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Specification of neurotransmitter receptor identity in developing retina: the chick ATH5 promoter integrates the positive and negative effects of several bHLH proteins

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SUMMARY

Genetic studies in *Drosophila* and in vertebrates have implicated basic helix-loop-helix (bHLH) transcription factors in neural determination and differentiation. In this report, we analyze the role that several bHLH proteins play in the transcriptional control of differentiation in chick retina. Our experimental system exploits the properties of the promoter for the β3 subunit of the neuronal acetylcholine receptors, important components of various phenotypes in the CNS of vertebrates. The β3 subunit contributes to define ganglion cell identity in retina and its promoter, whose activation is an early marker of ganglion cell differentiation, is under the specific control of the chick *atonal* homolog ATH5. Functional analysis of the ATH5 promoter indicates that interactions between ATH5 and several other bHLH transcription factors underlie the patterning of the early retinal neuroepithelium and form a regulatory cascade leading to transcription of the gene for β3. ATH5 appears to coordinate the transcriptional pathways that control pan-neuronal properties with those that regulate the subtype-specific features of retinal neurons.

Key words: Chicken, Retina, Neuronal bHLH proteins, Neuronal determination, Transcriptional control

INTRODUCTION

The assembly of neuronal circuits in the vertebrate nervous system is anticipated by the orderly differentiation of a vast array of diverse neurons whose phenotypes include such essential traits as neurotransmitter receptor specificity and membrane excitability. Among several transcription factor families that play crucial parts in neurogenesis (reviewed in He and Rosenfeld, 1991; Edlund and Jessell, 1999), the basic helix-loop-helix (bHLH) factors emerge as important regulators of neuronal identity.

In *Drosophila*, bHLH factors encoded by the *Achaete-scute* and *atonal* proneural genes are the main intrinsic determinants of neural fate and render neural precursors competent to form distinct sensory organs (reviewed by Campos-Ortega, 1993; Jan and Jan, 1993). Numerous *Achaete-scute* (ASH) and *atonal* (ATH) homologs have been identified in vertebrates (reviewed by Anderson and Jan, 1997; Lee, 1997). They are sequentially expressed during ontogenesis and there is evidence that the products of the early or upstream genes may be required for the expression of the late or downstream genes (Ma et al., 1996; Cau et al., 1997; Roztocil et al., 1997; Fode et al., 1998; Ma et al., 1998). *ASH1* and the three neurogenins (*Ngn1,2,3*; *ATH4c,a,b*) are among the earliest bHLH genes expressed in the developing nervous system and they are thought to act as early determination factors in proliferating precursors (Guillemot and Joyner, 1993; Jasoni et al., 1994; Gradwohl et al., 1996; Ma et al., 1996; Sommer et al., 1996), while *ATH2* genes such as *Nex1* and *NeuroD* are likely involved at later stages of differentiation (Bartholoma and Nave, 1994; Lee et al., 1995). *NeuroM*/*ATH3* is transiently expressed in newborn neurons that are about to embark on their migration to the outer layers, and its product may provide a functional link between the early and the late bHLH genes (Roztocil et al., 1997; Takebayashi et al., 1997; Fode et al., 1998).

bHLH regulatory cascades, including instances of epistasis among bHLH family members, have been established by dissecting the processes leading to the acquisition of pan-neuronal properties (reviewed by Lee, 1997). In addition, recent studies indicate that the *atonal* and *Achaete-scute* vertebrate homologs confer subtype-specific properties to neurons, thus inextricably linking neural determination and the specification of neuronal identity (reviewed by Brunet and Ghysen, 1999). The compartmentalization of distinct bHLH gene products in the nervous system anlage has suggested that elements of particular neuronal specificities may be assigned from very early on in determination (reviewed by Chitnis, 1999). A spatial complementarity between the expression patterns of ASH1 and of the neurogenins appears to be the rule in most proliferating neuroepithelia (Ma et al., 1997) and these factors have indeed been shown to function in the ontogeny of distinct classes of progenitors. ASH1 is essential for the generation of olfactory, telencephalic and autonomic neurons (Guillemot et al., 1993; Casarosa et al., 1999; Torii et al., 1999;

Fode et al., 2000). In peripheral autonomic lineages, it appears to promote the maturation of committed but undifferentiated neuronal precursors (Sommer et al., 1995). The factor is required, in concert with Phox2, for acquisition of the noradrenergic phenotype in neural crest cells (Hirsch et al., 1998; Lo et al., 1999; reviewed by Goridis and Brunet, 1999). The neurogenins have neural determination functions in cranial sensory lineages (Fode et al., 1998; Ma et al., 1998; Ma et al., 1999) and in the dorsal forebrain (Fode et al., 2000). They can induce the expression of pan-neuronal as well as of specific sensory markers in neural crest cells (Perez et al., 1999).

These studies leave open the question of whether bHLH factors that are required for the expression of certain phenotypic properties directly regulate genes that underwrite these properties. Likewise, it is not known whether and how different members of the bHLH family might interact to coordinate the assembly of the phenotypic components that define a particular neuronal identity. While sequential interactions are better understood, little is known about interactions between parallel processes that may exist to bring together different subprograms and integrate them into the overall program of neurogenesis. Heterodimerizing bHLH factors are well-suited to coordinating such programs because their large repertoire of specificities enables them to modulate target-gene activation, to control their own syntheses and to impose links between sequential and parallel expression patterns. To understand more about the transcriptional logic of such systems, it is necessary to identify the cis-regulatory domains of bHLH genes and those of their target genes. It is also necessary to analyze the interactions taking place between various members of the neuronal bHLH family, as well as those between bHLH factors and terminal differentiation genes.

In previous reports, we have clarified several aspects of the genetic circuitry underwriting the ganglion cell phenotype in the avian retina (reviewed in Matter and Ballivet, 2000). We have shown that expression of the β3 subunit of a neuronal nicotinic acetylcholine receptor subtype is confined to the ganglion cells (Hernandez et al., 1995; Matter et al., 1995), to which it may impart specific neurotransmitter receptivity and membrane excitability (Forsayeth and Kobrin, 1997; Groot-Kormelink et al., 1998; Palma et al., 1999). The promoter conferring stringent neuronal specificity upon the gene for this subunit is under the direct control of bHLH proteins and is able to discriminate accurately between related members of the bHLH transcription factor family (Hernandez et al., 1995; Roztocil et al., 1998). The β3 subunit promoter, whose activation is an early marker of ganglion cell differentiation (Matter et al., 1995), is therefore a useful tool for identifying the transcription factors specifying ganglion cell identity. Here, we show that it is under the direct and specific control of chick ATH5, the avian ortholog of the recently isolated *Xenopus* ATH5 factor, whose expression in retina appears to bias progenitor cells towards a ganglion cell fate (Kanekar et al., 1997). To characterize the cascade of gene regulations leading to expression of the β3 subunit, we isolated the cis-regulatory region of the *ATH5* gene. We determined that in retinal cells it is activated by Ngn2, NeuroM, NeuroD and ATH5 itself, and repressed in a dominant-negative mode by ASH1. These results are fully corroborated by the expression patterns of the corresponding genes in the course of retina development. They demonstrate that a bHLH transcription factor can directly regulate terminal differentiation genes in neurons and suggest that the *ATH5* gene integrates the effect of several sequentially expressed bHLH factors to coordinate the specification of ganglion cell identity within the overall program of retinogenesis. ATH5 may thus act as a coupling device between the transcriptional pathways that regulate pan-neuronal properties and those that control the subtype-specific features of retinal neurons.

MATERIALS AND METHODS

Cloning of the chicken ATH5 cDNA and gene

The degenerate oligonucleotides AT5a (5′ ggaattcatgcarggnctgaa-yaccgc) and AT5b (5′ ggaattcatgatgtamgacagngccat) bracketing the bHLH domain of *Xenopus* ATH5 (GenBank U93171; Kanekar et al., 1997) were used in standard Taq polymerase PCR conditions to amplify chicken DNA. The amplified DNA (about 115 bp in length) was gel-purified, digested with the restriction endonuclease *Eco*RI and cloned into pBluescript SK$^-$ (Stratagene). The purified insert from one transformant whose sequence was closely similar to that of *Xenopus* ATH5 was labeled with ^{32}P by random priming and used to screen an embryonic (E12) chick neuroretina cDNA library, home-made in the vector λgt10. The inserts of positive recombinant phage were subcloned, sequenced and found to encode ATH5. The insert extending furthest in 5′ was used as probe to screen a chicken genomic library (Stratagene, 946401) in the vector λ Fix2. The DNA extracted from several positive recombinant phage was mapped with restriction enzymes and appropriate gene fragments were subcloned and sequenced, yielding the 1700 bp located immediately upstream of the cDNA's 5′ end. There were no differences between gene and cDNA in the subsequent 5′ untranslated (455 bp) and coding sequences (456 bp). The GenBank Accession Number for ATH5 is AJ001178.

Cloning of the chicken neurogenin genes

The degenerate oligonucleotides Uni10 (5′ ggaattcgcgagcgsaa-ccgsatgca) and Uni11 (5′ ggaattcagrgcccagatgtaitt), corresponding to highly conserved blocks of residues located on either side of the bHLH region in vertebrate *atonal* homologs, were used to PCR amplify chicken DNA. The amplified DNA (about 140 bp in length) was purified, digested and subcloned as above. Eighteen transformants were sequenced and found to encode several isolates each of the bHLH regions in NeuroD (GenBank, Y09596), NeuroM (GenBank, Y09597) and Nex1/ATH2. In addition, several inserts encoded Ngn-like sequences. A mixture of these was labeled and used to screen a chicken genomic library, as above. Inserts from a dozen positive recombinant phage were mapped, subcloned, sequenced and found to encode the avian *Ngn1*, *Ngn2* and *Ngn3* genes. The coding sequences for the Ngn1 and Ngn2 proteins have been allocated GenBank Accession Numbers AJ012660 and AJ012659.

Primer extension

A 21-mer antisense oligonucleotide (complementary to nucleotides –528 to –549, relative to the initiator ATG of the *ATH5* gene) was phosphorylated with [γ^{32}P]ATP and hybridized to 2 μg of poly(A)$^+$ RNA from chick neuroretina (E6) or from optic tectum (E8). Annealing, reverse transcription and gel electrophoresis of the extension products were carried out as described by Hernandez (Hernandez et al., 1995).

Eukaryotic expression plasmids for ATH5 and the neurogenins

The pEMSV plasmid, which puts cloned sequences under the transcriptional control of the mouse sarcoma virus long terminal repeat, was used throughout to express the ATH5, NeuroM, NeuroD,

ASH1 (GenBank U01339; Jasoni et al., 1994) and Ngn2 cDNAs in transfection and co-transfection experiments.

Reporter plasmids for the ATH5 and β3 promoters

A fragment of the ATH5 gene –912 bp in length, bounded by *Xba*I and *Bst*XI restriction sites and including the 67 5′-most bp in the ATH5 cDNA – was subcloned in the proper orientation at the unique *Sma*I site of vector p00-CAT and at the unique *Not*I site of vector p00-*lacZ* to yield, respectively, p00-ATH5-CAT and p00-ATH5-*lac*Z. The similarly constructed p00-β3-CAT and p00-β3-*lacZ* plasmids bear the 143 bp promoter of the gene for the neuronal acetylcholine receptor β3 subunit and have been described previously (Roztocil et al., 1998).

Expression and purification of the ATH5 protein, gel mobility shift analysis

PCR primers were designed to amplify the DNA fragment encompassing the bHLH region (A_{38}-R_{105}) of ATH5 and introduce *Bgl*II sites at both ends. The amplified DNA was cloned in the appropriate orientation at the unique *Bgl*II site in the vector pDS-13 (Stüber et al., 1990), to yield pDS-ATH5. Upon induction with IPTG, bacterial cultures transformed with this plasmid express a recombinant protein (M_r approx. 30×10^3) consisting of the His-tagged mouse DHFR protein fused to the bHLH domain of ATH5. The fusion protein was affinity-purified on Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen). Band shift analysis was performed as described in Roztocil et al. (Roztocil et al., 1998).

Probes for in situ hybridization

^{35}S-labeled sense and antisense riboprobes were synthesized from linearized pBluescript derivatives, using T7 or T3 RNA polymerase as appropriate (Riboprobe Systems, Promega). ASH1 (Jasoni et al., 1994), NeuroM (Roztocil et al., 1997), ATH5 and Delta1 (GenBank U26590; Henrique et al., 1997) riboprobes encompassed the whole of the respective coding sequences while the Ngn2 riboprobe was limited to the bHLH region and short flanking sequences.

Cell cultures, transfection, CAT and β-galactosidase assays

Chick embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Neuroretina and optic tectum were dissected from stage 23 (E3.5) to stage 38 (E12) embryos and cells were prepared and transfected with CAT or *lacZ* reporter plasmids as described previously (Matter-Sadzinski et al., 1992; Matter et al., 1995). In transfection experiments with a single construct, we used 1 μg of plasmid DNA per 10^6 cells. In co-transfection experiments with two or three constructs, 1 μg of reporter plasmid was mixed, respectively, with 0.5 μg or 1.0 μg (0.5 μg of each construct) of expression or control vectors per 10^6 cells. In all instances, the ratio of DNA to lipofectin was 1/4. Secondary cultures of retinal glioblasts were prepared from E6 neuroretina and transfected essentially as described by Matter-Sadzinski et al. (Matter-Sadzinski et al., 1992) and Roztocil et al. (Roztocil et al., 1998). In each experiment, an aliquot of cells was transfected with pSV-CAT, and the resulting chloramphenicol acetyl transferase (CAT) activity was arbitrarily set at 100. The activities obtained in parallel with other constructs were calculated relative to this value. 25-100 μg of cytosolic proteins were used in CAT assays such that the proportion of acetylated [^{14}C]chloramphenicol in cells transfected with pSV-CAT did not exceed 70%. The means and s.d. values were calculated from data obtained in at least five independent experiments. Cells transfected with β-galactosidase reporter plasmids were plated into the chambers of a poly-DL-ornithine-coated plastic chamber slide. 24 hours after transfection, X-gal staining was performed as described by Hernandez et al. (Hernandez et al., 1995). Blue cells were counted in 20-30 grid areas that each contained about 10^2 positive cells upon transfection with pSV-*lacZ*. Tissue culture reagents were from Life Technologies and plasticware from Nunc.

Electroporation of genetic material in the eye of living embryos

We have developed an electroporation procedure to transfer DNA constructs into cells of the in situ retinal neuroepithelium. Chick embryos were collected at stage 22-23 (E3.5) and immersed in phosphate-buffered saline buffer. An embryo was positioned between two electrodes mounted on a home-made micromanipulator. The lens was removed and 1-2 μl of a DNA solution (1 μg/μl) was microinjected into one eye chamber, the contralateral eye serving as control. The embryo was subjected to five 50 V pulses of 70 ms duration (interpulse interval, 200-500 ms). The electroporated embryos were incubated in DMEM culture medium for 2 hours at 37°C, and the retinas were then dissected and cultured as floating explants for 22 hours at 37°C. Visualization and identification of β-galactosidase-positive cells was as described in Matter et al. (Matter et al., 1995).

Northern blot analysis

Twenty electroporated retina explants were rinsed twice in ice-cold phosphate-buffered saline and lysed in guanidine thiocyanate (Sambrook et al., 1989). Total RNA was isolated, gel fractionated (2 μg/lane) and hybridized as described in Matter et al. (Matter et al., 1990). Isolation and analysis of total RNA from neuroretinas and from transfected cell cultures were as described in Hernandez et al. (Hernandez et al., 1995) and Roztocil et al. (Roztocil et al., 1998). Blots were quantified with a Phospho-Imager.

In situ hybridization

In situ hybridization on tissue sections was performed as described by Roztocil et al. (Roztocil et al., 1997). Transfected retinal cells were stained for β-galactosidase and processed for in situ hybridization as described by Matter-Sadzinski et al. (Matter-Sadzinski et al., 1992). Following hybridization, sections or dissociated cells were dipped in liquid photographic emulsion (Kodak NTB-2) and exposed for about two weeks.

[^{3}H]Thymidine and BrdU labeling

Cells that had been transfected with a *lacZ* reporter plasmid were cultured in medium containing 5 μCi/ml [^{3}H]thymidine, as indicated in Results. They were stained for β-galactosidase, processed for autoradiography (Matter et al., 1995) and exposed for one day. Neuroretinas were dissected, rinsed in HBSS, incubated for 30 minutes in DMEM containing 100 μM BrdU and chased for 30 minutes in DMEM. The explants were fixed, embedded in paraffin, sectioned and processed for the immunodetection of BrdU (Boehringer).

RESULTS

Molecular cloning of avian atonal homologs

Each of the known *atonal* homolog (ATH) genes assumes a distinctive spatial and temporal expression pattern in the course of development and in the adult. Those ATH genes that are transcribed in dividing progenitors are of particular interest, as the factors they encode may regulate key steps in neural determination. We initiated a search for such early genes by amplifying avian genomic DNA sequences with primers encoding highly conserved peptide motifs (RERNRMH and NYIWAL) within the bHLH domain of several known ATH proteins. In addition to numerous fresh isolates of the avian NeuroM, NeuroD and Nex1 sequences, the screen yielded novel clones that were closely similar to those encoding the bHLH domains of the neurogenins (Ngns), a set of three mammalian ATH proteins expressed in proliferating neuroepithelia (Gradwohl et al., 1996; Ma et al., 1996). The

Fig. 1. The close relationship between the avian *atonal*-related factors is shown by alignment of their bHLH regions. Conserved and subclass-specific motifs are respectively highlighted in red and blue. The *Achaete-scute* homolog ASH1 is a more distant relative.

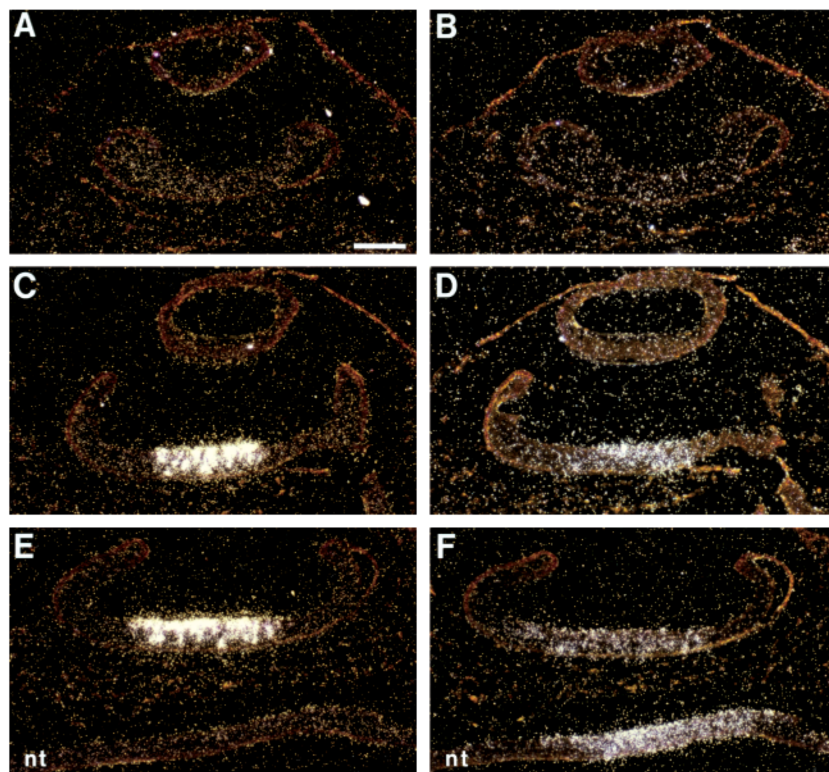
neurogenin inserts were used as probes to screen a chick genomic library and isolate the corresponding genes.

The avian ortholog of the recently characterized *Xenopus* *ATH5* and mouse *ATH5* (*Atoh7* – Mouse Genome Informatics) genes (Kanekar et al., 1997; Brown et al., 1998), was obtained in similar fashion by amplification of genomic bHLH sequences with primers encoding the ATH5-specific peptide motifs MQGLNTA and MALSYIM. The cloned bHLH domain was then used as a probe to isolate the chick *ATH5* gene from a genomic library and a full-length cDNA from an embryonic retina library. We found that in the neurogenins and in ATH5 the coding sequences were entirely contained within a single exon, a feature shared by the other ATH genes (*NeuroM*, *NeuroD*, *Nex1*) whose genomic organization has been established.

As shown in Fig. 1, the eight available avian ATH factors share extensive sequence homology (red symbols) in the bHLH region and possess specific peptide motifs (blue symbols) that distinguish the various ATH subclasses.

ATH5 and Ngn2 have very similar onsets and domains in the developing retina

The spatial and temporal expressions of ATH5 and Ngn2 were examined by in situ hybridization in the course of chick embryonic development. Much as for *Xenopus* and mouse *ATH5* (Kanekar et al., 1997; Brown et al., 1998), chick ATH5 mRNA was found to be restricted to the developing retina (Fig. 2), except for a tiny population of ventricular cells located in the ventral



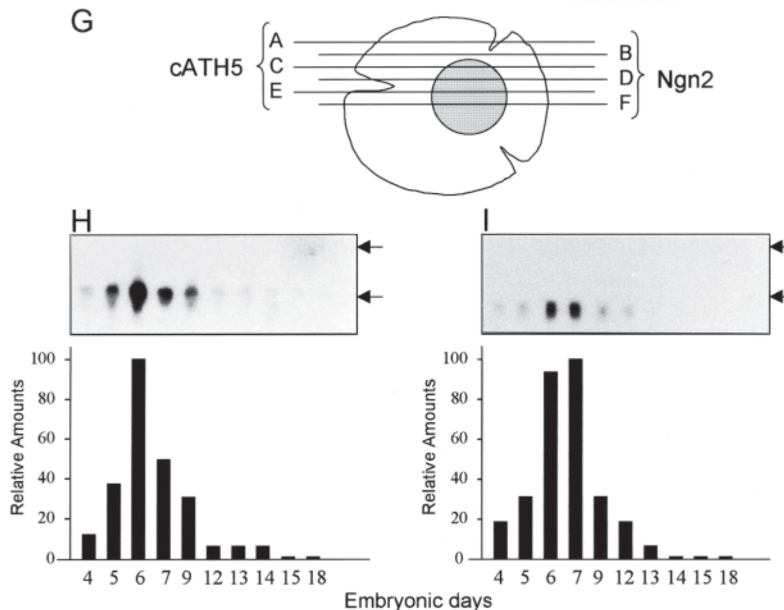


Fig. 2. Coincident expression of chick ATH5 (cATH5) and Ngn2 in the developing retina. (A-F) Adjacent sections of a stage 18 (E2.5) chick retina were hybridized with ATH5 (A,C,E) or Ngn2 (B,D,F) RNA probes. ATH5 and Ngn2 are expressed in coincident domains in central retina. Expression of ATH5 is exclusively detected in the retina, whereas Ngn2 is also expressed in the neural tube (nt, F). Scale bar: 250 µm. (G) The section planes and the central domain that expresses both ATH5 and Ngn2. (H,I) Northern blot of total RNA isolated in the course of retina development. ATH5 (H) and Ngn2 (I) mRNAs are detected at high levels from E5 until E9. The ATH5 and Ngn2 mRNAs peak, respectively, around stage 28 (E6) and 28-31 (E6-E7). The 18S and 28S ribosomal RNAs migrate as indicated (arrows).

domain of the spinal cord and hindbrain (M. T. O., unpublished observations). Ngn2, like its mammalian counterpart, has a much broader expression pattern in the CNS than ATH5 (Fig. 2F and M. T. O., unpublished observations). Cells expressing ATH5 and/or Ngn2 are detected in the central retina at stage 14-15, whereas the first postmitotic ganglion cells appear in the same region at stage 17-18 (Prada et al., 1991; McCabe et al., 1999). When adjacent sections encompassing the peripheral and central regions of stage 18 retina were hybridized with probes specific for ATH5 and for Ngn2 (Fig. 2A-F), the expression patterns defined identical domains in the dorsocentral region of the retinal neuroepithelium, where the first ganglion cells are being generated.

Expression of ATH5 and Ngn2 in the course of retinogenesis was assessed by northern blot of total RNA (Fig. 2H,I). The steady-state levels of the ATH5 and Ngn2 mRNAs follow similar kinetics. mRNA levels rapidly increase between stages 23 (E4) and 28 (E6), culminate at stage 28-31 and then decrease rather abruptly to low values on E9 and beyond. The rapid decrease in its mRNA level after E6 indicates that ATH5 is preferentially expressed in progenitors of early-born neurons. Ngn2 mRNA is maintained at high level until E7, suggesting that it may be expressed in precursor cells of both early- and late-born neurons.

ATH5 and ASH1 have mutually exclusive expression domains in the retinal neuroepithelium

Although the onset of ASH1 expression in the early retina coincides with those of ATH5 and Ngn2, their domains are strikingly different: by stage 18 ASH1 exactly surrounds the central region where both ATH5 and Ngn2 are being expressed (Fig. 3A,B). The expression level of ASH1 as detected by in situ hybridization is lower in stage 18 retina than in the dorsal half of the spinal cord or than in the optic tectum. This is probably the reason why early expression of ASH1 in retina went unnoticed in a previous study (Jasoni et al., 1994). As development proceeds to stage 25 (E5), the annular expression domain of ASH1 moves to the periphery and ATH5 expression expands within the confines bounded by ASH1 (Fig. 3C). By E5, expression of ASH1 is detected in the central region and from then on until E10 the expression domains of the two genes are intermingled throughout the retina. When assessed by northern blot of total RNA, ASH1 mRNA level increases between E4 and E5, is maintained at high levels between E5 and E10 and then decreases to low values on E12 and beyond (data not shown).

ATH5 is expressed in retinal progenitors and in newborn ganglion cells

ATH5 expression expands to the periphery in parallel with retina maturation. By stage 29 (E6), at the peak of ATH5 expression, there are high densities of ATH5-labeled cells on the ventricular aspect of the retina, indicating expression in proliferating progenitors (Fig. 4A-C). NeuroM, a bHLH protein solely expressed in post-mitotic neurons, is strongly expressed at this stage in individual cells scattered throughout the thickness of the retina (Fig. 4D and Roztocil et al., 1997). From E7 on, the level of ATH5 transcripts in the proliferative zone rapidly decreases while ATH5-expressing cells accumulate in the newly formed ganglion cell layer (GCL, Fig. 4E,F). Extinction of ATH5 expression in the inner nuclear layer coincides with the end of cell proliferation as detected by BrdU incorporation (Fig. 4G,H). In the GCL, ATH5 expression is

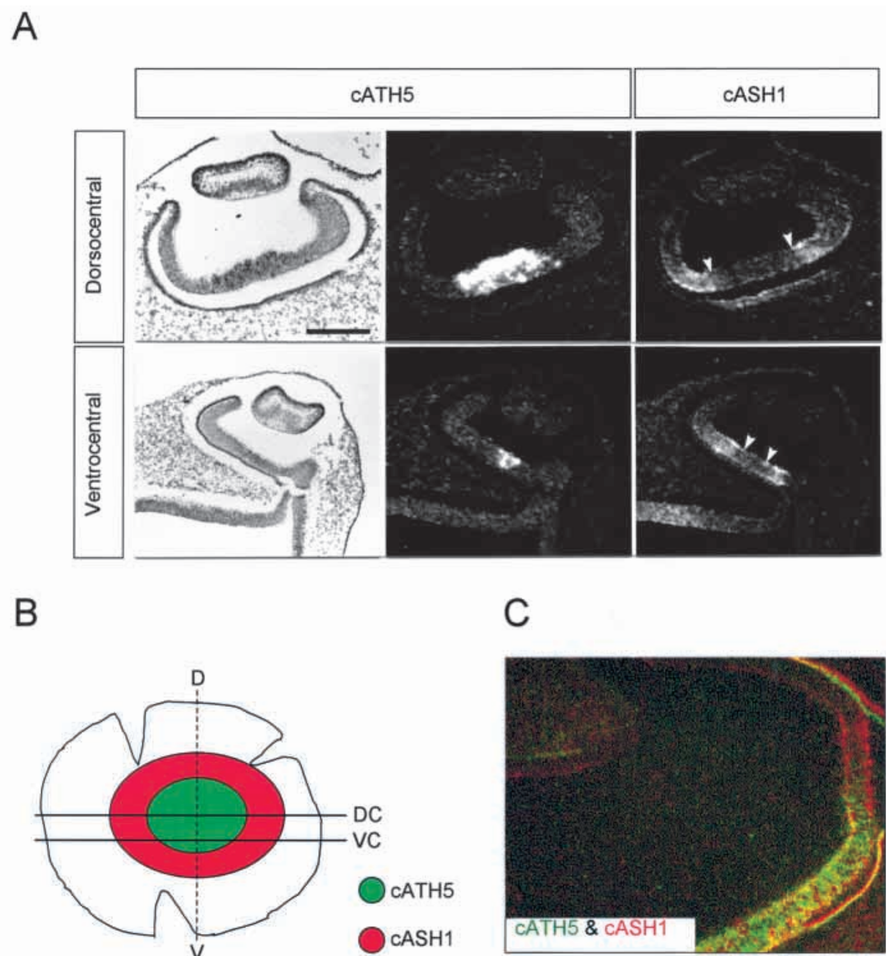


Fig. 3. The expression patterns of chick ATH5 (cATH5) and ASH1 (cASH1) define mutually exclusive domains in the early retina neuroepithelium. (A) Adjacent sections of a stage 18 (E2.5) eye were hybridized with ATH5 (bright- and darkfield) or ASH1 (darkfield) RNA probes. ATH5 is detected in central retina and ASH1 in an annular sector at the periphery. In dorso-central (DC) and ventro-central (VC) sections, the ATH5 and ASH1 domains are adjacent and non-overlapping (arrowheads). Scale bar: 400 μm. (B) The ATH5 and ASH1 domains in stage 18 (E.2.5) retina. (C) Adjacent sections of a stage 25 (E5) eye were hybridized with ATH5 or ASH1 riboprobes. In situ hybridization autoradiographs were assigned false colors and superimposed. At this stage, both ATH5 (green) and ASH1 (red) transcripts are detected in the central retina, whereas ASH1 is expressed in an annular sector at the periphery. The lens is visible in outline (top left). Melanin in the pigmented epithelium diffracts in dark field.

transient and ceases around E12 when differentiation of the GCL neurons is complete (Fig. 4G). By stage 32 (E8), NeuroD is expressed in the GCL (Roztocil et al., 1997) and thus NeuroD and ATH5 are coexpressed for a few days in this cell layer. No expression of ASH1 is detected in the developing GCL (Jasoni et al., 1994, and data not shown).

Sequential expression of bHLH genes during specification of retinal ganglion cells

Activation of the β3 subunit promoter, an early event in ganglion cell induction and differentiation (Matter et al., 1995), is under the direct control of bHLH factors (Hernandez et al., 1995; Roztocil et al., 1998). We first examined if cells in which the β3 subunit promoter was active were also expressing ATH5. Stage 25-26 (E5) retinal cells were transfected with a β3-promoter/*lacZ*-reporter plasmid, allowed to express the reporter gene for 24 hours and hybridized with a ATH5 probe (Fig. 5A). Most β3-positive cells were found to express ATH5, even though at this stage ATH5-expressing cells only represent about one-third of the total retinal cell population (Fig. 5B). In contrast, very few β3-positive cells expressed ASH1, providing further evidence that ASH1 and ATH5 are expressed in essentially distinct pools of precursor cells. Interestingly, ASH1 labeling in the rare cells that were both ASH1 and β3 positive was generally much weaker than in single-labeled cells.

About 30-40% of β3 positive cells express Ngn2, consistent with the high proportion (~75%) of cells that co-express Ngn2 and ATH5 (see Fig. 8F). The preferential expression of NeuroM in β3-positive cells (Fig. 5B) reflects the transient accumulation of this factor in newborn ganglion cells, which constitute the large majority of postmitotic cells at stage 25-26 (Prada et al., 1991). Similar proportions (~40%) of β3-positive, postmitotic cells express ATH5 or NeuroM (Fig. 5C), suggesting that a significant fraction of newborn ganglion cells coexpress these two factors. The *Delta1* gene is transiently expressed in postmitotic retinal cells (Henrique et al., 1997). We find that its expression is more frequent in β3-positive cells, confirming that Delta1 and NeuroM both mark the set of cells that have just stopped proliferating. In contrast, only a few β3-positive cells had begun expressing the late-onset NeuroD factor at stage 25-26 (data not shown). In summary, the β3 promoter is induced early on in ~15% (Matter et al., 1995) of the total pool of retinal progenitors and most β3-positive cells express ATH5. After these cells have left the mitotic cycle, they transiently continue to express ATH5, along with NeuroM and Delta1.

ATH5 regulates the β3 promoter in retinal cells

There is a key regulatory E-box element in the 143 bp β3 promoter (Roztocil et al., 1998). Gel mobility shift analysis indicates that the ATH5 protein binds to that promoter in vitro and that it specifically interacts with the E-box (Fig. 6A). The first retinal cells with an active β3 promoter are detected at stage 20 (Matter et al., 1995). From stage 24-25 on, promoter activity rapidly increases to culminate at stage 26-27 and then decreases to reach a stable low level by E8 (Fig. 6B and Matter et al., 1995). The burst of activity of the β3 promoter in early retina precisely coincides with the transient expression of ATH5 (Fig. 2H). If ATH5 plays a critical role in the induction of the gene for the β3 subunit, we reasoned that its overexpression in early retinal cells might force precocious activation of the β3 promoter. To test this notion, retinal cells were co-transfected at stage 23 (E3.5) with a β3-promoter/CAT-reporter plasmid and a ATH5

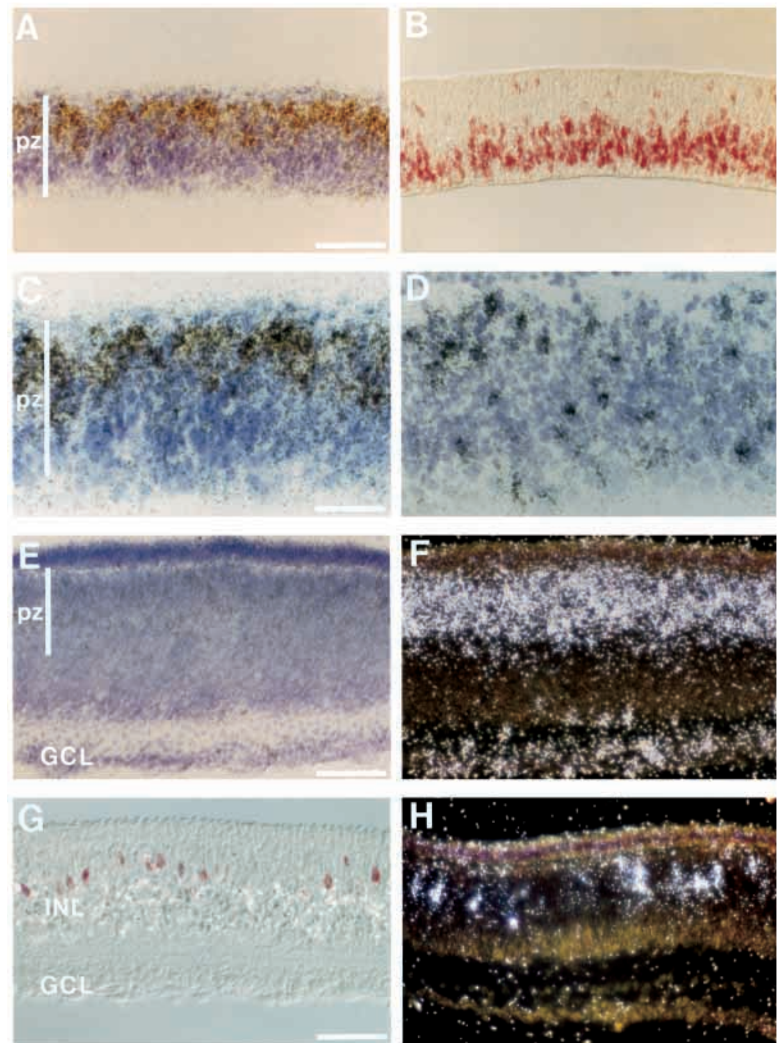


Fig. 4. ATH5 is expressed in progenitors and in the newly formed ganglion cell layer. (A-D) Stage 29 (E6) retina. (A) When ATH5 expression has reached peak levels and has expanded to the periphery, transcripts accumulate on the ventricular side of the retina. (B) The inner limit of the proliferative zone (pz) was revealed by pulse-labeling S-phase nuclei with BrdU for 30 minutes. (C) ATH5 transcripts are homogeneously distributed within the outer part of the pz whereas, at the same stage, NeuroM is strongly expressed in individual cells scattered throughout the thickness of the retina (D). (E,F) At E9, the levels of ATH5 transcripts have markedly decreased (E) and expression, as visualized by darkfield optics (F), occurs in two separate domains: there is a rather homogeneous distribution of transcripts throughout the pz and in the newly formed ganglion cell layer (GCL). (G,H) At E12, ATH5 is no longer expressed in the GCL. Some individual ATH5-expressing cells are still scattered in the inner nuclear layer (INL, H), along with a few BrdU-labeled cells (G). Scale bars: 80 µm in A,B; 40 µm in C,D; 50 µm in E,F; 70 µm in G,H.

expression vector, and allowed to express the transgene for 24 hours. Promoter activity was strongly stimulated in these cells and reached levels similar to that detected in cells transfected at stage 26-27 (E5) (Fig. 6B). Similarly, co-transfection of stage 23 retinal cells with a β3-promoter/lacZ-reporter plasmid and the ATH5 expression vector resulted in a tenfold increase in the number of β-galactosidase-positive cells. The β3 promoter was only activated in cells that overexpressed ATH5, excluding the possibility that activation results from an indirect effect mediated by cell-cell interactions (Fig. 6C).

At stage 26-29 (i.e., at the peak of β3 promoter activity and ATH5/Ngn2 expression), overexpression of ATH5 had no significant influence on promoter activity, suggesting that in precursors of ganglion cells the level of endogenous ATH5 protein was no longer limiting for β3 expression. From stage 30 onwards, the decrease of β3 promoter activity followed the decrease of endogenous ATH5 expression and could not be reversed by forced expression (Fig. 6B). Thus ATH5 activates the β3 promoter in a subset of progenitors, but late-stage maintenance in differentiated ganglion cells is ATH5-independent. These features agree quite well with the expression pattern of ATH5, whose mRNA all but disappears by E12 (Fig. 2H), whereas β3 expression continues into adulthood (Hernandez et al., 1995). We also tested whether the β3 promoter responded to overexpression of Ngn2 in retinal cells transfected at stage 23, and no significant effect was detected (Fig. 6D). In addition, ATH5 was found capable of activating β3 in newborn neurons that do not normally express either ATH5 or β3. For instance, although the β3 promoter is silent in the telencephalon, it is efficiently transactivated in stage 35 (E9) telencephalic cells by forced ATH5 expression. In contrast, forced expression of Ngn2, ASH1, NeuroM or NeuroD does not transactivate β3 in telencephalic cells (Fig. 6D; Roztocil et al., 1998), nor does ATH5 activate the β3 promoter in retinal glioblasts (Fig. 6D). Thus, it appears that ATH5 specifically regulates the β3 promoter, but only in neuronal precursors and newborn neurons.

ATH5 stimulates β3 in vivo

We next examined the topographic distribution of β3-positive

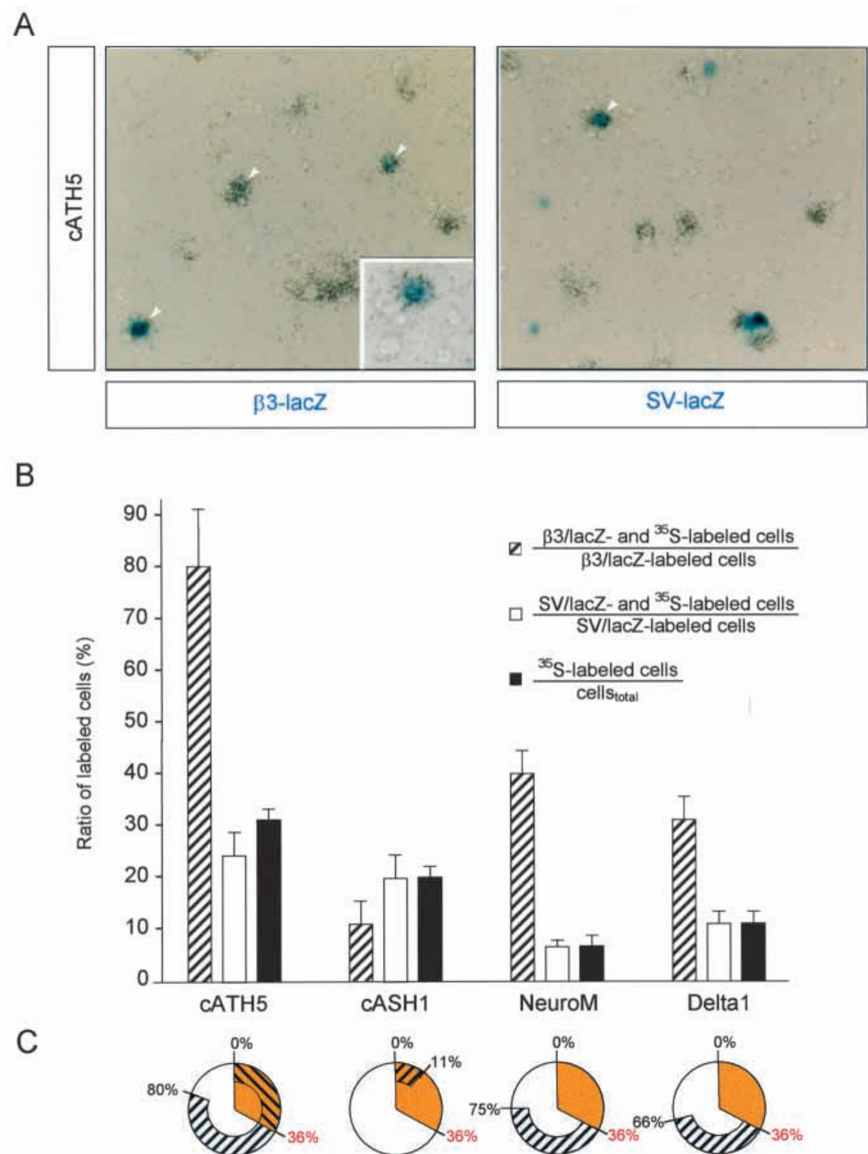


Fig. 5. Co-expression of the genes for the β3 subunit of the nicotinic acetylcholine receptor and bHLH proteins in retinal cells. (A,B) Retinal cells isolated at stage 25-26 (E5) were transfected with β3- or SV40-promoter/*lacZ*-reporter plasmids. (A) β-galactosidase-positive cells were revealed after 24 hours in culture and expression of chick ATH5 (cATH5) was detected by in situ hybridization. Arrowheads indicate double-labeled cells (β-galactosidase- and ^{35}S-positive). Inset shows one such cell at higher magnification. (B) The proportions of single- and double-labeled cells (β-galactosidase and/or ^{35}S-positive) were determined by cell counting after hybridization with ATH5, chick ASH1 (cASH1), NeuroM or Delta1 probes. Note that for each probe the proportions of ^{35}S-labeled cells in the total (black bars) and in the SV40-positive cell populations (white bars) are closely similar, indicating that the control SV40 promoter is equally active in all retinal cell types. In contrast, β3-positive cells express ATH5, NeuroM and Delta1 preferentially, whereas ASH1 expression is significantly under-represented (striped bars). (C) The pie charts visualize the population of β3-expressing cells which, at stage 26-28, represent approx. 15% of the total cell population (Matter et al., 1995). The orange sectors represent proliferating cells (approx. 36% of the β3-expressing cells, Matter et al., 1995). The hatched sectors represent cells expressing the probed genes (bHLH or Delta1, as indicated). Note that ATH5 is expressed both in dividing and in postmitotic cells. The small fraction of β3-expressing cells that do not express ATH5 may represent ganglion cells that have completed their differentiation (see Fig. 4). ASH1 is expressed in proliferating cells (Jasoni et al., 1994), whereas NeuroM and Delta1 are expressed in postmitotic cells (Roztocil et al., 1997; Henrique et al., 1997).

cells in retina that had been electroporated at stage 23 with a DNA mixture containing both a β3-promoter/*lacZ*-reporter construct and a ATH5 expression vector. Numerous ATH5-induced β3-positive cells were detected at the periphery of the retina (Fig. 7A), a region where β3 is not expressed until stage 30 in normal development (Matter et al., 1995). The observation that ATH5 activates a transfected β3 promoter in dissociated retinal cells and in retina explant prompted us to examine whether it can also induce expression of the endogenous β3 gene in retina. Stage 23 embryos were electroporated after a ATH5 expression vector had been microinjected into one optic cup, the contralateral eye serving as control. Dissected retinas were cultured as explants for 24 hours, total RNA was isolated from the control and transfected retinas and the presence of β3 mRNA was assessed by northern blot hybridization. As shown in Fig. 7B, the electroporated transgene caused the transfected retinas to accumulate β3 mRNA, whereas, as expected at this stage of development, β3 mRNA could not be detected in the control retinas. The signals correspond in sizes and ratio to the β3 mRNA species normally detected in E6 retina (Hernandez et al., 1995).

Identification of the ATH5 cis-regulatory domain

In order to outline the regulatory cascade leading to β3 expression and thus to the specification of ganglion cell identity, we next isolated the cis-regulatory domain of the ATH5 gene (Fig. 8A). We fused it to the CAT and lacZ reporter genes and tested its activity by transfection. Whereas it is silent in neurons from other brain compartments (e.g., optic tectum) or in non-neuronal cells (e.g., glioblasts, myoblasts and myotubes, Fig. 9B and data not shown), the domain displayed a robust promoter activity at early stages in the developing retina. The activity peaked between E5 and E7, in exact coincidence with the transient accumulation of the endogenous ATH5 mRNA (Figs 8B and 2H). In agreement with the expression pattern of ATH5 in retina (Fig. 4), the cis-regulatory domain displayed promoter activity both in proliferating retinal precursors and in newborn neurons (Fig. 8C-E). To confirm the specificity of the cloned ATH5 cis-regulatory domain, we transfected stage 28 retinal cells with a ATH5-promoter/*lacZ*-reporter plasmid and scored for *lacZ* expression and for endogenous ATH5 expression (Fig. 8F). All *lacZ*-positive cells contained ATH5 mRNA, indicating that the cloned ATH5 cis-regulatory domain faithfully reproduces the expression pattern of the endogenous *ATH5* gene in retina. In addition, we found that the large majority of cells that activate the ATH5 cis-regulatory domain also contain *Ngn2* mRNA, whereas only a relatively small fraction express ASH1 (Fig. 8F). Interestingly, β-galactosidase activity was consistently much weaker in cells expressing ASH1 than in cells that expressed ATH5 or Ngn2.

The ATH5 promoter is positively regulated by ATH5, Ngn2, NeuroM and NeuroD

The *ATH5* gene is active in proliferating precursors as well as

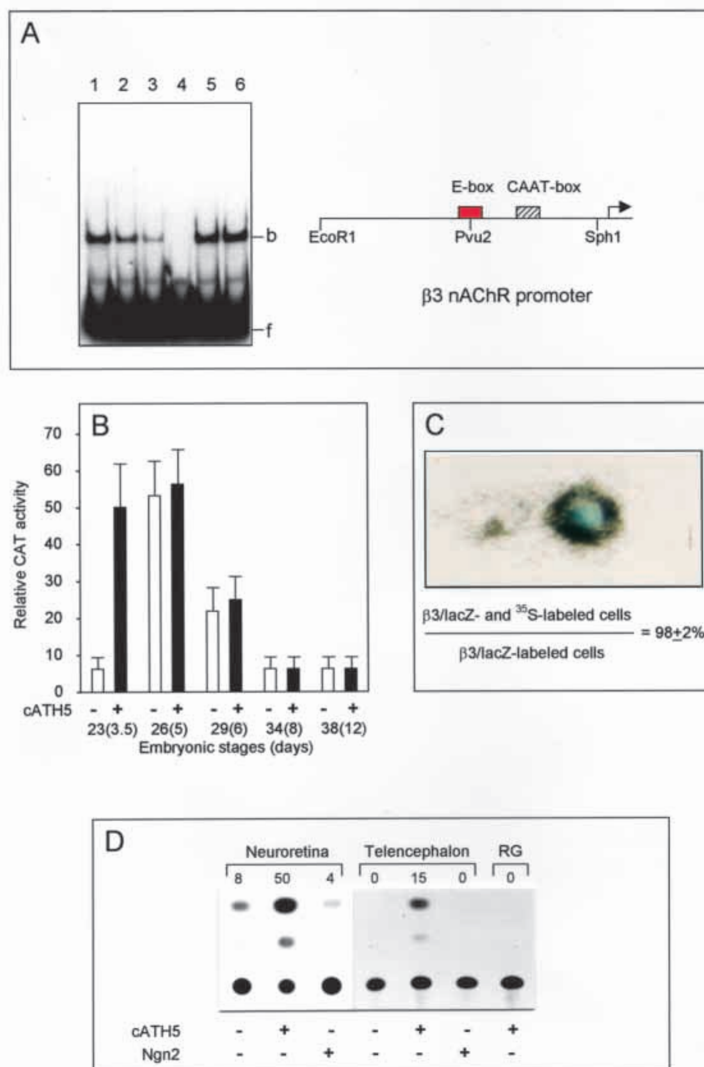


Fig. 6. ATH5 is a direct regulator of the gene for the β3 subunit of the nicotinic acetylcholine receptor (nAChR). (A) The bacterially expressed ATH5 protein binds the β3 promoter at the E-box. The protein-DNA complex (lane 1) is efficiently competed by 3× and 9× excess unlabeled β3 promoter (lanes 2,3). In contrast, 3× and 9× excess unlabeled, *Pvu*2-digested β3 promoter (lanes 5,6) do not prevent complex formation. Lane 4, negative control with no ATH5 protein. b (bound) and f (free) β3 promoter DNA. (B) Transactivation of the β3 promoter by chick ATH5 (cATH5). A β3-promoter/CAT-reporter plasmid was co-transfected with a ATH5 expression vector (EMSV-ATH5) or a control expression vector (EMSV-0) into retinal cells isolated between stages 23 (E3.5) and 38 (E12). Cells were assayed for CAT activity 24 hours after transfection. The CAT activity obtained upon transfection with the SV40-promoter/CAT-reporter plasmid is arbitrarily set at 100 for each developmental stages, and activities of the β3 promoter are given relative to this value. Note the strong transactivation of the β3 promoter at stage 23, whereas ATH5 has no significant influence on promoter activity at later stages. (C) Co-expression of overexpressed ATH5 and β3/*lacZ*. A β3-promoter/*lacZ*-reporter plasmid was co-transfected with a ATH5 expression vector into retinal cells isolated at stage 23. β-galactosidase-positive cells were revealed after 24 hours in culture and ATH5 expression was detected by in situ hybridization. The picture shows one of the many cells heavily labeled with ^{35}S (cytoplasm) and X-gal (nucleus). β3 promoter activity was only detected in cells overexpressing ATH5.
(D) CAT assay of stage 23 retinal cells, stage 34 telencephalic cells and retinal glioblasts (RG) transfected with a β3-promoter/CAT reporter plasmid in the presence (+) or absence (–) of a ATH5 or Ngn2 expression vectors. Cells were assayed for CAT activity 24 hours after transfection.

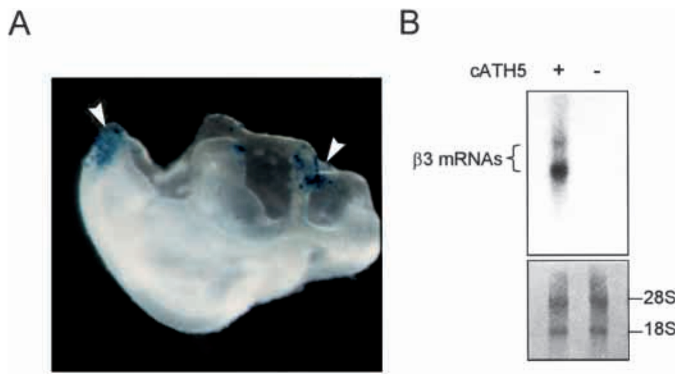


Fig. 7. ATH5 regulates β3 in vivo. (A) The β3-promoter/*lacZ*-reporter plasmid and the chick ATH5 (cATH5) expression vector were electroporated into the eye-cup of a stage 23 embryo and the retina was then grown for 24 hours as an explant. Note the presence of β-galactosidase-positive cells at the periphery of the retina (arrowheads). (B) ATH5 overexpression induces expression of the endogenous gene for the β3 subunit of the nicotinic acetylcholine receptor in retina. Northern blot of RNA isolated from ATH5-electroporated (+) or from control retinal explants (−) hybridized with a ^{32}P-labeled β3 probe. 28S and 18S rRNAs were stained with Methylene Blue.

in newborn neurons and its cis-regulatory domain contains seven E-box elements (Fig. 8A), raising the possibility that the gene is regulated by sequentially expressed bHLH transcription factors. We first examined the role of the ATH5 protein itself and found that it enhanced the activity of the ATH5 promoter in retinal cells at stage 28-30 (Fig. 9A). This self-stimulatory loop may help make ATH5 expression independent from inductive signals and/or overcome inhibitory signals. As assessed by expression of the ATH5-promoter/*lacZ*-reporter plasmid, the relatively weak activity of the ATH5 promoter in stage 28-29 retinal cells can be significantly enhanced by ATH5 overexpression. Forced expression of *Ngn2*, *NeuroM* and *NeuroD* also enhanced the activity of the ATH5 promoter (Fig. 9A), in remarkable agreement with the observation that these genes and ATH5 are coexpressed at various stages in the course of retinogenesis. In glioblasts selected from stage 28 retina, none of the neuronal bHLH genes are expressed above background levels and the transfected ATH5 promoter is silent (Fig. 9B). Forcing

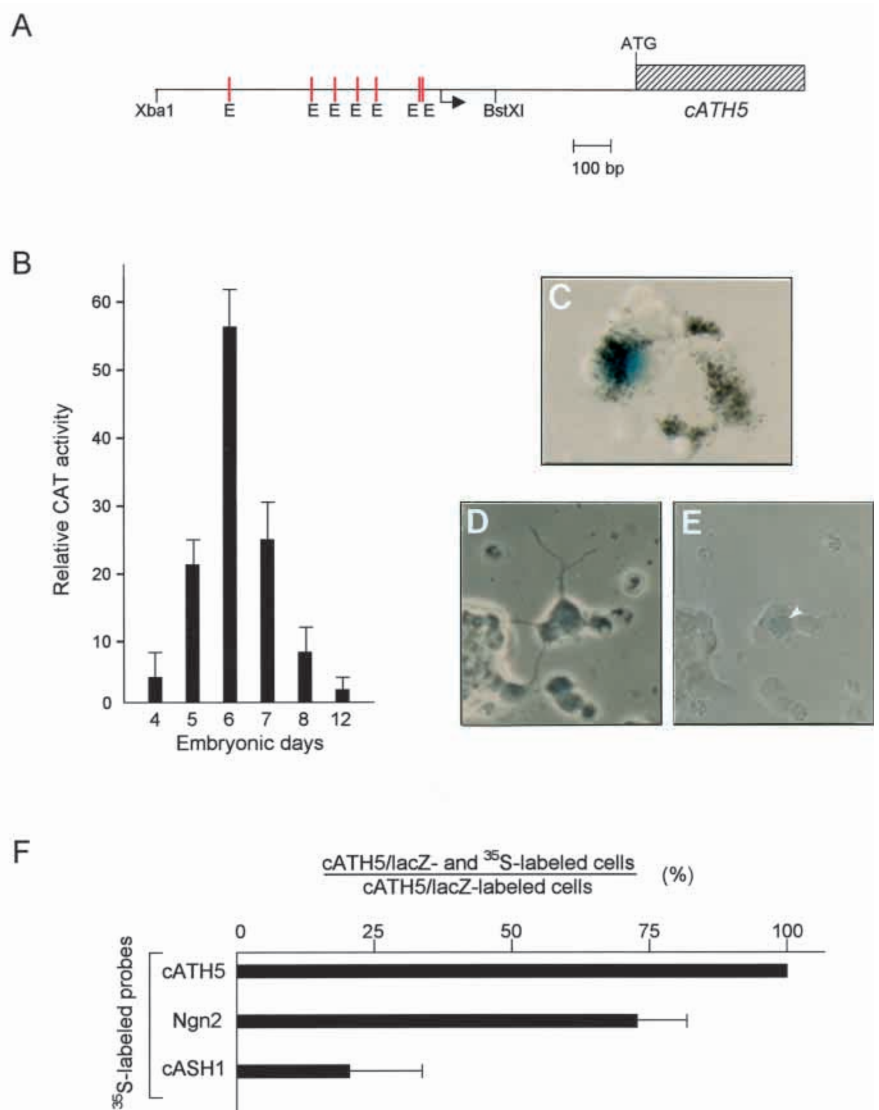


Fig. 8. Identification of the cis-regulatory domain of the chick *ATH5* (*cATH5*) gene. (A) Schematic structure of the 5′-flanking region extending 1301 bp upstream of the initiator ATG. The transcription initiation site (arrow) has been localized 640 bp upstream of the initiator ATG. The positions of seven E-box elements are indicated. (B) A restriction fragment, 912 bp in length, extending between the *Xba*1 and *Bst*X1 sites in A was fused to the gene for CAT. The construct was transfected into retinal cells isolated between stages 23 (E3.5) and 38 (E12) and the cells were assayed for CAT activity 24 hours later. The activity obtained upon transfection with the SV40-promoter/CAT-reporter plasmid is arbitrarily set at 100 for each developmental stage, and activities of the ATH5 promoter are given relative to this value. Note that CAT activity exactly follows the kinetics of endogenous ATH5 mRNA accumulation (Fig. 2H). (C-E) Stage 28-30 (E6) retinal cells were transfected with the ATH5-promoter/*lacZ*-reporter plasmid and grown in vitro for 24 hours. [^{3}H]thymidine was added to the culture medium 1 hour before revealing β-galactosidase activity. (C) One of many cells whose nuclei were labeled by β-galactosidase and by [^{3}H]thymidine. (D,E) Phase-contrast and brightfield views of a ganglion-like cell whose nucleus (arrowhead) was β-galactosidase positive. In general, the promoter fragment had a stronger activity in proliferating cells than in newborn neurons, as shown here. (F) Stage 28-30 retinal cells transfected with a ATH5-promoter/*lacZ*-reporter plasmid and grown in vitro for 24 hours were hybridized with ATH5, Ngn2 or chick ASH1 (cASH1) riboprobes. The single- and double-labeled cells were quantified by cell counting. 99%±1 of β-galactosidase-positive cells expressed ATH5.

expression of ATH5, Ngn2, NeuroM or NeuroD in these cells was sufficient to activate the co-transfected ATH5 promoter (Fig. 9B). Moreover, the transfected ATH5 promoter behaved much as the native promoter does, as evidenced by the finding that the endogenous ATH5 promoter also responded to misexpressed Ngn2 (Fig. 9B, inset). We find that the *ATH5* gene is not expressed and the transfected ATH5 promoter is inactive in the optic tectum and in the telencephalon even though Ngn2, NeuroM and NeuroD are all expressed in these brain compartments during development. Moreover, overexpression of these bHLH proteins in tectal cells does not activate the ATH5 promoter (data not shown). In the retina, the *ATH5* gene is not expressed and the ATH5 promoter is inactive beyond stage 38 (E12) (Figs 2, 8). The maturing retina, however, continues expressing NeuroM and NeuroD (Roztocil et al., 1997). Overexpression of these bHLH proteins in E12 retinal cells do not activate the ATH5 promoter. Therefore, it appears that the capacity of Ngn2, NeuroM and NeuroD to activate ATH5 expression is restricted to the differentiating retina.

Regulation of ATH5 by NeuroM is dependent on exit from the cell cycle

Since the *ATH5* gene appears to be regulated by bHLH proteins that are expressed at successive stages of neurogenesis, we examined whether its induction by ATH5, Ngn2 or NeuroM requires prior exit from the cell cycle. Retinal glioblasts were co-transfected with the ATH5-promoter/*lacZ*-reporter plasmid and the ATH5, Ngn2 or NeuroM expression vectors. In addition, proliferating cells were labeled with [^{3}H]thymidine during the 24 hours period allowed for *lacZ* expression (Fig. 9C). The ATH5 and Ngn2 proteins activated the ATH5 promoter both in proliferating and in postmitotic cells, whereas induction by NeuroM was restricted to postmitotic cells. NeuroM can thus stimulate ATH5 expression during its own short period of activity (Roztocil et al., 1997), when cells have

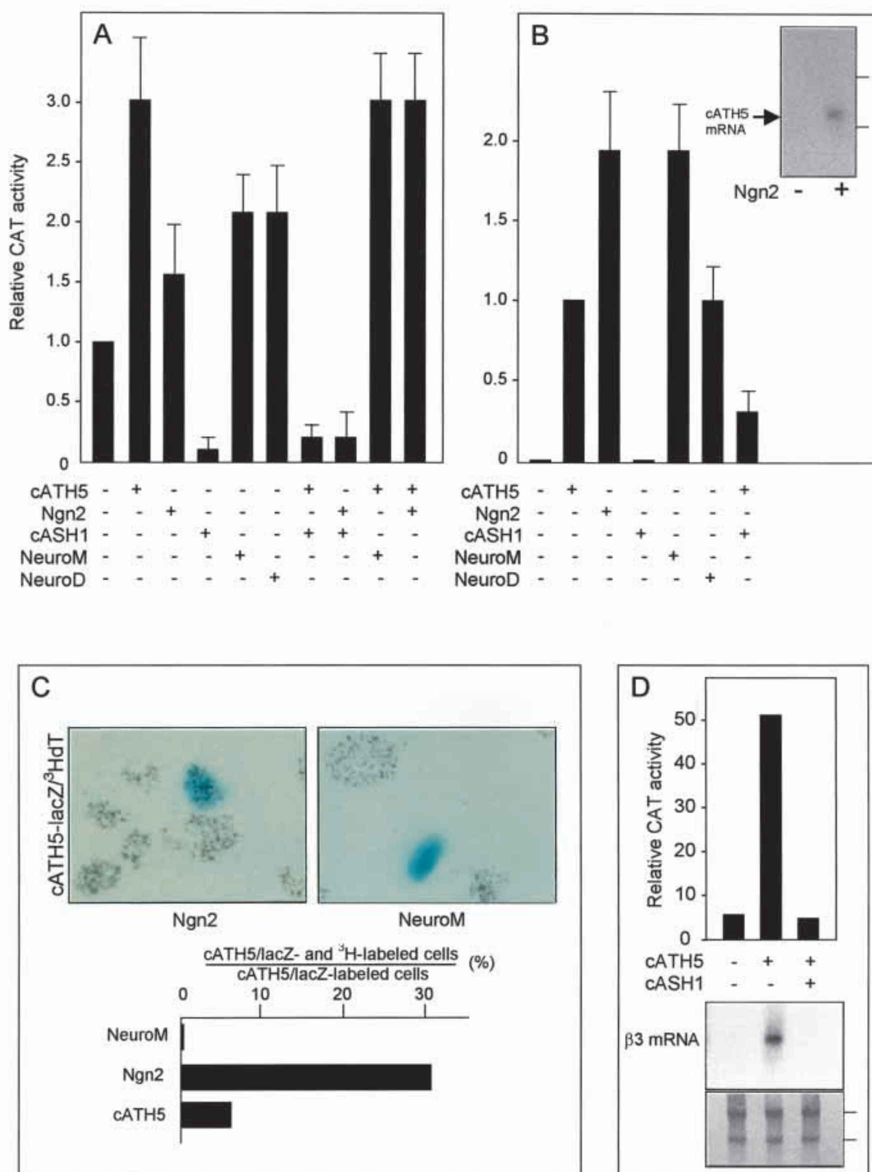


Fig. 9. bHLH proteins modulate the activity of the chick ATH5 (cATH5) cis-regulatory domain. (A,B) A ATH5-promoter/CAT-reporter plasmid was co-transfected with one or a pair of bHLH expression vectors into stage 28-30 (E6) retinal cells (A) or into retinal glioblasts (B). Cells were assayed for CAT activity 24 hours (A) or 48 hours (B) after transfection. Activities obtained upon co-transfection with a ATH5-promoter/CAT-reporter plasmid plus a negative control expression vector (A) or a ATH5 expression vector (B) are arbitrarily set at 1.0. Inset shows ATH5 probe hybridization to a blot of RNA isolated 48 hours after cell transfection in the presence (+) or absence (–) of an Ngn2 expression vector. The endogenous *ATH5* gene is transcribed in the cells that misexpress Ngn2. (C) Activation of the ATH5 promoter in proliferating or postmitotic retinal glioblasts by forced expression of ATH5, Ngn2 or NeuroM. Cells co-transfected with a ATH5-promoter/*lacZ*-reporter plasmid and the appropriate expression vector were cultured for 24 hours in the presence of [^{3}H]thymidine. The proportions of double-labeled cells (β-galactosidase and [^{3}H]thymidine-positive) were determined by cell counting. Note that Ngn2 and ATH5 activated the ATH5 promoter in [^{3}H]thymidine-positive cells, whereas NeuroM did so only in postmitotic cells. (D, upper panel) chick ASH1 (cASH1) inhibits the stimulatory activity of ATH5 upon the β3 promoter. A β3-promoter/CAT-reporter plasmid was co-transfected with ATH5 alone or with ATH5 plus ASH1 expression vectors into stage 23 (E3.5) retinal cells. Cells were assayed for CAT activity 24 hours after transfection. (D, lower panel) ATH5 and ASH1 overexpression modulates expression of the endogenous gene for the β3 subunit of the nicotinic acetylcholine receptor in retina. Stage 23 retinas were electroporated in vivo with ATH5 or ATH5 plus ASH1 vectors. Total RNA was assayed for β3 mRNA after 24 hours in explant culture.

stopped proliferating but have yet to mature into fully differentiated neurons.

ASH1 exerts a dominant-negative effect upon the ATH5 promoter

As detailed in Fig. 9A, forced expression of ASH1 resulted in a marked decrease in the intrinsic activity of the ATH5 promoter. Moreover, when the *ATH5* or the *Ngn2* genes were co-expressed with ASH1 in stage 28-30 retinal cells, ASH1 abolished the stimulation due to *ATH5*/*Ngn2* (Fig. 9A) and a similar inhibitory activity was evident in retinal glioblasts (Fig. 9B). The strong dominant-negative effect that ASH1 exerts over the ATH5 promoter apparently does not result from unspecific interactions between bHLH factors because overexpression of Ngn2 or NeuroM does not influence the stimulatory activity of ATH5 (Fig. 9A). The dominant-negative effect of ASH1 is promoter-specific, since ASH1 does not affect the bHLH-regulated nicotinic acetyl choline receptor α1 subunit core promoter (Roztocil et al., 1998). It is remarkably congruent with the observed, mutually exclusive expressions of ATH5 and ASH1 in the retinal neuroepithelium (Fig. 3) and may be one of the mechanisms that contain ATH5 expression within a subset of retinal progenitors. When stage 23 retinal cells were co-transfected with the β3-promoter/CAT-reporter plasmid and with the ASH1 and ATH5 expression vectors, the ATH5-mediated induction of the β3 promoter was completely abolished (Fig. 9D). We have examined whether ASH1 also mediates inhibition of the endogenous β3 gene. Stage 23 embryos were electroporated after expression vectors had been microinjected into one optic cup, the contralateral eye serving as control. As shown in Fig. 9D, the electroporated ATH5 gene causes the retina to accumulate β3 mRNA, whereas no accumulation takes place when ATH5 and ASH1 are electroporated together. This clearly shows that ASH1 does influence the expression of ATH5-regulated genes in vivo and that ASH1 exerts a dominant-negative effect over β3 gene expression. These results are consistent with the mutually exclusive expression of β3 and ASH1 (Fig. 5). The functional properties of the cloned and endogenous β3 promoter have thus been defined both in acutely isolated retinal neurons and in vivo and the results obtained in the two systems are remarkably consistent.

DISCUSSION

We have investigated the developmental mechanisms underlying the specification of neurotransmitter receptor identity in ganglion cells. The evidence, both from gene expression patterns and from promoter activity analysis, indicates that the *atonal* homolog ATH5 is the natural activator of the gene for the β3 subunit during retinogenesis. Interactions between ATH5 and several other bHLH factors underlie the patterning of the early retinal neuroepithelium and contribute to define the competence of retinal progenitors to generate ganglion cells. The activity of the ATH5 promoter is positively and negatively regulated by bHLH factors that are sequentially expressed during development. Because ATH5 is part of the tightly regulated genetic program specifying ganglion cell identity and also responds to bHLH proteins known to be widely expressed within the CNS, it provides a link between the regulatory pathways controling subtype-specific and pan-neuronal genes. Thus, ATH5 appears to coordinate different aspects of ganglion cell specification within the overall program of retinogenesis.

ATH5 activates the gene for the β3 subunit of the nicotinic acetylcholine receptor in developing retina

In the developing retina, the stringent neuron subtype-specific expression of the β3 gene is a tightly regulated part of the genetic program specifying ganglion cell identity. The β3 promoter, however, has a relatively simple organization: it is devoid of multipartite elements whose combinations could integrate complex transcriptional codes and a single E-box mediates its neuronal specificity. The E-box and its flanking sequences suffice to select the appropriate bHLH proteins from among a variety of related factors (Roztocil et al., 1998). These properties suggested that expression of the β3 gene was controlled by a particular bHLH transcription factor whose expression pattern was similar to that of β3 and which might function as a coupling device between the specification of ganglion cell identity and the overall program of neurogenesis. Recent studies have shown that expression of the frog and mouse *atonal* homologs of ATH5 is restricted to the retina and to a very few other regions of the nervous system (Kanekar et al., 1997; Brown et al., 1998). Likewise, we find that expression of ATH5 is confined to the developing retina and to a tiny cell population in the ventral neural tube. We show here that ATH5 is expressed in a pool of proliferating progenitors and in newborn ganglion cells. Its transient expression in the newly formed ganglion cell layer coincides with the onset of β3 expression in this layer (Fig. 4; Hernandez et al., 1995; Matter et al., 1995). Time-course experiments demonstrate that ATH5 expression and activation of the β3 promoter in ATH5-expressing cells follow precisely the same kinetics (Figs 2H, 5, 6B; Matter et al., 1995), and that both culminate in the period when the majority of ganglion cells are born. These convergent lines of evidence indicated that ATH5 may induce β3 expression during specification of ganglion cell identity. This was demonstrated by transfection in retina explants and in dissociated retinal cells: forced expression of ATH5 one day before endogenous expression takes place induced a precocious transcription of the endogenous β3 gene and a robust transactivation of an exogenous β3 promoter (Figs 6B, 7). This effect was found not to be limited to the retina, as ATH5 misexpression also induced ectopic activation of the β3 promoter in populations of newborn central neurons, whereas Ngn2, NeuroM and NeuroD did not. Taken together, our data thus strongly suggest that ATH5 is the natural activator of β3 during retinogenesis. How does the β3 promoter selects ATH5 from among the other members of the bHLH protein family? Binding at the E-box is not sufficient to confer activity to bHLH factors (Davis et al., 1990) and sequences flanking the E-box also play an important role (Weintraub et al., 1994). For example, NeuroD/Beta2 regulates the insulin promoter through interaction with the CATCTG E-box (Naya et al., 1995), and mutation of one central base pair in this element (CAgCTG) does not affect binding or promoter activity (Whelan et al., 1990, Naya et al., 1995). In contrast, although NeuroD/Beta2 binds the β3 CAGCTG E-Box – the same sequence as the functional insulin promoter mutant – it does not regulate the β3 promoter (Roztocil et al., 1998). We have previously shown

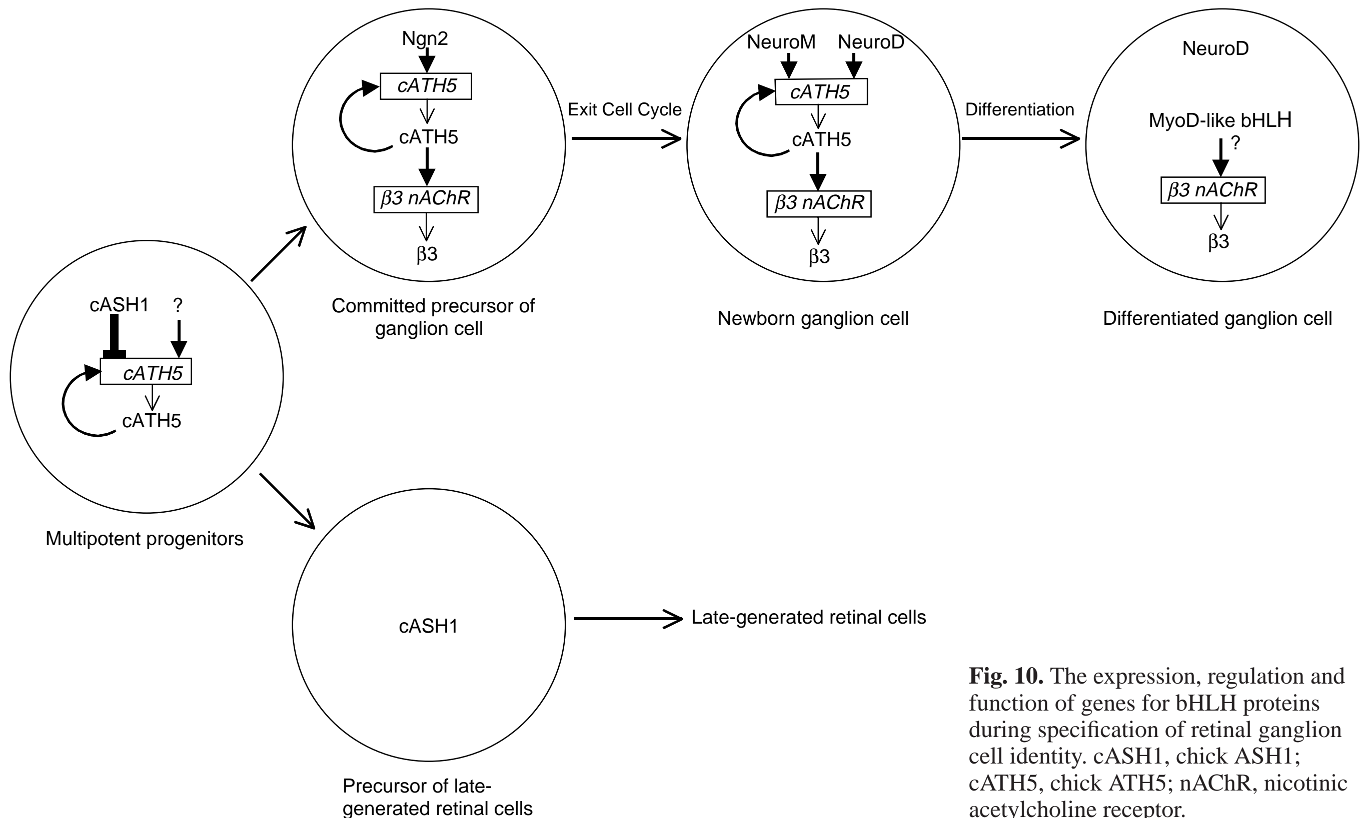


Fig. 10. The expression, regulation and function of genes for bHLH proteins during specification of retinal ganglion cell identity. cASH1, chick ASH1; cATH5, chick ATH5; nAChR, nicotinic acetylcholine receptor.

that the sequence located in 3′ of the β3 E-box participates in the selection of bHLH proteins (Roztocil et al., 1998). Furthermore, by swapping protein domains we have obtained preliminary evidence suggesting that the specificity of a bHLH protein for the β3 promoter may be, at least in part, encoded by its HLH domain. The selection of ATH5 might thus occur through a dimerization process as well as at the level of DNA-protein interactions through the basic domain (D. Skowronska-Krawczyk, L. M. S., M. B., J. M. M., unpublished observations). Additional experiments will be required to identify the putative heterodimerizing partners of ATH5 and to demonstrate that they participate directly in the regulation of the β3 promoter.

The expression patterns of several bHLH genes define the domain of ganglion cell determination

Induction of ATH5 expression occurs in the central retina about half a day prior to the appearance of the first ganglion cells (Prada et al., 1991; McCabe et al., 1999), and a similar delay is also seen in the mouse (Brown et al., 1998). The onsets of ATH5 and Ngn2 expression coincide in the central retina and their common expression domain is bounded by an annular region expressing ASH1 (Figs 2, 3). This mutually exclusive expression pattern resembles the neuroepithelial regionalization that occurs in most parts of the mammalian and avian CNS (Ma et al., 1997; M. T. O., unpublished observations). Our results suggest that the patterning of the early retinal neuroepithelium develops as the consequence of the antagonistic effects of ASH1 and ATH5/Ngn2 upon the ATH5 promoter, leading to the formation of two distinct pools of progenitors expressing either ATH5 or ASH1 (Fig. 10). ATH5 expression is a part of the genetic network enabling early retinal progenitors to make ganglion cells, while ASH1 expression defines the multipotent mitotic progenitors that will yield later-born retinal cells. Both types of progenitors are thought to segregate from a common pool of *ATH5*/*ASH1* competent cells as a result of changes in the relative expression levels of these two genes, precursors of ganglion cells being selected out by enhancement of ATH5 expression. After ATH5 reaches expression levels sufficient both to overcome the inhibitory effect of ASH1 and to activate β3, the ATH5-expressing progenitors may progress from a transient status to the state of fully committed ganglion cell precursors (Fig. 10). That there are transition states along the ganglion cell determination pathway is indicated by (1) the presence of low but significant fractions of cells expressing ASH1 and activating either the ATH5 or the β3 promoters; and (2) the finding that only about one third of ATH5-positive cells express the β3 subunit (Figs 5, 8). This dynamic changes in the status of precursors are restricted to a short period of development and overexpression of ATH5 at E5-E6 (i.e. at the peak of β3 and ATH5 expression) had no influence on β3 promoter activity (Fig. 6B). We surmise that at this stage of development, the endogenous level of ATH5 is no longer limiting within the pool of β3-positive cells, a pool that cannot be expanded because of the high level of ASH1 expression in other cell types. Induction of β3 by ATH5 in a subset of proliferating precursors certainly marks one of the earliest detectable steps of ganglion cell specification (Matter et al., 1995 and Figs 5C, 6B, 7) and highlights an interesting aspect of neurogenesis: cell determination or fate commitment may include expression of a terminal differentiation gene. The divergence of the ganglion

cell lineage may represent the first of several possible branch points on a pathway along which initially multipotent progenitors progress to produce distinct retinal cell-types (Cepko, 1999). Such branch points would generate neurons of different identities in proper number and order by restricting the competence of early progenitor lineages and by preserving a population of multipotent ASH1-expressing progenitors for the generation of later-born neurons. Overexpression of ATH5 in the developing *Xenopus* retina results in an increase in ganglion cells and a decrease in amacrine, bipolar and Muller glia cells (Kanekar et al., 1997). We have shown that overexpression of *ATH5* in early retinal cells markedly stimulates transcription of the gene for β3 and expands its expression domain, but that these effects can be effectively antagonized by ASH1 overexpression (Fig. 9D). Thus, because of its dominant-negative effect upon ATH5 and β3, ASH1 may help contain the domain of ATH5 and β3 expression, thereby preventing the whole pool of retinal progenitors from entering the ganglion cell differentiation program. If ASH1 is part of the genetic program keeping production of ganglion cells under control, we would expect that this population of neurons might increase in the retina of *Mash1* (*Ascl1* – Mouse Genome Informatics) knockout mice. Unfortunately, these mutant mice die before retina development is completed (Guillemot et al., 1993) and no comparative analysis of the proportions of ganglion cells in the wild-type and *Mash1*-null retina explants has been reported (Tomita et al., 1996). Other mechanisms, in conjunction with ASH1, may prevent untimely and excessive production of ganglion cells. In particular, the Delta-Notch signaling pathway is involved in the process whereby ganglion cell progenitors become newborn neurons (Henrique et al., 1997). The β3-expressing cells are among the first retinal precursors to leave the mitotic cycle (Matter et al., 1995) and Delta1 is expressed in nascent β3-positive ganglion cells (Fig. 5), from where it may sustain expression of Notch in neighboring progenitors, thereby keeping these cells in an uncommitted state.

Regulatory interactions between bHLH transcription factors during specification of ganglion cell identity

Our data suggest that the patterns of ATH5/Ngn2 and ASH1 expression in the retinal neuroepithelium define two distinct cell lineage domains and that specification of the β3 component of ganglion cell identity depends on the establishment of the ATH5 expression domain. The autoactivation of ATH5 may play an important role in initiating an autonomous program of ganglion cell differentiation, but it may not be sufficient for its long-term maintenance. As revealed by in situ hybridization, the onsets of Ngn2 and ATH5 expression coincide and Ngn2 is expressed in the majority of ATH5-positive cells (Figs 2, 8F). Moreover, we have shown that Ngn2 positively regulates the cloned ATH5 promoter in retinal cells, and activates the endogenous gene in retinal glioblasts. We do not know yet if Ngn2 is involved in the induction of ATH5 expression but it may, at least, contribute to the maintenance of ATH5 expression in proliferating progenitors (Fig. 10). ATH5 is transiently expressed in newborn neurons and other bHLH proteins may control its regulation at later stages of retinogenesis. In the CNS, the transient expression of NeuroM marks cells that have just left the mitotic cycle and, in keeping with this rule, newborn ganglion cell transiently express this factor (Roztocil et al., 1997). Because the capacity of NeuroM to stimulate the ATH5 promoter is restricted to postmitotic cells (Fig. 9C), NeuroM may transiently ensure ATH5 expression in newborn ganglion cells. NeuroD, whose onset in the ganglion cell layer occurs later than that of NeuroM (Roztocil et al., 1997), may exert its demonstrated ability to activate ATH5 (Fig. 9A) at the ultimate stages of ganglion cell differentiation. It is unclear, however, why several different bHLH proteins should be required for the positive regulation of ATH5. One reason might be that the autostimulatory capacity of ATH5 is inhibited at some stage and needs to be relayed by other factors. Another possibility is that Ngn2, NeuroM and NeuroD cooperate with ATH5 to overcome the negative effect of ASH1 and to enhance the overall level of ATH5 expression. Molecular mechanisms whereby ASH1 may exert a dominant-negative effects are still unclear. Gradwohl et al. have shown that heterodimers containing ASH1 and Ngn2 do not bind to E-box elements (Gradwohl et al., 1996). Similarly, ASH1 and ATH5/Ngn2 may also form heterodimers that do not interact with the E-boxes in the ATH5 and β3 promoters. Alternatively, ASH1 may bind to these E-boxes and thus prevent binding and activation by ATH5. The ATH5 promoter has a more complex organisation than the β3 promoter. At least four of the seven E-box elements in the ATH5 promoter are functional and mutational analysis indicates that a particular E-box may preferentially react with a particular bHLH protein (J. H., L. M. S., J. M. M., M. B., unpublished observations). A rather complex interplay between these elements may thus enable the ATH5 promoter to integrate the effects of stimulatory (e.g., ATH5, Ngn2, NeuroM, NeuroD) and inhibitory (e.g., ASH1) factors.

In transactivation assays, the ATH5 promoter fails to respond to bHLH factors after retinal cells have differentiated, a change in promoter properties that is remarkably congruent with the absence of ATH5 expression in the developed retina. As ASH1 is not expressed in mature retina, the mechanism whereby ATH5 is repressed at late stages of retina development must differ from those operating during neurogenesis. Likewise, the β3 promoter no longer responds to ATH5 after ganglion cells have completed their differentiation and we surmise that late in development a different transcriptional code maintains β3 expression. The proven ability of the myogenic factor MyoD to stimulate β3 transcription in differentiated neurons (Roztocil et al., 1998) suggests that the putative regulators of β3 in mature retina share some functional properties with MyoD.

ATH5, Ngn2 and ASH1 operate within the context of a general program of retinogenesis

Our results provide some insights into the molecular and cellular interactions contributing to the formation of two different pools of progenitors in the retinal neuroepithelium. However, the origin of the early patterning still remains an unresolved issue because we do not know the nature of the initial signals inducing expression of the *ATH5*, *Ngn2* and *ASH1* genes. In the embryonic spinal cord of chick and fish, the differential expressions of ASH1 and of the neurogenins along the dorsoventral axis appears to result at least in part from extrinsic determinants (Blader et al., 1997; Schneider et al., 1999). It would be interesting to establish whether tissues in contact with the neuroretina (e.g., the presumptive pigmented

epithelium) can influence regionalization in the retinal neuroepithelium. FGF has been shown to influence the differentiation of ganglion cells (Guillemot and Cepko, 1992; Zhao and Barnstable, 1996), and it has recently been reported that the activation of FGF receptors is required for the ganglion cell differentiation front to progress from center to periphery (McCabe et al., 1999), suggesting a possible contribution of FGF to the patterning of the retinal neuroepithelium. The ATH5 promoter assay we describe should be a useful tool with which to assess the effects of extrinsic cues on induction of ATH5 expression in retinal cells. Another possibility is that induction of both ATH5 and ASH1 results from a downregulation of the inhibitory *Hes* genes. In mice, Hes1 represses expression of ASH1, ATH5 and the neurogenins (Ishibashi et al., 1995; Brown et al., 1998; Ma et al., 1998), and we found that this factor represses activity of the ATH5 promoter in chick retinal cells (unpublished data). In agreement with these findings, we observed that avian Hes1 (Takebayashi et al., 1994; Palmeirim et al., 1997) recedes from the central retina at the onset of ATH5, Ngn2 and ASH1 expression (B. Barabino, L. M. S., J. M. M. and M. B., unpublished observations). The predominant expression of ASH1 at the periphery of the ATH5/Ngn2 central domain raises the possibility that transcription of the *ASH1* gene might be less sensitive to the inhibitory effect of Hes1 than the *ATH5* or *Ngn2* genes. Fode et al. have shown that Ngn1 and Ngn2 activities are required to repress mASH1 expression in the dorsal telencephalon (Fode et al., 2000). A similar relationship between Ngn2 and ASH1 may exist in the avian retina. In this scheme, Ngn2 would downregulate ASH1 and upregulate ATH5, thus leading to the predominant expression of Ngn2 and ATH5 in the central retina, at the peak of ganglion cell generation. On the other hand, the capacity of ASH1 to repress ATH5 expression may contribute to preserve a pool of ASH1-expressing cells in the central domain. Although the domains of ATH5/Ngn2 and ASH1 expression are initially sharply segregated in the central retinal neuroepithelium (Fig. 3A), dynamic changes in expression patterns occur in the course of retinogenesis. ATH5 expression (and in its wake the ganglion cell determination domain) expands to the periphery, but it remains bounded by an annular zone of ASH1 expression (Fig. 3C). As development proceeds, the ATH5- and ASH1-expressing domains partially overlap. Within the boundary zone, ASH1-expressing cells are always located at the peripheral edge, suggesting an interesting homology with the ciliary marginal zone in *Xenopus* retina (Perron et al., 1998). This dynamic zone may plausibly coincide with the front of ganglion cell differentiation as defined by McCabe et al. (McCabe et al., 1999) and is likely to be the playground for a number of signals initiating and mediating cellular interactions crucial for cell determination. Behind the boundary zone, ATH5-expressing precursors would cease proliferating and enter a ganglion cell differentiation program, whereas ASH1-expressing cells would continue to proliferate and replenish the progenitor pool of later born retinal cells. As a result of this process, the ATH5 and ASH1 expression domains finally overlap and the intermingled ATH5- and ASH1-expressing cells represent, on E5, respectively, approx. 30% and approx. 20% of the retinal cell population (Fig. 5B).

A complex dynamic equilibrium between positive and negative cross-regulations may thus contribute to the patterning of the retinal neuroepithelium. The experimental model we have developed should help analyse in greater details the functional interactions between transcription factors and genes involved in retinogenesis. In addition, our results highlight the many potential similarities between the cellular and molecular mechanisms leading to pattern formation in the retina and those that operate within proneural domains in *Xenopus* and zebrafish embryos and in the proneural strips of the *Drosophila* eye.

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