Function of RRM Domains of *Drosophila melanogaster* ELAV: RNP1 Mutations and RRM Domain Replacements With ELAV Family Proteins and SXL

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ABSTRACT

Members of the ELAV family of proteins contain three RNA recognition motifs (RRMs), which are highly conserved. ELAV, a *Drosophila melanogaster* member of this family, provides a vital function and exhibits a predominantly nuclear localization. To investigate if the RNA-binding property of each of the ELAV RRMs is required for ELAV's *in vivo* function, amino acid residues critical in RNA binding for each RRM were individually mutated. A stringent genetic complementation test revealed that when the mutant protein was the sole source of ELAV, RNA-binding ability of each RRM was essential to ELAV function. To assess the degree to which each domain was specific for ELAV function and which domains perhaps performed a function common to related ELAV proteins, we substituted an ELAV RRM with the corresponding RRM from RBP9, the *D. melanogaster* protein most homologous to ELAV; HuD, a human ELAV family protein; and SXL, which, although evolutionarily related, is not an ELAV family member. This analysis revealed that RRM3 replacements were fully functional, but RRM1 and RRM2 replacements were largely nonfunctional. Under less stringent conditions RRM1 and RRM2 replacements from SXL and RRM1 replacement from RBP9 were able to provide supplemental function in the presence of a mutant hypomorphic ELAV protein.

ELAV family member proteins are characterized by three RNA recognition motifs (RRMs, also RBD), the first two of which are in tandem and the third of which is separated by an interdomain hinge region. The RRM consists of 80–90 amino acid (aa) residues with two highly conserved short motifs, an RNP1 octamer and an RNP2 hexamer, and is found in numerous proteins involved in post-transcriptional processes (Kenan *et al.* 1991; Birney *et al.* 1993; Burd and Dreyfuss 1994). The crystal structures of RRMs from U1A, hnRNP A1, U2B″, and SXL have revealed that the tertiary structure of the RRM domain consists of four β pleated sheets packed against two α-helices (Nagai *et al.* 1990; Price *et al.* 1998; Handa *et al.* 1999). Within the ELAV family of proteins, the three RRM domains are highly conserved, whereas the N-terminal domain and the hinge can vary (reviewed in Okano and Darnell 1997). An evolutionary analysis of the RRM domains has revealed a close association between RRM1 and RRM2, suggesting that they arose by a duplication event, but the third RRM domain was on a separate branch of the phylogenetic tree (Birney *et al.* 1993). Outside the ELAV family, the closest related RRM domains were found in the protein encoded by *Drosophila melanogaster Sex-lethal* gene, which contains two tandem RRMs that are related to the first two ELAV RRMs (Birney *et al.* 1993).

Current data indicate that ELAV family members perform diverse functions in post-transcriptional processing of RNA (reviewed in Antic and Keene 1997). We have shown that *D. melanogaster elav* regulates neural-specific isoforms, a role consistent with its exclusively neural expression and predominantly nuclear localization (Robinow and White 1991; Koushika *et al.* 1996; Yannoni and White 1997). The vertebrate members of the *elav* family, as well as RBP9, a *D. melanogaster elav* family protein, have been implicated in functions related to mRNA stability and translatability (Antic *et al.* 1999; Kim-Ha *et al.* 1999) and ELAV has also been shown to autoregulate (Samson 1998). An intriguing feature of the RRM-containing molecules is their modular nature in that many of them have two or more RRMs. Thus, the individual RRM's contribution to the protein function has been a subject of a number of studies. The RRMs within an individual protein can be either related or diverse. Where tested, RRMs within a protein do not appear to be functionally equivalent (Caceres and Krainer 1993; Mayeda *et al.* 1998). Studies involving mutant proteins in which critical amino acids have been mutated or proteins with deletions of individual RRM domains are generally assayed using *in vitro* RNA binding as the primary test for the function. In a few cases, however, *in vivo* function has also been studied, for example, *D. melanogaster* NONA (Rendahl *et al.* 1996), U2AF (Kanaar *et al.* 1993), and SR proteins (Mayeda *et al.* 1998). Functional properties of the mutant RRM-

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containing proteins of the ELAV family of protein have not been tested *in vivo*.

Although most RRMs show some basal RNA binding, a single RRM has been shown to be sufficient for specific RNA binding. In the case of U1A (Scherly *et al.* 1990a,b; Allain *et al.* 1996), the N-terminal RRM was responsible for U1 snRNA stem loop II recognition. For U1 70K (Query *et al.* 1989), the single RRM region mediates specific binding to its U1 snRNA target and the RRM2 of the yeast poly(A)-binding protein is primarily responsible for its poly(A)-binding property (Deardorff and Sachs 1997).

Experiments described in this paper were designed to assess the contribution of individual RRM domains to ELAV function. ELAV is ideally suited for this functional analysis as the biological function can be assayed using transgenes that are driven by the *elav* promoter and that mimic the expression of the endogenous gene (Yao and White 1994). Moreover, functional rescue can be attempted under stringent conditions, where the transgene is the only source of ELAV in the fly. In addition, less stringent functional assays can also be used where the transgene-generated ELAV supplements a mutant ELAV protein.

In this report we address the following.

1. Is the RNA-binding property of each individual domain necessary for *in vivo* ELAV function?
2. Are the 13 amino acid residues that are unique to ELAV RRM1 loop3 crucial to the ELAV-related vital function?
3. Can the RRM domains of closely related *D. melanogaster* proteins RBP9 and SXL functionally substitute for ELAV's domains in ELAV function?

To answer the first question, we generated transgenes in which amino acid residues critical in RNA binding within RNP1 motif for each RRM individually were mutated. To answer the second question a transgene expressing a mutant ELAV protein with the 13 amino acid residues deleted was generated. The third question was addressed by constructing transgenes in which the RBP9 or SXL RRM domains replaced the corresponding RRM domains within the context of the ELAV protein. Considering the wealth of knowledge amassed about the functional aspects of SXL (Burtis 1993), which occupies a central position in the sex determination cascade of *D. melanogaster*, the functional comparison of SXL and ELAV domains could be rewarding.

MATERIALS AND METHODS

Construction of the AGD mutation and -13aa *elav* plasmids: Site-directed mutagenesis (Kunkel *et al.* 1987, 1991) was employed to mutagenize a 2.5-kb genomic fragment containing the *elav* open reading frame (ORF) in pBluescript, termed pKS2.5hst. The AGD mutagenic oligos introduce a diagnostic *Ngo*MI restriction site. The mutagenic oligo sequences are listed in Table 1.

PCR mutagenesis to generate domain replacements: The domain replacement method was adapted from Zhong and Bajaj (1993). The PCR-based technique utilizes oligos that contain complementary sequences of both the donor domain cDNA and *elav* cDNA. These oligo sequences are listed in Table 1. Three rounds of PCR successively generate the domain replacement PCR fragment flanked with ELAV sequence. The first round generates the donor domain with small flanking *elav* sequence tags. The second round consists of two separate reactions that extend into the 5′ or 3′ direction of *elav* cDNA sequence. For this step, one domain replacement oligo is used with a distal *elav*-specific oligo. The third and final step consists of using the two products from step two to prime off one another in the initial cycle and then using the two distal *elav* oligos to generate a larger product in subsequent cycles. The resulting PCR product has sufficient flanking *elav* sequence to facilitate subcloning into the *elav* genomic rescue construct. *Pwo*I polymerase (Boehringer Mannheim, Indianapolis) was used in all amplification steps to minimize misincorporation. The PCR conditions were modified from Zhong and Bajaj (1993), only by decreasing the temperature and duration of the denaturing steps.

Plasmid construction of *elav* expression vector, 9kbC: The domain replacement PCR fragments were cloned into pKS2.5hst, which consists of the ELAV ORF within a 2.5-kb genomic fragment in pBluescript. The following restriction sites in pKS2.5hst were used to facilitate PCR fragment insertion: for S1/2, *Sac*II and *Pfl*MI sites; for ES1, ES12, and EK12, *Sac*II and *Bst*XI sites; for ES2, *Pfl*MI and *Bst*XI sites; and for ER3 and EH3, *Bst*XI and *Nsi*I sites. A 3.5-kb, *Xma*I/*Xba*I fragment from pKS2.5hst^{DR} (domain replacement) was ligated with a 7.7-kb *Pst*I/*Xba*I pCaSpeR fragment and a 5.8-kb elav genomic *Pst*I/*Xma*I to generate 9 kbC. This 8.5-kb *elav* genomic rescue construct has been reported previously (Yao and White 1991).

The pCaSpeR vector had been modified previously by subcloning in the gene for kanamycin resistance (Kn, aminoglycoside-3′-phosphotransferase) to decrease ligation background. Oligos KanF GCGGACGTCTGCGTTGTCGGGAAGATG and KanR GCGGACGTCGGGAAGATGCGTGATCTG were used to PCR amplify a 1.3-kb fragment containing the Kn gene from pACYC177 (New England Biolabs, Beverly, MA); the PCR product was subsequently cleaved with *Aat*II and subcloned into the *Aat*II site in pCaSpeR.

Mutation detection enhancement analysis, plasmid sequencing: Prior to sequencing, pKS2.5hst^{DR} clones were preselected by performing mutation detection enhancement (MDE) gels (AT Biochem, Malvern, PA) to identify any demonstrably aberrant clones. Sequencing was performed with the Sequenase 7.0 kit (United States Biochemical, Cleveland) or by Prizm PCR sequencing (Perkin-Elmer/Cetus, Norwalk, CT) in conjunction with the Brandeis sequencing facility.

Drosophila culture and stocks: Flies were raised at 25° on standard culture medium. The genetic symbols for standard marker genes and balancers are as in Lindsley and Zimm (1992). The transgenes either used or generated for this study are described below.

Generation of germline transformants: Germline transformants were produced by injecting *Df(1)w/y w; Ki* Δ*2-3*/+ embryos deficient for the *white* gene function and harboring one copy of transposase (Robertson *et al.* 1988). Transformants were selected on the basis of red eye color and transgenic lines were established by standard procedures. A list of transgenes, with abbreviations as used in the text, and the proteins they encode is provided below.

$P\{w^+ = elav^{DMORF}\}$: $elav^{DMORF}$ is the parent vector for all the

TABLE 1

Oligos used in mutagenesis

1AGD	GCCGGACATAGTTAACATCGCCGGCGCCCAGACTTTGGCCC
2AGD	CCGCTTATCGAATCGAATATCGCCGGCTCCTTTCGTCTGCGTA
3AGD	GTTGGTCATCGAAACGTCGCCGGCGCCCTTGCACTGATTC
-13aa	CAGACTTTGGCCCTTGCTCTTGTCGCGTATCAGCTTC
K1F	CTACTTGCCGCAAACAATGTCGCAGGACGAGATCCGT
K2R	GGTGCTGCCGGGCGTATTGGCGAACTTGACGGTTATG
K3F	GGCCAGTGGGCCTGGCGGCGGCTGGTGCATTTTCGTCT
K3R,	GCTGACCTGCAGCACCCGGTTGCCAAGTGTGTAGCCG
H3F	TGGGCCTGGCGGCGCGTATTGCATCTTTGTCTACAACC
H3R	AGCTGACCTGCAGCACCCGGTCTCCCAGGCGGTACCCG
S1F	TGTCAACTACTTGCCGCAAGACATGACCGATCGCGAGC
S1/2R,	CCAGACTTTGGCCCTTGCTATAGTCTCGCATGATTCTG
S1R	TAATGGCATCGGACGACGGACGTGCATAGGAAACCTT
S2F	AGGCGCCAACCTTTATGTGACCAATCTGCCGCGTACC
S2R	TGGTGCTGCCGGGCGTATTAGCCAACCGGACGGACAGC

transgenes below. It encodes a wild-type ELAV protein. A 8.5-kb *elav* genomic sequence includes promoter and coding sequence in the w^+ *P*-element vector pCaSpeR (Yao and White 1991).

$P\{w^+ = elav^{RBD60}\}$: $elav^{RBD60}$ encodes a protein with N-terminal deletion in *elav* coding sequence (Yao *et al.* 1993).

$P\{w^+ = elav^{1AGD}\}$: $elav^{1AGD}$ encodes a protein with two point mutations designated 1AGD (Y205A and F270D) in the first RRM.

$P\{w^+ = elav^{2AGD}\}$: $elav^{2AGD}$ encodes a protein with two point mutations designated 2AGD (V292A and F294D) in the second RRM.

$P\{w^+ = elav^{3AGD}\}$: $elav^{3AGD}$ encodes a protein with two point mutations designated 3AGD (V445A and F447D) in the third RRM.

$P\{w^+ = elav^{-13}\}$: $elav^{-13}$ encodes a protein with the 13-amino-acid loop3 deletion in the first RRM.

$P\{w^+ = ER1\}$: *ER1* encodes a chimeric protein in which the first RRM of ELAV is replaced with the first RRM of RBP9 (domain swap limits are as in Figure 1B).

$P\{w^+ = ER12\}$: *ER12* encodes a chimeric protein in which the first and second RRMs of ELAV are replaced with the first and second RRMs of RBP9 (domain swap limits are as in Figure 1B).

$P\{w^+ = ER3\}$: *ER3* encodes a chimeric protein in which the third RRM of ELAV is replaced with the third RRM of RBP9 (domain swap limits are as in Figure 1B).

$P\{w^+ = EH3\}$: *EH3* encodes a chimeric protein in which the third RRM of ELAV is replaced with the third RRM of HuD (domain swap limits are as in Figure 1B).

$P\{w^+ = ES1/2\}$: *ES1/2* encodes a chimeric protein in which the carboxy half of the first RRM of ELAV is replaced with the carboxy half of the first RRM of SXL (domain swap limits are as in Figure 1B).

$P\{w^+ = ES1\}$: *ES1* encodes a chimeric protein in which the first RRM of ELAV is replaced with the first RRM of SXL (domain swap limits are as in Figure 1B).

$P\{w^+ = ES2\}$: *ES2* encodes a chimeric protein in which the second RRM of ELAV is replaced with the second RRM of SXL (domain swap limits are as in Figure 1B).

$P\{w^+ = ES12\}$: *ES12* encodes a chimeric protein in which the first and second RRMs of ELAV are replaced with the first and second RRMs of SXL (domain swap limits are as in Figure 1B).

Immunoblot analysis: For the immunoblot analysis, $elav^{e5}/elav^{e5}$; $elav^{RBD60}/elav^{RBD60}$ females were crossed to *w*/*Y*; Tg/Tg males; *e5* is a protein null allele, RBD60 encodes an ELAV protein generated by the $P\{w^+ = elav^{RBD60}\}$ transgene with a deletion of the amino-terminal A/Q-rich region, and Tg denotes the transgene expressing mutant ELAV protein. F1 adult males of the genotype $elav^{e5}/Y$; $elav^{RBD60}/$; Tg/+ were collected. Immunoblot analysis was done essentially as previously described (Yao *et al.* 1993). A total of 10 μg of soluble protein extract from adult head was subjected to SDS-PAGE and electroblotted to a nitrocellulose filter. A 1:1000 dilution of rat anti-ELAV polyclonal serum was visualized using the ECL Western blotting detection kit (Amersham, Piscataway, NJ).

Viability rescue assays: For viability assays, $elav^{e5}$ *w sn*/balancer or $elav^{ts1}$ *w sn*/balancer virgin females were crossed to *w*/*w*; Tg/balancer males, where *e5* is a null allele and *ts1* is temperature-sensitive allele of the *elav* locus, *w* and *sn* are cuticular markers affecting eye color and bristle morphology, respectively, and Tg refers to the autosomal transgene being tested. Balancer chromosomes are dominantly marked and multiply inverted to suppress recombination (for more information, see Lindsley and Zimm 1992). The progeny of these crosses were counted. Percentage of rescue for *e5* = number of $elav^{e5}$ *w sn*/*Y*; Tg males/number of $elav^{e5}$ *w sn*/*w* females. Percentage of viability rescue for *ts1* = number of $elav^{ts1}$*w sn*/*Y*; Tg *males*/number of $elav^{ts1}$*w sn*/*w* females. All crosses were done at 25°.

Immunocytochemistry: For immunocytochemistry, $elav^{e5}/elav^{e5}$; $elav^{-13}/+$ females were crossed to *w*/*Y*; Tg/Tg males. Third instar wandering male larvae were collected by inspecting gonads. The larval brains were dissected, squashed, and processed for ELAV immunoreactivity as previously described (Yannoni and White 1997). For immunolocalization, mouse anti-ELAV mAb 5D3C5 hybridoma supernate (dilution of 1:5 to 1:10) was used. Secondary antibody conjugated to FITC (dilution 1:50) was from Jackson ImmunoResearch Laboratories (West Grove, PA). Images were obtained on a Zeiss Axiophot microscope equipped with fluorescence and phase contrast.

RESULTS

Generation of mutations that knock out RNP1-mediated RNA binding: Figure 1A shows a schematic of ELAV protein that depicts the A/Q-rich N-terminal domain, the three RRM domains, and the hinge between RRM2 and RRM3. In Figure 1B, a comparison of amino acid

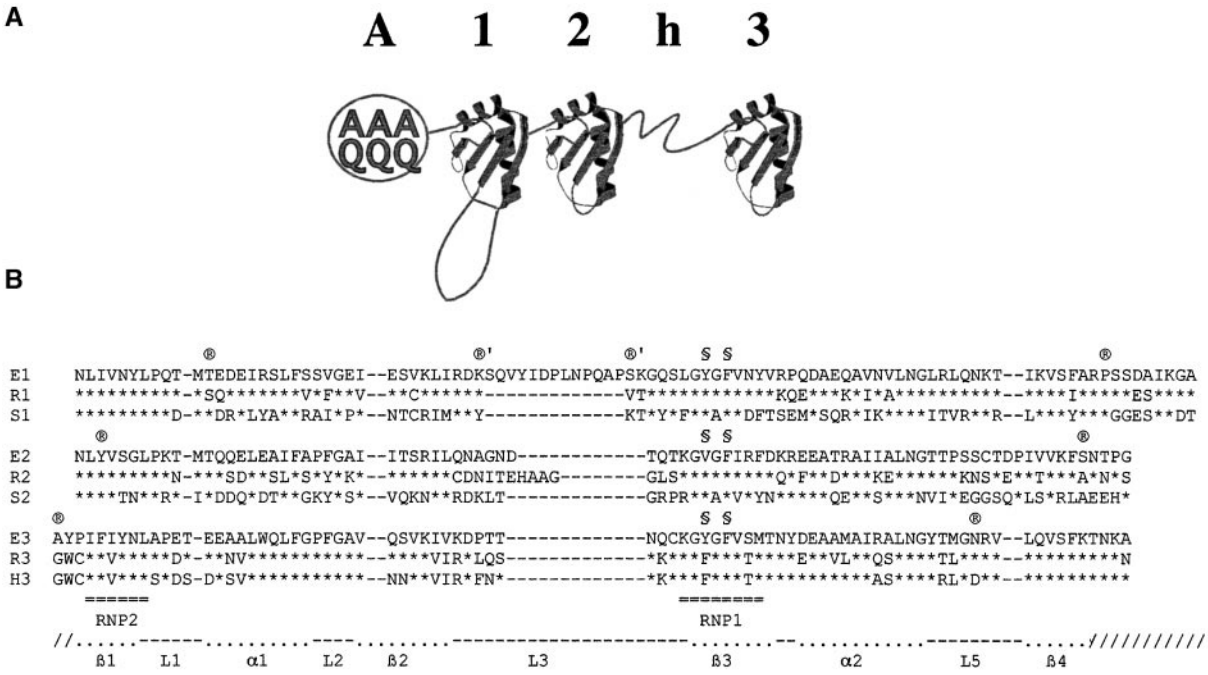

Figure 1.—(A) Schematic representation of the ELAV fusion protein, highlighting the domain structure as follows: A, Ala- and Gln-rich amino-terminal domain; 1, RRM1; 2, RRM2; h, hinge domain; and 3, RRM3. The RRM ribbon structure of U1A protein (Nagai *et al.* 1990) has been adapted for illustrative purposes of the ELAV domains. No specific knowledge of ELAV structure is implied. (B) Comparison of the amino acid sequences of the three ELAV RRMs with RBP9 and SXL. E, ELAV; R, RBP9; S, SXL; H, HuD. E1, S1, and R1 include the intradomain sequence between RRM1 β4 and RRM2 β1. At the bottom, the limits of RNP1, RNP2, and the β-sheets, α-helices, and loops are delineated. § indicates the mutated residues to generate AGD mutations. ® indicates boundaries of domain replacements for each RRM domain, ®′ shows the junction of amino half of S1 to carboxy half of E1, and the left boundary of ES1/2 is the same as ES1.

sequence of the RRMs of ELAV, RBP9 (Kim and Baker 1993), SXL (Bell *et al.* 1988), the third RRM of HuD (Szabo *et al.* 1991), the limits of consensus sequences RNP1 and RNP2, and secondary structure alignments are shown.

To generate ELAV mutants that specifically knock out individual RRM binding ability, the amino acids most likely to mediate RNA binding in each of the RNP1 motifs were mutated as described in materials and methods. These mutations consisted of two point mutants in solvent-exposed residues of RNP1 that are separated by a Gly and were designated as 1AGD (Y205A and F207D), 2AGD (V292A and F294D), and 3AGD (Y445A and F447D). Each AGD mutation is expected to selectively impair the RNA-binding ability of the RRM, without destroying the overall domain structure. The mutated Phe is the most conserved among the known RRMs and has been demonstrated to interact directly with the nucleotide base (Merrill *et al.* 1988), by a base stacking interaction for U1A N-terminal RRM (Oubridge *et al.* 1994) and for SXL (Handa *et al.* 1999). A and D substitutions were chosen as they dramatically alter the aromatic residues. To express these mutant ELAV proteins in the fly, we chose an 8.5-kb truncated version of the *elav* gene $P\{w^+ = elav^{DMORF}\}$, which provides a robust rescue of *elav* function (Yao *et al.* 1993). Transgenic fly stocks were generated by *P*-element-mediated transformation and several independent lines were established.

That the mutant proteins were expressed in the transgenic lines was confirmed by immunoblot analysis. For all mutant transgenes used in this study, flies of the genotype $elav^{e5}$; $elav^{RBD60}$; Tg were analyzed; *e5* is a protein null allele, *RBD60* is an *elav* allele that generates a functional protein in which most of the N-terminal auxiliary domain is deleted, and Tg denotes the transgene under consideration. The RBD60 protein provides an appropriate genetic background for this assay as it provides function and migrates faster than the wild-type ELAV (Yao *et al.* 1993). Therefore, the protein produced by the transgene is readily distinguished from RBD60 band (Figure 2) and even if the mutant protein is nonfunctional, $elav^{e5}$; $elav^{RBD60}$; Tg flies survive. Protein extracts from fly heads were subjected to immunoblot analysis and probed with anti-ELAV polyclonal antibody. As can be seen in Figure 2A, 1AGD, 2AGD, and 3AGD proteins generated by the transgenes used in this study are stable and seem to be present at a level comparable to the RBD60 protein.

Functional analysis of RNP1 mutants: The ability of each mutant protein to provide ELAV-associated viability function was assessed under two conditions. First, the transgene was challenged to rescue a null *elav* allele,

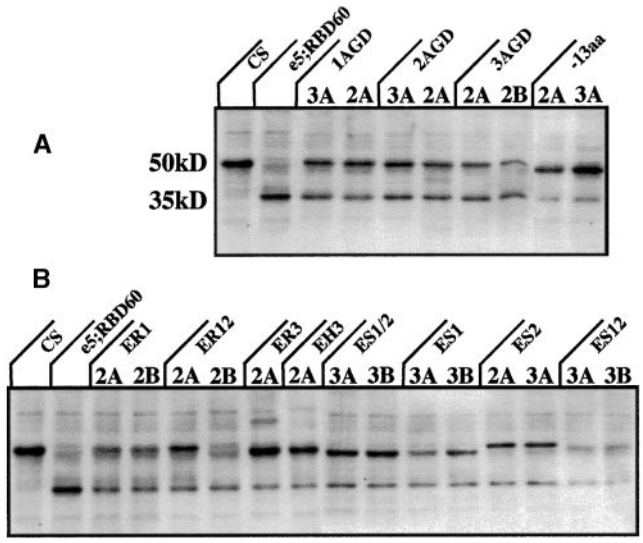

Figure 2.—Expression of *elav*^{AGD} and *elav*^{DR} transgenes. ELAV protein expression by the ELAV mutant expressing transgenes was assessed by immunoblot analysis. Immunoblots with 10 μg total adult head protein per lane were probed with rat polyclonal anti-ELAV. All mutant transgenes were examined in the genotype *elav*^{e5}*w sn*; *elav*^{RBD60}/Tg, where *elav*^{e5} is an *elav* null allele, *elav*^{RBD60} is an *elav* transgene that produces a functional protein with a large N-terminal deletion, and Tg is the mutant transgene to be tested. The protein produced by the mutant transgene is distinct from the smaller sized ELAV^{RBD60} protein (37 kD). The two lanes for a given construct represent two independent autosomal insertion lines. (A) Immunoblot of the AGD transgenic flies. Lane 1, Canton-S; lane 2, *elav*^{e5}*w sn*; *elav*^{RBD60}; other lanes, *elav*^{e5}*w sn*; *elav*^{RBD60}; Tg as indicated. (B) Immunoblot of the domain replacement transgenic flies. Lane 1, Canton-S; lane 2, *elav*^{e5}*w sn*; *Tf(2)elav*^{RBD60}; other lanes, *elav*^{e5}*w sn*; *elav*^{RBD60}; Tg as indicated.

elav^{e5}, where the transgene-generated protein is the only ELAV protein in the organism. Rescue under these same conditions with a transgene expressing the wild-type ELAV ORF (*elav*^{DMORF}) ranges between 50 and 90% (Yao *et al.* 1993). In the second paradigm, the transgene was asked to provide supplemental function to a hypomorphic *elav* allele, *elav*^{ts1}, which has a suppressed nonsense mutation at position 419W in RRM3 between RNP2 and RNP1 (Samson *et al.* 1995). *elav*^{ts1} flies survive at room temperature (22°) but do not survive at 25°, although a low percentage of survivors are seen depending on the culture conditions. The most critical temperature-sensitive period during development of *elav*^{ts1} flies is during the embryonic period when the concentration of nontruncated form of protein is low compared to concentration in the wild type (Samson *et al.* 1995).

Table 2 shows the results of viability assays using two independent transgenic lines for each of the AGD transgenes with the two *elav* alleles *elav*^{e5} and *elav*^{ts1}. Notably, as seen from lack of viability of *elav*^{e5}/*Y*; *elav*^{AGD} class of flies, none of the ELAV^{AGD} mutant proteins can support the viability function. Thus the RNA binding mediated by the RNP1 of each RRM is essential for ELAV function. The ELAV^{1AGD} and ELAV^{2AGD} proteins are also unable to provide supplemental function as the *elav*^{ts1}/*Y*; *elav*^{1AGD} or *elav*^{ts1}/*Y*; *elav*^{2AGD} flies do not survive. However, *elav*^{e5}/*Y*; *elav*^{3AGD} flies survive at a modest rate (∼10%), suggesting that ELAV^{3AGD} mutant protein is able to provide supplemental function to the ELAV^{ts1} protein.

The 13 amino acids in loop3 of ELAV RRM1 are not essential for ELAV function: When RRM1 of ELAV is compared to other ELAV family members and SXL, a sequence of 13 amino acids in loop3 stands out as being an unusual feature of ELAV alone (Figure 1B; Birney *et al.* 1993). Moreover, this sequence was preserved along with the rest of the RRM domain region in the *D. virilis* ELAV protein (Yao and White 1991). The significance of loop3 in the function of different proteins has been variable; in the case of U1A and U2B″ it has been implicated as a prime determinant of their cognate RNA, while some proteins have little or no loop3 (Gorlach *et al.* 1994).

To test the significance of these 13 amino acid residues, a transgene, *elav*$^{-13}$, encoding an ELAV protein deleted for these 13 amino acids was created and transformant flies bearing this transgene were generated. Figure 2A shows an ELAV immunoblot of total head protein from rescued *elav*^{e5}; *elav*$^{-13}$ flies. Viability analysis shows a robust rescue of both *e5* and *ts1* alleles (Table 2). Thus, although unique, the 13 amino acids are not essential for ELAV's vital function. The *elav*^{e5}; *elav*$^{-13}$ adult flies appear normal and are fertile. This result allows us to disregard the ELAV-specific 13 amino acid residues in loop3 from future functional considerations. Notably, the elimination of 13 amino acids increases the homology between RRM1 for ELAV, RBP9, and SXL significantly (Figure 1B).

Generation of RRM domain replacement mutants: The individual domains of the ELAV family of proteins show a high degree of sequence identity, yet the proteins perform diverse post-transcriptional functions. In Figure 1B the sequences of RRM domains of RBP9 and SXL are compared to ELAV. The experiments described in this section were undertaken to assess the degree to which each domain was specific for the ELAV function and which domains perhaps performed a function common to all ELAV proteins. The strategy we used consisted of substituting an ELAV RRM with the corresponding RRM from another protein. For RRM1 and RRM2 replacements we chose RBP9 and SXL, both from *D. melanogaster*, RBP9 as the protein with the highest homology to ELAV RRMs and SXL as a protein evolutionarily related to ELAV, but not in the ELAV family. RRM3 analyses were performed with RBP9 and HuD, a human ELAV family member (Szabo *et al.* 1991).

To simplify the nomenclature for chimeric proteins, we denote ELAV, RBP9, SXL, and HuD as E, R, S, and H, respectively, and a chimeric protein, for example, in which RRM1 is replaced with SXL RRM1, is denoted as

TABLE 2

Genetic complementation of *elav^{e5}* and *elav^{ts1}* by AGD and *elav^{-13}* transgenes

		elav^{e5}			*elav^{ts1}*			
Transgene	Line	No. rescued males	No. control females	Rescue (%)	No. rescued males	No. control females	Rescue (%)	No. ts1 males
DMORF	1	214	410	52	388	422	92	0
	2	110	114	96	104	114	91	0
1AGD	3A	0	384	0	0	563	0	0
	2A	0	350	0	0	324	0	0
2AGD	3A	0	303	0	17	457	4	0
	2A	0	428	0	7	464	2	5
3AGD	2A	0	274	0	34	354	10	1
	2B	0	365	0	44	468	9	0
elav^{-13}	2A	191	417	46	316	354	89	2
	3A	106	138	77	42	29	145	0

Transgenes encoding mutant proteins were tested for functional complementation with *elav* alleles: *elav^{e5}* and *elav^{ts1}*. *w/Y*; Tg/*balancer* were crossed to *elav^{x} w sn/balancer* (x denotes the *elav* allele, Tg stands for the transgene). Rescue (%) = (number of rescued males of genotype *elav^{x} w sn/Y*; Tg/+)/(number of control females of genotype *elav^{x} w sn/w*; Tg/+) × 100. For details see materials and methods.

ES1. The boundaries of each domain are shown in Figure 1B; the domain replacements consisted of precise placement of a domain in the related RRM. Transgenes encoding the following chimeric proteins were created to study RRM1 and RRM2 function: *ER1* and *ER12* with RBP9 RRMs, *ES1/2* (amino half of RRM1 from SXL replacing amino half of ELAV), *ES1*, *ES2*, and *ES12* with SXL RRMs. When both RRM1 and RRM2 were replaced (*ER12* and *ES12*), the replacement included the short amino acid intradomain region which is well conserved. To study RRM3 chimeric proteins, transgenes encoding ER3 and EH3 were constructed. Transformant lines for all transgenes were established.

That these transgenics express proteins was validated by immunoblot analysis using an anti-ELAV polyclonal serum. All domain replacement proteins were observed and were present at levels compatible with the expression level of the parental transgene (Figure 2B). We tested several independent lines in viability rescue assays for each domain replacement protein; in Table 3 we have chosen to show the lines that demonstrated rescue and, where applicable, lines that did not demonstrate any rescue under the most stringent condition. We also noticed that the transgene-generated protein levels showed only minor variations and did not necessarily correlate with rescue properties described below.

RRM3 from either RBP9 or HuD shows functional homology: The overall similarity of ELAV RRM3 to R3 and H3 is 75 and 70% (Figure 1). Both RNP1 and RNP2 sequences are identical in RBP9 and HuD. When compared to ELAV, 6/8 and 5/6 residues are identical; furthermore, the ones that are not identical are conservative changes. Not surprisingly, the rescue analysis in Table 3 shows a robust rescue of *e5* (*ER3*: 66%; *EH3*: 65%). Thus the third RRM domains of RBP9 and HuD can readily replace E3 as the chimeric proteins are fully functional.

RRM1 and RRM2: The rescue of *e5* allele with ER1 was line dependent. Two transgenes yielded a modest 4 and 6% rescue, whereas several others did not (Table 3). Those transgenes that conferred *e5* rescue also conferred a robust *ts1* rescue (69 and 58%). This suggests that in the context of the ELAV molecule a domain swap of R1 for E1 is somewhat functional; however, the low <10% rescue of *e5* suggests that this protein is still a severe hypomorph. *ES1* was ineffective in *e5* rescue (Table 2), but one of the lines did yield a modest rescue of *ts1* (8%), suggesting that *ES1* is severely impaired in function. The impairment may come from the replacement of the carboxy half of RRM1, as *ES1/2* in which only the amino half is replaced conferred partial rescue of the *e5* allele (28 and 35%) and complete rescue of the *ts1* allele (70 and 90%).

One of the three *ES2* transgenes rescued the *e5* allele (5%), and two transgenes rescued the *ts1* allele (17 and 11%). No rescue under either condition was seen by *ER12* or *ES12* transgenes.

Subcellular localization of mutant proteins: Do the RNP1 mutations, AGD1, AGD2, and AGD3 affect the subcellular localization of ELAV? We have shown that in the wild type, ELAV protein localizes predominantly to the nucleus and that the nuclear localization of ELAV is important for its function (Yannoni and White 1997, 1999). Therefore, one reason for the nonfunctionality of the AGD mutations could be that the subcellular localization is disrupted. To analyze the subcellular localization, larvae of the genotype *e^{5}/Y*; *elav^{-13}*; Tg were selected as described in materials and methods.

TABLE 3

Genetic complementation of *elav*^{e5} and *elav*^{ts1} by domain replacement transgenes

		elav^{e5}			*elav*^{ts1}			
Transgene	Line	No. rescued males	No. control females	Rescue (%)	No. rescued males	No. control females	Rescue (%)	No. ts1 males
ER1	29	26	467	6	219	326	67	0
	2A	17	455	4	163	252	65	0
	2B	0	930	0	1	861	0	0
ER12	2A	0	436	0	0	346	0	0
	2B	0	481	0	0	291	0	0
	52	0	227	0	0	299	0	0
ER3	2A	267	297	90	245	370	66	0
EH3	2A	222	339	65	342	354	97	2
ES1/2	3A	69	490	14	253	359	70	1
	3B	122	433	28	228	254	90	13
	42	80	230	35	145	191	76	3
ES1	3A	549	0	0	26	345	8	3
	42	338	0	0	0	310	0	2
	45	517	0	0	0	310	0	0
ES2	2	0	514	0	53	312	17	0
	2A	16	337	5	31	284	11	0
	3A	0	483	0	5	261	2	0
ES12	3A	0	440	0	0	305	0	0
	25	0	355	0	0	273	0	0
	32	0	426	0	0	290	0	0

Transgenes encoding mutant proteins were tested for functional complementation with *elav* alleles: *elav*^{e5} and *elav*^{ts1}. Males *w*/*Y*; Tg/balancer were crossed to *elav*^{x}*w sn*/balancer (x denotes the *elav* allele, Tg stands for the transgene). Rescue (%) = (number of rescued males of genotype *elav*^{x} *w sn*/*Y*; Tg/+)/(number of control females of genotype *elav*^{x}*w sn*/*w*; Tg/+) × 100. For details see materials and methods.

ELAV immunoreactivity in the larval neurons was analyzed using ELAV mAb 5D3C5 as described. Briefly, in the genotype analyzed, there are two ELAV proteins, the mutant protein produced by the transgene of interest and ELAV^{-13}, which is deleted for the epitope recognized by the monoclonal 5D3C5, but is functional. The ELAV immunoreactivity in the nucleus was evidenced for proteins AGD1, AGD2, and AGD3 and appeared characteristic of wild-type ELAV (Figure 3A). Thus aberrant localization is not involved in the functional impairment of AGD mutant proteins.

We also analyzed the subcellular localization of chimeric proteins: ES2, ER3, and EH3. These specific ones were chosen as they could be analyzed with the same paradigm as described for the AGD mutants. Chimeric proteins that replace E1 could not be analyzed as the mAb recognizes the loop3 in E1. The ER3 and EH3 proteins localized primarily to the nucleus as is the case with wild-type protein (Figure 3B, c–f). However, the ES2 protein was found to localize to both the nucleus and the cytoplasm (Figure 3B, a and b).

DISCUSSION

elav-encoded function, although vital at the organismal level, is not vital for cell survival (Campos *et al.* 1985). The *elav*-null embryonic nervous system has a disorganized appearance in that many processes are irregular as evidenced in defective commissures. Otherwise the embryos and the neuronal soma appear normal. Our studies have demonstrated that ELAV regulates neural-specific alternative splicing of at least three broadly expressed genes, *nrg*, *ewg*, and *arm*. In each case, the level of a neural-specific isoform-encoding transcript is influenced by ELAV levels (Koushika *et al.* 1996, 2000). Additionally, by analogy with other family members, ELAV could also affect mRNA stability by interacting with the 3′ untranslated region or regulating translatability.

Although the full extent of *elav*-modulated genes is currently not known, it is reasonable to suggest that the misregulation of a cohort of *elav*-regulated genes could collectively disable the embryonic nervous system. In this discussion our focus is on the ELAV-RNA interaction, which is the point of convergence for all *elav*-related functions. Because of our studies on ELAV's role in alternative splicing, ELAV's similarity to SXL, and the domain replacement studies in this report, we take advantage of the wealth of knowledge related to SXL-RNA interactions to interpret our results.

RNA binding of each RRM is essential to ELAV function: The analysis of AGD mutations revealed that aro-

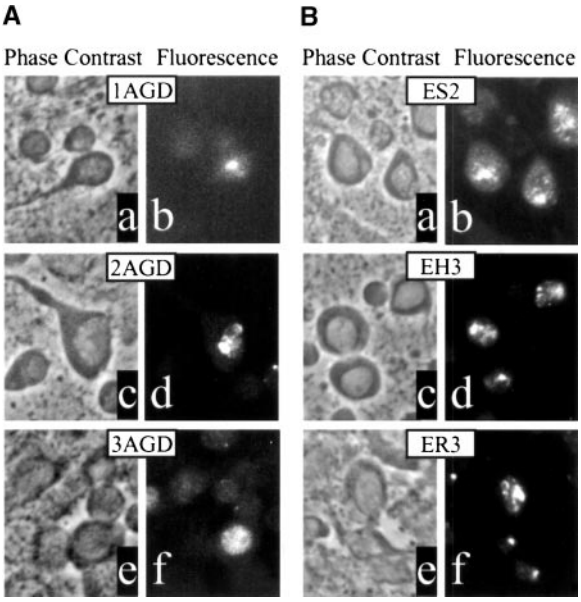

Figure 3.—Subcellular localization of mutant ELAV proteins. Central nervous system squash preparations from third instar larvae were immunoreacted with ELAV mAb 5D3C5. The antibody does not recognize ELAV^{-13aa} and, therefore, only the mutant ELAV is visualized in the genotype, *elav*^{e5}*w sn*; *elav*$^{-13}$/Tg, where *elav*^{e5} *is elav* null, *elav*$^{-13}$ is an *elav* transgene with 13-amino-acid deletion in loop3 of RRM1, and Tg is the mutant transgene to be visualized. (A) Phase contrast and immunofluorescent images for 1AGD (a and b), 2AGD (c and d), and 3AGD (e and f). (B) Phase contrast and immunofluorescent images for ES2 (a and b), EH3 (c and d), and ER3 (e and f).

matic amino acid substitutions in the RNP1 domain of each RRM result in proteins unable to carry out their *in vivo* function, as they show no rescue on their own. A recent report on the crystal structure of SXL complexed with *tra* target RNA further validates the importance of aromatics in protein-RNA interactions (Handa *et al.* 1999). This SXL study demonstrated that Phe in both RRM1 and RRM2 and Val in RRM2, precisely the amino acids mutated in the present study, are involved in stacking interactions with RNA (Handa *et al.* 1999). The complete conservation of these amino acids between ELAV and SXL, and the effectiveness with which the substitution mutations in these residues disable the protein, suggest that these mutations abrogate or at least greatly diminish the RNA-protein interaction. As the AGD mutation in the third RRM also did not provide rescue on its own, the RNA-binding ability of the third RRM is also essential. However, 3AGD did provide modest rescue in conjunction with the hypomorphic allele *ts1*, suggesting that the 3AGD can associate with either the target RNA or in some other way facilitate the ELAV^{ts1}-RNA interaction. These data indicate that each RRM contributes to the ELAV function through its RNA-binding property.

The AGD mutations did not appear to perturb subcellular localization of mutant ELAV. Thus RNA binding by ELAV, as disrupted by these mutations, is not essential for nuclear localization.

RRM domain replacement analysis: Domain replacement is a form of mutagenesis that samples many mutations at once while increasing the likelihood of maintaining the basic structure and stability of the protein. In assays used in this study, the RRM3 domain replacement proteins were fully functional. The complete functional rescue by ER3 and EH3 leads to the conclusion that the RRM3 domains are functionally homologous. It also demonstrates a considerable degree of pliancy within the amino acids, as in the case of RBP9 RRM3, where there are 21 amino acid changes in 80 residues. Moreover, 2 conservative amino acid changes in the RNP1 sequence, Tyr to Phe and Ser to Thr, that are in positions demonstrated to contact RNA bases in the SXL/*tra* RNA cocrystal (Handa *et al.* 1999), apparently do not significantly alter ELAV target recognition.

In contrast to RRM3 replacements, the RRM1 and RRM2 replacements provided at best a marginal rescue. The ER1 protein provided a modest rescue of *e5* allele (~5%) and robust rescue of *ts1* allele (~65%), while ES1 provided only a supplemental function in the *ts1* allele rescue (~8%), and no function in the *e5* rescue. The rescue demonstrated in the *ts1* background by these proteins suggests that they can participate in some protein-RNA interactions, albeit at a lower than normal efficiency. It is also interesting to note that in contrast to ES1, ES1/2 provided rescue of both *e5* and *ts1* alleles, underscoring the possible importance of amino acids in the C-terminal half of RRM1 for functional specificity. Similar to ES1, ES2 also exhibits the ability to provide supplemental function in the *ts1* background, but no ability to rescue *e5* allele.

The low function of the RRM1 replacement was surprising given the high homology among these proteins. ELAV and SXL RRM1 and RRM2 are strikingly similar. While SXL specifically interacts with the UGUUUU UUU sequence, SXL can also presumably recognize a poly(U) stretch without the intervening G, since it is lacking in some *in vivo* SXL targets (Wang and Bell 1994; Bashaw and Baker 1997; Kelley *et al.* 1997). ELAV-like proteins have been reported to bind to a variety of poly(U)-rich sequences *in vitro* (Levine *et al.* 1993; Gao *et al.* 1994; Liu *et al.* 1995; Chung *et al.* 1996; Ma *et al.* 1996; Jain *et al.* 1997; Myer *et al.* 1997; Wu *et al.* 1997; Peng *et al.* 1998; Sokolowski *et al.* 1999), further suggesting similar modes of target recognition. The data from Handa *et al.* (1999) allow an assessment of homology between RRM1 and RRM2 of ELAV and SXL among just the amino acid residues that participate directly in RNA binding. The six residues from the RNP1 and RNP2 sequences of both RRMs that are involved in base stacking interactions in the SXL-RNA crystal are conserved in ELAV (Handa *et al.* 1999). The only other residue involved in base stacking, Arg195, lies in loop5 of RRM1 and is not conserved in ELAV (Thr). Of the

amino acid side groups that contact either the backbone or base of the SXL target RNA, 8 of 12 are identical. This homology of RNA-interacting residues argues for a similar mechanism of RNA recognition. The RBP9 RRM1's similarity to ELAV is even more striking, since RNP1 and RNP2 domains are identical to ELAV's and overall it differs in only 15 of 80 residues (discounting the 13 amino acids in loop3).

Based on the SXL model it is reasonable to entertain the idea that RRM1 and RRM2 of ELAV together make up a single binding site. Single domain replacement proteins ES1 (8%), ES2 (17%), and ER1 (67%) were able to provide some supplemental function. However, when both RRM domains were replaced as in ES12 and ER12, the chimeric protein was completely nonfunctional. This could perhaps suggest that in a single domain replacement chimeric protein, the remaining ELAV domain is able to serve as the main anchoring domain. An untested possibility is that the concentration of ES12 and ER12 proteins in the nucleus was insufficient to support function. It is also possible that in these chimeras, inter-RRM1-RRM2 interactions are compromised, but conservation of the two SXL RRM1 residues that interact with RRM2 (Tyr131 and Lys197) in both ELAV and RBP9 makes it unlikely.

Given the homology between corresponding domains of ELAV, RBP9, and SXL, results of our domain replacement studies are indeed puzzling. On the one hand, RRM3 replacements were fully functional, which is consistent with the notion that it acts as a module. On the other hand, the very limited function of RRM1 and RRM2 replacements suggests that perhaps the function of these two domains is distinct from that of RRM3 and involves additional intra- and/or interprotein-RNA or protein-protein interactions. Moreover, these results imply that although the RNA-binding property of each RRM is essential for ELAV function, residues other than the RNP1 and RNP2 must also be important for the specificity of ELAV function.

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