

Transport and Localization Elements in Myelin Basic Protein mRNA

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Abstract. Myelin basic protein (MBP) mRNA is localized to myelin produced by oligodendrocytes of the central nervous system. MBP mRNA microinjected into oligodendrocytes in primary culture is assembled into granules in the perikaryon, transported along the processes, and localized to the myelin compartment. In this work, microinjection of various deleted and chimeric RNAs was used to delineate regions in MBP mRNA that are required for transport and localization in oligodendrocytes. The results indicate that transport requires a 21-nucleotide sequence, termed the RNA transport signal (RTS), in the 3' UTR of MBP mRNA. Homologous sequences are present in several other localized mRNAs, suggesting that the RTS represents a general transport signal in a variety of different cell types. Insertion of the RTS from MBP mRNA into

nontransported mRNAs, causes the RNA to be transported to the oligodendrocyte processes. Localization of mRNA to the myelin compartment requires an additional element, termed the RNA localization region (RLR), contained between nucleotide 1,130 and 1,473 in the 3' UTR of MBP mRNA. Computer analysis predicts that this region contains a stable secondary structure. If the coding region of the mRNA is deleted, the RLR is no longer required for localization, and the region between nucleotide 667 and 953, containing the RTS, is sufficient for both RNA transport and localization. Thus, localization of coding RNA is RLR dependent, and localization of noncoding RNA is RLR independent, suggesting that they are localized by different pathways.

ESTABLISHING and maintaining the intricate intracellular organization of eukaryotic cells requires targeting of newly synthesized components to appropriate subcellular compartments. There are well characterized pathways for targeting protein components to the membrane-bound compartments of the secretory pathway and the nucleus (Pfeffer and Rothman, 1987; Görlich and Mat-taj, 1996) and for targeting mRNAs encoding secretory and membrane proteins (Blobel and Dobberstein, 1975) to the endoplasmic reticulum (ER)¹ via the nascent polypeptide. The ribosome was initially thought to play a passive role in targeting mRNA to the ER membrane, but recent evidence suggests that the ribosome regulates targeting by increasing the affinity of the signal recognition particle

(SRP) for ER membrane components (Bacher et al., 1996).

The first evidence that subcellular localization of cytosolic proteins could also be achieved through localization of their mRNAs came from the observation that myelin basic protein (MBP) mRNA was enriched in isolated myelin (Colman et al., 1982). Later research demonstrated localization of MBP mRNA to the peripheral processes and myelin of oligodendrocytes in vivo (Kristensson et al., 1986; Verity and Campagnoni, 1988) and in vitro (Holmes et al., 1988; Shiota et al., 1989; Barbarese, 1991). This provided evidence that mRNA encoding a protein synthesized on free polysomes was spatially localized within the cell.

RNA localization has also been demonstrated in a variety of other systems. In *Drosophila*, localization of bicoid and nanos RNA is required for establishment of the anterior/posterior axis of the embryo (for review see St Johnston and Nüsslein-Volhard, 1992). In *Xenopus*, several different RNAs are localized during creation of the dorsal/ventral axis in the oocyte (for review see Melton et al., 1989). In motile fibroblasts (Singer et al., 1989) and in terminally differentiated neurons (Garner et al., 1988; Bruckenstein et al., 1990; Kleiman et al., 1990; Crino and Eberwine, 1996), several specific mRNAs are localized

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1. *Abbreviations used in this paper:* ER, endoplasmic reticulum; GFP, green fluorescent protein; MAP, microtubule-associated protein; MBP, myelin basic protein; PLP, proteolipid protein; RLR, RNA localization region; RTS, RNA transport sequence; SRP, signal recognition particle; UTR, untranslated region.

within the cell. Localization of mRNAs has been extensively reviewed (Steward and Banker, 1992; Wilhelm and Vale, 1993; St Johnston, 1995).

With the exception of mRNAs encoding signal sequences, which are cotranslationally targeted to the ER by the sequence of the nascent polypeptide in conjunction with SRP, most of the characterized *cis*-acting signals for RNA localization are found in the 3' untranslated region (UTR) of the mRNA. *Cis*-acting signals have been defined for localization of bicoid (Macdonald and Struhl, 1988; Macdonald et al., 1993), nanos (Wharton and Struhl, 1991; Gavis and Lehmann, 1992), oskar (Ephrussi and Lehmann, 1992; Kim-Ha et al., 1993), Vg1 (Mowry and Melton, 1992), β -actin (Kislauskis et al., 1994), cyclin B (Dalby and Glover, 1993), K10 (Cheung et al., 1992), and even-skipped (Davis and Ish-Horowitz, 1991). In *Drosophila*, localization of oskar and bicoid mRNAs are controlled by multiple RNA elements that control different steps in the pathway (Kim-Ha et al., 1993; Macdonald et al., 1993).

One function of mRNA localization is to ensure localized synthesis of the encoded protein. This implies that translation is repressed until the mRNA becomes localized. Translation is known to be tightly regulated in many oocytes and embryos, where specific mRNAs are not translated until fertilization or egg activation (for review see Curtis et al., 1995). Some of these translationally repressed maternal mRNAs, like nanos mRNA in *Drosophila*, are also localized. Nanos protein is not expressed from unlocalized nanos mRNA (Gavis and Lehmann, 1994). The oskar protein functions in localization of nanos mRNA as well as enhancing translation of nanos mRNA. In addition, during localization of oskar mRNA, no oskar protein can be detected (Kim-Ha et al., 1995). Translational repression and localization of oskar mRNA are controlled by separated elements in the 3' UTR of oskar mRNA.

These observations have led to the hypothesis of localization-dependent translation (Gavis and Lehmann, 1994; Kim-Ha et al., 1995), in which translation of specific mRNAs is dependent on their subcellular localization. In this context, the term RNA localization, which was originally used to describe the nonuniform, steady-state distribution of an mRNA in a cell, can be more narrowly defined as recognition of the correct subcellular destination of the transported mRNA. Thus, RNA localization can be considered a sequential process involving translational repression, transport, localization, and localization-dependent translation.

MBP mRNA localization in oligodendrocytes occurs through a multi-step pathway that has been defined using microinjection experiments (Ainger et al., 1993). The pathway includes assembly of the RNA into granules in the perikaryon, anterograde transport along cellular processes, and localization within the myelin compartment. The work described here takes advantage of the fact that granule assembly, transport, and localization occur in spatially distinct subcellular compartments (the perikaryon, processes, and myelin compartment, respectively) of the oligodendrocyte. By deleting various regions of MBP mRNA and analyzing the subcellular distribution of the injected RNA, it is possible to delineate discrete elements in MBP mRNA that are specifically required for different steps in the localization pathway.

Materials and Methods

Cell Culture

Mouse oligodendrocytes were isolated from mixed primary brain cell cultures and grown as described previously (Ainger et al., 1993).

Reagents

Restriction enzymes and RNA polymerases were obtained from New England BioLabs (Beverly, MA), Promega (Madison, WI), and Stratagene (La Jolla, CA). RNasin and transcription buffers were from Promega. Digoxigenin-UTP was purchased from Boehringer Mannheim (Indianapolis, IN). RNA molecular weight markers were from GIBCO BRL (Gaithersburg, MD). pSP64 and pGEM3 vectors were from Promega. pBluescript IISK was from Stratagene. Diethylpyrocarbonate (DEPC), mineral oil, monoclonal anti-digoxigenin, and ammonium sulfate were from Sigma Chemical Co. (St. Louis, MO). Fluorochrome-conjugated secondary antibodies were from Chemicon International, Inc. (Temecula, CA) and Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Oligonucleotides were from National Biosciences (Plymouth, MN). Anti-BIP (endoplasmic reticulum chaperonin) antibody was from StressGen Biotechnologies Corp. (Victoria, BC, Canada).

Recombinant DNA and In Vitro Transcription

Transcription templates were prepared from a plasmid containing rat 14 kD MBP (Roach et al., 1983) originally subcloned into pSP64 poly A vector. Digestion with EcoRI and transcription with SP6 RNA polymerase gave an RNA with the first 1473 bases of MBP mRNA. For the other truncations, full length MBP cDNA was subcloned into the EcoRI site of pBluescript IISK to create pKS3. Transcription with T3 RNA polymerase resulted in incorporation of a short length of vector-derived sequences from the polylinker on the 5' end of all RNAs. Digestion with SalI, PvuII, and BstEII gave RNAs with the first 666, 953, and 1,131 bases, respectively, of rat MBP. HindIII-cut plasmid was used to transcribe full length MBP mRNA containing plasmid-derived polyadenosine and a short 3' sequence from the polylinker. To make RNA with a translational frameshift, the XmaI to HindIII fragment of pKS3 was subcloned into pGem3. The plasmid was linearized with BamHI, filled in with the Klenow fragment of DNA polymerase I, and religated. The plasmids were grown in *dam*⁻ *Escherichia coli* and assayed for a newly generated ClaI site. The reading frame shifts to -1 after 13 amino acids of MBP. The polypeptide terminates after an additional 27 out-of-frame amino acids. The 3' UTR of MBP was subcloned as a SalI/HindIII fragment into SalI/HindIII-cut pGem3. The plasmid was linearized with HindIII and transcribed with T7 RNA polymerase. Similarly, the SalI/PvuII fragment of MBP 3' UTR was subcloned into SalI/EcoRV-digested pBluescript IISK, digested with PvuII, and transcribed with T7 RNA polymerase. This produced an RNA with a 280-nucleotide 3' extension of vector-derived sequences. To construct chimeric RNAs, the 3' UTR and the SalI/PvuII fragments of MBP cDNA were subcloned into SalI-linearized and SalI/SmaI-digested, respectively, *Xenopus laevis* globin cDNA (the gift of D. Melton of Harvard University, Cambridge, MA; pSP64-X β M). To make RNA, the chimeric plasmids were digested with EcoRV and SmaI, respectively, and transcribed with SP6 RNA polymerase. To make RNA containing the RNA transport sequence (RTS), an oligonucleotide containing the RTS sequence with SpeI and KpnI linkers (CTAGTGCCAAGGAGCCAGAGAGCATGGGTAC) was cloned into SpeI/KpnI-digested pBluescriptII (SK) to generate pRTS, which was digested with SspI and transcribed with T3 RNA polymerase. To construct proteolipid protein (PLP)-RTS, chimeric RNA, PLP cDNA (the gift of F. Smith, Shriver Center, Waltham, MA) was digested with SpeI and KpnI, removing 560 nucleotides from the PLP 3' UTR, and ligated to SpeI/KpnI-digested pRTS. The resulting plasmid was linearized with KpnI; blunt ends were created with the Klenow fragment and transcribed with T3 RNA polymerase. β -Actin cDNA was the gift of Dr. J. Pachter of the University of Connecticut Health Center. Protamine 2 cDNA was obtained from Dr. N. Hecht, Tufts University (Medford, MA). Green fluorescent protein (GFP) cDNA was purchased from GIBCO BRL and subcloned into a plasmid containing a T7 promoter for transcription and a sequence of 80 A's downstream of the cDNA insertion site. To construct GFP-RTS an oligonucleotide containing the RTS sequence was inserted between the cDNA open reading frame and the poly A tail. All RNAs were subjected to electrophoresis in gels containing formaldehyde (Sambrook et al., 1989).

to ensure that the injected RNA was of the appropriate size. RNA was labeled by addition of digoxigenin-UTP or fluorescein-UTP during transcription reactions according to manufacturer's instructions.

Microinjection and Immunocytochemistry

RNA was injected into cells maintained at room temperature for a period of not more than 45 min, as described previously (Ainger et al., 1993). To allow time for transport and localization of the injected RNA, cells were incubated for 15 min at 37°C, in medium containing 2% newborn calf serum to digest extracellular RNA that leaked into the medium during the injections and to reduce nonspecific background. To visualize digoxigenin-labeled RNA, cells were fixed in 4% paraformaldehyde in PBS and incubated with anti-digoxigenin antibodies as previously described (Ainger et al., 1993), or using a mouse monoclonal anti-digoxigenin antibody (1:75 dilution), followed by detection with a fluorescein-conjugated goat anti-mouse IgG (Chemicon International). MBP was visualized using rabbit polyclonal anti-MBP followed by Texas red-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.).

Microscopy and Image Processing

Laser scanning confocal microscopy was performed with an MRC-600 scanning system (Bio Rad, Cambridge, MA) mounted on a microscope (Axioskope; Zeiss, Oberkochen, Germany) equipped with a variety of infinity-corrected high numerical aperture objectives.

Computer Analysis of Sequences

Computer analysis of sequence information was performed with the Genetics Computer Group program (1991 Version 7; Madison, WI). Dr. M. Zuker (Ottawa University, Ottawa, Canada) kindly provided an updated version of the MFOLD program. Rat 14 kD MBP (Roach et al., 1983) and mouse 14 kD MBP (Takahashi et al., 1985) were used for sequence comparisons. The other sequences listed in Table I are available from GenBank/EMBL/DDJB index accession numbers as follows: human MBP, M13577; rat MOBP81-A, X87900; rat glial fibrillary acidic protein (GFAP), K01347; human N-type calcium channel α -1 (Ca-N), M94172; mouse microtubule-associated protein (MAP) 2A, M21041; bovine GABA(A) receptor (GABARA), X05717; bovine nitric oxide synthase, (NOS) M95674; rat activity-regulated cytoskeleton-associated protein (ARC), U19866; rat neurogranin (RC3), L09119; mouse protamine 2, X14004; pufferfish NCAML1, Z71926; rat c-jun, X17163; rat atrophin-1 related protein (rARP), U44091; human clathrin light chain b, M20469 and J04174; rat furosemide sensitive K-Cl cotransporter (KCC1), U55815; mouse protein kinase C α , M25811; human chromogranin A, J03483; human glycogen phosphorylase, J03544; mouse cyrtestin, X64227; mouse heparin binding protein 44 (HBP44), D00622; human LIM kinase, D26309; human insulin like growth factor I receptor (IGFRI), X04434; mouse RNA-binding protein (FLI-2), M99167; HIV-2 tat.vpr, J04542; hepatitis C virus NS-5 region, Z35506.

Results

Microinjection Assay of Modified RNAs

Previous work has shown that MBP mRNA microinjected into oligodendrocytes is assembled into RNA granules that are transported along the processes and localized to the myelin compartment, while control mRNAs (globin and actin) are assembled into RNA granules that remain in the perikaryon (Ainger et al., 1993). The differential distribution of these mRNAs is presumably controlled by some aspect of their structure. In this study, deletion analysis of MBP RNA was used to identify sequences or structures that are required for transport and/or localization. Digoxigenin-labeled RNAs were synthesized by *in vitro* transcription and microinjected into cultured oligodendrocytes. Cells were fixed and labeled by immunofluorescence with both anti-digoxigenin antibody to visualize the injected RNA and anti-MBP antibody to visualize the overall cell morphology.

The unique morphology of oligodendrocytes in primary culture makes it possible to spatially resolve three stages in the RNA sorting pathway: (a) granule assembly in the perikaryon; (b) transport in the processes; and (c) localization in the myelin compartment. Therefore, by analyzing the subcellular distribution of microinjected RNA it is possible to determine if a particular RNA is assembled into granules in the perikaryon, transported in the processes and/or localized to the myelin compartment. In this regard it is important to appreciate that the very features (thin, flattened morphology, unfurled myelin compartment) of oligodendrocytes in culture that make them suitable for studies on intracellular RNA trafficking are different from oligodendrocytes in the intact nerve where the myelin compartment is wrapped spirally around the myelinated axon and where intracellular RNA trafficking occurs in three dimensions and may be influenced by signals from the axons that are being myelinated or from other surrounding cells. The oligodendrocyte in culture recapitulates those aspects of RNA trafficking that are intrinsic to the cell. However, RNA trafficking may be regulated in more complex ways *in vivo*. These caveats notwithstanding, the geographic separation of perikaryon, processes, and myelin compartment in oligodendrocytes in culture provides means of resolving the various steps in intracellular trafficking of RNA.

Representative cells illustrating the observed distribution patterns are shown in Fig. 2, where the distribution patterns of the injected RNA are shown in the left column and the staining patterns with antibody to MBP to reveal the overall cell morphology are shown in the right column. Three distinct RNA distribution patterns were observed (illustrated in Fig. 2, A, C, and E). In Fig. 2 A, the injected RNA is present in granules in the perikaryon, processes, and myelin compartment, indicating that the RNA is assembled into granules, transported, and localized. In Fig. 2 C, RNA is present in granules in the perikaryon and processes but not in the myelin compartment, indicating that the RNA is assembled into granules and transported but not localized. In Fig. 2 E, RNA is present in granules in the perikaryon but not in the processes or myelin compartment, indicating that the RNA is assembled into granules but not transported or localized.

The microinjection assay employed here is based on the assumption that the behavior of exogenous RNA microinjected into oligodendrocytes accurately reflects the intracellular trafficking pathway of endogenous RNA synthesized in oligodendrocytes. Microinjection directly into the perikaryon makes it possible to analyze RNA transport and localization without the confounding variables of transcription, post-transcriptional processing, and nuclear export. However, the amount of exogenous RNA injected into the perikaryon may be substantially greater than the amount of endogenous MBP mRNA in the cell. This raises the possibility that certain steps in the intracellular trafficking pathway may become saturated and the quantitative distribution of exogenous RNA in the different subcellular compartments shortly after injection may not accurately reflect the steady state distribution of endogenous MBP mRNA in the cell. Nevertheless, on a qualitative level, appearance of injected RNA in granules in the perikaryon provides evidence for granule assembly, in the

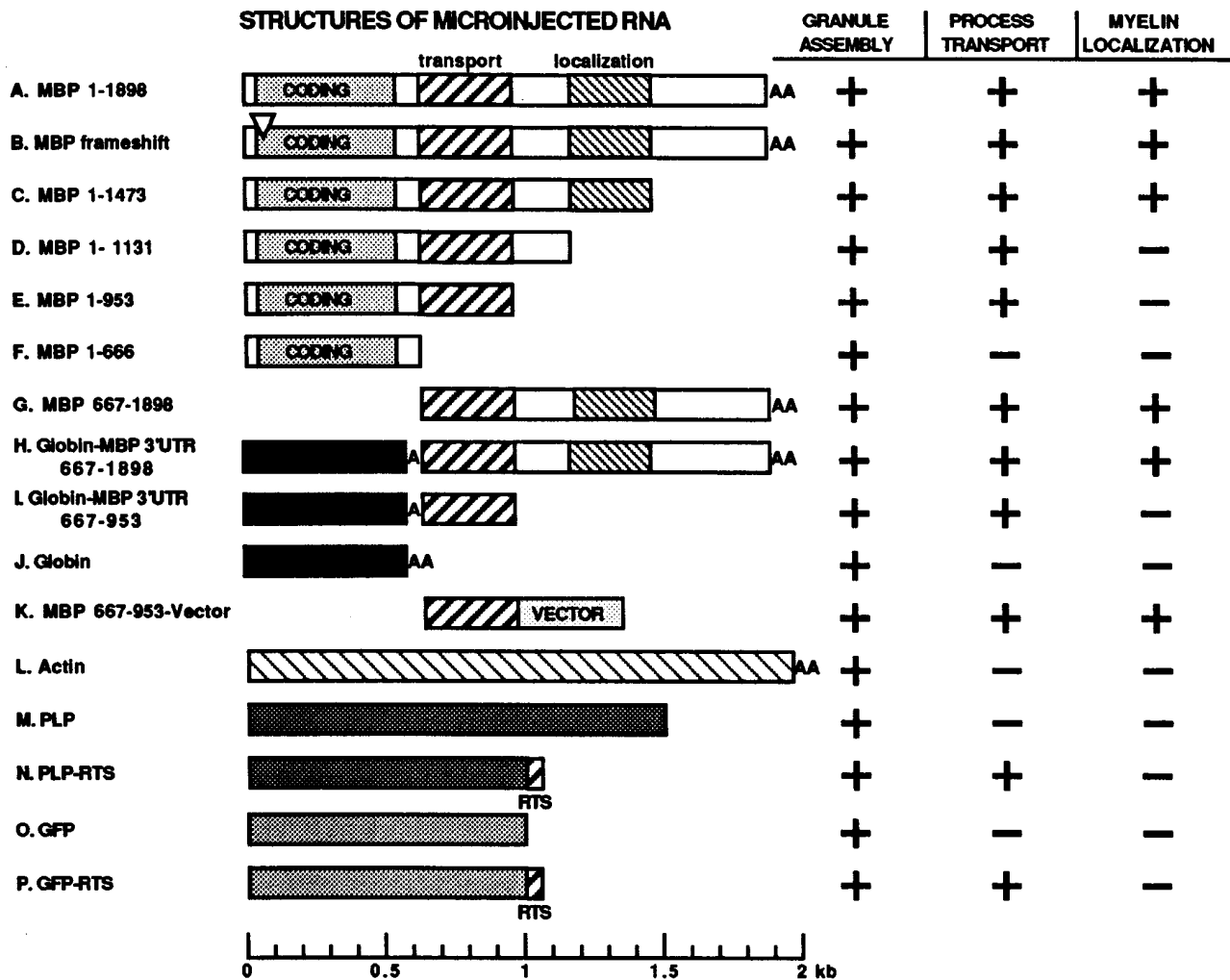


Figure 1. Intracellular localization of microinjected RNAs. (A) Full length MBP. (B) Frameshifted full length MBP. (C) EcoRI-truncated MBP. (D) BstEII-truncated MBP. (E) PvuII-truncated MBP. (F) SalI-truncated MBP. (G) SalI-fragment of MBP 3' UTR. (H) Chimeric construct of *X. laevis* β -globin with SalI fragment of MBP 3' UTR. (I) Chimeric construct of *X. laevis* β -globin with SalI-PvuII fragment of MBP 3' UTR. (J) *X. laevis* β -globin. (K) SalI-PvuII fragment of MBP 3' UTR with vector-derived sequences. (L) Mouse β -actin. (M) Mouse PLP. (N) Mouse PLP-RTS. (O) GFP. (P) GFP-RTS. Plus (+) indicates that this distribution was observed in the majority of several hundred injected cells.

distal processes provides evidence for transport, and in the myelin compartment provides evidence for localization.

The microinjection assay is also subject to a certain amount of inherent variability. This may reflect the delicacy of the microinjection process, the fragility of the cells, variations in the precise injection location, or morphological and developmental variation among oligodendrocytes in culture. Because of this variability, it was necessary to microinject several hundred cells with each RNA and to evaluate the distribution pattern observed in the majority of the cells. The results obtained for each RNA were compared with size-matched positive and negative controls that were microinjected at the same time into cells from the same preparation. Each RNA was assayed in at least three different preparations of oligodendrocytes. In most experiments, a small proportion (<20%) of the injected cells exhibited no transport or localization. This may mean that some of the microinjected cells died after injection or that some of the cells are not transport competent. In gen-

eral, >70% of the cells injected with a particular RNA displayed a consistent distribution pattern comparable to one of the three patterns illustrated in Fig. 2.

The structures of the various RNAs tested in the microinjection assay are diagrammed in Fig. 1, with the distribution pattern observed in the majority of injected cells tabulated for each RNA. When full length, polyadenylated MBP mRNA (Fig. 1 A) was injected into oligodendrocytes, granules were observed in the perikaryon, processes, and myelin compartment (this pattern is illustrated in Fig. 2, A and B), indicating that this RNA contains signals for granule assembly, transport, and localization, respectively. The distribution pattern was not affected by insertion of a frameshift mutation into the coding region of MBP mRNA (Fig. 1 B), deletion of 425 nucleotides (including the poly A tail) from the 3' end (Fig. 1 C), or deletion of the coding region (Fig. 1 G). Previous experiments demonstrated that the presence or absence of a cap at the 5' end did not affect the distribution pattern of microin-

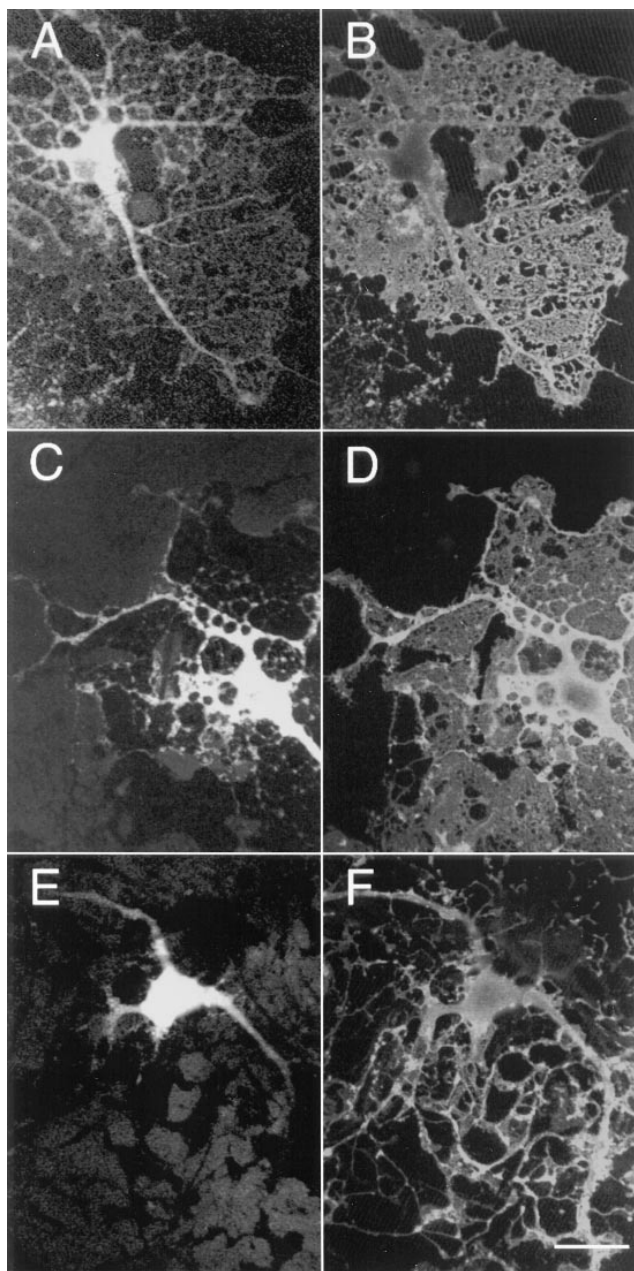


Figure 2. Representative mouse oligodendrocytes microinjected and immunofluorescently labeled with both anti-digoxigenin (A, C, and E) and anti-MBP antibodies (B, D, and F). A and B show an oligodendrocyte injected with RNA of Fig. 1 A. The cell exhibits granule formation, transport along the processes, and localization to the myelin membranes. This pattern is representative of cells injected with RNAs of A, B, C, G, H, and K in Fig. 1. C and D show an oligodendrocyte injected with RNA of Fig. 1 D. The cell exhibits granule formation and transport along the processes but without localization to the myelin membrane. This pattern is representative of cells injected with RNAs of D, E, I, N and P in Fig. 1. E and F show an oligodendrocyte injected with RNA of Fig. 1 F. The cell exhibits granule formation but without transport along the processes or localization to the myelin membrane. This pattern is representative of cells injected with RNAs of F, J, L, M and O in Fig. 1. The extracellular staining in C and E is due to RNA leaking from the microinjection needle and adhering nonspecifically to the glass coverslip in regions not occluded by the cell. Bar, 25 μ m.

jected mRNA (Ainger et al., 1993). Thus, granule assembly, transport, and localization do not require the 5' cap, the nascent MBP polypeptide, the coding region, the poly A tail, or the 3' 425 bases of the MBP mRNA.

When the 3' UTR region from position 1131–1473 was deleted from MBP mRNA (Fig. 1 D), granules were observed in the perikaryon and processes but were absent from the myelin compartment (this pattern is illustrated in Fig. 2, C and D), indicating that this region contains signals required for localization but not for granule assembly or transport. Deletion of the region from position 953–1131, also in the 3' UTR (Fig. 1 E), showed the same distribution as Fig. 1 D, indicating that this region also is not required for granule assembly or transport. It is not known if this region is required for localization, since, in the absence of the region from position 1131–1473, localization did not occur.

When the 3' UTR region from position 667–953 of MBP mRNA was deleted (Fig. 1 F), granules were observed in the perikaryon but not in the processes or myelin compartment (this pattern is illustrated in Fig. 2, E and F), indicating that this region contains signals required for granule assembly, but not for transport. The same distribution was observed when control RNAs encoding globin, a cytosolic protein (Fig. 1 J), GFP, a soluble protein (Fig. 1 O), actin, a cytoskeletal protein (Fig. 1 L), or PLP, a myelin membrane (Fig. 1 M), were injected.

When the entire 3' UTR region of MBP mRNA (containing the putative transport and localization signals delineated above) was attached to globin mRNA (Fig. 1 H), granules were observed in the perikaryon, processes, and myelin compartment, indicating that this region is sufficient to confer transport and localization on a heterologous mRNA. When the region from position 667–953 (containing the putative transport signal but not the localization signal) was attached to globin mRNA (Fig. 1 I), granules were observed in the perikaryon and processes but not in the myelin compartment, indicating that this region is sufficient to confer transport but not localization on a heterologous mRNA. These results delineate two distinct regions in the 3' UTR of MBP mRNA: one region (667–953) that contains signals required for transport, and a second region (1131–1473) that contains signals required for localization.

When RNAs smaller than \sim 500 bases were injected into oligodendrocytes, they dispersed nonspecifically throughout the perikaryon, processes, and myelin compartment, presumably due to diffusion (data not shown). Therefore, to assay specific transport and/or localization signals, it was necessary to inject RNAs $>$ 500 bases and to use appropriate size, matched controls. Accordingly, to assay the transport and localization properties of the 3' UTR region from 667–953 in the absence of other MBP mRNA sequences, the RNA was extended with nonspecific, vector-derived RNA sequences (Fig. 1 K). With this RNA, granules were observed in the perikaryon, processes, and myelin compartment (Fig. 2 A) indicating that, in the absence of a coding region, the region from 667–953 is sufficient for both transport and localization. This is in contrast to the results with RNA containing the region from 667–953 attached to the coding region from either MBP mRNA (Fig. 1 E) or globin mRNA (Fig. 1 I), which was transported but

not localized. There are two possible explanations: either the coding region contains elements that inhibit localization in the absence of the specific localization region from MBP mRNA, or the vector sequences in Fig. 1 *K* contain elements that mediate localization.

Sequence Analysis of Transport and Localization Elements in MBP mRNA

Sequence comparison methods were used to identify conserved elements within the transport and localization regions delineated by the deletion analysis described above. In the case of the transport element, we reasoned that RNA transport may use similar mechanisms in different systems. We have shown previously that microinjected MBP mRNA is transported in neuroblastoma cells (which do not express endogenous MBP mRNA), as well as in oligodendrocytes (Ainger et al., 1993), indicating that the transport machinery is conserved in different cell types. It follows that the transport signal may also be conserved in different transported RNAs. In most other systems it is not possible to differentiate RNA transport from RNA localization, because they are not spatially separated as in oligodendrocytes. However, it is reasonable to assume that RNAs that are localized are also transported and thus may contain transport signals. Therefore, we compared the sequence of MBP mRNA with sequences of mRNAs that are localized in other systems, including myelin-associated/oligodendrocytic basic protein (MOBP 81A), which is localized to oligodendrocyte processes (Holz et al., 1996); GFAP, which is localized in astrocyte processes (Sarchy et al., 1989); MAP2A and activity regulated cytoskeleton-associated protein (ARC), which are localized to dendrites of hippocampal neurons (Garner et al., 1988; Link et al., 1995; Lyford et al., 1995); neurogranin (RC3), which is localized in neurons (Landry et al., 1994); N-type calcium channel (Ca-N), GABA receptor α subunit (GABAR(A)), and nitric oxide synthase (NOS), which are localized to dendritic growth cones (Crino and Eberwine, 1996); and protamine 2, which is localized in spermatocytes (Braun et al., 1989).

As depicted in Table I, a 21-nucleotide sequence (from 794 to 814) within the transport region in the 3' UTR of rat 14 kD MBP, which is conserved in mouse and human MBP mRNA, is homologous to sequences from the 3' UTR of MOBP 81A, GABAR(A), and protamine 2, the coding regions of GFAP, Ca-N, MAP 2A, and ARC, and the 5' UTR of NOS and RC3. The regions of homology have 13 or more identical bases within the 21-base segment. In the case of MOBP 81A and MAP 2A, the homologous sequences are present in localized mRNA isoforms but absent from nonlocalized isoforms (Holz et al., 1996; Kindler et al., 1996), suggesting that the region containing this sequence is necessary for transport of these mRNAs, as it is for MBP mRNA. In the case of protamine 2, the homologous sequence is present in a region of the mRNA that is required for localization in spermatocytes (Braun et al., 1989). Furthermore, protamine 2 mRNA (containing the homology region) is transported when microinjected into oligodendrocytes (data not shown), indicating that the 3' UTR of protamine 2 mRNA contains sequences that can mediate transport in oligodendrocytes. In the case of

Table I. RTS Homology in mRNAs

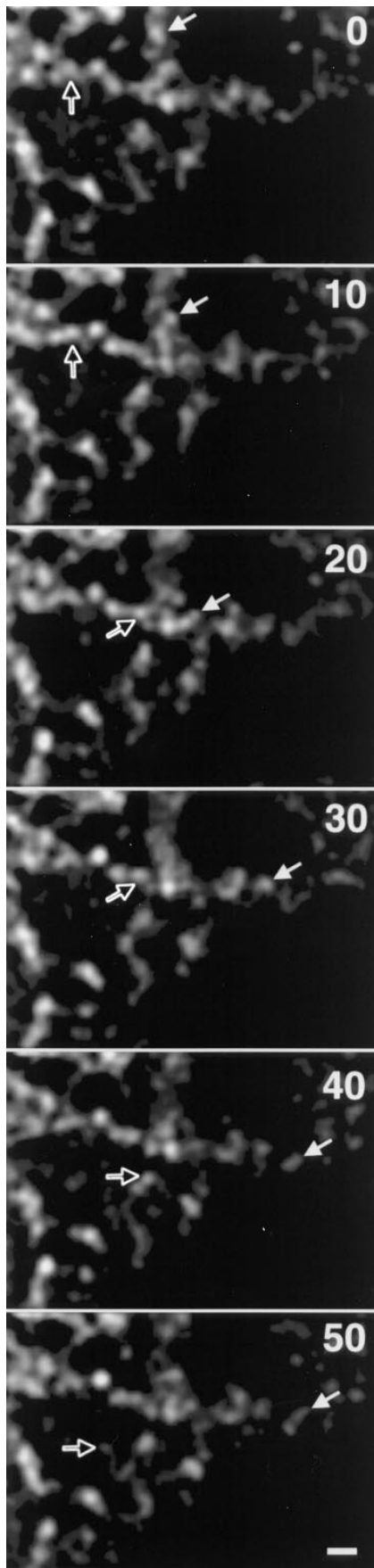
Species	mRNA	Region	RTS sequence
Transported RNAs			
Rat	MBP	3'UTR	GCCAAGGAGCCAGAGAGCAUG
Mouse	MBP	3'UTR	GCCAAGGAGCCAGAGAGCAUG
Human	MBP RTS1	3'UTR	GCCAUGGAGGCACACAGC UG
Human	MBP RTS2	3'UTR	GCUGCAGAGACAGAGAGGACG
Rat	MOBP81A	3'UTR	ACCCCGGAGACACAGAGCAUG
Rat	GFAP	ORF	GCCAAGGAGCCACCAACACUG
Human	Ca-N	ORF	GCCAAGGAGCCAGAGAGGGUG
Mouse	MAP2A	ORF	GCCAAGGAGUCAGAGAGAGAU
Bovine	GABAR(A)	3'UTR	GAGAGGGAGCCAGAGAGCAAA
Bovine	NOS	5'UTR	CACGAGGAGCCACAGAGCAGA
Rat	ARC	ORF	GCUGAGGAGGAGGAGAUCAUU
Rat	RC3	5'UTR	GCCAAGGAGCCUACACACCGG
Mouse	protamine 2	3'UTR	GCCAAGGAGCCACGAGAUUCG
Other RNAs			
Fugu	NCAML1	ORF	GCCCAGGAGCCAGAGAACAUA
Rat	c-jun (AP-1)	3'UTR	GGCGAGGAGCCAGAGAGCAGC
Rat	rARP	ORF	GCCGAGGAGCCAGAGAGCCCU
Human	clathrin	ORF	ACCCAGGAGCCUGAGAGCAUC
Rat	KCC1	ORF	GCCAAGGAGCCAAAGGAGCAG
Mouse	PKC α	5'UTR	GCCAGCGAGCCAGAGAGCCGG
Human	chromogranin	ORF	GACCAGGAGCUGGAGAGCCUG
Human	phosphorylase	ORF	GCCAAGGAGCCAGACUGCUUC
Mouse	cyritestin	ORF	GCCAAGGAGGCAGAGACACAA
Mouse	HBP44	3'UTR	UCCCUAGAGCCAGAGCGCAUG
Human	LIMK	ORF	GCCAUGGAGCCAGAGAGUGAG
Human	IGFR1	ORF	GACCUGGAGCCAGAGAACAU
Mouse	FLI-2	ORF	GCCAAGGAGCCAGAACAGCUG
HIV-2	tat, vpr	ORF	UUGAAGGAGCCAGAGAGCUCA
Hepatitis C	NS-5	ORF	GCAAGGGGGCCAGAGAGCAUC
	Consensus		GCCAAGGAGCCAGAGAGCAUG
	Consensus	ORF	A K E P E S M

RTS homology in mRNAs known to be transported and in other mRNAs. All sequences are from mature RNAs. The database accession numbers are given in Material and Methods. The species and location of the RTS homology are indicated. Bases identical to the consensus are given in standard type (non-bold face). Single letter amino acid code of the consensus reading frame is shown below the consensus RTS sequence. Note that the number of matches in protamine 2 and MAP 2A mRNAs can be increased by single base insertions or deletions.

ARC, activity-related cytoskeleton-associated protein; Ca-N, N-type calcium channel α 1; GABAR(A), γ amino butyric acid receptor α subunit; GFAP, glial fibrillary acidic protein; HBP, heparin-binding protein; HIV, human immune deficiency virus; IGFR, insulin-like growth factor receptor 1; KCC1, furosemide-sensitive K-Cl cotransporter; LIMK, LIM kinase; MOBP, myelin-associated/oligodendrocytic basic protein; NCAM, neural cell adhesion molecule; NOS, nitric oxide synthase; PKC, protein kinase c; rARP, rat atrophin-1-related protein; RC3, neurogranin.

GFAP, Ca-N, GABAR(A), NOS, ARC, and RC3, the regions required for localization have not been identified experimentally. The 21-nucleotide sequence present in these different transported mRNAs represents a putative transport element and thus is designated the RTS.

Close inspection of the 21-nucleotide RTS consensus sequence shown in Table I reveals that it contains two partially overlapping, homologous decanucleotide sequences: GCCAAGGAGC and GCCAGAGAGC, differing only by inversion of an AG dinucleotide in the center of the sequence. Most of the transported mRNAs in Table I contain perfect or almost perfect matches to one or the other of these decanucleotide sequences. It is possible that transport requires both sequences or that one or the other of these sequences by itself may be sufficient to mediate RNA transport. This has not been tested experimentally.



If the RTS is a general transport signal, the presence of an RTS homology region in a particular RNA may be indicative of transport. A search of the Genbank/EMBL/DBJ database revealed several additional RNAs with RTS homology regions, as shown in Table I. It is not known whether these RNAs are transported. However, the presence of the RTS homology region suggests that they may be and that the RTS may constitute a general RNA transport signal that is used in a variety of different systems.

Although most previously described elements required for RNA localization are found in 3' UTRs, some of the RTS homologies were found within coding regions, as indicated in Table I. Surprisingly, in all cases the translational reading frame was conserved with respect to the RTS, although there were differences in the encoded peptide sequences due to noncanonical bases within the RTS. The significance of conservation of reading frame in relation to RTS function is not known.

The RTS in MBP mRNA is flanked by U-rich repeats (not shown in Table I) that have a consensus of CUUUS-UUU (where S = C or G). There are three repeats in rat, mouse, and human MBP mRNA. Some of the RTS-containing mRNAs in Table I also have U-rich sequences flanking the RTS. In addition, there is a GGACT motif 8–12 bases downstream of the RTS in rat, mouse, and human MBP mRNAs. This motif has been shown to be involved in localization of β -actin mRNA to the lamellipodia of fibroblasts (Kislauskis et al., 1994). The relevance of sequences flanking the RTS to MBP mRNA transport has yet to be examined experimentally.

Microinjection Assay of RTS Function

To test the function of the RTS in the context of a heterologous mRNA, the 21-nucleotide RTS sequence was attached to the 3' end of PLP RNA (Fig. 1 M) or GFP RNA (Fig. 1 O), neither of which is transported in oligodendrocytes. The chimeric PLP-RTS RNA (Fig. 1 N) and GFP-RTS RNA (Fig. 1 P) were both assembled into granules that were transported to the processes but not localized to the myelin compartment. This indicates that the RTS is necessary and sufficient for transport along the processes but not for localization to the myelin compartment.

Insertion of the RTS into either PLP or GFP mRNA results in redistribution of the RNA from the perikaryon to the processes. This is interpreted as evidence that the RTS mediates directed RNA transport in the processes. However, it could be argued that PLP and GFP RNA contain perikaryon retention signals that are disrupted by insertion of the RTS, allowing the RNA to move to the pro-

Figure 3. Time-lapse confocal imaging of RNA movement in oligodendrocyte microinjected with fluorescent PLP-RTS RNA. PLP-RTS RNA was fluorescently labeled and microinjected into oligodendrocytes. Time-lapse confocal images were collected at 10-s intervals. The region shown contains several oligodendrocyte processes. The cell body is out of the frame to the left. The majority of the labeled granules were immobile during the time interval shown. Two granules (indicated by *open* and *closed arrows*) exhibited sustained vectorial motion in an anterograde direction during the time course shown. Bar, 1 μ m.

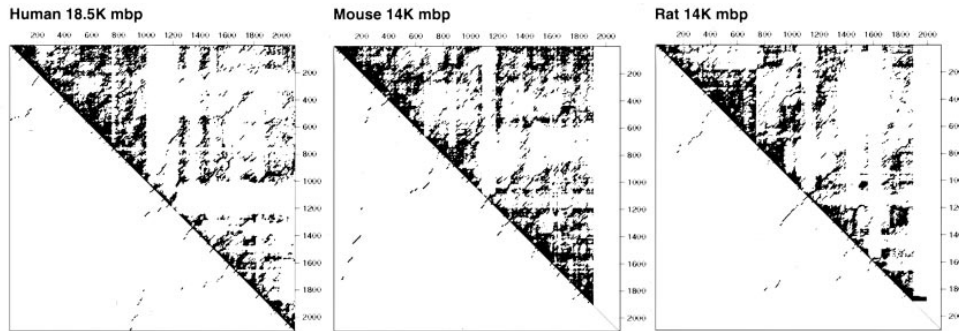
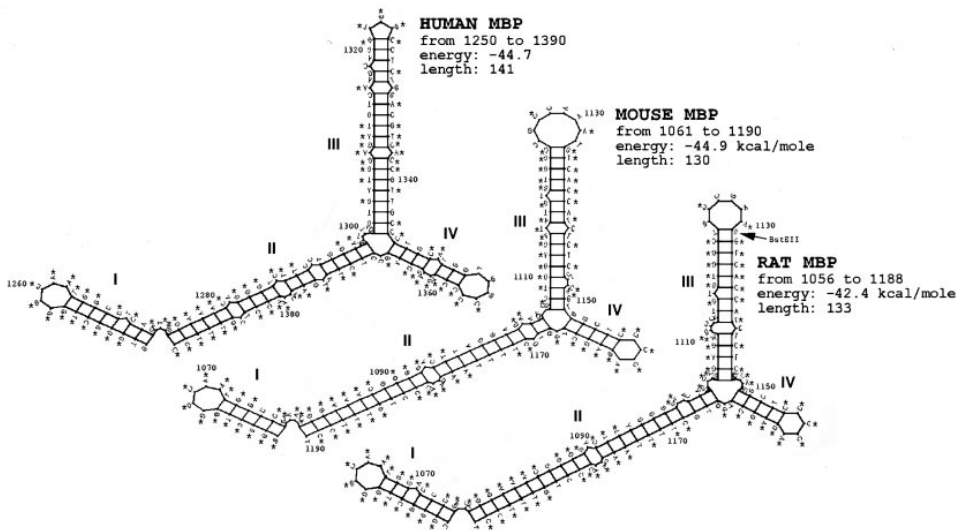
A**B**

Figure 4. Secondary structure predictions for MBP mRNA. (A) Energy dot plots of predicted secondary structures in mRNAs encoding rat 14-kD, mouse 14-kD, and human 18.5-kD MBP. Suboptimal structures within 10 kcal/mole of the optimal structures are plotted above the diagonal. Sparsely populated vertical and horizontal regions indicate possible stable structures. Optimal structures are plotted below the diagonal. (B) Predicted secondary structures for rat 14 kD MBP mRNA from base 1,056 to 1,188, mouse 14-kD MBP mRNA from 1,061 to 1,190, and human MBP 18.5-kD mRNA from base 1,250 to 1,390. Asterisks denote bases conserved in rat, mouse, and human MBP mRNAs. The BstEII site used to create the RNA in Fig. 1 D is indicated on the rat structure.

cesses by passive diffusion. To determine if RTS RNA in the processes exhibits directed transport or passive diffusion, fluorescently labeled PLP-RTS RNA was microinjected and visualized in living cells by time lapse confocal microscopy. A series of frames showing portions of several processes from an injected oligodendrocyte is shown in Fig. 3. The majority of the labeled granules in the processes are immobile. However at least two labeled granules (indicated by arrows) exhibit sustained anterograde vectorial motion (mean displacement, 0.1–0.2 $\mu\text{m}/\text{sec}$) during the time course shown. Although this does not rule out diffusion as a possible mechanism for RNA movement, it does provide evidence for directed RNA transport of at least some RTS-RNA-containing granules.

Predicted Secondary Structure of the MBP 3' UTR

While RNA transport may be a general phenomenon involving conserved sequences in different mRNAs in different systems, localization of MBP mRNA to the myelin compartment of the oligodendrocyte is likely to be a cell

type-specific phenomenon because of the unique morphology and biochemical composition of the myelin membrane. If localization of MBP mRNA to the myelin compartment occurs by the same mechanism in oligodendrocytes from different species, it might involve RNA sequences or structures that are conserved among MBP mRNAs from different species. Sequence comparisons of the RNA localization regions (RLR) from human, mouse, and rat MBP mRNA did not reveal any particularly highly conserved regions (data not shown). Overall, the RLR was $\sim 70\%$ homologous among the three species, which is similar to the overall homology in the 3' UTR.

RNA secondary structure can be conserved despite considerable sequence divergence. To identify potential conserved RNA secondary structures, the sequences of rat, mouse, and human MBP mRNA were analyzed using the MFOLD program of Jacobson and Zuker (1993). The results of the secondary structure predictions are shown in Fig. 4. Suboptimal energy dot plots for rat 14-kD MBP mRNA, mouse 14 kD MBP mRNA, and human 18.5-kD MBP mRNA are shown in Fig. 5 A. In these plots, poten-

tially stable structures are indicated by sparsely populated horizontal and vertical areas. A single, well determined structure can be discerned in the region corresponding to the RLR (1,056 to 1,188) in rat MBP mRNA and in the corresponding region of mouse MBP mRNA (1,061 to 1,190). Two well determined structures can be discerned within the human MBP mRNA: the first region (1,004 to 1,248) is just downstream of the RTS region (927 to 967); the second region (1,250 to 1,390) corresponds to the regions of predicted, stable secondary structure in rat and mouse MBP mRNA. Predicted structures for the RLR regions of rat, mouse, and human MBP mRNAs are shown in Fig. 4 B. The overall structure and the distance from the RTS are conserved despite significant sequence divergence among the three species. Truncation of the RNA at the BstEII site shown in rat MBP mRNA structure interferes with localization (as shown by the RNA in Fig. 1 D), suggesting that disruption of the potential structure in MBP mRNA by deletion interferes with localization. We propose that some or all of the predicted secondary structures shown in Fig. 5 may comprise an element necessary for localization of MBP mRNA to the myelin membranes.

To estimate the significance of the predicted secondary structures in the MBP mRNAs, several other mRNAs (2',3'-cyclic nucleotide 3'-phosphodiesterase [CNPase; Bernier et al., 1987], Vg1 [Weeks and Melton, 1987], and GFAP) were analyzed with the MFOLD program. These mRNAs were selected because one (CNPase) is expressed by oligodendrocytes, while the other two are transported mRNAs expressed in other cell types. The number, shape, and positions of predicted secondary structures in these mRNAs were all clearly different than the MBP mRNAs (data not shown), suggesting that the predicted secondary structure in the RLR of MBP mRNA is significant.

Discussion

The subcellular trafficking pathway for MBP mRNA in the oligodendrocyte involves three distinct sorting steps: (a) assembly into granules, (b) transport along the processes, and (c) localization to the myelin compartment. The results presented here identify specific regions in the 3' UTR of MBP mRNA that are required for the last two of these steps. The first is a 21-nucleotide sequence, the RTS, that is necessary and sufficient for transport of mRNA along oligodendrocyte processes. Homologous sequences are found in other transported mRNAs, suggesting that the RTS represents a general signal to direct RNAs to the transport apparatus of the cell. The second is a region, the RLR, with predicted stable secondary structure, that is required for localization of MBP mRNA to myelin. The RLR may be involved in anchoring or stabilizing MBP mRNA in the myelin compartment of the oligodendrocyte.

It is generally assumed that the function of transport and localization of MBP mRNA *in vivo*, is to target MBP expression to the developing myelin sheath in the CNS. Thus, the RTS and RLR in the 3' UTR of MBP mRNA may be required for normal myelination *in vivo*. In this regard, a transgene consisting of a truncated cDNA fragment containing the coding region, RTS and RLR of mouse 14-kD MBP mRNA (equivalent to the RNA in Fig.

1 C) was able to completely rescue the shiverer mutation, a deletion of the endogenous MBP gene (Kimura et al., 1989), suggesting that these regions are sufficient for normal targeting of MBP expression *in vivo*. The presence of the RTS in localized isoforms of MOBP 81A RNA (Holz et al., 1996) suggests that the RTS functions to target expression of other proteins, in addition to MBP, in oligodendrocytes.

RTS homologous sequences are found in a variety of different RNAs, some of which are known to be localized in other cell types. If the RTS represents a *cis*-acting transport signal that is conserved in different RNAs, it presumably interacts with a cognate *trans*-acting factor that is expressed in different cell types. *In vitro* binding experiments indicate that the RTS sequence interacts specifically with hnRNP A2 (Hoek et al., personal communication), a member of a family of hnRNP proteins that shuttle between the nucleus and cytoplasm. Furthermore, antisense suppression of hnRNP A2 in oligodendrocytes inhibits transport of microinjected MBP mRNA (Hoek et al., personal communication). These results suggest that hnRNP A2 binding to the RTS is required for transport of RTS-containing RNA in oligodendrocytes. Since hnRNP A2 is expressed in a variety of different cell types, and since RTS-homologous sequences are found in a variety of different mRNAs, hnRNP A2 binding to RTS homologous sequences may be a general feature of RNA transport.

In several RNAs, an RTS-homologous sequence is located within the coding region. In each case the translational reading frame is conserved. This may be a consequence of the constraints of coding for both protein sequence and RNA transport, or it may represent a protein structural element common to these proteins. The strongest evidence that an RTS-homologous sequence within a coding region mediates RNA transport comes from work on localization of alternatively spliced forms of MAP2 mRNA (Kindler et al., 1996). The RTS-homologous sequence in MAP2 mRNA is contained within a coding exon that is included in localized MAP2 mRNA isoforms but excluded from nonlocalized isoforms.

The RLR is required for localization of RNAs that contain a protein-coding region. In the absence of a coding region, the region containing the RTS is sufficient for both transport and localization. It appears that the presence of a coding region *per se* renders localization RLR dependent rather than specific sequences within the coding region, since localization was RLR dependent for RNAs containing either the MBP or globin-coding regions. It is formally possible that any 5' flanking RNA adjacent to the RTS will render localization RLR dependent. However, a more likely explanation is that the function of the coding region in translation regulates the distribution of the RNA such that localization of coding mRNA occurs by a different pathway than localization of noncoding RNA. As defined in this work, localization involves translocation from the processes to the myelin. This presumably requires dissociation from the transport apparatus in the processes and, in the case of coding mRNAs, association with the translational machinery in the myelin compartment. Translocation across the boundary between these two compartments is RLR dependent for coding RNA and RLR independent for noncoding RNA. One possible explanation is

that coding RNA associates with ribosomes, which may render translocation RLR dependent, while translocation of ribosome-free noncoding RNA is RLR independent. This would be analogous to the role of ribosomes in regulating recognition of the correct compartment for localization in the secretory pathway (Bacher et al., 1996).

The RLR contains a predicted stable secondary structure at a conserved position in rat, mouse, and human MBP mRNA. The limited phylogenetic data available makes it difficult to assess the accuracy of the predicted structures. Also, the MFOLD program, which was used to predict the secondary structures, uses only conventional Watson/Crick base pairing and G-U pairs. Nuclear magnetic resonance studies have shown that non-Watson/Crick base pairs and triplets can also contribute substantial energy to RNA structures (Wimberly et al., 1993). Determining the significance of the predicted secondary structures will require additional experimental evidence.

All the RNAs used in this study assembled into granules in the perikaryon soon after injection. The granules represent the unit of RNA transport and localization. Granules are novel organelles that have been visualized with several different methods in both live and fixed oligodendrocytes (Ainger et al., 1993) as well as in a variety of other cell types (Ferrandon et al., 1994; Wang and Hazelrigg, 1994; Forristall et al., 1995). Endogenous MBP RNA-containing granules in oligodendrocytes contain many components of the translational machinery, suggesting that they also represent the unit of RNA translation (Barbarese et al., 1995). Thus, the granule represents a common unit for transport, localization, and translation of RNA. The mechanism(s) responsible for coordinate regulation of these functions within the granule are not known.

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