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# ISOLATION OF DISTANTLY RELATED MEMBERS IN A MULTIGENE FAMILY USING THE POLYMERASE CHAIN REACTION TECHNIQUE

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**Summary:** We have designed a strategy to isolate and identify genes (cDNAs) coding for distantly-related members within a large multigene family. We have used limited protein sequence information data to delineate conserved regions where members of a supergene family are related. Comparison of the nucleotide sequences of such conserved areas defined consensus sequences that were used for the synthesis of deoxynucleotide primers. Two forward and two reverse primers were synthesized, and four separate pairs of primer combinations were used under low stringency in polymerase chain reactions (PCR) to generate amplified DNA products. The PCR products were directionally cloned into the phage vector M13mp18. Each of four libraries was screened with radiolabeled PCR product generated using a pair of primers different from those used to generate the library. Using this approach on the supergene family of ligand-gated ion channels, we were able to isolate and identify two novel subunits of neurotransmitter-operated ion channels. © 1990 Academic Press, Inc.

Responses of neurons to excitatory or inhibitory neurotransmitters are mediated through ligand-operated receptor/channel complexes. Transmitters such as acetylcholine (ACh) or glutamate are excitatory by gating cation-specific ion channels; transmitters like $\gamma$-aminobutyric acid (GABA) through $GABA_A$ receptors and glycine are inhibitory by opening chloride-specific channels. Receptor purification and more recently cDNA cloning studies revealed part of the structural complexity of the receptors for ACh, GABA, and glycine (1-5). These receptors consist of several subunits forming a multigene family (6). Using low stringency hybridization techniques, multiple cDNA clones encoding subunits of the ACh, GABA, and glycine receptors have been isolated (7-22). Comparison of the cDNA derived amino acid sequences for the other known ligand-gated channels showed regions of similarity among subunits indicating that they belong to the same supergene family (6). Two subfamilies were apparent. The first contains subunits of the nicotinic ACh receptors ($\alpha$1-6, $\beta$ 1-4, $\gamma$, $\delta$, $\epsilon$) (1-3,7-18); the second contains several subunits of the $GABA_A$ receptor ($\alpha$1-6, $\beta$1-3, $\gamma$1 , $\gamma$2, $\delta$) (4,19-22), and the 48 kD subunit of the glycine

**Abbreviations:** Ach: acetylcholine; bp: base pair(s); Denhardt's solution: 200 $\mu$g/ml of ficoll, 200 $\mu$g of polyvinyl pyrrolidone and 200 $\mu$g/ml of bovine serum albumin; dNTP: deoxyribonucleoside triphosphate; DTT: dithiothreitol; $GABA_A$ receptor: type A receptor of $\gamma$-aminobutyric acid; kb: kilobase; kD: kilodalton(s); LB: luria broth; PCR: polymerase chain reaction; SDS: sodium dodecyl sulfate; SSPE: 150 mM NaCl, 10 mM $NaH_2PO_4$, 1 mM EDTA, pH 7.4.

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receptor (5). In each subfamily there is 60-80% identity among members of the same group of $\alpha$, $\beta$, or $\gamma$ subunits, and 40-60% identity between members of different groups. Members of the ACh subfamily and the GABA/glycine subfamily are approximately 20-30% identical. Amino acid sequence alignment of subunits reveals four regions that are particularly conserved, with several identical amino acid positions around conservative substitutions. This supergene family of ion channels may be larger and contain more members than the ones known.

We employed the relatedness of these ion channel genes to design a strategy for isolating new candidates of ligand-gated ion channels. We generated pairs of forward and reverse primers corresponding to four conserved regions and used the polymerase chain reaction (PCR) technique to synthesize four different populations of short DNA fragments that should be enriched for potential ion channel subunits. Directional cDNA libraries were constructed in M13. To eliminate "false" positives, each library was screened for candidate clones using a radiolabeled PCR probe that had been generated with a different pair of primers. We isolated several clones encoding parts of subunits of ligand-gated ion channels, including two novel members of the GABA/glycine family.

## MATERIALS AND METHODS

**RNA and DNA isolation and handling:** Standard recombinant DNA techniques were used unless otherwise indicated (23,24). Rat brain RNA was isolated using the guanidinium/CsCl method (25) and RNA samples were stored at -80°C until use.

**Oligonucleotide primers:** The oligonucleotides used as primers were synthesized on a ABI 380B DNA synthesizer (Applied Biosystems).

**cDNA synthesis:** A modified procedure of Frech and Joho was used to synthesize cDNA as template for subsequent amplification (26). Forty $\mu$g of total brain RNA in 10 $\mu$l $H_2O$ was heated to 65°C for 5 minutes and subsequently diluted to 50 $\mu$l containing 50 mM TrisHCl (pH8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM dNTP, 0.1 mg/ml bovine serum albumin (BSA), 1600 U/ml RNasin (Promega), 10 $\mu$g random primer (hexamers), and 400 U reverse transcriptase of moloney leukemia virus (Bethesda Research Laboratories). The reaction mixture was incubated for one hour at 37°C. After alkaline hydrolysis the cDNA was precipitated with ethanol and used as template for PCR (27).

**Polymerase chain reaction:** Four reactions were run, each using cDNA made from 10 $\mu$g of total RNA with one of the following primer combinations: A and CC, A and M1 (both species), B and CC, and B and M1 (Figure 1 and 2). Each reaction was performed in 50 $\mu$l containing 18 mM TrisHCl (pH8.0), 50 mM KCl, 10 mM $MgCl_2$, 1.2 mM DTT, 0.3 mM dNTPs, 1 $\mu$g of each primer, and 2.5 U TaqI DNA polymerase (Perkin Elmer Cetus). Incubations were done in a DNA Thermal Cycler (Perkin Elmer Cetus). One cycle consisted of a denaturation step (95°C for 60 seconds), an annealing step (48°C for 80 seconds), and an elongation step (68°C for 180 seconds). After 32 cycles, a 10 $\mu$l aliquot of each reaction was run on a 5 % polyacrylamide gel. DNA fragments between 100-400 bp, 300-1100 bp, 100-500 bp, and 300-1000 bp, for the A-CC, A-M1, B-CC, and B-M1 products, respectively, were extracted and one tenth of the material was used for further amplification. After 24 more cycles, the PCR products were electrophoresed through 1.2% agarose (Seakem GTG, FMC) and DNA fragments within similar size ranges as above were electroeluted, extracted with phenol, and precipitated with ethanol. The samples were digested with EcoRI and HindIII and subsequently precipitated three times with isopropanol.

**Library construction and screening:** EcoRI/HindIII digested PCR DNA samples (6-12 ng) were ligated to HindIII and EcoRI cut M13mp18 (120 ng) in a total volume of 5 $\mu$l. The ligation products from the A-CC, A-M1, B-CC, and B-M1 reactions were used to transform E. coli XL-1 Blue

(Stratagene) and subsequently plated at a low density (a few hundreds pfu per 100 mm Petri dish) on the same bacteria. For production of radioactive probes 0.5 μl of PCR products (after 32 cycles) were used as templates for further amplification (six cycles) in a reaction mixture supplemented with α-[$^{32}$P]-dCTP (40 μC/100 μl reaction). The [$^{32}$P]-labeled PCR products A-CC, A-M1, B-CC, and B-M1 were used to screen the M13 libraries B-M1, B-CC, A-M1, and A-CC, respectively. Hybridization was performed in 5xSSPE, 5xDenhardt's solutions, 0.1 % SDS, with 2-3x$10^6$ cpm/ml of heat-denatured radiolabeled probe at 60°C for 12 to 18 hours. Filters were washed in 0.5xSSPE at 65°C. Candidate clones were picked and grown, and single stranded DNA was prepared for DNA sequencing (23,24).

A cDNA library prepared from rat brain poly(A)$^+$ RNA in lambda gt10 was used to isolate the full-length clone for the δ subunit of the $GABA_A$ receptor (28). An oligonucleotide (51 nucleotides in length) complementary to the nucleotide sequence underlined in Figure 3 was used as a radiolabeled screening probe.

## RESULTS

**Cloning Strategy to Isolate Ligand-gated Ion Channel Genes.** Figure 1 schematically depicts a prototype mRNA molecule encoding a subunit of a ligand-gated ion channel. The relative position of the four regions that are most conserved among all known subunits are shown as boxes, and the invariant amino acid positions within these regions

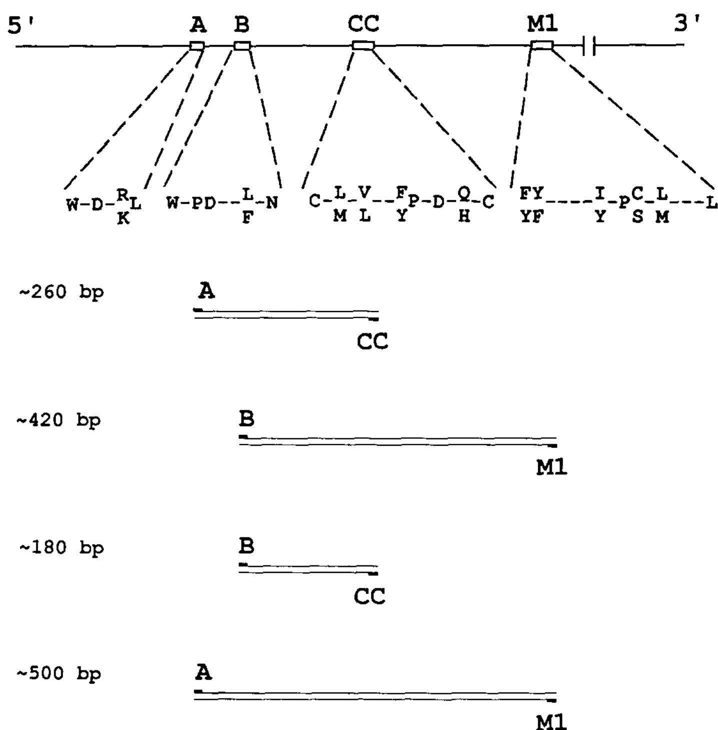


**Figure 1**
Strategy for cloning ligand-gated ion channel genes. The top part of the picture schematically depicts a prototype mRNA showing the relative positions of the four conserved regions used to design the forward and reverse primers. The invariant amino acid positions within these regions are indicated below using the single letter code. The bottom part of the figure shows the four types of PCR products generated and their respective lengths in base pairs. Sequences between B and CC are common to all four products and served as hybridization probes for the isolation of ligand-gated ion channel cDNAs.

are indicated below. Two forward primers corresponding to regions A and B, and two reverse primers complementary to regions CC and M1 were used to generate the four different PCR products schematically depicted in the lower part of Figure 1. Figure 2 shows the nucleotide sequences of the regions that were compared to deduce the consensus sequences used for the synthesis of the primers. Care was taken to generate primers that would be able to form perfect matches at positions of invariant amino acids. We also attempted to create 3' ends that would allow base pairing without mismatches

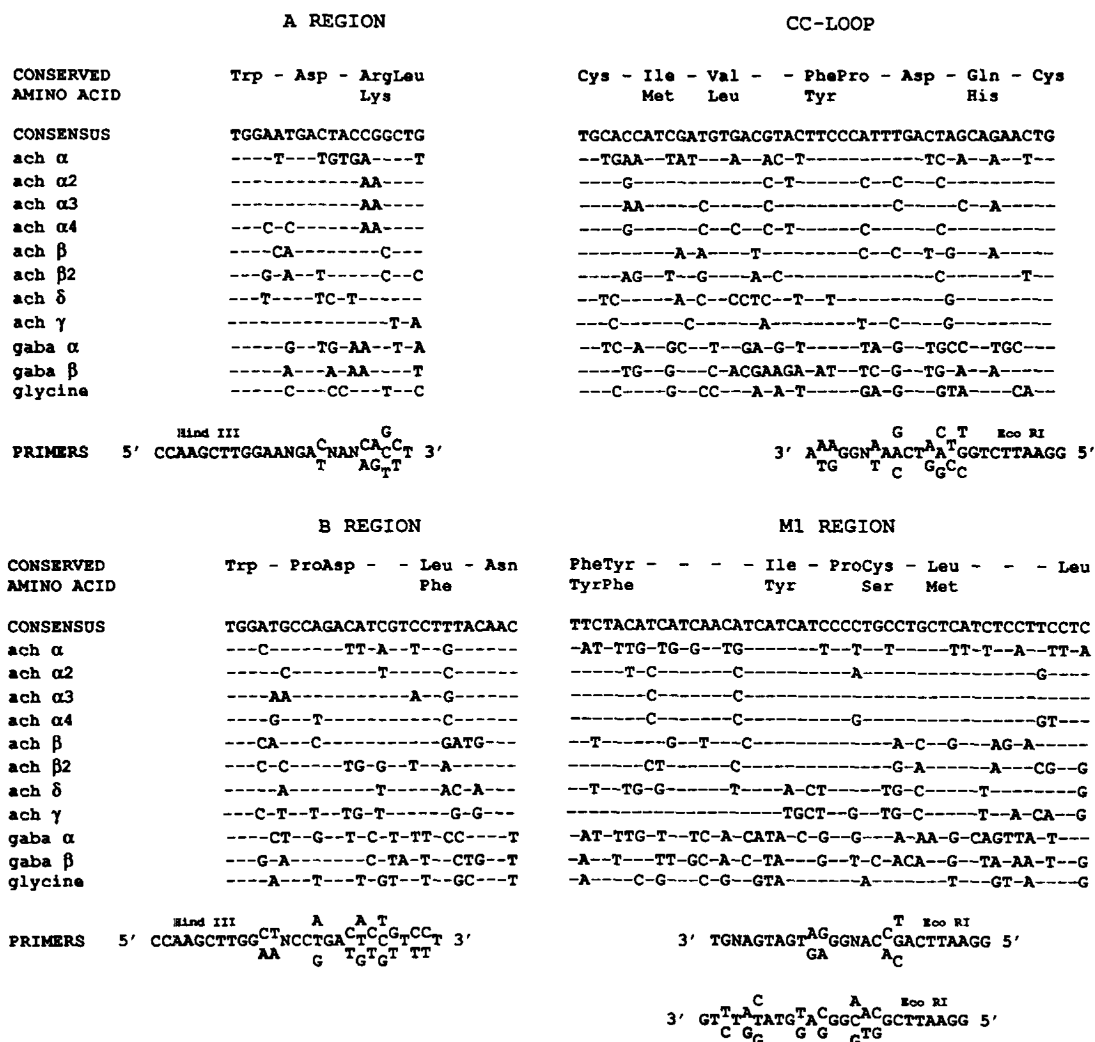


Figure 2
Design of forward and reverse primers for PCR. The nucleotide sequences corresponding to the four conserved regions are aligned below a consensus sequence. ACh α, β, γ, and δ are subunits of the nicotinic acetylcholine receptor of *Torpedo californica* (1-3). ACh α2-4, and β2 are subunits of the rat neuronal nicotinic ACh receptor (12-15). GABA α and β, and glycine are subunits of the bovine brain $GABA_A$ receptor (4), and the 48 kD subunit of the rat spinal chord glycine receptor (5). The primer sequences are shown below (N=A, G, C, and T). For the M1 region two primers were generated, one corresponding to the ACh receptor family, the other to the GABA/Glycine family.

to increase the likelihood of specific priming. These considerations led to highly degenerate primers that ought to hybridize and prime DNA synthesis on RNA or DNA templates encoding unknown, yet related ion channels. The degree of degeneracy for primers A, B, and CC was 3072, 13824, and 3456-fold, respectively. Primer M1 was a mixture of two independently synthesized oligonucleotides with 384 and 576-fold degeneracies. Nucleotides encoding a HindIII and an EcoRI restriction site at the 5' end of the forward and reverse primers, respectively, plus one or two extra nucleotides were introduced creating two different restriction sites at the upstream (HindIII) and downstream end (EcoRI) of the amplified DNA molecules. This permitted the generation of fragments with asymmetric ends and allowed directional cloning of the PCR products (Figure 1). Using non-stringent annealing conditions in conjunction with highly degenerate primers during the PCR assay, we expected a substantial degree of "false" priming that might lead to amplified DNA molecules unrelated to members of the ion channel supergene family. As can be seen on Figure 1, potential ion channel molecules contain overlapping DNA sequences (from B to CC in Figure 1). This area of overlap should be present in all four PCR products (A-CC, A-M1, B-CC and B-M1). However, "false" priming should not lead to amplified DNA molecules sharing sequences with PCR products from different groups, since in each group a different pair of primers was used. The fact that candidate clones in all four groups should overlap in the B-CC region was used to further enrich and clone isolates bearing this region.

RNA was isolated from brains of adult rats and used as template for randomly primed cDNA synthesis. The cDNA was used with a forward and a reverse primer in a PCR assay to generate four reaction products outlined in Figure 1. The amplified DNA was selected according to size on a preparative agarose gel. DNA between 100-400 bp (A-CC), 300-1100 bp (A-M1), 100-500 bp (B-CC), and 300-1000 bp (B-M1) was electroeluted and double-digested with HindIII and EcoRI to generate asymmetric ends for directional cloning in M13mp18. The A-CC, A-M1, B-CC, and B-M1 libraries were screened with [$^{32}$P]-labelled PCR products B-M1, B-CC, A-M1, and A-CC, respectively. From each library several hundred plaques were screened. Approximately five percent of the plaques gave positive hybridization signals of varying intensities.

**Ligand-gated Ion Channel Clones Isolated through PCR.** A total of 43 clones in M13mp18 were isolated, 13 from the A-CC, 11 from the A-M1, 9 from the B-CC, and 10 from the B-M1 library. DNA sequences were obtained from all 43 inserts. Since each of the forward primers contained a HindIII restriction site, the upstream (5' side) end of the cloned fragments were adjacent to the universal sequencing primer on M13mp18. The established DNA sequence should, therefore, be of sense direction and immediately

downstream of the forward primer used. The sequence was analyzed for the presence of an open reading frame, and the derived amino acid sequence was tested in the corresponding region for similarity to known ligand-gated ion channels subunits. The DNA and the translated amino acid sequences were also compared to the nucleotide and protein sequence data bank.

Thirteen clones were found to encode ion channel sequences. Seven isolates (five from A-CC and two from B-CC) are identical to parts of the β2 subunit of the nicotinic acetylcholine receptor. Three sequences (two from B-CC and one from B-M1) are identical to parts of the α2 subunit and one sequence (from A-CC) is identical to part of the γ2 subunit of the $GABA_A$ receptor. The remaining two isolates revealed new DNA sequences.

One (from B-CC) is closely related to the α1 (48 kD) subunit of the glycine receptor. A stretch of 37 amino acids between the B and the CC region is identical at 29 positions. This region is identical to the α2 subunit of the glycine receptor (29). The second isolate (from B-CC) shows similarities to the different known subunits of the GABA/glycine group. The remaining thirty clones do not show any relationship to ion channel genes known so far. The sequences of 13 of them are identical to parts of either the 18S or 28S ribosomal RNA genes. Two isolates (one from A-CC and one from A-M1) are derived from a mRNA encoding a rat cerebellar $Ca^{2+}$-binding protein (30). Another two isolates (one from A-M1 and one from B-M1) encode part of the nd1 gene for the mitochondrial NADH reductase (31). Thirteen clones had no homology to the superfamily of ligand-gatedion channels and could not be identified with anything known in the nucleotide or protein sequence data bank.

**Isolation of cDNA Encoding a New $GABA_A$ Receptor Subunit.** We synthesized an oligonucleotide complementary to the coding sequence of the isolate that showed similarity to the family of the GABA/glycine receptors. This oligonucleotide was used to screen several rat brain cDNA libraries. A clone with a 1.8 kb insert was isolated under high stringency conditions. The DNA sequence and the derived amino acid sequence are shown in Figure 3. A methionine at nucleotide position 50 (position 1 in Fig. 3) is followed by an open reading frame encoding a protein of 449 amino acids. The initiator methionine is flanked by a Kozak consensus sequence for initiation of translation (32). The derived amino acid sequence shows four hydrophobic stretches that are putative transmembrane segments and two conserved cysteine residues (the cysteine-loop). These features were highly reminiscent of all known ligand-gated ion channels. Comparison with members of the GABA/glycine family revealed a high degree of similarity. In particular, our isolate is nearly identical to the recently cloned δ subunit of the $GABA_A$ receptor

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  -49 GAATTCCCTGGGGCTAGCCGGACCTGTCCCGCGCACAGCCCGCAAGGCCATGGACGTTCTGGGCTGGCTGCTGCTGCCGCTCCTTCTGCTGTGCACGCAGCCGCACCATGGCGCCAGAGC
    1                                                  MetAspValLeuGlyTrpLeuLeuLeuProLeuLeuLeuLeuCysThrGlnProHisHisGlyAlaArgAl

   72 AATGAATGACATTGGGGACTACGTGGGCTCCAACCTGGAGATATCCTGGCTCCCCAACCTGGATGGACTAATGGAGGGCTACGCCCGAAACTTCCGACCAGGCATTGGAGGTCCTCCAGT
   25 aMetAsnAspIleGlyAspTyrValGlySerAsnLeuGluIleSerTrpLeuProAsnLeuAspGlyLeuMetGluGlyTyrAlaArgAsnPheArgProGlyIleGlyGlyProProVa

                                                                                                 T
  192 GAATGTGGCGCTTGCCCTAGAGGTGGCCAGCATTGACCACATCTCAGAAGCAAATATGGAATACACCATGACAGTGTTCCTGCACCAGAGCTGGCGAGACAGCAGGCTGTCCTACAACCA
   65 lAsnValAlaLeuAlaLeuGluValAlaSerIleAspHisIleSerGluAlaAsnMetGluTyrThrMetThrValPheLeuHisGlnSerTrpArgAspSerArgLeuSerTyrAsnHi
                                                                                            ArgAla

                                                                                    A
  312 TACCAACGAGACCCTGGGCCTGGATAGCCGCTTCGTGGACAAGCTGTGGCTCCCTGACACCTTCATTGTGAATGCCAAGTCTGCCTGGTTCCATGATGTGACCGTGGAAAACAAGCTTAT
  105 sThrAsnGluThrLeuGlyLeuAspSerArgPheValAspLysLeuTrpLeuProAspThrPheIleValAsnAlaLysSerAlaTrpPheHisAspValThrValGluAsnLysLeuIl
                                                                                    ValCysLeuVal

  432 CCGCCTACAGCCCGACGGTGTGATTTTATACAGCATCCGCATCACCTCCACAGTGGCCTGTGACATGGACCTTGCCAAGTACCCCATGGACGAGCAGGAGTGCATGCTGGACCTGGAGAG
  145 eArgLeuGlnProAspGlyValIleLeuTyrSerIleArgIleThrSerThrValAlaCysAspMetAspLeuAlaLysTyrProMetAspGluGlnGluCysMetLeuAspLeuGluSe

  552 CTATGGCTACTCTTCTGAGGACATTGTCTATTATTGGTCAGAAAACCAGGAGCAGATCCACGGGCTGGACAGGCTGCAACTGGCCCAGTTCACTATCACCAGTTACCGCTTCACCACGGA
  185 rTyrGlyTyrSerSerGluAspIleValTyrTyrTrpSerGluAsnGlnGluGlnIleHisGlyLeuAspArgLeuGlnLeuAlaGlnPheThrIleThrSerTyrArgPheThrThrGl

  672 GCTGATGAACTTCAAATCAGCTGGCCAGTTCCCTCGACTCAGCTTACACTTCCAGCTTCGGAGGAACCGGGGTGTCTACATCATCCAGTCTTACATGCCCTCTGTCCTCCTGGTTGCCAT
  225 uLeuMetAsnPheLysSerAlaGlyGlnPheProArgLeuSerLeuHisPheGlnLeuArgArgAsnArgGlyValTyrIleIleGlnSerTyrMetProSerValLeuLeuValAlaMe

  792 GTCCTGGGTCTCCTTCTGGATTAGCCAAGCAGCAGTGCCTGCCAGAGTATCTCTAGGCATCACCACTGTGCTGACAATGACCACACTCATGGTTAGTGCCCGCTCCTCCCTCCCGCGGGC
  265 tSerTrpValSerPheTrpIleSerGlnAlaAlaValProAlaArgValSerLeuGlyIleThrThrValLeuThrMetThrThrLeuMetValSerAlaArgSerSerLeuProArgAl

  912 TTCTGCTATCAAGGCTCTGGATGTGTATTTCTGGATCTGCTATGTCTTCGTGTTTGCTGCCCTGGTGGAGTATGCATTTGCCCACTTCAATGCTGACTACAGGAAGAAACGGAAAGCCAA
  305 aSerAlaIleLysAlaLeuAspValTyrPheTrpIleCysTyrValPheValPheAlaAlaLeuValGluTyrAlaPheAlaHisPheAsnAlaAspTyrArgLysLysArgLysAlaLy

 1032 GGTCAAGGTCACGAAGCCAAGGGCAGAGATGGACGTGAGGAACGCCATTGTCCTCTTCTCCCTCTCTGCTGCTGGGGTCAGCCAGGAGTTGGCTATCTCCCGCCGTCAAGGCCGGGTCCC
  345 sValLysValThrLysProArgAlaGluMetAspValArgAsnAlaIleValLeuPheSerLeuSerAlaAlaGlyValSerGlnGluLeuAlaIleSerArgArgGlnGlyArgValPr

                                                                G
 1152 TGGGAACCTCATGGGTTCCTATAGGTCTGTAGAAGTGGAGGCAAAGAAGGAGGGGGGTCCCGCCCAGGAGGCCCAGGAGGCATCCGTTCCAGACTCAAACCCATCGATGCAGACACCAT
  385 oGlyAsnLeuMetGlySerTyrArgSerValGluValGluAlaLysLysGluGlyGlySerArgProGlyGlyProGlyGlyIleArgSerArgLeuLysProIleAspAlaAspThrIl
                                                               ValPro

 1272 CGACATCTATGCCCGCGCTGTGTTCCCGGCAGCCTTTGCAGCAGTCAACATCATCTACTGGGCGGCGTATACCATGTGACGGCAGTGCGTACACCACATGACGGCTTTACATGTACCACG
  425 eAspIleTyrAlaArgAlaValPheProAlaAlaPheAlaAlaValAsnIleIleTyrTrpAlaAlaTyrThrMet

 1392 TGCGGTGGTTGCCACAAACTCCTGGAGGAACAGCCCTCTCCGTGGATCCCAAAGTGGGACAGAGAGGAGCGTCCGCTCCCTGGGCTTTACCTCAATTTTGCTCTACCAGCCTGCACTTCC

 1512 TGTAGATGGAGCAGGATGGGCTTTGCACAGAGGGCCATTAGTTGGCCTGTCCCTGAAGCTGAGCCTGCACTAAGGTGCCCTTGGGAGCTGTGAAGTTTTAGACTCAGGATGGGCCTGATT

 1632 TCTGGGCCTTTGCTCTGTGGGATCAGGATCAGAGAGAAAGAGAGTAGGGGTGGATGACCACTCTCATGGTGAACTGGAGGAGAAGGGCATTATGAGGCCTCTTTGGTCCCAGGGAATTC
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**Figure 3**
Nucleotide and derived amino acid sequence of the $\delta$ subunit of the $GABA_A$ receptor. The cDNA (including EcoRI linkers) has a length of 1799 base pairs. The longest open reading frame encodes a protein of 449 amino acids. The conserved regions A, B, CC, and M1 are outlined by heavy lines below the amino acid sequence. The thin line below the nucleotide sequence indicates the area chosen for the synthesis of the screening oligonucleotide. The three regions where the encoded amino acid sequence differs from the recently reported $\delta$ subunit are located at nucleotide positions 227-233, 390-402, and 1211-1218. A dot (.) above the nucleotide sequence indicates a base missing and a letter shows the position of an extra nucleotide in the reported sequence.

(22). The amino acid sequence is 45 to 50% identical to the GABA $\alpha$, GABA $\beta$, and glycine 48 kD subunits.

There are three regions where the encoded amino acid sequence of our clone differs from the reported GABA $\delta$ subunit (see Figure 3). In all three cases the changes appear to be due to two frameshifts (a deletion and an insertion of a nucleotide) close together in the nucleotide sequence. The sequences in between the frameshifts in our isolate and in the reported sequence are identical. The amino acid sequences in the first two regions of our clone are identical or similar to the other subunits of the GABA/glycine family. The third region is in an area where the subunits show low homology. The fact that we have determined the nucleotide sequence in these regions on both strands of the cDNA and that two of the three regions are related to the other GABA/glycine sequences, suggests that our DNA sequence is probably correct. Furthermore, the second region of difference where the reading frame of four amino acids is altered has been isolated

twice. The cloned PCR product that was used to derive the sequence for the screening oligonucleotide had been amplified between B and CC primers. Its nucleotide sequence is identical to the full-length cDNA isolate. The fact that in these regions the nucleotide sequences are identical except for the positions causing the frameshifts makes it unlikely that we are dealing with true genetic variants.

## DISCUSSION

We have used sequence information among different distantly related members of a supergene family to design a cloning strategy for unknown family members. We have applied this strategy to find new sequences encoding potential subunits of neurotransmitter-gated ion channel complexes. Of 43 candidate clones analyzed, thirteen encoded ion channel sequences. Two isolates carried sequences for new types of ion channel genes. The sequence of one isolate was used to clone the corresponding full-length cDNA which turned out to be the recently isolated $\delta$ subunit of the $GABA_A$ receptor. The second isolate is identical to the $\alpha 2$ subunit of the glycine receptor differing at eight positions over a stretch of 37 amino acids from the $\alpha 1$ subunit.

These results demonstrate that the strategy developed can be successfully applied to the isolation of still unknown members of large supergene families, like that of ligand-gated receptor-channel complexes.

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