Region-specific alternative splicing in the nervous system: Implications for regulation by the RNA-binding protein NAPOR

WENQING ZHANG, HAIYING LIU, KYOUNGHA HAN, and PAULA J. GRABOWSKI
Howard Hughes Medical Institute and Department of Biological Sciences, A507LH University of Pittsburgh, Pittsburgh, Pennsylvania 15260, USA

ABSTRACT
Alternative RNA splicing generates extensive proteomic diversity in the nervous system, yet few neural-specific RNA binding proteins have been implicated in splicing control. Here we show that the biochemical properties and spatial expression of mouse neuroblastoma apoptosis-related RNA-binding protein (NAPOR; also called NAPOR-1) are consistent with its role in the regulation of the exon 5 and exon 21 splicing events of the N-methyl-D-aspartate (NMDA) receptor R1 transcript. NAPOR, which is closely related to CUG binding protein 2 (CUG-BP2), promotes exon 21 and represses exon 5 splicing in functional coexpression assays. These NMDA mRNA isoforms are distributed, in vivo, in a region-specific manner in rat brain, such that high levels of exon 21 selection and exon 5 skipping coincide with high NAPOR mRNA expression in the forebrain. Within the forebrain, this spatial correspondence is most striking in the visual cortex. In contrast, low NAPOR expression coincides with the reciprocal pattern of alternative splicing in the hindbrain. Complementary experiments demonstrate a tissue-specific distribution of NAPOR, CUG-BP, and other highly related proteins within the nervous system as assayed by probing forebrain and hindbrain nuclear extracts with monoclonal antibody, mAb 3B1. Thus, NAPOR may be one of a group of closely related proteins involved in splicing regulation within the brain. An intronic RNA element responsible for the silencing of exon 21 splicing is identified by mutational analysis and shown to bind directly to recombinant NAPOR protein, suggesting a model in which exon 21 selection is positively regulated by an antirepression mechanism of action.

Keywords: alternative splicing; CUG-binding proteins; forebrain; hindbrain tissue-specific nuclear extracts; N-methyl-D-aspartate receptor

INTRODUCTION
The control of gene activity by alternative RNA splicing is important for cellular function and development. Yet, the determinants of cell and tissue-specific splicing mechanisms are poorly understood. In the mammalian nervous system, alternative splicing generates extensive molecular diversity, where hundreds of thousands of distinct proteins are generated from far fewer genes (Black, 2000; Graveley, 2001). Neuron-specific protein isoforms play important roles in learning and memory, cell communication, and neural development. Protein functional properties are frequently modulated in the nervous system by cell, tissue, or developmental variations in alternative splicing (Grabowski & Black, 2001).

The spliceosome is the active arrangement of small nuclear ribonucleoprotein particles (snRNPs), which aligns the splice site boundaries and serves as the catalytic machinery for splicing. The relative efficiency of spliceosome assembly on multiple introns of a complex pre-mRNA is instrumental in determining patterns of alternative splicing, as well as the accuracy of individual splicing events. Whether an internal exon is constitutively spliced or subject to exon skipping is determined by the strength of the individual splice sites flanking the exon in combination with the effects of splicing enhancer and/or repressor elements (Burge et al., 1999; Smith & Valcarcel, 2000). Serine/arginine-rich (SR) protein splicing factors and hnRNP proteins, which in specific combinations interact with such regulatory elements, have been shown to exhibit some tissue-specific variations (Kamma et al., 1995; Hanamura et al., 1998). However, these previous studies do not reveal a striking pattern of enrichment, or deficiency, of these factors in nervous system tissue, leaving open the possibility that subtle combinatorial differences in these proteins may contribute to splicing regulation in many tissues.
A number of RNA-binding proteins have been implicated in the regulation of neural-specific splicing events. Nova-1 is a splicing activator with three hnRNP K homology (KH) domains, and its expression pattern is brain specific (Buckanovich et al., 1996; Buckanovich & Darnell, 1997). Targets of Nova-1 regulation include the neural exons of the GlyR complex (Min et al., 1997), and its expression increases with postnatal age (Markovtsov et al., 2000). In Nova-1 knockout mice, the ratio of the exon-included and exon-skipped mRNA products is disrupted in the corresponding midbrain and hindbrain regions where Nova-1 protein has been genetically depleted.

The poly(A)-tract-binding protein (PTB, also called hnRNP I) is a negative regulator of alternative splicing affecting multiple targets in the nervous system and muscle (Valcarcel & Gebauer, 1997). PTB has four RNA recognition motif type (RRM) RNA-binding domains, and down-regulates neural exon selection by direct binding to intronic repressor elements in a variety of transcripts, including the c-src tyrosine kinase, GABA_A receptor γ2, Clathrin light chain B, and NMDA R1 receptor exon 5 (Grabowski & Black, 2001). The low levels of PTB expression in the mature rodent (Ashiya & Grabowski, 1997; Lillevall et al., 2001) or human (Markovtsov et al., 2000) brain are consistent with the idea that PTB represses neural-enriched splicing events in nonneural cells. In addition, PTB is implicated in the coordinate regulation of some neural-enriched splicing events during cerebellum development, as its expression decreases with increasing postnatal age (Wang & Grabowski, 1996; Zhang et al., 1999). The neural-enriched paralog of PTB, nPTB (also called brPTB), shows a complementary pattern of expression such that its expression increases with postnatal age (Markovtsov et al., 2000; Polydorides et al., 2000). Furthermore, nPTB interacts with Nova-1 in a yeast two-hybrid assay and counteracts the positive effects of Nova-1 on alternative splicing in transient coexpression assays (Polydorides et al., 2000).

KSRP (for KH-type splicing regulatory protein), is a neural-enriched RNA-binding protein identified from a neuronal splicing extract through its binding to the intronic enhancer of the c-src transcript, where it functions to promote the assembly of the intronic enhancer complex (Min et al., 1997). HnRNPs F and H also associate with the intronic enhancer preferentially in a neural splicing extract, although the expression patterns of these proteins are not strongly tissue specific (Chou et al., 1999).

In Drosophila, the embryonic lethal abnormal vision (ELAV) RNA-binding protein, exhibits a neuron-specific expression pattern and is believed to play an important role in the regulation of alternative splicing of neuroglian (Koushika et al., 1996). In mammals, the ELAV-related Hu-type RNA-binding proteins HuB, HuC, and HuD have a neural-specific expression pattern, are pre-dominantly cytoplasmic, and associate with a distinct subset of mRNAs (Keene, 2001). Their characterized functions involve the control of mRNA stability and translation (Antic & Keene, 1997; Lazarova et al., 1999). Additionally, a distinct set of RNA-binding proteins, termed Musashi, is expressed in the precursors of mitotically active neurons. Musashi proteins are implicated in translational control and pre-mRNA processing, although the detailed functions of these proteins are poorly understood (Nakamura et al., 1994; Sakakibara et al., 1996; Sakakibara & Okano, 1997).

Here we document the brain region-specific alternative splicing of exons 5 and 21 of the N-methyl-D-aspartate (NMDA R1) receptor, and show evidence of a regulatory role for mouse Neuroblastoma apoptosis-related RNA-binding protein (NAPOR). Mouse NAPOR (accession number AAD13763; also called mouse NAPOR-1) was identified by its increased expression in a neuroblastoma cell line during apoptosis (Choi et al., 1998). Human NAPOR is encoded by a single gene, which gives rise to three mRNA isoforms by alternative splicing, NAPOR-1, -2, and -3 (Li et al., 2001). NAPOR contains three RRM-type RNA-binding domains highly related to human CUG-binding protein (CUG-BP) and ELAV-type RNA-binding protein 3 (ETR3-type) factors (also called CELF factors; Ladd et al., 2001). In the protein databases, orthologs of these mammalian factors are found in Caenorhabditis elegans, Drosophila, Xenopus, and Danio rerio. The Drosophila orthologs are called Bruno factors, which have been functionally characterized as translational regulators (Good et al., 2000).

CUG-BP (formerly hNAB50) was initially identified as an RNA-binding activity having a preference for the CUG triplet repeats associated with CUG expansions in myotonic dystrophy (Timchenko et al., 1996b). CUG-BP was cloned by use of the specific monoclonal antibody, mAb 3B1, and found to be comprised of a 49-kDa isoform, CUG-BP1, which is predominantly cytoplasmic, and a 51-kDa isoform, CUG-BP2, which is predominantly nuclear (Timchenko et al., 1996a). Subsequently, CUG-BP1 was shown to promote splicing of exon 5 of the cardiac troponin T (cTNT) pre-mRNA, and to interact directly with an intronic enhancer element rich in CUG repeats (Phillips et al., 1998). The human ETR3 factors (also called CELF, for CUG-BP and ETR3-like factors) show a similar behavior toward cTNT exon 5, and the induction of these proteins in culture during the differentiation of myoblast cells correlates with an increase in cTNT exon 5 splicing (Ladd et al., 2001).

The NAPOR, CUG-BP, and ETR3 factors are widely expressed in mammalian tissues, but some isoforms appear to be enriched in muscle or neural tissue. By use of nuclear extracts prepared from different regions of rat brain, we show here that these protein factors, by virtue of their reactivity with mAb 3B1, are distributed in...
a forebrain-enriched, hindbrain-deficient pattern. This tissue-specific pattern of expression coincides with the in vivo pattern of splicing of NMDA R1 exons 5 and 21 in the forebrain, and agrees well with the functional effects of NAPOR on these exons in coexpression assays. Finally, we identify an intrinsic repressor of exon 21 selection and demonstrate that NAPOR interacts directly and specifically with this RNA region. These results point to a regulatory role for NAPOR in the alternative splicing of the NMDA R1 receptor. NMDA R1 glutamate receptors are important mediators of excitatory transmission in the mammalian brain, and their functions are crucial for synaptic transmission and plasticity, brain development, learning, and memory. Alternative splicing of exons 5, 21, and 22 generates at least seven mRNA isoforms, which differ in their physiological and pharmacological properties, or subcellular distribution (Zukin & Bennett, 1995).

RESULTS

Mouse NAPOR functions as a positive regulator of exon 21 and as a negative regulator of exon 5 in transient coexpression assays

Of the positives identified in a yeast two hybrid screen for interacting partners of PTB, mouse NAPOR was selected for further study to explore its possible role in alternative splicing in the nervous system based on its reported brain-specific mRNA expression pattern (Choi et al., 1998, 1999). The striking similarity of mouse NAPOR to ETR-3 and CUG-BP2 (also called NAPOR-3) is shown in the protein alignment of Figure 1. Database entries for the set of NAPOR-related mouse proteins and their corresponding mRNAs are summarized in Table 1. Figure 1 also shows two of the CUG-BP1-related factors, CUG-BP isofrom LYLQ, and mouse deadenylating factor, which is identical to CUG-BP1 (also called EDEN-BP). Note that mouse NAPOR is most closely related to human NAPOR-3 (accession number AAK72223) with the exception of one residue (position 283). To explore the functional effects of NAPOR on alternative splicing in the nervous system, splicing reporter minigene with neural exons were expressed individually in the presence and absence of NAPOR, and effects on alternative splicing assayed by RT-PCR. The nonneural mouse cell line, C2C12, was chosen for these experiments due to its high transfection efficiency.

Striking effects of NAPOR were observed for two alternative exons of the NMDA R1 receptor, exons 5 and 21 (Fig. 2A). In the absence of NAPOR, exon 21 is spliced predominantly by exon skipping, whereas coexpression of NAPOR strongly promotes exon 21 selection (Fig. 2, lanes 1, 4, and 5). Plasmids expressing rat PTB, or Lac Z in the same backbone vector, serve as negative controls for the effect of NAPOR, as exon 21 selection is not increased under these conditions (Fig. 2, lanes 2, 3, 6, and 7). Western blot analysis serves to verify protein expression from pcDNA/NAPOR in nuclear extracts of the transfected cells (Fig. 2A, inset). Nuclear expression of PTB in the transfection experiments was also confirmed by western blotting (data not shown). We cannot rule out possible negative effects of PTB coexpression on the wild-type exon 21 splicing reporter, because such effects would be difficult to detect based on the low ratio of exon-selected to exon-skipped mRNA products generated in the control samples lacking PTB.

In contrast, the NMDA E5 splicing reporter shows predominant exon 5 selection in the absence of NAPOR (Fig. 2, lanes 8, 11, and 14), whereas coexpression of NAPOR negatively regulates this exon (Fig. 2, lanes 12 and 13). Coexpression of PTB also results in the negative regulation of exon 5 (Fig. 2, lanes 9 and 10), whereas the control Lac Z plasmid has no effect on the splicing pattern (Fig. 2, lanes 15

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*Allelic differences and sequencing errors cannot be ruled out.*
The quantitative effects of NAPOR on exons 5 and 21 are summarized in Figure 2B. At the highest ratio of pcDNA/NAPOR to splicing reporter plasmid (6/1), NAPOR promotes a 57.5% increase in exon 21 selection, and a 45.6% decrease in exon 5 selection. Thus, NAPOR has opposite effects on the exon 5 and exon 21 alternative splicing events of the NMDA R1 receptor transcript.

The irregular spatial expression of NAPOR mRNA in adult rat brain corresponds to sites of NMDA R1 exon 21 selection and exon 5 skipping in vivo.

If NMDA R1 exons 5 and 21 are targets of NAPOR regulation, the spatial expression pattern of NAPOR should correspond to sites of exon 21 selection and...
The cerebral cortex. That is, translation with sites of NAPOR expression was observed. primers directed to common sequences in NAPOR, 3B in the forebrain (Fig 54). The rat has a striking brain-region-specific pattern in the adult, of the brain characterized in these tissues. The most striking spatial correspondence of exon 21 expression and exon 21 splicing (Fig 3C, top panel). To further investigate the spatial relationship between NAPOR mRNA expression and exon 21 splicing, expression was assayed in three subregions of the cerebral cortex. These results show that NAPOR expression tracks with exon 21 selection in the prefrontal and temporal cortex, whereas, NAPOR expression is reduced in the basal ganglia where exon 21 splicing is also reduced (Fig. 3C, right panels). Furthermore, exon 5 splicing is largely the reciprocal of that of exon 21 in these tissues.

The most striking spatial correspondence of exon 21 splicing and NAPOR mRNA expression is observed in the visual cortex, which is a subregion of the cerebral cortex (vc, schematic of Fig. 3B). In this experiment, RNA samples from the visual cortex of four rat littermates, designated S1, S2, S3, and S4, were compared to that of the cerebellum from the same animals. In the visual cortex, exon 21 splicing is maximized and

![Figure 2](https://example.com/figure2.png)

**FIGURE 2.** Alternative exons 5 and 21 of the NMDA R1 receptor transcript are sensitive to regulation by NAPOR in coexpression assays. A: Top: Splicing reporter plasmids containing NMDA R1 exon 5 (NMDA E5) or exon 21 (NMDA E21) were cotransfected into mouse C2C12 myoblasts with a pcDNA/NAPOR expression plasmid (NAPOR), pcDNA/PTB (PTB), or control plasmid pcDNA/Lac Z (Lac Z). Lanes – are mock transfected with backbone plasmid; wedge indicates 4/1 or 6/1 mass ratio of protein expression plasmid to splicing reporter. Bottom: Schematic of splicing reporter constructs. NMDA E21 (also called E21wt) contains the entire region of the rat NMDA R1 transcript between exons 20 and 22. NMDA E5 is a chimeric minigene containing NMDA exon 5 and portions of flanking introns (thick lines) inserted between constitutive GABA A γ2 receptor exons (crosshatched; GABA receptor introns are indicated as thin lines). Exon regions amplified by RT-PCR are indicated in nucleotides below each splicing reporter; intron lengths are shown below. RT-PCR products are resolved on 6% polyacrylamide, 5 M urea sequencing gels. Inset: Western blot analysis was used to verify pcDNA/NAPOR expression in the nuclei of the transfected cells by using an antibody specific for the Xpress epitope tag of the transfected plasmid (–, empty pcDNA vector; wedge indicates a 1/4 and 1/6 ratio of pcDNA/NAPOR to NMDA E21 splicing reporter). B: Bar graph illustrates the effect of NAPOR coexpression on alternative exon selection. Black bars: Effect on exon 21 selection in the absence of NAPOR (–) or in the presence of a 4/1 or 6/1 ratio of NAPOR to splicing reporter plasmid. White bars: Effect on exon 5 selection. The mRNA fragments of the experiment of A and repeat reactions (in total, n = 6) were quantified by phosphorimager analysis.
FIGURE 3. NAPOR mRNA isoform expression in adult rat brain is asymmetric: High expression in the forebrain coincides with sites of high exon 21 selection and exon 5 skipping. A: Sequence alignment of cDNAs encoding mouse NAPOR (accession number AF09069) and mouse ETR3 (accession number Y18298) showing the unique 5' untranslated region of NAPOR (5' UTR, boxed region). Only 5' proximal cDNA sequences are shown. The start site of translation is underscored. Primers are indicated as arrows. Primers A4 and C amplify NAPOR specifically. Primers A1 and C amplify NAPOR, ETR3, and CUG-BP2 mRNAs. Broken lines signify that the mRNA sequence is continued but not shown.

B: Assay for NAPOR mRNA expression. Left: Schematic of adult rat brain with forebrain structures indicated: cerebral cortex, hippocampus (hp), prefrontal cortex (pc), basal ganglia (bg), temporal cortex (tc), and visual cortex (vc, shaded box). Hindbrain structures, cerebellum, and spinal cord are also shown. Right: Total RNA is isolated from postnatal day 28 Sprague–Dawley rat tissues and used in RT-PCR reactions to amplify NAPOR-related mRNAs (top panel), NAPOR (middle panel), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; bottom panel) mRNAs. Fetal brain tissue is from late embryos. Samples tested from forebrain structures are indicated in bold type.

C: Assay for alternatively spliced mRNA transcripts of the NMDA R1 receptor. Primers specific for the flanking exons of NMDA R1 exon 5 (NMDA E5) or exon 21 (NMDA E21) were used to amplify the set of exon selected (L) and exon skipped (S) mRNAs as illustrated in schematic from RNA samples used in the experiment of B. DNA products are resolved on 6% polyacrylamide, 5 M urea sequencing gels. The cerebral cortex is highlighted as the region of high exon 21 selection and low exon 5 selection. This pattern of splicing is largely reversed in the cerebellum. Also tested are bg, pc, and tc subdivisions of the cerebral cortex. NAPOR and control GAPDH expression were assayed from the same RNA samples.
Dual splicing regulatory functions of NAPOR

An irregular distribution of NAPOR, CUG-BP, and ETR3 proteins in nuclear extracts of rat forebrain and hindbrain tissue

Whereas the 5′ untranslated region of the NAPOR mRNA is a highly specific feature of this transcript, which allows sensitive detection by RT-PCR (see above, Fig. 3), antibodies that distinguish rodent NAPOR from CUG-BP2, ETR3, and related proteins are not presently available. Nonetheless it is of interest to investigate the distribution of this protein family in rat brain. For this purpose, we took advantage of the monoclonal antibody mAb 3B1 to characterize protein levels in nuclear extracts prepared from rat cerebral cortex (forebrain) and cerebellum (hindbrain) tissue using western blot analysis. mAb 3B1 has been shown to recognize CUG-BP and its protein relatives in mammals and Drosophila (gift of M. Swanson; Timchenko et al., 1996a; Good et al., 2000). Nuclei were purified from these tissues using sucrose gradient centrifugation, and extracts prepared by salt extraction of the nuclear pellets. These results show that whereas the two extracts contain similar levels of the U1 snRNP 70K protein, there is a substantially higher level of mAb 3B1 reactive protein in the cerebral cortex relative to the cerebellum nuclear extract (Fig. 5). In comparison to the reference panel, which contains known amounts of recombinant NAPOR protein probed with the mAb 3B1 antibody, we estimate that there is at least a 20-fold higher level in the forebrain, compared to the hindbrain extract.

The striking difference in mAb 3B1-reactive protein expression observed in the forebrain and hindbrain nuclear extracts is not precisely mirrored in the mRNA analysis shown in the top panel of Figure 3B, where common primers for NAPOR and ETR-3 were used. This discrepancy may be explained by differences in the sensitivities of the assays, or because total RNA was used as the template for the RNA analysis. The Western blot analysis shown is a direct measure of nuclear protein expression, and, unlike mRNA analysis, should take into account any regulated protein expression that occurs at the level of translation, or subcellular localization.

Identification of an intronic RNA element responsible for the silencing of exon 21 splicing

To investigate the RNA control elements involved in the positive regulation of exon 21 splicing, intron deletions in the NMDA E21 splicing reporter were constructed and assayed for the effects of NAPOR coexpression (Fig. 6A). The downstream intron deletion (intron 21) had the most significant effect on the splicing pattern (construct E21-3). Upon deletion of intron 21 nucleotides 107–1746, the splicing pattern switches almost exclusively to exon 21 selection, and there is a small effect of NAPOR coexpression (Fig. 6A, lanes 4–6). The high level of exon 21 selection makes it difficult to precisely monitor positive effects of NAPOR. The control substrate E21wt shows the strong positive effect of NAPOR (Fig. 6A, lanes 1–3). The increase in exon 21 selection shown in Figure 6A, lane 4, indicates that this mRNA region contains an intronic splicing silencer element. In contrast, deletion of 891 nt of the upstream intron, intron 20, has a modest effect on the splicing pattern, and the positive effect of NAPOR on exon 21 splicing is similar to E21wt (substrate E21-5; Fig. 6A, lanes 10–12). Furthermore, the increase in exon 21 selection is retained even when the two intronic dele-

FIGURE 4. Strong exon 21 selection and exon 5 skipping coincide spatially with NAPOR expression in the visual cortex, and this pattern is largely reversed in the cerebellum where NAPOR expression is low. Top four panels: RNA samples from four rat siblings (S1–S4, 5 weeks postnatal) were tested in RT-PCR reactions with primers specific for the flanking exons of GABA_α_ receptor γ2 (GABA_α_ γ2), Clathrin light chain B (Clathrin EN), NMDA R1 exon 5 (NMDA E5), and exon 21 (NMDA E21). Exon selected (L) and exon skipped (S) mRNAs as illustrated in schematic are resolved in 6% polyacrylamide, 5 M urea sequencing gels. Bottom right: NAPOR and nPTB mRNA expression is assayed by RT-PCR in the same RNA samples.

exon 5 splicing minimized, whereas in the cerebellum this pattern is largely reversed (Fig. 4, middle panels). Here, NAPOR, but not nPTB, expression tracks with exon 21 splicing (Fig. 4, bottom right). In the same samples, splicing of the neural exon of Clathrin light chain B is essentially invariant in the visual cortex and cerebellum, whereas the neural exon of the GABA_α_ receptor γ2 shows modest variation with higher exon selection in the cerebellum (Fig. 4, top panels). Of this group of neural splicing events, only NMDA exon 21 shows a positive correlation with sites of NAPOR expression.
In the context of this intronic splicing silencer, compared to a 59% increase for E21wt, the efficiency (Fig 6A, lanes 7–9). Consequently, the role of intron 21 in alternative splicing was further investigated. To guide the design of additional mutations in intron 21, we performed a sequence alignment of the human (accession number Z32774) and rat (accession number AY090615) NMDA R1 genes to identify conserved regions, as intronic regulatory elements would be expected to reside in conserved sequence regions. A high degree of conservation was observed for the first 705 nt of intron 21, in which 74% of the residues in this region are identical for the rat and human genes. The importance of the first 705 nt of intron 21 was tested by constructing a splicing reporter containing this portion of intron 21, E21-9, and assaying for effects on alternative splicing in the C2C12 cell line. As a length control, a bacterial sequence was inserted into the E21-4 plasmid, to generate E21-18. This experiment shows that, in the absence of NAPOR coexpression, the presence of the 107 to 705 region of intron 21 results in a significant decrease in exon 21 selection (Fig. 6B, lanes 1 and 4). In contrast, the control sequence of the same length has little or no effect on the splicing pattern (Fig. 6B, lanes 1 and 7). Thus, the 5’ portion of intron 21 must contain a splicing silencer element. Interestingly, the positive regulatory effect of NAPOR on exon 21 selection can be detected only in the presence of the 107 to 705 region of intron 21 (Fig. 6B, lanes 4–6). In the context of this intronic splicing silencer, NAPOR promotes a net increase in exon 21 selection similar to that of E21wt (average of 46% increase for E21-9, compared to 59% increase for E21wt).

To further map the splicing silencer region of intron 21, smaller deletions were generated and tested for splicing repression activity in the presence and absence of NAPOR (Fig. 6C, lanes 4–12). Because the positive effects of NAPOR are similar for the E21-9 and E21-13 minigenes, the RNA element most likely resides within the 149 to 441 region of intron 21.

**NAPOR binds directly to the intron 21 splicing silencer**

Is the intron 21 splicing silencer required directly or indirectly for the positive regulatory effects of NAPOR on exon 21 selection? In one scenario, NAPOR may promote exon 21 selection independently of the splicing silencer theoretically by interacting with a splicing enhancer element. Alternatively, NAPOR may promote exon 21 selection through direct binding to the intron 21 repressor element in such a way that the negative function of the element is neutralized. To assess these possibilities, we prepared recombinant NAPOR and tested its ability to bind directly to the intron 21 silencer region using a mobility gel shift assay. The results of the gel mobility shift assay show that NAPOR binds directly and with the highest efficiency to the region between nt 149 and 440 (Fig. 7A, lanes 4–6). Under these conditions, essentially all of the RNA substrate is shifted into a NAPOR-bound complex at a 67 to 1 molar ratio of protein to RNA substrate calculated as the ratio 0.4 \( \mu M \) NAPOR/0.006 \( \mu M \) RNA substrate. NAPOR also binds to the flanking sequences but with moderate efficiency (Fig. 7A, lanes 1–3 and 7–9). A 201-nucleotide region...
**A:** The repression of exon 21 splicing requires the downstream intron. Splicing reporter constructs are schematically shown at bottom. Numbers indicate RNA lengths in nucleotides. Each splicing reporter is expressed in C2C12 cells in the absence (lanes –) or in the presence of NAPOR. Wedge indicates a 4/1 or 6/1 ratio of pcDNA-NAPOR to splicing reporter plasmid. Alternative splicing products are assayed by RT-PCR with specific primers; arrows indicate exon lengths amplified. The mRNA fragments of the experiments shown and repeat reactions (in total, n = 3) were quantified by phosphorimager analysis. The percent exon 21 selection with and without NAPOR coexpression is summarized at right ± standard deviations; delta represents percent exon 21 selection in the presence of NAPOR minus percent exon 21 selection in the absence of NAPOR.

**B:** Splicing repression activity coincides with a highly conserved region of intron 21 from nt 107 to 705 (E21-9). In the absence of NAPOR coexpression, exon 21 selection is reduced from 97.2 ± 1.0% (without insertion, E21-4) to 48.4% (with insertion, E21-9), relative to the control insertion (ctrl) of a bacterial sequence of the same length (E21-18, 93.7% exon 21 selection). NAPOR upregulates the E21-9 substrate, but little or no effect of NAPOR is observed for the intron 21 deletion construct or the control insertion. Delta represents the change in exon 21 selection upon overexpression of NAPOR.

**C:** The activity responsible for splicing repression is contained between intron 21 nt 149 and 440. To further define the region of intron 21 responsible for splicing repression activity, a nested series of deletion mutants was constructed and assayed for exon 21 selection in the presence and absence of NAPOR coexpression as described above.
of intron 20 (I20ctrl) shows little or no binding to NAPOR (Fig. 7A, lanes 10–12). RNA competitors corresponding to the most efficient and least efficient binding substrates (149–440 and I20ctrl, respectively) were then applied to the binding reactions to test for specific binding to the splicing silencer sequences. This experiment clearly shows that the self-competitor efficiently blocks formation of the NAPOR complex (Fig. 7B, lanes 2–4), whereas the nonbinding control fragment, I20ctrl, has little or no effect (Fig. 7B, lanes 6–8).

DISCUSSION

NAPOR exhibits dual effects on alternative splicing and is enriched in the forebrain

Sequence-specific RNA binding proteins play important roles in the control of alternative splicing. Yet our understanding of these control mechanisms is limited by the ability to identify relevant cell- and/or tissue-specific machineries. This problem is particularly acute in the nervous system, where alternative splicing is extensive, but where relatively few neural-specific, or neural-enriched, RNA-binding proteins have so far been identified. Here we show evidence for the functional roles of the neural-specific RNA binding protein mouse NAPOR in the regulation of the exon 5 and exon 21 alternative splicing events of the NMDA R1 receptor. A model and supporting evidence is summarized in Figure 8. NAPOR promotes exon 21 selection and exon 5 skipping in coexpression assays and binds directly to an intronic repressor of exon 21 splicing. Furthermore, the high forebrain, low hindbrain expression of the corresponding mRNA isoform coincides closely with the in vivo pattern of splicing of NMDA R1 exons 5 and 21, consistent with the functional effects of NAPOR coexpression on these splicing events. In a previous report, the expression of murine NAPOR mRNA was reported to be generally brain specific in the adult rat (Choi et al., 1999). The results shown here confirm and extend this previous report.

Multiple NAPOR-related factors may be involved in alternative splicing regulation in the nervous system

A unique feature of the present study involves the use of nuclear extracts from forebrain and hindbrain to demonstrate the tissue-specific distribution of NAPOR, CUGBP, and ETR3 proteins, which are recognized by the monoclonal antibody mAb 3B1 (Fig. 5). These protein isoforms, all approximately 50 kDa in molecular weight, show higher expression in the forebrain, compared to the hindbrain. Based on their similar polypeptide sequences (see Fig. 1) and the inability at present to distinguish individual isoforms, we cannot rule out the
Dual splicing regulatory functions of NAPOR

FIGURE 8. Summary of results and model for tissue-specific alternative splicing of the NMDA R1 receptor transcript. The top diagram of NMDA R1 pre-mRNA illustrates predominant exon 21 selection and exon 5 skipping in the forebrain as observed by analysis of spliced mRNAs. Alternative exons are labeled; flanking exons are unlabeled. The bottom diagram illustrates predominant exon 21 skipping and exon 5 selection in hindbrain. Splicing patterns are shown with reference to high forebrain, low hindbrain expression of NAPOR. Recombinant NAPOR protein binds directly and specifically to an intronic repressor (shaded bar) shown in this study to repress exon 21 selection. In a previous study, the intronic repressor adjacent to exon 5 (crosshatched bar) was characterized in functional splicing assays, and shown to bind directly to PTB and CUG-BP in rat brain nuclear extracts (Zhang et al., 1999). The negative effect of NAPOR on exon 5 splicing and the positive effect on exon 21 splicing is demonstrated by coexpression assays. See discussion.

 possibility that many or all of these isoforms are involved in alternative splicing regulation in the nervous system. These proteins may have overlapping functions, and sequence differences that lie for the most part in the divergent domain outside of RRMs 2 and 3 (see Fig. 1) might modulate interactions with different protein partners leading to changes in function. In a previous study, the human CUG-BP, ETR-3, and CELF3-5 isoforms were found to have positive effects on muscle specific exon inclusion, and differential effects of some of the isoforms were observed in the presence of a truncated regulatory region (Ladd et al., 2001). Future work will be required to fully explore the functional differences among these NAPOR-related factors, their interacting partners, and their precise spatial and temporal expression patterns in the brain.

Nova-1 is another RNA-binding protein that functions in the regulation of alternative splicing in the nervous system. Nova-1 has a region-specific pattern of expression in the mouse brain, but in contrast to NAPOR, Nova-1 has low expression in the forebrain, whereas high expression is observed in the midbrain and hindbrain (Buckanovich et al., 1996). Additionally, the GABA<sub>A</sub> receptor γ2 transcript, which is a known target of Nova-1 regulation, is insensitive to the effects of NAPOR coexpression (W. Zhang & P.J. Grabowski, unpubl.).

An intronic regulatory element that silences exon 21 splicing interacts specifically with recombinant NAPOR

The RNA sequence responsible for the control of NMDA exon 21 selection is identified in the present study as a highly conserved region of intron 21 that behaves as splicing silencer in the absence of NAPOR coexpression. To a first approximation, the intron 21 splicing silencer element maps within the first 705 nt of intron 21, and the first 440 nt appear to account for most of its activity. Curiously, NAPOR binds most efficiently to the first 440 nt of intron 21, and the full enhancing effect of NAPOR is only evident when these nucleotides are intact in the splicing reporter. With respect to this intronic repressor element, it is not understood how NAPOR promotes exon 21 selection. At present, these results suggest an antirepression mechanism of action in which NAPOR functions by displacing or otherwise neutralizing the functions of inhibitory factors that bind to the intronic repressor. Alternatively, the positive effect of NAPOR might be mediated through an as yet unidentified splicing enhancer element near or within exon 21.

An antirepression mechanism of action has previously been proposed to account for effects of nPTB (Markovtsov et al., 2000). That is, with respect to the downstream control sequence (DCS) of the c-src transcript, nPTB might function as an antirepressor resulting in increased N1 exon selection in neural cells. There is more efficient binding of the DCS to nPTB, compared to PTB, and each of these might interact with different protein partners. Consequently, in neural cells, nPTB might preferentially bind to the DCS RNA region and thereby disengage repressor activities.

The results shown here can be compared and contrasted to the muscle specific cTNT exon 5 splicing event. Alternative splicing of cTNT exon 5 is positively regulated by coexpression of the highly related human CUG-BP and ETR3 factors, and these proteins bind directly to splicing regulatory elements flanking exon 5.
(Philips et al., 1998; Ladd et al., 2001). However, in contrast to the present study, the regulatory elements flanking cTNT exon 5 function as intronic enhancers (Ryan & Cooper, 1996). At the sequence level, the cTNT intronic enhancer region shows no significant homology to that of the NMDA intron 21 repressor described here. Both of these sequences, however, contain multiple CUG triplets, which are enriched in the preferred RNA-binding sequences for CUG-BP, ETR3, and BRUNOL factors (Timchenko et al., 1996b; Lu et al., 1999; Ladd et al., 2001). It should be noted that these regulatory sequences contain imperfect CUG repeats, unlike the perfect repeats characterized to form double-stranded RNA hairpins and shown to interact with the muscleblind proteins (Miller et al., 2000). A physiological role for CUG-BP in the regulation of alternative splicing is indicated by experiments that document abnormalities in cTNT alternative splicing in striated muscle of patients with myotonic dystrophy (Philips et al., 1998). In addition, CUG-BP is implicated in a splicing event involving a novel exon found in the 3' untranslated region of the myotonic dystrophy protein kinase gene (Tiscornia & Mahadevan, 2000).

The identification of NAPOR as an interacting partner of PTB in a yeast two-hybrid screen raises the question of the relevance of this interaction in mammalian cells. In the absence of an RNA template, these proteins interact weakly as assayed by coimmunoprecipitation or by protein overlay experiments (H. Liu & P.J. Grabowski, unpubl.). Consequently, an interaction between NAPOR and PTB may be enhanced by an RNA template containing binding motifs for each of these factors. Curiously the 3' splice site region just upstream of exon 5 of the NMDA R1 receptor has been characterized as an intronic splicing silencer, which binds directly to PTB, nPTB, and CUG-BP in nuclear extracts (Zhang et al., 1999). In this previous study, we identified a group of neural-enriched protein bands, approximately 50 kDa in molecular weight, as CUG-BP based on their reactivity with the monoclonal antibody 3B1 and due to their UV crosslinking specificity for sequences containing imperfect CUG repeats. With the new information shown here, it is likely that these crosslinking proteins include NAPOR, based on their striking enrichment in the rat brain, compared to HeLa nuclear extracts. This idea is also consistent with the negative effect of NAPOR on exon 5 selection. Nonetheless, how PTB, nPTB, and NAPOR participate in the silencing of exon 5 selection is poorly understood.

**NAPOR and CUG-BP-related factors are implicated in diverse biological processes**

The RNA-binding-protein families containing NAPOR, CUG-BP, ETR3, BRUNOL, CELF, and EDEN-BP are highly related to each other at the level of sequence and domain organization. Interestingly, these RNA-binding proteins appear to be involved in posttranscriptional control mechanisms important for cell differentiation and development. In mammals, NAPOR is implicated in brain development due to its increased expression during apoptosis (Choi et al., 1998, 1999). In *Drosophila*, BRUNOL is an ovarian protein believed to function as a translational repressor in the control of the precise spatial and developmental timing of oskar protein expression. Its role as a translational repressor is indicated by the observation that BRUNOL associates specifically with BRUNOL regulatory elements (BREs) in the 3' untranslated region of oskar mRNA, and because the loss of BRUNOL expression or the mutation of BREs leads to the derepression of oskar protein synthesis and an embryonic lethal phenotype (Kim-Ha et al., 1995; Webster et al., 1997). In mammals, CUG-BP is believed to play important roles in the differentiation of skeletal muscle, based on the correlation of skeletal muscle abnormalities and overexpression of RNA CUG repeats both in transgenic mice and in patients with CUG triplet repeat expansions (Mankodi et al., 2000). The embryo deadenylation element binding protein (EDEN-BP; also called deadenylation factor; see Fig. 1) is a *Xenopus* CUG-BP believed to function in embryonic development, and evidence for its role in the deadenylation of c-Jun mRNA has recently been shown (Paillard et al., 2002).

**Biological implications of brain-region-specific alternative splicing of the NMDA R1 receptor**

Alternative splicing of the NMDA R1 receptor transcript contributes in important ways to the functional diversity and subcellular localization of NMDA type glutamate receptors in mammalian brain. The protein segment encoded by exon 21 is believed to be particularly important for localization of (heteromeric) NMDA R1 receptors, at the postsynaptic membrane of neurons, as this region is both required and sufficient for trafficking of the (homomeric) receptor to the plasma membrane in a cell culture model system (Ehlers et al., 1995). Furthermore, interactions of the exon 21 protein segment with neurofilament L may affect subcellular localization by anchoring the receptor at the cell cytoskeleton (Ehlers et al., 1998). What are the biological implications for NMDA R1 receptor function regarding prominent exon 21 skipping in hindbrain regions observed in this and previous studies (Laurie & Seeburg, 1994; Laurie et al., 1995; Wang & Grabowski, 1996)? Functional NMDA R1 receptors have been characterized previously in the hindbrain, where they are believed to operate at the synapse; however, it has recently been shown that Purkinje cells of the adult cerebellum stain positive for the presence of the NMDA R1 receptor in the cell body and cytoplasm of the proximal dendrites (Petralia et al., 1994).
In a recent study, the mechanism by which the NMDA R1 receptor induces excitotoxicity was investigated by expressing truncated forms of the receptor and assaying for effects in cultured cells (Rameau et al., 2000). Pertinent to the results shown here, carboxy terminal truncations of the NMDA R1 receptor, including the region encompassing exon 21, were shown to protect CHO cells and primary cultures of cortical neurons from excitotoxicity in their assays, possibly by disrupting the assembly of heteromeric receptors with various NR2 subunits. Thus, the results shown here, together with these previous studies, raise the intriguing possibility that regulated alternative splicing of the NMDA R1 receptor may contribute to the cell or brain region specific excitotoxic effects of the receptor.

**MATERIALS AND METHODS**

**Splicing reporter and protein expression plasmids**

The wild-type exon 21 splicing reporter (E21wt) contains the complete genomic sequence from the NMDA R1 receptor from exon 20 to exon 22 (accession number AY090615). The DNA fragment was generated by reverse transcription from genomic DNA by polymerase chain reaction (PCR) with primers (5′–3′, 3021-R1, ATATATGAAATTCGGCTGGAGGAGCTGC, and 3255A-R1, ATATATGAAATTCGGCTGGAGGAGCTGC) and inserted into the EcoRI site of the mammalian expression vector pBPSVPA (Nasim et al., 1990). Mutant derivatives were generated from E21wt. PCR reactions were carried out with 0.25 μg rat genomic DNA, or 0.05 μg E21 wt DNA, 1 μM primers, 0.2 mM deoxynucleotides, 0.125 U Pfu DNA polymerase (Stratagene) in a 10–μL volume. Cycling parameters were: 20–25 cycles of 94°C, 1 min; 60°C, 1 min; and 72°C, 1 min, with a final elongation of 72°C, 10 min. PCR products were gel purified prior to cloning. All constructs were verified by restriction mapping and DNA sequencing.

The NAPOR mammalian expression vector, pcDNA/NAPOR, was constructed as a fusion protein with a 6× histidine and X-Press epitope tag at the amino terminus. The mouse NAPOR coding region (accession number AAD13763) was subcloned by PCR into the BamHI site of pcDNA4/HisMax vector (Invitrogen) using the yeast library clone, pACT2/mNAPOR, as template. PCR conditions were used as above with primers (5′–3′, A AATGGATCTGACTGAGA GCTTTGG, and B, A AATGGATCTGACTGAGA GCTTTGG). The PTB expression vector was also constructed in the pcDNA4/HisMax vector using the full-length rat isoform (accession number X74565).

**Transient coexpression assays**

C2C12 cells were grown in DMEM, 10% (v/v) fetal bovine serum. For each transfection, 1.0 × 10⁶ cells were seeded on 60-mm plates to achieve 60–80% confluency. The pcDNA/NAPOR, pcDNA/PTB, or pcDNA/Lac Z expression vector was cotransfected with splicing reporter plasmid at a ratio of 4/1 or 6/1. Each transfection contained a total of 3.5 μg of plasmid DNA. Plasmids were mixed with 300 μL Opti-Mem followed by the addition of an equal volume of Opti-Mem mixed with 23 μL Lipofectamine (Gibco BRL). After 30 min incubation at room temperature, 2.4 mL of Opti-Mem were added and the mixture dispensed to one plate of cells. Cells were washed with serum-free DMEM immediately before applying the DNA–Liposome complex to the plate.

Total RNA was isolated with RNAzol (Biotex) 48 h after transfection followed by treatment with RQ1 DNase (Promega). Total RNA purified from each transfection, 1 μg, was reverse transcribed in a 20-μL reaction containing 400 ng hexanucleotide random primers, 0.5 mM dNTPs, 80 U M-MLV reverse transcriptase (Promega), and 0.01 M DTT. Reactions were incubated at 37°C for 1 h, followed by 75°C, 10 min to terminate the reactions. Each PCR reaction, 10 μL total volume, was performed with 1/20th of the reverse transcription reaction, 0.2 μM primers, 0.2 mM dNTPs, 2 mM MgCl₂, 2 U Taq polymerase with 1 μCi [32P]dCTP. Cycling parameters included: denaturation at 94°C, 1 min, annealing at 60°C, 1 min, and elongation at 72°C, 1 min for 30 cycles, followed by a final step at 72°C, 10 min. A portion of each reaction (1 μL) was resolved on 6% polyacrylamide/5 M urea gels. Data were quantified directly from the gel using a Fuji phosphorimager system.

**RNA isolation from rat tissues and RT-PCR analysis**

Total RNA from rat tissues was extracted using RNAzol (Biotex). Each tissue sample, 250 mg, was ground to power in liquid N₂ with a motor and pestle and lysed with tight pestle 10–15 times after adding 3 mL RNAzol. The homogenate was extracted using chloroform and the RNA was precipitated by isopropanol, and washed with 75% ethanol. The conditions used for RT-PCR are as described above, except that 25 cycles were used. Primer pairs are indicated in Table 2.

**Nuclear extract preparation from rat forebrain and hindbrain tissues**

Nuclear extracts were prepared from two dozen, 28-day Sprague–Dawley rats (Zivic Miller). Freshly dissected cerebral cortex (forebrain) or cerebellum (hindbrain) tissue was minced and transferred to two tubes containing 22.5 mL of ice cold homogenization buffer (10 mM HEPES, pH 7.6, 15 mM KCl, 1 mM EDTA, 2.2 M sucrose, 0.5 mM spermine, 0.5 mM spermidine, 0.5 mM DTT). Tissue was homogenized three to five times at maximum speed in a Con-Torque (Eberbach). Homogenate was centrifuged at 27,000 rpm (SW28 rotor) for 1 h, 4°C, with (cerebellum) or without (cerebral cortex) a sucrose cushion. The sucrose cushion used to isolate nuclei from cerebellum contained 10 mL of 10 mM HEPES, pH 7.6, 15 mM KCl, 1 mM EDTA, 2.0 M sucrose, 10% glycerol, 0.15 mM spermine, and 0.5 mM spermidine. After centrifugation, the supernatants were removed and the nuclear pellets rinsed twice in 0.5 mL of Buffer C (20 mM HEPES, pH 7.6, 20 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol, 1.5 mM MgCl₂). Nuclear pellets were resuspended in 0.6 mL of Buffer C containing 0.23 M KCl (final concentration) and incubated on ice for 30 min, followed by centrifugation at 14,000 rpm, for 10 min. Proteinase inhibitors
(10 μg/mL benzamidine, 1.5 μg/mL aprotinin, 15 μg/mL bovine trypsin inhibitor, and 0.5 μg/mL leupeptin) were present in all solutions. Supernatant was removed, divided into small aliquots, flash frozen on dry ice, and stored at −80°C.

**RNA binding assays**

Gel mobility shift assays for RNA binding were carried out with approximately 0.016 μM RNA substrate (50,000 cpm) and protein between 0.4 and 0.8 mM, final concentration in a 25 μL solution containing Buffer M (50 mM KCl, 10 mM HEPES, pH 7.6, 10% glycerol, 2 mM EDTA, 0.025% NP40, 500 μg/mL tRNA, and 1 mM DTT). All RNA binding reactions were carried out for 1 h at 30°C. Heparin, 0.4 mg/mL final concentration, was added to each completed RNA binding reaction and incubated at 30°C, 10 min. Loading dye, 3 μL, was added and 8 μL of each sample was loaded onto a 5% polyacrylamide (80:1 acrylamide: bisacrylamide), 50 mM Tris-glycine gel. Electrophoresis was carried out at 200 V, 3.5 h at 4°C.

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Region-specific alternative splicing in the nervous system: implications for regulation by the RNA-binding protein NAPOR.

Wenqing Zhang, Haiying Liu, Kyoungha Han, et al.

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