J Neuropathol Exp Neurol
Vol. 64, No. 12
December 2005
pp. 1037–1045
Copyright © 2005 by the American Association of Neuropathologists, Inc.

ORIGINAL ARTICLE

HuR mRNA Ligands Expressed After Seizure

Cara J. Westmark, PhD, Françoise A. Gourronc, PhD, Virginia B. Bartleson, BS, Ümit Sayin, PhD, Saswati Bhattacharya, PhD, Tom Sutula, MD, PhD, and James S. Malter, MD

Abstract

HuR is a ubiquitously expressed AU-rich element (ARE)-binding protein that interacts with and stabilizes selective early response gene (ERG) mRNAs after cell activation or stress. To date, approximately 20 mRNAs have been unambiguously defined as HuR ligands. Given the discordance between the large number of ERG mRNAs and those few defined as ligands, we applied in vitro selection to isolate a broad range of HuR mRNA ligands using postseizure mouse hippocampal tissue. Selected mRNAs were converted into cDNA libraries and sequenced. Using this approach, we have identified over 600 novel, putative HuR mRNA ligands. These genes code for a variety of proteins, including transcription factors, signaling molecules, and kinases, but many have unknown function. Consistent with the means of their selection, several of these HuR ligands are differentially expressed in hippocampus after seizure. These results demonstrate a biochemical approach to identify and characterize the diverse repertoire of ligands for HuR and other regulatory mRNA-binding proteins.

Key Words: Early response gene, Epilepsy, HuR, Pentylenetetrazole.

INTRODUCTION

Early response genes (ERGs) comprise a subset of genes whose activation and expression is triggered by changing environmental conditions. In general, ERGs code for key regulators, including transcription factors, kinases, receptors, and cytokines. Under resting conditions, these mRNAs are weakly expressed through a combination of transcriptional repression and rapid mRNA decay. Within minutes of cell activation, cascades of ERGs are sequentially expressed and translated with resulting alterations in cellular physiology.

The decay rate of many ERG mRNAs is attenuated after cell activation or stress, contributing to their rapid accumulation and pulsatile expression. Regulated ERG mRNA decay often depends on the cis/trans interaction between 3′-untranslated region (UTR) stability elements and cytoplasmic proteins. Perhaps the best example is the binding of AU-rich elements (AREs) to sequence-specific mRNA-binding proteins. AREs are structurally diverse, with class I AREs consisting of one to 3 copies of the AUUUA pentamer within a uridine-rich region and class II AREs containing at least 2 overlapping copies of the UUAUUUA(U/A)(U/A) nonamer within an uridine-rich region, whereas class III AREs lack the typical AUUUA elements but possess uridine-rich sequences (1). All 3 classes of AREs are destabilized in resting cells, probably through interactions with the AU-mRNA-binding proteins AUF1, TTP, and/or KSRP that culminate in degradation by the exosome (2). After cell activation, ARE mRNAs combine with stabilizing mRNA binding protein(s) that oppose the function of AUF1, TTP, and KSRP and prolong mRNA half-life. The best characterized of these binding proteins is HuR, a ubiquitously expressed member of the ELAV-like family of RNA-binding proteins (3) that are involved in neural development and plasticity (4). HuR selectively binds to and stabilizes ARE-containing mRNAs, including c-fos (5), cyclins A and B (6), vascular endothelial growth factor (7), TNF-α (8), p21 (9), and cyclooxygenase-2 (COX-2) (10) mRNAs. HuR likely associates with ARE-containing mRNAs in the nucleus and protects the bound mRNAs from RNase attack during and after export to the cytoplasm (11).

HuR contains 3 RNA recognition motifs (RRMs) and a novel shuttling sequence termed the hinge region, or HuR nucleocytoplasmic shuttling sequence (HNS) (amino acids 190–244) between the second and third RRMs. The first 2 RRMs mediate ARE recognition (12), whereas the third RRM has been implicated in poly(A) tail binding (13) and is required for ARE-mediated mRNA stabilization (11). The HNS domain, containing a nuclear localization signal and nuclear export signal, mediates nuclear/cytoplasmic shuttling (14). The HNS of HuR contains a basic sequence (205-RRFGGPVHHQAQRFRF-220) similar to the bipartite nuclear localization signal consensus sequence found in nucleoplasmin and HIV-1 Rev (14). HuR migrates across the nuclear membrane through 2 mRNA export receptors, transportin 2 (Trn2), and chromosome maintenance 1 (CRM1) (15). Heat shock-induced stress disrupts the Trn2/HuR interaction shifting export solely to the CRM1 pathway. The existence of 2 distinct HuR nuclear export pathways, which are differentially used on cell stress, may provide a rapid means to elevate cytoplasmic HuR levels and thus stabilize ERG mRNAs responsive to HuR.

Given the importance of HuR in regulating a small number of ERG mRNAs, we asked what the full repertoire of HuR ligands might be. We reasoned that ARE containing mRNAs could be selected by HuR affinity chromatography

From the Department of Pathology and Laboratory Medicine (CJW, FAG, VBB, SB, JSM), Waisman Center for Developmental Disabilities; and the Department of Neurology (US, TS), University of Wisconsin, Madison, Wisconsin.

Send correspondence and reprint requests to: James S. Malter, MD, University of Wisconsin, Waisman Center for Developmental Disabilities, Rm. T509, 1500 Highland Ave., Madison, WI 53705; E-mail: jsmalter@facstaff.wisc.edu

This work was supported by National Institutes of Health R01 Grants MH 61666 and AG 10675 (JSM).

from a complex mix of total cellular mRNA and converted to cDNA for identification by sequencing. To ensure adequate ERG mRNA representation in the starting material, we used a rodent epilepsy model. Seizures were induced in mice with pentylenetetrazol (PTZ), which is associated with increased mRNA levels of several early response genes in the hippocampus such as c-fos, c-jun, NGFI-A (also known as zif/268, Egr-1, and Krox 24) (16), and tissue plasminogen activator (17), as well as the late response genes dynorphin, neuropeptide Y (16), and NeuroD-related factor (18).

Based on sequencing of the affinity selected mRNAs, HuR likely interacts with at least 600 mRNA ligands in post-seizure mouse hippocampus. These mRNAs code for a variety of critical proteins, including transcription factors, cytokines, cell surface receptors, and signaling molecules with several having been previously defined as ERGs. To prove that HuR selection predicts ERG status, 3′-UTR sequencing of the library showed a large number of ARE-containing clones. Considering the rapidly accumulating data regarding post-transcriptional regulation by HuR, many of the clones identified here are likely ERG mRNAs regulated by 3′-UTR AREs.

MATERIALS AND METHODS

Materials

The pMal protein fusion and purification system was purchased from New England BioLabs (Beverly, MA). The Universal RiboClone cDNA synthesis system and pGEM-T vector were from Promega (Madison, WI) and the ThermalAce DNA polymerase and pcDNA3.1 directional topo expression kit from Invitrogen (Carlsbad, CA). The HotStarTaq DNA polymerase and Omniscript RT were ordered from Qiagen (Valencia, CA). Pentylenetetrazol (PTZ), protein A Sepharose (catalog no. 9424), RNase T1 and protease inhibitor cocktail were from Sigma (St. Louis, MO), oligo$(dT)_{25}$ magnetic beads from Dynal (Lake Success, NY), oligo(dT)-cellulose from Ambion, Inc. (Austin, TX), and TRI-reagent from Molecular Research Center, Inc. (Cincinnati, OH). The enhanced chemiluminescence (ECL^+) Western blotting detection kit and radioisotope [α-^{32}P]dCTP (3000 Ci/mmol) were from Amersham Life Science (Cleveland, OH), the nylon transfer membrane was from Fisher Scientific (Pittsburgh, PA), and the QuikHyb hybridization solution was supplied by Stratagene (La Jolla, CA). Oligos were synthesized by Gibco BRL Life Technologies (Gaithersburg, MD).

pMal/HuR Cloning, Expression and Purification

The plasmid pGEX2T/HuR (3) was a kind gift from Dr. Henry Furneaux (Memorial Sloan-Kettering Cancer Center, New York). The HuR gene was polymerase chain reaction (PCR)-amplified (94°C 1 minute, 60°C 1 minute, 72°C 1 minute) with HotStarTaq DNA polymerase per the manufacturer's protocol for 35 cycles. The forward primer 5′-AGTCGAAGGATTTCTATGTCTAATGGTTATGAAGACCA-3′ incorporated an XmnI site into the PCR product and the reverse primer 5′-AGTCAAGCTTTTATTTGTGGGACTTGTTGG-3′ a HindIII site. The HuR PCR product was digested with XmnI and HindIII and cloned into the corresponding sites of the pMal-c2 vector. pMal/HuR was transformed into XL-1 Blue competent cells and grown to A600 of 0.5, induced with IPTG to a final concentration of 0.3 mM, grown an additional 2 hours, and the cell pellet from 1 L of cells was resuspended in 50 mL gel shift assay (GSA) buffer containing 15 mM HEPES (pH 8), 10 mM KCl, 10% glycerol, and 1 mM DTT and frozen overnight at –20°C. The cell suspension was thawed on ice, sonicated at setting 3 for 8 × 15 seconds bursts on ice with a sonic dismembrator 550 from Fisher Scientific (Pittsburgh, PA), centrifuged 9,000 × g for 30 minutes at 4°C. The cleared lysate was applied to 6 mL packed amylose resin previously equilibrated in GSA buffer. Protein binding proceeded for 60 minutes at 4°C with rotation. The amylose resin was washed 6 times with 10 mL GSA buffer and maltose binding protein (MBP)/HuR was eluted twice with 10 mL GSA buffer containing 20 mM maltose for 10 minutes at 4°C. The maltose was removed by ion exchange chromatography over DE52 resin. The MBP/HuR protein was bound to the DE52 in GSA buffer, washed with 6 column volumes of GSA buffer, and eluted with GSA buffer containing 0.2 M KCl. Protein concentration was determined by Bradford assay and protein purity by SDS-PAGE. Aliquots of protein were frozen at –80°C.

Seizure Induction and mRNA Isolation

Adult male C57BL/6 mice (7–8 weeks old) from Harlan were injected with 50 mg/kg body weight pentylenetetrazol (PTZ) (or saline for control animals) and monitored for signs of behavioral seizure activity, including altered responsiveness to environmental stimuli, irregular tonic–clonic movements of the extremities, and alterations in postural tone. All of the mice injected with PTZ had seizures, which included tonic–clonic seizures. The seizures subsided 1 hour after injection, and only mice that exhibited at least one of the ictal behaviors after injection were included in the study. The seizures induced death in 25% of the mice, which were discarded. One hour post-PTZ injection, the mice were decapitated and the brains were rapidly removed, partitioned into cortex, hippocampus, and hemisphere fractions, and flash-frozen in liquid nitrogen. Animal handling and tissue isolation were performed in accordance with National Institutes of Health and University of Wisconsin–Madison guidelines for experimentation with animals. Frozen hippocampal tissue was homogenized and resuspended in Tri-Reagent at a concentration of approximately 30 mg tissue per 1 mL Tri-Reagent. RNA was prepared according to the manufacturer's procedure with a few modifications. Two additional RNA extractions after the TRI-reagent phase separation were performed, one extraction with an equal volume of water-saturated phenol/chloroform and the other with an equal volume of chloroform. The RNA was precipitated and resuspended in formamide for Northern blot analysis or resuspended in water for poly(A) mRNA selection with oligo(dT)-cellulose. The conditions for denaturing formaldehyde/agarose gel electrophoresis, radiolabeled cDNA probe preparation, and Northern blot analysis have been previously described (19).

HuR Selection of mRNA

Fifty micrograms of MBP/HuR protein was bound to 25 μL packed amylose resin equilibrated in GSA buffer for

© 2005 American Association of Neuropathologists, Inc.

90 minutes at 4°C with mixing. The MBP/HuR-amylose resin was washed twice with 1 mL GSA buffer and incubated with 500 ng heat-denatured PTZ-treated mouse hippocampal mRNA (isolated from five pooled hippocampi) and 80 units RNasin in 1 mL reaction volume for 2 hours at 4°C with mixing. The resin was washed three times with 1 mL cold GSA buffer and bound mRNA was eluted at 37°C for 10 minutes with 200 μL GSA buffer containing 0.5 M LiCl. MBP/HuR selected mRNA was heat-denatured and mixed with 25 μL oligo$(dT)_{25}$ magnetic beads for one hour at room temperature with mixing.

cDNA Library Construction, Amplification, and Screening

The oligo$(dT)_{25}$ magnetic beads with bound mRNA were washed twice with 50 μL first-strand synthesis buffer. For the first-strand DNA synthesis in a final volume of 25 μL, the oligo$(dT)_{25}$ magnetic beads with bound mRNA were mixed with 15 μL water, 5 μL 5× first-strand buffer, and 40 units RNasin and heated for 5 minutes at 42°C before the addition of 2.5 μL sodium pyrophosphate (40 mM) and 1.5 μL AMV-RT (20 units/μL). The reaction was incubated at 42°C for 60 minutes with gently mixing every 10 minutes to resuspend the magnetic beads. The second-strand DNA synthesis reaction in a final volume of 125 μL contained: 25 μL first-strand reaction, 50 μL second-strand 2.5× buffer, 6.25 μL acetylated BSA (1 mg/mL), 3.13 μL DNA polymerase I (9.2 units/μL), 0.63 μL RNase H (2 units/μL) and 40 μL water. Second-strand synthesis proceeded for 2 hours at 14°C and the beads were resuspended every 10 minutes. The reaction was heated for 10 minutes at 70°C, placed on ice, mixed with 4 units of T4 DNA polymerase for 10 minutes at 37°C to polish the ends, and treated with 10 μL of 200 mM EDTA. The oligo$(dT)_{25}$ magnetic beads with bound double-stranded DNA were washed twice with 50 μL T4 DNA ligase buffer and ligated with 5 pmol adaptor in a 30 μL reaction containing 1× T4 DNA ligase buffer plus 0.1 mg/mL BSA and 0.3 units/μL T4 DNA ligase. To prepare the adaptor, the complimentary oligos *CACC*GGCGGCCGCTCGAGTCTAGA and p-TCTAGACTC-GAGCGGCCGCC were annealed (10 nmoles each) in 10 mM Tris pH7.5, 100 mM NaCl, 1 mM EDTA by heating at 65°C for 10 minutes followed by slow cooling to room temperature. The ligation reactions were incubated at 15°C overnight, heated at 70°C for 10 minutes, set on ice, and the beads were washed three times with 50 μL PCR buffer. The cDNA library was amplified with ThermalAce DNA polymerase (Invitrogen) to produce blunt-end PCR products by a 2-step PCR process (20). First, the cDNA attached to the Dynal beads was mixed on ice with 100 ng of the forward primer 5′-*CACC*GGCGGCCGCTCGAGTCTAGA-3′, 2 ng of the reverse primer 5′-GATTAACCCTCACTAAAGGGAT_{15}-3′, 100 ng of the reverse primer 5′-GATTAACCCTCACTAA-AGGGA-3′, PCR buffer and ThermalAce DNA polymerase per the manufacturer's instructions, and heated at 95°C for 2 minutes to release the second strand. The supernatant was transferred to a fresh tube and amplified (30°C 15 minutes, 40°C 15 minutes, 72°C 15 minutes, 95°C 2 minutes [95°C 1 minute, 72°C 5 minutes, 16 cycles], 72°C 30 minutes). Second, 5-μL aliquots of the initial PCR reaction were reamplified with 100 ng of the forward 5′-*CACC*GGCGG-CCGCTCGAGTCTAGA-3′ and reverse 5′-GATTAACCCT-CACTAAAGGGA-3′ primers (95°C 3 minutes [95°C 1 minute, 72°C 5 minutes, 16 cycles], 72°C 30 minutes). The PCR reactions were combined, extracted with a 50/50 mixture of phenol/chloroform, extracted with chloroform, and precipitated with ethanol in the presence of 50 μg glycogen. The final pellet was resuspended in 50 μL water, and 4 μL of the amplified cDNA library was ligated with the pcDNA 3.1 vector per Invitrogen's protocol for the pcDNA3.1 directional topo expression kit and transformed into chemically competent TOP10 *Escherichia coli*. Approximately 1000 colonies resulted from 4 μL of the amplified cDNA library and were screened for inserts by PCR with primers against the vector T7 and BGH sequences and HotStarTaq polymerase (95°C 15 minutes to lyse and inactivate nucleases [94°C 1 minute, 60°C 1 minute, 72°C 1 minute, 30 cycles], 72°C 10 minutes). The PCR products were analyzed on 1% agarose gels and ranged in size from several hundred base pairs to 4.7 kb. Plasmids were purified from colonies containing inserts with Qiagen's plasmid mini kits (20 μg capacity) and sequenced. Over 1700 clones were sequenced, aligned to the NCBI database by BLAST homology searches (21) and categorized into families based on protein function.

c-fos Reverse Transcriptase–Polymerase Chain Reaction

Samples were collected during the HuR selection procedure and analyzed for the presence of c-fos mRNA, a seizure-induced positive control. RNA samples (2.5 ng PTZ-treated mouse hippocampal mRNA [0.5% total], 5 μL MBP/HuR-amylose resin flow-through [0.5% total], 1 μL MPB/HuR-amylose resin elution [0.5% total] and 1 μL oligo(dT)25 flow-through [0.5% total]) were reverse-transcribed (RT) with Omniscript RT and 500 ng oligo(dT) primer per Qiagen's recommendations at 37°C for 60 minutes. Aliquots (5 μL) of the RT reactions and the 2 rounds of library amplification (1 μL each) were PCR-amplified with the primers 5′-GGC-AGAACCCTTTGATGACTTC-3′ and 5′-AGCCCGGAGTA-CAGGTGACCA-3′ designed against the mouse c-fos oncogene (GenBank accession no. V00727). Amplification (94°C 1 minute, 60°C 1 minute, 72°C 30 sec, 35 cycles) produced a 206-bp c-fos fragment.

RESULTS

To characterize the repertoire of HuR mRNA ligands, we developed an affinity method for the selection of ARE-containing mRNAs from total cellular mRNA. Recombinant HuR bound to a solid support was mixed with total mRNA isolated from mouse hippocampal lysates. After extensive washing, retained mRNAs were eluted and converted into a cDNA library. To induce ERG expression, mice were injected with the convulsant pentylenetetrazol (PTZ), which induces tonic–clonic seizures by antagonizing inhibitory GABA receptors (22). One hour post-PTZ injection, total RNA was isolated from the hippocampus and analyzed by Northern blotting with a cDNA probe against c-fos. This prototypical ERG is rapidly upregulated by seizure (16). As

© 2005 American Association of Neuropathologists, Inc.

shown in Figure 1A, there was no detectable c-fos mRNA in control tissue from the hippocampus, cortex or hemispheres; however, ample c-fos mRNA was present in the hippocampus as well as the cortex and hemispheres after PTZ treatment. Therefore, mRNA was isolated by oligo(dT) chromatography from PTZ-treated animals and used as starting material for the HuR affinity chromatography.

HuR was expressed as a fusion protein with MBP (42 kD) (Fig. 2) and bound to amylose resin that acted as a solid support for the in vitro selection of mRNA ligands. Based on Coomassie blue staining, the fusion protein was >95% pure. The ability of the resin to select ARE-containing mRNAs was monitored by following the coisolation of a known amount of full-length, radiolabeled GM-CSF mRNA (23), a known HuR ligand, which was added to the starting mRNA. Thus, the MBP/HuR/amylose resin was incubated with increasing concentrations of heat-denatured hippocampal mRNA (50, 300, 1,000 ng) spiked with radiolabeled GM-CSF mRNA, washed,

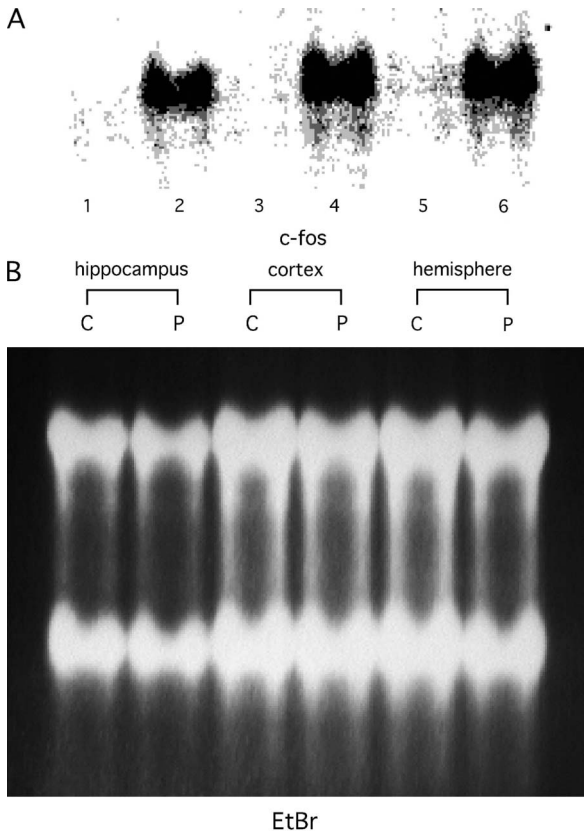

FIGURE 1. Pentylenetetrazol (PTZ) upregulates c-fos mRNA expression in mouse brain. Mice were injected with 50 mg/kg body weight pentylenetetrazol (P) (even numbered lanes) or with saline for control animals (C) (odd numbered lanes) and hippocampus (lanes 1 and 2), cortex (lanes 3 and 4), and hemisphere (lanes 5 and 6) tissue was isolated 1 hour later. RNA was isolated and analyzed as described in the ''Materials and Methods.'' **(A)** Northern blot of the c-fos-specific hybridization signal. **(B)** Ethidium bromide-stained agarose gel demonstrating equivalent total RNA loads for control versus PTZ-treated mice.

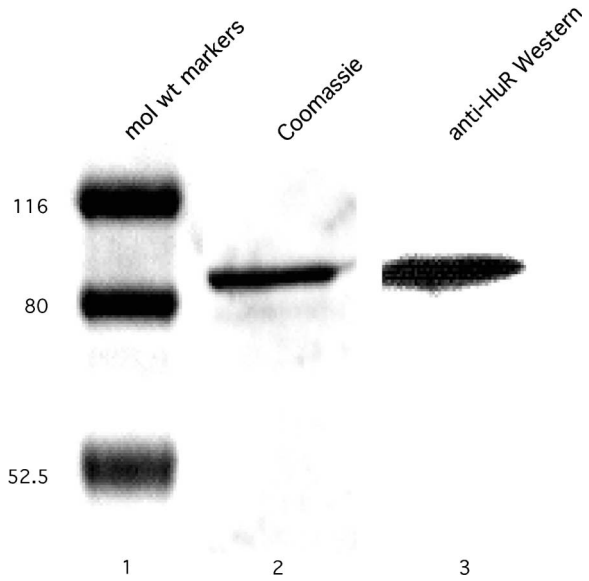

FIGURE 2. SDS-PAGE and Western blot analysis of MBP/HuR fusion protein. Molecular weight markers (lane 1) and MBP/HuR (lanes 2 and 3) were analyzed by 12% SDS-PAGE followed by staining with Coomassie blue dye (lane 2) or Western blotting with anti-HuR/19F12 monoclonal antibody (1:2000) and staining with ECL$^+$ reagents (lane 3).

and eluted in 0.5 M KCl. Aliquots from the flowthrough, washes, elutions, and residual MBP/HuR resin were counted in a scintillation counter and the cpms in the individual fractions normalized to the total cpm input and plotted against the fraction number (Fig. 3). When 50 ng (Fig. 3B) or 300 ng hippocampal mRNA (Fig. 3C) spiked with radiolabeled GM-CSF mRNA was selected on the MBP/HuR/amylose resin, >72% of GM-CSF mRNA was retained after multiple washes and eluted with 0.5 M salt. When the concentration of cold hippocampal mRNA was increased to 1 μg, 64% of the GM-CSF counts were in the flow-through fraction and 24.4% in the elutions (Fig. 3D). Control-binding reactions to amylose resin alone (Fig. 3A) showed that a very small amount of radiolabeled GM-CSF mRNA was retained by the column (2.1%), whereas nearly 90% was present in the flowthrough. Therefore, the column showed high specificity and sensitivity toward ARE+ mRNAs. The progressively reduced GM-CSF mRNA retention and elution as hippocampal mRNA was increased likely reflects competition for a limited number of HuR molecules. Thus, for affinity chromatography, we chose an intermediate amount of input mRNA from postseizure hippocampus (500 ng) to ensure rare transcripts would not be lost.

After selection, mRNAs were bound to oligo(dT)$_{25}$ magnetic beads for the solid-phase synthesis of the cDNA library as described in the "Materials and Methods." The presence of c-fos mRNA was used as a positive control for seizure induction (Figs. 1;4, lane 4) and was followed over the course of selection and library construction. Unexpectedly, a significant amount of c-fos mRNA was in the flowthrough fraction from the MBP/HuR/amylose resin (lane 5), presumably as a result of saturation of the HuR-binding sites with

© 2005 American Association of Neuropathologists, Inc.

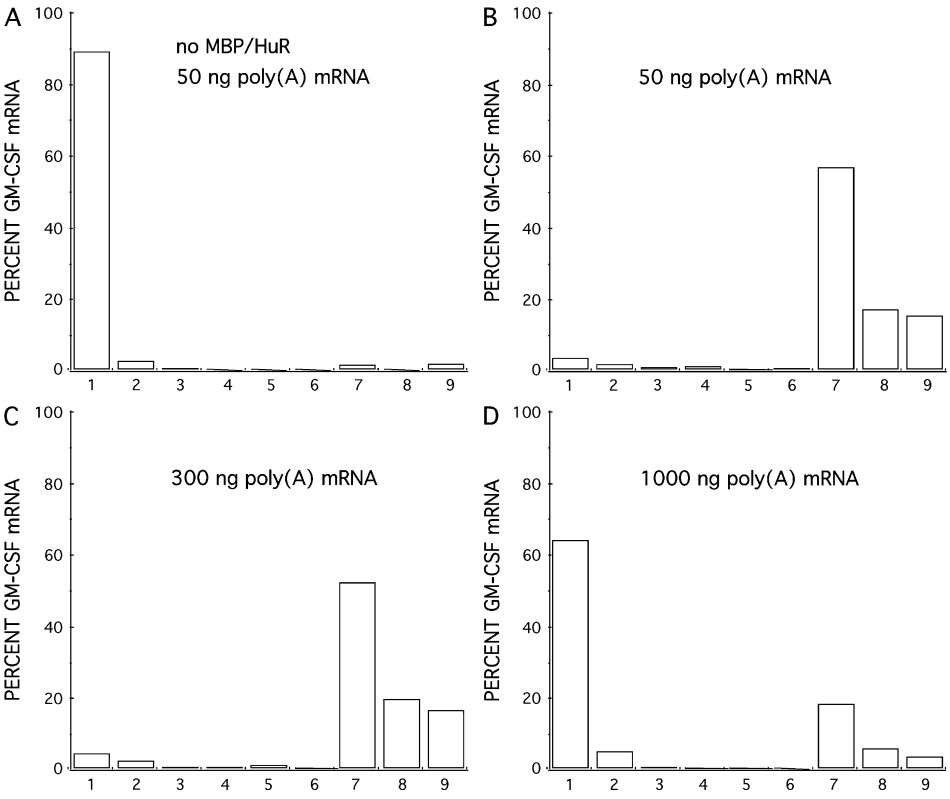

FIGURE 3. Radiolabeled GM-CSF mRNA elutes from MBP/HuR/amylose resin with high salt. Poly(A)-selected mRNA (50 ng, **A, B**; 300 ng, **C**; and 1 μg, **D**) from mouse hippocampus was spiked with radiolabeled GM-CSF mRNA and bound to MBP/HuR/amylose resin **(B–D)** or to resin alone **(A)** similarly to the HuR selection procedure described in the ''Materials and Methods.'' The MBP/HuR/amylose resin was washed 5× with 1 mL of GSA buffer and bound mRNAs were eluted 2× with 200 μL warm GSA buffer containing 0.5 M KCl. The flowthrough (column 1), wash (columns 2–6), and elution (columns 7–8) fractions were collected as well as the remaining resin (column 9) and monitored for GM-CSF mRNA by scintillation counting.

competing endogenous ARE mRNAs. However, c-fos mRNA was present in the high salt amylose resin elution (Fig. 4, lane 6) and retained on the oligo(dT) beads (Fig. 4, lane 7). Finally, c-fos DNA was detected in both the first (lane 8) and second (lane 9) rounds of library amplification.

The resulting cDNA library was transformed into TOP10 *E. coli* and the clones sequenced and analyzed for homology with the NCBI nonredundant and EST-mouse databases and categorized into families based on protein function. A partial list of clones involved in apoptosis, transcription, and cell regulatory events is given in Table 1. A comprehensive list of the isolated HuR ligands is available on request. Analysis of the 472 cDNAs with homology to the nonredundant NCBI database revealed that 78% contained isolated AUUUA pentamers, canonic AREs, and/or uridine-rich stretches (3 or more U_4 repeats or at least one U_6 sequence). Thus, these appear to be genuine HuR-binding partners. However, given the recent demonstration that HuC, a related ARE-binding protein, interacts with zip-code elements (24), the inclusion of actin, which contains a zip-code element, and other cytoskeleton mRNAs is also predictable. Of note, we did not isolate any clones previously identified as HuR ligands (25), although we did detect c-fos mRNA in the cDNA library by reverse transcriptase–polymerase chain reaction (RT-PCR). The absence of known RNAs bound by HuR in the library is most likely the result of lower levels of their expression or loss in library construction. The current database reflects the sequencing of approximately 1,700 clones and should be viewed as a partial and not complete list of HuR mRNA ligands.

DISCUSSION

Epilepsy is a recurrent seizure disorder caused by aberrant electrical signaling in the brain, which can result in neurodegeneration. Repeated and/or prolonged seizures cause

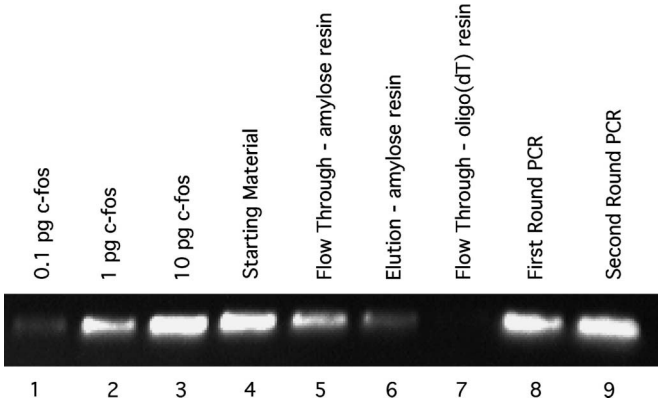

FIGURE 4. c-fos mRNA from pentylenetetrazol-treated mouse hippocampus binds to the MBP/HuR/amylose resin and is present in the amplified cDNA library. RNA samples were collected from the starting material (lane 4), MBP/HuR/amylose resin flowthrough (lane 5), MBP/HuR/amylose resin elution (lane 6) and oligo$(dT)_{25}$ flowthrough (lane 7) and reverse-transcribed (RT) as described in the ''Materials and Methods.'' Aliquots of the RT reactions, the 2 rounds of library amplification (lanes 8–9) and c-fos plasmid DNA (lanes 1–3) were polymerase chain reaction (PCR)-amplified and analyzed on a 1% agarose gel in TBE. A 206 base-pair PCR product was visualized by ethidium bromide staining.

© 2005 American Association of Neuropathologists, Inc.

TABLE 1. HuR Ligands With Known/Potential Role as Early Response Genes

Clone Name	Genus	Accession No.	ARE Class
Apoptotic chromatin condensation inducer in the nucleus mRNA	Mouse	NM_019567	2
BCL2/adenovirus E1B 19kDa-interacting protein 1 mRNA*	Mouse	XM_128520	1
Beclin 1 mRNA	Mouse	NM_019584	1
bHLHZip transcription factor BIGMAX beta mRNA	Mouse	AF213671	ND
Crystallin mRNA	Mouse	NM_016669	1
Cyclin-dependent kinase 4 mRNA	Mouse	BC052694	3
Cyclin-dependent kinase 5 mRNA*	Mouse	NM_007668	1
DnaJ heat shock protein 40 homolog mRNA*	Mouse	NM_178055	3
E4F transcription factor 1 mRNA	Mouse	BC031757	3
Ferritin heavy chain mRNA	Mouse	NM_010239	ND
Ferritin light chain 1 mRNA	Mouse	BC019840	ND
FK506 binding protein 1a mRNA*	Mouse	NM_008019	2
General transcription factor 2B mRNA	Mouse	NM_145546	1
Heat shock protein mRNA	Mouse	BC018378	1
Heat shock protein 1, alpha, mRNA	Mouse	NM_010480	3
Heat shock protein 1, beta, mRNA	Mouse	NM_008302	3
Heat shock protein 70 binding protein mRNA	Mouse	AF338351	ND
Heat shock protein 8 mRNA	Mouse	BC006722	3
Heterogeneous nuclear ribonucleoprotein A/B mRNA*	Mouse	NM_010448	1
Immediate early response 2 mRNA*	Mouse	BC002067	3
Inhibitor of DNA binding 3 mRNA*	Mouse	NM_008321	3
Interferon regulatory factor 3 mRNA	Mouse	BC050882	ND
MEK binding partner 1 mRNA*	Mouse	AF082526	1
NCK-associated protein 1 mRNA	Mouse	NM_016965	3
N-myc downstream regulated 2 mRNA*	Mouse	NM_013864	ND
Non-POU-domain containing, octamer binding protein, mRNA*	Mouse	BC005465	3
Nuclear DNA binding protein mRNA*	Mouse	NM_020558	1
5′, 3′-nucleotidase cytosolic mRNA	Mouse	NM_015807	ND
Par-6 (partioning defective 6) homolog alpha mRNA	Mouse	BC049593	ND
rhoB gene mRNA*	Rat	NM_022542	1
RNA polymerase II polypeptide C mRNA	Mouse	BC002023	1
TAF12 RNA polymerase II mRNA*	Mouse	NM_025579	1
Transferrin mRNA	Mouse	NM_133977	ND

*, The 3′-untranslated region (UTR) comprises 36% to 84% of the gene sequence. ND, not determined.

hippocampus sclerosis, presumably through excessive activation of excitatory glutamate receptors. Our goal in these studies was to identify ERGs expressed after seizure that bind to HuR. ERG mRNAs rapidly accumulate in response to cell signaling or damage such as seizures. Although transcriptional upregulation is involved, posttranscriptional processes, including mRNA stabilization, also play an important role. In the case of ERGs, many of the mRNAs contain AU-rich elements (AREs) in their 3′-UTRs, which modulate mRNA stability as well as translational efficiency. HuR is one of the best characterized of the ARE-binding proteins and functions in the stabilization and localization of many ERG mRNAs. Thus, we were interested in identifying HuR mRNA ligands expressed after seizure and in developing a database of ERG mRNAs important in synaptic plasticity, seizure induction, and response to neuronal injury.

Multiple approaches, including systematic evolution of ligands by exponential enrichment (SELEX) (26) and SNAAP (isolation of specific nucleic acids associated with proteins) (27), which involve in vitro binding reactions, as well as immunoprecipitation of endogenous RNA-binding protein/mRNA complexes directly with monoclonal antibodies and analysis by microarrays (28) have been used to discern the protein/mRNA interactions important for posttranscriptional gene regulation. We have used AUF1 (29) and HuR affinity chromatography to create cDNA libraries of ARE-enriched mRNAs. There are limitations and advantages with each of these techniques. The in vitro-binding reactions may not be representative of actual in vivo protein/mRNA interactions will not detect binding if ancillary proteins are necessary and involve PCR amplification steps. Immunoprecipitation also has limitations as the epitope bound by the antibody may not be accessible in the mRNA complex or mRNAs that are not directly bound by the protein may be coimmunoprecipitated. Microarrays offer the advantage of rapid analysis but are confined in the number of cDNAs spotted on the chip, whereas the brute force sequencing approach we have used is time-consuming and labor-intensive but identifies novel sequences that are not immobilized on all arrays. Overall, these techniques will complement each other and contribute to our understanding of the protein/mRNA interactions that control posttranscriptional regulation of ERGs.

© 2005 American Association of Neuropathologists, Inc.

We have generated a cDNA library of HuR mRNA ligands coding for a variety of proteins involved in diverse cellular functions. The majority of the clones contained class I, II, or III-type AREs. However, 22% of the isolated mRNAs lacked AREs, suggesting there is some flexibility in the Hu recognition sequence. Indeed, recent work has shown that 1) HuC interacts with zip-code elements found in cytoskeletal mRNAs subject to directed intracellular transport (24), 2) HuD and HuB preferentially bind to poly(U) (30), and 3) HuR binds to both U-rich (31), and a conserved UC-rich motif within the 3′-UTR of the androgen receptor mRNA (32). Bakheet et al have compiled a human ARE-containing mRNA database (ARED) and estimate that 8% of human mRNAs contain functional AREs (33). To date, the regulation of only a tiny percentage of those mRNAs has been studied. HuR is the most extensively characterized ARE-binding protein and yet less than two dozen HuR ligands have been previously described (25). Thus, our list of over 600 putative HuR ligands from postseizure hippocampus should facilitate progress in categorizing the full diversity of posttranscriptionally regulated, HuR-dependent genes.

Clones coding for apoptosis-, cell regulatory-, and transcription factor-related proteins were present in the library, and a subset of these clones with known or potential roles as ERGs are listed in Table 1. Many contained extensive 3′-UTR sequences, indicative of posttranscriptionally regulated genes.

TABLE 2. HuR Ligands Differentially Expressed in Neurodegeneration

Clone Name	Regulation	System*	Literature Reference
14-3-3 protein gamma-subtype mRNA	Down	SE/rat	D17447 (38)
Alpha-tubulin mRNA	Up	SE/rat	V01227 (38)
Bone morphogenic protein	Down	SE/mouse	(45)
Calmodulin 1 mRNA	Down	TLE/human	J04046 (36)
Calmodulin-dependent phosphatase mRNA	Up	TLE/human	L14778 (36)
Carboxypeptidase E	Up	IER/rat	NM_01312 (39)
Cathepsin B mRNA	Down	TLE/human	M14221 (36)
Clathrin heavy chain mRNA	Up	KA/rat	(46)
Craniofacial development protein I mRNA	Down	APP(Sw)/mouse	(47)
Cyclin-dependent kinase 4 mRNA	Up	KA/rat	(48)
Cyclin-dependent kinase 5 mRNA	Up	ECS/mouse/rat	(49, 50)
Ectonucleotide pyrophosphatase/phosphodiesterase 2 mRNA	Up	APP(Sw)/mouse	(47)
Eukaryotic translation initiation factor 3, subunit 8	Up	IER/rat	NM_019646 (39)
Ferritin heavy chain mRNA	Up	SE/rat	U58829 (38)
GAPDH mRNA	Up	SE/rat	M17701 (38)
Glucose-6-phosphatase	Up	SE/rat	(40)
Growth factor inducible immediate early protein (pip92) mRNA	Up	NMDA/mouse	(51)
Growth factor inducible immediate early protein (pip92) mRNA	Down	SE/mouse	(45)
HCNP mRNA	Up	Aging/rat	(52)
Heat shock-like (70 kD) protein mRNA	Down	SE/rat	M19942 (38)
Myelin proteolipid protein mRNA	Up	SE/rat	X62611 (38)
Nasal embryonic LHRH factor	Down	IER/rat	NM_057190 (39)
Neurogranin mRNA	Down	TLE/human	Y09689 (36)
Nucleobindin 1	Up	SE/rat	(40)
Prion protein mRNA	Up	TLE/human	M13667 (36)
Prosaposin mRNA	Up	GCI/rat	(53)
Pyruvate kinase M mRNA	Down	TLE/human	M23725 (36)
RhoB mRNA	Up	FI/mouse	(34)
rho GDP-dissociation inhibitor 1 mRNA	Down	TLE/human	X69550 (36)
Ribophorin 1	Up	IER/rat	NM_013067 (39)
Ribosomal protein L10a mRNA	Up	SE/rat	X93352 (38)
Ribosomal protein L14	Up	SE/rat	(40)
Ribosomal protein S9 mRNA	Up	SE/rat	X66370 (38)
RNA polymerase II subunit 3 mRNA	Up	APP(Sw)/mouse	(47)
Superior cervical ganglia-10 mRNA	Down	TLE/human	S82024 (36)
Tissue inhibitor of metalloproteinase 2	Up	IRE/rat	NM_021989 (39)
VASP/Ena mRNA	Up	Seizure/rat	(54)
Vimentin mRNA	Up	FCD/human	(42)

APP/Sw, amyloid precursor protein with Swedish mutation; ECS, electroconvulsive seizure; FCD, focal cortical dysplasia; FI, focal ischemia; GCI, global cerebral ischemia; IER, Ihara's epileptic rat; KA, kainic acid; NMDA, N-methyl-D-aspartic acid; SE, poststatus epilepticus; TLE, temporal lobe epilepsy.

© 2005 American Association of Neuropathologists, Inc.

Consistent with neuronal death associated with seizures, we have cloned 2 distinct mRNAs that code for apoptosis-related BCL2-interacting proteins (BCL2/adenovirus E1B 19 kD-interacting protein 1 and beclin 1 mRNAs) as well as the mRNAs for ACINUS, a protein involved in apoptotic chromatin condensation, and RhoB, which is upregulated in ischemia-damaged neurons (34) and ultraviolet-exposed NIH/3T3 (35). The latter message coimmunoprecipitated with HuR and was posttranscriptionally stabilized by UVL (35).

It is unlikely that the full spectrum of HuR-associated mRNAs has been identified here. We hypothesize that those associated with neuronal response to injury or cell death are likely to be upregulated. Several of the housekeeping ribosomal protein mRNAs (L4, L9, L10), and translation elongation factor 1 alpha were isolated here and from mitogen-activated human peripheral blood mononuclear cells with AUF1 affinity chromatography (29). The selection of the same genes by 2 different ARE-binding proteins in varied stress-induced systems suggests the validity of the experimental approach as well as commonality of cell response to activation. Consistent with the seizure model here, many of the mRNAs selected are differentially regulated in hippocampus by epilepsy, ischemia, glutamate neurotoxicity, and aging (Table 2).

Gene expression profile analysis by several laboratories have identified genes that are differentially regulated in temporal lobe epilepsy (36), human cortical dysplasia (37), and postseizure rat hippocampus (38), as well as rat models for temporal lobe epilepsy (39) and status epilepticus (40). Many of the differentially expressed genes played roles in apoptosis, suggesting that seizures perturb or trigger programmed cell death. In addition, mossy fiber sprouting is associated with status epilepticus; therefore, genes involved in axonal growth and regeneration would be expected to be upregulated under these conditions (41–43). We have identified many of the same cDNAs that may be involved in apoptosis and mossy fiber sprouting as these laboratories (Table 2). We have isolated clones associated with formation of the actin cytoskeleton and extracellular matrix, including several isoforms of actin, tubulin, and myelin basic protein. Of note, myelin basic protein is involved in axonal sprouting and has increased mRNA expression in the injured adult mouse central nervous system (44). Thus, based on our own and its relationship to others' data, we believe it likely that most of the mRNAs identified here are both legitimate ARE ligands and subject to posttranscriptional gene regulation by HuR.

Epilepsy is a chronic disorder present in 0.5% to 1% of the population. Head injury, infection, stroke, tumor, and degenerative disorders such as Alzheimer disease can precede the development of seizures. The interaction of multiple gene products likely leads to the disorder and knowledge of the predisposing genes will aid in the development of better therapeutics. In conclusion, we have identified a diverse repertoire of HuR mRNA ligands in a mouse epilepsy model. Many of these genes play a role in apoptosis, mossy fiber sprouting, and cytoskeletal organization and, therefore, could potentially contribute to the loss of synaptic plasticity and seizure induction observed in epilepsy. Future studies are required to demonstrate the precise HuR-binding sites in these mRNAs, the signaling mechanism of seizure-induced ERG upregulation, and the role these selected genes exert in epilepsy and neurodegeneration.

ACKNOWLEDGMENTS

The authors thank Dr. Henri Furneaux for pGEX2T/HuR plasmid and anti-HuR antibody (Memorial Sloan-Kettering Cancer Center, New York); Dr. Charles Nicolet and his staff at the University of Wisconsin Biotechnology Center for advice regarding automated sequencing reactions and for running the sequencing gels; and members of our laboratories, particularly Dr. Sohail Qureshi of the Sutula laboratory, for their thoughtful comments.

REFERENCES

1. Chen CY, Shyu AB. AU-rich elements: Characterization and importance in mRNA degradation. Trends Biochem Sci 1995;20:465–70
2. Chen CY, Gherzi R, Ong SE, et al. AU binding proteins recruit the exosome to degrade ARE-containing mRNAs. Cell 2001;107:451–64
3. Ma WJ, Cheng S, Campbell C, et al. Cloning and characterization of HuR, a ubiquitously expressed Elav-like protein. J Biol Chem 1996;271: 8144–51
4. Perrone-Bizzozero N, Bolognani F. Role of HuD and other RNA-binding proteins in neural development and plasticity. J Neurosci Res 2002;68: 121–26
5. Peng SS, Chen CY, Xu N, et al. RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. Embo J 1998;17:3461–70
6. Wang W, Caldwell MC, Lin S, et al. HuR regulates cyclin A and cyclin B1 mRNA stability during cell proliferation. Embo J 2000;19:2340–50
7. Levy NS, Chung S, Furneaux H, et al. Hypoxic stabilization of vascular endothelial growth factor mRNA by the RNA-binding protein HuR. J Biol Chem 1998;273:6417–23
8. Dean JL, Wait R, Mahtani KR, et al. The 3′ untranslated region of tumor necrosis factor alpha mRNA is a target of the mRNA-stabilizing factor HuR. Mol Cell Biol 2001;21:721–30
9. Wang W, Furneaux H, Cheng H, et al. HuR regulates p21 mRNA stabilization by UV light. Mol Cell Biol 2000;20:760–69
10. Dixon DA, Tolley ND, King PH, et al. Altered expression of the mRNA stability factor HuR promotes cyclooxygenase-2 expression in colon cancer cells. J Clin Invest 2001;108:1657–65
11. Fan XC, Steitz JA. Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs. Embo J 1998;17:3448–60
12. Chung S, Jiang L, Cheng S, et al. Purification and properties of HuD, a neuronal RNA-binding protein. J Biol Chem 1996;271:11518–24
13. Ma WJ, Chung S, Furneaux H. The Elav-like proteins bind to AU-rich elements and to the poly(A) tail of mRNA. Nucleic Acids Res 1997;25: 3564–69
14. Fan XC, Steitz JA. HNS, a nuclear-cytoplasmic shuttling sequence in HuR. Proc Natl Acad Sci U S A 1998;95:15293–98
15. Gallouzi IE, Steitz JA. Delineation of mRNA export pathways by the use of cell-permeable peptides. Science 2001;294:1895–1901
16. Yount GL, Ponsalle P, White JD. Pentylenetetrazole-induced seizures stimulate transcription of early and late response genes. Brain Res Mol Brain Res 1994;21:219–24
17. Popa-Wagner A, Fischer B, Platt D, et al. Delayed and blunted induction of mRNA for tissue plasminogen activator in the brain of old rats following pentylenetetrazole-induced seizure activity. J Gerontol A Biol Sci Med Sci 2000;55:B242–48
18. Konishi Y, Matsu-ura T, Mikoshiba K, et al. Stimulation of gene expression of NeuroD-related factor in the mouse brain following pentylenetetrazol-induced seizures. Brain Res Mol Brain Res 2001;97: 129–36
19. Westmark CJ, Malter JS. Extracellular-regulated kinase controls beta-amyloid precursor protein mRNA decay. Brain Res Mol Brain Res 2001; 90:193–201
20. Lambert KN, Williamson VM. cDNA library construction from small amounts of RNA using paramagnetic beads and PCR. Nucleic Acids Res 1993;21:775–76

 © 2005 American Association of Neuropathologists, Inc.

21. Altschul SF, Gish W, Miller W, et al. Basic local alignment search tool. J Mol Biol 1990;215:403–10
22. Golarai G, Cavazos JE, Sutula TP. Activation of the dentate gyrus by pentylenetetrazol evoked seizures induces mossy fiber synaptic reorganization. Brain Res 1992;593:257–64
23. Jarzembowski JA, Rajagopalan LE, Shin HC, et al. The 5′-untranslated region of GM-CSF mRNA suppresses translational repression mediated by the 3′ adenosine-uridine-rich element and the poly(A) tail. Nucleic Acids Res 1999;27:3660–66
24. Snee M, Kidd GJ, Munro TP, et al. RNA trafficking and stabilization elements associate with multiple brain proteins. J Cell Sci 2002;115: 4661–69
25. Brennan CM, Steitz JA. HuR and mRNA stability. Cell Mol Life Sci 2001; 58:266–77
26. Tuerk C, Gold L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science 1990;249:505–10
27. Trifillis P, Day N, Kiledjian M. Finding the right RNA: Identification of cellular mRNA substrates for RNA-binding proteins. RNA 1999;5: 1071–82
28. Tenenbaum SA, Carson CC, Lager PJ. Identifying mRNA subsets in messenger ribonucleoprotein complexes by using cDNA arrays. Proc Natl Acad Sci U S A 2000;97:14085–90
29. Bhattacharya S, Giordano T, Brewer G, et al. Identification of AUF-1 ligands reveals vast diversity of early response gene mRNAs. Nucleic Acids Res 1999;27:1464–72
30. Park-Lee S, Kim S, Laird-Offringa IA. Characterization of the interaction between neuronal RNA-binding protein HuD and AU-rich RNA. J Biol Chem 2003;278:39801–8
31. Lopez de Silanes I, Zhan M, Lal A, et al. Identification of a target RNA motif for RNA-binding protein HuR. Proc Natl Acad Sci U S A 2004;101: 2987–92
32. Yeap BB, Voon DC, Vivian JP, et al. Novel binding of HuR and poly(C)-binding protein to a conserved UC-rich motif within the 3′-untranslated region of the androgen receptor messenger RNA. J Biol Chem 2002;277: 27183–92
33. Bakheet T, Frevel M, Williams BR, et al. ARED: Human AU-rich element-containing mRNA database reveals an unexpectedly diverse functional repertoire of encoded proteins. Nucleic Acids Res 2001;29: 246–54
34. Trapp T, Olah L, Holker I, et al. GTPase RhoB: An early predictor of neuronal death after transient focal ischemia in mice. Mol Cell Neurosci 2001;17:883–94
35. Westmark CJ, Bartleson VB, Malter JS. RhoB mRNA is stabilized by HuR after UV light. Oncogene 2005;24:502–11
36. Becker AJ, Chen J, Paus S, et al. Transcriptional profiling in human epilepsy: Expression array and single cell real-time qRT-PCR analysis reveal distinct cellular gene regulation. Neuroreport 2002;13: 1327–33
37. Kim SK, Wang KC, Hong SJ, et al. Gene expression profile analyses of cortical dysplasia by cDNA arrays. Epilepsy Res 2003;56: 175–83
38. Hendriksen H, Datson NA, Ghijsen WE, et al. Altered hippocampal gene expression prior to the onset of spontaneous seizures in the rat post-status epilepticus model. Eur J Neurosci 2001;14:1475–84
39. Arai M, Amano S, Ryo A, et al. Identification of epilepsy-related genes by gene expression profiling in the hippocampus of genetically epileptic rat. Brain Res Mol Brain Res 2003;118:147–51
40. Lukasiuk K, Kontula L, Pitkanen A. cDNA profiling of epileptogenesis in the rat brain. Eur J Neurosci 2003;17:271–79
41. Khrestchatisky M, Ferhat L, Charton G, et al. Molecular correlates between reactive and developmental plasticity in the rat hippocampus. J Neurobiol 1995;26:426–36
42. Taylor JP, Sater R, French J, et al. Transcription of intermediate filament genes is enhanced in focal cortical dysplasia. Acta Neuropathol (Berl) 2001;102:141–48
43. Tang Y, Lu A, Aronow BJ, et al. Genomic responses of the brain to ischemic stroke, intracerebral haemorrhage, kainate seizures, hypoglycemia, and hypoxia. Eur J Neurosci 2002;15:1937–52
44. Jensen MB, Poulsen FR, Finsen B. Axonal sprouting regulates myelin basic protein gene expression in denervated mouse hippocampus. Int J Dev Neurosci 2000;18:221–35
45. Lahteinen S, Pitkanen A, Knuuttila J, et al. Brain-derived neurotrophic factor signaling modifies hippocampal gene expression during epileptogenesis in transgenic mice. Eur J Neurosci 2004;19:3245–54
46. Konopka D, Nowicka D, Filipkowski RK, et al. Kainate-evoked secondary gene expression in the rat hippocampus. Neurosci Lett 1995;185:167–70
47. Stein TD, Johnson JA. Lack of neurodegeneration in transgenic mice overexpressing mutant amyloid precursor protein is associated with increased levels of transthyretin and the activation of cell survival pathways. J Neurosci 2002;22:7380–88
48. Ino H, Chiba T. Cyclin-dependent kinase 4 and cyclin D1 are required for excitotoxin-induced neuronal cell death in vivo. J Neurosci 2001;21: 6086–94
49. Chen J, Zhang Y, Kelz MB, et al. Induction of cyclin-dependent kinase 5 in the hippocampus by chronic electroconvulsive seizures: role of [Delta]FosB. J Neurosci 2000;20:8965–71
50. Newton SS, Collier EF, Hunsberger J, et al. Gene profile of electroconvulsive seizures: Induction of neurotrophic and angiogenic factors. J Neurosci 2003;23:10841–51
51. Chung KC, Shin SW, Yoo M, et al. A systemic administration of NMDA induces immediate early gene pip92 in the hippocampus. J Neurochem 2000;75:9–17
52. Matsukawa N, Tooyama I, Kimura H, et al. Increased expression of hippocampal cholinergic neurostimulating peptide-related components and their messenger RNAs in the hippocampus of aged senescence-accelerated mice. Neuroscience 1999;88:79–92
53. Yokota N, Uchijima M, Nishizawa S, et al. Identification of differentially expressed genes in rat hippocampus after transient global cerebral ischemia using subtractive cDNA cloning based on polymerase chain reaction. Stroke 2001;32:168–74
54. Kato A, Ozawa F, Saitoh Y, et al. vesl, a gene encoding VASP/Ena family related protein, is upregulated during seizure, long-term potentiation and synaptogenesis. FEBS Lett 1997;412:183–89

© 2005 American Association of Neuropathologists, Inc.