Developmental function of Elf-1: an essential transcription factor during embryogenesis in *Drosophila*

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The *Drosophila* transcription factor Elf-1 binds to a *cis*-acting element that is essential for neuronal expression of the *Dopa decarboxylase gene (Ddc)*. Elf-1 also stimulates transcription from the *Ddc* and *Ultrabithorax* promoters *in vitro*. To investigate the function of this factor *in vivo* we have screened for mutations and identified the Elf-1 gene as *grainyhead (grh)*, a previously known embryonic lethal locus. Elf-1/*grh* mutations cause late embryonic lethality accompanied by multiple defects in the cuticle and head skeleton. Using *Ddc-lacZ* gene fusions, we show that these mutations affect *Ddc* expression in the embryo. Surprisingly, however, epidermal expression is disrupted, whereas neuronal expression remains unaffected. Analysis of the mutant phenotype indicates that Elf-1 coordinately regulates multiple genes involved in the differentiation of epidermal structures. The results highlight the importance of genetic analysis in the study of proteins required for developmental regulation of gene expression.

[Key Words: *Drosophila* development; epidermal/neural differentiation; transcription factor; *Ddc*; *Ubx*]

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Differential gene expression is a key facet of the generation of cell diversity during development. Considerable progress has been made toward understanding how the early specification of the *Drosophila* body plan is brought about through a hierarchy of transcription factors (see Akam 1987; Scott and Carroll 1987; Ingham 1988). However, little is known about the molecules that act subsequently to bring about the differentiation of the appropriate morphological structures. We have been studying a process that occurs late in the differentiation pathway, namely the expression of the *Drosophila Dopa decarboxylase gene (Ddc)*. Identifying the protein factors required for correct expression of this gene will provide a model for tissue-specific gene expression and will help in elucidating the pathway between the early-acting pattern formation genes and the final differentiated state of the organism.

Ddc is expressed in at least two tissues at the end of embryogenesis, the nervous system and the epidermis (Wright et al. 1976; Livingstone and Tempel 1983; Valles and White 1986; Wright 1987). In the former, the enzyme is required for the synthesis of the neurotransmitters serotonin and dopamine, and in the latter it is needed for hardening and coloring the cuticular structures. Expression of *Ddc* in the two tissues involves at least partially separate regulation, since deletion and point mutations

in this gene have been identified that specifically affect its expression in one or the other tissue (Scholnick et al. 1986; Bray et al. 1988, 1989; Johnson et al. 1989). For example, altering 2 bp in the *cis*-acting element I sequence (−72 to −57 bp upstream of *Ddc* transcription start) leads to a gene that can be expressed in epidermal cells but not in the nervous system when reintroduced into the germ line by P-element transformation. This sequence therefore shows the characteristics of a central nervous system (CNS)-specific *cis*-acting element.

A protein has been purified that binds to element I in a manner affected by the mutations that abolish neuronal expression (Bray et al. 1989). These results suggest that the binding factor Elf-1 may mediate the CNS-specific expression of *Ddc* through interaction with element I. Separate *in vitro* studies on the purified protein (also called NTF-1; Biggin and Tjian 1988; Dynlacht et al. 1989) have shown that it can stimulate transcription from the *Ddc* promoter and from the promoters of the *Ultrabithorax (Ubx)* and *fushi tarazu (ftz)* genes. Moreover, an Elf-1-binding site has been detected in the *engrailed (en)* gene (Soeller et al. 1988). Both element I and the related sequence in *en* are highly conserved in diverged *Drosophila* species, suggesting a fundamental role in the regulation of these genes (Bray and Hirsh 1986; Kassis et al. 1989).

The observed interactions of Elf-1 with both *Ddc* and the three other genes appear anomalous in some respects. *Ddc* is expressed late in development and might be viewed as an end product because the enzyme it en-

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codes catalyzes the formation of molecules that are characteristic of the fully differentiated cell. *Ubx*, *ftz*, and *en* act early during embryogenesis and are involved in patterning of embryonic structures. Furthermore, the expression pattern of Elf-1 in the nervous system is not fully consonant with its proposed role as a *Ddc* regulator (Bray et al. 1989). We therefore wanted to investigate the role of Elf-1 during development. If the aim is to understand the mechanisms of tissue-specific gene expression, it is essential to discover how the absence or alteration of a transcription factor affects development and specifically how it affects *in vivo* expression of the genes to which it binds *in vitro*. We have taken a genetic approach to this problem and report the isolation of Elf-1 mutations and describe their effects on embryonic development and on expression of *Ddc*, *Ubx*, *ftz*, and *en*.

Results

Identification of Elf-1 mutations

The gene encoding Elf-1 maps to position 54F on the second chromosome in *Drosophila* (Bray et al. 1989; Dynlacht et al. 1989). Two chromosomal deficiencies that remove sequences in this region have been isolated: *Df(2R)Pcl^{7B}* extending from 54E8 to 55C1 and *Df(2R)Pcl^{11B}* removing 54F6–55C1-3 (Duncan 1982). Using anti-Elf-1 antibodies, we tested whether these deficiencies, which we refer to as *Df7* and *Df11*, respectively, remove the Elf-1 gene. Nuclear Elf-1 protein can clearly be detected in *Df11* embryos but is absent from *Df7* embryos (Fig. 1). In both instances the homozygous deficiency embryos can be easily distinguished as they are poorly differentiated and smaller than wild type. As *Df11* does not remove the gene encoding Elf-1, but *Df7* removes part or all of it, we were able to use these two overlapping deficiencies to isolate mutations in a very small interval of the chromosome. We used an F2 lethal screen (see Materials and methods) on the basis of the assumption that Elf-1 is an essential protein so that mutations would be lethal in combination with *Df7*. All mutations that were lethal in *trans* to *Df7* were then tested with *Df11* to identify alleles that survived in combination with the latter deficiency.

From a screen of 3260 chromosomes, 13 were identified that satisfied these criteria. Inter se complementation analysis permitted assignment of the mutations to four complementation groups (one to four alleles per group). These groups were assayed for aberrations in the Elf-1 gene by using antibodies to screen for mutations that affected expression of the Elf-1 antigen. To make identification of the 1/4 of the progeny that were homozygous mutant possible, we used a balancer chromosome marked with a transposon expressing the β -galactosidase gene. The progeny were analyzed for Elf-1 and β -galactosidase expression in double label experiments. All four alleles of one complementation group (B28, B32, B35, and B37) showed altered Elf-1 expression in homozygotes (i.e., the class of embryos that lacked β -galactosidase; Fig. 2). In three of these no Elf-1 protein was

Figure 1. Elf-1 is not present in *Df7* embryos. Elf-1 expression was visualized in wild-type (A), *Df11* (B), and *Df7* (C) embryos with an anti-Elf-1 monoclonal antibody and a horseradish peroxidase-conjugated anti-mouse secondary antibody. A graphic map of the deficiencies is represented (D). Nuclear staining is visible in wild-type and *Df11* embryos but not in *Df7* embryos. Embryos were collected and aged in parallel and are all at approximately stage 14 (Campos-Ortega and Hartenstein 1985) of embryonic development. The homozygous deficiency embryos were identified on the basis of their poorly differentiated phenotype. In all embryos, anterior is to the left; the plain of focus is on the ventrolateral epidermis in A and B and on the ventral epidermis in C.

detectable and in the fourth (B28) Elf-1 was absent from the epidermis but not from the nervous system.

To confirm that the alleles correspond to mutations within the Elf-1 gene it would be desirable to show by transformation that Elf-1 DNA rescues the mutations. We have not attempted these experiments because preliminary mapping of the Elf-1 gene shows that it is large, spanning at least 30 kb (S.J. Bray, unpubl.), which makes transformation problematic. Instead, we characterized the lesion of one allele at the molecular level. Gradient denaturing gel electrophoresis was used to identify gene fragments that had altered base composition compared

Figure 2. Mutations eliminate Elf-1 expression. Embryos were incubated with anti-Elf-1 monoclonal antibodies visualized with an FITC-conjugated secondary antibody (A,C) and anti- β -galactosidase antibodies visualized with a rhodamine-conjugated secondary antibody (B,D). In the first embryo (A,B), Elf-1 is detected in the nuclei of all epidermal cells (A) and β -galactosidase is detected in select cells, principally in the nervous system (B). The second embryo is a mutant as revealed by the absence of β -galactosidase (D) and has no detectable Elf-1 (C). Embryos are progeny from the cross $^{*}/CyO-lacZ \times Df7/CyO-lacZ$, where * represents the mutagenized chromosome and $CyO-lacZ$ is a CyO balancer chromosome carrying a P-element insertion bearing $lacZ$. β -Galactosidase therefore identifies three of four of the progeny ($^{*}/CyO-lacZ$, $Df7/CyO-lacZ$, and $CyO-lacZ/CyO-lacZ$), as represented in B and is not detectable in the $^{*}/Df7$ mutant embryos (D). The allele shown is B37. In the original test for Elf-1 mutants the different alleles were scored blind. The embryos are stage 14; anterior is to the left and dorsal is up.

to the parental chromosome (Curtis et al. 1989; Gray et al. 1991). Altered fragments were identified in two of the four alleles (B32 and B37; data not shown), and one of these, B37, was cloned from homozygous mutant embryos using polymerase chain reaction (PCR) to amplify the appropriate fragment. The DNA sequence of the altered fragment revealed a deletion of 7 bp from the Elf-1-coding sequence leading to a frameshift and thus to a truncated protein (Fig. 3A). In vitro studies with truncated Elf-1 proteins demonstrate that truncations in the vicinity of the mutation interfere with the ability of the protein to bind DNA (Fig. 3B) even when not removing the DNA-binding domain per se (data not shown). The B37 mutation occurs in a common exon downstream of the sites of alternative splicing in Elf-1 and thus affects all forms of the protein.

Phenotype of Elf-1 mutants

The four Elf-1 alleles are all embryonic lethal. The most severe alleles, B32 and B37, show a similar phenotype when homozygous and in combination with *Df7*, suggesting that they lead to complete or near-complete inactivation of the protein. The embryos, which die at the end of embryogenesis, have apparently normal segmentation but have altered head skeleton and cuticular structures. The most notable aberration is the granular nature of the head skeleton (Fig. 4B), but cuticle formation is also abnormal. For example, when removed from the vitelline membrane, mutant embryos are abnormal in shape and appear flaccid and ameboid, in contrast to the normally elongated hatched larva. The abnormalities are also evident in cuticle preparations (Fig. 4), which manifest distended bulges, rupture easily, and generally appear misshapen. Further defects are apparent in the

mouth parts (Fig. 4B) and in the setal belts of the ventral cuticle (Fig. 4C): Both the mouth hooks and the denticles are poorly differentiated, and the denticles frequently appear disordered, with cases of reversed polarity and of two denticles formed in the same location. Clearly, genes regulated by Elf-1 are involved in many aspects of cuticle specialization.

The tracheal network also appears abnormal (data not shown). The main trunks are farther apart than usual, appear patchy, and rarely connect to the posterior spiracles. Patchiness is most likely the result of only parts of the tracheal tree filling with gas, suggesting a failure of some segmental tracheae to form, to interconnect, and/or to fill with air because of structural defects. Because the tracheae are specializations of the ectoderm that secrete chitin, these results suggest further that Elf-1 regulates multiple genes involved in the production of cuticle and cuticular derivatives by ectodermal cells.

In strong contrast to these cuticular defects, the formation and development of the nervous system appears normal in Elf-1 mutant embryos, according to both morphological criteria and the distribution of specific antigens detected with antibodies. For example, the axonal scaffold detected by anti-horseradish peroxidase antibodies appears normal, as does the location of *ftz*- or *Ubx*-expressing cells (Fig. 5). The expression of *even-skipped* and *en* have also been examined and appear to be wild type (data not shown).

Elf-1 is grainyhead

The cytological location of Elf-1 did not correspond to that reported for any known gene. However, once the phenotype of Elf-1 mutations had been characterized we realized that it was very similar to that reported for a

Figure 3. Molecular lesion in Elf-1 allele B37. (A) DNA sequence of the B37 allele reveals a deletion of 7 bp in the coding sequence of Elf-1. Bases shown in parentheses (right) are absent from mutant as compared to wild type. This deletion causes a frameshift that will result in premature termination of the encoded protein. (B–D). In vitro DNA-binding studies with truncated Elf-1 show that the product of allele B37 would be inactive in DNA binding. Plasmids containing truncated cDNA clones (D) were transcribed and translated in vitro (C), and the polypeptide products were tested for DNA-binding activity (B). Proteins encoded by a cDNA clone that is truncated at the 3' end are not able to bind DNA fragments in vitro, although proteins encoded by clones with 5' truncations still bind DNA. In the diagram of cDNA clones (D) the starting cDNA is shown at the top. The open rectangle indicates coding sequence, and the site of the mutation in allele B37 is indicated. Truncated cDNAs are shown below the diagram, with numbered lines representing sequences used to produce the correspondingly numbered proteins shown in B and C. Proteins produced by in vitro transcription and translation of truncated cDNA clones in the presence of [³⁵S]methionine were analyzed on an SDS–polyacrylamide gel (C). Binding activities of unlabeled products synthesized in parallel were tested by using a ³²P-labeled oligonucleotide encompassing the Elf-1-binding site (B). The DNA/protein complexes, separated from unbound DNA using a native acrylamide gel, are detected with the products of plasmids 1, 6, 7, and 8 only. The unbound oligonucleotide has been run off the gel. The label detected at the bottom edge results from a nonspecific binding activity in the extract. Similar results were obtained in two other experiments with different truncated cDNA clones.

gene in a screen for embryonic lethal genes on the second chromosome (Nüsslein-Volhard et al. 1984; R. Tearle, pers. comm.). This gene, referred to as *l(2)IM45* and subsequently named *grainyhead* (*grh*), mapped by recombination to a position on the chromosome proximal to Elf-1. Nevertheless, we crossed the Elf-1 alleles to available

grh alleles to test whether they might represent the same gene. All four Elf-1 alleles fail to complement, indicating that Elf-1 does correspond to *grh* (Table 1). Therefore, in accordance with convention, we suggest that the gene encoding Elf-1, which has also been called NTF-1, should be referred to as *grh*. For the purposes of this paper we will refer to the protein encoded by the locus as Elf-1 and the gene as Elf-1/*grh*.

Effect of Elf-1/*grh* mutations on *Ddc* expression

Elf-1 was isolated on the basis of its binding to element I, which has a major role in nervous system expression of *Ddc* (Bray et al. 1989). Surprisingly, endogenous *Ddc* expression, as assayed by antibody staining, is wild type in the nervous system of Elf-1/*grh* mutant embryos (Fig. 6C). Our ability to detect failures in *Ddc* expression was confirmed by using a strain with a *Ddc* gene that is disabled in neuronal expression because of a 2-bp mutation in element I (Fig. 6B). We conclude that Elf-1 does not mediate the nervous system expression of *Ddc*, which is so severely impaired when element I is mutated.

Ddc is also expressed in the epidermis during late stages of embryogenesis when the cuticle is being secreted. To analyze the effect of Elf-1/*grh* mutations on *Ddc* expression in this and other tissues, we used a *Ddc-lacZ* reporter gene construct (see Materials and methods). Expression of β-galactosidase can be assayed by using a histochemical stain that overcomes the problem of cuticle interfering with antibody detection methods. Like the endogenous *Ddc* gene, this construct is expressed in the epidermis and nervous system in wild-type embryos (although this fusion gene only leads to expression of β-galactosidase in a subset of the neurons normally expressing *Ddc*; data not shown). β-Galactosidase is also detectable in the gut, but this activity is endogenous at least in part because it occurs in embryos that do not contain the fusion gene. Elf-1/*grh* mutant embryos, which can be distinguished by their head skeleton abnormalities, retain expression in the nervous system but do not express β-galactosidase in the epidermis (Fig. 7). These results were obtained with two different Elf-1/*grh* alleles (B37 and B32), including the allele that results in a truncated Elf-1 protein. We conclude that Elf-1 is required for the expression of *Ddc* in the embryonic epidermis.

Effects of Elf-1/*grh* mutations on expression of other genes

Three other genes containing Elf-1-binding sites have been identified: *Ubx*, *ftz*, and *en* (Soeller et al. 1988; Dynlacht et al. 1989). Antibodies were used to analyze the expression of *Ubx* and *ftz* in Elf-1/*grh* mutant embryos. Expression of both genes appears normal in the nervous system and epidermis: No gross changes in the domains of *Ubx* or *ftz* expression are seen in Elf-1/*grh* mutant embryos, and no alterations in the cellular pattern can be detected (e.g., Fig. 5). Given the number of cells expressing *Ubx* in each segment, however, it re-

Figure 4. Cuticle preparations from *Elf-1/grh* alleles reveal multiple defects. Aspects of cuticle pattern of wild-type (wt) and *Elf-1/grh* (*grh*) embryos. (A) Ventrolateral aspect shows that the cuticle of *Elf-1/grh* mutant embryos is bulging and distended compared to that of wild type. (B) Head structures: The head skeleton of *Elf-1/grh* embryos is reduced and has an unusual globular appearance. The ventral arm of the head skeleton is indicated by an arrowhead; the small arrows mark the mouth hooks, which are much smaller than usual in the mutant. (C) Ventral aspect of cuticle in A3 showing denticles: The *Elf-1/grh* mutants have disordered and small denticles. The embryos shown are *grh*^{B37}/*Df7*. Phase-contrast photographs.

mains possible that *Ubx* expression might be altered in a small number of individual cells. For our analysis of *en* expression we used an *en-lacZ* gene fusion construct (Hama et al. 1990). In both the nervous system and epidermis of mutant embryos we observed the same segmentally repeating pattern of β -galactosidase expression as seen in wild-type embryos (Fig. 8). The expression of *en-lacZ* in the epidermis of *Elf-1/grh* mutant embryos shows that although there is no epidermal *Ddc-lacZ* expression, epidermal cells are still present in the mutant embryo. However, at late stages of embryogenesis the levels of β -galactosidase activity do not appear to be quite wild type in the mutant embryos. They are enhanced in subcuticular peripheral nervous system cells relative to epidermal cells. Although this may reflect a requirement for *Elf-1*, it is most likely the result of an increase in the accessibility of subcuticular structures to the β -galactosidase substrate because of flaws in the mutant cuticle. Prior to cuticle deposition (stage 15) both the levels and pattern of *en-lacZ* expression appear to be wild type (data not shown). In summary, the results with

Ubx, *ftz* and, possibly, *en* provide additional evidence that *Elf-1* protein binding in vitro does not correlate with effects of *Elf-1/grh* mutants in vivo.

Discussion

In vitro vs. *in vivo* functional assays of the *Elf-1* transcription factor

The results presented here emphasize the importance of testing the essential functions of a transcription factor in the organism. The molecular properties and *in vitro* actions of DNA-binding proteins can suggest a potential role, but their actual function during development can only be ascertained by perturbing these proteins *in vivo*. For this purpose, we have taken advantage of the sophisticated genetics of *Drosophila*.

Elf-1 was identified on the basis of its *in vitro* interaction with a sequence element that is essential for transcription of the *Ddc* gene only in the nervous system of *Drosophila* (Bray et al. 1988, 1989). *Elf-1* was subse-

Figure 5. Axonal scaffold and distribution of *Ubx* and *ftz* proteins appear unaffected by *Elf-1/grh* mutations. The expression of three antigens in wild-type (A,C,E) and *Elf-1/grh* mutant (B,D,F) embryos was analyzed. The anti-horseradish peroxidase antibody recognizes an antigen present on the axonal scaffold of wild-type (A) and *Elf-1/grh* mutant (B) embryos. No differences are distinguishable in mutant embryos. Expression of *Ubx* (B,D) and *ftz* (E,F) proteins also appears identical in wild-type and mutant embryos. Mutant embryos were of the genotype *grh*^{B37}/Df7 and were identified by the absence of the β -galactosidase-expressing *CyO* balancer chromosome in double-label experiments as described in Fig. 2. Similar results were obtained with alleles B32 and B35.

quently shown to be capable of activating *in vitro* transcription from this and other developmentally regulated genes, *Ubx* and *ftz* (Dynlacht et al. 1989). To test the hypothesis that this protein is responsible for activating expression of these genes *in vivo* we have generated mutations in the *Elf-1* gene. These mutations are embryonic

lethal, indicating that *Elf-1* is essential, and correspond to a previously defined lethal locus, *grh*. Using *Ddc-lacZ* fusion genes we have shown that the *Elf-1/grh* mutations do have an effect on expression from the *Ddc* promoter in the embryo. However, contrary to predictions, this effect is limited to the epidermal expression of *Ddc*; there is no apparent effect on *Ddc-lacZ* or endogenous *Ddc* expression in the nervous system of *Elf-1/grh* mutant embryos. No change in *Ubx* or *ftz* expression was detectable either. How can the disparate *in vitro* and *in vivo* results be reconciled?

Point mutations in the *cis*-acting element I of *Ddc* prevent *Ddc* expression in the embryonic CNS even though mutations in the putative *trans*-factor, *Elf-1*, do not. Clearly we must postulate the existence of another factor necessary for *Ddc* expression in the CNS. The simplest hypothesis is that this putative CNS factor interacts with element I, without involvement of *Elf-1*. There are examples in several genes of two or more factors binding to overlapping or identical sites in the reg-

Table 1. *Elf-1* is allelic to *grh*

Alleles tested	<i>Cy</i> ⁻ progeny	<i>Cy</i> ⁺ progeny
B37 × <i>grh</i> ³⁰⁰	395	0
B32 × <i>grh</i> ³⁰⁰	392	0
B35 × <i>grh</i> ³⁰⁰	441	0
B28 × <i>grh</i> ³⁰⁰	297	0
B37 × <i>grh</i> ³⁷⁰	351	0
B32 × <i>grh</i> ³⁷⁰	182	0
B37 × <i>grh</i> ³⁸⁰	465	0
B32 × <i>grh</i> ³⁸⁰	148	0

Figure 6. *Ddc* expression in the nervous system of Elf-1/*grh* mutant embryos is wild type. The CNS was dissected from embryos of wild-type (A), strain m1 (B), and Elf-1/*grh* (C) mutant [*grh*^{B37}/*Df7*] genotypes, and the levels of *Ddc* expression were visualized with an anti-*Ddc* antiserum and a rhodamine-conjugated anti-rabbit secondary antibody. Strain m1 is a P-element transformant strain described previously (Bray et al. 1989), which contains a *Ddc* gene with 2-bp mutations in element I, an Elf-1-binding site. Mutations in element I disrupt *Ddc* expression in the nervous system (B); mutations in Elf-1 have no effect on *Ddc* expression (C). Similar results were obtained with three other Elf-1/*grh* alleles.

ulatory DNA (e.g., Poellinger and Roeder 1989; Stanojevic et al. 1989; Pierani et al. 1990). The alternative possibility, that the CNS factor acts with Elf-1 and can stabilize the binding between mutant Elf-1 and element I, seems unlikely as all four different alleles of Elf-1/*grh* fail to cause any changes in neuronal *Ddc* expression. An additional possibility that must be considered is the presence of maternally provided Elf-1, which can function to turn on *Ddc* expression in the CNS in the absence of zygotic protein. However, maternal Elf-1 has not been detected. The mRNA and protein first appear in embryos during germ-band shortening (Bray et al. 1989; Dynlacht et al. 1989). We are thus led to postulate the existence of an unidentified CNS factor even though Elf-1 is the only detectable protein in embryonic extracts that binds to element I in vitro (Bray et al. 1989). The putative CNS factor could have failed detection if it is only present in a small number of cells, for example, the *Ddc*-expressing neurons of the CNS. Detection and/or disruption of spatially compartmented molecules are inherent limitations of in vitro studies with extracts from complex cell populations, such as whole embryos.

Unlike their effect on expression in the nervous system, mutations in the *cis*-acting element I have little, if any, detectable effect on *Ddc* expression in the epidermis (Scholnick et al. 1986; Bray et al. 1988; S.J. Bray, unpubl.). This seems paradoxical, in view of the fact that these mutations interfere with in vitro binding of Elf-1 to element I (Bray et al. 1988; 1989) and that Elf-1 is needed for normal expression in the epidermis in vivo. Either Elf-1 acts on *Ddc* indirectly in the epidermis, or element I is not the only Elf-1 binding site in the *Ddc* promoter. It is not uncommon to find multiple binding sites for a single transcription factor within the regulatory DNA of a gene. In several instances one or more of the sites appear to be functionally redundant (e.g., Dudler and Tra-

vers 1984; Topol et al. 1985; Stanojevic et al. 1989; Jiang et al. 1991; Regulski et al. 1991). We have recently scanned the entire *Ddc* gene with an in vitro binding assay and have detected additional strong Elf-1-binding sites (F.C. Kafatos, unpubl.). Furthermore, if large deletions are introduced into the regulatory DNA of the *Ddc* gene, the presence of element I does influence the levels of expression in the epidermis, albeit weakly (Bray et al. 1988). These data suggest that there are redundant Elf-1-binding sites within the *Ddc* gene; it remains to be determined whether it is necessary to mutate more than one Elf-1-binding site to alter *Ddc* expression in the embryonic epidermis or whether a single high-affinity site is crucial. It is instructive that interaction with element I led to the cloning of Elf-1, even though this is neither the strongest interaction of which Elf-1 is capable nor, apparently, the crucial interaction in vivo.

Our results raise questions about the significance of Elf-1 binding to the *Ubx*, *ftz*, and *en* promoters. Demonstration of in vitro binding and transcriptional activation by this factor (also called NTF-1) was based on nuclear extracts prepared from embryos (Biggin and Tjian 1988; Dynlacht et al. 1989). Nevertheless, Elf-1 does not appear to have an essential role in the transcription of *Ubx* or *ftz* in any part of the embryo. The Elf-1/*grh* mutations also leave expression of *en* largely unaffected. It seems likely that the *en* Elf-1-binding site is functionally important because, like the *Ddc* element I, it is conserved in a distantly related *Drosophila* species (Kassis et al. 1989). Given its similarity to the element I sequence, this might also be a target for the putative CNS factor, while its binding to Elf-1 itself is fortuitous. This argument could also hold for the Elf-1-binding sites in *Ubx* and *ftz*, although these show less clear sequence similarities to *Ddc* element I and may have no functional role in mediating in vivo expression of *Ubx* and *ftz*.

Figure 7. Elf-1 is required for *Ddc* expression in the epidermis. β -Galactosidase expression from a *Ddc-lacZ* fusion gene is detectable in embryos with wild-type head skeletons (A,B) but not in embryos with defective head skeletons characteristic of *Elf-1/grh* mutations (C,D). A *Ddc-lacZ* gene inserted on chromosome 2L was recombined onto chromosomes carrying *Elf-1/grh* mutations. Embryos from a *Ddc-lacZ grh/CyO* $\times$ *Df7/CyO* cross were examined for *Ddc* promoter activity by using a histochemical stain for β -galactosidase. The epidermis of the embryos has been slit open, the internal tissue removed, and the cuticle and underlying tissues flattened. The arrowhead in D indicates a small amount of gut tissue remaining in which β -galactosidase expression is detectable. Two *Elf-1/grh* alleles (B32 and B37) were used and gave identical results. Mutant embryos were allele B37.

One can never discount the possibility of subtle defects, but we have not observed any malformations in the embryonic nervous system associated with *Elf-1/grh* mutations, even though Elf-1 is normally expressed within some cells in this tissue. We do not know what role, if any, this factor plays in the nervous system. Clearly, it is neither necessary nor sufficient for expression of *Ddc*, *Ubx*, *ftz*, or *en* in the embryonic nervous system. Expression of these genes appears unaffected by *Elf-1/grh* mutations, and Elf-1-expressing cells that do not express the genes exist in the wild type (Bray et al. 1989 and unpubl.). However, Elf-1 might have an essential neuronal function later in development. Critical evaluation of the role of Elf-1 during later stages of development will require generation of mosaic nervous systems containing patches of mutant tissue or isolation of *Elf-1/grh* alleles that are temperature sensitive or only affect postembryonic development.

Involvement of Elf-1 in cuticular morphogenesis

In contrast to the results on the nervous system, we have

obtained clear evidence that Elf-1 is required in the embryonic epidermis and that it coordinates, directly or indirectly, the expression of multiple genes involved in the complex process of cuticular morphogenesis. *Ddc* is one of the genes regulated by Elf-1 in this tissue, but additional target genes clearly exist. Null *Ddc* mutants have underpigmented mouth hooks and denticle belts, but no other obvious cuticular defects, and can survive into larval instars if manually removed from the vitelline membrane (Valles and White 1986; Wright 1987). *Elf-1/grh* mutants have disorganized cuticular structures and weak cuticles and fail to survive if removed from the vitelline membrane. Both the pigmented sclerotized structures and the colorless cuticle that ensheathes the body, as well as the tracheae, are affected by *Elf-1/grh* mutations. On the other hand, the underpigmentation of cuticular structures is not as severe in *Elf-1/grh* mutants as in the case of *Ddc* null mutations. This suggests that even within the epidermis Elf-1 may not be required in all cells for *Ddc* expression. Indeed, antibody staining reveals residual *Ddc* protein in some cells surrounding the head skeleton of *Elf-1/grh* mutant embryos (data not shown).

Figure 8. An *en-lacZ* fusion gene is expressed in the epidermis of *Elf-1/grh* mutant embryos. β -Galactosidase is detectable in stripes in the epidermis from both wild-type (A) and mutant (B) *en-lacZ*, *grh*^{B37}/*Df7* embryos, showing that *Elf-1* is not essential for the epidermal expression of *en*. A P-element strain (*ryxho25*) containing an insertion of an *en-lacZ* fusion gene at the *en* locus expresses β -galactosidase in the equivalent pattern to that of the wild-type *en* gene (Hama et al. 1990). This locus was crossed onto *Elf-1/grh* mutant chromosomes by recombination, and expression of β -galactosidase in the epidermis was assayed in embryos of wild-type and mutant phenotype as in Fig. 7. All four *Elf-1/grh* alleles were tested for effects on *en-lacZ* and gave results identical to those for allele B37 presented here.

Although we do not know which genes, other than *Ddc*, are direct or indirect *Elf-1* targets in the epidermis, the mutant phenotypes suggest several candidates. For example, flimsy, friable cuticles as found in *Elf-1/grh* mutants are also seen in embryos lacking *Punch* and *l(2)amd* (see Wright 1987). The product of *l(2)amd*, which appears to be involved in the formation of colorless cuticle (Wright 1987), is closely related to the *Ddc* enzyme and presumably arose from a gene duplication (Eveleth and Marsh 1986). Regulation by *Elf-1* may predate the duplication that gave rise to the modern *Ddc* and *l(2)amd* genes. *Punch* and *Elf-1/grh* mutations cause similar defects in both the tracheal system and epidermis (Reynolds and O'Donnell 1987). The *Punch* product catalyzes the synthesis of a cofactor required by several enzymes including tyrosine hydroxylase. The similarity between the phenotypes of *Punch* and *Elf-1/grh* may indicate that *Elf-1* regulates *Punch* or, alternatively, that it regulates the enzymes that require the *Punch* product. It is important to note, however, that while *Elf-1/grh*-deficient embryos manifest some phenotypic characteristics similar to those of *Punch* and *l(2)amd* embryos, they do not exhibit all of the same defects. If *Elf-1* is involved in regulating the expression of such genes it is most likely only affecting expression in certain tissues and cell types, as is the case with its effect on *Ddc*.

Other aspects of the *Elf-1/grh* phenotype appear novel, suggesting that *Elf-1* may act on as yet unknown genes. This is the case for the head skeleton abnormalities. Unlike mutations affecting cuticle synthesis, which cause loss of pigmentation and sturdiness of the head skeleton without affecting the pattern, the head skeleton of *Elf-1/grh* mutants has a very different structure, appearing globular rather than linear. This may represent misregulation of genes required for synthesis of structural components, or it could reflect a problem in the communication between the cells that secrete the structure. A

problem in cell communication or the interpretation of positional information is also suggested by the disordered array of the denticles within the denticle belts of the ventral cuticle. Studies of mosaics indicate that local cell interactions are important in the interpretation of positional information in the epidermis (Gergen and Wieschaus 1985). A breakdown in these local communications could explain the sporadic denticles with reversed polarity and the disordered appearance of the denticles.

Now that many of the molecules involved in the hierarchy determining the early stages of embryonic patterning have been identified, attention is shifting to what occurs downstream of these molecules to lead to the morphological complexity of the organism. *Elf-1* appears to be a very downstream regulatory component. The identification of the gene encoding this transcription factor, coupled with the immunochemical and molecular tools available for its study, should lead to an understanding of how *Elf-1* regulates the final stages of embryonic epidermal differentiation and the complex morphogenesis of the cuticle.

Materials and methods

Drosophila strains

For a description of marker and balancer chromosomes, see Lindsley and Grell (1968) and Lindsley and Zimm (1985, 1990). *Df(2R)Pc1*^{7B}/*CyO* and *Df(2R)Pc1*^{11B}/*CyO* were obtained from I. Duncan (1982), and *grh* alleles [*b pr cn wx*^{wt}*grh*³⁰⁰ *bw*/*CyO bw* and *b pr cn wx*^{wt}*grh*³⁷⁰ *bw*/*CyO Roi bw*] were from R. Tearle and were generated by ethylmethane sulfonate (EMS) mutagenesis. The *en-lacZ* transformant line *ryxho25* was from the laboratory of T. Kornberg (Hama et al. 1990). All other stocks were from the collection maintained at The Biological Laboratories (Harvard University). Flies were cultured as described previously (Smolik-Utlaut and Gelbart 1987). Unless otherwise

stated, crosses were maintained at 25°C. Preparation of cuticles for phase microscopy was as described by Wieschaus and Nüsslein-Volhard (1986).

Mutagenesis

Two hundred aged males isogenic for *dp cn bw* second chromosome were exposed to 0.3% EMS (vol/vol) in 0.3 M sucrose for 24 hr (Lewis and Bachar 1968) and were then mated to *Sp B L/CyO* virgin females (10 males and 30 females per bottle). F1 *dp cn bw/CyO* males were mated individually to two *Df(2R)Pcl^{7B}/CyO* virgin females and maintained at 29°C. A total of 3260 single matings were set up, of which 5.2% were sterile. Each cross was scored for absence of straight-winged (*Cy⁺*) flies indicative of a lethal mutation in the region uncovered by the deficiency. Forty-five vials were identified, which lacked *Cy⁺* progeny, 1.4% of the total. The 45 putative lethal mutations were retested against *Df(2R)Pcl^{7B}/CyO* at 29°C, and 35 of those tested still failed to produce *Cy⁺* progeny. These alleles were then crossed at 29°C to *Df(2R)Pcl^{11B}/CyO* to identify the subset of alleles that were not located within the region removed in this deficiency. Thirteen alleles were identified that did give *Cy⁺* progeny from this cross; these alleles were then mated inter se at 29°C to assign the mutations to complementation groups. The alleles were subsequently tested for temperature sensitivity and were found to be lethal at all temperatures. All further crosses were maintained at 25°C.

Immunohistochemistry and immunofluorescence

Immunological detection of proteins in embryos was carried out essentially as described previously (Bray et al. 1989), except that the buffer used for the fixation was 160 mM KCl, 40 mM NaCl, 4 mM Na₃EGTA, 1 mM spermidine-HCl, 0.4 mM spermine-HCl, 0.2% β-mercaptoethanol, and 30 mM PIPES (pH 7.4). Embryos were staged according to Campos-Ortega and Hartenstein (1985). The primary antibodies used were as follows: Anti-Elf-1 monoclonal Bf1 diluted 1 : 6 (Bray et al. 1989), anti-*Ddc* rabbit antiserum diluted 1 : 50 (Beall and Hirsh 1987), anti-β-galactosidase rabbit antiserum diluted 1 : 2000 (gift of R. Holmgren), anti-*ftz* rabbit antiserum diluted 1 : 300 (gift of Sean Carroll), and anti-*Ubx* monoclonal FP3.38 diluted 1 : 6 (White and Wilcox 1984). Fluorescent secondary antibodies were DTAF-conjugated sheep anti-mouse diluted 1 : 100 (Jackson Immunologicals) and rhodamine-conjugated goat anti-rabbit diluted 1 : 250 (TAGO). FITC-conjugated anti-horseradish peroxidase was from Cappel. For visualization of immunofluorescence, embryos were mounted in Citiflor. Horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse used at 1 : 1000, Bio-Rad) were detected with 0.5 μg/ml of diaminobenzidine as substrate in the presence of 0.03% NiSO₄ and 0.06% H₂O₂. The stained embryos were dehydrated with an ethanol series (30%, 70%, 100%), rinsed twice in isopropanol and once in xylene, and finally transferred to a drop of DePeX (BDH). Stained embryos were photographed on a Zeiss axiophot microscope with DIC optics.

For analysis of late embryonic structures the embryos were dissected with tungsten needles and the tissues were fixed in 4% formaldehyde in fixation buffer for 20 min in depression-well plates. Following fixation the dissected tissues were treated in the same way as whole embryos except all manipulations were carried out in depression-well plates.

Analysis of the molecular lesion in allele B37

The protocols for standard molecular biology techniques were as described in Sambrook et al. (1989). Restriction enzymes and

other enzymes used for molecular biology were purchased from New England Biolabs or Boehringer Mannheim Biochemicals except where indicated otherwise. Oligonucleotides were synthesized by A. Khoury on a Cyclone Plus synthesizer. The following section describes some of the specific methods used.

The fragment of Elf-1 containing a lesion in allele B37 was identified by using the procedures described by Gray et al. (1991; see also Fischer and Lerman 1983; Curtis et al. 1989). Briefly, genomic DNA from adults of balanced stocks carrying the mutant alleles and from the parental strains was digested with *Hae*III and electrophoresed on a formamide/urea gradient denaturing acrylamide gel. The DNA was electroblotted from the gel onto nylon filters and probed with Elf-1 cDNA fragments. A band with altered mobility in strain B37 compared to the parental chromosomes was detected. This fragment was localized to a 1-kb genomic *Eco*RI fragment (the genomic library was constructed by using DNA from the isogenic *dp cn bw* strain used for the mutagenesis and was kindly provided by R. Blackman and W. Gelbart) which contained five *Hae*III fragments. Most of the fragments were very small, and only two appeared likely to correspond to the one detected on the denaturing gels. Probes were prepared from both fragments by using PCR to identify the *Hae*III fragment with altered mobility. Primers for PCR were synthesised corresponding to 21-bp sequences located just outside the *Hae*III fragment, with the aim of amplifying the sequence from genomic DNA of mutant embryos.

Nucleic acids were prepared from 10 homozygous B37 embryos by grinding frozen embryos in a 50/50 mixture of phenol/grind buffer [7 mM urea, 2% SDS, 10 mM Tris-Cl (pH 8), 1 mM Na₂EDTA, 0.35 mM NaCl; 25 μl of buffer per 10 embryos] that had been prewarmed to 60°C. A half volume of chloroform was then added. The aqueous phase was then re-extracted with chloroform, and the nucleic acids were precipitated with ethanol. Half of the resuspended nucleic acids were taken for PCR amplification, using Amplitaq and the conditions recommended by the manufacturer (Perkin-Elmer Cetus). Annealing was at 60°C (1 min), extension was at 72°C (1 min 45 sec), and melting was 94°C (1 min). Thirty cycles of amplification were carried out, and the tubes were then incubated at 25°C to allow full renaturation. Any ragged or protruding ends on the PCR products were filled in by using Klenow enzyme, and the products were analyzed on a 10% native polyacrylamide gel in 1× TBE buffer. Staining of the gel with ethidium bromide revealed a major band of appropriate molecular weight which was excised from the gel for cloning and subsequent sequencing (using U.S. Biochemical Sequenase; Tabor and Richardson 1987). A total of eight different clones, which were derived from two independent PCR amplification reactions, were sequenced.

In vitro binding of truncated Elf-1 proteins

The cDNA clones used to produce truncated Elf-1 proteins were from a series of exonuclease III deletions of Elf-1 cDNA clone N prepared for sequencing the cDNA (Bray et al. 1989). The procedures used to produce the proteins in vitro by in vitro transcription and in vitro translation were also described, as were the conditions for DNA-binding assays and analysis of the protein products on SDS-polyacrylamide gels.

lacZ fusion genes and β-galactosidase detection

The strain containing the *en-lacZ* fusion gene was *ryxho25* from the laboratory of T. Kornberg (Hama et al. 1990), which has the transposon inserted in the *en* locus at 48A on chromosome 2L. This locus was recombined onto chromosomes carrying the

Elf-1/*grh* alleles (genotype *dp cn grh bw*) by scoring for recombination between *cn* (43E) and *bw* (59D4-E1) with the chromosome *net dpp^{ho} dp Sp cn en² bw*. The presence of the *ryxho25* transposon could also be detected because it results in a wing-vein phenotype when in *trans* to an *en* mutation. Males carrying recombinant chromosomes with the *ryxho25* locus were then tested for lethality over *grh* alleles. Several different recombinant chromosomes were recovered for each of the four Elf-1/*grh* alleles; and in all cases, at least two different chromosomes were tested for effects of Elf-1/*grh* mutations on *en-lacZ* expression.

The strain containing the *Ddc-lacZ* fusion (*Ddc-lacZ.H10*) was one of a series of *Ddc-lacZ*-expressing strains, some of which have been described previously (Bray et al. 1988). The starting plasmid (DH43G) contained P-element transposon ends flanking an *hsp70/lacZ* fusion gene and an *Adh* gene (for a selectable marker). The *hsp70/lacZ* fusion contained 1167 bp of *hsp70* sequences from the *PstI* site (+89) in the 5'-untranslated leader sequence to the *BglII* site in the coding sequence (+1256). *PstI* linkers were ligated onto a fragment encompassing the *Ddc* promoter from a *BglII* site at -2200 to a *HpaI* site in the 5'-untranslated sequences (+174 bp), and this was cloned into the *PstI* site in the *hsp70* leader sequence. The fusion therefore occurred in the untranslated sequences downstream of the transcription start site and incorporated the start of translation from the *hsp70* gene. The host strain used for transformation was *Adh^{tn23} cn pr*, and methods were as described previously (Bray and Hirsh 1986). In the strain *Ddc-lacZ.H10* the P-element is inserted on the second chromosome (2L) close to *cn* (43E). This locus was recombined onto chromosomes carrying Elf-1/*grh* alleles (*dp cn grh bw*) by scoring for recombination between *dp* and *bw* with the chromosome *net dpp^{ho} dp Sp cn en² bw* as described above, and subsequently scoring for *grh* and the *lacZ* transposon on the recombinant chromosomes. In all cases, at least two different recombinant chromosomes were used to study the effects of each *grh* allele on *Ddc-lacZ* expression.

Histochemical detection of β -galactosidase activity was based on procedures described previously (Simon et al. 1985; Glaser et al. 1986). Activity was assayed in embryos at ~22–24 hr, that is, at about the time when many wild-type embryos were hatching. Those that had not hatched were dissected manually from the chorion and vitelline membrane and all embryos were slit longitudinally with tungsten needles before fixation. Fixed embryos were incubated in staining solution [0.2% 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside, 10 mM sodium phosphate (pH 7.2), 150 mM NaCl, 1 mM MgCl₂, 3.1 mM K₄[Fe(CN)₆], 3.1 mM K₃[Fe(CN)₆], 0.3% Triton X-100] for 30 min (*en-lacZ*) or overnight (*Ddc-lacZ*) at 37°C. The gut and other internal organs were then dissected away from the cuticle, leaving the epidermis and musculature attached. The cleaned cuticles were flattened under coverslips in Immumount (Shandon) and viewed on a Zeiss axiophot microscope by using bright-field optics and photographed with Kodacolor Gold film, ASA 100.

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