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Isolation, Characterization, and Localization of Human Genomic DNA Encoding the β1 Subunit of the $GABA_A$ Receptor (GABRB1)

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Genomic DNA that encodes the β1 subunit of the human γ-aminobutyric acid$_A$ ($GABA_A$) receptor was cloned and mapped. Exons and flanking introns (>14 kb) were sequenced to determine the structural organization of the gene. The gene was localized on human chromosome 4, in bands p12–13. The β1 subunit is encoded by a relatively large gene (>65 kb) on nine exons. In contrast to other conserved regions of the subunit polypeptide, the proposed channel-forming domain (M2) is derived from more than one exon. The organization of exons was compared with that of the genes that code for subunits of nicotinic acetylcholine receptors. There is no evidence for conservation of gene structure between these two members of the proposed gene superfamily. However, intron–exon junctions were found to be conserved precisely between subtypes of $GABA_A$ receptor subunits.

INTRODUCTION

In mammalian brain, synaptic inhibition of neuronal activity is achieved mainly through the action of γ-aminobutyric acid (GABA) on $GABA_A$ receptors. The $GABA_A$ receptors are ligand-gated anion channels that mediate chloride conductance at the postsynaptic membrane. The operation of these channels can be modulated allosterically by a range of excitatory and depressant agents, e.g., benzodiazepines and barbiturates (Olsen and Venter, 1986). Preparations of purified $GABA_A$ receptors are tetrameric or pentameric glycoproteins, composed of at least two polypeptide types (Stephenson, 1988; Olsen and Tobin, 1990). However, the precise subunit composition and stoichiometry of individual receptor proteins are currently unknown.

Recently, the isolation and expression of cDNA clones have revealed an unexpected diversity of $GABA_A$ receptor subunit types. Several classes of subunits (designated α, β, γ, δ) that share 25–35% amino acid sequence identity with each other have been identified. Additional subunits, which are more closely related to existing sequences (70–80% identity), have been classified as subtypes (α2–6, β2,3, γ2). At least some of these subunits have been demonstrated to confer characteristic properties to the pharmacology and physiology of the receptor (see Olsen and Tobin, 1990, for review; Verdoon *et al.*, 1990; Luddens *et al.*, 1990; Ymer *et al.*, 1990).

The sequences of cDNA clones suggest that $GABA_A$ receptor subunits may share a number of structural features with the subunits of nicotinic acetylcholine (nACh) receptors (Schofield *et al.*, 1987). Together with the strychnine-sensitive glycine receptor (Grenningloh *et al.*, 1987), the subunits of each receptor type exhibit amino acid sequence homologies that are low but significant. In addition, each subunit has the potential to form a Cys–Cys domain and four membrane-spanning regions (M1–M4) at equivalent positions of the sequence of each subunit. On the basis of these similarities, it has been proposed (Schofield *et al.*, 1987; Grenningloh *et al.*, 1987) that the three receptor types may belong to a gene superfamily of ligand-gated ion channels. It is therefore of interest to compare the structural features of subunit genes that may be conserved among the different receptor families.

Genes that encode nACh receptor subunits have been isolated from a wide range of vertebrate and invertebrate sources (Buonanno *et al.*, 1989; Boulter *et al.*, 1990; and references therein). These studies have revealed that several features of gene organization are conserved for all subunit types in all species that have been examined. However, little is known of the struc-

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession Nos. M59212, M59213, M59214, M59215, and M59216.

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ture of genes that encode $GABA_A$ and glycine receptor subunits. To compare the gene structures of different receptor families, and as a first step toward exploring the regulation of subunit expression, we have isolated genomic DNA that encodes $GABA_A$ receptor subunits. Very recently, a study describing the first gene structure of a $GABA_A$ receptor subunit, the mouse δ subunit, was reported (Sommer *et al.*, 1990). Here, the structural features of a gene that encodes the human $\beta 1$ subunit (GABRB1) are compared with the exon organization of related receptor genes.

MATERIALS AND METHODS

Materials

Enzymes used for the cloning and sequencing of DNA were purchased from New England Biolabs, Bethesda Research Laboratories, and Promega Corporation. Two genomic libraries, containing inserts derived from partial *Mbo*I digestion of human leukocyte or fibroblast DNA and cloned into EMBL3 or λFIX, were obtained from Clontech Laboratories and Stratagene, respectively. Oligonucleotides (20- to 30-mers) were synthesized on a Model 380B DNA synthesizer (Applied Biosystems).

Screening Genomic Libraries

Phage were plated at approximately 50,000 plaques/15-cm plate and transferred in duplicate onto Hybond N filters (Amersham). The DNA was fixed to the filters by successive blotting for 2 min in 0.5 *M* NaOH, 1.5 *M* NaCl and 1.0 *M* Tris–Cl (pH 7.4), 1.5 *M* NaCl. Filters were baked for 2 h at 80°C and washed for 2 h at 50°C with 0.1% SDS, 2× SSC (1× SSC is 0.15 *M* NaCl, 0.015 *M* sodium citrate). The plaques were screened with random primer-labeled cDNA probes ($0.5–2.5 \times 10^9$ dpm/μg) or end-labeled oligonucleotides ($3–6 \times 10^6$ dpm/pmol). The hybridization solutions for screening with cDNA probes contained 50% formamide, 5× SSC, 20 m*M* Tris–Cl (pH 7.6), 1× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidine, 0.02% bovine serum albumin), 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA. Filters were incubated with cDNA probes for 14 h at 42°C and washed at 60°C with 0.2× SSC, 0.1% SDS. The hybridization solutions used for screening with oligonucleotide probes contained 6× SSC, 1× Denhardt's solution, 0.05% sodium pyrophosphate, and 100 μg/ml denatured salmon sperm DNA. Filters were incubated with oligonucleotide probes for 14 h at 10°C below T_m and washed at 10°C below T_m with 6× SSC, 0.05% sodium pyrophosphate (T_m is 2°C (A+T) + 4°C (G+C); Dalbadie-McFarland *et al.*, 1982). The filters were exposed to Kodak XAR-5 film at −70°C.

Cloning of GABRB1 Fragments

The amplified genomic library that was used initially for screening contained human leukocyte DNA cloned into the vector EMBL3. Approximately 0.5×10^6 plaques were screened at high stringency with a 1761-bp *Bsm*I/*Bgl*II fragment of a cDNA that encoded the complete bovine $GABA_A$ receptor $\beta 1$ subunit (Schofield *et al.*, 1987). The fragment included 50 and 285 bp of 5′ and 3′ untranslated sequences, respectively. A total of six hybridizing clones were purified and characterized further by restriction fragment mapping and Southern blotting with the cDNA probe. Five of the six clones contained unique inserts. An oligonucleotide corresponding to coding sequence at the 3′ end of a human $\beta 1$ cDNA clone (bases 1473–1502; Schofield *et al.*, 1989) hybridized to one of the clones (λB1). Following subcloning and sequencing, the single hybridizing fragment of the λB1 insert was found to contain 342 bp of sequence that is identical to the 3′ end of the human $\beta 1$ cDNA coding sequence (Figs. 1a and b). The exon also includes at least 289 bp of 3′ untranslated sequence. The length of untranslated 3′ sequence on the human $\beta 1$ transcript is currently unknown. Consequently, the remaining 4 kb of 3′ sequence on the λB1 insert was not characterized further.

To detect upstream inserts that overlapped λB1, the remaining clones were probed with a 700-bp *Kpn*I/*Sal*I fragment derived from the 5′ end of the λB1 insert. The clones were also probed with an 820-bp *Apa*I fragment of the bovine $\beta 1$ cDNA clone (bp 226–1046). The latter probe would detect only exons that were directly upstream of the exon on the λB1 insert. A single clone, λB3, hybridized with both probes. The λB3 insert contained three exons, composed of 138, 153, and 245 bp, respectively. These exons correspond to $\beta 1$ cDNA sequences that are upstream and contiguous with the exon on the λB1 insert (Figs. 1a and b).

The 225-bp *Apa*I fragment of bovine $\beta 1$ cDNA was used to rescreen the library (1×10^6 plaques) for clones containing the 5′ end of the gene. No hybridizing clones were obtained. The probe was therefore used to screen a second genomic library. This amplified library contained inserts that were derived from human fibroblast DNA and were cloned into the vector λFIX. Twelve hybridizing clones were isolated from 1×10^6 plaques. All of these clones also hybridized with an oligonucleotide that corresponded to bases 1–19 of the human $\beta 1$ cDNA sequence. One of these clones (λB11) was characterized further and was found to contain three short exons (80, 92, and 68 bp) corresponding to the start of the human $\beta 1$ cDNA sequence (Figs. 1a and b).

A probe homologous to those exon sequences of GABRB1 that remained to be cloned was prepared from the bovine β1 cDNA by the polymerase chain reaction (PCR). A region of the bovine cDNA (bp 295–592) was amplified using oligonucleotides that corresponded to the ends of both bovine and human sequences (sense, TATACWCTCACCATGTATTTC; antisense, TTTCRATCTCCAGGGTGCAGT). The amplified DNA was used to rescreen 1×10^6 phage of the new library, and a total of 29 hybridizing clones were isolated. The oligonucleotides used to generate the PCR probe (sense and antisense primers) hybridized to 9 and 4 of these clones, respectively. None of the clones hybridized with both oligonucleotides. The inserts from selected clones (λB56 and λB48) were characterized further and each was found to contain a single exon (Figs. 1a and b). These exons (221 and 83 bp, respectively) were identical in sequence to the human β1 cDNA and completed the cloning of exons encoding the β1 subunit. Other clones obtained from these screenings (λB49, λB54) contained additional intron sequence (Fig. 1b). However, hybridization of all clones with probes (0.7–4.3 kb) derived from intron sequences at the 3′ ends of λB11, λB48, and λB49 inserts, or 5′ ends of λB56, λB54, and λB3 inserts, revealed that there was no further overlap between the 4 segments of cloned inserts (Fig. 1c).

Polymerase Chain Reaction

PCR was used to prepare probes and to confirm the presence of exons in isolated clones. Regions of DNA were amplified from plasmid DNA (100 ng) or purified λ clones (10^5 plaque forming units) using the GeneAmp system (Perkin–Elmer Cetus). A cycle of 94, 55, 72°C (each for 2 min) was repeated 30 times for optimal amplification.

Subcloning and Sequencing

DNA that was prepared from λ clones was digested with the restriction enzymes *Kpn*I, *Sal*I, *Bgl*II, and *Eco*RI, using all combinations of single and double digests. The digested DNA was fractionated by electrophoresis on 0.8% agarose gels. Fragments that contained exons of interest were identified by Southern blot analysis (see below). Following electrophoresis on 0.8% LMP agarose (Bethesda Research Laboratories), the appropriate DNA fragments were excised and subcloned into the vector pVZ1 (Henikoff and Eghtedarzadeh, 1987). For each subclone, sets of ordered deletions were generated using a modification of the Erase-a-Base system (Promega). Using this modification, treatment of the insert DNA with exonuclease III, S1 nuclease, and Klenow fragment (Henikoff, 1987) was followed by electrophoresis of the sample on 0.8% LMP agarose. The DNA fragments that were deleted appropriately were excised and ligated in the agarose medium. This modification resulted in a substantial increase in the proportion of subsequent transformants that contained plasmid DNA of the expected size. The vector inserts were sequenced from single-stranded templates. Sequencing was performed by the dideoxy termination method, using a *Taq* polymerase sequencing system (Promega) designed for use on the 370A (Gocayne *et al.*, 1987) or 373A automated DNA sequencing system (Applied Biosystems). Nucleotide sequence data were collated and analyzed using the Suite software package (IntelliGenetics).

Southern Blot Analysis

Samples of DNA (1–5 μg) were digested with restriction enzymes and fractionated by electrophoresis on 0.8% agarose gels. The DNA was transferred and fixed to Hybond N membranes (Amersham), following the recommendations of the manufacturer. The blots were hybridized with random primer-labeled or end-labeled probes under the conditions described for screening genomic libraries.

Localization of GABRB1

The chromosome location of GABRB1 was determined by fluorescence *in situ* hybridization. Probe labeling and hybridization reactions were performed as described previously (Lichter *et al.*, 1990). Briefly, a human genomic clone in EMBL3, containing 15 kb of GABRB1 (λB1; Fig. 1), was labeled with biotin-11-dUTP by nick translation (Brigati *et al.*, 1983) and used for hybridization to metaphase chromosomes prepared by standard techniques. Chromosome *in situ* suppression (CISS) hybridization (Lichter *et al.*, 1988) was performed by denaturing and preannealing 4 μg human placental DNA, 6 μg salmon sperm DNA, and 50–80 ng labeled λB1 DNA before application of the probe cocktail to denatured chromosome spreads. Following overnight incubation and posthybridization washes, the specimens were incubated with blocking solution and detected with 5 μg/ml fluorescein isothiocyanate (FITC)-conjugated avidin (Vector Laboratories). Chromosomal DNA was counterstained by propidium iodide, and images of metaphase specimens and hybridization signals were obtained using a laser-scanning confocal microscope. The map position of the gene is expressed in terms of the fractional length of the total chromosome relative to the terminus of the short arm, designated pter-to-

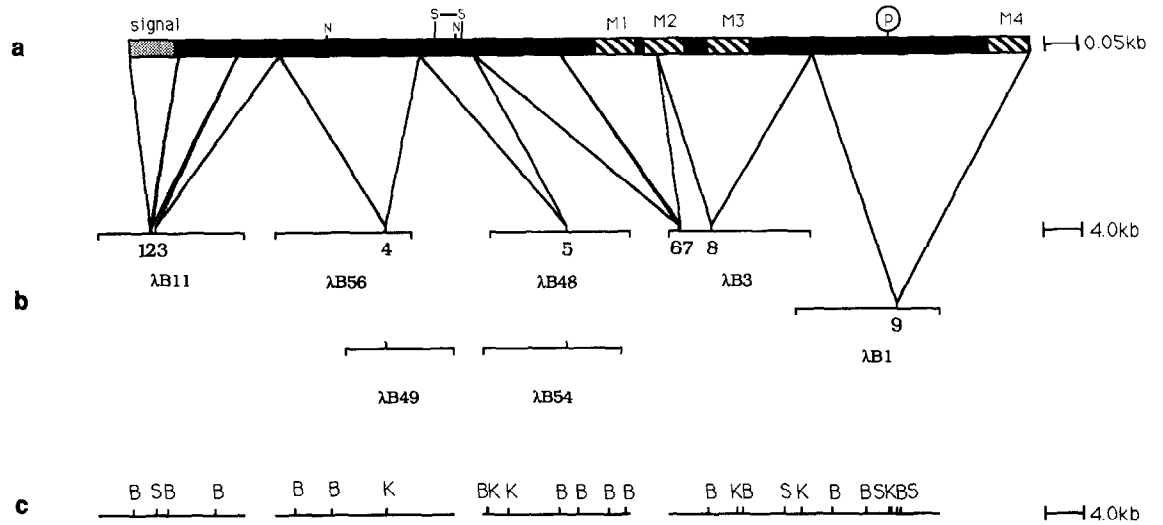

FIG. 1. Correspondence between the gene and the cDNA product of the human $GABA_A$ receptor $\beta 1$ subunit. (**a**) A representation of the human $\beta 1$ cDNA sequence (30). Bases of the cDNA that correspond to the start and end of individual exons are indicated by oblique lines that join the cDNA to the gene fragments (see **b**, below). Structural features of the $\beta 1$ subunit protein that have been predicted from the cDNA sequence are also illustrated. The signal sequence is thought to be cleaved from the mature polypeptide. Potential sites of N-linked glycosylation are marked N. The β-structural loop bounded by cysteine residues 136 and 150 is represented by S–S. The four proposed membrane-spanning, hydrophobic sequences (M1–M4) are highlighted by hatched boxes. A consensus sequence for cAMP-dependent phosphorylation is marked P. (**b**) Cloned fragments of human genomic DNA. The horizontal bars indicate the chromosomal fragments that were isolated from human genomic libraries. Within these fragments, the positions of exons (1–9) are marked by vertical bars. (**c**) Composite restriction maps of the cloned genomic fragments. B, S, and K represent the restriction enzymes *Bgl*II, *Sal*I, and *Kpn*I, respectively.

qter. The range of pter-to-qter values was obtained by analyzing 15 extended chromosomes. Chromosome identification was confirmed by cohybridization with a probe, B31, that is specific for chromosome 4 (Bates *et al.*, 1990).

Assignment to a precise cytogenetic band location was obtained by cohybridization of the λB1 probe to chromosome spreads with digoxigenin-labeled PCR products that were generated from human genomic DNA using an *Alu* DNA primer. *Alu* PCR products produce an R-banding pattern consistent with conventional R-banding patterns (Baldini and Ward, 1991). The banding signal for digoxigenin *Alu* hybridization was detected with anti-digoxigenin antibody labeled with rhodamine (Boehringer Mannheim), whereas the biotinylated λB1 probe was detected with FITC-labeled avidin. The *Alu*-digoxigenin hybridization banding and the λB1 hybridization signals were imaged separately using a Zeiss axioskop epifluorescence microscope coupled to a cooled CCD camera (Photometrics). The gray-scale images were then pseudocolorized and merged electronically to form a composite image as described elsewhere (Baldini and Ward, 1991). The camera was controlled and images were merged using an Apple Macintosh IIX computer running custom software (developed by T. Rand, Yale). Photographs were taken directly from the computer monitor.

RESULTS AND DISCUSSION

Cloning, Mapping, and Sequencing of β1 Gene Fragments

Probes prepared from a cDNA that encodes the $\beta 1$ subunit of the bovine $GABA_A$ receptor were used to screen human genomic libraries at high stringency (see Materials and Methods). A total of 47 clones were isolated and selected inserts were characterized by restriction mapping, Southern blotting, and DNA sequence analysis. Insert DNA from 5 of these clones (λB11, λB56, λB48, λB3, and λB1) contained 9 exons that encode the complete human $\beta 1$ subunit protein (Figs. 1a and b). Two other clones (λB49 and λB54) contained additional intronic sequence. The remaining clones contained either sequence described in Fig. 1b or inserts derived from the genes of distinct β-subunit subtypes (see below). The overall length of the gene between exons 1 and 9 is greater than 65 kb.

The strategies used to sequence each of the exons are outlined in Fig. 2. The sequences of exons and flanking introns were obtained from both strands following ordered deletion of subcloned fragments. The sequences of the 9 exons are presented in Fig. 3. All of the exons were flanked by canonical acceptor and donor splicing sites (Breathnach and Chambon, 1981). The nucleotide sequence of the spliced exons is iden-

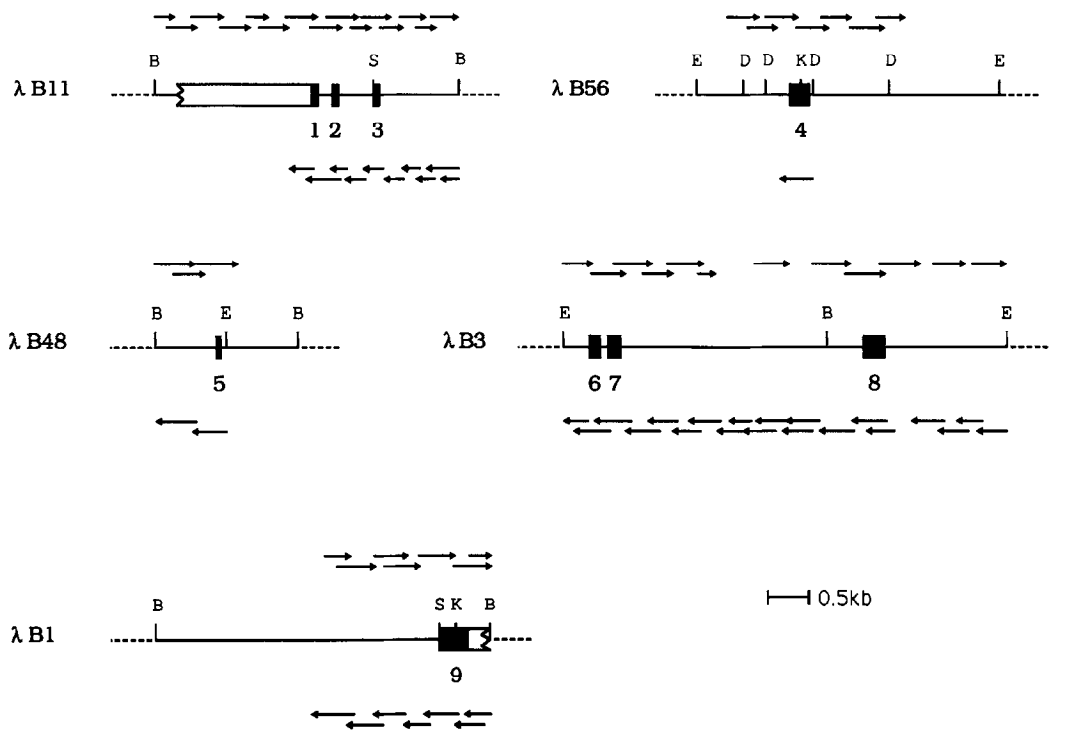

FIG. 2. Strategy used to sequence the exons of GABRB1. Selected fragments of cloned insert DNA, derived from *Bgl*II or *Eco*RI digests, were subcloned into the phagemid pVZ1 in both orientations. Sets of ordered deletions were generated from each subclone, and a single oligonucleotide was used to prime all of the sequencing reactions. The λ clone from which each subclone was derived has been indicated. The arrows represent examples of the direction and extent of sequence obtained. The exons that encode the β1 subunit are represented by filled boxes and are numbered 1–9. The horizontal bars represent intron sequences and the unfilled boxes signify untranslated exon (λB1) or undefined upstream sequence (λB11). B, *Bgl*II; S, *Sal*I; K, *Kpn*I; E, *Eco*RI; D, *Dra*I. The gene fragments, totaling 14.4 kb in length, were sequenced with an average redundancy of 5.1. A total 8.2 kb, including the exons and flanking introns, was sequenced in both directions.

tical to the sequence of a human β1 cDNA clone (Schofield *et al.*, 1989), with the exception of a single base substitution on exon 2, at base pair A329 (Fig. 3). This substitution (T for C) changes the deduced amino acid sequence in the region of the N-terminus, by replacing Pro_{10} with Ser. It is notable that Ser is also found at this position in the deduced amino acid sequences of bovine and rat β1 subunits (Schofield *et al.*, 1987; Ymer *et al.*, 1989).

As we have not yet been able to clone all of the larger intron sequences between exons 3 and 6, it was necessary to demonstrate that the nucleotide sequences of exons 4 and 5 matched perfectly with the corresponding regions of the human β1 cDNA. The amino acid sequences that are encoded by exons 4 and 5 are very highly conserved between the three subtypes of β subunits that are known to exist in bovine and rat brain (Ymer *et al.*, 1989; Lolait *et al.*, 1989a). However, a comparison of the nucleotide sequences that correspond to exons 4 and 5 indicates that, for each exon, the rat β1 sequence varies from the β2 or β3 sequences at greater than 10% of the bases (Ymer *et al.*, 1989). There is no reason to assume that similar comparisons between the subtypes of human β subunits would vary substantially from this figure. It is most likely, therefore, that the cloned exons described here encode the β1 subunit and reside on a single contiguous gene.

The Organization of β1 Exons Compared with That of Related Genes

Subunits of GABA$_A$ receptors and strychnine-sensitive glycine receptors share a number of structural similarities with the subunits of nACh receptors (Schofield *et al.*, 1987; Grenningloh *et al.*, 1987). The amino acid sequence conservation between these different subunit types is most pronounced in the proposed extracellular and hydrophobic regions of the polypeptides. These include a Cys–Cys domain and four potential transmembrane regions (M1–M4) at homologous positions of each subunit protein. In all of the subunit types, M1 contains an invariant proline residue, while M2 is composed of several hydrophilic residues that may form the lining of the ion channel. It has been proposed that the distinct receptor types

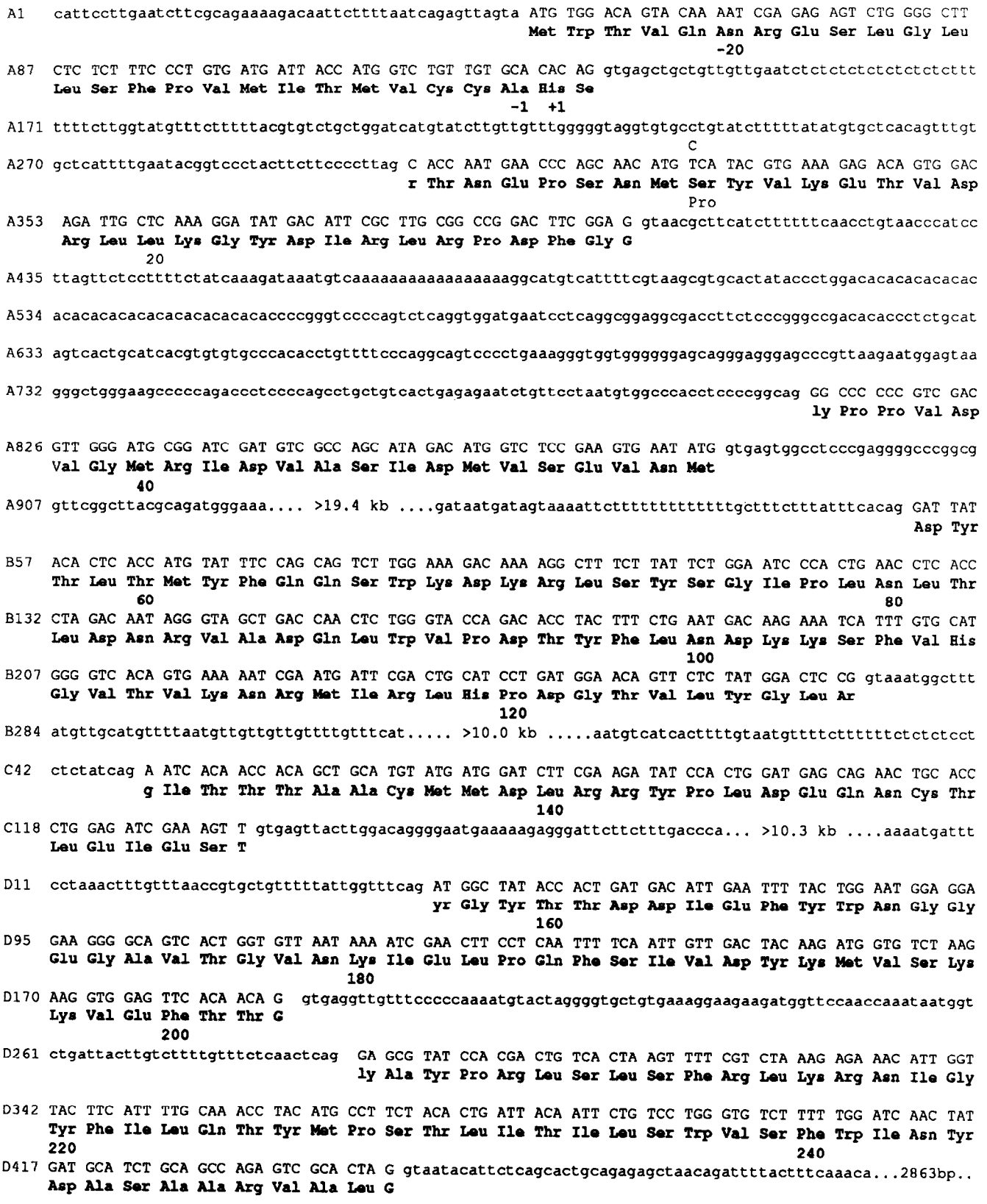

FIG. 3. Sequences of exons and flanking introns for GABRB1. The nucleotide sequences of exons and introns are written in uppercase and lowercase letters, respectively. The deduced amino acid sequence is written in boldface letters below the corresponding codons. The amino acids are numbered below the sequence, from the proposed amino terminus of the mature β1 polypeptide. Amino acids that constitute the putative signal peptide are signified by negative numbers. The nine exons are illustrated on six nucleotide sequences (A–F). The individual sequences are separated by dotted lines that represent large introns. The lengths, or minimum lengths, of these introns have been indicated. The nucleotide sequences (A–F) have been numbered to the left and each starts from the first base of 5′ flanking intron sequence. Comparisons of the exon sequences with the sequence of a human β1 cDNA (30) indicate only one mismatch. This mismatch (at A329) and its effect on the deduced amino acid sequence are indicated.

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E1   ......ggaacttaatagtggcaccttcagctaagtgttgtctttctctttcacag GA ATC ACG ACG GTG CTT ACA ATG ACA ACC ATC
                                                              ly Ile Thr Thr Val Leu Thr Met Thr Thr Ile
                                                                                          260
E83  AGC ACC CAC CTC AGG GAG ACC CTG CCA AAG ATC CCT TAT GTC AAA GCG ATT GAT ATT TAT CTG ATG GGT TGC TTT
     Ser Thr His Leu Arg Glu Thr Leu Pro Lys Ile Pro Tyr Val Lys Ala Ile Asp Ile Tyr Leu Met Gly Cys Phe
                                                                 280
E158 GTG TTT GTG TTC CTG GCT CTG CTG GAG TAT GCC TTT GTA AAT TAC ATC TTC TTT GGG AAA GGC CCT CAG AAA AAG
     Val Phe Val Phe Leu Ala Leu Leu Glu Tyr Ala Phe Val Asn Tyr Ile Phe Phe Gly Lys Gly Pro Gln Lys Lys
                                             300
E233 GGA GCT AGC AAA CAA GAC CAG AGT GCC AAT GAG AAG AAT AAA CTG GAG ATG AAT AAA GTC CAG gtaagatattaaata
     Gly Ala Ser Lys Gln Asp Gln Ser Ala Asn Glu Lys Asn Lys Leu Glu Met Asn Lys Val Gln
                         320
E311 ttcctaacaatattcttgttaaatttatcagcatc.... 17.8 kb ....caggcaaaggtcctgcaacttgtgtccgagcctgttctttttgccat

F48  cag GTC GAC GCC CAC GGT AAC ATT CTC CTC AGC ACC CTG GAA ATC CGG AAT GAG ACG AGT GGC TCG GAA GTG CTC
         Val Asp Ala His Gly Asn Ile Leu Leu Ser Thr Leu Glu Ile Arg Asn Glu Thr Ser Gly Ser Glu Val Leu
                         340
F123 ACG AGC GTG AGC GAC CCC AAG GCC ACC ATG TAC TCC TAT GAC AGC GCC AGC ATC CAG TAC CGC AAG CCC CTG AGC
     Thr Ser Val Ser Asp Pro Lys Ala Thr Met Tyr Ser Tyr Asp Ser Ala Ser Ile Gln Tyr Arg Lys Pro Leu Ser
     360                                                                                         380
F198 AGC CGC GAG GCC TAC GGG CGC GCC CTG GAC CGG CAC GGG GTA CCC AGC AAG GGG CGC ATC CGC AGG CGT GCC TCC
     Ser Arg Glu Ala Tyr Gly Arg Ala Leu Asp Arg His Gly Val Pro Ser Lys Gly Arg Ile Arg Arg Arg Ala Ser
                                                                 400
F273 CAG CTC AAA GTC AAG ATC CCC GAC TTG ACT GAT GTG AAT TCC ATA GAC AAG TGG TCC CGA ATG TTT TTC CCC ATC
     Gln Leu Lys Val Lys Ile Pro Asp Leu Thr Asp Val Asn Ser Ile Asp Lys Trp Ser Arg Met Phe Phe Pro Ile
                                             420
F348 ACC TTT TCT CTT TTT AAT GTC GTC TAT TGG CTT TAC TAT GTA CAC tgaggtctgttctaatggttccatttagactactttcct
     Thr Phe Ser Leu Phe Asn Val Val Tyr Trp Leu Tyr Tyr Val His
                         440
F432 cttctattgtttttaaccttacaggtccccaacagcgatactgctgtttctcgaggtaagagattcagccatccaattggttttaggtcttgcatatc

F531 agttttattactgcaccatgtttacttcaaaaagacaaaacaaaaaaaaaattatttttccagtctaccgtggtccaggttatcagctctttaagagct

F630 ctattaattgccatgtttacaaacaaacacaaagagagaagttagacaggta
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FIG. 3—*Continued*

that share these similarities may be encoded by a superfamily of related genes (Schofield *et al.*, 1987; Grenningloh *et al.*, 1987). Any conservation between the subunit gene structures of different receptor types would provide strong support for this proposal. Although the gene structure of a glycine receptor subunit has not yet been reported, genes that encode nACh receptor subunits have been isolated from a variety of sources. Of these, representative examples were selected to illustrate the organization of exons in different subunit genes. Following optimal alignment of these amino acid sequences, it is observed that the positions of three exon boundaries are conserved precisely between the genes of all nACh receptor subunits (Fig. 4). A fourth junction is also found at variable positions within the sequence of the proposed signal peptide.

The amino acid sequence homology between the subunits of GABA$_A$ and nACh receptors is greatest over a region extending from the start of the mature polypeptide to the end of M3. This region is 298–307 amino acid residues in length, for the examples illustrated in Fig. 4. Over this stretch, 70 residues are conserved between the different nACh receptor subunits. Of these, 32 are found at positions equivalent to those of the human GABA$_A$ receptor β1 subunit. It is possible, therefore, to obtain an accurate comparison of exon organization for the different subunit genes. Only the first exon of each gene encodes amino acid sequence that has little homology between subunits. It is therefore difficult to make direct comparisons of exon junctions within this region. However, there are significant differences between the gene structures of the two subunit types in this region. In contrast to nACh receptor genes, the junction between exons 1 and 2 of GABRB1 is not within the sequence of the proposed signal peptide. In addition, the junction is a nonintegral number of codons from the corresponding junctions on nACh receptor genes. This would suggest that the existence of introns in this region of GABRB1 and nACh receptor genes arose from independent insertions rather than from a common ancestor (Rogers, 1989). Over most of the remaining polypeptide length, amino acid sequence homologies permit an accurate comparison between the positions of exon boundaries. As illustrated in Fig. 4, it was determined that there is no conservation of sites for exon junctions between the subunit genes of the two receptor types. If the two receptor types described here are derived from a common ancestral gene, any

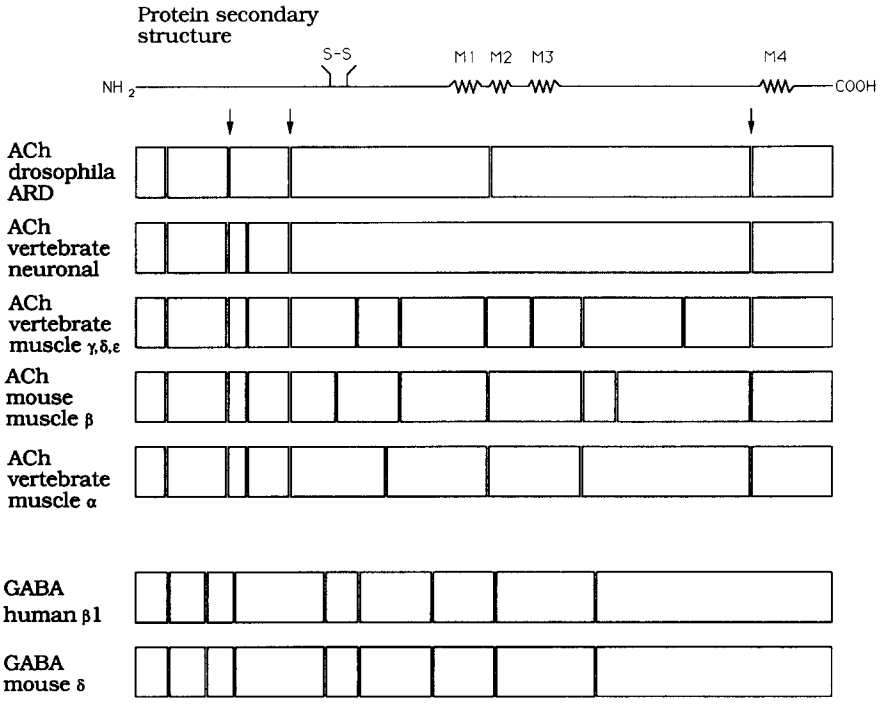

FIG. 4. Comparison of the gene structures of nACh and $GABA_A$ receptor subunits. The nACh receptor subunits that were selected for comparison are representative of the differing gene structures retrieved from GenBank. The different categories (and selected examples) are: *Drosophila* ARD (28); vertebrate neuronal (rat $\alpha 2$ (36)); vertebrate muscle γ, δ, ϵ (human γ (31)); mouse muscle β (9); vertebrate muscle α (human α (23)). The mouse $GABA_A$ receptor δ subunit is from (33). The deduced amino acid sequences of the nACh and $GABA_A$ receptor subunits were analyzed using the Genalign program of an IntelliGenetics software package. Gaps were introduced to maximize homology. Protein sequences that are encoded by distinct exons are represented by boxes. The uppermost line represents regions of predicted secondary structure that are common to all subunit proteins (see legend to Fig. 1 for definitions). The arrows indicate the position of exon boundaries that are conserved precisely between all of the nACh receptor subunits.

introns within that gene have not been retained by both receptor types during the courses of their evolution.

Following the cloning of human nACh receptor α and δ subunits, it was noted that the protein sequences encoded by different exons corresponded to proposed structural domains (Shibara *et al.*, 1985; Noda *et al.*, 1983). Indeed, comparative studies have indicated that functional domains are frequently encoded by single exons (Blake, 1985). The exons that encode the proposed membrane-spanning regions are not split by introns in any of the nACh receptor genes that have been characterized to date. GABRB1 encodes the Cys–Cys loop, M1, M3, and M4 on distinct exons. However, the DNA encoding the M2 region of the $\beta 1$ subunit is interrupted by intron sequence. Recently, it was determined that the Cys–Cys domain of the mouse nACh receptor β subunit is also encoded by two exons (Buonanno *et al.*, 1989). It is therefore unlikely that the location of structural or functional domains for ligand-gated ion channels can be predicted from the absence of exon boundaries.

Although the organizations of exons that encode $GABA_A$ and nACh receptor subunits are dissimilar, we have observed that the junctions between introns and exons are conserved between $GABA_A$ receptor subunits. The positions of the four introns between exons 4 and 8 of GABRB1 are conserved precisely in the equivalent sequences of the gene that encodes the human $GABA_A$ receptor $\beta 3$ subunit (unpublished data). While this work was in progress, the gene structure of the mouse $GABA_A$ receptor δ subunit was reported (Sommer *et al.*, 1990). This gene (~13 kb) is considerably smaller than GABRB1. However, the eight intron–exon junctions of GABRB1 are found at positions equivalent to those of the mouse δ subunit gene (Fig. 4). It would appear that the eight introns were contained within a common ancestral gene prior to the divergence of β and δ subunits. Such strict conservation of gene structure is also observed within the family of neuronal nACh receptor subunit genes (Boulter *et al.*, 1990). It will be of interest to compare these gene structures with the genes that encode distinct $GABA_A$ and glycine receptor subunits, particularly those of lower organisms. Such information may help determine the time course and mechanism through which the diversity of receptor subunits arose over the course of evolution.

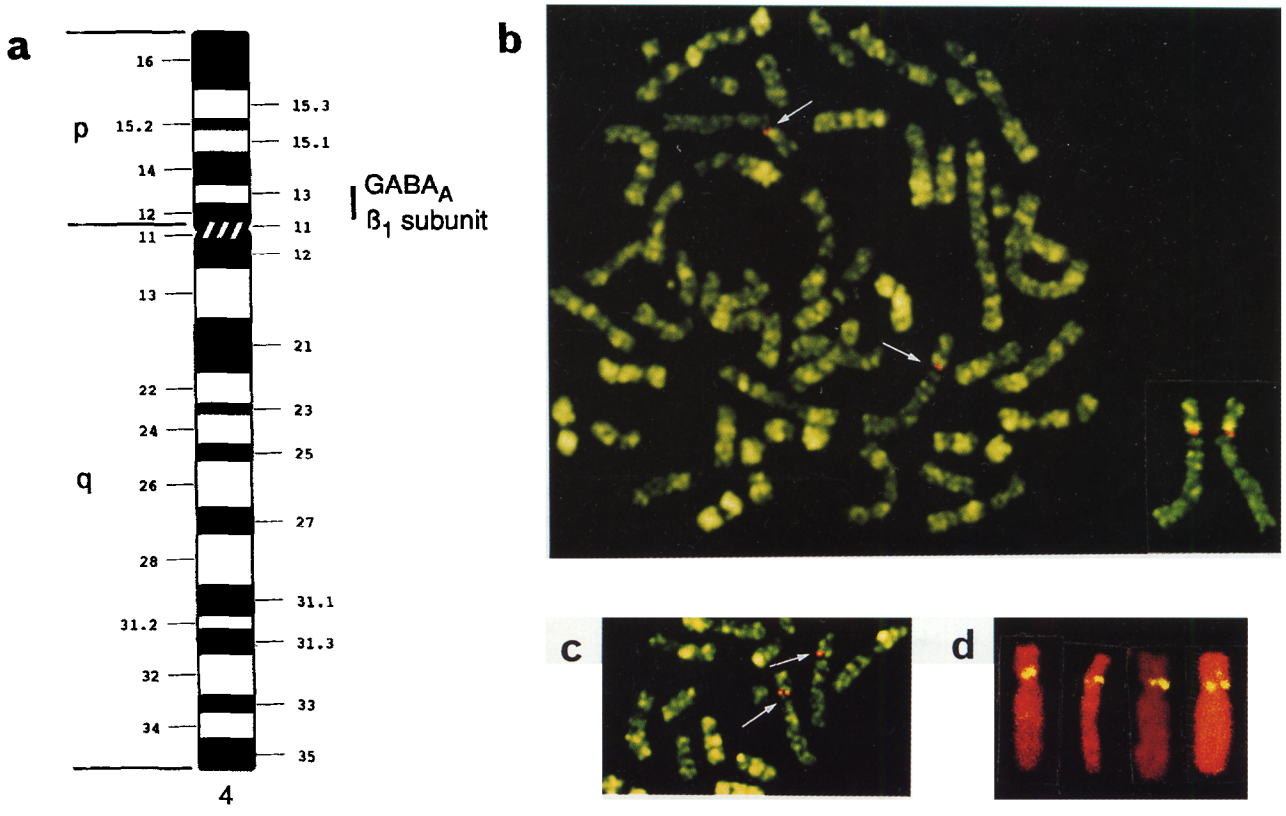

FIG. 5. Chromosomal localization of GABRB1 by fluorescence *in situ* hybridization. (a) An R-banded idiogram of human chromosome 4 depicting the localization of GABRB1 to 4p12–13. (b) Metaphase spread after cohybridization with a biotinylated β1 subunit probe (λB1) and digoxigenin-labeled PCR products produced using an *Alu* primer. The *Alu* PCR products produce an R-banding pattern that is pseudocolored yellow. The hybridization signal for the GABRB1 probe is seen in red (white arrows). (c) Partial R-banded metaphase spread showing the GABRB1 probe hybridization signal on chromosome 4 (white arrows). (d) A montage of unbanded chromosomes 4, displaying the GABRB1 probe hybridization signal as detected by FITC-labeled avidin. Here, chromosomes were counterstained with propidium iodide.

Chromosomal Localization of GABRB1

This study confirms a recent report by Buckle and colleagues (1989) that mapped a GABA$_A$ receptor β subunit to the same locus. The latter study used a full-length human β1 cDNA probe, but did not report the location of other β-subunit genes. As a consequence, the subtype of β subunit that had been localized could not be determined with certainty. Here, a fragment of genomic DNA, containing an exon of GABRB1 that has relatively low homology to other β subunits, has provided unequivocal evidence for the location of GABRB1. The location of GABRB1 on chromosome 4 was determined by fluorescence *in situ* hybridization (Lichter *et al.*, 1990). The 15-kb genomic clone was labeled with biotin and hybridized to metaphase chromosomes. The specimens were then incubated with FITC-conjugated avidin. The location of GABRB1 on chromosome 4 was established first by the position of the fluorescein-labeled probe and expressed in terms of fraction length of the chromosome relative to the terminus of the p arm, pter-to-qter (Lichter *et al.*, 1990). The pter-to-qter value range for GABRB1 on chromosome 4 is determined to be 0.22–0.26 (Figs. 5a and d). The precise cytogenetic band location was confirmed by direct visualization of GABRB1 by a two-color fluorescence *in situ* cohybridization, using biotinylated λB1 probe with digoxigenin-labeled *Alu* PCR products. The *Alu* PCR products were generated from genomic DNA using an *Alu* DNA primer (Baldini and Ward, 1991). The *Alu* PCR products yield an R-banding pattern that is imaged and subsequently merged with the images generated from the λB1 hybridization probe. The merged image permits direct visualization of GABRB1 on a precise band of the short arm of chromosome 4. GABRB1 is located on 4p12–13 (Figs. 5b and c). The position of GABRB1 does not correspond with the positions of any inherited human neurological diseases known at present (O'Brian, 1990).

The three GABA$_A$ receptor β subunits contain a consensus sequence for cAMP-dependent phosphorylation (Schofield *et al.*, 1987; Ymer *et al.*, 1989; Lolait *et al.*, 1989a). Phosphorylation of GABA$_A$ receptors

by protein kinase A (Kirkness *et al.*, 1989; Browning *et al.*, 1990) may play a role in the regulation of receptor activity (Heuschneider and Schwartz, 1989). Such a regulatory role may be of functional importance and could explain why β subunits are expressed in most brain regions that contain $GABA_A$ receptors (Lolait *et al.*, 1989b; Mohler *et al.*, 1990). However, each subtype of β subunit displays a characteristic pattern of expression in brain (Ymer *et al.*, 1989; Lolait *et al.*, 1989b; Mohler *et al.*, 1990); e.g., β1 transcripts are detected only in the granular cell layer of rat and bovine cerebella (Mohler *et al.*, 1990; Siegel, 1988). In addition to providing structural information for comparison with related receptor genes, the work described here represents a first step toward the characterization of β subunit gene expression. Future work will focus on those elements of the genes that control the distinct regional patterns of expression for different receptor subunits.

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REFERENCES

1. Baldini, A., and Ward, D. C. (1991). *In situ* hybridization banding of human chromosomes with *Alu*-PCR products: A simultaneous karyotype for gene mapping studies. *Genomics* **9:** 770–774.
2. Bates, G. P., MacDonald, M. E., Baxendale, S., Sedlacek, Z., Youngman, S., Romano, D., Whaley, W. L., Allitto, B. A., Poustka, A., Gusella, F., and Lehrach, H. (1990). A yeast artificial chromosome telomere clone spanning a possible location of the Huntington disease gene. *Am. J. Hum. Genet.* **46:** 762–765.
3. Blake, C. C. F. (1985). Exons and the evolution of proteins. *Int. Rev. Cytol.* **93:** 149–182.
4. Boulter, J., O'Shea-Greenfield, A., Duvoisin, R. M., Connolly, J. G., Wada, E., Jensen, A., Gardner, P. D., Ballivet, M., Deneris, E. S., McKinnon, D., Heinemann, S., and Patrick, J. (1990). α3, α5, and β4: Three members of the rat neuronal nicotinic acetylcholine receptor-related gene family form a gene cluster. *J. Biol. Chem.* **265:** 4472–4482.
5. Breathnach, R., and Chambon, P. (1981). Organization and expression of eukaryotic split genes coding for proteins. *Annu. Rev. Biochem.* **50:** 349–383.
6. Brigati, D. J., Myerson, D., Leary, J. J., Spalholz, B., Travis, S. Z., Fong, C. K. Y., Hsiung, G. D., and Ward, D. C. (1983). Detection of viral genomes in cultured cells and paraffin-embedded tissue sections using biotin-labeled hybridization probes. *Virology* **126:** 32–50.
7. Browning, M. D., Bureau, M., Dudek, E. M., and Olsen, R. W. (1990). Protein kinase C and cAMP-dependent protein kinase phosphorylate the β-subunit of the purified $GABA_A$ receptor. *Proc. Natl. Acad. Sci. USA* **87:** 1315–1318.
8. Buckle, V. J., Fujita, N., Ryder-Cook, A. S., Derry, J. M. J., Barnard, P. J., Lebo, R. V., Schofield, P. R., Seeburg, P. H., Bateson, A. N., Darlison, M. G., and Barnard, E. A. (1989). Chromosomal localization of $GABA_A$ receptor subunit genes: Relationship to human genetic disease. *Neuron* **3:** 647–654.
9. Buonanno, A., Mudd, J., and Merlie, J. P. (1989). Isolation and characterization of the β and ϵ subunit genes of mouse muscle acetylcholine receptor. *J. Biol. Chem.* **264:** 7611–7616.
10. Dalbadie-McFarland, G., Cohen, L. W., Riggs, A. D., Morin, C., Itakura, K., and Richards, F. H. (1982). Oligonucleotide-directed mutagenesis as a general and powerful method for studies of protein function. *Proc. Natl. Acad. Sci. USA* **79:** 6409–6413.
11. Gocayne, J., Robinson, D. A., FitzGerald, M. G., Chung, F.-Z., Kerlavage, A. R., Lentes, K.-U., Lai, J., Wang, C.-D., Fraser, C. M., and Venter, J. C. (1987). Primary structure of rat cardiac beta-adrenergic and muscarinic cholinergic receptors obtained by automated DNA sequence analysis: Further evidence for a mutigene family. *Proc. Natl. Acad. Sci. USA* **84:** 8296–8300.
12. Grenningloh, G., Rienitz, A., Schmitt, B., Methfessel, C., Zensen, M., Beyreuther, K., Gundelfinger, D. E., and Betz, H. (1987). The strychnine-binding subunit of the glycine receptor shows homology with nicotinic acetylcholine receptors. *Nature* **328:** 215–220.
13. Henikoff, S. (1987). Unidirectional digestion with exonuclease III in DNA sequence analysis. *In* "Methods in Enzymology" (R. Wu, Ed.), Vol. 155, pp. 156–165, Academic Press, San Diego.
14. Henikoff, S., and Eghtedarzadeh, M. K. (1987). Conserved arrangement of nested genes at the *Drosophila Gart* locus. *Genetics* **117:** 711–725.
15. Heuschneider, G., and Schwartz, R. D. (1989). cAMP and forskolin decrease γ-aminobutyric acid-gated chloride flux in rat brain synaptoneurosomes. *Proc. Natl. Acad. Sci. USA* **86:** 2938–2942.
16. Kirkness, E. F., Bovenkerk, C. F., Ueda, T., and Turner, A. J. (1989). Phosphorylation of γ-aminobutyrate (GABA)/benzodiazepine receptors by cyclic AMP-dependent protein kinase. *Biochem. J.* **259:** 613–616.
17. Lichter, P., Cremer, T., Borden, J., Manuelidis, L., and Ward, D. C. (1988). Delineation of individual human chromosomes in metaphase and interphase cells by *in situ* suppression hybridization using recombinant DNA libraries. *Hum. Genet.* **80:** 224–234.
18. Lichter, P., Tang, C.-J. C., Call, K., Hermanson, G., Evans, G. A., Housman, D., and Ward, D. C. (1990). High-resolution mapping of human chromosome 11 by *in-situ* hybridization with cosmid clones. *Science* **247:** 64–69.
19. Lolait, S. J., O'Carroll, A.-M., Kusano, K., Muller, J.-M., Brownstein, M. J., and Mahan, L. C. (1989a). Cloning and expression of a novel rat $GABA_A$ receptor. *FEBS Lett.* **246:** 145–148.
20. Lolait, S. J., O'Carroll, A.-M., Kusano, K., and Mahan, L. C. (1989b). Pharmacological characterization and region-specific expression in brain of the β2- and β3-subunits of the rat $GABA_A$ receptor. *FEBS Lett.* **258:** 17–21.
21. Luddens, H., Pritchett, D. B., Kohler, M., Killisch, I., Keinanen, K., Monyer, H., Sprengel, R., and Seeburg, P. H. (1990). Cerebellar $GABA_A$ receptor selective for behavioural alcohol antagonist. *Nature* **346:** 648–651.
22. Mohler, H., Malherbe, P., Draguhn, A., and Richards,

J. G. (1990). $GABA_A$-receptors: Structural requirements and sites of gene expression in mammalian brain. *Neurochem. Res.* **15:** 199–207.

23. NODA, M., FURUTANI, Y., TAKAHASHI, H., TOYOSATO, M., TANABE, T., SHIMIZO, S., KIKYOTANI, S., KAYANO, T., HIROSE, T., INAYAMA, S., AND NUMA, S. (1983). Cloning and sequence analysis of calf cDNA and human genomic DNA encoding α-subunit precursor of muscle acetylcholine receptor. *Nature* **305:** 818–823.
24. O'BRIAN, S. J. (Ed.) (1990). "Genetic Maps: Locus of Complex Genomes," Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
25. OLSEN, R. W., AND TOBIN, A. J. (1990). Molecular biology of $GABA_A$ receptors. *FASEB J.* **4:** 1469–1480.
26. OLSEN, R. W., AND VENTER, J. C. (Eds.) (1986). Benzodiazepine/GABA receptors and chloride channels: Structural and functional properties. *In* "Receptor Biochemistry and Methodology," Vol. 5, A. R. Liss, New York.
27. ROGERS, J. H. (1989). How were introns inserted into genes? *Trends Genet.* **5:** 213–216.
28. SAWRUK, E., HERMANS-BORGMEYER, I., BETZ, H., AND GUNDELFINGER, E. D. (1988). Characterization of an invertebrate nicotinic acetylcholine receptor gene: The *ard* gene of *Drosophila melanogaster*. *FEBS Lett.* **235:** 40–46.
29. SCHOFIELD, P. R., DARLISON, M. G., FUJITA, N., BURT, D. R., STEPHENSON, F. A., RODRIGUEZ, H., RHEE, L. M., RAMACHANDRAN, J., REALE, V., GLENCORSE, T. A., SEEBURG, P. H., AND BARNARD, E. A. (1987). Sequence and functional expression of the $GABA_A$ receptor shows a ligand-gated receptor superfamily. *Nature* **328:** 221–227.
30. SCHOFIELD, P. R., PRITCHETT, D. B., SONTHEIMER, H., KETTENMANN, H., AND SEEBURG, P. H. (1989). Sequence and expression of human $GABA_A$ receptor α1 and β1 subunits. *FEBS Lett.* **244:** 361–364.
31. SHIBARA, S., KUBO, T., PERSKI, H. J., TAKAHASHI, H., NODA, M., AND NUMA, S. (1985). Cloning and sequence analysis of human genomic DNA encoding γ subunit precursor of muscle acetylcholine receptor. *Eur. J. Biochem.* **146:** 15–22.
32. SIEGEL, R. E. (1988). The mRNAs encoding $GABA_A$/benzodiazepine receptor subunits are localized in different cell populations of bovine cerebellum. *Neuron* **1:** 579–584.
33. SOMMER, B., POUSTKA, A., SPURR, N. K., AND SEEBURG, P. H. (1990). The murine $GABA_A$ receptor δ subunit gene: Structure and assignment to human chromosome 1. *DNA Cell Biol.* **9:** 561–568.
34. STEPHENSON, F. A. (1988). Understanding the $GABA_A$ receptor: A chemically gated ion channel. *Biochem. J.* **249:** 21–32.
35. VERDOON, T. A., DRAGUHN, A., YMER, S., SEEBURG, P. H., AND SAKMANN, B. (1990). Functional properties of recombinant rat $GABA_A$ receptors depend upon subunit composition. *Neuron* **4:** 919–928.
36. WADA, K., BALLIVET, M., BOULTER, J., CONNOLLY, J., WADA, E., DENERIS, E. S., SWANSON, L. W., HEINEMANN, S., AND PATRICK, J. (1988). Functional expression of a new pharmacological subtype of brain nicotinic acetylcholine receptor. *Science* **240:** 330–334.
37. YMER, S., DRAGUHN, A., WISDEN, W., WERNER, P., KEINANEN, K., SCHOFIELD, P. R., SPRENGEL, R., PRITCHETT, D. B., AND SEEBURG, P. H. (1990). Structural and functional characterization of the γ1 subunit of $GABA_A$/benzodiazepine receptors. *EMBO J.* **9:** 3261–3267.
38. YMER, S., SCHOFIELD, P. R., DRAGUHN, A., WERNER, P., KOHLER, M., AND SEEBURG, P. H. (1989). $GABA_A$ receptor β subunit heterogeneity: Functional expression of cloned cDNAs. *EMBO J.* **8:** 1665–1670.