Interactions between *dorsal* and helix–loop–helix proteins initiate the differentiation of the embryonic mesoderm and neuroectoderm in *Drosophila*

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The *dorsal* (*dl*) morphogen has been implicated in the establishment of the embryonic mesoderm, neuroectoderm, and dorsal ectoderm in *Drosophila*. Here we show that the simultaneous reduction in the levels of *dl* and any one of several helix–loop–helix (HLH) proteins results in severe disruptions in the formation of mesoderm and neuroectoderm. Certain triple heterozygous combinations essentially lack mesoderm as a result of a block in ventral furrow formation during gastrulation. HLH proteins that have been implicated previously in sex determination and neurogenesis (*daughterless*, *achaete*, and *scute*) are shown to be required for the formation of these embryonic tissues. Evidence is also presented that *dl*–HLH interactions involve the direct physical association of these proteins in solution mediated by the *rel* and HLH domains. We discuss the striking parallels in mesoderm formation and sex determination.

[Key Words: *dorsal* morphogen; helix–loop–helix proteins; *Drosophila*; embryonic mesoderm; neuroectoderm]

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In *Drosophila*, the specification of dorsoventral polarity is linked to the establishment of diverse embryonic tissues. The ventral-most cells of the embryo form mesoderm, those in ventrolateral regions differentiate into the neuroectoderm, and cells in the dorsal half of the embryo form dorsal ectoderm. A cascade of maternally expressed genes establish a nuclear gradient of *dorsal* (*dl*) protein, which functions as a morphogen by regulating gene expression in a concentration-dependent manner (Roth et al. 1989; Jiang and Levine 1993). *dl* is a member of the *rel* family of transcription factors, which also includes the mammalian transcriptional activator NF- κ B and the chicken proto-oncogene *c-rel* (Steward 1987; Ip et al. 1991). Like the other members of the *rel* family, *dl* is regulated at the level of nuclear transport. The protein is initially distributed throughout the cytoplasm of unfertilized eggs and early embryos. Approximately 90 min after fertilization, *dl* protein in ventral and ventrolateral regions enters the nuclei, whereas protein in dorsal regions remains in the cytoplasm. This gradient in subcellular localization gives way to an absolute gradient, so that by the beginning of cellularization there are higher levels of *dl* in ventral versus dorsal regions (Roth et al. 1989; Rushlow et al. 1989; Steward 1989).

Peak levels of *dl* in ventral regions activate the mesoderm determinants *twist* (*twi*) (Thisse et al. 1988) and *snail* (*sna*) (Boulay et al. 1987). The promoters of both genes contain multiple, low-affinity *dl*-binding sites that

directly mediate expression in the early embryo (Jiang et al. 1991; Pan et al. 1991; Thisse et al. 1991; Ip et al. 1992a). *twi* is a member of the helix–loop–helix (HLH) family of transcription factors (Murre et al. 1989) and is required for the activation of downstream mesodermal genes (Leptin 1991). *sna* is a zinc finger protein that acts as a repressor and blocks the expression of neuroectodermal regulatory genes in the presumptive mesoderm, thereby restricting them to lateral regions (Kosman et al. 1991; Leptin 1991; Rao et al. 1991).

Low levels of *dl* in lateral regions activate the expression of *rhomboid* (*rho*), which encodes a putative transmembrane receptor required for the differentiation of a subset of the ventral epidermis that arises from the neuroectoderm (Bier et al. 1990). A 300-bp enhancer in the *rho* promoter is sufficient to direct lateral stripes of expression within the presumptive neuroectoderm. This enhancer contains high-affinity *dl*-binding sites and closely linked E-box sequences (HLH-binding sites) (Ip et al. 1992b). Mutations in either the *dl* sites or E boxes result in catastrophic reductions in *rho* promoter activity, suggesting that the two classes of transcription factors function multiplicatively to activate *rho* expression (see below). The *rho* enhancer also contains *sna*-binding sites, which are responsible for repressing its expression in the presumptive mesoderm where there are high levels of *dl* (Ip et al. 1992b). *sna* represses the expression of additional neuroectodermal regulatory genes, including

lethal of scute (T3) (Campuzano et al. 1985; Cabrera et al. 1987; Romani et al. 1987). It also restricts the expression of *single-minded* (*sim*) (Crews et al. 1988; Thomas et al. 1988) and the m7 gene of the Enhancer of split complex [E(spl)] (Knust et al. 1987). These observations prompted the proposal that *sna* plays a key role in establishing the boundary between embryonic mesoderm and neuroectoderm (Kosman et al. 1991; Leptin 1991; Rao et al. 1991).

dl-HLH interactions are not only important for the regulation of *rho* in the neuroectoderm, but they also participate in the establishment of the mesoderm. For example, *dl/+;twi/+* double heterozygotes display variable disruptions in mesoderm differentiation, similar to those observed in *twi* mutants (Simpson 1983). There is evidence that these disruptions arise from abnormal patterns of *twi* and *sna* expression (Kosman et al. 1991). A detailed molecular analysis revealed that *twi* binds to the *sna* promoter and functions multiplicatively with *dl*-binding sites to ensure strong, uniform expression of *sna* in the presumptive mesoderm (Ip et al. 1992a).

Here, we present evidence that *dl* interacts with additional members of the HLH family, including the maternal products of *daughterless* (*da*) (Caudy et al. 1988b; Cronmiller et al. 1988) and the *achaete-scute* complex (AS-C). Dosage-sensitive interactions between *dl*, *da*, AS-C, and *twi* are required for the specification of both the embryonic mesoderm and neuroectoderm. Various heterozygous combinations of these genes result in narrower limits of *twi* and *sna* expression. Reduced limits of *sna* cause ventral derepression of neuroectodermal regulatory genes normally restricted to lateral regions, including *rho*, T3, m7, and *sim*. Direct evidence that *dl*-HLH interactions are required for the activation of gene expression in the presumptive neuroectoderm was obtained by mutagenizing E boxes contained in the *rho* enhancer. Finally, evidence is presented that *dl*-HLH interactions involve direct physical associations mediated by the *rel* and the HLH domains of these two distinct classes of transcription regulators.

Results

dl-da interactions are required for mesoderm formation

Dosage-sensitive interactions between *dl* and *twi* were identified previously by analyzing *dl/+;twi/+* double heterozygotes (Simpson 1983; Kosman et al. 1991). These experiments involved mating *dl/+* females with *twi/+* males. Half of the embryos contained reduced levels of both proteins and showed severe disruptions in mesoderm formation. We decided to use this approach to determine whether *dl* interacts with any other members of the HLH family. Particular efforts centered on HLH genes known to be expressed in early embryos, during the time when *dl* initiates the dorsoventral pattern. *da* was tested first because it is maternally expressed and ubiquitously distributed in early embryos (Cronmiller and Cummings 1993).

All embryos derived from matings of *dl/+;da/+* females and normal males display abnormal patterns of *twi* and *sna* expression (Fig. 1), although the disruptions are somewhat variable from embryo to embryo. Figure 1, A–L, displays average distortions in the expression patterns. The expression limits of *twi* and *sna* are narrower than in wild type (Fig. 1, cf. E and K with B and H). Normally, both genes are expressed in the ventral-most 18–20 cells, but in the double heterozygotes the limits include just 10–12 cells. In addition, there are gaps in expression near the cephalic furrow (Fig. 1E,J,K), which persist during germ-band elongation (Fig. 1F,L). These gaps correlate with an expansion of the cephalic furrow, which is normally restricted to dorsolateral and lateral regions but extends into the presumptive mesoderm in double heterozygotes (Fig. 1F,L; data not shown). These alterations in the *twi* and *sna* patterns are similar to those observed in *twi/+* embryos derived from *dl/+* females (Kosman et al. 1991).

Dosage-sensitive interactions between *dl* and *da* depend solely on maternally expressed *da* products. Double heterozygotes obtained by mating *dl/+* females and *da/+* males are viable and display completely normal *twi* and *sna* expression patterns (data not shown).

dl interacts with AS-C in mesoderm determination

Certain HLH proteins encoded by the AS-C, such as T4, are also ubiquitously distributed in early embryos (Cabrera et al. 1987; Romani et al. 1987). These proteins have been shown to interact genetically with *da* (Dambly-Chaudière et al. 1988) and form heterodimers that are potent transcriptional activators (Cabrera and Alonso 1991; Van Doren et al. 1991). For these reasons, we examined the possibility that *dl* might interact with AS-C.

All embryos derived from matings of *Df(AS-C)/+;dl/+* females and normal males show the same defects as those observed when maternal *dl* and *da* products are reduced (Fig. 1). In particular, there is a narrowing in the *twi* and *sna* expression limits and a gap in the patterns near the presumptive cephalic furrow during cellularization that persists during germ band elongation (data not shown).

Triple heterozygotes show a virtual loss of mesoderm

To determine whether *dl*-HLH interactions are redundant or cumulative, we analyzed the *twi* and *sna* expression patterns in triple heterozygotes. *dl/+;da/+* or *Df(AS-C)/+;dl/+* females were mated with *twi/+* males. In these matings half of the resulting embryos contain reduced levels of *dl* and two HLH proteins. Both classes of triple heterozygotes show similar severe disruptions in *twi* and *sna* expression (Fig. 2). During cellularization there are gaps in expression along the anteroposterior axis (Fig. 2A,D), and the limits are dramatically narrower, with expression spanning just the ventral-most 3–10 cells (Fig. 2B,E). The gap in expression near the cephalic furrow is greatly expanded as compared

Figure 1. *twi* and *sna* expression in double heterozygotes for *dl* and *da*. Embryos were obtained by mating double-heterozygous females with Canton-S males. The embryos are oriented with anterior to the left. Expression patterns were visualized after in situ hybridization using digoxigenin-UTP-labeled RNA probes. (A–C) *twi* expression in wild-type (Canton-S) embryos. During cellularization, *twi* is expressed in a ventral band of 18–20 cells (B, ventral view) that extends throughout the poles (A, lateral view). This domain of expression represents the presumptive mesoderm and mesectoderm. *twi* expression is sustained in the mesoderm during germ band elongation (C, lateral view). (D–F) *twi* expression in embryos derived from *dl*⁸; +/+; *da*^{IIB31} females. At cellularization, the *twi* pattern is narrower than in wild type and spans only 12–14 cells (E, ventral view). In addition, there is a gap of expression in the vicinity of the presumptive cephalic furrow. Staining is detected in the invaginated mesoderm during germ band elongation (F). Identical results were obtained with a different *dl* mutation (*dl*⁶; data not shown). (G–I) *sna* expression patterns in wild-type embryos. During cellularization the *sna* pattern spans the ventral-most 18 cells (H, ventral view) but is repressed at the poles (G, lateral view). There is transient expression of *sna* in the invaginated mesoderm (I, lateral view). (J–L) *sna* expression in embryos derived from *dl*⁸; +/+; *da*^{IIB31} females. During cellularization, *sna* expression is restricted to a ventral band of just 8–10 cells (K, ventral view). Moreover, there is a gap near the presumptive cephalic furrow (J, lateral view). This gap is sustained during germ band elongation (L, lateral view). Identical results were obtained in similar experiments using the null allele *dl*⁶ (data not shown).

with double heterozygotes (Fig. 2B and E; cf. with Fig. 1E and K). During germ band elongation there is nearly a complete loss of mesoderm invagination, such that internal *twi* and *sna* expression is restricted to posterior regions near the posterior midgut (PMG) (Fig. 2C,F). The ventral furrow fails to form in the triple heterozygotes (Fig. 3A,B), and the posterior patches of invaginated cells appear to arise through a process of ingression during germ band elongation (see Discussion).

These disruptions are not observed in embryos con-

taining reduced doses of various HLH genes. For example, double heterozygotes for *da* and *twi* or AS-C and *twi*, as well as triple heterozygotes for *da*, AS-C, and *twi* show completely normal patterns of *twi* and *sna* expression (data not shown).

Neuroectoderm gene expression in *dl*/HLH heterozygotes

Previous studies suggest that *dl*–HLH interactions are

Figure 2. *twi* and *sna* expression in triple heterozygotes for *dl*, *da*, *twi* and *dl*, AS-C, *twi*. Double heterozygotes for *dl*, *da* or *dl*, AS-C females were mated with *twi*^{+/+} males, and the resulting embryos were hybridized with *twi* and *sna* RNA probes. (A–C) *twi* expression in triple heterozygotes derived from a cross involving *Df(1)260-1* (AS-C)/+; *dl*⁸/+ females and *twi*^{ID96}/+ males. By cellularization, the *twi* expression pattern includes a discontinuous band of just seven to eight cells. The most severe loss of expression occurs in the region of the presumptive cephalic furrow (A, lateral view; B, ventral view). These embryos lack a ventral furrow, and there is a near loss of invaginated mesoderm during germ band elongation (C, lateral view). Identical results were obtained with the *dl*⁶ and *sc*¹⁰⁻¹ alleles. In addition, a similar pattern was observed in *dl*⁸, *da*^{11B31}, *twi*^{ID96} triple heterozygotes (data not shown). (D–F) *sna* expression in triple heterozygotes for *dl*⁸, *da*^{11B31}, and *twi*^{ID96}. By cellularization, *sna* expression is restricted to discontinuous patches of just three to eight cells in the ventral-most regions (D, lateral view; E, ventral view). This pattern is sustained during germ-band elongation (F, lateral view), although there is virtually no invaginated mesoderm. A similar defective *sna* pattern was observed in triple heterozygotes for *dl*⁸, *Df(1)260-1* (AS-C), and *twi*^{ID96} (data not shown).

important for the regulation of neuroectodermal genes in lateral regions of cellularizing and gastrulating embryos (Ip et al. 1992b). We have investigated this issue further by examining the expression of various neuroectodermal regulatory genes in *dl*/HLH heterozygotes. Included in this study are three genes involved in neuronal differentiation; the m7 gene of the E(spl) complex, the T3 gene of AS-C, and *sim*. In addition, *rho* expression was examined as it is one of the earliest markers for the neuroectoderm (Bier et al. 1990).

Each of these genes is expressed in ventrolateral regions that normally invaginate during gastrulation in embryos derived from *dl*^{+/+}; *da*^{+/+} or *Df(AS-C)*^{+/+}; *dl*^{+/+} females (Fig. 4, cf. B, E, H, and K with A, D, G, and J). Ventral derepression is particularly severe in the presumptive cephalic furrow where disruptions in the *twi* and *sna* patterns are most extreme (i.e., Fig. 1E,K). Triple heterozygotes [*twi*^{+/+} embryos derived from either *dl*^{+/+}; *da*^{+/+} or *Df(AS-C)*^{+/+}; *dl*^{+/+} females] show severe ventral shifts of the *sim*, m7, and T3 patterns, with expression extending into the ventral-most regions of the presumptive mesoderm (Fig. 4C, F, L). These shifts in expression correlate with the progressive narrowing of the *sna* pattern (i.e., Fig. 2E), which is probably responsible for excluding *sim* and m7 from the ventral-most regions. This correlation is particularly striking in triple heterozygotes, where the only ventral regions lacking *rho* and T3 coincide with residual patches of *sna* expression (Fig. 4I, L; see Fig. 2E).

dl-HLH interactions appear to be important for activating *sim*, m7, and T3 expression in lateral regions

where there are low, limiting amounts of *dl*. Double and triple heterozygotes show a shift in expression, indicating that reduced levels of *dl* and HLH proteins are unable to define the normal dorsal limits of expression. For example, T3 is normally expressed in a series of “hemistripes” that include an average of four to six cells (i.e., Fig. 4J). Simple derepression of T3 into the presumptive mesoderm should result in T3 stripes that encompass ~28 cells in ventral and ventrolateral regions. However, these stripes include only 20–22 cells (Fig. 4L), indicating that the dorsal limits of the T3 pattern have shifted ventrally. Activation of *rho* does not appear to be sensitive to reductions in *dl* and HLH products. The dorsal limits of the *rho* pattern are essentially normal in triple heterozygotes, so that the expanded pattern encompasses nearly the entire ventral half of the embryo (~36 cells; Fig. 4I). This corresponds to the normal lateral stripes of *rho* expression in the neuroectoderm (each stripe is eight to nine cells in width) plus the presumptive mesoderm. It is conceivable that *rho* is buffered against changes in the levels of *dl* and HLH proteins because its promoter contains numerous *dl*-binding sites that are closely linked to several classes of E-box sequences (see below).

Role of E boxes in neuroectoderm gene expression

Additional evidence that *dl*-HLH interactions are important for neuroectoderm gene expression was obtained by analyzing the expression of the *rho* NEE, a 600-bp enhancer located between –2.2 and –1.6 kb upstream from the transcription start site. The NEE directs lateral

Figure 3. Triple heterozygotes lack a ventral furrow. (A) *sna* expression in a wild-type gastrulating embryo (ventral view). The 18 ventral-most cells invaginate through the ventral furrow. The cephalic furrow (arrowheads) does not extend through the presumptive mesoderm. (B) *sna* expression in a triple heterozygote for *dl*⁸, *da*^{l1B31}, *twi*^{lD96}. This embryo is about the same age as the one shown in A. Staining is restricted to terminal regions and a patch in the presumptive abdomen. There is no ventral furrow, and the cephalic furrow (arrowheads) extends through the presumptive mesoderm. The same result was obtained in triple heterozygotes for *dl*⁸, *Df(1)260-1* (AS-C), *twi*^{lD96}.

stripes of expression when attached to a *lacZ* reporter gene (Fig. 5A; Ip et al. 1992b). It contains four *dl*-binding sites that mediate activation in ventral and ventrolateral regions and four *sna*-binding sites that are responsible for repression in ventral regions. In addition, the NEE contains several different E boxes.

The dorsal limits of NEE expression are essentially normal in *dl*/+; *da*/+ and *Df(AS-C)*/+; *dl*/+ double heterozygotes (Fig. 5B). The slight ventral derepression of the pattern probably results from disruptions in the *sna* repressor. Perhaps the NEE is buffered against reductions in *dl* and HLH expression because of functionally redundant E boxes.

To determine whether divergent HLH proteins might function redundantly to regulate the lateral expression of the NEE, we have mutagenized the E1, E2, E3, and E4 E boxes. Previous studies have shown that mutations in the E4 and E5 E boxes reduce, but do not abolish, NEE activity (Ip et al. 1992b). A mutagenized NEE lacking all four E boxes is completely inactive in embryos undergoing cellularization (data not shown). However, like the wild-type NEE, it is activated in the ventral-most regions of precellular embryos (Fig. 5C), prior to the time when the *sna* repressor is first detected. Evidence that *dl* is

sufficient for activation of the NEE in the presumptive mesoderm was obtained by analyzing the expression of the mutagenized NEE in *sna* mutants (Fig. 5D). Expression is restored in ventral, but not lateral, regions of cellularizing embryos; this corresponds to the time when the same enhancer is inactive in normal embryos. These results suggest that *dl*-HLH interactions are required to direct expression in lateral regions where there are low levels of *dl*. High levels of *dl* are sufficient for activation in ventral regions, but this is obscured in wild-type embryos by the *sna* repressor.

The *dl* protein associates with HLH proteins in vitro

To understand the molecular nature of *dl*-HLH interactions, we determined whether the two classes of proteins can form heteromeric complexes. Radiolabeled full-length *twi*, *da*, and T4 proteins were synthesized using a cell-free transcription-translation system (Fig. 6A) and incubated with an unlabeled, bacterially expressed glutathione *S*-transferase (GST)-*dl* fusion protein. The GST-*dl* protein contains the amino-terminal 378 amino acid residues of the *dl* protein, which includes the entire *rel* domain. After immunoprecipitation with anti-*dl* antiserum, the immune complexes were analyzed by denaturing polyacrylamide gel electrophoresis. Labeled *twi* and T4, but not *da*, were recovered by immunoprecipitation with anti-*dl* antiserum only in the presence of the GST-*dl* fusion protein (Fig. 6B). The same results were obtained with two different anti-*dl* antisera and in the presence of ethidium bromide to avoid nonspecific binding resulting from contaminating DNA (Lai and Herr 1992). These results suggest that *dl* can form an activation complex with some, but not all, HLH proteins.

Because *da* is unable to interact in solution with *dl*, we decided to substitute its HLH domain with the one from T4, which can associate with *dl*. We constructed a chimeric *da* protein where the carboxy-terminal region (amino acids 553–710) containing the HLH domain was replaced by the corresponding carboxy-terminal region (amino acids 100–345) of T4. This chimeric *da*-T4 protein, containing the T4 HLH domain, now interacts with *dl* in the coimmunoprecipitation assay (Fig. 6C). These results suggest that selective interactions between *dl* and certain HLH proteins are mediated by the *rel* and HLH domains.

Discussion

The results presented in this study suggest that the establishment of the embryonic mesoderm and neuroectoderm depends on dosage-sensitive interactions between *dl* and a number of divergent HLH proteins. Some of these latter proteins have been implicated previously in sex determination and neurogenesis; this study provides the first evidence that HLH proteins such as *da* are also required for the specification of mesoderm. Triple heterozygotes containing reduced levels of *dl*, *da*, and *twi* (or *dl*, AS-C, and *twi*) proteins essentially fail to form a ventral furrow and are almost completely devoid of in-

Figure 4. *sim*, *m7*, *rho*, and T3 expression in double and triple heterozygotes. (A–C) *sim* expression. The wild-type pattern includes two one-cell-wide ventrolateral stripes along the A–P axis (A, ventral view). These stripes coincide with the presumptive mesectoderm and are separated by the presumptive mesoderm. The *sim* stripes shift ventrally and are closer together in double heterozygotes for *dl*⁸ and *da*^{IIB31} (B, ventral view). In addition, there are discontinuities in the stripes. *sim* expression is lost in anterior regions of triple heterozygotes for *dl*⁸, *da*^{IIB31}, and *twi*^{ID96}, and the remaining stripes are discontinuous and shifted to ventral regions (C, ventral view). (D–F) *m7* expression. After cellularization, the wild-type pattern includes two one-cell-wide ventrolateral stripes that are virtually identical to the *sim* pattern (D, ventral view). These stripes are shifted ventrally in *dl* and *da* double heterozygotes (E, ventral view). In addition, the *m7* pattern is joined in the vicinity of the presumptive cephalic furrow. The stripes include three to four cells in this embryo because it is younger (precellular) than the one in D. Initially, the *m7* stripes are broad, but they are refined during cellularization. There is a severe ventral shift of the pattern in *dl*, *da*, and *twi* triple heterozygotes (F, ventral view). In addition, expression is lost in anterior regions. (G–I) *rho* expression. The wild-type pattern includes two ventrolateral stripes that are 8–10 cells wide and span most of the presumptive neuroectoderm (G, ventral view). These lateral stripes are expanded slightly toward more ventral regions in *dl* and *da* double heterozygotes (H, ventral view). The expression pattern is nearly continuous in the region of the cephalic furrow. There is a severe ventral derepression of the pattern in *dl*, *da*, and *twi* triple heterozygotes (I, ventrolateral view). (J–L) T3 expression. The wild-type T3 pattern includes two discontinuous lateral stripes in the ventral half of the presumptive neuroectoderm (J, ventral view). In *dl* and *da* double heterozygotes, these lateral stripes are shifted ventrally and the pattern is continuous in the vicinity of the cephalic furrow (K, ventrolateral view). In *dl*, *da*, and *twi* triple heterozygotes there is a severe ventral derepression of the pattern (L, ventral view).

vaginated mesoderm. Biochemical studies suggest that the interactions between *dl* and HLH proteins appear to be mediated by the conserved *rel* and HLH domains, respectively.

The role of *dl*–HLH interactions in development

The *sna* expression pattern coincides with the presumptive mesoderm, and the sharp lateral borders of the pattern help to define the boundary between mesoderm and neuroectoderm (Boulay et al. 1987; Kosman et al. 1991;

Leptin 1991; Rao et al. 1991). It has been proposed that *dl*–*twi* interactions permit both high levels of *dl* in ventral regions and intermediate levels of *dl* in ventrolateral regions to activate *sna* to the same extent. According to this view, the shallow *dl* gradient triggers a somewhat steeper *twi* gradient, and these function multiplicatively to define the sharp on/off borders of *sna* expression (Ip et al. 1992a).

Interactions between *dl*, *da*, and AS-C are also important for specifying the presumptive mesoderm. The defective patterns of *twi* and *sna* expression observed in

Figure 5. E boxes are required for neuroectoderm expression of the *rho* promoter. *rho-lacZ* fusion genes were expressed in different genetic backgrounds. The expression patterns were visualized by in situ hybridization using a digoxigenin-UTP-labeled *lacZ* antisense RNA probe. The organization of the 600-bp NEE is indicated below each embryo, including the location of relevant E boxes as well as *dl*- and *sna*-binding sites. (A) The *rho* NEE pattern in a wild-type embryo (ventral view). Expression is restricted to lateral stripes in the presumptive neuroectoderm, similar to the endogenous pattern (cf. with Fig. 4G). (B) NEE expression in a *dl*⁸, *da*^{IIB31} double heterozygote. There is a derepression of the pattern in ventral regions, similar to the endogenous *rho* pattern observed in this genetic background (cf. with 4H). (C) Expression of a mutagenized NEE containing knockout mutations in the E1, E2, E3, and E4 E boxes. In this wild-type precellular embryo (lateral view) there is weak expression in ventral regions, similar to the initial pattern of the endogenous *rho* gene. However, during cellularization, expression is completely lost, indicating the importance of the E boxes for expression in the neuroectoderm. (D) Mutagenized NEE (same as C) in a cellularized *sna*[−] embryo (lateral view). There is strong expression in the presumptive mesoderm, indicating that the *dl*-binding sites are sufficient to mediate expression in ventral regions. The same NEE is inactive in comparable wild-type embryos (data not shown).

double heterozygotes for *dl* and either *da* or AS-C are similar to those seen in double heterozygotes for *dl*, *twi* (Fig. 1; Kosman et al. 1991). *dl-da* and *dl-AS-C* interactions may be distinct from the *dl-twi* interaction in that both *da* and AS-C products are homogeneously distributed throughout the early embryo. Thus, it would appear that the shallow *dl* gradient can establish sharp patterns of target gene expression by interacting with either localized or ubiquitous HLH proteins. However, the pre-

cise on/off borders of the *sna* pattern might rely on specific *dl-twi* interactions; reduced levels of *da* and/or T4 cause a narrowing of the *twi* pattern and perhaps a concomitant change in *sna* (see Fig. 1).

dl-HLH interactions are also important for the specification of the neuroectoderm. The dorsal limits of *sim*, *m7*, and *T3* expression are shifted ventrally in double and triple heterozygotes (see Fig. 4). These results suggest that *dl*-HLH interactions are required for the low

Figure 6. *dl* forms complexes with *twi* and T4 but not *da*. (A) ³⁵S-Labeled *dl*, *twi*, *da*, and T4 proteins synthesized in a reticulocyte extract. The proteins were fractionated on an SDS-polyacrylamide gel. Molecular mass markers are indicated at left. (B) Immunoprecipitation assays. *dl*, *twi*, *da*, and T4 proteins were incubated with an unlabeled GST-*dl* fusion protein and then immunoprecipitated with an anti-*dl* antiserum. Precipitated complexes were fractionated on an SDS-polyacrylamide gel and visualized by autoradiography. (+) Proteins present in each mixture (top); (*) radiolabeled in vitro synthesized proteins. For example, lane 3 shows the immunoprecipitation complex obtained when the GST-*dl* fusion protein was incubated with labeled *twi*. Lane 2 shows the same experiment, except that the GST-*dl* fusion protein was not added to the mixture. (C) Immunoprecipitation assays using a chimeric *da* protein. A similar experiment as described in B was performed using a chimeric *da* protein (referred to as *da*-T4) where its carboxy-terminal region containing the HLH domain was replaced by the carboxy-terminal region of T4 containing the T4 HLH domain.

levels of *dl* in ventrolateral and lateral regions of the early embryo to activate target genes in the presumptive neuroectoderm. However, *rho* expression is relatively insensitive to dose, possibly owing to the close linkage and large number of *dl*- and HLH-binding sites in the *rho* NEE.

A surprising finding of this study is that HLH proteins implicated previously in sex determination and neurogenesis are also essential for the determination of the mesoderm and neuroectoderm. *da* is a critical component of sex determination. Maternally encoded *da* products interact with both *sis-a* (which encodes a leucine zipper protein) and *sis-b* (the product of the T4 gene) to activate *Sex lethal* (*Sxl*) expression in precellular embryos (Cline 1989). In vitro binding assays suggest that *da* and T4 form heterodimers (Cabrera and Alonso 1991; Van Doren et al. 1991); perhaps these bind to the *Sxl* promoter and activate its transcription by interacting with *sis-a* protein bound to neighboring sites. Like *dl*-HLH interactions, the *da*/T4-*sis-a* interaction is dosage sensitive. A twofold difference in the levels of *sis-a* and T4 determines whether *Sxl* is on (females) or off (males). Similarly, a twofold reduction in the levels of *dl*, *da*, *twi* or *dl*, T4 (and/or T5), and *twi* result in a near on/off switch in *sna* expression (see Fig. 3). It is conceivable that the *sis-a*-*da*/T4 interaction is similar to *dl*-HLH interactions and involves direct protein associations (Fig. 6) and cooperative binding to DNA (Jiang and Levine 1993).

Mechanism of *dl*-HLH interactions

There are two types of models that could account for

dosage-sensitive interactions between *dl* and HLH proteins: cooperative binding to DNA or transcriptional synergism. According to the latter model, *dl* and HLH proteins bind independently of one another but separately contact different rate-limiting components of the transcription complex. We favor the view that cooperative binding is the prime determinant of *dl*-HLH interactions because gel-retardation assays have demonstrated cooperative binding of *dl* and T4 to neighboring sites on a short DNA fragment (Jiang and Levine 1993), and the present study indicates direct physical associations of the proteins.

The results of the in vitro binding assays (Fig. 6) suggest that *dl* associates directly with a broad spectrum of HLH proteins. These complexes could bind to the promoters of target genes and activate transcription in a cooperative manner. For example, the *sna* promoter might be activated by a combination of *dl*-*twi* and *dl*-T4 complexes. However, a potential limitation of this model is that dosage-sensitive interactions are also observed for *dl* and *da*, yet these proteins fail to interact in coprecipitation assays. Additional DNA-binding assays offer a potential explanation for this observation (J. Jiang and M. Levine, unpubl.). A mixture of *da* and T4 proteins greatly increases cooperative binding of *dl* to neighboring sites, although *da* alone has no effect on *dl* binding. This increase in *dl* cooperativity is the result of the enhanced binding activity of T4, which binds quite poorly as a homodimer but binds well as a heterodimer (Cabrera and Alonso 1991; Van Doren et al. 1991). Thus, it would appear that *da* interacts with *dl* indirectly by facilitating the binding of HLH proteins (e.g., *twi* and T4) that can directly contact *dl*.

Evolutionary conservation of *rel*-HLH interactions

The interactions that we have described for *dl*, a member of the *rel* family of transcription factors, and HLH proteins might be evolutionarily conserved. Coprecipitation of the *twi* and T4 proteins was obtained with a truncated *dl* protein that contains little more than the conserved *rel* domain. Moreover, *twi* and T4 were found to specifically associate with *dl*, and both proteins are virtually unrelated except for the conserved HLH domain. These observations suggest that conserved sequences in *dl* and HLH proteins are responsible for the interaction. If so, this interaction might apply to other systems involving members of the *rel* and HLH families such as the mammalian immunoglobulin κ -light-chain enhancer (Picard and Schaffner 1984) that contains closely linked NF- κ B- and HLH-binding sites.

Materials and methods

Fly stocks

The following fly stocks were used in this study. Wild type: Canton-S strain. *dl* mutants: *dl*⁸ and *dl*⁶ are strong alleles that result in the loss of detectable protein (Roth et al. 1989). *da*: *da*^{11B31} is a null allele (Caudy et al. 1988a). AS-C: *Df(1)260-1* is a deficiency that spans the entire AS-C (Dambly-Chaudière et al. 1988). *sc*¹⁰⁻¹ disrupts the T4 and T5 genes in the AS-C (Villares and Cabrera 1987). *twi*: *twi*^{1D96} causes the loss of detectable protein (Simpson 1983). *sna*: *sna*^{11G05} is a strong allele (Simpson 1983).

All matings and embryo collections were performed at 25°C.

Whole-mount in situ hybridization

Expression patterns were visualized in whole-mount preparations of embryos by in situ hybridization using antisense RNA probes labeled with digoxigenin-UTP and antidigoxigenin antibodies conjugated to alkaline phosphatase (Tautz and Pfeifle 1989; Jiang et al. 1991; Kosman et al. 1991). The stained embryos were photographed using Nomarski optics.

Site-directed mutagenesis

Point mutations were created in the E1, E2, E3, and E4 E boxes in the *rho* NEE using site-directed mutagenesis, as described previously (Ip et al. 1992a). The following mutagenic oligonucleotides were used: E1, AAGCTTCAGC (the normal sequence is AACATTTGGC); E2, GAGCAGAACG (the normal sequence is GACAAGTGGC); E3, CGCCAAGCTT (the normal sequence is CGCATATGTT); E4, ACGCGTTGTT (the normal sequence is AGCACATGTT).

The E-box consensus sequence is underlined. The mutations in the E3 and E4 sites are the same as described by Ip et al. (1992b; formerly called t1 and t2).

P-element transformation

The mutagenized NEE was cloned into the P-element vector pWHL upstream of the *hsp70* basal promoter (Driever et al. 1989). This plasmid (together with the Δ 2,3 transposase helper plasmid) was injected in *w*⁶⁷ embryos. Three independent germline transformed strains were analyzed.

Expression of bacterial GST-dl fusion protein

Bacterial *dl* protein was prepared as a fusion with GST using the pGEX KG expression plasmid and the *Escherichia coli* strain HB101 as described by Smith and Johnson (1988), Guan and Dixon (1991), and Ip et al. (1992a). The GST-*dl* fusion protein contains the amino-terminal 378-amino-acid residues of *dl*, which span the entire *rel* domain. The GST-*dl* protein that was used retains normal binding activity based on DNase I footprint assays (data not shown).

In vitro transcription and translation

In vitro-translated proteins were prepared from plasmids containing the entire *dl*-, *twi*-, *da*-, and T4-coding sequences under the control of either the T7 (pAR) or SP6 (pBS) promoter. Linearized plasmids (1 μ g) were used as templates for in vitro transcription reactions, which were done at 37°C for 1 hr with 50 units of T7 or SP6 RNA polymerase in 40 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 0.1 mg/ml of BSA, 0.5 mM each ATP, CTP, UTP, 0.05 mM GTP, 0.5 mM GpppG, and 80 units of RNase inhibitor in a final volume of 50 μ l. The reaction products were extracted with phenol/chloroform, ethanol precipitated, and analyzed by agarose gel electrophoresis. Typically, this procedure generated ~1 μ g of RNA.

These RNAs (0.5 μ g each) were translated at 30°C for 1 hr in a rabbit reticulocyte lysate (35 μ l) in the presence of 0.02 mM amino acid mixture (minus methionine), 4 μ l of [³⁵S]methionine (1200 Ci/mmol, 10 mCi/ml), and 40 units of RNase inhibitor in a final volume of 50 μ l. The reaction products were analyzed after fractionation in SDS-polyacrylamide gels.

Coimmunoprecipitation assays

Aliquots of the GST-*dl* protein (100–300 ng) were mixed with ~50,000 cpm of ³⁵S-labeled protein prepared by in vitro translation. The mixtures were incubated at room temperature for 30 min in 50 μ l of NET-gel buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA at pH 8.0, 0.25% gelatin); some of the experiments involved the addition of ethidium bromide, as described by Lai and Herr (1992). The mixture was then treated with protein A-agarose (30 μ l) in a final volume of 230 μ l of NET-gel buffer at 4°C for 1 hr. The supernatant was incubated with a preabsorbed anti-*dl* antiserum (5 μ l) at 4°C for 12 hr. Afterwards, protein A-agarose was added (40 μ l) and incubated at 4°C for 2 hr. After centrifugation, the pellet was washed twice with NET-gel buffer, twice with RIPA buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS), and once in 10 mM Tris-HCl (pH 7.5) and 0.1% NP-40. Each wash was performed with 1 ml of buffer for 15 min at 4°C. The final pellet was resuspended in 20 μ l of SDS gel-loading buffer (50 mM Tris-HCl at pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol), boiled for 3 min, and analyzed by autoradiography after fractionating on SDS-polyacrylamide gels.

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