Am. J. Hum. Genet. 61:273–278, 1997

INSIGHTS FROM MODEL SYSTEMS
Embryonic Lethal Abnormal Visual RNA-Binding Proteins Involved in Growth, Differentiation, and Posttranscriptional Gene Expression

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Cell growth and differentiation in mammalian tissues are regulated by tight control of gene expression at the transcriptional, posttranscriptional, and translational levels. Although transcription is the primary level of regulation of gene expression, it has become clear that several levels of posttranscriptional RNA processing play important roles in regulating the final outcome of protein production. Processing of eukaryotic pre-mRNA, including polyadenylation, capping, and splicing, as well as transport of RNAs, affect the availability of mature mRNA for translation. In addition, the localization, stability, and translatability of cytoplasmic mRNAs affect both quantitative and qualitative aspects of final gene expression.

Although many genes have been shown to influence organismal development through transcriptional regulation, relatively few have been implicated in regulation of cell growth or differentiation at posttranscriptional levels. As might be expected, RNA-protein interactions play key regulatory roles in postranscriptional gene expression. One gene whose product acts at the level of RNA processing was discovered in a genetic screen of the fruit fly *Drosophila melanogaster*. This gene, named *elav* (pronounced ella-vee) for the embryonic lethal abnormal visual phenotype, is essential for the development and maintenance of the nervous system (Campos et al. 1985; Robinow and White 1988). ELAV protein and its vertebrate homologues represent a subfamily of the RRM (RNA recognition motif) superfamily of RNA-binding proteins (reviewed in Kenan et al. 1991; Burd and Dreyfuss 1994). Genetic findings in the fly have been extended to the study of human ELAV proteins by application of molecular, biochemical, and combinatorial selection methods. Together, these approaches link ELAV proteins to posttranscriptional regulation of gene expression during growth and differentiation of many cell types.

Received May 9, 1997; accepted for publication June 4, 1997.

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This article represents the opinion of the authors and has not been peer reviewed.

0002-9297/97/6102-0004$02.00

Whereas *Drosophila* ELAV recently has been implicated in alternative splicing (Koushika et al. 1996), neuronal ELAV proteins from vertebrates may also act in the cytoplasm to modulate either the translation of specific mRNAs or their rate of turnover (Levine et al. 1993; Gao and Keene 1996; Jain et al. 1997; Myer et al. 1997). Unstable mRNA species encode a variety of proteins that regulate cell growth and differentiation. The synthesis of c-fos mRNA, for instance, represents an early step in the activation of the cell cycle in previously quiescent cells, and the duration of this step is limited by rapid degradation of c-fos mRNA (Schiavi et al. 1992, and references therein). A signal that confers rapid turnover of this and many other mRNA species includes the pentanucleotide, AUUUA, often present in multiple copies in the 3′ UTRs of unstable mRNAs (for review see Chen and Shyu 1995, and references therein). In some cases, this sequence is sufficient to make a normally long-lived mRNA, such as the β-globin mRNA, unstable (Shaw and Kamen 1986). The intrinsic instability of mRNAs encoding proteins that induce cell proliferation or differentiation represents an important regulatory mechanism and allows for precise temporal control of the expression of such proteins as c-fos, c-myc, the Id transcriptional regulator, or the glucose transporter, GLUT1. As described below, the ELAV proteins bind specifically to AU-rich sequence elements located in 3′ UTRs, raising the possibility that these proteins can alter the fate of bound mRNAs. Indeed, a widely expressed 32-kD protein implicated in mRNA stability and known to bind to AU-rich sequences in 3′ UTRs of c-myc and c-fos mRNAs (Vakalopoulou et al. 1991) was recently shown to be an ELAV protein (Myer et al. 1997).

ELAV Genes in Vertebrates and Invertebrates

Cloning of the *Drosophila elav* gene (Robinow et al. 1988), as well as other vertebrate ELAV homologues, demonstrated that they encode highly conserved proteins containing three RRMs, each of which encompasses ~90 amino acid residues, and represent the core structure of a functional RNA-binding domain (Chambers et al. 1988; Query et al. 1989; reviewed in Keene and Query 1991; Kenan et al. 1991; Burd and Dreyfuss

Table 1

***elav*-Like Genes in Vertebrates and Invertebrates**

Name	Homologue	Species	Tissue Distribution	Developmental Expression	Reference
ELAVL1[a]	*elrA*	*Xenopus*	Ubiquitous	All stages	Good (1995)
	HuR	Human			Ma et al. (1996)
	mHuA	Mouse			Okano and Darnell (1997)
	MelG	Mouse			Author's unpublished data
ELAVL2[a]	*elrB*	*Xenopus*	Brain, testes, ovaries	Up to gastrulation and in tadpole	Good (1995)
	Xel-1	*Xenopus*			Perron et al. (1995)
	Hel-N1	Human			Levine et al. (1993)
	mHuB	Mouse			Okano and Darnell (1997)
	MeI-N1	Mouse			Abe et al. (1996*b*)
	Rel-N1	Rat			King et al. (1994)
ELAVL3[a]	*elrC*	*Xenopus*	Nervous system	Late gastrula	Good (1995)
	HuC	Human			Szabo et al. (1991)
	PLE21	Human			Sakai et al. (1994)
	mHuC	Mouse			Abe et al. (1996*a*)
ELAVL4[a]	*elrD*	*Xenopus*	Nervous system	Late neurula	Good (1995)
	HuD	Human			Szabo et al. (1991)
	mHuD	Mouse			Okano and Darnell (1997)
elav		*Drosophila*	CNS and peripheral nervous system	Birth of neurons (all stages)	Robinow et al. (1988)
rbp9		*Drosophila*	CNS	Late 3d-instar larva	Kim and Baker (1993)
Cel-1		*Caenorhabditis elegans*	Nervous system	Larval and adult stages	Author's unpublished data

[a] Name endorsed by HUGO GDB Nomenclature.

1994). ELAV proteins also contain auxiliary regions—the N-terminus and the hinge region located between the second and the third RRM (Szabo et al. 1991; Levine et al. 1993; King et al. 1994), which presumably mediate interactions with other cell components (Gao and Keene 1996). The discovery of the neural-specific ELAV protein in *Drosophila,* as well as identification of its functional importance for the development and maintenance of the nervous system, was followed by an extensive search for *elav*-like RRM-containing genes that might participate in differentiation processes. Numerous cloning attempts led to the discovery of four different *elav*-like genes in vertebrates, another *elav* homologue in *Drosophila,* and one in *Caenorhabditis elegans* (table 1). Each of the four vertebrate *elav*-like genes shows tissue specificity and a unique pattern of developmental mRNA expression, as has been demonstrated in *Xenopus* and zebrafish (Good 1995; table 1).

Analysis of the three human neuronal *elav*-like genes—Hel-N1 (*elrB*), HuC (*elrC*), and HuD (*elrD*)—demonstrated that their pre-mRNAs can be processed by alternative splicing (see review by Cooper [1997], in this issue of the *Journal*), resulting in both further diversification of the gene products and altered tissue-specificity (Gao et al. 1994; King 1994; Sekido et al. 1994; Liu et al. 1995; Abe et al. 1996*a;* King and Dropcho 1996; Steller et al. 1996); for instance, the alternatively spliced form of Hel-N1, called "Hel-N2" (Gao et al. 1994), is most similar to HuR (Ma et al. 1996) and is expressed in various cultured cell lines including neuronal precursors but not in mature neurons (Gao and Keene 1996; authors' unpublished data). Since alternatively spliced hinge segments of the ELAV proteins are presumed to be protein-protein interaction sites, it is possible that all of the mammalian isoforms of ELAV proteins interact with the same set of RNA species but with different sets of cellular proteins. These interactions might convey ELAV messenger ribonucleoprotein (mRNP) complexes into different functional contexts.

The existence of four highly conserved *elav*-like genes and their multiple expressed isoforms in vertebrates is intriguing and, most likely, functionally important. However, spontaneous mutations in vertebrate *elav*-like genes have not been identified, and none has been generated experimentally. It will be interesting to learn both whether these genes are essential and to what extent the different genes and their isoforms overlap in function.

RNA Targeting of ELAV Proteins

Although developmental and genetic studies suggest the importance of ELAV genes in the differentiation and maintenance of the nervous system, a major insight into their function was revealed by study of their sequence-specific binding, by use of RNA-combinatorial selection methods (see accompanying sidebar). In these experiments, purified recombinant Hel-N1 and Hel-N2 proteins were used to select RNA molecules in vitro from

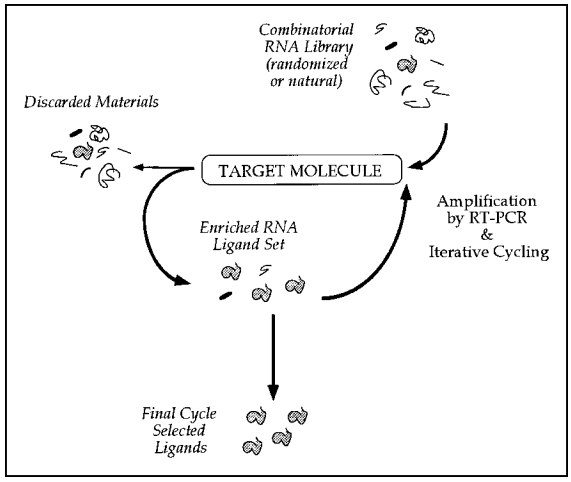
a heterogenous pool of synthetic RNA species (Levine et al. 1993; Gao et al. 1994; Keene 1996; Andrews and Keene, in press). In vitro–selected RNAs were amplified and subjected to several iterative rounds of reselection and reamplification until a clear "RNA recognition consensus sequence" was obtained (Tsai et al. 1991). Selected RNA molecules were purified, amplified by reverse transcription and PCR, cloned, and sequenced as described elsewhere (reviewed in Conrad et al. 1996, and accompanying articles). This approach revealed that both Hel-N1 and Hel-N2 proteins bind to RNA sequences containing short stretches of uridylate residues interspersed with other nucleotides (Levine et al. 1993; Gao et al. 1994). These sequences resembled the AU-rich sequences observed in the 3′ UTRs of c-myc, c-fos, and GM-CSF mRNAs, elsewhere shown to mediate mRNA degradation (Shaw and Kamen 1986). In subsequent studies, sequences selected by recombinant Hel-N1 from a pool of natural mRNAs derived from a brain polyA$^+$ library also contained short stretches of uridylates flanked by A, G, or C, which was consistent with the randomized RNA-selection experiments (Levine et al. 1993; Gao et al. 1994). The majority of these mRNAs identified in the cDNA databases encoded proteins with known roles in the regulation of cell growth. These combinatorial selection results prompted a series of in vitro binding experiments, which demonstrated unequivocally that Hel-N1 has high specificity for the 3′ UTRs of mRNAs containing these AU-rich elements (Levine et al. 1993; King et al. 1994). Similar experiments, performed with HuD, HuC, and HuR (Liu et al. 1995; Abe et al. 1996*a;* Chagnovich et al. 1996; Chung et al. 1996; Ma et al. 1996; Chung et al. 1997; Myer et al. 1997), demonstrated that all ELAV-like proteins share similar RNA-binding specificity. Biochemical experiments further support a role for mammalian neuronal ELAV proteins in the regulation of cytoplasmic mRNA metabolism. These proteins bind to a subset of poly(A)$^+$ mRNA in vivo, forming the mRNP complexes that associate with ribosomes during translation (Gao and Keene 1996; authors' unpublished data). To date, ELAV proteins represent the only example of RNA-binding proteins of unknown specificity for which combinatorial libraries have been used to elucidate their cellular RNA targets; but more are expected to emerge.

Cellular Dynamics of Mammalian ELAV Proteins

In *Drosophila,* the ELAV protein is apparently expressed only in nuclei (Robinow and White 1991; Kim and Baker 1993), consistent with a role in posttranscriptional nuclear events such as splicing and, possibly, RNA stability. Recent findings have implicated *Drosophila* ELAV in the generation of an alternatively spliced, nervous system–specific isoform of neuroglian protein

Evolution in the test tube

Crucial insights into the function of RNA-binding proteins came from the use of in vitro selection methods. Starting with a protein of interest, such as ELAV, and a vast number of combinations of synthetic RNA molecules, constituting a combinatorial "shape library," one can carry out in the test tube a process much like natural selection. RNA ligands whose structures fit the protein target molecule are selected through iterative cycles of binding and amplification; the result is the evolution of the most "fit," high-affinity target binders (Joyce 1992; Fitzwater and Polisky 1996). RNA "shape libraries" have been prepared with either a randomized sequence set or naturally occurring nucleic acids, as described by Gao et al. (1994). Selected molecules of this kind, called "aptamers" because they are "apt" to bind to the target, can also be derived by starting from combinatorial libraries consisting of DNA, peptides, or small organic compounds (reviewed in Gordon 1994; Janda 1994; Kenan et al. 1994).



Using various combinatorial libraries, one can select aptamers that will specifically inhibit metabolic pathways in cells or pathogens. This combinatorial approach has been termed "pharmacological genetics," because it allows one to probe gene structure and function by using aptamers to interfere with the function of a gene product, even one that has never been characterized. In both academic and commercial laboratories, combinatorial chemistry has begun to yield novel compounds, some with therapeutic promise. Large-scale plans are being developed to apply high-throughput screening of combinatorial libraries to derive inhibitors of gene products of unknown function as they appear in the genome databases (Kenan et al. 1994; Lander 1996).

(Koushika et al. 1996). However, ELAV proteins of vertebrates demonstrate both nuclear and cytoplasmic distribution (Barami et al. 1995; Gao and Keene 1996; authors' unpublished data), consistent with their involvement in posttranscriptional regulation of gene expression, including RNA stability or translatability (Jain et al. 1997; Myer et al. 1997). The cytoplasmic staining detected both in the cell body and in dendrites of cortical neurons and medulloblastoma tumor cells shows discrete granular RNP distribution (Gao and Keene 1996; authors' unpublished data). ELAV-containing mRNPs localize along microtubule tracks both in cell bodies and in the processes of cortical neurons. This association with the cytoskeleton was found to be essential for the association of ELAV mRNP complexes with ribosomes (authors' unpublished data), consistent with findings that mRNA transport, localization, and translation involve interactions with the cytoskeleton (for review, see St. Johnston 1995, and references therein). It is possible that association of mRNAs with ELAV proteins, as mediated through 3′ UTR binding, not only influences mRNA localization and translation but also affects their stability. The precise mechanisms that underlie these processes remain unknown, and it is unclear whether one of these processes is primary and whether it might account for the others. The well-documented examples addressing stability of histone mRNAs (Sive et al. 1984; Graves et al. 1987), β-tubulin mRNA (Pachter et al. 1987), c-myc mRNA (Linial et al. 1985), or c-fos mRNA (Schiavi et al. 1994) suggest that these mRNAs are degraded cotranslationally. Whatever the cause, it has become evident that the stability of many mRNAs and their active translation are coupled (for review, see Jacobson and Peltz 1996, and references therein). Therefore, ribosomes have been proposed to be one of the *trans*-acting factors that influence mRNA half-life, most likely in concert with other proteins. However, few other *trans*-acting factors that affect mRNA stability have been identified or functionally characterized. The presence of ELAV proteins in mRNA particles that associate with ribosomes and that are localized along microtubules suggests that these RNA-binding proteins influence and possibly couple the processes of mRNA localization, translation, and stability.

Effects of elrB/Hel-N1 on Gene Expression and Cell Differentiation

Direct effects of vertebrate neuronal ELAV proteins on mRNA metabolism have been demonstrated by use of cell-culture transfection assays. Ectopic expression of Hel-N1 protein in 3T3 L1 cells, which can be chemically induced to differentiate into adipocytes, showed an enhanced-differentiation phenotype, in parallel with a dramatic increase in the stability and translatability of the insulin-dependent glucose transporter (GLUT1) mRNA (Jain et al. 1997). Hel-N1 was found to bind to an AU-rich element present in the 3′ UTR of GLUT 1 mRNA and to recruit GLUT1 mRNA into active polysomes (Jain et al. 1997).

Hel-N1, like the *Drosophila* ELAV protein, appears to influence neuronal differentiation when ectopically expressed in cultured cells. For example, human embryonal teratocarcinoma (hNT2) cells differentiate into neurons in response to retinoic acid treatment, but, when these cells are transfected with Hel-N1 cDNA, the differentiated phenotype is enhanced, as measured by an increased number of terminally differentiated cells (authors' unpublished data). Although Hel-N1 was insufficient by itself, in the absence of retinoic acid treatment, to induce terminal differentiation, it was found to bind to the 3′ UTR of a differentiation-specific neurofilament M (NF-M) mRNA and to increase its translatability. Therefore, it is possible that binding of Hel-N1 to differentiation-specific mRNAs increases their expression, thereby affecting cellular programs that induce terminal differentiation.

Neuronal ELAV Proteins and Human Disease

Human neuronal ELAV proteins are expressed in tumors consisting of cells with neuroendocrine features such as small-cell lung cancer (SCLC). Interestingly, some patients with SCLC appear to develop an autoimmune response against neuronal antigens, including neuronal ELAV proteins (HuD, HuC, and Hel-N1). Anti-Hu antibodies and Hu-reactive B-cell lymphocytes (Dalmau et al. 1990; Szabo et al. 1991) penetrate the blood-brain barrier of these patients and lead to both the destruction of neural tissue and the development of neurological autoimmune disorders manifested by dementia, cerebellar degeneration, brainstem encephalitis, or myelitis (Dalmau et al. 1990; for review, see Darnell 1996). Therefore, the consequent brain dysfunction is considered to be a "remote effect of cancer" (reviewed in Anderson et al. 1987) and is presumed to result from an immune response against the ectopically expressed neuronal antigens present on a tumor that is outside the immune-privileged CNS.

The pathology of the paraneoplastic diseases suggests an important role of neuronal ELAV-like proteins in homeostatic functioning of the human nervous system. Findings that these proteins have high affinity for AU-rich sequences found in 3′ UTRs of many growth-regulatory mRNAs, their association with polysomes together with the granular mRNP distribution, and localization along neuronal processes suggest that ELAV proteins regulate localized mRNA expression. This type of regulation of protein production may be especially important in neuronal cells where rapid and specific responses to

particular stimuli are essential for neuronal plasticity, including the protein synthesis associates with the long-term memory (Gao and Keene 1996; Mayford et al. 1996).

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