A bHLH transcriptional network regulating the specification of retinal ganglion cells

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Summary

In the developing retina, the production of ganglion cells is dependent on the proneural proteins NGN2 and ATH5, whose activities define stages along the pathway converting progenitors into newborn neurons. Crossregulatory interactions between NGN2, ATH5 and HES1 maintain the uncommitted status of ATH5-expressing cells during progenitor patterning, and later on regulate the transition from competence to cell fate commitment. Prior to exiting the cell cycle, a subset of progenitors is selected from the pool of ATH5-expressing cells to go through a crucial step in the acquisition of a definitive retinal ganglion cell fate. The selected cells are those in which the upregulation of NGN2, the downregulation of HES1 and the autostimulation of ATH5 are coordinated with the progression of progenitors through the last cell cycle. This coordinated pattern initiates the transcription of ganglion cell-specific traits and determines the size of the ganglion cell population.

Key words: Retinogenesis, Retina patterning, Basic helix-loop-helix, Transcription, Chick embryo

Introduction

The early development of the vertebrate central nervous system crucially depends on the timely generation of specific classes of neurons at distinct positions and in appropriate numbers. A large set of different regulatory proteins are known to be implicated in neurogenesis, and considerable progress has been made in piecing together the pathways specifying both the generic neuronal traits and the subtype identity traits (Anderson, 2001; Bertrand et al., 2002; Jessell, 2000). However, the underlying transcriptional networks through which neuronal identity, number and position are coordinately regulated remain poorly defined.

The retina is one region of the central nervous system in which the conversion of progenitor cells into particular classes of neural cells is quite well understood (Cepko, 1999; Harris and Holt, 1990; Reh and Levine, 1998). Retina ontogenesis is geared to generate glia and six classes of retinal neurons from an undifferentiated neuroepithelium, according to a program that controls proliferation, specification, exit from the cell cycle and differentiation. Cell differentiation initiates in the inner layer of the central optic cup and progresses radially to the peripheral edge of the retina. A characteristic feature of vertebrate retinogenesis is that the different retinal cell types are generated in a fixed sequence. Retinal ganglion cells (RGC) differentiate first, followed in overlapping phases by amacrine cells, horizontal cells, cone photoreceptors, rod photoreceptors, bipolar cells and, finally, Müller glial cells.

The generic programs of neuronal differentiation are regulated in vertebrates as in *Drosophila* (Anderson and Jan, 1997) by members of the basic helix-loop-helix (bHLH) class of transcription factors. The *achaete-scute* homologue ASH1 and the three neurogenins (NGN1-NGN3) are among the earliest bHLH genes expressed in the developing nervous system and they are thought to act as proneural genes. A spatial complementarity between the expression of ASH1 and of the neurogenins appears to be the rule in most proliferating neuroepithelia and these factors have a role in the ontogeny of distinct classes of progenitors (Bertrand et al., 2002). In the developing retina, most of the broadly expressed neurogenic bHLH proteins are likewise implicated in the generation of distinct classes of neurons (Inoue et al., 2002; Vetter and Brown, 2001), but it is unresolved whether these factors act individually or combine to promote particular neuronal phenotypes. Likewise, the molecular mechanisms that control the timing of their expression and/or function are poorly defined.

The *atonal* homologue ATH5 is almost exclusively expressed in the developing retina. Initially cloned and analysed in *Xenopus* (Kanekar et al., 1997), its mouse (Brown et al., 1998), zebrafish (Masai et al., 2000), chicken (Liu et al., 2001; Matter-Sadzinski et al., 2001) and human (Brown et al., 2002) orthologues have also been identified. In the mouse,

inactivation of the *Ath5* gene results in a retina lacking most RGCs and, as a consequence, in optic nerve agenesis (Brown et al., 2001; Wang et al., 2001). Although ATH5 is directly involved in its own regulation and is able to activate genes that define neuronal identity traits (Liu et al., 2001; Matter-Sadzinski et al., 2001; Skowronska-Krawczyk et al., 2004), the mechanism for integrating ATH5 expression into a coherent program of RGC specification and differentiation has not been elucidated.

Lineage tracing studies have led to the hypothesis that retinal progenitors pass through a series of different competence states during which they sequentially produce different types of neural cells (Livesey and Cepko, 2001). This model suggests that progenitors may be limited to producing certain types of neurons at a given time in the course of retinogenesis. Here, we attempt to define at the molecular level the time frame and cellular context in which progenitors yield RGCs in the developing chick retina. Specifically, we identify several of the stages along the pathway leading to the conversion of progenitors into newborn RGCs. We show how the interplay between ATH5 and other bHLH proteins controls the transitions between stages and coordinates RGC specification with the patterning of progenitor cells. We highlight a program that operates during the two main phases of ATH5 expression, coordinating the selection of RGC precursors and the induction of RGC-specific traits with cell cycle exit. The first phase involves crossregulatory interactions between ATH5, NGN2, ASH1 and HES1 proteins that allow the expansion of pools of progenitors, contribute to their progressive intermingling and maintain ATH5 expression below the level required for inducing RGC differentiation. The second phase initiates when RGC progenitors are dispersed throughout the retina. The coordinated upregulation of NGN2 and downregulation of HES1 contribute to the progression of progenitors through the last cell cycle and create a suitable environment for efficient ATH5 autostimulation. The ATH5 protein upregulates its own expression and initiates the transcription of RGC-specific traits. Cells committed to the RGC fate then exit the cell cycle and express post-mitotic neuronal markers. In sum, we show how a subset of progenitors is selected from the pool of ATH5-expressing cells to enter the specification pathway at the proper time for RGC genesis.

Materials and methods

Reporter plasmids for the ATH5 and β3 promoters

A fragment of the ATH5 gene, 912 bp in length and bounded by *Xba*I and *Bst*XI restriction sites (GenBank AJ630209) was subcloned in the proper orientation at appropriate sites in the vectors p00-CAT, p00-lacZ and p00-GFP to yield, respectively, p00-ATH5-CAT, p00-ATH5-lacZ and p00-ATH5-GFP. The similarly constructed p00-β3-lacZ plasmid bears the 143 bp promoter of the gene encoding the neuronal acetylcholine receptor β3 subunit and has been described previously (GenBank X83740).

Eukaryotic expression plasmids for ATH5, NGN2 and HES1

The pEMSV plasmid (Matter-Sadzinski et al., 2001), which puts a cloned sequence under the transcriptional control of the mouse sarcoma virus long terminal repeat, was used throughout to express the ATH5, NGN2 and HES1 cDNAs in co-transfection and electroporation experiments.

Northern blot

Ten electroporated central and peripheral retina explants were lysed in guanidine thiocyanate. Total RNA was isolated, gel fractionated (2 μg/lane) and hybridized as described by Matter-Sadzinski et al. (Matter-Sadzinski et al., 2001).

In situ hybridization

^{35}S-labelled antisense riboprobes were synthesized and in situ hybridization on tissue sections were performed as described by Matter-Sadzinski et al. (Matter-Sadzinski et al., 2001). To correlate the expression level of a particular gene with ATH5 or β3 promoter activity, 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., 2001). For quantification (Fig. 1O), silver grains were counted in 50 radial sectors (~1300 μm^2 each) corresponding to a visual angle of ~3°.

[^{3}H]-thymidine and BrdU labelling

To label the S phase, transfected cells were incubated in medium containing 5 μCi/ml [^{3}H]-thymidine for 3 hours (stage 22-23) or 1 hour (stage 29-30) at the end of the 24 hours expression period. They were stained for β-galactosidase and processed for autoradiography (Matter-Sadzinski et al., 2001). Neuroretinas were dissected and incubated for 45 minutes in medium containing 100 μM BrdU and chased for 15 minutes. The explants were fixed, embedded in paraffin wax, sectioned and processed for in situ hybridization and for immunodetection of BrdU (Roztocil et al., 1997).

Cell cultures, transfection, CAT and β-galactosidase assays

Chick embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Neuroretina from stage 22-23 to stage 38 embryos were dissected and dissociated into single cells that were transfected with CAT, lacZ or GFP reporter plasmids. All transfections were carried out using the lipofectin reagent (InVitrogen), as described by Matter-Sadzinski et al. (Matter-Sadzinski et al., 1992). In all instances, the ratio of DNA to lipofectin was 1:4. In transfection experiments using a single construct, we transfected 1 μg of reporter plasmid per 10^6 cells. In co-transfection experiments using two or three constructs, 1 μg of reporter plasmid was mixed, respectively, with 0.5 μg or 2×0.5 μg expression vectors per 10^6 cells. Negative controls consisted of 1.0 μg reporter plasmid and 1.0 μg empty expression vector per 10^6 cells. Quantification of the chloramphenicol acetyl transferase (CAT) activity obtained with pATH5-CAT and identification of β-galactosidase-positive cells (lacZ) were as described by Matter et al. (Matter et al., 1995) and Matter-Sadzinski et al. (Matter-Sadzinski et al., 2001).

Electroporation of genetic material in the retina

Retinas were prepared from embryonic eyes collected at stages 22-23. Electroporations were as described by Matter-Sadzinski et al. (Matter-Sadzinski et al., 2001). Briefly, whole retinas or dissected peripheral and central sectors were immersed at room temperature in phosphate-buffered saline containing a reporter plasmid and/or expression vectors (100 μg/ml of each construct). Electroporation consisted of five 50 V/cm pulses of 50 mseconds duration spaced 1 second apart. The electroporated tissues were cultured as floating explants for 24 hours at 37°C. GFP- and β-galactosidase-positive cells were revealed or tissues were frozen in liquid nitrogen prior to RNA extraction.

Single-cell collection and RT-PCR

Cells transfected with the ATH5-promoter/GFP reporter plasmid were plated into poly-DL-ornithine-coated plastic petri dishes (30 mm in diameter). Twenty-four or 48 hours after transfection, individual GFP-positive cells were collected by aspiration with a glass micropipette mounted on a micromanipulator. Single-cell RT-PCRs were

performed according to Brady and Iscove (Brady and Iscove, 1993). Each cell was collected in 10 µl of a buffer containing 50 mM Tris HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT supplemented with 10 U of RNAsin (Promega), and immediately frozen in liquid nitrogen. Single cells were thawed on ice, vortexed for 10 seconds, spun down, heated to 65°C for 1 minute, vortexed again and then incubated on ice for 1 minute. For reverse transcription, dNTPs and Nonidet P-40 were added to final concentrations of 0.5 mM each dNTP, 0.5% NP-40, supplemented with 50 ng random DNA hexamers and 10 U RNAsin. 5 U of DNAse 1 (Gibco) was added and the mix was incubated at 37°C for 30 minutes to destroy genomic DNA. DNAse 1 was inactivated by 10 minutes incubation at 65°C. The mix was aliquoted in two fractions, both of which were again treated with 10 U of RNAsin and one of which received 200 U of Superscript II reverse transcriptase (Gibco). Both samples were incubated at 25°C for 10 minutes, at 37°C for 1 hour and at 68°C for 10 minutes. The whole reaction mixes were then used as templates in a first PCR performed with ExTaq polymerase (TaKaRa) and the complete set of external primers (0.2 mM final concentration of each primer) designed for amplifying the genes of interest (see Table S1 in the supplementary material). Initial denaturation at 94°C for 3 minutes was followed by 35 cycles consisting of denaturation at 94°C for 40 seconds, annealing at 56°C for 40 seconds, elongation at 72°C for 1 minute, and a final elongation at 72°C for 3 minutes. The second PCR was performed separately for each gene of interest using the internal primers specific for this gene (4 mM final concentration of each primer) and 0.1 volume of the first PCR as template. PCR conditions were as described above. When using the ATH5 internal primers (see Table S1 in the supplementary material), PCRs were performed with templates originating from both the actual and the mock reverse transcriptions. Only those cells that yielded no amplification of the negative control sample were selected for expression of the other genes of interest.

Results

The early retinal neuroepithelium is patterned by distinct bHLH expression domains

The spatial analysis of bHLH gene expression in early retina reveals a remarkable diversity of progenitor populations (Fig. 1). At stage 14 (E2), the structural continuum between the retinal neurepithelium and the neural tube is still in place, and HES1 transcripts accumulate in discrete territories of both structures (Fig. 1A). In the inner layer of the eyecup, the HES1 expression domain includes the periphery of the presumptive neuroretina and there is no marked accumulation of HES1 transcripts in the central region, where the first ATH5- and NGN2-expressing cells appear at stage 15-16 (Fig. 1B,C). During the next 12 hours – between stages 15 and 18 – a robust accumulation of HES1 transcripts is taking place in a peripheral domain expanding to the anterior edge of the retina (Fig. 1E,J), whereas the central region expresses HES1 at a low level, except for a few cells located on the vitreous side (Fig. 1E,I). As revealed by cell counting on serial sections of stage 18 retinas, ~88% of cells (370±20 cells/section) in the central domain express ATH5 and these cells represent ~26% of the total retinal cell population (Fig. 1K, Fig. 2A) (Skowronska-Krawczyk et al., 2004). About 1.5% of cells express ATH5 at a high level and they, as well as the sparse cells (~1%) expressing the post-mitotic bHLH Neuro M (Fig. 1H,M), are evenly distributed throughout the central retina. The Neuro M-expressing cells comprise the first set of newborn neurons, and their homogeneous distribution (Fig. 1H) indicates that neurogenesis is initiated at the same rate throughout the central

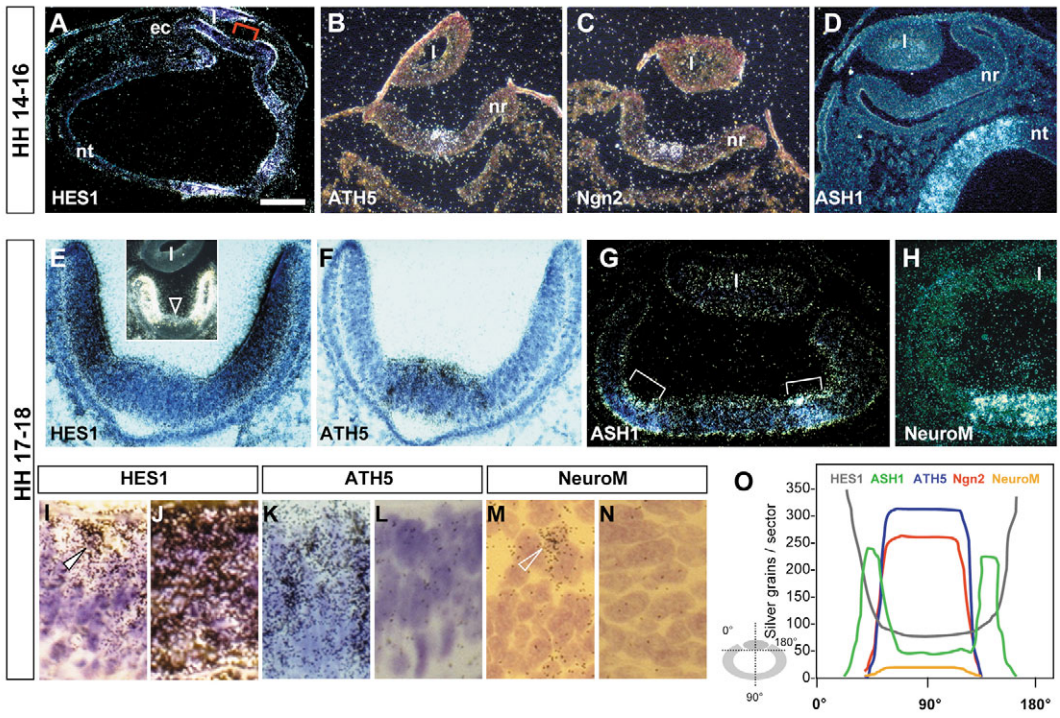


Fig. 1. Several bHLH factors pattern the early retinal neuroepithelium. (A) At stage 14, HES1 transcripts accumulate in discrete domains in the eyecup (ec) and neural tube (nt). There is no detectable accumulation of HES1 transcripts in the central region (red bracket) of the presumptive retina. (B,C) The first ATH5- and NGN2-expressing cells are detected in the central retina (nr) at stage 15. (D) At stage 16, ASH1 transcripts are not detected in retina. (E) At stage 17, a robust accumulation of HES1 transcripts is taking place throughout the peripheral retina (J). In the central retina, a few cells located on the vitreous side express HES1 at a high level (arrowheads in E,I). (F,K) Most cells in the central retina express ATH5 and those expressing ATH5 strongly are mostly located on the vitreous side. (H) At stage 18, the sparse cells expressing Neuro M are scattered across the central retina (arrowhead in M). There are no cells expressing ATH5 or Neuro M in the HES1 domain (F,H,L,N). (O) Quantification of in situ hybridization. Adjacent retinal sections were hybridized with the indicated bHLH riboprobes at stage 18. The ATH5, Neuro M and NGN2 domains coincide in the central retina and they abut on the peripheral HES1 domain. ASH1 is detected in an annular sector (G, brackets) at the interface between the HES1 and ATH5 domains. l, lens. Scale bar: 140 µm in A; 80 µm in B,C; 100 µm in D-H.

domain. The ATH5 and NGN2 domains precisely coincide at this stage, and they border the peripheral HES1 expression domain (Fig. 1O). Expression of ASH1 is undetectable in retina until stage 18, at which point an annular ASH1 region surrounds the central domain (Matter-Sadzinski et al., 2001) and overlaps the posterior edge of the HES1 domain (Fig. 1D,G,O). Very few cells expressing ATH5 or NGN2, and no Neuro M positive cells were found in the ASH1 or HES1 expression domains (Fig. 1L,N), confirming that the early retinal neuroepithelium is patterned in discrete progenitor domains between stages 14 and 18.

Dynamic changes in spatial patterning of progenitor cells precede RGC differentiation

The concentric expression pattern of the bHLH genes is maintained until stage 26 (E5) (Fig. 2). However, from stage 23 onwards, dynamic changes are taking place. Between stages 18 and 26, the retina diameter increases about threefold and expansion of the ATH5 and NGN2 expression domains parallels the growth of the whole retina (Fig. 2A-E). HES1 expression, on the whole, remains complementary to that of ATH5, with transcript levels maintained very high at the periphery and low in the centre (Fig. 2C). At stage 23, the central retina still contains isolated cells expressing HES1 at a high level (Fig. 2L). Contrasting with the mutually exclusive domains established at earlier stages, the anterior margin of the ATH5 domain now overlaps the posterior HES1 region, where the levels of HES1 transcripts are decreasing (Fig. 2H,I). Moreover, from stage 23 onwards, the NGN2 domain expands more peripherally than that of ATH5, suggesting that NGN2 expression is less sensitive than ATH5 to inhibition by HES1 (Fig. 2J,K), and thus precedes the onset of ATH5 expression as both domains expand to the periphery. Expansion of the NGN2 and ATH5 domains is paralleled by changes in the expression pattern of ASH1. At stages 23-26, an annular ASH1 expression region is still surrounding the ATH5 domain but ASH1- and ATH5/NGN2-expressing cells are intermingled in the centre (Fig. 2E,F). At stages 28-30 (E6), distinct progenitor domains are no longer detected and ATH5-expressing cells are distributed throughout the retina, except at the ciliary margin

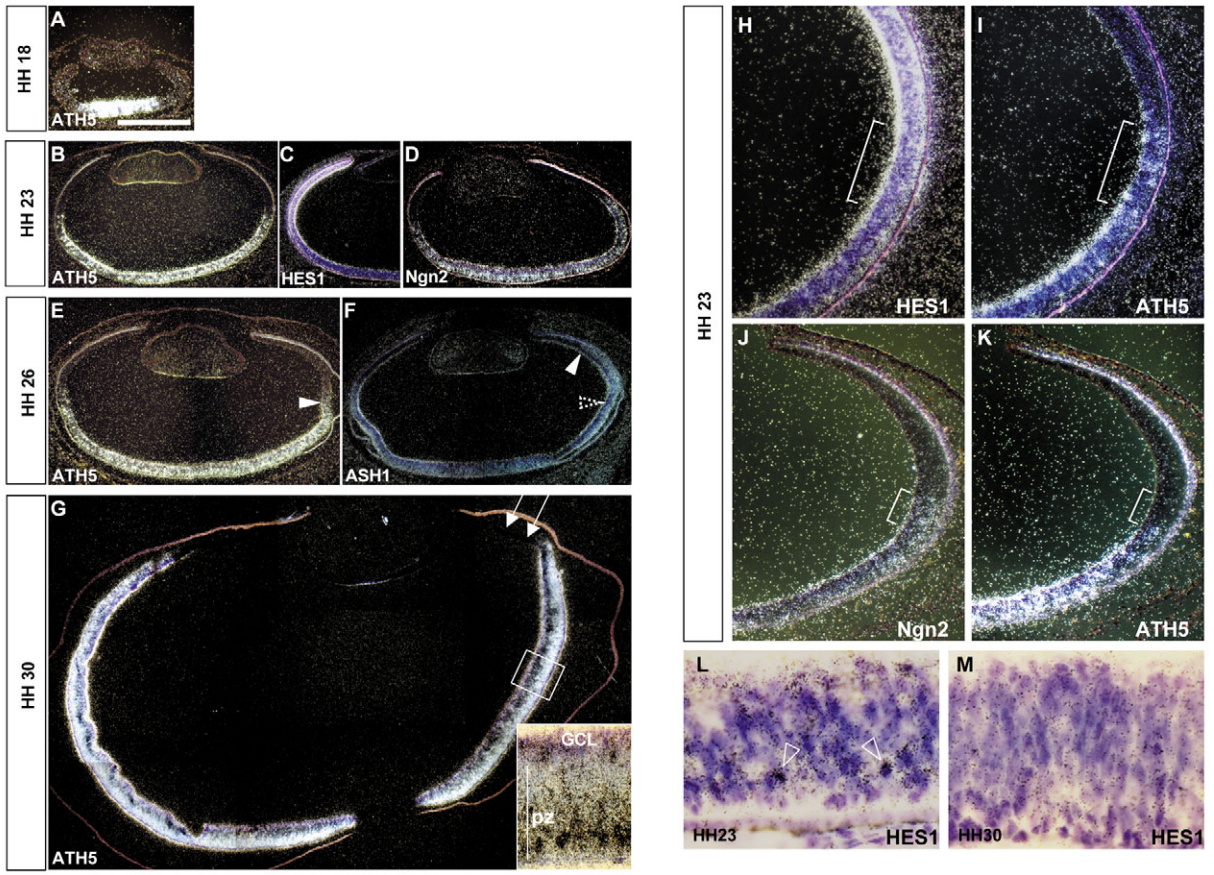


Fig. 2. Growth of the retina is accompanied by changes in the patterning of progenitor cells. (A,B,E) Between stages 18 and 26, the ATH5 domain expands in register with the threefold increase in retina diameter. (C) HES1 transcripts are abundant at the periphery and sparse in the central region. Scattered cells expressing HES1 at a high level are detected in the central retina (arrowheads in L). The HES1 and ATH5 expression domains are complementary (B,C), but the anterior margin of the ATH5 domain overlaps the posterior HES1 region, where HES1 transcript levels are decreasing (brackets in H,I). ATH5 and NGN2 transcripts accumulate in the posterior retina (B,D). The NGN2 domain extends beyond that of ATH5 (brackets in J,K). At stage 26, ASH1- and ATH5-expressing cells are interspersed in the posterior retina. ASH1 extends beyond ATH5 (arrowheads in E,F). At stage 30, ATH5 transcripts are distributed throughout the whole retina (G), except at the ciliary margin (arrows). ATH5 transcripts are not evenly distributed across the retina. They are abundant on the ventricular side of the proliferative zone (pz) (inset in G). At stage 30, HES1 expression is downregulated both in the peripheral and in the central retina (M). Sections in L and M were counterstained with Toluidine Blue. Scale bar: 380 μm in A-G; 40 μm in L; 60 μm in M; 240 μm in H,I; 150 μm in J,K.

(Fig. 2G). Expansion of the ATH5 domain to the periphery is paralleled by a strong increase in accumulation of ATH5 mRNA (Fig. 2G) and coincides with the downregulation of HES1 in central and peripheral retina (Fig. 2M). Thus, dynamic changes in the expression profile of progenitor cells at domain boundaries and in the central retina lead to a blending of different precursor sets, to a progressive blurring of borders and to the merging of formerly discrete domains.

The spatiotemporal expression of ATH5 is regulated at the promoter level

The cis-regulatory region of the chick *ATH5* gene extending 775 bp upstream of the translation initiation codon (GenBank AJ630209) contains important regulatory elements and this region drives reporter activity in those cells where in vivo *ATH5* mRNA accumulation takes place during retina development (Matter-Sadzinski et al., 2001) (J.H., L.M.-S., D. Skowronska-Krawczyk, J.-M.M. and M.B., unpublished). To determine whether the isolated promoter reproduces the spatiotemporal pattern of gene regulation at early developmental stages, a mix of an ATH5-promoter/*lacZ*-reporter plasmid with a CMV-promoter/GFP reporter construct was electroporated in retinas at stage 22-23. After 24 hours, GFP-positive cells were distributed throughout the central and peripheral retina, whereas *lac*$^+$ cells were confined to the central domain, as expected at this early stage (Fig. 3B,C). The rather low density of *lac*$^+$ cells in this experiment is due to the relative insensitivity of the X-gal assay, which, as we show below, only detects promoter activity in cells that express ATH5 at a high rate.

Single-cell transcription analysis reveals the stages of a progression along the RGC specification and differentiation pathway

The isolated ATH5 promoter region provides a unique means of identifying ATH5-expressing progenitor cells, some of which will become committed to the RGC lineage. Whereas in situ hybridization suffices to colocalize promoter activity and expression of a single gene in individual cells (Matter-Sadzinski et al., 2001), the single-cell RT-PCR approach is necessary for detecting the co-expression of multiple genes. To monitor the dynamics of bHLH expression in individual cells during the period of RGC specification and differentiation, acutely dissociated cells from stage 22-23 (E3.5) and 26 (E5) retinas were transfected with the ATH5-promoter/GFP-reporter plasmid singly or in combination with a NGN2 expression vector, plated into tissue culture dishes and cultured for 24 or 48 hours. The time of cell collection thus approximately corresponded to E4.5, E5.5 and E6 in vivo. One-hundred and sixty GFP-positive cells were collected and single-cell RT-PCRs were performed to produce collections of cDNA fragments representing the mRNA of single ATH5-expressing cells. To examine the combinations in which the selected genes were expressed, cells from the five groups generated by the experiment (Fig. 4B) were independently tested by second rounds of PCR, using appropriate (see Table S1 in the supplementary material) sets of primers (Fig. 4A, Fig. 5A,B). All GFP-positive cells expressed ATH5, as expected (Fig. 4A), but there was a high degree of heterogeneity and striking temporal changes in the expression profiles of the unselected genes HES1, Delta 1, Neuro M, β3nAChR and BRN3C. At early stages (E4.5), we did not find any cells co-expressing ASH1 and ATH5 (0/20), confirming that these two proneural genes are initially expressed in separate sets of early progenitors. At later stages (E5.5-E6), three out of 28 cells expressed both genes (e.g. cell 149), two of them expressing Neuro M as well (data not shown). The large majority (~80%) of cells transfected at stage 22-23 and collected 24 hours later were expressing HES1, evidence that most ATH5-expressing cells are proliferating progenitors. This proportion decreased to ~5% when stage 22-23 cells were kept for 48 hours in culture or when cells were

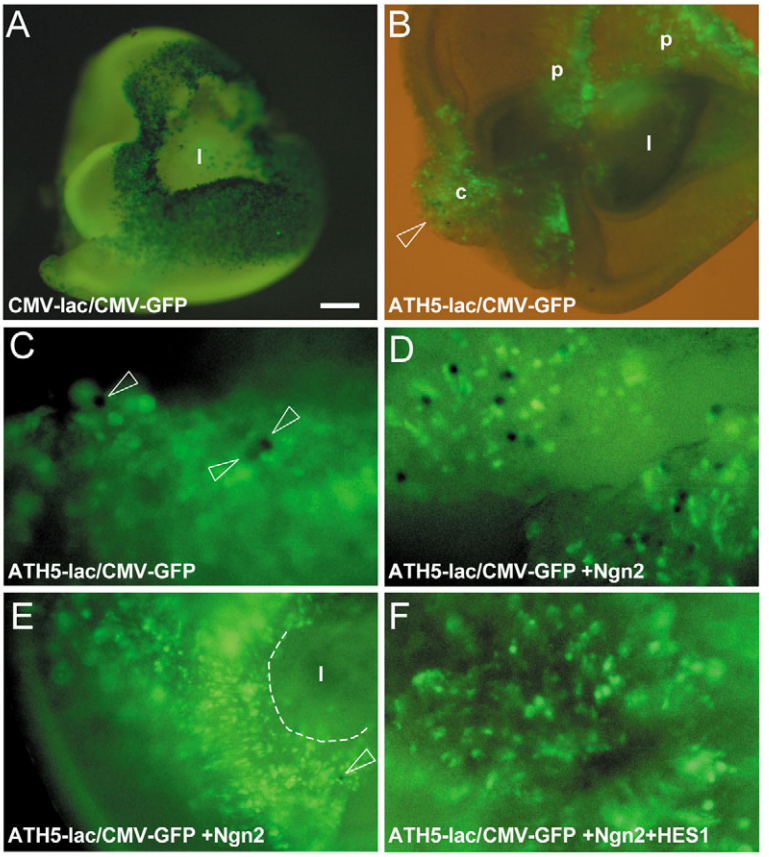
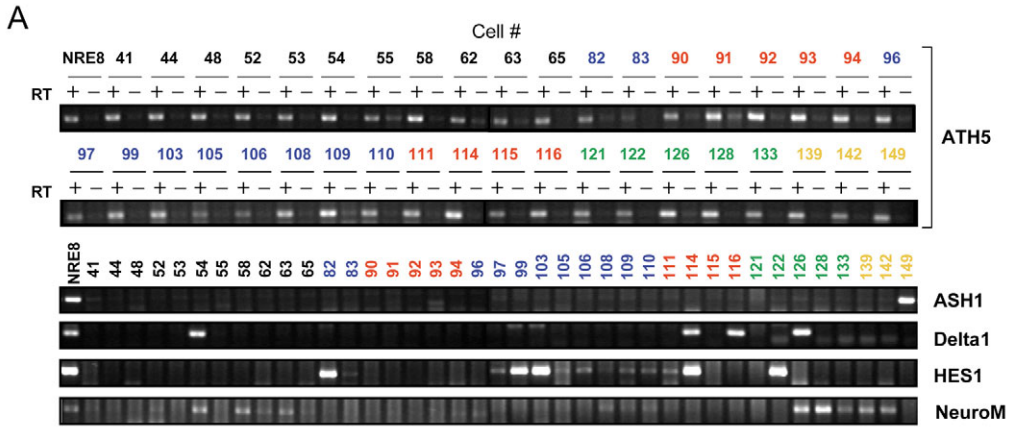


Fig. 3. Activity of the electroporated ATH5 promoter in stage 22-23 retina. (A) When controlled by the ubiquitous CMV promoter, GFP and *lac* reporters are both expressed in the electroporated peripheral retina. (B-F) The ATH5-promoter/lac and the CMV-promoter/GFP reporter plasmids were electroporated alone (B,C), in combination with a NGN2 expression vector (D,E) or in combination with NGN2 and HES1 expression vectors (F). (B) GFP-positive cells are distributed throughout the peripheral (p) and central (c) retina, whereas *lac*$^+$ cells are confined to the central region (arrowhead). (C) *lac*$^+$ cells (arrowheads) are sparse in the central retina. (D) Overexpression of NGN2 increases the proportion of *lac*$^+$ cells in the central, but not in the peripheral retina (arrowhead in E). (F) No *lac*$^+$ cells were detected when both NGN2 and HES1 were overexpressed in the central retina. Data presented in each panel are representative of at least five independent experiments. l, lens. Scale bar: 170 μm in A; 120 μm in B; 30 μm in C; 40 μm in D,F; 80 μm in E.



B

stage	collection
22-23 (E3.5)	E4.5
22-23 (E3.5) + Ngn2	E4.5
22-23 (E3.5)	E5.5
22-23 (E3.5) + Ngn2	E5.5
26 (E5)	E6

C

	E4.5		E5.5-E6
		+ Ngn2	
HES1$^+$	18/22	4/18	2/38
NeuroM$^+$	2/22	2/18	34/56
Delta1$^+$	0/11	2/9	2/19
ASH1$^+$	0/11	0/9	3/28

Fig. 4. Transcriptional analysis of ATH5-expressing single cells. Stage 22-23 (E3.5) retinal cells were transfected with an ATH5-promoter/GFP-reporter plasmid either singly or in combination with a vector expressing NGN2. They were cultured for either 24 (E4.5) or 48 hours (E5.5). Stage 26 (E5) retinal cells were transfected with the ATH5-promoter/GFP-reporter plasmid and cultured for 24 hours (E6). Individual GFP-positive cells were collected and processed for single-cell RT-PCR using the primers listed in Table S1 (see supplementary material). (A) Representative transcriptional profiles obtained with a set of 39 cells from the five groups generated by the experiment, as identified by the colour code in B. RT-PCRs of total RNA isolated from E8 retina (NRE8) were used as positive controls for each set of primers. (C) Ratios of HES1-, Neuro M-, Delta 1- and ASH1-positive cells to the total number of cells tested for expression of these genes.

transfected at stage 26 (Fig. 4C). Conversely, 10% of cells transfected at stage 22-23 were found to express ATH5 and Neuro M after 24 hours in culture. This proportion increased to ~60% when stage 22-23 cells were cultured for 48 hours (e.g. cell 128), or when stage 26 cells were cultured for 24 hours (e.g. cell 54). The expansion of the population expressing Neuro M is paralleled by a proportional decrease in the number of HES1-expressing cells as RGC precursors exit the mitotic cycle, a process culminating at E6. Because no cells co-expressing HES1 and Neuro M were found in the collection (0/60), whereas numerous single cells expressed neither HES1 nor Neuro M (22/60), these two proteins must be expressed at distinct stages separated by a lag. Interestingly, overexpression of NGN2 at stage 22-23 leads, after 24 hours, to a drastic decrease in the proportion of HES1-positive cells, but does not induce the precocious generation of Neuro M-positive cells (Fig. 4C), resulting instead in the accumulation of cells that express neither HES1 nor Neuro M. The proportion of Neuro M-positive cells increased to ~60% when stage 22-23 cells overexpressing NGN2 were cultured for 48 hours. Some cells co-expressed Delta 1 and HES1 (e.g. cell 114) and Delta 1 expression was seen both in the presence of Neuro M (e.g. cell 126) and in cells that expressed neither Neuro M nor HES1 (e.g. cell 116), indicating that the Notch ligand is expressed soon after the downregulation of HES1 but prior to the onset of Neuro M expression, a result that is consistent with previous in situ hybridization studies (Henrique et al., 1997; Roztocil et al., 1997). The small proportion of Delta 1-positive cells (4/39; Fig. 4A) suggests that this gene is expressed very transiently in differentiating RGCs.

Co-expression of ATH5, Neuro M and of the RGC-specific genes β3nAChR and BRN3C was mostly detected in stage 26 cells cultured for 24 hours, indicating that these post-mitotic cells are newborn RGCs (Fig. 5A,B). The presence of β3 transcripts in cells that do not express Neuro M (e.g. cells 49, 136) is consistent with a previous report demonstrating that the β3 promoter is activated in cells that are still proliferating (Matter et al., 1995). This is supported by the following additional findings: when retinal cells were transfected at stage 24 with a β3-promoter/*lacZ* reporter plasmid, low levels of HES1 transcripts were detected in ~10% of *lac*$^+$ cells (Fig. 5C, part a). The β3 promoter generally was more active in cells that did not express Neuro M and its activity decreased in newborn RGCs (Fig. 5C, parts b and c), in keeping with previous studies showing that the β3 promoter peaks between E5 and E6 – i.e. about 12 hours before the maximum level of ATH5 expression is reached (Matter et al., 1995; Matter-Sadzinski et al., 2001). No ATH5-positive single cell expressed BRN3C (0/20) when stage 22-23 cells were cultured for 24 hours (data not shown). BRN3C expression was seen only when stage 22-23 cells were cultured for 48 hours, or when stage 26 cells were cultured for 24 hours and all BRN3C-positive cells expressed Neuro M (e.g. cells 39 and 132). The small proportion of Neuro M-positive cells expressing BRN3C (3/17; Fig. 5 and data not shown) suggests that this gene is turned on when cells have exited the cell cycle and relatively late after the onset of Neuro

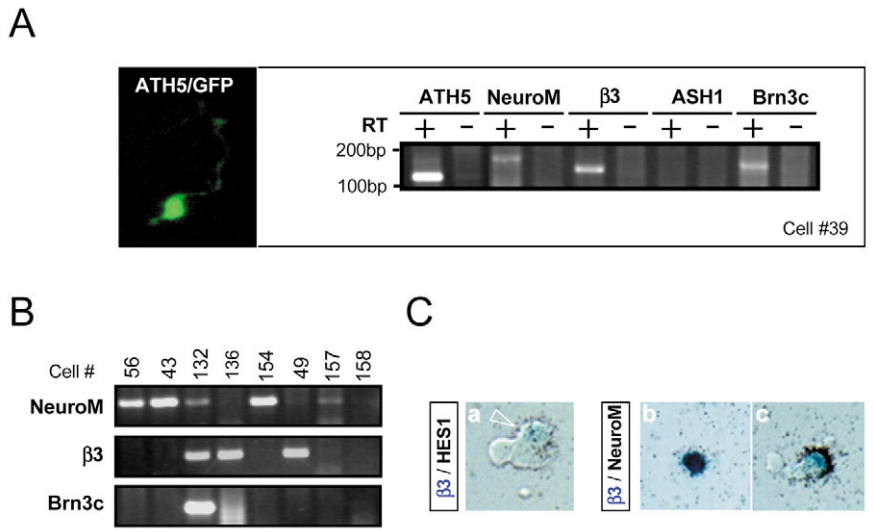


Fig. 5. Co-expression of RGC-specific genes and bHLH transcription factors in newborn RGCs and RGC precursors. Cells were transfected with an ATH5-promoter/GFP-reporter plasmid at stage 26 (E5) and cultured for 24 hours. (A, right) Transcriptional profile of a newborn RGC. This neuron-like GFP-positive cell (left) expresses ATH5, Neuro M, β3 and BRN3C, but not ASH1. (B) ATH5-expressing cells do not always co-express Neuro M, β3 and BRN3C. (C) Colocalization of β3 promoter activity and HES1 or Neuro M expression. Cells were transfected with a β3-promoter/*lacZ*-reporter plasmid at stage 24. After 24 hours in culture, *lacZ*-expression was revealed and cells were processed for in situ hybridization with (a) HES1- or (b,c) Neuro M-specific riboprobes. Arrowhead in a indicates a double-labelled cell.

M expression. This result is congruent with the reported presence of BRN3C in migrating and differentiated RGCs (Liu et al., 2000). Overall, the particular combinations of genes and their temporal expression profiles, as revealed by single-cell PCR are in good agreement with the genetic sequence of development in the ciliary margin of *Xenopus* retina (Perron et al., 1998). As judged by their expression profiles, ATH5-expressing cells fall into three main groups. First, there are HES1-expressing proliferating cells. Second, there are cells that do not express HES1 but express β3 and/or Delta 1. These are presumably passing through the last cell cycle. Third, there are post-mitotic cells expressing Neuro M. These cells correspond to newborn RGCs and some of them express BRN3C. Cells showing mixed status most probably were captured at the juncture between two phases (e.g. cells co-expressing HES1 and Delta 1) or in a transient state prior to acquiring a definite progenitor status (e.g. cells co-expressing ATH5 and ASH1).

Transcription of the ATH5 gene is regulated in several distinct phases

We then asked how ATH5 expression is regulated along the course of RGC specification. Promoter activity and accumulation of mRNA follow the same kinetics, indicating that the differential expression of ATH5 during retina development is regulated at the transcriptional level (Matter-Sadzinski et al., 2001) (Fig. 6A). The similar proportions of retinal cells expressing ATH5 at stages 18 (~26%) and 29-30 (~33%) (Skowronska-Krawczyk et al., 2004) suggest that the expression of ATH5 essentially reflects changes in the transcription rate within a roughly constant cell fraction, thus validating the analysis of promoter activity in the course of early retina development. The analysis revealed that transcription of the ATH5 gene is regulated in three sequential phases (Fig. 6). The first phase extends from E2 to E5 and is marked by low promoter activity. Because ATH5 and NGN2 contribute to the regulation of the ATH5 promoter (Fig. 3) (Matter-Sadzinski et al., 2001), we asked whether overexpression of these proteins might modify promoter activity at early developmental stages. Retinal cells were co-transfected at stages 22-23 or 23-24 with an ATH5-promoter/CAT reporter plasmid and an expression vector encoding either ATH5 or NGN2. Whereas overexpression of NGN2 resulted in a strong increase in promoter activity, ATH5 had no significant effect (Fig. 6A). In a complementary experiment, the number of *lac*$^+$ cells markedly increased when the NGN2 expression vector was mixed with the ATH5-promoter/*lacZ* reporter plasmid prior to electroporation in stage 22-23 retina (Fig. 3D). This effect was not detected when an ATH5 expression vector was mixed to the ATH5 promoter/*lacZ* reporter plasmid. Activation of the *ATH5* gene by NGN2 was independently demonstrated by electroporating the NGN2 expression vector into stage 22-23 central retinas, which led to a strong accumulation of endogenous ATH5 mRNA (Fig. 7B), whereas that transcript was barely detectable in the control. These results suggest that ATH5 expression is weak in early retina because NGN2 is expressed at an insufficient rate and because ATH5 is inefficient at activating its own promoter at early stages. Significant changes in the behaviour of the ATH5 promoter were detected at stage 25-26. The transfected promoter, like the endogenous one, is still poorly active but ATH5 overexpression upregulates it to the level it would normally reach on E6, indicating that the cellular context is now permissive for ATH5 autostimulation (Fig. 6A). This transition coincides with the suppression of HES1 expression in the majority of ATH5-expressing cells (Fig. 4). Promoter activity rapidly increases after stage 26 and can be further enhanced by overexpression of either ATH5 or NGN2, the effect of ATH5 being more pronounced than that of NGN2 (Fig. 6A). At stage 29-30, ATH5 transcripts accumulate in ~33% of retinal cells and transfection by the CAT reporter reveals a robust increase in promoter activity. The *lacZ* reporter, however, labels only ~10% of the transfected cells (Fig. 6B,D). This discrepancy comes from a difference in the sensitivity of the techniques. Compared with the CAT assay, which monitors promoter activity in virtually all ATH5-expressing cells, X-gal staining only detects expression of the reporter gene above a threshold level, which was not reached at this stage in about two-thirds of ATH5-expressing cells (Fig. 6C). This was confirmed by ATH5 overexpression, which resulted in a threefold increase in promoter activity, as measured by CAT assay, and raised the proportion of *lac*$^+$ cells

to ~30%, suggesting that upregulation of promoter activity by ATH5 brings the whole population of ATH5-expressing cells above the threshold for X-gal detection (Fig. 6C). A similar analysis was performed at stage 22-23 using NGN2 overexpression. Whereas at this stage ~30% of retinal cells accumulate ATH5 transcripts, X-gal staining reveals promoter activity in only ~1% of cells (Fig. 6B), the majority of which are weakly stained (Fig. 6D). NGN2 overexpression enhanced ATH5 promoter activity about 10-fold to the level found at E6 (Fig. 6A), but only increased the proportion of cells stained with X-gal to ~6% (Fig. 6B,D). In most ATH5-expressing cells, the level of promoter activity remains below the threshold for detection by the X-gal reagent (Fig. 6C).

A rapid decrease in the activity of the ATH5 promoter was detected between E6 and E9, marking the transition between the second and third phases in the regulation of ATH5 (Fig. 6A). At stages 34 and 37, the promoter is poorly active and no longer responds to overexpression of ATH5 or NGN2. Thus, it appears that the ability of the ATH5 protein efficiently to stimulate its own expression in a subset of competent progenitors is restricted to the narrow time window (E5-E7) when most RGC precursors are born.

HES1 is a dominant-negative regulator of the ATH5 promoter

Next, we asked why ATH5 does not efficiently stimulate its own expression at early stages of retinogenesis. The mutually exclusive domains of ATH5 and HES1 in early retina (Figs 1, 2) suggested that HES1 interfered negatively with ATH5 expression. Inhibition of the ATH5 promoter by HES1 was demonstrated by co-transfection of an ATH5 promoter/CAT reporter plasmid and a HES1 expression vector in retinal cells at stages 22-23 and 30. At both stages, promoter activity was reduced to the background level (Fig. 7A). We tested whether HES1 overexpression influences activation by transfected NGN2 and ATH5. We found both in electroporated retina and in transfected retinal cells that HES1 overexpression prevents activation of the ATH5 promoter by either proneural proteins, indicating that HES1 acts as a dominant-negative effector (Fig. 3F, Fig. 7A). We then assessed whether endogenously expressed HES1 is able to block the NGN2-mediated activation of the ATH5 gene. We separated the central from the peripheral regions of retinas at stage 22-23, electroporated the tissues with either a NGN2 or an empty expression vector, and cultured them as explants for 24 hours (Fig. 7B). As

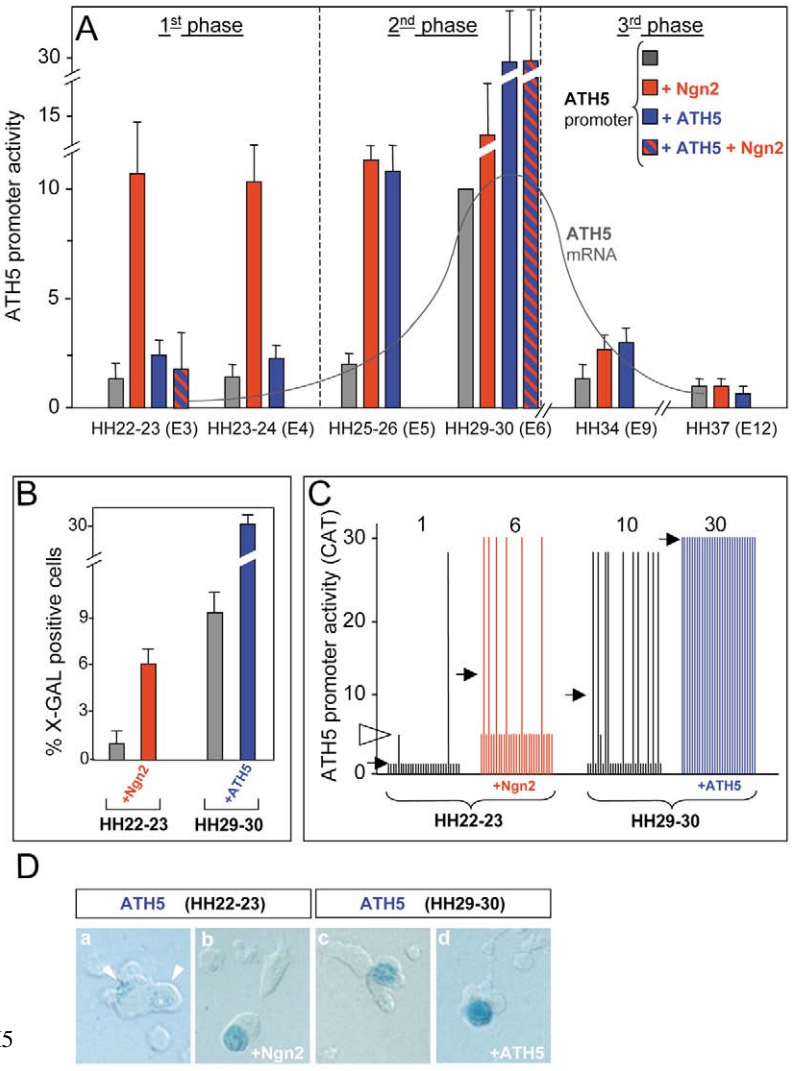


Fig. 6. Regulation of the ATH5 promoter during retinogenesis. (A) Retinal cells isolated at stages 22 (E3) to 37 (E12) were transfected with an ATH5-promoter/CAT-reporter plasmid singly or in combinations with ATH5 and/or NGN2 expression vectors. Cells were assayed for CAT activity 24 hours after transfection. ATH5 transcription is passing through three phases in the course of retinogenesis. During the first phase (HH22-HH24), the promoter is weakly active and responds strongly to NGN2 overexpression, except in the presence of ATH5. During the second phase (HH25-HH30), upregulation of promoter activity coincides with a transient increase in ATH5 mRNA (curve). ATH5 and NGN2 both enhance promoter activity and ATH5 becomes dominant over NGN2. The third phase (HH34 and beyond) sees a decrease in ATH5 mRNA and is marked by the inability of either proneural protein to transactivate the promoter. (B) Retinal cells isolated at stages 22-23 and 29-30 were transfected with an ATH5-promoter/*lacZ*-reporter plasmid singly or in combinations with NGN2 or ATH5 expression vectors. *lac*$^+$ cells were counted after 24 hours in culture. The number of *lac*$^+$ cells obtained upon transfection with a control SV40-promoter/*lacZ*-reporter plasmid at each stage is set at 100 and cell numbers are given relative to this value. (C) Schematic representation of promoter activity as revealed by X-gal and CAT assays. Approximately 30% of cells express ATH5 at stages 22-23 and 29-30. The horizontal arrows indicate average promoter activity as measured by CAT assay, the open arrowhead marks the threshold for X-gal detection. At stage 22-23, promoter activity is low and only one in 30 ATH5-expressing cells is detected by X-gal. Overexpression of NGN2 increases promoter activity 10-fold but only six out of 30 ATH5-expressing cells are stained with X-gal. At stage 29-30, the whole population of ATH5-expressing cells is stained with X-GAL upon ATH5 overexpression. (D) At stage 22-23, most cells are weakly stained with X-GAL (arrowheads in a). Overexpression of NGN2 strongly enhances promoter activity (b) and the number of X-gal stained cells. At stage 29-30, cells display strong promoter activity (c) and overexpression of ATH5 enhances staining intensity (d).

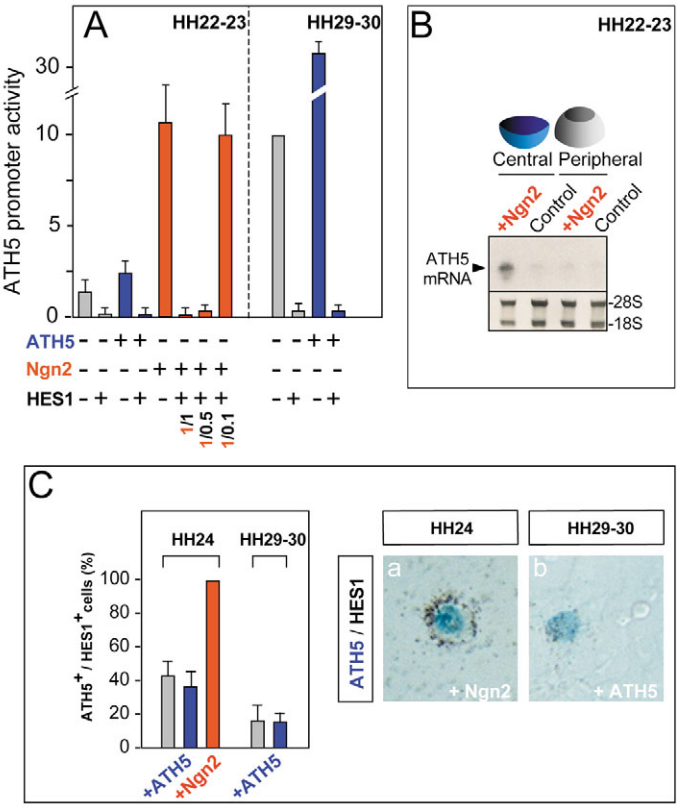


Fig. 7. HES1 exerts a dominant-negative effect upon the ATH5 promoter. (A) Retinal cells at stages 22-23 or 29-30 were transfected with an ATH5-promoter/CAT-reporter plasmid alone or with different combinations of the ATH5, NGN2 and HES1 expression vectors. NGN2 and HES1 expression vectors were co-transfected in different ratios, as indicated. (B) Peripheral and central regions of retina were dissected at stage 22-23. They were electroporated with NGN2 and control expression vectors and cultured as explants for 24 hours. The presence of ATH5 mRNA was detected by northern blot hybridisation. Overexpression of NGN2 upregulated ATH5 expression in the central but not in the peripheral retina. (C) Retinal cells at stages 24 or 29-30 were transfected with an ATH5-promoter/*lacZ*-reporter plasmid singly or in combinations with ATH5 or NGN2 expression vectors. *lac*$^+$ cells were revealed and processed for in situ hybridization with a HES1-specific riboprobe. Overexpression of NGN2 increased the relative number of double-labelled cells, indicating that the NGN2 protein can activate the ATH5 promoter in cells that express HES1 (a), unlike the ATH5 protein (b).

revealed by northern blot hybridisation, overexpression of NGN2 led to accumulation of endogenous ATH5 mRNA in the central but not in the peripheral retina, indicating that NGN2 is able to stimulate ATH5 transcription where most cells express HES1 at low levels, whereas the high rate of HES1 expression at the periphery precludes activation of the ATH5 gene. Consistent with this view, very few *lac*$^+$ cells were found at the periphery of a retina electroporated with a mix of ATH5-promoter/*lac* reporter plasmid and NGN2 expression vector (Fig. 3E), whereas the number of lac$^+$ cells was much increased in the central region (Fig. 3D). To quantify the strength of the HES1 inhibition, stage 22-23 retinal cells were co-transfected with different ratios of the NGN2 and HES1 expression vectors. We found that above a ratio of 2:1, NGN2 overcomes the inhibitory effect of HES1 and activates the ATH5 promoter (Fig. 7A). We then reasoned that if the inhibitory interactions between HES1 and the proneural proteins are concentration dependent, they could account for the expression pattern at the domain boundary where, from stage 23 onwards, HES1 is downregulated and NGN2 expression expands ahead of the ATH5 domain (Fig. 2H-K). To test this prediction, stage 24 retinal cells were transfected with the ATH5-promoter/*lacZ* reporter, either alone or together with an ATH5 or NGN2 expression vector. Twenty-four hours later, *lac*$^+$ cells were revealed and cells were processed for in situ hybridisation with a ^{35}S-labelled HES1-specific riboprobe. Overexpression of NGN2 increased the relative number of double-labelled cells, whereas ATH5 did not (Fig. 7C). Congruent with the in vivo expression pattern, NGN2 overexpression was able to activate the ATH5 promoter in cells expressing HES1 at low levels, whereas ATH5 could not (Fig. 7C). These results suggest that NGN2, being less sensitive than ATH5 to HES1 inhibition, contributes to the expansion of the ATH5 domain when expression of HES1 begins to wane. Moreover, this finding implies that ATH5 expression is driven by NGN2, rather than by ATH5, in cells co-expressing HES1 and ATH5 (Fig. 4). Finally, the inability of ATH5 to activate its own promoter in the presence of HES1 explains why ATH5 is a weak activator at early stages: the low but significant expression of HES1 in the central retina between stages 22 and 25 is probably sufficient to prevent autostimulation.

ATH5 and NGN2 compete to regulate the ATH5 promoter

In early retina, NGN2 is a strong activator of ATH5 transcription, whereas overexpression of ATH5 only results in a modest increase in promoter activity. Conversely, ATH5 is a more potent activator than NGN2 at stage 29-30 (Fig. 6A). We therefore wondered what effects would the overexpression of both transcription factors have on the activity of the ATH5 promoter. When retinal cells were co-transfected at stage 22-23 with an ATH5-promoter/CAT-reporter plasmid and expression vectors encoding both the NGN2 and ATH5 proteins, stimulation by NGN2 was abolished and promoter activity remained low. By contrast, overexpression of ATH5 at stage 30 demonstrates that ATH5 has now become an efficient positive regulator of its own promoter: at this stage, the promoter is upregulated to the same high level whether ATH5 is overexpressed alone or in combination with NGN2 (Fig. 6A).

These results suggest that ATH5 and NGN2 may compete for the same regulatory elements, and that ATH5 is the dominant activator in the absence of HES1. At early stages, because of the presence of HES1, ATH5 cannot upregulate its own expression but can efficiently compete with NGN2. Mutational analysis showing that the two proteins are using the same E-box elements to mediate their effects (Fig. 1S) and chromatin immunoprecipitation (ChIP) experiments demonstrating that both the ATH5 and NGN2 proteins bind the ATH5 promoter in vivo at stage 22-23 and stage 29-30 (Skowronska-Krawczyk et al., 2004) are in favour of this hypothesis. In addition, the co-transfection results suggest that autostimulation is in large part responsible for the upregulation

of ATH5 expression at stage 30, whereas at earlier stages ATH5 may contribute to the downregulation of its own expression.

NGN2 upregulates promoter activity in proliferating cells and drives ATH5-expressing cells beyond the last S phase

The presence of ATH5 transcripts in ~26% of stage 18 neuroepithelial cells (Fig. 1) (Skowronska-Krawczyk et al., 2004) and of HES1 transcripts in most of the ATH5-expressing cells at stage 22-23 (Fig. 4), taken together with previous findings (Liu et al., 2001; Matter-Sadzinski et al., 2001), provide ample evidence that the endogenous *ATH5* gene is transcribed in proliferating cells. The differential response of the ATH5 promoter to the NGN2 and ATH5 proteins in the course of retina development (Fig. 6) and the different sensitivities of these proteins towards HES1 (Fig. 7) suggest their implication at distinct moments in progenitor commitment to the RGC fate. To analyse how stimulation of the ATH5 promoter correlates with the proliferative status, NGN2 or ATH5 expression vectors were transfected together with an ATH5 promoter/*lacZ*-reporter plasmid at the stages when each factor exerts its major effect and the transfected cells were pulsed with [^{3}H]-thymidine at the end of a 24-hour culture period (Fig. 8A). Forced expression of NGN2 at stage 22-23 led to a 3.5-fold increase in double-labelled cells and to a sevenfold enlargement of the non-radioactive *lac*$^+$ cell population. On the whole, NGN2 overexpression diminished by half the [^{3}H]-thymidine-labelling index of cells that had an active ATH5 promoter when compared with controls (i.e. from ~34% to ~17%). This result reveals the dual effect of overexpressed NGN2, which both stimulates ATH5 expression in proliferating cells and significantly increases the pool of non-dividing cells that have an upregulated ATH5 promoter (Fig. 8A), indicating that NGN2 drives cells out of the S phase.

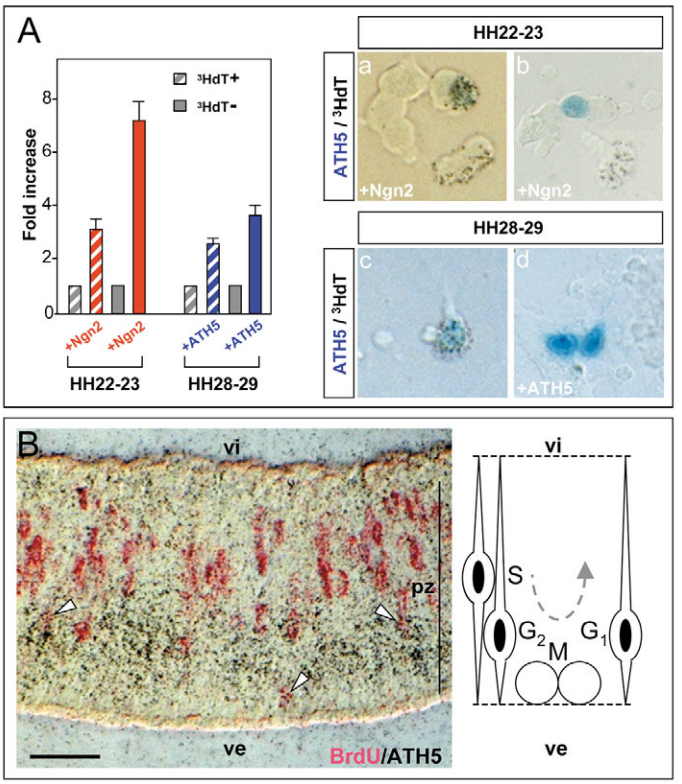


Fig. 8. ATH5 expression is upregulated during the last S phase. (A) Retinal cells isolated at stages 22-23 and 28-29 were transfected with an ATH5-promoter/*lacZ*-reporter plasmid singly or in combinations with NGN2 or ATH5 expression vectors and pulse-labelled with [^{3}H]-thymidine at the end of a 24-hour culture period. (Left) The number of *lac*$^+$ cells counted when the reporter plasmid was transfected alone is set at 1. (Right) At stage 22-23, overexpression of NGN2 enhances promoter activity in proliferating cells (a) and increases the pool of nonradioactive cells whose ATH5 promoter is upregulated (b). At stage 28-29, *lac*$^+$ cells whose promoter is strongly upregulated are unlabelled (d). The detection of double-labelled cells (c) and their increased number upon ATH5 overexpression indicate that ATH5 promoter activity is upregulated during the S phase. (B, left) A retina at stage 29-30 was pulse-labelled for 45 minutes with BrdU and chased for 15 minutes. Transverse sections were hybridized with an ATH5-specific riboprobe. Most BrdU-positive cells are in S phase and their nuclei are located on the vitreous side (vi) of the pz. ATH5 transcripts accumulate on the ventricular side (ve) of the pz in the region where cells are in the G1 and G2 phases of the cell cycle. A few BrdU-positive nuclei are located in this region (arrowheads). (Right) Schematic of mitosis in the pz. Scale bar: 40 μm.

ATH5 transcripts accumulate within the proliferative zone before cell cycle exit

Single-cell transcription analysis has revealed a lag period between the downregulation of HES1 and the upregulation of Neuro M, suggesting that the ATH5 autostimulatory pathway might be activated before the onset of Neuro M expression. In this context, we wanted to know at which point of the cell cycle is autostimulation able to drive the upregulation of ATH5 expression. When stage 28-29 retinal cells transfected with the ATH5 reporter plasmid were pulsed with [^{3}H]-thymidine for 1 hour, ~25% of *lac*$^+$ cells were in S phase and forced expression of ATH5 led to a twofold increase of this fraction and to a 3.5-fold enlargement of the population of non-radioactive cells (Fig. 8A). Most cells (>90%) that had robust promoter activity, as evidenced by strong X-gal staining, were found in the pool of non-radioactive cells both in the control and after ATH5 overexpression, indicating that cells bearing an upregulated ATH5 promoter do not re-enter the S phase (Fig. 8A). In situ hybridisation combined with anti-BrdU immunohistochemistry revealed a strong accumulation of ATH5 transcripts within the narrow region of the proliferative zone (Fig. 2G, Fig. 8B) where nuclei reside during the G1- or G2-phases of the cell cycle. When a 45-minute BrdU pulse is followed by a 15 minutes chase, the few BrdU-positive nuclei labelled with silver grains have already moved back towards the ventricular side of the retina (Fig. 8B, arrowheads). Taken together, our results suggest that increased promoter activity via the autostimulatory pathway begins during the last S phase and that the accumulation of ATH5 transcripts peaks as cells leave the S phase and enter the G2 phase.

Discussion

bHLH factors are required for the generation of a full array of the retinal neurons, but how they contribute to neuroepithelium patterning, cell commitment and cell cycle exit has remained elusive. Here, we use an approach combining single-cell mRNA profiling and promoter analysis to clarify the sequence

of molecular events leading to neural progenitor commitment. In particular, we define the transcriptional program determining the stages through which neural cells progress as they convert from progenitors into newborn RGCs. We find that spatial cell patterning and RGC commitment correlate with the two main phases of ATH5 expression. During the period of patterning, crossregulatory interactions between HES1, NGN2 and ATH5 keep ATH5 expression low, thereby maintaining the uncommitted status of ATH5-expressing cells and enabling the expansion and intermingling of pools of progenitors initially partitioned in distinct domains. Once progenitors are properly distributed throughout the retina, about one-third of ATH5-expressing cells become committed to acquire a definitive RGC fate immediately before exiting the cell cycle. This requires a tight coordination between downregulation of HES1, upregulation of NGN2, cell progression through the last S-phase and the upregulation of ATH5. Cells that upregulate ATH5 expression initiate transcription of early RGC-specific traits, then exit the cell cycle and express Neuro M and other post-mitotic RGC-specific genes. Our study highlights how changes in the transcriptional patterns correlate with the progression of progenitors through the last cell cycle and with their commitment to the RGC fate, underlining the role of HES1 as a key prompt of the molecular events leading to RGC genesis.

Spatiotemporal progenitor patterning and RGC genesis

A specific feature of retinogenesis is that it proceeds from the centre to the periphery such that all seven retinal cell types are distributed at the proper ratio throughout the retina. At early stages of development, the retinal neuroepithelium is subdivided into two developmentally distinct territories. Low levels of HES1 transcripts outline a broad region of the posterior retina where ATH5, NGN2 and ASH1 are expressed, whereas a robust accumulation of HES1 transcripts throughout the anterior retina prevents the onset of proneural gene expression. HES1 functions similarly at the onset of neurogenesis in the olfactory placode, where it circumscribes a domain of Mash1 expression (Cau et al., 2000). It thus appears that HES1 is acting, much like *hairy* in *Drosophila*, as a prepattern gene (Skeath and Carroll, 1991). Neurogenesis starts within a rather broad central region defined by expression of ATH5, NGN2 and Neuro M. Cells expressing ATH5 at a high level and Neuro M-positive cells are evenly distributed throughout the neurogenic domain, indicating that the first newborn RGCs are produced with similar frequency throughout the central retina. In the posterior retina, cells that initiated expression of proneural genes are initially organized in two separate domains corresponding to two retinal lineages: cells that express NGN2/ATH5 constitute the progenitor pools from which early-born retinal neurons will emerge, whereas ASH1-expressing cells form a pool for late-born neurons (Brown et al., 1998; Jasoni et al., 1994; Matter-Sadzinski et al., 2001). The opposite effects of NGN2 on ATH5 and ASH1 expression combined with the inhibitory activity of ASH1 on ATH5 transcription (Akagi et al., 2004; Fode et al., 2000; Matter-Sadzinski et al., 2001) account for the distribution of ASH1 and ATH5/NGN2 cells in two distinct progenitor domains, the more peripheral expression of ASH1 perhaps reflecting its lower sensitivity towards HES1. The initial patterning of the posterior retina resembles the neuroepithelial partitioning detected in other areas of the developing CNS (Bertrand et al., 2002; Jessell, 2000). However, whereas in other CNS regions the refining of borders is essential for the precise spatial generation of different classes of neurons along the dorsoventral axis, the blurring of borders and intermingling of initially distinct progenitor pools are necessary for a proper spatial distribution of neurons and glia throughout the retina. Although ATH5/NGN2 and ASH1 expressions are mutually exclusive, a small fraction of ATH5-expressing cells co-express ASH1 (Fig. 4) (Matter-Sadzinski et al., 2001), indicating that they are in a transient state prior to acquiring a definite progenitor status. Because the ATH5, NGN2 and ASH1 genes crossregulate and display different sensitivities towards HES1, we suppose that various balances between these four factors may mediate alternate fate choices. Such dynamic regulatory interactions are, in part, responsible for the progressive loss of patterning in the posterior retina. The ATH5/NGN2 domain remains restricted to the posterior retina until E4 and expands to keep pace with growth of the whole retina at a rate similar to that reported for the differentiation of RGCs (McCabe et al., 1999). Despite significant changes in the expression pattern of ATH5, similar proportions of retinal cells express this gene at stages 18 and 29-30, suggesting that ATH5-expressing cells propagate at a rate comparable with that of the other progenitors during the period of patterning.

Even though the population of ATH5-expressing cells is established at E2.5, only a small fraction of these will differentiate into RGCs until E4 (Prada et al., 1991; Rager, 1980; Waid and McLoon, 1995). Retinogenesis is controlled by components of the Notch pathway (Perron and Harris, 2000; Vetter and Brown, 2001), which may employ two strategies to keep the majority of cells in the central retina from differentiating during the patterning period. Cells that express proneural genes may promote the upregulation of HES1 in neighbouring cells, thereby preventing them from expressing proneural genes. The proximity in central retina of individual cells that highly express HES1 or ATH5 is indeed indicative of ongoing lateral inhibition. However, cells strongly expressing Notch effectors are rare in the posterior retina (Fig. 1I, Fig. 2L), whereas a high proportion of ATH5-expressing progenitors co-express HES1 (Fig. 4). Thus, it appears that the low level of HES1 in cells that have already initiated NGN2 and ATH5 expression suffices to prevent the upregulation of these genes. The proliferative state is thereby maintained in most ATH5-expressing cells, as required to ensure the proper ratio of RGC progenitors in the posterior retina and as expected of HES genes, which function to keep neuroepithelial cells undifferentiated, thereby regulating the size and cell architecture of brain structures and retina (Hatakeyama et al., 2004; Ishibashi et al., 1995; Takatsuka et al., 2004; Tomita et al., 1996). In anterior retina, progenitor cell patterning becomes evident by E4 and the expansion of proneural gene expression proceeds, much as in zebrafish (Masai et al., 2000), in a wave-like fashion as HES1 expression recedes to the retinal margin. The ASH1 and NGN2 expression domains expand to the periphery at similar rates, whereas the progression of the ATH5 domain is slightly delayed (Fig. 2). The full patterning of the retina accomplished around E6 coincides with the upregulation of proneural gene expression throughout the retina and with the peak of RGC production.

To analyse how ATH5 is regulated along the course of RGC specification, we used a promoter region extending 775 bp upstream of the initiation codon. The cloned sequence accurately reproduces the activity and the mode of regulation of the endogenous promoter. It contains essential regulatory elements that are well conserved across distant vertebrate species (Brown et al., 2002; Hutcheson et al., 2005; Skowronska-Krawczyk et al., 2004), but it is unclear whether the different species use similar strategies to regulate ATH5 expression. Whereas a proximal cis-regulatory region of the *Xenopus* Xath5 gene suffices, much as in the chick retina, to drive retina specific reporter gene expression in a bHLH-dependent manner, the mouse ATH5 promoter appears to be regulated differently (Hutcheson et al., 2005). It is tempting to speculate that the different modes regulating ATH5 across species may account for differences in the spatiotemporal progenitor patterning of the retinal neuroepithelium. Differences in the developments of the anterior and posterior retinas may have permitted the evolution of a specialized structure such as the macula.

Multiple functions of NGN2 in the specification of RGCs

Our study reveals that NGN2 acts at different regulatory levels during RGC specification. In early retina, NGN2 is a principal regulator of ATH5 expression and exerts this function through direct activation of ATH5 transcription and through crossregulatory interactions with HES1. In addition, NGN2 drives ATH5-expressing cells out of S phase. Whereas the capacity of NGN2 to promote cell cycle arrest is part of its panneuronal activities and is in evidence in other compartments of the developing CNS (Farah et al., 2000; Ma et al., 1996; Novitch et al., 2001), its capacity to activate ATH5 expression is largely retina specific. The quasi-simultaneous onset of NGN2 and ATH5 expression in the central retina shortly after formation of the eye cup (Fig. 1), the capacity of NGN2 to activate ATH5 transcription (Figs 6, 7) (Matter-Sadzinski et al., 2001) and to bind the ATH5 promoter (Skowronska-Krawczyk et al., 2004) at the early stages of development suggest that NGN2 may be directly involved in the activation of ATH5 expression. Our finding that the expansion of the NGN2 domain towards the anterior edge of the retina precedes that of ATH5 argues in favour of this interpretation. In the retina of the Ngn2$^{-/-}$ mouse, the much increased expression of ASH1 (Akagi et al., 2004) and the downregulation of ATH5 (D. Skowronska-Krawczyk and J.M.M., unpublished) when compared with the wild type, may result from an increase in the population of ASH1-expressing cells at the expense of the ATH5/NGN2 progenitors, thus underlining the importance of NGN2 in establishing and maintaining a pool of ATH5-expressing cells. Both the NGN2 and ATH5 genes fail to be activated in the retinal precursors of the Pax6$^{-/-}$ mouse and Pax6 has been proposed to regulate NGN2 directly in the mouse retina (Brown et al., 1998; Marquardt et al., 2001). There are multiple E-boxes but no consensus Pax6 binding site in the chicken ATH5 promoter, and therefore we favour the idea that Pax6 regulates ATH5 via NGN2. The expression of NGN2 in many regions of the nervous system anlage where ATH5 is not detected and the demonstration that recruitment of NGN2 on the ATH5 promoter is retina specific (Skowronska-Krawczyk et al., 2004) provide evidence that a retina-specific context accounts for the capacity of NGN2 to activate ATH5 expression. The ability of bHLH factors to regulate the development of distinct neurons has been proposed to depend upon the cellular contexts in which they function (Perron et al., 1999). In retina, this context may be determined, among other possibilities, by the balance between NGN2 and HES1, as we show that HES1 inhibits the NGN2-mediated activation of ATH5 in a dose-dependent manner (Fig. 7). Likewise, the upregulation of NGN2 correlates with the dowregulation of HES1 (Fig. 2C) (Matter-Sadzinski et al., 2001). Moreover, single cell transcriptional analysis reveals that overexpressing NGN2 diminishes the pool of cells that co-express ATH5 and HES1 (Fig. 4C), an indication that NGN2 may contribute to the downregulation of HES1 in early neural progenitors, thereby providing a cellular environment permissive for ATH5 autostimulation.

The upregulation of both NGN2 and ATH5 occurs later in development, around E6, but by then ATH5 has become the main regulator of its own transcription (Fig. 6) (Matter-Sadzinski et al., 2001). NGN2 occupies the ATH5 promoter similarly at E3 and at E6 (Skowronska-Krawczyk et al., 2004), suggesting that it still directly participates in the control of ATH5 transcription. However, its main contribution to ATH5 expression may occur through other, indirect regulatory pathways. As ATH5-expressing progenitors exit the cell cycle, NGN2 promotes the expression first of Neuro M and then of Neuro D (Novitch et al., 2001; Perron et al., 1999; Roztocil et al., 1997) both stimulators of ATH5 promoter activity (Matter-Sadzinski et al., 2001). These distinct functions of NGN2 in the ontogenesis of RGCs illustrate how, depending on specific combinations of transcription factors and of other cellular components, neurogenic proteins may contribute to neuronal identity.

Molecular interactions between bHLH transcription factors control ATH5 expression during progenitor patterning

How does the retina prevent the differentiation of cells that have initiated ATH5 expression and preserve an expanding pool of progenitors during a period of highly dynamic patterning and considerable tissue growth? The interplay between the molecular mechanisms underlying patterning and those controlling the rate of RGC differentiation appears to be an integral part of retina development. Among the different regulatory pathways that are involved, the transcriptional network regulating ATH5 has a pivotal role. In early retina, the *ATH5* gene is transcribed at a low rate in ATH5-expressing progenitors and its forced expression initiates the precocious transcription of β3 in these cells (Matter-Sadzinski et al., 2001), indicating that they are competent for the expression of this early RGC marker. The appropriate ATH5 dose is controlled through a complex interplay between positive (NGN2) and negative (HES1) regulators. We show that individual progenitors co-express HES1 and ATH5, and that HES1 represses the ATH5 promoter, thereby demonstrating that an effector of Notch helps maintain a low rate of ATH5 transcription during progenitor patterning, in agreement with the role of Xnotch pathway components in the regulation of Xath5 function (Schneider et al., 2001). The precise dose of HES1 is crucial during this period; when the level of HES1 is too high expression of ATH5 is suppressed, whereas the lack

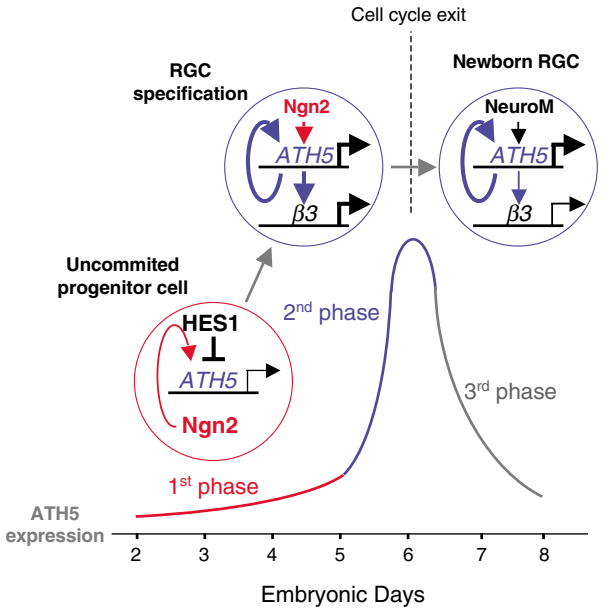


Fig. 9. Interacting transcriptional patterns as retinal cells go through three consecutive phases during the conversion of progenitors into newborn RGCs.

of HES1 leads to the precocious differentiation of retinal cells. In the posterior retina, HES1 is expressed at levels that are high enough to prevent ATH5 autostimulation, but low enough to allow NGN2-mediated expression of ATH5 (Fig. 9). As revealed by overexpression experiments, NGN2 is a potent activator of ATH5 in retinal progenitors, but its low expression level normally leads to low ATH5 levels. Moreover, NGN2 is counteracted by ATH5 itself, which acts as a dominant-negative inhibitor of NGN2 in cells expressing HES1, thereby directly contributing to the negative control of its own expression. This competition between NGN2 and ATH5 may occur at the promoter level as both factors bind the ATH5 promoter in stage 22-23 retinas (Skowronska-Krawczyk et al., 2004) and require the E-boxes E2 and E4 (see Fig. S1 in the supplementary material). Although HES1 represses the ATH5 promoter and prevents efficient autostimulation in retinal progenitors (Fig. 7), it does not prevent the binding of either NGN2 or ATH5 to the ATH5 promoter. We surmise that ATH5 activity at early stages may be repressed by heterodimerization with HES1, as reported for other bHLH proteins (Alifragis et al., 1997). This mechanism of inhibition is consistent with the fact that overexpression of ATH5 in retinal progenitors does not overcome HES1-mediated inhibition of the ATH5 promoter, whereas NGN2 does so in a dose-dependent manner (Fig. 7). Thus it appears that a subtle balance and interplay between NGN2, ATH5 and HES1 is responsible for maintaining ATH5 expression below the level needed to trigger cell commitment and direct expression of RGC markers.

RGC commitment requires the coordinated downregulation of HES1 and upregulation of NGN2 and ATH5 during the last cell cycle

During early retina development, ~30% of progenitors express ATH5 but only a fraction of these become RGCs. When and how is this subset committed to the RGC fate? On E6, at the peak of RGC genesis, ATH5-positive cells are distributed throughout the whole retina and a robust accumulation of ATH5 transcripts takes place within the proliferative zone. This phase of transcriptional regulation is characterized by a marked increase in ATH5 promoter activity as ATH5 autostimulation becomes dominant in the absence of HES1. ATH5-expressing progenitors progressively downregulate HES1 between E5 and E6 (Fig. 4), yet only one third of them will upregulate ATH5 on E6. We suppose that they are those that overexpression experiments conducted on E5 reveal to be permissive for ATH5 autostimulation (Fig. 6A). They may have been selected on E5 for RGC commitment because they were at the appropriate phase of the cell cycle when downregulation of HES1 was initiated. Whereas forced expression of NGN2 halves the [^{3}H]-thymidine-labelling index, it led to a fourfold decrease in the pool of cells co-expressing ATH5 and HES1 (Fig. 7A, Fig. 4C), suggesting that a significant fraction of S-phase cells have completed the downregulation of HES1. The accumulation of ATH5 transcripts in the proliferative zone where G2 and G1 cells reside and the presence of some S-phase cells expressing high levels of ATH5 (Fig. 8) indicate that ATH5 upregulation begins during the last S phase and peaks before cell cycle exit. Taken together, our results suggest that downregulation of HES1 and entry into S phase generate the proper conditions for ATH5 autostimulation, causing the ATH5 protein to accumulate during a very short period at levels sufficiently high to help RGC precursors withdraw from the cell cycle (Fig. 8) (Ohnuma et al., 2002) and trigger or boost expression of RGC-specific target genes (e.g. BRN3C and β3, respectively; Fig. 9). The expression of both β3 and BRN3C is stimulated when ATH5 is overexpressed and their in vivo activation coincides with increased ATH5 transcription (Liu et al., 2001; Matter-Sadzinski et al., 2001). As a case in point, the in vivo binding of ATH5 on the β3 promoter and robust β3 promoter activity are detected when ATH5 is upregulated (Matter-Sadzinski et al., 2001; Skowronska-Krawczyk et al., 2004).

Our results position HES1 as an important prompt at distinct stages in the sequence of events leading to the specification of RGCs. By interacting with the pathways that regulate ATH5 transcription, it prevents high-level ATH5 expression at the time of progenitor patterning, a function essential for establishing and maintaining the pool of ATH5-expressing cells. Its timely downregulation during the last cell cycle releases the autostimulatory activity of the ATH5 protein in a subset of progenitors and thus controls the timing of their commitment and perhaps the size of their pool.

Cells that upregulate ATH5 exit the cell cycle (Fig. 8) and start expressing the post-mitotic factor Neuro M. There is a time lag between the downregulation of HES1 and the expression of Neuro M (Fig. 4). Overexpression of NGN2 in early retina leads to the precocious accumulation of ATH5-positive cells expressing neither HES1 nor Neuro M, and drives these progenitors out of S phase, presumably in G2 or in G1, until they withdraw from the cell cycle and start expressing Neuro M. Thus, although the onset of Neuro M expression coincides with the transient upregulation of NGN2 and ATH5 in the developing retina (Roztocil et al., 1997; Matter-Sadzinski et al., 2001), it appears not to be regulated directly by these proteins, a notion supported by the absence of ATH5 and NGN2 binding on the Neuro M promoter in developing retina (Skowronska-Krawczyk et al., 2004) (D. Skowronska-

Krawczyk and J.M.M., unpublished) and by the finding that induction of Xath3 transcription by Xngn1 requires de novo protein synthesis (Perron et al., 1999). This also suggests that additional factors are required for Neuro M expression, which may not yet be present in early ATH5-expressing cells. Overexpression of NGN2 prevents early ATH5 progenitors from re-entering the S phase but is not sufficient to promote their precocious cell cycle exit. Likewise, overexpression of Xath5 at early stages of *Xenopus* retinogenesis produces extra RGCs that are all born at the appropriate time (Ohnuma et al., 2002). Thus, it is only when the coordinated upregulation of NGN2 and ATH5 coincide with the build up of cdk inhibitors (Dyer and Cepko, 2001; Ohnuma and Harris, 2003) that progenitors may leave the cell cycle in G1, enter G0 and begin expressing Neuro M. Expression of ATH5 remains strong in newborn RGCs and at this stage Neuro M contributes to its regulation (Fig. 9).

About two-thirds of ATH5-expressing cells fail to upregulate ATH5 expression and to acquire RGC traits. We reason that although these cells suppressed HES1, they only accessed their last S phase on E6, too late for properly upregulating ATH5 and thus missing the time-window when most RGCs are produced. Cell-fate tracing experiments suggest that they may become other retinal cell types (Yang et al., 2003) and the finding that a fraction of progenitors co-express ATH5 and ASH1 on E6 indicates ongoing alternate fate opportunities (Fig. 4) (Matter-Sadzinski et al., 2001). Even though all uncommitted ATH5-expressing progenitors are competent to upregulate ATH5 (Fig. 6C), not all of them can be made to adopt the RGC fate by forced expression of ATH5 (Liu et al., 2001). Overexpression of Xath5 at later stages of *Xenopus* retinogenesis does not change the proportion of RGCs and increases the number of photoreceptor and bipolar cells (Moore et al., 2002). It has been proposed that the ability of bHLH factors to promote the development of distinct retinal neurons depends upon the timing of their expression and/or function (Moore et al., 2002; Morrow et al., 1999). If this was the case, the set of genes regulated by ATH5 would change over time to comprise genes specific for later-born neurons. Establishing the compendium of ATH5 transcriptional targets should help answer the question of whether ATH5 is dedicated solely to the production of RGCs or whether it also promotes the development of other retinal subtypes.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/17/3907/DC1

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Table S1. Primers used		
Gene	External primers	Internal primers
ATH5	5′ ATH5 5′cttgctgaagcagagagatacag3′ 3′ ATH5 5′ctaattagctatttgaaagtgttcag3′	5′ ATH5 5′gagaatggattaaccttcactgtg3′ 3′ ATH5 5′aatattcgctgtgcataaggatca3′
Neuro M	5′ Neuro M 5′ctttgcttaacatctcccgtc3′ 3′ Neuro M 5′cgagccctgaacctctccaacc3′	5′ Neuro M 5′caaagccaaggagatggcagag3′ 3′ Neuro M 5′cctcgatgctgtcgtgctcttc3′
HES1	5′HES1 5′cgtgctgggcaaataccg3′ 3′HES1 5′gtggtaggaggcagttgga3′	5′HES1 5′caatgagtgcatgaacgagg3′ 3′HES1 5′gttgagccagatgtgcgg3′
Delta 1	5′Delta 1 5′aaaaactgtgagctgagtgc3′ 3′Delta 1 5′cactgggctccattagcac3′	5′Delta 1 5′tgtgctgatggaccgtgc3′ 3′Delta 1 5′gctggaactgcagtaatcg3′
β3nAChR	5′β3AChR 5′tccattcggtacatttccaggc3′ 3′β3AChR 5′gggcttgttgtatgttagcgtg3′	5′β3AChR 5′catcaggcaggttgtccaagac3′ 3′β3AChR 5′tctacaaagtgctgttcagccac3′
ASH1	5′ASH1 5′gacgagcacgacgccgtcag3′ 3′ASH1 5′cgggagagggcgcttatctc3′	5′ASH1 5′gttactcccacgacatgaactc3′ 3′ASH1 5′tcagaaccagctggtgaagtcg3′
BRN3C	5′BRN3C 5′ctctggccgctgtggatatag3′ 3′BRN3C 5′ttgaggtggtccaggagctc3′	5′BRN3C 5′catccgttcaagccggatgc3′ 3′BRN3C 5′cctgcgcgggctgatggtg3′