Neuron-specific ELAV/Hu proteins suppress HuR mRNA during neuronal differentiation by alternative polyadenylation

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ABSTRACT
The ubiquitously expressed RNA-binding protein HuR increases the stability and translation of mRNAs encoding growth regulatory proteins that promote proliferation in a variety of cell types. However, the three neuron-specific ELAV/Hu proteins, HuB, HuC and HuD, while binding to the same types of mRNAs, are required instead for neuronal differentiation, and it becomes difficult to reconcile these contrary functions when all four Hu proteins are expressed in the same neuron. HuR mRNA exists as three alternatively polyadenylated variants, a 1.5-kb testes-specific mRNA isoform, a ubiquitous 2.4-kb isoform and a 6.0-kb isoform that we now show is induced during neuronal differentiation and appears to be neuron-specific. This 6.0-kb neuron-specific mRNA isoform is inherently less stable and produces less HuR protein than the ubiquitous 2.4-kb mRNA. Furthermore, we show that neuronal HuB, HuC and HuD, as well as HuR itself, can bind at the 2.4-kb mRNA polyadenylation site, and when overexpressed can affect alternative polyadenylation to generate an extended HuR 3'-UTR that is translationally suppressed. We propose that the regulation of HuR protein expression by alternative polyadenylation allows neurons to post-transcriptionally regulate mRNAs-encoding factors required for proliferation versus differentiation to facilitate neuronal differentiation.

INTRODUCTION
Post-transcriptional processes have been shown to regulate the growth and development of cells and organisms. After being produced via transcription, the mRNA precursor (pre-mRNA) begins a journey towards the cytoplasm and eventual translation to a functional protein. The pre-mRNA must be spliced, polyadenylated and transported to the cytoplasm where it is subjected to many processing events including localization, degradation, editing and eventual translation (1). All of these steps are carefully coordinated and regulated by a vast array of RNA Binding Proteins (RBPs) and microRNAs (miRNAs) that contribute to proper gene expression (2,3). Indeed, many RBPs, including the ELAV/Hu family of RBPs, have been shown to have crucial roles at multiple steps along the mRNA processing pathway, assuring proper protein production in a coordinated manner (4–7).

The ELAV/Hu proteins belong to the AU-rich element (ARE) family of RBPs and consist of 3 RNA Recognition Motifs (RRMs) with RRM 3 by a variable hinge region that contains nuclear localization and export signals (4,8–10). Hu proteins bind to AREs in the 3‘-untranslated regions (UTRs) of target messages to translocate them to the cytoplasm and up-regulate their mRNA stability and translation (11–15). Indeed, Hu family members are among the few ARE-binding proteins associated with increased mRNA stability and translation. However, more recent evidence has shown that they can also regulate other post-transcriptional processes such as splicing and polyadenylation, suggesting a broader role in mRNA processing than once appreciated (7,16–21).

There are four known members of the mammalian ELAV/Hu RBP family including the ubiquitously expressed HuR and the neuron-specific HuB, HuC and HuD (8–10,22). HuR is an essential gene that is required for proper embryonic development and survival, emphasizing the importance of post-transcriptional regulation during disease and development (23,24). HuR is known to be involved in a large number of proliferative responses (9,25–27) and has been implicated in several human cancers including colon, cervical, breast and renal cell carcinoma (28–31). HuR shuttles between the cytoplasm and nucleus but in homeostatic cells is found predominantly in the nucleus. However, upon stimulation
and/or perturbation it can translocate to the cytoplasm of cells, a phenomenon that has been correlated with cell cycle regulation and aggressive phenotypes in a large number of cancer cells (25,31–34).

While the HuR protein is ubiquitously expressed, its mRNA exists in three different isoforms that arise via alternative polyadenylation (25,35,36). Thus, while the mRNA exists in three different isoforms that arise via number of cancer cells (25,31–34), they differ in the length of their 3′-UTRs. The predominant transcript variant is a 2.4-kb mRNA isoform that is expressed in most mammalian tissues and is found in the majority of cell types. The smallest 1.5-kb mRNA isoform appears to be expressed primarily in the testes (25). Previous Northern blot analysis from our lab suggests that the longer 6.0-kb mRNA isoform is expressed exclusively in the brain of both mouse and humans, indicating the possibility that HuR is alternatively regulated in these tissues, where coincidently the other three Hu family members are also expressed (25).

While HuR is expressed throughout the embryo, during neuronal development, embryonic cells also express HuB, C and D, the three neuron-specific Hu family members (37–39). These three proteins are known to regulate stability and translation of certain early response gene transcripts involved in growth and differentiation (13,37,40–45). Indeed, the neuron-specific Hu family members have been shown to be involved in neuronal differentiation (37,40,42,46–48). These proteins also shuttle between the cytoplasm and nucleus but are predominantly found in the cytoplasm of neuronally derived cells, localizing to the axons and dendritic spines of differentiated neurons, where HuR has also been found to be expressed (49). Interestingly, while the four Hu RBPs are highly homologous to each other, HuR appears to regulate growth promoting activities while the neuron-specific forms appear to drive differentiation (8). This creates a conundrum to explain why an ELAV/ Hu family member functioning to increase proliferation would be expressed in terminally differentiated neurons, and leads us to suggest that perhaps HuR, while ubiquitously expressed in all cell types, is differentially regulated in neural cells.

In this study, we identify the neuron as the natural environment for the investigation of the HuR 6.0-kb mRNA isoform. We show that HuR mRNA is alternatively polyadenylated in neuronal tissues to produce the 6.0-kb neuron-specific isoform and that this alternative polyadenylation correlates with increased expression of the neuron-specific Hu family members during differentiation. In addition, we report that all four Hu family members are capable of binding to HuR mRNA near the 2.4-kb polyadenylation site, and driving its alternative polyadenylation to produce the 6.0-kb neuron-specific isoform. As a result of this alternative polyadenylation event, HuR mRNA becomes much less stable and produces less HuR protein during neuronal differentiation, presumably lowering the proliferation potential and allowing differentiation to occur via the actions of the other neuron-specific Hu family members.

### MATERIALS AND METHODS

#### Cell lines

All cell lines were obtained from ATCC. HEK293 and HEK293T cells were maintained in DMEM with 10% FBS. P19 cells were maintained on gelatin coated dishes in Alpha-MEM with ribonucleosides and deoxyribonucleosides supplemented with 7.5% bovine calf serum and 2.5% fetal calf serum. Transfections were performed using the standard protocol for Lipofectamine 2000 (Invitrogen) with a 3:1 Lipofectamine to DNA ratio.

#### P19 differentiation

For neuronal differentiation, one million P19 cells in 10 ml of media were plated into 10 cm bacterial-grade petri dishes in the presence or absence of 5 μM all-trans-retinoic acid (RA; Sigma) for 4 days. Cell aggregates were dispensed with trypsin/EDTA, replated on tissue culture dishes coated with gelatin and allowed to differentiate for up to 6 days. Where indicated, to enrich for neuronal cell types, cultures were treated with (β-D-Arabinofuranosyl) cytosine (AFC; Sigma) beginning 2 days after replating. In a separate enrichment protocol, the overlying neuronal cells were gently washed off the underlying glial cells before harvest.

#### RNA extraction

Trizol (Invitrogen) was used for all RNA extractions according to the manufacturer’s protocol.

#### PCR

Reverse transcription was performed with the iScript cDNA synthesis kit (Bio-Rad). Semiquantitative PCR was carried out in the linear range of amplification (determined independently for each primer set) and products were resolved on 1% agarose gels and stained with ethidium bromide. Quantitative real-time PCR was performed using a Roche Lightcycler with Sybr green detection (Invitrogen) and the ΔΔCt analysis method, using cytoplasmic polyA binding protein (PABP) mRNA for normalization. All real-time PCR primer sets were designed so the products would span multiple exons (except for HuR-NS which was in the 3′-UTR), and amplification of a single product of the correct size was confirmed by melting curve analysis and agarose gel electrophoresis. A list of real time primers used can be found in Supplementary Table S2.

#### Northern blot

Northern blot analysis was conducted as described (25). First, 15 μg of total RNA was separated on agarose gels containing 6% formaldehyde and transferred to charged nylon membranes (Millipore). Membranes were probed with PCR fragments corresponding to the HuR neuron-specific UTR, HuR ORF or B-actin ORF, all of which were labeled with [32P]CTP using the Prime-It II Random Primer Labeling Kit (Stratagene). 18 and 28S rRNA loading controls were detected by ethidium bromide staining. Hybridization products were detected...
by PhosphorImager and scanned using the Typhoon System (Molecular Dynamics).

**Plasmid constructs**

The open reading frames of eGFP, PABP, HuR, HuB (HelN-1), HuC and HuD were PCR amplified from existing constructs with primers that incorporated a C-terminal 1× FLAG epitope and TA cloned into pGEMT (Promega) before being sub-cloned into the MCS of pCDNA3 or pCDNA3.1 mammalian expression vectors. For the HuR expression vector with various MCSs, Plasmid constructs were performed using the Dual-Luciferase Reporter Assay System (Promega).

**RESULTS**

**Tissue-specific expression of HuR mRNA isoforms**

While HuR protein expression appears to be ubiquitous, previously published Northern blot analysis from our lab suggests that the 3 HuR mRNA isoforms differ in their tissue distribution (25). The 2.4-kb HuR mRNA isoform is detected in almost all human and mouse tissues tested, suggesting that it is nearly ubiquitous in its expression, and is the predominant form of mRNA expressed in most tissues and cell types (25,35). The shortest 1.5-kb HuR mRNA isoform appears to be expressed predominantly in the testis with minor expression in heart and spleen. We were most intrigued, however, by the longer 6.0-kb mRNA isoform that appears exclusively in the brains of both mice and humans (25), as this is where the other Hu family members, B, C and D, are also predominantly expressed.

To confirm tissue-specific expression of the longer 6.0-kb mRNA isoform, we utilized an online microarray expression database of multiple tissues and cell types (50). The BioGPS gene portal hub (http://www.biogps.gnf.org) contained six probes from the GeneAtlas MOE430 (gcma) data set that matched murine ELAVL1 (HuR) mRNA (51). Of the six probes, two recognized mRNA sequence common to all three HuR isoforms (pan-HuR), while three mapped exclusively to the longer 6.0-kb mRNA's 3′-UTR (Supplementary Figure S1). One probe mapped to an intron between exons 2 and 3 and was excluded from our analysis. To aid in the analysis, samples were grouped according to organ system as shown in Figure 1A and B, with underrepresented systems grouped together into a miscellaneous category. The identities of the individual samples are listed in Supplementary Table S1. In support of our published Northern blot analysis (25), the longer 6.0-kb transcript (Figure 1A) was expressed almost exclusively in brain and other neuronal tissues, while the 'pan-HuR' probe detected mRNA to varying degrees in all tissues (Figure 1B), but appeared to be much lower in most neuronal tissues. Additional samples exhibited a moderate level of 6.0-kb mRNA expression but this could be explained by the presence of neurons in these complex tissues. Similar results were seen with the other probes (Supplementary Figure S1).

**Intrigued by these expression patterns, we next examined the expression of the 6.0-kb mRNA isoform in relation to the co-expression of the neuron-specific Hu family members HuB, HuC and HuD. Using the BioGPS gene portal hub, we identified probes within the**

**mRNA decay rates**

mRNA levels were determined by real-time quantitative PCR at 0, 1, 2, 4, 6 or 8 h after addition of 5μg/ml Actinomycin D. GAPDH mRNA was used for normalization. Exponential decay curves were fit to points representing the means of three to four biological replicates, and half-lives were calculated based on the equations describing those best-fit curves.

** Luciferase expression**

HEK293T cells were transfected with equimolar amounts (to account for the differences in UTR lengths) of the various HuR UTR firefly reporters, with the difference made up with PCDNA3. pRL was included to control for transfection efficiency. After 24 h, luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega).
MOE430 database that recognize the other three Hu family members. We then analyzed the correlation of expression of the various Hu family member probes in the different tissues. As shown in Figure 1C, expression of the neuron-specific 6.0-kb mRNA isoform was highly correlated with co-expression of the other Hu family members ($r = 0.53 \pm 0.14$), while the pan-HuR probes recognizing the HuR sequence common to all isoforms were negatively correlated with all three of the neuron-specific Hu family members as well as the HuR 6.0-kb mRNA isoform ($r = -0.35 \pm 0.11$). These data confirm that the 6.0-kb isoform of HuR mRNA is a neuron-specific (NS) isoform and this finding raises the question of the functional role and mechanism of HuR’s own mRNA alternative polyadenylation in neuronal tissues.

**Expression of the HuR mRNA isoforms during P19 neuronal differentiation**

Because of the high correlation between the expression levels of the 6.0-kb HuR mRNA isoform and the other Hu family members, which are expressed almost
exclusively in neurons, we investigated the temporal expression of HuR mRNA during neuronal differentiation of P19 embryonic carcinoma cells. While this system produces cultures containing glial as well as neuronal cells, the P19 system is widely used to recapitulate the events of neuronal differentiation (52,53). When P19 cells were exposed to 5 μM retinoic acid (RA) for 4 days in suspension culture (Day 4/0) and then replated onto tissue culture dishes for 2–6 days (Days 4/2, 4/4, 4/6) they underwent neuronal differentiation as evidenced by the formation of neuronal processes (Figure 2A), and the expression of many early neuronal markers including the neuronal Hu family members (HuB, C, D) and Neuroglycan C (Figure 2B). However, as differentiation progressed, the glial marker GFAP also showed a dramatic increase, highlighting the cellular heterogeneity of the culture. In addition to an increase in differentiation markers, there was also dramatic down-regulation of the stem cell marker Oct3 (Figure 2C), consistent with the cells progressing through differentiation.

Because, it is impossible to interrogate the shorter HuR mRNA isoforms via PCR without detecting the longer one as well, northern blot analysis was used to determine the expression pattern of HuR mRNA during P19...
differentiation. As shown in Figure 3A, a probe specific for the 6.0-kb mRNA isoform (HuR NS PCR product from Figure 3B) detected only a very weak band in both undifferentiated P19 cells as well as control (Ctrl) differentiations. However, its expression increased dramatically as the P19 cells differentiated in response to RA (Full blot shown as Supplementary Figure S2). When a probe recognizing the HuR ORF was used (HuR ORF PCR product from Figure 3B), similar results were seen for the 6.0-kb mRNA isoform, while the expression of the 2.4-kb mRNA isoform remained apparently unchanged in response to differentiation, likely due to the heterogeneous nature of the cultures and the increasing presence of proliferative cell types. Semi-quantitative PCR recapitulated these findings and revealed that the 6.0-kb HuR mRNA isoform was induced during neuronal differentiation with an expression pattern similar to that of the related Hu family member HuC mRNA and before the late neuronal differentiation marker Neuroligin 3 (Figure 3B). In addition, appearance of the 6.0-kb mRNA also correlated with induction of HuB and HuD protein expression (Figure 3C) as detected by the 3A2 antibody.

Effect of alternative polyadenylation on HuR mRNA stability

Alternative polyadenylation of HuR mRNA during neuronal differentiation results in the addition of 3.6 kb of UTR sequence that may contain post-transcriptional regulatory elements affecting the subsequent stability and/or translatability of the HuR mRNA. To elucidate the effects of potential downstream post-transcriptional regulation, we investigated the relative stability of the longer 6.0-kb mRNA isoform as compared to the 2.4-kb mRNA isoform. As shown in Figure 4A, when day 4/6 differentiated P19 cultures were treated with the transcriptional inhibitor Actinomycin D and mRNA levels measured at various time points, the neuron-specific HuR mRNA isoform was inherently unstable with a half-life of 4–5 h, while the 2.4-kb mRNA expressed in undifferentiated P19 cells was extremely stable, with a half-life greater than the time course of the experiment. Interestingly, while the 6.0-kb mRNA isoform has a similar half-life in both differentiated and undifferentiated P19 cultures (4.5 ± 0.6 versus 5.0 ± 1.5 h, respectively), total measured HuR mRNA stability decreased upon differentiation, most likely due to the increased proportion of the much less stable 6.0-kb transcript which is virtually undetectable in undifferentiated P19 cells (Figure 3).

Effect of HuR 6.0-kb mRNA 3'-UTR on exogenous HuR protein production

We next investigated the impact of the additional 3'-UTR on HuR protein expression by generating constructs in which the HuR open reading frame (ORF) was fused to either the ubiquitous 2.4-kb 3'-UTR or various forms of the 6.0-kb neuron-specific 3'-UTR, including one in which the 2.4-kb polyadenylation recognition sequence (PAS) was mutated (Δ2.4PAS), and one in which the 6.0-kb polyadenylation sequence was deleted (Δ6.0PAS). HEK293T cells were transiently transfected with equimolar amounts of the constructs and HuR protein expression was analyzed 24 h later using Western blotting. As shown in Figure 4B, while transfection with all constructs increased the level of HuR protein expression over endogenous levels (Null, Ctrl), inclusion of the 6.0-kb 3'-UTR decreased exogenous HuR protein expression.

Figure 3. The 6.0-kb HuR mRNA isoform is expressed during retinoic acid induced neuronal differentiation of P19 embryonic carcinoma cells. P19 cells were differentiated in the presence (RA) or absence (Ctrl) of 5 μM RA for 4 days, then replated and allowed to differentiate for 2–6 days. At each time point, total RNA was isolated via Trizol. (A) Northern Blot showing increasing expression of the 6.0-kb mRNA isoform with either a neuron-specific probe (HuR NS from B) or a probe detecting all HuR mRNA isoforms (HuR ORF from B). β-Actin and ribosomal RNA (18S/28S) were used as loading controls. MW = molecular weight as determined by position of rRNA 18S and 28S bands (B) Semiquantitative PCR to confirm results of Northern blots shown in A. PCR products show consistent expression of the HuR ORF but increased expression of the 6.0-kb HuR neuron-specific mRNA isoform (HuR NS). Neuron-specific mRNAs HuC and Neuroligin 3 (Neuro3) were used to verify the progression of P19 neuronal differentiation. (C) Western blot analysis of Hu protein expression showing induction of neuronal-specific HuB and D during P19 differentiation. GapDH served as a loading control.
expression by ~30%, when compared to the ubiquitous 2.4-kb UTR construct. Mutation of the 2.4-kb (Δ2.4PAS) or deletion of the 6.0-kb (Δ6.0PAS) polyadenylation sequences from the 6.0-kb UTR had little additional effect on HuR expression levels.

Effect of alternative polyadenylation of HuR mRNA on endogenous protein production

We also investigated the effect of HuR mRNA alternative polyadenylation on endogenous HuR protein levels during P19 cell neuronal differentiation; however we initially saw no change in HuR protein levels (Figure 3C). We reasoned that because P19 cultures consist of a mixture of undifferentiated and mixed differentiated cells, the effects on HuR protein production may have been masked. To test this possibility, differentiating P19 cultures were treated with the mitotic inhibitor (β-D-Arabinofuranosyl) cytosine (AFC) to enrich the cultures for non-dividing, differentiated neuronal cells. As shown in Figure 4C, when the P19 cultures were treated for 6 days with AFC to enrich for neuronal cells, a concomitant decrease in HuR protein expression was observed, while the HuR 6.0-kb mRNA actually increased in relative expression. To rule out the possibility that this decrease in HuR protein was due to AFC treatment per se, untreated day 4/6 RA cultures were washed gently with PBS to separate the neuronal cells from the underlying cells (a practice common with the P19 human counterparts NTera2 cells). Once again, when compared to either undifferentiated P19 cells or the underlying glial cells remaining on the plate, differentiated neurons that were washed off the plate, showed decreased HuR protein expression (Figure 4D). These results suggest that there is a decrease in HuR protein levels in differentiated neurons, which correlates with increased expression of the HuR 6.0-kb mRNA isoform.

Binding of the Hu family members to the HuR 3′-UTR

We next investigated the potential mechanism underlying the polyadenylation switch of HuR’s 6.0-kb mRNA isoform. The correlation between the expression of the 6.0-kb mRNA isoform and the expression of the other family members during P19 differentiation (Figure 3) was intriguing given the recently discovered ability of the Hu family members to regulate alternative polyadenylation of the SV40 Late (SVL) and calcitonin/calcitonin gene-related peptide (CGRP) mRNAs (17). It was suggested that binding of Hu proteins to Uridine-rich (U-rich) sequences near the canonical polyadenylation site of these mRNAs prevents access of the polyadenylation machinery to the site, resulting in use of an alternative downstream polyadenylation site. Interestingly, the HuR mRNA also contains many U-rich sequences near the 2.4-kb mRNA’s polyadenylation site (Figure 5A) suggesting that perhaps its alternative polyadenylation can be regulated by the neuron-specific Hu family members.

To begin to explore the possibility of the Hu family members regulating the polyadenylation of HuR mRNA, we first investigated whether Hu family proteins could bind to the U-rich sequences located near the 2.4-kb
polyadenylation site. Using the mRNA sequences shown in Figure 5A (mapped to the HuR 3'-UTR in Figure 5D), we conducted radiolabel transfer via UV cross-linking followed by immunoprecipitation to determine the binding sites of each of the family members. Interestingly, while no binding was seen with control extracts (PC3 and GFP), all four Hu family members were able to strongly bind fragments flanking (Fragments 4 and 6) or containing (Fragment 5) the polyadenylation recognition signal and cleavage site, while showing minimal binding to fragments further removed from those sites (Figure 5B). This binding was specific, as it was competed by a cold RNA competitor (Fragment 1) or positive (Fragment 4) probe. Band intensities from PhosphorImager were quantified and normalized for percent of control binding. (D) UCSC Genome Browser view of hELAVL1 (HuR) 3'-UTR showing published HuR binding sites near the 2.4-kb polyadenylation signal as determined by CLIP or PAR-CLIP analysis. Gel shift fragments used in this study are shown at top and the HuR 2.4-kb mRNA PolyA (PAS) and cleavage site (CS) are shown above the HuR 6.0-kb neural-specific (NS) and 2.4-kb (ubiq) mRNA for reference.

Regulation of HuR mRNA polyadenylation by Hu family members

To determine if the Hu family members can indeed regulate HuR polyadenylation site selection in vivo HEK293T cells were transiently transfected with DNA constructs expressing exogenous FLAG-tagged Hu
proteins and quantitative real-time PCR was used to detect expression of the HuR 6.0-kb mRNA transcript. As shown in Figure 6A, when Hu family member proteins were overexpressed for 24 h there was a >2-fold increase in the expression of the longer 6.0-kb HuR mRNA isoform while overall HuR mRNA levels remained unchanged. Overexpression of an unrelated protein (GFP) or of a control RNA binding protein (PABP) had no effect on HuR mRNA isoform expression, indicating that the increase in the 6.0-kb HuR transcript was due to a specific effect of the Hu proteins. Similar results were obtained when Hu-expressing HEK293 stable cell lines were used (Supplementary Figure S3). Taken together, these data suggest that increased expression of Hu family members can indeed drive alternative polyadenylation of the HuR mRNA in vivo.

However, as mentioned previously, Hu family members have also been implicated in many other aspects of mRNA regulation including splicing, stability and translation. Indeed, our analyses presented here, as well as PAR-CLIP analysis from our lab and others, confirm that there are multiple Hu binding sites in the HuR 3'-UTR, both in the ubiquitous, as well as the neuron-specific sequences (Supplementary Figures S4 and S5). It was therefore possible that the increase in the 6.0-kb mRNA isoform was instead due to an effect on mRNA stability following overexpression of the Hu family members. To investigate this possibility, we examined HuR mRNA half lives in HEK293 cells stably overexpressing the Hu family members. Stable overexpression of HuR, B or C had no significant effect on the half-life of the HuR 6.0-kb mRNA isoform (Figure 6B), supporting our hypothesis that the increase in the HuR 6.0-kb mRNA isoform is attributable to a change in polyadenylation. Furthermore, while we have been able to show direct binding to the HuR mRNA, we have been unable to definitively prove that the effect on polyadenylation is direct, due in part to the multitude of Hu binding sites found within the HuR mRNA (see Supplementary Figure S4).

Regulation of HuR mRNA translation

To investigate the effect of Hu overexpression on translation, we generated luciferase constructs containing various portions of the HuR 3'-UTR fused to the firefly luciferase ORF (Figure 6D). Equimolar amounts of these constructs were then transfected into HEK293T cells, using a separate renilla luciferase construct as a transfection control. As shown in Figure 6C, inclusion of the neuron-specific UTR sequence (0-4818) greatly downregulated luciferase expression when compared to the ubiquitous UTR (1-1256) construct. Removal of the common UTR sequence from the longer constructs (1253–4818) further downregulated expression, with all constructs showing significantly decreased expression when compared to the 2.4-kb transcript’s 3'-UTR (0–1256; P < 0.05). Interestingly, overexpression of HuR significantly downregulated luciferase expression from the construct containing the neuron-specific 3'-UTR (0–4818), which includes the 2.4-kb polyadenylation site but had minimal

Figure 6. Overexpression of the neuron-specific Hu Family members (HuB, HuC and HuD) affects alternative polyadenylation of HuR mRNA. (A) Real time PCR results of transiently transfected HEK293T cells expressing the indicated FLAG tagged constructs demonstrating increased expression of alternatively polyadenylated HuR 6.0-kb mRNA, while maintaining overall HuR levels (Total). GapDH mRNA level was used as a control. Values were calculated as fold change ratios using ΔCt values ±SD. The means and SD's are represented from three independent experiments. Asterisks indicates P < 0.05 as determined by Fisher’s least significant difference (LSD) procedure. (B) Overexpression of Hu family members does not affect mRNA stability of the 6.0-kb HuR isoform. Stable HEK293 cells overexpressing the indicated constructs were treated with the transcriptional inhibitor Actinomycin D and total RNA collected from 0–8 h. HuR mRNA levels were determined via real time PCR and mean half lives were calculated via linear regression and shown ±SD from 4 independent experiments. (C) HEK293T cells were transiently transfected with equimolar amounts of firefly luciferase constructs containing the indicated portion of the HuR 3'-UTR (depicted in D) along with renilla luciferase to control for transfection efficiency. Experiments were done in the absence (Ctrl) or presence (HuR) of HuR overexpression and luciferase ratios normalized to the empty vector (pLuc) expression. Asterisks indicates P < 0.05 as determined by unpaired Student’s t-test.
effect on other constructs lacking the polyadenylation signal, causing a slight decrease in 1239–2646 expression, and an increase in expression of 3775–4469 (Figure 6C).

Moreover, overexpression of the other Hu family members had a similar effect on the full-length (0–4818) neuron-specific construct (data not shown).

In conclusion, our results suggest that while HuR mRNA exists as three separate isoforms, the ubiquitous 2.4-kb isoform is expressed in the majority of cell types, and hence is the relevant species for most studies while the 6.0-kb isoform is clearly neuron-specific, and therefore, has a restricted natural environment. Furthermore, we have shown that the 6.0-kb isoform of HuR arises from neuron-specific alternative polyadenylation which appears to occur through the actions of the neuron-specific Hu family members, HuB, C and D. In the neuron, this 6.0-kb mRNA is much less stable and its expression results in a decrease in HuR protein production. Interestingly, while the presence of the neuron-specific UTR is sufficient to downregulate HuR expression, the Hu family members appear to be able to further repress the 6.0-kb isoform providing another layer of post-transcriptional control during neuronal development.

DISCUSSION

HuR is well documented to exert its post-transcriptional control by up-regulating the expression of multiple mRNAs (2,4). In this study, however, we focused on the post-transcriptional regulation of the HuR mRNA itself. Our study has revealed a unique mechanism by which the neuron-specific Hu family members HuB, C and D can regulate the alternative polyadenylation of HuR mRNA during neuronal differentiation. Our results coincide with a recent publication showing HuR can similarly auto-regulate its own polyadenylation, a mechanism proposed to help maintain elevated HuR levels in proliferating cancer cells (36). Overall, these findings are consistent with a regulatory model by which differentiating neuronal cells may utilize the neuron-specific Hu family members to keep HuR’s proliferative potential in check. According to this model, by regulating polyadenylation site selection of the HuR mRNA, and thereby decreasing the stability of HuR mRNA and resultant protein expression, the neuron-specific ELAV/Hu family members would be able to post-transcriptionally control HuR’s growth promoting properties during a time when they are also contributing to the process of neuronal differentiation.

We previously demonstrated that human and mouse HuR mRNA exists in three isoforms in a tissue-specific manner (25). A 2.4-kb mRNA isoform of HuR is expressed in almost all tissues and cell types examined, a shorter 1.5-kb mRNA isoform exists in the testes, while in brain the predominant form detected is ~6.0 kb. In the present study, we have cloned the full length HuR mRNA representing the 6.0-kb isoform and investigated its expression and regulation during neuronal differentiation. These data, combined with previous data from our lab, as well as existing microarray data, strongly indicate that HuR mRNA exists predominantly as a 2.4-kb isoform in the majority of cells and tissues. However, in neurons, HuR mRNA is found as a 6.0-kb transcript variant that arises from alternative polyadenylation. Corroborating these findings is the strong correlation observed between expression of the neuron-specific ELAV/Hu family members and the alternative polyadenylation of the HuR mRNA. These observations led us to investigate the mechanism and functional outcome of this alternative polyadenylation of HuR mRNA during neuronal differentiation.

Using the P19 system of retinoic acid induced neuronal differentiation, we were able to show that the expression of the 6.0-kb neuron-specific mRNA isoform is upregulated during neuronal differentiation and that a switch in preferred polyadenylation site correlates with the induction of expression of the other Hu family members. The correlation with the neuron-specific family members is intriguing given the fact that the Hu family members have been implicated in alternative polyadenylation of other gene transcripts and exemplifies the neuron-specific nature of the 6.0-kb mRNA isoform. While a more recent study suggests that the longer 6.0-kb transcript is simply a minor mRNA isoform that is only detectable by PCR (35), previous studies using microarray analysis have also found the longer transcript expressed exclusively in the nervous system (56–58). Our results strongly suggest that while this longer mRNA isoform can be detected at very low levels in other cell types, it is highly expressed in the nervous system where its extended 3′-UTR functions to keep HuR protein expression in check. Furthermore, based on these findings, neurons provide a more appropriate biological context in which to study the post-transcriptional regulation of this 6.0-kb mRNA isoform of HuR.

Recently, the Hu RBP family members have been reported to be involved in regulating alternative polyadenylation of mRNA by blocking cleavage and polyA addition at sites that are flanked by U-rich sequences resulting in use of an alternative polyadenylation site (17). Our investigation has revealed that HuR mRNA contains multiple U-rich sequences flanking the ubiquitous 2.4-kb polyadenylation site and we were able to show that overexpression of each of the three Hu family members induces alternative polyadenylation of the HuR mRNA, while not affecting mRNA stability. Furthermore, we have been able to show direct binding of HuB, C and D to sites near the 2.4-kb polyadenylation site, potentially blocking access of the polyadenylation machinery, as has been reported for the SV40 Late (SVL) and calcitonin/calcitonin gene-related peptide (CGRP) mRNAs in vitro (17). Indeed, we have also shown that the Hu neuron-specific family members, as well as HuR itself, can bind to multiple regions of the HuR 6.0-kb 3′-UTR, in addition to the 2.4-kb polyadenylation site, consistent with other studies that have studied HuR autoregulation (35,36,59). However, from our studies in HEK293 and HEK293T cells, it appears that the neuronal ELAV/Hu family members are more efficient than HuR itself at promoting HuR’s alternative polyadenylation. Although this observation
remains to be rigorously tested, it would support the hypothesis that the switch in HuR’s polyadenylation is a neuron-specific event that regulates HuR protein levels at a time when terminal differentiation is taking place.

While previous studies using reporter constructs have suggested that HuR overexpression stabilizes its own 6.0-kb mRNA isoform (35,59), our results, along with recently published results (36), show that HuR is involved in a negative feedback loop, at least in relation to its 6.0-kb mRNA isoform. This discrepancy is most likely due to the fact that previous studies were not conducted in the context of the full length UTR, and instead relied on fragments of the UTR. Indeed, in our study, while the full length, neuron-specific mRNA isoform was down regulated in the presence of HuR, there was variability in the response of the isolated fragments, including one that showed an increase in stability/translation in response to HuR protein expression. These discrepancies highlight the importance of studying post-transcriptional regulation in the context of the full-length UTR and in a biologically relevant cell type. While UTR fragments are useful in elucidating the actual sites of protein binding and potential regulation, when taken out of structural and cellular contexts functional results obtained by fragment analysis alone may be misleading.

Moreover, it is also important to determine that the mRNA isoform being studied is actually expressed in the cells/tissue of interest. Many studies of HuR mRNA and other mRNAs have relied on reporters and probes derived from alternative UTR sequences, when in fact the cells being studied may not normally express that particular mRNA isoform. Our study suggests that while the 6.0-kb isoform of HuR mRNA can be detected by PCR in most cells, it is likely a minor species of mRNA and its biological relevance is likely restricted to neuronal cells and the nervous system, while the 2.4-kb isoform appears to be the more predominant and relevant mRNA isoform for other cell types. This issue arises because databases often arbitrarily list only one mRNA isoform, not necessarily the most common or the most functionally relevant. Given that recent studies suggest that 40–50% of all human and mouse genes contain multiple polyadenylation sites and can undergo alternative polyadenylation (60–63), this phenomenon and the above may be more widespread than previously appreciated.

Finally, we have been able to show through a combination of reduced mRNA half-life and/or reduced translation, that the 6.0-kb neuron-specific mRNA transcript of HuR produces much less protein than the ubiquitous 2.4-kb isoform. While this appears to be in part due to negative feedback via the Hu family members, it is possible that other post-transcriptional regulators, including miRNAs and the RNA-induced silencing complex (RISC), may play a role as well. Indeed, while we were able to manipulate HEK293 cells to induce the alternative polyadenylation function, the effect was slight compared to P19 cells, and we saw no effect on the overall HuR protein levels in HEK cells, suggesting that indeed some other neuron-specific factors and/or environment are likely needed for the full effect. The net result, however, appears to lead to decreased HuR protein in differentiated neurons. This observation is supported by evidence from another study that looked at RBP levels during aging in a variety of tissues (64). While HuR protein was easily detectable in most tissues, it was reduced in both neurons and muscle, and with increasing age, decreased further in abundance in the nervous system.

This provides a plausible explanation to address the apparent conundrum of why all four Hu family members are expressed in terminally differentiated neurons. Because HuR is a growth-promoting factor, we hypothesize that down regulation of HuR is necessary for proper terminal differentiation to occur. Indeed, correlation of the expression of the 6.0-kb neuron-specific HuR mRNA isoform with expression of the other Hu family members supports this hypothesis. It is conceivable that while maintaining a stem cell-like phenotype, HuR expression is necessary for promoting growth and proliferation to support self-renewal of the stem cells. In fact, it has been previously reported that HuR is required for maintenance of murine progenitor cells (23). This is in direct contrast to the established role of the neuronal Hu family members (HuB, HuC and HuD) that have been shown to be necessary for neuronal differentiation in a variety of model systems. Therefore, by placing HuR’s polyadenylation and regulation under the control of the neuron-specific ELAV/Hu family members, there is a potentially more secure mechanism to activate differentiation. By down regulating HuR expression in neurons when HuB, C and D are upregulated, HuR’s growth promoting influence may be mitigated and neuronal differentiation allowed to progress. This extra layer of control would also ensure that when conditions are not favorable for differentiation, HuR expression could be maintained for continued growth and proliferation of the progenitor cell.

Overall, the role of the ELAV/Hu family members in alternative polyadenylation during neuronal development is likely to involve numerous downstream mRNA targets that encode factors crucial to the balance between proliferation and differentiation. Indeed, it has been observed that 3’-UTRs of mRNAs expressed in neuronal tissues of both mouse and drosophila progressively lengthen throughout development, presumably through global changes in polyadenylation (65,66). Investigating the mechanism that leads to this global switch in polyadenylation and how the downstream mRNA targets of RBPs are globally regulated will advance our understanding of neuronal differentiation.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online: Supplementary Figures 1–5 and Supplementary Tables 1 and 2.

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