The neuron-specific RNA-binding protein ELAV regulates neuroglian alternative splicing in neurons and binds directly to its pre-mRNA

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Drosophila melanogaster neural-specific protein, ELAV, has been shown to regulate the neural-specific splicing of three genes: neuroglian (nrg), erect wing, and armadillo. Alternative splicing of the nrg transcript involves alternative inclusion of a 3'-terminal exon. Here, using a minigene reporter, we show that the nrg alternatively spliced intron (nASI) has all the determinants required to recreate proper neural-specific RNA processing seen with the endogenous nrg transcript, including regulation by ELAV. An in vitro UV cross-linking assay revealed that ELAV from nuclear extracts cross-links to four distinct sites along the 3200 nucleotide long nASI; one EXS is positioned at the polypyrimidine tract of the default 3' splice site. ELAV cross-linking sites (EXSs) have in common long tracts of (U)-rich sequence rather than a precise consensus; moreover, each tract has at least two 8/10U elements; their importance is validated by mutant transgene reporter analysis. Further, we propose criteria for ELAV target sequence recognition based on the four EXSs, sites within the nASI that are (U) rich but do not cross-link with ELAV, and predicted EXSs from a phylogenetic comparison with Drosophila virilis nASI. These results suggest that ELAV regulates nrg alternative splicing by direct interaction with the nASI.

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Posttranscriptional mechanisms that control differential splicing of nascent transcripts and regulate mRNA stability, transport, and translation are fundamental to regulating gene activity. Members of the ELAV family proteins found in Drosophila, Caenorhabditis elegans, and vertebrates have been implicated in cytoplasmic RNA regulation and nuclear differential splicing [for review, see Antic and Keene 1997]. With the exception of one subclass within this family, HuA, their expression is largely restricted to the nervous system [Okano and Darnell 1997]. Posttranscriptional regulation is especially critical in the nervous system with its enormous cellular diversity in which neurons differ from each other in morphology, biochemical phenotypes, and function.

Drosophila melanogaster ELAV, the first identified member of the ELAV family, is specifically expressed in all neurons and has a predominantly nuclear localization [Robinow and White 1988, 1991; Yannoni and White 1997]. elav gene provides a vital function that is important to differentiation and maintenance of neurons [for review, see Yao et al. 1993]. ELAV has been implicated in the nuclear processing of transcripts of three genes: nrg, erect wing, and armadillo [Koushika et al. 2000]. All three genes are characterized by broad expression of the transcript and protein and neural-specific alternative splice forms. In each case, the level of the neural-specific spliced transcript correlates with the level of ELAV [Koushika et al. 2000]. Although we have shown that ELAV promotes the formation of neural-specific splice forms, the role of ELAV in this process has not been defined.

ELAV proteins are characterized by a minimal structure of two tandem RNA recognition motifs (RRM), followed by a hinge domain and a terminal RRM [Birney et al. 1993]. Moreover, RNA binding of each RRM in ELAV serves a nonredundant function [Lisbin et al. 2000]. Thus far within the ELAV family, ELAV alone has been implicated in splicing. All other members of ELAV family have been ascribed roles in cytoplasmic RNA processing [Jain et al. 1997; Myer et al. 1997; Fan and Steitz 1998; Levy et al. 1998; Peng et al. 1998; Antic et al. 1999; Ford et al. 1999], including the D. melanogaster RBP9 protein [Park et al. 1998; Kim-Ha et al. 1999]. ELAV has also been implicated in autoregulation [Samson 1998]. Out-

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side of the ELAV family, the first two tandem RRMs of ELAV are related to the well-studied *D. melanogaster* Sxl protein [Robinow et al. 1988; Birney et al. 1993; Lisbin et al. 2000]. Sxl protein regulates alternative splicing as well as translation of genes in the sex determination cascade [McKeown and Madigan 1992; Bashaw and Baker 1997]. The RNA target sequences of Sxl show stretches of U (Sosnowski et al. 1989; Inoue et al. 1990). Reported RNA targets for the vertebrate ELAV proteins are poly(U)-rich sequences of varying lengths interrupted with single (A) residues [for reviews, see Antic and Keene 1997; Wang and Tanaka Hall 2001].

Alternative splicing in neurons was first discovered in the CA/CGRP gene encoding two peptides that differ in the terminal exon: calcitonin in thyroid cells and CGRP in specific neurons [Amara et al. 1982; Crenshaw et al. 1987]. Since then, alternative splicing has been found to be a common mechanism used in the generation of isoforms in a variety of functionally important neuronal genes. Although the biological significance of much of the alternative splicing is not always readily evident, in many instances it is thought to be important in the fine tuning of neuronal function. In certain cases, however, the significance of specific isoforms has been shown. For example, isoforms of the potassium channel encoded by the *Drosophila* Shaker locus show distinct physiological properties [Iverson et al. 1988; Iverson and Rudy 1990], and only the neuronal isoform of agrin has the ability to cluster acetylcholine receptors at the neuromuscular junction [Ferns et al. 1992].

Studies on factors that influence neuronal alternative splicing have revealed that similar to other tissue-specific splicing, certain ubiquitously present RNA-binding proteins are important to splicing regulation [for reviews, see Horowitz and Krainer 1994; Grabowski 1998]. Data from several systems suggest that differential concentration of specific RNA-binding proteins, or modulation of their activity by posttranslational modifications as is the case with SR proteins, is crucial to splicing regulation [for review, see Manley and Tacke 1996]. These regulatory proteins, along with other proteins that they either recruit or are in complex with, recognize specific enhancer sequences in the pre-mRNA to mediate specific splicing events [Black 1992].

Although most of the neuron-specific splicing model systems have failed to reveal participation of neuron-specific regulatory proteins, it appears that at least in some instances, strictly neuron-specific factor is involved as an essential component of the regulatory complex. Recently, Nova-1, a member of the KH-type RNA-binding protein in mammals with an expression exclusively within specific brain regions, has been shown to be required for alternative splicing of receptor pre-mRNAs for glycine α2 exon 3A and GABA_A exon γ2L receptor. Nova-1 binds in vitro to these pre-mRNAs in a sequence-specific manner [Buckanovich et al. 1996; Jensen et al. 2000]. Our data suggest that *Drosophila* ELAV, exclusively present in all neurons, influences splicing [Koushika et al. 2000].

We initiated this study to assess the role of ELAV in *nrg* neuron-specific splicing. Two *nrg* spliceforms are generated from the *nrg* transcript by 3′ splice site (3′SS) choice between the penultimate default exon [D] and the ultimate neural exon [N] (Fig. 1A). This results in *Nrg* protein isoforms with distinct cytoplasmic domains [Hortsch et al. 1990]. Initially we set out to define the sequences within the *nrg* transcript that were sufficient to mimic the endogenous *nrg* alternative splicing. A reporter minigene, comprised of the alternative spliced *nrg* intron [nASI] placed upstream of β-galactosidase gene, was constructed, transformed, and analyzed in vivo in transgenic flies. We show that this minigene reporter produces β-galactosidase in neurons, and that the neural-specific processing is responsive to ELAV levels. Having defined the regulated intron as sufficient for recreating neural-specific splicing, we asked if ELAV bound directly to the intron in vitro. We show that ELAV in embryonic nuclear extracts cross-links to a subset of the poly(U)-rich sequences found throughout the 3.2 kb intron sequence. The ELAV cross-linking sequences (EXS) are strongly poly(U) in character but do not share a precise consensus sequence; rather, the length appears to be critical. The reporter gene simultaneously deleted for the EXS sequences shows reduced signal compared to the parent reporter gene. In addition, to allow an evolutionary comparison, we cloned and sequenced the nASI from *D. virilis*, which aided in the analysis of sequences essential in the splicing regulation and provided a context for positioning of the ELAV cross-linking sites along the intron. Based on the data presented in this paper, we present a working model for ELAV’s role in *nrg* alternative splicing.

## Results

### nASI from *D. melanogaster* and *D. virilis*

The nASI diagrammed in Figure 1 was cloned and sequenced from *D. melanogaster* (~3.2 kb). The nASI sequence was also cloned and analyzed from *D. virilis* (~4.3 kb), with the expectation that a phylogenetic comparison could yield information about essential intron sequences and potential target sequences for ELAV regulation. An antibody specifically recognizing the neural-specific Nrg also recognizes a protein in the *D. virilis* photoreceptors and larval brain [data not shown]; thus, neural-specific splicing is likely to be conserved in *D. virilis*. The 5′ splice site (5′SS) of the common exon [C] and the 3′SS of the neural exon [N] are close to the *Drosophila* consensus [Fig. 1B]. However, the 3′SS of the default exon [D] differs from the consensus sequence in that the polypyrimidine tract (PPT) is separated from the 3′SS by an [A]-rich sequence, and obvious candidates for a branchpoint sequence are lacking in proximity to the PPT (Fig. 1B). This character of the default 3′SS is conserved in both *Drosophila* species, suggesting this feature may be functionally significant. A 13-nt block of poly(U)-rich homology (Fig. 1B, outlined box) appears within the default PPT and stands out as a potential ELAV-binding site, as it is reminiscent of the *tra* target peptide...
sequence suggested for the ELAV-relative Sxl protein (Sosnowski et al. 1989; Inoue et al. 1990; Handa et al. 1999). Furthermore, two blocks of nonpoly(U) sequences showed significant homology. D. melanogaster and D. virilis introns have extensive regions of poly(U) sequence, which are thought to be ELAV family member targets (for reviews, see Antic and Keene 1997; Wang and Tanaka Hall 2001). However, outside of the default PPT, no blocks of AU-rich or poly(U)-rich sequence identity were evident.

**Reporter transgenes CNV and CNVt express specifically in neurons**

To study in vivo the regulated alternative splicing of the nrg transcript, we constructed a minigene reporter from the P-element vector, pCV (Mottes and Iverson 1995) and a 3.3-kb fragment containing the D. melanogaster nasi (Koushika et al. 1996). The resulting construct, termed CNV, consists of the Hsp70 promoter, driving expression of the nasi, followed by the *Escherichia coli* LacZ open-reading frame and Hsp70 trailer (Fig. 2A). CNVt is identical to CNV except that the tubulin 3′ trailer sequence has replaced those of the Hsp70 trailer. The nrg intron sequences were inserted such that the exon C lies in frame with an open-reading frame that initiates upstream within the transcribed region of the Hsp70 promoter, and the exon N lies in frame with the LacZ open-reading frame. Therefore, only neural-specific splicing of the nasi sequence will generate β-galactosidase.

CNV transcription was induced by subjecting the animals to an initial 35°C heat shock, followed by a 38°C heat shock. The mild heat shock protects splicing from the effects of the subsequent heat shock (Yost and Lindquist 1986; Mottes and Iverson 1995). Two hours after heat shock, CNV wandering third instar larvae stained intensely in the brain lobes and the ventral ganglion (Fig. 2B, panel 1), as well as in the eye imaginal discs above each brain lobe, but not in nonneuronal tis-
sues. X-gal staining in the developing eye disc is coincident with the photoreceptor neuron field behind the morphogenetic furrow (Fig. 2C, left side).

In control (lacking the CNV transgene) and in non heat shocked CNV adult flies, X-gal staining occurs predominantly in the gut indicative of endogenous β-galactosidase (Fig. 2B, panels 2,3). After heat shock, however, CNV transgenic adults show staining of the nervous system as well (Fig. 2B, panels 4–6). Strong X-gal staining is observed in the antennae, optic lobes, brain, and thoracic ganglion, but not in nonneuronal tissues. Similar results were seen with CNVt (see Fig. 6A, below; data not shown).

**Figure 2.** β-galactosidase expression from CNV minigene. **(A)** CNV minigene construct. The Hsp70 promoter drives the transcription of the nAS1 (shown in red) in frame with the downstream LacZ reporter gene. Splicing to the proximal 3'SS leads to the translation of a short peptide, whereas splicing to the distal 3'SS leads to the generation of β-galactosidase. **(B)** CNV expression in adults and larvae was subjected to heat-shock treatment, dissected, and stained at 2 h. Variations in the treatment as noted. (1) The larval cuticle was teased apart and the entire larva was bathed in the staining solution. Note the intense staining of the brain (Br) and in the developing photoreceptors in the eye discs (Ed). Mh, mouth hooks. (2) No CNV. (3) CNV without heat shock. (4) CNV, 35°C pre heat shock only. (5) CNV, heat shock, dissected after 1 h. (6) CNV, heat shock, except 2 h at 38°C. Note that controls 2 and 3 show only the endogenous staining, but in the CNV flies after heat shock, brain, antennae, retina, and the thoracic ganglion also stain. **(C)** β-Galactosidase expression from the CNV is reduced in photoreceptors with reduced ELAV. Third instar CNV larval brain and eye disc stained for β-galactosidase in two genetic backgrounds for elav: elav<sup>+</sup>; CNV (wild-type ELAV level) and elav<sup>e5</sup>; elav<sup>edr</sup>; CNV (reduced ELAV) expression in the photoreceptors. Each eye disc was photographed with attached brain lobe. The two left eye discs with normal ELAV levels show strong staining in the photoreceptor field of the eye disc behind the morphogenetic furrow (arrowhead) as well as staining of the attached brain lobes. Note that the right eye discs with reduced ELAV expression show a significant reduction in the staining compared to the left eye discs with wild-type ELAV expression. **(D)** Global expression of ELAV leads to global β-galactosidase expression from the CNV transgene. Third instar larvae were heat shocked, dissected, fixed, and stained. In panels 1 and 2, larval cuticle is cut open in the anterior and the whole animal is stained, whereas in panels 3, 4, and 5, larva is dissected and displayed with the brain attached to the mouth hooks and the digestive track (G). CNV larvae (1,3); CNV; Hsp–ELAV larvae (2,4); CNV; Hsp–Sxl (5) larvae. Note that only larvae carrying both CNV and Hsp–ELAV depict global staining (2,4).
**ELAV regulates CNV transgene processing**

The processing of the CNV transgene indicates that sequences within nASI are competent to generate the neuron-specific isoform similar to that observed for the endogenous nrg. Previously we had shown that reduced expression of ELAV in the eye disc caused a concomitant reduction in the neural-specific protein isoform, Nrg\(^{130}\) but not in the ubiquitous protein isoform, Nrg\(^{167}\) [Koushika et al. 1996]. To test if the transcript generated by the CNV transgene is regulated by ELAV, we examined the CNV transgene expression in the third instar larval eye discs with genetically reduced levels of ELAV expression in the photoreceptors [Fig. 2C]. Eye discs attached to brain lobes of the genotypes +; edr\(^{-}\)/Hsp–Sxl\(^{-}\) and edr\(^{-}\)/H9252-galactosidase were fixed concurrently and stained for β-galactosidase activity. In Figure 2C, a pair of wild-type eye disc/brain lobes on the left show robust X-gal staining in the photoreceptor field behind the morphogenetic furrow (solid arrowhead), whereas there is substantial reduction in the staining of the photoreceptors in the pair on the right with reduced ELAV. Thus, the neural processing of the CNV transgene is reduced with reduced levels of ELAV. Note that X-gal staining is not reduced in the edr\(^{-}\)/Hsp–Sxl\(^{-}\)/CNV brain lobes, where ELAV protein levels are not altered significantly.

Because ectopic expression of ELAV induces the neural-specific Nrg isoform, we tested if it would also induce CNV processing to generate ectopic β-galactosidase. Larvae of the genotype Hsp–ELAV/CNV and control larvae CNV and Hsp–Sxl/CNV were heat shocked, dissected, and stained. On heat shock, Hsp–ELAV, Hsp–Sxl, and CNV are all transcribed globally. Hsp–Sxl is a control for nonspecific effects that could arise from the ectopic expression of a poly[U]-binding protein. Neural-specific staining is seen in the CNV controls [Fig. 2D, panels 1,3], whereas global X-gal staining is observed in Hsp–ELAV/ CNV larvae [Fig. 2D, panels 2,4]. Control Hsp–Sxl/CNV larvae do not show global X-gal expression [Fig. 2D, panel 5]. These data suggest that ELAV is sufficient to alter the processing pathway of the CNV-derived transcript outside the nervous system. This is confirmed at the level of RNA processing in which ectopic ELAV leads to the appearance of the neuron-specific spliced CNV transcript in nonneural tissue [see below].

**CNV transgene splicing**

RT–PCR was used to analyze the processing of CNV transcripts from heat-shocked CNV transgenic adult flies. Sequence analysis confirmed proper splice site usage for the default and neural-specific spliced transcripts (data not shown).

RNase protection assays were used to analyze RNA processing of the CNV minigene in adult flies. RNA was isolated from CNV fly heads and abdomens after 1-, 3-, 6-, and 20-h post heat shock [Fig. 3A]. Heads contain the fly brain and are enriched for neurons, whereas abdomen is neuron poor as it has only sensory and enteric neurons. For RNase protection assays, antisense probe HCNZ was used [Fig. 3C]. Neural-specific spliced CNV transcripts are abundant in heads relative to abdomens. This is consistent with (1) the enrichment of neural tissue in adult heads, and therefore ELAV levels as well, and (2) relatively few neurons in the abdomen [Fig. 3B]. Whether default splicing also occurs in neurons is not known. At 1 h post heat shock, the ratio of neural-spliced protected RNA signal to nonneural-spliced protected RNA signal was 0.56 for heads and 0.09 for abdomens [Fig. 3D, lower panel]. This translates to a molar ratio of neural spliced CNV RNA to total CNV RNA of 28% for heads and 5.9% for abdomens. In addition, the decay rates for the two CNV RNAs appear to be similar so that the RNA ratio stays relatively constant for a number of hours [Fig. 3D, lower panel]. Endogenous nrg transcripts or any transcript from the endogenous β-galactosidase gene were not detected in this experiment.

To analyze effects of ectopic expression of ELAV on CNV RNA processing in nonneural tissue, RNase protection assays were performed on third instar larvae carrying just CNV or both CNV and Hsp–ELAV [Fig. 3E]. After heat shock, the CNS was dissected out and RNA was extracted from the remaining tissue. In parallel, larvae ectopically expressing Hsp–Sxl were similarly processed. In the absence of ELAV, there is little neural-specific splicing, however, in the presence of ectopic ELAV, neural-specific processing of the CNV transcript is greatly enhanced. Ectopic Sxl had no effect on neural-specific splicing.

**ELAV cross-links to four regions of the nASI**

We searched for in vitro ELAV-binding sites within the nASI sequences, as these sequences were sufficient for ELAV regulation. Thirteen overlapping RNAs (1–13, size range 200–380 nt) that span the entire 3200-nt length of the nASI were tested in the UV cross-linking assay with Drosophila embryonic nuclear extracts [Fig. 4A,B]. Nuclear extracts were made from 6- to 18-hour-old Canton S embryos. To identify unambiguously cross-linked ELAV, we generated two parallel extracts that differed only in the source of ELAV protein, D. melanogaster ELAV (50 kD) and D. virilis ELAV (55 kD) [Fig. 4C]. D. virilis ELAV is fully functional in D. melanogaster in vivo [Yao and White 1991]. RNAs 1 to 13 were tested in the UV cross-linking assay with the dual embryonic nuclear extracts [Fig. 4B]. ELAV cross-linking, indicated by the 50- and 55-kD bands (arrowheads) for the dual extract lanes, occurs with RNAs 2, 6, 10, and 12, but not with the other nine RNAs. RNA 6 contains the default 3′SS and the U-rich element conserved between the two Drosophila species. Robust non-ELAV signals in the range of 40–45 kD are ubiquitous and appear to coincide roughly in intensity with the poly(U) content of the RNA tested [i.e., RNA 3 has no poly(U) content and no 40–45 kD band]. These bands, presently of unknown identity, are most intense in lanes where ELAV cross-linking occurs.

Further confirmation that the cross-linked species is
ELAV, comes from the immunoprecipitation of ELAV protein from nuclear extracts, which is radiolabeled only on cross-linking with a radiolabeled RNA. Both the 50- and 55-kD ELAV within the extracts became radiolabeled when immunoprecipitated after cross-linking with RNA6 but not with non-ELAV cross-linking RNA8 (Fig. 4D).

To determine the linear range for the cross-linking signal, increasing amounts of the RNA 6' or the non-ELAV cross-linking RNA 2' [see Fig. 5A] were added to 1.5 mg/mL extract. The ELAV cross-linked species is detected for RNA 6' even at the lowest concentration of RNA (0.1 nM). However, the RNA 2' cannot be detected even at the highest concentration, although it is also rich in poly(U) sequences [Fig. 4E].

The concentration of nuclear extract was varied and the input RNAs kept constant [Fig. 4F]. RNA 6' cross-linking to ELAV is not detectable at 0.5 mg/mL nuclear extract [Fig. 4F, lane 1], barely detectable in the 1 mg/mL nuclear extract [Fig. 4F, lane 2], but strong at 2 mg/mL and above. The ELAV noncross-linking RNA 9' is either not detectable or only faintly detectable at all concentrations of nuclear extract tested, even though it also has substantial poly(U) content.

Sequences responsible for the ELAV cross-linking

To further limit the sequences within RNAs 2, 6, 10, and 12, which are responsible for the cross-links to ELAV, a set of deletions was tested for each of the four ELAV cross-linking RNAs [Fig. 5]. Deletions were made in the most poly(U)-rich regions within each RNA; their sequences are listed in Table 1.

Analyses of the nASI RNA 2

Deletions A, B, and C were made in RNA 2 [Fig. 5A]. The ELAV cross-linking signal was unaffected by deletions A and B [2ABC], but was abolished by A, B, and C together [2ΔABC] and C...

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**Figure 3.** RNA processing of the CNV transgene. (A) RNAse protection analysis of CNV transcripts. Heads and abdomens from 2- to 5-day-old CNV flies collected with no heat shock [N] or at 1-, 3-, 6-, or 20-h post 38°C heat shock. RNAse protection assay was performed as described in Materials and Methods with 10 μg of total head or abdomen RNA per lane. The antisense probe used for the protection analysis is termed HCNZ, for Hsp70 C exon/N exon lacZ (C). 321 nt are protected for a neural-specific splice, HCNZ, and 192 nt are protected for the default splice, HC [or unspliced]. The rp49 probe controls for total RNA loaded. Size markers are pBR322/MspI ladder. [B] Immunoblot with α-ELAV antibodies to show ELAV level in 10 μg of total protein from adult heads, H, and abdomens, A. [D] The data from A was analyzed and quantitated on a PhosphorImager to show relative amounts of neural-processed CNV RNA. (Top graph) HCNZ-protected RNA amounts for both head and abdomen in arbitrary units. The HCNZ RNA levels decrease in the hour after heat shock. (Bottom graph) The ratio of HCNZ to HC stays relatively constant for several hours as both neural and default RNAs appear to decay with similar rates. Note: In independent experiments using an HCl probe [I, intron], the unspliced CNV, which would be seen as the HC-protected species in the figure above, is present, but in very low amounts [data not shown]. [E] Ectopic expression of ELAV induces neural-specific processing of CNV transcripts in nonneuronal tissue. Third instar larvae were heat shocked, and 1 h later, the CNS was dissected out and the remaining tissue subjected to RNA extraction. RNA [5μg] was subjected to RNAse protection analysis with the HCNZ antisense probe [C]. Fly heads with CNV transgene alone (−) or with Hsp–Sxl transgene [hsS] have only reduced levels of the neural-specific transcript. However, the ectopic expression of ELAV when CNV is expressed with Hsp–ELAV [hsE] leads to increased neural processing of the CNV transcripts. [F] Deletion/mutation of EXS elements within the nASI leads to a reduction in neural-specific splicing. RNA from heat-shocked CNV and CNV–ΔEXS fly heads was extracted 2 h after heat shock and used in RNAse protection analysis. The ratios of HCNZ/HC from two insertion lines of CNV [1,2] are reduced threefold in two insertion lines of the CNVΔEXS [2-1, 2-4].
Deletion C was examined further by deleting either the left or the right half (2/H9004 CLH and 2/H9004 CRH). Both RNAs show reduced ELAV cross-linking, but neither eliminated the signal entirely, suggesting that both halves contain elements that contribute to the interaction with ELAV. The results with two smaller RNAs, 2/H11032 and 2/H11032/H11032 (Fig. 5A), are consistent with the deletion analysis, with only RNA 2/H11032/H11032, which contains the C sequence, cross-linking to ELAV (Fig. 5E).

Analyses of the nASI RNA 6  To analyze RNA 6, in addition to generating deletions, we mutated the default PPT with point mutations designed to disrupt the poly[U] character (Table 1; Fig. 5B). p2 and p3 have two and three point mutations in the AU4AU3A element 15 nt upstream of the PPT and in the conserved 13-nt block in the PPT, respectively. p5 has all the five point mutations.

As shown in Figure 5B and E, RNAs 6', 6'', and 6ΔB that delete terminal 5', terminal 3' regions, and the default PPT, respectively, show robust, reduced, and much-reduced ELAV cross-linking, suggesting the presence of two cross-linking sites—the default PPT and a site within 6''. The strong cross-linking to the PPT is further supported by robust ELAV signals with RNAs that retain the PPT (6', 6''ΔC, and 6'∈) and weak signals by RNAs that do not retain the PPT (6ΔB and 6'ΔB). Further, PPT mutant p5 also greatly diminishes ELAV cross-linking (6p5, 6'p5, and 6''p5). The second 3' cross-linking site is within ΔA, as those RNAs in which A is deleted.
and PPT is mutated (p5) abolish the ELAV signal (6 ΔAp5 and 6'p5), whereas deletions that retain the ΔA still show weak cross-linking (6p5 and 6'p5).

To assess the relative importance of the PPT and the upstream AU4AU3AU, we analyzed cross-linking of RNAs 6 Δp2, 6 Δp3, and 6 Δp5. ELAV cross-linking is slightly diminished in 6p2 and greatly diminished in 6p3, but eliminated only in 6p5. This suggests that disruption of the U8 in the PPT is more deleterious to ELAV binding than disruption of the AU4AU3A element (6Δp2), but both elements contribute to binding.

Analyses of the nASI RNA 12 of the four primary deletions made in RNA 10, only deletion B (10ΔB) eliminated the ELAV cross-linking signal, whereas the other three deletions combined (10ΔACD) had no effect [Fig. 5C; Table 1]. Deletion of just the left half of the 10ΔB sequence (10ΔBLH) was sufficient to abolish the ELAV cross-linking signal. RNA 10', harboring the ΔB sequence, generates an ELAV cross-linking signal (Fig. 5E).

Analyses of the nASI RNA 12 RNA 12 had the least amount of poly(U) sequence, 12ΔA, eliminated ELAV cross-linking in the 311 nt RNA 12. [E] UV cross-linking with RNAs from A, B, C, and D with large 5' or 3' deletions and RNAs 9 and 13. RNA sizes in nt: (2ΔC) 146, (2ΔC/ΔH) 112, (6ΔC) 198, (6'ΔC) 147, (6'ΔC) 144, (10ΔB) 200, (12ΔD) 129, (13ΔC) 106.

Figure 5. Deletion analysis to determine sequences responsible for ELAV UV cross-linking. Drosophila embryonic extracts were cross-linked with [α-32P]UTP nASI RNAs and processed as explained in Materials and Methods. For each RNA, deletion map schematic and dual-lane cross-linking data as in Figure 4 are shown. Deletion and point mutant sequences are given in Table 1. Arrowheads indicate the 50-kD ELAV [left lane] and 55-kD ELAV [right lane]. (A) The 358 nt RNA 2 has three internal deletions, A, B, and C. RNAs deleted for C, 2ΔC, and 2ΔABC lack the ELAV cross-linking signal. Deletions of the left half 2ΔCLH or right half 2ΔCRH of C reduce but do not eliminate the ELAV signal. [B] The 319 nt RNA 6 has three internal deletions, A, B, and C, and truncated RNA 6', 6'', and 6' have large 5', 3', and both 5' and 3' deletions, respectively. The default PPT region is deleted by ΔB and is disrupted by triple point mutant p3, whereas the nearby AU4AU3A element is disrupted by double point mutant p2. Point mutant locations are indicated by marks on the line drawings. Data indicate the primary ELAV cross-linking site is the PPT and adjacent 5' sequence and a suboptimal site 5' in the ΔA region of 6''. [C] Four deletions were made in the 322 nt RNA 10. Deletions A, C, and D, 10ΔACD, have no effect on ELAV cross-linking; however, deletion of B, 10ΔB, and just the left half of B, 10BLH, is sufficient to abolish ELAV cross-linking. (D) Deletion of part of the only significant poly(U) sequence, 12ΔA, eliminated ELAV cross-linking in the 311 nt RNA 12. [E] UV cross-linking with RNAs from A, B, C, and D with large 5' or 3' deletions and RNAs 9 and 13. RNA sizes in nt: (2ΔC) 146, (2ΔC/ΔH) 112, (6ΔC) 198, (6'ΔC) 147, (6'ΔC) 144, (10ΔB) 200, (12ΔD) 129, (13ΔC) 106.
RNA 6 sequence, AUUUAUUUUAUUUAUUUAAACUCAUCUUUUAUAAACUAAAGUAUUUUUUGGUUUUGGUUUGUUUUUUCACUCUCUUGAUUUCCUUUGCCACAUUUUGUUUUUGGUUUUAUAAAGUAAUAAUUUUAUUUGUUU, is a sequence implicated in ELAV cross-linking (Figs. 1B, 5B). RNA 9’ (Table 1) did not cross-link to ELAV in nuclear extracts (Figs. 4F, 5E), demonstrating that this poly(U) tract is insufficient on its own as an ELAV target in the nuclear extracts.

Deletion/mutation of EXS affects reporter expression

To test if the two blocks of poly(U) in 6’ are important for neural-specific splicing, minigene CNVtp5, which incorporated the same five point mutations as 6’p5 (Table 1), was generated in the parent CNVt vector. The splicing was assayed as before. Larvae of the genotype CNVtp5, along with the control CNVt, were heat shocked and assayed for X-gal staining 4 and 20 h later. The X-gal signal in CNVtp5 brain and eye disc was reduced when assayed 4 h after the heat shock, and the reduction in samples assayed 20 h after the heat shock was significant when compared to the CNVt controls (Fig. 6A–D). The reduction in reporter signal validates the importance of the poly(U)-sequence elements within the polypyrimidine tract of the default 3’SS.

To test if further diminishing of poly(U) content contributes to splicing regulation, we generated a transgene, CNV△EXS, in the CNV parent background, in which in addition to the 6’p5 mutations, the other three EXSs were deleted. Thus, effectively, all of the EXSs were either deleted or mutated. When assayed, the X-gal staining was reduced even in 4 h after heat shock sample, consistent with the idea that these sites contribute to ELAV regulation (Fig. 6E–F).

We analyzed RNA processing of the mutant transgenes in adult heads 2 h after heat shock using RNA-seq protection and the HCNZ antisense probe. Two insertion lines of germ-line-transformed flies were examined for each transgene, along with two lines of CNV. The nervous system processing was reduced in CNV△EXS lines (Fig. 3F), and quantitation by comparing ratios of protected probes HCNZ:HC showed a threefold reduction in HCNZ in CNV△EXS mutants. Analysis of two lines of CNVtp5 showed a twofold reduction (Lisbin 1999).

Derivation of putative ELAV target sequence

We analyzed the four EXS sequences as defined by the analysis above. 2ΔC, 6AB, 10ABLH, and 12ΔA constitute core deletions within EXS2, EXS6 EXS10, and EXS12, respectively (see Table 1), and together with the poly(U)-rich sequences that do not cross-link to ELAV, the sequence patterns that ELAV recognizes were deduced. We did not find precisely conserved sequences. Rather, the common feature in each EXS was the presence of long, 59% to 66% U content and interrupted by A, C, or G residues at apparently random positions. Tracts with a minimum of 8/10Us, with only single-base interruptions, were found in each EXS (Fig. 7D, green shapes). EXS12, which has the least poly(U) content of the four EXSs, has two blocks of 8/10Us that contain no more than four consecutive Us, whereas all the others have runs of five to eight consecutive Us. Additionally, most 8/10U tracts are preceded by a 5’A.

Further, the presence of at least two 8/10U tracts in each EXS is important. The inspection of sequences within the nasI site that do not cross-link, despite having

Table 1. Sequences of the deletions and point mutations in Figure 5

<table>
<thead>
<tr>
<th>RNA #</th>
<th>X-link</th>
<th>Deletions or point mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>2ΔA</td>
<td>++</td>
<td>AUUUUUCACUAAUUAUUUUUUU</td>
</tr>
<tr>
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<td>++</td>
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<td>+/-</td>
<td>AUUUUGUGAUCUUAACUAAUUAAU</td>
</tr>
<tr>
<td>2ΔCLH</td>
<td>++</td>
<td>AUUUUGUGAUCUUAACUUAAUUAAU</td>
</tr>
<tr>
<td>2ΔCRH</td>
<td>++</td>
<td>AUUUUUCAGUGUAUUUAUAAUUAA</td>
</tr>
<tr>
<td>6A</td>
<td>+</td>
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</tr>
<tr>
<td>10B</td>
<td>NO</td>
<td>AUUUUUCAGUGUAUUUAUAAUUAA</td>
</tr>
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<td>AUUUUUCAGUGUAUUUAUAAUUAA</td>
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<tr>
<td>12A</td>
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</tr>
</tbody>
</table>

6p2 is a point mutant in 6p5, 6p2 and 6p3 are in bold and underlined.

6p5 is a point mutant in 6p5, 6p2 and 6p3 are in bold and underlined.

6p5 is a point mutant in 6p5, 6p2 and 6p3 are in bold and underlined.
stretches of poly(U) content very similar to EXS, reveals that the noncross-linkers are shorter and do not contain two 8/10U stretches embedded in them (Fig. 7D). In RNA2/H11032, six Us are followed by six bases and then 11 Us, the longest continuous stretch of poly(U) in the nASI; still, it does not show cross-linking to ELAV (Figs. 5E, 7D). Also compare the poly(U) element in RNA 9/H11032 with the PPT portion of EXS6 (Fig. 7D). This leads us to propose that the specificity of ELAV binding may be in part attributable to the requirement for multiple-binding sites.

Using at least two 8/10U tracts as the minimum criterion, we analyzed the D. virilis nASI sequence for candidate ELAV-binding sites. Three sequences that most fit the EXS model lie in the positions shown in Figure 7C.

Discussion

Our previous studies have indicated that the ELAV, a pan-neural protein, is likely to regulate splicing in neurons. Pan-neural regulators of splicing have not been identified previously. In fact, other factors that, while not tissue-specific, are enriched in some tissues relative to others, are more commonly responsible for regulated splicing [Manley and Tacke 1996]. In contrast, only a few examples of tissue-specific splicing factors have been documented. The best studied of tissue-specific splicing factors are Sxl and Tra, the two proteins in the sex determination pathway that are expressed in female but not male tissue [McKeown and Madigan 1992]. Other examples are KH-domain RNA-binding proteins PSI and Nova-1. PSI, expressed in somatic but not germ-line tissue, suppresses transposase splicing in the somatic tissue [Siebel et al. 1994, 1995], and Nova-1, expresses specifically in certain brain regions [Buckanovich et al. 1996].

nASI has the determinants for nrg alternative splicing

We set out to determine if the nASI had all of the elements necessary for proper expression and regulation. The expression pattern of the CNV transgene conformed to the expression of neural-specific isoform Nrg180 [Hortsch et al. 1990]. This finding was confirmed at the level of the CNV RNA (Fig. 3). Furthermore, ELAV was both necessary and sufficient for the neural-specific expression of the β-galactosidase in the photoreceptor neurons, as previously observed for Nrg180 (Koushika et al. 1996). Two other considerations support the conclusion that the 3’UTR or the reporter coding sequence is not the
major regulating factor. First, reporter transgenes that were constructed with either \textit{LacZ} or GFP (green fluorescent protein) and have HSP 70 trailer, tubulin trailer, or actin trailer show similar expression patterns (data for GFP reporter or actin trailer not shown). Second, the mutated transgenes that show reduced reporter expression have the same 3’/H11032 UTR (Fig. 6). Thus, we can eliminate from consideration any other sequences from the \textit{nrg} gene and the non-\textit{n} ASI sequences in the \textit{CNV} vector as playing an essential role in ELAV regulation, including the 3’/H11032 UTR.

**ELAV target recognition**

In these studies, endogenous ELAV from a crude supernatant of embryonic nuclei was used in UV cross-linking experiments with radiolabeled \textit{nrg} RNAs. We pursued this strategy for ELAV cross-linking as it presented a more in vivo-like situation in an in vitro assay. The initial studies identified four ELAV cross-linking RNAs: 2 (358 nt), 6 (319 nt), 10 (322 nt), and 12 (311 nt). Deletions coupled with cross-linking assays were used to further limit the EXS within the cross-linking RNAs (Fig. 5).

The deduced ELAV target recognition sequences each have at least two blocks of 8/10Us, with an adjacent 5’ A nucleotide. The finding of 8/10Us, as at least part of the likely ELAV target sequence, is highly relevant in light of ELAV’s homology with Sxl, particularly in the RRM 1 and 2 residues that directly contact RNA. That ELAV is likely to be similar to Sxl in its RNA recognition mechanism has been suggested by Handa et al. (1999) who described the cocrystal structure data of the Sxl RRMs with the target \textit{tra} RNA, UGUUUUUUUU. We propose that ELAV RRMs 1 and 2 recognize these 8/10U tracts. The structure of the ELAV target sequence may help explain, for example, why ELAV and Sxl, expected to have con-
siderable overlap in target recognition, apparently do not interfere with each other's targets.

Whereas much of the ELAV cross-linking data with the nASI RNAs appear to be all or none, the deletion analysis suggests that weak ELAV binding can occur in suboptimal sites, as evidenced by the data from 6', 6Δ, and 2ΔCRH. These constructs have only one 8/10U but have at least one additional 7/10U element that may allow ELAV to bind, although with reduced affinity.

That each EXS has more poly[U] in addition to the 8/10U tracts suggests that there is more to ELAV–RNA interaction that perhaps involves ELAV RRM3 or auxiliary proteins. The cross-linked protein bands in the range of 40 kD are candidates, because they appear to be most intense in RNAs in which ELAV cross-linking occurs (Fig. 4A), although impaired ELAV binding does not necessarily diminish the 40-kD species (Fig. 5, 6Δp5, 6'p5, and 10Δb). In the case of 2ΔCRH, in which ELAV cross-linking is impaired but in which two 8/10U still remain, we suggest that the remaining U-tract is insufficient for another component of target recognition.

Within the ELAV family of proteins, homology is highest in the three RRM s (≥70%), particularly in the residues inferred to contact RNA. Therefore, RNA recognition is likely to be very similar among ELAV family members. We recently showed that RRM3 of Rd9 or HUD can functionally replace the ELAV RRM3 in vivo, but that RRM1 or RRM2 replacements were largely non-functional [Lisbin et al. 2000]. In vitro RNA selection procedures on vertebrate ELAVs have shown an RNA-binding preference for interrupted stretches of poly(U) [Levine et al. 1993; Gao et al. 1994; Abe et al. 1996]. Target sequences have been reported for ELAV relatives HuD, Hel-N1, and HuR, consisting of 3' UTR AU-rich elements (ARE), which are implicated in mRNA stability. These elements are comprised of predominantly poly[U] tracts with interspersed A residues. The ELAV cross-linking data is consistent with some reports on vertebrate ELAV proteins, such as GLUT-1 3' UTR [Jain et al. 1995], HPV-1 3' UTR [Sokolowski et al. 1999], TNF-α ARE [Ford et al. 1999], c-fos ARE and not c-jun ARE [Peng et al. 1998]. Other targets reported, for example Myer et al. [1997], which provide in vivo evidence for short AREs interacting with vertebrate ELAV, may represent a different mode of target recognition.

Results from the studies with the nASI minigenes, in which the poly[U] nature of the EXS6 is disrupted, suggest that the default PPT plays a regulatory role and it is likely to occur through the interaction with ELAV. The minigene with deletion/mutation of all four EXSs (CNVΔEXS) also expresses weakly in the nervous system. Many factors could contribute to the residual neural-specific expression from the mutated minigenes. These include additional ELAV-binding sequences not recognized in the UV cross-linking assay, or transcript sequences not included in the minigene, or the deletions themselves could also affect non-ELAV-regulated splicing properties of the nASI. The results from the CNVΔEXS would have to be confirmed further with individual deletions before a definitive conclusion can be drawn. Collectively, these data point to the importance of ELAV's interaction with the nASI in neural-specific splicing.

Model for splicing regulation

Taken together, the presence of EXS sites within the nASI and its ability to mimic the regulatory aspects of the endogenous nrg, strongly suggests that ELAV influences splicing regulation by direct binding to the nASI. ELAV could be affecting alternative splicing directly and/or affect polyadenylation of the D exon, and as a result, the splicing choice between two terminal exons. For example, in the case of the well-studied Calcitonin gene, regulation of the alternative splicing of the two terminal exons occurs by influencing polyadenylation [Lou et al. 1996]. Furthermore, nuclear polyadenylation and splicing of the terminal intron have been shown to be linked events [Niwa et al. 1990; Niwa and Berget 1991].

Our data point to the PPT of the default 3'SS that is included within the EXS6 as playing a pivotal role in splicing regulation. This is similar to the scenario for the alternative splicing of tra, or the Sex-lethal autoregulated intron, in which Sxl targets the default PPT [Sosnowski et al. 1989; Valcarcel et al. 1993]. The Sex-lethal autoregulated intron is perhaps a more apt comparison with the nASI because its of similar size and multiple Sxl-binding sites, and Sxl autoregulation also does not occur through a simple blockage model of the default 3'SS [Sakamoto et al. 1992; Horabin and Schedl 1993]. Could ELAV additionally regulate polyadenylation of the default transcript? There are three possible polyadenylation sites downstream of the exon D positioned 1959, 2650, and 2673 nt from the 5'SS. None of the sites reside within the conserved elements between D. melanogaster and D. virilis or have conserved elements in close proximity. A CNV minigene with a deletion at site 1959 has no effect on reporter expression [data not shown]. Nevertheless, current data does not allow us to rule out regulation at the level of polyadenylation.

We envision a model in which, in the absence of ELAV, the default 3'SS is solely used, despite lacking a consensus branch-point sequence and a proximal PPT. We hypothesize that the conserved intron elements, along with the default exon and polyadenylation site(s) and perhaps other as yet unrecognized sequences, conspire to positively maintain splicing exclusively to an otherwise weak default 3'SS. In the presence of ELAV, however, this positive maintenance is disrupted or partially disrupted, either by directly competing with poly[U]-binding proteins, or by countering the effects of the conserved sequences, leading to 3'SS recognition of the more consensus-like but distal neural-specific 3'SS. This model has predictive value. For example, the exclusive use of the default 3'SS could be compromised by deletion of one or both conserved intron elements. Further studies will be needed to address these questions.
Materials and methods

Cloning and transgene construction

To construct \( P(w^{+} = \text{CNV}) \), \( n_{\text{ASI}} \) was cloned from cosmid 48C2 (Madueno et al. 1995) by PCR using two sets of oligos complimentary to the flanking exon sequences resulting in pBlueScript KS-\( n_{\text{ASI}} \) (see Koushika et al. 1996). The 3325 base genomic region of the \( n_{\text{rg}} \) gene included in \( \text{CNV} \) extends from the 3' 59 nt of the C exon to the 5' 56 nt of the N exon. The \( n_{\text{ASI}} \) was cloned into the CaSpeR transformation vector, pCV, which contains the gene for \( \beta \)-galactosidase under the control of Hsp70 promoter (Mottos and Iverson 1995). To facilitate cloning, site-directed mutagenesis (Kunkel et al. 1991) of KS-\( n_{\text{ASI}} \) was performed to insert a KpnI site into exon C and a NotI site in exon N of \( n_{\text{ASI}} \). The deletions and point mutants of \( \text{CNVtp5} \) and \( \text{CNV}\Delta\text{EXS} \) were generated by site-directed mutagenesis (Kunkel et al. 1991) of KS-\( n_{\text{ASI}} \) and subsequent cloning into pCV. \( \text{CNVtp5} \) was mutated with oligos EXS6p2 and EXS6p3, sequentially, and \( \text{CNV}\Delta\text{EXS} \) was generated by further mutation with oligos \( \Delta\text{EXS2} \), \( \Delta\text{EXS10} \), and \( \Delta\text{EXS12} \). The sequences are: EXS6p2, CTTTTAATTGCACTCTATATATATATATATATA TTTGTATC; EXS6p3, TTATATATTTTGTAATCTTCTTCTTCTTTTTCTAACGCT; \( \Delta\text{EXS2} \), CTACAACTGCTATTACCACTGATGACGATT; \( \Delta\text{EXS10} \), TTAAGCC TATTGTGTAGATAAAGGAAAACATGGTTTAC; \( \Delta\text{EXS12} \), GTATAATTTCACTATTATTTATATTACCTGATGGTGTTGCC TGACAA. To construct \( P(w^{+} = \text{hs}-\text{ELAV}) \), a 1.8 kb cDNA fragment was fitted with EcoRI linkers and subcloned into the EcoRI site in pHsp70. Next, the 3.5-kb Xbal fragment, containing the \( \text{Hsp70}-\text{elav} \) cDNA-\( \beta \)-tubulin trailer, was inserted into the Xbal site of CaSpeR (Pirrotta 1988). A \( \text{D. virilis} \) genomic library (gift of J. Belote, Department of Biology, Syracuse University, NY) was screened with \( n_{\text{rg}} \) cDNA fragment spanning the \( n_{\text{ASI}} \) as described [Yao and White 1991]. The \( \text{D. virilis} \) clone was then sequenced.

Germ-line transformation and genetic crosses

Flies were raised at room temperature or at 25°C. Below are listed abbreviations of the alleles and transgenes used followed by their descriptions: \( \text{elav}^{e5} \) is a null allele at the \( \text{elav} \) locus, \( \text{elav}^{\text{DvORF}} \) \( P(w^{+} = \text{elav}^{\text{DvORF}}) \) is a transgene expressing \( \text{D. virilis} \) ELAV protein under the control of \( \text{elav} \) promoter [Yao and White 1991], \( \text{elav}^{a6} \) is a specific insert of \( \text{elav}^{\text{MOmORF}} \) \( P(w^{+} = \text{elav}^{\text{MOmORF}}) \), a genomic \( \text{elav} \) transgene [Yao and White 1991]; in the \( \text{elav}^{a6} \), \( \text{elav} \) expression is specifically reduced in photoreceptors [Koushika et al. 1996]. \( \text{Hsp–Sxl} \) \( P(w^{+} = \text{Hsp–Sxl}) \) and \( \text{Hsp–ELAV} \) \( P(w^{+} = \text{Hsp70–ELAV}) \) express Sxl and ELAV cDNAs, respectively, under the \( \text{hsp70} \) promoter control CyOUBGF is a balancer chromosome CyO with an inserted transgene \( P(w^{+} = \text{Ub–GFP}) \). \( \text{Ub–GFP} \) expresses green fluorescent protein under the control of the ubiquitin promoter.

Germ-line transformants were generated for the \( P(w^{+} = \text{CNV}) \), \( P(w^{+} = \text{Hsp–Sxl}) \) [a gift from L. Bell, Department of Biological Sciences, University of Southern California], and \( P(w^{+} = \text{Hsp–ELAV}) \) constructs as described previously [Lisbin et al. 2000]. To generate larvae with reduced \( \text{ELAV} \) expression in the eye and \( \text{CNV} \) transgene, \( \text{elav}^{e5} / \text{elav}^{e5} \); \( \text{elav}^{a6} / + \) females were crossed to \( \text{CNV} \) males. Male larvae of genotype \( \text{elav}^{e5} / \text{Y} ; \text{elav}^{a6} / + \) were used for further analysis.

To generate flies with \( \text{CNV} \) and \( \text{Hsp–ELAV} \) or \( \text{Hsp–Sxl} \), \( \text{CNV} \) females were crossed to \( \text{Hsp–ELAV/CyOUBGFP} \) or \( \text{Hsp–Sxl/CyOUBGFP} \). Larvae of genotype \( \text{CNV}/\text{Hsp–ELAV} \) or \( \text{CNV}/\text{Hsp–Sxl} \) were selected from siblings carrying the GFP balance by absence of GFP fluorescence.

Heat-shock and \( \beta \)-galactosidase activity assays

Adults were heat shocked according to Mottos and Iverson (1995) for 1 h at 35°C followed by 1 h at room temperature [22°C] and the primary heat shock of 38°C for the stated time. Third instar wandering larvae were heat shocked for 30 min at 35°C, for 40 min at room temperature, and for 40 min at 38°C. At stated times after heat shock, the samples were dissected in 1X PBS on ice, fixed in 10% formalin for 3 min and stained in Fe/NaP with 1/40 of 8% X-gal in DMF [Hiromi et al. 1985; Yao and White 1994]. For each transgene, at least two independent inserts were analyzed.

RT–PCR/RNAse protection

Total RNA was isolated from flies or larvae using TRIzol reagent [GIBCO BRL/Life Technologies]. For RT–PCR, AMV reverse transcriptase was used to generate cDNA from total CVN RNA with probes complementary to lacZ (LacR, GGCGGAT TAAGTGGGATAAG), the default exon (DR, CTTTGCGTC CATATTGGC), or the intron [IR, TTCCAACTGACAGTAC]. The cDNAs were amplified subsequently by a standard PCR with an Hsp70 oligo (HCF, GCTCTAGAAGTAACCGCAACAAAG), generating HCNZ, HCD, and HCI, respectively. The PCR products were XbaI cut, and the XbaI/blunt fragments were cloned into pKS+, generating pKS+ HCD, pKS+ HCNZ, and pKS+HCI and sequenced with Prizm [Perkin-Elmer Cetus].

For RNAse protection assays, pKS+ HCNZ was linearized with XbaI and used as template for antisense probe. The HCNZ antisense probe is 381 nt, 321 nt are protected by neural-spliced RNA and 192 nt are protected by default or nonspliced RNA. rp49 antisense probes control for total RNA [Hardin et al. 1990], with 65 of the 113 nt protected in the processed RNA. In vitro transcriptions were performed with T3 RNA polymerase in the presence of [\( \alpha\)-\( ^32 \)P]UTP. The transcribed RNA was gel isolated and eluted with standard procedures. RNAse protection assays were performed essentially as reported in Hardin et al. [1990]. 0.2 or 1 x 10^6 cpm probe (10^6 cpm/µg) was added to 5 or 10 µg total RNA in 80% deionized formamide, heated to 95°C for 3 min, and hybridized at 45°C for 16 h. After treatment with RNAse T1 [3 units] and RNAse A [12 µg], and Proteinase K digestion [60 µg], each RNA sample was phenol:chloroform extracted and precipitated with 2.5 volumes of EtOH. Samples were run out on 5% sequencing gels, dried, and analyzed on a PhosphorImager [Molecular Dynamics]. Assessment of neural-specific RNA levels from different lines were derived by comparing ratios of the HCNZ:HC signal.

In vitro transcriptions and template construction

All \( \text{CNV} \) template fragments were generated by PCR; primer sequences are given in Table 2. The “forward” oligos contain a KpnI site at their 5' end, which facilitated KpnI/blunt cloning into pKS+ vectors. Some templates had subsequent deletions and point mutations introduced by site-directed mutagenesis [Kunkel et al. 1991], for mutated sequences, see Table 1. The template vectors were linearized with XbaI before transcription.

In vitro transcription reactions with T3 RNA polymerase were performed using standard protocols. For a 10-µL reaction, 2.5 µL of 800 Ci/µm mole [\( \alpha\)-\( ^32 \)P]UTP, 1 µL of 50 µM UTP, and 1 µL each of 10 mM ATP, 10 mM CTP, and 10 mM GTP were used. Transcribed RNAs were gel purified on 5% polyacrylamide-uracil gels.
Table 2. Oligos used to PCR amplify nASI RNAs

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Drosophila embryo nuclear extract
Preparation of Drosophila embryo soluble nuclear fraction is a scaled-down version of Kamakaka et al. [1991]. Six- to eighteen hour embryos (at 25°C) were harvested for each genotype. The eggs were stored for up to 60 h at 4°C. The two genotypes used were Canton S and elav+ w; hsp68/Sxl. Two different RNA-binding activities for the AU-rich element are evident, with one activity being specific for elav+ w; hsp68/Sxl plasmids. The other activity is more general and is found in both Canton S and elav+ w; hsp68/Sxl embryos. This work was supported by NIH grants NS36179 and GM33205.

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