Two Distinct Temperature-Sensitive Alleles at the *elav* Locus of Drosophila Are Suppressed Nonsense Mutations of the Same Tryptophan Codon

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ABSTRACT

The Drosophila gene *elav* encodes a 483-amino-acid-long nuclear RNA binding protein required for normal neuronal differentiation and maintenance. We molecularly analyzed the three known viable alleles of the gene, namely $elav^{ts1}$, $elav^{Flj1}$, and $elav^{Flj2}$, which manifest temperature-sensitive phenotypes. The modification of the $elav^{Flj1}$ allele corresponds to the change of $glycine_{426}$ (GGA) into a glutamic acid (GAA). Surprisingly, $elav^{ts1}$ and $elav^{Flj2}$ were both found to have $tryptophan_{419}$ (TGG) changed into two different stop codons, TAG and TGA, respectively. Unexpectedly, protein analysis from $elav^{ts1}$ and $elav^{Flj2}$ reveals not only the predicted 45-kD truncated ELAV protein due to translational truncation, but also a predominant full-size 50-kD ELAV protein, both at permissive and nonpermissive temperatures. The full-length protein present in $elav^{ts1}$ and $elav^{Flj2}$ can *a priori* be explained by one of several mechanisms leading to functional suppression of the nonsense mutation or by detection of a previously unrecognized ELAV isoform of similar size resulting from alternative splicing and unaffected by the stop codon. Experiments described in this article support the functional suppression of the nonsense mutation as the mechanism responsible for the full-length protein.

MANY ts mutations have been reported to cause amino acid substitutions in open reading frames (*e.g.*, BAER *et al.* 1989; TH'NG *et al.* 1990; MASAI and HOTTA 1991; MULLINS and RUBIN 1991; JOHNSON *et al.* 1993; SAVILLE and BELOTE 1993), and many have successfully been engineered *in vitro*, leading to amino acid substitutions or, occasionally, to the insertion of one or two amino acids (see, *e.g.*, ENGELMAN and ROSENBERG 1987; KIPREOS *et al.* 1987). This is consistent with the fact that ts mutations are mostly caused by mutagenic agents generating missense mutations rather than by mutagens causing frame shifts or larger molecular rearrangements (reviewed in SUZUKI 1970). The resulting thermolability of the mutated proteins is only one of the reasons these alterations can be associated with ts phenotypes. Modified forms of protein whose activity, binding, folding, *etc.*, is affected by temperature constitute additional possibilities. For instance, both the decreased affinity (K_m) of N-myristoyltransferase for myristoyl-Coa (JOHNSON *et al.* 1993) and the defective assembly of the catalytic RNA M1 and its protein cofactor C5 into a functional RNAse P holoenzyme (BAER *et al.* 1989) are due to ts missense mutations.

Two notable cases of ts mutations that do not correspond to minor nucleotide substitution/additions have been reported. First, ts alleles of the Drosophila genes *white* and *Ubx* are caused by the insertion of transposable elements within intronic regions (BINGHAM and CHAPMAN 1986; ASHBURNER 1989), presumably affecting the production of mature transcripts in a temperature-dependent fashion. Second, a nonsense mutation in the Drosophila gene *sevenless* leads to a ts mutation that has been suggested as being due to reinitiation of translation downstream of the stop codon, resulting in a temperature-sensitive N-terminally truncated protein (MULLINS and RUBIN 1991). In this study, we analyzed three $elav^{ts}$ mutations and found that while one of them ($elav^{Flj1}$) corresponds to a missense mutation, the other two ($elav^{ts1}$ and $elav^{Flj2}$) are surprisingly due to nonsense mutations in the codon for $tryptophan_{419}$ of the 483-amino-acid-long ELAV.

The *elav* gene provides a function necessary for normal differentiation and maintenance of the Drosophila nervous system (for a review, see YAO *et al.* 1993). Most mutations at the locus are embryonic recessive lethal, and homozygous mutant embryos show abnormal neurites, except for three alleles ($elav^{ts1}$, $elav^{Flj1}$, and $elav^{Flj2}$), which allow development up to the adult stage. Each of the viable alleles exhibits temperature-sensitive phenotypes. Two of them, $elav^{Flj1}$ and $elav^{Flj2}$, were induced in a genetic screen for behavioral mutants (HOMYK *et al.* 1980) and show clear temperature-sensitivity of their pleiotropic phenotype. These mutants were reported to have good flying and hopping abilities at permissive temperature (22°), but to be flightless and uncoordinated when raised at the restrictive temperature (29°) (HOMYK

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et al. 1980; HOMYK and GRIGLIATTI 1983). The allele $elav^{Flij1}$ was described as more severe than $elav^{Flij2}$. The $elav^{Flij1}$ mutants have abnormal electroretinograms and no optomotor response whether raised at 22° or 29°, while most of the flies carrying the $elav^{Flij2}$ mutation have a normal ERG and optomotor response at 22°. In addition, the morphology of photoreceptor cells and optic lobes of $elav^{Flij1}$ flies is abnormal in flies raised at 29° but not at 20° (HOMYK *et al.* 1985). The mutation $elav^{ts1}$ was independently recovered from an EMS screen for lethal *elav* alleles (CAMPOS *et al.* 1985) and shows an accentuated ts phenotype. At 25°, the emerging adults are uncoordinated and die within a few days, and the stock survives only when raised at 18°. The morphology of the visual system is abnormal at 18° and, to a greater extent, at 25° (CAMPOS *et al.* 1985).

The structure of *elav* protein (ELAV) was deduced from the study of two cDNAs: cDNA-1 from an embryonic library and cDNA-2 from an adult head library (ROBINOW *et al.* 1988). Both cDNAs encode a 483-amino-acid open reading frame (ORF1) corresponding to a polypeptide with an A/Q-rich N-terminus, adjacent to three domains approximately 80 amino acids long containing ribonucleoprotein consensus sequences (RNP-CS; BANDZIULIS *et al.* 1989) that are characteristic of proteins that bind RNA. This class of RNA binding domain is referred to as either an RNA binding domain (RBD; for a review, see BANDZIULIS *et al.* 1989) or RNA recognition motif (RRM; KENAN *et al.* 1991; MATTAJ 1993). Members of this family of RNA binding proteins have diverse binding specificities and affinities and play roles in a variety of processes related to RNA metabolism (for reviews, see BANDZIULIS *et al.* 1989; KENAN *et al.* 1991; MATTAJ 1993). The *elav* gene is the first identified member of a neuron-specific RNA binding multigene family that exists also in vertebrates, including humans (SZABO *et al.* 1991; KIM and BAKER 1993; KING *et al.* 1994; SAKAI *et al.* 1994; GOOD 1995; PERRON *et al.* 1995; G. MANLEY and H. FURNEAUX, personal communication).

In this study, we analyzed molecular alterations in three *elav* ts mutations and found that one of them ($elav^{Flij1}$) corresponds to an amino acid substitution, consistent with the traditional view of ts mutations. In contrast, we found that in two independant mutations, $elav^{ts1}$ and $elav^{Flij2}$, tryptophan$_{419}$ TGG is changed into two different stop codons, TAG and TGA, respectively. Furthermore, analysis of protein extracts from flies of the genotypes $elav^{ts1}$ and $elav^{Flij2}$ revealed the presence of two ELAV forms. One of these forms has an apparent size of 45-kD, consistent with the presence of the premature stop codon mutations, and the other has an apparent size of 50-kD, identical to the size of normal ELAV. The full-size ELAV protein in $elav^{ts1}$ and $elav^{Flij2}$ flies might be explained by alternative splicing, where the mutant stop codon is absent in one of the spliced forms. Under this interpretation, the wild-type gene encodes two distinct proteins that comigrate in a denaturing gel. The possible presence of two forms of ELAV protein in the $elav^{ts1}$ and $elav^{Flij2}$ extracts is intriguing, as previous studies had never suggested two ELAV isoforms. An alternative possibility is that the wild-type gene encodes only one protein and that the full-size ELAV found in $elav^{ts1}$ and $elav^{Flij2}$ results from the functional suppression of the nonsense codon—for instance, by RNA editing, translational readthrough, translational hopping, or frameshifting (for reviews, see MURGOLA 1985; VALLE and MORCH 1988; CATTANEO 1991). We use the term *suppression* to refer to these possible mechanisms. Experiments to differentiate between these possibilities are described.

MATERIALS AND METHODS

Constructions: We refer to the location of nucleotides in the *elav* clones according to their position in the 9285 nucleotide published sequence (ROBINOW *et al.* 1988) and to the location of restriction sites by the position of the first nucleotide of the recognition site. The 8.5-kb fragment included between nucleotides 757 and 9285 (fused to termination of transcription and polyadenylation signals) is sufficient for transformation rescue (Figure 1; YAO and WHITE 1991). The 483-amino-acid-long open reading frame (nucleotides 7099–8546) found in cDNA-1 is contained in the third exon, except for the A of the ATG initiation codon (nucleotide 4890).

Isolation and sequencing of genomic fragments containing *elav* ORF from $elav^{ts1}$, $elav^{Flij1}$, and $elav^{Flij2}$ flies: A 2.9-kb *Hin*dIII-*Eco*RI (6378–9280) genomic fragment, containing the *elav* ORF, was cloned for each of the three *elav* alleles by creating a "mini" library of 2.9-kb *Hind*III-*Eco*RI fragments into pBluescriptKS+ for each genotype and screening with the wild-type *elav* cDNA. Several clones containing the 2.9-kb *elav* ORF fragment were identified for each mutant and are referred to as p2.9HEts1, p2.9HEJ1, and p2.9HEJ2. For each *elav* mutation, the entire ORF was sequenced with Sequenase DNA polymerase (U.S. Biochemicals) in both directions for at least two individual clones.

Plasmid p2.6SE containing the 2.6-kb *Xma*I-*Eco*RI (6639–9280) *elav* genomic fragment (YAO and WHITE 1991) was modified by filling in the *Bam*HI site of the vector backbone, yielding p2.6SEhst. The wild-type 1.8-kb *Xma*I-*Bam*HI fragment of p2.6SEhst was replaced with that from each of the constructs p2.9HEts1, p2.9HEFliJ1, and p2.9HEFliJ2, generating p2.6SEts1hst, p2.6SEJ1hst, and p2.6SEJ2hst. A 3.4-kb *Xma*I-*Xba*I fragment (containing the mutated *elav* ORF and the α1-tubulin trailer sequences) was purified from these plasmids and subjected to a three-fragment ligation together with the 5.9-kb *Pst*I-*Xma*I *elav* genomic fragment and transformation vector pCaSpeR (detailed in YAO and WHITE 1991).

Construction of *elav* transformation vectors carrying new mutations: Plasmid pe336 differs from the wild-type $elav^{DmORF}$ construct (YAO and WHITE 1991) by the insertion of oligonucleotide 5′-CCGAATTCGG-3′ between nucleotides 8357 and 8358, inducing a frame shift mutation in the *elav* ORF. In order to build pe336, a pBluescript derivative (pe325) containing the *Sau*3AI-*Bam*HI (8102–8402) *elav* fragment was partially digested with *Pvu*II and religated in the presence of the oligonucleotide, generating a set of recombinant plasmids among which pe330 was identified as carrying the linker between nucleotides 8357 and 8358. Plasmid pe333 was generated by using the *Bst*XI-*Bam*HI (8269–8402) fragment from pe330 to replace the wild-type *Bst*XI-*Bam*HI fragment in pe332, a pUC18 derivative that contains a *Sma*I-*Xba*I insert

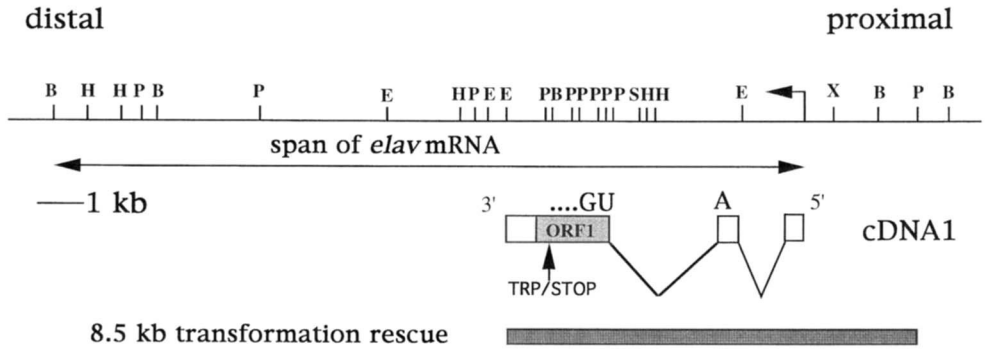

FIGURE 1.—Structure of the *elav* locus. A restriction map of the *elav* locus is shown as a continuous line. The 9.3 kb *Bam*HI-*Eco*RI at the 5′ end of the locus (first 5′ *Bam*HI site and second 5′ *Eco*RI site on the figure) has been entirely sequenced (ROBINOW *et al.* 1988). Transcripts extend much further downstream, as demonstrated by Northern and cDNA analysis. The structure and splicing pattern of cDNA-1 is shown below, as well as the 8.5 kb *Pst*I-*Eco*RI genomic fragment that rescues *elav* null mutations. B: *Bam*HI, E: *Eco*RI, H: *Hin*dIII, P: *Pst*I, S: *Sma*I, X: *Xba*I.

composed of the *elav Sma*I-*Eco*RI (6639–9280) fragment fused to transcription termination and polyadenylation signals of the α1 tubulin gene (YAO and WHITE 1991). A three-way ligation between the 3.4-kb *Sma*I-*Xba*I fragment from pe333, the 5.8-kb *Pst*I-*Sma*I (757–6639) *elav* fragment, and the 7.8 pCaSpeR cut with *Pst*I and *Xba*I yielded pe336.

Plasmid pe339 differs from the wild-type $elav^{DmORF}$ construct (YAO and WHITE 1991) by the insertion of oligonucleotide 5′-CTAGCTAGCTAG-3′ between nucleotides 8650 and 8657. The mutation affects ORF located downstream of *elav* ORF1. It shortens a 74-amino-acid-long ORF (nucleotides 8449–8670; Figure 6B, frame a) by 6 amino acids. It also modifies and splits a 131-amino-acid-long ORF (nucleotides 8462–8854; Figure 6B, frame b) into a series of short ORFs (from 5′ to 3′): 65, 3, 1, 27, 15, and 9 amino acids long, respectively. The 4-amino-acid-long ORF in reading frame c that is modified by the mutation is unlikely to be relevant (Figure 6B). The mutagenized plasmid was a Bluescript derivative, p3.4SX, containing the same 3.4-kb *Sma*I-*Xba*I fragment as its derivative, pe333 (see above; YAO and WHITE 1991). The oligonucleotide was inserted at the *Nsi*I site (8649) of the *elav Sma*I-*Eco*RI (6639–9280) fragment by cutting p3.4SX with *Nsi*I, treating with T4 DNA polymerase, and ligating with the linker, generating pe337. Plasmid pe337 was partially cut with *Xba*I and cut to completion with *Sma*I, and the 4.2-kb *Xba*I-*Sma*I *elav* fragment (2518–6639) was inserted, leading to a Bluescript derivative, pe338, containing nucleotides 2518–9280 of *elav* with the *in vitro* generated mutation, and fused to the termination of transcription and polyadenylation signals of the α1 tubulin gene. The final step for the construction of pe339 was to replace the 7.6-kb *Xba*I fragment of pe336 with the analogous *Xba*I fragment of pe338.

The sequence of the mutations was verified by dideoxy-sequencing with *Taq* DNA polymerase. As expected, the mutation in pe336 changes the wild-type 5′-. . . GGCAGCTGTT . . .-3′ into 5′-. . . GGCAGCCGAATTCGGCTGTT . . .-3′, where the inserted nucleotides are underlined. As expected, pe339 contains the oligonucleotide with a stop codon in all three possible open reading frames. However, the sequence of the mutation is 5′-. . . AAATCTAGCTAGCTAGGGTAT . . .-3′, and differs from the predicted 5′-. . . AAATACTAGCTAGCTAGTGGTAT . . .-3′, where the inserted oligonucleotide is underlined and the missing nucleotides are bold.

Protein preparation and immunoblot analysis: Protein extracts were made by homogenizing adult heads (10–30) or embryos (50–100) at 4° in 100–500 μl 1× PBS, 0.5% NP40, 1 mM EGTA pH 8, PMSF 0.2 mM, pepstatin 1 μg/ml, leupeptin 0.5 μg/ml. Extracts were spun for 10 min in a microcentrifuge and supernatants were collected. In the case of embryonic extracts, determination of the concentrations was performed using the Bio-Rad protein assay reagent. In the case of head samples, loadings were performed according to the number of heads (usually 10–15 per lane). Proteins were separated on 12% SDS-polyacrylamide (37.5:1) minigels (9 × 5 × 0.5 cm) and transferred to nitrocellulose in a semidry blotting apparatus for 30 min at 150 mA. ELAV immunodetection was performed using either colorimetric or chemiluminescence (Amersham ECL) methods according to manufacturers' instructions. A rat anti-ELAV polyclonal antibody (ROBINOW and WHITE 1991) or a mouse anti-ELAV monoclonal antibody (1:5000 dilution, a gift from G. RUBIN) were used for primary incubation. The secondary antibodies were either alkaline phosphatase-conjugated anti-rat IgG or anti-mouse IgG (Sigma or Amersham) or peroxidase-conjugated anti-mouse IgG (Amersham NNA 931). Comparisons between the amounts of protein loaded in each lane were performed by detecting β-tubulin immunoreactivity with chemiluminescence, via a 1:10,000 mouse primary antibody (Sigma) and 1:10,000 peroxidase-conjugated anti-mouse secondary antibody (Amersham NA 932).

Approximate quantification of the amount of ELAV protein in a given test sample was performed by preparing an immunoblot with twofold serial dilutions of the test and the reference extracts. The ratio between the dilution factors of the reference/test extract lanes that gave best matching signal intensities were considered to reflect the ratio of ELAV protein abundance between reference and test extracts.

Germline transformations: Embryos from the stock $y\ w^{67c23}$ were coinjected with 200 ng/ml pCaSpeR derivatives and 50 ng/ml pπ25.7 (KARESS and RUBIN 1984; PIRROTTA 1988; THUMMEL *et al.* 1988). Standard procedures were used to identify the transformants and homozygous or balanced stocks generated (Table 1).

Genetic analysis: All crosses were maintained on standard cornmeal medium. The ts mutants $elav^{ts1}$, $elav^{Flf1}$, and $elav^{Flf2}$ were previously described (HOMYK *et al.* 1980; HOMYK and GRIGLIATTI 1983; CAMPOS *et al.* 1985; HOMYK *et al.* 1985). $elav^{5}$ is an amorphic allele and $elav^{DmORF}$ is the *D. virilis* minigene, capable of providing *elav* function (YAO and WHITE 1991; YAO *et al.* 1993). In order to test for $elav^{null}$ rescue by one copy of an *elav* transgene (*Tf*), females $elav^{5}/elav^{5}$; $elav^{DmORF}/CyO$, were mated to $y\ w^{67c23}$; *Tf*/*Tf* or $y\ w^{67c23}$; *Tf*/*CyO* males carrying the transgene (*Tf*) to be tested, at 18° and, for some of the transformant lines, at 25° (see Table 1). The males eclosing from this cross must carry a transgene that provides $elav^{+}$ function; that is, either they have straight wings and

TABLE 1

Characteristics of the transformant lines

Line	Elav immunoreactivity (kD)	Rescue of *elav*^{null}
FliJ1-2	50	NT
FliJ1-5	50	No*
FliJ1-6	50	No†
FliJ1-7	50	No‡
FliJ1-13	50	No
FliJ2-2	45 and 50	No
FliJ2-3	45 and 50	No
TS1-12	45 and 50	No
TS1-13	45 and 50	No
TS1-271	NT	No*
TS1-272	45 and 50	No†
336-10	47	No
336-11	47	No
336-14	NT	No
336-19	NT	No
336-26	NT	No
336-30	NT	No
339-17	50	100%*
339-Sp17	50	92%
339-Sp9	50	87%

The size of the protein produced from the transgenes was determined by immunoblot analysis of *Drosophila* head protein extracts as detailed in MATERIALS AND METHODS. Rescue experiments with one copy of transgene were performed at 18° as described in MATERIALS AND METHODS. In the case of lines *FliJ1-7, TS1-12, TS1-13, TS1-272,* and *339-17,* crosses were also performed at 25° and yielded the same results. Two copies of *FliJ1-7* were necessary to rescue the *elav*^{5} mutation (see text). NT, not tested. Several minigene insertions were recessive-lethal, presumably because their insertion generated a mutation in a vital gene.

* Homozygous lethal.

† No rescue with two copies.

‡ Rescue with two copies.

carry *elav*^{DvORF}, or they have curly wings and are rescued by the tested transgene. The percent rescue as listed in Table 1 is number of males carrying the transgene/number of males carrying *elav*^{DvORF}.

Rescue with two copies of transgenes was tested for the transformed lines *FliJ1-6, FliJ1-7,* and *TS1-272,* in which the transposons are inserted on the second chromosome. The following crosses were performed at 18° to test for the viability of males *elav*^{5}*/Y; Tf/Tf.* Females *FM7a/FM7a; CyO/Sco* were mated with *y w*^{67c23}*/Y; Tf/Tf* males. F1 males *FM7a/Y;Tf/Sco* were then mated to *elav*^{5}*/elav*^{5}; *elav*^{DvORF}*/CyO* females. *elav*^{5}*/FM7a; Tf/CyO* female progeny were mated to males from the stock *y w*^{67c23}*/Y; Tf/Tf,* and the progeny scored for males *elav*^{5}*/Y; Tf/Tf.* When they survived, such males were mated to females *elav*^{5}*/FM6l.* The progeny of this cross yielded only *Bar* females, confirming that two doses of this transposon are necessary for rescue of *elav*^{5}. Females homozygous for the *elav*^{5} allele and for the *FliJ1-7* transposon were obtained by backcrossing these *Bar* females (*elav*^{5}*/FM6l; FliJ1-7/+*) to *elav*^{5}*/Y; FliJ1-7/FliJ1-7.* The rare surviving females *elav*^{5}*/elav*^{5}; *FliJ1-7/FliJ1-7* were sterile. Therefore, males *elav*^{5}*/Y; FliJ1-7/FliJ1-7* were mated to their sisters *elav*^{5}*/FM6l, FliJ1-7-/FliJ1-7.* After a few generations, a few fertile females *elav*^{5}*/elav*^{5}; *FliJ1-7/FliJ1-7* were found and were used to establish a stock *elav*^{5}*/elav*^{5}*/Y; FliJ1-7/FliJ1-7.*

In order to quantify the rates of rescue by the *FliJ1-7* transposon, the following crosses were established: females *FM7a/FM7a; CyO/Sco* were mated to males *elav*^{5}*/Y;FliJ1-7/FliJ1-7.* Female *elav*^{5}*/FM7a; FliJ1-7/CyO* progeny from this cross were mated to males *y w*^{67c23}*/Y; FliJ1-7/FliJ1-7.* Rescue by one copy of *FliJ1-7* was never obtained. The percentage of rescue by two copies of *FliJ1-7* was calculated as the ratio between the number of males *elav*^{5}*/Y; FliJ1-7/FliJ1-7* and the number of females *elav*^{5}*/FM7a; FliJ1-7/FliJ1-7.*

Analysis of *elav* RNA using reverse transcription PCR: Total RNA from adult heads was isolated by the guanidine-HCl method (COX 1968), followed by chloroform extraction and ethanol precipitation. Six independent annealing reactions of 500 ng *elav*^{FliJ2} RNA to 60 ng of oligonucleotide E6 5′-TGCACTGATTCGTTGTGG-3′ (complementary to nucleotides 8424–8407 of *elav*) were performed as in LO *et al.* (1994). The annealing mixes were suplemented with $MgCl_2$, dNTPs and RNasin as in LO *et al.* (1994), and their volume brought up to 20 μl. For each reaction, 1 unit of avian myeloblastosis reverse transcriptase (Promega) was added to 10 μl of the sample, and the remaining 10 μl were used as a control. The 12 tubes were incubated at 42° for 40 min. The reactions were brought up to 25 μl 20 mM Tris pH 8.4, 25 mM KCl, 1.5 mM $MgCl_2$, 0.05 mM dNTPs, and 0.4 ng of oligonucleotide E7 5′-CACACGCCAGTTAACAAG-3′ (complementary to nucleotides 8169–8187 of *elav*) was added to each tube. The reactions were overlaid with 14 μl liquid wax, incubated 10 min at 95°, and therafter maintained 10 min at 80° in a thermal cycler, during which 0.5 unit of *Taq* polymerase (Promega) was added. Cycling was performed for 60 cycles at 95° for 1 min, 53° for 1 min, 72° for 2 min. The products of the reactions were analyzed on an agarose gel and by Southern blot hybridization with a probe corresponding to *elav* genomic DNA. None of the reactions where reverse transcriptase was omitted gave any detectable product, while, as expected, the reverse transcription PCRs all yielded a product of approximatively 240 bp, which hybridized to the *elav* probe. These PCR products were pooled and purified on Promega Wizard PCR prep DNA purification column. The ends of the products were polished with T4 DNA polymerase before cloning into the pCR-Script SK(+) vector, according to the instruction of the manufacturer (Stratagene). The sequence of the insertions contained in five independent clones was determined by dideoxysequencing using *Taq* DNA polymerase.

RESULTS

Temperature-sensitive *elav* mutations are clustered within the third RNA binding domain of the coding sequence: The open reading frame encoding ELAV is contained in a single exon, except for the first nucleotide of its ATG initiating codon (ROBINOW *et al.* 1988). Since temperature-sensitive mutations often correspond to DNA alterations leading to minor changes in the structure of the protein, we cloned the 2.9-kb restriction fragment *Hin*dIII-*Eco*RI (Figure 1, and MATERIALS AND METHODS) from the stocks *elav*^{ts1}, *elav*^{FliJ1}, and *elav*^{FliJ2} and determined their nucleotide sequence. In each case, we found a different point mutation presumably altering the protein structure (Figure 2). The allele *elav*^{FliJ1} has glycine (G) codon 426 (GGA) changed into a glutamic acid (E) codon (GAA). The alleles *elav*^{ts1} and *elav*^{FliJ2} have tryptophan (W) codon 419 (TGG) changed into stop codons (TAG and TGA, respectively). This translational truncation is expected to result in a mu-

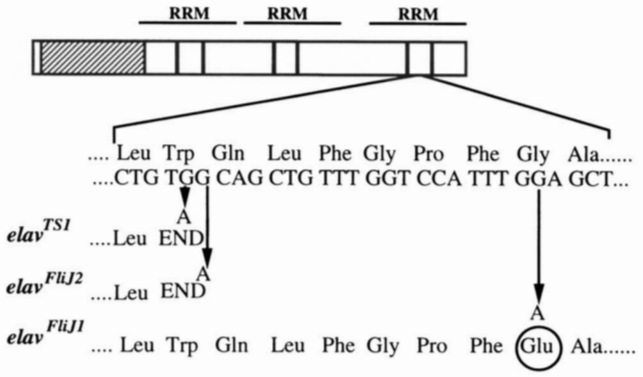

FIGURE 2.—The *elav*^{ts} mutations. The wild-type ELAV protein is shown as a box. The N-terminus of ELAV contains a Glutamine-Alanine (Q-A) rich region (amino acids 24–126). The protein contains three RRMs (RNA recognition motif). Each RRM is characterized by two conserved motifs about 30 amino acids apart, a hexapeptide (RNP2, most N-terminal), and an octapeptide (RNP1, most C-terminal), represented here as solid vertical bars. The nucleotide sequence (nucleotides 8349–8378) and the corresponding amino acid sequence (amino acids 418–427) in the region of the mutations are shown. Tryptophan (W) 419 and Glycine (G) 426 are altered in *elav*^{ts1}/*elav*^{FlixJ2} and in *elav*^{FlixJ1}, respectively.

tant ELAV polypeptide that is 65 amino acids shorter than the wild-type polypeptide.

Immunoblot analysis reveals two forms of proteins in *elav*^{ts1} and *elav*^{FlixJ2} mutants: The *elav* gene encodes a 50-kD polypeptide that can be detected by immunoblot analysis both in embryos and in adult heads. Two different ELAV-antibodies used in this study recognize the first RNA binding domain (M. J. LISBIN, unpublished data), and thus we expected them to recognize the C-terminally truncated ELAV forms anticipated in the *elav*^{ts1} and *elav*^{FlixJ2} protein extracts.

We performed immunoblot analysis of mutant protein extracts. In wild type and *elav*^{FlixJ1}, a normal size protein is synthesized in similar amounts both in embryos (unshown) and in adult heads (Figure 3A). This

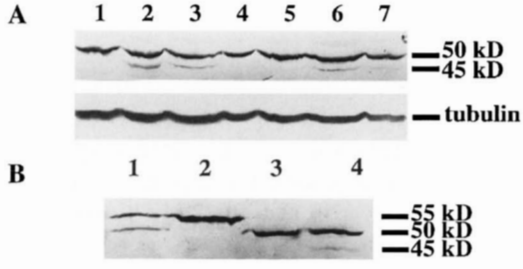

FIGURE 3.—Analysis of *elav* expression in adult head protein extracts of the temperature-sensitive mutants, using the affinity purified rat anti-ELAV antibody. Tubulin was used as an internal loading control. *D. melanogaster* and *D. virilis* ELAV migrate as 50-kD and a 55-kD polypeptides, respectively. The truncated ELAV migrates as a 45-kD polypeptide. A: (1) *elav*$^{+}$, 25°; (2) *elav*^{ts1}, 18°; (3) *elav*^{ts1}, 25°; (4) *elav*^{FlixJ1}, 18°; (5) *elav*^{FlixJ1}, 25°; (6) *elav*^{FlixJ2}, 18°; (7) *elav*^{FlixJ2}, 25°. B: all extracts from flies raised at 25° (1) *elav*$^{+}$/*elav*$^{+}$/*Y*; *elav*^{DvORF}/*CyO*, (2) *elav*^{f5}/*elav*^{f5}/Y; *elav*^{DvORF}/*CyO*, (3) *elav*$^{+}$/*elav*$^{+}$/Y; (4) *elav*^{ts1}/*elav*^{ts1}/Y.

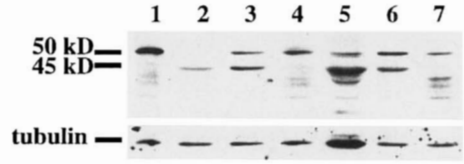

FIGURE 4.—Analysis of *elav* expression in embryonic protein extracts of the temperature sensitive mutants raised at 25°, using the affinity purified rat anti-ELAV antibody. Tubulin was used as an internal loading control. ELAV migrates as a 50-kD protein, and the truncated ELAV migrates as a 45-kD protein. Degradation products are seen below the 45-kD bands in some of the lanes. The age of the embryos is indicated for each sample: (1) *elav*$^{+}$, 12–15 hr; (2) *elav*^{ts1}, 6–9 hr; (3) *elav*^{ts1}, 12–15 hr; (4) *elav*^{ts1}, 18–21 hr; (5) *elav*^{FlixJ2}, 6–9 hr; (6) *elav*^{FlixJ2}, 12–15 hr; (7) *elav*^{FlixJ2}, 18–21 hr.

result is consistent with the single amino acid substitution predicted for the *elav*^{FlixJ1} allele. The patterns of expression are identical at 18° and 25° (Figure 3A for the adult head extracts).

In contrast, *elav*^{ts1} and *elav*^{FlixJ2} do not show the predicted pattern. In adult head protein extracts of *elav*^{ts1} and *elav*^{FlixJ2}, an unexpected full-size 50-kD protein and a less abundant 45-kD protein, consistent with the predicted truncated ELAV protein, are observed (Figure 3A). The 50-kD protein contributes >90% of the total ELAV immunoreactivity, while the 45-kD protein accounts for <10% of the ELAV immunoreactivity. This pattern of expression is identical at 18° and 25° for both *elav*^{ts1} and *elav*^{FlixJ2} genotypes (Figure 3A). Similarly, in embryonic extracts of 0–24-hr-old embryos raised at 18° and 0–18-hr-old embryos raised at 25°, a predominant 50-kD band and a minor 45-kD band are observed (data not shown). The two forms of embryonic ELAV have the same apparent molecular weight as the two forms of ELAV detected in adult heads (data not shown).

In order to monitor the expression of the two ELAV forms during the course of embryogenesis, we analyzed extracts of staged embryos aged 6–9 hr, 12–15 hr, and 15–18 hr. Similar patterns were observed with both *elav*^{ts1} and *elav*^{FlixJ2} embryos (Figure 4). In the 6–9-hr-old embryos, the 45-kD ELAV form is predominant, and the 50-kD ELAV represents only 5–10% of the total immunoreactivity (Figure 4, lanes 2 and 5). In the 12–15-hr-old embryos, the 45-kD and the 50-kD ELAV forms have similar relative abundances (Figure 4, lanes 3 and 6). In the 18–21-hr-old embryos, the ELAV expression pattern is identical to the pattern detected in adult head samples, the 50-kD ELAV now being the predominant form and the 45-kD ELAV being only 5–10% of the total immunoreactivity (Figure 3A and Figure 4, lanes 4 and 7). Thus, early in development, the 45-kD protein is the main form of ELAV, but as development proceeds the 50-kD form becomes predominant.

To verify that ELAV-antiserum specifically recognizes a single 50-kD protein, we used a *D. virilis* minigene that encodes a larger ELAV protein (55 kD) sufficient for normal *elav* function (YAO and WHITE 1991). Immu-

noblot analysis of head protein extracts from flies carrying this *D. virilis elav* minigene and no endogenous locus showed that the immunoreactivity is specific for ELAV, as only a single band at 55 kD is observed (Figure 3B), excluding the possibility that the 50-kD polypeptide is encoded by some other gene.

A formal possibility that could account for the two forms of ELAV found in $elav^{ts1}$ and $elav^{FliJ2}$ is that the *elav* transcript is alternatively spliced and that in wild type, two protein isoforms of similar sizes are encoded by the differentially spliced RNAs. The missense mutations of $elav^{ts1}$ and $elav^{FliJ2}$ would lie in the alternative intron, and the ELAV form translated from the alternatively spliced mRNA would therefore remain unchanged in the mutants. Another formal possibility is that only one form of ELAV exists in wild type, and that the 50-kD proteins in $elav^{ts1}$ and $elav^{FliJ2}$ are generated by the functional suppression of the mutations. The experiments detailed below were undertaken to distinguish between these possibilities.

Minigenes carrying the ts mutations express the same ELAV forms as the genomic $elav^{ts}$ alleles: To further characterize the expression of *elav,* we analyzed *elav* mutant transgenes reintroduced into flies by P-mediated transformation (RUBIN and SPRADLING 1982; SPRADLING and RUBIN 1982). The transgenes contain an 8.5-kb *Pst*I-*Eco*RI *elav* genomic fragment corresponding to 2.3 kb of 5′ nontranscribed sequences and 6.2 kb of transcribed sequences, including the entire open reading frame and 736 bp beyond the translational stop (Figure 1). Although the 8.5-kb fragment represents a truncated version of the *elav* locus, it provides a robust rescue of *elav* null mutations when fused to efficient termination of transcription and polyadenylation signals (YAO and WHITE 1991).

Multiple individual genomic inserts were obtained for each transgene (Table 1). They were analyzed by immunoblots to characterize the ELAV proteins that they express and genetically to test their ability to rescue an amorphic *elav* mutation, $elav^{5}$. The proteins expressed from the transgene were analyzed in the absence of other *elav* alleles when possible. In the case of the transgenes that do not provide functional rescue, expression was monitored both in a *D. virilis* and in a *D. melanogaster* background.

The expression and/or function of all the lines was examined at 18° and, in some cases, at 25° (Table 1). No significant difference between the independent lines corresponding to a given transgene was observed. The $elav^{FliJ1}$ transgenes produce a 50-kD ELAV protein in adult heads, similar to the genomic $elav^{FliJ1}$ allele (Table 1). The same two forms of protein (50 kD and 45 kD) are produced from the $elav^{ts1}$ and the $elav^{FliJ2}$ transgenes (Table 1) and the $elav^{ts1}$ and the $elav^{FliJ2}$ genomic alleles. At 18°, for all three types of transgene constructs, the level of expression of the 50-kD ELAV is significantly lower (two- to fourfold factor) than the level of expression from the endogenous $elav^{ts1}$, $elav^{FliJ1}$, or $elav^{FliJ2}$ alleles (Figure 5, A–C). The 45-kD protein characteristic of $elav^{ts1}$ and $elav^{FliJ2}$ is also present at reduced levels in the transformant flies compared to the mutant flies (Figure 5, B and C). Similar to the temperature-sensitive alleles themselves, the overall profile of expression of the three transgenes is identical at 18° and 25° (data not shown).

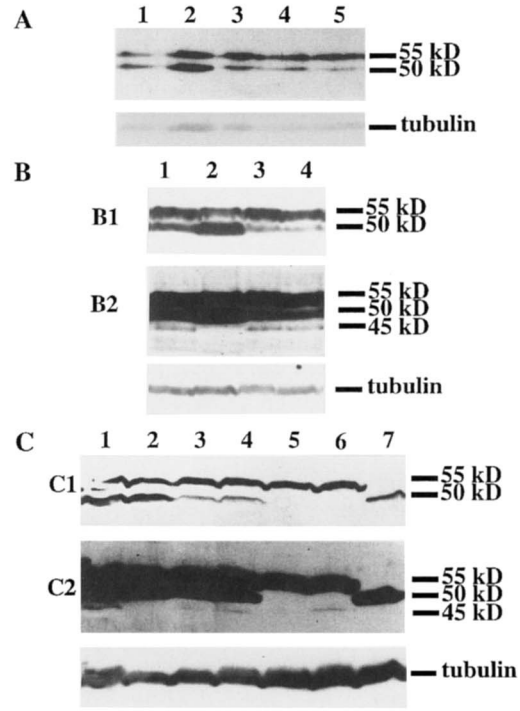

FIGURE 5.—Expression of ELAV from the transgenes: immunoblot analysis of head protein extracts (10–20 heads) from males raised at 25° with the monoclonal mouse anti-ELAV antibody. Tubulin is used as an internal loading control. *D. melanogaster* and *D. virilis* ELAV migrate as 50-kD and a 55-kD polypeptides, respectively. The truncated ELAV migrates as a 45-kD polypeptide. A: (1) $elav^{FliJ1}/Y$; $elav^{DvORF}/+$, (2) *y w*/*Y*; $elav^{DvORF}/CyO$, (3) $elav^{5}/Y$; *FliJ1-5*/$elav^{DvORF}$, (4) $elav^{5}/Y$; *FliJ1-6*/$elav^{DvORF}$, (5) $elav^{5}/Y$; *FliJ1-7*/$elav^{DvORF}$. B: (1) $elav^{ts1}/Y$; $elav^{DvORF}/+$, (2) *y w*/*Y*; $elav^{DvORF}/CyO$, (3) $elav^{5}/Y$; *TS1-12*/$elav^{DvORF}$, (4) $elav^{5}/Y$; *TS1-13*/$elav^{DvORF}$. B1 is a short exposure, and B2 a long exposure of the same immunoblot. C: (1) $elav^{FliJ2}Y$; $elav^{DvORF}/+$, (2) *y w*/*Y*; $elav^{DvORF}/CyO$, (3) $elav^{5}/Y$; *FliJ2-2*/$elav^{DvORF}$, (4) $elav^{5}/Y$; *FliJ2-3*/$elav^{DvORF}$, (5) $elav^{5}/Y$; $elav^{DvORF}/CyO$, (6) $elav^{5}/Y$; *336-10*/$elav^{DvORF}$, (7) $elav^{5}/Y$; *339-17*/+. C1 is a short exposure and C2 a long exposure of the same immunoblot.

The rescue of an amorphic *elav* mutation ($elav^{5}$) with one copy of the transgene (*Tf*) was attempted (see MATERIALS AND METHODS). We examined about 300 progeny for each cross; we were able to detect rescue occurring at a rate of about 1%. At 18° we obtained a few "escaper" males that were phenotypically compatible with a genotype $elav^{5}/Y$; *Tf*/*CyO,* three for *FliJ1-5,* one for *FliJ1-6,* two for *FliJ1-7,* two for *TS1-12,* and one for *TS1-272.* The males obtained from the crosses involving *FliJ1-5, FliJ1-6,* and *FliJ1-7* had irregular facets. Their genotype was not further verifed as all died soon after

emergence. Since the immunoblot analysis indicates that the transgenes produce reduced levels of ELAV, rescue of *elav* function at 18° with two copies of transgenes was attempted for the transgene inserts *FliJ1-6, FliJ1-7,* and *TS1-272.* Males of the genotype $elav^{e5}$/Y; *Tf/ Tf* (*Tf* stands for any of the transgene insert) were obtained only in the case of *FliJ1-7.* Rare females $elav^{e5}$/$elav^{e5}$; *FliJ1-7/FliJ1-7* were obtained in subsequent generations (see MATERIALS AND METHODS) and were sterile. We propagated males $elav^{e5}$/Y; *FliJ1-7/FliJ1-7* and females $elav^{e5}$/*FM6l; FliJ1-7/FliJ1-7* as a stock. After a few generations, probably due to modification of the genetic background, we found a few fertile females $elav^{e5}$/$elav^{e5}$; *FliJ1-7/FliJ1-7,* and were able to maintain a stock $elav^{e5}$/$elav^{e5}$/Y; *FliJ1-7/FliJ1-7* at 18°. The level of expression of ELAV in heads of $elav^{e5}$/$elav^{e5}$/Y; *FliJ1-7/FliJ1-7* flies is lower (about twofold) than in wild-type flies (not shown). At 18°, and to a larger extent at 25°, the flies of this stock have an altered deep pseudopupil and disorganized facets. At 18°, although two copies of transgenes proved to rescue $elav^{e5}$ at 70%, we did not recover flies carrying one copy of the transgene as the sole source of *elav* function (less than 1% rescue). At 25°, the rate of rescue was lower than at 18° (12%), and again no rescue was obtained with a single copy of the transposon. The *FliJ1-7* transgene constitutes the most severe existing viable *elav* mutation.

The mutant transgene $elav^{336}$ that introduces a stop codon in ORF1 produces only one truncated protein: One possibility to explain why $elav^{ts1}$ and $elav^{Flij2}$ produce two ELAV forms is that the wild-type *elav* allele encodes two immunologically related proteins of identical sizes: ELAV, corresponding to cDNA-1 (CAMPOS *et al.* 1987; ROBINOW *et al.* 1988), and ELAV* encoded by a hypothetical differentially spliced mRNA, whose intron contains the stop codons. Since the mutant transgenes $elav^{ts1}$ and $elav^{Flij2}$ are capable of producing the 50-kD ELAV, the presumptive alternative exon must arise from genomic sequences within the 8.5-kb fragment (Figures 1 and 6). The 0.9 kb downstream of the nonsense mutations within the 8.5-kb fragment was searched for open reading frames that could constitute the alternative exon. Three ORFs were identified: 74 residues (nucleotides 8449–8670), 131 residues (nucleotides 8462–8855), and 67 residues (nucleotides 8960–9160). The other ORFs in this region are at most 42 amino acids long, much shorter than the size requirement for the potential alternative exon.

In order to test for alternative splicing, we constructed a plasmid, pe336, which introduces a stop codon in ORF1 (encoding ELAV) without affecting any of the putative ELAV*'s ORFs. In this transgene, a 10-mer oligonucleotide is inserted between codon 420 and 421 of ORF1 (Figure 6A). The modified ORF1 is predicted to encode a protein 26 residues shorter than ELAV (457 *vs.* 483 amino acids), and whose 37 most C-terminal residues differ from those encoded by ORF1 (Figure 6A). Because of its proximity to the $elav^{ts1}$ and $elav^{Flij2}$ mutations and its location far upstream of the three potential alternative ORFs, it should leave ELAV*'s ORF unaffected (Figure 6B). We also constructed a plasmid pe339, which alters two of the three ORFs (74 and 131 amino acids long; see above and MATERIALS AND METHODS) that are potentially translated if *elav* mRNA is alternatively spliced.

The two types of corresponding transgenes, $elav^{336}$ and $elav^{339}$, were examined for *elav* expression and function. Immunoblot analysis of head proteins extracted from flies raised at 25° reveals that $elav^{336}$ generates low levels of a truncated ELAV protein (5–10% of the normal level), whose size is consistent with that of the predicted truncated protein (Table 1 and Figure 5C). No 50-kD protein is observed. This transgene fails to rescue *elav* null mutations. In contrast, $elav^{339}$ encodes a normal size ELAV protein (Table 1 and Figure 5C) and rescues *elav* null mutations fully.

The *elav* RNA produced in $elav^{Flij2}$ is complementary to the genomic sequence: One of the possibilities to explain the fact that $elav^{Flij2}$ and $elav^{ts1}$ produce full-size ELAV proteins is that the nonsense mutations are edited to sense codons. To test this possibility, we examined the sequence of five independent clones obtained by reverse transcription PCR of $elav^{Flij2}$ RNA. All five sequences match the sequence of the $elav^{Flij2}$ genomic DNA.

DISCUSSION

The *elav* ts mutations: The molecular lesions in three viable, temperature-sensitive *elav* alleles were analyzed. In the $elav^{Flij1}$ allele, G_{426} is changed to E. In $elav^{ts1}$ and $elav^{Flij2}$ the W_{419} codon TGG is changed into two different stop codons, TAG and TGA, respectively. All three temperature-sensitive *elav* mutations map within the third RNA binding domain and cluster in a 22-nucleotide-long stretch between the RNP2 and RNP1 motifs. The functional importance of the mutated amino acids is emphasized by the fact that they map to an 11-amino-acid-long stretch (LWQLFGPFGAV, where the bold amino acids correspond to the mutations), which is conserved between all known members of the *elav*-related proteins in Drosophila, human, and Xenopus. In fact, the 11 amino acids are identical between the two Drosophila proteins (ELAV and RBP9) and three of the four human ELAV-related proteins (HuD, HuC, and PL21), and they differ only by one amino acid (LWQMFGPFGAV *vs.* LWQLFGPFGAV) in Hel-N1 and the Xenopus homologue (SZABO *et al.* 1991; KIM and BAKER 1993; KING *et al.* 1994; SAKAI *et al.* 1994; GOOD 1995; PERRON *et al.* 1995; G. MANLEY and H. FURNEAUX, personal communication).

The three-dimensional structure of U1snRNP-A protein, also an RRM protein, has been determined both by crystallography and NMR (NAGAI *et al.* 1990; HOFFMAN *et al.* 1991; OUBRIDGE *et al.* 1994). According to

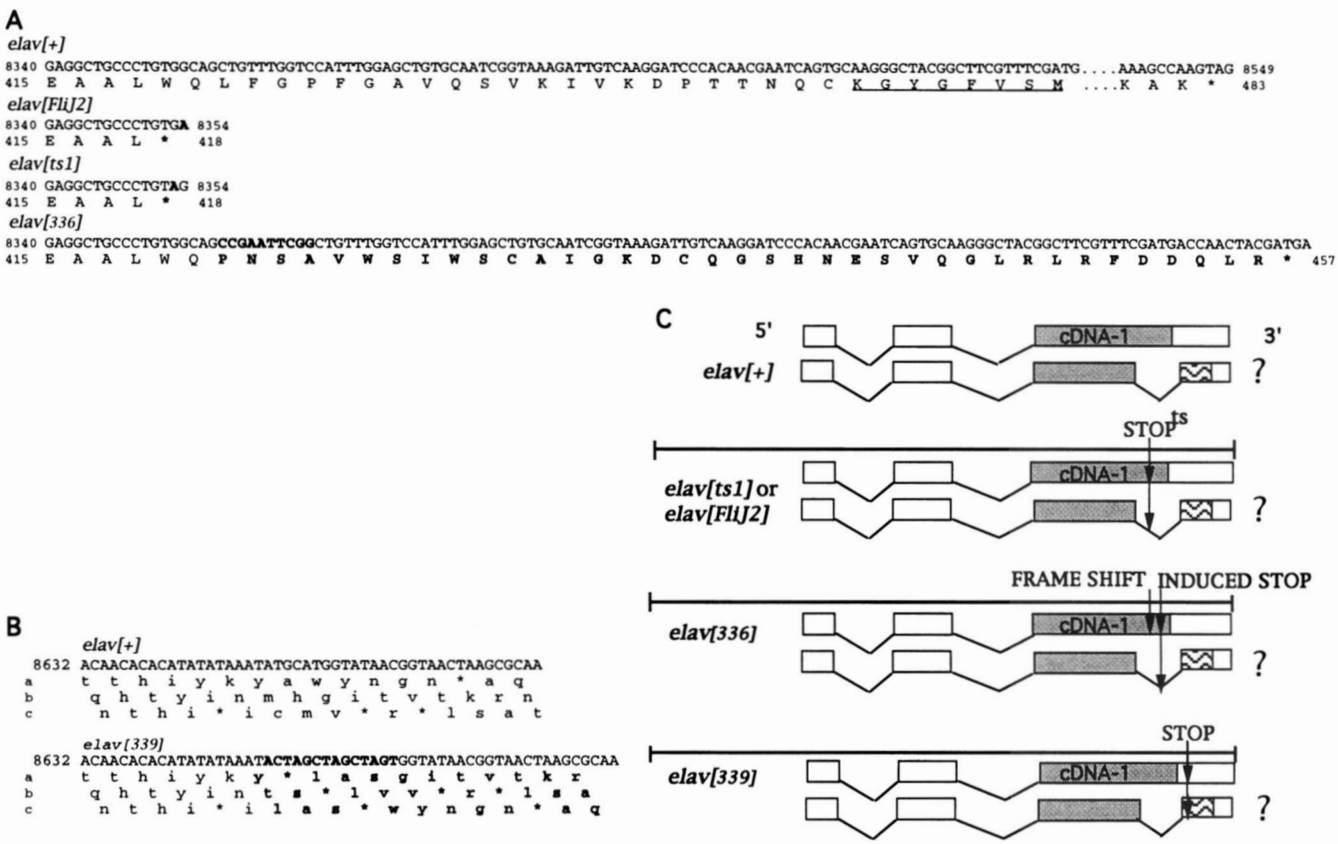

FIGURE 6.—Structure and predicted effect on *elav* expression of the mutant minigenes. A: Nucleic acid sequence of 3′ end of *elav* ORF1 (starting at nucleotide 8340) and conceptual translation of the sequences (starting at amino acid 415) in wild-type *elav,* and in the mutant transgenes $elav^{ts1}$, $elav^{FliJ2}$, and $elav^{336}$. The RNP1 of the third RRM is underlined. B: Nucleic acid sequence of wild-type *elav* and mutant $elav^{339}$ transgenes. The conceptual translation in three frames is indicated below the sequence and is specifically indicated in lower case to emphasize the hypothetical use of these ORFs. C: Predicted effects of *elav* mutations on the RNA corresponding to ELAV and on the hypothetical mRNA encoding ELAV* (see text). The structure of *elav* mRNA as deduced from the structure of cDNA-1 is represented at the top of the figure, where ORF1 is shown as a gray box. The hypothetical alternatively spliced mRNA and ORF specific for the hypothetical ELAV* protein are diagrammed below. The solid lines indicate the location of the genomic DNA fragments contained in the minigenes that were tested in our experiments, relative to the structure of the ORFs. Four of the transgenes ($elav^{+}$, $elav^{ts1}$, $elav^{FliJ2}$, $elav^{336}$, and $elav^{339}$, respectively), the mutations that they carry (arrows), the effect of the mutations on the ORFs of both *elav* mRNA, and the presumptive alternatively spliced *elav* mRNA are also shown.

this structure, the alteration of the ts alleles is inferred to lie within the region encoding the first alpha helix of the RRM, a region that does not directly contact RNA. G_{426} is a preferred consensus amino acid in loop 2 leading into the $\beta 2$, as suggested by NMR studies of the *Sxl* protein RRM2 and the hnRNPC RRM (BANDZIULIS *et al.* 1989; GÖRLACH *et al.* 1992; LEE *et al.* 1994). On the other hand, W_{419} in that position of a helix turn is very rare, being specific to the *elav*-subfamily. W_{419} is in a position that corresponds to position R_{25} in RRM of U2B″ which is involved in protein-protein interaction (SCHERLY *et al.* 1990; BENTLEY and KEENE 1991). A different amino acid in this position could compromise the function of the protein.

Biological activity of the mutant ELAV proteins: Both the genomic *elav* mutations and the mutant *elav* minigenes show profiles of ELAV protein(s) that are not affected by temperature (Figure 3, and data not shown), indicating that neither the mutant forms of ELAV protein are thermolabile, nor is the process leading to the generation of two forms of ELAV, in the case of $elav^{ts1}$ and $elav^{FliJ2}$, temperature-sensitive. Rather, this finding suggests that the mutant proteins themselves are functionally impaired. For instance, ELAV affinity for its targets may be affected by the mutations at nonpermissive temperature.

As opposed to nonmutated *elav* transgenes, none of the mutant transgenes were able to rescue $elav^{null}$ mutations when present as a single copy. However, we were able to obtain rescue of $elav^{null}$ mutations with two copies of *FliJ1-7*. These flies show very clear temperature sensitivity: 70% rescue at 18° and 12% rescue at 25° (Table 1) and abnormal eye morphology at 18° and to a greater extent at 25°. Consistent with their extreme phenotype, these flies have a lower level of ELAV expression (about twofold) compared to flies carrying an $elav^{FliJ1}$ mutation. Our attempts to rescue $elav^{null}$ with two copies of *FliJ1-6* and *TS1-272* failed,

but the number of flies that we examined may not have been sufficient.

We attribute the difficulty of the *elav*^{ts} minigenes to rescue *elav*^{null} to the lower than normal level of ELAV expression from the minigenes and the impaired functional activity of the protein products themselves. This insufficient expression is consistent with finding fewer females than males carrying two copies of *FlijI-7* transgenes as their only source of *elav* function, reflecting dosage compensation in males. Dependence of the *elav* phenotype on the copy number of hypomorphic alleles has previously been reported (CAMPOS *et al.* 1985; YAO *et al.* 1993).

Nonsense mutations at position 419 of ELAV appear to be suppressed: Surprisingly, analysis of the nonsense mutants *elav*^{ts1} and *elav*^{FlijI2} shows that both the predicted 45-kD protein and an unexpected 50-kD normal size ELAV protein are produced.

We considered the idea of alternative splicing as a mechanism producing these two forms of ELAV because (1) alternative splicing occurs within the 9 kb downstream of ORF1 that belong to the *elav* locus (CAMPOS *et al.* 1987); (2) alternative splicing is widely used as a mechanism to generate multiple protein isoforms (for reviews, see BREITBART *et al.* 1987; RIO 1993); and (3) alternatively spliced protein forms have been reported for other members of the ELAV protein family (SZABO *et al.* 1991; KIM and BAKER 1993; GAO *et al.* 1994; LIU *et al.* 1995).

We also considered the possibility that the full-length ELAV in the *elav*^{ts1} and *elav*^{FlijI2} mutants is generated by suppression of the stop codon because: (1) both *elav*^{ts1} and *elav*^{FlijI2} alter W_{419}, suggesting that that these mutations might have been selected because they can be suppressed by an inherent mechanism able to identify the sequence surrounding the mutations (for reviews, see MURGOLA 1985; VALLE and MORCH 1988; CATTANEO 1991); and (2) the specificity of the phenotype of *elav*^{FlijI2} *vs.* *elav*^{ts1} (CAMPOS *et al.* 1985; HOMYK *et al.*1985) and the similarity of the ELAV pattern of expression in the two mutants suggest that they encode distinct impaired proteins (see above). Thus, different amino acids might be substituted for the conserved W_{419} (see above) in the mutant *elav*^{ts1} and *elav*^{FlijI2} proteins. This could result from suppression of the stop codons, either by editing or readthrough of the nonsense codon to yield insertion of different amino acids. Although both ribosomal hopping and frameshifting are formal possibilities for nonsense suppression, we think that they are unlikely to be occurring, because they would give rise to forms of ELAV whose third RRM is significantly altered, most likely totally disrupting the protein function, and, in the case of frameshifting, whose size would be incompatible with our data.

To distinguish between the possibilities of alternative splicing *vs.* suppression, we first analyzed *TS1* and *FlijI2* mutant transgenes that span the 8.5-kb genomic fragment from *elav* sufficient to rescue an *elav* null mutation. The mutant transgenes generated the same two forms of ELAV as the genomic mutant genes, indicating that the 8.5-kb genomic fragment contains all the coding information for the two forms of ELAV protein to be produced. Second, we analyzed a mutant transgene, *elav*^{336}, with a frameshift mutation in ORF-1 mapping between the mutations in *elav*^{ts1}/*elav*^{FlijI2} (codon 419) and *elav*^{FlijI} (codon 426). This frame-shift mutation introduces a stop codon at position 457 (wild-type stop, 483) and thus should result in a truncated protein. The putative ELAV* should remain unaffected, assuming that the presumed alternatively spliced intron includes the frame shift. Transgene *elav*^{336} expresses a single truncated protein of about 47 kD whose level of expression is very low, probably as a consequence of the instability of the mutated RNA or protein. Since only a truncated ELAV product is detected, we conclude that unless the frameshift mutation alters sequences essential for normal splicing of the presumed alternative intron, a single form of ELAV is encoded by the *elav* gene.

To determine whether the frame shift mutation of *elav*^{336} might alter splicing signals, we searched the sequence in the vicinity of this mutation for the presence of a Drosophila consensus 3′ splice sites (MOUNT *et al.* 1992). A sequence matching 10/15 nucleotides was found, ^{8344}CTGCCCTGTGGCAG|CT^{8359}, which is modified into CTGCCCTGTGGCAG|CC in the *elav*^{336} transgene (Figure 6A). However, this is an unlikely splicing site for the following reasons:

1. As opposed to the 3′ splicing sites found in the database, which are consistently G-poor, it is buried in a G-rich region (MOUNT 1993). Moreover, if this site were used, none of the three possible ORFs that it would introduce in the ELAV* sequence would be long enough to generate a protein of size similar to the size of ELAV*.

2. The *D. virilis elav* gene encodes a protein identical to *D. melanogaster* ELAV in the region corresponding to the RNA binding domains (YAO and WHITE 1991). We searched *D. virilis* sequences for the potential ORFs identified in *D. melanogaster elav,* but did not find similarities, even under low stringency comparisons.

3. We searched for experimental evidence for splicing. Several *elav* cDNA clones from an embryonic library and a head cDNA library were characterized and found to match the structure of the genomic DNA (M.-L. SAMSON, unpublished results). We identified a minimum of seven different head and seven different embryonic cDNA clones in which the region corresponding to nucleotides 8090–8781 is intact. Consistently, RNAse protection assays of adult head and pupal RNA between nucleotides 8324 and 8472 reveals only an unspliced protected RNA (data not shown).

Thus, taken together, our data suggest that *elav* encodes a single protein and that, in the mutants *elav*^{ts1} and *elav*^{FlijI2}, translation of the mutated RNA generates

high levels of a truncated and unstable 45-kD ELAV and, at a lower level, a stable 50-kD ELAV arising from the suppression of the stop codons. Presumably, the original W_{419} is substituted by a different amino acid in each mutant, since the mutant phenotypes are distinct.

We found that five independent head mRNA *elav*^{Flif2} mRNAs were all complementary to the genomic *elav*^{Flif2} sequence. Thus, editing of the stop codon seems to be unlikely to be the mechanism responsible for the suppression. However, we cannot completely rule out this possibility, because it might be that the edited mRNA would be a minor species in the bulk of *elav* mRNAs. Suppression by translational readthrough or editing are the most likely mechanisms to account for our observations.

The suppression mechanism is able to act similarly on the two different stop codons (TAG and TGA, respectively, for *elav*^{ts1} and *elav*^{Flif2}). Such a case of suppressed nonsense mutations has been reported for *nina-E*, which encodes Drosophila's major rhodopsin (WASHBURN and O'TOUSA 1992). All three nonsense codons at position 309 of the *nina-E* ORF are suppressed, restoring partial or total function.

A variety of previous examples of suppression have been reported that proved to function with low efficiencies (see, *e.g.*, XUE and COOLEY 1993). The kinetics of ELAV embryonic expression also support low levels of suppression. When ELAV protein is synthesized at a high level, as in early stages of neuronal differentiation, the 45-kD form predominates over the 50-kD form. A similar pattern would probably be observed during the second wave of neuronal differentiation that occurs during metamorphosis. As development proceeds, the stable 50-kD ELAV form progressively accumulates, and its levels reach and eventually surpass that of the unstable 45-kD ELAV, generating the protein patterns observed in late embryogenesis and in adults.

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