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) 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 β-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. A2RE-like sequences are present in other localized mRNAs including mouse MAP2A, mouse protamine 2, and rat gliial 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. 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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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 280 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-isooamyl alcohol extraction, concentrated by vacuum centrifugation, and electrophoresed on a 4% agarose, 2.2% 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. Kira (Cold Spring Harbor Laboratories). Transformed cells were induced with isopropyl-β-D-thiogalactopyranoside, harvested by centrifugation, and lysed in 50 mM Tris-HCl, pH 8, containing 2 mM EDTA, 100 μg/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 purification at NaCl in the buffer. The concentrated sample was then loaded on a Sepharyl 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 to isolate 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 MgCl₂ 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, 32P-labeled with T4 polynucleotide kinase (New England Biolabs), which cuts just 3' to the poly(A) tail. The double-stranded inserts, which were synthesized on an oligoribonucleotide synthesizer (Pacific Oligos, Lismore, Australia), included the Sac1 and Xba1 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. Both 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 BamHI (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 (Epianal Technologies, Madison, 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 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/C6 mice as described previously (18, 19, 27). Cells grown on coverslips were microinjected using an Eppendorf Micromanipulator (Eppendorf, Westbury, NY) and fixed with 1% paraformaldehyde in 0.1 M sodium cacodylate (Carl Zeiss, Oberkochen, Germany) using phase contrast optics. The RNA was mixed with Texas Red-conjugated dextran (10 kDa), filtered through a 0.2 μm 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 CaCl₂, 1 mM MgCl₂, 25 mM N-acetylcysteine, 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 in 0.1% Nonidet P-40 for 10 min 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-digoxigenin; 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 × 63 (1.4 numerical aperture) lens. All injected cells that survived the subsequent treatment, as judged by the presence of the labeled dextran, were used to permit the determination of the distribution of the digoxigenin and fluorescein isothiocyanate-labeled exogenous RNA. The imaging 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) containing 1 mM EDTA and a final concentration of 8 μM phosphorothioate oligoribonucleotide 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, CCTTCTCTCCTCTCATCAGGGA, was synthesized by the Molecular Core Facility at the University of Connecticut Health Center.

In control experiments, the corresponding sense oligoribonucleotide (inverted complement) was used. Following oligoribonucleotide treatment, the hnRNP A2 levels in the cells were examined by electrophoblotting and immunofluorescence microscopy, and microinjection experiments were used to study transport of MBP RNA (see above). For electrophoblotting, 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 to establish whether the entire 21-nucleotide A2RE was required for this association.

**Construction of Vectors and in Vitro Transcription—** The plasmid vector pNK7 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. SacI and XbaI 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 SacI and XbaI 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. Both 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 BamHI (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 (Epianal Technologies, Madison, 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 an aliquot of each was subjected to electrophoresis on an agarose-formaldehyde gel to verify purity and estimate the RNA concentration.
Mutational Analysis of the hnRNP A2 Response Element

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 oligoribonucleotide 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 5'- 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 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 and A3, as associated directly with the A2RE (26).

| Oligoribonucleotide | Sequence          |
|---------------------|------------------|
| A2RE                | GCGAGCGGAGGAGGAG |
| A2RE11              | GCGAGCGGAGGAGGAG |
| A2REmd              | GCGAGCGGAGGAGGAG |
| A2RE1G              | GCGAGCGGAGGAGGAG |
| A2RE11 G1A          | GCGAGCGGAGGAGGAG |
| A2RE11 G2U          | GCGAGCGGAGGAGGAG |
| A2RE11 C2G          | GCGAGCGGAGGAGGAG |
| A2RE11 A4G          | GCGAGCGGAGGAGGAG |
| A2RE11 A5G          | GCGAGCGGAGGAGGAG |
| A2RE11 96C          | GCGAGCGGAGGAGGAG |
| A2RE11 97A          | GCGAGCGGAGGAGGAG |
| A2RE11 98G          | GCGAGCGGAGGAGGAG |
| A2RE11 99G          | GCGAGCGGAGGAGGAG |
| A2RE11 10U          | GCGAGCGGAGGAGGAG |
| A2RE11 C11U         | GCGAGCGGAGGAGGAG |
| MAP9A               | GCGAGCGGAGGAGGAG |
| GAP9AR(A)           | GCGAGCGGAGGAGGAG |
| ARC                 | GCGAGCGGAGGAGGAG |
| MOPB61A             | GCGAGCGGAGGAGGAG |
| NS                  | GCGAGCGGAGGAGGAG |

or if a subsection would suffice.

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), γ-amino butyric acid receptor α subunit. The nonspecific sequence (NS) has the same overall composition as A2RE.

Although untreated cells typically transported microinjected RNA into the peripheral myelinating processes, treatment with sense oligonucleotides complementary to 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 (~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
Transversional mutations introduced at positions 3, 4, 5, and 6 on hnRNP A2 binding. 

Counts were made of the number of cells which had transported the labeled RNAs after oligonucleotide treatment. As depicted in Fig. 6G, 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. Oligonucleotides with the A2RE-like sequences from the neural proteins MAP2A, γ-amino butyric acid receptor α 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 32P 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. 7A). Another oligonucleotide with randomized sequence (nonspecific sequence 1 of Hoek et al. (26)) was also cross-linked at about half the level of the A2RE (Fig. 7B).

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. Oligonucleotides with A2RE-like sequences from other localized messages also cross-linked to rat brain hnRNP A2. The A2RE-like sequences from ARC, γ-amino butyric acid receptor α 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 posttranslationally modified in a way that diminishes binding of RNA to the nonspecific site.

Addition of a 50-fold excess of nonspecific oligonucleotide 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 oligonucleotides. Again, MAP2A and ARC oligonucleotides bound at least as well as A2RE, but the γ-amino butyric acid receptor α subunit, and particularly MOBP81A, bound more weakly (Fig. 7B).

Other A2RE-like Sequences Support Transport—As discussed above, UV cross-linking/gel retardation and magnetic particle binding experiments have shown that oligonucleotides 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 trans- portability on GFP message. The results presented in Fig. 5...

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 transversional mutations introduced at positions 3, 4, 5, and 6 on hnRNP A2 binding. NS, nonspecific sequence.
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 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. 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 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.

**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.

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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 to 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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