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Hel-N1/Hel-N2 proteins are bound to poly(A)$^+$ mRNA in granular RNP structures and are implicated in neuronal differentiation

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SUMMARY

Human proteins Hel-N1 and Hel-N2 contain three RNA recognition motifs (RRMs), and are members of a family of proteins highly homologous to *Drosophila* ELAV, which is essential for neuronal differentiation. Both proteins bind to A+U-rich 3′ untranslated regions of a variety of growth-related mRNAs in vitro. Here we demonstrate that in medulloblastoma cells derived from childhood brain tumors, Hel-N1 and Hel-N2 are mainly expressed in the cytoplasm, but are detectable in the nucleus. Both proteins are associated with polysomes and can be UV-crosslinked to poly(A)$^+$ mRNA in cell extracts. In the cytoplasm the Hel-N1 protein family resides in granular structures that may contain multiple protein molecules bound to each mRNA. Evidence supporting this multimeric ribonucleoprotein (RNP) model includes in vitro reconstitution and competition experiments in which addition of a single RRM (RRM3) can alter complex formation. As in medulloblastoma cells, the Hel-N1 protein family is present in granular particles in the soma and the proximal regions of dendrites of cultured neurons, and colocalizes with ribosomes. In addition, we demonstrate that expression of the Hel-N1 protein family is up-regulated during neuronal differentiation of embryonic carcinoma P19 cells. Our data suggest that the Hel-N1 protein family is associated with the translational apparatus and implicated in both mRNA metabolism and neuronal differentiation. Furthermore, our findings open the possibility that these proteins participate in mRNA homeostasis in the dendrites and soma of mature neurons.

Key words: 3′ Untranslated region, mRNA stability, RNA binding protein, Autoimmunity, Paraneoplastic disease

INTRODUCTION

Mammalian cell differentiation involves the regulation of gene expression at many levels, both transcriptional and post-transcriptional. Identification of the genes involved and elucidation of their molecular functions are essential for understanding complex differentiation pathways. We have identified a human protein, Hel-N1, and its counterpart in rat, Rel-N1, which are implicated in cell differentiation and development in the central nervous system (CNS) (King et al., 1994). Rel-N1 and Hel-N1 were cloned from cDNA libraries on the basis of their strong sequence similarity to the *Drosophila* ELAV protein, which is essential for the early development of the central nervous system (CNS). Deletions at the *elav* locus give rise to an embryonic lethal phenotype with numerous defects in the CNS, including the failure of neuroblasts to differentiate fully (Campos et al., 1985; Jiminez and Campos-Ortega, 1987). However, the molecular mechanisms underlying the functions of ELAV, Hel-N1 and related family proteins are unknown.

ELAV and related proteins are distinct from other cellular factors involved in neuronal differentiation, in part because they function at the post-transcriptional level of gene regulation via three RNA recognition motifs (RRMs). It has been demonstrated that RRMs can constitute the core structure of functional RNA binding domains (Query et al., 1989). There are more than 200 RRM-containing proteins that are implicated in many aspects of RNA metabolism, including RNA splicing, transport and translation (reviewed by Kenan et al., 1991; Birney et al., 1993; Burd and Dreyfuss, 1994).

Previously we identified Hel-N2, which differs from Hel-N1 by lacking 13 amino acid residues between the second and the third RRMs. This 13 amino acid segment is predicted to interact with other proteins and may distinguish functional properties of Hel-N2 from those of Hel-N1. Interestingly, Hel-N2 is highly expressed in human brain tumor cells in contrast to Hel-N1, which is more predominant in normal adult brain (Gao et al., 1994). Using an in vitro randomized RNA selection procedure we have identified the A+U-rich RNA sequences that Hel-N1/Hel-N2 prefer for binding. These in vitro selected sequences match well with the sequences found in brain mRNAs selected by Hel-N1 from combinatorial 3′UTR libraries (Levine et al., 1993; Gao et al., 1994). The mRNAs that Hel-N1/Hel-N2 can bind in vitro include those encoding immediate early proteins (such as c-myc and c-fos), other transcription factors and signal transduction proteins. Together with the analogy of Hel-N1 to ELAV, these studies suggested a potential role for Hel-N1/Hel-N2 in the regulation of expression of a subset of growth-regulatory messages during neuronal differentiation (Gao et al., 1994).

A number of other proteins highly homologous to ELAV

(Robinow et al., 1988) and Hel-N1 have been identified in various species, including HuD (Szabo et al., 1991) and HuC/ple21 (Szabo et al., 1991; Sakai et al., 1994) in man, RBP9 in flies (Kim and Baker, 1993), and corresponding clones in mouse, zebrafish and *Xenopus* (Abe et al., 1994; Good, 1995) and in *Caenorhabditis elegans* (C. Carson and J. D. K., unpublished). Among the 16 members of this family reported to date, the degree of homology in the RRMs is exceedingly high, particularly for amino acids known to be critical for RNA recognition and specificity of binding (Kenan et al., 1991). Most of these proteins are expressed in the nervous system, indicating that they may also play a role in neural development and differentiation. The Hel-N1 protein family has been detected in testes and ovaries also (King et al., 1994; Good 1995; Gao, 1995). However, little is known about the subcellular localization of these proteins, the nature of the RNA species to which they are bound in vivo, or their functions.

In this report, we demonstrate that the expression of the Hel-N1 protein family in rat brain is neuron-specific. In normal rat neurons the Hel-N1 protein family is localized in the nucleus and the cytosol, as well as in dendrites, suggesting potential roles in RNA transport and localization. In human brain tumor cells Hel-N1/Hel-N2 proteins are mainly present in the cytosol, where they are bound to poly(A)$^+$ mRNA and are associated with polysomes, suggesting their involvement in translation or mRNA stability. In addition, the Hel-N1 protein family is shown to reside in granular particles in the cytosol and along the proximal regions of dendrites, but is not detectable in axons. Furthermore, the expression of the Hel-N1 protein family is dramatically increased during neuron-specific differentiation of embryonic carcinoma P19 cells induced by retinoic acid. We propose that the Hel-N1 protein family is bound to a subset of cellular mRNAs and, possibly, is involved in their translation or stability. Given their constitutive expression in postmitotic cells, these proteins may also participate in mRNA homeostasis within dendrites and soma of mature neurons.

MATERIALS AND METHODS

Cell lines

Human medulloblastoma cells were maintained in suspension in RPMI 1640 medium with 10% fetal bovine serum (Hyclone) (Bigner et al., 1988). Cells were passaged once every 2 days to maintain a density of less than 2×10^6 cells per ml. Mouse embryonal carcinoma P19 cells were maintained in α-MEM supplemented with 7.5% (v/v) bovine serum and 2.5% fetal calf serum. For inducing differentiation, monolayer P19 cells were trypsinized, and seeded at 10^6 cells per 100 mm bacteriological grade Petri dishes in α-MEM medium with 0.5 µM retinoic acid (RA) or 1% dimethyl sulfoxide (DMSO). After 3-4 days in culture, cell aggregates were collected and reseeded in tissue culture plates without inducing drugs for an additional 1-4 days before western blotting or immunofluorescence analysis. Rat cortical cells taken from day 16 rat embryo were cultured on polylysine-coated chamber slides for 1-2 weeks before fixation. All cells were grown at 37°C in a 5% CO_2 incubator.

Antibodies

Rabbit anti-Hel-N1 antibody was raised as described previously (Gao et al., 1994). Mouse monoclonal antibodies against microtubule-associated protein-2 (MAP-2) and glial fibrillary acidic protein (GFAP) were purchased from Boehringer Mannheim Inc. Monoclonal anti-hnRNP C antibody was provided by Dr G. Dreyfuss. Monoclonal anti-GAP-43 antibody (91E12) was provided by Dr P. J. H. Skene. Antibody to Fragile X Mental Retardation (FMR) protein was provided by Dr S. T. Warren. Human anti-ribosomal P antibody was purchased from Immunovision Inc.

western blot analysis

All the protein samples were boiled for 5 minutes in 1× Laemmli buffer, resolved by 10% polyacrylamide-SDS gel electrophoresis and then transferred to nitrocellulose. Hel-N1/Hel-N2 or hnRNP C proteins were detected by rabbit anti-Hel-N1 or mouse anti-hnRNP C antibodies in TBST buffer according to the ECL procedure (Amersham). In some cases, ECL was replaced by more quantitative western analysis using ^{125}I-conjugated Protein A as secondary detection reagent.

Preparation of S16 extracts

Human medulloblastoma cells were harvested by centrifugation at 3,000 ***g*** for 5 minutes. Cell pellets were washed three times with RNase-free cold phosphate buffered saline (PBS), and resuspended gently in 200 µl lysis buffer (10 mM HEPES (pH 7.4), 150 mM KCl, 5 mM $MgCl_2$, 0.2% NP-40, 40 units RNasin/ml, 1 mM DTT, 10 mM VRC; vanadyl/ribonucleotide complex) in a 1.5 ml Eppendorf tube. After 5 minutes on ice, nuclei were pelleted by centrifugation at 12,000 ***g*** for 10 minutes at 4°C. The clarified supernatant was taken as S16 extracts.

Polysome profile analysis by sucrose density gradient

Human medulloblastoma cells S16 supernatants were prepared as described above. In the case of EDTA treatment, 5 mM $MgCl_2$ was replaced by 10 mM EDTA. S16 extracts were loaded onto the top of sucrose gradient (15% to 45% (w/v) sucrose) in 100 mM KCl, 5 mM $MgCl_2$ (or 10 mM EDTA), 10 mM HEPES (pH 7.4) and centrifuged at 250,000 ***g*** for 1.5 hours at 4°C using SW41 rotor (Beckman). Fractions were collected for 200 µl per Eppendorf tube. The absorbance values at 254 nm were determined on DMS 80 UV spectrophotometer (Varian Inc.). Every six small fractions were combined into a sample, and analyzed on a western blot using rabbit anti-Hel-N1 antibodies.

UV-crosslinking of Hel-N1/Hel-N2 to poly(A)$^+$ mRNAs and oligo(dT)-cellulose purification

Human medulloblastoma cell S16 extracts were separated into S130 fractions and polysome fractions (Brewer and Ross, 1990). The polysome fractions were resuspended in UV-crosslinking buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA, 1 mM DTT, 40 units RNasin/ml). The supernatant after 12,000 ***g*** centrifugation for 5 minutes in the cold room were stored in 6-well culture dishes on ice. The samples were irradiated by a UVs-54 lamp (UV 254 nm, 0.16 amps, Ultra-violet Products Inc.) at 10 cm for 10 minutes. SDS was added to the samples at a final concentration of 0.1% at room temperature and NaCl was added to a final concentration of 0.45 M. The samples were heated to 65°C for 10 minutes, quick-cooled to room temperature, and passed through an oligo(dT)-cellulose column pre-equilibrated with washing buffer (150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.1% SDS). The column was washed with 5× column bed volume of washing buffer. The bound proteins were eluted with buffer containing 1 mM EDTA, 10 mM Tris-HCl (pH 7.5), 0.1% SDS and analyzed by western blotting.

Immunofluorescence of cultured cells

Medulloblastoma cells were centrifuged onto a glass slide in a cytocentrifuge. Cells were washed in PBS, and fixed with PLP (10 mM periodate, 75 mM lysine and 2% paraformaldehyde) for 15 minutes at the room temperature. For cultured rat cortical cells or P19 cells, 4% paraformaldehyde in PBS with 0.12 M sucrose was used as a fixative. After washing with PBS, cells were permeabilized in 0.1% Triton X-100 (in PBS) for 15 minutes, then incubated in blocking buffer (1% BSA, 1% goat serum, 0.05% Tween-20, 5 mM EDTA) for 1 hour. Cells were treated with rabbit anti-Hel-N1 and mouse anti-hnRNP C (or other monoclonal) antibodies in blocking buffer at a dilution of 1:100 to 1:400 for 30 minutes at room temperature, followed by incu-

bation for 30 minutes with Texas Red- or FITC-conjugated anti-rabbit or anti-mouse IgG antibodies (Jackson Immunoresearch) at a dilution of 1:50 to 1:150. The cells were washed in PBS, mounted on glass slides and observed under epifluorescence microscopy (Zeiss). Photographs were taken using Tmax-400 films (Kodak).

Sectioning of rat brain

Rat brain coronal section slides were prepared from fresh frozen rat brain tissue, and cut at 4 µm per section as previously described (Haynes et al., 1984). Immunostaining was identical to that described above for cultured cells.

Mobility shift analysis of protein-RNA interactions

[^{32}P]UTP-labeled RNAs were incubated with partially purified Hel-N1, RRM3, or both, in 50 µl of RNA binding buffer (20 mM KCl, 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.05% NP-40, 2.5% polyvinyl alcohol, 1 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-amino-ethyl-ether)-*N*,*N*,*N*′,*N*′-tetracetic acid (EDTA), 0.5 mg of yeast tRNA per ml, 50 µg poly(A) per ml, 1 mM DTT, 0.125 mg of bovine serum albumin (BSA) per ml, 0.4 mM VRC, 80 units of RNasin per ml) at room temperature for 10 minutes. RNA-protein complexes were resolved by electrophoresis through a 3.2% or 6% nondenaturing polyacrylamide gel with 0.5×TBE (final concentrations of 45 mM Tris-boric acid (pH 7.5), 1 mM EDTA) as the running buffer. Gels were pre-run at 4°C for 1 hour at 12 V/cm gel prior to loading the reaction mixtures, and electrophoresis proceeded for an additional 3-4 hours at a constant voltage in the cold room. The gels were transferred to filter paper (Whatman) and exposed for autoradiography.

RESULTS

Multiple forms of Hel-N1 proteins are detected mainly in the cytosol of human medulloblastoma cells

Human medulloblastoma is the most common pediatric primary brain tumor that appears to arise from the primitive bipotential cells in the cerebellum (Bigner et al., 1988). Using RT-PCR analysis we have previously reported the presence of Hel-N1 and its related form, Hel-N2, in human medulloblastoma cells and in small cell lung cancer cells (Gao et al., 1994). The upper panel in Fig. 1 shows a western blot analysis of medulloblastoma cell extracts using rabbit antibodies raised against recombinant Hel-N1. Consistent with our previous RT-

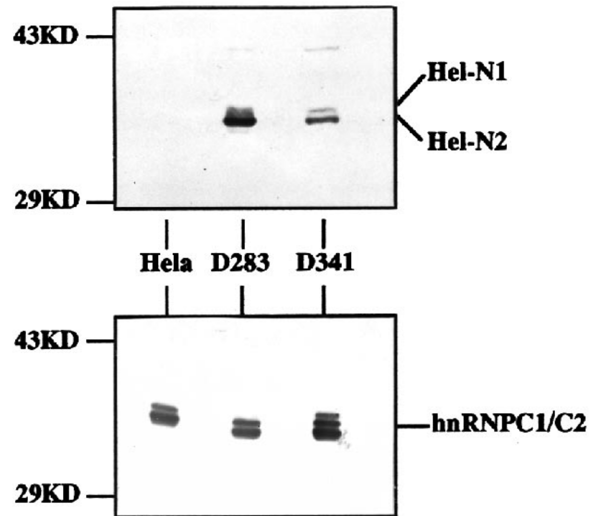

Fig. 1. Detection of Hel-N1/Hel-N2 proteins in human medulloblastoma cell lines D283 and D341 using western blotting. Upper panel: rabbit anti-Hel-N1 antibody; lower panel: monoclonal anti-hnRNP-C antibody 4F4. The numbers at the left indicate the positions of standard molecular mass markers (in kDa).

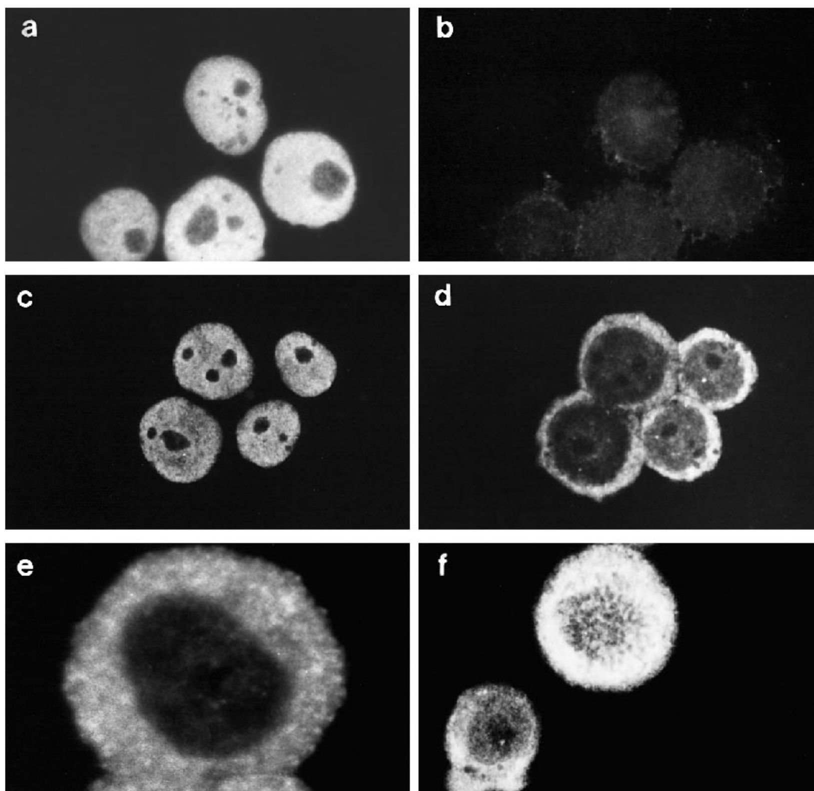

Fig. 2. Expression patterns of Hel-N1/Hel-N2 proteins in human medulloblastoma cells detected by indirect immunofluorescence. (a and c) Anti-hnRNP C antibody (4F4); (b) prebleed rabbit serum, same field as a; (d) rabbit anti-Hel-N1 antibody, same field as c. Cells were stained with anti-Hel-N1 antibody to demonstrate the granular distribution of Hel-N1/Hel-N2 in the cytosol of interphase cells (e) and during mitosis (f). All of the pictures were taken under ×100 oil-immersion objective.

PCR study, Hel-N1/Hel-N2 proteins (migrating at approximately 36-37 kDa) were expressed in human medulloblastoma cells, but not in HeLa cells. A faint band near 45 kDa detected by Hel-N1 antibody may represent another member of the Hel-N1 protein family, which has been observed in a variety of tissue types (C. Carson and J. D. K., unpublished). An hnRNP-C-specific monoclonal antibody was used to probe the same western blot and demonstrated that equal amounts of protein were loaded in each lane (Fig. 1, lower panel). The extra hnRNP-C band in cell line D341 is probably the phosphorylated form. By peptide mapping using V8 protease we also demonstrated that the major band in the upper panel of Fig. 1 corresponds to Hel-N2, which lacks 13 amino acid residues in a "hinge region" between the second RRM and the third RRM compared with Hel-N1 (data not shown). Interestingly, the ratio of Hel-N2 to Hel-N1 in the D283 cell line as determined by densitometry quantification is greater than 14. However, this ratio is reversed in adult brain in that Hel-N1 is predominant and Hel-N2 is barely detectable (Gao et al., 1994). In addition to results from the study using P19 cells presented below, these data suggest that the ratio of Hel-N1 to Hel-N2 may correlate with the differentiation state of cells, but this aspect will require further investigation.

To examine the subcellular localization of Hel-N1/Hel-N2 proteins in medulloblastoma cells, we performed double-labeling immunofluorescence experiments, as shown in Fig. 2. As a control, anti-hnRNP C antibody demonstrated the distinct localization of hnRNP C proteins to the nucleus (a and c). Fig. 2b shows background staining of human medulloblastoma cells using a pre-bleed rabbit serum as control. On the other

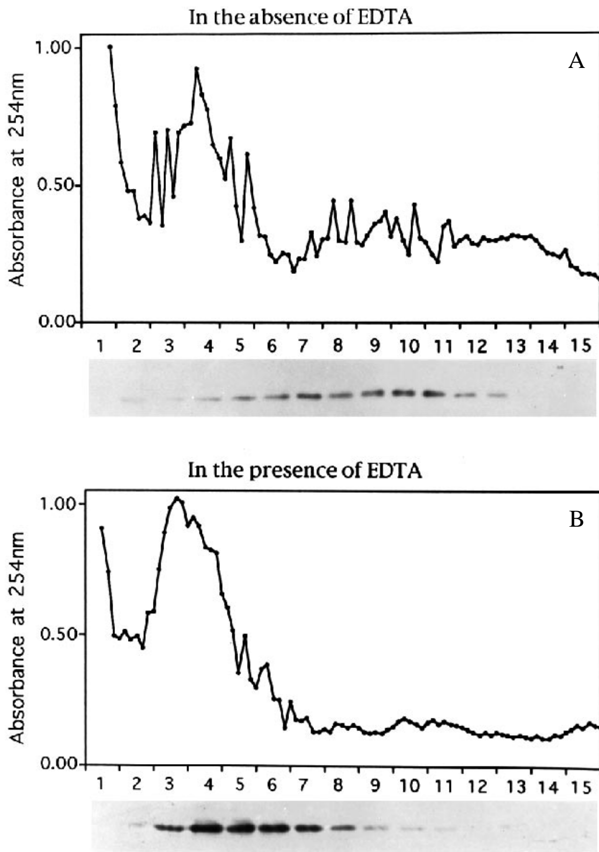

Fig. 3. Polysome profiles and the sedimentation of Hel-N1/Hel-N2 proteins on 15% to 45% sucrose gradients. (A) Separation in the absence of EDTA; (B) separation in the presence of 10 mM EDTA. Numbers 1-15 indicate each fraction that corresponds to each lane on the western blot underneath the polysome profile. The peak of Hel-N1/Hel-N2 sedimentation comigrates with the polysome peaks between fractions 7 and 12.

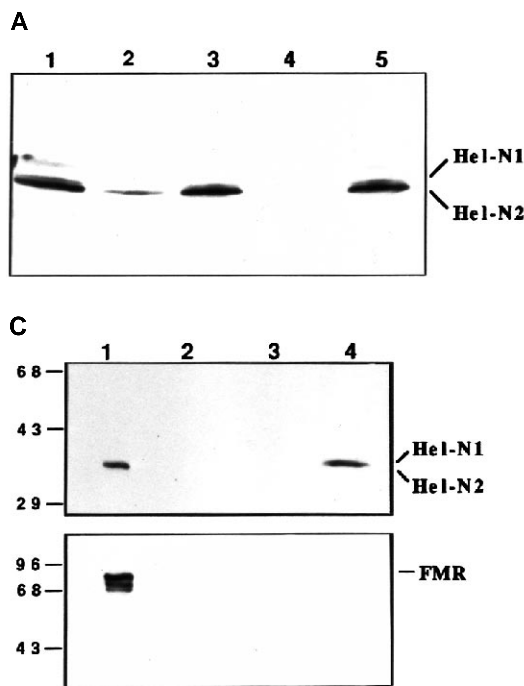

Fig. 4. Demonstration of direct contact between Hel-N1/Hel-N2 proteins and poly(A)$^+$ mRNA using UV-crosslinking. (A) Hel-N1 mRNPs in whole cell extracts bound to oligo(dT)-cellulose and detected by western blotting using anti-Hel-N1 antibody. Lane 1, total lysate; lane 2, flow through in the presence of 150 mM NaCl; lane 3, bound proteins in the presence of 150 mM NaCl; lane 4, flow through in the presence of 450 mM NaCl; lane 5, bound proteins in the presence of 450 mM NaCl. (B) Experimental procedure for the experiments shown in C. (C) Hel-N1/Hel-N2 proteins UV-crosslinked to poly(A)$^+$ mRNA. Lane 1, 10% of total polysome fraction treated with UV irradiation; lane 2, no UV-crosslinking and treated with 0.1% SDS; lane 3, treated with 0.1% SDS before UV-crosslinking; lane 4, UV-crosslinking before treatment with 0.1% SDS. Molecular mass is given on the left in kDa.

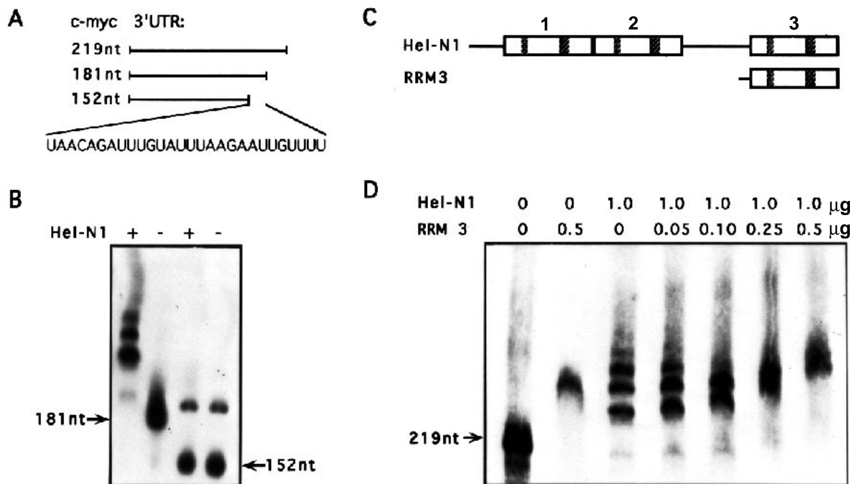

Fig. 5. Hel-N1 and the c-*myc* 3′untranslated region (3′UTR) form RNP multimers, which can be blocked by addition of Hel-N1 RRM3 fragment.
(A) Schematic representation of c-*myc* 3′UTR. Numbers on the left indicate the length of each transcript. The sequence shown is the core sequence to which Hel-N1 binds. (B) Gel shift analysis of Hel-N1 binding to the c-*myc* 3′UTR. + and – indicate the presence or absence of Hel-N1 in each lane. (C) Schematic representation of Hel-N1 and RRM3. The large boxes represent the three RRMs of Hel-N1 and the hinge region connecting RRM2 and RRM3. The small and the large shaded boxes represent RNP-2 hexamer and RNP-1 octamer consensus sequences. (D) Competition gel shift analysis of Hel-N1 and RRM3 proteins binding to the c-*myc* 3′UTR. Hel-N1 is four times larger than RRM3, resulting in a molar ratio of 1:2 in the last lane.

hand, Hel-N1/Hel-N2 proteins were observed mainly in the cytosol, but were detectable in the nucleus also (Fig. 2d). Interestingly, it is evident that Hel-N1/Hel-N2 proteins reside in granular structures in the cytosol, as seen best in the enlarged picture (Fig. 2e). This granular expression pattern of Hel-N1/Hel-N2 was also observed in normal brain sections and in cultured neurons (see below). During mitosis, Hel-N1/Hel-N2 proteins appear to be diffused throughout the medulloblastoma cells, but remain in granular structures (Fig. 2f).

Hel-N1/Hel-N2 proteins associate with polysomes in the cytosol of medulloblastoma cells

On the basis of in vitro RNA selection from combinatorial libraries (reviewed by Kenan et al., 1994) we found that Hel-N1/Hel-N2 can bind to A+U-rich sequences present in the 3′UTRs of various cellular mRNAs, including many that encode growth-regulatory proteins (Levine et al., 1993; Gao et al., 1994). To investigate whether Hel-N1/Hel-N2 proteins are related to the translation or stability of mRNA, we studied their association with polysomes in cell lysates. A 130,000 ***g*** post-ribosomal supernatant (S130) and polysome pellet from human medulloblastoma cell postnuclear extracts were separated on a 30% sucrose cushion (Brewer and Ross, 1990). The majority of Hel-N1/Hel-N2 proteins was present in this crude polysome fraction as determined by polyacrylamide-SDS gel electrophoresis and western blotting using rabbit anti-Hel-N1 antibodies, while as a control the RRM protein La remained in the S130 fraction. To demonstrate further the association of Hel-N1/Hel-N2 proteins with polysomes, 15% to 45% sucrose polysome gradients were performed using medulloblastoma

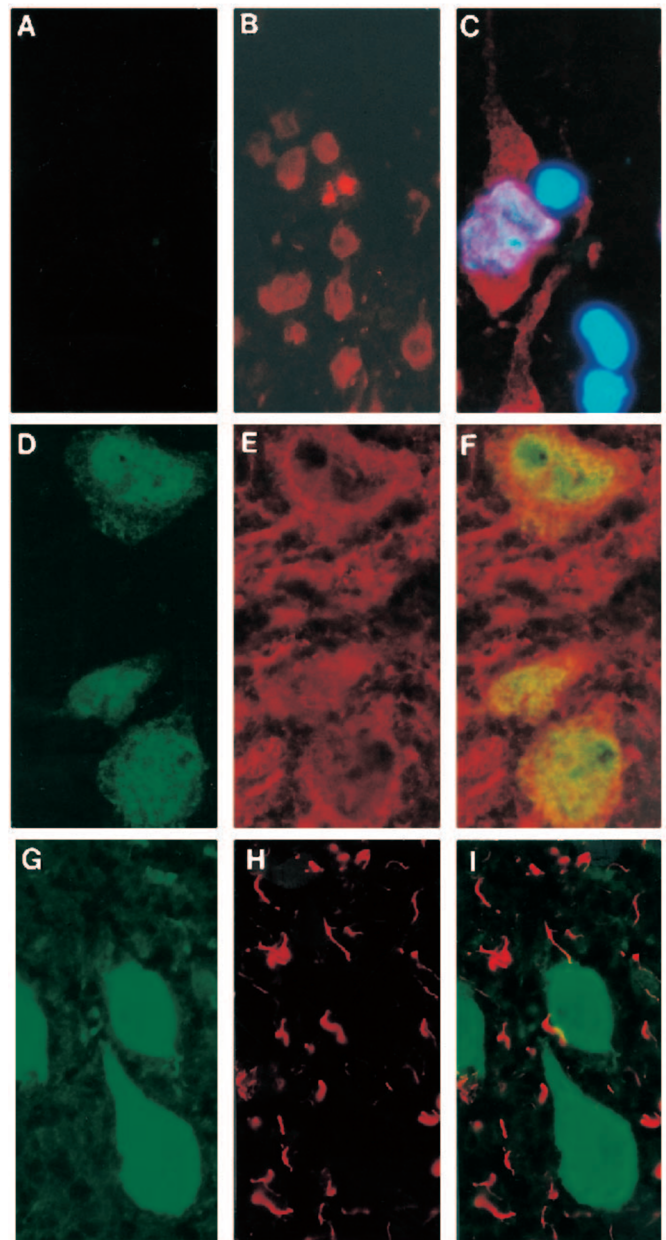

Fig. 6. Rel-N1 proteins are expressed exclusively in neurons in rat brain sections. Photos show cells in cerebral cortex layers.
(A) Normal rabbit serum. (B) Rabbit anti-Hel-N1 antibody showing the staining in layers I and II (×40). (C) Double staining with anti-Hel-N1 antibody (red) and DNA dye Bis Benzamide (blue).
(D) Anti-Hel-N1 antibody staining. (E) Anti-MAP-2 antibody staining of same field as in D. (F) Superimposition of anti-Hel-N1 staining (green in D) and anti-MAP-2 antibody staining (red in E).
(G) Anti-Hel-N1 antibody staining. (H) Anti-GFAP antibody staining of the same field as in G. (I) Superimposition of anti-Hel-N1 staining (green in G) and anti-GFAP staining (red in H). Photos in C-I are shown under oil at ×100 magnification.

cell extracts. As shown in Fig. 3, in the absence of EDTA, the majority of Hel-N1/Hel-N2 proteins migrated throughout the gradient, but were concentrated in the portions containing polysomes. EDTA was used to disrupt polysomes as shown by the disappearance of the polysome peaks between fractions 7 and 12 in Fig. 3B. Accordingly, the majority of Hel-N1/Hel-N2 proteins migrated in the upper portion of the gradient near monosomes. As a negative control, La protein remained in the top fractions in both the presence and absence of EDTA. In addition, confocal double immunofluorescence using anti-Hel-N1 and anti-P ribosomal antibodies indicated that most Hel-N1/Hel-N2 proteins and ribosomes were colocalized in the cytosol of medulloblastoma cells (data not shown). These results demonstrate that a significant portion of Hel-N1/Hel-N2 proteins is associated with polysomes in human medulloblastoma cells.

Hel-N1/Hel-N2 proteins are bound to poly(A)$^+$ mRNAs in medulloblastoma cells

To determine whether Hel-N1/Hel-N2 proteins are bound to poly(A)$^+$ mRNAs in vivo, medulloblastoma cell S16 extracts were passed through oligo(dT)-cellulose columns, and analyzed using western blotting. As shown in Fig. 4A, lane 3, the majority of Hel-N1/Hel-N2 proteins remained bound to oligo(dT)-cellulose in the presence of 150 mM NaCl. Since poly(A) binds to oligo(dT) under these conditions, the binding of Hel-N1/Hel-N2 proteins to the oligo(dT) column is likely due to binding of these proteins to the poly(A)$^+$ mRNA. Hybridization of poly(A) to oligo(dT) is enhanced under conditions of high salt. Therefore, when 450 mM NaCl was used more Hel-N1/Hel-N2 were bound to the oligo(dT) column (lanes 4 and 5 compared to lanes 2 and 3), which is consistent with the conclusion that Hel-N1/Hel-N2 proteins are bound to poly(A)$^+$ mRNA. In control experiments, we found that the purified Hel-N1 protein itself has affinity for oligo(dT)-cellulose, but that 0.1% SDS abolished the binding. To examine further whether Hel-N1/Hel-N2 proteins are directly bound to polysome-associated poly(A)$^+$ mRNA in vivo, we performed UV-crosslinking experiments on polysome fractions as outlined in Fig. 4B. Without UV crosslinking or when treating the samples with SDS prior to UV crosslinking, Hel-N1/Hel-N2 proteins did not bind to oligo(dT)-cellulose (Fig. 4C, lane 2 and lane 3), indicating the dissociation of Hel-N1/Hel-N2 from the poly(A)$^+$ mRNA by SDS. On the other hand, after UV crosslinking, Hel-N1/Hel-N2 proteins remained bound to oligo(dT)-cellulose even after SDS treatment. These results demonstrate that Hel-N1/Hel-N2 proteins are in direct association with poly(A)$^+$ mRNAs in these medulloblastoma cell extracts (Fig. 4C, lane 4). As a control, the Fragile X Mental Retardation (FMR) protein, predicted to be a RNA binding protein (Ashley et al., 1993; Siomi et al., 1993), could not be UV cross-linked to poly(A)$^+$ mRNA under these conditions (Fig. 4C, lower panel). It is notable that Hel-N1/Hel-N2 proteins were UV-crosslinked to poly(A)$^+$ mRNA at a high efficiency of approximately 10-15%. This high efficiency is consistent with the fact that uridylates are highly photoreactive, and the RNA binding surfaces of Hel-N1/Hel-N2 RRMs contain highly conserved aromatic amino acids that are engaged in direct contact with RNA (Kenan et al., 1991; Oubridge et al., 1994). Therefore, this high crosslinking efficiency is consistent with our previous finding that Hel-N1/Hel-N2 prefer binding to A+U-rich mRNA sequences (Levine et al., 1993; Gao et al., 1994). We conclude that Hel-N1/Hel-N2 proteins are bound directly to poly(A)$^+$ mRNA in vivo and a portion is associated with polysomes in the cytosol of human medulloblastoma cells.

Granular RNPs appear to consist of multiple Hel-N1/Hel-N2 proteins and multiple mRNA species

Previously we demonstrated by direct immunoprecipitation, gel shift analysis and in vitro RNA selection that Hel-N1 binds to a subset of A+U-rich 3′UTRs, including transcripts of c-*myc*, c-*fos* and CREB2 (Levine et al., 1993; Gao et al., 1994). It was observed that Hel-N1/Hel-N2 and mRNA transcripts can form multimeric complexes in vitro. In Fig. 5B, gel mobility shift analysis demonstrates a typical experiment in which Hel-N1 can complex with a 181 nt c-*myc* 3′UTR transcript, but not with a 152 nt transcript, confirming our previous immunoprecipitation RNA-binding study (Levine et al., 1993). In addition, this result demonstrates that the 29 nt sequence shown in Fig. 5A is a core sequence for multimer assembly between Hel-N1 and the c-*myc* 3′UTR transcript. Hel-N2, which possesses identical RRM sequences, has the same RNP multimerization properties as Hel-N1. The binding specificity has been demonstrated by competition gel shift analysis in which Hel-N2-selected A+U-rich RNAs could effectively compete with the c-*myc* 3′UTR for binding to Hel-N2 (Gao, 1995). These Hel-N1/Hel-N2 selected RNAs strongly resemble the 29 nt sequence shown in Fig. 5A. We postulate that this region may function as a high-affinity nucleation site for Hel-N1/Hel-N2 binding around which other Hel-N1/Hel-N2 molecules interact to form multimeric RNP complexes. The granular distribution of Hel-N1/Hel-N2 in the cytosol may represent multiple Hel-N1/Hel-N2 molecules bound to various mRNA species forming unique RNP granules. Interestingly, the formation of RNP multimers in vitro could be prevented by addition of recombinant RRM3 (Fig. 5D). RRM3 alone bound to the c-*myc* 3′UTR transcript, but multiple bands did not form (lane 2). Since Hel-N1 is four times larger than RRM3, the molar ratio of RRM3 versus Hel-N1 in the far right lane of Fig. 5D is 2:1. Under these conditions, it appears that a mosaic RNP complex was formed between Hel-N1 and/or RRM3 and c-*myc* 3′UTR transcripts, which migrated to a unique position (Fig. 5D, last lane). These findings suggest that RRM3 competes at an overlapping RNA binding site with Hel-N1. Therefore, it is evident that RRM3 functions as a dominant negative molecule in vitro by altering the formation of RNP multimers containing full-length Hel-N1. This result is consistent with a role for RRM3 in binding directly to the c-*myc* 3′UTR (Levine et al., 1993), and suggests that multiple Hel-N1 proteins can bind to A+U-rich 3′UTRs in mRNP complexes. It is probable that such RNA-protein complexes account for the granular appearance observed by immunofluorescence.

The Hel-N1 counterpart in rat, Rel-N1, is a neuron-specific protein in the central nervous system

It is important to determine whether the Hel-N1/Hel-N2 RNP granules observed in medulloblastoma cells are present in normal neurons. Previously, in situ RNA hybridization was used to demonstrate that the Hel-N1 counterpart in rat (Rel-N1) is expressed heterogeneously in a subset of cells throughout rat brain, and is abundant in regions of the hippocampus and the cerebral cortex (King et al., 1994). Immunofluorescence of rat brain coronal sections unambiguously demon-

strated that Rel-N1 proteins are expressed only in neurons (Fig. 6). Hel-N1 and Rel-N1 are extremely homologous, share 96% identity and anti-Hel-N1 antibodies recognize Rel-N1 proteins by western blotting. The immunofluorescence pattern of Rel-N1 in rat brain essentially corresponds to the distribution of Rel-N1 mRNA observed by King et al. (1994). For example, these proteins are strongly expressed in neurons in hippocampal CA3-CA4 fields of Ammon's horn, while only low level expression was observed in the CA1 region and in the dentate gyrus. Strong Rel-N1 immunostaining was observed in all layers of the cerebral cortex. Fig. 6A shows only background staining with prebleed rabbit serum, while Fig. 6B shows the staining of the interface between the cerebral cortex layer I and layer II, where a large number of cells were stained in layer II. Fig. 6C shows double labeling with anti-Hel-N1 antibody (red) and the DNA-specific dye, Bis Benzamide (blue). It is evident that only a subset of cells in the cortex expressed Rel-N1 proteins. These cells were further demonstrated to be neurons by positive costaining with neuron-specific anti-MAP-2 antibody, but negative costaining with glial cell-specific anti-GFAP antibody. Fig. 6D shows the Rel-N1 staining in both the nucleus and the cytosol, while Fig. 6E shows the staining of the same field with neuron-specific anti-MAP-2 antibody, which stains neuron cell bodies and dendrites. Superimposition of Fig. 6D and E (shown in F) demonstrates that Rel-N1-positive cells are also MAP-2-positive cells. Furthermore, Rel-N1-positive cells (Fig. 6G) did not costain with anti-GFAP antibody (Fig. 6H and I). These studies demonstrate with certainty that Rel-N1-positive cells in rat brain are neurons. In contrast to what happens in growing tumor cells, it appears that Rel-N1 proteins are expressed strongly in the nucleus of rat neurons (Fig. 6C, D, G) and presumably in dendrites (Fig. 6C), which is further demonstrated below, using cultured rat cortical neurons.

Rel-N1 proteins are expressed in granules along dendrites of rat cortical neurons, and partially colocalize with ribosomes

We studied the expression pattern of Rel-N1 proteins in isolated rat cortical neurons by indirect immunofluorescence. Rat cortical cells were taken from day 16 rat embryo brains, where Rel-N1 mRNA is known to be expressed (U. Atasoy and J. D. K., unpublished). As shown in Fig. 7A and B, only a subset of cortical cells was stained as Rel-N1-positive. As observed with the brain sections shown in Fig. 6, Rel-N1-positive cells did not costain with the glial cell-specific antibody, anti-GFAP (Fig. 7C); instead, they costained with neuron-specific antibody, anti-MAP-2, as shown by the yellow image generated by superimposing Rel-N1 and MAP-2 (compare Fig. 7D, E, F). This demonstrates that Rel-N1-positive cells among cultured rat cortical cells are indeed neurons. Rel-N1 was detected in the soma and dendrites of neurons, as marked by MAP-2 antibodies, which stain only neuron cell bodies and dendrites, but not axons. Using the axon-specific antibody, anti-GAP-43 (Goslin et al., 1988), we also found that Rel-N1 proteins were expressed in the proximal regions of dendrites, but were not detectable in axons. Similar conclusions were reached using cultured rat hippocampal neurons (unpublished), where Rel-N1 proteins were expressed at a high level in proximal regions of the dendrites, and were barely detectable at the distal regions of dendrites. In addition, the branched regions of dendrites appear to possess concentrated amounts of Rel-N1 proteins. The subcellular expression pattern of Rel-N1 proteins in hippocampal neurons resembles that of the known poly(A)$^+$ mRNA distribution pattern reported by Kleiman et al. (1993).

Fig. 7G shows a confocal image emphasizing the dendrites of a cultured rat cortical neuron stained for Rel-N1 proteins. As in medulloblastoma cells (Fig. 2), the Rel-N1 RNP granules in rat cortical neurons are evident, and partially colocalized with ribosomes, which stained with anti-ribosomal P antibody (Fig. 7G, H, I). Combining the studies presented above showing that Hel-N1/Hel-N2 proteins are directly bound to poly(A)$^+$ mRNAs and associated with polysomes (Figs 3 and 4), these results are consistent with the suggestion that Rel-N1 (and presumably Hel-N1) proteins play a role in translation or stability of mRNA in dendrites.

The Hel-N1 protein family is up-regulated during differentiation of mouse embryonic carcinoma P19 cells to a neuronal phenotype

To examine the functional role of the Hel-N1 protein family during neuronal differentiation, we investigated their expression in mouse embryonic carcinoma P19 cells (Bain et al., 1994). P19 cells were maintained in culture in an undifferentiated state (Fig. 8a, panel A) and were induced to differentiate into either neuron-like or muscle-like cells, depending on the chemical inducer. After treatment with 1% dimethylsulfoxide (DMSO), aggregated P19 cells acquired characteristics of cardiac and skeletal muscle (Fig. 8a, panel B) (McBurney et al., 1982). On the other hand, aggregated P19 cells differentiated into neuronal, glial and fibroblast-like cells upon treatment with 0.5 μM retinoic acid (RA) (Fig. 8a, panel C) (Jones-Villeneuve et al., 1982). The cells with round cell bodies and long processes shown in Fig. 8a, panel C, are known to possess many of the properties of mammalian neurons, such as morphology, post-mitotic state, the presence of synaptic structures and expression of a number of neurotransmitters, enzymes and receptors (Bain et al., 1994). Prior to forming their differentiation monolayer, P19 cells expressed barely detectable levels of the mouse Hel-N1 protein family, as shown by western blotting (Fig. 8b). Cellular aggregation alone did not induce increased expression of these proteins, as shown in the second lane in Fig. 8b. On the other hand, aggregated P19 cells, after 4 days in RA or DMSO-containing medium were plated onto culture dishes for an additional 1-4 days. RA-treated cells showed a dramatic increase in the expression of the mouse Hel-N1 protein family, while in DMSO-treated cells, their expression actually decreased (Fig. 8b). Further analysis demonstrated that the increased expression of these proteins in the aggregated P19 cells occurred by the second day in RA-containing medium (data not shown). It is interesting to note that the mRNAs encoding both MASH-1 protein and the EGF receptor are also expressed at 2 days post-RA treatment (Johnson et al., 1992; Joh et al., 1992). MASH-1 and EGF receptor are important regulatory genes during neuronal differentiation and are potential binding targets of the mouse Hel-N1 protein family (King et al., 1994). Consistent with the western blotting results presented in Fig. 8b, indirect immunofluorescence also demonstrated that the Hel-N1 protein family was expressed at a much higher level in differentiated P19 neurons than in monolayer P19 cells (see

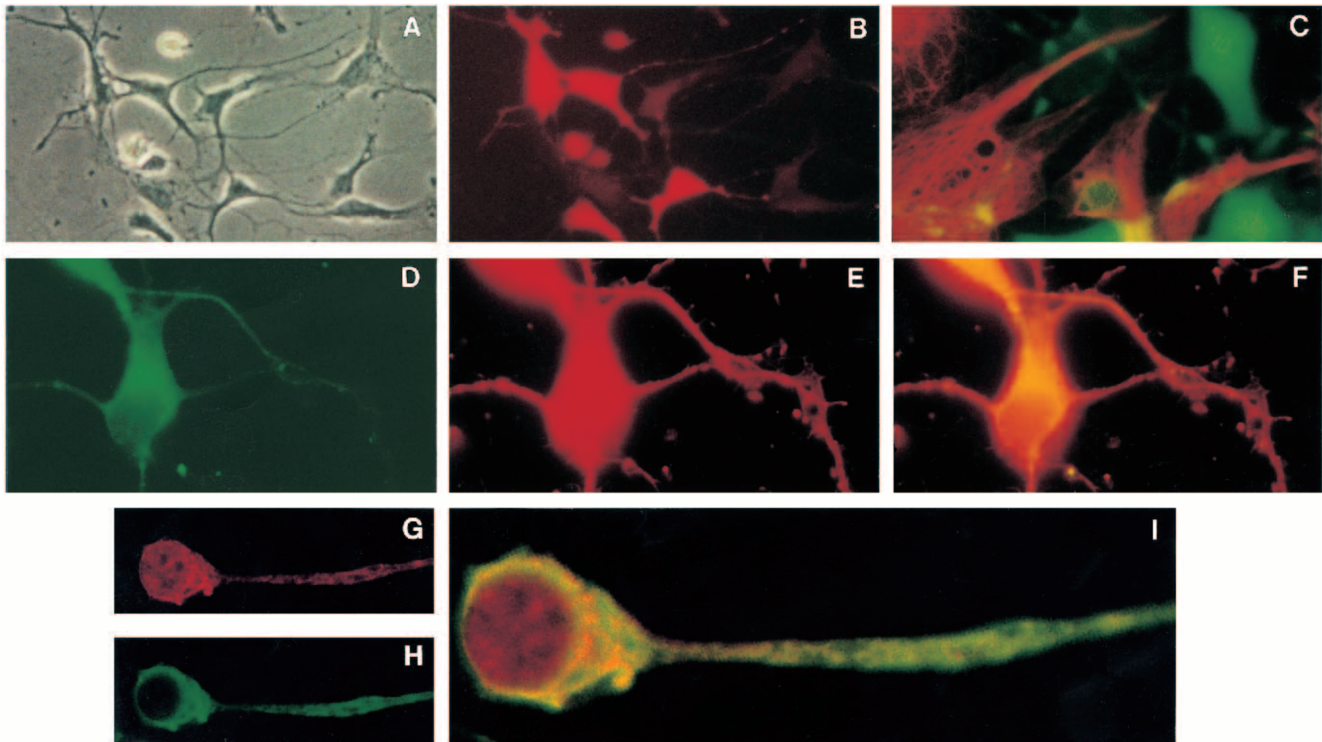

Fig. 7. Expression of Rel-N1 proteins in isolated rat cortical neurons. (A) Phase-contrast showing the morphology of rat cortical cells. (B) Anti-Hel-N1 antibody staining of the same field. (C) Double staining with anti-Hel-N1 antibody (green) and glial-specific anti-GFAP antibody (red). (D) Staining with neuron-specific anti-MAP-2 antibody. (E) Staining of the same neuron as in D, using anti-Hel-N1 antibody. (F) Superimposition of Hel-N1 staining (red) and MAP-2 staining (green). (G) Anti-Hel-N1 staining of a rat cortical neuron. (H) Anti-ribosomal P antibody staining of same neuron as in G. (I) Superimposition of staining with anti-Hel-N1 (red) and anti-P ribosomal antibodies (green). Images in G, H and I were made by laser confocal immunomicroscopy.

Fig. 8a, panels E and F), while the glial and fibroblast-like cells showed only background staining. Furthermore, mouse counterpart Hel-N1 proteins were also expressed in neurites of P19 neurons (Fig. 8a, panel F). These results suggest that the increased expression of these proteins was restricted to RA-induced neuron-like cells. This conclusion is further strengthened by the finding that the expression of the mouse Hel-N1 protein family was not up-regulated in RA-treated monolayer P19 cells (data not shown), which are known to retain endodermal and mesodermal properties (Mummery et al., 1986). Furthermore, as in human medulloblastoma cells and in mouse cortical neurons, the Hel-N1 protein family in P19 neuron cell bodies and neurites showed a granular RNP pattern. These studies suggest that the Hel-N1 protein family participates in neuronal differentiation, presumably by regulating the metabolism of a subset of mRNAs involved in neurogenesis.

DISCUSSION

RNA binding and potential target RNAs of Hel-N1 protein family

Much effort has been devoted to studying transcriptional regulation during neuronal differentiation, but less is known about regulatory processes affecting differentiation at the post-transcriptional level. Here we report analysis of the mRNA-binding proteins, Hel-N1/Hel-N2, and their involvement in neuronal differentiation. On the basis of a series of in vitro selection experiments using combinatorial RNA libraries, we found that Hel-N1/Hel-N2 prefer binding to A+U-rich sequences. One potential binding target resides in the A+U-rich 3′UTR of mRNAs encoding various growth regulatory proteins, these A+U-rich sequences have been implicated in the degradation of mRNAs (Shaw and Kamen, 1986). In vitro binding studies demonstrated that the Hel-N1 protein family could bind specifically to a subset of mRNA 3′UTRs, including those encoding c-myc, c-fos, GM-CSF, Id, CREB2, cdc42^{Hs}-binding tyrosine kinase and others (Levine et al., 1993; King et al., 1994; Gao et al., 1994; Liu et al., 1995). In the present study we further demonstrated that the Hel-N1 protein family is directly bound to poly(A)$^+$ mRNA and a portion is associated with polysomes, indicating their involvement in mRNA metabolism with potential roles in translation or mRNA stability. This notion is also supported by a series of transfection experiments demonstrating the biological effects of Hel-N1. For example, ectopic expression of Hel-N1 in 3T3-L1 preadipocytes caused accelerated differentiation to adipocytes and increased expression of the glucose transporter protein (GLUT1) whose mRNA could bind to Hel-N1 in vitro (Jain et al., unpublished data). Together with the data presented here showing that: (1) the expression of the Hel-N1 protein family is dramatically up-regulated during neuronal differentiation of P19 cells, and (2) the striking homology between Hel-N1 and ELAV that has been implicated in fly neuronal differentiation, we suggest a role for Hel-N1/Hel-N2 proteins in post-transcriptional gene regulation and neuronal differentiation. All genetic and biochemical data to

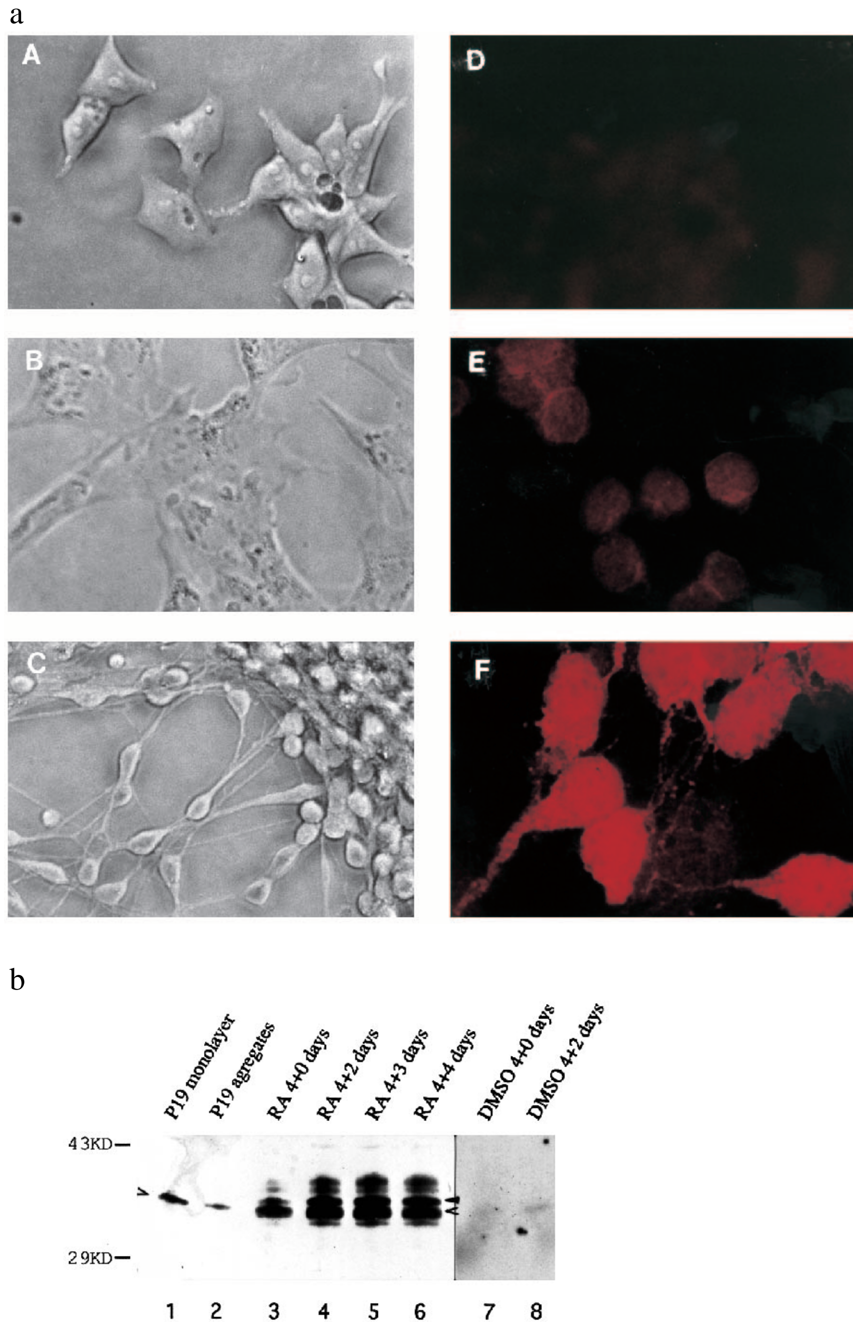

Fig. 8. (a) Expression pattern of mouse Hel-N1 protein family in P19 cells. (A) Normal monolayer P19 cells. (B) Dimethylsulfoxide-induced P19 cells. (C) Retinoic acid (RA)-induced P19 cells. (D) RA-differentiated P19 neurons stained with normal rabbit prebleed serum. (E) Monolayer P19 cells stained with anti-Hel-N1 antibody. (F) Staining of RA-induced differentiated P19 cells using anti-Hel-N1 antibody. (b) Induction of mouse Hel-N1 protein family during neuronal differentiation of P19 cells detected by immunoblotting. The filled arrowhead corresponds to mouse Hel-N1 protein; the open arrowhead corresponds to mouse Hel-N2 protein. Molecular mass markers are indicated at the right (in kDa). Growth periods following drug treatment are indicated at the top. Aggregated cells were harvested 4 days after 0.5 μM RA or 1% DMSO, designated as RA4 or DMSO4, then further incubated with drug for 1-4 days, designated as: RA4+1 days, RA4+2 days, RA4+3 days, RA4+4 days; and DMSO4+1days, DMSO4+2 days.

date concerning ELAV and the Hel-N1 protein family are consistent with this hypothesis.

Subcellular localization of the Hel-N1 protein family

Determining the subcellular localization of Hel-N1/Hel-N2 proteins is a key for understanding the molecular functions of these proteins. Hel-N1/Hel-N2 proteins are mainly expressed in the cytosol of human medulloblastoma cells, consistent with our finding that Hel-N1/Hel-N2 proteins bind to poly(A)$^+$ mRNAs and associate with polysomes, and may be involved in translation or stability of mRNA. The presence of the Hel-N1 protein family in testis and oocytes (King et al., 1994; Good, 1995; Gao, 1995) suggests that these proteins may also function in oogenesis and during early embryonic development, in which translational regulation is well known to play an important role (reviewed by Curtis et al., 1995). Further analysis using rat brain sections and cultured cortical cells demonstrated that the Hel-N1 protein family is expressed in both the nucleus and the cytosol of neurons, but not in other cell types. More importantly, these proteins are also present in RNP granules of dendrites, in a pattern that corresponds to the distribution of poly(A)$^+$ mRNAs (Bruckenstein et al., 1990; Kleiman et al., 1993). Since Hel-N1/Hel-N2 proteins can form multimers on A+U-rich 3′UTRs, it is possible that the granular appearance of Hel-N1/Hel-N2 is due to the presence of concentrated multiple Hel-N1/Hel-N2 proteins on mRNA in the mRNP complexes. For unknown reasons, the Hel-N1 protein family is more abundant in the nuclei of neurons than in the nuclei of medulloblastoma tumor cells. In general, the intracellular localization and transport of mRNAs are known to be

closely related to their expression as mediated by translational control (reviewed by Johnston, 1995). Thus, the localization pattern of the Hel-N1 protein family in neurons suggests that these 3′UTR-binding proteins may also be involved in the transport of a subset of mRNAs from the nucleus to the soma and dendrites. In addition, it appears that only a portion of the Hel-N1 protein family is colocalized with ribosomes (Fig. 7H, I, J) in dendrites. Polysome profile analysis using a sucrose gradient also demonstrated that only a portion of Hel-N1/Hel-N2 proteins comigrates with polysomes (Fig. 3). These studies raise the possibility that the Hel-N1 protein family and presumably the bound mRNAs are separated into two pools, one of which is associated with polysomes. The regulation of the switch between the two pools might be important for the regulation of gene expression in dendrites.

Differences between Hel-N2 and Hel-N1

As described previously, Hel-N2 is identical to Hel-N1, except for a 13 amino acid segment that is missing between the second and the third RRMs of Hel-N2 (Gao et al., 1994). Expression of Hel-N2 is predominant in human medulloblastoma cells while Hel-N1 is predominant in normal adult brain, which suggests that Hel-N1 and Hel-N2 might function differently. Consistent with this possibility, the ratio of the rat Hel-N1 counterpart protein to the Hel-N2 counterpart protein appears to be reversed during P19 cell neuronal differentiation. As shown in Fig. 8, it is apparent that the protein corresponding to Hel-N2 was predominant in the proliferating P19 cells, while the ratio was dramatically changed after neuronal differentiation. No difference has been found between Hel-N1 and Hel-N2 binding specificity or the multimerization property on c-*myc* 3′UTR. We proposed previously that the 13 amino acid segment and surrounding residues may mediate protein-protein interactions in the mRNP complex.

It has been generally believed that proteins are synthesized in the cell bodies of neurons and transported to intracellular domains through an active protein transport mechanism. However, the discovery that polysomes are present in dendrites has provided an alternative mechanism, which involves the targeting of mRNAs to specific intracellular regions and the localized production of corresponding proteins (Steward and Banker, 1992). We have found that Hel-N1 protein family members are present in dendrites and are capable of binding to a subset of mRNA 3′UTRs, including those encoding many immediate early proteins, receptors, and intracellular signaling molecules. Therefore, it is possible that Hel-N1 is involved in translation of certain mRNAs in dendrites and cell bodies as a functional aspect of neuronal plasticity.

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