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# The RNA-binding Protein HuD Is Required for GAP-43 mRNA Stability, GAP-43 Gene Expression, and PKC-dependent Neurite Outgrowth in PC12 Cells

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The RNA-binding protein HuD binds to a regulatory element in the 3′ untranslated region (3′ UTR) of the GAP-43 mRNA. To investigate the functional significance of this interaction, we generated PC12 cell lines in which HuD levels were controlled by transfection with either antisense (pDuH) or sense (pcHuD) constructs. pDuH-transfected cells contained reduced amounts of GAP-43 protein and mRNA, and these levels remained low even after nerve growth factor (NGF) stimulation, a treatment that is normally associated with protein kinase C (PKC)-dependent stabilization of the GAP-43 mRNA and neuronal differentiation. Analysis of GAP-43 mRNA stability demonstrated that the mRNA had a shorter half-life in these cells. In agreement with their deficient GAP-43 expression, pDuH cells failed to grow neurites in the presence of NGF or phorbol esters. These cells, however, exhibited normal neurite outgrowth when exposed to dibutyryl-cAMP, an agent that induces outgrowth independently from GAP-43. We observed opposite effects in pcHuD-transfected cells. The GAP-43 mRNA was stabilized in these cells, leading to an increase in the levels of the GAP-43 mRNA and protein. pcHuD cells were also found to grow short spontaneous neurites, a process that required the presence of GAP-43. In conclusion, our results suggest that HuD plays a critical role in PKC-mediated neurite outgrowth in PC12 cells and that this protein does so primarily by promoting the stabilization of the GAP-43 mRNA.

## INTRODUCTION

In addition to transcriptional factors, RNA-binding proteins play a critical role in the developmental control of gene expression. Among these is ELAV (embryonic lethal abnormal vision), an RNA-binding protein identified in *Drosophila*, where the gene is required for normal development and maintenance of the nervous system (Campos *et al.*, 1985; Robinow *et al.*, 1988). In higher vertebrates and mammals, four members of the ELAV-like family have been identified. These are also referred to as Hu proteins, namely HuR (also known as HuA), HuB (Hel-N1), HuC, and HuD, because these are targets of anti-Hu antibodies present in the sera of patients with paraneoplastic encephalomyelitis (Dalmau *et al.*, 1992). HuR is ubiquitously expressed (Ma *et al.*, 1996), while HuB, HuC, and HuD are expressed uniquely in the nervous system. Recent studies indicate that overexpression of neural ELAV-like proteins is sufficient to induce neuronal differentiation in vitro and in vivo (Wakamatsu and Weston, 1997; Akamatsu *et al.*, 1999; Antic *et al.*, 1999; Kasashima *et al.*, 1999). While the exact function and targets of ELAV/Hu proteins remain to be fully elucidated, it seems likely that this family of RNA-binding proteins controls neuronal differentiation by selectively modulating the expression of neural-specific, growth-associated genes.

The growth-associated protein GAP-43 is expressed in neurons primarily during the initial establishment and regeneration of neural connections (Skene, 1989; Benowitz and Routtenberg, 1997). During the development of the nervous system, GAP-43 expression correlates with axonal growth in

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all neural pathways examined (Benowitz and Perrone-Bizzozero, 1991). Both transcriptional and posttranscriptional mechanisms control GAP-43 gene expression in neurons. Basic helix-loop-helix proteins (bHLH) interact with sequences in the promoter region to regulate the neural-specific expression of the gene (Nedivi *et al.*, 1992; Eggen *et al.*, 1995; Chiaramello *et al.*, 1996; Kinney *et al.*, 1996). In addition, posttranscriptional mechanisms control GAP-43 gene expression in PC12 cells (Federoff *et al.*, 1988; Perrone-Bizzozero *et al.*, 1991, 1993) and in hippocampal neurons in vivo (Cantallops and Routtenberg, 1999). In PC12 cells, NGF induces the stabilization of the GAP-43 mRNA via a mechanism that depends on protein kinase C (PKC) activation but does not require de novo protein synthesis; Perrone-Bizzozero *et al.*, 1991; Perrone-Bizzozero *et al.*, 1993). Moreover, GAP-43 mRNA stability depends on the interaction of highly conserved sequences in the 3′ untranslated region (3′ UTR) of the mRNA (Kohn *et al.*, 1996) and neuronal-specific RNA-binding proteins (Kohn *et al.*, 1996; Irwin *et al.*, 1997). One of these GAP-43 mRNA-binding proteins was recently identified as the ELAV-like protein HuD (Chung *et al.*, 1997). This protein binds to the GAP-43 3′ UTR within a region that contains a U-rich TPA-responsive element that controls mRNA stability (Tsai *et al.*, 1997).

In view of HuD's neuronal-specific expression (Okano and Darnell, 1997; Clayton *et al.*, 1998), developmental regulation (Wakamatsu and Weston, 1997), and binding properties (Chung *et al.*, 1997), we proposed that this RNA-binding protein was involved in the posttranscriptional regulation of the GAP-43 gene during neuronal differentiation. To test this hypothesis, we measured the levels of the GAP-43 mRNA, protein, and neurite outgrowth in PC12 cells expressing either high levels of HuD antisense RNA (pDuH) or HuD sense RNA and protein (pcHuD). Using antisense methods, we found that HuD is required for the control of GAP-43 mRNA stability and induction of GAP-43 expression and neurite outgrowth, in response to PKC activation by NGF or phorbol esters. Alternatively, overexpression of HuD in PC12 cells was sufficient to increase GAP-43 mRNA stability, protein expression, and GAP-43-dependent process outgrowth. These results confirm the role of this RNA-binding protein in the posttranscriptional control of the GAP-43 gene and emphasize the significance of this ELAV-like protein in early events in neural development.

## MATERIALS AND METHODS

### *Plasmid Preparation*

The pDuH and pcHuD constructs were prepared using HuD cDNA from the pGEX-HuD construct (Chung *et al.*, 1996). The HuD fragment was removed from the pGEX vector with *Bam*HI and ligated into the same restriction enzyme site in pcDNA3 (Invitrogen, Boston, MA). After amplification, plasmids were screened for antisense (pDuH) orientation of the HuD cDNA. To prepare pcHuD, the HuD cDNA was cloned in the sense orientation in pcDNA3, downstream of the myc-tag sequence (EQKLISEEDL) engineered adjacent to the *Hin*dIII site in the vector.

### *Generation of Stable Transfectants*

Transfection experiments were performed on the original PC12 cell line (Greene and Tischler, 1976) and on two different PC12 cell clones isolated by Dr. Richard Burry, namely the PC12-N21 clone, which contains GAP-43 and exhibits a typical phenotypic differentiation in response to NGF (Burry and Perrone-Bizzozero, 1993), and the GAP-43 deficient subclone PC12-N36, which does not differentiate in the presence of the growth factor (Tsai *et al.*, 1997). Cells were grown at 37°C, 5% $CO_2$, in RPMI-1640 media supplemented with 7.5% horse serum and 2.5% fetal calf serum (Perrone-Bizzozero *et al.*, 1993). All cell transfections were performed by electroporation as previously described (Tsai *et al.*, 1997). Cells transfected with pcDNA3 vectors were selected with the neomycin analog G418 (500 $\mu$g/ml). Stable transfectants of PC12-N36 cells with the GAP-43 cDNA in pMEP4 vector (Invitrogen) were maintained with hygromycin B (150 $\mu$g/ml, Calbiotech, San Diego, CA). Cotransfection experiments used double selection with both G418 and hygromycin B.

### *Cell Morphology*

Control and transfected PC12 cells were cultured on poly-lysine-coated dishes and treated with either nerve growth factor (NGF; 100 ng/ml), 12-O-tetradecanoylphorbol-13-acetate (TPA; 160 nM), or dibutyryl cyclic AMP (cAMP; 1 mM). After treatment, cells were grown for 48 or 96 h, then fixed with 4% paraformaldehyde (PFA). Phase contrast micrographs were obtained using a Zeiss Axiovert microscope (Carl Zeiss, Inc, Thornwood, NY). In some experiments, cells were stained with 0.1% Coomassie blue (Fisher, Norcross, GA) in 50% methanol/10% acetic acid to aid in neurite visualization as previously described (Perrone-Bizzozero *et al.*, 1986). Cells were classified as undifferentiated, if they were round with no processes, or differentiated, if they were bipolar or polygonal with one or more processes longer than the diameter of the cell body. At least 300 cells were analyzed per condition. Statistical analyses were performed using a two-tailed Student's *t* test.

### *Immunocytochemistry*

PC12 cells were grown for 48 or 96 h on poly-L-lysine-coated Labtek slides (Nunc, Milwaukee, WI), then fixed with 4% PFA for 30 min at room temperature. Fixed cells were incubated for 20 min at room temperature, in buffer containing 2% bovine serum albumin (BSA), in Tris-buffered saline (TBS) at pH 7.4, with 0.1% Triton (T) X-100 (BSA-TBST). GAP-43 was detected with a sheep polyclonal antibody (Benowitz *et al.*, 1988) at 1:250 dilution. Cells were incubated with the primary antibodies for 2 h, then incubated with donkey antish-eep-FITC (1:50, Sigma Immunochemicals, St. Louis, MO) in BSA-TBST for 1 h in the dark. Coverslips were rinsed with phosphate buffered saline (PBS) and mounted onto glass slides with PermaFluor aqueous mounting media (Immunon, Pittsburgh, PA). Photographs were taken on a Zeiss Axiovert microscope at 400X magnification. For some experiments, images were scanned using an Olympus microscope (BX60) with color video camera (Optronics DEI-470), and GAP-43 immunofluorescence was measured using the Bioquant image analysis software (R and M Biometrics, Nashville, TN). Measurements were determined for control cells and NGF-induced cells. Data were collected from at least three fields per condition and cell type.

### *Generation of HuD-specific Antibodies*

Because none of the antibodies available against ELAV/Hu proteins recognize specific members of this family (King *et al.*, 1994; Barami *et al.*, 1995), we generated polyclonal antibodies ($\alpha$-HuD) using an N-terminal peptide that is uniquely present in HuD (SNNRNCPSP-MQTGA, Research Genetics, Huntsville, AL). The specificity of the antibody for HuD is shown in Figure 1A.

### *Western Blot Analysis*

Proteins from control and transfected cell lines were extracted using a simultaneous RNA/DNA/protein isolation reagent (Tri reagent, Sigma, St. Louis, MO) and solubilized in 0.1% SDS. Aliquots con-

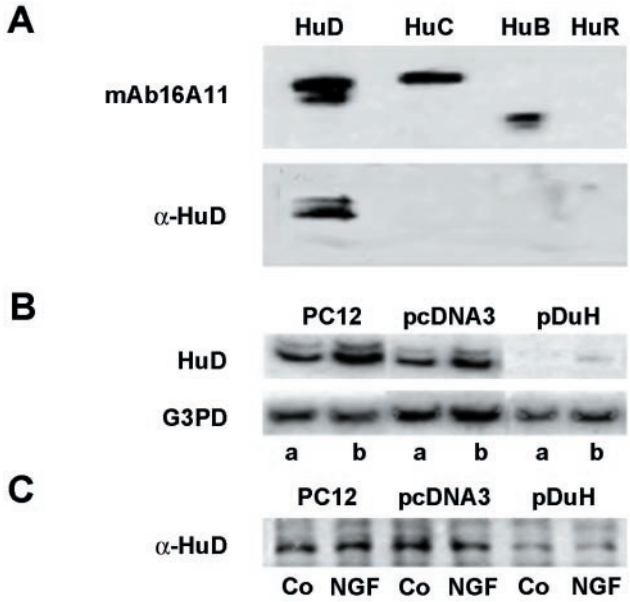


**Figure 1.** Effect of antisense RNA on the levels of HuD mRNA and protein. (A) Specificity of the anti-HuD antibody. Recombinant GST-HuD, GST-HuR, GST-HuC, and 6XHis-tagged-HuB proteins were purified and 500 ng of each protein was analyzed by Western blots using the mAb16A11 or our anti-HuD polyclonal antibodies, using chemiluminescent reagents. The same gel was exposed to both antibodies. (B) RT-PCR analysis of RNAs isolated from pDuH, pcDNA3, and untransfected PC12-N21 cells. Aliquots containing 0.5 $\mu$g (a) and 1 $\mu$g (b) of RNA per 50 $\mu$l reaction were amplified using rat specific HuD primers. The RNA was diluted 20-fold for G3PD amplification. (C) Western blot analysis of HuD in control cells and cultures transfected with pDuH or pcDNA3. The level of HuD protein in both control and NGF-treated cells was detected with our HuD specific antibody. Coommassie blue staining demonstrated an equal protein loading in all lanes (data not shown).

taining 50 $\mu$g protein were separated on 10% (wt/vol) polyacrylamide gels and electrotransferred onto Immunoblot PVDF membranes (Bio-Rad Laboratories, Hercules, CA). HuD levels were determined using our antipeptide antibody, while GAP-43 was detected using a polyclonal antibody to rat GAP-43 as previously described (Perrone-Bizzozero *et al.*, 1996). Specific protein bands were visualized using Renaissance Western blot chemiluminescence reagent (Dupont NEN, Boston, MA). Loading variations in protein levels in each band were corrected using either the density of the Coomassie Blue staining or a monoclonal anti-$\beta$-tubulin clone TUB 2.1 (Sigma, St. Louis MO). The amount of protein was determined densitometrically using the FotoAnaylyst system (Fotodyne, Inc., New Berlin, WI).

### *Northern Blots and Quantitative RT-PCR*

After RNA extraction, 15 $\mu$g total RNA from each sample was run on 1.1% formaldehyde-agarose gels, as described by Perrone-Bizzozero *et al.*, (1993). Membranes were probed for GAP-43 mRNA or glycerol-3-phosphate dehydrogenase (G3PD) mRNA using $^{32}$P-radiolabeled cDNA probes generated using random priming (Prime-a-Gene, Promega, Madison, WI).

RT-PCR reactions were performed using the Titan One Tube RT-PCR System (Roche Molecular Biochemical, Indianapolis, IN). Reaction components were as described in the manufacturer's protocol, with the addition of 1 $\mu$Ci $^{32}$P $\alpha$-dCTP per PCR reaction. Cycle and quantity titrations were used to determine the linear range of amplification for each set of primers used. RT-PCR reactions using HuD and GAP-43 primers and 0.5 and 1.0 $\mu$g RNA were within the linear range of response between 18 and 26 cycles (data not shown). Similarly, reactions were quantitative for G3PD using a 20-fold dilution of the total RNA and the same PCR conditions. The temperature profile for PCR reactions was 45 min at 45°C, 2 min at 94°C, 22 cycles of 94°C for 30 s, 55°C for 1 min, and 68°C for 2 min, followed by a final cycle of 7 min at 68°C. Primers used for RT-PCR reactions were: 5′ATAAGTAAGGGTGAGAAATTCAGG3′ and 5′TGCTTAATATGGCCTTATGGCG3′ (Steller *et al.*, 1996) for rat HuD, 5′GGAATAAGGATCCGAGGAGGAAAGGAG3′, 5′CTTAAAGTTCAGGCATGTTCTTGGT3′ (Basi *et al.*, 1987; Karns *et al.*, 1987) for rat GAP-43, and 5′CCCACGGCAAGTTCAAC3′ and 5′TGGCAGGTTTCTCCAGGCGGC3′ (accession number XO2231) for G3PD. Rat-specific HuD primers were derived from nonconserved sequences in the 3′ UTR and did not detect human HuD sequences (data not shown). The Protein Chemistry Laboratory of the University of New Mexico Health Sciences Center prepared all primers. PCR reactions were performed in a Perkin Elmer-Cetus (Wellesley, MA) Gene Amp System 9600. Reaction products were analyzed on 5% acrylamide-TBE gels (Bio-Rad) and quantitated using a phosphorimager (Storm 860, Molecular Dynamics) and ImageQuant software.

### *GAP-43 mRNA Stability Analysis in Transfected Cell Lines*

To determine the effect of HuD on the stability of GAP-43 mRNA, PC12-N36 cells were cotransfected with the full-length GAP-43 in pMEP4 (Kohn *et al.*, 1996) and pcHuD, pDuH, or pcDNA3. RNA decay assays were performed as previously described (Tsai *et al.*, 1997). Briefly, cultures were treated with 5 $\mu$M cadmium, which causes an 8- to 10-fold activation of the human metallothionein-IIA promoter in the pMEP4 vector. After induction, cadmium was removed, and cells were collected at various intervals. RNA was isolated with the Tri reagent according to the manufacturer's recommended protocol. Northern blots were probed with radiolabeled cDNAs for GAP-43 and G3PD. The intensity of mRNA bands was determined either densitometrically or using a phosphorimager. Loading variations were corrected by normalizing optical densities of the GAP-43 bands with those of G3PD. The half-life ($T_{1/2}$) of the GAP-43 transcripts was calculated from the slope of the plot of logarithm of relative mRNA levels versus time of decay, using linear regression analysis (Perrone-Bizzozero *et al.*, 1993).

### *HuD Immunoprecipitation Studies*

The in vivo interaction of the GAP-43 mRNA and HuD protein was investigated using a combination of immunoprecipitation and RT-PCR analysis that was recently developed for other ELAV-like proteins (Antic *et al.*, 1999). HuD was immunoprecipitated from cell extracts essentially as we have previously described for the separation of UV-cross-linked RNA-protein complexes from in vitro binding reactions (Chung *et al.*, 1997). Briefly, cell extracts were prepared using a buffer containing 1% NP-40, 10 mM Tris-HCl, pH 7.5, 1% BSA, 150 mM NaCl, and 2 mM EDTA. Our anti-HuD antibody was added at 1:100 dilution, and antigen-antibody complexes were separated using Protein-G-beads (Sigma). RNAs were purified from the beads using RNA Aqueous kit (Ambion, Austin TX) and RT-PCR reactions for GAP-43 were performed as indicated before.

## RESULTS

### *Antisense HuD RNA Decreases HuD and GAP-43 Expression in PC12 Cells*

To begin our assessment of HuD protein function in GAP-43 gene expression and neuronal differentiation, we generated

stable transfectants of PC12 cells with the pcDNA3 vector containing human HuD sequences in the antisense orientation (pDuH). Transfection experiments were performed using the original PC12 cell line (Greene and Tischler, 1976) and PC12-N21, a clone that shows a robust differentiation in response to NGF (Burry and Perrone-Bizzozero, 1993). To avoid selection of a particular cell phenotype, stable transfectants of both the original PC12 cell line and the N21 clone were selected without further cloning and used in parallel for all the experiments. Given that both transfected lines showed identical phenotypic responses in our studies, representative results from each of these PC12 cells are shown herein.

HuD specific primers and antibodies were used to evaluate the levels of endogenous mRNA and protein in control and transfected PC12 cells. Our HuD polyclonal antibodies specifically recognized recombinant human HuD (Figure 1A) and endogenous rat protein (Figure 1C), but they did not react against the neuronal ELAV-like proteins HuC and HuB or with the ubiquitously expressed HuR. In contrast, the anti-Hu mAb16A11 antibody (Marusich *et al.*, 1994; Wakamatsu and Weston, 1997) recognized all three neuronal ELAV proteins, HuB, HuC, and HuD, but did not react against HuR. The presence of HuR in the blot was confirmed using an antibody that specifically recognizes this ELAV-like protein (Wang *et al.*, 2000; data not shown). Rat-specific HuD primers (Steller *et al.*, 1996) were used to determine the levels of the endogenous mRNA in the presence of exogenous human sequences. As reported by Steller *et al.*, (1996), we detected a major and a minor PCR product for HuD in PC12 cells, of 360 and 400 bp, respectively (Figure 1B). These sizes correspond to two of the mRNA splicing variants, HuD and HuDpro (Liu *et al.*, 1995). Analysis of HuD mRNA levels in the different cells indicated that this mRNA was significantly reduced (by 75%) in pDuH transfected cells relative to untransfected (PC12) or vector only (pcDNA3) transfected cells (Figure 2A). In agreement with the RT-PCR data, Western blots revealed that the antisense treatment resulted in decreased levels of HuD protein in pDuH cells (Figure 1C). Although ~ 30% of residual HuD remained in the cells, the decrease was specific for this protein and did not affect the levels of other ELAV-like proteins in the cells (data not shown). In agreement with Steller *et al.*, (1996), we found that NGF did not affect the expression of this protein within the first 24 h of treatment. As the NGF-dependent stabilization of the GAP-43 mRNA does not require newly synthesized proteins, it is likely that HuD is the previously described translation-indepedent factor involved in this process (Perrone-Bizzozero *et al.*, 1993).

Having established the effects of HuD antisense RNA on HuD levels, we used Western and Northern blots to evaluate its effects on GAP-43 gene expression. Analysis of mRNA and protein levels in untransfected PC12, pDuH, and pcDNA3 transfectants demonstrated that all three cell types produced GAP-43 mRNA and protein (Figure 2). However, the endogenous GAP-43 mRNA levels in pDuH cells were only at 30% of the levels observed in the other cell types (Table 1). Thus, the low levels of GAP-43 in pDuH cells correlated well with residual HuD expression in these cultures. When PC12 cells were treated with NGF for 24 h, all cells responded with an approximate 2- to 3-fold induction of the mRNA and protein. However, in pDuH cells, this induction still left GAP-43 levels at 30% of those found in NGF-treated untransfected PC12 cells, which were equivalent to the levels of the protein in PC12 cells in the absence of NGF (Figure 1C). This result suggests that the residual HuD protein present in pDuH-transfected cells is not sufficient to mediate the NGF-dependent stabilization of the mRNA.

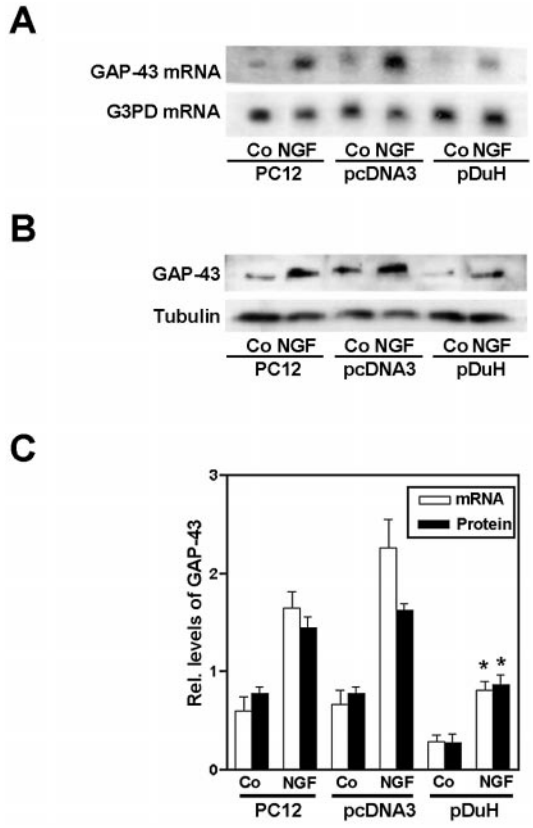


**Figure 2.** GAP-43 mRNA and protein levels in untransfected, pcDNA3 and pDuH cells. PC12 cells and PC12 N-21 cells were treated with NGF for 24 h, and total RNA and protein were isolated as described in MATERIALS AND METHODS. (A) Aliquots containing 15 $\mu$g total RNA were separated on a 1.2% agarose formaldehyde gel and transferred onto nylon membranes. Blots were probed with radiolabeled cDNAs for GAP-43 and G3PD. (B) Fifty $\mu$g each protein sample was separated on 10% polyacrylamide gels and transferred to PVDF membranes. Blots were probed with GAP-43 polyclonal antibody and tubulin antibody. Proteins were visualized using a chemiluminescent reaction. (C) Relative changes in the levels of GAP-43 mRNA and protein in both control and NGF-treated cells. Data shown are the mean ± SEM for three separate experiments using both PC12 and PC12-N21 cells. Asterisks denote significant differences ($p < 0.05$).

### *Decreased HuD Expression Results in the Destabilization of the GAP-43 mRNA*

We have shown previously that HuD binds to an element in the GAP-43 3′UTR that controls the stability of the mRNA (Chung *et al.*, 1997). Thus, it is likely that a decrease in HuD expression could affect the rate of degradation of the mRNA.

**Table 1.** Correlation between HuD levels and GAP-43 mRNA half-life and protein expression in PC12 cells

| PC12 Cells | HuD levels[a] (% control) | mRNA Half-life[b] ($T_{1/2}$ h) | % Change in $T_{1/2}$[c] | GAP-43[d] (% control) |
|---|---|---|---|---|
| pDuH antisense transfected cells | 30 | 3.4 ± 0.4 | 60 | 30 |
| pcDNA3 transfected cells (vector) | 100 | 5.1 ± 0.3 | 110 | 100 |
| Untransfected PC12 cells (control) | 100 | 5.0 ± 0.4 | 100 | 100 |
| pcHuD transfected cells | 300 | 9.0 ± 0.4 | 200 | 200 |

[a] Relative levels of endogenous and exogenous HuD protein measured by Western blot analysis.

[b] GAP-43 mRNA half-life ($T_{1/2}$, mean ± S.E.M.) was measured as described in MATERIALS AND METHODS.

[c] Percent changes were calculated relative to control untransfected cells.

[d] GAP-43 protein levels were measured by Western blot analysis and were expressed relative to control cells.

To evaluate this possibility, we examined the half-life of the GAP-43 mRNA in control and pDuH transfected cells (Figure 3). For these studies, a GAP-43 deficient PC12 subclone, PC12-N36, was cotransfected with full length GAP-43 cDNA in the inducible expression vector pMEP4 (Kohn *et al.*, 1996) and with either pDuH or pcDNA3. As in previous studies, cells were exposed for 16 h to 5 $\mu$M cadmium to induce high levels of GAP-43 mRNA via activation of the metallothionein promoter in pMEP4 (Tsai *et al.*, 1997). After induction, cadmium was removed and the RNA harvested at various time points. As shown in Figure 3, the GAP-43 mRNA in pcDNA3 transfected cells decayed with a half-life of 5 h, similar to that observed in untransfected PC12 cells. In pDuH cells, however, the half-life of the mRNA decreased to ~ 3 h, indicating that GAP-43 mRNA stability is linked to the levels of HuD protein in the cell (Table 1).

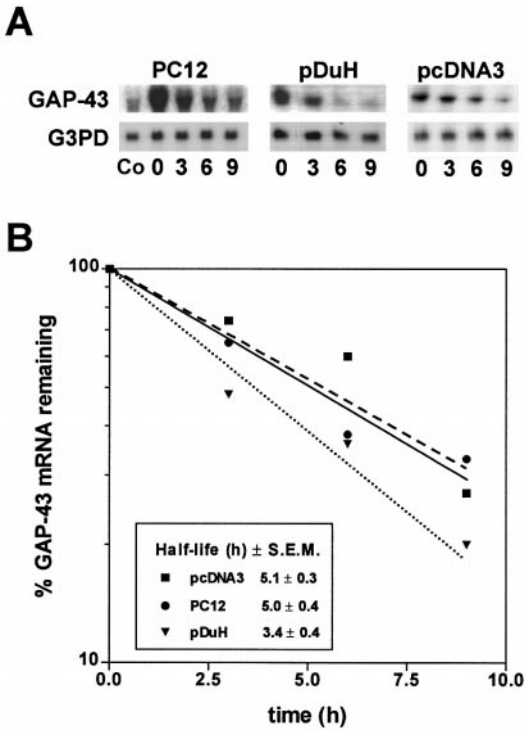


**Figure 3.** Effect of HuD antisense on the half-life of the GAP-43 mRNA. PC12-N36 cells were transfected with the full length GAP-43 in pMEP4, which contained the inducible metallothionein IIa promoter. Some cultures were cotransfected with pDuH or pcDNA3. After cadmium induction, mRNA decay assays were performed as indicated in MATERIALS AND METHODS. (A) Northern blots of a representative GAP-43 mRNA decay assay were probed with labeled cDNAs for GAP-43 and G3PD. The control lane (Co) shows the level of the mRNA due to the basal activity of the promoter in the absence of cadmium induction. (B) The levels of the GAP-43 mRNA were normalized to G3PD and were expressed relative to time 0 to calculate the half-life of the mRNA.

### *Defective Neurite Outgrowth in PC12 Cells Expressing Low Levels of HuD*

In view of the observed defect in GAP-43 gene expression in cells transfected with antisense HuD RNA, subsequent experiments examined the morphological differentiation of pDuH-transfected cells in response to NGF (Figure 4). Since GAP-43 is involved in early stages of neurite outgrowth, we examined the changes in cell morphology during the first 2 days of NGF exposure. At this time, neurites are still immature and elongate about one cell body length (Jacobs *et al.*, 1986). Unlike untransfected PC12 cells, we found that pDuH cells did not respond to NGF with increased outgrowth of neurites. After 48 h of stimulation, pDuH cells exhibited only very short stubby processes (Figure 4A), which failed to elongate even with longer NGF exposures (data not shown). In addition, pDuH cells did not exhibit the characteristic flattening and increase in cell body size that normally accompanies PC12 differentiation. Immunocytochemical analysis of GAP-43 expression in the cells (Figure 4B) demonstrated that the defect in neurite outgrowth correlated with a decrease in GAP-43 levels in pDuH cells. In unstimulated control PC12 and pDuH cells, GAP-43 was localized diffusely throughout the cytoplasm. After two days of NGF treatment, GAP-43 was still present in the cell body of PC12 cells, but more intense staining was seen in the processes and growth cones. In contrast, NGF-treated pDuH cells showed cytoplasmic localization of GAP-43 with intense staining in the perinuclear region. Analysis of the intensity of the immunofluorescence indicated that pDuH cells contained significantly lower levels of GAP-43 than wild-type PC12 cells and that these levels did not change in the presence of NGF (data not shown).

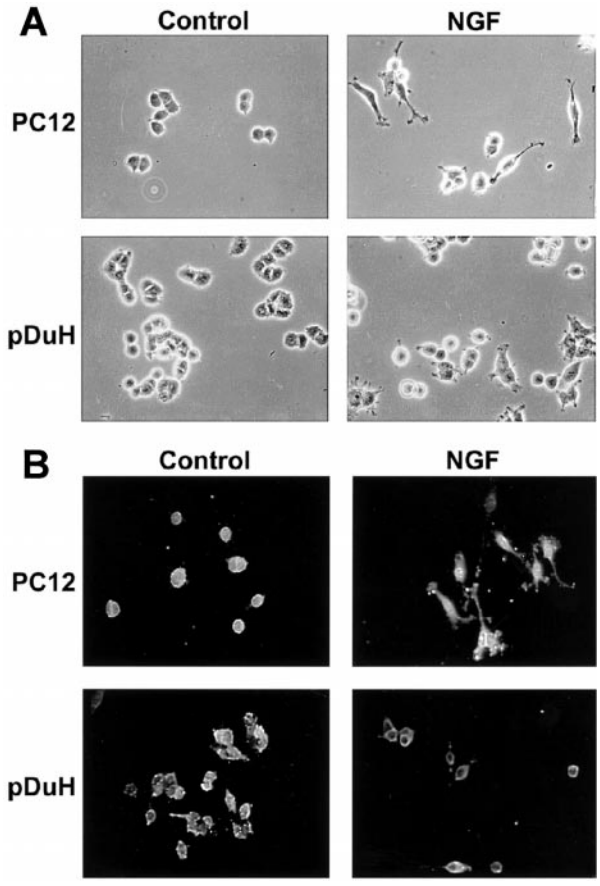


**Figure 4.** HuD antisense treatment (pDuH) blocks neurite outgrowth and GAP-43 expression in NGF-treated PC12 cells. Control and pDuH-transfected cells derived from the original PC12 cell line and the PC12-N21 clone were grown for 48 h in the presence or absence of NGF (100 ng/ml). Cells were then fixed with 4% PFA and processed for GAP-43 immunostaining using FITC-labeled secondary antibodies, as described in MATERIALS AND METHODS. (A) Phase contrast micrographs of untransfected PC12 cells and cells transfected with pDuH. Cells were photographed using an Axiovert Zeiss microscope at 400X magnification. (B) GAP-43 immunofluorescence in control and in pDuH transfected PC12-N21 cells. Images were taken using an Olympus BX-60 microscope at 400X magnification. Note that the HuD antisense RNA (pDuH) prevented neurite extension in both PC12 cell lines.

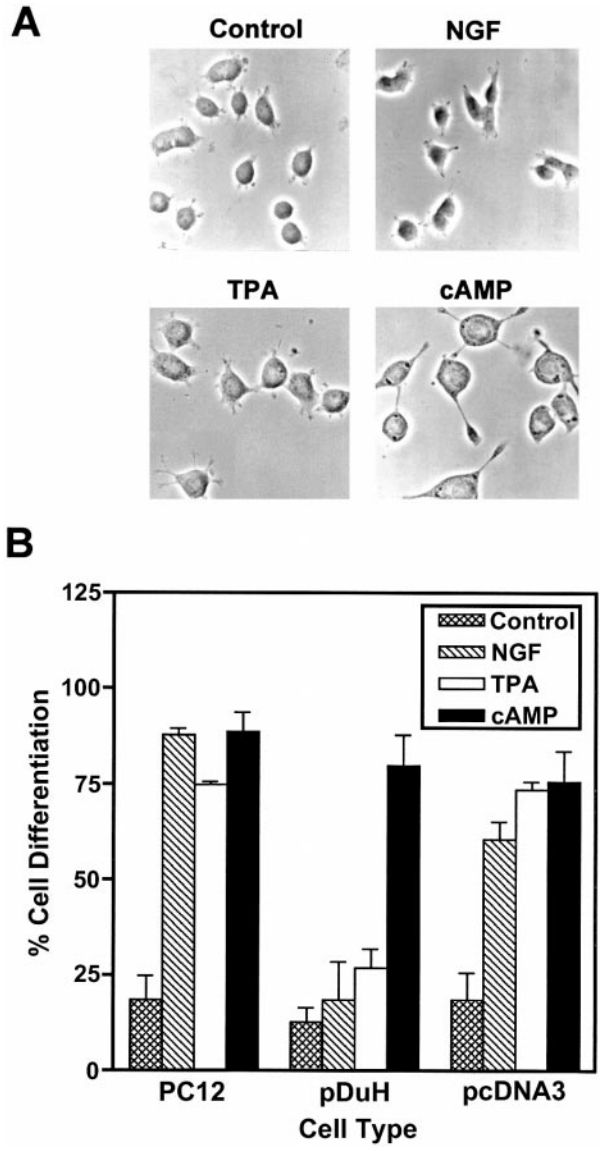


**Figure 5.** Effects of various agents on the differentiation of PC12-N21 cells expressing HuD antisense (pDuH). Cells were grown for 48 h in the presence of various agents before they were fixed with 4% PFA as follows: control, untreated; NGF, 100 ng/ml; TPA, 160 nM; dibutyryl-cAMP, 1 mM. (A) Microphotographs of cells cultured under different conditions. (B) Analysis of morphological differentiation of untransfected PC12 cells, pcDNA3, and pDuH cells in response to various agents. At least 300 cells per condition were counted and analyzed, as described in MATERIALS AND METHODS.

### *HuD Protein Is Required for Phorbol Ester-dependent, But Not for cAMP-dependent, Neurite Outgrowth*

To evaluate the extent of the defect observed in pDuH cells, additional studies compared the responses of pDuH, PC12, and pcDNA3-transfected cells to various agents that are known to mimic the effect of NGF on cell differentiation (Figure 5). Exposure of pDuH cells to the phorbol ester TPA failed to induce neurite outgrowth and resulted only in the extension of short stubby processes. In contrast, pDuH cells exhibited normal process outgrowth in the presence of dibutyryl-cAMP, an agent that induces neurite outgrowth via a different signaling pathway (Greene *et al.*, 1986; Burry, 1998). As expected, untransfected PC12 cells and pcDNA3-transfected cells exhibited normal process outgrowth in response to all agents (Figure 5B).

Phorbol esters are known to mimic the effect of NGF on GAP-43 expression (Perrone-Bizzozero *et al.*, 1993). Therefore, it was conceivable that the lack of response of pDuH

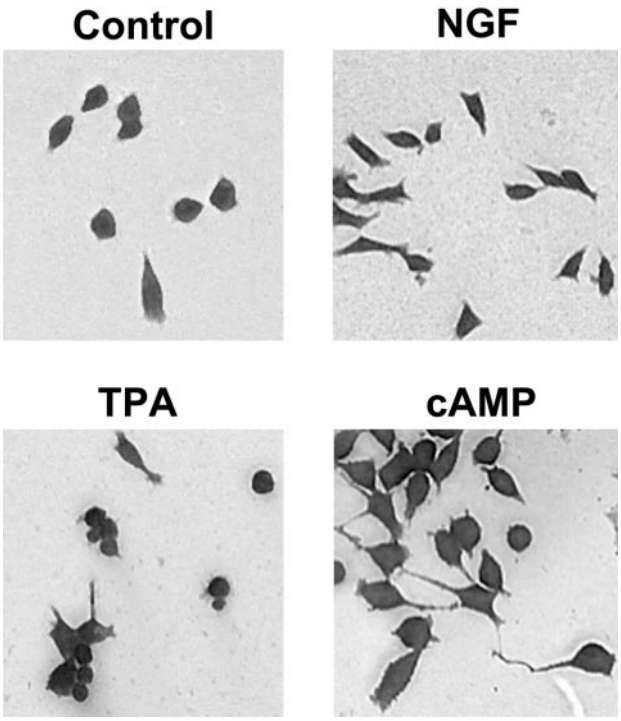


**Figure 6.** Morphological differentiation of pDuH-transfected cells mimics that of GAP-43 deficient PC12 cells. The GAP-43 deficient PC12 cell clone N36 (Tsai *et al.*, 1997) was cultured under the same conditions as those described in Figure 5. After 96 h, cells were fixed and neurite outgrowth was visualized with Coomassie blue staining. Photographs were taken at 400X magnification. Quantitative analysis of cell morphology is shown in Table 2.

cells to TPA could be due to the low levels of GAP-43 protein in the cells (Figure 2). To test this idea, we exposed a GAP-43-deficient PC12 cell clone, PC12-N36, to similar treatments. As shown in Figure 6 and Table 2, PC12-N36 cells, like pDuH-transfected cells, did not exhibit significant process outgrowth in response to NGF or TPA but differentiated normally in response to cAMP. The absence of GAP-43 mRNA in the PC12-N36 cells was confirmed using Northern blots (not shown) and RT-PCR (Figure 8C, lane c). The finding that neither pDuH cells nor GAP-43-deficient PC12 cells were able to differentiate in response to PKC activators suggest that HuD-mediated process outgrowth is selectively triggered by this signaling pathway and that it requires the presence of GAP-43 in the cells.

### *Overexpression of HuD Increases GAP-43 mRNA Stability, Gene Expression, and Neurite Outgrowth in the Absence of NGF*

To further address the role of HuD in GAP-43 gene expression and PC12 differentiation, we generated two additional stable PC12 cell lines expressing HuD mRNA in the sense orientation (pcHuD). The original PC12 line and PC12-N36 cells were transfected with pcHuD and stable transfectants were used for gene expression and morphometric studies (Figure 7). Analysis of GAP-43 expression in these cultures indicated that pcHuD cells contained higher levels of the mRNA and protein under basal conditions and that these levels did not increase upon NGF stimulation (Figure 7A,B). pcHuD cells also contained 2–3 fold higher levels of HuD than untransfected PC12 cells (Figure 7B) and showed spontaneous process outgrowth in the absence of NGF (Figure 7C). Consistent with the observed up-regulation of GAP-43 expression in pcHuD cells, mRNA decay assays indicated that overexpression of HuD protein increased the half-life of the GAP-43 mRNA from 5 h to 9 h (Figure 8, Table 1). To confirm that HuD was bound to the GAP-43 mRNA in vivo, we used a combination of immunoprecipitation and RT-PCR. This procedure was successfully used to demonstrate the interaction of other ELAV/Hu proteins to their target mRNAs (Antic *et al.*, 1999). As shown in Figure 8C, GAP-43 mRNA could be detected in the pellet after HuD immunoprecipitation (lane b). These results suggest that HuD protects the GAP-43 mRNA from ribonuclease attack by binding to its 3′ UTR. Supporting this idea, we have recently found that a GAP-43 mRNA molecule that is lacking the HuD binding site is not stabilized by the RNA-binding protein (Beckel-Mitchener *et al.*, unpublished observations).

### *HuD-induced Neurite Outgrowth Requires the Presence of GAP-43*

To evaluate whether the effect of HuD on neurite outgrowth was due to its effects on GAP-43 levels, we over-

**Table 2.** Effect of different agents on the percentage of control and transfected PC12-N21 and N36 cells exhibiting neurites

| Cell type and vector | Control % | NGF % | TPA % | cAMP % |
|---|---|---|---|---|
| PC12-N21, untransfected | 18 ± 5 | 87 ± 2 | 75 ± 1 | 88 ± 5 |
| PC12-N21, pDuH | 13 ± 4 | 18 ± 9 | 26 ± 5 | 79 ± 7 |
| PC12-N36, untransfected | 1 ± 0.7 | 6 ± 1 | 10 ± 2 | 65 ± 5 |
| PC12 N36, pcHuD | 1 ± 0.6 | 1 ± 1 | 1 ± 0.5 | 2 ± 1 |
| PC12-N36, pcDNA3 | 4 ± 3 | 4 ± 2 | 6 ± 2 | 65 ± 8 |
| PC12-N36, pGAP-43 | 8 ± 2 | 70 ± 5 | 75 ± 10 | 75 ± 12 |
| PC12-N36, pGAP-43 + pcHuD | 80 ± 4 | 87 ± 6 | 83 ± 7 | 40 ± 10 |

PC12-N21 and PC12-N36 cells were stably transfected as indicated in MATERIALS AND METHODS. Cells were cultured for 4 days in the presence of the various agents, as described in Figure 5. The percentage of cells with neurites was determined for 4 fields per condition. Values represent the mean ± S.E.M.

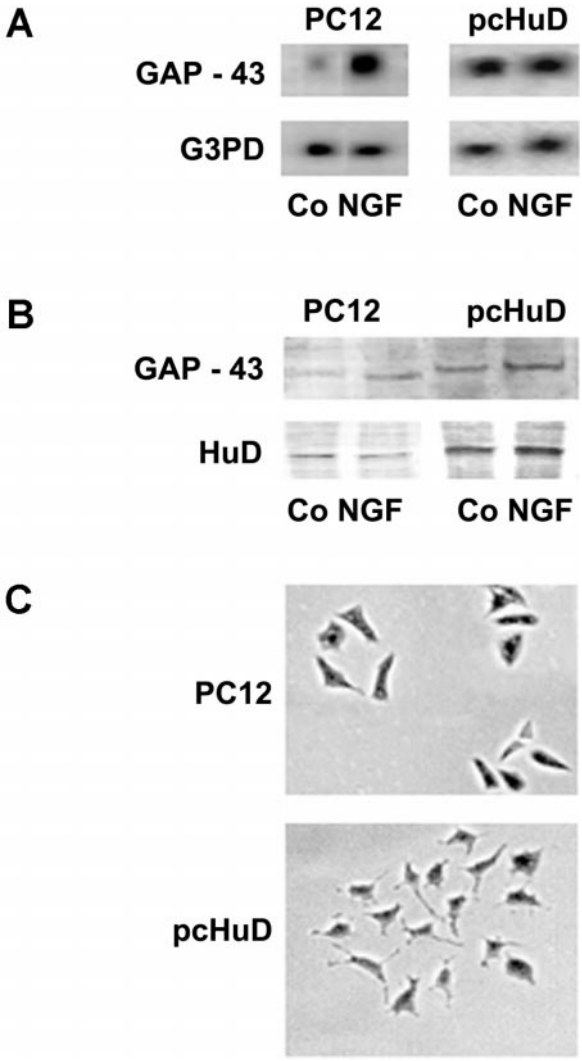


**Figure 7.** Effect of overexpression of HuD on GAP-43 expression and PC12 cell differentiation. (A) Untransfected PC12-N21 and pcHuD-transfected cells were grown for 24 h in the presence or absence of NGF, and the total RNA was analyzed by Northern blots as described in Figure 2. (B) Western blot analysis of GAP-43 and HuD proteins in the same control and pcHuD-transfected cells. (C) PC12-N21 and pcHuD-transfected cells were grown in culture for 96 h in the absence of NGF. Cells were fixed with 4% PFA and photographed at 400X magnification.

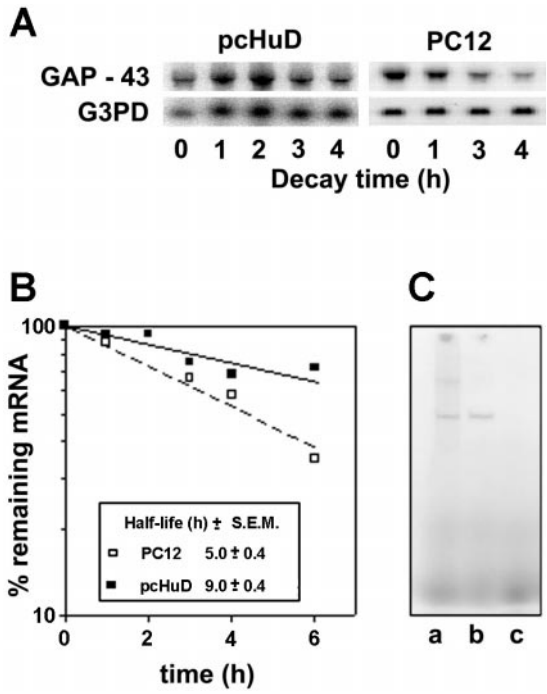


**Figure 8.** Overexpression of HuD increases the stability of GAP-43 mRNA. (A) Northern blots show the decay of the transfected GAP-43 mRNA in control PC12-N36 cells and in cells cotransfected with pcHuD. (B) Stability of the GAP-43 mRNA in pcHuD transfected PC12-N36 cells. The half-life of the mRNA was determined as indicated in MATERIALS AND METHODS. (C) In vivo binding of HuD to the GAP-43 mRNA. Cell extracts were prepared from untransfected (lane c) and GAP-43-transfected PC12-N36 cells (lanes a and b) as indicated in MATERIALS AND METHODS. HuD protein was immunoprecipitated with our specific antibodies (1:100 dilution) and GAP-43 mRNA in the sample was detected by RT-PCR. Lane a: RT-PCR from cell extracts without immunoprecipitation, b: RT-PCR of HuD immunoprecipitated materials, and c: RT-PCR of untransfected PC12-N36 cells.

expressed HuD in PC12-N36 cells that do not contain any endogenous GAP-43. Cells were transfected with pcDNA3 vector alone or with vector containing HuD sequences (Figure 9A, pcDNA3 and HuD, respectively). To control for the effect of GAP-43 in neurite outgrowth, some PC12-N36 cultures were also transfected with GAP-43 in the pMEP-4 vector (GAP-43), or were cotransfected with pcHuD and pMEP4-GAP-43 (HuD + GAP-43). GAP-43 expression in these transfected cultures was induced using 2.5 $\mu$M CdCl2 as described by (Neve *et al.*, 1999). When transfected alone, neither HuD nor GAP-43 was able to cause significant process outgrowth in the cells. Only the combination of HuD and GAP-43 resulted in extensive neurite outgrowth in the absence of NGF (Figure 9A). Similar results were observed in the presence of NGF or the phorbol ester TPA (Figure 9B). Cultures expressing GAP-43 (GAP-43 and pcHuD + GAP-43) showed a robust neurite outgrowth in response to these agents while those without GAP-43 (pcDNA3 and HuD) did not. As noted before, in the presence of decreased amounts of GAP-43 (Figure 4A) or in the absence of this protein (Figure 5A), cells exhibited process outgrowth only upon stimulation with dibutyryl-cAMP. The only cell type upon which cAMP had no effect was the pcHuD-transfected PC12 N36 cells, suggesting that excess HuD in the absence of GAP-43 may interfere with this signaling pathway. Altogether these results support the notion that HuD is a stabilizing factor for the GAP-43 mRNA and that GAP-43 expression is critical to mediate HuD's effects on neurite outgrowth.

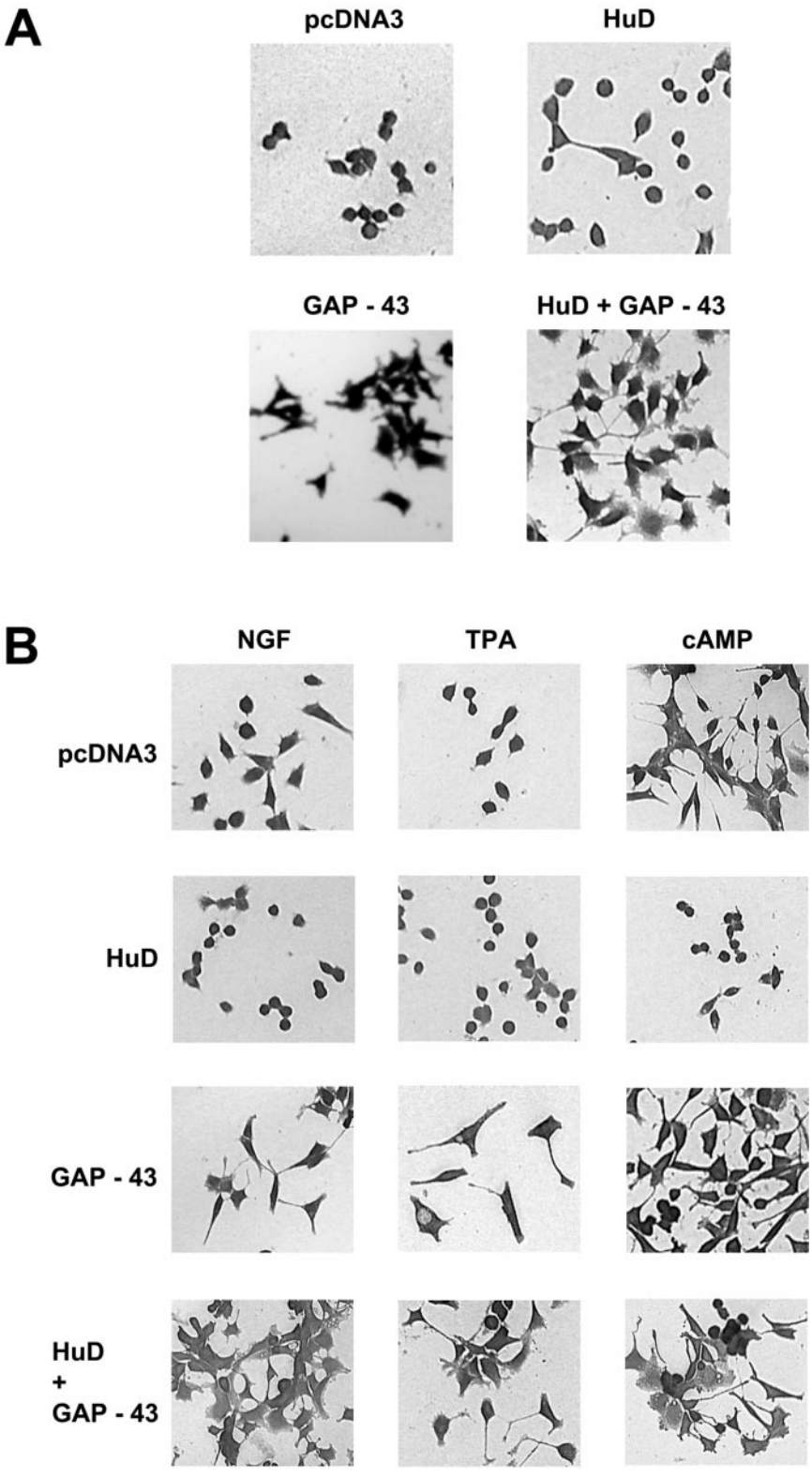


**Figure 9.** HuD-induced neurite outgrowth requires the presence of GAP-43. The GAP-43 deficient PC12-N36 clone was transfected with the pcDNA3 vector alone or with the vector containing HuD sequences (pcDNA3 and HuD, respectively). Cells were also transfected with GAP-43 in the pMEP-4 vector (GAP-43) or were cotransfected with pcHuD and pMEP4-GAP-43 (HuD + GAP-43), to examine the effect of both proteins in neurite outgrowth. Panels in A show the morphology of the cells in the absence of any stimulation, while panels in B show the effect of various agents on cell morphology. All cells were cultured as described in Figure 5. Photographs were taken at 400X magnification.

## DISCUSSION

The control of GAP-43 gene expression occurs through both transcriptional and posttranscriptional mechanisms, with the latter mediated by changes in the stability of the mRNA (Perrone-Bizzozero *et al.*, 1991; Perrone-Bizzozero *et al.*, 1993). In this study, we used antisense and sense RNA strategies to investigate the role of the RNA-binding protein HuD in GAP-43 mRNA stability and expression in PC12 cells. We found a tight correlation between the effect of HuD on GAP-43 gene expression and neurite outgrowth. PC12 cells expressing HuD antisense RNA (pDuH) had decreased levels of both GAP-43 mRNA and protein and failed to extend neurites in response to NGF and phorbol esters. These findings are in agreement with previous studies from our laboratory demonstrating that phorbol ester treatment mimics the effect of NGF on GAP-43 gene expression and mRNA stability and process outgrowth (Perrone-Bizzozero *et al.*, 1993). Moreover, HuD's binding site is located within the TPA-responsive element in the GAP-43 3′UTR and the phorbol ester increases its affinity for these sequences (Chung *et al.*, 1997; Tsai *et al.*, 1997). Based upon these results, we propose that HuD is an essential factor for controlling GAP-43 mRNA stability, GAP-43 expression, and neurite outgrowth in response to PKC activation.

HuD is a member of the ELAV family of RNA binding proteins first discovered in *Drosophila* (Campos *et al.*, 1985; Szabo *et al.*, 1991). In *Drosophila*, the *elav* gene is required for the normal development and maintenance of the nervous system (Campos *et al.*, 1985). Likewise in vertebrates, the neuronal ELAV-like proteins HuB, HuC, and HuD have been implicated in promoting the differentiation of neuronal cells (Wakamatsu and Weston, 1997). While all four ELAV-like proteins are important for neuronal differentiation, it is becoming apparent that their functional properties may be different. These RNA-binding proteins show a distinct pattern of expression during development, with HuD and HuR being the first to be expressed in neuronal progenitors, followed by HuC and HuB (Wakamatsu and Weston, 1997; Okano and Darnell, 1997; Clayton *et al.*, 1998) Likewise, these proteins map to different neuronal populations in the developing and adult rat CNS and PNS (Okano and Darnell, 1997; Clayton *et al.*, 1998) and appear to control different mRNAs (Aranda-Abreu *et al.*, 1999; Antic *et al.*, 1999).

Multiple pathways are capable of signaling the expression of proteins required for neurite outgrowth in PC12 cells (Kaplan and Stephens, 1994; Burry, 1998). Here we found that decreases in HuD expression by antisense RNA treatment selectively affected the signaling cascade activated by NGF and phorbol esters while leaving intact the cellular response to cAMP. Because GAP-43 gene expression is also dependent on PKC activity but independent of cAMP (Perrone-Bizzozero *et al.*, 1993), these results suggest that the effect of HuD on neurite outgrowth may be mediated by its direct action on GAP-43 expression. While it has been possible to dissociate GAP-43 expression and neurite outgrowth in specific GAP-43 deficient PC12 cell clones (Baetge and Hammang, 1991; Burry and Perrone-Bizzozero, 1993) and in cAMP-treated pDuH cells (Figure 6), it is apparent that neurite outgrowth in the absence of GAP-43 is not normal. GAP-43 deficient PC12 cells display reduced membrane excitability and altered cell adhesion properties (Gribkoff *et al.*, 1995; Meiri *et al.*, 1996). A similar phenotype was observed in other GAP-43 deficient PC12 clones in the presence of forskolin (Burry and Perrone-Bizzozero, 1993). Altogether these results suggest that neurite extension is normally linked to the amount of GAP-43 in the cells, and this process is controlled by HuD.

Besides GAP-43, HuD is known to bind the 3′ UTRs of other mRNAs expressed in neurons, such as those for c-fos, tau, N-myc, and $p21^{waf1}$ (Chung *et al.*, 1997; Joseph *et al.*, 1998; Aranda-Abreu *et al.*, 1999; Lazarova *et al.*, 1999). Therefore, it is likely that the expression of other genes may be affected by the presence of HuD antisense or sense transcripts. Notwithstanding, we believe that the observed effect of HuD on PC12 cell differentiation is mediated primarily by changes in GAP-43 gene expression for the following reasons. First, GAP-43 is involved in early events in neurite outgrowth occurring within the first 48 h after NGF treatment (Yankner *et al.*, 1990; Perrone-Bizzozero *et al.*, 1993; Aigner and Caroni, 1995), a period in which we found that HuD was required for cell differentiation (Dobashi *et al.*, 1998). As mentioned earlier, HuD's effect on neurite outgrowth, like GAP-43, was found to depend on PKC activity but was independent of cAMP (Table 2). In fact, HuD was unable to induce process outgrowth in a PC12 cell clone that does not express GAP-43 (Figure 9), suggesting that GAP-43 is required for some of the effects of HuD on neuronal differentiation. In this regard, it is noteworthy that, within the first 24 h of culture, HuD induced GAP-43 levels but did not affect the levels of other proteins associated with NGF induction or those whose expression is regulated by ELAV-like proteins, such as the microtubule associate protein $\tau$ and neurofilament M (Anderson *et al.*, 2000).

The results presented here suggest that HuD controls GAP-43 gene expression by increasing the stability of the mRNA. The effect of HuD on GAP-43 mRNA stability was demonstrated in cells containing levels of HuD protein that were either a 3-fold lower (pDuH) or a 3-fold higher (pcHuD) than untransfected PC12 cells (Table 1). Likewise, other members of the ELAV family have been shown to control the stability of cellular mRNAs. Overexpression of HuR, the ubiquitously expressed ELAV protein, was found to stabilize ARE-containing mRNAs such as those for c-fos and c-jun (Fan and Steitz, 1998b; Peng *et al.*, 1998). In the case of HuR, mRNA stabilization depended on the nuclear-cytosolic shuttling of the protein (Fan and Steitz, 1998a). Although we cannot exclude similar mechanisms of action for HuD, we favor the idea that HuD acts mainly by stabilizing GAP-43 mRNA at the cytoplasmic level. Analysis of the distribution of this protein in different subcellular fractions demonstrated that HuD is enriched in polysomes (Kohn *et al.*, 1996). Similarly, the ELAV-like proteins Hel-N1 and Hel-N2 were found to localize to polysomes (Gao and Keene, 1996), where they control mRNA stability and translation (Antic *et al.*, 1999). As shown in Figure 2, the GAP-43 mRNA and protein were similarly reduced in control and in NGF-induced pDuH cells, suggesting that HuD's effects on GAP-43 protein may be mediated by its effect on the levels of its mRNA. Thus, while Hel-N1 (HuB) participates in

the control of both types of posttranscriptional processes (i.e., mRNA stability and translation), our results suggest that HuD affects primarily GAP-43 mRNA stability without having any translational effects.

In conclusion, our results indicate that HuD is essential for controlling GAP-43 mRNA stability, GAP-43 expression, and PKC-dependent neurite outgrowth in PC12 cells. Based on these observations, we propose that HuD contributes to the induction of GAP-43 expression and neurite outgrowth in vivo. HuD is one of the first markers expressed in neuronal cells, at the onset of process outgrowth (Barami *et al.*, 1995; Wakamatsu and Weston, 1997). Likewise, GAP-43 is expressed in neurons in association with the initial stages of process outgrowth (Skene, 1989; Benowitz and Routtenberg, 1997; Oestreicher *et al.*, 1997). In addition, there is an excellent correlation between the levels of HuD and GAP-43 mRNA in different areas of the CNS and PNS during brain development (Szabo *et al.*, 1991; Okano and Darnell, 1997; Clayton *et al.*, 1998) and nerve regeneration (Anderson *et al.*, submitted), and between the levels of expression of GAP-43 and HuD in PC12 cells (Table 1). Once HuD is expressed in the cell, additional mechanisms may control its function. Because HuD is a substrate of PKC (H. M. Furneaux, unpublished observations) and because phorbol esters increase the binding of HuD to the GAP-43 mRNA (Tsai *et al.*, 1997), it is likely that PKC controls HuD function in vivo. While we are investigating these issues, it is becoming clear that ELAV-like proteins are important posttranscriptional regulators of nervous system-specific genes in a broad array of species, from invertebrates to humans (King *et al.*, 1994; Wakamatsu and Weston, 1997; Akamatsu *et al.*, 1999; Antic *et al.*, 1999; Kasashima *et al.*, 1999).

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