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Messenger ribonucleoprotein complexes containing human ELAV proteins: interactions with cytoskeleton and translational apparatus

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SUMMARY

Mammalian ELAV proteins bind to polyadenylated messenger RNAs and have specificity for AU-rich sequences. Preferred binding sites in vitro include the AUUUA pentamer and related sequences present in the 3′ untranslated regions of many growth regulatory mRNAs. Human ELAV (hELAV) proteins have been implicated in post-transcriptional regulation of gene expression by their effects on the stability and translatability of growth regulatory mRNAs.

We have examined the intracellular localization of ELAV proteins in neurons and in tumor cells of neuronal origin using indirect immunofluorescence, confocal microscopy and biochemical separation. Mammalian neuronal ELAV proteins are found predominantly in the cytoplasm of cells in mRNP complexes termed α complexes which, when associated with polysomes, form large and high density β complexes, as assayed by glycerol and accudenz gradients, respectively. Puromycin, cytochalasin or EDTA treatments disrupt β complexes causing the release of α complexes, which then appear, by confocal microscopy, as large hELAV mRNP granules associated with microtubules. Association of partially purified hELAV mRNP α complexes with microtubules was confirmed by in vitro reconstitution assays. Furthermore, colchicine treatment of cells suggested that association of hELAV mRNP α complexes with microtubules is also necessary for the formation of β complexes.

Our data suggest a model in which a subset of mRNAs is associated with microtubules as ELAV mRNP particles (α complexes) which, in turn, associate with polysomes to form a translational apparatus (β complex) that is, through polysomes, associated with the microfilament cytoskeletal network. hELAV proteins in these mRNP granules may affect post-transcriptional regulation of gene expression via the intracellular transport, localization and/or translation of growth regulatory mRNAs.

Key words: ELAV, Hel-N1, Hu, RRM, RNA-binding protein, mRNP, Cytoskeleton, Translation

INTRODUCTION

RNA molecules are usually present in cells as ribonucleoprotein (RNP) complexes, which both protect the RNAs and enable their interaction with other cellular components. Proteins that bind RNA have been grouped into families based on the possession of sequence motifs implicated in RNA binding (Mattaj, 1993). The largest known family of RNA binding proteins contains an RNA recognition motif (RRM) of approx. 80 amino acids (Kenan et al., 1991; Burd and Dreyfuss, 1994). Most members of the RRM superfamily are phylogenetically conserved and are involved in RNA metabolism at different levels of gene expression. For example, many nuclear RRM proteins are found in relatively small and homogeneous RNP particles (small nuclear RNPs, snRNPs) that have been purified and whose function, which is predominantly in splicing, has been largely defined (Kramer, 1996). On the other hand, numerous nuclear RRM proteins are found in heterogeneous particles (heterogeneous nuclear RNPs, hnRNPs), which have been difficult to purify and therefore difficult to study. These proteins have been mainly implicated in nuclear pre-mRNA processing events (Dreyfuss et al., 1993). Recently, some hnRNP proteins were demonstrated to shuttle between the nucleus and the cytoplasm and some were implicated in mRNA transport (Pinol-Roma and Dreyfuss, 1993; Michael et al., 1995).

RNA transport and localization have been shown to be dependent on the integrity of the cytoskeleton (St Johnston, 1995, and references therein), which may provide a 3-D framework for mRNP function. Certain mRNAs have been found localized in large granules, which are associated with the cytoskeleton and are transported down the processes to the periphery of oligodendrocytes (Ainger et al., 1993). For example, localization of actin mRNA in fibroblasts requires intact microfilaments (Sundell and Singer, 1991); transport and anchoring of Vg1 RNA in *Xenopus* oocytes requires intact microtubules and microfilaments, respectively (Yisraeli et al., 1990), whereas transport and localization of neuronal mRNAs is microtubule-dependent (Litman et al., 1994; Bassell et al., 1994; Ainger et al., 1993). In addition, interactions between the cytoskeleton and certain mRNAs have been shown to be mediated through their 3′ untranslated regions (UTRs)

(Kislauskis et al., 1993; Mayford et al., 1996). Despite the fact that mRNA localization and transport has become better understood in recent years, questions of whether these processes are controlled by *cis*-elements in mRNAs or depend upon associated proteins have not been resolved. In addition, some mRNAs have been shown to colocalize with the proteins they encode (St Johnston, 1995, and references therein), suggesting localized regulation of mRNA translation. The regulation of gene expression in local regions can be particularly important in neuronal cells where rapid responses to synaptic activity may govern neuronal plasticity (Steward, 1994; Mayford et al., 1996). Although mRNA localization, transport and translation have been individually studied in great detail, a functional connection between these processes has not been established. In this study, we used metabolic inhibitors to investigate the association between mRNPs containing neuronal ELAV RRM proteins, the cytoskeleton and the translational apparatus.

The *elav* gene of *Drosophila melanogaster* encodes an interesting member of the RRM family of proteins because genetic deletions have shown that it is essential for the development and maintenance of neurons (Campos et al., 1985; Robinow and White, 1991). *Elav* counterparts have been cloned from the nervous system, as well as from other tissues in a number of metazoans, including humans, chicken, mouse, rat and *Xenopus* (reviewed in Antic and Keene, 1997, and references therein; Szabo et al., 1991; King et al., 1994; Good et al., 1995). All ELAV proteins contain three highly conserved RRMs; the proteins differ in their N termini and in the hinge region connecting the second and the third RRM. Sequence similarity between *Drosophila* ELAV and the Sex-lethal splicing factor (Bier et al., 1988; Robinow and White, 1991) led to the suggestion that ELAV proteins are involved in splicing. Indeed, *Drosophila* ELAV protein has recently been implicated in the alternative splicing and thus generation of the neural-specific isoform of neuroglian (Koushika et al., 1996). However, the distribution of vertebrate ELAV proteins both in the nucleus and in the cytoplasm of neurons (Gao and Keene, 1996), their binding to AU-rich sequence elements in 3′ UTRs of growth regulatory mRNAs in vitro (reviewed in Antic and Keene, 1997, and references therein) and association with poly(A)$^+$ mRNA in vivo (Gao and Keene, 1996) implicates their involvement in cytoplasmic post-transcriptional regulation of gene expression. In fact, it has been recently shown that they might affect mRNA stability (Jain et al., 1997; Myer et al., 1997), translatability (Jain et al., 1997; reviewed in Antic and Keene, 1997) and, possibly, RNA editing (Anant et al., 1997).

Given the profound biological effects of ELAV proteins and their implicated roles in gene expression, we have analyzed hELAV RNP complexes. We report that hELAV proteins bind to a subset of mRNAs forming mRNP complexes (α complexes), which are microtubule-associated and are able to interact with the translational machinery. Association of hELAV mRNP α complexes with polysomes results in the formation of large (β) complexes which, through polysomes, associate with the microfilament framework of the cytoskeleton. Therefore, human neuronal ELAV proteins bind to a subset of polyadenylated mRNAs, and in association with other proteins, form mRNP particles, which are involved in translation of mRNAs via their cytoskeletal association.

MATERIALS AND METHODS

Cell culture

Human medulloblastoma D283 cell line (ATCC HTB-185) was propagated in 1× Improved MEM Zinc Option Medium (ZO-MEM) (GIBCO Laboratories, formula #86-0194AJ) supplemented with 10 mM Hepes, 0.22% sodium bicarbonate and 10% FBS (HyClone Laboratories, Inc.). Human small cell lung carcinoma lines SCC89-LY (SCC) and OH-3 were obtained from D. Sidransky and S. B. Baylin, respectively (Johns Hopkins University). They were grown in RPMI-1640 containing 1% glutamine and 20% FBS. Rat pheochromocytoma cells, PC12, were purchased from ATCC (CRL-1721). All cells were maintained at 37°C with 5% CO_2 and were grown in suspension or as loosely attached cells. A routine procedure in propagation of these suspension cultures was to remove dead cells from the culture after 5-6 passages (Dr H. S. Friedman, Duke University, personal communication). Lymphocyte separation medium (LSM) (Organon Teknika, PA) was used for this separation: cells were resuspended in 20 ml of medium loaded over 20 ml of LSM and centrifuged for 15 minutes at 1500 ***g*** at room temperature. Dead cells were pelleted and live cells accumulated at the interphase. When we treated these suspension cultures with metabolic inhibitors, this procedure was performed routinely after each treatment to make sure that cells remained viable and that drugs did not cause cell death.

Drug treatments of cells

Rat cortical neurons were kindly provided by Drs Manisha Patel and James McNamara (Duke University Medical Center). Cultures of embryonal rat cortical neurons were treated with metabolic inhibitors 15 days after plating, prior to detergent extraction and fixation (as described below). All treatments were performed for 1 hour at the following concentrations: 1 mM puromycin; 20 μg/ml colchicine (Bassell et al., 1994); and 5 μg/ml cytochalasin D (Bassell et al., 1994).

In medulloblastoma D283 cells, translation was inhibited by treatment with either puromycin (1 mM for 3 hours), or sodium fluoride (10 mM for 1-2 hours), or emetine (50 μg/ml for 3 hours). Indirect inhibition of translation was also achieved by treatment with cytochalasin B (10 μg/ml for 3 hours) or cytochalasin D (5 μg/ml for 3 hours), which were intentionally used to disrupt microfilaments. The structure of microtubules was disrupted by treatment with colchicine at 20 μg/ml for 3 hours. All chemicals were obtained from Sigma Chemicals Co. The drug concentrations were titrated and the duration of treatments were optimized experimentally so that metabolic processes would be affected in the majority of cells.

Preparation of cell extracts

Cell extracts were made in buffer B4 containing 50 mM Tris, pH 7.6, 150 mM KCl, 3 mM $MgCl_2$, 0.1% Triton X-100 and 0.01% sodium deoxycholate, 1 mM DTT, protease inhibitors (1 mM PMSF, 1 μg/ml leupeptin, 40 μg/ml bestatin, 2 μg/ml α_2-macroglobulin and 1 μg/ml pepstatin, purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN) and 200 units/ml rRNasin (Promega). Approximately 10^8 cells were resuspended in 1 ml of B4 and lysis was monitored by Trypan Blue (GIBCO Laboratories) staining of remaining nuclei. Nuclei and cell debris were pelleted at 800 ***g*** for 8 minutes at 4°C. The supernatant was collected and further centrifuged at 12000 ***g*** for 10 minutes at 4°C. The pellet was designated as the 'microsomal' fraction (M) and the supernatant represented the postmicrosomal cytoplasmic fraction (C). The 'microsomal' pellet was resuspended in 1 ml of B4 buffer for further analysis.

A nuclear fraction was prepared from the pellet obtained after the first centrifugation as follows: nuclei and cell debris were gently resuspended in 2 ml of buffer B4 without detergents and layered over 1.5 ml of 1.5 M sucrose in B4. Nuclei were pelleted through the

sucrose at 1500 ***g*** for 10 minutes at 4°C while cell debris remained at the interphase. Purified nuclei were lysed in 1 ml of buffer B4 by freeze-thawing and chromosomal DNA was digested with 50 units/ml RQ DNase (Promega) at 4°C for several hours. Remaining small DNA fragments were pelleted and the supernatant was used as nuclear extract. Total cell extracts were made by resuspending cells in the B4 buffer, freeze-thawing and sonicating until the solution became clear. The extract was further treated with 10 units/ml RQ DNase (as described above), to digest any remaining DNA fragments, and was centrifuged at 12000 ***g*** for 10 minutes at 4°C. The supernatant was collected and used as a total cell lysate. Protein concentration was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

Fixation for immunocytochemistry and in situ hybridization

Cells were grown in chamber slides (Nunc, Inc., Naperville, IL) coated with poly-L-lysine solution (Sigma Chemicals), as recommended by the manufacturer. To investigate the association of mRNPs with the cytoskeleton, cells were Triton-extracted prior to fixation with 4% paraformaldehyde (Bassell et al., 1994).

In situ hybridization

Prior to hybridization, cells were rehydrated by several PBS/5 mM $MgCl_2$/0.2 mM VRC washes. Oligo(dT50) was labeled at the 3′ end with digoxigenin-11-dUTP using terminal transferase (Boehringer Mannheim Biochemicals) and in situ hybridization was performed as described previously (Bassell et al., 1994). Poly(A)$^+$ mRNA was detected using sheep anti-digoxigenin primary antibody (Boehringer Mannheim Biochemicals) and anti-sheep-Texas Red conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., PA). Cells were subsequently stained with anti-Hel-N1 antibody and anti-rabbit FITC-conjugated secondary antibody. Cells treated with RNases (20 μg/ml RNase A and 2.8 μg/ml RNase T1) were used as a negative control. Immunofluorescence was monitored and documented on a Zeiss Axioscope using 100× magnification under oil immersion and two filters: green BP 546/12 and blue BP450-490.

Indirect immunofluorescence assays

Detergent-extracted and fixed cells were washed with PBS and exposed to antibodies as described previously (Gao and Keene, 1996). Immunofluorescence was monitored and documented using a confocal Zeiss LSM 410 inverted microscope with ArKr laser (Omnichrome series 43); 63× oil immersion Plan-Apochromat NA 1.4 lens and a computer software by Zeiss.

Gradient analysis of mRNP complexes

Cytoplasmic and nuclear mRNP complexes were analyzed by accudenz density (Accurate Chemical and Scientific Corp., NY) gradient centrifugation (Rickwood et al., 1982) and glycerol velocity centrifugation. Both gradient media were dissolved in the B4 buffer without detergents. Usually, 4.5 ml of the linear 10%-60% accudenz gradient was formed using a polystaltic pump and gradient maker (Buchler Instruments, A Labconco Company, Fort Lee, NJ); 0.2 ml of the cytoplasmic extract (equivalent of 20×10^6 cells) was loaded on top. To analyze mRNPs in the nuclear fraction, 1 ml of nuclear extract (equivalent of 10^8 cells) was loaded on top of 4 ml of an accudenz gradient. Samples were centrifuged for 44 hours at 26,000 rpm in a Beckman SW50.1 rotor at 4°C. Linear 10%-50% glycerol gradients were prepared similarly except that the volumes were doubled and a Beckman SW41 rotor was used at 19000 rpm for 2 hours at 4°C. After centrifugation, 200 μl fractions were collected from the top using an Auto Densi-Flow IIC collector (Buchler Instruments, A Labconco Company, Fort Lee, NJ). A sample (50 μl) of each fraction from cytoplasmic extracts gradients was loaded onto an SDS-acrylamide protein gel and analyzed by immunostaining of western blots. Gradient fractions containing nuclear extract were precipitated with 15% TCA and total proteins were then analyzed by SDS-PAGE and immunoblotting.

A sample of each fraction collected from accudenz gradients was used to measure refractory index using a refractometer (American Optical Corp. Buffalo, NY) and density was calculated according to the formula: $\rho=3.242\eta-3.323$ (Rickwood et al., 1982). The refractory index (η) values were corrected for each fraction due to the fact that buffer B4 and not water was used to dissolve accudenz.

Partial purification of α complexes for microtubule binding assay

To enrich cell extracts for α complexes, medulloblastoma suspension culture was treated with 1 mM puromycin for 3 hours. A cytoplasmic cell extract was made, loaded over accudenz density gradients, and fractions with the density 1.23-1.30 g/ml containing α complexes were pooled, diluted with 2 volumes of buffer B4 and recovered by differential centrifugation. After centrifugation in the SW41 rotor, at 20000 rpm for 15 hours at 4°C, pelleted α complexes were further separated on polysomal 10%-30% sucrose gradients. Gradients were centrifuged in an SW60 rotor at 25,000 rpm for 2 hours at 4°C. After determining OD_{260} for each fraction, they were then precipitated with 15% TCA and analyzed on denaturing protein gels by immunoblotting and silver staining. Alternatively, fractions containing hELAV complexes were pooled and used as partially purified α complexes.

Polymerization of tubulin and binding of α complexes

Polymerization of endogenous tubulin was performed by adding 1 mM GTP and 10 μM taxol to the medulloblastoma cytoplasmic extract in B4 buffer and incubation at 37°C for 1 hour. During this incubation period, binding of endogenous hELAV complexes to endogenous assembled microtubules was occurring as well. Binding of partially purified α complexes to reconstituted microtubules was performed in the following way: purified tubulin (in 80 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP, pH 6.8) was obtained from Cytoskeleton Inc. (Denver, CO). The reaction mixture was assembled at room temperature in 100 μl of PEM buffer (20 mM PIPES, pH 6.9, 1 mM $MgCl_2$, 1 mM EGTA, 1 μM DTT, 1 mM GTP) containing 10 μg of tubulin, 10 μg of BSA and 20 μM taxol, which was used to stabilize polymerized microtubules. Polymerization was performed at 37°C for 5 minutes. Partially purified α complexes were then added to pre-assembled microtubules and incubated for another 10 minutes at 37°C. Microtubule-bound complexes, from both assays, were pelleted by centrifugation at 50,000 rpm, 22°C for 30 minutes in the Beckman TL-100 ultracentrifuge using a TLA 100.3 rotor. In both experiments, pellets were resuspended in B4 buffer and samples of both the supernatants and pellets were analyzed by immunoblotting. Remaining supernatants and pellets were further analyzed by accudenz gradient centrifugation. Controls were performed in the same way except that either the GTP and taxol or the tubulin, GTP and taxol were omitted from the reaction.

Protein gels, western blotting, and quantitative analysis of western blots

Protein samples were separated on 15% acrylamide-0.2% bis-acrylamide denaturing gels and were electrophoretically transferred to Hybond-ECL nitrocellulose membranes (Amersham Life Science Inc.). Immunostaining was visualized by ECL (Amersham). For quantitative analysis, each western blot was electronically scanned and relative quantities of proteins were determined using ImageQuant program (Molecular Dynamics).

Source of antibodies

The rabbit polyclonal Hel-N1 antibody was produced in our laboratory (Gao et al., 1994). Antibody against the ribosomal protein L22 was generously provided by J. A. Steitz (Toczyski et al., 1994). A monoclonal antibody against the poly(A)-binding protein (Gorlach et al., 1994) and the monoclonal antibody against hnRNP C protein

(4F4) were kindly provided by G. Dreyfuss. Human anti-ribosomal P0/P1/P2 antibodies were purchased from Immunovision Inc. (Little Rock, AR), and were used to detect ribosomes in indirect immunofluorescence and immunoblotting assays. Monoclonal antibodies against β-tubulin were obtained from Amersham Life Sciences. Actin filaments were stained either using a monoclonal antibody (Amersham Life Sciences) or FITC-labeled phalloidin (Molecular Probes). Human autoimmune Go serum was used to detect La protein. All secondary antibodies used in this study (Jackson ImmunoResearch Laboratories, Inc., PA) were F(ab′)2 fragments with minimal species cross-reactivity. Secondary antibodies used for immunostaining of western blots were conjugated to horse radish peroxidase and were purchased from Amersham Life Sciences.

Source of the recombinant Hel-N1 protein and in vitro reconstitution assay

Recombinant Hel-N1 was expressed in *E. coli* as a g10-tagged fusion protein and was partially purified (Levine et al., 1993). RNA binding assays were performed as described previously (Levine et al., 1993; Gao et al., 1994). Recombinant protein and the complex reconstituted between the recombinant Hel-N1 and in vitro-transcribed RNAs was analyzed using accudenz gradient centrifugation. RNA molecules used in this assay were either full-length c-myc or interferon α mRNAs with or without 3′UTRs, or only their corresponding 3′UTRs. The shift in protein mobility in accudenz gradients was observed only when reconstitution was performed with the corresponding RNAs containing 3′UTRs.

RESULTS

Granules containing ELAV proteins in cells of neuronal origin are associated with microtubules and contain a subset of poly(A)$^+$ mRNA

Human neuronal ELAV proteins have been found associated with poly(A)$^+$ mRNA in cell extracts (Gao and Keene, 1996) and can bind in vitro to mRNA transcripts containing AU-rich sequence elements (reviewed in Antic and Keene, 1997; Levine et al., 1993; King et al., 1994; Gao et al., 1994; Liu et al., 1995). Granular cytoplasmic staining with antisera prepared against Hel-N1 (human ELAV like neuronal protein) is presumed to represent ELAV mRNP complexes, some of which colocalize with ribosomes (Gao and Keene, 1996). The structural properties of these putative ELAV RNPs have not been examined and it is not known whether ELAV proteins associate with poly(A)$^+$ mRNA in conjunction with ribosomes or if mRNP particles assemble and then attach to ribosomes during translation. If ELAV proteins affect mRNA stability and translatability, as suggested in several studies (reviewed in Antic and Keene, 1997), the dynamics of their assembly with the translational apparatus and the cytoskeleton could be key to understanding their function. In addition, the demonstration that poly(A)$^+$ mRNA is associated with microtubules in neuronal cells (Bassell et al., 1994; Litman et al., 1994) underlines the importance of investigating the interaction between ELAV RNPs and cytoskeletal components. In order to address these questions and to study the dynamics of ELAV RNP function, we performed morphological and biochemical analyses of ELAV RNP complexes in cells of neuronal origin.

To investigate involvement of ELAV proteins/RNPs in metabolic processes we treated embryonal cortical neurons with chemical inhibitors that abrogate translation or disrupt the integrity of the cytoskeleton. We monitored changes in the distribution of ELAV proteins by immunofluorescence and confocal microscopy of cells that were detergent-extracted prior to fixation. This procedure is conventionally used to localize components that are associated with the Triton-insoluble cytoskeleton (Lenk et al., 1977). In all cases, control cells were compared with cells treated with chemical inhibitors. Treatment of embryonal rat cortical neurons with puromycin (Fig. 1B), which is known to cause dissociation of ribosomes into subunits, resulted in dramatic reorganization of the ELAV-containing fine granules observed in untreated cells (Fig. 1A) into large ELAV granules (Fig. 1B). These large granules were associated with the detergent-insoluble cytoskeleton and were localized in the neuronal cell body and in processes. Treatment of cortical neurons with cytochalasin D (5 μg/ml for 1 hour), which causes dissociation of microfilaments, resulted in a similar reorganization of ELAV granules (not shown). On the other hand, dissociation of microtubules by treatment of cortical neurons with colchicine (20 μg/ml for 1 hour) alone or in combination with puromycin resulted in loss of cytoplasmic ELAV staining (not shown), suggesting an association of ELAV-containing granules with microtubules. It is important to note that concentrations of inhibitors required to achieve disruption of cytoskeletal components or reorganization of mRNPs were 5-10 times higher than concentrations used for non-neuronal cells in other studies (Sundell and Singer, 1991). This resistance of embryonal cortical neurons to concentrations of drugs that otherwise perturb the organization of cytoskeletal components in non-neuronal cells has been reported previously (Bassell et al., 1994). Our experimental conditions were identical to those described by Bassell et al. (1994) and we found that 90% of the cells in the population were consistently affected by the drug treatment and they showed similar staining patterns.

To further analyze these ELAV-containing granules, we examined human medulloblastoma neuronal tumor cells because they grow rapidly, are easy to propagate and constitutively express ELAV proteins. As observed above with cortical neurons, hELAV proteins (stained red) in Triton-extracted medulloblastoma cells appeared as small granules (arrow in Fig. 2A), which colocalized with tubulin (stained green) resulting in the yellow/orange appearance (Fig. 2A). Interestingly, reorganization of the fine hELAV-containing granules into larger granules (Fig. 2B,C; arrowheads) was observed after cytochalasin D treatment (Fig. 2B) or after puromycin treatment (Fig. 2C), and these large granules were localized along microtubule tracks (green). The arrow in Fig. 2C points to fine granules, remaining after the puromycin treatment, which also distributed along microtubules. Much like cortical neurons, medulloblastoma cells treated with both colchicine and puromycin did not display hELAV cytoplasmic staining after detergent extraction (not shown). Also, when medulloblastoma tumor cells were treated with metabolic inhibitors under the conditions described for embryonal cortical neurons (1 hour treatments) only 10-20% of cells were affected. 1 hour treatment of these tumor cells with higher concentrations of inhibitors did not increase the proportion of cells affected in the population. Therefore, to avoid toxic effects of these inhibitors, we extended the duration of each treatment, rather than increasing the concentration, and monitored the effects by immunofluorescence of cytoskeletal components and hELAV mRNPs. Depolymerization of

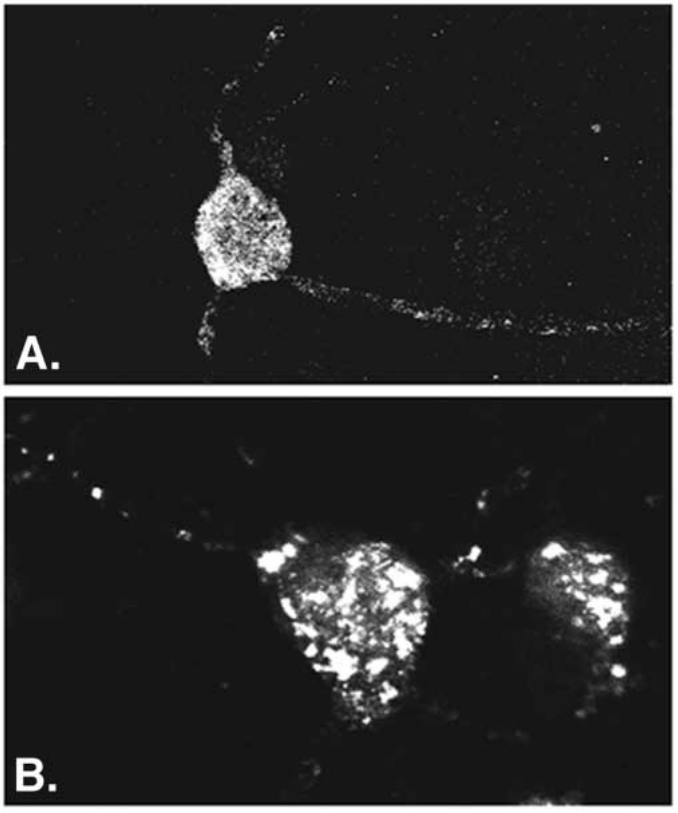

Fig. 1. Distribution of ELAV-like proteins in untreated and puromycin-treated rat embryonal cortical neurons. Cultures of cortical neurons (15 days after plating) were extracted for 1 minute with 0.2% Triton X-100 in cytoskeletal buffer prior to fixation and staining with anti-Hel-N1 antibody. Staining was detected using secondary antibody coupled to Texas Red. Images presented here are 0.5 μm thick sections, which were detected using the confocal microscope and a 568 nm laser beam. (A) Untreated cortical neuron. ELAV-like proteins show fine granular distribution in the cell body and processes. (B) Cortical neurons treated with puromycin (1 mM for 1 hour) prior to detergent extraction and fixation. Note reorganization of ELAV-like proteins from fine granular distribution into large granular structures, observed both in the cell body and processes.

microtubules was observed in 90% of cells after the 3 hour treatment with 20 μg/ml of colchicine (not shown). Similar percentages of cells showed redistribution of hELAV mRNPs after the 3 hour treatment with 1 mM puromycin, and approximately 60% of cells lost filamentous actin after the 3 hour treatment with 5 μg/ml of cytochalasin D (not shown). To confirm the expected conventional effects of these treatments, cytoskeletal components were stained after each treatment and (1) actin filaments were found to be intact after the colchicine treatments; (2) microtubules were intact after the cytochalasin treatments; and (3) puromycin treatments did not affect the integrity of either microfilaments or microtubules (not shown). The experiments with medulloblastoma cells were repeated numerous times and similar effects were consistently observed. Taken together, these data suggest that ELAV complexes may associate with and move along microtubules in medulloblastoma cells, as well as in isolated embryonal cortical neurons.

To determine whether stained ELAV granules in medulloblastoma cells represent RNP complexes containing poly(A)$^+$ mRNA, we performed *in situ* hybridization using oligo-dT as a probe (Fig. 3). The experiment showed that large hELAV granules, which formed after puromycin treatment (Fig. 3A-C), contained poly(A)$^+$ mRNA (Fig. 3A and C); compare to cells that were subjected to RNase digestion (Fig. 3D). These findings revealed that all hELAV granules (Fig. 3B) colocalized with poly(A)$^+$-containing, mRNP particles (yellow/orange staining in Fig. 3C; arrowheads). However, a population of the poly(A)$^+$-containing granules shown in Fig. 3A and C (arrows) did not colocalize with hELAV granules, indicating that hELAV proteins are not associated with all poly(A)$^+$ mRNA, but rather with a subset of mRNAs. The same result was observed with cytochalasin-treated cells (not shown).

Characterization of hELAV mRNPs in cell lines of neuroendocrine origin

To perform comparative analysis of ELAV mRNPs, we examined four cell lines of neuroendocrine origin for the expression of ELAV proteins: human medulloblastoma D283; human small cell lung carcinoma (SCLC) lines OH3, and SCC89-LY (SCC); and rat pheochromocytoma, PC12 cells. Medulloblastoma D283 cells expressed the highest detectable amount of hELAV proteins (Fig. 4A). However, we observed a significant decrease in the relative amounts of hELAV proteins in D283 cells after they had been repeatedly passaged in cell culture for several months (Fig. 4A; compare passage 6 (p6) with the passage 300 (p300)). It is interesting to note that the polyclonal rabbit anti-Hel-N1 antibody reacted with a protein(s) of a smaller size in rat PC12 and human SCLC cells than those detected in the medulloblastoma cell lines. These smaller proteins may represent unidentified members of the ELAV family or members with post-translational modifications, which affect their mobility in denaturing protein gels.

The intracellular distribution of hELAV proteins in medulloblastoma D283 cells was analyzed by subcellular fractionation (Fig. 4B). Analysis of hELAV proteins using cell equivalents showed a predominantly cytoplasmic localization (95%), and the majority of these were found in the postmicrosomal supernatant (C), with some being associated with the 'microsomal' fraction (M). Purified nuclei (N) contained a small amount (5%) of hELAV proteins. Further fractionation of the postmicrosomal supernatant (C) by centrifugation at 100,000 ***g*** showed that the hELAV proteins were pelleted and were absent from the S100 (data not shown), suggesting their involvement in large complexes. In contrast, the majority of the nuclear material reactive with the anti-Hel-N1 antibody remained in the S100 supernatant (data not shown), suggesting it might not be part of a large RNP complex.

Given the apparently large size of the hELAV complexes, gradient fractionation was used to examine their homogeneity. hELAV complexes were analyzed in both nuclear and cytoplasmic extracts, by accudenz density gradient centrifugation (Fig. 4C), which allowed us to distinguish between the lower density free-protein components and higher density ribonucleoproteins, including ribosomes. Although some hELAV complexes were present in the nucleus, the

majority of hELAV proteins (74%) detected in nuclear extracts were found at the top of the gradients, presumably in a free form (Fig. 4C, compare with the densities of the RNA-free recombinant Hel-N1 protein). On the other hand, cytoplasmic fractions (Fig. 4C) showed more heterogeneity, but contained three distinct hELAV populations: (1) free protein (peak close to the top of the gradient with the density 1.13-1.19 g/ml); (2) high density hELAV complexes (1.33-1.36 g/ml, peak close to the bottom of the gradient), which comigrated with ribosomes (as determined by the distribution of the L22 ribosomal protein (Fig. 4C, L22, bottom panel) and were designated as β complexes; and (3) intermediate density complexes (1.19-1.32 g/ml), which migrated in the middle of the gradient, between the free protein and β complexes, and were designated as α complexes. Interestingly, poly(A)-binding protein was found to have a distribution in accudenz gradients similar to the hELAV proteins (not shown), which is consistent with the finding that ELAV complexes contain poly(A)$^+$ mRNA. However, the proportion of poly(A)-binding protein found in α complexes (48%) was much greater than hELAV proteins (17% as shown in Table 1), which further suggests that hELAV mRNPs represent only a subpopulation of total cellular mRNPs. As expected, RNP complexes reconstituted using recombinant Hel-N1 protein and in vitro transcribed mRNAs migrated at a density of approximately 1.19-1.22 g/ml in accudenz gradients (marked by upward arrows in Fig. 4C, top panel).

Cytoplasmic extracts from other cell lines were examined for hELAV complexes by accudenz density gradient centrifugation and similar results were obtained (Fig. 4D). Interestingly, in cell lines containing only small amounts of hELAV proteins (D283, p>300 and SCLC, OH3), the proteins were predominantly (55-70%) found in the high density β complexes (1.32-1.36 g/ml), suggesting maximal utilization of available ELAV protein in association with polysomes. When these mRNP complexes were further analyzed by linear 10%-50% glycerol gradients, hELAV proteins and L22 ribosomal protein comigrated throughout the gradients, further confirming the association of hELAV complexes with polysomes (not shown).

To determine whether hELAV complexes in medulloblastoma D283 cells (both the p6 and p>300) are free or associated with RNAs, cytoplasmic extracts were treated with ribonuclease A. Partial sensitivity to RNase digestion (see Fig. 7) indicated that at least a portion of these ELAV complexes are likely to contain RNA because a significant portion (70%) of the proteins shifted to the top of accudenz gradient and had the same density as the recombinant Hel-N1 protein. Incomplete digestion may have resulted from the presence of abundant amounts of endogenous cytoplasmic RNA, which competed for the available RNase. This may also indicate that components in the complex partially protected the ELAV-associated RNA from digestion.

In summary, the experiments described above demonstrated a predominantly (95%) cytoplasmic localization of ELAV proteins and their engagement in high density mRNP complexes, which colocalized with polysomes in glycerol and

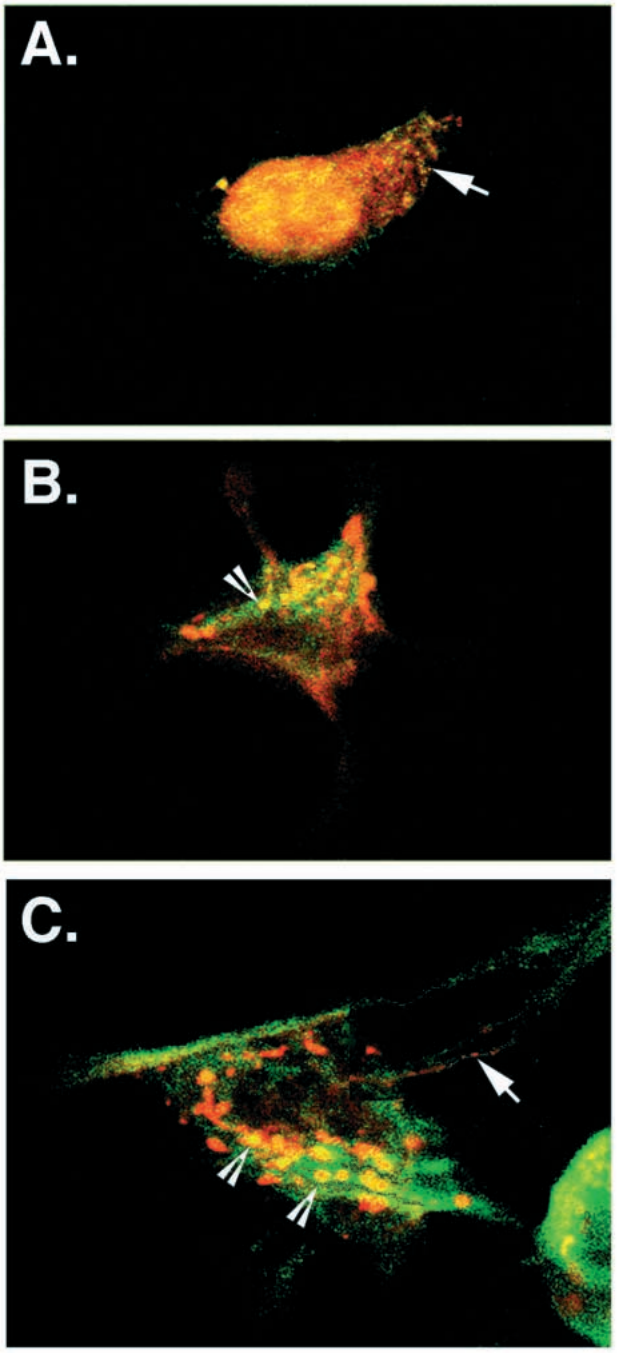

Fig. 2. Confocal microscopy images of medulloblastoma cells that were Triton-extracted prior to fixation and double stained for ELAV proteins (red) and tubulin (green). Anti-Hel-N1 antibody and secondary antibody coupled to Texas Red was used to stain ELAV proteins, and anti-tubulin antibody and secondary antibody coupled to FITC was used to stain microtubules. All images are 0.9 μm thick sections, which were detected under 568 nm laser beam (for Texas Red) and 488 nm laser beam (for FITC). The images shown were obtained by merging red and green channels. (A) Untreated, control cells. hELAV proteins are stained as fine granules localizing along microtubules. (B) Cells treated with cytochalasin D (5 μg/ml) for 3 hours. hELAV proteins are reorganized into large granules, which localize along microtubules. (C) Cells treated with 1 mM puromycin for 3 hours. As in cytochalasin D-treated cells, ELAV proteins reorganized into large granules which are microtubule associated after the puromycin treatment. Arrows point to fine hELAV granules and arrowheads to large hELAV granules formed after cytochalasin or puromycin treatment.

accudenz density gradients. While a previous report (Gao and Keene, 1996) demonstrated association of hELAV proteins with total poly(A)$^+$ mRNA by UV-crosslinking of medulloblastoma cell extracts, our study demonstrated that hELAV proteins are associated with only a subset of cellular mRNAs in vivo, and not with total poly(A)$^+$ mRNA. Furthermore, comparative analysis of four cell lines of neuroendocrine origin demonstrated similar colocalization of hELAV proteins and polysomes in gradients. These results are in agreement with more recent reports implicating hELAV proteins in regulating the stability and translatability of certain growth regulatory mRNAs (Jain et al., 1997; Myer et al., 1997).

hELAV mRNPs are associated with the translational machinery

Considerable evidence has suggested a functional link between mRNA stability and translation. For example, studies on the stability of histone mRNAs, β tubulin mRNA, c-myc mRNA or c-fos mRNA, suggest that these mRNAs are degraded co-translationally (for a review, see Jacobson and Peltz, 1996, and references therein). In order to understand the role of hELAV proteins in one of these two processes we investigated the dynamic association of hELAV mRNPs with the translational machinery as well as with the cytoskeleton.

To analyze the association of hELAV proteins with polysomes, cytoplasmic extracts of medulloblastoma cells (from passage 6) were prepared in B4 buffer lacking Mg^{2+}, but with 20 mM EDTA to dissociate ribosomes. The integrity of the hELAV complexes and polysomes was monitored by changes in their density and/or size, as measured by mobility in accudenz and glycerol gradients, respectively (Figs 5B and 6B). In comparison to the untreated, control samples (Figs 5A and 6A), ribosomes were degraded and the majority of hELAV proteins were found in smaller size and lower density complexes after EDTA treatment. Note in Figs 5B and 6B, the shift from the high density peak close to the bottom of the gradient (β complexes) to the lower density, α complexes, in the middle portion of the gradient (for quantitation see Table 1). Identical results were obtained after cells were treated with 10 mM NaF for 2 hours (not shown). Since it is known that NaF inhibits generation of ATP (Colombo et al., 1965), and therefore affects the integrity of ribosomes and cell metabolism in general, several specific inhibitors of translation were used to further investigate the association between hELAV mRNP complexes and ribosomes.

Dissociation of ribosomes into subunits by puromycin treatment of medulloblastoma cells (Fig. 5C, L22 staining, glycerol gradient) resulted in the appearance of hELAV α complexes of intermediate density (1.19-1.32 g/ml), (compare Fig. 5C with 5A, accudenz gradient, Hel-N1 staining; compare Fig. 6B, puromycin graph, with 6A; and see Table 1 for quantitative analysis). In contrast to puromycin, emetine affects translation irreversibly by inhibiting translocation

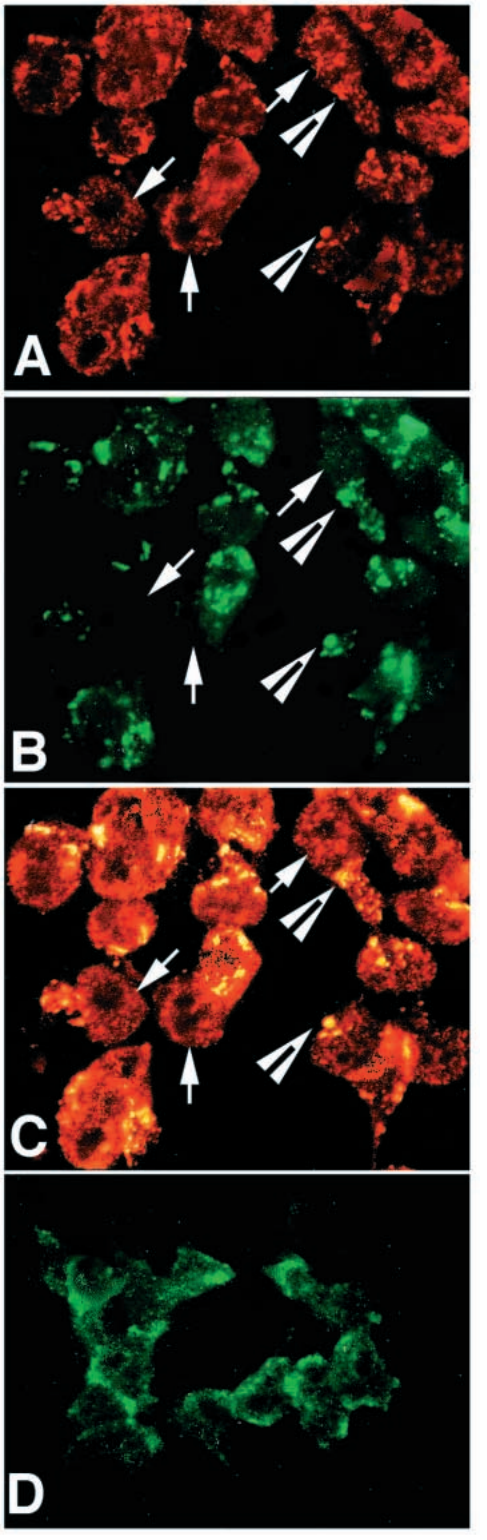

Fig. 3. ELAV-containing cytoplasmic granules present in puromycin-treated medulloblastoma cells contain a subset of poly(A)$^+$ mRNA. Medulloblastoma cells were treated with 1 mM puromycin for 3 hours (A, B and C), Triton extracted prior to fixation and then stained for hELAV proteins with anti-Hel-N1 antibody (green, B) and for poly(A)$^+$ mRNA with anti-digoxigenin antibody (red, A). The superimposed images of green and red are shown in C. (D) Control medulloblastoma cells treated with RNase A and T1 prior to fixation, staining and fluorescence, stained both for poly(A)$^+$ mRNA and hELAV. Immunofluorescence was monitored and documented on a Zeiss Axioscope using 100× magnification under oil immersion and two filters: green BP 546/12 and blue BP450-490. Arrowheads point to granules containing both poly(A)$^+$ mRNA and hELAV proteins, and small arrows point to granules containing only poly(A)$^+$ mRNA. Note the absence of red poly(A)$^+$ staining in RNase-treated sample (D).

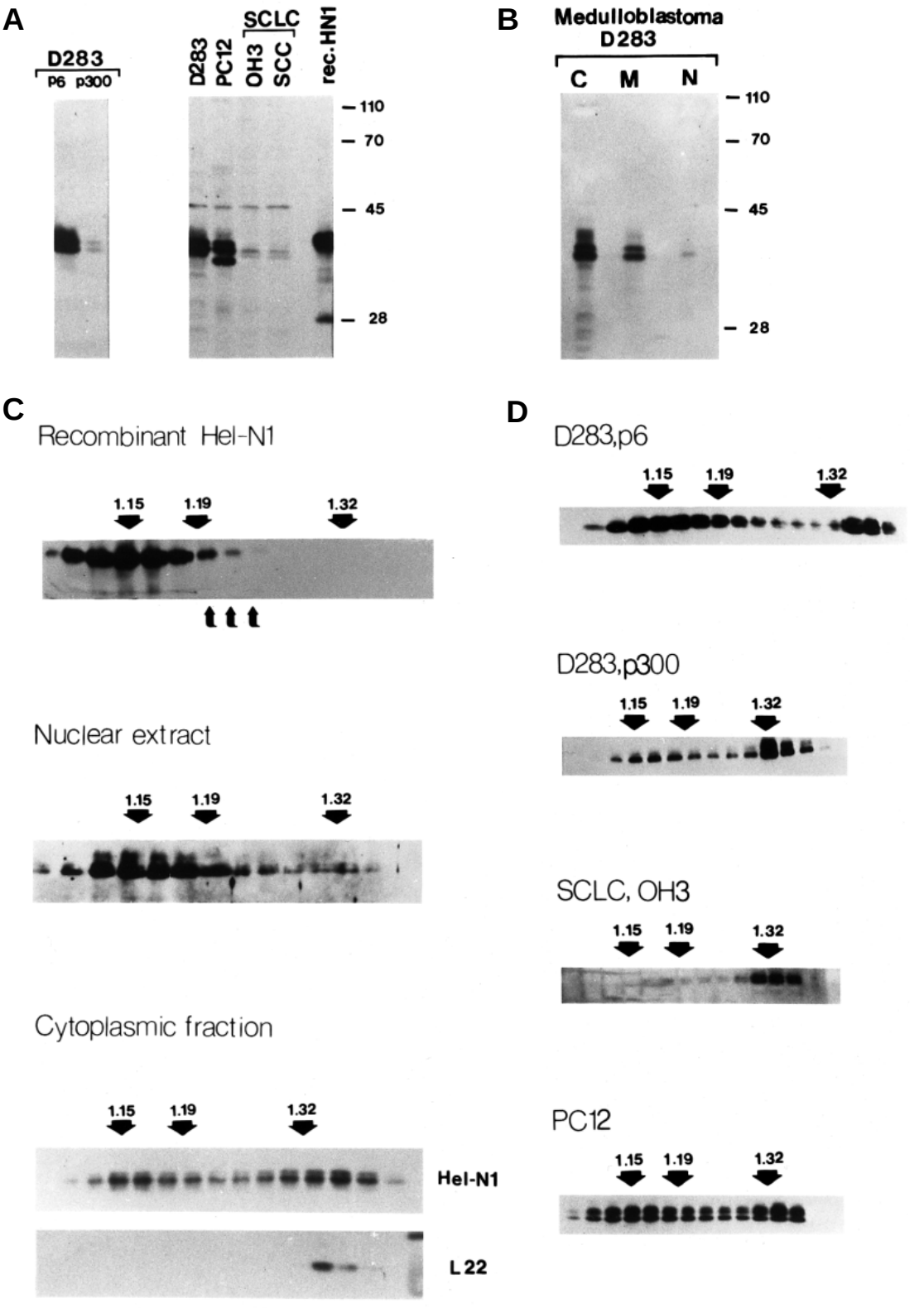

Fig. 4. Expression of the ELAV family of proteins in human medulloblastoma D283 cells (passage 6 (p6) and passage 300 (p300)); rat pheochromocytoma cells (PC12); and human small cell lung carcinoma cells (SCLC): OH-3 and SCC89-LY (SCC) cell lines. (A) Identical quantities of total cell extracts of the four cell lines were used and each contains different amounts and different forms of ELAV proteins. Mobility of hELAV proteins was compared to the recombinant Hel-N1 protein (rec.HN1). (B) Subcellular distribution of human ELAV proteins in medulloblastoma cells: cell equivalents of the cytoplasmic fraction (C), the 'microsomal' fraction (M) and purified nuclei (N) were analyzed by immunoblotting with anti-Hel-N1 antibody. (C) Comparison of the migration of the recombinant Hel-N1 protein or the recombinant Hel-N1 protein bound to c-myc mRNA (upward arrows in the top panel) with that of nuclear (equivalent of 10^8 cells) and cytoplasmic (equivalent of 5×10^6 cells) fractions from medulloblastoma cells by accudenz density gradient centrifugation and immunoblotting. The top of each gradient is shown with the first lane on the left side of each gel. Upper short arrows represent densities of corresponding fractions of each gradient and lower long arrows pointing upward indicate density of recombinant Hel-N1 when prebound in vitro to the 3′ UTR transcript of c-myc mRNA (1.19-1.22 g/ml). Cytoplasmic hELAV proteins are found in three distinct populations: one forming a peak at the top of the gradient with the density 1.15 g/ml, representing free hELAV proteins; one forming a peak at the bottom of the gradient with the density greater than 1.32 g/ml, comigrating with ribosomes (L22 staining) and representing β complexes; and one population migrating throughout the middle part of the gradient representing heterogeneous α complexes. Nuclear hELAV proteins are predominantly (74%) found in the free form (top of the gradient, middle panel) although some heterogeneous complexes with higher densities (up to 1.32 g/ml) are detected. Nuclear fractions did not contain ribosomes (not shown). (D) Cytoplasmic fractions from the four cell lines in A, analyzed by density gradient centrifugation and immunoblotting using anti-Hel-N1 antibody. Upper short arrows represent densities of corresponding fractions of each gradient.

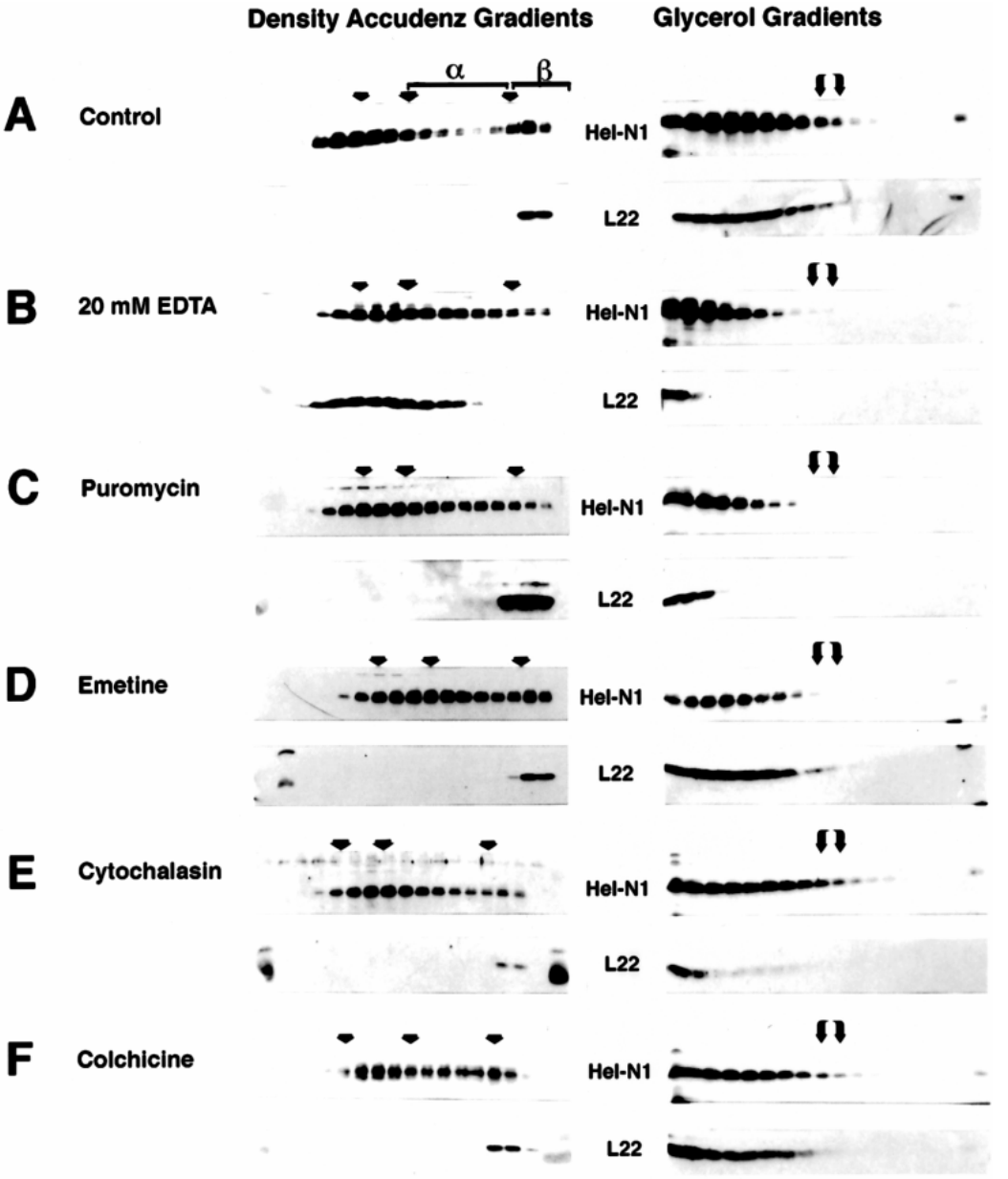

Fig. 5. Cytoplasmic hELAV α mRNP complexes from medulloblastoma cells associate with polysomes to form the translational apparatus designated as β complexes. Medulloblastoma cells were pooled, divided into six identical parts and then subjected to drug treatments. Cytoplasmic extracts were made and an equivalent of 20×10^6 cells was loaded onto gradients. Density-accudenz (left column) and velocity-glycerol (right column) gradient centrifugation and immunoblot analysis of each gradient fraction for hELAV proteins, using anti-Hel-N1 antibody, and ribosomal protein L22, using anti-L22 antibody. Upper short arrows in the left column show fractions with densities 1.15, 1.19 and 1.32 g/ml, left to right, respectively (as shown in Fig. 4C,D). First left lane in each panel represents the top of the gradient. Recombinant Hel-N1 protein bands at 1.15 g/ml (Fig. 4C) and therefore hELAV proteins found at the top of each gradient (left upper short arrow) probably represent free proteins. hELAV complexes have densities between 1.19 g/ml and 1.32 g/ml (between the second and the third upper short arrow: α complexes), and when associated with polysomes their density is greater than 1.32 g/ml (after the third upper short arrow, polysome-associated mRNPs, β complexes). More hELAV complexes are associated with polysomes in translationally active, untreated cells, control (A) and thus are found in β complexes. Translational inhibition by puromycin (1 mM for 3 hours) (C) or by cytochalasin B (10 μg/ml for 19 hours) treatment (E) of cells, as well as treatment of extracts with EDTA (B) resulted in complete disassembly (B and C) or significant reduction (E) of polysomes (as detected in glycerol gradients by L22 staining of B, C and E) and concomitant appearance of hELAV α complexes (Hel-N1 staining, accudenz gradients, B, C and E). Inhibition of translation by emetine (50 μg/ml for 3 hours) (D), on the other hand, did not disrupt polysomes (L22 staining, D) and thus it did not disrupt β complexes, but it resulted in accumulation of hELAV α complexes (Hel-N1 staining, D). Colchicine treatment of cells (20 μg/ml for 3 hours) (F) did not affect polysomes (L22 distribution in both accudenz and glycerol gradients). However, ELAV α complexes accumulated in these cells as shown in F, accudenz gradient. Upper long arrows in the right column indicate the position of large VSV particles, which were used as a size marker (approx. 600 S), in glycerol gradients.

(Grollman, 1968), thus leaving polysomes intact and still associated with mRNA. Indeed, preservation of polysomes was observed in these experiments (Fig. 5D, L22 staining, glycerol gradient) after emetine treatment for 3 hours. hELAV β complexes remained intact as well and they comigrated with polysomes in both glycerol and accudenz gradients. Interestingly, an increase in α complexes was observed after emetine treatment (Fig. 5D, Hel-N1 staining, accudenz gradient; compare Fig. 6C with 6A; and see Table 1) in spite of the fact that polysomes remained intact. This result was expected because, while ribosomes are kept translationally inactive and immobilized on mRNAs during the emetine treatment, hELAV proteins are recruited from the pool of free protein (Table 1) to bind newly synthesized mRNAs, forming α complexes. These newly assembled hELAV mRNP α complexes were presumably accumulating in the cytoplasm of

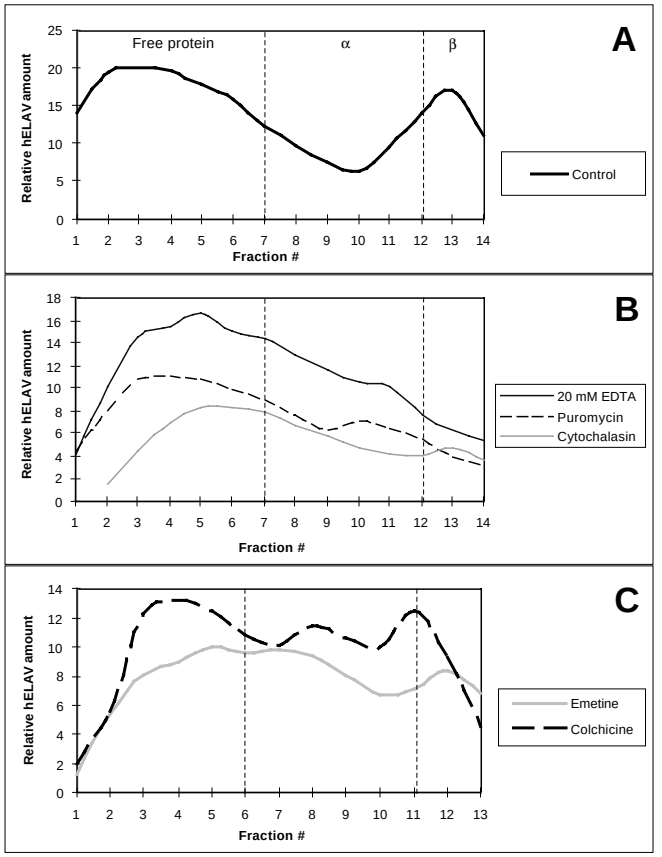

Fig. 6. Quantitative distribution of hELAV proteins in the accudenz density gradients shown in Fig. 5. Each gel was electronically scanned and quantitated using ImageQuant program (Molecular Dynamics). Graphs were generated using Microsoft Exel. The y-axis represents the relative quantity of hELAV proteins and the x-axis represents gradient fraction number. Dotted lines correspond to the upper short arrows, shown in Figs 4 and 5, which mark the position of fractions with the density 1.19 g/ml (left line) and 1.32 g/ml (right line). (A) Distribution of hELAV proteins in untreated, control medulloblastoma cells. (B) Distribution of hELAV proteins following EDTA, puromycin and cytochalasin treatment (C) Distribution of hELAV proteins following colchicine and emetine treatment.

cells, perhaps due to the unavailability of ribosomes. Hence, both the disassembly (caused by puromycin treatment) and immobilization (caused by emetine treatment) of the translational apparatus resulted in the appearance of hELAV α complexes (Table 1).

The experiments described above show that hELAV proteins associate with mRNAs to form heterogeneous mRNPs (α complexes). Apparently the association of these mRNPs with polysomes results in the formation of the high density β complexes. Quantitative analysis of density gradients, by scanning of blots shown in Fig. 5, demonstrated that hELAV RNPs remaining in these high density fractions after dissociation of ribosomes (Table 1, EDTA or puromycin) represented residual α complexes which were trailing to the bottom of the gradient (Fig. 6B). Despite this fact, the majority of hELAV mRNPs found in these high density fractions are associated with polysomes, and thus, represent β complexes. The observed heterogeneous distribution of α complexes in

Table 1. Quantitation of the hELAV proteins distributed across the accudenz density gradients

	Free protein	α complexes	β complexes
Control	61%	17%	22%
20 mM EDTA	58%	34%	8%
Puromycin	61%	32%	7%
Cytochalasin	52%	36%	12%
Emetine	44%	34%	22%
Colchicine	44%	34%	22%

Immunoblots of accudenz gradients stained with anti-Hel-N1 antibody, as shown in Fig. 5, were electronically scanned and quantitated using ImageQuant program (Molecular Dynamics). The sum of values obtained from one gradient represents the total amount of hELAV proteins, which were distributed in three forms: (1) free protein, (2) α complexes and (3) β complexes (as shown in Fig. 6). The quantity of hELAV proteins representing each form is shown as a proportion of the total hELAV in the gradient (see Fig. 6). Based upon the density of each gradient, the first six (Fig. 6C) or seven (Fig. 6A and B) fractions represent free protein. Fractions between the two vertical dotted lines in Fig. 6 represent α complexes, while the last two or three fractions containing ribosomes (Fig. 5) represent β complexes.

accudenz density gradients, is probably due to their association with a variety of mRNAs and other proteins resulting in the formation of mRNP complexes with various densities.

hELAV mRNPs, cytoskeleton and translation

Treatment of cells with cytochalasin is known to disrupt microfilaments. Treatment of cells with cytochalasin B (10 μg/ml) or with cytochalasin D (5 μg/ml) for 3 hours caused the disruption of microfilaments in 60-70% of medulloblastoma cells, as monitored by immunofluorescent staining of actin. To obtain a more uniform population of affected cells for biochemical analysis, we extended the duration and found that treatment of cells with cytochalasin B (10 μg/ml; Fig. 5E) or cytochalasin D (5 μg/ml) for 19 hours (not shown) caused the effect in greater than 90% of cells. These long treatments did not cause cell death in the culture, as monitored by separation of dead and live cells through the LSM medium, consistent with the reports in the literature that cells treated with cytochalasin for 24 hours do not die (Cooper, 1987; Litman et al., 1994). The cytochalasin treatments are also known to induce partial dissociation of polysomes from the cytoskeleton (Ornelles et al., 1986). The rate of protein synthesis in these experiments is directly dependent on the number of polysomes that remain bound to the cytoskeleton (Ornelles et al., 1986). In our experiments, a proportion of polysomes remained intact after cytochalasin B (10 μg/ml) treatment for 19 hours (Fig. 5E, light trailing of L22, glycerol gradient) and hELAV proteins were mainly present in α complexes of intermediate density (Fig. 5E, compare to 5A; compare Fig. 6B, cytochalasin graph, with 6A; and see summary in Table 1). Therefore, as observed with puromycin (Figs 5C, 6B; Table 1), an increase in the abundance of hELAV α complexes was observed after treatment of cells with cytochalasin (Fig. 5E; 6B; and Table 1). This result correlated with the dissociation of polysomes from the disrupted microfilament network (Fig. 5E, glycerol gradient, L22 staining; see Table 1). In addition, the observed increase in α complexes may be partially attributed to the assembly of new hELAV mRNPs (Table 1, see decrease in the amount of free hELAV proteins), which were

not efficiently recruited to the partially disassembled translational apparatus. It is interesting to note that hELAV α complexes, in cells treated with either puromycin or cytochalasin, reorganized into large mRNP granules which were associated with microtubules (Figs 1, 2 and 3).

Treatment of cells with colchicine, which prevents assembly of microtubules (Andreu and Timasheff, 1986), did not affect the distribution of polysomes (Fig. 5F, L22 staining, glycerol gradient). However, hELAV mRNP α complexes also accumulated in these cells (Fig. 5F, compare to 5A; compare Fig. 6C to 6A; see Table 1), while a significant portion remained associated with polysomes in the form of β complexes (Table 1). This result suggests that interactions between hELAV mRNP α complexes and polysomes, and therefore formation of β complexes, were impaired. This observation, together with immunofluorescence data in which cells were simultaneously treated with colchicine and puromycin, suggests that association of hELAV α complexes with microtubules is essential for their presentation to and association with the components of the translational machinery. In addition, as documented in Fig. 5E and Table 1, intact microfilaments were necessary for the integrity of polysomes, and thus for the assembly and integrity of β complexes. Therefore, we conclude that association of hELAV α mRNPs with microtubules and polysomes with microfilaments is essential for the translation of hELAV-bound mRNAs. Interestingly, these findings are consistent with our recent data showing that ectopic expression of Hel-N1 in 3T3L1 cells can affect mRNA translatability (Jain et al., 1997; reviewed in Antic and Keene, 1997).

Puromycin-dissociated hELAV complexes remain associated with RNA

The data presented above (Fig. 4D; Table 1) demonstrate that cytoplasmic hELAV proteins are mainly engaged in β complexes, which are partially sensitive to RNase treatments (not shown) in translationally active cells. We also showed that hELAV-containing granules observed in puromycin- and cytochalasin-treated cells contain poly(A)$^+$ mRNA. To determine whether large hELAV mRNP granules that accumulate in cells treated with translational inhibitors represent α ribonucleoprotein complexes or are devoid of RNA, accudenz gradient fractions of puromycin-treated cells (Figs 5C and Fig. 7A) were pooled, treated with RNase A and re-analyzed on accudenz density gradients (Fig. 7B). hELAV α complexes from the first 10 fractions, as well as the majority of complexes from fractions 11-13, were sensitive to RNase, resulting in the release of free hELAV proteins. Therefore, this experiment showed that hELAV α complexes appearing after translational inhibition with puromycin (Figs 5C and 7A) or emetine (not shown) still contain RNA. It is likely that this bound RNA represents various species of mRNA rather than ribosomal RNA, since these complexes have either been dissociated from ribosomes or accumulated in the cytoplasm while ribosomes or ribosomal subunits remained intact (Fig. 5A,C,D,E; L22 staining in accudenz gradients). It is also interesting to note that RNA-containing hELAV α complexes banded in accudenz gradients at a similar position to RNP complexes formed in vitro using recombinant Hel-N1 and c-myc mRNA (Fig. 4C). As a control, a sample of combined fractions 8-10 was not treated with RNase A and the resultant complexes banded at the original density (not shown).

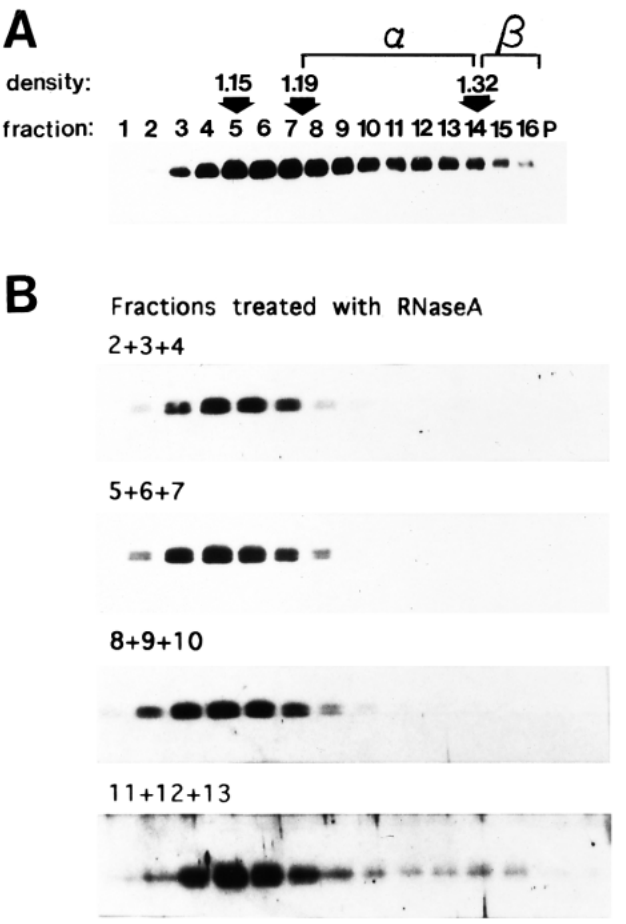

Fig. 7. hELAV α complexes released from polysomes by puromycin treatment contain RNA. (A) Puromycin treatment of cells for 3 hours resulted in the appearance of ELAV α complexes with intermediate density (1.19-1.32 g/ml in accudenz gradients). (B) Accudenz gradient fractions 2-4, 5-7, 8-10 and 11-13 containing ELAV α complexes (from A) were dialyzed against buffer B4, treated with RNaseA (50 μg) for 6 hours on ice, loaded over fresh accudenz gradients and analyzed by immunoblotting with Hel-N1 antibody.

The data presented above demonstrate that a subset of mRNAs of neuronal cells are found in hELAV mRNP α complexes, which in association with polysomes, form a translational apparatus (β complexes). When the α complexes were partially purified by sequential gradient purification and differential centrifugation (as described in Materials and methods), they were found to be heterogeneous and larger than 35 S. Separation of these complexes on polysomal gradients and their analysis by silver staining of SDS-polyacrylamide protein gels revealed that they contain at least 20 polypeptides (data not shown).

hELAV α complexes associate with microtubules in vitro

Experiments described above suggest that hELAV α complexes are associated with microtubules and that this association is important for their interaction with the

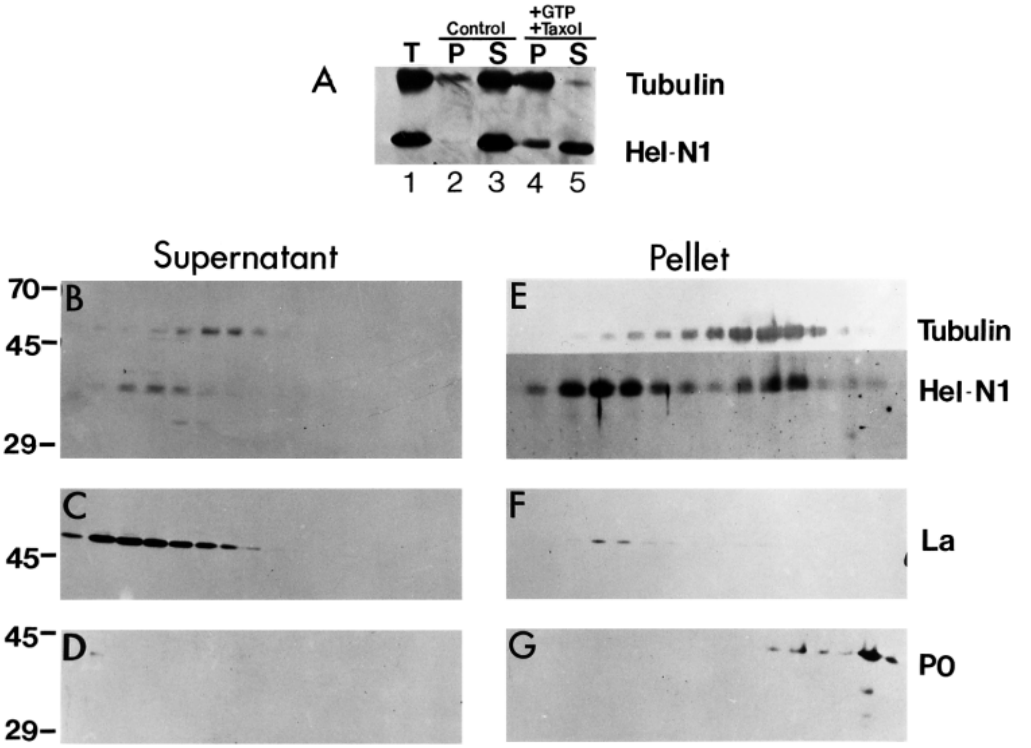

Fig. 8. Binding of hELAV RNP complexes to microtubules by reconstitution in medulloblastoma cell extracts. Immunoblotting analysis: (A) Lane 1, total amount (T) of tubulin and ELAV proteins in the cell extract used for in vitro reconstitution of microtubules; lanes 2-5, extracts incubated without GTP and taxol (control, lanes 2 and 3) or extracts incubated with GTP and taxol (lanes 4 and 5) were centrifuged and the pellets (P, lanes 2 and 4) or the supernatants (S, lanes 3 and 5) were analyzed for the presence of tubulin and ELAV proteins. Supernatants (B-D) and pellets (E-G) from the experiment shown in A, were further analyzed on accudenz density gradients. Each gradient fraction was examined for tubulin, ELAV (Hel-N1 staining), La and ribosomal P proteins by immunoblotting. B-D are obtained by staining of one blot with several antibodies: (B) anti-tubulin and anti-Hel-N1, (C) anti-La and (D) anti-P0. (E-G) represent one blot probed with: anti-tubulin and anti-Hel-N1 antibodies (E), anti-La antibody (F) and anti-P0 antibody (G). Lanes on the left side of each panel from B to G represent the top of the gradients.

translational apparatus. This is consistent with the observation that microtubules participate in mRNA localization in neurons (Bassell et al., 1994; Litman et al., 1994). To conclusively demonstrate this association, we reconstituted microtubules and hELAV complexes using extracts of medulloblastoma cells. Since cell extracts were prepared on ice, endogenous microtubules were dissociated, and thus all cellular components that are microtubule-associated were released. Therefore, to perform the reconstitution assay, endogenous tubulin was polymerized into microtubules by incubating cell extracts at 37°C in the presence of GTP and taxol. During this incubation, subcellular components which bind to microtubules will form a heavy microtubule-associated complex that can be pelleted by centrifugation (Vallee and Collins, 1986). After centrifugation, pellets and supernatants were analyzed for distribution of hELAV and control proteins by immunoblotting (Fig. 8). In control samples, cell extracts were incubated at 37°C without GTP and taxol (Fig. 8A, lanes 2 and 3) and hELAV complexes did not pellet after centrifugation. However, in the presence of GTP and taxol (Fig. 8A, lanes 4 and 5; Fig. 8B,E), a portion of hELAV proteins was pelleted. Similar results were obtained after probing of the same blot with an antibody against the ribosomal P proteins (not shown but represented in Fig. 8D,G). Probing of the blot with control anti-La antibody revealed that La protein stayed in the supernatant (not shown but represented in Fig. 8C,F). To investigate the association of these proteins with specific complexes, we analyzed supernatants and pellets obtained after the microtubule reconstitution assay using accudenz density gradients. It is evident that both hELAV (Hel-N1 staining in Fig. 8B) and La (Fig. 8C) proteins, which remained in supernatants, banded at low density (top of the gradient) and did not comigrate with tubulin (Fig. 8B). However, pelleted hELAV proteins were found to band at low (1.12-1.16g/ml) and high (1.26-1.29 g/ml) densities (Fig. 8E, Hel-N1 staining). The low density fraction probably represents free protein, while the higher density fraction comigrated with assembled microtubules (Fig. 8E) as an RNP complex since it was sensitive to RNase treatment (not shown). Although very little La protein was detected on the same blot, it was present in low density fractions (Fig. 8F). Probing the same blots with anti-ribosomal antibody showed that ribosomes were not present in the supernatant (Fig. 8D, P0 staining). Pelleted ribosomes banded at density 1.33 g/ml (Fig. 8G) and did not comigrate with hELAV-microtubule complexes, further demonstrating the specificity of the interaction of hELAV mRNPs with microtubules. It is interesting to note that in the presence of GTP and taxol, α complexes dissociated from high density (>1.33 g/ml) β complexes which, in control cell extracts, comigrated with ribosomes. It is also evident that a portion of the hELAV proteins was released from complexes after addition of GTP and taxol and they migrated in these gradients as free protein, which was not associated with microtubules (Fig. 8E, Hel-N1 staining at the top of the gradient). Based upon these data, it is possible that free hELAV proteins did not bind to microtubules or, alternatively, microtubules were saturated with hELAV complexes leaving a portion of the hELAV protein unbound. The association of α complexes with microtubules was confirmed by binding of partially purified α complexes (as described in Materials and methods) to microtubules following in vitro assembly (data not shown).

The data presented above demonstrate specific and direct association of hELAV mRNP complexes with microtubules. Analysis of pellets and supernatants after reconstitution suggested that interaction of hELAV mRNPs with microtubules is specific because La protein, which is another RNA-binding protein containing three RRMs, did not bind to assembled microtubules. However, finding that both ribosomes and hELAV mRNPs were pelleted after reconstitution might appear to be at variance with our finding that polysomes were not associated with microtubules (Fig. 5E). To investigate the specificity of this interaction, we analyzed both the supernatants and pellets, following in vitro reconstitution, using accudenz gradients, which allowed separation of complexes with different densities. We found that only pelleted hELAV mRNPs comigrated with microtubules while ribosomes banded at a higher density and a small amount of La protein banded at a low density in the gradients (Fig. 8E,F,G). Thus, ribosomes (polysomes) were co-pelleted not because of their association with microtubules, but rather because of their large size. These results also strongly argue against the possibility that hELAV mRNP complexes were nonspecifically trapped in microtubules and then released during resuspension. If this was the case we would expect to find ribosomes, as well as other RNP particles such as La RNPs, similarly trapped in the same microtubule-associated complex, and this did not occur (note the different distributions of La, P0 and Hel-N1 in Fig. 8E,F,G). Also, if RNP particles became nonspecifically trapped and subsequently released during resuspension, one would expect to find some hELAV mRNPs in the supernatants (Fig. 8B). However, only free hELAV proteins were observed (Fig. 8B). Therefore, the data demonstrate the specific interaction of hELAV mRNPs with microtubules and confirm the immunofluorescence data (Fig. 2) showing localization of hELAV mRNPs along microtubule tracks in detergent-extracted cells.

DISCUSSION

Untranslated sequences in eukaryotic mRNAs have been shown to be involved in the control of mRNA transport, localization, stability and translation. We have examined human neuronal ELAV proteins, which are predominantly localized in the cytoplasm (Gao and Keene, 1996) and are able to bind, with high affinity, to AU-rich sequences in 3′UTRs of certain growth regulatory mRNAs in vitro. Recent findings have implicated human ELAV proteins in stability and translatability (reviewed in Antic and Keene, 1997; Myer et al., 1997; Jain et al., 1997) of mRNAs via interactions with AU-rich 3′UTR sequences. Similarly to proteins involved in splicing in the nucleus, hELAV proteins were found to reside in distinct cytoplasmic granules of cortical neurons and cultured medulloblastoma cells (Figs 1 and 2; Gao and Keene, 1996), implying a role in mRNA localization/transport, translation or stability. To characterize these granules biochemically and to determine their involvement in metabolic processes, we explored the association of human ELAV proteins into mRNP complexes, as well as their association with cellular components such as nuclei, ribosomes, microtubules and microfilaments. We fractionated RNP complexes containing hELAV proteins and found them to be heterogeneous in nature (Figs 4 and 5). However, it was possible to discern three forms of cytoplasmic hELAV proteins by density gradient analysis: (1) free protein, (2) mRNP particles (α complexes) and (3) mRNPs associated with polysomes (β complexes), while hELAV proteins detected in nuclear fractions predominantly migrated as free protein.

Although the cellular dynamics of hELAV proteins are not well understood, it is possible that free proteins bind to mRNAs in the nucleus, forming α complexes, which are then exported to the cytoplasm where they bind to microtubules and associate with polysomes to form β complexes. The strongest evidence for a dynamic transition between α and β complexes is that inhibition of translation with puromycin caused a distinct reduction in β complexes and a concomitant increase in α complexes (Fig. 6; Table 1). Similarly, another translational inhibitor, emetine, caused a change in the distribution of α and β complexes (Figs 5 and 6; Table 1). We interpret these results to suggest that preformed β complexes remained intact after the addition of emetine which immobilized the translational machinery, while hELAV proteins and mRNAs combined to form new α complexes, which then accumulated in the absence of free ribosomes (Table 1).

A similar effect (accumulation of hELAV α complexes and preservation of β complexes) was observed after the colchicine treatment (Fig. 6C; Table 1). We demonstrated the association of hELAV mRNP α complexes with microtubules (Figs 2 and 8), as well as the association of polysomes with microfilaments (Fig. 5E; Table 1). Our findings suggest that translational components were not able to associate with hELAV mRNPs when microtubules have been dissociated. It is possible that association of hELAV mRNPs with microtubules is important either (1) for the transport, and therefore for the delivery of mRNAs to the translational machinery, or (2) it is necessary for interaction of mRNA with ribosomal subunits during formation of an active translational apparatus. The observed preservation of β complexes after colchicine treatment (Table 1) suggests that the translational apparatus itself is not associated with microtubules. On the other hand, partial sensitivity of β complexes to cytochalasin is consistent with their association with microfilaments (Table 1). These interpretations are consistent with several studies showing that essential components of the translational apparatus are associated with the cytoskeleton. For example, cap-binding protein, aminoacyl-tRNA synthetases, initiation factors, and elongation factors have been found associated with the cytoskeletal matrix (Barbarese et al., 1995, and references therein). It has also been demonstrated that mRNA is associated with microtubules in neuronal cells and that 3′UTRs of certain mRNAs contain zip-codes which apparently are involved in localizing mRNAs to specific cellular compartments (Kislauskis et al., 1993).

For multi-targeted mRNA-binding proteins like ELAV, it is likely to prove difficult to define homogeneous classes of mRNPs with high molecular precision until new technologies are developed. The technical challenges presented by heterogeneous RNPs in mammalian cells are not easily solved by genetics, biochemistry or cell biological methods alone, but rather by a combination of these approaches. In this study, we have demonstrated that metabolic inhibitors, together with specific antibodies, can be used to reconstruct certain structural and functional aspects of cytoplasmic RNPs and to manipulate

their organization. Thus, by controlled disruption of individual cellular components we have reconstructed events that may indicate functional relationships between hELAV proteins, their bound mRNAs and the cellular components. Like the pre-mRNP nuclear speckles containing SR proteins involved in splicing (Spector, 1996, and references therein), the cytoplasmic hELAV granules appear to represent mRNP particles assembled prior to entering into an RNA processing event such as translation, storage or degradation.

Recent findings demonstrated involvement of the Sex-lethal, another developmentally regulated RRM protein in *Drosophila*, in both the nuclear (alternative splicing) and cytoplasmic (translation) post-transcriptional processing events, thus controlling sex-determination in flies (Bashaw and Baker, 1997; Kelley et al., 1997). ELAV proteins could be analogous to Sex-lethal in having dual functions. While the pool of nuclear hELAV proteins may be involved in post-transcriptional mRNA processing events such as alternative splicing in the nucleus, as suggested recently for *Drosophila* ELAV (Koushika et al., 1996), cytoplasmic hELAV proteins may be involved in translation and/or stability of mRNAs in the cytoplasm (Jain et al., 1997). Further purification of ELAV mRNPs and more refined reagents will be needed to fully understand the mechanism of action of these important cell proteins which have been implicated in modulating mRNA expression during the growth and differentiation of metazoan organisms.

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REFERENCES

Ainger, K., Avossa, D., Morgan, F., Hill, S.J., Barry, C., Barbarese, E. and Carson, J.H. (1993). Transport and localization of exogenous myelin basic protein mRNA microinjected into oligodendrocytes. *J. Cell Biol*. **123**, 431-441.

Anant, S., Giannoni, F., Antic, D., DeMaria, C.T., Keene, J.D., Brewer, G. and Davidson, N.O. (1997). AU-rich RNA binding proteins Hel-N1 and AUF1 bind apolipoproteinB mRNA and inhibit posttranscriptional C to U editing. *Nucleic Acids Symposium Series* **34** (in press).

Andreu, J.M. and Timasheff, S.N. (1986). Tubulin-colchicine interactions and polymerization of the complex. *Ann. NY Acad. Sci*. **466**, 676-689.

Antic, D. and Keene, J.D. (1997). ELAV RNA-binding proteins involved in growth, differentiation and post-transcriptional gene expression. *Am. J. Hum. Gen*. **61,** 273-278.

Barbarese, E., Koppel, D.E., Deutscher, M.P., Smith, C.L., Ainger, K., Morgan, F. and Carson, J.H. (1995). Protein translation components are colocalized in granules in oligodendrocytes. *J. Cell Sci*. **108,** 2781-2790.

Bashaw, G.J., and Baker, B.S. (1997). The regulation of the *Drosophila msl-2* gene reveals a function for *sex-lethal* in translational control. *Cell* **89,** 789-798.

Bassell, G.J., Singer, R.H. and Kosik, K.S. (1994). Association of poly(A)$^+$ mRNA with microtubules in cultured neurons. *Neuron* **12,** 571-582.

Bier, E., Ackerman, L., Barbel, S., Jan, L. and Jan, Y. N. (1988). Identification and characterization of a neuron-specific nuclear antigen in *Drosophila*. *Science* **240,** 913-916.

Burd, C.G. and Dreyfuss, G. (1994). Conserved structures and diversity of functions of RNA-binding proteins. *Science* **265,** 615-621.

Campos, A.R., Grossman, D. and White, K. (1985). Mutant alleles at the locus elav in *Drosophila melanogaster* lead to nervous system defects. A developmental-genetic analysis. *J. Neurogenet.* **2,** 197-218.

Colombo, B., Felicetti, L. and Baglioni, C. (1965). Inhibition of protein synthesis by cycloheximide in rabbit reticulocytes. *Biochem. Biophys. Res. Commun.* **18,** 389-395.

Cooper, J.A. (1987). Effects of cytochalasin and phalloidin on actin. *J. Cell Biol.* **105,** 1473-1478.

Dreyfuss, G., Matunis, M.J., Pinol-Roma, S. and Burd, C.G. (1993). hnRNP proteins and the biogenesis of mRNA. *Annu. Rev. Biochem.* **62,** 289-321.

Gao, F.-B., Carson, C., Levine, T. and Keene, J. (1994). Selection of a subset of mRNAs from combinatorial 3′ untranslated region libraries using neuronal RNA-binding protein Hel-N1. *Proc. Nat. Acad. Sci. USA* **91,** 11207-11211.

Gao, F.-B. and Keene, J.D. (1996). Hel-N1/Hel-N2 proteins are bound to poly(A)$^+$ mRNA in granular RNP structures and are implicated in neuronal differentiation. *J. Cell Sci.* **109,** 579-589.

Good, P.J. (1995). A conserved family of elav-like genes in vertebrates. *Proc. Nat. Acad. Sci. USA* **92,** 4557-4561.

Gorlach, M., Burd, C.G. and Dreyfuss, G. (1994). The mRNA poly(A)-binding protein: localization, abundance, and RNA-binding specificity. *Exp. Cell Res.* **211,** 400-407.

Grollman, A.P. (1968). Inhibitors of protein biosynthesis. *J. Biol. Chem.* **243,** 4089-4094.

Jacobson, A. and Peltz, S.W. (1996). Interrelationships of the pathways of mRNP decay and translation in eukaryotic cells. *Annu. Rev. Biochem.* **65,** 693-740.

Jain, R.G., Andrews, L.G., McGowan, K.M., Pekala, P.H. and Keene, J.D. (1997). Ectopic expression of Hel-N1, an RNA-binding protein, increases glucose transporter (GLUT1) expression in 3T3-L1 adipocytes. *Mol. Cell. Biol.* **17,** 954-962.

Kelley, R.L., Wang, J., Bell, L. and Kuroda, M.I. (1997). *Sex lethal* controls dosage compensation in *Drosophila* by a non-splicing mechanism. *Nature* **387,** 195-199.

Kenan, D.J., Query, C.C. and Keene, J.D. (1991). RNA recognition: towards identifying determinants of specificity. *Trends Biochem. Sci.* **16,** 214-220.

King, P.H., Levine, T.D., Fremeau, R.T., Jr. and Keene, J.D. (1994). Mammalian homologs of *Drosophila* ELAV localized to a neuronal subset can bind in vitro to the 3′ UTR of mRNA encoding the Id transcriptional repressor. *J. Neurosci.* **14,** 1943-1952.

Kislauskis, E.H., Li, Z., Singer, R.H. and Taneja, K.L. (1993). Isoform-specific 3′-untranslated sequences sort α-cardiac and β-cytoplasmic actin messenger RNAs to different cytoplasmic compartments. *J. Cell Biol.* **123,** 165-172.

Koushika, S.P., Lisbin, M.J. and White, K. (1996). ELAV, a Drosophila neuron-specific protein, mediates the generation of an alternatively spliced neural protein isoform. *Curr. Biol*. **6,** 1634-1641.

Kramer, A. (1996). The structure and function of proteins involved in mammalian pre-mRNA splicing. *Annu. Rev. Biochem.* **65,** 367-409.

Lenk, R., Ransom, L., Kaufmann, Y. and Penman, S. A. (1977). Cytoskeletal structure with associated polyribosomes obtained from HeLa cells. *Cell* **10,** 67-78.

Levine, T.D., Gao, F.B., King, P.H., Andrews, L.G. and Keene, J.D. (1993). Hel-N1: an autoimmune RNA-binding protein with specificity for 3′ uridylate-rich untranslated regions of growth factor mRNAs. *Mol. Cell. Biol.* **13,** 3494-3504.

Litman, P., Barg, J. and Ginzburg, I. (1994). Microtubules are involved in the localization of tau mRNA in primary neuronal cell cultures. *Neuron* **13,** 1463-1474.

Liu, J., Dalmau, J., Szabo, A., Rosenfeld, M., Huber, J. and Furneaux, H. (1995). Paraneoplastic encephalomyelitis antigens bind to the AU-rich elements of mRNA. *Neurology* **45,** 544-550.

Mattaj, I.W. (1993). RNA recognition: a family matter. *Cell* **73,** 837-840.

Mayford, M., Baranes, D., Podsypanina, K. and Kandel, E.R. (1996). The 3′-untranslated region of CaMKIIa is a cis-acting signal for the localization and translation of mRNA in dendrites. *Proc. Nat. Acad. Sci. USA* **93,** 13250-13255.

Michael, W.M., Choi, M. and Dreyfuss, G. (1995). A nuclear export signal in hnRNP A1: a signal-mediated, temperature-dependent nuclear protein export pathway. *Cell* **83,** 415-422.

Myer, V.E., Fan, X. C. and Steitz, J.A. (1997). Identification of HuR as a protein implicated in AUUUA-mediated mRNA decay. *EMBO J.* **16,** 2130-2139.

Ornelles, D.A., Fey, E.G. and Penman, S. (1986). Cytochalasin releases

mRNA from the cytoskeletal framework and inhibits protein synthesis. *Mol. Cell. Biol.* **6,** 1650-1662.

Pinol-Roma, S. and Dreyfuss, G. (1993). hnRNP proteins: localization and transport between the nucleus and the cytoplasm. *Trends Cell Biol.* **3,** 151-155.

Rickwood, D., Ford, T. and Graham, J. (1982). Nycodenz: a new nonionic iodinated gradient medium. *Anal. Biochem.* **123,** 23-31.

Robinow, S. and White, K. (1991). Characterization and spatial distribution of the ELAV protein during *Drosophila melanogaster* development. *J. Neurobiol.* **22,** 443-461.

Spector, D.L. (1996). Nuclear organization and gene expression. *Exp. Cell Res.* **229,** 189-197.

Steward, O. (1994). Dendrites as compartments for macromolecular synthesis. *Proc. Nat. Acad. Sci. USA.* **91,** 10766-10768.

St Johnston, D. (1995). The intracellular localization of messenger RNAs. *Cell* **81,** 161-170.

Sundell, C.L. and Singer, R.H. (1991). Requirement of microfilaments in sorting of actin messenger RNA. *Science* **53,** 1275-1277.

Szabo, A., Dalmau, J., Manley, G., Rosenfeld, M., Wong, E., Henson, J., Posner, J.B. and Furneaux, H.M. (1991). HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding doains and is homologous to Elav and Sex-lethal. *Cell* **67,** 325-333.

Toczyski, D.P., Matera, A.G., Ward, D.C. and Steitz, J.A. (1994). The Epstein-Barr virus (EBV) small RNA EBER1 binds and relocalizes ribosomal protein L22 in EBV-infected human B lymphocytes. *Proc. Nat. Acad. Sci. USA* **91,** 3463-3467.

Vallee, R.B. and Collins, C.A. (1986). Purification of microtubules and microtubule-associated proteins from sea urchin eggs and cultured mammalian cells using taxol, and use of exogenous taxol-stabilized brain microtubules for purifying microtubule-associated proteins. *Methods Enzymol*. **134,** 116-127.

Yisraeli, J. K., Sokol, S. and Melton, D. A. (1990). A two-step model for the localization of maternal mRNA in *Xenopus* oocytes: involvement of microtubules and microfilaments in the translocation and ancoring of Vg1 mRNA. *Development* **108**, 289-298.