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Gene expression profiling identifies *Hes6* as a transcriptional target of ATOH1 in cochlear hair cells

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Abstract ATOH1 is a basic Helix-Loop-Helix transcription factor crucial for hair cell (HC) differentiation in the inner ear. In order to identify ATOH1 target genes, we performed a genome-wide expression profiling analysis in cells expressing *ATOH1* under the control of a tetracycline-off system and found that *HES6* expression is induced by ATOH1. We performed in situ hybridisation and showed that the rise and fall of *Hes6* expression closely follow that of *Atoh1* in cochlear HC. Moreover, electrophoretic mobility shift assays and luciferase assays show that ATOH1 activates *HES6* transcription through binding to three clustered E boxes of its promoter.

Keywords: Ear, inner; Hair cells; ATOH1 protein; Basic Helix-Loop-Helix transcription factors; Hairy and enhancer of split 6 (Drosophila) protein, mouse

1. Introduction

The vertebrate inner ear consists of the cochlea for audition, and the vestibular end organs including the utricle, saccule and three semi-circular canals, for balance. Each of these structures contains a sensory epithelium made of supporting cells and mechanosensory hair cells (HC) [1]. They are produced by a prosensory equivalence group located in the zone of non-proliferating cells in the cochlea, initially marked by expression of the proneural gene *Atoh1* (Drosophila atonal homolog 1) [2,3]. As the equivalence group develops, some cells upregulate *Atoh1* expression and complete differentiation as HC. It has been shown that ATOH1 is both necessary and sufficient for HC differentiation [2,4].

ATOH1 belongs to the tissue-specific class 2 of bHLH (basic Helix-Loop-Helix) factors which perform their biological function by forming heterodimers with ubiquitously expressed class 1 bHLH factors such as E12 and E47. The heterodimers bind to regulatory sequences called E boxes (CAnnTG) and transcriptionally activate their target genes [5]. Only two genes have been shown to be direct downstream targets of ATOH1: *Mbh1* in commissural neurons [6] and *Nr2f6* in cerebellar granule cell progenitors [7]. ATOH1 also regulates its own expression through binding to a 3′ located enhancer [8]. Despite its critical role in development, the molecular mechanisms by which ATOH1 orchestrates HC differentiation remain elusive.

HES factors (Hairy-Enhancer of Split homologs) are also bHLH proteins. *HES1* and *HES5* are expressed in supporting cells of the cochlea and non-sensory regions [9,10]. They are thought to prevent the differentiation of progenitors of HC as a result of lateral inhibition. Another HES factor, HES6, has been recently described in HC [11]. HES6 is unique among HES factors as it promotes, instead of inhibiting, neuronal and myogenic differentiation [12–15] probably through antagonizing the HES1 and HES5 functions [15,16].

In order to identify ATOH1 target genes, we performed a genome-wide expression profile analysis in cells expressing *Atoh1* under the control of a tetracycline (TET)-off system, and found that *HES6* expression is induced by ATOH1. The rise and fall of *HES6* expression during development closely follow that of *Atoh1* in cochlear HC. Moreover, ATOH1 directly regulates *HES6* expression in vitro through binding to three E boxes of its promoter.

2. Materials and methods

All primers used are described in Supplementary Table 1.

2.1. Animal procedures

OF1 and CD1 mice were purchased from Charles River Laboratories. Animal procedures were approved by the Comite Regional d'Ethique pour l'Experimentation Animale.

2.2. Microarray expression analysis

We used U2OS (human osteosarcoma) cells stably transfected with *Atoh1*-HA-tagged cDNA under the control of a TET responsive element (pUHD10-3 vector) [17]. ATOH1 expression is induced by removing TET. Cells were plated (2×10^6 cells per 10 cm diameter plate) in the presence of TET for 24 h, then the medium was replaced with or without TET. After replacing the medium, the experiment was stopped at $t = 8$ or 24 h. Total RNA extraction and labelled cRNA synthesis were performed as recommended by Affymetrix using 1 μg of total RNA. Data analysis is described in the Supplementary material.

2.3. RT-PCR

Stably transfected U2OS cells were grown in DMEM with and without TET for 0, 2, 4, 6, 8 or 24 h before extraction for time-course analysis of target-gene expression. RNA extractions were performed using

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Abbreviations: bHLH, basic Helix-Loop-Helix; EMSA, electrophoretic mobility shift assay; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pairs; kb, kilo base; TET, tetracycline; HC, hair cells; NE, nuclear extract; ISH, in situ hybridisation

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Trizol reagent (Invitrogen) and reverse transcription-polymerase chain reaction (RT-PCR) using Superscript II and Taq polymerase (Invitrogen).

2.4. Plasmid constructs

The mouse *Atoh1* coding sequence was cloned in the pCMV-sport6 expression vector. The E47 cDNA inserted into the same vector was purchased as I.M.A.G.E. clone #4505427. The 1.2 kilo base (kb) *HES6* promoter was cloned into pXP1 (GenBank GI:3929277). See supplementary data for more details. The E boxes were mutated as in EMSA probes (see Supplementary data) by site-directed mutagenesis (Quik-Change, Stratagene). ISH probes were cloned into the pCRII-Topo vector (Invitrogen).

2.5. Cell culture

U2OS cells were grown in DMEM + 10% FCS + 1 mg/ml TET. Transfection and luciferase assays were carried out as previously described [18]. Each condition was performed in duplicate and results presented here are the mean of four experiments. Nuclear extracts (NEs) were prepared in HeLa cells as previously described [18], then characterized by Western Blot using monoclonal anti-mouse MATH1/ATOH1 (Hybridoma bank) and rabbit polyclonal anti-E47 (N-649, Santa Cruz Biotechnology Inc.) antibodies.

2.6. EMSA

Electrophoretic mobility shift assay (EMSAs) were performed as previously described [19], using [γ-^{33}P]-dCTP. Double-stranded DNA oligonucleotides were end-labelled with [γ-^{33}P]-dCTP. Binding reactions were carried out for 45 min on ice using 20 μg of NEs, 0.5 ng of probe, and 2 μg d*I*/d*C* in the binding buffer. Supershifts were obtained adding 2 μg anti-HA (Roche). Competition assays were 100-fold molar excess of unlabelled oligonucleotides. Samples were submitted to electrophoresis on a cold 5% polyacrylamide gel in 0.5X TBE at 120 V. Dried gels were analysed by Phosphorimager and ImageQuant v5.2 (Molecular Dynamics).

2.7. Non-radioactive in situ hybridisation

Heads of CD1 embryos and mice were fixed in 4% formaldehyde and cryosectioned (7- to 10-μm-thick frozen sections). In situ hybridisation (ISH) was performed as previously described [20]. Hybridisation solution contained 5X SSC, 50% formamide, 0.2 mg/ml yeast tRNA, 100 μg/ml heparin, 1X Denhardt's solution, 0.1% Tween 20 and 5 mM EDTA, and hybridisation was performed at 68 °C. The *Atoh1* probe was a gift of P. Gray, Department of Neurobiology, Harvard Medical School.

3. Results

3.1. Gene expression profiling in cells expressing ATOH1

We assessed gene expression in U2OS cells expressing *Atoh1* under the control of a TET-off system using the HG-U95 Affymetrix GeneChips. Twenty-four hours after ATOH1 induction, 73 genes showed increased expression (Supplementary Table 1). Eleven of them showed upregulation within 8 h (Table 1). We used RT-PCR to assess their expression in two independent *Atoh1*-U2OS clones at 24 h and confirmed that they are all upregulated in the presence of ATOH1 (data not shown; see Fig. 1a for *HES6*). We then analysed the time-course of their expression, measuring every 2 h after TET removal. We were able to detect upregulation as early as 2 h after *Atoh1* induction for four genes: *CHRNA1*, *SECTM1*, *SNAI2*, and *HES6* (Table 1 and Fig. 1b for *HES6*), indicating a fast regulation by ATOH1.

The induction of *HES6* as an immediate response to ATOH1 expression in the U2OS cells together with its colocalization with *Atoh1* in HC [11] suggest it is directly regulated by ATOH1 during inner ear development.

3.2. ATOH1 regulates Hes6 expression

We found that *Hes6* is expressed in the cochlea from E14.5 (data not shown; [11]) in the zone of non-proliferating cells, to early post-natal stage in HC (Fig. 2). *Hes6* was expressed in hair cells of the apex at P1, a time when *Atoh1* begins to be downregulated. At the base, *Atoh1* was silenced and *Hes6* was still weakly expressed. No expression of *Atoh1* nor *Hes6* was detected at P3. Therefore, *Hes6* mRNA expression closely follows that of *Atoh1* in HC, in agreement with the hypothesis that *Hes6* is directly regulated by ATOH1.

The 1.2 kb sequence located 5′ upstream of the *HES6* transcription start site (human NM_018645, NT_005120; mouse NM_019479, NT_078297) showed 38% identity between mouse and human (Fig. 3). It contained a TATA box and five conserved ATOH1 putative binding sites (E boxes), here named E1 to E5. E1–E4 are clustered in −1130 to −860 base

Table 1
Genes upregulated by ATOH1 in U2OS cells at 8 and 24 h

Probe	Gene		Complementary blast analysis	24 h[a]		8 h		Onset of upregulation by ATOH1(h)
				Log ratio	*p* Value	Log ratio	*p* Value	
49052_at	–	CDNA FLJ14388 fis, clone HEMBA1002716	WISP1; WNT1 inducible signaling pathway protein 1	5.4	0.000001	4.4	0.000006	6
34143_at	*CHRNA1*	Cholinergic receptor, nicotinic, alpha polypeptide 1		4.6	0.000010	3.2	0.000014	2
82144_at	–	CDNA FLJ14388 fis, clone HEMBA1002716	WISP1; WNT1 inducible signaling pathway protein 1	4.4	0.000001	2.4	0.000143	
39268_at	*KCNF1*	Potassium voltage-gated channel, subfamily F, member 1		3.5	0.000352	2.8	0.000023	8
41475_at	*NINJ1*	Ninjurin 1		2.9	0.000000	2.9	0.001226	6
41045_at	*SECTM1*	Secreted and transmembrane 1		2.6	0.000001	2.5	0.000050	2
77603_at	*SHF*	Src homology 2 domain containing F		2.5	0.000000	1.4	0.000001	6
63992_at	*SNAI2*	Snail homolog 2		2.5	0.000001	1.4	0.000000	2
49633_at	*HES6*	Hairy and enhancer of split 6		2.1	0.000000	1.7	0.000003	2
36533_at	*PTGIS*	Prostaglandin I2 (prostacyclin) synthase		2.0	0.000059	1.9	0.000499	4
51162_at	*SYT7*	Synaptotagmin VII		1.7	0.000008	1.2	0.000608	8
37117_at	*ARHGAP8*	Rho GTPase activating protein 8		1.6	0.000015	1.4	0.000333	8

[a]Mean of two experiments.

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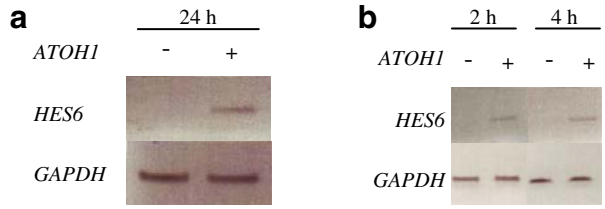

Fig. 1. RT-PCR showing the induction of *HES6* expression by ATOH1 in U2OS cells. (a) *HES6* expression after 24 h in the absence (−) or presence (+) of ATOH1. (b) *HES6* mRNA was detected both 2 and 4 h after the induction of ATOH1 expression. The housekeeping gene *GAPDH* was used as a RT control.

pair (bp) while E5 is located in the proximal promoter (−167 bp).

We placed the *HES6* promoter in front of a luciferase reporter gene and performed transient transfection in *Atoh1*-U2OS cells. ATOH1 expression caused an increase in luciferase activity (Fig. 4, lane 2). This activation was lost with a shorter promoter construct containing only E5 (lane 3), indicating that E5 is not sufficient for regulation by ATOH1. When E1, E2 or E4 was mutated in the promoter, there was a significant loss of the luciferase activity (compare lanes 4, 5, 7 to lane 2). On the contrary, E3 alteration alone or together with E4 did not affect the

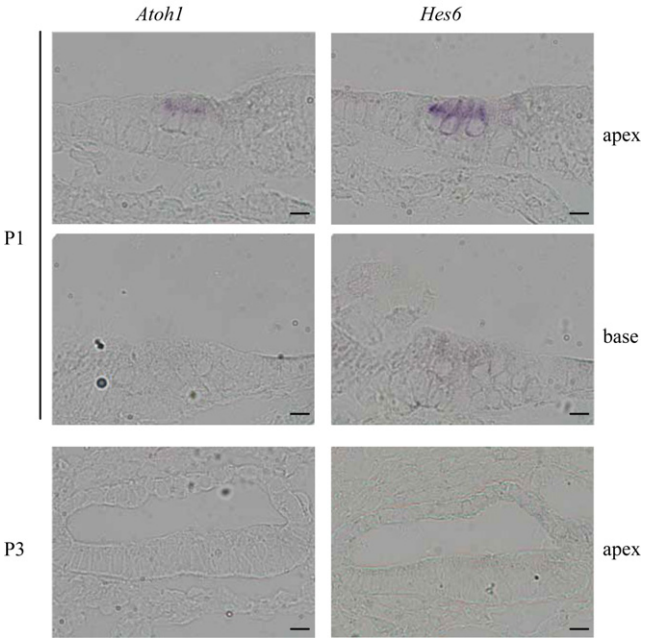

Fig. 2. In situ hybridisation showing *Hes6* and *Atoh1* expression in cochlear hair cells at post-natal stages P1 and P3 (adjacent sections). Scale bars 10 μm.

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human TGGGGTCCGAGAAGGCGTCTCCCGTTCCGGCTCCTCCACACAGCGTCTGGACTGACGCCGGGGCAGGAGCCGGGCAGGGGTCCCCAGGACGCTGCCCGAG -1105
mouse T-----CTAAAGCAGCAGCCGAAATCCAAGAACGCTGGAGCAGAGCCAGGGGTTCCGAGGGGGCGCG---CGCGCGCGCACACACA------CACACACA -1184
      *     *  *    **  *     * *  *  *       *** * * **  *  **  *****  *   ** **  *    * **        * *

human GGCGCGGCCCCACCGCCCACGCACCCCGCCCGCGGCCAAGGAGGAACCCAGCAGATGGGCGGCCCGCCGCGATGGGGGAGTCGCGGGCGTCTGGCCGGGAC -1005
mouse CACACACACACAC-ACACACACACCCCTCCCGCGGCGAGGCAGGGCC-GGCAGATGGACCGCCGACGACGCGGGG-----CTTGGGCGTCTGGCCGACAC -1091
        * *   * ***  * *** ****** ******** * *  *  **  ******** * ***  *  **  ***      *  ************** **
                                                  E1
human AAGCG--CCCGCCGCCTGCCCGCCGGACAAAGGGTGCGAGACAAAGGCGCGGCCAACACCTGTCGCTCTTGGCGCGGCCGCCGCTTTTCTCGCTTTTCTC -907
mouse AGGCGTGCCTGTTGCA-GCCTGCAGGACAAAGCCAGCTAGACAAAGGCGCGGCCAACACCTGTCGCTCCTGGC-CGGCC--------TGCCGCTTTTCTC -1001
      * ***  ** *  **  *** ** ********   ** ********************************** **** *****        *  **********
                                                  E2
human CGCCGAAGCGGCCGCAGCTGCCAACTGTCACGGGCATCAAGTTAC-AGGACCAGACGGCAGCTTCGGC----------------------------GGGCC -835
mouse TGCGGAAGCGGCTGCAGCTGCCAACTGTCACGGGCATCAAGTTACTGGGGCCAGACAGCAGCCTCGGCCTGCAGGAACCAAGAATAGAAGGGGGAGGAGC -901
       ** ******** ************************************  ** ****** ***** *****                          **  *
                     E3      E4
human TGGGAGGC----GGGGCGCGGAGGACGAGG-------AGGGGA--------GGAGGGAGGCGGGCG-----GGGGGCAGTGGAA--GGGAGGGAGGGGGC -761
mouse TGAGAGGGACTGGGGGTAGTGGGGATGGGGGACTCTAAGGGGACTCGCGGGGAAGGGAGGTGAGCGTGGAGAGGGGTTGGGGAACAGGGATGTGTGAGAC -801
      ** ****      ****    * *** * **       ******        * ******* * ***      ****  * ****  **** *   * * *

human CCC------GGGGGCCAAAGGGGACTAGAGGGAGATGGGGGA-GCTCAGAGGGGGGCGGGGCTGGTGACCC-----GCAGAGCCCAGGGGGAGAGCGGCG -673
mouse CACCGCAAAGAGGATGCGGAGGAACTAGAGGGAAATGAGAGGCGCTTAGGCGGGTACGGGG-TGATGAACAAGGAAGGAGAAGCTTGACTGGGGGCAGTT -702
      * *      * **       ** ********** *** * *  *** **  ***  ***** ** *** *     * ***  *  *    * * ** * *

human CGGGCGGG-----------------------------AGCACCCGGGACCCCGCCCCACTAGGACTCCAGTGCTGGCGGAGGGCGGCGTCGGGG--GTCC -604
mouse GGGGTGGGGTTAAAATAGACAAGGTAGTGGTTGGGTAAGTGACTTAGAGCACAGAGAAATAGAGATTTAGTAAGAACGACCGCGGACCTTGGAGTAAGAA -602
       *** ***                              **   *   ** * *     * ***   *  ***     **   *  * * * ** *

human GGCCC--GGCGTCTGGACGAGTCCGGAAGAA-ACTCAGGGGCCGCCCCGCCACCGCTGCCCAATGGACCC-TGCCTCCCCGGGGCTTGGCAGCCACCCCA -508
mouse GGTTATAGGTGTCTTAATGGGTCCAAAAGGACACACAAGGGTC---CTGTTCCTGCTGCTAAATTGGCTCGTACTTTCCCCGGACTTTGCACTCTCGCCA -505
      **      ** ****  * * ****  *** * ** ** *** *   * *   * *****  *** * * * * * * * *** ** *** ***  * * ***

human GCCGGGACCTGAAACCCCT-CTAGGTCCAGAGACCGCGGACTTGACTGTGGCTCTGCACACCAGGGACGCATCTCCTGGGGCCAGTG----GCCCCAGCG -413
mouse CCGGTGACCCGAAGCCCCTTCCAGGTCCCGAGGTCACGGACTTGACTTTGGCACTGCCCTGTCTGAAGTCAGCTC-TGGGACTGGAGCGCTATTCTCGAG -406
       * * **** *** ***** * ****** ***  * ********** **** **** *     * *  ** *** **** *  * *       *  * *

human CTCAACTCTGGAGGCGCCATCGGCGAAGGAG------GGAAATGAATGAATTAATGGGTGAATGAATGAGTGG-ACGGACGGACGGACGGACGGGTGGAC -320
mouse AGCACTTTTGGTGAACCAAGGGAAGCAAATGCCCTGTAGGAATGGATGGGTGAAGAGGTGGGTGGAAGGGCGGCATGGATAGATATACGGACCGAAGGAT -306
        **  * *** *   * *  *  * *  *       * **** ***  * **  ****  ** * * * ** * ***  **   ****** *  ***

human GCCT------------GCTCGCACGCGCGGCAGATCAGTGGTCCTAGAGCAGCAGACGGAGAGAAACAAGCGCGGGCAGGGCCGCAGCTACCCGGGGTCG -232
mouse GTATAGCTAAGTCCGAGCTTACAGGCA-GGCAGGTCTGGCCTCATAACTCAGCGAAAGGAAGTACAAGCACACCAGCAAGAGAGAAGCCG---GAGGTCA -210
      *  *            ***  ** **  ***** ** *   ** **   ****  * ***   * *    * *  *** *   * ***     * ****

human CCGCCCCGGTGCCGCCCCCGCGGGGGTCTAGGGAAGGCCGGGCTGGGGGCCCCGCCTAGCTCAGCCAGCTGCCCGACCGCCAGAGCCGCTGGGCCGGACA -132
mouse CTGCCCAGGAGTCCTGCCCGGCCTAAGTGGCAGGAGGTCTGGCTGTGGGCTCCGCC-----CCGCCAGCTGCCAGA------GACCCGCGGGGCCTGACA -121
      * **** ** * *   ****           * *** * ***** **** *****     * ********** **      ** **** ***** ****
                                                  E5
human AAGCGCGCTGCCCCGATTGGCTGCAGCGCGCCTCGCGGTCGGCCGCCCCGGGCCCGCGCGGCCAATCGGCGCATTGAGATGCAAATAAGCGGCTATAAAA -32
mouse AAGCGCGCCGCTCTCATTGGCTACAGGCTGCCTCACGGCTGGCCTCTCCGACCACGCACAGCCAATCGGTGCATTGACATGTAAATAAGTTGCTATAAAA -21
      ******** ** *  ******* ***   ***** ***  **** * ***  * *** * ********* ******* *** *******  *********

human GGGGCGGGACCGCGGCGGGCCGGAAGCCGCGAGGAGCGCGGACGGCTGGGCTGCTGCTGGGCGGCCGCGGGGCAGCGGAGGGCGCCGGCACTCCGGTCCC 2068
mouse TGGGCGGGGTCCCG-CGGGCCGGAAGACTC--------CGGAGAGCTAG--------TGGG--GCCTCCGGGCTGCGA-------------------CC 2040
       *******  * ** ********** * *         ****  *** *         ****  *** * **** ***                    **
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Fig. 3. ClustalW (EMBL-EBI http://www.ebi.ac.uk/clustalw/) alignment of the promoter of *HES6* between mouse and human. Conserved E boxes are highlighted. The TATA box and the transcription start sites are indicated in bold. The EMSA probes are underlined.

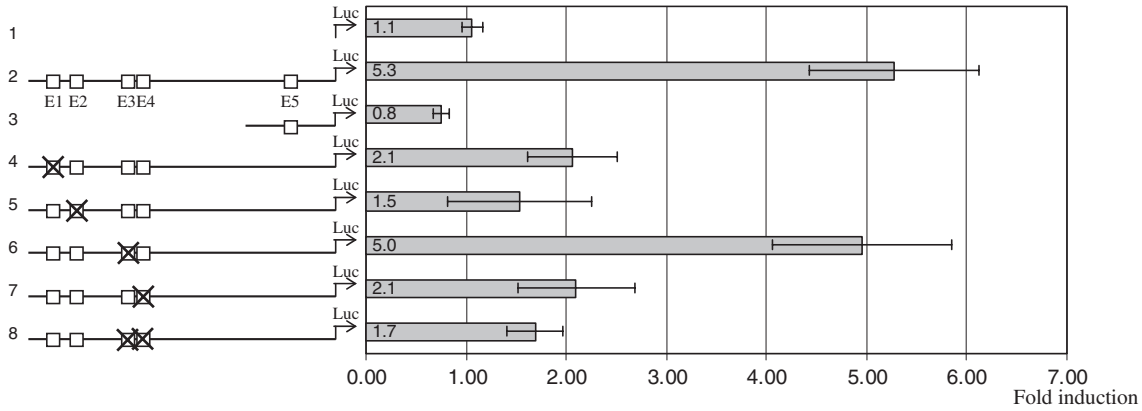

Fig. 4. *HES6*-promoter-mediated luciferase activation by ATOH1. The constructs used were: (1) empty luciferase vector; (2) *HES6* promoter −1.2 kb; (3) proximal promoter −750 bp; (4–8) E1, E2, E3, E4, E3 + E4 mutated respectively. The luciferase activity is shown as the fold induction

fold induction (compare lane 6 to 2, and 8 to 7). Thus, ATOH1 activates *HES6* transcription through three conserved clustered E boxes: E1, E2 and E4.

To determine whether this regulation occurs through direct binding of ATOH1 to the E boxes, we performed EMSAs. When the probe containing the E1 box was incubated with a

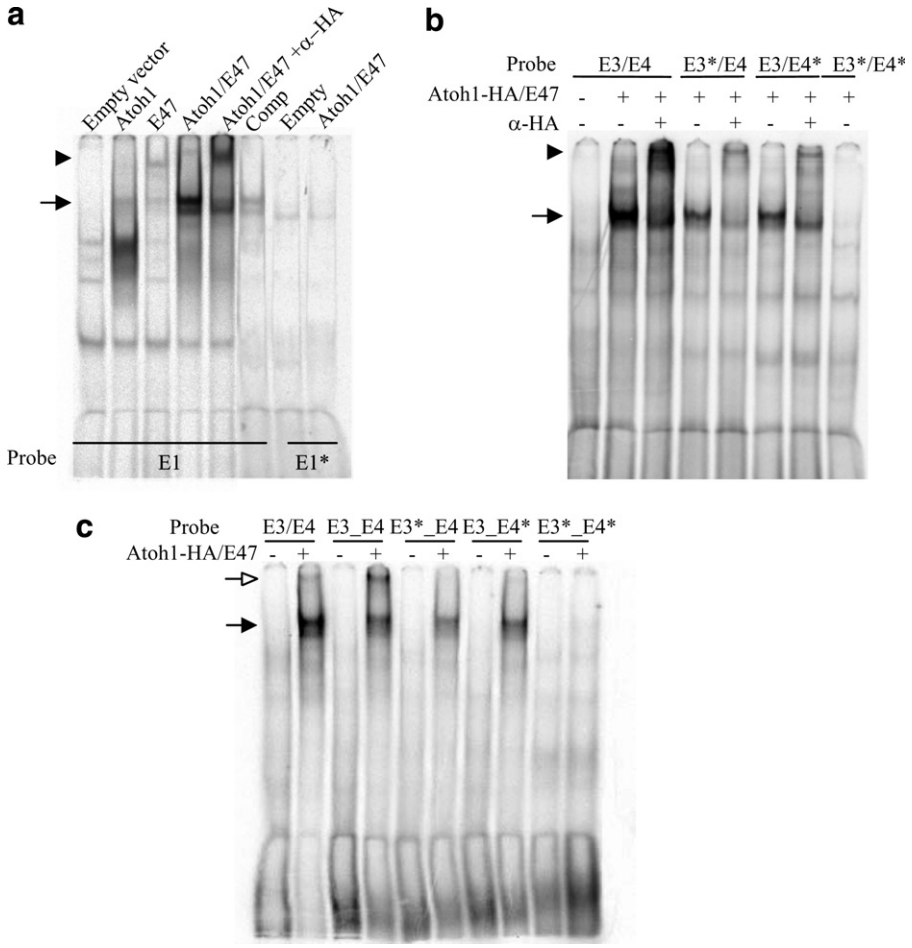

Fig. 5. Physical interaction between ATOH1/E47 and the E boxes of the promoter of *HES6* by EMSA. (a) Interaction between probe E1 and NE of cells transfected with empty vector, *Atoh1*-HA, E47, *Atoh1*-HA + E47. *Atoh1*-HA/E47 interaction on the probe resulted in a shift (arrow) and a supershift (arrowhead) when incubated with an anti-HA antibody. Competition (comp) was performed using a 100× excess of cold probe. There was no shift when E1 was mutated (E1*). (b) Interaction between ATOH1-HA/E47 and the probe containing both E3 and E4 (E3/E4) (arrow) confirmed by the supershift (arrowhead). (c) Comparison of the shifts obtained on the E3/E4 probe and the probe containing E3 and E4 separated by 13 bp (E3_E4). A higher molecular weight shift is indicated by a white arrow. * Indicates that the corresponding E box is mutated on the probe. The unbound probe is not shown.

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NE containing ATOH1 and E47, a specific mobility shift was observed (Fig. 5a, arrow). Supershift assays showed the specific binding of ATOH1 to the probe (arrowhead). ATOH1/E47 heterodimers were no longer able to bind to the probe when the E box E1 was mutated (E1*). Similar results were obtained with the E2 probe (data not shown).

E boxes E3 and E4 are separated by only 1 bp. When the EMSA probe containing these two E boxes was incubated with a NE containing ATOH1/E47, a band shift was observed (Fig. 5b, arrow). Preincubation with the antibody directed against HA-tagged ATOH1 resulted in a supershift (arrowhead). The shift and supershift were conserved when only one E box was mutated but was lost when both were mutated, indicating that the heterodimers can bind to either E3 or E4. However, the close proximity of the two E boxes could result in steric hindrance and prevent the binding of two ATOH1/E47 heterodimers (one to each E box), as we found for the *CHRNA1* promoter [21]. We therefore designed a probe where E3 and E4 are separated by 13 bp (Fig. 5C, E3_E4). The shift was still present, and we observed a supplementary shift of higher molecular weight (white arrow). This higher shift was specifically abolished when only one of the E boxes was mutated, indicating that it is likely to result from the binding of two heterodimers. This result indicates that steric hindrance prevents the binding of two ATOH1/E47 heterodimers to the E3 and E4 E boxes of the native sequence.

4. Discussion

Our findings show that ATOH1 directly activates the transcription of *HES6* in vitro through binding to three clustered E boxes (E1, E2 and E4) located in its promoter. The expression profile of *Hes6* closely follows that of *Atoh1* and therefore the basal-to-apical gradient of differentiation of cochlear HC [3]. This observation, as well as the direct regulation shown in vitro, indicates that ATOH1 is likely to be the main regulator of *Hes6* expression in HC in vivo. We also detected *Hes6* mRNA in the spiral ganglia during embryogenesis (data not shown) in agreement with its earlier expression in delaminating neurons of the E9.5 otocyst [22].

Several other class 2 bHLH have the capability to regulate *Hes6* expression: *Ngn1* or *Xath3* injection promotes *Hes6* upregulation in Xenopus [14], and *Hes6* upregulation follows that of *MyoD* during muscle regeneration [23]. Moreover, MASH1 can bind on the same clustered E boxes than ATOH1 [24], indicating that this region is possibly involved in multiple bHLH regulations of *Hes6*.

In the inner ear, HES6 might be involved in the differentiation of HC. In fact, it promotes neuronal differentiation in the neural plate [14], the retina [15] and in the cortex [25,26]. However, unlike Atoh1, Hes6 overexpression in the Kölliker's organ of post-natal rat cochlea does not lead to HC differentiation [11]. As HES6 has been shown to act synergistically with proneural genes [16], lack of expression of ATOH1 in the targeted cells might prevent a HES6-mediated trans-differentiation into HC. Our results show that a bHLH-factor cascade similar to that observed in neuronal tissues [27] may take place in the inner ear during development of the sensory epithelia.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.08.059.

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