

# The Bearded box, a novel 3' UTR sequence motif, mediates negative post-transcriptional regulation of *Bearded* and *Enhancer of split* Complex gene expression

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## SUMMARY

During the development of the *Drosophila* adult peripheral nervous system (PNS), inhibitory cell-cell interactions mediated by the Notch receptor are essential for proper specification of sensory organ cell fates. We have reported previously (M. W. Leviten, E. C. Lai and J. W. Posakony (1997) *Development* 124, 4039-4051) that the 3' untranslated regions (UTRs) of many genes involved in Notch signalling, including *Bearded* (*Brd*) and the genes of the *Enhancer of split* Complex (*E(spl)-C*), contain (often in multiple copies) two novel heptanucleotide sequence motifs, the Brd box (AGCTTTA) and the GY box (GTCTTCC). Moreover, the molecular lesion associated with a strong gain-of-function mutant of *Brd* suggested that the loss of these sequence elements from its 3' UTR might be responsible for the hyperactivity of the mutant gene. We show here that the wild-type *Brd* 3' UTR confers negative regulatory activity on heterologous transcripts *in vivo* and that this activity requires its three Brd box elements and, to a lesser extent, its GY box. We find that Brd box-mediated regulation decreases both transcript and

protein levels, and our results suggest that deadenylation or inhibition of polyadenylation is a component of this regulation. Though *Brd* and the *E(spl)-C* genes are expressed in spatially restricted patterns in both embryos and imaginal discs, we find that the regulatory activity that functions through the Brd box is both temporally and spatially general. A *Brd* genomic DNA transgene with specific mutations in its Brd and GY boxes exhibits hypermorphic activity that results in characteristic defects in PNS development, demonstrating that *Brd* is normally regulated by these motifs. Finally, we show that Brd boxes and GY boxes in the *E(spl)m4* gene are specifically conserved between two distantly related *Drosophila* species, strongly suggesting that *E(spl)-C* genes are regulated by these elements as well.

Key words: *Drosophila*, neurogenesis, sensory organ development, 3' untranslated region, RNA stability, translational control, post-transcriptional regulation, Brd box, GY box, *Bearded*, *Enhancer of split*

## INTRODUCTION

The adult peripheral nervous system (PNS) of *Drosophila melanogaster* has been a fruitful model for investigating cellular and molecular mechanisms of pattern formation, cell-cell communication and cell fate specification. External mechanosensory bristles constitute the majority of the adult PNS, and are distributed over the body surface of the fly in a regular and highly stereotyped pattern. Each of these sensory organs is composed of four cells, a bipolar sensory neuron and three non-neuronal accessory cells, which are clonally derived from a single sensory organ precursor (SOP) cell (Hartenstein and Posakony, 1989). SOPs become determined during late larval and early pupal development within the imaginal discs and histoblast nests (Huang et al., 1991; Usui and Kimura, 1993). The spatially patterned activities of the proneural genes *achaete* (*ac*) and *scute* (*sc*), both of which encode basic helix-loop-helix (bHLH) transcriptional activators, define the proneural clusters, groups of cells that are competent to adopt the SOP fate (Cabrera and Alonso, 1991; Cubas et al., 1991;

Skeath and Carroll, 1991; Van Doren et al., 1992). Proper spatial activity of the proneural genes is dependent upon negative regulation by *extramacrochaetae* (*emc*) and *hairy* (*h*) (Cubas and Modolell, 1992; Ohsako et al., 1994; Orenic et al., 1993; Van Doren et al., 1992, 1994). Within each proneural cluster, inhibitory cell-cell interactions mediated by the neurogenic genes allow only a single cell to become stably committed to the SOP fate (Dietrich and Campos-Ortega, 1984; Hartenstein and Posakony, 1990; Parks and Muskavitch, 1993; Schweisguth and Posakony, 1992, 1994; Tata and Hartley, 1995). This process, known as lateral or mutual inhibition, is largely controlled by signalling through the Notch (N) receptor (reviewed by Artavanis-Tsakonas et al. (1995)). The inhibited cells of a proneural cluster adopt an epidermal fate indistinguishable from that of the remaining cells of the epidermis.

Genes of the *Enhancer of split* Complex (*E(spl)-C*) function downstream of the N receptor, and are directly activated by the Suppressor of Hairless (Su(H)) protein in response to N activity (Bailey and Posakony, 1995; Lecourtois and

Schweisguth, 1995). Seven transcription units in the E(spl)-C encode bHLH repressor proteins, while an additional member of the complex, *m4*, encodes a novel protein that is also likely to have a role in lateral inhibition (Klambt et al., 1989; Schrons et al., 1992). The E(spl)*m4* protein is predicted to be structurally related to the small protein encoded by *Bearded (Brd)* (Leviten et al., 1997), which acts genetically as an antagonist of N-mediated signalling in the adult PNS (Leviten and Posakony, 1996). Gain-of-function alleles of *Brd* confer mutant phenotypes that mimic at the cellular level those caused by loss-of-function mutations in the neurogenic genes (Leviten and Posakony, 1996). Finally, *Brd* and several E(spl)-C genes are subject to direct transcriptional activation by the proneural proteins *ac* and *sc*, and are co-expressed in all or a subset of proneural clusters in developing imaginal discs (Bailey and Posakony, 1995; de Celis et al., 1996; Singson et al., 1994).

Recently, we described two novel sequence motifs, the Brd box (AGCTTTA) and the GY box (GTCTTCC), that are present in the 3' untranslated regions (3' UTRs) of *Brd* and multiple genes of the E(spl)-C (Leviten et al., 1997). Like known sequence elements that function in post-transcriptional regulation, both of these motifs are found in a single orientation and specifically in the 3' UTRs of the genes that include them. Moreover, genetic evidence from *Brd* gain-of-function alleles implicates its 3' UTR in post-transcriptional negative regulation. Specifically, mutant *Brd* transcripts that are truncated in the 3' UTR due to a transposon insertion are present at elevated steady-state levels (Leviten et al., 1997). The region deleted in the mutant transcripts includes two of the three Brd boxes and the GY box normally found in *Brd* mRNA, suggesting that these motifs may function to destabilize the wild-type transcript.

A number of recent studies have elucidated multiple roles for 3' UTRs in negative regulation of gene activity. Some 3' UTRs control transcript localization, thereby regulating exposure to translational repressors. For example, the 3' UTR of *nanos (nos)* mediates localization of *nos* transcripts to the posterior pole of the *Drosophila* embryo. Unlocalized transcripts are thought to be bound in the 3' UTR by a ubiquitously distributed repressor protein whose action is inhibited only at the posterior pole (Dahanukar and Wharton, 1996; Gavis et al., 1996). UTRs can also control the polyadenylation state of the transcript. Many mRNAs are translationally inactive until they undergo additional cytoplasmic polyadenylation, a process controlled by cytoplasmic polyadenylation elements (CPEs) (reviewed by Curtis et al. (1995)). Other 3' UTR elements destabilize transcripts. For example, AU-rich elements (AREs) confer rapid transcript turnover (reviewed by Chen and Shyu (1995)). AREs trigger mRNA degradation by a deadenylation mechanism, but deadenylation-independent degradation pathways have also been described (reviewed by Beelman and Parker (1995)). Finally, some 3' UTR motifs directly confer translational repression. In the nematode *Caenorhabditis elegans*, the *tra-2* sex-determining gene is subject to negative translational regulation mediated by two 28-nucleotide (nt) direct repeat elements (DREs) located in its 3' UTR (Goodwin et al., 1997). The DREs are bound by a protein factor called DRF that appears to be a direct translational repressor of *tra-2*.

In the present study, we establish that the *Brd* 3' UTR confers negative regulatory activity on heterologous reporter genes *in vivo*. This activity is spatially and temporally general, affects both RNA and protein levels, and is strongly dependent on the

integrity of the Brd boxes. We also find that the GY box may have a role in translational control that is synergistic with Brd box function. We show that a *Brd* genomic DNA transgene with mutations in its Brd and GY boxes causes characteristic gain-of-function defects in adult PNS development, demonstrating that *Brd* itself is normally regulated by these motifs. Finally, sequence comparison of the 3' UTRs of the *D. melanogaster* and *D. hydei E(spl)m4* genes reveals that Brd box and GY box motifs are both specifically conserved during *Drosophila* evolution, strongly suggesting that these elements are important for regulation of E(spl)-C expression as well.

## MATERIALS AND METHODS

### *Drosophila* stocks

Flies were cultured on standard yeast-cornmeal-molasses-agar medium. Mark Van Doren provided the P[*w*<sup>+</sup>, *ac-lacZ-SV40t*] transgenic lines (Van Doren et al., 1992). Mobilization of P-element transposon insertions was performed using TMS as a genomic source of transposase; phenotypic interactions with *N* were investigated using *N<sup>81kl</sup>*; both are described in Lindsley and Zimm (1992).

### Plasmid construction

#### Wild-type and mutant *Brd* 3' UTRs

A 347-base pair (bp) fragment of *Brd*, which includes the entire *Brd* 3' UTR as well as 142 bp of sequence downstream of the polyadenylation site, was PCR amplified from a *Brd* genomic DNA subclone (kindly provided by A. Singson) using primers that added a 5' *KpnI* site and a 3' *EcoRI* site. The PCR product was cloned into pBluescript KS(+), fully sequenced and designated the *wtBrd* 3' UTR fragment. The Transformer site-directed mutagenesis kit (Clontech) and appropriate oligonucleotide primers were then used to change all three Brd box motifs from AGCTTTA to ATAGGGA and the GY box motif from GTCTTCC to GTAGGAA. Following complete sequencing to verify directed mutations, the mutant *Brd* 3' UTR fragments were designated *Brdmut123*, *GYmut* and *Brd+GYmut*, corresponding respectively to a *Brd* 3' UTR with mutations in all three Brd boxes, in the single GY box alone and in all four motifs.

#### *ac-lacZ* P-element transformation constructs

A P-element transformation vector containing unique pairs of restriction sites both upstream and downstream of a *lacZ* reporter gene was constructed as follows. A 3.3-kilobase (kb) *lacZ* fragment was obtained by digesting CaSpeR*lacZ* (Margolis et al., 1994) with *BamHI* and *DraI* and cloned into the *BamHI/SmaI* sites of pUC19. This fragment was excised by digestion with *BamHI* and *KpnI* and cloned into the *BamHI/EcoRI* sites of the CaSpeR P-element transformation vector (Pirrota, 1988) along with a 200 bp *KpnI/EcoRI* stuffer fragment. This plasmid thus contains unique *PstI* and *BamHI* sites upstream of *lacZ* and unique *KpnI* and *EcoRI* sites downstream. A 0.9 kb *ac* promoter subclone containing flanking *PstI* and *BamHI* sites has been described by Van Doren et al. (1992). The four versions of the *Brd* 3' UTR described above were cloned into the vector as *KpnI/EcoRI* fragments, to generate *ac-lacZ-wtBrd*, *ac-lacZ-Brdmut123*, *ac-lacZ-GYmut* and *ac-lacZ-Brd+GYmut*.

#### *arm-lacZ* P-element transformation constructs

A 1.8 kb *arm* promoter subclone (Vincent et al., 1994) was inserted into pRSETA as an *XhoI* (partial)/*BamHI* fragment in order to add a 5' *PstI* site. *arm-lacZ* transformation constructs were then generated by replacing the *ac* promoter with the *arm* promoter in the above *ac-lacZ* constructs to generate *arm-lacZ-wtBrd*, *arm-lacZ-Brdmut123*, *arm-lacZ-GYmut* and *arm-lacZ-Brd+GYmut*. *arm-lacZ-SV40t* was created by inserting the 1.8 kb *arm* promoter fragment into the unique *PstI/BamHI* sites of CaSpeR*lacZ*.

### *Brd* genomic P-element transformation constructs

Site-directed mutagenesis was used to introduce a single base change 16 nt downstream of the stop codon, thus creating an *EcoRV* site (GGAAATCC to GGATATCC). Mutagenesis was performed on a *SalI/EcoRI* fragment of *Brd* genomic DNA (the 5' *SalI* terminus of this fragment is in the 5' UTR of the gene; see Leviten et al., 1997) and on the *wtBrd* and *Brd+GYmut* 3' UTR fragments. All mutations were confirmed by completely sequencing the plasmid inserts. The two versions of the *Brd* 3' UTR were then introduced into the *SalI/EcoRI* *Brd* subclone as *EcoRV/EcoRI* fragments. A 6.0 kb *XbaI/SalI* *Brd* promoter fragment (gift of A. Singson) and the *SalI/EcoRI* *Brd* coding+3' UTR fragments were then cloned by three-way ligation into the *XbaI/EcoRI* sites of CaSpeR.

The sequences of oligonucleotides used for site-directed mutagenesis are available upon request.

### Germline transformation

The P-element transformation constructs described above were coinjected with a  $\Delta 2-3$  helper plasmid into the recipient strain *w<sup>1118</sup>* (Rubin and Spradling, 1982). For each construct, a minimum of 5, but typically 7-12, independent homozygous transgenic lines were analyzed.

### $\beta$ -galactosidase activity staining

Imaginal discs and other organs were dissected from late third-instar larvae and stained for  $\beta$ -galactosidase activity as described (Romani et al., 1989). Preparations were dehydrated in an ethanol series and mounted in Epon.

### Northern blot analysis

Total RNA was prepared from staged embryos (2-14 hours and 14-24 hours) and late third-instar larvae using TriZol (Gibco BRL). Poly(A)<sup>+</sup> RNA was isolated using the FastTrack 2.0 kit (Invitrogen). RNA was fractionated on 1% formaldehyde gels, transferred to Nytran filters (Schleicher and Schuell) and probed for *lacZ* or *rp49* transcripts. Radioactive probes were generated using [ $\alpha$ -<sup>32</sup>P]dCTP, the Prime-It kit (Stratagene) and either a 3.3 kb *lacZ* fragment from CaSpeRlacZ (see above) or a 0.65 kb fragment of *rp49* (O'Connell and Rosbash, 1984) as template, followed by chromatography over G50-Sephadex (Sigma). Hybridized filters were exposed to X-ray film (Kodak X-OMAT AR) with two intensifying screens at -80°C. Quantitation was performed using a phosphorimager and ImageQuant software (Molecular Dynamics).

### Western blot analysis

Total protein extracts were prepared from staged embryos (2-14 hours and 14-24 hours), whole late third-instar larvae, or dissected anterior mass (composed primarily of brain, imaginal discs and salivary glands) from late third-instar larvae by homogenization in 8 M urea/10%  $\beta$ -mercaptoethanol and boiling for 8 minutes, followed by a 10-minute centrifugation to give a clear supernatant. These extracts were separated on 7% polyacrylamide gels, transferred to Immobilon P filters (Millipore) and probed with monoclonal antibodies to  $\beta$ -galactosidase (Promega) at a 1:10,000 dilution and groucho (gift of S. Artavanis-Tsakonas) at a 1:40 dilution. Antibody binding was visualized using a secondary antibody linked to horseradish peroxidase (Bio-Rad) and enhanced chemiluminescence (National Diagnostics) exposure of X-ray film (Kodak X-OMAT AR). Quantitative western blot analysis was performed using a rabbit secondary antibody followed by incubation with <sup>125</sup>I-Protein A (Amersham).

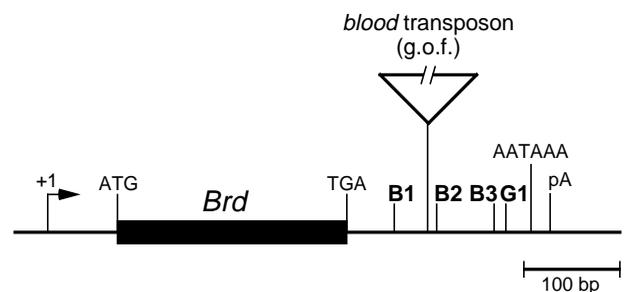
## RESULTS

### The *Brd* 3' UTR confers Brd box-dependent negative regulation in proneural clusters

To investigate potential regulatory activities of the *Brd* 3' UTR,

we tested its ability to influence the expression of a heterologous transcript. *Brd* is expressed in proneural clusters under the direct control of the *ac* and *sc* transcriptional activators (Singson et al., 1994). An *ac-lacZ-SV40t* transgene, containing the *ac* proximal promoter, a *lacZ* reporter gene, and the 3' UTR and flanking sequences of the SV40 t antigen gene, was previously shown to be expressed at high levels in all proneural clusters of the late third-instar larval wing imaginal disc (Van Doren et al., 1992). The SV40 t 3' UTR is compatible with high-level transgene expression in *Drosophila* (Thummel et al., 1988) and has been used extensively as a generic 3' UTR for reporter constructs. Here, we investigated whether *ac-lacZ* reporter activity was significantly altered when the SV40 t 3' UTR was replaced with a wild-type *Brd* 3' UTR (*ac-lacZ-wtBrd*). To ensure proper polyadenylation of reporter gene transcripts, we made use of a fragment of genomic DNA that contains the entire *Brd* 3' UTR along with 142 bp of sequence downstream of the polyadenylation site (Fig. 1). We found that the *Brd* 3' UTR exhibits strong negative regulatory activity with respect to the SV40 t 3' UTR (Fig. 2A,B). In even the most strongly expressing *ac-lacZ-wtBrd* transgenic lines, reporter activity was detected in only a vestige of a proneural cluster pattern (Fig. 2B). Many other insertions of this construct displayed undetectable reporter gene expression. The negative regulatory activity of the *Brd* 3' UTR appears to be uniformly potent in all proneural clusters.

To test whether Brd box elements are specifically required for this negative regulation, we constructed a version of the *Brd* 3' UTR in which the three Brd boxes were mutated (see Fig. 1). Lacking knowledge of the particular bases that might be critical for Brd box function, we changed the core five nucleotides of this motif by non-complementary transversion



	<u>Wild-type</u>	<u>Mutant</u>
<b>Brd box (B):</b>	<b>AGCTTTA</b>	<b>ATAGGGA</b>
<b>GY box (G):</b>	<b>GTCTTCC</b>	<b>GTAGGAA</b>

**Fig. 1.** Brd box and GY box sequence motifs in the *Brd* 3' UTR. Filled box represents the *Brd* protein coding region, with start and stop codons indicated. The transcription start site (+1) is indicated by a horizontal arrow; the polyadenylation signal (AATAAA) and polyadenylation site (pA) are also shown. Bold letters mark the positions of Brd box (B) and GY box (G) motifs in the 3' UTR. Dominant gain-of-function (g.o.f.) alleles of *Brd* (*Brd<sup>l</sup>* and derivatives; see Leviten and Posakony (1996)) are associated with an insertion into the 3' UTR of a transposable element of the *blood* family, as shown (Leviten et al., 1997). To create the mutant 3' UTRs used in this study (see Materials and Methods), the core 5 nt of each Brd box or the last 5 nt of the GY box (underlined) were changed as indicated.

mutations (AGCTTTA to ATAGGGA). We found that the activity of the *ac-lacZ-Brdmut123* transgene was greatly elevated throughout all proneural clusters of the wing imaginal disc relative to the *ac-lacZ-wtBrd* transgene (Fig. 2B,C). The *Brdmut123* construct displays quantitatively lower levels of expression than the *SV40t* construct, as these discs must be stained for longer periods in order to give a comparable reporter signal. The mutant *Brd* 3' UTR may retain residual negative regulatory activity relative to the *SV40t* 3' UTR, or perhaps the greater length of the latter indirectly supports higher expression levels. Nevertheless, it is clear that the *Brd* 3' UTR possesses strong negative regulatory activity in proneural clusters, and that this regulation is specifically dependent on the integrity of the Brd boxes.

### Brd box-mediated negative regulation is both spatially and temporally general

We tested whether negative regulation by the *Brd* 3' UTR is restricted to proneural clusters by making use of a more ubiquitously expressed transgene. A 1.8 kb fragment of the *armadillo* (*arm*) promoter was previously shown to direct expression of a *lacZ* reporter gene in virtually all cells throughout development (Vincent et al., 1994). In the first set of experiments, we compared the activities of *arm-lacZ* reporter transgenes containing the *SV40t*, *wtBrd* or *Brdmut123* 3' UTRs described above.

We observed that the *wtBrd* 3' UTR drastically reduces reporter gene activity with respect to the *SV40t* 3' UTR in all tissues examined. Reduced expression levels were found throughout all imaginal tissues, as well as the salivary glands, fat bodies and other larval organs (c.f. Fig. 3A,F,K and Fig. 3B,G,L). Low levels of reporter gene activity were still apparent in salivary glands (Fig. 3L) and in the vicinity of the morphogenetic furrow of the eye imaginal disc (Fig. 3G), but this may reflect a higher activity of the *arm* promoter in these cells, since even the *arm-lacZ-SV40t* construct exhibits elevated reporter expression near the morphogenetic furrow. Thus, the regulatory activity of the *wtBrd* 3' UTR is both spatially general and relatively uniform.

We demonstrated that this ubiquitous negative regulatory activity is largely mediated by the Brd boxes. Mutation of all three of these motifs (*arm-lacZ-Brdmut123*) yielded high levels of reporter gene expression throughout all tissues examined (Fig. 3C,H,M), indicating that the general activity of the *Brd* 3' UTR, like that in the proneural clusters, is specifically dependent on its Brd box elements. We further found that the Brd box-dependent activity is temporally general; for example, negative regulation of reporter gene expression was observed throughout embryonic development (data not shown).

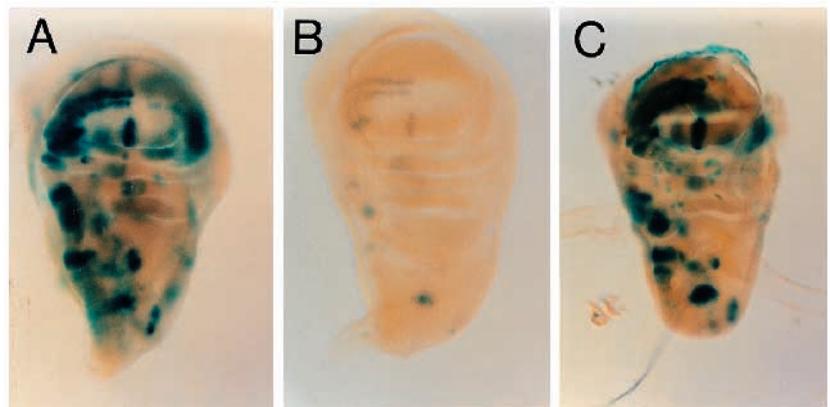
To examine the potential role of the GY box, we analyzed the effect of mutations in this motif on regulation by the *Brd* 3' UTR (see Fig. 1). The last five bases of the GY box were mutated by non-complementary transversions (GTCTTCC to GTAGGAA), either alone (*GYmut*) or in conjunction with the triple Brd box mutation (*Brd+GYmut*). These 3' UTRs were then assayed for activity as part of *arm-lacZ* reporter trans-

genes. At this level of analysis, we are unable to discern a role for the GY box in regulation by the *Brd* 3' UTR. The *GYmut* construct (Fig. 3D,I,N) behaved similarly to the *wtBrd* construct (Fig. 3B,G,L), and the activity of the *Brd+GYmut* transgene (Fig. 3E,J,O) resembled that of the *Brdmut123* transgene (Fig. 3C,H,M). These results do not rule out the possibility that the GY box has an activity that is quantitatively more subtle, or much more spatially or temporally specific, than that of the Brd box.

### Negative regulation by the Brd box motif affects steady-state levels of both RNA and protein

Having established a role for the Brd box in some form of negative regulation, we next addressed the level or levels at which this motif acts. In addition, we sought to identify a role for the GY box by quantitative analysis. We prepared embryo RNA and protein from a mixture of four to six representative transgenic lines for each *arm-lacZ* construct and analyzed reporter gene transcript and protein levels relative to the ubiquitously expressed endogenous genes *rp49* and *groucho*, respectively (Fig. 4, Table 1).

First, it is noteworthy that relative transcript levels differ between the total and poly(A)<sup>+</sup> RNA populations. Mutation of the Brd boxes causes a modest increase in the amount of total *lacZ* reporter gene transcript (~1.5-fold), but a greater increase (~2.3-fold) in the relative amount of polyadenylated transcript (Table 1). These results demonstrate that Brd box-mediated regulation affects steady-state transcript levels and suggest that deadenylation or inhibition of polyadenylation is one component of this regulation. While our northern blot analysis does not exclude a role for Brd boxes in transcriptional regulation, it is consistent with a post-transcriptional function in promoting RNA turnover. We find that Brd box activity has an even greater effect on reporter protein accumulation. Specific



**Fig. 2.** Integrity of Brd box sequences is required for a negative regulatory activity of the *Brd* 3' UTR in proneural clusters. (A-C)  $\beta$ -galactosidase activity staining of late third-instar wing imaginal discs. (A) An *ac-lacZ* transgene utilizing the 3' UTR and flanking sequences of the *SV40t* antigen gene (*ac-lacZ-SV40t*) exhibits strong reporter activity in all proneural clusters. (B) The most strongly expressing lines carrying a reporter transgene that includes the wild-type *Brd* 3' UTR (*ac-lacZ-wtBrd*) display only weak activity in proneural clusters. Other insertions of this transgene lack detectable reporter activity. (C) Mutation of the three Brd boxes in the *Brd* 3' UTR, as in the *ac-lacZ-Brdmut123* transgene, leads to greatly elevated reporter activity throughout all proneural clusters. Stainings shown are representative of 5-8 independent homozygous lines for each construct; (A) *SV40t* lines were stained for 4 hours, while (B) *wtBrd* and (C) *Brdmut123* lines were stained for 7 hours.

mutation of these elements causes a 3- to 5-fold increase in steady-state protein levels (Fig. 4, Table 1). This result indicates that Brd boxes have an additional role in regulating translation, beyond the effect attributable to transcript level differences.

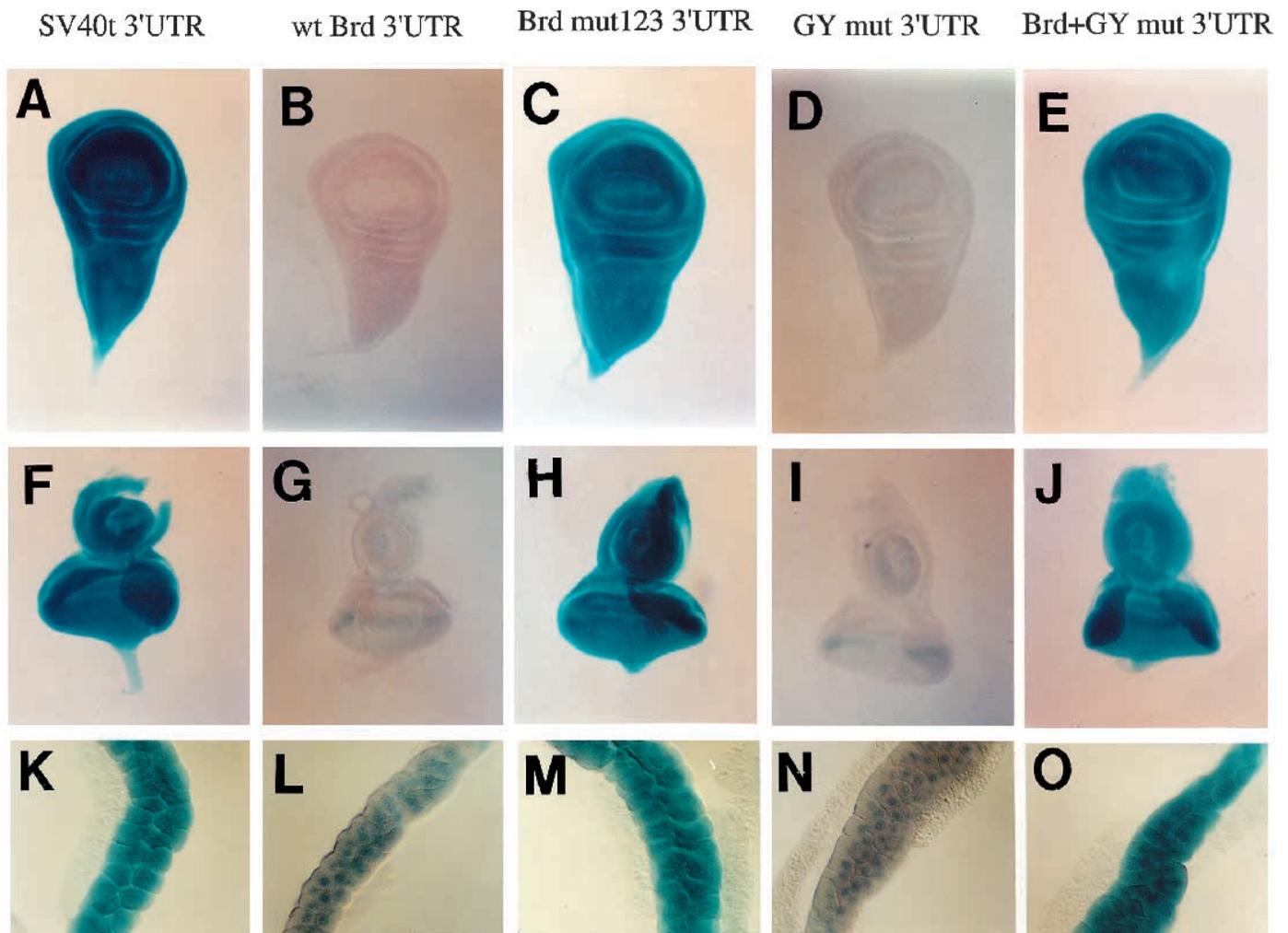
By this quantitative analysis, we also detected small effects resulting from mutation of the GY box motif. In particular, we observed a 1.5- to 2-fold increase in reporter gene protein levels from the *Brd+GYmut* transgene (in which both Brd and GY motifs are mutant) relative to the *Brdmut123* construct (which is mutant only for the Brd boxes). However, differences of this magnitude were not observed when the *GYmut* construct was compared to the *wtBrd* transgene. This suggests that there may be a synergistic effect on translation when both motifs are mutant.

### ***Brd* is subject to negative regulation mediated by the Brd and GY boxes**

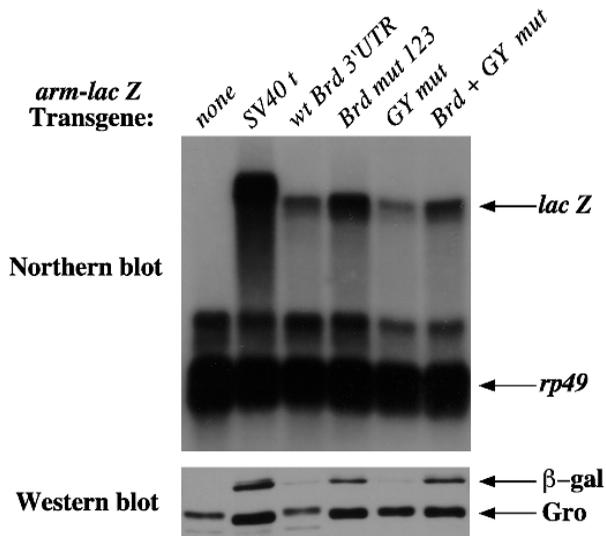
Our findings that the wild-type *Brd* 3' UTR confers negative

regulation on heterologous transgenes, and that this activity is dependent on specific sequence motifs located therein, prompted us to investigate whether normal adult PNS development requires this mode of regulation. In particular, we sought to determine whether *Brd* itself is subject to regulation by the Brd and GY box motifs.

As flies that are deleted for the endogenous *Brd* gene lack a detectable mutant phenotype (Leviten and Posakony, 1996), it has not been possible to test how much genomic DNA sequence is necessary to provide a normal level of *Brd* activity. However, our studies of *Brd* promoter function have indicated that as little as 1.5 kb of upstream sequence is sufficient to drive reporter gene expression in imaginal discs in a pattern that is coincident with endogenous *Brd* expression (Singson et al., 1994). We constructed wild-type and mutant versions of a *Brd* genomic DNA transgene, each containing 6.0 kb of upstream sequence, the entire *Brd* protein coding region, and 350 bp of



**Fig. 3.** Negative regulation mediated by Brd boxes is spatially general. (A-E)  $\beta$ -galactosidase activity staining of late third-instar wing imaginal discs, (F-J) eye-antenna discs, (K-O) salivary glands. (A,F,K) An *arm-lacZ-SV40t* transgene is expressed at high levels throughout most cells of all imaginal and larval tissues. (B,G,L) An *arm-lacZ-wtBrd* transgene displays very low reporter activity throughout these tissues. (C,H,M) Mutation of the three Brd boxes (*arm-lacZ-Brdmut123* transgene) leads to high levels of reporter activity throughout these tissues. (D,I,N) Mutation of the single GY box (*arm-lacZ-GYmut* transgene) does not detectably alter reporter expression levels relative to *arm-lacZ-wtBrd*. (E,J,O) Mutation of all four motifs (*arm-lacZ-Brd+GYmut* transgene) does not detectably alter reporter expression levels relative to *arm-lacZ-Brdmut123*. Stainings shown are representative of 5-12 independent homozygous lines for each construct, and were performed in parallel for 35 minutes each.



**Fig. 4.** Brd boxes mediate negative regulation of steady-state levels of both transcript and protein. Shown is an analysis of the expression of *arm-lacZ* reporter transgenes bearing different 3' UTRs and flanking sequences (see Table 1 for quantitation). (Top) Northern blot analysis of poly(A)<sup>+</sup> RNA isolated from 2-24 hour embryos. mRNA was isolated from a mixture of 4-5 representative transgenic lines for each construct and probed to detect both *lacZ* and *rp49* transcripts. The SV40 t antigen gene 3' UTR is ~500 bp longer than the *Brd* 3' UTRs, hence the larger size of the *arm-lacZ-SV40t* transcript. Note that *lacZ* transcripts containing mutations in the Brd boxes (*Brdmut123* and *Brd+GYmut*) are present at higher levels relative to those with wild-type Brd boxes (*wtBrd* and *GYmut*). *w<sup>1118</sup>*, the recipient strain for germline transformation, is used here as the source of control RNA ('none'). (Bottom) Western blot analysis of total protein from 14-24 hour embryos probed to detect both  $\beta$ -galactosidase and groucho. Blots of 2-14 hour embryo protein extracts show similar relative levels of  $\beta$ -galactosidase accumulation (data not shown). Note that the difference in the relative levels of reporter protein expressed from transgene constructs bearing wild-type vs. mutant Brd boxes is greater than the differences in relative mRNA levels (top).

3' UTR and flanking sequence (either *wtBrd* or *Brd+GYmut* 3' UTRs, as described earlier). These transgenes were analyzed for their phenotypic effects in otherwise wild-type flies; that is, with two endogenous copies of *Brd* present.

Two of the seven homozygous lines carrying the *Brd+GYmut* transgene displayed fully penetrant bristle tufting phenotypes at one or more macrochaete positions (Fig. 5). In contrast, of 11 independent homozygous lines bearing the wild-type transgene, none exhibited any penetrant *Brd* phenotype. That two independent *Brd+GYmut* lines with characteristic *Brd* gain-of-function phenotypes were obtained indicates that these effects are not likely to be dependent on transgene insertion in rare, extremely highly expressed regions of the genome.

Our detailed examination indicates that the mutant transgene confers a phenotype that is qualitatively indistinguishable from that associated with known *Brd* gain-of-function alleles. Ectopic bristles in the mutant lines include both sockets and shafts, and are thus likely to represent complete sensory organs. All ectopic bristles are present as tufts in the normal positions of single sensory organs (i.e., no bristles were found in ectopic locations), indicating that the extra bristles arise from the normal comple-

**Table 1. Quantitation of RNA and protein expression from *arm-lacZ* transgenes**

	Brdmut123 wtBrd	GYmut wtBrd	Brd+GYmut wtBrd
2-24 hour embryo total RNA	1.54±0.22	0.93±0.16	1.28±0.25
2-24 hour embryo poly(A) <sup>+</sup> RNA	2.34±0.17	1.32±0.10	2.31±0.28
2-14 hour embryo total protein	2.83±0.51	1.07±0.05	5.93±1.62
14-24 hour embryo total protein	4.77±2.24	1.30±0.55	6.07±1.78

Accumulation of RNA and protein products from *arm-lacZ* reporter transgenes was quantitated relative to the endogenous controls *rp49* (RNA) and *groucho* (protein) using a Molecular Dynamics phosphorimager (see Fig. 4). Values represent the ratio of the normalized (control-corrected) signal for each mutant construct to that of the fully wild-type construct. A minimum of three independent blots were quantitated for each data set; means and standard deviations are shown.

ment of proneural clusters. Finally, we tested whether the phenotypic effects of the mutant *Brd* transgene are sensitive to reduction of *N* function, as is the case with gain-of-function alleles of the endogenous *Brd* gene (Leviton and Posakony, 1996). Female flies of both of the *Brd+GYmut* transgene lines with penetrant *Brd* phenotypes display strong enhancement of these phenotypes when made heterozygous for a null allele of *N* (data not shown). We also applied this test to three mutant transgene lines that lack penetrant bristle phenotypes in the wild-type genetic background. Of these, one line now exhibits a fully penetrant bristle tufting phenotype when heterozygous for a *N* null allele, one displays a partially (~30%) penetrant effect and the third continues to lack a detectable mutant bristle phenotype (data not shown). These results indicate that a *Brd* transgene carrying mutations in the Brd box and GY box elements of its 3' UTR behaves as a gain-of-function allele, and interferes with adult PNS development in a characteristic manner that is sensitive to changes in *N* dosage.

Finally, we further investigated the capacity of the *wtBrd* and *Brd+GYmut* genomic transgenes to confer gain-of-function phenotypes by mobilizing them in the germline and screening progeny flies for dominant bristle tufting effects. We found that it was virtually impossible to generate such phenotypes starting with two different insertions of the *wtBrd* transgene. Out of more than 1600 progeny examined, only a single fly (0.06%) displayed bristle tufting. A single tuft containing three bristles was present in this individual. In contrast, starting with two *Brd+GYmut* transgenes, we found that 2.2% of more than 1800 progeny exhibited dominant bristle tufts. Moreover, most of the phenotypes observed with these mobilized mutant transgenes were quantitatively more severe than that observed with the wild-type transgene. Many flies had several macrochaete positions tufted, and tufts often included five or more bristles. These data further establish that a *Brd* genomic DNA transgene with mutations in its Brd and GY boxes has hypermorphic activity capable of interfering with normal sensory organ development.

### Evolutionary conservation of Brd box and GY box sequence elements

The experiments described above provide evidence that the Brd box and GY box sequence elements are required for proper regulation of *Brd* expression. The concentration of these same motifs in the 3' UTRs of most of the genes of the E(spl)-C strongly suggests that they are functionally important for E(spl)-C regulation as well. To investigate this question further, we

sequenced the *E(spl)m4* gene from a distantly related species, *Drosophila hydei* (Maier et al., 1993). *D. hydei* is approximately 60 million years separated from *D. melanogaster*, sufficient evolutionary time for significant divergence of sequences that are not functionally constrained (Beverley and Wilson, 1984; Caccone and Powell, 1990). Alignment of the 3' UTRs of these *E(spl)m4* homologues reveals that they are indeed highly diverged; however, 'islands' of evolutionarily conserved sequence remain (Fig. 6). These islands include both of the Brd boxes and both GY boxes that we described earlier in the *D. melanogaster* gene (Leviten et al., 1997), in the same GY-Brd-GY-Brd order. One Brd box and one GY box heptanucleotide are fully conserved, while the remaining Brd box and GY box in *D. hydei* are 1 bp variants (TGCTTA and GTCTCT) of the canonical sequences. Substantial insertions/deletions flank the first GY box and first Brd box, further indicating that the sequences surrounding these motifs are in considerable flux. Elsewhere, sequence flanking the Brd and GY elements is conserved as well. The interspecific conservation of these motifs in homologous genes strongly supports the hypothesis that Brd and GY boxes are important for E(spl)-C regulation.

## DISCUSSION

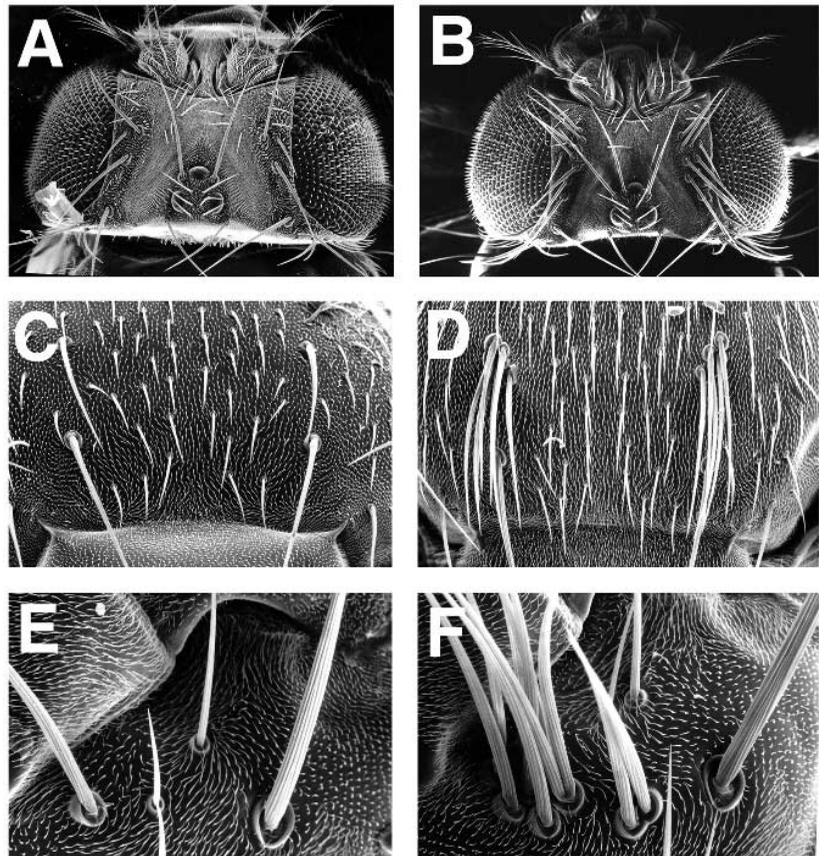
### Negative regulation mediated by specific sequence elements in the *Brd* 3' UTR

We have shown here that, as suggested by our previous study (Leviten et al., 1997), the 3' UTR of the *Drosophila Brd* gene exhibits negative regulatory activity in vivo. In comparison with the SV40 t antigen 3' UTR, a 'generic' 3' UTR that supports high-level *lacZ* reporter activity when expressed from the *ac* and *arm* promoters, the *Brd* 3' UTR permits much lower levels of reporter gene expression. We also demonstrated that this negative regulation is mediated at least in part by specific sequence motifs found in the *Brd* 3' UTR; primarily by three repeats of the 'Brd box' (AGCTTTA) and to a lesser extent by the 'GY box' (GTCTCC). A *Brd* 3' UTR carrying mutations in these sequence elements yields strongly elevated levels of reporter gene activity compared to that supported by the wild-type *Brd* 3' UTR.

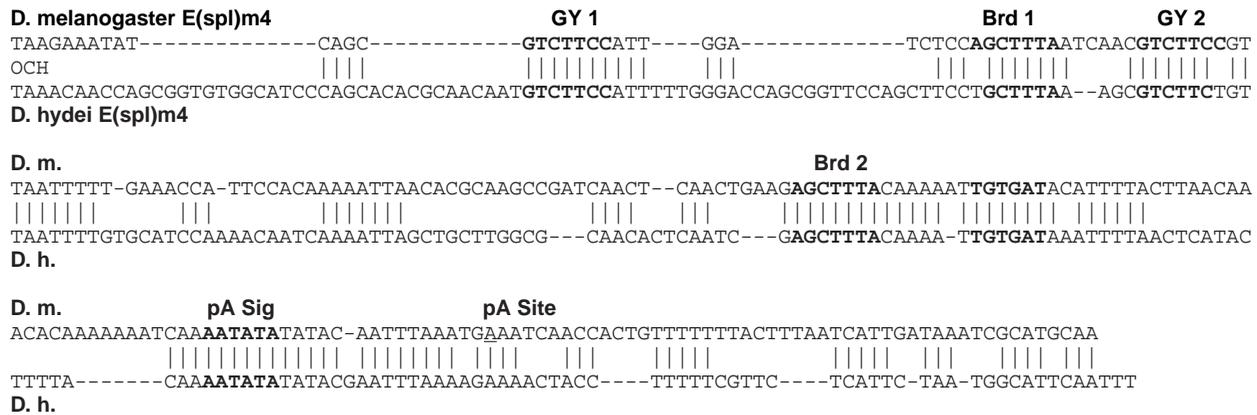
In accord with these results, we found that a *Brd* genomic DNA transgene with mutations in its Brd and GY boxes, but not the wild-type transgene, is readily capable of causing phenotypic defects in the adult PNS that mimic those characteristic of *Brd* gain-of-function alleles. These alleles are associated with an elevated accumulation of *Brd* transcript (Leviten et al., 1997), strongly suggesting that the mutant transgene likewise exhibits elevated *Brd* gene activity. Despite the clear functional difference between the wild-type and mutant transgenes in this assay, only a minority of mutant transgenic lines displayed gain-of-function phenotypes. Our interpretation is that mutation of the Brd and GY boxes raises *Brd* gene activity to near a threshold, beyond which PNS development (specifically, lateral inhibition in

proneural clusters) becomes compromised; insertion site-specific position effects would then determine whether a phenotypic defect is observed. We note that, since the presence of three doses of the wild-type *Brd* locus fails to cause a detectable mutant phenotype (Leviten and Posakony, 1996), *Brd* gain-of-function effects appear to result from at least a two-fold increase in *Brd* activity.

Our results establish that *Brd* is normally regulated by its Brd box elements (and probably by its GY box motif) and that such regulation is important for proper specification of cell fates in adult PNS development. The E(spl)-C functions in many of the same PNS cell fate decisions as are affected by *Brd* gain-of-function alleles, and several genes of this complex are co-expressed with *Brd* specifically in imaginal disc proneural clusters, under overlapping transcriptional control (Bailey and Posakony, 1995; de Celis et al., 1996; Singson et al., 1994). These genes include not only those that encode



**Fig. 5.** Bristle multiplication phenotype of flies carrying a *Brd* transgene with mutations in its Brd and GY boxes. Shown are scanning electron micrographs of (A,B) heads, (C,D) nota and (E,F) pleurae dissected from adult flies. (A,C,E) Wild-type (*w<sup>118</sup>*) flies display singularized mechanosensory bristles in all of these regions of the body surface. (B,D,F) Two of seven lines carrying a *Brd* genomic DNA transgene with mutations in its three Brd boxes and single GY box display fully penetrant bristle multiplication (tufting) phenotypes including strong effects on (B) head (orbital and ocellar) macrochaetes (line *mut3*), (D) anterior dorsocentral macrochaetes (line *mut3*) and (F) anterior sternopleural macrochaetes (line *mut5*). None of 11 lines homozygous for a wild-type *Brd* transgene exhibited any bristle multiplication. Note that as is characteristic of *Brd* gain-of-function phenotypes (Leviten and Posakony, 1996), supernumerary bristles are present as tufts at the normal locations of single bristles; sensory organs are never found in ectopic territories. Tufts generally contain 4-7 supernumerary bristles.



**Fig. 6.** Sequence alignment of the 3' UTRs of *D. melanogaster* and *D. hydei E(spl)m4*. Vertical lines connect identical nucleotides in the two sequences (only regions containing at least three consecutive identical bases are shown); bold letters highlight specific conserved regions. The seven nucleotides of both GY box 1 and Brd box 2 (Leviten et al., 1997) are fully conserved between these homologous genes; single-base changes are found in the first position of Brd box 1 and the last position of GY box 2. Note that, in most cases, nucleotides flanking the Brd box and GY box motifs are also conserved, but that the sequence is highly diverged outside of these regions. One exception is the conserved hexamer TGTGAT (just downstream of Brd box 2), also indicated in bold. We have found that this sequence is present in either one or two copies in the 3' UTRs of several E(spl)-C genes (E. C. L. and C. Burks, unpublished observations), and we are currently investigating its possible functional significance. The region including and surrounding the putative polyadenylation signal sequence (pA Sig) is also well conserved. We have determined by sequence analysis of an apparently full-length cDNA clone (unpublished observations) that *D. melanogaster E(spl)m4* transcripts are polyadenylated 16 nt downstream of this element (pA Site; underlined A), strongly suggesting that it functions as the polyadenylation signal in this transcription unit, despite its non-canonical sequence (AATATA instead of AATAAA).

bHLH transcriptional repressors, but also *E(spl)m4*, which encodes a small protein related to the Brd gene product. The presence of Brd box and GY box sequence elements, often in multiple copies, in the 3' UTRs of most E(spl)-C genes makes it highly likely that these genes are subject to the same negative regulatory activity as *Brd* (Leviten et al., 1997). Further support for this conclusion is provided by our finding that Brd and GY box sequences are specifically conserved in the 3' UTRs of the *E(spl)m4* homologues from two widely diverged species.

### Brd boxes mediate a temporally and spatially general mode of negative regulation

Transcript accumulation from *Brd* and the genes of the E(spl)-C occurs in a spatially and temporally restricted pattern in both embryos and larvae. In particular, the transcriptional activity of these genes is precisely controlled in space and time by the proneural bHLH activator proteins and by Su(H) (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995; Singson et al., 1994). Nevertheless, using an *arm-lacZ* reporter gene, we have shown that the *Brd* 3' UTR, and specifically the Brd box motif, confers negative regulation on heterologous transcripts in most, if not all, cells throughout development. A general mode of post-transcriptional negative regulation is mediated by AU-rich elements (AREs) (reviewed by Chen and Shyu (1995)). These elements, typified by AUUUA motifs, are thought to confer transcript instability via a deadenylation mechanism. It is interesting to note that the AUUUA sequence would be largely included in the Brd box motif, AGCUUUA. It is possible that the Brd box represents a bipartite site that is bound by both an ARE-binding protein and another factor. At least ten different proteins have been shown to bind with high affinity to AREs and/or AUUUA repeats in vitro, though only one of them has been shown to stimulate RNA degradation in an in vitro assay (Brewer, 1991; Chen and Shyu, 1995). Perhaps one of these identified proteins functions on Brd boxes and thus requires a partner protein.

However, it is equally possible that the Brd box mediates a wholly distinct form of negative post-transcriptional regulation.

The observation that most or all cells are able to respond to Brd boxes (and possibly to the GY box as well) suggests that some genes expressed outside of proneural clusters may be regulated by these motifs as well. In this regard, we were interested to find that three other genes that encode negative regulators of PNS development also contain these sequences in their 3' UTRs. In particular, *kuzbanian (kuz)* and *extramacrochaetae (emc)* each include single Brd boxes, while *hairy (h)* contains a GY box (Ellis et al., 1990; Garrell and Modolell, 1990; Rooke et al., 1996; Rushlow et al., 1989). *emc* also includes four copies of a GY box-related sequence (GTTTTCC) in its 3' UTR, which may be relevant for its regulation. *kuz* has functions in SOP selection and lateral inhibition, so its expression certainly includes proneural clusters. However, *emc* and *h* are expressed in spatial patterns that are largely complementary to proneural clusters in the leg and wing imaginal discs, and are thus possible examples of genes regulated by the Brd box (and possibly the GY box) in territories outside the clusters. Interestingly, the *emc* and *h* proteins, as members of the HLH family, are structurally related to the E(spl)-C bHLH proteins. In contrast, *kuz* encodes a metalloprotease/disintegrin protein of the ADAM family (Rooke et al., 1996).

We note that *Achaetous (Ach)*, a dominant gain-of-function allele of *emc*, is associated with a transposon insertion, which results in a truncated transcript. The truncated mRNA lacks the codons for the C-terminal 42 amino acids, as well as the entire wild-type 3' UTR (Garrell and Modolell, 1990). *Ach* is the only known gain-of-function allele of *emc* and we speculate that the loss of 3' UTR sequences (including the Brd box and the GY-like motifs) may contribute to this hypermorphic effect.

### The mechanism of Brd box-mediated regulation

Clearly, much remains to be learned about the mechanism under-

lying Brd box-mediated post-transcriptional regulation. In the current study, we have shown that these sequence elements exert a negative effect on steady-state transcript levels in both total and polyadenylated RNA populations, and also negatively regulate steady-state protein levels. Since a greater quantitative effect is observed at the protein level, it is possible that translational repression is the primary function of the Brd box, with effects on transcript stability representing a secondary, indirect effect. For example, the presence of Brd boxes may cause a transcript to be inefficiently loaded onto ribosomes (or actively inhibited therefrom); unloaded transcripts might be secondarily more susceptible to degradation. However, it is also conceivable that the Brd box motif exerts its primary effect on the polyadenylation state of a transcript; for instance, by controlling the length of the poly(A) tail. Deadenylation itself can promote mRNA degradation, while increases or decreases in the length of the poly(A) tail can activate or repress translation, respectively (Bouvet et al., 1994; Salles et al., 1994). Finally, it is possible that Brd boxes have independent effects on different regulatory levels. Whatever the mechanism of Brd box function, it is clear that it does not depend upon specific sequences in the 5' UTR or other regions of the gene, as we have shown that heterologous reporter genes expressed from two different promoters are subject to efficient Brd box-mediated negative regulation.

Any consideration of the mechanism of Brd box or GY box regulation will necessarily address the possibility of *trans*-acting factors that mediate their action. There is of course ample precedent for conserved sequence motifs in RNA representing protein binding sites, and it is reasonable to imagine that the Brd box and/or GY box elements are bound by sequence-specific RNA-binding proteins. Ongoing UV-crosslinking studies are aimed at identifying proteins with specificity for these sites. However, we are also struck by the length (seven nucleotides) of the strict consensus sequences defined by the Brd box and GY box motifs, which is unusual by the standard of typical RNA-binding proteins. Although we cannot rule out the possibility that other slightly degenerate versions of these motifs may also have equivalent functions, their conservation between *D. melanogaster* and *D. hydei* suggests that the identity of all or nearly all of the nucleotides is important for Brd box and GY box function. It is thus useful to consider the possibility that these elements represent sites of RNA:RNA interaction. The nematode *lin-14* and *lin-28* genes are known to be translationally repressed via sequence motifs in their 3' UTRs which are bound by small RNAs encoded by the *lin-4* gene (Ha et al., 1996; Lee et al., 1993; Moss et al., 1997), providing a clearly relevant precedent. An additional possibility is that Brd boxes and/or GY boxes form part of a larger RNA secondary structure that is itself the site of protein or RNA interaction, so that these motifs are not directly bound by any *trans*-acting factors. However, a preliminary computer analysis of some of the transcripts that bear Brd and GY boxes does not reveal these elements to be consistently associated with any obvious predicted secondary structures (unpublished observations).

### Multiple modes of regulation for genes involved in adult PNS development in *Drosophila*

Cell fate choices during adult sensory organ development in *Drosophila* are characteristically sensitive to relatively small changes in the activities of the regulatory genes involved. For example, three of the very small number of haplo-insufficient

loci in the genome (*N*, *Dl* and *H*; Lindsley et al. (1972)) have essential roles in this process, and many other strong gene-dosage sensitivities have been documented (e.g., Botas et al. (1982) and Ashburner (1982)). This suggests the need for precise regulation of the expression of genes that direct PNS development. The transcriptional control of a number of these genes has been well studied, but their possible post-transcriptional regulation has not been characterized. The present work reveals a role for specific 3' UTR sequence motifs, shared by many genes involved in sensory organ development, in negative post-transcriptional regulation. Such an activity may serve generally to maintain rapid responsiveness to transcription-level modulation. In addition, such regulation may be necessary for the proper spatial or cell-type-specific activity of these genes. For example, while the promoters of E(spl)-C bHLH repressor genes are directly activated by proneural proteins (Kramatschek and Campos-Ortega, 1994; Singson et al., 1994), their RNA and protein products do not accumulate to high levels in the SOP, where proneural activity is highest. Although we did not detect strong spatial variation in Brd box-mediated regulation, it may nevertheless be involved in such cell-type-specific negative control. Other sequence elements and/or *trans*-acting factors may augment Brd box function at very specific times or in specific cells that are not resolved by the reporter gene activity assay used in this study.

We have shown here that loss of Brd box/GY box-mediated regulation from a single gene, *Brd*, is sufficient to cause phenotypic defects in the adult PNS. Extrapolation from this example leads us to imagine that much more severe developmental consequences would result if all of the genes that include these motifs were to be relieved of such regulation. Thus, it seems clear that both transcriptional and post-transcriptional modes of control are critical for the normal development of the *Drosophila* PNS.

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