The RNA-binding protein HuD: a regulator of neuronal differentiation, maintenance and plasticity

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Summary
mRNA stability is increasingly recognized as being essential for controlling the expression of a wide variety of transcripts during neuronal development and synaptic plasticity. In this context, the role of AU-rich elements (ARE) contained within the 3' untranslated region (UTR) of transcripts has now emerged as key because of their high incidence in a large number of cellular mRNAs. This important regulatory element is known to significantly modulate the longevity of mRNAs by interacting with available stabilizing or destabilizing RNA-binding proteins (RBP). Thus, in parallel with the emergence of ARE, RBP are also gaining recognition for their pivotal role in regulating expression of a variety of mRNAs. In the nervous system, the member of the Hu family of ARE-binding proteins known as HuD, has recently been implicated in multiple aspects of neuronal function including the commitment and differentiation of neuronal precursors as well as synaptic remodeling in mature neurons. Through its ability to interact with ARE and stabilize multiple transcripts, HuD has now emerged as an important regulator of mRNA expression in neurons. The present review is designed to provide a comprehensive and updated view of HuD as an RBP in the nervous system. Additionally, we highlight the role of HuD in multiple aspects of a neuron's life from early differentiation to changes in mature neurons during learning paradigms and in response to injury and regeneration. Finally, we describe the current state of knowledge concerning the molecular and cellular events regulating the expression and activity of HuD in neurons. BioEssays 28:822–833, 2006. © 2006 Wiley Periodicals, Inc.

Introduction
In recent years, the role of the 3' untranslated region (3'UTR) has become increasingly recognized in modulating mRNA stability, localization and translation in several cell biological aspects. More specifically, the 3'UTR can control mRNA expression at the earliest stages of oogenesis, embryogenesis and tissue development (see for review Refs 1,2). Post-transcriptional events have been implicated in the regulation of short-lived mRNAs encoding specific cytokines, growth-factors and immediate-early response genes (see for review Ref. 3). Importantly, post-transcriptional mechanisms are not limited to short-lived transcripts since they are also important for the control of various mRNAs encoding proteins involved in metabolic activities including for example, the regulation of parathyroid hormone mRNA stability by calcium and phosphate.⁴ In parallel to the discovery of additional mRNAs being regulated via post-transcriptional mechanisms, there is also an increase in the number of diseases of the cardiovascular, immune and nervous systems that are associated with mutations in the 3'UTR of specific mRNAs or with misregulation of RNA-binding proteins (RBP) that interact with 3'UTRs (see for review Refs 3,5–8).

Within the nervous system, the role of RBP in all aspects of neuronal function is not only becoming evident but is also...
gaining importance. This is likely related to the fact that amongst cell types, neurons present an architectural challenge because of their relatively small cell body size and extensive network of projections and connections. As we learn more about the basic mechanisms underlying neurogenesis, neurite elongation, synapse formation and plasticity, we find that RNA-binding proteins are not only involved in all these events but that they are in fact essential and, hence, act as key components, to ensure their appropriate unfolding and completion. Consequently, it is not surprising to find direct associations between neurodegenerative diseases and conditions such as spinal muscular atrophy, amyotrophic lateral sclerosis and Fragile X syndrome, and perturbation in RNA regulation and/or RBP activity (see for review Refs 3,5,6,8,9).

There are a number of well-characterized RBP that have been shown to assume specific roles in normal neuronal development and function (see Table 1). Among these RBP, the cytoplasmic polyadenylation element binding (CPEB) protein, zipcode-binding protein (ZBP), Fragile-X mental retardation protein (FMRP) and Staufen are important for mRNA transport along dendrites or axons. In addition to transporting specific target transcripts, CPEB, ZBP and FMRP all have translational regulatory roles such that translation is inhibited during transport and activated at the final destination by specific signals. Besides regulating transport and translation, RBP are known to control mRNA degradation. To date, the majority of these, including specifically AUF1 (see Table 1), have been shown to stimulate or facilitate mRNA decay. In comparison, the Hu family of RBP, of which HuD is the focus of this review, are among the few known proteins that stabilize transcripts in the cytoplasm.

Over the last 15 years, there has therefore been growing interest in the function and regulation of the Hu family of RBP, which is directly involved in development of paraneoplastic encephalomyelitis and sensory neuropathy syndromes. These syndromes result from the expression of neuronal proteins by tumor cells in the body, typically small-cell lung cancer tumors. Ectopic expression of these ordinarily immune-privileged proteins results in production of onconeuronal antibodies that attack the nervous system and lead to the development of autoimmune neurologic diseases. The antibody produced in the above disorder was termed anti-Hu based on the name of the patient in which the antibody was discovered. The Hu antibody was used to identify HuD and other Hu proteins (see further).

Amongst the different family members, HuD is recognized as one of the earliest markers of neuronal cells as well as being an essential regulator of neuronal differentiation and survival. Of particular interest and of potential clinical relevance is the recent association between polymorphisms in the HuD gene and age-at-onset of Parkinson’s disease. A total of nine single nucleotide polymorphisms (SNP) within the human HuD locus region were genotyped in this study, of which two, one in intron 2 and one in exon 8, showed

### Table 1. Neuronal RNA-binding proteins

<table>
<thead>
<tr>
<th>RBP</th>
<th>Domains</th>
<th>Function and recognized element</th>
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</thead>
<tbody>
<tr>
<td>Hu (HuB, HuC and HuD)</td>
<td>3 RRM</td>
<td>mRNA stabilization, Cell-cycle regulation and neurite extension, ARE (AUAUA, see text)</td>
</tr>
<tr>
<td>AUF1 (p37, p40, p42, p45)</td>
<td>2 RRM</td>
<td>mRNA destabilization, ARE</td>
</tr>
<tr>
<td>Cytoplasmic polyadenylation element binding (CPEB) protein</td>
<td>2 RRM</td>
<td>Polyadenylation and translation activation (derepression), mRNA dendritic transport, Synaptic plasticity, CPE (UUUUUAU)</td>
</tr>
<tr>
<td>Musashi (MsI1 and 2)</td>
<td>2 RRM</td>
<td>Translation repression, Neuronal development</td>
</tr>
<tr>
<td>Zipcode-binding protein (ZBP1 and 2)</td>
<td>1 RRM, 4 KH</td>
<td>mRNA transport, Translation repression, Growth-cone dynamics, Zipcode</td>
</tr>
<tr>
<td>Human K homology-type splicing regulatory protein (KSRP) or rat MARTA-1</td>
<td>4 KH</td>
<td>mRNA dendritic transport, mRNA degradation</td>
</tr>
<tr>
<td>NOVA-1 and NOVA-2</td>
<td>3 KH</td>
<td>Splicing regulation, Neuronal survival, UCA(U/C) stem-loop repeats</td>
</tr>
<tr>
<td>Fragile X mental retardation protein (FMRP)</td>
<td>2 KH, 1 RGG</td>
<td>Translation repression and mRNA transport, Synaptic structure and plasticity, G quartet structure</td>
</tr>
<tr>
<td>Staufen (Stau1 and 2)</td>
<td>5 dsRBD</td>
<td>Dendritic mRNA targeting, Double-stranded RNA</td>
</tr>
</tbody>
</table>
moderately to strongly significant effects on age-at-onset of Parkinson's disease. Here, we therefore present a comprehensive and updated view of HuD and its role in multiple aspects of a neuron's life including the initial phenotypic commitment and differentiation, as well as synaptic remodeling during learning paradigms and in response to injury and regeneration. Additionally, we describe the current state of knowledge concerning the molecular and cellular events regulating the expression, activity and function of HuD in neurons.

The HuD Gene, its transcripts and encoded proteins

As mentioned, HuD was the first of the Hu proteins to be identified as the anti-Hu antigen in patients with paraneoplastic encephalomyelitis and sensory neuronopathy. The Hu protein family also includes HuB (Hel-N1), HuC (Hel-N3) and HuR (HuA). Whereas HuR is ubiquitously expressed and HuB is found in neurons and gonads, expression of HuC and HuD is restricted to neurons. Due to the presence of three highly conserved RNA-recognition motifs (RRM) (see below and for review 45,46), HuD shares a high degree of homology with the Drosophila proteins ELAV (responsible for the Embryonic Lethal Abnormal Vision phenotype; see Fig. 1) that is required for the normal development and maintenance of the nervous system.

HuD is encoded by a relatively large gene (~146 Kb) located on chromosome 1 in humans (1p34) and chromosome 4 in mice (49.5 cM). As shown in Fig. 2, some of the exons are separated by large intronic regions. The localization of the HuD gene in the mouse genome was based on syntenic regions between human and mouse, suggesting that the flanking genes are conserved between species. The gene consists of three putative non-coding exon 1 variants, termed 1a, 1b and 1c, and several common coding exons (2, 3, 4, 5 and 8; see Fig. 2). The first and second RRM are encoded by exons 2 and 3, and exons 4 and 5, respectively, while the third RRM is encoded by exon 8. The HuD gene is also subject to alternative splicing of exons 6 and 7 which affects the length of the hinge region linking the second and third RRM. This results in the three different transcripts and molecular forms, termed HuDpro, HuD and HuDmax, of which HuDpro and HuD are the major variants. The resulting proteins have molecular masses ranging between 37 and 43 kDa and are characterized by the three RRMs and unique nuclear export signals located within the hinge region. With the exception of the N-terminal domain and the hinge region, the protein sequences between the different Hu proteins are very similar (70 to 85% identity; see Fig. 1).

HuD's RNA-recognition motifs (RRM) and target transcripts

As shown in Figs 1 and 2, all Hu proteins contain three RRM. RRMs are found in various proteins involved in RNA processing and turnover, including hnRNP A1 involved in splicing and transport of RNA, poly(A)-binding protein (PABP) involved in transcript stability and translation, and the Drosophila ELAV protein involved in RNA splicing. The RNP motifs that bind the RNA are indicated by underlined blue letters. The exons encoding each protein region are indicated above the sequence alignment and the splicing between the exons is indicated by the slanted bar. The underlined and red residue (R236) corresponds to the methylated arginine in HuD and HuR.
RRM is a conserved structure of ~80–90 residues containing two consensus ribonucleoprotein (RNP) motifs separated by 25–35 amino acids that interact directly with the RNA. The RNP motifs, octameric RNP-1 and hexameric RNP-2, each contain three conserved aromatic residues that are implicated in RNA interactions in a number of different RBP including proteins that bind pre-mRNA, mRNA, pre-rRNA as well as small nuclear and heteronuclear RNAs. The consensus structure of RRM consists of $\beta_1$-$\alpha_1$-$\beta_2$-$\beta_3$-$\alpha_2$-$\beta_4$ and the location of the RNP motifs in the first and third $\beta$-strands of the RRM is highly conserved. \(^{(45,51)}\) Although the structure of RRM is well preserved, only a few residues found mostly in the RNP motifs are highly conserved. Consequently, it is the variable regions between the RNP motifs and RRM that tend to lend sequence specificity to the RBP. Similarly to other RRM-containing RBP, the first and second HuD RRM consist of the consensus RRM structure and form a cleft between them where the RNA is bound specifically between the $\beta$-sheets. \(^{(52)}\) Crystal structure experiments revealed that the residues within the first and second HuD RRM that interact with the RNA, are conserved between HuD and ELAV proteins. \(^{(52)}\)

Hu proteins recognize and bind specifically to well-described AU-rich elements (ARE) found within the 3' UTR of approximately 1 in 20 human genes \(^{(53,54)}\) and directly implicated in RNA turnover. \(^{(30,53,55,56)}\) The AREs are separated into three classes based on their sequence and structure. Class I AREs consist of one to three dispersed AUUUA motifs separated by U-rich regions while class II AREs consist of multiple clusters of AUUUA motifs. Class III AREs, although less-well defined, consist mainly of U-rich sequences and do not contain the AUUUA pentamer. \(^{(57)}\) The different classes of AREs appear to instruct distinct modes of mRNA decay. Class I and III ARE-directed mRNA degradation involves simultaneous deadenylation of mRNAs resulting in a pool of transcripts of homogeneous lengths, termed distributive deadenylation, which is followed by degradation of the mRNA body. In contrast, Class II ARE-directed mRNA degradation consists of complete deadenylation of one transcript resulting in a pool of mRNAs of heterogeneous lengths, termed processive deadenylation. \(^{(58)}\) Recently, HuD-RNA crystal structure and in vitro binding assays have determined that the consensus sequence recognized by RRM1 and RRM2 is X-U/C-U-X-X-U/C-?U-U/C, where the "?" denotes questionable binding of the C in this location. \(^{(52,59)}\) Consequently, HuD has been demonstrated to bind and stabilize transcripts with CU- and U-rich sequences as well as class II and III AU-rich elements. The structure of the RRM allows for the binding of any number of domains and RNA conformations. \(^{(52)}\) Similarly to HuR, HuD, while able to bind all classes of ARE, retains some sequence specificity and preference such that it will not bind a transcript based solely on the presence of an ARE. \(^{(60)}\)

Unlike many other ARE-binding proteins that principally act to destabilize transcripts, such as AUF1 family members (Table 1), Hu proteins act to stabilize ARE-containing transcripts thereby significantly prolonging their half-life. \(^{(39,42,61,62)}\) In vitro binding studies performed with HuD have demonstrated that the RNA is bound primarily by the first RRM while the second RRM, which also binds the RNA, functions mostly to stabilize the RNA–protein complex. \(^{(52,63)}\)
The third RRM, in addition to participating in maintaining RNA–protein complex stability, binds to poly(A) tails. In the latter case, the length of the poly(A) tail correlates with the overall binding efficiency of Hu proteins. In contrast, the third RRM of mouse HuB and HuC could not bind to poly(A) ribohomopolymers and generally showed very little RNA-binding activity. There are, however, some inconsistencies between studies as another study performed with mouse HuC demonstrated that the third RRM could specifically bind to poly(A)-sepharose beads. The different results obtained with each Hu family member may simply reflect different technical approaches. In that context, the studies performed with HuD used complete in vitro transcribed GAP-43 transcripts with differing lengths of poly(A) tails as opposed to poly(A) nucleotide chains, and could consequently be considered a more accurate representation of the binding activity of the third RRM. Alternatively, it is possible that the third RRM is also involved in protein–protein interactions rather than direct RNA-binding, as suggested by Kasashima et al. (2002), who showed that HuD, HuB and HuC could form dimers via the third RRM.

As the variety of cis-acting elements recognized by the RRM have increased, so have the number of transcripts that interact with HuD. Accordingly, HuD has been shown to bind and stabilize several developmentally regulated transcripts including c-fos, c-myc, N-myc, p21waf1, neuroserpin and MARCKS. Interestingly, and in agreement with its role in neuronal differentiation, many of the transcripts bound by HuD play a key role in the formation of neuronal processes such as GAP-43 and tau. In this context, our studies have recently shown that HuD also binds and stabilizes acetylcholinesterase (AChE) transcripts during neuronal differentiation. Since in addition to its well-recognized function in neurotransmission, AChE can also stimulate neurite outgrowth (Refs 42, 75, 76 and references therein), it appears therefore that HuD binds and regulates the stability of a subset of transcripts with similar functional roles that is key for neuronal development and plasticity.

Although the principal function of HuD is to stabilize its target transcripts and increase their half-life, several studies performed with HuD or other members of the Hu family suggest that HuD is also involved in other aspects of mRNA regulation (see Figure 3). The variable linker domain separating the second and third RRMs has been suggested to contain a novel nuclear export signal that allows HuD to shuttle target transcripts out of the nucleus into the cytoplasm. In addition, the first and second RRM and part of the linker domain have been shown to interact with the primary mRNA export receptor TAP/NXF1 in cultured neuronal cells. In fact, previous studies have shown that neurite elongation is severely impeded when shuttling of HuD is blocked, suggesting that HuD plays an important role in localizing its target transcripts to the cytoplasm. Once in the cytoplasm, HuD and its cargo are targeted to axons, in the case of tau mRNA, and growth cones, in the case of GAP-43 mRNA.

Specific subcellular localization of the HuD-mRNA granules appears to involve the KIF3A microtubule-associated motor protein. Along these lines, HuD was shown to bind in an RNA-dependent manner to insulin-like growth factor mRNA-binding protein 1 (IMP1). IMP1 is the mouse ortholog of chicken ZBP1 which is known to have a role in mRNA transport and translation regulation (see Table 1). Furthermore, HuD has also been shown to colocalize with...
ribosomes in dendrites of pyramidal neurons of the CA1 and CA3 regions of the hippocampus. Finally, HuD-bound mRNAs are also targeted to ribosomes and polysomes, similarly to HuB and neurofilament-M mRNA. These studies suggest therefore that HuD could also be involved, either directly or indirectly through binding with other RBP with translational functions, in stimulating translation of its target transcripts. In this context, a recent study has shown that HuB can interact with hnRNP K and both can bind to p21 mRNA 3'UTR. This later work also demonstrated that hnRNP K alone decreased the translation of this transcript, whereas HuB could block this effect and indirectly increase translation. Given the similarities in Hu protein function and target transcripts, it is possible that HuD has similar functions.

**Functional role of HuD in neurons**

The neuronal Hu proteins are known to be some of the earliest markers of the neuronal phenotype since they appear to suppress neuroblast proliferation and promote neuronal differentiation. In situ hybridization experiments performed in developing and adult mouse and rat cerebral and cerebellar cortex revealed a distinct expression pattern for mRNAs encoding HuB, HuC and HuD. During embryonic development, HuD expression can be localized to cells exiting the cell cycle in the ventricular zone, and to those migrating in the intermediate zones and undergoing terminal differentiation. In the adult brain, HuD-positive neurons correspond primarily to projection neurons found in the neocortex, hippocampus, entorhinal cortex and cerebellum. Similarly, HuD is very strongly expressed in the ventral motoneurons of the spinal cord, the sensory neurons of the dorsal root ganglia (DRG) and sympathetic neurons in their ganglia. At the subcellular level, HuD is detected in the cell body, axon and growth cones. Generally, HuB and HuD have a similar pattern of expression which is somewhat different from HuC. Although expression of HuB and HuD overlap, they appear to have similar and opposing functions during neuronal development depending on the context. For example, they can both stimulate neuronal differentiation and neurite outgrowth but have opposing roles in progenitor cell self-renewal, such that HuB positively and HuD negatively affects this capacity.

Studies in which the expression level of HuD has been manipulated by either overexpression or downregulation, have highlighted the significance of HuD in stabilizing various mRNA in neurons. Specifically, HuD binds a subset of transcripts that encode proteins involved in neuronal development. Several recent studies have shown that overexpression of HuD increases the rate and length of neurite outgrowth in several different types of neuronal cells, including in both primary cultures and neuronal cell lines. By contrast, decreased HuD expression results in an inhibition of neurite extension. Together, these results indicate that HuD is an important regulator of neurite elongation and morphological differentiation. Expression of HuD in non-neuronal cells does not stimulate the development of processes, suggesting that pluripotent cells must already be engaged along a neuronal cell fate in order for HuD to be effective in promoting morphological differentiation. This effect is specific to neuronal Hu proteins, as HuB and HuC can also stimulate morphological differentiation, while HuR is unable to stimulate neurite extension in neural crest cells.

Recently, HuD transgenic and HuD knockout mice have been generated and characterized. HuD transgenic mice specifically expressed higher total amounts of HuD in the forebrain, particularly in all subregions of the hippocampus including in dentate granule cells that do not normally express HuD. Interestingly, in both HuD-overexpressing and -deficient mice, there are no apparent morphological or structural defects in the adult brain. However, extension of some cranial nerves is transiently impaired during embryogenesis in HuD-deficient mice. In comparison, loss-of-function alleles of Drosophila elav are embryonic-lethal and hypomorphic mutations result in morphological and structural defects of the eye. Given that there are three neuronal Hu proteins with similar functions and one ELAV, it is possible that expression of the other members of the Hu family are compensating for the loss of HuD in knockout animals. Although there are no apparent morphological defects, HuD-deficient mice display motor/sensory defects such as an abnormal clasping reflex of the hindlimbs when suspended by the tail. The abnormal clasping reflex suggests a defect in sensory and motor functions which is also confirmed by poor performance in rotarod experiments. Both of these mouse models of aberrant HuD expression have initially been used to confirm some of the in vitro and cell culture data and to further elucidate the importance of HuD to neuronal development and function.

During early developmental stages, HuD appears to be involved at multiple levels of cell lineage commitment, differentiation and survival. Studies using HuD knockout mice showed that, during embryogenesis, the absence of HuD is related to an increase in the progenitor cells’ ability to renew, and to a decrease in their ability to exit the cell cycle and morphologically differentiate into neurons. Cells that are not leaving the cell cycle are also more likely to undergo apoptosis since levels of apoptosis are elevated in HuD-deficient mice. In adult mice, the number of subventricular zone progenitor cells is greater in the HuD knockout mice. These studies suggest therefore, that HuD is important for promoting the exit from the cell cycle, for negative regulation of proliferation and stimulation of the differentiation process. In addition, an increased level of HuD in cultures of neural crest cells precipitates neurotrophin dependence. Similarly, HuD overexpression in mouse embryonic stem cells can
double the number of cells with long neurites and expressing GAP-43 only when the cells are induced to differentiate with retinoic acid. Another study has demonstrated the co-localization of HuD with GAP-43 mRNA in growth cones. In this context, we suggest that HuD expression in regenerating neurons results in a concomitant increase in GAP-43 transcript levels.

The significance of HuD in the regulated expression of mRNAs and proteins involved in the growth and formation of neuronal projections, suggests that it is also important to other aspects of axonal and dendritic functions in the adult nervous system. A few distinct and complementary studies have clearly demonstrated that spatial learning tasks can enhance HuD expression in hippocampal neurons resulting in a concomitant increase in GAP-43 transcript levels. Another experimental paradigm, single trial contextual fear conditioning, also demonstrated an association between HuD expression and learning. In the latter study, HuD expression was increased in different regions of the hippocampus namely, in the hilar region of the dentate gyrus, as well as in the CA3 and CA1 regions. Along similar lines, increased expression of HuD in the hippocampus of transgenic mice overexpressing HuD, stimulates GAP-43 mRNA expression and stabilization as well as AChE mRNA expression. More specifically, over-expression of HuD in dentate granule cells, where HuD is not normally expressed, results in a marked increase in GAP-43 mRNA levels via post-transcriptional mRNA stabilization of newly synthesized, but normally degraded, transcripts.

Consequently, atypical expression of HuD in neurons may have important regulatory effects on the expression of its target transcripts and, thus, a significant downstream effect on neuronal function.

Given its significant role in growth and differentiation, HuD has also been suggested to have a role in axonal regeneration. Recent findings have shown that following nerve crush of DRG sensory neurons and facial motoneurons, HuD protein and transcript levels increase within 7 days of the injury and remain elevated for up to 21 days. This increase in HuD expression is accompanied by a dramatic increase in the mRNA levels of GAP-43, a known target of HuD. In regenerating neurons of the DRG, HuD protein colocalized with GAP-43 transcripts as well as with ribosomal RNA. A recent study has also demonstrated the co-localization of HuD and GAP-43 mRNA in growth cones. In this context, we have found that exogenous expression of human HuD in the rat superior cervical ganglion (SCG) results in the maintenance of AChE and GAP-43 mRNA levels following axotomy (unpublished data). Together, these findings demonstrate that, in addition to its role during neuronal development, HuD expression and function correlate with plasticity of the nervous system both in response to injury and during learning.

Further studies performed with the HuD-transgenic and -deficient mice are necessary to strengthen these correlations into direct or indirect involvement of HuD.

**Molecular events regulating expression of HuD and its function**

Despite the well-established involvement of HuD in the control of a subset of neuronal transcripts involved in neuronal differentiation and plasticity, there appears to be a lack of information concerning the nature of the molecular and cellular mechanisms that regulate expression of HuD in neurons. This is important since a number of recent studies point to mRNA stability as a consequence of functional antagonism between stabilizing and destabilizing proteins (see for review Ref. 102). This is best exemplified by studies performed with the ubiquitous Hu family member HuR, whose tissue expression overlaps with AUF1, a family of four isoforms that are predominantly destabilizing (Table 1).

Using a similar approach, competition for transcript-binding sites was also demonstrated within the AUF1 family. In this context, it is important to note that AUF1 has also been reported to have mRNA-stabilizing activity. Thus, it appears that competition for target transcripts and the eventual outcome for their longevity are largely determined by the relative abundance of the different RBPs. Accordingly, it becomes important to gain a better understanding of the events presiding over HuD expression in neurons.

**Transcriptional and post-transcriptional regulatory mechanisms.**

The HuD gene is divided into eight coding exons beginning with exon 2 (see Figs 1 and 2). A comparison of the available cDNA sequences in GenBank (NM_010488, BC052451 and BC052451) shows the presence of three alternate non-coding exon1 splice variants termed exon1a, 1b and 1c (see Fig. 2). Presently, it is unclear whether a single promoter is responsible for transcription of this large gene with splicing of exon1 variants occurring subsequently or whether there are multiple promoter choices driving specific expression of exon 1 variants. Although the HuD 5’ regulatory region of the mammalian gene has yet to be isolated and characterized, studies performed with the *Xenopus laevis* HuD homologue, elrD, have shown that there are two possible promoter regions upstream of exon 2. The most 5’ promoter region is upstream of a non-coding exon 1 and the second promoter region is located within the first intron. Both
promoter regions have been shown to drive transcription in a neuronal-dependent fashion and to contain neuron-specific transcription factor binding sites (E-box and neuron-restrictive silencer factor site). Similarly, characterization of the human HuB 5’ regulatory region demonstrates the presence of cis-acting elements that can direct tissue-specific expression in neurons. Analysis of the zebrafish HuC 5’ regulatory region resulted in the identification of multiple E-box sequences, a putative Myt1-binding site and two GC boxes that could also drive neuron-specific expression of HuC. The Drosophila ELAV 5’ flanking region including enhancer domains within an intronic region, also confers neural-specific expression patterns to this gene. Accordingly, the specific and early neuronal expression of HuD along with the presence of alternate 5’ regulatory regions strongly suggest that expression of HuD is regulated by transcriptional mechanisms involving selective cis-acting elements and trans-acting factors that are specific to neurons (see Ref. 87). In agreement with this view, a recent study demonstrated that the transcriptional rate of HuD is significantly altered in neurons subjected to changes in the levels of thyroid hormone.

In addition to transcriptional regulatory events, the relative abundance of HuD may also be regulated by post-transcriptional mechanisms (see Figure 3). Northern blot analysis has revealed the presence of two HuD transcripts of ~3.7 and 4.4 kb in neurons. These are thought to originate from alternative polyadenylation or splicing to a putative downstream non-coding exon (exon 9). Alignment of available cDNA sequences indeed reveals the presence of alternate 3’UTRs. Amongst the different Hu proteins, the 3’UTRs are unique to each transcript. In contrast, specific sequences coding for stem-loop structures and putative ARE within the HuD 3’UTR are highly conserved between different species. In addition, the 3’UTR of the Drosophila homologues (ELAV and SxL) are important for the appropriate regulation of these transcripts. More specifically, HuB, ELAV and SxL have been shown to autoregulate their expression through interactions with their own 3’UTR. Recent studies have shown that ELAV expression is dependent on the presence of a long UTR at its 3’ end. An ELAV-binding site was in fact found within the elav mRNA located within an alternative non-coding 3’ exon. The authors of this study proposed that ELAV protein binds to elav mRNA 3’UTR when protein levels reach a certain threshold and, in this case, block further protein production. Such a similar negative autoregulatory loop also can be assigned to SxL protein, which can downregulate its own expression by binding to specific regions within the sxl 3’UTR. Together, these observations are coherent with the notion that, in addition to transcriptional events (see paragraph above), post-transcriptional mechanisms operating through the 3’UTR are also important for controlling HuD expression.

Post-translational regulatory mechanisms
Although the abundance of RBP is important to ultimately control the fate of specific transcripts, other factors such as the RBP affinity and function are also implicated. In a recent study, we have shown that HuD did not actively bind to one of its target transcripts until neuronal differentiation was stimulated, even though significant levels of HuD protein were present in undifferentiated PC12 cells and these levels did not change upon differentiation (see Refs 42,49,62). This suggests that additional factors affect the ability of HuD to stabilize target transcripts. In this context, several recent studies have begun to elucidate the pathways regulating post-translational modification of the Hu family of RNA-binding proteins including phosphorylation and methylation, and to determine the impact of these modifications on their activity. Notably, NGF-induced differentiation and stress-related stimuli can indirectly regulate HuD and HuR function, respectively, by a PKC-mediated signaling cascade. Recently, a series of indirect experiments led to the suggestion that threonine phosphorylation of the neuronal Hu proteins (HuB, HuC and HuD) by PKCζ isoenzyme results in the re-distribution of the proteins and in an increased stabilization of GAP-43 mRNA, a well-known HuD target.

In addition to phosphorylation, a recent study has shown that arginine residues in the HuR hinge region are methylated by the methyltransferase CARM1 in response to lipopolysaccharide. Arginine methylation is another common modification of some RBP that results in modification in the pattern of protein–protein interactions. Along those lines, it has also recently been demonstrated in PC12 cells that HuD is methylated on the corresponding arginine (Arg236, see Fig. 1) by the same methyltransferase, and that methylation results in decreased RNA-binding activity of HuD. In these studies, methylation-resistant mutant HuD increased expression of some target transcripts such as p21cip1/waf1, and resulted in increased neuronal differentiation of PC12 cells. Although these cell culture studies suggest that phosphorylation and methylation can regulate HuD function directly or indirectly, it should be noted that these studies have been performed in vitro. Whether post-translational modifications are also occurring in vivo still remains to be determined. Nonetheless, these studies indicate that post-translational modifications are also likely to be important in regulating the binding and functional activity of HuD on target transcripts.

Finally, the fate of target transcripts may also depend on other factors that might modulate the binding activity and specificity of HuD. These include the structure and sequence of the target transcripts and the interaction with additional RBPs. The transcript itself can, in many cases, modify the function of RBP (see for review Refs 52,122). An example of this was given above with the poly(A) tail since the presence and length of the poly(A) tail can alter the binding of the RRM to
the ARE.\cite{64,67} The presence of HuD in RNP granules and the suggested multimerization of HuD implies that interactions with other proteins or RBPs can modulate the activity of HuD and its specificity in a manner analogous to the interactions of transcription factors on promoter elements.\cite{47,79,85,123} This may explain in part the identification of numerous RBPs interacting with a single transcript such as GAP-43, AChE and the early-response genes c-myc and c-fos (see for review 102).\cite{42,61,62,124}

Conclusion and perspective

To date, there is compelling evidence indicating that, through its ability to bind and stabilize a specific subset of transcripts, HuD plays an essential role in the development and maintenance of neuronal phenotype. The downstream effects of this specific activity include the stimulation of morphological differentiation of newly committed and developing neurons. Additionally, HuD expression and binding activity have recently been shown to increase during nerve regeneration as well as with learning and memory. The ability of HuD to directly control these key molecular and cellular events indicates that HuD may act as an important regulator of neuronal differentiation and function. Therefore, a thorough understanding of the mechanisms affecting the expression, activity and function of HuD in neurons appears warranted in order to increase our basic knowledge of the events regulating neuronal differentiation, maintenance and plasticity. Ultimately, this knowledge could prove useful for the development of novel therapeutic strategies aimed at manipulating, pharmacologically or through gene therapy approaches, the levels of RBPs that may be beneficial for the treatment of several neurological diseases and conditions.

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References


Review articles


