dorsal–twist interactions establish *snail* expression in the presumptive mesoderm of the *Drosophila* embryo

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The first step in the differentiation of the *Drosophila* mesoderm is the activation of two regulatory genes, *twist* (*twi*) and *snail* (*sna*), in ventral regions of early embryos. *sna* is a transcriptional repressor that is uniformly expressed throughout the presumptive mesoderm. Its sharp lateral limits help to establish the boundary between the mesoderm and neuroectoderm. Genetic studies suggest that *sna* is a target of the *dorsal* (*dl*) morphogen, and this interaction provides a model for determining how a morphogen gradient establishes a sharp, on/off threshold response. We present evidence that *dl* and *twi* directly activate *sna* expression. Site-directed mutagenesis of *dl*- and *twi*-binding sites within defined regions of the *sna* promoter suggest that the two proteins (containing the Rel and helix–loop–helix domains, respectively) function multiplicatively to ensure strong, uniform expression of *sna*, particularly in ventral–lateral regions where there are diminishing amounts of *dl*. These results are consistent with the possibility that the sharp *sna* borders are formed by multiplying the shallow *dl* gradient and the steeper *twi* gradient.

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A cascade of maternally active genes results in the regulated nuclear transport of the *dorsal* (*dl*) morphogen in the early *Drosophila* embryo (Roth et al. 1989; Rushlow et al. 1989; Steward 1989; for review, see St. Johnston and Nüsslein-Volhard 1992). The *dl* protein is initially distributed throughout the cytoplasm of unfertilized eggs and early embryos, but protein present in ventral regions enters the nuclei ~90 min after fertilization. An as yet uncharacterized cue in ventral regions of the perivitelline fluid stimulates the *Toll* receptor which, in turn, results in nuclear transport (Hashimoto et al. 1988; Stein et al. 1991). This local activation of *Toll* triggers a subcellular gradient of *dl*, such that protein in ventral regions is predominantly nuclear, while it is mainly cytoplasmic in dorsal regions. The protein is equally partitioned between cytoplasm and nuclei in lateral regions. This gradient in nuclear transport gives rise to an absolute gradient by cellularization, such that there are overall higher levels of the protein in ventral versus dorsal regions. In this report we consider how the *dl* concentration gradient establishes different territories of gene expression along the dorsal–ventral axis.

dl functions as a sequence-specific DNA-binding protein that directly regulates the transcription of zygotic target genes (for review, see Govind and Steward 1991; Ip and Levine 1992). The two best characterized targets are *zerknüllt* (*zen*) and *twist* (*twi*). *zen* encodes a homeo box protein that is required for the differentiation of the am-

nioserosa (Doyle et al. 1986; Rushlow et al. 1987), an embryonic tissue that arises from the dorsal ectoderm. *zen* is initially expressed in the dorsal half of the embryo but is derepressed throughout ventral regions of mutant embryos derived from *dl*[−] mothers. The *cis*-regulatory element that keeps *zen* off in ventral regions maps ~1.3 kb upstream from the transcription start site (Doyle et al. 1989). This ventral repression element contains four *dl*-binding sites, and mutations in these sites cause ventral expression, similar to the derepression of the pattern observed in *dl*[−] mutants (Ip et al. 1991; Jiang et al. 1992). Thus, *dl* appears to directly repress *zen* transcription, and there is genetic evidence that it may also repress other target genes, including *decapentaplegic* (*dpp*) and *tolloid* (*tld*) (St. Johnston and Gelbart 1987; Ray et al. 1991; Shimell et al. 1991).

Although both high levels of *dl* in ventral regions and low levels in lateral regions repress *zen*, only high levels are sufficient to activate *twi*. Recent studies have identified a number of *dl*-binding sites in the *twi* promoter, and at least several of these are required for activation (Jiang et al. 1991; Pan et al. 1991; Thisse et al. 1991). The affinities of these binding sites are at least fivefold lower than those present in the *zen* promoter (Jiang et al. 1991; Thisse et al. 1991), which suggests that binding affinities play an important role in establishing the dorsal–ventral limits of target gene expression. The high-affinity sites in the *zen* promoter permit repression in lateral regions

where there are low levels of *dl*, whereas the low-affinity sites in *twi* restrict expression to ventral regions where there are high levels. It is not known how *dl* functions as both a repressor of *zen* and an activator of *twi*, although there is evidence that *dl* might interact with different neighboring factors in the *zen* and *twi* promoters (Doyle et al. 1989; Pan et al. 1991).

twi acts in concert with a second regulatory gene, *snail* (*sna*), to initiate the differentiation of the mesoderm (Boulay et al. 1987; Thisse et al. 1987; Leptin and Grunewald 1990). Mutations in either gene cause a similar failure of ventral furrow formation and loss of mesoderm. *twi* and *sna* are expressed in overlapping but noncoincident patterns in the early embryo (Alberga et al. 1991; Kosman et al. 1991; Leptin 1991). *twi* is expressed in a graded fashion, with peak levels present in ventral regions and progressively lower levels in ventral-lateral regions. Expression extends into the presumptive mesoderm and includes at least the ventral-most cells of the neuroectoderm. In contrast, *sna* expression abruptly ends at the lateral limits of the presumptive mesoderm. Double-staining studies indicate that these sharp *sna* borders coincide with the boundary between the mesoderm and neuroectoderm (Kosman et al. 1991). Another difference between the *sna* and *twi* patterns is that *sna* is not graded but, instead, is uniformly expressed throughout the presumptive mesoderm. The establishment of the sharp *sna* pattern represents a critical early step in the differentiation of the embryonic mesoderm.

Genetic studies suggest that *twi* and *sna* play different roles in mesoderm formation (Kosman et al. 1991; Leptin 1991). *twi* encodes a basic helix-loop-helix protein that appears to function primarily as a transcriptional activator (Thisse et al. 1988; Murre et al. 1989), and in *twi*⁻ mutants there is a failure to activate genes required for mesoderm differentiation, such as the *Drosophila* homolog of MyoD (*nautilus*) (Michelson et al. 1990). In contrast, *sna* encodes a zinc finger protein (Boulay et al. 1987) that functions mainly as a repressor. *sna* restricts the expression of a number of mesodermal and neuroectodermal regulatory genes to lateral regions of the early embryo. These include *lethal of scute* (*T3*), *rhomboid* (*rho*), *single-minded* (*sim*), and genes of the *Enhancer of split* complex (Nambu et al. 1990; Kosman et al. 1991; Rao et al. 1991). In *sna*⁻ mutants these genes are expressed in both lateral and ventral regions. Recent evidence suggests that the sharp lateral limits of *sna* expression define the mesoderm/neuroectoderm boundary by directly repressing one or more of these genes (Kasai et al. 1992). Disruptions in *sna*-binding sites present in the *rho* promoter cause otherwise normal *rho-lacZ* fusion genes to be expressed in both lateral and ventral regions (Ip et al. 1992). Thus, an early step in mesoderm formation is the activation of *sna*, which functions to exclude an alternative pathway of differentiation (mesoderm/neuroectoderm).

Previous genetic studies have implicated *dl* and *twi* as the key activators of *sna* expression. *sna* is not initiated in *dl*⁻ mutants, and there is a severe reduction in the

levels of *sna* expression in *twi*⁻ mutants (Kosman et al. 1991; Ray et al. 1991). *dl* and *twi* function in a dosage-sensitive manner to activate *sna*, because there is a narrowing of the *sna* pattern in embryos that contain half the amount of *dl* and *twi* proteins (*dl*⁺/*twi*⁺ double heterozygotes) (Kosman et al. 1991). The simplest model for *sna* regulation is that the *dl* gradient is the primary activator of both *twi* and *sna*. *twi* then acts in concert with *dl* to drive optimal expression of *sna*.

In an effort to determine how *dl* and *twi* regulate *sna* we have performed a detailed analysis of its promoter. We present evidence that *dl* and *twi* directly activate *sna* transcription. Activation by *dl* depends on a distal promoter element, located between -1.9 kb and -900 bp, which contains a total of 10 *dl*-binding sites. The sequential removal of these *dl* sites causes a progressive narrowing and eventual loss of the pattern. *twi* binds to a proximal-promoter element located between -250 and -50 bp and augments *sna* expression. This regulation by *twi* is important for high levels of *sna* expression in ventral-lateral regions where there are diminishing amounts of *dl*. Site-directed mutagenesis of the *dl*- and *twi*-binding sites suggests that the two proteins function multiplicatively to ensure strong, uniform levels of *sna* expression throughout the presumptive mesoderm.

Results

Genetic regulation of the early *sna* pattern

In situ hybridization assays suggest that *twi* and *sna* are activated at about the same time during embryogenesis, by the onset of nuclear cycle 13 (Thisse et al. 1988; Alberga et al. 1991; Kosman et al. 1991; Leptin 1991). *sna* RNAs are initially expressed at low levels in the ventral-most 12–14 nuclei (Fig. 1A). The *sna* pattern becomes quite intense by the onset of nuclear cycle 14 and includes 18 nuclei, but the lateral limits are still fuzzy (Fig. 1B). By mid-cycle 14, the pattern becomes strong and uniform throughout the presumptive mesoderm and exhibits the characteristic sharp boundary (Fig. 1C, arrow). Previous studies have shown that this mature pattern fails to form in *twi*⁻ embryos (Kosman et al. 1991; Ray et al. 1991). An altered *sna* pattern can be seen by mid-cycle 14 (Fig. 1F; cf. with C). There is a failure to activate expression in ventral-lateral regions, and the overall levels of staining remain low. Only the ventral-most 12–14 cells are stained, and the lateral borders remain fuzzy (Fig. 1F, arrow).

The *dl* morphogen has been implicated as an activator of *sna* because mutant embryos derived from *dl*⁻ mothers lack *sna* RNA and protein. To obtain additional evidence that *dl* is important for the initiation of *sna*, we have examined the expression of a "sensitized" *sna-lacZ* fusion gene in *dl*⁻/*dl*⁺ heterozygotes. This sensitized *sna* promoter is truncated and lacks several *dl*-binding sites (see below). It does not direct a completely authentic *sna* pattern, although the levels of expression are essentially normal. The staining pattern is restricted to the ventral-most 12–14 cells, and the lateral limits are jagged

Figure 1. *dl* and *twi* activate *sna*. All embryos are oriented with anterior to the left and display ventral views, except for *B*, which shows a lateral view. (A) Wild-type nuclear cycle 13 embryo showing the *sna* RNA pattern. The levels of expression are low and the limits are fuzzy. (B) Lateral view of an embryo at the onset of nuclear cycle 14. There are increased levels of *sna* RNA and the limits include ~18 cells; however, the borders are still fuzzy. (C) Wild-type cellularizing embryo showing the *sna* RNA expression pattern. It includes the ventral-most 18 cells, with homogeneous staining throughout the presumptive mesoderm. Staining ends abruptly at the boundary between the mesoderm and neuroectoderm (arrow). (D) Precellular P transformant expressing a sensitized *sna-lacZ* fusion gene (A1.6, see Fig. 2). The staining pattern was determined by hybridizing the embryo with a *lacZ* antisense RNA probe, followed by histochemical staining. This *sna* promoter fragment lacks some of the *dl*-binding sites present in the full-length promoter and, as a result, directs a narrower pattern of expression (just 12 cells rather than 18 cells). However, the levels of expression are essentially normal. (E) A precellular embryo derived from a *dl*⁺/*dl*⁻ heterozygous female carrying the same *sna-lacZ* fusion gene shown in *D*. There is a dramatic disruption of reporter gene expression, suggesting that *dl* might play a direct role in activating *sna* expression. (F) *sna* expression pattern in a precellular *twi*⁻ embryo. There is a severe reduction in the levels of expression, and the lateral borders are fuzzy (arrow). The staining pattern is narrower than normal and includes just 12–14 cells. There is an even more dramatic reduction in the levels of expression in slightly older embryos [Kosman et al. 1991].

(Fig. 1D; cf. with C). When this *sna* promoter is expressed in eggs obtained from *dl* heterozygotes, there is a dramatic reduction in the levels of expression (Fig. 1E). In addition, the residual pattern has a salt-and-pepper appearance, such that neighboring cells display all or no levels of staining. Because *dl* heterozygotes show no detectable change in *twi* staining (data not shown), this result suggests that *dl* might directly activate *sna* expression.

5'-Flanking sequences direct an essentially normal sna pattern

The expression patterns of various *sna* promoter-*lacZ* fusion genes were examined in P-transformed embryos, as summarized in Figure 2. The largest of this series of

fusions contains 6 kb of 5'-flanking sequence, whereas the smallest includes only the proximal-most 250 bp of the promoter. *lacZ* fusion genes containing either 6 or 2.8 kb of the *sna* promoter display patterns of expression that are nearly identical to the endogenous *sna* gene, although the expression levels obtained with the 2.8-kb fusion are somewhat reduced as compared with the 6-kb fusion. Reporter gene expression was assayed in whole-mount preparations of P-transformed embryos by use of a digoxigenin-UTP-labeled *lacZ* antisense RNA probe. Expression is first detected in nuclear cleavage cycle 13 embryos (data not shown), and staining coincides with the presumptive mesoderm by cycle 14 (Fig. 3A). The lateral limits are sharp (Fig. 3A, open arrow), but perhaps not quite as sharp as the endogenous *sna* RNA pattern (see Fig. 1C). Staining extends throughout the poles (ar-

Figure 2. Summary of *sna-lacZ* fusion genes. The first set of seven fusion genes was prepared by sequentially deleting 5' sequences of the *sna* promoter. These fragments were inserted into the pCaSper-AUGbetagal P-transformation vector (Thummel et al. 1988). The second and third sets of promoter fusions were placed upstream of the HSP70 minimal promoter in the pWHL transformation vector. The restriction sites of the 5' series are 6.0 kb, *Bam*HI, 2.8 kb, *Nae*I, 2.2 kb, *Acc*I, 1.6 kb, *Eco*RV, 1.3 kb, *Eco*RI, 0.9 kb, *Nco*I, 0.25 kb, *Bam*HI. The 3' deletions (second series) contain the same 5' site (*Nae*I at -2.8 kb). The 3' restriction sites are *Aha*II, -50 bp; *Pfl*MI, -100 bp; *Bsp*MI, -180 bp; *Bam*HI, -250 bp. The HR construct includes a 400-bp *Hinc*II-*Eco*RV *sna* promoter fragment, which spans the region from -2.0 to -1.6 kb. This fragment was inserted into both the pWHL vector containing only the heterologous HSP70 promoter (HR) and the same vector containing the proximal 250 bp of the *sna* promoter (HR-p). (TD-p) A 400-bp *Pst*I-*Dra*I *sna* fragment (from -1.9 to -1.5 kb) that

was placed in the pWHL vector with the 250-bp *sna* proximal region. (RP) A 600-bp *Eco*RV-*Pvu*II fragment (-1.6 to -1.0 kb), that was placed either in pWHL alone or in the vector with proximal *sna* promoter sequences (RP-p). (RE-p) A 300-bp *Eco*RV-*Eco*RI fragment (-1.6 to -1.3 kb) placed in the pWHL vector with the proximal promoter. (p) Proximal promoter sequences from -250 to -50 bp. The expression levels and patterns are indicated at right. The broad expression patterns usually include ~18 cells in the ventral regions plus polar staining. The narrow patterns usually include 12–14 cells and have fuzzy borders. Abbreviations: (A) *Acc*I; (B) *Bam*HI; (E) *Eco*RI; (Na) *Nae*I; (Nc) *Nco*I; (P) *Pvu*II; (R) *Eco*RV; (Ra) *Rsa*I.

rowheads), although the endogenous *sna* RNA is lost from the poles by the onset of gastrulation. This staining persists during gastrulation and the rapid phase of germ band elongation (Fig. 3B) but is lost shortly thereafter. Expression reappears during neurogenesis and includes all of the neuroblasts that give rise to the central nervous system (CNS) (Fig. 3C). Later, expression is observed in the differentiating neurons of the peripheral nervous system (PNS) (Fig. 3D). These dynamic patterns of neuronal expression are similar to those observed for the endogenous *sna* gene (Alberga et al. 1991; Kosman et al. 1991; Leptin 1991).

In summary, these results suggest that 2.8 kb of *sna* 5'-flanking sequence contains most of the *cis*-regulatory elements responsible for both the early and late phases of the normal *sna* pattern. Hereafter, we focus on the regulation of the early (mesodermal) pattern and no longer consider neuronal expression.

5' Truncations

A 2.2-kb *sna-lacZ* fusion gene directs a pattern of expression that is similar, but not identical, to those obtained with the 6- and 2.8-kb promoters. Strong staining is observed in the presumptive mesoderm (Fig. 4A,B) and at the poles, but the lateral limits are slightly narrower and fuzzier than those observed for the larger fusions.

Further deletions cause a narrowing of the pattern and the loss of polar expression. The staining pattern obtained with a 1.6-kb *sna-lacZ* fusion gene is restricted to the ventral-most 12–14 cells and does not encompass the poles (Figs. 1D and 4C). Thus, the 600-bp region between -2.2 and -1.6 kb appears to be important for directing expression in ventral-lateral regions and the poles. The removal of an additional 300 bp from the 5' end causes a dramatic reduction in the levels of expression (Fig. 4D). Further 5' deletions, yielding the 900- and 250-bp *sna-lacZ* fusion genes, cause an almost complete loss of expression (data not shown). These results suggest that there are at least two regulatory elements located in distal regions of the *sna* promoter. One of these is located between -2.2 and -1.6 kb and is required for expression in polar and ventral-lateral regions. The second element is located between -1.6 kb and -900 bp and is required for activation in ventral regions.

3' Deletions

The next set of fusion genes that was examined involved deleting 3' sequences from proximal regions of the *sna* promoter (summarized in Fig. 2). The 5' limit of these deletions is at -2.8 kb, which directs an essentially normal *sna* pattern when all of the 3' sequences from the *sna* promoter are intact. The four 3' deletions were at-

Figure 3. 5'-Flanking sequences can direct an essentially normal *sna* pattern. Progressively older P-transformed embryos that express either the 6-kb (A–C) or 2.8-kb (D) *sna-lacZ* fusion gene. The embryos are oriented with anterior to the left, and the staining patterns were determined by in situ hybridization. (A) Ventral–lateral view of a cellularizing P-transformed embryo expressing the 6-kb *sna-lacZ* fusion gene. The expression pattern spans the entire presumptive mesoderm and shows nearly normal lateral borders (arrow). Expression extends throughout the poles (arrowheads). (B) Lateral view of an elongating embryo expressing the 6-kb *sna-lacZ* fusion gene. Staining is detected throughout the invaginated mesoderm. (C) Dorsal view of an extended embryo expressing the 6-kb fusion gene. By this time, the initial expression in the mesoderm has disappeared, and a new pattern appears in the neurogenic region, which includes all of the segregating neuroblasts. (D) Lateral view of a germ-band-retracted embryo expressing the 2.8-kb *sna-lacZ* fusion gene. Staining is detected in the PNS.

tached to a minimal *hsp70-lacZ* fusion gene containing only 50 bp of the *hsp70* promoter. The first of the 3' *sna*

deletions that was tested lacks the proximal-most 50 bp of the *sna* promoter, as well as leader sequences (the W0.05 fusion; see Fig. 2). This *sna-hsp70* chimeric promoter directs a pattern of expression that is essentially identical to the one obtained with the 2.8-kb *sna-lacZ* fusion gene (Fig. 5A). The removal of an additional 50 bp of the *sna* promoter (W0.1) also has little, if any, effect on the *sna* pattern (Fig. 5B). These results suggest that the first 100 bp of *sna* promoter-proximal sequences, such as the TATA box, do not play a specific role in *sna* regulation.

Further deletions of the *sna* promoter disrupt reporter gene expression. Deleting an additional 80 bp of the proximal promoter, thereby removing all sequences up to –180 bp (W0.18), causes a sporadic staining pattern (Fig. 5C). The ventral–lateral limits are not altered, but some of the cells within these limits show little or no staining. The affected cells appear to be randomly distributed within the *sna* expression domain, with those present in the ventral-most regions showing reduced staining as often as those present in ventral–lateral regions. An even more pronounced salt-and-pepper staining pattern is observed when an additional 70 bp is removed from the *sna* promoter (Fig. 5D). In this case, only 10–20% of the cells within the *sna* domain show any expression. Interestingly, the ventral–lateral limits do not appear to be altered. The lateral-most cells that are stained (Fig. 5D, arrows) bracket the normal *sna* pattern, which encompasses a band of ~18 cells.

In summary, the results obtained by deleting 3' regions of the *sna* promoter suggest that there is at least one *cis*-regulatory element, located between –250 and –100 bp upstream from the transcription start site, which is essential for ensuring uniformly high levels of expression throughout the *sna* domain.

Defining the limits of the *cis*-regulatory elements

The 5'- and 3'-deletion assays suggest that the *sna* promoter contains at least three *cis*-regulatory elements (see Fig. 7C, below). To pinpoint the limits of these elements more accurately, various internal regions of the *sna* promoter were deleted, and upstream sequences were attached to the minimal *hsp70* promoter (see the last set of *lacZ* fusion genes summarized in Fig. 2). The limits of the distal-most element [the polar and lateral activator, (PLA)] were determined by attaching two overlapping 400-bp regions, from –2 to –1.6 kb and –1.9 to –1.5 kb, to a truncated *sna-lacZ* fusion gene containing just 250 bp of 5'-flanking sequence (the HR-p and TD-p fusions; see Fig. 2). Both direct strong expression at the poles (Fig. 6A), but only the TD-p fusion (–1.9 to –1.5) shows broad ventral staining (data not shown). Polar staining was also observed when the –2- to –1.6-kb region was attached to the *hsp70* minimal promoter (the HR fusion), although there were overall lower levels of expression (data not shown). These results suggest that *sna* promoter sequences located between –2 and –1.6 kb are sufficient for polar expression. In addition, a 100-bp sequence between –1.6 and –1.5 kb is critical for ventral expression.

Figure 4. Distal sequences are important for the levels and limits of expression. All embryos are at the nuclear cleavage cycle 14 stage and are oriented with anterior to the left. A–D contain progressive 5' truncations, as indicated in the lower right corner of each panel (i.e., A and B contain the 2.2-kb *sna-lacZ* fusion gene). (A) Ventral and (B) lateral views of embryos expressing the 2.2-kb fusion gene. Staining includes nearly the entire presumptive mesoderm (~18 cells), but the borders are fuzzier than those observed for the larger fusion genes or the endogenous *sna* pattern. (C) Embryo expressing the 1.6-kb *sna-lacZ* fusion gene. The expression pattern is narrower than normal and includes just the ventral-most 12 cells (see Fig. 1D). Staining does not extend through the poles, as is observed for the larger fusions. (D) Embryo expressing the 1.3-kb fusion gene, which directs very weak staining in the ventral-most 12 cells. Neither of the smaller fusion genes (900 and 250 bp) directs consistent patterns of expression.

The limits of the ventral activation (VA) element were confirmed by attaching *sna* promoter sequences located between -1.6 and -1 kb to both the *hsp70* minimal promoter and the -250 -bp truncated *sna-lacZ* fusion gene (RP and RP-p, respectively; see Fig. 2). There is strong, uniform staining in ventral regions when proximal *sna* promoter sequences are used (Fig. 6B). Staining includes the ventral-most 12–14 cells but does not extend through the poles. This pattern is very similar to that observed for the truncated 1.6-kb *sna-lacZ* fusion gene (Fig. 1D), suggesting that sequences located between -1 kb and -250 bp are not required for activation in ventral regions. A sporadic staining pattern was observed when the VA element was attached to the *hsp70* minimal promoter (Fig. 6C), rather than proximal *sna* promoter sequences. These results suggest that optimal expression depends on interactions between the VA element and proximal sequences [the augmentation element (AE)].

The smallest minipromoter that directs a reasonable ventral pattern is only 550 bp in length (the RE-p fusion; Fig. 2) and was obtained by attaching a 300-bp sequence from the VA element (from -1.6 to -1.3 kb) to the 250-bp *sna* proximal promoter containing the AE. This minipromoter directs expression in the ventral-most 10–12 cells (Fig. 6D). The pattern is similar to the one obtained with the intact 600-bp VA element, although staining is not completely uniform, and the ventral–lateral limits are a bit narrower (cf. with Fig. 6B).

DNA-binding assays

As a first step toward determining whether *dl* and *twi*

directly regulate *sna*, DNase I protection assays were done with *dl* and *twi* fusion proteins. The DNA fragments that were used in these experiments span the distal PLA and VA elements (-2.2 kb to -900 bp), as well as the proximal AE (-250 to -100 bp). The fusion proteins were prepared in the pGEX vector system (Smith and Johnson 1988) and purified on glutathione-affinity beads. A total of 10 *dl*-binding sites were identified in the region from -2 to -1 kb. An example of the *dl*-binding assays is shown in Figure 7A. This particular region contains three of the *dl* sites (d7, d8, and d9). Two *twi*-binding sites were identified within the AE (Fig. 7B).

The locations and sequences of the *dl*- and *twi*-binding sites are summarized in Figure 7C. A cluster of four *dl*-binding sites are located within the limits of the PLA element, whereas six sites occur within or near the VA element. The sequences of these 10 *dl*-binding sites conform to the consensus sequence based on previous studies with *zen*, *twi*, and *rho* promoters (Ip et al. 1991, 1992; Jiang et al. 1991; Pan et al. 1991; Thisse et al. 1991). In general, one of the half-sites includes the sequence GGG, whereas the other half-site contains at least 1 or 2 C residues. The two half-sites are usually separated by 4 or 5 AT-rich residues. The two *twi*-binding sites include E-box sequences (CANNTG), although the t2 site is unusual in that it contains two central A residues. Usually, the central residues correspond to G and/or C residues (Lenardo et al. 1987).

In vivo relevance of *dl*- and *twi*-binding sites

Site-directed mutagenesis was done to establish a direct

Figure 5. Proximal-promoter sequences are required for optimal expression. All embryos are undergoing cellularization, oriented with anterior to the *left*, and display ventral views. They contain progressively larger 3' deletions, as indicated in the *lower right* corner of each panel (i.e., A lacks the first 50 bp of the proximal promoter). All fusion genes contain the same 5' site at -2.8 kb. (A and B) Embryos expressing *sna-lacZ* fusion genes lacking either 50 or 100 bp of proximal sequences. They direct essentially normal patterns of expression. (C) Embryo expressing a fusion gene lacking the first 180 bp of the *sna* proximal promoter. There is a disruption in the pattern, in that a significant fraction of cells in the ventral domain are unstained. (D) Embryo expressing the fusion gene lacking the first 250 bp of proximal sequences. There is an even more severe salt-and-pepper pattern, with few cells stained. However, the lateral limits of expression (arrows) appear nearly normal and span the ventral-most 16–18 cells.

link between individual *dl*- and *twi*-binding sites and the genetic regulation of the *sna* pattern. The first experiments involved disrupting *dl*-binding sites in the PLA,

within the context of an otherwise normal 2.8-kb *sna-lacZ* fusion gene. Mutations in two of the four *dl* sites (*d1* and *d4*) in the PLA resulted in a staining pattern that does not extend throughout the anterior and posterior poles, as is seen for the wild-type promoter (Fig. 8B; cf. with A). This suggests that polar expression depends on the direct interaction of *dl* protein present at the poles with the binding sites in the PLA. Interestingly, mutations in the *d1* and *d4* sites have no effect on the ventral staining pattern, suggesting that the *dl* protein present at the poles is functionally distinct from the protein in middle body regions. It is conceivable that these distinct activities involve interactions between *dl* and the *torso* (*tor*) tyrosine kinase activity at the poles. Alternatively, perhaps the *tor* terminal system controls an unknown regulatory factor that binds near the *dl* sites and interacts with *dl* to direct optimal expression at the poles.

The next series of mutations involved the use of the RP-p minipromoter, which contains the 600-bp VA element attached to the 250-bp *sna-lacZ* fusion gene. This minipromoter contains a total of six *dl*-binding sites (all in the VA) and two *twi* sites (both in the AE). Mutations in two of the *dl* sites, *d5* and *d10*, did not alter the staining pattern driven by the minipromoter (Fig. 8C; cf. with Fig. 6B). However, mutations in five of the six *dl* sites cause a substantial reduction in expression (data not shown). Mutations that abolish the two *twi* E boxes in the proximal AE result in a sporadic salt-and-pepper staining pattern (Fig. 8D). This pattern is similar to those obtained by deleting AE sequences (i.e., Fig. 5C), suggesting that the interaction of the *twi* protein with the AE is essential for uniform transcription of the *sna* promoter throughout the presumptive mesoderm.

Evidence for an interaction between the proximal *twi*-binding sites and the distal *dl* sites was obtained by creating double mutants. The simultaneous inactivation of the two *twi* sites in the AE and the *d5* and *d10* *dl* sites in the VA results in a catastrophic reduction in transcription (Fig. 8F; cf. with E). Most P-transformed embryos that carry this mutagenized minipromoter show no staining whatsoever, and the embryo shown in Figure 8F is one of the very few that contains residual staining. This result suggests that *dl* and *twi* act in concert to activate transcription (see Discussion). Mutations in the two *dl* sites alone have virtually no effect on the staining pattern (Fig. 8C), probably because the other four *dl* sites are still intact. Although mutations in the *twi* sites produce an erratic pattern, the overall levels of expression are not dramatically altered. However, the simultaneous removal of both the *dl* and *twi* sites causes more than a 10-fold reduction in the levels of expression (cf. Fig. 8F with E). Although it is difficult to quantify the absolute levels of expression, it should be noted that all of the *sna-lacZ* fusion genes shown in Figures 6 and 8 have been stained in parallel, thereby permitting a reasonable estimate of comparative levels of expression.

Discussion

The establishment of the *sna* expression pattern is some-

Figure 6. Identification of the three *cis*-regulatory elements. P-transformed embryos express *lacZ* fusion genes containing different pieces of the *sna* promoter. All embryos are at nuclear cleavage cycle 14 and are oriented with anterior to the *left*. The particular fusion gene that each embryo expresses is indicated at *lower right* (see Fig. 2 summary). (A) Embryo expressing the HR-p fusion gene, which contains the distal PLA element (–2 to –1.6 kb) attached to proximal sequences spanning the AE. Staining is restricted to the poles. (B) Embryo expressing the RP-p fusion gene, which contains the VA element (–1.6 to –1 kb) attached to proximal AE sequences. Strong staining is observed in the ventral-most 12–14 cells. (C) Embryo expressing the RP fusion gene, which is identical to the one shown in B, except that the VA element is attached to the minimal HSP70 promoter rather than the *sna* proximal AE. A sporadic, salt-and-pepper staining pattern is observed. (D) Embryo expressing the smallest *sna* minipromoter, which contains a 300-bp region of the VA (–1.6 to –1.3 kb) attached to proximal AE sequences. The expression pattern is weaker and contains fuzzier lateral borders than that obtained when the entire VA (–1.6 to –1 kb) is used.

what different from the regulation of *twi* and the interaction of the *bicoid* (*bcd*) morphogen with *hunchback* (*hb*) (Driever and Nüsslein-Volhard 1989; Struhl et al. 1989). Both the *dl*–*twi* and *bcd*–*hb* interactions are responsible for transducing broad gradients into steeper ones. The lateral *sna* borders are considerably sharper than either the *twi* or *hb* borders and are reminiscent of the on/off anterior margins seen for refined *even-skipped* and *fushi tarazu* pair-rule stripes (Frasch et al. 1987; Lawrence et al. 1987). The picture that is emerging for the regulation of *sna* is summarized in Figure 9. The broad *dl* concentration gradient triggers the expression of *twi*, which shows a somewhat steeper gradient than the input *dl* pattern. This response of *twi* to *dl* is probably comparable to the *bcd*–*hb* interaction, which has been proposed to involve cooperative interactions of the morphogen with multiple binding sites in the *hb* promoter (Driever et al. 1989; Struhl et al. 1989). The broad *dl* gradient then acts in concert with the steeper *twi* gradient to direct an even sharper *sna* pattern. The single biggest drop in the steep *twi* gradient has been shown to coincide with the *sna* border (Kosman et al. 1991). In principle, the multiplication of the *dl* and *twi* slopes could produce a sharp *sna* border, and the demonstration of a catastrophic reduction in *sna* expression when both classes of binding sites are disrupted is consistent with this possibility.

dl–*twi* interactions

Mutations in *dl*- and *twi*-binding sites result in severely reduced levels of expression, although mutations in ei-

ther of these sites alone have little or no effect on the *sna* pattern. The nature of the *dl*–*twi* interaction is unknown. It is possible that the two proteins directly contact one another to create an “activation surface” that is qualitatively more efficient in activating transcription than either protein alone. Alternatively, perhaps the two proteins interact independently with different rate-limiting basal factors of the transcription complex. We are currently investigating the possibility that *dl* and *twi* might form multimeric complexes in solution. It is likely that *dl* interacts with additional activators present in proximal regions of the *sna* promoter because proximal deletions cause a more severe disruption of the pattern than point mutations in the two *twi*-binding sites (i.e., cf. Figs. 5D and 8D).

Whatever the nature of the *dl*–*twi* interaction, it appears to be essential for driving optimal expression of *sna*. Interestingly, the 1.2 kb of *twi* 5'-flanking sequence that is sufficient to direct an essentially normal *twi* expression pattern (Jiang et al. 1991; Pan et al. 1991; Thisse et al. 1991) lacks general E-box consensus sequences and, in particular, lacks *twi*-binding sites (R. Park, unpubl.). Thus, it would appear that the differential response of the *twi* and *sna* promoters to the *dl* concentration gradient involves *dl*–*twi* interactions, which occur for *sna* but not *twi*. The *dl*–*twi* interaction is responsible for allowing both high and intermediate levels of *dl* protein (in ventral vs. ventral-lateral regions) to work equally effectively in activating *sna* expression. Further support for this view is the pattern of *sna* expression observed in *twi*[–] mutants. The altered *sna* pattern is quite similar to the initial pattern seen in early wild-type embryos, with

Figure 7. Identification of *dl* and *twi*-binding sites. DNase I footprint assays with pGEX-*dl* and pGEX-*twi* fusion proteins and DNA fragments from the *sna* promoter. (A) A 600-bp DNA fragment corresponding to the VA element (−1.6 to −1 kb) was ³²P-labeled with Klenow, incubated with increasing amounts of the pGEX-*dl* fusion protein, digested with DNase I, and electrophoresed on a polyacrylamide-urea gel. Three regions of protection, which correspond to the d7–d9 *dl*-binding sites, are shown here. (B) A 200-bp fragment spanning the AE (from −250 to −50 bp) was ³²P-labeled and incubated with increasing amounts of the pGEX-*twi* fusion protein. There are two regions of protection, which correspond to the t1 and t2 *twi*-binding sites. (Lanes G + A) The G + A sequence of the fragment; (lanes 0) the digestion patterns without the addition of fusion protein. (C) Summary of the *cis*-regulatory elements and protein-binding sites. The PLA corresponds to the 400-bp region from −2 to −1.6 kb that is required for expression in polar and ventral-lateral regions. The VA element includes the 300-bp region from −1.6 to −1.3 kb and is required for activation in the ventral-most 12 cells. Finally, the proximal AE includes the region from −250 to −50 bp

and is required for strong expression in ventral and ventral-lateral regions. A total of 10 *dl*- and 2 *twi*-binding sites were identified within and near these elements. The *dl* site has about fivefold higher affinity than the other sites based on gel mobility-shift assays. The sequences of these sites are shown below. Core consensus sequences are underlined.

graded expression in ventral and ventral-lateral regions (see Fig. 1). Sharp on/off patterns of gene expression in mammalian hematopoietic lineages may involve similar interactions, as many B-cell-specific enhancers contain both E boxes and binding sites for NF- κ B (related to *dl*) (Lenardo et al. 1987).

Redundancy of *dl*-binding sites

The *twi* and *sna* promoters contain a remarkable number of *dl* activator sites. There are 10 binding sites in the *sna* promoter and at least 5 (and maybe as many as 10) sites in *twi* (Jiang et al. 1991; Pan et al. 1991; Thisse et al. 1991). Perhaps activation requires only several binding sites, but the additional sites ensure a reproducible and uniform response to the *dl* concentration gradient. Alternatively, each *dl*-binding site might be contributing qualitatively distinct aspects of the *sna* and *twi* expression patterns. Some of the sites may be important for polar expression, others may mediate activation in ventral regions, and yet others may be required for expression in ventral-lateral regions. The results presented in this study are consistent with both types of models.

Redundancy of the binding sites is suggested by the

results obtained with a 2.2-kb *sna-lacZ* fusion gene bearing a 100-bp internal deletion between −1.6 and −1.5 kb (data not shown). This deletion removes the d5, d6, and d7 *dl*-binding sites but, nonetheless, has only a minor effect on the levels of expression (at most a twofold reduction as compared with the intact 2.2-kb fusion gene). This observation suggests that the d5–d7 sites are more or less dispensable (see Fig. 7C summary). However, the results obtained with *sna* minipromoters suggest that the d5, d6, and d7 sites directly mediate activation. The 300-bp region from −1.6 to −1.3 kb, which contains these three sites, directs strong ventral expression when placed upstream of the proximal AE. Moreover, the 400-bp region from −1.9 to −1.5 kb directs weak ventral expression while sequences from −2 to −1.6 kb fail to drive such expression, suggesting that the 100-bp interval containing d5–d7 is essential for ventral activation.

Not all of the *dl*-binding sites are interchangeable and direct the same aspects of the *sna* pattern. For example, the distal-binding sites (d1–d4) are important for mediating expression in ventral-lateral regions where there are only intermediate levels of *dl* protein. Truncated promoters lacking these binding sites are expressed only in the ventral-most regions where there are peak levels of

Figure 8. Point mutations in individual *dl*- and *twi*-binding sites. P-transformed embryos express various *sna-lacZ* fusion genes with mutations in individual binding sites. All embryos are oriented with anterior to the left. The diagram above each embryo shows the fusion gene that is expressed and the identities of mutagenized binding sites (indicated by circled X). Lateral views of embryos expressing the normal (A) and mutagenized (B) 2.8-kb *sna-lacZ* fusion gene are shown. Inactivation of the d1 and d4 *dl*-binding sites causes reduced expression at the poles (arrows). (C) Ventral view of an embryo expressing the RP-p fusion gene (-1.6 to -1 kb attached to proximal sequences) containing mutations in two of the six *dl*-binding sites (d5 and d10). There is no significant change in expression relative to the unmutagenized fusion gene (see Fig. 6B). (D) Ventral view of an embryo expressing the same RP-p fusion gene as in C, except that the two *twi*-binding sites (t1 and t2) were mutagenized instead of the *dl* sites. This causes a slightly sporadic pattern of expression, but there is only a twofold reduction in the overall levels of staining. (E) Lateral view of an embryo expressing the wild-type RP-p fusion gene. The levels of staining are comparable to that obtained with the 2.8-kb *sna-lacZ* fusion gene, although expression does not extend to the poles (see A and B). (F) Lateral view of an embryo expressing the RP-p fusion gene containing mutations in both the *dl* d5 and d10 sites and the *twi* t1 and t2 sites. Expression is virtually abolished, and the residual staining observed here represents one of the few embryos that shows any expression at all. It was not possible to display a ventral view of this embryo, because such an angle obscures the low levels of staining. Residual staining is amplified in lateral views.

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Figure 9. *dl*–*twi* interactions establish the homogeneous, sharp *sna* expression pattern. The results of genetic analyses and promoter fusion assays suggest that the *dl* gradient is the primary activator of both *twi* and *sna*. *twi* is expressed in a somewhat steeper gradient than *dl*. *dl* and *twi* then act in concert to augment *sna* expression and establish its sharp on/off pattern.

morphogen. When alone, the distal sites are able to drive expression only at the poles, and for them to mediate expression in ventral–lateral regions, at least three of the more proximal binding sites (d5–d7) must also be present. It is curious that the distal binding sites (such as the high-affinity d1 site) alone are unable to activate transcription, whereas the lower-affinity sites are sufficient for activation. Perhaps the arrangement or spacing of the sites influences *dl* activity. By analogy to NF- κ B (Baeuerle and Baltimore 1989; Ghosh et al. 1990; Kieran et al. 1990), it is conceivable that *dl* associates with another, as yet unknown, subunit; the activities of different binding sites might reflect the interactions of *dl* homo- versus heteromultimers.

Levels vs. limits

Previous studies on the interaction of the *bcd* morphogen with the *hb* promoter led to the suggestion that the affinities of the binding sites are crucial for setting the limits of expression, whereas the total number of sites contribute to the overall levels (Driever et al. 1989; Struhl et al. 1989). The results presented in this study are essentially consistent with this view. The 10 *dl*-binding sites located in distal regions of the *sna* promoter are important both for defining the ventral–lateral limits of the pattern and for driving high levels of expression. 5' Truncations that sequentially remove the *dl* sites result first in a narrowing of the pattern and, ultimately, in the complete loss of expression. The removal of the distal sites results in a narrowing of the pattern, presumably because only peak levels of *dl* (in the ventral-most 10–12 cells) are able to fill the sites present in proximal regions. As observed for *bcd*, it would appear that multiple *dl*-binding sites are required for activation. In the case of *sna*, a minimum of four sites, d5–d8, are needed for directing relatively strong levels of expression. A truncated promoter containing just two sites, d9 and d10, drives only very weak expression. In summary, both *dl* and *twi* are direct activators of *sna*, and the *dl*–*twi* interaction ensures uniform expression of *sna* in response to the *dl*

gradient. Enhanced expression in ventral–lateral regions is important for establishing the sharp *sna* border.

Materials and methods

Molecular cloning

A 300-bp *sna* cDNA fragment, which includes the 5' end, was used to screen a λ genomic DNA library (see Jiang et al. 1991). The upstream flanking regions were isolated and subcloned for subsequent sequencing. The first 2.8 kb of 5'-flanking sequence of the *sna* promoter was sequenced by use of the Sequenase kit, as suggested by the manufacturer (U.S. Biochemical, Cleveland OH).

Construction of transformation plasmids

The experiments described in this study involved the use of two different injection vectors, pCaSper–AUGbetagal (Thummel et al. 1988) and pWHL (see Driever et al. 1989). Both contain the *white* gene as a marker. The first vector contains the alcohol dehydrogenase translation initiation sequences. The *sna* 5'-truncation series (see Fig. 2) includes the *sna* transcription start site and the first 100 bp of untranslated leader sequence. *sna* promoter fragments containing either 6 or 3 kb of 5'-flanking sequence were first subcloned into the pGEM7Zf(+) plasmid (Promega, Madison WI). The 5' ends of the various fragments are indicated in the legend to Figure 2, and the 3' ends used the unique *KpnI* site in the polylinker of the vector. The fragments were cloned into the P-transformation vectors after digestion with *Bam*HI and *Kpn*I; the *Bam*HI site was blunted when necessary. The pWHL vector contains the HSP70 minimal TATA element, and the *sna* fragments were directly blunt-end ligated into the *Stu*I site upstream of the promoter. The internal deletions were prepared by sequential cloning of the fragments into the pGEM7 vectors, and the fusion promoters were then ligated into the transformation plasmid.

Site-directed mutagenesis

The appropriate *sna* pGEM7 subclones were transformed into the CJ236 strain of *Escherichia coli* to obtain single-stranded DNA templates containing uracil. The mutant oligonucleotides were annealed, and the complementary strands were synthesized in vitro by use of T4 polymerase, as suggested by the manufacturer (Bio-Rad Muta-gene kit). The double-stranded plasmids were transformed into the DH5a strain, and the mutated DNAs were confirmed by sequence analysis. The relevant sequences of the mutagenic oligonucleotides are as follows: d1, AAAGAGCTCTACAGAA; d4, GTGCGCATGCGTGCGC; d5, TCCTAGATGGTTAACC; d10, GTTGTAAGGATGCATC; t1, CGCAAAGCTTCGAG; t2, CCACAATATTGCGA. The bold nucleotides correspond to the mutagenic substitutions.

Detection of reporter gene expression

At least two, and usually three, independent transformants were examined for each *sna* fusion gene tested in this study. Appropriately staged P-transformed embryos were fixed with formaldehyde and stained for *lacZ* reporter gene expression by use of the digoxigenin-detection system (Boehringer Mannheim) as described previously (Jiang et al. 1991). An antisense *lacZ* riboprobe was hybridized with whole-mount preparations of P-transformed embryos, and the expression patterns were vi-

sualized with a histochemical staining method [anti-digoxigenin antibodies conjugated to alkaline phosphatase].

DNA-binding assays

dl and *twi* fusion proteins were prepared from bacterial extracts by use of the pGEXKG expression vector, which contains glutathione S-transferase (Smith and Johnson 1988). The *dl*-pGEX fusion gene was made with a 1.2-kb *NdeI*-*SacI* *dl* cDNA fragment, which was obtained from the pAR*dl*-378 T7 expression plasmid described by Ip et al. (1991). This DNA fragment encodes the amino-terminal 378-amino-acid residues of the *dl* protein, which includes the entire *rel*/NF- κ B homology region. The fragment was blunt-ended with T4 DNA polymerase and inserted into the *SmaI* site of the pGEX vector. The pGEX-*twi* fusion protein was prepared by inserting a 1.6-kb *NdeI*-*EcoRI* *twi* cDNA fragment into the expression vector. This fragment was obtained from a pAR T7 expression plasmid containing the entire *twi*-coding sequence (D. Kosman, unpubl.). The *twi* fragment obtained from this T7 recombinant was blunt-ended and inserted into the *SmaI* site of the pGEX vector. Fusion proteins were prepared as described (Smith and Johnson 1988), with the following modifications. Induced bacteria were pelleted by centrifugation and resuspended in 1/50 volume of lysis buffer (25 mM HEPES at pH 7.5, 20 mM KCl, 2.5 mM EDTA, 1% Triton X-100, 1 mM DTT, 0.5 mM PMSF, 1 μ g/ml of pepstatin, 1 μ g/ml of leupeptin). Resuspended bacteria were subjected to three freeze-thaw cycles and then sonicated for 30 sec. A 1/10 volume of 5 M NaCl was added and placed on ice for 15 min. The lysate was spun at 12,000g for 15 min at 4°C. Fusion proteins were affinity purified by adding 1/10 volume of glutathione-agarose beads (Sigma) and incubating for 1 hr at 4°C. The beads were pelleted by centrifugation and washed three times with 20 volumes of lysis buffer and once with 20 volumes of 50 mM Tris (pH 8). Fusion proteins were eluted from the beads by the addition of 1 volume of 50 mM Tris (pH 8), 10 mM glutathione. The glutathione was removed by dialyzing three times against 250 volumes of buffer B (25 mM HEPES at pH 7.5, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 6 mM MgCl₂, 10% glycerol), and the protein was stored at -70°C. DNase I footprinting was done essentially as described by Hoey and Levine (1988). *sna* promoter sequences located between -2.0 and -1.6 kb, -1.6 and -1.0 kb, and -250 and -50 bp were ³²P-labeled with Klenow and gel purified. Binding reactions were done with pGEX fusion proteins and 100,000 cpm of labeled DNA in 50 μ l of footprinting buffer (25 mM HEPES at pH 7.5, 50 mM KCl, 3 mM MgCl₂, 1 mM EDTA, 0.5% NP-40, 10 μ g/ml of poly[d(I-C)], 10% glycerol).

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