The forkhead transcription factor Foxi1 directly activates the AE4 promoter

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Intercalated cells are highly specialized cells within the renal collecting duct epithelium and play an important role in systemic acid–base homoeostasis. Whereas type A intercalated cells secrete protons via an apically localized H^+-ATPase, type B intercalated cells secrete HCO_3^-. Type B intercalated cells specifically express the HCO_3^-/Cl^- exchanger AE4 (anion exchanger 4), encoded by *Slc4a9*. Mice with a targeted disruption of the gene for the forkhead transcription factor Foxi1 display renal tubular acidosis due to an intercalated cell-differentiation defect. Collecting duct cells in these mice are characterized by the absence of intercalated cell markers including AE4. To test whether *Slc4a9* is a direct target gene of Foxi1, an AE4 promoter construct was generated for a cell-based reporter gene assay. Co-transfection with the Foxi1 cDNA resulted in an approx. 100-fold activation of the AE4 promoter construct. By truncating the AE4 promoter at the 5′-end, we demonstrate that a fragment of approx. 462 bp upstream of the transcription start point is sufficient to mediate activation by Foxi1. Sequence analysis of this region revealed at least eight potential binding sites for Foxi1 in both sense and antisense orientation. Only one element was bound by recombinant Foxi1 protein in bandshift assays. Mutation of this site abolished both binding in bandshift assays and transcriptional activation by co-transfection of Foxi1 in the reporter gene assay. We thus identify the AE4 promoter as a direct target of Foxi1.

Key words: anion exchanger 4 (AE4), differentiation, Foxi1, intercalated cell, kidney, transcription.

INTRODUCTION

The molecular events leading to normal kidney development and function are very complex. For most of these processes, regulators have been identified, many of which are transcription factors [1]. One major step in kidney development is the differentiation of the highly specialized intercalated cells in renal-collecting ducts. Intercalated cells play an essential role in acid–base homoeostasis and regulate extracellular pH [2] by the fine adjustment of net tubular bicarbonate transport. The pH of extracellular fluid is 7.4 and varies by less than 0.05 pH unit despite a large metabolic acid challenge. Type A intercalated cells secrete protons via the apically located vacuolar-type H^+-ATPase into the lumen [3] and reabsorb bicarbonate via the basolateral HCO_3^-/Cl^- exchanger AE1 (anion exchanger 1) [4,5]. Bicarbonate-secreting type B intercalated cells reabsorb acid via a basolateral H^+-ATPase. The molecular identity of the protein that mediates apical bicarbonate transport in type B intercalated cells is still under debate. Candidates are pendrin, a multifunctional transporter mutated in Pendred syndrome [6], and the anion exchanger AE4, a chloride/bicarbonate transporter of the SLC4 (solute carrier 4) family [7–10], which is specifically expressed in type B intercalated cells. The importance of intercalated cells for acid–base homoeostasis is underlined by the fact that mutations in genes encoding proteins that are involved in proton and bicarbonate reabsorption by these cells can cause hereditary dRTA (distal renal tubular acidosis) [11–15].

Differentiation of intercalated cells takes place during the first few weeks of postnatal life, when different marker proteins involved in acid–base homoeostasis like carbonic anhydrase II, H^+-ATPase, pendrin, AE4 and AE1 begin to be expressed [16,17]. Interestingly, the collecting duct epithelium of mice with a targeted disruption of *Foxi1* lacked cells with immunoreactivity for pendrin, AE1, the vacuolar H^+-ATPase, and the anion exchanger AE4, indicating an intercalated cell-differentiation defect. As a consequence, the $Foxi1^{-/-}$ mouse model has dRTA [18]. Initially, Foxi1 was described to play an important role in early development of the inner ear in mice [19]. However, Foxi1 in the kidney was shown to be expressed in the distal tubules of the kidney as well [20]. Foxi1, also known as HFH3, Fkh10 and FREAC6, is a member of the family of forkhead transcription factors. Forkhead transcription factors were initially discovered in *Drosophila* [21]. Since then numerous genes encoding forkhead transcription factors have been described. Structurally they encode a subgroup of the helix–turn–helix class of proteins and contain a forkhead DNA-binding domain [22]. X-ray crystallography of HNF-3 (hepatocyte nuclear factor 3) bound to its target DNA showed that helix H3 of the DNA-binding domain fills out the major groove of DNA [23]. Less is known about the target genes of these transcription factors and the respective promoter binding sites. As immunoreactivity for AE4 was absent from mice deficient in Foxi1, we addressed the question as to whether Foxi1 regulates the gene encoding AE4 (*Slc4a9*). We show that Foxi1 directly binds to the *Slc4a9* promoter *in vitro* and provide evidence that a single element within the *Slc4a9* promoter is sufficient to mediate transcriptional activation by Foxi1.

EXPERIMENTAL

Plasmid construction

The full-length murine Foxi1 cDNA cloned into the pCMVsport6 vector (IMAGE3601768) was obtained from the RZPD (Berlin, Germany). The 5′-region of the AE4 gene was amplified by PCR

Abbreviations used: AE1, anion exchanger 1 (etc.); dRTA, distal renal tubular acidosis; EMSA, electrophoretic mobility-shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Gdnf, glial cell line-derived neurotrophic factor; GST, glutathione S-transferase; HEK-293T, human embryonic kidney 293T; mut-oligo, mutated oligonucleotide.

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using genomic C57/BL6 mouse DNA as a template. Three different promoter constructs were amplified comprising nucleotides −3183/−1 (promH1), −2055/−1 (promH2) and −462/−1 (promH3), the 'A' of the initiation codon ATG of murine AE4 being +1 (NCBI accession number NM_172830; nt 36 is A of the translation start ATG). Numbers of the respective constructs are given in 5′ direction of +1 on genomic level. Forward primers used were 5′-accgcggacgcgtggtcttagggagcggctctagcga-3′ for promH1, 5′-accgcggacgcgtagtgctgcagcctcccaagcagtt-3′ for promH2 and 5′-accgcggacgcgtaaccttctgtttccctttcccgcccta-3′ for promH3. The reverse primer was 5′-actcgagcctggaaagacttgcacaaatcct-3′ for all fragments. PCR products were cloned into pBluescript KS+ (Stratagene) and subcloned via HindIII/SacI into the pGL3-Basic vector, which contains the gene encoding luciferase (Promega). The pGL3-Basic vector lacks eukaryotic promoter and enhancer sequences and thus allows testing as to whether a given sequence can drive the expression of a luciferase reporter cassette. A 5′-truncated promH3-variant, promH3Δ, including nucleotides −221/−1, was amplified with the forward primer 5′-atacatggtaccgcaaggtcagacttgatgcaca-3′ and the reverse primer 5′-atacataagcttcctggaaagacttgcacaaatcc-3′ and subcloned into pGL3-Basic via KpnI and HindIII. The triple point mutation −258T → G, −257T → G and −256T → G within promH3 (promH3 mut) was cloned in two steps by PCR via primer 1 (5′-atacatggtaccaaccttctgtttccctttcc-3′) and primer 2 (5′-tatgttttgcgtccccagacaggagta-3′) as primers in one PCR and primer 3 (5′-tactcctgtctggggacgcaaaacata-3′) and primer 4 (5′-atacataagcttcctggaaagacttgcacaaatcc-3′) in a second PCR. Both amplicons served as a template for a fusion PCR using the flanking primers 1 and 4. After digestion with the restriction enzymes KpnI and HindIII, the PCR product was cloned into the pGL3-Basic vector.

Bacterial expression of mouse Foxi1

The coding region of the mouse Foxi1 cDNA was amplified by PCR using the primers mFoxi1f (5′-agaattctaatgagctccttcgacctcccagcg-3′) and mFoxi1r (5′-aaagcttagtcgacctagacttcagtgccttccct-3′) and cloned via EcoRI and HindIII into the pGEX-KG expression vector (Amersham Biosciences). The resulting plasmid pGEX-KG-mFoxi1 coding for a fusion protein of GST (glutathione S-transferase) and Foxi1 was transformed into *Escherichia coli* BL21. Cells were grown in 500 ml of Lennox L broth base containing 200 μg/ml ampicillin to a D_{600} of 0.8. Subsequently, cells were induced under constant shaking with 1 mmol of isopropyl β-D-thiogalactoside for 4 h at 37 °C. The cells were harvested and resuspended in 10 ml of ice-cold PBS, lysed by sonication and centrifuged at 20000 ***g*** for 15 min at 4 °C. The GST fusion protein was purified from the supernatant using glutathione–Sepharose 4B beads according to the manufacturer's instructions (Amersham Biosciences) and was used for EMSA (electrophoretic mobility-shift assay) (see below).

EMSAs

Single-stranded oligonucleotides were ordered custom-made (MWG, Ebersberg, Germany), annealed in 10 mM Tris/HCl (pH 7.5) and 60 mM NaCl and stored at −20 °C. The oligonucleotide design resulted in 5′-overhangs. For EMSAs, double-stranded oligonucleotides were labelled using Klenow polymerase and [α-^{32}P]dATP. Unincorporated nucleotides were removed by gel filtration with Probe Quant G-50 micro columns (Amersham Biosciences). Labelled oligonucleotides were stored at −20 °C in 10 mM Tris/HCl (pH 7.5), 1 mM EDTA and 60 mM NaCl. Binding was done in a 12 μl reaction mixture with 20 mM Hepes (pH 7.4), 80 mM NaCl, 20 mM KCl, 2 mM dithiothreitol, 1 μg of Cot-1 DNA and 6 μl of recombinant GST–Foxi1 fusion protein. A protease inhibitor cocktail (Complete; Roche) was added according to the manufacturer's specifications. Reaction mixtures were incubated for 30 min on ice, 1 μl of the labelled oligonucleotides (40000 c.p.m.) was added and the reaction mixtures were subsequently incubated for another 30 min at room temperature (21 °C). Complexes were resolved by non-denaturing 8 % (w/v) PAGE in 0.5 × Tris/borate/EDTA (45 mM Tris base, 45 mM boric acid and 1 mM EDTA) at 4 °C at 20 V/cm for 4 h. The gels were dried, analysed with the Fujix BAS 2000 bioimaging system using the Tina™ software (Raytest) and exposed to BioMax MS films (Kodak) or Hyperfilm (Amersham Biosciences). The oligonucleotides used for EMSAs are listed in Table 1.

Table 1 Oligonucleotides used for EMSAs

Abbreviations: F, forward; R, reverse.

Function	Oligonucleotide	Sequence
EMSA	−462/−435F	5′-aaccttctgtttccctttccctg-3′
	−462/−435R	5′-atggcagggaaagggaaacaga-3′
	−428/−387F	5′-agcctagttatttattagtttcttgtttgtccaa-3′
	−428/−387R	5′-cacatttggacaaacaagaaactaataaataact-3′
	−358/−332F	5′-ttgttgataaccaaacagtcct-3′
	−358/−332R	5′-atccaggactgtttggttatca-3′
	−332/−302F	5′-tcgtggtgttaatatttgacacagga-3′
	−332/−302R	5′-gttgtcctgtgtcaaatattaacacc-3′
	−270/−243F	5′-tactcctgtctgtttacgcaaaa-3′
	−270/−243R	5′-tatgttttgcgtaaacagacagg-3′
	−180/−153F	5′-agattagttttacaaacaggtc-3′
	−180/−153R	5′-ctgctgacctgtttgtaaaact-3′
	−153/−122F	5′-ggggtcagagaccaaatagaggaggag-3′
	−153/−122R	5′-acttctcctcctctatttggtctctg-3′
	−43/−1F	5′-ctaggatacacagctcccaggatttgtgcaagtctttc-3′
	−43/−1R	5′-cctggaaagacttgcacaaatcctgggagctgtgtatc-3′
Competition −270/−243	F	5′-tactcctgtctgTTTacgcaaaacata-3′
	R	5′-tatgttttgcgtAAAcagacaggagta-3′
Mut-oligo −270/−243	F	5′-tactcctgtctgGGGacgcaaaacata-3′
	R	5′-tatgttttgcgtCCCcagacaggagta-3′
Mut-oligo −270/−243 for labelling	F	5′-tactcctgtctgGGGacgcaaaa-3′
	R	5′-tatgttttgcgtCCCcagacagg-3′

Cell culture and transfection

HEK-293T (human embryonic kidney 293T) and COS-7 cells were grown on 35-mm-diameter tissue culture dishes at 37 °C with 5 % CO_2 in Dulbecco's modified Eagle's medium with 10 % (v/v) foetal calf serum containing 100 units/ml penicillin and 100 μg/ml streptomycin to approx. 80 % confluence. They were then transfected with 0.5 μg of Foxi1 cDNA and 1 μg of promoter plasmid using the FuGENE-6 reagent (Roche) according to the manufacturer's instructions. Cells were harvested 24–36 h later.

Luciferase assay

The luciferase activity of transfected cells was determined using the Luciferase reporter gene assay, high sensitivity (Roche). Transfected cells were washed twice with PBS, dissolved in 170 μl of lysis buffer supplied with the kit and transferred to 1.5 ml reaction tubes. Cell debris was pelleted by centrifugation at 17000 ***g***. Then, 50 μl aliquots of the supernatants were transferred into individual wells of a 96-well microtitre plate. Light emission was measured for 15 s in a luminometer (EG&G Berthold Microlumat LB96 P) after injection of 100 μl of luciferase reagent. Light emission was integrated over time. Each experiment was performed in triplicate and repeated at least three times.

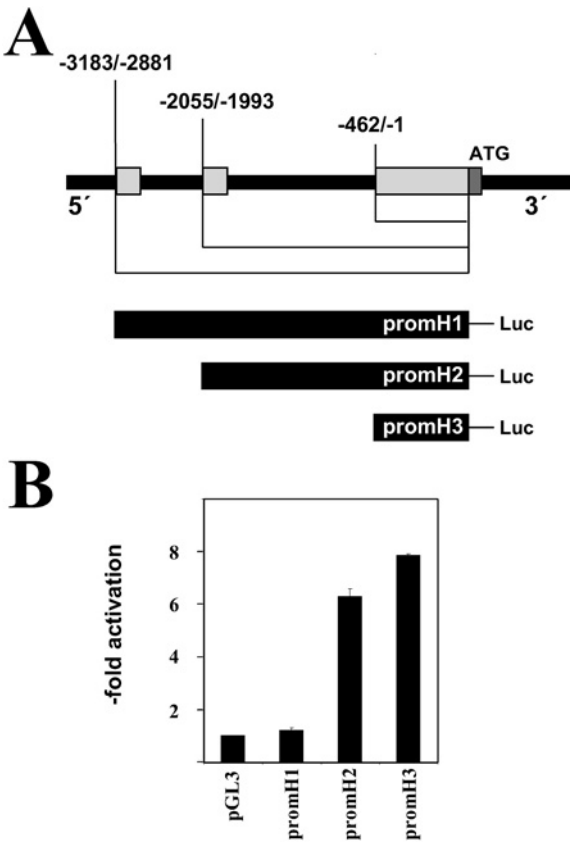

Figure 1 The AE4 promoter region

(**A**) Database search identified three regions within the 5′-region of the *Ae4* gene that are conserved between mice and human indicating that these regions may be functionally important. The genomic regions promH1, promH2 and promH3, which include one, two or three conserved regions, were cloned into a luciferase expression vector. (**B**) Transcriptional activity of the promoter constructs in HEK-293T cells was determined by a luciferase-based reporter gene assay. Activity is shown as fold activation beyond basal activity of the pGL3 vector. The experiment was performed in triplicate and was repeated twice.

Real-time PCR

Transcript levels of AE4 and Foxi1 were quantified in a quantitative PCR using the SYBR Green PCR Master Mix (Applied Biosystems) and the Rotor-Gene real-time cycler (Corbett Research, Sydney, Australia). The cycling method used was: step 1, 95 °C for 5 min; step 2, 95 °C for 10 s; step 3, 60 °C for 15 s; and step 4, 72 °C for 20 s; steps 2–4 were repeated 40 times. Each sample was amplified in duplicate and gave consistent results in two independent experiments. Amplification efficiency was normalized to that of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) transcripts. The primers used are as follows: hGAPDH-F, 5′-catcttcttttgcgtcgcca-3′; hGAPDH-R, 5′-ttaaaagcagccctggtgacc-3′; hFoxi1-F, 5′-caagaaggtgccccgcgacgaggac-3′; hFoxi1-R, 5′-ctgattttctcttccttttcctgc-3′; mFoxi1-F, 5′-ggccgacaacctttccctttt-3′; mFoxi1-R, 5′-tgcgaaagtttccgttgtca-3′; hAE4-F, 5′-gctaccatgagatgggacgggcag-3′; and hAE4-R, 5′-gtcacctcctctaggaatgcatcc-3′.

RESULTS

The 5′-region of the AE4 gene displays basal promoter activity

A database search identified three regions within the 5′-region of the AE4 gene that are conserved between mice and human (schematically presented in Figure 1A), indicating a regulatory function. The first conserved element is located approx. 3 kb upstream of the translational start site and comprises the nucleotides −3183/−2881, while the second homology region comprises nucleotides −2055/−1993. The third region was localized between positions −462/−1. To characterize these homology regions functionally they were amplified by PCR using genomic DNA from the mouse strain C57/BL6 as a template. Amplicon promH1 (−3008/−1) includes all the three conserved regions, promH2 (−2005/−1) comprises the two proximal homology regions and promH3 (−462/−1) encompasses the region directly upstream of the start (schematically represented in Figure 1A). First, the promoter constructs promH1, promH2 and promH3 were tested with regard to their transcriptional activity in a cell-based luciferase assay. Briefly, 24 h after transfection of HEK-293T cells, luciferase activity was measured and values were compared with the basal activity of the pGL3 vector, which was set to 1. PromH1 displayed only very low activity (1.2-fold induction), whereas promH2 showed an approx. 6-fold and promH3 an approx. 8-fold increased basal activity (Figure 1B). Thus the region spanning 462 bp 5′ of the translational start site seemed to be sufficient to confer basal promoter activity.

Foxi1 activates the AE4 promoter

In a second step, it was tested whether Foxi1 is a direct activator of the AE4 promoter. Again, 24 h after transfection of HEK-293T cells, luciferase activity was measured. No significant luciferase activity was observed after transfection with the pGL3-Basic vector and the pCMVsport6 vector. Co-transfection of the Foxi1 cDNA with the pGL3-Basic vector also did not result in activation. In contrast, cells transfected with the promH1 construct showed approx. 19-fold higher luciferase activity when co-transfected with 0.1 μg of Foxi1. Activation by Foxi1 was dose-dependent, being approx. 34-fold with 0.25 μg of Foxi1 cDNA and approx. 74-fold with 0.5 μg of Foxi1 cDNA (Figure 2A). Subsequently, the Foxi1-dependent activation of the truncated AE4 promoter constructs promH2 and promH3 was tested in HEK-293T cells. Both constructs were strongly activated by co-expression with Foxi1 (Figure 2B). Mean activation in this experiment was 88-fold for promH1, 142-fold for promH2 and 135-fold for promH3 relative to the respective promoter activity without Foxi1, which was set to 1. Activation of the AE4 promoter by Foxi1 was specific, as co-transfection of the Foxi1 cDNA with a myoglobin promoter construct [24] did not result in measurable activation of the myoglobin promoter (Figure 2B). These experiments were repeated with COS-7 cells (Figure 2C). Activation of the AE4 promoter construct in COS-7 cells was 78-fold for promH1, 85-fold for promH2 and 111-fold for promH3 relative to the respective promoter activity without Foxi1 which was again set to 1. Again, the empty pGL3 vector did not show significant activation upon co-transfection with Foxi1. Thus the region directly 5′ of the AE4 gene appeared to be sufficient for both basal promoter activity and Foxi1 transactivation. As analysed by quantitative PCR, HEK-293T cells did not express significant levels of endogenous AE4 and Foxi1 (results not shown). To test whether transfection of Foxi1 in HEK-293T cells is sufficient to induce AE4 expression, total RNA was extracted from Foxi1-transfected and -untransfected HEK-293T cells. Alhough expression analysis by real-time PCR did show high levels of Foxi1 transcripts, expression of AE4 after Foxi1 transfection was not detected (results not shown).

Foxi1 binds to a consensus motif within promH3

Forkhead proteins are a family of transcription factors that share identity in the winged helix DNA-binding domain, the

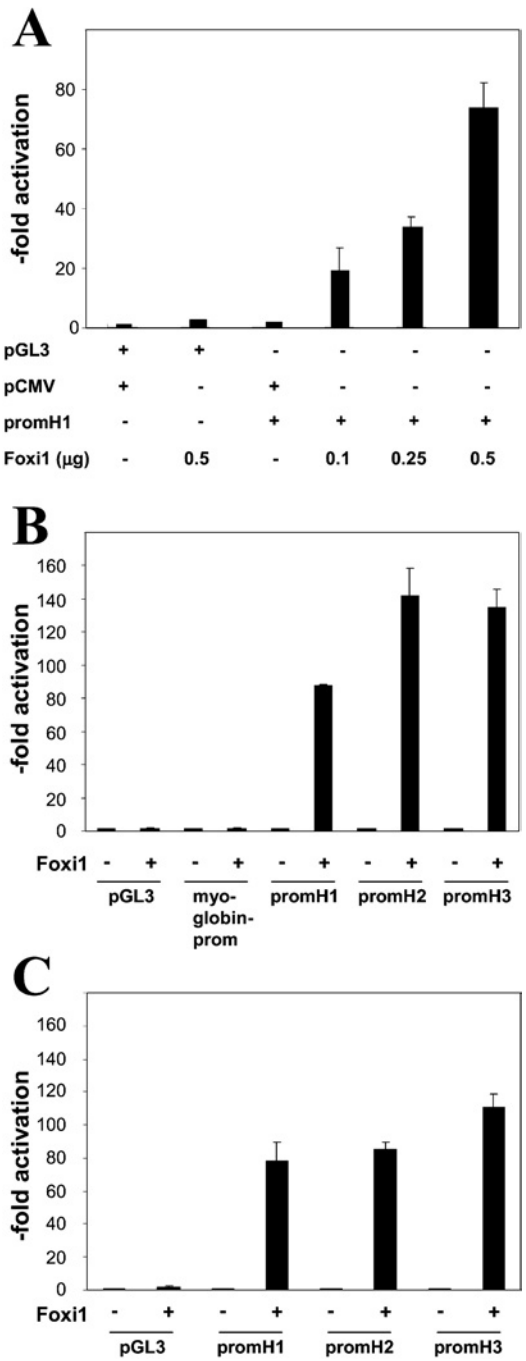

Figure 2 Transcriptional activities of the promoter constructs in HEK-293T cells determined by a luciferase-based reporter gene assay

Activities of the promoter constructs after co-transfection with Foxi1 are shown as fold activation compared with basal activity of the respective promoter construct, which was set as 1. Each experiment was performed in triplicate and was repeated at least twice. (**A**) pGL3 (1.0 μg of DNA) and pCMV (0.5 μg of DNA) vector served as a negative control. HEK-293T cells were transfected with 1.0 μg of promH1 and increasing amounts of Foxi1 cDNA as indicated. (**B**, **C**) Transfection of the AE4 promoter constructs promH1, promH2 and promH3 (1.0 μg of DNA each) in HEK-293T cells (**B**) and COS-7 cells (**C**) with pCMV (−) or with the pCMV-Foxi1 cDNA (0.5 μg) (+). All promoter constructs were strongly activated by co-transfection with Foxi1. Co-transfection of the pGL3 vector (1 μg) or the myoglobin promoter (1 μg of DNA) with Foxi1 did not result in significant luciferase activity.

Figure 3 Nucleotide sequence of the 5′-end of the AE4 gene (nt −462/−1)

Eight potential binding motifs in either sense or antisense direction were identified within the sequence as used for the promH3 construct, if stringency criteria were limited to a minimal core sequence TxTTT in the sense and antisense orientation. Motifs are highlighted by boldface letters. The oligonucleotides used for EMSA experiments are framed.

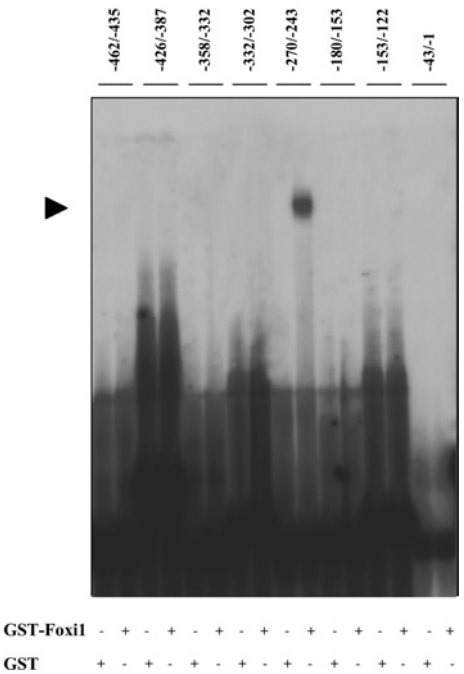

Figure 4 EMSA of recombinant Foxi1 with radioactively labelled oligonucleotides spanning putative Foxi1-binding sites

Labelled oligonucleotides were incubated with either bacterially expressed GST–Foxi1 (+) or bacterially expressed GST (−). Seven of these putative motifs did not show significant Foxi1 binding. A new band corresponding to Foxi1–DNA complexes was only formed with oligonucleotides −270/−243 (arrowhead).

so-called forkhead domain [23]. By *in vitro* binding site selection, the consensus binding site for Foxi1 was shown to consist of the core sequence T(g/a)TTT(g/a)(t/c) [20]. Eight potential binding motifs in either the sense or antisense direction were identified within the promH3 promoter, if stringency criteria were limited to a minimal core sequence TxTTT in sense and antisense orientations (Figure 3). To test whether these elements are capable of binding GST–Foxi1 fusion protein, an EMSA was performed with radioactively labelled oligonucleotides spanning these putative Foxi1-binding sites. Seven of the putative motifs did not show Foxi1 binding as no differences could be observed between the control lane with bacterially expressed GST and the respective lane after incubation with GST–Foxi1 (Figure 4). In contrast, protein–DNA complexes were formed between oligonucleotide −270/−243 of promH3 and GST–Foxi1, resulting in a shifted complex, which was not detected in the respective control lane and thus identified a binding site in proximity to the start ATG of the AE4 gene. The sequence of the binding element is

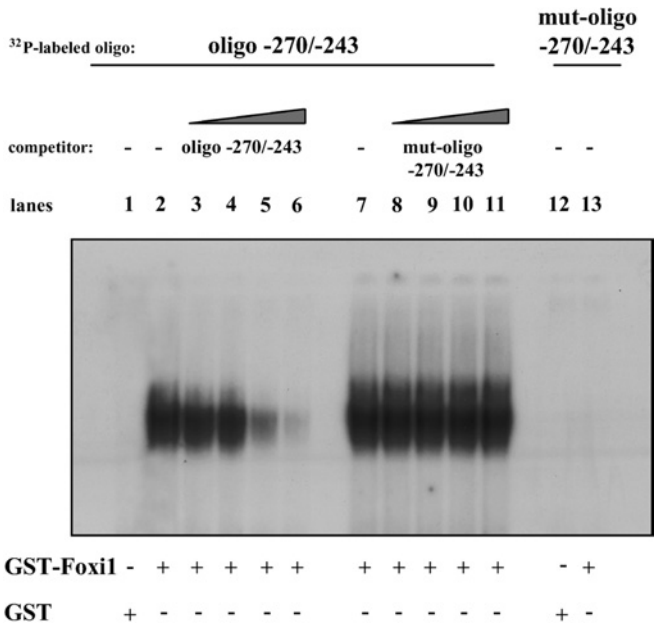

Figure 5 Specificity of Foxi1 binding to the motif −270/−243

Whereas GST–Foxi1 binding to radioactively labelled oligonucleotide −270/−243 could be competed with increasing amounts of the unlabelled oligonucleotide (lane 3, 5-fold excess of unlabelled oligonucleotides; lane 4, 10-fold; lane 5, 50-fold; and lane 6, 100-fold), competition was not achieved with a mut-oligo, where the three central thymidines in positions −258/−256 had been replaced by guanines (mut-oligo −270/−243) (lane 8, 5-fold excess of unlabelled mut-oligo; lane 9, 10-fold; lane 10, 50-fold; and lane 11, 100-fold). The ^{32}P-labelled mut-oligo −270/−243 did not bind to GST–Foxi1 (lanes 12 and 13). Lane 1, labelled oligonucleotide −270/−243 in the presence of GST without Foxi1; lanes 2 and 7, labelled oligonucleotide −270/−243 in the presence of GST–Foxi1 without competitor.

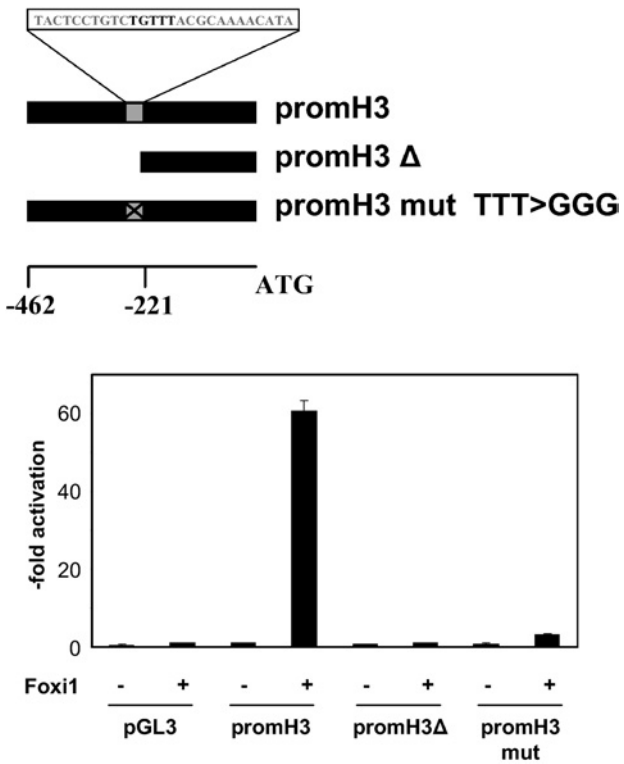

Figure 6 The motif −270/−243 is a Foxi1-response element

Reporter plasmid promH3Δ lacking the identified binding motif −270/−243 and a mutant promH3 (promH3 mut), where the core sequence TTT in positions −258/−256 had been replaced by GGG, were analysed in the cell-based luciferase reporter assay. Cells transfected with the respective plasmids did not show induced luciferase activity in case of promH3Δ, and the mutated variant had low residual Foxi1-dependent transactivation activity (3-fold). Basal activity of the respective promoter construct without Foxi1 was defined as 1.

tactcctgtcTgTTTacgcaaaacata. The specificity of Foxi1 binding to this motif was confirmed by a dose-dependent competition in the presence of 5-, 10-, 50- and 100-fold excess of the unlabelled oligonucleotide (Figure 5, lanes 3–6). Competition was not achieved with a mutated oligonucleotide (mut-oligo), where the three central thymidines in positions −258/−256 had been replaced by guanines (lanes 8–11). Moreover, a ^{32}P-labelled mut-oligo −270/−243, where the central thymidines were mutated, did not bind to GST or GST–Foxi1 (lanes 12 and 13). These results indicated that Foxi1 binds to the identified AE4 promoter site in a sequence-specific manner.

The binding motif −270/−243 plays an essential role in Foxi1-dependent AE4 promoter transactivation

To test whether the identified binding motif is important for promoter activity, a 5′-truncated variant of promH3 lacking the Foxi1-binding site (promH3Δ) and a mutant promH3 (promH3 mut), in which the core sequence TTT in positions −258/−256 was replaced by GGG, were cloned into the pGL3-Basic vector. In our cell-based luciferase reporter assay, the truncated construct did not induce any luciferase activity and the mutated variant showed only residual Foxi1-dependent transactivation activity (3-fold) (Figure 6). This experiment demonstrates that the identified Foxi1-binding site in the AE4 promoter plays a critical role for activation of promH3 in this cell-based assay.

DISCUSSION

The organization of polarized epithelia into distinct tubules is a hallmark of the mammalian kidney. For the formation of renal tubules, dynamic interactions with the branching ureteric bud that forms the collecting duct system are essential [25]. First insights into the processes that lead to terminal differentiation of the collecting duct epithelium came from the observation that Foxi1-knockout mice exhibited dRTA and lacked expression of the intercalated cell markers pendrin, the V-type H^+-ATPase, AE1 and AE4 due to a differentiation defect of these cells [18,26]. It was proposed that embryonic precursor cells of the ureteric bud form a transient cell population, which further differentiates into intercalated cells upon expression of Foxi1 [18]. In the present study, we have identified the AE4 promoter as a direct target of Foxi1, which is strongly activated in a dose-dependent manner in a cell-based reporter *in vitro* assay. Previously, other members of the forkhead transcription factor family have been shown to be involved in kidney development as well. In early stages of kidney differentiation, Foxc1 exerts an inhibitory effect on a pathway leading to the production of the Gdnf (glial cell line-derived neurotrophic factor). Gdnf is important for the early differentiation of metanephric mesenchymal cells [27]. Another example is Foxd1, which is involved in the differentiation of renal stromal cells [28]. Our results provide evidence that in the collecting duct, Foxi1 does not only act as an upstream regulator of intercalated cell differentiation, but also directly targets the expression of proteins like AE4 that are expressed in fully differentiated intercalated cells. As AE4 is a chloride/bicarbonate exchanger, which is expressed in mature intercalated cells, it has been speculated that AE4 is involved in the regulation of extracellular acid–base homoeostasis by these cells [8,9]. Further studies are required to investigate the regulation of AE4

expression according to different metabolic demands and whether Foxi1 is involved.

According to our results, Foxi1 binds to the motif TGTTTAC within the AE4 promoter. This site fits well with the predicted DNA-binding consensus sequence TrTTTry (r = A/G and y = C/T), which had been identified by *in vitro* high-affinity binding site selection assays with partially degenerate oligonucleotides using recombinant Foxi1 [20], and is the first report of a Foxi1-binding site within a native promoter. Both the deletion and the targeted mutagenesis of this consensus sequence abolished Foxi1-dependent transactivation in our system, indicating that the motif −270/−243 is essential for Foxi1-dependent transactivation of the AE4 promoter. While none of the other oligonucleotides used for the EMSAs contained exactly the TGTTTAC motif, oligonucleotide −426/−387 and oligonucleotide −180/−153 include motifs that fit with the predicted Foxi1-binding sequence TrTTTry [20]. As neither oligonucleotide bound Foxi1 *in vitro*, other nucleotides in close proximity to the core sequence obviously are important for binding as well. Hence systematic mutation of surrounding nucleotides in future studies should delineate the exact requirements for DNA binding of Foxi1. This will facilitate the identification of Foxi1-binding sites in other promoters of genes that are expressed in intercalated cells and may allow us to find a correlation between binding sequences and the time course of the expression of the respective genes during collecting duct development. Indeed, the analysis of the binding affinity of pha-4, a *Caenorhabditis elegans* orthologue of FoxA, for different motifs with a consensus sequence similar to that in our study helped to elucidate the differentiation processes in the pharynx. The authors demonstrated that the order of appearance of pharyngeal genes depends on the affinity of pha-4 for the respective promoters [29].

Apart from AE4, as shown in the present study, Foxi1 was shown to activate the kidney-specific promoter of AE1 and the pendrin promoter as well [18], but the respective Foxi1-binding sites have not yet been identified. Activation of AE1 or pendrin promoter constructs by Foxi1 in COS-7 cells was low compared with the activation of the AE4 promoter construct in our study. This difference cannot be attributed to a cell line-specific effect, as the magnitude of the response of the AE4 promoter construct in COS-7 cells was in the same range as in HEK-293T cells (Figures 2B and 2C). However, as the genomic fragments used may represent the physiological promoter to a varying degree, it is difficult to compare the activation of the different promoter constructs by Foxi1. To test whether transfection of Foxi1 in HEK-293T cells is sufficient to induce AE4 expression, we extracted total RNA from Foxi1-transfected and -untransfected cells and performed reverse-transcription PCR followed by real-time PCR. Neither Foxi1 nor AE4 was expressed endogenously in HEK-293T cells. Transfection with a Foxi1 cDNA construct whose expression was verified by real-time PCR did not result in significant up-regulation of AE4 transcription (results not shown), thus demonstrating that expression of Foxi1 alone is not sufficient for initiation of AE4 transcription in these cells.

The kidney-specific AE1 isoform is localized at the basolateral side of type A intercalated cells [5], whereas pendrin resides in the apical region of type B intercalated cells [30]. For AE4, the cellular and subcellular localization is still under debate. It was shown to be expressed only in type B intercalated cells of rabbit [8] and mouse [18] kidney, but has been detected in both type B and type A intercalated cells of rat and rabbit by others [9]. If all of these proteins are regulated by Foxi1, elaborate mechanisms have to be postulated that enable the turning off of the respective promoter in one or the other intercalated cell type to guarantee their exclusive expression pattern. Our findings point towards repressive elements in the 5′-region of the AE4 promoter that will be addressed in future studies, as promH1, which extends approx. 3 kb upstream of the translational start site, is less active alone or after co-transfection with Foxi1 in comparison with the shortened constructs promH2 and promH3.

Taken together, our results identify Foxi1 as a direct activator of the AE4 promoter. Another prospect for the future is the elucidation of target genes for Foxi1 in the inner ear, as disruption of Foxi1 interferes with inner ear development [19].

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