Characterization and localization of the *even-skipped* protein of *Drosophila*

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On the basis of homeo box cross-homology we have isolated the pair-rule gene *even-skipped* (*eve*) of *Drosophila*. The *eve* transcription unit appears to be less than 1.5 kb in length, and encodes a single mRNA of ~1.4 kb. The nucleotide sequence of genomic and cDNA clones indicates that the *eve* protein is composed of 376 amino acid residues, and that its homeo domain shares only ~50% amino acid identity with the homeo domains of previously characterized genes. Using antibodies raised against a β -galactosidase fusion protein we show that the *eve* protein is distributed in a series of seven transverse stripes at the cellular blastoderm stage, and is localized primarily within the nuclear regions of those embryonic cells that express the gene. After gastrulation, seven weakly stained stripes of *eve* expression appear, resulting in a transient pattern that consists of a total of 14 evenly spaced stripes. Both the original and new stripes gradually disappear during germ band elongation. A second expression pattern emerges during neurogenesis, whereby *eve* protein is detected in discrete subsets of neurons in each of the ventral ganglia.

Key words: *Drosophila/even-skipped* homeo box / nucleotide sequence/pair-rule genes/protein localization

Introduction

The specification of morphologically diverse body segments in *Drosophila* involves the activities of segmentation genes and homeotic genes. Segmentation genes divide the embryo into a repeating series of homologous segment primordia (Nusslein-Volhard and Wieschaus, 1980). Homeotic genes establish the diverse pathways by which each of these embryonic segments acquires a distinct adult phenotype (Lewis, 1978; Kaufman *et al.*, 1980). Three classes of segmentation genes have been identified: gap genes, pair-rule genes and segment polarity genes (Nusslein-Volhard and Wieschaus, 1980). Of these, the pair-rule genes have been characterized in the greatest detail. Mutations in any of the nine known pair-rule genes usually result in embryos that lack pattern elements in alternating segments (Nusslein-Volhard *et al.*, 1985). The spatial and temporal patterns of pair-rule gene expression are stringently regulated. The distributions of transcripts encoded by *fushi tarazu* (*ftz*) (Hafen *et al.*, 1984), *hairy* (*h*) (Ingham *et al.*, 1985), *engrailed* (*en*) (Kornberg *et al.*, 1985; Weir and Kornberg, 1985; Fjose *et al.*, 1985), *paired* (*prd*) (Kilcherr *et al.*, 1986) and *even-skipped* (*eve*) (Harding *et al.*, 1986; MacDonald *et al.*, 1986) have been examined in embryo tissue sections by *in situ* hybridization using the cloned genes as probes. Each of these genes shows a characteristic periodic (or 'zebra') pattern of expression during early embryonic develop-

ment. In some cases there appears to be a close correlation between the regions of wild-type embryos that contain transcripts encoded by pair-rule genes and the embryonic segments or portions of segments that are most disrupted in mutants for these genes.

A central problem in the specification of positional information in *Drosophila* is how pair-rule genes come to be expressed in specific regions of the developing embryo. It has been proposed that selective patterns of pair-rule gene expression depend on regulatory interactions among these genes (Howard and Ingham, 1986; Carroll and Scott, 1986; Harding *et al.*, 1986; MacDonald *et al.*, 1986). For example, *en* transcripts are not detected in the middle body region of *eve*⁻ embryos, which suggests that *eve*⁺ products are somehow required for the initiation or maintenance of the *en* expression pattern in wild-type embryos (Harding *et al.*, 1986; MacDonald *et al.*, 1986). Additional examples of possible regulatory interactions have been described in detail (Howard and Ingham, 1986; Carroll and Scott, 1986; Harding *et al.*, 1986; Rushlow *et al.*, 1987). The emerging view from these studies is that pair-rule gene expression involves a complicated network of cross-regulatory interactions. Thus, a combination of pair-rule gene products might act in concert to regulate the expression of a given pair-rule gene.

The pair-rule gene *eve* appears to be particularly important for the overall segmentation pattern since *eve*⁻ embryos lack all segmental subdivisions in the middle body region (Nusslein-Volhard *et al.*, 1985). In contrast, null mutations in the other pair-rule genes do not completely eliminate segmentation. It has been shown that the patterns of *en* and *ftz* expression are altered in *eve*⁻ embryos (Carroll and Scott, 1986; Harding *et al.*, 1986; MacDonald *et al.*, 1986). These results suggest that *eve* plays a key role in a hierarchy of cross-regulatory interactions among pair-rule genes. Thus, the *eve*⁻ phenotype appears to result not only from the absence of *eve*⁺ products, but also as an indirect consequence of altered expression patterns of other pair-rule genes. Here we report the nucleotide sequence, and putative amino acid sequence, of *eve*. Information concerning the expression and subcellular localization of the *eve* protein was obtained by indirect immunofluorescence, using antibodies specific for the *eve* moiety of a β -galactosidase/*eve* fusion protein. During early periods of embryonic development the *eve* protein shows a pair-rule distribution pattern, which is similar to that observed for the *eve* transcript. A distinct pattern of expression is detected during neurogenesis, whereby *eve* protein is detected in discrete subsets of neurons in each of the ventral ganglia. The *eve* protein is localized within the nuclear regions of those embryonic cells that express the gene.

Results

eve was isolated in a previous screen of a *Drosophila* DNA library for clones that cross-hybridize with the homeo box region of the homeotic gene, *Sex combs reduced* (Levine *et al.*, 1985). One of the clones that was obtained in this screen (called S72) maps to the 46C region of the second chromosome, within the limits

of a small deficiency [Df(2R)*eve*^{1.27}] (Nusslein-Volhard *et al.*, 1985) which uncovers the *eve* locus (data not shown). Further evidence that S72 corresponds to *eve* has been obtained by MacDonald *et al.* (1986), who showed that an X-ray induced *eve* mutation, *eve*^{3.77.17} (Nusslein-Volhard *et al.*, 1985), deletes a ~25-bp portion of the S72 protein coding region. Moreover, several *eve* mutations result in altered expression of the S72 protein (M.Frasch and M.Levine, in preparation). In particular, no S72 protein is detected in embryos homozygous for an EMS-induced *eve* mutation (Nusslein-Volhard *et al.*, 1985) that abolishes *eve* gene function (see Figure 6, below). Together, these observations strongly suggest that S72 corresponds to *eve*.

A restriction map of an 8.2-kb genomic DNA fragment from the phage λ S72 clone that contains the *eve* homeo box homology is shown in Figure 1. A 1.1-kb *Pst*I subclone of S72 was used to screen a phage λ gt10 cDNA library, which was prepared with poly(A)⁺ RNA from 3–12 h wild-type embryos (Poole *et al.*, 1985). The largest of the cDNAs that was obtained (called pS72-6) is 1.1-kb in length and contains most of the *eve* protein coding sequence (see below). The *eve* genomic and cDNA restriction maps are similar, suggesting that the *eve* transcription unit is relatively small (Figure 1).

Molecular organization of *eve*

Northern analysis using poly(A)⁺ RNA from embryo populations of different stages indicates that *eve* encodes a single mRNA of ~1.4 kb (Figure 2a). Primer extension (McKnight and Kingsbury, 1982) and S1 nuclease protection (Berk and Sharp,

1977) analyses were done to identify the 5' end of this transcript. For these studies, poly(A)⁺ RNA was obtained from extracts of 2–3 h embryos since this is the period of peak *eve* expression (Harding *et al.*, 1986; MacDonald *et al.*, 1986) (Figure 2a). A 34 nucleotide synthetic oligomer was used for the primer extension experiment. The nucleotide sequence and location of the primer within the *eve* gene are shown in Figure 4. The extended RNA product that results from the use of this primer has a length of 90 nucleotides (Figure 2b). The 5' end of the *eve* mRNA is indicated in the map shown in Figure 2, and in the nucleotide sequence of *eve* shown in Figure 4. The probe used for S1 nuclease protection analyses was obtained from a 123-bp *Pst*I–*Hinf*I genomic DNA fragment. The nucleotide sequence and location of this probe within *eve* is shown in Figure 4. After hybridization with embryonic poly(A)⁺ RNA and treatment with S1 nuclease, the probe was reduced to a length of 80 nucleotides (Figure 2c). Thus, the primer extension and S1 nuclease experiments indicate the same transcription start site, which is located only 378-bp upstream from the start of the homeo box coding region (see Figure 4). These results, together with a comparison of genomic and cDNA nucleotide sequences, indicate that the *eve* transcription unit is interrupted by a single 71-bp intron and appears to be less than 1.5 kb in length (summarized in Figure 3).

Nucleotide sequence of *eve*

DNA templates that were used for dideoxynucleotide sequencing (Sanger *et al.*, 1977) are shown in Figure 1b. The sequenc-

Fig. 1. Restriction maps of genomic and cDNA clones. (a) The uppermost restriction map corresponds to S72, which is an 8.2-kb *Eco*RI genomic DNA fragment that contains the entire *eve* transcription unit. Transcription is from left to right. An enlargement of a 1.1-kb *Pst*I–*Bam*HI fragment that contains the homeo box is shown below the restriction map of S72. This region is compared with a restriction map of the pS72-6 cDNA. The location of the homeo box within the genomic and cDNA fragments is indicated by the solid bars. (b) DNA templates used for nucleotide sequence analysis. The solid portion of the central horizontal line corresponds to the pS72-6 cDNA, whereas the dashed portions correspond to flanking genomic DNAs. The horizontal arrows show the regions that were sequenced by the dideoxy method (Sanger *et al.*, 1977). The solid arrows correspond to cDNA templates, whereas the dashed arrows indicate the genomic DNA templates that were sequenced. The 5' genomic regions that were sequenced include portions of a 1.8-kb *Xho*I fragment and a ~400-bp *Pst*I/*Pvu*II fragment [see maps in (a)]. The 3' genomic DNA templates used for sequencing derive from the *Bam*HI–*Pvu*II fragment that is just 3' to the 1.1-kb *Pst*I–*Bam*HI fragment (see S72 maps, above). Portions of the cDNA and genomic clones were also sequenced by the chemical cleavage method (Maxam and Gilbert, 1980) in order to resolve several ambiguities. Restriction enzymes: B, *Bam*HI; H, *Hinf*I; P, *Pst*I; Pv, *Pvu*II; R, *Eco*RI; X, *Xho*I.

Fig. 2. Identification of the transcription start site. (a) Northern analysis of *eve* RNA. Poly(A)⁺ RNA was extracted from wild-type embryo populations at 0–2, 2–3, 3–6, 6–12, and 12–24 h after fertilization. These RNAs were fractionated in an agarose/formaldehyde gel, transferred to nitrocellulose, and hybridized with the *eve* cDNA probe (pS72-6). A single 1.4-kb transcript was detected, which was most abundant in the RNA sample from 2–3 h embryos. (b) Identification of the 5' terminus of the *eve* transcript by primer extension. The 34 nucleotide synthetic oligomer used in this analysis is shown in the map below the autoradiograms in (b) and (c). The primer was annealed to poly(A)⁺ RNA from 2–3 h embryos, extended with reverse transcriptase, and electrophoresed in an acrylamide gel. The extended product is 90 nucleotides in length, which is the distance from the end of the primer to the transcription start site [indicated by the +1 in the map below (b) and (c)]. Lane 1, 10 units of reverse transcriptase; lane 2, 20 units of reverse transcriptase. Mol. wt markers are shown in the leftmost lane. (c) S1 nuclease protection. The location of the S1 probe used in this analysis is shown in the map below the autoradiograms in (b) and (c). The single-stranded probe was hybridized with poly(A)⁺ RNA from 2–3 h embryos, digested with S1 nuclease, and electrophoresed in an acrylamide gel. The protected DNA fragment is 80 nucleotides in length, which indicates the distance to the transcription start site. Lane 1, digestion of RNA–DNA hybrids with 100 units of S1 nuclease; lane 2, 50 units of S1 nuclease; lane 3, 25 units of S1 nuclease; lane 4, the deoxyguanosine + deoxyadenosine sequence of the S1 probe. Mol. wt markers are shown in the rightmost lane.

Fig. 3. Molecular organization of *eve*. This summary is based on a comparison of genomic and cDNA sequences, as well as 5' mapping studies using S1 nuclease and primer extension. The transcription unit is over 1450 bp in length. Putative protein coding regions are indicated by the solid bars, whereas those regions that are transcribed but untranslated are indicated by the open bars. The homeo box is indicated by the cross-hatches. The 5' end of the homeo box coding sequence is located 378-bp downstream from the transcription start site. There is a well-conserved 'TATA' sequence located 28-bp upstream from the transcription start site. The gene is interrupted by an intron, which is 71 bp in length and located between +239 and +310. As a result of incomplete second-strand synthesis, the *eve* cDNA lacks at least 250 bp of 3' transcribed sequences. The nucleotide sequence of this region was determined from appropriate genomic DNA templates (see Figure 1b). There are no conserved splice sites within this 250-bp interval, which also contains a possible polyadenylation signal (at +1458). Cleavage and polyadenylation near this site would result in a ~1.4-kb mRNA, which is in agreement with the Northern data shown in Figure 2a. Since S1 nuclease protection studies have not been done with the 3' region, it is possible that additional *eve* transcribed sequences occur further downstream.

ed interval includes the entire *eve* cDNA (pS72-6), as well as the genomic regions that immediately flank the 5' and 3' ends of the cDNA. The first AUG codon is located 94-bp downstream from the 5' end of the cDNA and is in frame with an open reading frame (ORF) that extends throughout the length of the cDNA (Figure 3). Since the cDNA does not contain the 3' portion of the *eve* transcript, it was necessary to derive the sequence of putative carboxy-terminal amino acid residues from the nucleotide sequence of appropriate genomic DNAs (see Figure 1). The extended ORF in the cDNA sequence continues for an additional 87 bp before interruption by a stop codon. Thus, the total length of the ORF is 1128 bp. If translation begins with the first AUG of this ORF, then a protein consisting of 376 amino acid residues and a molecular weight of ~40 kd would result. The complete amino acid sequence of this protein is shown in Figure 4.

The putative *eve* protein contains a divergent homeo box sequence (homeo domain), which shares only ~50% amino acid identity with each of several homeo domains that have been characterized previously, including those associated with *ftz* (McGinnis *et al.*, 1984; Scott and Weiner, 1984), *en* (Poole *et al.*, 1985; Fjose *et al.*, 1985) and the homeotic gene *Antennapedia* (*Antp*) (McGinnis *et al.*, 1984; Scott and Weiner, 1984). The *eve* homeo domain is located within the amino terminal half of the protein (amino acid residues 70–130), which is in contrast to the locations observed for *ftz*, *en*, and *Antp* where the homeo domains are closer to the carboxy terminus (Poole *et al.*, 1985; Laughon and Scott, 1984; Schneuwly *et al.*, 1986).

Preparation of antibodies against a β -gal/*eve* fusion protein

The expression and subcellular localization of the *eve* protein was examined with polyclonal antibodies against a β -galactosidase/*eve* fusion protein made in *Escherichia coli*. The gene fusion that was constructed for preparation of the hybrid protein involved the use of the pUR 291 plasmid (Ruther and Muller-Hill, 1983), which allows insertion of a foreign coding sequence at the 3' end of the bacterial *lacZ* gene. The strategy used for constructing the *lacZ/eve* recombinant is shown in Figure 5a. The predicted mol. wt of the hybrid protein is 145 kd, of which ~30 kd corresponds to the *eve* gene. The 858-bp *eve* cDNA fragment that was fused with the *lacZ* gene contains 284 of the 376 codons that encode the *eve* protein.

Bacterial cells containing the β -galactosidase/*eve* recombinant plasmid were induced to express the hybrid protein by addition of isopropyl-1-thio- β -D-galactoside (IPTG). Extracts from induced cells were fractionated on an SDS polyacrylamide gel, and

TTGGCCGCTCCACCGACGGCGGCCATTTCCTGCAGAGCGCAGCGGTATAAAAGGGCGGGGCTGGCTGAG
 ★
 +1
AGCAGCACACTCGAGCTGTGACCGCCGACAGTCAACAATACTGCCTTCGTTAATATCCTCTGAATAAGC
 MetHisGlyTyrArgThrTyrAsnMetGluSerHisHisAla 14
 CAACTTTGAATCACAAGACGCATACCAACATGCACGGATACCGAACCTACAACATGGAGAGCCACCATGCC
 ★
 HisHisAspAlaSerProValAspGlnLysProLeuValValAspLeuLeuAlaThrGlnTyrGlyLysPro 38
 CACCACGACGCCAGTCCCGTGGACCAAGCCCTGGTTGTGGACCTCTTGGCCACCCAGTACGGCAAGCCC
 GlnThrProProProSerProAsnGluCysLeuSerSerProAspAsnSerLeuAsnGlySerArgGlySer 62
 CAGACACCGCTCCCTCGCCAAATGAATGCCTATCCAGTCCGGATAACTCCTTGAACGGCAGCCGCGGCTCG
 GluIleProAlaAspProSerValArgArgTyrArgThrAlaPheThrArgAspGlnLeuGlyArgLeuGlu 86
 GAGATTCCCGCCGACCCGTCGGTACGCCGCTATCGCACCGCCTTACCCGTGACCACTGGGTGCGCTTGGAG
 LysGluPheTyrLysGluAsnTyrValSerArgProArgArgCysGluLeuAlaAlaGlnLeuAsnLeuPro 110
 AAGGAGTTCTACAAGGAGAACTACGTGTCCCGTCCCGTCCGCTGCGAACTGGCCGCCAGCTGAACCTCCCG
 GluSerThrIleLysValTrpPheGlnAsnArgArgMetLysAspLysArgGlnArgIleAlaValAlaTrp 134
 GAGAGCAGCATCAAGGTGTGGTTCCAGAACCGCGCATGAAGACAAGCGTCAGAGGATCGCCGTGCGCTGG
 ProTyrAlaAlaValTyrSerAspProAlaPheAlaAlaSerIleLeuGlnAlaAlaAlaAsnSerValGly 158
 CCTACGCAGCCGTCTACTCCGATCCCGCTTCGCGCGCTCCATCCTCAGGCCGCGGCCAACAGCGTGGGC
 MetProTyrProProTyrAlaProAlaAlaAlaAlaAlaAlaAlaAlaAlaValAlaThrAsnPro 182
 ATGCCCTATCCGCCCTACGCCCCCGCTGCTGCGCGCGCTGCTGCGCGCGCGCTGCCGTGGCCACCAATCCG
 MetMetAlaThrGlyMetProProMetGlyMetProGlnMetProThrMetGlnMetProGlyHisSerGly 206
 ATGATGGCCACCGGAATGCCCCCGATGGGCATGCCCCAGATGCCACAATGCAGATGCCCGGACACTCGGGA
 HisAlaGlyHisProSerProTyrGlyGlnTyrArgTyrThrProTyrHisIleProAlaArgProAlaPro 230
 CATGCCGGCCATCCCTCGCCCTACGGACAGTACCGCTACACGCCCTACCACATCCCCGCCCGCCCGCGCCG
 ProHisProAlaGlyProHisMetHisHisProHisMetMetGlySerSerAlaThrGlySerSerTyrSer 254
 CCACATCCCGCTGGTCTCATATGCATCATCCGCACATGATGGGATCCAGCGCCACGGGATCGTCTGACTCC
 AlaGlyAlaAlaGlyLeuLeuGlyAlaLeuProSerAlaThrCysTyrThrGlyLeuGlyValGlyValPro 278
 GCGGTGCGCGCGGCTTTTGGCGCTCTGCCCTCCGCCACCTGCTATACCGGACTGGGTGTGGGTGTGCC
 LysThrGlnThrProProLeuAspLeuGlnSerSerSerSerProHisSerSerThrLeuSerLeuSerPro 302
 AAGACCCAGACGCCCGCGCTGGATCTGCAGTCTGCTCATCGCCGCACTCCTCCACGCTGTCTGCTCTGCCA
 ValGlySerAspHisAlaLysValPheAspArgSerProValAlaGlnSerAlaProSerValProAlaPro 326
 GTGGATCCGATCAGCCAAGGTGTTGACCGCAGTCCAGTGGCTCAATCCGCTCCATCAGTTCCTGCTCCC
 AlaProLeuThrThrThrSerProLeuProAlaProGlyLeuLeuMetProSerAlaLysArgProAlaSer 350
 GCTCCACTGACCACCACCGCCGCTGCCGCTCCCGCTCCTGATGCCAGTGCCAAGCGGCTGCTCC
 AspMetSerProProProThrThrThrValIleAlaGluProLysProLysLeuPheLysProTyrLysThr 374
 GACATGTCCCGCGCCGACGACAACCTGTGATTGGCGAGCCCAAGCGAAGCTCTTCAAGCCCTACAAGACT
 GluAlaStop 376
 GAGGCGTAAGCCCGGATCCACACACTCTCTCCCCCCCCCATGCTCCCCAAAAGATTGTACAACTAG
 TCTTAGTCAGCCTCATCTATTTATTCGCGAAGATTGTACAGATTGTAGAGTAGCTAATTGTAGTCATAATTA
 AGGCGCAAAATCAAATTAAGAAATAAA

Fig. 4. Nucleotide sequence of *eve*. The complete nucleotide sequence of the *eve* cDNA (pS72-6) is shown. This cDNA is not full-length and lacks sequences from both termini. The cDNA does not contain the first six nucleotides from the 5' end of the *eve* mRNA, and is missing at least 250 bases from the 3' end. The nucleotide sequence of these missing regions was obtained from genomic DNAs, which are underlined in the above sequence. The nucleotide labeled +1, corresponds to the transcription start site. There is a well-conserved 'TATA' sequence located 28-bp upstream from the transcription start site. The first ATG is located 100 bases downstream from the start site and is in frame with the longest open reading frame, which extends for 376 amino acid residues. The amino acid sequence of this reading frame is shown above the nucleotide sequence. The numbers in the right column refer to the amino acid residues. The vertical arrow, between nucleotides 238 and 239, indicates the location of the splice junctions of a 71-bp intron (see Figure 3). The homeo domain is boxed bracket the S1 nuclease probe used in Figure 2c.

Fig. 5. Preparation of antibodies to an *eve*/β-galactosidase fusion protein. (a) Plasmid used for preparing an *eve* fusion protein. An 858-bp *HinfI*–*EcoRI* fragment from the *eve* cDNA (pS72-6) was inserted into the pUR 291 plasmid. The cross-hatches correspond to the homeo box. The fusion protein should contain 284 of the 376 amino acid residues that comprise the *eve* protein. The fusion protein contains a total of 1323 amino acid residues, and should have a mol. wt of 145 kD. The first 1023 amino acid residues derive from the *lacZ* gene, whereas several residues result from the polylinker of the vector (labeled 'pUR'). Residues 1026–1310 derive from the *eve* cDNA. (b) SDS–acrylamide electrophoresis, and Western analysis, of extracts from *E. coli* that express the β-gal/*eve* fusion protein. **Lane 1**, total extract from IPTG-induced cells that contain the pUR 291/*eve* plasmid. **Lane 2**, total proteins from induced cells that contain the pUR 291 plasmid without the *eve* cDNA insert. **Lane 3**, Western transfer of total proteins from a replicate of lane 2 after staining with a 1:10 000 dilution of whole serum from a rabbit immunized with the β-gal/*eve* fusion protein. **Lane 4**, Western transfer of total proteins from a replicate of lane 1 after staining with affinity-purified anti-*eve* antibody. **Lane 5**, Western transfer of total proteins from a replicate of lane 2 after staining with the affinity-purified antibody.

the β-galactosidase/*eve* hybrid protein was found to migrate with an apparent mol. wt of ~150 kD (Figure 5b), which roughly corresponds to the mol. wt predicted from the nucleotide sequence. The fusion protein was electroeluted from preparative SDS acrylamide gels and used to immunize rabbits. *eve* antibodies were affinity purified from the immune serum as described in Materials and methods. The affinity purified antibodies that were obtained react specifically with the *eve* portion of the fusion protein, and do not react with the β-galactosidase moiety (Figure 5b). This antibody preparation was used to localize *eve* protein

in whole-mount preparations of fixed wild-type and *eve*[−] embryos (Figure 6). As can be seen, the antibody is specific for the native *eve* protein and does not appreciably cross-react with other antigens. Strong periodic staining is observed in wild-type embryos undergoing cellularization and germ band elongation (Figure 6b and d), whereas labeling above background levels is not detected in embryos homozygous for the *eve*^{R13} point mutation (Figure 6a and c).

Distribution of *eve* protein during early embryogenesis

Previous studies have shown that the *eve* transcript is localized within seven transverse stripes, which display a periodic distribution along the anterior-posterior axis of embryos undergoing cellular blastoderm formation and gastrulation (Harding *et al.*, 1986; MacDonald *et al.*, 1986). During somewhat later periods, including the initial stages of germ band elongation, the *eve* transcript is localized within 14 transverse stripes (MacDonald *et al.*, 1986). The *eve* protein shows a similar distribution pattern, and is localized almost exclusively within the nuclear compartment of those cells that express the gene, as described below.

Staining above background levels is first detected during cleavage stage 12, ~45 min prior to the onset of gastrulation (Figure 7a). At this time each nucleus shows an equal level of staining. It is possible that the staining results from cross-reaction of the antibody with other antigens, however, such staining is not observed in *eve*^{R13} homozygotes at similar stages of development (data not shown). Moreover, *eve* transcripts show a similar distribution pattern at this time (Harding *et al.*, 1986). In addition, yolk nuclei are also strongly stained, which persists throughout gastrulation and germ band elongation. A localized staining pattern is first detected at the beginning of cleavage stage 14, whereby nuclei located in the posterior two-thirds of the embryo are more strongly stained as compared with those located more anteriorly. A rather sharp anterior boundary of staining is detected at ~69% egg length (see arrow, Figure 7b). Within several minutes nuclei located at ~55% and ~25% egg length show lower levels of staining, resulting in three broad bands of expression (Figure 7c). The anterior-most band of staining has already begun to separate into three narrower stripes. In addition, there is an overall gradient in the level of staining along the anterior-posterior axis, with stronger expression in the anterior regions. By mid-stage 14 a periodic pattern begins to emerge, but is not yet fully resolved (Figure 7d). The anterior-most and posterior-most stripes are more intense as compared with the others, and in addition, the fifth and sixth expression stripes have not yet separated. During cellularization the mature zebra pattern is established, whereby each of the seven transverse stripes is equally intense and encompasses the same number of nuclei (Figure 7e). Shortly thereafter, the posterior-most band broadens and includes an average of approximately six cells, whereas the six more anterior stripes encompass an average of four cells each.

A periodic pattern of *eve* expression persists over the course of gastrulation and the early stages of germ band elongation. Figure 8a shows a ventral view of a gastrulating embryo. During this time there is a narrowing of the expression stripes such that each encompasses approximately three cells and is separated from adjacent stripes by five less strongly stained cells. In contrast to earlier stages, the anterior border of each stripe is sharply defined. As germ band elongation begins seven new stripes of expression can be detected in addition to the original stripes (Figure 8, b–d). Each of these new stripes is one to two cells wide and is much weaker than the original stripes (small arrows, Figure 8b). During germ band elongation both the original stripes

Fig. 6. Localization of *eve* proteins in wild-type and *eve*⁻ embryos. Whole-mount embryos were stained with affinity-purified anti-*eve* antibodies and rhodamine-conjugated anti-rabbit IgG. Embryos were collected from balanced stocks carrying the *eve*^{R13} mutation, which is an EMS-induced allele that abolishes *eve* gene function (Nusslein-Volhard *et al.*, 1985). *eve*^{R13} homozygotes and wild-type embryos were simultaneously stained with the anti-*eve* antibody. (a) Late cleavage stage 14 *eve*⁻ embryo. Staining is not detected above background levels. (b) A wild-type embryo of similar age. The protein is distributed in a series of seven equally spaced transverse stripes along the anterior-posterior axis. (c) An *eve*⁻ embryo undergoing germ band elongation. As for earlier stages, no specific staining is observed. (d) A wild-type embryo of similar age as that shown in (c). The seven stripes of expression persist along the germ band. For details of the wild-type expression pattern see Figures 7–9.

and new stripes gradually disappear (Figure 8d and e). However, this decline in the expression pattern is not uniform in that the anterior-most stripe is the first to be lost (Figure 8d). Prior to the completion of germ band elongation each of the remaining stripes encompasses only one to two cells.

During elongation, a new site of staining is detected near the posterior-most region of the germ band, which includes the presumptive proctodeum. After the germ band is fully extended and the middle body stripes of expression are no longer observed, this staining remains intense (Figure 8e) and persists throughout embryogenesis. During germ band shortening these cells form a ring that surrounds the anal plate (arrow, Figure 9b).

Distribution of eve protein during neurogenesis

By the beginning of germ band shortening (~7–8 h after fertilization) *eve* is expressed within the neurogenic region of the germ band. Figure 9a shows an embryo undergoing germ band shortening (~9–10 h after fertilization), whereby *eve* protein is detected in discrete clusters of cells located along both sides of the ventral midline. Each cluster includes ~4–6 cells, and there is one such cluster on each side of the ventral midline per segmental repeat. In addition, there is a single stained cell anterior to each of the clusters. Just before germ band shortening is complete, additional cells become stained by the antibody. The new cells are located laterally and somewhat posterior to the original clusters (arrows, Figure 9a). After germ band shortening, the original and lateral clusters each comprise about six to seven neurons (Figure 9c). At this time, the final number of cells that

express *eve* protein within each ganglion of the ventral cord is fixed. The ventral cord is composed of three subesophageal, three thoracic and nine abdominal ganglia. Each hemi-ganglion of the ventral cord is composed of ~300 neurons (Poulson, 1950). Only ~13–15 neurons are stained by the *eve* antibody in each of the three thoracic and first seven abdominal ganglia. Fewer neurons are stained within the subesophageal ganglia, as well as the eighth and ninth abdominal ganglia. Based on the timing and pattern of expression, it appears unlikely that all of the *eve*-expressing neurons of each ventral ganglion are clonally related or derive from the same neuroblast progenitor.

In order to better define the positions of the labeled neurons within the ventral cord, whole-mount embryo preparations were double-stained with rhodamine-conjugated anti-*eve* antibody and fluorescein-conjugated anti-peroxidase antibody (Figure 9e and f). Anti-peroxidase antibodies stain neuronal projections and therefore clearly delineate the commissures of the ventral cord (Jan and Jan, 1982) (see Figure 9f). The neurons that comprise the central cluster of *eve* staining are associated with the posterior commissure of each ventral ganglion, whereas the lateral clusters are located just in front of the anterior commissure of the neighbouring ganglion (compare Figure 9e and f). This pattern of *eve* expression persists throughout embryonic and larval development (data not shown).

At the time when neurogenic expression of *eve* is first detected, the antibody also stains clusters of cells along the dorsal rim of the germ band (Figure 9b). Each such cluster consists of two to three cells. During dorsal closure (~10–11 h after fertiliza-

Fig. 7. Legend overleaf.

Fig. 8. Legend overleaf.

tion), these cells move together and form two parallel rows along the dorsal midline. Each segment contains one cluster of these cells on each side of the midline. Examination of the dorsal staining with high magnification shows that each segment also contains a weakly stained ring of cells that is located dorso-laterally (Figure 9d). Double staining with anti-peroxidase antibodies indicates that neither the dorsal clusters nor the dorso-lateral rings correspond to peripheral neurons (data not shown). However, nerve endings are detected within or near the dorso-lateral rings. Based on their locations, the dorsal clusters and/or the dorso-lateral rings could correspond to portions of the dorsal vessel (Hartenstein and Campos-Ortega, 1985).

Discussion

We have described the sequence, expression and subcellular localization of a protein encoded by the pair-rule gene *eve*. The *eve* protein contains a divergent homeo box sequence, shows a periodic distribution pattern during early embryonic stages, and accumulates in the nucleus. The protein is encoded by a 1.4-kb mRNA, which is the only *eve* transcript that is detected in Northern assays using a cloned cDNA sequence as a probe. Nucleotide sequence analysis of genomic and cDNA clones suggests that the *eve* transcription unit is interrupted by a single intron of 71 bp and is less than 1.5 kb in length. Thus, *eve* is the smallest of the seven homeo box genes that have been characterized in detail (Bender *et al.*, 1983; Garber *et al.*, 1983; Scott *et al.*, 1983; Laughon and Scott, 1984; Poole *et al.*, 1985; Kuroiwa *et al.*, 1985; Mlodzik *et al.*, 1985; Regulski *et al.*, 1987), and its simple organization is similar to that described for the pair-rule gene *ftz* (Laughon and Scott, 1984).

Comparison of *eve* with other homeo box proteins

There is evidence that homeo box gene function involves a sequence-specific DNA binding activity, which is specified by the homeo domain of the encoded proteins (Desplan *et al.*, 1985). The demonstration that homeo box proteins are localized within the nucleus is consistent with such a DNA binding activity, and provides further support for the proposal that these genes control pathways of development by directly modulating the expression of certain 'target' genes. It has been shown previously that proteins encoded by the homeotic gene *Ultrabithorax* (*Ubx*) (White and Wilcox, 1984; Beachy *et al.*, 1985) as well as the pair-rule genes *ftz* (Carroll and Scott, 1985) and *en* (DiNardo *et al.*, 1985), are localized in the nucleus. Here we have shown that the *eve* protein is also restricted to the nucleus. *eve* has been shown to influence the expression patterns of several other pair-rule genes (Harding *et al.*, 1986; MacDonald *et al.*, 1986; Rushlow *et al.*, 1987). The basis for this regulation is not known.

Fig. 7. Distribution of *eve* protein in blastoderm stage embryos. Whole-mount preparations of fixed embryos were stained with affinity purified anti-*eve* antibodies and visualized with rhodamine-conjugated goat anti-rabbit antibodies. Embryos are orientated so that anterior is to the left. The bar in (e) corresponds to 50 microns. (a) Cleavage stage 12 embryo (~2 h after fertilization). All of the nuclei are stained. (b) Early stage 14 embryo. The nuclei in the posterior two-thirds of the embryo show stronger staining than those located in more anterior regions. There is a sharp boundary of staining at ~69% egg length (arrow). (c) Mid-stage 14 embryo. A periodic pattern of expression begins to unfold. The numbers refer to the seven transverse stripes that are observed at later stages (see below). (d) Stage 14 embryo that is several minutes older than the one shown in (c). The fifth and sixth expression stripes have not yet separated. (e) Cleavage stage 14 embryo after cellularization. A periodic staining pattern consisting of seven stripes can be seen. The bright fluorescence seen in the central regions of the embryos shown in (b), (c) and (d) results from staining of the yolk nuclei.

However, since *eve* contains a homeo domain and is localized in the nucleus, it is possible that this regulation occurs at the level of transcription.

The *eve* homeo domain is distinct from the 10 *Drosophila* sequences that have been reported previously (McGinnis *et al.*, 1984; Scott and Weiner, 1984; Poole *et al.*, 1985; Kuroiwa *et al.*, 1985; Mlodzik *et al.*, 1985; Regulski *et al.*, 1985; Hoey *et al.*, 1986; Regulski *et al.*, 1987), and shares only 51% amino acid identity with the most related of these sequences, which includes the *Scr* homeo domain (Kuroiwa *et al.*, 1985). Nonetheless, several features of the *eve* homeo domain are more strongly conserved. In particular, 12 of 16 amino acid residues located near the carboxy ends of the *eve* and *Scr* homeo domains are identical. It is interesting that this is the region of greatest homology among all of the homeo box sequences that have been reported, and includes the putative DNA binding recognition helix (Laughon and Scott, 1984) (residues 111–119 of the amino acid sequence of the *eve* protein shown in Figure 4). Aside from the homeo domain the *eve* protein shares few homologies with any of the five full-length homeo box protein sequences that have been reported (Laughon and Scott, 1984; Poole *et al.*, 1985; Hogness *et al.*, 1985; Schneuwly *et al.*, 1986; Regulski *et al.*, 1987). Although, as for *en* (Poole *et al.*, 1985), the *eve* protein contains an alanine repeat and regions of high serine content. Moreover, as has been shown for several of the homeo box proteins, *eve* contains a high proportion of proline residues.

Correspondence between the sites of *eve* protein accumulation and the domains of *eve* gene function

Weak *eve* mutant alleles result in embryos that lack pattern elements in alternating segments (Nusslein-Volhard and Wieschaus, 1980; Nusslein-Volhard *et al.*, 1985). The periodic distribution of *eve* RNA and protein observed in wild-type embryos at early stages of development is consistent with this mutant phenotype. There appears to be a good correspondence between the regions deleted in weak *eve* mutant embryos and the sites where *eve* products show the highest levels of accumulation in wild-type.

Unlike other pair-rule genes, embryos that completely lack *eve*⁺ product show no overt segmentation in the middle body region (Nusslein-Volhard *et al.*, 1985). The periodic distribution of *eve* transcripts in alternating regions of wild-type embryos does not provide a simple explanation for this extreme *eve*⁻ phenotype (Harding *et al.*, 1986). In previous studies it has been shown that *eve* influences the expression of *ftz* and *en*, which

Fig. 8. Distribution of *eve* protein in embryos undergoing gastrulation and germ band elongation. Embryo whole-mounts are oriented so that anterior is to the left. (a) Ventral view of a gastrulating embryo. Seven stripes of expression can be seen, although staining of the anterior-most stripe is partially obscured by the cephalic furrow. (b) Vento-lateral view of an embryo at an early stage of germ band elongation. Weakly stained expression stripes (small arrows) alternate with the more strongly stained originals. (c) Magnified, lateral view of an embryo at a similar stage as that shown in (b). The arrows show weakly stained nuclei that occur between the stronger stripes of expression. Note that the anterior margins of the strong expression stripes are sharply delineated. (d) Lateral view of an embryo at a later stage of germ band elongation. Each of the expression stripes has narrowed and encompasses only 1–2 cells. The anterior-most stripe has almost disappeared. (e) Lateral view of an embryo that has completed germ band elongation. The arrow indicates a region of strong staining near the posterior end of the germ band. The original pair-rule expression pattern is no longer seen. Abbreviations: cf, cephalic furrow; pr, proctodeum; ptf, posterior transverse furrow; vf, ventral furrow. The bar in (e) corresponds to 50 microns, and the bar in (c) corresponds to 25 microns. The same magnification was used for the photomicrographs shown in (a), (b) and (d).

Fig. 9. Distribution of *eve* protein during germ band shortening and dorsal closure. Embryo whole-mounts are oriented so that anterior is to the left. (a) Ventro-lateral view of an embryo undergoing germ band shortening (~9–10 h after fertilization). The arrows show weakly stained clusters of neurons that arise 1–2 h after the first appearance of the more strongly stained clusters. (b) Dorso-lateral view of the same embryo shown in (a). Labeled cells occur at the dorsal rim of each middle body segment. The arrow indicates the staining of the developing anal plate. (c) Ventral view of an embryo that has completed germ band shortening. Each hemi-ganglion of the ventral cord shows three distinct foci of staining. The staining that occurs within the subesophageal ganglia is not seen in this plane of focus. (d) Dorsal view of the third through eighth abdominal segments of an embryo undergoing dorsal closure (about 10–11 h after fertilization). Each segment shows a cluster of two to three stained cells on each side of the dorsal midline, and a pair of weakly stained rings that are located dorso-laterally. (e) Ventral cord of an embryo at a similar stage as that shown in (d). This embryo was stained simultaneously with rhodamine-conjugated anti-*eve* antibodies and fluorescein-conjugated anti-peroxidase antibodies. The rhodamine stained neurons that contain *eve* protein can be seen (compare with c). Weak fluorescein staining of the ventral commissures is also detected. (f) Same as (e) except that different filters were used in order to detect fluorescein. The neuropile and peripheral neurons were stained with anti-peroxidase antibodies. Abbreviations: A1, A2,, first abdominal ganglion, second abdominal ganglion, etc. The bar in (c) represents 50 microns, and the same magnification was used in (a) and (b). The bar in (f) represents 40 microns and the same magnification was used for (d) and (e).

suggests that the extreme *eve*⁻ phenotype involves altered expression patterns of other pair-rule genes (Harding *et al.*, 1986; MacDonald *et al.*, 1986). Nonetheless, it seems paradoxical that *eve* could regulate a pair-rule gene such as *ftz*, when in wild-type, *ftz* and *eve* are most strongly expressed in complementary sets of embryonic cells (Harding *et al.*, 1986; MacDonald *et al.*, 1986). The *eve* protein distribution patterns presented in this study provide several possible explanations for the regulation of pair-rule gene expression by *eve*, and consequently for the extreme *eve*⁻ phenotype.

Most of the embryonic nuclei are stained by the *eve* antibody during pre-cellular stages of development (see Figure 7), which suggest that the *eve* protein at least transiently accumulates in each segment primordium. *eve* might generally influence the patterns of pair-rule gene expression as a result of this pervasive distribution of *eve* protein during early development. Of the three pair-rule genes that have been examined (*ftz*, *en* and *eve*), *eve* proteins are the first to be detected during embryogenesis. This early appearance of *eve* protein is consistent with the previous demonstration that *eve* influences the establishment of the *ftz* and *en* expression patterns, but there is no reciprocal requirement for *ftz* or *en* activity in establishing the *eve* pattern (Harding *et al.*, 1986). It is possible that the early appearance of *eve* protein involves a translational control mechanism since *eve* and *ftz* transcripts appear at about the same time during development (cleavage stage 10–11), yet *eve* protein is detected ~30 min prior to the first appearance of *ftz* proteins. It must be cautioned that the early detection of the *eve* protein involved the use of an antibody prepared against a β -galactosidase fusion protein which included the homeo box and an alanine repeat (see Figure 5). Thus, antibodies raised against this fusion protein might cross-react with other homeo box proteins, or with proteins such as *en* that contain an alanine repeat. However, staining above background levels is not observed in *eve*⁻ embryos at any time during development (Figure 6).

There are additional explanations for the regulation of pair-rule gene expression by *eve*. For example, yolk nuclei are strongly stained by the *eve* antibody during early stages of development. Cytoplasmic bridges connect the centrally located yolk nuclei with the peripheral somatic nuclei in pre-cellular blastoderm stage embryos (Foe and Alberts, 1983). Consequently, expression of *eve* in the yolk nuclei could influence the fates of somatic nuclei, including those that do not express the gene. Finally, we have shown that *eve* protein transiently accumulates in a series of 14 stripes along the germ band, which is in agreement with recent transcript distribution studies (MacDonald *et al.*, 1986). Thus, *eve* is expressed (at least weakly) in each of the middle body segment primordia. The regions where *eve* protein is detected at low levels probably coincide with the periodic domain of *ftz* expression (see arrows, Figure 8c). The regulatory influence that *eve* exerts over *ftz* and/or *en* might be mediated by these low levels of *eve* protein.

Neurogenic expression of *eve*

Previous genetic analyses concerning the spatial and temporal domains of pair-rule gene activity have centered on the embryonic epidermis. Here we have shown that the *eve* protein is detected in discrete subsets of the neurons that comprise each ganglion of the ventral cord. Thus, there is a dramatic shift in the *eve* expression pattern that was not predicted from genetic studies of *eve* activity. During early embryonic development, *eve* protein shows a pair-rule distribution pattern, which disappears during germ band elongation and is replaced by a pattern whereby

the protein is detected in certain neurons from each of the middle body segments (see Figure 9). Proteins encoded by other pair-rule genes have also been shown to accumulate in the embryonic CNS (White and Wilcox, 1984; Beachy *et al.*, 1985; Carroll and Scott, 1985; DiNardo *et al.*, 1985), and in the case of *ftz* there is a similar transition from a pair-rule to segmental pattern of expression (Carroll and Scott, 1985). In contrast to *ftz*, *eve* proteins persist in the CNS throughout embryonic and larval development (unpublished results). It is possible that *eve* controls aspects of neuronal morphogenesis such as migration, cell death or axonal guidance. However, there is currently no evidence that supports or rejects this view. It should be possible to assess whether *eve* products affect neurogenesis by examining the morphology of those neurons that normally express the gene in an *eve* temperature sensitive mutant (Nusslein-Volhard *et al.*, 1985), after shifting to the restrictive temperature at different times during development.

The finely localized expression of *eve* protein in discrete subsets of neurons, as well as in non-neuronal cells (see Figure 9b and d), raises the intriguing possibility that pair-rule genes can specify rather specific cellular identities. Genetic studies concerning *eve* activity during early embryogenesis suggest that *eve* directs relatively large groups of cells to follow a uniform pathway of segment morphogenesis. It is possible that *eve* specifies more restricted patterns of positional identity than have been proposed on the basis of previous genetic analyses, since subtle changes in cell identity might be obscured by the strong pair-rule phenotype of *eve* mutant embryos. Consistent with this possibility is the observation that the different cells of an *eve* expression stripe show quantitative differences in the level of staining by the *eve* antibody (for example, see Figure 8c). Further studies, including analysis of genetic mosaics, will be required to determine the extent to which *eve* specifies diverse pathways of morphogenesis at the level of the single cell.

Materials and methods

Transcript mapping

Primer extension analyses were performed essentially as described previously (McKnight and Kingsbury, 1982). The 34 nucleotide primer shown in Figure 4 was ³²P-labeled at its 5' terminus with polynucleotide kinase, and hybridized with 2 μ g of embryonic poly(A)⁺ RNA in 0.5 M NaCl; 10 mM Pipes, pH 6.4 for 6 h at 52°C. The primer–RNA hybrid templates were extended with reverse transcriptase for 1 h at 42°C in 50 mM Tris, pH 8.0; 10 mM DTT; 6 mM MgCl₂; 24 μ g/ml Actinomycin D; 0.5 mM deoxy-NTPs. The products were electrophoresed in a 6% polyacrylamide/8 M urea gel. The DNA probe used for S1 nuclease protection experiments was obtained from a 123-bp *Pst*I–*Hinf*I genomic DNA fragment. After ³²P-labeling with polynucleotide kinase, the probe was electrophoresed in a non-denaturing gel in order to separate the strands (Maxam and Gilbert, 1980). Single-stranded probe was hybridized with poly(A)⁺ RNA for 12 h at 37°C in 50% formamide; 0.67 M NaCl; 67 mM Pipes, pH 6.4; 1 mM EDTA. The DNA–RNA hybrids were digested with S1 nuclease for 30 min at 37°C in 0.25 M NaCl; 30 mM NaOAc; 1 mM ZnSO₄; 5% glycerol. The resulting products were electrophoresed in a 6% polyacrylamide/8 M urea gel.

Expression and purification of a β -gal/*eve* fusion protein

The pUR291/*eve* cDNA plasmid shown in Figure 5a was grown in cultures of bacterial 71-18 cells (Ruther and Muller-Hill, 1983), which were induced to express the hybrid protein by addition of IPTG. Induced cultures were suspended in 50 mM Tris, pH 7.9; 0.2 M NaCl; 2 mM EDTA; 2 mM β -mercaptoethanol; 1 mM PMSF, and incubated with 0.2 mg/ml of lysozyme for 20 min on ice. After a freeze-thaw cycle, Triton X-100 was added to a final concentration of 1%, and the bacterial suspension was sonified 3 $\times$ 30 s each with a Branson sonifier. The extract was centrifuged through a sucrose cushion containing 40% sucrose in 10 mM Tris, pH 8.0; 0.2 M NaCl; 1 mM EDTA; 1 mM PMSF for 30 min at 13 000 rpm in a Sorvall HB-4 rotor. The pellet was resuspended in SDS sample buffer, and the fusion protein was purified in preparative SDS–polyacrylamide gels. SDS was removed from the electroeluted protein using AG11A8 resin (purchased from Biorad) (Kapp and Vinogradov, 1978).

Affinity purification of anti-eve antibodies

A New Zealand White Rabbit was immunized with 300 µg of gel-purified fusion protein in a double emulsion containing complete Freund's adjuvant (Sigma) and 2% Tween 80 (Sigma). After two booster injections the rabbit was bled (6 weeks after the first immunization), and antibodies directed against β -galactosidase were removed from the serum by adsorption with purified β -galactosidase immobilized on CNBr-activated Sepharose 4B (Pharmacia) (March *et al.*, 1974). The antiserum was further absorbed with total proteins from IPTG-induced 71–18 cells containing the pUR 291 plasmid vector without the *eve* cDNA insert, and also with total protein from 0–1 h embryos coupled to Sepharose. *eve* antibodies were affinity purified by incubation with Sepharose-bound β -galactosidase/*eve* fusion protein.

Immunofluorescence staining of embryos

Embryos were fixed and stained with the antibody essentially as described by Dequin *et al.* (1984), with the following modifications. After methanol treatment, embryos were rehydrated in PBS and incubated in 10% BSA; PBS for 1 h at 4°C. All antibody incubations and washes were performed in PBS; 0.5 M NaCl; 0.1% Tween 80 (Sigma). A TRITC-conjugated goat anti-rabbit IgG (Jackson labs, Avondale, PA) was used for detection at a dilution of 1:300 after pre-absorption with a total embryo protein extract coupled to Sepharose (see above).

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