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ARTICLE

Localization of Two Enzymes of the Tetrahydrobiopterin Biosynthetic Pathway in Embryonic Chick Retina

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SUMMARY Tetrahydrobiopterin (BH4) is an essential co-factor for the biosynthesis of catecholamine-type neurotransmitters and of nitric oxide (NO). The expression of the enzymes catalyzing the first two steps of the BH4 biosynthetic pathway was studied in the developing chicken retina by in situ hybridization and immunocytochemistry. GTP-cyclohydrolase-I (GTP-CH-I) and 6-pyruvoyl-tetrahydropterin synthase (PTPS) were already expressed in the undifferentiated and proliferating retina of E7. At stage E11 both enzymes were expressed in photoreceptors, amacrine cells, displaced amacrine cells, and ganglion cells, and in the plexiform layers in which synaptic connections take place. At stage E18 the labeling was comparable to E11 but appeared to be more concentrated in photoreceptors and ganglion cells. **(J Histochem Cytochem 50:265–274, 2002)**

KEY WORDS
BH4
chick retina
development
PTPS
GTP-cyclohydrolase I
in situ hybridization
immunolabeling
tyrosine hydroxylase
nitric oxide synthase

Pteridines are widely distributed compounds in both plants and animals (including humans), in which they control important processes. Tetrahydrobiopterin (BH4) serves as a co-factor for the biosynthesis of nitric oxide (NO) and catecholamine and indolamine neurotransmitters [3,4-dihydroxyphenylalanine (L-DOPA), norepinephrine, epinephrine, and serotonin] (for review see Nichol et al. 1985). The biosynthetic pathway (Figure 1) of BH4 from GTP involves three enzymes. The first and rate-limiting enzyme is GTP-cyclohydrolase I (GTP-CH-I; E.C. 3.5.4.16) converting GTP (1) to dihydroneopterintriphosphate (2), which is subsequently transformed into (3) by 6-pyruvoyl tetrahydropterin synthase (PTPS; E.C. 4.6.1.10). The 6-pyruvoyl tetrahydropterin is then reduced in two steps to BH4 (4) by sepiapterin reductase (SPR; E.C.1.1.1.153) (Auerbach and Nar 1997; Nagatsu and Ichinose 1999). Rare human mutations in GTP-CH-I and PTPS genes were shown to cause severe neurological defects (Thöny and Blau 1997; Ichinose et al. 1999).

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It was our aim to localize two enzymes of the biosynthetic pathway of BH4, GTP-CH-I, and PTPS, on both the protein and the mRNA level in a differentiating neuronal system. Neuronal embryonic chick retina is a favorable experimental system for investigation of developmental patterns of the CNS, because it is an extension of the embryonic brain and therefore a part of the CNS and can be easily dissected out at various stages of development, which are well characterized. The prenatal retina develops from a single layer of multipotent neuroepithelial precursor cells into a distinct three-layered tissue composed of several types of specialized neurons and glia cells. The chick retina has no vascular system, which makes both dissection and observation easier (for an overview see Mey and Thanos 2000).

The localization of tyrosine hydroxylase, a BH4-dependent enzyme that is rate-limiting in the biosynthesis of dopamine, has been studied in chicken retina (Ballesta et al. 1984), in which it was found to be involved in the processing of visual information (Parkinson and Rando 1983). NO and its biosynthetic enzyme nitric oxide synthase (NOS) are also expressed in the neural retina, where their role is still under investigation (Fujikado et al. 1997; Roth 1997; Goureau et al. 1999).

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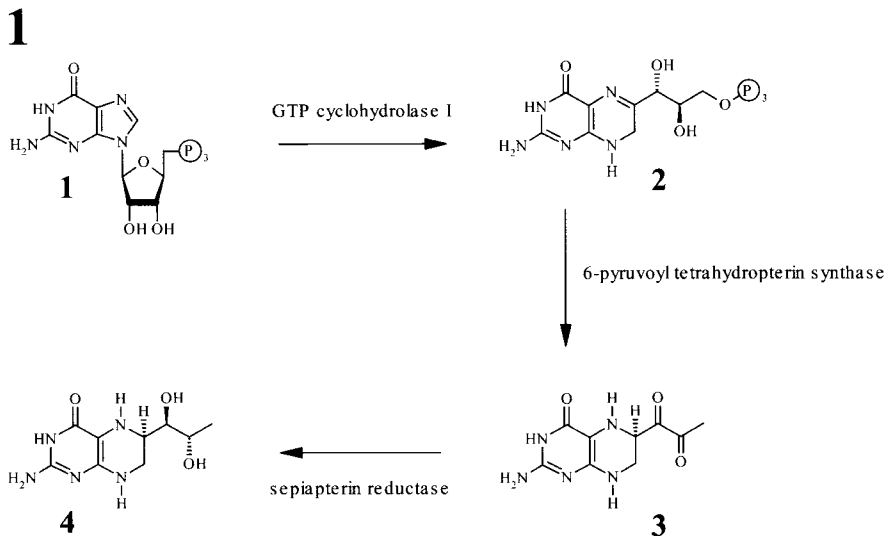

Figure 1 Biosynthetic pathway of BH4: (1) GTP, (2) dihydroneopterin triphosphate, (3) 6-pyruvoyl-tetrahydropterin, (4) BH4.

Figure 2 (**A**) Sequence of GTP-CH-I in situ antisense probe. (**B**) Sequence of PTPS in in situ antisense probe.

2A

GGTACCCAGC ACAATGTTGG GGGTATTCCG GGAAGACCCA AAGACCCGTG AAGAGTTCTT GACGCTCATC AGGAGCTGAA GCTGTTGTTC TCTGAATGAG CTC

B

ACAAAAACCT GGATAAGGAT GTGCCCTACT TCGCTGAGGT GGTGAGCACC ACAGAGAACG TTGCAGTGTT CATCTGGGAA AACCTCAAGA GGCTCCTGCC TGCGGGAATG CTTTATAAAG TCAAAGTGTA TGAAACGACC AGAACATTGT GGTCTATAAA G

Materials and Methods

Animals

All experiments were conducted according to the regulations established by the ethics committee of Tel Aviv University.

Fertilized White Leghorn eggs were obtained at Day 1 from the animal facility of the university and could be kept at 4C for up to 1 week. The eggs were incubated in a humid egg incubator at 38C. Experiments were performed at the embryonic ages of 7 days (E7), 11 days (E11), and 18 days (E18). Eggs were staged according to Hamburger and Hamilton (1992).

Materials

pBluescript SK (−) and bacterial strain *E. coli* XL1 Blue were from Stratagene (La Jolla, CA). The expression plasmid pMal-c2 was from New England Biolabs (Beverly, MA). Restriction enzymes (Kpn I, SacI), T3 RNA polymerase, and RNase A were from Promega (Madison, WI). T7 RNA polymerase, proteinase K, digoxigenin-labeled UTP in nucleotide mixture, blocking solution, alkaline phosphatase-conjugated anti-digoxigenin antibodies, and BM purple were from Roche Diagnostics (Penzberg, Germany). [α-^{35}S]-UTP (1000 Ci/mmol) was from Amersham (Freiburg, Germany) and NTB2 emulsion was from Eastman Kodak Company (Rochester, NY). Goat anti-rat FITC-conjugated second antibody was from Sigma (Deisenhofen, Germany), goat anti-rabbit FITC-conjugated antibody, FITC-conjugated anti-chicken IgY antibody, and lissamine–rhodamine (LRSC)-conjugated Affinipure goat anti-rabbit IgG (H+L) were from Jackson ImmunoResearch (West Grove, PA), and biotin-conjugated goat anti-mouse IgG + IgA + IgM was from Zymed Laboratories (South San Francisco, CA). BrCN-activated Sepharose 6MB was purchased from Pharmacia Biotech (Freiburg, Germany). Tissue freezing medium was purchased from Jung (Nussloch, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany) or Sigma.

Cloning, Sequencing, and Expression of PTPS

Total RNA was purified from chicken liver and was used as a template for reverse transcriptase polymerase chain reaction. Based on an alignment of PTPS sequences from various species, two oligonucleotides were designed (AGG AGC CGC GGT ACC ACA AAA ACC TGG ATA AGG ATG TGC and CTC AGA AAT GTT TTG AGC TCC TTT ATA GAC CAC ATT GTT CYG GTC C). Using these oligonucleotides as primers, a segment of the cDNA of chicken PTPS was amplified, cloned, and sequenced. The complete cDNA of PTPS was obtained by rapid amplification of cDNA ends (RACE) using the RACE kit from GIBCO (Paisley, UK). The complete open reading frame was inserted into the expression plasmid pMal-c2. This genetic construct yielded PTPS as a fusion protein to the C-terminus of the maltose-binding protein of *E. coli*. This fusion protein was purified on mal-

tose agarose according to the manufacturer's instructions (New England Biolabs) and was used for immunization of a rabbit.

Antibodies

Monoclonal rat antibodies against chicken GTP-CH-I were a gift from I. Ziegler (Munich, Germany). Cell culture supernatant was concentrated 25 times by ultrafiltration and directly used for labeling.

Polyclonal chicken yolk anti-mouse GTP-CH-I was prepared as previously described (Jensenius et al. 1981). The dilution used was 1:30 to 1:50. PTPS antibody was prepared by immunization of rabbits with PTPS protein (0.17 mg/immunization). The antiserum to PTPS was affinity-purified on a PTPS–agarose column prepared by coupling PTPS to BrCN-activated Sepharose 6MB (20 mg PTPS/g dry BrCN-activated Sepharose 6MB). Diluted antiserum (1:10) was loaded on the column and developed according to the manufacturer's manual. Fractions were checked by Western blotting techniques. The antiserum was used undiluted or two times diluted.

Tissue Preparation

Dissected retinas were fixed in 4% paraformaldehyde (PFA) in PBS for 1 hr at room temperature (RT). Cryosections used for immunocytochemistry (IHC) and in situ hybridization (ISH) were infiltrated for cryoprotection with 4% sucrose for 2 hr at 4C, followed by 20% sucrose and 5% glycerol in PBS at 4C overnight. Fixed tissues were transferred to Tissue Freezing Medium and quickly frozen in liquid nitrogen. Sections were placed on subbed slides (1% gelatin containing 0.3% chromium potassium sulfate) and stored at −20C or −70C. For ISH, slides were cleaned with 1 M HCl and ethanol and baked overnight at 240C before coating. For IHC, paraffin sections of 6–10 μm were obtained as previously described (Polak and Van Noorden 1983).

Immunocytochemistry

Cryosections were dried for 2 hr at RT, rehydrated in PBS, and incubated in PBS with 1% BSA for 30 min at RT. Sections were incubated with the primary antibody overnight at 4C in a humid chamber. For double-labeling experiments, the sections were incubated with a mixture of the two antibodies. They were then washed three times with PBS and incubated with the second antibody for 1–3 hr at RT. In double-labeling experiments the second antibodies were applied successively. Sections were incubated with LRSC-conjugated goat anti-rabbit (1:200), washed, and then incubated with FITC-conjugated rabbit anti-chicken IgY (1:50). After three washings in PBS, the sections were stained with bis-benzimide (Hoechst 33258) for 30 min, washed, and mounted with galvanol. Observations and photography were carried out with a Zeiss Axioplan microscope. Control experiments were performed without the first antibody. Alternatively, sections were incubated with 5% normal goat serum instead of primary antibody. Pictures were scanned with Presto Page Manager, processed with Adobe Photoshop to adjust the size and arranged and labeled in PowerPoint.

Immunohistochemistry

Paraffin-embedded retinas were used for staining with the monoclonal rat anti-chicken GTP-CH-I antibody. Thin (10-μm) paraffin sections were deparaffinized and rehydrated. To block residual endogenous peroxidase activity, the sections were incubated for 5 min with 3% hydrogen peroxide in PBS. Slides were then washed three times with PBS for 5 min, treated with 1% BSA in PBS for 10 min at RT, and washed again in PBS. The monoclonal rat anti-chicken GTP-CH-I antibody was applied overnight at 4C. After three washes with PBS, the slides were incubated with a biotinylated goat anti-rat IgG (1:100) for 1 hr at RT. Excess antibody was removed by washing three times with PBS. Peroxidase-conjugated streptavidin was added for 30 min at RT. After washing, sections were stained for peroxidase reaction by incubation with a mixture of diaminobenzidine and hydrogen peroxide for 5–10 min. The slides were counterstained with Accustain (Harris' hematoxylin) for 30 sec, cleared, and mounted with galvanol. Controls were performed without the primary antibody. Observation and photography were carried out as described above.

RNA Probe Preparation

For preparation of a GTP-CH-I-probe, a segment of 103 bases (Figure 2A) from the central part of the open reading frame of GTP-CH-I-cDNA from chicken was inserted into the plasmid pBluescript SK−. A segment of 161 bases (Figure 2B) was used for the PTPS probe and was also cloned between the KpnI and SacI sites. Both cDNA probes are part of the coding region of the respective enzymes. They were selected to have a G/C content of about 50–60% and a length of 100–200 bp. The oligonucleotide primers, which were used in the reverse transcriptase PCR for GTP-CH-I-probe, were selected from a published cDNA sequence of the chicken GTP-CH-I (GenBank accession number Z49267). For PTPS, an alignment of the amino acid sequence of PTPS from rat, mouse, and human was performed. Oligonucleotide primers were selected to match the cDNA in areas of high protein homology. The resulting cDNA amplificate was sequenced on both strands. The deduced amino acid sequence showed a high grade of homology to the mammalian enzymes. Both sense and antisense probes were prepared using either T3 or T7 RNA polymerase and labeled with [^{35}S]-UTP or digoxigenin–UTP according to the protocol of the manufacturer.

Radioactive ISH

Frozen retina sections were used for ISH with ^{35}S-labeled probe as described by Phillips et al. (1997). The sections were washed several times, permeabilized, and then hybridized overnight at 50C with ^{35}S-labeled sense or antisense RNA (1×10^6 cpm/section). After several washing steps, sections were dehydrated and coated with a thin layer of NTB2 emulsion. The emulsion was developed after 6–8 days. Sections were counterstained with Harris' hematoxylin if required, mounted using galvanol, and observed and photographed as described above.

Non-radioactive ISH

Frozen retina sections were dried for several hours at 40C. Prehybridization treatments included the following steps: incubation for 20 min in 0.2 M hydrochloric acid, washing with distilled water and two times with PBS, permeabilization with PBS containing 0.3% Triton X-100 for 15 min, washing with PBS, 30-min incubation with 1 μg/ml proteinase K at 37C and quenching with 0.1 M glycine for 5 min, fixation with 4% PFA for 20 min, washing with PBS, acetylation with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, and dehydration by a water/ethanol series (50%, 70%, 95%, 100%). Prehybridization was carried out in 4 × SSC and 50% formamide. The sections were hybridized overnight at 55C with digoxigenin-labeled sense or antisense PTPS RNA (~50 ng probe/section). The hybridization buffer consisted of 40% formamide, 5% dextran sulfate, 1 × Denhardt's solution, 1 mg/ml yeast tRNA, 10 mM DTT, and 4 × SSC. The sections were then washed for 15 min at 52C with 50% formamide/2 × SSC, followed by washing at 37C for 15 min with 2 × SSC and then twice with 1 × SSC. To remove unhybridizided RNA, the sections were incubated for 30 min at 37C with 20 μg/ml RNase A and washed twice for 30 min at 45C with 0.1 × SSC.

Immunological detection was performed according to the Boehringer manual using BM purple as substrate. Slides were mounted using galvanol and observed and photographed as described above.

Results

Cloning, Sequencing, and Expression of PTPS

Based on a sequence comparison of published PTPS genes from rat, mouse, human, salmon, and *Drosophila melanogaster*, oligonucleotides were designed for PCR amplification affording a segment of the cDNA for chicken PTPS which was amplified, cloned, and sequenced. The complete cDNA of PTPS was obtained by rapid amplification of cDNA ends (RACE). The complete open reading frame was expressed in *E. coli* and was used to prepare polyclonal antisera in rabbits.

Localization of GTP-CH-I

GTP-CH-I was detected in sections of embryonic chicken retina with a polyclonal chicken anti-mouse antibody against GTP-CH-I and a second antibody conjugated to FITC. At early development (E7) when the retina is still undifferentiated (Figure 4C), GTP-CH-I was present all over the tissue (Figure 4A). However, more labeling was seen at the areas that eventually differentiate into the ganglion cell layer (GCL) and photoreceptor cells. At E11, the retina was more differentiated and the specific layers were easily recognized (Figure 4F). Stronger labeling was detected at the GCL but also in some cells in the inner part of the inner nuclear layer (INL) (Figure 4D). Staining with the monoclonal rat anti-chicken GTP-CH-I and peroxidase-conjugated second antibody E11 retina confirmed the same pattern of expression as shown in Figure 3A.

In E18 retina, the layers are well defined (Figure 4I). Most of the GTP-CH-I immunoreactivity was in the outer retina, outer plexiform layer (OPL), and outer nuclear layer (ONL), in the GCL and in some of the displaced cells in the inner plexiform layer (IPL) (Figure 4G).

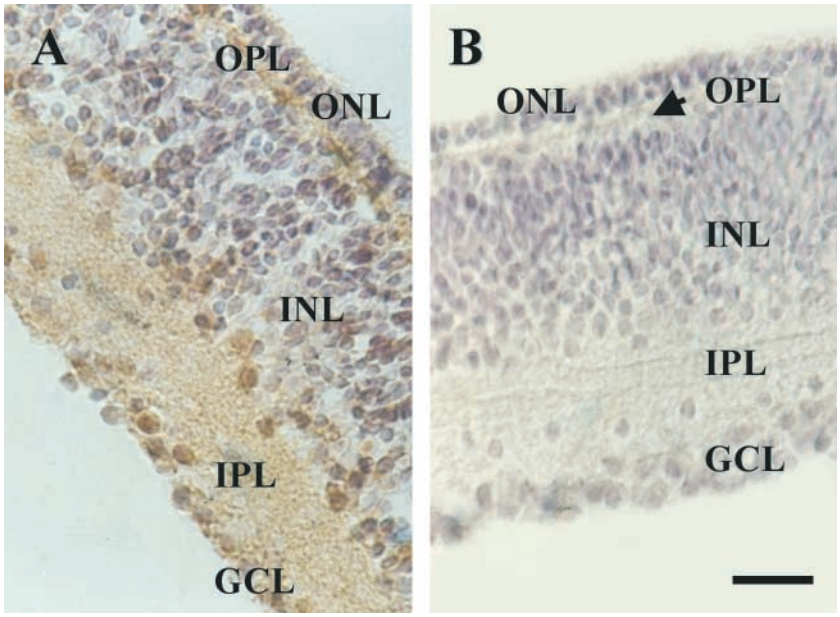

Figure 3 Immunolabeling of GTP-CH-I in E11 retina paraffin sections (**A**) treated with monoclonal anti-GTP-CH-I and detected using the streptavidin–peroxidase method (brown), counterstained with Harris' hematoxylin (purple). (**B**) Negative control, counterstained with Harris' hematoxylin. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; PE, pigmented epithelium. Bar = 30 μm.

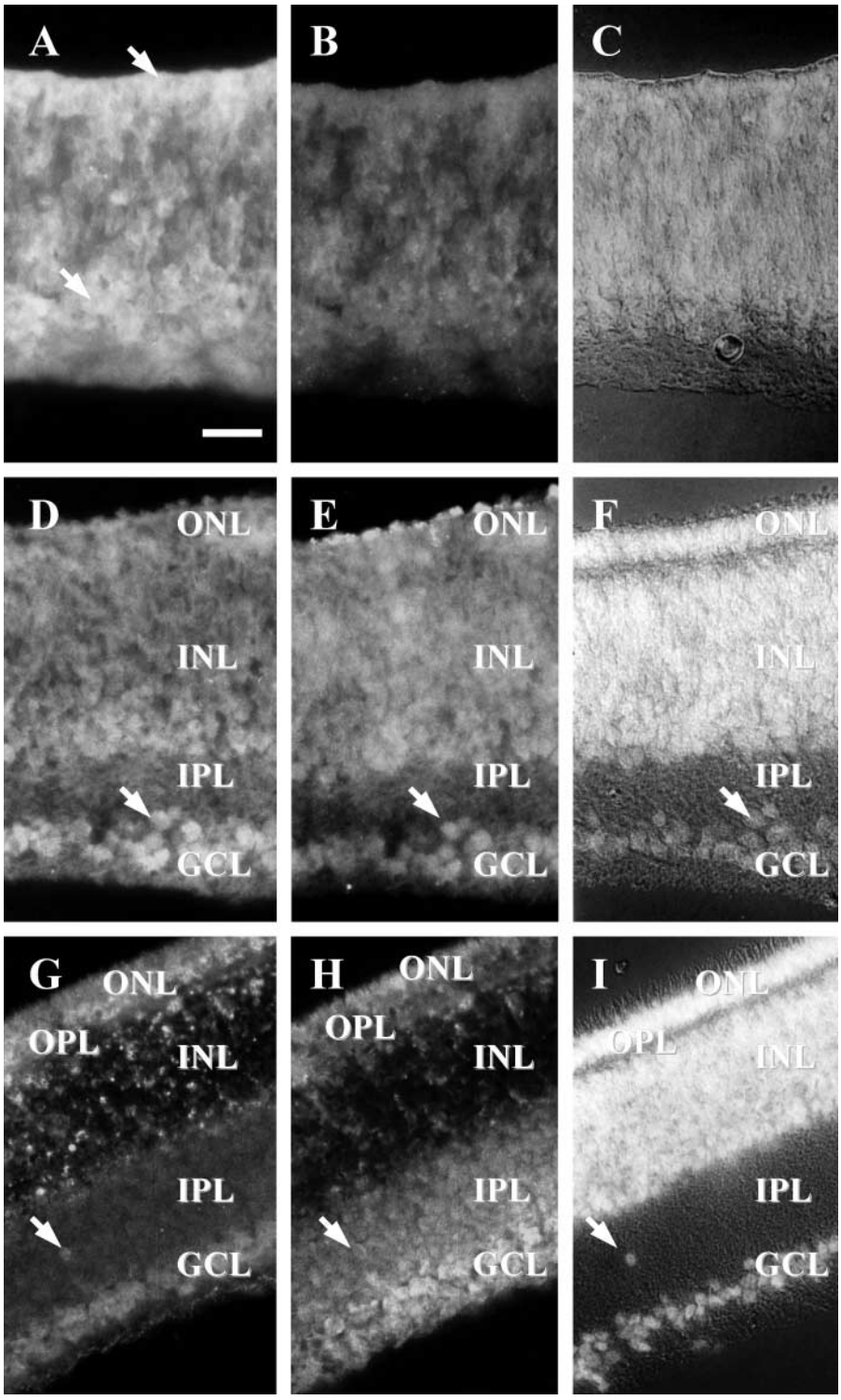

Figure 4 Doub e abe ing of GTP-CH-I and PTPS during development of the chick retina. Cryosections of (**A**) E7 retina labeled with anti-GTP-CH-I and FITC immunofluorescence; note the stronger labeling at the areas (white arrows) that will become GCL and photoreceptors. (**B**) E7 retina labeled with anti-PTPS and LRSC-immunofluorescence; compare with similar labeling in Figure 3A. (**C**) E7 retina labeled with Hoechst 33258 fluorescence (Nomarski micrograph). (**D**) E11 retina labeled with anti-GTP-CH-I and FITC immunofluorescence. (**E**) E11 retina labeled with anti-PTPS and LRSC immunofluorescence. (**F**) E11 retina labeled with Hoechst 33258 fluorescence (Nomarski micrograph). (**G**) E18 retina labeled with anti-GTP-CH-I and FITC immunofluorescence, white arrows point to displaced cells in the IPL. (**H**) E18 retina labeled with anti-PTPS and LRSC immunofluorescence. (**I**) E18 retina labeled with Hoechst 33258 fluorescence (Nomarski micrograph). Bar = 20 μm.

Localization of PTPS

Specific immunoreactivity was observed using affinity-purified polyclonal rabbit anti-chicken PTPS serum (tested by Western blotting). Labeling was detectable all over the retina at E7, with higher density at the areas that differentiate to GCL and photoreceptor cells (Figure 4B). At the later embryonic stages, E11 and E18, more labeling was seen at the outer retina, ONL, OPL, and also at the GCL (Figures 4E and 4H).

Co-localization of GTP-CH-I and PTPS

GTP-CH-I and PTPS were expressed in the same layers, as confirmed by double-labeling experiments. The similar pattern of expression was present in all embryonic stages tested (E7, E11, and E18). As previously described, the expression was stronger at the outer layers of the retina and at the GCL. Distinct cells at the IPL (probably amacrine cells) were also labeled with both antibodies (arrows in Figures 4D, 4E, 4G, and 4H). The localization of the immunoreactivity was over the entire cytoplasm of the cell bodies (Figure 5).

ISH of GTP-CH-I mRNA

To show that the same cells in which GTP-CH-I was localized also express it, we performed a series of ISH experiments on frozen retina sections. Figure 6 shows the pattern of ^{35}S hybridization on sections from three different stages of embryonic retina using a [^{35}S]-RNA probe complementing the central part of chicken GTP-CH-I mRNA (Figure 2A). At E7 the retina was undifferentiated, the neuronal layers were not stratified, and typical plexiform layers did not yet exist. At this age there was hybridization of the antisense probe across the width of the retina (Figure 6A). At E11 the plexiform layers and distinct nuclear layers were formed. Hybridization was higher at the outer layers of the retina (Figure 6C). This pattern was more pronounced at E18 (Figure 6E); most of the grains appeared to be concentrated at the ONL and OPL. Figures 6B, 6D, and 6F show the low level of hybridization seen with the sense probe.

ISH PTPS mRNA

Transcripts of PTPS, the second enzyme in the biosynthetic pathway of BH4, were localized by ISH using a ^{35}S-labeled probe or a non-radioactive digoxigenin-labeled probe. PTPS mRNA was found, like GTP-CH-I mRNA, over the entire retina tissue at E7, but more profoundly at the areas that differentiate to GCL and photoreceptor cells (Figures 7A, 7B, and 8A). Later in development, at E11, hybridization was predominantly at the outer retina (Figures 7D, 7E, and 8B). At E18, most of the hybridization was located at the ONL and OPL (Figures 7F, 7G, and 8C).

Discussion

We have shown that enzymes of the BH4 biosynthetic pathway are expressed in the embryonic chick retina during development. GTP-CH-I immunoreactivity was previously demonstrated in rat (Dassesse et al. 1997; Hirayama and Kapatos 1998; Hwang et al. 1998), mouse (Nagatsu et al. 1995), and human brain (Nagatsu et al. 1999). However, no data were available on the expression of GTP-CH-I and PTPS in the various layers of embryonic chick retina during differentiation.

Co-expression of both enzymes in E7 retina was found over the whole tissue. At this early stage, cell division is still going on and the expression of GTP-CH-I and PTPS protein increases in the areas where ganglion cells and photoreceptor cells will be formed.

At E11, these layers are already established. In the GCL it was easy to observe single ganglion cells that were all double labeled with anti-GTP-CH-I and anti-PTPS. Double-labeled cells were also found in the inner part of the INL, in which amacrine cells are present. The localization of both protein immunoreactivities was over the perikaryon and the enzymes appeared to be cytosolic, as expected.

In E18 retina, the results once more confirmed the co-expression of GTP-CH-I and PTPS. Both proteins were localized in the inner and outer parts of the retina and in a population of displaced cells in the IPL.

In E7 retina it was possible to show the simultaneous presence of GTP-CH-I and PTPS on both levels, message (mRNA) and protein. During the development of the retina, the protein localization and mRNA expression of PTPS are always present in the same layers.

The results of GTP-CH-I staining showed that only in the outer layers and the mRNA expression corre-

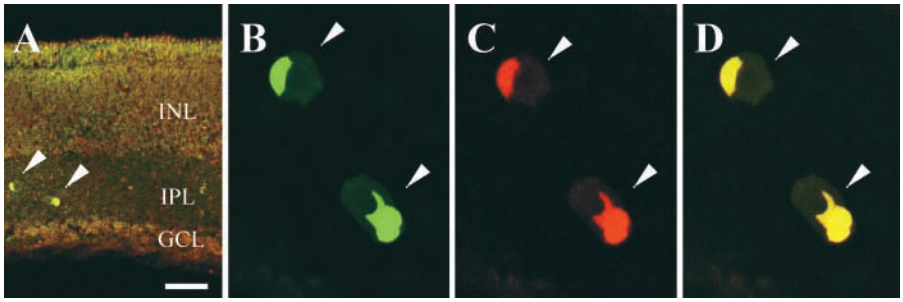

Figure 5 Immunolabeling of GTP-CH-I and PTPS of E18 retina observed in displaced amacrine cells (confocal microscopy). **(A)** Overview through the section with overlapping fluorescence; the two cells marked by white arrows are the same as in **B–D**. **(B)** Observation at 488 nm; cells are labeled with anti-GTP-CH-I and FITC immunofluorescence. **(C)** Observation at 568 nm; cells are labeled with anti-PTPS and LRSC immunofluorescence. **(D)** Overlapping observation; yellow fluorescence shows labeling of both enzymes at the same location. Bar = 20 μm.

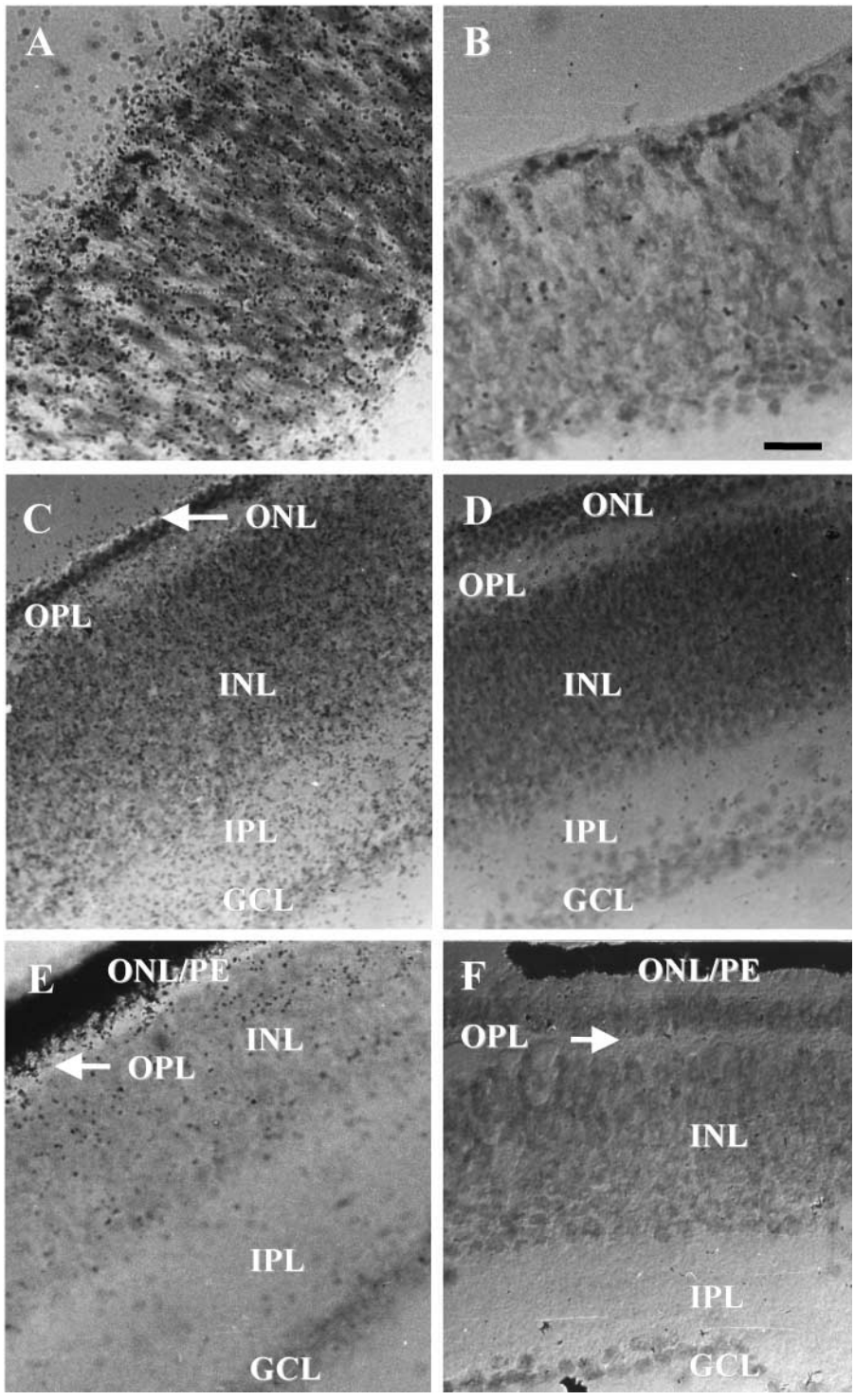

Figure 6 Loca ization of GTP-CH-I mRNA in embryonic chick retina by ISH with a ^{35}S-labeled probe. (**A**) E7 retina hybridized with antisense probe. (**B**) E7 retina control, hybridized with sense probe. (**C**) E11 retina hybridized with antisense probe. (**D**) E11 retina control, hybridized with sense probe. (**E**) E18 retina hybridized with antisense probe. (**F**) E18 retina control, hybridized with sense probe. Bar = 20 μm.

spond with immunological staining. In the INL and the GCL no hybridization with GTP-CH-I antisense probe was visible. GTP-CH-I immunostaining was proved in these layers. The reason for labeling of protein but not mRNA might be a low concentration of mRNA. Another reason might be a regulation of GTP-CH-I via mRNA, because GTP-CH-I has a rate-limiting role in BH4 biosynthesis (Thöny et al. 2000).

TH catalyzes the initial reaction in the biosynthesis of catecholamine neurotransmitters, by the hydroxylation of tyrosine to L-DOPA (Nagatsu et al. 1964). L-DOPA is then transformed into dopamine, the main

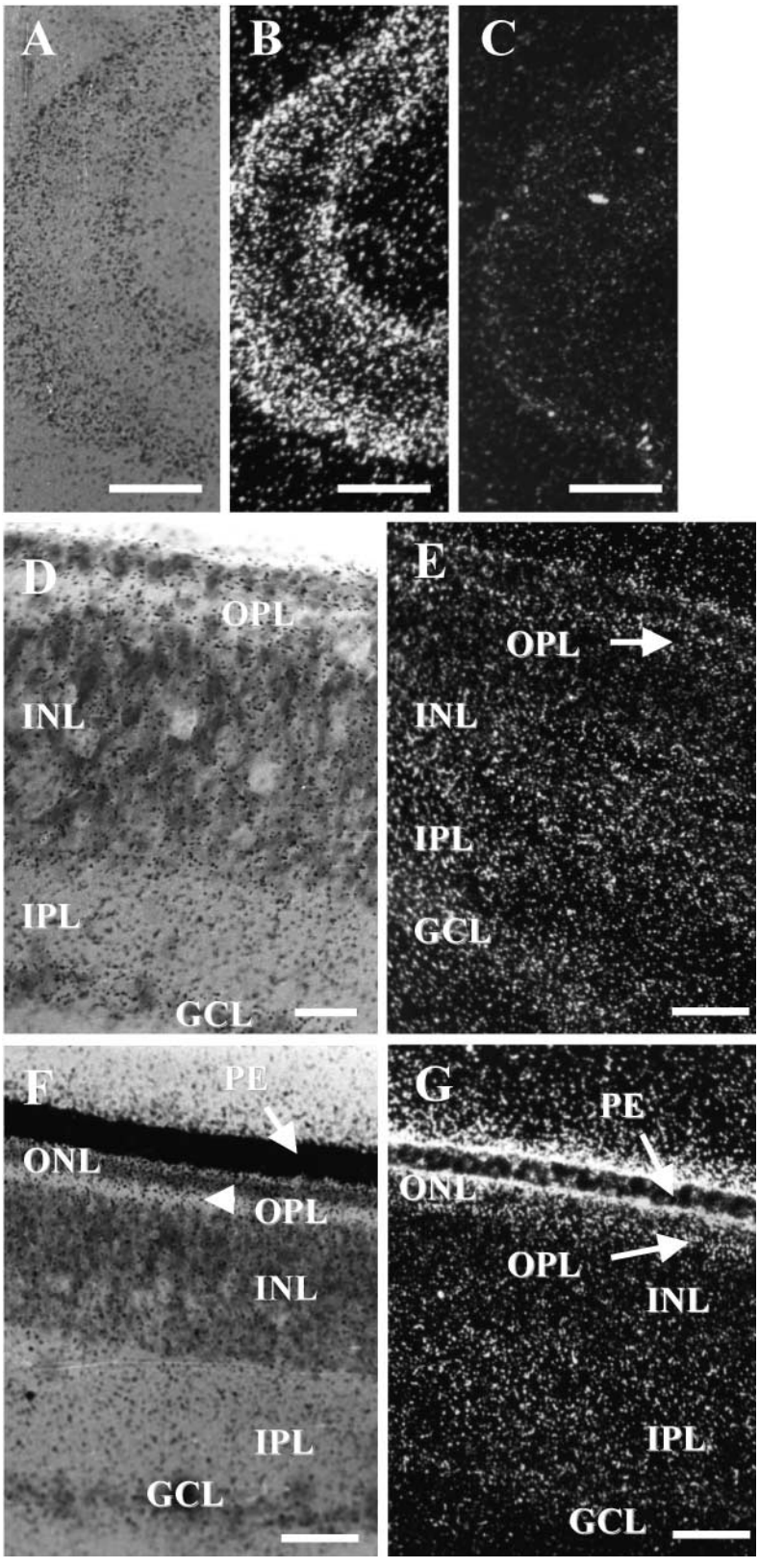

Figure 7 Localization of PTPS mRNA in embryonic chick retina by ISH with a ^{35}S-labeled RNA probe. (**A**) E7 retina hybridized with antisense probe, brightfield image. (**B**) E7 retina hybridized with antisense probe, darkfield. (**C**) control, E7 retina hybridized with sense probe, darkfield. Bars = 90 μm. (**D**) E11 retina hybridized with antisense probe, brightfield. Bar = 20 μm. (**E**) E11 retina hybridized with antisense, darkfield. Bar = 30 μm. (**F**) E18 retina hybridized with antisense probe, brightfield. (**G**) E11 retina hybridized with antisense probe, darkfield. Bars = 45 μm.

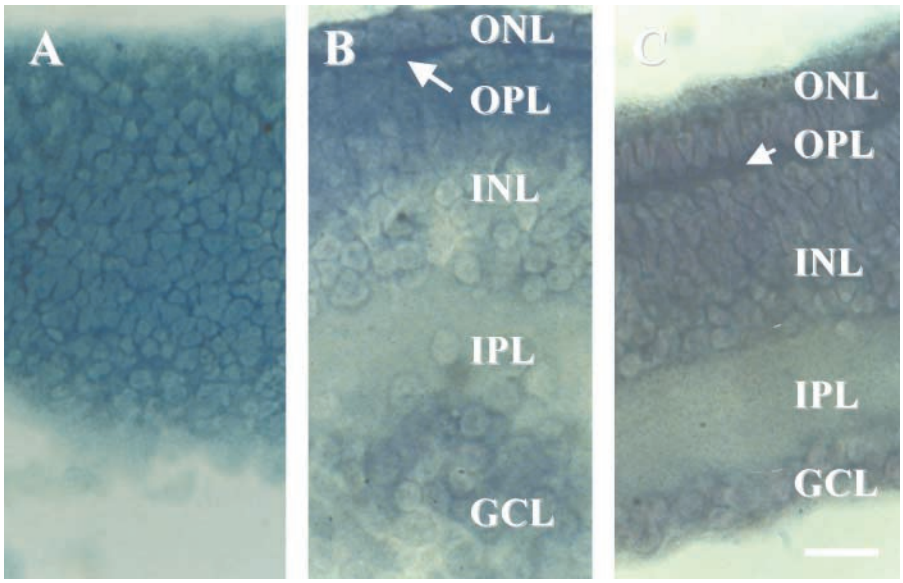

Figure 8 ISH on frozen sections of embryonic retina using a digoxigenin-labeled PTPS probe. Staining with antisense RNA at (**A**) E7 retina, (**B**) E11 retina, and (**C**) E18 retina. Bar = 20 μm.

catecholamine in the retina, which is suggested to be involved in the processing of visual information (Parkinson and Rando 1983; Stenkamp et al. 1994; Rohrer et al. 1995) as well as in postnatal ocular growth.

TH requires BH4, and their co-localization during development was recently shown in the nervous system of *D. melanogaster* (Krishnakumar et al. 2000). Ballesta et al. (1984) described TH immunoreactivity in adult chick retina. They localized TH in amacrine cells in the INL and in displaced cells in the IPL. These results were validated by other groups (Teakle et al. 1993), and it was found that TH started to be generated in chick retina at E13–14 (Gardino et al. 1993; Dos Santos and Gardino 1998). We found that the biosynthetic enzymes of BH4 are already expressed at E7. In E11 retina, the cells that expressed GTP-CH-I and PTPS enzymes are at the inner part of the INL and in displaced amacrine cells in the IPL, which may eventually develop into dopaminergic amacrine cells.

Another function of BH4 is its role as a co-factor for NOS. The localization of nNOS (also known as NOS-I) in chick retina after hatching was shown in subtypes of amacrine cells and ganglion cells (Fischer and Stell 1999). The localization of nNOS and eNOS (also known as NOS-III) during development was shown in other studies (Goureau et al. 1997). Both enzymes were found over the entire retina at E6 and E8, albeit at low levels (Goureau et al. 1997). This observation corresponds with our findings about GTP-CH-I and PTPS expression at E7. At E12, NOS activity was revealed in the PRL, the OPL, the INL (eNOS in the inner part), and the GCL (Goureau et al. 1997), which resembles the distribution of the BH4 pathway enzymes. The expression of GTP-CH-I and PTPS mentioned in this study was analogous to NOS expression shown during development (Goureau et al. 1997).

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