Heterogeneous Nuclear Ribonucleoprotein (hnRNP) E1 Binds to hnRNP A2 and Inhibits Translation of A2 Response Element mRNAs

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Heterogeneous nuclear ribonucleoprotein (hnRNP) A2 is a trans-acting RNA-binding protein that mediates trafficking of RNAs containing the cis-acting A2 response element (A2RE). Previous work has shown that A2RE RNAs are transported to myelin in oligodendrocytes and to dendrites in neurons. hnRNP E1 is an RNA-binding protein that regulates translation of specific mRNAs. Here, we show by yeast two-hybrid analysis, in vivo and in vitro communoprecipitation, in vitro cross-linking, and fluorescence correlation spectroscopy that hnRNP E1 binds to A2RE RNA in an hnRNP A2-dependent manner. hnRNP E1 is colocalized with hnRNP A2 and A2RE mRNA in granules in dendrites of oligodendrocytes. Overexpression of hnRNP E1 or microinjection of exogenous hnRNP E1 in neural cells inhibits translation of A2RE mRNA, but not of non-A2RE RNA. Excess hnRNP E1 added to an in vitro translation system reduces translation efficiency of A2RE mRNA, but not of nonA2RE RNA, in an hnRNP A2-dependent manner. These results are consistent with a model where hnRNP E1 recruited to A2RE RNA granules by binding to hnRNP A2 inhibits translation of A2RE RNA during granule transport.

INTRODUCTION

Intracellular trafficking of RNA molecules provides a mechanism to maximize expression of the encoded proteins in specific subcellular compartments and minimize ectopic expression in the cell. This implies that translation is inhibited during RNA transport and activated once the RNA reaches its destination, a mechanism referred to as localization-dependent translation. Several proteins involved in suppression of translation during RNA transport have been identified in Drosophila (Gavis and Lehmann, 1994; Kim-Ha et al., 1995; Rongo et al., 1995; Dahanukar and Wharton, 1996; Smibert et al., 1996, 1999; Dahanukar and Walker, 1999), and in mammalian cells (Hüttelmaier et al., 2005). However, the molecular mechanism of translation regulation during RNA trafficking has not been determined.

We have characterized a specific RNA-trafficking pathway mediated by the trans-acting trafficking factor heterogeneous nuclear ribonucleoprotein (hnRNP) A2 that binds to a characteristic 11 nucleotide (nt) cis-acting trafficking element (GC-CAAGGAGCC), termed the A2 response element (A2RE), found in a variety of transported RNAs. Molecules of A2RE RNA and hnRNP A2 protein assemble into large ribonucleoprotein complexes, termed RNA granules, containing cognate RNA-binding proteins, components of the translational machinery, and molecular motors. RNA granules are transported along microtubules to distal dendrites where the RNA is localized and translation is activated (Ainger et al., 1993, 1997; Barbarese et al., 1995; Carson et al., 1997; Hoek et al., 1998; Mouland et al., 2001). The A2RE sequence was initially identified in myelin basic protein (MBP) mRNA (Ainger et al., 1993, 1997), which is preferentially localized to dendrites and myelin in oligodendrocytes (Colman et al., 1982; Zeller et al., 1985; Verity and Campagnoni, 1988; Gillespie et al., 1990). Immunostaining of mature oligodendrocytes reveals that MBP expression is higher in the myelin compartment and lower in the perikaryon (Sternberger et al., 1978; Hartman et al., 1979; Roussel and Nussbaum, 1981; Hardy et al., 1996), implying that translation of MBP RNA is suppressed during transport and activated only when the granules reach the myelin compartment (Barbarese et al., 1995).

Translation of specific mRNAs can be regulated by trans-acting RNA binding proteins that bind to cis-acting sequences in the RNAs. During erythrocyte differentiation, translation of lipoxygenase mRNA is inhibited by hnRNP E1 and hnRNP K, which bind to the RNA through a repetitive cis-acting element, termed the differentiation control element (DICE) (Ostareck et al., 1997; Reimann et al., 2002). When hnRNP E1 and K bind to the DICE sequence, recruitment of the 60S ribosomal subunit is blocked, thereby inhibiting translation. In the final stages of reticulocyte differentiation, suppression of translation of lipoxygenase mRNA is relieved when hnRNP E1 and hnRNP K dissociate from the RNA (Ostareck et al., 2001). Either hnRNP E1 or hnRNP K is sufficient to inhibit translation of DICE-containing RNA in...
vitro. Binding of hnRNP E1 to folate receptor, renin, and collagen I mRNAs has the opposite effect, leading to stabilization and/or enhanced translation of the bound mRNAs (Xiao et al., 2001; Persson et al., 2003; Thiele et al., 2004).

Here, we have used a combination of in vitro and in vivo approaches to analyze hnRNP E1–hnRNP A2 interactions and their roles in A2RE mRNA trafficking in neural cells.

**MATERIALS AND METHODS**

**Yeast Two-Hybrid Analysis**

Yeast two-hybrid identification of binding partners for hnRNP A2 was performed as described previously (Kosturko et al., 2005). Full-length mouse cDNA encoding hnRNP A2 was used as bait to screen the Matchmaker Frettransformed human brain cDNA library from a 37-year-old male (BD Biosciences, Franklin Lakes, NJ). Clones were screened as described in Kosturko et al., (2005) and maintained as Escherichia coli transformants and also as pure DNA. DNA sequencing was performed by the University of Connecticut Health Center Molecular Core Facility (Farmington, CT) or commercially (Acgt, Wheeling, IL). The 5’-terminal sequences were used to identify genes by a BLAST search of GenBank. Clones of interest were sequenced to their 3’ ends.

**Coimmunoprecipitation of hnRNP E1 and hnRNP A2 Proteins**

hnRNP E1 and hnRNP A2 proteins were synthesized by in vitro transcription-translation using a rabbit reticulocyte lysate (Lytell) and [35S]methionine (Amersham). The full-length mouse cDNA encoding hnRNP A2 was used as bait to screen the Matchmaker Frettransformed human brain cDNA library. The hnRNP E1 clone identified by yeast two-hybrid analysis was cloned into pGEX-KG for expression in *Escherichia coli*. The fusion protein was purified by affinity chromatography on glutathione-Sepharose 4B (Pharmacia, Piscataway, NJ).

**Recombinant hnRNP A2 and hnRNP E1**

Full-length mouse cDNA for hnRNP A2 (residues 1-341) (Brumwell et al., 2002) and N-terminal (1-189) and C-terminal (189-341) fragments of hnRNP A2, full-length human hnRNP E1 (1-356) cDNA (obtained from Dr. Mathias Hentze, European Molecular Biology Laboratory, Heidelberg, Germany), and N-terminal (1-173) and C-terminal (173-356) hnRNP E1 fragments were amplified and cloned into the pET100/D-TOPo vector downstream of the hexahistidine-tag (Invitrogen, Carlsbad, CA). These constructs were used to transform *E. coli* BL21 (DE3) cells grown to an *A*~*opt*~ of 0.6 before induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside. Bacteria were lysed using a French press. Lysates were centrifuged at 12,000 × g for 20 min at 4°C, and supernatants were applied to a metal affinity column (Talon; BD Biosciences, Franklin Lakes, NJ). Clones were screened as described in Kosturko et al., (2005) and maintained as E. coli transformants and also as pure DNA. DNA sequencing was performed by the University of Connecticut Health Center Molecular Core Facility (Farmington, CT) or commercially (Acgt, Wheeling, IL). The 5’-terminal sequences were used to identify genes by a BLAST search of GenBank. Clones of interest were sequenced to their 3’ ends.

**In Vitro Transcription**

For localization studies, fluorescent RNA was prepared from template DNA (RTS11) consisting of the coding region of green fluorescent protein (GFP) with the 11-nucleotide A2RE sequence from MBP mRNA (previously called RFS; Ainger et al., 1997) inserted into the 3’ UTR. UTR. Alexa Fluor 546-conjugated dextran (Invitrogen) was coinjected into control B104 cells and with full-length recombinant hnRNP E1, hnRNP A2, or GFP-A2RE RNA. Alexa Fluor 546-conjugated dextran (Invitrogen) was coinjected to identify injected cells and to quantify the volume/relative amount of mRNA injected into each cell as described previously (Kwon et al., 1999). In all cases, the RNA concentration in the injection mix was ~100 ng/μl.

**Microinjection, Immunofluorescence, and Image Acquisition and Analysis**

Microinjection of cells plated on glass-bottomed dishes (MatTek, Ashland, MA) was performed using an Ultramicroinjection Apparatus (Ferdinand, Hamburg, Germany). Alexa Fluor 488-conjugated UTP-labeled RNA was injected into the cytoplasmic compartment. Injected cells were examined by confocal laser scanning microscopy after fixation with 3.7% formaldehyde and immunostaining with antibodies to hnRNP E1/E2. Cells were fixed for cytoskeleton (Cytoskeleton, Inc.) extraction using the method of Biegel and Pachter (1991), with minor modifications (Kosturko et al., 2005). Treated cells were fixed with formaldehyde, permeabilized with detergent, incubated with a blocking reagent and successively with primary and secondary antibodies. Mouse monoclonal antibody to hnRNP A2 (EF67) (used at 1:50 (Xiao et al., 2000) and 1:200 (Gammack and Andino, 1997) were generous gifts from Dr. Asok Antony (Indiana University, Bloomington, IN) and Dr. Raul Andino (University of California, San Francisco, San Francisco, CA, respectively). Both antibodies cross-react with both hnRNP E1 and hnRNP E2 because the proteins share 90% sequence homology (Lefers et al., 1995). Fluorescein-conjugated and rhodamine-conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA).

**In Vivo Translation and RNA Stability Assays**

In the first protocol, B104 cells were microinjected with cDNA containing the cytomegalovirus promoter and hnRNP E1 coding sequence to achieve hnRNP E1 overexpression and with capped and polyadenylated GFP RNA containing the A2RE element in its 3’ UTR (GFP-A2RE), or capped and polyadenylated GFP RNA containing the A2RE element in its 3’ UTR (GFP-A2RE). Capsid proteins A2RE and A2RE were included with full-length A2RE RNA, A2RE RNA, and GFP-A2RE RNA. Alexa Fluor 546-conjugated dextran (Invitrogen) was coinjected to identify injected cells and to quantify the volume/relative amount of mRNA injected into each cell as described previously (Kwon et al., 1999). In all cases, the RNA concentration in the injection mix was ~100 ng/μl.
In the second protocol, B104 cells were injected with capped and polyadenylated GFP-A2RE or GFP-A8G RNA with or without recombinant hnrNP E1 protein or BSA, and with Alexa Fluor 546-conjugated dextran. GFP-A2RE RNA and GFP-A8G RNA concentrations in the injection mix were 100 and 110 ng/μl, respectively. BSA and hnrNP E1 concentrations in the injection mix were 0.1 and 0.075 μg/μl, respectively.

In both protocols, fluorescence intensities of dextran and of GFP synthesized over a 6-h time period after injection of the RNA were monitored by dual channel confocal microscopy and quantified using Adobe Photoshop software (Adobe Systems, Mountain View, CA). For quantification of fluorescence intensities, a square frame of fixed dimensions was positioned over the area of the cell body, exclusive of the nucleus, to obtain two independent, nonoverlapping samples of fluorescence intensity for each cell. The mean intensity of pixels within the frame was calculated for each channel. Similarly, the frame was positioned over nonoverlapping areas outside the cell to obtain two independent measurements of background fluorescence intensity for each cell. The average value of background intensity was subtracted from the average value of cytoplasmic intensity for the green channel. The amount of GFP accumulated in each cell, expressed as 488-nm fluorescence intensity units, was plotted as a function of the amount of dextran (as a measure of injected volume of RNA) into that cell, expressed in 546-nm fluorescence intensity units.

To analyze stability of the injected RNA, a nuclear/cytoplasmic partition assay, based on the observations of Nikolov and Dabevo (1965), was used. B104 cells were injected with fluorescent cyamine (Cy)-5-UTP–labeled GFP-A2RE RNA with or without recombinant hnrNP E1 protein or RNAase A. GFP-A2RE RNA concentration in the injection mix was 50 ng/μl and that of hnrNP E1 and RNAase A, 0.06 μg/μl and 0.2 μg/μl, respectively. The nuclear and cytoplasmic distribution of fluorescent components corresponding to labeled nucleotides and intact RNA, respectively, was monitored by fluorescence confocal microscopy over a period of 6 h.

**In Vitro Translation Assay**

Capped and polyadenylated RNAs encoding GFP containing either A2RE or A8G sequences were prepared as described above. RNA (150 ng/Reaction) was translated using the Flexi RRL (Promega) and Transluc TRNA (Promega) from Promega (Madison, WI) according to the manufacturer’s instructions. In some experiments, the translation mixture also contained luciferase mRNA (250 ng/Reaction) provided with the kit as an internal control. In some cases, recombinant hnrNP A2 or recombinant hnrNP E1 was added to the reaction mixture. The reaction mixture was analyzed by Western blotting using anti-biotin antibody and horseradish peroxidase-conjugated secondary antibody. The intensities of the biotin-labeled GFP band and biotin-labeled luciferase bands were measured using Adobe Photoshop software.

**Fluorescence Correlation Spectroscopy (FCS)**

Full-length recombinant hnrNP A2 (1-341) and N (1-189) and C (189-341) subfragments of hnrNP A2 were conjugated with Alexa Fluor 488 tetrafluoro-orthophenyl ester (Invitrogen), which has an excitation maximum at 495 nm and emission maximum at 519 nm. Full-length recombinant hnrNP E1 (1-1100) and N (1-173) and C (173-356) subfragments of hnrNP E1 were conjugated with the megastokes dye DY-520XL succinimidyl ester (Dyomics, Jena, Germany), which has an excitation maximum at 520 nm and emission maximum at 570 nm. Free dye was removed by passing the conjugated proteins over a free column (Bio-Rad), precipitated with 7.5 M ammonium acetate, and washed with 70% ethanol.

**RESULTS**

**Yeast Two-Hybrid Analysis of hnrNP E1 Binding to hnrNP A2**

A yeast two-hybrid screen of a human brain cDNA library using hnrNP E1 as bait identified several different binding partners for hnrNP A2 (Kosturko et al., 2005). High-stringency conditions were used to select true positive clones. To distinguish between proteins that bind specifically to hnrNP A2, plasmids from the clones of interest were used to transform yeast containing one of three different plasmids: bait (hnrNP A2), vector alone, or false bait (laminin), and the number of ADE2 “HIS3” Lac z” cotransformants was scored. Positive clones (30 from the original 50) bound to the bait better than to vector alone or to false bait. These clones were analyzed further by restriction endonuclease digestion of the PCR-amplified insert. Clones with unique digestion patterns were subjected to sequence analysis and BLAST searches. One of the clones corresponded to a nearly full-length copy of heterogeneous ribonucleoprotein (hnrNP) E1, an RNA-binding protein containing three K-homology (KH) domains (accession no. BC039742). The protein encoded by this clone, E1-25, corresponds to hnrNP E1 lacking the N-terminal 25 amino acids, representing a portion of the first KH domain. The yeast two-hybrid analysis indicates that hnrNP E1 interacts with hnrNP A2 when both proteins are overexpressed in yeast nuclei.

**Coimmunoprecipitation of hnrNP E1 and hnrNP A2**

To determine whether hnrNP E1 interacts with hnrNP A2 in vitro, binding of hnrNP E1-25 protein to hnrNP A2

\[
K_d = \frac{[A]_0 - [A]_{obs}}{[A]_{obs} - [A]_{in}}
\]

where \([A]_0\) is the total concentration of molecule A, determined by autocorrelation analysis of data from channel A; \([A]_{obs}\) is the total concentration of molecule B, determined by autocorrelation analysis of data from channel B; and \([A]_{in}\) is the concentration of the AB complex, determined by cross-correlation analysis of data from channels A and B. As a positive control, the apparent \(K_d\) for binding of donkey anti-mouse IgG antibody (Alexa Fluor 488-labeled) to mouse IgG (Alexa Fluor 647-labeled) measured by cross-correlation FCS was ~5 nM (the range of \(K_d\) values for antigen-antibody binding is 10−7–10−11). As a negative control, the apparent \(K_d\) for binding of BSA (Alexa Fluor 647-labeled) to hnrNP A2 (Alexa Fluor 488-labeled) was ~500 nM.

Several limitations related to instrument sensitivity and reagent preparation can potentially reduce the extent of cross-correlation signal and therefore lead to underestimation of apparent \(K_d\) values measured by cross-correlation FCS. With our Confocor II instrument the average number of photons detected as each labeled molecule traverses the FCS observation volume is relatively small, particularly for the green channel, which means there is a significant probability that some labeled molecules will traverse the volume without being detected, leading to reduced cross-correlation and underestimation of binding affinities. The average number of fluorophores per labeled protein molecule is also relatively small (2–3), which means that a significant proportion of molecules will be unlabeled, reducing the extent of cross correlation and leading to underestimation of the binding affinity. If some molecules are denatured, degraded, or otherwise inactivated during purification or conjugation this may reduce the extent of cross correlation leading to underestimation of binding affinities. This may be particularly problematic in the case of the C terminus of hnrNP A2 where purification required denaturation and renaturation. Because the relative contributions of these various factors are difficult to determine, the binding affinities measured by dual channel cross correlation FCS should be considered “apparent” \(K_d\) values with the understanding that they may represent underestimates of the actual affinities. To determine whether hnrNP E1 interacts with hnrNP A2 for in vitro binding studies, sense GATC(GCCAAGGGACCC, and antisense GATC(GGCCTTCGCGC) oligonucleotides containing six A2RE sequences were methylated and cloned into the BamHI site of pLITMUS28 (New England Biolabs, Beverly, MA). One clone (pA2RE12) contained two copies of the insert in a tandem head to tail orientation. To generate A2RE RNA (285 nt) containing 12 A2RE sequences pA2RE12 DNA was linearized with Stul and transcribed with T7 polymerase (Epichem Technologies, Madison, WI). To generate control nonA2RE RNA (149 nt) pLITMUS28 without the insert was linearized with Stul and transcribed with T7 polymerase. Both reactions were performed in the presence of Alexa Fluor 488-5-UTP (Invitrogen), yielding labeled RNA products that were purified by MicroBio-Spin P-30 Tris RNase-free column (Bio-Rad), precipitated with 7.5 M ammonium acetate, and washed with 70% ethanol.

**HnRNP E1 Inhibits Translation**

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protein was analyzed by coimmunoprecipitation using proteins synthesized by in vitro translation. [35S]Methionine-labeled hnRNP A2-c-myc fusion protein synthesized in vitro was mixed with unlabeled hnRNP E1-25-HA (hnRNPE1 bearing an HA epitope) or with a positive control protein, hnRNP A2-HA synthesized in vitro. Previous yeast two-hybrid analysis indicated that hnRNP A2 can interact with itself (Cartegni et al., 1996; Kosturko et al., 2005). After immunoprecipitation with anti-HA antibody, the complexes were analyzed by gel electrophoresis and autoradiography. Figure 1A shows that radiolabeled hnRNP A2 coprecipitated with both hnRNP E1 and with hnRNP A2. This indicates that binding can be detected between relatively small amounts of hnRNP E1 and hnRNP A2 synthesized in an in vitro translation system. Binding must be relatively tight and the off rate relatively slow for the proteins to remain bound during the multiple washing steps in the coimmunoprecipitation procedure.

Western blotting of endogenous proteins present in the in vitro translation lysate (RRL) did not detect hnRNP A2 (our unpublished data) but did detect hnRNP E1 (Figure 6E). The latter did not prevent newly synthesized hnRNP E1 from binding to newly synthesized hnRNP A2. Indeed, endogenous hnRNP E1 in RRL did not bind hnRNP A2 in coimmunoprecipitation assay (Figure 6E). During the final stages of reticulocyte differentiation, suppression of translation of lipoygenase mRNA (which is mediated by hnRNPs E1 and hnRNPK) is relieved, suggesting that hnRNP E1 and/or hnRNPK in reticulocytes are modified in some manner preventing them from suppressing translation of lipoygenase mRNA (Ostareck-Lederer et al., 1996; Ostareck-Lederer and Ostareck, 2004). It is possible that such modifications prevent binding of RRL-endogenous hnRNP E1 to newly synthesized hnRNP A2.

To determine whether hnRNP E1 associates with hnRNP A2 in vivo, antibodies to hnRNP E1 were added to a solubilized cell homogenate of cultured oligodendrocytes. The control consisted of a sample in which normal rabbit serum was added to a similar aliquot of the same cell homogenate. Immune complexes and proteins associated with them were isolated by centrifugation after binding to protein A-conjugated agarose beads. Complexes were solubilized and subjected to SDS-PAGE and Western blotting (left and middle lanes). An aliquot of homogenate before any addition (right lane) was run on the same gel. The presence of hnRNP A2 in the cell homogenate and in the immune complexes was revealed with anti-hnRNP A2 and a secondary peroxidase conjugated antibody. Figure 1B shows that hnRNP A2 was present in homogenate (right lane), in anti-hnRNP E1 immune complexes (left lane), but not in normal rabbit serum complexes (middle lane). This suggests that oligodendrocyte-endogenous hnRNP E1 associates with hnRNP A2 in vivo, either directly or through an intermediate.

Sulfo-SBED Cross-linking/Biotin Transfer between hnRNP A2 and hnRNP E1

Demonstrating interaction between two proteins by yeast two-hybrid analysis and coimmunoprecipitation does not prove direct binding between the two proteins because both yeast nucleoplasm, oligodendrocyte cytoplasm and reticulocyte lysates contain many other proteins that could mediate indirect interaction. To determine whether hnRNP E1 binds directly to hnRNP A2, binding was analyzed using purified recombinant proteins and a cross-linking/biotin transfer assay (ProFound Sulfo-SBED; Pierce Chemical). HnRNP A2 was conjugated to Sulfo-SBED, an amine group cross-linking reagent that contains a thiol-cleavable disulfide linkage, a biotin moiety, and a photoreactive aryl azide group. Sulfo-SBED-conjugated hnRNP A2 was incubated with either full-length recombinant hnRNPE1 or BSA as a negative control. Sulfo-SBED–conjugated hnRNP A2 was UV cross-linked to its binding partners. Subsequent reduction of the disulfide bond in Sulfo-SBED results in transfer of the biotin moiety from hnRNP A2 to its binding partner. Biotin-labeled proteins were identified by SDS-PAGE and Western blotting using horseradish peroxidase-streptavidin. Figure 1C shows that in the reaction containing Sulfo-SBED–conjugated hnRNP A2 and full-length hnRNPE1, biotin is transferred...
molecules were determined by cross-correlation analysis of
Concentrations of bimolecular complexes containing both
terminated with DY-520XL. Total concentrations of individual
(1-173) or C (173-356) -terminal fragments of hnRNP E1,
488, was incubated with either full-length hnRNP E1 or N-
hnRNP E1, full-length hnRNP A2, labeled with Alexa Fluor
with Alexa Fluor 488. To identify hnRNP A2 binding sites in
bated with either full-length hnRNP A2 (1-341) or N- (1-189)
length hnRNP E1 (1-356), labeled with DY-520XL, was incu-
measured in this way (Table 1).

nant hnRNP A2 and subfragments of the two proteins were
bound proteins, as described in

molecules, whereas cross-correlation data provide a measure of
concentrations for each of the two differentially labeled mol-
ations will be detected in both channels. Cross-correlation
the FCS observation volume, correlated fluorescence fluctu-
ferentially labeled molecules can be analyzed simulta-
aneously by dual-channel FCS. If two differentially labeled
molecules are bound to each other as they diffuse through the
FCS observation volume, correlated fluorescence fluctu-
ations will be detected in both channels. Cross-correlation
analysis is used to measure the concentration of bimolecular
complexes containing both molecules. Thus, autocorrelation
data for each channel separately provides a measure of total
concentrations for each of the two differentially labeled mol-
eules, whereas cross-correlation data provide a measure of
the concentrations of bound proteins. The K_d for the inter-
action can be calculated from the concentrations of free and
bound proteins, as described in Materials and Methods. Bind-
ing affinities between recombinant hnRNP E1 and recombi-
hnRNP A2 and subfragments of the two proteins were
measured in this way (Table 1).

To identify hnRNP E1 binding sites in hnRNP A2, full-
length hnRNP E1 (1-356), labeled with DY-520XL, was incu-
bated with either full-length hnRNP A2 (1-341) or N- (1-189)
or C (189-341) -terminal fragments of hnRNP A2, labeled with Alexa Fluor 488. To identify hnRNP A2 binding sites in
hnRNP E1, full-length hnRNP A2, labeled with Alexa Fluor 488,
was incubated with either full-length hnRNP E1 or N-
(1-173) or C (173-356) -terminal fragments of hnRNP E1,
labeled with DY-520XL. Total concentrations of individual
labeled molecules in each reaction were determined by au-
tocorrelation analysis of data from individual channels.
Concentrations of bimolecular complexes containing both
molecules were determined by cross-correlation analysis of
data from both channels. Apparent K_d values determined for
each pairwise combination of differentially labeled mole-
cules are shown in Table 1.

Full-length hnRNP A2 binds to full-length hnRNP E1
(apparent K_d = 22 nM). HnRNP A2 contains two separate
binding sites for hnRNP E1, one site located in the N-
terminal portion of the molecule and one site located in the
C-terminal portion. Both the N- and C-terminal fragments of
hnRNP A2 bind more strongly to the N-terminal fragment of
hnRNP E1 than to the C-terminal fragment. Individual sub-
fragments of hnRNP A2 bind hnRNP E1 slightly tighter than
the full-length protein, indicating negative cooperativity be-
tween the two sites in the intact protein. Either the confor-
mation of full-length hnRNP A2 somehow reduces affinity
for binding of hnRNP E1 to the N- and C-terminal binding
sites or binding of hnRNP E1 to one site interferes with
binding to the other site.

HnRNP E1 also contains two separate binding sites for
hnRNP A2, a strong binding site located in the N-terminal
portion of the molecule and a weaker binding site located in the
C-terminal portion. Both subfragments of hnRNP E1 bind
more strongly to the C-terminal fragment of hnRNP A2
than to the N-terminal fragment. However, full-length
hnRNP E1 binds more strongly to the N terminus of hnRNP
A2 than do either the N-terminal or the C-terminal frag-
ments of hnRNP E1 alone, suggesting positive cooperativity
between the two hnRNP A2 binding sites in full-length
hnRNP E1. Binding of hnRNP A2 to one site may alter the
conformation of hnRNP E1 to facilitate binding to the second
site, or one hnRNP A2 molecule bound to one site may
dimerize with a second hnRNP A2 molecule bound to the
second site, decreasing the off rate for dissociation from
hnRNP E1. These results indicate that hnRNP A2 and
hnRNP E1 each have two potential interaction surfaces that
can mediate cooperative combinatorial molecular interac-
tions between the two proteins. Complexes between hnRNP
A2 and hnRNP E1 can form in different ways, depending on
which particular interaction surfaces are involved. Further-
more, each protein contains two separate binding sites for
the other protein, which means the stoichiometry of hnRNP
A2:hnRNP E1 complexes can vary from 1:2 to 2:1. Because
both hnRNP A2 and hnRNP E1 can also interact with other
molecular partners in vivo, binding of the two proteins in
live cells may be affected by other proteins if specific molec-
ular interaction surfaces of hnRNP A2 and/or hnRNP E1 are
occupied by alternative binding partners.

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<table>
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<th>Table 1. Apparent K_d values (nanomolar) for binding between hnRNP A2, and hnRNP E1</th>
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<tr>
<td></td>
<td>hnRNP E1 (1-356) (full)</td>
</tr>
<tr>
<td>hnRNP A2 (1-341) (full)</td>
<td>22 ± 2.1</td>
</tr>
<tr>
<td>hnRNP A2 (1-189) (N-terminal)</td>
<td>9 ± 1.3</td>
</tr>
<tr>
<td>hnRNP A2 (189-341) (C-terminal)</td>
<td>15 ± 3.4</td>
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Average K_d values were calculated from at least three separate
experiments for each pairwise combination of proteins. Full-length
hnRNP A2 and subfragments of hnRNP A2 were conjugated with
Alexa Fluor 488 and measured in channel A. Full-length hnRNP E1
and subfragments of hnRNP E1 were conjugated with DY-520XL
and measured in channel B.

Intracellular Concentrations of hnRNP A2 and hnRNP E1 in Oligodendrocytes

Because the apparent K_d value for binding of hnRNP E1 to
hnRNP A2 in vitro is 22 nM, we measured the concentra-
tions of hnRNP E1 and hnRNP A2 in oligodendrocytes to
determine whether the proteins are present in high enough
concentrations to interact with each other in vivo. Quantita-
tive Western blotting was performed by comparing the in-
tensities of the hnRNP E1 and hnRNP A2 bands for known
numbers of oligodendrocytes with band intensities for

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known amounts of purified recombinant hnRNP E1 and
hnRNP A2 proteins. A similar analysis of hnRNP E1 and
hnRNP A2 in B104 cells showed that they contain twice
the amount of hnRNP E1 and half the amount of hnRNP
A2 compared with oligodendrocytes. The concentrations
of each protein in the nucleus and cytoplasm of oligodendro-
cytes were calculated based on immunofluorescence
staining of the two proteins and estimates of nuclear and
cytoplasmic volumes. The results (Table 2) indicate that
concentrations of hnRNP E1 and hnRNP A2 in the cyto-
plasm of oligodendrocytes are in excess over the K_d for
binding, which means that binding between hnRNP E1
and hnRNP A2 can occur in the cytoplasm of oligoden-
drocytes.

### Table 2. Nuclear and cytoplasmic hnRNP A2 and hnRNP E1 in
oligodendrocytes

<table>
<thead>
<tr>
<th>Molecules/cell</th>
<th>Nuclear conc. (μM)</th>
<th>Cytoplasmic conc. (μM)</th>
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<tbody>
<tr>
<td>hnRNP A2^d</td>
<td>102 × 10^6</td>
<td>117</td>
</tr>
<tr>
<td>hnRNP E1^e</td>
<td>1.56 × 10^6</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

^a Measured by quantitative Western blotting with known numbers
of cells and recombinant hnRNP A2 and hnRNP E1 as standards.
^b Nuclear volume of an oligodendrocyte was estimated to be 1 pl.
^c Cytoplasmic volume of an oligodendrocyte was estimated to be 9 pl.
^d Nuclear:cytoplasmic partition coefficient for hnRNP A2 is 20:1
(determined by immunofluorescence confocal microscopy and sub-
cellular fractionation).
^e hnRNP E1 was not detected in the nucleus of oligodendrocytes by
immunofluorescence and confocal microscopy.

Because hnRNP E1 binds to hnRNP A2, which binds to
A2RE RNA, it is possible that hnRNP E1 is recruited to
A2RE RNA through binding to hnRNP A2. To test this
possibility, recombinant hnRNP E1 labeled with Alexa Fluor
647 was incubated in the presence or absence of unlabeled
recombinant hnRNP A2 with a synthetic RNA containing 12
repeats of the A2RE sequence or a control non-A2RE RNA
labeled with Alexa Fluor 488. Recruitment of hnRNP E1 to
RNA was measured by cross-correlation FCS (Figure 2).
With non-A2RE RNA a low amount of nonspecific hnRNP
E1 binding was detected with or without unlabeled hnRNP
A2. With A2RE RNA hnRNP E1 recruitment was signifi-
cantly enhanced in the presence of hnRNP A2, indicating
that hnRNP E1 is recruited to A2RE RNA through binding
to hnRNP A2. The binding curve seems sigmoidal and was
fitted to the Hill equation with apparent K_d = 14.3 and Hill
coefficient b = 4.7. The apparent K_d for hnRNP E1 recruit-
ment to A2RE RNA in the presence of hnRNP A2 is com-
parable to the apparent K_d for binding of hnRNP E1 to
hnRNP A2, suggesting that hnRNP E1 is recruited to A2RE
RNA through binding to hnRNP A2 bound to A2RE se-
quencies. Positive cooperativity indicated by the Hill coeffi-
cient may reflect cooperative binding of hnRNP E1 to N- and
C-terminal binding sites on hnRNP A2 or may be an assay-
specific phenomenon reflecting the fact that the test RNA
contains reiterated A2RE sequences and thus can bind mul-
tiple hnRNP A2 molecules, each of which can bind hnRNP
E1. Homotypic interactions between adjacent hnRNP E1
molecules recruited to the RNA could also contribute to
positive cooperativity.

### Colocalization of hnRNP E1 and hnRNP A2 in Granules
in Oligodendrocytes

The binding studies described above indicate that hnRNP
E1 can bind to hnRNP A2 in vitro and in vivo. To deter-
mine whether the proteins interact in neural cells, colo-
calization of hnRNP E1 and hnRNP A2 was analyzed in
oligodendrocytes. Both hnRNP E1 and hnRNP A2 shuttle
between the nucleus and the cytoplasm (Dreyfuss et al.,
2002), and their interaction could potentially take place in
either compartment. Immunostaining of oligodendrocytes
was performed using a monoclonal anti-hnRNP A2 anti-
body and polyclonal anti-hnRNP E1 antibodies. The anti-
hnRNP E1 antibodies cross-react with both hnRNP E1 and
hnRNP E2 because these proteins are 90% homologous
(Leffers et al., 1995; Gamarnik and Andino, 1997; Xiao
et al., 2001). Immunostaining for hnRNP E1/E2 showed a
punctate pattern throughout the soma and dendrites with
little staining of the nucleus (Figure 3B). In contrast,
hnRNP A2 was most abundant in the nucleus with punc-
tate staining in the soma and dendrites (Figure 3A; Brum-
well et al., 2002). Furthermore, although hnRNP A2-posi-
tive puncta were distributed uniformly throughout the
oligodendrocyte dendrites, hnRNP E1/E2-positive puncta
showed a nonuniform distribution with higher density of
puncta in the cell body and proximal dendrites and lower
density in distal dendrites.

To determine whether hnRNP E1/E2 was colocalized with
hnRNP A2 in granules, coimmunostaining for both proteins
was performed using differential fluorophores for
their detection. Oligodendrocytes were first exposed to an
extraction buffer to remove non-CSK–associated compo-
nents (Biegel and Pachter, 1991). A high-magnification view
of a distal portion of a dendrite (Figure 3B, inset) shows
three populations of granules: granules containing both
hnRNP E1/E2 and hnRNP A2, granules containing only hnRNP A2, and granules containing only hnRNP E1/E2. Single granule ratiometric analysis of clearly resolved granules in the dendrites (Figure 3C) indicates that hnRNP E1/E2 (green intensity) and hnRNP A2 (red intensity) are colocalized in ~40% of the granules (>6700 granules analyzed in four independent experiments). The hnRNP A2+/hnRNP E1/E2− granule population and the hnRNP E1/E2+/hnRNP A2− granule population constituted 27 and 33%, respectively, of the total population of granules analyzed. Colocalization of hnRNP A2 and hnRNP E1/E2 in the cell body could not be analyzed because individual granules were not resolved in this compartment due to their high density. These data indicate that a subpopulation of hnRNP A2 granules in proximal and medial dendrites also contains hnRNP E1/E2.

Colocalization in the same granules does not provide evidence for direct interaction between hnRNP A2 and hnRNP E1. However, because in vitro analysis indicates that hnRNP A2 binds to hnRNP E1 it is likely that colocalization of hnRNP A2 and hnRNP E1 in the same granules is mediated by direct binding between the two proteins (see Discussion).

### Colocalization of hnRNP E1 with A2RE mRNA in Granules in Oligodendrocytes

In oligodendrocytes, ~90% of hnRNP A2-positive granules located in dendrites contain A2RE RNAs (Kosturko et al., 2005). To determine whether A2RE RNA granules also contain hnRNP E1/E2, fluorescently labeled A2RE RNA (546-nm fluorescence intensity; Figure 3D) was injected into the cell body and allowed to assemble into granules before fixing and staining for hnRNP E1/E2 (detected by 488-nm fluorescence intensity of the secondary antibody; Figure 3E). The inset in Figure 3E shows a medial portion of a dendrite where the majority of granules contains A2RE RNA and hnRNP E1/E2. Single granule ratiometric analysis of cells injected with A2RE RNA (Figure 3D) and immunostained for hnRNP E1/E2 (Figure 3E) indicates that 50% of A2RE RNA granules contain hnRNP E1/E2 (Figure 3F) (7000 granules analyzed in three independent experiments). These data indicate that hnRNP E1/E2 is associated with a subset of...
A2RE RNA granules, presumably recruited by binding to hnRNP A2.

**Translation of A2RE mRNA Is Inhibited by hnRNP E1 In Vivo**

Translation was analyzed in vivo using two protocols involving coinjection of GFP RNA into B104 neuroblastoma cells along with fluorescent dextran as a marker for the amount of RNA injected into each cell. The ratio of injected RNA to synthesized GFP provides a measure of translational efficiency. Because the amount of RNA injected into each cell is variable, translational efficiency can be measured over a range of RNA concentrations, which allows for determination of dose response and threshold or saturation effects due to under- or overexpression, respectively. Significant variability in translational efficiency was observed among different cells, which may reflect differences in the injection site or physiological differences among cells. However, if enough cells are analyzed this experimental approach can provide more information about translational efficiency in individual cells than conventional transfection/overexpression assays for in vivo translation.

In the first protocol, control B104 cells were injected with GFP-A2RE RNA, which contains a specific hnRNP A2 binding site (the A2RE sequence), or with GFP-A8G RNA, which differs from A2RE RNA by a single base change in the A2RE, eliminating specific hnRNP A2 binding. Cells injected with both RNAs exhibited comparable translational efficiencies over a range of RNA concentrations (Figure 4, A and C, respectively). In B104 cells overexpressing hnRNP E1, synthesis of GFP from GFP-A2RE RNA was reduced (Figure 4B), but synthesis of GFP from GFP-A8G RNA was not affected (Figure 4D). These data indicate that hnRNP E1 inhibits translation of A2RE RNA, presumably by binding to hnRNP A2.

In the second protocol, B104 cells were injected with GFP-A2RE RNA and hnRNP E1 cDNA, or BSA as a control. In cells injected with GFP-A2RE RNA and BSA, GFP was synthesized normally (Figure 5A). However, in cells injected with GFP-A2RE RNA and hnRNP E1, GFP synthesis was reduced (Figure 5B). The ratio of GFP:RNA (0.08) was 10-fold less than in control cells, indicating that hnRNP E1 inhibits translation of A2RE RNA.

In cells injected with GFP-A8G RNA and BSA (Figure 5C) or hnRNP E1 (Figure 5D) synthesis of GFP was comparable under both conditions. The ratio of GFP:RNA was 0.7 for cells injected with GFP-A8G RNA in the presence of hnRNP E1. These results indicate that hnRNP E1 inhibits translation of A2RE RNA but does not affect translation of A8G RNA. Because the A8G mutation interferes with binding of hnRNP E1 (Munro et al., 1999) and also prevents translation inhibition by hnRNP E1, this suggests that translation inhibition by hnRNP E1 requires hnRNP A2 binding.

To determine whether reduced GFP expression in the presence of hnRNP E1 was due to RNA degradation, we used a nuclear/cytoplasmic partition assay based on the observation that intact RNA is excluded from the nucleus, whereas products of RNA degradation diffuse into the nucleus (Nikolov and Dabeva, 1985; Ifrim, personal communication). Fluorescently tagged GFP-A2RE RNA injected with hnRNP E1 (Figure 5E, b and e) and without hnRNP E1 (Figure 5E, a and d) into the cytoplasm of B104 cells was...
excluded from the nucleus up to 6 h after injection, indicating that hnRNP E1 does not increase degradation of GFP-A2RE RNA. In both cases, the ratio of nuclear to cytoplasmic fluorescence intensity (0.39–0.55) did not vary significantly during the 6-h period (Figure 5F), but it was different from that of cells injected with RNA alone and RNAse A (1.5–2.3). In that case, fluorescence accumulated in the nucleus (Figure 5E, c and f), indicating that the RNA was degraded by RNAse. These data indicate that reduced GFP expression in the presence of hnRNP E1 is not due to RNA degradation.

Figure 5. Effect of added hnRNP E1 on in vivo translation. Control B104 cells were coinjected with A2RE-GFP RNA and BSA (A), GFP-A2RE RNA and hnRNP E1 (B), GFP-A8G RNA and BSA (C), and GFP-A8G RNA and hnRNP E1 (D). All RNAs were polyadenylated, and all injection mixtures contained Alexa Fluor 546-conjugated dextran. Newly synthesized GFP was visualized in the green channel, and Alexa Fluor 546-conjugated dextran in the red channel. (D) Average intensity in each channel was determined and plotted for each cell. Each dot represents one cell. A representative experiment is shown were 43, 52, 36, and 28 cells in which analyzed in A, B, C, and D, respectively. B104 cells were injected with Cy-5–labeled GFP-A2RE RNA (E, a and d), Cy-5–labeled GFP-A2RE RNA and hnRNP E1 (E, b and e), and Cy-5–labeled GFP-A2RE and RNAse A (E, c and f), and imaged 30 min (E, a–c) and 6 h (E, d–f) after injection. The ratio of the average fluorescence intensity in the nucleus and the cytoplasm was calculated (F). The nuclear/cytoplasmic ratio of cells injected with RNA alone and RNA with hnRNP E1 was significantly smaller than that of cells injected with RNA and RNAse at both time points. Significance was assessed at p < 0.01.

Inhibition of A2RE RNA Translation In Vitro by hnRNP E1 Is hnRNP A2 Dependent

To determine whether inhibition of translation by hnRNP E1 requires hnRNP A2, GFP-A2RE or GFP-A8G RNA was translated in vitro using an RRL system in the presence or absence of hnRNP A2 and increasing amounts of hnRNP E1 (Figure 6). The amount of RNA (150 ng) in each reaction was within the linear dose-response range (previously determined) to facilitate analysis of differences in translation efficiency (Kwon et al., 1999). The concentration range of re-
combinant hnRNP E1 tested was comparable to that used by Ostareck et al. (1997) in a similar assay. In the absence of hnRNP A2, hnRNP E1 had no effect on translation of GFP-A2RE RNA (Figure 6A). In the presence of hnRNP A2 (100 nM), increasing concentrations of hnRNP E1 inhibited translation. Inhibition of translation reached a maximum of ~50% inhibition at 600 nM hnRNP E1 (Figure 6, B and D). Translation of a control RNA, luciferase (luc) RNA, was not significantly affected by the combined presence of hnRNP A2 and hnRNP E1 (Figure 6D) or by the combined presence of GFP-A2RE RNA, hnRNP A2, and hnRNP E1 (Figure 6B, double panel). When GFP-A8G RNA, which lacks specific binding to hnRNP A2 (Munro et al., 1999), was used in the assay in the presence of hnRNP A2, translation was not affected by hnRNP E1 (Figure 6C). Translation of luciferase RNA in the presence of GFP-A8G RNA, hnRNP A2 and increasing amounts of hnRNP E1 remained constant (our unpublished data). RRL contains protein(s) that are recognized by antibodies to hnRNP E1/E2 and that have the apparent molecular weight of hnRNP E1 (Figure 6 E, lane 1). Immunoreactive hnRNP E1/E2 present in RRL did not precipitate with anti-hnRNPs A2 antibodies in the absence or presence of recombinant hnRNP A2 (rhnRNP A2) (Figure 6E, lanes 3 and 4, respectively). In contrast, recombinant hnRNP E1 (rhnRNP E1) (Figure 6D, lane 2) coprecipitated with anti-hnRNP A2–hnRNP A2 complexes when added to RRL (Figure 6E, lane 5) or when in a saline solution with hnRNP A2 (Figure 6E, lane 6). These data suggest that hnRNP E1-like protein in RRL does not associate with hnRNP A2 and does not contribute to hnRNP A2-dependent inhibition of translation of A2RE RNA.

**DISCUSSION**

The results presented here indicate that hnRNP E1 binds to hnRNP A2 in vitro and is colocalized with hnRNP A2 and A2RE RNA in granules in oligodendrocytes. Sequence-specific binding of hnRNP A2 to A2RE RNA mediates recruitment of hnRNP E1, which inhibits translation of A2RE RNA in an hnRNP A2-dependent manner in vitro and in vivo.

Molecular interactions of hnRNP A2 and hnRNP E1 are complex and may involve quaternary structure. Both proteins exhibit homotypic as well as heterotypic interactions (Kim et al., 2000). The sequence of hnRNP E1 is 90% homologous to hnRNP E2, which must be present in multimeric form to bind to poliovirus RNA efficiently and permit cap-independent translation (Bedard et al., 2004). Our finding that hnRNP E1 binds to hnRNP A2 is consistent with the known properties of both proteins (Kamma et al., 1999; Makeyev and Liebhaber, 2002). HnRNP E1 and hnRNP E2 both contain three hnRNP KH domains. The N-terminal portion of hnRNP E2 (consisting of the first two KH domains and the two exons after the second KH domain) is necessary for dimerization and interaction with other hnRNPs (Kim et al., 2000). Our finding that hnRNP E1 binds to hnRNP A2 is consistent with the known properties of both proteins (Kamma et al., 1999; Makeyev and Liebhaber, 2002). HnRNP E1 and hnRNP E2 both contain three hnRNP KH domains. The N-terminal subfragment of hnRNP E1 contains a high-affinity binding site for hnRNP A2 and is consistent with a dimerization role for KH domains in hnRNP E1. Despite their sequence homology, hnRNP E1 and E2 have distinct functions and therefore may display different binding properties (Zhu et al., 2002).
Direct binding can be analyzed by a variety of fluorescence techniques, including fluorescence resonance energy transfer, bimolecular fluorescence complementation, and cross-correlation FCS. Each of these techniques involves analysis of binding interactions between exogenous fluorescent molecules injected or expressed in live cells. However, if intracellular concentrations of unlabeled endogenous proteins are high, fluorescently labeled exogenous molecules will interact predominantly with unlabeled endogenous molecules, thereby reducing the interactions of fluorescent molecules. Because intracellular concentrations of endogenous hnRNP A2 are extremely high, direct binding between hnRNP A2 and hnRNP E1 may be difficult to detect in live cells by fluorescent techniques.

Interaction of hnRNP E1 with hnRNP A2 seems to occur in cytoplasm where both proteins are colocalized in granules. Both proteins are believed to shuttle between the cytoplasm and the nucleus (Dreyfuss et al., 2002), but hnRNP E1/E2 was not detected in the nucleus of oligodendrocytes by immuno-fluorescence. This result is in accord with the observation of Gamarnik and Andino (1997) who did not detect hnRNP E1 in nuclei of HeLa cells, but contrary to that of Chkheidze and Liebhaber (2003) who did detect hnRNP E1 in the nucleus of the same cells and could not reconcile their observation with that of Gamarnik and Andino (1997). A study by Zhu et al., (2002) in brain sections reported that in neurons hnRNP E1 and E2 were detectable in the cytoplasm but not the nucleus. The subcellular distribution of hnRNP E1 may depend on its phosphorylation state, as reported for hnRNP K, which shifts from the nucleus to cytoplasm upon phosphorylation (Habelhah et al., 2001).

By analogy with other KH domain proteins (hnRNP K and ZBP), the function of hnRNP E1 may depend on its state of phosphorylation. Phosphorylation of hnRNP K and ZBP is mediated by c-src and derepresses translation of specifically silenced mRNAs by inhibiting binding of hnRNP K to the DICE sequence and ZBP to zip code sequence, respectively, in inhibited mRNAs (Ostareck-Lederer et al., 2002; Hüttermayer et al., 2005). HnRNP E1 also exists in a phosphorylated form in vivo. The in vitro binding of the phosphorylated form to poly(C) is significantly less than that of its non-phosphorylated form (Leffers et al., 1995). It is not known whether homotypic or heterotypic interactions of hnRNP E1 are affected by phosphorylation state. Coimmunoprecipitation data indicate that endogenous hnRNP E1 in RRL does not bind hnRNP A2 while recombinant hnRNP E1 does. These differences may be due to posttranslational modifications.

The mechanism of hnRNP A2-dependent inhibition of translation by hnRNP E1 is not known. Binding of hnRNP E1 and hnRNP K to the DICE sequence of 15-lipoxygenase mRNA inhibits translation by blocking recruitment of the 60S ribosomal subunit (Ostareck et al., 2001). Our data support a model in which hnRNP E1 is recruited to A2RE RNA by binding to hnRNP A2, which in turn is bound to the A2RE sequence. The A2RE RNA used in this study (GFP-A2RE RNA) does not contain a DICE sequence, but hnRNP E1 bound to hnRNP A2 may inhibit translation by a similar mechanism. Alternatively, hnRNP E1 could inhibit translation of A2RE RNA by inhibiting the cap-dependent translation enhancer function of hnRNP A2 (Kwon et al., 1999). However, this cannot explain the full extent of inhibition, because translation of GFP-A2RE RNA in the presence of hnRNP E1 and hnRNP A2 is less than with hnRNP E1 alone, indicating that inhibition of translation of A2RE RNA by hnRNP E1 and hnRNP A2 is greater than stimulation of translation by hnRNP A2 alone.

The extent of inhibition of translation by hnRNP E1 was greater in vivo than in vitro. Inhibition of translation of A2RE RNA in vitro reached a maximum of 50% at 600 nM hnRNP E1. Increasing the concentration of hnRNP E1 further did not result in greater inhibition. In comparison, injection or overexpression of hnRNP E1 in B104 cells inhibited translation of A2RE RNA almost completely. One possible explanation for this difference is that the concentration of hnRNP A2 (100 nM) added to the reticulocyte lysate used for in vitro translation may be subsaturating relative to the concentration of A2RE RNA (100 nM), so that some RNA molecules may not be associated with hnRNP A2 or may not have the necessary complement of hnRNP A2. Concentrations of hnRNP A2 (100 nM) and hnRNP E1 (100–1200 nM) in the in vitro assay were both greater than their Kd for binding (22 nM) in vitro. Translation inhibition was proportional to hnRNP E1 concentration up to a maximum at 600 nM, which represents a sixfold molar excess of hnRNP E1 over hnRNP A2. This suggests that either multiple hnRNP E1 molecules bind to each hnRNP A2 molecule or the in vitro translation lysate contains other components that compete with hnRNP A2 for binding to hnRNP E1. The simplest explanation is that translation in vitro is unaffected or enhanced when hnRNP A2 alone binds to A2RE RNA but inhibited when hnRNP A2::hnRNP E1 complex binds to A2RE RNA. Thus, the extent of translation inhibition is determined by the amount of hnRNP A2::hnRNP E1 bound to each A2RE RNA molecule.

Translation of A2RE RNA in vivo was almost completely inhibited by coinjecting exogenous hnRNP E1 along with the RNA. The concentration of hnRNP E1 in the injection needle was 2 μM. If the volume injected is ~1/10 of the total cytoplasmic volume, the final intracellular concentration of injected hnRNP E1 will be 1/10 the concentration in the needle (~200 nM). This means that the total concentration of endogenous plus injected hnRNP E1 in the cytoplasm of injected cells (~0.5 μM) would be increased almost twofold compared with the concentration in control cells and would be comparable to the concentration of recombinant hnRNP E1 necessary to achieve 50% inhibition in the in vitro translation assay. Total cytoplasmic concentration of hnRNP A2 in control and injected cells (5.9 μM compared with 100 nM in the in vitro translation assay) is in excess over hnRNP E1 if it is all available for binding exogenous hnRNP E1. Availability of hnRNP A2 and hnRNP E1 in the neural cell cytoplasm or the reticulocyte lysate is not known, but it is probably different in the two systems, because the composition of the reticulocyte lysate differs from the composition of neural cell cytoplasm in terms of types and concentrations of molecular species present. For example, RNA binding proteins such as La autoantigen, hnRNP A1, and hnRNP A2 are either absent or present in very small amounts in reticulocyte lysate relative to mammalian nucleated cells (Meerovitch et al., 1993; Svitkin et al., 1996; this study), and their methylation or phosphorylation patterns may be different. Thus, the difference in extent of inhibition of translation in vitro and in vivo probably reflects molecular differences between reticulocyte lysate and B104 cell cytoplasm.

In other systems, inhibition of translation by hnRNP E1 and hnRNP K is reversible upon phosphorylation of hnRNP K by c-src kinase (Ostareck-Lederer et al., 2002). However, hnRNP E1 is not a substrate for c-src kinase. The recombinant hnRNP E1 used in this study presumably does not contain posttranslational modifications, but it does inhibit translation of A2RE RNA, suggesting that unphosphorylated hnRNP E1 may be the active form in translation inhibition. It is not known whether other posttranslational mod-
ifications of hnRNP E1 affect its binding to hnRNP A2 or its role in translation regulation. Translation of A2RE RNA may be activated by phosphorylation of hnRNP E1 or removal of hnRNP E1 from the RNA granules by some other mechanism. The relative paucity of hnRNP E1-containing granules in the distal dendrites suggests that hnRNP E1 is released from RNA granules when they become localized in the myelin compartment. Thus, the mechanism for localization dependent translation of A2RE RNA in oligodendrocytes may involve dissociation of hnRNP E1 from RNA granules.

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