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Differential Expression of AP-2α and AP-2β in the Developing Chick Retina: Repression of *R-FABP* Promoter Activity by AP-2

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ABSTRACT **Retinal fatty acid binding protein (R-FABP) is the avian counterpart of murine brain FABP implicated in glial cell differentiation and neuronal cell migration. R-FABP is highly expressed in the undifferentiated retina and brain of chick embryos. We have previously shown by in vitro studies that the AP-2 transcription factor binds to a consensus AP-2 binding site in the *R-FABP* promoter region. Based on the expression pattern of AP-2 in the developing retina and on mutational analysis of the AP-2 binding site in DNA transfection experiments, we proposed that AP-2 could be involved in the down-regulation of *R-FABP* transcription. Here, we describe the cDNA isolation of two members of the AP-2 family expressed in the chick retina, AP-2α and AP-2β. We show that *R-FABP* mRNA and the AP-2 factors are expressed in mutually exclusive patterns in the differentiating retina: whereas AP-2α and AP-2β are selectively expressed either in amacrine, or in amacrine and horizontal cells, respectively, *R-FABP* mRNA is found in Müller glial cells and/or bipolar cells. Furthermore, a decrease in *R-FABP*-dependent expression is obtained upon cotransfection of primary retinal cultures with AP-2 expression vectors and a CAT reporter construct. The early and cell-specific expression of AP-2α and AP-2β in the developing retina suggest a role for this transcription factor family in the early steps of amacrine and horizontal cell differentiation. Repression of the *R-FABP* gene in these cells may be an important component of their developmental program. *Dev Dyn 1999;214:195–206.*** **© 1999 Wiley-Liss, Inc.**

Key words: retinal development; retinal fatty acid binding protein; AP-2; amacrine cells; DNA transfection

INTRODUCTION

To better understand the molecular mechanisms of retinal development, we are studying genes whose expression patterns suggest a role in cell specification and differentiation. Chick retinal development begins shortly after formation of the optic vesicle on embryonic day (ED) 2 and is complete by the time of hatching on ED21. The mature retina contains six different neuronal cell types (photoreceptor, amacrine, horizontal, bipolar, ganglion, and interplexiform) and a single type of glial cell, the Müller glial cell. These cells arise from multipotent progenitor cells that are predominantly localized to a region of active mitosis called the ventricular zone, located next to the retinal pigment epithelium (Fekete et al., 1994). Post-mitotic cells migrate from the ventricular zone to their appropriate strata, eventually resulting in the characteristic lamellar organization seen in the mature retina.

The chicken retinal fatty acid binding protein (R-FABP) gene may play a role in retinal development. Brain FABP, the mammalian orthologue of R-FABP, has been implicated in murine brain development, specifically in the establishment and maintenance of the radial glial fiber system guiding neuronal migration (Feng et al., 1994; Kurtz et al., 1994). R-FABP is highly expressed in the primitive neuroectodermal cells of the undifferentiated chick retina from ED3 to ED7, as well as in the developing brain. There is a 50-fold decrease in transcript levels from ED7 to ED19 in the retina (Godbout, 1993; Godbout et al., 1995). R-FABP, expressed during a period of active neuronal migration in the retina, may play an analogous role to that of B-FABP in the brain.

In a previous study of the regulation of the chicken *R-FABP* gene, we found multiple sites of protein–DNA interaction within 205 bp of 5′ flanking DNA (Bisgrove et al., 1997). One of these, located 59 bp upstream of the *R-FABP* transcription initiation site, corresponded to the consensus AP-2 DNA binding site, GCCNNNGGC. Supershift experiments using antibodies to AP-2, and methylation interference experiments, indicated that an AP-2-like transcription factor binds to this region. Surprisingly, mutation of the AP-2 consensus site did not result in a decrease in *R-FABP* transcription activity upon DNA transfection of ED5 retinal cultures, suggesting that AP-2 might function as a repressor rather than an activator of *R-FABP* transcription. Further support for this hypothesis came from Western blots showing an increase in AP-2 levels from ED7 to ED16 in the retina, in contrast to *R-FABP* mRNA

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levels, which undergo a dramatic decrease during this time.

Three genes encoding members of the AP-2 family have been isolated in mammals: *AP-2*α, *AP-2*β, and *AP-2*γ. The spatial and temporal expression profiles of the AP-2 factors during embryogenesis suggest that they are important for normal development. Severe malformations of the face and skull, as well as abnormalities of the sensory organs, result from inactivation of the *AP-2*α gene in mouse (Schorle et al., 1996; Zhang et al., 1996). Eye development in the *AP-2*α-null embryos is severely perturbed, with morphological defects apparent in the optic cups as early as E10.5 (P. Mitchell, personal communication). *AP-2*β gene knock-out mice die one to two days postnatally because of polycystic kidney disease (Moser et al., 1997a). No ocular defects were reported in this paper. Recently, Drosophila AP-2 has been shown to be expressed in distinct regions of the embryonic head including the visual system, suggesting a role in the development and differentiation of the central nervous system (Monge and Mitchell, 1998).

The expression pattern of chicken AP-2 in early embryos from stage 5 (ED1) to stage 28 (ED5.5) suggests a role in the budding out of facial prominences and limb buds (Shen et al., 1997). To define the role of the AP-2 transcription factors in retinal development, we have isolated and sequenced the *AP-2* cDNAs present in an ED16 chick retina library. Here, we examine the cellular distribution of AP-2α and AP-2β in the developing chick retina by in situ hybridization and immunofluorescence analyses and show that *R-FABP* mRNA is excluded from retinal cells that express AP-2. We also show that overexpression of AP-2 downregulates *R-FABP* promoter activity.

RESULTS

Isolation of AP-2α and AP-2β cDNAs From Chick Retina

To identify the *AP-2* genes expressed in the chick retina, we screened an ED16 retina cDNA library with human EST probes specific for *AP-2*α and *AP-2*β, as well as with the previously cloned mouse *AP-2*γ cDNA. Three *AP-2*α cDNA clones, four *AP-2*β clones, but no *AP-2*γ clones, were obtained upon screening 2×10^5 bacteriophage. None of the *AP-2*α cDNAs were full-length, based on sequence analysis. The sequence of the coding region was identical to that of the previously described chicken *AP-2*α (Shen et al., 1997), although there were a few alterations in the 3′ untranslated region. None of the *AP-2*α clones represented *AP-2*α*B*, a dominant negative alternative splice form of *AP-2*α described in mouse (Buettner et al., 1993).

Both strands from one of the *AP-2*β clones containing the entire open reading frame were completely sequenced. The portion of chicken *AP-2*β cDNA encoding the open reading frame is 85% and 83% identical to that of the human and mouse *AP-2*β cDNAs, respectively. Fig. 1 shows an alignment of the predicted amino acid sequence of chicken AP-2β with that of mouse and human AP-2β. Chicken and human AP-2β are 98% identical at the amino acid level (440/449 amino acids). Eight of the nine amino acid substitutions are conservative. Six of the substitutions are located in the N-terminal half of the protein, which contains the transactivation domain. The DNA binding/dimerization domain contains three amino acid differences, at positions 373, 425, and 427. A similar level of identity is observed with mouse AP-2β, except that the murine sequence has one less codon than either human or chicken AP-2β (codon 425).

The predicted molecular mass of chicken AP-2β is 49 kDa, similar to that of the 437 amino acid chicken AP-2α, predicted to be 48 kDa. The predicted amino acid sequences of chicken AP-2α and AP-2β are 71% identical (83% similar) over their entire length (Fig. 2). The region of highest sequence conservation lies within the C-terminal half (DNA binding/dimerization domain) with 84% identical residues (94% similar). There are also long stretches of conserved residues within the proline-rich N-terminal transactivation domain, particularly between residues 40–100, where AP-2α and AP-2β are 83% identical (87% similar). This region was shown to be essential for the transactivation function of human AP-2α (Williams and Tjian, 1991).

AP-2α and AP-2β mRNA and Protein in the Developing Embryo

*AP-2*α and *AP-2*β transcript levels in chick tissues at different developmental stages were analyzed by Northern blotting (Fig. 3A). Probes specific to either *AP-2*α or *AP-2*β transcripts were used for this analysis. Three main forms of the *AP-2*α transcript were observed in the retina, ranging in size from 2.5 to 4 kilobases (kb). Four *AP-2*β transcripts of different sizes were present in the retina (1.8, 2.2, 3.5, >5 kb). Levels of both *AP-2*α and *AP-2*β mRNA peaked at ED10 in the retina. Transcripts were barely detectable at ED5.5, the earliest stage tested. Although at lower levels than in the retina, the *AP-2*α and *AP-2*β transcript patterns in the ED5.5 to ED10 brain were similar to those observed in the retina, with a significant decrease in levels at ED16. *AP-2*β mRNA was relatively abundant in the developing kidney, with two to three times higher levels at ED16 compared to ED7. Of note, only the 2.2 kb form of *AP-2*β was expressed in kidney, suggesting that the three other forms of *AP-2*β mRNA found in brain and retina are neural tissue-specific. *AP-2*β mRNA was not detected in either heart or liver, while low levels of *AP-2*α mRNA were observed in heart and liver on overexposed films, decreasing from ED5/6 to ED16.

Next, we analyzed AP-2 protein levels in the ED7 to ED16 retina by Western blotting of whole cell lysates. The polyclonal AP-2 antibody used for this analysis recognizes both AP-2α and AP-2β, based on its ability to immunoreact with either recombinant (our unpublished results) or in vitro translated AP-2α and AP-2β (Moser et al., 1997a, Fig. 3) . Low levels of AP-2 protein were observed at ED7, followed by a rapid increase at

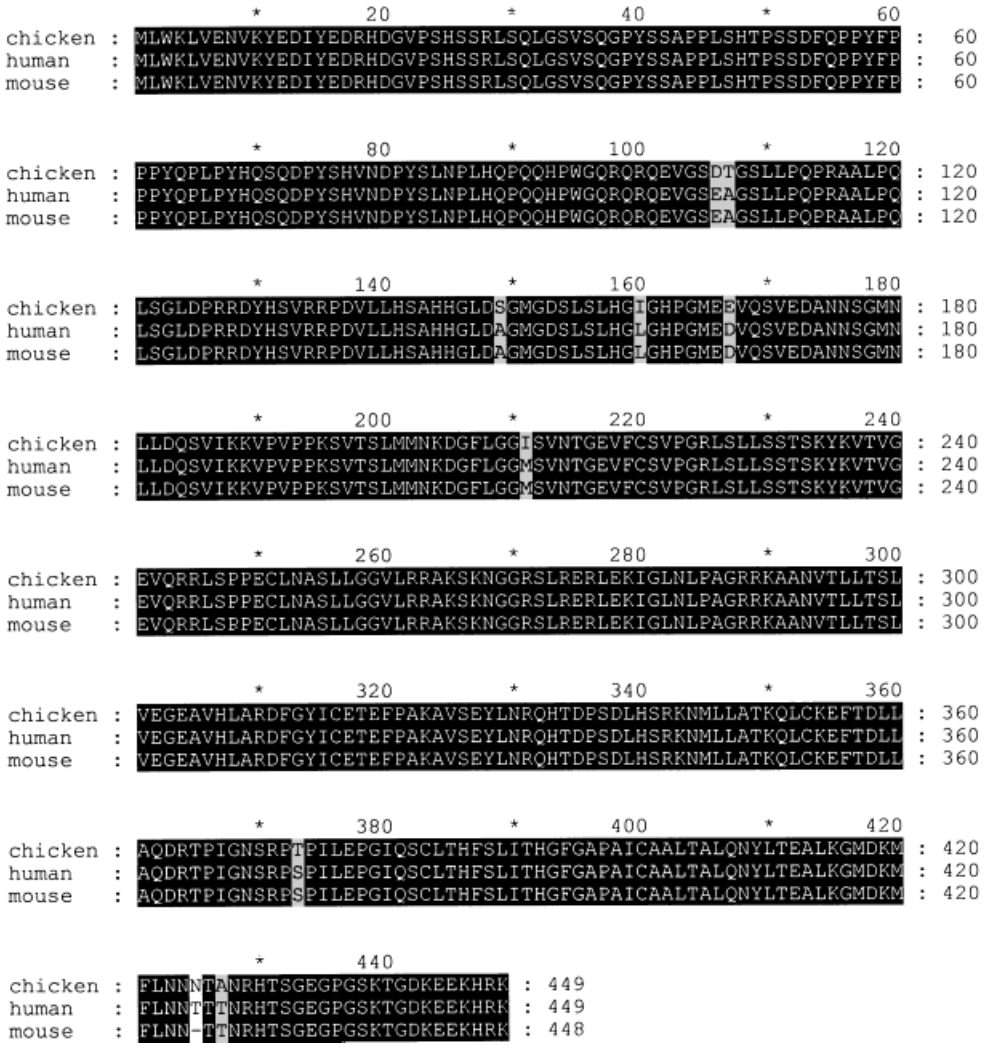

Fig. 1. Comparison of the chicken, human, and mouse AP-2β amino acid sequences. Identical residues are indicated in the black boxes and conserved residues in the shaded boxes. A dash at position 425 of the murine sequence was inserted in order to preserve sequence alignment. The following residues were considered similar: G, A, S, T; E, D, Q, N; R, K, H; V, M, L, I; F, Y, W.

ED8 and ED9, to reach maximal levels at ED9/ED10 (Fig. 3B). The reduction in AP-2 levels at ED16 reflects the decrease in *AP-2* transcript levels between ED10 and ED16.

Inhibition of *R-FABP/CAT* Reporter Gene Expression by AP-2

In a previous study, we found that an AP-2-immunoreactive transcription factor was binding to a consensus AP-2 recognition site within the promoter of the *R-FABP* gene (Bisgrove et al., 1997). Transfection of pCAT reporter constructs with mutagenized AP-2 binding sites into undifferentiated ED5 retinal cultures (i.e. AP-2-negative) did not produce a significant decrease in CAT activity, suggesting that AP-2 down-regulates rather than up-regulates *R-FABP* expression. A repressor role for AP-2 was corroborated by Northern and Western blot analyses, indicating that increased expression of AP-2 correlated with decreased levels of *R-FABP* mRNA (Godbout, 1993; Bisgrove et al., 1997). To directly test the effect of AP-2 on *R-FABP* promoter activity, we co-transfected AP-2 expression constructs with an *R-FABP/CAT* reporter construct containing 135 bp of *R-FABP* 5′ flanking DNA including the consensus AP-2 recognition site (Fig. 4A). Four AP-2 expression constructs were prepared, with the coding regions of AP-2α or AP-2β in the sense or anti-sense orientation. Co-transfection of either AP-2α or AP-2β in the sense orientation decreased CAT activity by 50% compared to the anti-sense expression vectors (Fig. 4B) (AP-2α, $P=0.01$; AP-2β, $P=0.0002$). No decrease in CAT activity was observed upon co-transfection of either pCAT-basic vector or a construct containing a mutagenized AP-2 recognition site (data not shown). Overexpression of the AP-2 expression vectors was verified by Western blot analysis of transfected cell lysates (Fig.

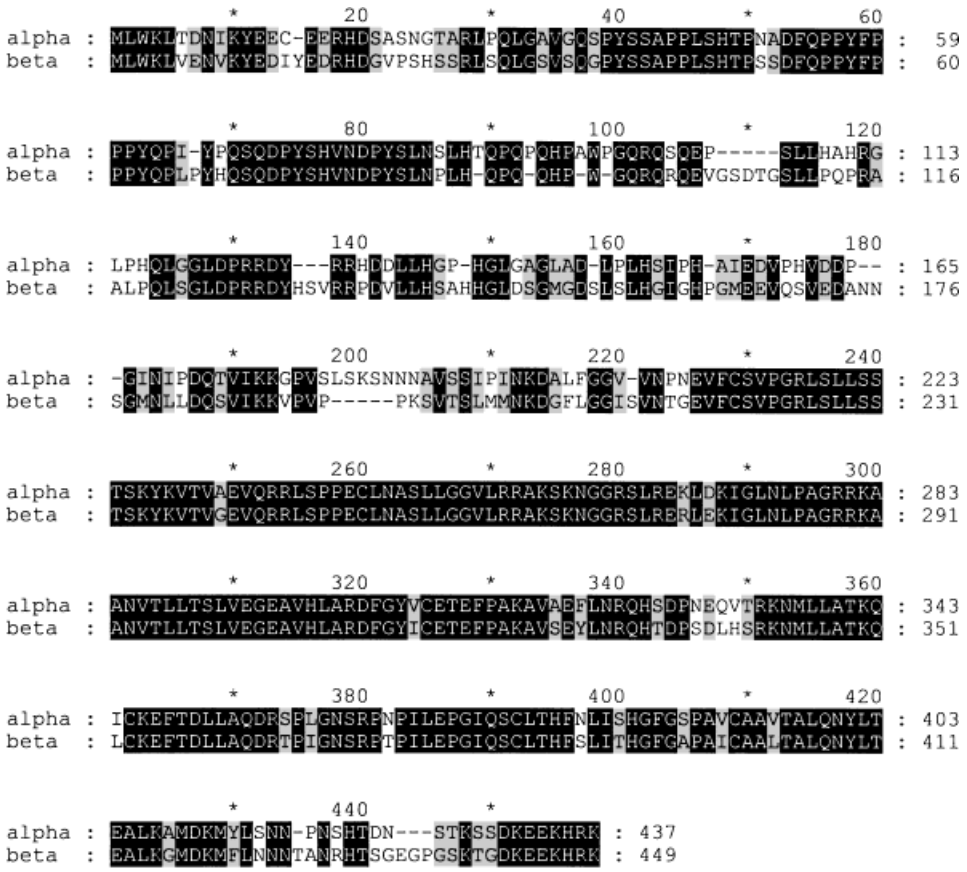

Fig. 2. Comparison of chicken AP-2α and AP-2β amino acid sequences. The predicted amino acid sequences of chicken AP-2α and AP-2β were aligned, inserting dashes to maximize alignment. Black boxes indicate identical residues while shaded boxes indicate conserved residues. Similar residues are defined in Fig.1.

4C). Consistency in the amount of transfected DNA from experiment to experiment was monitored by Southern blot analysis of Hirt DNA (Fig. 4D). These results indicate that AP-2α and AP-2β are capable of repressing *R-FABP* promoter activity in vivo.

Localization of *AP-2α*, *AP-2β*, and *R-FABP* Transcripts in the Retina

If AP-2 can down-regulate *R-FABP* transcription, then AP-2 positive cells should have reduced levels of *R-FABP* mRNA. We therefore studied the distribution of *AP-2α*, *AP-2β*, and *R-FABP* mRNA in the developing retina by in situ hybridization. No *AP-2* transcripts were detected in the ED3.5 (stage 21) retina, in contrast to *R-FABP* transcripts, which were abundantly expressed throughout the tissue (data not shown). By ED5, *AP-2α*- and *AP-2β*-positive cells (purple) were scattered throughout the inner neuroblastic layer (INBL) of the posterior retina (Fig. 5a,c), while *R-FABP* mRNA remained abundant throughout the retina (Fig. 5b). At ED7, the inner plexiform layer is beginning to form in the posterior retina and is not yet present in the anterior retina. The number of *AP-2α*- and *AP-2β*-positive cells was higher in the ED7 retina, with the zone of positive cells extending further towards the anterior retina (Fig. 5d,f). Positive cells were primarily confined to the inner nuclear layer (INL). The retina differentiates from the posterior to the anterior region and from the inner to the outer layer. AP-2-positive cells in both ED5 and ED7 are therefore located in the most differentiated aspect of the retina. In comparison, *R-FABP* transcripts were widespread throughout the INL as well as in the ventricular (proliferative) zone (VZ) of ED7 retina (Fig. 5e). By ED11, the inner and outer plexiform layers have formed, generating the multilayered appearance of the mature tissue consisting of three nuclear layers separated by plexiform layers. The *AP-2α* transcripts were specifically found in the vitreal (inner) half of the INL, where amacrine cell bodies are located (Fig. 5g). *AP-2β* transcripts were found in amacrine cells, as well as in a thin layer of cells in the INL along the outer plexiform layer, identified as horizontal cells based on their characteristic position within the retina (Fig. 5i). *AP-2β* transcripts were also detected in a subset of cells in the ganglion cell layer, likely displaced amacrine cells because of their location

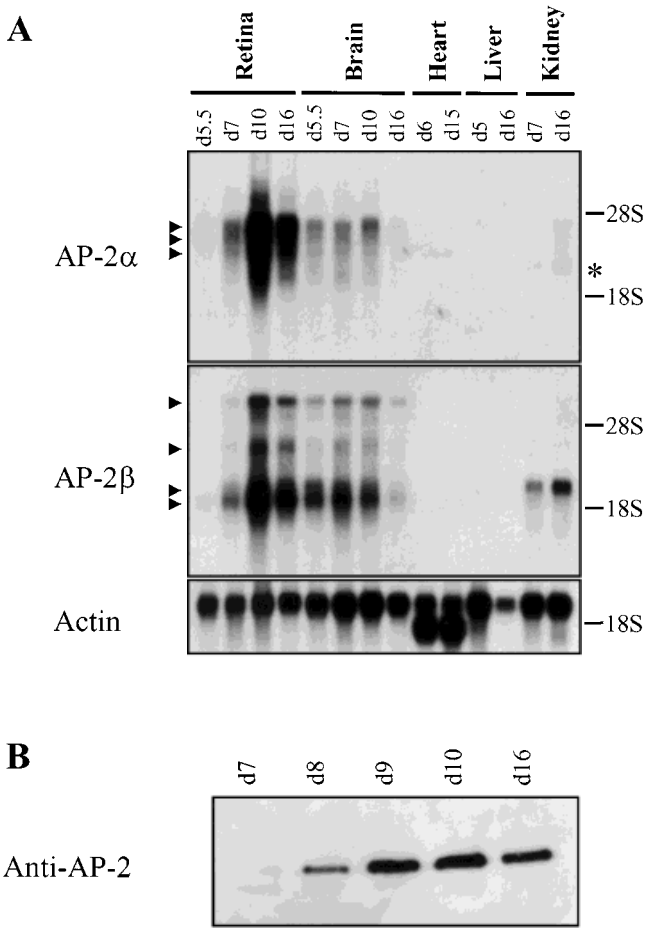

Fig. 3. Northern and Western blot analyses of AP-2α and AP-2β expression. **A:** Northern blots were prepared from poly(A)$^+$ RNA extracted from retina (ED5.5, 7, 10, 16), brain (ED5.5, 7, 10, 16), heart (ED6, 15), liver (ED5, 16), and kidney (ED7, 16). The filter was sequentially hybridized with ^{32}P-labelled: (i) 400 bp *AP-2*β cDNA, (ii) 400 bp *AP-2*α cDNA, and (iii) actin DNA. The filter was stripped after each hybridization. The 28S and 18S rRNA markers are indicated on the side. The *AP-2*α and *AP-2*β transcripts are indicated by the arrowheads. The asterisk indicates residual signal from the AP-2β hybridization. The extra bands obtained with the actin probe represent tissue-specific actin mRNAs. **B:** Western blots were prepared from ED7, ED8, ED9, ED10, and ED16 total chick retina extracts (100 μg protein/lane). The proteins were electrophoretically separated by SDS-PAGE and transferred to a nitrocellulose filter. The filter was incubated with polyclonal anti-human AP-2 antibody at a 1:1000 dilution. The molecular mass of AP-2 is ~50 kDa.

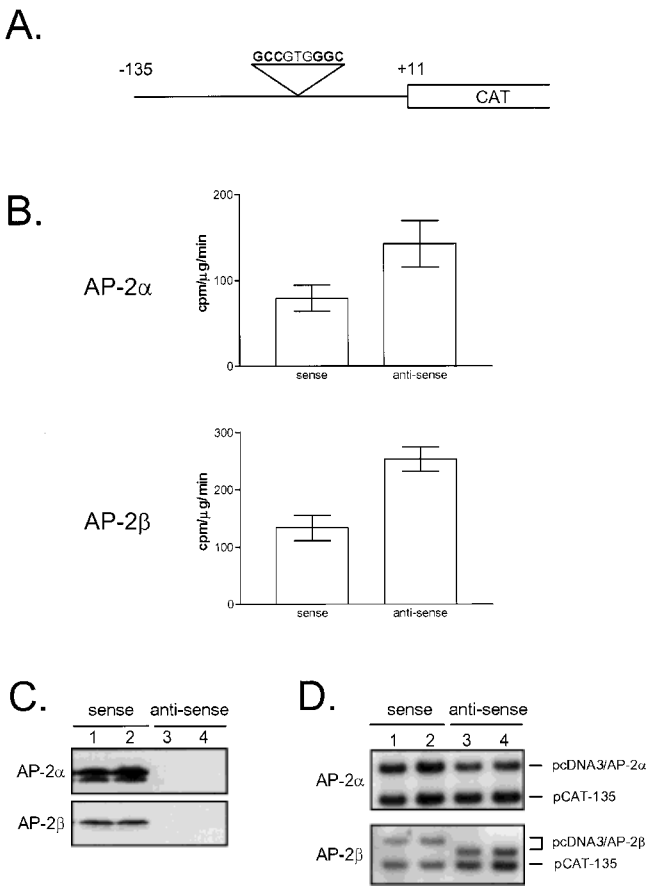

Fig. 4. Overexpression of AP-2α and AP-2β represses *R-FABP* promoter activity. **A:**Map of the pCAT-135 plasmid containing the *R-FABP* promoter region from −135 to +11 bp linked to the CAT reporter gene. The AP-2 consensus binding site (indicated in bold-face type) is located 59 bp upstream of the transcription initiation site. **B:** Co-transfection of ED5 primary retinal cultures with AP-2α and AP-2β expression constructs and pCAT-135. Level of CAT activity obtained upon co-transfection of sense and anti-sense pcDNA3/AP-2α and pcDNA3/AP-2β expression constructs with pCAT-135 was measured as cpm/μg cell extract/min. The results shown represent the average of four experiments. The standard deviations are indicated by the error bars. **C:** Western blot analysis of AP-2α and AP-2β levels in co-transfected retinal cultures. Retinal culture lysates were electrophoresed in a 10% SDS-PAGE gel and blotted onto a nitrocellulose filter. AP-2α and AP-2β were detected using anti-AP-2 antibody. The results from two separate experiments are shown, with the AP-2 constructs in both sense (lanes 1 and 2) and anti-sense (lanes 3 and 4) orientations. **D:** Southern analysis of *Bam*HI-digested Hirt DNA from co-transfected retinal cultures. The blot was probed with pCAT-Basic, which hybridizes to both the pCAT-135 construct and to the expression constructs. The results from two experiments are shown with AP-2α and AP-2β in the sense (lanes 1 and 2) and anti-sense (lanes 3 and 4) orientations.

immediately next to the inner plexiform layer, if not slightly within it, as previously described (Génis-Gálvez et al., 1977). In contrast to the *AP-2* transcripts, *R-FABP* mRNA in the ED11 retina is confined to the outer half of the INL (Fig. 5h). The nuclei of Müller glial cells are found predominantly in the middle of the INL, while bipolar cell bodies are in the outer half of the INL. The dual in situ hybridization experiments shown in Fig. 5j and 5k demonstrate the mutually exclusive distribution of *R-FABP* (brown) mRNA and either *AP-2*α (Fig. 5j) or *AP-2*β (Fig. 5k) (purple) mRNA in the ED11 retina, dividing the INL into an inner AP-2(+)/R-FABP(−) region and an outer AP-2(−)/R-FABP(+) region.

In the mouse brain, radial glial cells express B-FABP during granule cell migration (Feng et al., 1994; Kurtz et al., 1994). Müller glial cells represent a type of radial glial cell specific to the retina and may serve a role similar to that of brain radial glial cells (Willbold and Layer, 1998). Carbonic anhydrase II (CA-II) has been

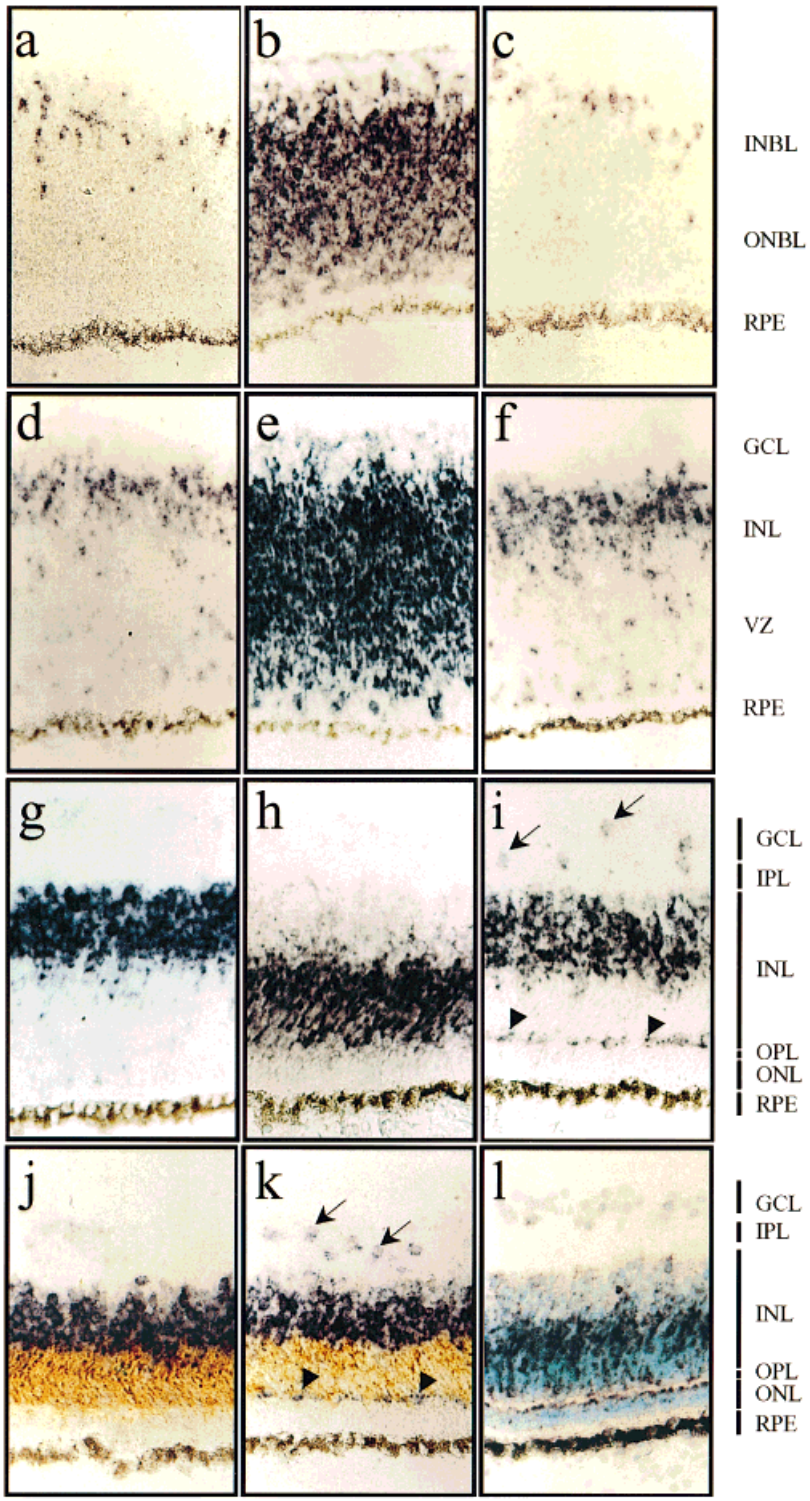

Fig. 5. Localization of *AP-2α*, *AP-2β* and *R-FABP* transcripts in the developing chick retina. Non-radioactive in situ hybridization was performed to determine which cells in the developing retina express *AP-2α*, *AP-2β*, and *R-FABP* mRNA. Adjacent sections of ED5 (**a–c**), ED7 (**d–f**), ED11 (**g–l**) retina were hybridized with DIG- or fluorescein-labelled AP-2α (**a,d,g**), AP-2β (**c,f,i**), R-FABP (**b,e,h**) and CA-II (**l**) antisense RNA. The signal was detected by alkaline-phosphatase-coupled secondary antibody, generating a purple or brown color. Panel l was counterstained with ethyl green to show the cell layers. Panels **j** and **k** represent sections dually hybridized with either AP-2α (purple) (**j**) or AP-2β (purple) (**k**), and R-FABP (brown) antisense RNA. Displaced amacrine cells are indicated by arrows and the horizontal cell layer by arrowheads (**i,k**). The sections are oriented with the retinal pigment epithelium at the bottom and ganglion cells on top. GCL: ganglion cell layer; INBL: inner neuroblastic layer; INL: inner nuclear layer; IPL: inner plexiform layer; ONBL: outer neuroblastic layer; ONL: outer nuclear layer; OPL: outer plexiform layer; RPE: retinal pigment epithelium; VZ: ventricular zone. Photomicrographs were taken at 200× magnification using a Nikon Diaphot 300 microscope.

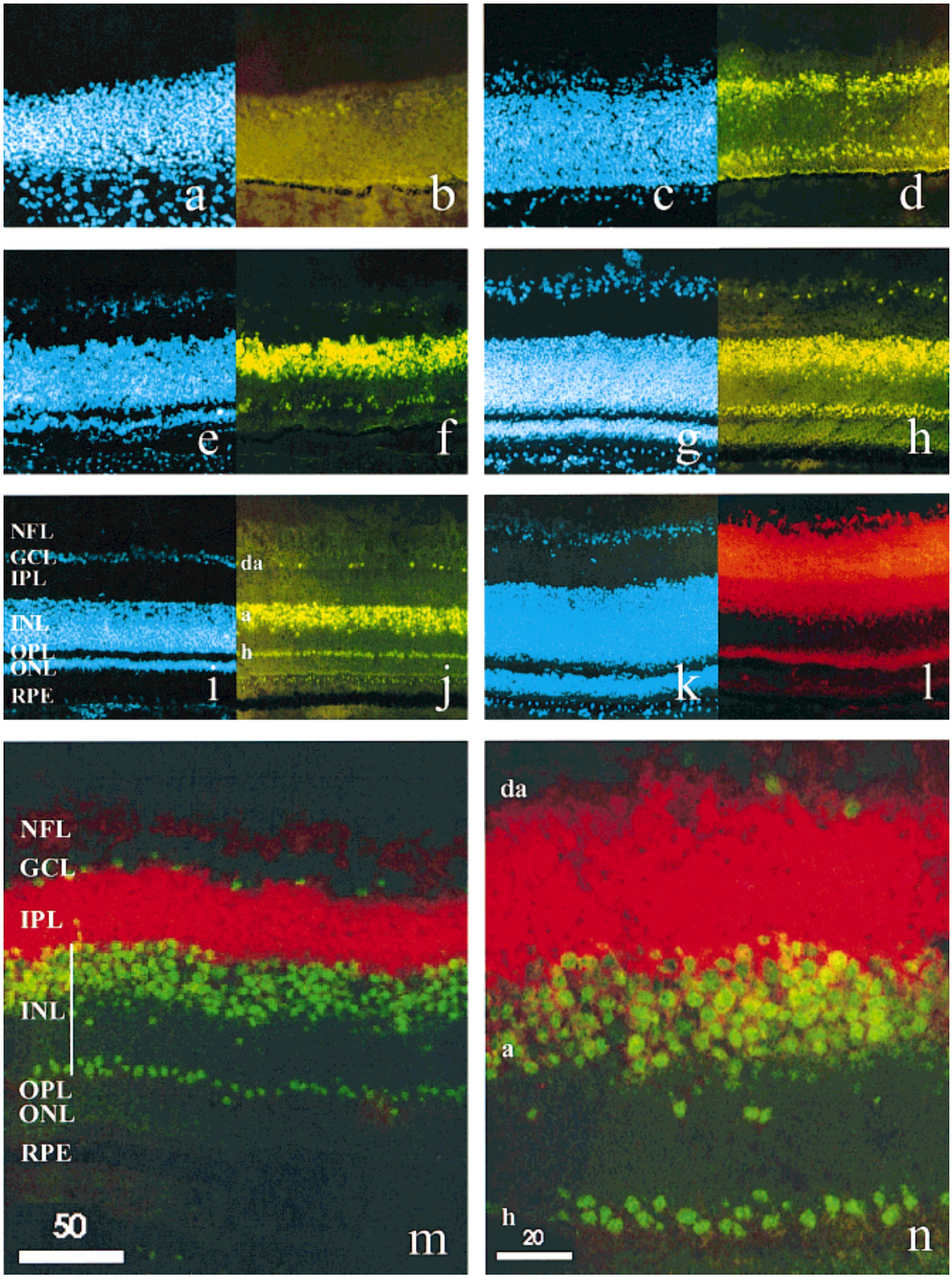

Fig. 6. Immunofluorescent analysis of AP-2 in the developing chick retina. Sections from each developmental stage were stained with anti-AP-2 antibody (with FITC-conjugated secondary antibody, green) (**b, d, f, h,** and **j**) and counterstained with the fluorescent dye Hoescht 33258 to label the nuclei blue (**a, c, e, g,** and **i**). Retinal sections were prepared from: (a,b) ED5; (c,d) ED7; (e,f) ED10; (g, h) ED16; and (i, j) ED19 stage embryos. The retinal layers are indicated in i, while the positive cell layers are labeled in j. **k,l:** ED16 retina stained with Hoescht 33258 (k) and monoclonal anti-syntaxin (TRITC-labeled secondary antibody, red) (l). Photomicrographs were taken at 200× magnification using a Nikon Diaphot 300 microscope. **m,n:** Confocal microscope images representing dual AP-2 (green) and syntaxin (red) immunofluorescent staining of ED16 retina are shown at 200× (m) and 600× (n) magnification. Confocal images were obtained using a Molecular Dynamics Multiprobe 2001 microscope. Scale bars, (m) 50 µm and (n) 20 µm. NFL: nerve fiber layer; GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; RPE: retinal pigment epithelium; da: displaced amacrine cells; a: amacrine cells; and h: horizontal cells.

shown by others to be specifically expressed in the Müller glial cells of the differentiated retina (Vardimon et al., 1986). We carried out in situ hybridization with a CA-II riboprobe to determine whether *CA-II* and *R-FABP* mRNA have similar distribution profiles. Fig. 5l shows an ED11 retinal section hybridized to CA-II antisense RNA (purple). The section was counterstained with ethyl green in order to visualize all three nuclear layers. *CA-II* mRNA was primarily found in the middle of the INL, the usual location of Müller glial cell nuclei. As shown in Fig. 5h, *R-FABP* mRNA is also concentrated in the middle of the INL; however, it extends throughout the outer half of the INL where bipolar nuclei are located, suggesting a broader distribution for the *R-FABP* transcript.

Immunofluorescent Analysis of AP-2 in the Retina

To analyze the cellular distribution of AP-2 during retinal development, we performed immunofluorescence analysis of retinal sections at various developmental stages. As mentioned earlier, the antibody used in these experiments recognizes both AP-2α and AP-2β. There were occasional AP-2 positive cells distributed along the inner border of the ED5 posterior retina (Fig. 6a,b). As expected, the AP-2 transcription factor was restricted to the nucleus. A band of AP-2 positive cells, several nuclei deep, was found in the INL bordering the inner plexiform layer of ED7 retina (Fig. 6c,d). The AP-2-expressing cells extended into the anterior retina nearly to the ciliary epithelium, thinning to a single layer of cells in the mid-peripheral retina. A discontinuous band of nuclei was also found immediately vitreal to the presumptive outer plexiform layer, the location of horizontal neurons. Expression in this region was restricted to the posterior third of the retina. The positive cells in the INL between the two bands of AP-2 expression are presumably migrating amacrine cells. Migrating cells would still be expected at ED7 because amacrine neurogenesis continues until ED10 (Prada et al., 1991). As with the in situ hybridization experiments, positive cells were also observed in the ganglion cell layer and likely represent displaced amacrine cells.

By ED10, there was a greater number of AP-2-expressing amacrine cells, with most, if not all of the cells in the vitreal half of the INL being AP-2 positive. A positive signal was also detected in the INL bordering the outer plexiform layer where horizontal cells are located (Fig. 6e,f). As well, AP-2 was present in the putative displaced amacrine cells that line the outer border of the ganglion cell layer. Similar results were obtained at ED16 when the retinal strata are more defined (Fig. 6g,h). There was a sharp band of expression approximately 10 cells wide spanning the inner third of the INL. The staining of the horizontal cell layer was more uniform than at ED10, with a depth of three to four cells. The staining of amacrine and horizontal cells at ED19 had condensed into tighter layers, with AP-2-positive amacrine cells spanning approximately six nuclei in the posterior retina, while AP-2-positive horizontal cells spanned one to two nuclei (Fig. 6i,j).

Syntaxin, a protein involved in synaptic vesicle docking, is a marker of amacrine and horizontal cells (Barnstable et al., 1985; Alexiades and Cepko, 1997). Immunostaining of an ED16 retinal section with anti-syntaxin revealed extensive staining of the inner plexiform layer, as expected for a protein involved in synaptic function (Fig. 6k,l). Staining of the outer plexiform layer was somewhat weaker. Cytoplasmic staining of amacrine and horizontal cells by anti-syntaxin can be observed in the inner nuclear layer. To determine if syntaxin and AP-2 co-localize, we carried out double immunofluorescent labeling. Co-localization of cytoplasmic syntaxin (red) and nuclear AP-2 (green) to the same cells can be seen in Fig. 6m,n. These data provide further evidence that the AP-2 positive cells in the INL are amacrine and horizontal cells, as expected based on their position.

DISCUSSION

In recent years, considerable progress has been made in identifying genes that are developmentally regulated in the retina (Freund et al., 1996). Functional analyses of some of these genes have generated valuable information regarding the molecular mechanisms controlling cell proliferation and cell fate determination in the developing retina (Fini et al., 1997; Reh and Levine, 1998). For example, experiments by Austin et al., (1995) have shown that the transmembrane receptor Notch-1 and its ligand Delta affect the growth and differentiation of retinal cells along the ganglion pathway. However, many gaps remain in our understanding of the cascade of events underlying the retinal maturation process.

The elevated levels of *R-FABP* mRNA in the chick retina from ED3 to ED7, followed by a dramatic decrease in transcript levels from ED7 to ED19, suggest that the transcription of the *R-FABP* gene must be tightly regulated in the developing embryo (Godbout, 1993). The presence of a consensus AP-2 binding site in the *R-FABP* promoter and the binding of an AP-2-immunoreactive nuclear protein to this region indicate that one or more members of the AP-2 family are likely involved in the regulation of the *R-FABP* gene (Bisgrove et al., 1997). In our earlier study, we demonstrated the presence of AP-2 mRNA and protein in the chick retina (Bisgrove et al., 1997). At that time, we were not able to identify which *AP-2* genes were expressed in the retina because the different members of the AP-2 family had not been cloned in chicken. The sequence of chicken *AP-2*α cDNA has since been reported by Shen et al. (1997), and here we have cloned *AP-2*β from a chick retina cDNA library. We now show that both the *AP-2*α and *AP-2*β genes are expressed at elevated levels in the chick retina. The temporal profiles of both genes are similar, with barely detectable levels in the undifferentiated ED5 retina and a dramatic increase in transcript

and protein levels from ED7 to ED9/10. Maximal levels are observed at ED9/10, when few retinal cells are proliferating and the great majority of cells are committed to a specific differentiation pathway (Prada et al., 1991). Moser et al. (1997a) did not detect either *AP-2*α or *AP-2*β mRNA in the undifferentiated retina of day 15 mouse embryos by in situ hybridization, although transcripts were detected in the adult mouse eye by RT-PCR (Moser et al., 1995). These results suggest a similar expression profile for the AP-2 factors in mammalian and avian retina. Based on their patterns of expression, we propose that the AP-2 factors are required during the early steps of retinal differentiation.

The *AP-2*α and *AP-2*β transcripts are also expressed in the developing brain and kidney, although levels of *AP-2*α mRNA are considerably lower in these tissues than in the retina. The *AP-2* transcripts are either barely detectable in heart and liver (*AP-2*α) or not detectable (*AP-2*β). Multiple forms of the *AP-2*α and *AP-2*β transcripts are observed in retina and brain, likely representing alternative splicing events or usage of different transcription initiation sites, as previously observed for murine *AP-2*α (Meier et al., 1995). In contrast, there is only one major form of the *AP-2*β transcript in kidney, suggesting that the additional forms found in retina and brain may be neural tissue-specific.

Both *AP-2* genes are selectively expressed in subsets of retinal cells in the chick embryo: AP-2α is restricted to amacrine cells while AP-2β is specifically found in amacrine, horizontal, and putative displaced amacrine cells. AP-2α and AP-2β were detected in the ED5 retina but not at ED3.5. Neurogenesis of amacrine and horizontal cells begins at ED3 and ED4, respectively (Prada et al., 1991). It is unlikely that AP-2 plays a role in the specification of these cell lineages because AP-2 appears to be induced shortly after the cells are committed to a particular lineage. However, the early and cell-specific expression of AP-2α and AP-2β suggest a role for AP-2 in determining the initial events associated with amacrine and horizontal cell differentiation. Others have reported the presence of both AP-2α and AP-2β in specific cell types, leading to the hypothesis that combinations of AP-2 factors may cooperate to regulate the expression of specific sets of genes (Moser et al., 1997b). Some of the genes activated or repressed by the combination of AP-2α and AP-2β in amacrine cells may therefore be different from those activated or repressed by AP-2β in horizontal cells.

Analysis of both *AP-2*α-null mice and chimeric mice indicates a role for AP-2α in several morphogenic pathways including eye formation, limb pattern formation, and development of the neural tube, head, and body (Schorle et al., 1996; Zhang et al., 1996; Nottoli et al., 1998). The ocular defects in these mice are so severe and appear so early that it has not been possible to assess whether AP-2α plays a role in retinal differentiation. In contrast to *AP-2*α gene knock-outs, *AP-2*β-null mice show no abnormalities other than renal malformation (Moser et al., 1997a). However, the mouse retina is still undifferentiated when the *AP-2*β-/- mice die of polycystic kidney disease one to two days after birth. Expression of amacrine and horizontal cell differentiation markers occurs primarily after birth (Grün, 1982; Cepko et al., 1996). A role in retinal differentiation may therefore only be identified if the lifespan of *AP-2*β-/- mice can be extended. Alternatively, different members of the AP-2 family may be able to compensate for each other in some cell types or tissues, and retinal abnormalities may not be observed unless multiple *AP-2* genes are inactivated.

Although no *AP-2*γ cDNA clones were obtained by screening an ED16 chick retina library, Oulad-Abdelghani et al. (1996) detected *AP-2*γ mRNA in the adult mouse retina by in situ hybridization, specifically in the inner nuclear layer and ganglion cell layer, although the cells expressing this transcript were not identified. *AP-2*γ was not expressed in retinal precursor cells of day 11.5 mouse embryos, as determined by in situ hybridization (Chazaud et al., 1996). Our Northern blot analysis of chick retina using mouse *AP-2*γ cDNA as the probe generated a barely detectable signal (data not shown), suggesting that AP-2γ is not expressed or weakly expressed in the chick retina.

Examination of the regulation of the *AP-2* genes to determine how AP-2 expression is directed to horizontal and amacrine cells may lead to insight into the nature of the factors involved in the determination and differentiation of these two classes of neurons. In some cell types, such as P19 embryonal carcinoma and NT2 teratocarcinoma, AP-2 expression is inducible by retinoic acid (Philipp et al., 1994; Lüscher et al., 1989). These cells undergo neuronal differentiation in response to retinoic acid. Induction of AP-2 could therefore couple response to retinoic acid with the regulation of genes involved in neuronal differentiation. It will be interesting to determine if retinoic acid, previously implicated in retinal development (Dräger and McCaffery, 1996), can up-regulate AP-2 in differentiating amacrine and horizontal cells. If retinoic acid participates in this process, it is likely to be in concert with differentially expressed proteins involved in retinoic acid metabolism. It is noteworthy that, unlike other retinal cells, the processes of amacrine, horizontal, and displaced amacrine cells are all confined to their neighboring plexiform layer, providing a commonality among these cell types. Transcription factors restricted to retinal cells with this property may be involved in *AP-2* regulation.

Although the AP-2 family has most commonly been associated with transcriptional activation (Duan and Clemmons, 1995; Dyck and Fliegel, 1995), it has also been shown to repress the transcription of several genes (Gaubatz et al., 1995; Getman et al., 1995; Chen et al., 1996). If members of the AP-2 family are involved in the repression of *R-FABP* transcription, one would expect *R-FABP* mRNA to be excluded from cells expressing AP-2. Northern blot analysis of *AP-2*α, *AP-2*β , and

R-FABP indicates a negative correlation between *AP-2α/β* and *R-FABP* mRNA levels during retinal development, with high R-FABP/low AP-2 in the undifferentiated retina and low R-FABP/high AP-2 in the differentiated tissue. Furthermore, in situ hybridization and immunofluorescent analyses show mutually exclusive expression patterns for the AP-2 factors and *R-FABP* transcripts starting at ED10. While *R-FABP* mRNA is abundantly and widely expressed in the undifferentiated retina from ED3.5 to ED7, the transcript becomes restricted to the outer portion of the inner nuclear layer with differentiation. This region of the retina contains cell bodies from Müller glial cells and from bipolar cells. *R-FABP* transcripts are excluded from amacrine and horizontal cells, in contrast to AP-2α and AP-2β, which are selectively expressed in these cells. *R-FABP* mRNA is also absent in photoreceptors and ganglion cells, neither of which expresses AP-2, suggesting that other transcription factors are involved in *R-FABP* repression in these cell types.

In addition to the correlative studies showing that *R-FABP* mRNA is excluded from AP-2-expressing cells, our DNA transfection experiments indicate that AP-2α and AP-2β can directly repress *R-FABP* promoter activity in vitro. Overexpression of either AP-2α or AP-2β in primary retinal cultures resulted in a 50% decrease in the activity of the *CAT* reporter gene under the control of the *R-FABP* promoter containing the AP-2 recognition site. There was no decrease in CAT activity when either AP-2α or AP-2β antisense expression constructs were used, or when a pCAT construct carrying a mutagenized AP-2 site was tested.

Murine brain FABP, expressed in radial glial cells, is involved in the establishment of the radial glial fiber system guiding neuronal migration and may also play a role in relaying inductive signals for neuronal differentiation (Feng et al., 1994; Kurtz et al., 1994). Similar to B-FABP, R-FABP (initially expressed in neuroectodermal precursor cells and later restricted to Müller glial cells and/or bipolar cells) may be involved in the formation of a migratory system for retinal neurons. The production of R-FABP by Müller glial cells, which span the entire width of the retina, may play a role in the positioning and differentiation of all neuronal cell types in this tissue.

In summary, we have used two approaches to determine whether members of the AP-2 family downregulate *R-FABP* transcription. First, we identified the AP-2 factors that are expressed in the retina and demonstrated mutually exclusive expression profiles for two members of this family and *R-FABP* transcripts. Second, we showed by DNA transfection analysis that overexpression of either AP-2α or AP-2β in primary retinal cultures results in a decreased activity from the *R-FABP* promoter. These data suggest that these two AP-2 factors can repress *R-FABP* transcription. Given the number of genes shown to be regulated by AP-2 in different systems, repression of the *R-FABP* gene by AP-2α and AP-2β probably represents only one component of their overall transcriptional regulation program in the retina. A number of transcription factors are expressed in the retina; however, little is known regarding their mechanisms of action and the nature of their target genes. The identification of two AP-2 transcription factors specifically expressed in the amacrine and horizontal cells of the retina, in conjunction with the discovery that *R-FABP* likely represents a target gene for these transcription factors, provide the first steps towards the elucidation of one of the molecular pathways underlying retinal differentiation.

EXPERIMENTAL PROCEDURES

Screening of the ED16 Chick Retina cDNA Library

Preparation of the cDNA library was as previously described (Godbout, 1993), except that the cDNA was produced from ED16 chick retina poly(A)$^+$ RNA. Approximately 2×10^5 bacteriophage were sequentially hybridized with the following: (i) a 1.2 kb *Eco*RI/*Hin*dIII human *AP-2α* cDNA fragment (IMAGE EST clone ID 142203, Genome Systems Inc, St. Louis, MO), (ii) a 1.5 kb *Eco*RI/*Hin*dIII *AP-2β* cDNA fragment derived from human adult retina (IMAGE EST clone ID 362684, Genome Systems Inc) and (iii) mouse *AP-2γ* cDNA (a kind gift from R. Buettner, University of Regensburg Medical School, Germany). Filters were washed at 45°C in 0.1× SSC and 0.1% SDS. Positive clones were purified by additional rounds of screening. The cDNA ends of positive clones were sequenced using an ABI 310 automated sequencer and the sequences analyzed using the BLAST program of the National Center for Biotechnology Information, NIH. The sequence of the *AP-2β* cDNA was generated by sequencing both strands of a cDNA containing the entire coding region. Sequence data from this article have been deposited with the EMBL/GenBank Data libraries under Accession No. AF065140.

Northern Blot Analysis

Poly(A)$^+$ RNA was isolated from retina, brain, heart, liver, and kidney at the developmental stages indicated in the legend to Fig. 3.

The mRNAs were electrophoresed in a 6% formaldehyde–1.5% agarose gel in MOPS buffer and transferred to nitrocellulose. The filter was sequentially hybridized to a 400 bp *Pst*I fragment derived from the 3′ end of chicken *AP-2α* cDNA and to a 400 bp PCR-amplified DNA fragment extending from bp 301–700 of chick *AP-2β* cDNA. Both probes were selected on the basis of their low level of similarity to other members of the AP-2 family. Filters were washed at 45°C in 0.1× SSC, 0.1% SDS. The filter was hybridized to mouse actin cDNA to control for lane to lane variation in mRNA levels.

Western Blot Analysis

Chick retinas were dissected at various stages between ED5 and ED19, and were immediately frozen in

liquid nitrogen. Whole cell lysates were prepared by lysing thawed retina in 4 volumes of 1× SDS-PAGE buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol). Sample viscosity was reduced by several passes through a 21 gauge syringe and then clarified by ultracentrifugation. The Bradford assay was used to quantitate the protein content of each extract (Bio-Rad Laboratories, Hercules, CA). Cell lysates (100 µg protein/lane) were electrophoresed in a 10% SDS-PAGE gel and transferred to nitrocellulose. Blots were stained with 0.005 % CTPS (copper phthalocyanine 3, 4′, 4″, 4‴ tetrasulfonic acid in 12 mM HCl) to mark the position of the standards. The filter was incubated with affinity-purified anti-human AP-2 polyclonal antibody at a 1:1000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA). Antigen–antibody interactions were visualized with the ECL detection system (Amersham Pharmacia Biotech, Uppsala, Sweden) using horseradish peroxidase-conjugated anti-rabbit IgG antibody at a 1:100,000 dilution (Jackson ImmunoResearch, West Grove, PA).

In Situ Hybridization

Sense and antisense riboprobes labeled with digoxigenin (DIG) or fluorescein were synthesized by in vitro transcription with T3 or T7 RNA polymerase according to the manufacturer's protocol (Boehringer Mannheim, Indianapolis, IN). An 850 bp chick *AP-2*α partial cDNA extending from bp 826 to 1676 (GenBank Accession No. U72992) served as the template for synthesis of the AP-2α riboprobe. A 1.2 kb *AP-2*β cDNA fragment containing the entire coding region (GenBank Accession No. AF065140) served as the template for the AP-2β riboprobe. The R-FABP riboprobe has been described previously (Godbout et al., 1995). Whole embryos (ED5) or eyes (ED7 and ED10) were fixed overnight in 4% PBS-buffered paraformaldehyde at 4°C, cryoprotected overnight in 30% sucrose and embedded into O.C.T. (Tissue-Tek, Miles Inc., Elkhart, IN). Frozen sections were pretreated essentially as recommended by Boehringer Mannheim, except that the sections were dehydrated through an ethanol gradient after acetylation. The sections were then prehybridized at 55°C in hybridization solution minus the probe (40% formamide, 10% dextran sulphate, 1× Denhardt's solution, 4× SSC, 10 mM DTT, 1 mg/ml yeast tRNA, and 1 mg/ml denatured herring sperm DNA). Riboprobes were denatured at 70°C for 10 minutes prior to overnight hybridization with the sections at 55°C. Posthybridization washes were as previously described (Belecky-Adams et al., 1997). Sections were blocked with PBS containing 2% blocking reagent (Boehringer Mannheim) and 0.3% Triton X-100. DIG (or fluorescein) label was detected using an alkaline phosphatase (AP)-conjugated anti-DIG (or anti-fluorescein) antibody at a 1:1000 dilution with polyvinyl alcohol enhancement as described (Jowett, 1997).

Immunofluorescence

Frozen sections were prepared by embedding freshly dissected chick eyes in O.C.T., followed by cryostat sectioning (6 µm). All sections were in the nasolateral plane and intersected the lens. The sections were fixed in acetone, washed with PBS, and blocked in 10% skim milk. Manipulations involving fluorescent reagents were performed in the dark. Sections were incubated with a 1:100 dilution of rabbit polyclonal anti-AP-2 antibody (Santa Cruz Biotechnology) or a 1:100 dilution of monoclonal anti-syntaxin antibody (Sigma Immunochemicals, St. Louis, MO). Immunoreactivity was detected using a 1:100 dilution of the secondary antibodies, FITC-conjugated anti-rabbit IgG antibody (Boehringer Mannheim), or TRITC-labeled anti-mouse IgG antibody (Jackson ImmunoResearch). Control slides were exposed to the secondary antibodies only. Slides were counterstained with the fluorescent nuclear stain Hoescht 33258 (Molecular Probes). Slides were mounted with Fluorosave (Calbiochem) and visualized using a Nikon Diaphot 300 microscope equipped with epifluorescence attachment. Confocal images were obtained using a Molecular Dynamics Multiprobe 2001 microscope (Biological Sciences Microscopy Unit, University of Alberta).

DNA Transfection Analysis

Expression plasmids were prepared by inserting the coding regions of chicken *AP-2*α and *AP-2*β into pcDNA3 (Invitrogen, La Jolla, CA) in the sense and anti-sense orientations. To prepare the AP-2α construct, an *Eco*RI fragment extending from bp 1–1675 of the previously cloned chicken *AP-2*α cDNA (a kind gift from J. Richman, University of British Columbia, Canada) was inserted into the *Eco*RI site of pcDNA3. To prepare the AP-2β construct, the coding region of the *AP-2*β cDNA obtained from our ED16 chick retina library was PCR-amplified with *pfu* polymerase (Stratagene, La Jolla, CA) and the primers: 5′-*ATG*CTCTGGAAACTG-GTTG-3′ and 5′-*TCA*TTTTCTGTGTTTCTCTTCC-3′ (start and stop codons are italicized). The product was cloned into the *Eco*RV site of pcDNA3 and sequenced to verify that no mutations were introduced by PCR. The *R-FABP* promoter reporter construct (pCAT-135) and calcium phosphate mediated transient transfections into ED5 primary retinal cultures have been previously described (Bisgrove et al., 1997). Co-transfections were carried out using 5 µg of reporter plasmid and 10 µg of either pcDNA3/AP-2α or pcDNA3/AP-2β. Overexpression of AP-2α and AP-2β was confirmed by Western blotting of whole cell lysates of transfected cells. Transfection efficiency was monitored by preparation of Hirt DNA from the transfected cells, followed by Southern blotting using pCAT-Basic (Promega, Madison, WI) as the probe.

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