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The regulation of *empty spiracles* by *Abdominal-B* mediates an abdominal segment identity function

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The *empty spiracles* (*ems*) homeo box gene is required for the development of the *Drosophila* larval filzkörper, which are structural specializations of the eighth abdominal segment. Filzkörper development is also dependent on the function of the homeotic selector gene *Abdominal-B* (*Abd-B*). Here, we show that *ems* is a downstream gene that is transcriptionally regulated by *Abd-B* proteins. This regulation is mediated by an *Abd-B*-dependent *ems cis*-regulatory element that in early- to mid-stage embryos is activated only in the eighth abdominal segment. Genetic epistasis tests suggest that both *ems* and *Abd-B* are required in combination for the specification of the filzkörper primordia. In a general sense, these results also provide evidence that the hierarchical level immediately downstream of the homeotic genes contains additional homeo domain transcription factors that define subsegmental domain identities.

[*Key Words*: *empty spiracles*; *Abdominal-B*; *Drosophila*; homeotic; homeo domain]

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The eight homeotic genes of the *Antennapedia* and *Bithorax* complexes (collectively called the homeotic complex or HOM-C) (Lewis 1978; Kaufman et al. 1980) assign identities to the segments of the abdomen, thorax, and posterior head of the larva of *Drosophila melanogaster*. Mutations in these genes transform whole body segments or parts of segments into the form of other segments. They thus act as master regulatory or selector genes, which instruct cells to assume position-specific fates, presumably by regulating a battery of subordinate realisator or downstream genes that actually construct segmental structure and morphology (García-Bellido 1977; Lewis 1978). Over the past decade, much light has been shed on the mechanisms of action of the homeotic selector genes, but the identity and nature of the presumed downstream targets under the control of the homeotic genes have remained obscure. Ultimately, answering the question of how the homeotic genes impose positional identities and thereby control the development of unique structures and morphologies in different segments depends on identifying these target genes and understanding how the homeotic genes coordinate their expression to generate segmental diversity.

In general, the ectopic expression of a given HOM-C gene causes the transformation of segmental identities toward the identity normally specified by the gene. This principle is demonstrated dramatically in experiments that use heat shock promoters to direct ectopic expression of HOM-C cDNAs (Schneuwly et al. 1987; Gibson and Gehring 1988; Kuziora and McGinnis 1988a; Mann and Hogness 1990). For example, ectopic expression of the *Deformed* gene (*Dfd*) causes head and thoracic segments to develop maxillary and mandibular structures like mouth hooks and cirri, whereas ectopic expression of the *Antennapedia* gene (*Antp*) induces head-to-thorax transformations. These experiments suggest that the homeotic selector genes are able to autonomously coordinate the expression of downstream genes to induce the development of their subordinate structures in ectopic positions.

In keeping with their genetically defined roles as master regulatory genes, the proteins encoded by the homeotic selector genes act as transcriptional regulators (for review, see Levine and Hoey 1988), and, at the structural level, contain very similar versions of the highly conserved homeo domain motif (McGinnis et al. 1984; Scott and Weiner 1984; Regulski et al. 1985). The 60-amino-acid homeo domain mediates sequence-specific DNA binding for those homeotic genes that have been tested (Beachy et al. 1988; Desplan et al. 1988; Hoey and Levine 1988; Affolter et al. 1990; Ekker et al. 1991; Dessain et al. 1992) and is structurally related to the helix-turn-helix motif of prokaryotic DNA-binding transcriptional regulators (Laughon and Scott 1984; Otting et al. 1990; Kissinger et al. 1990). Thus, homeotic genes are thought to target subordinate genes via the sequence-specific binding of the homeo domain and other interacting cofactors, and control these target genes at the level of transcription.

Despite the extensive study of the homeotic selector genes, little is currently known about the nature and number of the transcription units that they regulate or about the DNA elements that mediate such regulation. What is known involves primarily cross-regulatory and

autoregulatory interactions between members of the HOM-C. Posterior members of the HOM-C are known to repress the transcription of members with more anterior boundaries of expression (Hafen et al. 1984; Struhl and White 1985; Wedeen et al. 1986). *Dfd* and *labial* (*lab*) are both known to autoregulate positively in the ectoderm, and *Utrabithorax* (*Ubx*)autoactivates its own expression in the visceral mesoderm (Bienz and Tremml 1988; Kuziora and McGinnis 1988a; Chouinard and Kaufman 1991).

To date, only a few putative downstream target genes of the homeotic selector genes have been found. There is good evidence that *Ubx* and *abdominal-A* (*abd-A*) control the expression of the transforming growth factor-β (TGF-β) homolog *decapentaplegic* in the visceral mesoderm and that *abd-A* controls the expression of the *int-1* homolog *wingless*, also in the visceral mesoderm (Immerglück et al. 1990; Reuter et al. 1990). The activation of these putative growth factors in the mesoderm by *Ubx* and *abd-A* is thought to mediate morphoregulatory induction across germ layers into the adjacent endoderm. Two other genes that may be regulated by *Ubx* and *abd-A* in the central nervous system have been identified using a chromatin immunoprecipitation technique but as yet have no known genetic function (Gould et al. 1990). Finally, the *spalt major* gene has been shown to be negatively regulated by *Antp* in imaginal disc cells (Wagner-Bernholz et al. 1991).

Are there a large or small number of downstream genes targeted by the homeotic genes, and to what extent are these targets shared? Are these genes themselves all regulatory proteins or are some of them structural? How are they expressed to give rise to different morphologies in each segment? In this paper we attempt to answer some of these questions by exploring how the homeotic selector gene *Abdominal-B* (*Abd-B*) controls the development of a unique structure in the eighth abdominal segment (A8) of the larva. *Abd-B* is a homeotic selector gene required for the development of the posterior abdominal segments (Lewis 1978; Sanchez-Herrero et al. 1985; Casanova et al. 1986). Genetic and molecular studies on the locus have revealed that *Abd-B* has at least two separate functions carried out by two protein isoforms that contain the same homeo domain (Casanova et al. 1986; DeLorenzi et al. 1988; Kuziora and McGinnis 1988b; Sanchez-Herrero and Crosby 1988; Celniker et al. 1989; Zavortink and Sakonju 1989; Boulet et al. 1991). The longer m isoform is expressed at high levels in parasegment 13 (the posterior compartment of A7 and the anterior compartment of A8) and much lower levels in parasegments 12–10. In A8, loss of the *m* function of *Abd-B* has its most dramatic effect. *Abd-B*$^{m-}$ larvae exhibit a transformation of A8 toward a more anterior abdominal segment identity, which includes transformations of the ventral denticle belts and elimination of dorsal structures such as the posterior spiracles. The smaller, or r, form of Abd-B protein is expressed in parasegments 14 and 15 and is required for the correct development of those metameres.

When ectopically expressed under heat shock promoter control, *Abd-B* homeo domain-containing proteins can partially transform more anterior segments toward A8 identity (Kuziora and McGinnis 1991; M. Kuziora, in prep.). Here, we show that *Abd-B* controls the development of one of the A8-specific structures, the filzkörper, by the activation of the downstream patterning gene *empty spiracles* (*ems*). *ems* is a homeo box gene with at least two patterning functions during embryonic development. One of these functions is exerted in A8, where mutations in *ems* result in loss of the filzkörper, the specialized structures that line the tracheal openings in the posterior spiracles of the larva (Jürgens et al. 1984; Dalton et al. 1989; Walldorf and Gehring 1992). By use of a DNA-binding assay we have been able to identify an *ems* upstream regulatory element that directs *Abd-B*-dependent expression of a *lacZ* reporter gene in transgenic embryos. The evidence suggests that *ems* acts to define subsegmental positional information in A8 and that the combined expression of *ems* and *Abd-B* are crucial determinants of filzkörper development.

Results

Correlation of ems *and* Abd-B *phenotypes*

A *Dfd/Abd-B* chimeric gene (which contains the *Abd-B* homeo box substituted for that of *Dfd*) can induce the homeotic transformation of dorsolateral regions of anterior larval segments towards A8 identity when ectopically expressed under the control of the *hsp70* promoter (*hsp70–Dfd/Abd-B*; Kuziora and McGinnis 1991). These transformations are recognized principally by the ectopic development of components of the posterior spiracles in *hsp70–Dfd/Abd-B* animals (Fig. 1B). *hsp70–Abd-B* genes of both the *m* and *r* forms also induce similar segmental transformations involving ectopic spiracular structures (M. Kuziora, in prep.). The most conspicuous of the ectopic spiracular structures is filzkörper, the fuzzy, filter-like material that normally lines the atrium of the tracheal openings (Fig. 1B,D). These results are consistent with the idea that the *Abd-B* homeo domain is responsible for targeting specific downstream genes that induce the development of posterior spiracle structures in the larva. A good candidate for such a downstream gene is *ems*. Animals carrying *ems* null mutations (Fig. 1C) do not develop filzkörper, although the remaining spiracular structures are intact (Jürgens et al. 1984; Dalton et al. 1989; Walldorf and Gehring 1992). The *ems* gene is also required for the formation of filzkörper in ectopic positions: Animals carrying both an *ems* null mutation and *hsp70–Abd-B*^{m} do not develop ectopic filzkörper after heat shock treatment (data not shown; see Materials and methods).

Transcriptional regulation of ems *in* Abd-B *mutant and transgenic embryos*

If *ems* modulates filzkörper development under the control of *Abd-B*, we would expect elements of the *ems* expression pattern to be altered or missing in *Abd-B* mu-

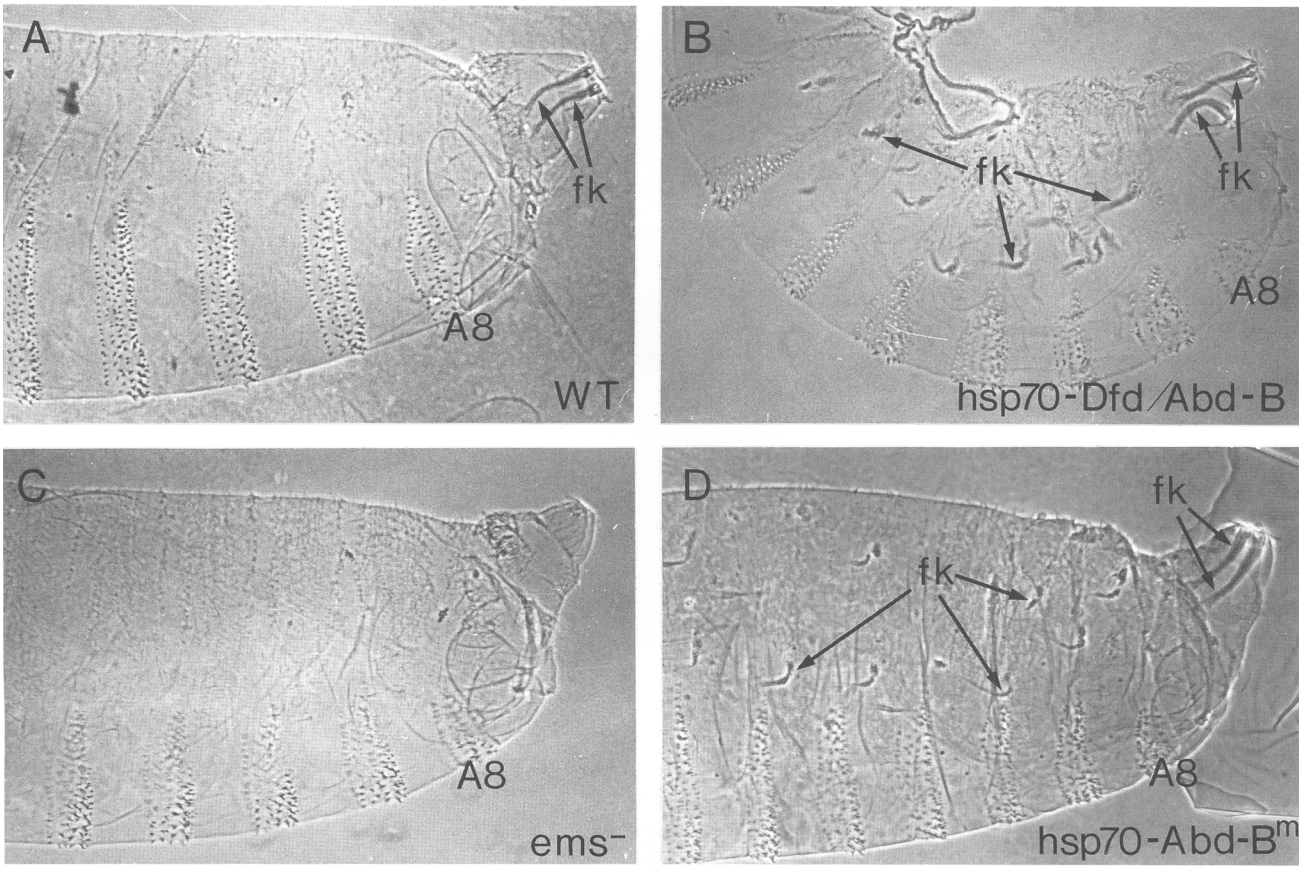

Figure 1. Cuticular phenotypes of *hsp70–Dfd/Abd-B*, *hsp70–Abd-B*^{m}, and *ems*$^-$ larvae. Cleared cuticle preparations were photographed under phase-contrast microscopy. The tail and posterior abdomen are shown viewed from a lateral aspect with anterior to the *left*. The eighth abdominal segment denticle band is marked (A8). (*A*) Wild-type (WT) cuticle has one pair of filzkörper (fk, arrows) in the posterior spiracles. (*B*) After a 1-hr heat shock during late blastoderm stage, an *hsp70–Dfd/Abd-B* larva develops ectopic filzkörper (arrows) in more anterior segments. (*C*) An *ems*$^-$ larva exhibits a complete lack of filzkörper in the posterior spiracles. (*D*) After a 1-hr heat shock at late blastoderm stage, a *hsp70–Abd-B*^{m} larva develops ectopic filzkörper (arrows) in more anterior segments.

tants and ectopic expression of *ems* in *hsp70–Abd-B* transgenic embryos. To test this, we looked at *ems* transcript expression using the whole-mount in situ technique of Tautz and Pfeifle (1989) on mutant and transgenic embryos.

ems normally has a dynamic pattern of expression that begins at the cellular blastoderm stage with an anterior stripe at ~80% egg length as measured from the posterior pole (Dalton et al. 1989; Walldorf and Gehring 1992). This evolves into a metameric pattern that arises during germ-band elongation as lateral epidermal patches of transcripts around the primordia of the tracheal pits. At the end of germ-band extension two larger, more abundant bilateral patches of *ems* transcription are activated in A8 (Fig. 2A). These large dorsolateral A8 patches are in the position expected for the anlagen of the posterior spiracles and filzkörper (Jürgens 1987). In *Abd-B*$^{m-}$ embryos, the A8 patches of *ems* transcription are never established even though the remainder of the *ems* transcription pattern is normal (Fig. 2B). The failure to activate *ems* transcription in the dorsolateral patch of A8 cells correlates with the lack of filzkörper formation in *Abd-B*$^{m-}$ larvae. Figure 2 also shows the expression pattern of *ems* transcripts in *hsp70–Dfd/Abd-B* and *hsp70–Abd-B*^{m} embryos (Fig. 2C,D). After a 1-hr heat shock at late blastoderm to early gastrulation stages, *ems* transcription can be activated in both transgenic lines throughout the epidermis of the embryo. The ectopic expression of *ems* is variable, ranging from high global epidermal expression, as shown in Figure 2, to large dorsolateral patches of segmentally reiterated epidermal expression superimposed on a low level of global epidermal *ems* transcription. Ectopic expression of Dfd protein in *hsp70–Dfd* strains does not result in ectopic *ems* transcriptional activation in comparably staged embryos (G. Vezina, unpubl.).

DNA fragments at the ems *locus that bind Abd-B homeo proteins*

The above results are consistent with Abd-B homeo domain-containing proteins having either a direct or an indirect effect on the activation of *ems* transcription in posterior spiracular anlagen. If the activation is direct,

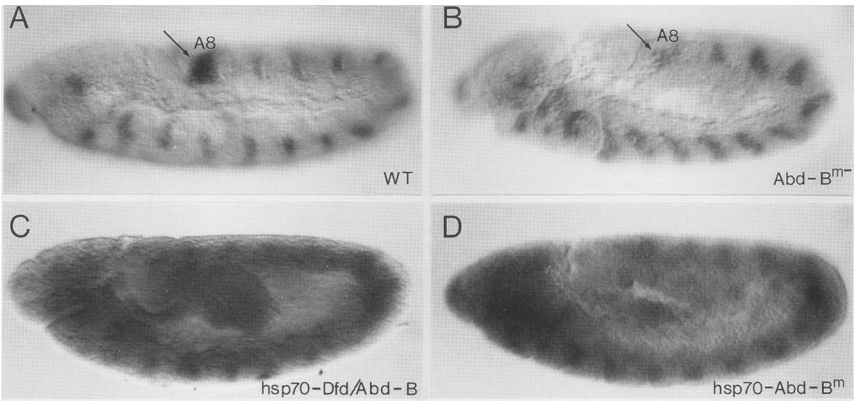

Figure 2. Transcriptional regulation of *ems* in *Abd-B* mutant and transgenic embryos. *ems* transcripts were detected with digoxigenin-labeled probes in late germband extended embryos, which were photographed under Nomarski optics. Embryos are viewed laterally with anterior to the *left*. (*A*) A wild-type (WT) embryo has a reiterated metameric pattern of *ems* expression. Note the larger, more intense, dorsolateral patch of *ems* transcripts (arrow) in the eighth abdominal segment (A8). (*B*)An *Abd-B*$^{m-}$ embryo does not activate *ems* transcription in a comparable dorsolateral patch in A8 (arrow) but still retains the metameric pattern. (*C*) *hsp70–Dfd/Abd-B* and (*D*) *hsp70–Abd-B*^{m} embryos that have been heat-shocked for 1 hr during blastoderm through early gastrulation stages (2.5–4.5 hr) activate *ems* transcription throughout the epidermis of the developing animal.

we might expect to find DNA near the *ems* transcription unit that has preferential binding affinity for Abd-B homeo domain-containing proteins. To test this idea we used an immunoprecipitation assay (McKay 1981) to identify restriction fragments of *ems* genomic DNA that have high affinity for the chimeric Dfd/Abd-B protein (the same protein expressed by *hsp70–Dfd/Abd-B*, i.e., Dfd with the Abd-B homeo domain).

Dfd/Abd-B protein was produced in *Escherichia coli* and partially purified from soluble lysates over a DNA affinity column as described in Dessain et al. (1992). The protein was incubated with end-labeled restricted fragments of λE4 [a 14-kb genomic clone that contains the *ems* transcription unit along with 9 kb of 5′- and 2 kb of 3′-flanking DNA (Dalton et al. 1989)], and immunoprecipitated with anti-Dfd rabbit serum (Jack et al. 1988). As a comparison and a control, we performed the same assay using wild-type Dfd protein purified in the same manner. Figure 3A shows the results of an experiment in which λE4 DNA was cut with four restriction enzymes, and the immunoprecipitated fragments were separated on a 5% polyacrylamide gel. In this assay, Dfd/Abd-B protein binds with highest affinity to a 1-kb *Eco*RI–*Xho*I fragment that maps ~2 kb 5′ of the *ems* promoter—a fragment that is not detectably bound by Dfd protein. The *E. coli*-produced Dfd protein does possess binding activity, as shown by its preferential affinity for a 0.3-kb *Xho*I–*Hind*III fragment that maps 3′ of the *ems* transcription unit (Fig. 3B).

The DNA fragment with high affinity for the Abd-B *homeo domain provides posterior spiracle expression in transgenic embryos*

At this point we were curious to know whether the *ems* DNA region with high affinity for the Dfd/Abd-B protein contained any *Abd-B*-dependent enhancer-like activity. To test this we subcloned a 1.2-kb *Eco*RI fragment that includes sequences spanning the region from −2.0 to −3.2 upstream of the *ems* transcription start (see Fig. 3) in front of a *lacZ* reporter gene with the *hsp70* basal promoter (from the HZ50PL construct of Hiromi and Gehring 1987). This construct, designated HZems1.2, was introduced into the germ line of flies via P-element transformation (Rubin and Spradling 1982). Several independent lines with the *HZems1.2* insert were obtained.

Figure 4 shows the developmental expression of *lacZ* in *HZems1.2* lines as detected by immunohistochemical staining of β-galactosidase (β-gal) protein. β-Gal is first observed at the end of germ-band extension in two bilateral patches of epidermal cells in dorsolateral A8 (Fig. 4A,B). These patches of reporter gene expression are in the region that contains the primordia of the posterior spiracles and include most or all of the cells that express *ems* in the dorsolateral A8 patch (Fig. 2). Later in embryogenesis, beginning at stage 14 (Campos-Ortega and Hartenstein 1985), additional β-gal expression is initiated in dorsal cells in the anterior of the embryo (Fig. 4C, D). In fully developed embryos, these β-gal-expressing cells lie just dorsal to the pharyngeal muscles. The identity of these cells is currently unknown, and we are uncertain whether they have any relationship to the developing tracheal system. During a brief period when *HZems1.2* is initially activated in its anterior domain, ems protein can also be detected in the same anterior dorsal region, but then is no longer present. The more persistent β-gal expression in this region from *HZems1.2* is likely to be attributable to the relatively stable character of the β-gal protein (Hiromi and Gehring 1987). The A8 pattern of β-gal expression is still present at stage 14 and subsequent stages of embryogenesis and is localized in the cells lining the atrium of the posterior spiracles, which corresponds to the position of the cells that will give rise to the filzkörper (Fig. 4C,D).

The 1.2-kb fragment behaves as an Abd-B *response element*

To test whether the activation of the *HZems1.2* transgene in A8 requires *Abd-B* function, the construct was crossed into an *Abd-B*$^{m-}$ background. In one-quarter of the embryos from parents that are heterozygous for the

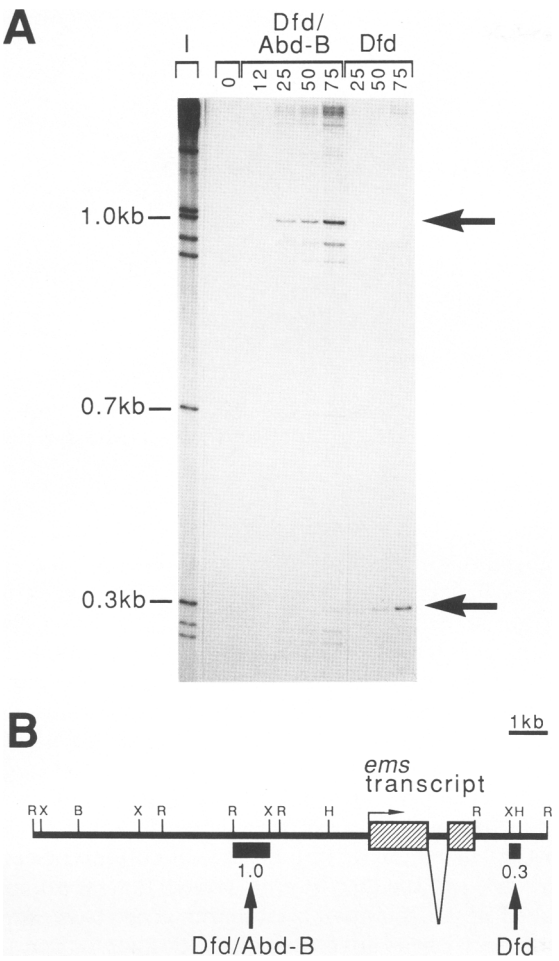

Figure 3. Immunoprecipitation of DNA fragments at the *ems* locus that bind Dfd/Abd-B and Dfd proteins. (*A*) Autoradiograph of an immunoprecipitation experiment. *ems* genomic clone λE4 was digested with *Eco*RI, *Xho*I, *Bam*HI, and *Hind*III, end-labeled with ^{32}P, incubated with Dfd/Abd-B and Dfd proteins, immunoprecipitated with anti-Dfd antiserum bound to *S. aureus* cells, and resolved on a 5% denaturing polyacrylamide gel. Binding reactions contained either no protein (0), or the indicated amounts in nanograms of purified Dfd/Abd-B or Dfd proteins. Input DNA fragments (I) are shown in lane *1*. Fragment lengths in kilobases (kb) are indicated at *left*. The arrows at *right* point out fragments that are bound with high affinity. Dfd/Abd-B protein binds a 1-kb fragment (top arrow) with highest affinity; this fragment is not detectably bound by wild-type Dfd protein, which preferentially binds a 0.3-kb fragment (bottom arrow). (*B*) Restriction map of the *ems* genomic clone λE4 showing the location of high affinity fragments detected in the immunoprecipitation experiment (solid boxes below the line) in relation to *ems*-coding regions (hatched boxes). Vertical arrows underneath the solid boxes indicate which protein binds to the fragment; the size of the fragment (in kb) is also indicated. Dfd/Abd-B protein binds to a 1-kb (1.0) fragment that is located ~2.5 kb 5′ of the *ems* transcription start. Wild-type Dfd protein binds to a 0.3-kb fragment located 3′ of *ems*. (R) *Eco*RI; (X) *Xho*I; (B) *Bam*HI; (H) *Hind*III.

Abd-B^m mutation and homozygous for the *HZems1.2* reporter gene, β-gal expression is not detected in A8 (Fig. 5D). At later stages, *Abd-B^{m-}* embryos can be identified by the lack of posterior spiracles (Fig. 5E); although β-gal expression is not observed in the eighth abdominal segment, these embryos still activate expression of the reporter gene in the dorsal anterior region.

To test whether *Abd-B* homeo domain protein expression is sufficient to ectopically activate embryonic expression of the *HZems1.2* reporter gene, the transgene was crossed into *hsp70–Dfd/Abd-B*, *hsp70–Abd-B^m*, and *hsp70–Abd-B^r* backgrounds. Figure 5, A–C shows that ectopic expression of β-gal can be induced in *hsp70–Abd-B* lines by a 1-hr heat shock administered at late blastoderm to early gastrulation stages. All three of the *hsp70–Abd-B* homeo box genes are capable of activating ectopic expression of the *HZems1.2* reporter construct. In general, the chimeric *Dfd/Abd-B* gene activates the most abundant production of ectopic *HZems1.2* expression, which is observed in reiterated segmental patches of ectoderm in germ-band-extended embryos (Fig. 5A). Ectopic expression of *HZems1.2* is also observed in head segments, especially in the procephalon. Weak expression is observed in scattered epidermal cells throughout the embryo. Both *hsp70–Abd-B^m* and *hsp70–Abd-B^r* are also capable of activating ectopic expression of *HZems1.2*, but to lower levels than *hsp70–Dfd/Abd-B* (Fig. 5).

To test whether the *ems* genetic function itself is required for the activation of *HZems1.2* reporter gene expression, the transgene was crossed into *ems* mutant backgrounds. In *ems$^-$* embryos, which can be identified by their altered spiracle morphology, β-gal is still expressed in both the posterior spiracle primordia and the dorsal anterior (Fig. 5F). Thus, *ems* activity is not required for the activation of *HZems1.2*.

Previous work has shown that the *hsp70–Dfd/Abd-B* chimeric gene can also ectopically activate endogenous *Abd-B* transcripts in the dorsolateral epidermis of more anterior segments (Kuziora and McGinnis 1991). Thus, it seemed possible that the activation of the *HZems1.2* reporter gene might be accomplished indirectly through the action of endogenous Abd-B proteins, rather than directly by the protein produced by the *hsp70–Dfd/Abd-B* chimeric gene. To obtain evidence addressing such a possibility, we performed experiments to test how rapidly ectopic transcription from *HZems1.2* is induced after heat shock induction of *hsp70–Dfd/Abd-B* expression, and whether *HZems1.2* activation precedes or follows the appearance of ectopic endogenous Abd-B proteins.

Embryos carrying one copy of the *hsp70–Dfd/Abd-B* chimeric gene and one copy of the *HZems1.2* reporter gene were collected for 2 hr. After varying intervals, they were heat-shocked for 15 min. The embryos were allowed to recover another 15 or 30 min and were then fixed. They were then incubated with either a digoxigenin-labeled RNA probe for *lacZ* or an antibody probe for endogenous Abd-B proteins [we used a monoclonal antibody against Abd-B that recognizes an epitope outside of the homeo domain and thus does not recognize the Dfd/Abd-B protein (Celniker et al. 1989)]. In embryos heat-shocked between 5.5 and 7.5 hr after egg lay, ectopic

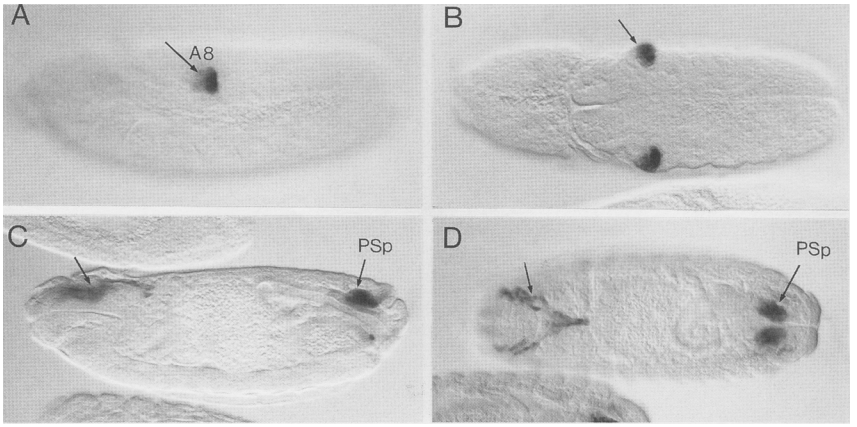

Figure 4. Filzkörper region expression from the *HZems1.2* reporter gene. β-Gal protein was detected by immunoperoxidase staining; embryos were photographed under Nomarski optics, with anterior to the *left*. (*A*) Lateral view of a late germband-extended (7.5-hr) embryo; β-gal can be detected in a dorsolateral patch of epidermal cells (arrow) in the eighth abdominal segment (A8). This patch corresponds to the position of the *Abd-B*-dependent patch of *ems* transcripts in A8 (see Fig. 2). (*B*) Dorsal view of an embryo at the germband-extended stage. Note bilateral patches of β-gal expression in A8 (arrow). (*C*) Lateral view of a 10.5-hr embryo; β-gal expression seen in the atrium of the posterior spiracles (PSp) is now also expressed in dorsal anterior cells (unlabeled arrow). (*D*) A dorsal view of an embryo at the same stage as in *C*.

lacZ transcripts can be detected in a small number of embryos even at the 15-min recovery time point. More abundant ectopic *lacZ* transcription can be seen in the majority of embryos after a 30-min recovery (data not shown). Ectopic Abd-B protein, however, is not detected in embryos at either the 15- or the 30-min recovery time points (data not shown). Thus, ectopic *HZems1.2* transcripts are activated in the absence of detectable levels of ectopic endogenous Abd-B protein. This, in combination with the rapid induction of *HZems1.2* transcripts, argues for a direct interaction between the Dfd/Abd-B protein and the *Abd-B*-dependent regulatory element in the *HZems1.2* construct.

Abundant ectopic ems *expression is not sufficient for filzkörper induction*

We wanted to determine whether ectopic *ems* expression alone is sufficient to induce ectopic filzkörper or whether *Abd-B* and/or other genes that are *Abd-B* dependent are required in combination with *ems*. That is, does *ems* act in a strict hierarchy or in a combinatorial hierarchy with *Abd-B*? *ems* is normally expressed in many anterior embryonic segments that develop no filzkörper. It is possible, however, that the level of *ems* expression in more anterior regions is insufficient for filzkörper specification, or that filzkörper specification requires a significant spatial expansion of the pre-existing subsegmental pattern. Thus, the role of Abd-B might be simply to boost or broaden the expression of *ems* in A8 to trigger filzkörper development. To address these questions we constructed transgenic fly lines that carry an *ems* cDNA (Dalton et al. 1989) fused to the *hsp70* promoter to observe the effects of ectopic *ems* expression. After a heat shock, ems protein can be detected with anti-ems antiserum (Dalton et. al. 1989) at high levels throughout *hsp70–ems* embryos. The level of ems protein induced in *hsp70–ems* embryos immediately after a single, 1-hr heat shock is high enough to completely obscure the endogenous ems protein pattern and remains so for at least 2.5 hr. By 5 hr after heat shock, the level of ectopically expressed ems in most embryos is lower than the ems protein abundance in the endogenous pattern (data not shown).

To assess the effects of ectopic *ems* activation, 1-hr heat shocks were administered at different times during embryonic development and cuticle preparations were made of terminal embryos. At all times, the effect of *hsp70–ems* expression is lethal. At no time, however, are ectopic filzkörper induced. Embryos that received a single 1-hr heat shock at any stage between 2.5 and 7.5 hr after egg lay exhibit a similar range of cuticular defects. Over 60% of the *hsp70–ems* larvae that were heat-shocked at the cellular blastoderm stage have severe head defects; head involution is often incomplete, and head skeleton elements are fused and mangled. On rare occasions, we have observed what appeared to be ectopic antennal sense organs in the head region of *hsp70–ems* embryos, but at such a low frequency (<1%) that the specificity of this effect is highly questionable. Generic problems with head involution are rarely seen (8% penetrance) in heat-shocked wild-type control larvae, most of which (84%) go on to hatch and develop normally. In addition to head defects, >90% of *hsp70–ems* larvae also have posterior spiracle defects (Fig. 6). In the defective posterior spiracles, the filzkörper are fat, rounded, and often enlarged, and the protuberances that bear the posterior spiracles (the stigmatophores) are greatly reduced or absent. The hairs at the base of the stigmatophores (the Fell), the hairs that encircle the spiracular openings (spiracular hairs), and other characteristic features of the posterior spiracles are missing. We have not observed posterior spiracle defects of this kind in control larvae (wild type, *hsp70*–Abd-B strains, *hsp70–Dfd* strains) that were heat shocked in the same manner.

Discussion

ems *is a downstream gene of* Abd-B

Genetic and molecular data support the model that *Abd-B* and other HOM-C genes control the identity and

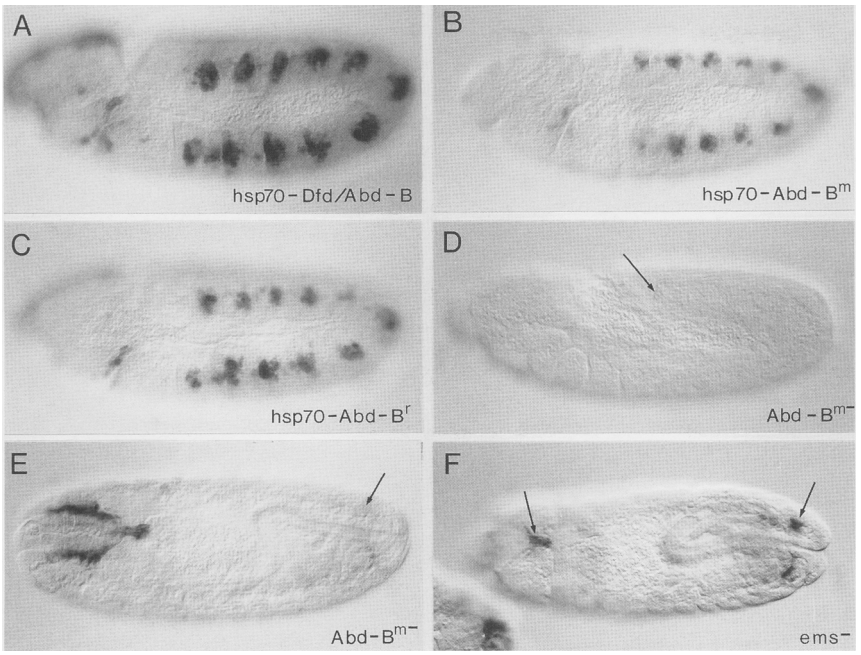

Figure 5. *Abd-B*-dependent activation of the *HZems1.2* reporter gene. (*A*) A lateral view of a *hsp70–Dfd/Abd-B* embryo, which also carries two copies of the *HZems1.2* reporter gene. This extended germ-band embryo was heat-shocked for 1 hr during the late cellular blastoderm stage and then processed for immunohistochemical detection of β-gal protein. β-Gal is strongly expressed in reiterated epidermal patches. *hsp70–Abd-B^m* (*B*) and *hsp70–Abd-B^r* (*C*) embryos carry two copies of the *HZems1.2* reporter gene, have been treated in the same manner as the embryos in *A*, and also express β-gal protein in ectopic reiterated patches. (*D*) A lateral view of a *Abd-B^{m-}* embryo at the extended germ-band stage, which carries two copies of the *HZems1.2* reporter gene. β-Gal is not expressed in A8 (arrow) (cf. Fig. 4A). (*E*) A dorsal view of an 11-hr *Abd-B^{m-}* embryo with two copies of *HZems1.2*. Note that β-gal is expressed in dorsal anterior cells but not in the position of the posterior spiracles that do not develop (arrow) (cf. Fig. 4C). (*F*) A dorsal view of a 10.5-hr *ems$^-$* embryo, which carries two copies of the *HZems1.2* reporter gene. Despite the altered morphology of expressing cells in the region of the posterior spiracle primordia (and in the dorsal anterior region), β-gal expression can still be detected (arrows). All embryos are depicted with anterior to the left and were photographed under Nomarski optics.

morphology of segments in the larva through the regulation of a largely undefined group of downstream genes. These experiments provide strong evidence that *ems* is a downstream gene that mediates an important segmental identity function of *Abd-B*. By transcriptionally activating *ems* in dorsolateral patches of cells in the eighth abdominal segment of germ-band embryos, *Abd-B* is able to promote the development of a unique structure, the filzkörper, at the posterior tracheal openings of the larva.

The *Abd-B* regulatory effect on *ems* is exerted at least in part through an upstream DNA fragment that provides expression in filzkörper primordia. This fragment was identified by its preferential binding affinity for the Abd-B homeo domain in immunoprecipitation assays. In extended germ-band embryos, this regulatory element, which we call *ems*–ARFE (for Abd-B response filzkörper element), is active only in the eighth abdominal segment in the posterior spiracle primordia. The *ems*–ARFE element requires the function of the *Abd-B^m* gene product for its normal activity, and Abd-B m and other Abd-B homeo domain-containing proteins are sufficient to activate *ems*–ARFE in dorsolateral regions of other segments. The *ems*–ARFE element is only A8 specific at the extended germ-band stage, as it is later activated in dorsal anterior cells that are associated with the pharyngeal apparatus. *ems*–ARFE-directed expression is also detected in all of the segmentally reiterated spiracles of the adult (B. Jones, unpubl.).

The temporal and spatial pattern of expression conferred by the *ems*–ARFE raises a number of intriguing questions about its regulation. That the *ems*–ARFE does not direct expression throughout the domain of *Abd-B* expression indicates that other factors are acting to restrict its activity. The restriction of *ems*–ARFE-directed expression to dorsolateral A8 probably results from multiple factors acting on the *ems*–ARFE; likely candidates include genes that are regionally expressed along the dorsoventral axis and genes that restrict expression within the segment along the anterior-posterior axis (e.g., segment polarity genes).

The fragment containing the *ems*–ARFE element was isolated on the basis of its binding affinity for the Abd-B homeo domain and contains multiple binding sites for Abd-B homeo domain-containing proteins (A. Gonzalez-Reyes and B. Jones, unpubl.). *lacZ* transcripts directed by *ems*-ARFE are detected shortly after heat shock induction of *hsp70–Dfd/Abd-B* expression. The rapidity of this induction suggests that the *ems*–ARFE element is regulated directly by the binding of Abd-B homeo domain proteins, which will make it quite useful in elucidating the mechanisms that allow differential regulation by different members of the HOM-C homeo domain protein class.

It is interesting that the Abd-B r protein is also capable of activating the *ems*–ARFE element. This result and the ability of the *hsp70–Abd-B^r* gene to induce ectopic posterior spiracle structures (M. Kuziora, in prep.) are consistent with the idea that the r protein can act as a transcriptional activator as well as in its previously suggested role as a repressor. Genetic analysis and expression studies show that the Abd-B r protein isoform suppresses the function and expression of other ho-

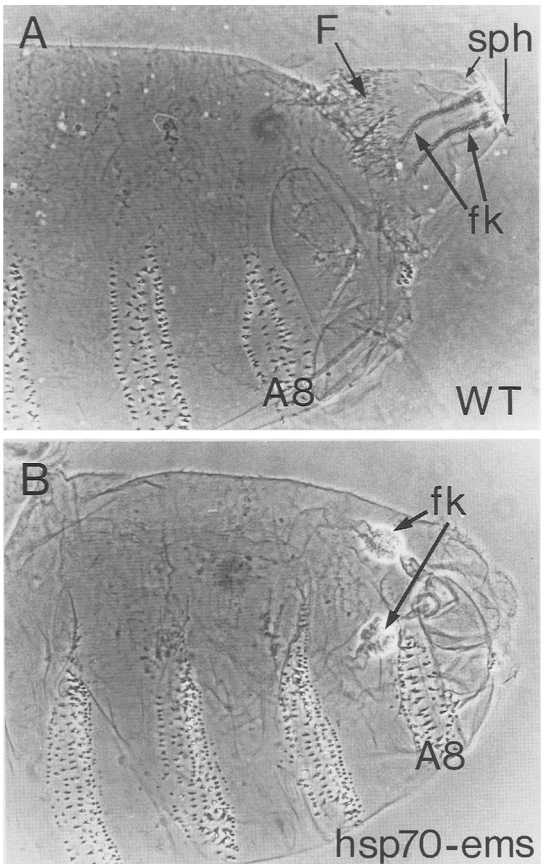

Figure 6. Cuticular phenotype of *hsp70–ems*. Cleared cuticle preparation of the tail region of wild-type (WT) and *hsp70–ems* larvae are depicted viewed from a lateral aspect, with anterior to the *left* under phase-contrast microscopy. (*A*) Wild-type larvae have a pair of protruding stigmatophores bearing the posterior spiracles. Inside the stigmatophores are a pair of filzkörper (fk). The spiracular openings are encircled by spiracular hairs (sph), and the dorsal stigmatophore base has a patch of characteristic large hairs called Fell (F). (*B*) An *hsp70–ems* larva that was heat-shocked for 1 hr at the late cellular blastoderm stage (3 hr after egg lay). The filzkörper appear fat and rounded, and the protruding stigmatophore is strikingly reduced. The Fell, spiracular hairs, and other characteristic features of the posterior spiracles are missing. (Tail region terminology is from Jürgens 1987.)

meotic genes in parasegments 14 and 15 (Casanova et al. 1986; Casanova and White 1987; A. Gonzalez-Reyes and G. Morata. pers. comm.). We believe that *Abd-B*^{r} does not normally activate the *ems*–ARFE or filzkörper in A9 as a result of the lack of the cells in the appropriate dorsolateral position, where A9 is truncated.

The role of ems *in* Drosophila *development*

Like *Abd-B* and other HOM-C genes, the *ems* gene encodes a homeo domain transcription factor. The evidence here indicates that expression of the *ems* transcription factor is capable of specifying filzkörper only in a specific context, that is, in dorsolateral epidermis in combination with abundant amounts of Abd-B homeo domain protein. Thus, *ems* does not act as an autonomous selector gene for filzkörper identity, requiring the activity of *Abd-B* as well as other combinatorial inputs. The striking reduction of posterior spiracle structures along with the expansion of filzkörper in *hsp70–ems* indicates that *ems* expression is actually incompatible with the normal development of many of the other spiracular components.

This phenotypic effect of *hsp70–ems*, and the expression pattern of the *ems*–ARFE suggests that a general role of *ems* in the development of the tracheal system may be to specify a positional identity that is equivalent to a connection between internal tracheal trunk and an exterior opening. This idea is supported by the posterior spiracle phenotype of *ems* mutants, which is described most accurately as a deletion of the entire spiracular atrium, resulting in an animal in which the tracheal trunks are no longer connected to the posterior spiracular openings; the absence of the distinctive filzkörper is only the most obvious sign of the mutant defect. Whether *ems* has a required role in the development of the adult spiracles/tracheal system is currently under investigation.

The *ems* gene is also required for the formation of sensory structures in the larval head, most notably in the antennal sense organs and the dorsomedial and dorsolateral papillae of the antennomaxillary complex (Jürgens et al. 1984; Dalton et al. 1989; Cohen and Jürgens 1990; Walldorf and Gehring 1992). Cohen and Jürgens (1990) have argued that the development of the segments that bear these head structures is specified by the combinatorial activity of *ems* and two other genes: *orthodenticle* (*otd*), a homeo box gene whose expression overlaps *ems* expression in the head (Finkelstein and Perrimon 1990), and *buttonhead* (*btd*). All three genes are required for the development of the antennal sense organ, whereas the dorsomedial papillae require *ems* and *otd* and the dorsolateral papillae require *ems* and *btd*. Our experiments with *hsp70–ems* are consistent with the idea that *ems* function requires combinatorial input from other genes such as *otd* and *btd*. Ubiquitous expression of *ems* throughout the embryo does not produce ectopic antennomaxillary structures outside of the head region. Ubiquitous expression of *ems* does, however, severely disrupt the development of the head skeleton. This phenotype could possibly be attributable to the inappropriate expression of *ems* with *otd*, *btd*, and other developmental genes in neighboring domains in the head.

The data presented here, along with previous results, argue that the two developmental roles of *ems*, early antennal/mandibular region development and later spiracular atrium development, are distinguished by *ems* interactions in different combinatorial codes with known transcription factors. A code for antennal/mandibular region development results when *ems* is coexpressed with other head determination genes like *otd* and *btd*. A later code for spiracular atrium development in the eighth abdominal segment results when *ems* is activated by and coexpressed with the homeotic selector

gene *Abd-B*. The conservation of *ems*-like genes and some of its proposed *Drosophila*-coding partners in mice and humans [*Emx1* and *Emx2* (Simeone et al. 1992a); *Otx* and *Abd-B*-like Hox genes (Simeone et al. 1992b; McGinnis and Krumlauf 1992)], coupled with the apparent genetic conservation of overlapping patterns of expression for some of these putative coding partners, suggests the intriguing possibility that some regulatory relationships and combinatorial interactions involving *ems* have been conserved since the common ancestor of flies and mammals.

Materials and methods

Fly strains

Fly culture and crosses were performed according to standard procedures. The *hsp70–Dfd/Abd-B* flies used were the A48 line of *P[HS–Dfd/(Abd-B HD) ry*$^{+}$*]*, which is a second chromosome lethal insert balanced over *CyO*, described in Kuziora and McGinnis (1991). The *hsp70–Abd-B*^{m} strain M10B is a viable X chromosome insert of *P[HS–Abd-B*$^{pAB713(m)}w^{+}$*]*. This construct contains an α class cDNA (Kuziora and McGinnis 1988b) that encodes the m protein of *Abd-B* (Kuziora and McGinnis 1988b; Sanchez-Herrero and Crosby 1988; Celniker et al. 1989; Zavortink and Sakonju 1989). The *hsp70–Abd-B*^{r} strain R7 is a third chromosome lethal insert of *P[HS–Abd-B*$^{pAB728(r)}w^{+}$*]* balanced over *TM3 Sb*. A β-class cDNA (Kuziora and McGinnis 1988b) that encodes the r protein (Kuziora and McGinnis 1988b; Sanchez-Herrero and Crosby 1988; Celniker et al. 1989; Zavortink and Sakonju 1989) was used in this construct. Both strains utilized the pHSBJ heat shock promoter vector (described below) and were constructed and kindly provided by M. Kuziora (University of Pittsburgh, PA). Multiple lines generated with these constructs give consistent cuticular phenotypes (M. Kuziora, in prep.; our own observations) The *ems* null mutant strain *ems*^{9H83}*/TM3 Sb Ser* (Jürgens et al. 1984; Dalton et al. 1989) was obtained from Eric Wieschaus. The *Abd-B* strains provided by G. Morata were as follows: *m* flies were *Abd-B*^{M5}*/TM1*, and *r* flies were *Abd-B*$^{x23\text{-}1}$*/TM6b* as described in Casanova et al. (1986). Unless otherwise stated, wild-type embryos were of the *cn; ry*^{506} strain used for transformations and were obtained from G. Rubin (University of California, Berkeley). These strains were crossed into *HZems1.2* lines as described below.

Larval cuticular phenotypes

Embryos were collected at 25°C on molasses agar plates for time periods described in the text. Heat shocks were administered by submerging these plates wrapped in parafilm in a 37°C water bath for the indicated length of time. Embryos were allowed to continue development for >24 hr at 25°C. Unhatched larvae were dechorionated, dissected from the vitelline membrane, fixed, and cleared of internal tissue in Hoyer's/lactic acid as described by Wieschaus and Nüsslein-Volhard (1986). To test whether *ems* is required for the formation filzkorper in ectopic positions in *hsp70–Abd-B*^{m} larvae, the following strain was constructed: *P[HS–Abd-B*$^{pAB713(m)}w^{+}$*]M10B; ems*^{9H83}*/TM3 Ser*. Embryos (2 to 4 hr) were heat shocked for 2 hr, and their cuticles were examined after 24 hr of development, looking for ectopic filzkörper and cuticular markers characteristic of *ems* mutant larvae (lack of antennal sense organs and lack of A8 filzkörper). Of 168 larvae counted, the 57 larvae that were *ems*$^{-}$ did not have ectopic filzkörper, 32 larvae that were *ems*$^{+}$ had ectopic filzkörper, and the remaining 79 *ems*$^{+}$ larvae did not develop ectopic filzkörper. The low penetrance of the filzkörper phenotype in an *ems*$^{-}$ background suggests that having only one functional copy of *ems* reduces the filzkörper forming activity of *hsp70–Abd-B*^{m}.

Transcript localization

Embryos were collected and heat-shocked as described. After recovery for the appropriate time at 25°C, embryos were prepared for whole-mount in situ localization of transcripts by the method of Tautz and Pfeifle (1989) using DNA probes; a modified version of this protocol was used for RNA probes. The *ems* cDNA probe (E4cDNA12 described in Dalton et al. 1989) was labeled with digoxigenin–dUTP (Boehringer-Mannheim) using the random primer method of Feinberg and Vogelstein (1983). An RNA probe was used to detect *lacZ* transcripts and was prepared as follows. The *Kpn*I–*Sal*I fragment of HZ50PL (Hiromi and Gehring 1987), which contains the entire open reading frame of the *lacZ* gene, was cloned into pBluescript (Stratagene). Digoxigenin-labeled antisense-RNA was generated from the *Sac*I linearized plasmid using T7 polymerase (Boehringer-Mannheim) and the solutions and protocols in Boehringer-Mannheim's DIG RNA Labeling Kit. After detection of the transcripts, embryos were cleared in xylene and mounted in Permount (Fischer).

Immunoprecipitation assay

Dfd and Dfd/Abd-B proteins were produced in *E. coli* and purified over a DNA affinity column as described in Dessain et al. (1992). The immunoprecipitation assay was based on the protocol of McKay (1981), as modified in Dessain et al. (1992). λE4 DNA (Dalton et al. 1989), digested with *Eco*RI, *Xho*I, *Bam*HI, and *Hind*III and end-labeled with ^{32}P, was used in the binding reactions with Dfd and Dfd/Abd-B proteins. Binding reactions were performed in 50 ml of binding buffer [20 mM HEPES (pH 7.6), 210 mM KCl, 6.25 mM $MgCl_2$, 0.25 mM EDTA, 1 mM dithiothreitol, 10% glycerol (wt/vol), 100 mg/ml of sonicated herring sperm DNA, and 0.05% NP-40] with protein and 1 fmole of the ^{32}P-labeled digest. After 30 min on ice, the protein–DNA complexes were immunoprecipitated using anti-Dfd rabbit serum (Jack et al. 1988) bound to *Staphylococcus aureus* cells (Calbiochem) as described previously (Dessain et al. 1992). Immunoprecipitated DNA fragments were resolved on a 5% denaturing polyacrylamide gel and detected by autoradiography.

P-element constructs and transformation

The *ems* enhancer–reporter construct pHZems1.2 was constructed as follows. A 1.2-kb *Eco*RI fragment (containing the highest affinity Dfd/Abd-B protein-binding fragment) from the λE4 genomic clone (Dalton et al. 1989) was subcloned into the *Eco*RI site of pBluescript (Stratagene). This insert was then excised with *Xba*I and *Kpn*I in the flanking polylinker sequences and subcloned into the *Xba*I and *Kpn*I sites in the polylinker of the enhancer test vector HZ50PL (Hiromi and Gehring 1987), maintaining the same orientation of the *Eco*RI fragment with respect to the promoter.

The *hsp70–ems* vector pDMHSems was constructed as follows. First, a heat shock shuttle vector pHSBJ was constructed that contains the pBluescript polylinker located between the *hsp70* promoter and 3′-untranslated sequences of the *Adh* gene. The 750-bp *Adh* sequence was excised with *Xba*I from the vector pRmHa-3 (Bunch et al. 1987) and ligated into the *Xba*I site of pBluescript. The *hsp70* promoter was cut out of the *hsp70–Dfd/Abd-B*v ector pMK526 (Kuziora and McGinnis 1991) with *Kpn*I

and *Sac* end-filled with T4 polymerase, and inserted into the blunted *Xho*I site of the *Adh* containing pBluescript plasmid. The resulting vector pHSBJ has seven unique cloning sites located between the *hsp70* promoter and the *Adh* 3′ sequences containing polyadenylation signals and cleavage sites; this heat shock shuttle cassette can be cut out with flanking *Not*I sites. The *ems* cDNA (Dalton et al. 1989) was inserted into the *Eco*RI site in the polylinker of pHSBJ, and the resulting *ems* heat shock cassette was cut out with *Not*I and subcloned into the P-element injection vector pDM30 (Mismer and Rubin 1987) creating plasmid pDMHSems.

To obtain transgenic strains pHZems1.2 and pDMHSems were coinjected separately with the helper plasmid pπ25.7wc (Karess and Rubin 1984) into *cn; ry*^{506} embryos as described by Rubin and Spradling (1982). Three independent lines were isolated carrying the HZems1.2 insert: *P[HZems1.2 ry*$^{+}$*]*^{A} on the third chromosome; *P[HZems1.2 ry*$^{+}$*]*^{B} on the second chromosome; and *P[HZems1.2 ry*$^{+}$*]*^{C} on the third. All three have the same *ems*-related expression pattern elements; the *C* strain also has non*ems*-related expression elements (in the hindgut and foregut), which we presume to be attributable to position effects. The injection of pDMHSems generated a single viable insert on the X chromosome designated *P[hsp70–ems ry*$^{+}$*]*^{A}. Two additional strains (*P[hsp70–ems ry*$^{+}$*]*^{B} on the second chromosome and *P[hsp70–ems ry*$^{+}$*]*^{C} on the third chromosome) were generated by transposition of *P[hsp70–ems ry*$^{+}$*]* using a genomic source of transposase (Robertson et al. 1988). *P[HZems1.2 ry*$^{+}$*]* lines were crossed into mutant backgrounds by use of standard genetics. The following strains were produced. *P[HS-Abd-B*$^{pAB713(m)}$*w*$^{+}$*]M10B;P[HZems1.2 ry*$^{+}$*]*^{B}. *P[HZems1.2 ry*$^{+}$*]*^{B}; *P[HS–Abd-B*$^{pAB728(r)}$*w*$^{+}$*]R7/TM3 Ser. P[HZems-1.2 ry*$^{+}$*]*^{B}*;Abd-B*^{M5}*/MKRS. P[HZems1.2 ry*$^{+}$*]*^{B}; *Abd-B*$^{x23\text{-}1}$*/MKRS. P[HZems1.2 ry*$^{+}$*]*^{B}*;ems*^{9H83}*/TM3 Ser.* Some experiments were performed using the F1 embryos from single crosses of *P[HZems1.2 ry*$^{+}$*]* lines against *hsp70–Abd-B* lines.

Immunohistochemical detection of proteins in embryos

Embryos were prepared and stained using horseradish peroxidase (HRP) immunohistochemistry as described previously (Jack et al. 1988). Mouse monoclonal anti-β-galactosidase antibody (Promega) was used at a 1 : 500 dilution. Mouse monoclonal anti-Abd-B antibody 1A2E9 (Celniker et al. 1989) was used at a 1 : 1 dilution of supernatant. A goat anti-mouse antibody conjugated to biotin (Jackson Immunoresearch) was used as the secondary antibody followed by HRP detection with Vector Laboratory's ABC system. Anti-ems antiserum was used as described previously (Dalton et al. 1989). Embryos were cleared in methyl salicylate and mounted in Permount (Fischer).

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