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Antagonism of Notch signaling activity by members of a novel protein family encoded by the *Bearded* and *Enhancer of split* gene complexes

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SUMMARY

Cell-cell signaling through the Notch receptor is a principal mechanism underlying cell fate specification in a variety of developmental processes in metazoans, such as neurogenesis. In this report we describe our investigation of seven members of a novel gene family in *Drosophila* with important connections to Notch signaling. These genes all encode small proteins containing predicted basic amphipathic α-helical domains in their amino-terminal regions, as described originally for Bearded; accordingly, we refer to them as Bearded family genes. Five members of the Bearded family are located in a newly discovered gene complex, the *Bearded* Complex; two others reside in the previously identified *Enhancer of split* Complex. All members of this family contain, in their proximal upstream regions, at least one high-affinity binding site for the Notch-activated transcription factor Suppressor of Hairless, suggesting that all are directly regulated by the Notch pathway. Consistent with this, we show that Bearded family genes are expressed in a variety of territories in imaginal tissue that correspond to sites of active Notch signaling. We demonstrate that overexpression of any family member antagonizes the activity of the Notch pathway in multiple cell fate decisions during adult sensory organ development. These results suggest that Bearded family genes encode a novel class of effectors or modulators of Notch signaling.

Key words: Brd family, Notch signaling, Amphipathic helix, *Drosophila melanogaster*, Sensory organ development, Neurogenesis

INTRODUCTION

Cell-cell signaling via the Notch (N) receptor has emerged as a fundamental mechanism of developmental cell fate specification in metazoans (see for review Artavanis-Tsakonas et al., 1999; Greenwald, 1998; Kimble and Simpson, 1997). Substantial progress has been made in the past ten years or so in unraveling the structure and operation of this signaling system, and one of the most fruitful settings for these studies has been the adult peripheral nervous system (PNS) of the fruit fly *Drosophila melanogaster*.

The adult fly PNS includes more than 6000 external mechanosensory organs that are principally manifest as stereotyped arrays of bristles covering most of the body surface. Each bristle organ is composed of five distinct differentiated cells that are derived from a common sensory organ precursor, or SOP (Gho et al., 1999; Hartenstein and Posakony, 1989). SOPs, in turn, are selected from among small groups of cells known as proneural clusters (Cubas et al., 1991; Skeath and Carroll, 1991). These clusters are functionally defined as groups of cells that express proneural genes (*achaete* (*ac*), *scute* (*sc*), and *daughterless* (*da*)), which encode basic helix-loop-helix (bHLH) transcriptional activators that confer neural potential (Cabrera and Alonso, 1991; Van Doren et al., 1992). Inhibitory cell-cell interactions mediated by the N pathway are essential for the singularization of the SOP cell fate within each proneural cluster, and are further required to generate cell fate asymmetry in at least three of the subsequent divisions of the SOP lineage (reviewed in Posakony, 1994).

In our present understanding of the N pathway as it acts in most cell fate decisions in *Drosophila* neurogenesis, interaction between the N receptor and its ligand, Delta (Dl), results in activation of the transcription factor Suppressor of Hairless (Su(H); Fortini and Artavanis-Tsakonas, 1994; Furukawa et al., 1992; Jarriault et al., 1995; Schweisguth and Posakony, 1992; Tamura et al., 1995). Su(H) then directly activates transcription of multiple genes of the *Enhancer of split* Complex (E(spl)-C; Bailey and Posakony, 1995; Furukawa et al., 1995; Lecourtois and Schweisguth, 1995); this complex includes seven genes that encode bHLH transcriptional repressors (Delidakis and Artavanis-Tsakonas, 1992; Klämbt et al., 1989; Knust et al., 1992). This basic structure for the N pathway is known to be widely conserved among metazoan phyla (Artavanis-Tsakonas et al., 1999); nevertheless, much remains to be learned about this key signaling system. In particular, there has been intense recent interest in identifying other N-regulated targets of Su(H), in determining the identity and function of modulators

of N pathway activity, and in elucidating the nature of feedback mechanisms that may operate in N signaling.

We have earlier reported our genetic and molecular analyses of *Bearded* (*Brd*) (Leviten et al., 1997; Leviten and Posakony, 1996). Gain-of-function alleles of *Brd* cause bristle multiplication and bristle loss phenotypes indistinguishable from those conferred by loss-of-function mutations in genes of the N pathway (Leviten and Posakony, 1996). *Brd* encodes a novel small protein that is distantly related to the product of the *E(spl)m4* gene, a non-bHLH member of the E(spl)-C; both proteins include a predicted basic amphipathic α-helical domain (Klämbt et al., 1989; Leviten et al., 1997). The phenotype of *Brd* gain-of-function mutants, the observation that both *Brd* and *E(spl)m4* are expressed specifically in imaginal disc proneural clusters under direct proneural protein control (Bailey and Posakony, 1995; Singson et al., 1994), and the finding that *m4* is an integral member of the N pathway [being a direct target of transcriptional activation by Su(H) in response to N receptor activity (Bailey and Posakony, 1995)], all strongly indicated a role for these genes in N signaling. However, molecularly characterized deletions of the *Brd* locus do not cause a detectable mutant phenotype (Leviten and Posakony, 1996), and no specific lesions or mutant phenotypes have been described for *m4*, which suggested that these genes have functions that extensively overlap those of other, as yet unidentified, genes.

In this report, we identify five new *Drosophila* paralogs of these genes, all of which encode small proteins that, like Brd and E(spl)m4 (Leviten et al., 1997), contain predicted basic amphipathic α-helical domains. The new paralogs include three *Brd*-like genes that encode nearly identical transcripts (*Brother of Brd* (*Bob*) *A*, *B* and *C*), as well as two *E(spl)m4*-like genes (*Twin of m4* (*Tom*) and *E(spl)mα*). Surprisingly, we find that *Bob A*, *B* and *C*, *Tom*, and *Brd* are all located within a 30-kb interval at cytological location 71A1-2, and thus define a new gene complex that we have named the *Brd* Complex (Brd-C). Thus, the seven known members of the *Brd* family of genes are found within two widely separated gene clusters, the Brd-C and the E(spl)-C.

We find that the Brd family genes each include a high-affinity upstream binding site for the proneural bHLH activator proteins, and that transcripts from all but *E(spl)mα* are also likely to participate in a novel form of regulation involving the formation of RNA:RNA duplexes with proneural gene transcripts (Lai and Posakony, 1998). Moreover, all possess at least one high-affinity binding site for Su(H) in their proximal upstream regions, and thus may be subject to direct transcriptional regulation by this key component of the N pathway. Consistent with this, we show that Brd family genes are expressed in a variety of territories in imaginal tissue that correspond to sites of active N signaling. Finally, we demonstrate that over- or mis-expression of all Brd family genes (including both *Brd*-related and *m4*-related members) interferes specifically with multiple N-mediated cell fate decisions during adult PNS development. Taken together, our results indicate that the Brd family genes are likely to be integral members of the N pathway, and to play important roles as effectors or modulators of this pathway.

MATERIALS AND METHODS

Drosophila stocks

The following GAL4 driver lines were used for over-/misexpression studies by the GAL4/UAS method (Brand and Perrimon, 1993; Phelps and Brand, 1998): *sca-GAL4* (gift from Yuh Nung Jan; Hinz et al., 1994; Nakao and Campos-Ortega, 1996); 109-68 (gift from Yuh Nung Jan; Frise et al., 1996); *GMR-GAL4* (gift from Matt Freeman; Freeman, 1996); *ey-GAL4* (unpublished; gift from Tom Serano and Gerald Rubin); MS 1096 (gift from Ethan Bier; Capdevila and Guerrero, 1994; Milán et al., 1998); *hs-GAL4* (Bloomington Stock Center; Brand et al., 1994). The A101 and A1-2-29 *lacZ* enhancer trap lines are described by Bellen et al. (1989) and Bier et al. (1989), respectively. The *dpp-lacZ* reporter line (BS 3.0) is described by Blackman et al. (1991) and was a gift from Nora Ghbeish.

Cloning of *Bob* and *Tom*

BLAST searches (Altschul et al., 1997) of the GenBank database (Benson et al., 1999) identified EST CK02476 (AA141792), containing an ORF with similarity to *Brd*, and the overlapping ESTs EST36 (AA433222) and LD05688 (AA246754), containing an ORF with similarity to *E(spl)m4*. Primers were used to amplify most of the sequence of these ESTs by PCR from genomic DNA; the products were then used as probes to screen a cDNA library representing 4- to 8-hour embryonic poly(A)$^+$ RNA in pNB40 (gift from Nick Brown; Brown and Kafatos, 1988) and a genomic DNA library in bacteriophage EMBL3 (gift from Ron Blackman). Multiple cDNA and genomic DNA clones were obtained for both genes; representative sequences have been submitted to GenBank. We note that *Bob* and *E(spl)mα* are severely under-represented in the 4- to 8-hour pNB40 library: while both genes are highly expressed during this period of embryonic development (our unpublished observations; Wurmbach et al., 1999), *Bob* clones are present at <1/10,000, and we were unsuccessful in identifying any *E(spl)mα* clones. By contrast, *Tom* cDNAs represent >1/100 clones in this library.

Genomic DNA mapping

Bob and *Tom* probes were hybridized successively to a filter array of *Drosophila* P1 genomic DNA clones (Genome Systems). The positive clones were found to be part of an overlapping set in the 71A1-2 region of the left arm of chromosome 3 (BDGP), the known cytological location of *Brd* (Leviten et al., 1997; Leviten and Posakony, 1996). One P1 clone (DS 05763) was found to contain all three genes and was subsequently used for detailed mapping. Long PCR (20 kb^{PLUS} system, Boehringer Mannheim) was used to localize *Bob* near STS Dm2452 and *Tom* and *Brd* to the vicinity of STS Dm2122, and to determine the distance between these STSs. Positive PCR reactions were then confirmed in wild-type (w^{1118}) genomic DNA; all distances were found to be identical, except for a polymorphism in the *Tom-Brd* intergenic region. Sequence analysis identified a transposable element of the *suffix* class (see FlyBase, 1998) in one of two phage genomic DNA clones and in the P1 clone, but not in genomic DNA.

Plasmid construction

To create Brd family gene expression constructs, we used PCR to amplify the coding regions and 8-10 nt of 5′ UTR sequence (to provide translational initiation context) of *Brd*, *E(spl)m4*, *Bob*, *Tom* and *E(spl)mα*. PCR products containing upstream *Bam*HI and downstream *Sal*I sites were subcloned into pBluescript and fully sequenced. These fragments were then excised with *Bam*HI and *Xho*I and cloned into the *Bgl*II and *Xho*I sites of the pUAST vector (Brand and Perrimon, 1993). Sequences of oligonucleotide primers used for PCR amplification are available upon request.

Germline transformation

P element-mediated germline transformation was carried out as described by Rubin and Spradling (1982), using w^{1118} as the recipient strain.

DNA-binding assays

GST-Su(H) fusion protein was purified as described by Bailey and Posakony (1995). Rabbit reticulocyte lysate preparations of Daughterless (Da) and Achaete (Ac) proteins were a gift from Mark Van Doren (Van Doren et al., 1991). Electrophoretic mobility shift assays (EMSAs) were performed as described by Van Doren et al. (1991) and by Bailey and Posakony (1995). Sequences of the oligonucleotide probes tested are as follows.

Brd E1: GAGACCGAGAAA**CACCTG**CGCGCTAGGACT
CTCTGGCTCTTT**GTGGAC**GCGCGATCCTGA

Bob E1: ATTCAAATTAGG**CAGGTG**TAATATAACTCA
TAAGTTTAATCC**GTCCAC**ATTATATTGAGT

Tom E1: TGTTTGTGCAAC**CACCTG**CAGGCAGTCTGC
ACAAACACGTTG**GTGGAC**GTCCGTCAGACG

mα E1: ACCAAGGAA**CACCTG**CCCCGTATC
TGGTTCCTT**GTGGAC**GGGGCATAG

Brd S2: ATACT**CTCCCACG**ACGAA
TATGA**GAGGGTGC**TGCTT

Bob S2: CAATT**TTCTCACA**CTATG
GTTAA**AAGAGTGT**GATAC

Tom S3: AATCA**CGTGGGAA**ACATA
TTAGT**GCACCCTT**TGTAT

mα S1: ATTGT**TTCCCACA**CTCGT
TAACA**AAGGGTGT**GAGCA

mα S2: GGTGT**CGTGAGAA**ATTTT
CCACA**GCACTCTT**TAAAA

mα S3: GAATG**CGTGGGAA**TGGTC
CTTAC**GCACCCTT**ACCAG

RNA duplex assays

Wild-type (PB wt) and mutant (PB mut) proneural box-containing RNA probes derived from the *ato* 3′ UTR were constructed as follows. The following pairs of oligonucleotides were synthesized, annealed, filled in with Klenow fragment, and cloned into the *Eco*RV site of pBS+:

PB wt:
CCTAGCCTAAATGGAAGACAATGATTAAGACTAAGGAAGACAA**TG**TAAAAGCACC
ATCGGATTTACCTTCTGTTACTAATTCTGATTCCTTCTGTT**AC**ATTTTCGTGG**GG**

PB mut:
CCTAGCCTAAATTTCCTCAAATGATTAAGACTAATTCCTCAAA**TG**TAAAAGCACC
ATCGGATTTAAAGGAGTTTACTAATTCTGATTAAGGAGTTT**AC**ATTTTCGTGG**GG**

These oligonucleotides represent nt 1412-1464 in the *ato* 3′ UTR (GenBank accession L36646), except that the polyadenylation signal has been destroyed by a 2-nt mutation (**TG**, in bold) to facilitate their use in reporter constructs (E. C. L., unpublished results). PB mutant oligonucleotides contain non-complementary transversions of the central 7 bp of each proneural box (underlined). Labeled sense strand RNA probes were synthesized on PB plasmid templates by linearizing with *Eco*RI and transcribing with T3 polymerase in the presence of [^{32}P]dUTP. Unlabeled GY box-containing RNAs were made as follows. For *E(spl)m4*, the 3′ UTR was subcloned from a full-length cDNA clone in pNB40 (unpublished) as a *Stu*I/*Eco*RI fragment and cloned into the *Hin*cII and *Eco*RI sites of pBS. pBS subclones of the wild-type *Brd* 3′ UTR and a mutant version containing a 5-bp mutation in the GY box were described by Lai and Posakony (1997). Sense strand RNAs were synthesized by linearizing *Brd* 3′ UTR plasmids with *Bst*BI and the *E(spl)m4* 3′ UTR plasmid with *Eco*RI, and transcribing with T3 polymerase.

In vitro assays of RNA duplex formation were carried out largely according to the method of Ha et al. (1996). Reaction mixtures typically contained 5 μl (out of a standard 100 μl transcription reaction) of GY box RNA, 1 μl of labeled PB RNA (out of a standard 20 μl transcription reaction), and 1.5 μl of 5× annealing buffer (5×: 100 mM Hepes pH 8.0, 25 mM $MgCl_2$, 25% glycerol, 5 mg/ml yeast tRNA) containing 0.5 μl RNAsin/reaction. Mixtures were incubated at room temperature for 2 hours, standard loading dye was added, and the RNAs were separated on 1.7% agarose gels. Gels were dried and subjected to autoradiography to visualize labeled RNA.

Histochemistry

Staining to detect β-galactosidase activity was carried out as described by Romani et al. (1989).

Immunohistochemistry

Double-labeling with fluorescent secondary antibodies was performed as described by Kavaler et al. (1999) after using the following primary antibodies: Rabbit anti-β-galactosidase (Jackson Laboratories), diluted 1:200; mAb 22C10 (Developmental Studies Hybridoma Bank, University of Iowa), diluted 1:100; mAb 9F8A9 (mouse anti-Elav, Developmental Studies Hybridoma Bank), diluted 1:100; rabbit anti-D-Pax2 polyclonal antiserum (gift from Markus Noll), diluted 1:50; anti-Prospero monoclonal antibody (gift from Chris Doe), diluted 1:4.

In situ hybridization

Digoxigenin-labeled antisense RNA probes were generated by linearizing pNB40 cDNA clones for *Brd*, *Bob*, *Tom*, and *E(spl)m4* with *Hin*dIII and transcribing with T7 polymerase, and by linearizing an *Eco*RI/*Xho*I genomic DNA subclone of *E(spl)mα* with *Xho*I and transcribing with T7 polymerase.

In situ hybridization to imaginal tissue was performed as described by Sturtevant et al. (1993). For simultaneous visualization of *dpp-lacZ* expression and in situ hybridization patterns, several modifications were made to this protocol. First, the proteinase K treatment was reduced to 4 minutes duration. Second, anti-β-galactosidase monoclonal antibody (Promega, diluted 1:400) was added along with the anti-digoxigenin antibody. After primary antibodies were removed by washing, anti-β-galactosidase was detected by incubation with biotinylated goat anti-mouse antibody (Vector, diluted 1:200), a series of washes, and finally incubation with 10 μg/ml BODIPY-avidin (Molecular Probes, A2641). Following another series of washes, the alkaline phosphatase-conjugated anti-digoxigenin antibody was detected using Sigma *FAST* Fast Red TR/Naphthol AS-MX tablets, as directed. Tissue was then further dissected and mounted in Gel/mount (Biomeda). In situ hybridization and *dpp-lacZ* signals were captured separately on a Nikon Microphot-FXA microscope, and images were overlaid in Adobe Photoshop.

RESULTS

The *Brd* Complex and the *E(spl)* Complex each contain multiple Brd family genes

We have previously reported that *Brd* and *E(spl)m4* encode related small proteins containing putative basic amphipathic α-helices (Leviten et al., 1997). Recently, the sequences of *Drosophila* ESTs encoding apparent paralogs of both *Brd* and *E(spl)m4* have been deposited in the GenBank database (Benson et al., 1999; Harvey et al., 1998; Kopczynski et al., 1998; Schmid and Tautz, 1997). PCR products containing these EST sequences were used as probes to isolate full-length cDNA and genomic DNA clones for both genes, which we have named *Bob* (Brother of Brd) and *Tom* (Twin of m4), respectively. In addition, we have cloned and sequenced genomic DNA that includes the previously identified *E(spl)mα* locus (Schrons et al., 1992) and found that its predicted protein product is also strongly related to that of *E(spl)m4* (Klämbt et

A

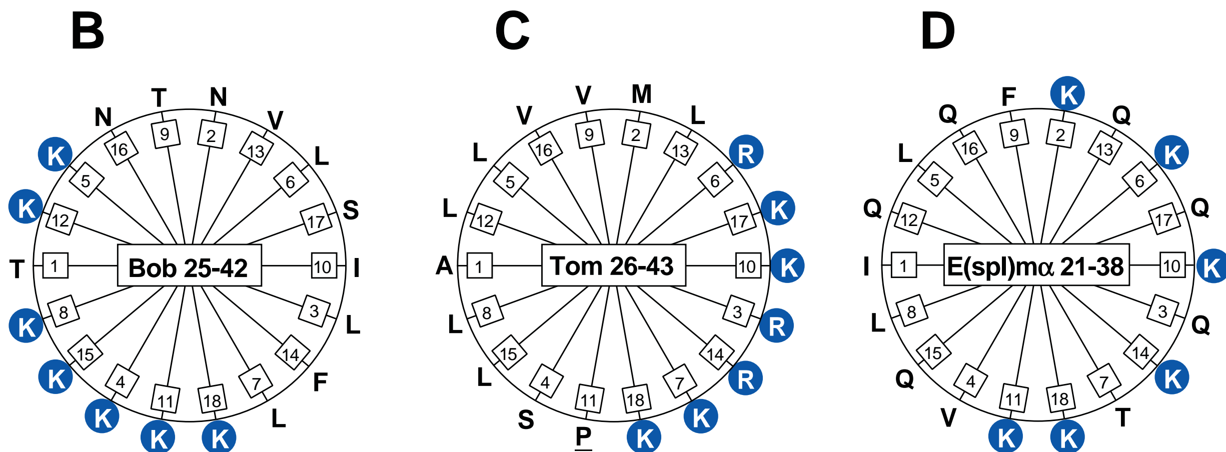

Fig. 1. Predicted primary and secondary structure of Brd family proteins. (A) Alignment of amino acid sequences. Identities are indicated by vertical lines, + signs represent conservative substitutions. Predicted basic amphipathic α-helical domains are shown in bold; putative phosphorylation sites for protein kinase C (PKC) and casein kinase II (CK II) are shown in blue and red, respectively. Note the identity of the four underlined residues (QXLKN) to the right of the amphipathic helix domains in Brd and E(spl)m4. The sequence labeled Bob is the predicted product of the *Bob A*, *B* and *C* genes of the Brd-C; that labeled Bob D is the predicted product of a putative fourth *Bob* gene identified by the EST clone CK02476 (BDGP; Kopczynski et al., 1998). The Bob D sequence differs at two positions (underlined) from the other Bobs; note that the second of these changes (K to Q) makes Bob D even more similar to the Brd protein. (B-D) Helical wheel plots illustrating predicted basic amphipathic α-helical domains in (B) Bob, (C) Tom, and (D) E(spl)mα. Note the presence of a proline (P) residue (underlined) at position 11 in the plot for Tom. Comparable plots for Brd and E(spl)m4 are in Leviten et al. (1997).

al., 1989). Similar findings concerning the *E(spl)mα* gene have been made independently by Wurmbach et al. (1999).

The predicted amino acid sequences of what we will refer to as Brd family proteins are aligned in Fig. 1A. We classify these proteins as Brd-like (Brd and Bob) or m4-like (m4, mα, and Tom), based on their relative sizes and degree of amino acid similarity. Although there are a few well-conserved regions in these proteins, particularly within the C-terminal half of the longer m4-like proteins, it is obvious that Brd family members are not in general highly related at the primary structure level. We have, however, noted previously that Brd and m4 are related by secondary structure, since a domain located near the N-terminus in both proteins is predicted to form a basic amphipathic helix (Leviten et al., 1997). We find that similar N-terminal domains in Bob (Fig. 1B) and E(spl)mα (Fig. 1D) are likewise strongly predicted to form basic amphipathic helices, while a proline residue in the center of the corresponding region of Tom (Fig. 1C) suggests that its 'helix' may be kinked or separated into two helices. The strong basic amphipathic character of these N-terminal domains of Brd family proteins may be considered a defining structural feature. Brd family proteins also share certain classes of consensus phosphorylation sites, namely protein kinase C (PKC) sites in their N-terminal regions and casein kinase II (CK II) sites in their C-terminal portions (Fig. 1A). The similar placement of these consensus sites in the context of otherwise weakly related amino acid sequences suggests that they may be relevant for the regulation of Brd family protein function.

We next localized the *Bob* and *Tom* genes to the *Drosophila* genome physical map using a filter grid library of P1 clones [Berkeley Drosophila Genome Project (BDGP) and Genome Systems]. Interestingly, *Bob* and *Tom* map to a set of P1 clones

covering cytological region 71A1-2, the known chromosomal location of *Brd* (Leviten et al., 1997; Leviten and Posakony, 1996). Our characterization of this genomic region, using a combination of P1 and lambda bacteriophage genomic DNA clones, is summarized in Fig. 2. The *Tom* gene was found to lie only about 2 kb upstream of the previously described *Brd* transcription unit (Leviten et al., 1997; Singson et al., 1994), with *Bob* about 20 kb upstream of *Tom* (Fig. 2A). Surprisingly, the genomic DNA corresponding to the *Bob* EST and cDNA clones is triplicated, such that three distinct, tandemly arranged genomic loci have the capacity to encode nearly identical *Bob* transcripts. We have observed this triplicated structure in two independent, overlapping lambda phage genomic DNA clones. Significantly, small sequence differences in the transcribed portions of the different *Bob* genomic loci are also represented in our cDNA clones, allowing us to conclude that at least two copies are transcriptionally active in wild-type flies (see legend to Fig. 2). We have arbitrarily designated the three gene copies *Bob A*, *B*, and *C* (Fig. 2A), but will subsequently refer to the encoded transcripts and proteins collectively as '*Bob*', as we currently do not have the means of distinguishing them in vivo. Analysis of the *Bob A-Tom* intergenic region by northern blots failed to reveal additional small transcription units that might represent candidates for other Brd family genes; we have not, however, exhaustively surveyed the regions flanking *Bob* or *Brd*. Thus, a minimum of five Brd family genes are contained within an approximately 30-kb interval that we refer to as the *Brd* Complex (Brd-C), and this complex contains both *Brd*-like (*Brd* and *Bob*) and *m4*-like (*Tom*) genes. The E(spl)-C contains at least two *m4*-like genes, *m4* and *mα*.

Common transcriptional and post-transcriptional regulatory elements in genes of the Brd-C and E(spl)-C

The *Brd* gene is a known target of direct transcriptional activation by the proneural proteins, via a single high-affinity binding site in its proximal upstream region (Singson et al., 1994). Extensive studies of the transcriptional regulation of E(spl)-C genes have revealed that the proximal upstream regions of *m4*, *mα*, and six of the seven bHLH genes each contain high-affinity binding sites not only for proneural proteins, but also for Su(H) (Bailey and Posakony, 1995; Eastman et al., 1997; Kramatschek and Campos-Ortega, 1994; Lecourtois and Schweisguth, 1995; Nellesen et al., 1999; Singson et al., 1994; Wurmbach et al., 1999). For several of these genes, including *Brd* and both bHLH genes and *m4* in the E(spl)-C, promoter-reporter transgenes have been used to demonstrate that these binding sites are indeed essential in vivo for proper transcriptional activation (Bailey and Posakony, 1995; Kramatschek and Campos-Ortega, 1994; Lecourtois and

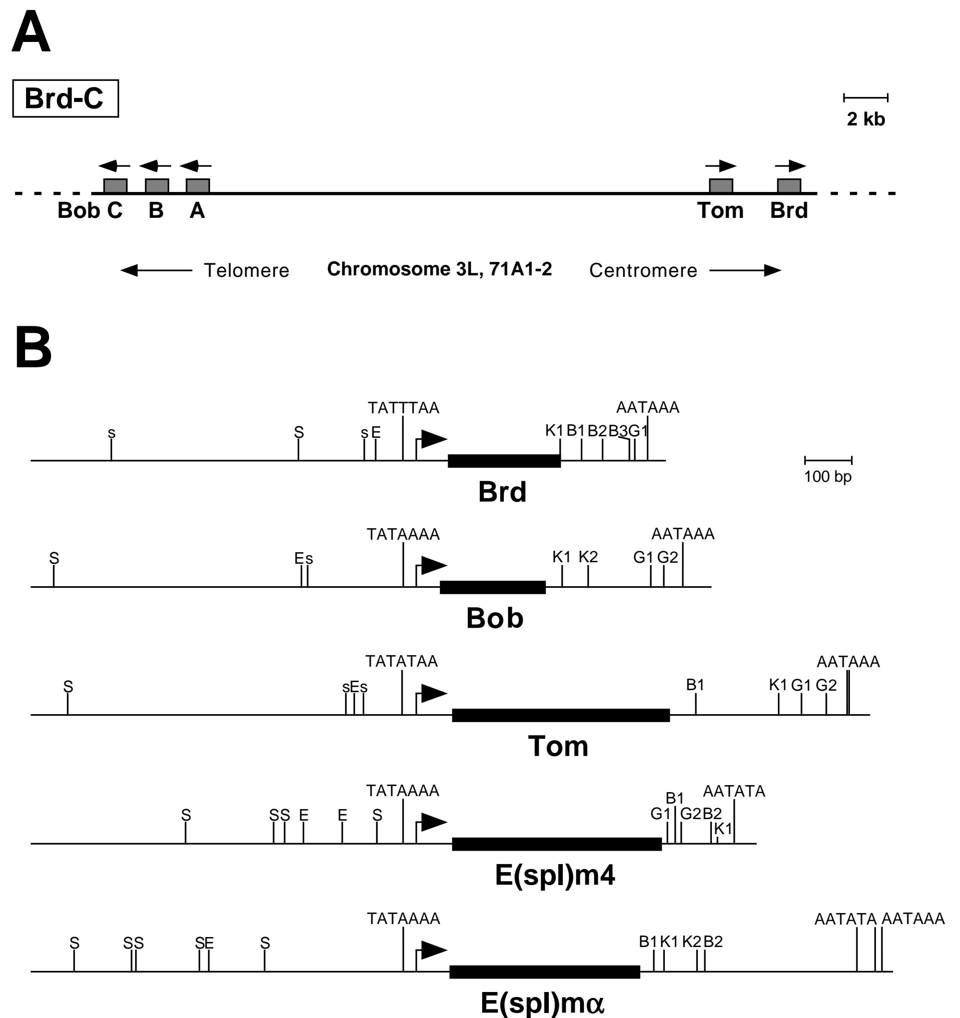

Fig. 2. (A) Physical organization of a new gene complex in *Drosophila*, the Brd-C. Shaded boxes (not to scale) indicate Brd family genes; arrows show direction of transcription. Dashed lines indicate that the left and right limits of the complex have not been determined. Orientation of the Brd-C on the left arm of chromosome 3 is shown. (B) Comparison of the physical structure of Brd family genes. Genes are aligned by their transcription start sites (arrows). Shown to scale are the locations and extents of coding (black boxes) and regulatory (vertical lines) sequences. The sequences of cDNA and genomic DNA clones of both *Bob* and *Tom* are colinear, establishing that these transcription units, like *Brd* (Leviten et al., 1997), are intronless. TATA boxes and polyadenylation signals are indicated. Upstream transcriptional and 3′ UTR post-transcriptional regulatory motifs common to these genes are marked as follows: S, high-affinity Su(H) binding site (YGTGRGAR); s, possible lower-affinity Su(H) site (RTGDGAR); E, E box proneural bHLH activator binding site (RCAGSTG); B, Brd box (AGCTTTA); K, K box (TGTGAT); G, GY box (GTCTTCC). Note the strong tendency of lower-affinity Su(H) sites to be in close proximity to proneural binding sites. The diagram of *Bob* refers to *Bob B* and *Bob C* (see A), *Bob A* does not include the upstream motifs shown. Comparison of our cDNA and genomic DNA sequences for *Bob* indicates that *Bob C* and probably *Bob A* (at least) are transcriptionally active in vivo.

Schweisguth, 1995; Singson et al., 1994). Thus, the combination of high-affinity binding sites for both types of activator appears to be a hallmark of many N pathway-regulated genes, particularly those involved in neurogenesis (Nellesen et al., 1999). In the present study, we find that high-affinity binding sites for both classes of activator are also present in the promoters of all Brd family genes, suggesting that they too represent transcriptional targets of the N pathway.

Heterodimeric proneural protein complexes such as Ac/Da and Sc/Da bind with high affinity in vitro to E boxes of the class RCAGSTG (Cabrera and Alonso, 1991; Murre et al., 1989; Van Doren et al., 1991); however, proximal E box sites in proneural target genes (proximal proneural response elements, or PPREs) typically fit the more restricted consensus GCAGGTGK (Singson et al., 1994). We find that the proximal upstream regions of *Bob*, *Tom*, and *m*α all contain sequences conforming to this latter consensus (Fig. 2B). We performed electrophoretic mobility shift assays (EMSAs) with oligonucleotides containing these sequences and found that they behave as high-affinity binding sites for Ac/Da heterodimers in direct binding assays, and that they compete efficiently for binding with the previously characterized *Brd* E1 site (Singson et al., 1994) (Fig. 3, lanes 1-7). The similar locations and in vitro binding properties of these E box sequence elements suggest that all Brd family genes, like *Brd* and *m4*, are direct targets of transcriptional activation by the proneural proteins.

Su(H) binds with high affinity to sequences of the class YGTGRGAA (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995; Tun et al., 1994). Binding sites of this type have been shown to be essential for transcriptional activation of *E(spl)m4* in response to N pathway activity (Bailey and Posakony, 1995), and five such sites are present in the proximal upstream region of *E(spl)m*α (Nellesen et al., 1999; Wurmbach et al., 1999). We find that the upstream regions of *Bob* and *Tom* each contain a single site fitting this consensus, while the upstream region of *Brd* contains the variant CGTGGGA**G**. Analysis of these sites using purified GST-Su(H) in direct binding EMSAs demonstrates that *Brd*, *Bob*, *Tom*, and *m*α each contain at least one high-affinity Su(H) binding site (Fig. 3, lanes 8-13). Thus, Brd family genes and E(spl)-C bHLH repressor genes share the characteristics of having PPRE-class E boxes and high-affinity Su(H) sites located in their proximal upstream regions (Fig. 2B; see Nellesen et al., 1999). However, the presence of multiple high-affinity Su(H) sites upstream of most E(spl)-C genes, including the bHLHs, *m4* and *m*α, suggests that E(spl)-C genes may be more sensitive to N pathway activity than the genes of the Brd-C.

Brd and most genes of the E(spl)-C (including both bHLH genes and *m4*) are also subject to common modes of negative post-transcriptional regulation via defined sequence motifs present in their 3′ UTRs (Fig. 2B). In particular, we have previously demonstrated that K boxes (TGTGAT) and Brd boxes (AGCTTTA), which are broadly distributed within the 3′ UTRs of these genes, mediate negative regulation of transcript accumulation and translational efficiency (Lai et al., 1998; Lai and Posakony, 1997; Leviten et al., 1997). We have identified two Brd boxes and two K boxes in the 3′ UTR of *m*α (see also Wurmbach et al., 1999), a K box and a Brd box in the 3′ UTR of *Tom*, and two K boxes in the 3′ UTR of *Bob* (Fig. 2B). Moreover, the second K box in *Bob* is directly adjacent to a CAAC motif, a sequence that has been implicated in augmentation of regulation by an associated K box (Lai et al., 1998). *Bob*'s 3′ UTR does not contain a canonical Brd box, but does contain a 7/7 match to a variant of the Brd box (TGCTTTA) found in the *D. hydei* ortholog of *E(spl)m4* (Lai and Posakony, 1997). Overall, the presence of canonical K box and Brd box sequences in the 3′ UTRs of *Bob*, *Tom* and *m*α strongly suggests that most genes of the Brd-C and E(spl)-C are subject to the same two modes of negative post-transcriptional regulation.

RNA:RNA duplexes form between the 3′ UTRs of Brd family transcripts and proneural gene transcripts

A third class of conserved 3′ UTR sequence motif, the GY box (GTCTTCC), is also shared by *Brd* and genes of the E(spl)-C (Lai and Posakony, 1997, 1998; Leviten et al., 1997). Although the precise function of this motif is poorly understood, we have

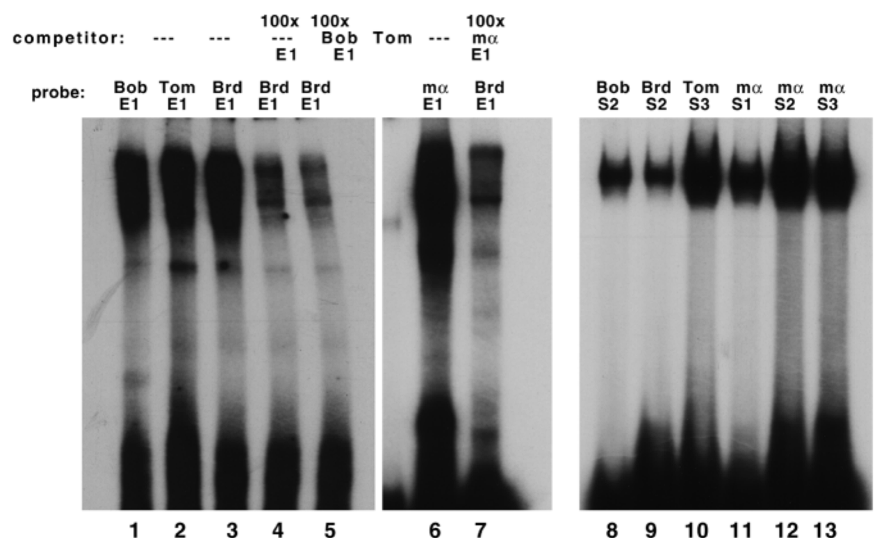

Fig. 3. Binding sites for proneural proteins and for Su(H) upstream of Brd family genes. Shown are electrophoretic mobility shift assays (EMSAs) to detect binding of in vitro translated Ac/Da hetero-oligomers (lanes 1-7) or purified GST-Su(H) (lanes 8-13) to labeled oligonucleotide probes representing candidate E box [E (GCAGSTGK); lanes 1-7] or high-affinity Su(H) [S (YGTGRGAR); lanes 8-13] binding sites (see Fig. 2B; sites are numbered in order upstream of the transcription start). Proneural protein complexes bind efficiently to newly identified E box sites (E1s) upstream of *Bob*, *Tom*, and *E(spl)m*α (lanes 1, 2, 6; see Fig. 2B), compared to the previously characterized *Brd* E1 site (lane 3; see Singson et al., 1994); these sites are also efficient competitors of binding to *Brd* E1 (lanes 4, 5, 7). All three Brd-C genes have at least one high-affinity Su(H) binding site upstream (lanes 8-10; see Fig. 2B). *m*α sites S1, S2 and S3 (lanes 11-13; see Fig. 2B) are representative of the high-affinity Su(H) sites upstream of this gene; sites S4 and S5 have the same core sequence (TGTGGGAA) as S1.

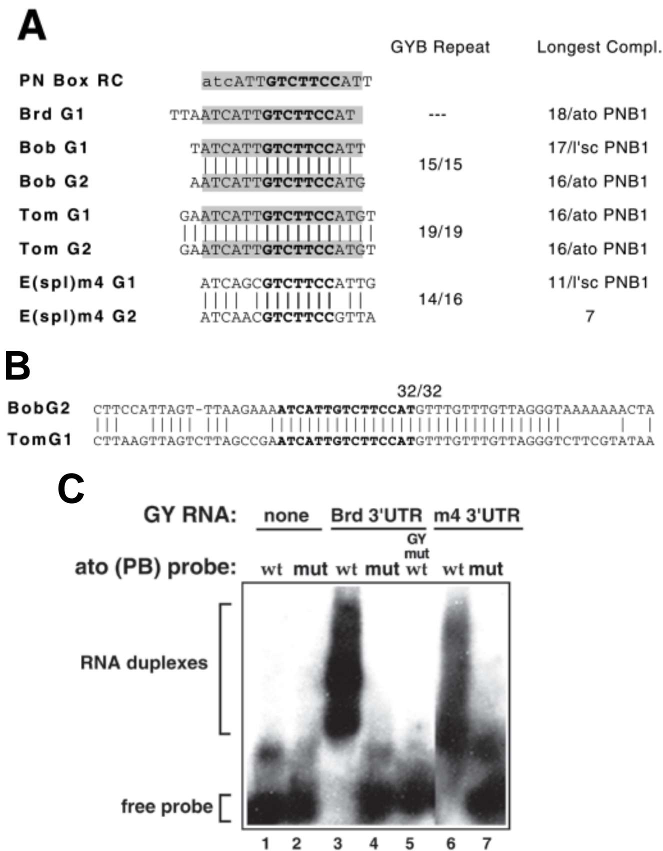

Fig. 4. Extended sequence complementarity between GY box motifs in the 3′ UTRs of Brd-C genes and proneural boxes in the 3′ UTRs of proneural genes (see Lai and Posakony, 1998).
(A) Aligned on the left are the GY box (G) motifs found in four Brd family genes; core GY box heptamer (GTCTTCC) is in bold. GY box elements are part of lengthy direct repeats in *Bob*, *Tom* and *E(spl)m4,* as indicated in the middle column. Remarkably, all GY box motifs in all Brd-C genes share a common 15-nt sequence (shaded) with perfect complementarity to the region of the first proneural box (PN Box) sequence in *l'sc* and *ato*; the reverse complement (RC) of this latter sequence is shown for comparison. The column on the right lists the longest uninterrupted complementarity between each GY box motif and any proneural box; PNB1 is the first proneural box sequence in the indicated proneural gene. Note that Brd-C genes have a minimum of 16 nt of perfect complementarity to either *ato* or *l'sc*. (B) Alignment of portions of the 3′ UTRs of *Bob* and *Tom*, showing a 32/32 identity that includes the common 15-nt GY box-containing sequence identified in A (bold). (C) In vitro assays of RNA:RNA duplex formation between GY box (GY) sequences of Brd family genes and proneural box (PB) sequences of proneural genes. Labeled *ato* 3′ UTR wild-type (wt; lanes 1, 3, 5, 6) or mutant (mut; lanes 2, 4, 7) probes were incubated either alone (none; lanes 1, 2) or with unlabeled *Brd* (wild type, lanes 3, 4 or GY box mutant, lane 5) or *E(spl)m4* (wild-type; lanes 6, 7) 3′ UTRs, and complexes were resolved by non-denaturing agarose gel electrophoresis. RNA:RNA duplex-containing structures are recognizable by their reduced mobility compared to free probe.

speculated that it has a likely role in forming RNA:RNA duplexes with a complementary sequence motif (the proneural box, AATGGAAGACAAT) found in the 3′ UTRs of proneural genes, including *ac*, *lethal of scute* (*l'sc*), and *atonal* (*ato*) (Lai and Posakony, 1998). We find that the 3′ UTRs of both *Bob* and *Tom* each contain a pair of GY boxes (Figs 2B, 4A).

Closer examination of the GY boxes of *Bob*, *Tom* and *Brd* revealed an unexpected degree of sequence identity in the nucleotides flanking the GY box heptamer in Brd-C genes (Fig. 4A). The GY boxes of *Tom* are found within a 19/19 direct repeat in the *Tom* 3′ UTR, while *Bob*'s GY boxes fall within a 15/15 direct repeat in its 3′ UTR. Moreover, an exact 16-bp sequence including a GY box is common to the 3′ UTRs of *Brd*, *Bob*, and *Tom*, and all five GY boxes in these Brd-C genes are contained within an exact 15/15 identity (shaded in Fig. 4A). It is striking that this latter sequence is exactly complementary to a 15-nt sequence shared by proneural boxes located in the *ato* and *l'sc* 3′ UTRs (Fig. 4A). That the GY boxes of all Brd-C genes should share such an exceptional relationship with the proneural boxes of divergent proneural genes located on different chromosomes (*ato* and *l'sc*) strongly suggests that these complementary sequence elements are subject to common constraint. We also note that the two GY boxes in the 3′ UTR of *E(spl)m4* are more related to the extended GY box consensus just described than are the GY boxes of most E(spl)-C bHLH transcripts. Thus, the constraint on *m4*'s GY boxes similarly appears to extend well beyond the core seven nucleotides of this motif, in a way that is also evidently connected to the proneural box sequence. Finally, we have found that the 3′ UTR segments containing the second GY box of *Bob* and the first GY box of *Tom* are related by an extraordinary 32-nt exact identity (Fig. 4B). That members of distinct subfamilies of the Brd gene family should share such an extended GY box-containing identity further underscores the sequence constraint associated with this motif, and may suggest the existence of a common 'partner' gene for *Bob* and *Tom* that carries a complementary sequence.

We directly tested the capacity of a synthetic RNA representing a 50-nt region of the *ato* 3′ UTR (including both proneural boxes) to interact with RNAs representing either the full-length *Brd* or full-length *m4* 3′ UTR using a gel shift assay. [^{32}P]dUTP-labeled *ato* probes were incubated with unlabeled *Brd* or *m4* 3′ UTRs at room temperature, and complexes were resolved by non-denaturing agarose gel electrophoresis (Fig. 4C). We found that RNA:RNA duplex-containing structures, recognizable by their reduced mobility, formed spontaneously and efficiently between wild-type RNA partners (Fig. 4C, lanes 1, 3, 6). Comparable RNA pairs in which one partner contains clusters of point mutations in either the proneural boxes (lanes 2, 4, 7) or the GY box (lane 5) were found to be incapable of forming such structures. We conclude that the complementary 3′ UTR sequence motifs found in proneural genes and Brd family genes mediate the formation of RNA:RNA duplexes in vitro. Since transcripts of members of the proneural gene family and the Brd gene family co-accumulate in all developmental settings where neurogenesis occurs, we suggest that these RNA:RNA duplexes also form in vivo, although the possible regulatory consequences of this association remain to be determined.

Brd family genes are expressed at multiple sites of active N signaling

We next examined the postembryonic expression patterns of Brd family genes by in situ hybridization (Fig. 5). In wing imaginal discs of third-instar larvae, *Brd* and *E(spl)m4*

transcripts have previously been observed to accumulate specifically in the full complement of sensory organ proneural clusters (Fig. 5B,C; Bailey and Posakony, 1995; Leviten et al., 1997; Nellesen et al., 1999; Singson et al., 1994). Similarly, the complex pattern of *E(spl)m*α expression in the wing disc (Fig. 5D; Wurmbach et al., 1999) includes proneural clusters, although *m*α transcript accumulation in the clusters consistently appears broader and more diffuse than that of *Brd* or *m4* (Fig. 5B-D). In addition, *m*α transcripts appear in a narrow stripe along the dorsoventral boundary of the wing pouch, as well as along wing vein borders (Fig. 5D). In contrast, we find that neither *Bob* nor *Tom* exhibit any patterned expression in the wing disc, although *Tom* may be generally expressed at a very low level in this tissue (Fig. 5A,E). To demonstrate that the failure to observe specific wing disc expression of the endogenous *Bob* and *Tom* genes is not due to a detection problem, we performed control experiments in which transcripts from *UAS-Bob* or *UAS-Tom* transgenes, activated by a *scabrous* (*sca*)-*GAL4* driver, were assayed using the same probes. As shown in Fig. 5, the characteristic proneural cluster pattern of *sca-GAL4* activity in the wing disc is readily revealed by both the *Bob* (Fig. 5O) and *Tom* (Fig. 5T) probes in these experiments.

In the eye imaginal disc, four of the five Brd family genes studied here are expressed in the vicinity of the morphogenetic furrow (see Singson et al., 1994), the exception being *Bob*, which is not detectably expressed in either the eye or antenna discs (Fig. 5F-J). As it became clear from these in situ hybridization analyses that the qualitative eye disc expression patterns of the four genes are distinct, we carried out double-labeling experiments to examine the precise register of *Brd*, *m4*, *m*α, and *Tom* expression with respect to the furrow marker *decapentaplegic (dpp)-lacZ* (Fig. 5K-N). We find that transcripts from the different Brd family genes accumulate with distinct spatial profiles relative to the morphogenetic furrow. *Brd* is expressed in two closely spaced

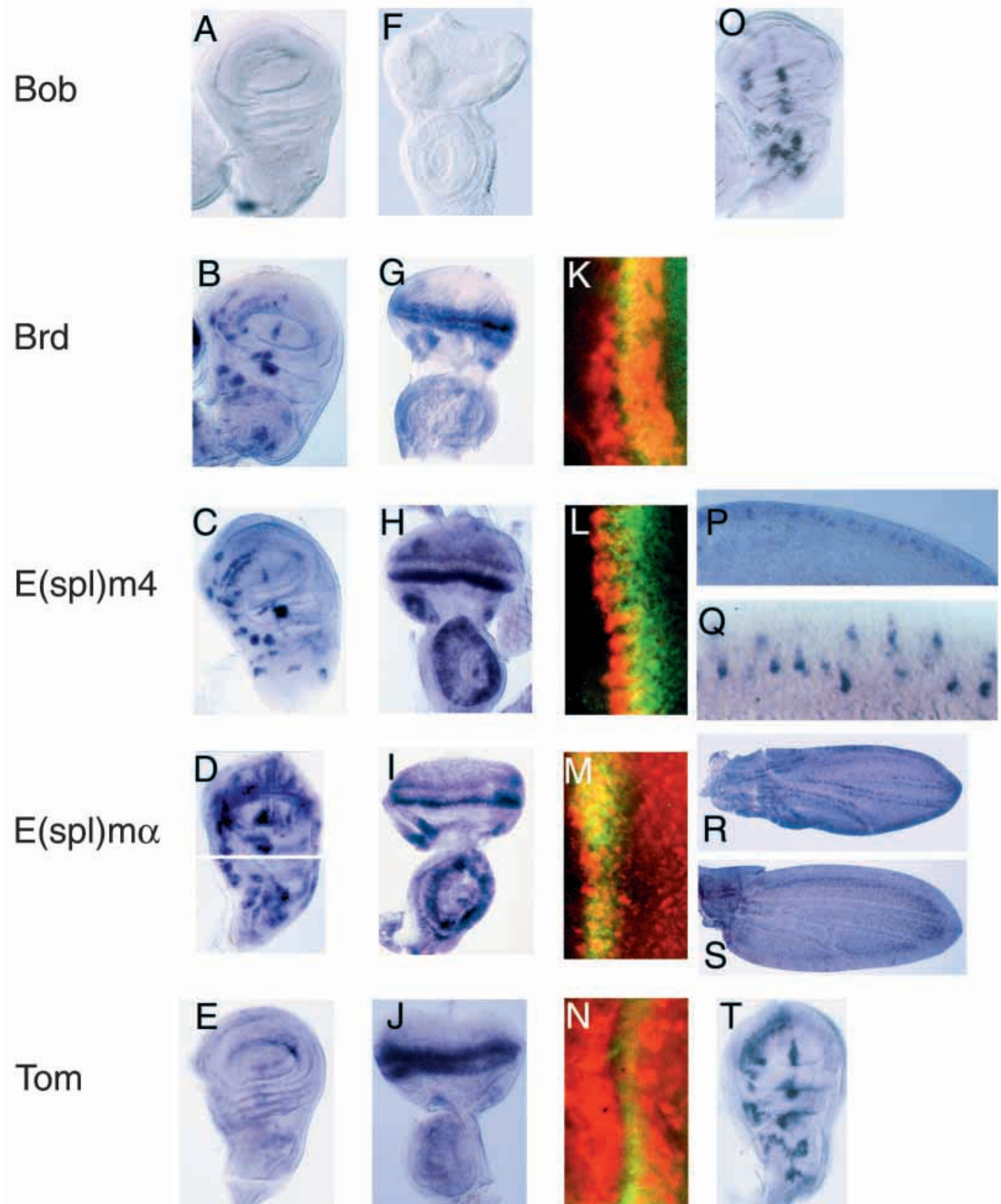

Fig. 5. Patterns of transcript accumulation from Brd family genes in developing imaginal tissue. Whole-mount in situ hybridization, using a digoxigenin-coupled antisense RNA probe for the indicated gene, was applied to wing (A-E,O,T) and eye-antenna (F-N) imaginal discs from late third-instar larvae and to pupal wings (P-S). Genotypes: (A-J,P-S) wild type (w^{1118}); (K-N) *dpp-lacZ*; (O) *sca-GAL4::UAS-Bob*; (T) *sca-GAL4::UAS-Tom*. K-N show detail of transcript patterns in the vicinity of the morphogenetic furrow of the developing retina (anterior is to the left), in eye discs subjected to both in situ hybridization (red; to detect transcripts) and labeling with anti-β-galactosidase antibody (green; to detect expression of the *dpp-lacZ* furrow marker). (O,T) Transcripts from both *Bob* and *Tom* are readily detected in wing discs when these genes are expressed under the control of the *sca-GAL4* driver, indicating that our inability to detect patterned expression of the two genes in wild-type wing discs (A,E) is not due to technical failure. (P) Pupal wing aged 16 hours APF at 18°C (approximately 8 hours APF at 25°C). (Q) Higher magnification view of anterior margin of wing in P. (R) Pupal wing at 24 hours APF (25°C). (S) Pupal wing at 30 hours APF (25°C).

stripes, one just anterior to, and one within and posterior to, the *dpp* furrow stripe (Fig. 5K). Transcripts from *m4*, by contrast, appear in a strong band that is largely just anterior to the zone of *dpp-lacZ* expression (Fig. 5L). *mα* shows expression in a pattern that overlaps, and extends posterior to, the marker stripe (Fig. 5M). Finally, *Tom* expression somewhat resembles that of *Brd*, in that its transcripts accumulate in two stripes lying anterior and posterior to the *dpp-lacZ* stripe (Fig. 5N).

We also examined the pattern of transcript accumulation from these genes during pupal wing development, to assess their possible expression in sensory organ lineages and in the vicinity of the developing wing veins. The members of the Brd-C are not expressed at detectable levels in the pupal wing at 8 hours after puparium formation (APF), although *Brd* and *Tom* transcripts are present in the large clusters of proximal campaniform sensilla at this time (data not shown). By contrast, *m4* and *mα* in the E(spl)-C display both proximal campaniform expression (not shown) and specific wing margin expression at 8 hours APF (Fig. 5P,Q and data not shown). We find that *m4* transcripts accumulate in a set of anterior wing margin cells at this stage (Fig. 5Q). Based on their spacing, they are likely to represent cells in the lineage of the chemosensory organs that appear in dorsal and ventral rows on the margin. Since transcripts from *E(spl)mγ* have recently been shown to accumulate in these organs (Nellesen et al., 1999), it appears that at least one Brd family member and at least one bHLH gene in the E(spl)-C share this aspect of their expression. We find that *mα* is expressed at this time (8 hours APF) in a broad domain of wing margin cells that includes cells of the posterior as well as the anterior margin, and also in an incomplete wing vein boundary pattern (data not shown). This latter observation prompted us to examine the accumulation of *mα* transcripts in later pupal wing discs (Fig. 5R,S). At 24 hours APF, *mα* is indeed expressed in a largely complete pattern consisting of thin rows of cells at all vein/intervein boundaries (Fig. 5R). This is highly reminiscent of the pattern of transcript accumulation from both *N* and the bHLH gene *E(spl)mβ* at approximately the same time (24-28 hours APF; de Celis et al., 1997). In addition, *mα* transcripts remain present throughout the wing margin (both posterior and anterior) at this stage. *mα* expression in the pupal wing is highly dynamic, however: By 30 hours APF, its transcripts have nearly gone from the margin, are excluded from vein/intervein borders, and appear instead in the veins themselves and in non-vein wing blade tissue (Fig. 5S). Taken together, these observations strongly suggest that at least one Brd family member may have a role in wing vein development.

In summary, we find that in developing imaginal tissue, Brd family members are expressed specifically in multiple territories in which N signaling-dependent cell fate decisions take place.

Overexpression of any Brd family gene interferes with PNS and eye development

Our previous studies of gain-of-function alleles of *Brd* demonstrated that overexpression of this gene causes adult phenotypes closely resembling those conferred by loss-of-function mutations in N pathway genes (Leviten et al., 1997; Leviten and Posakony, 1996). These phenotypes include both bristle multiplication and bristle loss; the former is due to the specification of supernumerary SOPs, while the latter is caused

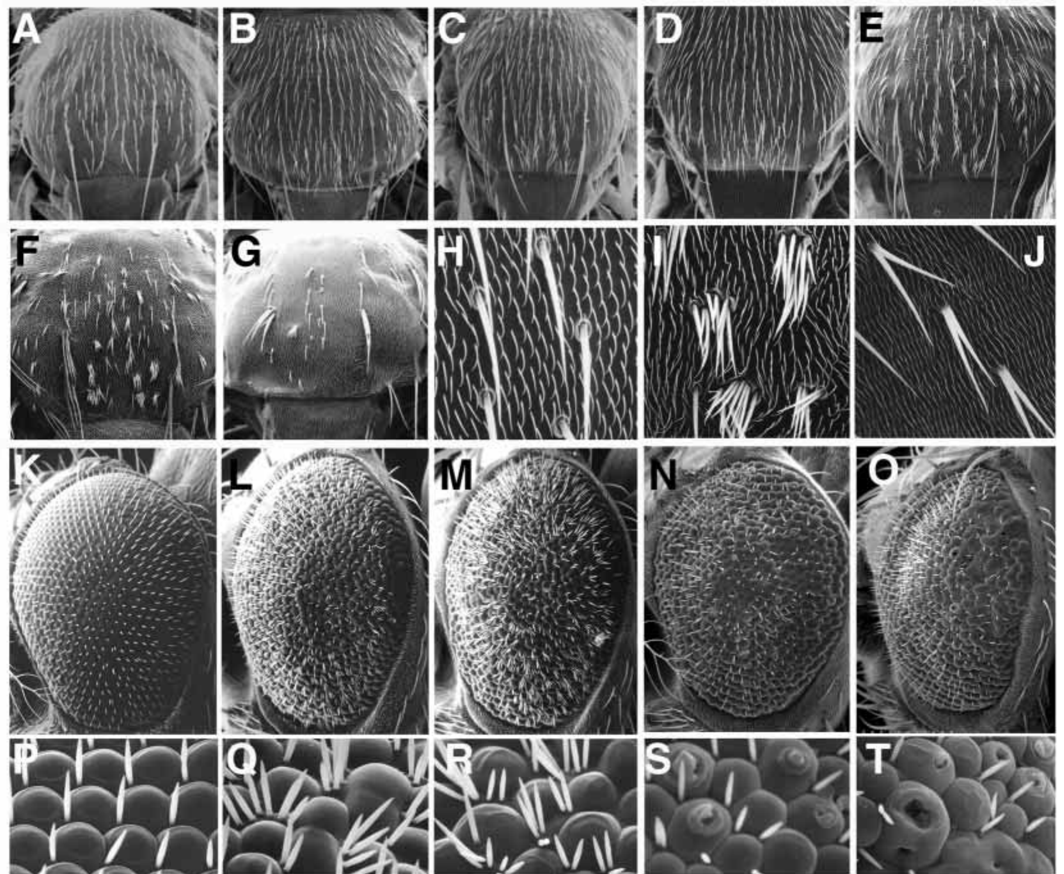

Fig. 6. Phenotypic effects of over- or misexpression of Brd family genes on the development of the adult PNS. (A-T) Scanning electron micrographs of the dorsal thorax (A-I), dorsal abdomen (J), and compound eyes (K-T) of adult female flies. Genotypes are as follows: (A,H) wild type; (B) *sca-GAL4::UAS-Brd*; (C) *sca-GAL4::UAS-mα*; (D) *sca-GAL4::UAS-m4*; (E) *sca-GAL4::2xUAS-m4*; (F,I) *sca-GAL4::2xUAS-Bob*; (G) *sca-GAL4::2xUAS-Tom*; (J) *109-68::2xUAS-Bob*; (K,P) *GMR/+*; (L,Q) *GMR::2xUAS-Brd*; (M,R) *GMR::2xUAS-Tom*; (N,S) *ey-GAL4::2xUAS-m4*; (O,T) *ey-GAL4::2xUAS-Brd*.

by inappropriate allocation of cell fates within the bristle lineage (Leviten and Posakony, 1996). We were interested to determine if overexpression of other Brd family genes could similarly interfere with cell fate specification events controlled by N pathway activity. To do so, we inserted the protein coding regions of all five Brd family genes into the pUAST vector, and examined the ability of these transgene constructs to interfere with adult development using the GAL4-UAS system.

The *sca-GAL4* driver described above, which expresses GAL4 in proneural clusters as well as in the bristle lineage, was used to assess the effect of Brd family overexpression on adult peripheral neurogenesis. We found that all five Brd family genes tested in this way are capable of inducing defects in adult PNS development, although the different UAS transgenes clearly differ in their phenotypic strength (Fig. 6A-I). *Brd* itself has relatively mild effects in this assay, though as noted above characterized hypermorphic alleles of *Brd* have potent effects on PNS development (Leviten and Posakony, 1996). Seven out of 10 lines carrying one copy of *sca-GAL4* and one copy of *UAS-Brd* exhibited completely penetrant PNS defects, including tufting (multiplication) of many head macrochaetes and some notum macrochaetes, frequent doubling or mild tufting of up to a third of the notum microchaetes, and mild increases in microchaete density (Fig. 6B). All 10 *UAS-m4* transgenic lines generated phenotypes similar to those conferred by *UAS-Brd*, although the overall severity of the effects (i.e., degree of bristle multiplication) was slightly greater (Fig. 6D); a further increase in the degree (number of bristles per position) and extent (number of affected positions) of bristle tufting was observed with two copies of *UAS-m4* (Fig. 6E). Ten of ten *UAS-mα* lines also caused defects in PNS development, the severity of which was typically intermediate between those caused by *Brd* and by *m4* (Fig. 6C). These results indicate that the E(spl)-C contains at least two genes that are not only structurally related to *Brd*, but also share with *Brd* the property that their overexpression interferes with lateral inhibition in proneural clusters.

By comparison with the results with *Brd*, *m4*, and *mα*, we found that overexpression of *Bob* and *Tom* each cause much stronger mutant phenotypes (Fig. 6E-I). With one copy of *UAS-Bob*, all 10 lines yield a strong tufting or lethal phenotype, with bristle tufting extending to most macrochaetes and microchaetes, as well as occasional bristle loss (data not shown). *UAS-Tom* causes the most severe effects of all Brd family members when expressed under the control of *sca-GAL4*, with most lines giving high percentages of lethality at late pupal/pharate adult stages. The relatively infrequent escapers typically exhibit strong tufting of nearly all macrochaetes and microchaetes and frequently display some degree of bristle loss, especially on the legs (data not shown). Bristle loss phenotypes are significantly more severe with two copies of *Bob* (Fig. 6F) or two copies of *Tom*; rare pharate adults of the latter genotype often exhibit nearly complete loss of notum microchaetes (Fig. 6G). The phenotypic progression from bristle tufting to bristle loss as the level of *Bob* or *Tom* activity is increased is strongly reminiscent of that observed previously with gain-of-function alleles of *Brd* (Leviten and Posakony, 1996). Finally, we note that flies homozygous for both *sca-GAL4* and *UAS-Brd* or *UAS-m4* display phenotypes that are typical for flies heterozygous for *sca-GAL4* and *UAS-Bob* or *UAS-Tom* (data not shown). We suggest that collectively these results indicate that all five Brd family genes tested here have qualitatively similar, but quantitatively graded, effects on adult peripheral neurogenesis. The strength of their phenotypic activities can be rank ordered as follows: *Brd<mα<m4<Bob<Tom*.

We have also characterized the effects of overexpression of these genes in other postembryonic settings where N signaling is required for growth and patterning. We find that high-level expression of Brd family genes (typically using two or more copies of the *UAS* effector construct) anterior to the morphogenetic furrow of the eye disc [using *eyeless (ey)-GAL4* as the driver] causes strong defects in ommatidial assembly and organization, as manifested by substantial ommatidial fusion and lens pitting in adult eyes (Fig. 6N-O,S-T). These external phenotypes are consistent with interference with N-dependent events in retinal development (Cagan and Ready, 1989). In contrast to our observations on bristle development, we find that *UAS-Brd* (Fig. 6O,T) and *UAS-m4* (Fig. 6N,S) yield the strongest phenotypes in combination with *ey-GAL4*. Expression of Brd family genes posterior to the morphogenetic furrow, using *GMR-GAL4* as the driver, results in strongly roughened adult eyes (Fig. 6K-M) with defects that include tufting of the interommatidial bristles (Fig. 6P-R) and excess photoreceptors (not shown). Thus, overexpression of Brd family genes interferes with the proper specification of multiple cell types in distinct imaginal tissues.

In contrast, we find that overexpression of the five Brd family genes we have tested (under the control of either the MS 1096 (Capdevila and Guerrero, 1994; Milán et al., 1998) or 69B (Brand and Perrimon, 1993) GAL4 drivers) does not appear to alter wing vein fates or the overall integrity of the wing margin, although sensory organ fates in each of these territories are easily altered. Thus, for example, we observe increased density and multiplication of campaniform sensilla along wing vein L3, as well as wing margins with extremely disorganized sensory organ arrays that feature both double-shaft bristles and loss of bristle structures (data not shown). Despite this, the non-sensory vein and margin tissue itself appears normally patterned.

Overall, our results indicate that different N pathway-controlled cell fate decisions are differentially sensitive to Brd family overexpression, and that neurogenesis (such as in the sensory organs and the eye) is apparently the process most sensitive to the levels of activity of these genes.

Overexpression of Brd family genes interferes with multiple binary cell fate decisions controlled by the N pathway

We next examined the cellular basis of mutant phenotypes in the adult PNS caused by Brd family overexpression (Fig. 7), in order to relate them to the previously characterized cell fate transformations caused by reduction or loss of N signaling during PNS development (reviewed in Posakony, 1994). We performed most of these analyses in late third-instar larvae and early pupae bearing a single copy of the *sca-GAL4* driver and two copies of either *UAS-Bob* or *UAS-Tom*.

To assay the status of SOP specification within imaginal disc proneural clusters, we made use of the A101 *lacZ* enhancer trap insertion in the *neuralized* (*neu*) gene. A101 is an early marker for the SOP cell fate; in wild-type discs, it labels a single nucleus in each proneural cluster (Figs 7A, 8A). We find

that overexpression of *Bob* (Fig. 7B) or *Tom* (Fig. 7C) leads to significant increases in the numbers of A101-positive cells in third-instar wing imaginal discs, with all of the supernumerary SOPs being confined to the positions of normal proneural clusters. Thus, the inappropriate activity of either *Bob* or *Tom* is capable of interfering with the normal restriction of the SOP fate within proneural clusters, as has been previously documented for *Brd* (Leviten and Posakony, 1996).

Next, we examined the specification of cell fates within the SOP lineage. Since conditions of *Tom* overexpression that result in massive bristle loss in the adult are associated with increased numbers of SOPs (Fig. 7C), we inferred that the deficit in cuticular structures was likely due to cell fate transformations within the bristle lineage rather than to loss of SOPs. To investigate this, we made use of a number of cell type-specific markers for external sensory organs (Fig. 8A), including A1-2-29 (a *lacZ* enhancer trap marker specific for the A cell progeny, the socket and shaft cells), anti-D-Pax2 antibody (which labels the shaft and sheath cells), anti-Prospero (Pros) antibody (a sheath cell marker), mAb 22C10 (labels the shaft cell and neuron), and anti-Elav antibody (a neuron-specific marker).

In the wild-type pupal notum at 36 hours APF, a double-label analysis using A1-2-29 (β-galactosidase) and mAb 22C10 reveals a regular array of microchaetes, each containing two A1-2-29-positive nuclei and two clearly 22C10-positive structures, the differentiating shaft and the neuronal axon (Fig. 7D-F). Under conditions of *Tom* overexpression (Fig. 7G-I), which results in strong tufting of macrochaetes and extensive microchaete loss (see Fig. 6G), we find massive clusters of β-galactosidase-expressing cells at the positions of many macrochaetes, but virtually no β-galactosidase-positive microchaete cells (Fig. 7G). In these same territories, large mats of 22C10-labeled axons are observed (Fig. 7H). A different double-label analysis, using anti-D-Pax2 and anti-Elav antibodies, reveals in the wild-type notum at 30 hours APF one large and one small D-Pax2-positive nucleus (the shaft and sheath cells, respectively) and one Elav-positive nucleus (the neuron) associated with each bristle (Fig. 7J-L). Under conditions of *Tom* overexpression (Fig. 7M-O), we find large territories of the notum devoid of D-Pax2-positive nuclei (Fig. 7M); instead, these regions are found to contain large clusters of Elav-positive neurons (Fig. 7N). Many (tufted) macrochaete positions serve as internal controls for this latter analysis, as they clearly contain groups of shaft and sheath nuclei as well as neurons (Fig. 7M-O). However, the number of neurons in these clusters is often greater than the number of shaft or sheath cells, indicating that there is a bias towards neuronal fates even at positions of macrochaete tufting. Single labeling of similar *Tom*-overexpressing nota with anti-Pros also shows a strong deficit of sheath cells in the microchaete field (not shown). Qualitatively similar results are obtained when the anti-D-Pax2/anti-Elav and anti-Pros analyses are applied to nota overexpressing *Bob* (not shown), although the loss of cells expressing D-Pax2 and Pros is quantitatively less severe, as expected from the comparative adult phenotypes (see Fig. 6F,G).

We interpret these data as demonstrating that the adult bristle loss phenotype caused by overexpression of *Tom* or *Bob* reflects defects at multiple steps of sensory organ development (summarized in Fig. 8B). First, failure to restrict the SOP fate to a single cell in each proneural cluster (as indicated by A101 as well as by the large clusters of cells expressing lineage markers at both macrochaete and microchaete positions); second, failure to specify the pIIA precursor cell fate (as indicated by the loss of markers for either of its progeny; positivity for A1-2-29 and large D-Pax-2-positive nuclei); third, failure to specify the sheath cell fate (as marked by the loss of small D-Pax-2-positive nuclei and Pros-positive cells). Thus, *Tom*- or *Bob*-induced bristle loss represents the loss of three non-neuronal cell types and their apparent conversion to neurons (Fig. 8B). Such a ‘four-neuron’ phenotype has previously been shown to represent the complete failure of N signaling within the bristle lineage, as observed with temperature-sensitive alleles of *N* (Hartenstein and Posakony, 1990) and *Dl* (Parks and Muskavitch, 1993). Finally, we note that when certain GAL4 drivers (*sca-GAL4*, *hs-GAL4*, or 109-68, another GAL4-expressing insertion in *sca*) are used in combination with *UAS-Bob* or *UAS-Tom*, we occasionally observe a clear ‘double shaft’ phenotype, in which two shafts are present at the expense of the socket cell (Fig. 6J). This effect, representing the symmetric division of the pIIA precursor cell (see Fig. 8A), has been observed previously under conditions of decreased N pathway activity (Bang and Posakony, 1992; Schweisguth and Posakony, 1994). Thus, the bristle tufting, double shaft, and bristle loss phenotypes resulting from overexpression of Brd family genes can all be correlated with a loss of N pathway function, affecting multiple binary cell fate choices in adult sensory organ development (Fig. 8).

DISCUSSION

A new family of genes involved in N signaling

Our laboratory has previously characterized gain-of-function mutations of *Brd*, which genetically act antagonistically to signaling via the N receptor (Leviten and Posakony, 1996). The molecular cloning of the *Brd* locus revealed that it encodes a novel small protein with limited but significant similarity to the predicted product of *E(spl)m4* (Leviten et al., 1997), a known target of direct transcriptional activation by the N pathway (Bailey and Posakony, 1995). Here we have shown that *Brd* and *m4* are the founding members of a substantial new gene family in *Drosophila* that includes five additional structurally and functionally related genes: *Bob A*, *Bob B*, *Bob C*, *Tom* and *E(spl)mα*. Moreover, we have found that *Brd*, *Bob* and *Tom* are part of a newly recognized gene cluster, the Brd-C. Although we have not yet defined genetic lesions that yield loss-of-function mutant phenotypes for Brd family genes (see below), a large body of evidence from previous and current studies links these genes definitively to cell-cell communication via the N pathway.

First, Brd family genes are expressed specifically in multiple developmental settings in which N signaling determines cell fates (this paper; our unpublished observations; Leviten et al., 1997; Singson et al., 1994; Wurmbach et al., 1999). Postembryonically, these include the sensory organ proneural clusters of the imaginal discs (*Brd, E(spl)m4, E(spl)mα*); the lineage of at least some types of sensory organs (*E(spl)m4* and probably *E(spl)mα*); the vicinity of the morphogenetic furrow of the developing retina (*Brd, Tom, E(spl)m4, E(spl)mα*); the

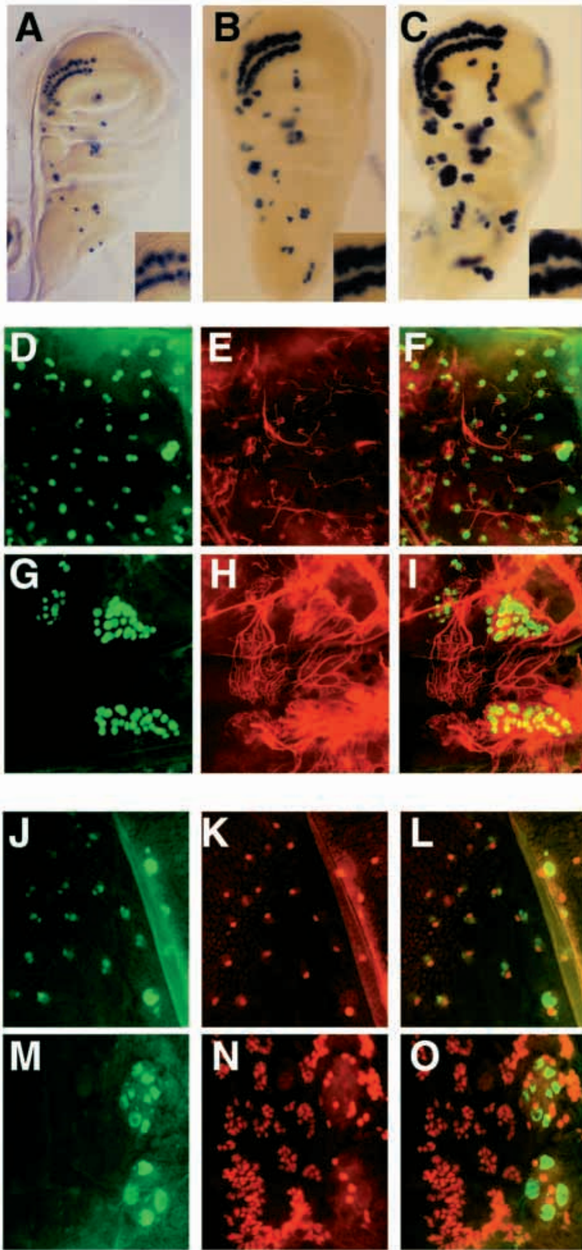

Fig. 7. Cellular basis of Brd family over- or misexpression phenotypes in the adult PNS. Genotypes: (A) *A101/+*; (B) *sca-GAL4::2xUAS-Bob; A101/+*; (C) *sca-GAL4::2xUAS-Tom; A101/+*; (D-F) *A1-2-29/A1-2-29*; (G-I) *A1-2-29/A1-2-29; sca-GAL4::2xUAS-Tom*; (J-L) *wild type*; (M-O) *sca-GAL4::2xUAS-Tom*. (A-C) Wing imaginal discs from late third-instar larvae, stained for β-galactosidase activity expressed from the A101 enhancer trap insertion; insets show detail of a portion of the anterior wing margin. (D-I) Pupal nota at 36 hours APF, double-labeled with anti-β-galactosidase antibody (green; to detect activity of the A1-2-29 enhancer trap insertion) and mAb 22C10 (red; a marker for sensory neurons and shaft cells). (D,G) Green channel; (E,H) red channel; (F,I) Combined red and green channels. (J-O) Pupal nota at 30 hours APF, double-labeled with anti-D-Pax2 (green; a marker for the shaft and sheath cells) and anti-Elav (red; a marker for sensory neurons) antibodies. (J,M) green channel; (K,N) red channel; (L,O) Combined red and green channels.

dorsal/ventral boundary of the larval wing disc (*E(spl)m*α), and vein/intervein boundaries in the pupal wing (*E(spl)m*α). In the embryo, all of these genes are expressed at peak levels throughout the ventral neuroectoderm during times of neuroblast segregation.

Second, certain Brd family genes are known to be integral members of the N pathway, and it is likely that all of them are. In particular, *E(spl)m4* has been shown to be subject to direct transcriptional activation by Su(H) in response to N receptor activity in imaginal disc proneural clusters (Bailey and Posakony, 1995). The presence of multiple high-affinity binding sites for Su(H) in the proximal upstream region of *E(spl)m*α, its reduced expression in a *Su(H)*$^{-}$ background, as well as its responsiveness to activated N, all make it extremely likely that this gene as well is a component of the N pathway (this paper; Nellesen et al., 1999; Wurmbach et al., 1999). In this report we have documented that the *Brd*, *Bob* and *Tom* genes of the Brd-C each contain at least one high-affinity Su(H) site in their upstream regions, strongly suggesting that they, too, share the property of direct transcriptional regulation by this key element of the N pathway. We also believe it is significant that the members of the Brd family appear to share multiple modes of both transcriptional regulation [by the proneural proteins and by Su(H)] and post-transcriptional regulation (via Brd, K, and GY boxes) with another family of genes intimately connected with N signaling, namely the E(spl)-C bHLH repressor genes (this paper; Bailey and Posakony, 1995; Kramatschek and Campos-Ortega, 1994; Lai et al., 1998; Lai and Posakony, 1997; Lai and Posakony, 1998; Lecourtois and Schweisguth, 1995; Leviten et al., 1997; Nellesen et al., 1999; Singson et al., 1994; Wurmbach et al., 1999).

Finally, and perhaps most importantly, overexpression of each of the five Brd family genes studied here (counting *Bob* as one gene) is capable of interfering with multiple N pathway-mediated cell fate decisions (this paper; Leviten et al., 1997; Leviten and Posakony, 1996). These phenotypes correlate well with those caused by gain-of-function mutations of *Brd*, which further display strong dosage-sensitive genetic interactions with other genes involved in N signaling, including *N*, *neu*, and *Hairless* (Leviten and Posakony, 1996). The accumulated evidence leads us to conclude that members of the Brd family function in the determination of cell fates controlled by the N pathway.

Function of Brd family genes

The Brd family genes encode novel proteins with limited similarity to each other, and thus far have no apparent homologs in other species. However, our comparison of five different Brd family proteins in this study shows that each is predicted to contain a basic amphipathic α-helical domain near its N terminus (see Fig. 1). We believe it is likely that this motif is central to the biochemical function of all of these proteins. Basic amphipathic α-helices have been shown to function as protein-protein interaction domains (most notably as calmodulin-binding domains), and can also promote interaction with or insertion into cell membranes (Segrest et al., 1990).

The commonality of an N-terminal basic domain in all Brd family proteins, along with their similar phenotypic effects when over- or mis-expressed, suggests that they may have a

common biochemical mechanism of action and may interact with a common target or targets. The conserved C-terminal extension found in the m4-related proteins (m4, mα, and Tom; see Fig. 1A) further suggests that this subfamily may have additional functions that are not shared by the shorter Brd-related proteins (Brd and Bob). In particular, it is possible that the terminal DRW$^{V}/_{A}$QA motif in these proteins, by analogy with the conserved C-terminal domain of the E(spl)-C bHLH proteins (which recruits the co-repressor Groucho), may also mediate protein-protein interactions (see below). A similar possibility exists for the shared PVXFXRTXXGTFFWT motif (see Fig. 1A).

If the gain-of-function effects we report here are indicative of the normal direction of Brd family protein function (i.e., they are normally antagonists of N pathway activity), and if all members of the Brd gene family are indeed targets of transcriptional activation by this pathway, as we have hypothesized, then Brd family proteins are excellent candidates to mediate a negative feedback mechanism in N signaling. However, we stress that a full understanding of Brd family protein function must ultimately incorporate loss-of-function genetic data, which, owing to apparent functional overlap among these genes, we do not currently possess. Thus, it is entirely possible that overexpression of Brd family proteins, rather than reinforcing or exaggerating their wild-type activity, instead causes a 'dominant negative' effect; in this case, these proteins may normally function as positive effectors of N signaling.

An important issue concerning the function of Brd family proteins is whether they exert their effects on N signaling on the sending or receiving side of the process, or both. We have obtained preliminary evidence which suggests that overexpression of Brd family genes is able to exert a cell non-autonomous effect on lateral inhibition in proneural clusters (our unpublished observations), consistent with the possibility that these proteins can antagonize the ability of a cell to send an inhibitory signal. This is of considerable interest, since relatively little is known about the detailed structure and

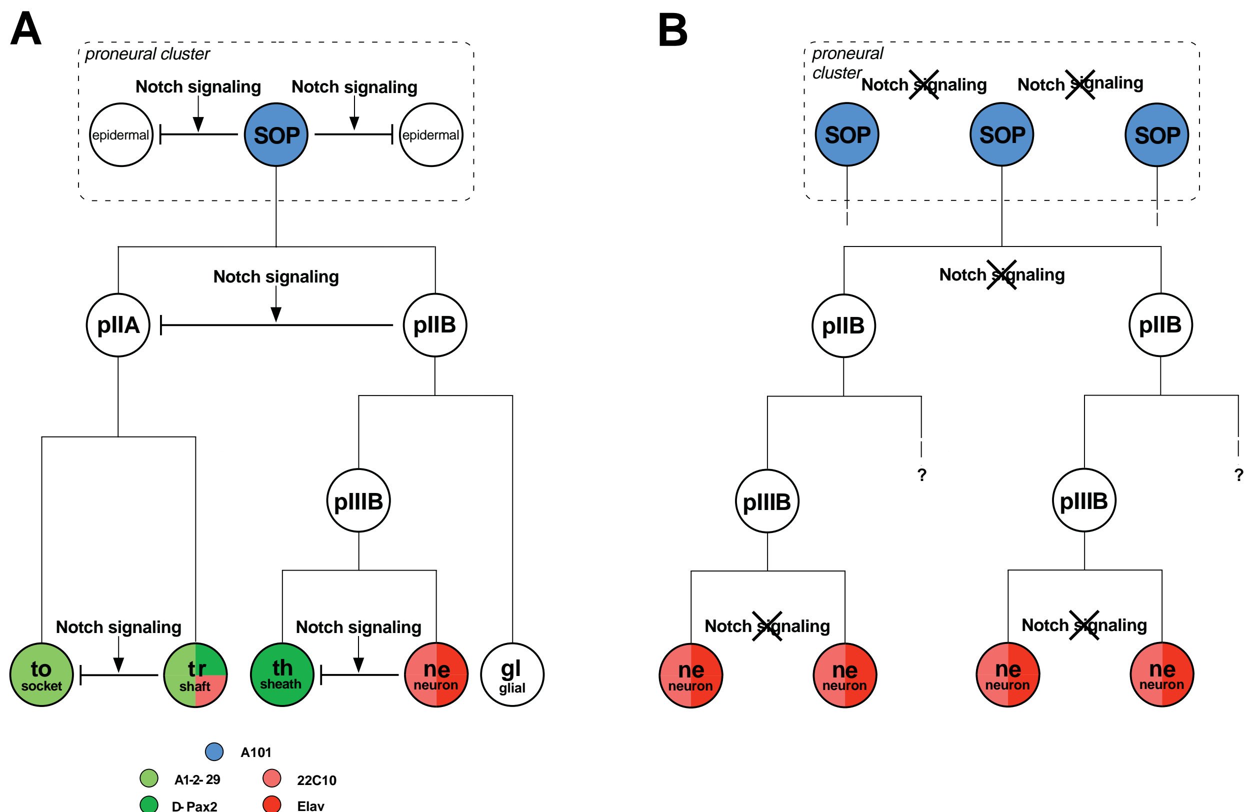

Fig. 8. Summary model of the effects of Brd family over- or misexpression on N signaling activity during development of adult mechanosensory bristles. (A) Wild-type bristle development. N signaling is utilized throughout the development of external mechanosensory organs, both in the proneural cluster (top) and at multiple divisions in the bristle lineage, to effect binary decisions between alternative cell fates (see Posakony, 1994). Specificities of cell-type markers used in this study (see Fig. 7) are indicated. The lineage shown is that recently proposed by Gho et al. (1999). to, tormogen; tr, trichogen; th, thecogen. (B) Effects of over-/misexpression of *Tom*. All cell fate defects observed under these conditions (see Figs 6, 7) can be accounted for by inhibition of N pathway activity. Bristle tufting at macrochaete positions (see Fig. 6G) is associated with specification of supernumerary SOPs (see Fig. 7C, G-I, M-O) and can be attributed to a failure of N signaling in the proneural clusters (top); bristle loss phenotype at microchaete positions (see Fig. 6G) is associated with loss of three non-neuronal cell fates in the SOP lineage and the appearance of supernumerary sensory neurons (see Fig. 7G-I, M-O), and can be accounted for by a successive failure of N signaling in the proneural clusters, in the pIIA/pIIB decision (middle), and in the thecogen/neuron decision (bottom). We have not assayed for possible alterations of the fate of the fifth cell in the bristle organ (the glial or soma sheath cell; Gho et al., 1999; Hartenstein and Posakony, 1989).

function of the N pathway upstream of the N receptor. For the cell fate choices studied in this report, possible candidates for Brd family targets thus include the transmembrane protein Dl, which appears to be the primary ligand for the N receptor in PNS development (Parks and Muskavitch, 1993; Zeng et al., 1998), and Kuzbanian, a metalloprotease that has recently been reported to be cleave Dl (Qi et al., 1999). The gain-of-function results we have presented here are consistent with the possibility that the Brd family proteins may antagonize the activity of one of these molecules.

Finally, we point out that the strong complementarity between the 3′ UTRs of Brd family genes and proneural genes via GY boxes and proneural boxes, respectively, along with the observation that multiple Brd family genes are expressed at each site of proneural gene expression, strongly suggests that the mRNAs of Brd family genes also function to regulate neural development, via the formation of RNA:RNA duplexes with proneural transcripts (Lai and Posakony, 1998). Thus, both mRNA and protein products of Brd family genes have the capacity to be involved separately in regulating neurogenesis. We are currently analyzing both RNA-mediated and protein-mediated functions of this family by investigating the regulatory effects of the postulated RNA duplexes and by identifying protein partners that interact with Brd family proteins.

Why Brd family genes have been difficult to identify

Drosophila neurogenesis is one of the most extensively scrutinized of developmental processes. Similarly, the N pathway plays an essential role in the development of many different tissues, and has been and continues to be a very intensively studied signal transduction cascade. It is thus perhaps surprising that the existence of a substantial family of Brd-type genes, at least some and possibly all of which are components of the N pathway, has only now come to our attention. However, this apparent paradox is not difficult to rationalize, since several features of this set of genes effectively shield them from identification by most conventional means.

First, Brd family genes are probably not readily amenable to traditional loss-of-function genetics, in part because multiple members of the family are co-expressed in multiple settings. Indeed, no mutant phenotypes have been detected in flies that are null for any single Brd family gene. For example, flies homozygous for characterized deletions of the *Brd* locus are viable and apparently wild-type in phenotype (Leviten and Posakony, 1996); a homozygous fly line bearing a P-element insertion in the proximal upstream region of *Tom* is similarly unaffected (BDGP; our unpublished observations); the triplication of the *Bob* transcription unit in genomic DNA suggests that mutation of an individual *Bob* gene may have little effect; and extensive mutagenesis screens and genetic analyses of the E(spl)-C have suggested that *E(spl)m4* and *E(spl)mα* (along with the bHLH repressor genes) are individually nonessential (Delidakis et al., 1991; Preiss et al., 1988; Schrons et al., 1992; Ziemer et al., 1988). Recently, we have determined that embryos deficient for the known extent of the Brd-C exhibit comparatively mild mutant phenotypes with respect to embryonic neurogenesis; however, all Brd family genes [including *E(spl)m4* and *E(spl)mα*, not deleted in this genotype] are expressed at high levels throughout the embryonic ventral neuroectoderm (our unpublished observations; Knust et al., 1992; Wurmbach et al., 1999). All of these data suggest that there is a large degree of functional overlap amongst Brd family genes.

Second, Brd family genes may well escape detection by systematic gain-of-function genetic screens using mobile enhancer elements (Rørth et al., 1998), due to the strong negative regulation conferred by the 3′ UTRs of these genes (Lai et al., 1998; Lai and Posakony, 1997). Indeed, we have been unable to generate mutant phenotypes under a variety of conditions with multiple copies of P[*Hs-Brd*] or P[*Hs-m4*] heat-shock transgenes that include substantial or complete 3′ UTR sequences (our unpublished observations; Leviten et al., 1997).

Third, the paralogous members of the Brd family in *Drosophila* are sufficiently diverged from each other to largely preclude the possibility of identifying additional members by low-stringency hybridization or degenerate PCR. One Brd family gene (*Tom*) was in fact originally identified in a screen designed specifically to isolate rapidly evolving genes (Schmid and Tautz, 1997), suggesting that it may not even be possible to identify potential orthologs in data from the various genome sequencing projects based on sequence alone.

In summary, the relatively invisible nature of the Brd family genes poses a serious obstacle for identifying any remaining family members in *Drosophila*, or, perhaps more interestingly, for identifying orthologs of these genes in other taxa. In spite of this, we have reason to believe that such additional *Drosophila* paralogs and even vertebrate homologs may exist. Our analysis of certain recently identified genes in the E(spl)-C indicates that both *E(spl)m2* and *E(spl)m6* (see Wurmbach et al., 1999) encode divergent m4-like proteins, and further characterization of the Brd-C may reveal additional genes. The complete sequence of the *Drosophila* genome will of course prove invaluable in helping to identify any remaining family members.

The number, regulation, and in vivo activities of Brd family genes all indicate their importance as elements of the N cell-cell signaling system. Further investigations should elucidate the specific biochemical and cell biological functions of their products.

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Notes added in proof

(1) The GenBank accession numbers for the sequence data reported in this paper are: *Tom/Brd* region genomic DNA, U13067; *Bob A, B, C* region genomic DNA, AF208486.

(2) Genomic sequence data recently released by Celera Genomics (GenBank accession number AC014109) reveals the existence of an eighth distinct Brd family gene in *Drosophila*, which we have accordingly named *Ocho*. *Ocho* encodes a clearly recognizable member of the m4 subfamily of Brd family proteins, and is most related to Tom, E(spl)mα, and E(spl)m4. *Ocho* lies only about 2 kb downstream of *Brd*, and is thus a member of the Brd-C.

(3) Overexpression phenotypes for *E(spl)m4* and *E(spl)mα* have also recently been described by Apidianakis et al. (1999, *Mech. Dev.* **86**, 39-50).