates that the Hoxd-11 product can replace Abd-B in specifying filzkorper differentiation. This suggests a striking functional conservation with respect to the *Drosophila Abd-B*, not observed in the other homologs. It might suggest that Hoxd-11 has conserved some of the functional features of the ancestral *Abd-B* gene.

In summary, our results strongly suggest a hierarchy of effects for the *Hoxd-8−d-11* genes which correlates with their expression pattern. As the *Hox* genes are expressed more posteriorly in the mouse, they affect more posterior segments in *Drosophila*, that is, they suppress more posterior *Drosophila* homeotic genes. Thus, this aspect of the colinearity, the correlation between expression patterns and functional hierarchy appears to be a general feature of the Hox/HOM complexes. The lowest and highest genes in the hierarchy are those specifying the two extreme regions of the body: the acron (anterior part of the head) and the telson. In the telson neither the expression of any homeotic (Gibson and Gehring, 1988; González-Reyes and Morata, 1990; González-Reyes *et al.*, 1990; Mann and Hogness, 1990) nor that of the *Hox* genes so far tested has any effect, indicating that telson specific genes suppress them. Contrarily, head morphogenesis is affected by both homeotic and *Hox* genes, indicating that head specific genes are suppressed by HOM/Hox products. The chromosomal location of the head and telson specific genes is unknown but it is unlikely that they are in lineal order with respect to the *Hox/HOM* complexes. Yet they conform to the correlation between expression pattern and phenotypic suppression/posterior prevalence.

We do not know the reasons for the conservation of this hierarchy. Why the expression patterns of homeotic genes, which presumably depend on *cis*-regulatory elements, correlate with the phenomenon of phenotypic suppression, which likely depends on the nature of the homeoproduct, e.g. binding affinity to target genes, is quite an enigma. In this respect, however, it is worth considering that the *Hoxd-9−d-11* genes have a similar degree of homology (Izpisúa-Belmonte *et al.*, 1991) with respect to *Drosophila Abd-B* and have probably arisen by tandem duplications of an ancestral *Abd-B* gene. Yet, they interact differently with *Drosophila* homeotics depending on their relative position and pattern of expression. Perhaps the duplication mechanism is generating *Hox* genes which, because of the manner in which *cis*-regulatory sequences are duplicated, are driven to function in more posterior regions of the body, where they have to compete with pre-existing homeoproducts. Under these circumstances, the newly duplicated genes can only have a function, and hence the possibility of an evolutionary advantage, if the proteins they encode have greater affinity for target sites than pre-existing products. This would ultimately lead to the correlation genetic order/expression pattern/phenotypic suppression which occurs in *Drosophila* and that we show here also applies to the *Hox* genes.

At the moment it is not clear whether the phenotypic suppression/posterior prevalence phenomenon has a functional significance. The cases reported in *Drosophila* (Gibson and Gehring, 1989; González-Reyes and Morata, 1990; Mann and Hogness, 1990; González-Reyes *et al.*, 1992) describe situations which do not occur *in vivo*. However, it might be different in organisms in which developmental specifications are established sequentially, such as short germ insects or vertebrates. In these, temporal progression in the activation of *Hox* genes, in parallel with the appearance of more posterior regions of the body, leads to largely overlapping expression domains (Duboule, 1991). It has been proposed (Duboule, 1992) that the phenotypic suppression/posterior prevalence phenomenon may be used to functionally inactivate a co-existing homeoproduct without having to switch off transcription. The persistence of phenotypic suppression in *Drosophila* may be an atavistic trait.

Origin of insect cephalic patterns

We observe a thoracic transformation of the head segments as a consequence of the activity of *Hox* genes. This is especially clear in the case of the transformations caused by the *Hoxd-10* gene where we often find two, and sometimes three, thoracic-like belts in the place of the normal cephalic structures. The presence of these belts correlates with the ectopic induction of *tsh* activity in the head. Although at a first glance these transformations appear to be like those reported in cases of ectopic expression of *Drosophila* thorax-determining genes (Gibson and Gehring, 1988; González-Reyes *et al.*, 1990), which also induce *tsh* expression in the head (Roder *et al.*, 1992), we fail to observe any ectopic activity of the endogenous genes, like *Antp* or *Scr*, which specify thoracic patterns. Thus, it appears that in the absence of homeotic information, the cephalic patterns become thoracic-like. It then follows that the cephalic patterns are built on a thoracic scaffold, which probably represents the primordial insect pattern. As we point out above, this is consistent with the presence of *tsh* activity in the head of *Hoxd-10* embryos, for *tsh* is expressed in the trunk in the absence of homeotic information (Roder *et al.*, 1992). Thus *tsh* expression may result from a default state of homeotic genes and may be indicative of the ground segment pattern.

A similar argument applies to the cephalic structures (sclerotic plates) appearing in parasegment 14 of *Abd-B*$^-$ embryos, which result from the ectopic expression of an as yet unidentified head specific gene (Lewis, 1978; Struhl, 1983). The elimination of the sclerotic plates, which belong to compartment A8p (Casanova *et al.*, 1986), is normally accompanied by the appearance of an extra thoracic-like belt in A9a, the other element of parasegment 14. The suppression of the cephalic gene by the presence of the Hoxd products results in a thoracic-like pattern in parasegment 14.

Material and methods

Generation of the transgenic lines

The *hsp70−Hox* fusion genes were constructed as follows: a cDNA fragment containing the *Hoxd-8* coding region was isolated from pSGH4.3 (Zappavigna *et al.*, 1991) by digestion with *Bam*HI, and cloned into the *Bgl*II site in the polylinker of the pCaSpeR vector (Thummel and Pirrotta, 1991). Similarly, a cDNA fragment containing the *Hoxd-9* coding region was isolated from pSGH4C (Zappavigna *et al.*, 1991) by partial digestion with *Bam*HI and cloned into the *Bgl*II site in the polylinker of the pCaSpeR vector. A cDNA fragment including the *Hoxd-10* open reading frame was excised from pSGH4D (Zappavigna *et al.*, 1991) by digestion with *Eco*RI and cloned into the *Eco*RI site in the pCaSpeR polylinker. The *Hoxd-11* complete open reading frame has been reconstructed by blunt end cloning the product of the fusion of an *Apa*LI−*Not*I genomic fragment containing the 5′ part of the *Hoxd-11* coding sequence with a *Not*I−*Eco*RI subfragment of a partial cDNA clone into the *Sma*I site of pGEM7 (Izpizua-Belmonte *et al.*, 1991). This fragment was removed by an *Eco*RI−*Bam*HI double digestion and recloned into the polylinker of the pSG5 expression vector (Green *et al.*, 1988). Finally, the *Eco*RI−*Bam*HI fragment obtained from pSG5/Hoxd-11 has been cloned into the *Eco*RI−*Bgl*II sites of the pCaSpeR polylinker. All constructions were co-injected with the pUCHSIIΔ2:3 transposase encoding plasmid (Mullins *et al.*, 1989) into *Df(1)w*^{1118} embryos as previously described (Rubin and Spradling, 1982). A minimum of three independent integrations were tested for each construction. Embryos and larvae of the different lines were inspected for possible morphological effects of the *Hox* transgenes independent of heat shock activation and no significant alteration of the wild type pattern was found.

Mutant chromosomes and stocks

The following mutant alleles were used: *Scr*^{C1} and *Antp*$^{NS+C3}$ (Struhl, 1982), *Abd-B*^{M1} (Casanova *et al.*, 1986) are considered as null mutations. The *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. As the *hsp70−Hox* gene constructs used in this study contain the *white*$^+$ (*w*$^+$) gene, stocks were made incorporating all these mutant chromosomes in a *w*$^-$ background.

The *ems−β*-gal line is a gift of Dr W.McGinnis and has been described in Jones and McGinnis, 1993. The *ems* enhancer fragment it contains drives β-galactosidase expression in the precursor cells of filzkorpers, granulous structures that form part of the larval spiracles in the A8 segment. This transgene is up regulated by *Abd-B* (Jones and McGinnis, 1993).

The *hsp70−Antp* stock H4 has been described in Gibson and Gehring (1988). It is homozygous viable and located in the third chromosome.

Heat shock treatments

To make results from different experiments comparable, the heat shock protocol was always the same. Embryos were collected after a short egg-laying period of 2 h and the heat treatment initiated when they were 4 ± 1 h of age. They were given a 45 min pulse at 37°C followed by 2 h recovery and another 45 min 37°C pulse. The same treatment did not produce any morphological alterations in wild type controls.

We cannot monitor the presence of Hox protein products due to the lack of specific antibodies, but heat shocked embryos were stained by the whole mount *in situ* method (Tautz and Pfeifle, 1989) using *Hox* specific probes and show a high and uniform level of *Hox* RNA after the treatment. Untreated embryos show no label. We do not know the stability of the Hoxd proteins, but *Drosophila* homeoproteins last several hours after one heat shock (González-Reyes and Morata, 1990; Sánchez-Herrero *et al.*, 1994) and the mouse Hoxa-5 product, for which there is a specific antibody, last about 3 h after 1 h heat pulse (Zhao *et al.*, 1993). As we are administering two heat shock pulses separated by a 2 h interval, we are reasonably certain there is high level of Hoxd products between 4 and at least 8 h of development, when the larval epidermal patterns are established (Gibson and Gehring, 1989; González-Reyes and Morata, 1990)

Larval cuticle preparations

We used the standard method of van der Meer (1977) as modified by Wieschaus and Nusslein-Volhard, 1986.

Antibody staining and whole mount in situ

The conditions of fixation and staining were as described in Macias *et al.* (1990). The polyclonal anti-*Dfd* antibody was provided by W.McGinnis, the anti-*Antp* and anti-*Scr* by W.Gehring. The anti-*abd-A* was described in Macias *et al.* (1990). To recognize the Ubx and Abd-B products we used the monoclonal antibodies developed by White and Wilcox (1984) and by Celniker *et al.* (1989), respectively.

The double staining X-gal and anti-*Abd-B* antibody was performed taking the following precautions. The X-gal reaction should be done first using the standard procedure. Embryos to be stained with the X-gal reaction cannot be stored at low temperature as the reaction does not work, possibly due to inactivation of β-galactosidase. Also, after removing the vitelline membrane with the 1:1 heptane/methanol solution embryos should be rehydrated immediately in the appropriate buffer. Then proceed with the antibody staining protocol.

The whole mount *in situ* hybridization with non-radioactive probes was done as in Tautz and Pfeifle (1989) with minor modifications, using the Boehringer Kit 1093 657.

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