

## Mutational Analysis of a Heterogeneous Nuclear Ribonucleoprotein A2 Response Element for RNA Trafficking\*

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Cytoplasmic transport and localization of mRNA has been reported for a range of oocytes and somatic cells. The heterogeneous nuclear ribonucleoprotein (hnRNP) A2 response element (A2RE) is a 21-nucleotide segment of the myelin basic protein mRNA that is necessary and sufficient for cytoplasmic transport of this message in oligodendrocytes. The predominant A2RE-binding protein in rat brain has previously been identified as hnRNP A2. Here we report that an 11-nucleotide subsegment of the A2RE (A2RE11) was as effective as the full-length A2RE in binding hnRNP A2 and mediating transport of heterologous RNA in oligodendrocytes. Point mutations of the A2RE11 that eliminated binding to hnRNP A2 also markedly reduced the ability of these oligoribonucleotides to support RNA transport. Oligodendrocytes treated with antisense oligonucleotides directed against the translation start site of hnRNP A2 had reduced levels of this protein and disrupted transport of microinjected myelin basic protein RNA. Several A2RE-like sequences from localized neuronal RNAs also bound hnRNP A2 and promoted RNA transport in oligodendrocytes. These data demonstrate the specificity of A2RE recognition by hnRNP A2, provide direct evidence for the involvement of hnRNP A2 in cytoplasmic RNA transport, and suggest that this protein may interact with a wide variety of localized messages that possess A2RE-like sequences.

Vectorial transport and localized translation of mRNA in the cytoplasm affords a mechanism for establishing an asymmetric distribution of cytosolic proteins in cells. This is particularly important in oogenesis and embryonic development (1–8). In somatic cells, some mRNAs are also transported to discrete locations within the cell. These include myelin basic protein (MBP)<sup>1</sup> mRNA, which is localized in the myelinating periphery

of oligodendrocytes (9–11), microtubule-associated protein 2A (MAP2A) and tau, which are localized in neurites (12–14), and  $\beta$ -actin mRNA, which is localized in the leading edge of fibroblasts (15–17).

An active, multistep, cytoplasmic transport pathway has been delineated for MBP mRNA in cultured oligodendrocytes. MBP transcripts microinjected into the soma assemble into granules that move out along the myelin-forming processes (18, 19). Transport of these granules is dependent on the presence of kinesin and intact microtubules (20). Similar RNA-rich granules have been observed in other cells (21–24).

A *cis*-acting sequence sufficient and necessary for MBP mRNA transport has been identified within a 21-nucleotide stretch of the 3'-untranslated region. Incorporation of this segment, the hnRNP A2 response element (A2RE, formerly termed the RNA transport sequence (25)), into heterologous RNAs mediates transport into the processes and also enhances translation.<sup>2</sup> A2RE-like sequences are present in other localized mRNAs including mouse MAP2A, mouse protamine 2, and rat glial fibrillary acidic protein (25), suggesting that the A2RE represents a general signal for RNA transport.

Earlier we used biotin-labeled oligoribonucleotides containing the A2RE to isolate proteins that bind specifically to this RNA sequence (26). Affinity matrices were created by attaching these oligoribonucleotides to streptavidin-labeled magnetic particles, which were added to subcellular protein fractions prepared from a variety of rat tissues. The principal proteins bound to immobilized A2RE, but not to randomized sequences, were hnRNP A2 (26) and two isomers of hnRNP A3.<sup>3</sup> Subsequent binding experiments with purified protein showed that hnRNP A3 had little affinity for A2RE in the absence of hnRNP A2 (26).

The initial aim of the work communicated here was to define the nucleotides within the A2RE that are essential for binding to hnRNP A2 and to determine if other nonoligodendroglial, localized messages that possess A2RE-like elements also bind hnRNP A2. These experiments led to the identification of an 11-base subsequence of the A2RE that bound to hnRNP A2 as strongly as the A2RE and to the observation that many single nucleotide changes within this sequence greatly diminished this interaction. The strength of the RNA-hnRNP A2 interaction was directly correlated with the efficiency of cytoplasmic RNA transport in oligodendrocytes. Antisense oligonucleotide treatment of oligodendrocytes caused a reduction in hnRNP A2 levels and a concomitant decrease in cytoplasmic transport of MBP RNA providing additional evidence for the involvement of this protein in RNA transport. A2RE-like sequences from neuronal mRNAs were shown to both bind hnRNP A2 and support

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<sup>1</sup> The abbreviations used are: MBP myelin basic protein; GFP, green fluorescent protein; A2RE, hnRNP A2 response element; hnRNP, heterogeneous nuclear ribonucleoprotein; MAP2A, microtubule-associated protein 2A; ARC, activity-related cytoskeleton-associated protein; MOBP, myelin-associated/oligodendrocyte basic protein; PIPES, piperazine-*N,N'*-bis(2-ethansulphonic acid); HPLC, high pressure liquid chromatography; dig, digoxigenin.

<sup>2</sup> Kwon, S., Barbarese, E., and Carson, J. H. (1999) *J. Cell Biol.* **147**, 247–256.

<sup>3</sup> K. S. Hoek and R. Smith, unpublished data.

cytoplasmic transport of RNA in oligodendrocytes, suggesting that other mRNAs may also be transported by a mechanism involving hnRNP A2.

#### EXPERIMENTAL PROCEDURES

**Mutant Oligoribonucleotide Synthesis and Characterization**—Oligoribonucleotides were obtained from Oligos Etc, Inc. (Wilsonville, OR). All incorporated 3'-linked biotin to permit attachment to streptavidin-conjugated superparamagnetic particles (Roche Molecular Biochemicals). These oligoribonucleotides were purified by the supplier, but further analysis was performed to establish that they contained no free biotin and could bind equally to the magnetic particles. In most experiments the magnetic particles were saturated with the biotinylated RNA with the presence of excess, unbound RNA confirmed by agarose gel electrophoresis of the supernatants. Significant variations in biotinylation were also excluded by two other methods. First, dot blots of equivalent amounts of RNA, as assessed by their absorbance at 260 nm, were developed with streptavidin-conjugated alkaline phosphatase (Sigma). Second, equal amounts of the biotinylated oligoribonucleotides were added to equivalent aliquots of magnetic particles, and the excess RNA was washed away. The particles were then treated with RNase-free proteinase K to hydrolyze the streptavidin, and the released RNA was purified by phenol-chloroform-isoamyl alcohol extraction, concentrated by vacuum centrifugation, and electrophoresed on a 4% agarose, 2.2 M formaldehyde gel. No significant variations in the amounts of the ethidium bromide-stained RNAs were evident.

**hnRNP A2 Isolation**—Rat brain protein extracts were prepared as described previously (26). Human hnRNP A2 was expressed in *Escherichia coli* BL21(DE3) by using a plasmid (based on pET-9c) kindly supplied by Dr. A. Krainer (Cold Spring Harbor Laboratories). Transformed cells were induced with isopropyl- $\beta$ -D-thiogalactopyranoside, harvested by centrifugation, and lysed in 50 mM Tris-HCl, pH 8, containing 2 mM EDTA, 100  $\mu$ g/ml lysozyme, and 0.1% Triton X-100. The soluble fraction was dialyzed against Buffer A (50 mM Tris-HCl, pH 8.5, 0.2 mM EDTA, 5% w/v glycerol) before concentrating 10-fold by chromatography on DEAE-cellulose (Whatman, United Kingdom) and one step elution with 0.5 M NaCl in the above buffer. The concentrated solution was then loaded on a Sephacryl S-300 column (Amersham Pharmacia Biotech). Fractions containing hnRNP A2 were purified using a linear 10–50% acetonitrile gradient in 0.1% trifluoroacetic acid on a C4 reverse-phase HPLC column (Vydac, Hesperia, CA). Isolated hnRNP A2 was stored in low ionic strength solutions to avoid precipitation. Oligoribonucleotide-labeled superparamagnetic particles were used for isolation of RNA-binding proteins as described previously (26).

**RNA Protein Cross-linking**—Rat brain proteins to be used in UV cross-linking experiments were removed from A2RE-linked magnetic particles by incubation in 300 mM MgCl<sub>2</sub> for 1 h at 4 °C. Eluted proteins were dialyzed against 10 mM HEPES, pH 7.5, containing 40 mM NaCl and 5% glycerol. Equimolar amounts of oligoribonucleotide, <sup>32</sup>P-labeled with T4 polynucleotide kinase (New England Biolabs, Beverly, MA), and protein were mixed in binding buffer (10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 4% w/v glycerol, 0.1% w/v Triton X-100, and 1 mM dithiothreitol) and incubated for 30 min on ice. The reaction mixtures were then irradiated with 250 mJ of 254-nm light in a Bio-Rad GS Genelinker UV chamber. The samples were run on 12% SDS-polyacrylamide gels using standard methods. Competition experiments were performed by preincubation with a 50-fold excess of unlabeled RNA for 20 min prior to the addition of <sup>32</sup>P-labeled oligoribonucleotide.

**Construction of Vectors and *In Vitro* Transcription**—The plasmid vector pNKT7 was obtained from Dr. S. Kwon, University of Connecticut Health Center. This vector contains the green fluorescent protein (GFP) open reading frame under the control of the bacteriophage T7 promoter. *Sac*I and *Xba*I sites allowed insertion of additional sequences between the GFP open reading frame and the segment encoding the poly(A) tail. The double-stranded inserts, which were synthesized on an oligonucleotide synthesizer (Pacific Oligos, Lismore, Australia), included the *Sac*I and *Xba*I restriction sites on the ends of the 11- or 21-nucleotide segments incorporating the A2RE or A2RE-like sequences. The sequences of these inserts are in Fig. 1. *E. coli* were transformed with these vectors, and the plasmids were isolated by standard methods. All vectors were sequenced to ensure the integrity of the inserted sequences.

Digoxigenin (dig)-labeled cRNA was generated as described previously (18). The purified plasmids were linearized with BSAW1 (New England Biolabs), which cuts just 3' to the poly(A) tail, and used for *in vitro* transcription in the presence of dig-labeled UTP (Roche Molecular Biochemicals) with an Ampliscribe kit (Epicenter Technologies, Madi-

son, WI). The resultant RNAs were precipitated with 7.5 M ammonium acetate; the pellet washed with 70% ethanol and then dissolved in diethyl pyrocarbonate-treated water. They were further purified on polyacrylamide gel matrix columns (Micro Bio-Spin Columns P-30 Tris, RNase-Free; Bio-Rad) to remove any remaining free oligonucleotides and salts, and an aliquot of each was subjected to electrophoresis on an agarose-formaldehyde gel to verify purity and estimate the RNA concentration.

**RNA Microinjection and Visualization**—Cultures of mouse oligodendroglia were produced from brains of newborn C57Bl/6 mice as described previously (18, 19, 27). Cells grown on coverslips were microinjected using an Eppendorf Micromanipulator (Eppendorf, Westbury, NY) attached to a Zeiss Axiophot inverted microscope (Carl Zeiss, Oberkochen, Germany) using phase contrast optics. The RNA was mixed with Texas Red-conjugated dextran (10 kDa), filtered through a 0.2  $\mu$ m spin filter (Millipore, Bedford, MA), and introduced into cells using microinjection needles produced in a Flaming Brown micropipette puller (Sutter Instruments, San Raphael, CA). Only relatively large oligodendrocytes with well developed processes were injected. After injection, coverslips were incubated at 37 °C for 30 min to allow peripheral transport to occur.

The cells were washed in PIPES-buffered saline (PIBS) at pH 7.0 (120 mM NaCl, 5 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 25 mM D-glucose, and 20 mM PIPES) and then fixed for 20 min in 3.7% paraformaldehyde (Sigma) in PIBS. After washing with PIBS, the cells were permeabilized by incubation for 2 min in 0.1% Nonidet P-40 (Sigma) diluted in the same buffer, washed, and then incubated in 5% goat serum in PIBS for 10 min. To visualize dig-labeled RNA, the cells were incubated for 1–2 h in the primary antibody (mouse anti-digoxin; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), washed extensively, incubated in secondary antibody (fluorescein isothiocyanate-conjugated goat anti-mouse IgG; Jackson ImmunoResearch Laboratories) for 1–16 h, and washed with PIBS. Finally, 70% glycerol containing an anti-fading agent (1,4-diazabicyclo[2,2,2]octane) was added to the cells before they were viewed on a Zeiss AxioScope fluorescence microscope and imaged using a Zeiss LSM 410 confocal microscope equipped with a  $\times$  63 (1.4 numerical aperture) lens. All injected cells that survived the subsequent treatment, as judged by the presence of the labeled dextran, were imaged to permit the comparison of the distribution of dextran and fluorescein isothiocyanate-labeled exogenous RNA. The imaged cells, typically 30 or more for each RNA, were scored as positive or negative for transport by three independent observers using well established criteria (18, 25).

**Antisense Treatment of Oligodendrocytes**—Cultures of mouse oligodendrocytes were incubated for 18 h in defined culture medium (28) that contained a final concentration of 8  $\mu$ M phosphorothioate oligonucleotide directed against the translation start site of hnRNP A2 (20). Although hnRNP A1 and A2 share considerable sequence similarities, there is little within this domain. The antisense sequence used for these experiments, CTTTCTCTCCATCGCGGA, was synthesized by the Molecular Core Facility at the University of Connecticut Health Center. In control experiments, the corresponding sense oligonucleotide (inverse complement) was used. Following oligonucleotide treatment, the hnRNP A2 levels in the cells were examined by electrophoresis and immunofluorescence microscopy, and microinjection experiments were used to study transport of MBP RNA (see above). For electrophoresis, approximately equal numbers of cells were used. The blots were developed with a mouse antibody to hnRNP A2 (a gift from Dr. William Rigby, Dartmouth; used at a dilution of 1:1000) and rabbit polyclonal antibody to the 65-kDa ribophorin I (a gift from Dr. Gert Kreibich, New York University; used at a 1:1000 dilution) (29) as a control for protein loading followed by horseradish peroxidase-conjugated secondary antibodies. For confocal microscopy the cells were fixed and treated with the above antibody to hnRNP A2, followed by a fluorescein isothiocyanate-conjugated goat anti-mouse IgG secondary antibody (Jackson ImmunoResearch Laboratories).

#### RESULTS

**An 11-Nucleotide A2RE Subfragment Binds hnRNP A2**—Previous experiments have shown that in the presence of heparin A2RE binds to a small group of rat brain proteins, including predominantly hnRNP A2 and two isoforms of hnRNP A3 (26). The A2RE sequence is comprised of three imperfect, overlapping tandem repeats (Fig. 1). Our first objective in the experiments reported here was therefore to establish whether the entire 21-nucleotide A2RE was required for this association



Oligoribonucleotide	Sequence
A2RE	GCCAAGGAGCCAGAGCAUG
A2RE11	GCCAAGGAGCC
A2REmid	GGAGCCAGAGA
A2RE3'	CAGAGAGCAUG
A2RE11 G1A	<u>ACCAAGGAGCC</u>
A2RE11 C2U	<u>GUCAAGGAGCC</u>
A2RE11 C3G	<u>GCCAAGGAGCC</u>
A2RE11 A4C	<u>GCCACGGAGCC</u>
A2RE11 A5C	<u>GCCACGGAGCC</u>
A2RE11 G6C	<u>GCCAAGGAGCC</u>
A2RE11 G7A	<u>GCCAAGGAGCC</u>
A2RE11 A8G	<u>GCCAAGGAGCC</u>
A2RE11 G9A	<u>GCCAAGGAGCC</u>
A2RE11 C10U	<u>GCCAAGGAGCC</u>
A2RE11 C11U	<u>GCCAAGGAGCC</u>
MAP2A	GCCAAGGAGCCAGAGCAUG
GABAR(A)	<u>GCAAGGAGCCAGAGCAAAA</u>
ARC	<u>GCAAGGAGCCAGAGCAUCAU</u>
MOBP81A	<u>ACCCCGAGCAACAGAGCAUG</u>
NS	<u>CAAGCACCGAACCCGCAACUG</u>

FIG. 1. Sequences of oligoribonucleotides used in these experiments. Bases that differ from those in the A2RE are in **bold** and underlined. Mutations were introduced at each nucleotide in A2RE11 as transversions (C3G, A4C, A5C, G6C) or transitions (G1A, C2U, G7A, A8G, G9A, C10U, C11U). GABAR(A),  $\gamma$ -amino butyric acid receptor  $\alpha$  subunit. The nonspecific sequence (NS) has the same overall composition as A2RE.

or if a subsection would suffice.

Three 11-nucleotide fragments were synthesized comprising the 5'- and 3'-halves and the middle of the A2RE (Fig. 1). To facilitate comparison between these oligoribonucleotides, identical amounts of magnetic particles were added to an excess of the biotin-labeled RNAs ensuring saturation of the streptavidin binding sites. Saturation was verified by showing that there was free oligonucleotide in the supernatant after removal of the particles and that use of higher RNA concentrations resulted in no increase in the binding of hnRNPs A2 and A3, as measured by Coomassie Blue staining of SDS-polyacrylamide gels. It was further established, by reverse-phase HPLC, that the synthesized oligoribonucleotides contained no free biotin that could have caused variable amounts of RNA to be bound to the particles.

When bound to the magnetic particles each of these oligoribonucleotides bound hnRNP A2 in rat brain extracts; the binding was strongest for the 5'-segment (A2RE11) and lowest for the middle segment (Fig. 2), reflecting the base differences in the 3'- and middle 11-nucleotide segments and particularly the absence of three 3'-nucleotides in the latter. The A2RE11 reproducibly bound hnRNP A2 a little stronger than the full-length A2RE and might thus also be expected to act as an RNA transport signal if association with this protein is a prerequisite for transport. No direct attempt was made to determine if a still shorter oligoribonucleotide would bind as well as the A2RE, but as mutations at or close to the ends of this segment diminish binding to the protein, most, if not all, of the A2RE11 appears to be needed.

The binding of the two hnRNP A3 polypeptides to the A2RE fragments and to mutant A2RE sequences discussed below paralleled that of hnRNP A2, reinforcing the view that these proteins together form a complex with the RNA. In the discussion below we have, however, focused our attention on hnRNP A2 because earlier studies showed that isolated hnRNP A2, but not A3, associated directly with the A2RE (26).

**A2RE11 Mutations Reduce hnRNP A2 Binding**—The sequence requirements for binding to hnRNP A2 have been further explored by following the effects of single nucleotide changes within the A2RE11. The initial nucleotides targeted were those conserved in the A2RE-like sequences from several different transported RNAs (see below), and the later targets were the poorly conserved nucleotides in these sequences (Fig. 1). For the conserved residues the modifications introduced were transitions (*i.e.* purine to purine or pyrimidine to pyrim-

idine) as their conservation suggested that these mutations might not be well tolerated, whereas the latter were subject to the less conservative transversions (purine to pyrimidine and vice versa). These modified 3'-biotin-conjugated oligoribonucleotides were attached, through the biotin-streptavidin interaction, to magnetic particles, which were then added to rat brain protein extracts as described in the previous section.

The effects of base substitutions on hnRNP A2 binding varied markedly from one position to another. Seven transitional mutants all diminished hnRNP A2 and A3 binding, but whereas transitions in positions 1, 2, 7, 10, or 11 still had detectable hnRNP A2 binding, transitions at positions 8 and 9 had little or no detectable hnRNP A2 binding (Fig. 3A). Similarly, transversion of position 3 had very little effect on hnRNP A2 binding, but transversions at positions 4 and particularly 5 and 6 markedly diminished hnRNP A2 binding (Fig. 3B).

**Mutations Interfere with RNA Transport**—The A2RE, A2RE11, and several of the mutant sequences that showed little binding in the magnetic particle experiments were inserted in the 3'-untranslated region of DNA encoding GFP. The RNAs were transcribed *in vitro* in the presence of dig-conjugated UTP to facilitate detection in fixed cells, purified, and then microinjected into the soma of mature, cultured oligodendrocytes, together with Texas Red-labeled, 10-kDa dextran. The dextran spreads uniformly through the cell, and the fluorescence associated with it allows identification of injected cells and visualization of their processes. Laser scanning confocal micrographs of typical transporting and nontransporting cells are shown in Fig. 4.

Previous studies using this assay have revealed that RNAs containing the A2RE are transported in more than 70% of injected cells (25). Conversely, RNAs lacking the A2RE are transported in less than 15% of injected cells (25). Thus, the dynamic range of the microinjection assay is from 15 to 70%. RNA containing A2RE11 was transported as efficiently as RNA containing the complete A2RE, indicating that the A2RE11 is sufficient to mediate RNA transport (Fig. 5). RNAs containing the mutations G6C, A8G, and G9A (Fig. 1) were transported at levels comparable to RNAs lacking a recognizable transport sequence. Thus, there is a direct correlation between hnRNP A2 binding and cytoplasmic transport.

Using a previously described antisense method (20), we undertook to inhibit hnRNP A2 translation in oligodendrocytes and examine their capacity to transport microinjected MBP RNA. Western blot analysis indicated that antisense oligonucleotide treatment reduced hnRNP A2 levels to approximately 50% of untreated control cultures (Fig. 6F, results from five experiments), with a corresponding reduction in hnRNP A2 immunostaining. A small decline ( $\sim 15\%$ ) was also consistently observed in hnRNP A2 levels in oligodendrocyte cultures treated with sense oligonucleotides.

Although untreated cells typically transported microinjected RNA into the peripheral myelinating processes, treatment with phosphorothioate-modified oligonucleotides complementary to the translation start site of hnRNP A2 changed the RNA distribution. Typically, RNA immunofluorescence was restricted to the cell body and proximal cytoplasmic trunks (Fig. 6, A and B) with few granules detected in the distal cytoplasmic web. Although not transported, the microinjected RNAs were concentrated into granular aggregates (Fig. 6, B and C). Of those antisense-treated cells that did transport RNA, the granules appeared less abundant than in either untreated or sense oligonucleotide-treated control cells.

Control cultures were treated with the corresponding sense oligonucleotide and appeared indistinguishable from untreated cultures. In most injected cells, RNA granules were observed in

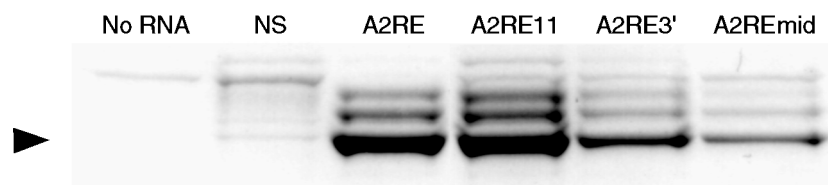


FIG. 2. **Deletion analysis of A2RE binding to hnRNP A2.** Magnetic particles bearing the biotinylated sequences A2RE, A2RE11, A2RE3', A2REmid, a randomized nonspecific sequence (NS), or no RNA were used to isolate proteins from rat brain extracts. Bound proteins were removed for analysis on Coomassie Blue-stained SDS-polyacrylamide gels. The position of hnRNP A2 (36 kDa) is shown (arrowhead). The two bands immediately above hnRNP A2 have been identified by protein sequencing as isoforms of hnRNP A3 (K. S. Hoek and R. Smith, unpublished data).

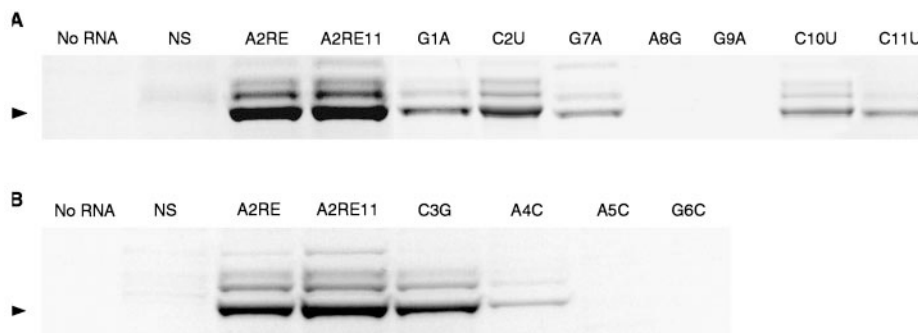


FIG. 3. **Mutational analysis of A2RE binding to hnRNP A2.** 3'-biotinylated oligoribonucleotides were synthesized with mutated sequences and used in magnetic particle experiments; bound proteins were removed and analyzed by electrophoresis as described previously. Unlabeled magnetic particles and magnetic particles labeled with a randomized nonspecific sequence were used as controls. The position of hnRNP A2 is shown (arrowhead). *A*, the effect of transitional mutations introduced at positions 1, 2, 7, 8, 9, 10, and 11 on hnRNP A2 binding. *B*, the effect of transversal mutations introduced at positions 3, 4, 5, and 6 on hnRNP A2 binding. NS, nonspecific sequence.

both major and minor cytoplasmic trunks and scattered within the membrane sheets extending from these processes (Fig. 6, *D* and *E*). Several other control oligonucleotides used under the same conditions have produced similar results (20). As reported previously for untreated cells, approximately 20% exhibited only perinuclear staining (25).

Counts were made of the number of cells which had transported the labeled RNAs after oligonucleotide treatment. As depicted in Fig. 6*G*, antisense treatment reduced the percentage of transporting oligodendrocytes compared with sense-treated cultures. When cultures were fixed, stained for MBP, and their relative sizes compared by phase microscopy and MBP immunofluorescence, there were no statistically significant differences observed in cell sizes and no qualitative differences in cell appearance that would distinguish between the different treatments.

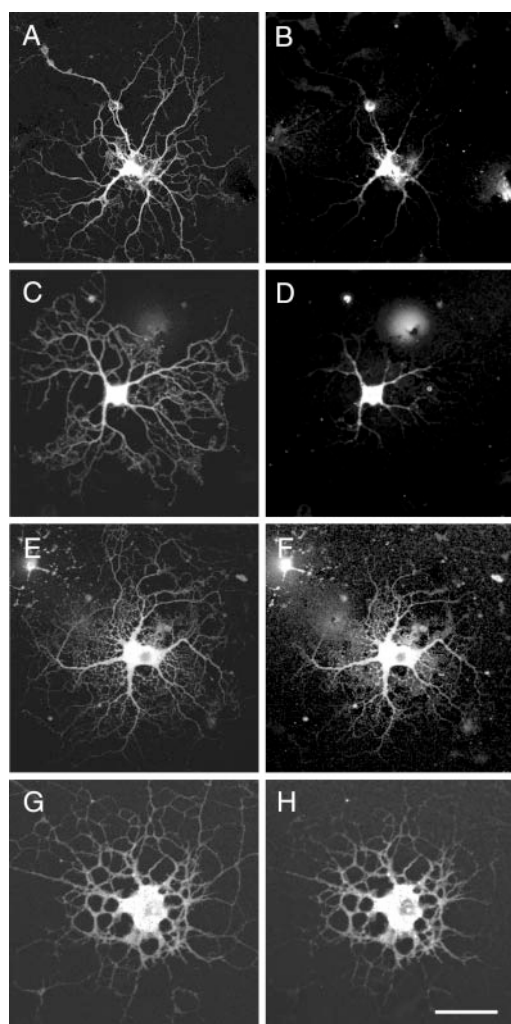
**Other A2RE-like Sequences Bind hnRNP A2**—Other localized mRNAs contain A2RE-like sequences (25), raising the possibility that these RNAs are transported by the same pathways as MBP mRNA in oligodendrocytes. Evidence for this hypothesis comes from the observation that MAP2A mRNA including the A2RE-like region is localized, whereas splicing variants lacking this sequence are not (25, 30). It was therefore of particular interest to see if A2RE-like sequences from other RNAs could also bind to hnRNP A2. Oligoribonucleotides with the A2RE-like sequences from the neural proteins MAP2A,  $\gamma$ -amino butyric acid receptor  $\alpha$  subunit, activity-related cytoskeleton-associated protein (ARC), and myelin-associated/oligodendrocyte basic protein (MOBP81A) were synthesized as 3'-biotin conjugates for attachment through streptavidin to magnetic particles. Oligonucleotides were end-labeled with  $^{32}$ P for detection on SDS-polyacrylamide gels following UV cross-linking in the presence of recombinant human hnRNP A2 or the affinity-purified A2RE-binding proteins from rat brain.

The A2RE was readily cross-linked to purified, recombinant hnRNP A2 (Fig. 7*A*). Another oligoribonucleotide with randomized sequence (nonspecific sequence 1 of Hoek *et al.* (26)) was also cross-linked at about half the level of the A2RE (Fig. 7*B*).

This is consistent with biosensor data showing that, in the absence of heparin, this protein possesses two oligonucleotide binding sites, one of which is nonspecific and one is specific for the A2RE. When added to protein extracts of rat brain, UV cross-linked, and run on SDS-polyacrylamide gels and autoradiographed, the radiolabeled A2RE comigrated with radiolabeled A2RE cross-linked to recombinant human hnRNP A2. Oligoribonucleotides with A2RE-like sequences from other localized messages also cross-linked to rat brain hnRNP A2. The A2RE-like sequences from ARC,  $\gamma$ -amino butyric acid receptor  $\alpha$  subunit, and MAP2A bound as well as the A2RE, but MOBP81A bound less strongly. No cross-linking of nonspecific RNA to the rat brain hnRNPs A2 and A3 was observed, in contrast to the binding to recombinant hnRNP A2; this suggests that when a complex is formed with hnRNP A2 and the two hnRNP A3 isoforms the nonspecific site may be occluded leaving only the specific site available for RNA binding. It is also possible that hnRNP A2 purified from rat brain is post-translationally modified in a way that diminishes binding of RNA to the nonspecific site.

Addition of a 50-fold excess of nonspecific oligoribonucleotide prior to UV irradiation diminished binding as anticipated because one of the two sites on the protein binds RNA nonspecifically. A similar excess of the A2RE eliminated binding of the radiolabeled RNA (data not shown). Association of neural A2RE-like sequences with hnRNP A2 was confirmed by isolation of this protein from rat brain extracts with magnetic particles bearing these oligoribonucleotides. Again, MAP2A and ARC oligoribonucleotides bound at least as well as A2RE, but the  $\gamma$ -amino butyric acid receptor  $\alpha$  subunit, and particularly MOBP81A, bound more weakly (Fig. 7*B*).

**Other A2RE-like Sequences Support Transport**—As discussed above, UV cross-linking/gel retardation and magnetic particle binding experiments have shown that oligoribonucleotides with the base sequences of several of these potential transport signals bind strongly to hnRNP A2. We therefore investigated whether these sequences could also confer transportability on GFP message. The results presented in Fig. 5

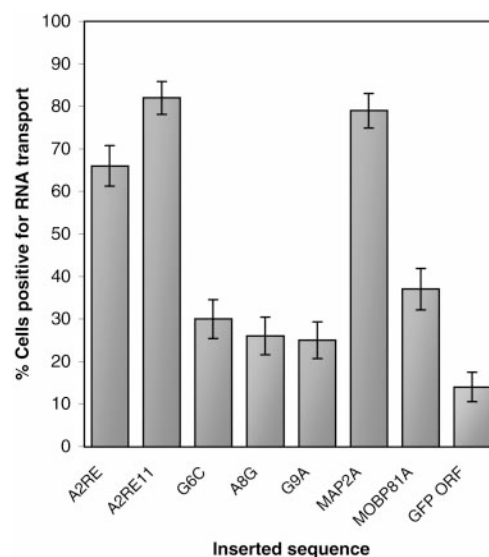


**FIG. 4. Confocal laser scanning microscope images of four representative mouse oligodendrocytes.** Cells were microinjected with dig-labeled RNA and immunofluorescently labeled with both anti-dioxin monoclonal antibodies (*B*, *D*, *F*, and *H*) to highlight the position of injected RNA within the cell and 10-kDa Texas Red-labeled dextran (*A*, *C*, *E*, and *G*) to show cell morphology. *A* and *B* and *C* and *D* show cells injected with RNA containing mutated A2RE11 (C3G and A8G, respectively); the injected RNA is largely confined to the cell body with minimal RNA transport into the processes. *E* and *F* and *G* and *H* show cells injected with RNA containing the MAP2A A2RE-like sequence and A2RE, respectively, exhibiting significant transport of the RNA into the processes. Scale bars, 25  $\mu\text{m}$ .

show that a chimera containing the MAP2A A2RE-like sequence inserted into GFP RNA promoted RNA transport in cultured oligodendrocytes as effectively as the A2RE or A2RE11. MOBP81A was less effective in promoting transport; about 40% of the cells transported this RNA. This is greater than the 15% that transport the GFP message lacking a transport signal yet below the 75–80% that transport RNAs containing the A2RE from MBP mRNA. Taken together with the binding studies referred to above, these data indicate that the MOBP81A A2RE-like sequence is relatively less effective in interacting with hnRNP A2 and this results in compromised cytoplasmic transport.

#### DISCUSSION

hnRNP A2 is a component of the nuclear hnRNP core particles that package nascent pre-mRNA presumably without regard for nucleotide sequence (31). But hnRNP A2 is also capable of binding to specific DNA and RNA sequences, including the telomeric sequence (32–34) and the A2RE (26). As shown



**FIG. 5. Transport of heterologous RNAs in mouse oligodendrocytes.** The proportion of cells that transported RNA is shown for RNAs containing the indicated oligoribonucleotides inserted in the GFP open reading frame (*ORF*). A2RE and A2RE11 facilitate RNA cytoplasmic transport of GFP RNA, whereas RNAs containing the mutated A2RE11 sequences exhibit message transport only slightly above the levels of GFP cRNA, which lacks a transport sequence. The A2RE-like sequence in MAP2A was sufficient to promote transport, whereas the proportion of cells positive for transport was significantly reduced for the corresponding sequence from MOBP81A mRNA. For each construct at least 30 cells were scored as positive or negative for RNA transport. The error bars show the statistical standard deviations.

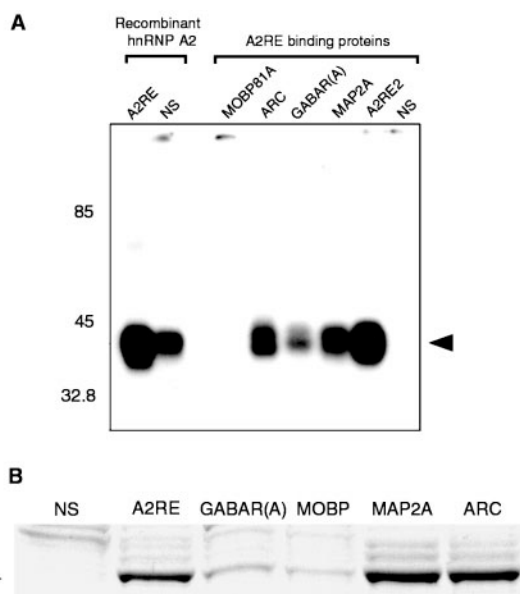
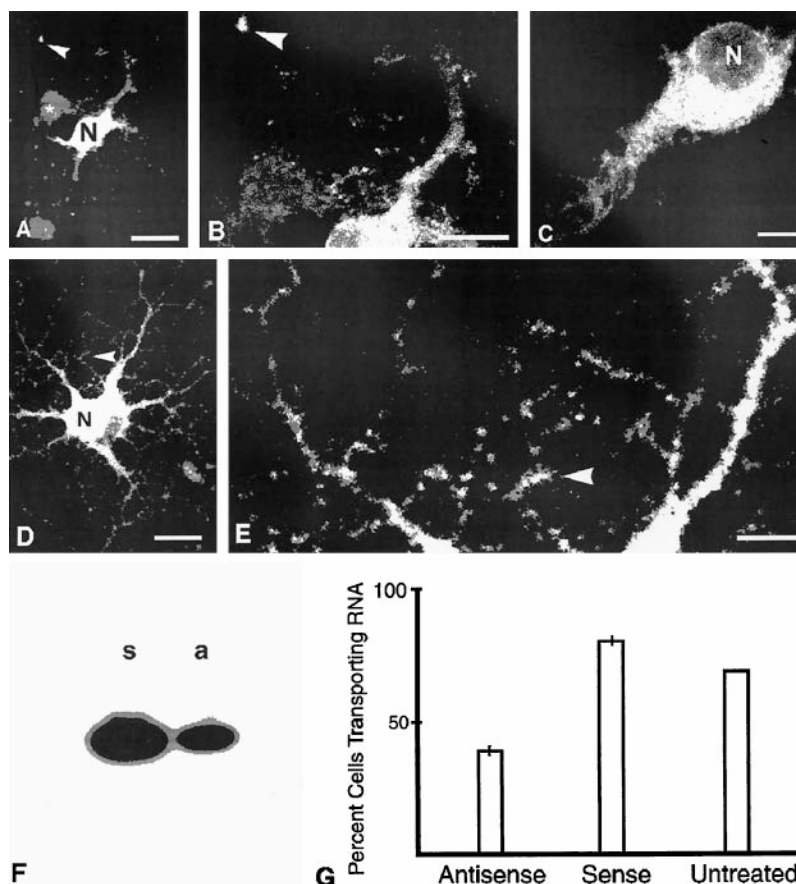
previously (26), nonspecific binding is eliminated by the addition of heparin, whereas it reduces but does not abrogate association of hnRNP A2 with the A2RE. This observation has been confirmed by our recent resonant mirror biosensor studies, which have shown that the protein possesses one nonspecific oligoribonucleotide binding site and another that is selective for the A2RE and cognate sequences.<sup>4</sup> Our mutational analysis highlights the specificity of the A2RE interaction with hnRNP A2; some single nucleotide changes have no effect, whereas others eliminate this interaction. Thus, hnRNP A2 binds the A2REs bearing the C2U, C3G, and C10U mutations almost as strongly as the native A2RE but not those with the mutations A5C, G6C, A8G, and G9A.

Although the earlier deletion analysis (25) showed that the 21-nucleotide A2RE is sufficient and necessary for mRNA cytoplasmic trafficking, our experiments demonstrate that a subfragment, the 5' 11 nucleotides, can both bind to hnRNP A2 and confer transportability on an RNA that is not otherwise transported. Examination of the A2RE and the A2RE-like sequences of other localized mRNAs (25) also reveals tandem, overlapping sequences that have high sequence identity. So, for example, the 5'-half of the A2RE has the sequence GCCAAGGAGCC; overlapping by one nucleotide with this segment is the 3'-sequence CAGAGAGCAUG, in which the common nucleotides have been underlined. The mutations G1A and C2U diminished the protein binding but had far less effect than mutations in many other positions, suggesting that the core recognized by the protein may be CAAGGAGC. But some variation within this segment is tolerated as illustrated by the corresponding regions, CAAGGAGU and UGAGGAGG, of the MAP2A and ARC RNAs, respectively, which bind hnRNP A2 at least as strongly as the A2RE. The AGGAG sequence is conserved in all of the oligoribonucleotides in Fig. 1 that bind

<sup>4</sup> J. Shan, K. S. Hoek, G. J. Kidd, T. P. Munro, D. J. Winzor, D. R. Hall, A. Schindeler, and R. Smith, submitted for publication.



**FIG. 6. Effects of antisense oligonucleotide treatment on MBP RNA transport.** A–C, antisense-treated cells. A, RNA staining is primarily restricted to the perinuclear cytoplasm with only one or two spots of staining away from the cell body (arrowhead). Background fluorescence is enhanced here to show cell outline; the asterisk indicates adjacent, uninjected cell. N, nucleus. B, higher magnification image; C, in an image of a second treated cell, perinuclear RNA staining is granular. D and E, sense-treated control cell. Microinjected RNA is detected in the perinuclear and peripheral cytoplasm; E, at higher magnification, RNA granules are present in both large and small processes. F, Western blot of hnRNP A2 levels (36-kDa band) in sense (s) and antisense (a) oligonucleotide-treated cells. G, histogram showing the proportion of oligodendrocytes transporting microinjected MBP RNA after treatment with antisense, sense, and no oligonucleotides. Cells were scored as transporting by comparison with A and D. Only intact cells showing significant staining were counted. Values are mean and S.E. for six experiments (antisense, 148 cells total), five experiments (sense, 89 cells total), and one experiment (untreated control, 63 cells; similar control values were previously reported by Ainger *et al.* (18)). Scale bars in A and D, 20  $\mu$ m; B, C, and E, 2  $\mu$ m.



**FIG. 7. Other A2RE-like sequences bind hnRNP A2.** A, radiolabeled RNA probes (Fig. 1) were incubated with purified recombinant human hnRNP A2 or A2RE-binding proteins purified from rat brain and UV cross-linked prior to analysis by SDS-polyacrylamide gel electrophoresis and autoradiography. The resulting RNA-protein complexes are shown (arrowhead). B, biotinylated RNA probes (Fig. 1) were used in a magnetic particle experiment. RNA-bound proteins were removed and analyzed by electrophoresis as above (Fig. 2). Beads labeled with nonspecific sequence (NS) were used as a control. The position of hnRNP A2 is shown (arrowhead).

strongly to hnRNP A2 (with the exception of the G7A mutant, which binds, albeit less strongly than the A2RE) and is modified in all those that show markedly lower binding.

Despite this sequence repetition, deletion of the 3' 10 nucleotides of the A2RE does not lower the RNA binding to hnRNP A2; A2RE11 binds as tightly as the whole A2RE. This observation reinforces the view that the footprint of the RNA on the protein arises from a segment of 6–8 nucleotides. This association could, however, still be modulated by interactions with nucleotides outside this core.

Although our earlier studies (26) had demonstrated a strong and sequence-specific interaction between hnRNP A2 and the A2RE, a sequence known to be necessary for cytoplasmic RNA trafficking, no direct link was established between this interaction and RNA transport. This link is provided by the RNA transport experiments reported here. RNA segments that bind strongly to hnRNP A2, A2RE, A2RE11, and the cognate MAP2A sequence, when attached to GFP RNA result in efficient transport into the processes of oligodendrocytes. By contrast, oligoribonucleotides such as G6C, A8G, and G9A, which show little or no interaction with hnRNP A2, do not support GFP RNA transport.

Treatment of cultured oligodendrocytes with antisense, but not sense, oligoribonucleotides directed against the hnRNP A2 translational start site causes a moderate, reproducible reduction of the concentration of hnRNP A2. There is a concomitant decrease in MBP RNA transport, providing further evidence that A2RE-mediated transport is hnRNP A2-dependent. Although some artifacts are possible with antisense protocols (35), they predominantly involve sense effects or nonspecific effects of exposure to oligonucleotides. At the concentrations used here, treatment with the sense oligonucleotide had no detectable effect on RNA transport, and neither oligonucleotide had obvious toxicity affecting myelin membrane morphology or oligodendrocyte size.

The existence of high cytoplasmic concentrations of hnRNP A2 in some neurons suggests that these cells may use a path-

way for RNA transport which parallels that in oligodendrocytes. This possibility is reinforced by the presence in neurons of several mRNAs that are known to be localized (12, 14, 17, 36–40). Several of these localized messages contain A2RE-like sequences, which we have shown bind as efficaciously to hnRNP A2 as the A2RE (Fig. 7). Additionally, our studies of the transport of RNA containing the ARC and MOBP81A A2RE-like sequences support the conclusion, drawn from the above studies of the A2RE11 mutants, that there is a direct correlation between transport and hnRNP A2 binding. Confirmation of the involvement of these mRNAs in an hnRNP A2-mediated transport in neurons will require direct observations on these cells.

An hnRNP-based transport system may also operate for other messages, such as that for protamine 2 in testis, and for viral messages (25). Several viral mRNAs contain A2RE-like sequences, and our preliminary studies indicate that some, including those from HIV *vpr* and *gag* genes, bind to hnRNP A2 as efficiently as the A2RE and mediate RNA transport in oligodendrocytes.

In summary, hnRNP A2 may have multiple roles in RNA processing and trafficking. In the nucleus it is a component of core particles that bind pre-mRNA, whereas in the cytoplasm it plays a role in transport of some localized mRNAs as indicated by the following evidence: (i) hnRNP A2 binds specifically to the segment of MBP mRNA that is necessary and sufficient for cytoplasmic transport in oligodendrocytes; (ii) modifications of this A2RE that abrogate hnRNP A2 binding also interfere with RNA transport; (iii) exogenous A2RE-containing RNA injected into oligodendrocytes colocalizes with hnRNP A2 in cytoplasmic granules; (iv) reduction of hnRNP A2 levels in oligodendrocytes by treatment with antisense, but not sense, oligoribonucleotides interferes with transport of A2RE-containing RNA; (v) hnRNP A2 binds A2RE-like sequences in other localized messages; and (vi) hnRNP A2 is abundant in the cytoplasm of many brain cells in which mRNAs are known to be localized.

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