Evidence for 3′ Untranslated Region-Dependent Autoregulation of the Drosophila Gene Encoding the Neuronal Nuclear RNA-Binding Protein ELAV

Marie-Laure Samson

Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, Nebraska 68198-4525

Manuscript received January 21, 1998
Accepted for publication June 24, 1998

ABSTRACT

The Drosophila locus *embryonic lethal abnormal visual system* (*elav*) encodes a nuclear RNA-binding protein essential for normal neuronal differentiation and maintenance of neurons. ELAV is thought to play its role by binding to RNAs produced by other genes necessary for neuronal differentiation and consequently to affect their metabolism by an as yet unknown mechanism. ELAV structural homologues have been identified in a wide range of organisms, including humans, indicating an important conserved role for the protein. Analysis of *elav* germline transformants presented here shows that one copy of *elav* minigenes lacking a complete 3′ untranslated region (3′ UTR) rescues null mutations at *elav*, but that two copies are lethal. Additional *in vivo* experiments demonstrate that *elav* expression is regulated through the 3′ UTR of the gene and indicate that this level of regulation is dependent upon ELAV itself. Because ELAV is an RNA-binding protein, the simplest model to account for these findings is that ELAV binds to the 3′ UTR of its own RNA to autoregulate its expression. I discuss the implications of these results for normal *elav* function.

THE Drosophila gene *embryonic lethal abnormal visual system* (*elav*) is the first identified member of a conserved multigene family with homologues in many species, including humans (Robinow *et al.* 1988; Szabo *et al.* 1991; Kim and Baker 1993; King 1994; King *et al.* 1994; Manley 1994; Sakai *et al.* 1994; Good 1995; Perron *et al.* 1995; Abe *et al.* 1996a,b; Ma *et al.* 1996; Steller *et al.* 1996; U. Atasoy, J. Watson and J. D. Keene, personal communication). The two Drosophila family members so far identified, *elav* and *rbp9*, are strictly expressed in neuronal nuclei (Campos *et al.* 1987; Kim and Baker 1993). Null and severe mutations at the *elav* locus cause altered neurite organization and are embryonic lethal (Campos *et al.* 1985; Jimenez and Campos-Ortega 1987). The lack of *elav* function in an embryo does not prevent the generation of neurons, but rather leads to their abnormal differentiation. Flies carrying temperature-sensitive *elav* mutations develop normally at permissive temperature, but initiate neuronal degeneration when shifted to nonpermissive temperature, showing that *elav* function is also necessary for neuronal maintenance (Campos *et al.* 1985; Homyk *et al.* 1985). No mutations of the *rbp9* gene have been reported.

The products of *elav* and of the other members of this gene family contain three RNA recognition motifs, referred to as RRMs, that are present in a large number of proteins involved in diverse processes of RNA metabolism and translation (for reviews, see Kenan *et al.* 1991; Mattaj 1993; Burd and Dreyfuss 1994). Because ELAV is a nuclear RNA-binding protein, it seems likely that it promotes normal neuronal differentiation by modulating the metabolism of RNA produced from genes whose expression is required in neurons. The mechanism of this proposed modulation remains unknown.

Several genes homologous to *elav* have been described in vertebrates, including humans. On the basis of sequence similarity, the vertebrate ELAV sequences can be split into four groups, referred to here as ELAV-A, -B, -C, and -D, following the nomenclature introduced by Good (1995). Human, mouse, and Xenopus have one each of these four ELAV subtypes. Additionally rat ELAV-B and -D as well as zebrafish and chicken ELAV-A, -C, and -D have been identified (Szabo *et al.* 1991; King 1994; King *et al.* 1994; Manley 1994; Sakai *et al.* 1994; Good 1995; Perron *et al.* 1995; Abe *et al.* 1996a,b; Ma *et al.* 1996; Steller *et al.* 1996; Hirotaka and Darnell 1997; Wakamatsu and Weston 1997; U. Atasoy, J. Watson and J. D. Keene, personal communication). Amino acid sequence identities range from 85–99% among the members of each group. Interestingly, each structural group has distinct tissue-specific and subcellular localizations. The human, Xenopus, and mouse ELAV-A members are ubiquitously transcribed (Good 1995; Ma *et al.* 1996; U. Atasoy, J. Watson and J. D. Keene, personal communication). In contrast, the

Address for correspondence: Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, 984525 Nebraska Medical Center, Omaha, NE 68198-4525.
E-mail: msamson@molbio.unmc.edu

ELAV-B, -C, and -D types are neuronal (Szabo *et al.* 1991; Levine *et al.* 1993; King *et al.* 1994; Sakai *et al.* 1994; Good 1995; Perron *et al.* 1995, 1997; Abe *et al.* 1996a,b; Steller *et al.* 1996). Low levels of Xenopus, mouse, and rat ELAV-B and mouse ELAV-D transcripts are also detected in testis/ovaries (King *et al.* 1994; Good 1995; Abe *et al.* 1996a,b). Human, mouse, and Xenopus ELAV-B are found both in the cytoplasm and to various extents in the nucleus, possibly reflecting shuttling between those compartments (Abe *et al.* 1996a,b; Gao and Keene 1996; Perron *et al.* 1997). Human ELAV-C and -D are mostly nuclear (Szabo *et al.* 1991; Sakai *et al.* 1994). Many of these proteins can bind U/AU-rich sequences *in vitro* (Levine *et al.* 1993; Gao *et al.* 1994; Liu *et al.* 1995; Abe *et al.* 1996a,b; Chagnovich and Cohn 1996; Chung *et al.* 1996, 1997; Ma *et al.* 1996, 1997; Jain *et al.* 1997; Myer *et al.* 1997), but this binding depends upon different RRMs in the proteins, respectively the third RRM domain of human ELAV-B (Hel-N1), the first RRM of mouse ELAV-C (mHuC), and the first two RRMs of human ELAV-D (HuD). The third RRM of ELAV-A, -C, and -D (human HuR, mouse HuC, and human HuD) shows different specificity, and binds poly(A) *in vitro* (Abe *et al.* 1996a; Ma *et al.* 1997).

The sequences of the two Drosophila proteins, ELAV (Robinow *et al.* 1988) and RBP9 (Kim and Baker 1993), are similarly related to each of the four classes of human ELAV sequences (52–54% amino acid identity in the case of ELAV and 56–61% amino acid identity for RBP9). On the basis of their strict neuronal nuclear localization (Robinow and White 1991; Kim and Baker 1993; Yannoni and White 1997), it can be inferred that they are more related to the ELAV-C or -D types, although ELAV is singled out by its systematic and exclusive neuronal expression. Together, the different subcellular localizations and the diversity of *in vitro* binding specificities underline differences in the function of ELAV-A, -B, -C, and -D proteins, but the strong structural conservation indicates the possibility that all mediate the same general function in RNA metabolism, possibly triggering differentiation of specific cell types, both for the exclusively neuronal and the ubiquitous members of the family.

Analysis of the ELAV family suggests two possible functions for ELAV-related genes. First, these proteins have been proposed to be implicated in the modulation of mRNA turnover/translation, processes that are intimately interdependent (reviewed in Jacobson and Peltz 1996). This model was proposed because ELAV family members from all four ELAV subtypes bind AU-rich sequences *in vitro.* These AU-rich binding sites resemble and in some instances correspond to the loosely defined ARE sequences typically present in the 3′ untranslated region (3′ UTR) of short-lived mRNA (Chen and Shyu 1995). Consistent with a role in mRNA turnover/translation, the human Hel-N1 protein is associated with polysomes in neuroblastoma and medulloblastoma cells (Chagnovich *et al.* 1996; Gao and Keene 1996), while overexpression of Hel-N1 in a preadipocyte cell line leads to upregulation of the rate of GLUT1 mRNA stability and translational inititation (Jain *et al.* 1997). Second, it was proposed that ELAV functions as an alternative splicing factor. The basis for this model is the influence of the *elav* genotype (*elav* ectopic expression versus *elav* gene deletions) on the ratio between the neuronal and nonneuronal forms of neuroglian protein that derive from alternatively spliced mRNAs (Koushika *et al.* 1996). Although performed *in vivo*, these experiments do not exclude an indirect effect of ELAV, for example on the stability of a neuroglian-specific splicing factor.

These disparate views are not necessarily contradictory, but require better documentation. It is worth noting the parallel between *elav* and another Drosophila gene, *Sex-lethal* (*Sxl*). Both genes are required for initiation and maintenance of developmental pathways: neuronal differentiation in the case of *elav*, and sex determination in the case of *Sxl.* Both encode related proteins that contain RRMs and show high affinity for poly U *in vitro. Sxl* autoregulates, and the data presented here show that *elav* also autoregulates. The molecular mechanism of action of *Sxl* protein (SXL) is better understood than that of *elav.* SXL has multiple activities in the regulation of pre-mRNA splicing and in translation, presumably through interactions with different transcript regions (Bashaw and Baker 1997; Granadino *et al.* 1997; Kelley *et al.* 1997).

In this article, I analyze the regulation of *elav* expression using functional *elav* transgenes carrying an altered 3′ UTR. Remarkably, these transgenes are associated with dosage-dependent lethality and behave as neomorphic *elav* mutations. I show that the *elav* 3′ UTR confers gene dosage independence on *elav* expression and that *elav* expression is autoregulated. Given the properties of ELAV, autoregulation is most likely to occur through its direct binding to the 3′ UTR of *elav* mRNA.

MATERIALS AND METHODS

Transformation vectors: A partial sequence of 9285 nucleotides has been published for the *elav* gene (Robinow *et al.* 1988). The gene extends about 8 kb 3′ to this sequenced fragment (Campos *et al.* 1987). The positions of nucleotides in the gene are referred to according to their positions in the published sequence.

The transformation vector pe350 that carries a 15.5-kb genomic fragment (see Figure 1) was built as follows: The *Bam*HI-*Eco*RI (8402–9280) fragment of pe332 (Samson *et al.* 1995), a pUC18 derivative that contains a *Sma*I-*Xba*I insert composed of the *elav Sma*I-*Eco*RI (6639–9280) fragment fused to transcription termination and polyadenylation signals of the β1 tubulin gene, was replaced by the 7.5-kb *Bam*HI restriction fragment ending at position 8402, yielding pe348. A 10.1-kb *Sma*I-*Xba*I (composed of the 9.3-kb *elav Sma*I-*Bam*HI beginning at nucleotide 6639 fused to transcription termination and

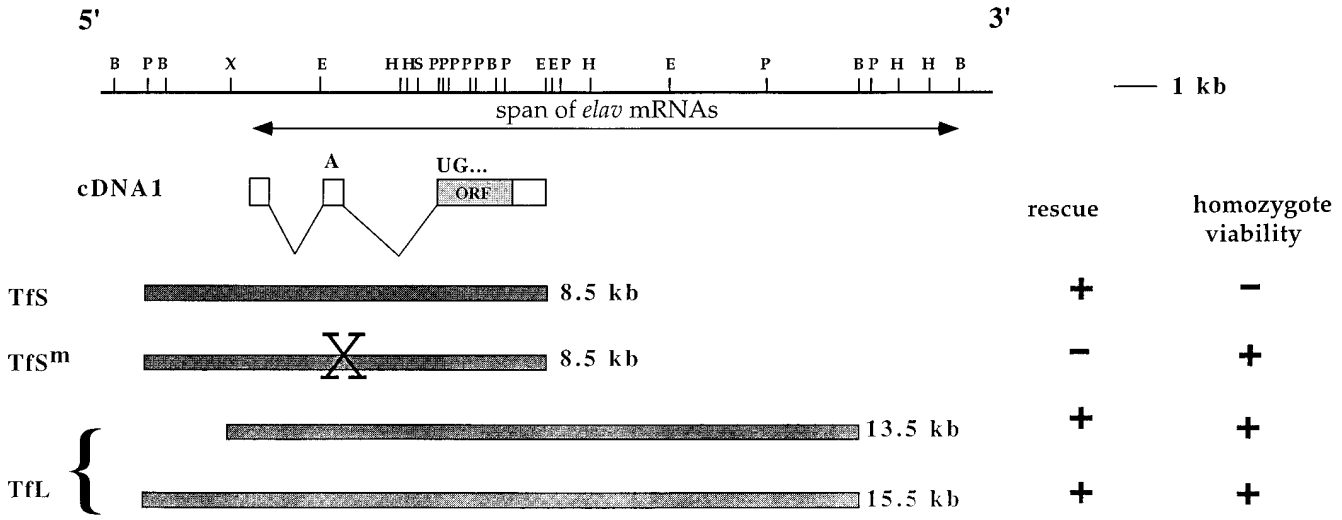

Figure 1.—Structure of the *elav* locus. A restriction map is shown as a continuous line. The structure and splicing pattern of cDNA 1 (a partial embryonic cDNA, one of the two similar *elav* cDNAs characterized) and its translational AUG initiation codon are shown. Transcripts extend much farther downstream, spanning over 16 kb, as shown, but their detailed structures have not been determined (Yao *et al.* 1993). The structure and properties of the four classes of tested transformants (see Table 1 for details), respectively, correspond to the 8.5-kb *Pst*I-*Eco*RI genomic fragment, mutant versions of this fragment, the 13.5-kb *Xba*I-*Bam*HI genomic fragment, and the 15.5-kb *Pst*I-*Bam*HI genomic fragment. TfL, long-transformants (function and 3′ UTR); TfS, short-transformant (function and truncated 3′ UTR); TfS^m, mutant-short-transformant (little or no function and truncated 3′ UTR). B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sma*I; X, *Xba*I.

polyadenylation signals of the β1 tubulin gene) was purified from pe348. A three-way ligation between the 5.9-kb *Pst*I-*Sma*I (757–6639) *elav* fragment, the 10.1-kb *Sma*I-*Xba*I fragment from pe348 and the 7.8-kb pCaSpeR cut with *Pst*I and *Xba*I yielded pe350. A similar strategy was used to build pe353, after replacing the wild-type *Bst*XI-*Bam*HI fragment (8269–8402) from pe332 by the homologous fragment from the *elav*^{FliJ2} allele.

Germline transformations: Embryos from the stock *y w*^{67c23} were coinjected with 200 ng/μl transformation vectors and 50 ng/μl pπ25.7 (Karess and Rubin 1984; Pirrotta 1988; Thummel *et al.* 1988). Standard procedures were used to identify the transformants and generate stocks.

Transformed lines: The 8.5-kb genomic fragment included between nucleotides 757 to 9285 (fused to termination of transcription and polyadenylation signals) is sufficient for transformation rescue (*DmORF* series; Yao and White 1991). Transformed Drosophila stocks of the 336, 339, FliJ1, FliJ2, and TS1 series carry the 8.5-kb fragment, modified by the insertion of an oligonucleotide or by point mutations (Samson *et al.* 1995). The *DvORF* lines also contain the *Drosophila melanogaster* 8.5-kb genomic fragment, where most of the *D. melanogaster* coding sequence is replaced by its *D. virilis* counterpart, generating a larger ELAV protein (Yao and White 1991). Transformed lines 17 and 20 carry the 8.5-kb genomic DNA, where the most 5′ intron is deleted, and transformed lines 7 and 22 carry the 8.5-kb genomic DNA depleted of its two introns (a gift from K.-M. Yao and K. White).

The lines P15.2 and P62.2 (a gift from K. White), carry a 13.5-kb-long transforming genomic fragment whose 5′ end corresponds to nucleotide 2528 of the *elav* gene, and are similar to those described by Campos *et al.* (1987). The lines 350 and 353 carry a 15.5-kb-long transforming genomic fragment whose 5′ end corresponds to nucleotide 757 of *elav*. Lines of the 350 series carry a wild-type sequence, while line 353 carries the mutation *elav*^{FliJ2}, as does the FliJ2 series (Samson *et al.* 1995).

Genetic analysis: All crosses were maintained on standard cornmeal medium at 25°, or in specified instances at 18°. Fertility and viability of specific phenotypes were measured as detailed in the table legends. The hypothesis that the flies carrying two transgenes have normal viability was tested using a chi-square test with 1 d.f., comparing expected and observed values of the number of progeny carrying two transgenes and the number of progeny carrying no or one transgene.

Protein preparation and immunoblot analysis: Drosophila head protein extracts were prepared and resolved as previously described (Samson *et al.* 1995). All flies were raised at 25°. Immunodetection was performed using chemiluminescence (Amersham ECL, Arlington Heights, IL). A mouse anti-ELAV monoclonal antibody (ascites fluid obtained after injection of hybridoma cell line 9F8A9 provided by G. Rubin) was used for primary incubation (1:5000 dilution). The secondary antibody was peroxidase-conjugated anti-mouse IgG (Boehringer 1317377, 1:5000 dilution; Boehringer Mannheim, Mannheim, Germany). An internal loading control was performed by detecting β-tubulin immunoreactivity via a 1:10,000 mouse primary antibody (Sigma, St. Louis, MO) and 1:10,000 peroxidase-conjugated anti-mouse IgG secondary antibody. Quantification was done with a computing laser densitometer (ImageQuant software; Molecular Dynamics, Sunnyvale, CA).

RESULTS

Functional *elav* transgenes missing the 3′ UTR are homozygous lethal, while transgenes containing the 3′ UTR are homozygous viable: To analyze the structure of *elav* and its function, *elav* constructs were reintroduced into the Drosophila germline. An 8.5-kb genomic fragment rescues *elav* mutations, although it does not include the entire *elav* 3′ UTR (Figure 1). Nine indepen-

TABLE 1

Properties of the stocks carrying *elav* transgenes

X chromosome	Names	Insert sizes (kb)	Status of the *elav* insert	References	Efficiency of rescue with one copy of transgene (%)	Homozygous viable stock	Survival of flies in heterozygous stocks: Surviving females with two doses of transgene	Surviving males with two doses of transgene	Females and males with one dose of transgene
	TfL								
y elav^{+} *w*	*350-83-1*	15.5	WT	1	92	Yes			
y elav^{+} *w*	*350-83-2*	15.5	WT	1	109	Yes			
y elav^{+} *w*	*P15.2*	13.5	WT	2, 3	54	Yes			
y elav^{+} *w*	*P62.2*	13.5	WT	2, 3	112	Yes			
	TfL^{m}								
y elav^{+} *w*	*353-66-2*	15.5	*elav*^{FliJ2} mutation	1, 5	5 at 25°, 35 at 18°	Yes			
	TfS								
y elav^{+} *w*	*Tf(2)DmORF3*	8.5	WT	4	97	No	0	0	295
elav^{e5}	*Tf(2)DmORF3*	8.5	WT	4	NT	No	8[a]	3[a]	372
elav^{e5} *w sn*	*Tf(2)DmORF3*	8.5	WT	4	NT	No	0	0	616
y elav^{+} *w*	*Tf(3)DmORF2*	8.5	WT	4	94	No	0	0	334
elav^{e5}	*Tf(3)DmORF2*	8.5	WT	4	NT	No	18[a]	8[a]	157
y elav^{+} *w*	*339-17*	8.5	Point mutation	5	100	No	3[a]	0	343
y elav^{+} *w*	*7*	8.5	No introns	2	98	No	0	0	227
y elav^{+} *w*	*22*	8.5	No introns	2	88	No	8[a]	1[a]	341
y elav^{+} *w*	*17*	8.5	No 1st intron	2	98	No	11[a]	0	467
y elav^{+} *w*	*20*	8.5	No 1st intron	2	87	No	1[a]	0	321
y elav^{+} *w*	*Tf(2)DvORF2*	8.5	*D. virilis*	4	81	No	0	0	448
elav^{e5}	*Tf(2)DvORF2*	8.5	*D. virilis*	4	NT	No	0	0	261
y elav^{+} *w*	*Tf(3)DvORF1*	8.5	*D. virilis*	4	115	No	2[a]	0	406
	TfS^{m}								
y elav^{+} *w*	*336* (7 lines tested)	8.5	Altered ORF	5	<1	Yes			
y elav^{+} *w*	*FliJ1* (3 lines tested)	8.5	*elav*^{FliJ1} mutation	5	<1 at 18°	Yes			
y elav^{+} *w*	*FliJ1-5*	8.5	*elav*^{FliJ1} mutation	5	<1 at 18°	No	0	0	404
y elav^{+} *w*	*FliJ2* (2 lines tested)	8.5	*elav*^{FLiJ2} mutation	5	<1 at 18°	Yes			
y elav^{+} *w*	*TS1* (3 lines tested)	8.5	*elav*^{ts1} mutation	5	<1 at 18°	Yes			
y elav^{+} *w*	*TS1-271*	8.5	*elav*^{ts1} mutation	5	<1 at 18°	No	0	0	200

See Figure 1 for the location of the ELAV encoding ORF and the structure of the transgenes. References: 1, this article; 2, K. White, personal communication; 3, Campos *et al.* 1987; 4, Yao and White 1991; 5, Samson *et al.* 1995. The ability of one copy of a transgene to provide *elav* function was determined by mating females *elav*^{e5}*/FM61* to males *y w/Y*; *Tf/balancer*, where *elav*^{e5} is a null *elav* allele, *FM61* is a recessive-lethal-bearing *FM6* chromosome (Campos *et al.* 1985), and *Tf* is an *elav* transgene. No males can emerge from this cross, unless the *Tf* provides *elav* function. The efficiency of rescue was calculated as the ratio between males *elav*^{e5}*/Y*; *Tf/+* (rescued) and females *elav*^{e5}*/y w*; *Tf/+* (not rescued). Determination of fertility, defined as the ability of flies to give rise to adult progeny, was performed by individually mating 10 flies of a given genotype to 4–6 flies of the stock *y w*^{67c23}. WT, wild type; NT, not tested.

[a] Sterile flies.

dent lines carrying such transgenes [transformant-short (*TfS*)], were analyzed. In all cases, attempts to establish homozygous stocks carrying two copies of a given *TfS* failed, whether in a wild-type or a null *elav* background. In balanced heterozygous stocks carrying these constructs, flies with two copies of the transgene represent 0–10% of the total progeny, as opposed to the expected 33%, and they are invariably sterile (Table 1). In contrast flies carrying one *TfS* copy are obtained in the expected number in the progeny of the crosses (Table 1, see rescue of $elav^{e5}$), indicating that a single copy of *TfS* has no deleterious effect. Transgenes carrying mutated *elav* forms [mutant transformant-short (TfS^m)] that fail to provide $elav^+$ function were also tested. Most (15 out of 17) are homozygous viable (Table 1). The two exceptions, *FliJ1-5* and *TS1-271*, probably correspond to recessive lethal mutations generated by the insertion of the transgene.

To test whether the lethality/sterility observed in the *TfS* homozygotes can be accounted for by the truncation of the *elav* 3′ UTR, the effect of constructs carrying a larger *elav* 3′ UTR was analyzed. Constructs including a 6-kb fragment of the 3′ UTR [transformant-long (*TfL*)] rescue $elav^{null}$ function and, in contrast to the *TfS*, can be recovered and maintained as homozygotes. Two lines of a 13.5-kb construct (Campos *et al.* 1987) and two lines of a 15.5-kb construct were tested (Figure 1 and Table 1).

Combinations of different functional *elav* minigenes lacking the normal 3′ UTR induce high levels of lethality or sterility: To exclude further the possibility that the homozygous lethality of the *TfS* was due to the generation of deleterious mutations, males and females carrying different *TfS* insertions were mated and their progeny examined. A representative sample of the results of 116 crosses is shown in Table 2.

Effects of varying severity were found in progeny carrying two different *TfS* insertions. In the majority of these crosses, a significant deficit of flies carrying two transgenes was observed (7 of 10 cases, crosses 3, 4, 8, 10, 13, 19, and 20, Table 2). When they could be recovered as adults, flies carrying two *TfS* had reduced fertility. In less extreme cases, flies carrying two *TfS* did not show significantly decreased viability, but still showed decreased fertility (crosses 5, 15, and 18, Table 2). Overall, viability was reduced in males more than in females, and fertility was decreased in females more than in males. Deleterious effects were seen both in normal and $elav^{null}$ backgrounds.

The sterility of the flies carrying two *TfS* transgenes was evidenced in multiple ways (data not shown): some adults died within 24 hr of eclosion leaving no progeny, some females did not lay eggs, while the embryos of other females failed to hatch. In some instances, only female fertility was affected, while males were normal (for instance, see crosses 5 and 13, Table 2).

As a control, functional *TfS* transgenes were combined with functional *TfL* or nonfunctional TfS^m transgenes. Expected numbers of flies carrying either one or two transgenes were obtained in all of these crosses (Table 2). The fertility of both male and female progeny was normal, with the exception of females carrying two transgenes arising from cross 12 (Table 2). The lowered fertility of these females is unrelated to the transgene, because a similar cross involving the same transgene in a different genetic background yielded fertile females (cross 7, Table 2). The consequence of *X* chromosome-dependent genetic background, or perhaps a maternal effect, on the outcome of these crosses becomes apparent by comparing reciprocal crosses, as for instance crosses 15 and 20 (Table 2). These lead either to a significant deficit of flies carrying two transgenes (cross 20), or to normal viability of the sterile females carrying two transgenes (cross 15).

In summary, the effect of combinations of two different insertions of *TfS* is similar to the effect of homozygosity for a given transgene insertion. In each case, the presence of two *TfS* elements leads to abnormal phenotypes ranging from sterility to lethality. The milder effects observed when combining different *TfS* presumably reflect some aspect of the genetic background.

The 3′ UTR is a regulator of ELAV protein levels: Because two copies of the *TfS* minigenes lead to abnormalities, but two copies of *TfL* minigenes containing an *elav* 3′ UTR are normal, it seemed possible that the *elav* 3′ UTR was modulating *elav* expression. I therefore looked at the dosage dependence of *elav* expression respectively from a *TfL* minigene (*350-83-1*), from a *TfS* minigene [*Tf(2) DmORF3*], and from the endogenous locus, all of which provide full *elav* function (Tables 1 and 3). Surprisingly, immunoblot analysis shows that the same amount of ELAV protein is produced from one or two doses of the endogenous *elav* locus, and from one or two doses of the *TfL* transgene *350-83-1* (Figure 2, lanes 1–4 and 9–11). In contrast, expression from the *TfS* transgene, missing the 3′ UTR, is directly proportional to its copy number (Figure 2, lanes 5–8). Thus the 3′ UTR confers gene copy number independence of ELAV expression.

Not all *TfS* produce less protein than the endogenous locus. Two copies of the fully functional *TfS* minigene *Tf(2) DmORF3* yield lower amounts (about 50%) of protein than wild-type flies (Figure 2). However, the same transgene inserted in a different location [*Tf(2) DmORF2*] yields normal amounts of ELAV (when present as two copies) but is also expressed in a gene dosage-dependent fashion (not shown).

There is an inverse correlation between expression of the endogenous *elav* locus and expression of the *D. virilis* minigene missing the 3′ UTR: Immunoblot analysis was used to examine the expression of ELAV protein in flies carrying different doses of the endogenous *elav* gene and of an *elav* minigene. A *D. virilis* minigene, which encodes a 55-kD ELAV containing an

TABLE 2

Effect on viability and/or fertility of two *elav* transgenes

Cross no.	Parents		Progeny						
	Females	Males	Females carrying two transgenes	Males carrying two transgenes	Females carrying zero or one transgene	Males carrying zero or one transgene	Segregation of flies with two transgenes	Fertile females	Fertile males
1	*TfS*-1	*TfL*	112 (113)	122 (113)	110 (113)	110 (113)	Expected	10	10
2		TfS^m	81 (82)	81 (82)	83 (82)	84 (82)	Expected	10	10
Stock		*TfS*-1	0 (50)	0 (50)	150 (100)	150 (100)	Aberrant	NA	NA
3		*TfS*-2	25 (29)	14 (29)	92 (88)	104 (88)	Aberrant	2	4
4		*TfS*-2bis	35 (52)	28 (52)	136 (104)	112 (104)	Aberrant	1	2
5		*TfS*-3	35 (31)	26 (31)	61 (62)	64 (62)	Expected	0	10
6	*TfS*-2	*TfL*	79 (79)	75 (79)	82 (79)	79 (79)	Expected	8	9
7		TfS^m	88 (88)	87 (88)	93 (88)	84 (88)	Expected	10	10
8		*TfS*-1	39 (47)	32 (47)	158 (141)	146 (141)	Aberrant	3	8
Stock		*TfS*-2	0 (56)	0 (56)	167 (111)	167 (111)	Aberrant	0	0
9		*TfS*-2bis	0 (31)	0 (31)	107 (61)	77 (61)	Aberrant	0	0
10		*TfS*-3	44 (54)	17 (54)	186 (163)	189 (163)	Aberrant	2	1
11	*TfS*-2bis	*TfL*	34 (35)	37 (35)	39 (35)	32 (35)	Expected	10	10
12	($elav^{null}$ background)	TfS^m	79 (75)	78 (75)	69 (75)	0 (0)	Expected	4	10
13		*TfS*-1	31 (42)	35 (42)	133 (126)	95 (84)	Aberrant	0	10
14		*TfS*-2	15 (33)	5 (33)	78 (65)	98 (65)	Aberrant	0	0
Stock		*TfS*-2bis	18 (30)	8 (30)	77 (61)	80 (61)	Aberrant	0	0
15		*TfS*-3	27 (22)	19 (22)	73 (66)	34 (44)	Expected	0	0
16	*TfS*-3	*TfL*	95 (89)	85 (89)	80 (89)	98 (89)	Expected	10	10
17		TfS^m	88 (88)	87 (88)	93 (88)	84 (88)	Expected	10	10
18		*TfS*-1	41 (43)	39 (43)	80 (86)	99 (86)	Expected	1	5
19		*TfS*-2	51 (47)	2 (47)	171 (142)	154 (142)	Aberrant	0	0
20		*TfS*-2bis	59 (40)	0 (40)	80 (80)	102 (80)	Aberrant	0	0
Stock		*TfS*-3	3 (60)	0 (60)	180 (121)	180 (121)	Aberrant	0	0

TfS-1 stock: *y* $elav^+$ *w/ y* $elav^+$ *w/Y*; *Tf(2)DmORF3/CyO*. *TfS*-2 stock: *y* $elav^+$ *w/y* $elav^+$ *w/ Y;Tf(3)DmORF2/TM3Sb*. *TfS*-2bis stock (same insert as in *TfS*-2): $elav^{e5}$ *w sn/* $elav^{e5}$ *w sn/ Y*; *Tf(3)DmORF2/TM3Sb*. *TfS*-3 stock: *y* $elav^+$ *w/ y* $elav^+$ *w/ Y*; 339-17/CyO. *TfL* stock: *y* $elav^+$ *w/ y* $elav^+$ *w/Y*; *350-83-1/350-83-1*. TfS^m stock; *y* $elav^+$ *w/ y* $elav^+$ *w/ Y*; *FliJ1-7/FliJ1-7*. See Figure 1 and Table 1 for the detailed properties of the transgenes. For each category of progeny, the numbers of flies observed are given; numbers of flies expected are given in parentheses. Expected numbers for each class of progeny were calculated as a fraction (predicted by Mendelian rules) of the total progeny from a cross. Chi-square values were calculated to test the hypothesis that flies carrying two transgenes were obtained at expected rates ($P < 0.05$). The number of fertile flies among the 10 tested individuals carrying two transgenes is indicated (when <10 of those flies were obtained, all were tested). NA, not applicable.

TABLE 3

Sex specificity of the rescue efficiency of an *elav*^{null} mutation by transgenes providing moderate levels of *elav*$^{+}$ function

Transgene	Size (kb)	Status of the *elav* insert	Rescue of *elav*^{e5} in females (%)	Rescue of *elav*^{e5} in males (%)	Conditions of rescue
Tf(2)DmORF3	8.5	WT	87	107	25°, 1 copy
Tf(2)DvORF2	8.5	*D. virilis*	84	79	25°, 1 copy
P62-2	13.5	WT	120	105	25°, 1 copy
350-83-1	14.5	WT	89	111	25°, 1 copy
353-66-2	14.5	*elav*^{Flil2} mutation	<1	5	25°, 1 copy
353-66-2			5	35	18°, 1 copy
FliJ1-7	8.5	*elav*^{Flil1} mutation	<1	<1	25°, 1 copy
FliJ1-7			<1	<1	18°, 1 copy
FliJ1-7			6	47	25°, 2 copies
FliJ1-7			18	88	18°, 2 copies

The *elav*^{ts1}, *elav*^{Flil1}, and *elav*^{Flil2} mutations are temperature-sensitive hypomorphic mutations (Samson *et al.* 1995). The properties of the transgenes are documented in Figure 1 and Table 1. The comparison of a transgene's ability to provide *elav* function in males *vs.* females was tested by mating females *elav*^{e5}/*FM6l* to *elav*^{e5}/*Y*; *Tf/balancer*. Males and *Bar*$^{+}$ females arise from such crosses only when the transgene provides *elav* function. Rescue efficiency in females and males was calculated as the ratio of *Bar*$^{+}$ females to *Bar* females/2 and the ratio of males to *Bar* feamles/2, respectively. In the case of the transgene *FliJ1-2*, which provides little or no function when present as one copy (Samson *et al.* 1995), the assay was also performed by crossing males *elav*^{e5}/*Y*; *FliJ1-7/FliJ1-7* with females *elav*^{e5}/*FM7a*; *FliJ1-7/CyO*. Sex-specific rescue efficiency was calculated as the ratio of *Bar*$^{+}$ females to *Bar* females/2 and the ratio of *Bar*$^{+}$ males to *Bar* males/2, respectively.

extended N terminus compared to its 50-kD *D. melanogaster* counterpart, was used (Yao and White 1991). The *D. virilis* transgene differs from the *D. melanogaster* gene by the absence of the 3′ UTR, as well as the replacement of *D. melanogaster* sequences with those of *D. virilis* in the 460 nucleotides most 3′ of the second intron and through most of the open reading frame (ORF; Figure 1; Yao and White 1991). Similar to the *TfS* 8.5-kb *D. melanogaster* transformants that provide *elav*$^{+}$ function, the *D. virilis* transformants that provide *elav*$^{+}$ function are homozygous lethal or sterile (Table 1).

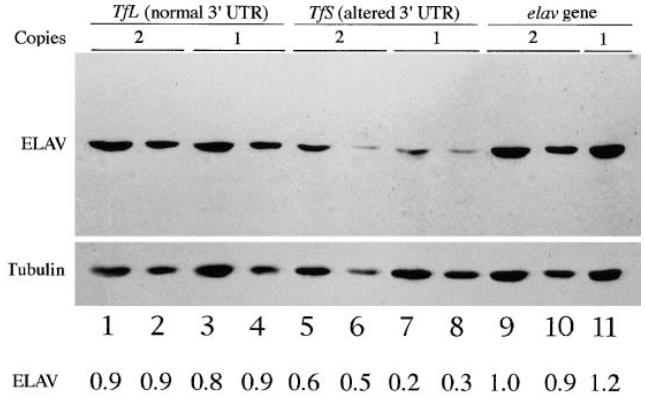

Figure 2.—Production of ELAV in flies carrying one or two copies of *elav* minigene or gene: immunoblot analysis with the mouse anti-ELAV monoclonal antibody of protein extracts from the heads of males (10 μl of protein extracts at about 1 μg/μl in odd-numbered lanes and 5 μl in even-numbered lanes). Tubulin is used as an internal loading control. (1 and 2) *elav*^{e5}/*Y*; *350-83-1*/*350-83-1*, (3 and 4) *elav*^{e5}/*Y*; *350-83-1*/+, (5 and 6) *elav*^{e5}/*Y*; *Tf(2)DmORF3*/*Tf(2)DmORF3*, (7 and 8) *elav*^{e5}/*Y*; *Tf(2)DmORF3*/+, (9 and 10) *y w*/*y*$^{+}$ *sc Y*, (11) *y w*/*Y*. The *Y* chromosome *y*$^{+}$ *sc Y* carries a translocation of the *X* chromosome including *elav*$^{+}$. The amount of ELAV protein in each band, as determined by laser scanner densitometry, is indicated below the gel in arbitrary units. Quantification of one other gel gives consistent results.

When combining the two types of *elav* loci, I found that each locus affects the expression of the other. First, the truncated *D. virilis elav* minigene alone produces ELAV in direct relationship to its copy number (Figure 3, lanes 1, 2, and 7), as does the *D. melanogaster* minigene (Figure 2, lanes 5–8). However, expression of the *D. virilis* minigene is lowered in genetic combinations where the ratio between the copy numbers of the *D. virilis* and *D. melanogaster* loci is ≤1 (Figure 3, lanes 3, 4, and 6); the lower the ratio, the greater the reduction. Second, as demonstrated, the endogenous *elav* locus alone produces ELAV in a dose-independent fashion (Figure 2, lanes 9–11, and Figure 3, lanes 8 and 9). However, protein expression from the endogenous *elav* locus is reduced in genetic combinations where the ratio between the copy numbers of the *D. virilis* and *D. melanogaster* loci is ≥1 (Figure 3, lanes 3, 4, and 5); the higher the ratio, the greater the reduction.

Interestingly, the *D. melanogaster* gene and the *D. virilis* transgene do not respond coordinately to regulation. When the ratio between the copy numbers of the *D. virilis* and *D. melanogaster* loci is at its lowest (flies carrying one copy of the *D. virilis* minigene and two copies of the *D. melanogaste*r gene), the *D. virilis*

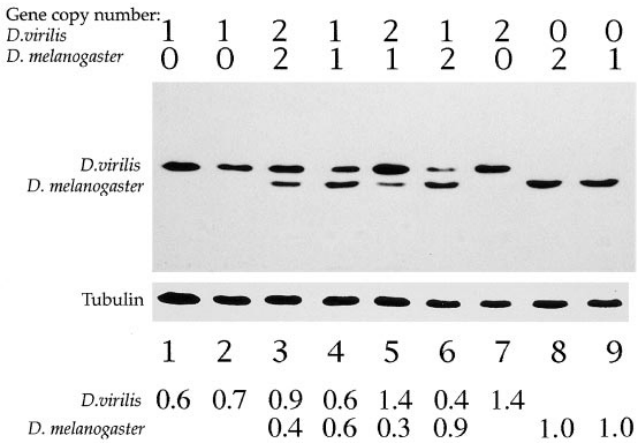

Figure 3.—Production of ELAV in flies carrying zero, one, or two copies of the *D. virilis* minigene (encoding a 55-kD protein) and zero, one, or two copies of the *D. melanogaster* gene (encoding a 50-kD protein): immunoblot analysis with the mouse anti-ELAV monoclonal antibody of protein extracts from the heads of females (10 μl of protein extracts at about 1 μg/μl in lane 1 and 5 μl in other lanes). Tubulin is used as an internal loading control. (1 and 2) $elav^{e5}/elav^{e5}$; $elav^{DvORF}/CyO$, (3) +/+; $elav^{DvORF}/elav^{DvORF}$, (4) $elav^{e5}/+$; $elav^{DvORF}/CyO$, (5) $elav^{e5}/+$; $elav^{DvORF}/elav^{DvORF}$, (6) +/+; $elav^{DvORF}/CyO$, (7) $elav^{e5}/elav^{e5}$; $elav^{DvORF}/elav^{DvORF}$, (8) +/+, (9) $elav^{e5}/+$. The amount of ELAV protein in each band, as determined by laser scanner densitometry, is indicated below the gel in arbitrary units. Quantification of two other gels gives consistent results. Note that the amount of *D. virilis* protein produced from the flies carrying one copy of *D. virilis* transgene and one copy of *D. melanogaster* minigene is close to, but slightly lower than the expected value.

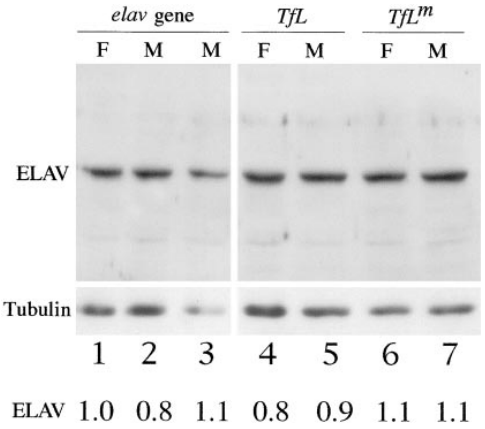

Figure 4.—Production of ELAV in males *vs.* females: immunoblot analysis with the mouse anti-ELAV monoclonal antibody of protein extracts from the heads of males (10 μl of protein extracts at about 1 μg/μl per lane, except 5 μl in lane 3). Tubulin is used as an internal loading control. (1) females $elav^{+}/elav^{+}$, (2 and 3) males $elav^{+}/Y$, (4) females $elav^{e5}/elav^{e5}$; *350-83-1/350-83-1*, (5) males $elav^{e5}/Y$; *350-83-1/350-83-1*, (6) females $elav^{e5}/elav^{e5}$; *353-66-2/353-66-2*, (7) males $elav^{e5}/Y$; *353-66-2/353-66-2*. The amount of ELAV protein in each band, as determined by laser scanner densitometry, is indicated below the gel in arbitrary units.

transgene produces about 60% (0.4/0.6 or 0.7) of normal *D. virilis* ELAV levels, but the *D. melanogaster* gene is normally expressed. In contrast, when the ratio reaches its highest (flies carrying two copies of the *D. virilis* minigene and one copy of the *D. melanogaster* gene), the *D. melanogaster* gene produces only about 30% (0.3/1) of normal *D. melanogaster* ELAV levels, but the *D. virilis* minigene is normally expressed. Indeed, there is an inverse correlation between the levels of the *D. virilis* ELAV and *D. melanogaster* ELAV (Figure 3, lanes 3–6). This suggests the existence of different, but correlated, modes of regulation of the *D. virilis* transgene and the endogenous *D. melanogaster* locus.

***elav* minigenes have differential effects, but are similarly expressed in males and females:** As noted previously, viability is more affected in males and fertility is more affected in females carrying two *TfS* transgenes (Table 2). Since the genomic *elav* locus is located on the *X* chromosome, it seemed plausible that these differences might reflect dosage compensation of *elav* transgenes when integrated on autosomes. When dosage compensated, a given *X*-linked gene is expressed at levels roughly twice as high in males as in females (Kelley and Kuroda 1995).

To examine this possibility, the level of *elav* function provided by transgenes was compared in males *vs.* females. Two situations were found regarding the efficiency of rescue by *elav* transgenes. First, transgenes carrying normal *D. melanogaster elav* sequences providing 25–100% of the normal ELAV level (Table 3) fully rescue $elav^{null}$ function similarly in males and females (Table 3). Second, transgenes that carry mutated *elav* minigenes encoding an impaired ELAV protein and providing partial function rescue significantly better (at least five-fold) in males than in females (Table 3). I also examined levels of ELAV expression in males and females carrying either a *TfL* minigene that fully rescues (*350-83-1*, Table 3) or a mutant transgene TfL^{m} that partially rescues (*353-66-2*, Table 3). While, as seen by Yao *et al.* (1993), there is some fluctutation in the level of ELAV protein between males and females, there is clearly much less than a twofold difference, relative to the control (Figure 4). Thus males and females express similar levels of ELAV protein in heads (Figure 4). It is a possibility that differential expression between males and females occurs earlier in development or in a subset of neurons, leading to the differential activity of *elav* transgenes in males and females. The sexual dimorphism of defects due to *elav* misexpression is reminiscent, for instance, of the sexual dimorphism seen with some alleles of *mushroom body miniature* that leads to central brain lesions (de Belle and Heisenberg 1995) or of the homeotic gene *Transabdominal* (Celniker and Lewis 1993). For these genes as for *elav*, the sexual dimorphism is unexpected because the genes are not, at least directly, implicated in sex determination, and the observation remains unexplained to date.

DISCUSSION

The long 3′ UTR is an important functional part of *elav*: Detection of *elav* RNA and protein *in situ* shows that the expression of the gene is restricted to neurons (Robinow *et al.* 1988; Robinow and White 1991). Multiple developmentally regulated mRNA transcripts have been detected (Campos *et al.* 1987; Yao *et al.* 1993) that span approximatively 16 kb. Alternative forms differing in their 3′ UTRs exist (Figure 1; Campos *et al.* 1987; Robinow *et al.* 1988; Yao *et al.* 1993; M.-L. Samson, unpublished data). The two characterized *elav* cDNAs are each about 2.5 kb long. Both have a full length 5′ end, contain the same ORF, and are truncated at their 3′ end. These cDNAs each terminate within five nucleotides of the sequence 5′-A_6GA_5-3′ (noncoding strand), suggesting that reverse transcription initiated in this region of the RNA rather than at its true 3′ polyadenylated end (Robinow 1989). The size of *elav* transcripts (4.7 kb to 9.5 kb) is consistent with an extended 3′ UTR. The complexity of RNA transcript patterns, their temporal regulation, and the differential expression of *elav* RNA in different parts of the nervous system are in contrast to the ubiquitous and continuous expression of the 50-kD ELAV in neurons (Robinow and White 1991; Samson *et al.* 1995). In this article, the functional importance of the *elav* 3′ UTR in the regulation of *elav* expression of the gene is directly demonstrated by analyzing the properties of *elav* minigenes.

Minigenes containing 8.5 kb of the *elav* gene including the ORF but lacking the normal 3′ UTR provide apparently normal function when present as a single copy, although flies carrying two of these short minigenes have reduced viability and / or fertility. The dosage properties of the *TfS* minigenes differ from those of the endogenous locus and from those of the *TfL* transgenes carrying an additional 7 kb of the *elav* 3′ UTR, because multiple copies of the latter are viable. In addition, TfS^m 8.5-kb minigenes with alterations (point mutations or oligonucleotide insertions) that eliminate *elav* function are homozygous viable, indicating that the 8.5-kb DNA fragment *per se* is not responsible for the observed defects. The defects resulting from the presence of two copies of *TfS* thus directly reflect the absence of the normal *elav* 3′ UTR.

The 3′ UTR is responsible for gene dosage independence of *elav* expression: Insight into the function of the *elav* 3′ UTR was gained by monitoring ELAV protein expression levels produced from the endogenous gene and from minigenes. Surprisingly, ELAV expression was found to be independent of *elav* gene dosage. Males carrying one (*y w*/*Y*) or two (*y w*/y^+ *sc Y*) copies of the gene express similar levels of ELAV, as do females carrying one *vs.* two doses of the *elav* gene (unpublished results). Similar to the endogenous *elav* locus, transgenes carrying the 3′ UTR show dosage independence of *elav* expression. In contrast, *TfS* minigenes produce ELAV in a dosage-dependent fashion. Thus, the 3′ UTR is responsible for normalizing ELAV amount produced, regardless of the gene copy number.

It is interesting to note that, before generation of an ELAV antibody, Bier *et al.* (1988) proposed that the monoclonal antibody Mab44C11 identified *elav* protein, on the basis of the pattern of embryonic staining, the size of the corresponding antigen, and the absence of this antigen in *elav* mutants. However, reservations were expressed because the quantity of ELAV protein did not depend upon the copy number of the *elav* gene. The data presented here explain this discrepancy, because they provide direct evidence for gene dosage independence of *elav* expression. Mab44C11 thus is a *bona fide* ELAV antibody. Furthermore, the gene dosage independence of *elav* expression mediated via the 3′ UTR suggests the existence of a feedback regulation mechanism by which control of ELAV synthesis occurs.

The *elav* gene autoregulates: The possible autoregulation of *elav* was investigated by producing increasing amounts of a 55-kD *D. virilis* ELAV from a minigene (without 3′ UTR) and examining levels of ELAV produced from the endogenous *elav* gene (with 3′ UTR) encoding the 50-kD *D. melanogaster* ELAV.

The experiment reveals that the amounts of *D. melanogaster* ELAV and *D. virilis* ELAV are inversely correlated, in a fashion that works to maintain ELAV levels within a set range, indicating that both the *D. melanogaster* gene and the *D. virilis* minigene autoregulate. Autoregulation is consistent for a gene whose function is required not only for the initiation of differentiation, but also for its maintenance (for a review, see Yao *et al.* 1993). However, since *elav* gene and transgene expression are inversely correlated, the data suggest distinct mechanisms of autoregulation.

The specific autoregulation of the *elav* endogenous gene must depend upon sequences that are specific to the *D. melanogaster* gene. Candidates are the 3′ UTR, and possibly the region of the *D. melanogaster* gene corresponding to 460 nucleotides most 3′ of the second intron and most of the ORF, because they are replaced in the *D. virilis* transgene by the homologous *D. virilis* sequences. While the data do not allow exclusion of the region containing the second intron and the ORF of *elav*, there is no evidence for a regulatory role either. I favor the model in which autoregulation of the *D. melanogaster* gene depends upon the 3′ UTR, shown in this article to confer gene dosage independence.

I propose that a second mechanism is responsible for the modulation of the level of the *D. virilis* minigene. It is likely that the *D. melanogaster* gene is also sensitive to this second level of autoregulation. The ratio of *D. virilis* and *D. melanogaster* ELAV levels is therefore the result of the balance between these regulatory mechanisms that work together to maintain ELAV levels within a set range.

ELAV protein contains three RNA recognition motifs diagnostic of a family of RNA-binding protein impli-

cated in RNA metabolism and translation (Burd and Dreyfuss 1994). Indeed, ELAV binds homoribopolymers, as well as RNA corresponding to the 3′ UTR of its own gene *in vitro* (C. D. Borgeson and M.-L. Samson, unpublished results). One of the mouse ELAV homologues, Mel-N1, also binds its own 3′ UTR *in vitro* (Abe *et al.* 1996b). On the basis of these properties, the possibility can be considered that 3′ UTR-dependent autoregulation occurs through the direct binding of ELAV to the 3′ UTR of *elav* RNA. Thus, it may be that ELAV promotes normal neuronal differentiation by binding to the 3′ UTR of specific RNAs, influencing some aspect of their metabolism. *Cis*-regulatory elements present in the 3′ UTR of mRNAs are known to influence the initiation of translation, intracellular localization, and multiple pathways of mRNA decay (reviewed in Sachs 1993; Curtis *et al.* 1995; Dreyfuss *et al.* 1996). Such functions are consistent with the properties of RRM-containing proteins and could account for the role of ELAV.

The *TfS* behave as dosage-dependent neomorphic *elav* mutations: The distinctive and unsual genetic properties of the transgenes shed some light on the abnormalities associated with the presence of two *TfS* copies. First, two copies of the *TfS* remarkably lead to abnormal fertility/viability, while one copy fully rescues $elav^{null}$ function without conferring any deleterious effects. Second, the defects associated with the presence of two transgenes are seen independently of the $elav^{+}$ or $elav^{null}$ genetic backgrounds. Third, although the lowered viability of the individuals carrying two transgenes could be explained by altered *elav* expression, their increased sterility and the sex specificity of the defects are not easily explained by altered *elav* function, which is thought to be limited to development and maintenance of the nervous system. This third point suggests that a process other than that affected by *elav* might be altered in the flies carrying two *TfS* transgenes.

Therefore, it seems that the *TfS* produce or induce a molecule that is toxic to the flies past a given threshold. As shown, transgene dosage itself (DNA) does not explain the deleterious effects of two copies of *TfS* minigenes. The data presented suggest that the nuclear ELAV protein normally binds its 3′ UTR, leading to autoregulation, by modulating a currently undetermined process in the metabolism of its RNA. The absence of a complete 3′ UTR in the *TfS* alters this regulation, and it is thus possible that the observed deleterious effects seen in flies carrying two *TfS* minigenes result from the elevation of ELAV levels. Although I did not detect increased levels of ELAV in the proteins of head extracts from flies carrying two copies of transgenes, it is possible that elevated ELAV expression could happen either earlier in development and/or in a subset of neurons. Alternatively, it is possible that the production of an abnormal *elav* RNA, and not of the final gene product, triggers the deleterious phenotypes associated with two *TfS* copies. Going past a threshold of *TfS* RNA reached when two copies of *TfS* are present could lead to the observed deleterious effects, presumably by titrating a factor that binds the *TfS* RNA and forms an unprocessable complex. Clearly, additional experiments are required to test these possibilities.

Elucidating how the interaction of ELAV with the *elav* 3′ UTR regulates the expression of the gene will contribute substantially to an understanding of ELAV function in promoting neuronal differentiation, assuming that the gene modulates the metabolism of RNA "targets" involved in neuronal differentiation in the same way it influences its own expression. Moreover, such understanding will also contribute more generally to the understanding of 3′ UTR function. The minigenes provide a means to monitor the function of the *elav* 3′ UTR *in vivo*, via assessing their viability in various genetic combinations. This tool, that has to my knowledge no equivalent in other systems, should greatly facilitate the analysis of *elav* function.

I thank B. Chase, M.-P. Furrer, L. Rabinow, and M. Wegnez for constructive comments on the manuscript, and H. Furneaux and J. Keene for communication of unpublished results. This work was supported by a Basic Research grant no. FY96-0995/FY97-0605 from the March of Dimes Birth Defect Foundation, and a University of Nebraska Medical Center seed grant no. 22-271-81201.

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Communicating editor: L. L. Searles