Gene Expression Patterns 5 (2005) 411–421

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Distinct developmental expression of *Drosophila* retinoblastoma factors

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Received 22 October 2003; received in revised form 7 September 2004; accepted 8 September 2004
Available online 26 October 2004

Abstract

Retinoblastoma (RB) tumor suppressor proteins are important regulators of the cell cycle and are implicated in a wide variety of human tumors. Genetic analysis of *RB* mutations in humans and in model systems indicates that individual RB proteins also have distinct functions in development. Specific target genes or mechanisms of action of individual RB proteins in developmental contexts are not well understood, however. To better understand the developmental activities of the two RB family members in *Drosophila*, we have characterized endogenous expression patterns of Rbf1 and Rbf2 proteins and transcripts in embryos and imaginal discs. These gene products are coexpressed at several stages of development, however, spatial and temporal differences are evident, including partly complementary patterns of expression in the embryonic central nervous system.

Keywords: Retinoblastoma; *Drosophila*; Rbf; Transcriptional repression

Retinoblastoma (RB) tumor suppressor proteins regulate the cell cycle, differentiation, apoptosis, and growth by controlling the transcription of key genes that in turn regulate those processes. In mammals, the RB family comprises the RB, p107, and p130 proteins. Numerous studies have focused on the roles of RB in disease states, but all of the RB proteins play important roles in development as well. The mouse, RB, p107 and p130 are expressed in dynamic patterns during development and are associated with neurogenesis, hematopoiesis, and eye formation (Cobrinik et al., 1996; Jacks et al., 1992; Zacksenhaus et al., 1996; Jiang et al., 1997; Nikitin et al., 1999). RB is expressed in both mitotically active and inactive regions of the central nervous system (CNS), while p107 expression is limited to areas containing proliferating cells, and p130 expression is relatively low and diffuse. $RB^{-/-}$ embryos show elevated apopotosis and failure to arrest cellular divisions in the nervous system (Jacks et al., 1992). The phenotypes exhibited by p130, p107, and RB mutants are complex and are highly dependent on the genetic background; homozygous mutants are lethal in some backgrounds but not others (LeCouter et al., 1998a,b). Interpretation of mutant phenotypes is further complicated by the cell non-autonomy of RB activity in the mouse, whereby some central nervous system effects were found to be cell non-autonomous, dependent on disruption in placental function (Wu et al., 2003; MacPherson et al., 2003).

A common feature of RB family proteins is the 'pocket domain', the region required for controlling E2F- factor mediated transcription (Chow and Dean, 1996). The pocket domain comprises two sub-domains, the A and B regions, characterized by the presence of cyclin fold motifs, which mediate numerous protein–protein interactions required for RB function. Biochemical studies indicate that RB acts as an adapter to link a variety of effector proteins into complexes to control transcription (Welch and Wang, 1995; Zhang et al., 2000). RB itself is not a DNA-binding protein, but represses transcription by interacting with the DNA binding E2F transcriptional factor comprised of E2F and DP proteins, which directly recognize cognate promoter

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doi:10.1016/j.modgep.2004.09.005

elements in responsive genes (Weintraub et al., 1992). In vertebrates, there are six E2F proteins (E2F1-6) that can complex with one of two DP proteins (DP1-2) to form a functional heterodimer (Dyson, 1998). The best-studied E2F complexes, containing E2F1, are potent activators of gene transcription by RNA polymerase II. When RB-E2F complexes bind, however, E2F target promoters are repressed. Interactions between E2F and RB are regulated by changes in RB phosphorylation mediated by cyclins D and E in association with cyclin dependent kinases (Hinds et al., 1992; Kato et al., 1993; Ewen et al., 1993). RB phosphorylation leads to release of E2F factors and relief of repression. The RB protein p130 is also regulated by proteolytic turnover, which may explain the need for new synthesis at critical points in development (Tedesco et al., 2002).

The *Drosophila* RB system contains fewer components than the vertebrate counterpart, but essential features appear to be conserved. Two RB family proteins, Rbf1 and Rbf2, interact with one DP protein and two E2F proteins, E2F1 and E2F2 (Dynlacht et al., 1994; Du et al., 1996; Cayirlioglu et al., 2001; Stevaux et al., 2002; Dimova et al., 2003). Cyclin D and E proteins regulate Rbf1 activity, as with vertebrate RB (Xin et al., 2002). Although fewer studies of RB proteins have been carried out in *Drosophila* compared to vertebrates, the relative simplicity of this model system may facilitate analysis of developmental functions of RB proteins.

In cell culture assays, Rbf2 appears to preferentially act through E2F2, a factor that has been suggested to play a dedicated repressive role, while Rbf1 interacts with both E2F1 and E2F2 (Stevaux et al., 2002). Mutants for *rbf1* show deregulated E2F transcription of cell cycle regulated promoters such as *RNR* and *PCNA*, and exhibit ectopic entry into S phase (Du and Dyson, 1999). Rbf1 has also been suggested to control the rate of S phase progression and has been implicated in control of cellular growth, as opposed to proliferation (Xin et al., 2002). No mutation in *rbf2* has yet been reported, but misexpression of Rbf2 together with E2F2 in *Drosophila* wing and eye discs blocks cell entry into S phase and disrupts development (Stevaux et al., 2002). Rbf function has been closely studied in control of eye imaginal disc development in the third instar larvae. In the eye disc, a progressively moving indentation, the morphogenetic furrow, marks a wave of differentiation that sweeps across the disc. Mitosis is suppressed in the furrow, and just posterior to the furrow a synchronized mitosis, regulated by the Rbf pathway, occurs in a narrow band (Wolff and Ready, 1993; Xin et al., 2002). Following passage of the morphogenetic furrow, cells undergo terminal differentiation into various ommatidial components, and exit the cell cycle (Wolff and Ready, 1993). Rbf factors are also involved in control of apoptosis in the embryo and control of origin of replication firing during endoreplication in follicle cells (Bosco et al., 2001; Cayirlioglu et al., 2001). These studies reinforce the notion that central RB protein functions are widely conserved and are employed at many points in development.

Of ~13,500 genes examined, expression of only one was elevated in Rbf2 depleted *Drosophila* SL2 cells but not in Rbf1 depleted cells, and expression of that gene was further elevated in cells depleted for both Rbf1 and Rbf2, suggesting that there are few, if any, Rbf2 specific target genes expressed in these cells (Dimova et al., 2003). The extent to which *Drosophila* Rbf proteins serve overlapping or distinct functions in the developing animal remains poorly understood, however. In this study we examine the temporal and spatial Rbf1 and Rbf2 transcript and protein expression patterns during *Drosophila* development, noting points at which these genes may play overlapping or discrete roles. Rbf1 is present at all stages of embryonic development, while Rbf2 shows a peak level of expression during early stages. In the eye disc, both are markedly reduced following the initiation of terminal differentiation.

1. Results

1.1. Generation of anti-Rbf1 and Rbf2 antibodies

Antibodies were raised against synthetic peptides corresponding to an internal epitope of Rbf1 (amino acids 452–465) and the C-terminus of Rbf2 (amino acids 763–783). Expression plasmids encoding full-length Rbf1 (pET-Rbf1) and Rbf2 (pET-Rbf2) were created for protein over-expression in *E. coli*. Characterization of the recombinant protein expression and the α-Rbf1 and α-Rbf2 antibodies is shown in Fig. 1. Western blots performed with α-Rbf1 antibodies detected a single protein of ~90 kDa in 0–12 h

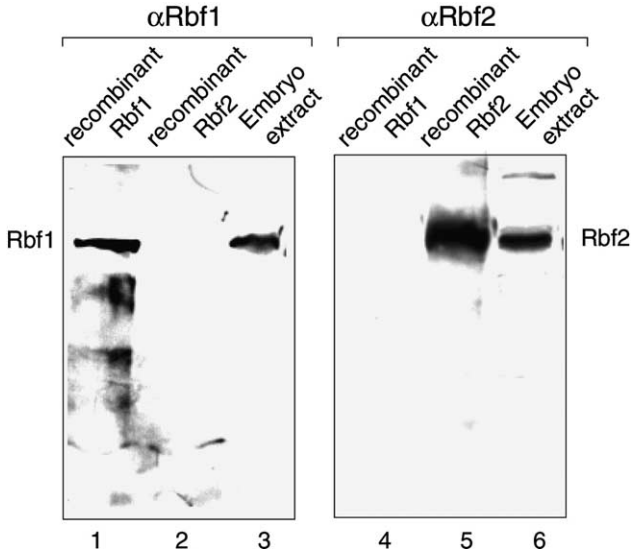

Fig. 1. Rbf antibodies specifically recognize their cognate proteins in *Drosophila* embryo extracts. Anti-Rbf1 (lanes 1–3) and anti-Rbf2 (lanes 4–6) sera were tested against recombinant Rbf1 (lane 1, 4), recombinant Rbf2 (lane 2, 5), and endogenous Rbf proteins in embryonic nuclear extracts (lanes 3, 6).

Drosophila embryo nuclear extracts (Fig. 1, lane 3) that comigrated with the major band present in the recombinant Rbf1 sample (Fig. 1, lane 1). No proteins were detected in the recombinant Rbf2 sample (lane 2), demonstrating that the α-Rbf1 antibodies specifically recognize Rbf1 and do not cross-react with Rbf2. Similar Western blot experiments performed with α-Rbf2 antibodies revealed a cluster of proteins around 85 kDa in embryo extracts (Fig. 1, lane 6, and Fig. 2). The 85 kDa proteins co-migrate with the protein detected in the recombinant Rbf2 sample (Fig. 1, lane 5), and nothing was detected in the recombinant Rbf1 sample (Fig. 1, lane 4). Therefore, both the α-Rbf1 and α-Rbf2 antibodies specifically recognize their cognate full-length Rbf target proteins in *Drosophila* embryo nuclear extracts. Independently generated monoclonal antibodies to Rbf1 and Rbf2 (Stevaux et al., 2002) detected species of the same size in immunoprecipitates produced using our α-Rbf1 and α-Rbf2 antibodies (data not shown).

1.2. Rbf1 and Rbf2 expression during Drosophila embryonic development

After verifying that the Rbf1 and Rbf2 antibodies specifically recognize their cognate target proteins, an analysis of Rbf1 and Rbf2 protein expression during embryogenesis was performed. Whole cell extracts were prepared from Canton S embryos harvested at 2 h intervals for Western blot analysis using α-Rbf1 and α-Rbf2 antibodies. As shown in Fig. 2A, Rbf1 protein was expressed at relatively uniform levels throughout the 0–20 h time period, whereas a peak of Rbf2 expression was observed between 4 and 10 h followed by a gradual decline in levels during the remainder of the time course. Rbf1 was also detected in adults (both male and female); however, Rbf2 protein expression was significantly reduced in adult females, and was undetectable in adult males (Fig. 2B). These results indicate that Rbf1 and Rbf2 proteins are present at different levels during the course of *Drosophila* embryonic development, consistent with a previous report in which antibody specificity was not shown (Stevaux et al., 2002). These differences in expression levels, together with differential expression patterns (below), are consistent with the idea that the two proteins may have different functions during embryogenesis.

We examined the tissue distribution of Rbf1 and Rbf2 protein expression during embryonic development by whole mount immunohistochemistry (Fig. 3). Both Rbf1 and Rbf2 proteins are widespread during early development. Comparing staining with control preimmune sera to that of postimmune indicated that in the serum samples, the reactive antibodies were present only following exposure

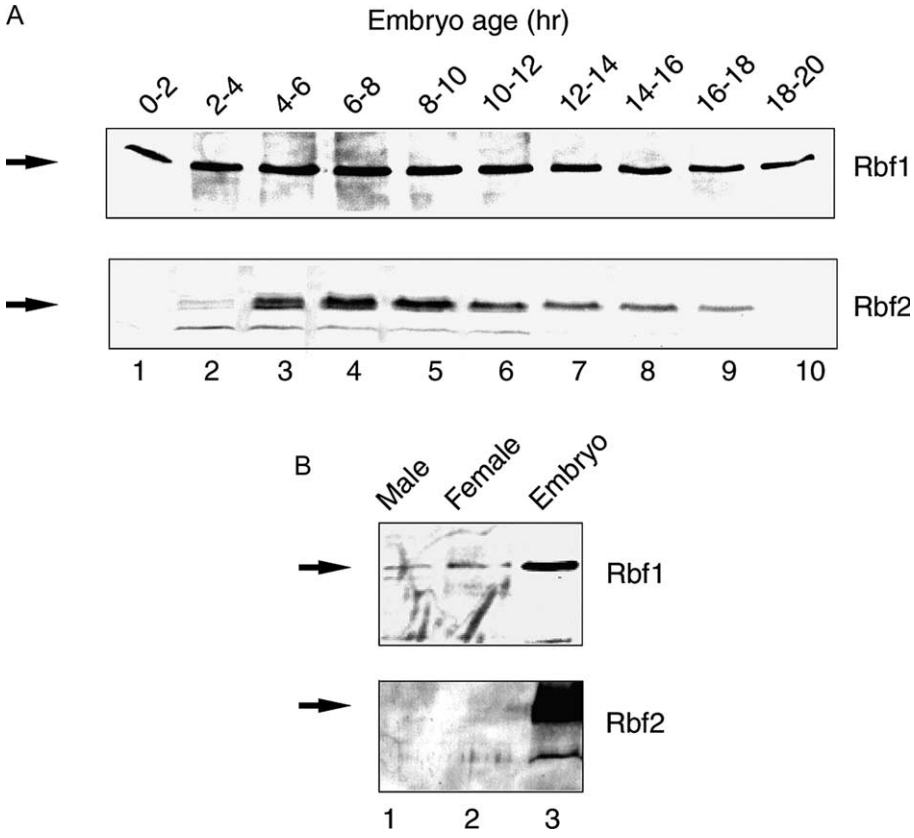

Fig. 2. Rbf1 and Rbf2 are expressed in dynamic patterns during development. Whole cell extracts were prepared from *Drosophila* embryos (top) collected at the indicated time points and were probed for Rbf1 and Rbf2 by Western blot analysis. The bottom panels indicate the levels of Rbf1 and Rbf2 in extracts prepared from adult flies. 180 μg of total protein was used for detection of Rbf2 in adults, compared to 50 μg for Rbf1.

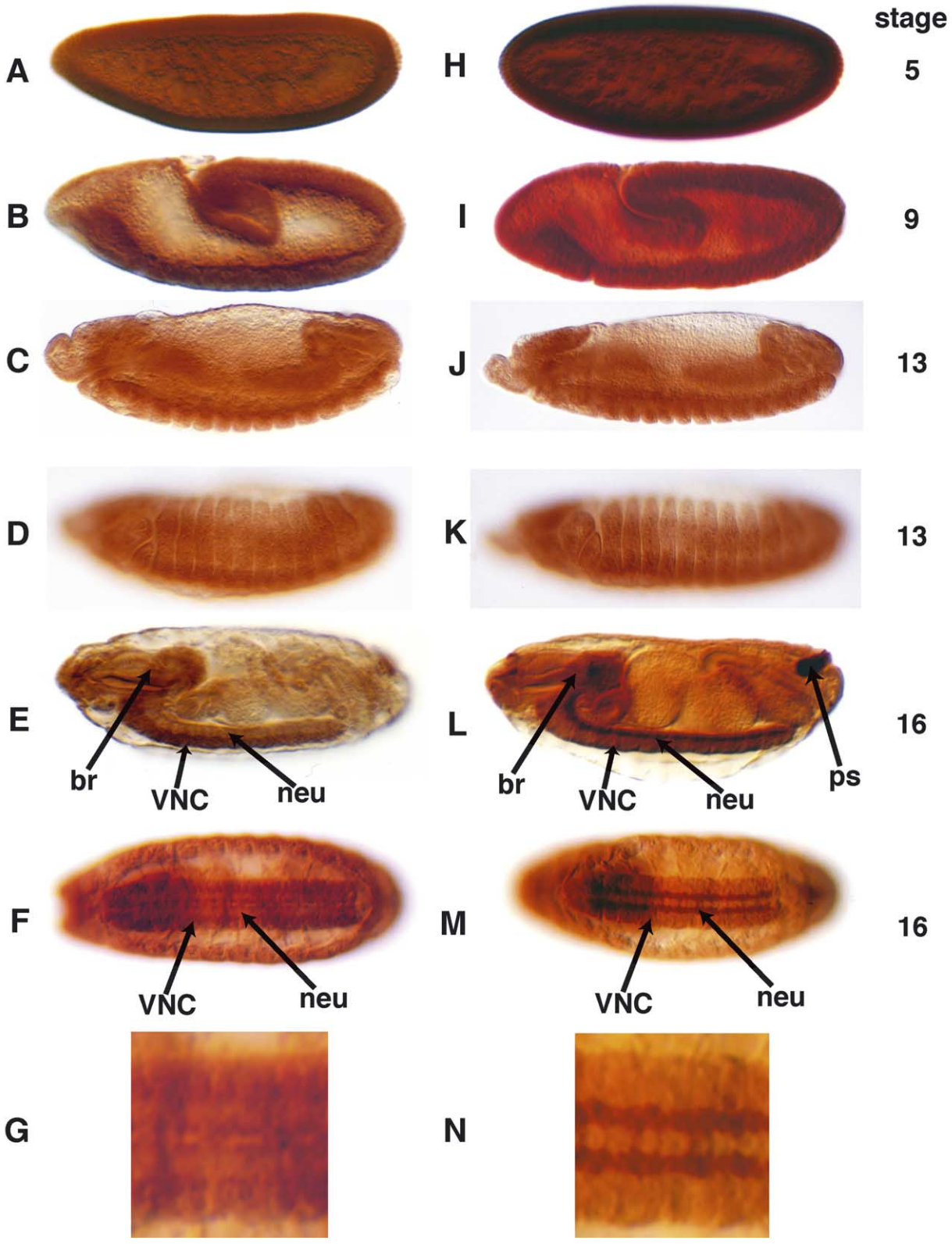

Fig. 3. Dynamic patterns of Rbf protein expression revealed by whole mount immunohistochemistry of *Drosophila* embryos. Embryos were stained with anti-Rbf1 (A–G) or anti-Rbf2 (H–N). Widespread, overlapping expression patterns early in development later give way to complementary patterns of expression in the central nervous system (high magnification of ventral nerve cord shown in G and N). A surface plane of focus is shown for stage 13 only, and shows widespread epidermal staining (C,I). Stages of development are indicated at right. All embryos are oriented anterior to left and dorsal up except F, G, M and N, which are oriented with the ventral surface to viewer. Abbreviations: br, supraesophageal ganglion (brain); VNC, ventral nerve cord; neu, neuropile; ps, posterior spiracle.

Fig. 4. Specificity of α-Rbf1 (B) and α-Rbf2 antibodies (D). Stage 9 embryos were stained with 1:500 dilutions of preimmune (A,C; pre) or post immunization (B,D imm) rabbit serum. Ectopic Gal4-Rbf2 expressed in a central circumferential blastoderm stripe is readily detected by anti-Rbf2 antiserum (E), providing a further measure of the specificity of this serum.

to the target antigens. In an additional control embryo, the expression of a Gal4-Rbf2 fusion protein in a central blastoderm stripe demonstrated further the specificity of this antiserum (Fig. 4). Following germ band shortening, both Rbf1 and Rbf2 are concentrated in the gut, epidermis, and developing CNS, with CNS staining continuing late into embryonic development. Intriguingly, a pronounced difference in the CNS distribution of Rbf1 and Rbf2 is observed following condensation of the ventral nerve cord (Fig. 3E–G,L–N). Rbf2 is detected at high levels in the neuropile. The longitudinal connectives and lateral commissures stain darkly relative to the surrounding nerve (Fig. 3L–N). In contrast, the neuropile is less well stained by anti-Rbf1 antibodies (Fig. 3E–G). The lighter stained connectives and commissures stand out in relief against the darker cord. The Rbf1 CNS staining was absent in one-quarter of embryos derived from *rbf1*$^{\Delta 14}$ null mutant stocks, consistent with the expected absence of zygotic expression in one half of the male embryos (data not shown). The pattern of Rbf1 and Rbf2 neuronal expression is similar to the distinct, but overlapping, pattern of RB and p107 expression in the mouse CNS (Jiang et al., 1997). In the mouse CNS, a knockout mutation in *RB*, but not *p107*, leads to elevated apoptosis and specific CNS defects (Jacks et al., 1992; Lee et al., 1996) that suggest these proteins, and perhaps the Rbf1 and Rbf2 counterparts, have distinct functions in development of this tissue.

RNA expression patterns were also examined using antisense probes for *rbf1* and *rbf2* in in situ hybridizations (Fig. 5). In both cases, the RNA pattern detected by the full-length probe was confirmed using probes for only 5′ and 3′ sequences (data not shown). Both RNAs were widespread early in development (Fig. 5A–C,G–I). The entire germ band is stained until the beginning of segmentation and germ band shortening. Beginning with germ band retraction, high levels of *rbf1* mRNA become limited to the midgut, hindgut, and the developing Malpighian tubules (Fig. 5D–F). The gut expression is maintained at least until late embryonic stages. Although the proventriculus is darkly stained, there is little other staining of the foregut. Subsequent to germ band retraction, *rbf2* mRNA becomes limited to the developing CNS before disappearing late in development (Fig. 5I–L).

Unlike the patterns of *rbf1* transcripts in the embryo published online by the Berkeley *Drosophila* Genome Project (http://www.fruitfly.org/cgi-bin/ex/insitu.pl), we did not observe a virtually complete absence of *rbf1* transcripts at stage 4–6. However, other aspects of *rbf1* and *rbf2* mRNA distribution are in agreement with those studies, including the strong gut expression of *rbf1*.

Early patterns of mRNA closely mirror distributions of proteins, while the later high levels of *rbf1* transcription in gut are not reflected in similar protein accumulations. The identical results obtained with 5′ and 3′ probe sequences suggest that the patterns we observe do in fact correspond to *rbf1* and *rbf2* transcripts. Lack of protein accumulation in regions of active *rbf* transcription may indicate proteolytic turnover, as has been seen for p130 (Tedesco et al., 2002).

1.3. rbf1 and rbf2 expression in imaginal discs

RNA expression patterns in the third instar larval imaginal disks were also examined by in situ hybridizations (Fig. 6A–C,E–G). The expression patterns of both family members were quite similar. Transcripts for each were present relatively uniformly throughout the leg, wing, and haltere disks, similar to the pattern observed for the proteins (data not shown). The eye-antennal disc, in contrast, showed a distinct pattern. Both *rbf1* and *rbf2* were expressed at highest levels in a pair of stripes flanking the morphogenetic furrow, in a region where previously asynchronous cells enter a coordinated

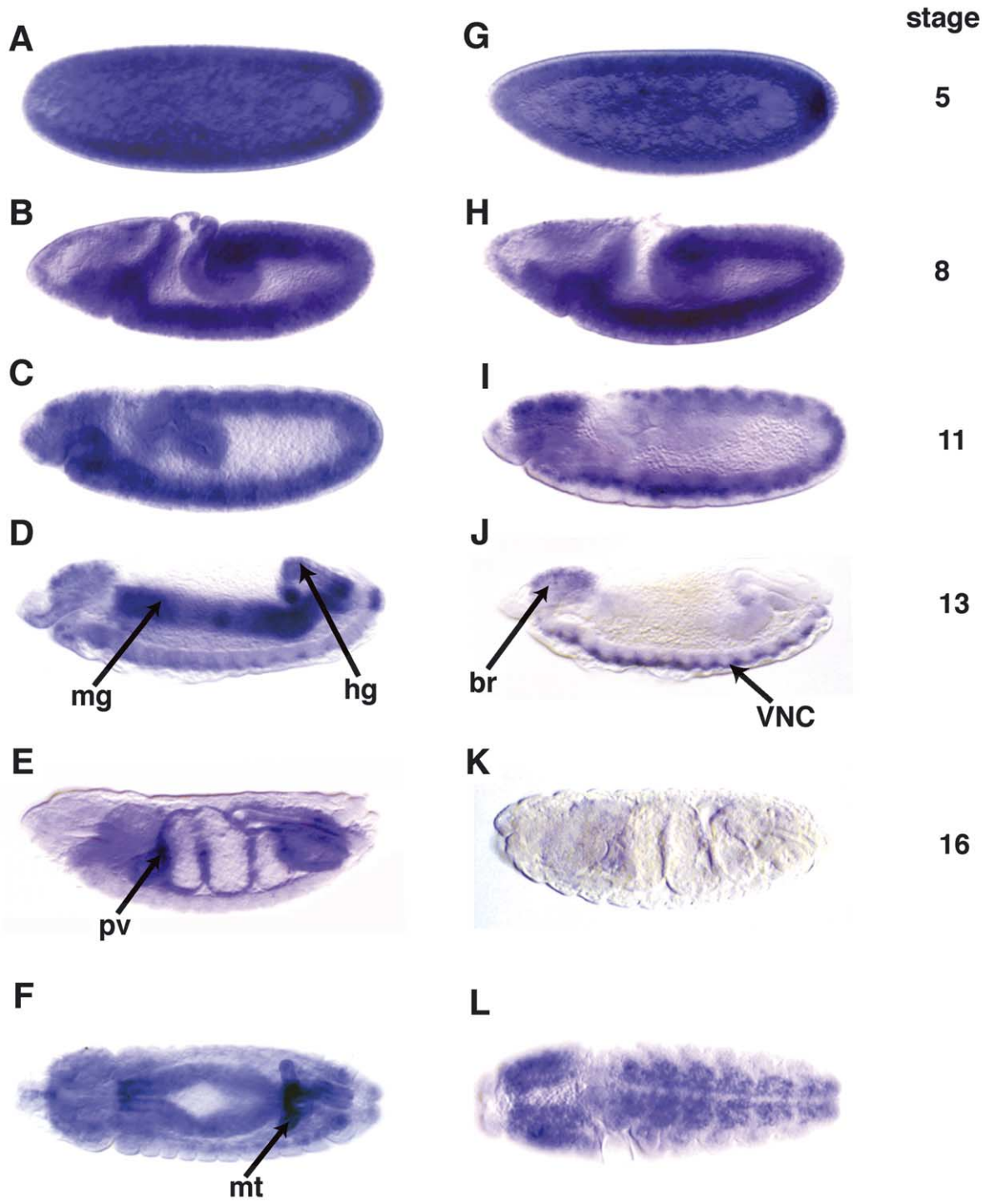

Fig. 5. *rbf1* and *rbf2* transcript distribution in embryos revealed using whole mount in situ hybridization. Embryos were stained using probes to *rbf1* (A–F) or *rbf2* (G–L). Stages of development are shown at right. Early widespread, overlapping patterns of expression of *rbf1* and *rbf2* later give rise to more specific patterns by stage 13. Arrows highlight specific gut expression of the *rbf1* gene and nervous system expression of the *rbf2* gene. The view shown in L highlights the restriction of the *rbf2* pattern to the developing CNS. All embryos are oriented anterior to left and dorsal up, except for the dorsal views of a stage 13 (F) and a stage 11 (L) embryo. Abbreviations: mg, midgut; hg, hindgut; pv, proventriculus; mt, Malpighian tubules; br, supraesophageal ganglion (brain); VNC, ventral nerve cord.

mitotic event. In the most posterior parts of the eye disk, levels of *rbf1* and *rbf2* transcripts were low or undetectable. Anterior to the furrow, transcript levels were higher. Protein was detected within and anterior to the morphogenetic furrow (Fig. 6G,H). Most cells in posterior regions of the disc had undetectable levels of Rbf1 and Rbf2, suggesting that Rbf proteins are not required in these stably differentiated cells. The identity

of the few cells that maintain protein levels in this region has not been determined.

The observation that transcript levels are higher in two stripes flanking the morphogenetic furrow is consistent with increased transcription of *rbf1* and *rbf2* in cells entering S phase. The increased levels of *rbf1* and *rbf2* transcripts in these cells are not apparently associated with dramatic increases in protein levels, as was noted for *rbf1* expression in the gut. Higher magnification views of Rbf1 and Rbf2 protein expression in the region of the furrow confirm that total Rbf1 protein levels do not reflect the transient increases of mRNAs (Fig. 6I,J).

2. Discussion

2.1. Distinct developmental roles for Rbf1 and Rbf2?

In the mouse, RB family members have both distinct and partially overlapping functions and patterns of expression (Jiang et al., 1997). The lack of redundancy among RB proteins at several stages of development can be accounted for by differential expression patterns of these proteins as well as functional dissimilarities, such as preferential interaction with certain E2F family members. Different sets of E2F target genes are derepressed on mutation of RB or p107 and p130 (Hurford et al., 1997). Evidence for such preferential interaction comes from a recent microarray study found that in a human cell line, RB protein is generally associated with promoters bound by E2F1 (Wells et al., 2002). Another possible mechanism by which functional specificity might be achieved is through RB protein interaction with DNA binding factors other than E2F proteins.

Likewise, several lines of evidence lead to the conclusion that *Drosophila* Rbf1 and Rbf2 are likely to have distinct roles in development. Previous work has identified functional differences between the two proteins, for instance, Rbf1 is a more abundant and more potent repressor in cell culture assays, and this protein generates more severe phenotypes when overexpressed in *Drosophila* (Stevaux et al., 2002). In addition, Rbf2 appears to interact preferentially with E2F2, while Rbf1 interacts with both E2F1 and E2F2 (Stevaux et al., 2002). Both Rbf1 and Rbf2 appear to play roles in regulation of some genes, for example, both can be found binding to the *PCNA* and *Polα* promoters in cell culture assays (Stevaux et al., 2002), and as we show, both genes are coexpressed in many tissues during embryonic development (Figs. 3 and 5). In a survey of genes misregulated in SL2 cells when depleted of either or both of Rbf1 and Rbf2, few if any genes specifically regulated by Rbf2 were identified, although a requirement for Rbf2 was unmasked in cells depleted for Rbf1, suggesting significant functional redundancy (Dimova et al., 2003). However, Rbf2 was unable to complement Rbf1 reduction on targets that were E2F1, but not E2F2 regulated, consistent with a specific requirement for E2F1/Rbf1 for some genes. These cultured cell studies do not address the question of whether the two proteins have specific developmental functions. However, the lethality of *Rbf1* mutations indicates that Rbf2 cannot completely substitute for Rbf1 function in vivo, consistent with the non-redundancy of the two proteins. The temporal variation of Rbf2 protein during embryogenesis (Fig. 2, Stevaux et al., 2002), in contrast to the more constant levels of Rbf1, is also consistent with Rbf2 playing a distinct role in development. Alternatively, the induction of protein levels might merely reflect the need for higher total levels of Rbf proteins, rather than a specific requirement for Rbf2, and the failure of Rbf2 to complement Rbf1 mutations could result from reduced quantity of Rbf proteins. Importantly, we show here for the first time that the *Drosophila* Rbf1 and Rbf2 proteins exhibit distinct patterns of expression at certain points in development (Fig. 4). These findings raise the possibility that Rbf2 serves a specific function in the embryonic CNS, either supplementing the low levels of Rbf1, or providing a distinct activity in these cells. Reduced Rbf1 activity in neurons may be requisite for enhanced Rbf2 expression, consistent with the increased levels of Rbf2 seen in Rbf1 depleted SL2 cells (Dimova et al., 2003).

The observation that Rbf2 protein is present at high levels in the neuropile, largely composed of axon bundles, was unexpected. Current models of RB function have highlighted the transcriptional repression activities and association with replicating DNA, phenomena occurring only in the nucleus. Although it remains a possibility that our antibody is detecting another protein in the neuropile, we have demonstrated that there is little, if any, cross-reactivity when this serum is applied to Western analyses. Furthermore, BLAST analysis of the peptide sequence used to generate our antibody results in no hits other than Rbf2 in the database of predicted proteins. These results suggest Rbf2 may have a novel, non-nuclear role.

2.2. Transcriptional induction of rbf1 and rbf2 in eye imaginal disc development.

During development of the adult eye, as individual photoreceptor cell clusters differentiate, an indentation in the eye imaginal disc, the morphogenetic furrow, sweeps across the disc from posterior to anterior. As the furrow approaches, cells that have previously been undergoing asynchronous mitosis arrest in G1. A subset of cells posterior to the furrow then undergo another, synchronized round of mitosis, which is easily identified by BrdU labeling, and corresponds to an upregulation of the *cyclinE* and *PCNA* genes (Richardson et al., 1995; Du, 2000). The induction of the *rbf1* and *rbf2* genes in the eye imaginal disc in the cells that are flanking the morphogenetic furrow (Fig. 6A,E) suggests the transcriptional induction of these genes is linked to the mitotic program. Within the furrow, misexpression of Cyclin E protein can drive cells into

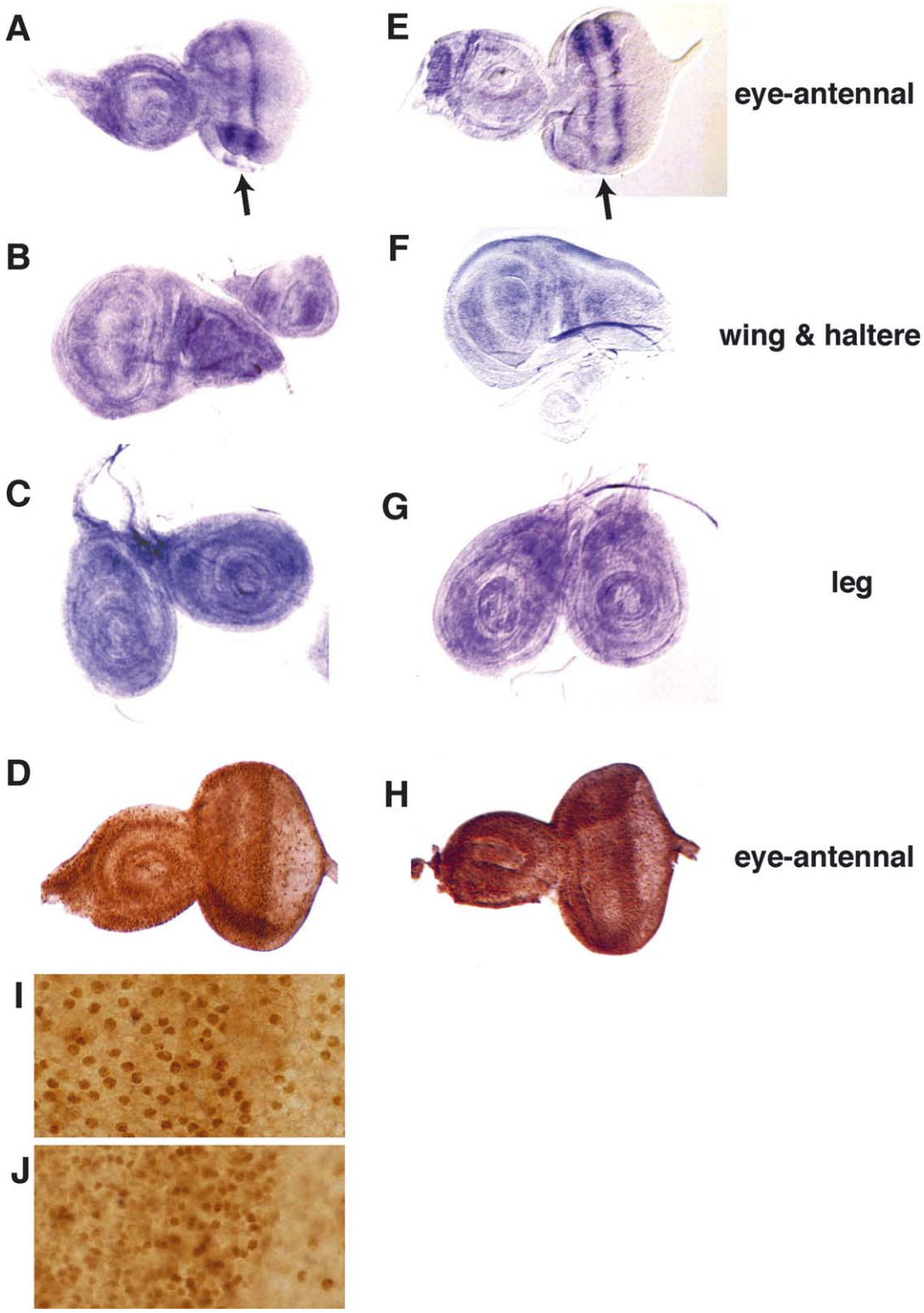

Fig. 6. Distribution of *rbf1* and *rbf2* transcripts and proteins revealed by in situ hybridization and antibody staining of imaginal disks. Disks from third instar larvae were stained using RNA probes to *rbf1* (A–C) or *rbf2* (E–G). Shown are eye-antennal disks (A,E); wing and haltere disks (B,F); and leg disks (C,G). A and E are oriented anterior to the left, with the arrows marking position of the morphogenetic furrow. Protein distribution in eye-antennal disks revealed by immunohistochemical staining with antibodies to Rbf1 (D) and Rbf2 (H), revealing widespread protein in a region from the anterior end to just posterior to the morphogenetic furrow, and lower levels and fewer staining cells in the posterior portion of disk containing nonmitotic determined cells. High magnification of posterior limit of anti-Rbf1 staining at apical (I) and basal (J) focal planes shows a largely nuclear pattern, with a marked decrease in staining in posterior, differentiating cells. Differences in levels of Rbf1 protein in anterior nuclei were not detected, unlike the pattern of *rbf1* transcript.

S phase, suggesting that Rbf proteins normally are involved in the imposition of G1 phase arrest (Richardson et al., 1995; Crack et al., 2002). The pulse of *rbf1* and *rbf2* transcription in the posterior portion of the furrow, in the same region that has upregulated *cyclinE* expression and is undergoing replication, may serve to resupply the cells with new stores of unmodified, repression-competent Rbf protein, ensuring a cessation of mitotic cycling. However, posterior regions containing differentiated cells showed low to undetectable levels of Rbf1 and Rbf2 (Fig. 6D,H). Therefore, if Rbf proteins play a role in setting the terminally differentiated states of these cells, their activity might involve transient interaction with promoters that establishes a long-lived repressive state that does not depend on the continued presence of Rbf protein, analogous to the establishment of repressive Polycomb complexes by transiently expressed transcriptional regulators in the early embryo (reviewed in Mahmoudi and Verrijzer, 2001; Simon and Tamkun, 2002).

Many mechanistic studies on transcriptional repressors have focused on a few promoters in cell culture or in vitro. Yet regulation of transcription often takes place in the context of development, where repression may vary according to cell type, stage of development, or the particular promoter involved (Lunyak et al., 2002; Strunk et al., 2001). Although these parameters affect essential features of repression in biological systems, the functional significance of such diversity is still poorly understood. Over thirty years after Knudson's seminal observations (Knudson, 1971), in which he postulated that a tumor suppressor activity lay behind a rare form of eye cancer, we still do not understand why some tissues are especially sensitive to inactivation of RB function. *Drosophila* and mammalian RB proteins have tissue specific patterns of expression and functions, thus a major goal will be to identify promoter, stage and tissue specific Rbf repression complexes. The characterization of Rbf activities in the development of *Drosophila* is likely to provide valuable information not only about the action of Rbf, but also to give insights into developmental and cancer suppressive roles of the mammalian RB pathway.

3. Experimental procedures

3.1. Plasmids

rAll PCR amplifications were done using *Pfu*I DNA polymerase (Stratagene) and protein coding sequences and all regulatory elements introduced were completely sequenced. The cDNAs pLD02906 and pLD45859, encoding Rbf1, and pLD15806 encoding Rbf2 were obtained from Resgen (Invitrogen). Published work with Rbf1 has described a form of the protein that is terminates at residue 797 (Stevaux et al., 2002). Genomic sequence predicts an additional ~50 amino acid C terminal extension to produce a protein of 845 residues, including residues that are similar in the mammalian p107 homolog (S. Keller, unpublished observations), and we confirmed the sequence of the gene by sequencing Berkeley EST LD 45859. To generate a clone containing the complete coding region the *rbf1* clone pLD02906 was amplified using the primers 5′GAGGTCGG TACCATGAGCGA GCCTGACCCGCAGG3′ and 5′GGG GAATCTAGATT AACTAATTACTAAGCGGCCGCTGTCTCGTGTTCTC-CTCCTTCG3′. The PCR product was digested with *Kpn*I and *Xba*I and cloned between the *Kpn*I and *Xba*I sites of a modified pBluescript(SK+) vector (pBluescript(Not$^-$), in which the *Not*I site was removed, M. Sutrias-Grau, unpublished) generating pBS-Rbf1. The pET15b+adaptor vector was created by inserting the double stranded oligonucleotide 5′TATGGGTACCGAAT TCGCTAGCACTAGTG3′ and 5′ATCCACTAGTGCTAG CGAATTCGGTACCCA3′ between the *Nde*I and *Bam*HI sites of pET15b (Novagen). The *Kpn*I/*Xba*I insert fragment from pBS-Rbf1 was isolated and cloned between the *Kpn*I and *Spe*I sites of pET-15b+adaptor, generating pET-Rbf1, which encodes the Rbf1 protein fused to the N-terminal peptide MGSSHHHHHHSSGLVPRGSHMGT. The peptide includes a hexahistidine tag and a thrombin cleavage site. A *Not*I site was introduced downstream of the coding sequence, adding the peptide sequence AAA to the C terminus of the predicted protein product. The *rbf2* clone pLD15806 was first amplified using 5′ CTTTCAAAAGAT TCTGGAGT TGGTGGTGCGCTACG3′ and 5′ GGGGAA TCTAGAT-TAACTAACTATTAAGCGGCCGCGATCA CTGAAA GCTGGCGACGC3′. The PCR product and the primer 5′ GAGGTCGGTACCATGGAGACTTGTGA AGTGGA GG3′ were then used to amplify the same *rbf2* plasmid template. The product of this amplification was digested with *Kpn*I and *Xba*I and cloned into *Kpn*I/*Xba*I digested pBlue-Script(Not$^-$) to create pBS-Rbf2, into pKreg (Nibu et al., 1998) to create pKr-Rbf2, and into *Kpn*I/*Spe*I digested pET-15b+adapter to create pET-Rbf2. These *Rbf2* clones contain a silent mutation removing an internal *Xba*I site, and a *Not*I site added downstream of the coding region, adding the peptide sequence AAA to the C terminus of the predicted protein product. The clone pET-Rbf2 also places the N-terminal peptide MGSSHHHHHHSSGLVPRGS HMGT at the N terminus of the bacterially expressed Rbf2 protein.

3.2. Transgenic Gal4-Rbf2 line

Transgenic lines were constructed by injection of pKreg-*Rbf2* into y,w^{67} embryos and were detected by w^+ eye color.

3.3. Antibodies and whole mount immunohistochemistry

Peptides were synthesized by either MSU Macromolecular Structure Facility or the Keck Foundation Biotechnology Resource Laboratory (Yale University). Peptides were conjugated to keyhole-limpet hemocyanin (KLH) and injected with TitreMax-Gold (Pierce) into rabbits.

Rabbits were boosted with a second set of injections 4 weeks later, and sera were collected at intervals of about 2 weeks. The peptide sequences used were QAEIRNKP-DIDLKRC (Rbf1) and CNDLMRETKRPNILRRRQLSVI (Rbf2). The Rbf2 peptide differs from the Rbf2 protein at position 13 (should be T rather than I), however the antibody generated was confirmed to be specific for the Rbf2 protein (Figs. 1 and 4). Embryos were collected, fixed and stained as previously described (Small et al., 1992). Antibody localization used the Vectastain kit (Vector Labs). Primary antibody dilutions were 1:500 for embryo staining and 1:1000 for imaginal disc staining. Imaginal discs were dissected from third instar larvae in chilled PBT and fixed in 4% formaldehyde in 9 mM potassium phosphate, pH 6.8, 13.5 mM NaCl, 40 mM KCl, 1.8 mM $MgCl_2$ for 15 min at room temperature. Following the HRP reaction, individual discs were mounted in 50% glycerol [1:1 v:v with H_2O].

3.4. Transcript localization

Digoxigenin labelled antisense probes were synthesized from the template pBS-Rbf1 and pBS-Rbf2 by in vitro transcription with T3 RNA polymerase. Embryos were fixed and stained as described previously (Small et al., 1992). Imaginal discs were fixed as the embryos except without the heptane and gentle, rather than vigorous shaking.

3.5. Testing Rbf1 and Rbf2 Specific Antibodies

To test the specificity of each antiserum, Rbf1 and Rbf2 recombinant proteins were overexpressed in *E. coli* BL21 codon plus cells from the pET-Rbf1 and pET-Rbf2 plasmids described above by induction with 0.4 mM IPTG. Total cell extracts were prepared from the induced bacterial cells by sonication. One microgram of total proteins were subjected to SDS PAGE separation and transferred to nitrocellulose membrane for Western blot analysis. Endogenous Rbf proteins were identified in Western blots from 70 μg of total nuclear proteins/lane prepared from 0–12 h old *Drosophila* (Canton S) embryos as described (Kamakaka et al., 1991). Membranes were blocked in 4.5% milk prepared in TBS Tween (10 mM Tris, pH 7.6, 100 mM NaCl, 0.05% Tween), and hybridizations were carried out for 1 h at room temperature using appropriate dilution of the antibodies in 4.5% milk. Membranes were washed three times (5 min each) in TBS Tween after each hybridization. Rbf1 antibodies were used in 1:2000 dilution and Rbf2 antibodies were used in 1:3000. Horseradish peroxidase (HRP) linked donkey anti-rabbit antibodies (Amersham) were used in 1:5000 dilution and the proteins were detected using the ECL detection system (Amersham).

3.6. Developmental expression of Rbf1 and Rbf2 proteins

To profile Rbf1 and Rbf2 proteins during embryo development, wild type Canton S embryos were collected at 2 h intervals and aged to cover the course of embryonic development. Total extracts were prepared from aged embryos, and 50 μg of total proteins were tested for Rbf1 and Rbf2 proteins in Western blot analysis. To test for the presence of Rbf2 protein in adults, 180 μg of total proteins were used.

Acknowledgements

We thank D. Herrington and E. Fernandez Villatoro for technical assistance. This research was supported by a grant from the Michigan State University Foundation through the Gene Expression in Disease and Development Focus Group initiative to D.N.A and R.W.H and NIH R01 GM59805 to R.W.H.

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