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The chicken *RaxL* gene plays a role in the initiation of photoreceptor differentiation

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SUMMARY

The paired type homeodomain gene, *Rax*, was previously identified as a key molecule in early eye formation in mice and humans. We report the expression patterns of two Rax family members from chicken, *Rax* and *RaxL*, and on the function of *RaxL* in photoreceptor development. Both *Rax* and *RaxL* are expressed in early retinal progenitor cells, with *Rax* being expressed at a significantly higher level than *RaxL*. At the time that photoreceptors begin to form, *RaxL* appears at a relatively high level in a subset of cells within the zone of proliferating progenitor cells. Subsequently, it is expressed in cells migrating to the photoreceptor layer, where it is highly expressed during the initial, but not late, stages of photoreceptor differentiation. To test the function of *RaxL*, a putative dominant-negative allele of *RaxL* comprising a fusion of the *engrailed* repressor domain and a region of *RaxL* (EnRaxLΔC) was introduced in vivo into the early chick eye using a retroviral vector. EnRaxLΔC, but not the dominant negative *Rax* (EnRaxΔC), caused a significant reduction in expression of early markers of photoreceptor cells. Examination of the transactivation activity of RaxL on a reporter construct bearing a canonical photoreceptor-specific enhancer element showed that RaxL exhibited significant activation activity, and that this activity was severely diminished in the presence of EnRaxLΔC. The effect on photoreceptor gene expression in vivo was specific in that other cell types were unaffected, as was general proliferation in the retina. The reduction in numbers of cells expressing photoreceptor markers was probably due to decreased survival of developing photoreceptor cells, as there was increased apoptosis among cells of the retina expressing dominant-negative *RaxL*. We propose that *RaxL* plays a role in the initiation of differentiation, and also possibly commitment, of photoreceptor cells in the chicken retina.

Key words: Photoreceptors, Rx, Rax, RaxL, Retina, Homeodomain protein, Cell fate, Cell differentiation, Chicken

INTRODUCTION

The vertebrate retina is an exquisitely sensitive organ for light detection and information processing. Vision begins with the reception of various wavelengths of light by the photoreceptor cells that line the back of the retina. Photoreceptor cells are highly specialized for the capture of light and for phototransduction, the conversion of light into a biochemical signal. Phototransduction takes place in a series of membranous discs comprising a unique structure: the photoreceptor outer segment. The details of this biochemical process have been well studied and provided us with the first appreciation of a complex signal transduction process. However, the details, and even some of the fundamental aspects of the development of these cells are much less well understood. From lineage analyses, it is known that photoreceptors derive from multipotent retinal progenitor cells (Turner and Cepko, 1987; Holt et al., 1988; Wetts and Fraser, 1988). These progenitor cells generate all six retinal neuronal cell types, and a retinal glial cell type, in a sequential fashion (reviewed by Altshuler et al., 1991). How the retina generates the appropriate cell types at each developmental time to form a functional retina with the correct ratio of each cell type remains an interesting issue. We have begun to understand the mechanisms of how each cell type is generated, including the photoreceptor cells (Cepko et al., 1996; Morrow et al., 1998; Levine et al., 2000).

Rods, which comprise the majority of photoreceptors in rodents and in humans, are the most light-sensitive photoreceptor cell types. They are susceptible to degeneration, in some cases, because of mutations that effect the development of rods. Studies of human diseases (reviewed by Clarke et al., 2000) and a comprehensive analysis of genes expressed in photoreceptor cells (Blackshaw et al., 2001) have provided us with a source of candidate genes for the study of photoreceptor development. Cone photoreceptor development remains somewhat more mysterious. Cone photoreceptors are much less sensitive to light, but are active in the light intensities typical of daylight and of our brightly lit night. Cones provide us with high acuity vision, because of their high density in several regions of the retina and the high density of cells that compute the information from the photoreceptors and report it

to the brain. Cone photoreceptors are also susceptible to degeneration, particularly in the prevalent human disease of the elderly, age related macular degeneration (reviewed by Weber, 1998). It is thus of great interest to learn how both rods and cones develop, not only to provide us with a basic understanding of retinal development, but also to allow for replacement of these cells in retinal degenerations, and/or to provide us with other points at which to intervene in disease processes.

Many types of birds have a need for high-acuity vision during the day. They typically have cone photoreceptors as the major photoreceptor cell type. Such is the case with chickens, which have a rod-free, cone-rich central zone, similar to that of humans (Morris, 1982; Bruhn and Cepko, 1996). We have been investigating the development of rod and cone photoreceptors in chickens and in mammals. In the chick, we and others have found two Rax genes, *Rax*/*Rx* and *RaxL* (Ohuchi et al., 1999). The *Rax* gene is expressed in all retinal progenitor cells, which is similar to the expression of mouse *Rax* (Furukawa et al., 1997a). By contrast, we found that *RaxL* is expressed in both retinal progenitor cells and early developing photoreceptors. *RaxL* homologs, including *rx1* and *rx2*, have been previously reported in zebrafish and medaka. In zebrafish, *rx1* and *rx2* are expressed in cone, but not rod, photoreceptors (Chuang et al., 1999). We provide evidence that chick *RaxL* is required for the earliest stage(s) of photoreceptor development, most probably by acting at the stage of commitment to the photoreceptor fate, and/or at the earliest stages of photoreceptor differentiation. We also report the presence of a second Rax gene (*RAX2*) in humans that may be the human homolog of *RaxL*. Wang, Zack and their colleagues have also identified this human gene and have found mutations in this gene in several individuals with retinal degenerations (D. Zack, personal communcation). However, the significance of these mutations has not yet been established.

MATERIALS AND METHODS

Isolation of chick *Rax* and *RaxL* cDNAs

The cDNA encoding the homeodomain of chick *RaxL* was isolated by RT-PCR from E3 chick eyes using degenerate primers based on the mouse *Rax* sequence. The full-length chick *Rax* and *RaxL* cDNAs were further isolated by screening a random-primed chick E6-E8 retinal library using the cDNA fragment encoding the *RaxL* homeodomain. The full-length chick *Rax* cDNA (pKScRax) was constructed by ligating 5′ and 3′ partial cDNA clones of pSKcRax into the *Eco*RI site of pBluescriptKS. The plasmid pKScRax contains the *Rax* open reading frame, 175 bp of the 5′ untranslated region (UTR) and 101 bp of the 3′ UTR. The full-length *RaxL* cDNA clone, pSKcRaxL, contains *RaxL* ORF, 214 bp of the 5′ and 256 bp of the 3′ UTR.

Plasmid constructions

The *Bbs*I/*Eco*RI and *Bsa*I/*Eco*RI DNA fragments encoding oar/paired-tail motif deletion of *Rax* and *RaxL* were PCR amplified using TGAGAAGACCCCATGCACCCTCCCGGC and GAGAATTCCATGGCTCCCAGGGGCTG, and AAGGTCTCAGATGTTCCTCAATAAGTG and GAGAATTCCATGGGCTGCATGCCCTG as prime pairs, and were subcloned into *Nco*I/*Eco*RI site of pSlaxEn vector to generate pSlaxEnRaxΔC and pSlaxEnRaxLΔC, respectively. The *Nco*I/EcoRI DNA fragment encoding the homeodomain of RaxL was PCR amplified using AACCATGGCTGCTGCTGAGGAGGAACAGCCC and GAGAATTCGGACAGCATGGGGGTGTCGTG as primers and subcloned into pSlaxEn vector to generate pSlaxEnRaxLHD. The *Cla*I DNA fragments of pSlaxEnRaxΔC, pSlaxEnRaxLΔC and pSlaxEnRaxLHD were subsequently cloned into pRCAS(B) retroviral vector (Hughes et al., 1987) to generate pRCASEnRaxΔC, pRCASEnRaxLΔC and pRCASEnRaxLHD, respectively. The same fragments were also subcloned into pCS2 expression vectors (Rupp et al., 1994; Turner and Weintraub, 1994) to generate pCSEnRaxΔC, pCSEnRaxLΔC and pCSEnRaxLHD, respetively. The RaxL expression vectors were generated as follows: the DNA fragment encoding the N-terminal region of RaxL was PCR amplified using CGACCATGGAGATGTTCCTCAATAAGTGT and GTGCCCGCCATAGGGGGG as primers; the *Nco*I/*Afl*III fragment of this PCR product together with *Afl*III/*Sac*II(blunted) DNA fragment encoding the C-terminal region of RaxL were ligated into the *Nco*I/*Eco*RV site of pSlax21 (Chen et al., 1999) to product pSlaxcRaxL vector; the *Cla*I fragment and *Cla*I/*Spe*I fragment of pSlaxcRaxL were further subcloned into the *Cla*I site of pRCAS(A) and the *Cla*I/*Xba*I region of pCS2 to generate pRCAS (A) cRaxL retroviral and pCScRaxL expression vectors, respectively. The Rax expression vector was generated as follows: the *Bbs*I/*Eco*RI DNA fragment encoding Rax ORF was PCR amplified from pKScRax using TGAGAAGACCCCATGCACCCTCCCGGC and M13 reverse primers and subcloned into *Nco*I/*Eco*RI locus of pSlax21 to generate pSlaxcRax; the *Cla*I DNA fragment of pSlaxcRax was further subcloned into *Cla*I site of pCS to generate pCScRax expression vector. The *Rca*I fragment of mouse *Crx* was cloned into the *Nco*I locus of pSlax21 to generate pSlaxmCrx. The *Cla*I fragment of pSlaxmCrx was further subcloned into pRCAS(A) to construct pRCAS(A)mCrx retroviral vector. In situ hybridization probes specific to *Rax* and *RaxL* were transcribed from pKScRaxspl and pKScRaxLspl, respectively, which includes 5′ UTR, 3′ UTR and the homeodomain deleted coding regions of *Rax* and *RaxL*, respectively. The pKScRaxspl was constructed by ligating two *Eco*RI/*Bam*HI PCR fragments into *Eco*RI site of pBluescriptKS. These two PCR fragments were amplified from pSKcRax using T7 primer and GCGGATCCCTCCTCGTCCGACGGCTTCCC primer pair, T3 primer and AAGGATCCAGCCGCTCCCCGCAGGCG primer pair. The pKScRaxLspl was constructed in a similar way in that T3 (GCGGATCCTTCCTCCTCAGCAGCAGCTGG) and T7 (AAGGATCCAACCGGCCGCCCATGACG) were used as two PCR primer sets.

Electroporation

Plasmid DNA containing 0.05% of Fast Green was injected into the right optic vesicle of Hamburger-Hamilton stage 9 to stage 11 chick embryos in ovo. Immediately after injection, the embryo was subjected to electroporation using the Tokiwa CUY-21 square electroporator with 10 mV for three cycles of 50 mseconds pulse and 950 mseconds chase.

In situ hybridization

Whole-mount and section in situ hybridization were performed as described (Chen and Cepko, 2000). Flat-mount in situ hybridization was performed as described (Bruhn and Cepko, 1996) with the following modification. The flat-mounted retinal tissues were hybridized overnight at 70°C with digoxigenin-labeled RNA probes of specific cell markers together with the fluorescein-labeled RNA probe of engrailed repressor domain. After hybridization, the retinas were washed and blocked as described and incubated overnight at 4°C with 1:2000 dilution of AP-conjugated anti-digoxigenin antibody (Roche Diagnostics Coporation) in TBST and 1% heat-inactivated sheep serum. Retinas were washed several times in TBST and further detected with NBT and BCIP until the desired purple signal developed. The developing reaction was stopped by washing three times with TBST (pH 5.5) and heating at 70°C for 2 hours in the same buffer to dissociate anti-digoxigenin Ab. The pictures of the retinas with the first in situ signal were taken before detecting the second

signal. To detect the second signal, the heat-inactivated retinas were blocked in TBST and 10% sheep serum for 2 hours and incubated overnight at 4°C with 1:2000 dilution of AP-conjugated anti-fluorescein antibody (Roche Diagnostics Corporation) in 1% sheep serum/TBST. After washing in TBST, the second in situ signal was detected with BCIP alone until the desired blue color developed. The developing reaction was stopped in TBST (pH 5.5) and pictures of the retinas with both the first and second in situ signals were taken. To further detect the total viral infection, the retinas were further subjected to 3C2 mAb staining based on the protocol described in the Immunostaining section, after treatment at 70°C for 2 hours to dissociate anti-Fluorescein Ab.

Cell transfection and CAT assays

COS cells were grown in DMEM with 10% fetal calf serum. Five micrograms of pRET1-CAT reporter (Furukawa et al., 1997b), 1 μg of pSVβ (Clontech), 5 μg of pCScRaxL with increasing amounts of pCSEnRaxΔC, pCSEnRaxLΔC or pCSEnRaxLHD, and decreasing amounts of pCS2 to make a total 26 μg of plasmid DNA were transfected onto 10 cm dishes using Superfect as the transfection reagent according to the manufacture's protocol (Qiagen). Cells were harvested for a CAT assay 48 hour post-transfection as described (Chen et al., 1996). Five to 10 μl of cell extract without heat treatment were used for measuring the β-galactosidase activity at room temperature in 1 ml of Z buffer (60 mM Na_2HPO_4/40 mM NaH_2PO_4/10 mM KCl/1mM $MgSO_4$) containing 1 μl of β-mercaptoethanol and 0.5 mg/ml of ONPG. The reactions were stop with 0.5 ml of 1 M Na_2CO_3 and OD420 were measured. The OD420 value, which reflects the transfection efficiency of each extract, was used to normalize the CAT value from each transfection.

The generation of visinin monoclonal antibodies and western blot analysis

Monoclonal antibodies to chick visinin were generated by Maine Biotechnology Service Incorporation (S. Bruhn and C. Cepko, unpublished) using purified chick visinin protein as an antigen (gift from Dr A. Polans) (Polans et al., 1993). One of the visinin mAbs (7G4) was deposited into The Developmental Studies Hybridoma Bank at the University of Iowa. For western blot analysis, chick retinas were harvested and sonicated in whole cell extract buffer (20 mM HEPES pH7.6/150 mM NaCl/0.5 mM DTT/0.2 mM EGTA/0.2 mM EDTA/25% glycerol) with proteinase inhibitor cocktail (Roche Diagnostics Corporation). The cell lysates were collected after centrifugation for 15 minutes at 4°C and the protein concentration was determined by Bradford analysis (BioRad protein assay) using bovine serum albumin as a standard. The retinal extracts containing 25 μg protein were run on a 10% precast SDS-PAGE gels and transferred to a nitrocellulose membrane according to the manufacture's protocol (Invitrogen). The transferred nitrocellulose membrane was stained with Ponceau S to confirm that an equal amount of protein was loaded and transferred in each lane before blocking with 5% nonfat milk in PBST (0.1% Tween-20 in PBS). Two-thousand-fold dilution of visinin mAb ascites fluid was used as a primary antibody and peroxidase-conjugated goat anti-mouse IgG (1:4000 dilution) (Jackson Immunoresearch Laboratory) was used as a secondary antibody. The western blot signal was further detected with ECL reagent (Amersham).

Immunostaining

Retinal cryosections (20 μm) were blocked with 5% sheep serum/0.02% TritonX-100/PBS for 30 minutes at room temperature. The sections were subsequently incubated with visinin mAb (1:100 dilution of hybridoma culture supernant) for 1 hour. After several washes in PBS, the sections were incubated in biotinylated anti-mouse IgG (1:500 dilution) (Vector) for another hour. The Vectastain ABC kit (Vector) and DAB peroxidase substrate kit (Vector) were further used for amplifying and detecting the signal according to manufacture's protocol.

Retina dissociation and FACS analysis

Papain (100 units/ml) (Worthington Biochenical Corporation) was first activated in Hank's balanced salt solution (HBSS) containing 10 mM HEPES pH 7.6, 2.5 mM cysteine and 0.5 mM EDTA for 15 minutes at 37°C. Dissected chick retinas were incubated in activated papain solution for 40 minutes at 37°C. Retinal pellets were gently triturated and incubated in 0.1 mg/ml of DNaseI/HBSS for 10 minutes. The dissociated retinal cells were further washed twice with HBSS and fixed in 4% paraformaldehyde for 5 minutes at room temperature. The protocol for antibody staining on fixed cells is the same as staining tissue sections. The Cyt2-conjugated anti-rabbit IgG and Cyt3-conjugated anti-mouse IgG (1:500 dilution) (Jackson Immunoresearch Laboratories) were used for secondary antibodies. After two washes with PBS, the cells were suspended in 1% formaldehyde/PBS for FACS analysis.

TUNEL assay

Viral-infected retinas were fixed in 4% paraformaldehyde/PBS and embedded in OCT compound (Tissue-Tek) after cryoprotection in 30% sucrose solution. Cryosections (20 μm) were subjected to the TUNEL assay using the in situ cell death detection fluorescein kit (Roche) according to the manufacture's protocol. Retinal sections were then further stained with 3C2 mAb and Cyt3-conjugated goat anti-mouse IgG (1:400 dilution) (Jackson Immunoresearch Laboratories) to visualize the viral infected areas.

RESULTS

Isolation of chick *Rax* and *RaxL* cDNAs

Full-length chick *Rax* cDNAs were isolated from a chick E6-E8 retinal library using a RT-PCR fragment encoding the homeodomain region of chick Rax. Two distinct Rax cDNAs, *Rax* and *RaxL*, were isolated. The open reading frame (ORF) of *Rax* encodes a 316 amino acid protein. The *RaxL* cDNA encodes an ORF containing 228 amino acid residues. Sequence comparison of Rax and RaxL showed that they share 100% amino acid identity in their paired-type homeodomains. Scattered sequence similarity was also found in the region C-terminal to the homeodomain, including the conserved oar/paired-tail motif, which has been found in several paired-type homeobox genes (Furukawa et al., 1997a; Mathers et al., 1997). Unlike mouse Rax and chick Rax, the chick RaxL contains very little sequence that is N-terminal to the homeodomain. Moreover, the highly conserved octapeptide, identified in some of the paired-domain and homeodomain proteins including mouse Rax and chicken Rax, is missing in RaxL. During the course of our studies, the *Rax* and *RaxL* cDNAs were reported by Ohuchi et al. (Ohuchi et al., 1999). The cDNA of *Rax* that we isolated has roughly 0.15 kb more 5′ UTR sequence than the published *Rax* and some base pair differences throughout the cDNA. Our *RaxL* isolate contains roughly 0.2 kb more sequence information in both the 5′ and 3′ UTRs. The sequences of both *Rax* and *RaxL* have been submitted to GenBank with the Accession Numbers AF420600 and AF420601, respectively.

Through searches of the human EST and genome databases, a second human Rax *(RAX2)* gene was found and located on human chromosome 19. The corresponding EST was also isolated from a human retinoblastoma cell line (I.M.A.G.E. clone ID3344166). An amino acid sequence comparison showed 93% identity in the homeodomain region between RaxL and the human protein (RAX2). Scattered similarity was

also found outside of the homeodomain. Interestingly, like RaxL, the RAX2 exhibited a very short sequence N-terminal to the homeodomain, and was lacking the octapeptide (Fig. 1A). Based on these sequence similarities, this gene is probably the human homolog of *RaxL*.

The expression patterns of *Rax* and *RaxL* in early chick embryos

The expression patterns of *Rax* and *RaxL* in early chick embryos (Hamburger-Hamilton stage 8 to stage 20) were analyzed by whole-mount in situ hybridization. To avoid cross-hybridization between *Rax* and *RaxL* through their conserved homeodomain regions, specific RNA probes with the homeodomain regions deleted were used. *Rax* RNA was detected in the anterior neural folds at stage 8 (data not shown). By stage 11, *Rax* was expressed in the entire forebrain region (Fig. 1B) and highly concentrated in the optic vesicles and the ventral midline structure, the infundibulum (Fig. 1C, arrowhead and arrow, respectively). By stage 12, when the optic vesicles have formed, the *Rax* signal remained strong in the optic vesicles and in the infundibulum, but became weak in the anterior and dorsal forebrain (Fig. 1D,E). By stage 14, the expression of *Rax* was confined to the retina and ventral diencephalon (Fig. 1F,G arrowhead and arrow, respectively). The retinal and ventral diencephalon expression of *Rax* persisted to stage 20, the oldest stage we have analyzed by whole-mount in situ hybridization (Fig. 1H,I, and data not shown).

RaxL was expressed in an overlapping but not identical pattern to that of *Rax*. The transcript of *RaxL* was first found at stage 9 in the ventral anterior neural tube (data not shown). By stage 11, *RaxL* was highly expressed in the ventral optic vesicles, while the signals in the anterior forebrain and infundibulum were barely detectable (Fig. 1J,K). In contrast to *Rax*, by stage 12, *RaxL* was expressed strongly in the optic vesicles, very weakly in the infundibulum, and at undetectable levels in the dorsal and anterior forebrain (Fig. 1L,M). By stage 14, *RaxL* was expressed only in the retina and no infundibulum expression was observed (Fig. 1N,O compare with Fig. 1G arrow). Interestingly, the early retinal expression of both *Rax* and *RaxL* was not uniform throughout the optic vesicle. *RaxL* was expressed in a high ventral to low dorsal gradient transiently from stage 13 to stage 17 (Fig. 1N,P, and data not shown). By contrast, *Rax* was expressed at a high level in both dorsal and ventral domains and at a low level in the middle region of the retina at similar stages (Fig. 1F,H, and data not shown).

The expression patterns of *Rax* and *RaxL* in the developing retina

As both *Rax* and *RaxL* expression was observed in the eyes of early chick embryos, a further detailed analysis of retinal expression was carried out on retinal sections from embryonic day 5 (E5) to E19 (Fig. 2). The *Rax* transcript was detected in the majority of retinoblasts at a high level in the E5 retina (Fig. 2A). By E6, two domains with undetectable *Rax* expression were observed. One was adjacent to the pigment epithelium, presumably comprising differentiating photoreceptor cells, and the other

Fig. 1. The amino acid sequence alignment of chick RaxL (cRaxL) and human RAX2 (hRAX2) (A). Identical amino acid residues between these two proteins are indicated by asterisks. Gaps required for optimal alignment are represented by dashes. The homeodomain is underlined. (B-Q) Whole-mount in situ hybridization of *Rax* (B-I) and *RaxL* (J-Q) on Hamburger-Hamilton stage 11 (B,C,J,K), stage 12 (D,E,L,M), stage 14 (F,G,N,O), and stage 15 (H,I,P,Q) chick embryos. (B,D,J,L) Dorsal views of the embryos; (C,E,K,M) ventral views of the same embryos. (F,H,N,P) Lateral views; (G,I,O,P) Magnified frontal or ventral views. The arrowheads and arrows indicate the retina and ventral diencephalon, respectively.

was adjacent to the vitreous, presumably comprising ganglion cells (Fig. 2B, arrows). This pattern is consistent with the observation that mouse *Rax* is highly expressed in proliferating retinal progenitors and is downregulated in differentiated retinal cells (Furukawa et al., 1997a). At E7, the *Rax* transcript was found in a small population of cells residing in the future inner nuclear layer (INL) throughout the retina, which are likely to be the remaining retinal progenitors (Fig. 2C,D). *Rax* expression was further restricted into a narrow domain in the INL at E9 (Fig. 2I). At E11, when almost all retinal progenitor cells have become postmitotic, we observed a low level of *Rax* signal in the middle of the INL (Fig. 2J). Because a low level expression of *Rax* was found in Muller glial cells of the mouse retina (Furukawa et al., 2000), it is likely that this small population comprises the remaining retinal progenitors and/or differentiating Muller glial cells. However, the identity of these *Rax*-expressing cells needs further characterization. A faint *Rax* signal remained at E14 (Fig. 2K) and became undetectable at E19, immediately before hatching (Fig. 2L). We found no *Rax* expression in the retina of post-hatched chicks at one month of age (P30) (data not shown). Based on the in situ hybridization analysis of *Rax*, which shows a similar expression pattern to that of the mouse *Rax* gene, and given the amino acid sequence similarity of Rax and mouse Rax, chick *Rax* is likely to be the homolog of the mouse *Rax* gene.

The *RaxL* transcript was detected at a lower level than *Rax* throughout the retina at E5 (Fig. 2E). By E6, the *RaxL* non-expressing domain was seen in the ganglion cell region (Fig. 2F, arrow). However, unlike *Rax*, the *RaxL* signal remained in the developing photoreceptor layer at this stage (compare Fig. 2B,F). As development proceeded, some retinal cells expressing higher levels of *RaxL* appeared near the pigment epithelium at E7 (Fig. 2G), presumably the progenitors fated to be photoreceptors. This pattern of expression in the future outer nuclear layer (ONL) persisted (Fig. 2H). The increase in staining of the future ONL progressed from the center to the periphery (compare Fig. 2H,G) in a pattern that coincides with the overall temporal developmental of the retina. As development progressed, two expression domains of *RaxL* resulted; one weak expression zone overlapping that of *Rax* in the INL, representing the remaining retinal progenitor cells (Fig. 2H, arrowhead), and one strong expression zone located in the future ONL, most likely representing the developing photoreceptor cells (Fig. 2H arrow). This two-domain expression pattern of *RaxL* persisted until E11 with decreasing signal in the INL and increasing signal in the ONL (Fig. 2M,N). By E14, *RaxL* was detected only in photoreceptors, and this expression was downregulated to an undetectable level by E19 (Fig. 2O,P). No *RaxL* expression was found in the P30 chick retina (data not shown). The expression of *RaxL* in retinal progenitor cells, and later at a high level in developing photoreceptors, suggests a key role for *RaxL* in the early stage of photoreceptor development.

RaxL is required for photoreceptor cell development

To determine whether *RaxL* plays a role in photoreceptor

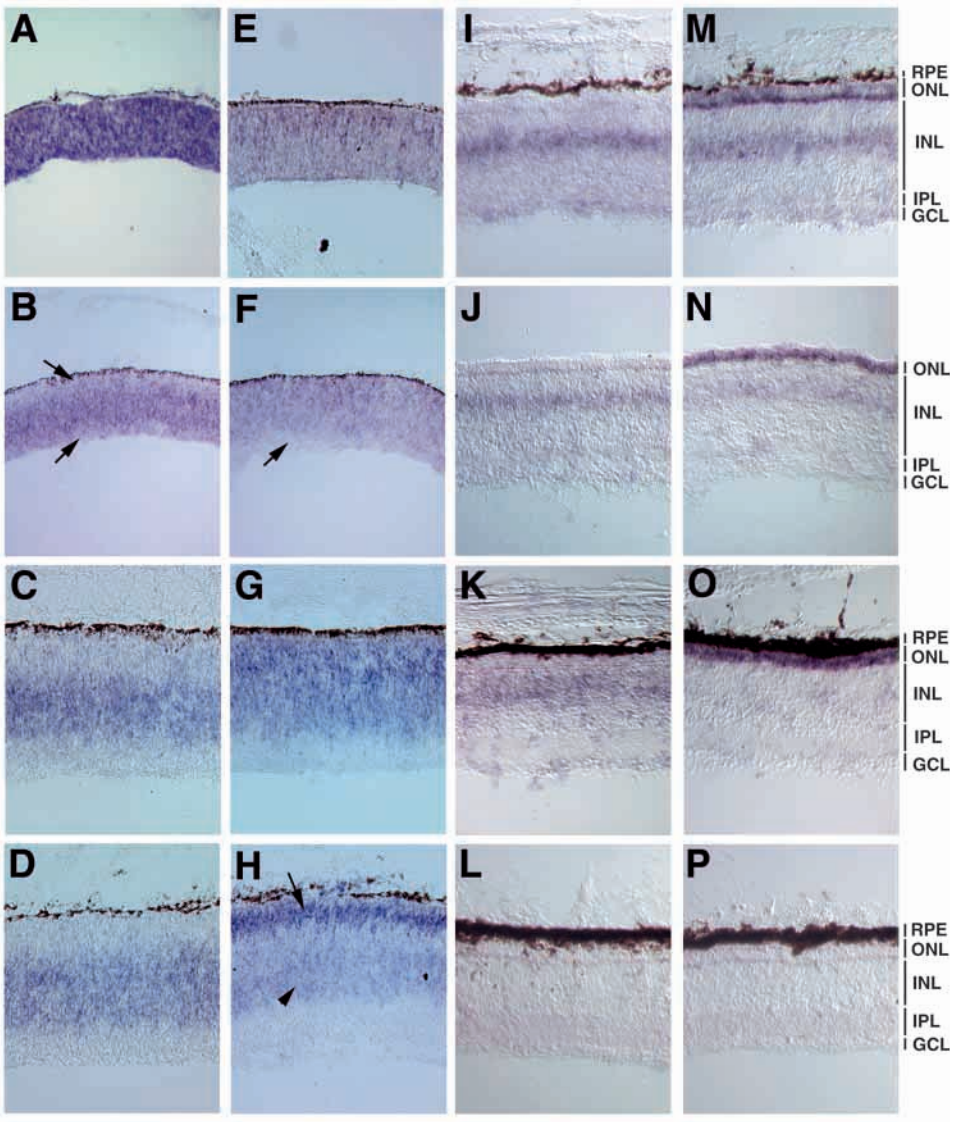

Fig. 2. In situ hybridization of *Rax* (A-D,I-L) and *RaxL* (E-H,M-P) on retinal sections of E5 (A,E), E6 (B,F), E7 (C,D,G,H), E9 (I,M), E11 (J,N), E14 (K,O), and E19 (L,P) chick embryos. (C,G) Sections from the peripheral region; (D,H) sections from the central region of the E7 retina. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium. In B and F, arrows indicate Rax and RaxL non-expressing domains, respectively. In H, strong expression of RaxL in developing photoreceptor cells is indicated by an arrow and the weak expression zone in INL is indicated by an arrowhead.

development, we ectopically expressed full-length RaxL protein in optic vesicles using a retroviral expression vector. The optic vesicles of chick embryos were infected with a RaxL retrovirus at Hamburger-Hamilton stage 10 and infected retinas were harvested between E6 and E7. The development of photoreceptor cells was analyzed using the photoreceptor marker, visinin, by flat-mount in situ hybridization (Yamagata et al., 1990). We detected no ectopic expression of *visinin*-expressing cells in the RaxL infected retina (data not shown), suggesting the *RaxL* is not sufficient to promote photoreceptor cell fate choice. We then examined if *RaxL* is necessary for photoreceptor cell development by introducing a putative dominant-negative allele of *RaxL*. As RaxL shares the identical amino acid sequence in the homeodomain region with Rax, dominant-negative *RaxL* could potentially interfere with both *RaxL* and *Rax* functions. To minimize this possibility and maintain as much *RaxL* specificity as possible, we made a fusion construct containing the engrailed repressor domain and *RaxL* with deletion only of the oar/paired-tail motif (EnRaxLΔC). The similar fusion construct has been shown as a dominant negative allele of *Xrx1* (*Xenopus* homolog of *Rax*) in *Xenopus* embryos (Andreazzoli et al., 1999). The EnRaxLΔC retroviral vector was electroporated into optic vesicles of Hamburger-Hamilton stage 10 chick embryos. After electroporation, the EnRaxLΔC transfected retinal cells should produce EnRaxLΔC virus, which subsequently infects neighboring retinal cells to create some viral infected patches in the retina. As we have found that electroporation with high concentrations of DNA can lead to a nonspecific small eye phenotype, we electroporated the viral construct at the low concentration of 0.1-0.2 μg/μl. The infected retinas were harvested at E7.5-E8, and the expression of endogenous visinin and exogenous EnRaxLΔC was analyzed by double in situ hybridization using visinin and engrailed probes, respectively. The infected retinas showed the same overall *visinin* pattern as control non-electroporated retinas. However, 73% (11 out of 15) of the retinas electroporated with the EnRaxLΔC had several patches with low *visinin* expression (Fig. 3A). These patches were within the infected areas, detected with an engrailed probe (Fig. 3B in greenish blue). In some cases, there was no reduction in *visinin* expression in infected areas. This could be due to a low level expression of the EnRaxLΔC protein, which could occur because of the interference of some viral integration sites. We found no correlation between the size and location of infected patches and the reduction of *visinin*. To examine if viral infection grossly altered retinal morphology by affecting progenitor cell proliferation, a concern because *RaxL* is expressed in retinal progenitors, the retina was sectioned after double in situ hybridization. Fig. 3C shows that the viral infected patches spanned the entire thickness of the retina and that the retinal thickness remained normal within those patches. However, there were fewer cells expressing *visinin* transcript within the EnRaxLΔC virus-infected domains. To further investigate whether EnRaxLΔC interfered with the proper development of photoreceptor cells, or just visinin marker gene expression, we examined the expression of another photoreceptor-specific gene, *RXRγ* (*Rxrg*) (Hoover et al., 1998). *Rxrg* exhibited a uniform pattern

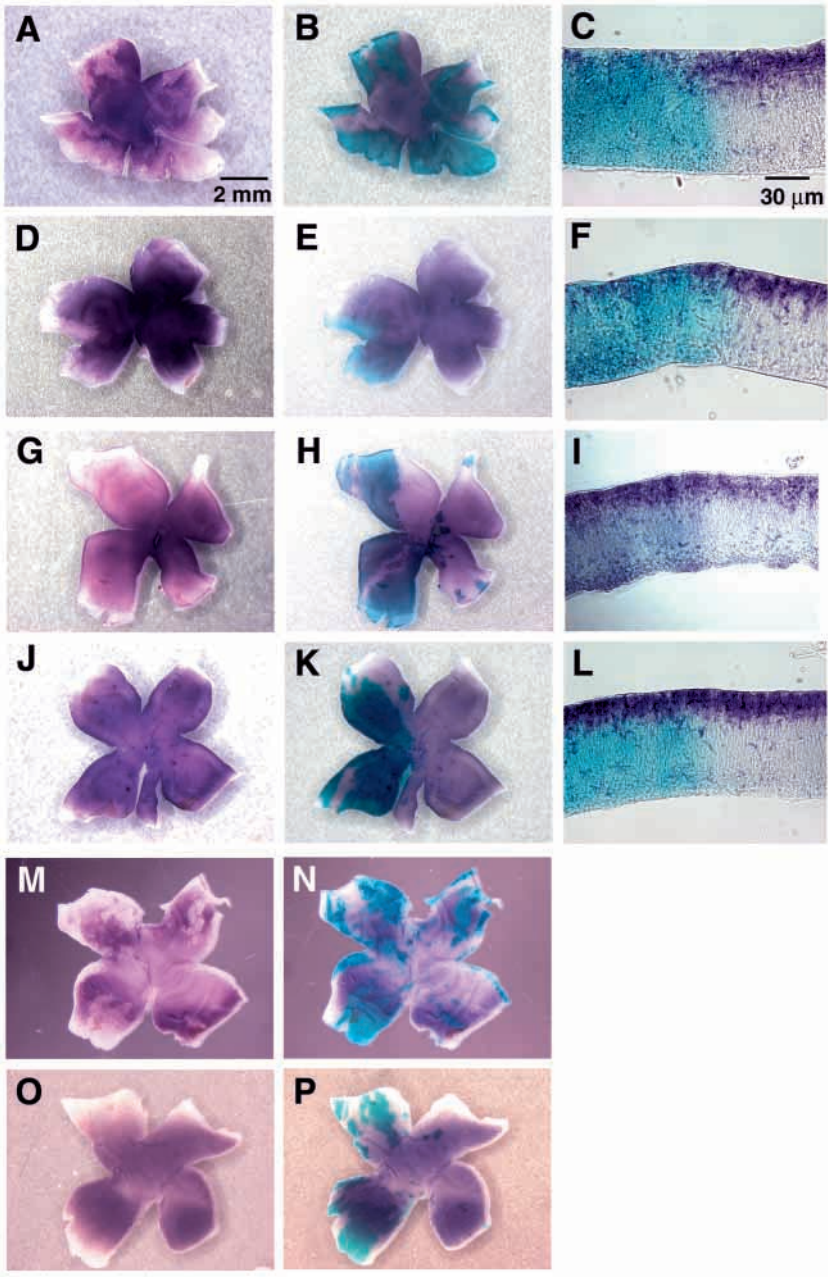

Fig. 3. Flat-mount in situ hybridization of E7.5 chick retinas electroporated with EnRaxLΔC (A-F), EnRaxΔC (G-L), EnRaxLHD (M,N), and EnIrx (O,P) viral constructs. Endogenous visinin (A-C,G-I,M-P) and *Rxrg* transcripts (D-F,J-L) are represented by the purple stain and exogenous EnRaxΔC, EnRaxLΔC, EnRaxLHD and EnIrx by the green-blue stain. The sections of the retina samples in B,E,H,K are shown in C,F,I,L, respectively. Scale bars: in A, 2 mm for all the flat-mount images; in C, 30 μm for C,F,I,L.

of expression in the control, uninfected E7.5 retina (data not shown). However, when the retina was electroporated with EnRaxLΔC viral construct, the virus infected patches showed significant reduction of *Rxrg* expression in 82% (14 out of 17) of the infected retinas (Fig. 3D-F). The fact that two independent photoreceptor specific genes were reduced by EnRaxLΔC, makes it likely that the development of photoreceptor cells is affected by EnRaxLΔC. These results suggest that *RaxL* is required for the development of photoreceptors, and that introducing EnRaxLΔC did not alter retinal cell proliferation.

To determine whether EnRaxLΔC interfered specifically with *RaxL*, we electroporated a similar viral construct, EnRaxΔC, containing the engrailed repressor domain and *Rax*, with deletion of the oar/paired-tail motif. Seven and 16 infected retinas were tested for *visinin* and *Rxrg* expression, respectively. We found all the retinas tested exhibited normal *visinin* and *Rxrg* expression within the EnRaxΔC infected patches (Fig. 3G-I and 3J-L, respectively). These results strongly suggest that *RaxL*, but not *Rax*, is required for photoreceptor cell development. Interestingly, we observed a similar photoreceptor phenotype with 100% penetrance when we introduced a dominant-negative allele comprising the engrailed repressor domain fused with the *RaxL* homeodomain (EnRaxLHD) (Fig. 3M,N). However, when a control viral construct, EnIrx, which carries the homeodomain of *Irx* fused to the engrailed repressor domain (Bao et al., 1999), was introduced into chick retina, the normal *visinin* and *Rxrg* expression was observed within the EnIrx infected patches (Fig. 3O,P, and data not shown).

RaxL is not required for non-photoreceptor cell development in the retina

In order to examine whether the effects on retina cell differentiation by EnRaxLΔC virus were specific to photoreceptors, the expression of markers of other cell types were analyzed. The expression of *Brn3a* (now known as *Pou4f1*) the ganglion cell marker (Liu et al., 2000), *Chx10*, the bipolar cell marker (Belecky-Adams et al., 1997; Chen and Cepko, 2000), and *Pax6*, which marks horizontal, amacrine and ganglion cells (Belecky-Adams et al., 1997), were analyzed on EnRaxLΔC infected retina. Virus infected E9 retinas, including seven retinas for *Brn3a*, 6 retinas for *Chx10* and 8 retinas for *Pax6*, were analyzed. None of these markers were affected by expression of EnRaxLΔC (Fig. 4). We also found no changes on the expression of these markers when EnRaxLHD was introduced into chick retina (data not shown). These results strongly suggest that the *RaxL* gene is required for proper development of photoreceptor cells, but not other retinal neurons.

EnRaxLΔC functions as a dominant negative form of *RaxL*

We have shown that EnRaxLΔC, which theoretically acts as a dominant negative allele of *RaxL*, blocks normal photoreceptor differentiation. To determine whether EnRaxLΔC indeed functions as a dominant negative allele of *RaxL*, the transactivation activities of RaxL and EnRaxLΔC were analyzed using a reporter construct encoding the chloramphenicol acetyltransferase (CAT) gene driven by five copies of the Ret1 enhancer element (RET1-CAT) (Fig. 5A). The Ret1/PCE1 site, an enhancer element present in many photoreceptor specific genes, is required for photoreceptor specific expression of these genes (Kikuchi et al., 1993). Fig. 5B shows that RaxL transactivated the RET1-CAT reporter construct 53-fold above the control expression vector when transiently transfected into COS cells, suggesting that RaxL is a strong transcriptional activator which can transactive photoreceptor specific genes through the Ret1 enhancer element. By contrast, Rax transactived the same reporter construct more weakly (ninefold above the control vector) (Fig. 5B), suggesting that Rax might recognize different enhancer elements that perhaps function in progenitor cells. To test the dominant-negative activity of EnRaxLΔC, the RET1-CAT construct was transiently co-transfected with vectors expressing RaxL and/or various engrailed-fusion constructs into COS cells. The CAT activity of cell extracts was assayed 48 hours after transfection. In the presence of an increasing amount of EnRaxLΔC, the activation activity of RaxL was reduced in a dose-responsive manner. An equal amount of

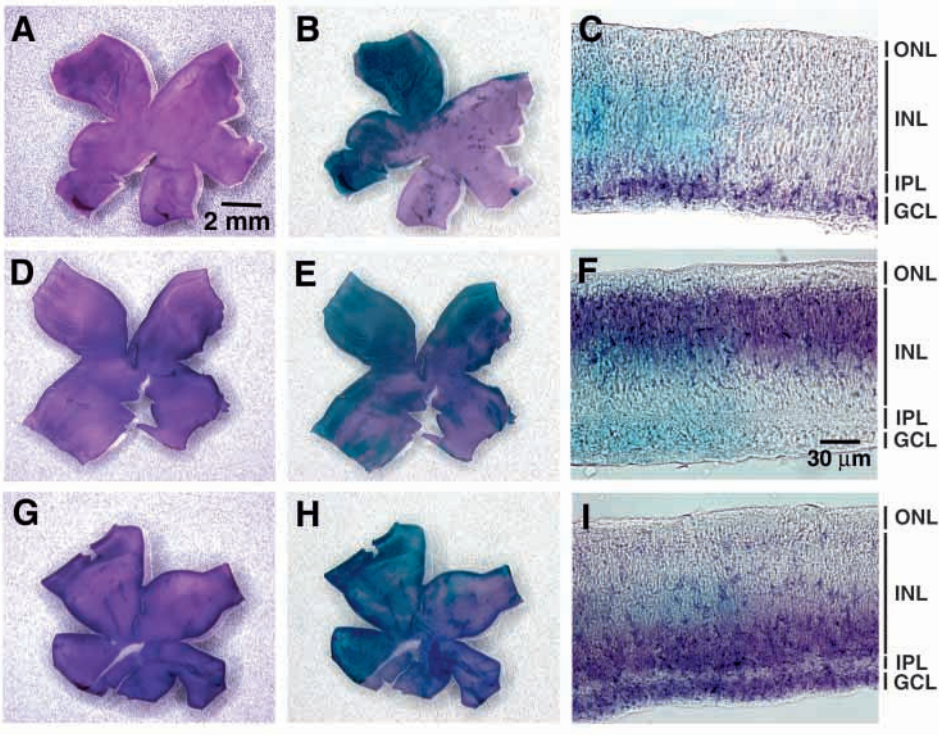

Fig. 4. Flat-mount in situ hybridization of E9 chick retinas electroporated with EnRaxLΔC viral construct. The signals of endogenous *Brn3a* (A-C), *Chx10* (D-F) and *Pax6* (G-I) transcripts are shown in purple and EnRaxLΔC viral transcript is shown in green-blue. The sections of the retina samples in B,E,H are shown in C,F,I, respectively. Scale bars: in A is 2 mm for A,B,D,E,G,H; in F, 30 μm for C,F,I. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer.

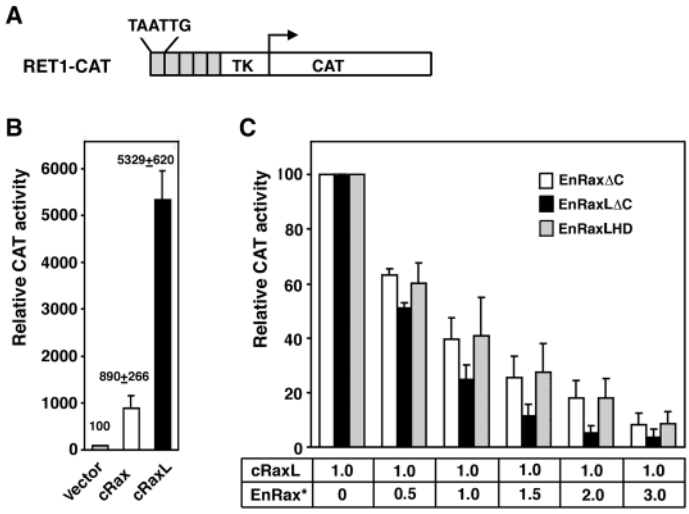

Fig. 5. CAT activities were assayed in COS cells transiently transfected with the RET1-CAT reporter construct (A) together with Rax or RaxL expression vectors (B) or with combinations of different ratios of expression vectors as indicated (C). The CAT activity from each transfection was normalized for internal transfection efficiency (see Materials and Methods). The relative CAT activity was normalized to the value from empty vector (B) or from RaxL transfection alone (C). The data presents the average value of triplicates. EnRax* represents EnRaxΔC, EnRaxLΔC or EnRaxLHD indicated in C.

EnRaxLΔC repressed the RaxL activation activity to 25.3%, and three times more EnRaxLΔC further repressed the activity of RaxL to 3.9% (Fig. 5C). This decrease of CAT activity is not due to the overexpression of a homeodomain protein, as cells transfected with four doses of RaxL showed similar CAT activity to those transactivated by a single dose of RaxL (data not shown). EnRaxLΔC repressed the transactivation of RaxL specifically, as EnRaxLΔC showed no effect on the activity of a CAT reporter driven by the SV40 enhancer elements (data not shown). These data suggest that EnRaxLΔC functions as a dominant negative form of *RaxL*, and that expression of EnRaxLΔC inhibits the endogenous *RaxL* activity. Interestingly, we also found dominant negative activities of EnRaxΔC and EnRaxLHD on RaxL transactivation activity in a dose-related manner (Fig. 5C). The dominant negative effects of EnRaxΔC and EnRaxLHD were similar but weaker than EnRaxLΔC.

Overexpression of *RaxL* rescues the photoreceptor phenotype induced by EnRaxLHD

We have demonstrated that EnRaxLΔC can inhibit the transcription activity of RaxL in tissue culture cells, suggesting that the phenotype observed by expressing EnRaxLΔC in the chick retina is due to a block of endogenous RaxL activity. If this assumption is correct, the dominant negative phenotype created by EnRaxLΔC should be rescued by coexpression of RaxL in ovo. As the phenotype created by EnRaxLΔC did not show full penetrance, we decided to use the dominant-negative EnRaxLHD, which is more effective, and thus facilitate the interpretation of a rescue experiment. We electroporated EnRaxLHD alone or together with RaxL into chick optic vesicles. The EnRaxLHD and RaxL retroviral constructs carry the type B and type A envelope proteins, respectively, which allows co-infection of both viruses into the same cells. After the detection of EnRaxLHD virus, we stained the infected retinas with the 3C2 mAb, which recognizes a matrix core protein of Rous Sarcoma virus (Potts et al., 1987). The 3C2 mAb recognizes both viruses. When EnRaxLHD alone was electroporated, we observed strong inhibition of the visinin signal, which correlated with EnRaxLHD infected patches, detected by engrailed expression. The staining pattern of 3C2

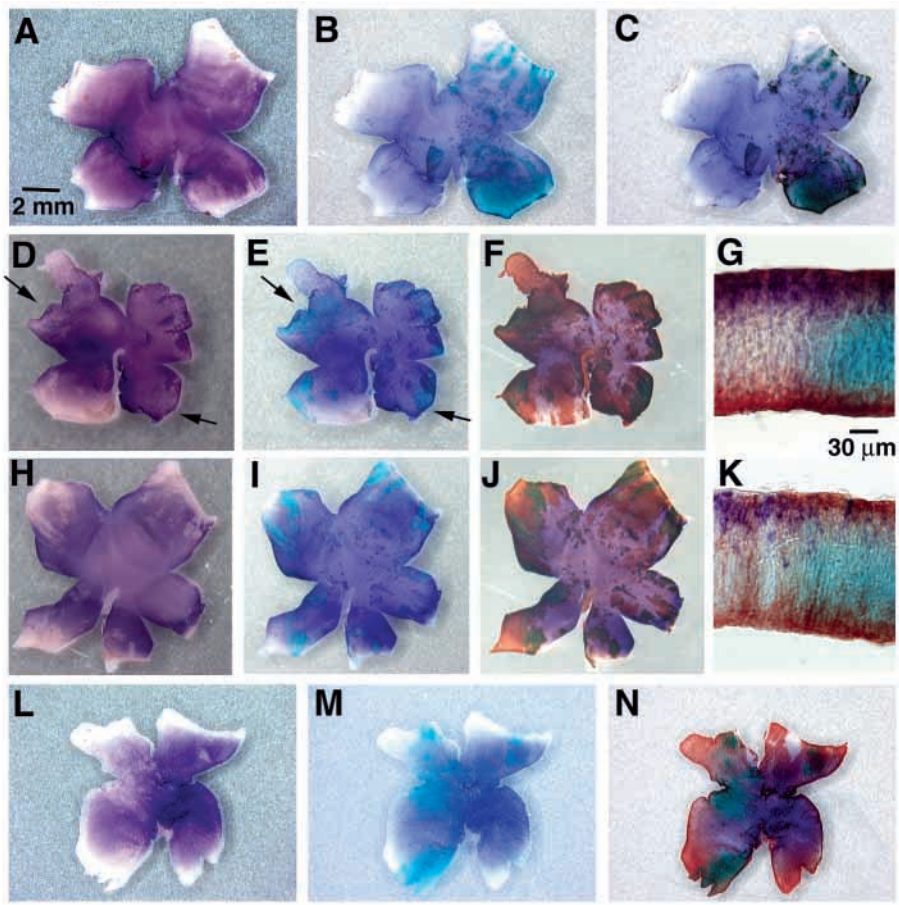

Fig. 6. Flat-mount in situ hybridization of E7.5 chick retinas electroporated with EnRaxLHD alone (A-C), EnRaxLHD plus RaxL (D-G), EnRaxLHD plus Rax (H-K) or EnRaxLHD plus mouse Crx (L-N) viral constructs. The sections of the retina samples in F,J are shown in G,K, respectively. The signals of endogenous *visinin* transcripts are shown in purple, EnRaxLHD viral transcript is shown in green-blue, and 3C2 mAb, which stains all viral infected patches, is shown in brown. The arrows in D,E indicate the *visinin* expression is partially rescued by RaxL. Scale bars: in A, 2 mm for A-F,H-N; in G, 30 mm for G,K.

mAb perfectly matched the engrailed staining pattern (Fig. 6A-C). This observation allows us to assume that 3C2 stained areas with no engrailed signal represents the RaxL-only infected region. However, the patches with engrailed signal may express EnRaxLHD virus alone, or express both EnRaxLHD and RaxL viruses. To ensure that most of the EnRaxLHD infected cells were also infected with RaxL virus, we electroporated three times as much RaxL viral construct as EnRaxLHD construct when co-electroporation was performed. Fig. 6D-G show that when both EnRaxLHD and RaxL were introduced, the inhibitory effect on *visinin* expression by EnRaxLHD was dramatically reduced (Fig. 6D,E, arrows). These data demonstrated that ectopic expression of RaxL rescued the dominant-negative phenotype generated by EnRaxLHD. This rescue was specific to RaxL. Chick Rax can not rescue the photoreceptor phenotype (Fig. 6H-K). Similarly, when the mouse Crx viral construct, encoding another paired-type homeodomain protein, which is required for photoreceptor maturation but not for initial photoreceptor cell generation (Furukawa et al., 1997b), was introduced with EnRaxLHD, no rescue of *visinin* expression was found (Fig. 6L-N). These in vivo rescue results strongly suggest that the inhibition of photoreceptor gene expression was due to a block of endogenous *RaxL* activity. The fact that *Crx* could not rescue or bypass *RaxL* function suggests that *RaxL* is required before *Crx* function during photoreceptor cell development. This is consistent with the idea that *RaxL* is required in the early stage of photoreceptor cell generation.

EnRaxLHD caused a reduction in cells expressing photoreceptor markers

The reduction in the expression of photoreceptor specific genes could be due to EnRaxLΔC interfering with proper photoreceptor differentiation and/or photoreceptor survival. To further address these issues, we took advantage of the consistent penetrance of EnRaxLHD to quantify the number of photoreceptors following expression of EnRaxLHD. Two monoclonal antibodies (mAb) against chick visinin were generated, 6H9 and 7G4, which exhibited specificity for chick visinin (S. Bruhn and C. Cepko, unpublished). They behaved similarly in both western blots and immunohistochemical assays, with the results of 7G4 shown in Fig. 7A-C. 7G4 recognized a single band of 24 kDa from chick retinal extracts by western blot analysis, which corresponds to the predicted size of chick visinin (Fig. 7A). A low level of visinin protein was evident at E5.5 and the level increased gradually as more photoreceptors differentiated between E6.5 and E8.5 (Fig. 7A). This time course is consistent with the expression profile of *visinin* transcripts (Bruhn and Cepko, 1996). The immunostaining on retinal sections further demonstrated that the visinin mAb recognized differentiating and mature photoreceptors, which are localized to the developing ONL in differentiating (E7) and mature (E18) retinas (Fig. 7B,C). These analyses demonstrate that the visinin mAb is a reliable early marker of photoreceptors.

To quantify the number of photoreceptor cells, optic vesicles were electroporated with EnRaxLHD, RaxL or control RCAS retroviral constructs at Hamburger-Hamilton stage 10 and infected retinas were harvested and dissociated at E8. FACS analysis was performed on dissociated retinal cells after staining with 7G4 mAb against visinin and antiserum against p27, an Avian Leukemia Viral protein (SPAFAS) (Fig. 7D,E). As viral infections only occurred in some patches of the retina and we expected the action of EnRaxLHD to be cell autonomous, only infected cells were scored for visinin expression. The percentage of visinin and p27 double-positive cells among the viral infected population (p27 positive) was calculated after a total of 250,000 cells were counted from each retina. In two independent experiments, 10.8% and 15.7% (on average) of control virus infected retinal cells were visinin-positive photoreceptors. However, when the retina was infected with the EnRaxLHD virus, the percentage of photoreceptors

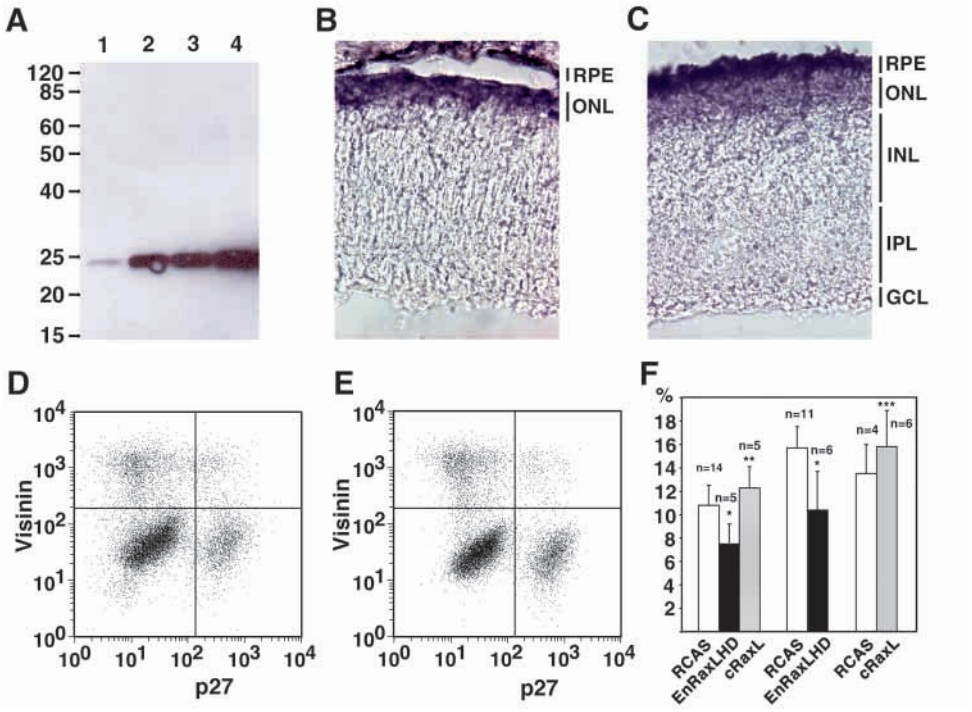

Fig. 7. Western blot with anti-visinin mAb showing a 24 kDa visinin protein in E5.5, 6.5, 7.5 and 8.5 chick retinal extracts (A, lanes 1 to 4). The photoreceptors in the ONL of E7 (B) and E18 (C) retina are stained with visinin mAb. The examples of FACS analyses of RCAS- (D) and EnRaxLHD- (E) infected retina are shown, with the upper-right quadrants representing the visinin and p27 (virus infected) double-positive population; and the upper- and lower-right two quadrants representing all viral-infected cells (p27 positive). The average percentages of double-positive cells among all viral infected population are indicated in the F. *n* indicates the retina sample number. *P* values are based on the Student's *t*-test of two-tailed distribution as follows: *, $P<0.005$; **, $P=0.152$; ***, $P=0.226$. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium.

was significantly decreased to average 7.5% and 10.4%, respectively (Fig. 7F). We found no significant change in the VC1.1-positive population, which comprises amacrine and ganglion cells, when the retina was infected with EnRaxLHD virus (data not shown). Interestingly, we found a slight increase in visinin-expressing photoreceptors in retinas infected with RaxL virus (from average 10.8% and 13.5% to 12.3% and 15.8%, respectively) (Fig. 7F). Overexpression of RaxL thus slightly increased the number of photoreceptors, and interfering with the endogenous *RaxL* by overexpression of the dominant negative EnRaxLHD led to a significant reduction of differentiating photoreceptor cells in the retina.

EnRaxLHD induces apoptosis

Results from both the FACS analysis and whole-mount in situ hybridization showed that decreasing the number of differentiating photoreceptors did not lead to an increase of the other retinal cell types scored following introduction of dominant negative *RaxL*. These data suggested that interfering with the normal function of *RaxL* did not induce a change in retinal cell fates. The reduction in differentiating photoreceptors could then be a block in photoreceptor cell differentiation and/or induction of photoreceptor cell death, or an effect on proliferation that affects only photoreceptor cells. The latter case is very unlikely, as photoreceptors are made by a multipotent progenitor (Fekete et al., 1994) and thus other cells would be affected, as would general proliferation, if this were the case. Nevertheless, to test the effect of dominant negative *RaxL* on cell proliferation, the anti-phospho-Histone H3 antibody was used to detect mitotic cells on E7.5 retinal sections electroporated with the EnRaxLHD viral construct. The virus infected patches were visualized with 3C2 mAb (Fig. 8, red). At E7.5 very few M-phase cells were found in or near the ventricular surface (green nuclei in Fig. 8A), and there was no significant difference between virus-infected patches and adjacent non-infected areas (Fig. 8A,B). We also found that EnRaxLHD had no significant effect when scored for phospho-Histone H3 staining on E5.5 and E6.5 retinas when there were more mitotic cells (data not shown). These data suggest that the decrease in differentiating photoreceptor cells by EnRaxLHD was not due to interference with progenitor cell proliferation. We then examined the possibility that reduction was due to apoptosis. The TUNEL assay was performed on E7.5 retinal sections electroporated with EnRaxLHD or control EnIrx viral constructs. TUNEL-positive cells were found only occasionally in normal E7.5 retinal sections. Very few TUNEL-positive cells were found in control EnIrx infected retina (Fig. 8C,D) and non-infected patches in EnRaxLHD infected retina (Fig. 8E,F). However, many TUNEL-positive cells were observed in the EnRaxLHD infected patches (Fig. 8E, green and yellow dots). The same viral construct induced no apoptosis when electroporated into chick brain (Fig. 8G,H), suggesting that overexpression of EnRaxLHD does not lead to non-specific apoptosis. The specific increase of apoptosis in EnRaxLHD infected retina provides an explanation for the decreased number of photoreceptor cells. Interestingly, we found that the TUNEL-positive cells were not concentrated in the photoreceptor layer, but spanned the radial thickness of the retina.

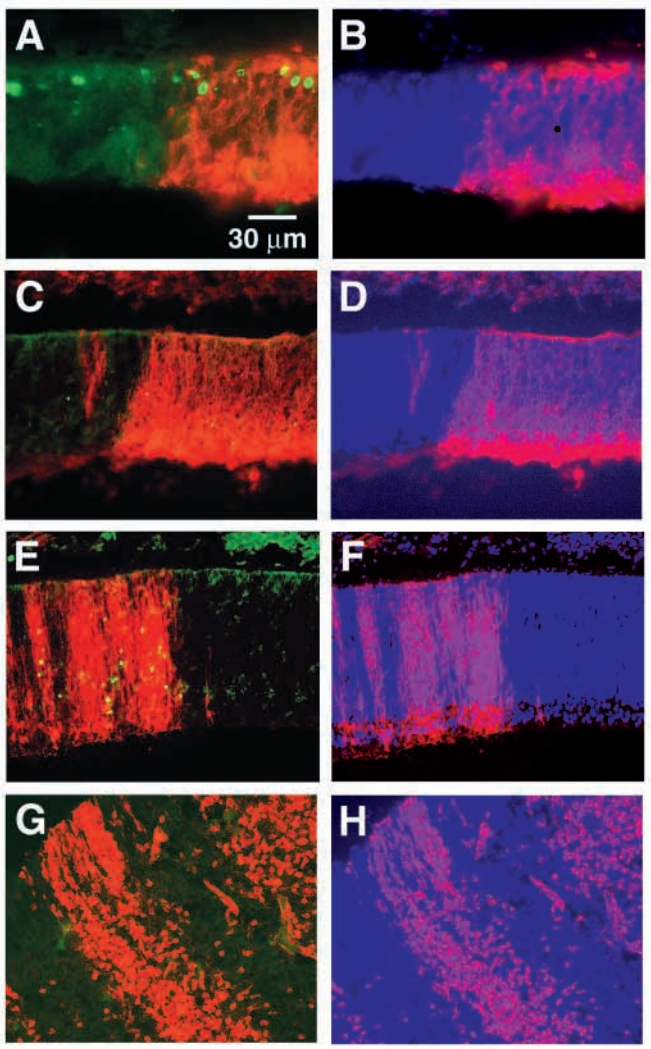

Fig. 8. Phospho-Histone H3 staining (A,B) and TUNEL analysis on E7.5 retinal (C-F) and brain (G,H) sections infected with EnRaxLHD virus (A,B,E-H) or control EnIrx virus (C,D). The phospho-Histone H3 or TUNEL-positive cells are shown in yellow or green, viral-infected cells are shown in red and nuclei stained with DAPI are in blue. The red and green merged images are presented in A,C,E,G. The same fields with red and blue merged images are presented in B,D,F,H, respectively.

DISCUSSION

We have investigated the expression patterns of *Rax* and *RaxL* and performed functional analyses of *RaxL*. Our data indicate the *RaxL* is required for the early steps in the development of photoreceptor cells.

Rax and *RaxL* are expressed in overlapping, but not identical, patterns

We have isolated cDNAs encoding two members of chick Rax family, *Rax* and *RaxL*. *Rax* is highly expressed in the optic vesicles, retinal progenitor cells, and the ventral diencephalon, in a pattern similar to that of the mouse *Rax/Rx* (Furukawa et al., 1997a; Mathers et al., 1997) and the published chicken *Rax* gene (Ohuchi et al., 1999). However, contrary to the previous report that *RaxL* is highly expressed in the developing retina and ventral diencephalon (Ohuchi et al., 1999), we found that

RaxL is expressed in the optic vesicles and retinal progenitor cells, but is absent from the ventral diencephalon. We reason that this difference is due to the specificity of the *RaxL* probe used in each study. The *RaxL* probe used previously contains the homeodomain, which shares 96% nucleotide (173 out of 180 nucleotides) identity to the *Rax* homeodomain region. The *RaxL* probe containing the homeodomain region can recognize both *Rax* and *RaxL* transcripts, and therefore can cross-hybridize with *Rax* in the ventral diencephalon. The fact that we do not observe *RaxL* in the ventral diencephalon allows us to conclude that the *RaxL* expression pattern resembles that of the zebrafish homologs *rx1* and *rx2*. *rx1* and *rx2* are expressed in the optic primordium and are absent from the ventral midline of the diencephalon. More interestingly, similar to *RaxL*, *rx1* and *rx2* are also downregulated as the retina differentiates, except in the ONL where they continue to be expressed at high levels in photoreceptors. The photoreceptor cells where *rx1* and *rx2* expressed are cones, but not rods (Chuang et al., 1999). *RaxL* is also expressed in cones as cones comprise 80% of chick photoreceptors and the *RaxL*-expressing population comprises the majority, if not all, of the photoreceptors. However, we cannot determine if *RaxL* is also expressed in rods. We speculate that *RaxL* homologs are expressed in cone, but not rod, photoreceptor cells in vertebrates. Such conserved expression pattern and gene sequences suggest an important function for *RaxL* in photoreceptor development. In mammals, the expression of a human *RaxL* homolog (*RAX2*) in a retinoblastoma cell line further suggests a role of *RaxL* in retina development. In addition, mutations in the human *RAX2* gene have been found in individuals with photoreceptor degeneration, which, if shown to be causal, would further establish the importance of *RaxL* homologs in photoreceptor cell development and/or function (D. Zack, personal communication). A mouse *RaxL* homolog has not been isolated. Surprisingly, the human *RAX2* syntenic region is missing in the mouse genome (T. Matsuda and C. Cepko, unpublished). It is possible that the mouse *RaxL* homolog is located in a different location in the mouse genome, and is expressed at very low abundance because it is expected to be in cone photoreceptors, which comprise only 2.2% of retina cells in the mouse (Young, 1985). It is also possible that the mouse has no *RaxL* homolog. The function of *RaxL* may be carried out by the mouse *Rax/Rx* gene, as mouse *Rax/Rx* has been reported to be expressed in photoreceptor cells and can transactivate photoreceptor specific genes (Kimura et al., 2000).

The expression pattern of *RaxL* suggests a role in early developing photoreceptors

Photoreceptor cells develop in a temporal gradient from the central to the peripheral retina. In the peripheral chick E7 retina, a subset of retinal cells that express a high level of *RaxL* spans the retinal epithelium, except in the differentiating ganglion cell layer. As development proceeds, centrally located *RaxL*-expressing cells become concentrated in the photoreceptor layer. This pattern is consistent with *RaxL* being expressed in mitotic progenitors that are in the process of producing photoreceptors, and/or in newly postmitotic photoreceptors. The high level expression of *RaxL* in such populations places *RaxL* at an important point in early photoreceptor development.

Chick photoreceptor genesis is reported to begin sometime between E3 and E5 in different studies (Kahn, 1974; Spence and Robson, 1989), with the bulk of photoreceptor genesis occurring between E5 and E6 (Prada et al., 1991; Belecky-Adams et al., 1996). Photoreceptors do not differentiate morphologically until E9.5, when the inner segments appear (Meller and Tetzlaff, 1976). The outer segments appear on E13 (Meller and Tetzlaff, 1976), and the synapses from photoreceptors to bipolar cells are evident on about E18 (Hughes and LaVelle, 1974). As discussed above, we found that *RaxL* is expressed in developing photoreceptors, but not in mature photoreceptors on E19, suggesting that *RaxL* is not required for the maintenance or survival of mature photoreceptors. Furthermore, apoptosis was observed as early as E7.5 when proper *RaxL* function was blocked, also indicating that *RaxL* is required for an early step in photoreceptor development.

EnRaxLHD interferes with the function of *RaxL* but not *Rax*

There are two populations of retinal progenitor cells expressing the *RaxL* transcript. One is the majority of retinal progenitors, which expresses a low level of *RaxL* and a high level of *Rax*. The other population is a small subset of cells that expresses a high level of *RaxL*. Our data show that overexpression of a fusion construct, EnRaxLHD, interferes with survival of a subset of cells located predominantly in the middle retinal layer. This is the area where mitotic progenitor cells reside and thus it is possible that EnRaxLHD interferes with survival of a subset of mitotic cells. These may be the same cells that express high levels of *RaxL* in this area, and we would propose that these are the cells that are in the process of producing photoreceptor cells. We believe that it would be this subset of cells, rather than all progenitor cells, based upon the observations that EnRaxLHD does not interfere with general progenitor proliferation, as the number of mitotic cells and the overall thickness of infected areas, as well as differentiation of other retinal cell types, were not significantly affected. Alternatively, the dying cells located in the middle of the retina following transduction with EnRaxLHD are newly produced, postmitotic cells that are fated to be photoreceptor cells. It is not known if cells in this state would be located in this area as there are no markers for cells that are newly postmitotic and fated to be photoreceptors. Although photoreceptor cells are usually located in the outer nuclear layer, it is possible that they briefly reside in the middle of the retina prior to migrating to the future outer nuclear layer. It is curious that murine cones do display an inward migration prior to undergoing full differentiation in the mouse (Rich et al., 1997).

Despite the identical amino acid sequence in the homeodomain regions of *Rax* and *RaxL*, dominant-negative EnRaxLΔC, which included most of the *RaxL* sequence outside of homeodomain region, seemed to maintain it's specificity and interfere mainly with the function of *RaxL*, and not *Rax*. It is possible that the enhancer sequence recognized by *Rax* in progenitor cells is different from that recognized by *RaxL* in early photoreceptors. The finding that RaxL transactives the photoreceptor specific Ret1 enhancer element more efficiently than Rax supports this idea. However, this idea remains to be confirmed after the identification of the authentic binding elements of *Rax* and *RaxL*. It is also possible that the

expression level of *Rax* is higher than that of *RaxL* in the retinal progenitor cells and that the expression level of EnRaxLΔC was not high enough to interfere with *Rax* function. Alternatively, the function of *Rax* in progenitor cells may be dispensable since other paired-type homeodomain genes, e.g. *Pax6* and *Chx10* are highly expressed in retinal progenitor cells. The similar dominant-negative construct, EnRaxΔC, which contained most of *Rax*, had no effect on photoreceptor cell differentiation, further supporting the notion that the sequence outside of the homeodomain region provides significant specificity in ovo. Although EnRaxΔC can interfere with the transactivation activity of RaxL when assayed on a simplified reporter construct (RET1-CAT) in tissue culture cells, it appears not to function as a dominant-negative allele of *RaxL* on complex photoreceptor promoters in ovo. Our finding that interference with the endogenous *RaxL* activity by overexpression of EnRaxLΔC disturbs an early step in photoreceptor development, but not the general progenitor pool, suggests that only the progenitor population in the process of producing photoreceptor cells, or newborn photoreceptor cells, is affected. Without the proper activity of *RaxL*, photoreceptor cells cannot differentiate properly and, as a result, undergo apoptosis.

Other transcription factors are required for photoreceptor cell differentiation

Several photoreceptor-specific transcription factors have been identified over the last several years. Among them, *neuroD* (now known as *Neurod1*) a basic helix-loop-helix gene, is expressed in retinal photoreceptors transiently in chick and is sufficient to generate more photoreceptors when overexpressed in chick retina (Yan and Wang, 1998). In mice, *Neurod1* is expressed in retinal progenitor cells as well as in developing photoreceptor and amacrine cells, and is maintained in a subset of mature photoreceptors. Analysis of a *Neurod1* knockout mouse and overexpression of *Neurod1* in rats shows that it is not required for the initial formation of photoreceptor cells (Morrow et al., 1999). Thus the role of *Neurod1* in photoreceptor cell development is not the same in chick and mouse, or perhaps it is not required in chick or mouse photoreceptor development. Further studies are needed to clarify its role.

Crx, an otx-like homeodomain gene, is expressed in newly generated photoreceptors, including both cones and rods, as well as at a low level in bipolar cells in mice and a high level in bipolar cells in zebrafish (Furukawa et al., 1997b; Chen et al., 1997; Liu et al., 2001). Interestingly, in zebrafish, *Crx* is expressed in mitotic cells presumably fated to produce photoreceptor cells, while in murine retinal cells, the expression of *Crx* appears to be initiated in cells that are fated to be photoreceptors, just after exit from the cell cycle. The timing of chick *Crx* expression appears to be the same at it is in mouse (T. Furukawa, personal communication). Functional studies in rodents have shown that *Crx* is required for a high level of expression of many photoreceptor specific genes. It is required for maturation, but not for the initial generation, of photoreceptors (Furukawa et al., 1997b; Livesey et al., 2000). Another important transcription factor in photoreceptor development is *Nrl*, a basic motif- leucine zipper transcription factor. *Nrl* is expressed in rod, but not cone, photoreceptors (Swain et al., 2001). It physically interacts with *Crx* and synergistically transactivates the rhodopsin promoter in vitro (Mitton et al., 2000). Analysis of *Nrl* mutant mice has revealed that it is a critical determinant of early rod photoreceptor cell development (Mears et al., 2001). A similar function is ascribed to *Nr2e3* (also known as *PNR*), which encodes a ligand-dependent retinal nuclear receptor. *Nr2e3* is expressed in photoreceptor cells (Kobayashi et al., 1999), and mutations in *Nr2e3* lead to an increased number of cone cells in mice and the enhanced S cone syndrome, a disorder of photoreceptor cells, in humans (Haider et al., 2000; Haider et al., 2001). We provide evidence that a *Rax* family member, *RaxL*, is required for the initial generation of photoreceptors in chick. *RaxL* is expressed in cone photoreceptors. We hypothesize that *RaxL* and *Nrl* are required for the early stages of cone and rod cell fate determination, respectively. Later in development, as cones and rods take up their final stages of differentiation, *Crx* plays the major role in supporting photoreceptor-specific gene expression. Overexpression of *Crx* failed to rescue the photoreceptor phenotype induced by a dominant-negative allele of *RaxL*, further supporting the idea of early role of *RaxL* in photoreceptor development.

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