Isolation and Characterization of a Skate Retinal GABA Transporter cDNA

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Purpose: The inhibitory neurotransmitter γ-aminobutyric acid (GABA) is believed to play a crucial role in the processing of information within the vertebrate retina. Extracellular concentrations of GABA are thought to be tightly regulated by carrier-mediated transport proteins in neurons and glial cells. The purpose of this work was to isolate the gene that encodes one of these transport proteins in the skate retina.

Methods: cDNA clones were isolated from a skate retinal cDNA library using a mouse retinal GABA transporter (GAT1) cDNA as a probe. The PCR technique was used to fill sequence gaps, and 5’ and 3’ RACE were employed to amplify the 5’ and 3’ untranslated regions. The amplified fragments were subcloned into a T-vector. Blots containing RNA from 10 different tissues were probed to determine the size of the transcript and the tissue distribution.

Results: Sequence analysis revealed that the skate retinal GABA transporter cDNA shared 72% identity with the mouse GABA transporter-1 at the DNA level and 80% identity at the amino acid level. Multiple sequence alignments showed that our sequence is closest to the Torpedo GABA transporter-1. Two transcripts, 4.5 and 7 kb, were detected in retina and possibly brain by RNA blot analysis. Forty-four introns were detected in the skate GABA transporter gene.

Conclusions: We successfully isolated a full length GABA transporter cDNA from the retina of the skate. The size of the full length sequence of the skate retinal GABA transporter is in agreement with the size of the smaller transcript detected on RNA blots. The larger transcript observed on the RNA blot may be the result of either alternative splicing or utilization of a downstream poly A signal.

GABA (γ-aminobutyric acid) is a major inhibitory neurotransmitter in the mammalian brain and is widely distributed throughout the nervous system [1,2]. GABAergic transmissions are involved in many aspects of normal neuronal function, and dysfunction of GABAergic transmission is associated with numerous neurological and psychiatric diseases [3-5]. In the retina, GABA may be involved in the regulation of the dynamic range of retinal neurons, the establishment of the surround portion of the receptive field of visual neurons, the establishment of color opponency, the mediation of movement detection, and may also be involved in the process of visual adaptation [6]. GABA is thought to be released by certain classes of horizontal and amacrine cells [7-14]. Unlike the neurotransmitter acetylcholine, which is degraded by the enzyme acetylcholinesterase in the synaptic cleft, the actions of GABA are thought to be terminated by its rapid re-uptake via GABA transporters into presynaptic neurons and surrounding glial cells [15-18]. GABA transporters also are proposed to have an additional function. Electrophysiological studies show that GABA release from catfish horizontal cells may be mediated also by GABA transporters [19-21] since a portion of the GABA release from the horizontal cells of these species is Ca2+-independent, but does require Na+, an ion crucial to the process of GABA transport.

Four different GABA transporter cDNAs (GAT-1-4) have been isolated from rat and mouse brain (cf. [22] for review). Mouse GAT-1 is the homologue of rat GAT-1. Mouse GAT-2 is the homologue of BGT-1 (initially isolated from Madin-Darby canine kidney cells and which transports betaine as well as GABA). Mouse GAT-3 is the homologue of rat GAT-2. Mouse GAT-4 is the homologue of rat GAT-3. GABA transporters from other species such as human [23-25], Torpedo californica [26,27], and Manduca sexta [28] also have been cloned. All these transporters share 50-70% identity in their predicted amino acid sequences [22,29], and they transport GABA with high affinity in a sodium- and chloride-dependent manner, but they show distinct distribution patterns [30-34] and pharmacological properties [35-40].

We have undertaken to clone GABA transporters from a tissue source that offers the potential to correlate molecular biological analyses with function in the cells in which the transporters are normally expressed. In this respect, the retina of the skate offers a particularly favorable model system in which to compare the nature and distribution of GABA transporters with their function in enzymatically isolated cells as well as in the intact isolated retinal preparation. In the present study, we report the isolation from a skate retinal cDNA library of a GABA transporter cDNA, named SGAT-1 due to its high degree of similarity to other GAT-1 transporters. We also describe its tissue distribution as revealed by northern blot analysis, and discuss the gene structure of this GABA transporter.

METHODS

Isolation of Skate Retinal GABA Transporter cDNAs— The skate retinal cDNA library used in this study has been previously described [41]. A low-stringency screening protocol

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was used to isolate GABA transporter cDNAs from the skate retina library. Approximately $10^6$ plaque-forming units were spread on Petri dishes and lifted onto nylon filters (Micron Separations, Westborough, MA) for screening. A mouse retinal GAT-1 clone, kindly provided by Dr. Vijay Sarthy (Northwestern University Medical School, Chicago, Illinois), was used as the probe. The probe fragment was excised from its vector, gel purified, labeled with $\alpha$-32P-dATP by nick translation (Nick Translation Kit, Boehringer Mannheim, Indianapolis, IN) and used to probe the filters. Hybridization was performed at 37 °C in a solution containing 30% formamide, 1.5X SSPE (1X SSPE = 150 mM NaCl, 10 mM NaH$_2$PO$_4$, pH 7.4, 1 mM EDTA), 0.5% nonfat dry milk, 2% SDS and 0.5 mg/ml salmon sperm DNA. The filters were washed sequentially at 45 °C for 30 min each in solutions containing 2X SSC (1X SSC = 150 mM NaCl, 15 mM C$_6$H$_5$Na$_3$O$_7$, pH 7.0), 0.5% SDS, and 0.5X SSC, 0.5% SDS. Primary screening of the library under these conditions yielded 63 potential clones. Twenty potential clones were selected for further purification and yielded three clones (c13-4a, c12-2b and c11-1) representing portions of a putative GABA transporter mRNA. One clone was used as a probe to screen the remaining 43 primary clones under high stringency conditions (hybridization solution contained 50% formamide, 5X SSC, 1X Denhardt’s solution, 0.5% SDS, and 0.5 mg/ml salmon sperm mRNA; 45 °C). The filters were washed at 55 °C sequentially in buffers containing 0.5% SDS plus 2X SSC, 0.5X SSC and 0.1X SSC for 30 min each. One additional clone (c7-1) was isolated in this screen.

**Polymerase Chain Reaction (PCR) Screening and Cloning of cDNA Fragments**— Sequences that were not represented in any of the isolated cDNA clones were obtained by PCR amplification from the cDNA library. The PCR was performed with 10 µl (7 x 10$^7$ pfu) of the cDNA library and 200 ng each of 5' and 3' primers in a 100 µl reaction mixture. The primary amplification was carried out with two external primers (upstream primer pGAT25, 5'-TTCTCATACCGG-GTTGGTCTTTG-3' and downstream primer pGAT26, 5'-CGCAAGTTATGAAACCTT-3'). Figure 1). A second amplification was performed using the primary PCR products as templates and two sets of nested primers (upstream pGAT21, 5'-GAAACTCATCCACCAGCCAG-3' and downstream pGAT22, 5'-ATACGGGTTGGTCTTT-3', Figure 1). Final PCR products were cloned into a T-vector produced by digesting pBluescriptII KS (Stratagene, Carlsbad, CA) with EcoRV and adding 3' Ts by incubation with Taq polymerase for 30 min at 72 °C in Taq buffer containing only dTTP. PCR products were ligated into this vector at a 10:1 molar ratio of PCR product to vector, and plasmids containing an insert were selected from ampicillin-resistant transformants by disruption of $\beta$-galactosidase activity. Clones were restriction mapped and then sequenced using vector-specific primers.

**Amplification of 3' and 5' Untranslated Regions by RACE**— Total RNA was isolated from skate retina using Trizol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer’s protocol. For 5' RACE, gene-specific primers (pGAT27: 5'-CGAGACCCAGCCTTTGGAAC-3', pGAT35: 5'-CATCCAGAACAGATGTCAGG-3', and pGAT32: 5'-'CCACACGGCTTTTCGGTT-3') were selected in clone c7-1 (Figure 1). First strand cDNA was reverse-transcribed from mRNA using pGAT27 as a primer and a polyC tail was added to the 3' end. Anchor primer (5'-CUACUACUACUGCCACCGCTGACTAGTACGGGIIG-GGIIkkkGGk-3'), containing the sequence complementary to the polyC and a multiple cloning sequence (UAP 5'-CUAUCACUCACAGCCACCGCTGACTAGTAC-3'), and pGAT35 were used to amplify the 5' end sequence. The second round PCR was performed on the product by using UAP and nested primer pGAT32 (Figure 1) as a pair and the final product was subcloned into T vector.

For 3' RACE, cDNA was reverse transcribed from mRNA by using an oligo-dT adapter primer MIN109 (5'-GGCCACGCCGTCTGACTAGTAC-3'). Specific cDNA was then amplified by PCR using gene-specific primer pGAT52 in clone c13-4a and an adapter primer MIN121 (5'-GGCCACGCGCGGTCTGACTAG-3'). The locations of the primers are indicated in Figure 1.

**Amplification of the Open Reading Frame by RT-PCR**— The first strand cDNA was synthesized with gene-specific primer pGAT59 (5'-CATATTTTTGTTGAGGAGTGTG-3') located at the 3' untranslated region (Figure 1). The full length of the coding region was amplified by primer pGAT66 (5'-GCCCGATCTAAAGCCATGGCCACCAACAG-3') and primer pGAT68 (5'-TACACTTAATAAAAAGATCTACATATGCTTCGTCCTTG-3') using an Expand Long Template PCR kit (Boehringer Mannheim) according to the manufacturer’s protocol. The primers introduced a BglII site at each end of the open reading frame. The PCR product was cut with BglII and cloned into the BglII site of the pSP64T vector [42]. The orientation of the insertion was confirmed by PCR using primers Sp6 and pGAT41 (5'-CACAAGAACCAGCGATGG-3').

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Figure 1. Diagram depicting the relative position of six partial clones with respect to the RT-PCR fragment. Arrows represent the location and orientation of primers used in PCR, RACE and RT-PCR. Dark lines denote nucleic acid sequence of each clone, and the gray line indicates the incompletely sequenced 3' RACE product. (ATG: start codon; TAG: stop codon).
Sequencing—All isolated clones were restriction mapped and further characterized by direct DNA sequencing. Sequencing was performed by the dideoxy chain termination technique (Sequenase kit, United States Biochemical, Cleveland, OH) using a double-strand sequencing protocol involving α-32P-dATP incorporation. Sequencing reactions were routinely analyzed on 5% denaturing polyacrylamide gels. Atypical sequencing gel yielded 300-400 bases. Sequence data were digitized and analyzed with PCGene software ( IntelliGenetics, Mountain View, CA).

Sequence Analysis Using PCGene Software—Amino acid sequence translated from compiled skate retinal GAT-1 cDNA was aligned with all GAT-1 amino acid sequences from different species available in the GenBank database by using the Clustal alignment function and a dendrogram was constructed afterwards. The alignment parameters were as follows: K-tuple value: 1, Gap penalty: 5, Window size: 10, Filtering level: 2.5, Open gap cost: 10, Unit gap cost: 10. The homology of each domain of SGA T-1 was compared to those of mouse GAT-1-4 as well.

Northern (RNA) Blot Analysis—Total RNA was isolated from skate retina, brain, heart, stomach, kidney, spleen, liver, skeletal muscle, and skin using Trizol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer’s protocol. Forty micrograms of each total RNA was run on a 1% agarose gel containing 6.7% formaldehyde in buffer of 20 mM 3-[N-morpholino]propanesulfonic acid, pH 7.0, 8 mM sodium acetate, 1 mM EDTA. The gel was stained with ethidium bromide and the RNA was transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) by capillary diffusion in 2X SSC. Transcript size was determined by comparison to an RNA molecular weight ladder (Life Technologies).

The blot was probed with α-32P-labeled 1 kb fragment that contains 800 bp of the 3’ end of the open reading frame. The probe was prepared by random priming (Boehringer Mannheim random primed DNA labeling kit) and hybridized to the blot under high stringency conditions. The hybridization solution contained 50% formamide, 5X SSC, 1X Denhardt’s solution, 0.5% SDS, and 0.5 mg/ml salmon sperm DNA; the hybridization was performed at 45 °C. The blot was washed at 65 °C sequentially in buffers containing 0.5% SDS plus 2X SSC, 0.5X SSC and 0.1X SSC for 30 min, coupled to X-ray film (Kodak X-Omat AR, Rochester, NY) at -70 °C for 5 days.

Figure 2 (Right). Nucleotide (GenBank Accession number AF049108) and amino acid sequences of the skate retinal GABA transporter. The 12 putative transmembrane domains are in bold face and underlined, and numbers denoting them are on the left. Potential N-glycosylation sites are indicated by asterisks. Potential phosphorylation sites by protein kinase C are indicated by triangles, and by casein kinase II are indicated by dots underneath their residues. Intron positions are indicated by arrows above the nucleotide sequence, and the poly(A) signal is double underlined. The number of the nucleotides starting from the 5’ RACE product is labeled on the right side and the number of the deduced amino acids is labeled below, starting at the ATG. The dashed line represents the unsequenced portion of the 3’ RACE product. Alternative versions of the sequences are available.
with intensifying screens.

**Determination of Intron Locations by PCR Amplification**— Fragments of skate genomic DNA were amplified by PCR using an Expand long template PCR kit (Boehringer Mannheim). The reactions were performed with 250 ng genomic DNA and 200 ng of each primer in a 50 µl reaction mixture. The putative intron positions were determined by comparing the intron positions in mouse and human GAT-1 and the primers were selected using the PCGene program. Amplification of fragments from cDNA clones was used as positive controls. PCR products containing the GAT-1 introns were either directly sequenced or subcloned into pGEM-T vector (Promega, Madison, WI) and then sequenced using vector-specific primers.

**Southern Blot Analysis of Skate Genomic DNA**— Genomic DNA was isolated from the brain of a single skate (Raja erinacea) by published protocols [43]. Fifteen microgram aliquots were digested with each of the following enzymes: BamHI, EcoRI, Ncol, PstI and XbaI. The enzymes were chosen based upon analysis of the entire SGAT cDNA sequence; no sites for these enzymes were detected. After complete digestion, each digested DNA sample was run on a 0.8% agarose gel and subsequently transferred to a Nytran Plus membrane (Schleicher & Schuell) by capillary diffusion in 0.4 M NaOH and 10X SSC.

The blot was probed under moderate stringency conditions with a DNA fragment containing the entire open reading frame. The fragment was released from the RT-PCR clone by BglII digestion and labeled with α-32P-dATP by nick translation. The blot was probed for 24 h at 42 °C in a solution containing 50% formamide, 1.5X SSPE, 0.5% nonfat dry milk, 2% SDS and 0.5 mg/ml salmon sperm DNA. The blot was washed at 55 °C in buffers containing 0.5% SDS plus 2X SSC for 30 min and then in 0.5% SDS plus 0.5X SSC for 15 min, and coupled to Kodak Biomax MS film at -70 °C for 7 days with intensifying screens.

### RESULTS

**Isolation of GABA Transporter cDNA Clones**— Low stringency screening of a skate retinal cDNA library with a mouse retinal GAT-1 clone [44] yielded 63 primary clones. Twenty of these were selected for further purification, and three partial clones with high sequence homology to the mouse retinal GAT-1 were finally obtained. The 600 bp clone, c11-1, contained predicted transmembrane domains III to VII (Figure 1). The 1 kb clones, c13-4a and c12-2b, contained 800 bp of the open reading frame from the middle of transmembrane domain VIII to the C terminus and 296 bp (clone c13-4a) or 79 bp (clone c12-2b) of 3’ untranslated region (Figure 1). Because these clones did not cover the entire coding region, the remaining 43 primary clones were screened under high stringency conditions using c11-1 as a probe. Another partial clone, c7-1 (Figure 1), which covered transmembrane domains I to VII, was isolated. However, the middle portion of the cDNA (between transmembrane domains VII and VIII) was still missing. To obtain this portion of cDNA, the PCR technique was employed to amplify this missing fragment directly from the skate retinal cDNA library. A 386 bp fragment was produced with 103 bp and 72 bp overlap with clones c11-1 and c13-4a (Figure 1), respectively. Its sequence showed a high degree of homology to mouse retinal GAT-1 cDNA. The remainder of the sequence was completed by RACE. 5’ RACE produced a 769 bp fragment which completed the 5’ end of the coding region and contained a 680 bp 5’ untranslated region as well. 3’ RACE generated 2 kb of the 3’ untranslated region containing a poly (A) signal. The full coding region was obtained by RT-PCR and subcloned into the SP64T vector. The relative position of the clones is diagrammed in Figure 1, and the primers used for the PCR, RACE and RT-PCR are

### Table I. COMPARISON OF AMINO ACID SEQUENCES OF SKATE AND MOUSE GABA TRANSPORTERS

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<th>Percentage of Identity</th>
<th>Percentage of Similarity</th>
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</tr>
<tr>
<td>mGAT1</td>
<td>100</td>
</tr>
<tr>
<td>mGAT2</td>
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</tr>
<tr>
<td>mGAT3</td>
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<tr>
<td>mGAT4</td>
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</table>

The predicted amino acid sequence of the skate GABA transporter was aligned with mouse GAT-I-4. The numbers in red (left of ‘100’ diagonal) are the percentage of identity calculated by dividing the number of identical amino acids by the total number of amino acids. Numbers in blue (right of ‘100’ diagonal) are the percentages of similarity that include identical amino acids and conserved substitutions.
indicated also. All of the clones were fully sequenced (indicated by dark lines in Figure 1) except for the 3' RACE product (gray line in Figure 1), where only 200 bp of both the 5' and 3' ends were sequenced.

Figure 2 shows sequences compiled from the different clones as well as the translation of the predicted open reading frame of 1,797 bp. A number of features identify this as a member of the GABA transport family. First, hydrophobicity analysis [45] revealed 12 regions that may represent transmembrane domains (bold face and underlined in Figure 2); thus the secondary structure prediction for the skate retinal GABA transporter is very similar to that of other members of the Na+ - and Cl- -dependent co-transporter family. Second, there is a large extracellular loop between transmembrane domains III and IV. Third, six consensus sites for potential phosphorylation obtained by PCGene analysis are present on the cytoplasmic N- and C- terminal regions and intracellular loop II. There is a protein kinase C phosphorylation consensus site (S5, indicated by triangles) and a casein kinase II phosphorylation consensus site (T22, indicated by dots) at the N terminus. Another protein kinase C phosphorylation consensus site was found at intracellular loop II (T35), and two protein kinase C phosphorylation consensus sites (T557 and S561) and one casein kinase II phosphorylation consensus site (T591) were located at the C terminus. Furthermore, three potential N-glycosylation sites (N175, 180, 183, indicated by asterisks in Figure 2) [46] were found on the large extracellular loop between potential membrane domains III and IV. Thus the predicted 12 transmembrane domain structure characteristic of GABA transporters is likely to be conserved in the protein coded by this sequence.

Homology of Skate GABA Transporter to Other GABA Transporters—To determine the relationship of the isolated skate GABA transporter to other GABA transporters, the amino acid sequence was aligned and compared with mouse GAT-1-4 using the Clustal program in the PCGene software (Figure 3). The percentage of identity was calculated as the number of identical amino acid residues divided by the total number of amino acids after the alignment. The percentage of similarity included conservative substitutions. The comparison revealed that the isolated skate GAT clone shared the greatest homology with mouse GAT-1, with over 80% identity at the amino acid level as compared with only 55% identity to mouse GAT-2-4. If the conservative substitutions are counted also, the percentage of similarity is increased to 90% to mouse GAT-1, while only 65% compared to mouse GAT-2-4 (Table I). Consequently, the isolated cDNA is designated as SGA-T-1. The comparison of the identity of each domain demonstrated that the regions from the middle of the first transmembrane domain to the beginning of the second transmembrane domain (aa61-81; IGLGNVWRFPYLCNKNGGGAF) and the fifth extracellular loop, loop IX (aa442-456: GL YVFKLF-DYYSAS) are the most conserved; the N and C termini are most variant (Figure 3).
Figure 5. Dendrogram of a multiple amino acid sequence alignment of all the GABA transporter sequences available in the database. The alignment was created with Clustal program of PCGene. The dendrogram plots the similarity of the sequences to each other based on the Dayhoff MDM-78 amino acid similarity matrix. The distances of the horizontal lines represent the similarity of one GABA transporter compared to the next one (HGA T: human brain; RGA T: rat brain; MGA T: mouse brain; MRGA T: mouse retina; TGA T: Torpedo electromotor nucleus; SGA T-1: skate retina; DBGT: dog betaine/GABA transporter).

SGAT-1 showed extensive homology with GAT-1 sequences obtained from human, rat, and mouse brain, mouse retina, and Torpedo electromotor nucleus (Figure 4). This high degree of homology is maintained throughout the species examined and distributed throughout the whole sequence. Similar to Torpedo GAT-1, SGAT-1 lacks a lysine at position 37. A dendrogram, based on multi-alignment of the full SGAT-1 protein sequence with all GABA transporters available in the database clearly revealed that SGAT-1 belongs to the GAT-1 group and it is closest to Torpedo GAT-1 (Figure 5) with 94% amino acid identity and 97% similarity.

Tissue distribution of SGAT-1— The tissue distribution of SGAT-1 expression was examined by northern (RNA) blot analysis. Total RNA was isolated from a number of skate tissues and probed with the c13-4a clone (Figure 1), covering the C-terminal of the GABA transporter. This probe recognized two transcripts (4.5 and 7.0 kb) in the retina and possibly brain (Figure 6A). Probe hybridization was faint but detectable for brain RNA. The amount of RNA loaded in each lane is represented by the ethidium bromide staining of the ribosomal RNA bands. The lower amount of brain RNA loaded into the lane may account for the lower degree of signal detected in brain as compared to retina (Figure 6B). Probe hybridization was not evident in RNA from heart, stomach, kidney, liver, spleen, skin, or skeletal muscle, in spite of the relatively long exposure time (5 days). The smaller transcript (4.5 kb) was more strongly labeled than the larger (7.0 kb) transcript (Figure 6A).

The Gene Structure of SGAT-1— Figure 7 shows a Southern blot of skate genomic DNA digested with the restriction enzymes BamHI, EcoRI, NcoI, PstI and XbaI, and probed with the SGAT-1 coding region. The averaged sum of labeled fragment sizes is about 30 kb. However, the transcripts detected on the RNA blot were only 4.5kb and 7.0kb. This suggests the presence of introns in this gene.

The putative intron positions were estimated by aligning the SGAT-1 with mouse and human GAT-1, since the intron positions in these two species have been determined, although they are not identical [23,47]. Fourteen pairs of primers were made and the PCRs were performed on the skate genomic DNA. All fourteen amplified fragments were longer than control fragments amplified from the cDNA, indicating that at least fourteen introns were present in the SGAT-1 gene. Two large introns (10 kb) were found between predicted transmembrane domains IX and XI. The precise positions of all introns were determined by DNA sequencing and are indicated in Figure 2 by arrows. One intron is located prior to the ATG translation initiation site. Table II shows the size of the various introns and the sequences of the exon-intron junctions. All determined splice donor and acceptor sequences contained the consensus [48] splice signal GT at 5' and AG at 3' of each intron except the first intron in the 5' untranslated region, which is GC at 5' and AG at 3'.

Several GABA transporter cDNAs have been cloned from both vertebrate and invertebrate animals [35,36,38,47,49]. These transporters appear to be members of a larger family of sodium- and chloride-dependent neurotransmitter transporters, which include carriers for glycine, proline, taurine, dopamine, and serotonin [18,50,51]. The common features of this family are a secondary structure believed to be composed of 12
hydrophobic transmembrane domains, both amino- and carboxy- termini located in the intracellular side, and putative glycosylation sites on the large extracellular loop between transmembrane domains III and IV.

Starting with low stringency screening of a skate retinal cDNA library using a mouse retinal GAT-1 probe, and then using PCR from a cDNA library and RACE amplification of the 5' and 3' untranslated regions, we cloned a GAT-1-like GABA transporter, named SGAT-1. It contains 598 amino acids and has one amino acid deletion in the N-terminus compared to mouse retinal GAT-1. This deletion is located at K37. Like other members in the Na+- and Cl- -dependent transporter family, hydrophobicity analysis shows that SGAT-1 contains 12 putative transmembrane domains and a large extracellular loop between transmembrane domains III and IV. Sequence alignment reveals high homology (over 80% amino acid identity) to the GAT-1 from other species, including human, rat, mouse, and Torpedo. This homology is throughout the whole sequence. The most conserved region is S55 to F86, corresponding to the second half of transmembrane domain I through the first half of transmembrane domain II, where 31 amino acids are identical. This result is consistent with previous studies showing a high degree of similarity in this region [18]. The second most highly conserved region is the fifth extracellular loop and the first half of transmembrane domain X (Y444-E464). Three potential glycosylation sites located between transmembrane domains III and IV are conserved. Utilization of these three glycosylation sites was recently confirmed in rat GAT-1 by N-glycosylation screen mutagenesis [50]. The potential intracellular phosphorylation sites by protein kinase C and casein kinase II also were conserved. All the critical residues (Typtophan 68, 222, 230, Arginine 69 and Glutamate 101) necessary for the function and GABA binding [52-54] are well conserved.

To determine the distribution of mRNA encoding SGAT-1, northern blots were carried out using skate tissue. Two transcripts were detected only in skate retina and very faintly in brain. The larger transcript is about 7.0 kb and the smaller transcript is about 4.5 kb. The size of the small transcript is similar to the transcripts detected in human, rat, and Torpedo GAT-1 [25,27,35]. Adding the 680 bp of the 5' untranslated region obtained by 5' RACE, 2 kb of the 3' untranslated region obtained by 3' RACE, and the 1.8 kb of the coding region, yields a transcript 4.5 kb in size. This is in agreement with the size of the smaller transcript detected by northern blot analysis. The 7.0 kb band is not likely to be the transcript of another member in the Na+- and Cl- -dependent transporter family even though there is high homology throughout this family, because the hybridization was done under high stringency conditions. However, it may result from initiation of transcription at an upstream site or utilization of a poly(A) signal downstream from the predicted poly A site. Alternatively, it may indicate the existence of an alternative splicing mechanism. Multiple transcription initiation sites have been reported in rat liver betaine/GABA transporter [55]. It is also worth noting that Nelson et al. detected a 6.5 kb band when rat brain mRNA was probed with human GAT-1 [25].

The dendrogram of the multiple amino acid alignment revealed that the SGAT-1 belongs to the GAT-1 group and appears to be most closely related to the Torpedo GABA transporter (TGAT-1). In situ hybridization studies in the electromotor nucleus of Torpedo show that TGAT-1 is restricted to glial cells [27]. However, studies examining the distribution of GAT-1 in mammalian brain have suggested that GAT-1 is primarily, although not exclusively, found in neuronal populations [38,56,57]. In situ hybridization and immunoreactive investigations examining the distribution of GAT-1 within the mammalian retina have shown that GAT-1 is mainly associated with amacrine cells and ganglion cells, and weakly expressed by Müller cells [32,44,58-61]. In salamander retina, polyclonal antibodies directed against the C-terminus of rat GAT-1 stain amacrine cells, interplexiform cells, and cells in the ganglion cell layer, as well as a subpopulation of bipolar cells, but no labeling is seen in either horizontal or Müller cells [62]. This raises the question of which cells in the retina of the skate express the SGAT-1 protein and what its precise role might be. Electrophysiological experiments have provided clear evidence for the presence of
GABA transporters in both horizontal cells (neural elements receiving input directly from the photoreceptors), and Müller cells (the radial glial cells) in the skate retina [63,64]. Autoradiographic experiments also have demonstrated the presence of a GABA transport mechanism in Müller cells of the skate. It has been suggested that the GABA transporter of horizontal cells may regulate the release as well as uptake of GABA [63,65]. In this context it is interesting to note that, in all species so far examined, GAT-1 transporters have not been observed to be present in horizontal cells. It may be that horizontal cells possess a distinct GABA transport mechanism geared specifically for the dual role of uptake and release. The cellular localization of SGAT-1 in skate retina needs to be further investigated by in situ hybridization and immunolocalization studies. Its precise physiological role remains to be established.

The gene structure of SGAT-1 was determined by Southern blot and PCR techniques. Fourteen introns were identified from the skate genomic DNA and precisely located. The total intron size is about 40 kb. Adding the transcript size of the 4.5 kb signal detected on the RNA blot, the gene size would be over 44 kb. The averaged sum of labeled fragment sizes is about 30 kb, which is smaller than the total intron size. The difference in the estimated sizes is very likely due to the presence of restriction enzyme sites within the two large introns. Since the probe only contained the coding sequences, those internal fragments were not accounted for. Like mouse GAT-1 gene, there is an intron in the 5′ untranslated region. The position of the first intron in the coding region is similar to that of the mouse gene, while the remaining intron positions in the coding region are in agreement with the human gene. The first intron located prior to the translation initiation site. The position of the first intron in the coding region needs to be further investigated by in situ hybridization and immunolocalization studies. Its precise physiological role remains to be established.

The gene structure of SGAT-1 is complex. It has 14 introns with the first intron located prior to the translation initiation site. The position of the first intron in the coding region is similar to that of the mouse gene, while the remaining intron positions in the coding region are in agreement with the human gene. Most exons encode only one transmembrane domain. It has been proposed that genes evolve via intron-mediated recombination of exon modules that code for functional or structural elements [67-69]. However, there is no absolute correlation between intron-exon structure, topological organization, or functionality since the 12 putative transmembrane domains of the mouse and human GABA transporters are encoded by 10 and 11 exons, respectively. In the SGAT-1 gene, like the human GAT-1 gene, the 12 putative transmembrane domains are coded by 11 exons.

In summary, a GAT-1 like cDNA, named SGAT-1, was cloned from the skate retina. It codes for a 598 amino acid sequence that has over 80% identity to other GAT-1s. The predicted secondary structure is also very similar to that of other GAT-1s. RNA blot shows that SGAT-1 is mainly expressed in the retina along with a weak expression in the brain. Like the human and mouse GAT-1 gene, the gene structure of SGAT-1 is complex. It has 14 introns with the first intron located prior to the translation initiation site. The first intron position in the coding region is similar to that of the mouse gene, while the remaining intron positions in the coding region are in agreement with the human gene. The cellular localization of the SGAT-1 messenger is currently being examined by in situ hybridization.

### ACKNOWLEDGEMENTS

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### REFERENCES


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#### Table II. Size and Junction Sequences of Introns in the Skate GAT1 Gene

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<th>Intron</th>
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<td>mouse</td>
</tr>
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<td>tccaagGGGCA</td>
<td>mouse / human</td>
</tr>
<tr>
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<td>tccaagGGCGG</td>
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</tr>
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</tr>
<tr>
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<td>ctgcagGGCA</td>
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</tr>
<tr>
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<td>cccagGTGGT</td>
<td>human / mouse</td>
</tr>
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<tr>
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</tr>
<tr>
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<td>ttcagGTCCCTG</td>
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<tr>
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</table>

The introns in the SGAT-1 gene were obtained by PCR from the skate genomic DNA and the intron positions were determined by sequencing. The exon sequence is shown in the upper case and the intron sequence is shown in the lower case. The intron positions in the SGAT-1 coding region were compared with that in the human and mouse GAT-1 genes as shown in the rightmost columns.


27. Swanson GT, Umbach JA, Gundersen CB. Glia of the cholinergic electromotor nucleus of Torpedo are the source of the eDNA encoding a GAT-1-like GABA transporter. J Neurochem 1994; 63:1-12.


