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Analysis of an *even-skipped* rescue transgene reveals both composite and discrete neuronal and early blastoderm enhancers, and multi-stripe positioning by gap gene repressor gradients*

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SUMMARY

The entire functional *even-skipped* locus of *Drosophila melanogaster* is contained within a 16 kilobase region. As a transgene, this region is capable of rescuing *even-skipped* mutant flies to fertile adulthood. Detailed analysis of the 7.7 kb of regulatory DNA 3′ of the transcription unit revealed ten novel, independently regulated patterns. Most of these patterns are driven by non-overlapping regulatory elements, including ones for syncytial blastoderm stage stripes 1 and 5, while a single element specifies both stripes 4 and 6. Expression analysis in gap gene mutants showed that stripe 5 is restricted anteriorly by *Krüppel* and posteriorly by *giant*, the same repressors that regulate stripe 2. Consistent with the coregulation of stripes 4 and 6 by a single *cis*-element, both the anterior border of stripe 4 and the posterior border of stripe 6 are set by zygotic *hunchback*, and the region between the two stripes is ‘carved out’ by *knirps*. Thus the boundaries of stripes 4 and 6 are set through negative regulation by the same gap gene domains that regulate stripes 3 and 7 (Small, S., Blair, A. and Levine, M. (1996) *Dev. Biol*.175, 314–24), but at different concentrations. The 3′ region also contains a single element for neurogenic expression in ganglion mother cells 4-2a and 1-1a, and neurons derived from them (RP2, a/pCC), suggesting common regulators in these lineages. In contrast, separable elements were found for expression in EL neurons, U/CQ neurons and the mesoderm. The *even-skipped*3′ untranslated region is required to maintain late stage protein expression in RP2 and a/pCC neurons, and appears to affect protein levels rather than mRNA levels. Additionally, a strong pairing-sensitive repression element was localized to the 3′ end of the locus, but was not found to contribute to efficient functional rescue.

Keywords

even-skipped; CNS; Pattern formation; Segmentation; Translational control; Chromatin

INTRODUCTION

A primary pair-rule gene, *even-skipped* (*eve*) is expressed in a striped pattern in precellular embryos, as well as in several tissues at later stages of embryogenesis (Frasch et al., 1987; Macdonald et al., 1986). *eve* function is required in the early segmentation network to establish

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odd-numbered parasegment primordia, as well as to establish all of the parasegment boundaries (Macdonald et al., 1986). *eve* first appears as a single broad band in early nuclear division cycle 12, which then begins to split into stripes. By nuclear cycle 14, *eve* is expressed as a regular pattern of seven stripes (the early stripes) in the primordia of the odd-numbered parasegments. This pattern of broad early stripes then gives way during cycle 14 to narrow 'late' stripes with sharply demarcated anterior borders, where the segment polarity gene *engrailed* will be expressed (Ingham et al., 1988; Lawrence et al., 1987). *eve* is also required for *engrailed* expression in the anterior-most cell row of the even-numbered parasegments, where weak *eve* expression is observed (the minor stripes) at the same time as the late stripes. In addition, stripe 1 is required for cephalic furrow formation (Vincent et al., 1997) and *eve* function is required for proper germband extension (Irvine and Wieschaus, 1994).

Previous studies focused on *cis*-regulatory elements upstream of the *eve*-coding region. Individual elements were defined for early stripes 2 and 3, and a single element for all seven late stripes. Early stripe 7 can be driven by a region that includes either the stripe 2 or the stripe 3 element (Goto et al., 1989; Harding et al., 1989; Small et al., 1996).

Detailed analysis of *eve* expression in gap and pair-rule mutants established that gap genes regulate the early stripes directly, while pair-rule genes are required for the proper expression of late stripes (Frasch and Levine, 1987). Reporter transgenes driven by elements for stripes 2, 3 and 7 give the same response in gap gene mutants as the endogenous gene (Goto et al., 1989). The stripe 2 regulatory element requires both the Bicoid protein and the *hunchback* (*hb*) gap gene product for its activation, while the anterior and posterior borders are formed by the repressive action of *giant* (*gt*) and *Krüppel* (*Kr*), respectively (Small et al., 1991,1992; Stanojevic et al., 1989,1991; Wu et al., 1998). Stripes 3 and 7 are activated by ubiquitously distributed factors including *D-STAT* (Hou et al., 1996; Yan et al., 1996), and their borders are set through negative regulation by *knirps* (*kni*) and *hb* (Small et al., 1996; Stanojevic et al., 1989).

Expression of the *eve* late stripes is driven by a single upstream element. This 'late element' is regulated by the pair-rule genes *paired* (Fujioka et al., 1995,1996) and *runt* (Goto et al., 1989) as well as by early *eve* expression (Fujioka et al., 1995; Goto et al., 1989; Harding et al., 1989). The early, broad stripes of Eve protein act in a concentration-dependent manner to repress both the activator *paired* as well as repressors of late element expression. The repressors are sensitive to lower Eve concentrations, generating a narrow zone at the edge of each early stripe where a late stripe is activated (Fujioka et al., 1995). Early *runt* stripes overlap the posterior portion of early *eve* stripes and provide 'polarity' by preventing late expression there (Fujioka et al., 1995).

As germband extension proceeds, the seven late *eve* stripes begin to fade, while a new, 8th stripe appears in the posterior region (Frasch et al., 1987; Macdonald et al., 1986). The anterior border of this stripe corresponds with that of engrailed stripe 15 (Lawrence et al., 1987). While the germband is shortening, *eve* is expressed as a ring surrounding the anal plate (Frasch et al., 1987) and continues to be expressed there after shortening is complete. Posterior embryonic *eve* expression is apparently conserved through evolution. In the grasshopper, the *eve* homolog is expressed at the germband stage in a ring of tissue at the anal plate, as well as in patterns similar to those in *Drosophila* in identified neurons and in the dorsal mesoderm (Patel et al., 1992,1994). Additionally, *eve* homologs in *Caenorhabditis elegans* (Ahringer, 1996) and in zebrafish (Joly et al., 1993) were shown to function in the specification of posterior cell fates while, in the mouse, posteriorly biased expression is seen in the primitive streak and the tail bud (Bastian and Gruss, 1990; Dush and Martin, 1992).

Patterned *eve* expression is observed in the developing nervous system (Frasch et al., 1987; Patel et al., 1989). Ganglion mother cells (GMCs) 1-1a and 7-1a express *eve* at stage 10, and continue to do so while dividing to produce the aCC/pCC sibling neurons and the U/CQ/fpCC neurons, respectively (Bossing et al., 1996; Broadus et al., 1995). At early stage 11, expression is seen in GMC 4-2a. This GMC divides to produce the RP2 neuron, which continues to express *eve*, and the RP2 sibling, which extinguishes *eve* expression (Broadus et al., 1995). At late stage 12, expression occurs in a lateral cluster of neurons (EL cells; Patel et al., 1989) derived from neuroblast 3–3 (Schmidt et al., 1997). These cells maintain *eve* expression at high levels throughout embryogenesis. The CNS function of *eve* was analyzed in a subset of *eve*-expressing neurons using a temperature-sensitive *eve* allele (Doe et al., 1988). Removal of *eve* function during CNS development was found to change the axonal projections of the aCC and RP2 neurons. This led to the suggestion that *eve* controls the fates of these neurons, since their axons showed a preferred alternate morphology. From stage 11 onwards, *eve* is also expressed in a small subset of cells in the dorsal mesoderm, including some pericardial cells (Frasch et al., 1987) and the dorsal-most somatic muscle (Bodmer, 1993), DA1 (nomenclature of Bate, 1993).

Here, we show that a 15.6 kb genomic region is sufficient to rescue the lethality of *eve* null mutants, and we describe the localization of regulatory elements required for *eve* expression in several tissues. We show that previously uncharacterized early stripe elements are negatively regulated by the gap genes, with a separable elements for stripes 1 and 5 but a composite element for stripes 4 and 6. Stripes 4 and 6 are regulated by the same gap genes as stripes 3 and 7, but apparently at quite distinct concentrations. Neuronal elements are also separable for some lineages, but composite for others, suggesting an underlying commonality of upstream regulators. The 3′ untranslated region (UTR) is required for efficient late stage protein expression, while a chromatin control region at the edge of the locus did not apparently facilitate function.

MATERIALS AND METHODS

Drosophila strains

Injection procedures were as described previously (Rubin and Spradling, 1982) with some modifications (Fujioka et al., 1999). The alleles used for mutant analysis were hb^{14F}, Kr^{2}, gt^{X11}, kni^{1}, tll^{G}, *Df(2R)eve*, eve^{3} (eve^{R13}), eve^{1} (eve^{ID19}), $runt^{LB5}$, $hairy^{I22}$, prd^{4}, $fish^{87}$, *mrl (stat92E)*, ftz^{KMQ}, $Df(2L)ed^{sZ1}$*(for slp)* and *odd[7L]*

Construction of transgenes

All *lacZ* reporter constructs (unless noted) were made using a modified C3D vector (Fujioka et al., 1996) in which *KpnI* and *Xba* I sites were introduced downstream of the α-tubulin polyadenylation signal, providing cloning sites for the 3′ deletion fragments. All 3′ deletions were cloned into a modified pSP72 vector flanked 5′ by a *Kpn*I site (also by *Not*I) and 3′ by an *Xba*I site, then transferred to the C3D vector. This places each fragment in an orientation and position relative to the promoter like that in the endogenous gene. In order to test the *eve* 3′ untranslated region (UTR) with the RP2+aCC/pCC element, a modified C3D vector was used in which *Not*I and *Xba*I sites were introduced upstream of *lacZ*, carrying either the α-tubulin 3′ UTR, or the *eve* 3′ UTR from +1306 (*Bst*U1 site) to +1542 (*Kpn*I site). The 5′ endpoint of the *eve* 3′ UTR was chosen based on a previous report (Kosman and Small, 1997). The RP2 element, from +7843 (*Eco*RI site) to +9235 (*Eco*RI site), was placed in opposite orientation relative to its normal 5′ to 3′ direction. Five to ten independent transgenic lines were analyzed for each *lacZ* construct.

For rescue experiments, the region +1.85 to either +8.4 or +9.2 kb was added downstream of the *eve*-coding region in the localized rescue construct E+L-*eve* (Fujioka et al., 1995), so that the contiguous region was restored exactly as in the endogenous gene, from −6.4 to either +8.4 or +9.2 kb. Many of the rescued lines initially showed a faint, difficult to detect eye color. To avoid this problem, the region upstream of the *Dra*I site in the *mini-white* gene promoter was replaced with Glass activator (Ellis et al., 1993) binding sites (a kind gift of Bruce Hay), resulting in much stronger *white* expression in the eye, and no detected changes in *eve* expression in embryos. Details are available on request.

Embryo analysis

In situ hybridization to whole-mount embryos using digoxigenin (DGG)-labeled antisense mRNA probes was performed as described (Tautz and Pfeifle, 1989). This was followed by antibody staining with anti-Eve antibody (kindly provided by Manfred Frasch). Biotinylated secondary antibody was detected using streptavidin-conjugated horseradish peroxidase (Chemicon International Inc.), as described (Mullen and DiNardo, 1995). Hatching rates were determined 36 hours after collection by counting both hatched and unhatched egg casings. Unhatched embryos were collected and subjected to cuticle preparation without devitellinization (data in Table 2; to determine phenotype of rescued embryos, cuticles were prepared prior to hatching). Briefly, after dechorionation, embryos were washed three times with the PBS/0.1% Tween 20, transferred onto slides, dried by blotting, and a 1:1 mixture of Hoyer's reagent and lactic acid was added. The embryos were cleared by incubation at 55°C. All other incubations were at room temperature.

Sequencing of the eve^{R13}-coding region

eve^{R13} stocks were obtained from two different laboratories. Embryo DNA (a mixture of wild-type and eve^{R13} chromosomes) were extracted as described (Jowett, 1986). PCR reactions were done using primers from the transcription initiation site (+1) and from +1540, downstream of the polyadenylation site. PCR-amplified DNA from at least four independent reactions with template DNA from each stock were sequenced. After discovery of a single mutation in the eve^{R13} reactions, relative to wild-type sequences (Ludwig and Kreitman, 1995), which eliminates a *Pvu*II site at +488, these PCR products were also cloned into the pSP72 vector (which does not have a *Pvu*II site), and *Pvu*II digestion was used as a selection for eve^{R13} DNA. Two independent clones were sequenced in both directions, and the alteration at the *Pvu*II site was present in both. Genomic DNAs from two eve^{R13} lines and a wild-type line were cut by *Xho*I and *Pvu*II and subjected to Southern analysis, using a probe made from the same region (+7 to +1542). With wild-type DNA, fragments were detected of the expected sizes, 406, 75 and 1138 bp. With eve^{R13} DNA, which contains both wild-type and mutant chromosomes, an additional fragment of about 1213 bp was detected, consistent with the absence of the *Pvu*II site and confirming the point mutation on the *R13* chromosome.

RESULTS

Characteristics of stripe elements

Previously, the regulatory elements for early stripes 1 and 5 were localized to between +6.6 and +8.4 kb, and for stripes 4 and 6 to between +4.8 and +6.6 kb (Sackerson et al., 1999). To test if each of the stripes could be driven individually by separable elements, deletions were made in each region in the context of a *lacZ* reporter (the deletions are summarized in Fig. 1). The results showed that there are separable elements for stripes 1 and 5. The stripe 1 element was localized to between +6.6 and +7.4 kb (Fig 1 #35, Fig 2A), and the stripe 5 element between +7.4 and +8.4 kb (Fig 1 #30, Fig 2B). The apparent extension of *lacZ* RNA expression into the posteriorly adjacent parasegment is due at least in part to the sensitivity of the assay. Endogenous protein expression can also be seen to extend into these regions, albeit at relatively

low levels. Shortening the stripe 5 element to +7.9 kb (Fig. 1 #32) gave weaker but correctly localized expression, while shortening it to +8.2 kb (Fig. 1 #31) did not decrease the level of expression. Interestingly, both stripes 4 and 6 were driven by the region +4.5 to +5.2 kb (Fig. 1 #21), and also by the region +4.8 to +5.8 kb (Fig. 1 #37, this gave somewhat lower level expression than the former fragment), while the shared region of +4.8 to +5.2 kb (Fig. 1 #22) gave weak expression of both stripes, suggesting that these stripes share regulatory inputs.

Genetic interactions with segmentation genes

In order to investigate the regulatory mechanisms responsible for establishment of these stripes, a *lacZ* reporter transgene carrying the elements for stripes 1 and 5 (+6.6 to +8.4 kb; Fig 1 #40, Fig 2C) and one carrying the stripe 4+6 element (+4.0 to +5.2 kb; Fig 1 #20, Fig 2D) were individually crossed into several gap and pair-rule mutant backgrounds.

In zygotic *hb* mutants, stripe 1 is weakened while stripe 5 is unaffected (or may be weakened slightly). Stripe 4 expands anteriorly and stripe 6 expands posteriorly, suggesting that *hb* sets the outside borders of expression of the stripe 4+6 element (Fig. 2E,F). The weakened expression may be explained by broadened Knirps expression (Hülskamp et al., 1990; Kraut and Levine, 1991b), since *kni* negatively regulates this element (see below).

In *Kr* mutant embryos, stripe 5 expands anteriorly and weakens (Fig. 2G), suggesting that its anterior border is defined by *Kr*. The weakening is consistent with an anterior shift of *gt* expression (Eldon and Pirrotta, 1991;Kraut and Levine, 1991a), which represses this element and appears to set the posterior border (see below). Alternatively, reduced expression of stripe 5 might be due to a change in the relative levels of activators (see below) and repressors within the stripe. Stripe 4 may also be weakened (Fig. 2H) and stripe 6 expands anteriorly. The latter effect is consistent with the reduction in *kni* expression that occurs in *Kr* mutants (Pankratz et al., 1989), since *kni* represses the 4+6 element (see below). The weakening of stripe 4 may be due to an anterior shift of the remaining *kni* expression. Alternatively, it may indicate that activators of the stripe 4+6 element are affected in *Kr* mutants (perhaps in common with those of stripe 5).

In *kni* mutant embryos, the 4+6 reporter is expressed in a single broad stripe covering the region between stripes 4 and 6, inclusive (Fig. 2J). Thus *kni* defines both the posterior border of stripe 4 and the anterior border of stripe 6. Stripe 5 is posteriorly shifted, overlapping with stripe 6, and weakened (Fig. 2I), indicating that *kni* also plays a role in regulating this stripe, possibly through its effects on the patterns of other genes, such as *Kr* (Gaul and Jäckle, 1987; see above and Discussion).

In *gt* mutant embryos, stripe 5 expands posteriorly (Fig. 2K). While there is no apparent effect on stripe 4, stripe 6 is weakened (Fig. 2K,L), consistent with the posterior expansion of *kni* expression (Eldon and Pirrotta, 1991), which sets its anterior border (above). Stripe 1 may also be weakened.

In *tll* mutant embryos, all stripes are posteriorly shifted, especially stripe 6 (Fig. 2M,N), and stripe 7 is missing (Frasch and Levine, 1987), consistent with the shift of other gap gene expression patterns previously described (Bronner and Jäckle, 1991; Kraut and Levine, 1991a; Pankratz et al., 1989). *tll* may help to set the posterior border of stripe 6, since stripe 6 expands posteriorly in the mutant. However, this may be due to the absence of the posterior *hb* domain (Bronner and Jäckle, 1991), since *hb* appears to define this border (above).

There is no strong effect on the boundaries of stripe 1 expression in any of these gap gene mutants.

We also tested the effects of pair-rule mutations on the early stripe elements (data not shown). In *runt* embryos, endogenous Eve stripe 5 expression is delayed and weakened (Frasch and Levine, 1987). While the expression of *lacZ* driven by the stripe 5 element is slightly delayed, it recovers later. In *eve* null mutants, stripe 1 expression is somewhat weakened. In *ftz*, *prd*, *h*, *odd* and *slp*, there are no apparent effects on any of the stripes.

Consistent with previous results suggesting that *fish-hook* may be a direct positive regulator of early *eve* stripes 4, 5 and 6 (Ma et al., 1998; Nambu and Nambu, 1996; Russell et al., 1996), *lacZ* expression in these stripes showed some reduction in the mutant (not shown). When the *marelle* gene (encoding D-STAT) was removed both maternally and zygotically, stripe 5 element expression disappeared, while stripe 1 was unaffected (data not shown), consistent with previous observations (Hou et al., 1996; Yan et al., 1996).

Neuronal elements

eve is expressed in ganglion mother cells (GMCs) and neurons in the central nervous system. Deletion analysis of the 3′ region showed that distinct regulatory elements exist for some aspects of this pattern (the constructs used are summarized in Fig. 1). A regulatory element for GMC 7-1a and the CQ neurons was localized to between +3.5 and +4.3 kb (Fig 1 #14, Fig 3A,B), which overlaps the regulatory element for anal plate ring expression (see below). An element was identified for expression in GMCs 4-2a and 1-1a, and later in their derivatives the RP2, aCC and pCC neurons, between +7.9 and +9.2 kb (Fig 1 #27, Fig 3C,D). Truncating the latter element at +8.4 (Fig. 1 #33) reduced the overall level of expression, while truncating at +8.6 (Fig. 1 #34) did not cause an obvious reduction. Changing the 5′ endpoint to +8.2 (Fig. 1 #28) resulted in a complete loss of activity.

Either of the regions +1.5 to +3.2 kb (Fig 1 #4, Fig 3E) or +1.9 to +4.8 (Fig. 1, #11) could drive *lacZ* expression in EL cells, while neither the fragment +1.5 to +2.6 (Fig. 1 #3) nor +2.6 to +4.8 (Fig. 1 #10) could do so. This suggests a requirement for sequences both upstream and downstream of +2.6 kb. In fact, the region of overlap between the active regions, +1.9 to +3.2 (Fig. 1 #12) or to +3.0 (Fig. 1 #13), which still overlaps the early anal plate regulatory element (see below), was sufficient to drive *lacZ* expression in EL cells.

Stage-specific post-transcriptional regulation

Endogenous Eve expression normally persists strongly in the developing CNS through stage 15 (Fig. 4A–D). The same was true for rescue transgenes (see below) containing the region −6.4 to +8.4 kb. In contrast, when the RP2+a/pCC element was used to drive *lacZ*, although all transgenic lines showed quite high level expression at stage 11, the level of staining faded by stage 15 (Fig. 3C,D). Since the *eve* 3′ untranslated region (UTR) was not contained in the *lacZ* transgenes, we reasoned that it might be required for high level expression in these neurons, particularly after stage 11. To test this possibility, we constructed a vector containing the *eve* 3′ UTR (see Materials and Methods) in place of the α-tubulin 3′ UTR of the original vector. In this construct, and in controls with the original 3′ UTR, the element was placed upstream of *lacZ*, in order to reduce ectopic expression due to position effects. The relative levels of mRNA and protein were compared at late stage 11 and at stage 15 in multiple lines for each construct. Endogenous *eve* mRNA is expressed strongly at stage 11 as a 6-cell cluster (consisting of aCC, pCC, the two CQs and two of unknown origin; Broadus et al., 1995) and slightly more weakly in the RP2 neuron (Fig. 4A), while the apparent level drops by stage 15 in all cells, especially in RP2 (Fig. 4C). Eve protein is expressed strongly through stage 15 (Fig. 4B,D), with no apparent reduction at the later stage. Staining for *lacZ* mRNA in transgenic lines without the *eve* 3′ UTR appeared similar to that of the endogenous mRNA at stage 11, marking the aCC/pCC and RP2 neurons strongly (Fig. 4E). Like *eve* mRNA, the level dropped by stage 15 (Fig. 4G), perhaps more severely than did the *eve* mRNA, particularly in the aCC/

pCC cluster. Likewise, *lacZ* protein at stage 11 showed strong expression in all of the cells (Fig. 4F). However, unlike endogenous Eve, expression at stage 15 was quite severely reduced relative to the earlier stage (Fig. 4H).

With the *eve* 3′ UTR, the transgene showed fairly strong mRNA expression at stage 11 (albeit perhaps weaker than with the α-tubulin 3′ UTR), and again the level had dropped by stage 15 (Fig. 4I,K). The protein level at stage 11, like that of the mRNA, appeared to be slightly lower than without the *eve* 3′ UTR (Fig. 4J, compared to F). However, in contrast to transgenes with the α-tubulin 3′ UTR, with the *eve* 3′ UTR the level of *lacZ* protein staining at stage 15 increased relative to stage 11 (Fig. 4L, compared to J), resulting in protein expression that was clearly higher than without the *eve* 3′ UTR (Fig. 4L, compared to H). This was true for multiple lines with each construct, with the staining performed in parallel with complete sets of embryos, as shown in Fig. 4. Although there is some variation from one neuromere to another, the strong trend in all neuromeres, as well as in all lines examined, is that protein levels are consistently higher at stage 15 with the *eve* 3′ UTR than without it. This maintenance of high protein levels at stage 15 despite an apparent decrease in mRNA levels (relative to stage 11) is similar to the situation with endogenous Eve, indicating that there is a mechanism to maintain the level of protein that requires the *eve* 3′ UTR. Since the *eve* 3′ UTR causes either no change or a slight decrease in mRNA levels at stage 15, relative to the α-tubulin 3′ UTR, while protein levels are quite strongly increased, the mechanism would seem to involve translational control.

Localization of other elements

Expression in the anal plate ring is driven by sequences between +2.6 and +4.8 kb (Fig 1 #10, Fig 3F). Detailed analysis revealed complicated regulation in this region. Posterior expression starts as the germband becomes fully elongated as an ‘8th stripe’ driven by the *eve* late element (Goto et al., 1989). This expression fades at stage 10 (when *eve*-positive GMCs appear) but, at early stage 11, expression from the +2.6 to +4.8 element begins. This expression may overlap the posterior extent of the earlier 8th stripe expression, or may lie just posterior to it (data not shown). This element can be partially separated into two overlapping elements, one for early anal plate ring (early APR) expression through germband shortening, and another for expression after dorsal closure is completed (late APR). Late without early APR expression can be driven by the region from +3.5 to +4.3 (Fig. 1 #14), a region also sufficient to drive CQ neuronal expression (described above). Early APR expression can be driven by the region +2.6 to +3.5 (Fig. 1 #15), but this also drives weak late APR expression, indicating some redundancy in its specification. The region +3.2 to +4.0 (Fig. 1 #16) drives, in addition to late APR expression, weak early APR expression. The weakness of the latter indicates that something upstream of +3.2 is required for the proper level of early APR expression.

Mesodermal cells that include pericardial and other muscle precursors express *eve*. The element for this expression was found to lie between +5.8 and +6.6 kb (Fig 1 #36, Fig 2G), which does not overlap other elements. However, at the time of dorsal closure, expression from this element becomes weaker, unlike that of endogenous *eve* (see Discussion).

The fragment from +1.5 to +3.2 kb (Fig. 1 #4) drove strong *lacZ* expression in the *ftz* domain in stage 7–9 embryos, where *eve* is not normally expressed strongly. In the endogenous *eve* gene, the activity of this element may be suppressed by the upstream late element (Sackerson et al., 1999). The *ftz* stripe element can be shortened to between +1.5 and +2.6 (Fig 1 #3, Fig 2I), while the region +1.5 to +1.9 (Fig. 1 #2) drives very weak *ftz* domain expression. This shorter fragment also gives short-lived and faint *lacZ* expression in GMC 1-1a at stage 10.

The region +7.9 to +9.2 kb contains a strong pairing-sensitive repression (PSR) element. When this fragment was present upstream of the promoter, 50–70% of homozygous viable lines showed weaker *mini-white* expression in homozygotes than in heterozygotes, as reflected in

the eye color (data not shown; normally, homozygotes show stronger expression due to the increased copy number). A similar effect was seen even when the element was placed 3′ of the *lacZ*-coding region, 3.8 kb downstream of the promoter, or when it was present in its normal position downstream of the promoter, in the EVE92 rescue construct. The fragment was dissected into two parts, +7.9 to +8.6 (Fig. 1 #34) and +8.6 to +9.2 kb (Fig. 1 #29). The former fragment, when placed upstream of the *mini-white* promoter, showed PSR in one out of 12 lines (8%), while the latter (Fig. 1 #29) caused PSR in 5 out of 7 lines (71%). Most, if not all, of the PSR activity is therefore separable from the minimal element for RP2+aCC/pCC expression.

Complete rescue of *eve* function

Since all *eve* regulatory elements seem to be localized between −6.4 and +9.2 kb, we tested whether this region, which is flanked by DNaseI hypersensitive sites (Sackerson et al., 1999), can rescue the lethality of *eve* mutants. We tested both the region −6.4 to +9.2 (EVE92) and the region −6.4 to +8.4 (EVE84, Table 1). We used the latter endpoint because it also gave a complete pattern of *lacZ* reporter expression (above), albeit with possibly reduced levels in the nervous system (in aCC/pCC and RP2 neurons). Transgenic lines with insertions on the 3rd chromosome were crossed into either *Df(2R)eve* [*Df(eve)*] or eve^{R13} (*R13*) mutant backgrounds, generating flies of the genotypes *Df(eve)*/*CyO*, P[*wg-lacZ* (or *hb-lacZ*)]; P[EVE]/P[EVE] and *b, R13/CyO*, P[*wg-lacZ* (or *hb-lacZ*)]; P[EVE]/P[EVE]. *R13* is an apparent null mutation that truncates the protein within the homeodomain (see below). One transgenic insertion on the 2nd chromosome was recombined onto these *eve* mutant chromosomes. Adults from the *R13* lines were scored for both a wild-type wing phenotype (non-*CyO*) and the *black* (*b*) phenotype (indicating *R13* homozygotes). *Df(eve)* lines were stained with anti-Eve antibody to determine the pattern and level of Eve expression. In addition, cuticles from both lines were analyzed to determine the overall degree of rescue (see Materials and Methods).

Df(eve) is a deficiency that includes at least three lethal complementation groups (O'Brien et al., 1994) and, therefore, as expected, was not rescued by *eve* transgenes. However, both the EVE92 and EVE84 transgenes rescued the lethality of *R13* (Table 1). For EVE92, two lines were analyzed. One (EVE92-H) produced adults with both the wild-type wing and *black* phenotypes (always together) at 21.4% of the total adult population, while the other line (EVE92-G) gave these phenotypes at 0.7%. For EVE84, seven lines were analyzed, and all showed the rescued phenotype at frequencies of between 15% and 32%. The Eve expression pattern and the cuticle phenotype of each of the efficiently rescued lines were indistinguishable from wild type (data not shown, see Table 2 for details). Thus the rescued phenotype did not differ significantly between EVE84 and EVE92 based on cuticle and expression patterns. The line EVE92-G, which did not rescue efficiently, showed a weak hypomorphic *eve* cuticle phenotype (Nusslein-Volhard et al., 1985). Each of these transgenes (except EVE92-G) also rescued the heterozygous mutant combination *Df(eve)*/*R13* to adulthood at a frequency of between 21% and 36%. When rescued *R13*/*R13* flies were self-crossed, they showed poor fertility, while rescued *Df(eve)*/*R13* showed normal fertility, indicating the presence of recessive mutations on the *R13* chromosome that affect fertility. In many cases, rescue to adulthood required two copies of the transgene, for both *R13*/*R13* and *Df(eve)*/*R13*. Cuticles of the single copy rescued lines showed a high frequency of defects characteristic of *eve* hypomorphic mutants (Table 2, P+).

Even though the transgenes rescued *R13* lethality, they failed to rescue the eve^{ID19} mutant (*ID19*) to adult viability. *ID19* is a hypomorphic allele that has a single amino acid substitution in the homeodomain (Frasch et al., 1988). However, the heteroallelic combination *ID19*/*Df(eve)* was efficiently rescued (Table 1), indicating the existence of at least one additional recessive lethal mutation on the *ID19* chromosome.

Many of the lines carrying rescue constructs showed very faint eye color from the mini-*white* gene (some lines required aging to identify transgenic flies). To avoid this problem, Glass activator binding sites were introduced upstream of the *mini-white* gene (see Methods). Lines with this construct (EVEG84) showed strong eye color without apparent effects on the efficiency of either transformation or rescue, in combination with either *R13*/*R13*, *R13*/*Df (eve)* or *ID19*/*Df(eve)*.

eve^{R13} protein is truncated within the homeodomain

R13 shows a null cuticle phenotype and was found to lack detectable Eve protein expression (Frasch et al., 1988). *eve* RNA is present and we do detect very weak protein staining under some circumstances (data not shown). Since this mutant was used extensively in our analysis of *eve* regulatory function, we sequenced the *R13*-coding region (see Materials and Methods). A single alteration from the wild-type sequence was found: a C-to-T transition, which would create a termination codon in place of Gln 106. Fortuitously, this change also eliminates a *Pvu*II restriction site, and Southern analysis showed that *R13* DNA (from stocks of two different laboratories) was missing the *Pvu*II site (not shown, see Materials and Methods). This mutation is consistent with the *R13* null phenotype, as the resulting truncated protein would be missing the ‘recognition helix’ (helix 3) of the homeodomain required for DNA binding, as well as a transcriptional repression domain (Han and Manley, 1993). The very low level of antibody staining in mutant embryos suggests that this truncated protein is relatively unstable.

DISCUSSION

Concentration-dependent regulation of *eve* by gap genes

Recent studies showed that regulatory elements for all known aspects of *eve* expression are located within a 15.6 kb region, spanning −6.4 to +9.2 kb from the transcription start site (Sackerson et al., 1999). Based on the regions of overlap of larger transgenes, these studies suggested that early stripes 1 and 5 might be driven by the region from +4.8 to +8.4 kb, and stripes 4 and 6 by the +4.8 to +6.6 kb region. In order to determine whether these stripes were regulated independently, and to define minimal elements required for each of them, we further dissected these regions. Elements for stripes 1 and 5 proved to be separable, while a single element drives stripes 4 and 6 (see Fig. 1 for details). Extensive analysis has been done of how gap genes regulate early *eve* stripes 2, 3 and 7 (Goto et al., 1989; Harding et al., 1989; Small et al., 1991, 1992, 1996; Stanojevic et al., 1989, 1991). We now have an opportunity to compare their regulation with that of the remaining stripes. The growing knowledge of segmentation genes in other insects (Brown et al., 1997; Patel et al., 1992, 1994), when combined with these data, may provide an understanding of how pattern formation has evolved. As a first step in this direction, we examined how the stripe elements are regulated by gap genes. Consistent with a composite element driving stripes 4 and 6, both the anterior border of stripe 4 and the posterior border of stripe 6 are determined by zygotic *hb* expression. In addition, in a *kni* mutant, the isolated stripe 4+6 element drives expression throughout the interstripe region. The spatial and temporal expression patterns of zygotic *hb* and *kni* (Kraut and Levine, 1991b) are consistent with the products of these loci exerting direct repression on the element. Similarly, the anterior and posterior borders of expression of the stripe 5 element are set by *Kr* and *gt*, respectively. Again, these regulators are expressed in an appropriate pattern to direct repression of this stripe element. More subtle effects of these mutations and of *tll* on these elements are consistent with the above interpretation, when previously observed crossregulation among the gap genes is taken into account (see Results). Thus, as for stripes 2, 3 and 7, much of the spatial regulation of stripes 4, 5 and 6 appears to be due to repression by gap gene products. The sequences of these regulatory elements contain potential binding sites for the gap gene products that we suggest may directly regulate them (our unpublished observations). However, further analysis will be required to determine if these regulatory interactions are indeed direct.

The above observations concerning regulation of stripes 4 and 6 include a striking parallel with the regulation of stripes 3 and 7. The stripe 7 element is not separable from that of stripe 3, although full activation of stripe 7 requires sequences outside of the minimal stripe 3 element (Small et al., 1996). Like the 4+6 element, a combined stripe 3+7 element directs expression throughout the interstripe region in a *kni* mutant, and both the anterior and posterior borders (of stripes 3 and 7, respectively) are set by *hb*-dependent repression (Small et al., 1996). Thus, an intriguing situation exists in which the stripe 4+6 element is repressed by a higher concentration of Knirps protein than is the stripe 3+7 element and, at the same time, by a lower concentration of Hunchback protein. The differential sensitivity of these elements to repressor concentrations might be due to simple mechanisms, such as differential affinities of binding sites, or to more complex mechanisms, such as combinatorial interactions with different cofactors. Whatever the mechanism, this differential sensitivity is precise enough to allow three gap protein domains (those of Knirps and the anterior and posterior Hunchback domains), acting as repressor gradients, to regulate the positioning of eight distinct expression boundaries, thus helping to define four of the early stripes of *eve* expression.

In a similar vein, stripe 5 is negatively regulated by the same gap genes that regulate stripe 2. The *Kr* domain represses both the posterior border of stripe 2 and the anterior border of stripe 5, while the anterior and posterior domains of *gt* expression are involved in setting the anterior and posterior borders of stripes 2 and 5, respectively.

Previous computer modeling of the regulatory circuitry upstream of *eve* predicted specific negative regulatory interactions of gap genes on stripes 4 and 5 (Reinitz and Sharp, 1995). This model predicted that *kni* would be the primary regulator of both the posterior border of stripe 4 and the anterior border of stripe 5, and that *Kr* and *gt* would repress the anterior border of stripe 4 and the posterior border of stripe 5, respectively. Two of these proposals are consistent with our data, and two are not. Rather than *Kr* setting the anterior boundary of stripe 4 (at a high concentration), it sets that of stripe 5 (at lower concentration). Similarly, rather than setting the stripe 5 anterior boundary at high concentration, a lower concentration of *kni* sets the stripe 6 anterior boundary. Apparently, the independent behavior of the stripe elements allows one enhancer to essentially 'ignore' a high repressor concentration, while a different stripe enhancer responds to the same repressor at a lower concentration. Further analysis of specific binding sites within identified enhancers may support more detailed models of *eve* regulation.

Recently, several genes were reported to show stripe-specific effects on *eve* activation. *lacZ* expression driven in stripes 4, 5 and 6 by the *eve* 3′ region were weakened in a *fish-hook* mutant (*fish*) (Ma et al., 1998). It was also shown that the product of this gene can bind within this large regulatory region, as determined by gel mobility shift assays. While we observed that expression from the minimal elements also showed some reduction in *fish* embryos, expression from these elements is clearly activated by other proteins as well. In a *marelle* mutant (encoding *D-STAT*), *lacZ* expression from a stripe 3 element was seen to be weakened (Hou et al., 1996; Yan et al., 1996). We observed that D-STAT is a primary activator of stripe 5, since expression from the stripe 5 element was absent in this mutant. Consistent with a direct effect on this element, we find several consensus sequences for D-STAT binding within the +7.4 to +8.2 kb region (M. F. and J. B. J., unpublished observations).

None of the gap and pair-rule mutants that we tested had a strong effect on stripe 1 element expression (see Results). *hb* mutants weakened reporter gene expression, but not severely. In a *buttonhead* mutant (*btd*), endogenous *eve* expression in the stripe 1 region was seen to be reduced (Vincent et al., 1997). In beetles, as in *Drosophila*, *eve* forms stripes with anterior borders that coincide with parasegment boundaries but, rather than forming multiple stripes at once, stripe 1 is formed first, followed by sequential progression toward the posterior (Patel et al., 1994). Further analysis of the regulation of stripe 1 may reveal regulatory relationships that

predated the divergence of Diptera. Recent analyses of *eve* stripe elements among *Drosophila* species (Fujioka et al., 1996; Ludwig and Kreitman, 1995; Ludwig et al., 1998; Sackerson, 1995) suggested that many of the regulatory mechanisms are evolutionarily conserved. The growing body of information from various species may soon support detailed hypotheses for how the regulatory mechanisms of segmentation evolved.

Nervous system regulation and *eve* 3′ UTR function

eve is expressed in the nervous system, initially in GMCs 1-1a, 4-2a and 7-1a, and later in the aCC/pCC, RP2, CQ and EL neurons. We were unable to separate elements for GMC 1-1a, its cellular progeny the aCC/pCC neurons, GMC 4-2a and its progeny neuron RP2. This was surprising, since these cells originate from different neuroblasts. A single element drives *lacZ* expression strongly in these neurons at least through stage 11. However, by stage 15, transgene expression is reduced, particularly at the protein level, when both endogenous Eve expression and expression from rescue constructs (in an eve^{-} background) remain strong. The *eve* 3′ UTR, which the initial *lacZ* transgenes did not contain, appears to affect the efficiency of translation in these cells. Transgenic lines in which the standard 3′ UTR (from the α-tubulin gene) is replaced by that of *eve*, while they show reduced mRNA levels at stage 11 and similar levels at later stages, give *lacZ* protein levels that remain high through stage 15 in the RP2 and aCC/pCC neurons. The *eve* 3′ UTR was previously reported to confer a rapid turnover rate in early cycle 14 (Kosman and Small, 1997) of the blastoderm stage. Thus it appears that the *eve* 3′ UTR has functions in controlling protein levels in several tissues, at various stages, and probably through multiple mechanisms.

Elements for EL cells and for GMC 7-1a and its progeny CQ neurons were also localized. However, the CQ and EL elements overlap those for posterior region expression and for even-numbered parasegment expression, respectively (see Fig. 1 and below), suggesting that common activators may be utilized in these different tissues.

Expression in posterior regions and the mesoderm

An element for muscle precursor cell expression is separable from those of other tissues. However, its expression becomes weaker than that of endogenous Eve at stage 15, as observed for the RP2+aCC/pCC element. The *eve* 3′ UTR may provide for a high level of protein expression in this tissue, at a similar time to that in the nervous system.

Expression in the posterior region of the embryo is apparently a highly conserved feature of *eve* function, since it is shared by *eve* homologs in *C. elegans*, zebrafish and mice (Ahringer, 1996; Bastian and Gruss, 1990; Dush and Martin, 1992; Joly et al., 1993). While it was reported that posterior structures are not affected in eve^{ID19} mutants at the non-permissive temperature (Sato and Denell, 1986), it remains a possibility that *eve* has some function in this region. *eve* homologs have been shown to have important functions in specifying posterior cell fates in *C. elegans* and zebrafish (Ahringer, 1996; Joly et al., 1993). The regulation of *eve* expression in the posterior region is complex. Initially, the late stripe element is responsible for expression in this region, which appears as an 8th stripe corresponding to parasegment 15 (Lawrence et al., 1987; Frasch et al., 1987; Macdonald et al., 1986). Later, expression is driven in a ring near the posterior end of the embryo by two separable elements, one active through germband retraction and the other after dorsal closure. The latter expression corresponds to the anal plate ring.

Just downstream of the *eve*-coding region (+1.5 to +2.6 kb) lies an element that, when assayed by itself, drives *lacZ* expression strongly in the even-numbered parasegments, where only very weak *eve* expression is normally observed. As suggested previously, the upstream late element may be responsible for long-range repression of these ‘*ftz*-like stripes’ (Sackerson et al.,

1999) in the endogenous *eve* gene. The biological function of this element, if any, is unclear, although *eve* expression does extend into this region, where it is required to clear *odd-skipped* expression from the anterior *ftz* domain, allowing activation of *engrailed* (Fujioka et al., 1995). This element may serve a function in this context.

Rescue of the *eve* mutant phenotype

The regulatory DNA that we have characterized downstream of the transcription unit, in combination with upstream regions previously analyzed, is sufficient to functionally rescue *eve* null mutants. In most cases, a single copy of the rescue transgene was not sufficient for full rescue. Many mutant embryos exhibited a weak *eve* hypomorphic phenotype when they carried only one copy of the transgene, suggesting that transgene expression is below that of the endogenous gene when inserted at most chromosomal locations. This might indicate that the transgene is missing a general enhancer of early *eve* expression. Alternatively, sequences within the Pelement vector may repress *eve* expression at early stages. It is also possible that a chromosomal environment exists around the *eve* locus that is required for full activity which most insertion sites do not provide. The PSR element described below might participate in providing such an environment.

The genomic region downstream of the RP2+aCC/pCC element causes strong pairing-sensitive repression (PSR) of the *mini-white* gene. Similar PSR is observed when *Polycomb*-group gene responsive elements are introduced into the genome with *mini-white*. Recently, it was reported that a region from the *engrailed* gene that exhibits PSR is bound directly by the *Drosophila* YY1 homolog, Pho, encoded by the *pleiohomeotic* gene (Brown et al., 1998). Consensus sites for YY1/Pho binding, as well as for GAGA factor, which are also seen in the *engrailed* element, exist within this region (M. F., J. Kassis, and J. B. J., unpublished observation). Consistent with chromatin-based regulation of *eve*, a *Polycomb*-group protein, Polyhomeotic, was found to bind to polytene chromosomes in the region of the *eve* locus (DeCamillis et al., 1992), and *eve* expression in the NB4-2 lineage was seen to be affected by *Polycomb*-group activity (Weigmann and Lehner, 1995). Nonetheless, the function of the *eve* PSR element is unclear, since rescue transgenes that lack it do not show abnormal *eve* expression, and since including it did not appear to enhance either expression or rescue (see Results). It is possible that the PSR element is only required in the context of the *eve* locus, perhaps to prevent inappropriate activation of *eve* by enhancers from a neighboring gene, or of a neighboring gene by *eve* enhancers (see Sackerson et al., 1999). Other regions of the *eve* locus are also capable of repressing *mini-white* expression, since the −6.4 to +8.4 kb transgenes consistently gave transformants with very weak eye color. The utilization of Glass activator binding sites to enhance *mini-white* expression facilitated identification of transformants, but these also showed weak eye color relative to other *Glass-mini-white* transgenes. Although this repression was not consistently pairing sensitive, it may represent a function that is redundant with that of the PSR region in some aspect of *eve* regulation.

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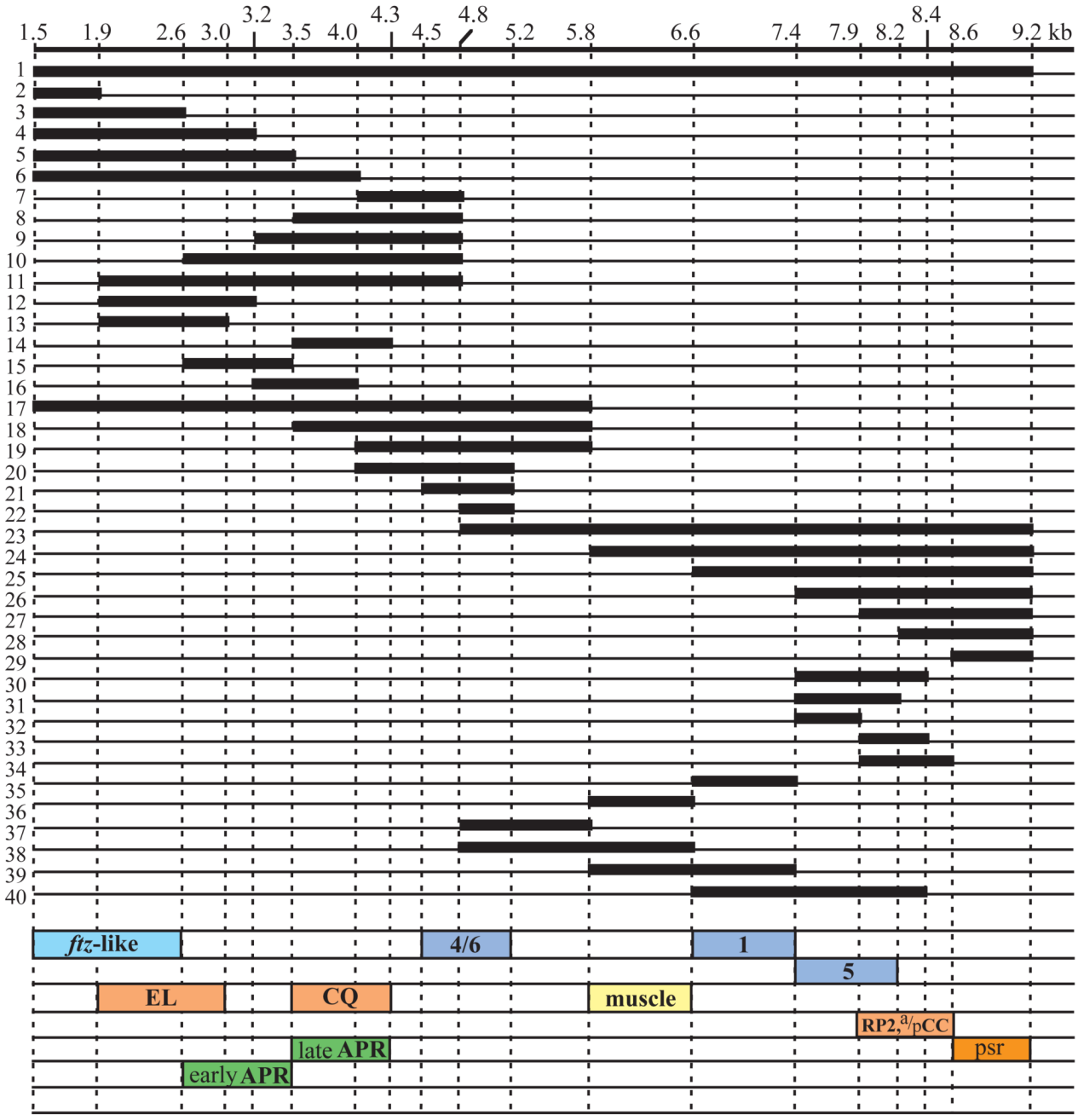

Fig. 1.

A deletion analysis of the *eve* 3′ region. Numbers along the top show distance (in kb) to the transcription start site of the *eve* locus. Regions of the sequence included in each construct are indicated by thick lines, and deleted regions by thin lines. All fragments were placed downstream of an *eve* promoter-*lacZ* reporter in a pCaSpeR-based vector (see Materials and Methods). Five to ten independent transgenic lines were analyzed for each construct. Boxes at the bottom indicate minimal elements required to drive the following *lacZ* expression: *ftz*-like, 7 stripes in the even-numbered parasegments; EL, EL neurons in the central nervous system; CQ, GMC 7-1a and CQ neurons; early APR, early anal plate ring; late APR, late anal plate ring; 4/6, blastoderm stripes 4 and 6; muscle, mesodermal precursors; 1, blastoderm stripe 1;

5, blastoderm stripe 5; RP2,a/pCC, GMCs 4-2a and 1-1a, and neurons RP2, aCC and pCC. PSR indicates the region causing pairing-enhanced repression of the *mini-white* gene.

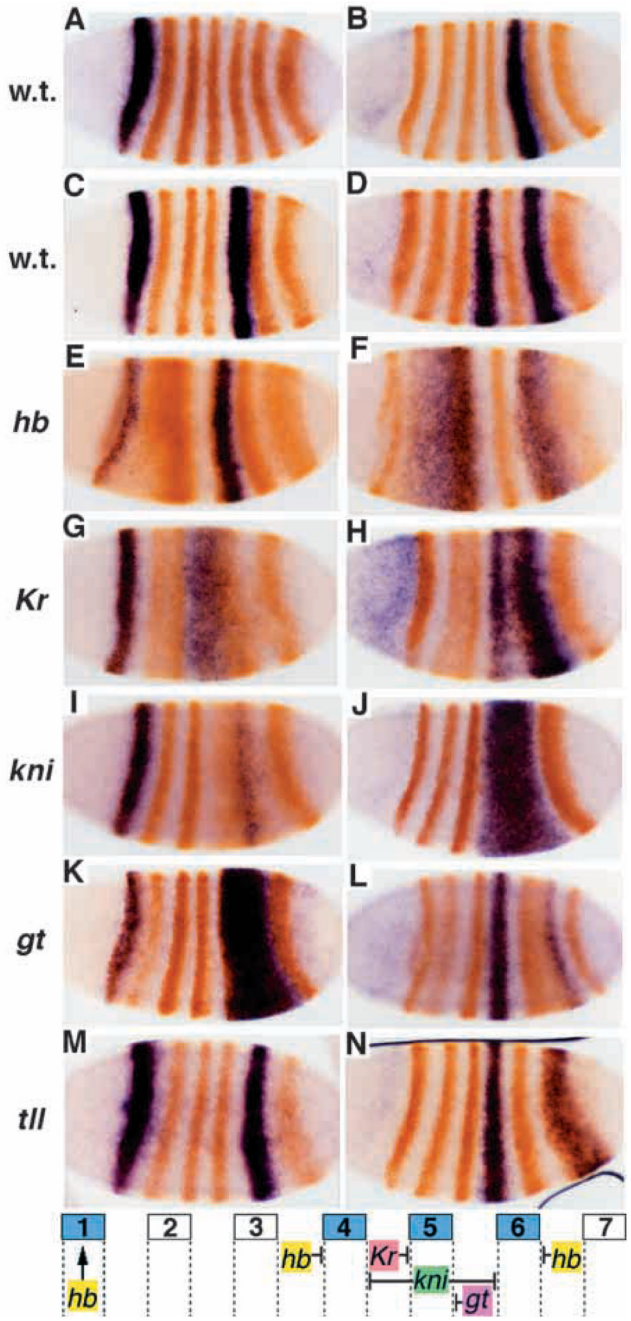

Fig. 2.

Elements for early stripes 1, 5 and 4+6 are negatively regulated by gap genes. In situ hybridization was performed for *lacZ* mRNA (blue), followed by staining with anti-Eve antibody (orange). (A) *lacZ* expression driven by the region +6.6 to +7.4 kb; (B) by the region +7.4 to +8.4 kb; (C) by the region +6.6 to +8.4 kb; (D) by the region +4.0 to 5.2 kb. The transgenic lines in C and D were crossed into several gap gene mutant backgrounds and stained as above. Staining patterns in these mutant embryos are shown below the corresponding wild-type pattern: *hb* (E,F), *Kr* (G,H), *kni* (I,J), *gt* (K,L) and *tll* (M,N). A summary of the apparent gap gene regulatory influences on stripes 1, 4, 5 and 6 is diagrammed at the bottom.

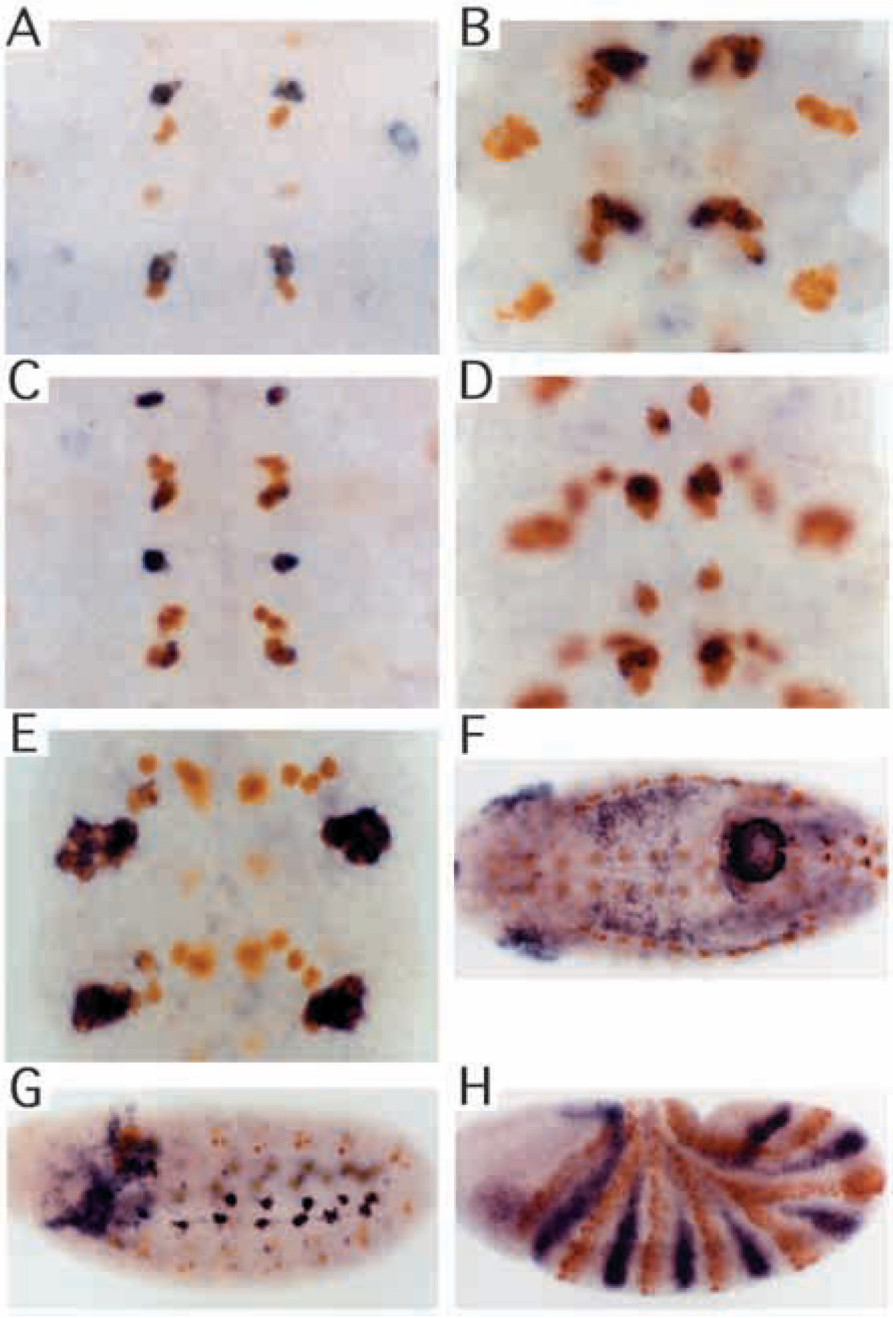

Fig. 3.

Isolated elements drive various components of tissue-specific expression. In situ hybridization was performed for *lacZ* mRNA (blue), followed by staining with anti-Eve antibody (orange). (A) *lacZ* expression in GMC 7-1a progeny at early stage 11 driven by the region + 3.5 to +4.3 kb. (B) *lacZ* expression in CQ neurons at stage 15 in the same transgenic line as in A. (C) *lacZ* expression in neurons RP2, aCC, and pCC at stage 11 driven by the region +7.9 to +9.2 kb. Note that at this stage *lacZ* expression is strong. (D) *lacZ* expression in RP2, aCC and pCC neurons at stage 15 in the same transgenic line as in C. Overall expression of *lacZ* at this stage is faint. (E) *lacZ* expression in EL neurons at stage 15 driven by the region +1.9 to +3.2 kb. (F) *lacZ* expression in the anal plate ring at stage 12 driven by the region +2.6 to +4.8 kb. (G)

lacZ expression in muscle precursor cells at stage 11 driven by the fragment +5.8 to +6.6 kb. (H) *lacZ* expression in the anterior portion of even-numbered parasegments driven by the region +1.5 to +2.6 kb.

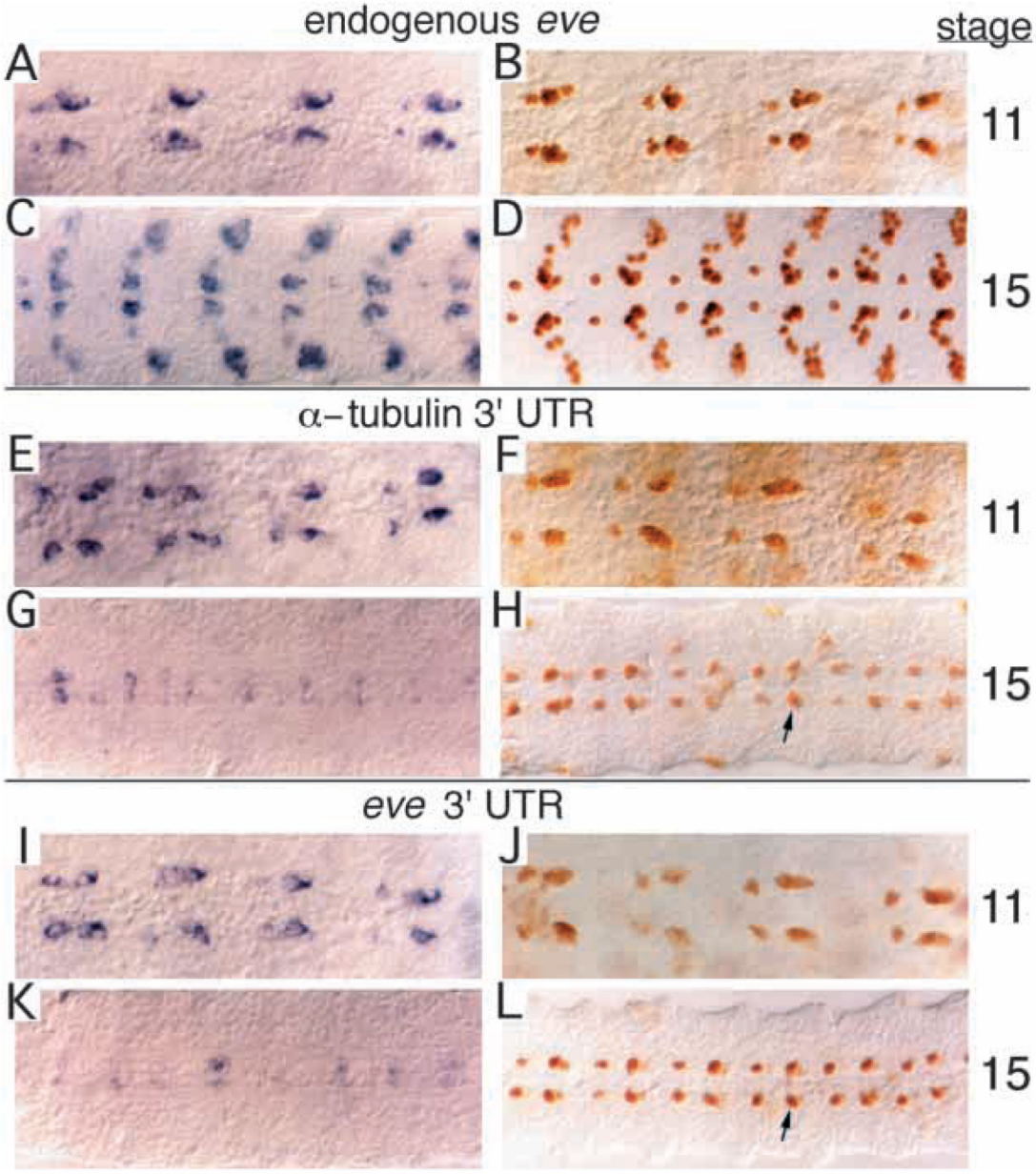

Fig. 4.

The *eve* 3′ UTR is required for high level protein expression in RP2 and aCC/pCC neurons at later stages. (A-D) Wild type; (E-H) transgenic line carrying *lacZ* construct driven by the +7.9 to + 9.2 kb fragment, with the α-tubulin 3′ UTR (See Materials and Methods); diffuse ectopic expression due to a position effect is visible. (I-L) Transgenic line carrying *lacZ* construct driven by the same +7.9 to + 9.2 kb fragment, but with the *eve* 3′ UTR; again, there is diffuse ectopic expression, particularly at stage 11. (A,C,E,G,I,K) mRNA expression visualized by in situ hybridization with appropriate probe; (B,D,F,H,J,L) protein expression visualized by staining with appropriate antibodies. (A,C) *eve* mRNA; (B,D) Eve protein; (E,G,I,K) *lacZ* mRNA; (F,H,J,L) β-galactosidase protein. Expression in RP2, aCC and pCC neurons, at stage 11 (E,F,I,J); at stage 15 (G,H,K,L). Arrows, a representative neuron in a representative line,

showing increased protein staining at stage 15 with the *eve* 3′ UTR (in L) relative to that with the α-tubulin 3′ UTR (in H).

Table 1

The region −6.4 to +8.4 kb can rescue the lethality of *eve* mutants

		% rescued (no. scored)		
		R13/R13	***R13/Df(eve)***	***ID19/Df(eve)***
EVE92	G	0.7 (136)	2.0 (525)	ND
	H	21.4 (341)	23.1 (981)	ND
EVE84	A	29.0 (376)	32.2 (421)	28.1 (466)
	C	15.8 (183)	21.3 (1058)	22.9 (280)
	D	17.2 (174)	34.2 (372)	33.1 (320)
EVEG84	A	28.1 (473)	35.6 (988)	18.5 (417)
	B	28.8 (163)	31.1 (283)	ND
	D	32.3 (572)	34.9 (1224)	26.9 (579)
	E	19.3 (351)	25.8 (1161)	ND

Two copies of each rescue construct were crossed into the *eve* mutant background indicated at the top. EVE92 carries the −6.4 to +9.2 kb fragment, EVE84 has a 3′ endpoint of +8.4 kb instead and EVEG84 is the same as EVE84, except that it has Glass activator binding sites upstream of the *mini-white* gene, which strongly enhance the eye color of transgenic flies. Each letter designation in the second column indicates an independent insertion. All were homozygous viable, and all were on chromosome III except EVEG84-E, which was on chromosome II and was recombined onto each *eve* mutant chromosome prior to the experimental cross. Adult flies either *R13*/*R13*, *R13*/*Df(eve)* or *ID19/Df(eve)* were identified by their wild-type (non-*Curly*) wing phenotype. The percentages of adult flies showing this phenotype are shown. The total number of flies counted is shown in parentheses.

Table 2

Hatching rates and cuticle phenotypes of rescued *eve* embryos

		Mutant Background	Hatching Rate (%)	Cuticle phenotype (%)					No. scored
				Wild type	Severe def.	Mild def.	Undeveloped	*wg*	
EVE84	A	*Df(eve)*	54.8(1229)	1.3	0.1	0.1	23.2	20.5	644
		R13	62.5 (566)	4.2	0.0	0.3	12.4	20.6	217
		Df(eve) P+	35.9(1305)	9.8	5.6	5.8	42.9	NA	321
	C	*Df(eve)/R13*	59.4(2000)	12.4	0.0	0.0	28.2	NA	128
		R13	61.1(1413)	12.2	2.6	0.0	24.1	NA	332
		Df(eve) P+	46.0(3639)	1.5	0.0	22.8	29.7	NA	246
	D	*Df(eve)*	58.0(1170)	2.7	0.0	0.4	17.2	21.7	611
		R13	61.0 (515)	2.4	0.2	0.0	20.3	16.1	181
		Df(eve) P+	40.8 (719)	10.1	16.8	6.2	26.1	NA	258
EVEG84	A	*Df(eve)*	69.8 (927)	0.0	0.0	0.0	10.2	20.0	454
		R13	64.6(1572)	2.2	1.0	0.4	13.3	18.5	251
		Df(eve) P+	64.3(1119)	1.5	5.3	2.2	26.7	NA	315
	B	*Df(eve)**	26.1 (712)	22.3	7.3/1.3	4.2	38.8	NA	298
		R13	64.2 (662)	3.7	0.0	0.0	12.5	19.6	221
		R13 P+	47.8(1165)	6.9	26.1	5.7	13.5	NA	174
	D	*Df(eve)*	64.9(1012)	1.6	0.9	0.0	8.5	24.1	190
		R13	67.4(1238)	1.9	0.0	0.0	7.8	22.9	337
		Df(eve) P+	59.1(1193)	1.6	7.3	3.3	28.7	NA	376
	E	*Df(eve)*	75.5 (961)	0.2	0.1	0.1	6.3	17.8	289
		R13	68.5 (954)	0.6	0.3	0.1	3.4	27.1	111
EVE92	G	*Df(eve)*	47.3(1540)	17.4	4.1	20.0	11.2	NA	335
		R13	14.1 (895)	4.9	0.0	2.7	78.4	NA	355
	H	*Df(eve)*	45.6 (851)	14.5	3.1	0.3	36.5	NA	192
		R13	68.0 (872)	12.0	0.3	0.8	18.9	NA	125
		Df(eve) P+	55.9(1168)	16.4	7.1	0.0	20.6	NA	326
		R13 P+	48.0(2020)	6.0	33.4	0.0	12.6	NA	190

Transgenic constructs and lines are as in Table 1, and are indicated in the first column. Either one or two copies of each rescue construct were crossed into the indicated *eve* mutant background. Crosses in which all of the progeny carried one copy of the rescue transgene are indicated by *P+* following the *eve* genotype. All others carried two copies of the transgene, except EVEG84 B Df(*eve*)*, where both parents carried one copy of the transgene over a *TM3* balancer (used because lines homozygous for the transgene had low fertility); therefore, some *eve*-deficient progeny did not receive a copy of the transgene, which may account for cuticles with a lawn phenotype (% shown after / in severe def. column). The numbers in parentheses under hatching rate indicate the total number of eggs assayed. The hatching rate was determined about 36 hours after the end of the collection, then cuticles were prepared from unhatched eggs. Phenotypes of rescued embryos were determined by preparing cuticles prior to hatching, and they were indistinguishable from wild type (not shown) for all homozygous lines except EVE92-G, which did not rescue efficiently. Severe def. embryos showed a denticle phenotype characteristic of strong to intermediate *eve* hypomorphs (pair-rule defects to somewhat less severe). Mild def. embryos resembled weak *eve* hypomorphs (missing 1 or 2 denticle bands or had partial deletions of denticle bands in a pair-rule pattern). The *wg* phenotype is due to the homozygous *wg-lacZ* marked balancer. NA under *wg* indicates the absence of this balancer in the cross. The total number of cuticles scored is shown in the far right column.