Ectopic expression of *Drosophila* ELAV and human HuD in *Drosophila* wing disc cells reveals functional distinctions and similarities

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Summary

***Drosophila* ELAV and human HuD are two neuronal RNA binding proteins that show remarkable sequence homology, yet differ in their respective documented roles in post-transcriptional regulation. ELAV regulates neural-specific alternative splicing of specific transcripts, and HuD stabilizes specific mRNAs that are otherwise unstable due to AU-rich elements (AREs) in their 3′ untranslated region (UTR). AREs are major determinants of transcript stability in mammalian cells. The role of each of these proteins was investigated and compared, by ectopically expressing them in *Drosophila* imaginal wing disc cells, which lack endogenous expression of either protein. The effect of the ectopic expression of ELAV and HuD was assessed on two sets of green fluorescent protein reporter transgenes, which were all driven with a broadly expressing promoter. Each set consisted of three reporter transgenes: (1) with an uninterrupted open reading frame (ORF); (2) with a constitutively spliced intron inserted into the ORF; and (3) with the intron *n*ASI whose splicing is regulated in neurons by ELAV, inserted into the ORF. The two sets differed from each other only in their 3′UTR: *Heat-shock-protein-70Ab* (*Hsp70Ab*) trailer with ARE-like characteristics or *Actin 5C* (*Act5C*) trailer. Our results show that: (1) both ectopically expressed ELAV and HuD can enhance expression of transgenes with the *Hsp70Ab* 3′UTR, but not of transgenes with *Act5C* 3′UTR; (2) this enhancement is accompanied by an increase in mRNA level; (3) only ELAV can induce neural-specific splicing of *n*ASI; and (4) although HuD is localized primarily to the cytoplasm, ELAV is localized to both the cytoplasm and the nucleus.**

Key words: ARE, Transgenic studies, Neuron-specific, Alternative splicing, Transcript stability, RRM

Introduction

The primary RNA transcript in eukaryotic cells undergoes processing in the nucleus to generate an mRNA that subsequently gets transported to the cytoplasm where it is translated, degraded or stored. In its journey, an individual RNA is always in a dynamic ribonucleoprotein complex that determines its fate at each step. Diverse proteins that associate with specific mRNAs to promote specific outcomes have been identified. Among these post-transcriptional regulators is a group of proteins belonging to the ELAV/Hu-family of RNA binding proteins (reviewed by Antic and Keene, 1997; Brennan and Steitz, 2001; Keene, 1999). The vertebrate members of ELAV/Hu-family proteins and the *Drosophila* protein RBP9, have been implicated in mRNA stability, translatability and polyadenylation (Antic and Keene, 1997; Brennan and Steitz, 2001; Keene, 1999). Thus far, among the ELAV/Hu-family proteins, ELAV alone has been implicated in regulation of neural-specific alternative splicing (Koushika et al., 1996); additionally, ELAV has been shown to autoregulate its expression (Samson, 1998). Thus, the ELAV/Hu-family proteins seem to serve diverse functions in post-transcriptional regulation.

The predominant function so far associated with the ELAV/Hu-family proteins is to act as regulators of mRNA stability through their interaction with the previously defined AU-rich elements (AREs) that usually reside within the 3′ untranslated region (UTR) of the transcripts. ARE sequences were identified as being important in the rapid degradation of many mRNAs including the transcripts of the immediate early response gene c-*fos* and human granulocyte macrophage colony stimulating factor gene (Chen and Shyu, 1995). AREs are AU rich, generally but not always contain multiple copies of AUUUA pentanucleotide, and range in size between 50-150 nucleotides (Chen and Shyu, 1995). Current evidence indicates that the neuronal vertebrate ELAV-proteins, Hel-N1, HuC and HuD, as well as the ubiquitously expressed HuR stabilize ARE-containing mRNAs (Levine et al., 1993; Brennan and Steitz, 2001; Keene, 1999). Interestingly, RBP9, a *Drosophila* protein, is reported to destabilize transcripts through ARE sequences (Kim-Ha et al., 1999; Park et al., 1998).

ELAV/Hu-family proteins are composed of three classic RRM (RNA recognition motif) domains, and a hinge region separates the first two tandem RRMs from the third RRM. Recent studies have shown the hinge region to include nuclear import/export signals (Atasoy et al., 1998; Fan and Steitz, 1998; Yannoni and White, 1999). The hinge region is also likely to be important for protein-protein interactions and may conceivably provide some of the functional specificity to individual members of this family.

ELAV, a *Drosophila* neural-specific protein, provides a vital function that is essential to the development and maintenance of neurons. Studies in our laboratory have demonstrated that

ELAV is essential to the formation of neural specific splice forms of three genes, *neuroglian* (*nrg*), *erect wing* (*ewg*) and *armadillo* (*arm*), in *Drosophila* (Koushika et al., 2000). These three genes regulated by ELAV are ubiquitously expressed, but also generate neural-specific isoforms. The two isoforms of *nrg*, neural-specific Nrg_{180} and ubiquitous Nrg_{167}, are generated by 3′ exon choice. In the default splicing, the common exon is spliced to the penultimate exon, while in neurons, at least some splicing occurs to the last exon yielding a neural-specific isoform. We have shown that the regulated alternatively spliced intron of *nrg* (*n*ASI), which spans the sequence between the common and the neural-specific exon, contains all the sequences necessary for the splicing regulation (Lisbin et al., 2001).

Despite their extensive homology at the amino acid level, based on current data, *Drosophila* ELAV and human HuD (Szabo et al., 1991) have been associated with different functions. The amino acid identity between these proteins for RRM1, RRM2 and RRM3 is 74%, 63% and 71%, respectively. The main difference appears to be in the hinge region where only 16% identity is seen. In addition to the different functions that these proteins serve, they show distinct patterns of localization within cells. The neuron-specific HuD is primarily cytoplasmic in cultured cell lines, but it appears to be equally distributed in both the cytoplasm and nucleus in neurons of the hippocampus (reviewed by Antic and Keene, 1997). The transgenic expression of HuD in *Drosophila* neurons also shows cytoplasmic expression; however, endogenous or transgene-expressed ELAV is mainly nuclear (Yannoni and White, 1999). Also, ectopically expressed ELAV in *Xenopus* neural tube cells localizes primarily to the nuclei (Perron et al., 1997).

Intrigued with the functional differences between ELAV and HuD, we investigated and compared their properties in the same cells on the same RNA substrates in wing imaginal disc cells of *Drosophila*. We chose wing imaginal disc cells because they are devoid of endogenous ELAV, and because our previous studies show that ectopic ELAV expression in these cells is sufficient for the generation of the neural-specific isoforms of *nrg*, *ewg* and *arm*, the three known ELAV-regulated genes (Koushika et al., 2000). ELAV and HuD were ectopically expressed in the wing discs using the GAL4/UAS system as described (Brand and Perrimon, 1993; Koushika et al., 1996). Effects of ELAV and HuD expression were assayed in transgenic fly strains carrying transgenes in which generally expressed *Ubiquitin-63E* (*Ubi-p63E*) promoter (Lee et al., 1988) was used to drive green fluorescent protein (GFP) coding sequence. Transgenes with introns in the ORF were constructed with either *n*ASI, a regulated alternatively-spliced intron from the *nrg* gene, or GI, a constitutively-spliced artificial intron (Mottes and Iverson, 1995). In the *n*ASI- or GI-containing transgenes, the splicing of the intron was essential for GFP expression. To study the effect of 3′UTR, each transgene was constructed with two distinct 3′UTRs from *Drosophila* genes, *Heat-shock-protein-70Ab* (*Hsp70Ab*) and *Actin 5C* (*Act5C*). Our objective was to examine (1) whether HuD, like ELAV, is capable of inducing neural-specific splicing of the *n*ASI in non-neural tissue or the two differ in this ability; and (2) if ectopically-expressed HuD and/or ELAV influence the level of GFP produced from the reporter genes through distinct 3′UTRs.

Materials and Methods

Transgene construction

To construct *pP{UGA}* and *pP{UGH}*, a *GFP* S65T cDNA sequence was PCR amplified from *pRSET B* vector (a gift of R. Y. Tsien) using the primers 5′-GCGCGGCCGCAAGTAAAGGAGAAGAACTTT-3′ and 5′-GCTCTAGATTATTATTTGTATAGTTCATCC-3′. This PCR fragment contains the entire GFP S65T coding sequence but lacks the first two codons, including the translation initiation codon. The PCR fragment was digested with *Not*I and *Xba*I, and cloned into the *Not*I and *Xba*I sites of *pBluescript* (Stratagene). The *Xba*I-*Pst*I fragment from *pP{CaSpeR-hs/act}* (Pirrotta, 1988), containing an *Act5C* 3′UTR sequence, was inserted downstream to the cloned *GFP* cDNA through the *Xba*I and *Pst*I sites to generate *pGA*. The *Not*I-*Eco*RI fragment from *pGA*, which contains *GFP* cDNA and *Act5C* 3′UTR sequences, was joined to the Myc-tag coding sequence of *pP{WUM2}* (Heck et al., 1993) through the *Not*I and *Eco*RI sites to give the final construct of *pP{UGA}*. The resulting *pP{UGA}* is an *Ubi-p63E* promoter-driven *GFP* cDNA followed by an *Act5C* 3′UTR. The translation is initiated from the ATG codon of the *pP{WUM2}*, and the GFP is expressed as a Myc-tag fusion protein. Similarly, the *Xba*I-*Pst*I fragment from *pP{CV}* (Mottes and Iverson, 1995) containing an *Hsp70Ab* 3′UTR sequence was inserted into the *Xba*I and *Pst*I sites downstream to the cloned *GFP* cDNA to create *pGH*. The *Not*I-*Eco*RI fragment from *pGH* containing *GFP* cDNA and *Hsp70Ab* 3′UTR sequences was cloned between the *Not*I and *Eco*RI sites of *pP{WUM2}*, generating *pP{UGH}*. The *pP{UGH}* is the same as *pP{UGA}* except for the 3′UTR sequence.

To create *pP{UgGA}* and *pP{UgGH}*, the GI intron was PCR amplified from *pP{GI}* (Mottes and Iverson, 1995) using the primers 5′-GCGGTACCCAGGTAAGTTAGTAGATAG-3′ and 5′-AACGAC-GGGATCGTTTGC-3′. The GI intron fragment was *Kpn*I/*Not*I-digested and inserted into the *Kpn*I and *Not*I sites of *pP{UGA}* to generate *pP{UgGA}*. The same GI intron fragment was inserted into the *Kpn*I and *Not*I sites of *pP{UGH}* to create *pP{UgGH}*. In both *pP{UgGA}* and *pP{UgGH}*, the GI intron is inserted between the Myc-tag and *GFP* coding sequences so that the GFP expression is dependent on GI intron splicing.

To construct *pP{UnGA}* and *pP{UnGH}*, an oligonucleotide-mediated site-directed mutagenesis (Kunkel et al., 1987) was performed on an *n*ASI cloned in *pBluescript* (Stratagene). The oligonucleotide 5′-AATCCGTGAGTTCAGGTACCAAACCGGG-CGTGG-3′ was used for the mutagenesis to create a *Kpn*I site upstream of the *n*ASI. The *n*ASI was excised from the vector by cutting with *Kpn*I and *Not*I, and cloned between the *Kpn*I and *Not*I sites of *pP{UGA}* generating *pP{UnGA}*. Similarly, the *Kpn*I-*Not*I *n*ASI fragment was inserted into the *Kpn*I and *Not*I sites of *pP{UGH}* to give *pP{UnGH}*. Both *pP{UnGA}* and *pP{UnGH}* have the *n*ASI between the Myc-tag and *GFP* coding sequences. In both cases, the expression of GFP requires neural splicing of *n*ASI.

Germline transformation

Df(1)w/y w; Ki $p^{p}\Delta 2$*-3/+* embryos were injected with the construct DNA (Robertson et al., 1988; Rubin and Spradling, 1982). The germline transformants were recovered based on [w^{+}] eye color, and transgenic lines were established by standard procedures.

Genetic crosses

Flies were reared at 25°C. For nomenclature of transgenes, see Table 1; for structure of transgenes see Fig. 1.

To examine the effect of HuD and ELAV ectopic expression on GFP reporter expression, *UxGX/CyO; dpp-GAL4/TM6B Tb* females were crossed to *UAS-elav*^{2e2} or *UAS-HuD*^{7a}*; UAS-HuD*^{6c} males, where *UxGX* represents one of the following GFP reporters: *UGA*, *UGH*, *UgGA*, *UgGH*, *UnGA* and *UnGH*. *UAS-HuD*^{7a} and *UAS-HuD*^{6c} are different insertions of the same transgene. Progeny larvae, ectopically ELAV-expressing (genotype *UxGX/UAS-elav*^{2e2}*; dpp-GAL4/+*) or ectopically

Table 1. Transgenes used in this study

Transgene (FlyBase ID)	Text name*	Promoter	Intron in ORF (splicing)	Coding	3′UTR	Source
P{w$^{+mW.hs}$ GAL4$^{dpp.blk1}$ =GAL4-dpp.blk1} (FBtp0000365)	*dpp-GAL4*	*dpp*		GAL4		Stock center†
P{w^{+mC} elav^{UAS}=UAS-elav} (FBtp0007320)	*UAS-elav*	*UAS*		ELAV		Koushika et al., 1996
P{w^{+mC} HuD^{UAS}=UAS-HuD} (FBtp0011873)	*UAS-HuD*	*UAS*		HuD		Yannoni and White, 1999
P{w^{+mC} GFP$^{S65T.UAS}$=UAS-GFP.S65T} (FBtp0001403)	*UAS-GFP*	*UAS*		GFP		Stock center†
P{w$^{+mW.hs}$ GFP^{UGA}=UGA}	*UGA*	*Ubi-p63E*	NO	GFP	*Act5C*	This paper
P{w$^{+mW.hs}$ GFP^{UGH}=UGH}	*UGH*	*Ubi-p63E*	NO	GFP	*Hsp70Ab*	This paper
P{w$^{+mW.hs}$ GFP^{UgGA}=UgGA}	*UgGA*	*Ubi-p63E*	GI (constitutive)	GFP	*Act5C*	This paper
P{w$^{+mW.hs}$ GFP^{UgGH}=UgGH}	*UgGH*	*Ubi-p63E*	GI (constitutive)	GFP	*Hsp70Ab*	This paper
P{w$^{+mW.hs}$ GFP^{UnGA}=UnGA}	*UnGA*	*Ubi-p63E*	*n*ASI (neural)	GFP	*Act5C*	This paper
P{w$^{+mW.hs}$ GFP^{UnGH}=UnGH}	*UnGH*	*Ubi-p63E*	*n*ASI (neural)	GFP	*Hsp70Ab*	This paper

*Abbreviation used in the text.
†Bloomington *Drosophila* Stock Center, IN, USA.

HuD-expressing (genotype *UxGX/UAS-HuD^{7a}; dpp-GAL4/UAS-HuD^{6c}*) were identified by GFP expression and *Tb*$^{+}$ phenotype, and used for GFP fluorescent analysis and in situ hybridization. *UxGX*/+ larvae were used as controls, and *UAS-GFP*/+*; dpp-GAL4*/+ larvae were used to show the *dpp-GAL4* expression pattern.

For imunofluorescent ectopic ELAV and HuD localization, *dpp-GAL4/TM6B Tb* females were crossed to *UAS-elav^{2e2}* or *UAS-HuD^{7a}; UAS-HuD^{6c}* males to obtain *UAS-elav^{2e2}*/+*; dpp-GAL4*/+ (ectopic ELAV expressing) and *UAS-HuD^{7a}*/+*; dpp-GAL4/UAS-HuD^{6c}* (ectopic HuD expressing) larvae which were identified by *Tb*$^{+}$ phenotype.

Fluorescence analysis

For larval fillet preparations, wandering third instar larvae were dissected in a dissecting chamber containing phosphate-buffered saline (PBS) and the gut and Malpighian tubules were removed. The dissected larvae were washed in PBS, fixed in 4% formaldehyde in PBS for 5 minutes at room temperature (RT), washed in PBS, then mounted with 70% glycerol in PBS. Photographs were taken using Leica MZFLIII dissection microscope and Nikon N6006 camera loaded with Kodak Elite Chrome 400 film.

To analyze GFP expression, wing discs of wandering third instar larvae were dissected in PBS, fixed in 4% formaldehyde in PBS for 5 minutes at RT, washed in PBS, then mounted with 70% glycerol in PBS. GFP fluorescence was analyzed under Zeiss Axiophot fluorescence microscope. Fluorescence images were obtained using Roper Scientific SenSys CCD camera. At least 20 discs of each genotype were analyzed.

For immunofluorescent analysis, wing discs of wandering third instar larvae were dissected in PBS and fixed in 4% formaldehyde in PBS for 1 hour at RT. The wing discs were washed in 0.3% Triton X-100 in PBS (PBTx), treated with 100 μg/ml RNase A in PBTx for 30 minutes at 37°C, washed in PBTx, and blocked in 5% normal goat serum for 30 minutes at RT. Mouse anti-ELAV mAb 7D and mouse anti-HuD mAb 16A11 (a gift of H. Furneaux) were used as primary antibodies at dilutions of 1:100 and 1:250, respectively, in PBTx. A fluorescein-conjugated goat anti-mouse immunoglobulin antibody (Jackson ImmunoResearch Laboratories) was used as secondary antibody at a dilution of 1:100 in PBTx. Primary and secondary antibody incubations were carried out overnight at 4°C. Nuclear staining was achieved by mounting the tissues with 70% glycerol in PBS containing 1 μg/ml propidium iodide. Confocal images were collected on a Bio-Rad MRC-600 confocal microscope.

In situ hybridization

A digoxigenin-labeled antisense RNA probe was prepared by in vitro transcription using digoxigenin-11-UTP (Boehringer Mannheim) and T3 RNA polymerase (Boehringer Mannheim). A *GFP* cDNA cloned into *pBluescript* plasmid (Stratagene) was used as a template. The probe size was decreased as described (Lehmann and Tautz, 1994).

Anterior halves of wandering third instar larvae were dissected and inverted in PBS, then fixed in 4% formaldehyde in PBS for 25 min at RT. The fixed larval heads were washed in PBS containing 0.1% Tween 20 (PBTw), treated with 8 μg/ml proteinase K in PBTw for 10 min at RT, washed in PBTw, and fixed in 4% formaldehyde in PBTw for 25 min at RT. Hybridization of the *GFP* RNA probe was performed as described (Lehmann and Tautz, 1994). Hybridization signals were detected with an anti-digoxigenin Fab fragments conjugated to alkaline phosphatase (Boehringer Mannheim) using 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim) as substrates. The stained wing discs were dissected and mounted with 70% glycerol in PBS. Photographs were taken using Zeiss Axioskop microscope and Zeiss MC80 camera loaded with Kodak Elite Chrome 160T film.

Results

Expression of reporter transgene constructs in the third instar larvae

All reporter genes depicted in Fig. 1 were driven by *Ubi-p63E* promoter and expressed GFP. In these transgenes, *U* represents *Ubi-p63E* promoter, *n* and *g* represent the regulated *nrg* intron *n*ASI and a generic intron GI, respectively; *G* represents *GFP*, and *A* and *H* represent the *Act5C* and *Hsp70Ab* 3′UTRs, respectively. *Ubi-p63E* promoter was chosen because it drives expression in a tissue-general manner (Lee et al., 1988). It contains the 5′UTR of the *Ubi-p63E* gene, including an intron 5′ to the beginning of the ORF. Because the constitutively spliced intron is 5′ to the ORF and is common to all transgenes, it is not depicted in Fig. 1. In transgenes containing introns within the ORF, the regulated intron (*n*ASI) or the generic intron (GI) are inserted within the ORF, such that splicing of the introns is necessary for GFP production. Thus, neural splicing is necessary for transcripts containing the *n* intron, and constitutive splicing is necessary for transcripts containing the *g* intron. The regulated intron *n*ASI was chosen as it contains all the sequences necessary for ELAV-mediated regulation (Lisbin et al., 2001). The generic intron GI was chosen as a control, since it is constitutively spliced in all tissues (Mottes and Iverson, 1995). GI has *Drosophila* consensus 3′ and 5′ splice sites, branch point sequence, and a 30 nucleotide polypyrimidine tract located between the branch point sequence and the 3′ splice site (Mottes and Iverson, 1995).

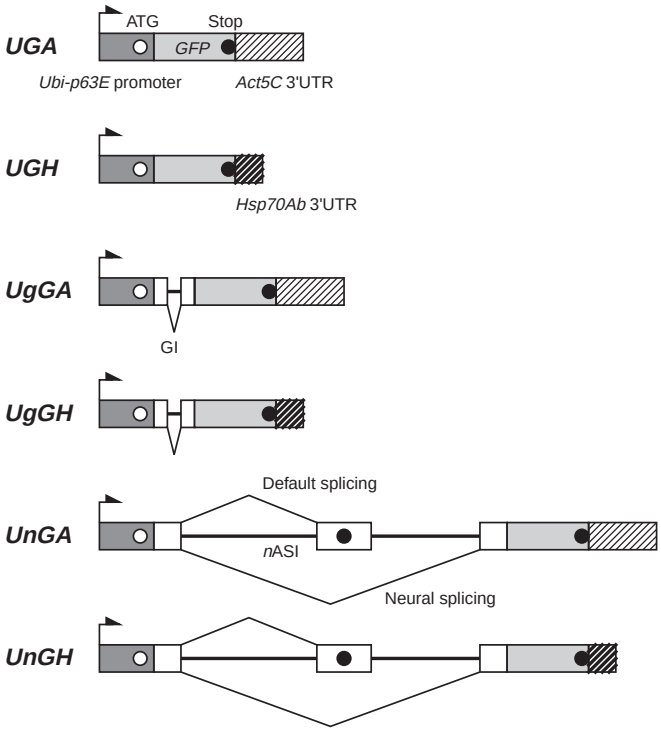

Fig. 1. Structure of GFP reporter transgenes. Boxes represent exons, and solid bars represent introns. Unfilled and filled circles represent translation start and stop codons, respectively. Arrows show transcription start sites. All the transgenes are driven by *Ubi-p63E* promoter and have *Ubi-p63E* 5′UTR with an intron (intron not shown) which is 5′ to the open reading frame (ORF) starting at ATG. The ORF encodes a Myc-tag fusion GFP. In *UGA* and *UGH*, the ORF is not interrupted by an intron, whereas in *UgGA* and *UgGH*, a generic intron GI (Mottes and Iverson, 1995) is inserted within the ORF. In *UnGA* and *UnGH*, an alternatively spliced intron from *nrg* (*n*ASI) is inserted at the same position as GI, necessitating neuron-specific splicing of the intron for GFP expression. *UGA*, *UgGA*, and *UnGA* have *Act5C* 3′UTR, while *UGH*, *UgGH*, and *UnGH* have *Hsp70Ab* 3′UTR. This figure is not drawn to scale.

Reporter genes *UGA*, *UnGA* and *UgGA* have *Act5C* 3′UTR, and *UGH*, *UnGH* and *UgGH* have *Hsp70Ab* 3′UTR. We chose *Hsp70Ab* trailer because it potentially has an ARE-like sequence structure. It is AU-rich (76% AU) and it has four AUUUA motifs (Fig. 2A). Moreover, it is known that the transcripts of *Drosophila Hsp70* genes are highly unstable at normal temperature, and at least for one of the *Hsp70* genes, the signal for rapid degradation resides in its 3′UTR (Petersen and Lindquist, 1989). The *Act5C* trailer was chosen for comparison because it does not have ARE-like characteristics; it has a lower AU content (58%) and has a single AUUUA motif (Fig. 2B). Although no direct analysis on stability of *Act5C* trailer has been published, *Act5C* mRNA is likely to be stable compared with *Hsp70* mRNAs. In *Drosophila* oocytes *Act5C* transcripts are more stable than *Heat-shock-protein-83*, *nanos* and *string* transcripts, three transcripts that are known to be highly unstable (Bashirullah et al., 1999) (R. L. Cooperstock and H. D. Lipshitz, personal communication). Another factor in favor of *Hsp70Ab* and *Act5C* 3′UTR is that the endogenous *Hsp70Ab* and *Act5C* genes are indeed expressed in the wing imaginal disc cells; *Hsp70Ab* is expressed in response to heat shock and *Act5C* is expressed constitutively.

We analyzed the GFP expression from the two sets of reporter genes (*UGA* and *UgGA*, and *UGH* and *UgGH*) that are expected to express broadly, with an aim to compare effects of the insertion of a generic intron and the two different trailers. The GFP fluorescence was analyzed in third instar larval fillet preparations from which the autofluorescing gut and Malpighian tubules were removed. *UGA* and *UgGA* expressed GFP broadly in all larval tissues as expected from the *Ubi-p63E* promoter (Fig. 3A, data for UGA not shown). The expression from these transgenes was similar, showing that the generic intron is spliced efficiently in all tissues. However, in *UGH* and *UgGH* larvae, a weaker GFP signal was observed in most tissues, but a strong expression was seen in the brain and

A ***Hsp70Ab***

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UAAGGCCAAAGAGUCUAAUUUUUGUUCAUCAAUGGGUUAUAACAUAUGGGUUAUAUUAUA   60
AGUUUGUUUUAAGUUUUUGAGACUGAUAAGAAUGUUUCGAUCGAAUAUUCCAUAGAACAA  120
CAAUAGUAUUACCUAAUUACCAAGUCUUAAUUUAGCAAAAAUGUUAUUGCUUAUAGAAAA  180
AAUAAAUUAUUUUAUUUGAAAUUUAAAGUCAACUUGUCAUUUAA                  223
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B ***Act5C***

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CAUGAAGAUCAAGAUCAUUGCCCCGCCAGAGCGCAAGUACUCUGUCUGGAUCGGUGGCUC   60
CAUCCUGGCUUCGCUGUCCACCUUCCAGCAGAUGUGGAUCUCCAAGCAGGAGUACGACGA  120
GUCCGGCCCCUCCAUUGUGCACCGCAAGUGCUUCUAAGAAGGAUCGCUUGUCUGGGCAAG  180
AGGAUCAGGAUCGGGAUGGUCUUGAUUCUGCUGGAGGAGGAGGAGGAGAAGUCGAGGAAG  240
CAGCAGCGAAAGUGCAAGUGCGAGUGGUGGAAGUUUGGAGUGCAGCACAACAAAAUCAAC  300
AACAACACCAACUACAAGAUGAAAAGAGCGGAACCACCUGCACACCAUCAUCACUAUCAU  360
CAUCGUUUUGGGCGCAUGUUGUGUGGUUCCAGCGUAUUAAUAUAAUUAAUUUAUUCCACA  420
UGAGAUAUGAUAUGAUAUACUAUGUAUUUUUUGUUUUUUUUUUAUUUGUAAACCUUUAAU  480
AUAACAAGAACUACAAAAAAGUGAAAAUGAGCGAAAAUGCAUAUUCUGCCAUUCCACACA  540
CACACCAACAACACCCAACACACGCACACCCACAAGCUUACACACACACACAUUCGCGGC  600
AUGACAAGGACAUCAAGAUAAAGAAGAACUUAAAGAAGAUAUUUCCCAAAGCGCAAAAAG  660
AACACACACACAUUGCAAAACACAAACAACACACUAGCGUUUUGUACAAUUCGUCAGCAA  720
CCUUAUGUAUUAUUUUUUAAUUAUGAUGUAAUUAUAAACAAAGUGAAACAAAAAUAUGAA  780
AACAAAAAGGAAAAUCAAAUCUGUCUUCUCUU                               812
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Fig. 2. The 3′UTR sequences used in this study. (A) The *Hsp70Ab* 3′UTR sequence (*UGH*, *UgGH*, and *UnGH*) contains four AUUUA motifs that are often found in AREs. The AU content of the sequence is 76%. The sequence was obtained from the *pP{CaSpeR-hs}* sequence (Pirrotta, 1988). The 3′ end of the sequence is an expected approximate polyadenylation site at 220 bases downstream to the *Hsp70Ab* stop codon (Dellavalle et al., 1994). (B) The *Act5C* 3′UTR sequence (*UGA*, *UgGA*, and *UnGA*). The sequence has a single AUUUA motif and 58% AU content. The sequence was obtained from the *pP{CaSpeR-hs/act}* sequence (Pirrotta, 1988) and is terminated at an expected approximate polyadenylation site (655 bases downstream to the *Act5C* stop codon) (Bond and Davidson, 1986). The AUUUA motifs are boxed. The underlined sequences are protein coding in the original genes, but constitute 3′UTR in our constructs. All the transgenes have a common 77-base sequence between the *GFP* stop codon and the sequences shown here.

salivary glands, and a very strong signal was seen in male gonads (Fig. 3B, data for *UGH* not shown). Again, both *UGH* and *UgGH* expressed similarly. Although we analyzed all the four transgenes throughout these studies, we show results from only *UgGA* and *UgGH*, as *UGA* behaved similar to *UgGA* and *UGH* behaved similar to *UgGH* in all our experiments.

The differences between the expression of transgenes with the *Hsp70Ab* 3′UTR (*UGH*, *UgGH*) and the *Act5C* 3′UTR (*UGA*, *UgGA*) are noteworthy as the two sets of transgenes differ only in their 3′UTR. The non-uniform distribution of GFP signal in the *UgGH* larvae could be due to differentially higher levels of transcription, stabilization, degradation, and/or translatability of the mRNA selectively in certain tissues. Since *UgGA* does not show a similar differential expression pattern, the possibility of higher levels of transcription is unlikely. These observations are consistent with the relative higher stability of mRNAs with *Act5C* 3′UTR.

Next, we analyzed the expression from transgenes requiring neural-specific splicing for GFP synthesis. In *UnGA* larvae, the nervous system cells showed a bright GFP signal, although a weak but discernable signal was seen in male gonads (Fig. 3C). Additionally, a very low basal level of expression that is barely above background, cannot be ruled out in most other tissues (Fig. 3C). In *UnGH* larvae, a very bright signal was also seen in male gonads in addition to the nervous tissue, and again, a just above background signal cannot be ruled out for many other tissues (Fig. 3D). The neural expression of *UnGA* and *UnGH* is expected because the *n*ASI splicing, which requires ELAV, should occur only in neurons; however, high levels of expression in *UnGH* male gonads is surprising as ELAV is not expressed in male gonads. We expect that the selective high signal in male gonads with *UgGH* and *UnGH* is due to the specific RNA processing molecules that probably exist within these cells. The very low level of general expression of *UgGH* and *UnGH* must be due to some baseline neural splicing occurring in all cells.

We also analyzed the expression of both sets of transgenes in the wing imaginal discs, as we planned to use that tissue for our ectopic expression studies. In the wing disc, uniform low level GFP expression was seen for *UgGA* and *UgGH* (Fig. 4I,L). GFP signal observed in the wing discs of *UnGA* or *UnGH* was extremely low, but discernable when compared with the control disc without transgenes (compare Fig. 4C,F with A).

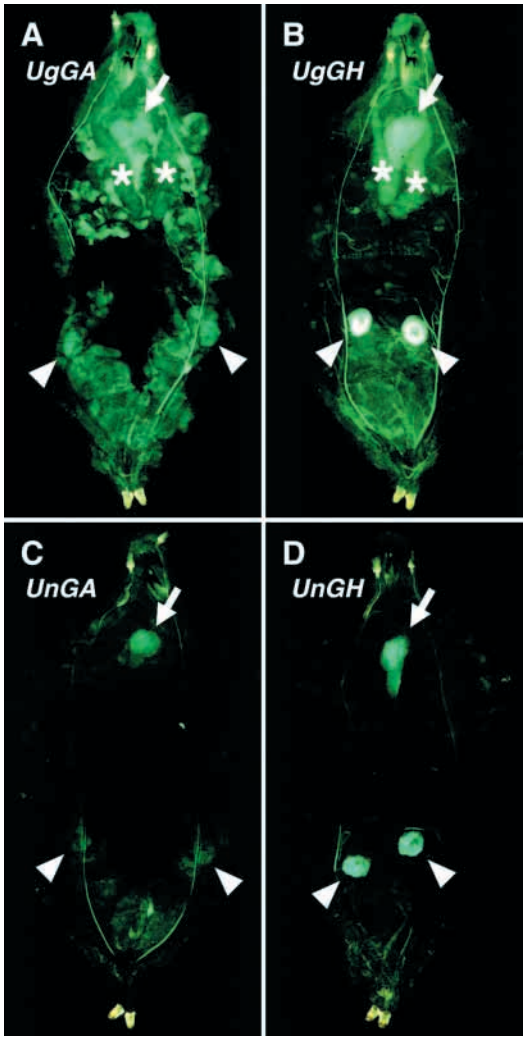

Fig. 3. Expression of GFP reporter transgenes in third instar larvae. (A) *UgGA* larva shows broad GFP signal in most tissues implying ubiquitous transcription from the *Ubi-p63E* promoter. (B) In the *UgGH* larva, the GFP signal is weaker in most tissues compared with the *UgGA* larva. However, strong expression is observed in the CNS and salivary glands, and a very strong expression is seen in the male gonads. (C) In the *UnGA* larva, GFP signal is primarily in the CNS. However, a very low level of expression is also found in other tissues including the male gonads. (D) In the *UnGH* larva, GFP signal is similar to *UnGA*, but strong signal is seen also in the male gonads. Arrow, CNS; asterisk, salivary gland; arrowhead, male gonad.

In contrast to ELAV, ectopic HuD expression does not effect neural-specific intron splicing in wing disc cells

To determine functional similarities and differences between ELAV and HuD, we studied their effects on the GFP reporter transgenes described above. To express ELAV and HuD in wing imaginal disc, we chose *dpp-GAL4* driver, as it expresses in wing discs in a stereotypic pattern (Staehling-Hampton et al., 1994). The expression pattern of *dpp-GAL4* visualized using *UAS-GFP* in wing disc cells shows a wide band of cells that runs in the middle along the dorso-ventral axis (Fig. 4B).

We examined wing discs ectopically expressing HuD and ELAV as described in Materials and Methods. Previous studies have shown that ELAV is sufficient in the wing disc cells for neural splicing of *nrg*, *ewg* and *arm*, its three identified targets (Koushika et al., 2000); however, whether ectopically-expressed HuD will also influence neural splicing of the *n*ASI was not known. As expected, the control ELAV-expressing wing discs show GFP signal along the GAL4-expressing band of cells from both *UnGA* and *UnGH* transgenes (Fig. 4E,H). In contrast, in HuD-expressing wing discs, the signal was uniformly low with either *UnGA* or *UnGH* (Fig. 4D,G) and comparable with controls when there was no HuD ectopic expression (Fig. 4C,F). Thus, ectopic expression of HuD does not induce neural specific splicing.

Previously, using a different GAL4 driver we have demonstrated that ectopically-expressed ELAV was sufficient to induce the neural protein isoform of endogenously expressed genes *nrg* and *ewg* in wing disc cells (Koushika et al., 2000).

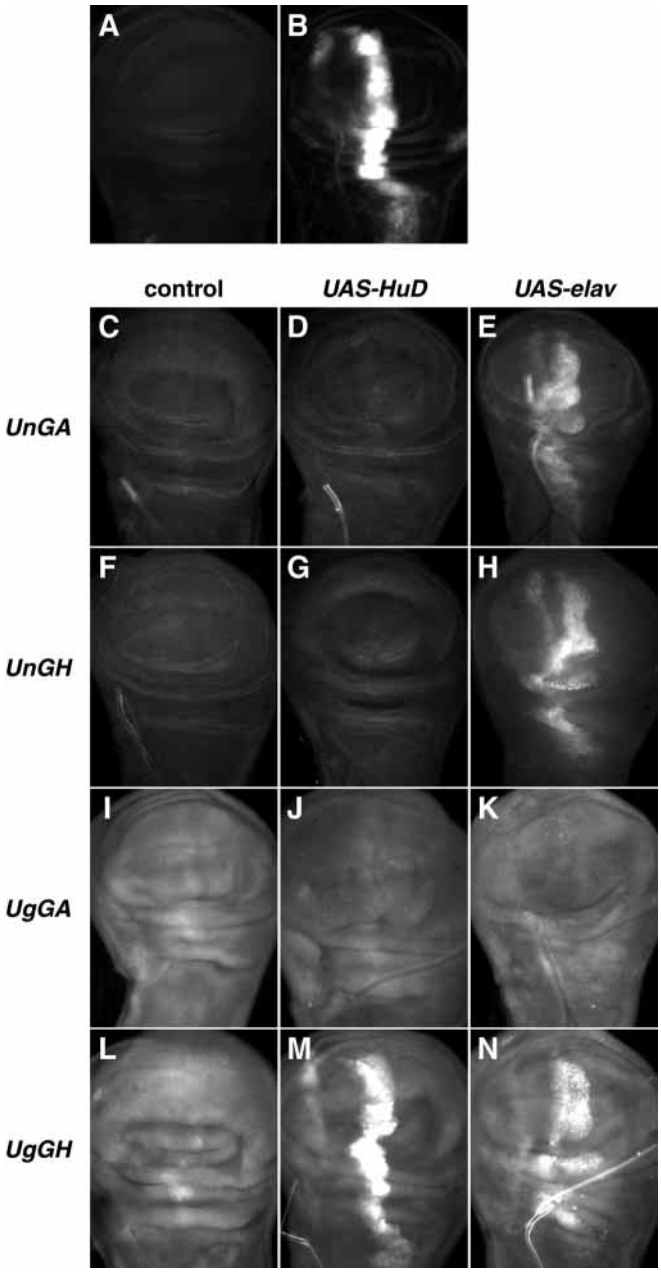

Fig. 4. Effects of HuD and ELAV ectopic expression on GFP reporter expression in the third instar larval wing disc. The ectopic expression is achieved by expressing either *UAS-HuD* or *UAS-elav* using *dpp-GAL4* as a driver. (A) Wild-type (Canton-S) wing disc shows background level of fluorescence. (B) The expression pattern of *dpp-GAL4* driver in the wing disc is visualized using *UAS-GFP*. (C-N) Effect of ectopic HuD (D,G,J,M) and ELAV (E,H,K,N) are examined for *UnGA* (D,E), *UnGH* (G,H), *UgGA* (J,K) and *UgGH* (M,N) expression. Basal GFP signal of *UnGA* (C), *UnGH* (F), *UgGA* (I) and *UgGH* (L) is shown as control. Ectopically expressed HuD has no discernable effect on either *UnGA* (D) or *UnGH* (G) expression, while ectopic ELAV expression leads to strong increase in GFP signal along *dpp-GAL4* expression pattern for both reporters (E,H). On *UgGA* expression, neither HuD nor ELAV have any obvious effect (J,K). In contrast, ectopic expression of either HuD or ELAV strongly enhances *UgGH* expression (M,N). For the genotype of each wing disc, see Materials and Methods.

In that system, ectopically-expressed HuD was also unable to generate neural protein isoforms, Nrg_{180} or 116-kDa EWG (data not shown). Thus, HuD fails to induce neural isoforms, in *Drosophila* wing disc cells, of either the endogenous genes, *nrg* or *ewg*, or the GFP reporter genes in which the splicing of *n*ASI is essential for reporter expression.

Both ELAV and HuD enhance GFP signal from transgenes with *Hsp70Ab* 3′UTR

Next we asked whether the ectopic expression of HuD and/or ELAV influences the level of GFP from the intron-less transgenes, or those with generic intron. This could happen as a consequence of stabilization of the transcript by HuD and/or ELAV. Both *UgGA* and *UgGH* cause relatively uniform expression in the wing disc cells (Fig. 4I,L). Wing discs ectopically expressing HuD or ELAV were examined. In *UgGA* wing discs, ectopic expression of HuD or ELAV still resulted in a uniform GFP expression (Fig. 4J,K). In contrast, in *UgGH* wing discs, ectopic expression of either HuD or ELAV resulted in strong enhancement of the GFP signal along the *dpp-GAL4* expression band (Fig. 4M,N). The enhanced signal was somewhat stronger with HuD than with ELAV. Since *UgGA* and *UgGH* transgenes differ only in the 3′ trailer, the enhancement of GFP signal specifically seen in *UgGH* is likely to be due to its *Hsp70Ab* trailer. The enhanced GFP signal could result from increased stability or translatability of the transgene generated message. Given that HuD has been shown to stabilize transcripts, we suggest that, in the wing discs, both HuD and ELAV can stabilize mRNAs with the *Hsp70Ab* trailer, perhaps through its ARE-like sequence.

In situ RNA analysis of transgenes *UgGA* and *UgGH* with ectopically expressed ELAV and HuD

To determine if the enhanced GFP signal in ELAV- or HuD-expressing wing discs is accompanied by increased transcript level, in situ hybridization was performed as described in Materials and Methods. GFP transcript of *UgGH* was compared with *UgGA* in wing imaginal discs that also ectopically expressed ELAV or HuD. As shown in Fig. 5, the expression of HuD and ELAV resulted in a higher transcript signal in the band of GAL4-expressing cells in *UgGH* wing discs (Fig. 5C,D), but not in *UgGA* wing discs (Fig. 5A,B). This result is consistent with the notion that ELAV and HuD stabilize mRNAs with *Hsp70Ab* 3′UTR. However, selective increased transcription from the *UgGH* transgene somehow facilitated by the *Hsp70Ab* 3′UTR only when ELAV or HuD are expressed remains a possibility.

Localization of ectopically expressed ELAV and HuD in wing disc cells

We were interested to see whether ectopically-expressed ELAV in the wing disc cells is localized mainly in nuclei, similar to endogenous or overexpressed localization in neurons, or whether it showed perturbed localization. To analyze the localization of ectopic ELAV and HuD, wing discs expressing ELAV or HuD were immunostained using anti-ELAV or anti-

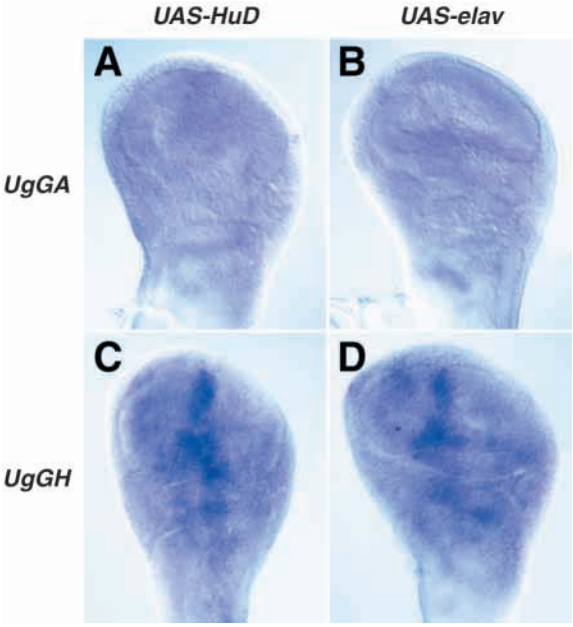

Fig. 5. Effect of HuD and ELAV ectopic expression on *UgGA* and *UgGH* reporter expression. *UgGA* and *UgGH* expression was examined at the RNA level by in situ hybridization in the third instar larval wing discs. No apparent change in *UgGA* mRNA level is observed when either *UAS-HuD* (A) or *UAS-elav* (B) are ectopically expressed using *dpp-GAL4*. However, in *UgGH*-expressing discs, the mRNA level is increased along the *dpp-GAL4* expression pattern by ectopic expression of either *UAS-HuD* (C) or *UAS-elav* (D).

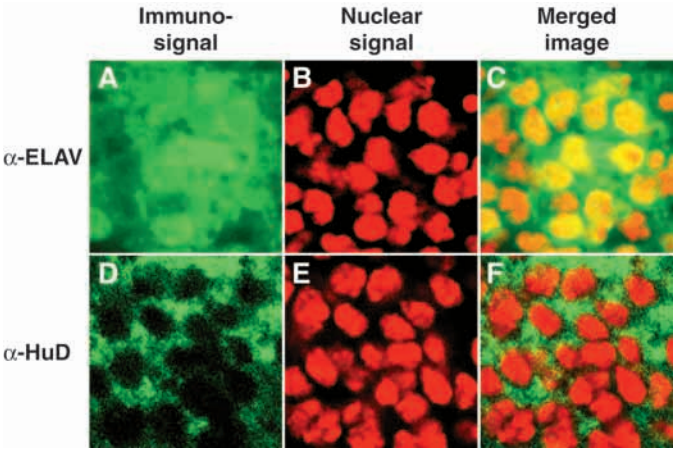

Fig. 6. Localization of ectopic HuD and ELAV in the wing disc cells. Third instar larval wing disc cells ectopically expressing HuD or ELAV under the control of *dpp-GAL4* driver were dissected and immunoprocessed with anti-HuD and anti-ELAV antibodies. Propidium iodide was used to visualize nuclei. Confocal images were collected for wing pouch cells. Immuno-signal in green (A,D); propidium iodide signal in red (B,E); merged images (C,F). ELAV shows both nuclear and cytoplasmic localization (A,B,C). In contrast, HuD shows mainly cytoplasmic localization (D,E,F). For details see Materials and Methods.

HuD monoclonal antibodies. The samples were analyzed by confocal microscopy after propidium iodide staining to visualize the nuclei. ELAV expressed in the wing disc cells was localized to both the cytoplasm and the nucleus, with a robust signal in the cytoplasm (Fig. 6A-C). This is a clear departure from the mainly nuclear localization of endogenous ELAV in neurons or even overexpressed ELAV in neurons. HuD in wing disc cells was localized predominantly to the cytoplasm (Fig. 6D-F) similar to what is seen in the vertebrate cell cultures (Wakamatsu and Weston, 1997) and in *Drosophila* neurons (Yannoni and White, 1999). The difference in localization of ectopically expressed ELAV in non-neural cells could potentially explain its role in stabilizing the *Hsp70Ab* 3′UTR-containing RNAs. Conversely, the inability of HuD to splice the *n*ASI, could be largely due to the cytoplasmic localization of HuD.

Discussion

Drosophila ELAV and human HuD are two closely related proteins of the ELAV/Hu-family of RNA-binding proteins. In spite of their striking structural similarity, they appear to perform distinct functions in RNA regulation. In these studies we address this dichotomy by assaying their function in wing imaginal disc cells of *Drosophila* larvae, which lack endogenous expression of either ELAV or HuD. Collectively, the experiments reported here show that in wing disc cells both ELAV and HuD can enhance expression of transgenes with the *Hsp70Ab* 3′UTR, but not of transgenes with *Actin5C* 3′UTR. Additionally, they show that only ELAV can induce neural-specific splicing. Thus, HuD and ELAV can be similar in one functional aspect, yet be distinct in another aspect.

Hu proteins have been shown to bind ARE sequences through RRM domains (Chung et al., 1996; Levine et al., 1993). The structural similarities between the RRM domains of ELAV and HuD predict that they ought to share RNA recognition specificity, as the residues that contact RNA are conserved between the first two RRM domains with the exception of a single conservative substitution (Wang and Tanaka Hall, 2001). Furthermore, the RRM3 domain of HuD, and that of its closely related *Drosophila* homologue RBP9, can fully functionally substitute the RRM3 domain of ELAV using viability rescue of a null allele as a criterion (Lisbin et al., 2000). Thus, the RRM domains in the two proteins could be expected to behave similarly. However, functional tests on chimeric proteins with RRM1 and RRM1RRM2 from RBP9 yielded rescue values of about 6% for RRM1 substitution, while no rescue was obtained with a RRM1RRM2 substitution, suggesting that other amino acids in addition to RNA contact residues are required for function (Lisbin et al., 2000), as these were precise RRM substitutions in the context of ELAV protein.

Ectopically expressed ELAV and HuD enhanced GFP signal from *UGH* or *UgGH* transgenes, but not *UGA* or *UgGA* transgenes. The increase in GFP signal was accompanied by an enhancement of transcript level. A straightforward explanation is that *Hsp70Ab* 3′UTR is acting as a HuD-responsive element and that HuD is acting true to its documented function in the mammalian cells. An alternative possibility that there is selective increased transcription from the transgenes with *Hsp70Ab* 3′UTR when ELAV or HuD are expressed is unlikely but cannot be formally ruled out. Furthermore, the increase in transcript level suggests that increased translatability of the mRNA is not the likely cause of enhancement of GFP signal.

That ELAV mimics HuD in the wing disc cells raises an intriguing question. Does ELAV also stabilize certain transcripts in neural cells? Strong GFP expression in the central nervous system observed in *UGH* and *UgGH* larvae (Fig. 3B, data for *UGH* not shown) could be explained by endogenous ELAV which stabilizes the *GFP* transcript. We tried to address this issue by overexpressing ELAV in the nervous system and assessing the GFP signal from the *UGH* transgene with several GAL4 drivers (data not shown). No enhancement was observed. We believe that in *Drosophila* neurons *UGH* signal is not further enhanced by overexpression of ELAV, with a caveat that a slight enhancement would not have been necessarily discerned in this assay.

Several arguments led us to speculate that *Hsp70Ab* 3′UTR behaves as an ARE in this assay. First, both HuD and ELAV have been shown to interact with A/U-rich sequences (Chung et al., 1996; Lisbin et al., 2001; Park et al., 2000), and Hu proteins have been shown to stabilize mRNAs containing AREs (reviewed by Brennan and Steitz, 2001; Keene, 1999). Second, *Hsp70Ab* 3′UTR has potential ARE-like composition and it is unstable at non-heatshock temperatures. If *Hsp70Ab* 3′UTR is indeed acting as an ARE-like element, it suggests that *Drosophila* cells possess the molecular components necessary for the HuD-mediated mechanism for ARE-related turnover and could be used to study this process in vivo. This is consistent with the idea that the mechanism of ARE-mediated mRNA turnover is conserved from yeast to humans (Vasudevan and Peltz, 2001).

Our studies have focussed on the role of ELAV in regulated neural-specific splicing in *Drosophila* neurons. We first identified *nrg* as an ELAV-regulated gene by demonstrating that ELAV is essential in neurons and is sufficient in non-neural wing disc cells for the formation of a neural-specific isoform of Nrg (Koushika et al., 1996). The interpretation of this finding as ELAV being required for neural-specific spliceform generation was subject to the criticism that it could also be explained by selective stabilization of the neural-specific transcript or increased stability of protein. Subsequent studies on *nrg* splicing and on the splicing of *ewg*, a second downstream target of ELAV, have reinforced the role of ELAV in splicing regulation. These follow-up studies demonstrated: (1) a requirement of *ewg* introns for ELAV-mediated regulation (Koushika et al., 2000); (2) similar tissue-specificity for expression of reporter gene constructs that report on ELAV-mediated splicing with several distinct 3′UTRs (Lisbin et al., 2001); and (3) ELAV-binding sites within the *nrg* regulated intron, *n*ASI (Lisbin et al., 2001). The current study provides further convincing evidence that the generation of neuron-specific isoforms is at the level of splicing. First, HuD expression leads to the stabilization of *UgGH* transcripts, yet fails to induce *UnGH* expression. Second, ELAV expression does not affect the level of *UgGA* expression, but still induces *UnGA* expression in a non-neural tissue. Together, these results show that the basal level production of the neural-specific spliceform in non-neural wing disc cells is not sufficient to induce expression through selective mRNA stabilization, and that HuD appears to be lacking the ability to mediate neural splicing in these cells. These findings underscore the functional divergence of these two proteins in these cells.

In this study, stabilization function correlates with cytoplasmic localization and splicing correlates with nuclear localization of ELAV and HuD. Both ELAV and HuD appear to be comparably expressed in the cell, as assessed by their effect on *UGH* and *UgGH* expression, and both are expressed in the cytoplasm, but only ELAV shows significant localization in the nucleus. Thus, the inability of HuD to mediate splicing could be explained by its mainly non-nuclear localization, allowing the possibility that if localized to the nucleus it could mediate splicing. Consistent with this finding are our previous results with certain ELAV mutant proteins that localize mainly to the cytoplasm in neurons. These proteins are unable to provide ELAV's vital function, but their function is partially restored when forced into the nucleus by tagging them with an exogenous nuclear localization signal (Yannoni and White, 1999). We envision that inherent differences between ELAV and HuD result in differential interactions with factors that they encounter in the wing disc cells. These different interactions contribute to the differences in function and localization. For example, recently several proteins that associate with HuR have been identified; at least two of these have been implicated in nuclear export of HuR (Brennan et al., 2000; Gallouzi et al., 2001).

Regulation of alternative RNA splicing and mRNA stability are key processes that influence qualitative and quantitative aspects of proteins produced from a gene. Individual ELAV/Hu-family proteins have evolved to control these and other regulatory steps. This study signifies the importance of cellular context and the proteins that ELAV/Hu-family proteins associate with to the specificity of its function and suggests that evolution has tailored ELAV-family proteins along with the cellular context for specific post-transcriptional processes.

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