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A novel invertebrate $GABA_A$ receptor-like polypeptide

Sequence and pattern of gene expression

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A full-length complementary DNA has been isolated, from the fresh-water mollusc *Lymnaea stagnalis*, that encodes a polypeptide (which we have named ζ) that exhibits between 30% and 40% identity to vertebrate $GABA_A$ and glycine receptor subunit sequences. The locations of seven introns have been determined in the corresponding gene and six of these occur at similar relative positions as those found in vertebrate $GABA_A$ receptor genes. RNase protection studies have revealed that the transcript for this Lymnaean polypeptide is present at highest levels in the adult nervous system but that it can also be detected in peripheral tissues.

cDNA cloning; $GABA_A$ receptor-like subunit; Gene organization; Invertebrate ligand-gated ion channel; *Lymnaea stagnalis*; RNase protection

1. INTRODUCTION

Electrophysiological studies on identified molluscan neurons [1,2] have strongly suggested that γ-aminobutyric acid (GABA) can bind to, and cause the opening of, chloride-ion channels that are similar to the well-characterized vertebrate GABA type A ($GABA_A$) receptors. In support of this proposal, we have recently described [3] the cloning, from the fresh-water snail *Lymnaea stagnalis*, of a complementary DNA (cDNA) that encodes a $GABA_A$ receptor-like subunit. The predicted amino-acid sequence of this polypeptide displays significantly greater identity to β subunits ($\sim$ 50%) than to any other subunit type of the vertebrate brain $GABA_A$ receptor. Functional expression of the molluscan cDNA alone, in *Xenopus* oocytes, results in the formation of GABA-activated, bicuculline- and picrotoxin-sensitive chloride-ion channels. Furthermore, the molluscan polypeptide is able to functionally replace vertebrate β subunits in $GABA_A$ receptors containing, in addition, the bovine α1 subunit [3]. This latter observation strongly suggests that Lymnaean GABA-gated chloride channels exist in vivo as hetero-oligomers. We have, therefore, begun to isolate cDNAs that encode additional Lymnaean $GABA_A$ receptor-like subunits. This report describes the isolation of a full-length cDNA, and genomic sequences, for one such molluscan polypeptide. We also present data on the developmental and tissue-specific expression of the corresponding gene.

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Abbreviations: cDNA, complementary DNA; GABA, γ-aminobutyric acid; PCR, polymerase chain reaction; RNase, ribonuclease.

2. MATERIALS AND METHODS

2.1. *Isolation of a molluscan full-length $GABA_A$ receptor-like cDNA*

Five degenerate oligonucleotides: $D1_a$ (5′-ATTGTCGACTITCX-TGGGTX(A/T)(C/G)XTT(C/T)TGG-3′; where X = G, A, T and C, and I = inosine), $D1_b$ (5′-ATTGTCGACTIAG(C/T)TGGGTX(A/T)(C/G)XTT(C/T)TGG-3′), $D3_a$ (5′-ACGGTCGACGCIA(A/G)(A/G)AAIAC(A/G)AAX(A/G)C(A/G)(A/T)A(A/G)CA-3′), $D3_b$ (5′-ACGGTCGACGCIGC(A/G)AAIAC(A/G)AAX(A/G)C(A/G)(A/T)A(A/G)CA-3′) and $D3_c$ (5′-ACGGTCGACGCI(C/G)(A/T)(A/G)AAIAC(A/G)AAX(A/G)C(A/G)(A/T)A(A/G)CA-3′), which recognize DNA sequences that encode parts of the first (primers $D1_a$ and $D1_b$) and third (primers $D3_a$, $D3_b$ and $D3_c$) membrane-spanning domains of $GABA_A$ receptor subunits, respectively, were used to amplify first-strand cDNA synthesized from *L. stagnalis* egg-mass (mixed-stage) poly(A)$^+$ RNA using oligonucleotide $R_oR_iT_{17}$ [3] as primer. Six separate polymerase chain reactions (PCRs), each using one D1 and one D3 primer, were performed; these contained (in 50 μl): 50 mM KCl, 10 mM Tris-HCl (pH 9 at 25°C), 1.5 mM $MgCl_2$, 200 μM each dNTP,

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0.1% (v/v) Triton X-100, 0.01% (w/v) gelatin, ~ 20 ng of first-strand cDNA, 25 pmol of each primer, and 2.5 units of *Thermus aquaticus* DNA polymerase (Promega). Amplification was for 35 cycles of 94°C for 1 min (denaturation), 55°C for 1 min (annealing), and 72°C for 1 min (extension), with a final extension at 72°C for 10 min. The products of the six reactions were pooled, and DNA fragments of ~ 200 bp in size were cloned into M13mp19, using the *Sal*I site (underlined) that was incorporated into the 5′ end of each primer, and sequenced. Three novel cDNAs were isolated and the sequence of one of these was used to design oligonucleotides (GRS1–GRS3) that were employed to generate corresponding full-length cDNAs (see below).

The 3′ end of the cDNA was amplified using the RACE technique [4]. Briefly, first-stage PCR was performed with $R_oR_idT_{17}$-primed egg-mass first-strand cDNA as template and primers R_o [3], which corresponds to part of $R_oR_idT_{17}$, and GRS1 (5′-AGTGCCTGGAATTC-TGTCGACGGGACTGCTCACGGTGCTGACCATG-3′). Reaction conditions were as above except that 1 nmol of T4 gene 32 protein (United States Biochemical) was included, and amplification was for 40 cycles of 94°C for 1 min (denaturation) and 72°C for 3 min (annealing and extension), with a final extension at 72°C for 10 min. For second-stage PCR, primers R_i [3], which also corresponds to part of $R_oR_idT_{17}$, and GRS2 (5′-ACCCAAAGGTCGACTCTAGATGCT-GCCCTGCCCAGGGTTTCCTAC-3′) were used. Reaction conditions were as described for the first-stage amplification except that 2

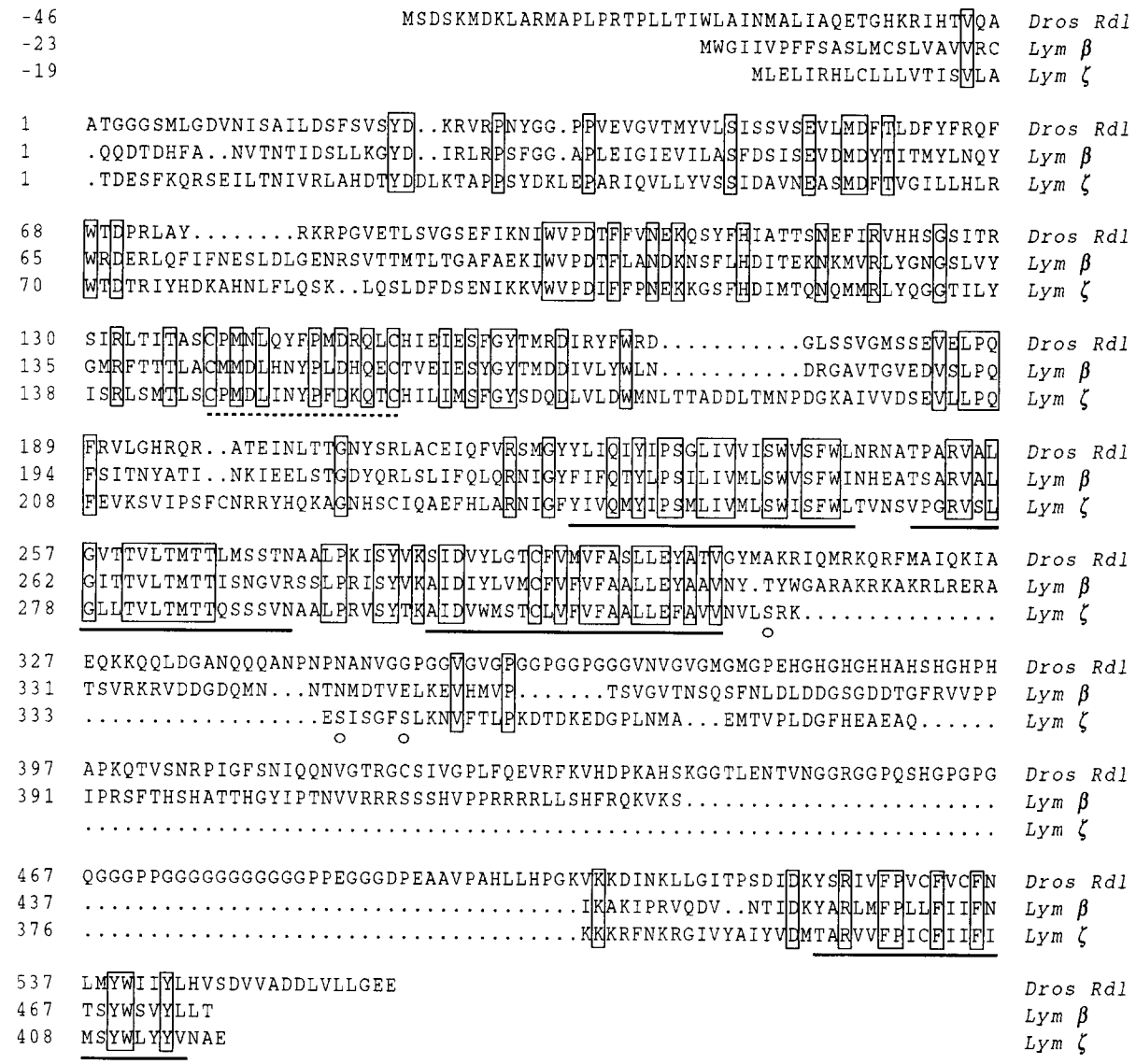

Fig. 1. Alignment of the amino-acid sequence of the molluscan (*L. stagnalis*) $GABA_A$ receptor-like ζ subunit (*Lym ζ*) with those of the Lymnaean $GABA_A$ receptor β subunit (*Lym β*; [3]) and the *Drosophila* $GABA_A$ receptor-like *Rdl* subunit (*Dros Rdl*; [16]). The sequences (in single-letter code) were aligned using the computer program PILEUP [26]; dots denote gaps that have been introduced to maximize the alignment. Positions at which the same amino acid is found in all three sequences are boxed. Residues are numbered from the mature amino-terminus of each polypeptide and signal peptides [9] are indicated by negative numbering. The dicysteine loop in the presumed extracellular domain, that is found in all $GABA_A$ receptor subunits, is indicated by a broken line, and the four putative membrane-spanning domains (M1–M4) are underlined. Potential protein kinase C (serines 330 and 339) and multifunctional calmodulin-dependent protein kinase II (serine 334) phosphorylation sites, in the putative large intracellular loop of the ζ subunit, are marked by open circles. The polypeptide sequence of the Lymnaean $GABA_A$ receptor-like ζ subunit was derived from one (plLG13.PCR1) of three amplified full-length cDNAs, the sequence of which has been given the EMBL accession number X71357.

μl of the first-stage PCR products were substituted for the first-strand cDNA. Products were cloned into M13mp18 using the *Xba*I site incorporated into GRS2 (underlined) and the *Hin*dIII site of primer R_i.

The 5′ end of the cDNA was amplified directly [5,6] from a *L. stagnalis* egg-mass cDNA library constructed in λZAPII. Briefly, amplification was performed on DNA from ~ 10^7 p.f.u. using one of two vector-based primers (either λZAPa: 5′-CGAGGTCGACGGTATC-GATAAGCTTGATAT-3′ or λZAPb: 5′-CGGTGGCGGCCGCTC-TAGAACTAGTGGATC-3′) in combination with GRS3 (5′-TCGT-GTAGGAATTCGTCGACAGGGCAGCATTGACGCTTGAGCT-TT-3′). Reaction conditions were identical to those for 3′-end cDNA amplification. Products were cloned into M13mp18, using the *Sal*I site incorporated into GRS3 (underlined) and the *Eco*RI site in the linker that was used in the construction of the cDNA library, and sequenced.

Finally, full-length cDNAs were amplified using primers GRS4 (5′-TTTTCCCTGCAGCTCGCCAATCATGTTGGAATTAATTA-GAC-3′) and GRS5 (5′-ACTTGCTGTCGACATCATTGCCTCGA-AATACACTACTTG-3′) which were based on sequences flanking the coding region. The reaction contained 25 pmol of each primer and ~ 20 ng of $R_0R_1dT_{17}$-primed egg-mass first-strand cDNA. Amplification was for 40 cycles of 94°C for 1 min (denaturation), 65°C for 1 min (annealing), and 72°C for 2 min (extension), followed by a final extension at 72°C for 10 min. Products were cloned into pBluescript KS+ (Stratagene), using restriction endonuclease sites (underlined) incorporated into GRS4 and GRS5, and sequenced.

2.2. *Isolation of genomic DNA sequences and mapping of intron/exon boundaries*

Approximately 5×10^5 clones of an amplified *L. stagnalis* genomic library, constructed in λEMBL3, were screened with a 1,429 bp *Pst*I/*Sal*I cDNA fragment that encodes all of the molluscan polypeptide. Hybridization was in 6 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7), 0.5% (w/v) SDS, 5 × Denhardt's solution, and 100 μg/ml yeast tRNA at 60°C for 24 h. The final wash conditions were 0.1 × SSC, 0.1% (w/v) SDS, at 60°C, for 30 min. One clone (λlLG13.1) was isolated and purified; DNA was prepared from this and sequenced directly using a linear amplification method [7] and oligonucleotide primers that were designed using the sequence of a full-length cDNA.

2.3. *In vitro transcription, oocyte injection and electrophysiology*

RNA for the molluscan polypeptide was transcribed from three independent full-length cDNAs, after linearization of the appropriate plasmid with *Sal*I, using T7 RNA polymerase (Pharmacia). In vitro transcription of other RNAs, oocyte injection, and electrophysiological methods were essentially as described previously [3,8].

2.4. *RNase protection*

A full-length cDNA (plLG13.PCR1) in pBluescript KS+ was digested with *Xho*I, to release a 381 bp fragment that encodes the majority of the fourth putative membrane-spanning domain and the 3′-untranslated region, and subsequently religated. The resultant plasmid was linearized with *Xba*I, which cuts within that part of the DNA encoding the carboxy-terminal part of the third membrane-spanning domain, and an [α-^{32}P]UTP-labelled anti-sense RNA was generated using T3 RNA polymerase (Pharmacia). The probe (253 nucleotides) comprises 224 nucleotides which are complementary to the region that encodes all of the large intracellular loop, and 29 nucleotides of the pBluescript KS+ polylinker; the latter permits protected fragments to be distinguished from undigested probe. The radiolabelled RNA was purified after electrophoresis in a 6% (w/v) polyacrylamide/urea gel, and RNase protection was performed using an RPA II kit (Ambion) according to the manufacturer's instructions. Each hybridization (45°C overnight) contained ~ 2.3 fmol (~ 7×10^4 cpm) of probe and either 10 μg of total RNA from different developmental stages and tissues or 10 μg of yeast total cellular RNA (as control). RNases were then added to give final concentrations of 0.09 units/ml for RNase A and 18 units/ml for RNase T1. Protected fragments were detected, after electrophoresis in a 6% (w/v) polyacrylamide/urea gel, by autoradiography. Note that the concentration of each RNA was quantified by ultra-violet spectroscopy to ensure that equal amounts were used, and the integrity was checked prior to use by electrophoresis in a 1% (w/v) agarose/formaldehyde gel.

3. RESULTS AND DISCUSSION

DNA sequence analysis of a 187 bp cDNA fragment, that was generated by degenerate PCR, revealed the presence of an open reading frame that encodes a portion of a polypeptide which exhibits significant identity (48%) to the second membrane-spanning domain (M2) and flanking sequences of the Lymnaean $GABA_A$ receptor β subunit [3]. This amino-acid sequence contains 7 of the 8 residues of the octapeptide (TTVLTMTT; single-letter code) which is present within the M2 segment of all $GABA_A$ receptor subunits and ligand-binding subunits of the glycine receptor; the presence of this domain in the molluscan polypeptide strongly suggests that it is part of a chloride-selective channel. The sequence of the PCR-generated fragment was subsequently used to design oligonucleotide primers which were employed in two variants of the PCR technique [4–6] to amplify three independent full-length cDNAs.

The complete sequence of one of the full-length

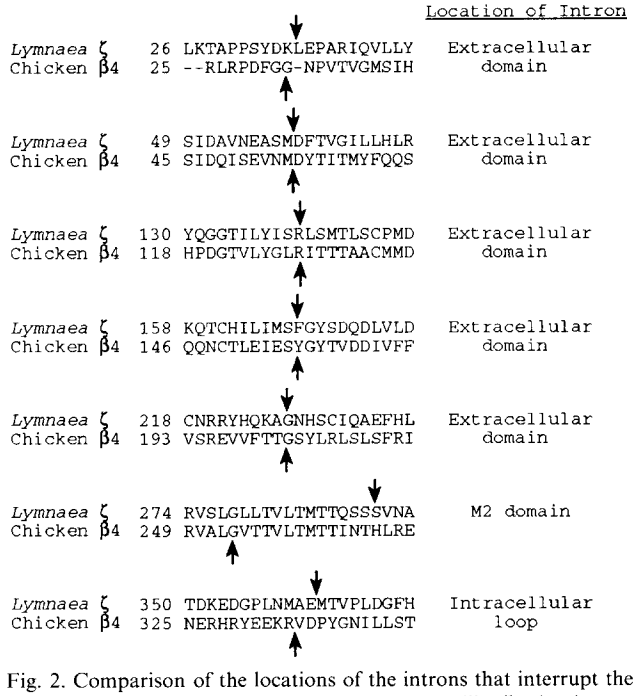

Fig. 2. Comparison of the locations of the introns that interrupt the coding region of the Lymnaean $GABA_A$ receptor-like ζ-subunit gene with those found in vertebrate $GABA_A$ receptor subunit genes. Note that only seven intron positions were determined for the molluscan gene. The relative locations of these (denoted by arrows), and those that interrupt the chicken $GABA_A$ receptor β4-subunit gene [19], are shown in aligned segments of the two amino-acid sequences (in single-letter code); dashes represent gaps that have been introduced to maximize the alignment. The amino-acid numbering for each polypeptide corresponds to that of the mature subunit and numbers refer to the left-most residue on each line.

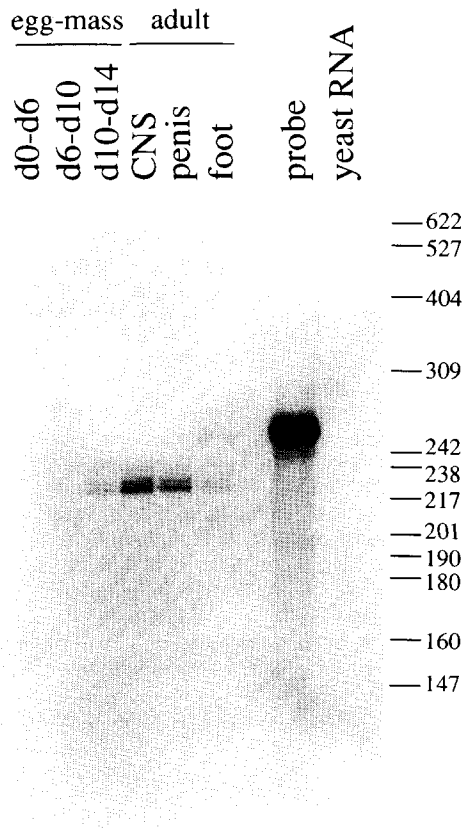

Fig. 3. RNase protection analysis of the Lymnaean $GABA_A$ receptor-like ζ-subunit transcript during development and in different tissues. Protection was performed using total RNA from 0- to 6-day egg-mass (d0–d6), 6- to 10-day egg-mass (d6–d10), 10- to 14-day egg-mass (d10–d14), adult nervous system (CNS), adult penis, and adult foot. Also shown are the undigested probe and the result of a control hybridization performed using yeast total cellular RNA. Size markers are *Msp*I-digested pBR322 restriction fragments that have been end-labelled with [γ-^{32}P]ATP, and run in parallel.

cDNAs (plLG13.PCR1) was determined; this contains an open reading frame of 1,311 bp that predicts a mature polypeptide of 418 amino acids (Fig. 1), with a calculated M_r of 47,880, and a 19 amino-acid signal peptide [9]. The mature protein exhibits significant sequence identity to vertebrate $GABA_A$ (30–33% to α subunits, 35–36% to β subunits, 31–33% to γ subunits, and 35% to the δ subunit) and glycine (40–41% to α subunits and 35% to the β subunit) receptor polypeptides [10–14]. It also displays 35%, 36%, 37%, and 41% identity to the vertebrate $GABA_A$ receptor-like ρ1 and ρ2 subunits [15], the previously-reported Lymnaean $GABA_A$ receptor β subunit [3], and the *Drosophila* $GABA_A$ receptor-like subunit named *Rdl* [16], respectively. Since we are unaware of any evidence in support of the existence of glycine receptors in molluscs, we presume that the polypeptide whose cDNA we have cloned is a novel GABA-gated chloride-channel subunit which we name ζ (zeta). Such a hypothesis is not so unlikely since the *Drosophila Rdl* subunit does not exhibit strong sequence similarity to any one type of vertebrate $GABA_A$ receptor subunit [17], yet it forms homo-oligomeric GABA-gated, bicuculline-insensitive, picrotoxin-sensitive chloride channels when the corresponding cDNA is expressed in *Xenopus* oocytes [18].

Interestingly, the Lymnaean ζ subunit contains in the presumed extracellular domain, in addition to the pair of cysteine residues found in all $GABA_A$ and glycine receptor subunits, a second pair of cysteines (C218 and C231) that are found in a very similar position to those in subunits of the vertebrate glycine receptor. These two cysteines are separated by 12 residues in the molluscan polypeptide, 10 in glycine receptor α subunits [11–13], and 11 in the glycine receptor β subunit [14]. It is also noteworthy that the ζ polypeptide has an 11 amino acid insertion in the presumed extracellular domain when compared to the Lymnaean $GABA_A$ receptor β subunit and the *Drosophila Rdl* subunit (residues 182–192; Fig. 1).

The positions of seven introns in the molluscan ζ-subunit gene have been determined. When these are compared to those of vertebrate $GABA_A$ receptor genes, which are known to have a conserved structure [19], six are found in identical or similar relative positions (Fig. 2). The seventh is found within the sequence that encodes the M2 domain, but it is located 39 nucleotides further 3′ compared to that found in vertebrate $GABA_A$ receptor genes [19] and the Lymnaean $GABA_A$ receptor β-subunit gene [3]. Interestingly, the corresponding intron in the *Drosophila Rdl*-subunit gene is also shifted; however, in this case, it interrupts the DNA sequence that encodes the third membrane-spanning domain [20]. The similarity in the organizations of the molluscan ζ-subunit gene and vertebrate $GABA_A$ receptor genes supports the proposal that the ζ polypeptide is a component of an invertebrate GABA-gated ion channel/receptor.

To test whether the Lymnaean ζ subunit is capable of forming a functional homo-oligomeric ion channel, RNA was transcribed in vitro from three different full-length cDNAs and injected, individually, into *Xenopus* oocytes. The sensitivity of these oocytes to the application of GABA, glycine, nicotine, L-glutamate, kainate, quisqualate, ibotenate, *N*-methyl-D-aspartate, histamine and 5-hydroxytryptamine was then investigated. No significant current responses to any of these agonists (each at up to 1 mM except for ibotenate which was used at up to 100 μM) were detected, using any of the RNAs (S.H. Zaman, unpublished results). In vitro-transcribed RNA for the ζ subunit was also co-injected with RNA transcribed from either the Lymnaean $GABA_A$ receptor β-subunit cDNA [3], the bovine $GABA_A$ receptor α1-subunit cDNA [8] or the bovine $GABA_A$ receptor β1-subunit cDNA [8]. Co-expression did not markedly affect the functional properties of Lymnaean $GABA_A$ receptor β-subunit homo-oligomeric receptors nor did

it result in functional molluscan (ζ-subunit)/bovine hybrid receptors (S.H. Zaman, unpublished results).

RNase protection experiments revealed two protected bands (one strong and one weak), that appeared to differ in size by a few nucleotides when hybridizations were performed with either 6- to 10-day or 10- to 14-day egg-mass RNA, or with adult nervous system, penis or foot RNA (Fig. 3). The same two bands were also seen when in vitro-transcribed sense RNA from one of the full-length cDNAs (plLG13.PCR1) was hybridized with the same radiolabelled RNA probe (data not shown). The size of the stronger band ($\sim$ 224 nucleotides) corresponds to that part of the probe which is specific for the molluscan ζ-subunit transcript. The presence of a slightly larger (weaker) protected fragment is thought to be due to incomplete RNase digestion of the short (29-nucleotide) vector sequence which was included to distinguish protected fragments from undigested probe. No protection was observed when the probe was hybridized to yeast total cellular RNA, and no signal was detected when 0–6-day egg-mass RNA was used (Fig. 3), even after an exposure of 7 days (data not shown). These data indicate that the ζ-subunit gene is developmentally regulated, and that while it is expressed at highest levels in the adult nervous system, significant amounts of the corresponding transcript are also present in peripheral tissues.

In summary, we have isolated a cDNA for a molluscan (*L. stagnalis*) $GABA_A$ receptor-like subunit (ζ) that does not appear to be the partner for the previously-reported [3] *Lymnaea* $GABA_A$ receptor β subunit. Thus, if the ζ subunit is, indeed, a component of a GABA-gated ion-channel receptor, then the mollusc would appear to contain at least two different $GABA_A$-like receptors. This is consistent with electrophysiological studies on other invertebrates, such as the moth *Manduca sexta*, that have revealed the existence of both bicuculline-sensitive and bicuculline-insensitive GABA receptors in nervous tissue (reviewed in [21]). However, there is considerable evidence to suggest that, in the molluscan nervous system, not only GABA but also glutamate [22,23] and acetylcholine [2,24] can gate chloride-selective channels. In addition, data in support of the existence of invertebrate histamine-gated chloride-ion channels has been presented [25]. The possibility remains, therefore, that the ζ polypeptide is not a GABA-gated chloride-ion channel/receptor subunit but a component of some other receptor complex.

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