Molecular mechanisms of epithelial cell-specific expression and regulation of the human anion exchanger (pendrin) gene

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1Laboratory of Developmental Nephrology, Department of Physiology and Biophysics, Faculty of Medicine and 2The Rappaport Family Institute for Research in the Medical Sciences, Technion-Israel Institute of Technology; and 3Pediatric Nephrology Unit, Rambam Medical Center, Haifa, Israel

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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 294: C1261–C1276, 2008. First published March 5, 2008; doi:10.1152/ajpcell.00486.2007.—Pendrin, a Cl−/anion exchanger encoded by the gene PDS, is highly expressed in the kidney, thyroid, and inner ear epithelia and is essential for bicarbonate secretion, iodide accumulation, and endolymph ion balance, respectively. This study aimed to define promoter regulatory elements essential for renal, thyroid, and inner ear epithelial cell-specific expression of human PDS (hPDS) and to explore the effect of ambient pH and aldosterone on hPDS promoter activity. Endogenous pendrin mRNA and protein were detected in renal HEK293, thyroid LA2, and inner ear VOT36 epithelial cell lines, but not in the fibroblast cell line, NIH3T3. A 4.2-kb hPDS 5′-flanking DNA sequence and consecutive 5′-deletion products were cloned into luciferase reporter vectors and transiently transfected into the above cell lines. Distinct differences in expression/activity of deduced positive/negative regulatory elements within the hPDS promoter between HEK293, LA2, and VOT36 cells were demonstrated, with only basal activity in NIH3T3 cells. Acidic pH (7.0–7.1) decreased and alkaline pH (7.6–7.7) increased hPDS promoter activity in transfected HEK293 and VOT36, but not LA2 cells. Aldosterone (10−8 M) reduced hPDS promoter activity in HEK293 but had no effect in LA2 and VOT36 cells. These pH and aldosterone-induced effects on the hPDS promoter occurred within 96-bp and 89-bp regions, respectively, which likely contain distinct response elements to these modulators. Acidic pH and aldosterone decreased, and alkaline pH increased, endogenous pendrin mRNA level in HEK293 cells. In conclusion, pendrin-mediated HCO3− secretion in the renal tubule and anion transport in the endolymph may be regulated transcriptionally by systemic pH and aldosterone.

acid-base transport; aldosterone; kidney; inner ear

The gene PDS, also known as SLC26A4 (National Center for Biotechnology Information accession no. NM_000441.1), encodes the chloride/anion exchanger pendrin (11). PDS is most highly expressed in the kidney, thyroid, and inner ear. Mutations in human PDS (hPDS) result in Pendred syndrome, an autosomal recessive disorder, which is a major cause of congenital deafness and thyroid goiter (4, 33). PDS consists of 21 exons and is located on chromosome 7q31.1 (11). Pendrin, which is a member of the SLC26A anion exchanger superfamily (27), is a 780 amino acid membrane protein with 12 putative trans-membrane domains (11, 22). Pendrin is capable of exchanging chloride with bicarbonate (44), formate (40), nitrate (10), iodide (41), and thiocyanate (30).

In the kidney, pendrin is located in the apical membrane of β-intercalated cells of the cortical collecting duct (CCD). Pendrin has been shown to play a role in acid-base balance by secreting HCO3− into the tubular lumen in exchange for Cl− (14, 36) as well as in blood pressure regulation by reabsorbing Cl− in exchange for secreted HCO3− (32, 50). In the thyroid, pendrin resides on the apical membrane of thyrocytes and, by its activity as a Cl−/I− exchanger, is essential for I− efflux from the cell and accumulation in the follicular lumen (35, 41, 58). In the inner ear, pendrin, possibly by secreting HCO3− into the endolymphatic sac, is thought to play a major role in maintaining endolymph ion balance and pH, and to be essential for normal development of the inner ear (6, 12).

The kidney represents an excellent experimental system for investigating mechanisms of epithelial cell-specific gene expression. The specialized absorptive and/or secretory function of each tubule segment depends on its structural arrangement and on the specific pattern of gene expression in each tubular cell type. The promoters of several transporter and channel genes including those encoding aquaporin 2 (29), Na+/phosphate cotransporter (42), Na+/K+/Cl− cotransporter (20), chloride channels (47), and magnesium channels (8) as well as the promoters of nephrin (56) and cadherin (19) genes have been cloned and shown to direct kidney-specific expression in vitro and/or in transgenic mice. Several transcription factors and elements including myc-associated zinc finger protein and Krüppel-like factor (48), hepatocyte nuclear factor 3 (46), and hepatocyte nuclear factor 1α and 1β (1) were found to be involved in kidney-specific expression of the CIC-K1 chloride channel, thiazide-sensitive NaCl cotransporter, and cadherin genes, respectively. The cis-acting peroxisome proliferator response element was found to play a role in renal epithelial cell-specific expression of the Mg2+ channel, paracellin-1, gene (8). However, very little is known about the regulatory elements or nephron-specific transcription factors responsible for cell-specific expression of transporter and channel genes within the kidney.

Three thyroid-specific transcription factors, thyroid transcription factors 1 and 2 (TTF-1, TTF-2) and Pax-8, have been shown to confer exclusive thyroid expression of several genes, including thyroglobulin (Tg), thyroperoxidase, and TSH-receptor genes, and govern the development, differentiation, and the unique three-dimensional structure and function of the thyroid gland (5, 7, 34). Na+/I− symporter TSH-responsive factor 1 was found to be involved in the transcriptional regul-
loration of Na\(^+\)/I\(^-\) symporter expression in the thyroid (45). In the inner ear, the promoters of several channel genes including the genes encoding the Cl\(^-\) channel ClC-Kb (26) and the water channel aquaporin-2 (37), as well as the gene encoding the tight junction protein claudin-14 (2), have been shown to direct inner ear-specific expression in vitro and/or in transgenic mice. As yet, the promoter region of the human pendrin gene has not been characterized and the molecular mechanisms for its epithelial cell-specific activity in the kidney as well as in the thyroid and inner ear have not been investigated.

Several factors are known to control acid-base transport in various nephron segments including the CCD. These factors include, among others, systemic pH, body K\(^+\) and Cl\(^-\) stores, and extracellular fluid volume as well as hormones such as aldosterone, thyroid hormone, and angiotensin II (15, 17, 39). While the modulation of renal acid-base transport has been thoroughly investigated at the tubular and cellular levels, the molecular mechanisms of this modulation are poorly understood. Systemic pH and aldosterone modulate activity of several ion transport processes involved in acid-base transport in the CCD, including pendrin activity (14, 36, 50). It has been shown that acid loading diminishes and alkali loading increases pendrin protein expression and activity in the rodent kidney (14, 31, 53). Administration of the aldosterone analog, deoxycorticosterone, resulted in upregulation of PDS mRNA and protein expression in mouse kidney (50). Nevertheless, it has not been established whether the pH- and aldosterone-induced effect on pendrin activity occurs at the protein or DNA/RNA level, and the direct effect of ambient pH and aldosterone on the PDS promoter has not been explored.

Thyroid function and Na\(^+\)/I\(^-\) symporter activity are known to be regulated by several factors including TSH, I\(^-\), and Tg (7). Northern blot analysis (35) has demonstrated induction of PDS expression by low concentration of Tg in the rat thyroid cell line FRTL-5. In addition, TTF-1, whose action is a prime factor in controlling thyroid differentiation in vivo, was found to regulate the expression of pendrin in vitro (5). There is evidence that the transcription factor Foxi1 is an upstream regulator of the pendrin gene during inner ear development (18). Moreover, mutations in the Foxi1 binding sites on the hPDS promoter abolish Foxi1-mediated transcriptional activation of hPDS and result in enlarged vestibular aqueduct syndrome (57). However, the cellular and molecular mechanisms controlling pendrin activity in the thyroid and inner ear are essentially unknown.

The purpose of the present study was to define promoter regulatory elements essential for renal, thyroid, and inner ear epithelial cell-specific expression of hPDS. We also explored the effect of modulators of acid-base transport on hPDS promoter activity in kidney, thyroid and inner ear cells. We demonstrate the existence of distinct patterns of pendrin promoter activity in renal, thyroid, and inner ear cells. We show that acidic pH decreases, and alkaline pH increases, hPDS promoter activity in renal and inner ear cells and that aldosterone has an inhibitory effect on the hPDS promoter in renal cells. In addition, we show that this pH- and aldosterone-induced effect occurs via distinct response elements on the hPDS promoter located within 96-bp and 89-bp regions, respectively. Finally, we demonstrate that acidic pH and aldosterone decrease whereas alkaline pH increases endogenous PDS mRNA levels in renal cells. These findings provide the first direct evidence that systemic pH and aldosterone may regulate pendrin activity at the transcriptional level.

### MATERIAL AND METHODS

#### Cell Lines

Opossum kidney (OK) cells (provided by Dr. J. Green, Technion, Haifa, Israel), human embryonic kidney (HEK293) cells (provided by Dr. K. Skorecki, Technion, Haifa, Israel), mouse distal convoluted tubule (MDCT) cells (provided by Dr. P. Friedman, University of Pittsburgh, Pittsburgh, PA), mouse cortical collecting duct (M1) cells (provided by Dr. Brian Harvey, University College Cork, Ireland), human thyroid follicular carcinoma-derived (LA2) cells (provided by Dr. Z. Kraiem, Technion, Haifa, Israel), and mouse embryonic fibroblast (NIH3T3) cells were grown in monolayer and maintained in DMEM/F-12 supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Biological Industries, Beit Ha’Emek, Israel) at 37°C in a humidified atmosphere of 95% air-5% CO\(_2\). Mouse embryo ventral otocyst-derived (VOT36) cells (provided by Dr. M. Holley, University of Sheffield, Sheffield, UK) were grown and maintained in MEM with Glutamax (GIBCO-Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum, 50 U/ml IFN-γ (PeproTech, Rocky Hill, NJ), 100 IU/ml penicillin, and 100 μg/ml streptomycin at 33°C in a humidified atmosphere of 95% air-5% CO\(_2\).

#### RT-PCR Analysis

Total RNA isolated from the cell lines mentioned above with TRI-reagent (MRB, Cincinnati, OH) was reverse transcribed using Moloney murine leukemia virus (MLLV)-RT and random hexamers (Promega, Madison, WI). The resultant cDNA was amplified by polymerase chain reaction using FastStart Taq DNA polymerase (Roche, Mannheim, Germany) with the intron-spanning primers F1/R1 (Table 1), from exons 3 and 6, respectively, highly homologous between human and mouse PDS. The resultant cDNA was separated by 1% agarose gels. A similar procedure was carried out with actin primers F6/R3 (Table 1) as control.

Mineralocorticoid receptor (MR) expression was examined in a similar manner with primers F7/R5 (Table 1), which were intron-spanning. The resultant cDNAs were amplified using FastStart Taq DNA polymerase (Roche, Mannheim, Germany) with the intron-spanning primers F1/R1 (Table 1), from exons 3 and 6, respectively, highly homologous between human and mouse PDS. The resultant cDNA was separated by 1% agarose gels. A similar procedure was carried out with actin primers F6/R3 (Table 1) as control.

### Table 1. Primers complementary to various segments of the human PDS gene, 5’-flanking region, and other regions used in PCR

<table>
<thead>
<tr>
<th>Primer No.</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>5'-TACGAGTCAAAGGAGTGG-3’</td>
</tr>
<tr>
<td>F2</td>
<td>5'-ACAAATATTCCCCCTCAGAC-3’</td>
</tr>
<tr>
<td>F3</td>
<td>5'-CTAGCTTGTGACACAAACAA-3’</td>
</tr>
<tr>
<td>F4</td>
<td>5'-GATTGTCCAGAAGGATGAC-3’</td>
</tr>
<tr>
<td>F5</td>
<td>5'-TGAGCTGCCCAGCAGAGATG-3’</td>
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<tr>
<td>F6</td>
<td>5'-TGCCCGGTCGCCAGGAGGCTATCTA-3’</td>
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<tr>
<td>F7</td>
<td>5'-ACTGGATCTCCAGAGCCTTC-3’</td>
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<td>F8</td>
<td>5'-TTCTCTTATGAGGCTGCTG-3’</td>
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<tr>
<td>F9</td>
<td>5'-GATTCCACTAAGATTCAAAGGCA-3’</td>
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<td>F10</td>
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<td>F11</td>
<td>5'-GATTTCAGATGAGTTGAACATC-3’</td>
</tr>
<tr>
<td>F12</td>
<td>5'-CAGACAGAGCTGATTGTA-3’</td>
</tr>
<tr>
<td>F13</td>
<td>5'-GACGCCTGGGGGGCCGAAGAATATTGTCAGCAATAG-3’</td>
</tr>
<tr>
<td>R1</td>
<td>5'-CCTGAAAGATCCCTACATG-3’</td>
</tr>
<tr>
<td>R2</td>
<td>5'-CTAAGCGGAGGACTGCG-3’</td>
</tr>
<tr>
<td>R3</td>
<td>5'-CTAGAGAACTCGGTGGAGAATGAGG-3’</td>
</tr>
<tr>
<td>R4</td>
<td>5'-GCTGTACTGCTGCAGGAGG-3’</td>
</tr>
<tr>
<td>R5</td>
<td>5'-GCAATCTTCTCTCCAGGAGC-3’</td>
</tr>
<tr>
<td>R6</td>
<td>5'-GGAATTCTTATGAGGCTGCTGACTTACATTAGGGAAAGGCGC-3’</td>
</tr>
</tbody>
</table>

Base pairs in bold represent site-specific mutation sequences.

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spanning (exons 3 and 4) in both human and mouse DNA, with 95% base resemblance.

**Immunofluorescence Microscopy**

HEK293, LA2, VOT36, and NIH3T3 cells grown on Lab-Tek chamber slides (Nunc, Naperville, IL) were fixed with 4% paraformaldehyde for 30 min at room temperature. Cells were washed twice with PBS, and after permeabilization with 0.2% Triton X-100 for 30 min and blocking with 10% normal donkey serum (Santa Cruz Biotechnology, Santa Cruz, CA) for 15 min, the cells were incubated with a previously characterized (21) rabbit polyclonal anti-pendrin antibody (provided by Dr. Dominique Eladari, Université René Descartes, Paris, France) at a final dilution 1/300 for 60 min at room temperature. Cells were then washed three times with PBS, followed by incubation with FITC-conjugated donkey anti-rabbit IgG (Santa Cruz Biotechnology) at a final dilution 1/300 for 60 min at room temperature. Membranes were incubated with UltraCruz Mounting Medium containing 4,6-diamidino 2-phenylindole dihydrochloride for nuclear staining (Santa Cruz Biotechnology). Immunostained slides were visualized with a Zeiss inverted Axiovert 135 microscope set (Carl Zeiss, Germany) with the appropriate filters.

**Immunoprecipitation Followed by Western Blot Analysis of Cell Lines**

Pendrin protein was immunoprecipitated from 1 × 10^7 HEK293, LA2, VOT36, and NIH3T3 cells grown in monolayer in appropriate media using the Protein G-based Immunoprecipitation Kit (Roche) according to the manufacturer’s instructions using goat anti-human/mouse pendrin antibody (Santa Cruz Biotechnology). The immunoprecipitated protein extracts were separated on 4–20% gradient SDS-polyacrylamide gels (Pierce, Cheshire, UK) and electrotransferred (40 V for 90 min) to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). Membranes were blocked with 5% BSA in TBS, pH 7.4, for 1 h at room temperature, followed by incubation with primary antibody diluted 1:50 in TBS containing 2% BSA and 0.1% Tween 20 (Sigma-Aldrich, St. Louis, MO) overnight at 4°C. After four 10-min washes in TBS containing 0.1% Tween 20, membranes were incubated with 1:50,000 dilution of donkey anti-goat IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology) in TBS containing 2% BSA for 1 h at room temperature. Blots were washed as above and assayed with ECL using SuperSignal West Pico Chemiluminescent Substrate (Pierce) to visualize bound antibodies.

**Cloning of hPDS 5′-Flanking DNA**

Using the published sequence of hPDS cDNA (accession no. NM-000441.1), a DNA fragment of 4.2 kb in the 5′-flanking sequence region, to but not including the hPDS translation start site (ATG) (Fig. 1A), was synthesized using PCR with human genomic DNA as template and primers F2/R2 (Table 1). The 3′, transcription initiation site flanking, 968-bp end of this fragment contained the first exon and intron of the hPDS gene, and it terminated 40 bp upstream of the ATG, which begins 4 bp into exon 2 (Fig. 1B). In a similar manner, a DNA fragment of 3.2 kb in the 5′-flanking sequence region, to but not including the hPDS transcription initiation site (Fig. 1B), was synthesized using PCR with human genomic DNA as template and primers F2/R4 (Table 1). To minimize the possibility of PCR-generated mutations, the High Fidelity Long Range PCR system (Roche) was used. The fragments produced were TA cloned into pCR-XL-TOPO vector (Invitrogen) and sequence-verified. Computer analysis (13, 38) of the 5′-flanking region of hPDS from the gene’s translation start site was carried out, and putative transcription factor binding sites were identified.

**Generation of Promoter/Reporter Constructs**

Both 5′-flanking sequences described above (4185 bp and 3217 bp) were subcloned into pGL3-basic vector (Promega) upstream to a luciferase reporter gene (and named pL4 and pL3TI, respectively) (Fig. 1B). In addition, a set of six deletion fragments decreasing in size from the 5′ end were prepared by PCR with human genomic DNA as a template and were cloned upstream to the luciferase reporter gene in pGL3-basic vectors. The 786-bp DNA fragment in pL0.75 is completely within exon 1 and intron 1 of the PDS gene. B: deletion analysis of the human PDS promoter region. Left: a schematic representation of the 4.2-kb DNA fragment stretching to the transcription initiation site (ATG) codon and the 3.2-kb DNA fragment stretching to the transcription initiation (TI) site. Both are attached 5′ to the luciferase reporter gene in the pGL3-basic vector. Right: analysis of the human PDS promoter region in HEK293 cells. Cells were transfected with 0.3 μg pGL3-basic or 0.3 μg pGL3-basic containing human PDS promoter fragments beginning at the TI (pL3TI) or the ATG codon (pL4) sites of the gene. Luciferase activity was measured after 48 h. To control for transfection efficiency, cells were cotransfected with 0.3 μg pCH110, and luciferase activity was normalized to β-galactosidase activity. Activity of reporter plasmids is expressed as %pGL3-basic vector activity. Values are presented as means ± SE of 3–5 independent experiments, each performed in quadruplicate. No significant difference was observed between the relative luciferase activities of the two hPDS promoter fragments tested.

**Expression and Regulation of the Human Pendrin Gene**

Fig. 1. A: DNA constructs of the human pendrin gene (hPDS) promoter in pGL3-basic vectors. Promoter fragments of decreasing size from the 5′ end were prepared by PCR with human genomic DNA as a template and were cloned upstream to the luciferase reporter gene in pGL3-basic vectors. The 786-bp DNA fragment in pL0.75 is completely within exon 1 and intron 1 of the PDS gene. B: deletion analysis of the human PDS promoter region. Left: a schematic representation of the 4.2-kb DNA fragment stretching to the transcription initiation site (ATG) codon and the 3.2-kb DNA fragment stretching to the transcription initiation (TI) site. Both are attached 5′ to the luciferase reporter gene in the pGL3-basic vector. Right: analysis of the human PDS promoter region in HEK293 cells. Cells were transfected with 0.3 μg pGL3-basic or 0.3 μg pGL3-basic containing human PDS promoter fragments beginning at the TI (pL3TI) or the ATG codon (pL4) sites of the gene. Luciferase activity was measured after 48 h. To control for transfection efficiency, cells were cotransfected with 0.3 μg pCH110, and luciferase activity was normalized to β-galactosidase activity. Activity of reporter plasmids is expressed as %pGL3-basic vector activity. Values are presented as means ± SE of 3–5 independent experiments, each performed in quadruplicate. No significant difference was observed between the relative luciferase activities of the two hPDS promoter fragments tested.
expression and regulation of the human pendrin gene

Epithelial cell lysates as follows: 160 μg of total protein. The reaction mixture was incubated for 30 min at 37°C or until light intensity of the reaction was immediately read in a luminometer. Luciferase activity was determined by subtraction of background activity using blank reactions containing no luciferase reporter construct.

Plasmid pCH110 (Pharmacia, Uppsala, Sweden) containing a LacZ gene driven by the cytomegalovirus promoter was used to normalize -galactosidase activity, which was measured in identical conditions. The parental DNA template was digested with Dpn I overnight, and the mutated plasmid was transformed into competent Escherichia coli strain DH5α. Subsequently, small- and large-scale plasmid preparations were performed using Genopure Plasmid Maxi kit (Roche). The presence of the mutation was verified by DNA sequencing.

Transient Transfections and Reporter Gene Assays

In all transfection experiments, luciferase reporter constructs were purified using Genopure Plasmid Maxi kit. Empty pGL3-basic vector containing no insert was used as negative control. The pGL3-control plasmid containing an SV-40 enhancer/promoter was used as positive control. Promoter activity was estimated from the ratio of luciferase activity in pGL3-control plasmid to promoterless pGL3-basic vector.

Plasmid pCH110 (Pharmacia, Uppsala, Sweden) containing a LacZ gene driven by the cytomegalovirus promoter was used to normalize transcription efficiency. HEK293, MDCT, M1, LA2, VOT36, and NIH3T3 cells were plated in quadruplicate (5 × 10⁴ cells/well) in 24-well dishes in serum-containing media. Cotransfections were performed using Fugene 6 (1.2 μg/well; Roche) with 0.3 μg reporter plasmid and 0.3 μg pCH110, and cells were incubated at 37°C for 48 h. For enzymatic assays, cells were washed with PBS (150 mM NaCl, 15 mM sodium phosphate, pH 7.4) and lysed by incubation in 200 μL/well M-Per (Pierce) for 5 min at 37°C. Lysed cells were centrifuged, and supernatant was aliquoted (50 μL/well) into 96-well plates. Luciferase Assay Reagent (30 μL; Promega) was automatically added, and the light intensity of the reaction was immediately read in a luminometer (Lucy, Anthos, Austria) for a period of 10 s. Luciferase activity was normalized to β-galactosidase activity, which was measured in identical cell lysates as follows: 160 μL of ONPG substrate (Sigma-Aldrich) were added to 30 μL of cell lysate in each well of a 96-well plate. The reaction mixture was incubated for 30 min at 37°C or until yellow color developed. β-Galactosidase activity was measured by performing a spectrophotometer with a 405-nm filter. Measurements of luciferase and β-galactosidase were performed using Genopure Plasmid Maxi kit (Roche).

In some experiments, 48 h after transfection, growth media were replaced and cells were washed with PBS and exposed to fresh media containing 0.2% bovine serum albumin instead of 10% fetal calf serum to prevent artifacts (hormones, growth factors, etc.) arising from the serum. These media had acid pH (7.0–7.1) titered from 1 M HCl solution, normal pH (7.35–7.45) or alkaline pH (7.6–7.7) titered from 1 M NaHCO₃ solution. In other experiments, the fresh medium (pH 7.35–7.45) contained aldosterone (from a stock solution of 10⁻⁸ M in ethanol; Fluka, Sigma-Aldrich) at a final concentration of 10⁻⁶ M. Control experiments for aldosterone studies were carried out with 0.1% ethanol. Cells were exposed to experimental media for 24 h and analyzed as explained above.

Quantitative Real-Time PCR Analysis of Cell Lines

Confluent HEK293 cells were plated in six-well dishes (10⁴ cells/well). After 24 h in serum-containing regular media, cells were washed with PBS and placed in media with acidic pH (7.0–7.1), normal pH (7.35–7.45), or alkaline pH (7.6–7.7) containing 0.2% BSA, or in media supplemented with 10⁻⁸ M aldosterone. Following 24 h exposure to these media, cells were washed with PBS, and total RNA was prepared using Tri Reagent (Sigma-Aldrich) followed by phenol/chloroform extraction and isopropanol precipitation. Reverse transcription of 2 μg of total RNA was carried out using MMLV (Promega) with random hexamer primers (Promega). Quantitative PCR experiments were performed using TaqMan Universal PCR Master Mix (Roche Molecular Systems) with Assay on Demand primers (Applied Biosystems, Foster City, CA) for gene expression analysis of human PDS (no. Hs 00166504_m1), with human TATA box binding protein (TBP) (no. Hs 99999910_m1) serving as a housekeeping gene for normalization. All measurements were performed in triplicate in an ABI Prism 7000 Cycler (Applied Biosystems).

RESULTS

Cloning of the 5'-Flanking Region of hPDS

Using the published sequences of PDS cDNA, we localized the human, rat, and mouse pendrin genes on chromosomes 7q31 (accession no. NM-000444.1), 6 (accession no. NM_019214.1), and 12 (accession no. NT_039548.4), respectively. Multiple sequence alignments of pendrin genes, using CLUSTALW engine (http://www.ebi.ac.uk/clustalw), revealed high sequence similarity between the species tested, which was maximal (over 55% resemblance) near the ATG codon.

A DNA fragment corresponding to the hPDS 5'-flanking region, stretching over 4.2 kb from −40 relative to the PDS translation start site, was isolated (Fig. 1A). Computer analysis of this fragment (13, 38) revealed an abundance of SP1 elements throughout this GC-rich region and no TATA box motif. Putative binding sites for several hepatocyte nuclear factors and GATA factors, known to confer renal-specific expression of several genes (1, 46), as well as binding sites for the thyroid transcription factors TTF-1, TTF-2, and Pax8, all conferring thyroid-specific expression of genes (34), were found in the 5'-flanking region of hPDS.

Epithelial Cell-Specific Expression of Pendrin mRNA

RT-PCR analysis detected expression of endogenous PDS mRNA in the renal cell lines HEK293, M1, and MDCT, but not OK, as well as in the thyroid cell line LA2 and the inner ear cell line VOT36 (Fig. 2A). PDS mRNA was not detected in squamous epithelial SCC-25 or in the fibroblast cell line NIH3T3.

Epithelial Cell-Specific Expression of Pendrin Protein

Immunofluorescence with pendrin-specific primary antibody and FITC-labeled secondary antibody detected pendrin protein expression in HEK293, LA2, and VOT36 but not in NIH3T3 cells (Fig. 2B). Antibody specificity was demonstrated by the
absence of staining in all cell lines when the primary antibody was omitted (data not shown).

Immunoprecipitation with pendrin-specific antibody and protein G, followed by Western blot analysis with pendrin-specific antibody and horseradish peroxidase-labeled secondary antibody, detected pendrin protein expression in HEK293, LA2, and VOT36 but not in NIH3T3 cells (Fig. 2C).

On the basis of these findings, the PDS-expressing renal cell lines HEK293, M1, and MDCT, thyroid cell line LA2, and the
inner ear cell line VOT36 served as recipient cells in the next set of transfection experiments, and NIH3T3 cells were used as a negative control.

**Epithelial Cell-Specific Activity of the hPDS Promoter**

To verify that the 5′-flanking region of human PDS contains a functional cell-specific promoter, reporter gene assays were carried out. We first examined whether the 968-bp end of the 4.2-kb region [containing the first exon and intron of hPDS (Fig. 1B)], located 3′ to the transcription initiation site, included any elements with transcriptional activity. For this purpose, the 4.2-kb hPDS 5′-flanking region beginning at the ATG, as well as the 3.2-kb fragment beginning at the transcription initiation site, were cloned in sense orientation upstream to a luciferase reporter gene in pGL3-basic vectors. The resulting plasmids, designated pL4 (4145-bp insert) and pL3TI (3177-bp insert), were transiently transfected into the PDS-expressing cell line HEK293. As shown in Fig. 1B, both constructs induced a similar level of reporter gene activity, a finding that excluded the presence of any regulatory elements of functional significance on the 3′ 968-bp end of the 4.2 kb insert. Thus further analysis of the hPDS 5′-flanking region was carried out using the translation initiation site as the 3′ end point of the hPDS 5′-flanking region, rather than the transcription initiation site.

In the next set of experiments, the plasmid containing the 4.2-kb hPDS 5′-flanking region, pL4, as well as three 5′-deletion products similarly cloned in sense orientation upstream to a luciferase reporter gene in pGL3-basic vectors, designated pL2.6 (2655-bp insert), pL2 (2020-bp insert), and pL1.4 (1393-bp insert), were transiently transfected into the PDS-expressing cell lines HEK293, M1, and MDCT (renal), LA2 (thyroid), and VOT36 (inner ear). Reporter gene activities were compared with those in transfected fibroblast cell line NIH3T3, which does not express PDS mRNA.

As shown in Fig. 3, HEK293, LA2, and VOT36 cells, which demonstrated the highest levels of endogenous PDS mRNA (Fig. 2A), displayed markedly increased levels of luciferase activity when transfected with pL4, pL2.6, pL2, and pL1.4, compared with activity in the control fibroblast cell line, NIH3T3, which showed no endogenous PDS mRNA expression (Fig. 2A) and negligible induction of luciferase activity (Fig. 3). A mild increase in luciferase activity was observed in M1 and MDCT cell lines (Fig. 3), which also showed lower levels of endogenous PDS mRNA (Fig. 2A). Nevertheless, a different pattern of reporter gene activity was evident between cells displaying maximal activity, namely, a difference between HEK293 cells, on one hand, and LA2 and VOT36 cells, on the other hand. While all of these cell lines transfected with pL4 (4 kb), pL2.6 (2.6 kb), or pL2 (2 kb) displayed a significant and gradual increase in luciferase activity when the insert size was decreased, a further truncation from 2.6 kb to 1.4 kb caused a significant reduction in luciferase activity in LA2 and VOT36 cells but not in HEK293 cells (Fig. 3).

Taken together, these results indicated that the 4-kb hPDS promoter fragment cloned in pL4 contained a promoter that was active in renal, thyroid, and inner ear cells. The lack of stimulation in NIH3T3 cells suggested that the activity of this PDS promoter fragment was kidney-, thyroid-, and inner ear epithelial cell-specific.

The much higher level of induction in HEK293 cells, when compared with induction in M1 and MDCT cells, prompted us to select HEK293 cells as experimental renal cells along with LA2 (thyroid) and VOT36 (inner ear) cells in subsequent experiments.

**Deletion Analysis of the hPDS Promoter**

To further explore the 5′-flanking region of hPDS and to investigate the different pattern of reporter gene activities in renal, thyroid, and inner ear cells, a second set of deletion

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**Fig. 3.** Deletion analysis of the human PDS promoter region in epithelial cell lines. Cells were transfected with 0.3 μg pGL3-basic or 0.3 μg pGL3-basic containing human PDS promoter fragments of decreasing size from the 5′ end (see Fig. 1). Luciferase activity was measured after 48 h. To control for transfection efficiency, cells were cotransfected with 0.3 μg pCH110, and luciferase activity was normalized to β-galactosidase activity. Activity of reporter plasmids is expressed as %pGL3-basic vector activity. Values are presented as means ± SE of 3–5 independent experiments, each performed in quadruplicate. Markedly increased levels of luciferase activity were evident in renal HEK293, thyroid LA2, and inner ear VOT36 epithelial cells compared with the activity in the control fibroblast cell line, NIH3T3. A mild increase in luciferase activity was observed in renal epithelial MDCT and M1 cells. A different pattern of reporter gene activity was evident between cells displaying maximal activity (see text for details).
constructs was tested (Fig. 1A), two within pL1.4 (designated pL0.75 and pL1.0) and one between pL1.4 and pL2 (designated pL1.7). All seven constructs were transiently transfected into HEK293, LA2, and VOT36 cells. As evident from Fig. 4, the highest level of luciferase activity resulted from transfection of a 2-kb insert-containing plasmid (pL2) in all of the cell lines tested. Nevertheless, decreasing the size of this fragment by 300 bp (pL1.7) caused a 75% and 65% reduction in luciferase activity in LA2 and VOT36 cells, respectively, which remained unchanged or mildly decreased when the fragment was further shortened to 1.4 kb (pL1.4) and 1.0 kb (pL1). On the other hand, decreasing the 2.0-kb fragment size to 1.7 kb and 1.4 kb did not considerably change luciferase activity in HEK293 cells. A marked decrease (60%) in luciferase activity, however, occurred in this cell line when the fragment size was shortened to 1 kb (pL1). In all three cell lines, another marked decrease (50–80%) was noted when the fragment was further shortened to 0.75 kb (pL0.75).

In all three cell lines, lengthening the 2.0-kb fragment by 600 bp (pL2.6) resulted in a mild (20–25%) or no reduction of luciferase activity. However, further lengthening of the fragment by 2000 bp (pL4) markedly diminished reporter gene activity in HEK293, LA2, and VOT36 cells when compared with activity generated by pl2.0. No significant differences in luciferase activity were observed between the various fragments tested were demonstrated in control NIH3T3 cells.

To further explore the 2-kb 5′-flanking region of hPDS, which displayed the highest promoter activity in all three cell lines, HEK293, LA2, and VOT36, an orientation study was performed in these cells. When the 2-kb promoter fragment in pGL3 vector was reversed, luciferase activity was 60–70% lower than activity induced by the same sequence in sense orientation (data not shown). This result demonstrated that the cloned 2-kb fragment (pL2) was orientation-dependent in all three cell lines.

Taken together, these results suggested that positive regulatory elements (PRE), most likely located between positions −1433 and −1044 in renal cells, and between −2060 and −1726 in thyroid and inner ear cells, as well as a negative regulatory element (NRE), likely positioned between −4185 and −2060 in all three cell lines, were involved in PDS promoter activity (Fig. 4).

Deletion Analysis of the PRE-Containing DNA Regions in Cell Lines

To further define the PREs located within the 389-bp (between −1433 and −1044) and the 334-bp (between −2060 and −1726) promoter sequences of functional importance in renal cells and thyroid/inner ear cells, respectively, fine deletion analysis of these segments was carried out. For this purpose, a third set of deletion constructs was tested (Fig. 1A): three between pL1 and pL1.4 (designated pL1.1, pL1.2, and pL1.3) and two between pL1.7 and pL2 (designated pL1.8 and pL1.9).

First, the five constructs including and between pL1 and pL1.4 (pL1, pL1.1, pL1.2, pL1.3, and pL1.4) were transiently transfected into renal HEK293 cells. As seen in Fig. 5A, pL1.4 showed high levels of luciferase activity. A 50% reduction in luciferase activity was evident when this fragment was shortened to 1.3 kb (pL1.3), which remained unchanged with further shortening of the fragments to 1.2 kb (pL1.2), 1.1 kb (pL1.1), and 1 kb (pL1).

Next, the four constructs including and between pL1.7 and pL2 (pL1.7, pL1.8, pL1.9, and pL2) were transiently transfected into thyroid LA2 and inner ear VOT36 cells. Fig. 5B illustrates that luciferase activity in LA2 cells was highest with the constructs containing 2-kb (pL2) and 1.9-kb (pL1.9) inserts. Truncation of the promoter to 1.8 kb (pL1.8) diminished reporter activity by 40%. Further shortening of the promoter to 1.7 kb (pL1.7) caused little change in luciferase activity. As shown in Fig. 5C, luciferase activity in VOT36 cells was maximally induced by pL2 (2 kb) and markedly diminished as the insert was truncated to 1.9 kb (pL1.9). Further decrease in size to 1.8 kb (pL1.8) and 1.7 kb (pL1.7) did not significantly change reporter gene activity.

Taken together, these findings provide evidence for the presence of putative PREs within the 91-bp region (between −1433 and −1342) in renal HEK293 cells, within the 114-bp region (between −1952 and −1838) in thyroid LA2 cells, and, finally, within the 108-bp region (between −2060 and −1952) in inner ear VOT36 cells (Fig. 5).

In silico analysis of these putative PRE-containing regions, using promoter analysis programs (13, 38), revealed a variety of potential transcription factor binding sites. A search for known kidney-specific transcription factor binding sites in the region between −1433 and −1342 yielded putative hepatocyte nuclear factor 3 and Wilm’s tumor 1 binding sites. A similar search for thyroid-specific transcription factor binding sites in the region between −1952 and −1838 revealed TTF-1 and TTF-2 binding sites. No known inner ear-specific transcription factor binding sites were found in the region between −2060 and −1952.

Mutation Analysis of the PRE-Containing DNA Region in LA2 Cells

As indicated above, the putative PRE-containing region between −1952 and −1838 identified in the deletion analysis conducted in the thyroid LA2 cells (Fig. 5) was found to harbor TTF-1 and TTF-2 binding sites. These binding sites were located between −1946 and −1938 and between −1942 and −1933, respectively.

It is noteworthy that Dentice and colleagues (5), using small interfering RNA studies, identified on the rat Pds promoter a putative TTF-1 binding site, which was involved in thyroid-specific expression of pendrin in rat FRTL-5 cell line. However, in silico sequence comparison performed by us, using the CLUSTALW engine, detected no significant homology between the rat Pds promoter area found by Dentice et al. (5) to contain a TTF-1 binding site and the corresponding location on the hPDS promoter (data not shown).

To further investigate the PRE-containing DNA region between −1952 and −1838, and to explore the possible role of the TTF binding sites identified within this region in thyroid-specific expression of hPDS, mutation analysis was performed. For this purpose, mutations encompassing a 13-bp region, which includes the TTF-1 and TTF-2 binding sites between −1946 and −1938 and between −1942 and −1933, respectively, were introduced into the 1.9-kb-containing plasmid pL1.9 (Fig. 6A). The resulting plasmid, termed pL1.9mut, as
Fig. 4. Deletion analysis of the hPDS promoter region in HEK293 (A), LA2 (B), VOT36 (C), and NIH3T3 (D) cells. Cells were transfected with 0.3 μg pGL3-basic or 0.3 μg pGL3-basic containing hPDS promoter fragments of decreasing size from the 5’ end (see Fig. 1). Luciferase activity was measured after 48 h. To control for transfection efficiency, cells were cotransfected with 0.3 μg pCH110, and luciferase activity was normalized to β-galactosidase activity. Activity of reporter plasmids is expressed as %pGL3-basic vector activity. Values are means ± SE of 3–5 independent experiments, each performed in quadruplicate. The highest level of luciferase activity resulted from transfection of the 2-kb insert-containing plasmid (pL2) in renal, thyroid, and inner ear cells. However, a different pattern of reporter gene activity was demonstrated between HEK293 cells, on one hand, and LA2 and VOT36 cells on the other hand (see text for details). Only basal luciferase activity was detected in NIH3T3 cells. PRE, putative positive regulatory element; NRE, putative negative regulatory element.
well as pL1.9 were transiently transfected into LA2 cells. As demonstrated in Fig. 6B, luciferase activity in cells transfected with pL1.9mut was diminished by 90% when compared with activity induced by pL1.9. These results suggest that the TTF-1 and/or TTF-2 binding sites between positions −1946 and −1938 and between −1942 and −1933, respectively, may play a key role in thyroid-specific activity of the hPDS promoter.

**Effect of Ambient pH on hPDS Promoter Activity**

Systemic pH is known to affect H^+ and HCO_3^- transport in the CCD (17, 39), and ambient pH has been shown to modulate pendrin mRNA and protein expression in this nephron segment (14, 31, 53). Furthermore, it has been suggested that pendrin is involved in acid-base balance in the endolymphatic sac (6, 12). However, the exact molecular mechanisms of these actions are unknown. Therefore, we examined the direct effect of ambient pH on the hPDS promoter. For this purpose, HEK293, LA2, and VOT36 cells were transiently transfected with plasmids pL4, pL2, pL1.4, and pL1 and exposed to acidic pH (7.0–7.1), normal pH (7.35–7.45), or alkaline pH (7.6–7.7). As demonstrated in Fig. 7, exposure of transfected HEK293 and VOT36 cells to acidic pH caused a 30–40% decrease in luciferase activity driven by 4-kb, 2-kb, and 1.4-kb hPDS promoter fragments when compared with conditions of normal pH, whereas only a minimal acid-induced inhibition was observed in reporter gene activity driven by the 1-kb promoter fragment. The exact opposite occurred when transfected HEK293 and VOT36 cells were exposed to alkaline pH, namely, a 30–45% increase in luciferase activity driven by fragments 4 kb, 2 kb, and 1.4 kb, along with a minute increase in activity driven by the 1-kb promoter fragment. No such pH-induced effect on PDS promoter activity was evident in similarly transfected LA2 cells.

These results suggest that ambient pH likely plays a role in modulating pendrin activity at the transcriptional level in

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**Fig. 5.** Deletion analysis of the hPDS promoter region in HEK293 (**A**), LA2 (**B**), and VOT36 (**C**) cells. Cells were transfected with 0.3 μg pGL3-basic or 0.3 μg pGL3-basic containing hPDS promoter fragments of decreasing size from the 5′ end (see Fig. 1). Luciferase activity was measured after 48 h. To control for transfection efficiency, cells were cotransfected with 0.3 μg pCH110, and luciferase activity was normalized to β-galactosidase activity. Activity of reporter plasmids is expressed as %pGL3-basic vector activity. Values are means ± SE of 3–5 independent experiments, each performed in quadruplicate. The highest level of reporter gene activity resulted from transfection of 1.4-kb-, 1.9/2-kb-, and 2-kb-insert-containing plasmids in renal, thyroid, and inner ear cells, respectively. Truncation of each of these fragments by −0.1 kb led to marked reduction in reporter gene activity, which remained unchanged with further shortening of the fragments. *P < 0.01.

**Fig. 6.** Mutation analysis of the hPDS promoter region in the thyroid cell line LA2. **A:** sequence comparison of pL1.9 and pL1.9mut using FinchTV (version 1.4.0; http://www.geospiza.com/finchtv/). Depicted in blue is the area mutated in pL1.9 to produce pL1.9mut. **B:** mutation analysis of the region containing the transcription factors 1 and 2 (TTF-1 and TTF-2) binding sites in LA2 cells. Cells were transfected with 0.3 μg pGL3-basic or 0.3 μg pGL3-basic containing hPDS promoter fragments pL1.9 and pL1.9mut. Luciferase activity was measured after 48 h. To control for transfection efficiency, cells were cotransfected with 0.3 μg pCH110, and luciferase activity was normalized to β-galactosidase activity. Activity of reporter plasmids is expressed as %pGL3-basic vector activity. Values are means ± SE of 3–4 independent experiments, each performed in quadruplicate. Luciferase activity was diminished by 90% in cells transfected with the mutated construct, pL1.9mut. *P < 0.01.
kidney and inner ear, and is without effect in the thyroid. In addition, it appears that a cis-acting element(s) affected by pH on the hPDS promoter is probably located within the 389 bp between −1433 bp and −1044 bp upstream to the hPDS translation start site and may have an identical location in the kidney and inner ear.

To further define the pH-response element located within the 389-bp promoter region between −1433 bp and −1044 bp, fine deletion analysis of this region was performed. For this purpose, the plasmids pL1, pL1.1, pL1.2, pL1.3, and pL1.4 (Fig. 8A) were transfected into HEK293 and VOT36 cells that were exposed to acidic, normal, and alkaline pH, as above. As demonstrated in Fig. 8B, a similar pattern of reporter gene activity was evident in both cell lines. While transfection of pL1.4 (1.4 kb), pL1.3 (1.3 kb), pL1.2 (1.2 kb), and pL1.1 (1.1 kb) resulted in similar magnitude of acidic pH-induced inhibition and alkaline pH-induced stimulation of luciferase activity in HEK293 and VOT36 cell lines, further truncation of the fragment to 1.0 kb (pL1) caused a marked reduction in acidic pH- and alkaline pH-induced effects in both cell lines.

These findings provide evidence for the presence of a putative acid-base response element (ABRE) within the 96 bp between −1140 bp and −1044 bp on the hPDS promoter in the kidney and the inner ear (Fig. 8A). In silico analysis of this region (13, 38) detected no transcription factor binding sites or regulatory elements known to be directly influenced by pH changes.

Effect of Aldosterone on hPDS Promoter Activity

Aldosterone is known to affect acid-base transport in the CCD (15) and to modulate pendrin protein and mRNA expression in this nephron segment (50). However, the molecular mechanisms of this aldosterone-induced effect on pendrin activity are unknown.

Hence, we examined the direct effect of aldosterone on the hPDS promoter. To this end, HEK293 as well as LA2 and VOT36 cells were transiently transfected with plasmids pL4, pL2, pL1.4, and pL1 and exposed to 10⁻⁸ M aldosterone. As shown in Fig. 7, exposing transfected HEK293 cells to aldosterone caused a marked (30–35%) decrease in pL4-, pL2-, and pL1.4- and pL1.2- plasmid luciferase activity. These decreases were significant compared with control luciferase activity in the absence of aldosterone. Furthermore, these decreases were maximal in cells transfected with 1.4-kb to 4-kb PDS promoter fragments and diminished with promoter shortening. **P < 0.001.

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pL1.4-driven luciferase activity. This decrease greatly diminished in cells transfected with pL1. Aldosterone had no effect on medium pH, which remained identical to the pH in the control medium (7.35–7.45). Aldosterone had no effect on PDS promoter activity in transfected LA2 and VOT36 (Fig. 7). Taken together, these results suggest that aldosterone may directly affect the hPDS promoter and transcriptionally modulate pendrin activity in the kidney, but not in thyroid and inner ear. The hPDS promoter likely contains an aldosterone-induced element, which appears to be located in the 389-bp region between −1342 and −1044.

To further analyze the aldosterone-induced element residing within this 389-bp promoter region, fine deletion analysis of this region was performed. To this end, plasmids pL1, pL1.1, pL1.2, pL1.3, and pL1.4 (Fig. 8C) were transfected into HEK293 cells, which were exposed to 10⁻⁸ M aldosterone (D) for 24 h. Subsequently, luciferase activity was measured. To control for transfection efficiency, cells were cotransfected with 0.3 μg pCH110, and luciferase activity was normalized to β-galactosidase activity. Data represent the %change in luciferase activity in cells exposed to experimental media (with acidic pH, alkaline pH, or aldosterone) relative to cells exposed to control medium (pH 7.35–7.45 without aldosterone). Values are means ± SE of 3–5 independent experiments, each performed in quadruplicate. Acidic pH-induced inhibition and alkaline pH-induced stimulation of luciferase activity were evident in HEK293 cells and VOT36 cells, which markedly decreased when the fragment size was shortened from 1.1 kb to 1 kb (B). Aldosterone-induced inhibition of luciferase activity was demonstrated in HEK293 cells, which markedly diminished when the fragment size was truncated from 1.3 kb to 1.2 kb (D). *P < 0.01.

These data indicate that a putative aldosterone-induced element likely resides within the 89-bp between −1342 and −1253 on the hPDS promoter in the kidney (Fig. 8C). In silico analysis (13, 38) revealed a single MR binding site within this region. To support our findings on the effect of aldosterone on the pendrin gene promoter, we tested whether the cells responsive to this effect of aldosterone harbor the MR. As shown in Fig. 9, RT-PCR analysis detected MR mRNA in aldosterone-responsive HEK293 cells but not in LA2 and VOT36 cells that showed no response to this hormone. MR is known to mediate aldosterone action by binding the hormone and translocating from the cytoplasm to the nucleus, where the receptor modulates gene expression by functioning as a transcription factor (3, 28). The association of the aldosterone-dependent effect on the pendrin gene promoter in renal HEK293 cells with the evidence for the presence of MR in these cells suggests that aldosterone influences pendrin gene activity in the kidney directly via the genomic pathway.

Fig. 8. Effect of ambient pH on the hPDS promoter in HEK293 and VOT36 (A and B) and of aldosterone on the hPDS promoter in HEK293 cells (C and D). A and C: schematic representation of the DNA constructs of hPDS promoter in pGL3-basic vectors used in these experiments. ABRE, putative acid-base response element; AIE, putative aldosterone-induced element. B and D: cells were transfected with 0.3 μg pGL3-basic or 0.3 μg pGL3-basic containing segments of decreasing size corresponding to the 5'-flanking region of hPDS (pL1.4, pL1.3, pL1.2, pL1.1, and pL1). Cells were exposed to acidic pH (7.0–7.1), normal pH (7.35–7.45), or alkaline pH (7.6–7.7) (B) or to 10⁻⁸ M aldosterone (D) for 24 h. Subsequently, luciferase activity was measured. To control for transfection efficiency, cells were cotransfected with 0.3 μg pCH110, and luciferase activity was normalized to β-galactosidase activity. Data represent the %change in luciferase activity in cells exposed to experimental media (with acidic pH, alkaline pH, or aldosterone) relative to cells exposed to control medium (pH 7.35–7.45 without aldosterone). Values are means ± SE of 3–5 independent experiments, each performed in quadruplicate. Acidic pH-induced inhibition and alkaline pH-induced stimulation of luciferase activity were evident in HEK293 cells and VOT36 cells, which markedly decreased when the fragment size was shortened from 1.1 kb to 1 kb (B). Aldosterone-induced inhibition of luciferase activity was demonstrated in HEK293 cells, which markedly diminished when the fragment size was truncated from 1.3 kb to 1.2 kb (D). *P < 0.01.
Effect of Ambient pH and Aldosterone on Endogenous PDS mRNA Level in Renal Cells

To further explore the influence of ambient pH and aldosterone on transcription of the pendrin gene, we examined the effect of these modulators on endogenous PDS mRNA levels in renal HEK293 cells. For this purpose, cells were grown to confluence and were exposed to acidic pH (7.0–7.1), normal pH (7.35–7.45), alkaline pH (7.6–7.7), or to medium containing aldosterone (10^{-8} M) for 24 h. Subsequently, cells were lysed and total RNA was extracted and analyzed by real-time PCR for PDS mRNA levels following normalization to the housekeeping gene encoding TBP (TATA-box binding protein). As shown in Fig. 10, acidic pH and aldosterone decreased endogenous PDS mRNA levels by 20–30%, whereas alkaline pH increased these levels by 20%, when compared with conditions of normal pH.

These findings further indicate that PDS gene expression is subject to transcriptional regulation by pH changes and aldosterone.

DISCUSSION

In the present study, we describe a transcriptional analysis of the promoter of the human pendrin gene. We demonstrate that interplay between PREs and NREs may determine renal-, thyroid-, and inner ear epithelial cell-specific expression of pendrin (Fig. 4) in an orientation-dependent manner. Furthermore, we show that, although pendrin is expressed in these three organs, its transcriptional regulation differs among them and involves different DNA regions within the gene’s 5’-flanking region. Moreover, we demonstrate that TTF-1 and/or TTF-2 binding sites, located between -1946 and -1938 and between -1942 and -1933, respectively, on the hPDS promoter are essential for the activity of this promoter in thyroid LA2 cells (Fig. 6).

In addition, we demonstrate that ambient pH affects hPDS promoter activity in renal and inner ear cells and that aldosterone has a kidney-specific modulatory action on this promoter (Figs. 7 and 8). We provide evidence that a 96-bp region between positions -1140 bp and -1044 bp and a 89-bp region between positions -1342 bp and -1253 bp may play key roles in the modulation of hPDS promoter activity by ambient pH and aldosterone, respectively (Fig. 8). These findings were further supported in vitro by demonstrating an acidic pH- and aldosterone-induced decrease and alkaline pH-induced rise in endogenous pendrin mRNA levels in renal cells (Fig. 10).

Epithelial Cell-Specific Expression of hPDS

Identification of regulatory elements and signals governing PDS gene expression in renal, thyroid, and inner ear epithelia may shed light on the molecular mechanisms of bicarbonate secretion, iodide accumulation, and endolymph balance, respectively, and may significantly improve our understanding of epithelial cell differentiation and function in these organs in both health and disease.

Although cis-acting regulatory elements involved in regulating gene transcription may be dispersed throughout the gene locus, often the proximal promoter region contains elements sufficient for high levels of tissue-specific gene transcription. These elements may function as binding sites for tissue-restricted proteins that arbitrate transcriptional activation in expressing cells. In an effort to identify cis-acting elements involved in regulating kidney-, thyroid-, and inner ear-specific expression of hPDS, we focused our study on the 5’-flanking region of this gene.

We isolated a 4.2-kb genomic sequence corresponding to the 5’-flanking region of hPDS and, using reporter gene assays, defined regions necessary for epithelial cell-specific gene transcription. In our study, we examined pendrin mRNA and protein expression as well as PDS promoter activity in renal, thyroid, and inner ear cell lines of epithelial origin as well as in a fibroblast cell line. Our results indicate specific PDS expression and promoter activity in cell lines originating in embryonic kidney (HEK293), distal convoluted tubule (MDCT), and collecting duct (M1), and no expression in a cell line of proximal tubular origin (OK) (Figs. 2 and 3). PDS expression
was also detected in thyroid LA2 and inner ear VOT36 epithelial cells, but not in the epithelial cell line SCC25 or the fibroblast cell line NIH3T3 (Fig. 2). Taken together, these results suggest involvement of the 4.2-kb 5’t-flanking region of the PDS gene in driving epithelial cell-specific expression in distal parts of the nephron, as well as in the thyroid and inner ear. Our in vitro findings showing PDS expression in renal cell lines of distal but not proximal tubular origin, indicating nephron segment-specific expression of PDS confined to the distal, but not proximal, tubule, are in accordance with in vivo studies (21, 54) demonstrating no expression of pendrin in the proximal tubule of the mouse kidney. These studies and our findings contradict another study (44) showing pendrin mRNA and protein expression in the proximal tubule of the rat kidney. The discrepancies between these data may be related to species differences (opossum, mouse, and rat), methodological differences, or other factors.

It should be noted that our immunofluorescence experiments detected pendrin protein in the membrane as well as in the cytoplasm of renal HEK293, thyroid LA2, and inner ear VOT36 cells (Fig. 2B). A likely explanation for the cytoplasmic pendrin staining is that this staining may represent a nonfunctional fraction of pendrin residing in the endoplasmic reticulum, a finding commonly observed in thyroid carcinoma-derived cell lines (43).

Deletion analysis of reporter gene constructs containing hPDS promoter DNA transfected into the PDS expressing cell lines HEK293, LA2, and VOT36 showed distinct induction of reporter gene activity, with an expression pattern that differed between the cell lines tested (Fig. 4). The highest levels of promoter activity were evident in all PDS-expressing cells using a 2-kb hPDS 5’t-flanking DNA fragment. However, this activity diminished in LA2 and VOT36 with increasing or decreasing DNA length, whereas in HEK293 cells a decrease in promoter activity was evident when DNA length was increased or when the DNA length was shorter than 1.4 kb. Hence, these findings suggest that an NRE between −4185 and −2060 controls PDS promoter activity in renal, thyroid, and inner ear cells. A PRE between −1433 and −1044 controls PDS promoter activity in renal cells, whereas a PRE between −2060 and −1726 controls activity of this promoter in thyroid and inner ear cells.

Further detailed analysis of the two PRE-containing regions controlling hPDS promoter activity in renal and in thyroid/inner ear cells, respectively (Fig. 5), revealed a 91-bp sequence (between −1433 and −1342), a 114-bp sequence (between −1952 and −1838), and a 108-bp region (between −2060 and −1952), which seem to be responsible for modulating kidney-, thyroid-, and inner ear-specific hPDS transcription, respectively.

Mutation analysis targeting the TTF-1 and TTF-2 binding sites, which were found by computer analysis (13, 38) to reside in the 114-bp sequence shown by us to be responsible for thyroid-specific hPDS transcription (Fig. 5), clearly demonstrated that the TTF binding sites at positions −1946 to −1938 and between −1942 to −1933, respectively, are essential for the activity of the hPDS promoter in human thyroid-derived LA2 cells (Fig. 6). Several kidney-specific transcription factor binding sites, including hepatocyte nuclear factor 3 and Wilm’s tumor 1 binding sites but no known inner ear-specific transcription factor binding sites, were identified on the 91-bp and 108-bp sequences shown by us to be responsible for kidney- and inner ear-specific hPDS transcription, respectively.

Future in-depth studies are needed to examine the role of the PRE-containing regions identified by us in the epithelial cell-specific activity of the hPDS promoter and to verify the involvement of the transcription factor binding sites outlined above and other, yet to be identified sites in controlling the activity of this promoter.

Regulation of the Pendrin Gene

Acid-base transport in various nephron segments, including the CCD, is modulated by a variety of hormonal and nonhormonal factors (15, 17, 39). Systemic pH regulates activity of several transport pathways participating in acid-base transport in the CCD, including pendrin activity (14, 24, 36). Immunolocalization studies (14, 31, 53) in acid-loaded rats and mice have demonstrated diminished levels of pendrin protein in the apical membrane of intercalated cells accompanied by a shift of the protein to the cytosol, as well as decreased expression of PDS mRNA. In HCO3−-loaded animals, pendrin protein was exclusively expressed in the apical membrane (53). In addition, acid loading resulted in a decline in the relative abundance of pendrin-positive cells, whereas alkali loading increased the number of these cells (53). Pendrin was not detected in the kidneys of PDS-knockout mice, and tubules from alkali-loaded PDS knockout mice failed to secrete HCO3− (36). These studies have suggested that pendrin plays an important role in acid-base regulation and that this anion exchanger is essential for HCO3− secretion following HCO3− loading. However, the exact molecular mechanisms underlying the alterations in pendrin expression and activity in response to pH changes and whether these alterations occur at the level of the pendrin gene have not been investigated.

Pendrin is thought to play a major role in endolymph ion balance in the inner ear (12). Immunohistochemical analysis demonstrating coexpression of pendrin, vacuolar H+−ATPase, and carbonic anhydrase II in epithelial cells of the murine endolymphatic sac (6) suggests that pendrin may exert its action on the endolymph by controlling acid-base balance in this fluid compartment, which is essential for normal hearing. However, nothing is known about the factors modulating pendrin activity in the inner ear and about their mechanisms of action.

In the present study, we have used the powerful model of cell lines transfected with DNA fragments of interest corresponding to the pendrin gene promoter to directly examine the involvement of this promoter in the modulation of pendrin activity by known modulators of pendrin activity, including pH changes and aldosterone. This model is of great advantage since it permits highly controlled investigation and detailed analysis of the control mechanisms involved in cell-specific regulation of the PDS gene. More importantly, in contrast with in vivo studies (14, 31, 50, 53) where the actions of the various modulators of pendrin activity (pH, aldosterone, volume status, etc.) on this anion exchanger are intermingled and hard to differentiate from one another, the in vitro model, used by us, of transfected cell lines enables the dissection and isolation of the specific action on the pendrin gene of each of the factors known to modulate pendrin activity independently of the other factors.
Effect of ambient pH on the hPDS promoter. In our study, we show that ambient pH affects PDS promoter activity in renal and inner ear cells (Figs. 7 and 8). Specifically, acidic pH decreases and alkaline pH increases PDS promoter activity in HEK293 and VOT36 cells. pH changes did not affect PDS promoter activity in the thyroid cell line LA2. These findings are in line with the modulatory effects of acidosis and alkalosis on pendrin protein activity observed in the rodent kidney at the tubular and cellular levels (14, 31, 50, 53) and demonstrate, for the first time, that systemic pH may directly regulate pendrin-mediated, renal tubular HCO$_3^-$ secretion at the transcriptional level. In addition, these data suggest that systemic pH may modulate endolymph pH by directly regulating transcription of the pendrin gene. The exact molecular mechanisms, whereby this pH-induced effect on the PDS promoter is achieved, remains to be explored. Li and colleagues (25) have shown that the proline-rich tyrosine kinase 2 (Pyk2), a member of the focal adhesion kinase family, is directly activated by acidic pH and that Pyk2 activation is required for acidic activation of c-Src kinase and the proximal tubular Na$^+$/H$^+$ exchanger 3 (NHE3). The authors suggest that Pyk2 may serve as a pH sensor that, via phosphorylation of c-Src, provides subsequent activation of the MAPK and JNK signaling pathways, which, in turn, increase transcription of the NHE3 gene (16, 25). There is evidence that the proximal tubular, basolateral-bound Na$^+$/HCO$_3^-$ cotransporter, NBC, may be regulated by a similar pathway (9).

It is possible that a similar pH-sensing mechanism exists in more distal nephron segments, including the CCD, as well as in other organs in which pH is tightly controlled, such as the endolymphatic sac of the inner ear (6), and that such a mechanism is responsible for the acid and alkal modulation of the pendrin gene promoter demonstrated by us.

In our study, we found that the 96-bp 5′-flanking region of PDS between −1044 and −1140 harbors a putative pH-response element that is active in transfected renal and inner ear cells (Fig. 8). Computer analysis (13, 38) of this DNA region revealed a variety of putative transcription factor binding sites whose binding proteins are known to be regulated by the MAPK signaling pathways. These include, among others, binding sites for Elk-1, c-Jun, ATF-2, CREB, NFAT-4, c-Myc and N-Myc. This finding raises the possibility that, similar to its effect on other H$^+$ and HCO$_3^-$ transporting pathways, the MAPK pathway may also be involved in pH-induced modulation of the pendrin gene promoter. Alternatively, a direct effect of ambient pH-mediated alterations in intracellular pH on this pH-response element residing on the PDS promoter cannot be excluded. Further studies are needed to elucidate the exact location and the molecular nature of this novel pH-response element.

Our findings on the effects of ambient pH on the hPDS promoter in transfected renal cells were further supported by our experiments demonstrating an acid-induced inhibition and an alkali-induced stimulation of endogenous PDS mRNA levels in renal HEK293 cells (Fig. 10). The combined use of these in vitro models in our study has been of great advantage since it has permitted careful analysis of the molecular pathways responsible for the regulation of pendrin activity by pH and has demonstrated that the pH-induced effect on this anion exchanger involves modulation at the DNA as well as RNA levels.

Effect of aldosterone on the hPDS promoter. Acid-base transport in the distal segments of the nephron, including the CCD, is known to be regulated by aldosterone (15, 17). Aldosterone stimulates proton secretion in the CCD by activating several transport processes, including epithelial Na$^+$ channels in the apical membrane of principal cells as well as H$^+$/ATPase and H$^+$/K$^+$-ATPase activity in the apical membrane and Cl$^-$/HCO$_3$ exchange activity in the basolateral membrane of α-intercalated cells (15, 17). These actions of aldosterone, which result in net excretion of acid in the distal tubule, are mediated by a variety of genomic mechanisms involving the action of aldosterone-induced regulatory proteins as well as by nongenomic pathways (23, 52, 55). Verlander and colleagues (50) demonstrated upregulation of pendrin mRNA and protein in mouse kidney in response to administration of the aldosterone analog, deoxycorticosterone. To explain this effect of aldosterone, which is not in line with the acid-secreting properties of this hormone, the authors have proposed that in the in vivo model they used, pendrin attenuated the aldosterone-induced metabolic alkalosis by augmenting HCO$_3^-$ secretion. Nevertheless, the direct effect of aldosterone on the PDS gene has not been investigated.

Using the model of transfected cell lines, we have isolated the effect of aldosterone on the pendrin gene promoter, and we demonstrate that this hormone directly inhibits PDS promoter activity in renal cells (Figs. 7 and 8). Aldosterone had no effect on reporter gene activity in transfected thyroid and inner ear cells. This finding demonstrates, for the first time, that aldosterone may regulate pendrin-mediated HCO$_3^-$ secretion at the transcriptional level. Moreover, our finding of isolated, direct aldosterone-induced inhibition of the pendrin gene promoter [as opposed to the complex aldosterone/alkalosis-induced stimulation of pendrin activity demonstrated in vivo (50)] is in concert with the H$^+$-secreting and HCO$_3^-$-retaining actions of this hormone (15, 17). The PDS promoter-inhibiting action of aldosterone was not secondary to pH changes in the medium (see RESULTS), and it appeared to involve the 89-bp region between −1342 and −1253 of the PDS 5′-flanking region (Fig. 8).

In line with this kidney-specific inhibitory effect of aldosterone on hPDS promoter activity was our finding demonstrating an aldosterone-induced decrease in endogenous pendrin mRNA levels in renal HEK293 cells (Fig. 10), as well as our finding of MR mRNA expression in HEK293 cells but not in thyroid LA2 and inner ear VOT36 cells (Fig. 9).

Taken together, these findings support the notion that the action of aldosterone on the pendrin gene in the kidney may represent a direct genomic effect rather than a nongenomic one. Computer analysis (13, 38) of the functionally significant, aldosterone-sensitive 89-bp region on the hPDS promoter disclosed a single MR binding site within this region. The potential role of this binding site in the aldosterone-dependent effect or the possibility that the aldosterone-induced element demonstrated in our study is a novel transcription factor binding site remains to be explored.

It has been proposed that in addition to the role of pendrin in acid-base balance, mediated by HCO$_3^-$ secretion into the tubular lumen (36, 53), this anion exchanger may also play an important role in blood pressure regulation by reabsorbing Cl$^-$ in exchange for secreted HCO$_3^-$ (32, 49, 50). Administration of the aldosterone analog, deoxycorticosterone, resulted in hyper-
tension in wild-type PDS(+/+) mice but not in knockout PDS(−/−) mice, suggesting that pendrin may play a role in the pathogenesis of mineralocorticoid-induced hypertension (50). Moreover, it has been known that Cl− depletion increases and Cl− excess decreases pendrin protein abundance in the rodent kidney (32, 49, 51). Hence, our findings on the direct effect of aldosterone on pendrin gene transcription and a possible, to be explored, role of Cl− on transcription of this gene may be of major biological importance and may improve our understanding of blood pressure control at the gene level.

The modulators of acid-base balance used in the present study, namely, ambient pH and aldosterone, had no effect on the hPDS promoter in the thyroid cell line LA2. This finding is not surprising considering the facts that acid-base conditions are not known to exert an effect on thyroid hormone metabolism and that the thyroid gland does not contain aldosterone-responsive epithelial cells. Pendrin functions in the thyroid gland as an exchanger of Cl− and I−. Hence, future studies examining the modulatory effect of these two anions on pendrin activity and exploring whether such an effect occurs at the transcriptional level will be of great importance.

Conclusions

In conclusion, interplay between positive regulatory elements and more distally located negative regulatory elements on the 5′-flanking region of the pendrin gene may determine epithelial cell-specific expression of this gene in the kidney, thyroid, and inner ear. The TTF-1 and TTF-2 binding sites successively located between positions −1946 and −1938 and between −1942 and −1933, respectively, may play a key role in the activity of the hPDS promoter in the thyroid.

Acidic pH and aldosterone decrease, and alkaline pH increases, hPDS promoter activity by acting on distinct response elements on this promoter, which remain to be identified and characterized. Our findings provide the first direct evidence that pendrin-mediated Cl−/HCO3− exchange in the renal tubule, iodide accumulation in the thyroid, and endolymph ion balance in the inner ear may be differentially regulated at the transcriptional level by systemic pH and aldosterone.

Future studies using transgenic animals may verify whether the activity and regulation of the hPDS promoter fragments, examined in the cell culture model, demonstrate the expected organ and epithelial cell activity in the intact organism, where the transgene is exposed to a more physiologically relevant environment (i.e., hormones, proteins, etc.). Once established, the transgene is exposed to a more physiologically relevant organ and epithelial cell activity in the intact organism, where examined in the cell culture model, demonstrate the expected activity and regulation of the hPDS promoter fragments, examined in the cell culture model, demonstrate the expected organ and epithelial cell activity in the intact organism, where the transgene is exposed to a more physiologically relevant environment (i.e., hormones, proteins, etc.). Once established, the transgene is exposed to a more physiologically relevant organ and epithelial cell activity in the intact organism, where examined in the cell culture model, demonstrate the expected activity and regulation of the hPDS promoter fragments, examined in the cell culture model, demonstrate the expected organ and epithelial cell activity in the intact organism, where the transgene is exposed to a more physiologically relevant environment (i.e., hormones, proteins, etc.). Once established, the transgene is exposed to a more physiologically relevant organ and epithelial cell activity in the intact organism, where examined in the cell culture model, demonstrate the expected activity and regulation of the hPDS promoter fragments, examined in the cell culture model, demonstrate the expected organ and epithelial cell activity in the intact organism, where the transgene is exposed to a more physiologically relevant environment (i.e., hormones, proteins, etc.). Once established, the transgene is exposed to a more physiologically relevant organ and epithelial cell activity in the intact organism, where examined in the cell culture model, demonstrate the expected activity and regulation of the hPDS promoter fragments, examined in the cell culture model, demonstrate the expected organ and epithelial cell activity in the intact organism, where the transgene is exposed to a more physiologically relevant environment (i.e., hormones, proteins, etc.). Once established, the transgene is exposed to a more physiologically relevant organ and epithelial cell activity in the intact organism, where

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