Phylogenetically Conserved Binding of Specific K Homology Domain Proteins to the 3'-Untranslated Region of the Vertebrate Middle Neurofilament mRNA*

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As axons mature, neurofilament-M (NF-M) expression rises, contributing to maturation of the axonal cytoskeleton and an expansion in axon caliber. This increase is partly due to a rise in NF-M mRNA stability. Such post-transcriptional regulation is often mediated through the binding of specific proteins to the 3'-untranslated region (3'-UTR) of mRNAs. Vertebrate NF-M 3'-UTRs are remarkably well conserved, prompting us to test whether similar proteins bind the 3'-UTRs of different vertebrate NF-Ms. Identification of such proteins could lead to insights into the regulation of NF-M expression during development and in response to trauma or disease. Ultraviolet cross-linking analysis of proteins isolated from adult frog (Xenopus laevis), mouse, and rat brains revealed three ribonucleoprotein complexes (97, 70, and 47 kDa) that were present in all species and bound specifically to NF-M 3'-UTRs. Affinity purification of NF-M 3'-UTR-binding proteins from rat brain followed by mass spectrometry and immunoprecipitation assays identified heterogeneous nuclear ribonucleoprotein (hnRNP) K and hnRNP E1 as the proteins forming the 70- and 47-kDa complexes, respectively. These RNA-binding proteins of the KH domain family recognize CU-rich motifs identical to ones present in NF-M 3'-UTRs. Ultraviolet cross-linking assays performed on Xenopus embryos at different stages of neural development demonstrated that whereas hnRNP K binding occurred only at the most mature stages of axon development. Since hnRNP E is known to stabilize mRNAs, these results raise the hypothesis that these proteins may contribute to the increases in cytoplasmic levels of NF-M mRNA that accompany axonal maturation.

Neurofilaments (NFs)1 are the most abundant structural component of vertebrate axons. They are made of neuronal intermediate filament (nIF) proteins, which include peripherin, α-internexin, and the low, middle, and high molecular mass NF triplet proteins (NF-L, -M, and -H, respectively). In adult neurons, the stoichiometry of these proteins is tightly controlled and if unbalanced can lead to the formation of Lewy bodies and axonal degeneration (1). During development, changes in nIF expression are linked to successive phases of axon development, including neurite initiation in PC12 cells (2), the rapid growth phase of axon elongation (3, 4), and the expansion of axon caliber that follows synaptogenesis and myelination (5–8). Such changes in nIF expression are regulated, at least in part, by post-transcriptional mechanisms, which include changes in mRNA stability (9, 10) as well as in translation and localization (11–13). Aberrant post-transcriptional regulation of nIF expression plays a role in a number of neurodegenerative disorders, including amyotrophic lateral sclerosis (14).

In eukaryotes, the 3'-untranslated region (3'-UTR) of mRNAs harbors many of the cis regulatory elements critical for cytoplasmic post-transcriptional control of gene expression (15–17). Among nIFs, the role of the 3'-UTR in post-transcriptional regulation has been studied best for NF-L. Its 3'-UTR contains a 45-nucleotide-long destabilizing element that overlaps with the 3' end of the coding domain (18, 19) and binds a specific protein, p190RhoGEF (20). RNA electrophoretic mobility shift assays (EMSAs) have also implicated as yet unidentified poly(C)-binding proteins in the formation of ribonucleoprotein (RNP) complexes with the NF-L 3'-UTR (21).

NF-M is also post-transcriptionally regulated (9–11), but much less is known about the specific proteins that bind its 3'-UTR than is known for NF-L. Competitive EMSAs have implicated AU-rich element (ARE) RNA-binding proteins (21), and one such protein, Hel-N1 (also known as HuB), binds non-canonical AREs in the NF-M 3'-UTR (21). It also enhances NF-M translation when transfected into a human teratocarcinoma cell line (22). The 3'-UTRs of vertebrate NF-Ms, from fish to mammal, contain extensive stretches of exceptionally high adenine-cytosine content (23, 24), suggesting that many more trans factors must bind the 3'-UTR than are implied by the presence of the non-canonical AREs. For example, within the domains conserved among vertebrate NF-M 3'-UTRs lies a pyrimidine-rich sequence matching the consensus sequence for binding of hnRNP K homology (KH) domain RNA-binding proteins.

In this study, we set out to learn more about which proteins bind the NF-M 3'-UTR in the nervous system. Using EMSA and SDS-PAGE of brain proteins cross-linked to the NF-M 3'-UTR RNA by ultraviolet irradiation, we found evidence for cross-linking: hnRNP, heterogeneous nuclear ribonucleoprotein; MS/MS, tandem mass spectrometry; LC-Q-TOF, liquid chromatography-quadrupole time-of-flight.

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1 The abbreviations used are: NF, neurofilament; NF-M, middle molecular mass neurofilament protein; NF-L, low molecular mass neurofilament protein; NF-H, high molecular mass neurofilament protein; nIF, neuronal intermediate filament; UTR, untranslated region; GEF, guanine exchange factor; EMSA, electrophoretic mobility shift assay; RNP, ribonucleoprotein; ARE, AU-rich element; KH, K homology; IBC, Incubation Buffer C; GFP, green fluorescent protein; UVCL, ultraviolet cross-linking; hnRNP, heterogeneous nuclear ribonucleoprotein; MS/MS, tandem mass spectrometry; LC-Q-TOF, liquid chromatography-quadrupole time-of-flight.
at least three RNP complexes that are conserved between frog and rodent. In Xenopus, formation of one of these conserved complexes was developmentally regulated. These conserved complexes included three specific KH domain proteins (hnRNPs K, E1, and E2) that have been demonstrated to play key roles in post-transcriptional gene regulation in a variety of non-neuronal systems. Although these KH domain proteins had been previously observed in neurons (25), their targets in the nervous system were unknown. Our study provides evidence of their binding a specific, developmentally regulated neuronal mRNA, thus offering important clues about their possible functions within the nervous system.

**EXPERIMENTAL PROCEDURES**

**Cloning of Xenopus and Rat NF-M 3′-UTR**—The Xenopus NF-M 3′-UTR was extended to its 3′ end by 3′ rapid amplification of cDNA ends (26) from adult Xenopus laevis brain mRNA using a sense primer (TATGGATAGTGGAGAAGAT) targeted to the 3′-UTR of the Xenopus NF-M mRNA sequence in GenBank™ (accession number U85969). This sequence was located 230 nucleotides upstream from the 3′ end of the published sequence between positions 2967 and 2988 (27). The rapid amplification of cDNA ends was cloned into pGEM-T Easy (Promega, Madison, WI), and four of these clones were sequenced.

cDNA from adult Xenopus brain was next used as a template for PCR to obtain three additional cDNA clones that spanned overlapping regions from the full-length Xenopus NF-M 3′-UTR. X1 (684 bp) contained the full-length NF-M 3′-UTR plus the last 100 bp of the coding region (sense primer, GCACGCGAAGCTTGGAGACGCAGAGGACGT; antisense primer, GATGACTGCTGTATCTTCTAATACCATACCCAT). X2 (557 bp) began at the stop codon at the beginning of the 3′-UTR (sense primer, GCGCGAATTCCATAAGAAGAGGCAGCCG; antisense primer, GCATGCAAGCTTGGATATTTTCAATATAACCTTTTGG). X3 (657 bp) contained the last 100 bp of the coding region (sense primer, GCGCGAATTCAAAGTGGAAGAGCATGAGGAGACTG; antisense primer, GCATGCAAGCTTGAGCAAGGTTCACTACATAAACGTTTTG). The sequences of these 3′-UTRs as well as those from human (GenBank™ accession number NM005382), mouse (GenBank™ accession number Z12152) from the last 100 bp of its coding region (sense primer, GCGCGAAGCTTGGAGAGCTAGGAGACGGAGTCCCTTTG), and the nuclear polyA(α) signal (antisense primer, ATGCTCTAGACAAGAACTGCTGTTGGACAGTGCGCG, and the beads were washed six times with 200 l of prewarmed Buffer H (10 mM Tris-HCl, pH 8.4, 1 mM EDTA, 2.5 mM NaCl, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone) at 4 °C, which was fixed and stained with Coomassie Blue, dried, and exposed to a phosphor screen for 24 h and then stored at 4 °C.

**In Vitro Transcription Reactions**—Cytosolic extracts were affinity-purified by binding to in vitro transcribed X3S RNA affinity-purified streptavidin beads (New England Biolabs). To prepare the affinity matrix, 360 nmol of X3S RNA (made by T7, Epicenter Technologies, Madison, WI) was synthesized in 200 l of Buffers A (10 mM Tris-HCl, pH 7.2, 1 mM EDTA, 50 mM NaCl, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone) containing 8 nmol of a 3′-biotinylated oligo (5′-CTAGTGCCTTCCTCCATGAGATTCGCCCATACTACTATATACATATATAGGAGGTCCCGACGCGATACTGCTGCTCCTCTCCTTTG) to the nuclear polyA(α) signal (antisense primer, ATGCTCTAGACAAGAACTGCTGTTGGACAGTGCGCG, and then purified by agarose gel electrophoresis. To generate radiolabeled RNA transcripts, 1 μg of each DNA template was added to in vitro transcription reactions (30) containing 18-fold-labeled oligo (5′-TCATGCTCTTCCTCCATGAGATTCGCCCATACTACTATATACATATATAGGAGGTCCCGACGCGATACTGCTGCTCCTCTCCTTTG) that was complementary to the 5′ end of the X3S probe. The RNA and oligo were then hybridized together by incubating them at room temperature over a 2-h period. The RNA/biotinylated oligo hybrid was then bound to 400 μl of magnetic streptavidin beads (New England Biolabs) that had been precleared with IBC. The binding reaction was performed by incubating these beads together with the RNA/biotinylated oligo hybrid mixture in a final volume of 400 μl for 2–3 h on a Nutator at 4 °C. The RNA-bound beads were washed three times with SDS-PAGE buffer, Ultralink (254 nm, 1650 mJ of total energy; Stratalinker, Agilent Technologies), and then air dried. The RNA was melted the RNA/oligo hybrid to release RNA-protein complexes. The RNA and oligo were then hybridized together by incubating them at room temperature for 2 h and then stored at 4 °C.

Proteins that bind to the NF-M 3′-UTR were purified from 2 mg of adult rat brain cytosolic extract. To clear the extract of proteins that bind nonspecifically to the beads, it was incubated for 1–2 h at 4 °C on a Nutator with 600 μl of prewarmed beads lacking any RNA. The incubated beads were collected after centrifugation at 4 °C. The incubated beads were washed in 100 μl of prewarmed Buffer H (10 mM Tris-HCl, pH 8.4, 1 mM EDTA, 2.5 mM NaCl, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone) at 4 °C, which melted the RNA/oligo hybrid to release RNA-protein complexes. The proteins that bound specifically to the RNA were distinguished from those that bound nonspecifically to the beads by analyzing aliquots from all the wash fractions and the eluates on silver-stained 8% polyacrylamide gels. To confirm the RNA binding of the proteins, the eluates from the RNA beads were pooled, concentrated by precipitation with trichloroacetic acid (35, 36), resuspended in SDS sample buffer, and separated by 8% SDS-PAGE. Proteins were visualized by staining with BioSafe Coomassie Blue (Bio-Rad) following the manufacturer’s instructions. The stained gel was sent to the University at Albany proteomics core facility for protein identification by mass spectrometry. Designated bands were excised and subjected to in situ alkyla...
cysteines and in-gel tryptic digestion, and the digest solution was injected onto a nanospray LC-Q-TOF 2 tandem mass spectrometer (Waters-Micromass). For protein identification, tandem spectra or MS/MS spectra were searched against the National Center for Biotechnology Information (NCBI) non-redundant data base under the Rodent Taxonomy.

Western Blots—For Western blots, samples were separated by 8% SDS-PAGE and transferred to nitrocellulose membrane (Schleicher and Schuell) at 75 V for 3 h at 10 °C (34, 37). After blocking for 90 min at room temperature (38), the membranes were incubated overnight at 4 °C with one of the following antibodies: a 1:1,000 dilution of three separate rabbit polyclonal antibodies specific for hnRNP E1 (anti-PCBP1), hnRNP E2 (anti-PCBP2), or both (anti-PCBP1 and -2) (39); a 1:500 dilution of rabbit polyclonal anti-hnRNP K (Santa Cruz Biotechnology, Santa Cruz, CA); or blocking solution without primary antibody that served as a control. After washing, blots were incubated for 3 h at room temperature in secondary antibody (alkaline phosphatase–conjugated anti-rabbit IgG, 0.4 μg/ml, Kirkegaard and Perry, Gaithersburg, MD) and then processed for the detection of alkaline phosphatase activity with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium following conventional procedures (30).

Immunoprecipitation of UV Cross-linked RNP Complexes—Radiolabeled RNP complexes were formed as described for the UVCL assay. After digestion of RNA and digestion, the volume of the reaction increased to 500 μl, and 5 μl of anti-hnRNP K or anti-hnRNP E1 and E2 antibody was added and incubated overnight at 4 °C on a Nutator. The antibody-bound RNP complex was incubated with 50 μl of Protein A-Sepharose beads (Sigma) for 2 h after which the beads were washed with Buffer A. Immunoprecipitated RNP complexes were eluted by boiling the beads in 50 μl of SDS sample buffer and separated on an 8% Tricine gel. The gel was stained with Omnigene Blue and then dried and exposed to a phosphor screen. The images were captured using a STORM 860 PhosphorImager, and then the same gels were exposed to X-Omat x-ray film for 10 days.

RESULTS

The 3′-UTRs of NF-M mRNAs Are Highly Conserved and Contain Pyrimidine-rich Sequences That May Function as cis Regulatory Sequences—Because the sequence of the Xenopus NF-M 3′-UTR in GenBank™ (accession numbers U85969/ U85970) was nearly 300 nucleotides shorter than that of other vertebrates and lacked a nuclear termination and poly(A) signal, we suspected that more of the 3′-UTR was nearly 300 nucleotides shorter than that of other vertebrate NF-Ms (mouse, chick, rat, and human). The full-length Xenopus NF-M 3′-UTR was remarkably similar to that of other vertebrate NF-Ms (mouse, chick, rat, and human). The full-length Xenopus NF-M 3′-UTR was compared in length (593 nucleotides) to that of other vertebrate NF-Ms (mouse, chick, rat, and human). The full-length Xenopus NF-M 3′-UTR was remarkably similar to that of other vertebrate 3′-UTRs both because they had a canonical nuclear termination and polyadenylation signal 20 nucleotides upstream from the poly(A) tail and because they yielded a 3′-UTR that was comparable in length (593 nucleotides) to that of other vertebrate NF-Ms (mouse, chick, rat, and human). The full-length Xenopus NF-M 3′-UTR was remarkably similar to that of other vertebrate 3′-UTRs both because they had a canonical nuclear termination and polyadenylation signal 20 nucleotides upstream from the poly(A) tail and because they yielded a 3′-UTR that was comparable in length (593 nucleotides) to that of other vertebrate NF-Ms (mouse, chick, rat, and human).

This high degree of sequence conservation strongly suggests that the NF-M 3′-UTR plays an important functional role, perhaps providing cis elements involved in the recruitment of regulatory trans factors, such as RNA-binding proteins. Consistent with this idea, we found within the conserved regions several pyrimidine-rich stretches (underlined in Fig. 1) that strongly resembled cis elements that bind poly(C)-sensitive RNA-binding proteins (17, 40–44).

trans Factors in the Adult Xenopus Brain Formed Specific RNP Complexes with the Xenopus NF-M 3′-UTR—The presence of highly conserved domains in the NF-M 3′-UTR suggested that the 3′-UTR is involved in the formation of specific RNP complexes. To test this hypothesis, we performed EMSAs on cytosolic extracts from adult Xenopus brain using two overlapping radio-labeled RNA probes (Fig. 2A). To avoid interference from poly(A)-binding proteins, both probes were terminated just upstream of the AAUAAA polyadenylation signal. The first probe (X2S) spanned the entire remaining 3′-UTR beginning with the stop codon. The second probe (X3S) spanned this same region but also extended further upstream to include the last 100 nucleotides of the coding domain. We made this second probe because a comparable region of the coding domain of NF-L mRNA is essential for the formation of RNP complexes (18–21).

The migration of both the X2S and X3S RNA probes shifted when incubated with adult frog brain cytosolic extract, indicating the formation of a RNP complex (Fig. 2B). Because the shifts were indistinguishable between the two probes, we concluded that the last 100 nucleotides of the coding region are unlikely to be involved in complex formation. We further concluded that RNP complex formation was specific because both the intensity and the shift of the band representing the complex were reduced with increasing amounts of specific competitor RNA (i.e. non-radioactive X3S probe; Fig. 2C, lanes 3–6).

Because the last 100 nucleotides of the coding domain were involved in RNP complex formation, we used UVCL to determine how many proteins are involved in formation of these RNP complexes. With 60 μg of extract, both the X2S and X3S probes yielded the same three complexes, which had apparent molecular masses of 94, 70, and 47 kDa (Fig. 3A). The formation of identical complexes with the two probes further supported our conclusion from the EMSA results that RNP complexes formed independently of the last 100 nucleotides of the NF-M coding domain.

Because in UVCL assays, the radiolabeled RNA probe must compete with endogenous RNAs still bound to the extracted proteins, the detection of complexes by the exogenously added probe can be influenced by the ratio of probe to extract. We thus performed UVCL over a wide range of protein concentrations (10–350 μg) using a fixed amount of labeled probe. Over this range, we detected the same three RNP complexes (Fig. 3B) with a peak in the intensity of the labeling at 200 μg of total protein.

To test the specificity of formation of each of the three RNP complexes, increasing amounts of specific competitor or nonspecific competitor RNA (15–350-fold molar excess over labeled probe) were added to the UVCL reactions. Increasing the amount of specific competitor RNA (non-radioactive X3S RNA) markedly reduced the signal from the three RNP complexes starting with a 75-fold molar excess (Fig. 3C, lanes 2–5), whereas addition of nonspecific competitor RNA over this range had very little effect (Fig. 3C, lanes 6–9). These results demonstrated that the 94-, 70-, and 47-kDa RNP complexes are formed by specific interactions between RNA-binding proteins in the brain and the Xenopus NF-M mRNA 3′-UTR.

Formation of the 47-kDa RNP Complex Is Developmentally Regulated in Xenopus—The formation of the three RNP complexes were studied in UVCL assays with the X3S probe at four developmental stages in Xenopus (stages 18, 29/30, and 37/38 and adult) that correlate with the changes in NF-M expression that accompany successive phases of axonal development (45).

Stage 18 represents an early stage of neurite outgrowth before any NF-M is expressed. Stage 29/30 represents a stage when many axons are actively growing, but relatively few synapses have yet formed, and NF-M expression is low. Stage 37/38 represents a stage when tadpoles can swim. At this stage, although numerous functional synapses have formed, rela-
tively few axons are myelinated, axon caliber remains small, and levels of NF-M expression are moderate. The adult represents the stage when axons are fully myelinated, axonal caliber is expanded, and NF-M expression is at its peak.

Formation of the RNP complexes varied during development. Whereas the 94- and 70-kDa RNP complexes were observed at all stages (Fig. 4), the 47-kDa complex was observed only in the adult (Fig. 4, lane 4). An additional 98-kDa RNP complex (Fig. 4, lanes 1–3, white arrowhead) was observed only in larval and tadpole stages (stages 18–37/38) but was absent in the adult. Thus, formation of the 47-kDa complex appeared to be adult-specific, whereas the 98-kDa complex was specific to the developing animal.

NF-M mRNA 3′-UTR Forms Similar RNP Complexes in X. laevis, Rat, and Mouse—The high degree of sequence conservation among vertebrate NF-M 3′-UTRs suggests that the proteins that bind to them are likely to be conserved as well. To test this prediction, we performed UVCL assays with the Xenopus X3S and rat R3S probes using cytosolic extracts from adult rat and mouse brains. With these extracts, the Xenopus X3S probe formed 94-, 70-, and 47-kDa RNP complexes (Fig. 5A, lanes 3 and 4) that were indistinguishable in size from those formed using Xenopus extracts (Fig. 5A, lane 1). An additional 56-kDa RNP complex also formed (Fig. 5A, lanes 3 and 4) with rat and mouse extracts that was missing in frog. In reciprocal assays, the rat R3S probe formed the same RNP complexes (Fig. 5A, lanes 2 and 5) as the Xenopus X3S probe did with all the brain extracts.

In competition experiments, increasing the specific competitor (Fig. 5B, lanes 2–5) reduced formation of the 94-, 70-, and 47-kDa complexes more readily than did the nonspecific competitor (Fig. 5B, lanes 6–9), whereas it reduced the 56-kDa complex at nearly the same rate (Fig. 5B, white arrowhead). Thus, the 94-, 70-, and 47-kDa RNP complexes appear to form

![Figure 1](attachment:image.png)

**Figure 1.** The 3′-UTR of NF-M mRNA is highly conserved. Multiple sequence alignment of the 3′-UTR of NF-M mRNAs from five organisms shows the high degree of sequence conservation among them. The black and the gray shading represents nucleotides conserved among all five and in four of the five species, respectively. Each sequence begins with the stop codon. Black lines represent conserved pyrimidine-rich stretches. The hnRNP E consensus binding sequence is marked by asterisks. The dotted black line represents a sequence motif similar to the 15-lipoxygenase differentiation control element of rabbit 15-lipoxygenase mRNA.

| Species   | Sequence |
|-----------|----------|
| Human     | UAGGUGAUA AAGGCAAC GA AU |
| Mouse     | UAGGUGAUA AAGGCAAC GA AU |
| Rat       | UAGGUGAUA AAGGCAAC GA AU |
| Chick     | UAGGUGAUA AAGGCAAC GA AU |
| Xenopus   | UAGGUGAUA AAGGCAAC GA AU |

The high degree of sequence conservation among vertebrate NF-M 3′-UTRs suggests that the proteins that bind to them are likely to be conserved as well. To

- [Human](attachment:sequence1.txt)
- [Mouse](attachment:sequence2.txt)
- [Rat](attachment:sequence3.txt)
- [Chick](attachment:sequence4.txt)
- [Xenopus](attachment:sequence5.txt)
with higher affinity than the 56-kDa complex. These results demonstrated that proteins in rat and mouse brain can form similar RNP complexes with the frog and rat NF-M 3′-UTRs.

**KH Domain RNA-binding Proteins Bind the NF-M mRNA 3′-UTR**—To identify specific proteins that bind the 3′-UTR of NF-M, we combined mass spectrometry with purification of RNA-binding proteins using an RNA affinity substrate (46, 47). Because the protein data bases for rodent are more complete than for *Xenopus*, we purified proteins from rat rather than frog. We chose the rat over mouse because the larger brain of frog. We purified proteins from rat rather than mouse because the larger brain of *Xenopus* would provide more starting material. This approach is justified by the conservation of the frog and rat NF-M 3′-UTR sequences as well as by the similarity of the UVCL results.

As the affinity substrate, we used *in vitro* transcribed X3S RNA attached to paramagnetic streptavidin beads via an intervening 3′-biotinylated oligo that was complementary to the 5′ end of the X3S transcript. This method of attaching the RNA to the beads would permit RNP complexes to be eluted gently by melting the oligo/RNA hybrid at 40 °C with reduced salt. This approach greatly reduced the background contamination from other proteins binding nonspecifically to the beads. The specifically bound proteins (Fig. 6A, lane E) were distinguished from the nonspecific ones by preabsorbing the protein extracts with beads lacking RNA and then comparing on SDS-polyacrylamide gels the eluate from the RNA-beads (Fig. 6A, lane E) with that from beads lacking RNA (Fig. 6A, lane BO). Three bands, with apparent molecular masses of 70, 66 and 43 kDa, were eluted specifically in sufficient quantity for mass spectrometry. A fourth specific band (90 kDa), which was a good candidate for the 94-kDa RNP complex, was visible on silver-stained gels (not shown) but in quantities too small for mass spectrometry.

The other three bands yielded peptides (Table I) with MS/MS spectra that led to the unambiguous identification of these proteins as known KH domain RNA-binding proteins. The 70-
and 66-kDa bands shared four identical peptides found in hnRNP K, and the 66-kDa band yielded an additional peptide that was unique to either the 47- or the 56-kDa UVCL RNP complex or both. A similar immunoblot probed with anti-hnRNP K also confirmed that the 70- and 66-kDa bands were hnRNP K (data not shown). Because of their size, we predicted they would correspond to the 70-kDa UVCL RNP complex. These predictions were confirmed by further Western blot and immunoprecipitation assays.

The 70-, 56-, and 47-kDa RNP Complexes Observed in UVCL Experiments Are Formed by hnRNP K, hnRNP E2, and hnRNP E1, Respectively—To test whether hnRNP K and the hnRNP Es were indeed the proteins involved in the formation of the 70-, 56-, and 47-kDa RNP UVCL complexes, we performed Western blots and immunoprecipitation experiments with UVCL RNP complexes formed between rat brain extracts and the X3S probe. The experiments were done with rat extracts because none of our antibodies cross-reacted with Xenopus (data not shown). Western blots of UVCL reactions between rat brain and the X3S probe were probed with anti-hnRNP K, anti-hnRNP E1, and anti-hnRNP E2 (Fig. 7A, lane 2), anti-hnRNP E1 (Fig. 7A, lane 3), anti-hnRNP E2 (Fig. 7A, lane 4), and anti-hnRNP K (Fig. 7A, lane 5). The immunoblot was then overlaid with x-ray film to reveal the positions of the radiolabeled UVCL bands (e.g., lane 1) relative to those that reacted with each of the antibodies (lanes 2–5). The 70-, 56-, and 47-kDa radiolabeled bands overlaid precisely on those that reacted with the antibodies to hnRNPs K, E1, and E2, respectively. The identities of these RNP complexes were further confirmed by immunoprecipitating the UVCL reactions with the hnRNP K and the hnRNP E1 and E2 antibodies. As expected, the 70-kDa complex was specifically immunoprecipitated by anti-hnRNP K (Fig. 7B, lane 3), and the 56- and 47-kDa complexes were specifically immunoprecipitated by anti-hnRNP E1 and E2 (Fig. 7B, lane 2).

**DISCUSSION**

The 3′-UTRs of vertebrate NF-Ms are strikingly well conserved from fish to mammal (23, 24). Such ancient highly conserved sequences occur within the 3′-UTRs of cytoskeletal mRNAs about 10 times more frequently than they do in those of enzymes, hormones, and hormone receptors (24), suggesting that 3′-UTRs play an especially important role in post-transcriptional regulation of cytoskeletal genes such as NF-M. In this study, we demonstrated that not only are the NF-M 3′-UTR sequences conserved but so are the RNP complexes that
they form. Three conserved RNP complexes (94, 70, and 47 kDa) were found in UVCL assays using various reciprocal combinations of rat and frog NF-M 3′-UTRs with brain extracts from different species. In rat, two of these conserved complexes contained the KH domain, poly(C)-sensitive RNA-binding proteins, hnRNP K (70-kDa complex), and hnRNP E1 (47-kDa complex). An additional 56-kDa complex that was present only in rodent proved to be hnRNP E2. Although these proteins are well known outside the nervous system, ours is the first report of their binding a specifically neuronal target RNA. In Xenopus, 47-kDa complex formation was restricted to adult brain, implicating hnRNP E in a novel role of regulating expression of a neuron-specific gene during axonal maturation.

The identification of hnRNPs K and E1/E2 is supported by the presence within the NF-M 3′-UTR of sequences known to bind these proteins. In humans, CCUCC is the consensus binding sequence for hnRNP E (17, 40, 42). In NF-M, this sequence is within a CCUCCUC motif, which also matches the binding site for hnRNP E within RNAs of the androgen receptor (42), renin (43), tyrosine hydroxylase (41, 44, 48), and erythropoietin (44). This motif in NF-M is further nested within a longer motif similar to the 15-lipoxygenase differentiation control element, which is recognized by both hnRNP E and hnRNP K (49, 50). A 15-lipoxygenase differentiation control element sequence also serves as the minimal binding element for binding of hnRNPs E1 and E2 to α-globin RNA (40). Thus, this region (underlined by a dotted line in Fig. 1) represents the most likely site for the binding of both hnRNP E and hnRNP K. That these sites overlap further suggests these proteins may bind either as a complex or bind differentially to NF-M RNA in different populations of neurons.

Although poly(C)-sensitive RNP complexes form with NF-L and NF-H mRNAs (21), our finding of such complexes with NF-M was at first unexpected because of the two earlier studies that had pointed to ARE proteins as the most likely candidates (21, 22). We, on the other hand, found no hint of ARE proteins in the mass spectrometry analysis of our affinity-purified proteins, even though the masses of the hnRNP Es are comparable to those of HuB and several other ARE proteins. One possibility is that the absence of ARE proteins in our study was due to differences in our NF-M 3′-UTR probes. Both earlier studies used a full-length 3′-UTR, including AU-rich sequences at the poly(A) signal, which Antic et al. (22) found was essential for HuB binding. In making our probe, we deleted the poly(A) signal to avoid detecting and purifying proteins that bind ubiquitously to the poly(A) region of RNAs. By doing so, we may have removed nucleotides essential for the binding of ARE proteins while keeping the poly(C) elements intact. Interestingly many ARE proteins bind to destabilize RNAs (51), whereas binding of hnRNPs K and E tend to stabilize them. Thus, in identifying poly(C)-binding proteins we may have also uncovered the other side of the coin of regulating NF-M mRNA stability.

In the rat, immunoprecipitation and Western blots with well characterized antibodies unambiguously confirmed the mass spectrometry data. Unfortunately, because these antibodies failed to cross-react in Xenopus, similar confirmation was not possible in frog. Nevertheless the reciprocal formation of comigrating complexes in rat and frog argues strongly that the complexes in these two species contain homologous proteins. From cDNA cloning, we know that homologs of both hnRNPs E (52) and K (53) exist in Xenopus, although the precise nature of the expression of their various forms still needs to be characterized more fully. Thus, we feel safe in referring to these proteins in Xenopus as hnRNP K and E homologs.

Both hnRNP K and the hnRNP Es are well known regulators of the stability and translation of cytoplasmic mRNAs in a wide range of non-neuronal cell types (50, 54, 55). The precise role these proteins play in any one instance, however, varies with context, meaning that the same protein can have different effects on several RNAs in the same cell or on the same RNA in response to varying extracellular signals. In one model, hnRNP K acts as a docking platform on the RNA to interact with molecules from various signaling pathways (54). hnRNP K can also interact with hnRNP E. For example, they, together with YB-1, bind the renin mRNA 3′-UTR to regulate its stability (56). Other mRNAs whose stability is regulated by hnRNPs E1 and E2 include those of α-globin (57, 58), α1(I) collagen (59, 60), tyrosine hydroxylase (61), and the androgen receptor in testis (42). hnRNP Es can also help regulate mRNA stability through interactions with other proteins binding elsewhere along the mRNA. For example, in the stabilization of α1(I) collagen mRNA (60), hnRNP E binding the 3′-UTR interacts both with poly(A)-binding protein to prevent loading of the degradosome and with an unidentified factor bound to the 5′-UTR to circularize the mRNA. This latter interaction would both protect the RNA from degradation and increase translation. Alternatively hnRNP K and hnRNP E1 can also silence translation, as in the case of the 15-lipoxygenase mRNA, where they bind a CU-rich differentiation control element within the 3′-UTR (49). Because these same proteins can play multiple roles, how they regulate NF-M expression remains an important unanswered question.

Answering this question will require learning when and where in the nervous system these proteins bind NF-M mRNA endogenously. In rat cortex, both hnRNPs E1 and E2 are expressed in neurons but not in astrocytes (25), demonstrating that the expression of both hnRNP Es, like that of NF-M, is neuronal. Within neurons, however, hnRNPs E1 and E2 likely play separate physiological roles since they respond differentially to extracellular signals. For example, in the cortical response to hypoxia and ischemia, hnRNP E1 expression increases via the activation of p38 mitogen-activated protein kinase, and the increase in E1 is also associated with a decrease in p70/S6K activity (62). Because E1 binds hypoxia-inducible factor-1α (63), it may contribute to the hypoxia response in neurons.
kinase, whereas that of hnRNP E2 decreases via activation of protein kinase C. In Drosophila, the hnRNP E homolog mushroom-body expressed (mbe) is found preferentially in mushroom body neurons and is one of the first genes expressed during neural differentiation (62). Its overexpression protects transgenic flies from the neurodegenerative effects of expressing human ataxin-1 (63). This connection between hnRNP E and neuronal injury may be relevant for NF-M since its expression also changes dramatically in response to injury both in frog (64) and in mammal (65). In rat, these changes reflect alterations in the stability of NF-M mRNA (10, 66).

The possibility that a hnRNP E might help to stabilize NF-M mRNA is also suggested by our observation that formation of the 47-kDa RNP complex in frog is restricted to mature brain and in trauma (5, 181–196). The inability of exogenous NF-M 3′-UTR to bind hnRNP E in embryos may be due either to the relatively lower levels of hnRNP E expression in young embryos than in older animals (52) or possibly to phosphorylation of hnRNP E, which would inhibit binding (67). The presence in embryos of a 98-kDa complex that is missing in adult suggests that other RNA-binding proteins may be involved in regulating NF-M as well. Characterization of how the endogenous NF-M messenger RNP complexes change and influence NF-M expression will be essential for providing insights into the role of these RNA-binding proteins in neuronal development.

One possibility is that these RNA-binding proteins may provide a means for neurons to coordinate expression among the various nIF subunits in response to extracellular signals. All nIF 3′-UTRs contain pyrimidine-rich sequences, and many of these match the consensus sequence for binding hnRNPs E and K. Thus, these proteins might be recruited to other nIF mRNAs as well. Variations among nIF 3′-UTRs outside the similar pyrimidine-rich areas might conceivably alter the partners that these proteins interact with, individualizing the response of each nIF to the same extracellular signals. This could be important for balancing the changes in nIF subunit stoichiometry that occur during axonal outgrowth and in trauma (5, 64, 68, 69), many of which are regulated post-transcriptionally (5, 9, 10, 69, 70). The composition of endogenous messenger RNP complexes changes with the physiological state of many cells (71), and KH domain proteins complexing with nIF mRNAs might represent one such dynamic set of complexes. Two other KH domain proteins, Nova and the fragile X mental retardation protein, participate in mRNA-regulatory networks that these proteins interact with, individualizing the response to, and disease but also into how these KH domain proteins might integrate multiple signaling pathways to regulate expression of functionally related genes.

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