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Distinctive Patterns of $GABA_A$ Receptor Subunit mRNAs in 13 Cell Lines

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We have investigated the $GABA_A$ receptor mRNA composition in 13 cell lines, using 13 subunit-specific oligo-primers (α1-6, β1-3, γ1-3, and δ) and reverse transcriptase PCR amplification. Cell lines (B35, B65, B103, B104, RINm5F, Rat1, PC12, C6, C17, C27, βTC3, NB41A3, AtT-20), derived from diverse tissue origins, were investigated in order to identify homogeneous cellular sources with distinctive $GABA_A$ receptor subunits. Fifteen $GABA_A$ receptor subunits have been cloned from mammalian tissue (those listed above plus the retinal subunits ρ1 and ρ2). This multiplicity of $GABA_A$ receptor subunits underlies the diverse pharmacology of the $GABA_A$ receptor. Attempts to understand the regulation and pharmacology of individual subunits and of the heterooligomeric receptor combinations have been impeded by a lack of pure populations of cells expressing $GABA_A$ receptor subunits. Permanent cell lines provide such a resource. Each $GABA_A$ receptor subunit mRNA, α1-5, β1-3, γ1-3, and δ, was detected in at least one cell line. All cell lines examined contained detectable levels of at least one $GABA_A$ receptor subunit mRNA. Each cell line contained distinctive combinations of subunit mRNAs. None of the cell lines examined contained detectable amounts of α6 mRNA. These cell lines, which transcribe $GABA_A$ receptor subunit mRNAs, provide useful cellular sources for transcriptional and pharmacological studies. Our data also suggest that endogenous $GABA_A$ receptor subunit mRNAs may be present in cells that are routinely used for transfection studies, and that this expression might confound interpretation of the studies. In the following companion article, we have looked for functional $GABA_A$ receptor Cl^- ion channels in these cell lines, using the patch-clamp technique (Hales and Tyndale, 1994).

[Key words: $GABA_A$ receptors, PCR, cell lines, neuroblastomas, NB41A3, C6, AtT-20, PC12, RINm5F, βTC3, fibroblasts]

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Cell lines allow the study of a homogeneous population of cells, thus permitting the examination of such issues as the influence of drug and growth factor treatments on protein and mRNA levels, as well as providing a tissue source for studies of transcriptional regulation (von Blankenfeld et al., 1990). While cell lines may or may not accurately represent *in vivo* cell types, they offer many advantages for the study of protein and mRNA regulation, subunit assembly, and pharmacology. Many investigators have used permanent cell lines to study the function and regulation of neurotransmitter receptors (e.g., Lukas, 1989; Hales et al., 1992).

GABA type A ($GABA_A$) receptors are ligand-gated Cl^- channels modulated by a variety of drugs (Burt and Kamatchi, 1991; Hales and Olsen, 1994). They are found in the CNS and the periphery, both in neuronal and non-neuronal cell types (Bormann and Clapham, 1985; Bormann and Kettenmann, 1988; Olsen and Tobin, 1990).

Studies of the purified nicotinic ACh receptor and $GABA_A$ receptor proteins suggest that the $GABA_A$ receptor is a pentameric membrane-spanning channel of assorted subunit composition (Anand et al., 1991; Olsen and Tobin, 1990). To date, 15 mammalian $GABA_A$ receptor subunits have been cloned and expressed (α1-6, β1-3, γ1-3, δ, and ρ1-2; for review, see Tyndale et al., 1994). Evidence for heterogeneity of the $GABA_A$ receptor complex arises from immunohistochemical, *in situ* hybridization and ligand binding techniques, as well as autoradiography, immunochemical and immunoprecipitation studies (Olsen et al., 1990; Houser, 1991; McKernan et al., 1991; Wisden et al., 1992; Bureau and Olsen, 1993; Endo and Olsen, 1993; Mertens et al., 1993). The identities of the individual subunits that combine *in vivo* to provide this heterogeneity, and their respective $GABA_A$ receptor pharmacology are unknown. These combinations have not been identified, partly because of the difficulty in identifying pure populations of homogeneous cells in which to assess subunit composition.

Functional $GABA_A$ receptors have been reported in only two cell lines, the murine hypothalamic GT1-7 cell line and the human IMR-32 neuroblastoma cell line (Hales et al., 1992; Anderson et al., 1993; Noble et al., 1993). In addition, the β3 subunit mRNA has been detected in several cell lines of diverse tissue origin (Kirkness and Fraser, 1993).

The cell lines were chosen on the basis that their disparate tissue origins might provide distinct subunit patterns. We investigated eight cell lines derived from the CNS. The NB41A3 are a murine neuroblastoma cell line, while the B35, B65, B103, and B104 cells, derived from nitrosoethylurea-induced rat brain

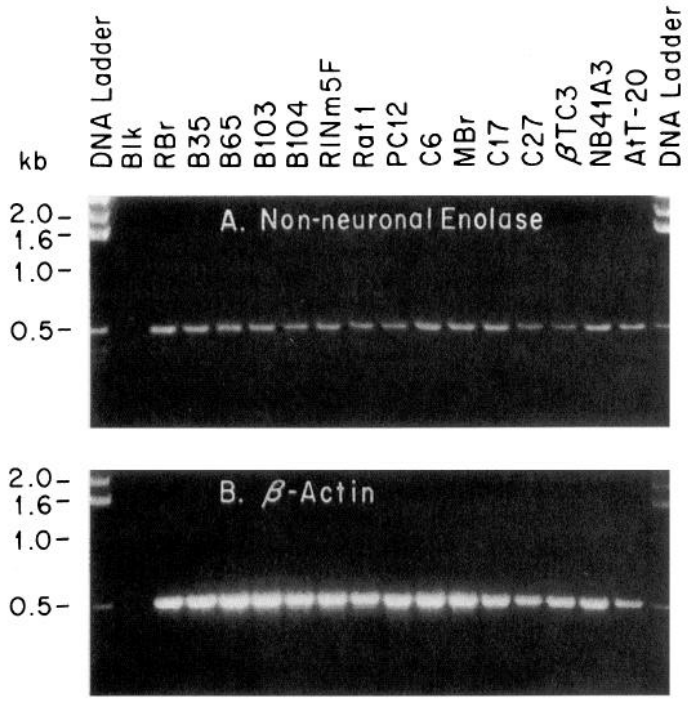

Figure 1. PCR products from control primers. PCR products using (*A*) non-neuronal enolase (25 cycles) and (*B*) β-actin (25 cycles) primers with templates from cDNA synthesis blanks (*Blk*), rat brain (*RBr*), mouse brain (*MBr*), and 13 cell lines; 1 kb DNA ladders were run in the outer lanes.

tumors, are thought to be neuronal in that they can produce regenerative action potentials and contain enzymes for neurotransmitter synthesis (Schubert et al., 1974). The C17 and C27 cell lines were obtained from murine cerebellar tissue with an immortalizing oncogene in a retroviral vector (Ryder et al., 1990). They were of interest since they might transcribe the exclusively cerebellar α6 subunit (Wisden et al., 1992). Recent reports have identified $GABA_A$ receptors in glial cells; therefore, we investigated the C6 cell line, which was cloned from a rat glioma induced by *N*-nitrosomethylurea (Benda et al., 1968; Bormann and Kettenmann, 1988; Hösli et al., 1990; Bovolin et al., 1992).

We also investigated seven cell lines derived from the periphery. The pituitary contains functional $GABA_A$ receptors, which appear to be involved in the regulation of hormone secretion (Jones et al., 1992; Valerio et al., 1992; Vincens et al., 1992). We therefore investigated the AtT-20 cell line, which secrets ACTH, and was derived from a mouse pituitary tumor (Buonassisi et al., 1962). We studied the PC12 cell line, a rat adrenal pheochromocytoma, because of the existence of $GABA_A$ receptors in the adrenal medulla (Greene and Tischler, 1976; Bormann and Clapham, 1985; Peters et al., 1989; Hales and Tyndale, 1994). The endocrine pancreas contains functional $GABA_A$ receptors, which prompted our investigation of the RINm5F and βTC3 cell lines (Rorsman et al., 1989). The RINm5F cell line is derived from a rat pancreatic islet cell tumor, while the βTC3 cell line is an insulinoma cell line derived from a transgenic mouse into which an SV40 T-antigen gene, linked to the insulin promotor, had been introduced (Gazdar et al., 1980; Efrat et al., 1988). We also examined a cell line in which we did not expect to detect $GABA_A$ receptor mRNAs, the Rat1 fibroblast cell line.

Materials and Methods

Tissue culture. Cells were cultured in 75 cm^2 tissue culture flasks at 37°C in 5% CO_2, 95% air and 100% humidity. NB41A3 cells from the American Type Culture Collection (ATCC, CCL 147) were grown in Ham's F-10 medium supplemented with 2.5% (v/v) fetal calf serum (FCS), 15% (v/v) horse serum (HS). AtT-20 (ATCC CCL 89), B35, B65, B103, B104, Rat1, and PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM; 4.5 gm/liter glucose) supplemented with 10% (v/v) FCS. C17 and C27 cells were grown in DMEM (4.5 gm/liter glucose) supplemented with 10% (v/v) FCS, 5% (v/v) HS, and 2 mM glutamine. βTC3 cells were grown in DMEM (4.5 gm/liter glucose), supplemented with 2.5% (v/v) FCS and 15% (v/v) HS. RINm5F cells were grown in RPMI medium 1640 supplemented with 10% (v/v) FCS. C6 (ATCC CCL 107; clonal line:C62BD) cells were grown in 45% DMEM (1 gm/liter glucose) and 45% Ham's F-12, supplemented with 10% (v/v) FCS. In addition, all culture media contained 5×10^4 IU/liter penicillin and 50 mg/liter streptomycin. All tissue culture materials were obtained from GIBCO–Bethesda Research Labs (Gaithersburg, MD).

RNA isolation and cDNA synthesis. At approximately 80% confluency, culture media were aspirated and cells were washed three times in phosphate-buffered saline. Total cellular RNA was extracted directly from the flasks using the acid guanidium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). First-strand cDNA synthesis reactions (500 μl) contained reaction buffer (GIBCO–Bethesda Research Labs), 10 μg of total cellular RNA, 3.1 U of random hexamers (Pharmacia, Uppsala, Sweden), 10 U of RNasin (Promega, Madison, WI), 10 mM DTT, 2000 U of reverse transcriptase (M-MLV; GIBCO–Bethesda Research Labs), and 0.5 mM nucleotide triphosphates (Pharmacia). For an estimation of yield, 9.5 μl of the reaction mixture was removed and 0.5 μl of ^{32}P-dCTP (Amersham, Arlington Heights, IL) was added. The reaction mixtures were incubated in parallel at 37°C for 2 hr. cDNA-incorporated ^{32}P-dCTP was separated from free label by NICK column (Pharmacia) chromatography. cDNA yield was calculated following scintillation counting. Control blanks for cDNA synthesis contained no RNA. Paired flasks of cells were grown for the RNA isolation and PCR studies presented in this article and the electrophysiological studies that are presented in the following companion article (Hales and Tyndale, 1994).

Polymerase chain reaction amplification. For polymerase chain reaction (PCR) amplifications, samples (100 μl) were brought to final concentrations of (in mM) 10 Tris (pH 8.3), 50 KCl, 2.5 $MgCl_2$, 0.5 nucleotide triphosphate, 4% DMSO, 50 pmol of both the forward and reverse primers, and 30 ng of cDNA template. After heating at 95°C for 5 min, 1.0 U of Amplitaq DNA polymerase (Perkin Elmer, Branchburg, NJ) was added and the samples were overlaid with 80 μl of mineral oil. An initial amplification cycle was run that consisted of denaturing at 94°C for 1 min, annealing at 55°C for 2 min, and extending at 72°C for 2 min in a Coy Thermal Cycler. PCR was then carried out for 30 and 40 cycles, where each cycle consisted of denaturing at 94°C for 45 sec, annealing at 55°C for 60 sec, and extending at 72°C for 60 sec. After PCR, the DNA products were analyzed by electrophoresis in 1% agarose and 2% NuSieve GTG agarose (FMC, Rockland, ME) gels containing 0.5 μg/ml ethidium bromide. Oligonucleotide primer sequences (α1-6, β1-3, γ1-3, δ, non-neuronal enolase, and β-actin) and locations can be found in Table 1. The ρ subunits were not investigated as they differ from the other $GABA_A$ receptor subunits in that they (1) have been found predominantly in retina and (2) are insensitive to bicuculline and barbiturates (Cutting et al., 1991; Shimada et al., 1992).

Southern blotting. Electrophoretically separated DNA products were denatured, transferred to Zeta-Probe membranes (Bio-Rad, Richmond, CA), and prepared for screening according to the manufacturer's instructions. The membranes were hybridized at 42°C with ^{32}P random-primed cDNA probes for 15 hr in hybridization buffer (50% formamide, 5× Denhart's, 5× SSPE, 0.1% SDS, 100 μg/ml salmon sperm DNA). The membranes were washed in 0.15× SSC and 0.1% SDS at 25°C for 20 min, followed by four washes at 65–75°C for 30 min each. Exposure times varied from 10 min to 1 hr.

Control studies. PCR amplification was carried out at 40 cycles with 30 ng of template in order to determine which subunits were transcribed in the cell lines. In preliminary studies 40 cycles was deemed appropriate based on the following: (1) subunits were detected after 40 cycles that were not seen after 30 cycles of PCR (e.g., see Table 3), but additional subunits were not detected after 50 cycles; and (2) the comparison between amplification from rat brain regions with published *in situ* studies

Table 1. Primer sequences for PCR

Subunit	Direction	Primer sequence	Starting at base	Size (bp)
$\alpha 1$	Forward	5′-ATCTTTGGGCCTGGACCCTCATTCT-3′	29	580
	Reverse	5′-CGGGCTGGCTCCCTTGTCCACTC-3′	608	
$\alpha 2$	Forward	5′-GAGGACAAAATTGAGCACTTGCA-3′	3	345
	Reverse	5′-GAGTTGTTAAGTCGAAGGATATT-3′	347	
$\alpha 3$(i)	Forward	5′-GAATCAAGACGACAAGAACCT-3′	91	601
	Reverse	5′-CAGATTTGTTCTTCCCAAGAG-3′	691	
$\alpha 3$(ii)	Forward	5′-TTTATTCTTGGACTCTTGGGAAGAACA-3′	659	484
	Reverse	5′-CTTCATCTCCAGGGCCTCTGGTACCT-3′	1143	
$\alpha 4$(i)	Forward	5′-CTGGACCAAAGGCCCTGAGA-3′	609	503
	Reverse	5′-TTTTCCTTCAGTACTGGGGCAGCTG-3′	1112	
$\alpha 4$(ii)	Forward	5′-TTTAAACGAATCCCCAGGACAGAA-3′	87	387
	Reverse	5′-TGCCATTTCTCATAATTCTAA-3′	475	
$\alpha 5$	Forward	5′-ACTTTGGCTTTTCACAAATGCCAA-3′	80	292
	Reverse	5′-AGAAGGTTGAGAGGGAGACGTT-3′	371	
$\alpha 6$	Forward	5′-AGGGTGACCTGACCTGGCATTTCAGTGAACCATAGG-3′	−31	476
	Reverse	5′-TCATGGTGTACAGGATCGTTCCA-3′	445	
$\beta 1$	Forward	5′-ACAGTACAAAATCGAGAGAGTTTG-3′	7	664
	Reverse	5′-TCCACCTTCTTGGACACCATCTTG-3′	671	
$\beta 2$	Forward	5′-ATAAACTCATCACCAAGAAAGTTG-3′	644	515
	Reverse	5′-AAGTCCCATTACTGCTTCTGATGT-3′	1158	
$\beta 3$	Forward	5′-TGGAGCACCGTCTGGTCTCCAGGA-3′	641	419
	Reverse	5′-TCGATCATTCTTGGCCTTGGCTGT-3′	1059	
$\gamma 1$	Forward	5′-AGTACAAGTGGAAAAAGCCC-3′	650	465
	Reverse	5′-TCAGCTTCCTGTCTCTGGTGGTT-3′	1114	
$\gamma 2_{L\&S}$	Forward	5′-GTGGAGTATGGTACCCTGCACTATTTTGTG-3′	1048	311
	Reverse	5′-CAGAAGGCGGTAGGGAAGAAGATCCGAGCA-3′	1358	
$\gamma 3$	Forward	5′-TGCTCGGTCCAGGAGGGTAGA-3′	48	591
	Reverse	5′-CTGATCAGCTGCCTCAACTGAATTTTT-3′	639	
δ	Forward	5′-GACTACGTGGGCTCCAACCTGGA-3′	88	398
	Reverse	5′-ACTGTGGAGGTGATGCGGATGCT-3′	485	
NNE	Forward	5′-ACTCCGAGACAATGATAAGACCC-3′	134	504
	Reverse	5′-AGGTGCGAATCCACCCTCATCA-3′	637	
β-Actin	Forward	5′-CACCACAGCTGAGAGGGAAATCGTGCGTGA-3′	603	517
	Reverse	5′-ATTTGCGGTGCACGATGGAGGGGCCGGACT-5′	1120	

Numbering is from the ATG. Published sequences used to design primers and putative exons (E) that they span: $\alpha 1$ (Khrestchatisky et al., 1989; Lolait et al., 1989; E1-6), $\alpha 2$ (Khrestchatisky et al., 1991; E1-4), $\alpha 3$ [Malherbe et al., 1990; $\alpha 3$(i):E1-6; $\alpha 3$(ii):E6-8], $\alpha 4$ [Wisden et al., 1991; $\alpha 4$(i):E6-8, $\alpha 4$(ii):E1-4], $\alpha 5$ (originally described as $\alpha 4$ by Khrestchatisky et al., 1989; Malherbe et al., 1990; Pritchett and Seeburg, 1990; E1-4), $\alpha 6$ (Lüddens et al., 1990; E1-4), $\beta 1$ (Ymer et al., 1989; E1-6), $\beta 2$ (Ymer et al., 1989; E6-9), $\beta 3$ (Lolait et al., 1989; Ymer et al., 1989; E6-8), $\gamma 1$ (Ymer et al., 1990; E6-8), $\gamma 2$ (Shivers et al., 1989; E8-9), $\gamma 3$ (Knoflach et al., 1991; Herb et al., 1992; E1-6), δ (Shivers et al., 1989; Zhao and Joho, 1990; E1-5), NNE (non-neuronal enolase; Sakimura et al., 1985), and β-actin (Nudel et al., 1983).

(data not shown) suggested that 40 cycles would detect mRNAs of high and low abundance.

The oligo-primer pairs may amplify with different efficiencies, suggesting that comparisons of mRNA levels between different subunits, based on the amount of PCR product produced, may be inappropriate. However, PCR products for individual oligo-primer pairs between cell lines can be compared as the problem of varying efficiencies has been eliminated. Thus, amplification was also carried out at 30 cycles in order to identify cell lines with higher or lower levels of any one subunit.

No differences were observed between cells harvested at 50–100% confluency with respect to the levels of $GABA_A$ receptor subunit PCR products/30 ng total cDNA (data not shown). All experiments were subsequently performed on cells harvested at approximately 80% confluency. All of the cell lines described maintained the same level and composition of $GABA_A$ receptor mRNAs, as detected by PCR, over at least three different passage numbers. The figures demonstrate results from representative experiments.

Due to the possible detection, by PCR, of contaminant cDNA/RNA, we used multiple controls. (1) RNA blanks that were taken through the cDNA synthesis steps were used as controls for PCR. These blanks were used in every PCR reaction, for every set of primers. (2) Samples without primers were run for every cDNA template for each PCR experiment. (3) Primers for ubiquitous mRNAs were also used to ensure that the RNA isolation and cDNA synthesis had been successful. (4) Rat and/or mouse brain (as appropriate) positive controls were run for each experiment as the cell lines are derived from one of these two species. For many of the $GABA_A$ receptor subunits only sequence data from the rat was available for design of the primer sets. While there is high sequence identity between the cloned mouse and rat $GABA_A$ receptors, mouse brain cDNA as well as rat brain cDNA was used as a positive control to ensure that all of the primer sets hybridized to cDNA of both species.

Specificity of the $GABA_A$ receptor primers. Specificity of the $GABA_A$ receptor primers was tested as follows. (1) For each set of primers, PCR DNA was cut with restriction endonucleases and the appropriate size products were produced (data not shown). (2) Southern blotting was performed on PCR derived DNA products. The primers designed for individual $GABA_A$ receptor subunit mRNAs amplified products of the expected size (see Table 1; Figs. 2, 3, 5–10) and hybridized specifically with the appropriate labeled cDNA. In addition, the cDNA probes did not hybridize to bands amplified from inappropriate primer sets. (3) Primers were able to amplify products of the correct size from the appropriate $GABA_A$ receptor cDNA clones and not from others (data not shown).

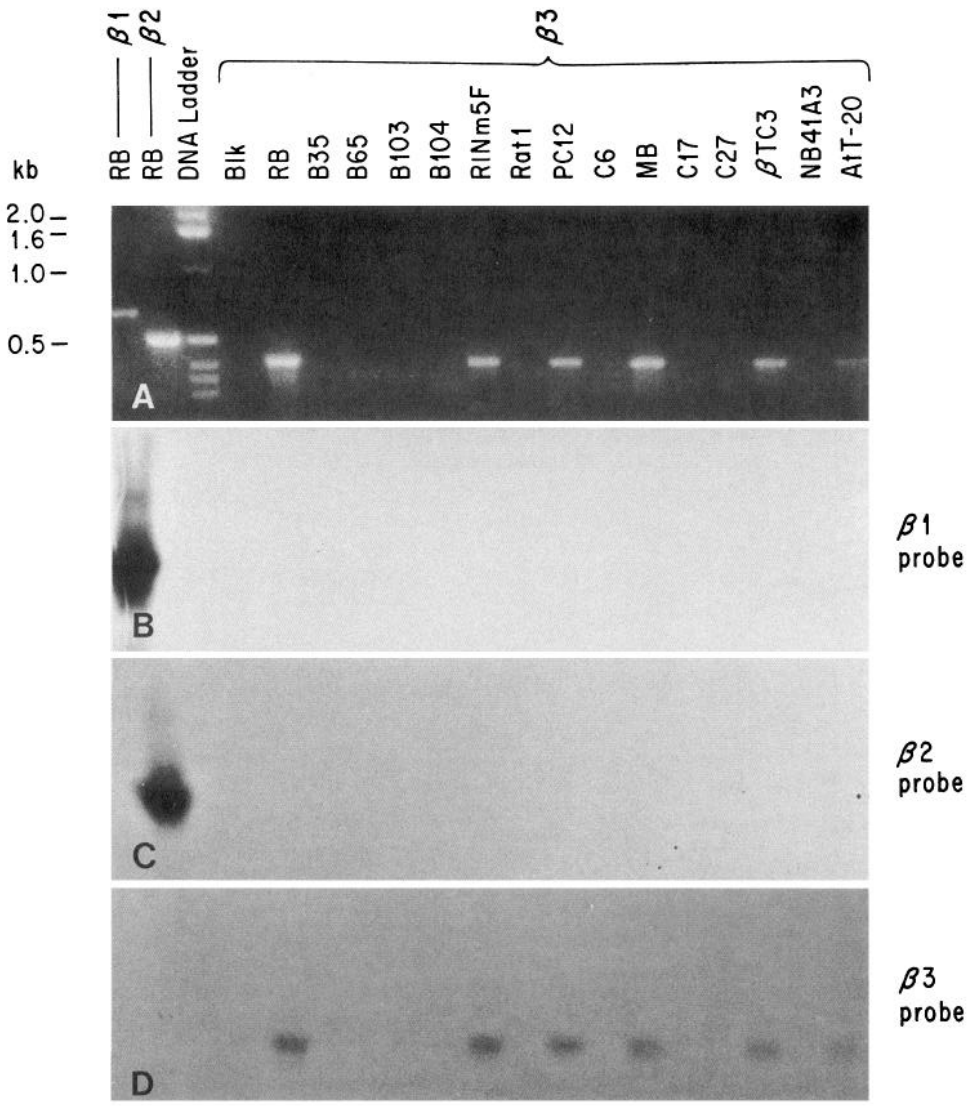

Figure 2. PCR products using $GABA_A$ receptor subunit β3 primers and cell line cDNA templates for 30 cycles of amplification. Positive controls include the β3 primers against rat brain (*RB*) and mouse brain (*MB*) cDNA templates while the negative controls include an RNA blank (*Blk*). A 1 kb DNA ladder was run in *lane 3*. Southern blots with β1 (*B*), β2 (*C*), and β3 cDNA (*D*) probes were performed. β1 (*lane 1*) and β2 (*lane 2*) primer sets were used with rat brain cDNA template to generate DNA products as negative and positive controls for the Southern blots.

Since cDNA clones for $GABA_A$ receptor α3 and α4 subunits were unavailable for testing the specificity of the α3 and α4 primers, two sets of primers for each of these subunits were designed. Both primer sets amplified the correctly sized DNA products, which, upon digestion with restriction endonucleases, produced the expected products. α3 PCR product was found in RINm5F, βTC3, B104, C17 cells using either α3(i) or α3(ii) primer sets, while α4 PCR product was found in RINm5F and βTC3 cells with either α4(i) or α4(ii) primer sets (Table 2). In addition, low-stringency Southern blotting with $GABA_A$ receptor ^{32}P-labeled α-cDNA clones (α1, α2, α5) detected the appropriately sized DNA products from PCR using the α3 and α4 primers (data not shown).

All of the primer sets produced PCR DNA products of the same size from rat and mouse brain cDNA (e.g., Figs. 1–3). In addition, rat cDNA probes hybridized to the DNA products produced from mouse brain cDNA templates (e.g., Figs. 2, 3).

Genomic DNA contamination of cellular RNA. The possibility of genomic DNA contamination of total cellular RNA was determined by DNase treatment of at least one RNA sample for each cell line. No differences were observed between DNAase treated or nontreated RNA samples in the level or composition of $GABA_A$ receptor mRNAs detected by PCR in any cell line. Therefore, DNase treatment was not routinely performed. In addition, $GABA_A$ receptor intron–exon structure has been characterized for the mouse δ subunit, the human β1 subunit, part of the human β3, and the chicken β4 (Sommer et al., 1990; Kirkness et al., 1991; Lasham et al., 1991; Kirkness and Fraser, 1993). These subunits display highly conserved exon boundaries. By aligning subunits of known gene structure with those of unknown structure, the location of the introns in the unstudied subunit genes can be estimated (Feng and Doolittle, 1987). The primer sets were therefore designed to amplify across multiple introns (the postulated introns that each set crosses can be found in the Table 1 notes). If PCR amplification of genomic DNA contamination had been present, a product of altered size would have been expected as the primers span multiple introns.

Results

PCR DNA products were detected from all of the cell lines with non-neuronal enolase and β-actin primers (Fig. 1). Negative controls lacking RNA in the cDNA synthesis do not demonstrate any detectable DNA bands (Fig. 1), while positive controls containing total mouse or rat brain cDNA demonstrate detectable DNA bands.

All of the primer sets were run individually against cDNA from each of the cell lines (30 and 40 cycles) as demonstrated with the β3 primers (Figs. 2, 3). The β3 primer set at 30 cycles of PCR amplification demonstrated detectable DNA products only in RINm5F, βTC3, PC12, and AtT-20 cell lines, as well as the rat and mouse total brain samples (Fig. 2*A*). After 40 cycles the same four cell lines, and no additional cell lines,

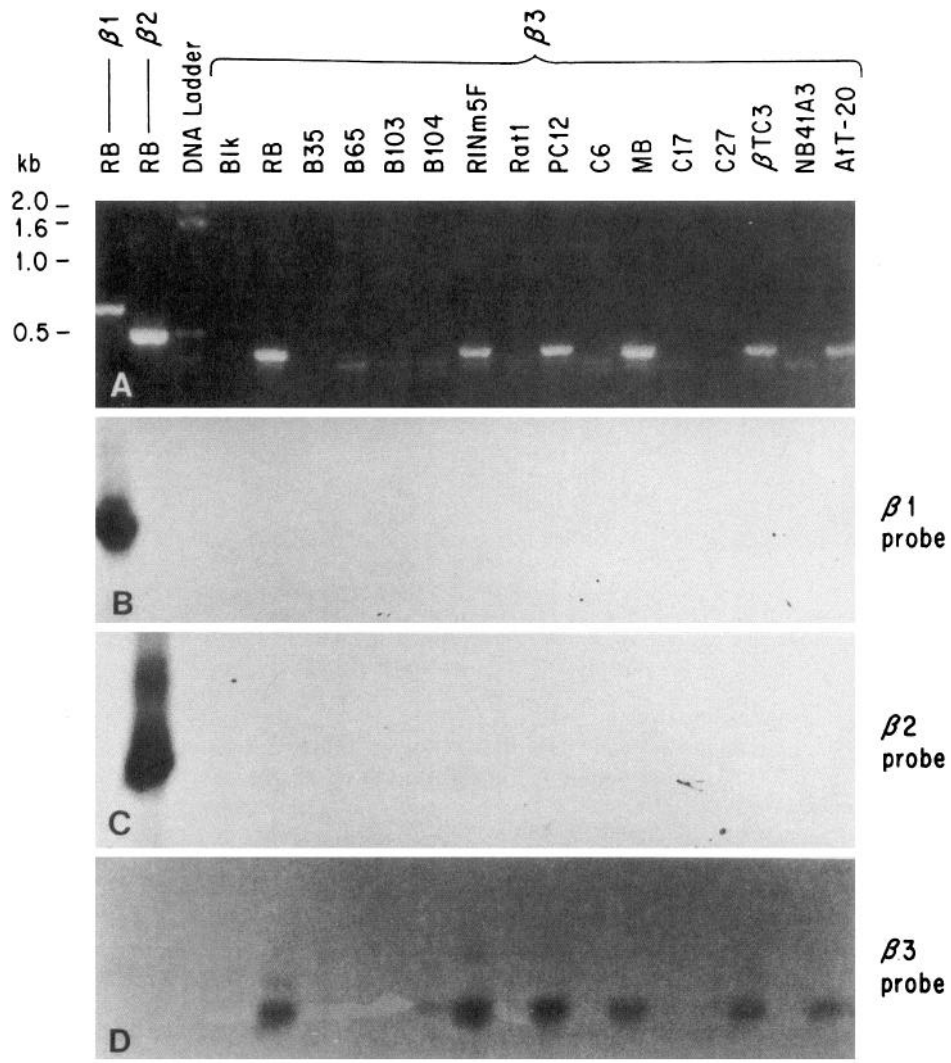

Figure 3. PCR products using $GABA_A$ receptor subunit β3 primers and cell line cDNA templates for 40 cycles of amplification. Positive controls include the β3 primers against rat brain (*RB*) and mouse brain (*MB*) cDNA templates while the negative controls include an RNA blank (*Blk*). A 1 kb DNA ladder was run in *lane 3*. Southern blots with β1 (*B*), β2 (*C*), and β3 (*D*) cDNA probes were performed. β1 (*lane 1*) and β2 (*lane 2*) primer sets were used with rat brain cDNA template to generate DNA products as negative and positive controls for the Southern blots.

demonstrated β3 DNA products (Fig. 3*A*). Primer sets, β1 and β2, were used in PCR with rat brain cDNA template as controls for the Southern blotting (Figs. 2*A*, 3*A*). The β3 cDNA probe hybridized to the β3 primer generated PCR products in the Southern blots but not to β1 or β2 PCR products (Figs. 2*D*, 3*D*). The β1 and β2 cDNA probes only hybridized to the lanes containing the corresponding primer sets and not to the lanes with β3 primer (Figs. 2*B,C*; 3*B,C*). These data suggest that the β-primers amplify products only from their corresponding mRNAs.

These results are typical of the experiments that were run with

Table 2. Cell lines in which individual $GABA_A$ receptor subunit mRNAs are found

Subunit	Cell line
α1	RINm5F, B35, B65, B103, B104
α2	RINm5F, B35, B65, B103, C17
α3	RINm5F, βTC3, B104, C17
α4	RINm5F, βTC3
α5	RINm5F, AtT20
β1	RINm5F
β2	RINm5F, B35, B65, B103, B104, C6
β3	RINm5F, βTC3, PC12, AtT20
γ1	RINm5F, βTC3, B35, B65, B103, B104, C17
$\gamma 2_L$	RINm5F, B35, B65, B103, PC12, C17, C6, C27
$\gamma 2_S$	RINm5F, B35, B65, B103, PC12, C17, C6, C27
γ3	RINm5F
δ	RINm5F, βTC3, B35, B65, B014, PC12, AtT20, NB41A3, C17, C6, C27, Rat1

$GABA_A$ receptor subunit mRNAs found by PCR after 40 cycles of amplification.

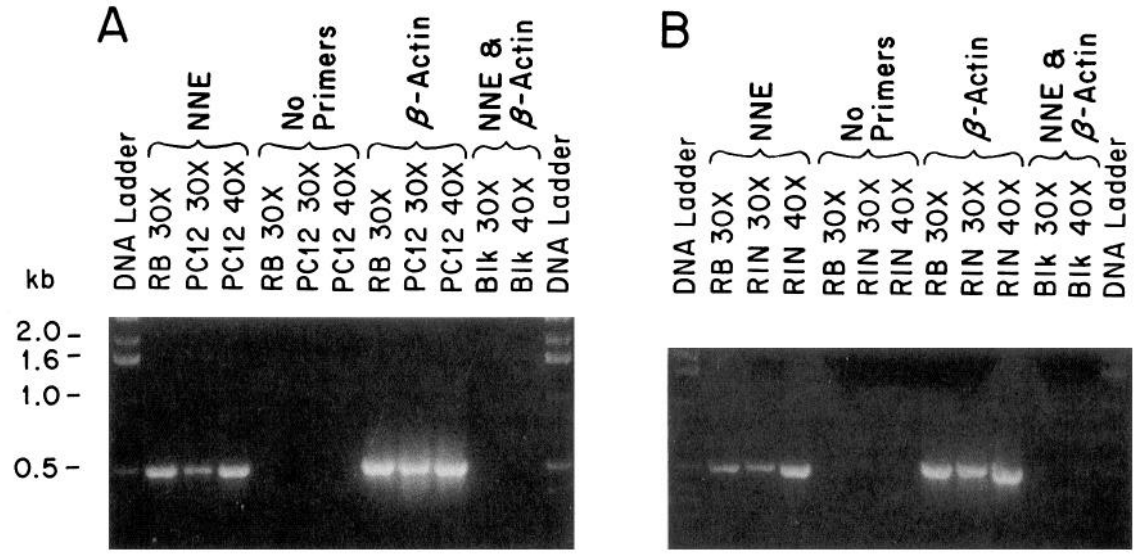

Figure 4. Control PCR amplifications PC12 and RINm5F templates for 30 and 40 cycles of amplification. Non-neuronal enolase (*NNE*) primers (*lanes 2–4*), no primers (*lanes 5–7*), β-actin primers (*lanes 8–10*) and NNE and β-actin primers (*lanes 11, 12*) were used. cDNA templates used were rat brain (*RB*), PC12 (*A*), RINm5F (*B*), or RNA blanks (*Blk*); 1 kb DNA ladders were run in the outer lanes.

the cell lines in that even at 40 cycles of amplification only a few of the cell lines demonstrated each of the $GABA_A$ receptor subunits. These data also suggest that generalized $GABA_A$ receptor subunit contamination of the samples was very unlikely.

In addition to running each primer set against all of the cell lines, the PCR was performed for each cell line with all of the primer sets. $GABA_A$ receptor subunit PCR products are demonstrated from a cell line that contains very few subunits (PC12; Figs. 4*A*, 5, 7, 9) and one that contains the greatest variety of $GABA_A$ receptor subunit mRNAs (RINm5F; Figs. 4*B*, 6, 8, 10). Once again, the subunit primer sets amplify a band of the expected size (Table 1) that can be hybridized specifically by a probe from the corresponding cDNA clone alone, and not by cDNA clones of other subunits. At 30 cycles of amplification the RINm5F cell line shows α2-4, β3, and δ while the PC12 cell line only demonstrates β3 and δ. However, with 40 cycles of PCR the RINm5F cell line demonstrates α1-5, β1-3, γ1-3, and δ subunits, in contrast to the PC12 cell line, which only demonstrates β3, γ2, and δ. Some cell lines display a large variety of detectable $GABA_A$ receptor subunit mRNAs while some demonstrate very few subunits (Table 3). These data attest to the absence of generalized contamination.

Table 2 summarizes the PCR at 40 cycles of amplification for each subunit. Each subunit, except α6, was found in at least one cell line. Most of the subunits, however, were found in a limited number of lines. For example, one or more α subunit(s) was found in 5 of the 13 cell lines, one or more β subunit(s) was found in 6 of the 13 lines, while γ subunits were found in 8 of the 13 cell lines. The most prominent subunit was the δ subunit, which was found in 12 of the 13 cell lines tested.

Table 3. $GABA_A$ receptor subunit mRNAs transcribed in each cell line

		$GABA_A$ receptor subunit mRNA	
Origin	Cell line	30 cycles	40 cycles
Brain			
Neuroblastoma	NB41A3	δ	δ
	B35	α1, β2	α1,2, β2, γ1,$2_{L\&S}$, δ
	B65	α1, β2	α1,2, β2, γ1,$2_{L\&S}$, δ
	B103	α1, β2	α1,2, β2, γ1,$2_{L\&S}$
	B104	β2	α1,3, β2, γ1, δ
Cerebellum	C17		α2,3, γ1,$2_{L\&S}$, δ
	C27		$\gamma 2_{L\&S}$, δ
Glioma	C6		β2, $\gamma 2_{L\&S}$, δ
Periphery			
Pituitary	AtT20	β3	β3, δ
Adrenal Medulla	PC12	β3, δ	β3, $\gamma 2_{L\&S}$, δ
Fibroblast	Rat1		δ
Endocrine pancreas	RINm5F	α2,3,4, β3, δ	α1,2,3,4,5, β1,2,3, γ1,$2_{L\&S}$,3, δ
	βTC3	α4, β3, δ	α3,4, β3, γ1, δ

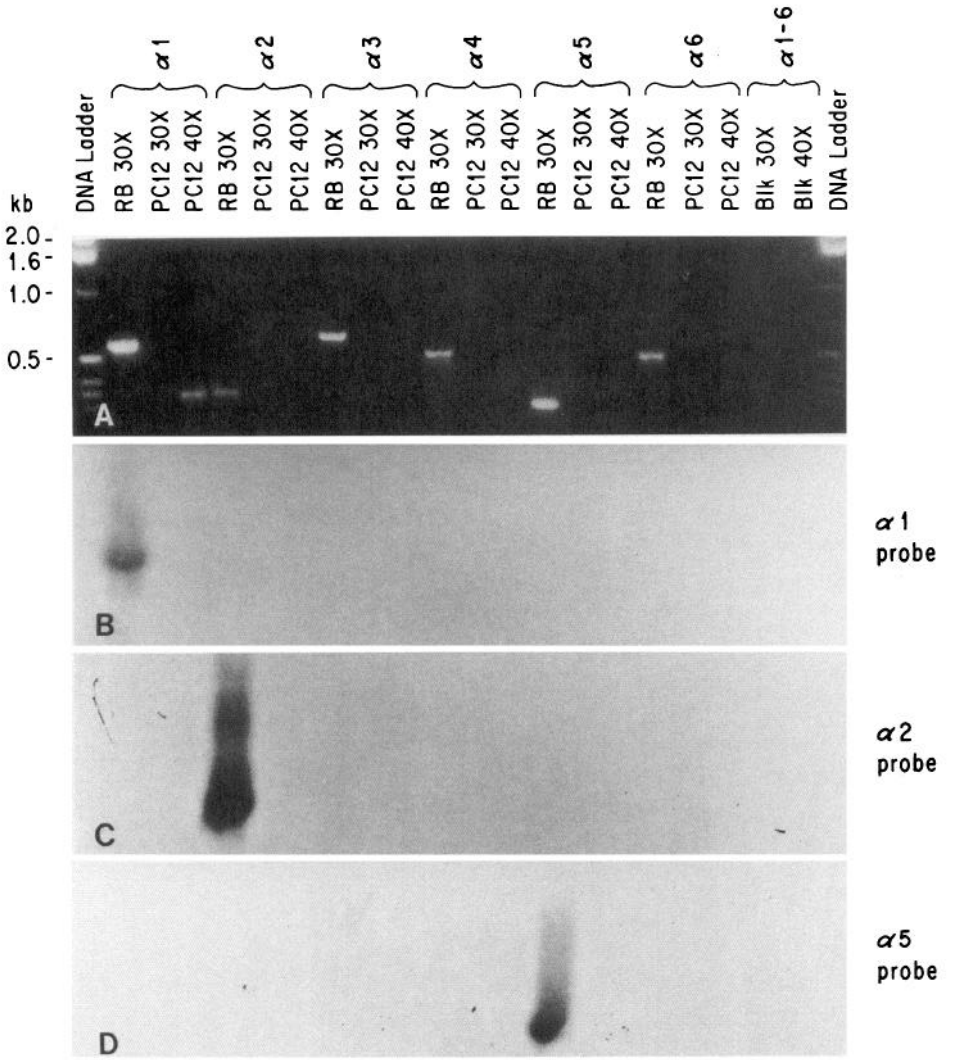

Figure 5. PCR products using GABA$_A$ receptor α subunit–specific primers and PC12 cDNA template. Rat brain template at 30 cycles was used as a positive control while PC12 template was amplified for 30 and 40 cycles. RNA blanks were run with the primers as negative controls (*lanes 20* and *21*); 1 kb DNA ladders were run in the outer lanes. Southern blots were performed with α1 (*B*), α2 (*C*), and α5 (*D*) cDNA probes.

Table 3 summarizes our data by cell line. At 30 cycles, we detected a limited number of subunits in any of the cell lines. After 40 cycles of amplification many more subunits can be detected, however most of the lines still demonstrate a limited repertoire of subunits. Tables 2 and 3 clearly demonstrate that the cell lines each contain a different although overlapping subunit mRNA composition.

Discussion

We have examined 13 cell lines for the presence of GABA$_A$ receptor subunit mRNAs. The α1-5, β1-3, γ1-3, and δ subunit mRNAs were each identified in at least one cell line, and in turn, each cell line examined contained at least one GABA$_A$ receptor subunit mRNA. None of the cell lines contained detectable amounts of α6.

A comparison of the subunit mRNAs found in each cell line

PCR amplification may proceed with different rates from individual primer pairs, making a direct comparison between distinct subunits inappropriate. For example, we cannot say for certain that there are more α2 than α1 mRNAs in RINm5F cells, based on the appearance of the α2 PCR product at 30 cycles versus the α1 product at 40 cycles, as α2 PCR amplification may simply proceed more efficiently. It is still possible, however, to compare individual subunit mRNAs between cell lines. For example, an α2 PCR product appears after 30 cycles from the RINm5F cell line while not until 40 cycles in the B103 cell line, suggesting the presence of more α2 mRNA in the RINm5F cells, than in the B103 cells.

Expected subunit composition based on tissue origin

The subunits found in a previously described cell line with functional GABA$_A$ receptors, the GT1-7 cells, suggests that cell lines may contain at least some subunits that are found in cell types from which they were derived (Hales et al., 1992; Favit et al., 1993). This comparison is difficult for many of the cell lines described here, however, as the subunits found in the tissues from which the cell lines were derived (e.g., pancreas and adrenal medulla) are unknown. In addition, the CNS subunit composition varies widely within tissues, and the CNS location from which most of the tumor lines are derived, is unknown. The cell types within regions may also have distinctive subunit combinations, and the cell lines may represent a cell type that is a minor component of the tissue of origin.

The cell lines B35, B65 and B103 demonstrate detectable levels of α1, α2, β2, γ1, γ2, and δ (no δ in B103 cells). The α1,

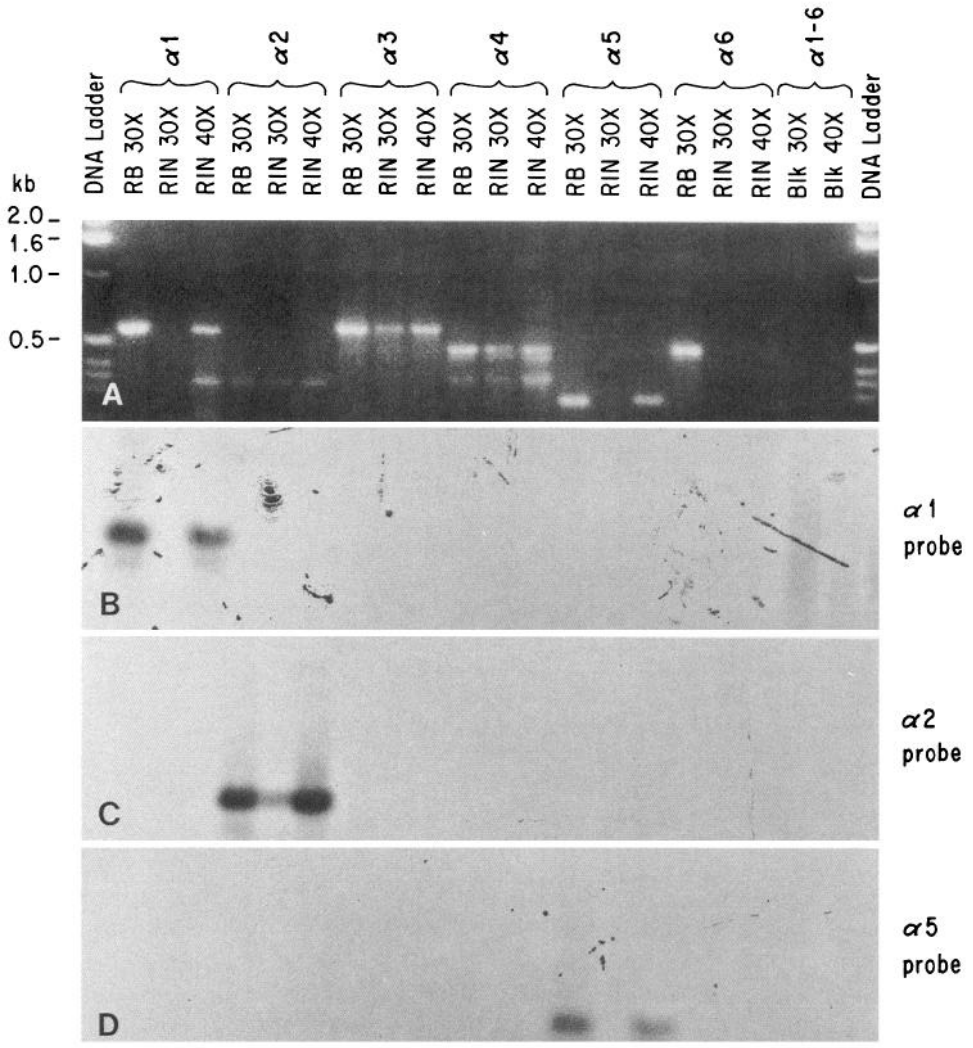

Figure 6. PCR products using $GABA_A$ receptor α subunit–specific primers and RINm5F template. Rat brain template at 30 cycles was used as a positive control while RINm5F template was amplified for 30 and 40 cycles. RNA blanks were run with the primers as negative controls (*lanes 20 and* 21); 1 kb DNA ladders were run in the outer lanes. Southern blots were performed with α1 (*B*), α2 (*C*), and α5 (*D*) cDNA probes.

α2, β2, γ1, and γ2 subunits are prominent in the CNS, found together in regions such as the globus pallidus, inferior colliculus and midbrain (Wisden et al., 1992). These areas may represent the tissue of origin for these cell lines, and in turn, the cell lines may be useful for modeling receptors from these regions. B104 cells contain slightly different $GABA_A$ receptor mRNAs (α1, α3, β2, γ1, and δ) and have a different morphology from the B35, B65 and B103 cells (Schubert et al., 1974). This combination is not common to any one CNS region and may represent a minor cell type.

The cerebellar lines, C17 and C27, do not transcribe α6, suggesting that they are not of granule cell origin. However, the combination of α2, α3, γ1, γ2, and δ (Table 3) is not found in stellate/basket, Purkinje, or Bergmann glia cells, suggesting that the C17 and C27 cells do not represent these major cerebellar cell types (Wisden et al., 1992).

The individual subunit mRNAs found in the cells of the endocrine pancreas are unknown. The two pancreatic lines (βTC3 and RINm5F), derived in different ways and from different species, both contain similar subunits (α3, α4, β3, γ1, and δ common to both). These findings suggest that the two cell lines may contain an endocrine pancreas-like subunit composition.

Another reason that the cell lines may not demonstrate subunits that are found *in vivo* is that many of these cell lines contain a mixed phenotype (e.g., neuronal and glial characteristics) and are mostly undifferentiated cells, suggesting that they resemble embryonic cells. However, δ, which is found in 12 of the 13 cell lines, is not a prominent subunit in embryonic development, while α3, which is a prominent embryonic subunit, is found in only three of the 13 cell lines (Laurie et al., 1992; Table 2). The comparisons of cell line composition and embryonic *in situ* data, however, may be misleading, since many of the cell lines are derived from peripheral tissues where the embryonic subunit composition is unknown.

The undifferentiated nature of some of the cell lines may become an advantage if treatment (e.g., NGF) of the cells causes them to differentiate toward diverse phenotypes (Pollock et al., 1990). This characteristic of the cell lines may be very useful for subunit transcriptional regulation and phenotypic subunit combination studies.

Functional $GABA_A$ receptors in the cell lines

All of the cell lines demonstrate at least one $GABA_A$ receptor subunit mRNA. These findings prompted us to examine which of these cell lines contained functional GABA-gated Cl^- channels, in order to identify combinations of subunits that were functional. In the following companion article, we tested nine

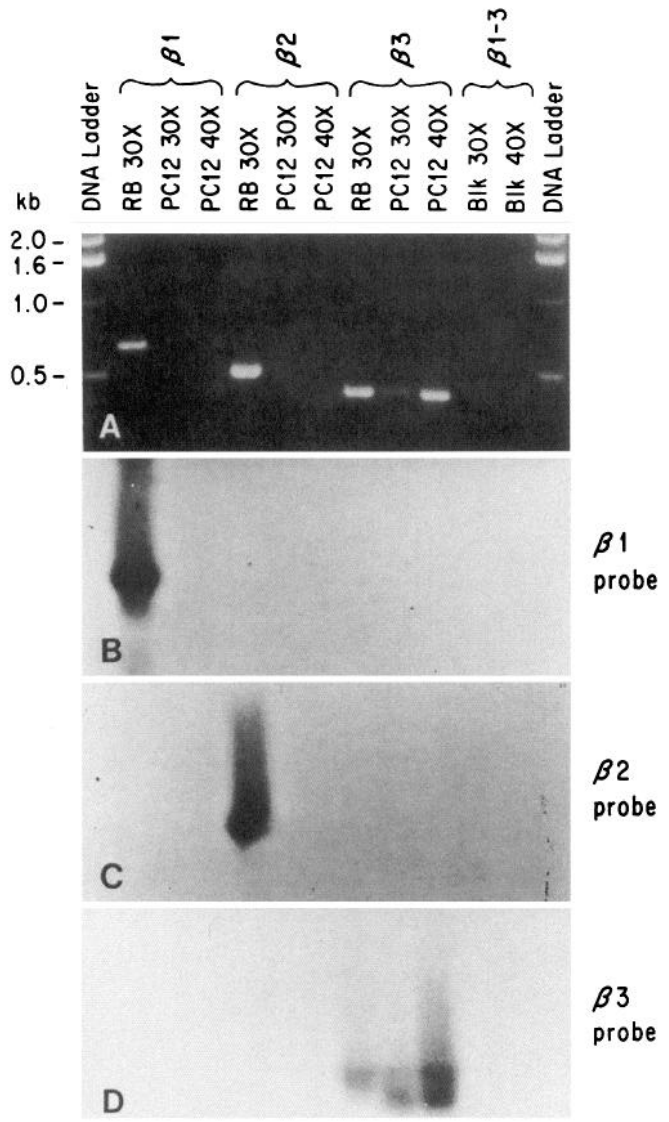

Figure 7. PCR products using $GABA_A$ receptor β subunit–specific primers and PC12 cDNA template. Rat brain template at 30 cycles was used as a positive control while PC12 template was amplified for 30 and 40 cycles. RNA blanks were run with the primers as negative controls (*lanes 11* and *12*); 1 kb DNA ladders were run in the outer lanes. Southern blots were performed with β1 (*B*), β2 (*C*), and β3 (*D*) cDNA probes.

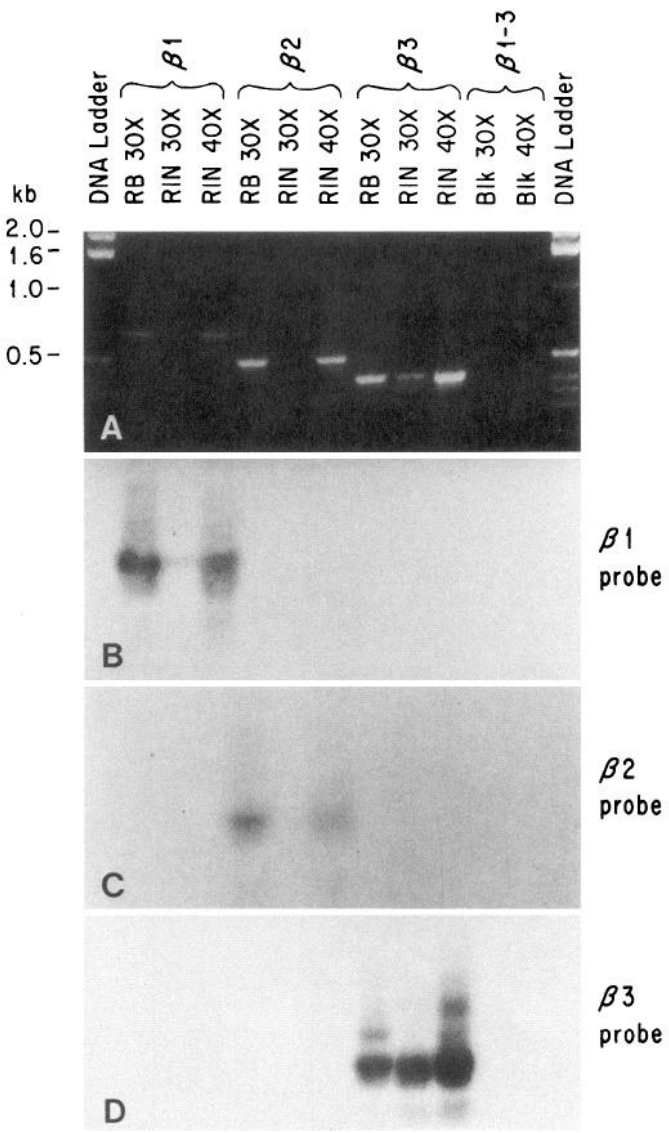

Figure 8. PCR products using $GABA_A$ receptor β subunit–specific primers and RINm5F template. Rat brain template at 30 cycles was used as a positive control while RINm5F template was amplified for 30 and 40 cycles. RNA blanks were run with the primers as negative controls (*lanes 11* and *12*); 1 kb DNA ladders were run in the outer lanes. Southern blots were performed with β1 (*B*), β2 (*C*), and β3 (*D*) cDNA probes.

of the cell lines for GABA-gated Cl^- channels, using the whole-cell configuration of the patch-clamp technique. Only two of the cell lines, the RINm5F and βTC3 cells, responded to GABA (Hales and Tyndale, 1994). However, none of the other cell lines tested, even though they contained $GABA_A$ receptor subunit mRNAs (Table 3), expressed functional $GABA_A$ receptor Cl^- channels. These included the B65, B104, PC12, AtT-20, NB41A3, C17, and C6 cells (Hales and Tyndale, 1994). The Rat1 cells were not tested as the NB41A3 cells also contain only the δ subunit mRNA (and in greater quantity than the Rat1 cells; Table 3), and did not respond to GABA. Likewise, the C27 cells were not tested because they show an even more limited subunit mRNA composition than their sibling line, the C17 cells (Table 3), which did not demonstrate functional channels. In addition, Kasckow et al. (1992) did not find functional $GABA_A$ Cl^- channels in the B35, B65, or B103 cells.

What makes a functional GABA-gated chloride channel?

Many factors might contribute to why cell lines with subunit mRNAs are not producing detectable channels. These include (1) lack of subunit translation, (2) incorrect subunit combinations, (3) inability to assemble or localize channels correctly, (4) insufficient amounts of mRNAs, and/or (5), defective mRNAs. These alternative explanations are discussed below.

(1) Do the cell lines translate receptor mRNA? While the B35, B65, B103, and B104 cell lines contained $GABA_A$ mRNAs, $GABA_A$ receptor binding sites and α subunit immunoreactivity, they do not demonstrate functional channels (Table 3; Napias et al., 1980; Kasckow et al., 1992; Hales and Tyndale, 1994). Likewise, the C17 cells have mRNAs (Table 3), bind ^{3}H-muscimol and ^{3}H-flunitrazepam (Sapp, Tyndale, and Olsen, unpublished observations), but do not have functional channels.

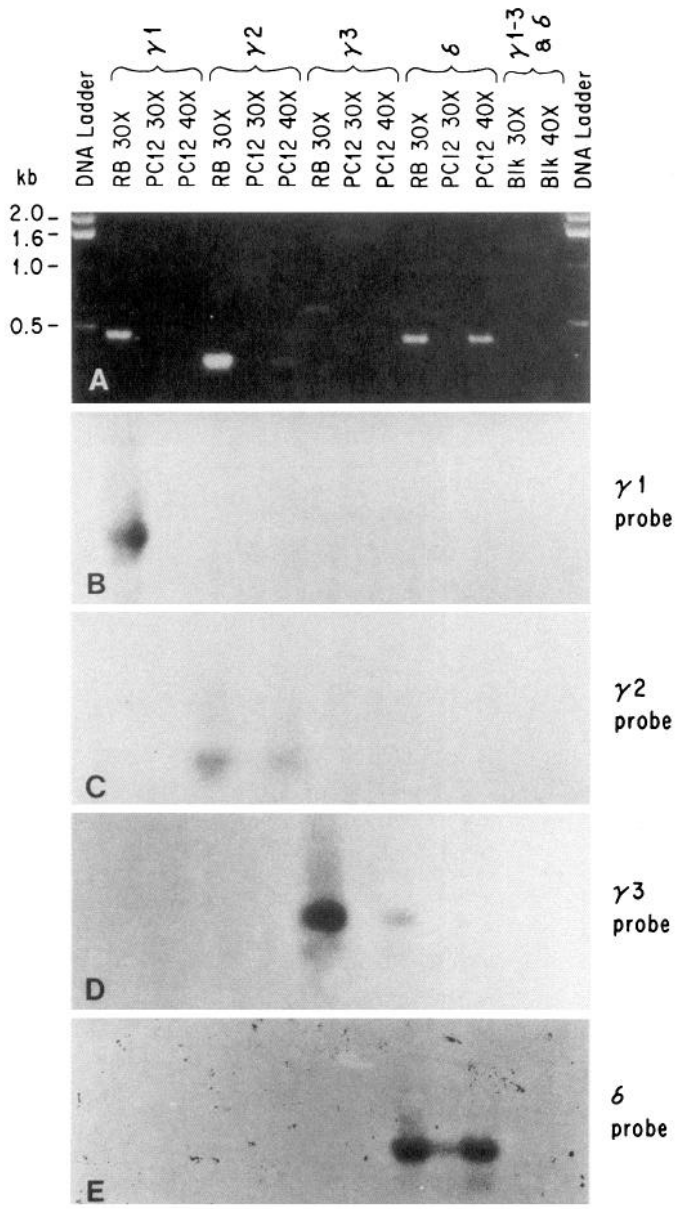

Figure 9. PCR products using $GABA_A$ receptor γ and δ subunit–specific primers and PC12 cDNA template. Rat brain template at 30 cycles was used as a positive control while PC12 template was amplified for 30 and 40 cycles. RNA blanks were run with the primers as negative controls (*lanes 14* and *15*); 1 kb DNA ladders were run in the outer lanes. Southern blots were performed with γ1 (*B*), γ2 (*C*), γ3 (*D*), and δ (*E*) cDNA probes.

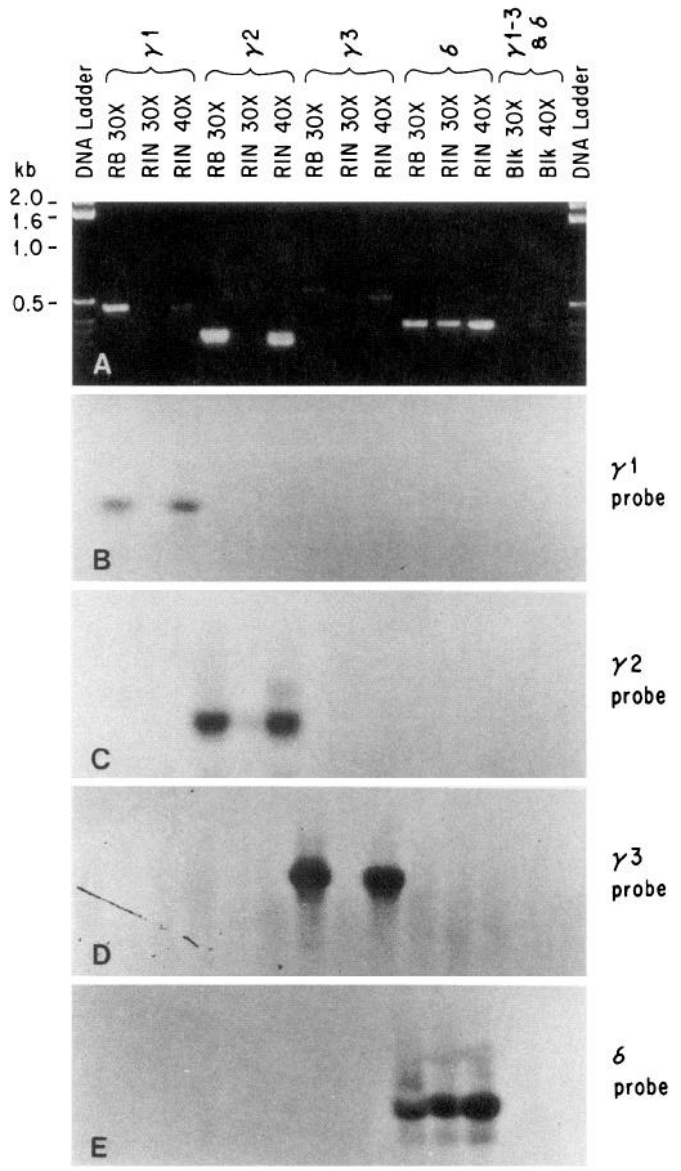

Figure 10. PCR products using $GABA_A$ receptor γ and δ subunit–specific primers and RINm5F template. Rat brain template at 30 cycles was used as a positive control while RINm5F template was amplified for 30 and 40 cycles. RNA blanks were run with the primers as negative controls (*lanes 14* and *15*); 1 kb DNA ladders were run in the outer lanes. Southern blots were performed with γ1 (*B*), γ2 (*C*), γ3 (*D*), and δ (*E*) cDNA probes.

These results indicate that translation of the subunit mRNAs in cell lines lacking channels can occur.

(2) Do the cell lines contain appropriate subunit combinations? Subunit composition may be responsible for the lack of channels in some cell lines that do not contain an α or β subunit (e.g., no channels in lines lacking an α subunit: PC12, AtT-20, NB41A3, C6; or a β subunit: C17; Table 3). However, it is unlikely that incorrect subunit combinations account for the lack of channels in some of the cell lines, as they produce combinations that are believed to form functional channels (α, β, and γ: e.g., B35, B65, B103, B104; Table 3; Pregenzer et al., 1993). Both lines containing functional channels (βTC3 and RINm5F), transcribe at least one member of each subunit class (α, β, γ, and δ). However, cell lines without functional channels also transcribe α, β, γ, and δ subunits (e.g., B35, B65, B104).

(3) Can the cell lines correctly assemble and target the receptors? It has been shown that different subunits can direct cellular localization of the $GABA_A$ receptor (Perez-Velazquez and Angelides, 1993). It is possible that some cell lines could be incorrectly targeting the receptor to a membrane other than the plasma membrane, or are simply unable to localize or assemble channels into the plasma membrane. The PC12 cells, which are derived from $GABA_A$ receptor-expressing chromaffin cells, do not demonstrate functional $GABA_A$ receptors. Correctly localized channels of multimeric composition (the nicotinic ACh receptor) have, however, been identified in PC12 cells (Pollock et al., 1990; Hales and Tyndale, 1994). The possibility that

receptors may indeed assemble, but desensitize too rapidly to be detected cannot be excluded.

(4) Do the cell lines have sufficient levels of mRNA? In the following companion article, the RINm5F and βTC3 cell lines both exhibited responses to GABA application (Hales and Tyndale, 1994). In βTC3 cells, GABA was too small to characterize pharmacologically. The βTC3 cells have fewer subunits (Table 3) and lower levels of some of them (α3, Table 3; δ, data not shown). GABA-evoked currents activated small currents in only 50% of the βTC3 cells while all RINm5F cells responded to the amino acid, suggesting that lower levels of mRNAs might be reflected as decreased channel activity (Hales and Tyndale, 1994). The cell lines studied here that were without channels, but in which we can detect subunit mRNAs by PCR, may be making functional channels below the level of detection.

(5) Are the mRNAs in cell lines defective? The $GABA_A$ receptor mRNAs detected by PCR may contain minor nucleotide changes that effect their function. For example, mutations may be directly inactivating or, more likely, may disfavor translocation or correct assembly of the complex. However, many of the cell lines without functional receptors contain multiple subunits; therefore, several mutations would probably be required to eliminate function.

We conclude that the most likely explanation(s) for the cell lines having subunit mRNAs, but lacking detectable channels, is nonfunctional subunit combinations and/or insufficient levels of mRNAs. This could be clarified further using immunohistochemical, antisense and subunit transfection techniques. As so few of the cell lines have functional channels, while demonstrating subunit mRNAs, it is possible that there is a selection against $GABA_A$ receptors in the cell lines; that is, functional $GABA_A$ Cl^- channels may be incompatible with cell division.

Endogenous $GABA_A$ receptor subunit mRNAs in cell lines

As described above, the B35, B65, B103, and B104 cell lines contain $GABA_A$ mRNAs, $GABA_A$ receptor binding sites and α subunit immunoreactivity, but do not demonstrate functional channels (Table 3; Napias et al., 1980; Kasckow et al., 1992; Hales and Tyndale, 1994). These findings suggest that these cell lines can synthesize the receptor proteins but do not contain the appropriate combinations and/or sufficient amounts of the subunits for detectable channel activity. Addition of even a single subunit, by transfection for example, may be enough to restore channel activity when combined with the endogenous subunits. $GABA_A$ receptor subunits have been identified in unlikely cell types such as the Rat1 fibroblast cells (Table 3) and the kidney 293 cells (Kirkness and Fraser, 1993). In fact, every cell line tested here had at least one subunit mRNA (Table 3), suggesting that many cell lines used routinely to transfect $GABA_A$ receptor subunits may already contain endogenous subunits. The endogenous subunits may confound the interpretation of results found with multimeric transfections, and may be of particular importance in explaining some of the homomeric receptor studies. Endogenous subunits in cell lines without detectable channels may combine with single transfected subunits to form detectable channels, mistakenly characterized as homomeric channels. In addition, while the levels of endogenous subunits may be low compared to the levels of those transfected, they may contribute distinctive pharmacology (e.g., γ2, Pregenzer et al., 1993), allow for channel assembly, or direct cellular localization (Perez-Velazquez and Angelides, 1993).

In conclusion, we have identified the $GABA_A$ receptor subunits in 13 cell lines using PCR. Every cell line examined contained at least one subunit mRNA, even from the Rat1 fibroblast cell line. Two of the 13 cell lines demonstrated functional responses to GABA (Hales and Tyndale, 1994). These lines will now provide an homogeneous tissue source for studying many facets of $GABA_A$ receptors such as subunit assembly and composition, subunit regulation, transcription, translation, and pharmacology.

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