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extradenticle determines segmental identities throughout *Drosophila* development

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SUMMARY

extradenticle (*exd*) and the homeotic selector proteins together establish segmental identities by coordinately regulating the expression of downstream target genes. The inappropriate expression of these targets in *exd* mutant embryos results in homeotic transformations and aberrant morphogenesis. Here we examine the role of *exd* in adult development by using genetic mosaics and a hypomorphic *exd* allele caused by a point mutation in the homeodomain. *exd* continues to be essential for the specification of segmental identities, consistent with a continuing requirement for *exd* as a cofactor of the homeotic selector proteins. Loss of *exd* results in the homeotic transformation of abdominal segments to an A5 or A6 segmental identity, the antenna and arista to leg, and the head capsule to dorsal thorax or notum. Proximal leg structures are particularly sensitive to the loss of *exd*, although *exd* does not affect the allocation of proximal positional values of the leg imaginal disc. Using heat-shocks to induce expression of a *hsp70-exd* fusion gene, we show that, in contrast to the homeotic selector genes, ubiquitously high levels of *exd* expression do not cause pattern abnormalities or segmental transformations.

Key words: *extradenticle*, homeotic transformation, clonal analysis, *Drosophila*, pattern formation, leg development

INTRODUCTION

In *Drosophila*, development occurs in two distinct phases, an embryonic period that leads to the larva and a period of imaginal development that gives rise to the adult. Both the larval and adult body patterns are segmented. Homeotic genes of the Antennapedia (ANT-C) and bithorax (BX-C) complexes specify segmental identities throughout both phases of development (reviewed by Duncan, 1987; Kaufman et al., 1990). Each homeotic selector gene is expressed in a distinct spatial and temporal pattern. If a given homeotic gene is absent or is expressed outside its normal domain, the morphology of affected segments is altered to reiterate that of other segments. The homeotic selector genes encode homeodomain-containing transcription factors that specify different developmental fates by regulating the expression of distinct sets of downstream genes (reviewed by Botas, 1993).

We have shown that *extradenticle* (*exd*) plays an important and singular role in regulating homeotic gene activity (Peifer and Wieschaus, 1990a; Rauskolb et al., 1993; Rauskolb and Wieschaus, 1994). *exd* mutant embryos show homeotic transformations, although the ANT-C and BX-C genes are expressed normally and function genetically in the appropriate segments. Instead, in *exd* mutants the activity of the homeotic selector genes is altered, resulting in aberrant morphology. In the visceral mesoderm, *exd* and the homeotic selector genes coordinately regulate some of the same target genes (Rauskolb and Wieschaus, 1994). In the absence of *exd* the homeotic proteins are incapable of regulating these targets. *exd* also encodes a homeodomain protein and thus the requirements for *exd* activity may reflect a direct interaction between *exd* and the selector proteins on the target DNA (Rauskolb et al., 1993). Indeed, in vitro studies have demonstrated cooperative interactions between *exd* and *Ultrabithorax* and *exd* and *abdominal-A* (Chan et al., 1994; van Dijk and Murre, 1994). Together, these experiments suggest that *exd* is a ubiquitous cofactor required in the embryo for the interaction of various homeodomain proteins with many of their targets.

Given the dramatic effect of *exd* on selector gene activity in the embryo, it was important to determine whether *exd* is also required to specify segmental identities at post-embryonic stages of development. Because null alleles of *exd* are embryonic lethal, we used genetic mosaics to investigate the requirements for *exd* function at later stages of development. We extended this analysis to whole animals and developing discs, using a hypomorphic allele whose larval lethality was circumvented by increased wild-type dosage in the mother. Lastly, we used heat-shock constructs to determine whether ubiquitous expression of *exd* is sufficient to alter cell fates. These experiments demonstrate that *exd*, like the homeotic selector genes, is required for the specification of segmental identities throughout development.

MATERIALS AND METHODS

Fly stocks

exd mutants are described by Wieschaus et al. (1984) and by Katzen

(1990). The following enhancer trap lines were used: *rK111* (insertion in *odd-skipped*; Cohen, 1993), *rK568* (insertion in *apterous*; Cohen et al., 1992), and *P(Lac, ry⁺)A R30b* (insertion in *teashirt*; Fasano et al., 1991). Balancer chromosomes and all other mutants are described by Lindsley and Zimm (1992).

Generation and analysis of *exd* mosaic flies

To produce *exd* gynandromorphs, heterozygous *y w exd^XP11 f/FM6* or *y w exd^EM5 f/FM6* females were crossed to *In(1)w^vC/Y* males. All female progeny surviving to adulthood were scored as either *exd* or *FM6*, mosaic or nonmosaic (Table 1). To assess mosaic frequency in the *FM6* controls, clones were identified on the basis of male phenotypic characteristics in XO clones. The frequency of male structures in controls ranged from 39% in the first thoracic legs to 25.5% in the analia; the average was 30.7%. Since the distribution of XO clones is random, the clone frequency in any given structure is about 30%, similar to that seen in previous experiments (Wieschaus et al., 1992). Mitotic clones of *exd* were produced as described by Wieschaus et al. (1992) except females of the genotype *y w exd^XP11 f/FM6* or *y w exd^EM5 f/FM6*, or in controls *y w f/y w f*, were crossed to *M(1)O^sp/Y* males.

Sequencing of mutant alleles

DNA was isolated from homozygous mutant embryos (*exd^XP11, exd^YO12, exd^EM25, exd^EM54, exd^WM*), heterozygous adults (*exd^EM52/ exd^WM* and *exd^EM5/FM7 exd⁺*), or homozygous mutant larvae (*exd^EM5*) and the *exd* coding region was PCR amplified as described by Peifer and Wieschaus (1990b). Amplified DNA was cloned into pGEM-T (Promega). At least three independent clones were sequenced for each allele, and in most the entire coding region. A silent nucleotide polymorphism allowed the *EM* series of mutants to be distinguished from *FM7*, allowing unambiguous identification of clones encoding the mutant allele. A silent polymorphism at nt 1184 confirmed that *exd^XP11* and *exd^EM25* are independently derived.

HS:exd expression constructs and heat-shocks

Three *HS:exd* constructs with different 3′ UTRs were made; details available upon request. *HS:exd1* contains the *exd* coding region and *hsp70* 3′ untranslated sequences. *HS:exd2* is identical to *HS:exd1*, but has about 400 bp of untranslated 3′ *exd* sequences added. *HS:exd3* is similar to *HS:exd2*, but contains the poly(A) tail of *Ubx*. Transgenic flies were generated as described by Rauskolb et al. (1993). The *HS:exd1*-4AX transgene, on the X chromosome, and autosomal lines *HS:exd2*-7A and *HS:exd3*-9A are homozygous viable. To assay for rescue of *exd* mutant embryos, the *HS:exd1*-4AX transgene was recombined with a chromosome carrying *exd^XP11* to generate the stock *w HS:exd1*-4AX *exd^XP11/FM7/ Dp(1;Y)shi⁺Y#1*.

Six different lines carrying *HS:exd1* were heat-shocked for 1 hour at 37°C. Development continued at room temperature. Ages tested were 0-19, 19-42.5, 42.5-69 hours and 2.9-4.6, 4.6-6.5, 6.5-7.8 days. No differences in phenotype or viability were noted between transformant lines and *w* controls. Other treatments in which several lines each carrying *HS:exd1*, *HS:exd2*, or *HS:exd3*, hemizygous or homozygous, were tested included the following: 0- to 3-day old animals were heat-shocked for 1 hour at 37°C every 24 hours until eclosion; white pupae were heat-shocked twice daily for 1 hour at 37°C; and, to induce persistent *exd* expression, white pupae were placed at 33°C for 5 days. *HS:exd* flies did not display any developmental abnormalities under any of these conditions.

Detection of expression patterns

Whole-mount in situ hybridizations were carried out as described by Rauskolb et al. (1993). Imaginal discs from male (*exd^EM5*) and female (*exd⁺*) mature third instar larvae were dissected and β-galac-tosidase expression patterns determined as described by Irvine et al. (1991).

RESULTS

Uniform expression of *exd⁺* suppresses the phenotype of null mutations

The homeotic transformations produced by loss of zygotic *exd* are most obvious in the anterior two-thirds of the embryo (Wieschaus et al., 1984; Peifer and Wieschaus, 1990a). The head is defective and thoracic segments T2 and T3 are transformed anteriorly toward T1. The abdominal segments are transformed posteriorly; segments A1 through A4 are transformed toward A5. The most posterior abdominal segments
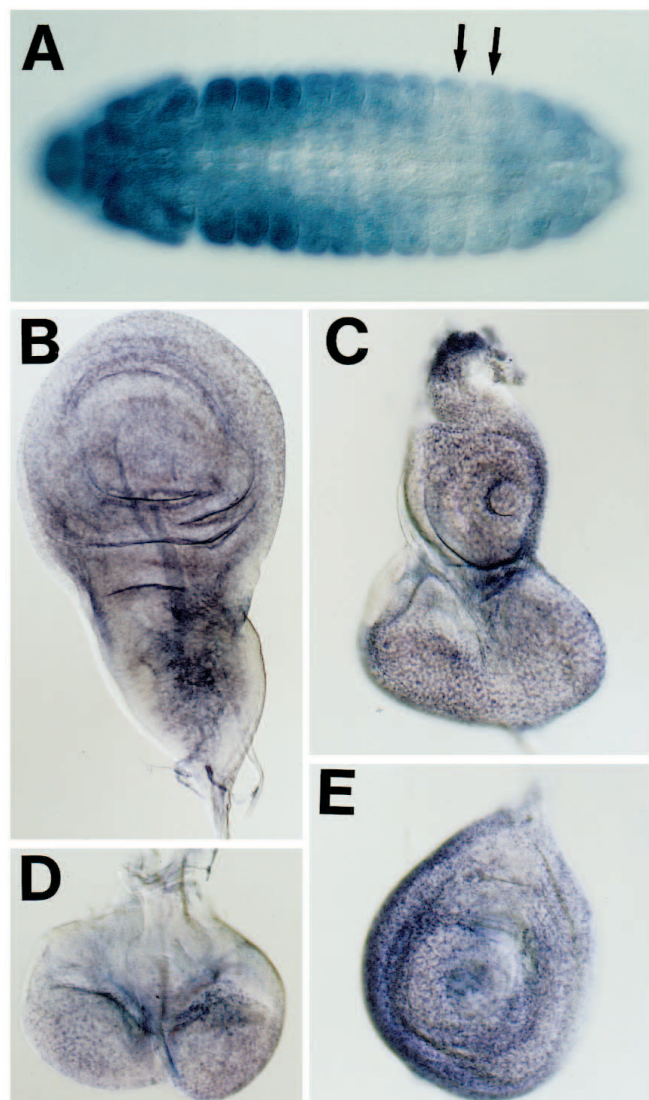
Fig. 1. *exd* is uniformly expressed in imaginal discs. *exd* RNA expression at mid-embryogenesis and in imaginal discs as revealed by whole-mount in situ hybridization. (A) Wild-type embryo at stage 13 demonstrating that *exd* is expressed at a distinct level in each segment. Lowest levels of expression are observed in segments A5 and A6 (arrows). *exd* expression is uniform in (B) wing, (C) eye-antennal, (D) genital, and (E) leg imaginal discs.
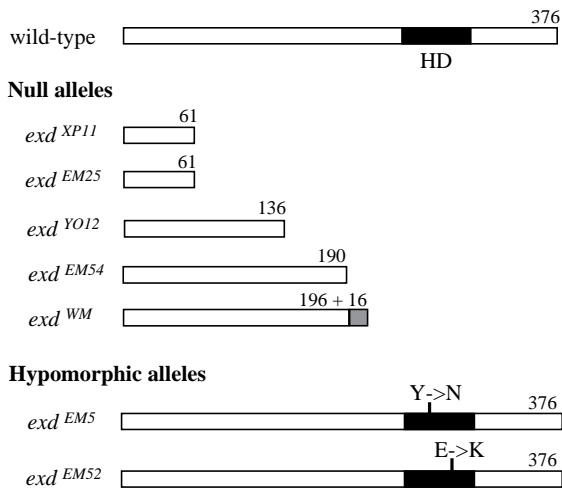
Fig. 2. Molecular analysis of *exd* alleles. The bar schematically represents the 376 amino acid *exd* protein, including the homeodomain (HD). Null alleles have the following lesions: *exd^{XP11}* nt (nucleotide) 390 C→T; *exd^{EM25}* nt 390 C→T; *exd^{YO12}* nt 615 C→T; *exd^{EM54}* nt 777 C→T; *exd^{WM}* deletes the region from nt 36 of the intron 790/791 to nt 813. This last lesion results in a frameshift, adding 16 novel amino acids after amino acid 196. All null mutations introduce a premature stop codon, thereby giving rise to a truncated protein of the indicated length. Hypomorphic *exd^{EM5}* alters nt 978 from T→A and *exd^{EM52}* alters nt 1008 from G→A. These result in missense mutations within the homeodomain, as indicated. We have also now sequenced *exd* genomic DNA. The *exd* gene has three small introns at nts 471/472, 790/791, and 1049/1050. All contain multiple stop codons, and using consensus acceptor and donor sites, no likely alternate splice possibilities are apparent. The region C-terminal to the homeodomain does not contain any introns, ruling out the possibility that *exd* is alternatively spliced in a manner similar to the vertebrate homologs *pbx1* and *pbx3* (Monica et al., 1991).

and telson are normal. These transformations are somewhat reminiscent of the modulated pattern of zygotic *exd* expression during mid-to-late embryonic stages (Fig. 1A; Rauskolb et al., 1993). High levels of *exd* transcripts are observed in the head and thoracic segments, while levels decline posteriorly; the lowest levels are observed in A5 and A6.

The homeotic transformations described above are produced by deletions and apparently amorphic alleles at the locus (Peifer and Wieschaus, 1990a). Sequence analysis of five such alleles reveals lesions consistent with their amorphic nature; *exd^{XP11}*, *exd^{YO12}*, *exd^{EM25}*, *exd^{EM54}*, and *exd^{WM}* result from the introduction of a premature stop codon or a frameshift that truncates the protein before the homeodomain (Fig. 2).

The mutants *exd^{EM5}* and *exd^{EM52}* are not embryonic lethal. We have examined *exd^{EM52}* in *trans* to the null alleles *exd^{XP11}* and *exd^{WM}*. *exd^{EM52}/exd^{XP11}* animals die as pharate adults with no obvious cuticular defects, while *exd^{EM52}/exd^{WM}* animals sometimes eclose as morphologically normal adults, but always die within a day or two. Transheterozygotes of the two embryonic viable alleles, *exd^{EM5}/exd^{EM52}*, survive to adulthood at about 25% the frequency of their *exd^{EM5}/FM7* siblings, but often die within 24 hours. Minor cuticular defects, including an absence of coxal bristles on the

mesothorax and missing or reduced palps, occur. Sequence analysis of these two hypomorphic alleles revealed that they each carry a missense mutation in the homeodomain (Fig. 2). Both mutations occur in residues that are not part of the global homeodomain consensus, but which are conserved between *exd* and its vertebrate homologs (Rauskolb et al., 1993).

The embryonic cuticular phenotypes associated with *exd* null alleles are observed only in embryos from heterozygous mothers; embryos derived from attached-X mothers, which carry two wild-type copies of *exd*, die with a normal cuticular pattern (Wieschaus and Noell, 1986). Because maternally supplied *exd* RNA is uniformly distributed in the egg, we have argued that a particular spatial pattern of *exd* expression is irrelevant for rescuing activity, and thus the distribution of *exd* itself does not appear to provide positional information (Rauskolb et al., 1993). To test this view, we used the *hsp70* promoter to drive ubiquitous zygotic expression of *exd*. Three *HS:exd* constructs, differing in their 3′ UTRs (see Materials and Methods), rescue *exd* null embryos to almost normal morphology following a single heat-shock at the onset of gastrulation (Fig. 3). Notably, ectopic *exd* expression has no obvious effect on the development of wild-type embryos.

These results raise questions about the significance of the modulated expression pattern of *exd* that arises during mid-embryogenesis. This late expression pattern may reflect a requirement for *exd* during post-embryonic stages of development. Northern analysis indicated that *exd* is expressed at all stages of development (Rauskolb et al., 1993). Furthermore, in situ hybridization to imaginal discs from mature third instar larvae reveals that *exd* is expressed uniformly in the eye-antennal, leg, wing, haltere, and genital discs (Fig. 1). Thus, the patterned *exd* expression in the late embryo may mark the beginning of a later role for *exd* in specifying the segmental identities of adult structures.

exd mosaics survive to adult stages but with reduced viability

To analyze post-embryonic requirements for *exd*, we generated gynandromorph mosaics in which the male *exd* mutant regions of the adult cuticle were marked with *yellow*(*y*) and *forked* (*f*). The survival of these *exd* mosaic flies was greatly reduced. When an amorphic allele (*exd^{XP11}*) was used, *exd* mosaics were recovered at only 18% of the control *FM6*

Table 1. Recovery of *exd* gynandromorphs surviving to adulthood

	Experiment A		Experiment B	
	control*	*exd^{XP11}*†	control*	*exd^{EM5}*‡
Number nonmosaics	2035	1693	370	352
Number mosaics	790	116	202	78
Mosaic survival§	1.00	0.18	1.00	0.41

*Control flies are of the genotype *FM6/In(1)w^{vC}* and serve to control for ring-X loss and recovery of *exd* mosaics.

†The genotype of these flies is *y w exd^{XP11} f/In(1)w^{vC}*.

‡The genotype of these flies is *y w exd^{EM5} f/In(1)w^{vC}*.

§Mosaic survival = (number of *exd* mosaics)/(number of *exd* nonmosaics) × (number of control nonmosaics)/(number of control mosaics). The control mosaic survival rate was normalized to 1.00.

Table 2. Frequencies of *exd^{XP11}* and *exd^{EM5}* clones in various structures of surviving adult gynandromorphs

Structure	No. *exd^{XP11}* clones/ no. times scored	Frequency of *exd^{XP11}* clones (%)*	No. *exd^{EM5}* clones/ no. times scored	Frequency of *exd^{EM5}* clones (%)
Head				
pv†	3/232	**1.3**	25/154	**16.2**
Ocellar†	2/232	**0.9**	26/156	**16.7**
Postorbital†	0/232	**0.0**	27/154	**17.5**
Antenna	8/232	**3.4**	27/156	**17.3**
Palpus	1/231	**0.4**	20/152	**13.2**
Proboscis	3/232	**1.3**	18/156	**11.5**
Thorax				
pst†	1/227	**0.4**	2/155	**1.3**
Humerus	5/226	**2.2**	8/154	**5.2**
Scutellum	1/227	**0.4**	3/155	**1.9**
anp†	1/227	**0.4**	2/155	**1.3**
Wing	0/228	**0.0**	4/158	**2.5**
sp†	2/230	**0.9**	0/151	**0.0**
T1 leg	5/230	**2.2**	12/155	**7.7**
T2 leg	4/232	**1.7**	7/156	**4.5**
T3 leg	5/230	**2.2**	12/156	**7.7**
Abdomen				
A1 tergite	13/232	**5.6**	9/155	**5.8**
A2 tergite	29/232	**12.5**	25/156	**16.0**
A3 tergite	40/230	**17.4**	31/156	**19.9**
A4 tergite	50/231	**21.6**	36/155	**23.2**
A5 tergite	48/224	**21.4**	29/156	**18.6**
A6 tergite	52/226	**23.0**	28/156	**17.9**
A7 tergite	36/217	**16.6**	26/156	**16.7**
Genitalia	30/232	**12.9**	21/154	**13.6**
Analia	37/232	**15.9**	19/154	**12.3**

*The frequency of clones in the *FM6* control gynandromorphs was about 30% for any given structure.

†These structures are particular bristles. pst, presutural; pv, postvertical; anp, anterior notopleural; sp, sternopleura.

For a description of adult cuticular structures see Bryant (1978).

frequency, while with the hypomorphic allele *exd^{EM5}* the mosaic survival was 41% (Table 1). The viability of mosaics depended on the distribution of *exd* mutant tissue (Table 2). In the surviving *exd^{XP11}* mosaics, *exd^−* cells were seldom obtained in the head and thorax. By contrast, the frequencies of *exd^−* patches in the abdomen and genitalia (5.6% to 23%) were closer, although still below, control frequencies (30%; Table 2). Similar results were obtained with *exd^{EM5}*, except that structures were more often *exd^−*, particularly in the head (Table 2).

The low recovery of *exd^−* cells in adult head and thoracic structures presumably reflects a higher requirement for *exd* in the regions of the embryo giving rise to these structures. Given the large size of the *exd* clones in gynandromorphs, the focus of this lethality need not be the head or thoracic disc precursors themselves; it could be the central nervous system or another internal tissue. When mitotic recombination is used to produce smaller, later clones restricted to individual discs, the recovery of *exd* cells in the head and thorax increases, but is still reduced (Table 3). Most of the reduction in clone frequency in the mitotic recombination experiments can be attributed to the failure to obtain clones in certain regions of each disc. Moreover, recovered clones were frequently associated with transformations of segmental identity (see below). Thus, although not required for the viability of most imaginal cells, *exd* does play an essential role in the development of adult structures.

Loss of *exd* leads to homeotic transformations in the adult abdomen

The abdominal segments are distinguished by unique features of the tergites (dorsal cuticle). The A1 tergite has small bristles; the A5 and A6 tergites have uniform pigmentation in males; the A5 tergite has trichomes (fine hairs) of characteristic density; the A6 tergite essentially lacks trichomes; and the A7 tergite is small and dumbbell-shaped (Bryant, 1978; Karch et al., 1985; Sanchez-Herrero et al., 1985). Homeotic transformations were associated with *exd* clones in both gynandromorph and mitotic recombination experiments. In *exd^{XP11}* mosaic flies, the segmental identity of clones in A1 through A4 is transformed posteriorly toward A5 or A6. In gynandromorphs, this transformation is evidenced by the darker cuticle pigmentation and trichome density characteristic of posterior segments (Fig. 4). Most often these clones have a trichome density characteristic of A5. However, clones were also recovered with regions devoid of trichomes, indicative of a partial transformation toward A6. The clones behave cell autonomously, as only cells marked with *y* and *f* are transformed. *exd* also affects tergite differentiation less specifically. Small *exd^{XP11}* clones within a tergite differentiated normally, but larger clones encompassing most or all of a hemitergite often differentiate abnormally, resulting in the absence of part of the tergite.

In contrast to the posterior transformations associated with clones in A1-A4, *exd^{XP11}* clones within A6 and A7 are transformed anteriorly. Clones within A6 are transformed toward A5 as evidenced by the presence of trichomes, which are not

Table 3. Frequency of *exd* mitotic clones in various adult structures

	Head	Humerus	Wing	Tergites	Sternites	Genitalia	Analia
ywf	0.53	0.11	0.19	3.29	1.22	0.14	0.11
	(61/116)	(7/64)	(12/64)	(28/8.5)	(11/9)	(12/84)	(10/87)
	100%	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**
exd^{XP11}	0.30	0.02	0.14	1.55	0.15	0.13	0.04
	(34/115)	(2/88)	(12/88)	(31/20)	(3/20)	(11/87)	(3/84)
	56%	**18%**	**74%**	**47%**	**13%**	**89%**	**31%**
exd^{EM5}	0.70	0.06	0.24	3.53	1.00	0.14	0.10
	(78/111)	(5/88)	(21/88)	(53/15)	(15/15)	(10/71)	(7/72)
	134%	**55%**	**126%**	**107%**	**82%**	**100%**	**85%**

The top value is the frequency at which clones were obtained. Values in parentheses are the actual no. of clones/no. of adults examined. The **percentages** reflect the frequency of *exd* clones relative to the frequency of *ywf* control clones (normalized to 100%).

normally found in the A6 tergite (Fig. 4E). In wild-type flies the A7 tergite is present in females and absent in males. Clones of *exd^{XP11}* in A7 develop in both gynandromorph and mitotic recombination mosaics, even though in gynandromorphs the tissue is male in genotype (Fig. 4E). These ectopic A7 tergites have a morphology characteristic of A5 or A6, indicating that they are transformed anteriorly. The only segment where *exd* clones have no effect is in A5, where segmental identity remains normal.

The genital disc forms the most posterior structures of the abdomen. Female genital structures are derived from A8, male genitalia from A9, and both male and female analia from A10 (Schüpbach et al., 1978). In wild-type females only A8 and A10 segment derivatives develop, and in males only A9 and A10 develop. *exd* clones in the female genitalia show the same anterior-ward transformation observed in A6 and A7. The tiny A8 tergite is enlarged and has bristles characteristic of more anterior abdominal tergites (Fig. 5A). The vaginal plate is also abnormal with multiple rows of thorn-like bristles and fusions along the midline. Occasionally we also observe an ectopic ninth tergite in females with *exd* null clones, implying that the normally absent A9 has been transformed to a more anterior fate (Fig. 5B). In the male primordia the effects of *exd* are more subtle. In gynandromorphs, when the entire genital disc is male and *exd^-*, the genitalia and analia remain as a ball of internalized cuticle and do not fuse with the more anterior abdominal segments, regardless of whether they are wild-type or *exd^-* (Fig. 5C). These unfused male genitalia and analia are apparently normal, including such structures as the clasper, penis and anal plate. When the genitalia of a gynandromorph is composed of both female *exd^+* and male *exd^-* cells, it fuses with the exoskeleton, and the *exd^-* cells make morphologically normal male structures. Clones restricted to the analia are normal in both gynandromorphs and mitotic recombination experiments; *exd* does not affect the development of A10 in either males or females. Thus, the major homeotic transformation caused by *exd* appears to be restricted to the female region of disc, that is, to abdominal segment A8. The transformation of A8 is anterior-ward, similar to the effect of *exd* in A6 and A7.

Flies mosaic for the hypomorphic *exd^{EM5}* allele show only a subset of the homeotic transformations observed with *exd^{XP11}* mosaics. Abdominal segments A1-A5 are unaffected and clones within A6 most often retain their appropriate identity; they are transformed toward A5 in about one-third of the cases (Fig. 4). In contrast, *exd^{EM5}* clones within A7 are always transformed anteriorly toward A6, as evidenced by their differentiation into a tergite lacking trichomes (Fig. 4F). In the genitalia, *exd^{EM5}* and exd^{XP11} clones also have similar phenotypes; the same transformations are observed in the female eighth tergite and vaginal plate and *exd^{EM5}* gynandromorphs display the same effects on male genitalia.

exd mutations cause homeotic transformations in the head

The most obvious effect of amorphic *exd* clones in the head are transformations of the antenna to leg-type structures. Although clones in the proximal antenna are relatively normal, clones in the third antennal segment and arista are transformed toward leg, as evidenced by the presence of bracted bristles

and tarsal claws, cuticular structures associated only with legs (Fig. 6A,B). In the gynandromorph mosaics, we could rule out that these antennae are transformed toward the first thoracic (T1) leg because sex combs, a characteristic feature of male T1 legs, are never associated with them. Rather, in several instances a large apical bristle was associated with the antennal leg, indicative of a transformation toward a T2 identity (Fig. 6B).

Another effect of *exd* on head development is the failure to recover *exd* cells in certain head structures. *exd* null cells never give rise to recognizable vibrissae, orbital, postorbital, or palp bristles. Most often these structures are deleted, although sometimes they are replaced by masses of *y f* tissue of indeterminate identity. For example, among the surviving gynandromorphs, two *exd^{XP11}* clones were recovered in the ocellar region and each was associated with the deletion of the postvertical bristle and perturbation of one ocellus. The structures were replaced by clonal tissue of thinner density cuticle that lacked trichomes, reminiscent of genital tissue. In the mitotic recombination analyses, ectopic *exd* structures are more frequent. We often found a *y f* outgrowth on the back of the head that had a cuticular texture, pigmentation and density of trichomes reminiscent of the dorsal thorax or notum (Fig. 6C). One *exd^{XP11}* clone was recovered in the
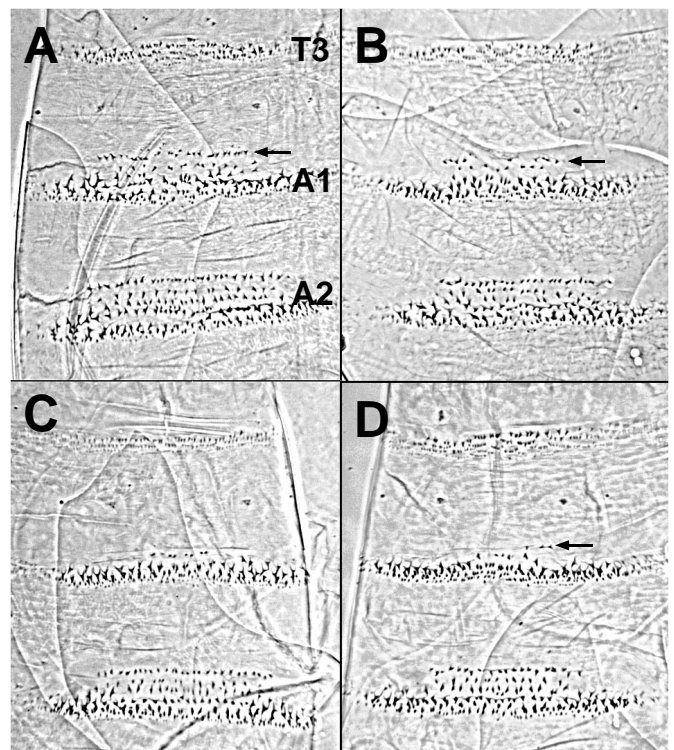
Fig. 3. *Hsp70*-induced *exd* expression suppresses the *exd* embryonic cuticular phenotype. Ventral denticle pattern of first instar larvae. The segments shown are T3, A1, and A2. (A) *exd^{XP11}* mutant embryo, showing the clear transformation of A1 to A2 as evidenced by the extra row of denticles (arrow). (B) *exd^{XP11} HS:exd1* embryo raised at 18°C. Note that A1 is partially rescued as there are fewer extra denticles (arrow). (C) *exd^{XP11} HS:exd3* and (D) *exd^{XP11} HS:exd1* embryos heat-shocked at the onset of gastrulation. These embryos are effectively rescued as only a couple of ectopic denticles remain in A1 (arrow).

Fig. 4. Homeotic transformations associated with *exd* clones in the abdomen. *exd* clones observed in the tergites of gynandromorphs; identical phenotypes are observed with mitotic clones. In some panels, the clone boundary is designated by a dashed line, the *exd* mutant tissue starred. Posterior is right. (A) *exd*^{XP11} clone in A1. In contrast to the surrounding wild-type tissue (arrow), the bristles of this clone are larger and thicker (arrowheads) and the cuticle more darkly pigmented, indicative of a transformation toward A5. (B) *exd*^{EM5} clone in A1. This clone is not transformed as the bristles remain small and thin (arrowheads) and the cuticle lightly pigmented. (C) *exd*^{XP11} clone in A4. This clone is transformed towards A5 as evidenced by the dark cuticular pigmentation and sparsely spaced trichomes. (D) *exd*^{EM5} clone in A4. The pigmentation of the cuticle and trichome density are appropriate for A4; this clone is not transformed. (E) *exd*^{XP11} clone in A6 and A7. The clone in A6 differentiates trichomes in a region normally devoid of them, and hence is transformed toward A5. In A7 a tergite devoid of trichomes develops, even though the clonal tissue is genotypically male, and thus the clone is transformed toward A6. Sometimes clones within A7 are transformed toward A5. (F) *exd*^{EM5} clone in the abdomen. Both A6 and A7 are completely composed of mutant tissue; note that a seventh tergite develops.
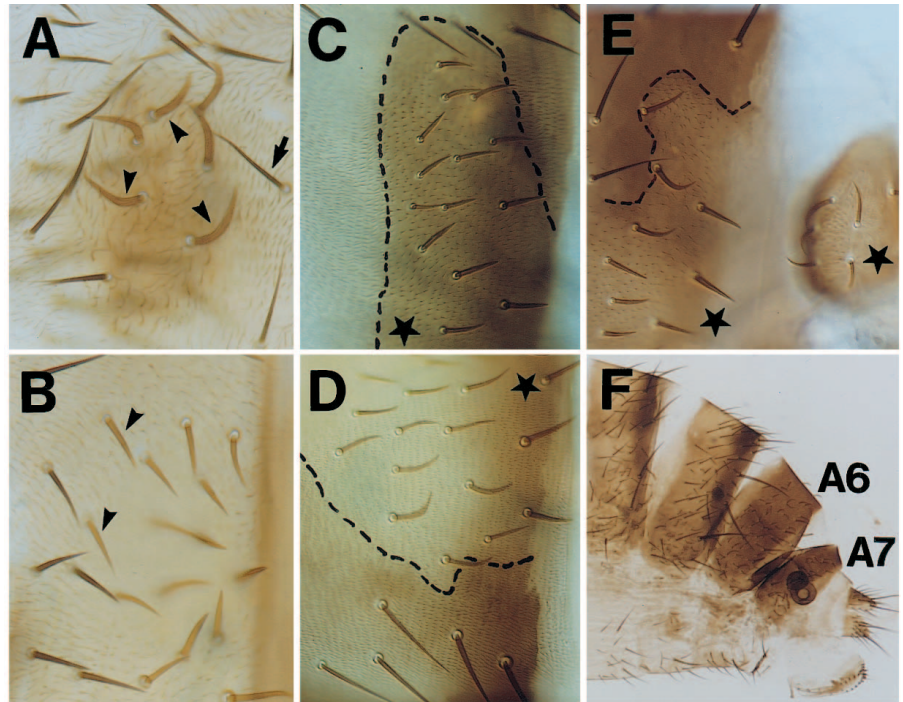
labial palp of the proboscis (Fig. 6D,E). This mutant tissue is transformed toward leg, as the bristles within the clone have bracts, found normally only on distal leg structures. The labial disc derivative in the head also shows a reduced frequency of clones; clones survive only in the prementum region of the proboscis where they form protrusions of tissue with ectopic bristles.

In contrast to the limited regions of the head in which *exd*^{XP11} clones could be recovered, *exd*^{EM5} clones in gynandromorphs could encompass any region of the head, to the extent that half of the fly head could be mutant for *exd*^{EM5} (Fig. 6F). In the antenna, *exd* clones cause a size reduction and slight thickening of the arista (Fig. 6F), which may indicate a partial transformation toward leg. Similar effects are observed in the smaller mitotic recombination clones. Clones in and near the ocellar region are associated with the loss of the ocellar and postvertical bristles (Fig. 6F). Occasionally, clones near the vertical bristle are associated with ectopic bristles like those seen in *exd*^{XP11} clones, and in one case the adjacent occipital region was reduced. Clones are recovered at a reduced frequency in the region of the vibrissae; in those recovered, the large vibrissal bristles are deleted (Fig. 6F). Clones in the cuticular structures derived from the labial disc are normal.

Fig. 5. Homeotic transformations associated with *exd* clones in the genitalia. Posterior is left. (A) *exd* null mitotic clones in A8 are transformed anteriorly. The tergite in this female is larger than the normal A8 tergite and heavy bristles characteristic of more anterior segments are present (arrowhead). (B) *exd* null clones in A9 can transform this segment to a more anterior fate. Normally A9 does not develop in females; here A9 is clearly present (arrowhead). (C) An *exd* null clone in a gynandromorph. The male genitalia and analia are internalized (arrowhead).
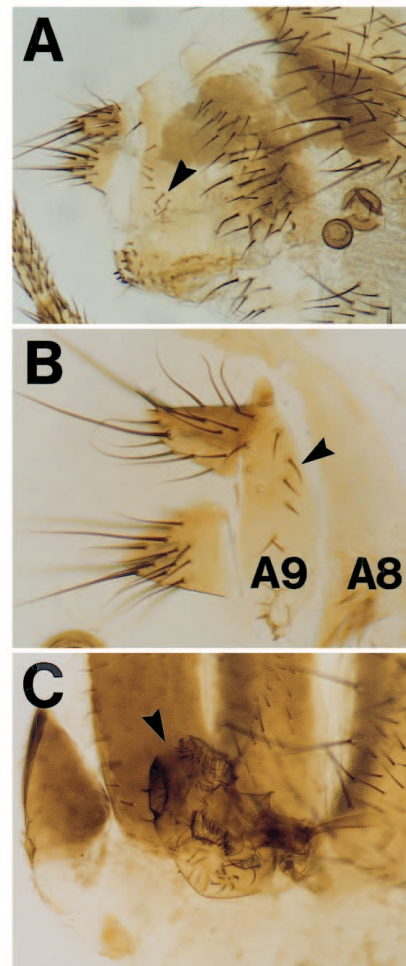
## A proximal-distal sensitivity to *exd* during leg development

In the thorax, *exd* mutant tissue derived from the notal region of the wing disc and from the humeral disc is normal, although clone survival is reduced (Table 3). The only occasional effect is on the spacing of bristles, which seems to be decreased in mutant clones.

By contrast, *exd* dramatically affects the development of the legs. Since the frequency of clones in the legs of gynandromorphs was low (Table 2) and they gave similar, if not identical, phenotypes to mitotic clones, only mitotic *exd* clones will be discussed in detail. Interestingly, the phenotype of *exd* clones is different depending on whether the clone is located distally or proximally in the leg (Fig. 7). Clones of cells mutant for either the null *exd^{XP11}* or the hypomorphic *exd^{EM5}* allele survive and participate in normal patterning of distal tarsal regions of the leg. In contrast, clones located in proximal regions either do not survive, grossly distort pattern and reduce

leg segment size, or make ectopic clusters of bristles in regions of the leg normally devoid of bristles (Figs 7, 8). The recovery of clones is graded along the proximal-distal axis and seems to depend on the strength of the allele (Fig. 7). One point that deserves note is that the segmental identities of mutant legs are not affected. For example, a clone in the second thoracic leg can include the apical bristle, a structure characteristic of this leg. Thus, although the pattern of proximal leg segments is distorted, homeotic transformation of proximal leg tissue to another segmental identity (i.e. leg to antenna or leg to genitalia), or transformation to more distal leg fates, is not observed.

A possible proximal-distal bias in the requirement for *exd* activity is also revealed in non-mosaic animals mutant for the hypomorphic allele. *exd^{EM5}* mutants normally die during larval stages. However, if the maternal component of *exd* is increased, occasional pharate adults are obtained which can be dissected from the pupal case. These flies lack proximal leg

Fig. 6. *exd* clones in adult heads. (A) *exd* null clone in the antenna and arista. The clonal tissue is transformed toward leg (arrowheads). The wild-type antenna (a) and arista (r) are designated. The vibrissal bristles are also highly disorganized. (B) Higher magnification of the clone in A showing the distal claw (arrow) and the large apical bristle (arrowhead) indicative of T2 identity. (C) *exd* null clone protruding from the back of the head (arrowhead). The clonal tissue is darkly pigmented with heavy bristles. (D) *exd^{XP11}* clone in the proboscis (arrowhead). The bristles of this clone have bracts, shown in E (arrowhead), cuticular structures associated only with leg bristles. (F) Hypomorphic *exd^{EM5}* clone making up half of a gynandromorph head (on right). The arista is shortened and appears thickened (arrowhead) compared to the wild type. The ocellar (oc), postvertical (pv) and large vibrissal (v) bristles are absent.
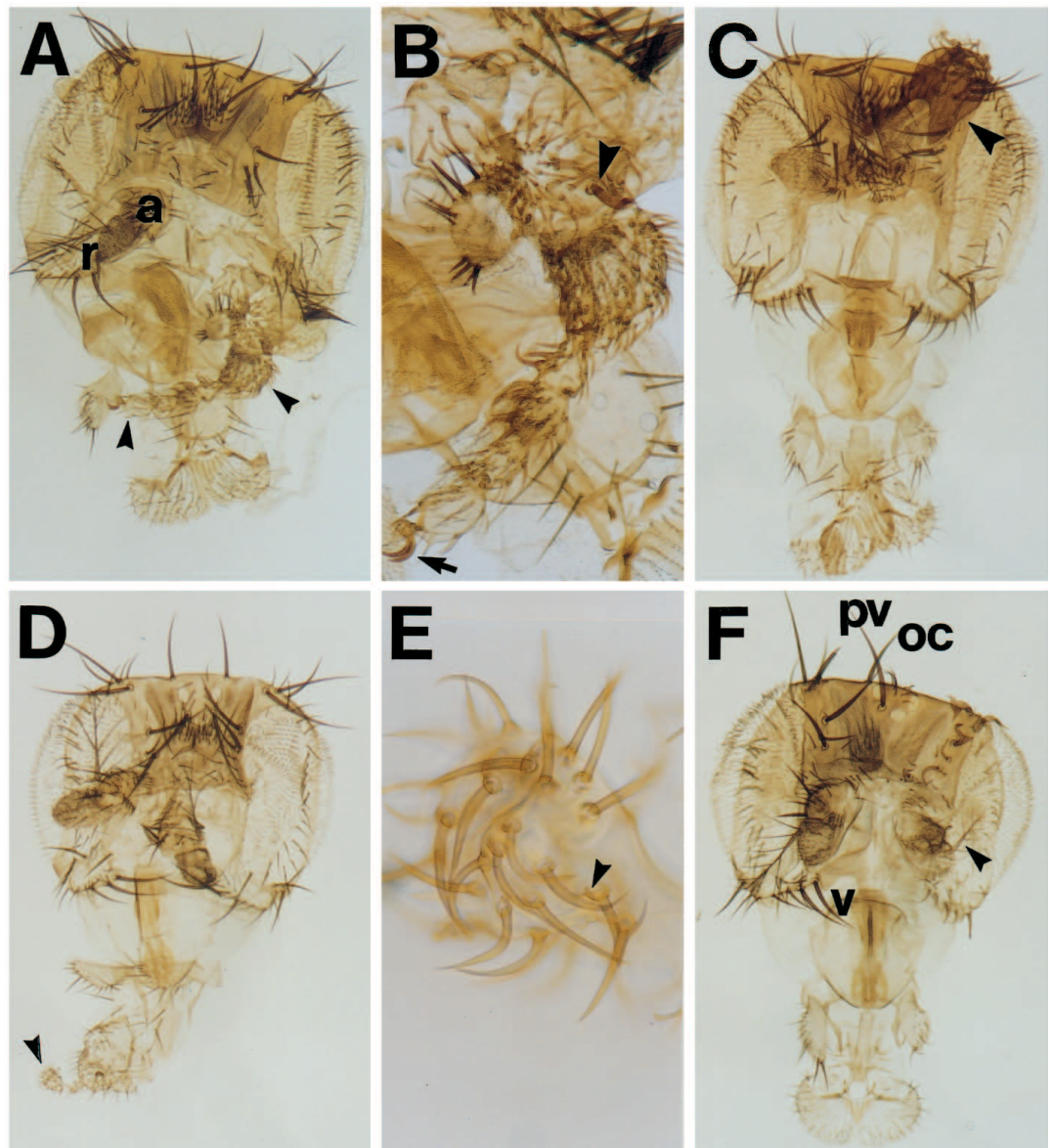
structures, while distal ones are normal. This phenotype is more easily visualized in exd^{EM5} gynandromorphs (Fig. 8A). The coxa and trochanter are absent, while the femur and tibia are reduced in length. Distal structures, the tarsal segments, remain unaffected.

To understand further this phenotype, and to determine whether *exd* may be involved in establishing proximal-distal positional values in the leg primordia, we used various enhancer trap lines as molecular markers of positional values in the leg imaginal discs and examined their expression patterns in discs from exd^{EM5} mutant third instar larvae. Normally, *teashirt* is expressed in a ring filling the coxa and trochanter segments, and in the peripodial membrane (Fig. 8B; Cohen, 1993). In exd^{EM5} discs this pattern is unaltered (Fig. 8C). Expression patterns of additional molecular markers such as *odd-skipped*, expressed in a ring in each leg segment, and *apterous*, expressed in the fourth tarsal segment (Cohen et al., 1992), remain normal in exd^{EM5} discs (data not shown). Thus, although the coxa and trochanter are deleted in the adult cuticular structures of exd^{EM5} mutant flies, the establishment of positional values in the leg primordia remains unaffected.
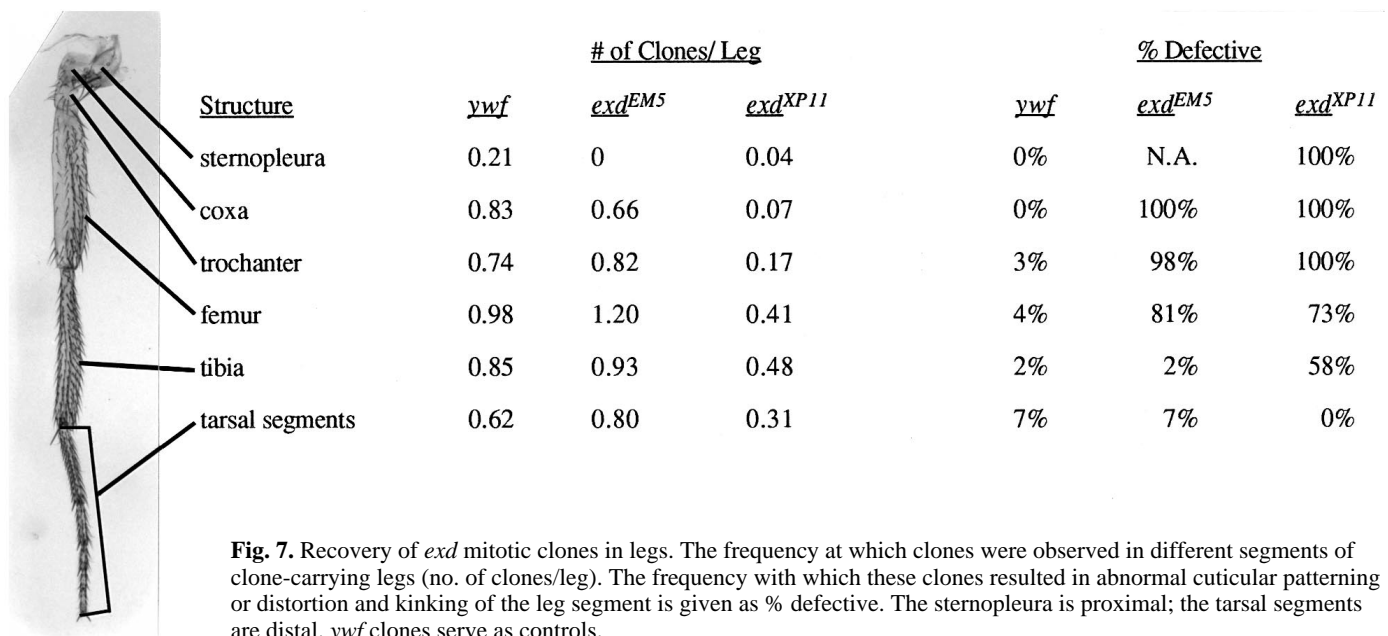
Ubiquitous expression of *exd* does not alter segmental identities

Our analysis of *exd* clones has indicated that loss of *exd* in certain regions of the animal results in homeotic transformations. Intriguingly, in the abdomen, the loss of *exd* results in the transformation of A1-A4 and A6-A9 to an A5/A6 segmental identity, precisely those segments in the embryo that have the lowest levels of *exd* expression. To determine whether the modulated expression of *exd* during embryogenesis is essential for normal patterning in the adult, we tested whether increased levels of *exd* could alter segmental identities in the abdomen or elsewhere in the fly. We used the *hsp70* promoter to drive high and ubiquitous *exd* expression throughout development and examined the effects.

First instar larvae transgenic for *HS:exd* were heat-shocked for 1 hour at 37°C every day until the adult flies eclosed. *HS:exd* flies treated in this manner are viable and do not display any homeotic transformations or other developmental aberrations. We also attempted to induce persistent ubiquitous expression of *exd* by placing *HS:exd* larvae at 33°C until the completion of pupal development. Although the *HS:exd* and the *white* control flies do not eclose, dissection of the *HS:exd* flies from their pupal cases revealed that this treatment does not alter segmental identity. The fact that these *HS:exd* constructs are capable of rescuing *exd* embryos (see above) indicates that they make functional *exd* protein upon heat-shock. Thus, although the loss of *exd* can result in homeotic transformations, ubiquitous expression of *exd* does not appear to have morphological consequences.

DISCUSSION

The studies presented here demonstrate that *exd*, like the homeotic selector genes, not only establishes segmental identities during embryonic development, but also during adult morphogenesis. Consistent with its genetic requirement, *exd* is expressed throughout embryogenesis and in imaginal discs, the primordia of many adult cuticular structures (Fig. 1; Rauskolb et al., 1993). We show that in the adult, *exd* clones often do not display their appropriate segmental identity, adopting instead a morphology characteristic of another segment. Clones of a hypomorphic *exd* allele displayed a subset of the phenotypes observed with a null allele, presumably reflecting differential requirements for *exd* activity in different cell fate decisions. Molecular characterization of the *exd* alleles revealed that, while *exd* null mutations would result in a truncated protein, the two hypomorphic mutations contain missense mutations within the *exd* homeodomain, thereby perhaps affecting the ability of *exd* to bind to its target sequences.

		# of Clones/ Leg			% Defective	
Structure	*ywf*	exd^{EM5}	exd^{XP11}	*ywf*	exd^{EM5}	exd^{XP11}
sternopleura	0.21	0	0.04	0%	N.A.	100%
coxa	0.83	0.66	0.07	0%	100%	100%
trochanter	0.74	0.82	0.17	3%	98%	100%
femur	0.98	1.20	0.41	4%	81%	73%
tibia	0.85	0.93	0.48	2%	2%	58%
tarsal segments	0.62	0.80	0.31	7%	7%	0%

Fig. 7. Recovery of *exd* mitotic clones in legs. The frequency at which clones were observed in different segments of clone-carrying legs (no. of clones/leg). The frequency with which these clones resulted in abnormal cuticular patterning or distortion and kinking of the leg segment is given as % defective. The sternopleura is proximal; the tarsal segments are distal. *ywf* clones serve as controls.

exd is necessary but not sufficient to establish segmental identities

Clones of *exd* null mutant cells in the abdomen always adopt an A5 or A6 identity, regardless of their segmental location. Normally, abdominal segmental identities are specified by the BX-C homeotic selector genes *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*), and *Abdominal-B* (*Abd-B*) (reviewed by Duncan, 1987). Each of the first eight abdominal segments (A1-A8) is specified by unique combinations and levels of expression of these three genes. Specific levels of embryonic homeotic gene expression correlate with specific morphological consequences in the adult. The fifth and sixth abdominal segments arise from cells expressing high levels of *abd-A* and low levels of *Abd-B* (Celniker et al., 1989; DeLorenzi and Bienz, 1990; Karch et al., 1990; Macias et al., 1990; Boulet et al., 1991). Clearly these genes do not require *exd* in order to specify the identity of A5, as *exd* clones within A5 always retain their appropriate segmental identity. By contrast, correct identity specification of A1-A4 and A6-A8 requires both the BX-C genes and *exd*. In the absence of *exd* these segments are all transformed toward A5/A6. We do not think that these adult transformations result from selector gene mis-expression, since the homeotic selector genes are expressed normally in *exd* mutant embryos in a variety of different tissues (Peifer and Wieschaus, 1990a; Rauskolb and Wieschaus, 1994), but this possibility cannot be excluded. Nor can the homeotic genes simply be inactive; a deficiency for the BX-C results in the transformation of all abdominal segments toward a second thoracic identity (Lewis, 1978). Instead, we postulate that in the absence of *exd* the homeotic selector proteins inappropriately regulate the expression of genes involved in adult morphogenesis, analogous to the mis-regulation of target genes by the homeotic proteins in *exd* mutant embryos (Rauskolb and Wieschaus, 1994). Our results argue that during imaginal development the homeotic genes *Ubx*, *abd-A*, and *Abd-B* all require *exd* in order to correctly regulate their downstream targets.

Notably, *exd* expression levels in the abdomen of the late embryo are graded, with the lowest levels in A5 and A6 (Fig. 1; Rauskolb et al., 1993). Although *exd* clones in the abdomen are transformed toward the segments with the lowest levels of *exd*, the actual level of *exd* expression does not appear to be instructive. Induction of high ubiquitous *exd* expression does not alter segmental morphology, suggesting that different *exd* levels may play, at most, only a subtle role in dictating different segmental identities. Nonetheless, *exd* expression levels do reflect the differential requirement for *exd* in each of the segments. A differential requirement for *exd* activity was also observed with our gynandromorphs, as the head and thorax, those regions in the embryo with the highest levels of *exd* expression, are particularly sensitive to the loss of *exd*.

exd functions in the head

Clones of *exd* in the head result in homeotic transformations and phenotypes reminiscent of those observed with mutations in some ANT-C genes, suggesting that *exd* may also act together with these genes to specify segmental identities. For example, a clone of *exd* tissue in the labial palp

gave rise to leg tissue, which is similar to, albeit less complete than, the transformation observed with mutations in *proboscipedia* (Kaufman, 1978). Furthermore, *exd* clones often result in deletion of head structures, which has also been associated with mutations in *Deformed* (Merrill et al., 1987). Taken together, these results imply that *exd* may also interact with *proboscipedia* and *Deformed* in establishing segmental identities.

Although *exd* acts as a cofactor of the homeotics in the specification of many segmental identities, the specification of the antenna and arista by *exd* must occur differently. In the absence of *exd*, both structures are transformed toward leg. No known homeotic selector gene is expressed in the region of the eye-antennal imaginal disc that will give rise to the antenna and arista. However, if *Antp* or *Ubx* is ectopically expressed in the eye-antennal disc, these structures are transformed toward leg (Frischer et al., 1986; Jorgensen and Garber, 1987; Schneuwly et al., 1987; Gibson and Gehring, 1988; Mann and Hogness, 1990). Thus, *exd* may negatively regulate the expression of a homeotic selector gene in the eye-antennal imaginal disc. Alternatively, *exd* may function independently of the homeotic selector genes in the antenna and arista, analogous to the independent regulation of a downstream gene by *exd* in the visceral mesoderm (Rauskolb and Wieschaus, 1994). Thus, in the eye-antennal disc, *exd* may independently repress the expression or function of leg promoting genes, similar to how *Antp* acts in the leg imaginal discs to repress the function of head-promoting genes (Struhl, 1981).

Differential sensitivities to *exd* during leg development

exd is required for the correct patterning of proximal leg structures but not distal structures. Although it is clear that there is a differential proximal-distal sensitivity to *exd*, this sensitivity is not reflected in an underlying graded expression pattern of *exd*; *exd* transcripts are uniformly distributed in leg imaginal discs (Fig. 1). Furthermore, the absence of *exd* does not affect the proximal-distal nature of a group of cells (Fig. 8), but rather whether these cells will differentiate into an appropriate leg structure. Although homeotic transformations were not observed with *exd* leg clones, it is still possible that their identity is altered in such a way that they are prevented from contributing to the normal pattern of the leg. In this regard, it is interesting to compare *exd* clones to *Antp* clones in the first and third legs. Like *exd* clones, abnormal *Antp* clones are associated with atypical cuticular patterns and an absence of leg structures, giving the leg a truncated appearance (Struhl, 1981). In addition, in the second leg, proximal *Antp* clones are transformed toward antenna, while distal clones develop normally (Struhl, 1981, 1982). Together, the analyses of *exd* and *Antp* clones suggest that proximal leg structures are inherently more sensitive to alterations in homeotic gene function. Thus, the effects of *exd* on leg development may again be consistent with alterations in homeotic gene activity.

exd acts in concert with many homeotic selector genes

We previously showed that *exd* acts together with the homeotic
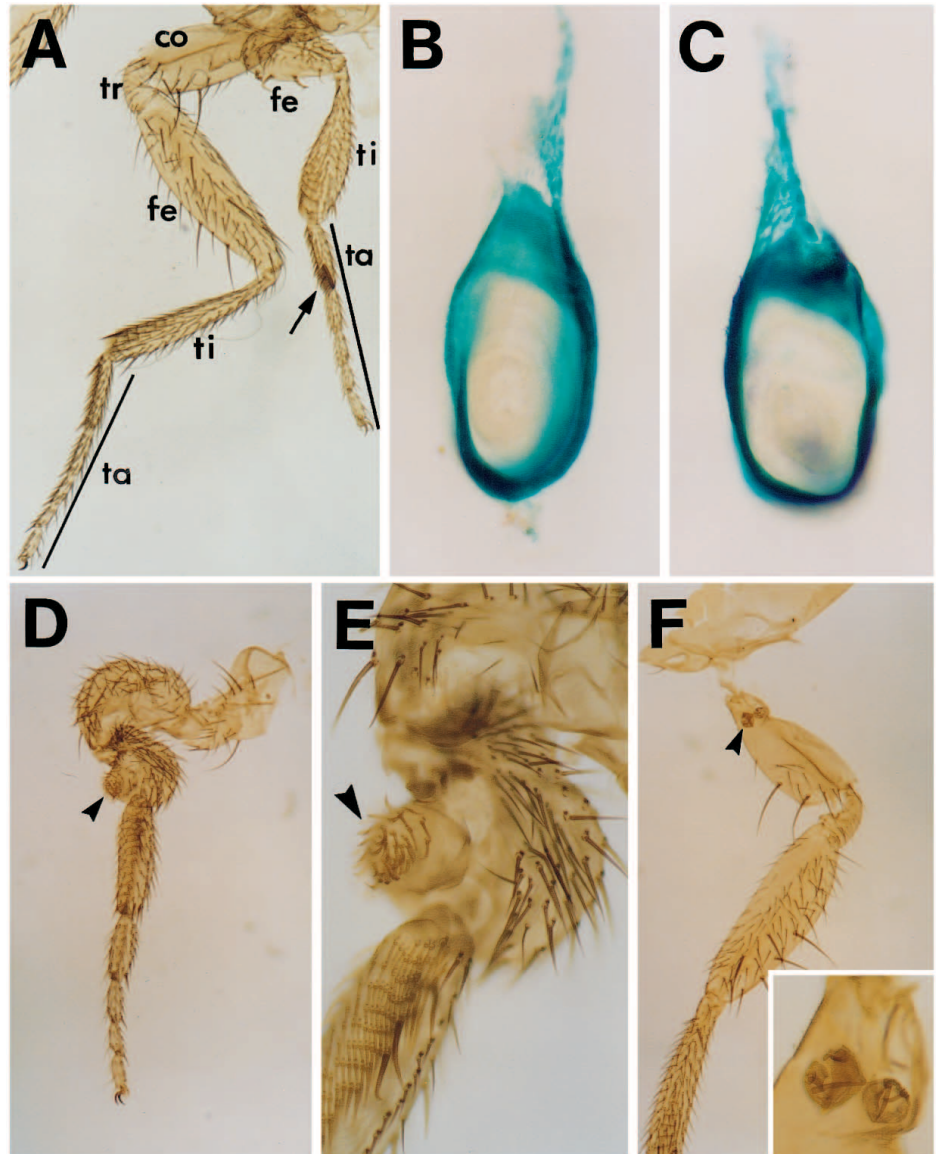
Fig. 8. *exd* affects the development of legs. (A) *exd*^{EM5} gynandromorph clone in the first thoracic leg. The proximal leg segments, the coxa (co) and trochanter (tr) are absent, and the femur (fe) and tibia (ti) are abnormal. The distal tarsal (ta) segments are normal. Sex-combs are found on the *exd* mutant leg (arrow) because the genotype of this tissue is male, whereas the wild-type leg tissue is female. (B) *teashirt* enhancer trap expression in wild-type and (C) *exd*^{EM5} mutant third instar leg imaginal discs. In both discs, expression is observed in the primordia of the coxa and trochanter segments. (D) *exd* null mitotic clone in a leg. The patterning of the femur (arrowhead) is severely disrupted. (E) Higher magnification of the clone in D. (F) Abnormal *exd* null clone in the coxa (arrowhead), also shown at high magnification (inset).

selector proteins ANTP, UBX, and ABD-A in the regulation of genes that execute morphogenetic events in the embryo (Peifer and Wieschaus, 1990; Rauskolb and Wieschaus, 1994). In the absence of *exd*, the activity of these homeotic selector proteins is altered such that they no longer appropriately regulate their target genes, resulting in altered morphogenesis. In vitro studies have provided a molecular model for these in vivo findings. *exd* and the homeotic selector proteins cooperatively bind DNA; *exd* alters the DNA-binding specificity of UBX and ABD-A (Chan et al., 1994; van Dijk and Murre, 1994). Although no such interactions were found between *exd* and ANTP on the target DNA used in these in vitro studies, our previous studies strongly suggested that *exd* modulates ANTP function (Rauskolb and Wieschaus, 1994). Our results presented here suggest that *exd* also affects the morphological outcome of *proboscipedia*, *Deformed*, *Ubx*, *abd-A*, and *Abd-B* activities during adult development. Given the overlap in the expression of and requirement for *exd* and the homeotic

selector genes, *exd* appears to be an indispensable factor that cooperates with most of the homeotic selector proteins in many cell fate decisions throughout development.

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Notes added in proof

The accession number for the *excl* genomic sequence is U33747.

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