Volume 331, number 3, 211–216 FEBS 13022 October 1993

Molecular cloning reveals the existence of a fourth γ subunit of the vertebrate brain $GABA_A$ receptor

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Received 24 July 1993

We have isolated a cDNA, from the chicken, that encodes a fourth type of γ subunit of the vertebrate brain $GABA_A$ receptor. The mature polypeptide (which we name $\gamma 4$) displays 67%, 69% and 70% identity, respectively, to the rat $\gamma 1$, $\gamma 2$ and $\gamma 3$ subunits. In the developing chicken brain, the $\gamma 4$-subunit mRNA is first detected at embryonic day 13; the transcript level then increases progressively during embryogenesis. In situ hybridization reveals that the $\gamma 4$-subunit mRNA is abundant in several brain regions, including the ectostriatum, nucleus rotundus and hyperstriatum ventrale, which are involved in visual processing and learning.

cDNA cloning; Chick brain; $GABA_A$ receptor γ subunit; Developmental gene expression; Hybridization, in situ; Visual processing

1. INTRODUCTION

γ-Aminobutyric acid (GABA) type A ($GABA_A$) receptors are multisubunit (probably pentameric) membrane-spanning complexes, that each contain an intrinsic chloride-selective ion channel, which mediate postsynaptic inhibition in the vertebrate brain. In mammals, complementary DNA (cDNA) cloning studies have revealed the existence of four types of subunit (named α, β, γ and δ), three of which occur in a variety of isoforms ($\alpha 1$–$\alpha 6$, $\beta 1$–$\beta 3$, $\gamma 1$–$\gamma 3$). Subtypes of the $GABA_A$ receptor are thought to be generated by the association of different combinations of these polypeptides, with most receptors containing an α, a β and either a γ or a δ subunit [1,2]. Unfortunately, attempts to elucidate receptor subtype compositions have, so far, been relatively unsuccessful. While a complete immunological characterization of $GABA_A$ receptors has not been possible because of a lack of specific antibodies for each subunit, in situ hybridization localization of all of the receptor mRNAs in the mammalian brain has been performed [3,4]. Although the latter studies have provided good evidence that one major $GABA_A$ receptor subtype is comprised of $\alpha 1$, $\beta 2$ and $\gamma 2$ subunits, no other three-subunit (i.e. α, β, and γ or δ) combinations could easily be deduced. However, the frequent co-localization of pairs of mRNAs (for example, those encoding the $\alpha 2$ and $\beta 3$, $\alpha 5$ and $\beta 1$, $\alpha 4$ and δ, and $\alpha 2$ and $\gamma 1$ subunits), was observed [3,4]. One plausible explanation for the inability to derive receptor subtype compositions from the in situ hybridization results is that other $GABA_A$ receptor polypeptides remain to be identified. We report here the sequence of a fourth $GABA_A$ receptor γ subunit, that has been deduced from a cloned full-length cDNA, and describe the developmental and spatial patterns of expression of the corresponding gene in avian brain.

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Abbreviations: Cbm, cerebellum; cDNA, complementary DNA; E, ectostriatum; GABA, γ-aminobutyric acid; HV, hyperstriatum ventrale; PCR, polymerase chain reaction; RSd, nucleus reticularis superior, pars dorsalis; Rt, nucleus rotundus; TeO, optic tectum.

2. MATERIALS AND METHODS

2.1. *Isolation of a full-length $GABA_A$ receptor $\gamma 4$-subunit cDNA*

First-strand cDNA was synthesized from embryonic day 20 chicken (*Gallus domesticus*) whole-brain poly(A)$^+$ RNA using the oligo(dT)-containing primer $R_0R_1dT_{17}$ [5]. A $GABA_A$ receptor $\gamma 4$-subunit partial cDNA was amplified from this using two degenerate oligonucleotides, 5′-TTAGAATTCTCTC(A,C)TGGGT(G,A,T,C)TC(G,A,T,C)TT-(T,C)TGG-3′ and 5′-GTAGGTCGAC(G,A)AA(G,A,T,C)AC(G,A)-AA(G,A,T,C)AC(G,A)AA(G,A)CA-3′, which are based on the DNA sequences that encode the peptide sequences ITILSWVSFW (located within the first membrane-spanning domain) and CFVFVF(L or M)AL (which is within the third membrane-spanning domain) of vertebrate $GABA_A$ receptor β subunits [6,7], in the polymerase chain reaction (PCR) as described [8]. The cDNA fragment (182 bp) was then used to screen ~1×10^6 clones of an amplified chicken cDNA library, constructed in λgt10 using a combination of embryonic day 12 and day 16 whole-brain poly(A)$^+$ RNA, at high stringency. Hybridization was in 6 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7), 0.1% (w/v) SDS, 5 × Denhardt's solution, 100 μg/ml yeast tRNA and 10% (w/v) dextran sulphate, at 65°C overnight; the final wash conditions were 0.1 × SSC, 0.1% (w/v) SDS, at 65°C for 1 h. 12 positively-hybridizing plaques were detected and purified; the insert of one of these was subcloned as an *Eco*RI fragment into pBluescript II SK+ (Stratagene), deleted using the Erase-a-Base system (Promega), and sequenced on both strands using an automated Applied Biosystems Model 373A DNA sequencer and universal dye primers.

2.2. *Northern blotting*

Total RNA was isolated from the whole brains of embryonic chicks at different developmental stages using guanidine isothiocyanate [9]. Approximately 50 μg of each RNA was electrophoresed in a 0.8% (w/v) formaldehyde/agarose gel, and transferred to Hybond-N nylon membrane (Amersham). Blots were hybridized with either an oligonucleotide (γ4T, 5′-GCTTTCCTGCTGCTGTGCTCCAGTGGCTTC-TTGTTTCCCACCAGG-3′), which is complementary to the DNA sequence that encodes part of the large intracellular loop, or a 496 bp cDNA fragment that encodes from the end of the third membrane-spanning domain to the carboxy-terminus of the γ4 subunit, and which also contains 114 bp of 3′-untranslated sequence. The oligonucleotide was 3′-end labelled with [α-^{32}P]dATP (6000 Ci/mmol; New England Nuclear) using terminal deoxynucleotidyltransferase (Gibco BRL), and the cDNA was labelled with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham) using a Prime-It II kit (Stratagene). The oligonucleotide hybridization and wash conditions were as previously described [8]. Hybridization with the cDNA (0.25 ng/ml of hybridization buffer) was performed in 6 × SSC, 0.1% (w/v) SDS, 5 × Denhardt's solution, 25 mM sodium phosphate, pH 7, 1 mM sodium pyrophosphate, 100 μg/ml polyadenylic acid and 100 μg/ml yeast tRNA, at 65°C for 18 h. The final wash conditions were 0.1 × SSC, 0.1% (w/v) SDS at 65°C for 1 h. After autoradiography, blots were stripped in 5 mM Tris-HCl, pH 8, 2 mM EDTA, 0.1 × Denhardt's solution at 65°C for 1 h, and then rehybridized with an ~1.9 kb chicken β-actin cDNA. The hybridization and wash conditions were the same as those used for the γ4-subunit cDNA.

2.3. *In situ hybridization*

In situ hybridizations were performed essentially as described [10] using the 45-base anti-sense oligonucleotides γ4T (see above), and γ2T, 5′-CATTTGAATAGTAGCTGATCGAGGTCGGATGTCAA-TTGTGGGTGC-3′ [11], which recognizes both alternatively-spliced forms of the chicken γ2-subunit mRNA. Briefly, oligonucleotides were 3′-end labelled with [α-^{35}S]dATP (~1200 Ci/mmol; New England Nuclear) using terminal deoxynucleotidyltransferase (Gibco BRL) to specific activities greater than 2×10^9 cpm/μg. Hybridizations (200 μl of buffer per slide) were performed, on horizontal 10 μm serial sections of embryonic day 18 chick whole brains, in 50% (v/v) formamide, 4 × SSC, 5 × Denhardt's solution, 25 mM sodium phosphate, pH 7, 1 mM sodium pyrophosphate, 100 μg/ml polyadenylic acid, 120 μg/ml heparin, 200 μg/ml acid/alkali-cleaved salmon sperm DNA, 20 mM dithiothreitol, 10% (w/v) dextran sulphate, with 1,250 cpm/μl of the appropriate radiolabelled oligonucleotide, at 42°C overnight. Sections

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Rat γ1                                                    MGSGKVFLFSPSLLWSQTRGVRLIFLLLTLHLGNC   -1
Rat γ2                                                MSSPNTWSTGSTVYSPVFSQKMTLWILLLLSLYPGFTS   -1
Rat γ3                                                      MAAKLLLLLCLFSGLHA   -1
Chk γ4                                                    MPAMVLLLCLALGPALRSARC   -1

Rat γ1  .IDKADDEDDEDLTMNKTWVLAPKIHEGDITQILNSLLQGYDNKLRPDIGVRPTVIETDVYVNSIGPVDP   69
Rat γ2  ...QKSDDDYEDYASNKTWVLTPKVPEGDVTVILNNLLEGYDNKLRPDIGVKPTLIHTDMYVNSIGPVNA   67
Rat γ3  RSRRVEEDDSEDSPSNQKWVLAPKSQDTDVTLILNKLLREYDKKLRPDIGIKPTVIDVDIYVNSIGPVSS   70
Chk γ4  ..ESTEEYDYDYLSINKTWVLTPKAQETDATQILNSLLKNYDNKLRPDIGIKPTFIDVDIYVNSIGPVSV   68

Rat γ1  INMEYTIDIIFAQTWFDSRLKFNSTMKVLMLNSNMVGKIWIPDTFFRNSRKSDAHWITTPNRLLRIWSDG  139
Rat γ2  INMEYTIDIFFAQTWYDRRLKFNSTIKVLRLNSNMVGKIWIPDTFFRNSKKADAHWITTPNRMLRIWNDG  137
Rat γ3  INMEYQIDIFFAQTWTDSRLRFNSTMKILTLNSNMVGLIWIPDTIFRNSKTAEAHWITTPNQLLRIWNDG  140
Chk γ4  IQMEYTIDIFFAQTWYDRRLRFNSTLKALTLNTNMVSRIWIPDTFFRNSKRADSHWITTPNQLLRIWNDG  138

Rat γ1  RVLYTLRLTINAECYLQLHNFPMDEHSCPLEFSSYGYPKNEIEYKWKKPSVEVADPKYWRLYQFAFVGLR  209
Rat γ2  RVLYTLRLTIDAECQLQLHNFPMDEHSCPLEFSSYGYPREEIVYQWKRSSVEVGDTRSWRLYQFSFVGLR  207
Rat γ3  KILYTLRLTINAECQLQLHNFPMDAHACPLTFSSYGYPKEEMIYRWRKNSVEAADQKSWRLYQFDFMGLR  210
Chk γ4  KVLYTLRLTIEAECLLQLQNFPMDTHSCPLVFSSYGYPREEIVYRWRRYSIEVSDQRTWRLYQFDFTGLR  208

Rat γ1  NSTEISHTISGDYIIMTIFFDLSRRMGYFTIQTYIPCILTVVLSWVSFWINKDAVPARTSLGITTVLTMT  279
Rat γ2  NTTEVVKTTSGDYVVMSVYFDLSRRMGYFTIQTYIPCTLIVVLSWVSFWINKDAVPARTSLGITTVLTMT  277
Rat γ3  NTTEIVTTSAGDYVVMTIYFELSRRMGYFTIQTYIPCILTVVLSWVSFWIKKDATPARTTLGITTVLTMT  280
Chk γ4  NTSEVLRTGAGEYMVMTVSFDLSRRMGYFAIQTYIPCILTVVLSWVSFWIKRDSTPARTSLGITTVLTMT  278

Rat γ1  TLSTIARKSLPKVSYVTAMDLFVSVCFIFVFAALMEYGTLHYFTSNNKGKTTRDRKLKSKTSVS......  343
Rat γ2  TLSTIARKSLPKVSYVTAMDLFVSVCFIFVFSALVEYGTLHYFVSNRKPSKDKDKK.KKNPAPT......  340
Rat γ3  TLSTIARKSLPRVSYVTAMDLFVTVCFLFVFAALMEYATLNYYSSCRKPTIRKKKTSLLHPDSTRWIPDR  350
Chk γ4  TLSTISRKHLPRVSYITAMDLFVSVCFIFVFAALMEYATLNYYLVGNKKPLEHSSRKARLPPAGAQVMPSF  348

Rat γ1  PGLHAGS.............TLIPMNNIS.MP.....QGEDDYGYQCLEGKDCATFFCCFEDCRTGSWREG  395
Rat γ2  IDIRPRS.............ATIQMNNATHLQ.....ERDEEYGYECLDGKDCASFFCCFEDCRTGAWRHG  393
Rat γ3  ISLQAPSNYSLLDMRPPPPVMITLNNSMYWQ.....EFEDTCVYECLDGKDCQSFFCCYEECKSGSWRRG  415
Chk γ4  TAIN.................INININMHWPPEIEEDEDDDPGSPCLEGKECERFFCCIEDCQTGMWREG  401

Rat γ1  RIHIRIAKIDSYSRIFFPTAFALFNLVYWVGYLYL                                      430
Rat γ2  RIHIRIAKMDSYARIFFPTAFCLFNLVYWVSYLYL                                      428
Rat γ3  RIHIDVSELDSYSRVFFPTSFLLFNLVYWVGYLYL                                      450
Chk γ4  RVRIHISRLDSYSRVFFPTAFLLFNIVYWIAYLYL                                      436
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Fig. 1. Alignment of the chicken $GABA_A$ receptor γ4-subunit sequence with those of mammalian $GABA_A$ receptor γ subunits. The amino-acid sequence (in single-letter code) of the chicken γ4 subunit (Chk γ4) has been aligned with those of the rat γ1 (Rat γ1), γ2S (Rat γ2) and γ3 (Rat γ3) subunits [15–17] using the computer programme PILEUP [25]; dots denote gaps that have been introduced to maximize the alignment. Positions at which all four of the sequences are identical are boxed. Amino acids are numbered from the proposed mature amino-terminal residues; the signal peptides [12] are indicated by negative numbering. Proposed membrane-spanning domains are marked by lines below the sequences, and potential phosphorylation sites [26,27] within the large intracellular loop region of the chicken γ4 subunit for casein kinase I (serine-332), protein kinase C (serine-331, and serine-332) and multifunctional calmodulin-dependent protein kinase II (serine-412) are indicated by open circles. The sequence of the cDNA, from which the chicken γ4-subunit sequence was deduced, has been given the EMBL accession number X73533.

were washed in 1 × SSC at 55°C for 1 h, briefly rinsed in 1 × SSC followed by 0.1 × SSC at room temperature, then dehydrated in an ascending ethanol series. Exposure to Fuji RX-100 X-ray film was at room temperature for 14 days. Slides were then dipped in Kodak NTB-3 emulsion, and left at 4°C for 50 days. Control experiments which contained, in addition, a 200-fold excess of the same unlabelled oligonucleotide failed to produce signals on autoradiographs (data not shown).

3. RESULTS AND DISCUSSION

Amplification of embryonic day 20 chicken whole-brain first-strand cDNA with two degenerate oligonucleotides that encode peptide sequences within the first and third membrane-spanning domains of vertebrate $GABA_A$ receptor β subunits resulted in the isolation of two new sequences. Serendipitously, one of these was found to encode part of a polypeptide that more closely resembles γ subunits than β subunits. Screening of an embryonic chicken whole-brain library with this partial cDNA yielded 12 positively-hybridizing clones. Upon DNA sequence analysis, one of these (~1.6 kb in length) was found to contain an open reading frame of 1,371 bp that specifies a mature polypeptide of 436 amino acids, with a predicted M_r of 51,001 daltons, and a signal peptide [12] of 21 residues (Fig. 1). The encoded product has all of the features of a ligand-gated ion-channel/receptor subunit, including four putative membrane-spanning segments and a pair of cysteines in the presumed amino-terminal extracellular domain. The sequence of the mature polypeptide is 69% identical to the previously-reported chicken $GABA_A$ receptor γ1 [13] and γ2 [14] subunits. In addition, the new subunit displays 67%, 69% and 70% identity to the rat $GABA_A$ receptor γ1 [15], γ2 [16] and γ3 [17] polypeptides; it exhibits significantly less identity (no greater than 48%) to the $GABA_A$ receptor α, β and δ [1,2], and the $GABA_A$ receptor-like ρ1 and ρ2 [18], subunits. Since isoforms of a given subunit typically exhibit between 70% and 80% identity to one another [1], and since species homologues of $GABA_A$ receptor subunits are usually at least 90% identical [6,8,13–15,19,20], we conclude that the cDNA we have isolated encodes a new member of the γ class of polypeptides which we name γ4.

It has previously been shown that the $GABA_A$ receptor γ2 subunits of human, bovine and rat [19], and mouse [20] and chicken [11] each exist in two forms (γ2S and γ2L) which arise by alternative splicing of the corresponding primary gene transcripts. In addition, we have recently demonstrated [8] that the chicken β2 subunit occurs in two forms (β2S and β2L) that are generated by the same mechanism. Since for both of these polypeptides the differences are found in the large intracellular loop region, we investigated whether the chicken γ4 subunit similarly exists in two forms. For this, the PCR was used with specific oligonucleotide primers to amplify first-strand cDNA, that was synthesized from either embryonic chicken day 18 or day 20

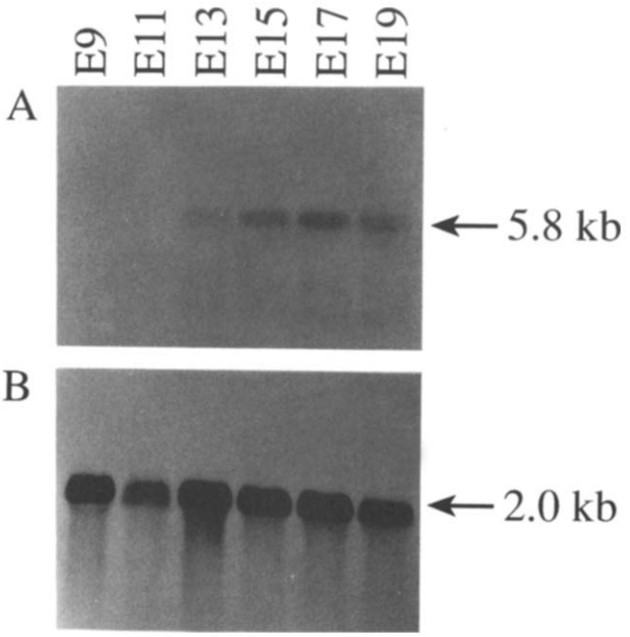

Fig. 2. Pattern of expression of the chicken $GABA_A$ receptor γ4-subunit gene during embryonic development. (A) Autoradiograph of a Northern blot of embryonic day 9 (E9) to embryonic day 19 (E19) chick whole-brain total RNA hybridized with a radiolabelled γ4-subunit transcript-specific oligonucleotide. (B) Autoradiograph of the same blot, after stripping off the γ4-subunit mRNA probe, rehybridized with a chicken β-actin cDNA. Exposure times to X-ray film were either 20 days (A) or 20 h (B). The sizes of the γ4-subunit and β-actin transcripts are indicated; these were determined by comparison with RNA size markers (not shown) that were run in parallel. Note that similar results were obtained in a series of three such developmental Northern blots when using either the same γ4-subunit mRNA oligonucleotide probe or a γ4-subunit cDNA fragment.

poly(A)$^+$ RNA, which encodes the majority of the large intracellular loop and which spans the sites of insertion in the β2L and γ2L subunits. With both RNAs only one product, which corresponds in size to that predicted from the cloned cDNA sequence, was detected (data not shown).

To examine the developmental pattern of expression of the γ4-subunit gene, Northern blots were hybridized with either a γ4-subunit-specific oligonucleotide or a γ4-subunit cDNA probe. In each case, a major band of ~5.8 kb was detected (Fig. 2A, and data not shown); weakly hybridizing RNAs of ~2.2 and ~3.1 kb were also observed. The γ4-subunit transcripts could not be detected at embryonic days 9 and 11; the mRNA is first observed at embryonic day 13. When the signals detected with either of the γ4-subunit-specific probes, at each developmental time-point, are normalized with respect to the level of β-actin mRNA (Fig. 2B), then the γ4-subunit steady-state mRNA level is seen to progressively increase during embryogenesis. It is noteworthy that the γ4-subunit mRNA appears much later in development than the γ2-subunit mRNA, which can be de-

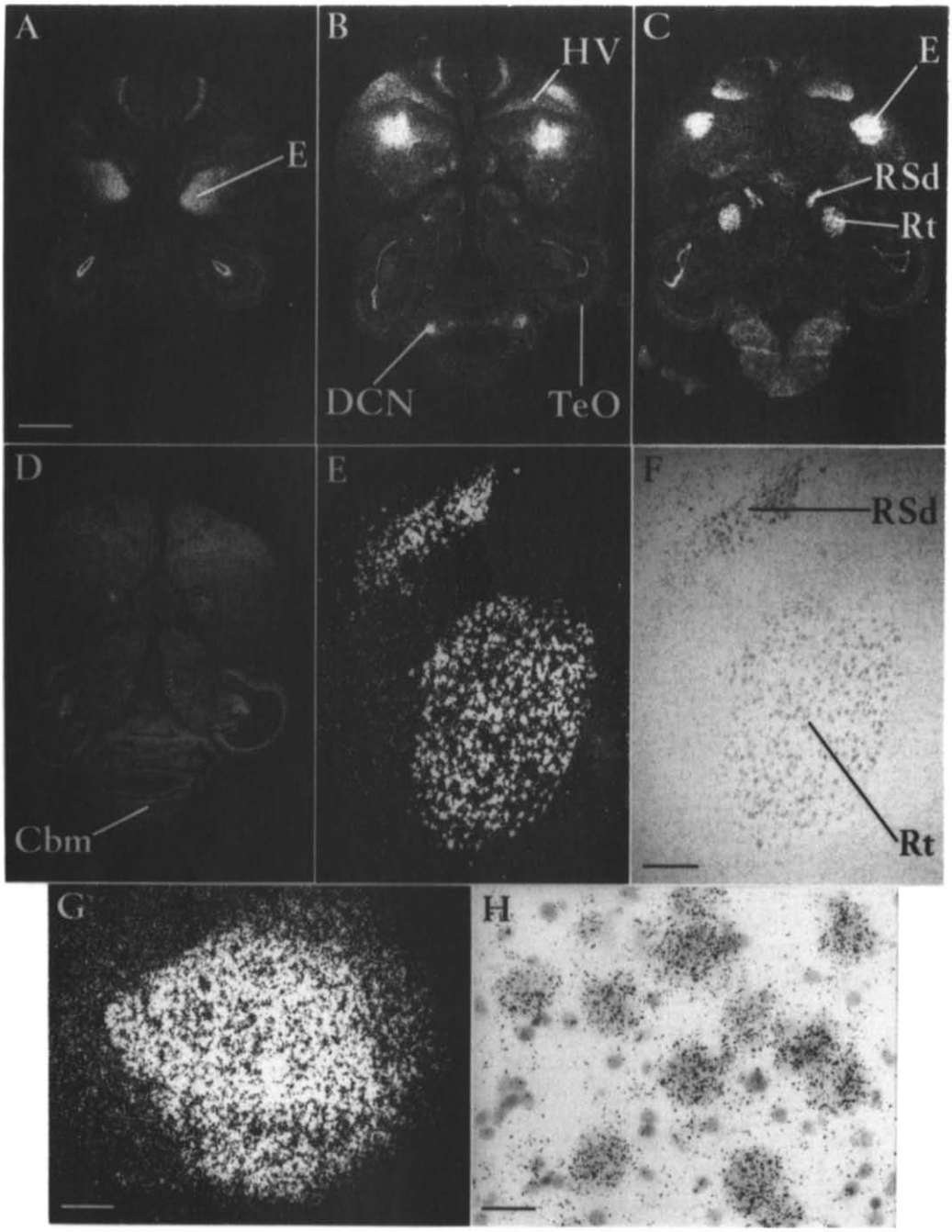

Fig. 3. Regional and cellular expression pattern of the $GABA_A$ receptor γ4-subunit gene in embryonic day 18 chick brain. In situ hybridization was performed using specific oligonucleotide probes (see section 2). (A–C) Regional expression of the γ4-subunit gene in three different horizontal sections; (D) regional expression of the γ2-subunit gene. Note that (B) and (D) are parallel sections. (E–H) Cellular localization of the γ4-subunit mRNA; (E) dark-field photomicrograph of the nucleus rotundus and nucleus reticularis superior, pars dorsalis; (F) light-field complement of the section shown in (E); (G) dark-field photomicrograph of the ectostriatum; (H) high-power magnification light-field photomicrograph of part of the nucleus rotundus. Scale bars: 0.25 cm (A–D), 286 μm (E–G), and 29 μm (H). *Abbreviations:* Cbm, cerebellum; DCN, deep cerebellar nuclei; E, ectostriatum; HV, hyperstriatum ventrale; RSd, nucleus reticularis superior, pars dorsalis; Rt, nucleus rotundus; TeO, optic tectum.

tected as early as embryonic day 9 (A. Müller and M. G. Darlison, unpublished results).

The regional pattern of expression of the $GABA_A$ receptor γ4-subunit gene in embryonic day 18 chick brain was examined by in situ hybridization and compared with that of the γ2-subunit gene (Fig. 3A–D). The γ4-subunit mRNA distribution is striking; high levels of transcripts are present in the ectostriatum, hyperstriatum ventrale, nucleus rotundus, nucleus reticularis superior, pars dorsalis, and the deep cerebellar nuclei. In

addition, weak labelling is evident in the optic tecta. The pattern detected with the γ2-subunit mRNA-specific probe (Fig. 3D), which was hybridized to parallel sections, is the same as that previously reported for 1-day-old chick brain [11]. The specificity of the γ4-subunit mRNA signal was confirmed by hybridization of sections with a second transcript-specific 45-base oligonucleotide, which yielded similar results (data not shown). Cellular resolution of the γ4-subunit mRNA signal (Fig. 3E–H), obtained using photographic emulsion, revealed that, as expected, the silver grains are clustered over cell bodies. At this level of resolution, not all of the cells in the nucleus rotundus, ectostriatum and nucleus reticularis superior, pars dorsalis, are labelled by the γ4-subunit-specific oligonucleotide; however, for the latter two areas, the majority of cells clearly contain γ4-subunit transcripts. In the nucleus rotundus, which contains both large and small cells, the γ4-subunit mRNA probe only seems to label the former (Fig. 3H). These are probably the same cells that have previously been shown [13] to express the γ2-subunit gene. Thus, it appears that the large cells possess more than one γ-subunit type and, hence, either a single receptor subtype with two different γ subunits or, perhaps more likely, two (or more) different subtypes.

The strong expression of the $GABA_A$ receptor γ4-subunit gene in the ectostriatum, nucleus rotundus, and hyperstriatum ventrale is of note since the former two brain regions play a major role in the processing of visual information (see, for example, [21,22]), and the latter is an area in which biochemical changes occur during the learning process known as 'imprinting' [23]. It is, therefore, tempting to speculate that, at least in the chicken, the γ4 subunit may form part of a receptor that has a function in visual processing and, perhaps, information storage and learning. The $GABA_A$ receptor α1-subunit gene has also previously been shown to be highly expressed in the ectostriatum and nucleus rotundus [24]. It will, therefore, now be interesting to determine which other $GABA_A$ receptor genes are transcribed in these areas, and then examine the pharmacological properties of heterologously-expressed receptors that contain the γ4 polypeptide and which occur in vivo.

In summary, we have described here the sequence and pattern of gene expression of a new member of the γ class of vertebrate brain $GABA_A$ receptor subunits. The existence of this polypeptide in the chicken prompts the question of whether mammals also possess a γ4 subunit. In this context, it is noteworthy that this laboratory has previously reported [7] the sequence of a β subunit from the chicken (named β4), for which no mammalian homologue has been described. There are two plausible explanations for this data. The first is that the β4 and γ4 subunits are specific to the chicken or, perhaps, avian species. The second is that these two polypeptides also exist in mammals but that they have yet to be identified. This latter possibility is the most tempting in the light of in situ hybridization studies that have revealed the co-existence, in mammalian brain, of pairs of $GABA_A$ receptor mRNAs [3,4]. Since the majority of vertebrate receptor subtypes are generally assumed to contain α, β, and either γ or δ subunits, it is plausible that the α2 and β3 subunits and/or the α5 and β1 subunits, which are thought to associate together [3], assemble with a mammalian homologue of the chicken γ4 subunit. Similarly, the α4 and δ subunits and/or the α2 and γ1 subunits, the mRNAs for which also co-localize [3,4], may assemble with a mammalian homologue of the chicken β4 subunit. Current studies are aimed at determining whether all of the mammalian $GABA_A$ receptor subunits have, indeed, been identified.

Acknowledgements: We thank Dr. Andrew Hicks (Gif-sur-Yvette) for the chicken brain cDNA library, Dr. Evita Mohr (Hamburg) for the chicken β-actin cDNA, Dr. Susanne Fehr (Hamburg) and Stefan Lenz (Hamburg) for advice on in situ hybridization, Dr. Katharina Braun (Magdeburg) and Dr. Stephen Hunt (Cambridge) for help with chick neuroanatomy, Sönke Harder (Hamburg) for DNA sequencing, and Marion Däumigen-Kullmann (Hamburg) for oligonucleotide synthesis.

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