Determinants of ELAV gene-specific regulation

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Abstract
How RNA-binding proteins recognize their complement of targets in a complex cellular environment remains poorly understood. Sequence degeneracy and redundancy of short motifs at genomic scales have mostly eluded predictions of specific target genes for gene-specific ELAV (embryonic lethal abnormal visual system)/Hu proteins that bind ubiquitous AU-rich motifs. Using the genetic tools of Drosophila, we have analysed binding properties of ELAV in vitro and ELAV-dependent regulation of its major target ewg (erect wing) in neurons. These studies reveal that an integral part of ELAV gene-specific regulation involves combinatorial binding to variably spaced short U-rich motifs on an extensive binding site.

Introduction
RNA-binding proteins comprise a major class in the proteome of eukaryotes. Important functions for RNA-binding proteins are indicated in the brain, since alternative splicing is particularly prominent in this organ [1]. A prominent family of prototype RNA-binding proteins expressed in neurons are comprised by ELAV (Embryonic Lethal Abnormal Visual system)/Hu proteins. Originally identified in Drosophila, the ELAV family in this organism contains two additional members expressed in neurons, Fne (Found in neurons) and RBP9 (RNA-binding protein 9). RBP9 is also expressed in gonads [2]. The human ELAV/Hu family consists of four members, HuB–HuD and HuR. All are expressed in neurons, whereas HuB is present in gonads and HuR is ubiquitous [3].

ELAV/Hu proteins contain three highly conserved RRMs (RNA-recognition motifs), whereby RRM3 is separated from closely joined RRM1 and RRM2 by a less-conserved hinge region (Figure 1). Compared with human Hu proteins, ELAV and RBP9 contain N-terminal AQ- (Ala-Gln) and NQ- (Asn-Gln) rich extensions respectively. ELAV and Fne also have additions of a few amino acids that separate RNP (ribonucleoprotein) 1 and RNP2 in RRM1. RBP9 has an alternatively spliced exon at a similar position in RRM2. HuB–HuD proteins are alternatively spliced in the hinge region, whereas HuR has alternative promoters that can add an N-terminal extension. In the 12 currently sequenced Drosophila species [4], the RNA-binding part of the ELAV family of proteins is largely identical with very few amino acid changes [2] (Figure 2).

ELAV/Hu family proteins have demonstrated roles at many steps of post-transcriptional regulation of gene expression ranging from nuclear events, such as alternative splicing and alternative polyadenylation, to cytoplasmic events such as the regulation of stability, localization and translation. Within this broad spectrum of regulating alternative mRNA processing, however, ELAV/Hu protein-dependent regulation is gene-specific and has been associated with specific biological processes in neurons such as neurite extension, guidance of axons and regulation of synaptic growth [3,5,6]. Similarly, ubiquitously expressed HuR has also been associated with specific functions in developing embryos and in response to hypoxia [3,7].

Despite the gene-specific roles of ELAV/Hu proteins, analysis of binding sites has not revealed sequence motifs that would allow for the prediction of binding to targets at a genomic scale. Major insights into ELAV/Hu protein target specificity in neurons comes from transgenic studies of ELAV-regulated splicing of its major target ewg (erect wing) [8,9].

Regulation of ELAV target genes in Drosophila
Currently, there are four target genes of ELAV known in Drosophila: ewg, a transcriptional regulator homologous with human NRF-1 (nuclear respiratory factor 1); nrg (neuroglian), a cell adhesion molecule homologous with human NCAM (neural cell adhesion molecule) L1; arm (armadillo), a cell adhesion molecule and transcriptional regulator of Wingless signalling, and ELAV itself as it auto-regulates [10]. In addition, ELAV has been shown to stabilize transcripts via 3′-UTR (untranslated region) sequences in ectopic expression experiments [11]. In ewg, nrg and arm, ELAV is both necessary in photoreceptor neurons and sufficient in non-neuronal wing imaginal discs for neuron-specific alternative splicing of these genes [12,13].

The best-studied targets of ELAV are nrg and ewg, where ELAV regulates the use of a terminal exon. In nrg, ELAV regulates the use of an alternative terminal exon that is distal to the canonical terminal exon. In this regulated intron of nrg, ELAV was shown to bind multiple sequences in vitro that are also important for ELAV-dependent regulation in vivo [14]. In ewg, neuronal splicing of the last intron is regulated by ELAV-mediated inhibition of a 3′-end-processing site in

Key words: alternative polyadenylation, alternative splicing, embryonic lethal abnormal visual system (ELAV), nervous system development.

Abbreviations used: arm, armadillo; ELAV, embryonic lethal abnormal visual system; EMSA, electrophoretic mobility-shift assay; ewg, erect wing; Fne, Found in neurons; nrg, neuroglian; RBP9, RNA-binding protein 9; RNP, ribonucleoprotein; RRM, RNA-recognition motif.

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**Figure 1 | Structure of ELAV/Hu proteins**
The core structure of ELAV/Hu proteins consisting of a short N-terminal extension, three RRMs and a hinge region are indicated in blue. Variable regions between Hu and Drosophila ELAV proteins are indicated in orange. Positions of RNP1 and RNP2 consensus sequences are indicated by black boxes at the bottom.

**Figure 2 | Evolutionary conservation of Fne and RBP9 in Drosophila spp.**
Alignment of RBP9 (A) and Fne (B) in the 12 Drosophila species indicates high evolutionary conservation. Identities are indicated in red, homologies are in orange, non-homologous changes are in green, insertions are in black and deletions are in white. Drosophila simulans is not included owing to irresolvable sequencing errors. D. mel, Drosophila melanogaster; D. sec, Drosophila sechellia; D. yak, Drosophila yakuba; D. ere, Drosophila erecta; D. ana, Drosophila ananassae; D. pse, Drosophila pseudoobscura; D. per, Drosophila persimilis; D. wil, Drosophila willistoni; D. moj, Drosophila mojavensis; D. vir, Drosophila virilis; D. gri, Drosophila grimshawi.

Given the low complexity of ELAV/Hu protein-binding sites, other scenarios than recognition of targets by mere diffusion could be imagined. For example, ELAV/Hu proteins could be recruited by the transcription machinery and deposited on nascent transcripts as U-rich sequences are transcribed. A number of reporter gene studies in cell culture and Drosophila, which are, however, overexpression studies, currently argue against this possibility [3,15].

### Combinatorial interactions among ELAV proteins
On the basis of the absence of any extensive sequence motifs that could explain specific recognition of targets for most RNA-binding proteins at genomic scales, it has been postulated that combinatorial interaction of RNA-binding proteins is key to gene-specific recognition [1]. Accordingly, ELAV/Hu proteins have been demonstrated to interact with each other in yeast two-hybrid assays and in in vitro X-linking experiments as well as in vitro [8,15,16]. In the absence of RNA, recombinant ELAV forms tetramers as shown by gel-filtration and X-linking experiments. Furthermore, HuD interacts with HuB and HuC, and itself in co-immunoprecipitation assays. In contrast, HuR is present as a monomer, suggesting that multimerization of ELAV/Hu proteins without binding to RNA might not be a conserved feature [16,17]. In the presence of RNA, multimerization is strongly favoured for both ELAV and Hu proteins, both in vitro and in vivo [3,8,16]. Exceptions, however, have been observed for Hu proteins in in vitro binding assays to short target RNAs or for the Xenopus HuR homologue ElrA [17–19]. In HuB and HuC, all RRMs contribute to the interaction properties in yeast to hybrid assays while in Drosophila ELAV interactions are mediated by the third RRM [15,16].

### RNA-binding properties of ELAV
An inherent property of ELAV to bind RNA in vitro is to form a defined and saturable complex as demonstrated by gel-filtration and EMSA (electrophoretic mobility-shift assay) experiments [8]. In these experiments, ELAV either binds to RNA and forms a complex or no binding is observed, e.g. with short RNAs. Stoichiometric titration EMSAs suggest formation of a dodecameric complex per RNA molecule. Multimerization upon binding to RNA has also been demonstrated for Hu proteins, but whether or not defined complexes form has not been elucidated [3]. Analysis of the ewg ELAV-binding site in vitro revealed the absence of a defined binding element. Rather, length and polyU content are important for high-affinity binding and complex formation. An extended binding site for ELAV in ewg is further demonstrated with segmentally labelled RNAs in UV cross-linking assays in nuclear extracts [9]. In the ewg ELAV-binding site, a number of U-rich motifs together are important for binding in vitro and for ewg splicing regulation in neurons. Mutations in individual
U-rich motifs have little effect. Within the ewg ELAV-binding site, the 3′ part has a higher affinity and serves to initiate complex formation from 3′ to 5′. Formation of an ELAV complex on an extended binding site probably explains that insertion of spacer sequences in reporter transgenes or the presence of small deletions in the ewg ELAV-binding site in closely related species have little effect on ELAV-mediated ewg intron 6 splicing regulation. These experiments, together with ELAV’s binding of single-stranded RNA, also exclude the possibility of any structural element being important for target recognition, which is supported further by the absence of phylogenetic conservation of any secondary structure in the ewg ELAV-binding site in closely related species from the suzukii and takahashii subgroups [8]. In conclusion, the ELAV-binding site in ewg is characterized by short and variably spaced polyU motifs spread over an extended binding site.

Future directions
An intriguing feature of ELAV/Hu proteins is the high conservation of the RRMs. Furthermore, the core of the ELAV proteins is basically identical in the 12 sequenced Drosophila species [2] (Figure 2). The degeneracy of the ELAV-binding site in ewg together with the fast evolution of intronic sequences suggests, however, that binding sites for ELAV/Hu proteins are not characterized by unique and extended sequence motifs that can be recognized with current prediction tools. More likely is a scenario whereby the composition of the sequence provides multiple binding modes to favour complex formation of ELAV/Hu proteins, or combinatorial binding of ELAV/Hu proteins with other RNA-binding proteins. Hence, characterization of the composition of ELAV/Hu RNP complexes in combination with a phylogenetic analysis of binding sites will be instrumental to reveal insights into the RNA binding codes for this family of RNA-binding proteins. Furthermore, the degeneracy of ELAV/Hu binding sites together with the high conservation of ELAV/Hu proteins also predicts that ELAV/Hu protein-mediated post-transcriptional gene regulation is likely to be evolutionarily more conserved than anticipated from sequence alignments.

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