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RESEARCH ARTICLE

Expression of AP-2δ in the Developing Chick Retina

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AP-2 is a family of transcription factors that play important roles during embryonic development. Two *AP-2* genes, *AP-2*α and *AP-2*β, have previously been characterized in chick retina. Here, we demonstrate that a third member of the chicken AP-2 family, *AP-2*δ, is primarily expressed in the retina and brain, with highest levels at embryonic days 7 to 11. By in situ hybridization and immunohistochemical analysis, we show that *AP-2*δ RNA and protein are found in a subset of ganglion cells in embryonic chick retina. Co-immunostaining with anti-Brn3a and anti–AP-2δ antibodies indicates that approximately one-third of Brn3a-positive ganglion cells express AP-2δ. *AP-2*δ RNA but not AP-2δ protein is also found in cells located in the outer half of the inner nuclear layer. The spatial and temporal distribution of AP-2δ protein in the retina suggests a transient role in a subset of late-born ganglion cells likely involving axonal trafficking or pathfinding. *Developmental Dynamics 237:3210–3221, 2008.* © 2008 Wiley-Liss, Inc.

Key words: AP-2; transcription factors; ganglion cells; Brn3a; immunostaining; in situ hybridization

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INTRODUCTION

AP-2 is an important family of transcription factors implicated in many aspects of development, cell differentiation and the regulation of cell growth and death (Hilger-Eversheim et al., 2000). Five *AP-2* genes have been isolated in mice and humans: *AP-2*α, *AP-2*β, *AP-2*γ, *AP-2*δ, and *AP-2*ε (Williams et al., 1988; Moser et al., 1995; Bosher et al., 1996; Chazaud et al., 1996; Zhao et al., 2001a; Feng and Williams, 2003; Tummala et al., 2003). In chicken, two *AP-2* genes have been isolated to date, *AP-2*α and *AP-2*β (Bisgrove et al., 1997; Shen et al., 1997; Bisgrove and Godbout, 1999). AP-2 proteins have a highly conserved C-terminal half, which contains the central and helix-span-helix domains involved in DNA binding and dimerization. The poorly conserved N-terminal half contains a proline- and glutamine-rich domain responsible for transactivation (Williams and Tjian, 1991a,b; Garcia et al., 2000; Wankhade et al., 2000). AP-2 has been shown to bind as homodimers or heterodimers to the consensus sequence 5′-GCC-NNNGGC-3′ (Hilger-Eversheim et al., 2000).

The function of AP-2 has been studied by generating mouse models lacking *AP-2*α, *AP-2*β, and *AP-2*γ. *AP-2*α knockout mice die perinatally and exhibit severe defects in cranial and body wall closure and skeletal structures (Schorle et al., 1996; Zhang et al., 1996; West-Mays et al., 1999). The ocular defects in these mice are so severe and appear so early, that it has not been possible to evaluate the function of AP-2α in retinal development. Recently, conditional knockout mice with *AP-2*α inactivation specific to the retina have been generated (Bassett et al., 2007). Surprisingly, these mice have no obvious retinal defects. The absence of a phenotype is likely due to compensatory effects by other members of the AP-2 family. In particular, AP-2α and AP-2β have very similar expression profiles in the developing retina (Bisgrove and Godbout, 1999; Bassett et al., 2007). In contrast to *AP-2*α knockout mice, *AP-2*β−/−

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mice do not exhibit defects in craniofacial and ocular structures, but die perinatally due to massive apoptosis of renal tubular epithelia (Moser et al., 1997). *AP-2*γ knockout mice die after gastrulation due to defective placental development (Auman et al., 2002; Werling and Schorle, 2002). Thus, even though AP-2 family members have overlapping expression patterns, disruption of individual *AP-2* genes reveals nonredundant roles during development. In humans, mutations generating dominant negative AP-2β cause Char syndrome characterized by patent ductus arteriosus with facial dimorphism and abnormal fifth digits (Zhao et al., 2001b).

*AP-2*δ cDNA was first isolated from fetal mouse head at embryonic day (E) 13 (Zhao et al., 2001a). Based on Northern blot analysis, the murine *AP-2*δ gene encodes two transcripts in embryonic and newborn mouse brain. AP-2δ is highly similar to other AP-2 proteins in the DNA-binding and dimerization domains. However, there is significant divergence from other AP-2 proteins in the N-terminal transactivation domain, with AP-2δ lacking residues that are critical for transcriptional activation in the other members of this family (Zhao et al., 2001a). In mouse, *AP-2*δ RNA is detected later than the other *AP-2* genes and is mainly found in the CNS as well as heart (Zhao et al., 2003). In the mouse retina, *AP-2*δ RNA is primarily detected in ganglion cells (Bassett et al., 2007). Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis has revealed *AP-2*δ transcripts in a wide variety of human tissues (Cheng et al., 2002).

In previous studies, AP-2α and AP-2β were shown to be restricted to two neuronal cell lineages in the developing chick retina, amacrine (AP-2α, AP-2β), and horizontal cells (AP-2β) (Bisgrove and Godbout, 1999; Hilger-Eversheim et al., 2000). To further investigate the expression of AP-2 transcription factors in the developing retina, we isolated *AP-2*δ cDNA from chick retina and generated anti–AP-2δ antibody. Here, we demonstrate that AP-2δ is preferentially expressed in the retina and brain during embryonic development. We also show that both *AP-2*δ RNA and AP-2δ protein are found in a subset of ganglion cells. In addition, we document the presence of *AP-2*δ RNA (but not the protein) in cells located in the outer half of the inner nuclear layer. Our data indicate that there is no overlap between AP-2α/β and AP-2δ expression patterns in the developing retina and that AP-2δ may play a specific role in ganglion cell differentiation.

RESULTS

Isolation of *AP-2*δ cDNA From Chick Retina

The human *AP-2*δ cDNA sequence was compared with sequences entered in the chicken expressed sequence tag (EST) databases to identify the chicken *AP-2*δ ortholog. An 804-bp EST was identified that encompassed the 3′ end of the human *AP-2*δ cDNA (NM_172238, 999 to 1,763 bp) with 83% identity at the nucleotide level. An additional 706 bp of 5′ *AP-2* sequence was generated by rapid amplification of cDNA ends (5′RLM-RACE). Comparison searches of the chicken genomic database using the 1,510-bp *AP-2*δ cDNA identified a hypothetical protein (LOC771525) encoded by a computer-generated 1,456-bp (XR_026774) cDNA sequence. The hypothetical chicken AP-2δ protein showed extensive similarity to human AP-2δ with the exception of the N-terminal region which showed wide divergence. Analysis of the genomic DNA encompassing the hypothetical chicken AP-2δ protein revealed that a region predicted to be an intron in the chicken gene was highly homologous to human exon 1. We therefore generated primers spanning the deduced chicken *AP-2*δ start (based on similarity with human *AP-2*δ) and stop codons to PCR-amplify the complete open reading frame of chicken *AP-2*δ. Poly(A)$^+$ RNA isolated from chick retina at E10 was used for this analysis. Cloning and sequencing of the resulting product revealed a 1,359-bp cDNA sequence encoding 452 amino acids. Comparison of the chicken and human AP-2δ revealed 86% identity at the nucleotide level and 98% identity (443/452) or 99% similarity (449/452) at the amino acid level (Fig. 1). The three nonconserved residues were located in the N-terminal half of the protein in the transactivation domain.

The predicted molecular mass of chicken AP-2δ is 50 kDa, similar to that of chicken AP-2α (48 kDa) and AP-2β (49 kDa). Comparison of AP-2δ and AP-2α/AP-2β amino acid sequences revealed little similarity in the N-terminal halves (or transactivation domain) of the proteins. In contrast, the C-terminal half of AP-2δ was 69–70% identical (86% similar) to that of AP-2α and AP-2β (Fig. 2). Eight residues located in the transactivation domain have previously been shown to be critical for AP-2 function (bold in Fig. 2; Wankhade et al., 2000; Hilger-Eversheim et al., 2000). Of these eight residues, only three are conserved in AP-2δ. In comparison, the transactivation domains of AP-2α and AP-2β are relatively well-conserved with both domains containing the eight residues critical for transactivation (Bisgrove and Godbout, 1999).

To generate the genomic structure of chicken *AP-2*δ, we aligned our *AP-2*δ cDNA sequence with the human and chicken genomic DNA sequences. As shown in Figure 3, the open reading frame of chicken *AP-2*δ is encoded by 8 exons spanning 54,789 bp on chromosome 3. In comparison, human *AP-2*δ is encoded by 8 exons spanning 59,490 bp on chromosome 6p12.1. All exons found in the chicken genome have matching exons in the human genome. Exon 2 (498 bp) was predicted to be split into two exons [labeled exons 2A (189 bp) and 2B (309 bp) based on the sequence of the chicken *AP-2*δ gene (accession no. NC_006090). However, PCR amplification of chicken genomic DNA using primers flanking exons 2A and 2B supports the absence of this predicted intron (see Supp. Fig. S1, which is available online).

As with the mouse and human *AP-2*β and *AP-2*δ genes (Williamson et al., 1996; Cheng et al., 2002), the chicken *AP-2*β and *AP-2*δ genes are closely linked to each other, and are separated by only 21 kb. Southern blot analysis using chicken genomic DNA and an N-terminal 550 bp *AP-2*δ cDNA fragment as the probe indicate that there is a single *AP-2*δ gene in the chicken genome (Supp. Fig. S2).

*AP-2*δ RNA in Embryonic Chick Tissues

We used both RT-PCR and Northern blot analysis to examine the expres-

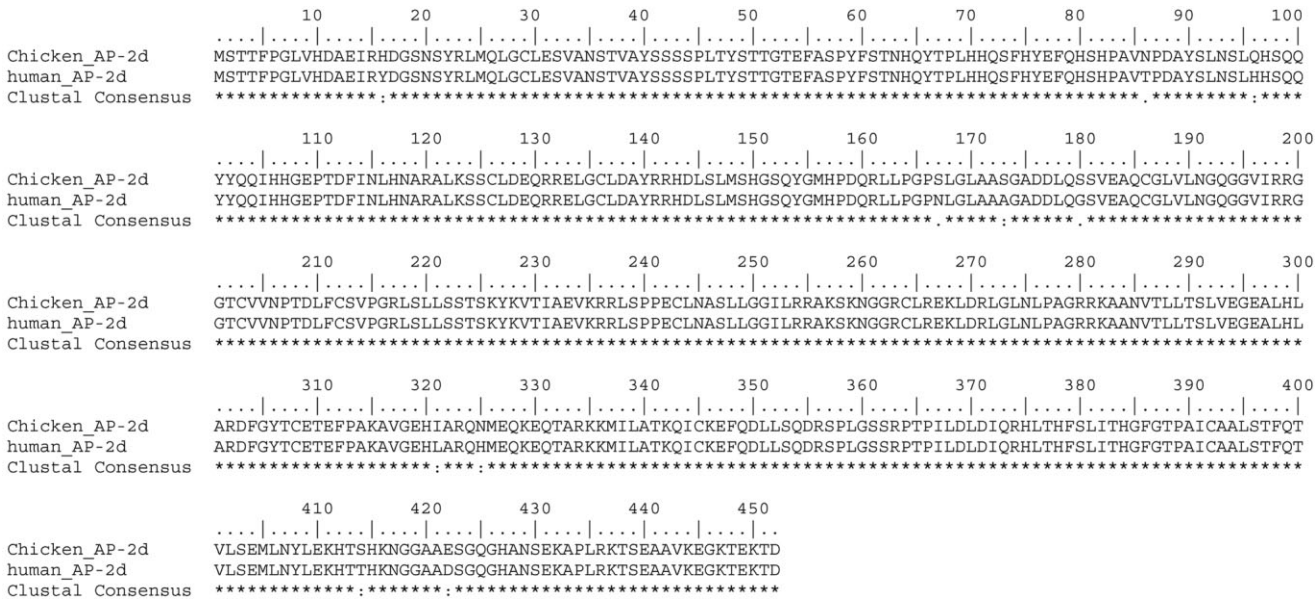

Fig. 1. Comparison of chicken and human AP-2δ amino acids. Identical residues are indicated by "*", similar residues are indicated by ":".

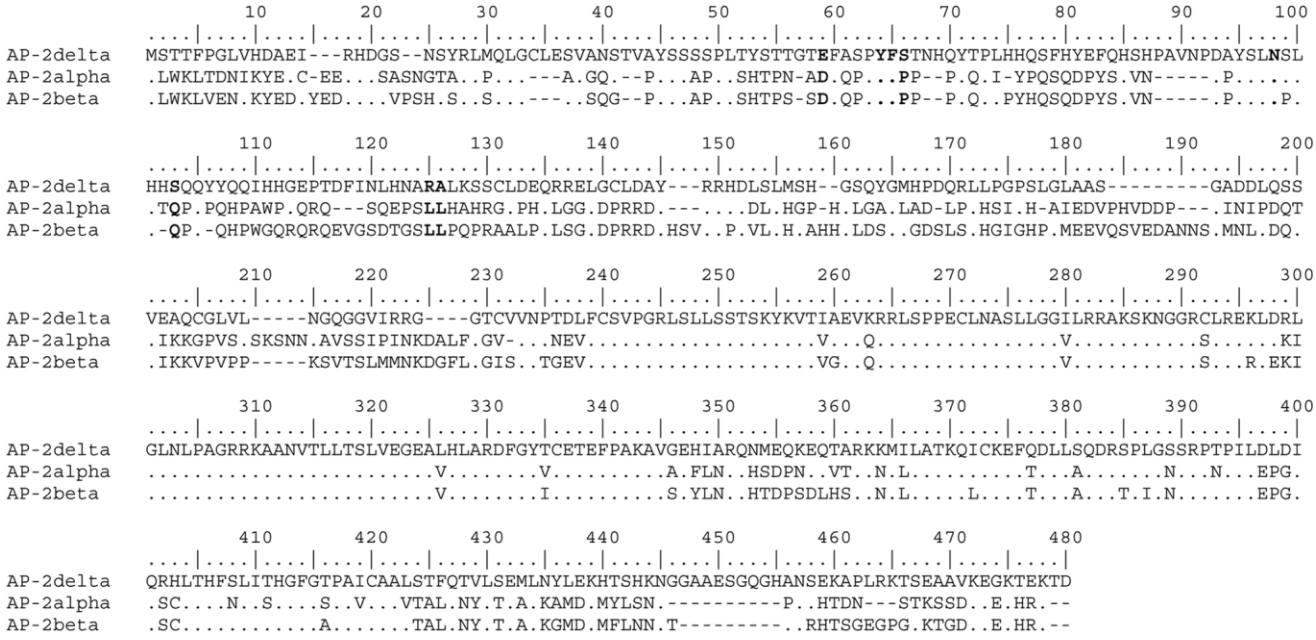

Fig. 2. Comparison of chicken AP-2δ, AP-2α, and AP-2β amino acids. Identical residues are indicated by "*", similar residues are indicated by ":" (conserved substitutions) and "." (semi-conserved substitutions). Previously identified critical residues for transactivation are in bold.

sion pattern of *AP-2*δ in the developing chick embryo. For RT-PCR, we included cDNAs derived from retina, brain, lung, heart, liver, kidney, and gut at different developmental stages. The primers used for the amplification were located at the 3′ end of the *AP-2*δ cDNA and generated a DNA fragment of 205 bp. As shown in Figure 4A, *AP-2*δ RNA is easily detected in E7, E10 and E16 retina and brain. *AP-2*δ is barely detectable or undetectable in all other tissues tested.

Northern blot analysis revealed an *AP-2*δ signal in retina and brain but not in any of the other tissues tested (lung, heart, liver, and kidney; Fig. 4B). Two different size transcripts were identified in retina and brain, at ~4 kb and ~5 kb. In retina, both forms were present at very low levels at E5, peaked at E7, and were significantly decreased by E16. In brain, both forms of *AP-2*δ peaked at E10 and were barely detectable at E16. These results suggest a transient role for AP-2δ during retina and brain development.

Distribution of *AP-2*δ RNA in the Developing Chick Retina

The *AP-2*δ RNA distribution pattern was analyzed by in situ hybridization

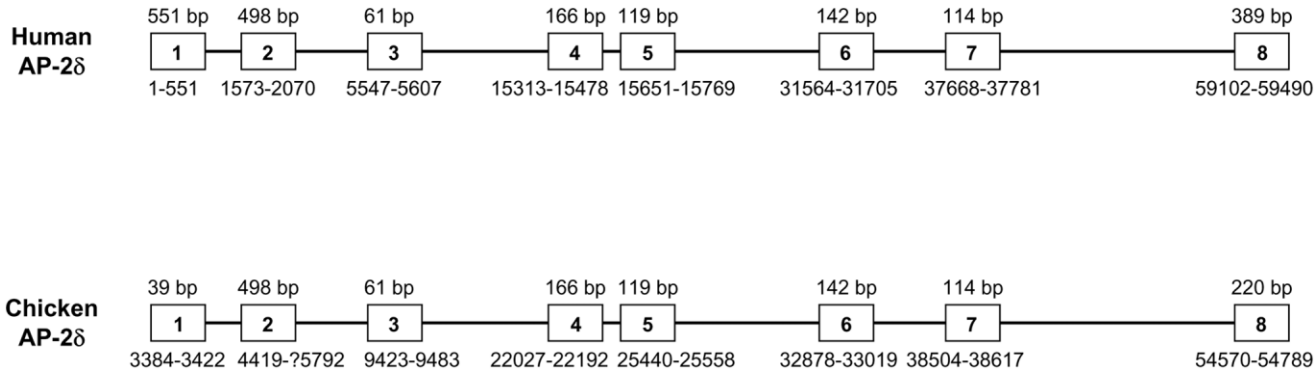

Fig. 3. Genomic DNA structure of chicken and human AP-2δ. Exon numbers are indicated in the boxes. The length of each exon is indicated on top of each box. The numbers under the boxes indicate the location of the exons relative to the genomic DNA. Numbering is based on the chicken *AP-2*δ gene sequence accession no. NC_006090. The chicken genomic DNA structure is predicted based on the sequence of *AP-2*δ cDNA. The indicated size of exon 1 (39 bp) reflects sequences up to the translation start site and does not include the 5′-UTR. The question mark (?) indicates the predicted presence of an intron within exon 2. As shown in Supp. Fig. S1, polymerase chain reaction amplification of chicken genomic DNA using primer pairs spanning predicted exons 2A and 2B failed to reveal the presence of an intron.

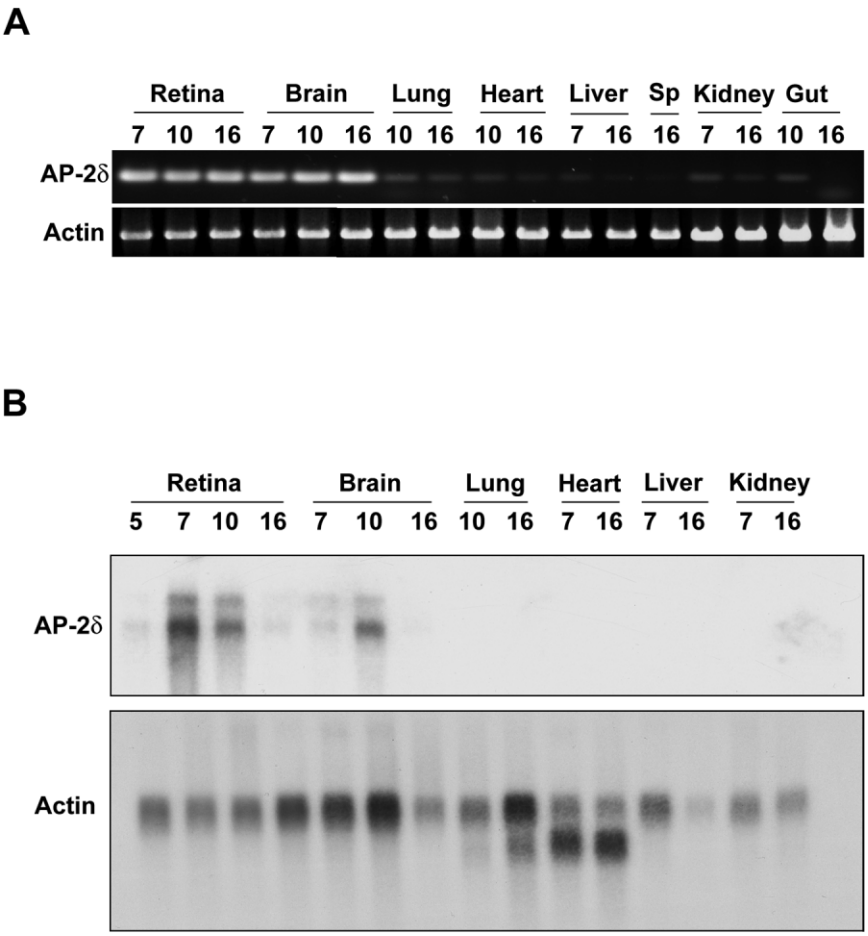

Fig. 4. Reverse transcriptase-polymerase chain reaction (RT-PCR) and Northern blotting of *AP-2*δ in embryonic chick tissues. **A:** RT-PCR analysis of *AP-2*δ using cDNA from chick retina, brain, lung, heart, liver, spleen (Sp), kidney, and gut at embryonic day (E) 7, E10, and E16 (upper panel). The cDNAs underwent 35 cycles of amplification. The same cDNA was used to amplify actin (lower panel). Twenty-five cycles were used for actin amplification. **B:** Northern blots were prepared using poly(A)$^+$ RNA purified from retina, brain, lung, heart, liver, and kidney at E5, E7, E10, and E16. The filter was sequentially hybridized with ^{32}P-labeled: (i) 1,359 bp *AP-2*δ cDNA and (ii) actin cDNA. The extra bands obtained with the actin probe represent tissue-specific actin mRNAs.

using tissue sections from retina at E5, E7, E10, and E15. Two nonoverlapping probes were used for this analysis, with the first probe encompassing the 5′ end of *AP-2*δ cDNA, from 1 to 549 bp, and the second probe corresponding to the 565 to 1,063 bp region in the middle of the *AP-2*δ cDNA. The expression patterns, described below, were identical for both probes.

At E5, when the majority (>80%) of cells are proliferating, scant *AP-2*δ-positive cells were found scattered throughout the retina (Fig. 5A). The *AP-2*δ distribution pattern was different from that of *AP-2*β, which is expressed in emerging amacrine cells (Bisgrove and Godbout, 1999) and *CA-II*, expressed in proliferating cells as well as emerging Müller glial cells (Vardimon et al., 1986), but similar to that of *Chx10-1*, expressed in emerging bipolar cells (Chen and Cepko, 2000). A significant increase in the number of *AP-2*δ-positive cells was observed at E7 when ~60% of cells are still proliferating (Prada et al., 1991; Fig. 5B). At this developmental stage, the ganglion cell layer (GCL) is readily apparent and separated from the inner nuclear layer by the inner plexiform layer in the central retina. *AP-2*δ-positive cells were found in the ganglion cell layer, with positive cells also scattered throughout the inner neuroblastic layer. Again, the distribution profile of *AP-2*δ in the inner neuroblastic layer most closely resembled that of *Chx-10-1*.

At E11, both the inner and outer

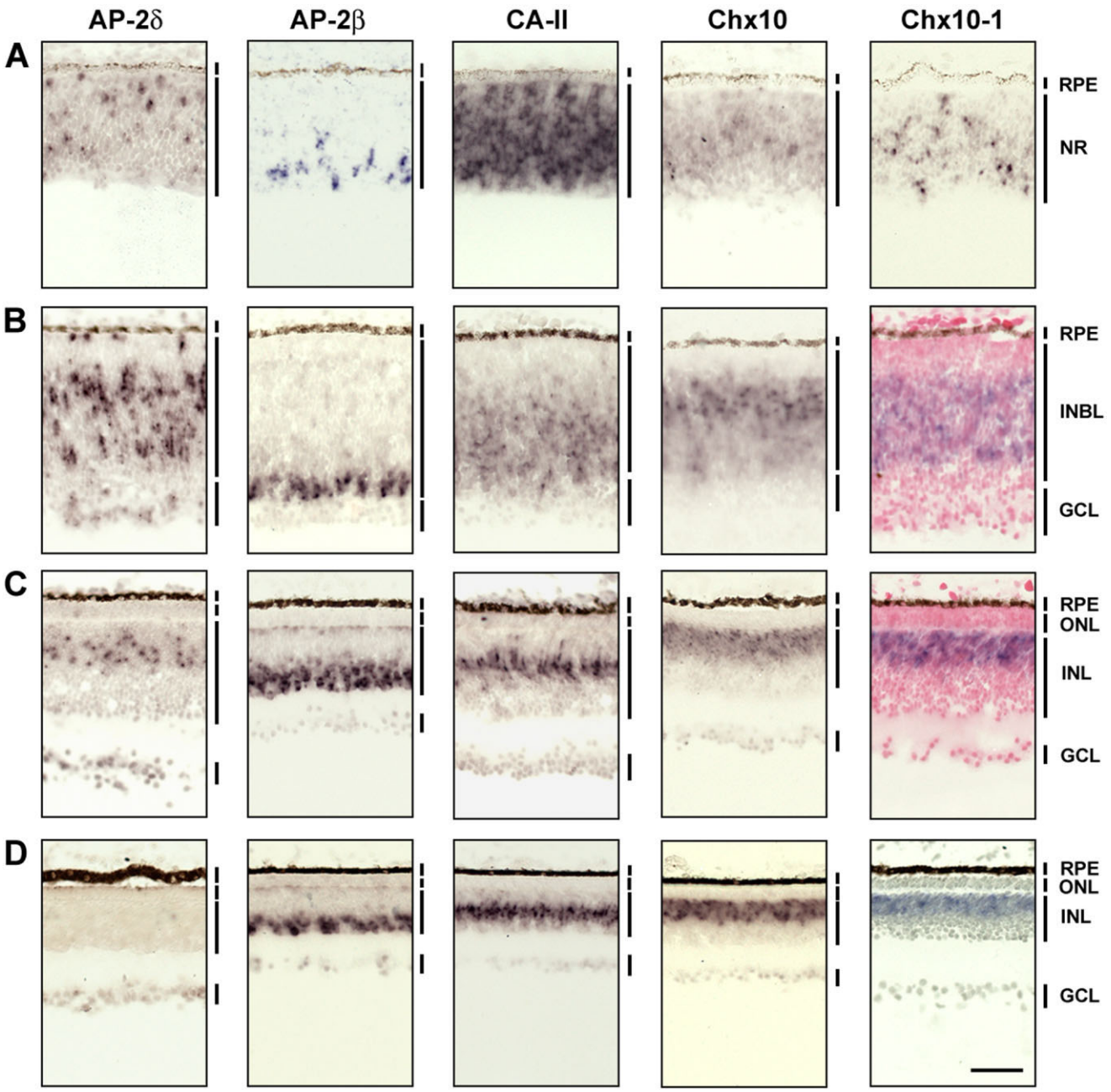

Fig. 5. In situ hybridization of *AP-2*δ in the developing chick retina. **A–D:** Retinal tissue sections at embryonic day (E) 5 (A), E7 (B), E11 (C), and E15 (D) were hybridized with DIG-labeled *AP-2*δ, *AP-2*β, *CA-II*, *Chx10*, and *Chx10-1* antisense RNA probes. The signal was detected by alkaline-phosphatase–coupled antibody, generating a purple color. Sections probed with digoxigenin (DIG) -labeled *Chx10-1* RNA at E7 and E11 were counterstained with eosin to show the cell layers. The section probed with *Chx10-1* RNA at E15 was counterstained with methyl green. All images shown are from the central half of the retina. RPE, retinal pigment epithelium; NR, neuroretina; INBL, inner neuroblastic layer; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer. Photographs were taken with a 20× lens using a Zeiss Axioskop 2 plus microscope. Scale bar = 50 μm.

plexiform layers of the retina have formed, generating the multi-layered appearance of the mature tissue which consists of three nuclear layers separated by plexiform layers. *AP-2*δ was detected in the ganglion cell layer as well as in the outer half of the inner nuclear layer (Fig. 5C). Based on its spatial distribution within the inner nuclear layer, and comparison with *AP-2*β, *CA-II*, *Chx10*, and *Chx10-1* staining patterns, *AP-2*δ RNA is most likely expressed in a subset of bipolar cells. In agreement with the Northern blot data, *AP-2*δ RNA was much less abundant at E15, with the signal primarily detected in the ganglion cell layer of the more differentiated parts of the retina (Fig. 5D). However, the *AP-2*δ signal remained strong in the most undifferentiated part of the retina, next to the ciliary epithelium, possibly reflecting the emergence of bipolar cells in this region (Supp. Fig.

S3). In contrast to *AP-2δ*, the *AP-2β*, *CA-II*, *Chx10*, and *Chx10-1* signals all remained strong at E15, indicating a more temporally restricted expression pattern for AP-2δ.

AP-2δ Protein Expression in the Developing Chick Retina

To study AP-2δ at the protein level in the developing chick retina, we generated anti–AP-2δ antibodies. To minimize the likelihood that these antibodies would recognize other members of the AP-2 family, we injected rabbits with GST fused to the poorly conserved N-terminus of AP-2δ (amino acids [aa] 1–199) (Fig. 2). Antibodies were purified by passing bacteria-pre-absorbed antiserum through a column conjugated to an AP-2δ-specific hydrophilic peptide (peptide 1, described under Experimental Procedures). The specificity of the anti–AP-2δ antibody was verified by overexpressing full-length AP-2α, AP-2β, and AP-2δ in HeLa cells and immunostaining a blot containing extracts from all three transfectants with anti–AP-2 antibodies. As shown in Figure 6A, anti–AP-2δ antibody recognized a protein expressed in cells transfected with AP-2δ, but not AP-2α or AP-2β. Monoclonal anti–AP-2α (3B5) antibody only recognized AP-2α, whereas anti–AP-2β (H87) antibody recognized AP-2β and to a lesser extent AP-2α.

Next, we examined AP-2δ expression in retinal tissue at E5, E7, E10, and E15. Strong bands at ~50 kDa were observed at E7 and E10, in agreement with the Northern blot data (Fig. 6B). Two weakly staining slower migrating bands (at ~65 and 70 kDa) were also detected in all four lanes. Peptide 1, used to affinity purify the anti–AP-2δ antibody, effectively blocked binding of the antibody to the 50 kDa band, but not to the higher molecular weight bands, thus confirming the identity of the 50-kDa band (data not shown). Analysis of additional tissues by Western blotting revealed a strong signal in the E7 and E10 brain (Fig. 6C). AP-2δ protein levels were considerably reduced in the E15 brain. Heart, stomach, and gut appeared negative even with longer exposure, whereas E5 head was weakly positive.

We also carried out immunohistochemical and immunofluorescence analyses of AP-2δ using retinal sections at different stages of development. As expected for a transcription factor, AP-2δ staining was restricted in the nucleus. However, in contrast to the in situ hybridization data, our antibody only detected a signal in the ganglion cell layer with a complete absence of immunostaining in the inner nuclear layer. Immunostaining with a second affinity-purified anti–AP-2δ antibody produced in a different rabbit generated the same expression profile as the first antibody (data not shown).

As predicted from the Northern and Western blot data, few AP-2δ–positive cells were observed at E5 (Fig. 7A). By E7, the percentage of the AP-2δ–positive cells was significantly higher with the great majority of these cells confined to the ganglion cell layer. The number of AP-2δ–positive cells remained high at E10 but was significantly reduced by E15. To confirm that the signal detected with our antibody was specific to AP-2δ, peptide 1 (AP-2δ peptide used for affinity purification) or peptide 3 (AP-2δ peptide shown not to bind to the anti–AP-2δ antiserum) was preincubated with anti–AP-2δ antibody before immunostaining. Whereas a 0.1 μM concentration of peptide 1 effectively blocked most of the AP-2δ signal, peptide 3 had no effect on signal intensity even at 1 μM (Fig. 7B). Together, the spatial and temporal distribution pattern of AP-2δ, combined with the peptide competition experiment, indicate that the signal detected in the ganglion cell layer is specific to AP-2δ.

To verify that AP-2δ–positive cells

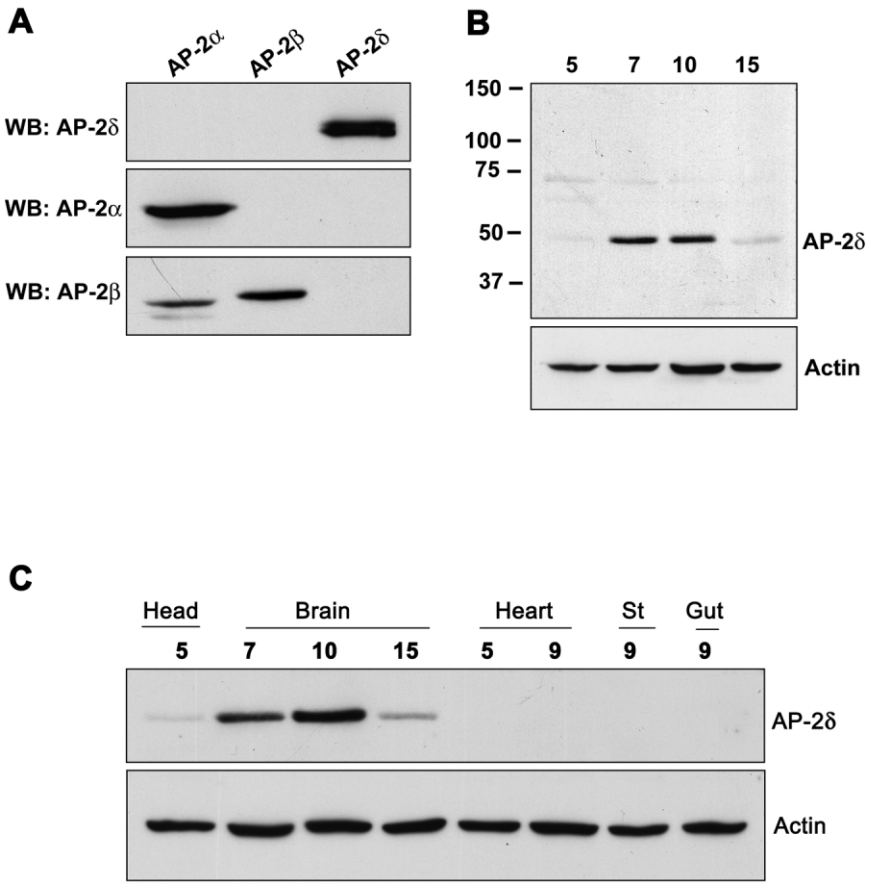

Fig. 6. Western blot analysis of AP-2δ. **A:** HeLa cells were transfected with 6 μg of p3Xflag-AP-2δ, pcDNA3-AP-2α or pcDNA3-AP-2β. The cells were lysed and proteins were separated in a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a nitrocellulose membrane. AP-2δ, AP-2α, and AP-2β proteins were detected by the corresponding antibodies. **B:** Twenty-five micrograms of protein lysates from chick retina at embryonic day (E) 5, E7, E10, and E15 were electrophoresed through a 10% SDS-PAGE gel and AP-2δ detected as described above. **C:** Fifty micrograms of protein lysates from chick head, brain, heart, stomach, and gut at the indicated developmental stage were electrophoresed through a 10% SDS-PAGE gel and immunostained with anti–AP-2δ antibody.

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were indeed ganglion cells, we carried out co-immunofluorescence analysis with AP-2δ, the ganglion-specific transcription factor Brn3a and neuronal class III β-tubulin TUJ1 antibodies. At E5, 85% of retinal cells are proliferating and ganglion cells constitute the great majority of nonproliferating cells (Dutting et al., 1983). Using TUJ1 as a marker of early ganglion cell differentiation, we found that between 4 and 9% of nonmigrating ganglion cells were AP-2δ–positive in the central half of the retina with the more differentiated regions of the retina having a higher percentage of AP-2δ/TUJ1-positive cells (Fig. 8). Migrating TUJ1-positive cells did not express AP-2δ. There were very few AP-2δ–expressing cells in the less-differentiated peripheral half of the retina. At E5, 23% of Brn3a-positive cells were AP-2δ-positive with virtually every AP-2δ–positive cell being Brn3a-positive. At E7, 29% of Brn3a-positive cells were AP-2δ–positive. The percentage of Brn3a-expressing cells that were positive for AP-2δ did not differ significantly in different regions of the retina, although there were fewer Brn3a- and AP-2δ-positive cells in the peripheral retina. By E11, when neurogenesis is almost complete and all three nuclear layers are well-demarcated, the great majority (84%) of cells in the ganglion cell layer were positive for Brn3a. AP-2δ was found in ~34% of Brn3a-positive cells throughout the retina (Fig. 8). A few Brn3a/AP-2δ–positive cells were observed at the inner edge of INL (indicated by arrowhead), likely representing displaced ganglion cells. At E15, only 14% of Brn3a-positive cells were AP-2δ–positive with all AP-2δ–positive cells being Brn3a-positive and 82% of cells in the ganglion cell layer being positive for Brn3a. There was no significant difference in the percentage of Brn3a/AP-2δ coexpressing cells in the peripheral and central retina. These results are summarized in Table 1.

TABLE 1. Results

	AP-2δ/Brn3a (%)	Brn3a/Hoechst (%)	AP-2δ/TUJ1 (%)
E5	23[a]	ND	4-9[a]
E7	29[b]	ND	ND
E11	34[c]	84[c]	ND
E15	14[d]	82[d]	ND

[a]These numbers were obtained by counting all AP-2δ–, Brn3a-, and TUJ1-positive cells in two representative retinal tissue sections.
[b]This number was obtained by counting the number of AP-2δ–positive cells in 230 Brn3a-expressing cells in three different regions of a retinal tissue section.
[c]These numbers were obtained by counting the number of AP-2δ–positive cells in 604 Brn3a-expressing cells and the number of Brn3a-expressing cells in 871 Hoescht-stained cells in five different regions of a retinal tissue section.
[d]These numbers were obtained by counting the number of AP-2δ-positive cells in 207 Brn3a-expressing cells and the number of Brn3a-positive cells in 201 Hoescht-stained cells in three different regions of a retinal tissue section.
Similar numbers were obtained in at least five different sections for each developmental stage analyzed.

Ganglion cells have been shown to undergo programmed cell death during chick retina development, with peak numbers of apoptotic cells in the ganglion cell layer observed at E10/11 (Cook et al., 1998). To address a possible association between AP-2δ expression and apoptosis, we co-stained retinal tissue sections with the TUNEL (deoxynucleotide transferase mediated dUTP-fluorescein nick end labeling) reagent (Roche) and anti–AP-2δ antibody. As shown in Supp. Fig. S4, there was no overlap between AP-2δ–positive cells and TUNEL-positive cells at ED11, suggesting that AP-2δ–expressing cells are not undergoing programmed cell death.

DISCUSSION

We have previously shown that AP-2α and AP-2β are specifically expressed in the amacrine and horizontal cell lineages of the developing chick retina (Bisgrove et al., 1997). Here, we describe a third member of the AP-2 family, AP-2δ, which has an even more restricted distribution pattern in the developing chick retina. Based on Northern and Western blots, AP-2δ levels are low in E5 retina, peak at E7-11, and are considerably reduced by E15. Immunostaining analysis indicates that AP-2δ protein is found in a subset of ganglion cells, suggesting a specialized role in these cells. As generation of ganglion cells is already well underway by E5 and virtually completed by E9 (Prada et al., 1991), we propose a role for AP-2δ in late-born ganglion cells.

Like human *AP-2*δ, chicken *AP-2*δ RNA encodes a protein of 452 amino acids. The structure of the human and chicken genes is similar, both consisting of eight exons. The chicken *AP-2*δ gene spans ~55 kb and is located on chromosome 3. Of interest, *AP-2*δ gene and *AP-2*β gene are closely linked in chicken (separated by 21 kb on chromosome 3) and mammals (human AP-2δ separated by 46 kb at 6p12 and mouse AP-2δ separated by 43 kb at 1A). This suggests that a tandem duplication event at this locus occurred before the divergence of birds and mammals during evolution.

Many cells express more than one type of AP-2. As AP-2 can form homodimers and heterodimers, different combinations of AP-2 family members may regulate different target genes. In this regard, it is noteworthy that AP-2δ is missing several residues believed to be critical for transactivation. Furthermore, AP-2δ exhibits greater DNA sequence preference than AP-2α, requiring an optimized AP-2 consensus sequence for binding (Zhao et al., 2001a). Although AP-2δ has been shown to heterodimerize with AP-2α in vitro (Zhao et al., 2001a), there is no overlap between AP-2δ and AP-2α/AP-2β expression patterns in the developing chick retina (Bisgrove and Godbout, 1999). AP-2δ may thus function as a homodimer or may heterodimerize with AP-2γ and AP-2ε, both of which are expressed in the eye, with *AP-2*γ RNA specifically detected in mouse ganglion cells (Bassett et al., 2007; Unigene, http://www.ncbi.nlm.nih.gov/sites/entrez).

Retinal ganglion cells are projection neurons that can be divided into dif-

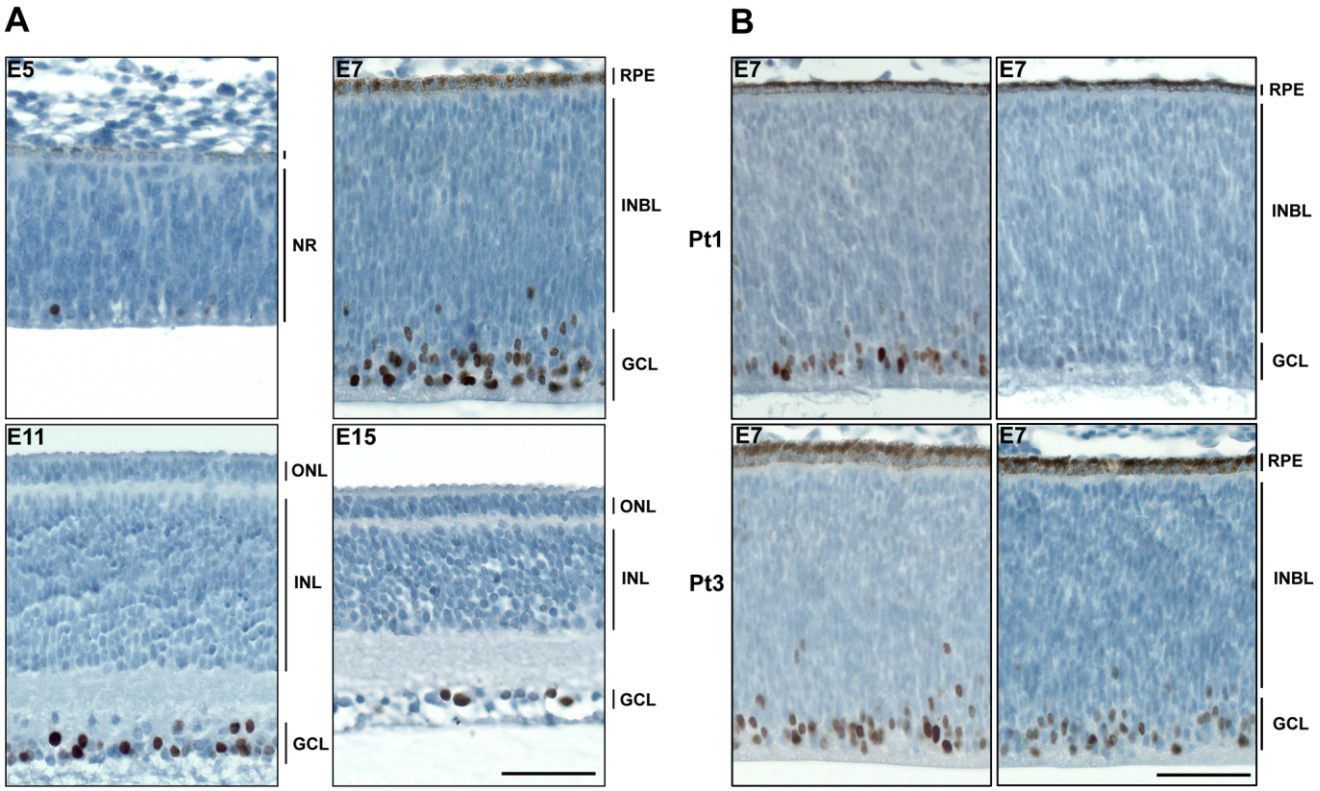

Fig. 7. Immunohistochemical analysis of AP-2δ in the developing chick retina. **A:** Retinal tissue sections from embryonic day (E) 5, E7, E11, and E15 were immunostained with rabbit anti–AP-2δ antibody. **B:** Retinal tissue sections from E7 were immunostained with AP-2δ antibody alone (left panels) or antibody preincubated with 0.1 μM peptide 1 (Pt1) or 1 μM peptide 3 (Pt3). The sections were counterstained with hematoxylin to label the nuclei. Photographs were taken with a 20× lens using a Zeiss Axioskop 2 plus microscope. The images shown are from the central half of the retina. Scale bar = 50 μm.

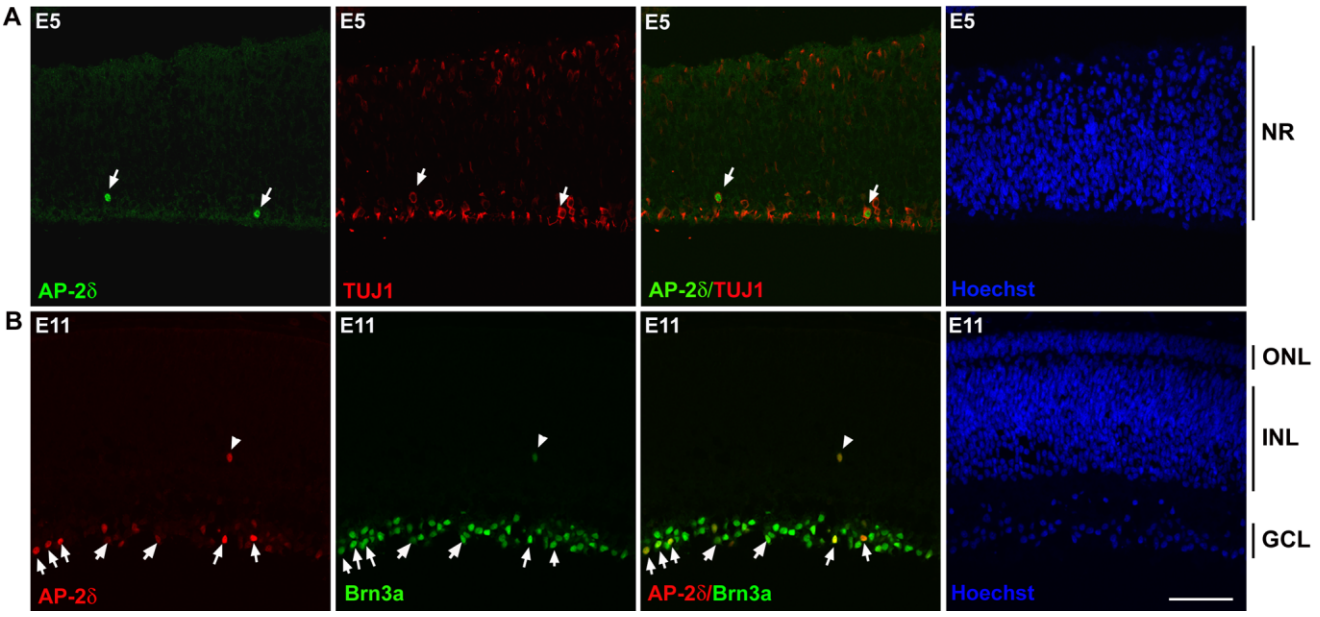

Fig. 8. Immunofluorescence analysis of AP-2δ in the developing chick retina. **A:** Retinal tissue sections from embryonic day (E) 5 were double-stained with anti–AP-2δ and anti-TUJ1 antibodies followed by secondary antibodies conjugated with Alexa 488 and Alexa 555, respectively. **B:** Retinal tissue sections from E11 were double-stained with anti–AP-2δ and anti-Brn3a antibodies followed by secondary antibodies conjugated with Alexa 555 and Alexa 488, respectively. A,B: Sections were counterstained with Hoechst 33342 to label the nuclei. Merged pictures show colocalization of AP-2δ and TUJ1 (A) or Brn3a (B). The arrowhead points to a displaced ganglion cell. The arrows indicate ganglion cells that are positive for AP-2δ and TUJ1 (A) or positive for AP-2δ and Brn3a (B). The images shown are from the central half of the retina. Photographs were taken with a Zeiss LSM510 confocal microscope equipped with a 20× objective. Scale bar = 50 μm.

ferent classes based on their pathfinding decisions, morphology, response properties and molecular differences (De Monasterio and Gouras, 1975; Rodieck and Brening, 1983; Wassle and Boycott, 1991; Xiang et al., 1995). The Brn3 POU4 class of transcription factors is critical for the differentiation and survival of projection neurons (Wang et al., 2002). Brn3b is the first member of the family to be expressed in the ganglion cells of mouse retina, followed by Brn3a and Brn3c (Xiang et al., 1995; Gan et al., 1999). In contrast, Brn3c is expressed in the ganglion cells of chick retina before Brn3a and Brn3b (Liu et al., 2000). Brn3a and Brn3b largely overlap at later stages of development and are expressed in the majority of ganglion cells (Xiang et al., 1995; Gan et al., 1999; Liu et al., 2000; Pan et al., 2005; Quina et al., 2005). Murine Brn3c is found in approximately half of Brn3a/Brn3b-positive cells (Xiang et al., 1995). We have shown that AP-2δ is expressed in a subset (14–34% depending on developmental stage) of Brn3a-positive ganglion cells in the chick retina. Furthermore, at the earliest stage of development tested, E5, AP-2δ was expressed in only 4–9% of nonmigrating ganglion cells and was not detected in migrating ganglion cells. As ganglion neurogenesis is well underway by E5, with 40–50% of ganglion cells being postmitotic (Prada et al., 1991), it is unlikely that AP-2δ plays a role in ganglion cell fate specification. Rather, we propose a role for this transcription factor in the differentiation of a subset of ganglion cells.

Retinal ganglion cells are located in the innermost layer of the retina. These cells send out their axons through the optic nerve to innervate specific targets in the brain. Once they have left the retina, the axons must make complex pathfinding decisions, with a fundamental decision being whether to cross the midline and project contralaterally, or avoid the midline and project ipsilaterally (Williams et al., 2004). Brn3a expression in mouse retinal ganglion cells is associated with a preference for the contralateral hemisphere (Quina et al., 2005). Restriction of AP-2δ to a subset of Brn3a-positive cells suggests a role for this transcription factor in pathfinding decisions. A recent two-hybrid screen to identify AP-2δ interacting proteins resulted in the identification of seven candidates: Fkbp8, Sfrs2, Kifc5a/Kifc1, Fip1l1, Cdh11, Pom121, and Ash2l (Tan et al., 2008). All these proteins are expressed in brain based on Unigene expression profiles, with Kifc5a specifically reported in the eye. Kifc5a is of particular interest in that it is a motor protein implicated in vesicle transport. As such, it may function as a motor for the transport of molecules required for axonal trafficking and/or pathfinding.

In contrast to the immunostaining data, in situ hybridization indicates that *AP-2*δ transcripts are found not only in the ganglion cell layer, but also in cells distributed in the outer half of the inner nuclear layer. The absence of an AP-2δ protein signal in the inner nuclear layer indicates that either the AP-2δ protein is not expressed in this retinal layer or the antibody does not recognize the form of AP-2δ expressed in this retinal layer. Similar to embryonic and newborn mouse brain (Zhao et al., 2001a), there are two *AP-2*δ transcripts in chick retina and brain based on Northern blotting. It is therefore possible that different forms of the transcript are expressed in the ganglion and inner nuclear layers. However, as the same signal was observed using two nonoverlapping in situ hybridization probes (spanning 1–549 bp and 565–1,063 bp, respectively), and the anti–AP-2δ antibody was generated using the N-terminal half of the AP-2δ protein (aa 1–199), it seems unlikely that the antibody would not recognize AP-2δ expressed in the inner nuclear layer unless the N-terminus of this protein is significantly altered by posttranslational modification or conformational changes. Another possibility is that the transcript expressed in the inner nuclear layer is not translated into protein. There are many ways of regulating translation; for example, through signals in the untranslated regions, secondary or tertiary RNA structures. The *AP-2*δ transcript expressed in the inner nuclear layer may not be translated into protein or may be translated into protein under very specific circumstances. Regardless of whether AP-2δ protein is modified or simply not expressed in the inner nuclear layer, the presence of this transcript in only a small subset of cells in the outer half of the inner nuclear layer suggests tight regulation at the transcript level.

In summary, we show that AP-2δ has a highly specific temporal distribution pattern in the chick retina, peaking at day 7 to 11 of embryonic development. AP-2δ protein is restricted to a subset of Brn3a-positive late-born ganglion cells, whereas *AP-2*δ RNA is found in ganglion cells as well as a small subset of cells in the outer half of the inner nuclear layer. The AP-2δ expression pattern in the ganglion cell layer suggests a role in the transcriptional regulation of proteins involved in the differentiation of a specific subset of ganglion cells. Overexpression and/or underexpression of AP-2δ in retina will uncover the functional role(s) of this transcription factor in the developing retina.

EXPERIMENTAL PROCEDURES

Isolation of *AP-2*δ cDNA

To obtain *AP-2*δ cDNA, poly(A)+ RNA was isolated from E10 retinal tissue and primers to the predicted sequence of chick *AP-2*δ (based on an EST cDNA sequence) were used to amplify the C-terminus of *AP-2*δ. The 5′ end of *AP-2*δ cDNA was extended using the FirstChoice RLM-RACE kit (Ambion). The nested antisense primers used for rapid amplification of 5′ *AP-2*δ cDNA ends were: 5′-CGTCTCTTCACCTCTGCAAT-3′ (inner primer) and 5′-AGAGGGAAGCATTGA GGCAT-3′ (outer primer), corresponding to the 5′ end (positions 222 and 257) of the chicken EST cDNA sequence. PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced. The GenBank accession number for chicken *AP-2*δ cDNA is EU740390.

RT-PCR and Northern Blot Analysis

One microgram of poly(A)$^+$ RNA from chick retina, brain, lung, heart, liver, spleen, kidney, and gut at E7, E10, and E16 was reverse transcribed using oligod(T) primer and Superscript reverse transcriptase (Invitrogen). Single-strand cDNAs were PCR-am-

plified using the following primers: 5′-GAATTCCAAGACCTTCTGA G-3′ (top strand) and 5′-GTTTTTGTGCGATGTGTGCT-3′ (bottom strand). A 200-bp PCR product (from 1,047 to 1,251 bp based on *AP-2*δ cDNA) was generated using these primers.

For Northern blotting, poly(A)$^+$ RNAs isolated from retina, brain, lung, heart, liver, kidney at E7, E10, and E16 were electrophoresed in a 6% formaldehyde-1.5% agarose gel in MOPS buffer and transferred to nitrocellulose. The filter was hybridized to a ^{32}P-labeled cDNA probe corresponding to the entire coding region of AP-2δ and washed under high stringency conditions. To ensure that the same amount of RNA was loaded into each lane, the filter was stripped and re-hybridized with ^{32}P-labeled actin cDNA.

In Situ Hybridization

Two nonoverlapping *AP-2*δ probes were generated for in situ hybridization, the first encompassing the N-terminus of chicken *AP-2*δ (1–549 bp), the other encompassing the central region (565–1,063 bp). The primers used to generate the N-terminus probe were 5′-ATGTCAACTACCTTTCCAGG-3′ (top strand) and 5′-CTCCACTGAGCTCTGCAAAT-3′ (bottom strand). The primers for the central probe were 5′-GTACTGAACGGACAAGGTGG-3′ (top) and 5′-CTTGACTCAGAAGGTCTTGG-3′ (bottom). The probes used to detect *AP-2*β and carbonic anhydrase II (*CA-II*) have been described previously (Bisgrove and Godbout, 1999; Witte and Godbout, 2002). *Chx10* and *Chx10-1* probes were generated by PCR amplification of E10 chick retina cDNA using 5′-GGCCCTGGTTTCTTCACGTG-3′ (top) and 5′-TACAGCTCCATATCTCAAACAC-3′ (bottom) for *Chx10* (1,241–2,186 bp) and 5′-AAAGATCCCGAAGACACAGC-3′ (top) and 5′-CGGGTGTGTAATAAATAAATGG-3′ (bottom) for *Chx10-1* (971–1,996 bp), respectively. PCR products were cloned into pBluescript vector and verified by sequencing.

Antisense riboprobes labeled with digoxigenin (DIG) were synthesized by in vitro transcription with T3 or T7 RNA polymerase (Roche) according to the manufacturer's directions. Tissues were fixed in 4% paraformaldehyde, cryoprotected in sucrose and embedded in OCT (Tissue-Tek) along the dorsal-ventral axis. Tissue sections (6–7 μm) were prehybridized at 55°C in 40% formamide, 10% dextran sulfate, 1× Denhardt's solution, 4× standard saline citrate (SSC), 10 mM dithiothreitol (DTT), 0.5 mg/ml yeast tRNA, and 0.5 mg/ml heat denatured herring testis sperm DNA. Riboprobes were hybridized to tissue sections overnight at 55°C. Tissue sections were washed and incubated with alkaline-phosphatase–conjugated anti-DIG antibody. The signal was detected with BCIP/NBT (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate). Pictures were taken using Zeiss Axioskop 2 plus microscope with a 20× objective.

Purification of GST Fusion Protein and Antibody Production

pGEX-4T2 encoding the N-terminus of chicken AP-2δ (1–598 bp) was transformed into BL21 bacteria. Expression of the fusion protein was induced with 1 mM IPTG for 3 hr at 30°C. Cells were lysed in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF) using a French Press. Cleared lysates were incubated with glutathione-Sepharose beads (Amersham Pharmacia Biosciences) and GST fusion protein was eluted in 10 mM glutathione (Sigma). Anti–AP-2δ antiserum was obtained by immunizing rabbits with recombinant GST-AP-2δ protein. Anti–AP-2δ antiserum was preabsorbed over an induced GST *E. coli* lysate column. Preabsorbed serum was then passed over Affi-gel 15 (Bio-Rad) conjugated to an AP-2δ–specific peptide (peptide 1, LVHDAEIRHDGSNSY) (GenScript Corporation).

Cell Transfections and Western Blot Analysis

The entire coding region of AP-2δ cDNA was generated by PCR amplification of E10 chick retina cDNA using primers flanking the start codon (5′-CTAGGCGGCCGCTATGTCAACTACCTTTCCAGG-3′) and stop codon (5′-CTAGAGATCTCTAGTCTGTCTTTTCTGTTTTG-3′) (1–1,359 bp). The PCR product was inserted into P3Xflag and verified by sequencing.

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics at 5% CO_2. Transfections were carried out with FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer's protocol. Cells were lysed in RIPA buffer and proteins separated in a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose membrane and AP-2α, AP-2β, and AP-2δ detected using anti–AP-2α (1:500 dilution; 3B5; DSHB), anti–AP-2β (1:500 dilution) (H87; Santa Cruz), and anti–AP-2δ antibody (1:1,000 dilution; described above).

For analysis of AP-2δ in chick retina, retinal tissues at E5, E7, E10, and E15 were homogenized in RIPA buffer. Proteins were separated in a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane and immunostained with rabbit anti–AP-2δ antibody (1:500 dilution) followed by goat anti-actin antibody (1:500 dilution).

Immunohistochemistry and Immunofluorescence Analysis of Tissue Sections

Chick retina tissues at E5, E7, E11, and E16 were fixed in formalin and embedded in paraffin. Tissues were deparaffinized in xylene, re-hydrated and microwaved in a pressure cooker for 20 min in 10 mM citrate/0.05% Tween-20 pH 6 for antigen retrieval. For immunohistochemistry, sections were stained with rabbit anti–AP-2δ antibody (1:2,000). The signal was detected using the DakoCytomation EnVision+ secondary system. Tissues were counterstained with the nuclear stain hematoxylin. Images were captured with a Zeiss Axioskop 2 plus microscope and 20× objective. For immunofluorescence analysis, frozen tissue sections were double-stained with rabbit anti–AP-2δ antibody and mouse anti-Brn3a antibody (1:400, Chemicon), followed by secondary antibodies conjugated with Alexa 555 or Alexa 488. Sections were counterstained with the nuclear fluorescent dye Hoechst 33342 (1:1,500). Apoptotic cells were stained using the In Situ

Cell Death Detection kit, fluorescein, following the manufacturer's directions (Roche). Images were captured by using a Zeiss LSM510 confocal microscope.

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