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Differential Expression of γ-Aminobutyric Acid$_A$ Receptor Subunits

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SUMMARY

A 1.8-kilobase (kb) cDNA clone for a β_1 subunit of the human γ-aminobutyric acid$_A$ (GABA$_A$) receptor has been isolated and sequenced. The longest open reading frame of the clone, pCLL610, contains nucleotide sequence encoding a portion of the putative signal sequence followed by 449 amino acids of the entire mature protein. The deduced amino acid sequence of pCLL610 differs from a recently described human β_1 subunit by a single amino acid. The amino acid sequences of the human GABA$_A$ receptor β_1 subunits share 98% identity with the β_1 subunits of the bovine and rat GABA$_A$ receptor, with the majority of the differences occurring in the intracellular loop between the M3 and M4 transmembrane regions of the protein. A single 11-kb transcript is observed in Northern blots of poly(A)$^+$ RNA isolated from rat brain probed with nick-translated pCLL610. In human brain, the pCLL610 probe recognized the 11-kb message, in addition to two other bands between 8 and 11-kb. Developmental studies of rat brain mRNA show that the message of the GABA$_A$ β_1 subunit is highest at birth, rapidly decreases, and reaches adult levels by 5 to 7 days of age. This is in contrast to the development of the α_1 subunit, which is low from days 1 to 5 and increases to adult levels by days 14 to 25. Relative levels of the mRNA for the α_1 and β_1 subunits vary among rat brain regions. The levels of mRNA for the α_1 subunit are similar in the cortex, hippocampus, and midbrain, whereas cerebellar levels are twice those in the cortex. The rank order of the relative amount of β_1 subunit message is hippocampus > cortex = midbrain > cerebellum. These data, taken with our previous study of the α_1 subunits of the GABA$_A$ receptor, suggest that the differences in the distribution and regulation of the α_1 and β_1 subunits may reflect a variety of subunit combinations forming the GABA$_A$ receptor. Heterogeneity in the GABA$_A$ receptor composition may provide a molecular basis for the diverse pharmacological properties associated with this receptor.

The GABA$_A$ receptor is a ligand-gated anion channel that is the site of action for several classes of important therapeutic agents (1). Benzodiazepines and barbiturates bind to unique sites on this multimeric complex and allosterically facilitate GABA-induced chloride conductance (2). The actions of these drugs on the GABA$_A$ receptor are thought to lead to their numerous pharmacological properties, which include anticonvulsant, anxiolytic, sedative, and hypnotic activities.

cDNAs for the multiple subtypes of α, β, and γ subunits (α_1–α_3, β_1–β_3, γ_1 and γ_2) (3–10) have been cloned. GABA-induced chloride conductance and the pentobarbital potentiation of the GABA response were observed in *Xenopus* oocytes injected with mRNA for α and β subunits, either singly or in combination (3, 4, 6, 10, 11). The potentiation of the GABA response by benzodiazepines, however, was only consistently observed when the γ_2 subunit was expressed with the α and β subunits (10).

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Compounds that bind to specific sites on the GABA$_A$ receptor complex have a variety of pharmacological effects. The particularly diverse pharmacological profile of ligands that interact with the benzodiazepine binding site led to the concept of benzodiazepine receptor subtypes (12) and the corollary, subtypes of GABA$_A$ receptors. The discovery of multiple forms of the α, β, and γ subunits suggests that the pharmacological heterogeneity of the GABA$_A$ receptor may be due, in part, to different forms of these subunits included in the receptor (4). In order to begin assessing the potential variations of the GABA$_A$ receptor composition, we have cloned a cDNA for a β_1 subunit of the human GABA$_A$ receptor and have used this clone to quantify the distribution of the β_1 subunit mRNA in rat brain and to determine the developmental profile of the message.

Materials and Methods

Library screening. Oligonucleotide probes were designed based on the nucleotide sequence of the β subunit of the bovine GABA$_A$ receptor (3). Our previous study showed a high degree of homology of the

ABBREVIATIONS: GABA, γ-aminobutyric acid; SSC, saline sodium citrate; MOPS, 3-(*N*-morpholino)propane sulfonic acid; kb, kilobase.

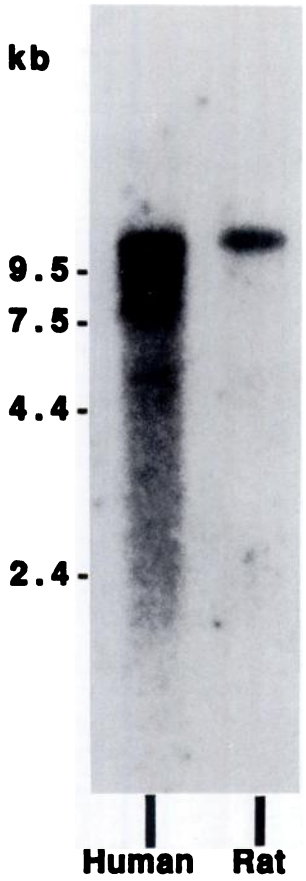


Fig. 1. Northern blot analysis of human and rat brain mRNA. Poly(A)$^+$ RNA was extracted from a section of human hippocampus and parahippocampal cortex or rat cerebral cortex. Aliquots (3 μg) of poly(A)$^+$ RNA were fractionated on a 1% agarose gel, transferred to nitrocellulose paper, and hybridized with ^{32}P-labeled pCLL610, as described in Materials and Methods. RNA size markers are shown on the left (kb).

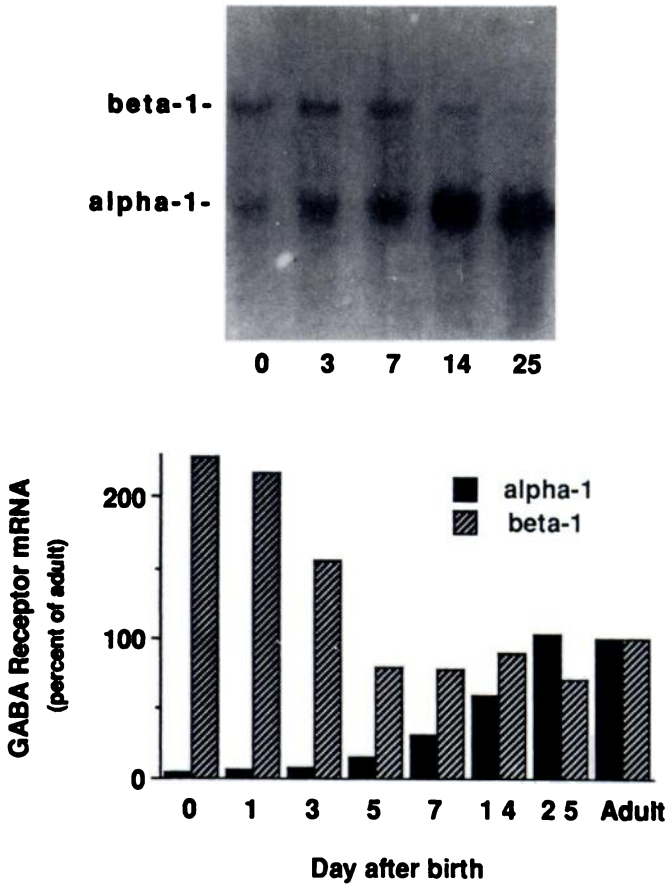


Fig. 2. Development of GABA$_A$ receptor α_1 and β_1 mRNA in rat whole brain. RNA was extracted from rat whole brain at varying times after birth and 3 μg of poly(A)$^+$ RNA were fractionated on a 1% agarose gel and transferred to nitrocellulose paper. *Upper*, example of a Northern blot hybridized with ^{32}P-labeled pCLL610 and pCLL800 (α_1 probe) (7). The filters were then stripped and hybridized with ^{32}P-labeled oligo(dT), as described in Materials and Methods, to quantitate the relative amount of mRNA in each sample. The autoradiograms were quantitated by densitometry and standardized to the level of oligo(dT). *Lower* means of two to four experiments expressed as the percentage of adult levels of α_1 or β_1 mRNA.

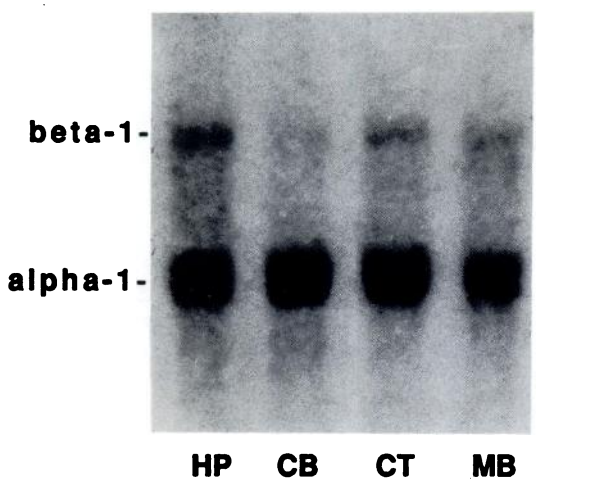
nucleotide sequence between the human and bovine GABA$_A$ receptor α_1 subunit (88%) (7); therefore, we used the exact bovine nucleotide sequence for our probes. Three probes (βU1, βU2, and βU3) were targeted for unique regions of the β subunit, whereas three probes (βC1, βC2, and βC3) were directed to areas of the β subunit that had significant amino acid homology with the α subunit. The oligonucleotides were synthesized using an Applied Biosystems 380B DNA synthesizer, utilizing β-cyanoethyl phosphoramidite chemistry, and were purified by preparative acrylamide gel electrophoresis.

βU1 GACTTTGGAG GGCCCCCCGT GGACGTCGGG ATGAGGATCG ATGTCGCCAG CATAGACATG
βU2 ATCGATGACA TTGAGTTTTA CTGGAATGGA GGAGAGGGCG CAGTAACAGG AGTGAATAAA ATTGAG
βU3 AACGAGACGA GCGGCTCTGA AGTGCTCACG GGCGTGGGAG ACCCCAAGAC CACCATGTAC
βC1 TTCATTTTGC AAACCTACAT GCCTTCCACA CTGATTACAA TTCTGTCGTG GGTGTCGTTT
βC2 GCAGCCAGAG TTGCACTAGG AATCACCACA GTGCTGACAA TGACCACCAT CAGCACTCAC
βC3 TGGTCCCGAA TGTTCTTCCC CATCACCTTT TCTCTTTTTA ACGTCGTTTA TTGGCTTTAC

A λgt11 cDNA library, constructed from a 7-year-old human cerebellum, was obtained from the American Type Culture Collection (Kamholz, Puckett and Lazzarini; ATCC No. 37435). The six oligonucleotides were pooled to screen 6.5 × 10^5 recombinant λgt11 plaques. The nitrocellulose plaque-lifts were hybridized for 18 hr at 62° in hybridizing solution containing 2× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) 10× Denhardt's (1× Denhardt's = 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 0.2% Ficoll 400), 1.7 mg/ml yeast RNA and 0.15 mg/ml salmon sperm DNA, with oligonucleotide probes end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Following hybridization, the filters were washed twice at room temperature with 2× SSC for 15 min each and then once with 2× SSC at 62° for 30 min to remove the nonspecifically bound radioactivity. The filters were dried and placed with Kodak X-OMAT AR film for 24 hr. Positive clones were identified and plaque purified. The positive λgt11 clones were digested with *Eco*RI, and the restriction fragments were purified on a 1% agarose gel and then ligated into a pBluescript KS(+) phagemid vector (Stratagene) that had been digested with *Eco*RI. The subclones were transformed into XL1-Blue bacteria (Stratagene) for subsequent manipulation.

DNA sequencing. Plasmid DNA was isolated using the alkaline lysis method and was purified by CsCl isopycnic gradient ultracentrifugation (13). Both strands of the cDNA were sequenced using the dideoxy chain termination method of Sanger *et al.* (14) using [α-^{32}P] dATP and multiple oligonucleotide primers.

RNA preparation. Human and Sprague-Dawley rat brain RNA were isolated using the method of Chirgwin *et al.* (15). Human tissue was obtained from the Department of Neurosurgery, Yale University School of Medicine, after partial resection of anterior hippocampus and parahippocampal cortex from epileptic patients. Rats were sacrificed by decapitation, and the brains were quickly removed to ice and dissected. Tissues were homogenized in 4 volumes of ice-cold 4 M guanidine isothiocyanate, 25 mM sodium acetate, pH 6.0, 0.5% β-mercaptoethanol. The homogenate was layered on a 5.7 M CsCl cushion and centrifuged at 150,000 × *g* for 20 hr at 20°. The RNA pellets were



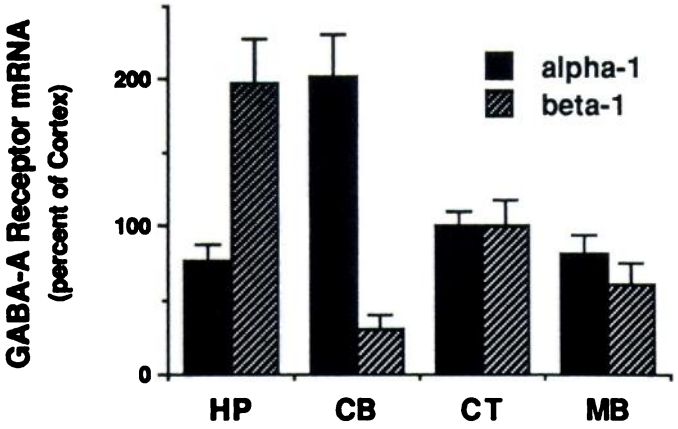


Fig. 3. Regional distribution of $GABA_A$ receptor and α_1 and β_1 subunit mRNA in rat brain. RNA was extracted from rat cerebral cortex (*CT*), hippocampus (*HP*), cerebellum (*CB*), and midbrain (*MB*). Poly(A)$^+$ RNA (3 μg) was fractionated on a 1% agarose gel and transferred to nitrocellulose paper. The Northern blots (*upper*) were probed with ^{32}P-labeled pCLL800 (α_1 subunit) and pCLL610 (β_1 subunit). The blots were then stripped and reprobed with a ^{32}P-labeled probe for β-actin, to quantitate the relative levels of mRNA in each sample. Autoradiograms of each blot were quantitated by densitometry and standardized to the levels of β-actin mRNA. The results (*lower*) are expressed as a percentage of the level of α_1 or β_1 signal in the cerebral cortex samples and are the mean ± standard error of three to five experiments.

resuspended in 0.3 M sodium acetate (pH 6.0), precipitated with 2.5 volumes of ethanol, and washed with 80% ethanol. The RNA pellets were resuspended in sterile water and poly(A)$^+$ RNA was purified by oligo(dT)-cellulose affinity chromatography (16). The yield of total RNA was approximately 0.4 mg/g of wet brain tissue, of which approximately 5% was poly(A)$^+$ RNA.

Northern blots. Poly(A)$^+$ RNA was separated on 1% agarose gels (containing 0.66 M formaldehyde, 0.2 M MOPS, pH 7.0, 50 mM sodium acetate, and 10 mM EDTA), and the RNA was transferred to nitrocellulose membranes (13). The Northern blots were hybridized at 42° for 16 hr in hybridization solution (2× SSC, 1× Denhardt's, 0.02 mg/ml salmon sperm DNA, 10 mM Tris, pH 7.4) containing 40% formamide, and ^{32}P-labeled probes generated by random priming with [α-^{32}P]dATP (Amersham). Either filters were hybridized sequentially with the α and β subunit clones or the hybridizations were carried out with both probes at the same time. These two procedures yielded equivalent results. The filters were washed three times for 20 min each in 2× SSC, 0.1% sodium dodecyl sulfate, at room temperature, followed by two washes for 20 min each with 0.1× SSC, 0.1% sodium dodecyl sulfate, at 55° to remove the nonspecifically bound radioactivity. The blots were dried and autoradiographed, and the relative densities of the positively hybridized bands were quantified with a Soft-laser densitometer. Although this method of quantitation does not yield absolute values, it is valid and reproducible when data obtained within a single blot are compared.

The amount of RNA applied to the gels was quantified by reprobing the filters with ^{32}P-end-labeled oligo(dT). Nitrocellulose filters were hybridized with 40 pmol of labeled oligo(dT)/40-cm^2 filter paper, in 5× SSC and 5× Denhardt's, for 2 hr at room temperature. The filters were washed four times for 5 min each with 2× SSC. The nitrocellulose papers were autoradiographed, and the amount of bound oligo(dT) was quantified by liquid scintillation counting of excised lanes. In some experiments, the amount of RNA on gels was standardized to the levels of β-actin mRNA, which was determined by hybridization of the nitrocellulose filters with a [α-^{32}P]-labeled β-actin and quantitation of the resulting bands. This method could not be used for the developmental studies, because the level of β-actin varies during early postnatal development (7).

Computer models. Nucleotide and amino acid sequences were analyzed and compared using the molecular biology computer package INTELLIGENETICS (Mountain View, CA).

Results

A human cerebellar cDNA library was screened for a β_1 subunit of the $GABA_A$ receptor with six oligonucleotide probes, and three plaques were found to hybridize with the pooled probes. These clones were plaque purified and screened with two groups of probes, a 5′ group (βU1, βU2, and βC1) and a 3′ group (βC2, βC3, and βU3). One clone hybridized with only the 5′ group of probes and two clones hybridized with both the 5′ and 3′ probes. The two clones that were positive with both the 5′ and 3′ probes were digested with *Eco*RI. One of the clones contained an internal *Eco*RI site and the digestion produced 1.3-kb and 0.5-kb fragments. This clone was partially digested with *Eco*RI to give a 1.8-kb fragment, which was ligated into the *Eco*RI site of a pBluescript KS(+) vector and designated pCLL610. Sequencing of pCLL610 revealed that the cDNA insert was 1797 nucleotides long, with 1368 in the longest open reading frame and 429 nucleotides in the 3′ noncoding region. The amino acid sequence of the longest open reading frame did not contain an amino terminal methionine.

To estimate the number of messages that could potentially encode the $GABA_A$ β_1 subunit, we made Northern blots from poly(A)$^+$ RNA isolated from human tissue (hippocampus and parahippocampal cortex) and rat cortex. Hybridization of the Northern blot with ^{32}P-random-primed pCLL610 showed a single band of approximately 11-kb in both tissues (Fig. 1). In addition to this 11-kb band, in the human samples pCLL610 also recognized two other mRNA bands between 8- and 11-kb.

The development of the message for the β_1 subunit was studied by preparing Northern blots of poly(A)$^+$ RNA isolated from rat whole brain at varying times after birth. A single 11-kb transcript was observed at all times studied. There was a time-dependent decrease in the levels of mRNA that hybridized with the β_1 clone; the signal was highest at birth, decreased rapidly, and reached adult levels by 5 to 7 days of age (Fig. 2). When the same blots were analyzed for levels of α_1 mRNA, the levels of this subunit message were found to be low during early postnatal development and increased to adult levels between 14 and 25 days, as previously described (7).

The regional distribution of the message for the α_1 and β_1 subunits was determined by Northern blot analysis of poly(A)$^+$ RNA isolated from various regions of rat brain and probing

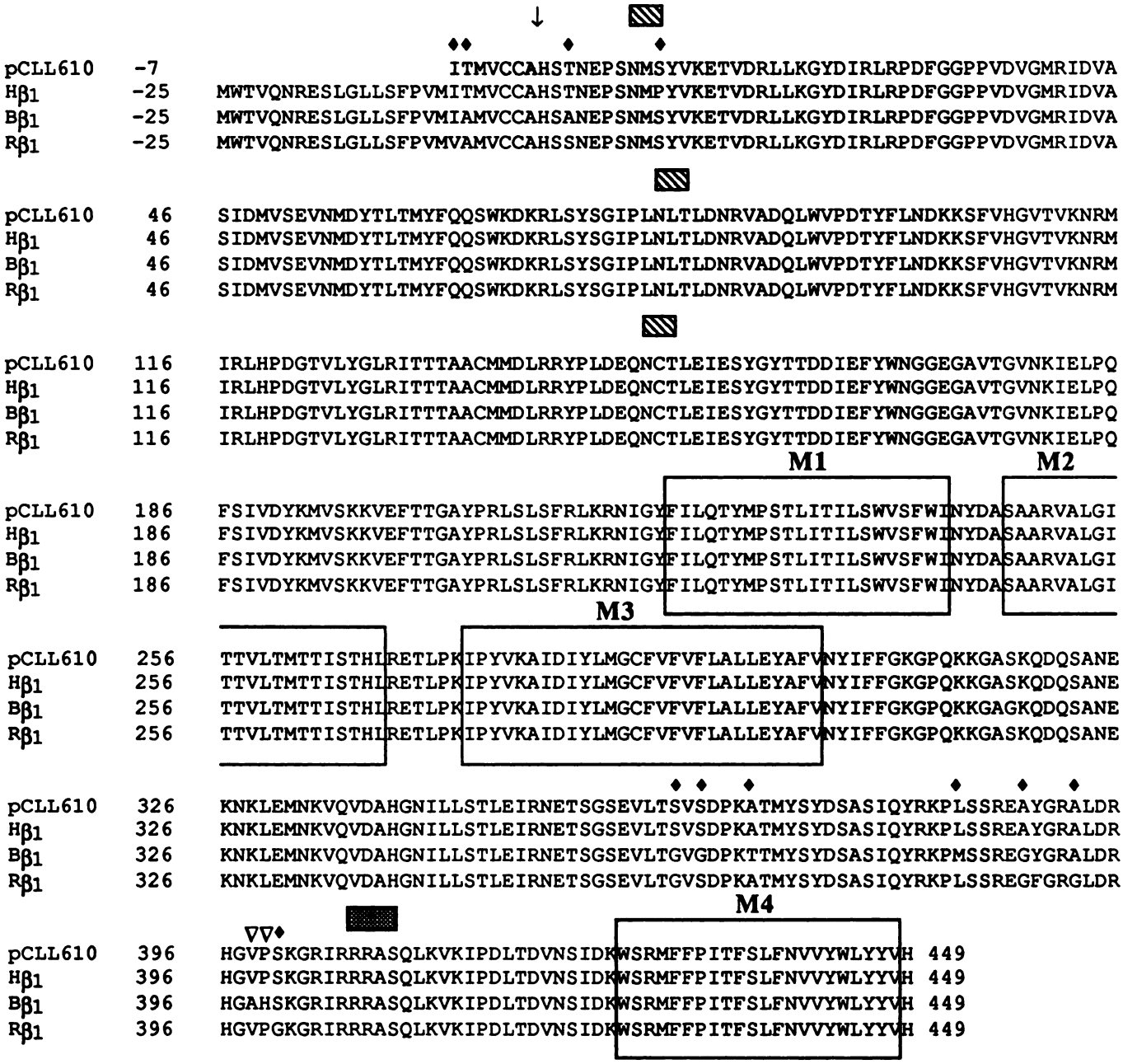


Fig. 4. Comparison of the deduced amino acid sequences for pCLL610, the human GABA$_A$ receptor β_1 subunit (Hβ_1; Ref. 9), bovine GABA$_A$ receptor β_1 subunit (Bβ_1; Ref. 3), and rat GABA$_A$ receptor β_1 subunit (Rβ_1; Ref. 5). *Boxed areas*, the predicted transmembrane regions M1–M4. ■, predicted *N*-glycosylation sites; ■, proposed cAMP-dependent phosphorylation site. ♦, conserved amino acid substitutions; ∇, divergent amino acid substitutions.

with ^{32}P-random-primed clones of the human GABA$_A$ receptor α_1 (pCLL800) (7) and β_1 subunits. Northern blots hybridized with the β_1 subunit probe produced a single 11-kb band in the cerebral cortex, hippocampus, midbrain, and cerebellum. Reprobing the blots with α_1 subunit probe resulted in a 3.9/4.3-kb doublet, both bands being of equal intensity in all four brain regions. Signals from the Northern blots were quantified using β-actin as an internal control. Analysis of these signals indicated that the amount of mRNA for the α_1 and β_1 subunits varied among the four rat brain regions studied (Fig. 3). The amount of α_1 mRNA was found to be similar among the cortex, hippocampus, and midbrain, but the level of α_1 mRNA in the cerebellum was approximately double that seen in the cortex. The relative levels of β_1 message were greatest in the hippocampus, followed by the cortex and midbrain, and the lowest amount of signal was found in the cerebellum (Fig. 3).

Discussion

We have isolated and sequenced a cDNA clone encoding a β subunit of the human GABA$_A$ receptor. pCLL610 is missing a start codon for the longest open reading frame; however, comparison of this sequence with a human β_1 subunit cDNA (9) suggests that pCLL610 contains the nucleotide sequence that encodes the last seven amino acids of the putative signal sequence and the nucleotide sequence encoding the entire mature protein. pCLL610 and the human β_1 subunit isolated by Schofield *et al.* (9) differ by five nucleotides, which results in a

variation of a single amino acid (Fig. 4); at residue 10, pCLL610 contains a serine where a proline occurs in the β_1 subunit. We isolated a second independent cDNA clone of the β_1 subunit of the human $GABA_A$ receptor, which also encodes for a serine at this position. It is unlikely that this single conservative substitution would alter the function of the subunit. Therefore, we suggest that pCLL610 encodes a human β_1 subunit and the difference between this clone and the β_1 clone isolated by Schofield *et al.* (9) may be due to multiple alleles of the human β_1 subunit. The human, bovine, and rat β_1 subunits of the $GABA_A$ receptor display a high degree of homology of the receptor among the three species (98% identity) (Fig. 4), with the majority of the differences lying in the intracellular loop between the M3 and M4 transmembrane regions. Of the nine substitutions, seven are conserved and two are nonconserved. At amino acid 398 a valine in the human and rat subunits is substituted by an alanine in the bovine subunit, and at amino acid 399 a proline appears in the human and rat subunits where a histidine occurs in the bovine subunit. This intracellular loop is the most divergent region between the α, β, and γ subunits (10) and among the multiple bovine α subunits (4). These amino acid substitutions do not affect the predicted general architecture of the β subunit. The putative extracellular disulfide β structural loop, three *N*-linked glycolsylation sites, and the cAMP-dependent phosphorylation site reported in the bovine β_1 subunit (3) also occur in the human sequence. The functional significance of the differences in the amino acid sequences will be determined when the physiology and pharmacology of various β_1 subunits are compared.

Significant amounts of mRNA for the $GABA_A$ receptor α and β subunits are present in both human and rat brain tissue. In agreement with previous studies (4, 7, 8, 17), Northern blots hybridized with α_1 subunit probes show two bands (3.9/4.3 kb). The 3.9- and 4.3-kb bands observed in rat brain regions using the α_1 subunit probe are similar in size to the message for the bovine α_3 and α_1 subunits, respectively (4). Blots hybridized with the β_1 probe show a single high molecular weight transcript (11-kb) in rat brain tissue and a predominant band of approximately the same size in the human tissue. These bands are similar in size to those observed by Ymer *et al.* (5) using β_1-specific oligonucleotides. In addition, the β_1 probe hybridization resulted in labeling of two other bands in human hippocampus and parahippocampal cortex. Multiple forms of the β_1 subunit mRNA have also been described in bovine brain (5) and it has been suggested that these forms may be due to different polyadenylation sites, which could produce varying lengths of the 3′ untranslated sequence.

The ontogeny of the mRNA for the β_1 subunit varies significantly from that of the α subunit and the [^{3}H]GABA binding site. The levels of β_1 subunit mRNA are highest at birth and decrease rapidly to adult levels by 5 to 7 days after birth. This developmental profile differs from those of the α_1 subunit and [^{3}H]GABA binding sites, which are both low at birth and increase to adult levels by 21 days of age (7, 18), suggesting that the α_1 and β_1 subunits are under different developmental regulation.

The relative amounts of the α_1 and β_1 subunit mRNAs hybridizing with our probes vary among the four rat brain regions examined. The differences in the blotting efficiencies of the 3.9/4.3- and 11-kb bands in our Northern blots prohibit us from rigorously comparing the ratios of the α_1 and β_1 messages among the brain regions; however, the relative distribution of the individual subunits was accurately assessed. Levels of the α_1 subunit message in the cerebellum are approximately twice that observed in the cortex, whereas mRNA levels are similar among the cerebral cortex, hippocampus, and midbrain. The relative distribution of the β_1 subunit message is more complex, with hippocampus > cortex = midbrain > cerebellum. The distribution of message for both subunits also differs from the relative regional distribution of the GABA binding sites. Studies examining the localization of [^{3}H]GABA and [^{3}H]muscimol binding have shown that the levels of these sites are highest in the cerebellum > cortex > hippocampus = midbrain (19–22). These data, along with *in situ* hybridization studies in the cerebellum (10, 17), suggest that the stoichiometry of the $GABA_A$ receptor subunits is more complex than the 2α:2β (3, 23) or α_1:β_1:γ_2 (10) models previously proposed.

The $GABA_A$ receptor complex contains a binding site for benzodiazepines, and previous pharmacological studies suggested that several subtypes of benzodiazepine binding sites exist in the central nervous system (for review, see Ref. 24). Differences in the relative distribution and ontogeny of the α_1 and β_1 subunits indicate that the combination of these subunits in $GABA_A$ receptors may vary, producing several subtypes of receptors. Heterogeneity in the ensemble of subunits forming the $GABA_A$ receptor could provide the molecular foundation for the pharmacological subtypes of $GABA_A$ receptors and may account for the diverse actions of agents that interact at this receptor.

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