Target specificity of neuronal RNA-binding protein, Mel-N1: direct binding to the 3′ untranslated region of its own mRNA

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ABSTRACT

We have identified cDNAs encoding Mel-N1, the mouse homologue of a human nervous system-specific RNA-binding protein, Hel-N1. Two major mRNA transcripts of Mel-N1 were detected predominantly in the adult mouse brain by Northern blot analysis. To gain insight into the RNA binding specificity of Mel-N1, we performed iterative *in vitro* RNA selection. The resulting *in vitro* selected RNAs were found to contain AU-rich sequences as well as a GAAA motif in the majority of clones. By means of *in vitro* binding assays we demonstrate that this GAAA sequence appears to significantly affect the Mel-N1 RNA-binding efficiency. Our studies further reveal that Mel-N1 can bind to its own 3′ untranslated region (3′UTR) as well as to the *c-fos* 3′UTR, and is localized predominantly in the cytoplasmic region in cells, suggesting that post-transcriptional autoregulation of Mel-N1 gene expression occurs *in vivo*.

INTRODUCTION

In eukaryotes, RNA-binding proteins have been found to play important roles in various aspects of post-transcription regulation of gene expression (1,2). One well-characterized group of RNA-binding proteins that have been shown to be involved in many RNA processing events, such as pre-mRNA splicing and polyadenylation, are those that contain an RNA recognition motif (RRM) (3–12). The RRM consists of ~90 amino acids, is structurally characterized by the presence of two α helices and four β strands (9,13,14), and functions as an RNA-binding domain (RBD) (10,15–19).

Recently, a group of nervous system-specific RNA binding proteins with RRM-type RBDs have been identified. Elav is a neuron-specific protein, of which deficiency causes abnormal neural development and leads to embryonic lethality in *Drosophila melanogaster* (20–22). It is also required for maintenance of the nervous system in adult flies (23,24). Rbp9 is another fly Elav-like protein which is also restricted to the nervous system (25). HuC and HuD, two human proteins which were initially identified as antigens recognized by autoimmune Hu antibodies of patients with paraneoplastic neurologic disorders (26,27) are related to Hel-N1, a human Elav homologue which can also be recognized by Hu antibodies (28). These proteins share extensive similarity to Elav and are believed to be members of the mammalian Elav homologue family.

The restricted expression of the Hu proteins and their rodent counterparts in the nervous system, suggest that like Elav, they may play important roles in neuronal cell regulation (29–31). Hel-N1 and HuD have been shown to bind to specific sequences within the 3′ untranslated regions (3′UTRs) of mRNAs which encode cell proliferation regulatory elements (28,30,32). The 3′UTRs of these mRNAs contain an AU-rich element (ARE) which is characterized by the presence of an AUUUA pentamer and are generally AU-rich. Since AREs have been demonstrated to influence mRNA stability (33–38), Hu proteins may regulate the expression of particular mRNAs by altering their stability, thereby contributing to neuronal differentiation and/or maintenance.

In this study, we report the cloning and sequence determination of a cDNA encoding a mouse Hu protein, Mel-N1, which is homologous to human Hel-N1. Using a Mel-N1 fusion protein, we performed iterative *in vitro* RNA selection analysis and show that Mel-N1 binds to RNAs containing ARE-like motifs, and that this binding may be further modulated by specific sequences downstream of the ARE-like motif. Moreover, we propose that autoregulated expression of Mel-N1 may occur via binding to the 3′UTR of its own mRNA.

MATERIALS AND METHODS

Screening and sequencing

A newborn mouse brain cDNA library (Stratagene) was screened according to previously described methods (39). Positive recombinant λ ZAPII phages were subjected to plasmid rescue using helper phage and plasmid DNA was obtained by conventional methods (39). Sequencing was performed using Sequenase version 2.0 kit (United States Biochemicals).

Northern blot analysis

A mouse MTN Blot (Clontech) was used for Northern blot analysis. The RNA from each tissue was checked both qualitatively and quantitatively with a human glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA (Clontech) control probe. Hybridization was performed as described under high stringency conditions (39). A Fuji BAS-2000 imaging analyzer and standard autoradiography were used to analyze the hybridization patterns.

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Table 1. *In vitro* selected RNA sequences by Mel-N1

Clones	Selected sequences[a]
13-2, 13-3, 18-1, 18-2, 20-1, 20-2, 21-1, 21-2, 22-1, 23-2, 27, 28-2, 28-3, 30, 31	**CAUU AUAAUUUG**AGU<u>GAAA</u>UGG GUUG
16	**CAUU AUAAUUUG**AGU<u>GAAA</u>UG GUUG
13-1	**CAUU UUAUUUGUUACGUUUA<u>GAAA</u>UCA GUUG**
28-1	**CAUU UUUAUUUG**GUACGUUA<u>GAAA</u>GCUA GUUG
22-2	**CAUU UGAUUUU**UUUUUUUCG GUUG
23-1	**CAUU UGAUUUU**UUUCG GUUG

[a]The first and last four nucleotides in the sequences are derived from the primers used in PCR amplification step. ARE-like sequences are bold-faced. The GAAA sequence observed with 18 of 20 clones are underlined. All clones contained <25 nt in the randomized region, possibly due to unknown error in the PCR amplification step.

Construction of Mel-N1 expression plasmids

DNA containing the Mel-N1 coding region was PCR-amplified using the following synthetic primers: 5′-TCG GAT CCA TGG AAA CAC AAC TGT C-3′ and 5′-CCG AAT TCG AGC TCA TTA GGC TTT G-3′. The amplified fragment was cloned into the *Eco*RI and *Bam*HI sites of a bacterial expression vector, pGEX-2T (Pharmacia). The resultant plasmid, pGEX-Mel-N1, was transformed into *Escherichia coli* XL1-blue, GST-Mel-N1 fusion protein was induced with 1 mM IPTG for 4 h and affinity-purified by glutathione–Sepharose. pEFT7-Mel-N1 was made by inserting the entire Mel-N1 coding region into the *Bam*HI site of pEF-BOS-T7, a derivative of pEF-BOS (40,41) kindly provided by M. Ohno of Kyoto University.

In vitro selection and RNA binding assay

In vitro selection using GST-Mel-N1 was performed as described previously (17) with the following modifications. During the last two rounds of selection, the KCl concentration was raised to 350 mM in the binding and washing buffers, and the final round of washing buffer contained 0.5 M urea. *In vitro* RNA synthesis was performed as described previously (17). RNA binding reaction mixture [containing ~0.3 μg GST-Mel-N1, labeled RNA (2 × 10⁴ c.p.m.), 1 μg yeast RNA and 55 U RNase inhibitor in the RNA binding buffer] was incubated at 20°C for 20 min followed by UV light irradiation and RNase digestion and then analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) as described previously (46). The efficiency of label transfer to GST-Mel-N1 with each selected RNA was calculated by densitometry using a Fuji BAS 2000 Image Analyzer. The GAAA sequence of Mel-N1-selected clone 27 was changed to CCCC sequence by PCR-based mutagenesis. Sequence confirmation of the resulting clone (mu27) however, revealed an additional single nucleotide deletion just upstream of the target sequence. FosA and FosB RNAs were synthesized using the following DNAs. FosA1 (sense): 5′-AAT TCG TTT TTA TTG TGT TTT CAA TTT ATT TAT TAA G-3′, FosA2 (anti-sense): 5′-GAT CCT TAA TAA ATA AAT TGA AAA CAC AAT AAA AAC G-3′, FosB1 (sense): 5′-AAT TCA TAT TTA TAT TTT TAT TTT ATT TTT TTC TAG-3′, FosB2 (anti-sense): 5′-GAT CCT AGA AAA AAA TAA AAT AAA AAT ATA AAT ATG-3′. Two pairs of sense and anti-sense DNAs (FosA1–FosA2 and FosB1–FosB2) were annealed and introduced into *Eco*RI and *Bam*HI sites of pBluescriptII SK⁺. The resultant plasmids were

digested with *Xba*I and used as templates for *in vitro* transcription with T7 RNA polymerase.

Transfections and immunofluorescence

HeLa and COS7 cells were plated 24 h before transfection at ~30% confluence on circular 15 mm coverglasses. Transfection was performed using Lipofectoamine (Gibco BRL). Twelve hours after transfection, cells were fixed, incubated with anti-T7 tag mouse monoclonal antibody (Novagen) and FITC-labelled anti-mouse immunoglobulin secondary antibody (DACO, F479), and examined under a Zeiss confocal laser microscope. Anti-T7 tag and anti-mouse immunoglobulin antibodies were used at dilutions of 1:1000 and 1:200 respectively. Cells were double-stained with DAPI to localize nuclei.

RESULTS

Cloning and tissue-specific expression of Mel-N1

During cDNA screening for mouse homologues of HuD from a brain cDNA library, we obtained a fragment which showed homology to the third RRM of Hel-N1 (31). Using that cDNA fragment as a probe, we screened another mouse brain cDNA library for longer clones. From ~5 × 10⁵ recombinants, two positive clones were isolated and sequenced. The longest insert had an open reading frame encoding a 360 amino-acid protein containing three RBDs (Fig. 1). The deduced protein showed 99% identity to the human Hel-N1 and was designated Mel-N1. Interestingly, the Mel-N1 cDNA contains a long AU-rich 3′UTR of ~1 kb. The AU content (66.2%) of the 3′UTR is significantly higher than that of the 5′UTR (26.6%) and the protein coding region (53.4%).

We examined the expression of Mel-N1 in mouse tissues by Northern blot analysis and found that two mRNA transcripts of 5.4 and 4.9 kb are expressed in almost equal amounts in the brain (Fig. 2). A weak 4.9 kb band was also observed in the testis RNA after prolonged exposure (data not shown), as is the case with the mouse HuD homologue, mHuD (31). Thus Mel-N1 is expressed predominantly in brain tissue, indicating that similar to its human counterpart, it is a nervous system-specific RNA-binding protein.

In vitro selection of RNAs with affinity for Mel-N1

To determine the RNA binding ability of Mel-N1, we constructed a GST fusion protein to take advantage of iterative *in vitro* ligand

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              10        20        30        40        50        60        70        80        90        100       110       120
   1 GCGCGGCGCGCCCAGCGTCACTGAGGCAGCAGCCGGTCGGTAGGGCGGGGGTTCCGTCGTGTTCCAGTCCGCCTTCCCGCCGCCGCCAGCGTGACTCCTCTGAGCGGTGCTGTCGGCCGGCG
 121 GCGCGAGCGTGGGAGCTGCAGCCGAGCCGCAGCAGCAGGTAATTGCTGCCATGGAAACACAACTGTCTAATGGGCCAACTTGCAATAACACAGCCAATGGTCCAACCACTGTAAACAACA
                                                        M  E  T  Q  L  S  N  G  P  T  C  N  N  T  A  N  G  P  T  T  V  N  N  N
 241 ACTGCTCCTCACCAGTTGACTCCGGGAACACAGAGGACAGCAAGACCAACCTAATAGTCAACTACCTTCCTCAGAACATGACACAGGAAGAACTAAAGAGTCTTTTTGGGAGCATTGGCG
       C  S  S  P  V  D  S  G  N  T  E  D  S  K  T  N  ┌───────────────┐ P  Q  N  M  T  Q  E  E  L  K  S  L  F  G  S  I  G  E
 361 AGATAGAGTCCTGTAAGCTTGTAAGAGACAAAATAACAGGGCAGAGCTTGGGTTACGGATTTGTGAACTACATTGACCCCAAGGATGCAGAGAAAGCTATCAACACTCTGAATGGATTGA
       I  E  S  C  K  L  V  R  D  K  I  T  G  Q  S ┌L  G  Y  G  F  V  N  Y┐ I  D  P  K  D  A  E  K  A  I  N  T  L  N  G  L  R
 481 GACTTCAAACCCAAAACAATAAAAAGTTTCCTATGCTCGCCCAAGCTCAGCCTCAATCAGAGATGCAAACTTATACGTCAGCGGGCCTTCCAAAAACCATGACCCAGAAGGAGCTGGAACAGC
       L  Q  T  K  T  I  K  V  S  Y  A  R  P  S  S  A  S  I  R  D  A  N ┌E  Y  V  S  G  L┐ P  K  T  M  T  Q  K  E  L  E  Q  L
 601 TGTTTTTCACAGTATGGGCGCATCATTACTTCTCGGATTCTTGTCGACCAGGTCACTGGCATATCAAGGGGGTGTAGGGTTTATTCGATTTGACAAGCGGATTGAGGCAGAAGAAGCTATCA
       F  S  Q  Y  G  R  I  I  T  S  R  I  L  V  D  Q  V  T  G  I  S ┌R  G  V  G  F  I  R  F┐ D  K  R  I  E  A  E  E  A  I  K
 721 AAGGCCTAAATGGCCAGAAACCTCCTGGTGCCACAGAGCCAATCACTGTAAAGTTTGCTAATAATCCAAGCCAAAAAACCAATCAGGCCATCCTTTCCCAGCTGTACCAGTCTCCAAACA
       G  L  N  G  Q  K  P  P  G  A  T  E  P  I  T  V  K  F  A  N  N  P  S  Q  K  T  N  Q  A  I  L  S  Q  L  Y  Q  S  P  N  R
 841 GAAGGTACCCAGGACCGCTAGCTCAGCAGGCACAGCGTTTTAGGTTGGACAATCTGCTCAATATGGCTTATGGAGTGAAGAGTAGGTTTTCTCCAATGACGATCGATGGAATGACCAGTC
       R  Y  P  G  P  L  A  Q  Q  A  Q  R  F  R  L  D  N  L  L  N  M  A  Y  G  V  K  S  R  F  S  P  M  T  I  D  G  M  T  S  L
 961 TGGCTGGAATTAATATTCCTGGGCACCCTGGAACAGGCTGGTGCATATTTGTGTACAACCTGGCGCCTGATGCAGATGAAAGTATCCTGTGGCAAATGTTTGGGCCTTTTGGAGCTGTCA
       A  G  I  N  I  P  G  H  P  G  T  G  W  C ┌E  S  V  Y  N  L┐ A  P  D  A  D  E  S  I  L  W  Q  M  F  G  P  F  G  A  V  T
1081 CCAACGTGAAGGTCATCCGTGACTTTAACACCAATAAGTGCAAAGGTTTTGGATTTGTGACTATGACAAACTATGATGAGGCGGCCATGGCGATAGCTAGCCTCAATGGATACCGCCTGG
       N  V  K  V  I  R  D  F  N  T  N  K  C ┌K  G  F  G  F  V  T  M┐ T  N  Y  D  E  A  A  M  A  I  A  S  L  N  G  Y  R  L  G
1201 GAGACAGAGTCCTGCAGGTCTCCTTTAAGACAAACAAAACGCACAAAGCCTAATGAGCTCTTGTCCTCAGTCCATTTATATATGAAAACTATACAACAAAAGCAAGTTAAGAGAAACTTT
       D  R  V  L  Q  V  S  F  K  T  N  K  T  H  K  A  *
1321 ATACATTAGTAAATGTCTTTGTAAGTCAGTGTTGAGATGGGGATAAATGACTACTTAGCATCCTAAGAAATATGTGAGATTTTTTTATTGCTAGTATTTGAATTAAAACTTCTTAAATATC
1441 TTTTATGTTTGAATATGGACAAGAGGTACAGGGTTTTTACCTGTCACATTGCATTCTATTGCCTTCTTTGAAGAAGGTGGACCTTTTAAAGTGTTTCAGCTAAGGGAAGACATTTCTTTT
1561 CTTTTTACATAACTGCCTTGATCCTGTGAGTAAGTACTGAGGCTTGTGTGTTGTAGCTCTTCAGTTGGTTGTCTTCCCCACCTCCTTATCTTTCCTTTTCTTTCTCTTTCTTTTGTCTTT
1681 GTTTTTTCCTTTCCATCTTTAATTAGCTTTGTGTTTGGTTTACATTTCAAGCATTGCTGTTATGTCTCAGAAAAGCATTTTGAAGTTTACATTTTTATTTATTGAAGTTACAAAACAGTAT
1801 TTATTTTGTAATTCTGATTTGGGCTGGGCAGGGGGGGCGACATTATAAACGCTTATTGTAAGAATACTGGAGAACTTTTCGTAAAGCAGTACCTTGCCAAAGAGATAAGAGCCTCTTTG
1921 ATGTGGGTTTAAAAAAAGCATCTATTTTTATAAAAAAGAAAATTTGGAGAAACTTTTTACTGGTCCTGGAACAAATATTTTGACTTGAATACTTTGAAAATCTCTTCATATGACACCTA
2041 GTGAGCTTTTAAAATTTACCAGGAAAATTTGCAACGGTTGGAAAATTTAGAAAGATTTATGATGTAGAAAAATACTTTTGAGATCTTTGTATGAGAGGAATTGAGTCAGTGAGGGAACACT
2161 GCTGGTTTCATTTTCGTAATCACCAGTGGGGCGTCTGATCATCCTGGTTGTTATGTGATAGATGGCTCACATTGATTTGTGATTTTGA
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Figure 1. Nucleotide sequence of the mouse Mel-N1 cDNA with the deduced amino acid sequence shown below. RNP1 and RNP2 consensus sequences are indicated by open and shaded boxes, respectively. Within the 3′ untranslated region, the pentanucleotide ATTTA and related sequences are underlined, and the GAAA tetranucleotide is bold-faced.
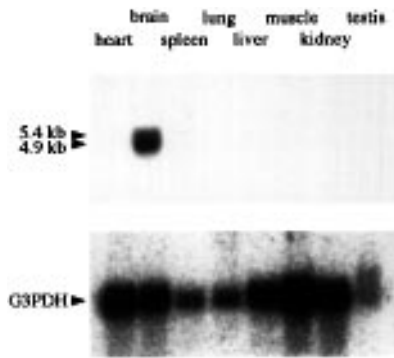

Figure 2. Tissue-specific expression of Mel-N1 mRNAs. Northern blot analysis was performed using poly(A)+ RNAs from the indicated mouse tissues. The same blot was reprobed with a glyceraldehyde 3-phosphate dehydrogenase probe for control.

selection from pools of random RNAs (15,17,42). After three rounds of binding and washing steps at moderate stringency, followed by two rounds at high stringency, RNAs bound by GST-Mel-N1 were reverse-transcribed and 20 cDNA clones were subjected to sequence analysis (Table 1). Of these, 15 clones comprised a single sequence with a motif resembling the core AUUUA element of ARE. Three other clones contained slightly different sequences which were also comprised of ARE-like elements. A tetranucleotide of GAAA was found downstream of the AU-rich region in all of the above 18 clones. The sequences of the remaining two clones were more varied, both contained an AUUUG followed by 7–11 consecutive polyuridines headed by an A residue. No GAAA sequence was found in either of these two clones. Enrichment of sequences displaying such similar features suggest that these motifs may represent the target sequences recognized by Mel-N1.

In vitro RNA binding of Mel-N1

To confirm the results obtained from the *in vitro* selection further, we selected four RNAs as representative Mel-N1-selected RNAs (clones 22-2, 23-1, 27 and 28-1) and directly tested Mel-N1 RNA binding ability by means of UV-crosslinking assays (Fig. 3). We found that GST-Mel-N1 was able to specifically bind to all of the RNAs. To evaluate the effect of the GAAA tetranucleotide on Mel-N1 RNA binding, we altered the sequence within clone 27 to CCCC by *in vitro* mutagenesis and checked the binding affinity of the mutated RNA (27m). Mel-N1 binding to the mutant RNA was reduced by ~3-fold as compared with that of the wild-type RNA, indicating that the GAAA sequence may exert a positive influence on Mel-N1 binding.

Mel-N1 binding to the *c-fos* ARE

To confirm previous observations that the human homologue of Mel-N1 binds to the *c-fos* 3′UTR and to determine the precise binding site within the 3′UTR, we examined Mel-N1 binding to two kinds of RNAs, FosA and FosB, which are derived from the
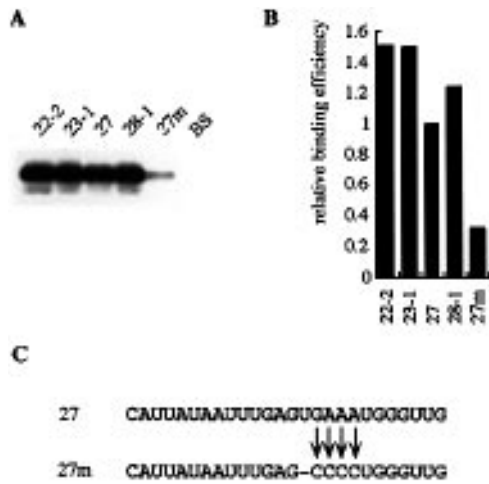
Figure 3. Mel-N1 binding to *in vitro* selected RNAs and the influence of the GAAA sequence on binding. (**A**) Mel-N1 binding to the *in vitro* selected RNAs. GST-Mel-N1 was incubated with the RNAs indicated above, UV-irradiated followed by RNase treatment, electrophoresed on a 12% SDS–polyacrylamide gel and autoradiographed. BS is RNA containing the multi-cloning site sequence of the plasmid Bluescript II. (**B**) Relative binding efficiency of each of the RNAs to Mel-N1 was determined using a Fuji BAS-2000 imaging analyzer. (**C**) RNA sequences of clone 27 and its derivative, 27m.
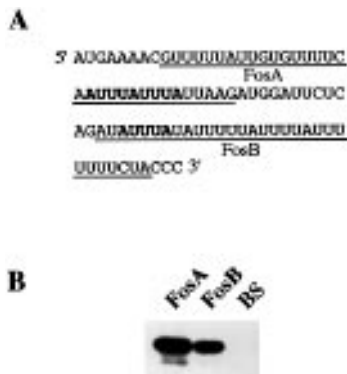
Figure 4. Mel-N1 binding to the *c-fos* ARE. (**A**) The ARE sequence within the 3′UTR of the mouse *c-fos* mRNA deduced by comparison with the human *c-fos* 3′UTR (44). The RNAs, FosA and FosB, which were used for crosslinking experiment are underlined. ARE core sequence (AUUUA) is in bold-face. (**B**) *In vitro* binding of Mel-N1 to FosA and FosB RNAs. GST-Mel-N1 was incubated with the RNAs indicated above, and then analyzed as described in Figure 3.

Figure 5. Mel-N1 binding to the 3′UTR of its own mRNA. (**A**) Schematic representation of RNAs used for *in vitro* RNA binding analysis. Open box indicates the Mel-N1 open reading frame of the cDNA sequence. Thick bars represent the RNAs, the numbers indicating the corresponding nucleotide positions in the cDNA sequence. (**B**) *In vitro* binding of Mel-N1 to the RNAs shown in (A). (**C**) Intensity of crosslinked bands were measured by a Fuji BAS2000 imaging analyzer and relative binding efficiencies were calculated. UTR-A RNA binding strength was taken as 1.0.

Autogenous binding of Mel-N1 to its 3′UTR

As mentioned above, the Mel-N1 3′UTR is rich in A and U residues. Close inspection revealed that there are six copies of the ARE core (AU_3A) and 12 of related sequences (AU_5A, AU_6A, AU_3G, AU_4G), within the 3′UTR (see Fig. 1). In addition, 11 copies of the GAAA motif are present in the 3′UTR, most of which are located near the ARE and ARE-like sequences. Since these types of sequences were selected *in vitro* and bound by Mel-N1, we examined whether Mel-N1 could bind to the 3′UTR of its own mRNA (Fig. 5) by dividing the Mel-N1 cDNA into three segments and synthesizing the corresponding RNAs. The results show that indeed Mel-N1 could bind the UTR-A and UTR-B RNAs derived from its 3′UTR. No significant binding was observed with ORF RNA derived from the coding region.

Cytoplasmic localization of Mel-N1

To elucidate the cellular localization pattern of Mel-N1, we designed an expression plasmid, pEFT7-Mel-N1, which is driven by an EF-1α promoter (40) with a T7 epitope tag sequence fused upstream to the full-length Mel-N1 coding region. This expression vector has been shown to give efficient yields of tagged protein in tissue culture cells (41). pEFT7-Mel-N1 was transiently transfected into HeLa and COS7 cells, and the cells harvested 48 h after transfection. These cell extracts were subjected to immunoblot analysis with a monoclonal antibody against the T7 tag. A discrete band of a size corresponding to the

mouse *c-fos* 3′UTR ARE and whose sequences are highly conserved between mice and humans (Fig. 4 and see 43,44). As the presence of ARE core sequences predicted, both FosA and FosB RNAs were bound specifically by Mel-N1. As judged from the intensity of the crosslinked bands, FosA seems to be bound ~2-fold more efficiently by than FosB, possibly due to the fact that FosA contains two overlapping ARE core repeats. These results demonstrate that Mel-N1 binds to the *c-fos* ARE and suggest that the ARE contains at least two Mel-N1 binding sites.
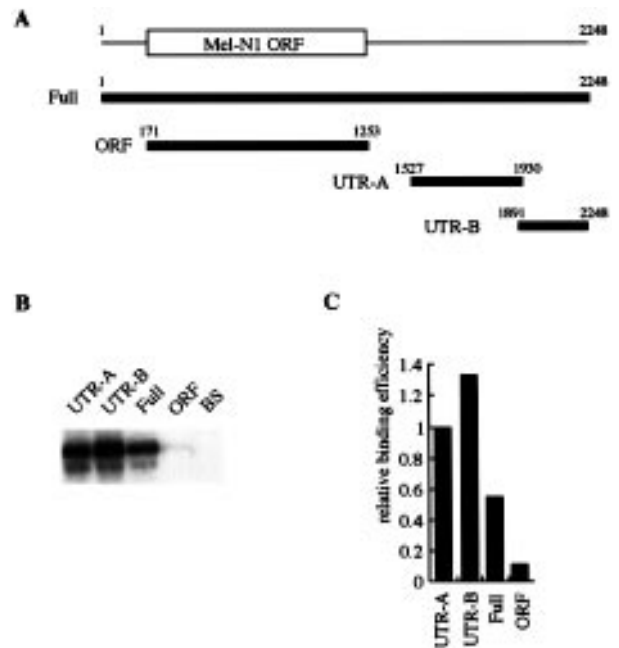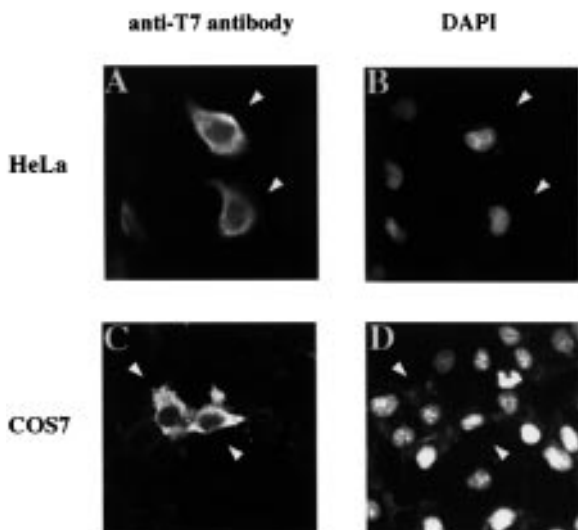
Figure 6. Cytoplasmic localization of Mel-N1. HeLa and COS7 cells were transiently transfected with pEF-T7-Mel-N1, reacted with anti-T7 tag monoclonal antibody and secondary FITC-labelled anti-mouse immunoglobulin antibody, and double-stained with DAPI. (**A** and **B**) HeLa cells; (**C** and **D**) COS7 cells. (A and C) immunofluorescence views (B and D) DAPI staining views.

tagged Mel-N1 was seen in extracts from both cell lines, indicating that the tagged Mel-N1 protein is efficiently produced in cells (data not shown).

Cells were transfected with pEFT7-Mel-N1, incubated with antibody against the tag and secondary FITC-labelled antibody, and observed under confocal laser microscope. In 70% of HeLa and 30% of COS7 cells which express Mel-N1, fluorescence could be observed only in the cytoplasmic region, complementary to the DAPI-stained region (Fig. 6). In the remaining Mel-N1 positive cells, which expressed much higher levels of Mel-N1, the fluorescence extended into the nuclei (data not shown). This appears to be the result of excessive Mel-N1 overexpression and may not reflect the physiological situation. Our results indicate that Mel-N1 localization occurs primarily in the cytoplasm.

DISCUSSION

The human HuD and Hel-N1 are among a new family of RNA binding proteins which are expressed in the nervous system and have been demonstrated to possess specific RNA binding ability *in vitro* (28,32). HuD binds to the ARE within the 3′UTR of c-*fos* mRNA, and Hel-N1 to the 3′UTR of c-*fos*, c-*myc* and granulocyte–macrophage colony-stimulating factor (GM-CSF) mRNAs. Although the precise sites on the mRNAs bound by Hel-N1 have yet to be determined, *in vitro* RNA selection analysis by Gao *et al.* (45) suggested that Hel-N1 and its alternative isoform Hel-N2 recognize specific sequences resembling the ARE core, AUUUA. They have shown that Hel-N1 can bind the 3′UTR RNAs of other growth-related mRNAs which contain ARE-like motifs.

In this study, we demonstrate that like its human counterpart, Mel-N1 specifically selects RNAs which contain ARE core-like sequences such as AU_3G, GU_3A, AU_4A and AU_5A. In most of the Mel-N1-selected RNAs, the ARE core-like sequences are accompanied by a downstream tetranucleotide GAAA motif, which has not been reported for the Hel-N1 and Hel-N2 selected

RNAs. We further show that Mel-N1 binding is reduced by ~3-fold upon disruption of this GAAA sequence. Examination of the Mel-N1 3′UTR revealed that interestingly, GAAA sequences occur in high frequency near ARE core-like sequences. Such GAAA sequences are also found in the vicinity of the mouse and human c-*fos* AREs (43,44) suggesting that this purine-rich sequence may act as a site to assist in the recognition of ARE by Mel-N1. In addition, we demonstrate for the first time that Mel-N1 can bind to the highly conserved region of the c-*fos* ARE which has been shown to function in mRNA destabilization. This finding confirms previous observations from work involving the human Hel-N1, and expands it to show that multiple Mel-N1 molecules may associate with the ARE during regulation of mRNA stability.

Two of the Mel-N1-selected RNAs contained an ARE core-like sequence, AU_3G, but no GAAA. Instead, they have long runs of U initiated by an A residue, AU_7 and AU_{11}. Our *in vitro* RNA binding analysis indicated that Mel-N1 bound these RNAs as efficiently as the RNAs containing the GAAA sequence. This suggests that the U-rich stretches may be involved in further strengthening the binding of Mel-N1 to the ARE core. Alternatively, Mel-N1 may recognize AU_7 and AU_{11} as a part of the ARE core, AUUUA. In any case, efficient RNA binding of Mel-N1 seems to require at least two stretches of three or more consecutive U residues as suggested by the data of Hel-N1-selected sequences (45).

An important discovery is that Mel-N1 can bind specifically to its own 3′UTR RNAs. In addition, we were able to demonstrate that Mel-N1 is localized predominantly in the cytoplasmic region. These results suggest that Mel-N1 may post-transcriptionally regulate the expression of its own mRNA in addition to other ARE-containing mRNAs and contribute to various aspects of neuronal differentiation and/or maintenance. It will be of great interest to examine whether such an autonomous binding occurs also with the human Hel-N1 and other related proteins. We further found that two other proteins, mHuC and mHuD, murine homologues of human HuC and HuD, were able to bind to both the Mel-N1-selected RNAs and the Mel-N1 3′UTR RNAs (R.A. and H.S., unpublished data). Whether or not these mHuC and mHuD also contain ARE-like sequences within their 3′UTRs remains yet unclear. Since the ARE has been shown to cause destabilization of mRNA, Mel-N1 and other related proteins might function as *trans*-acting factors which facilitate mRNA decay *in vivo*. Alternatively, they might act as mRNA stabilizing factors which compete with other potential destabilizing factors such as URBPs which have been shown to bind the c-*fos* ARE (44). It remains to be shown whether Mel-N1 affects the expression of ARE-containing mRNA *in vivo* and, if does, at which post-transcriptional level Mel-N1 and its closely related proteins exert their effect.

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