The Journal of Neuroscience, August 15, 1999, *19*(16):6907–6917

Embryonic Lethal Abnormal Vision-Like RNA-Binding Proteins Regulate Neurite Outgrowth and Tau Expression in PC12 Cells

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The embryonic lethal abnormal vision (ELAV)-like proteins are mRNA-binding proteins that regulate mRNA stability. The neuronal members of this family are required for neuronal differentiation. We identified the binding region of purified HuD protein to a target neuronal mRNA encoding for the tau microtubule-associated protein and demonstrated an *in vivo* interaction between the ELAV-like protein and its target tau mRNA. We show that treatment of neuronal cells with antisense oligodeoxynucleotides directed against HuD blocks the induction of neurite outgrowth and decreases the levels of tau mRNAs, indicating that the ELAV-like proteins are required for neuronal differentiation.

Key words: RNA-binding proteins; tau mRNA; mRNA stabilization; neurite outgrowth; antisense oligodeoxynucleotides; microtubules

The embryonic lethal abnormal vision (ELAV) gene is required for the development of the *Drosophila* nervous system (Campos et al., 1985; Jimenez and Campos-Ortega, 1987). In mutant flies the neuroblasts differentiate inappropriately, resulting in a defective nervous system. In normal flies, ELAV is expressed immediately after the neuroblasts withdraw from the cell cycle and continues to be expressed in adult postmitotic neurons (Robinow et al., 1988; Robinow and White, 1991). Continued expression of ELAV in adult neurons is essential for brain function, because temperature-sensitive ELAV mutants are incapacitated at nonpermissive temperatures (Homyk et al., 1985). The ELAV gene product encodes a protein with three characteristic RNA-binding motifs (RRMs) (Robinow et al., 1988). These motifs are found in many RNA-binding proteins that regulate gene expression (Kenan et al., 1991). In view of this, it is thought that ELAV promotes neuronal differentiation by selectively modulating the expression of required genes (Yao et al., 1993; Koushika et al., 1996). However, the mRNA targets regulated by ELAV have yet to be clearly elucidated.

ELAV homologs have been identified in human, mouse, rat, *Xenopus*, and birds (Abe et al., 1994; King et al., 1994; Good, 1995; Perron et al., 1995; Steller et al., 1996; Ma and Furneaux, 1997; Myer et al., 1997; Okano and Darnell, 1997; Wakamatsu and Weston, 1997). The human members of the family (HuD, Hel-N1, HuC, and HuR) are of particular interest because they were independently discovered as tumor antigens (Szabo et al., 1991; Sakai et al., 1994). A vital clue to the mechanism of action of these proteins in vertebrates was provided by the observation that they bind *in vitro* to U-rich regulatory elements in the 3′-UTRs (3′-untranslated region) of mRNAs. These U-rich elements were originally described by Shaw and Kamen (1986), who found that they direct the rapid turnover of mRNA. Thus, mRNAs that contain these elements have a very short half-life and are usually present at a very low steady-state level. The mechanism of this rapid turnover is not clear but has been ascribed to a specific endonuclease or an adenylate/uridylate-rich element (ARE)-dependent deadenylase (Chen and Shyu, 1995). The expression of these unstable mRNAs can be increased dramatically by factors that bind to the U-rich elements. Recent evidence has indicated that the ELAV-like proteins are such factors and that they selectively inhibit the decay of mRNAs that contain U-rich elements (Jain et al., 1997; Fan and Steitz, 1998; Levy et al., 1998; Peng et al., 1998).

The transition from neuroblast to postmitotic neuron is accompanied by a regulated increase in the stability of mRNAs that are required for terminal differentiation. The vertebrate ELAV-like proteins have been shown to bind to many such mRNAs *in vitro* (Gao et al., 1994; King et al., 1994; Chung et al., 1996). This suggests that the vertebrate ELAV-like proteins, like their *Drosophila* counterparts, play a critical role in neuronal differentiation. It was recently shown that overexpression of HuD in neural crest cells indeed stimulates their differentiation into mature neurons (Wakamatsu and Weston, 1997). Similarly, transfection with Hel-N1 caused increased translation of neurofilament M mRNA and induced formation of neurites in human teratocarcinoma cells (Antic et al., 1999). It is possible, however, that this does not reflect the normal physiological condition and was the result of an abnormally high HuD or Hel-N1 in the above cells. We therefore decided to examine whether the endogenous ELAV-like proteins are required for neuronal differentiation. In this investigation we used the PC12 model system, because PC12 cells are known to express at least one ELAV-like family member (Steller et al., 1996). We show that treatment of PC12 cells with antisense oligonucleotide directed against HuD blocks the induction of differentiation by nerve growth factor (NGF). In the course of these studies we also discovered that tau mRNA is a binding target of the ELAV-like proteins. Tau is a microtubule

Received Jan. 21, 1999; revised April 28, 1999; accepted June 1, 1999.

This work was supported by grants from the Basic Research Foundation (Israel Academy of Sciences and Humanities) and the German-Israeli Foundation (GIF), an AFIRST grant from the France-Israel Ministry of Science (I.G.), and National Science Foundation Grant IBN-9604175 (H.M.F.). I.G. is the incumbent of the Sophie and Richard S. Richard Professorial Chair in Cancer Research. We thank Dr. Eyal Schejter for his substantial help and comments on the confocal studies.

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(MT)-associated protein that is essential for neurite development and cell polarity in cultured neuronal cells. Our results show that tau mRNA levels are regulated by HuD. These observations strongly indicate that the vertebrate ELAV-like proteins are required for neuronal differentiation.

MATERIALS AND METHODS

Cell culture system. PC12 cells were grown in DMEM supplemented with 8% horse serum and 8% fetal calf serum at 37°C in an 8% CO_2 incubator. For treatment with NGF, 1.2–1.5 $\times$ 10^6 cells were plated on 90 mm collagen-coated dishes and grown in DMEM supplemented with 1% horse serum, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 50 ng/ml 7S NGF (Alamone, Jerusalem, Israel). NGF was added every 2 d.

Antisense treatment. The experiments were performed with r-HuD sense and antisense oligodeoxynucleotide (oligo) 5′-TGGATGTCGG TCCATTTGAC-3′ (15–34) (Steller et al., 1996) or with the unrelated AC6 antisense oligo (NRO) 5′-AAGCAAACAGCCTTACTC-3′ (Premont et al., 1992). There was no significant homology between the r-HuD antisense oligonucleotide and any other sequence in the database.

PC12 cells were plated on collagen-coated microtiter plates at a density of 1 $\times$ 10^5 cells per well and grown in DMEM supplemented with 10 μg/ml insulin, 10^{-8} M hydrocortisone, 5 μg/ml transferrin, 10 μg/ml somatostatin, and 10 μg/ml glycyl-L-hystidyl-lysine, 50 U/ml penicillin, and 50 μg/ml streptomycin (Sigma, St. Louis, MO). They were then treated with 50 μM unmodified antisense oligonucleotide in the presence of 50 ng/ml NGF for the specified time periods. The morphological appearance of the treated PC12 cells was observed by light microscopy. At the end of the experiment, RNA was isolated and amplified as described below.

Preparation of cell extracts and microtubules. S100 extracts were prepared in TGKED buffer [50 mM Tris, pH 7.5, 25% glycerol, 50 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 1 mM phenylmethanyl-sulfonyl fluoride (PMSF)]. Cells were homogenized in 2 vol of TGKED buffer, cleared of cell debris by centrifugation in a microfuge at 12,000 $\times$ *g* for 10 min at 4°C, and then centrifuged in a Beckman airfuge for 15 min at 100,000 $\times$ *g* in the cold. Extracts were stored as aliquots at −80°C. Protein concentrations were determined by the Bradford method and ranged from 5 to 10 μg/μl.

MTs were prepared from PC12 cells after two cycles of *in vitro* assembly (Shelanski et al., 1973). The final MT pellets were resuspended in TGKED buffer and stored as aliquots at −80°C until use.

Preparation of RNA transcripts. Plasmids encoding the F, G, H, I, and J fragments (Behar et al., 1995) were linearized with *Hin*dIII, *Hin*dIII, *Sac*I, *Sty*I, and *Sac*I, respectively. The plasmid Gdel was derived by PCR deletion of the 21 nucleotides AU-rich region from fragment G and linearized with *Bam*HI. Template DNAs were transcribed with the appropriate RNA polymerase in the presence of [^{32}P]-UTP (Amersham, Arlington Heights, IL). All transcripts were gel-purified as described previously (Behar et al., 1995).

RNA complex assay. Reaction mixtures (20 μl) contained 50 mM Tris, pH 7.0, 150 mM NaCl, 0.25 mg/ml tRNA (Boehringer Mannheim, Mannheim, Germany), 0.25 mg/ml bovine serum albumin (BSA), 30 fmol labeled RNA, and purified HuD protein, as indicated. After incubation at 37°C for 10 min, 5 μl of a dye mixture (50% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanol) was added, and 5 μl of the reaction mixture was then immediately loaded on a 1% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). The gel was then electrophoresed at 40 V for 2.5 hr, dried on DE-81 paper (Whatman) with a backing of gel-drying paper (Hudson City Paper), and exposed to XAR5 film (Kodak, Rochester, NY) for 6 hr at −70°C.

Nitrocellulose filter binding assay. Reaction mixtures (20 μl) contained 50 mM Tris, pH 7.0, 150 mM NaCl, 0.25 mg/ml BSA, 0.25 mg/ml tRNA (Boehringer Mannheim), 30 fmol radiolabeled mRNA, and purified HuD as indicated. After incubation for 10 min at 37°C, the mixtures were diluted 1:6 with buffer F (20 mM Tris, pH 7.0, 150 mM NaCl, 0.25 mg/ml tRNA) and filtered through nitrocellulose (BA85, Schleicher & Schuell, Keene, NH). The filter was washed twice with buffer F. Bound radioactivity was determined by Cerenkov counting.

RNase T1 selection assay. Reaction mixtures (20 μl; see preceding section for contents) were incubated for 10 min at 37°C. RNase T1 (5 U) (Calbiochem, La Jolla, CA) was added, and the reaction was allowed to continue for an additional 10 min. The mixtures were diluted 1:6 with buffer (20 mM Tris, pH 7.0, 150 mM NaCl) and filtered through nitrocellulose (BA 85, Schleicher & Schuell). The nitrocellulose filter was washed twice with buffer, and the bound RNA was eluted by phenol-chloroform extraction. The resultant RNA was mixed with formamide buffer, denatured at 65°C for 3 min, and analyzed by electrophoresis (12% polyacrylamide/urea gel). The gel was fixed with acetic acid/methanol/water 1:1:8, dried on DE-81 paper with a backing of gel-drying paper, and exposed to the XAR5 film at −70°C overnight.

Antibodies. High-titer polyclonal human antisera (1:1000), which specifically recognize ELAV-like proteins (Szabo et al., 1991), were purified and further analyzed by Athena Diagnostic (Boston, MA). These antibodies were initially used to screen a cDNA expression library and were found to encode for one gene product, HuD (Szabo et al., 1991) (GenBank accession no. M62843). Anti-HuD monoclonal antibodies (4 μg/ml) (Marusich et al., 1994) were prepared by immunization using peptide antigen. The tau-1 monoclonal antibodies (2.5 μg/ml) were obtained from (Binder et al., 1985) actin monoclonal antibodies (1:2000; Sigma), and monoclonal anti-tubulin (1:500; BioMakor). Secondary antibodies (goat anti-human and goat anti-mouse) were obtained from Jackson ImmunoResearch (West Grove, PA).

UV cross-linking assay and immunoprecipitation. α-[^{32}P]UTP-labeled RNA transcripts at the specified amounts (8 fmol, 2 $\times$ 10^5 cpm) were incubated with 10 μg of PC12 cells (S100 extracts), 10 μg of MT preparation, or 100 ng of glutathione *S*-transferase (GST)-HuD-purified fusion protein in a final volume of 0.02 ml. After 30 min at room temperature, heparin (Sigma) was added at a final concentration of 5 mg/ml, and the samples were irradiated at 0.5 J/cm^2 with a 254 nm UV light source (Spectrolinker XL-1500 UV cross-linker). After incubation with 1 mg/ml RNase A (Sigma) for 15 min at 50°C, samples were either directly analyzed on 12% SDS-PAGE or immunoprecipitated with polyclonal human antisera for 1 hr at 4°C. This was followed by incubation with protein A-Sepharose (Pharmacia, Piscataway, NJ) for 1 hr at 4°C. Complexes were collected, denatured at 65°C, and resolved by 12% SDS-PAGE.

Purification of GST-HuD proteins. An overnight culture of *Escherichia coli* BL 21, transformed with pGST-HuD (Chung et al., 1996), was diluted 1:50 in LB medium. At an OD_{600} of 0.4, the culture was induced with isopropyl β-D thiogalactopyranoside (0.1 mM). After 4 hr of further growth, the cells were spun down and resuspended in 10 ml of buffer A (50 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA). The cells were lysed by the addition of lysozyme (0.2 mg/ml) and Triton X-100 (1%). The lysate was centrifuged at 12,000 $\times$ *g* for 30 min, and the resulting supernatant was loaded onto a glutathione-agarose affinity column (13 mg of protein per milliliter of resin). The column was washed with buffer B (50 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA, 1% Triton X-100), and GST-HuD was eluted with 50 mM Tris, pH 8.0, containing 5 mM glutathione. Active protein was determined by RNA-complex formation. They were then pooled and stored at −70°C, as described previously (Chung et al., 1996).

Immunoblot analysis of PC12 protein extracts. For immunoblot analysis of PC12 proteins, cells were extracted in 1 vol of lysis buffer (50 mM Tris, pH 8.5, 1% Triton X-100, 5 mM EDTA, 0.15 M NaCl, 50 μg/ml PMSF). Cell extracts were cleared of cell debris by centrifugation for 10 min at 14,000 $\times$ *g* at 4°C.

Protein samples (25 μg) were resolved by SDS-gel electrophoresis, transferred to nitrocellulose filters, and reacted with specified antibodies at 4°C for 16 hr. They were then visualized by reaction with peroxidase-conjugated goat anti-human or goat anti-mouse secondary antibodies at room temperature for 1 hr and developed using the ECL chemiluminescence procedure.

Cell fractionation. PC12 cells were extracted under conditions that preserve preexisting MTs in the cells and allow for separation between MTs assembled *in vivo* and unassembled tubulin (Black and Kurdyla, 1983). Cultures were rinsed twice with Ca^{2+}-free and Mg^{2+}-free PBS, and the cells were collected and homogenized in 100 mM PIPES buffer, pH 6.9, containing 1 mM $MgSO_4$, 0.5% Triton X-100, and protease inhibitors [1 mM PMSF, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 10 mg/ml pepstatin (Sigma)] in the presence of MT-stabilizing reagents (4 M glycerol, 5% v/v dimethylsulfoxide, 5 μM Taxol, and 1 mM EGTA). After centrifugation of the homogenate at 12,000 $\times$ *g* in an Eppendorf centrifuge for 5 min, the supernatant was removed and centrifuged at 32,000 $\times$ *g* in a Beckman airfuge for 30 min at room temperature. Under these conditions the *in vivo* cellular composition is preserved: unpolymerized proteins remain in the supernatant, but the *in vivo* MT fraction, being insoluble, is found in the pellet (Black and Kurdyla, 1983; Litman et al., 1994).

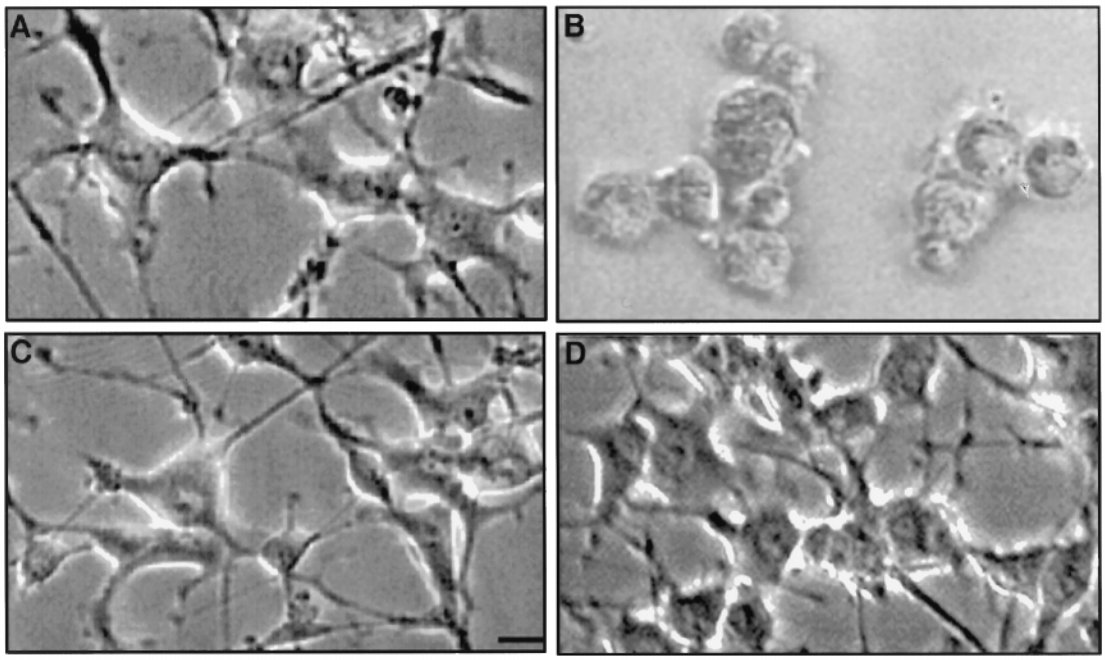

Figure 1. Effect of r-HuD antisense treatment on neurite outgrowth in PC12 cells. *A*, Control PC12 cells treated with NGF for 4 d. *B*, PC12 cells treated with NGF and r-HuD antisense oligonucleotides for 4 d. *C*, PC12 cells treated with NGF and unrelated AC6 antisense oligonucleotides. *D,* PC12 cells treated with NGF and sense r-HuD oligonucleotides for 4 d. Scale bar, 20 μm.

Immunoprecipitation analysis of PC12 cellular extracts. PC12 cells were lysed in 1 vol of lysis buffer (50 mM Tris, pH 7.5, 25% glycerol, 50 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 0.5% NP-40, 40 U/μl RNasin, 10 mM vanadyl complex) and cleared of cell debris by centrifugation at 14,000 × *g* at 4°C. Anti-Hu sera (1:1000) was added, and the mixture was incubated for 1 hr at 4°C and then for another hour at 4°C with protein A-Sepharose (Pharmacia; 10% final concentration). The immunocomplex was precipitated by centrifugation, washed, and processed for RNA isolation as described below.

Similar procedures were performed when MT fractions were used for immunoprecipitation experiments.

RNA isolation and RT-PCR analysis. RNA was isolated from total cell extracts or from the immunoprecipitated complex using RNAzol reagent (Biotecx Laboratories, Houston, TX). The extracted RNA was reverse-transcribed with random hexamers using the standard procedure in a 20 μl reaction mixture. Aliquots of 5 μl, from the RT mixture, were used for amplification, using the following primers: for r-HuD, 5′-CCAA CAAAGCCCACAAGTCC-3′ (1226–1245) and 5′-AATCCTTTCCT GGTACACCTCA-3′ (1410–1431) (Steller et al., 1996); for tau, 5′-GG TGAGGGATGGGGGTGGTA-3′ (2179–2198) and 5′-GTGACTGG CTCTCGTGGCA-3′ (2278–2297) (Sadot et al., 1994); for GADPH, 5′-GCCATCAACGACCCCTTCAT-3′ (118–137) and 5′-TTCACACC CATCACAAACAT-3′ (412–431) (Tso et al., 1985); for actin, 5′-GCACCACACTTTCTACAATGA-3′ (1585–1606) and 5′-GAACCG CTCATTGCCGATAGT-3′ (2537–2558) (Nudel et al., 1983) (this published sequence includes an intron between 1692 and 2155); for AC6, 5′-CTTCCAGATGAAGATCGGG-3′ (3289–3308) and 5′ AAGCAAA-CAGCCTTACTC 3′ (3778–3796) (Premont et al., 1992); for p75NGFR, 5′-GTCGTGGGCCTTGTGGCC-3′ (903–921) and 5′-CTGTGAGTT CACACTGGGG-3′ (1381–1400) (Radeke et al., 1987); for TrkA, 5′-CGTTGATGCTGGCTTGTGC-3′ (135–153) and 5′-GGAGAGATT CAGGTGACTGA-3′ (411–430) (Meakin et al., 1992).

The amplification program consisted of one cycle at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, at 55°C for 1 min, and at 72°C for 2 min.

Confocal microscopy analysis of PC12 cells. PC12 cells were grown on coverslips coated with collagen type 1. The cells were fixed with 4% paraformaldehyde in 4% sucrose for 30 min at room temperature. They were then permeabilized by incubation for 3 min in 0.5% Triton X-100, washed three times with PBS, and blocked with 1% BSA. The cells were incubated with the primary antibodies monoclonal anti-tubulin (1:500) (Bio-Makor) or anti-Hu sera (1:250), or a mixture of the two, for 24 hr at 4°C. They were then washed three times, each for 15 min, with PBS and incubated for 2 hr at room temperature with the secondary antibodies goat anti-mouse FITC (1:100) (Bio-Makor) and goat anti-human C_y3 (1:100) (Jackson ImmunoResearch) for anti-tubulin and anti-ELAV-like antibodies, respectively. The coverslips were mounted with Mowiol and visualized with the MRC-1024 confocal laser scanning imaging system (Bio-Rad, Richmond, CA) at 40× objective, using green and red filters for tubulin and human antibodies, respectively. The images were analyzed using software for the MRC-1024 confocal imaging system.

RESULTS

Effect of treatment with r-HuD oligo antisense on the morphology of PC12 cells

To determine whether inhibition of ELAV-like gene expression affects neurite outgrowth in PC12 cells induced by NGF, we focused on one member of the ELAV family of proteins, r-HuD, which is expressed in and has been cloned from PC12 cells (Steller et al., 1996). We applied r-HuD-specific antisense oligonucleotides (50 μM) to NGF-treated PC12 cells for up to 4 d and followed the effect on neurite outgrowth.

In untreated control cells, NGF treatment resulted in extension of neurites (Fig. 1*A*). In cells subjected to antisense treatment, neurite retraction was clearly evident from day 2 after the start of treatment, and after day 4 the cells exhibited the morphology of noninduced cells (Fig. 1*B*). Similar findings were recently obtained by Dobashi et al. (1998). Cells treated with sense oligo or with an unrelated oligo antisense (NRO) directed against adenyl cyclase type VI were not affected, i.e., their morphology was similar to that of untreated control cells (Fig. 1*C*,*D*). At this stage, replacement of the medium by fresh medium without the antisense oligos from the treated cells led to their full recovery. Similar results were reported when PC12 or primary cerebellar cells were treated with these concentrations of tau antisense (Caceres and Kosik, 1990; Hanemaaijer and Ginzburg, 1991).

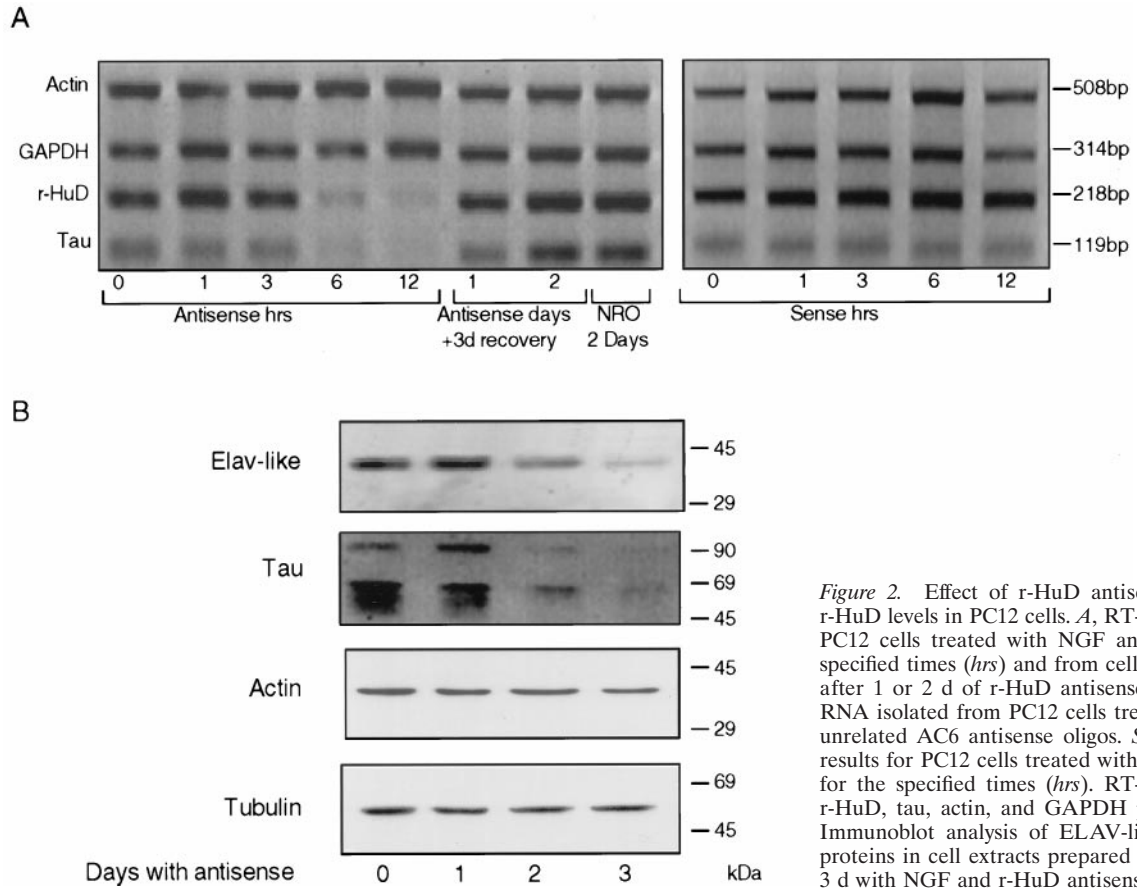

Figure 2. Effect of r-HuD antisense treatment on tau and r-HuD levels in PC12 cells. *A*, RT-PCR of RNA isolated from PC12 cells treated with NGF and r-HuD antisense for the specified times (*hrs*) and from cells allowed to recover for 3 d after 1 or 2 d of r-HuD antisense treatment. *NRO* indicates RNA isolated from PC12 cells treated for 2 d with NGF and unrelated AC6 antisense oligos. *Sense* panel shows RT-PCR results for PC12 cells treated with NGF and sense HuD oligo for the specified times (*hrs*). RT-PCR was performed using r-HuD, tau, actin, and GAPDH primers in each sample. *B*, Immunoblot analysis of ELAV-like, tau, actin, and tubulin proteins in cell extracts prepared from PC12 cells treated for 3 d with NGF and r-HuD antisense oligos.

Treatment with antisense to r-HuD decreases r-HuD and tau mRNAs and proteins

Because tau mRNA and proteins are involved in neurite outgrowth, we were interested in examining their response to treatment with antisense to r-HuD. PC12 cells were induced with NGF in the presence of r-HuD antisense oligos for the indicated periods. Total RNA was extracted from equal numbers of control and antisense-treated cells, and the amounts of r-HuD and tau mRNAs were determined by RT-PCR, using r-HuD- and tau-specific primers. One of the two primers used to amplify r-HuD was a downstream 3′ primer complementary to a sequence located in the 3′-UTR of r-HuD, a region that is divergent among the ELAV family members (Szabo et al., 1991; King et al., 1994; Steller et al., 1996) (GenBank accession no. L26405). Use of this primer yields an expected PCR fragment of 218 base pairs (bp). No PCR product was obtained when the RT step was omitted, indicating the absence of DNA contamination in the isolated RNA (data not shown). A decrease in the amounts of r-HuD and tau mRNAs was observed after the r-HuD antisense treatment (Fig. 2*A*). The decrease was already apparent after 6 hr of treatment but was more pronounced for tau mRNA than for r-HuD mRNA. After treatment for 12 hr, both the r-HuD and the tau signals had almost disappeared.

When the medium was replaced by fresh medium without the r-HuD antisense oligo, both r-HuD and tau mRNAs increased to the amounts observed in untreated control cells. As in the previous morphological experiment (Fig. 1), treatment of PC12 cells with r-HuD sense oligo or NRO-AC6 had no effect on the levels of the tested RNAs. Control mRNAs, monitored by GAPDH and actin primers, were not affected by the treatment.

Immunoblot analysis of protein extracts prepared from PC12 cells treated with r-HuD antisense showed that both ELAV-like and tau proteins were markedly decreased after 2 d (Fig. 2*B*). In addition, the amounts of actin and tubulin proteins were not affected by the antisense treatment, in line with the above results showing no reduction in actin mRNA. These results are in agreement with the morphological changes (neurite retraction) observed from 2 d after antisense administration (Fig. 1). Thus, the decrease in r-HuD RNA and ELAV-like proteins is correlated with the decrease in tau mRNA and proteins.

ELAV-like protein binds to tau mRNA *in vivo*

To determine whether the ELAV-like proteins bind to tau RNA *in vivo*, we prepared total cell extracts from NGF-treated PC12 cells and subjected them to immunoprecipitation with anti-HuD serum (see Materials and Methods). RNA was isolated from the immunoprecipitated pellet (P) and supernatant (S) fractions and analyzed by RT-PCR, using tau or control primers specific for AC6, p75NGFR, and TrkA (Fig. 3). AC6 encodes for adenyl cyclase type VI; p75NGFR and TrkA encode for low- and high-affinity receptors for NGF, respectively, all of which are expressed in PC12 cells (Radeke et al., 1987; Meakin et al., 1992; Premont et al. 1992), and their amplification products are observed when total PC12 RNA is used (Fig. 3, *lanes 8–9*).

The results show that tau mRNA was present in the immunoprecipitated complex formed with anti-HuD serum, whereas no AC6 products were observed (Fig. 3, *lane 1*). Assay of the supernatant fraction using tau and AC6 primers showed no tau-specific product but revealed positive reaction with AC6 primers (Fig. 3, *lane 3*). No tau or AC6 products were obtained in the immuno-

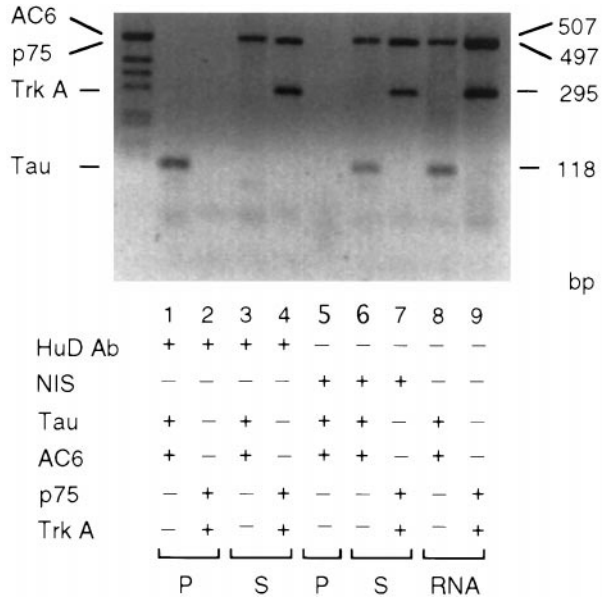

Figure 3. Binding of tau mRNA to ELAV-like proteins *in vivo*: immunoprecipitation of tau mRNA–ELAV-like complex, followed by isolation and RT-PCR analysis of RNA. PC12 cell extracts were immunoprecipitated with anti-Hu serum. The RNA isolated from the immunoprecipitated complex (*P*) and the remaining supernatant (*S*) were assayed by RT-PCR with tau and AC6 primers (*lanes 1, 3*) and with p75NGFR and TrkA primers (*lanes 2, 4*). *Lanes 5–7*, Cell extracts immunoprecipitated with nonimmune serum (*NIS*) were assayed with tau and AC6-specific primers (*lane 5, 6*) and with p75NGFR and TrkA primers (*lane 7*). RT-PCR products using total RNA isolated from PC12 cells were amplified with tau and AC6-specific primers (*lane 8*) and with p75NGFR and TrkA primers (*lane 9*). The sizes of RT-PCR products obtained with AC6, p75NGFR, TrkA, and tau primers are 507, 497, 295 and 118 bp, respectively.

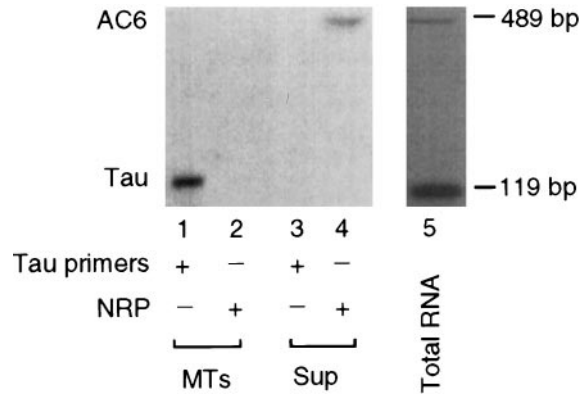

Figure 4. Association of tau mRNA-ELAV-like protein complex with MTs *in vivo*. Preexisting polymerized microtubules (*MTs*) and unpolymerized supernatant (*Sup*) fractions were prepared from PC12 cells. The MT fraction was immunoprecipitated by anti-Hu serum. RNA was isolated from the MT-immunoprecipitated complex and the initial supernatant, and analyzed by RT-PCR using tau-specific (*lanes 1, 3*) and AC6-specific NRP (*lanes 2, 4*) primers. *Lane 5* is similar to *lane 8* in Figure 3.

precipitated complex when nonimmune serum (NIS) was used (Fig. 3, *lane 5*). Using p75NGFR and TrkA primers, no products were observed in the immunoprecipitated complex formed with anti -HuD serum (Fig. 3, *lane 2*). Products were observed in the supernatant fractions remaining after immunoprecipitation with either HuD antibodies (Fig. 3, *lane 4*) or NIS (Fig. 3, *lane 7*). These results indicate that HuD antibodies precipitated the tau mRNA in the immunoprecipitated complex, whereas the other tested messages remained in the supernatant fraction.

The ELAV-like protein–tau mRNA complex is associated with microtubules *in vivo*

As a follow-up to the above experiment with total PC12 cellular fractions, and in light of previous evidence that tau mRNA is bound to MTs (Litman et al., 1994), it was of interest to determine whether the complex formed between the ELAV-like proteins and the tau mRNA is associated with the MT fraction in the cell. The polymerized MT fraction preexisting in the cells was isolated as described in Materials and Methods and then subjected to immunoprecipitation with anti-ELAV-like serum. RNA was isolated from the immunoprecipitated complex and from the initial supernatant (Sup) fraction and analyzed by RT-PCR. The results showed that tau mRNA is associated with the immunoprecipitated complex isolated from the MT fraction and is not detected in the unpolymerized supernatant fraction (Fig. 4, *lanes 1, 3*). When the same fractions were assayed with AC6 primers, amplification products were detected only in the supernatant (Fig. 4, *lane 4*). This association among tau mRNA, MTs, and ELAV-like proteins is in agreement with our previous results showing that proteins of 38 and 43 kDa that bound to the tau 3′-UTR were enriched in the MT fraction (Behar et al., 1995).

The colocalization of ELAV-like proteins with the MTs in PC12 cells was analyzed by confocal microscopy. PC12 cells were treated for 3 d with NGF, by which time a substantial neurite outgrowth was observed (Fig. 5). Staining of the cells with tubulin antibodies revealed a typical array of MTs (Fig. 5*A*,*B*), similar to that observed on staining with the anti-ELAV-like antibodies. The staining was observed in the cell bodies and extended into the neurites and growth cones. The overlap images between the fluorescent signals of tubulin and ELAV-like signals seen in Figure 5, *A* and *D,* and between *B* and *E*, are shown in *G* and *H*, respectively. The computerized image analysis of the pictures reveals overlapping of the two antibodies, suggesting that the ELAV-like proteins in PC12 cells associate with the MTs.

Control experiments showed no penetration of Cy3 or fluorescein signals into the opposite windows (Fig. 5, *C* and *F*, respectively), and no staining was observed when the primary antibodies were omitted (data not shown).

The 38–43 kDa tau mRNA-binding proteins correspond to protein of the ELAV-like family

A schematic outline depicting tau mRNA and the fragments of the 3′-UTR of tau mRNA used in this study (segments F, G, H, I, J) is presented in Figure 6. In an earlier study (Behar et al., 1995), we showed that RNAs transcribed from fragments G, H, and I cross-link to the 43 and 38 kDa proteins present in extracts prepared from rat brain and PC12 cells. No cross-linking was observed with other RNAs transcribed from tau coding or untranslated regions. In an attempt to determine whether the 38 and 43 kDa proteins are related to the ELAV-like protein family, we subjected the labeled cross-linked material to immunoprecipitation with serum specific for the ELAV-like protein family (Szabo et al., 1991). As shown in Figure 7, this serum precipitated labeled 43 kDa protein from UV cross-linked material formed between labeled RNA transcribed from fragment I and total proteins

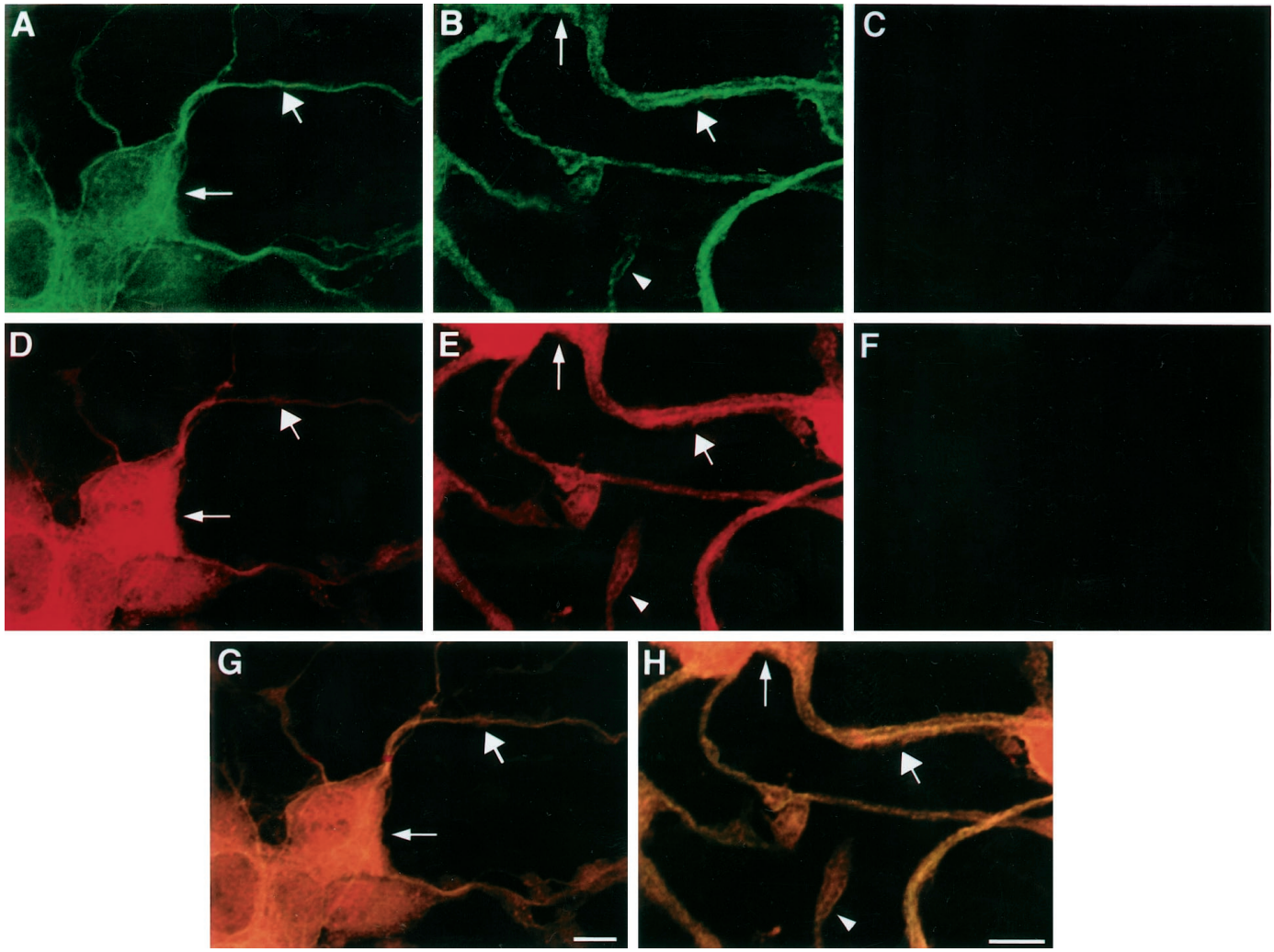

Figure 5. Confocal microscopy analysis of PC12 cells stained with tubulin and anti-ELAV-like antibodies. *A*, *B*, Confocal image of PC12 cells stained with tubulin antibodies. *D*, *E*, Confocal image of PC12 cells stained with anti-Hu serum. *C*, *F*, Control experiments showing no penetration of Cy3 or fluorescein signals into the opposite windows. *Thin arrow*, *wide arrow*, and *arrowhead* indicate cell body, neurite, and growth cone, respectively. Magnification: 5× for *A*, *D*, *G*; 6× for *B*, *E*, *H*. Scale bar, 5 μm.

extract (*lane 1*) or MT-enriched fraction (*lane 2*), both prepared from PC12 cells. The 43 kDa species was not precipitated by nonimmune serum (*lane 4*). In view of these results, we conclude that the previously identified 43 kDa tau mRNA-binding protein belongs to the ELAV-like protein family. This conclusion is based on the following argument. There are three neuronal members of the ELAV-like protein family, namely HuD, HuC, and HelN1 (Szabo et al., 1991; King et al., 1994; Sakai et al., 1994), which are indistinguishable when immunoprecipitated with anti-Hu sera or with mAb 16A11 (Szabo et al., 1991; Marusich et al., 1994). The lower cross-linked protein band (observed in the total PC12 extract) (Fig. 7, *lane 5*) was not precipitated by the antiserum. This may indicate that it is a fragment of the ELAV-like protein, which does not contain the antibody epitope. Alternatively, it may represent another RNA-binding protein, which does not belong to the ELAV-like protein family. The sequence of the recently cloned rat homolog r-HuD, expressed in PC12 cells, shares 99.5 and 95% identity at the protein and DNA levels, respectively, to human HuD (Steller et al., 1996).

Characterization of HuD–tau mRNA complex formation

Although it is clear that the ELAV-like proteins are bound to tau mRNA, it is possible that efficient binding requires the participation of co-factors in the extract. We therefore examined whether purified HuD can bind to a specific fragment of tau mRNA. Purified recombinant HuD was incubated with labeled RNA (Fig. 6, fragments *F* and *H*), and complex formation was assayed by gel retardation analysis, as described previously (Chung et al., 1996). HuD bound with high affinity to tau-H RNA but not to tau-F RNA (Fig. 8). No complexes were detectable in the absence of HuD or in the presence of 200 nM GST fusion carrier protein. Thus, the presence of purified HuD is sufficient to reconstitute the binding. The interaction between HuD and tau-H RNA was examined further by means of a quantitative RNA binding assay. We used the same method as that originally used for the R17 coat protein (Carey et al., 1983). A low concentration of labeled RNA was incubated with increasing concentrations of HuD protein as indicated. The reaction mixture was filtered through nitrocellulose, and the bound radioactivity was determined. As shown in

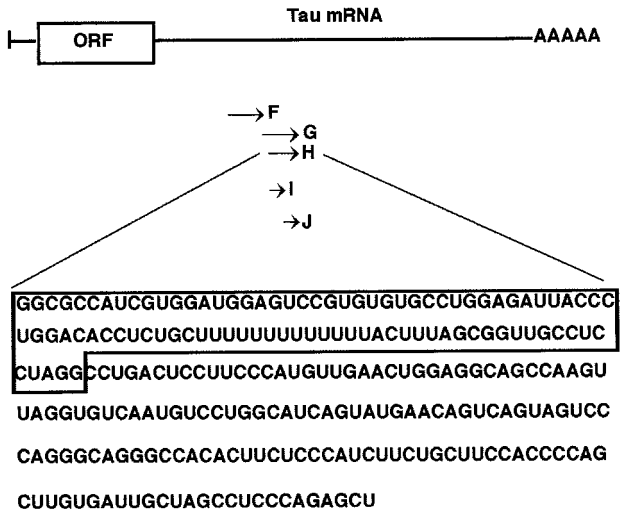

Figure 6. Structure of tau mRNA. The fragments (*F*, *G*, *H*, *I*, and *J*) used in this study correspond to nucleotides 1778–2175 (397 bp), 2175–2760 (624 bp), 2519–2760 (241 bp), 2519–2610 (91 bp), and 2610–2760 (150 bp), respectively (Sadot et al., 1994). The 241-nucleotide sequence of fragment H is presented, and the I fragment containing the U-rich segment is *boxed*. The U-rich 21 nucleotides are deleted from fragment G (Gdel).

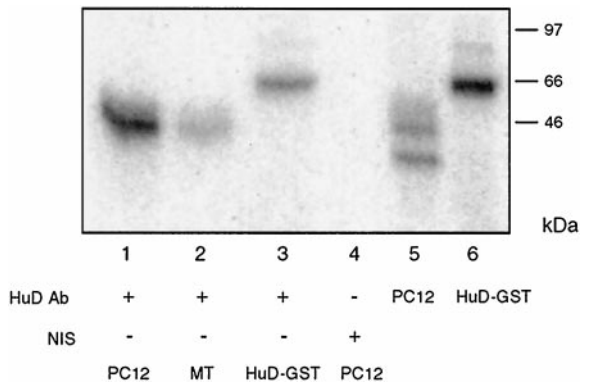

Figure 7. Immunoprecipitation of UV cross-linked complexes by anti-Hu ELAV-like antibodies: SDS gel analysis of immunoprecipitated complexes formed between PC12 cell extracts (*lane 1*), assembled MT preparations isolated from PC12 cells (*lane 2*), or purified HuD-GST (*lane 3*) UV cross-linked to [^{32}P]-labeled tau RNA fragment I. NIS is the complex formed with normal serum (*lane 4*). *Lanes 5* and 6 show unprecipitated PC12 extract and GST-HuD protein analyzed immediately after cross-linking.

Figure 9, formation of the tau-H–HuD complex was detectable at 3 nM and had a midpoint at ~100 nM. Complex formation with tau-F RNA was not detectable under these conditions. A plot of the log of complexed/free RNA versus the log of HuD concentration yields a straight line. At 50% complex formation the intercept on the *x*-axis corresponds to 131 nM (Fig. 9). Thus, the binding of HuD to tau mRNA is a simple molecular reaction with an apparent Kd of 131 nM. Similar kinetic data were recently obtained for GAP-43 mRNA (Chung et al., 1997).

HuD binds to a conserved U-rich segment in tau mRNA

To localize the HuD-binding region within the H segment of tau mRNA, we used the RNase T1 selection analysis that we have

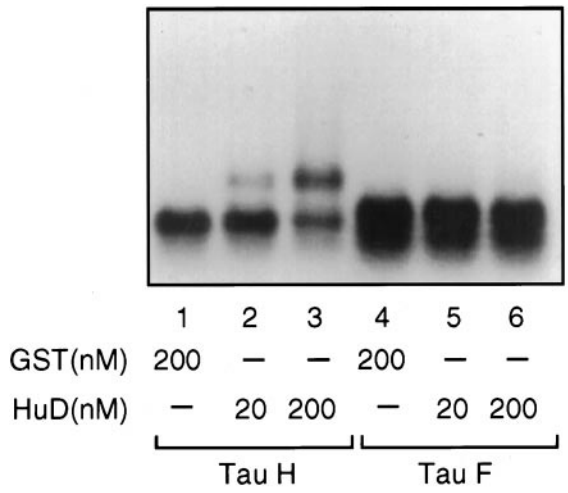

Figure 8. Purified HuD binds to tau mRNA. The indicated [^{32}P]-labeled RNAs were incubated with the indicated concentrations of GST or HuD. After incubation, the reaction mixture was resolved by gel electrophoresis in 0.8% agarose gel.

used previously to analyze other mRNAs. In this technique, the HuD–RNA complex is formed and is then digested with RNase T1. The specific RNA fragments bound to HuD are isolated by allowing the complex to be absorbed by nitrocellulose filter (under the conditions used, uncomplexed RNA passes through). The RNA fragments bound to HuD are then eluted from the nitrocellulose with phenol-chloroform (Chung et al., 1996). The pattern of digestion of tau-H RNA with RNase T1 is shown in Figure 10 (*lane 3*). The tau-H RNA contains two large RNase T1 oligonucleotides that are not resolved, because small RNAs migrate according to size and base composition. The two oligonucleotides can be distinguished by analysis of tau fragments I and J, which are subfragments of H (Figs. 6, 10). HuD was found to bind to the U-rich region present in fragments H and I (Fig. 10, *lanes 2, 5*) but not J (*lane 8*). Thus we can conclude that HuD binds within the U-rich segment located within fragment I (Fig. 6, *boxed area*). The selected species was resistant to further digestion with RNase T1. The precise assignation of the binding site cannot be made because of the close ladder observed in the lanes demonstrating the presence of the complex formed between HuD and RNA transcript. The reasons for the multiple bands are technical and stem from the length of the U-rich region, which causes stuttering of the RNA polymerase during synthesis of the transcript. This binding region is similar in sequence to those observed in other mRNAs (Chung et al., 1997; Jain et al., 1997; Joseph et al., 1998). Our assignment of the binding region was further validated by RNase T1 selection experiments using RNA transcribed from a Gdel fragment (*lanes 13–15*). Deletion of the 21 nucleotides of the U-rich region from fragment G (*lanes 10–12*), which is also included in fragment H (Behar et al., 1995), abolished the binding of purified HuD protein (*lane 14*). Similar results were observed when UV cross-linking assay was performed using RNA transcribed from the same fragments incubated with purified GST-HuD protein or protein extracts prepared from PC12 cells (Fig. 10, *A* and *B*, respectively).

DISCUSSION

The ELAV-like RNA-binding proteins are a conserved protein family involved in growth, differentiation, and post-transcriptional

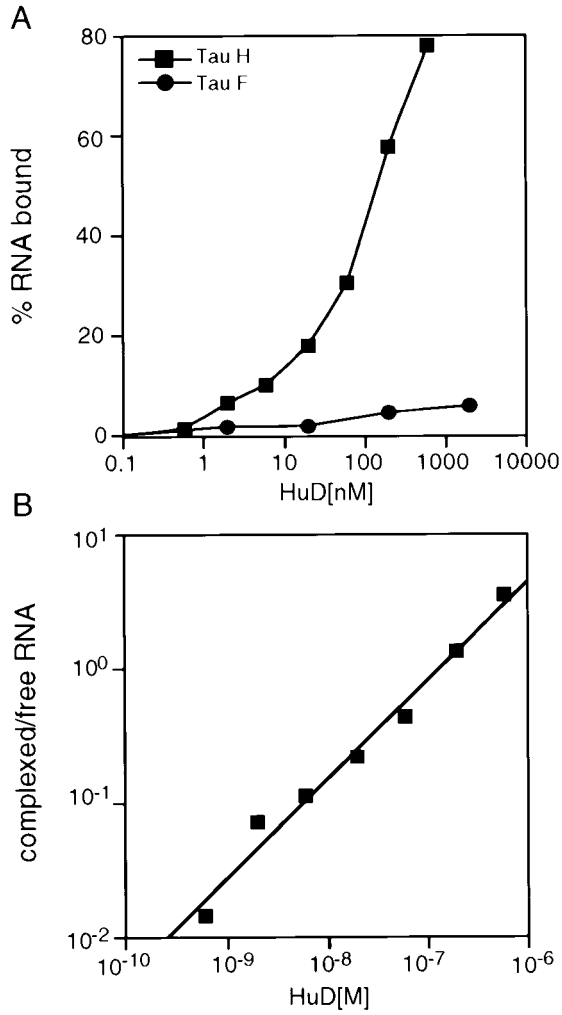

Figure 9. Affinity of HuD for tau mRNA. The affinity of purified HuD-GST for tau mRNA was determined by nitrocellulose filter binding assay, as described in Materials and Methods. *A*, Plot of percentage of bound RNA versus log of HuD concentration. *B*, Plot of log of ratio between complexed/free RNA versus log of HuD concentration.

gene expression (Antic and Keene, 1997; Good, 1997). Among these proteins HuD, HuC, and Hel-N1 are exclusively neuronal and are expressed in postmitotic neurons and in neuroendocrine tumors (Dalmau et al., 1992; King et al., 1994; Marusich et al., 1994). These proteins bind to U-rich elements found in a wide variety of mRNAs, and it was therefore suggested that such binding might target them for rapid degradation (Chen and Shyu, 1995). Recent findings suggest that ELAV-like proteins help to stabilize a specific subset of mRNAs, which may be involved in the mechanism leading to neuronal differentiation (Antic and Keene, 1997; Jain et al., 1997; Myer et al., 1997; Fan and Steitz, 1998; Levy et al., 1998; Peng et al., 1998).

In a previous study we identified neuronal RNA-binding proteins that specifically recognize the 3′-UTR region of tau mRNA (Behar et al., 1995) present in extracts prepared from rat brains and PC12 cells. In the present study we characterized these RNA-binding proteins as the ELAV-like proteins (Fig. 7) (Chung et al., 1996). This discovery might represent an important link between proteins that regulate neuronal differentiation at the post-transcriptional level and a target mRNA that encodes a neuron-specific protein required for differentiation. Furthermore, we demonstrated the interaction between the endogenous ELAV-like protein and tau mRNA *in vivo* (Figs. 3, 4). Inhibition of HuD expression in PC12 cells abolished their response to NGF-induced differentiation, with concomitant downregulation of tau mRNA (Figs. 1, 2). GAP-43 is another neuronal target mRNA that binds to and is stabilized by the ELAV-like protein (Chung et al., 1997).

The PC12 cell line serves as a useful model to study many steps of neuronal differentiation (Greene and Tischler, 1976). However, NGF induction in PC12 cells results in neurite extension and not in the elaboration of defined dendrites and axons. It therefore remains to be seen whether tau expression during the early stages of brain development is regulated by the ELAV-like proteins. The kinetics of ELAV-like protein induction during development are consistent with such a role, because these proteins are expressed immediately after the withdrawal from the mitotic cycle (Marusich et al., 1994; Barami et al., 1995), which happens before the induction of tau or GAP-43. Recent data indeed demonstrate that HuD protein binds to a conserved U-rich region within the 3′-UTR of $p21^{waf1}$ mRNA, a protein that is involved in arresting the cell cycle in PC12 cells, a requirement for initiating neuronal differentiation (Yan and Ziff, 1995, 1997). Taken together, these findings might suggest that the ELAV-like proteins may regulate the stability of a subclass of mRNAs in a time-specific manner, thereby regulating neuronal differentiation.

From the present study and recent work on Hel-N1 and HuR (Changnovich et al., 1996; Jain et al., 1997; Levy et al., 1998; Peng et al., 1998; Antic et al., 1999), it is clear that the ELAV-like proteins act by increasing the amounts of their target mRNAs and/or translatability, which may be linked in some cases. Our results demonstrate specific binding both *in vitro* and *in vivo* to a U-rich region located in tau 3′-UTR (Figs. 8–10). The current concept is that the ELAV-like proteins bind to the U-rich element and thus prevent the selective turnover of that mRNA. Unlike other mRNA targets, tau mRNA is a relatively stable molecule (Sadot et al., 1995). In a recent study, we showed that fragment H (Fig. 6) of the tau 3′-UTR, which contains the U-rich region, is responsible for tau mRNA stability and confers stability on heterologous c-*fos* after transfection into neuronal cells (Aronov and Ginzburg, 1999). It thus seems that in addition to inhibition of selective decay, the ELAV-like proteins may serve as linkers between a target mRNA and MTs, thus anchoring the message and protecting it from degradation. We have shown previously that localization of tau mRNA involves an association with the functional MT system. This was evident both in neuronal cells (Litman et al., 1994) and in microinjected *Xenopus* oocytes, where additional proteins are involved (Litman et al., 1996). In the present work we showed that ELAV-like proteins fractionate with MT (Fig. 4). In addition, confocal microscopy showed colocalization of the ELAV-like proteins on MTs and extension into the neurites of PC12 cells (Fig. 5). We therefore suggest that ELAV-like proteins may be a component of the RNA–protein particles that were recently described in the RNA localization pathway (Ainger et al., 1993; Wilhelm and Vale, 1993). These particles are localized near MTs and contain both the targeted mRNA and several additional protein factors, including protein synthetic machinery and possibly proteins involved in mRNA stabilization and anchorage (Hamill et al., 1994; Barbarese et al., 1995). In agreement with this notion is the finding that Hel-N1 is present in granular RNA protein structures that contain mRNA

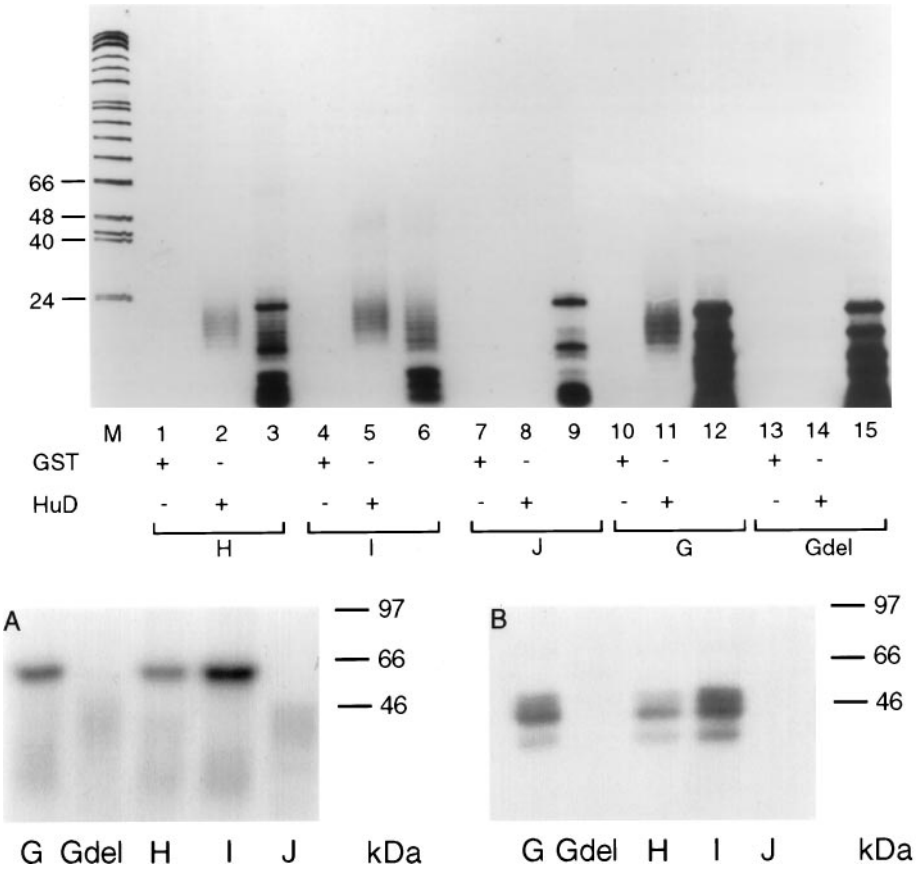

Figure 10. RNase T1 selection analysis of the HuD binding region in the tau-H fragment. RNase T1 selection assay was performed as described previously (Chung et al., 1996) with GST (*lanes 1, 4, 7, 10, 13*) or HuD (*lanes 2, 5, 8, 11, 14*), incubated with [^{32}P]-labeled *H*, *I*, *J*, *G*, and *Gdel* RNAs, respectively. The reaction mixture was treated with RNase T1 and filtered through nitrocellulose to select for the RNA/protein complexes. After washing, the fragments were eluted and resolved on a 12% denaturing polyacrylamide gel. *Lanes 3, 6, 9, 12*, and *15* are the total unselected RNase T1 digests of the indicated fragments. *A*, UV cross-linking assay with purified GST-HUD. *B*, UV cross-linking assay with PC12 protein extracts.

and ribosomes (Gao and Keene, 1996; Antic and Keene, 1998). Indeed a pleasing aspect of this model is that it may explain the diversity of ELAV-like proteins in neuronal cells. It is possible that the three neuronal members, HuD, HuC, and Hel-N1, are involved in different localization pathways. It is important to note, however, that there must be an additional level of specificity. For example, HuD binds *in vitro* to a large number of mRNAs that contain the U-rich element (Chung et al., 1996, 1997; Ma et al., 1997). Obviously, not all of these mRNAs are localized in the same cellular microdomains. Moreover, the ELAV-like proteins do not appear to differ significantly in the way they bind to particular RNA targets. It was shown recently that only a subset of mRNAs is associated with MTs as ELAV mRNP particles that associate with the translational apparatus (Antic and Keene, 1998). *In vivo*, therefore, it is likely that other conserved flanking sequences and *trans*-acting specific factors are involved.

Neuronal polarity, which is required for neuronal plasticity, depends on interaction between neuron-specific mRNAs and a family of proteins regulating their expression. Subcellular RNA localization has been described in germ cells, as well as in somatic cells such as fibroblasts, muscle cells, and neurons (Wilhelm and Vale, 1993; St. Johnston, 1995), as a mechanism responsible for creating the polarity that involves synergistic controls of translation, stabilization, and association with the cytoskeleton. Such controls are mediated primarily through the 3′-UTRs of the mRNAs. Regulation of the stability of these mRNAs is crucial during movement and establishment of the cell polarity as well as subsequent controlling of their levels at the different compartments (Gao, 1998).

Understanding of the interaction between tau mRNA and ELAV-like proteins may help to explain the control of tau mRNA stabilization, which is important for neuronal differentiation and its subcellular localization process. Although the molecular mechanism by which the ELAV-like proteins control the half-life of specific messages remains to be determined, our results show that these proteins bind to specific *cis*-sequences in the 3′-UTR and thus facilitate their binding to the MTs. This mechanism may shed light on the physiological function of the ELAV-like protein family in differentiation and maintenance of the neuronal system.

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