Barhl1 Regulatory Sequences Required for Cell-Specific Gene Expression and Autoregulation in the Inner Ear and Central Nervous System

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The development of the nervous system requires the concerted actions of multiple transcription factors, yet the molecular events leading to their expression remain poorly understood. Barhl1, a mammalian homeomain transcription factor of the BarH class, is expressed by developing inner ear hair cells, cerebellar granule cells, precerebellar neurons, and collicular neurons. Targeted gene inactivation has demonstrated a crucial role for Barhl1 in the survival and/or migration of these sensory cells and neurons. Here we report the regulatory sequences of Barhl1 necessary for directing its proper spatiotemporal expression pattern in the inner ear and central nervous system (CNS). Using a transgenic approach, we have found that high-level and cell-specific expression of Barhl1 within the inner ear and CNS depends on both its 5′ promoter and 3′ enhancer sequences. Further transcriptional, binding, and mutational analyses of the 5′ promoter have identified two homeoprotein binding motifs that can be occupied and activated by Barhl1. Moreover, proper Barhl1 expression in inner ear hair cells and cerebellar and precerebellar neurons requires the presence of Atoh1. Together, these data delineate useful Barhl1 regulatory sequences that direct strong and specific gene expression to inner ear hair cells and CNS sensory neurons, establish a role for autoregulation in the maintenance of Barhl1 expression, and identify Atoh1 as a key upstream regulator.

The mammalian Barhl1 and Barhl2 proteins belong to the BarH class of homeodomain transcription factors that are evolutionarily conserved in both invertebrate and vertebrate species ranging from Drosophila flies to humans. The factors of this class are usually expressed in the developing and adult nervous systems and are required for proper neural development and survival (4, 7, 12, 13, 15, 18–20, 24, 26–32, 34). In Drosophila, BarH1 and BarH2 are coexpressed in cells of the central nervous system (CNS) and the peripheral nervous system and are redundantly required for determining the subtypes of external sensory organs and photoreceptor and primary pigment cells during eye development (12, 13, 15). The vertebrate Barhl1 and Barhl2 genes similarly display overlapping yet distinct patterns of expression in the CNS and sensory organs, thereby also playing redundant as well as distinct roles during neurogenesis (4, 7, 18–20, 24, 26–29, 31, 32, 34). For instance, Xenopus Barhl2 is expressed in the developing neural plate and retina during Xenopus embryogenesis and plays a key role in patterning the neural plate and specifying retinal ganglion cells (27, 29).

The functions of mammalian Barh1 genes during murine neurogenesis have been extensively investigated previously. In the developing mouse inner ear, Barhl1 but not Barhl2 is specifically expressed in all hair cells of the cochlear and vestibular systems (4, 18). The targeted disruption of Barhl1 leads to hearing loss as a result of the age-related progressive degeneration of inner and outer hair cells in the organ of Corti (18), demonstrating a critical requirement for Barhl1 in the long-term maintenance of these sensory cells. In the developing CNS, Barhl1 and Barhl2 are expressed in the superior and inferior colliculi of the midbrain, the cerebellar granule cells and precerebellar neurons of the hindbrain, and the dorsal interneurons of the spinal cord (4, 19, 20, 26, 31, 32). Analyses of Barhl1-deficient mice have revealed a crucial role for Barhl1 in the migration and survival of cerebellar and precerebellar neurons and the long-term maintenance of superior collicular neurons as well (19, 20). In the spinal cord, Barhl1 inactivation results in no appreciable change, yet gain-of-function studies have implied a role for Barhl2 in the differentiation of dorsal commissural sensory neurons (19, 31, 32), suggesting a likely functional redundancy between Barhl1 and Barhl2 during spinal neurogenesis. In the developing retina, contrary to the inner ear, Barhl2 instead of Barhl1 is uniquely expressed in several inner retinal cell types and plays a role in the specification of glycinergic amacrine cells (26).

It is now clear that the exact functions of Barhl1 and Barhl2 factors depend to a large extent on the timing and location of their expression and activities. Thus, due to their distinct expression profiles, Barhl1 has a key role in maintaining cochlear hair cells whereas Barhl2 plays a role in specifying a retinal cell subtype (18, 26). Without doubt, proper spatial and temporal regulation of Barhl1 and Barhl2 expression is crucial for their normal function. At present, however, little is known about the molecular basis leading to their appropriate expression pat-
terns, despite their essential roles during sensorineural development. A recent transgenic analysis of Barhl2-flanking sequences identified a 3' enhancer that can be activated by the basic helix-loop-helix factor Atoh1/Math1 and can drive spinal cord-specific gene expression (31). In this study, we aimed to define Barhl1 regulatory sequences specific to inner ear hair cells and CNS areas. We found by transgenic analysis that 4.2-kb 5' and 3.4-kb 3' flanking sequences together are sufficient for recapitulating the endogenous Barhl1 expression pattern. The 4.2-kb promoter sequence is capable of driving specific gene expression to inner ear hair cells, the spinal cord, and the hindbrain, but high-level and midbrain-specific expression depends on a distal 1.7-kb 3' enhancer sequence. Furthermore, Barhl1 and Barhl2 are able to auto- and cross-activate the Barhl1 promoter by binding to two specific homeoprotein binding sites, and Atohl is directly and/or indirectly required for proper Barhl expression in the inner ear and CNS.

MATERIALS AND METHODS

Plasmid constructs. The transgenes were constructed by the excision of the Barhl1 5' arm-lacZ sequence from an earlier Barhl1 knockout intermediate construct, pSDFkIt/ZipA-5' arm (18), by using the NotI and Xhol restriction endonucleases. The released fragment was joined with a 3.4-kb 5' Barhl1 flanking sequence to yield the transgene construct Tg1 or a 1.7-kb proximal 3' flanking sequence to generate Tg2 or was not fused to any 3' flanking sequence to yield Tg3. Barhl1 and Barhl2 expression plasmids were constructed by inserting the full-length Barhl1 and Barhl2 CDSs into the pCDNA 3.1 expression vector (Invitrogen). To make the R1 luciferase reporter construct, the 4.2-kb 5' promoter sequence from R1 by using the KpnI and Xhol restriction enzymes and then ligated into the pGL3-Basic vector (Promega). The RS luciferase reporter plasmid was constructed by the excision of the 4.2-kb promoter sequence from R1 by using the Kpn1 and Xhol restriction enzymes, followed by ligation into the pGL3-Promoter vector (Promega). All other luciferase reporter constructs were derived from R1 or R5 plasmids by restriction digestion or PCR. The mutant constructs R10 to R12 were derived from R1 using PCR-based site-directed mutagenesis.

Generation of transgenic mouse lines. Transgenic mice were generated by standard procedures (14, 40). In brief, linear DNA fragments of transgenic constructs were purified by gel electrophoresis and microinjected into pronuclei of C57BL/6J × FVB/N zygotes. Embryos were transplanted into foster mothers, and founder animals were identified by PCR analysis. The founders were crossed with C57BL/6J mice to produce F1 progeny, which were subjected to histochemical in situ hybridization and immunostaining. To prepare chromatin DNA from mouse tissues, postnatal day 6 (P6) cerebella were dissected and incubated for 15 min at room temperature with 0.5 M acetic acid, 0.5 M sodium chloride, and 6% sodium dodecyl sulfate (SDS) and 50 mM EDTA to 0.1% Triton X-100, 50 mM KCl, 0.1 M dithiothreitol, and 0.1 M phenylmethylsulfonyl fluoride (PMSF) for 30 min at 4°C. Following the neutralization, the cerebella were washed with PBS-Tween and then with PBS. Antigen retrieval was achieved by heating the slides in 0.1 M sodium citrate buffer (pH 6.0) for 10 min. Endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide for 30 min. Nonspecific binding was blocked with 5% normal goat serum for 30 min at room temperature. Slides were incubated with primary antibodies for 2 h at room temperature. The slides were then washed with PBS and incubated with biotinylated secondary antibodies (1:200 dilution) for 1 h at room temperature. Slides were then washed with PBS and incubated with avidin-biotinylated peroxidase complex (ABC) for 1 h at room temperature. After washing, slides were incubated with 0.01% 3,3'-diaminobenzidine (DAB) and 0.02% H2O2 for 5 min to develop the color reaction, and then slides were counterstained with 0.1% methylene blue for 10 min. Slides were then washed, dried, and mounted with a coverslip.

RESULTS

5' and 3' flanking sequences involved in conferring the endogenous Barhl1 expression pattern. As a first step to define DNA sequences in the Barhl1 gene that can direct specific gene expression to the inner ear and CNS, we constructed a transgene (Tg1) containing approximately 4.2 kb of 5' flanking and 3.4 kb of 3' flanking DNA sequences fused to a lacZ reporter (Fig. 1A). Among the four founder lines obtained for this transgenic construct, three lines displayed lacZ expression with...
similar patterns in the inner ear and CNS (Fig. 1A). From embryonic day 13.5 (E13.5) to early postnatal stages, X-Gal staining and β-Gal immunofluorescence revealed that the transgene was expressed strongly in both inner hair cells and outer hair cells of the organ of Corti, as well as in hair cells of the saccule, utricle, and cristae of the vestibular system (Fig. 1C and 2B to H). Double immunolabeling showed that β-Gal was probably expressed in all hair cells that were immunoreactive with the hair cell-specific marker Myo6 (Fig. 2N to P) (10, 39), indicating that Tg1 was expressed in the inner ear in a pattern that recapitulated that of the endogenous Barhl1 gene (4, 18). Consistent with this notion, there was significant downregulation of Tg1 expression in inner hair cells at late postnatal and adult stages (Fig. 2I and J), similar to that of endogenous gene expression (see Fig. S1 in the supplemental material) (18).

In the developing CNS, Tg1 was expressed in the diencephalon, mesencephalon, rhombencephalon, and spinal cord, also in patterns similar to those of the endogenous gene (Fig. 1A and B and 2A and Q to T) (4, 19, 20). In particular, Tg1 was prominently expressed by the tectum of the midbrain, the anterior and posterior rhombic lips, the external granule layer of the cerebellum, the dorsal spinal cord, and precerebellar nuclei, including the pontine nucleus (PN), the cochlear nucleus (CN), and the lateral reticular (LR) nucleus (Fig. 1B and 2A and Q to T; also data not shown). Thus, the 4.2-kb 5′ flanking sequence and the 3.4-kb 3′ flanking sequence together were sufficient to recapitulate the spatial and temporal expression patterns of the endogenous Barhl1 gene in the inner ear and CNS.

Hair cell-specific transgene expression driven by a 4.2-kb Barhl1 promoter sequence. Given the sufficiency of Tg1 to drive strong reporter gene expression in the inner ear and CNS, we next investigated whether its 3′ flanking sequence was necessary for high-level tissue-specific gene expression. Two additional transgenes were constructed from Tg1 by deleting either the distal 1.7-kb 3′ flanking sequence from the 3′ end (Tg2) or the entire 3.4-kb 3′ fragment (Tg3) (Fig. 1A). Tg2 and Tg3 displayed indistinguishable patterns of β-Gal expression in the inner ear and CNS (Fig. 1A and D to G). In the inner ear, both Tg2 and Tg3 directed β-Gal expression specifically to hair cells of both the cochlear and vestibular systems (Fig. 1E and F), suggesting that the 4.2-kb promoter sequence alone was sufficient to confer hair cell-specific expression in the inner ear. However, the β-Gal activities in the inner ears of Tg2 and Tg3 animals were substantially weaker than that in the inner ears of Tg1 mice, and the expression was visible only in scattered hair cells (Fig. 2B to H and K to M; also data not shown), indicating the existence of an enhancer within the distal 1.7-kb 3′ flanking sequence that directs strong Barhl1 gene expression to the inner ear (Fig. 1A).

In the CNSs of Tg2 and Tg3 mice, there was also a dramatic reduction of β-Gal expression compared to that in Tg1 animals (Fig. 1B, D, and F). Moreover, β-Gal expression was completely absent from the tecta of Tg2 and Tg3 embryos, even though it was still present in the rhombencephalon and spinal cords (Fig. 1D and F), indicating a change in the tissue specificity of gene expression. Therefore, unlike in the inner ear, in the CNS the distal 1.7-kb 3′ flanking sequence of Barhl1 was not required not only for strong expression but also for tissue-specific expression.

Auto- and cross-activation of the Barhl1 promoter by Barh1 and Barh2. Given the expression of Tg3 in inner ear hair cells and CNS neurons and the expression of Barhl1 and/or Barh2 in the same cells (4, 18, 19, 26, 31), it is possible that the 4.2-kb Barhl1 promoter sequence may be auto- and cross-regulated by Barhl1 and Barh2. We tested this possibility by fusing the 4.2-kb promoter sequence to a luciferase reporter gene in the

**FIG. 1.** Expression patterns of the lacZ reporter driven by Barhl transgenes. (A) Schematics of the Barhl1 genomic locus, transgenic constructs, and transgene expression levels in various tissues. The black bars represent the three coding exons of Barhl1, and the positions of the initiator codon ATG and the stop codon TGA are indicated. Indicated also are the proximal (prox) and distal (dist) 3′ 1.7-kb fragments. Expression levels were scored as follows: ++++, strong; +, weak; and −, absent. (B to G) X-Gal staining of E13.5 whole-mount embryos containing the indicated transgene followed by clearing in benzyl alcohol-benzyl benzoate. Strong β-Gal immunofluorescence revealed that the Barhl1 and Barhl2. We tested this possibility by fusing the 4.2-kb Barhl1 promoter sequence to a luciferase reporter gene in the
pGL3-Basic vector (R1) and then measuring luciferase activity in transient transfection and transcription assays. The results of these assays revealed that both Barhl1 and Barhl2 could increase the luciferase activity by severalfold in the 293T human embryonic kidney cells and P19 embryonic carcinoma cells (Fig. 3A and C). Compared to the luciferase activity of the full-length construct, R1, activated by Barhl1 or Barhl2, the 5’ deletion construct R2 did not display any change of activity but R3 showed a sharp 7- to 10-fold drop in luciferase activity (Fig. 3A and B). Therefore, it appeared that all cis-acting elements necessary for Barhl1 and Barh2 activation were confined to a 1.2-kb region from bp 1786 to 589 (Fig. 3A). A 142-bp internal deletion in this region, from bp 1333 to 1192 (R4), resulted in a three- to fourfold decrease in luciferase activity, indicating the presence of a potential cis-acting element(s) in this short sequence. The findings from 3’ deletion analyses using the pGL3-Promoter vector supported these conclusions, since a deletion from bp 1021 to 1511 resulted in a moderate decrease in luciferase activity activated...
by Barhl1 and a deletion from bp −1512 to −1785 caused a complete loss of activity (compare data for R6, R7, and R8 in Fig. 3C and D). On the other hand, we found that a 494-bp segment from bp −1786 to −1293 (R9) could largely restore the luciferase activity (Fig. 3C and D), indicating the presence of a potential Barhl1 cis-acting element(s) in this region.

Identification of Barhl1 and Barhl2 binding sites in the Barhl1 promoter. The auto- and cross-activation of the Barhl1 promoter by Barhl1 and Barhl2 homeoproteins suggests the existence of Barh1 and Barhl2 cis-acting elements or binding sites in the promoter region. To identify these sites, we first determined whether Barhl1 and Barhl2 could bind to a consensus homeoprotein binding site, (C/G)TAATTG, that contains the TAAT core motif (5). EMSAs showed strong binding of Barhl1 to the consensus site, whereas mutations in this site, in particular those in the TAAT core motif, greatly diminished or completely abolished the binding activity (Fig. 4A to C). The DNA binding activities of Barhl1 and Barhl2 were specific since their binding to the consensus site could be inhibited by a 500-fold excess of unlabeled consensus sites but was not inhibited by the same amount of cold sites containing a mutation in the TAAT core motif (Fig. 4A, C, and D). Thus, Barhl1 and Barhl2 may bind to CTAATTG or similar homeoprotein binding sites to activate transcription from the 4.2-kb Barhl1 promoter sequence.

In vivo binding by Barhl1 to its own promoter. We verified the binding of Barhl1 to the putative homeoprotein binding motifs b1 and b2 by using a ChIP assay (16). Chromatin DNA was prepared from 293T cells cotransfected with the R1 promoter construct and a Barhl1 expression plasmid. An anti-Barhl1 antibody specifically immunoprecipitated promoter fragments containing the consensus binding sites, whereas mutations in this site, in particular those in the TAAT core motif, greatly diminished or completely abolished the binding activity (Fig. 4A to C). The DNA binding activities of Barhl1 and Barhl2 were specific since their binding to the consensus site could be inhibited by a 500-fold excess of unlabeled consensus sites but was not inhibited by the same amount of cold sites containing a mutation in the TAAT core motif (Fig. 4A, C, and D). Thus, Barhl1 and Barhl2 may bind to CTAATTG or similar homeoprotein binding sites to activate the expression of their target genes.

A sequence search in the 1.2-kb region from bp −1786 to −589, identified above as containing all the potential Barhl1 and Barhl2 cis-acting elements, yielded two CCTAATT motifs in reverse orientations between bp −1210 and −1204 (b1) and bp −1530 and −1524 (b2) (Fig. 3A and C). Either or both candidate Barhl1 and Barhl2 binding sites were missing from constructs R3, R4, and R7 to R9 by Barhl1. Each histogram represents the means ± SD of results from triplicate assays in a single experiment, and all experiments were repeated three times with similar results.

FIG. 3. Transcriptional activities of Barhl1 and Barhl2 on deletion constructs of the 4.2-kb Barhl1 5′ promoter sequence. (A) Schematic of 5′ and internal-deletion luciferase (Luc) reporter constructs (R1 to R4) and their relative activities activated by Barhl1 and Barhl2. All constructs were made with the pGL3-Basic vector. The ovals indicate putative Barhl1 and Barhl2 binding sites (b1 and b2). (B) Levels of activation (n-fold) of the luciferase activities of constructs R1 to R4 by Barhl1 and Barhl2. Each histogram represents the means ± standard deviations (SD) of results from triplicate assays in a single experiment, and all experiments were repeated three times with similar results. (C) Schematic of 3′ deletion luciferase reporter constructs (R5 to R9) and their relative activities activated by Barhl1. All constructs were made with the pGL3-promoter (p) vector. The ovals indicate putative Barhl1 and Barhl2 binding sites (b1 and b2). (D) Levels of activation of the luciferase activities of constructs R5 to R9 by Barhl1. Each histogram represents the means ± SD of results from triplicate assays in a single experiment, and all experiments were repeated three times with similar results.
that Barhl1 may bind to b1 and b2 sites of its own promoter in vivo to autoactivate the transcription of the endogenous Barhl1 gene.

Inhibition of Barhl1 binding and autoactivation activities by mutations of both homeoprotein binding motifs in the Barhl1 promoter. The binding of b1 and b2 sites by Barhl1 does not indicate their necessity for autoregulation, so we investigated whether they are required for this function by site-directed mutagenesis. When either site was mutagenized from CCTAATT to CCGTGGT in the R1 promoter construct to generate mutant constructs R10 and R11 (Fig. 5A), the mutant site lost its Barhl1 binding activity, as determined by ChIP (Fig. 5C). Consequently, Barhl1-induced luciferase activity from R10 and R11 was diminished by three- to fourfold compared to that from the R1 construct (Fig. 5D). When both b1 and b2 sites were simultaneously mutated in the R12 promoter construct (Fig. 5A), the construct lost its Barhl1 binding activity at both b1 and b2 sites and its Barhl1-induced luciferase activity was reduced to the basal level (Fig. 5C and D). Therefore, the presence of both b1 and b2 sites and their binding by Barhl1 appear to be necessary and sufficient for the autoactivation of endogenous Barhl1 gene expression.

Downregulation of Barhl1 expression in Atoh1 null mice. It has been shown previously that the expression of Barhl1 and Barhl2 is under direct and/or indirect regulation by Atoh1 in the spinal cord (2, 31). We therefore investigated the possible regulation of Barhl1 expression by Atoh1 in other CNS areas and the inner ear using Atoh1 null mice (1). In the embryonic mouse, the expression of Barhl1 overlaps only partially with Atoh1 expression. There is little embryonic expression of Atoh1 in the midbrain, and the expression of Barhl1 in the midbrains of Atoh1 null mice was unchanged compared to that in wild-type mice (Fig. 6A to D). In contrast, in areas in which neurons depend on Atoh1 for their differentiation, there was a near complete loss of Barhl1 expression in Atoh1 null mice (1). In the embryonic mouse, the expression of Barhl1 overlaps only partially with Atoh1 expression. There is little embryonic expression of Atoh1 in the midbrain, and the expression of Barhl1 in the midbrains of Atoh1 null mice was unchanged compared to that in wild-type mice (Fig. 6A to D). In contrast, in areas in which neurons depend on Atoh1 for their differentiation, there was a near complete loss of Barhl1 expression in Atoh1 null animals (Fig. 6D). This was particularly true for the CN and PN, which did not show any expression of Barhl1 in Atoh1 null mice. In other Atoh1-dependent nuclei of Atoh1 null mice, such as the external cuneate (EC) and LR nuclei, as well as in the cerebella, Barhl1 in situ hybridization showed some residual expression.
up to E18.5. This expression was presumably in postmitotic cells that had not yet degenerated in the absence of Atoh1.

Previous work has demonstrated that Atoh1 is required for the differentiation, but not the initial specification, of cochlear hair cells (1, 9) and that Atoh1 upregulation reaches the apex only around birth. Likewise, the apex was the last part of the cochlea to demonstrate Barhl1 upregulation, and the pattern of this upregulation precisely followed that of Atoh1 (inner hair cells before outer hair cells) (Fig. 6G and H). In contrast, Atoh1-lacZ null mice showed a different pattern of upregulation in the cochlea (outer hair cells before inner hair cells), and no expression whatsoever of Barhl1 in the Atoh1 null cochlea was detected (Fig. 6E, F, and I). These data suggest that Atoh1 may be directly and/or indirectly required for activating and/or maintaining Barhl1 expression in cochlear hair cells and neurons within the CN and PN. The reduced expression of Barhl1 in neurons of the cerebella and EC and LR nuclei of the Atoh1 null mice suggests that these cells can still upregulate Barhl1 in the absence of Atoh1 but are unable to maintain the expression either because they are dying or because they require Atoh1 to maintain the expression. In addition to Atoh1, Pou4f3/Bm3c is required for the maturation and survival of inner ear hair cells (8, 38, 39). In the ears of Pou4f3 null animals, strong Barhl1 protein expression persisted in remaining hair cells (Fig. 6J and K), indicating that Barhl1 expression was unlikely to be regulated by Pou4f3.

DISCUSSION

We have reported previously that Barhl1 is specifically expressed in inner ear hair cells, where it is required for the long-term maintenance of these cells (18), and in developing cerebellar granule cells, precerebellar neurons, and superior collicular neurons, where it is critically involved in migration and survival (19, 20). The experimental data reported here have further defined the Barhl1 regulatory sequences necessary for directing the appropriate spatiotemporal expression pattern of Barhl1. We have found by transgenic analysis that a
expression in the wild type (C) follows, to some extent, the expression of Barhl1 most notably the PN and CN. IC, inferior colliculus. (C and D) Barhl1 expression in the ear and brain at E18.5. (A and B) As indicated by the β-Gal activity expressed from the knock-in lacZ reporter, Atoh1 is expressed at high levels in the cerebellum (CB), as well as in the LR nucleus, the PN, the CN, and the EC and perifacial (PF) nuclei (A). In the Atoh1 null brain (B), there is some expression of the lacZ reporter, in particular in the perifacial neurons. However, there is a great loss of neurons and/or lacZ expression in the cerebellum and several nuclei, most notably the PN and CN. IC, inferior colliculus. (C and D) Barhl1 expression in the wild type (C) follows, to some extent, the expression of Atoh1 and is found in the cerebellum and the LR nucleus, PN, and EC nucleus and in some cochlear neurons. Barhl1 is also found in neurons that do not express Atoh1, such as those in the inferior colliculus and the superior colliculus (SC). Some neurons (perifacial nuclei) that are positive for Atoh1 do not show any Barhl1 in situ hybridization (ISH) signals. In the absence of Atoh1 (D), there is a complete loss of Barhl1 expression in the CN and PN and only residual expression in the EC and LR nuclei and the cerebellum. As expected, in areas (interior and superior colliculi) where there is no Atoh1 expression, there is no loss of Barhl1 signals. (E and F) In the ear (E), Barhl1 expression is strong but has not yet reached the cochlear apex at E18.5 (dotted line). Atoh1 null mutant ears (F) show no indication of Barhl1 expression, despite the fact that undifferentiated Atoh1-null hair cells exist in the apex (I). (G to I) Barhl1 upregulation very closely follows the upregulation of Atoh1, including the absence of expression in the apex and initial upregulation in inner hair cells (IHC) (G and H). In contrast, β-Gal expression in Atoh1 null mice is first detected in the equivalent of outer hair cell (OHC) precursors in the apex (J). (J and K) In the wild-type cochlea, immunostaining with a Barhl1 antibody (Ab) reveals Barhl1 protein expression in the inner and outer hair cells (J). This expression remains strong in residual hair cells of the Pou4f3 null cochlea (K). Scale bars, 1 mm (A to D), 100 μm (E to I), and 25 μm (J and K).

FIG. 6. Effect of Atoh1 and Pou4f3 inactivation on Barhl1 expression in the ear and brain at E18.5. (A and B) As indicated by the β-Gal activity expressed from the knock-in lacZ reporter, Atoh1 is expressed at high levels in the cerebellum (CB), as well as in the LR nucleus, the PN, the CN, and the EC and perifacial (PF) nuclei (A). In the Atoh1 null brain (B), there is some expression of the lacZ reporter, in particular in the perifacial neurons. However, there is a great loss of neurons and/or lacZ expression in the cerebellum and several nuclei, most notably the PN and CN. IC, inferior colliculus. (C and D) Barhl1 expression in the wild type (C) follows, to some extent, the expression of Atoh1 and is found in the cerebellum and the LR nucleus, PN, and EC nucleus and in some cochlear neurons. Barhl1 is also found in neurons that do not express Atoh1, such as those in the inferior colliculus and the superior colliculus (SC). Some neurons (perifacial nuclei) that are positive for Atoh1 do not show any Barhl1 in situ hybridization (ISH) signals. In the absence of Atoh1 (D), there is a complete loss of Barhl1 expression in the CN and PN and only residual expression in the EC and LR nuclei and the cerebellum. As expected, in areas (interior and superior colliculi) where there is no Atoh1 expression, there is no loss of Barhl1 signals. (E and F) In the ear (E), Barhl1 expression is strong but has not yet reached the cochlear apex at E18.5 (dotted line). Atoh1 null mutant ears (F) show no indication of Barhl1 expression, despite the fact that undifferentiated Atoh1-null hair cells exist in the apex (I). (G to I) Barhl1 upregulation very closely follows the upregulation of Atoh1, including the absence of expression in the apex and initial upregulation in inner hair cells (IHC) (G and H). In contrast, β-Gal expression in Atoh1 null mice is first detected in the equivalent of outer hair cell (OHC) precursors in the apex (J). (J and K) In the wild-type cochlea, immunostaining with a Barhl1 antibody (Ab) reveals Barhl1 protein expression in the inner and outer hair cells (J). This expression remains strong in residual hair cells of the Pou4f3 null cochlea (K). Scale bars, 1 mm (A to D), 100 μm (E to I), and 25 μm (J and K).

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are expressed in but not specific to hair cells (6, 11, 25, 33). Hence, the Barhl1 regulatory sequences defined here provide additional tools for conditional hair cell-specific gene knockout, the characterization of hair cell-acting mutants, and the study of properties of deafness gene products (25, 36, 41).

The observation that Tg2 exhibited much weaker expression than Tg1 and lost tectum expression suggests the existence of an enhancer element(s) within the 3’ distal 1.7-kb fragment that directs strong and tectum-specific Barhl1 expression. Interestingly, there is a 3’ enhancer present in the Barhl2 and Atoh1 loci that can be activated by Atoh1 and is critical for high-level reporter gene expression (11, 31). It is tempting to assume that Barhl1 and Barhl2 have similar 3’ enhancers given their similar spatiotemporal expression patterns during neurogenesis. However, a sequence comparison failed to identify any motif similar to the Barhl2 enhancer sequence in the 3’ distal 1.7-kb fragment of Barhl1. This finding may be hardly surprising since the Barhl2 enhancer is specific to the spinal cord (31) whereas the Barhl1 enhancer is required for high-level and tectum-specific expression. Therefore, the similar expression patterns of Barhl1 and Barhl2 may be controlled by a mechanism much more complex than anticipated, involving cis-regulatory elements common to several tissues as well as tissue-specific modular elements.

Reporter gene expression driven by the 4.2-kb 5’ promoter (Tg3) was shown to retain specificity to the inner ear, rhombencephalon, and spinal cord, albeit occurring at a much reduced level compared to that of Tg1. This low level of transgene expression may explain the scattered but nevertheless hair cell-specific expression within the inner ear. Alternatively, the scattered labeling of hair cells may reflect an altered mosaic expression pattern. In either case, the 5’ promoter must harbor cis-acting elements necessary for the low-level and yet tissue-specific expression. We utilized transcriptional, DNA binding, ChIP, and mutational assays to demonstrate that Barhl1 and Barhl2 can auto- and cross-activate gene expression from the 4.2-kb promoter, suggesting that Barhl1 expression may be maintained partly by a positive feedback mechanism in the hair cells and CNS. In Drosophila, misexpressed Barh1 and Barh2 could induce ectopic Barh1 and Barh2 expression in the eye imaginal disk, suggesting that once initiated by secreted signaling molecules, Barh1 and Barh2 expression is maintained by autoactivation in basal undifferentiated cells (22). Therefore, autoregulation appears to be evolutionarily conserved among Drosophila and mammalian BarH homologs, perhaps as a parsimonious mechanism of maintaining their expression.

Besides autoregulation, other parallel mechanisms must exist for the maintenance of Barh1 expression, as the knocked-in lacZ reporter retains its expression within the hair cells of homozygous mouse mutants (18). Obviously, regulatory factors activating the 3’ enhancer are involved. A sequence analysis of the 3’ distal 1.7-kb fragment revealed the existence of two E boxes (CAGCTG) that can be bound by Atoh1 (11, 31), suggesting Atoh1 as a possible upstream activator. Indeed, our data indicate that Barhl1 is in part regulated by Atoh1 and in part independent of Atoh1. More specifically, we found four different sets of neurons and cells with respect to Atoh1 and Barhl1 interaction: some cells did not show any Atoh1 expression but were positive for Barhl1 (superior and inferior colliculi). At the other extreme, there were cells that expressed Atoh1 but did not express Barhl1 (perifacial neurons). The cells that showed the coexpression of Atoh1 and Barhl1 seemed to fall into two categories. In one case (that of hair cells, CN, and FN), there was a complete loss of Barhl1 expression in Atoh1 null mice, suggesting the necessity of Atoh1 in controlling Barhl1 expression. In the second category (cerebellar cells and EC and LR nuclei), there was some residual upregulation of Barhl1 in Atoh1 null mice, and the loss of expression may reflect either the need for the continued presence of Atoh1 to maintain Barhl1 expression or the fact that these cells die and thus do not show appreciable levels of expression of Barhl1. Most interestingly, cochlear hair cells coexpressed both genes, and these hair cells die according to different time courses in the respective mutants (6, 18). An interesting possibility for future study in this case may be that the differential expression of Barhl1 in hair cells through an Atoh1 promoter may rescue hair cells that are otherwise lost in the Atoh1 null mutant.

In this work, we used a combination of in vivo and in vitro approaches to define two closely located Barhl binding sites, b1 and b2 (~300 bp apart), within the 4.2-kb 5’ promoter necessary for autoactivation. These two cis-acting elements are functionally and physiologically relevant. First, β-Gal expression driven by the 4.2-kb 5’ promoter was limited to the inner ear, rhombencephalon, and spinal cord, where endogenous Barhl1 and/or Barhl2 are expressed. Second, both b1 and b2 sites (CCTAATT) are AT-rich and closely resemble the canonical homeoprotein binding motif (C/G)TAATTG (5). Third, these sites can be occupied in vivo by Barhl1 not only in cultured cells but also in cerebellar neurons, as demonstrated by the ChIP assay. Finally, site-directed mutagenesis of both binding sites eliminated Barhl1 binding to and activation of the 4.2-kb 5’ promoter. It appears that both b1 and b2 sites are required for the maximal level of expression from the promoter. The mutation of either site greatly reduced the activation activity by Barhl1 but did not eliminate it, thereby suggesting a synergistic effect between these two cis-acting elements on the autoregulation of Barhl1 expression. Interestingly, a survey of the CCTAATT motifs among Barhl1 and Barhl2 orthologs identified one to three such sites within a 4-kb promoter region in each of the zebrafish, rat, mouse, and human Barhl1 and Barhl2 genes (see Fig. S2 in the supplemental material), indicating that these sites may be conserved across vertebrate species for auto- and cross-regulation.

Thus far, accumulated evidence shows that the BarH class of homeoproteins act as both positive and negative transcriptional regulators, depending on different target genes, cell types, and developmental contexts. We show in this study that both Barhl1 and Barhl2 can transactivate the Barhl1 promoter. In the organ of Corti-derived cells, however, Barhl1 was found to act as a transcriptional repressor (35). In a teratocarcinoma cell line, Barhl2 was observed to function either as an activator or as a repressor in regulating the expression of basic helix-loop-helix proneural genes (34). During mouse retinogenesis, Barhl2 acts as an activator of the specification of glycinergic amacrine cells but as a repressor of bipolar and Müller cell differentiation (26). The Barhl2 repressive activity is also required for specifying commissural interneuron identity during spinal cord development (31). Similarly, in Xenopus, proper
specification of retinal ganglion cells and neural plate patterning depend on the repressor function of Xbarhl2 (27, 29). In Drosophila, BarH1 and BarH2 are able to activate their own promoters but repress atonal expression during retinal neurogenesis (21, 22). Thus, BarH factors from Drosophila to mammals share a common property as dual functional transcriptional regulators of neurogenesis.

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