Control of *Drosophila* imaginal disc development by *rotund* and *roughened eye*: differentially expressed transcripts of the same gene encoding functionally distinct zinc finger proteins

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**SUMMARY**

The *Drosophila rotund* gene is required in the wings, antenna, haltere, proboscis and legs. A member of the Rac family of GTPases, denoted the *rotund racGAP* gene, was previously identified in the *rotund* region. However, previous studies indicated that *rotund racGAP* was not responsible for the *rotund* phenotypes and that the *rotund* gene had yet to be identified. We have isolated the *rotund* gene and show that it is a member of the *Krüppel* family of zinc finger genes. The adjacent *roughened eye* locus specifically affects the eye and is genetically separable from *rotund*. However, *roughened eye* and *rotund* are tightly linked, and we have therefore also isolated the *roughened eye* transcript. Intriguingly, we show that *roughened eye* is part of the *rotund* gene but is represented by a different transcript. The *rotund* and *roughened eye* transcripts result from the utilization of two different promoters that direct expression in non-overlapping domains in the larval imaginal discs. The predicted Rotund and Roughened Eye proteins share the same C-terminal region, including the zinc finger domain, but differ in their N-terminal regions. Each cDNA can rescue only the corresponding mutation and show negative effects when expressed in each others domain of expression. These results indicate that in addition to the differential expression of *rotund* and *roughened eye*, their proteins have distinct activities. *Rotund* and *roughened eye* act downstream of early patterning genes such as *dachshund* and appear to be involved in *Notch* signaling by regulating *Delta, scabrous* and *Serrate*.

**Key words:** Imaginal disc development, Zinc finger, *Drosophila melanogaster*, Dual promoters, *rotund, roughened eye*

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**INTRODUCTION**

The *Drosophila rotund* (*rn*) locus is recessive viable causing male and female sterility as well as defects in adult body structures (Cavener et al., 1986). These include defects in the antennae, wing, haltere and proboscis as well as fusion of all five leg tarsi into one fused tarsal-like segment. Analysis of third instar larvae imaginal discs revealed localized cell death in the regions giving rise to the affected adult structures (Kerridge and Thomas-Cavallin, 1988). The *rn* locus has previously been molecularly analyzed (Agnel et al., 1989) and a cDNA encoding a member of the Rac family of GTPase-activating proteins (GAP) was isolated from this genomic region (Agnel et al., 1992b). Since this gene was located in the *rn* genomic region it was denoted the *rotund racGAP* (*rnracGAP*), but molecular analysis of multiple *rn* alleles indicated that the *rnracGAP* is not responsible for the *rn* phenotypes (Agnel et al., 1992a). In fact, all studies to date instead point to an uncharacterized larger transcript as the likely candidate for the *rn* gene (Agnel et al., 1992a; Hoemann et al., 1996).

The closely linked *roughened eye* (*roe*) locus affects a late step in the development of the eye, and *roe* mutants display rough eye morphology and reduction of photoreceptors (Renfranz and Benzer, 1989). The *roe* gene is genetically separable from *rn*, but the two genes show complex complementation (Brand and Campos-Ortega, 1990; Kerridge and Thomas-Cavallin, 1988; Ma et al., 1996). This previously led to the suggestion that *rn* and *roe* may be ‘two classes of mutation of the same gene, each of them disrupting a subfunction’ (Ma et al., 1996). To address the tight link between these two adjacent loci we have isolated the *rn* and *roe* genes. Intriguingly, our results show that *roe* is part of the *rn* gene but is represented by a different transcript. These two transcripts encode predicted proteins with an identical C-terminal region, containing a Krüppel-type zinc finger domain, but with different N-terminal regions. *rn* and *roe* are expressed in non-overlapping domains in the larval imaginal discs. Each cDNA can rescue only the corresponding mutation and when misexpressed in each others domain of expression has negative effects. Our results indicate that these two loci are genetically separable not only because of their differential expression but also because of distinct activities of the Rn and Roe proteins. By analyzing the expression of a number of markers in the...
developing imaginal discs, we further show that \( r\) and \( roe \) act downstream of early patterning genes such as \( dachshund \), but may act to modulate Notch signaling by regulating expression of Delta, Scabrous and Serrate.

**MATERIALS AND METHODS**

Fly stocks

\( w^{118}, \) \( roe^3, \) UAS-lacZ, and \( p^+/cu \) (Bloomington Stock Center); \( r^n 89 \) (Couso and Bishop, 1998) identified as \( P 089 \) in Flyview stock collection (http://pbiol07.uni-muenster.de); \( r^n 16, \) \( r^n 19, \) \( r^n 20 \) (Agnel et al., 1989); \( ser\)–GAL4 (A. Bailey and G. M. Rubin); GMR-GAL4 (Hay et al., 1997); UAS-\( rin\#1, \) UAS-\( rin\#32, \) UAS-\( roe\#18, \) UAS-\( roe\#88 \) and \( r^n GAL4\) (this study). Mutations were maintained over standard balancers with lacZ or GFP markers.

**Isolation of \( r^n \) and \( roe \) cDNAs**

Using genomic fragment D (Agnel et al., 1989) (provided by R. Griffin-Shea) as a probe, three \( Drosophila \) cDNA libraries were screened for a total of 11 million plaques and colonies. A larval \( Agt1 \) cDNA library (Clontech) yielded a 1.3 kilobase pair (kb) positive clone (4H). Comparison of the 4H sequence with \( Drosophila \) genomic sequence revealed that the 4H cDNA was truncated on both ends owing to internal EcoRI sites. To obtain the remainder of the cDNA we used PCR to amplify a 700 bp fragment downstream of the 3′ EcoRI site and used this PCR fragment to screen the same larval library. From 4 million plaques a 2.3 kb clone (22-4) was isolated and sequenced. The compiled cDNA sequence (4H/22-4) contained a long RI site and used this PCR fragment to screen the same larval EcoRI sites. To obtain the remainder of the cDNA we used PCR to amplify a 700 bp fragment downstream of the 3′ EcoRI site and used this PCR fragment to screen the same larval library. From 4 million plaques a 2.3 kb clone (22-4) was isolated and sequenced. The compiled cDNA sequence (4H/22-4) contained a long open reading frame (ORF) encoding a putative protein of 945 amino acids (aa; GenBank AF395905). There are several putative start codons at the beginning of the ORF, one of which closely matches the consensus (Cavener and Ray, 1991). Owing to internal EcoRI sites at the 5′ of clone 4H and the 3′ of clone 22-4, the precise extent of the \( r^n \) gene was not determined. Immediately 3′ of clone 22-4 the genome sequence reveals a number of polyadenylation sites that likely are used as termination signals.

We used a 3′ fragment from \( r^n \) clone 22-4 (bp 2714-3658 of GenBank AF395905) as a probe to screen the larval cDNA library used for isolation of the \( r^n \) cDNA. This yielded 2 positive clones out of 5 million plaques. Both clones contained truncated \( roe \) cDNAs, corresponding to bp 332-2856 and 621-2856 (GenBank AF395904). Both inserts crossed the junction between exon 1 and exon 2 of the predicted \( roe \) gene, extending past the end of the Roe ORF. Since we did not obtain a full-length \( roe \) cDNA, we verified the structure of the \( roe \) transcript by amplifying part of it using RT-PCR. For this, RNA from \( w^{118} \) embryos was isolated and purified using RNAsol (Tel-Test, Inc.) and Qiagen Oligotex (Qiagen). We designed a primer in the predicted first exon, 5′ to stop codons in all three reading frames and followed by the predicted Roe start methionine (TAAATGGATCT-TGGACAGTGA), and 2 primers in exon 2 (ATGCCGAGCT-GGTGAACCT and TGGAGACGACGAGCTG). Using these primers, nested PCR was performed and a product of the predicted size was generated. Sequencing of this fragment was in agreement with our prediction for the intron/exon structure of \( roe \) (GenBank AF395904).

**Generation of UAS-\( r^n \) and UAS-\( roe \)**

\( r^n \) sequences corresponding to position 0-3373 (GenBank AF395905) of \( r^n \) cDNA, and \( roe \) sequences corresponding to 0-2160 of \( roe \) cDNA (GenBank AF395904) and 86 bp of upstream genomic DNA, were cloned into the pUAST vector (Brand and Perrimon, 1993). Three independent UAS-\( r^n \) and eight independent UAS-\( roe \) transgenic lines were generated using P-element transformation (Spradling and Rubin, 1982). These lines were tested for expression using GMR-GAL4 and all gave strong phenotypes indicating similar levels of expression.

**P-element analysis**

The insertion of the \( r^n 89 \) enhancer trap, a \( P[\text{lArB}] \) insert, was determined using standard plasmid rescue methods. This revealed that \( P[\text{lArB}] \) is inserted at position -440 bp upstream of the \( r^n \) cDNA (GenBank AF395905).

Conversion of \( P[\text{lArB}] \) in \( r^n 89 \) to \( P[GawB] \) was carried out as previously described (Sepp and Auld, 1999) with some modifications. Briefly, males of the genotype \( w^{118}, elav\text{C159}(GawB); w^{118}2-3,B \) were crossed to \( w^{118} \) females and their progeny screened for red-eyed males (indicating that the \( P[GawB] \) had mobilized onto the autosomes). These males were crossed singly to \( UAS\text{-GFP}/TM3,B \) and their progeny screened for the \( r^n \) expression pattern in larvae. From 30 lines screened, 3 independent insertions (\( r^n GAL4_{A85}, \) \( r^n GAL4_{A113} \) and \( r^n GAL4_{A14} \)) expressed GFP in the \( r^n \) pattern and subsequently failed to complement \( r^n \). The site of insertion and the orientation of \( P[GawB] \) was determined by PCR amplification and sequencing. In all three cases \( P[GawB] \) was inserted in the exact same position as \( r^n 89 \) \( P[\text{lArB}] \). For the rescue experiments \( r^n GAL4_{A85} \) was used. The three \( r^n GAL4 \) lines enhance the wing phenotype of \( Ser^1 \), common to many third chromosome balancer lines (not shown).

To verify that the \( r^n 89 \) and \( r^n GAL4_{A85} \) mutant phenotypes were due to the insertion of the \( P \) elements, we excised them by standard methods. For \( r^n 89 \), six independent revertants were isolated using their complementation of \( r^n \). Two independent revertant lines (\( r^n 1-5 \) and \( r^n 2-1 \)) were homozygous viable and showed no \( roe \) phenotype. They were further analyzed by PCR and sequencing to determine the structure at the P-element insertion site. In both cases the \( P \) element had imprecisely excised but left a 30 bp (\( r^n 1-5 \)) and 37 bp (\( r^n 2-1 \)) ‘footprint’ containing the expected direct duplication of the 8 bp \( P \) element target sequence and additional sequences from both ends of the \( P \) element. These ‘footprints’ are outside the identified \( r^n \) exons thus explaining why they reverse the \( roe \) phenotype. Additionally, four stronger independent alleles were identified, one of which, \( r^n 32-2 \) was analyzed in more detail. Southern blot analysis using multiple probes, revealed that \( r^n 32-2 \) retained \( P[\text{lArB}] \) but is deleted for 3′ flanking genomic DNA removing the first and part of the second \( r^n \) exon (Fig. 1A). For the reversion of \( r^n GAL4_{A85} \) a similar strategy was used and we obtained 5 independent revertant lines that complemented multiple \( r^n \) alleles, and in addition had lost the \( white \) marker and \( GAL4 \) expression.

**Analysis of \( roe^3 \)**

To identify the EMS-induced mutation in \( roe^3 \), we amplified a 1.5 kb genomic region covering the first exon of \( roe \) (primers were ATGCCAGAGCT-GGTGAAACTT and CCAATGGAGGCGGCTTCCA). Three independent PCR fragments using genomic DNA from \( w^{118}, \) \( roe^3/m^20 \) and \( p^+/cu \) were sub-cloned and three clones from each were sequenced (\( p^+/cu \) was used as a second control since the \( roe^3 \) parental chromosome could not be obtained). We found several conservative changes between \( roe^3 \) and each of the other two lines, but only one non-conservative change between \( roe^3 \) and both \( w^{118} \) and \( p^+/cu \). This was a nonsense C→T mutation resulting in a glutamine to amber stop codon change at aa position 191 (bp 629 in GenBank AF395904) in the Roe ORF (Fig. 1A). This mutation would truncate the predicted Roe protein and the mutant protein would lack the entire C-terminal region including the ZF domain.

**In situ hybridization and immunohistochemistry**

Standard in situ protocols were used to examine expression of \( r^n \) and \( roe \) (Tautz and Pfeile, 1989). We used three probes, 4H, containing \( r^n \) only sequences (0-1331of GenBank AF395905), \( roe \), containing the first exon of \( roe \) (0-785 of GenBank AF395904) and ZF, containing common 3′ sequences including the ZF domain (2016-3373 of GenBank AF395904). Sense probes showed no signal in embryos or larvae. For the roe rescue experiments, adult eyes were cryo-sectioned and immunostained for Elav, a marker for photoreceptors (O’Neill et al., 1994). More than 14 ommatidia from more than four flies per genotype were analyzed and the total number
of R1-7 photoreceptors determined. For epistatic analysis, third instar imaginal discs were immunostained using the following primary antibodies: anti-Elav (1:10), anti-Dac (1:25), anti-Boss (1:2000), anti-Sca (1:10), anti-Ser (1:1000), anti-Bab (1:2000) and anti-Dl (1:20).

RESULTS

Isolation of rotund and roughened eye

To isolate the rn cDNA we used genomic fragment D (Fig. 1A), shown to hybridize to the putative rn transcript (Agnel et al., 1989). The cDNA sequence indicates that rn encodes a Krüppel-type zinc finger (ZF) protein and contains six C2H2 ZFs. The predicted Rn protein has a high degree of homology to the predicted protein of Drosophila gene CG5557 (Adams et al., 2000), and to C.elegans Lin-29 (Rougvie and Ambros, 1995). Over the ~150 aa ZF domain these two proteins display 84-90% identity to Rn (Fig. 1C). Among mammalian proteins, a recently identified rat cDNA, Cas-Interacting Zinc finger (CIZ) (Nakamoto et al., 2000), displays the highest homology (59% in the ZF) to Rn. Rn and CG5557 also share a short C-terminal domain of high homology not found in the other proteins (Fig. 1C). In line with the complex genetics of this area, the alignment of the rn cDNA with the genomic sequence reveals that rn spans ~50 kb and extends on both sides of the mnracGAP (Fig. 1A).

The roe gene shows complex complementation with rn and a number of roe alleles are also rn (Agnel et al., 1989; Brand and Campos-Ortega, 1990; Kerridge and Thomas-Cavallin, 1988; Ma et al., 1996). The rn gene structure together with previous molecular work on rn alleles gave us some initial insight into the identity of roe. Particularly informative were the rnA2-2 and rn19 alleles. The rnA2-2 P-element excision allele (materials and methods) contains a deletion in the rn 5' region removing the first and part of the second exon of rn (Fig. 1A). Complementation analysis of rnA2-2 shows that it is a null allele of rn but does not cause roe phenotypes (see below). Furthermore, the rn19 allele, shown to contain a larger deletion in the rn 5' region (Agnel et al., 1989), acts as a rn null allele and, although it removes at least one other lethal complementation group, does not cause roe phenotypes. These results indicated the existence of roe-specific functions encoded in the genomic region proximal to the breakpoint of rn19 (Fig. 1A). One model could be the existence of roe specific exon(s) that are spliced and utilized specifically in the eye.

Fig. 1. (A) Genomic organization of the rn region. Insertion site of the three P elements is denoted by open triangles. The deletion in rnA2-2 is denoted by the extended line. Fragment D was isolated in the previous study (Agnel et al., 1989) and used to initiate the screen for rn. Putative promoters are depicted as angled arrows. The rn and roe transcripts are outlined and the ORFs designated by black boxes for both genes. The ZF domain is represented by gray shading. Deletions used in this study are indicated at the bottom and breakpoints, where known, are shown. Data for rn19 and rn20 are based on previous studies (Agnel et al., 1989). rn16 was described as a smaller deletion mapping to the 3' area (Agnel et al., 1989) but our work shows that it extends further, deleting both the common ZF coding exons and the roe-specific exons (not shown). The roei mutation (asterisk), is a glutamine to an amber stop codon. (B) Predicted protein structure of Rn and Roe. The N-termini are unique but the C-termini, containing most of the ZF domain, are identical. The glutamine, serine and alanine stretches are designated Q, S and A, respectively. (C) Comparison of Rn with other ZF proteins. Rn has a few close homologs in Drosophila (D.m.), C. elegans (C.e.) and rat. Numbers in circles are the percentage of identical amino acids between Rn and the other proteins in the ZF domain. Rn, Roe and Drosophila CG5557 further share a C-terminal region of homology not present in the other proteins (gray).
Molecular analysis of rotund and roughened eye mutations

The genomic structure of the rn locus that we propose fits well both with previous studies as well as with our molecular analysis of rn and roe alleles. First, rn16 and rn20 are deletions that show both rn and roe phenotypes, while the rn19 deletion only shows rn phenotypes (Agnele, et al., 1989). In agreement, rn16 deletes both the common ZF coding exons and roe-specific exons, rn20 deletes the whole region, and rn19 removes most of the rn-specific exons (Fig. 1A). Second, we sequenced roe1, a strong roe-specific allele, and show that it is the result of a nonsense mutation in the roe-specific exon. This mutation does not affect the common 3’ exons and explains why roe1 acts as a roe null allele but does not show rn phenotypes. Third, rn89, a lacZ-containing P-element transposon allele (Couso and Bishop, 1998) was shown to be inserted within the 5’ region of the rn gene. This explains why it only displays rn and not roe phenotypes. In addition, imprecise excision of rn89 yielded rn82-2, which contains a deletion of the first and part of the second rn exon (Fig. 1A). As expected, rn82-2 displays a rn null phenotype (Fig. 3C,I) but no eye phenotype. In agreement with this, in situ hybridization failed to detect any rn transcript in rn82-2 mutant discs (not shown). We further generated rnGAL4#5 by P-element conversion of rn89, rnGAL4#5 displays a stronger leg phenotype than rn89, possibly due to differences in the structure of the P element, but again no aberrant eye phenotype (not shown). Wild-type revertants of rn89 and rnGAL4#5 were generated that complement other rn alleles, verifying that in both cases the rn phenotype was caused by the P-element insertion.

Expression of rotund and roughened eye

We detect expression of rn and roe in developing imaginal discs, as well as in the embryonic and larval CNS. Here we will focus on the expression in the imaginal discs. Expression of rn commences during the early third larval instar in the leg, wing, haltere and antennal part of the eye-antennal imaginal disc (Fig. 2E-H). Expression of rn is observed as a ring in the leg and antenna discs and in the presumptive wing pouch and capitellum of wing and haltere discs respectively. In late third instar, expression of rn in the leg disc is no longer evident, but is maintained in the other discs (Fig. 2G). We also studied the expression of lacZ in both rn89 and in rnGAL4#5/UAS-lacZ larvae to determine rn expression. In both genotypes, expression of lacZ is in agreement with the rn in situ hybridization, except for the persistence of tarsal expression (Fig. 2A-D), but in neither line do we detect expression in the eye disc. Expression of roe commences in the third instar and the predicted Roe protein shares the C-terminal region, including the ZF domain except the first finger, with Rn but differs in the N-terminal region (Fig. 1B). It is interesting to note that the rn genomic structure was not revealed by the analysis of the sequences carried out by the Drosophila Genome Project (Adams et al., 2000). Although parts of the rn coding regions were identified (CG14600, CG14601, CG14603 and CG10040), the rn transcript was not predicted, probably because rn has several small exons spread over 50 kb. In contrast, the roe transcript was accurately predicted, short of one aa error in the splice junction between exons 1 and 2 (CG10040). At the submission of this study, the rn and roe cDNAs had not been isolated in the BDGP or RIKEN expressed sequence tag (EST) projects.

However, the fact that rn19 extends further distally, uncovering other complementation group(s), but does not produce roe phenotypes argues against eye-specific splicing of a long transcript originating from a promoter in the rn region. Instead, a more likely scenario would be the existence of an eye-specific promoter and exon(s). This notion was further supported by analysis of P-element insertions in the rn 5’ area that result in the rn phenotype and matching expression but not in the roe phenotype or eye disc expression (see below). These results prompted us to look for additional exons that could explain the molecular nature of the roe gene. By screening a larval cdna library with a rn 3’ probe and by subsequent PCR analysis we isolated the roe cdNA. The roe gene utilizes the same two 3’ exons as rn but contains a different 5’ exon (Fig. 1A). As a result
is confined to the eye part of the eye-antennal imaginal disc in a band of 4-6 cells at the morphogenetic furrow (Fig. 2I,K). We find no evidence of roe expression in other imaginal discs. The expression of rn and roe is in agreement with the observed phenotypes. For instance, rn mutants have defects in wings and halteres, and correspondingly rn is expressed in the appropriate presumptive regions in wing and haltere imaginal discs. In the leg, rn mutants display fusion of all 5 leg tarsi into one fused tarsal-like segment. In agreement with this, rn is expressed in a sub-distal ring that represents the presumptive tarsus, as revealed by the persistent tarsal expression of rn-driven lacZ in late third instar discs. Similarly, roe specifically affects the eye, and mutants have rough eyes and reduced numbers of photoreceptors (Ma et al., 1996). Accordingly, we observe expression of roe in the eye part of the eye-antennal imaginal disc but not in other imaginal discs. The mutually exclusive patterns of expression of rn and roe raised the issue of whether they may in fact negatively regulate each other. To determine this, we analyzed the expression of roe in rn mutant imaginal discs and conversely the expression of rn in roe mutant imaginal discs. These studies revealed no apparent changes in the expression of rn and roe when compared to wild type, indicating that there is no cross-regulation between rn and roe (not shown).

**Rescue of rotund**

Owing to the complexity of the rn locus we wanted to further verify the authenticity of our rn and roe cDNAs by rescue experiments. For the rn rescue we focused on the leg phenotype and used the rnGAL4/#5 line that shows strong leg phenotypes over rn20 (Fig. 3A,D,I). By providing rn function with UAS-rn, we observe rescue of the rnGAL4/#5/rn20 leg phenotypes, often to a level indistinguishable from the wild-type leg (Fig. 3F,I, P<0.001). We do not observe any dominant effect in the leg of UAS-rn in a heterozygous background (Fig. 3E,I).

The structure of the rn genomic region and the differential expression in imaginal discs explains why rn and roe can be genetically separated and affect different tissues. However, the rn and roe gene products are also different, and the first ZF is truncated in the Roe protein (Fig. 1B), intriguing given that the first finger of Krüppel-type ZF proteins has been shown to be involved in DNA-binding (Avram et al., 1999; Hamilton et al., 1998). Rn and Roe further differ in the N-terminal regions where they contain stretches of glutamine/serine (Roe) or alanine (Rn), often found in transcriptional activator and repressor domains respectively (Gerber et al., 1994; Lanz et al., 1995; Licht et al., 1994; Madden et al., 1993; Nowling et al., 2000). This raised the possibility that these two proteins may have different activities and may not be interchangeable. To address this issue we misexpressed roe in the leg disc and also attempted to rescue rn with roe. When roe is misexpressed in the developing leg disc using rnGAL4/#5, we noticed a negative effect with reduced number of tarsi, similar to rn mutants (Fig. 3).
(A,B) Expression of sev-GAL4, visualized by crossing to UAS-lacZ and staining for anti-β-gal (green), in relation to Elav (red) expression. Expression of sev-GAL4 commences posterior to the morphogenetic furrow in subsets of photoreceptors, as evident by the overlap with Elav. In addition, sev-GAL4 expression is observed in cells adjacent to the developing photoreceptors, most likely corresponding to mystery and cone cells. (C,D) Misexpression of rn (C) in UAS-rn/+;GMR-GAL4/+ and roe (D) UAS-roe/+;GMR-GAL4/+ both lead to disruptions in the morphology and size of the adult eye. These include an apparent loss of pigment cells and bristle cells, as well as the presence of patches of necrotic tissue (black).

Furthermore, in a rn mutant background (rn3G,I) we observe no evidence of rescue by UAS-roe (Fig. 3H,I).

**Rescue of roughened eye**

We also wanted to rescue roe mutants using the GAL4/UAS system. The roe rescue was complicated by the fact that we did not have a GAL4 insertion in the roe gene. This is especially relevant given the dynamic pattern of roe expression in the eye disc, with transient expression in a band of approx. 4-6 cells at the morphogenetic furrow (Fig. 2I,K). We were unable to identify a GAL4 line that would express precisely in the roe pattern and instead attempted to rescue roe using GAL4 drivers that would drive in photoreceptors. To this end, we tested several eye disc GAL4 driver lines for ectopic effects. Not surprisingly, strong pan-eye drivers such as GMR-GAL4 lead to dramatic phenotypes with loss of pigment and bristle cells (Fig. 4D). A novel sevenless-GAL4 (sev-GAL4) line that expresses GAL4 in the photoreceptors, cone and mystery cells (Fig. 4A,B) showed little if any sign of rough eye morphology when crossed to UAS-roe (not shown). Using sev-GAL4 crossed to UAS-roe in a roe null mutant background (rn16/rn20) we observe partial rescue of the eye phenotypes with increased eye size and reduced roughness (Fig. 5A-C). To quantify the roe rescue we counted the number of adult R1-7 photoreceptors in wild-type, mutant and rescued flies. These results confirm previous studies (Ma et al., 1996) and show that roe mutants have a reduced number of photoreceptors compared to wild type (Fig. 5E). In line with the apparent morphological rescue we find significantly increased numbers of photoreceptors in rescued flies when compared to mutants (P<0.04, Fig. 5E). Given that we were unable to use a GAL4 driver line that perfectly matched the dynamic expression of roe in eye discs, we believe that this partial rescue supports the proposed identity of the roe gene. As in the rn rescue experiments, we wanted to address whether rn is interchangeable with roe and could provide rescue activity in the eye. First we tested the activity of UAS-rn in the eye by misexpressing it using GMR-GAL4 and sev-GAL4. This leads to severe rough eye phenotypes with GMR-GAL4 (Fig. 4C) and little if any sign of rough eye morphology with sev-GAL4 (not shown). In a roe null mutant background (rn16/rn20) we find no evidence of rescue by adding UAS-rn (Fig. 5B-E).
roughened eye is part of the rotund gene

Molecular context for rotund and roughened eye activity

Previous studies suggested that *rn* and *roe* act late during development of their respective tissues, perhaps during terminal differentiation (Godt et al., 1993; Renfranz and Benzer, 1989). To further explore the function of *rn* and *roe* during leg and eye development, we have examined the expression of genes that play key roles during development of these tissues. We first studied the leg disc and analyzed genes whose expression abuts or overlaps that of *rn*. Dachshund (Dac), a nuclear factor required for normal leg development, is expressed at early stages of leg development in a ring pattern that abuts the early *rn*-expressing ring (M. I. G., S. A. Bishop and J. P. C., unpublished). Bric a brac (Bab), a BTB-domain containing transcription factor, has been suggested to be active late in limb development and is expressed in a similar pattern to *rn* in the leg (Godt et al., 1993). Furthermore, *bab* mutants show similar (though not identical) phenotypes to *rn* mutants in the tarsal segments of the leg (Godt et al., 1993). Interestingly, neither Dac nor Bab appears to be regulated by *rn* as revealed by staining of third instar leg discs (Fig. 6A,B; not shown). These results suggest that *rn* might act in parallel to, or downstream of, *dac* and *bab* to specify tarsal segment...
identity. Ser, a ligand for the Notch (N) receptor, is expressed in presumptive joint areas in larvae and pupa leg discs and controls the development of the leg joints (Bishop et al., 1999). In wild-type mid-third instar leg discs, Ser is expressed in the first tarsal fold, which coincides with the \( rn \)-expressing ring. In \( rn \), Ser is down-regulated in the tarsal ring but not outside it (Fig. 6C,D). In pupal leg discs, Ser expression, normally present in four stripes within the presumptive tarsal area (Fig. 6E), is present in fewer and less defined stripes in \( rn \) (Fig. 6F).

The \( roe \) rough eye phenotype is reflected in reduced numbers of photoreceptors present in adult ommatidia (Brand and Campos-Ortega, 1990) (this study). To determine whether \( roe \) mutants show early patterning defects in the eye-antennal disc, we analyzed expression of Dac, which plays an early role in the eye disc and is expressed in a broad domain spanning both sides of the morphogenetic furrow (MF) (Mardon et al., 1994). Since \( dac \) mutants have a more severe eye phenotype than \( roe \) we anticipated that Dac would not be regulated by \( roe \), and as expected we observe no change in the pattern of Dac staining in \( roe \) when compared to wild type (Fig. 7A,B). Next we analyzed third instar eye-antennal discs with antibodies to Elav and to Bride of Sevenless (Boss), a marker of R8 photoreceptors (Hart et al., 1990). In wild-type eye discs, Elav and Boss are expressed in a stereotyped pattern immediately posterior to the MF (Fig. 7C,E). In \( roe \) mutants, expression of Elav and Boss reveals abnormal photoreceptor differentiation with apparent gaps in the expression of both markers posterior to the MF (Fig. 7D,F). Elav expression also indicates that photoreceptor clusters frequently have fewer photoreceptors than normal (Fig. 7E,F). Expression of Elav and Boss further reveals an apparent failure of the MF to progress in a straight line from dorsal to ventral. The MF appears to progress more slowly in some areas, creating a wave-like appearance of developing photoreceptor clusters near the MF (Fig. 7C-F). These results indicate that \( roe \) function is centered around the MF, a notion that fits well with the strong but transient \( roe \) expression seen at the MF (Fig. 2I,K). We therefore analyzed markers expressed at the MF, and since \( roe \) has been shown to interact genetically with the \( N^{mol} \) mutation (Brand and Campos-Ortega, 1990), we examined expression of Delta (Dl), a N ligand (Vassil et al., 1987), and Scabrous (Sca), a secreted glycoprotein implicated in N signaling (Baker et al., 1990). In wild type, Dl and Sca are expressed in clusters of cells at the MF, and expression is maintained posterior to the MF in subsets of cells (Fig. 7G,I). In \( roe \) mutants, the punctate expression of Dl and Sca is lost at the MF and replaced by a diffuse band of expression. Posterior to the MF, expression is punctate but appears disorganized (Fig. 7H,J).

DISCUSSION

The \( rn \) and \( roe \) loci are tightly linked and this study reveals the underlying molecular basis for this linkage. Intriguingly, our work shows that \( roe \) is part of the \( rn \) gene and is represented by a related but distinct transcript. The rescue and misexpression experiments support the notion that \( rn \) and \( roe \) play different roles during imaginal disc development not only because of their differential expression but also because of distinct activities of the Rn and Roe proteins. These activities could involve different target DNA sequences and/or different transcriptional effects, perhaps based on their different ZF and glutamine/alanine-serine stretches.

Regarding the function of the \( rnracGAP \), both our work and previous studies argue against any involvement of \( rnracGAP \) in the \( rn \) or \( roe \) phenotypes (Agnel et al., 1989; Agnel et al., 1992a; Hoemann et al., 1996). In situ studies indicate that \( rnracGAP \) is only expressed at low levels in the imaginal discs during pupal stages (Agnel et al., 1989; Agnel et al., 1992a; Hoemann et al., 1996). In addition, there is no obvious difference in the severity of \( rn \) and \( roe \) phenotypes whether or not the \( rnracGAP \) is simultaneously removed. For instance, we have found no significant difference in the severity of \( rn \) leg phenotypes in \( rn^{20}/rn^{20} \) (that removes \( rn \), \( roe \) and \( rnracGAP \)) compared to \( rn^{19}/rn^{20} \) (\( rn^{19} \) does not remove \( rnracGAP \)). Similarly, \( roe^{2}/rn^{20} \) (\( roe^{3} \) has a premature stop codon in the \( roe \)-specific exon) displays as severe of an eye phenotype as \( rn^{20}/rn^{20} \) (not shown). Furthermore, we can rescue \( rn \) and \( roe \) mutants with the \( rn \) and \( roe \) cDNAs. Recent studies may indicate an involvement of \( rnracGAP \) specifically in male fertility, and high levels of \( rnracGAP \) expression have been observed in the adult testis (Agnel et al., 1989; Agnel et al., 1992a; Hoemann et al., 1996). The \( rn^{89} \) and \( rn^{GAL\#5} \) P-element insertions described here may provide useful starting materials for the generation of mutations specifically affecting the \( rnracGAP \) by local P-element mobilization.

Little is known about the genetic cascades within which \( roe \) and \( rn \) are acting. The results from eye-antennal imaginal discs indicate that \( roe \) acts at the morphogenetic furrow, as evident both from its expression and from the effects on Dl and Sca expression in \( roe \) mutants. Both Dl and Sca play roles in spacing the array of ommatidial precursors in the morphogenetic furrow (Baker et al., 1990; Baker and Zitron, 1995; Ellis et al., 1994), and it is interesting to note that the expression of \( roe \) at the furrow is not evenly distributed and appears stronger in clusters of cells (Fig. 2I). Genetic screens for modifiers of the \( N^{pp} \) mutation identified \( roe \) as an enhancer, and \( sca \) and Dl as suppressors of the \( N^{pp} \) eye phenotype (Brand and Campos-Ortega, 1990). Given the dynamics of N signaling, these results support models where Roe acts at the morphogenetic furrow, as evident in the MF (this study). To determine whether \( roe \) and \( rnracGAP \) have a role in the development of this region and for its subsequent patterning, as observed by the loss of Ser expression. Thus, the transient expression of \( rn \) in the leg might reveal that the intercalation of the presumptive tarsal region between the distal tip and medial leg regions occurs during early third instar.

It is increasingly common, even in invertebrates, to find genes that utilize two or more promoters (Gower et al., 2001; Krishnan et al., 1995; Li et al., 1999; Mevel-Ninio et al., 1995). Although this may lead to the generation of different proteins, it is often unclear whether the proteins have distinct activities. In fact, this issue is not easily resolved by traditional forward genetics and subsequent molecular analysis, since even if a locus can be genetically dissected into different subfunctions, this does not identify whether the different proteins have distinct activities. Perhaps the best way to test whether the variant proteins are...
interchangeable in vivo, is by cross-rescue in each others domain of expression. The rn gene is a clear example of a locus that utilizes both tissue-specific promoters and functionally distinct proteins to achieve functional diversity, a scenario likely to be observed more and more frequently in the post-genomic era.

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