Development 127, 3441-3455 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV8740

The *Enhancer of split* Complex of *Drosophila* includes four Notch-regulated members of the Bearded gene family

Eric C. Lai*,‡, Ruth Bodner* and James W. Posakony§

Division of Biology/Department of Cell and Developmental Biology and Center for Molecular Genetics, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0349, USA

*These authors contributed equally to this work

‡Present address: Department of Molecular and Cellular Biology/HHMI, University of California, Berkeley, CA 94720-3200, USA

§Author for correspondence (e-mail: jposakony@ucsd.edu)

Accepted 31 May; published on WWW 20 July 2000

SUMMARY

During *Drosophila* development, transcriptional activation of genes of the *Enhancer of split* Complex (E(spl)-C) is a major response to cell-cell signaling via the Notch (N) receptor. Although the structure and function of the E(spl)-C have been studied intensively during the past decade, these efforts have focused heavily on seven transcription units that encode basic helix-loop-helix (bHLH) repressors; the non-bHLH members of the complex have received comparatively little attention. In this report, we analyze the structure, regulation and activity of the *m1*, *m2* and *m6* genes of the E(spl)-C. We find that *E(spl)m2* and *E(spl)m6* encode divergent members of the Bearded (Brd) family of proteins, bringing to four (*mα*, *m2*, *m4* and *m6*) the number of Brd family genes in the E(spl)-C. We demonstrate that the expression of both *m2* and *m6* is responsive to N receptor activity and that both genes are apparently direct targets of regulation by the N-activated transcription factor Suppressor of Hairless. Consistent with this, both are expressed specifically in multiple settings where N signaling takes place. Particularly noteworthy is our finding that *m6* transcripts accumulate both in adult muscle founder cells in the embryo and in a subset of adepithelial (muscle precursor) cells associated with the wing imaginal disc. We show that overexpression of either *m2* or *m6* interferes with N-dependent cell fate decisions in adult PNS development. Surprisingly, while misexpression of *m6* impairs lateral inhibition, overexpression of *m2* potentiates it, suggesting functional diversification within the Brd protein family. Finally, we present our initial studies of the structure, expression and regulation of the newest member of the Brd gene family, *Ocho*, which is located in the recently identified Bearded Complex.

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

INTRODUCTION

The Notch (N) pathway is a conserved signal transduction module that is essential for the proper specification of cell fates in a wide variety of developmental contexts in metazoans (Artavanis-Tsakonas et al., 1999; Greenwald, 1998; Kimble and Simpson, 1997). Much of our understanding of N pathway structure and function has come from studies of adult sensory organ development in *Drosophila*, in which N-mediated cell-cell signaling is used at multiple steps to effect binary cell fate choices (Posakony, 1994). Interaction of the transmembrane N receptor with its ligand Delta results in cleavage of the intracellular domain of N (N^{IC}), which then translocates to the nucleus and acts as a transcriptional coactivator for the sequence-specific DNA-binding protein Suppressor of Hairless (Su(H)) (Jarriault et al., 1995; Lecourtois and Schweisguth, 1998; Schroeter et al., 1998; Struhl and Adachi, 1998). The Su(H)/N^{IC} complex activates expression of various target genes, the best characterized of which include multiple basic helix-loop-helix (bHLH) repressor genes of the *Enhancer of split* Complex (E(spl)-C)) (Bailey and Posakony, 1995; Furukawa et al., 1995; Lecourtois and Schweisguth, 1995). All of these proteins have been conserved structurally and functionally during metazoan evolution, and are considered core components of the N pathway (Artavanis-Tsakonas et al., 1999).

The E(spl)-C encompasses a genomic region that is particularly dense with transcription units, with 13 different genes resident within a 55 kb interval (Fig. 1A). These include the seven N-activated bHLH repressor genes just referred to (Delidakis and Artavanis-Tsakonas, 1992; Klämbt et al., 1989; Knust et al., 1992), as well as *groucho* (*gro*), which encodes a transcriptional co-repressor for the E(spl)-C bHLH proteins and for a number of other bHLH and non-bHLH repressors encoded elsewhere in the genome (Fisher and Caudy, 1998; Preiss et al., 1988). Both *E(spl)m4* and *E(spl)mα* encode members of the non-bHLH Bearded (Brd) family of small proteins containing predicted N-terminal basic amphipathic α-helical domains (Lai et al., 2000; Leviten et al., 1997). Overexpression of either protein can antagonize the activity of

the N pathway (Apidianakis et al., 1999; Lai et al., 2000). Thus, three distinct molecular functions related to N signaling are encoded in the E(spl)-C (bHLH DNA-binding repressor, Gro co-repressor, and Brd family). These findings suggest that care must be taken when interpreting the mutant phenotypes associated with E(spl)-C deficiencies, which have typically been attributed solely to loss of bHLH and *gro* gene function.

Such considerations also make clear that it is crucial to have a full understanding of the molecular nature of the E(spl)-C in order to understand its function. Accordingly, we have analyzed the structure, regulation and activity of the remaining non-bHLH genes in the E(spl)-C, namely *m1*, *m2*, and *m6* (Knust et al., 1987b; Nellesen et al., 1999; Wurmbach et al., 1999). We find that *E(spl)m2* and *E(spl)m6* encode additional members of the Brd protein family; that these genes are expressed specifically in a wide spectrum of locations where the N pathway is active; and that both are likely to be direct targets of transcriptional activation by Su(H) and thus integral members of the N pathway. We also present here our initial investigations of the structure, expression, and regulation of an eighth Brd family gene, *Ocho*, which is a member of the recently identified Bearded Complex (Brd-C), a second major clustering of Brd family loci (Fig. 1B; Lai et al., 2000).

MATERIALS AND METHODS

Fly stocks

sc^{10-1} is a compound mutation that inactivates both *ac* and *sc* function (Gelbart et al., 1999; Lindsley and Zimm, 1992). The transheterozygous combination $Su(H)^{SF8}/Su(H)^{AR9}$ (Schweisguth and Posakony, 1992) was used as a *Su(H)* null genotype. A third-chromosome insertion of the P element transgene P[ry^+, *Hsp70-N(intra)*] was used for constitutive, ubiquitous activation of the N pathway (Struhl et al., 1993). A101 is a *lacZ* enhancer-trap transposon insertion in the *neuralized* gene (Bellen et al., 1989; Boulianne et al., 1991; Price et al., 1993); it drives β-galactosidase expression specifically in sensory organ precursor cells and their progeny (Huang et al., 1991; Usui and Kimura, 1993). GAL4 driver lines used for mis- and over-expression studies (Brand and Perrimon, 1993; Phelps and Brand, 1998) include *GMR-Gal4* (Freeman, 1996), *sca-Gal4* (Hinz et al., 1994; Nakao and Campos-Ortega, 1996), and MS 1096 (Capdevila and Guerrero, 1994; Milán et al., 1998).

DNA sequencing and transcription unit analysis in the E(spl)-C and Brd-C

The cloning and sequencing of genomic DNA segments covering *E(spl)m1*, *m2* and *m6* (Fig. 1A), as well as the criteria for identifying their transcription start sites, have been described recently (Nellesen et al., 1999). A clot of seven larval/pupal-stage EST clones corresponding to *m1* have been sequenced by the BDGP/HHMI Drosophila EST Project (Rubin et al., 2000); a cDNA clone for *m2* was isolated from a library representing poly(A)$^+$ RNA from 4- to 8-hour embryos (Brown and Kafatos, 1988).

A putative transcription start site for *Ocho* was assigned by its distance downstream of a canonical TATA box (TATAAATA); the implied start site sequence is an 8/8 match (CAGCATCA) with those of *E(spl)m2* and *E(spl)m5* (see Nellesen et al., 1999).

Plasmid construction

UAS transgene constructs for misexpression studies (Brand and Perrimon, 1993; Phelps and Brand, 1998) were prepared as follows. PCR was used to amplify the coding regions of *m1*, *m2* and *m6* (along with 6-10 nucleotides (nt) of 5′ UTR sequence to ensure normal translation initiation) and to include appropriate flanking restriction sites. PCR products were digested with the following enzymes and cloned into the corresponding sites in pBS+ (Stratagene): *m1*, 5′ *Bam*HI, 3′ *Sal*I; *m2*, 5′ *Bam*HI, 3′ *Xho*I; *m6*, 5′ *Bam*HI, 3′ *Xba*I. The inserts were sequenced, excised and recloned into pUAST (Brand and Perrimon, 1993).

E(spl)mα, *m2* and *m6* reporter transgenes were constructed as follows. For *mα*, a small TATA box-containing PCR fragment from –68 to +35 (Nellesen et al., 1999) was cloned into pBS; this included an endogenous *Xho*I site on the 5′ end and a *Kpn*I site added to the 3′ end. A 1.0 kb *Bam*HI-*Xho*I genomic DNA fragment was added to this subclone to create a 1.1 kb *mα* promoter fragment. This was then cloned into Stinger, a new P element transformation vector that includes a nuclear-GFP reporter gene (L. Carver and J. W. P., unpublished data), as an *Xba*I-*Kpn*I fragment, to create an *mα-GFP* reporter transgene. A 2.7 kb *Eco*RI-*Xho*I genomic DNA fragment was added to the TATA box-bearing subclone to create a 2.8 kb *mα* promoter fragment. This was cloned into Pelican, a new P element transformation vector that includes a *lacZ* reporter gene (S. Barolo, unpublished), as an *Eco*RI-*Kpn*I fragment, to create an *mα-lacZ* reporter transgene. To create an *m2-lacZ* reporter transgene, a PCR product covering –2924 to +59 of *m2* (Nellesen et al., 1999) was subcloned into pBS as an *Eco*RI-*Xho*I fragment; the promoter-proximal 1.5 kb of this fragment was sequence-verified. This 3.0 kb *m2* promoter fragment was then cloned into Pelican as an *Eco*RI-*Xho*I fragment. To create an *m6-GFP* reporter transgene, a PCR product covering –2098 to +37 of *m6* (Nellesen et al., 1999) was cloned as an *Xba*I-*Xho*I fragment into Green Pelican, a new P element transformation vector that includes a non-nuclear-GFP reporter gene (S. Barolo and J. W. P., unpublished data). The Stinger, Pelican, and Green Pelican vectors will be described fully in a separate report (S. Barolo, L. Carver and J. W. P., unpublished data).

P element transgene constructs were injected into w^{1118} recipient embryos according to standard methods (Rubin and Spradling, 1982). For each UAS construct, 10 independent insertions were individually tested for their ability to confer mutant phenotypes upon activation by a GAL4 driver. For each reporter construct, 3-4 independent insertions were individually tested for their ability to direct a given pattern of expression; in all cases, only slight quantitative differences were observed between different insertion lines.

Electrophoretic mobility shift assay

GST-Daughterless (GST-Da) and GST-Achaete (GST-Ac) fusion proteins were purified as described previously (Singson et al., 1994; Van Doren et al., 1994). GST-Su(H) fusion protein was purified as described by Bailey and Posakony (1995). His-tagged Su(H) [6xHis-Su(H)] was purified by binding to Ni-NTA Agarose (Qiagen), according to the manufacturer's protocol. Electrophoretic mobility shift assays (EMSAs) were performed essentially as described by Singson et al. (1994) and by Bailey and Posakony (1995).

In situ hybridization, histochemistry and immunohistochemistry

In situ hybridization to detect endogenous transcripts was performed essentially as described by O'Neill and Bier (1994). However, the hybridization was performed at 65°C, in hybridization buffer adjusted to pH 5.0. Digoxigenin-labeled antisense RNA probes (Tautz and Pfeifle, 1989) were generated by linearizing the following plasmid subclones with the indicated restriction enzymes and transcribing with the indicated RNA polymerase: *mα*, *Eco*RI/*Xho*I genomic DNA subclone in pBS/*Xho*I/T7; *m2*, cDNA clone (coding region plus 3′ UTR) in pCRII-TOPO (Invitrogen)/*Sal*I/T7; *m4*, cDNA clone in pNB40/*Hin*dIII/T7; *m6*, 1.0 kb genomic DNA subclone (coding region plus 3′ UTR) in pBS/*Bam*HI/T3.

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

The double-labeling experiment shown in Fig. 3J-L was performed

as described by Kavaler et al. (1999), using the following antibodies at the indicated dilutions: primary, mouse anti-β-galactosidase (Promega, 1:300) and rabbit anti-GFP (Molecular Probes, 1:500); fluorescent secondary, goat anti-mouse IgG conjugated to Texas Red (Molecular Probes, 1:100) and goat anti-rabbit IgG conjugated to Oregon Green (Molecular Probes, 1:100).

Simultaneous detection of Twist or Cut protein and *E(spl)m6* transcripts utilized a modified protocol based on those of O'Neill and Bier (1994) and Sturtevant et al. (1993). Details are available upon request, or at our Web site (http://www.biology.ucsd.edu/labs/posakony).

Scanning electron microscopy

Scanning electron microscopy was carried out as described by Bang et al. (1991).

RESULTS

The E(spl)-C encodes four Brd family proteins

In an effort to gain a more complete understanding of the structure and function of the E(spl)-C, we have cloned and sequenced genomic DNA that includes the non-bHLH transcription units *E(spl)m1*, *E(spl)m2* and *E(spl)m6* (Knust et al., 1987b; Nellesen et al., 1999). In addition, we have completely sequenced the region reputed to contain '*E(spl)mX*' (originally placed between *m6* and *m7*; Delidakis and Artavanis-Tsakonas, 1992; Delidakis et al., 1991; Maier et al., 1993; Preiss et al., 1988), and found that it contains no substantial open reading frame. Moreover, our analysis of this *m6-m7* intergenic region by northern blot hybridization, using both total and polyadenylated RNAs from a variety of developmental stages, failed to reveal any corresponding transcripts (data not shown), suggesting that '*mX*' may not exist.

We have concluded (Figs 1A, 2A) that *m1* encodes a protease inhibitor of the Kazal family (see also Wurmbach et al., 1999), and that *m2* and *m6* encode members of the Brd family of small proteins that appear to act as effectors or modulators of N pathway activity (Lai et al., 2000; Leviten et al., 1997; Leviten and Posakony, 1996). Brd family proteins share a common secondary structural characteristic, a predicted basic amphipathic α-helix near their N termini (Lai et al., 2000; Leviten et al., 1997). We find that the predicted protein products of both *m2* and *m6* similarly contain highly basic domains with amphipathic character near their N termini (Fig. 2A,B). The basic domain of m2 is most similar to that of Tom (Lai et al., 2000), in that both contain a proline residue within this region (Fig. 2B). The basic domain in m6 is found at its extreme N terminus (Fig. 2A); it is likewise predicted to form a largely α-helical, strongly amphipathic structure (Fig. 2B). Thus, it is clear that a defining structural characteristic of Brd family proteins is present in both m2 and m6.

Classification of m2 and m6 as Brd family proteins is further bolstered by the presence of two short sequence domains that are widely shared by members of the family (Fig. 2A). First, we note that the motif NXANE(K/R)(L/M) is common to m6, mα and m4, while Tom, Bob and Brd contain related sequences at comparable positions. A second motif, (I/L/V)P(L/V)X(F/Y)XXTXXGTFFW, is found near the C terminus of mα, m2, m4 and Tom, while m6 contains the clearly related sequence VXXXXTXXGSFYW. However, we note that the motif DRW(A/V)QA found at the extreme C-termini of mα, m4 and Tom (Apidianakis et al., 1999; Lai et al., 2000) is not present in m2 and m6 (Fig. 2A). Our identification of *E(spl)m2* and *E(spl)m6* as members of the Brd family brings the number of Brd family genes in the E(spl)-C to four (see Fig. 1A).

Expression of E(spl)-C Brd family genes coincides with sites of N signaling

We analyzed by in situ hybridization the spatial patterns of transcript accumulation from *m1*, *m2*, and *m6* in wild-type imaginal tissue (Figs 3, 4). As shown in Fig. 3A-F, *m2* displays a complex pattern of transcript accumulation in late third-instar imaginal discs that is highly reminiscent of *mα* (see Fig. 6A; Lai et al., 2000; Wurmbach et al., 1999). Its sites of expression include proneural clusters (although at significantly lower levels compared to *mα*; Fig. 3A,B), the territories of the developing wing veins (Fig. 3A), the dorsoventral boundary of the wing disc (Fig. 3A), and the vicinity of the morphogenetic furrow of the developing retina (Fig. 3B), all specific locations where the N pathway is used to specify cell fates. We have also found that, like the E(spl)-C bHLH genes *mβ* (de Celis et al., 1998) and *m8* (Bishop et al., 1999), both *m2* and *mα* are expressed in pre-eversion (late third-instar) and everting [1-2 h after puparium formation (APF)] leg imaginal discs in a series of concentric rings that correspond to the distal borders of the leg segments (Fig. 3D-F,G-I), the specification of which is under the control of N signaling (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999).

Interestingly, neither *m2* nor *mα* appear to be expressed in the sensory organ precursors (SOPs) of proneural clusters of various larval imaginal discs. Although this feature of their activity is somewhat difficult to discern by in situ hybridization detection of transcripts from the endogenous genes, single 'holes' in each proneural cluster are readily observed in discs from animals carrying *mα* and *m2* promoter-reporter transgenes (Fig. 3J). Double-label analysis utilizing the SOP-specific enhancer-trap marker A101 (Bellen et al., 1989; Huang et al., 1991) demonstrates that the 'holes' in the cluster expression pattern correspond to SOPs (Fig. 3J-L).

E(spl)m6 displays a much more restricted pattern of transcript accumulation in the third-instar wing imaginal disc (Fig. 4A; see also Wurmbach et al., 1999). We find that it is expressed predominantly in a single large anterior patch of cells adjacent to the presumptive wing hinge region, with lower-level expression in a small group of cells near the proneural clusters for the dorsocentral macrochaetes and in certain other regions. No accumulation of *m6* transcripts is apparent in the presumptive wing blade region of the disc (Fig. 4A); likewise, *m6* is evidently not expressed in either the eye or antenna discs at third instar (Fig. 4B). We do observe comparatively weak but characteristic patterns of *m6* transcript in the haltere (Fig. 4C) and leg (not shown) discs at this stage. All of these spatial aspects of *m6* expression are fully reproduced by a GFP reporter transgene driven by a 2.1 kb *m6* promoter fragment (Fig. 4D-F and data not shown).

While Wurmbach et al. (1999) reported roughly the same major position of *m6* transcript accumulation in the wing disc as described above, they interpreted it to represent a portion of the notal anlage; i.e., part of the ectodermal imaginal epithelium. Our data indicate instead that these are adepithelial

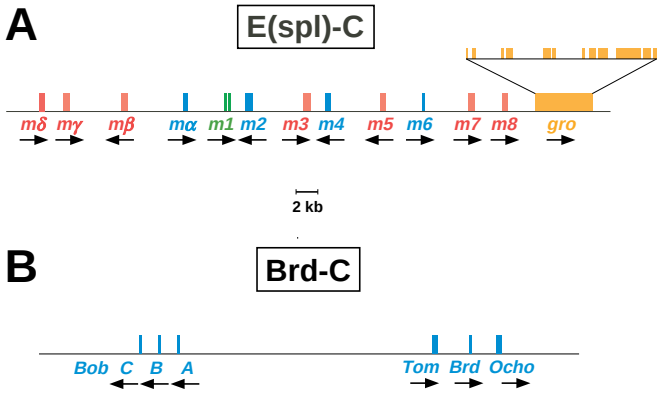


Fig. 1. Physical maps of the E(spl)-C and Brd-C. Protein coding regions of labeled genes are represented (to scale) by vertical boxes; directions of transcription are indicated by arrows. (A) Three distinct molecular functions related to the N pathway are encoded in the E(spl)-C, which includes seven bHLH repressor-encoding genes (red), four Brd family genes (blue), and the co-repressor-encoding gene *groucho* (*gro*, orange; the physical organization of the *gro* protein-coding sequences is also shown at a 3X-expanded scale). *E(spl)m1* (green) encodes a Kazal-type protease inhibitor, the function of which appears not to be related to the N pathway. This map is based on contiguous genomic DNA sequence covering the entire E(spl)-C, reported by Celera Genomics and BDGP (GenBank accession number AE003754). (B) A total of six Brd family genes (blue) have been identified in the Brd-C, including the triplicated *Bob* locus on the left and the cluster of *Tom-Brd-Ocho* on the right. This map is based on genomic DNA sequence data generated by us and by Celera Genomics and BDGP (GenBank accession numbers AF208486, U13067, and AE003532).

cells, a mesodermal population that function as precursors of the adult flight musculature (reviewed by Roy and VijayRaghavan, 1999). Double staining of late third instar wing, haltere and leg discs for *m6* transcript and either Twist (Bate et al., 1991) or Cut (Blochlinger et al., 1993) protein demonstrates that the very great majority of *m6*-positive cells are part of the adepithelial population and not the disc epithelium proper (Fig. 4K,L and data not shown). The specific association of *m6* and Twist expression is especially evident in certain linear arrangements of cells that appear away from the main body of the adepithelial population in the wing (Fig. 4L) and haltere (not shown) discs.

The above interpretation is further strengthened by our finding that in stage 13-15 embryos *m6* transcripts accumulate specifically in small numbers of mesodermal cells in each segment (Fig. 4M,N), in addition to specific cells in the CNS and midgut (data not shown). Double-label analysis at stage 15 using anti-Twist antibody demonstrates clearly that the *m6*-positive mesodermal cells are adult muscle founder cells, which include the precursors to the disc-associated adepithelial cells (Fig. 4N-P; Bate et al., 1991). Again, our findings contrast with those of Wurmbach et al. (1999), who evidently interpreted these cells as belonging to the peripheral nervous system. The specific expression of *m6* in the adult muscle founder cells in the embryo has particular significance, since the fates of these cells are specified in a N signaling-mediated process (see Discussion). A full description of the embryonic expression patterns of the Brd family genes, including *m6*, will be presented in a forthcoming report.

We also examined the expression of *m2* and *m6* during pupal wing development. At 24 h APF, accumulation of *m2* transcripts along wing vein-intervein borders (sites of active N signaling; de Celis et al., 1997; Huppert et al., 1997) is especially apparent, as is a continuous band of expression along both the anterior and posterior wing margins (Fig. 3C). We have previously described a very similar pupal wing expression pattern for *mα* at this same stage (Lai et al., 2000). We observe strong expression of *m6* in discrete cells of the anterior wing margin at 8 h APF (Fig. 4G,H). Although the pattern of labeled cells is not completely regular, they may be components of sensory organs, given their location in the anterior but not the posterior margin. At 24 h APF, *m6* transcripts are most notably present in pairs of posterior margin cells, which may be constituents of the large non-sensory hairs that develop on this margin (Fig. 4I,J).

Finally, we find that *m1* is not detectably expressed in the imaginal discs of third instar larvae, though in embryos it is expressed at high levels at the anterior constriction of the gut (data not shown; see Wurmbach et al., 1999), potentially consistent with a digestive function for its product like that of some other Kazal-type protease inhibitors (Roberts et al., 1995).

Binding sites for Su(H) and for proneural proteins in the *m2* and *m6* proximal upstream regions

A previous study from our laboratory revealed the presence of predicted high-affinity binding sites for proneural bHLH activator proteins and for Su(H) in the proximal upstream regions of *m2* and *m6* (Nellesen et al., 1999). Such binding sites are common to, and essential for normal activation of, a variety of E(spl)-C transcription units, including both bHLH and Brd family genes (Bailey and Posakony, 1995; Kramatschek and Campos-Ortega, 1994; Lecourtois and Schweisguth, 1995; Nellesen et al., 1999; Singson et al., 1994). In contrast, the proximal 1 kb of *m1*'s upstream region evidently lacks even predicted low-affinity binding sites of either type, suggesting that it is not a target of either proneural activators or the N pathway (Nellesen et al., 1999).

The upstream regions of all four E(spl)-C Brd family members contain an extended 'E box' sequence conforming to the consensus GCAGSTGKK, a known high-affinity binding site for proneural proteins (Fig. 5A; Bailey and Posakony, 1995; Lai et al., 2000; Nellesen et al., 1999; Singson et al., 1994). In addition, *m2* includes the sequence ACAGGTG, also a predicted proneural protein binding site (Bailey and Posakony, 1995; Nellesen et al., 1999; Van Doren et al., 1991). Using the electrophoretic mobility shift assay (EMSA), we find that oligonucleotide probes containing these sites from the *m2* and *m6* upstream regions are efficiently bound in vitro by hetero-oligomeric protein complexes formed by purified GST-Ac and GST-Da (Fig. 5C), as well as by GST-Sc/GST-Da complexes (data not shown). These observations suggest that *m2* and *m6* are possible targets of direct transcriptional activation by proneural proteins.

We have also investigated by EMSA the in vitro interaction of purified GST-Su(H) or 6xHis-Su(H) with a number of predicted Su(H) binding sites in the proximal upstream regions of *m2* and *m6*. Although Wurmbach et al. (1999) noted only a single putative Su(H) site in the promoter of *m2* and found none in *m6*, we instead find multiple predicted Su(H) binding sites in the upstream regions of both genes (Fig. 5B; Nellesen

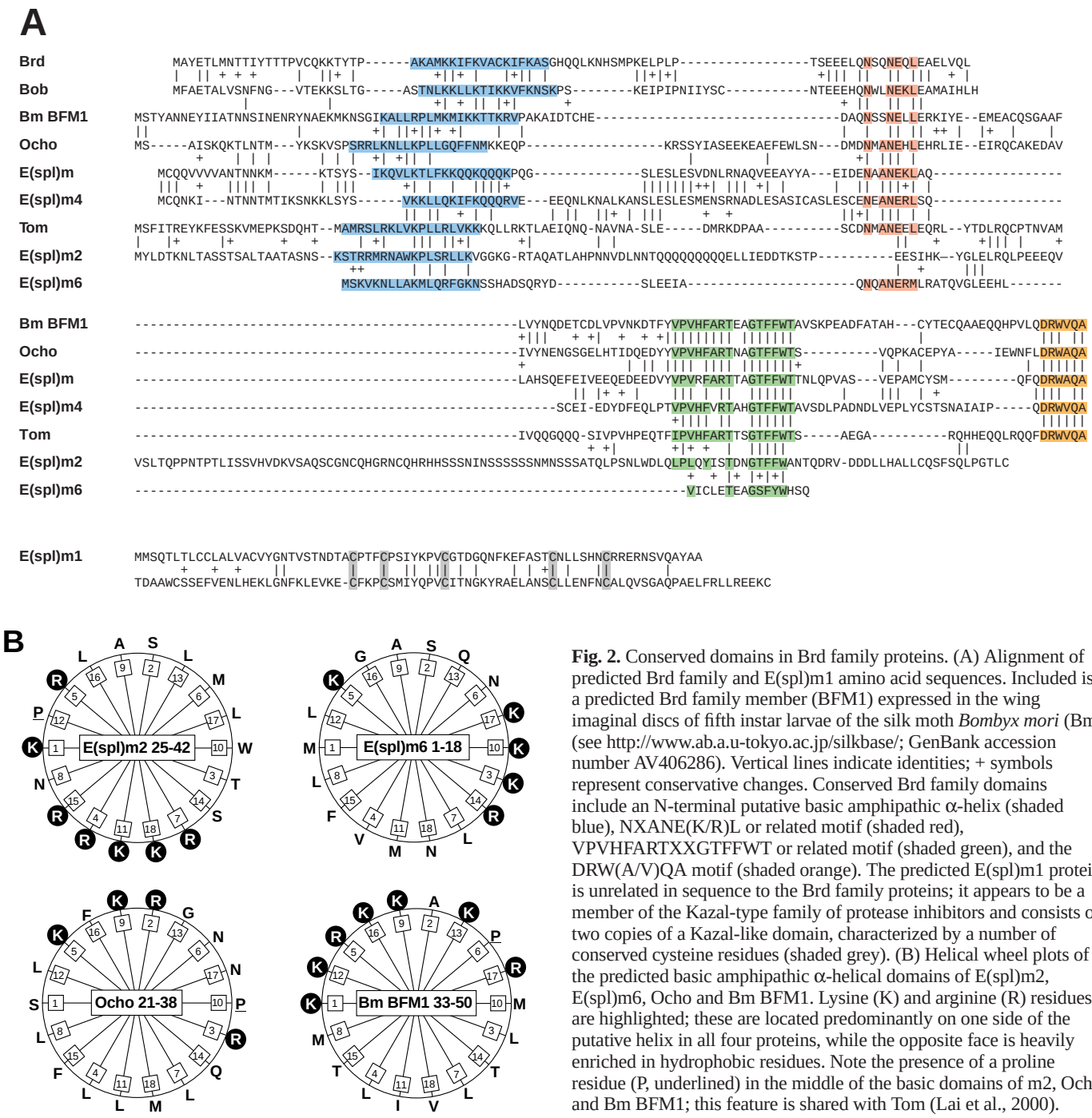


Fig. 2. Conserved domains in Brd family proteins. (A) Alignment of predicted Brd family and E(spl)m1 amino acid sequences. Included is a predicted Brd family member (BFM1) expressed in the wing imaginal discs of fifth instar larvae of the silk moth *Bombyx mori* (Bm) (see http://www.ab.a.u-tokyo.ac.jp/silkbase/; GenBank accession number AV406286). Vertical lines indicate identities; + symbols represent conservative changes. Conserved Brd family domains include an N-terminal putative basic amphipathic α-helix (shaded blue), NXANE(K/R)L or related motif (shaded red), VPVHFARTXXGTFFWT or related motif (shaded green), and the DRW(A/V)QA motif (shaded orange). The predicted E(spl)m1 protein is unrelated in sequence to the Brd family proteins; it appears to be a member of the Kazal-type family of protease inhibitors and consists of two copies of a Kazal-like domain, characterized by a number of conserved cysteine residues (shaded grey). (B) Helical wheel plots of the predicted basic amphipathic α-helical domains of E(spl)m2, E(spl)m6, Ocho and Bm BFM1. Lysine (K) and arginine (R) residues are highlighted; these are located predominantly on one side of the putative helix in all four proteins, while the opposite face is heavily enriched in hydrophobic residues. Note the presence of a proline residue (P, underlined) in the middle of the basic domains of m2, Ocho and Bm BFM1; this feature is shared with Tom (Lai et al., 2000).

et al., 1999). These include ‘canonical’ high-affinity sites of the class YGTGRGAAM (Bailey and Posakony, 1995; Lai et al., 2000; Lecourtois and Schweisguth, 1995; Tun et al., 1994) as well as sites that fit the more relaxed consensus RTGRGAR, which includes lower-affinity Su(H) binding sites (Lai et al., 2000; Nellesen et al., 1999). In addition, we identified sites that fit neither consensus, but are highly related to the canonical high-affinity sites, including CGTG**T**GAA (twice in *m2*; see Tun et al., 1994), CGTGGGA**T** (twice in *m6*), and CGTGGGA**C** (once in *m2*). We performed EMSAs on 15 oligonucleotide probes containing putative Su(H) binding sites located within the proximal 1.5 kb of the *m2* and *m6* upstream regions (Fig. 5B,D). We find that GST-Su(H) or 6xHis-Su(H) are bound in vitro to eight sites in the *m2* upstream region and four sites in the *m6* upstream region, including the variant sites noted above. Thus, the presence of multiple proximal Su(H) binding sites is a shared feature of both bHLH and Brd family genes of the E(spl)-C (see Nellesen et al., 1999). Our results suggest that both *m2* and *m6* are direct targets of transcriptional regulation by Su(H), consistent with their expression at multiple sites of N signaling activity, as described above.

Finally, we note that the 3′ UTRs of both *m2* and *m6* contain single copies of the K box (TGTGAT), a negative post-transcriptional regulatory motif previously observed to be

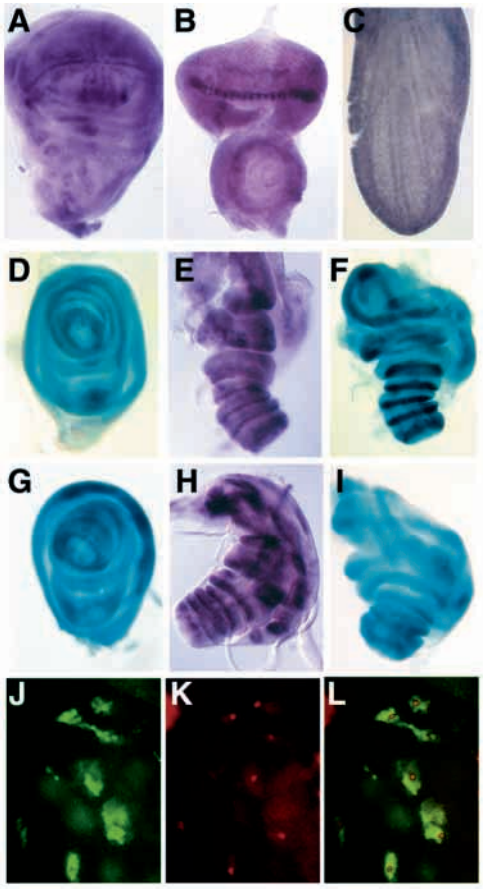


Fig. 3. Expression of *E(spl)m2* (A-F) and *E(spl)mα* (G-L) in imaginal tissues during larval and pupal development. (A-C,E,H) In situ hybridization using digoxigenin-labeled probes to detect accumulation of endogenous transcripts. (D,F,G,I) β-galactosidase activity staining to detect expression of promoter-*lacZ* reporter transgenes. (J-L) Double fluorescent antibody labeling to detect GFP (green) and β-galactosidase expressed by the A101 enhancer-trap marker (red). (A) *E(spl)m2* is expressed in a complex pattern in late third instar wing imaginal discs that includes proneural clusters, stripes associated with presumptive wing veins, and the dorsoventral boundary of the wing margin. (B) *m2* transcripts accumulate in the anlage of Johnston's organ in the antenna disc and in the vicinity of the morphogenetic furrow in the developing retina. (C) Expression of *m2* at the boundaries of developing wing veins is particularly evident in the pupal wing at 24 h APF. In late third instar leg discs, *m2-lacZ* reporter gene activity, though broad, is elevated in a series of concentric rings (D), which correspond to the distal portions of leg segments in the everting pupal leg at 1-2 h APF (F); transcripts from the endogenous *m2* gene accumulate in the same pattern (E). *E(spl)mα* is expressed in a similar pattern at leg segment boundaries, as revealed both by in situ hybridization to detect endogenous *mα* transcripts (H), and by staining for β-galactosidase activity to detect *mα-lacZ* reporter gene expression (G,I). (J-L) Transcriptional activity of *mα* is restricted to non-SOP cells of proneural clusters. Double antibody labeling with (J) anti-GFP (green) and (K) anti-β-galactosidase (red), of a late third-instar leg disc carrying an *mα-GFP* promoter-reporter transgene and the SOP-specific *lacZ* enhancer-trap marker A101 reveals that the expression of these markers is non-overlapping (L).

widely distributed among the 3′ UTRs of genes in both the Brd-C and the E(spl)-C (Lai et al., 2000; Lai et al., 1998). By contrast, the 3′ UTR of *m1* includes none of the regulatory sequence motifs we have found broadly represented in these genes; this includes the Brd box (Lai and Posakony, 1997; Leviten et al., 1997), K box (Lai et al., 1998), and GY box (Lai et al., 2000; Lai and Posakony, 1998; Leviten et al., 1997) elements.

Dependence of E(spl)-C Brd family gene expression on proneural and Su(H) activity

The normal patterns of *m2* and *m6* transcript accumulation, taken together with the presence of proneural and Su(H) binding sites in upstream DNA fragments sufficient to recapitulate these patterns, suggest that either or both genes may be subject to transcriptional regulation by these activators. To investigate this question further, we have examined the expression of the E(spl)-C Brd family genes (*mα*, *m2*, *m4* and *m6*) under a variety of mutant conditions, including the absence of *ac* and *sc* proneural gene function, the absence of *Su(H)* function, and finally constitutive N receptor activity (Figs 6, 7).

We have observed previously that the expression of both *Brd* and *E(spl)m4* in external sensory organ proneural clusters of third-instar imaginal discs is abolished in the absence of *ac-sc* function (see Fig. 6K,L; Singson et al., 1994). Here we have likewise found that proneural cluster expression of both *mα* and *m2* in wing discs is strongly diminished or eliminated under these conditions, although both genes maintain essentially normal levels of transcript accumulation along the dorsoventral boundary and in the vicinity of the developing wing veins (Fig. 6A,B and F,G). Similar results were obtained using promoter-*lacZ* reporter transgenes (Fig. 7A,B and D,E), indicating that proneural gene activity is essential for normal transcriptional activation of *mα* and *m2* in imaginal disc proneural clusters. In contrast, the main territory of *m6* expression in the wing disc (in the mesodermal adepithelial cells) was not strongly modified in either an *ac-sc* (Fig. 6O,P) or *atonal* (not shown) mutant background, although the low-level expression in the vicinity of the dorsocentral macrochaete proneural cluster (see Fig. 4A) appears to be reproducibly lost in the former condition (Fig. 6O,P). Thus, most transcript accumulation from *m6* in the wing disc is not strongly dependent upon proneural gene function, consistent with our finding that most of its expression appears not to be located within the disc epithelium.

We find that proneural cluster expression of *mα* behaves similarly to that of both *m4* (Bailey and Posakony, 1995) and *mγ* (Nellesen et al., 1999) and is maintained in *Su(H)*$^{-}$ imaginal tissue, both by in situ hybridization analysis of endogenous *mα* transcript accumulation (Fig. 6C,M) and by analysis of *mα* reporter transgene expression (Fig. 7C). At the same time, however, wing vein-associated and dorsoventral boundary expression of *mα* is largely lost in *Su(H)*$^{-}$ wing discs, indicating that *Su(H)* gene function is essential for activation of *mα* in these locations (Figs 6C, 7C). Thus, different aspects of *mα* expression, namely in proneural clusters and wing margin/vein territories, can effectively be uncoupled by loss-of-function mutations in the genes encoding either of its likely transcriptional activators.

m2 activity in the third-instar wing disc displays a very strong dependence on *Su(H)* function, as its dorsoventral

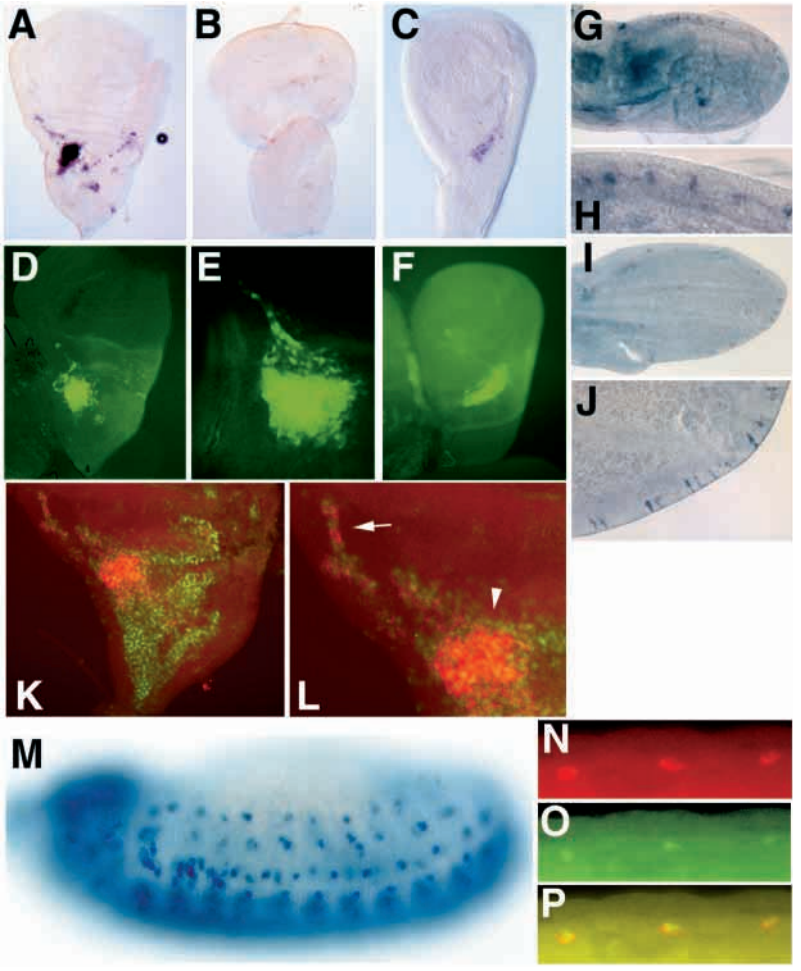


Fig. 4. Expression of *E(spl)m6* in imaginal tissues during larval/pupal development (A-L) and in mid-embryogenesis (M-P). *E(spl)m6* transcripts accumulate in a subset of adepithelial cells (see below) of the late third instar wing disc (A) and haltere disc (C), but not appreciably in the eye-antenna disc (B). Fluorescence activity of an *m6-GFP* promoter-reporter transgene recapitulates this adepithelial cell expression pattern in the wing (D, closeup in E) and haltere discs (F). (G-J) Accumulation of endogenous *m6* transcripts at the wing margin during early pupal development. *m6* is expressed in a small subset of cells at the anterior wing margin at 8 h APF (G, closeup in H; the large stained patch in the wing blade region of G is an artifact). At 24 h APF, expression of *m6* is detected in pairs of cells along the posterior wing margin (I, closeup in J). (K-P) Double labeling reveals co-accumulation of *m6* transcripts and Twist protein in imaginal discs and embryos. (K,L) In the wing imaginal disc, *m6* transcripts (red) are largely confined to a subset of the Twist-positive adepithelial cell population (green). Co-expression of the two markers is most clearly evident in linear groups of cells (arrow in L) connected to the main domain of *m6* expression (arrowhead in L). (M) *m6* transcripts accumulate in specific cells of the mesoderm of stage 13 to 15 embryos (stage 13 is shown), in a pattern resembling that of Twist accumulation (Bate et al., 1991). Double-label analysis of *m6* transcript (red, N) and Twist protein (green, O) accumulation in stage 15 embryos reveals cytoplasmic *m6* transcripts clearly surrounding Twist-positive nuclei (merged images in P), identifying these cells as adult muscle founders (Bate et al., 1991), which include the precursors to the adepithelial cells.

boundary and vein-associated expression appears to be lost in *Su(H)*$^{-}$ discs, while its already comparatively weak proneural cluster expression is greatly diminished and even eliminated at some sites (Fig. 6H, 7F). Again, we observe good agreement in the mutant background between the behavior of endogenous *m2* transcripts (Fig. 6H) and the activity of an *m2* promoter-reporter transgene (Fig. 7F), indicating that *Su(H)* function is required in trans for the proper transcriptional activation of the gene. The low-level residual expression of *m2* in *Su(H)*$^{-}$ proneural clusters indicates that it does not respond strongly to the elevated proneural activity in these territories. Its relatively weak expression in the clusters of wild-type discs may likewise indicate that its cis-regulatory apparatus is constructed in such a way as to make it less responsive to these activators, despite the presence of at least two high-affinity proneural protein binding sites (see Fig. 5).

The accumulation of *m6* transcripts in the third-instar wing disc was also found to be highly dependent on *Su(H)* gene activity, as it is largely abolished in the *Su(H)*$^{-}$ mutant background (Fig. 6Q). Staining of *Su(H)*$^{-}$ wing discs with anti-Twist antibody demonstrates that this effect is not due simply to a loss of the adepithelial cell population in the absence of zygotic *Su(H)* function (Fig. 6S). The comparatively low level of *m6* transcript near the dorsocentral macrochaete proneural cluster (see Fig. 4A) is apparently also eliminated in *Su(H)*$^{-}$ discs (Fig. 6Q).

All four E(spl)-C Brd family genes are activated in response to expression of an activated form of the N receptor

The dependence of various aspects of E(spl)-C Brd family gene expression on *Su(H)* function, coupled with our identification of multiple Su(H) binding sites in each of these genes' regulatory regions, strongly suggests that they are targets of transcriptional control by the N pathway. As a further test of this possibility, we assayed the responses of endogenous E(spl)-C Brd family genes to expression of N^{intra}, a constitutively activated form of the N receptor (Struhl et al., 1993), as we have done previously for *m4* (Bailey and Posakony, 1995). We find that all four genes are ectopically expressed in response to N^{intra}, although the spatial domain of the response is different for the different genes (Fig. 6D,I,N,R).

As previously, we found that proneural cluster expression of *m4* in the third-instar wing disc is strongly intensified, with a lower level of ectopic expression observed throughout much of the remainder of the disc (Fig. 6N). *mα* is also strongly ectopically expressed in all discs following induction of N^{intra}, possibly to a greater degree than for *m4* (Fig. 6D,E). Interestingly, some normal aspects of *mα* expression appear to be weakened following N^{intra} induction; this is most clearly illustrated by the apparent loss of the normally strong stripe of dorsoventral boundary transcript (Fig. 6A,D,E).

m2 is likewise broadly, though not as strongly, activated in

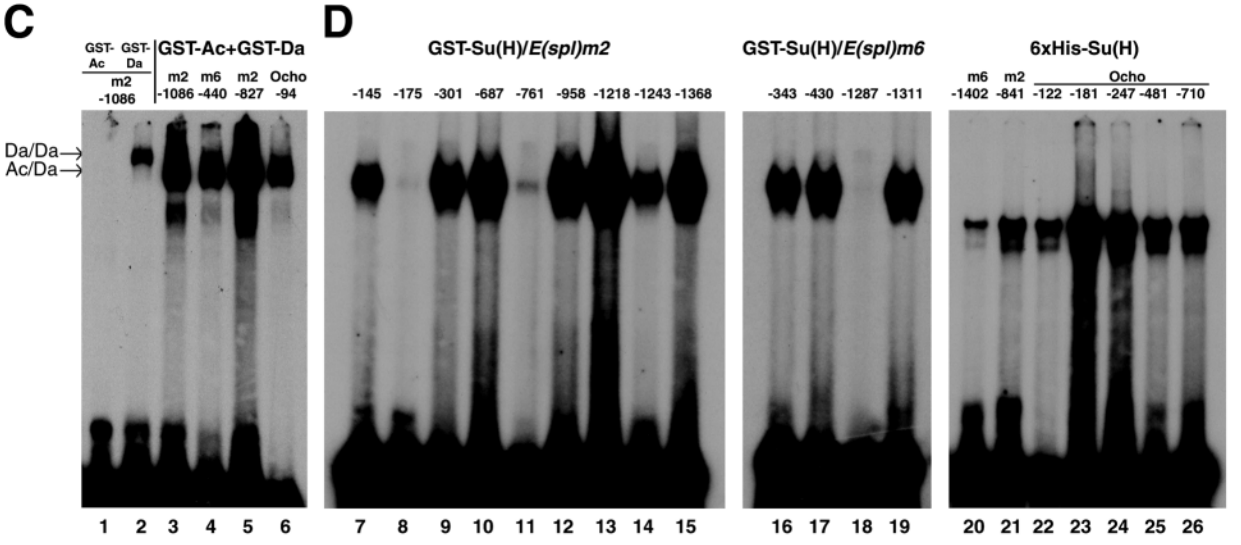
A

mα (-454')	ACGGG**GCAGGTGTT**CCTTG
m2 (-1086')	GCTGA**GCAGCTGTT**ATTGC
m4 (-161')	AAACG**GCAGGTGTG**TTTTT
m6 (-440')	GAGTG**GCAGCTGTT**TGCAA
Consensus	**GCAGSTGKK**
m2 (-827)	AGGGA**ACAGGTG**CTATT
Ocho (-94')	GGCAG**GCAGGTG**CAAGG

B

m2 (-1368)	TGCAG**TGTGGGAA**CCTGC
‡m2 (-1243')	AACGG**GATGGGAA**TCGGA
m2 (-1218)	CGGAG**CGTGTGAA**CCACA
m2 (-958')	TCGAG**TGTGGGAA**GGGGC
m2 (-841')	GCGAG**CGTGGGAC**TAAGA
§m2 (-761')	GATGCGGTGGGAGGAGGA
m2 (-687)	GGTAC**CGTGTGAA**TGTGA
m2 (-301)	ACGTG**CATGGGAA**AAGTA
§m2 (-175')	GCCGGGGTGAGAGGTGAG
‡m2 (-145)	CTGTG**TGTGAGAG**TGCAC
‡m6 (-1402)	CAATC**CGTGGGAT**GCTTA
m6 (-1311)	CACAT**CATGGGAA**AAACA
§m6 (-1287)	AAGTTAATGGGAGGAGAG
m6 (-430)	CGAAC**CGTGGGAT**ACTTT
m6 (-343)	TTATC**CATGGGAA**CCACA
Ocho (-710')	CAGCC**TGTGAGAA**TTTGT
Ocho (-481)	CTGAA**CGTGAGAA**TTCTC
Ocho (-247')	CTTGG**CGTGGGAA**CAGCA
Ocho (-181')	CGAAG**CGTGGGAA**TCTCG
Ocho (-122)	ACTAA**CGTGTGAA**ATTTC

Fig. 5. Binding sites for proneural proteins and for Su(H) in the proximal upstream regions of *E(spl)m2*, *E(spl)m6* and *Ocho*. (A) Predicted binding sites (bold) for proneural bHLH activators. (B) Candidate Su(H) binding sites (bold or underlined). ‡Sites bound only weakly or moderately by Su(H). §Possible sites (underlined) not bound significantly by Su(H). (C,D) Labeled oligonucleotide probes containing predicted proneural protein or Su(H) binding sites (A,B) were tested for binding in vitro by the corresponding purified proteins in the electrophoretic mobility shift assay (EMSA). (C) As observed previously with other well-characterized proneural protein binding sites (Van Doren et al., 1991), the m2 (–1086) site is not bound detectably by GST-Ac alone (lane 1), is bound weakly by GST-Da alone (lane 2; probably Da/Da homodimers), and is bound strongly by a combination of GST-Ac and GST-Da (lane 3; probably Ac/Da heterodimers: note the increased mobility of this complex relative to that observed with Da alone). The other three predicted binding sites (one each in *m2*, *m6* and *Ocho*) are also bound efficiently by Ac/Da protein complexes. (D) For Su(H), either purified GST-Su(H) or purified 6xHis-Su(H) was used in the EMSA. Of 10 possible Su(H) binding sites in *m2* tested, eight are bound significantly in vitro (lanes 7-15, 21; see summary in B); of five possible sites in *m6*, four are bound detectably (lanes 16-20); all five predicted sites in *Ocho* are bound efficiently (lanes 22-26).



the wing disc in response to expression of N^{intra} (Fig. 6I,J). As with *mα*, we observe under these conditions a suppression of *m2*'s stripe of transcript accumulation along the dorsoventral boundary, normally one of the stronger components of its expression (see Fig. 6F,G,J).

We find that transcript accumulation from the endogenous *m6* gene responds powerfully to expression of N^{intra}. Strong induction is observed throughout the adepithelial cell population (Fig. 6R), such that the normally restricted pattern of *m6* expression in the wing disc (Fig. 6O) now clearly resembles that of a variety of general adepithelial cell markers, including Twist (see Fig. 4K; Bate et al., 1991), Cut (Blochlinger et al., 1993), and D-Mef2 (Cripps et al., 1998). *m6* is also ectopically expressed in a regular pattern of cells in the developing retina, posterior to the morphogenetic furrow (data not shown). Our results for imaginal tissue contrast with those of Wurmbach et al. (1999), who failed to observe a response of *m6* expression, in the embryo, to activated N.

The capacity of all four Brd family genes in the E(spl)-C to respond to an activated form of the N receptor is fully consistent with the dependence of their normal expression patterns on *Su(H)* function, and with the presence of multiple binding sites for Su(H) in their upstream cis-regulatory DNA. Collectively, these data indicate that all four genes are normally regulated in imaginal tissue by N signaling activity, very likely as direct targets of Su(H).

Overexpression of *m2* and *m6* modulates N pathway activity

Since mis-expression or overexpression of five other members of the Brd gene family (*Brd*, *Bob*, *m4*, *mα* and *Tom*) interferes with N signaling-dependent events in imaginal development

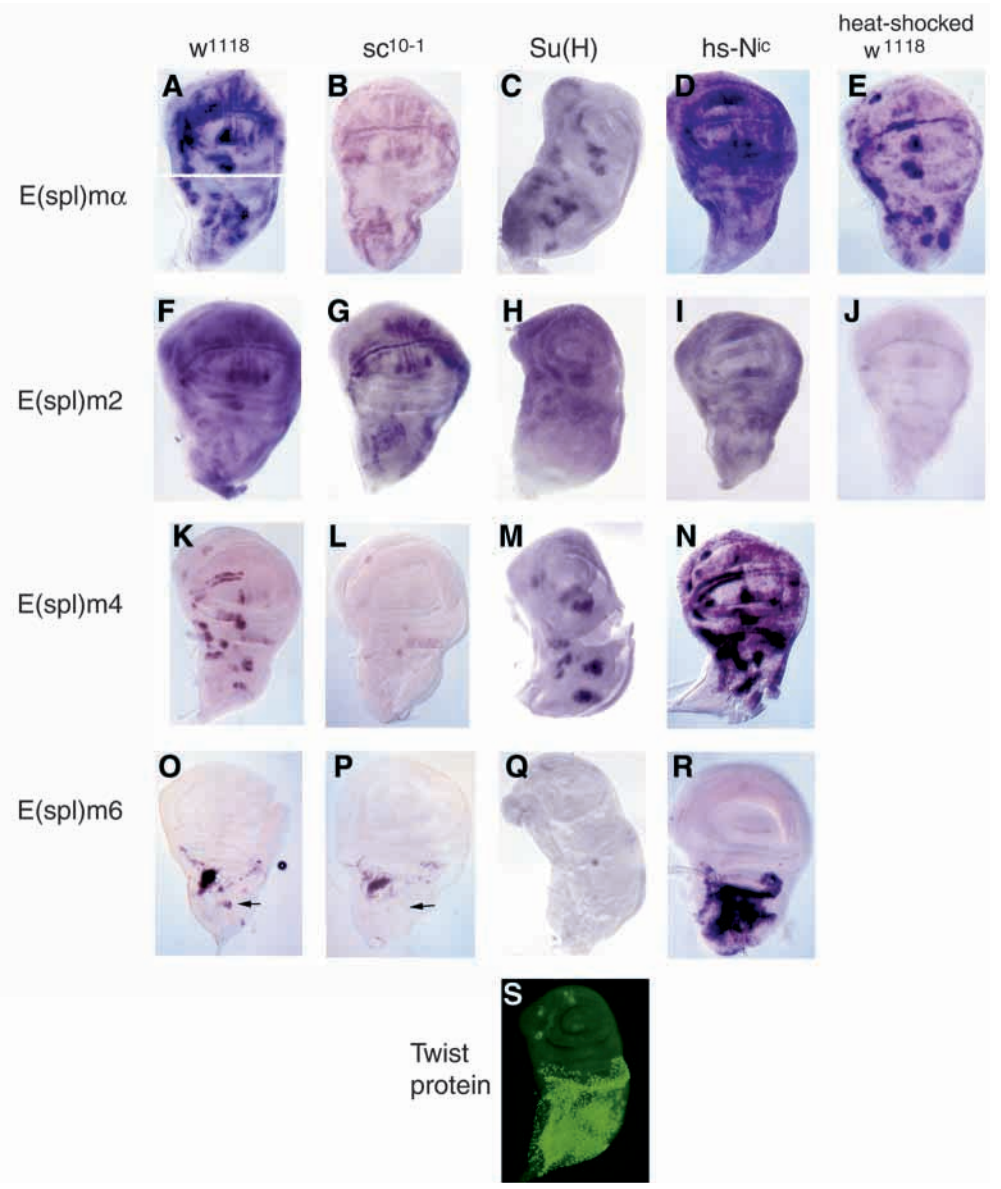


Fig. 6. E(spl)-C Brd family gene expression in late third instar wing imaginal discs depends on proneural gene and *Su(H)* function and N pathway activity. Expression patterns of *E(spl)mα* (A), *m2* (F), *m4* (K) and *m6* (O) in a wild-type (w^{1118}) background are shown for comparison. In the absence of *ac/sc* function (sc^{10-1}/Y; B,G,L,P), proneural cluster expression of *mα*, *m2* and *m4* (see Singson et al., 1994) is lost, while wing margin and wing vein-associated expression of *mα* and *m2* is largely unaffected. Expression of *m6* is largely normal in this background (P), although transcript accumulation in the vicinity of the dorsocentral macrochaete proneural clusters appears to be lost (arrows in O,P). In the absence of *Su(H)* function (C,H,M,Q,S), proneural cluster expression of *mα* and *m4* (see Bailey and Posakony, 1995) is maintained or enhanced (C,M), while cluster expression of *m2* is diminished (H). Dorsoventral boundary and wing vein-associated expression of both *mα* and *m2* are absent in the $Su(H)^{-}$ background (C,H). Expression of *m6* in the wing disc is strictly dependent on *Su(H)* activity (Q); this is not due simply to a failure of adepithelial cells to form in the mutant background, as shown by staining with anti-Twist antibody (S; compare with Fig. 4K). The reduction in the size of these mutant wing discs is due to the absence of *Su(H)* function (Schweisguth and Posakony, 1992). Upon ectopic activation of the N pathway using $hs\text{-}N^{intra}$ (D,I,N,R), expression of *mα*, *m2* and *m4* (see Bailey and Posakony, 1995) is strongly activated ectopically in the wing disc [D,I,N; compare with parallel staining of discs from heat-shocked wild-type (w^{1118}) larvae (E,J)]. *m6* is strongly expressed ectopically throughout the adepithelial cell population associated with the wing disc in response to $hs\text{-}N^{intra}$ (R; compare with expression of Twist in Fig. 4K).

(Apidianakis et al., 1999; Lai et al., 2000; Leviten et al., 1997; Leviten and Posakony, 1996), we were interested to determine if *m2* and *m6* have similar activities, especially in light of their divergent primary structures (see Fig. 2A). We made use of the GAL4-UAS system (Brand and Perrimon, 1993; Phelps and Brand, 1998) for targeted expression of these genes, employing a variety of GAL4 drivers including *sca-Gal4* (active in proneural clusters and in sensory organ lineages), *GMR-Gal4* (active in all cells posterior to the morphogenetic furrow of the developing retina), and MS 1096 (active throughout the wing pouch as well as in the lineage of some sensory organs).

We find that misexpression of *m6* produces a similar phenotype with respect to adult PNS development as misexpression of the five previously studied Brd family members, in that a mild degree of macrochaete bristle tufting is observed in flies carrying one copy each of *UAS-m6* and *sca-Gal4* (9 of 10 lines; data not shown). The relative potency of *m6* in this assay, as indicated by the number of bristle positions affected and the number of bristles in representative tufts, is less than that of *Brd*, previously the weakest Brd family gene by these criteria. However, increasing *m6* dosage (by including two copies of the *UAS-m6* transgene and raising the flies at 29°C) induces much stronger tufting phenotypes, including multiplication of both microchaetes (Fig. 8A,C) and macrochaetes (Fig. 8D). These phenotypes are correlated with increased numbers of A101-positive cells at normal proneural cluster positions, indicating that they are due to specification of supernumerary SOPs within the clusters (Fig. 8M,O). Similar effects on peripheral neurogenesis are produced when *m6* is misexpressed in the wing under control of the MS 1096 driver (Fig. 8G-L). The anterior wing margin displays an increased density of chemosensory organs (Fig. 8H,I), and

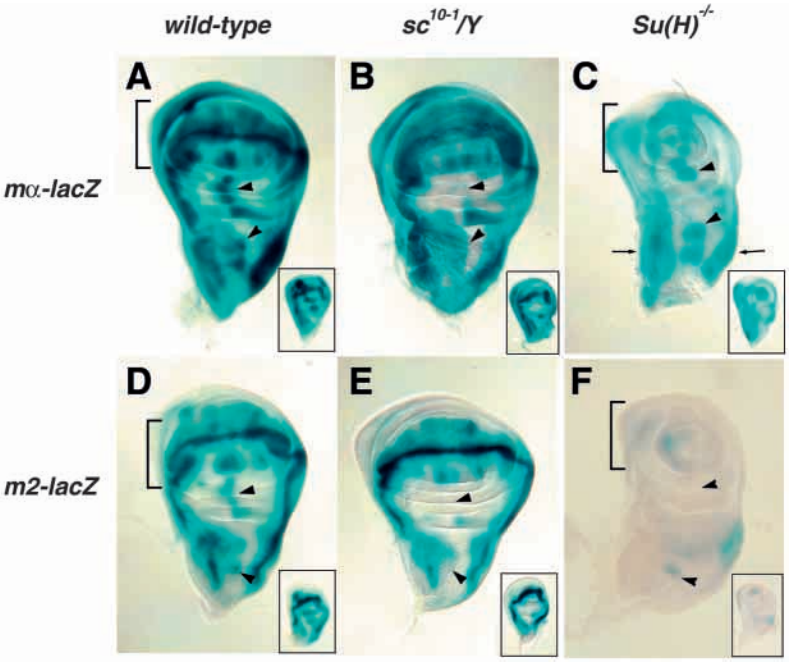


Fig. 7. Modulation of *mα-lacZ* (A-C) and *m2-lacZ* (D-F) reporter gene activity in the absence of proneural and *Su(H)* gene function. Shown are late third instar wing discs (main panels) and haltere discs (insets) stained to reveal β-galactosidase activity in wild-type (A,D), sc^{10-1}/Y (B,E) and $Su(H)^{-}$ (C,F) tissue. Promoter-*lacZ* constructs faithfully reproduce all aspects of *mα* (A) and *m2* (D) expression in these discs, including expression in proneural clusters, along the dorsoventral boundary, and in the vicinity of developing wing veins; as with the endogenous genes (see Fig. 6A,F), expression of *m2-lacZ* in proneural clusters is weaker than that of *mα-lacZ*. Specific loss of proneural cluster expression in the sc^{10-1} background is clearly observed for both *mα-lacZ* (B, arrowheads; compare with A) and *m2-lacZ* (E, arrowheads; compare with D) in both wing and haltere discs; other aspects of these expression patterns are essentially unchanged. Expression of *mα-lacZ* in proneural clusters is clearly maintained or elevated in $Su(H)^{-}$ tissue (C, arrowheads), while proneural cluster expression of *m2-lacZ* is nearly absent in this background (F, arrowheads). *mα-lacZ* also remains strongly active along both anterior and posterior lateral regions of the prospective notum in this mutant tissue (C, arrows). The activity of both reporters along the dorsoventral boundaries of the wing and haltere discs and along presumptive wing veins is abolished in the $Su(H)^{-}$ background (C,F, brackets; compare with A,D, respectively).

wing vein L3 bears increased numbers of campaniform sensilla (Fig. 8J-L). In addition, we often observe ‘double shaft’ structures on the anterior wing margin (representing the transformation of the socket cell to a second shaft cell; Bang and Posakony, 1992), indicating that misexpression of *m6* can also interfere with at least one cell fate decision in the bristle lineage. All of these effects on adult PNS development are consistent with antagonism of N signaling activity by *m6* misexpression (Posakony, 1994). Nevertheless, as is the case for the other Brd family members tested previously (with the possible exception of *mα*), misexpression of *m6* had no detectable effect on the overall integrity of the wing margin or on wing vein differentiation (see Lai et al., 2000), both of which are also dependent on the activity of the N pathway.

Strikingly, we find that overexpression of *m2* using *sca-Gal4* causes an oppositely directed effect on PNS development. With a single copy of the *UAS-m2* effector transgene, we observe loss of several notum macrochaetes; the most sensitive positions include the anterior and posterior dorsocentrals (data not shown). When higher levels of *m2* expression are induced as described above, we observe loss of a majority of notum and head macrochaetes (Fig. 8A,B), as well as a small degree of microchaete loss (not shown), a phenotype possibly indicative of N pathway hyperactivity (Bang et al., 1995; Schweisguth and Posakony, 1994). We confirmed this interpretation using the A101 enhancer-trap marker, which reveals that the SOPs of many notum macrochaetes fail to be specified under these conditions (Fig. 8M,N). Consistent with this effect on sensory organ development, overexpression of *m2* in the wing using the MS 1096 driver results in the analogous phenotype of wing vein truncation (Fig. 8E,F). This defect resembles the dominant phenotypes of *Hairless* or certain *Abruptex* mutants, which are also known to represent hyperactivity of the N pathway. Thus, *m2* is unique among Brd family members in the nature of its phenotypic effects in this overexpression assay, as it appears to potentiate the activity of, or the response to, the N pathway. In addition, *m2* has an apparent functional specificity not shared by other members of this family, as evidenced by its ability to affect wing vein fates.

Finally, in an effort to investigate a possible role for *E(spl)m1* in N signaling, we examined the phenotypic consequences of *m1* misexpression in a variety of developing imaginal tissues. We generated 10 independent fly lines bearing a *UAS-m1* transgene and found that these produce no detectable mutant phenotype when combined with *sca-Gal4*, *GMR-Gal4*, or MS 1096, even when the transgene is present in two copies (Fig. 8A and data not shown). As *m1* is not expressed in territories of N pathway activity (this paper; Wurmbach et al., 1999), evidently does not share either transcriptional (Nellesen et al., 1999) or post-transcriptional (this paper; Wurmbach et al., 1999) regulation with other members of the E(spl)-C (including both bHLH genes and Brd family genes), and does not yield a mutant phenotype in our misexpression assay, we conclude that it likely has no functional relationship to the N pathway.

A new member of the Brd gene family located in the Brd-C

BLAST searches (Altschul et al., 1997) of genomic DNA sequence data released by Celera Genomics (GenBank accession numbers AC014109 and AE003532) revealed the existence of a third new member of the Brd gene family, which we have named *Ocho* (eighth distinct family member identified; Lai et al., 2000), in a position only about 2 kb downstream of *Brd* (Fig. 1B). *Ocho* encodes a clearly recognizable member of the m4 subfamily of proteins (Lai et al., 2000), most closely related to mα, Tom, and m4 (Fig. 2). Like all other Brd family proteins, the predicted Ocho product

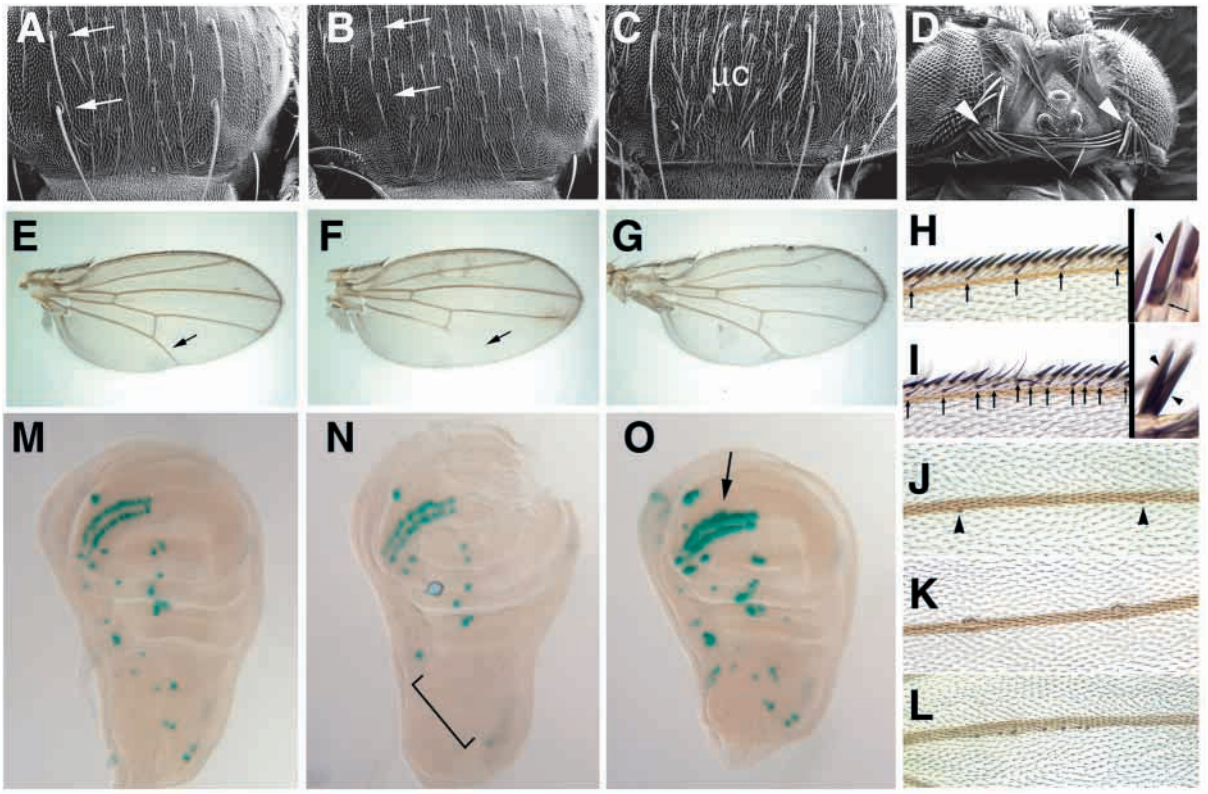


Fig. 8. Overexpression of *m2* and *m6* have opposite effects on cell fate decisions controlled by the N pathway. (A) Dorsal thorax of *sca-Gal4::2xUAS-m1* adult is indistinguishable from wild-type. Arrows point to dorsocentral macrochaetae. (B) Thorax of *sca-Gal4::2xUAS-m2* fly is missing many macrochaetae (arrows). (C) Thorax of *sca-Gal4::2xUAS-m6* fly displays many tufted sensory organs in the microchaete field (μc). (D) Head of *sca-Gal4::2xUAS-m6* fly exhibits many tufted macrochaetes (arrowheads). (E) Wing of *MS 1096/+* adult. Arrow points to the distal part of the L5 wing vein. (F) *MS 1096::2xUAS-m2* wing displays a truncated L5 vein (arrow). (G) *MS 1096::2xUAS-m6* wing has an irregular wing margin, but venation is relatively normal (except for occasional minor defects also observed in the *MS 1096/+* background). (H-L) High magnification views of the anterior wing margin (H,I) and L3 wing vein (J-L) of *sca-Gal4* (H,J) and *sca-Gal4::2xUAS-m6* flies (I-L). The latter genotype displays an increased density of chemosensory organs (arrows, I; compared with H) as well as many double-shaft mechanosensory organs. (H, right) Arrowhead points to the shaft of a stout mechanosensory bristle, arrow points to its socket; (I, right) two arrowheads point to double-shaft structure. In addition, overexpression of *m6* causes both 'tufting' of campaniform sensilla (K; compare with J arrowheads) as well as an increased density of these sensilla (L) on vein L3. (M-O) Effect of *E(spl)m2* and *m6* overexpression on activity of the A101 enhancer-trap marker in late third instar wing imaginal discs. (M) *A101/+* disc shows the normal pattern of SOPs. (N) *sca-Gal4::2xUAS-m2; A101/+* disc is missing many SOPs in the presumptive notum (bracket) and elsewhere. (O) *sca-Gal4::2xUAS-m6; A101/+* disc displays supernumerary SOPs, particularly evident along the anterior wing margin (arrow).

includes a basic amphipathic α-helical region near its N terminus that shares with the corresponding domains of Tom (Lai et al., 2000) and E(spl)m2 (this paper) the presence of a proline residue (Fig. 2A,B). Ocho also includes all three of the other conserved sequence domains found in Tom, mα and m4 (Fig. 2).

Uniquely among the known members of the Brd-C, *Ocho* has a strong concentration of predicted Su(H) binding sites in its proximal upstream region (Fig. 5B), a feature more typical of Brd family members in the E(spl)-C (this paper; Bailey and Posakony, 1995; Lai et al., 2000; Nellesen et al., 1999). Within the first 720 bp 5′ to the presumed *Ocho* transcription start site, there are five sequences fitting the high-affinity Su(H) site consensus YGTGDGAA (Bailey and Posakony, 1995; Lai et al., 2000; Nellesen et al., 1999; Tun et al., 1994). In addition, a predicted high-affinity binding site (GCAGGTG) for proneural bHLH activators is found quite close to the start site at position –94 (Fig. 5A). We verified by EMSA that all five predicted Su(H) binding sites upstream of *Ocho*, as well as the single predicted proneural protein binding site, are indeed bound in vitro by the respective purified fusion proteins (Fig. 5C,D). These results suggest that *Ocho* is a direct target of regulation both by proneural bHLH activators and by Su(H) and the N pathway.

By contrast to all other known Brd family genes, *Ocho* does not appear to include in its 3′ UTR any of the known or putative post-transcriptional regulatory sequence elements (Brd box, K box, or GY box) we have previously identified (Lai et al., 2000; Lai et al., 1998; Lai and Posakony, 1997, 1998; Leviten et al., 1997).

We next investigated by in situ hybridization the spatial pattern of *Ocho* transcript accumulation in imaginal discs of late third instar larvae (Fig. 9). Consistent with its possible regulation by proneural proteins and by N signaling, *Ocho* is expressed in external sensory organ and chordotonal organ proneural clusters in the wing (Fig. 9A), eye-antenna (Fig. 9B),

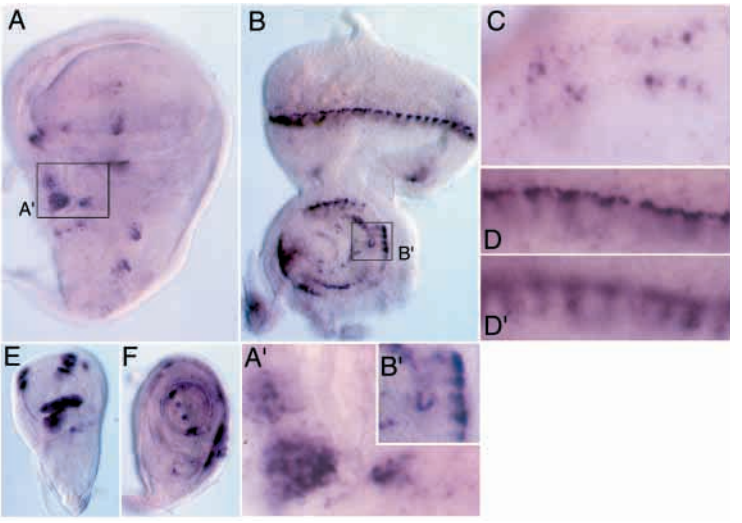


Fig. 9. Spatial pattern of *Ocho* transcript accumulation in imaginal discs of late third instar larvae. *Ocho* is expressed specifically in external sensory organ and chordotonal organ proneural clusters in the wing (A), eye-antenna (B), haltere (E), and leg discs (F), and in the vicinity of the morphogenetic furrow of the developing retina (B). Higher magnification views of the region shown boxed in A (A′), the anterior wing margin (C), and the furrow region of the retina (D) reveal an unusually punctate appearance of the in situ hybridization signal. This appears to be due to the predominant localization of *Ocho* transcripts to the constricted apical ends of the disc epithelial cells, which are columnar in overall morphology. *Ocho* transcripts accumulate in a single column of cells near the morphogenetic furrow (B); closeup views of this region at two different focal planes (D,D′) show that the great majority of the signal is at the apical tips of the retinal cells (D), with a lesser quantity of signal in the more basal perinuclear region (D′). Confocal microscopy of fluorescent in situ hybridization preparations (not shown) confirmed these observations. Similarly, *Ocho* transcripts accumulate in very thin stripes in the anlage of Johnston's organ in the antenna disc (B). When the antenna disc is mounted, the columnar epithelium flattens out, such that the apical ends of the cells are on the outside of each concentric ring of cells. A higher magnification view of the region boxed in B (B′) shows a very heavy concentration of hybridization signal at the apical tips of the cells (to the right), with little or none more basally (to the left).

haltere (Fig. 9E) and leg (Fig. 9F) discs. *Ocho* transcripts also appear in a very thin band in the vicinity of the morphogenetic furrow of the developing retina (Fig. 9B), evidently corresponding to a single column of cells. Strikingly, at most sites of its accumulation in imaginal disc epithelia, the majority of the *Ocho* transcript is apparently localized in very small apical 'dots' (Fig. 9A′,B′,C,D), with markedly less signal in more basal positions (Fig. 9B′,D′). We have not observed this same predominantly apical concentration of transcripts from other Brd family genes, and its significance and control in the case of *Ocho* are under investigation.

DISCUSSION

Members of two substantial gene families located in the E(spl)-C are regulated by the N pathway

We have described in this report our detailed characterization of *E(spl)m2* and *E(spl)m6*, two newly recognized members of the Brd gene family resident within the E(spl)-C. Along with the previously identified *E(spl)m4* (one of the two founding members of the family; Klämbt et al., 1989; Leviten et al., 1997) and *E(spl)mα* (Lai et al., 2000; Wurmbach et al., 1999), they bring the total number of Brd family members in this complex to four. Our extensive DNA sequence analysis of the previously uncharacterized regions of the E(spl)-C (this paper; Lai et al., 2000; Nellesen et al., 1999) indicated that it is unlikely that additional family members remain to be identified in the complex. The recent release of the complete sequence of the E(spl)-C by Celera Genomics and BDGP (GenBank accession numbers AC019694 and AE003754) also supports this conclusion.

Beginning with the demonstration that *m4* is a direct target of transcriptional activation by Su(H) in response to N receptor activity (Bailey and Posakony, 1995), we have presented multiple lines of evidence indicating that all four E(spl)-C Brd family genes are integral members of the N pathway (this paper; Lai et al., 2000; Nellesen et al., 1999). (1) All four genes contain multiple high-affinity binding sites for Su(H) in their proximal cis-regulatory regions. In the case of *m4*, it is known that the integrity of these sites is essential for its normal transcriptional activation and its responsiveness to activated N in vivo (Bailey and Posakony, 1995). (2) E(spl)-C Brd family genes are specifically expressed in a variety of territories that are sites of N-mediated signaling activity, including the proneural clusters of imaginal discs (*m4*, *mα*, *m2*; possibly *m6*), in the vicinity of the morphogenetic furrow of the eye (*m4*, *mα*, *m2*), along the dorsoventral boundary of the wing and haltere discs (*m2* and *mα*), at the boundaries of developing wing veins (*m2* and *mα*), at the boundaries of leg segment primordia (*m2* and *mα*), in adult muscle precursors in the embryo (*m6*), and in a subset of imaginal adepithelial cells (*m6*). (3) Expression of all E(spl)-C Brd family genes is modified by loss of *Su(H)* function as well as by gain of N receptor activity; the genes' responses under these conditions is wholly consistent with a normal role for the N pathway and Su(H) in activation of E(spl)-C Brd family gene expression. (4) Over- or mis-expression of each E(spl)-C Brd family gene has specific phenotypic effects on cell fate decisions controlled by the N pathway. Taken together, these data strongly corroborate the view that activation of E(spl)-C Brd family gene function is a major cellular response to N signaling activity in many different developmental settings where the N pathway is deployed.

This picture of the structure, expression, and regulation of the non-bHLH genes of the E(spl)-C differs substantially from that described earlier by Wurmbach et al. (1999). Several of the principal conclusions of that work – that the E(spl)-C harbors three classes of N-responsive genes; that *E(spl)m2* and *E(spl)m6* encode novel proteins unrelated to each other and to other products of the E(spl)-C; that the principal sites of *E(spl)m6* expression include the notum anlage of the wing disc; and that *E(spl)m6* is not regulated by N – are, we believe, inconsistent with the data we have presented here.

Comparative roles of bHLH repressor and Brd family genes in E(spl)-C function

The E(spl)-C is a genetically dense and complex locus that is

essential in many settings for the function of the N pathway. Historically, the best-studied members of this complex have been the seven bHLH repressor genes and *gro*, which encodes a co-repressor protein for the E(spl)-C bHLH repressors as well as for other repressors encoded elsewhere in the genome. As *gro* is the only gene within the E(spl)-C that is individually associated with a loss-of-function phenotype, the only avenue for revealing E(spl)-C bHLH gene function has been through the use of chromosomal deficiencies (de Celis et al., 1996; Delidakis et al., 1991; Knust et al., 1987a; Preiss et al., 1988; Schrons et al., 1992). Deficiencies that remove multiple bHLH genes display 'neurogenic' defects in ectodermal tissue, which can be partially rescued by supplying E(spl)-C bHLH gene function via transgenes (Delidakis et al., 1991; Schrons et al., 1992). Such studies have concluded that E(spl)-C bHLH genes are collectively required in a positive direction for a subset of N pathway functions, and that individual bHLH genes have highly overlapping or 'redundant' activities.

However, all E(spl)-C deficiencies that confer a mutant phenotype also delete one or more non-bHLH genes, including Brd family members. In spite of this, the observed mutant phenotypes have been almost entirely attributed to loss of bHLH gene function. This has remained the case even though one of the first genes shown to be a direct target of Su(H) in response to N receptor activity was in fact *E(spl)m4*, a non-bHLH gene and a member of the Brd family (Bailey and Posakony, 1995). We suggest that the results of experiments in which Brd family transgenes are 'added back' to various E(spl)-C deficiency genotypes may change our current picture of how E(spl)-C function is partitioned among its bHLH and non-bHLH members.

Expression of *E(spl)m6* in adult muscle lineages

Our finding that *E(spl)m6* is expressed in adult muscle founder cells in the embryo, and later in a subset of imaginal adepithelial cells, suggests for the first time a role for Brd family genes in N signaling in the mesoderm. Remarkably, the process by which different muscle founder cells are specified during embryogenesis closely parallels that which gives rise to specific cell types in external sensory organ lineages (see for review Posakony, 1994; Roy and VijayRaghavan, 1999). Within *twist*-expressing mesodermal domains, single muscle progenitor cells are selected by a N-dependent 'lateral inhibition' mechanism from 'pro-muscle' clusters of cells expressing *lethal of scute* (Carmena et al., 1995; Corbin et al., 1991). The muscle progenitor cell then divides asymmetrically in a *N*- and *numb*-dependent fashion (Ruiz Gómez and Bate, 1997), yielding either the founders of two different larval muscles, or one larval muscle founder and one adult muscle founder. In the latter case, the adult muscle founder cell is the N responder, while its larval muscle founder sister is inhibited from responding due to its inheritance of Numb protein. We observe expression of *m6* specifically in these N-responsive adult muscle founder progeny, consistent with our hypothesis that the gene is a direct target of activation by Su(H). Unlike their larval muscle founder sisters, which immediately commence fusion with surrounding cells to form myotubes, adult muscle founder cells delay differentiation and remain in a proliferative state during larval life. Like the persistence of Twist expression (Bate et al., 1991), *m6* expression is a specific property of this specialized muscle founder cell population.

It is striking that *m6* transcripts accumulate in only a regional subset of adepithelial cells associated with the third instar larval wing disc. No other gene identified to date exhibits such restricted expression within the adepithelial cell population. Since wing disc adepithelial cells contribute to a diverse set of adult thoracic muscles (Roy and VijayRaghavan, 1999), we are currently investigating the possibility that the *m6*-expressing subset has some particular significance with respect to the development or patterning of these muscles.

Diversification of Brd family gene structure and expression

To date we have identified a total of ten members of the Brd gene family, four in the E(spl)-C (*mα*, *m2*, *m4* and *m6*) and six in the recently discovered Brd-C (*Bob A*, *Bob B*, *Bob C*, *Tom*, *Brd* and *Ocho*) (this paper; Lai et al., 2000; Leviten et al., 1997; Nellesen et al., 1999). While there are good reasons to believe that these genes have significantly overlapping functions (Lai et al., 2000; Leviten and Posakony, 1996), the evolutionary creation and maintenance of so large a paralogous gene set indicates that there must be functional specialization of Brd family members in vivo. What is already clear is that this family has diversified considerably in both structure and expression.

It seems reasonable to postulate that an ancestral Brd family gene encoded a protein resembling the present-day E(spl)mα, E(spl)m4, Tom and Ocho products, with their four shared domains (see Fig. 2A). Though now significantly diverged in overall amino acid sequence, these paralogous proteins are very similar in size (138-158 aa) and have very similar domain organization. This proposal is supported by the existence of such an archetypal Brd family member (158 aa) in the silk moth *Bombyx mori* (Fig. 2). The Lepidoptera and Diptera diverged approximately 200 million years ago, indicating that the Brd gene family is at least this old.

The Brd and Bob proteins can be viewed as truncations of this archetypal Brd family protein (Lai et al., 2000), suggesting that a common ancestor of the *Brd* and *Bob* genes might have arisen by acquiring a premature termination codon. The *E(spl)m2* and *E(spl)m6* genes may have derived independently from an archetypal progenitor or progenitors; their predicted protein products can be seen to represent the loss of one [E(spl)m6] or two [E(spl)m2] of the four canonical domains, along with expansions or contractions in the length of non-conserved regions between the remaining domains. It is likely that these evolutionary changes in the domain composition of the Brd, Bob, m2 and m6 proteins contribute to functional diversity in this family.

The only structural element common to all eight Brd family proteins is the N-terminal basic amphipathic domain. These domains are themselves quite diversified and are classifiable into three groups: 'very strongly' amphipathic (Brd and Bob), 'less strongly' amphipathic (mα, m4 and m6), and proline-containing (m2, Tom and Ocho). Our observation that all Brd family proteins tested, with the exception of m2, induce qualitatively (but not quantitatively) similar phenotypes in GAL4-UAS misexpression experiments (i.e., interference with N pathway activity) suggests that they may interact with a common target, though the quality of the interaction may be influenced by the type of basic amphipathic domain present.

The diversity of expression patterns among Brd family genes

is no less striking. In both embryonic and imaginal tissue, these genes are deployed in a myriad of locations in which N signaling is used to elicit cellular responses and/or determine cell fates, and we have presented evidence that all Brd family genes are direct targets of transcriptional regulation by Su(H) (this paper; Bailey and Posakony, 1995; Lai et al., 2000). Nevertheless, the precise expression pattern of each Brd family member is unique, such that different combinations of Brd family genes are active at different sites of N pathway activity. Thus, the members of this family are differentially responsive to regulation by N receptor activity. Our observation that promoter-reporter constructs for all Brd family genes tested to date recapitulate the expression pattern of the corresponding endogenous gene (this paper; Bailey and Posakony, 1995; Singson et al., 1994) demonstrates that the selectivity of this response is mediated largely at the transcriptional level (Nellesen et al., 1999). Thus, we suggest that evolution of transcriptional cis-regulatory sequences has been a major mechanism for diversification of Brd family gene expression and probably function as well.

We are very grateful for the expert technical contributions of Tammie Stone and Gina Freschi, who performed the EMSAs. Scott Barolo and Lucy Carver generously provided the Pelican and Stinger reporter vectors. We thank April Fischer for assisting with the molecular characterization of *E(spl)m2*; BDGP and Celera Genomics for making *Drosophila* cDNA and genomic DNA sequences publicly available; Ethan Bier and the Developmental Studies Hybridoma Bank at the University of Iowa for antibodies; Brian Biehs for helpful advice on in situ hybridization and antibody staining; Bill McGinnis and Larry Goldstein for their generosity with microscope facilities; and Lucy Carver, Par Towb, and Rick Shoop for generous instruction on confocal microscopy. Scott Barolo provided valuable critical comments on the manuscript. This work was supported by NIH grant GM46993 to J.W.P.

REFERENCES

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389-3402.

Apidianakis, Y., Nagel, A. C., Chalkiadaki, A., Preiss, A. and Delidakis, C. (1999). Overexpression of the *m4* and *mα* genes of the *E(spl)-Complex* antagonizes Notch mediated lateral inhibition. *Mech. Dev.* **86**, 39-50.

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch signaling: Cell fate control and signal integration in development. *Science* **284**, 770-776.

Bailey, A. M. and Posakony, J. W. (1995). Suppressor of Hairless directly activates transcription of *Enhancer of split* Complex genes in response to Notch receptor activity. *Genes Dev.* **9**, 2609-2622.

Bang, A. G., Bailey, A. M. and Posakony, J. W. (1995). *Hairless* promotes stable commitment to the sensory organ precursor cell fate by negatively regulating the activity of the *Notch* signaling pathway. *Dev. Biol.* **172**, 479-494.

Bang, A. G., Hartenstein, V. and Posakony, J. W. (1991). *Hairless* is required for the development of adult sensory organ precursor cells in *Drosophila*. *Development* **111**, 89-104.

Bang, A. G. and Posakony, J. W. (1992). The *Drosophila* gene *Hairless* encodes a novel basic protein that controls alternative cell fates in adult sensory organ development. *Genes Dev.* **6**, 1752-1769.

Bate, M., Rushton, E. and Currie, D. A. (1991). Cells with persistent *twist* expression are the embryonic precursors of adult muscles in *Drosophila*. *Development* **113**, 79-89.

Bellen, H. J., O'Kane, C. J., Wilson, C., Grossniklaus, U., Pearson, R. K. and Gehring, W. J. (1989). P-element-mediated enhancer detection: a versatile method to study development in *Drosophila*. *Genes Dev.* **3**, 1288-1300.

Bishop, S. A., Klein, T., Arias, A. M. and Couso, J. P. (1999). Composite signalling from *Serrate* and *Delta* establishes leg segments in *Drosophila* through *Notch*. *Development* **126**, 2993-3003.

Blochlinger, K., Jan, L. Y. and Jan, Y. N. (1993). Postembryonic patterns of expression of *cut*, a locus regulating sensory organ identity in *Drosophila*. *Development* **117**, 441-450.

Boulianne, G. L., de la Concha, A., Campos-Ortega, J. A., Jan, L. Y. and Jan, Y. N. (1991). The *Drosophila* neurogenic gene *neuralized* encodes a novel protein and is expressed in precursors of larval and adult neurons. *EMBO J.* **10**, 2975-2983.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Brown, N. H. and Kafatos, F. C. (1988). Functional cDNA libraries from *Drosophila* embryos. *J. Mol. Biol.* **203**, 425-437.

Capdevila, J. and Guerrero, I. (1994). Targeted expression of the signaling molecule decapentaplegic induces pattern duplications and growth alterations in *Drosophila* wings. *EMBO J.* **13**, 4459-4468.

Carmena, A., Bate, M. and Jiménez, F. (1995). *Lethal of scute*, a proneural gene, participates in the specification of muscle progenitors during *Drosophila* embryogenesis. *Genes Dev.* **9**, 2373-2383.

Corbin, V., Michelson, A. M., Abmayr, S. M., Neel, V., Alcamo, E., Maniatis, T. and Young, M. W. (1991). A role for the Drosophila neurogenic genes in mesoderm differentiation. *Cell* **67**, 311-323.

Cripps, R. M., Black, B. L., Zhao, B., Lien, C. L., Schulz, R. A. and Olson, E. N. (1998). The myogenic regulatory gene *Mef2* is a direct target for transcriptional activation by Twist during *Drosophila* myogenesis. *Genes Dev.* **12**, 422-434.

de Celis, J. F., Bray, S. and Garcia-Bellido, A. (1997). Notch signalling regulates *veinlet* expression and establishes boundaries between veins and interveins in the *Drosophila* wing. *Development* **124**, 1919-1928.

de Celis, J. F., de Celis, J., Ligoxygakis, P., Preiss, A., Delidakis, C. and Bray, S. (1996). Functional relationships between *Notch*, *Su(H)* and the bHLH genes of the *E(spl)* complex: the *E(spl)* genes mediate only a subset of *Notch* activities during imaginal development. *Development* **122**, 2719-2728.

de Celis, J. F., Tyler, D. M., de Celis, J. and Bray, S. J. (1998). Notch signalling mediates segmentation of the *Drosophila* leg. *Development* **125**, 4617-4626.

Delidakis, C. and Artavanis-Tsakonas, S. (1992). The *Enhancer of split* [*E(spl)*] locus of *Drosophila* encodes seven independent helix-loop-helix proteins. *Proc. Natl. Acad. Sci. USA* **89**, 8731-8735.

Delidakis, C., Preiss, A., Hartley, D. A. and Artavanis-Tsakonas, S. (1991). Two genetically and molecularly distinct functions involved in early neurogenesis reside within the *Enhancer of split* locus of *Drosophila melanogaster*. *Genetics* **129**, 803-823.

Fisher, A. L. and Caudy, M. (1998). Groucho proteins: transcriptional corepressors for specific subsets of DNA-binding transcription factors in vertebrates and invertebrates. *Genes Dev.* **12**, 1931-1940.

Freeman, M. (1996). Reiterative use of the EGF receptor triggers differentiation of all cell types in the Drosophila eye. *Cell* **87**, 651-660.

Furukawa, T., Kobayakawa, Y., Tamura, K., Kimura, K., Kawaichi, M., Tanimura, T. and Honjo, T. (1995). Suppressor of Hairless, the *Drosophila* homologue of RBP-Jκ, transactivates the neurogenic gene *E(spl)m8*. *Jpn. J. Genet.* **70**, 505-524.

Gelbart, W. M., Crosby, M., Matthews, B., Chillemi, J., Twombly, S. R., Emmert, D., Bayraktaroglu, L., Smutniak, F., Kossida, S., Ashburner, M. et al. (1999). The FlyBase database of the Drosophila genome projects and community literature. *Nucl. Acids Res.* **27**, 85-88.

Greenwald, I. (1998). LIN-12/Notch signaling: lessons from worms and flies. *Genes Dev.* **12**, 1751-1762.

Hinz, U., Giebel, B. and Campos-Ortega, J. A. (1994). The basic-helix-loop-helix domain of Drosophila lethal of scute protein is sufficient for proneural function and activates neurogenic genes. *Cell* **76**, 77-87.

Huang, F., Dambly-Chaudière, C. and Ghysen, A. (1991). The emergence of sense organs in the wing disc of *Drosophila*. *Development* **111**, 1087-1095.

Huppert, S. S., Jacobsen, T. L. and Muskavitch, M. A. (1997). Feedback regulation is central to Delta-Notch signalling required for *Drosophila* wing vein morphogenesis. *Development* **124**, 3283-3291.

Jarriault, S., Brou, C., Logeat, F., Schroeter, E. H., Kopan, R. and Israel, A. (1995). Signalling downstream of activated mammalian Notch. *Nature* **377**, 355-358.

Kavaler, J., Fu, W., Duan, H., Noll, M. and Posakony, J. W. (1999). An essential role for the *Drosophila Pax2* homolog in the differentiation of adult sensory organs. *Development* **126**, 2261-2272.

Kimble, J. and Simpson, P. (1997). The LIN-12/Notch signaling pathway and its regulation. *Ann. Rev. Cell Dev. Biol.* **13**, 333-361.

Klämbt, C., Knust, E., Tietze, K. and Campos-Ortega, J. (1989). Closely related transcripts encoded by the neurogenic gene complex *Enhancer of split* of *Drosophila melanogaster*. *EMBO J.* **8**, 203-210.

Knust, E., Bremer, K. A., Vässin, H., Ziemer, A., Tepass, U. and Campos-Ortega, J. A. (1987a). The *Enhancer of split* locus and neurogenesis in *Drosophila melanogaster*. *Dev. Biol.* **122**, 262-273.

Knust, E., Schrons, H., Grawe, F. and Campos-Ortega, J. A. (1992). Seven genes of the *Enhancer of split* complex of *Drosophila melanogaster* encode helix-loop-helix proteins. *Genetics* **132**, 505-518.

Knust, E., Tietze, K. and Campos-Ortega, J. A. (1987b). Molecular analysis of the neurogenic locus Enhancer of split of *Drosophila melanogaster*. *EMBO J.* **6**, 4113-4123.

Kramatschek, B. and Campos-Ortega, J. A. (1994). Neuroectodermal transcription of the *Drosophila* neurogenic genes *E(spl)* and *HLH-m5* is regulated by proneural genes. *Development* **120**, 815-826.

Lai, E. C., Bodner, R., Kavaler, J., Freschi, G. and Posakony, J. W. (2000). Antagonism of Notch signaling activity by members of a novel protein family encoded by the *Bearded* and *Enhancer of split* gene complexes. *Development* **127**, 291-306.

Lai, E. C., Burks, C. and Posakony, J. W. (1998). The K box, a conserved 3′ UTR sequence motif, negatively regulates accumulation of *Enhancer of split* Complex transcripts. *Development* **125**, 4077-4088.

Lai, E. C. and Posakony, J. W. (1997). The Bearded box, a novel 3′ UTR sequence motif, mediates negative post-transcriptional regulation of *Bearded* and *Enhancer of split* Complex gene expression. *Development* **124**, 4847-4856.

Lai, E. C. and Posakony, J. W. (1998). Regulation of *Drosophila* neurogenesis by RNA:RNA duplexes? *Cell* **93**, 1103-1104.

Lecourtois, M. and Schweisguth, F. (1995). The neurogenic Suppressor of Hairless DNA-binding protein mediates the transcriptional activation of the *Enhancer of split* Complex genes triggered by Notch signaling. *Genes Dev.* **9**, 2598-2608.

Lecourtois, M. and Schweisguth, F. (1998). Indirect evidence for Delta-dependent intracellular processing of Notch in *Drosophila* embryos. *Curr. Biol.* **8**, 771-774.

Leviten, M. W., Lai, E. C. and Posakony, J. W. (1997). The *Drosophila* gene *Bearded* encodes a novel small protein and shares 3′ UTR sequence motifs with multiple *Enhancer of split* Complex genes. *Development* **124**, 4039-4051.

Leviten, M. W. and Posakony, J. W. (1996). Gain-of-function alleles of *Bearded* interfere with alternative cell fate decisions in *Drosophila* adult sensory organ development. *Dev. Biol.* **176**, 264-283.

Lindsley, D. L. and Zimm, G. G. (1992). The Genome of *Drosophila melanogaster*. San Diego: Academic Press, Inc.

Maier, D., Marte, B. M., Schafer, W., Yu, Y. and Preiss, A. (1993). Drosophila evolution challenges postulated redundancy in the E(spl) gene complex. *Proc. Natl. Acad. Sci. USA* **90**, 5464-5468.

Milán, M., Diaz-Benjumea, F. J. and Cohen, S. M. (1998). *Beadex* encodes an LMO protein that regulates Apterous LIM-homeodomain activity in *Drosophila* wing development: a model for *LMO* oncogene function. *Genes Dev.* **12**, 2912-2920.

Nakao, K. and Campos-Ortega, J. A. (1996). Persistent expression of genes of the *Enhancer of split* complex suppresses neural development in *Drosophila*. *Neuron* **16**, 275-286.

Nellesen, D. T., Lai, E. C. and Posakony, J. W. (1999). Discrete enhancer elements mediate selective responsiveness of *Enhancer of split* Complex genes to common transcriptional activators. *Dev. Biol.* **213**, 33-53.

O'Neill, J. W. and Bier, E. (1994). Double-label *in situ* hybridization using biotin and digoxigenin-tagged RNA probes. *Biotechniques* **17**, 870, 874-875.

Phelps, C. B. and Brand, A. H. (1998). Ectopic gene expression in *Drosophila* using GAL4 system. *Methods* **14**, 367-379.

Posakony, J. W. (1994). Nature versus nurture: asymmetric cell divisions in Drosophila bristle development. *Cell* **76**, 415-418.

Preiss, A., Hartley, D. A. and Artavanis-Tsakonas, S. (1988). The molecular genetics of *Enhancer of split*, a gene required for embryonic neural development in *Drosophila*. *EMBO J.* **7**, 3917-3927.

Price, B. D., Chang, Z., Smith, R., Bockheim, S. and Laughon, A. (1993). The *Drosophila neuralized* gene encodes a C3HC4 zinc finger. *EMBO J.* **12**, 2411-2418.

Rauskolb, C. and Irvine, K. D. (1999). Notch-mediated segmentation and growth control of the *Drosophila* leg. *Dev. Biol.* **210**, 339-350.

Roberts, R. M., Mathialagan, N., Duffy, J. Y. and Smith, G. W. (1995). Regulation and regulatory role of proteinase inhibitors. *Crit. Rev. Euk. Gene. Expr.* **5**, 385-436.

Romani, S., Campuzano, S., Macagno, E. R. and Modolell, J. (1989). Expression of *achaete* and *scute* genes in *Drosophila* imaginal discs and their function in sensory organ development. *Genes Dev.* **3**, 997-1007.

Roy, S. and VijayRaghavan, K. (1999). Muscle pattern diversification in *Drosophila*: the story of imaginal myogenesis. *Bioessays* **21**, 486-498.

Rubin, G. M., Hong, L., Brokstein, P., Evans-Holm, M., Frise, E., Stapleton, M. and Harvey, D. A. (2000). A *Drosophila* complementary DNA resource. *Science* **287**, 2222-2224.

Rubin, G. M. and Spradling, A. C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218**, 348-353.

Ruiz Gómez, M. and Bate, M. (1997). Segregation of myogenic lineages in *Drosophila* requires Numb. *Development* **124**, 4857-4866.

Schroeter, E. H., Kisslinger, J. A. and Kopan, R. (1998). Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature* **393**, 382-386.

Schrons, H., Knust, E. and Campos-Ortega, J. A. (1992). The *Enhancer of split* complex and adjacent genes in the 96F region of *Drosophila melanogaste*r are required for segregation of neural and epidermal progenitor cells. *Genetics* **132**, 481-503.

Schweisguth, F. and Posakony, J. W. (1992). *Suppressor of Hairless*, the Drosophila homolog of the mouse recombination signal-binding protein gene, controls sensory organ cell fates. *Cell* **69**, 1199-1212.

Schweisguth, F. and Posakony, J. W. (1994). Antagonistic activities of *Suppressor of Hairless* and *Hairless* control alternative cell fates in the *Drosophila* adult epidermis. *Development* **120**, 1433-1441.

Singson, A., Leviten, M. W., Bang, A. G., Hua, X. H. and Posakony, J. W. (1994). Direct downstream targets of proneural activators in the imaginal disc include genes involved in lateral inhibitory signaling. *Genes Dev.* **8**, 2058-2071.

Struhl, G. and Adachi, A. (1998). Nuclear access and action of Notch in vivo. *Cell* **93**, 649-660.

Struhl, G., Fitzgerald, K. and Greenwald, I. (1993). Intrinsic activity of the Lin-12 and Notch intracellular domains in vivo. *Cell* **74**, 331-345.

Sturtevant, M. A., Roark, M. and Bier, E. (1993). The *Drosophila rhomboid* gene mediates the localized formation of wing veins and interacts genetically with components of the EGF-R signaling pathway. *Genes Dev.* **7**, 961-973.

Tautz, D. and Pfeifle, C. (1989). A non-radioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98**, 81-85.

Tun, T., Hamaguchi, Y., Matsunami, N., Furukawa, T., Honjo, T. and Kawaichi, M. (1994). Recognition sequence of a highly conserved DNA binding protein RBP-Jκ. *Nucleic Acids Res.* **22**, 965-971.

Usui, K. and Kimura, K. (1993). Sequential emergence of the evenly spaced microchaetes on the notum of *Drosophila*. *Roux's Arch. Dev. Biol.* **203**, 151-158.

Van Doren, M., Bailey, A., Esnayra, J., Ede, K. and Posakony, J. (1994). Negative regulation of proneural gene activity: hairy is a direct transcriptional repressor of *achaete*. *Genes Dev.* **8**, 2729-2742.

Van Doren, M., Ellis, H. M. and Posakony, J. W. (1991). The *Drosophila extramacrochaetae* protein antagonizes sequence-specific DNA binding by *daughterless/achaete-scute* protein complexes. *Development* **113**, 245-255.

Wurmbach, E., Wech, I. and Preiss, A. (1999). The *Enhancer of split* complex of *Drosophila melanogaster* harbors three classes of Notch responsive genes. *Mech. Dev.* **80**, 171-180.