Spatial regulation of zerknüllt: a dorsal–ventral patterning gene in Drosophila

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Zerknüllt (zen) is one of approximately 10 zygotically active genes that control the differentiation of the dorsal–ventral (D/V) pattern during early embryogenesis in Drosophila. Past genetic analyses suggest that maternal factors repress the expression of zen in ventral regions, thereby restricting zen products to dorsal and dorsal–lateral regions of precellular embryos. Subsequent interactions with other zygotic D/V regulatory genes refine the zen pattern, restricting expression to the dorsal-most ectoderm. Here we describe the use of zen promoter fusions and P-element transformation to identify cis elements that are responsible for the complex spatial pattern of zen expression. The zen promoter shows a two-tier organization: Distal sequences mediate its initial response to maternal factors, whereas proximal sequences are responsible for the refinement of the pattern in older embryos. The distal regulatory element has the property of a silencer (or anti-enhancer) element and can act over a distance to repress ventral expression of a heterologous promoter. Also, we discuss evidence that proximal promoter sequences interact with factors that may be modulated by a cell–cell communication pathway.

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Embryonic cells follow diverse pathways of morphogenesis that are based on their spatial coordinates within the Drosophila embryo. This process depends on the activities of early-acting zygotic genes, which are expressed selectively along the anterior–posterior (A/P) [for review, see Ingham 1988; Levine and Harding 1989] and dorsal–ventral (D/V) body axes (e.g., Doyle et al. 1986; Thisse et al. 1987; St. Johnston and Gelbart 1987). Maternal factors initiate spatially restricted patterns of zygotic gene expression, which are further refined by subsequent cross-regulatory interactions among zygotic gene products during later stages of development. There is mounting evidence that the key A/P and D/V maternal factors are distributed asymmetrically and function as morphogen gradients [Driever and Nüsslein-Volhard 1988a; Driever and Nüsslein-Volhard 1988b; Steward et al. 1988]. A central problem regarding the control of positional information in Drosophila is how discrete patterns of zygotic gene expression emerge in response to crudely localized maternal cues. The zygotic D/V gene zerknüllt (zen) provides an excellent model system for the investigation of this problem.

Zen is required for the differentiation of dorsal tissues, including the amnioserosa and optic lobe (Wakimoto et al. 1984). zen embryos show a weakly ventralized phenotype, whereby the dorsal-most cells follow a lateral pathway of development (C. Rushlow and M. Levine, in press). As a result of this transformation in cell fate, several tissues that normally derive from the dorsal-most regions of the embryo fail to differentiate [Wakimoto et al. 1984]. The absence of the amnioserosa disrupts germ-band elongation, which in turn causes secondary defects in developing zen embryos. The zen protein contains a homeo box and binds to specific DNA sequences [Hoey and Levine 1988]; there is direct evidence that zen controls development by modulating gene expression at the level of transcription [Han et al. 1989].

During cleavage cycle 10–11, zen shows a broad dorsal-on/ventral-off pattern of expression, and zen products are distributed around 40% of the embryo's circumference. During cellularization and gastrulation, zen expression is lost from dorsal–lateral regions and is restricted to the dorsal-most cells of the middle body region, encompassing only 10% of the embryo's circumference. The distribution of zen products coincides quite closely with the regions of the embryonic fate map where zen gene function is most critical (i.e., presumptive optic lobe and amnioserosa).

The wild-type zen expression pattern includes two distinct phases: an early phase when zen products are distributed broadly, and a late phase when expression is restricted sharply to the dorsal-most regions. Analyses of zen expression in maternal and zygotic D/V mutants has provided considerable information concerning the trans-control of this dynamic pattern during development [Rushlow et al. 1987b; C. Rushlow and M. Levine, in press].
press). It appears that the initial, broad dorsal-on/ventral-off phase of \textit{zen} expression is under maternal control, whereas the subsequent refinement in expression involves interactions with other zygotic and maternal factors (Rushlow et al. 1987b, C. Rushlow and M. Levine, in press).

Most of the maternal genes required for the establishment of embryonic dorsal-ventral polarity have been identified in previous genetic screens (Anderson and Nüsslein-Volhard 1984; Schüpbach and Wieschaus 1989, for review, see Anderson 1987; Levine 1988). Recent studies suggest that one of these genes, \textit{dorsal}, plays a particularly crucial role in the specification of D/V positional identities during early development (Anderson et al. 1985; Steward 1987; Steward et al. 1988). Interactions among the products encoded by the maternal D/V genes are thought to result in a ventral-to-dorsal gradient of \textit{dorsal} protein (Steward et al. 1988; DeLotto and Spierer 1986; Chasan and Anderson 1989). Peak levels of \textit{dorsal} protein specify a ventral pathway of development, probably by activating the expression of zygotic regulatory genes such as \textit{twist} and \textit{snail} (Thiss et al. 1987). \textit{dorsal} may also control the D/V pattern by repressing the expression of other zygotic regulatory genes, such as \textit{zen} and decapentaplegic (\textit{dpp}), which control the differentiation of dorsal tissues. Consistent with this possibility is the finding that embryos derived from \textit{dorsal} females show ectopic expression of \textit{zen} in various regions (Rushlow et al. 1987b).

None of the zygotic D/V mutants that have been tested disrupt the initial dorsal-on/ventral-off pattern of \textit{zen} expression (C. Rushlow and M. Levine, in press). However, several of these mutants affect the refinement of the \textit{zen} pattern during later stages. For example, \textit{dpp} (Padgett et al. 1987) embryos show a premature loss of \textit{zen} expression, whereas \textit{short gastrulation}~{\textit{sog}}~ embryos (Wieschaus et al. 1984; Zusman et al. 1988) embryos show a persistence of \textit{zen} expression in dorsal-lateral regions. The pattern of expression that is observed is essentially identical to the pattern of the normal \textit{zen} gene located within the context of the ANT-C (data not shown; Rushlow et al. 1987b).

\textbf{Promoter fusions}

As indicated above, the 5'-flanking region required for normal \textit{zen} expression is \~1.6 kb in length. We used P-element transformation to analyze the in vivo activities of different portions of this 5' region. We began our analysis by attaching the entire 1.6-kb promoter fragment to the reporter gene \textit{lacZ}, as shown in Figure 1a. The 5' region of the \textit{zen} promoter includes the transcription start site, the 52-bp untranslated leader sequence, and the initiating ATG codon by in vitro mutagenesis (see Materials and methods). The newly created \textit{BamHI} site was created just downstream of the ATG codon in the \textit{bcd} transcription unit (Berleth et al. 1988). Because this fragment provides complete \textit{zen} gene function, it was anticipated that it contains all of the relevant \textit{cis}-regulatory elements required for normal \textit{zen} expression. To test this, we examined the distribution of the \textit{zen} protein encoded by the P-\textit{zen}+ transposon in a \textit{zen} null mutant background. The pattern of expression that is observed is essentially identical to the pattern of the normal \textit{zen} gene located within the context of the ANT-C (data not shown; Rushlow et al. 1987b).

\textbf{Results}

The \textit{zen} region of the Antennapedia complex (Kaufman et al. 1980; Wakimoto et al. 1984) contains two closely linked homeo box genes, previously called \textit{z1} and \textit{z2} (Rushlow et al. 1987a). Several lines of evidence suggest that \textit{z1} corresponds to \textit{zen}, whereas \textit{z2} is dispensable. By P-element transformation, we have shown that one copy of \textit{z1} can provide full \textit{zen}+ activity in a \textit{zen}− background (Rushlow et al. 1987a). Furthermore, \textit{z1}+, \textit{z2}− embryos are fully viable and survive to adulthood (Pultz et al. 1988). We will hereafter refer to \textit{z1} as \textit{zen}. The P-\textit{zen}+ transposon that rescues \textit{zen}− embryos contains a 4.4-kb genomic DNA fragment with 1.6 kb of 5'− and 1.5 kb of 3'-flanking sequences, as well as the \textit{zen}-coding region. The 5' region of the fragment includes virtually the entire interval between the 3' end of \textit{bicoid} (\textit{bcd}) and the 5' end of \textit{zen}. A detailed restriction map of this region is shown in Figure 1a. The distal 5' \textit{XbaI} site of the P-\textit{zen}+ transposon maps only \~80 bp downstream from the putative polyadenylation site of the \textit{bcd} transcription unit (Berleth et al. 1988). Because this fragment provides complete \textit{zen} gene function, it was anticipated that it contains all of the relevant \textit{cis}-regulatory elements required for normal \textit{zen} expression. To test this, we examined the distribution of the \textit{zen} protein encoded by the P-\textit{zen}+ transposon in a \textit{zen} null mutant background. The pattern of expression that is observed is essentially identical to the pattern of the normal \textit{zen} gene located within the context of the ANT-C (data not shown; Rushlow et al. 1987b).

\textit{zen} promoter fusions

Here we identify \textit{cis} control elements within the \textit{zen} promoter that mediate interactions with \textit{trans}-acting factors encoded or modulated by D/V genes. Different DNA fragments from the 5' end of the \textit{zen} transcription unit were attached to the reporter gene \textit{lacZ}, and integrated into the \textit{Drosophila} genome by P-element-mediated germ-line transformation (Rubin and Spradling 1982). The activities of the different promoter fragments were analyzed in transformed embryos by in situ hybridization using \textit{lacZ} RNA probes. We show that a distal region, between \~1.4 kb and \~1.2 kb upstream from the transcription start site, is responsible for preventing expression in ventral regions of early embryos. This distal promoter element probably interacts with one or more maternal factors, which possibly include the \textit{dorsal} protein. This element has the properties of a yeast silencer sequence because it acts over a distance to mediate specific repression of \textit{zen} and of a heterologous promoter. We also identify sequences within the proximal promoter that are responsible for the refinement of the \textit{zen} pattern during cellularization and gastrulation.
Figure 1. Summary of zen-lacZ promoter fusions. A 4.4-kb genomic DNA fragment that contains the zen coding sequence and 1.6 kb of 5' and 1.5 kb of 3'-flanking sequence is sufficient to fully rescue zen-embryos. (a) Restriction map of the 1.6-kb zen 5'-flanking region indicating relevant restriction sites used in the zen-lacZ fusions. zen 5'-flanking and untranslated leader sequences were fused to the lacZ coding sequence at a BamHI site. The numbers on the left indicate the amount of 5' zen sequences included in each fusion. Several full-length zen promoters that contained internal deletions were prepared also (ΔXmn, ΔNru, ΔRsa, and ΔAcc). Each of these fusions was cloned into either the Carnegie 20-derived vector or the CaSpeR P-element transformation vector or both, and analyzed as described in Materials and methods. Those fusions in the CaSpeR vector are indicated by an * on the left. (b) Map of the pC20XJ (derived from Carnegie 20) and CaSpeR P-element transformation vectors that contain the full-length (1.6 kb) zen-lacZ fusion promoter. The ry marker gene is contained on a 7.2-kb HindIII fragment, which includes the entire coding sequence and ~1.5 kb of 5' and ~0.5 kb of 3'-flanking sequences (Lee et al. 1987; Keith et al. 1987). The CaSpeR vector contains a truncated version of the w gene, with ~300 bp of 5'-flanking sequences and ~630 bp of 3'-flanking sequences, with a polylinker containing several unique restriction sites near the 5' end of w (V. Pirrotta pers. comm.; see Materials and methods for the use of these vectors.) [A] AccI, [B] BamHI, [E] EcoRI, [H] HindIII, [N] NruI, [R] RsaI, [P] PvuII, [S] ScaI, [X] XbaI, [Xm] XmnI.

significant lag between the time when zen RNAs and the protein can be detected (Doyle et al. 1986; Rushlow et al. 1987a). In situ hybridization methods permit the detection of zen transcripts as early as cleavage cycle 10–11, whereas the protein is not detected until the beginning of cleavage cycle 14, about 30–40 min later. A similar lag is observed for the expression of lacZ from the fusion promoters, suggesting that the zen-untranslated leader sequence might inhibit efficient translation. Because we were particularly interested in the early expression patterns, we assayed reporter gene expression by localizing lacZ RNAs in tissue sections of embryos.
obtained from transformed lines. The activities of each of the promoter fusions shown in Figure 1 were compared directly with the endogenous zen pattern by hybridizing adjacent sections with a zen cDNA probe.

1.6 kb is sufficient for a wild-type zen pattern
The early expression of a promoter fusion containing the entire 1.6-kb zen 5' fragment shows a dorsal-on/ventral-off pattern that is virtually identical to the endogenous zen gene. During cleavage cycle 13–14 zen and lacZ transcripts are detected along the dorsal surface of the embryo and wrap around both the anterior and posterior poles (Fig. 2a and b). Within the next 20 min, expression begins to be lost from the poles, and discontinuities in the labeling pattern appear near the presumptive cephalic furrow (Fig. 2c and d). There appears to be a slight delay in the loss of lacZ transcripts from the poles (as compared with zen transcripts), but this may be from an increased stability of the lacZ RNA.

By gastrulation, lacZ, but not zen, transcripts appear at low levels in several patches within the ventral mesoderm (arrows, Fig. 2d), whereas expression along the dorsal surface is normal. Ventral expression persists in the anterior midgut (AMG) and mesoderm during germ-band elongation (Fig. 2f). Unlike zen, the 1.6-kb fusion promoter is not expressed in pole cells (cf. Fig. 2e and f). These results indicate that 1.6 kb of zen 5'-flanking sequence is sufficient for the correct initiation of the zen pattern in early embryos, for the later loss of expression from the poles, and for maintenance of high levels of expression in dorsal cells. However, the 1.6-kb promoter shows low levels of expression in ventral tissues, and fails to direct expression in the pole cells. Late ventral expression does not appear to be a property of the zen promoter, but instead results from sequences contained

![Figure 2](https://example.com/figure2)

**Figure 2.** Expression of the full-length zen promoter. Embryos were prepared from a transformed line carrying the 1.6-kb zen–lacZ fusion in the ry vector, and adjacent sections were hybridized with a zen cDNA probe (left) and a lacZ probe (right). Sagittal sections are oriented so that anterior is to the left and dorsal is up. (a and b) Cleavage stage 12–13 embryo. Both zen (a) and lacZ (b) transcripts are detected along the dorsal surface and around the anterior and posterior poles. (c and d) Gastrulating embryo. zen and lacZ transcripts are confined to the dorsal-most regions. Note that expression is not uniform and gaps in the pattern are observed. Low levels of lacZ expression can be seen on the ventral surface (arrows, d). (e and f) Embryo near the completion of germ-band elongation. The ectopic expression of lacZ in the mesoderm is quite apparent. Dorsal expression becomes largely restricted to the differentiating amnioserosa. The pole cells within the posterior midgut invagination (PMG) are clearly labeled by the zen probe (e), but not by the lacZ probe (f). (AMG) anterior midgut invagination; (PC) pole cells; (PMG) posterior midgut invagination.
within the rosy (ry) transcription unit (see Fig. 1b and below).

ry contains a mesoderm enhancer element

Deletions of distal sequences of the zen promoter give progressively stronger expression in the ventral mesoderm of cellularizing and gastrulating embryos. To identify zen promoter sequences involved in this late ventral expression, we examined the activities of additional truncated promoters, as summarized in Figure 1a. Three of these promoters, which contain only 300, 200, and 42 bp of 5'-flanking sequences, give about the same pattern of expression, as summarized for the 200-bp promoter in Figure 3. During pre-cycle 14 stages, uniform expression is observed around the entire circumference of the embryo (data not shown). This expression pattern is very transient, and during cellularization, transcripts are lost from all regions of the embryo except the ventral-most 20-30%, which corresponds to the presumptive mesoderm. Little or no expression is observed in dorsal regions [Fig. 3b, compare with a]. During gastrulation, the ventral expression can be seen to coincide with the invaginating mesoderm [Fig. 3d], whereas zen expression is confined to the dorsal-most cells, which give rise to the amnioserosa [Fig. 3c].

The observation that as little as 42 bp of zen upstream sequences can direct mesoderm-specific expression was unexpected and not predicted by previous studies on the pattern of zen expression in various D/V mutants [Rushlow et al. 1987b; C. Rushlow and M. Levine, in press]. Consequently, we tested the possibility that this mesoderm expression is not a property of the zen promoter, but instead might involve fortuitous regulatory sequences present in the P-transformation vector. The ry gene is used as a marker for establishing transformed lines and resides just upstream from the zen-lacZ promoter fusions within the P-element vector [Rubin and Spradling 1982, see Fig. 1b]. Conceivably, enhancer elements contained within the ry transcription unit could influence the activities of the closely linked zen-lacZ fusions. To test this, we analyzed the pattern of ry expression in early, wild-type embryos [Fig. 4].

ry transcripts are not detected until cleavage cycle 14, considerably later than the first appearance of zen products [Fig. 4a and b]. At this time, expression extends along the ventral surface and wraps around the anterior pole. During the next 10–20 min, expression intensifies in ventral regions and comes to include the posterior pole as well as the anterior pole [Fig. 4c and d]. Polar and ventral expression persist through gastrulation and germ-band elongation. A horizontal section through a gastrulating embryo clearly displays the polar expression [Fig. 4e and f]. Examination of embryos undergoing germ-band elongation shows that the ventral expression corresponds to AMG invagination and mesoderm [Fig. 4g and h]. The expression seen in early embryos at the posterior pole becomes included in the posterior midgut invagination and corresponds to the anlagen of the Malpighian tubules. This tissue is a known site of ry expression in third-instar larvae [Clark et al. 1984]. Overall, this early, highly localized pattern of ry expression is
Figure 4. Embryonic expression of the *ry* gene. Tissue sections of wild-type (nontransformed) embryos were analyzed for expression of the endogenous *ry* gene. Sagittal sections are oriented so that anterior is to the left and dorsal is up. Bright-field photomicrographs are shown on the left, with corresponding dark-field photographs on the right. (a and b) Early cleavage cycle-14 embryo. *ry* transcripts are detected first along the ventral surface and more weakly around the anterior pole. (c and d) Cellular blastoderm embryo. Expression increases on the ventral surface and is also seen around both the anterior and posterior poles. No expression is detected on the dorsal surface. (e and f) Horizontal section of early gastrula. The polar expression is strong in regions corresponding to the presumptive AMG and PMG. (g and h) Embryo undergoing germ-band elongation. *ry* RNAs are detected in the mesoderm along the length of the germ band, extending to the PMG. Slightly stronger expression is seen in the AMG and extends around the anterior pole. Virtually no expression is detectable on the dorsal surface. |AMG| anterior midgut invagination; |CF| cephalic furrow; |ect| ectoderm; |ms| mesoderm; |PC| pole cells; |PMG| posterior midgut invagination.

virtually identical to the *lacZ* patterns observed for the 300-, 200-, and 42-bp *zen* promoters. It seems quite likely that the ventral expression of the different truncated *zen* promoters results from one or more enhancer elements contained within the *ry* transcription unit. An interesting implication of these results is that *ry* may be a target gene for a ventrally localized D/V gene such as *twist* (Thisse et al. 1987).

The distal region of the *zen* promoter is required for ventral repression

To eliminate the effects of the putative *ry* ventral enhancer on truncated *zen* promoters, we cloned several *zen*-*lacZ* fusions (see Fig. 1a) into the CaSpeR transformation vector, which contains the *white* (*w*) gene as a detectable eye color marker (Fig. 1b; V. Pirrotta, pers. com. 1987).
By in situ hybridization to embryo tissue sections, w was shown to be expressed in embryos undergoing germ-band elongation, but not in earlier stages of embryogenesis (Fjose et al. 1984). Therefore, we do not expect that w would have any enhancer-like effects on the early expression of zen-lacZ fusions. To confirm this we performed in situ hybridizations using a w RNA probe and found that w transcripts are detected first within the presumptive Malpighian tubules in embryos completing germ-band elongation (data not shown). When placed in the CaSpeR vector, the full-length 1.6-kb zen-lacZ promoter fusion shows a completely normal zen pattern; lacZ transcripts are not detected above background levels in ventral regions of embryos undergoing cellularization, gastrulation, or germ-band elongation (data not shown). This observation supports our view that the ventral expression seen in Figure 2d and f is not a property of zen regulatory sequences, but instead is because of ry sequences in the Carnegie 20 vector.

The 1.0-kb promoter fusion that lacks the distal 600 bp shows strong misexpression in ventral regions as early as transcripts can be detected (approximately cleavage cycle 11–12). Figure 5 compares the endogenous zen transcript pattern with the lacZ transcript pattern from the 1.0-kb fusion. The endogenous zen transcripts are seen along the dorsal surface and around the anterior and posterior poles of the embryo. In contrast, lacZ transcripts are detected in both dorsal and ventral regions, but do not extend around the poles (Fig. 5a and b). The ventral misexpression of the 1.0-kb promoter is transient, and only very low levels of ventral expression are detected after cellularization (Fig. 5d). High levels of lacZ expression are seen in the amnioserosa and optic lobe primordia during gastrulation and germ-band elongation (Fig. 5f) comparable to that seen with the endogenous zen gene (Fig. 5c). These results suggest that the distal region of the zen promoter contains sequences required for early ventral repression. It also appears that early ventral repression can be uncoupled from the later
maintenance and refinement of the *zen* pattern, because the 1-kb promoter shows normal loss of expression in lateral regions and continuous expression in the dorsal-most cells during cellularization and gastrulation (see below).

Additional promoter fusions were prepared to define more accurately the limits of the distal ventral repression sequence. A truncated 1.4-kb promoter shows normal dorsal-on/ventral-off expression (cf. Fig. 6a with Fig. 2a and b), whereas a 1.3-kb promoter shows early ventral expression, similar to that observed with the 1-kb promoter (cf. Fig. 6c with Fig. 5b). This result suggests that the 5' boundary of the repressor is located between -1.4 and -1.3 kb upstream from the *zen* transcription start site. The 5' and 3' boundaries of the repression element were delineated further by examining the activities of promoters containing internal deletions in distal regions (see Fig. 1a). A *zen* promoter fusion containing an internal deletion between approximately -1.5 and -1.1 kb (Δ*Nrul*) shows early ventral expression similar to the -1.3-kb truncation, indicating that sequences within this deletion are required for early ventral repression (Fig. 6d). In contrast, a deletion between approximately -1.2 and -820 bp (Δ*Xmn1*) does not have a discernible effect on the expression of the full-length promoter and gives early ventral repression (Fig. 6b). A more striking comparison between the Δ*Xmn1* pattern and the expression obtained with the truncated -1.3 kb promoter is obtained by examining cross sections (Fig. 6e and f). These results suggest that the maximum limits of the distal ventral repression element map between -1.4 and -1.2 kb. Preliminary experiments indicate that the Δ*RsaI* deletion (between -1.3 and -750 bp) also is not repressed ventrally, indicating that the region between -1.3 and -1.2 kb is critical for repression (data not shown). Note that the -1.4 kb and Δ*Xmn1* promoters are repressed ventrally and also show expression around the anterior and posterior poles. In contrast, the -1.3 kb and Δ*Nrul* promoters do not drive expression around the poles, indicating that the putative ventral repressor element and the polar expression element map to the same region of the *zen* promoter.

The ventral repression element can act on a heterologous promoter

Studies on truncated *zen* promoters indicate that the region from -1.4 to -1.2 kb is necessary for early ventral repression. To determine whether the distal region alone is sufficient for repression, we attached *zen* 5'-flanking sequences to a promoter that is expressed at uniform levels in both dorsal and ventral regions of precellular embryos. For this purpose we used the proximal promoter of the gap gene *hunchback* (*hb*), which is expressed transiently in the anterior 40% of early embryos in response to maternal *bcd* protein (Schröder et al. 1988). This pattern is obtained with a 750-bp region of the *hb* promoter attached to the *hsplO* basal promoter (G. Struhl, pers. comm.). Ventral repression of this *hb–hsp70* fusion promoter is obtained when distal *zen* sequences, from -1.6 to -1 kb, are placed upstream of the *hb* promoter (summarized on the bottom of Fig. 7; see Materials and methods). Figure 7 shows adjacent sec-

![Figure 6. Expression of intermediate truncations and distal internal deletions. Embryos were collected from transformants carrying the 1.4- and 1.3-kb truncations, or the Δ*Xmn1* and Δ*Nrul* internal deletions. All sections were hybridized with the lacZ probe and are oriented anterior to the left and dorsal up. Cross sections are oriented so that dorsal is up. (a) Precellular stage-14 embryo containing the 1.4-kb promoter. lacZ transcripts are detected along the dorsal surface and around the anterior and posterior poles. (b) Precellular embryo containing the Δ*Xmn1* internal deletion. lacZ transcripts are restricted to the dorsal surface and around the poles, as seen in a. Expression is not observed in ventral regions. (c) Precellular embryo carrying the 1.3-kb promoter. lacZ transcripts are distributed uniformly in both dorsal and ventral regions of the embryo. (d) Precellular embryo carrying the Δ*Nrul* internal deletion. As seen for the 1.3-kb promoter, lacZ is expressed in both dorsal and ventral regions. Note that the embryos in c and d do not show expression around the anterior and posterior poles. A comparison of cross sections through embryos containing the Δ*Xmn1* promoter (e) and 1.3-kb promoter (f) shows the extent of ventral derepression around the circumference of the embryo. These *zen–lacZ* fusions were analyzed in the *ry* vector.](https://www.journals.org/cshlpress/genesdev/1525_Figure6.png)
tions through two different embryos containing the zen--hb--hsp70 heterologous promoter. The tissue sections in Figure 7a and b were hybridized with a hb probe and show the normal pattern of endogenous hb products. Expression of the endogenous hb gene is initially detected broadly around the anterior pole and in a more restricted domain around the posterior pole. The hb--hsp70 fusion promoter directs expression in the anterior region but not in the posterior domain [G. Stuhl, pers. comm.; data not shown]. The sections in Figure 7c and d were hybridized with a lacZ probe and show the activity of the zen--hb--hsp70 heterologous promoter. Strong expression extends along the dorsal surface and wraps around the anterior pole. In contrast to the pattern of endogenous hb products, expression is not observed along the ventral surface in the middle body region. This result suggests that the distal zen sequences interfere with the ability of the hb promoter to drive expression in ventral regions.

The proximal region of the zen promoter is required for the refinement of the pattern

The proximal half of the zen promoter appears to contain the regulatory sequences responsible for both the maintenance of high levels of zen expression in dorsal tissues and the loss of expression from dorsal--lateral regions during cellularization and gastrulation. Truncations and internal deletions in distal regions of the zen promoter do not significantly disrupt expression in dorsal tissues [see Fig. 5f]. Furthermore, these promoters show a normal refinement of the zen pattern because expression becomes restricted to the dorsal-most 10% of the embryo’s circumference during gastrulation. In contrast, short promoter fusions, which include 300 bp or less of zen 5'-flanking sequences, are essentially inactive and fail to drive dorsal expression [see Fig. 3d]. Evidence that the maintenance and refinement of the zen pattern depends on regulatory sequences located within the first 660 bp of the zen promoter is shown in Figure 8. These embryos were transformed with a zen--lacZ fusion promoter containing the proximal-most 660 bp of zen 5'-flanking sequences. An essentially normal pattern of expression is observed along the dorsal surface of cellularizing embryos [Fig. 8a.] Strong expression is detected in middle body regions, with a gap separating the labeling in the presumptive amnioserosa and optic lobe, as is seen for the normal zen pattern. Examination of cross sections clearly reveals strong, refined expression in the dorsal-most ectoderm [Fig. 8b]. Because the experiment shown in Figure 8 was performed with the Carnegie 20

Figure 7. Ventral repression of a heterologous hb promoter. Sections were prepared from embryos transformed with a heterologous zen--hunchback (hb) promoter fusion, and hybridized with a hb probe [a and b] or a lacZ probe [c and d]. The heterologous promoter construct indicating the orientation of zen, hb, and hsp70 sequences is shown below the photomicrographs [see Materials and methods for construction]. All sections are oriented anterior to the left, dorsal up. [a and b] Endogenous hb expression before cellularization. hb transcripts are detected around the anterior pole, in both dorsal and ventral regions, extending to ~60% egg length. In a dark-field micrograph, weaker hb expression is also seen in a smaller domain at the posterior pole [b]. [c and d] Expression of the zen--hb heterologous promoter. Adjacent sections were hybridized with the lacZ probe. In the anterior--ventral region, lacZ transcripts do not extend as far along the ventral surface as on the dorsal surface [c and d]. The hb sequences do not direct expression at the posterior pole, as seen with endogenous hb expression [cf. b and d]. lacZ is weakly expressed along the dorsal surface and slightly around the pole, apparently the result of activation by distal zen sequences [d]. (PC) pole cells; [X] XbaI; [H] HindIII.

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transformation vector, lacZ expression is observed in the presumptive ventral mesoderm because of the ry enhancer. Because no dorsal expression is seen for the −300 and −200 bp truncated promoters, it appears that a dorsal enhancer lies between −660 and −300 bp.

To identify sequences that might mediate the refinement of the zen pattern, we examined the activity of a 260-bp internal deletion within the full-length 1.6-kb promoter between −300 and −42 bp (Δ Accl). The expression of this promoter appears normal during precellular stages [Fig. 9a] compared to the endogenous zen pattern [Fig. 9b]. However, after cellularization, an abnormal pattern can be observed as a result of a failure in the refinement process [cf. Fig. 9c with the zen pattern in Fig. 9d]. Strong expression of this deleted promoter persists in lateral regions well after the time when expression driven by the 660-bp promoter becomes restricted to the dorsal-most regions [see Fig. 8a]. Thus, it appears that there are sequences within the proximal region of the zen promoter that mediate the refinement of the zen pattern during cellularization and gastrulation. Moreover, it appears that this element can be uncoupled from the regulatory sequences required for maintaining normal levels of zen expression in the dorsal-most cells.

Discussion

We have shown that the zen promoter is composed of two distinct regulatory domains: a distal region, acted
on by maternal products, required for early ventral repression, and proximal sequences required for the refinement and maintenance of the zen pattern through interactions with other zygotic D/V gene products (summarized in Fig. 10). There is a close correlation between the abnormal patterns of expression obtained with defective zen promoters that disrupt these cis elements and the altered zen patterns seen in different maternal and zygotic D/V mutants [Rushlow et al. 1987b; C. Rushlow and M. Levine, in press].

Figure 10. Summary of zen-lacZ expression patterns. [a] The three columns represent the major aspects of the wild-type zen expression pattern. The amount of 5’ zen-flanking sequences for each promoter fusion is indicated left. The promoter displays a normal activity for this aspect of the zen pattern (+). The 0.3-, 0.2-, and 42-bp promoters are expressed at only very low levels in dorsal regions, and therefore the refinement of the pattern cannot be assessed (?). [b] Summary of the cis elements that constitute the zen promoter. The zen promoter can be separated into two domains, containing at least three major regulatory elements: [1] a distal sequence located between -1.4 and -1.2 kb that is responsible for early ventral repression; [2] a sequence located between -0.7 and -0.3 kb that is required for maintaining high levels of expression on the dorsal surface; and [3] proximal sequences that are required for repression in dorsal-lateral regions. The boundaries of this latter element are difficult to identify because truncated promoters give only weak dorsal expression. The dorsal protein either directly or indirectly represses early ventral expression, possibly by interacting with the distal ventral repression element of the zen promoter. The proximal promoter sequences control both the maintenance and refinement of the zen pattern and may be acted on by zygotic D/V gene products. At least some of these zygotic interactions are indirect, suggesting that a cell-cell communication pathway may be involved in the modulation of zygotic D/V gene activities.
The distal ventral repression element acts as an anti-enhancer

The early dorsal-on/ventral-off pattern of zen expression depends on a ventral repression (VR) element located between −1.4 and −1.2 kb upstream from the zen transcription start site (summarized in Fig. 10). Disruptions of VR sequences cause strong expression of zen promoter fusions in ventral regions of precellular embryos. Ectopic expression in ventral regions occurs quite early, at the time when zen transcripts are detected first at the dorsal surface during normal development (cleavage cycle 10–11). The VR element appears to function only transiently during early development, which is consistent with the suggestion that it is regulated by a maternal trans-acting factor. Older embryos show nearly normal patterns of expression from truncated zen promoters that lack the VR element (see Fig. 5). The boundaries of the VR element were determined by examining the expression of zen fusion promoters containing various internal deletions or distal truncations (summarized in Fig. 10).

Previous analyses of zen expression in dorsal—ventral mutants suggest that the maternal dorsal-group genes specify a repressor that keeps zen off in ventral regions (Rushlow et al. 1987b). Several lines of evidence suggest that dorsal may correspond to the maternal repressor. First, double mutant studies suggest that the primary role of the other dorsal-group genes is to localize dorsal activity to ventral regions (Anderson et al. 1985). Thus, the removal of any one of these genes results in the absence of active dorsal products, which is responsible for the observed derepression of zen in ventral regions of early embryos (Rushlow et al. 1987b). Second, the dorsal protein may correspond to a transcription factor because it shares extensive homology with the nuclear oncogene rel (Gilmore and Temin 1986; Steward 1987). And finally, it has been shown that the distribution of the dorsal protein is graded along the D/V axis, with peak levels present on the ventral surface (Steward et al. 1988). The finding that none of the known zygotic D/V mutants alter the initial zen pattern supports the notion that dorsal or other maternal factors repress zen expression directly (C. Rushlow and M. Levine, in press, K. Arora, pers. comm.).

Our working model is that the dorsal protein interacts with or alters an unknown sequence-specific DNA-binding protein (X), and the dorsal—X complex binds to the VR and represses zen expression. Preliminary studies suggest that tel is a promiscuous transcriptional regulator that lacks sequence specificity (Gelinas and Temin 1988). Protein X may be a ubiquitous factor, involved in regulating many genes, that has not been identified in previous genetic screens. The characterization of protein X may depend on biochemical methods, such as affinity purification using DNA sequences from the zen VR element.

The direct or indirect regulation of the zen VR element by dorsal is of considerable interest as a paradigm for the way in which target promoters make qualitative on/off choices in response to a morphogen gradient. The VR element is somewhat unusual because it is located at the distal end of the zen promoter, far from the transcription start site. Distantly located eukaryotic cis-repressor sequences often work through an indirect mechanism, whereby they interfere with the binding of positive control factors to a neighboring enhancer (for review, see Maniatis et al. 1987). The zen VR sequence does not appear to function in this way. Instead, the binding of repressor to the VR appears to act over a long distance to influence the activity of the basal promoter, similar to the activity of a yeast silencer sequence (Brand et al. 1984). The exact spacing of the VR relative to the transcription start site does not appear to be critical because a 1.6-kb promoter that contains internal deletions of several hundred base pairs shows normal ventral repression. An additional property that the VR shares with silencers is that it can act on different basal promoters. We have shown that the distal half of the zen promoter can repress the ventral expression of a heterologous hb promoter (Fig. 7).

Activation elements

The initiation of zen expression in precellular embryos may involve redundant regulatory sequences that are scattered at several sites within the zen promoter. The proximal half of the zen promoter is sufficient for initiation because truncated promoters [i.e., −1 and −0.7 kb] are activated ubiquitously in dorsal and ventral regions of cleavage cycle 11–12 embryos. At least some of these proximal sequences are not required for initiation, because the internal deletion ∆AccI does not affect the initial level of expression. In addition, the distal half of the zen promoter appears to contain weak activation sequences. The zen−hb heterologous promoter, which includes sequences between −1.6 and −1 kb, drives weak expression along the dorsal surface and around the posterior pole (see Fig. 7). The maintenance of zen expression after cellular blastoderm formation may depend on a discrete regulatory element (dorsal activation) located between −660 bp and −300 bp. Truncations of the zen promoter down to −300 bp essentially eliminate high levels of zen expression on the dorsal surface, whereas an internal deletion that removes sequences between −300 and −50 bp does not reduce expression. This proximal activation element can act independently as an enhancer. Within the context of the P-element vector, the zen promoter has a positive effect on ry expression, and causes ry to be expressed in the dorsal region of gastrulating embryos (H. Doyle, unpubl.). This enhancement activity functions from the 3’ end of the ry transcription unit and acts over a distance of at least 7 kb from the ry transcription start site (see Fig. 1b).

The distal region between −1.4 and −1.2 kb not only contains the VR element, but is also required for the transient activation of zen at the poles. zen is not expressed at the poles in embryos derived from the terminal class maternal mutants torso and trunk (Schüpbach and Wieschaus 1986), although the middle body
dorsal-on/ventral-off pattern is normal in these mutants (Rushlow et al. 1987b). torso or trunk products may directly or indirectly activate zen expression at the poles. Alternatively, they may function as anti-repressors. The dorsal protein has been shown to accumulate at the poles (Steward et al. 1988), and it is possible that torso or trunk products (or both) prevent dorsal from repressing zen at these sites. Thus, the absence of polar zen expression in torso and trunk mutants may result from the unmasking of a dorsal repressor activity.

Refinement of the pattern

The restriction of zen products to the dorsal-most 10% of the embryo's circumference depends on regulatory sequences located in the proximal region of the zen promoter. An internal deletion within this proximal region shows strong, persistent expression in dorsal—lateral regions of embryos after gastrulation (see Fig. 9). Despite our failure to identify precise limits for regulatory elements that mediate the refinement of the zen pattern, it is clear that these sequences can be uncoupled from the dorsal activation element. The Δ AccI deletion gives strong expression in dorsal regions of older embryos, but is defective in its ability to repress expression in lateral regions.

The patterns of zen expression obtained with defective promoters that disrupt proximal regulatory sequences are similar to the abnormal patterns observed in certain zygotic D/V mutants. For example, dorsal—lateral persistence of zen expression is observed in the zygotic D/V mutant sog (Wieschaus et al. 1984; C. Rushlow and M. Levine, in press). It is likely that sog exerts an indirect effect on the proximal promoter because mosaic studies have shown that sog⁺ activity is required in ventral regions of gastrulating embryos (Zusman et al. 1988). In contrast, there is a premature loss of zen expression in dpp—embryos, similar to that obtained with several of the truncated promoters. The regulatory effect that dpp exerts on zen is almost certainly indirect because dpp encodes a protein that is closely related to the secreted mammalian growth factor TGF-β (Padgett et al. 1987). Given the nature of the dpp product, and the site of genetic function of sog, it appears that the refinement and maintenance of the zen pattern depends on interactions among several zygotic D/V gene products and may be mediated through a cell—cell communication pathway.

Several mechanisms can be envisioned to account for the regulation of zen proximal promoter elements by zygotic D/V genes such as sog and dpp. One possible model is that a transcriptional activator maintains zen expression during gastrulation and germ-band elongation by interacting with dorsal activation sequences located between −660 and −300 bp. This putative activator may be distributed broadly in dorsal and dorsal—lateral regions, but absent in ventral regions. It is conceivable that the zen protein corresponds to the activating factor because there is a premature loss of zen transcripts in zen⁻ embryos (C. Rushlow, unpubl.). The binding of repressors to the lateral repression element within the proximal promoter may prevent the interaction of the activator with the neighboring activation sequences. According to this model, the spatial refinement of the zen pattern depends on the selective activity of the repressor in dorsal—lateral regions, which is superimposed on a more broadly distributed activator.

Two-tier organization of early-acting patterning genes

We have shown that the zen promoter contains two distinct regulatory domains. The distal region appears to respond directly to crudely localized positional cues, possibly involving the dorsal morphogen. The proximal half of the promoter functions later in development to refine the pattern, such that zen products become restricted to the dorsal-most region of gastrulating embryos. A similar two-tier promoter organization was described for the initiation and maintenance of expression of the segment polarity gene, engrailed [en] (DiNardo et al. 1988). Other examples are the promoters of the pair-rule genes eve and ftz, which contain two classes of regulatory elements. One class of elements establishes the basic seven-stripe patterns of eve and ftz expression. These stripes are sharpened and maintained in older embryos via a second class of autoregulatory elements located in the distal regions of the eve and ftz promoters (Hiromi et al. 1985; Hiromi and Gehring 1987; Harding et al. 1989; Goto et al. 1989).

A two-tier organization helps explain how early-acting patterning genes come to be expressed within discrete spatial limits in response to crudely localized positional cues. Such an organization of the zen promoter suggests that the dorsal morphogen serves to define broad on/off states of a number of zygotic D/V genes and that refinement to their final patterns involves cross-regulatory interactions. An additional implication of this model is that zen may be relatively upstream in a genetic hierarchy leading to D/V patterning. In principle, D/V genes further downstream in the hierarchy would only require the proximal zen promoter sequences for restricted expression in dorsal tissues. These genes would not be regulated directly by maternal factors, and consequently such genes need not contain the equivalent of the distal zen VR element.

Materials and methods

zen promoter fusions

zen promoter fragments were fused in-frame with the bacterial lacZ gene. The 3’ region of this fusion includes the entire 52-bp zen-untranslated leader sequence and the initiating ATG of the coding sequence. A BamHI site was created just 3’ of the ATG by in vitro mutagenesis. Two G residues were inserted by use of a 24-nucleotide primer with the sequence: AGAAAAATATCTCGGTATCCGCCCTC. [The inserted Gs are underlined, and the BamHI sites are indicated in brackets]. Mutants were made and selected in M13 as described previously (Zoller and Smith 1984) and analyzed by the dideoxy nucleotide sequencing method (Sanger et al. 1977). zen promoter fragments were cloned into a unique BamHI site located at the ninth...
The **zen** promoter

codon of the *lacZ* gene. The *lacZ* sequences were obtained from the pEL1 plasmid [Lawrence et al. 1987], which includes the tubulin polyadenylation sequences and a unique *XbaI* site at the 3' end. Most of the **zen**-*lacZ* fusions were cloned into the transformation vector pC20X, which is a modification of Carnegie 20 [Rubin and Spradling 1982] that contains a unique *XbaI* site. This transformation vector also contains a 900-bp α-tubulin 'stuffer' fragment that separates the *XbaI* site and the 3' end of the *ry* gene (kindly provided by J. Fischer, derived from pC20X made by P. Macdonald; see Lawrence et al. 1987). The **zen**-*lacZ* fusions were cloned into the *XbaI* site directly, or by blunt-ended ligation. Several of the fusions were cloned initially into pHSS7, which is a kanamycin-resistant vector that contains a poly linker flanked by NotI sites. The **zen**-*lacZ* fusions were subsequently cloned into the unique NotI site of a Carnegie 20 derivative, pDM30 [Mismer and Rubin 1987]. All constructs were oriented so that the direction of transcription of *ry* and the **zen**-*lacZ* genes was the same (see Fig. 1b). Transformation DNAs were injected into *ry*000 embryos according to standard methods [Rubin and Spradling 1982] by use of the pI25.7 helper plasmid [Kearsey and Rubin 1984]. The 0.2-kb **zen**-*lacZ* fusions were also cloned into the unique *XbaI* site of the CaSpeR transformation vector, which were injected into the *w* mutant stock, Df(1w,yw645c209) (vector and strain obtained from V. Pirrotta). The CaSpeR vector contains the *w* gene on a 4.1-kb *Spel* fragment, with ~300 bp of 5'-flanking sequences and 630 bp of 3'-flanking sequences. The 1.6- and 1.0-kb **zen** promoter fusions were oriented with their 5' ends near the 5' *P*-element end, so the direction of transcription is the same as *w* (see Fig. 1b). The 0.2-kb fusion was cloned in the opposite orientation, with its 5' ends adjacent to the 5' end of *w*.

Between 2 and 12 independent transformed lines were obtained for each of the **zen**-*lacZ* fusions analyzed in this study. The different lines for a given fusion exhibited essentially the same patterns of expression on the basis of a histochemical staining assay using anti-β-Gal antibodies. Two independent lines for each fusion were also analyzed by in situ hybridization to tissue sections, and the results presented were obtained for both lines.

The original *hb*-**hsp70**-*lacZ* fusion, called HB586, was made by G. Struhl. A 747-bp *HindIII*-MhuI fragment from −55 to −802 bp upstream of the proximal *hb* transcription start site was inserted into the unique *NotI* site of HZ50-PL [Hiromi and Gehring 1987; G. Struhl, pers. comm.]. The distal 600 bp of the *zen* promoter (from *XbaI* to *HindIII*) sites, see Fig. 1a) were inserted by blunt-ended ligation into the *XbaI* site of HB586. Transformants that contained the HB586 control construct were stained with an antibody against β-Gal and compared with the endogenous *hb* expression. The proximal 750 bp of the *hb* promoter show anterior β-Gal expression comparable to endogenous *hb* [Schröder et al. 1988; G. Struhl, pers. comm.; data not shown]. Several independent transformed lines that contained the **zen**-*hb*-*lacZ* fusion were stained with a β-Gal antibody, one of these was assayed also by in situ hybridization to better compare dorsal and ventral expression.

**In situ hybridization**

Staged embryos from transformed lines were collected and fixed, and tissue sections were prepared as described previously [Ingham et al. 1985; Harding et al. 1986]. A 1.9-kb *BamHI*-Saci fragment from the 5' region of the *lacZ* coding sequence was used as a probe to analyze the activities of each **zen**-*lacZ* promoter and of the **zen**-*hb* heterologous promoter. The **zen** probe that was used corresponds to a near full-length cDNA [Rushlow et al. 1987a]. These DNA fragments were cloned separately into pGEM-1 [Promega] and were labeled with 35S antisense RNA probes that were synthesized by T7 transcription. The *lacZ* and **zen** probes were hybridized to adjacent tissue sections, and autoradiography was done for 2–10 days. The *rY* probe was derived from an ~1-kb *BamHI*-PstI genomic DNA fragment that contained the 5' coding region cloned into pSP64 [a gift from Lee Dutton]. The 35S-labeled antisense RNA probe was synthesized with Sp6 polymerase and hybridized to sections from a balanced *kni*Δ*Abj* TM3, Sb stock. Autoradiographic exposure time was 10 days. The *hb* probe was a 2.4-kb genomic DNA fragment cloned into the *XbaI* site of pGEM-1, and T7 polymerase was used to make an antisense riboprobe after linearization with HindIII. Exposure time was 4 days for the *hb* probe, and 3 days for the *lacZ* probe on the **zen**-*hb* transformants.

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