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Down regulation of *extramacrochaetae* mRNA by a *Drosophila* neural RNA binding protein Rbp9 which is homologous to human Hu proteins

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ABSTRACT

Rbp9 is an RNA binding protein expressed mainly in the central nervous system of adult *Drosophila melanogaster.* Rbp9 shares a high degree of sequence similarity with human neural proteins referred to as Hu antigens. Hu antigens bind to U-rich mRNA destabilizing elements with a high affinity and, thus, have been implicated as regulators of mRNA stability. Using *in vitro* RNA binding assays, we found that Rbp9 binds strongly to poly U sequences. We then employed a Selex system to identify a consensus Rbp9 binding site (UUUXUUUU). Information obtained from the Selex results allowed the detection of two repeats of the Rbp9 consensus binding sequence in the 3′ untranslated region of *extramacrochaetae* mRNA. UV crosslinking experiments demonstrated that Rbp9 interacts specifically with *emc* mRNA. The requirement of Rbp9 protein in the down regulation of *emc* mRNA was confirmed by northern (RNA) analysis, which revealed that the level of *emc* mRNA increased 10-fold in *rbp9* mutant flies. Taken together with the *in vitro* RNA binding results, the genetic evidence obtained strongly supports the hypothesis that Rbp9 functions as a regulator of RNA stability.

INTRODUCTION

Regulation at the level of RNA processing is a general mechanism used to control expression of genes involved in many biological processes (1–3). Several RNA binding proteins that display specific developmental expression patterns have been implicated in this mode of regulation. However, the precise mechanism by which RNA binding proteins regulate expression of target gene is known only in a few cases (1,4). Of the known tissue-specific RNA binding proteins, Elav (5) and Rbp9 (6) of *Drosophila melanogaster* are particularly interesting, as they belong to a nervous system-specific family of RNA binding proteins that includes neural proteins from *Xenopus* (*elrA*, *B*, *C* and *D*) (7,8) and humans (HuC, HuD, HuR, Hel-N1 and Hel-N2) (9–11). The presence of multiple homologues in a single species and their common neural-specific expression likely reflect functional importance.

Although Rbp9 homologues are believed to function as post-transcriptional regulators of gene expression in the nervous system (6), the mechanism by which these proteins accomplish their biological function is not yet known. Genetic analysis of *elav* showed that it is essential for neuronal cell development and maintenance (12), but lack of information on an Elav-interacting protein(s) hampers the precise elucidation of *elav* function. Biochemical studies on human homologues of Rbp9 demonstrated that they bind to U-rich elements in untranslated regions (UTRs) of mRNAs that encode cell growth regulators (10,11,13–15). Because the U-rich elements have been implicated in the regulation of mRNA stability, it was suggested that the human Rbp9 homologues destabilize specific mRNAs and thus prevent cell proliferation. Whether these lines of evidence reflect the physiological function of this gene family remains to be tested *in vivo*.

In order to decipher the function of Rbp9, we determined the Rbp9 consensus binding sequence using a Selex system (16). We then identified the consensus sequences in the mRNA of *extramacrochaetae* (*emc*), a *Drosophila* gene involved in proneuronal cell differentiation (17), and detected a physical interaction between *emc* mRNA and Rbp9 protein. We also demonstrated that this interaction is essential for the down regulation of *emc* mRNA by analyzing *rbp9* mutant flies. These results suggest that Rbp9 regulates nervous system development by controlling the stability of mRNAs that encode regulators of cell proliferation and differentiation.

MATERIALS AND METHODS

Purification of a His-tagged Rbp9 protein expressed in *Escherichia coli*

The Rbp9 coding sequence with an altered initiation codon (to incorporate *Spe*I site) was amplified with PCR (polymerase chain reaction), fused in frame with six histidine residues of pEHB1 to make pEHrbp9, and transformed into a BL21 strain. Expression of the recombinant protein was induced with IPTG (0.05 mM) at a cell density of 0.7 (OD_{600}). After a 3-h induction at 37°C, cells were washed with HNE buffer (50 mM HEPES–KOH pH 7.6,

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0.25 M NaCl, 5 mM EDTA), then lysed with freezing and thawing in the presence of lysozyme (0.2 mg/ml) and Triton X-100 (0.1%). The lysate was then sonicated to complete the cell lysis and to reduce the viscosity by breaking down the nucleic acids. After centrifugation to remove cell debris, the soluble fraction was treated with PEI (polyethyleneimmine; 0.1% final concentration), and lipid and nucleic acids were removed by centrifugation at 15 000 *g* for 30 min. The supernatant was loaded onto a Ni^+-NTA column (Qiagen, Santa Clara, CA) which was washed with HNE buffer containing 10 mM immidazole, and bound protein was eluted with HNE buffer containing 200 mM immidazole. This procedure yielded a protein fraction that contained 95% recombinant Rbp9 protein, as confirmed by western (immunoblot) analysis with Rbp9-specific polyclonal antibodies (Ab). This recombinant Rbp9 protein was used for further studies.

Oligonucleotide column binding assay

Oligo rU–agarose, oligo rC–agarose and single-stranded DNA (ssDNA)–cellulose (Sigma, St Louis, MD) were equilibrated with RSB buffer [20 mM HEPES–KOH pH 7.6, 5% glycerol, 42 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$ and 1 mM β-mercaptoethanol]. After equilibration, each resin (100 µl) was incubated with 5 µg of recombinant Rbp9 protein at various NaCl concentrations ranging from 0.1 to 2 M. After 30 min of incubation at 4°C, the resin was washed five times with sodium phosphate buffer (50 mM $NaPO_4$, pH 7.6) containing the heparin (1 mg/ml) and the equivalent amount of NaCl used in the binding reaction. Aliquots (10 µl) of the column fractions were resuspended in protein sample loading buffer and analyzed by SDS–polyacrylamide (10%) gel electrophoresis (PAGE).

Selex

In order to synthesize templates for random RNA oligonucleotides, three DNA oligonucleotides were prepared as described in Tsai *et al*. (16) and used for PCR amplification. The oligonucleotide N_{25} (sequence 5′-TGG GCA CTA TTT ATA TCA ACN_{25} AAT GTC GTT GGT GGC CC-3′), which was used as a template in the PCR had a random sequence of 25 nucleotides (nt) in the middle. At the ends of the oligonucleotide were sequences complementary to the primers (Rev primer and T7 primer) used for PCR amplification. T7 primer (5′-CGC GGA TCC TAA TAC GAC TCA CTA TAG GGG CCA CCA ACG ACA TT-3′) contained the T7 promoter in addition to the complementary sequence that directed synthesis of RNA from the amplified PCR products *in vitro*. Both T7 and Rev primer (5′-CCC GAC ACC CGC GGA TCC ATG GGC ACT ATT TAT ATC AAC-3′) contained a restriction site at the 5′ end to facilitate cloning. *In vitro* transcription of the PCR products was carried out with the T7 RNA polymerase system (Ribomax, Promega, Madison, WI) as suggested by the manufacturer. After RNase-free DNase (Promega, Madison, WI) treatment (10 U for 90 min at 37°C), free nucleotides were removed from the synthesized RNAs with the use of Microcon 3 column (Amicon, Beverly, MA) filtration. In order to estimate the amount of RNA synthesized, a trace amount of [α-^{32}P]UTP (100 c.p.m./pmol) was added to the PCR reaction, and the amount of labeled nucleotides incorporated into RNA was determined using a scintillation counter (Wallac).

For affinity purification, recombinant Rbp9 protein (10 µg) was bound to Ni^+-NTA resin (20 µl), and random RNA oligonucleotides (10–40 µg) were added to the resin in 0.1 ml of RSB buffer containing bovine serum albumin (BSA) (50 ng/µl) and 0.3 M NaCl. After 30 min of incubation at room temperature, the resin was washed extensively with 0.3 M NaCl–RSB buffer. In order to elute the bound RNA, the resin was incubated with proteinase K (40 µg; Promega) for 20 min at 37°C, and RNAs were recovered from the supernatant. The eluted RNA was annealed to Rev primer and converted to complementary DNA (cDNA) with AMV reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions. The cDNAs were converted to double-stranded DNA and amplified by PCR with T7 and Rev primers. The amplified products were used as templates for *in vitro* RNA synthesis and affinity purification on a column containing immobilized Rbp9 protein. This whole process was repeated up to seven times. As a means of monitoring the enrichment of specifically bound RNAs after affinity purification, RNA loaded onto the affinity columns was labeled with a trace amount of ^{32}P, and the percentage of RNA bound to Rbp9 protein resin was calculated for each purification step.

Filter-binding assay

Nitrocellulose filters (Schleicher & Schuell, Keene, NH) were pretreated with 0.5 M KOH for 8 min at room temperature and neutralized in 0.1 M Tris–HCl (pH 8.0) for 20 min. ^{32}P-labeled RNA (0.5 µg) was mixed with recombinant Rbp9 protein (2 µg) in RSB buffer containing 0.3 M NaCl and BSA (200 µg/ml), and the mixture was incubated for 30 min at room temperature. The RNA–Rbp9 mixture was bound to a KOH-treated nitrocellulose filter on a Hoeffer (San Francisco, CA) slot blot system, and the filter was extensively washed with the binding buffer. Radioactivity retained on the nitrocellulose filters was analyzed either with a Phosphorimager (Molecular Dynamics) or scintillation counting.

Cloning of *emc* 3′ UTR

The 3′ UTR of *emc* mRNA (1156 bp between the termination codon and the polyadenylation signal) was prepared by PCR amplification of wild-type *Drosophila* cDNA with primers emcUTR5 (5′-TTT CTA GAG CGT GGA AAC ACC CAG-3′) and emcUTR3 (5′-TTT CTA GAA AGA GCT AGT GTT TGT TTT-3′). The amplified fragment digested with *Xba*I was cloned into the *Xba*I site of pBluescript SK+ (Stratagene) to make pSKemc and sequenced to confirm the absence of a mutation. RNA probe encoding the first 849 nt of the *emc* 3′ UTR (contains two putative Rbp9 binding sites between the nucleotide positions 434 and 463) was transcribed by T7 RNA polymerase from the pSKemc template linearized with *Sty*I.

UV crosslinking assay

UV crosslinking assays were performed as described (18). Recombinant Rbp9 protein (60 ng) was preincubated for 10 min with 10 µg of yeast tRNA in a 10 µl reaction mixture that contained 1 µl of 10X reaction buffer A (32 mM $MgCl_2$, 20 mM ATP, 1 mg/ml BSA, 60 mM HEPES–KOH pH 7.9). ^{32}P-labeled RNA probe (100 fmol) was added to the mixture, and the sample was incubated for an additional 10 min at room temperature. The sample was placed on ice and irradiated with UV light (10^5 erg/mm^2) with use of a Stratagene (La Jolla, CA) UV crosslinker. The RNA was

digested with RNaseA (30 µg) for 15 min at 37°C and mixed with protein loading buffer. Samples were boiled for 90 s and subjected to SDS–PAGE and autoradiography. For UV crosslinking competition assays, a 20–400-fold excess of competitor RNA oligonucleotides were added to the reaction mixtures together with the ^{32}P-labeled RNA probes. The RNA oligonucleotides used for this assay are shown in Table 2.

Northern analysis of RNA from *rbp9* mutant flies

A nucleic acid probe for *emc* was prepared by PCR amplification of *Drosophila* genomic DNA with primers emc5 (5′-GAGAATGCCGAGATGAAG-3′) and emc3 (5′-GAAAACGATCCAAGGGAC-3′). Poly(A)$^+$ RNA preparation from mutant flies and northern hybridization were carried out as described in Sambrook *et al.* (19).

RESULTS

Recombinant Rbp9 binds strongly to ribohomouridylate

To define the RNA binding specificity of Rbp9 protein, we have used ribohomopolymer and ssDNA affinity chromatography (Fig. 1A). A significant portion (25–50%) of Rbp9 protein loaded bound to both poly rU and ssDNA resins in a low salt RSB buffer (lanes 3 and 4 for poly rU; lanes 11 and 12 for ssDNA). Rbp9 bound more tightly to poly rU resin than ssDNA resin as Rbp9 binding to poly rU resin was not disrupted in the presence of high salt (2 M NaCl) (lanes 5 and 6) [compare with loss of Rbp9 binding to ssDNA resin (lanes 13 and 14) in 2 M NaCl]. In contrast, Rbp9 protein did not bind to the poly rC resin efficiently regardless of the salt concentration (lanes 7–10). These results indicated that Rbp9 is an RNA binding protein with a strong affinity for U-rich sequences.

The high-affinity of Rbp9 for U-rich sequence was confirmed with a filter-binding assay (Fig. 1B). Three kinds of ribohomopolymers (poly rC, poly rA and poly rU) were end-labeled with ^{32}P and incubated with Rbp9. Upon filtration through a pretreated nitrocellulose filter, only the RNA associated with Rbp9 was retained. In the presence of Rbp9, 100% of labeled poly rU was retained on nitrocellulose (lanes 1 and 2), while only 0.3 and 1.4% of poly rA and poly rC, respectively, were retained on the filters (lanes 3 and 4 for poly rA; lanes 5 and 6 for poly rC). Both types of experiments suggest that Rbp9 binds preferentially to U-rich sequences.

Determination of the Rbp9 target RNA sequence

To determine the target RNA binding sequence of Rbp9 protein, we used Selex as described in Materials and Methods. Random RNA oligonucleotides synthesized *in vitro* were incubated with histidine-tagged Rbp9 protein immobilized on Ni^{2+}-NTA resin. The amount of RNA recovered from the Rbp9 containing beads was <0.1% of the RNA used in the binding reaction, and most of the recovered RNA appeared to be bound non-specifically to Rbp9, as a similar portion of the starting RNA was also recovered when BSA was used instead of Rbp9. To further enrich for RNAs that were specifically bound, RNA oligonucleotides recovered from the Rbp9 resin were amplified as cDNA by RT–PCR, converted into RNA, and used for additional rounds of purification, and this whole process was repeated up to seven times. As shown in Figure 2A, the percentage of Rbp9 bound RNA increased after

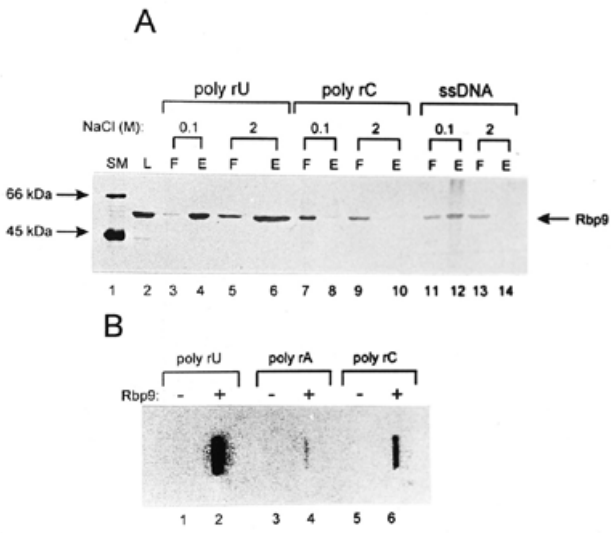

Figure 1. Rbp9 binding to poly rC, poly rU and ssDNA resins. (**A**) Oligo rU–agarose, oligo rC–agarose and ssDNA–cellulose were each incubated with 5 µg of recombinant Rbp9. The concentration of NaCl in the binding buffer is indicated. Both the flow-through (F) and eluted proteins (E) were analyzed by 10% SDS–PAGE and visualized by silver staining. The molecular size marker (SM) and one-fourth of the loaded protein (L) are shown in lanes 1 and 2. (**B**) The radiolabeled oligoribonucleotides (0.5 µg, each) are indicated above the lanes, and were filtered through KOH-treated nitrocellulose in the presence (+) or absence (–) of recombinant Rbp9 (2 µg).

each round of selection, reaching a maximum of 25% bound after seven selection cycles. The enrichment of Rbp9 bound RNA by *in vitro* Selex selection was confirmed with a filter-binding assay, which showed that 25% of the input RNA bound to the Rbp9 after seven cycles of selection (Fig. 2B, lanes 3 and 4). Again, only 0.1% of the input RNA bound to Rbp9 was retained on nitrocellulose after the first round of selection (lanes 1 and 2).

In order to determine the nucleotide sequence of the Rbp9-binding RNAs, we sequenced 30 independent PCR fragments amplified from RNAs enriched in the final round of selection. Among these were 15 clones that contained a stretch of at least of eight U residues (Table 1). In order to characterize the binding of Rbp9 to the selected RNAs, ^{32}P-labeled RNA prepared from #26 clone was analyzed by UV crosslinking in the presence of Rbp9. The selected RNA was crosslinked specifically to Rbp9 protein, as these interactions were inhibited by the addition of unlabeled poly U RNA, but not poly rC or poly rA RNAs (Fig. 2C). These results demonstrated once again the affinity of Rbp9 for poly U sequences.

Binding of Rbp9 to a synthetic Hel-N1 binding site

Although we determined the Rbp9 RNA binding sequence to be a simple U-stretch, a similar Selex study with Hel-N1, one of human Rbp9 homologues (20), identified RWUUUAUUUWR (R = A or G; W = A or U) as a consensus binding sequence. These results suggest either that the two proteins have different binding specificities or that the minimum requirement for Rbp9 binding is shared by the two consensus sequences. Therefore, we tested the affinity of Hel-N1 RNA oligonucleotides with various modifications of sequences using UV crosslinking as well as filter-binding assays. When we examined the binding affinity of

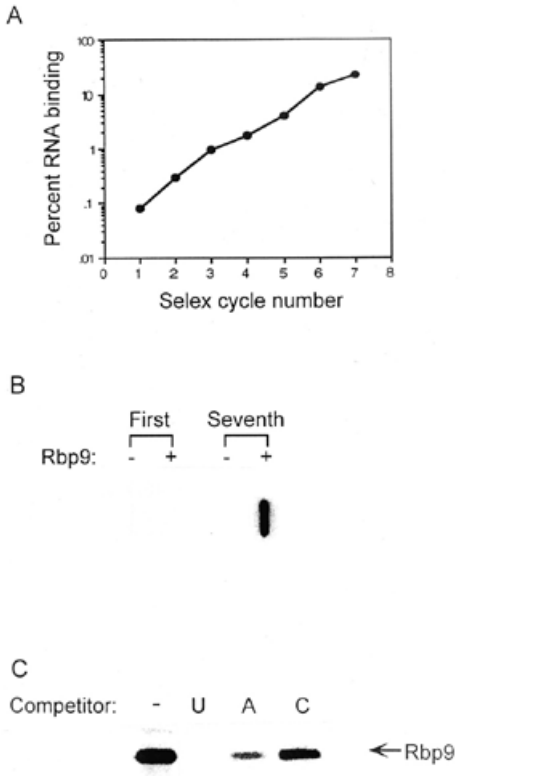

Figure 2. Enrichment of Rbp9-binding RNAs by the Selex system. (**A**) A pool of Rbp9-binding RNAs was enriched from a collection of random oligonucleotides by seven cycles of Selex. The percentage of bound RNA was calculated based on the ratio between the amount of radioactive RNA eluted and the total radioactive RNA used in the binding reaction. (**B**) RNAs recovered from the first and seventh cycles of Selex were assayed by filter-binding in the presence (+) and absence (–) of Rbp9 protein as described in Figure 1B. (**C**) 50 fmol of ^{32}P-labeled #26 RNA was UV cross-linked with 125 ng of Rbp9 with and without the competitor RNA. The competitor RNA used is indicated. Lane 1, no competitor; lane 2, poly rU (U, 2 µg); lane 3, poly rA (A, 2 µg); lane 4, poly rC (C, 2 µg).

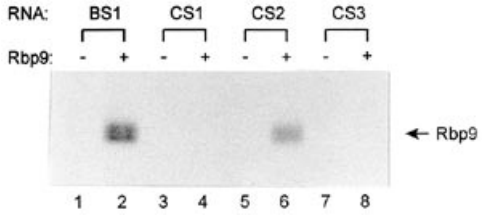

Figure 3. The binding specificity of Rbp9 protein. Rbp9 protein (125 ng) (lanes 2, 4, 6 and 8) was UV-crosslinked with ^{32}P-labeled ribooligonucleotides (6 ng) containing two repeats of the Hel-N1 binding RNA consensus sequence (BS1) or its mutant versions (CS1, CS2 and CS3). As a control for non-specific interaction, results obtained with a binding reaction that contained BSA (100 ng) instead of Rbp9 is shown (lanes 1, 3, 5 and 7).

Rbp9, an RNA oligonucleotide composed of two direct repeats of the Hel-N1 consensus sequence (Table 2; BS1) bound as efficiently as poly U RNA (Fig. 3). When we replaced the U-residues in the BS1 RNA with A-residues (Table 2; CS1), the RNA was no longer able to bind Rbp9 protein (lane 4). In order to test whether the context around the U-stretches or the length of the U-stretch is important for Rbp9 interaction, we made two mutant versions of BS1 RNA. In the first one, we changed the purine residues to 'C' (Table 2; CS2) without changing the U-stretch, while in the other we disrupted the U-stretches by replacing the U in the middle to C (Table 2; CS3). UV crosslinking assays showed that the purine to C change reduced the Rbp9 binding efficiency slightly (lanes 2 and 6). However, disruption of the U-stretch completely abolished Rbp9 binding (lane 8). In the filter-binding assay with identical RNAs, similar binding patterns were observed (data not shown). Therefore, Rbp9 protein appears to bind to a stretch of at least eight U residues, with a random nucleotide position in the middle (UUUXUUUU).

Table 1. Deduced RNA sequences from the cDNA clones isolated by Selex[a]

#1	GGGCCACCAACGACAUU**UCGUCUUUUUUUUUUUUUUUGAUAGGUUGAU**GUUGAUAUAAAUAGUGACCAUGGAUC
#2	GGGCCACCAACGACAUU**UCGUCUUUUUUUUUUUUUUUGAUAGGUUGAU**GUUGAUAUAAAUAGUGACCAUGGAUC
#4	GGGGCCACCAACGACAUU**UUUUUUUUUUUUUUUGCGCUUCUACCUCUCCCGC**GUUGAUAUAAAUAGUGACCAUGGAUC
#7	GGGGCCACCAACGACAUU**CCACCUUUUUUUUUUUUUGAGAUGGCUUU**GUUGAUAAAAUAGUGCCCAUGGAUC
#8	GGGGCCACCAACGACAUU**UUUUUUUUUUUUUUUUUUUAGGAACUCCGAGACNNNGUU**GUUGAUAUAAAUAGUGCCCAUGGAUC
#12	GGGGCCACGAACGACAUU**CUGUUUUUUUUUUUUUUUUAGCCGCGCUAUUGU**GUUGAUAUAAAUAGUGCCCAUGGAUC
#17	GGGGCCACCAACGACAUU**UACCGUUGAUCAUUUUUUUUUUUUUUUUUUUUUUGCUUA**GUUAAUAUAAAUAGUGCCCAUGGAUC
#18	GGGGCCACCAACGACAUU**UUUUUUUCCGUUUCUCAUUACUAUUG**GUUGAUAUAAAUAGUGCCCAUGGAUC
#19	GGGGCCACCAACGUCAUU**GUUUUUUUUUUUUUUUUUUUUGGGGUUUGUUCCUUCC**GUUGAUAUAAAUAGUGCCCAUGGAUC
#21	GGGGCCACAAACGACAUU**UUUUUUUUUUUUCUUAUGACGGUCCCUGUU**GUUGAUAUAAAUAGUGCCCAUGAUCGCGACAUCCCGCACAGCGGAUC
#22	GGGGCCACCAACGACAUU**UUUUUUUUUUUUUUUAGUUCUAUUCCGAAUGUG**GUUGAUAUAAAUAGUGCCCAUGGAUC
#23	GGGGCCACCAACGACAUU**ACGUUUUUUUUUUUUUGUUAGUAAGG**GUUGAUAUAAAUAGUGCCCAUGGAUC
#24	GGGGCCACCAACGACAUU**UUUUUUUUUUAGUACCCGAGCCCGAGAA**GUUGAUAUAAAUAGUGCCCAUGGAUC
#25	GGGGCCACCAACGACAUU**UUUUUUUUUUUUCUUAUGACGGUCCCUGUUC**GUUGAUAUAAAUAGUGCCCAUGGAUC
#26	GGGGCCACCAACGACAUU**UUUCUUUUUUUUUUUUUUUUUUUUCUAGUAGUGUUAAGGUAAG**GUUGAUAUAAAUAGUGCCCAUGGAUC
CS3	UUGAU**C**UAU**C**UUGAU**C**UUAU**C**UAGUU[d]

[a]From the cDNA clones isolated by Selex, RNA sequences transcribed from T7 promoter were deduced. Underlined bold letters are randomized region.

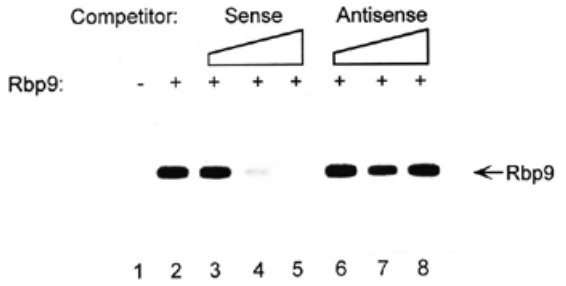

Figure 4. Binding of Rbp9 protein to the U-rich element of *emc* mRNA. UV crosslinking of Rbp9 protein to *emc* mRNA. Recombinant Rbp9 protein (60 ng) was UV-crosslinked to the 3′ UTR of *emc* mRNA. Rbp9 BS1 (lanes 3–5) and Rbp9 CS1 (lanes 6–8) were used in 40-, 120- and 400-fold excess as sense and antisense competitor RNAs, respectively.

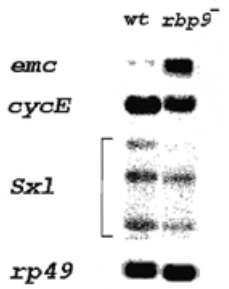

Figure 5. Northern analysis of *rbp9* mutant. The amount of *emc, cyclin E* and *Sxl* mRNAs in wildtype (*wt*) and *rbp9*$^{P[2567]}$ mutant (*rbp9*$^{-}$) flies were shown by northern hybridization. The 4.1, 3.1 and 1.9 kb adult female *Sxl* transcripts were shown in the *Sxl* blot. Poly(A)$^{+}$ RNA (2 µg) was loaded in each lane, and the amount of *rp49* transcript is shown as a loading control.

Table 2. RNA oligonucleotides used for UV crosslinking assay with Rbp9

Consensus sequence[a]	RWUUUAUUUWRRWUUUAUUUWR[b]
BS1	UUGAUUUAUUUUGAUUUUAUUUAGUU[c]
CS1	UUGAAAAAAAAAAGAAAAAAAAAAAGUU[d]
CS2	UUCCUUUCUUUUCCUUUUCUUUGCUU[d]

[a]Consensus binding sequence for Hel-N1 selected RNAs.
[b]W = A or U; R = A or G.
[c]Two direct repeats of different types of the Hel-N1 binding sequences were used.
[d]The mutated nucleotide positions are underlined.

Cloning of a target gene encoding Rbp9 binding RNA

The interaction *in vitro* between *Id1* and Hel-N1 (11), the human counterparts of *emc* and Rbp9, respectively, as well as the involvement of *emc* in *Drosophila* nervous system development, suggested *emc* mRNA as a strong candidate for an Rbp9-binding RNA. But, the Hel-N1 binding sequence (RWUUUAUUUWR), which would indicate their interaction, was not present in the *emc* mRNA. However, examination of the *emc* mRNA for the presence of the newly identified Rbp9 consensus binding sequence (UUUXUUUU) identified two UUUGUUUU sequences within the 3′ UTR located ~450 base pairs (bp) downstream of the stop codon. This finding prompted us to test whether these repeats in the *emc* mRNA are authentic Rbp9 binding sites. The affinity of Rbp9 to *emc* 3′ UTR was examined with UV crosslinking. As shown in Figure 4, the 3′ UTR of *emc* was crosslinked very efficiently to Rbp9 protein (lane 2). Rbp9 protein bound specifically to the UUUGUUUU repeats as increasing amounts of BS1 (Table 2) inhibited the crosslinking (lanes 3–5). However, CS1 (Table 2) had no effect on *emc* RNA-Rbp9 binding, even upon addition of a 400-fold excess of CS1 competitor (lanes 6–8).

Identification of the physical interaction between Rbp9 and *emc* mRNA prompted us to examine whether *emc* mRNA stability is regulated by the presence of Rbp9. To study the effect of an *rbp9* null mutation on *emc* expression, we prepared poly(A^{+}) mRNAs from wild-type and *rbp9*$^{P[2567]}$ mutant flies (22) and analyzed the level of *emc* mRNA by northern blot hybridization. As shown in Figure 5, the level of *emc* mRNA increased >10-fold in flies carrying the *rbp9* null allele as compared to that in wild-type flies. The level of a control RNA (*rp49*) was not altered. This result suggests that *rbp9* is required for down regulation of *emc* expression, possibly by destabilizing *emc* mRNA.

In order to examine the specificity of the Rbp9-mediated down regulation of *emc* mRNA, the effect of the *rbp9* mutation on the expression of other RNAs containing the putative Rbp9 binding consensus sequences was analyzed. We first examined the level of *cyclin E* mRNA (GenBank accession no. X75026), because it contains three Rbp9 binding sequences in the 3′ UTR (UUUUUG-UU, AUUUUUUU and UUUUAUUU at the nucleotide positions 2643, 3374 and 3702, respectively) and expressed in ovaries where Rbp9 is required for a proper germ cell differentiation (22). However, in contrast to the *emc* mRNA, the level of *cycE* mRNA did not increase in the *rbp9*$^{P[2567]}$ mutant (Fig. 5).

We also examined the effect of *rbp9* mutation on the levels of *Sxl* transcripts. *Sxl* expresses four adult female specific transcripts; the 4.1 kb large transcript, two 3.1 kb transcripts and 1.9 kb small germ-line dependent transcript. These transcripts differ at their 3′ ends. The large transcript contains 14 putative Rbp9 binding consensus sequences, and the intermediate-sized transcripts have eight binding sequences, but the small transcript has only one binding sequence. Despite the presence of a number of the binding sequences, the northern analysis revealed that the 3.1 and 1.9 kb transcripts were expressed normally in the *rbp9* mutant (only 1.2-fold decrease in the mutant). Therefore, Rbp9 did not act on the putative Rbp9 binding sequences in these *Sxl* and *cycE* mRNAs.

The effect of *rbp9* mutation on the 4.1 kb *Sxl* transcript is complex. Contrary to our expectation, the 4.1 kb transcript was decreased 3-fold in the *rbp9* mutant. However, this negative effect on the *Sxl* large transcript could be indirect. Because Emc protein represses *Sxl* by inhibiting the formation of Daughterless/Sisterless-b heterodimers (23), the elevated level of Emc may cause the down regulation of the *Sxl* large transcript in the *rbp9* mutant. These results suggest that not all Rbp9 binding consensus sequences interact with Rbp9 and that the specific interaction between Rbp9 and *emc* mRNA may require additional elements.

DISCUSSION

Rbp9 belongs to a large neural RNA binding protein family that contains *Drosophila* Elav (12) and human Hu proteins (9,10,21). Although these homologues share similarities in their predominant nuclear expression in nerve cells, genetic and biochemical studies reveal that a neuro-specific pre-mRNA processing may not be the

only function of the Rbp9 protein family. Especially, biochemical studies on human Hu proteins proposed a regulation of mRNA stability as one of their functions (10,11,13–15). However, whether the regulated degradation of target mRNAs is the genuine function of the Hu proteins *in vivo* has not been proved yet. Therefore, the 10-fold increase of *emc* mRNA in the *rbp9* null mutant provides strong evidence in support of the hypothesis that Rbp9 protein family functions as regulators of mRNA stability.

The role of *rbp9* in the regulation of mRNA stability suggests that Rbp9 protein would be localized in the cytoplasm, which is contrary to the previous observations showing nuclear-specific localization of Rbp9 and Elav proteins in nerve cells (6,12). However, our recent studies on *rbp9* mutants found that, in germ cells, Rbp9 is localized in the cytoplasm to regulate cell proliferation and differentiation (22). Besides, a small amount of human Hu protein is also localized in the cytoplasm (9,24).

The fact that Rbp9 is present both in the nucleus and the cytoplasm is particularly interesting, because a highly homologous Sxl protein has two different functions as a regulator of alternative splicing in the nuclei and a regulator of mRNA translation in the cytoplasm (25–28). Rbp9 and Sxl may utilize a similar mechanism in the regulation of RNA processing even though they are involved in different developmental processes. Therefore, in the down regulation of *emc* mRNA, Rbp9 may reduce the amount of *emc* mRNA by affecting its stability directly. But it is equally possible that degradation of *emc* mRNA is caused indirectly by an Rbp9-mediated translational inhibition as *msl-2* translation was repressed by Sxl protein (26,28).

Finally, our analysis of the Rbp9 binding consensus sequence using the Selex system, as well as our mutational analysis of the Hel-N1 binding sequence suggests that Rbp9 binds to a rather simple U-stretch. What appears to be more important for Rbp9 binding is the length of the U-stretch rather than the context that surrounds it. This rather simple binding specificity appears to be shared with other Rbp9 homologues and Sxl. The RNA recognition motifs (RRMs) of Sxl protein are very similar to those of Rbp9 protein (33% identical), and binds to AU_7 or AU_8 *in vitro* (29,30). This raises a question about their binding specificity: how do they bind to their specific target RNAs? Although Rbp9 and Sxl are expressed together in some developmental stages, each of them is involved in a distinct developmental process, thus they may have different target RNAs. As shown in this study, Rbp9 and Sxl do not regulate all the RNAs that contain the Rbp9 binding consensus sequences. Therefore, additional *cis*-elements may be required for the specific binding of these proteins to their target RNAs *in vivo*. In addition, the distinct domains of each protein may interact with specific co-factors (29). Therefore, the identification of these additional *cis*- and *trans*-acting factors is needed to understand the precise mechanism by which Rbp9 regulates the target RNAs.

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