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The RNA Binding Domain of Jerky Consists of Tandemly Arranged Helix-Turn-Helix/Homeodomain-Like Motifs and Binds Specific Sets of mRNAs

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A deficit in the Jerky protein in mice causes recurrent seizures reminiscent of temporal lobe epilepsy. Jerky is present in mRNA particles in neurons. We show that the N-terminal 168 amino acids of Jerky are necessary and sufficient for mRNA binding. The binding domain is similar to the two tandemly arranged homeodomain-like helix-turn-helix DNA binding motifs of centromere binding protein B. The putative helix-turn-helix motifs of Jerky can also bind double-stranded DNA and represent a novel mammalian RNA/DNA binding domain. Microarray analysis identified mRNAs encoding proteins involved in ribosome assembly and cellular stress response that specifically bound to the RNA binding domain of Jerky both in vitro and in vivo. These data suggest that epileptogenesis in Jerky-deficient mice most likely involves pathways associated with ribosome biogenesis and neuronal survival and/or apoptosis.

It was previously reported that deletion of the *jerky* gene in mice causes recurrent limbic seizures (9, 54). The manifestation and course of the seizure abnormality in Jerky-deficient mice are reminiscent of familiar temporal lobe epilepsy, an inherited and relatively benign form of temporal lobe epilepsy (3, 4, 7, 13, 18, 36, 37, 42). A de novo nonconservative mutation to a potential glycosylation site in the human homologue of *jerky* (*JRK*/*JH8*) in an epileptic patient was recently described (29), but a pathogenic role for this mutation in seizures has not been established yet.

Although *jerky* mRNA is detectable in various organs in the mouse, expression of the Jerky protein is restricted to the central nervous system (26). Jerky is found in both the nuclei and the cytoplasms of neurons. In the cytoplasm, Jerky is present in messenger ribonucleoprotein (mRNP) complexes and is not associated with ribosomes or actively translating mRNAs (26). These data suggest that Jerky may regulate the availability of mRNAs to the translational machinery in neurons. Jerky not only is part of mRNP complexes but also binds mRNAs directly with high affinity.

Besides Jerky, the absence of fragile X protein, another mRNA binding protein, can also cause seizures in human and mouse (8, 15, 31, 32, 55, 56). This protein regulates the polyribosome association of target mRNAs and is believed to control their translation (5, 12, 21, 23, 25, 44). These data suggest that a deficit in mRNA binding proteins may represent a novel disease mechanism of epilepsy.

To learn more about possible mechanisms that cause or increase the vulnerability to epileptic seizures in Jerky-deficient mice, we mapped the RNA binding domain of Jerky and identified mRNA species bound to the protein. Sequence analysis showed that the RNA binding domain of Jerky is similar to the DNA binding domain of centromere binding protein B (CENP-B). Structural studies revealed that the DNA binding domain of CENP-B consists of two helix-turn-helix (HTH) repeats. Jerky interacts with a large number of brain mRNAs, and the highest-affinity mRNA targets of Jerky could be clustered into functional groups.

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MATERIALS AND METHODS

Generation of *jerky* deletion constructs and production of truncated GST-Jerky fusion proteins. N-terminal deletion mutants of Jerky were produced by PCR using pCMV2-jerky (26) as a template. 5′ primers containing an *Eco*RI site corresponded to residues 49 to 54, 119 to 124, and 169 to 174, while the 3′ primer corresponded to the final six amino acids of Jerky. For C-terminal deletions, a primer corresponding to the first six amino acids of Jerky was used in conjunction with 3′ primers containing sequences corresponding to residues 113 to 118 and 163 to 168. Internal deletions were produced with 5′ primers corresponding to residues 49 to 54 and 119 to 124 and 3′ primers corresponding to residues 113 to 118 and 163 to 168. The *Eco*RI sequence on the 5′ primers allowed the cloning of various mutants in frame with the sequence encoding glutathione *S*-transferase (GST). PCR fragments were first cloned into the TOPO-PCR2.1 vector (Invitrogen, Carlsbad, Calif.) followed by recloning via *Eco*RI sites into pGEX-6P2 (Amersham, Piscataway, N.J.). The sequences of all mutant plasmids were confirmed by sequencing. Expression of mutant proteins was induced in BL21 *Escherichia coli* cells by 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). GST-Jerky was purified as described by Liu et al. (26).

RNA and DNA labeling and nucleic acid binding assays. mRNA (1 μg; Clontech, Palo Alto, Calif.) was 3′ end labeled by using T4 RNA ligase in the presence of [^{32}P]pCp (3,000 Ci/mmol; 10 mCi/ml) at 4°C overnight. For DNA labeling, mouse genomic DNA was first isolated from mouse embryonic stem cells by using DNAzol (Invitrogen). Then DNA was digested with *Sau*3A followed by labeling with Klenow polymerase in the presence of [α-^{32}P]dATP (3,000 Ci/mmol; 10 mCi/ml) at 37°C for 30 min. In some experiments, mRNA and cut DNA were preselected with GST-Jerky (see below) before labeling. To obtain RNA and DNA probes with identical specific activities, labeled and unlabeled RNA and DNA were mixed. Labeled RNA and DNA (10^6 cpm) were used to hybridize nitrocellulose blots containing various amounts of purified Jerky, Jerky$_{1\text{-}168}$, and Jerky$_{169\text{-}557}$ as described by Liu et al. (26). Filter binding assays with Jerky proteins were carried out as described by Liu et al. (26).

Selection of mRNAs with Jerky and oligonucleotide microarray analysis.

Mouse brain mRNA (1 μg; Clontech) was incubated with 0.5 μg of GST-Jerky or GST-Jerky$_{169-557}$ immobilized on agarose beads for 20 min in RNA binding buffer (50 mM LiCl, 10 mM Tris [pH 7.5], 1 mM EDTA) containing 2 μg of yeast tRNA at room temperature. Beads were washed five times with RNA binding buffer for 15 min each. Bound mRNA was then recovered by phenol-chloroform extraction followed by ethanol precipitation in the presence of 1 μl of glycogen (20 mg/ml). As internal controls, four *Bacillus subtilis* RNAs (dap, phe, thr, and trp) were synthesized from pGIBS-dap, -phe, -thr, and -trp plasmids (ATCC, Manassas, Va.) by using a MEGAscript T3 high-yield transcription kit (Ambion, Austin, Tex.). One microgram of input brain mRNA or Jerky-selected mRNAs derived from 1 μg of brain mRNA was mixed with 2×10^{-3} pmol of each of the four *B. subtilis* poly(A) RNAs, and cRNA was generated according to the manufacturer's instructions (Affymetrix, Santa Clara, Calif.). Briefly, double-stranded cDNA was synthesized with the SuperScript Choice system (Invitrogen) by using an oligo(dT) primer containing T7 polymerase promoter sequences (Genset, San Diego, Calif.). Next, the double-stranded cDNA was used as a template to synthesize cRNA with a BioArray high-yield RNA transcript-labeling kit (Enzo Diagnostics, Farmingdale, N.Y.) in the presence of biotinylated UTP and CTP. Purified (RNeasy minikit; Qiagen, Valencia, Calif.) and partially hydrolyzed cRNA was used to probe Affymetrix murine genome U74A arrays (MG-U74Av2) in a GeneChip hybridization oven and fluidics station. These experiments were repeated with three independently isolated and prepared pools of selected mRNAs. A high-resolution image of the hybridization pattern was obtained in a Hewlett-Packard GeneArray scanner with Microarray Suite software 5.0 (Affymetrix). Signals corresponding to individual mRNAs were normalized to the signal of the four spiked bacterial RNAs.

In vivo pulldown and Atlas arrays. Jerky-containing complexes were pulled down from human HEK293 and mouse Neuro-2A cells. Transfection with Jerky-expressing constructs was carried out with Lipofectamine (Invitrogen) and Superfect (Qiagen) for HEK293 and Neuro-2A cells (10^9), achieving approximately 50 and 3% transfection rates, respectively. Two days later, cells were harvested and washed three times with 10 volumes of phosphate-buffered saline. Cells were then lysed mildly with lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 30 mM EDTA, 0.5% Triton X-100) containing complete protease inhibitor cocktail for 45 min on ice. Nuclei were pelleted at 3,000 × *g* for 10 min at 4°C. The cytoplasmic supernatant was precleared for 1 h with 250 μl of protein A agarose. After centrifugation, the supernatant was cleared again with 200 μl of anti-Flag M1 agarose beads for 20 min (Sigma, St. Louis, Mo.). Following centrifugation, the supernatant was immunoprecipitated with 200 μl of anti-Flag M2 agarose bead (Sigma) for 3 h. The immunoprecipitated material was recovered by centrifugation and washed twice with 1 ml of wash buffer (50 mM Tris [pH 7.5], 150 mM NaCl) for 15 min at 4°C. Then the material was washed again with the wash buffer containing 50 U of RNase-free DNase I (Roche, Indianapolis, Ind.) in the presence of 200 U of RNasin (Promega, Madison, Wis.). Finally, the immunoprecipitated material was washed with wash buffer containing 200 U of RNasin and pelleted. Ten percent of the pellet was used for protein analysis. To recover the RNA content from the complexes, the rest of the pellet was first incubated with lysis buffer containing 100 μg of proteinase K (Life Technologies, Gaithersburg, Md.) and 200 U of RNasin at 37° for 15 min, followed by phenol-chloroform extraction, and the mRNA was ethanol precipitated. RNA was used in reverse transcription (RT)-PCR experiments (see below).

Human mRNA isolated from HEK293 Jerky immunocomplexes was also used in RT reactions to generate probes for Atlas nylon cDNA arrays (Atlas 1.2; Clontech). A mixture of specific primers corresponding to the Atlas array and provided by Clontech was used for the labeling. Arrays were hybridized in ExpressHyb solution (Clontech) at 68°C overnight and washed four times with a solution of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 1% sodium dodecyl sulfate (SDS) at 68°C for 30 min each, then with a solution of 0.1× SSC and 0.5% SDS at 68°C for 30 min, and finally with 2× SSC for 5 min at room temperature.

Quantitative RT-PCR assays. One-step real-time RT-PCR was performed with human and mouse mRNAs isolated from Jerky complexes. Reactions were carried out in an Opticon (MJ Research, Incline Village, Nev.) cycler with SYBR-Green I (Molecular Probes, Eugene, Oreg.) by using Superscript one-step RT-PCR Platinum *Taq* (Invitrogen) and gene-specific primers. Human gene-specific primer pairs were as follows: P1, 5′-CATCTACTCGGCCCTCATTC-3′ and 5′-AGACCAAAGCCCATGTCATC-3′; S100A, 5′-AGGGAGGCTGGAG ATCATTT-3′ and 5′-CAGAGTGCTGAACCCAGGA-3′; GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 5′-CGAGATCCCTCCAAAATCAA-3′ and 5′-TGTGGTCATGAGTCCTTCCA-3′; L27A, 5′-TAATGCTGGTGGTCTGC ATC-3′ and 5′-TGCTGAAGAATTTGGCCTTC-3′; S11, 5′-CACCCAAGGAG GCTATTGAG-3′ and 5′-GTGACCTTGAGCACGTTGAA-3′; SOD1 (superoxide dismutase), 5′-GAAGGTGTGGGGAAGCATTA-3′ and 5′-ACCTTTGC CCAAGTCATCTG-3′; actin, 5′-AGACCTGTACGCCAACACAG-3′ and 5′-G CGCAAGTTAGGTTTTGTCA-3′; tubulin, 5′-CCTACTGCATCGACAACGA G-3′ and 5′-TTCTTGGCATCGAACATCTG-3′; GNBR (guanine nucleotide binding protein β1), 5′-TACACCACCAACAAGGTCCA-3′ and 5′CAGTGTG TCCGGTAAACGTG-3′. Mouse gene-specific primer pairs were as follows: P1, 5′-ATCTACTCCGCCCTCATCCT-3′ and 5′-ATGAGGCTCCCAATGTTGA C-3′; GAPDH, 5′-CACTGAGCATCTCCCTCACA-3′ and 5′-GTGGGTGCA GCGAACTTTAT-3′; S11, 5′-ACTGGTAACGTCTCCATCCG-3′ and 5′-CCT GCACTCTCCAACTGTGA-3′; SOD1, 5′-TCTAAGAAACATGGTGGCCC-3′ and 5′-GTTTACTGCGCAATCCCAAT-3′; actin, 5′-GCTCTTTTCCAGCCTT CCTT-3′ and 5′-TGATCCACATCTGCTGGAAG-3′; karyopherin, 5′-CCTGA GGCTTGGAGAACAAG-3′ and 5′-TGCTTCAAAGCTGGAAACCT-3′; GNBR, 5′-GCGGGACACACAGGTTATCT-3′ and 5′-GAAGCATCACAAG CACCAGA-3′. PCR conditions and primers were optimized to produce single DNA products ranging from 100 to 400 bp, as demonstrated by melting curves and visualization in agarose gels. Calculations of threshold cycle and difference were based on the method described in the manual for the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, Calif.).

RESULTS

The N-terminal 168 residues of Jerky are necessary and sufficient for mRNA binding. It has been shown that Jerky binds mRNAs with high affinity (26). However, the actual sequence responsible for RNA binding remained elusive, as Jerky contains no known RNA binding domain. Here we used N-terminal deletion mutants to map the RNA binding domain of Jerky. The purity of the various GST-Jerky preparations following affinity purification was assessed on Coomassie blue-stained SDS-polyacrylamide gels, which showed single bands corresponding to the fusion proteins (data not shown). In binding assays, increasing amounts of GST-Jerky was incubated with labeled RNA generated from a pool of mRNAs preselected with Jerky (see Materials and Methods). RNA-protein complexes (bound fraction [B]) were retained on nitrocellulose membranes, while unbound (free [F]) RNA was captured by nylon membranes. As described previously (26), Jerky bound RNA with a low nanomolar affinity (K_d, 8 nM) (Fig. 1A). Removal of the first 48 residues from the N terminus of the 557-amino-acid Jerky resulted in a dramatic decrease in both affinity (K_d) and the amount of the bound fraction of RNA (B/B+F) (Fig. 1A). Nevertheless, Jerky$_{49-557}$ still bound RNA, as did Jerky$_{119-557}$, and a complete loss of RNA binding was seen only when 168 residues were removed from the N terminus of Jerky (Jerky$_{169-557}$) (Fig. 1A). This experiment demonstrated that the first 168 residues in Jerky are necessary for mRNA binding.

Figure 1B shows that the 168 N-terminal residues of Jerky are not only necessary but also sufficient for binding. Although the binding affinity of Jerky$_{1-168}$ was somewhat lower than that of full-length Jerky (20 versus 8 nM), they bound a similar amount of RNA (B/B+F = 0.5 to 0.6). Further dissection of the 1-168 domain again revealed the importance of the first 48 residues, as both the K_d and total binding of Jerky$_{49-168}$ were reduced compared to those of Jerky$_{1-168}$ (Fig. 1B). Removal of an additional portion of the N terminus (Jerky$_{119-168}$) further reduced both the affinity and total binding (Fig. 1B), as was seen with a similar deletion on the full-length protein (Jerky$_{119-557}$) (Fig. 1A). Since the 119-168 polypeptide still bound mRNA fragments, we tested whether deletion of residues between 119 and 168 has a negative effect on the binding of Jerky$_{1-168}$. Indeed, Jerky$_{1-118}$ bound less RNA than Jerky$_{1-168}$ (Fig. 1B). Taken together, these data demonstrated

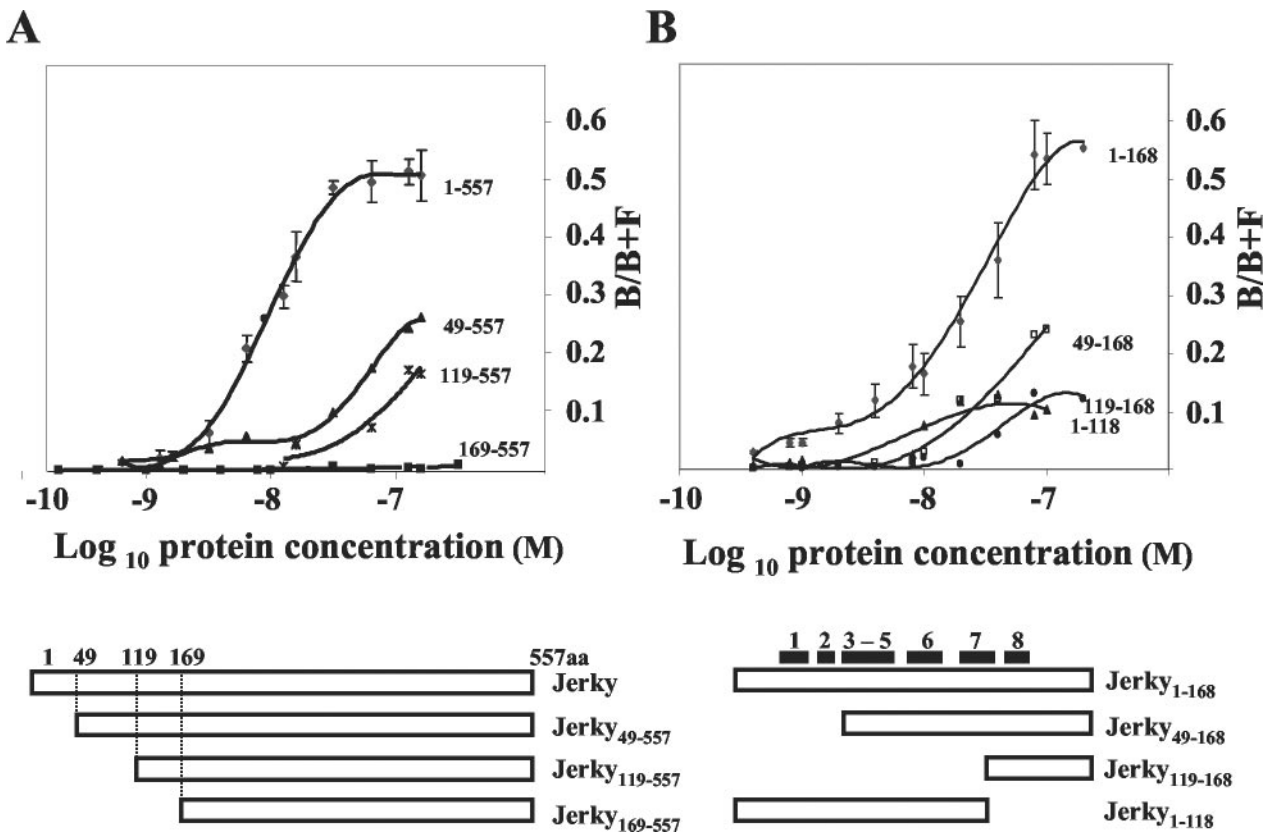

FIG. 1. Identification of the RNA binding domain in Jerky. Filter binding assays were performed with labeled mouse brain mRNA fragments and various Jerky mutants. (A) Mapping of the RNA binding domain in Jerky; (B) dissection of the RNA binding domain of Jerky. The results for full-length Jerky and $Jerky_{1-168}$ are from four independent experiments, three of them performed in duplicate. Data for the other Jerky mutants are from two independent measurements. Black boxes represent the putative α-helices identified in CENP-B (51).

that all three subfragments (residues 1 to 48, 49 to 118, and 119 to 168) contribute to the high-affinity RNA binding of $Jerky_{1-168}$.

As Fig. 2 shows, there is a high level of similarity between Jerky and CENP-B at their N termini. Since the N terminus of CENP-B is responsible for DNA binding, it was surprising that a homologous region in Jerky serves as an RNA binding domain. Figure 2 also shows the two homeodomain-like HTH structures within the N-terminal 129 residues of CENP-B that are both required for DNA binding (51). The first motif, besides the three canonical helices of homeodomain-like HTHs, contains two additional helices (helices 4 and 5) which are directly adjacent to helix 3. The second domain consists of three helices (helices 6 to 8) where the HTH is formed by helices 7 and 8 (51). There is a significant homology between Jerky and CENP-B within these helices (Fig. 2) suggesting that Jerky has a homeodomain-like HTH structure at its N terminus. Alignment of the two putative HTH sequences in Jerky with deletion mutants showed that the three subfragments (residues 1 to 48, 49 to 118 and 119 to 168) of $Jerky_{1-168}$, which all required for high-affinity binding, map on helices 1 and 2,

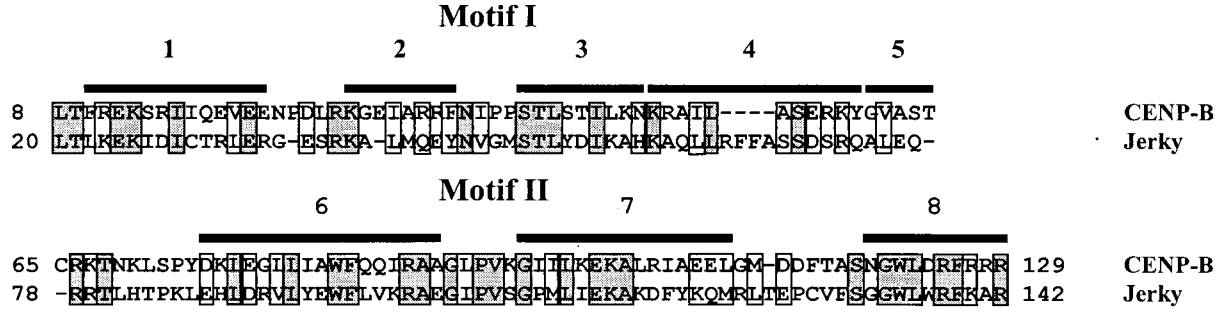

FIG. 2. Alignment of the mouse Jerky and human CENP-B sequences at the N-terminal regions of the proteins. Identical and similar amino acids are highlighted with shaded and open boxes, respectively. The two HTH structures in CENP-B (51) are labeled as motif I and II. Black boxes indicate the positions of individual helices.

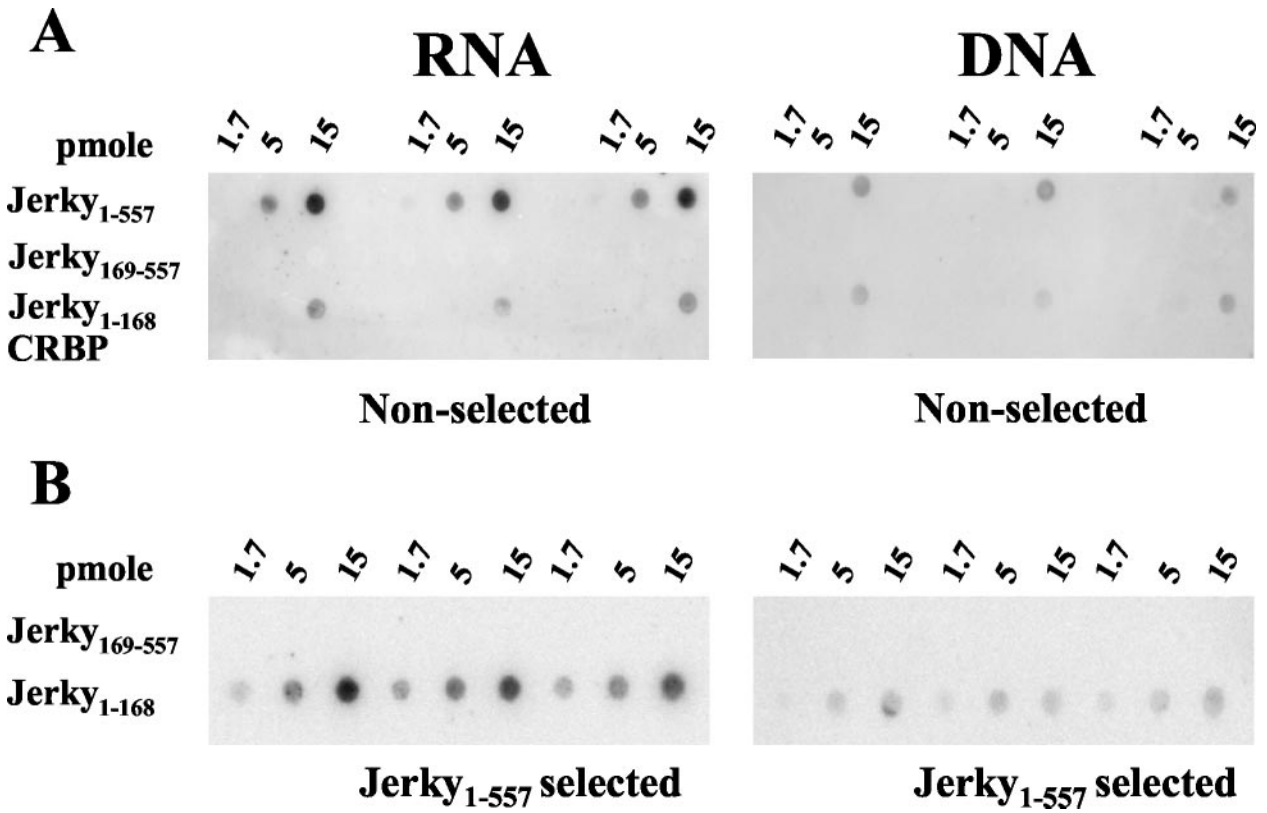

FIG. 3. Jerky binds both RNA and DNA. (A) Interaction of labeled mouse brain mRNA fragments and mouse dsDNA (right panel) with various amounts of GST-Jerky, GST-Jerky$_{169-557}$, and GST-Jerky$_{1-168}$ immobilized on nitrocellulose membranes. GST-CRBP was the negative control. (B) Interaction of brain mRNA fragments and mouse dsDNA preselected on GST-Jerky beads with GST-Jerky$_{1-168}$ and GST-Jerky$_{169-557}$.

helices 3 to 7, and helix 8, respectively (Fig. 1). These data suggest that disrupting any of the two HTH motifs greatly reduces the overall RNA binding of Jerky.

DNA binding of Jerky. As the Jerky RNA binding domain is homologous to the DNA binding domain of CENP-B, we tested whether Jerky can bind double-stranded DNA (dsDNA). Blots containing triplicate serial dilutions of various Jerky proteins were incubated with radiolabeled RNA and DNA probes with the same specific and total activities. This experiment included full-length Jerky, RNA binding-deficient Jerky (Jerky$_{169-557}$), the minimal Jerky RNA binding domain (Jerky$_{1-168}$), and a control protein (cytoplasmic retinoid binding protein [CRBP]) with no RNA binding activity (26). At 15 pmol, both Jerky$_{1-557}$ and Jerky$_{1-168}$ interacted with brain mRNAs more strongly than with fragmented mouse DNA (Fig. 3A). At one-third the protein level (5 pmol), only Jerky$_{1-557}$ incubated with RNA was visible (Fig. 3A). Consistent with previous experiments (Fig. 1), RNA binding of Jerky$_{1-168}$ was lower than that of Jerky$_{1-557}$ at equal protein concentrations. Similarly, Jerky$_{1-168}$ bound less DNA than Jerky$_{1-557}$. Indeed, DNA binding of Jerky$_{1-168}$ was barely detectable. Deletion of the N-terminal 168 residues (Jerky$_{169-557}$) abolished both RNA and DNA binding. To further assess the RNA and DNA binding of Jerky, binding experiments were repeated with Jerky$_{1-557}$-preselected RNA and DNA species (Fig. 3B). Labeled probes, prepared from preselected RNA and DNA with the same specific and total activities, were incubated with the RNA/DNA binding domain of Jerky (Jerky$_{1-168}$) and binding-deficient Jerky$_{169-557}$. Again, RNA binding resulted in a higher signal than DNA binding at identical Jerky$_{1-168}$ concentrations, and binding was not seen with Jerky$_{169-557}$. We concluded that Jerky can bind both RNA and DNA, but its binding of mouse mRNAs is stronger than that of fragmented mouse DNA. These data also showed that the same N-terminal 168 residues of Jerky are responsible for both RNA and DNA binding.

Microarray identification of mRNA species bound to Jerky in vitro. Next we asked the question of whether Jerky binds specific subsets of mRNAs. Tenenbaum and coworkers (52) recently demonstrated that the mRNA content of mRNP complexes can be determined by cDNA arrays. To identify mRNA targets for Jerky, brain mRNAs were captured by recombinant Jerky and the eluted RNA was used to probe Affymetrix MG-U74Av2 oligonucleotide microarrays. These arrays contain about 6,000 sequences from the Unigene database that have been functionally characterized and approximately 6,000 expressed sequence tag clusters. In parallel, identical MG-U74Av2 arrays were probed with input mRNA not selected by Jerky. To monitor labeling and hybridization, all samples were spiked with predetermined amounts of four different bacterial RNAs. This also allowed the normalization of signals; thus, arrays hybridized with various RNA pools could be directly compared. Microarrays probed with input mRNA showed strong fluorescence consistent with the hybridization of 50.14% of the 12,473 genes present on the array (Fig. 4). When

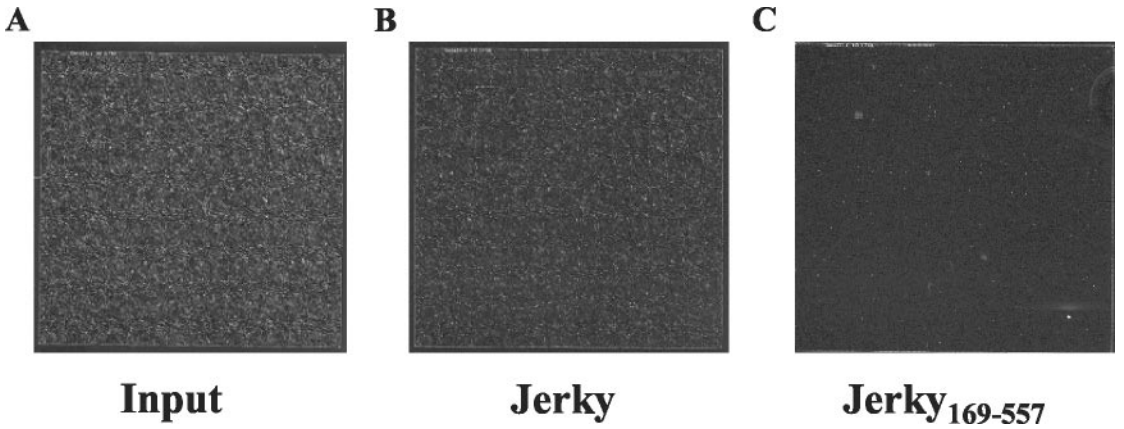

FIG. 4. Probing Affymetrix MG-U74Av2 arrays with mouse input brain mRNA and mRNAs selected with the indicated protein.

Jerky-selected mRNAs were used to probe arrays, fewer, but still a substantial number (34.0%), of the genes were hybridized. To control for nonspecific binding of mRNAs, a pool of brain mRNAs was also incubated with RNA binding-deficient Jerky ($Jerky_{169-557}$). Only 104 genes, representing 0.83% of the total, were present on microarrays. Also, the normalized average signal in these arrays was 3.03, a very low value compared to the average signals of 101.4 and 22.88 for arrays hybridized with input and Jerky-associated mRNAs, respectively. These data show that RNA binding-deficient Jerky does not bind a significant number of mRNAs. We concluded that the large majority of mRNAs captured by $Jerky_{1-557}$ bind to its RNA binding domain.

Microarrays were also used to characterize how deletions within the N terminus of Jerky alter the binding of individual mRNA species. For each individual mRNA, signal obtained on arrays probed with Jerky-selected RNA was plotted against the signal measured on arrays probed with mRNA selected with $Jerky_{1-118}$ and $Jerky_{119-557}$. As described earlier, these two mutants have reduced binding compared to full-length Jerky (Fig. 1A). Figure 5A shows that $Jerky_{1-118}$ bound the same species as $Jerky_{1-557}$, but RNA signals corresponding to this mutant were somewhat lower. $Jerky_{1-118}$ contains putative helices 1 to 5, which form the first HTH motif, but lacks helix 8, which is required for the second HTH motif (Fig. 1B). Another comparison shows that when this sequence was deleted ($Jerky_{119-557}$), resulting in a protein with only helix 8 intact, signals corresponding to most mRNA species became too low to be considered positive (signal is generally <1) (Fig. 5B). Nevertheless, species with high signal levels on arrays probed with Jerky-selected RNAs were also bound by this mutant. Finally, as Fig. 5C shows, no individual mRNAs that were bound to RNA binding-deficient $Jerky_{169-557}$ could be identified by microarray analysis.

After these initial microarray tests, new sets of brain mRNA samples were prepared and analyzed on three parallel arrays to specify the number and nature of mRNAs that can bind to Jerky. A total of 1,603 genes (12.85%) hybridized with Jerky-selected mRNA in all three arrays. Using triplicate arrays eliminated many genes with low signal levels. Also, tRNA was present during the selection procedure in these experiments to further increase specificity. tRNA does not interfere with overall binding of high-affinity targets but prevents the association of weakly bound mRNAs with Jerky (data not shown). Since signal strength in the selected pool is dependent on both binding and abundance, Jerky-selected mRNA signals were normalized to signals obtained with input RNAs (relative signal) (Fig. 6A). Based on the relative signal, mRNAs were ranked. As Fig. 6A shows, a small portion (114 mRNAs) of the ~1,600 selected mRNAs had a relative signal of more than 0.3. The large majority of Jerky-bound mRNAs had a low relative signal, 0.05 to 0.2. Importantly, signals measured in the input

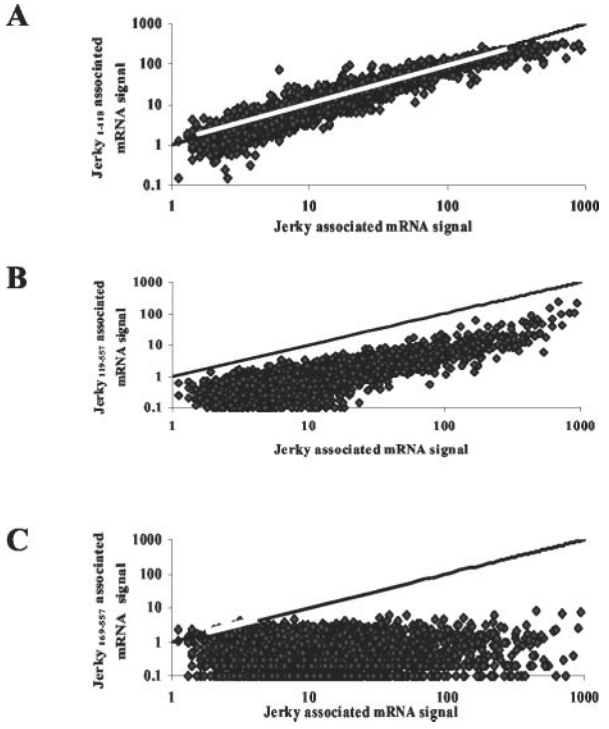

FIG. 5. Microarray analysis of mouse brain mRNAs captured by various Jerky mutants in comparison with mRNAs bound to full-length Jerky. Signal obtained with mRNAs selected with $Jerky_{1-118}$ (A), $Jerky_{119-557}$ (B), and $Jerky_{169-557}$ (C) was plotted against signal corresponding to mRNAs bound by full-length Jerky. The line represents identical signals on the arrays.

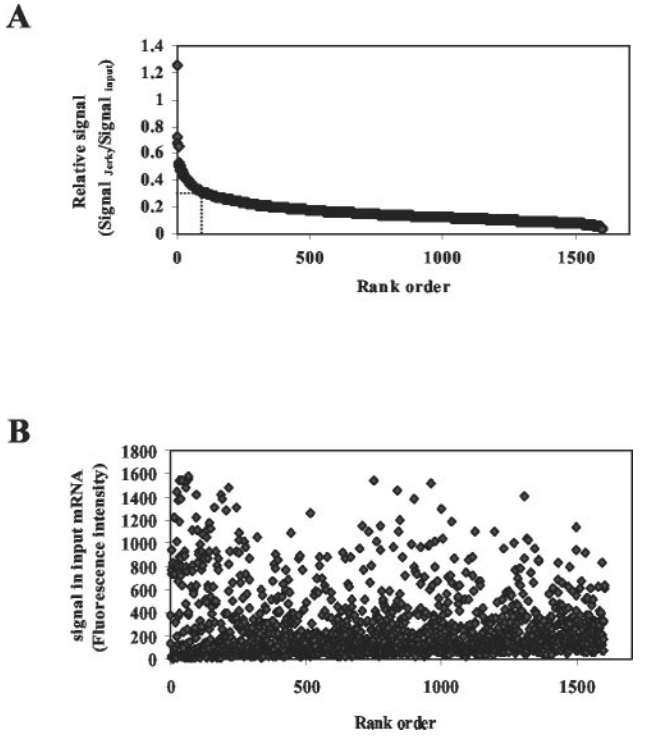

FIG. 6. (A) Distribution of signals normalized to abundance of all mouse brain mRNAs bound to Jerky. Relative signal is displayed as a function of rank in signal strength. The dotted line represents a relative signal of 0.3. A total of 114 mRNAs, including 60 that are functionally characterized, have a relative signal higher than 0.3. (B) Distribution of signal of selected mRNAs in input samples as a function of rank.

showed a similar distribution for high-, medium-, and low-ranking mRNAs (Fig. 6B), indicating that top-ranking mRNAs were not biased toward species with high abundance.

Since preferential targets of Jerky presumably have high relative signals, we further analyzed the first 114 highest-ranking targets (relative signal > 0.3), especially the 60 mRNAs which encode proteins with known functions (Table 1). The highest-ranking targets in this group of mRNAs had relative signals of 0.65 to 1.25, clustering around the theoretical maximum of 1.0, indicating that these species are captured very effectively by Jerky. Surprisingly, the majority of the top 60 functionally characterized targets were not neuron or brain specific even when mouse brain mRNA pools were used for selection. Interestingly, mRNAs encoding certain proteins, such as ribosomal proteins (14 mRNAs), cellular-stress-related proteins (7 mRNAs), and cytoskeletal proteins (6 mRNAs), were enriched in the group of preferential targets of Jerky (Table 1).

Interaction of in vitro-selected target mRNAs with Jerky in vivo. Selection of mouse mRNAs in the previous experiments was carried out with highly purified recombinant Jerky protein. To determine whether these mRNAs are associated with Jerky in cells, we employed HEK293T cells because they express many of the human homologues of the identified mouse targets and are also amenable to transfection to express Jerky. Jerky was expressed as a Flag-tagged protein to facilitate immunoprecipitation of Jerky-RNA complexes with a monoclonal anti-Flag antibody. Immunoprecipitation with Jerky antibodies was less specific and was not used in these experiments. The presence of Jerky in the immunocomplexes was verified by Western blotting (data not shown). Immunocomplexes were analyzed for the presence of mRNAs by Atlas 1.2 macroarrays.

The Atlas array contained ~1,000 functionally characterized human genes and a total of 12 genes hybridized with mRNA coimmunoprecipitated with Jerky from HEK293 cells (Table 2). The mouse homologues of three of these mRNAs (α_1-tubulin, GAPDH, and SOD1) ranked among the top 60 functionally characterized targets on the Affymetrix arrays (ranked 12, 17, and 33, respectively). The mouse homologue of another Jerky-associated mRNA encoding the small ribosomal protein L13a ranked 420 on the Affymetrix array. The remaining eight genes, which included the small ribosomal protein S9 and others that are involved in cellular stress response and apoptosis, such as heat shock proteins (27 and 90 kDa) and GST isoforms (microsomal and pi), were unique for the Atlas array, and their mouse homologues were not present on the Affymetrix array. However 13 other ribosomal proteins (besides L13a) and 6 other cellular-stress-related proteins (besides SOD) were among the group of preferential targets identified by Affymetrix arrays (Table 1). Also, besides the α_1-tubulin mRNA bound to Jerky both in vivo and in vitro, five other mRNAs encoding cytoskeletal proteins were among the in vitro targets (Table 1). Taken together, these data showed that the human homologues of all four Jerky-bound mouse mRNAs that could be tested on Atlas arrays were associated with Jerky in HEK293 cells.

Because Atlas arrays contained a limited number of genes, we extended these studies by probing Jerky immunocomplexes pulled down from HEK293 cells for the presence of additional mRNAs by real-time RT-PCR. In these experiments, levels of mRNAs from Jerky and control immunocomplexes were compared to identify mRNAs that specifically associated with Jerky (Table 3). To assess nonspecific association of mRNAs with immunocomplexes, we selected GNBR mRNA, which, despite its high abundance, was not among the Jerky-associated mRNAs on the Affymetrix array. Also, we measured the level of β_4-tubulin mRNA, which had a very low relative signal and was among the last of Jerky-selected mRNAs (ranked 1595 of the 1,603 selected mRNAs) (Fig. 6A). As Table 3 shows, there was a $<$2-fold difference in the level of GNBR and β_4-tubulin mRNAs in Jerky and control immunocomplexes, demonstrating that they are not specifically associated with Jerky in cells. In contrast, GAPDH and SOD mRNAs, targets that were among the top 60 targets encoding known proteins and were also shown to be associated with Jerky in HEK293 cells by Atlas arrays, were present at 7- and 10-times-higher levels in Jerky than in control immunocomplexes, respectively. Next we analyzed actin β and three ribosomal protein mRNAs (P1, L27A, and S11) that were among the preferential targets on the Affymetrix array. All of these mRNAs were present in a higher level in Jerky (3.8- to 6.8-fold) than in control immunocomplexes (Table 3). Although S100A mRNA was among the preferential targets, it was not found to be specifically associated with Jerky in HEK293 cells. Combining the Atlas array and RT-PCR results shows that Jerky interacts with most of its in vitro targets (eight of the nine tested) in HEK293 cells.

TABLE 1. In vitro mRNA targets with a relative signal higher than 0.3 encoding proteins with known functions

Rank[a]	Identifier	Gene product	Assignment to functional cluster
1	AI840733	Prostaglandin D2 synthase	
4	AF110520	Fas death domain-associated protein	Cellular stress-related/apoptosis associated protein
9	U29402	Ribosomal protein, large, P1	Ribosomal protein
10	Y12474	Centrin 3	
11	AV329607	Aldehyde dehydrogenase 2	
12	M28729	α_1-Tubulin	Cytoskeletal protein
13	AV007820	S100 calcium binding protein A13	
15	X52940	Cytochrome *c* oxidase, subunit VIIc	Cellular stress-related/apoptosis-associated protein
17	M32599	GAPDH	
19	X05021	Ribosomal protein L27a	Ribosomal protein
21	AW045418	Ribosomal protein L44	Ribosomal protein
23	AI851603	NADH dehydrogenase	
24	X51703	Ubiquitin B	
25	M28727	α_2-Tubulin	Cytoskeletal protein
26	U93864	Ribosomal protein S11	Ribosomal protein
28	M13441	α_6-Tubulin	Cytoskeletal protein
30	U11248	Ribosomal protein S28	Ribosomal protein
31	X04591	Creatine kinase, brain	
33	M35725	SOD-1, soluble	Cellular stress-related/apoptosis-associated protein
36	Y17223	ATP synthase, H^+ transporting	
38	AA880275	Metallothionein-I activator	
40	X80699	Ribosomal protein L26	Ribosomal protein
42	X17320	Purkinje cell protein 4	
43	M29015	Ribosomal protein L7	Ribosomal protein
45	X59382	Parvalbumin	
47	U41626	Split hand/foot deleted gene 1	
51	U28917	Ribosomal protein L13	Ribosomal protein
53	X60367	Retinol binding protein 1, cellular	
54	V00754	H3 histone, family 3A	
55	AF035939	Mago-nashi homolog	
57	U59282	ATP synthase, H^+ transporting	
58	K02928	Ribosomal protein L30	Ribosomal protein
60	AF058955	Succinate-coenzyme A ligase	
65	M27844	Calmodulin 2	
66	X52803	Peptidylprolyl isomerase A	
67	X53157	Cytochrome *c* oxidase, subunit Vb	Cellular stress-related/apoptosis-associated protein
68	AJ223066	Fatty acid binding protein 5	
70	L10244	Spermidine/spermine transferase	
73	AI845819	Retinoblastoma-binding protein 9	
75	X15962	Ribosomal protein S12	Ribosomal protein
78	U62674	H3 histone, family 2	
79	D00466	Apolipoprotein E	
82	M12481	β-Actin, cytoplasmic	Cytoskeletal protein
85	AI852230	Mitochondrial ribosomal protein L3	Ribosomal protein
86	AW048899	Ribosomal protein S19	Ribosomal protein
88	AF085809	Synapsin I	
89	AI851740	Actin-related protein 2/3 complex	Cytoskeletal protein
91	AV358770	Lysophospholipase 1	
92	AI852363	Cytochrome *c* oxidase, subunit XVII	Cellular stress-related/apoptosis-associated protein
95	V00835	Metallothionein 1	
97	U08440	Cytochrome *c* oxidase, subunit VIa	Cellular stress-related/apoptosis-associated protein
99	X01756	Cytochrome *c* somatic	Cellular stress-related/apoptosis-associated protein
100	U08354	Melanocortin 5 receptor	
101	AF020185	Dynein, cytoplasmic, light chain 1	Cytoskeletal protein
102	X06407	Translationally regulated transcript	
107	M93310	Metallothionein 3	
109	AF013715	Periplakin	
110	AA666635	Mitochondrial ribosomal protein L13	Ribosomal protein
113	AF043285	Ribosomal protein S7	Ribosomal protein
114	AF100956	Procollagen, type XI, alpha 2	

[a] Based on the relative signal on Affymetrix arrays.

Data also indicate that mouse Jerky can recognize targets regardless of whether they are mouse or human in origin.

Although these data showed that Jerky can recognize its in vitro targets in HEK293 cells, Jerky is a neuronal protein and may form complexes in HEK293 cells that do not exist in neuronal cells. Therefore, we tested Jerky-mRNA association in mouse Neuro-2A cells expressing Flag-tagged mouse Jerky. These mRNAs were mostly chosen from the set tested in the

TABLE 2. Atlas array identification of mRNAs present in Jerky immunocomplexes from transfected HEK293 cells

GenBank no.	Gene or gene product	Function	Rank[a]
M13267	Cytosolic SOD1	Cellular stress-related protein	33
X54079	27-kDa heat shock protein 1	Cellular stress-related protein	NA
X07270	90-kDa heat shock protein A	Cellular stress-related protein	NA
L19185	Peroxiredoxin 2	Cellular stress-related/apoptosis-associated protein	NA
J03746	Microsomal GST 1	Cellular stress-related/apoptosis-associated protein	NA
X15480	GST pi	Cellular stress-related/apoptosis-associated protein	NA
L22474	BCL2-associated X protein (BAX beta)	Apoptosis-associated protein	NA
X01677	GAPDH	DNA damage signaling/repair proteins and DNA ligases	17
U07418	*mut*L (*E. coli*) homolog 1	DNA damage signaling/repair proteins and DNA ligases	NA
X56932	Ribosomal protein L13a	Ribosomal proteins	420
U14971	Ribosomal protein S9	Ribosomal proteins	NA
K00558	α-Tubulin	Cytoskeletal proteins	12

[a] Rank of the mouse homolog in the Affymetrix array (see Table 1). NA, not applicable (not present on Affymetrix arrays).

HEK293 pulldown experiments (Table 3) and included five (P1, GAPHD, S11, SOD, and actin) that were among the top-ranking mRNAs selected with Jerky in vitro and two (GNBR and karyopherin) that were not bound to Jerky in vitro (Table 4). To identify mRNAs that specifically interact with the RNA binding domain of Jerky, neuronal cells expressing Flag-Jerky$_{169\text{-}557}$ were used as a control (rather than cells expressing no Jerky) in these experiments. Four (P1, GAPDH, S11, and SOD) of the five mRNAs that were among the top in vitro targets were present at 4.2- to 8.7-times-higher levels in Jerky than in Jerky$_{169\text{-}557}$ immunocomplexes. Interestingly, mRNAs with an 8.2- to 8.7-fold difference in Jerky and Jerky$_{168\text{-}557}$ immunocomplexes (P1, GAPDH, and S11) were on the very top of the target list (rank, 9 to 26), while SOD1, with a 4.2-fold difference, had a rank of 33. Moreover, actin mRNA, whose rank was the lowest (i.e., 82), showed no specific binding to Jerky. The difference in mRNA levels in Jerky/Jerky$_{169\text{-}557}$ immunocomplexes for the controls (GNBR and karyopherin) was within twofold and cannot be regarded as significant. We concluded that Jerky interacts with in vitro-identified target mRNAs in neuronal cells. In fact, there was a good correlation between the in vitro target rank and the specificity of the association in Neuro-2A cells.

DISCUSSION

The RNA binding domain of Jerky is homologous to HTH motifs found in CENP-B. Although Jerky binds RNAs with high affinity, none of the well-known RNA binding motifs could be located within its sequence. Well-characterized RNA binding domains include the RNA recognition motif, with a consensus sequence of (L/R)G(F/Y)(G/A)FVX(F/Y) (1, 20, 50), the arginine-rich motif (24), the K homology motif, with a core sequence of VIGXXGXXI (46), and the RGG box, which contains between 6 and 18 copies of closely spaced Arg-Gly-Gly (RGG) repeats (22). Instead of these domains, our mapping revealed that two HTH-like motifs, located at the N-terminal 168 residues, are responsible for the high-affinity RNA binding property of Jerky.

The HTH structure of the Jerky RNA binding domain is inferred from its high sequence similarity to the N terminus of CENP-B. The HTH structure of CENP-B$_{1\text{-}56}$ was first revealed by nuclear magnetic resonance (19). The CENP-B HTH structure could be superimposed on the Fushi tarazu homeodomain and the homeodomain-like structures found in Myb and RAP1 (19). Homeodomains contain three helical regions that are folded into a compact globular structure. Helices 1 and 2 lie parallel to each other and across from the third helix (2). Further, X-ray crystallography studies by Tanaka et al. (51) showed tandemly arranged two-HTH structures in CENP-B$_{1\text{-}129}$. This sequence shows high homology with the N-terminal 141 residues of Jerky. Since deletion analysis showed that the first 168 residues of Jerky are required and sufficient for mRNA binding, these data suggest that a tandem repeat of two HTH structures serves as the RNA binding domain of Jerky. Additional deletion mutants demonstrated

TABLE 3. RT-PCR identification of mRNAs present in Jerky immunocomplexes from transfected HEK293 cells

Rank[a]	Gene product	Difference (fold)[b]
9	P1	3.76
13	S100	1.79
17	GAPDH	7.26
19	L27A	5.57
26	S11	4.53
33	SOD1	10.13
82	β-Actin	6.77
1595	β_4-Tubulin	1.91
NA	GNBR	0.83

[a] See Table 1. NA, not applicable.
[b] Difference in mRNA levels in Jerky and control immunocomplexes, calculated as $2^{C(t)\text{vector} - C(t)\text{Jerky}}$, where $C(t)$ is the threshold cycle for target application.

TABLE 4. RT-PCR identification of mRNAs present in Jerky immunocomplexes from transfected Neuro-2A cells

Rank[a]	Gene product	Difference (fold)[b]
9	P1	8.663
17	GAPDH	8.663
26	S11	8.281
33	SOD1	4.192
82	β-Actin	1.061
NA	GNBR	2.000
NA	Karyopherin	1.610

[a] See Table 1. NA, not applicable.
[b] Relative difference in mRNA levels in Jerky and Jerky$_{169\text{–}557}$ immunocomplexes, calculated as $2^{[C(t)\text{vector} - C(t)\text{Jerky}]}/2^{[C(t)\text{vector} - C(t)\text{Jerky}169\text{–}557]}$, where $C(t)$ is the threshold cycle for target application.

that both of these putative HTH structures in Jerky have to be intact for high-affinity RNA binding. Database searches with the putative Jerky HTH motifs identified, in addition to CENP-B, six mammalian genes, indicating that such a structure can be found in a family of proteins (W. Liu and M. Toth, unpublished data). It may be predicted that Jerky-like proteins also bind RNA and that the Jerky family of proteins represents a novel group of RNA binding proteins.

HTH structures, including homeodomains and the homeodomain-like motifs of Myb and RAP1, are primarily known for their dsDNA binding. However, the homeodomain of the *Drosophila* Bicoid protein has recently been recognized as a functional RNA binding motif (11, 40). Although not entirely composed of α-helices as are homeodomains and homeodomain-like structures, the RNA binding domains of E1AV-TAT and the ribosomal protein L11 can also fold into HTH-like structures (41).

Although Jerky, similar to Bicoid, binds RNA and may assume an HTH structure, there are differences between these two proteins. While Jerky binds a large set of mRNAs, Bicoid interacts with only one known species, caudal mRNA (11, 40). Structurally, Bicoid contains a single homeodomain, while Jerky may harbor two adjacent homeodomain-like HTH motifs. To the best of our knowledge, no mammalian HTH structure with the ability to bind RNA has been identified, and the two tandemly arranged homeodomain-like HTH motifs in Jerky may represent a novel mammalian RNA binding domain.

Jerky binds both RNA and DNA. CENP-$B_{1\text{-}129}$ binds a specific dsDNA sequence called the B box. There is extensive homology between CENP-B and Jerky at their N termini. When tested with either total fragmented genomic DNA or a Jerky-preselected pool of genomic DNA fragments, Jerky showed an interaction which was less robust than its binding to total or Jerky-preselected mRNAs. Once DNA sequences that specifically bind Jerky are identified, it will be possible to determine if their binding affinities reach a level that is biologically relevant.

Data obtained with deletion mutants clearly showed that the 168 residues that are responsible for RNA binding and form the putative tandem HTH structure are also involved in binding dsDNA. Another HTH sequence which binds both DNA and RNA is the Bicoid homeodomain. Bicoid, by binding to specific DNA sequences, transcriptionally activates zygotic segmentation genes such as Hunchback in *Drosophila* (6, 10, 14, 48).

How is it possible that certain HTH structures can bind both RNA and dsDNA? The DNA binding of HTH can be easily explained by the accommodation of an α-helix, in particular helix 3, into the major groove of the DNA. It has also been shown that the third helix is crucial in binding of RNA by Bicoid (33). However, the major and minor grooves of RNA do not form a smooth surface to interact with α-helices, and the HTH structure may recognize partially unfolded RNA that is presented at specific stem-loop or bulged structures.

Jerky binds specific sets of mRNAs. Reviewing the target list obtained by Affymetrix arrays indicated the selection of specific sets of mRNAs by Jerky. In particular, mRNAs encoding proteins involved in ribosome biogenesis, cellular stress response and/or apoptosis, and cytoskeleton organization were enriched in the Jerky-selected mRNA pool. Most of the target mRNAs tested in subsequent pulldown experiments demonstrated an interaction with Jerky and specifically with its RNA binding domain in HEK293 and Neuro-2A cells. Enrichment of Jerky targets in Jerky immunocomplexes from Neuro-2A cells was somewhat higher and correlated better with the rank obtained in the in vitro binding. This may be due to potential differences in the protein composition of Jerky complexes formed in neuronal (Neuro-2A) and epithelial (HEK293) cells. In terms of function, Jerky-mRNA interactions in Neuro-2A cells are more relevant, as Jerky is a predominantly neuronal protein. Species differences could have also accounted for some of the differences. Indeed, in both the in vitro binding and Neuro-2A pulldown assays, complexes were formed between mouse Jerky and mouse mRNAs, while in the HEK293 pulldown experiment, the interaction was between mouse Jerky and human mRNAs.

Importantly, the interaction of Jerky with mRNAs encoding ribosomal proteins and proteins linked to cellular stress response and/or apoptosis has been confirmed in Neuro-2A cells. In contrast, the mRNA for the cytoskeletal protein β-actin did not interact with Jerky in these cells. Determining whether mRNAs that encode cytoskeletal proteins are genuine Jerky targets will require testing of additional members of this group of mRNAs in Neuro-2A cells.

Ribosomal proteins are required for ribosome biogenesis, and mRNAs for 14 and 2 of the total of approximately 70 ribosomal proteins were confirmed to be associated with Jerky in vitro and in vivo (in Neuro-2A cells), respectively. Regulating the expression of ribosomal proteins is challenging because the cell needs equimolar amounts of the individual components of the ribosome and ribosomal proteins. While downregulation of these proteins may interfere with translation, overexpression of ribosomal proteins, due to their highly charged nature, could endanger the cell unless they are assembled into ribosomes. Vertebrates utilize translation as a primary means of regulating ribosomal protein synthesis (28), and Jerky, by controlling mRNA accessibility, could be one of the regulatory factors.

A total of 11 targets of Jerky encode proteins involved in cellular stress response and/or apoptosis. Cells are constantly exposed to environmental, physical, or chemical stresses that can induce either a cellular response which promotes survival or apoptosis, characterized by a sequence of regulated events culminating in cell death. One of the key molecules in cell survival or apoptosis is SOD, which accelerates the formation of H_2O_2 from $O_2^{\cdot -}$ (17, 34, 45). The mRNA encoding SOD was associated with Jerky in vitro as well as in HEK293 and Neuro-2A cells. GST mRNAs, whose encoded proteins are believed to exert a critical role in cellular protection against oxidative stress, were also found to be associated with Jerky in vitro and in HEK293 cells. These proteins detoxify a variety of electrophilic compounds, including oxidized lipid, DNA, and catechol products generated by reactive oxygen species (16). Peroxiredoxin, another Jerky-target encoded protein, has similar functions (39). Two heat shock protein mRNAs were also among those that interacted with Jerky in HEK293 cells. It has been shown that heat shock proteins promote neuronal survival by facilitating both recovery and suppression of apoptosis (43). During apoptosis, cytochrome *c* enzymes are released

from the mitochondria (27, 30, 49), and five mRNAs encoding these proteins were bound to Jerky in vitro. Once in the cytosol, cytochrome *c* activates caspase 9, whose cleavage is followed by activation of downstream caspases and apoptosis (47). Bax, a Bcl-associated protein, has been shown to promote apoptosis (35), and its mRNA was among the Jerky-associated mRNAs in HEK293 cells. Finally, the mRNA for the Fas death domain-associated protein that is associated with Fas receptor and procaspases to form the "death-inducing signaling complex" (38) was found in Jerky complexes isolated from HEK293 cells. The synthesis of these proteins may be altered in Jerky-deficient cells, which in turn could increase the vulnerability of neurons to physical and chemical stressors.

It seems that association of these functionally related mRNAs with Jerky is not coincidental, because targets that could be analyzed were found in association with Jerky both in vitro and in vivo. Also, functionally similar targets were often found on both the Affymetrix and Atlas arrays. Although these data show that Jerky binds to mRNAs within cells, we do not know whether Jerky regulates these targets. It has been reported that Jerky is present in mRNA particles but is absent from ribosomal complexes (26), which suggests that Jerky may regulate the accessibility of bound mRNAs and their utilization by the translational machinery. However, it is also possible that Jerky controls the transport, localization, and/or stability of these mRNAs.

Epilepsy is a heterogeneous disorder, and mutations in many genes can lead to seizure disorders. Although several single-gene mutations in neuronal channels and/or receptors that cause epilepsies have been identified, seizures can be elicited by mutations in other genes whose protein products are not directly involved in membrane excitability (53). These include genes associated with neuronal migration defects and neurodegeneration. Jerky, as an RNA binding protein, may regulate the expression of numerous genes, and its absence could cause defects in several molecular or cellular pathways. The nature of mRNAs associated with Jerky suggests that the most likely pathways that can be affected are ribosome biogenesis and translation and neuronal survival and apoptosis.

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