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Gene discovery in the embryonic chick retina

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Purpose: The chick embryo is a powerful model system for the study of retinal development. However, analysis of gene expression in the chick retina has lagged behind biological studies. The purpose of this study was to identity and characterize genes expressed in the chick embryo retina as candidate molecules involved in the development and function of photoreceptors and other retinal cell types.
Methods: RNA from embryonic day (ED) 18 White Leghorn chick embryo retinae was used to generate an oligo dT-primed cDNA library. Bacterial colonies representing five thousand individual clones were arrayed onto nylon membranes using a microarray robot. Replicate membranes were hybridized with cDNA probes synthesized from ED 18 retina, brain and liver. Clones that appeared preferentially expressed in retina were identified by homology searches, and their spatial and temporal expression patterns were analyzed by in situ hybridization.
Results: Two hundred and seventy-two clones were identified. Approximately forty percent of the clones represented potential novel genes, including ESTs, hypothetical proteins and clones with no assigned identities. Furthermore, many genes were identified that are the putative chick orthologues of genes cloned from other species. We determined the expression pattern of several clones for which sequence homologies suggested possible roles in transcriptional regulation, apoptosis or intercellular signaling. Their corresponding mRNAs were expressed in the embryonic retina in topographically specific, developmentally regulated patterns.
Conclusions: We identified and characterized genes in the chick embryo retina using a combination of microarray analysis and in situ hybridization. Analysis of the expression patterns suggests involvement of several of these genes in key events during embryogenesis.

The chick embryo has a number of advantages for the experimental analysis of retinal development. The embryonic eye, for example, is easily accessible from very early stages of development, allowing surgical manipulation [1-5], infection of retinal progenitor cells with replication incompetent or replication competent viruses [1,6-10], and pharmacological intervention [11,12]. This has made possible significant progress in a number of areas of retinal development, including cell lineage, the role of various transcription factors in retinal patterning and cell differentiation, and the mechanisms controlling guidance of ganglion cell axons towards specific regions of the CNS. In addition, the chick embryo retina is well suited for in vitro studies, and has been used for decades for explant, reaggregation and dissociated cultures [13-16].

Despite its substantial strengths, however, the chick retina does have certain limitations as a model system, many of which arise from the lack of well-developed genetic and genomic resources. Only a limited number of genes expressed in the developing and adult chick retina have been identified, and in many cases their patterns of expression have not yet been analyzed. This has limited substantially the scope of studies that could take advantage of powerful techniques available for delivering reagents for gain and loss of function experiments in the chick embryo retina, including electroporation and avian-specific retroviral vectors [6-8,17,18]. As an initial approach to this problem, in the present study we have isolated and characterized numerous genes that may be relevant for the development of photoreceptor and other retinal cell types. The strategy involved using an embryonic chick retinal nylon membrane-based microarray for high throughput screening of mRNA expression, followed by sequence analysis of abundantly expressed genes and in situ hybridization to determine their temporal and spatial patterns of expression at several stages of embryonic development. We identified 272 clones that include a large number of genes that had previously been cloned but not described in the chick retina, as well as others that represent altogether novel genes. Topographically-specific, developmentally-regulated patterns of expression were observed for many of these genes, suggesting that they could be involved in key events during embryogenesis.

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METHODS

Embryonic day 18 (ED 18) chick retina library: All procedures involving chickens were carried out in accordance with the statement by the Association for Research in Vision and

Ophthalmology for the Use of Animals in Ophthalmic and Vision Research and was approved by the Animal Care and Use Committee at Johns Hopkins University. Neural retina tissue was dissected from ED 18 White Leghorn chick embryos and total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA), followed by polyA(+) RNA purification with the Message Maker kit (Gibco BRL, Rockville, MD). An oligo dT-primed cDNA library was constructed with the polyA(+) RNA using Superscript cDNA Library reagents (Gibco BRL), according to the manufacturer's protocols. The library contained $1.1x10^7$ primary clones, with an insert size range of 0.5-5 kb. The library was subsequently excised and converted to a PZL1 plasmid library (Gibco BRL), using endogenous CRE protein. Ninety percent of the viable colonies contained retina library inserts.

Generation of membrane-based microarrays: The ED 18 chick retina plasmid library was re-plated at low density, and 5,000 bacterial clones were randomly picked into 384-well plates, and cultured overnight in LB-ampicillin/8% glycerol. The bacterial cultures were arrayed in duplicate onto nylon membranes (Pall Biodyne B, Nunc, Rochester, NY) with a Microgrid II Robot (Biorobotics, Cambridge, England) using a 0.4 mm 384-well pin tool. The membranes were placed on an LB-ampicillin agar support, and the printed colonies were grown at 37 °C for 16-24 h. When the majority of the colonies on the membrane were visible, the membranes were treated with 0.5 M NaOH/1.5 M NaCl for 7 min, neutralized twice in 1 M Tris-HCl, pH 7.4 for 5 min, incubated in 0.5 M Tris-HCl (pH 7.4)/1.5 M NaCl for 4 min, and cross-linked with ultraviolet light (120 J/cm^2 for 30 s).

Probe generation for array analysis: Hybridization probes were synthesized from stage ED 18 brain, liver and retina tissues. Retinas were dissected under a microscope to exclude pigmented epithelium, lens and other extraretinal tissue. Tissue samples were processed immediately, or frozen in liquid nitrogen and stored at -80 °C. Total RNA was extracted using TRIZOL reagent (Invitrogen), the integrity and purity were assessed by gel electrophoresis and A_{260}/A_{280} absorbance ratios, and mRNA was then purified using the Message Maker kit (Gibco BRL). First-strand cDNA was synthesized from 2 μg of mRNA using SuperScript II reverse transcriptase (Invitrogen), with random hexamers and ^{33}P-labeled dCTP. The membranes were prehybridized with 5X Denhardts reagent in 6X SSC/1% SDS for 2 h at 68 °C in a rotating hybridization oven. $5x10^6$ dpm/ml of labeled probe was then added with 100 μg/ml herring sperm DNA and hybridized overnight at 65 °C. The membranes were washed twice with 2X SSC/1% SDS at room temperature for 5 min and then with 0.1X SSC/1% SDS at 65 °C for 20 min.

Array data analysis: Hybridized membranes were exposed to phosphoimager screens for 4 h to 3 days and acquisition of radioactive images was performed using the Cyclone phosphoimager (Packard, Boston, MA) and OptiQuant software. Spot-finding and image analysis on the scanned images was performed using Imagene software (Biodiscovery, Marina Del Rey, CA). Automatic flagging of spots that were empty (indistinguishable from background) or poor (due to non-specific background) allowed elimination of faulty data. Clones preferentially expressed in the retina were identified by the Genesight software analysis program (Biodiscovery) and were confirmed manually by visual inspection of the spots.

Database searches: The plasmids corresponding to differentially expressed genes were purified from the bacteria and sequenced using the M13 reverse primer, yielding on average 600 bp of useful sequence information. Many of the clones were sequenced in both directions, permitting full sequencing of the insert. Clones with no significant matches in the database or those with poor quality sequence were re-sequenced in the opposite direction using a degenerate primer 5'-(T)17(A+C+G)(A+C+G+T)-3'.

Following editing of the sequences to remove segments corresponding to the vector, database searches were performed using the available public databases, with the non-redundant (nr) Blastn or Blastx algorithms at the National Center of Biotechnology Information (NCBI) [19]. Due to the relatively small number of chicken genes deposited in the databases, we expected that most of the matches for our clone set would represent cross-species homologies. Consequently, low stringency cut-off values (expectation values [E] of $E=10^{-10}$) were used as the criteria for determining homology. It should be noted that various other reports have used less stringent criteria (e.g., 10^{-5}) [20]. We used a slightly more stringent E-value to reduce false matches while enabling us to identify potential homologies in our cross-species comparisons; typically, E-values of 10^{-15} or lower have been used in mammalian gene discovery efforts. A low complexity filter was used in the analysis. Domain and motif searches were performed using public online search tools. Additionally, all the matched sequence alignments were examined visually to exclude spuriously high probability values arising from repetitive sequences. Functional classification of the genes was based on the primary reported function obtained from the PubMed literature database (NCBI).

Probe synthesis for in situ hybridization: An approximately 400 bp insert PCR product was amplified from individual clone plasmids using a gene-specific forward primer containing a T3 RNA polymerase overhang (18-20 bp of perfect homology; T3 sequence: AAT TAA CCC TCA CTA AAG GGA GA) and a reverse primer corresponding to plasmid sequence (M13/pUC reverse primer: AGC GGA TAA CAA TTT CAC ACA GG). This amplification strategy resulted in the inclusion of a T7 RNA polymerase sequence from the plasmid. The resulting PCR product was gel-purified using β-agarase digestion. In vitro transcription was performed in the presence of digoxigenin-11-uridine-triphosphate (DIG-UTP) to produce DIG-UTP-labeled single-stranded antisense RNA (using T7 polymerase) or sense RNA (using T3 polymerase) probes using the DIG RNA Labeling Kit (Roche Molecular Biochemicals, Indianapolis, IN), according to the manufacturer's instructions. Transcript concentration was estimated by comparison against a DIG-labeled RNA control (Roche).

In situ hybridization: Tissues were dissected in RNase-free Hanks Balanced Salt Solution, fixed at 4 °C in 4% paraformaldehyde/PBS containing 5% sucrose, incubated in

an increasing concentration series of sucrose in 0.1 M phosphate-buffered saline (PBS), and embedded in a 2:1 mixture of 0.1 M PBS with 20% sucrose and OCT in PBS [21]. Seven and 10 μm thick cryosections were collected on Superfrost Plus slides, rinsed in PBS, treated with proteinase K (10 μg/mL) for 1 min, rinsed in PBS, and pre-hybridized for 2 h at 65 °C in 50% formamide, 5X SSC, 100 μg/ml heparin, 0.1% Tween 20, 1 mg/ml tRNA, 1X Denhardts, 0.1% CHAPS and 5 mM EDTA. Sections were then covered with 100 μl of 400 ng/ml probe in the prehybridization solution, and incubated overnight at 60 °C in a humidified chamber. Post-hybridization washes were as follows: (1) twice in a 50% formamide/2X SSC solution at 60 °C for 30 min each; (2) three times in 2X SSC at 37 °C for 5 min; (3) RNAse A and T1 RNAse in 2X SSC at 37 °C for 15 min; (4) 50% formamide, 0.1% CHAPS, in 2X SSC at 60 °C for 15 min; (5) 50% formamide, 0.1% Tween 20, in 0.2X SSC for 15 min. Hybridization was detected with the DIG Nucleic Acid Detection kit according to the manufacturer's instructions (Roche Molecular Biochemicals).

RESULTS

Classification of genes expressed in chick embryo neural retina: The microarray generated for this study included 5,000 randomly selected clones from a non-normalized embryonic day (ED) 18 chick retina cDNA library (see Materials and Methods for details). ED 18 retina represents a fairly advanced stage of development at which time all retina cell types have acquired a mature differentiated phenotype, and their synaptic connections have been established at the plexiform layers. To identify genes that are highly expressed and/or preferentially expressed in the retina, comparative hybridizations were performed with probes generated from ED 18 retina, brain, and liver. Although microarray studies can be used with appropriate statistical analysis to provide quantitative differential expression data, we chose to use them as a qualitative "first-pass" filter to rapidly identify potentially interesting genes, focusing further studies on their sequence and in situ hybridization analysis. This proved to be a rewarding strategy, yielding over 100 genes that had not previously been described in the chick retina.

Approximately 40% of the 5000 arrayed clones hybridized to the retina probe under the stringent conditions used. Of these, 272 hybridized more intensely to the retina probe than to the brain probe (or, in an initial smaller experiment, than to the liver probe), and were therefore selected for sequencing. Readable sequence of at least 300 bp was obtained for 236 clones. The sequences were classified into three main categories based on bioinformatic analysis (see Methods for a description of the criteria used): (1) previously cloned genes with known function from chick and other species, which were further classified into the ten functional groups in Table 1; (2) genes with unknown function including hypothetical proteins, predicted genes and ESTs with no homology to known genes ("ESTs/Unknown function" in Table 1); and (3) clones that were unclassifiable ("Unassigned identity/no homologs" in Table 1), including clones that had no matches in the database with the stringency criteria used, as well as clones that only had homologies to genomic regions. Categories 2 and 3 include clones that potentially represent novel genes.

Sequence analyses demonstrated that 188 (80%) of the clones were in categories 1 and 2, in that they matched genes or ESTs present in public databases (functional classification in Table 1 and Table 2). For the clones in category 1, 71 matched to previously cloned chick sequences whereas 65 were homologous to transcripts not previously cloned from the chick, suggesting that we have identified their chick orthologue (or a gene closely related to their orthologue). Their orthologues were found mostly in mammals, except the homologue of CRG (chick retina gene) 178 that was only present in another bird, the Japanese quail. As described below, there was little information in the literature about ocular expression and/or function of most of these homologues. As expected, we identified known retina-enriched genes in our clone database (for example, melanopsin, transducin). However, the total number of clones representing vision genes was low (4%). The reason why visual function genes were not abundant in the library is unclear, although it is apparent from in situ hybridization results (see below) that there were several examples of clones representing genes with clear photoreceptor expression.

For category 2 clones, we identified 25 clones that matched only to ESTs found in the chick, 8 clones that matched to ESTs found in mammals, and 19 were identified only as novel genes of unknown function (including predicted genes and hypothetical proteins). Some of the ESTs had weak similarity to known genes but the majority was "anonymous" in that they lacked recognizable predicted protein motifs, and their identity and function are currently unknown. Of interest, we have identified a large number of potentially novel genes in this study (clones in categories 2 and 3; clone and sequence information in Table 2). Approximately 40% of the clones with readable sequence belonged to category 3, having no signifi-

Table 1. Classification of sequenced CRG clones

Clones	%
EST's and unknown function	20
Unassigned identity/no homologs	19
Mitochondrial function	14
Metabolism	11
Intra- and extra-cellular signaling	10
Nuclear function	8
Translation	6
Trafficking and transport	4
Visual function	4
Development	2
Cell death and proliferation	1
Cytoskeleton	1

Cloned (category 1) and unknown (category 2) genes were classified into functional groups. The table shows the fraction of the genes placed in each classification.

TABLE 2. IDENTITY OF THE ISOLATED CHICK RETINA GENES

Clone	GenBank accession number of clone	Gene	GenBank accession number of match
CRG1	CD526884	Chick calmodulin	AF081672
CRG2	-	no match: putative novel gene	
CRG3	-	human lysosomal H+ ATPase (ATP6F)	XM001463
CRG4	CD526885	mouse EST 10 d whole embryo	AK011185
CRG5	-	insufficient sequence	
CRG6	-	Chick Pou1 POU transcription factor	U30520
CRG7	-	insufficient sequence	
CRG8	CD526916	Chick polyubiquitin gene UbII	X58195
CRG9	CD527057	human KIAA0728, which has 22% idenity to mouse dystrophin	XM0301820
CRG10	-	insufficient sequence	
CRG11	CD527024	Identical to chick EST (Normalized Chicken Pituitary/Hypothalamus/Pineal Library); and has 170 aa region with 43% sim to Xenopus transcription factor MTGR1-like protein	AAF63193, CAA67688
CRG12	CD526917	Chick cytochrome c oxidase subunit III	NC001323
CRG13	-	insufficient sequence	
CRG14	-	no match: putative novel gene	
CRG15	CD526925	Human FLJ10618	AK001480
CRG16	-	no match: putative novel gene	
CRG17	-	no match: putative novel gene	
CRG18	CD526938	Chick EST Bursa of Fabricius	AJ398436
CRG19	CD526988	low similarity to mouse EST (which has weak similarity to phosphoglucomutase 55/63 bp 67% id)	BB855428
CRG20	-	no match: putative novel gene	
CRG21	-	no match: putative novel gene	
CRG22	CD526947	Gallus gallus kinesin light chain, 3'UTR	U48359
CRG23	CD526905	Chick calretinin	X62866
CRG24	CD526953	no match: putative novel gene	
CRG25	CD526960	Homo Sapiens phosphatidylinositol 3-kinase regulatory gamma subunit	AF028785
CRG26	CD527038	chick EST 93J15 (AC091091); short region similar to Mus musculus transducin (beta)-like 2 (Tbl2)	AC091091 (EST), NM_013763 (transducin)
CRG27	CD526971	Chick EST Bursa Fabricuis	AJ451598
CRG28	CD526886	Chick ribosomal protein L5	X57016
CRG29	CD526975	human Heparan sulfate D-glucosaminyl 3-O sulfotransferase	AAB84388
CRG30	CD526903	similarity to human FLJ14225 protein, which has 3 polycystic kidney disease protein domains	XM051935
CRG31	CD526982	Homo Sapien hypothetical protein FLJ20113	NM_017670
CRG32	CD526902	similarity to human KIAA1691 protein and Drosophila tweety	XM042128, NP065710
CRG33	-	no match: putative novel gene	
CRG34	-	no match: putative novel gene (weakly similar to human MMS19 [DNA repair & transcriptional regulator, 153/214 aa 71% sim])	
CRG35	CD526901	Chick Jun-binding protein	L13234
CRG36	-	no match: putative novel gene	
CRG37	CD526987	no match: putative novel gene	
CRG38	CD526993	no match: putative novel gene	
CRG39	CD526999	Homo Sapiens amyloid beta (A4) precursor like protein 2	Q06481
CRG40	-	similar to human adhesion protein MEMD	Y10183
CRG41	CD526887	Chick EST from pituitary/hypothalamus/pineal	BI391243
CRG42	CD526898	Chick melanopsin	AY036061
CRG43	CD527005	Chick EST Bursa Fabricuis	AJ398056
CRG44	-	no match: putative novel gene	
CRG45	CD526899	Chick green-sensitive cone opsin	M88178
CRG46	CD527016	Rattus norvegicus corneal wound healing related protein	AF272892
CRG47	CD526900	similarity to mouse glutamyl-tRNA synthetase	X54327
CRG48	-	no match: putative novel gene	
CRG49	-	no match: putative novel gene	
CRG50	CD526904	Chick glyceraldehyde-3-phosphate dehydrogenase	AF047874
CRG51	CD526919	Chick glyceraldehyde-3-phosphate dehydrogenase	AF047874
CRG52	CD526922	human X chrom that contains a predicted protein dJ1189B24, which has homology to hypothetical yeast and C. elegans proteins	AL030996
CRG53	CD526923	spermatogenesis-associated protein SPATA2	NO_006029
CRG54	CD527022	Chick enolase alpha	D37900
CRG55	-	no match: putative novel gene	
CRG56	CD526924	chick EST from pituitary/pineal/hypothalamus library	BI392778
CRG57	CD527052	human cisplatin resistance-associated overexpressed protein	NM_016424
CRG58	-	no match: putative novel gene	
CRG59	-	insufficient sequence	
CRG60	CD526906	Chick transforming growth factor-beta2	X58071
CRG61	-	insufficient sequence	
CRG62	-	insufficient sequence	
CRG63	CD526907	Chick angiopoietin-2	GGA289777
CRG64	CD526908	Human GABA(A) receptor-associated protein	XM_036649.1
CRG65	CD527037	Rattus norvegicus adapter protein ATH-55 (93% id in 59 bp domain to Homo Sapiens HCNP)	AF277899
CRG66	CD526909	chick EST from T-cell library	AI982414
CRG67	CD526911	Human ribosomal protein 2	MN_002952
CRG68	CD526910	Chicken snap-25 gene, exon 1	CHKSNAP02
CRG69	CD527042	Chick EST Bursa Fabricuis	AJ393281
CRG70	CD527041	Chick reverse transcriptase VTGIII, pol-like	I50209
CRG71	-	no match: putative novel gene	
CRG72	-	no match: putative novel gene	
CRG73	CD527046	chick EST from eye library	AI438138
CRG74	CD527047	no match: putative novel gene	
CRG75	CD526920	Sus scrofa PI3 (pig phosphatidyl inositol triphosphate)	U88368
CRG76	CD526921	Chick enolase alpha	D37900
CRG77	CD527066	chick EST from pituitary/pineal/hypothalamus library, similar (80, e-14) to rat signal regulatory protein alpha	BI394443
CRG78	CD527051	Rattus Norvegicus assembly protein (AP50)	M23674
CRG79	CD526912	Chick EST Bursa of Fabricius	BC003394
CRG80	CD526913	Homo sapiens hepatocellular carcinoma-related putative tumor suppressor	AP205718
CRG81	-	insufficient sequence	BC015268
CRG82	CD526889	Chick EST Bursa Fabricuis	AJ395770
CRG83	CD526914	Homo sapiens KIAA1580 protein	XM_045271
CRG84	-	insufficient sequence	
CRG85	CD526888	Gallus gallus mRNA for ferritin H chain protein	Y14698
CRG86	CD526891	Gallus gallus Na+-dependent glutamate/aspartate transporter	AF154672
CRG87	-	Homo sapiens, Similar to KIAA0808	BC007636
CRG88	CD526892	Gallus gallus mRNA for carbonic anhydrase II CA-II	X04810
CRG89	CD526890	Chicken synaptosomal associated protein-25	M57957
CRG90	CD526893	Mus musculus ribosomal protein S18	NM_011296
CRG91	CD527071	Chick EST normalized chicken fat cDNA library	BI064730
CRG92	-	no match: putative novel gene	
CRG93	CD526926	Rattus norvegicus C2-HC type zinc finger protein r-MyT13 (neural zinc finger protein)	RNU67080
CRG94	-	chick EST from activated T cell library	AI980924
CRG95	CD526894	Homo sapiens nucleoporin 153 kD (NUP153)	XM_004272
CRG96	CD526932	no match: putative novel gene	
CRG97	CD526915	Homo sapiens mitogen-activated protein kinase kinase kinase 7 (MAP3K7)	XM_004499
CRG98	-	no match: putative novel gene	
CRG99	CD526895	Chick EST activated T cell library	AI981770
CRG100	CD526896	Gallus gallus defender against death protein 1 (DAD1)	GGU83833
CRG101	CD526897	Gallus gallus aryl hydrocarbon receptor	AF260832
CRG102	-	insufficient sequence	
CRG103	CD526918	Chick EST Bursa Fabricuis	AJ399283
CRG104	CD526939	Homo sapiens hypothetical protein FLJ12565	NM_022064.1
CRG105	CD526943	human lysosomal-associated protein transmembrane 4 alpha	BC000421
CRG106	CD526948	Chicken middle-molecular weight neurofilament fragment	X05558
CRG107	CD526955	Chick EST 5 d embryo	AL586665
CRG108	CD526962	no match: putative novel gene	
CRG109	-	insufficient sequence	
CRG110	CD526972	Chick macrophage migration inhibitory factor (MIF)	M95776

TABLE 2. CONTINUED

Clone	GenBank accession number of clone	Gene	GenBank accession number of match
CRG111	CD526976	Homo sapiens LATS (large tumor suppressor, Drosophila) homolog 1	XM_015547.1
CRG112	CD526983	Homo sapiens, Similar to poly(rC)-binding protein 3	BC012061
CRG113	CD526989	no match: putative novel gene (weak similarity to Homo sapiens nucleoporin 153 kD, 174 bp region with 78%)	
CRG114	CD526995	Homo sapiens, clone MGC:4491 IMAGE:2963838 Conserved gene telomeric to alpha globin cluster	BC012825
CRG115	-	insufficient sequence	
CRG116	CD527000	no match: putative novel gene	
CRG117	CD527007	Chicken mRNA for enolase alpha	D37900
CRG118	-	no match: putative novel gene	
CRG119	CD527017	Homo sapiens N-myc downstream regulated (NDRG1)	XM_005243.4
CRG120	-	insufficient sequence	
CRG121	CD527029	no match: putative novel gene (Weak similarity to Homo sapiens hypothetical protein MGC4054, 79/97 bp (81%id))	
CRG122	CD527034	no match: putative novel gene	
CRG123	CD527039	Chick EST Normalized Chicken Pituitary/Hypothalamus/Pineal Library	BI390526
CRG124	CD527043	Homo sapiens CDC42-binding protein kinase beta (DMPK-like)	XM_007257.4
CRG125	CD527048	Homo sapiens phosphofructokinase, liver (PFKL)	NM_002626.1
CRG126	CD527053	Homo sapiens genomic clone RP5-965K10	AC006015
CRG127	CD526931	no match: putative novel gene	
CRG128	CD527062	Chicken carbonic anhydrase II	Z14957.1
CRG129	CD527067	chick fat library EST	BI067608
CRG130	-	insufficient sequence	
CRG131	CD527075	Homo sapiens selenoprotein T	XM_018149.2
CRG132	CD527079	Mouse, Similar to ATPase, H+ transporting, lysosomal pump	BC014706
CRG133	CD526927	no match: putative novel gene	
CRG134	CD526933	Chinese hamster COP-coated vesicle membrane protein CHOp24	U26264.1
CRG135	CD526940	Chicken LDH-A lactate dehydrogenase A chain	X53828.1
CRG136	CD526944	Chicken carbonic anhydrase II	Z14957.1
CRG137	CD526949	Homo sapiens seizure related gene 6 (mouse)-like (SEZ6L)	NM_021115.1
CRG138	CD526956	Homo sapiens enhancer of polycomb 1 (EPC1)	XM_034740.1
CRG139	CD526963	no match: putative novel gene	
CRG140	CD526954	no match: putative novel gene	
CRG141	-	Chicken Cystatin	M95725
CRG142	CD526977	Human ribosomal RNA Methyl Transferase 2	MN012280
CRG143	CD526984	Homo Sapien FLJ25006 fis clone	AK057735
CRG144	CD526961	human cDNA FLJ14223 fis, similar to mouse Spnr RNA binding protein (spermatid perinuclear RNA binding protein (STRBP))	AK024285
CRG145	CD526996	Homo Sapiens Gamma transducing activity polypeptide 2 (GNGT2) 110/129 bp (85%) in 5' half'; 3' half matches to chicken subtractive eye library EST 182/182 bp (100%)	AAC98924.1, AI438124
CRG146	CD527001	Gallus gallus Glyceraldehyde-3-Phosphate dehydrogenase	AF047874.1
CRG147	CD527008	Homo Sapiens KIAA1484 protein 149/204 aa (72% sim)	AAH14678.1
CRG148	CD526966	Chicken EST normalized chicken pituitary/hypothalamus/pineal cDNA library	BI391486.1
CRG149	CD527018	mouse eye EST	BB470653
CRG150	CD527025	Similar to Homo sapiens CGI-130 protein	AAH03357
CRG151	-	insufficient sequence	
CRG152	CD527030	Gallus gallus heme oxygenase-1	U95209
CRG153	CD527035	Chicken Carbonic Anhydrase I	Z14957.1
CRG154	-	no match: putative novel gene	
CRG155	CD527044	Homo sapiens neuro-oncological ventral antigen (NOVA) 1	NM_006489.1
CRG156	CD527049	Homo Sapiens Phosphofructokinase	BC002536.1
CRG157	-	Chick EST normalized chicken pituitary/hypothalamus/pineal cDNA library	BI394412.1
CRG158	CD527058	chick 37LRP/p40 gene (37 kD Laminin receptor precursor/p40 ribosomal associated protein)	X94368.1
CRG159	CD527063	rabbit phosphofructo-1-kinase C 150/183 bp (81%)	U01154
CRG160	CD527068	Transmembrane serine proteinase ST14 (Suppresion of tumorigenicity 14) Rattus norvegicus 135/215 aa (45%sim)	NP_446087.1
CRG161	CD527072	chick EST activated T cell cDNA library	AI981800
CRG162	CD527076	Gallus gallus Glyceraldehyde-3-Phosphate dehydrogenase	AF047874
CRG163	-	insufficient sequence	
CRG164	-	insufficient sequence	
CRG165	CD526934	Chicken Acidic Ribosomal Phosphoprotein	L28704
CRG166	-	insufficient sequence	
CRG167	-	Homo sapiens EST dorsal root ganglion	BF941189
CRG168	CD526950	Chick EST Normalized Chicken Pituitary/Hypothalamus/Pineal Library	BI393538
CRG169	CD526957	Rattus Norvegicus aspartoacylase	NM_024399
CRG170	CD526967	no match: putative novel gene	
CRG171	CD526973	Homo sapiens NADH Dehydrogenase (ubiquinione) flavoprotein 1	NM_007103
CRG172	CD526978	chick EST from activated T cell library	AI980477
CRG173	-	insufficient sequence	
CRG174	CD526990	Gallus gallus carbonic anhydrase II	GGCAII12
CRG175	CD526997	Cyprinus carpio (carp) H(+)-transporting ATPase beta subunit	AB023582
CRG176	CD527002	no match: putative novel gene	
CRG177	CD527009	Homo Sapiens Gamma transducing activity polypeptide 2 (GNGT2) 110/129 bp (85%) in 5' half'; 3' half matches to chicken subtractive eye library EST 182/182 bp (100%)	XM_054432, AI438124
CRG178	-	Coturnix coturnix japonica (Japanese quail) thymosin beta	AJ301650, AJ395712
CRG179	CD527019	chick 37LRP/p40 gene (37 kD Laminin receptor precursor/p40 ribosomal associated protein)	X94368
CRG180	-	no match: putative novel gene	
CRG181	CD527028	no match: putative novel gene (low similarity to Rat Surfeit 1 46/51 (90%))	
CRG182	CD527031	no match: putative novel gene	
CRG183	-	no match: putative novel gene	
CRG184	CD527040	no match: putative novel gene	
CRG185	CD527045	no match: putative novel gene	
CRG186	-	no match: putative novel gene (small (56 bp) identity to human brain EST that is ataxin2-binding protein 1 (A2BP1))	
CRG187	CD527054	Chicken LDH-A lactate dehydrogenase A chain	X53828
CRG188	CD527059	chick voltage-dependent anion channel	AF268470.1
CRG189	CD527064	Chick Na+-dependent glutamate/aspartate transporter	AF154672
CRG190	CD527069	no match: putative novel gene	
CRG191	CD527073	G. domesticus carbonic anhydrase	Z14957
CRG192	CD527077	human protein IMAGE:3162799	AAH07586
CRG193	CD527080	no match: putative novel gene	
CRG194	CD526928	insufficient sequence	
CRG195	CD526935	Chicken purpurin mRNA (retinal retinol-binding protein precursor)	M17538
CRG196	CD526941	Chicken SCG10 (stathmin-like 2; NGF-induced microtubule-destabilizing factor)	L14938
CRG197	-	no match: putative novel gene	
CRG198	CD526951	similar to human polycystin-1, similar to polycystic kidney disease 1 homolog	XM_095613
CRG199	CD526958	sialyltransferase 7 protein	AAM81378
CRG200	-	Chicken muscle pyruvate kinase	J00903
CRG201	CD526968	Chick EST Normalized Chicken Pituitary/Hypothalamus/Pineal	BI394441
CRG202	CD526974	Chicken creatine kinase B	X03509.1
CRG203	CD526979	Homo sapiens similar to phosphoglycerate mutase 1	XM_012219.4
CRG204	CD526985	Mus musculus clathrin adaptor complex adaptor-related protein complex AP-1, mu subunit 1	NM_007456.1
CRG205	CD526994	chick elongation factor 1 alpha	L00677
CRG206	CD526998	Chicken LDH-A lactate dehydrogenase A chain	X53828.1
CRG207	CD527003	Gallus gallus glyceraldehyde-3-phosphate dehydrogenase	AF047874
CRG208	CD527010	Gallus gallus mitochondrial genome	X52392.1
CRG209	CD527013	EST chick Brain Library	AL587826
CRG210	CD527026	Chick 18S ribosomal RNA gene in 5'; remaining 0.5 kb matches to chick EST Chicken Brain Library	AF173612, AL588863
CRG211	-	insufficient sequence	
CRG212	CD527032	Chicken muscle pyruvate kinase	J00903
CRG213	CD527036	Chicken ubiquitin I	M14693
CRG214	CD527006	Normalized Chicken Muscle EST	BM489384
CRG215	-	insufficient sequence	

TABLE 2. CONTINUED

Clone	GenBank accession number of clone	Gene	GenBank accession number of match
CRG216	CD527050	no match: putative novel gene	
CRG217	CD527055	Chicken acidic ribosomal phosphoprotein	L28704.1
CRG218	CD527060	Chick EST Normalized Chicken Reproductive Tract cDNA Library	BM440566
CRG219	CD527065	3' is similar to C. familiaris mRNA for rod transducin beta subunit	Z75134.1
CRG220	CD527070	Homo sapiens similar to spastic ataxia of Charlevoix-Saguenay (sacsin)	XM_037469.1
CRG221	CD527074	Homo sapiens cDNA FLJ21267 fis	AK024920
CRG222	-	insufficient sequence	
CRG223	CD527081	Gallus gallus mitochondrial genome	X52392.1
CRG224	CD526929	no match: putative novel gene	
CRG225	CD526936	Chicken c-beta-3 beta-tubulin	M14228.1
CRG226	-	insufficient sequence	
CRG227	CD526945	Human branched chain alpha-ketoacid dehydrogenase kinase	NM_005881
CRG228	CD526952	Equine peptidylglycine monooxygenase and peptidylamidoglycolate lyase	D29625
CRG229	-	insufficient sequence	
CRG230	CD526964	Mouse mitochondrial malate dehydrogenase	NM_008617
CRG231	CD526969	Human protein kinase C-binding protein Zeta1/fasciculation and elongation protein	NP_005094
CRG232	-	insufficient sequence	
CRG233	CD526980	Chicken HT7 antigen (100%)	X52751
CRG234	CD526986	Rattus norvegicus for proteasomal ATPase (SUG1)	NM_031149
CRG235	CD526991	Mus musculus, N-ethylmaleimide sensitive factor	BC006627
CRG236	-	Chick EST Normalized Chicken Abdominal Fat Library	BM426094
CRG237	CD527004	no match: putative novel gene	
CRG238	CD527011	Gallus gallus GTP-binding protein (rhoB)	AF098515
CRG239	CD527014	Chick EST Normalized Chicken Pituitary/Hypothalamus/Pineal Library	BI389614
CRG240	CD527020	chick death associated protein 5 (homolog of translation initiation 4gamma, Rat translation repressor NAT1, human p97)	AF093110
CRG241	CD527023	no match: putative novel gene	
CRG242	-	insufficient sequence	
CRG243	CD527033	Gallus domesticus cystatin	M95725
CRG244	-	insufficient sequence	
CRG245	-	insufficient sequence	
CRG246	-	insufficient sequence	
CRG247	-	insufficient sequence	
CRG248	CD527056	Gallus gallus photopigment melanopsin	AY036061
CRG249	CD527061	Chicken GAPDH	K01458
CRG250	-	insufficient sequence	
CRG251	-	insufficient sequence	
CRG252	-	insufficient sequence	
CRG253	CD527078	chick mitochondria sequence	
CRG254	-	Stratagene Chick Embryo EST ROS017C05	AL585002
CRG255	CD526930	no match: putative novel gene	
CRG256	CD526937	no match: putative novel gene	
CRG257	CD526942	Chicken c-beta-3 beta-tubulin	M14228
CRG258	CD526946	Chick EST pgf1n.pk011.d24	BI067234
CRG259	-	no match: putative novel gene	
CRG260	CD526959	Chick calretinin	X62866
CRG261	CD526965	Human CGI-97 protein (LOC51119)	XM_037741
CRG262	CD526970	Mouse ribosomal protein S18	NM_011296
CRG263	-	insufficient sequence	
CRG264	CD526981	chick tyrosinase-related protein-1	AF003631
CRG265	-	insufficient sequence	
CRG266	CD526992	R. rattus ribosomal protein S18	X57529
CRG267	-	Gallus gallus prosaposin	AF108656
CRG268	-	insufficient sequence	
CRG269	CD527012	Human small fragment nuclease	NM_015523
CRG270	CD527015	human thyroid hormone receptor-associated protein complex TRAP150	AF117756
CRG271	CD527021	Chick EST pgp1n.pk013.b6	BI393988
CRG272	CD527027	no match: putative novel gene	

Identification of CRG clones (CRG1-CRG272) based on sequence homologies, as described in Methods. GenBank accession numbers for the chick clones are provided where available. The closest homolog and its corresponding accession numbers are also shown.

cant matches in the databases (48 clones), or matched ESTs and/or genes of unknown function (category 2, 52 clones). Genes in this group may represent bona fide novel genes, but could conceivably have diverged so significantly from genes in other species that a significant match could not be detected with the available sequences and databases. It is also possible that the lack of sequence homology of clones in this category may in some cases be due to 3' UTR sequences present in the clones, which typically contain larger regions of lower sequence homology than the coding regions of the same mRNA. Additional sequence information could facilitate identification of the clones in those hypothetical cases.

Developmental expression analysis: The temporal and spatial expression of a selection of these newly cloned genes was studied by in situ hybridization, using a developmental series of chick embryo retinas that included the following stages: ED 5, when neuroepithelial cells are actively proliferating, and some cells are beginning terminal mitosis and differentiation; ED 8, when cell proliferation is largely complete, particularly near the fundus of the retina, and most postmitotic retinal progenitors have relocated to their future laminar positions; ED 12 and ED 15, when the various cell types of the retina are undergoing cell differentiation and forming specific neuronal interconnections; and ED 18, when cell differentiation is essentially complete, and the retina has a mature organization [22,23]. For clarity of presentation, the description of the various genes that were analyzed by in situ hybridization will be presented following the classification described above. The frequency of each category is listed in Table 1. Control sense strand hybridization was negative in all cases (Figure 1F,J, Figure 2I,N, Figure 3D,K,O, Figure 4D,H,L,P, and Figure 5G,J,P,V).

ESTs: As described above, many of the clones had sequence matches to ESTs that did not contain motifs or domains that could indicate their identity (see Table 1 and Table 2). As one example, clone CRG73 was homologous only to an EST from a subtracted chick eye library. In our analysis, CRG73 expression appeared relatively low at early developmental stages, but became much higher (and more localized) as the retina matured. At ED 5 there were practically no detectable hybridization signals in the retinal fundus, although some positive cells could be seen at higher magnification (not shown). Signals were more evident at the retinal periphery, near the future ciliary epithelium (Figure 1A). This pattern remained largely unchanged by ED 8 (not shown), but at ED 12 ganglion cells were positive, and the inner nuclear layer INL (INL) showed scattered, but abundant positive cells (Figure 1B). The putative photoreceptor layer remained negative at this stage, as it did at ED 15 (Figure 1C). At the later stage, signals were very strong in the ganglion cell layer (GCL) and in the inner part of the INL, and even stronger in the central region of the INL, in marked contrast with the light staining in the INL adjacent to the outer plexiform layer (OPL; Figure 1C). By ED 18, positively hybridizing cells were found both in the ganglion layer and in the inner part of the INL at the periphery of the retina (Figure 1E), but only in ganglion cells at the midperiphery (not shown) and fundus (Figure 1D).

Clone CRG123 was homologous to an EST isolated from a normalized chicken pituitary/hypothalamus/pineal cDNA library. Neither the clone nor the EST has recognizable motifs or known homologues. The expression of CRG123 changed developmentally from widespread to cell-type specific. Strong signals were seen throughout the neural retina at ED 5, except in the region immediately adjacent to the RPE (Figure 1G). The overall pattern persisted at ED 8, although the periphery of the retina had much lighter hybridization signals than the fundus (not shown). Widespread expression in all retinal layers was noted at more advanced stages (illustrated for ED 15 in Figure 1H), with the outer half of the INL appearing darker than its inner half. Some cells in the INL showed a circular outline and very strong hybridization signals (Figure 1I); their identity remains unknown, but they were reminiscent of apoptotic cells [24].

Genes with unknown function: The first group in Table 1 also contains clones with significant homology to predicted genes or hypothetical proteins in the databases that have no established annotated function. We have performed expression analyses on those genes that contain sequence motifs that

could suggest possible roles in retina development. An example is clone CRG9, which is homologous to the predicted human protein KIAA0728. The presence of a dystrophin-like domain in KIAA0728 suggests that it, and by inference, CRG9, may belong to a group of dystrophin-like molecules that have been implicated in development [25]. In ED 5 embryos, hybridization signals were present throughout the fundal region of the neural retina (Figure 2A-B), but were more restricted at the periphery, where they predominated near the RPE and the vitreal chamber (Figure 2A,C). As the retina acquired some degree of lamination by ED 8, the putative ONL appeared negative in the fundal region of the eye adjacent to the optic nerve, while the INL remained positive throughout, with occasional cells appearing darker than their neighbors (Figure 2D,E). At the periphery (Figure 2E) there was some heterogeneity in expression throughout the retinal epithelium. In more mature retinas (illustrated for ED 18 in Figure 2F-H), hybridization signals were strong in ganglion and amacrine cells, and much weaker in the rest of the INL (Figure 2F,G). Hybridization was undetectable in photoreceptors at the periphery (Figure 2F), but was very strong in their inner segments at the fundus (Figure 2G,H).

CRG31 is homologous to the human hypothetical protein FLJ20113, a gene of unknown function that lacks any recognizable motif. Cognate ESTs are broadly distributed in human, mouse and bovine tissues. The most interesting aspects

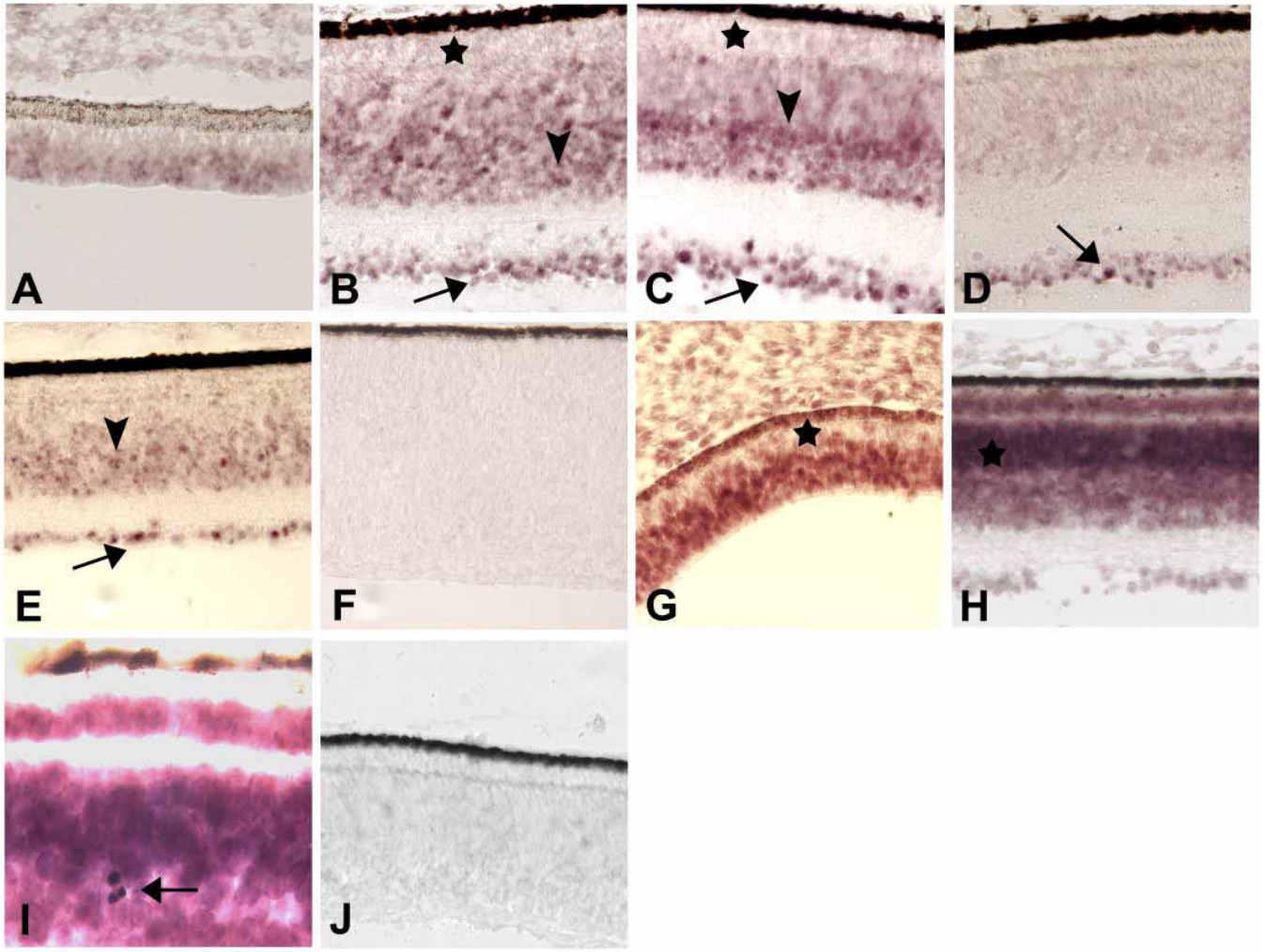


Figure 1. Topographically-specific, developmentally-regulated expression of novel chick retina ESTs. **A**-**F**: CRG73, homologous to an EST from a chick eye library. **A**: ED 5. Hybridization signals are detectable at the retinal periphery but not at the retinal fundus (not shown). **B**: ED 12. Positive cells are seen in the ganglion layer (arrow) and within the INL (arrowhead). The ONL (asterisk) is largely negative. **C**: ED 15. Expression is shown in the GCL (arrow) and the ONL (*), and strongly expressing cells in the middle part of the INL (arrowhead). Signals are weaker in the external half of the INL, adjacent to the ONL. **D**-**E**: ED 18. Positive cells in the INL at the retina periphery (**E**, arrowhead) but not at the fundus (**D**) or the midperiphery (not shown). Ganglion cells are positive throughout the retina (arrows in **D** and **E**). **G**-**K**: CRG123, homologous to an EST from a normalized chicken pituitary-hypothalamus-pineal cDNA library. **G**: ED 5. Hybridization signals are strong in the neural retina, except near the RPE (*). **H** and **I**: ED 15. Widespread expression in all retinal layers; the outer part of the INL (*) has stronger signals than its inner part. **I**: Higher magnification showing very dense, strongly positive cells with a circular outline (arrow) whose identity remains undetermined. **F** and **J** are sense control sections for CRG73 and CRG123, respectively.

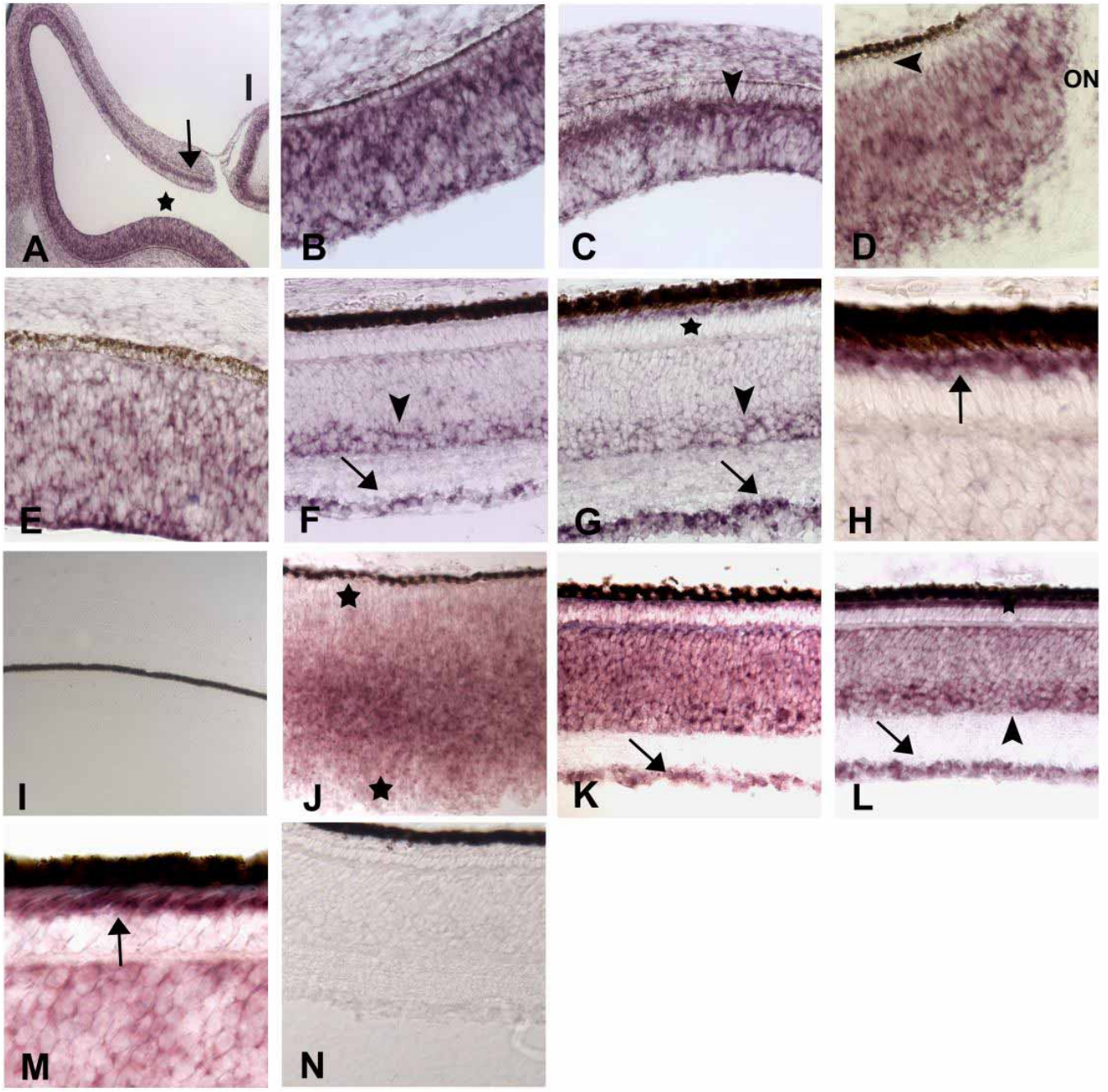


Figure 2. Developmentally regulated expression patterns of novel chick homologs. **A**-**I**: CRG9, homologous to dystrophin-like human predicted protein KIAA0728. **A**-**C**: ED 5. **A**: Low power view of the retina and lens (l). Signals are widespread at the fundus (**A***, **B**), but more restricted at the periphery (**A**, arrow, **C**). **D**: ED 8, fundus; the putative photoreceptor layer (arrowhead) is negative in the region adjacent to the optic nerve (ON), while the remaining neural retina appears very positive. **E**: ED 8, periphery; heterogeneous expression is seen in the retinal epithelium. **F**-**H**: ED 18. **F**, periphery, **G**-**H**, fundus. Cells in the GCL (arrows) and the amacrine layer (arrowheads) are positive throughout the retina and much weaker or absent in the rest of the INL. Photoreceptor cells appear negative at the periphery (**F**), but are very strong in the fundal region (**G**, *) as illustrated in more detail in panel **H** (arrow). **J**-**N**: CRG31, homologous to human hypothetical protein FLJ20013. **J**: ED 8. Widespread expression, predominating in the outer most regions of the neural epithelium (*). **K**: ED 15. GCL (arrow) and the INL have positive signals. **L** and **M**: ED 18. **L**: Ganglion cells (arrow), amacrine layer (arrowhead) and other cells scattered throughout the INL and in the photoreceptor layer (*) show strong signals. Photoreceptor signals can be seen at higher magnification to localize to the inner segment region of these cells (**M**, arrow). **I** and **N**: Sense control probes for CRG9 and CRG31, respectively.

of its expression were seen in ED 15-18 retinas, when very strong signals were observed in ganglion cells, and in the inner segments (but not the cell bodies) of photoreceptors (Figure 2K-M). Within the INL, expression was strong in many cells of the amacrine layer, in scattered cells in the region corresponding to glial cells of Müller, and in the region abutting the OPL (Figure 2K,L). In contrast to this selective pattern of expression, younger retinas (ED 5-8) showed a much more widespread pattern of expression, although the innermost and outermost aspects of the neuroepithelium appeared less positive than its center (Figure 2J).

Unassigned identity/no homologs: The third category of potentially novel genes is the 46 clones that had no significant matches in the databases to genes or ESTs. This category also included genes that had regions of similarity to mammalian genomic clones, but for which no other sequence similarities were found, making them currently unclassifiable. These clones (19% of total) could represent novel genes, or novel splice forms of previously cloned genes, or could correspond to expressed genes that were not previously identified due to low abundance in cDNA libraries (see Discussion). A representative clone is CRG92, which yielded practically no hybridization signals in the retina at ED 5 (not shown), and was only lightly and diffusely positive at ED 8 (Figure 3A); at this stage the fundus was stronger than the periphery (not shown). In a fully differentiated retina (ED 18), the outer part of the INL showed strongest signals, while ganglion cells and photoreceptor cells were lightly stained or negative (Figure 3C). A similar pattern was detectable at ED 15 (Figure 3B).

Newly cloned chick genes: Clones with significant matches to genes previously cloned in other species could be either the chick orthologue of such genes, or a chick gene closely related to the true orthologues. The categories of the various functions of these genes are listed in Table 1. We chose for further analysis genes whose involvement in the development of non-ocular tissues has been reported, but whose expression in the chick retina was unknown.

CRG111 is homologous to human LATS (large tumor suppressor, Drosophila) homolog 1, a serine/threonine kinase [26] that has been reported to suppress cell proliferation and induce apoptosis in mammalian and fly tissues [27]. As shown in Figure 3E, neural retinal signals appeared light (or absent) in the region adjacent to the RPE, but were stronger more vitreally at ED 5. The RPE was negative at the periphery, near the lens (Figure 3E), but appeared positive in the midperiphery (Figure 3F) and fundus (not shown). The periocular mesenchyme appeared positive as well (Figure 3F), as did the neural tube (not shown). By ED 8 the neural retina appeared fairly homogeneously positive (Figure 3G), although near the ora serrata, hybridization signals appeared strong towards the vitreal surface but negative adjacent to the RPE (not shown). The lens showed a negative anterior epithelium, but elongated fibers had perinuclear signals (Figure 3H). By ED 15 (not shown), and particularly by ED 18, maximum expression was seen in ganglion cells, with intermediate levels in the INL and weak staining in photoreceptors (Figure 3I). Analysis at high magnification showed heterogeneity both within the INL and the ONL (Figure 3J), with positive cells (*, arrowhead) alternating with negative ones.

CRG137 is homologous to human and mouse seizure-related gene SEZ-6, a brain-specific gene that is upregulated in response to convulsant drugs [28]. SEZ-6 expression has not been studied in the retina. The overall hybridization patterns with this probe changed from diffuse at early stages of development, to fairly localized in the differentiated retina. On ED 5 there were strong signals throughout the circumference of the retina, although they were stronger in its vitreal than its scleral side (Figure 3L). The extraretinal mesenchyme (Figure 3L) and the neural tube (not shown) were also positive. The retina (except for the extraretinal mesenchyme) maintained this pattern of expression at ED 8 (not shown). The ganglion cells, the INL and some cells in the photoreceptor layer appeared clearly positive at ED 12 (Figure 3M). Further restrictions in signal distribution were noted by ED 15 (not shown) and ED 18 (Figure 3N). In the latter case, intense signals were observed in many (but not all) cells in the amacrine region of the INL. Photoreceptors appeared negative, and the ganglion cells showed very light signals. Conspicuous signals were also detected in (or adjacent to) the OPL (Figure 3N).

CRG150 is homologous to human CGI-130 protein. This protein is predicted to contain a metal-dependent phosphohydrolase domain, found in enzymes involved in nucleic acid metabolism and signal transduction, including the visual transduction protein 3',5'-cGMP phosphodiesterase. Cognate ESTs have been isolated from human neural retina and numerous other tissues. The most striking observation in the chick retina was the exquisite localization of this gene to ganglion cells at ED 18, when there were only very faint hints of expression in some cells in the INL and essentially none in the ONL (Figure 4C). Expression also predominated in the GCL at intermediate stages of development (e.g., ED 12, Figure 4B), although there was also detectable expression in the INL, particularly in the region of amacrine cells. The ONL was negative. Expression was light and fairly diffuse at ED 5 (Figure 4A) and ED 8 (not shown).

The sequence of CRG177 has overall low homology to known genes, but does contain a 130 nucleotide region with 85% identity to human γ-transducin activity polypeptide 2 (GNGT2, cone transducin) and a second region of 180 bp that has 100% identity to an EST from a chick eye library. Therefore, part or all of this clone could be a chick orthologue of human cone transducin, or it may represent a novel transducin family member. A probe from the region homologous to the eye library EST demonstrated that the gene has late, markedly cell type-specific expression: strong signals were seen in photoreceptor inner segments on ED 18, but not in other photoreceptor regions or cell types or at any of the stages studied before ED 18 (Figure 4E-G).

CRG220 is homologous to the human gene sacsin. Mutations in sacsin lead to the neurological disease spastic ataxia of Charlevois-Saguenay [29], which features prominent myelination of retinal nerve fibers. As illustrated in Figure 4I-K, its overall pattern of expression in chick retina was fairly generalized throughout the developmental stages studied. At ED

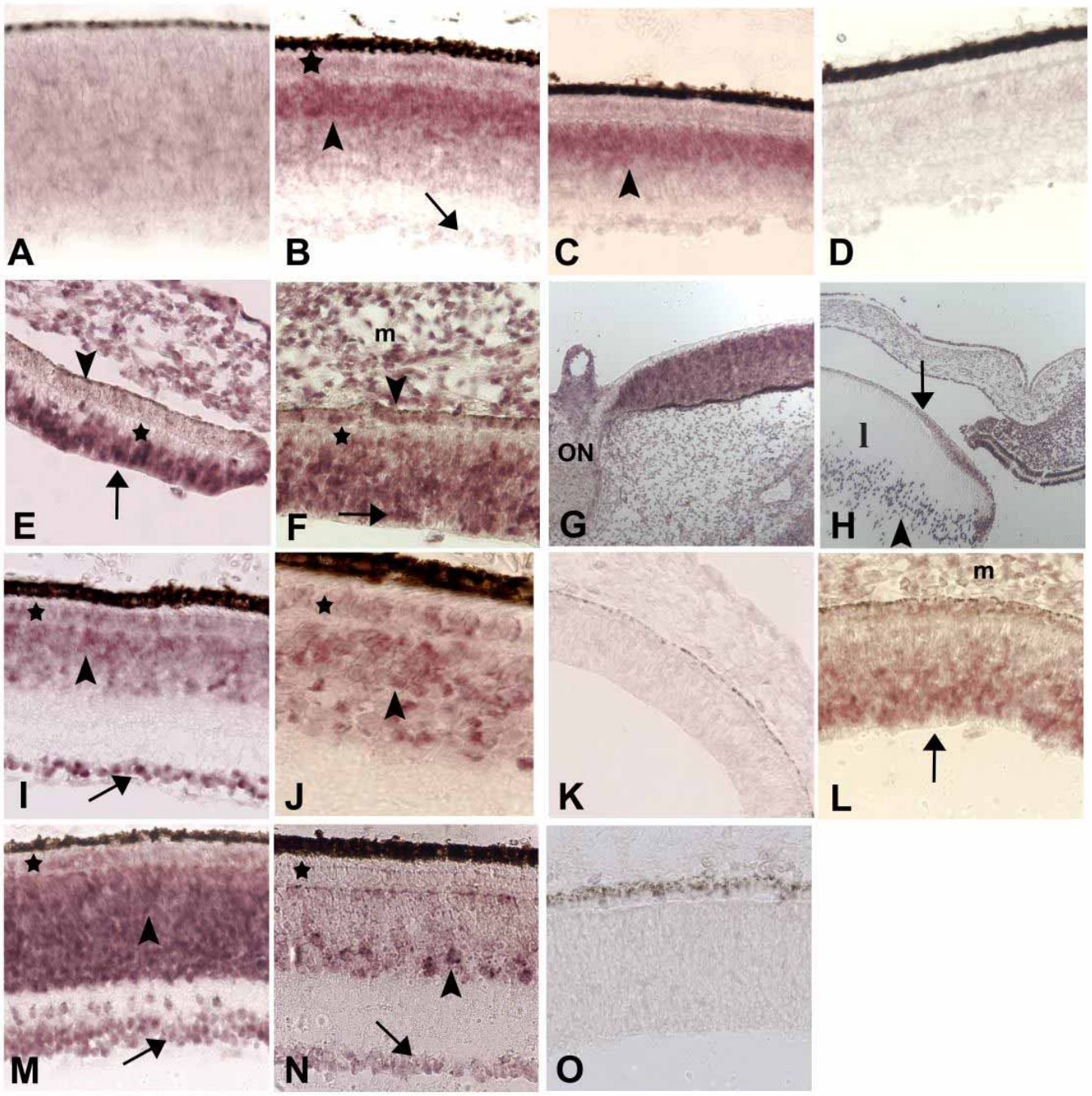


Figure 3. Developmentally-regulated expression patterns of novel chick retina genes. **A**-**D**: CRG92, unassigned identity. **A**: ED 8. Light and diffuse hybridization signals. **B**: ED 15. Outer part of INL (arrowhead) shows strong signals; ganglion cells (arrow) and the ONL (*) are very light or negative. **C**: ED 18. Similar pattern as ED 15. **E**-**K**: CRG111, homologous to human LATS. **E** and **F**: ED 5. Strongly positive retinal neural epithelium is seen towards the vitreal surface at the periphery (**E**) and fundal region (**F**, arrows), but not near the RPE (*). The RPE is negative at the periphery (**E**, arrowhead) but appears positive in the midperiphery (**F**, arrowhead). Note signal in the periocular mesenchyme (m in panel **F**). **G** and **H**: ED 8. **G**: Homogeneously positive fundus adjacent to the optic nerve (ON). **H**: Anterior segment of the eye. Negative anterior epithelium of the lens (l, arrow), and perinuclear signals (arrowhead) in elongating lens fibers. **I** and **J**: ED 18. Widespread expression is accompanied by stronger signals in the GCL (**I**, arrow) than in the INL (arrowhead) and ONL (*). Higher magnification analysis showing heterogenous signals in both layers (panel **J**); positive cells are indicated (*, arrowhead). **L**-**P**: CRG137, homologous to mouse seizure-related gene SEZ-6. **L**: ED 5. Signal expression throughout the retinal neural epithelium, which is stronger near the vitreal surface (arrow). The mesenchyme (m) is also positive. **M**: ED 12. Expression in ganglion cells (arrow), the INL (arrowhead) and some cells in the ONL (*). **N**: ED 18. Restricted expression pattern in mature retinas. Many (but not all) cells are positive in the amacrine layer (arrowhead), accompanied by lightly positive ganglion cells (arrows), and negative photoreceptors (*). **D**, **K**, and **O**: Sense control probes for CRG92, 111 and CRG137, respectively.

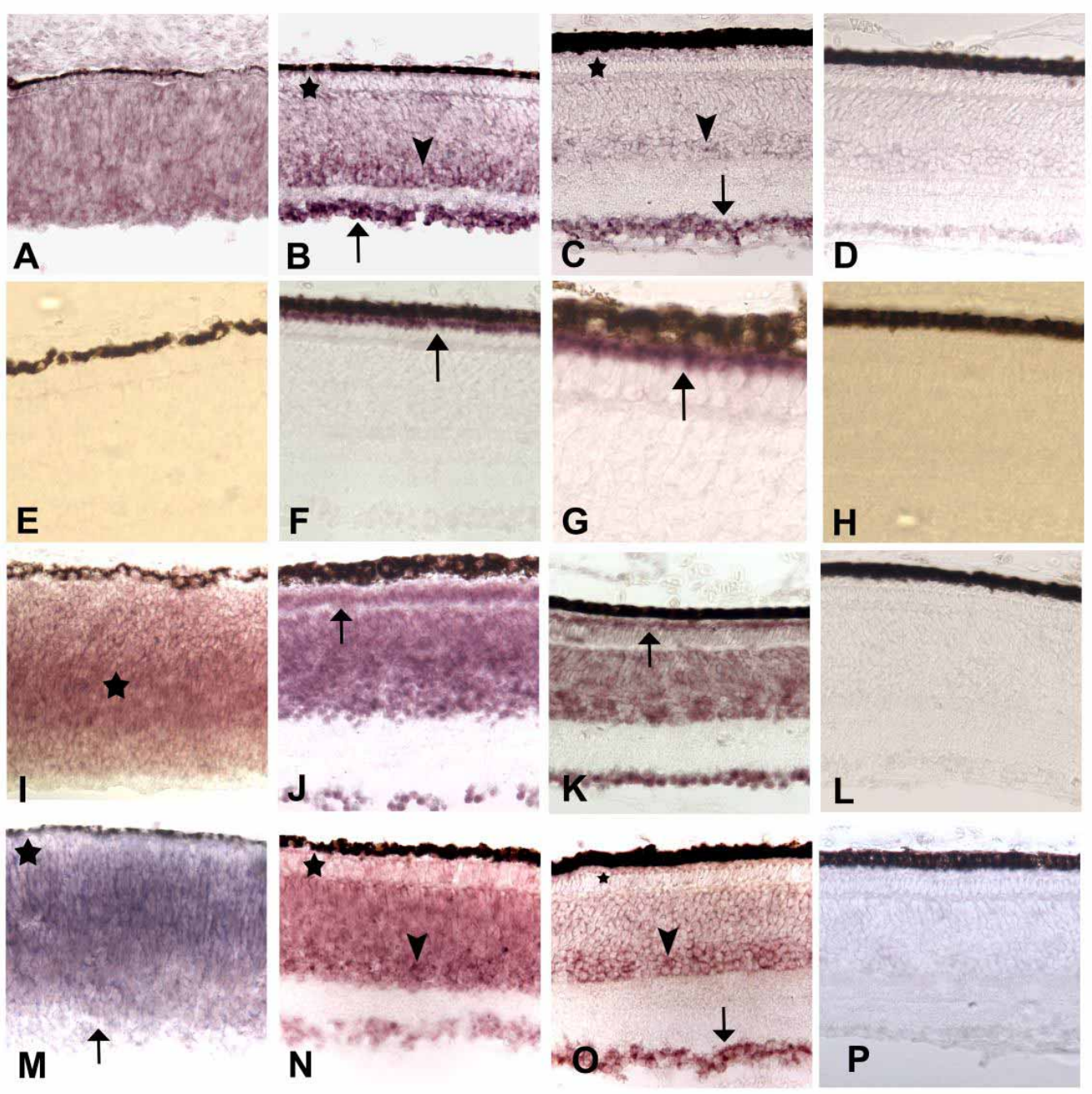
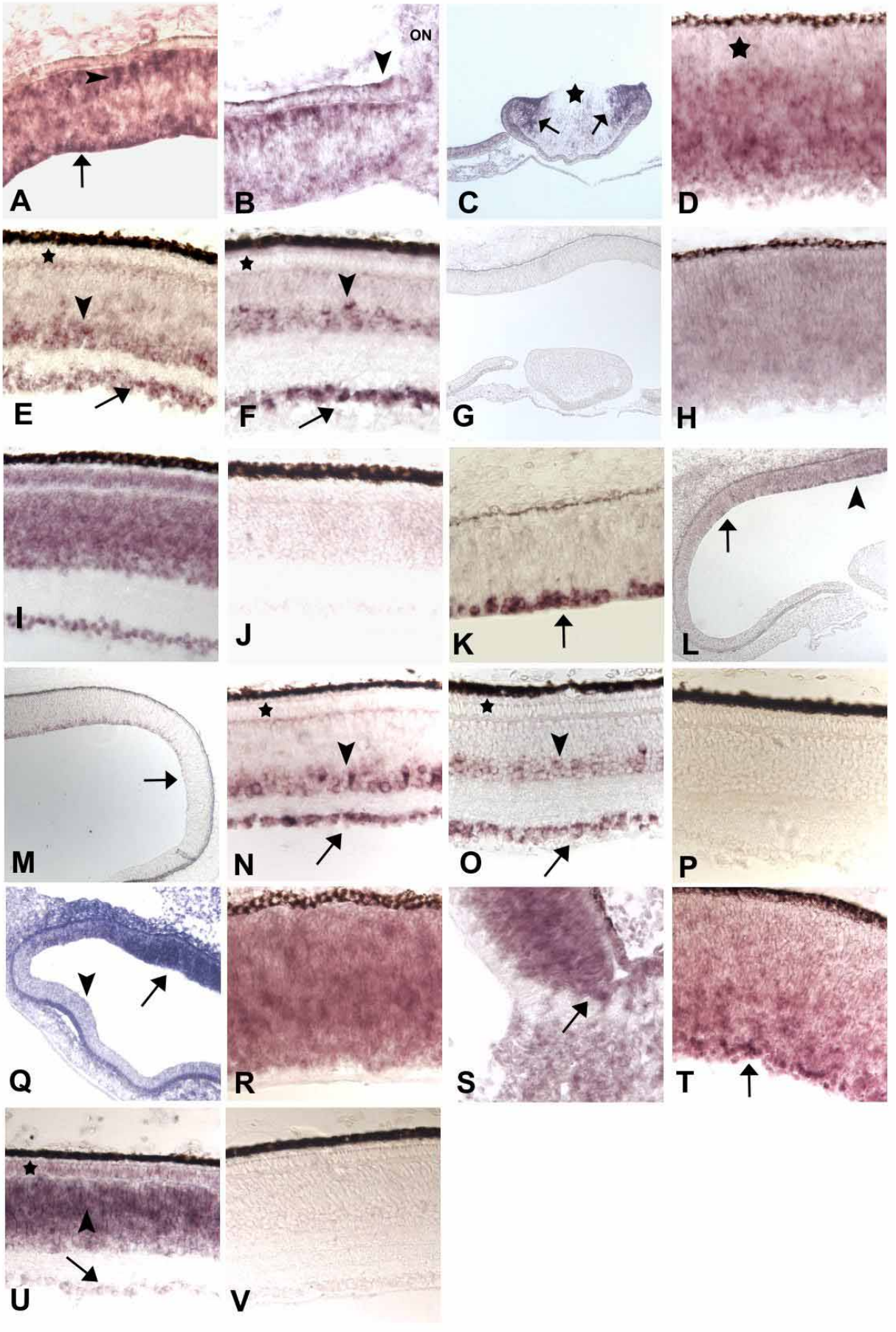


Figure 4. Developmentally regulated expression patterns of novel chick retina genes. **A**-**E**: CRG 150, homologous to human CGI-130. **A**: ED 5. Light and diffuse expression throughout the retinal neuroepithelium. **B**: ED 12. Expression predominates in GCL (arrow) and is also observed in the amacrine region of the INL (arrowhead). The ONL is negative (*). **C**: ED 18. Expression is almost completely restricted to the GCL (arrow). Very infrequent and light signals can be seen in the INL (arrowhead); the photoreceptor layer is negative (*). **E**-**H**: CRG177, homologous to human gamma transducin activity polypeptide 2; GNGT2, cone transducin. **E**: ED 15, no apparent expression. **F**: ED 18. Expression is restricted to the inner segment of photoreceptor cells (arrow); **G**: higher magnification of panel **F**. **I**-**L**: CRG220, homologous to the human gene sacsin. **I**: ED 8. Expression is widespread, although darker in the region of the future INL (*). **J** and **K**: ED 15, 18, respectively. Widespread expression. Signals are seen in photoreceptor cell bodies at ED 15 (**J**, arrow), but appear localized to inner segments on ED 18 (**K**, arrow). **M**-**P**: CRG231, human protein kinase C-binding protein Zeta1. **M**: ED 8. Expression appears less intense in the putative GCL (arrow) and photoreceptor layer (*). **N**: ED 15. Signals predominate in the GCL and the INL (arrowhead). Little expression in the ONL (*). **O**: ED 18. Ganglion cells (arrow) and some cells in the amacrine layer (arrowhead) are clearly positive. Photoreceptor cell bodies are negative (*), but the inner segments are positive. **D**, **H**, **L**, and **P**: Sense controls for CRG150, 177, 220 and 231, respectively.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8, for example, the only detectable heterogeneity was a somewhat darker signal in the future INL (Figure 4I). All cell layers showed clear signals in more differentiated retinas (Figure 4J,K). Some noteworthy details were the presence of patches with more intense signals in the INL, and the differential distribution of signals in the photoreceptors, which predominated in their cell bodies at ED 15 (Figure 4J) but were stronger in their inner segments on ED 18 (Figure 4K).

CRG231 is homologous to human protein kinase C-binding protein Zeta 1, also known as fasciculation and elongation protein (FEZ1). FEZ1 is believed to play a regulatory role in axonal guidance during *C. elegans* development [30] and is possibly involved in neuronal differentiation of PC12 cells [31]. In our study, a generally diffuse pattern of expression was observed in ED 5 (not shown) and ED 8 retinas (Figure 4M). In the latter case, however, the periphery appeared less stained than the fundus (not shown), and the ganglion cell region and putative ONL appear lighter than the rest of the neuroepithelium. Signals were predominantly localized to the INL and GCL at ED 12 (not shown) and ED 15 (Figure 4N), with little expression in the ONL. By ED 18 the ganglion cells were the most conspicuous positive cell type (Figure 4O); in the INL some amacrine cells appeared darker than the rest of its cells. Photoreceptor cell bodies were negative, but some inner segment had detectable signals (Figure 4O).

Known chick genes: The clones described in this section are identical to chick genes that have been previously characterized in non-retinal tissue (Table 1). The first such gene, CRG110, corresponds to the chicken gene for macrophage migration inhibitory factor (MIF), primarily known for its role in the immune response [32] but is also involved in chick lens differentiation [33]. The spatial expression pattern of CRG110 became substantially restricted during development. Expression in the ED 5 neural retina was widespread, but predominated near the RPE and the vitreal surface of the retina (Figure 5A). The RPE was largely negative (Figure 5A), but some signal could be seen in the RPE near the origin of the optic nerve (Figure 5B). As previously reported, the anterior epithelium of the embryonic lens was negative or lightly positive [33]; we observed that signal intensity increased markedly at the lens equator, and very strong signals were seen in the area where cells are known to become postmitotic and start to differentiate and elongate (Figure 5C, arrows), whereas mature lens fibers were weakly positive or negative (Figure 5C). Some expression was also observed in the extraretinal mesenchyme (Figure 5A) and in the neural tube (not shown). At ED 8 there was conspicuous absence of hybridization signals from the prospective ONL region; the rest of the retina showed widespread but somewhat uneven expression (Figure 5D). The pattern of expression in the lens remained as on ED 5 (not shown). As the retina matured, hybridization signals appeared predominantly localized to the GCL and the inner part of the INL (prospective amacrine cells), as illustrated for ED 12 and 18 (Figure 5E,F). The photoreceptor layer was negative.

CRG100 has significant sequence similarity to the chick Defender Against Death gene (DAD1), a protein that inhibits programmed cell death and enhances cellular proliferation in certain cell types [34,35]. DAD1 is part of the oligosaccharyltransferase enzyme complex that initiates N-linked glycosylation [36]. CRG100 showed little, if any, cell type-specificity of expression throughout development. Fairly widespread signals were seen in the retinal neuroepithelium at ED 5 (Figure 5H) and ED 8 (not shown). Similarly, all retinal layers showed hybridization in more matured retinas (illustrated for ED 18 in I) although ganglion cells appeared lighter than the remaining retinal layers. It is noteworthy that photoreceptor cell bodies were intensely stained, but their inner segments appeared devoid of signal.

Clone CRG196 was identified as stathmin-like 2 (SCG10-like), a member of a family of proteins that regulate microtubules and may play a role in axonal and dendritic outgrowth during neuronal development [37,38]. This probe yielded a very distinct hybridization pattern. On ED 5, when most other genes were either not expressed or expressed in fairly diffuse patterns, CRG196 signals were localized to a small population of cells adjacent to the vitreal surface of the retina, and only in its fundal region (Figure 5K,L). This localization and the large size of the cells suggest that they were newly gener-

Figure 5 (previous page). Developmentally-regulated expression of chick genes. **A**-**G**: CRG110, macrophage migration inhibitory factor, MIF. **A**, **B**, and **C**: ED 5. Widespread expression in the retinal neuroepithelium, particularly near the vitreal surface (**A**, arrow) and adjacent to the RPE (**A**, arrowhead). Restricted signals in the region adjacent to the RPE near the optic nerve (ON) in **B**; signals also detected in the RPE (arrowhead). The anterior epithelium of the embryonic lens appeared negative (**C**, *), but strong signals are seen at the lens equator (**C**, arrows). **D**: ED 8. The putative photoreceptor layer is negative (*), but expression is widespread, although a bit irregular in the rest of the retinal neural epithelium. **E** (ED 12), **F** (ED 18). Expression becomes restricted to the GCL (arrows) and the amacrine cell layer (arrowheads). The photoreceptor layer is negative (*). **H**-**J**: CRG100, homologous to defender against death, DAD1. This gene showed widespread expression throughout development, as illustrated for ED 5 (**H**) and ED 18 (**I**). **K**-**P**: CRG196, homologous to stathmin-like 2. **K** and **L**: ED 5. In fairly thin sections (**K**), expression appears completely restricted to cells which, based on their size and position, can be identified as recently generated retinal ganglion cells (arrow). This pattern is only seen in the fundal region (**L**, arrow); in this slightly thicker section, some signal can be observed in other regions of the fundal neuroepithelium (arrowhead). **M**: ED 8. Expression is restricted to retinal ganglion cells, but the domain of expression has spread peripherally (arrow). **N** (ED 15) and **O** (ED 18). Ganglion cell expression (arrows) is now accompanied by significant (but not uniform) expression in the amacrine cell layer (arrowheads). Photoreceptors are negative (*). **Q**-**V**: CRG233, chicken HT7 antigen. **Q**: ED 5. Expression is more intense at the fundus of the eye (arrow) and is practically undetectable at the periphery (arrowhead). **R**, **S**, and **T**: ED 8. Intense expression at the retinal fundus (**R**) with a clear demarcation with the ON (**S**, arrow). At the periphery (**T**) the future INL appears much lighter than the fundus (compare **T** and **R**), but heavily labeled ganglion cells can be seen (**T**, arrow). **U**: ED 15. INL is strongly positive, particularly on its outer half (arrowhead); photoreceptors show some signal (*), but ganglion cells are negative (arrow). **G**, **J**, **P**, and **V** are sense control images for CRG110, 100, 196 and 233.

ated ganglion cells [39,40]. It must be noted, however, that this apparent ganglion cell-specific pattern of expression was only observed in thinner tissue sections, whereas thicker sections also showed some signal in the fundal (but not the peripheral) retinal neuroepithelium (Figure 5L), as well as in the ciliary epithelium (not shown). Expression continued to be restricted to the GCL by ED 8, extending further into the periphery as compared to ED 5 (Figure 5M). As the retina matured, ganglion cells appeared strongly positive throughout the retina, but were accompanied by cells scattered in the region corresponding to putative amacrine cells that were also conspicuously positive (illustrated for ED 15 (Figure 5N) and ED 18 (Figure 5O)). The photoreceptor layer was negative at all time-points.

The CRG233 clone was identified as a fragment of the chicken HT7 antigen gene, which encodes a member of the immunoglobulin super gene family. HT7, also known as 5A11 and basigin, has been implicated in the formation and maintenance of the blood-retina barrier [41] as well as in cell-cell recognition during retinal development [42]. On ED 5, expression appeared stronger in the fundus than the periphery of the retina (Figure 5Q). The fundus continued to be very positive on ED 8 (Figure 5R), with a clear demarcation between the positive retina and the negative optic nerve (Figure 5S). The INL appeared to be much lighter at the periphery than in the fundus, but very darkly stained ganglion cells could be observed in this area (Figure 5T). The ciliary epithelium showed strong, polarized hybridization signals (not shown). As the retina differentiated (illustrated for ED15 in Figure 5U), the INL appeared strongly positive in its outer half, and somewhat lighter more internally; photoreceptors showed weak staining and ganglion cells appeared very lightly stained.

DISCUSSION

The goal of this study was to discover genes expressed in the embryonic chick retina, which could represent candidates for future experimental analysis in retinal development and function. Retinal gene expression was surveyed by differential library screening on robotically printed membranes. Genes abundantly and/or preferentially expressed in the retina were sequenced and analyzed by in situ hybridization to determine their developmental and spatial expression patterns. This gene discovery approach was successful because the majority of the selected clones had no assigned function and/or represented genes not previously identified in the chick. This successful outcome was achieved despite the relatively small number of clones screened (5000), and without a thorough quantitative comparison of expression ratios of genes in different tissues. Moreover, most of the genes that had been previously identified in the chick or other species had not been studied in the retina. Due to the highly specialized nature of the retina, genes involved in retinal homeostasis might be expected to be conserved across species, permitting the identification of chick genes by sequence similarities. In contrast, the anatomical differences between the chick and mammalian eye and the increased number of visual pigments in the chick retina may also result in the identification of genes specific to the avian retina. Even with moderately stringent statistical probability cut-off values to identify putative homologues in the databases, 40% of the clones in this study appeared to be novel, having no significant matches in the databases, or only matching ESTs or genes of unknown function. A caveat for the interpretation of these results is that the library was generated by oligo dT priming, which could have resulted in some messages being predominantly represented by their less well conserved 3' UTR. In some cases, the unknown genes did match weakly to genomic regions, but we considered these genes as "unassigned" since they did not have similarity to definitively predicted or cloned genes. Finally, because this was a first-pass sequencing project we were primarily concerned with obtaining sequence from the clones in order to facilitate homology searches, as has been done in the majority of published sequencing efforts. Sequencing of the entire coding region of the identified genes will be necessary before further investigations of their functional significance are undertaken.

It has been reported that the proportion of novel or "anonymous" clones found in a gene-hunting study varies amongst various species and tissues [43]. The frequency of anonymous clones in our clone set (40%) falls within the range of anonymous ESTs found by other groups [20]. It is of course conceivable that we may have failed in some cases to find significant matches for our genes because their homologues may have low expression, leading to their absence from the database, as well as due to the relatively low number of chick genes in the public databases. Additional investigations of the novel genes isolated in this study, including identification of the full-length sequences and manipulation of gene expression, will be important for determining whether any play critical roles in the development and function of the chick retina.

Developmental patterns of gene expression: From a developmental point of view, the group that contained the largest number of genes was characterized by detectable, although diffuse, expression in the neuroepithelium of ED 5 embryos. Some of these genes (e.g., CRGs 100, 123 and 220) retained a very generalized distribution even in mature retinas, suggesting that they may be associated with general metabolic or other "common" activities. In other cases, however, the diffuse pattern changed over development into a layer-specific distribution (e.g., CRGs 31, 69, and 110). Additional genes that were detectable on ED 5 already had a somewhat restricted expression at this stage; in some cases (e.g., CRG 233) expression was more intense in the fundal than in the peripheral region of the retina, and in others (e.g., CRGs 111 and 137) signals were stronger in the inner (vitreal) than in the outer regions of the retina. Four additional genes were very low or undetectable on ED 5, and appeared subsequently in layer-specific patterns or in a single cell type (e.g., CRG177, γ-transducin, which was undetectable through ED 15 and, when first observed in ED 18, was specifically restricted to photoreceptor cells). A unique expression pattern was seen with CRG196, which even at the earliest developmental stages studied appeared very intensely positive in cells that could be identified as newly generated ganglion cells; this identification [39,40] is based on their large size, position (adjacent to the inner limiting mem-

brane) and regional distribution (restricted to the fundal region of the eye on ED 5). When the above-mentioned temporal variations are evaluated from a cellular perspective, it appears noteworthy that the morphologically undifferentiated neuroepithelium (at ED 5) already expresses many genes that are later found in layer-specific patterns in the mature retina. While the meaning of these molecular similarities between neuropithelial cells and subsets of differentiated cells remain unclear, it is interesting that in several cases these genes are unevenly distributed within the retinal neuropithelium. Such results suggest that the "undifferentiated" neuroepithelium, although morphologically homogeneous and developmentally uncommitted [13], is in fact heterogeneous at the molecular level [6].

Another cellular feature of potential interest is that, in differentiated retinas, cells in the ganglion and amacrine layers are by far the ones that were strongly positive for the largest number of gene products; within the INL, the region adjacent to the IPL frequently had stronger signals than the region adjacent to the OPL (e.g., CRGs 9, 100, 110, 111, 137, 196, 233), while the opposite pattern was less frequent (CRGs 92, 123). Photoreceptor cells were only infrequently positive and, when they were, signals appeared in some cases in the inner segment (e.g., CRGs 177, 233) and in other cases in the perinuclear region of the cell bodies (CRG 100, and in some cells in CRG 123). The explanation for this relative lack of genes whose expression is enriched in photoreceptors is unclear. It remains to be determined whether these differences reflect biologically meaningful cell properties; ongoing studies using microarrays to analyze cDNAs from individual cells may provide insight to these questions (Bradford and Adler, in preparation).

Without detailed biological studies it is not possible to assess how expression of the identified genes contributes to retina development. It is noteworthy, however, that very powerful methods are now available to study the function of specific genes in the chick embryo retina, since reagents for loss of function or gain of function experiments can be readily delivered to retinal cells using lipid-mediated transfection [44], avian retroviral vectors [6-8] or electroporation [17,18]. These methods should allow direct testing of the working hypotheses that can be generated based on the developmental expression patterns and/or sequence characteristics of the genes that we have discovered.

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