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DEVELOPMENTAL AND TISSUE-SPECIFIC EXPRESSION OF THE ROD PHOTORECEPTOR cGMP-GATED ION CHANNEL GENE

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Probes against the retinal cGMP-gated cation channel were generated by PCR amplification of cDNA from rat and bovine retina. Southern and Northern analyses showed that the channel is encoded by a single gene that gives rise to a single mRNA species of 3.2 kb. Low levels of cGMP-gated channel RNA were detected in postnatal day 1 (PN 1) retinas and the amount increased to adult levels over the next two weeks of development. Screening of a number of tissues by Northern blot hybridization and by PCR amplification showed the channel to be expressed by heart and kidney as well as retina, but not by cerebellum, cerebral cortex, liver, muscle, olfactory bulb, spleen, testes or thymus.

A major step in the visual transduction pathway of mammalian rod photoreceptors is mediated by a cation channel activated by cGMP (1,2). A light-dependent decrease in cGMP concentration leads to closure of the cation channel, hyperpolarization of the cell membrane and a reduction in transmitter released.

The role of cGMP in transduction and the identification of the cGMP-gated ion channel was first defined electrophysiologically (3-5). Subsequent work led to the biochemical isolation of a membrane protein that retained some properties of the channel when reconstituted into lipid bilayers (6,7). Partial amino acid sequence of this protein allowed the cloning of a cDNA that, when expressed in *Xenopus* oocytes, encoded a cGMP-gated cation channel (8). It is not known whether the cGMP-channel is just one member of a family of such molecules found throughout the nervous system or even throughout the body. In several other tissues cGMP has been implicated in events leading to changes in membrane properties, possibly through the action of ion channels (9,10).

We have used the polymerase chain reaction (PCR) to generate probes against the mRNA for the rat retinal cGMP-gated channel. These probes have been used to investigate the tissue distribution of the expression this gene in the adult rat, as well as to determine when it is expressed during rod photoreceptor development.

MATERIALS AND METHODS

RNA Preparation. Total RNA from 8-10 pairs of rat retina/age group was isolated by a single step acid guanidinium -phenol-chloroform extraction (11) using RNAzol B (Cinna Biotecx). Total RNA from bovine retina and other tissues was isolated by same procedure.

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Reverse Transcription. The reaction was carried out at 37°C for 2 hr in 50 µl of reverse transcription buffer (50mM Tris-HCl, pH 7.6, 60mM KCl, 10mM $MgCl_2$, 1unit/ml RNase inhibitor (Promega), 50µg/ml actinomycin D) containing 0.5mM of each dNTP, 0.1mg/ml dT_{17}, 5 µg of total retinal RNA and 200 units of murine reverse transcriptase (BRL). The reaction mixture was extracted with phenol-chloroform, and the DNA then recovered by ethanol precipitation and dissolved in 50µl of H_2O.

Synthesis of Oligonucleotide Primers. The oligonucleotide primers were synthesized on an automated DNA synthesizer (Applied Biosystems 391) and purified by polyacrylamide gel electrophoresis followed by reverse phase extraction using a Sep-Pak18 column (Millipore). The forward (cGMP-A) and reverse (cGMP-B) primers corresponded to bases 1356-1385 and 2143-2111 of the published bovine rod cGMP gated channel cDNA sequence (Figure 1)(8). Primers (5'GTGGGGCGCCCCAGGCACCA3' [β-A] and 5'CTCCTTAATGTCACGCACGATTTC3' [β-B]) were synthesized and used to amplify a 548 base pair fragment of β-actin cDNA as a positive control in PCR amplification (12).

PCR Amplification. The reaction was carried out in a final volume of 100µl containing PCR amplification buffer (10mM Tris-HCl, pH 8.0; 50mM KCl; 1.5mM $MgCl_2$; 0.001% gelatin), 200µM of each dNTP, 50pmol of forward and reverse primers, 1-5 µl reverse transcription product, and 2.5 units of Ampli taq (Perkin-Elmer-Cetus). The reaction mixture was overlaid with 50µl of mineral oil and the amplification was carried out on a DNA thermal cycler (Techne) by a step program (92°C, 1 min, 50°C, 1 min, 72°C, 2 min) for 30 cycles followed by a final 10 min. extension at 72°C. The reaction mixture was recovered by extraction with 100µl of chloroform. 10-15µl of each PCR amplification reaction was analyzed on a 1.5% agarose gel containing 0.5µg/ml ethidium bromide.

Southern Blot Analysis. Aliquots (10µl) of PCR products were resolved on a 1% agarose gel containing 0.5µg/ml ethidium bromide, denatured, neutralized, transferred onto Nytran membrane (Schleicher & Schuell) and hybridized with ^{32}P-labelled probe by standard procedures (13). Genomic Southern analysis was carried out according to procedures mentioned above and included Eco RI, Hind III and Bam HI digested rat brain genomic DNA resolved on a 0.8% agarose gel.

Northern Blot Analysis. Electrophoresis and hybridization were carried out as previously described (14). For quantitation of developmental expression, films were exposed at room temperature without an intensifying screen. Sequential hybridization with different probes was carried out after washing the membrane in 0.5% SDS at 100°C, five times for 5 minutes each to strip off the previous probe. The uniformity of RNA loading was ascertained by UV-induced fluorescence of 28S and 18S rRNA after ethidium bromide staining. The size of mRNA species was obtained by a comparison with an RNA ladder (BRL) electrophoresed in a side well. Quantitation of hybridization signals and 28S rRNA fluorescent bands were carried out by densitometric scanning. Data were expressed in optical density units after normalization to 28S rRNA levels.

Probe Preparation. The cGMP gated channel PCR product, a 1.1 kb BamHI-EcoRI fragment corresponding to exons 6 to 8 of the a subunit of mouse transducin (Tα) (15) and a 1kb Pst1-XbaI γ-actin insert were resolved and labeled in 1% Seaplaque (FMC) low gelling agarose by random primer extension (16) with α-[^{32}P] dCTP (3000 Ci/mmol, Amersham) to a specific activity of about 10^9 cpm/µg DNA.

RESULTS

The amplification of both bovine and rat retinal cDNA using primers cGMP-A and cGMP-B consistently yielded a PCR product of the size (790 bp) predicted from the bovine rod photoreceptor cGMP gated channel cDNA sequence (Fig. 1, 2A). No PCR products were detected when reverse transcriptase was omitted, showing that the PCR products

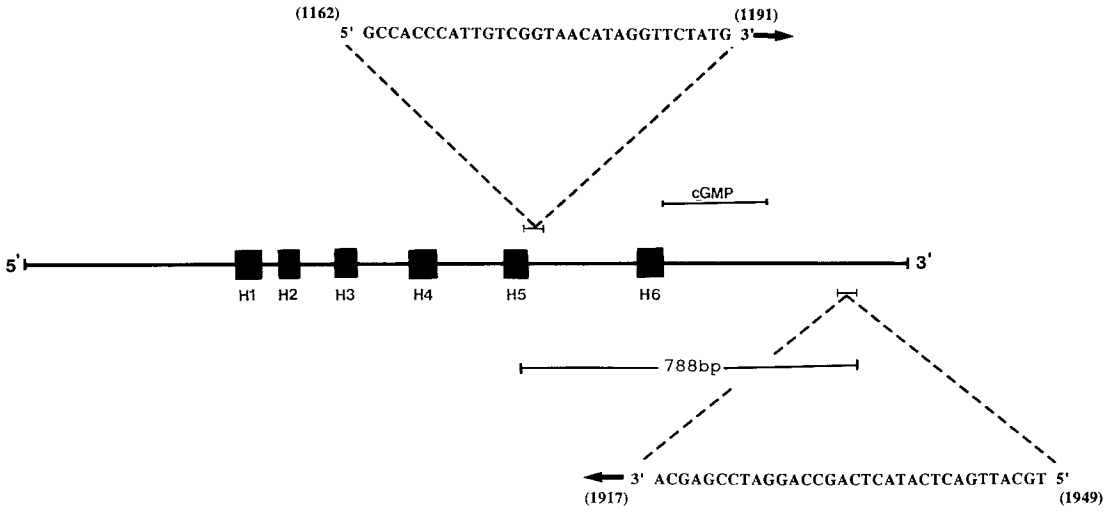


Figure 1. A schematic diagram of bovine cGMP-gated ion channel cDNA based on Kaup et al. (8) showing the position and sequence of forward (cGMP-A) and reverse (cGMP-B) primers. Filled boxes and the horizontal line represent sequences coding for a putative transmembrane domain and a putative cGMP binding region, respectively.

were mRNA dependent. Amplification of retinal as well as non retinal cDNAs using primers for β-actin consistently yielded the expected 548bp PCR product (Fig.2A).

Southern analysis using bovine or rat cGMP-PCR products as a probes revealed cross reacting bands corresponding to the positions of rat and bovine cGMP-PCR products (Fig. 2B and C). In each case, however, the intensity of cross hybridization of the heterologous probe was less. No hybridization signal was obtained in the lane containing the β-actin PCR product. Northern analyses of rat and bovine total RNA using bovine cGMP-PCR product identified a mRNA of 3.2 kb corresponding to the size reported for bovine rod cGMP gated channel mRNA (Fig.3A)(8).

A Southern analysis of rat genomic DNA digested with Eco RI, Hind III and Bam HI using rat cGMP-PCR product as probe revealed a single distinct hybridizing fragment of different size in each lane, suggesting the existence of a single gene with this sequence (Fig. 3B).

Northern analysis of total RNA from rat cerebral cortex, cerebellum, heart, kidney, muscle, olfactory bulb, spleen, testis, thymus and retina using rat cGMP-PCR product as a probe revealed a 3.2 kb band with heart, kidney and retina total RNA (Fig.4A). Heart and kidney contained an additional band of approximately 6.0 kb. Northern analysis using a ^{32}P-labelled probe from the mouse Tα gene revealed a mRNA of 2.6 kb present only in retina (Fig. 4B), confirming that this probe was specific under these conditions.

Quantitation of the developmental expression of rat rod cGMP gated channel and Tα genes was carried out on 25μg retinal total RNA/age group by a sequential hybridization, first with ^{32}P labelled rat cGMP-PCR (Fig.5A) product and then with ^{32}P BamHI-EcoRI mouse Tα insert (Fig5B). γ-actin mRNA was quantitated as a measure of a constitutive gene expression (Fig. 5C). 28S and 18S rRNA levels showed the uniformity of RNA loading (Fig. 5D). cGMP-gated channel mRNA was first detected at postnatal day 1

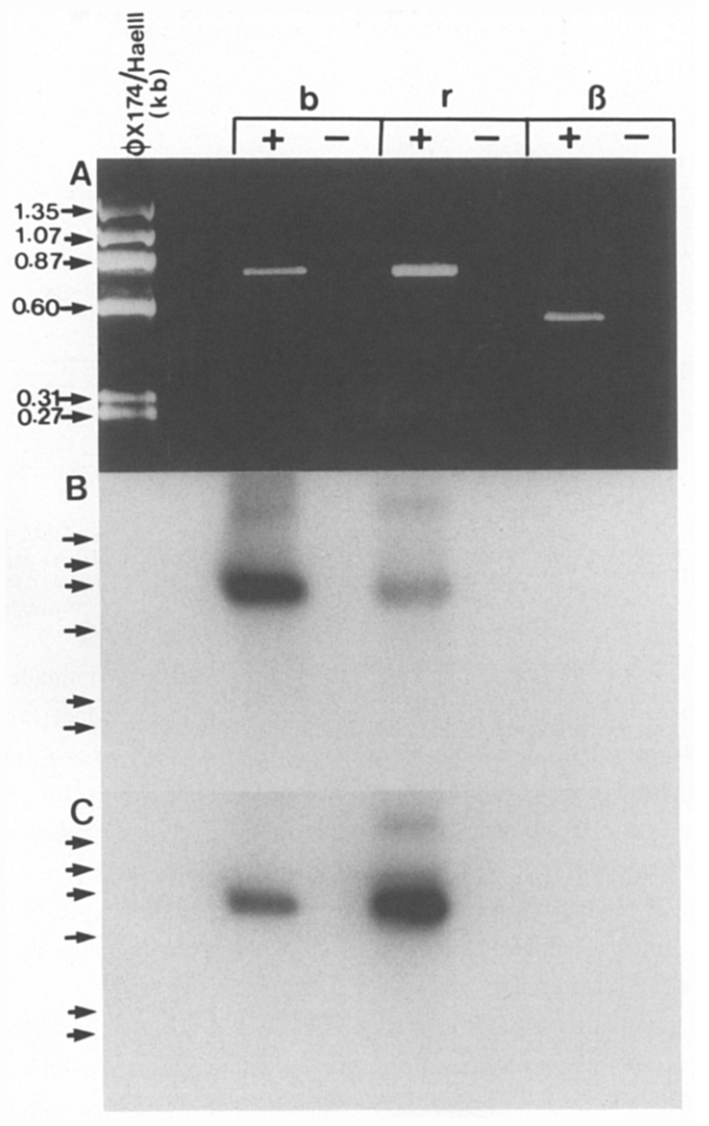


Figure 2. Analysis of PCR amplification by agarose gel electrophoresis (**A**) and by Southern analyses (**B**,**C**). PCR products, obtained from reverse transcribed retinal total RNA using primers cGMP-A/cGMP-B or β-A/β-B in the presence (+) or absence (-) of reverse transcriptase were resolved on a 1.5% agarose gel with 0.5μg/ml ethidium bromide. Southern analyses of PCR amplification (**A**) using bovine (**B**) and then rat (**C**) cGMP-PCR product as probes. Tracks: b, bovine retinal cDNA with cGMP-A/cGMP-B; r, rat retinal cDNA with cGMP-A/cGMP-B; β, rat retinal cDNA with β-A/β-B.

(PN1) and its levels increased 3-fold by PN3. The adult levels were reached at PN12 which was 25-fold higher than that at PN1. Tα mRNA was first detected at PN1, was below the level of detection at PN3 and PN6, and then increased from PN9 onward. There was an 11- and 24-fold increase in Tα mRNA levels at PN12 and PN19 respectively, in comparison to the level at PN9. γ-actin mRNA (2.3 kb) was detected at all ages studied and its levels were similar at PN1 and PN19.

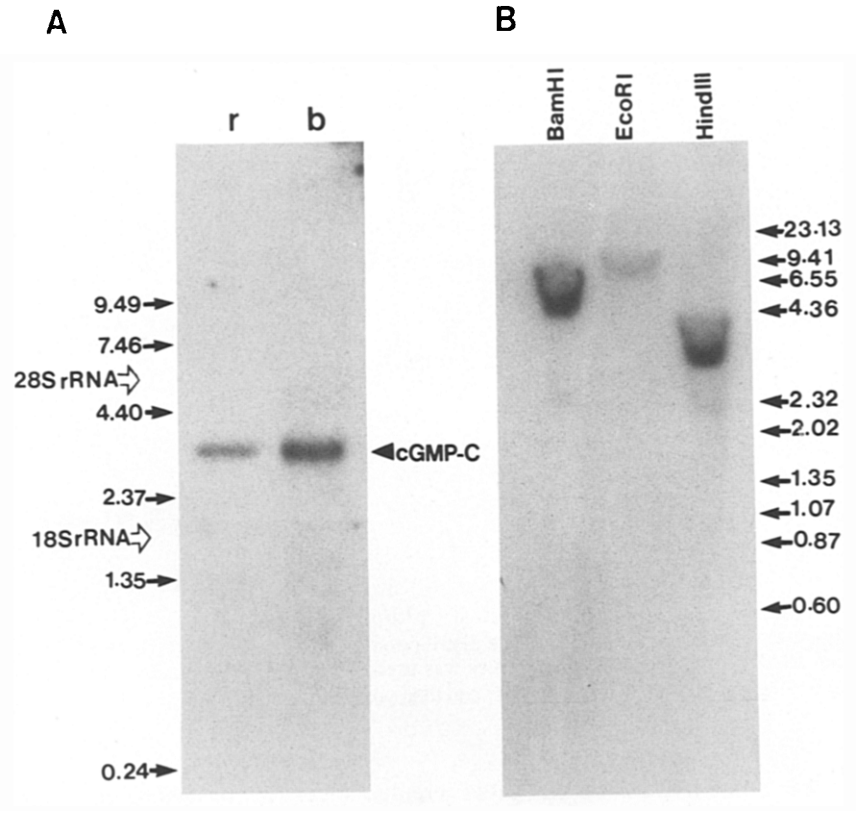


Figure 3. Northern analysis cGMP-gated ion channel (cGMP-C) (A) and Southern analysis of rat genomic DNA (B). Rat (r) and bovine (b) retinal total RNA (20μg) was electrophoresed and hybridized with ^{32}P-labelled bovine cGMP-PCR product. Molecular size markers correspond to RNA ladder (BRL). A blot of digested genomic DNA (20μg) was probed with ^{32}P-labelled rat cGMP-PCR product. Molecular size markers correspond to Hind III digested λ DNA and Hae III digested ϕX174 DNA.

DISCUSSION

The amplification of rat or bovine retinal cDNA consistently yielded a PCR product of the size (790 bp) predicted from the bovine cDNA sequence. The forward and reverse primers were selected to amplify a sequence in 3' region of cGMP-gated channel mRNA that encodes putative cGMP binding sites and is expected to be conserved across species. The authenticity of the PCR product was confirmed by the presence of restriction sites as predicted by bovine cDNA sequence, by identification of a 3.2 kb hybridization signal corresponding to the size of bovine rod cGMP-gated channel mRNA on a Northern analysis using either bovine or rat cGMP-PCR product as a probe and by sequence analysis of the PCR products (Figure 4A and data not shown). The divergence in the sequence of rat and bovine rod cGMP-gated channel mRNAs was evident from the decrease in the intensity of cross reactivity both in Southern and Northern analyses and by the variation in restriction sites in rat cGMP-PCR product in comparison to the bovine product.

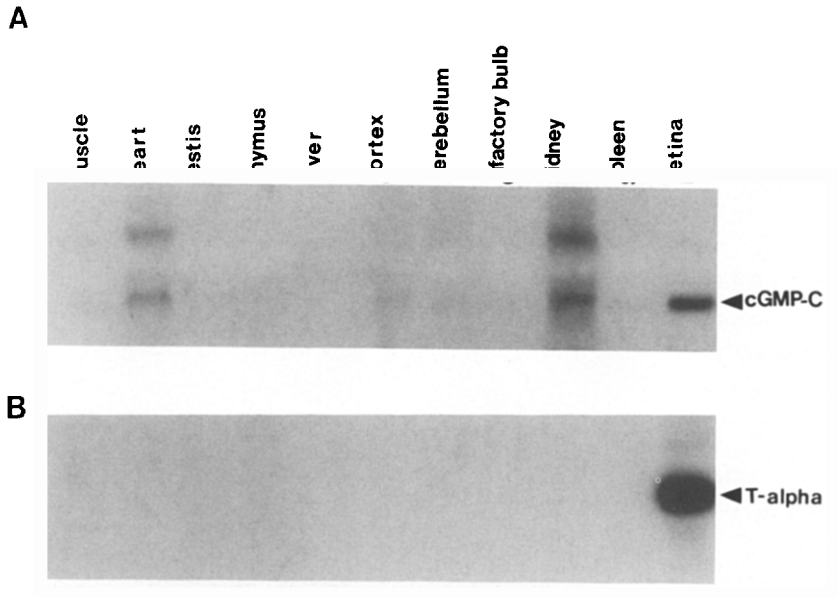
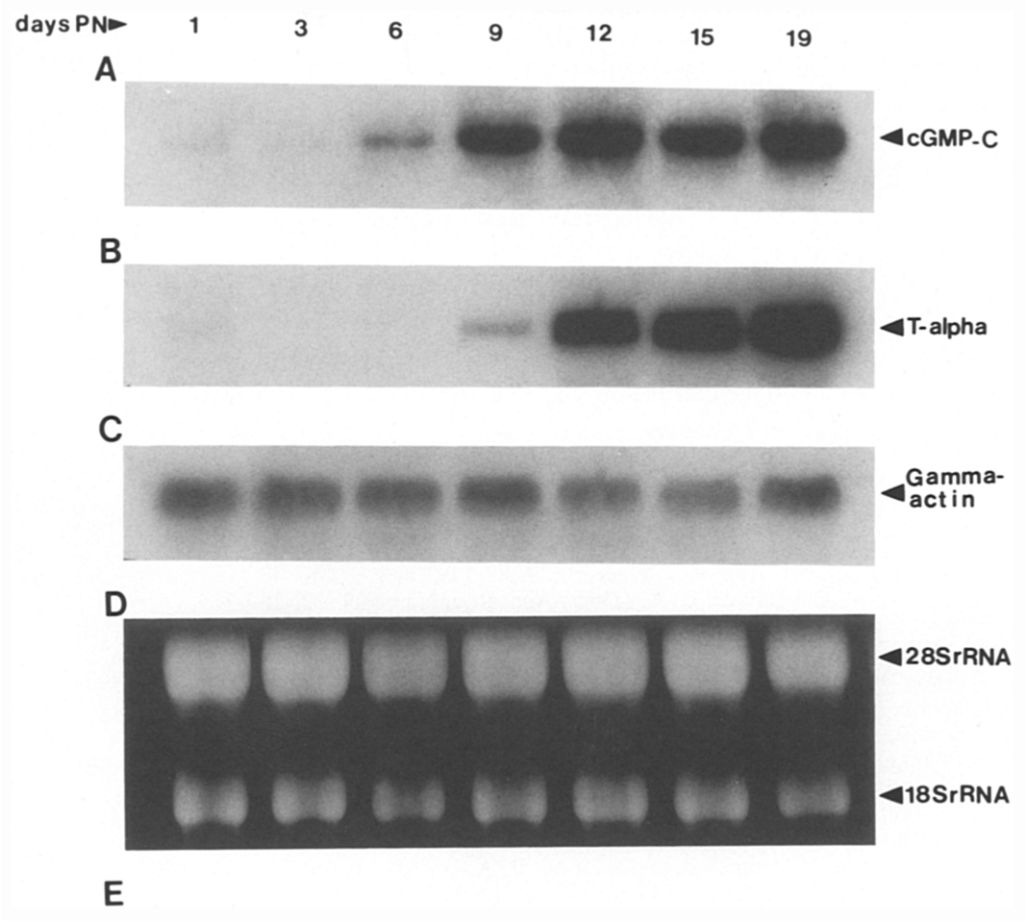


Figure 4. Northern analyses of tissue distribution of cGMP-C (A) and Tα (B) mRNA. Total RNA (20μg) from different tissues was used. The Northern blot was probed with ^{32}P labelled rat cGMP-PCR (A) product and then with ^{32}P labelled Tα probe (B).

The detection of cGMP-gated conductances in tissues other than rod photoreceptors, such as kidney (9) has led to the suggestion that there is a general class of channels gated by cyclic nucleotides (2). A hybridization signal corresponding to the 3.2 kb cGMP gated channel mRNA was detected in retina, heart and kidney total RNA, but not RNA from muscle, liver, testis, thymus, spleen, cortex, cerebellum and olfactory bulb. The hybridization signal with heart and kidney was present after stringent washing (0.1X SSC at 68°C for 1hr). Southern blot analysis of rat genomic DNA implies that cGMP-gated channel gene may be a single copy gene. This suggests that the cGMP-gated channel previously detected electrophysiologically in inner medullar collecting duct of kidney is a product of the same gene as retina (9). Similarly, detection of a 3.2 kb mRNA in heart suggests the expression of the same channel in this tissue. Since no such channel has yet been reported in heart this is a novel finding and requires further investigation.

The developmental expression of cGMP-gated channel and Tα genes was studied in rat retina. mRNA for the cGMP-gated channel was first detected at PN1 which correlates well with the expression of opsin, the first step in the transduction cascade, which is transcriptionally activated at PN1 (17). Tα mRNA was first detected at PN 1 but was below the threshold of detection at PN3 and PN6. It was detected again at PN9 and then increased over the second postnatal week. The onset of gene expression of the rod cGMP-gated channel , Tα and opsin at PN1, and the fact that the majority of rod photoreceptors become postmitotic between PN1 and PN2 (18-20), suggest that genes encoding proteins of the visual transduction pathway are among the earliest to become active during photoreceptor differentiation and may respond to similar or a common developmental



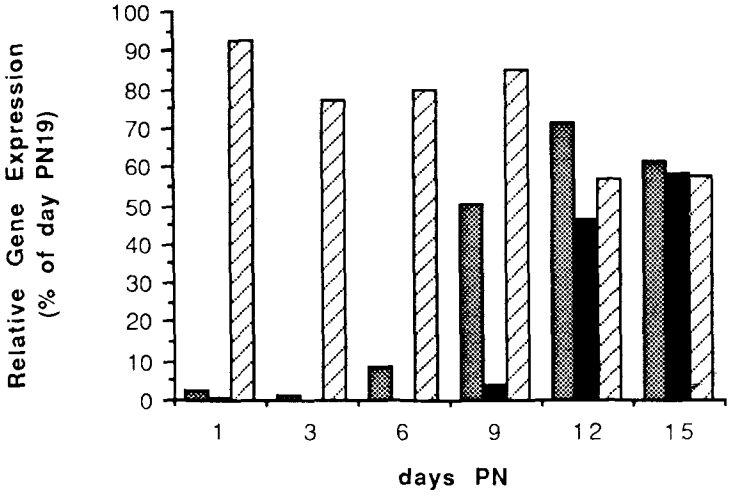


Figure 5. Northern analyses of developmental expression of cGMP-C and Tα mRNA. Total retinal RNA (25μg)/age group was used. The Northern blot was sequentially hybridized with ^{32}P labelled-rat cGMP-PCR product (A), -Tα probe (B) and - γ-actin probe (C). Uniformity of loading of total RNA is represented by UV induced fluorescence of ethidium bromide stained 28S and 18S rRNA bands (D). The developmental expression was quantitated in optical density units and represented as the % of PN19 after normalization to 28S rRNA levels (E). ▩ = cGMP-channel; ■ = Tα; ▨ = γ-actin.

cue(s). The different rates of increase in mRNA accumulation over the first two postnatal weeks suggest that additional cues may affect transcription rates or RNA stability. The understanding of all these cues may shed light on important aspects of rod photoreceptor differentiation.

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