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The promoter and multiple enhancers of the *pou4f3* gene regulate expression in inner ear hair cells

Masatsugu Masuda^{1,3}, Yan Li¹, Kwang Pak¹, Eduardo Chavez¹, Lina Mullen¹, and Allen F Ryan^{1,2}

¹Department of Surgery/Otolaryngology, VA Medical Center and UCSD School of Medicine, La Jolla, CA 92093 USA

²Department of Neurosciences, VA Medical Center and UCSD School of Medicine, La Jolla, CA 92093 USA

³Department of Otolaryngology, Kyorin University, Tokyo 181-8611, Japan

Abstract

Few enhancers that target gene expression to inner ear hair cells (HCs) have been identified. Using transgenic analysis of eGFP reporter constructs and bioinformatics, we evaluated the control of *pou4f3* gene expression, since it is expressed only in HCs within the inner ear, and continues to be expressed throughout life. An 8.5 kb genomic DNA fragment 5' to the start codon, containing three regions of high cross-species homology drove expression in all embryonic and neonatal HCs, and adult vestibular and inner HCs, but not adult outer HCs. Transgenes with 0.4, 0.8, 2.5 or 6.5 kb of 5' DNA did not produce HC expression. However, addition of the region from 6.5 to 7.2 kb produced expression in vestibular HCs and neonatal basal turn outer HCs, which also implicated the region from 7.2 kb to 8.5 kb in inner and apical outer HC expression. Deletion of the region from 0.4 to 5.5 kb 5' from the 8.5 kb construct did not affect HC expression, further indicating lack of HC regulatory elements. When the region from 1 to 0.4 kb was replaced with the minimal promoter of the *Ela1* gene, HC expression was maintained, but at a drastically reduced level. Bioinformatics identified regions of highly conserved sequence outside of the 8.5 kb, which contained POU4F3, GFI1, and LHX3 binding sites. These regions may be involved in maintaining POU4F3 expression in adult outer HCs. Our results identify separate enhancers at various locations that direct expression to different HC types at different ages, and determine that 0.4 kb of upstream sequence determines expression level. These data will assist in the identification of mutations in noncoding, regulatory regions of this deafness gene.

Keywords

Inner ear; hair cell; gene regulation; deletion analysis; POU4F3

Introduction

The inner ear hair cell (HC) plays a critical role in auditory and vestibular function. Many of the genes responsible for the remarkable structure and the unique functions of this highly specialized cell have now been identified e.g. [1,2]. In contrast, despite progress in identifying transcription factors (TFs) critical for inner ear development individually, there has been less progress in understanding how target genes are specifically regulated in HCs. Understanding the determinants of gene regulation has been an important target of research into cellular diversity in general, since it addresses fundamental issues related to cell identity [3]. Developing gene-based therapies for individual cell types is also enhanced by information on mechanisms of targeting gene expression. To elucidate mechanisms of cell-specific gene regulation, a study of *cis*-regulatory elements is commonly employed, because gene expression is regulated through the integrated action of many such elements, through the binding of specific TFs and TF-binding proteins.

Cis-regulatory elements can be divided into two categories: promoters immediately upstream to the transcription start site (TSS) and other *cis*-regulatory elements that are often localized at greater distance from the TSS, including enhancers, but also silencers, insulators, and tethering elements [4,5].

Promoters, enhancers and their associated TFs play a leading role in the regulation of gene expression, including the location, timing and degree of transcription e.g. [5,6]. Promoters and enhancers are small DNA segments, typically up to a few hundred base pairs in length, that serve as platforms for the recruitment of TFs through short, specific DNA motifs. Promoter and enhancer activity is highly correlated with occupancy by TFs and associated TF-binding proteins. These factors act in a cooperative manner, and different combinations of factors can result in significant differences in gene expression [5,7,8]. Promoters serve as the binding site for the core transcriptional complex. The core promoter is the minimal DNA sequence within the promoter that is sufficient to direct initiation of transcription [9], although it typically does not contribute to cellular specificity [10,11]. Enhancers act on the core transcriptional complex via protein-protein interaction, after being brought into proximity via DNA looping [5,6]. Mutations in regulatory regions can cause disease [12], including deafness e.g. [13], but they are difficult to identify without knowledge of their location within the noncoding sequence of the gene [14].

With respect to cell fate, binding of specific TFs to the enhancers of key genes is a primary means by which the adoption of a particular cell phenotype is regulated [5]. In the case of HCs, a primary regulator of their fate has been shown to be the basic helix-loop-helix (bHLH) TF ATOH1, in that induced expression of ATOH1 can convert nonsensory inner ear cells into HC-like cells or even HCs [15–18]. Once HCs are specified, ATOH1 expression is limited to this cell type in the inner ear. However, ATOH1 also controls the fate of other cell types in other tissues and organs, including cerebellar granule cells, Merkel cells, and gut epithelial cells [19–21].

Regarding targeting of ATOH1 expression to HCs, Helms et al. [22] determined that expression of the *atoh1* gene is itself regulated by multiple enhancers. These enhancers

target expression to several cell types, however, and enhancers that target expression specifically to HCs have yet to be identified. Boeda et al. [23] analyzed the gene encoding MYO7A, another protein expressed in HCs but also in additional sensory cells such as photoreceptors. Deletion analysis revealed multiple enhancers. They found that the 1.5 kb first intron of the gene was sufficient to drive expression in HCs, when combined with the 118 bp proximal promoter, suggesting the existence of one or more HC-specific enhancer(s) within the intron. To date, these HC-specific enhancer(s) have not been further localized.

Regulation of the *pou4f3* gene is also potentially useful for understanding how genes are controlled in HCs, since it is directly regulated by ATOH1 during development [24,25], and is expressed throughout the life of the HC, from lineage commitment through death [26,27]. We previously evaluated the control of *pou4f3* gene expression in HCs, using an 8.5 kb genomic DNA fragment 5' to the start codon of the *pou4f3* gene, linked to enhanced green fluorescent protein (eGFP) as a reporter in transgenic mice [24,28,29]. In prior papers, using homology analysis, we identified potential regulatory domains that contain clustered TF-binding sites, including ATOH1 and HC development-related TF recognition motifs, which are highly conserved across widely separated mammalian species (Fig. 1) [24]. Three highly conserved regions were identified within the 8.5 kb 5' region: 400 bp immediately 5' to the *pou4f3* ATG (proximal conserved region, PrCR); a 60 bp sequence 1.3 kb 5' to the ATG (distal conserved region I, DCR I), and a 280 bp sequence located 8.2 kb 5' (DCR II) to which ATOH1 binding was detected by chromatin immunoprecipitation (ChIP). However, homology analysis alone cannot identify regulatory sequences, since DNA sequence can be conserved for other reasons. To define the DNA elements involved in the functional regulation of *pou4f3* gene transcription in HCs, various portions of the 8.5 kb transgene were deleted, and transgenic mice expressing eGFP under the control of the fragments were generated. The animals were then assessed for eGFP expression patterns. Because the 8.5 kb transgene did not drive expression in adult outer HCs, we also evaluated additional genomic DNA for conserved regions with relevant TF-binding sites.

Materials and Methods

Transgene construction

The 8.5 kb transgene contained an 8.5 kb genomic fragment immediately 5' to the ATG of the murine *pou4f3* gene, with eGFP and SV40 termination sequences, as previously described [24]. This basic construct was modified by restriction enzyme deletion of specific fragments from the 5' end of the 8.5 kb, followed by re-ligation. The resulting transgenes were designated according to the length of the 5' DNA sequence fragments of the *pou4f3* gene employed (Fig. 2). The fragment lengths generated included DNA from the *pou4f3* ATG to 5': 0.4 kb (0.4 kb transgenic), 0.8 kb, 1.6 kb, 2.5 kb, 6.4 kb or 7.2 kb. In addition, deletion of the region from 0.4 to 5.5 kb 5' to the *pou4f3* ATG was performed, with re-ligation resulting in a total of 3.4 kb of 5' *pou4f3* DNA.

Finally, the 400 bp immediately 5' to the *pou4f3* ATG was edited from the 8.5 kb construct by restriction digest. The *Ela1* minimal promoter, which as defined in previous studies does not contribute to cell or organ specificity [10,11,30], was cloned by PCR from genomic

DNA using primers with appropriate restriction sites on their 5' ends. This was then ligated into the edited *pou4f3* construct.

Transgenic mouse generation and analysis

Each of the transgenes was used to generate transgenic mice on a C57BL/6 background by male pronuclear injection of fertilized oocytes and standard procedures, as previously described [31]. Genomic DNA samples from the resultant mice were screened by dot-blot using a biotinylated eGFP DNA probe. Mice with the highest level of hybridization were presumed to exhibit maximal transgene copy number, and 2–3 lines were chosen for breeding and further analysis.

To evaluate eGFP expression, transgenic mice were sacrificed at various ages, and tissues were microdissected and imaged on an Olympus FSX100 fluorescent microscope or a Leica MZFLIII fluorescent dissecting microscope with Spot RT3 digital cameras, or on a Zeiss LSM 880 confocal microscope. Fresh or 4% paraformaldehyde-fixed samples were used. To label actin, fixed tissue was stained with Texas Red-conjugated phalloidin, allowing imaging of cell junctions and HC stereocilia. DAPI was used to visualize nuclei. For quantitative analysis of eGFP expression, unfixed tissue was employed, since fixation reduces the level of eGFP fluorescence, and standardized exposure intervals were used. Relative expression levels were determined using NIH Image J and adjusted for exposure time. Analysis was primarily focused on neonatal animals, when expression levels are maximal for mice generated with the 8.5 kb construct.

Bioinformatic analysis

To identify highly conserved regions outside of the 8.5 kb, additional genomic sequence was compared across four widely separated mammalian species: mouse, human, dog and cow. These species have been estimated by DNA analysis studies [32] to be separated by 60–80 million years of evolution, since the radiation of the major mammalian families. 20 kb 5' to the ATG, the transcribed region of the gene, and 20 kb 3' to the polyadenylation signal were aligned to the corresponding regions of each species. Because the location of conserved elements vary widely across distantly related species, stepwise alignment of 500 bp segments were initially performed to identify elements with sequence similarity in the larger DNA fragments. Observed similarities were subjected to greater scrutiny to identify the extent and degree of sequence homologies. Once regions of homology between all four species had been identified, we evaluated TF-binding sites that were also present in all four species, using rVISTA (<http://genome.lbl.gov/vista/index.shtml>). We refer to such TFs as conserved TFs. We also evaluated two downstream targets of POU4F3 identified in previous studies: GFI1 and LHX3 [33,34], as well as TFs that we and others have previously found to influence HC development and *pou4f3* gene expression including ATOH1, TCF3 (also known as E47 or TFE2), SOX2, SIX1, HES1, NEUROD, NMYC, GATA3, ETV4, ETS2 and POU4F3 itself [24,28,29,35].

Within the 8.5 kb, the region from 6.4–7.2 kb 5' to the ATG of the mouse *pou4f3* ATG affected reporter gene expression in outer and vestibular HCs (see Results below). Because the sequence of this region was not highly conserved, we sought to identify common TFs

that might bind to this region using PROMO TF-binding pattern analysis [36,37], which has been shown to be unconstrained by sequence similarity for the identification of functionally conserved binding patterns. To concentrate only upon sites with a higher probability of TF-binding, the matrix dissimilarity rate was set at 3% (default is 15%). TF-binding sites in mouse 7.3, human 4.8, dog 5.5, and cow 7.2 kb 5' to the ATG were then analyzed. TFs for which there were fewer than 4 binding sites in the mouse sequence region were further evaluated for similarity of binding site position relative to the PrCR and DCR II.

Results

8.5 kb Transgenic

As previously described [24], robust eGFP expression was observed in HCs of the auditory and vestibular inner ear (Figs. 3a, 3b, 4a, 4d, 5a, 5d, 6a), beginning at the time of normal *pou4f3* mRNA expression, and continuing into the early postnatal period. Neonatal expression was noted in many other sensory organs including nasal and oral mucosa, Merkel cells in the skin (Fig. 7b, 7f), and various neurons including trigeminal, olfactory and dorsal root ganglion but not in retinal ganglion cells that normally express *pou4f3* [20,26,38,39]. However, presumably ectopic expression was observed in embryonic neurons of the spiral ganglion. Moreover, expression in outer HCs faded after the first postnatal week, and was absent after four weeks of age, unlike *pou4f3* mRNA which is well transcribed by HCs into adulthood and presumably for life [26].

0.4 kb Transgenic

With only 0.4 kb of 5' DNA, no eGFP expression was observed in HCs. However, in one line, bright eGFP expression was noted in neonatal inner pillar cells, which are located between the inner and outer HCs (Fig. 2). Although different in location, this expression was similar in level to that observed in the HCs of mouse lines that were generated using the 8.5 kb construct. In all three lines, eGFP expression outside of the inner ear was similar to that observed with the 8.5 kb construct.

0.8, 1.6, 2.5 and 6.4 kb Transgenics

Successively longer constructs up to 6.4 kb 5' to the ATG showed no eGFP expression in the inner ear, including in HCs. In the case of the 0.8 kb construct, neonatal eGFP expression outside of the inner ear was similar to that with 0.4 and the 8.5 kb construct. However, transgenics generated with the 1.6 kb and 2.5 kb constructs, both of which included DCR I at 1.3 kb 5' to the *pou4f3* ATG, showed no expression in or outside of the inner ear. eGFP was again observed outside of the inner ear in the 6.4 kb transgenic, although it was present only in Merkel cells.

7.2 kb Transgenic

The 7.2 kb construct showed neonatal inner ear eGFP expression in outer and vestibular HCs, but not in inner HCs (Fig. 4b, 4e, 5b, 5e, 5g-g'', 6b). The eGFP intensity in the cochlear HCs decreased from the cochlear base to the apex, and in both the basal turn cochlear and the vestibular HCs it was noticeably weaker than with the 8.5 kb construct. Expression was not observed in outer HCs beyond 4 weeks of age (Fig. 5h), as observed

with the 8.5 kb construct. Outside of the inner ear, weak eGFP was observed in the skin of the paws, but not in the roots of the vibrissae, the oral or nasal mucosa, or olfactory neurons (Fig. 7c, 7g).

These results suggest that the region from 6.4–7.2 kb contains regulatory sequences that direct gene expression in outer and vestibular HCs. TF-binding pattern analysis of this region by PROMO revealed one N-MYC and/or C-MYC binding site in the mouse (ACACGTGT), two in the cow (GCACGTGG, CCACGTGT), three in the dog (GCACGTGT, CCACGTGG, GCACGTGC) and one in the human (GCACGTGC) sequence. In the mouse, cow and dog, the most distal MYC site was similar in position, relative to DCR II (Fig. 8). It was somewhat more distant from DCR II in the human. This was the only TF-binding site that fulfilled the following conditions in the pattern analysis: (1) there were fewer than 4 binding sites in the mouse sequence 1 bp–7.3 kb 5' to the ATG of mouse *pou4f3* gene; (2) the binding site is within 6.4–7.2 kb 5' to the ATG of mouse *pou4f3*; and (3) the position of the site is relatively similar among species in relation to the position of DCR II.

3.4 kb Transgenic

The results above suggest that the region from 6.4 kb to 8.5 kb to the ATG of *pou4f3* gene contains HC-specific enhancers, while the region from 0.4 to 6.4 kb does not. To test this, the DNA between 0.4 kb and 5.5 kb 5' to the ATG was deleted. The resultant transgene (3.4 kb construct) directed eGFP expression to cochlear and vestibular HCs uniformly, with expression levels comparable to that seen with the 8.5 kb construct (Fig. 2, 4c, 4f, 5c, 5e, 6c). Outside of the inner ear, eGFP expression was also observed, although it was weaker than with the 8.5 kb construct (Fig. 7d, 7h). As with the 8.5 kb construct, outer HC expression declined after the first postnatal week.

Substitution of the PrCR for a minimal promoter resulted in weak eGFP expression in HCs

To assess the role of the PrCR, we replaced it with the minimal promoter of the *Ela1* gene, which does not contribute to cell- or organ-specificity. The transgene (8.5 kb/*Ela1*/eGFP) directed eGFP expression to cochlear and vestibular HCs and to cells outside of the inner ear with a pattern similar to that of the 8.5 kb transgenic. However, the intensity of expression was less than 0.1% of that observed with the 8.5 kb construct containing the PrCR (Fig. 2).

Variation in expression across transgene lines

We saw little variation in the cellular pattern of eGFP expression between different lines of the same transgene, with the exception of the 0.4 kb transgene as described above. This suggests that the other transgenes were too long to be affected by regulatory elements upstream from the insertion sites, and/or that no such sites were appropriately situated. In contrast, we noted variation in the expression level of eGFP between lines created with the same transgene. This was generally related to the intensity of the dot-blots used to confirm transgene integration, and thus presumably reflects transgene copy number. For one of the 8.5 kb lines, with particularly intense eGFP expression, HCs in epithelial explants did not survive as well in culture as did HCs in the lines with lower expression. GFP has been

reported to have some degree of cellular toxicity [40], which could explain this phenomenon.

Bioinformatic analysis from 20 kb 5' to 20 kb 3' to the *pou4f3* coding sequence

In addition to the three conserved region previously reported in 8.5 kb, (PrCR, DCR I, and DCR II), we identified additional conserved regions via homology analysis of the mouse, human, dog and cow *pou4f3* genes (Fig. 9). This analysis revealed that the single intron of the gene is 95–98% conserved across the four species. Another region of very high homology (96–98%) was identified ~11.1 kb 5' to the *pou4f3* ATG in mice (termed DCR III). Two additional regions of high homology were detected 3' to the coding sequence. These regions (90–94% and 88–96% homology across the four species) were respectively located from ~3.5 kb (3' DCR I) and ~7.3 kb (3' DCR II) 3' to the ATG. rVISTA TF-binding analysis of these regions revealed conserved binding sites for many of TFs known to be important for HC development and/or identified as binding to conserved regions located within the 5' 8.5 kb (DCRII, DCRI and PrCR). However, while no sites for ATOH1 were found in these new homology regions, multiple binding sites for GFI1 and LHX3 were present.

The DCR III, 3' DCRs, and the intron contain conserved binding sites for other TFs including GATA3, ETV4, ETS2, HES1, and/or NEUROD that enhance or reduce effects of ATOH1 overexpression to produce 8.5 kb transgenic and MYO7A positive cells in the neonatal cochlear epithelium [28,29].

Discussion

Various genomic regions regulate gene expression in HCs

We employed transgenic analysis of reporter constructs to identify 5' regions of the *pou4f3* gene that support gene expression in HCs, and bioinformatic homology analysis to identify potential regulatory sequences. The results provide the first functional analysis of regulatory regions for this important HC developmental [24,27] and human deafness [41] gene. The results indicate that separate enhancers at various locations direct expression to different HC types at different ages, and that ~400 bp of upstream sequence plays a critical role in determining *pou4f3* expression levels.

The ~400 bp PrCR contains a conserved TATA box and overlapping initiator element at ~300 bp 5' to the ATG. While it alone did not direct reporter expression to HCs, this region did produce appropriately targeted expression in many tissues outside the inner ear. It also appears able to interact with enhancers that direct gene expression to atypical cell types, since one transgenic line containing only this region upstream from eGFP produced strong expression in inner pillar cells. This is presumably the result of random insertion near a regulatory element not present in the *pou4f3* gene. It seems rather unlikely that this insertion occurred near a gene with inner ear expression limited to inner pillar cells. The results thus suggest that the 0.4 kb of immediate 5' DNA may predispose the construct for expression in inner ear sensory epithelia, without being sufficient to produce expression on its own; only interaction with another regulatory element produces targeted inner ear expression.

The function of the region from 0.4 to 6.4 kb 5' to the ATG is less clear. The additional sequence present in the 0.8 kb construct did not alter the pattern of expression seen with the 0.4 kb construct. However, constructs of 1.6 to 2.5 kb, which contain DCR I, did not direct gene expression to any of the tissues that normally express POU4F3, even those outside of the inner ear, while the 6.4 kb construct directed expression only to skin around the mouth and at the end of the digits. Thus, the sequences from 0.8 to 5.5 kb appear to contain suppressive elements. Longer constructs reversed this effect. While we can only speculate on the cause for this, it seems possible that interaction with enhancers in the longer construct may block any inhibitory regulatory sequences, perhaps by competitive protein interactions at the transcription initiation complex [42].

The lack of inner ear regulatory sequence in the 5' DNA region in between the immediate 5' 0.4 kb PrCR and 6.4 kb is further indicated by gene expression in the transgenic generated from the edited 3.4 kb construct (consisting of the PrCR plus 5.5–8.5 kb of 5' DNA) (Fig. 2, 4–6), which was similar to that of the entire 8.5 kb construct.

It can also be noted that transfection of nonsensory inner ear cells from 8.5 kb transgenics with TFs showing conserved binding sites in DCRI (located 1.3 kb 5' to the ATG) neither induced eGFP expression nor enhanced the ability of ATOH1 to induce eGFP expression, unlike some TFs with binding sites in the PrCR or DCR II [29].

Multiple enhancers direct *pou4f3* expression to different HC types

Our results with the 7.2 kb construct suggest that the sequence from 6.4 kb to 7.2 kb 5' to the ATG of *pou4f3* gene contains regulatory sequence sufficient to produce expression in vestibular HCs and neonatal basal turn outer HCs even though none of this sequence was highly conserved across mammalian species. While evolutionary sequence conservation analysis is an accepted criterion by which to identify regulatory sequence [43], it is known that substantial amounts of functional sequence, including tissue-specific enhancers, can show little or no sequence similarity across species [43–46]. This is due in part to the variability of many TF-binding sites [47,48]. Our results are consistent with this phenomenon.

To overcome the constraint of sequence similarity for identifying functionally conserved regions of DNA, we employed TF-binding pattern analysis across four mammalian species. This analysis suggested that an N- and/or C-MYC binding site was conserved among species (Fig. 8), and might participate in the competence of the 6.4–7.2 kb region to direct gene expression to early outer HCs and vestibular HCs. In general, the *myc* TF gene family exhibits a variety of functions including cellular proliferation, differentiation, and apoptosis. C-MYC interacts with the genes of hundreds of proteins, and plays roles in chromatin remodeling, DNA repair, replication, general transcription, and elongation [49], and N-MYC can functionally replace C-MYC [50]. In the inner ear, it has been found that N-MYC plays a role in HC development and fate: conditional deletion of the *n-myc* gene in the ear produces cochlear HCs without a distinction between inner and outer HCs [51]; moreover, *n-myc* transfection enhances the ability of ATOH1 to induce neonatal greater epithelial ridge cells to adopt a HC phenotype [29].

Sequences from 7.2 kb to 8.5 kb contain additional regulatory sequences which direct expression to inner HCs and more apical outer HCs, and enhances vestibular HC expression (Fig. 2, 4–6). This region contains DCR II, which exhibits two sequence motifs preferred by ATOH1, and that are conserved across mammalian species (Fig. 1c) [24]. These binding motifs are consistent with the report that ATOH1-regulated genes typically contain closely clustered ATOH1 binding motifs [52]. These motifs in DCR II are also overlapped by binding motifs for HES, a well known negative regulator of ATOH1 in HC development [53]. We have previously demonstrated direct binding of ATOH1 to DCR II using ChIP [24], and other TFs known to influence HC expression, including GATA3, and N-MYC also bound to this region [29]. These and our present findings strongly support our previous speculation that DCR II is an enhancer that directs gene expression to HCs, and that binding of combinations of TFs to DCR II maximally activate the *pou4f3* gene in HCs.

The DCR II also plays an additional critical regulatory role in HC gene expression, since transgenes containing the DCR II (i.e. the full 8.5 kb construct and the edited 3.5 kb construct) showed more robust eGFP expression in all inner ear HCs than was seen for the 7.2 kb construct. It is known that the presence of two enhancers with overlapping activity provides robustness to gene expression, and such dual transcriptional control can provide the phenotypic robustness that is essential for a highly deterministic process such as embryonic development [5]. The DCR II and the 6.4–7.2 kb region may exhibit a similar regulatory relationship. However, since we did not generate a transgenic in which only the DCR II was included, we can only speculate about this possibility.

Taken together, our data suggest that there are multiple enhancers that direct gene expression to HCs and to other tissues that normally express POU4F3. This is consistent with other developmental genes, which frequently are regulated by multiple enhancers, each controlling a specific spatiotemporal aspect of gene expression of the gene [5]. Boeda et al. [23] similarly suggested that there are different regulatory elements targeting *Myo7a* gene expression to inner HCs versus outer HCs.

The PrCR region strongly regulates *pou4f3* expression level

To further clarify the functional role of the PrCR, we substituted the minimal promoter from the *Ela1* gene for the PrCR. This minimal promoter does not contain the enhancers that normally direct expression of this elastase gene to the pancreas in mice, and it does not contribute to cell- or organ-specificity [10,11].

While the *pou4f3*/8.5 kb/*Ela1*/eGFP construct directed eGFP expression to HCs, the intensity of expression was dramatically (more than 100-fold) less than that with the original 8.5 kb construct or with the 7.2 kb construct. This reinforces our conclusion that the PrCR is not involved in targeting gene expression to HCs. Rather this region appears to play a significant role in determining the level of inner ear transcriptional activity. Lee and Wu [54] similarly demonstrated that heterologous *Drosophila* promoters produce highly different levels of transcriptional activity of the *yellow* gene. It should be noted that the PrCR contains a number of TF-binding sites other than those for an RNA polymerase II and the core transcriptional complex. Highly conserved binding sites for N-MYC, ETV4, and ETS2 were observed, and these TFs have been shown to enhance the induction of eGFP- and

myosin VIIa-positive cells in the cochlear sensory epithelium from *pou4f3*/8.5/eGFP mice by ATOH1 [29]. Huang et al. [55] demonstrated that the change of even a single TF-binding site near the core promoter of the *tert* gene can drastically alter its level of transcription. Deletion of one or more of these sites could explain the reduced level of eGFP expression seen in *pou4f3*/8.5 kb/*Ela1*/eGFP mice. Motif mutation would be required to elucidate this.

Comparison of conserved pou4f3 elements to enhancers of other HC-expressed genes

As discussed above, prior work has identified potential HC enhancer elements in the *atoh1* and *myo7a* genes [22,23]. We therefore aligned the sequences of the PrCR, DCR I and DCR II to regions containing HC enhancers for these genes. However, no significant sequence similarities were detected. This suggests that there is no general enhancer sequence that can direct gene expression to this cell type. However, our previous studies found that transfection of different TFs in combination with ATOH1 induced not only eGFP but also MYO7A expression in extrasensory cells of the inner ear. This suggests that the HC enhancers of different genes may bind similar combinations of TFs, despite a lack of overall sequence homology.

Conserved genomic regions outside of the pou4f3 8.5 kb construct

We previously speculated that regulatory sequence for late expression in outer HCs must be outside of the 8.5 kb transgene [24], since eGFP expression was not observed in *pou4f3*/8.5/eGFP adult outer HCs, although POU4F3 expression in outer HCs continues throughout life [26,27]. A single promoter can interact with multiple enhancers, and enhancers that interact with a promoter can change during development [43,44,56]. In addition to outer HC differences, our 8.5 kb construct transgenics show spatially different gene regulation from the physiological POU4F3 expression pattern, including ectopic eGFP expression in some regions of the embryo and absent gene expression in retinal ganglion cells [24] that normally express POU4F3 [39].

These findings indicate that regulatory elements important for *pou4f3* expression lie outside of our analyzed constructs. Supporting this speculation, our bioinformatic analysis revealed several additional sequence regions conserved among mammalian species, outside of the 5' 8.5 kb (Fig. 9, Table 1). In particular, the intron and the DCR III located ~11.1 kb 5' to the murine *pou4f3* ATG are most highly conserved. Interestingly, several of these regions identified by homology analysis contain binding sites not only for POU4F3 itself, but also for two outer HC-related TFs, GFI1 and LHX3 [33,34]. These regions are thus strong candidates for the regulation of POU4F3 expression in adult outer HCs. Additional transgenic analysis will be required to accomplish this.

Regulation of pou4f3 gene expression outside of the inner ear

Expression of POU4F3 is observed in many tissues outside of the inner ear, and these tissues exhibited bright eGFP expression even when only 400 bp of 5' DNA was included in transgenics. Since promoters generally do not direct expression to specific cells, this suggests that distal enhancers for these tissues are present in the DNA immediately upstream from the core promoter. This expression was suppressed with transgenes longer than 0.8 and up to 2.5 kb, and suppressed in most of these tissues with a transgene of 6.4 kb. While

expression was restored in the skin of the paws in the 6.4 kb transgenic, they exhibited lower levels of eGFP expression than was observed in the 0.4 kb or 8.5 kb transgenics. These data suggest that strong suppressor or silencer elements for cells outside of the inner ear are present in the region from 0.4 to 6.4 kb, and mildly suppressive elements in the region between 6.4 and 8.5 kb (perhaps right up to the 3' end of DCR II), elements which influence expression in tissues outside of the inner ear. Interestingly, expression outside of the ear was observed in all tissues but substantially reduced in the 3.4 kb transgenic relative to the 8.5 kb transgenic, even though inner ear expression was similar to that seen with the full 8.5 kb. In this transgenic the DNA from 0.4 to 5.5 kb 5' to the ATG was deleted. This may be related to a generally suppressive element between 6.4 and 8.5 kb as discussed above, or perhaps to conformational changes in the DNA between distal HC enhancers and the promoter.

Clinical implications

As noted above, in addition to changes in coding regions, mutations in the regulatory sequences of a gene can result in disease, as has been demonstrated for a variety of inherited conditions [15,17] and for deafness [16]. However, such mutations can be difficult to identify, since the regulatory sequences themselves are typically unknown outside of the core and proximal promoter located immediately prior to the expressed sequence. In the present study, our deletion analysis identified several regions that affect *pou4f3* expression in HCs as well as in non-auditory cells. Moreover, homology analysis identified highly conserved sequences that are potentially involved in regulation. Evaluation of these regions in patients suspected of mutations in the *pou4f3* region could identify sequence changes, the significance of which would otherwise go unrecognized.

Pou4f3 regulatory sequences also have the potential for use as drivers for HC gene therapy. However, the 8.5 kb construct is too large for most vectors. Our edited 3.5 kb construct is more appropriately sized, and could more readily be used to construct HC-specific gene delivery vectors, although such a vector would not deliver to adult outer HCs. This deficient could be amended if *pou4f3* outer HC regulatory sequences are identified in the future, as discussed above.

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Fig. 1.

Conservation of 5' *pou4f3* sequence regions and TF-binding sites across 4 mammalian species. A proximal conserved region (PrCR), a distal conserved region I (DCR I), and a second DCR II show high sequence similarity (respectively 87–92%, 77–88% and 72–88%) (a). In the PrCR (b) or DCR II (c), binding motifs for ATOH1, TFE2, GATA3, NMYC, ETS2, and ETV4 are also conserved. Their binding to the regions was previously confirmed by chromatin immunoprecipitation, while the combination of ATOH1 plus any of these other TFs induced *pou4f3* expression and HC induction more effectively than ATOH1 alone in the neonatal greater epithelial ridge. HES-binding motifs are also conserved, and this factor is known to reduce the effect of ATOH1 on induction of a HC phenotype

Fig. 2.

Schematic representations of the deletion constructs used to generate transgenics are shown on the left. Summaries of eGFP expression patterns in postnatal 2–4 day (p2–4) mice are indicated on the right. Transgenics generated with the 8.5 kb construct showed bright, homogenous eGFP expression in inner, early outer, and vestibular HCs (but not adult outer HCs), and in other tissues that normally express POU4F3, except for retinal ganglion cells. A transgenic generated with the 0.4 kb construct, one of three lines, showed bright eGFP expression in pillar cells. While no other inner ear expression was seen, all 0.4 kb lines showed expression comparable to the 8.5 kb construct in tissues outside the inner ear. The 1.6 and 2.5 kb constructs showed no eGFP expression at any location, while the 6.4 kb construct showed expression only in the skin of the digits and oral region. The 7.2 kb construct showed inner ear eGFP expression in early outer and vestibular HCs but not in inner HCs, indicating that the 6.4–7.2 kb region has competency to direct gene expression to these cells. When DNA between 0.4 kb and 5.5 kb was deleted, the edited 3.4 transgene directed expression to HCs and other tissues similar to the 8.5 kb construct. When the PrCR was replaced with the minimal promoter of the *Ela1* gene, and the transgene directed eGFP expression to cochlear and vestibular HCs, intensity was dramatically less than with the 8.5

kb construct. A much longer (more than 100-fold) exposure was required to generate the HC expression image shown

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Fig. 3.

Confocal images of isolated sensory epithelia, stained with Texas Red phalloidin, illustrate expression of eGFP in the organ of Corti (a) and the utricular macula (b) of postnatal day 3, 8.5 kb-eGFP mice. HCs express eGFP, but supporting cells do not. The scale bar in (a) = 100 μm , in (b) = 50 μm .

Fig. 4.

Overview of eGFP expression in postnatal day 3 transgenic mouse cochleae (a–c: side view, d–f: top view), under the control of the 8.5 (a, d), 7.2 (b, e), and 3.4 (c, f) kb *pou4f3* constructs. The cochlear capsule has been removed. Only cochlear HCs expressed eGFP. In the 7.2 kb-eGFP mice (b, e), eGFP intensity was progressively weaker from base to apex, and inner HCs did not express eGFP (see Fig. 5). The 3.4 kb construct (including the DCR II and the 6.4–7.2 kb region) showed intense and homogenous eGFP expression in inner and outer HCs (c, f). The scale bar in (a–f) = 300 μ m

Fig. 5.

High-magnification images of postnatal day 3 transgenic mouse organ of Corti under the control of 8.5 (a, d), 7.2 (b, e), and 3.4 (c, f) kb constructs. In the 7.2 kb-eGFP mice (b, e, g), inner HCs did not express eGFP, although their hair bundles are apparent with phalloidin staining (g-g''). In 6-week-old 7.2 kb-eGFP mice (h), outer HCs did not show eGFP expression, as was the case for adult 8.5 kb-eGFP mice [24]. Figure g'' and h are merged images of phalloidin (red), eGFP, and DAPI (blue). The scale bar in (a-f) = 100 μ m, (g) = 20 μ m, and (h) = 50 μ m

Fig. 6.

Expression of eGFP in postnatal day 3 transgenic mouse maculae and cristae of semicircular canals, under the control of 5' 8.5 (a), 7.2 (b), and 3.4 (c) kb constructs. Vestibular HCs with all constructs express eGFP, although it was significantly weaker in 7.2 kb-eGFP mice. Scale bar in (a, b, c) = 200 μ m

Fig. 7.

Expression of eGFP in postnatal day 3 transgenic mice at the root of vibrissae, the skin of the digits, and the nasal and oral mucosae. In wild type mice (a, e), eGFP expression (a', e') is not observed; while it is clear in 8.5 kb-eGFP mice (b, f). In 7.2 kb-eGFP mice (c, g), weak expression was observed in the digits, but not in the vibrissae and nasal or oral mucosae. In 3.4 kb-eGFP mice (d, h), it is present at all regions, although significantly weaker than in 8.5 kb-eGFP mice. The scale bar in (a, d, h) = 1 mm. Scale bar in (d) applies to (b, c). The scale bar in (h) applies to (e-h)

Fig. 8.

Conserved MYC-binding sites for 4 mammalian species. The deletion analysis suggested that murine 6.4–7.2 kb may contain TF-binding sites critical to direct gene expression in outer and vestibular HCs. TF-binding pattern analysis by PROMO was conducted. In the mouse, cow and dog, a distal MYC site was similar in position, relative to the distance from DCR II, although other sites are present in the cow and dog and a single site is somewhat more proximal from DCR II in the human. This was the only TF-binding site that fulfilled the following conditions in the pattern analysis: (1) there were fewer than 4 binding sites in the mouse sequence 1 bp–7.3 kb 5' to the ATG of mouse *pou4f3*; (2) the binding site was within 6.4–7.2 kb 5' to the ATG of mouse *pou4f3*; and (3) the position of the site was similar among species in relation to the position of DCR II

Fig. 9.

Homology analysis of the mouse, human, dog, and cow from 20 kb 5' to 10 kb 3' to the *pou4f3* coding sequence. In addition to the three conserved regions within the 8.5 kb (PrCR, DCR I, and DCR II), four additional conserved regions were identified. The single intron of the gene was highly conserved (95–98% homology across the four species). A third 5' distal region at ~11.1 kb 5' to the *pou4f3* ATG in mice (DCR III) was also highly conserved (96–98%), although as with DCR II at different locations relative to the ATG. Two regions of sequence homology were detected downstream from exon 2. The regions (90–94% and 88–96% homology across the four species) were located at ~3.5 kb (3' DCR I) and ~7.3 kb (3' DCR II) 3' to the ATG. Analysis of these regions across species revealed conserved binding sites for POU4F3 itself and for two of its outer HC-related downstream targets, LHX3 and GFII1 (Table 1)

Table 1

TFs predicted to bind to conserved regions in the *pou4f3* gene.

	5' to the ATG				3' to the ATG		
	5' DCR III	5' DCR II	5' DCR I	PrCR	Intron	3' DCR I	3' DCR II
SOX2					X		
SIX1					X	X	
ATOH1		X		X			
TFC3		X		X			
NMYC				X	X		
GATA3	X	X		X	X	X	X
ETV4	X			X	X	X	X
ETS2	X		X	X	X	X	X
HES1		X		X	X	X	
NEUROD	X	X		X	X	X	X
POU4F3	X		X		X	X	X
GFI1	X				X		X
LHX3	X				X	X	X

“X” indicates that the TF binds the conserved regions. Data for the PrCR, 5' DCR I and 5' DCR II previously published in Masuda et al. [28].