Highly Conserved Sequences Mediate the Dynamic Interplay of Basic Helix-Loop-Helix Proteins Regulating Retinogenesis*

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The atonal homolog 5 (ATH5) protein is central to the transcriptional network regulating the specification of retinal ganglion cells, and its expression comes under the spatiotemporal control of several basic helix-loop-helix (bHLH) proteins in the course of retina development. Monitoring the in vivo occupancy of the ATH5 promoter by the ATH5, Ngn2, and NeuroM proteins and analyzing the DNA motifs they bind, we show that three evolutionarily conserved E-boxes are required for the bHLH proteins to control the different phases of ATH5 expression. E-box 4 mediates the activity of Ngn2, ATH5, and NeuroM along the pathway leading to the conversion of progenitors into newborn neurons. E-box 1, by mediating the antagonistic effects of Ngn2 and HES1 in proliferating progenitors, controls the expansion of the ATH5 expression domain in early retina. E-box 2 is required for the positive feedback by ATH5 that underlies the up-regulation of ATH5 expression when progenitors are going through their last cell cycle. The combinatorial nature of the regulation of the ATH5 promoter suggests that the bHLH proteins involved have no assigned E-boxes but use a common set at which they either cooperate or compete to finely tune ATH5 expression as development proceeds.

Retina development in vertebrates relies on regulatory proteins, most of which are widely expressed in the developing nervous system. However, the expression of the basic helix-loop-helix protein atonal homolog 5 (ATH5) appears to be restricted to retina ontogenesis. ATH5 activates neurogenesis and is required for the production of retinal ganglion cells (RGCs) (1–4). It is transiently expressed during the period of development when RGCs are produced and underlies the pathway leading to the conversion of proliferating progenitors into newborn RGCs (5, 6). Retinotopic differences in the timing of RGC production may reflect the wave-like expression of ATH5 (5, 7). In zebrafish, a signal from the optic stalk appears to induce the first patch of ATH5-expressing cells, and the continued spread of ATH5 expression beyond that patch may require a cascade of cell-to-cell interactions (7, 8). It has been suggested that Sonic hedgehog derived from newborn RGCs drives a self-propagating wave of ATH5 expression and RGC production across the zebrafish retina (9, 10). In contrast, related experiments in zebrafish and chick highlight the importance of intrinsic factors for triggering ATH5 expression and neurogenesis (11–13).

The presence of consensus E-box binding sites in the highly conserved upstream sequence of the ATH5 gene suggests that bHLH transcription factors are directly involved in the regulation of ATH5. This idea is supported by experiments showing the selective binding of neuronal bHLH proteins to the upstream sequence of ATH5 and the simultaneous changes in the expression level of ATH5 in response to several of these proteins (5, 6, 14–16). These findings indicate a requirement for different combinations of bHLH proteins in regulating the different phases of ATH5 expression in the course of retina development. In proliferating progenitors, activation of ATH5 by Ngn2 is counteracted by HES1, which contributes to maintain expression of ATH5 at a low level in these uncommitted cells. When the cells enter their last cell cycle, the down-regulation of HES1 leads to the rapid, self-stimulated up-regulation of ATH5. The cells then exit the cell cycle, whereupon ATH5 and NeuroM cooperate to maintain ATH5 expression in the newborn RGCs (5).

The mechanism by which neuronal bHLH transcription factors select their targets in the developing nervous system has remained elusive. bHLH proteins form heterodimers through the interaction of the HLH domains. The basic regions act as sequence-specific DNA binding domains that recognize a binding site with the sequence CANNTG, the consensus E-box. A simple model for the role of neurogenic bHLH proteins posits that they regulate transcription by binding to the E-boxes in the regulatory regions of genes expressed in neurons. This basic model has several shortcomings. First, E-boxes occur frequently in the genome, not just in the regulatory regions of neural genes. Second, the many different subfamilies of bHLH proteins recognize the same canonical sequence. Mechanisms must, therefore, be in place to limit the potential of these proteins promiscuously to activate or repress genes.

Here, we monitor changes in the in vivo occupancy of the ATH5 proximal promoter by bHLH proteins during the course
of retina development. Dynamic changes in the binding of these proteins correlate with the different phases of ATH5 expression along the RGC specification and differentiation pathway. By analyzing the functional properties of a cis-regulatory region encompassing the ATH5 promoter, we show that three evolutionarily conserved E-boxes play a crucial role in regulating the ATH5 gene. Whereas Ngn2 requires all three E-boxes to activate ATH5 properly, two mediate autoregulation by ATH5, and one is sufficient for activation by NeuroM. In addition, one E-box mediates the antagonistic effects of HES1 and Ngn2. In sum, we demonstrate how differential occupancy at three regulatory sites modulates ATH5 transcription as development proceeds.

**EXPERIMENTAL PROCEDURES**

**ATH5 Promoter Constructions**—The subcloning of the chick ATH5 promoter and the identification of a single transcription initiation site have been described (6). Wild-type and mutated upstream sequences 912 bp in length and bounded by XbaI and BstXI restriction sites (GenBank™ AJ630209) were subcloned in the proper orientation at appropriate sites in the vectors p00-CAT, p00-lacZ, pEGFP, and pDsRed2-N1 (Clontech). In control experiments wild-type and mutated upstream sequences 2220 bp in length and bounded by EcoRI and BstXI restriction sites were subcloned in the vectors pEGFP and pDsRed2-N1. Mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene). Mutated promoter fragments were checked by sequencing and re-cloned at the EcoRI site of the p00-CAT vector (17, 18). The deletion Δ(−775; −235) in the 5′ region was generated by PCR from the ATH5 promoter fragment using a downstream primer flanking E-box 4 and corresponding to the region −235 to −214 and an upstream primer located in the region +146 to +125. The PCR fragment was subcloned in the proper orientation into the unique SmaI site of the p00-CAT vector and checked by sequencing.

**Eukaryotic Expression Plasmids for ATH5, Ngn2, NeuroM, and HES1**—The pEMSV plasmid (6), which puts a cloned 114 bp fragment of the chick HES1 cDNA under the control of a SV40 promoter, was subcloned into the unique SmaI site of the p00-CAT or lacZ reporter genes as described in Matter et al. (17) and Matter-Sadzinski et al. (18). When transfections were performed with only the reporter plasmid, we used 1 µg of DNA/10⁶ cells. In co-transfection experiments, we used 1 µg of reporter plasmid and 0.5 µg of expression vector per 10⁶ cells.

In all cases the ratio of DNA to Lipofectin (Invitrogen) was 1:4. 24 h after transfection, the cells were either fixed for X-gal staining or harvested and processed for CAT assay. Chloramphenicol acetyl transferase activity was determined using 100 µg of cytosolic proteins, and the activity of the wild-type ATH5 promoter was set arbitrarily at 100. In all experiments a promoter-less reporter plasmid (pCAT00 (18)) was included to serve as a negative control. Its very low background activity was subtracted from the experimental CAT activities. The means and S.D. values were calculated with data obtained in at least five independent experiments. Tissue culture reagents were from Invitrogen, and plastic-ware was from Nunc.

**Preparation of Retina Protein Extracts and Gel Mobility Shift Assay**—Tissues were dissected in cold phosphate-buffered saline, immediately frozen in liquid nitrogen, and stored at −70 °C or processed directly for extraction. Tissues were homogenized in a solution containing 100 mM Hepes, pH 7.4, 150 mM KCl, 5 mM MgCl₂, 5 mM EDTA, pH 8.0, 35% glycerol, 5 mM NaF, and a mixture of protease inhibitors (Sigma) to which was added 1 mM dithiothreitol, 0.1 mM benzamidine, 1.5 mM phenylmethylsulfonl fluoride, and 0.5 µg/ml leupeptin. The mixture of protease inhibitors contained 104 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 80 µM aprotinin, 2.1 mM leupeptin, 3.6 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64 and was diluted 100X in the solution. The tissues were then treated to four snap/freeze cycles and left to thaw 30 min under rocking agitation. Cellular debris were pelleted by centrifugation, and the supernatant was transferred to a clean tube. The protein concentration was determined by the Bradford assay (Bio-Rad protein assay). Bacterially expressed glutathione S-transferase fusion proteins of E47 and NeuroM were purified according to the manufacturer’s instructions (Amerham Biosciences). The probes were double-stranded DNA fragments of 70 bp end-labeled by fill-in of the 5′ overhang with the Klenow enzyme in the presence of [α-32P]dATP. The probes were purified using the QIAquick nucleotide removal kit (Qiagen). Each electrophoretic mobility shift assay reaction was set up by mixing 2 µg of bovine serum albumin, 2.5 µg of poly(dl-dc) with 20,000–50,000 cpm of probe in 25 mM Hepes, pH 7.6, 40 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 10% glycerol. Usually, 6–8 µg of whole-cell protein extract or 0.5–0.8 µg of purified proteins were used in a total volume reaction of 20 µl. Samples were incubated for 15 min on ice and loaded on 4% acrylamide, 0.25 × Tris borate-EDTA gels. After a run of 2.5 h at 180 V and 4 °C, the gels were fixed in 20% ethanol, 10% acetic acid, dried, and exposed overnight at −70 °C with an intensifying screen.

**Chromatin Immunoprecipitation Assays**—Chromatin immunoprecipitation assays were performed essentially as described in Skowronska-Krawczyk et al. (15) using purified antibodies raised against the bacterially expressed chicken ATH5, Ngn2, and NeuroM proteins. In a typical experiment retinas and optic tecta (the latter, a control tissue expressing Ngn2 and NeuroM but not ATH5) were dissected from HH23 to HH38 embryos and incubated in 1% formaldehyde solution with Dounce homogenization. Cross-linking was blocked, cells were incubated in a lysing solution, and the collected nuclei were sonicated to an average DNA length of 700 bp. Cross-linked chromatin was incubated in a solution containing affinity-purified antibody, and immune complexes were captured on protein A-coated Sepharose beads. Immunoprecipitated DNA sequences were quantified by real-time PCR using the iCycler iQ real-time PCR detection system (Bio-Rad) and a SYBR-Green-based kit for quantitative PCR (iQ Supermix; Bio-Rad). Immunoprecipitated DNA was quantified by comparison to a
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standard curve generated by serial dilution of input DNA, subtracting values obtained with a control antibody (anti-FLAG M2, Sigma). The data were plotted as the mean of at least two independent chromatin immunoprecipitation (ChIP) assays and three independent amplifications. Immunoprecipitation efficiency was calculated as the ratio of precipitated sequence over total amount of sequence in the input chromatin. The primers used for real-time amplifications were as follows: ath5fwd, GCTGGGAAGGTACTGGGAT; ath5rev, CTTAGTCAGATGATGAAGAA; gatafwd, GAGGCCTTGACGTCCCCCC------------CCC-CCCCCTTAG-GATCGTTTTAGGACTGT---TAAGACGGAAGCAGACAA-CTTCAGAGAGGTTTCGGTACAGACAGGTTGGGATTG-TTGTGTG

FIGURE 1. The proximal regulatory region of the ATH5 gene. A, the sequence extends −775 to +185 with respect to the transcription initiation site (arrow). The E-boxes are numbered E1 to E7. The base changes introduced to mutate the underlined regulatory sites are as shown. The probe sequences used for gel shift experiment (see Fig. 4, B and D) are shown with dashed underline. B, multispecies alignments of the ATH5 promoter sequences. Although E-box E4 is highly conserved in birds and mammals, it is absent in both X. laevis and X. tropicalis.

Electroporation of Genetic Material and Confocal Microscopy—Retina electroporations were as described in Matter-Sadzinski et al. (6) with some modifications. Briefly, embryonic eyes were collected at HH22–23, and the pigmented epithelium was removed. Stripped eyes were positioned in an electroporation chamber (BT 640, BTX, Genetronics) filled with 100 μl of phosphate-buffered saline containing reporter plasmids (0.2 μg/μl each) with or without expression vectors (0.1 μg/μl each). Electroporation consisted of five 10-V/cm pulses of 50-ms duration spaced 1 s apart. The polarity was then inverted, and the electroporation protocol was repeated once. The electroporated tissues were cultured as floating explants for 24 h at 37 °C. Identification of lac+ cells was as described in Matter-Sadzinski et al. (6). For confocal microscopy, retinas were fixed for 20 min in 4% paraformaldehyde, rinsed 24 h in phosphate-buffered saline (PBS) and mounted in PBS containing 43% glycerol, 21 mM 1,4-diazabicyclo[2.2.2]octane (Sigma). Red and green fluorescent cells were imaged with a confocal laser scanning microscope (LEICA SP2-AOB5) using a 20× NA 0.7 oil objective (Leica). An argon/krypton (Ar/ArKr) laser (488 nm line) was used for both GFP and RFP excitation. Optical sections of 0.8 μm were taken through a volume of the retina up to 50 μm in depth. Image data were acquired and stored as TIFF files using confocal software (Leica) or Imaris.

RESULTS

The 5′ Promoter Sequence of the Chick ATH5 Gene—We have reported the isolation of a proximal cis-regulatory region of the chick ATH5 gene that reproduces the expression pattern of the endogenous gene when transfected into the retina (6). We sequenced about 1300 base pairs upstream of the first ATG, determined the transcription start site, and identified several putative regulatory elements of which E-boxes were the most in evidence. As shown in Fig. 1A, there are seven E-boxes within the region upstream of the transcription start site. Numbered E1 to E7 in the proximal-to-distal direction, they are located,
The ATH5 Promoter Is Transiently and Sequentially Bound by bHLH Proteins during Retina Development — The expression of ATH5 goes through a sequence of phases during retina development, and the Ngn2, ATH5, and NeuroM proteins contribute differentially to ATH5 promoter activity (5). We wanted to determine how the binding profiles of these proteins to the ATH5 promoter correlate with changes in the kinetics of ATH5 expression in the developing retina.

ATH5 mRNA level is low and steady during a first phase that extends between HH18 and HH25 (staged according to Hamburger and Hamilton (19)); it then increases during a second phase (HH26 to HH30) that coincides with the period of development when the majority of RGCs are produced. Finally, ATH5 expression is much decreased in HH35 retina and reaches background level at HH38 (6).

ATH5 and Ngn2 are co-expressed (5, 6), and to analyze the binding of these proteins to the ATH5 promoter in the course of development, chromatin was prepared from retinas and optic tecta in the range HH22–23 to HH38, and ChIP was performed using antibodies directed against the N-terminal domains of ATH5 and Ngn2 (15). In retina the binding of Ngn2 and ATH5 was high at HH22–23 and HH29–30 and low at HH35 and HH38 (Fig. 2A). The proportion of ATH5-expressing cells in the retina remains constant between HH22 and HH30, whereas it is much decreased at HH35 and HH38 (Fig. 2B and Refs. 5 and 15). When ChIP efficiency was calculated relative to the proportion of ATH5-expressing cells, the binding of ATH5 was steady between HH22 and HH35, indicating that it may interact with its own promoter along the whole pathway leading to conversion of progenitors into newborn RGCs. In contrast, the decreased occupancy of Ngn2 at HH29–30 and HH35 suggests a role for this factor in progenitors (Figs. 2A, inset).

NeuroM is expressed in newborn RGCs (5). To analyze its binding to the ATH5 promoter, ChIP was performed using antibodies directed against the C-terminal domain of NeuroM (Fig. 2A). In retina the binding of NeuroM to the ATH5 promoter was low at HH22–23 and much enhanced at HH29–30. Although similar levels of NeuroM protein were detected by Western blot in HH29–30 retina and optic tectum, no binding of NeuroM was detected in the optic tectum (Fig. 2A), suggesting that its interaction with the ATH5 promoter is a retina-specific feature. The significant increase in the binding of the NeuroM protein to the ATH5 promoter between HH22–23 and HH29–30 is consistent with the co-expression of NeuroM and ATH5 in newborn RGCs (5). Normalized ChIP efficiency (Fig. 2A, inset) revealed a much increased binding of NeuroM at HH35 despite the down-regulation of ATH5 expression. At this stage NeuroM is expressed in the inner retina but not in the newly formed ganglion cell layer (GCL) (20), suggesting that it interacts with the ATH5 promoter in newborn RGCs on their way to the GCL. This idea is supported by the finding that RGCs may co-express NeuroM and Brn3c (5) and is consistent with the fact that at HH38, when the large majority of RGCs have migrated in the ganglion cell layer, the binding of NeuroM to the ATH5 promoter has decreased (Fig. 2A) despite a robust expression of NeuroM in the inner nuclear layer (20). However, because there are still significant amounts of NeuroM protein bound to the ATH5 promoter at HH38, we cannot exclude the possibility that the protein may also interact with the ATH5 promoter in cells unrelated to the RGC lineage. This notion is consistent with the fact that NeuroM is broadly expressed in newborn post-mitotic retinal neurons (20).

Taken together, these data indicate that different bHLH proteins interact with the ATH5 promoter during development. Dynamic changes in the binding of these factors correlate with the different phases of ATH5 expression, indicating that bHLH proteins directly participate in the regulation of the ATH5 gene. We next attempted to find out which of the E-boxes in the ATH5 promoter mediate bHLH activity and whether the different bHLH proteins are using different combinations of E-boxes.

Identification of Functional Regulatory Sequences within the ATH5 Promoter — To determine whether any of the E-boxes E1 to E7 played a role in ATH5 regulation, we mutated each of them. The activity of the mutant promoters was tested by transient transfection in chick retinal cells collected at HH29–30, the stage when ATH5 promoter activity is strongest (6) and during which most of the ganglion cells are produced (21). As shown in Fig. 3, we found that mutation of any of the distal E-boxes (E5, E6, or E7) had no significant effect on promoter activity. In contrast, mutation of E4 resulted in a complete loss of activity, indicating a crucial role in regulating ATH5. Similarly, mutation of E2 or E3 resulted in severe losses of activity, indicating a strong contribution of these elements (Figs. 3 and 4D), whereas mutation of E1 had no significant effect. Because E4 is necessary for promoter activity, we asked whether E4 alone is sufficient to drive ATH5 expression or whether it requires other E-boxes. We constructed a promoter where all E-boxes were mutated except E4 and assayed its activity in HH29–30 retinal cells. This mutant was clearly incapable of driving transcription (Fig. 3), demonstrating that E4 is necessary but not sufficient for ATH5 expression. We investigated the role of E4 in conjunction with other E-boxes by constructing a series of combined mutations. The presence of the distal E-boxes (E5–E7) in combination with E4 did not significantly boost activity as compared with E4 alone. In contrast, the addition of the proximal E-boxes (E1–E3) to E4 resulted in a strong enhancement of promoter activity. The recovered activity, however, was still lower than that of the wild type, suggesting that the distal E-boxes (E5–7) modestly contribute to promoter activity, their influence being detectable only when all three are mutated. In an attempt to identify other regulatory sequences and/or a minimal promoter, we performed a deletion of the entire promoter region upstream of E4. Removal of these sequences caused a severe loss in activity, suggesting that other elements besides the distal E-boxes reside in that region and are important for the regulation of ATH5. In addition, we found a strong reduction in activity when we mutated the TATA-box or the consensus site for the Sp1 transcription factor, indicating...
that both of these elements have a role in the transcription process. These results show that the ATH5 gene is under the control of a hierarchy of regulatory elements; a functional E-box at the position of E4 is indispensable for expression, but E4 does not operate without support from at least some of the proximal E-boxes, and optimal expression requires the contribution of other sites in addition to E-boxes.

The Context and Identity of E4 Are Essential for Promoter Activity—Several studies have shown that the sequences flanking an E-box contribute to the selection of the appropriate bHLH factors (22–25). The high degree of conservation of the sequences flanking E1, E2, and E4 (Fig. 1B) suggests that they are important for ATH5 expression. We mutated the base C, 5' of E4, from CCA-GATG to GCAGATG (EE in Fig. 4A) and found that the mutation severely diminished promoter activity, demonstrating that the wild-type base pair contributes to the interaction between site and factor(s) and plays a role essentially as important as the sequence within the E-box.

![Diagram of ATH5 promoter occupancy](image)

**FIGURE 2.** *In vivo* occupancy of the ATH5 promoter by ATH5, Ngn2, and NeuroM as a function of development. A, antibodies directed against ATH5, Ngn2, and NeuroM were used to immunoprecipitate cross-linked chromatin fragments prepared from HH22 to HH38 retina (NR) and optic tecta (OT). Immunoprecipitates were analyzed for the abundance of ATH5 regulatory sequences by quantitative PCR. Insets, ChIP efficiency relative to the proportion of ATH5-expressing cells shown in B. A, lower panel, Western blot showing accumulation of the NeuroM protein in the developing retina and optic tectum as compared with the constitutive TATA-binding protein (TBP). B, ATH5-expressing cells in the developing chick retina. Serial transverse sections were hybridized with an ATH5-specific antisense riboprobe. ATH5-expressing cells were counted, and the ratio of labeled to unlabeled cells was determined. C, binding sites of the ATH5, Ngn2, and NeuroM proteins in HH22–23 and HH29–30 retinas. A sequence extending about 1 kb upstream of the first coding ATG was tiled at 100-bp intervals using variable-length (65–75 bases) polynucleotides to keep a constant target Tm of 76 °C. Although the ChIP-on-chip approach does not allow perfect alignments between probe coordinates and E-box positions, the two or three probe-hybridizing sequences that encompass, respectively, E4 or E1/E2, reflect binding of bHLH proteins to these elements. These binding sites are not detected in chromatin immunoprecipitated from HH22–23 and HH29–30 optic tecta. Lower line drawing, schematic representation of the upstream region of the ATH5 gene. The converging arrows mark the extent of the DNA fragment amplified in A.
moter fails to be activated because the factors expressed in the HH29–30 retina cannot bind in the absence of E4. We investigated this possibility by monitoring the gel mobility shift of probes encompassing E3, E4, and flanking sequences. The tested proteins were the chick homologues of the ubiquitous bHLH protein E47, the neural bHLH factors NeuroM and ATH5, and protein extracts from stage HH29–30 retina. We found that all these preparations bound to the wild-type probe (Fig. 4B, lanes 1–5). In contrast, in the absence of E4 (mE4) binding of the purified bHLH factors was abolished, and binding of retinal extracts was severely diminished (Fig. 4B, lanes 6–10). When the central base pairs of E4 were mutated, the bindings of the ATH5 and NeuroM proteins were severely diminished or abolished, except for mutant ED, whose binding ability remained similar to the wild-type (Fig. 4B, lanes 11–20). In this mutant, the E-box has the same identity as the one that mediates the specific activity of the ATH5 protein for the β3 promoter (26).

ATH5, Ngn2, and NeuroM Use Different Combinations of E-boxes to Regulate the ATH5 Promoter—E4 is necessary for promoter activity, and ChIP experiments suggest that the ATH5, Ngn2, and NeuroM proteins bind to this element (Fig. 2C). Consistent with these findings, the mutation of E4 abolished promoter activity at HH22–23, HH29–30, and HH35, and overexpression of ATH5, Ngn2, and NeuroM had no positive effect upon the mE4 promoter at HH22–23 and HH29–30 (Fig. 5). Next, we examined the roles of E1 and E2 in mediating the bHLH-dependent activity of the promoter. The mE1/CAT reporter plasmid was transfected alone or with the ATH5, Ngn2, or NeuroM expression vectors in acutely dissociated retinal cells at HH22–23, HH29–30, HH35, and HH38. At HH29–30, the absence of E1 had essentially no effect on promoter activity and did not abolish the capacity of ATH5 to up-regulate its own promoter. In contrast, E1 was required to mediate the positive effect of Ngn2 upon the promoter at HH22–23. Moreover, in the absence of E1, overexpression of Ngn2 actually decreased promoter activity at HH29–30 (Fig. 5). This experiment indicates that positive feedback by ATH5 is largely responsible for the rapid up-regulation of ATH5 at HH29–30. To test whether the much decreased positive effect of Ngn2 upon the mE1 promoter was due to a decreased proportion of cells capable of activating the promoter, we transfected HH22–23 retinal cells with a mE1/lacZ plasmid. Surprisingly, we found that the proportion of lac+ cells was higher when using the mE1 promoter and that overexpressing Ngn2 led to a further increase in lac+ cells (Fig. 5, inset). To reconcile these results with those obtained by CAT assay, we postulate that the E1 mutation leads to a modest increase in promoter activity which is nevertheless sufficient for β-galactosidase to reach the threshold for X-gal detection. Although the population of lac+ cells revealed using the mE1 promoter is broader, data obtained by CAT assay (Fig. 5) suggest that activity of the mutated promoter in these cells was weaker than in lac+ cells revealed with the wt promoter. Experiments presented below suggest that the complex responses of the mE1 promoter may reflect antagonistic activities mediated by E1. Although E1 and E2 have the same sequence identity, the mE1 and mE2 promoters displayed different properties. Mutation of E2 markedly decreased pro-
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FIGURE 4. Sequence specificity at E3 and E4. **A**, mutations in E4 (boxed) and in its flanking sequences are underlined. The wt and mutant fragments were fused to the CAT gene and transfected into retinal cells isolated at HH29–30. The cells were assayed for CAT activity 24 h later. The CAT activity obtained upon transfection with the wt/CAT reporter plasmid is set arbitrarily at 10, and activities of the mutants are given relative to this value. **B**, the bacterially expressed bHLH proteins and retina protein extracts (lanes 2–5) all form stable electrophoretic mobility shift assay complexes with a probe encompassing E4 (sequence shown in Fig. 1A). Mutation of E4 in the probe disrupts the binding of bHLH proteins and retinal factors (lanes 7–10). Mutation of the central base pairs of E4 diminishes or abolishes the binding of NeuroM (lanes 12–14) and of ATH5 (lanes 17–19) except for mutation ED (lanes 15 and 20). Arrows indicate the positions of probes that bind retinal proteins (a), E47 (b), NeuroM (c), and ATH5 (d). C, co-transfection of the wt or mutant promoters with an expression vector encoding ATH5, Ngn2, or NeuroM into retinal cells at HH29–30. The cells were assayed for CAT activity 24 h later. The effects of bHLH proteins upon the wt promoter are presented in Fig. 5. Relative promoter activities are represented as the mean ± S.D. of at least 4 independent experiments (n ≥ 4) for the mutant promoters alone and of at least 3 independent experiments (n ≥ 3) for co-transfections. **D**, E4 is part of a well conserved regulatory element that does not involve the direct interaction of bHLH proteins. wt and mE3/CAT reporter plasmids were transfected in retinal cells at HH22–23 and HH29–30, and cells were assayed for CAT activity 24 h later. The CAT activity obtained upon transfection with the wt promoter at HH29–30 is set at 10. Mutating E3 affects the ATH5 promoter at HH29–30 but not at HH22–23. The sequences encompassing E3 are well conserved, but slight differences lead to the absence of E3 in mammals (see Fig. 18). When E3 in the avian promoter was mutated from CAGATG to the corresponding human CAGACT, activity was almost as high as the wt at HH29–30. Relative promoter activities are represented as the mean ± S.D. of at least 8 (n ≥ 8; HH29–30) and 3 (n ≥ 3; HH22–23) independent experiments. **Lower panel**, the E47 protein forms a stable electrophoretic mobility shift assay complex with a probe encompassing both E3 and E4 (sequence shown in Fig. 1A). Mutation of E4 disrupts the binding of the E47 protein, whereas mutation of E3 has no effect. The arrow indicates the position of probes that bind E47.

moter activity at HH22–23, HH29–30, and HH35, and overexpression of ATH5 and Ngn2 did not up-regulate the mE2 promoter (Fig. 5). No lac" cells were detected when retinal cells were transfected with a mE2/lacZ plasmid (Fig. 5, inset). These results indicate that E2 is required to mediate the activity of both ATH5 and Ngn2, a requirement that is consistent with the observed in vivo binding of these bHLH proteins to sequences encompassing E1 and E2 (Fig. 2C). The activity of the mE1 and mE2 promoters was up-regulated by NeuroM at HH29–30 and HH35, suggesting that, despite its ability to bind E1 and/or E2 (Fig. 2C), NeuroM does not absolutely require these elements to activate the promoter (Fig. 5). However, overexpression of NeuroM does not bring activity of the mE2 promoter at the level reached by the wt promoter (Fig. 5), showing that NeuroM cannot fully compensate for the inability of ATH5 and Ngn2 to act via E2. At HH38, NeuroM loses the capacity to enhance promoter activity (Fig. 5) despite significant binding to the promoter (Fig. 2A), suggesting that at this stage NeuroM mediates an inhibitory effect that may repress ATH5 expression in cells unrelated to the RGC lineage.

Antagonistic Activities Target E1—To compare the activity patterns of the mE1 and wt promoters, HH22–23 retinas were electroporated with mutant or wt/lacZ plasmids, and β-galac-
Figure 5. The ATH5 promoter is differentially driven by E1, E2, and E4 during retina development. Retinal cells isolated at HH22–23, HH29–30, HH35, and HH38 were transfected with wt, mE1, mE2, or mE4/CAT reporter plasmids alone or in combination with a Ngn2, ATH5, or NeuroM expression vector. The base changes introduced to mutate the E-boxes are as shown in Fig. 1A. Cells were assayed for CAT activity 24 h after transfection. Because the proportions of ATH5-expressing cells were similar at HH22–23 and HH29–30 (Fig. 2B), activity obtained upon transfection with the wt/CAT reporter plasmid at HH29–30 is set at 10, and activities of the mutants are given relative to this value. Mutating E1, E2, or E4 differentially modify the effects of bHLH proteins upon the promoter. Inset, retinal cells isolated at HH22–23 were transfected with wt, mE1, mE2, or mE1,2/lacZ reporter plasmids alone or in combination with a Ngn2 expression vector. Cells were cultured for 24 h, and lac + cells were revealed and counted. The number of lac + cells obtained upon transfection with a SV40/lacZ-reporter plasmid is set at 100. The proportion of lac + cells was higher when using the mE1 promoter and overexpressing Ngn2 led to a further increase in positive cells. No lac + cell (arrowheads) has been detected when using the mE2 promoter. At HH22–23 and HH29–30, relative promoter activities are represented as the mean ± S.D. of at least 7 independent experiments (n = 7) for the mutant promoters alone and from at least 3 independent experiments (n ≥ 3) for co-transfections. At HH35 and HH38, relative promoter activities are represented as the mean ± S.D. of at least 5 independent experiments (n = 5).

tosidase positive cells were revealed 24 h later. The proportion of lac + cells in the central retina was significantly higher in the absence of E1 and increased as with the wt promoter when Ngn2 was overexpressed (Fig. 6, A, B, and E). As with the wt promoter, no lac + cells were detected at the periphery when using the mE1 reporter plasmid alone (Fig. 6, C and E). Because lac + cells were much increased in the peripheral domain when overexpressed Ngn2 acted upon the mE1/reporter plasmid (Fig. 6, D and E), we compared distribution of fluorescent cells in HH23 retina electroporated with mutated or wt/GFP plasmids. Although both plasmids have strong activities in the central retina, fluorescent cells were detected at the periphery only in the absence of E1 (Fig. 6, inset, and data not shown). These cells were not detected when lacZ was used as a reporter, suggesting that activity of the mutated promoter was lower at the periphery than in the central region. This peripheral expansion of promoter activity suggests that E1 mediates an inhibitory activity that represses ATH5 expression outside the central domain. At HH22–23, the expression of HES1 is high in the peripheral retina and low in the central region, where HES1 and Ngn2 are coexpressed and where Ngn2 is known to overcome the inhibitory effect of HES1 in a dose-dependent manner (5). Whereas overexpression of HES1 in the wt promoter context led to the complete absence of lac + cells in the central retina, it merely provoked a modest decrease in the number of positive cells when acting upon the mE1 promoter (Fig. 6E), indicating that in the absence of E1 Ngn2 activates the mutated promoter even if HES1 is expressed at high levels. This finding is consistent with the capacity of Ngn2 to activate the mE1 promoter at the periphery, where the level of HES1 is high (Fig. 6, D and E). Overexpression of both Ngn2 and HES1 led to the complete absence of lac + cells when acting upon the wt promoter and to a decreased proportion of lac + cells with the mutant promoter (Fig. 6E), suggesting that the competition between the two factors is not fully abolished in the absence of E1. Because the mE1 promoter was found to be activated in a domain of the retinal neuroepithelium broader than that of the wt promoter, we wondered if mutating E1 did not decrease the specificity of the promoter. Using GFP and RFP reporter proteins, we tested whether activity of the mE1 and wt promoters is localized in electroporated retina. Although the promoter activity of the sequences extending ~0.9 and ~2.2 kb upstream of the transcription initiation site display similar specificities, the longer fragment is more active and was used to drive expression of the RFP gene. Thus, HH23 retinas were electroporated with mE1/GFP and wt2.2 kb/RFP reporter plasmids, and fluorescent cells were detected 24 h later.
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FIGURE 6. E1 mediates the antagonistic activities of Ngn2 and HES1. HH22–23 retinas were electroporated with a cytomegalovirus/GFP and wt, mE1, mE2, or mE1,2/lacZ reporter plasmids alone or in combination with Ngn2 and/or HES1 expression vectors. Lac⁺ and GFP⁺ cells were counted 24 h later. Lac⁺ cells were detected in the central (A) but not in the peripheral retina (C) when using the mE1 promoter. A few scattered fluorescent cells (C, upper inset) were detected anterior to the equator (C, lower inset, arrowheads) when HH22–23 retinas were electroporated with a mE1/GFP reporter plasmid. Lac⁺ cells were detected both in the central (B) and peripheral (D) regions when co-electroporating with the mE1 promoter and a vector expressing Ngn2. The data are representative of at least five independent experiments. Bars, 100 μm. E, overexpression of Ngn2 in the absence of E1 much increased the percentage of lac⁺ cells at the periphery. Overexpression of HES1 did not abolish accumulation of lac⁺ cells when using the mE1 promoter, but it did so when using the wt promoter. No lac⁺ cells were found when using the mE2 promoter. Arrowheads indicate that no lac⁺ cell has been found. Data are presented as the mean ± S.D.; five electroporated retinas were analyzed per condition, p, peripheral; c, central.

FIGURE 7. In the absence of E1, activity of the mutated promoter expands to the peripheral retina. A and B, HH22–23 retinas were electroporated with wt2.2 kb/RFP and mE1.0 kb/GFP reporter plasmids. C–F, HH22–23 retinas were electroporated with wt2.2 kb/RFP and mE1.0 kb/GFP reporter plasmids alone (C and D) or in combination with a HES1 expression vectors (E and F). Fluorescent cells were detected by confocal microscopy 24 h later. A and B, in the central retina the same cells are labeled with the GFP and RFP markers. Inset, co-localization of the mE1.0 kb and wt2.2 kb promoter activities. C and D, numerous GFP⁺ cells and very few RFP⁺ cells (arrowheads) were detected in the peripheral domain. E and F, numerous GFP⁺ cells and very few RFP⁺ cells were detected in the central domain when overexpressing HES1. The data are representative of at least five independent experiments. Bars, 50 μm.

...the dominant negative effect of HES1, we tested a promoter where both E1 and E2 had been mutated. A population of lac⁺ cells was detected in HH22–23 retinas electroporated with a mE1, mE2/lacZ plasmid, and overexpression of Ngn2 led to a modest increase in the size of this population (Fig. 5, inset). These results suggest that in the absence of inhibition by HES1 upon E1, the E4 site mediates a positive response to Ngn2 in early retina. Mutation of E2 was shown to prevent the positive feedback by ATH5 at HH29–30 (Fig. 5), i.e. when HES1 is down-regulated (5). To evaluate further the role of E2, HH28–29 retinas were electroporated with both a mE1, mE2.2 kb/GFP and a wt2.2 kb/RFP plasmid, and fluorescent cells were detected 24 h later. The faint fluorescence in the green canal of cells in which activities of the mutant and wt promoters co-localized was a further indication that the ATH5 promoter is not up-regulated in absence of E2 (not shown). Taken together, these results reveal that E1 and E2 fulfill different and complementary functions during retina development.

DISCUSSION

ATH5 occupies a pivotal place in a bHLH transcriptional network regulating the specification of RGCs and is under the spatiotemporal control of several neuronal bHLH proteins. Monitoring the in vivo occupancy of the ATH5 promoter by the ATH5, Ngn2, and NeuroM proteins and analyzing the functional properties of regulatory elements in the course of retina development, we have shown that dynamic changes in the binding and activity of these proteins set regulatory circuits...
where distinct bHLH proteins either cooperate or compete to control the different phases of ATH5 expression. Among the seven E-boxes found in the avian ATH5 proximal promoter region, only three are evolutionarily conserved (E1, E2, and E4) and essential for bHLH-mediated promoter activity. Ngn2 requires all three elements for full-strength activity, and it competes with HES1 specifically at E1 in proliferating progenitors. E2 mediates a positive feedback by ATH5 at the time when precursors are going through their last cell cycle and when the majority of RGCs are specified. E4 is sufficient for NeuroM to exert its positive effect upon the promoter in newborn RGCs (Fig. 8).

**bHLH-dependent Regulation of ATH5 during Retina Ontogenesis**—Our study has revealed that E1, E2, and E4 play different parts in the course of retina development. E4 is required for the continued expression of ATH5 along the pathway leading to the conversion of progenitors into newborn RGCs. Although E4 and its flanking sequences are very well conserved in birds and mammals (Fig. 1B), they are absent in *Xenopus laevis* and *X. tropicalis*, species in which expression of ATH5 relies on regulatory sequences acting independently of bHLH transcription factors (16). We show that activity of the chick promoter also depends on regulatory sequences that do not involve bHLH proteins, but we have no evidence indicating that the promoter may operate independently of bHLH proteins (Fig. 3). In contrast, E1 and E2 are evolutionarily well conserved in lower vertebrates and in mammals (Fig. 1 and Ref. 16). Although E1 and E2 are identical, it appears that they fulfill different tasks. E2 is required for activity of the Ngn2 protein during the first phase of ATH5 expression and mediates the positive feedback of ATH5 during the second phase. The binding of ATH5 to E2 (Fig. 2B) and the inhibitory effect of ATH5 on Ngn2 activity at HH22–23 (5) suggest, however, that E2 mediates competition between ATH5 and Ngn2 in early retina. E1 enhances the capacity of Ngn2 to activate the promoter in early retina and is not required for autostimulation by ATH5. In the absence of E1, the promoter is much less sensitive to the inhibitory effect of HES1. Consistent with this property, the mE1 promoter is activated in retinal neuroepithelium domains that do not express the wild-type gene. In early retina, expansion of the Ngn2 expression domain precedes that of ATH5 (5), and the mE1 promoter is active in the region that already expresses Ngn2 but has yet to express ATH5. Moreover, overexpression of Ngn2 expands the activity of the mutant promoter to the periphery where expression of HES1 is high. Although we have no direct evidence of the binding of HES1 to E1, we surmise that by orchestrating competition between HES1 and Ngn2, E1 may set a threshold for activation by Ngn2, thereby contributing to establishing the sharp border between the ATH5 and HES1 expression domains in early retina (5).

**An E-box Code Regulates the Interplay of bHLH Proteins**—At least four different bHLH proteins are involved in the regulation of avian ATH5, and we wished to know how the promoter integrates their functions during the different phases of ATH5 expression. The mechanisms by which neuronal bHLH transcription factors select their targets in the developing nervous system remain elusive. The basic region acts as a sequence-specific DNA binding domain that recognizes sites with the simple core consensus sequence CANNTG. The two central base pairs and the base pairs flanking the E-box contribute to the selection of appropriate bHLH proteins (28). In line with this finding, we found that both the identity and the position of E4 determine its stringent specificity. On the other hand, ATH5 and Ngn2 act through E-boxes (E2 and E4) that have different identities. Whereas the ATH5 protein binds these elements in early retina when expression of ATH5 is low, it needs to be up-regulated to bind the single E-box of sequence CAGCTG mediating the specificity of the β3 promoter (26, 29). Mutating the two central bases of E4 from GA to GC does not affect the binding of ATH5 to E2 (Fig. 2B) and the inhibitory effect of ATH5 on Ngn2 activity at HH22–23 (5). Precocious expansion of the expression domain of ATH5 in HES1−/− mice (27) suggests that competition between expression of proneural genes and HES1 also operates in the developing mouse retina.

**FIGURE 8.** Model illustrating the requirement by the different bHLH proteins for the E-boxes E1, E2, and E4 at HH22–23 (1st phase) and HH29–30 (2nd phase). A, about one-third of retinal cells express ATH5 between HH22–23 and HH29–30. At HH22–23 (first phase), cells express ATH5 at low level. At HH29–30 (second phase), ATH5 is up-regulated in about one-third of ATH5-expressing cells (5). At HH33–34, most RGCs have been born (21), and ATH5-expressing cells (5). At HH33–34, most RGCs have been born (21), and ATH5 expression is down-regulated. B, E1, E2, and E4 mediate stimulation by Ngn2 during the first phase, and this effect is counteracted by HES1 and ATH5. E2 and E4 mediate the positive feedback of ATH5 during the second phase. Other factors (e.g., the Sp1 protein) likely cooperate with bHLH proteins to regulate ATH5 during retina ontogenesis.
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gene selection is related to ATH5 protein level. E4 does not discriminate between the ATH5, Ngn2, and NeuroM proteins; likewise, E2 interacts with Ngn2 and ATH5 and mediates the activity of both these factors. The combinatorial nature of the regulation of the ATH5 promoter suggests that the bHLH proteins involved have no assigned E-boxes and that their specific activity arises from the differential use of a common set of regulatory elements. Although Ngn2 requires E1, E2, and E4 to activate the promoter, E2 and E4 suffice for autoregulation by ATH5. Moreover, E4 mediates activation by NeuroM, and HES1 requires E1 to repress the promoter (Fig. 8). Such a combinatorial code implies that bHLH proteins probably compete to regulate ATH5. We suppose that such competition (Fig. 5; see also Ref. 5) and dynamic changes in the expression pattern of the bHLH proteins underlie the fine tuning of promoter activity and lead to the consecutive phases of ATH5 expression. We do not exclude that heterodimerization may also contribute to the interplay of ATH5, Ngn2, and NeuroM. Their common requirement for E4 suggests that this element could mediate such heteromeric interactions.

Changes in the differential occupancy by various transcription factors in relation with changing levels of ATH5 expression (Fig. 8) coincide with chromatin remodeling of the ATH5 promoter. K4-dimethylation of histone H3, a modification known to reflect transcriptional competence, strikingly increases between HH22–23 and HH29–30, in exact register with the kinetics of ATH5 promoter activity (15). A few other areas of the central nervous system were found to express the ATH5 gene (6, 14, 31). In the chick embryo, ATH5 expression has been detected in the proliferating zone of a tiny ventral domain of the neural tube and in the outer layers of a discrete area in the hindbrain. However, the uninterrupted expression of ATH5 in proliferating progenitors, then in precursor cells going though their last cell cycle, and finally in newborn neurons appears to be a specific feature of the developing retina. Our study highlights how a set of bHLH transcription factors that are widely expressed combine to establish highly restricted regulatory circuits within a specialized part of the developing nervous system. The competition taking place between Ngn2 and HES1 (Fig. 8 and Ref. 5) exemplifies how a balance of factors having opposite activities is finely tuned for the proper expression of ATH5. Moreover, our study suggests the ambivalent role of NeuroM upon the ATH5 promoter. This factor positively regulates the ATH5 promoter in newborn RGCs and turns into a repressor (Figs. 2 and 5), perhaps through a change in dimerization partner, as ATH5 expression is down-regulated. The idea that NeuroM may negatively regulate ATH5 is consistent with a previous study (32) showing that when the Math3/NeuroM and NeuroD genes are both inactivated in mice, Math5 expression increases, and the population of RGCs expands. Although NeuroM accumulates at similar levels in HH29–30 retina and optic tectum, it is only in the retina that it binds the ATH5 promoter (Fig. 2). The lack of ATH5 expression in the optic tectum might correlate with a chromatin structure that prevents the binding of NeuroM to the ATH5 promoter. As a corollary, we suppose that proteins that may facilitate the binding of NeuroM in the developing retina are absent or inactivated in the optic tectum.

In sum, differential occupancy by various transcription factors (Fig. 8) is associated with chromatin remodeling of the ATH5 promoter (15) and underlies the fine tuning of ATH5 expression at consecutive stages of development. The dosage variations of the ATH5 protein that result may be required for the timely activation or repression of its target genes in the course of retina ontogenesis.

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