The Human Pendrin Promoter Contains two N₄ GAS Motifs with Different Functional Relevance

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Key Words
Interleukin 4 • Cytokine • ChIP • SLC26A4 • STAT6

Abstract
Background: Pendrin, an anion exchanger associated with the inner ear, thyroid and kidney, plays a significant role in respiratory tissues and diseases, where its expression is increased following IL-4 and IL-13 exposure. The mechanism leading to increased pendrin expression is in part due to binding of STAT6 to a consensus sequence (N₄ GAS motif) located in the pendrin promoter. As retrospective analyses of the 5' upstream sequence of the human pendrin promoter revealed an additional N₄ GAS motif (1660 base pairs upstream of the one previously identified), we set out to define its contribution to IL-4 stimulated changes in pendrin promoter activity.

Methods and Results: Electrophoretic mobility shift assays showed that STAT6 bound to oligonucleotides corresponding to both N₄ GAS motifs in vitro, while dual luciferase promoter assays revealed that only one of the N₄ GAS motifs was necessary for IL-4 –stimulated increases in pendrin promoter activity in living cells. We then examined the ability of STAT6 to bind each of the N₄ GAS motifs in vivo with a site-specific ChIP assay, the results of which showed that STAT6 interacted with only the N₄ GAS motif that was functionally implicated in increasing the activity of the pendrin promoter following IL-4 treatment. Conclusions: Of the two N₄ GAS motifs located in the human pendrin promoter region analyzed in this study (nucleotides -3906 to +7), only the one located nearest to the first coding ATG participates in IL-4 stimulated increases in promoter activity.

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Introduction

Pendrin is an anion exchanger encoded by the solute carrier family 26A4 gene (SLC26A4) family and loss of function mutations in this protein can result in nonsyndromic or syndromic sensorineural hearing loss, termed Pendred Syndrome [1-7]. Pendrin is expressed in a multitude of tissues, including the airway epithelium, although its basal mRNA expression is relatively low in the latter [8-20]. However, in the presence of the T-helper type-2 (T_h2) cytokines, interleukin (IL)-4 and IL-13, pendrin expression is rapidly and robustly increased [11, 12, 18, 21-24].

IL-4 and IL-13 are two of many small glycoproteins acting as signaling molecules in the humoral immune system. Once bound to their membrane-located receptor, the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signal transduction cascade is activated. This cascade involves activation of the receptor by phosphorylation, followed by the recruitment, binding, phosphorylation and activation of STAT monomers. Currently, seven STAT isoforms have been identified, and STAT6 is the isoform preferentially activated in the presence of IL-4. Once phosphorylated on tyrosine residue 641, STAT6 monomers dimerize, at which point they are able to translocate from the cytosol into the nucleus. The STAT6 homodimers then scan and preferentially bind to conserved DNA sequences referred to as interferon-γ activated sequences (GAS) motifs, which are defined as 5’ TTC (N4) GAA 3’, where N is any nucleotide [25, 26].

It has been shown that pendrin regulation by IL-13 within the airway epithelium impacts the volume of the airway surface liquid (ASL) [21]. We initiated experiments aimed to define the mechanism of IL-4 and IL-13 stimulated increases in pendrin expression, and found that both cytokines increase pendrin promoter activity [23]. In addition, we discovered that an N4 GAS motif that bound STAT6 was necessary for the increases in pendrin promoter activity. Further analysis of the 5’ upstream sequence of the human pendrin promoter revealed another N4 GAS motif. Therefore, in the present manuscript, we set out to investigate the contribution of this additional N4 GAS motif to IL-4 stimulated changes in pendrin promoter activity.

Materials and Methods

Materials

Penicillin, streptomycin, glutamine, and base medias were from Sigma Aldrich (St. Louis, MO; USA). Fetal bovine serum (FBS) was from Lonza (Cologne, Germany). IL-4 was from Invivogen (San Diego, CA; USA) and prepared in distilled water. Blasticidin, zeocin and the HEK-Blue™ IL-4/IL-13 (HEK-Blue™) cells were from Invivogen. The rabbit anti-STAT6 (catalogue# sc-621X) antibody used for electrophoretic mobility shift assays (EMSAs) and chromatin immunoprecipitation (ChIP) assays was from SantaCruz Biotechnology (Europe). The normal rabbit IgG (catalogue# 2729) used for ChIP assays was from Cell Signaling (Boston, MA; USA). All antibodies conjugated to an infrared-dye were from LICOR (Lincoln, NE; USA). Oligonucleotides for primers and EMSAs were purchased from Microsynth (Balgach, Switzerland) and the identities of all nucleotides corresponding to the human pendrin promoter were verified with sequencing (Microsynth) prior to use in experiments. Brightness and contrast adjustments were made to agarose gel and EMSA images with Adobe Photoshop.

Cell Culture

All cell lines were cultured at 37°C in a humidified incubator gassed with 5% CO₂ and maintained in plastic petri dishes. HEK-Blue™ cell media consisted of DMEM high glucose (4.5g/L) base media supplemented with 10% FBS, 10µg/mL blasticidin, 100µg/mL zeocin, 50U/mL penicillin, 50µg/mL streptomycin and 1.5% NaHCO₃. NCI-H292 cell media consisted of RPMI-1640 base media supplemented with 10% FBS, 4.5g/L D-glucose, 1.5g/L NaHCO₃, 2.38 g/L HEPES, 1mM sodium pyruvate, 50U/mL penicillin and 50µg/mL streptomycin. Media was replaced three times weekly for both cell lines.
Transient Transfection and Luciferase Assays

NCI-H292 cells were seeded in white 96-well Nunc™ ∆ Surface plates (Nunc; Langenselbold, Germany) and transiently transfected on the following day with polyethyleneimine (PEI). A transfection mix containing 120µg/mL PEI (pH 7.0), pGL3B vector (containing fragments of the human pendrin promoter subcloned upstream of the firefly luciferase gene), and pRL-TK vector (containing the thymidine kinase promoter upstream of the Renilla luciferase gene) was prepared in PEI buffer (150mM NaCl, 10mM HEPES, pH 7.0). Transfection mix was added to culture media at a 100µL:2500µL ratio. The final concentrations of pGL3B and pRL-TK vectors were 0.183fM and 0.076fM, respectively. 24 hours later, media containing transfection mix was replaced with media containing vehicle (distilled water), IL-4 (40ng/mL) or TNFα (40ng/mL). 48 hours later, the cells were prepared for the dual luciferase assay using the Dual Luciferase® Assay System (Promega). Media was removed and cells were washed once with phosphate buffered saline (PBS, 136.89mM NaCl, 2.69mM KCl, 3.21mM Na₂HPO₄, pH 7.4). 20µL of passive lysis buffer (Promega) were added to each well and incubated at room temperature (RT) for 15 minutes with agitation. 50µL of luciferase assay reagent (LAR) II was added and luminescence from the Firefly luciferase was detected for 10 seconds. 50µL of STOP & Glo reagent was then added to simultaneously quench the Firefly luciferase signal and activate the Renilla luciferase signal. The Renilla luciferase luminescence was also detected for 10 seconds. The experimental design was as follows: for each 96-well plate, at least 4 replicate wells were transfected and treated in the same manner. This was then repeated at least 3 times (each time, a newly passaged batch of cells was used). Addition of the LARIIL and STOP & Glo buffers, as well as detection of the luminescence was performed using the VICTOR™ X3 2030 multi-label reader from Perkin Elmer (Vienna, Austria).

3′-end biotin labeling

The following oligonucleotides were commercially synthesized (Microsynth) and biotinylated using the Biotin 3′ End DNA Labeling (Pierce; Rockford, IL; USA) kit according to the manufacturer’s instructions. The oligonucleotide sequences used for the wildtype  N₄ GAS motif 1 were: (forward) 5′-GATGCATCTTTCATGGAAAGAAGTCTCGTA 3′ and (reverse) 5′-TCAGGACTTCCTTCCAAGAATAGATGCATC 3′; and for the mutant N₄ GAS motif 1 were: (forward) 5′-GATGCATCTTTATTGGAAAGAAGTCTCGTA 3′ and (reverse) 5′-TCAGGACTTCCTTCCAAGAATAGATGCATC 3′. Nucleotides belonging to the N₄ GAS motif 1 are underlined, and the mutated nucleotides are in bold-face font. Mutation of N₄ GAS motifs in this way has been previously demonstrated to abrogate STAT6 binding [27, 28]. Following biotinylation, equal amounts of complementary oligonucleotides were annealed by heating to 95°C for 5 minutes and cooling down to RT over time.

Nuclear Extract Preparation & Electrophoretic Mobility Shift Assays (EMSAs)

HEK-Blue™ cells were treated for 15 minutes with either vehicle (water) or IL-4 (40ng/mL) at 37°C and fractionated into nuclear and cytoplasmic extracts using the NE-PER Nuclear and Cyttoplasmic Extraction Reagents kit (Pierce) according to the manufacturer’s instructions, except the following ratio of reagents were used: 500µL:27.5µL:40µL CERI:CERII:NER. Halt protease inhibitor cocktail (HPIC) was added to CERI and NER to a final concentration of 1X. Protein concentrations were determined by the RCDC protein assay (BioRad; Vienna, Austria).

The binding reaction contained 20mM HEPES-KOH pH 7.9, 0.5mM Tris pH 8.0, 50mM KCl, 10% glycerol, 50ng/µL poly dI•dC, 5mM dithiothreitol, and 0.15mM EDTA pH 8.0. Nuclear extract (5µg total) from vehicle or IL-treated cells was added and incubated on ice for 20 minutes. 25nM non-biotinylated (cold) oligonucleotides or 0.6µg anti-STAT6 antibody were then added and incubated on ice for 15 minutes. 1.2nM biotinylated oligonucleotides were then added and incubated for another 15 minutes on ice. Orange loading dye (LICOR) was added to the samples to a final concentration of 1X (to stop the reaction), and the entire reaction was then separated on 5% polyacrylamide native gels at 120V for 45 minutes. Samples were transferred to Biodyne® nylon membranes (Pierce) for 45 minutes at 380mA with stirring. All electrophoreses were carried out with ice-cold buffers. The membranes were cross-linked at 120 ml/cm² for 1 minute with UV light and subsequently blocked for 15 minutes with 1X Nucleic Acid Detection Blocking Buffer (Pierce). Membranes were then incubated for 15 minutes with a 1:1000 dilution of anti-strepaavidin-IRdye-800, followed by six, five minute washing steps with 1X Nucleic Acid Detection Wash Buffer. All incubations and washing steps were carried out with gentle agitation at RT and in the absence of light. The membranes were visualized with the ODYSSEY® infrared imaging system (LICOR).
Chromatin Immunoprecipitation (ChIP)

ChIP experiments were executed with the ChIP-IT® Express kit (Active Motif; La Hulpe, Belgium). 1x10^7 HEK-Blue™ cells on plastic 10cm Ø petri dishes were treated with IL-4 (40 ng/mL) or vehicle (water) for 15 minutes. Nucleoprotein complexes were cross-linked by treating the cells with 1% formalin (Sigma Aldrich) in serum free HEK-Blue™ media for 10 minutes at RT. To stop cross-linking, the formaldehyde containing media was replaced with 1X glycine-PBS (Active Motif), and cells were incubated for 5 minutes at RT. After removing the PBS solution, the fixed cells were collected by scraping in 1mL of PBS containing 5 µl of phenylmethanesulfonyl fluoride (PMSF). Cells were centrifuged at 600 x g at 4°C for 10 minutes, and the supernatant discarded. Nuclei were isolated from cells using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce), according to the manufacturer’s instructions with the exceptions described above. Nuclei were centrifuged at 1200 x g for 10 minutes at 4°C, and the supernatant discarded. All recovered chromatin was fragmented by simultaneous digestion with 20 Fast Digest Units each of Fast Digest restriction enzymes HindIII, BglII and XbaI (ThermoFisher Scientific) overnight at 37°C. Complete fragmentation was verified via PCR. Samples were centrifuged at 1200 x g for 5 minutes at 4°C and the supernatant discarded. Nuclei were then lysed with 350µL sample buffer (1% SDS, 10 mM EDTA, pH 8.0 in 50mM Tris HCl, pH 8.1) to recover cleaved chromatin. 10µL of this solution (input DNA) was set aside for later use. 10 to 150µL cleaved chromatin were then subjected to immunoprecipitation using 1 µg ChIP-validated anti-STAT6 antibody or rabbit IgG (negative control), together with 25µL protein G magnetic beads (Active Motif) overnight on a rotating wheel at 4°C. Samples were then washed once with 800µL ChIP Buffer 1 (Active Motif) and twice with 800µL ChIP Buffer 2 (Active Motif). Elution was performed using 50µL of Elution Buffer AM2 (Active Motif) for 15 minutes on a rotating wheel at RT. Next, reverse cross-linking was performed with 50µL Reverse Cross-link Buffer (Active Motif), and protein digestion was realized with the addition of 0.01 mg/mL Proteinase K (Active Motif). Protein digestion was then halted with 2µL Proteinase K stop solution (Active Motif). DNA from all samples, as well as that from the input DNA, was then subjected to PCR.

PCR reactions were performed with the KOD Hot Start DNA polymerase kit (Novagen). Each 50 µl reaction was composed of 1X PCR KOD Hot Start DNA polymerase buffer, 0.2 mM deoxynucleotide triphosphates (dNTPs), 1.5 mM (or 1.25 mM for the N_4 GAS motif 1 reaction) MgSO_4, 1 µM forward primer, 1 µM reverse primer; 0.02 U/µl KOD Hot Start DNA Polymerase and 1 µl template chromatin (or 0.1 µl for the input chromatin). The PCR parameters were as follows: 95°C for 2 minutes, followed by 35 cycles of 95°C for 20 seconds, 59.7°C for 10 seconds and 68°C for 3 seconds (HindIII and XbaI) or 5 seconds (BglII). PCRprimer sequences and expected product lengths are shown in Table 1.

Bioinformatic Analyses

The ~5000 base pair sequence upstream of the first coding ATG in the human pendrin gene (GenBank accession no. AC078937.1) was analyzed for transcription factor binding consensus motifs using MatInspector software (a module of the Genomatix software suite).
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Statistics

Significant differences between 2 experimental groups were determined using the two-tailed Student's t-test, whereas significant differences between 3 or more experimental groups were determined using a one-way ANOVA followed by Bonferroni’s Multiple Comparison Test. Differences were considered statistically significant where the p-value ≤ 0.05. Statistical analyses and the generation of graphs were performed with GraphPad Prism 5.0 software.

Results

We recently identified an N₄ GAS motif located 1803 nucleotides upstream of the pendrin open reading frame [23]. Further bioinformatical analysis of the 5′ upstream region of the pendrin promoter revealed an additional N₄ GAS motif located 3463 nucleotides upstream of the coding ATG (Fig. 1). The N₄ GAS motif located furthest from the ATG will heretofore be referred to as N₄ GAS motif 1 and the one closest to the ATG will be referred to as N₄ GAS motif 2.

We previously demonstrated that STAT6 binding to N₄ GAS motif 2 is necessary for IL-4 stimulated increases in human pendrin promoter activity and mRNA expression [23]. Therefore, we first set out to investigate if STAT6 also interacts with N₄ GAS motif 1. Using EMSAs, a single nucleoprotein complex (shift) was detected in HEK-Blue™ cell nuclear...
extracts stimulated with IL-4 and incubated with a biotinylated oligonucleotide probe containing the wild-type $N_G$ GAS motif 1 sequence (Fig. 2). This interaction was specific since the shift was not visible in the presence of a molar excess of unlabeled, wild-type oligonucleotide. As expected, no prominent nucleoprotein complexes were detected when using a biotinylated oligonucleotide probe in which $N_G$ GAS motif 1 was mutated. In addition, a single nucleoprotein complex was detected in HEK-Blue™ nuclear extracts treated with IL-4 in the presence of an anti-STAT6 antibody (supershift). From these experiments, we can conclude that STAT6 binds to an oligonucleotide bearing the sequence of $N_G$ GAS motif 1 in vitro.

Next, the functional relevance of $N_G$ GAS motif 1 in human pendrin promoter activity was assessed using dual luciferase reporter assays. Taking the aforementioned EMSA results into account, we hypothesized that $N_G$ GAS motif 1 contributes to IL-4 stimulated increases in pendrin promoter activity similarly to that observed with $N_G$ GAS motif 2. To this
end, we subcloned the 3912 base pair fragment of the human pendrin promoter shown in Figure 1 upstream of the firefly luciferase gene within the pGL3B vector. The N$_4$ GAS motifs were systematically mutated, and the ability of the constructs to drive firefly luciferase expression in the presence and absence of IL-4 was evaluated. Firstly, Figure 3A shows that IL-4 significantly increased firefly luciferase expression in cells transiently transfected with the 3912 base pair fragment containing the two wild-type N$_4$ GAS motifs. As expected, this increase was blocked after mutating N$_4$ GAS motif 2, as well as after mutating both N$_4$ GAS motifs within the same fragment. Interestingly, however, mutation of N$_4$ GAS motif 1 alone did not alter the response of the promoter fragment to IL-4. This was unexpected since STAT6 binds to both N$_4$ GAS motifs in vitro (as determined by the EMSAs), but only N$_4$ GAS motif 2 was required for IL-4 stimulated increases in pendrin promoter activity.

Since STAT6 bound to both N$_4$ GAS motifs in vitro (as determined by the EMSAs), but only N$_4$ GAS motif 2 was required for IL-4 stimulated increases in pendrin promoter activity,
we wondered whether STAT6 bound either of the two N\textsubscript{4} GAS motifs \textit{in vivo}. To do this, we turned to site-specific chromatin immunoprecipitation (ChIP), which combines the benefits of EMSAs and \textit{in vivo} footprinting, thereby allowing detection of the simultaneous association of transcription factors with individual sequences on chromatin. Following the design reported by Schuch et al. [29], we engineered a site-specific ChIP assay to simultaneously assess the \textit{in vivo} interaction of STAT6 with both N\textsubscript{4} GAS motifs present in the human pendrin promoter (Fig. 4A). Complete and specific digestion of the chromatin (with the use of specific restriction enzymes) is an essential prerequisite for site-specific ChIP, since this step should physically separate the regions of interest (the nucleotides expected to interact with a transcription factor; in this case, the two N\textsubscript{4} GAS motifs with STAT6). This aspect was verified using primer pairs designed to amplify regions overlapping the critical restriction enzyme sites (digestion control amplicons), as well as regions within the fragments containing the two N\textsubscript{4} GAS motifs (target amplicons). Figure 4B shows that only the target amplicons were generated following digestion of the chromatin, confirming that the isolated chromatin was completely digested and that the N\textsubscript{4} GAS motifs were located on separate, individual chromatin fragments. Figure 4C shows that STAT6 was immunoprecipitated only from the chromatin fragment encompassing N\textsubscript{4} GAS motif 2 and only from cells treated with IL-4. These results are consistent with those from the dual luciferase reporter assay and confirm that N\textsubscript{4} GAS motif 1 does not contribute to IL-4 –stimulated increases in pendrin promoter activity.

**Discussion**

At least 10 transcripts of varying sizes from the SLC26A4 gene are listed in the Ensemble database (www.ensemble.org, gene ID# ENSG00000091137), only five of which code for proteins. Of these 5 protein coding transcripts, only the one (Ensemble transcript ID# ENST00000265715) encoding a 780 amino acid long protein (from 21 exons) was originally linked to Pendred Syndrome [4]. Since then, the promoter controlling this particular open reading frame has been the subject of many investigations [18, 23, 30-38], as well as the present study.

It is becoming increasingly accepted that pendrin, an anion exchanger associated with the inner ear, thyroid and kidney, also plays a significant role in respiratory tissues/diseases [11, 12, 18, 21-24, 39-42]. We previously demonstrated that increases in pendrin promoter activity following IL-4 stimulation required the N\textsubscript{4} GAS motif 2 and involved binding of STAT6 [23]. Subsequent bioinformatical analyses of the human pendrin promoter revealed another N\textsubscript{4} GAS motif (referred to as N\textsubscript{4} GAS motif 1 in the present study), and we therefore set out to define the role of this motif in T\textsubscript{H}2-type cytokine –induced increases in pendrin promoter activity.

While EMSAs demonstrated STAT6 binding to oligonucleotides bearing the wild-type sequence of either N\textsubscript{4} GAS motif \textit{in vitro}, promoter activity assays clearly showed that only N\textsubscript{4} GAS motif 2 is necessary for IL-4 stimulated increases in promoter activity. Data from ChIP experiments showed that STAT6 associates only with N\textsubscript{4} GAS motif 2 \textit{in vivo}, and only in cells stimulated with IL-4. These data are consistent with previous data from our group showing that active STAT6 (phosphorylated at Tyr residue 641) localizes to the nucleus only after IL-4 treatment [23]. While STAT6 is able to bind N\textsubscript{4} GAS motif 1 \textit{in vitro}, the ChIP data suggest a different scenario \textit{in vivo}.

The promoters of other genes, including the thymus- and activation-regulated chemokine (TARC/CCL17) and adhesion receptor P-selectin promoters both harbor two N\textsubscript{4} GAS motifs, and the suppressors of cytokine signaling-1 (SOCS-1) promoter contains at least three N\textsubscript{4} GAS motifs [27, 28, 43]. For all of these promoters, the multiple N\textsubscript{4} GAS motifs cooperate to induce maximal transcriptional activation following IL-4 treatment. The two N\textsubscript{4} GAS motifs in the P-selectin promoter are separated by 87 nucleotides and are located only \textasciitilde140 nucleotides upstream of the coding ATG; in the TARC/CCL17 promoter, the two
N₄ GAS motifs are only 46 base pairs apart and ~190 base pairs upstream of the translational start site; and for SOCS-1, the three N₄ GAS motifs are separated from each other by 35 and 20 nucleotides, and are located ~600 base pairs upstream of the first open reading frame. In the human pendrin promoter, the two N₄ GAS motifs are separated by more than 1600 nucleotides, and located more than 1800 (N₄ GAS motif 2) and 3400 (N₄ GAS motif 1) base pairs upstream of the open reading frame (refer to Fig. 1). Therefore, the proximity of the N₄ GAS motifs to the coding ATG, as well as to each other, may impact their relevance for functional changes in promoter activity.

It is also possible that other nuclear proteins (transcriptional factors) may regulate the in vivo binding of STAT6 to either of the N₄ GAS motifs in the human pendrin promoter. It is well known that efficient induction and regulation of gene expression involving N₄ GAS motifs/STAT6 is highly dependent on the total transcriptional environment (both enhancer and repressor transcription factors), the activity of which can be altered by exogenous stimuli and cell type specific expression [44]. As such, potential transcription factor binding sites neighboring either of the N₄ GAS motifs in the pendrin promoter may explain the functional difference (in terms of promoter activity) we observed. The sequences that flank both N₄ GAS motifs in the human pendrin promoter do differ from one another, and the presence of potential transcription factor binding sites flanking these motifs and their contribution to IL-4 stimulated increases in pendrin promoter activity and gene expression could be of significant clinical value.

Although STAT6 has been described as having the highest affinity for the N₄ GAS motif, other STAT isoforms (STAT1, for example) have been shown to interact with these motifs [25, 26]. Therefore, N₄ GAS motif 1 could represent a site for binding of STAT isoforms other than STAT6. We have not detected activation of any other STAT isoform following IL-4 stimulation in HEK-Blue™ cells (data not shown), but stimuli that activate other STAT family members may interact with N₄ GAS motif 1 to regulate pendrin promoter activity. Further experimentation is required to answer these conjectures.

Conflicts of Interests

There are no conflicts of interest or disclosures.

Acknowledgements

C. Nofziger is supported by the Roche Postdoc Fellowship Program (#231). We sincerely thank Katrin Wagner for performing the transcription factor analysis, and Elisabeth Mooslechner for her expert secretarial assistance. This work was further supported by the FWF (P18608) and the FP-7 (PIRSES-GA-2008-230661) grants to M. Paulmichl.

References

1. Dossena S, Rodighiero S, Vezzoli V, Nofziger C, Salvioni E, Boccazzi M, Grabmayer E, Botta G, Meyer G, Fugazzola L, Beck-Pecco P, Paulmichl M: Functional characterization of wild-type and mutated pendrin (SLC26A4), the anion transporter involved in Pendred syndrome. J Mol Endocrinol 2009;43:93-103.

2. Dossena S, Vezzoli V, Cerutti N, Bazzini C, Tosco M, Sironi C, Rodighiero S, Meyer G, Fascio U, J PUR, Ritter M, Fugazzola L, Persiani L, Zorowka P, Storelli C, Pecco PB, Botta G, Paulmichl M: Functional characterization of wild-type and a mutated form of SLC26A4 identified in a patient with Pendred syndrome. Cell Physiol Biochem 2006;17:245-256.

3. Everett LA, Belyantseva IA, Noben-Trauth K, Cantos R, Chen A, Thakkar SI, Hoogstraten-Miller SL, Kachar B, Wu DK, Green ED: Targeted disruption of mouse Pds provides insight about the inner-ear defects encountered in Pendred syndrome. Hum Mol Genet 2001;10:153-161.
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4 Everett LA, Glaser B, Beck JC, Idol JR, Buchs A, Heyman M, Adawi F, Hazani i, Nassir E, Baexevanis AD, Sheffield VC, Green ED: Pendred syndrome is caused by mutations in a putative sulphate transporter gene (PDS). Nat Genet 1997;17:411-422.

5 Kopp P, Pesce L, Solis SJ: Pendred syndrome and iodide transport in the thyroid. Trends Endocrinol Metab 2008;19:260-268.

6 Pendred V: Deaf mutism and goiter. Lancet 1896;2:532.

7 Pera A, Dossena S, Rodighiero S, Gandia M, Botta G, Meyer G, Moreno F, Noziger C, Hernandez-Chico C, Paulmichl M: Functional assessment of allelic variants in the SLC26A4 gene involved in Pendred syndrome and nonsyndromic EVA. Proc Natl Acad Sci U S A 2008;105:18608-18613.

8 Alesutan I, Daryadel A, Mohebbi N, Pelzl L, Leibrock C, Voelld J, Bourgeois S, Dossena S, Noziger C, Paulmichl M, Wagner CA, Lang F: Impact of bicaaranite, ammonium chloride, and acetazolamide on hepatic and renal SLC26A4 expression. Cell Physiol Biochem 2011;28:553-558.

9 Bidart JM, Lacroix L, Evain-Brion D, Caillou B, Lazer V, Frydman R, Bellet D, Filetti S, Schlumberger M: Expression of Na+/I- symporter and Pendred syndrome genes in trophoblast cells. J Clin Endocrinol Metab 2000;85:4367-4372.

10 Bronckers AL, Guo J, Zandieh-Doulabi B, Iervroets TJ, Lyaruu DM, Li X, Wangemann P, DenBesten P: Developmental expression of solute carrier family 26a member 4 (Slc26a4/pendrin) during amelogenesis in developing rodent teeth. J Oral Sci 2011;119:185-192.

11 Di Valentin E, Crahay C, Garbach N, Hennuy B, Gueders M, Noel A, Foidart JM, Grooten J, Colige A, Piette J, Cataldo D: New asthma biomarkers: Lessons from murine models of acute and chronic asthma. Am J Physiol Lung Cell Mol Physiol 2009;296:L185-197.

12 Kuperman DA, Lewis CC, Woodruff PG, Rodriguez MW, Yang YH, Dolganov GM, Fahy JV, Erle DJ: Dissecting asthma using focused transgenic modeling and functional genomics. J Allergy Clin Immunol 2005;116:305-311.

13 Lacroix L, Mian C, Caillou B, Talbot M, Filetti S, Schlumberger M, Bidart JM: Na+/I- symporter and Pendred syndrome gene and protein expressions in human extra-thyroidal tissues. Eur J Endocrinol 2001;144:297-302.
24 Pedemonte N, Caci E, Sordo E, Caputo A, Rodon K, Pfeffer U, Di Candia M, Bandettini R, Ravazzolo R, Zegarra-Moran O, Galietta LJ: Thiocyanate transport in resting and IL-4-stimulated human bronchial epithelial cells: Role of pendrin and anion channels. J Immunol 2007;178:5144-5153.

25 Ehret GB, Reichenbach P, Schindler U, Horvath CM, Fritz S, Nabholz M, Bucher P: DNA binding specificity of different STAT proteins. Comparison of in vitro specificity with natural target sites. J Biol Chem 2001;276:6675-6688.

26 Schindler U, Wu P, Rothe M, Brasseur M, McKnight SL: Components of a STAT recognition code: Evidence for two layers of molecular selectivity. Immunity 1995;2:689-697.

27 Hebenstreit D, Luft F, Schmiedelechner A, Regl G, Frischau AM, Aberger E, Duschl A, Horejs-Hoeck J: IL-4 and IL-13 induce SOCS-1 gene expression in A549 cells by three functional STAT6-binding motifs located upstream of the transcription initiation site. J Immunol 2003;171:5901-5907.

28 Wirsberger G, Hebenstreit D, Posselt G, Horejs-Hoeck J, Duschl A: IL-4 induces expression of TARC/CCL17 via two STAT6 binding sites. Eur J Immunol 2006;36:1882-1891.

29 Schuch R, Agelopoulos K, Neumann A, Brandt B, Burger H, Korschning E: Site-specific chromatin immunoprecipitation: A selective method to individually analyze neighboring transcription factor binding sites in vivo. BMC Res Notes 2012;5:109.

30 Adler L, Efrati E, Zelikovic I: Molecular mechanisms of epithelial cell-specific expression and regulation of the human anion exchanger (pendrin) gene. Am J Physiol Cell Physiol 2008;294:C1261-1276.

31 Blomqvist SR, Vidarsson H, Fitzgerald S, Johansson BR, Ollerstam A, Brown R, Persson AE, Bergstrom G, Enerback S: Distal renal tubular acidosis in mice that lack the forkhead transcription factor Fox1. J Clin Invest 2004;113:1560-1570.

32 Blomqvist SR, Vidarsson H, Soder O, Enerback S: Epididymal expression of the forkhead transcription factor Fox11 is required for male fertility. EMBO J 2006;25:4131-4141.

33 Dentice M, Luongo C, Elefante A, Ambrosio R, Saltano S, Zannini M, Nitsch R, Di Lauro R, Rossi G, Fenzi G, Salvatore D: Pendrin is a novel in vivo downstream target gene of the TTF-1/Nkx2.1 homeodomain transcription factor in differentiated thyroid cells. Mol Cell Biol 2005;25:10171-10182.

34 Jonard L, Niasme-Grare M, Bonnet C, Feldmann D, Rouillon I, Louond N, Calais C, Catros H, David A, Dollfus H, Drouin-Garraud V, Duriez P, Eliot MM, Fellmann F, Francannet C, Gilbert-Dussardier B, Gehl CR, Goizet C, Horejs-Hoeck J, Mom T, Thuillier-Obstoy MF, Couderc R, Garabedian EN, Denoyelle F, Martin S: Screening of SLC26A4, FOXI1 and KCNJ10 genes in unilateral hearing impairment with ipsilateral enlarged vestibular aqueduct. Int J Pediatr Otorhinolaryngol 2010;74:1049-1053.

35 Xing M, Tokumaru Y, Wu G, Westra WB, Ladenson PW, Sidransky D: Hypermethylation of the Pendred syndrome gene SLC26A4 is an early event in thyroid tumorigenesis. Cancer Res 2003;63:2312-2315.

36 Yang T, Vidarsson H, Rodrigo-Blomqvist S, Rozenfeld J, Efrati E, Aberger E, Smith RJ: Transcriptional control of SLC26A4 is involved in Pendred syndrome and nonsyndromic enlargement of vestibular aqueduct (DFNB4). Am J Hum Genet 2007;80:1055-1063.

37 Rozenfeld J, Efrati E, Adler L, Tal O, Carrithers SL, Alper SL, Zelikovic I: Transcriptional regulation of the pendrin gene. Cell Physiol Biochem 2011;28:385-396.

38 Rozenfeld J, Tal O, Kladnitsky O, Adler L, Efrati E, Carrithers SL, Alper SL, Zelikovic I: The pendrin anion exchanger gene is transcriptionally regulated by uroguanylin: A novel enteroendothelial function. Am J Physiol Renal Physiol 2012;302:F614-624.

39 Ishida A, Ohta N, Suzuki Y, Kakehata S, Okubo K, Iida H, Shiraishi H, Izuhara K: Expression of pendrin and periostin in allergic rhinitis and chronic rhinosinusitis. Allergol Int 2012;61:589-595.

40 Yick CY, Zwinderman AH, Kunst PW, Groenewegen S, Vanoni et al.: The Human Pendrin Promoter Contains two N4 GAS Motifs

41 Zegarra-Moran O, Galietta LJ: Thiocyanate transport in resting and IL-4-stimulated human bronchial epithelial cells: Role of pendrin and anion channels. J Immunol 2007;178:5144-5153.

42 Ehret GB, Reichenbach P, Schindler U, Horvath CM, Fritz S, Nabholz M, Bucher P: DNA binding specificity of different STAT proteins. Comparison of in vitro specificity with natural target sites. J Biol Chem 2001;276:6675-6688.

43 Schindler U, Wu P, Rothe M, Brasseur M, McKnight SL: Components of a STAT recognition code: Evidence for two layers of molecular selectivity. Immunity 1995;2:689-697.

44 Goenka S, Kaplan MH: Transcriptional regulation by STAT6. Immunol Res 2011;50:87-96.