

Functional and regulatory interactions between *Hox* and *extradenticle* genes

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The homeobox gene *extradenticle* (*exd*) acts as a cofactor of *Hox* function both in *Drosophila* and vertebrates. It has been shown that the distribution of the Exd protein is developmentally regulated at the post-translational level; in the regions where *exd* is not functional Exd is present only in the cell cytoplasm, whereas it accumulates in the nuclei of cells requiring *exd* function. We show that the subcellular localization of Exd is regulated by the BX-C genes and that each BX-C gene can prevent or reduce nuclear translocation of Exd to different extents. In spite of this negative regulation, two BX-C genes, *Ultrabithorax* and *abdominal-A*, require *exd* activity for their maintenance and function. We propose that mutual interactions between Exd and BX-C proteins ensure the correct amounts of interacting molecules. As the *Hoxd10* gene has the same properties as *Drosophila* BX-C genes, we suggest that the control mechanism of subcellular distribution of Exd found in *Drosophila* probably operates in other organisms as well.

[Key Words: *extradenticle*; *Hox* genes; nuclear translocation; post-translational control]

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The homeotic genes determine the characteristic development (identity) of *Drosophila* body segments (Lawrence and Morata 1994; Lawrence and Struhl 1996). They encode proteins that contain a DNA-binding domain known as a homeodomain and regulate the transcription of target genes by binding to their regulatory sequences. Although the various homeotic genes determine different segment identities in vivo, the homeotic proteins show similar binding specificity in vitro (Hoey and Levine 1988) raising the problem of how the in vivo specificity is achieved.

One factor that contributes to the specificity of the homeotic function is the gene *extradenticle* (*exd*). Mutations in *exd* alter the identity of some body segments without altering the expression pattern of the homeotic genes (Peifer and Wieschaus 1990; Rauskolb et al. 1993). The Exd protein contains a homeodomain and acts as a cofactor of the homeotic proteins; in vitro binding experiments have also shown that Exd can cooperatively bind to DNA with the Ultrabithorax (Ubx) and Abdominal-A (Abd-A) proteins, increasing their affinity for some target sites (Chan et al. 1994; van Dijk and Murre 1994). Given the high degree of conservation of the Exd product (Rauskolb et al. 1993), it is likely that *exd* vertebrate homologs, the *Pbx* genes, also act as cofactors. In fact, there is evidence that the *Pbx* proteins interact with *Hox* proteins to mediate their binding specificity (Kamps et

al. 1990; Nourse et al. 1990; Monica et al. 1991; Flegel et al. 1993; Chang et al. 1994; Phelan et al. 1994; Lu et al. 1995).

Besides the evidence for molecular interactions with homeotic products, there are some results indicating distinct levels of regulation of *exd* function. In wild-type embryos the *exd* RNA is distributed uniformly throughout the body segments in early development, but at the extended germ-band stage there are fewer *exd* transcripts in the posterior abdomen than in the thorax (Rauskolb et al. 1993). As embryos deficient for the Bithorax Complex (BX-C) genes show high and uniform levels of transcripts in thorax and abdomen at this stage (Rauskolb et al. 1993), it suggests a negative regulation of *exd* transcription by the BX-C genes. In addition, there is another level of regulation: The Exd product accumulates in early embryos only in the cell cytoplasm and is later translocated to the nucleus (Mann and Abu-Shaar 1996; Aspland and White 1997). The nuclear accumulation is also spatially regulated; there is more nuclear Exd in the cells of the thoracic segments than in those of the abdominal segments, both in the epidermis and the CNS. In the imaginal discs the nuclear localization of Exd is restricted to the proximal regions of the legs, wings, halteres, and antennae and is cytoplasmic in the distal regions (Mann and Abu-Shaar 1996; Aspland and White 1997). Functional tests (Gonzalez-Crespo and Morata 1995; Rauskolb et al. 1995) indicate that *exd* is developmentally active only in the regions where its product is nuclear.

Mosaic analyses have also shown a general require-

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ment for *exd* in various adult patterns (Gonzalez-Crespo and Morata 1995; Rauskolb et al. 1995). The elimination of *exd* activity in clones of imaginal cells give rise to a large variety of homeotic phenotypes in the different body segments. One particularly intriguing phenotype is the antenna to leg transformation, characteristic of the dominant *Antp* mutations and of experiments forcing gain of *Antennapedia* (*Antp*) function (Schneuwly et al. 1987; Gibson and Gehring 1988; Kaufman et al. 1990). A similar *Antp*-like transformation is also produced when the BX-C genes *Ubx*, *abd-A* and *Abd-B* are expressed ectopically in the antenna (Mann and Hogness 1990; Kuziora 1993; Casares et al. 1996). The transformation induced by *abd-A* and *Abd-B* is surprising because these genes normally specify the development of abdominal segments (Sanchez-Herrero et al. 1985) that do not possess legs. Moreover, in other body regions the phenotype of *exd* mutant clones resembles the loss of function of some homeotic genes. For example, *exd*⁻ clones in the metanotum produce a transformation into mesonotum, a typical *Ubx*⁻ phenotype (Morata and Garcia-Bellido 1976), or a thoracic transformation of the head capsule that resembles a *labial*⁻ (*lab*⁻) phenotype (Gonzalez-Crespo and Morata 1995).

These observations suggest the existence of various interactions between *exd* and the homeotic genes, some of which are described in this paper. We show that the *Drosophila* proteins *Ubx*, *Abd-A*, and *Abd-B* and the mouse Hoxd10 protein can reduce or eliminate *exd* function by preventing nuclear translocation of Exd, suggesting that in normal development they modulate *exd* activity. This effect on *exd* provides an explanation for the unexpected phenotypes caused by ectopic expression of *abd-A* and *Abd-B*. Conversely, nuclear localization of Exd is necessary for the normal expression and function of *Ubx* and *abd-A*. We propose that there is a mechanism to ensure the appropriate relative amounts of interacting Hox/Exd products and that the BX-C genes contribute to this mechanism.

Results

Distribution and regulation of the Exd product

The distribution of *exd* transcripts during embryogenesis has been described (Rauskolb et al. 1993). *exd* RNA has both a maternal and a zygotic component; the maternal component is uniform and persists until embryonic stage 9. The expression of the zygotic component is modulated (Rauskolb et al. 1993), and as a result the overall pattern of *exd* transcripts is nonuniform, being higher in the thorax than in the abdomen. In the embryo Exd protein is detected at cellular blastoderm stage, but then it is exclusively cytoplasmic (Mann and Abu-Shaar 1996; Aspland and White 1997). After the extended germ-band stage accumulates predominantly in the nuclei of the thoracic segments in both the epidermis and the CNS, whereas there is a gradation of nuclear expression in the abdomen that diminishes toward the posterior end, where the Exd protein is detected only in the cytoplasm.

We have studied and compared the distribution of *exd* RNA and protein in embryos containing only either the maternal (*mat*⁺ *zyg*⁻) or the zygotic (*mat*⁻ *zyg*⁺) component of *exd*. Maternal *exd* RNA lasts for a few hours (Fig. 1A–C), and by stage 9 of embryogenesis it is undetectable, in agreement with previous work (Rauskolb et al. 1993). Protein produced by maternal RNA is stable; in

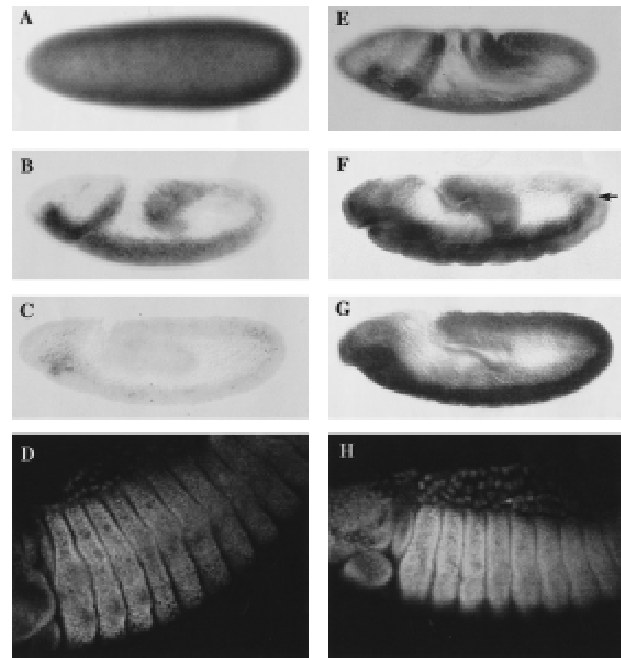


Figure 1. Distribution of *exd* RNA and protein in maternal and zygotic *exd* mutants. All panels show embryos with anterior to the left and dorsal up. (A–D) Embryos of *exd*^{y012} genotype and therefore, have Exd product exclusively from maternal origin (*mat*⁺ *zyg*⁻). (E–H) Embryos containing only Exd product of zygotic origin (*mat*⁻ *zyg*⁺). (A) *mat*⁺ *zyg*⁻ embryo at the blastoderm stage stained for *exd* RNA. The maternal product is ubiquitously distributed throughout the embryo. (B) *mat*⁺ *zyg*⁻ embryo at stage 8–9 of embryogenesis. Maternal *exd* RNA is still present, but at lower levels. (C) Stage 10 embryo of the same genotype in which the maternal RNA is almost undetectable. (D) *mat*⁺ *zyg*⁻ embryo after germ-band retraction stained for Exd protein. Although no maternal RNA is detectable in the embryo from stage 11 onwards, the protein is still present in later stages of embryogenesis. Note the nuclear localization of Exd in the cells of thoracic segments. (E) Stage 8 *mat*⁻ *zyg*⁺ embryo lacking the maternal contribution stained for *exd* mRNA. The zygotic product is present throughout the body. (F) Stage 11 embryo showing a modulation in the levels of zygotic RNA along the A/P axis. In the ectoderm there is very little expression in the more posterior segments, but note that there is mesodermal *exd* expression (arrow) that is not in register with the ectoderm. (G) Embryo of approximately the same age as in F stained with the anti-Exd antibody. The Exd zygotic protein is still present in the posterior segments of the embryo, where the mRNA is not detected anymore. (H) Stage 14 *mat*⁻ *zyg*⁺ embryo stained with anti-Exd antibody. The zygotic protein shows high levels of nuclear distribution in the thoracic segments and lower levels in the abdominal segments. The distribution of the zygotic protein is the same as the maternal one, but the levels of the maternal protein are lower (cf. D and H).

mat⁺ zyg⁻ embryos it can be detected until late stages of embryogenesis although at lower levels than the protein produced by zygotic expression. Most importantly, maternally derived protein translocates into nuclei (Fig. 1D) and the pattern of cells that do so is similar or identical to the wild type. This suggests that the protein translated from maternal RNA is functional, a result that is consistent with experiments showing that the presence of extra copies of the *exd* gene during oogenesis can rescue the *exd* zygotic phenotype (Peifer and Wieschaus 1990). It also indicates that regulation of protein distribution is not dependent on transcriptional control.

Zygotic *exd* RNA is first synthesized around stage 5 of embryogenesis. In mat⁻ zyg⁺ embryos, it is originally uniform but is later modulated (Fig. 1E,F). Transcripts cannot be detected in the posterior segments of the body after the extended germ-band stage, although there are differences between the ectoderm and mesoderm (Fig. 1F). Protein derived from zygotic RNA (Fig. 1G) can be detected shortly after initiation of transcription and translocates into nuclei beginning at stage 8–9. The pattern of cells with nuclear Exd is similar to wild type (Fig. 1H).

The finding that Exd is subjected to a subcellular regulation may provide an explanation for previous results concerning *exd* ectopic expression (Rauskolb et al. 1995). Heat shock-driven *exd* expression does not have a phenotypic effect, but it is able to rescue the zygotic phenotype of *exd* mutants (Rauskolb et al. 1995). The excess of product in the regions where it is not translocated to the nuclei may remain in the cytoplasm and therefore, not be functional. We have checked this hypothesis by making use of the GAL4/UAS method (Brand and Perrimon 1993) to generate embryos containing greater amounts of Exd in body regions where it is normally cytoplasmic. The Gal4-444 and *patched* (*ptc*)-Gal4 (Hinz et al. 1994) lines drive expression of the UAS-*exd* gene (Gonzalez-Crespo and Morata 1996) all over the body or in the anterior compartments respectively. We did not detect any morphological alteration in the cuticle of Gal4-444/UAS-*exd* or in *ptc*-Gal4/UAS-*exd* first instar larvae. Despite their normal phenotype, *ptc*-Gal4/UAS-*exd* embryos show high levels of Exd in the anterior part of every thoracic and abdominal segment (Fig. 2A) at the

germ-band elongation stage. By the time these embryos retract the germ band, the protein is translocated normally into the nucleus in the thoracic segments, but it is mostly cytoplasmic in the posterior abdominal segments (Fig. 2B). We also observed the normal low levels corresponding to this region, suggesting that the ectopic cytoplasmic Exd protein is unstable (Fig. 2B). These observations suggest that the nuclear transport of Exd is tightly regulated during embryonic development. There is a negative control in the posterior body segments that can cope with high levels of Exd product.

The Drosophila BX-C genes and the mouse Hoxd10 gene regulate the subcellular localization of Exd at the post-translational level

Because both *exd* transcripts and nuclear distribution of Exd protein changes along the A/P axis, it seems plausible that the BX-C genes are involved in regulating *exd* function. We therefore analyzed how the different BX-C genes affect Exd protein distribution.

We find that embryos homozygous for the deficiency *Df(3R)P9*, lacking the entire Bithorax complex (Lewis 1978), contain Exd at high levels in the nuclei of epidermal cells of both thoracic and abdominal segments (Fig. 3B). Only in the Keilins' organs of each segment does Exd remain cytoplasmic (Fig. 3A,B), as expected from previous results (Mann and Abu-Shaar 1996; Aspland and White 1997). This rise in the level of nuclear Exd in the abdominal segments (in comparison with the wild-type distribution) already indicates an involvement of the BX-C genes with the subcellular distribution of the product. In contrast, the thorax-determining genes *Sex combs reduced* (*Scr*) and *Antp* do not appear to affect Exd localization, as *Scr*⁻ *Antp*⁻ homozygous embryos exhibit normal distribution of Exd (not shown). Furthermore, high levels of Antp protein in *ptc*-Gal4/UAS-*Antp* embryos or of Deformed (*Dfd*) protein in *hsp70-Dfd* embryos fail to produce any significant effect on Exd subcellular localization.

To demonstrate that the effect of the BX-C genes on Exd nuclear translocation is not mediated by transcription, we studied Exd localization in *exd*^{Y012}; *Df(3R)P9* embryos only containing Exd of maternal origin. These embryos show lower levels of Exd protein than the wild type, but the protein is observed in the nuclei of all the segments, including the more posterior ones (Fig. 3C,D), and is uniformly distributed. This observation demonstrates that regulation of Exd subcellular localization by the homeotic genes occurs at the post-translational level and is independent of *exd* transcription.

To discriminate the roles of individual BX-C genes in the nuclear translocation of Exd, we looked at the distribution of Exd protein in two more BX-C mutant combinations: *Ubx*⁻ *abd-A*⁺ *Abd-B*⁺ and *Ubx*⁻ *abd-A*⁻ *abd-B*⁺. As shown in Figure 3E, embryos homozygous for the first combination, defective only for *Ubx* function, show an increased level of nuclear Exd in the first abdominal segment as compared to wild type. In more posterior abdominal segments the levels and distributions of Exd are

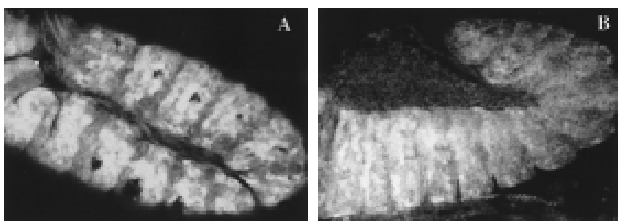


Figure 2. Ectopic expression of *exd*. (A) Stage 11 embryo with induced *exd* expression in the *ptc* domain stained for Exd protein. The levels of ectopic Exd are high in every anterior compartment. (B) Embryo with the same genotype as A shortly before completion of germ band retraction, close to stage 12, stained as in A. The high levels of Exd are not observed in the posterior end of the embryo where Exd is clearly cytoplasmic.

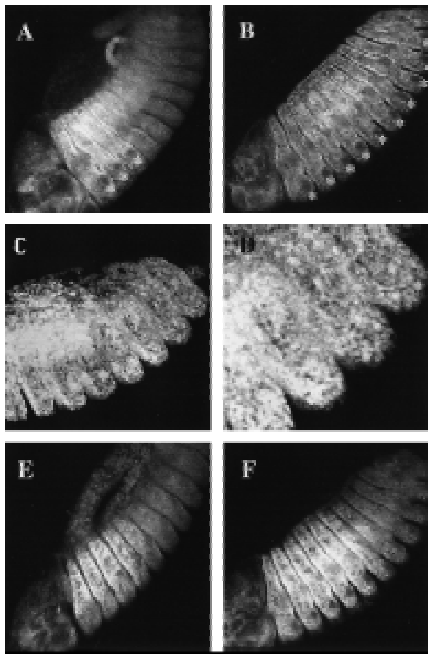


Figure 3. Effect of BX-C mutations on the subcellular localization of Exd. (A) Wild-type distribution (see text for details). Note the predominant nuclear localization in the three thoracic segments, except in the precursors of the Keilin's organs (*). (B) Exd distribution in homozygous *Df(3R)P9* embryos lacking the BX-C genes. Exd is nuclear in all the thoracic and abdominal segments. Because of the homeotic transformation, all thoracic and abdominal segments develop Keilin's organs whose precursor cells (*) have cytoplasmic Exd. (C) Exd localization in a *mat*⁺ *zyg*⁻ embryo also homozygous for *Df(3R)P9*. The product is nuclear and evenly localized in thoracic and abdominal segments. (D) Magnification of the posterior segments of the embryo in C. (E) Embryo homozygous for the *Ubx*^{placZ} mutation. The region of high nuclear expression of Exd extends to the A1 segment (cf. A). (F) Embryo homozygous for the *Ubx*^{MX9} *abd-A*^{M1} chromosome and, therefore, lacking the *Ubx* and *abd-A* functions. The region of high nuclear Exd expression now extends to segment A4 (cf. with A and E).

normal (Fig. 3E). In the second combination, lacking *Ubx* and *abd-A* functions, Exd is detected at high levels in the nuclei of the abdominal segments A1 to A4. Because in *Df(3R)P9* (*Ubx*⁻ *abd-A*⁻ *Abd-B*⁻) embryos Exd nuclear localization extends to A8, these results indicate that each BX-C gene is capable of preventing or reducing the nuclear translocation of Exd.

We have explored this property further by making use of the UAS/Gal4 system (Brand and Perrimon 1993) to induce ectopic expression of each of the three BX-C genes during the embryonic period. We describe the results for *Ubx*, although similar results were obtained for *abd-A* and *Abd-B*. The *ptc*-Gal4 line was used to drive a UAS-*Ubx* construct encoding the major form (Ia) of the Ubx protein (O'Connor et al. 1988; Kornfeld et al. 1989); in *ptc*-Gal4/UAS-*Ubx* embryos Gal4-induced Ubx protein is present in the anterior compartment of each segment in addition to the normal Ubx protein. The effects

on *exd* RNA and protein localization are illustrated in Figure 4. The distribution of *exd* RNA is not altered by ectopic or high levels of Ubx product. As shown in Figure 4, G-H, for the thoracic segments, the presence of Ubx protein in the anterior compartments does not affect *exd* transcription. We do not observe any intrasegmental modulation that would be expected if *Ubx* expression in the *ptc* domain were transcriptionally repressing *exd*. This normal *exd* RNA distribution observed cannot be the consequence of perdurance of the maternal RNA because at this stage the maternal *exd* RNA has already disappeared (Fig. 1C). In contrast, ectopic or high levels of Ubx protein clearly affect the subcellular localization of Exd. As shown in Figure 4, A-F, the level of nuclear Exd in the anterior compartments of the three thoracic segments is reduced, whereas it remains high in the posterior compartments. As the same result is observed after similar experiments with UAS-*abd-A* and UAS-*Abd-B* (data not shown), we conclude that each BX-C gene can

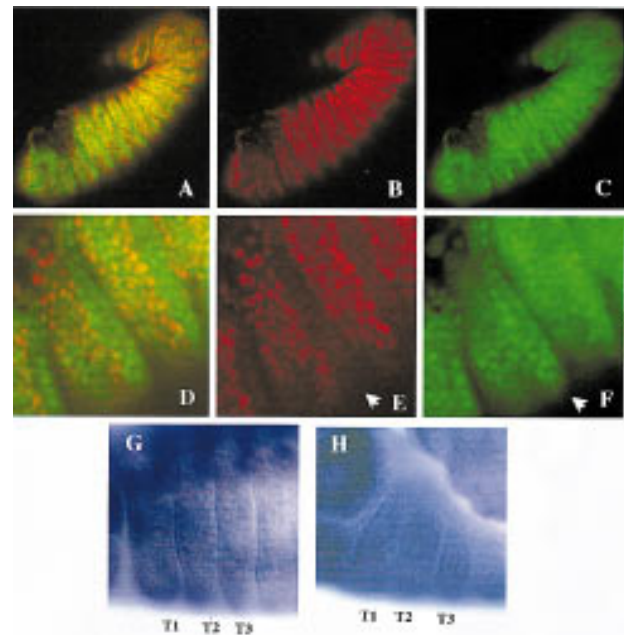


Figure 4. Ectopic expression of *Ubx* in embryos. (A-F) Exd antigen is in green, and Ubx is in red. (A) Embryo with *Ubx* ectopic expression in the *ptc* domain doubly stained for Exd and Ubx proteins. The domains of high nuclear expression of both proteins do not overlap. (B) Same embryo as A, showing ectopic Ubx protein in the anterior compartments. (C) *exd* expression in the same embryo as A. Nuclear Exd localization in the thoracic segments is restricted to the posterior compartment, where no ectopic Ubx is induced. (D) Higher magnification of the thoracic segments of the embryo in A, showing that Ubx and Exd are mutually exclusive. (E) *Ubx* expression induced in the thorax by the *ptc*-Gal4 driver. The posterior compartments do not show ectopic Ubx (arrow). (F) Expression of *exd* in the same region as in E. Nuclear protein is mostly confined to the posterior compartments (arrow). (G, H) *exd* RNA expression in the thoracic segments (T1, T2, and T3) of wild-type (G) and in *ptc*-Gal4/UAS-*Ubx* embryo (H), showing no significant differences.

inhibit nuclear translocation of Exd in the thorax, the region of maximal *exd* expression.

Although the experiments above indicate that any BX-C gene has the potential to inhibit Exd nuclear translocation, wild-type embryos show distinct levels of nuclear Exd along the abdominal segments that gradually decrease toward the most posterior segments, where Exd is almost entirely cytoplasmic. This indicates that at normal physiological levels the different BX-C genes prevent Exd nuclear transport to different extents. We have tested this possibility by comparing the effect of Ubx and Abd-Bm proteins when under the control of the same *Antp*-Gal4 driver. This driver will induce ectopic Ubx or *Abd-Bm* expression in the *Antp* domain, which extends from the posterior compartment of the first to the third thoracic segment (Wirz et al. 1986). As shown in Figure 5 *Antp*-Gal4/UAS-*Ubx* embryos, compared to the wild type, reared at 17°C show a reduction but not elimination of nuclear Exd in the second and third thoracic segments. In comparison, *Antp*-Gal4/UAS-*Abd-Bm* embryos, reared under the same conditions, show no detectable nuclear Exd, suggesting that the Abd-Bm product is more effective in preventing Exd nuclear transport.

The ability of BX-C genes to inhibit Exd nuclear transport can also be demonstrated in imaginal cells. In the following experiments we tested the effect of Ubx and the mouse *Hoxd10* gene, and *Abd-B* ortholog, for we have previously observed that the expression of *Hoxd10* in the fly produces morphological alterations suggestive of *exd* inactivation (see below). We generated clones of cells containing *Ubx*⁺ or *Hoxd10*⁺ activity and examined the subcellular localization of Exd protein in the cells of the antennal disc. Ubx-expressing cells were detected with anti-Ubx antibody and *Hoxd10*-expressing clones

were monitored by in situ hybridization with a *Hoxd10* probe. We find that expression of either Ubx or *Hoxd10* protein in antennal cells prevents nuclear translocation of Exd; in all of the *Ubx*⁺ clones found in the region where Exd is normally nuclear, it disappears from the nucleus (Fig. 6D–F). We find a similar result with *Hoxd10*⁺ clones (Fig. 6A–C), although in this case we cannot demonstrate the coincidental expression of the *Hoxd10* and the Exd products. However, patches of cytoplasmic *exd* expression appeared with the same frequency as those labeled by in situ hybridization in sibling discs, and they never appear in control non-heat-shocked larvae, which suggests strongly that the loss of nuclear Exd in the clones is due to *Hoxd10* activity. Furthermore, we tested the possibility of an effect of *Hoxd10*-expressing cells on *exd* transcription by in situ hybridization of the antennal discs with an *exd* RNA probe. We failed to observe any patch of cells lacking *exd* RNA (data not shown).

The inhibition of Exd nuclear transport by BX-C genes and Hoxd10 causes exd-like phenotypes

The experiments described above indicate that high levels of the BX-C products interfere with the translocation of Exd into the nucleus and presumably cause a functional inactivation of *exd*. This effect should be reflected in the appearance of *exd*-like mutant phenotypes. We have tested this by inducing ectopic Ubx expression with several Gal4 lines during embryonic development and examining the segmental transformations produced. In the presence of normal *exd* function, Ubx specifies the pattern of the first abdominal (A1) segment. In contrast, in embryos lacking *exd* function, Ubx specifies a pattern resembling a more posterior segment, of A3–A5 type (Peifer and Wieschaus 1990).

The results obtained with the Gal4-444 line, which induces high to moderate levels in the thoracic and abdominal segments, are shown in Figure 7. When grown at 17°C (the temperature at which the Gal4 product is less active) the thoracic and some head segments of Gal4-444/UAS-*Ubx* first instar larvae display A1 identity. This transformation (Fig. 7B) is expected for ectopic Ubx activity, in agreement with previous work (Gonzalez-Reyes and Morata 1990; Mann and Hogness 1990). However, when grown at 29°C, a temperature at which levels of Gal4 activity are higher, larvae of the same genotype develop all segments anterior to A2 with an A3–A5 pattern (Fig. 7C). This segment pattern resembles closely that found in the A1 segment of zygotic *exd*[−] larvae and is the same overall pattern observed after heat shock-inducing Ubx expression in zygotic *exd*[−] embryos (Peifer and Wieschaus 1990). These observations indicate that high levels of Ubx protein are able to produce an *exd*-like phenotype, in good agreement with the observed negative effect of BX-C genes on the nuclear translocation of Exd. We have also observed that the transformation into an A3–A5 pattern in Gal4-444/UAS-*Ubx* larvae can be partially reverted by adding an additional dose of *exd*⁺. In an experiment crossing fe-

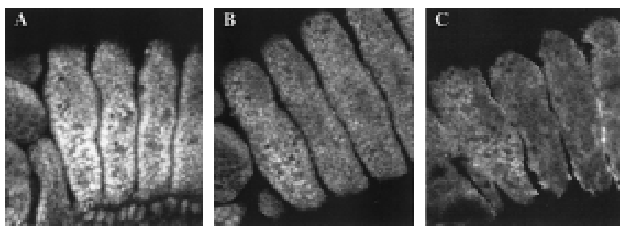
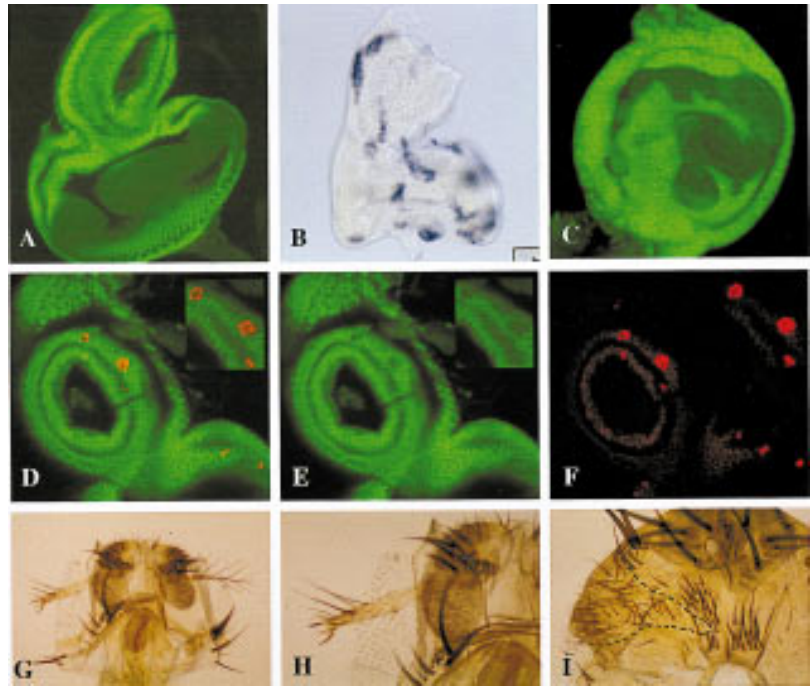


Figure 5. Ectopic expression of Ubx and *Abd-B* in embryos shows their different ability in preventing the nuclear translocation of Exd. (A) Stage 13 wild-type embryo showing the nuclear distribution of Exd in the three thoracic segments, aligned left to right. (B) Stage 13 embryo of genotype *Antp*-Gal4/UAS-*Ubx* reared at 17°C stained for Exd protein. The *Antp* domain occupies the posterior part of the first thoracic to the end of the third thoracic segment (see text). The moderate excess of Ubx protein in the second and third thoracic segments reduce, but do not eliminate, the nuclear transport of Exd. Note higher levels of nuclear Exd in the anterior region of the first thoracic segments, outside the *Antp* domain. (C) Stage 13 embryo of the genotype Gal4-*Antp*/UAS-*Abd-B*, also reared at 17°C. The presence of the Abd-Bm protein abolishes Exd nuclear expression, except in the anterior first thoracic segment.

Figure 6. Ectopic expression of *Hoxd10* or *Ubx* prevents the nuclear translocation of Exd in the antennal cells. (A) Confocal image with the expression of Exd in a wild-type eye-antennal disc. Exd is nuclear in the proximal and cytoplasmic in the more distal regions. (B) Eye-antennal disc dissected from a larvae of FLP; Arm>y>Gal4/UAS-*Hoxd10* genotype heat shocked at 37°C and stained for *Hoxd10* mRNA. Clones of *Hoxd10*-expressing cells can be detected all over the disc. (C) Confocal image of an eye-antennal disc from a larva of the same genotype and treated as in B, stained with anti-exd antibody. The patches of cytoplasmic Exd are likely to be clones of *Hoxd10*-expressing cells in which the nuclear translocation of Exd is prevented. (D–F) Clones of *Ubx*-expressing cells (red) affecting Exd (green) localization. (D) Confocal image of an eye-antennal disc with several clones containing ectopic *Ubx* activity stained for Exd and *Ubx*. No overlapping of the nuclear expression of both proteins is observed. (E) Confocal image of the same disc as D, stained for Exd and showing cytoplasmic expression. (F) The same portion of the disc as in E showing the clones containing *Ubx* expression. (G) Adult head with a clone of *Hoxd10*-expressing cells marked with γ and showing a leg transformation. (H) Higher magnification of the clone shown in G. Note the presence of two claws. (I) Clone of *Hoxd10*⁺ cells (encircled) in the head capsule exhibiting a transformation toward thorax.



males homozygous for the first chromosome Gal4-444 driver to UAS-*Ubx* homozygous males, all the progeny contain both the Gal4-444 and the UAS-*Ubx* chromosomes, and at 29°C the majority of the embryos (65 out of 72) develop the thoracic and A1 segments with an A3–A5 pattern. In comparison, in a similar cross in which parental males carry an additional dose of the *exd* gene in the Y chromosome, 33 out of 73 embryos develop thoracic segments of A1 type, whereas 40 develop an A3–A5 pattern. This suggests strongly that the presence of the extra dose of *exd* in the male embryos reduces the *exd*-like phenotype induced by the excess of *Ubx* protein.

Induction of high levels of *Ubx*, *Abd-A* and *Abd-B* products in the antennal cells is known to produce transformation into leg (this paper; Mann and Hogness 1990; Kuziora 1993; Casares et al. 1996). Because an antenna-to-leg transformation is also produced in *exd*⁺ clones (Gonzalez-Crespo and Morata 1995; Rauskolb et al. 1995), it is possible that any of the BX-C products may produce an inactivation of *Exd* in the antennal cells. The lack of specificity of this phenotype is further emphasized by previous work showing that the mouse *Hoxb9* gene (an *Abd-B* homolog) can induce the antenna-to-leg transformation (Malicki et al. 1993) and by our finding that a similar transformation is observed in *Distal-less* *Dll*-Gal4/UAS-*Hoxd10* flies in which the mouse *Hoxd10* gene is expressed in the *Dll* domain of the an-

tenna. *Hoxd10* also interferes with head and thorax development when expressed with other Gal4 lines (N. Azpiazu and G. Morata, unpubl.). To discriminate better

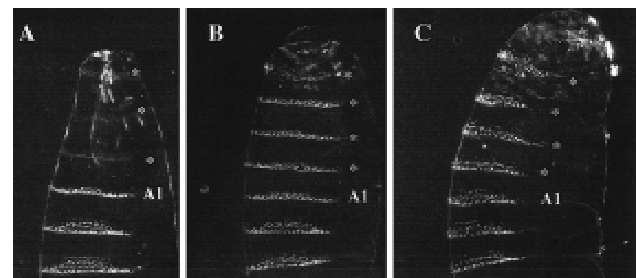


Figure 7. Different amounts of *Ubx* protein cause different transformations. (A) Ventral aspect of a wild-type larva showing head, thorax, and the first three abdominal segments. The first abdominal segment (A1) has a characteristic thin belt of denticles, whereas those of more posterior abdominal segments are wider and with a trapezoidal shape. (B) Larva of genotype Gal4-444; UAS-*Ubx*, grown at 17°C, in which *Ubx* has been overexpressed during embryogenesis (see text). At this temperature the three thoracic and one head segments (*) are transformed toward A1, and the A1 segment remains unaltered. (C) Larva of the same genotype as B grown at 28°C. The greater amount of *Ubx* protein produces a transformation of A1, the three thoracic segments, and one head segment (*) into an A3–A5 segment type.

the local effect of the *Hoxd10* gene, we generated flip-out clones (see Materials and Methods) of *Hoxd10*-expressing cells labeled with the cuticle marker *y*. These clones autonomously differentiate leg structures in the antenna (Fig. 6G–H). Moreover, in the head capsule they differentiate thoracic structures (Fig. 6I) resembling those produced by *lab*[−] clones, whereas in the thorax they alter the bristle arrangement. All of these phenotypic aspects are similar to those described for *exd*[−] clones (Gonzalez-Crespo and Morata 1995; Rauskolb et al. 1995).

exd function is necessary for maintenance of *Ubx* expression in the metathorax

The preceding results indicate that the BX-C genes negatively regulate *exd* function. However, previous work also indicates that the lack of *exd* function can produce phenotypes resembling loss of function of homeotic genes. Notably, *exd*[−] clones in the metanotum (Gonzalez-Crespo and Morata 1995) produce a transformation into mesonotum similar to that reported for the loss of *Ubx* function (Morata and Garcia-Bellido 1976). This suggests that in this region the normal function of *Ubx* requires *exd* activity.

Using an antibody directed against the *Ubx* protein we examined *Ubx* expression in haltere imaginal discs containing *exd*[−] clones induced at different times during larval development. We find that in *exd*[−] clones induced at 48–60 hr after egg laying (AEL), *Ubx* expression is abolished or greatly reduced (Fig. 8A–C) in the region of the disc that will develop into metanotum. In *exd*[−] clones induced later (72–84 hr AEL), *Ubx* expression is variable; some cells retain the *Ubx* antigen, whereas it is undetectable in others (Fig. 8D–F). As expected, *exd*[−] clones in the region of the disc giving rise to the haltere pouch,

where *Exd* is cytoplasmic, have no effect on *Ubx* expression. This observation indicates that *exd* is involved in the maintenance of *Ubx* activity in the trunk region of the segment (metanotum), but not in the appendage.

The involvement of *exd* in *Ubx* expression is supported by the next experiment in which we induced clones in the haltere disc, using the armFRT<*y*⁺<FRT/*Gal4* system, that express high levels of *exd* activity. In the metanotum, where cells normally possess *exd* function, the excess of *Exd* results in an increase of *Ubx* protein that can be readily detected (Fig. 9). We did not expect an effect of *exd*⁺ clones on the haltere appendage, but we found, first, that the excess of *Exd* protein results in its appearance in the nucleus (Fig. 9B), and second, that the presence of nuclear *Exd* in the appendage cells gives rise in many of those cells, although not in all of them, to an increase of *Ubx* protein above the normal levels (Fig. 9C). It appears that unlike embryonic cells, an increase in the amount of gene product in imaginal cells may result in the breakdown of the control mechanism of subcellular localization of *Exd*. A similar observation has been made recently in the leg disc (Gonzalez-Crespo and Morata 1996).

As it is known that *Ubx* negatively autoregulates its own expression, an excess of *Ubx* protein reduces *Ubx* transcription (Irvine et al. 1993; Casares et al. 1997), we searched for *Ubx* loss-of-function phenotypes associated with the increase of *Ubx* product observed in the *exd*⁺ clones in the haltere. A *Ubx*-like phenotype would have consisted of an easily scorable wing transformation marked with *y*. However, we failed to observe any *y*[−] clone showing wing transformation. Unfortunately, *y*[−] clones that do not produce a transformation into wing or mesonotum cannot be detected, but as we find many *y*[−] clones in other parts of the fly, we assume that they are

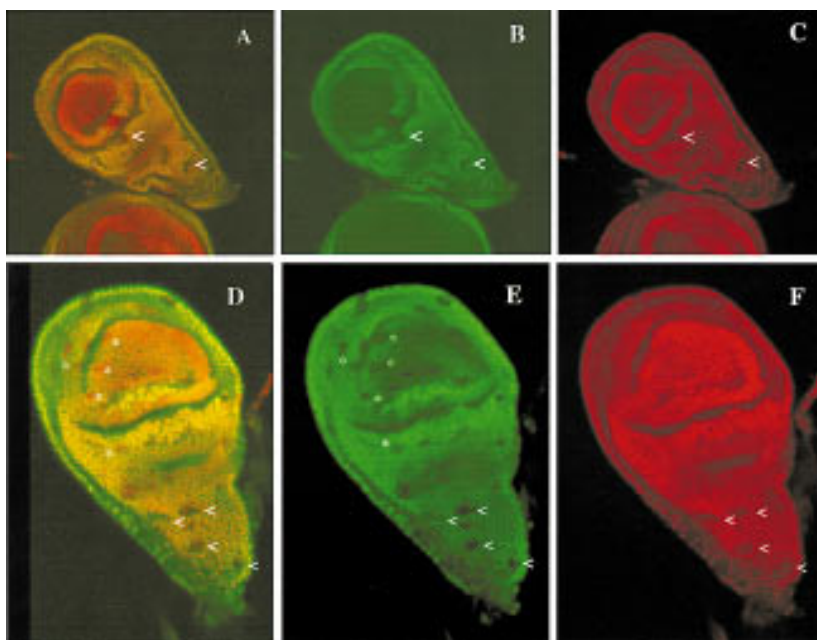


Figure 8. *exd* function is necessary to maintain *Ubx* expression in the haltere imaginal disc. (A–F) *Exd* protein is in green; *Ubx* is in red. Overlapping regions of expression appear yellow. (A–C) Confocal image of a haltere disc with *exd*[−] clones induced at 48–72 hr, doubly stained for *Exd* and *Ubx*. *exd*[−] clones are detected by the lack of *Exd* protein. The upper pointer (<) indicates an *exd*[−] clone crossing the border of nucleo/cytoplasmic *exd* expression. The portion of the clone in the appendage part contains *Ubx* protein, but in the proximal part of the clone *Ubx* protein is not detected. Other *exd*[−] clones showing loss of *Ubx* antigen are also visible in the thoracic part of the haltere (<). (B, C) The same clones in the green (*Exd*) and red (*Ubx*) channels. (D–F) Confocal image of another haltere disc with several small *exd*[−] clones induced at 72–96 hr of development and double-stained as in A–C. *exd*[−] clones in the haltere pouch (*) have no effect on *Ubx* expression, whereas many of those in the metanotum (<) reduces *Ubx* expression.

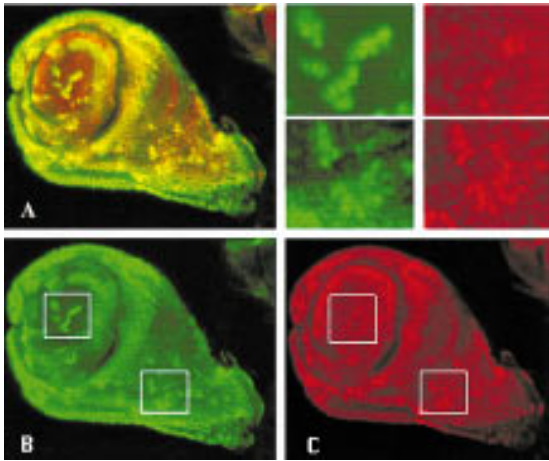


Figure 9. Effect of increasing levels of Exd on *Ubx* expression in the haltere imaginal disc. Confocal images of a third instar disc containing clones of excess of *exd* expression double-stained with anti-Exd (green) and anti-*Ubx* (red) antibody. (A) Double staining of the disc, where cells with both proteins appear yellow. Clones can be seen in the proximal and distal part of the disc and are detected by higher levels of the two proteins. (B) The same disc only showing Exd protein. (C) *Ubx* protein in the same disc to show the general correspondence between the increase of Exd and *Ubx* proteins. Insets in B and C are amplified in the upper right panel for a better assessment of the protein levels in the clones. Note that the excess of Exd protein in the clones in the pouch gives rise to nuclear Exd localization and that the increase of nuclear Exd produces an increase of *Ubx* protein, although the correspondence is sometimes not strictly cell by cell.

also present in the haltere, but do not produce a transformation.

Discussion

Regulation of *exd* expression and function by Hox genes

The *exd* gene has a maternal and a zygotic component, both of which contribute to *exd* function during embryogenesis. Our results show that the two components have the same pattern of distribution in the organism and in cells.

Examination of the distribution of the *exd* RNA and protein products in wild-type embryos reveals two levels of regulation. In the first place, there is transcriptional control, the number of transcripts in the posterior abdominal segments is lower than in the thorax, and this down-regulation depends on the presence of the BX-C genes (Rauskolb et al. 1993). This suggests that the BX-C genes are negative transcriptional regulators of *exd*. Obviously, transcriptional regulation during embryogenesis can affect only the zygotic component. In the second place, there is post-translational control, Exd protein is translocated to the cell nucleus in some body regions, whereas it remains in the cytoplasm in others (Mann and Abu-Shaar 1996; Aspland and White 1997). This process

operates in embryonic as well as in imaginal cells and appears to be a control mechanism of *exd* function, as only the nuclear protein is functional (Gonzalez-Crespo and Morata 1995; Rauskolb et al. 1995). During embryogenesis, as indicated by our *ptc*-Gal4/UAS-*exd* experiment (Fig. 2), this mechanism can keep excess Exd protein out of the nucleus, thereby impeding a developmental effect. In the experiments reported by Rauskolb et al. (1995), the heat shock-driven product can rescue *exd*⁻ phenotype in the thoracic segments because the nuclear translocation mechanism is operating in those segments and, therefore, the exogenous Exd product can be translocated to the nucleus. However, the heat shock has no effect in the abdominal region where Exd nuclear translocation is prevented.

Our results show that each of the three *Drosophila* BX-C genes and the mouse *Hoxd10* gene can prevent Exd nuclear transport and consequently reduce or eliminate *exd* function; thus, the BX-C genes are involved in the control of Exd subcellular localization. This control takes place at the post-translational level because the BX-C genes exert this role even in *mat*⁺ *zyg*⁻ embryos (Fig. 3) in which transcriptional control is not possible. The BX-C (and likely *Hoxd10*) genes do not act directly on *exd*, but trigger a control mechanism presumably activating some target genes responsible for the process. The lack of effect of GAL4 or heat shock-induced excess of Exd in the abdomen is due to the presence of the *abd-A* and/or *Abd-B* products that prevent Exd nuclear transport.

This regulation by the BX-C genes is also reflected in the pattern of subcellular localization of Exd in wild-type embryos (see Fig. 3A). In the thoracic segments Exd is predominantly nuclear, but becomes gradually more cytoplasmic toward the abdomen, and in the most posterior segment (A8) it is only cytoplasmic. This segment is part of the *Abd-B* domain and contains almost exclusively *Abd-B* product (Sanchez-Herrero et al. 1985). This suggests that the physiological levels of *Abd-B* in A8 are sufficient to prevent the nuclear translocation of Exd. More anterior segments contain low amounts of nuclear Exd coincidental with *Ubx* and *abd-A* proteins, indicating that the physiological levels of *Ubx* and *abd-A* products in those segments reduce but do not eliminate the nuclear translocation of Exd. This observation is consistent with the finding that *Ubx* and *abd-A* product possess the hexapeptide motif and interact with Exd (Johnson et al. 1995) and, as discussed below, the normal function of *Ubx* and *abd-A* requires *exd* activity, that is, Exd nuclear localization. In the case of *Ubx* there is a correlation between protein levels and nuclear translocation of Exd. In the T3 and A1 segments where *Ubx* is the only BX-C gene product present, the low amounts of *Ubx* protein in T3 correlate with the higher level of nuclear Exd, whereas the higher *Ubx* levels in A1 are coincidental with lower levels of nuclear Exd.

All together these results indicate that each BX-C protein has a different ability to suppress Exd nuclear translocation and, hence, *exd* function. It is higher in *Abd-B*, more moderate in *abd-A*, and lower in *Ubx*. It also sug-

gests that this property is used in normal development to modulate the amount of Exd in the nuclei of cells of different segments.

This negative control can be visualized at the phenotypic level. Both in embryos (Fig. 6) and in adult structures (Fig. 5, see also Fig. 1B–D in Casares et al. 1996), the Ubx, abd-A, Abd-B, or Hoxd10 products acting in ectopic places or in higher than normal expression levels can produce *exd*-like phenotypes. In fact, this property can readily account for previously unexplained results such as the antenna-to-leg transformation produced by inappropriate expression of abdominal genes like *abd-A* or *Abd-B* (Kuziora 1993; Casares et al. 1996). We propose that these transformations are produced by functional inactivation of *exd*.

Thus, considering all of the results, the BX-C genes appear to affect negatively both *exd* transcription (Rauskolb et al. 1993) and Exd nuclear transport mechanism. However, we believe that the effect on transcription may be a secondary consequence of the inhibition of Exd nuclear transport. This is based on the finding that ectopic expression of any BX-C gene affects the subcellular localization of Exd at a time when it has no detectable expression on zygotic transcription. We presume that *exd* may have an autoregulatory mechanism to maintain its own expression that requires nuclear localization of the product. This process is prevented by the BX-C genes and, as a consequence *exd* transcription, is abolished or diminished.

The negative control of *exd* function by the *Hox* genes, especially for *Ubx*, is intriguing, for there is evidence that Exd and Ubx proteins interact and Exd is a necessary cofactor for normal *Ubx* function. For example, the patterning function of *Ubx* in T3 and A1 segments depends on the presence of the sufficient amount of active Exd (Peifer and Wieschaus 1990), and the control of *dpp* expression by *Ubx* in the visceral mesoderm requires normal *exd* function (Chan et al. 1994; Rauskolb and Wieschaus 1994). In imaginal cells the elimination of *exd* function produces a *Ubx* phenotype in the metanotum, which we show is due to the *exd* requirement for *Ubx* maintenance. In the adult A1 segment the loss of *exd* function also alters the normal patterning role of *Ubx* (Gonzalez-Crespo and Morata 1995). In the A2–A4 segments, where the predominant role is played by *abd-A* (Sanchez-Herrero et al. 1985), *exd* mutant clones give rise to abnormal patterns (Gonzalez-Crespo and Morata 1995), indicating that normal *abd-A* function also requires *exd* activity.

Thus, on one hand, normal function of *Ubx* and *abd-A* is shown to require *exd* activity, and on the other, excess of Ubx and abd-A products is shown to repress *exd* activity. These two observations together suggest that Exd and Hox products have to be in each segment in the appropriate relative amounts. The current view about Exd/Hox interaction (Chan and Mann 1996) is that Exd and the different Hox proteins form heterodimers and the binding specificity and affinity to target DNA depend on the heterodimer itself. Increasing amounts of the homeotic product will probably result in some free

homeotic product in addition to the heterodimer. The free homeotic product, which by itself has little binding specificity (Hoey and Levine 1988), is able to bind DNA and may interfere with the activity of the heterodimer. Furthermore, it has been proposed recently (Pinsonneault et al. 1997) that by themselves the *exd* and *Hox* genes act as repressors while the Exd/Hox complexes act as activators. In such a manner of function any free Exd or Hox product would interfere with the normal function of the Exd/Hox complex. This situation calls for a mechanism to regulate precisely the relative number of interacting molecules.

exd and the maintenance of Ubx expression

Our results also indicate a hitherto unknown aspect of the maintenance of *Ubx* expression, that is, its dependence on *exd* function; the lack of *exd* activity results in the loss of Ubx product in the metanotum and, conversely, increasing amounts of Exd product anywhere in the disc produce a corresponding increase in the amount of Ubx product. Thus, Exd has the property of promoting *Ubx* expression. It may simply indicate that during imaginal development *exd* acts as transcriptional activator of *Ubx*. Another possibility is that Exd partakes as a cofactor with the Ubx protein in the process of *Ubx* maintenance, a role that it plays in autoregulatory circuits of other homeotic genes (Chan and Mann 1996; Pinsonneault et al. 1997). At this point we do not have evidence to favor any of these alternatives. It is of interest, however, that the regulation of *Ubx* expression appears to be different in the trunk region of the disc, where it requires *exd*, and in the appendage, where it does not. There is another aspect of the homeotic function that also discriminates clearly between the trunk region and the appendage: Ectopic expression of any of the three BX-C genes (Casares et al. 1996), and also of *Hoxd11* (N. Azpiazu and G. Morata, unpubl.), produce a nonspecific wing-to-haltere transformation, whereas the same BX-C genes produce specific transformations in the trunk region of the wing segment. Where the regions show specificity or lack of it coincides with the presence or absence of nuclear Exd.

The interactions between *Ubx* and *exd* may provide an explanation for the phenomenon of negative autoregulation of some homeobox genes like *Ubx* and others (Chouinard and Kaufman 1991; Heemskerk et al. 1991; Irvine et al. 1993; Casares et al. 1996). Negative autoregulation for *Ubx* has been described in embryos and in the haltere disc; the observation is that an excess of Ubx protein induced via heat shock (Irvine et al. 1993) or Gal4 (Casares et al. 1997) results in loss or reduction in the transcription of the endogenous *Ubx* gene. As we have observed that the excess of Ubx produces a loss of *exd* function and the latter is in turn necessary for *Ubx* maintenance in the metanotum, we propose that negative autoregulation may simply be the result of the negative effect that *Ubx* has on *exd* function. This hypothesis does not explain negative autoregulation in the appendage, where *exd* is not functional. The explanation that

we suggest is based on the recent model by Pinsonneault et al. (1997), proposing that the *Hox* genes by themselves act as repressors. If we assume that in the appendage *Ubx* transcription is maintained by an interaction of *Ubx* with another cofactor, the increase in the amount of *Ubx* product may produce free *Ubx* protein, which inhibits transcription. It is noteworthy that in our experiments the increase of *Ubx* product observed in the *exd*⁺ clones in the haltere appendage does not produce loss of *Ubx* function, as these clones do not show indication of *Ubx*⁻ phenotype. We speculate that in this case there is an increase of both *Exd* and *Ubx* product, which prevents the repressing function of *Ubx* alone.

Conservation of *Exd* nuclear transport

The conservation in the entire animal kingdom of the general organization of the *Hox* complex (Duboule and Dollé 1989; Graham et al. 1989) and of some properties of *Hox* genes, such as spatial colinearity (Gaunt et al. 1988; Duboule and Dollé 1989; Graham et al. 1989) or phenotypic suppression/posterior prevalence (Gonzalez-Reyes and Morata 1990; Bachiller et al. 1994), suggests that the general aspects of *Hox* function are also conserved. The *Exd* gene product is a general cofactor in *Drosophila* and is also highly conserved, not only in the homeodomain, but also in most of the protein sequence (Rauskolb et al. 1993). Moreover, similar cooperative interactions for DNA binding have been described at the molecular level for *Exd* and homeotic products in *Drosophila* and for the mouse *Hoxb7* and *Hoxb8* and *Pbx1* and *Pbx2* (vertebrate homologs of *exd*) gene products (van Dijk et al. 1995). Thus, the *exd/Hox* interaction appears to be conserved. To date, however, there is no indication that subcellular control of *Exd* localization occurs in organisms other than *Drosophila*. We have demonstrated that in *Drosophila* some *Hox* genes are involved in the nuclear translocation of *Exd* and consequently regulate *exd* function. We have also shown that one *Abd-B* mouse homolog, the *Hoxd10* gene, is able to prevent nuclear translocation of *Exd* when expressed in *Drosophila* imaginal cells. It also produces homeotic transformations in the *Drosophila* antenna, head capsule, and thorax, similar to those produced by the loss of *exd* function in those regions. This ability of *Hoxd10* may be indicative that the *Pbx* genes may also be subjected to a similar control by vertebrate *Hox* genes. It is possible that the *Hox* proteins modulate the intracellular location of *Pbx* proteins. The *Hoxd10* gene and perhaps other vertebrate *Hox* genes, such as *Hoxb9*, which also produces *exd*⁻-like phenotypes in *Drosophila* (Malicki et al. 1993), may activate or repress the set of genes that control *Pbx* translocation into the nucleus. The existence of such mechanism in vertebrates may be of importance in understanding the overall role of *Pbx* proteins. In humans the fusion protein E2A-*Pbx1* is responsible for the pre-B acute lymphoblastoid leukemia, and it would be of interest to know whether a nucleo/cytoplasmic control mechanism is compromised in cases like this.

Materials and methods

Drosophila mutant strains

The following homeotic and *exd* mutant lines were used in this study: *Ubx*^{MX9} *abdA*^{M1}, *Df(3R)P9*, *exd*^{Y012}/*FM7eve-lacZ*, *exd*^{Y012}/*FM7eve-lacZ*; *Df(3R)P9/Mc*. The *exd*^{Y012} allele was provided by Cordelia Rauskolb (Princeton University, NJ). Germ-line clones homozygous for *exd*^{Y012} were generated by using the yeast recombinase-based FLP-DTS system (Chou and Perrimon 1992). Recombination was induced in females carrying two copies of a FRT insertion in the X chromosome, and a single copy of the dominant female sterile mutation *ovo*^{D2}, in *trans* to the chromosome carrying the *exd*^{Y012} mutation. The FLP recombinase was provided by a 1.5-hr heat shock treatment of 24–48 hr larvae. In experiments combining mutant combinations for BX-C genes and *exd*, homozygous homeotic mutant embryos were identified using the appropriate antibodies to recognize the lack of homeotic gene product, and the zygotic *exd* mutant embryos were identified by using a *FM7/eve lacZ* balancer.

UAS/GAL4 strains

The Gal4/UAS system (Brand and Perrimon 1993) was used to drive the expression of the different BX-C proteins. The UAS-*Ubx* and UAS-*abd-A* lines drive the expression of the Ia form of *Ubx* (O'Connor et al. 1988; Kornfeld et al. 1989) and *abd-A*, respectively, and have been described previously (Mitchelson 1994; Casares et al. 1996). The UAS-*Abd-Bm* line drives the expression of the *Abd-Bm* form (Casanova et al. 1986; Zavortink and Sakonju 1989) and has also been described (Castelli-Gair et al. 1995). All of these lines were provided by Ernesto Sanchez-Herrero (CSIC-UAM, Madrid, Spain). The UAS-*Antp* was a gift from Eduardo Moreno (CSIC-UAM, Madrid, Spain). The UAS-*Hoxd10* line was generated in the laboratory using the 1.6-kb *Bam*HI cDNA fragment of the *Hoxd10* provided by D. Duboule (Zappavigna et al. 1991).

The following driver lines were utilized: GAL4-444 (general expression throughout the embryo, provided by Fernando Jimenez, CSIC-UAM, Madrid, Spain), or *ptc*-GAL4 [expression in the *ptc* domain, a gift from Campos-Ortega (see Hinz et al. 1994)]. Embryos from UAS/GAL4 crosses were collected at 29°C when used for antibody or in situ staining. To prepare larval cuticles the embryos were grown either at 29°C or 17°C.

To generate clones of ectopic expression of the *Hoxd10* gene we used the FLP; Armadillo [Arm-FRT>y>FRT-Gal4/UAS-*Hoxd10*] genotype (Sanson et al. 1996). For clones of ectopic expression of *Ubx* we used the FLP/actin-FRT>y>FRT-Gal4; UAS-*Ubx* genotype. Clones were induced by giving a heat shock at 37°C at convenient times during larval development and discs were dissected from mature third instar larvae. The arm-FRT>y>FRT-Gal4 transgenic flies were provided by P. Simpson (Sanson et al. 1996), and the heat shock-flipase stock (FLP122) by G. Struhl (Struhl and Basler 1993). Transgenic actin-FRT>y>FRT-Gal4 flies were provided by L. Zipursky (Pignoni and Zipursky 1997).

Preparation of larval cuticles

Eggs were collected on agar plates over 12-hr intervals and allowed to develop to larvae at the desired temperature (either 29°C or 17°C). They were dechorionated in bleach, fixed in methanol-heptane (1:1) for 40 sec, washed in methanol, rinsed in 0.1% Triton, mounted in a 1:1 mixture of Hoyer's mountant and lactic acid, and incubated at 60°C for 12 hr.

Antibody staining of embryos

Embryos were fixed for 20 min in a 4% paraformaldehyde–heptane solution (1:1) at room temperature and then washed in methanol. They were blocked in 10% BSA for 1 hr and incubated with the first antibody in PBT (PBS, 0.1% Tween 20) overnight at 4°C. Washes were performed in PBT, and the secondary antibody incubated for 2 hr in PBT at room temperature. The secondary antibodies were biotinylated and an avidin/biotin/horseradish peroxidase (HRP) complex was used for the detection (Vectastain from Vector Laboratories). The HRP substrate was diaminobenzidine (DAB) in PBS. Antibodies were provided by Sergio Gonzalez-Crespo (Centro de Investigación y Desarrollo–CSIC, Barcelona, Spain; anti-Exd), R. White (University of Cambridge, UK; anti-Ubx). β -Gal antibody (rabbit) was purchased from Cappel. After staining, the embryos were dehydrated and mounted in Permount. Photographs were taken on Zeiss Axiophot with Nomarski optics and Fujichrome 64T film.

In situ hybridization

The in situ hybridization with DIG-labeled probe to embryos was done as described in Tautz and Pfeiffle (1989), with slight modifications. The *exd* probe used was a 2.2-kb *Clal* fragment from the *exd* cDNA. To stain discs, they were dissected from third instar larvae in PBS, kept on ice for 30 min, and fixed in 4% paraformaldehyde during 20 min at room temperature. They were washed three times in PBS, incubated for 2 min in 0.25% glutaraldehyde on ice, washed in PBS, and fixed in 4% formaldehyde, 0.1% Triton X-100, 0.1% sodium deoxycholate for 20 min at room temperature. After a short wash in PBS, discs were rinsed in PBT, incubated with proteinase K (50 μ g/ml) for 3 min, postfixed in 4% paraformaldehyde during 20 min at room temperature, washed in PBT, and prehybridized at 48°C in hybridization solution for 1 hr. The following steps were done as for embryos. The *Hoxd10* probe used was a 1.6-kb *Bam*HI fragment from the *Hoxd10* cDNA.

Immunofluorescence staining

Imaginal discs were dissected from third instar larvae, fixed in 4% paraformaldehyde in PBS for 20 min at room temperature, postfixed in 4% paraformaldehyde, 0.1% Triton X-100, and 0.1% sodium deoxycholate in PBS for 20 min, washed in PBS for 5 min, and blocked in 1% BSA and 0.3% Triton X-100 in PBS for 1 hr. Discs were incubated in the first antibody (anti-Exd diluted 1/300) overnight at 4°C. Washes were performed with PBS, 1% BSA, and 0.3% Triton X-100, and the discs were incubated for 2 hr in the secondary biotinylated anti-rat antibody (Amersham). After washing the secondary antibody, a FITC–streptavidin complex (Jackson) was added for 2 hr and the discs were washed and mounted in PBS, 4% propylgalate. For embryos, the fixation was done as described previously (antibody staining for embryos), and the rest of the procedure was performed as for imaginal discs. Photographs were taken in a laser scan Zeiss microscope and subsequently processed using Adobe Photoshop.

Preparation of adult cuticle

The adult flies with appropriate phenotypes were dissected in water and cut into pieces. They were subsequently treated with 10% KOH at 95°C for 3–5 min to digest the internal tissues, washed with water, rinsed in ethanol, and mounted in Euparal. The preparations were photographed on a Nikon AFX-II microscope using Fujichrome 64T film.

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