A Rat Brain Bicistronic Gene with an Internal Ribosome Entry Site Codes for a Phencyclidine-binding Protein with Cytotoxic Activity

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The cloning and characterization of the gene for the fourth subunit of a glutamate-binding protein complex in rat brain synaptic membranes are described. The cloned rat brain cDNA contained two open reading frames (ORFs) encoding 8.9- (PRO1) and 9.5-kDa (PRO2) proteins. The cDNA sequence matched contiguous genomic DNA sequences in rat chromosome 17. Both ORFs were expressed within the structure of a single brain mRNA and antibodies against unique sequences in PRO1- and PRO2-labeled brain neurons in situ, indicative of bicistronic gene expression. Bicistronic vectors in which ORF1 and ORF2 were substituted by either two different fluorescent proteins or two luciferases indicated concurrent, yet independent translation of the two ORFs. Transfection with noncapped mRNA led to cap-independent translation of only ORF2 through an internal ribosome entry sequence preceding ORF2. In vitro or cell expression of the cloned cDNA led to the formation of multimeric protein complexes containing both PRO1 and PRO2. These complexes had low affinity (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801)-sensitive phencyclidine-binding sites. Overexpression of PRO1 and PRO2 in CHO cells, but not neuroblastoma cells, caused cell death within 24–48 h. The cytotoxicity was blocked by concurrent treatment with MK-801 or by two tetrahydroisoquinolines that bind to phencyclidine sites in neuronal membranes. Co-expression of two of the other subunits of the protein complex together with PRO1/PRO2 abrogated the cytotoxic effect without altering PRO1/PRO2 protein levels. Thus, this rare mammalian bicistronic gene coded for two tightly interacting brain proteins forming a low affinity phencyclidine-binding entity in a synaptic membrane complex.

A complex of four proteins purified from brain synaptic membranes was shown to have recognition sites for 1-glutamate, N-methyl-d-aspartate (NMDA),4 and other ligands characteristic of NMDA receptors in brain, including binding sites for the co-agonist glycine, the modulator spermine, the competitive antagonist (+)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), and the ion channel inhibitors thienylcyclohexypiperidine (TCP) and (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK-801) (1, 2). Reconstitution of the purified complex into planar lipid bilayer membranes leads to the formation of channels with four ion conductance levels upon activation by glutamate or NMDA in the presence of glycine (3). These conductances differ from either the predominant NMDA-activated receptor-ion channels of brain neurons or those formed by reconstitution of the NMDA receptor subunits (4), but are similar to those described for ion channels in rat spinal cord motor neurons (5).

The genes for three of the proteins in this complex have been cloned and expressed in heterologous cells (6–10). The gene GRINA for the glutamate-binding protein (GBP) subunit was identified as part of a "learning and memory" module of genes expressed in the entorhinal cortex of the mammalian brain (11), and as the gene responsible for mental retardation and epilepsy in infants with a gene duplication in chromosome 8q24.3 (12). Expression of GRINA in heterologous cells leads to activation of mitogen-activated protein kinases (13), i.e. it may be involved in signal transduction in neurons. Because of the potential role of GBP and of the associated membrane complex in cell signaling, there is a need to fully characterize all components of the complex and reconstitute the intact complex in cells lacking in its expression. The genes for two other components of the complex have been cloned, those for the glycine-binding and CPP-

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4 The abbreviations used are: NMDA, N-methyl-d-aspartate; CPP, (+)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid; BP, binding protein; GBP, glutamate-binding protein; hp, hairpin mRNA structure; inter-ORF, sequence between two open reading frames; IP, immunoprecipitation; IRES, internal ribosome entry sequence; MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine; MOPS, 3-(N-morpholino)propanesulfonic acid; ORF, open reading frame; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; TCP, thienylcyclohexypiperidine; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; CHO, Chinese hamster ovary; RFP, red fluorescence protein; EGFP, enhanced green fluorescent protein; (5)-4, (5)-1-methyl-1-phenyl-2,3,4-tetrahydroisoquinolinium chloride.
binding proteins. But the gene for the fourth subunit has not yet been cloned.

The fourth protein of the complex was identified on SDS-PAGE as an ~40-kDa protein. To complete the characterization of this complex of proteins, the cDNA for the fourth subunit was cloned, and a corresponding genomic sequence in rat genome was identified. The presence of two open reading frames (ORFs) in the cloned cDNA, the expression of both ORFs in a single mRNA in brain, and the translation in brain of the two proteins coded by the cDNA, led to the investigation of the mechanism of translation of both ORFs. Translation of both ORFs through an internal ribosome entry sequence (IRES) was identified, as was the need for the co-expression of the two proteins to create a functional protein, a phencyclidine-binding protein.

**EXPERIMENTAL PROCEDURES**

**Purification of the ~40-kDa Protein and Raising of Polyclonal Antibodies**—Synaptic membranes were prepared from two rat brains (14, 15), and membrane proteins were solubilized in 25 ml of Buffer A (1% CHAPS, 0.5% n-octylglucopyranoside, 10% glycerol, 10 mM potassium phosphate buffer, pH 7.4, plus protease inhibitors) (7) and subjected to centrifugation (100,000 × g for 1 h). The supernatant was then loaded onto an l-glutamate ReactiGel (Pierce) column, and the protein complex was eluted with buffer containing 5 mM NMDA (16). Following SDS-PAGE and silver staining of a single gel lane (6, 14, 15), the ~40-kDa band was identified, and the unstacked portion of the gel in the location of that band was cut. The protein was then electro-eluted in 2 mM EDTA, 40 mM Tris acetate buffer, pH 8.4, dialyzed against H2O, concentrated, and 100–200 µg mixed with either 0.5 ml of Freund’s complete or 0.5 ml of Freund’s incomplete adjuvant and used to immunize rabbits (17). Serum was collected, and antibodies were characterized (15–17).

**Screening of Brain cDNA Libraries with Anti-40-kDa Protein Antiserum**—A cDNA expression library from brain hippocampus in λZap was plated on Escherichia coli XL-1 Blue and screened using the antiserum to the ~40-kDa protein (1:1,000 dilution) as described (8–10). Approximately 10⁸ plaques were screened, and phages from eight positive plaques were re-screened until a homogeneous population of immunopositive recombinant phages was obtained. Recombinant λ DNA was subcloned into pBS phagemid (Stratagene).

**cDNA Sequencing, PCR Amplification, and Rapid Amplification of cDNA Ends (RACE)**—cDNA inserts of phage and plasmid clones were sequenced on both strands (Thermo Sequenase, Amersham Biosciences; fmol Promega). DNastar and Vector Suite software were used to analyze the sequences as follows: BLAST to search the National Center for Biotechnology Information, GenBank™, PSORT, TMBase, and SMART to determine various protein domains, organelle-targeting sequences, and protein family relationships. RNA structure 4.3 (18) was used to determine the tertiary structure and thermodynamic stability of mRNA structures.

PCR amplification of cDNA was performed (50 µl of DNA template, 250 µM of each dNTP, 0.2 µM of each primer described under “Results and Discussion,” 2.5 mM MgCl₂, and 2 µl of DNA polymerase mixture, 2 µl of Taq, 5 units/µl, and 1 µl of Pfu DNA polymerase, 2.5 units/µl). PCR were 30 cycles of 94°C (45 s), 55°C (90 s), and 72°C (2 min). The DNA was purified from agarose gels and either labeled with [32P]dCTP or ligated into pCR-Script (Stratagene) or pGEM-T Easy (Promega). Each PCR product was sequenced on both strands. Marathon-Ready cDNAs (Clontech) were used in 5’- and 3’-RACE of 1 cDNA. Reactions were 30 cycles at 94°C (30 s), 94°C (5 s), and 68°C (4 min), and products were cloned into pGEM-T Easy and sequenced.

**Gel Electrophoresis, Northern Blot, and Reverse Transcriptase (RT)-PCR Analyses of mRNA**—Gel electrophoresis of RNA synthesized by in vitro transcription of the cloned cDNA was performed by denaturing the RNA in 50% formamide, 6% formaldehyde and subjecting it to electrophoresis on 1.2% agarose gels containing 6% formaldehyde and either 40 mM Tris acetate, 1 mM EDTA (TAE), or 0.1 M MOPS, 40 mM sodium acetate, 5 mM EDTA, pH 7.

Northern blot analyses of total tissue RNA or poly(A⁺) RNA subjected to agarose gel electrophoresis (2 µg RNA/lane) were performed (8–10). Amplified DNA was labeled ([α-32P]dCTP, 3,000 Ci/mmol, random primer labeling, 5.6–6.1 × 10⁶ dpm) and hybridized with RNA. For RT-PCR, brain mRNA was isolated by tissue homogenization in RNAzol, extracting with chloroform, precipitating by isopropyl alcohol, primer annealing to RNA (2.5–10 μg), and adding Moloney murine leukemia virus RT (50 units/µl) or Moloney murine leukemia virus RT and Taq Plus Precision DNA polymerase (Promega). PCRs were 40 cycles of 95°C (1 min), 95°C (30 s), 60°C (30 s), and 68°C (2 min); products were resolved on 0.7% agarose gels.

**In Situ Hybridizations**—A 1.1-kb amplified fragment used for Northern blots was subcloned into PCR-script vector and used for in vitro transcription reactions to generate cRNA probes for in situ hybridization (19). The cRNA probes were synthesized in the presence of 35S-CTP and had a specific activity of 10⁸ cpm/µg RNA. In situ hybridization was performed on cryostat sections of rat brain (19).

**Generation of Antibodies to Peptide Sequences and Immunocytochemistry**—A peptide corresponding to amino acids 2–13 from the first putative ORF of the cloned cDNA (CEQSGGDALPTE) and one to amino acids 42–54 from the second (CRGYSIQHRRLVE) were synthesized, including a C-terminal cysteine (Quality Controlled Biochemicals). Purity was established by high performance liquid chromatography and mass spectrometry (>95%). Immunization with each peptide, determination of antibody selectivity, and immunocytochemical studies on serial brain sections (20 µm) were performed (19, 20). Alexa 458-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch, 1:200 dilution) was used.

**Reporter Vector Construction**—The following reporter vectors were constructed: (a) fusion proteins of enhanced green fluorescent protein (EGFP) with each of the two putative ORFs of the cloned cDNA; (b) dicistronic cDNA containing red fluorescence protein (RFP) in place of ORF1, the intervening sequence between ORF1 and ORF2 (inter-ORF), and EGFP in place of ORF2; (c) dicistronic cDNA containing firefly luciferase, inter-ORF, and Renilla luciferase; and (d) dicistronic cDNA
containing firefly and *Gaussia* luciferase in place of ORF1 and ORF2, respectively.

To construct the EGFP fusion proteins, ORF1 and ORF2 were amplified using reverse primers in which each stop codon was removed and the PCR products cloned at the BamHI site of pEGFP-N1 (Clontech). The PCR primer pair F1 (5'-GGGGTGCACGTTGCCCAGCTCAGGAA-3', SalI) and R1 (5'-GGGGATCCCTGTTCAAAAGGAAAGAGAG-3', BamHI) was used to amplify ORF1; the pair F2 (5'-GGGGACTCTCCCTTTCTTTTGAAACAATAG-3', SacI) and R2 (5'-GGGAAATCATGATAAAACGACACACATG-3', EcoRI) for the inter-ORF; the pair F3 (5'-GGGGAGCTCATGATTTCTACAGTGAA-3', SacI) and R3 (5'-GGGGATCTCTGCCCAGTTGAAATTGGCCAAG-3', BamHI) for ORF2; and the pair F2 and R3 for the combined sequence of inter-ORF and ORF2. PCRs were 33 cycles of 95 °C (30 s), 60 °C (1 min), 73 °C (2 min), and 73 °C (10 min) in 50 μl containing 200 ng of each dNTP, 2 units of Turbo Pfu polymerase, 200 ng of each primer, and 1 ng of plasmid template. All primer combinations amplified the correct size of DNA products and were digested with the corresponding restriction enzymes (underlined sequences in primers) and cloned into pEGFP-N1 pre-digested with the same enzymes.

Dicistronic reporter vectors containing RFP and EGFP in place of ORF1 and ORF2, respectively, including the inter-ORF sequence between RFP and EGFP, were generated by digesting the ORF of RFP (BglII/NotI), cloning it into phrGFP (Stratagene), then excising the RFP insert (BamHI), and cloning it into pEGFP (Sambrook, Amersham). The oligonucleotides 5'-AGCTTTTC-GGGAATTCAT-3', SacI) and R3 (5'-GGGGTGCACGTTGCCCAGCTCAGGAA-3', BamHI) were annealed, and 10 ng of this double-stranded DNA was ligated to 400 ng of pGL3 pre-digested with BglII/HindIII (removal of SV40 promoter). To this vector, the sequence 5'-CTTTACCAACAGTACCGGAATGCCAAGAAA-3', predicted to form a thermodynamically stable stem-loop hairpin (hp) in mRNA when combined with the palindromic sequence 3'-GAATAATGTGTCTAGTCATTGCTAAGAA-5', introduced 5 bases upstream of the initiation codon of firefly luciferase. Subsequently, the luciferase and hp-Luc sequences were cut from the respective vectors and inserted in B2 (Nhel site). The new vectors, pBLucMG and phpBLucMG, containing firefly luciferase, inter-ORF, and EGFP sequences, without or with the hp, were digested (EcoRI/NotI), and the EGFP ORF was excised and ligated to vectors pRL-null (Promega) and pRL-null, Promega) inserted, thus forming the dicistronic vectors pBLucMRL and phpBLucMRL.

A vector with firefly and *Gaussia* luciferase in place of ORF1 and ORF2, respectively, was constructed by cutting (HindIII/XbaI) the SP6 promoter sequence from pSinRep5 vector (Invitrogen) and ligating it to vector pLucMRL that was digested with HindIII/XbaI. The resulting vector, SBMRL, containing the SP6 promoter/firefly luciferase/inter-ORF/Renilla luciferase. The inter-ORF and Renilla luciferase were removed from SBMRL (BglII/Stul) and replaced with *Gaussia* luciferase ORF (BamHI/XbaI of vector pNEBR-X1Glu (New England Bio-labs)). The new vector, SBMRL-EBL, was digested (HindIII/AfeI) to remove firefly luciferase DNA and replace it with the combined firefly luciferase and inter-ORF sequence from SBMRL (HindIII/SmaI). The new vector, SMMRL, contained the SP6 promoter/firefly/inter-ORF/Renilla luciferase.

**Bicistronic Rat Brain cDNA for TCP-binding Protein**

*In Vitro Transcription and Translation of the Reporter Vectors and the Cloned cDNA*—The cloned cDNA was transcribed using the T7 promoter (mMessage mMACHINE T7 kit, Ambion). For the *in vitro* transcription and translation of the cloned cDNA, T<sub>3</sub> or SP6 RNA polymerase and reticulocyte lysate (TNT-coupled, Promega) plus phage cDNA or PCR-amplified fragments of phage cDNA were used. Reactions (reticulocyte lysate, 1 μg, and RNasin, 40 units/μl, final volume of 25 μl) were at 30 °C (90 min). Synthesized proteins were used in ligand binding assays or were labeled with [35S]methionine (1000 Ci/mmol) and analyzed by SDS-PAGE.

Noncapped mRNA containing firefly luciferase/inter-ORF/Renilla luciferase was synthesized using vector SMMRL (20 μg) cut with SspI, purified, and introduced as template (5 μg) in RiboMAX<sup>TM</sup> large scale RNA production system, SP6 (Promega). Synthesis of mRNA proceeded for 2 h in the absence of 7-methylguanosine nucleotide (2 h). The DNA was digested with DNase and the RNA purified.

**Cell Transfection with Cloned cDNA, Reporter Vectors, and Noncapped mRNA and Measurement of Luciferase Activity**—CHO and SK-N-SH cells were used for protein expression studies. CHO cells (2 ml, 1–2 × 10<sup>5</sup> cells/ml, Ham’s F-12 medium, 10% fetal bovine serum) were plated in either 35-mm dishes or 6-well plates, incubated for 18–36 h (50–80% confluence), washed (Opti-MEM reduced serum medium), and incubated 6-well plates, incubated for 18–36 h (50–80% confluence), washed (Opti-MEM reduced serum medium), and incubated for 5 h (37 °C) with 1 ml of Lipofectamine/plasmid DNA mixture (2.5–3 μl of Lipofectamine 2000, 1 μg of DNA in Opti-MEM). The transfection mixture was removed, and Ham’s F-12 with serum was added, and the cells were harvested 48 h later in phosphate-buffered saline containing 1 mM EDTA. The suspension was centrifuged, and the pellet was resuspended in buffer (in mM: EDTA, 150 NaCl, 0.1 dithiothreitol, 0.1 benzamidine, 0.1 benzamidine–HCl, 0.1 aminooxybenzene sulfonate, 10 Tris–SO<sub>4</sub>, pH 7.4, plus MINI protease inhibitor mixture tablet, Roche Applied Science), incubated at 0 °C for 15 min, sonicated, and then centrifuged (16,000 × g, 30 min). The supernatant was concentrated by filtration (10-kDa cutoff, Amicon) and subjected to SDS-PAGE and immunoblot analyses.

For transfection of CHO cells with *in vitro*-synthesized RNA, the RNA (2 μg) was mixed with Lipofectamine 2000 (1 μg/2.5 μl) in serum-free Dulbecco’s modified Eagle’s medium, treated with protease K (10 μg/ml, 50 °C, 1 h), heated at 95 °C for 30 min, cooled to 37 °C, and added to cell cultures (24-well plates). Following a 4-h incubation, the medium was changed to serum-containing Dulbecco’s modified Eagle’s medium, and the cells were incubated for 24 h before measuring luciferase activities.

Luciferase activities in cell extracts were measured using the dual-luciferase reporter assay (Promega). Luminescence was measured three times for 0.5 s (LumiCount, Packard Instrument Co.). Enzyme activity was expressed as relative luminescence units. Promoterless SMMRL vector was used as a transfection control in the RNA transfection experiments. Each
transfection experiment was performed at least twice using triplicate or quadruplicate transfections for each experiment.

SK-N-SH cells were cultured in 24-well plates pre-coated with poly-D-lysine and laminin (0.5 ml/well, 0.5–1.0 × 10^5 cells/well), grown for 5 days in serum-containing Ham’s F-12 (50–80% confluence), and then transfected as described above. The transfection mixture was removed and 0.5 ml of Ham’s F-12 serum added to each well, and 24 h from the start of transfection, the culture medium was changed to serum-free Ham’s F-12 (induction of differentiation). The cells were harvested 48 h later and lysed as above.

**Immunohistochemistry and Immunoprecipitation (IP) of Protein Complexes**—Immune labeling of SK-N-SH cells was performed as described (20). For double labeling with SYTOX Blue (Invitrogen) and antibody, SYTOX Blue was added after all antibody labeling reactions were completed. Excess SYTOX was removed by multiple washings with Tris borate-EDTA and PBS buffers, and the cells were examined by confocal microscopy (458 nm excitation for SYTOX; 568 nm for Alexa 568 dye-secondary antibody).

For the IP of proteins expressed in CHO cells, 50 µl of protein A-Trisacryl beads pre-washed with H2O and antibody-binding buffer (50 mM sodium borate, pH 8.2) were incubated with antibodies in binding buffer (200 µg/ml, 30 min, 23 °C), and the bound antibody was cross-linked to protein A by d-uccinimidyl suberate (21). Protein (500 µg) extracted from cells (in 1 ml buffer: 50 mM NaCl, 1 mM EDTA, 0.5% deoxycholate, 0.1% SDS, 0.5% Triton X-100, 1% Nonidet P-40, and 50 mM Tris-HCl, pH 7.6) was applied to the beads and incubated for 3 h (23 °C), and bound proteins were eluted as described (20, 21).

**Ligand Binding and Cell Viability Assays**—Binding of [3H]glycine (100 nM), [3H]TCP (50 nM), or L-[3H]glutamate (100 nM) to CHO cells, or CHO cells expressing protein A, was determined. Ligand binding was measured in the presence of 100 µM D-serine (for [3H]glutamate), 100 µM NMDA (for [3H]TCP), or [3H]glycine (100 nM) in MEM (for [3H]glutamate), and 100 µM or 1 mM MK-801 for [3H]TCP. Data were analyzed (2, 6, 14). When [3H]glycine was used, all samples were pretreated with 100 µM strychnine.

Cell viability was determined at either 48 h (CHO cells) or 72 h (SK-N-SH cells) post-transfection by measuring (Celltiter 96, Promega) the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (22).

**RESULTS AND DISCUSSION**

**Screening of a Brain cDNA Library and Cloning of a Bicistronic Rat Brain cDNA for TCP-binding Protein**

The antisera raised against the ~40-kDa protein subunit of the purified complex had a titer of 1:2,000, reacted with at least a concentration of synaptic membrane proteins as 10 ng (data not shown), and labeled a 52-kDa band in synaptic membrane proteins (supplemental Fig. S1). When the antisera was used to immunoprecipitate proteins from synaptic membranes, it precipitated an ~56-kDa protein (data not shown). Thus, the molecular size of the native protein was thought to be 52–56-kDa, with the ~40-kDa protein possibly being a degradation product.

Screening of a rat hippocampal cDNA expression library with the antisera identified several λ phage clones. The largest insert was 1.3 kb, and this was subcloned into pBS phagemid. Further library screening using the 1.3-kb cDNA insert as probe did not lead to the identification of clones with larger inserts. In addition, performance of RACE, either in the 5′ or 3′ direction, did not yield DNA sequence extensions.

Sequence of the cDNA on both strands in λ phage and pBS yielded identical sequences (GenBank™ accession number EF495200). There were five potential initiation ATG codons in the 5′-terminal region, four of which were followed by in-frame termination codons leading to short putative ORFs (Fig. 1A). The fifth initiation codon was associated with a putative
ORF (ORF1) 246 bp long. Following the termination codon of ORF1, there were eight potential initiation codons, seven of which were followed by termination codons. The eighth initiation codon was associated with an ORF (ORF2) 267 bp long (Fig. 1A). ORF2 started 410 bp downstream from the termination codon of ORF1 and was offset by a −1 frameshift.

Analysis of the structure of the 410-bp sequence between the two putative ORFs (18) predicted a structure with four Y-type stem-loops. The estimated Gibbs free energy of folding of the inter-ORF mRNA was −70 kcal mol\(^{-1}\), indicative of stable mRNA structures that would inhibit ribosome scanning through this region (23). Thus, it was not certain that translation of ORF2 could occur in vivo.

ORF1 encoded a protein of 81 amino acids with a predicted nonglycosylated molecular size of 8.9 kDa (PRO1) and, if ORF2 were translated, it coded for a protein containing 88 amino acids with a size of 9.5 kDa (PRO2). BLAST analyses indicated that the sequences for PRO1 and PRO2 were unique. In PRO1, there was a region of eight consecutive Ser followed by three acid repeats of SIYL (Fig. 1A). BLAST analyses performed using only the sequences containing the eight Ser residues revealed several proteins in plants and animals that had long tracts of Ser residues. Included among them were a rat brain and immune system phosphatase, PHLPP, a cell wall surface anchor family protein, and a retinoblastoma-binding protein 1-like protein. BLAST analyses also revealed several proteins in invertebrates and vertebrates with multiple SIYL repeats (frequency of 4 – 6 consecutive repeats). Included among them was a mouse cerebellar granule cell marker protein. None of these proteins were homologous to the predicted whole sequence of PRO1.

Hydropathy analyses of the inferred amino acid sequence of PRO1 indicated a possible signal peptide sequence in the N-terminal region, a single hydrophobic region forming a potential transmembrane domain (Fig. 1A), and a likely localization for PRO1 in membranes with the N-terminal in the extracellular environment. The sequence of PRO1 had one consensus glycosylation site within this extracellular domain (Fig. 1A). The deduced sequence of PRO2 did not contain consensus glycosylation sites, had a signal peptide between residues 1 and 24, two putative TM regions (Fig. 1A), and a likely topography in membranes with both N and C termini in the extracellular domain.

BLAST searches using the nucleotide sequence of the cloned cDNA against that of the rat genome revealed that the cDNA sequence between bases 10 and 995, i.e. the sequence covering all of ORF1 and most of ORF2, was 100% identical to the plus strand of the genomic sequence of Sprague-Dawley rat chromosome 17 (Fig. 1B), bases 28,879–29,864 (RGSC, NCBI). The cDNA sequence between 995 and 1291, representing the 3′-terminal sequence of ORF2 and the 3′-UTR, was 99% identical to the sequence on the same strand of rat chromosome 17, but starting 8,437 bases downstream of the first sequence, bases 38,301–38,597 (Fig. 1B). The two genomic sequences covered 98% of the total length of the cloned cDNA. The location of these two sequences on chromosome 17 appeared to represent a gene with two exons separated by a putative 8.4-kb intron. These sequences have not been identified previously as part of a specific gene. The presence of identical sequences in the cDNA and genomic DNA of chromosome 17 indicated that the multiple Ser and SIYL repeats in ORF1, and the inter-ORF and ORF2 sequences, were not the result of cloning artifacts.

A short section of the nucleotide sequence in cDNA, nucleotide 1007–1291, was 88 – 94% identical to a 284-bp sequence in rat, mouse, and human histone genes. In all occasions, the matches were to the inverted sequence of the sense strand of histone genes. Alignment of the sense strand of rat histone 2A and 2B cDNA with the cloned cDNA indicated only 22% overall identity and a lack of any long sequences that were identical to each other. Nor was there any homology between the protein sequences of histones and either the predicted sequence of PRO1 or PRO2.

A 1.1-kb fragment of the cDNA containing both ORF sequences was used as a probe for Northern blots and in situ hybridization studies. Hybridization of this probe with commercially available rat multiple tissue Northern blot (Clontech) labeled a 2.4-kb poly(A\(^{+}\)) mRNA in heart, liver, kidney, brain, lung, and skeletal muscle (Fig. 2A). Brain mRNA also contained an ∼1.8-kb band that hybridized with the probe. Liver, heart, and testis contained mRNA species around 3.5 kb and smaller than 1 kb that were labeled by the probe (Fig. 2A). A second probe representing an ∼500-bp sequence near the 5′ end of the cloned cDNA, i.e. avoiding the region homologous to histone genes, also labeled the 2.4-kb band in the mRNA from various tissues and the ∼1.8 kb in brain mRNA (data not shown).

The 2.4-kb mRNA labeled by the two probes was either an mRNA that did not correspond to the cloned cDNA or an abnormally migrating mRNA that corresponded to the cDNA. CHO cells that were not transfected with the cloned cDNA vector did not express any RNA that was labeled by the 1.3-kb probe (Fig. 2B). However, Northern blot analyses of total RNA from cells transfected with the cloned cDNA revealed two prominent bands, one of 2.5 and the other of 1.3 kb (Fig. 2B), suggestive of abnormal migration of the mRNA synthesized from the 1.3-kb cDNA.

To probe this further, mRNA was synthesized by in vitro transcription of the cloned cDNA and subjected to denaturing agarose gel electrophoresis in TAE buffer. Under these conditions, the mRNA had a prominent band between 1 and 1.3-kb and two weak, diffuse bands between 2 and 2.6 kb (data not shown). Denaturing electrophoresis of the same mRNA in a MOPS buffer system resolved the mRNA into a single species with an estimated size of 1.3 kb (Fig. 2C). The reasons for the abnormal migration of RNA species in different buffers are not known but may be indicative of the presence of different structures of RNA assumed under different conditions of treatment.

Evidence That Both ORF1 and ORF2 in the Cloned cDNA Are Transcribed and Translated in Brain—Sense and antisense cRNA probes synthesized using a 1.1-kb cDNA fragment were used for in situ hybridization. Highest levels of labeling by the antisense probe were in pyramidal neurons of the hippocampus and cerebral cortex, granule cell neurons of the dentate gyrus and cerebellum, and cells of the olfactory bulb and medial habenula (Fig. 3A). The sense cRNA probe did not hybridize with mRNA in brain (Fig. 3A). The pattern of labeling in brain was similar to that observed with cRNA probes for the other three subunits of the complex (24).
To determine whether both ORFs were present in brain mRNA, RT-PCR analyses were performed with sets of primers designed to amplify either the whole sequence of the cloned cDNA or that of only ORF1 or ORF2 (Fig. 3B). Amplification of λ phage cDNA using these primers led to the synthesis of amplicons with the predicted sizes (Fig. 3B). Reverse transcription of rat brain poly(A)- RNA and PCR amplification yielded amplicons that had identical sizes to those obtained with λ phage cDNA as the template (Fig. 3B). No amplification products were detected if RT was not included in the reaction. These results indicated that mRNA with the sequence of the 1.3-kb cDNA insert, including ORF1, ORF2, and the inter-ORF sequence, was present in brain as intact mRNA. Therefore, the translation of both ORFs in brain was not likely to be due to trans-splicing of the mRNA into a monocistronic RNA.

To probe for the expression of both PRO1 and PRO2 proteins in brain, antibodies were raised to unique immunogenic domains of the predicted sequence of each protein. The anti-PRO1 and anti-PRO2 antisera recognized their cognate peptides and did not react with the noncognate peptide (supplemental Fig. S2). Of the two antibodies, the anti-PRO2 was the more reactive against the respective cognate peptide. A mixture of the two antibodies at a 1:1,000 dilution each was used in immunoblot assays against whole cell particulate proteins from non-neuronal, nontransfected cells to test their specificity. Nontransfected CHO cells did not contain RNA corresponding to the cloned cDNA (Fig. 2B) and cell extracts from nontransfected CHO cells did not react with the anti-PRO1/anti-PRO2 antibodies (supplemental Fig. S3), indicative of appropriate antibody specificity.

Both anti-PRO1 and anti-PRO2 labeled most strongly an ~52-kDa protein in synaptic membranes (Fig. 4A). The anti-PRO1, however, labeled additional bands at 28, 41, 65, and 82 kDa, but less strongly than the 52-kDa protein. Both antibodies labeled an ~56-kDa protein band in the partially purified complex of four proteins from synaptic membranes (Fig. 4B). Finally, the purified IgG from each antiserum labeled PRO1-
like and PRO2-like proteins in brain hippocampus neurons (Fig. 4, C and D). Thus, both ORFs and the inter-ORF sequence were transcribed, and the mRNA for both ORFs was translated to the respective proteins in brain cells.

Estimates of Molecular Size of Brain PRO1 and PRO2—The molecular size of the labeled proteins in synaptic membranes and in the partially purified complex was larger than predicted on the basis of ORF1 or ORF2 sequences. CHO cells transfected with the cloned cDNA also expressed a 56-kDa membrane-associated protein recognized by the antibodies (Fig. 5A). The 52–56-kDa proteins might represent stable multimeric protein structures formed through hydrophobic interactions between two relatively hydrophobic small proteins, PRO1 and PRO2. To test this possibility, synaptic plasma membrane proteins were subjected to SDS-urea gel electrophoresis (25). The presence of 8 M urea caused some of the 52–56-kDa proteins in synaptic membranes to dissociate to lower molecular size species of 46, 27, and 10-kDa that were recognized by anti-PRO2 (Fig. 5B) and anti-PRO1 (data not shown). Other known membrane-associated proteins tend to form stable aggregates. For example, a protein homologous to the GBP subunit, the 34.6-kDa RECS1 protein, is relatively hydrophobic and forms multimeric complexes in SDS-PAGE (26).

The formation of aggregates composed of PRO1 and PRO2 was further explored by the following: (a) analyzing immunoprecipitated proteins from CHO cells transfected with the 1.3-kb cDNA; and (b) conducting in vitro transcription and translation using the cloned cDNA. Protein IP from extracts of transfected CHO cells using anti-PRO1 antibodies led to the isolation of a 52-kDa protein that was recognized by anti-PRO2; IP with anti-PRO2 yielded a 52-kDa protein that was labeled by anti-PRO1 (Fig. 5C). In IP and immunoblots, purified IgGs were used. Neither of the two proteins was precipitated by preimmune IgG (Fig. 5C), and no detectable proteins were precipitated from cells transfected with vector that did not contain the 1.3-kb cDNA insert (data not shown). The labeling by anti-PRO1 of proteins precipitated by anti-PRO2 was weaker than the labeling by anti-PRO2 of proteins precipitated by anti-PRO1 (Fig. 5C). This might have been due to either lower expression of PRO1 than PRO2, less efficient IP by anti-PRO2 compared with anti-PRO1, or less efficient labeling by anti-PRO1 versus that of anti-PRO2 (see Fig. 3C). However, because different populations of cells were transfected with the cloned cDNA and used for the IP and immunoblot studies, the absolute numbers of transfected cells and of protein expression was not expected to be identical. Thus, comparisons of the levels of expression of the two proteins in transfected CHO cells could not be made.
In a separate series of IP experiments, proteins extracted from CHO cells were immunopurified through anti-PRO1 antibody-derivatized matrices and resolved by SDS-urea PAGE. The 52–59-kDa proteins expressed in CHO cells were resolved under conditions of SDS-urea into lower molecular size species of 40–44, 18, and 9-kDa (supplemental Fig. S4). All protein bands reacted with the anti-PRO1/PRO2 antibodies. The effect of urea on the gel migration of these proteins suggested, once again, that the 52–59-kDa proteins were multimers of PRO1 and PRO2 resulting from hydrophobic interactions. The studies described above were indicative of the formation of heteromeric complexes of PRO1 and PRO2. To explore whether in vitro synthesized proteins had a tendency to form homomeric or heteromeric complexes of PRO1 and PRO2, three different constructs based on the cloned cDNA were used for in vitro transcription and translation in the presence of [35S]methionine. The DNA used in these reactions was generated by PCR amplification and contained only ORF1, only ORF2, or both ORF1 and ORF2 sequences downstream of the promoter (supplemental Table S2). SDS-PAGE of the proteins synthesized revealed an ~10-kDa labeled protein species for each condition. Yet, together with the 10-kDa protein, two other proteins with estimated sizes of 27 and 47 kDa were also formed under all conditions of in vitro transcription and translation (Fig. 6A). The larger molecular size proteins were less abundant than the 10-kDa protein and only became apparent after a prolonged exposure of the film to the radioactive gel (Fig. 6A). Thus, PRO1 and PRO2 exhibited a high tendency to form stable homo- or hetero-multimeric complexes.

When ORF1 or ORF2 was expressed separately in CHO cells as fusion proteins with EGFP (sequence of ORF1 or ORF2 cloned upstream of and in-frame with the sequence of EGFP), they each formed proteins with the expected molecular size, and there was no evidence of multimer formation (Fig. 6B). The 80-kDa protein was not observed when PRO2-EGFP was expressed by itself. The 80-kDa protein was also labeled by PRO1 antibodies (data not shown), indicative of the formation of multimeric complexes between PRO1 and the PRO2-EGFP fusion protein. Thus, if one of the two proteins was expressed as a non-fusion protein, it tended to form multimeric complexes with the other, even when the second protein was expressed as a fusion protein.

Evidence for Independent Translation of the Two ORFs—To determine whether any two ORFs separated by the inter-ORF sequence of the cloned cDNA could be translated at the same time, the sequences of ORF1 and ORF2 were removed from the cloned cDNA and replaced with those of RFP and EGFP, respectively, while leaving the 410-bp inter-ORF intact. Transfection of either CHO cells (Fig. 7, A–C) or of a neuronal cell line, SK-N-SH cells (Fig. 7, D–F), with this dicistronic vector led to the expression of both red and green fluorescent proteins in the same cells. The SK-N-SH cells were transferred to a serum-free medium after 24 h to induce differentiation into neuron-like cells. Nontransfected CHO cells exhibited weak background green fluorescence and essentially undetectable red fluorescence (supplemental Fig. S5). SK-N-SH cells did not have detectable background green or red fluorescence (data not shown). Despite a higher background of green fluorescence in nontransfected CHO cells, the transfected cells were easily identified by the presence of bright green and red fluorescence (Fig. 7, A–C). Both types of fluorescence were observed in the same cells. The same was true for transfected SK-N-SH cells but expression of the two fluorescent proteins in SK-N-SH was, generally, at a lower level than in CHO cells (Fig. 7, D–F). The expression of RFP and EGFP in the same cells was an indication of the translation of both ORFs in the same cell and over the same period of time, and of the fact that any two ORFs could replace those in the cloned cDNA.
The continued expression of ORF2, even when ORF1 expression was substantially inhibited, was suggestive of a possible engagement of ribosomes by the inter-ORF RNA sequence thus leading to independent translation of ORF2, i.e. a possible IRES within the inter-ORF sequence. To exclude other potential alternatives, such as the presence of a cryptic promoter within the inter-ORF sequence or a cap-dependent continuation of translation by either frame-shifting (27, 28), ribosome hopping (29), or leaky ribosome scanning (30), we synthesized in vitro noncapped mRNA that contained two luciferase ORFs, firefly and *Gaussia* luciferase, separated by the inter-ORF sequence. If transfection of CHO cells with in vitro transcribed mRNA led to the synthesis of *Gaussia* luciferase, then that would negate the possible presence of a cryptic DNA promoter. And, if following transfection with noncapped RNA, ORF2 *Gaussia* luciferase was predominantly or exclusively translated in comparison with firefly luciferase, then that would suggest that ORF2 translation was cap-independent and ORF1 was cap-dependent. The activity of firefly luciferase in RNA-transfected CHO cells was barely detectable above that in extracts from cells transfected with promoterless DNA vector used as control (relative luminescence units in RNA-transfected cells = 212.1 ± 3.4, n = 18, and in promoterless DNA-transfected cells = 170.8 ± 18, n = 12). On the other hand, the activity of *Gaussia* luciferase was very high in cells transfected with the noncapped mRNA and was much higher than that in lysates from cells transfected with the control promoterless DNA vector (relative luminescence units in RNA-transfected cells = 5910.1 ± 316.8, n = 18, and in promoterless DNA-transfected cells = 279.4 ± 22, n = 12; p < 0.0005, degrees of freedom = 28). Based on these results, translation of ORF1 in the cloned cDNA appeared to be cap-dependent, whereas that of ORF2 was cap-independent and was not due to the presence of a cryptic promoter. Translation of ORF2 rated by the 410-base inter-ORF sequence. In one version of this vector construct, a stem-loop hairpin with an estimated free energy of folding equal to −51 kcal mol⁻¹ was inserted immediately preceding the initiation codon of firefly luciferase. The DNA for the vector with the hairpin loop had the identical size on gel electrophoresis as that for the vector without the hairpin loop. The presence of the stable structure of the hairpin stem-loop in the mRNA impedes ribosomal scanning and mRNA translation (23). Thus, it was expected that firefly luciferase expression would be reduced when the hairpin stem-loop was present. If translation of ORF2 were dependent on that of ORF1, *Renilla* luciferase expression would also be reduced. In the presence of the hairpin structure, firefly luciferase activity in transfected CHO cells was significantly reduced by 75% of that in its absence (13,898 ± 1034 relative luminescence units with the hairpin versus 54,617 ± 6,067 without the hairpin; units ± S.E., n = 3, p = 0.03, two-tailed t test). On the other hand *Renilla* luciferase activity in the presence of the hairpin structure increased to 176% of that in its absence (1,724 ± 53.9 relative luminescence units with the hairpin versus 979 ± 102.7 without the hairpin). The increase in ORF2 expression was probably the result of reduced competition between ORF1 and ORF2 for translation by ribosomes. The key conclusion was that the two ORFs were translated concurrently, yet independently.

To explore whether translation of ORF2 was dependent upon translation of ORF1, a dicistronic vector was constructed in which ORF1 and ORF2 were replaced by firefly and *Renilla* luciferase, respectively. The two luciferase ORFs were sepa-

**FIGURE 6. Multimer complex formation by in vitro-synthesized PRO1 and PRO2 and lack of multimer formation by EGFP fusion proteins of PRO1 and PRO2.** A, fluorography of [³⁵S]methionine-labeled protein products obtained from in vitro transcription and translation of PCR-amplified ORF1, ORF2, and the combination of both. Sequences of the cloned λ phage cDNA representing ORF1, ORF2, and both ORFs were each amplified by PCR using the promoters shown in supplemental Table S2. Upstream primers for ORF1 and the combined ORF1/ORF2 contained the T₇ promoter; the ORF2 primer had the SP6 promoter. The proteins synthesized were subjected to SDS-PAGE (12% gel) and fluorography. Arrow, major radioactive protein bands. B, immunoblots of fusion proteins PRO1-EGFP and PRO2-EGFP expressed separately in transfected CHO cells (see text). Proteins in the particulate fraction were labeled by anti-PRO1, anti-PRO2, and anti-40-kDa protein antibodies. Arrows, major band labeled by all antibodies.
had the characteristics of an IRES-controlled translation process.

PRO1-PRO2 Co-expression Needed for Formation of a TCP-binding Protein—When either PRO1 or PRO2 was expressed singly in a cell-free transcription/translation system, there was no detectable ligand binding activity for L-[3H]glutamate, [3H]glycine, or [3H]TCP. When the intact 1.3-kb cDNA was transcribed and translated in vitro, an MK-801-sensitive [3H]TCP-binding site was detected (Fig. 8A) but no L-[3H]glutamate or [3H]glycine binding activity (data not shown). There was no [3H]TCP binding to the proteins in reticulocyte lysates in the absence of the cloned cDNA. These results indicated that PRO1 and PRO2 were both needed to form the TCP binding entity but did not form either glutamate- or glycine-binding sites.

The inhibitory concentration for 50% displacement (IC50) of [3H]TCP by MK-801 was estimated to be 1 μM. A small activation of [3H]TCP binding at low MK-801 concentrations (25–100 nM) was observed (Fig. 8A), possibly the result of cooperative interactions between binding sites. Low affinity binding sites for [3H]TCP are expressed in rodent brain, and some of these sites are activated by MK-801 (32).

Expression of the cloned cDNA in CHO cells also led to the appearance of MK-801-sensitive [3H]TCP-binding sites in membranes of transfected cells (Fig. 8B). The estimated IC50 for MK-801 displacement of [3H]TCP binding was 5 μM (Fig. 8B). Nontransfected cells had no MK-801-sensitive [3H]TCP binding (data not shown). Neither transfected nor nontransfected cells had any [3H]glycine or L-[3H]glutamate-binding sites (data not shown). Because of the TCP binding activity associated with PRO1/PRO2, the cloned gene was named the TCP-binding protein (TCP-BP) gene.

Induction of Cell Death in CHO Cells by Overexpression of TCP-BP—Many CHO cells died within 24–48 h post-transfection with the 1.3-kb cDNA. Neither competitive inhibitors of glutamate- (2-amino-5-phosphonopentanoic acid) nor of glycine (5,7-dichlorokynurenic acid)-binding sites had any effect on the cell death induced by overexpression of TCP-BP. All agents were added at high micromolar concentrations (100–500 μM) at the time of transfection and were maintained in the medium during the post-transfection period. On the other hand, addition of MK-801 under the same conditions protected cells from the effects of TCP-BP overexpression, and such protection was dependent on the concentration of MK-801 (Fig. 9A). The survival rate for cells transfected with vector lacking the TCP-BP insert (empty vector) was 79.8 ± 2.7% (±S.E., n = 5) that seen in cells that were never subjected to the transfection procedure, i.e. a 20% death rate among cells subjected to just the
transfection procedure. Cells transfected with the TCP-BP-containing vector in the absence of MK-801 had an average survival of 45.4% when compared with that of cells transfected with empty vector (values shown in Fig. 9A are based on comparison with cells transfected with empty vector considered as 100%). If the transfection efficiency of CHO cells with TCP-BP vector (equal to 50.9%; measured as EGFP fluorescence in cells co-transfected with pEGFPN1) is taken into account, the corrected estimate of cell death due to TCP-BP expression was nearly 100% of all transfected cells.

When MK-801 was present at concentrations greater than 150 μM, maximal survival of cells transfected with TCP-BP vectors was 84.5% (Fig. 9A). MK-801 was protective despite the fact that concentrations of MK-801 between 150 and 200 μM were partially toxic to CHO cells. CHO cells treated with 150 μM MK-801 and transfected with empty vector had reduced viability as compared with cells that were neither transfected nor treated with MK-801 (viability in the presence of MK-801 plus empty vector = 68.8 ± 4.0%, n = 5; empty vector alone = 79.8 ± 2.4%). Normalization of the MK-801 effect to the baseline survival of cells transfected with empty vector, i.e.

drug treatment control, yielded survival values for cells exposed to MK-801 and empty vector equal to 86.2 ± 5.0%. This rate was essentially identical to the estimated maximal survival for MK-801-treated and TCP-BP-transfected cells (Fig. 9A). Survival of cells transfected with TCP-BP in the presence of high concentrations of MK-801 was equal to 98% of that in the presence of MK-801 plus empty vector, i.e. complete protection by MK-801.

A series of 1-aryl-1,2,3,4-tetrahydroisoquinoline derivatives compete with [3H]MK-801 for binding to neuronal membrane NMDA receptor sites (33, 34). These compounds were tested as possible protectants against TCP-BP transfection (Table 1). All

FIGURE 9. Protection by phencyclidine analogs and by GBP and Gly-BP of CHO cells transfected with TCP-BP. A, reduction of CHO cell death associated with transfection with the TCP-BP gene through concurrent exposure to increasing concentrations of MK-801 and (S)-4-aryl tetrahydroisoquinoline. Curve fitting procedures and estimated constants for protection of cells by the compounds are presented in the text. B, reduction of CHO cell death associated with TCP-BP transfection upon concurrent transfection with pcDNA3.1 containing the cDNA for either Gly-BP (8) (left panel) or GBP (10) (right panel). The ratio of the amount of vector used in co-transfection was 1:1.

| Chemical Structure | Systematic Name | Inhibition of [3H]MK-801 Binding (K_i, μM) | Survival of TCP-BP-Transfected CHO Cells (%) |
|--------------------|-----------------|------------------------------------------|---------------------------------------------|
| 1-Phenyl-1,2,3,4-tetrahydroisoquinolinium chloride 0.921 ± 0.021 | 51.2 ± 5.0 |
| 1-(2,6-Dimethylphenyl)-1,2,3,4-tetrahydroisoquinolinium chloride 7.52 ± 0.20 | 42.9 ± 7.6 |
| 1-(2-Methylphenyl)-1,2,3,4-tetrahydroisoquinolinium chloride 0.514 ± 0.039 | 41.7 ± 7.3 |
| (S)-1-Methyl-1-phenyl-1,2,3,4-tetrahydroisoquinolinium chloride 0.0225 ± 0.0019 | 72.6 ± 3.7 |
| 8-Methyl-1-phenyl-1,2,3,4-tetrahydroisoquinolinium chloride 0.872 ± 0.063 | 51.2 ± 11.9 |
| 8-Methyl-1-(2-methylphenyl)-1,2,3,4-tetrahydroisoquinolinium chloride 0.0827 ± 0.0097 | 55.9 ± 7.7 |
| 8-Methyl-1-(2,6-dimethylphenyl)-1,2,3,4-tetrahydroisoquinolinium chloride 1.87 ± 0.25 | 36.9 ± 4.4 |

1 Synthesis of the tetrahydroisoquinolines described in (Refs. 34, 38, 39). For (S)-4 see also Ref. 40.  
2 Data on inhibition of binding from (Ref. 34).  
3 All values are means of three experiments and are calculated as % survival in comparison with empty vector only (84 ± 9.6% compared with no treatment). All compounds introduced at 175 μM. Transfection with TCP-BP was associated with 47.6 ± 5.4% survival, whereas TCP-BP transfection in the presence of 175 μM MK-801 had 71.4 ± 10.1% survival.
compounds were introduced into the culture medium at 175 μM concentration. In this study, transfection by TCP-BP reduced survival to 47.6% of that seen in cells transfected with empty vector. Only tetrahydroisoquinoline compounds that produced survival rates greater than 53% were considered as active. Two compounds were protective based on this criterion as follows: (S)-1-methyl-1-phenyl-1,2,3,4-tetrahydroisoquinolinium chloride (S)-4 and the racemic mixture of 8-methyl-1-(2-methylphenyl)-1,2,3,4-tetrahydroisoquinolinium chloride. These two compounds have the highest affinity for binding to (2-methylphenyl)-1,2,3,4-tetrahydroisoquinolinium chloride.

Interestingly increased (supplemental Fig. S7, line. SK-N-SH cells had low endogenous levels of TCP-BP and toxicity of TCP-BP overexpression (data not shown).

The presence of the complete sequence of the cDNA in rat genomic DNA indicated that the TCP-BP mRNA was a eukaryotic bicistronic mRNA. Bicistronic mRNAs in mammalian brain are rare but do exist (35); however, there is no demonstration that both ORFs of previously identified bicistronic brain mRNA are coordinately translated.

This study shows the coordinated, yet independent translation of the two ORFs. The mechanism of such translation was not because of trans-splicing of the mRNA into a monocistronic mRNA. The presence of a long intervening sequence with multiple termination codons between ORFs also negated the possibility that translation of ORF2 started from an initiation codon in close proximity to the termination codon of ORF1. Re-initiation of translation at the start of ORF2 through the continuous engagement of the ribosome was also unlikely as shown by transfecting CHO cells with in vitro synthesized, non-capped mRNA. There was no translation of ORF1 following transfection of CHO cells with in vitro synthesized, non-capped mRNA, while translation of ORF2 in the same mRNA proceeded normally. The results of transfection with synthesized mRNA also negated the possibility that there was a cryptic promoter in the inter-ORF DNA sequence. The mechanism favored to explain the translation of both ORF1 and ORF2 in TCP-BP was therefore that of re-initiation of translation by an IRES element (36, 37). The 410-bp inter-ORF sequence has the characteristics of IRES elements, i.e. length of ~400–600 nucleotides and low G and C content (27, 28).

The cloned 1.3-kb cDNA is therefore an example of a rare mammalian bicistronic gene that allows for coordinated expression in time and space of two small proteins. The need for coordinated expression of the two proteins might be linked to the fact that both PRO1 and PRO2 were needed to form a
phenycyclidine and MK-801-binding protein. The two proteins, when co-expressed, formed stable oligomers composed of PRO1 and PRO2. The size of the most abundant species of the oligomeric protein, 52–59 kDa, was suggestive of a hexamer made of the 8.9 and 9.5 proteins but with an undetermined stoichiometry of the two proteins. This study also provided preliminary evidence for the fact that the oligomeric TCP-BP was part of the glutamate/glycine-binding protein complex in neuronal membranes and that the TCP-BP interactions with either the GBP or Gly-BP subunits of the complex altered its cellular function. The latter was demonstrated as the elimination of cytotoxicity by TCP-BP when either GBP or Gly-BP was co-expressed in CHO cells. The mechanism for cytotoxicity by TCP-BP in cells is currently under investigation.

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