Groucho augments the repression of multiple Even skipped target genes in establishing parasegment boundaries

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INTRODUCTION

The development of multicellular organisms requires precise temporal and spatial control of gene expression. In Drosophila, segmentation is a well-studied example, wherein the embryo is subdivided along the anterior-posterior axis into repeating patterns of gene expression, which leads to the formation of morphological segments (Akam, 1987; Ingham, 1988). This process is controlled by a temporal hierarchy of zygotically active genes, the gap, pair-rule and segment-polarity genes (Nüsslein-Volhard and Wieschaus, 1980), in which spatial domains of expression are sequentially refined down to the single cell level (Fujioka et al., 1995; Manoukian and Krause, 1992). A pair-rule gene at the center of this process is even skipped (eve), which encodes a homeobox protein (Macdonald et al., 1986). Although like other pair-rule genes, it is expressed in a striped pattern that repeats every two segments, the eve null mutant phenotype at late embryonic stages is devoid of repeating pattern elements. This is due to the crucial role of eve in the establishment of all of the parasegment boundaries, which serve as organizational centers for the remainder of embryonic development (DiNardo et al., 1994). The even-numbered and odd-numbered parasegment boundaries are set up by distinct mechanisms (DiNardo et al., 1988), but the Even skipped (Eve) protein is required in both cases (Goto et al., 1989) for activation of the segment polarity gene engrailed (en) in the proper cell rows. en then marks and helps to maintain each parasegment boundary throughout later development (DiNardo and O’Farrell, 1987).

Although eve is required to activate en, Eve has been shown to have a potent repression activity in cultured Drosophila cells (Han et al., 1989; Jaynes and O’Farrell, 1988), and to repress transcription in vitro (Li and Manley, 1998; Manley et al., 1996). The apparent paradox can be explained by considering intermediary genes in the segmentation cascade. Eve represses three genes (Fujioka et al., 1995) that themselves repress en (Cadigan et al., 1994; Grossniklaus et al., 1992; Manoukian and Krause, 1993), as well as one gene that is required to activate en (DiNardo and O’Farrell, 1987). The combination of these activities allows the activation of en in a single-cell-wide stripe at the edge of each Eve stripe (Fujioka et al., 1995; Morrissey et al., 1991).

SUMMARY

Groucho acts as a co-repressor for several Drosophila DNA binding transcriptional repressors. Several of these proteins have been found to contain both Groucho-dependent and -independent repression domains, but the extent to which this distinction has functional consequences for the regulation of different target genes is not known. The product of the pair-rule gene even skipped has previously been shown to contain a Groucho-independent repression activity. In the Even skipped protein, outside the Groucho-independent repression domain, we have identified a conserved C-terminal motif (LFKPY), similar to motifs that mediate repression domain, we have identified a conserved C-terminal motif, which abolished binding to Groucho, showed a significantly reduced ability to rescue the even skipped null phenotype when driven by the complete even skipped regulatory region. Replacing this motif with a heterologous Groucho interaction motif restored the rescuing function of Even skipped in segmentation. Further functional assays demonstrated that the Even skipped C terminus acts as a Groucho-dependent repression domain in early Drosophila embryos. This novel repression domain was active on two target genes that are normally repressed by Even skipped at different concentrations, paired and sloppy paired. When the Groucho interaction motif is mutated, repression of each target gene is reduced to a similar extent, with some activity remaining. Thus, the ability of Even skipped to repress different target genes at different concentrations does not appear to involve differential recruitment or function of Groucho. The accumulation of multiple domains of similar function within a single protein may be a common evolutionary mechanism that fine-tunes the level of activity for different regulatory functions.

Key words: Repressor, Co-repressor, Embryogenesis, Segmentation, Drosophila
An alanine/proline-rich repression domain was mapped in Eve using transient transfection assays (Han and Manley, 1993; Jaynes and O’Farrell, 1991), and similar repression domains have been found in other proteins (Hanna-Rose and Hansen, 1996). For one of these repressors, Engrailed (En), the domain responsible for activity in transient assays was shown to provide only part of the repression activity in vivo (Tolkunova et al., 1998), while the major activity in vivo was attributable to a conserved peptide, eh1. The eh1 domain showed strong activity both in vivo (Jiménez et al., 1997; Smith and Jaynes, 1996) and on stably integrated target genes in cultured cells, but was virtually inactive in transient transfections of the same cells (Tolkunova et al., 1998), suggesting that these repression domains have significant mechanistic differences. Similar conclusions have been drawn from studies showing that some repressors can act in vivo over greater distances than others (reviewed by Gray et al., 1995).

The eh1 motif is found in several classes of homeodomain proteins (Smith and Jaynes, 1996), where it functions by binding the co-repressor Group of (Gro) to mediate repression activity (Jiménez et al., 1997; Jiménez et al., 1999; Mailhos et al., 1998; Tolkunova et al., 1998). The co-repressor function of Gro was first identified through its interaction with Hairy (Paroush et al., 1994). Subsequently, a diverse array of proteins with different types of DNA-binding domains have been shown to interact with Gro (Chen andCourrey, 2000; Fisher and Caudy, 1998; Parkhurst, 1998; Paroush et al., 1997), including dTc/Lef-1 (Cavallo et al., 1998) and Dorsal (Dubnicoff et al., 1997). Many of them have Gro interaction domains (GIDs) that contain sequence similarity either to the eh1 motif, such as Goosecoid and Pax proteins (Choi et al., 1999; Eberhard et al., 2000; Jiménez et al., 1999; Ren et al., 1999), or to the Hairy family’s GID (Fisher et al., 1996). The latter group includes Runt domain proteins (Aronson et al., 1997; Levanon et al., 1998) and Drosophila Hückebein (Goldstein et al., 1999). In contrast, the repression domain of Eve mapped in transient transfections was shown to act independently of Gro (Jiménez et al., 1997).

In this report, we show that Eve possesses a second, previously unrecognized repression domain, similar to those of Hairy, Runt and Hückebein, which interacts with Gro, mediates a Gro-dependent repression activity in early embryos, and is crucial for the function of Eve in segmentation. We have tested whether this Gro-dependent activity is required on target genes whose expression patterns show a sensitive dose-dependent response to Eve, and find that it is responsible for part, but not all, of their repression. Thus, Eve utilizes its two repression domains in concert to repress multiple target genes.

**MATERIALS AND METHODS**

**Plasmids**

For rescue experiments, a construct containing the endogenous eve locus from –6.4 to +8.4 kb (Fujio et al., 1999) was used. To create point or deletion mutants of the Eve C-terminal LFKPY motif, the region from +1093 to +1360 bp (Syl to SpeI) was amplified by nested PCR, subcloned and sequenced. Correctly mutated fragments were then transferred into the parental transgene vector. For yeast two-hybrid and His-tag pull-down assays, Eve C-terminal fragments were cloned into the vectors pAS2-1 or pET15b, respectively.

A Gro full-length cDNA in the vector pET3a (a gift from G. Jiménez) was used in His-tag pull-down assays, while a Gro full-length cDNA in pACT2 (a gift from M. Caudy and A. Fisher) was used for yeast two-hybrid assays.

**Construction of hb-HairyEve**

Sequences encoding either Eve amino acids 238-376 (Ndel to normal C terminus), for hb-HauryEve, or 238-366, followed by a stop codon, for hb-HairyEveA, were fused to hairy sequences encoding amino acids 1-268, then inserted into pCaSpeR4-hbh (Goldstein et al., 1999; Jiménez et al., 1997).

**Protein interaction assays**

Yeast two-hybrid assays and in vitro pull-down assays with GST-En and –Ene(F→E) were performed as previously described (Tolkunova et al., 1998). Recombinant derivatives of Eve containing a His6 tag were incubated with in vitro translated 35 S-labeled Gro at 4°C, and pulled down using TALON® Metal Affinity Resin (Clontech). After washing (50 mM NaPO4 (pH 7.0), 150 mM NaCl, 5 mM imidazole), bound Gro was eluted (50 mM NaPO4 (pH 7.0), 300 mM NaCl, 150 mM imidazole) and analyzed by autoradiography following 10% SDS-PAGE. The relative quantitation of signals (Fig. 1) was calculated using ImageMaster (Pharmacia Biotech AB).

**Drosophila strains and embryo analysis**

The alleles of eve used were Df(2R)eve and eve1 (eveDI9), both null alleles, and eve1 (eveDI9), a temperature-sensitive hypomorph. Mutations of grochou were: groE48, a point mutant that is a strong hypomorph; Df(3R)E(spl)82X2 (gro82X2), a deficiency encompassing m5, m7, m8 and gro; and groE77a, a putative antimorph (gifts from S. Artavanis-Tsakonas).

P-element-mediated transformation was performed as described previously (Fujio et al., 2000; Rubin and Spradling, 1982). Transgenic lines of hb-HairyEve inserted on the X-chromosome were maintained in males using an attached-X chromosome (C1)M3M3; insertions on the autosomes were kept as unbalanced stocks, selecting in each generation for transformant males and non-transformant females. Five lines (two X-linked) were generated that contained hb-HairyEve and three lines (one X-linked) that contained hb-HairyEveA. Each of these lines was crossed to yw females to assess female-specific lethality (progeny were scored for their sex and eye color). Two X-linked hb-HairyEve lines were crossed to gro82X2 or groE48 mosaic females, so that all female progeny carried the transgene; eggs were harvested in 1-3 hour collections, dechorionated in bleach and fixed in 4% formaldehyde, PBS and heptane for 15 minutes.

Embryos were fixed and stained for in situ hybridization as described previously (Tautz and Pfeifle, 1989). This was followed by antibody staining with anti-Eve antiserum (a generous gift from M. Frasch). Biotinylated secondary antibodies were detected using streptavidin-conjugated horseradish peroxidase (Chemicon International), as described (Mullen and Dinardo, 1995). For Sex lethal immunohistochemistry, embryos were preincubated for 1 hour at room temperature in PBS, 5% normal goat serum (Sigma) and 0.1% Triton (United Technologies Packard); incubated overnight at 4°C with a monoclonal antibody specific to the female form of Sex lethal (Bopp et al., 1991) in PBS, 5% normal goat serum and 0.1% Triton; washed extensively in PBS and 0.1% Tween (Sigma); preincubated for 1 hour in PBS, 0.1% BSA and 0.1% Tween; incubated 2-4 hours with goat α-rabbit alkaline phosphatase secondary antibody (Jackson Immunoresearch Laboratories); washed extensively in PBS and 0.1% Tween; stained in NBT/BCIP (Boehringer Mannheim); mounted in methylcarylate (JB-4, Polyscience); and examined under Nomarski optics. For cuticle preparation, embryos were collected for 4 hours, aged (for either 18 or 32 hours) and processed without devitellinization, as described previously (Fujio et al., 1999).

Embryos that lack maternal gro activity were derived from mosaic females with either gro82X2 or groE48 mutant germline clones, obtained using the FLP-DFS technique (Chou et al., 1993). Males
RESULTS

An Even skipped C-terminal conserved region interacts with Groucho

The Even skipped protein (Eve) has previously been shown to contain a Gro-independent repression domain (Jiménez et al., 1997). In a search of known repressors for sequence motifs similar to the previously identified Gro interaction domains (GIDs) in Hairy, Runt and Hückebein, we discovered that the Eve C-terminus contains such a motif (Fig. 1A, LFKPY), separated from the previously defined repression domain by over 100 amino acids. The motif is most similar to that in Hückebein, which also contains a phenylalanine at the first position of the core four amino acid motif (Goldstein et al., 1999), originally identified in the Hairy protein as WRPW (Fisher et al., 1996). Comparison with Eve homologs in other species has shown that the region immediately surrounding the motif is conserved (Fig. 1A).

Both in vitro (Fig. 1B, lane 4) and in yeast two-hybrid assays (Fig. 1C) the Eve C-terminus binds Gro. To test whether the conserved LFKPY residues are important for the Eve-Gro interaction, we introduced point mutations into the GID, as well as deleting it altogether. Mutations in the GID abolished the interaction (Fig. 1B,C), as did similar mutations in the Hairy, Runt and Hückebein GIDs (Aronson et al., 1997; Fisher et al., 1996; Goldstein et al., 1999; Paroush et al., 1994). As we detect no residual interaction in the two-hybrid assay, we attribute the in vitro signals from the mutated proteins, which are close to background, to nonspecific interactions. Thus, Eve contains a conserved GID at its C terminus, separate from its Gro-independent transient assay repression domain (TARD).

even skipped interacts genetically with groucho

If the Eve-Gro interaction is functionally significant, we might see a dose-dependent genetic interaction in Drosophila embryos between the genes encoding these proteins. To test this, we used an even skipped (eve) hypomorphic allele (eve\textsuperscript{1}, also known as ID19) in combination with several alleles of groucho (gro). This eve mutant encodes a protein with a single amino acid substitution in its homeodomain (Frash et al., 1988), and therefore a normal C-terminal region (including the GID). We crossed females heterozygous both for eve and for gro to males heterozygous for eve. As gro has a strong maternal contribution, this reduces the amount of Gro supplied to each of the progeny. Compared with progeny from a cross of eve heterozygotes with normal maternal gro dosage, the severity of defects among the homozygous eve progeny was increased (Fig. 2A; data not shown). The degree of the effect increased with the strength of the gro allele used.

The gro-enhanced cuticle defects are pair-rule in nature, affecting primarily the odd-numbered parasegments, and are similar to the defects seen in stronger alleles of eve (data not shown). Thus, they are consistent with Gro assisting Eve at the early stages of embryogenesis, during segmentation. This is also reflected in the patterns of expression of en and wingless (wg), two genes that are just downstream of eve in the segmentation cascade. Both patterns showed an increase in the severity of defects when gro function was reduced, over that
seen with the eve mutation alone (Fig. 2B). This effect is seen when wg and en are first initiated, and the defects are consistent with the known functions of eve in repressing wg and in setting correct parasegmental spacing during the process of segmentation (Frasch et al., 1988; Ingham et al., 1988). The defects seen at early stages persist to late stages of embryogenesis (Fig. 2B), consistent with the increased severity of cuticle defects seen when gro function is reduced.

The Gro interaction domain is essential for normal Even skipped function

If a direct Eve-Gro interaction is physiologically relevant, then specifically mutating the GID should strongly reduce Eve function. Previous work identified a 16 kb region from the eve locus (Sackerson et al., 1999) that could completely rescue eve-null mutants (Fujioka et al., 1999). We introduced separately into this transgene the small deletion and the two single amino acid changes that abolished the Gro interaction in vitro, and assayed the rescuing activity of the resulting constructs. Several lines were examined for each construct, and in each case, the ability to rescue viable adults was completely or almost completely abolished (Fig. 3A).

To determine whether the inability of GID-altered Eve to rescue eve mutants to adulthood was due to a deficiency in early Eve function, we examined embryonic cuticles from several lines (Fig. 3B). For the three GID mutants, the cuticle phenotype was similar to that of an eve hypomorph, with parasegmental defects in the pattern of abdominal denticle bands.

To rule out the possibility that the inability to rescue was due to aberrant protein accumulation, we stained embryos using polyclonal α-Eve antiserum. In most of the transgenic lines, for each of the GID changes protein staining was at least as robust as with the wild-type rescue construct (data not shown), showing that none of the protein changes reduced protein stability to any noticeable extent. Overall, the analysis of GID mutants showed that without the ability to interact with Gro, Eve function during segmentation is seriously impaired.

The GID mediates the genetic interaction between groucho and even skipped

If eve requires gro for its repressor function in segmentation, and if this requirement is mediated by the GID, then the rescuing ability of eve transgenes with mutations in the GID
Fig. 3. Alterations in the GID severely compromise the ability of Eve to rescue eve null mutations. (A) Rescue of eve null mutants to adulthood. Homozygous transgenic insertions expressing either the wild-type Eve-coding region (in the context of the entire eve locus) or a coding region with the indicated alterations in the LFKPY motif (altered amino acids are underlined) were crossed into two eve-null mutant backgrounds (D(f)eve1, eve1/eve2, see Materials and Methods). Similar results were obtained for both; the graph shows the combined data. Rescued mutant flies were identified by their lack of the balancer marker (Curly). For wild type, the graph shows the average and range of seven independent insertion lines; for GID mutants, results are shown for individual insertion lines. The numbers above the bars are the total number of flies scored. Note that each of the alterations, which abolish the Gro interaction, cause Eve to rescue very poorly or not at all. (B) Rescue of the embryonic cuticle pattern. Embryos from the transgenic lines in A were collected within 23 hours AED, and cuticles were prepared. Abdominal denticle bands were counted and classified into the following categories: mild to moderate (fewer than three ventral abdominal denticle bands fused or deleted; most embryos in this category had two to three denticle bands deleted) and moderate to severe (three or more denticle bands fused or deleted; most had three to four denticle bands deleted). For wild type, the average and range of results from seven lines are shown, and in each case, the total number analyzed is shown above the bar. Note that the percentage of defective embryos is high for each of the mutants, but low for the wild-type lines.

should not be further compromised by the genetic removal of gro. To test this, we crossed such altered transgenic copies of eve into an eve-null mutant background, and tested for a genetic interaction with gro, as before (Fig. 2). We compared rescue with two copies of the mutated transgene to one copy of the wild type transgene because these gave similar degrees of rescue, allowing us to directly compare their phenotypes. As Fig. 4 shows, a reduction of maternal gro caused an increase in severity of denticle band deletions when eve function was provided by the wild-type eve transgene. On average, more than one extra denticle band was deleted when gro was reduced. In contrast, when eve function was provided by transgenes encoding GID-mutated proteins, there was a considerably smaller effect of reducing gro. On average, less than an additional one-third of a denticle band was deleted per embryo in this case (see Fig. 4 legend for details). The latter genetic interaction may be due to an indirect effect of gro through other gene products such as Engrailed, which is known to interact physically and functionally with Gro (Jiménez et al., 1997; Smith and Jaynes, 1996; Tolkunova et al., 1998). Thus, the eve-gro genetic interaction can be attributed to a direct physical interaction between the proteins.

The Even skipped C terminus is a Groucho-dependent, transferable repression domain

As Eve binds DNA (through it homeodomain) and represses transcription, we explored the possibility that the Eve C terminus constitutes a repression domain that acts to directly recruit the co-repressor Gro. We first determined that the Eve C terminus is a transferable repression domain, using an in vivo repression assay (Jiménez et al., 1997). Previous studies have shown that expressing the pair-rule gene hairy prematurely, under the hunchback (hb) promoter, brings about repression of Sex lethal (Sxl) expression in the anterior of syncytial blastoderm stage embryos (Parkhurst et al., 1990). Presumably, the Hairy protein mimics the activity of Deadpan, a known Sxl repressor (Younger-Shepherd et al., 1992) that is structurally related to Hairy (Bier et al., 1992). Because Sxl is required for proper dosage compensation in females, this causes female-specific lethality (Parkhurst et al., 1990). In this assay, Hairy also represses Sxl when its own repression domain is substituted for by heterologous repression domains (Jiménez et al., 1997). We therefore generated transgenic lines carrying a hb-HairyEve construct, in which the repression domain of Hairy was replaced with the Eve C-terminal 139 amino acids, including the GID (see Materials and Methods). As shown in
Fig. 5A, Sxl is efficiently repressed at the anterior of transgenic female embryos expressing this fusion protein. Importantly, this repression leads to a partial female-specific lethality (only 50-70% of the expected number of females survived to adulthood; data not shown). Thus, the Eve C-terminal region is a functional repression domain.

To test the Gro-dependence of the Eve C terminus, we examined the ability of the transgene to repress Sxl in embryos lacking maternal gro (see Materials and Methods). groRX22 or groE48 germline mosaic females were crossed to males carrying an X-linked hb-HairyEve, thus ensuring that all female embryos inherit the transgene. Indeed, Sxl is expressed at full-level throughout all progeny examined (Fig. 5B). Notably, the Gro-dependent repression activity of Eve is mediated by the GID: when males carrying a chimeric hb-HairyEve∆ transgene, in which the LFKPY motif is deleted, were crossed to wild-type females, full-level Sxl expression is seen almost throughout all female embryos (Fig. 5C), and no female-specific lethality ensues. Thus, the Eve C terminus requires an intact GID for its repressor activity, and this activity is completely dependent on Gro.

Conversion of the Groucho-recruitment motif LFKPY to LWRPW

The Eve GID sequence is different from the Hairy GID, yet both can bind Gro in vitro, in yeast and in vivo. We therefore asked whether the two GIDs are functionally equivalent, or whether the differences in sequence reflect differing activities. First we tested whether WRPW could substitute for the Eve GID in vitro and in yeast. As shown in Fig. 1B, lane 3 (Ha) and Fig. 1C (Eve WRPW), the Hairy GID interacts more strongly with Gro than does the Eve GID. Second, we assayed for function in vivo, and found that an EveWRPW transgene can rescue eve null mutants to adulthood (Fig. 6A). The degree of rescue, although certainly greater than the GID mutants that abolish the Gro interaction (compare with Fig. 3A), is not as high as with wild-type Eve. Importantly, the inability of EveWRPW to rescue fully does not appear to be due to aberrant EveWRPW function in segmentation, as embryos rescued by EveWRPW showed very few cuticle defects (comparable to those rescued by wild type Eve, Fig. 6B). Thus, EveWRPW is able to rescue embryonic development, but, perhaps because of the stronger binding to

**Fig. 4.** The eve-gro genetic interaction is dependent on the GID. (A) Enhancement of eve partial rescue phenotypes by gro. On the left, cuticles of embryos with eve function provided by one wild-type transgenic copy (2nd chromosome genotype eve^3.P[Eve-wt]/eve^3) from mothers either wild type for gro (top) or heterozygous for gro (bottom, 3rd chromosome maternal genotype groE48/+). On the right, eve function is provided by two transgenic copies of Eve without the GID (2nd chromosome genotype eve^3.P[Eve-∆LFKPY]). Note that the reduction in Gro more strongly enhances the wild-type rescued phenotype. (B) For each population of embryos described in A, cuticle phenotypes were categorized according to the number of abdominal denticle bands deleted. The graph shows how the distribution of phenotypes changed in response to the reduction in maternal Gro. The length of the arrow above the graph indicates, for each population, how much the average number of denticle bands deleted was changed by the reduction of Gro. Note that the response to gro dosage was strongly reduced by removal of the GID.
Gro and Eve co-regulate early boundaries

Fig. 6. The Hairy GID can substitute for the Eve GID in early Eve function. (A) Rescue of eve null mutants to adulthood. Homozygous transgenic insertions expressing either the wild-type Eve-coding region (in the context of the entire eve locus) or with the Hairy-like LWRPW in place of the LFKPY motif were crossed into two eve null mutant backgrounds (Df(eve)/eve, eve/, see Materials and Methods). Similar results were obtained for each; the graph shows the combined data. Rescued mutant flies were identified by their lack of the balancer marker (Curly). For wild type, the graph shows the average and range of seven independent insertion lines (as in Fig. 3); for the Hairy substitution, results are shown for individual insertion lines. The numbers above the bars are the total number of flies scored. Note that while some WRPW-containing lines rescue to adulthood, they do not do so as efficiently or as consistently as does wild-type Eve. (B) Rescue of the embryonic cuticle pattern. Embryos from the indicated transgenic lines were collected within 23 hours AED, and cuticles were prepared. Abdominal denticle bands were counted and categorized as in Fig. 3. For wild type, the average and range of results from seven lines are shown, and in each case, the total number analyzed is shown above the bar. Note that the percentage of defective embryos is low for both the wild-type lines and the lines that carry the WRPW substitution (the scale here is much smaller than in Fig. 3B), indicating that embryonic Eve function is effectively provided by the Hairy GID.

Gro, is able only to rescue adult development partially (see Discussion).

Groucho directly assists Even skipped in establishing correct parasegment spacing

When comparing the activities of altered proteins in their normal context in vivo, it is desirable to have a reliable dose-sensitive assay for function. eve is weakly haplo-insufficient, showing a reduced viability when only one copy is present (Nusslein-Volhard et al., 1984). Although most single copy embryos have no apparent defects at the end of embryogenesis, most of them do have abnormally narrow odd-numbered parasegments at early stages of development (Fig. 7B). Conversely, introducing extra copies of eve using the rescuing transgene results in abnormally wide odd-numbered parasegments (Fig. 7C). The hypomorphic (pair-rule) eve phenotype arises from odd-numbered parasegments that are

Fig. 7. Effects of gene dose and the GID on the function of Eve in establishing parasegment spacing. In each panel, the yellow bar spans parasegment 3 and the green bar spans parasegment 4. (A) Embryo with one endogenous copy and two transgenic copies of eve (eve/CyO,hb-Z; P[en]) stained for en RNA (blue) and β-galactosidase protein (brown, balancer marker). The en pattern is indistinguishable from wild type: two transgenic copies provide the equivalent of one endogenous copy. (B) Embryo with one endogenous copy only (eve/CyO,hbZ) stained for En protein (brown). Note that the odd-numbered parasegments are slightly narrower than the even-numbered ones. (C) Embryo with two endogenous and two transgenic copies of eve (CyO,hb-Z; P[en]) stained as in A; the posterior extent of β-galactosidase staining indicates that it carries two copies of the balancer. Note that the odd-numbered parasegments are slightly wider than the even-numbered ones. (D) Embryo with one transgenic copy of eve only (Df(eve); P[en]) stained for wg RNA (purple) and En protein (brown). Severe spacing defects result from eve activity equivalent to about 1/2 of one endogenous copy. (E) eve hypomorphic embryo (eve/, grown at 25°C), stained for en RNA. Note the similarity to D. (F) Embryo deficient for endogenous eve, rescued with two copies of a GID-mutated transgene (Df(eve); P[en]) stained for en RNA. Note the similarity to D,E, and the lack of rescue relative to G,H. (G) Embryo deficient for endogenous eve, rescued with two copies of a transgene with a Hairy-like GID (Df(eve); P[en]; P[ve-LFKPA]), stained for en RNA. Note the similarity to D,E, and the lack of rescue relative to G. (H) Embryo deficient for endogenous eve, rescued with two copies of a wild-type transgene (Df(eve); P[en]; P[ve-WRPW]), stained for en RNA. Odd-numbered parasegments are slightly reduced, indicating activity about equal to one endogenous eve gene (as in B).
severely reduced in width (Fig. 7E), and are unstable, so that at later stages they are eliminated by processes that repair patterning defects. Thus, one copy of the endogenous gene is near a threshold of sufficiency for generating stable parasegments, and the spacing of parasegments is a dose-dependent assay for early eve function.

Two copies of the wild-type eve transgene, inserted at various chromosomal locations (Fujioja et al. 1999), do not completely rescue the parasegment spacing defects of eve null mutants. Rather, a homozygous transgenic genotype produces heterozygous eve (Fig. 7H versus 7B), while a single (heterozygous) eve transgene produces a hypomorphic phenotype (Fig. 7D). This provides a means with which to compare the activities of altered Eve proteins expressed in their normal pattern, in either one or two copies.

When we examined the early parasegment spacing in eve mutant embryos rescued by a homozygous transgenic gene with a point mutation in the GID, we found that odd-numbered parasegments were reduced, closely resembling an eve hypomorph (Fig. 7F versus 7E). This was consistently observed with each of the GID mutants that abolished the Gro interaction (data not shown). In contrast, in transgenic flies in which the wild-type Eve motif was replaced with the Hairy GID, odd-numbered parasegments were normal, or sometimes even slightly increased relative to even-numbered parasegments (Fig. 7G). Thus, the parasegment spacing parallels the relative strength of the in vitro interaction with Gro. The similarity in phenotype produced by one copy of the wild-type transgene and two copies of the GID-mutant transgene suggests that the activity of Eve is reduced about twofold by mutation of the GID. The more complete rescue by the Hairy GID-containing protein suggests that it has a stronger activity than does wild-type Eve, consistent with the in vitro interaction data of Fig. 1 and the embryonic pattern rescue (Fig. 6B). However, the apparently more complete rescue of embryonic pattern was not fully reflected in the ability to rescue to adulthood (Fig. 6A, see Discussion).

**Groucho acts with Even skipped on two target genes with distinct repression thresholds**

What is the significance of the two distinct Eve repression activities, only one of which is dependent on Gro? Gro could be required to repress a subset of Eve targets, whereas repression of other target genes might be Gro independent. Alternatively, the two repression activities might function cooperatively, in which case both activities might be required for repression of each target gene. Extensive molecular and genetic studies have identified several target genes that are likely to be directly repressed by Eve. The best characterized of these genes are sloppy paired (slp), paired (prd) and odd skipped (odd). The posterior boundaries of expression of slp and prd correspond to the anterior and posterior borders, respectively, of the odd-numbered en stripes. As these en stripes shift posteriorly, both when the dose of gro is reduced and when the GID is mutated, the boundaries of slp and prd

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**Fig. 8.** Eve and Gro collaborate to repress two target genes.

(A-E) Embryos were hybridized with probe for slp RNA (blue), followed by staining with α-Eve polyclonal antiserum (brown).

(F-H) Embryos were similarly probed for prd RNA and Eve protein.

(A,D,F) Wild type. (B,E,G) eve deficient, rescued by two copies of a GID-mutated transgene (genotype Df(eve), P[Eve-LFKPA]). Note the expansion of slp and prd expression anterior to each Eve domain (anterior is to the left in all cases). (C,H) eve deficient, rescued by two copies of a GID-altered transgene that preserves the Gro interaction (genotype Df(eve), P[Eve-WRPW]). Note the rescue of near-normal slp and prd expression. (I) Model of how Gro assists Eve in establishing parasegment boundaries. Reduction of Gro concentration in the syncytial blastoderm reduces the repression activity of Eve, causing changes similar to a reduction in eve genetic dose or eve function (Fig. 7). Concentration-dependent effects at the anterior edge of each Eve stripe include expanded prd and slp expression (lighter colored bars). Prd activates both en and wg, while Slp represses en. Thus, Slp (and other en repressors, such as Runt, which is expressed similarly to Slp at this stage) can effectively subdivide the prd domain into wg and en domains. wg is repressed by both Eve and En. It expands posteriorly when eve activity is reduced (Fig. 2B), then maintains its border with the posteriorly shifted en stripe while contracting to one cell row at later stages (Figs 2B, 7D). The border between wg and en becomes the parasegment boundary, and the overall width of the parasegment is determined by the location of this border. The shifted parasegment boundary is indicated by a broken line. The net effect of reducing Gro or mutating the GID is to reduce the width of the odd-numbered parasegments, and to sometimes expand the odd-numbered en stripes, as prd sometimes expands more than does slp. This is also similar to what is seen in eve hypomorphs, i.e. it is not specific to a reduction in Gro. There may also be effects at the posterior border of the Eve stripe, but these appear to be relatively minor; for example, ftz expression does not appreciably expand in the absence of eve (Fujioja et al., 1995) and odd still clears from the anterior-most cell row of the ftz stripe (an eve-dependent effect) when Gro is reduced, allowing activation of the even-numbered en stripes. This still occurs both when Gro is reduced and in eve hypomorphs, so this function of eve is less concentration dependent than is repression of prd and slp.
may be coordinately shifted. We examined slp and prd expression in embryos rescued with a GID-mutated transgene. Both slp (Fig. 8B,E) and prd expression (Fig. 8G) were expanded in the eve domains, relative to wild-type embryos (Fig. 8A,D,F). The degree of expansion of each gene correlates with the shift of en stripes seen in Fig. 7F. Furthermore, both the width of individual en stripes and their spacing are very similar to those in eve hypomorphs, such as that shown in Fig. 7E. Thus, the removal of the GID has an effect that is similar to that of a general reduction of eve activity on both targets, slp and prd. This expansion of slp and prd expression was reversed, in each case, when the Eve GID motif was replaced by that of Hairy (Fig. 8C,H). These results suggest that Gro is required by Eve to a similar degree for its repression activity on each of these genes.

Repression of another Eve target gene (odd) is required for the establishment of the even-numbered (fit-dependent) en stripes (Manoukian and Krause, 1992). Intriguingly, these are established more or less normally in embryos rescued by the GID-mutated transgene (Fig. 7F), and examination of odd expression in those embryos showed it to be normal in the even-numbered parasegments (data not shown). However, repression of odd and the establishment of even-numbered en stripes are also normal when eve function is reduced in other ways (e.g. in the hypomorph; Fig. 7E; data not shown), suggesting that a lower threshold of Eve activity is required for this eve function than for proper repression of slp and prd. Therefore, this assay did not allow us to fully assess the contribution of Gro to odd repression by Eve (see Discussion).

DISCUSSION

Here, we describe the discovery of a Gro-dependent repression domain in Eve, a homeodomain protein previously known to contain a Gro-independent repression activity. An emerging theme is that many transcriptional regulators have multiple domains that use distinct mechanisms to achieve similar end results. However, little is known about the functional relationships between such domains in the regulation of target genes during development. Eve is a central component of the segmentation cascade in Drosophila, which establishes repeating patterns of gene expression along the anterior-posterior axis of the embryo, and several specific target genes regulated by Eve are known. This, in conjunction with the ability to functionally substitute altered Eve proteins for endogenous Eve, using a rescuing transgene (Fujioka et al., 1999), allowed us to analyze how the distinct repression activities of Eve function in the regulation of its target genes.

A requirement for Groucho in developmental functions of Even skipped

We have shown that Eve contains a conserved motif at its C-terminus that resembles a subset of previously identified Gro interaction domains in Hairy, Runt and Hückebein. The C-terminal region of Eve interacts with Gro in vitro and in yeast, and a conserved pentapeptide is essential for the interaction (Fig. 1). The previously known repression domain of Eve, identified in cell culture assays (Han and Manley, 1993) and shown to be Gro independent (Jiménez et al., 1997), is not included in this C-terminal region. We established that Gro is involved in Eve function in two ways: (1) by showing a genetic interaction between mutations in the two genes during embryogenesis (Fig. 2); and (2) by removing the GID in the context of a transgene capable of rescuing eve null mutants to viability (Figs 3, 7). By both criteria, the Gro interaction is crucial to the required level of Eve activity in embryos. Without it, embryonic development is seriously perturbed, and there is almost no rescue of eve-null mutants to adulthood (Fig. 3). We further showed that the genetic interaction is mediated by physical interaction between the two proteins (Fig. 4). We established that the GID mediates repression in early embryos, and that this repression is Gro dependent (Fig. 5). These studies set the stage for an investigation of how Gro and Eve function together on specific target genes during development.

Groucho augments the repression of multiple Even skipped target genes

Previous studies have shown that the parasegment spacing defects of eve hypomorphs are the result of a reduced activity on at least two target genes, slp and prd (summarized by Fujioka et al., 1995; see also Raj et al., 2000). Eve represses each of these target genes, but within each early Eve stripe, which is about five to six nuclei wide, prd is expressed in a region of intermediate Eve concentration where slp is repressed. Thus, these two target genes are differentially sensitive to Eve’s repression activity. We examined how these target genes responded when the GID was mutated, and found that both expand their expression domains into regions of higher Eve concentration (Fig. 8). However, in eve-null mutants, both expand throughout the entire eve domain of expression (Fujioka et al., 1995; Gutjahr et al., 1993), so clearly both are still repressed to some extent without the GID. As expected from the fact that Eve contains a strong Gro-independent activity in early embryos (Jiménez et al., 1997), removal of the Gro-independent domain also has a strong effect on repression of these target genes (M. F., G. Yusibova, and J. B. J., unpublished). Thus, both of the repression domains of Eve contribute significantly to its activity, and we find no evidence of differential Gro activity on these target genes.

Another target gene of Eve that is repressed at this stage of development is odd. Repression of odd in the cells that give rise to the even-numbered en stripes is not apparently affected by mutating the GID. Thus, odd is formally a Gro-independent target of repression by Eve, in contrast to prd and slp. However, repression of odd is also insensitive to reducing the activity of eve genetically. Therefore, as odd is not a dose-sensitive target gene, either the Gro-dependent or -independent activity of Eve may be sufficient for complete repression of odd. Overall, in the early embryonic (segmentation) function of Eve, Gro may be a constitutive partner that contributes a substantial fraction of the repression activity on each target gene.

Functional distinctions among repression domains

Substituting the GID from Hairy for that of Eve provides a similar function in several assays, although it appears that there is a subtle difference in their activities. The Hairy GID is fully functional in segmentation, where its activity appears to be somewhat stronger than that of the Eve GID, consistent with the relative strengths of the in vitro interactions with Gro (Fig. 1). In embryos, both segmentation defects (Figs 6, 7) and
the ability to hatch (data not shown) are rescued by the Hairy GID, and the expression of downstream target genes is restored to normal or near normal (Fig. 8). However, the Hairy GID, in the context of Eve, may be improperly regulated or hyperactive at a later stage of development. With the Hairy GID, the ability of Eve to rescue eve mutant flies to adulthood is impaired (Fig. 6). Thus, in addition to the Hairy GID having a stronger activity than that of Eve, these two GIDs may respond differently to regulatory inputs, particularly at post-embryonic stages of development, where Eve is known to function in the regulation of neuroblast proliferation (Park et al., 1998; Park et al., 2001).

It is intriguing that although the GID of Eve provides a substantial amount of activity in vivo, it was not identified as a repression domain in transient assays in Drosophila S2 cells, even though Gro is abundant in these cells, and the GIDs from Hairy (Fisher et al., 1996) and Runt (Aronson et al., 1997) were shown to have activity in such assays. This situation is similar to that with the two En repression domains, where the activity of the GID (eh1) was not apparent in transient transfections, but was apparent in the same cells when stably integrated target genes were assayed (Tolkunova et al., 1998), consistent with its substantial contribution to En function in embryos (Smith and Jaynes, 1996). Thus, GIDs may not function as effectively in transient transfection assays as they do in vivo, for reasons that are still unclear.

Despite the identification of many repressors and several co-repressors, the ultimate targets of their action remain largely unknown. Consistent with the involvement of chromatin structure, histone deacetylases have been shown to interact with Gro family members, and to account for part of their repression function (Chen et al., 1999; Mannervik and Levine, 1999; Sun and Taneda, 2000).

In conclusion, Gro-mediated repression is an important aspect of the function of Eve, collaborating with the Gro-independent repression activity of Eve on multiple target genes. Their combined action during embryonic segmentation is required to provide a precise level of repression of each of the dose-dependent target genes for Eve.

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**Note added in proof**

A recent study identified an ‘LFKPY’ motif in the Brinker protein that interacts with Gro (Zhang et al., 2001).

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