PAX2 Activates WNT4 Expression during Mammalian Kidney Development*

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The transcription factor PAX2 is expressed during normal kidney development and is thought to influence outgrowth and branching of the ureteric bud. Mice with homozygous null Pax2 mutations have developmental defects of the midbrain-hindbrain region, optic nerve, and ear and are anephric. During nephrogenesis, PAX2 is also expressed by mesenchymal cells as they cluster and reorganize to form proximal elements of each nephron, but the function of PAX2 in these cells is unknown. In this study we hypothesized that PAX2 activates expression of WNT4, a secreted glycoprotein known to be critical for successful nephrogenesis. PAX2 protein was identified in distal portions of the “S-shaped” body, and the protein persists in the emerging proximal tubules of murine fetal kidney. PAX2 activated WNT4 promoter activity 5-fold in co-transfection assays with JTC12 cells derived from the proximal tubule. Inspection of the 5′-flanking sequence of the human WNT4 gene identified three novel PAX2 recognition motifs; each exhibited specific PAX2 protein binding in electromobility shift assay. Two motifs were contained within a completely duplicated 0.66-kb cassette. Transfection of JTC12 cells with a PAX2 expression vector was associated with a 7-fold increase in endogenous WNT4 mRNA. In contrast, Wnt4 mRNA was decreased by 60% in mesenchymal cell condensates of fetal kidney from mice with a heterozygous Pax2 mutation. We speculated that a key function of PAX2 is to activate WNT4 gene expression in metanephric mesenchymal cells as they differentiate to form elements of the renal tubules.

PAX2 belongs to the “paired box” family of transcription factors. Like other family members, it is thought to orchestrate the patterns of gene expression in specific cells during organ development. Homozygous inactivation of the Pax2 gene in mice causes malformation of the midbrain-hindbrain region, the optic nerve, and complete absence of the kidneys (1, 2). Although these observations clearly implicate PAX2 in the development of renal and neural tissue, little is known about its precise gene targets or the specific developmental processes in which it plays a role.

Studies thus far suggest that the developmental functions of PAX2 may be multiplex, activating distinct panels of genes in different cell lineages at different stages. During development of mammalian kidney, PAX2 first appears during the caudal descent of the nephric duct where it affects the fate of a cell (3). When the nephric duct arrives at about somite 26, a “ureteric bud” (UB) emerges from its wall and grows toward the adjacent lateral mesenchyme. This event is again orchestrated by PAX2 through activation of glial cell line-derived neurotropic Factor (GDNF) in the undifferentiated mesenchyme and activation of the GDNF receptor (RET) in UB cells (4). Thus, homozygous Pax2 knockout mice lack normal ureteric bud outgrowth and are unable to induce metanephrin kidneys (2).

A third function of PAX2 in developing kidney involves suppression of programmed cell death in ureteric bud cells. During normal development, the UB arborizes as it penetrates the metanephric mesenchyme, inducing nephrons to form at the tip of each branch. Final nephron endowment is determined by the extent of UB branching achieved at the point when new nephron formation comes to an end in the perinatal period. Mice and humans lacking one Pax2 allele have increased apoptosis of UB cells, reduced UB branching, and suboptimal final nephron number (5, 6). Delivery of a pro-apoptotic gene (Baxα) to the developing UB reduces arborization (7); conversely, the branching defect seen in Pax2 heterozygous mutants can be rescued by targeted expression of an anti-apoptotic gene (Bcl2) to the UB lineage or by administration of caspase inhibitors to pregnant mice bearing heterozygous mutant Pax2 fetuses (8).

Once within the mesenchyme, the arborizing UB delivers signals to nearby mesenchymal cells, inducing them to cluster at the tip of each UB branch. These induced mesenchymal cells express moderately high levels of PAX2 as they undergo a profound transformation into polarized epithelia, but the precise role of PAX2 during this process is unknown (2). Rapid cell division and coordinated cell movement result in twisting growth of an elongated “S-shaped” body with a central lumen lined by epithelial cells. Capillary ingrowth at one end allows formation of the glomerulus, and at its other end fusion with the UB branch tip affords outflow of luminal fluid into the common collecting system. Torban et al. (9) showed that PAX2 expression is associated with an increase in E-cadherin and suppression of vimentin levels in cultured fetal kidney cells, thus proposing that PAX2 might regulate the transition from mesenchyme to epithelium. However, the PAX2 gene targets that might mediate this transition remain elusive.

In 1994, Stark et al. (10) reported that formation of S-shaped bodies in the developing kidney is critically dependent on WNT4, a member of the WNT family of secreted glycoproteins. WNT4 is expressed by the

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*) This work was supported by Operating Grants 62903 and 12954 from the Canadian Institutes of Health Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: UB, ureteric bud; RT, reverse transcriptase; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; PBS, phosphate-buffered saline; E, embryonic day; mutant.

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induced metanephric mesenchymal cells as they cluster and undergo transformation into epithelia (10). Knock-out mice with homozygous inactivation of the Wnt4 gene exhibit initial outgrowth of the ureteric bud, induction of mesenchymal cell clustering, and local expression of Pax2 but no further development of S-shaped bodies (10, 11). In the absence of S-shaped body formation, UB branching comes to an abrupt halt, and the mutant Wnt4 mice are born anephric (10). In general, WNT4 is thought to transmit signals to nearby cells bearing cognate receptors, thereby organizing cell alignment and tissue patterns during development (12). Wnt4 was recently shown to be capable of activating the "canonical" β-catenin signaling pathway and therefore presumably regulates β-catenin-responsive gene targets, such as c-Myc, involved in cellular growth (13).

In this study, we hypothesize that a key function of Pax2 in the S-shaped body is to activate the Wnt4 gene. We show that Pax2 protein is present in the distal portion of the S-shaped body and persists in the emerging proximal tubular cells of fetal kidney. Transient transfection of a Pax2 expression vector into JTC12 cells (derived from the simian proximal tubule) stimulates transcriptional activity of the human Wnt4 promoter. We identify several unique recognition motifs in the Wnt4 promoter that specifically bind Pax2 protein and show that JTC12 cells stably transfected with Pax2 cDNA have increased endogenous Wnt4 expression. Finally, we demonstrate that endogenous Wnt4 expression is reduced in hypoplastic fetal kidneys of mice bearing a heterozygous Pax2 mutation.

MATERIALS AND METHODS

Cell Lines, Transfections, and Luciferase Assays—The monkey proximal tubule cell line (JTC12) was grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin. Cells were incubated at 37°C with 5% CO2 in a humidified environment. The activity of a 10.7-kb Wnt4 promoter was analyzed in transient transfections of the JTC12 cell line in the presence or absence of a Pax2 expression vector. Plasmid DNA preparation, cell culture, and transient transfections using FuGENETM 6 transfection reagent (Roche Applied Science) were carried out as described (14), with modifications. Briefly, cells at 50–70% confluency were co-transfected with 0.4 μg of pRSV-βgal and 0.8 μg of human Wnt4 promoter/pGL2Basic. Cells were harvested 48 h after transfection, lysed in a passive lysis buffer (Promega), and assayed for firefly luciferase and normalized for Renilla luciferase activity of a reporter plasmid that contained a minimal promoter of the firefly luciferase gene. All transfection experiments were run in triplicate, with at least three transfections per condition. The pGL2Basic vector serves as the Renilla luciferase control for each transfection experiment. In some experiments, a reporter plasmid containing a minimal promoter of the firefly luciferase gene was cotransfected with the expression construct for Pax2, and the results were normalized to Renilla luciferase activity of a control reporter plasmid that contained a minimal promoter of the luciferase gene. The ratio of firefly to Renilla luciferase activity was calculated for each transfection experiment and used to normalize for transfection efficiency. Data are presented as means ± SEM, with statistical analysis performed using one-way ANOVA with Tukey's post-hoc test. Differences were considered significant at p < 0.05.

Immunohistochemistry—Immunohistochemistry for Pax2 was performed on 4% paraformaldehyde-fixed, paraffin-embedded embryonic mouse kidney tissues as described previously (6). Briefly, 7-μm sections were deparaffinized, rehydrated, and incubated with 5 μg/ml of proteinase K followed by three rinses in PBS. Slides were incubated for 2–4 h at room temperature in 100 μl of prehybridization buffer (4× SET, 1× Denhardt's, 0.5 mg/ml salmon sperm DNA, 0.6 mg/ml yeast RNA) mixed with 50% formamide followed by overnight incubation at 52.5°C for 18 h in a humidified chamber. The cRNA probes were hybridized for 18 h at 4°C. Slides were then rinsed three times in 0.5× SSC at room temperature and treated with 20 μg/ml RNase A at 37°C. Slides were then rinsed four times in 3× SSC and three times at room temperature in 2× SSC, followed by dehydration in ethanol. Slides were dipped in the photographic emulsion, exposed, counterstained with hematoxylin-eosin, and photographed.

Electrophoretic Mobility Shift Assays (EMSAs)—Probes for EMSAs were prepared from synthetically generated oligonucleotides. The sequences of the probes are as below. Putative sense (A and B) and antisense (C) core recognition nucleotides are double-underlined. Mutations were created in conserved core Pax2 recognition nucleo-
tides for sites A, B, and C; these are shown in boldface. For A, 5′-TCG-GCCGGCGCAAGCAGCTGGCTGGAG-3′; for B, 5′-AGAGGCCCCGTCCTGTGAGAGAT-3′; and for C, 5′-CAGTCTACGTTCTATGATATGGTGCAACAG-3′; for A (mut), 5′-TCG-GCCGGCGCAAGCAGCTGGCTGGAG-3′; for B (mut), 5′-AGAGGCCCCGTCCTGTGAGAGAT-3′; and for C (mut), 5′-CAGTCTACGTTCTATGATATGGTGCAACAG-3′. Synthetic oligonucleotide probes to the Wnt4 promoter region were labeled by back filling with the Klenow fragment of DNA polymerase I using [α-32P]dCTP (3000 Ci/mmol; PerkinElmer Life Sciences). The Pax2 proteins were synthesized using a T7-coupled transcription/translation reticulocyte lysate system (Promega). The in vitro translated products were incubated with 32P-labeled probes at room temperature and then incubated with the radiolabeled probe for 20 min at room temperature. For competition experiments, reaction mixtures were preincubated at 15 min at room temperature and then incubated with the radiolabeled probe for 20 min at room temperature. For competition experiments, reaction mixtures were preincubated at 15 min at room temperature with unlabeled oligonucleotide prior to the addition of radiolabeled probes. Following the binding step, reaction mixtures were electrophoresed on a 6% polyacrylamide gel (acrylamide/bisacrylamide ratio, 29:1) in 0.25 M TBE buffer (22.25 mM Tris-HCl, 22.25 mM boric acid, and 1 mM EDTA) at 90 V at room temperature. The gels were dried and exposed to Kodak X-Omat film at room temperature.

Stable Transfection of JTC12 Cells and Western Immunoblotting—Subconfluent JTC12 cells were transfected with a PAX2 expression vector, pCDNA3.1/PAX2, or with an empty vector without the PAX2 insert, using the lipophilic transfection agent FuGENETM 6 (Roche Applied Science). After 48 h, cells were then selected in medium containing 400 μg/ml G418 for 2–3 weeks; surviving colonies were pooled. Protein in whole cell extracts were resolved on 10% SDS-polyacrylamide gels, electroblotted onto Hybond ECL membrane (Amersham Biosciences), and probed with rabbit anti-PAX2 polyclonal antibody (1:2000; Santa Cruz Biotechnology) for 20 min at room temperature, which will give rise to podocytes of the primitive glomerulus (Fig. 1A). PAX2 expression is evident in maturing tubules that stain for the simian JTC12 cell line, derived from proximal tubules and exhibit staining for the fluorescein-tagged proximal tubule marker, L. tetragonolobus lectin as do the Pax2-positive cells of fetal mouse kidney (D).

Results

PAX2 is Expressed in the Distal S-shaped Body and Persists in Fetal Proximal Tubule—Previous investigators have demonstrated Pax2 mRNA expression in induced mesenchymal cells as they cluster at the tip of each ureteric bud branch (2). Our immunohistochemical studies show that PAX2 protein expression is sustained in tubular cells of the distal loop of S-shaped bodies (Fig. 1A). However, PAX2 protein is conspicuously absent in epithelial cells at the proximal loop of the S-shaped bodies (Fig. 1B–D). The JTC12 cell line, derived from simian renal proximal tubule, also exhibits immunofluorescent staining for L. tetragonolobus lectin (Fig. 1C) and was used for the in vitro studies of PAX2 and Wnt4 below.

FIGURE 1. PAX2 protein expression in fetal mouse kidney. A, PAX2 protein was identified by immunohistochemistry in the distal portion of the S-shaped bodies (arrowheads) of E15.5 fetal mouse kidneys. B, in frozen sections of E16.5 fetal mouse kidney, Pax2 protein was evident in epithelial cells of developing tubules. Anti-PAX2 antibody was detected by immunofluorescence with Texas Red-labeled secondary antibody. C, simian JTC12 cells are derived from proximal tubules and exhibit staining for the fluorescein-tagged proximal tubule marker, L. tetragonolobus lectin as do the Pax2-positive cells of fetal mouse kidney (D).
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PAX2 Activates the WNT4 Promoter in Vitro—To determine whether PAX2 activates WNT4 promoter activity in vitro, a 10.7-kb 5′-flanking sequence of the human WNT4 gene (15) was cloned into a luciferase reporter vector and co-transfected with a full-length human PAX2b expression vector into cultured JTC12 cells (Fig. 2). Luciferase activity in cell extracts was assayed after 48 h. In the presence of PAX2b, transcriptional activity of the 10.7-kb WNT4 promoter was increased 5-fold.

Description of Putative PAX2-binding Sites in the WNT4 Promoter—To determine whether the WNT4 promoter contains plausible PAX2-binding sites, we screened the 10.7-kb 5′-flanking sequence of the human WNT4 gene (15) with MatInspector software. The recognition motif for PAX2 has been studied by others and appears to vary significantly from gene target to gene target. Nevertheless, a consensus sequence consisting of about 11 core nucleotides spaced over a stretch of 25 bp has been proposed (4). Of 11 putative sites with partial homology to this consensus sequence, only 5 were subsequently shown to specifically bind PAX2 protein. The first putative PAX2-binding motif (motif A in Fig. 3A) was identified close (−461 to −435 in sense orientation) to the transcriptional start site. It contained only 7 of the 11 core nucleotides of the PAX2 consensus sequence but was selected for study because it also exhibited homology with the “PAX5” recognition sites.

A second (motif B1 at −2428 to −2405) and third (motif C1 at −2460 to −2581) putative PAX2 recognition sequence were identified further upstream, positioned fairly closely together and in sense and antisense orientations, respectively. These two motifs contained 9 (motif B1) and 6 (motif C1) nucleotides of the PAX2 consensus sequence but was selected for study because it also exhibited homology with the “PAX5” recognition sites.

Most interestingly, motifs C1 and B1 lie within a 0.66-kb cassette of DNA (−5331 to −4672) that was completely duplicated further upstream.

**FIGURE 2.** PAX2 activates transcriptional activity of the human WNT4 promoter. JTC12 cells were transiently co-transfected with a pGL2 luciferase reporter vector driven by a 10.7-kb fragment of the human WNT4 gene 5′-flanking sequence and a mammalian expression vector containing the full-length human PAX2 cDNA. Luciferase activity was assayed after 48 h and expressed as a percent of basal promoter activity. Empty pGL2 vector luciferase activity was one-third of that in the presence of a WNT4 promoter. In the presence of exogenous PAX2, basal promoter activity was stimulated 5.4-fold.

**FIGURE 3.** Putative PAX2-binding sites in the human WNT4 promoter. A and B, the 10.6-kb 5′-flanking sequence of the human WNT4 gene was analyzed with MatInspector software for possible PAX2-binding motifs. Three of 11 potential PAX2 recognition motifs were subsequently shown to bind PAX2 protein in gel shift assays. Two motifs (B1 and C1) lie within a 660-bp cassette duplicated further upstream. C, the three putative unique PAX2-binding motifs are compared with other published sequences.
upstream (−7563 to −6904) (Fig. 3A). Thus, two additional putative PAX2 motifs (B2 and C2) were identified close together lying on opposite DNA strands, but these were duplicates of sites in the downstream cassette. The three unique putative PAX2-binding motifs and surrounding nucleotide sequences are listed in Fig. 3B and compared with PAX2 recognition motifs reported by others (Fig. 3C).

**EMSA**—To determine whether the three putative recognition motifs exhibited specific high affinity PAX2 binding, a series of gel shift experi-

**FIGURE 4. Electrophoretic mobility shift assays for PAX2 binding to putative recognition motifs in the WNT4 promoter.** A, PAX2 protein forms a unique complex with radiolabeled probe A (lane 1), probe B (lane 5), or probe C (lane 8) that disappear in the presence of anti-PAX2 antibody (lanes 2, 6, and 9, respectively). B, the radiolabeled PAX2-probe A complex is competed out by progressively increasing amounts of cold unlabeled probe A (lanes 2–7). Mutation of a single cytosine to an adenine within the putative PAX2 recognition motif significantly diminishes the PAX2-probe band (lanes 8 and 9). C, the radiolabeled PAX2-probe B complex is competed out by increasing amounts of unlabeled probe B (lanes 2–7). For the mutation of two core nucleotides of the putative PAX2 recognition motif in probe B, no complex formation is seen (lane 8). D, the radiolabeled PAX2-Probe C complex is competed out by increasing amounts of unlabeled probe C (lanes 2–6). Mutation of two core nucleotides in probe C eliminates complex formation (lane 8). E, although complex inhibition by unlabeled wild type oligonucleotides was evident at 25-fold excess (probe A), 50-fold excess (probe B), and 100-fold excess (probe C) (lane 4 (B), lane 4 (C), and lane 5 (D), respectively), mutant unlabeled oligonucleotides were ineffective competitors at similar molar ranges. Protein-DNA complexes were resolved on nondenaturing 6% polyacrylamide gels electrophoresed at room temperature in 0.25 × TBE. Complexes were visualized by drying the gel and exposing to film (Eastman Kodak Co.) at room temperature. The positions of free probe and protein-DNA complexes are indicated.
PAX2 protein level by Western immunoblotting. As seen in Fig. 5 culture medium. Stable transfectants were isolated and re-assessed for human JTC12 cells and in the stable JTC12/D andimal tubular marker, derived from the monkey proximal tubule lineage and express the prox-
nizes each of the three sites in sequence-specific fashion. beled wild type oligonucleotide competitors (Fig. 4 unable to inhibit complex formation in the same molar range as the unlabeled wild type oligonucleotides A–C (Fig. 4, E) Binding of PAX2 to probes A–C was competed out by increasing amounts of unlabeled wild type oligonucleotides A–C (Fig. 4, B–D). Competition was evident at 25-fold excess (probe A), 50-fold excess (probe B), and 100-fold excess (probe C) unlabeled wild type oligonucleotide. EMSAs were also performed with PAX2 protein and mutated oligonucleotides A (mut), B (mut), and C (mut) (Fig. 4E). Mutation of a single core nucleotide in the putative recognition motif of probe A significantly reduces complex band intensity (Fig. 4B, lanes 9 versus 2). When core nucleotides of motif B and C were mutated, complex formation was completely abrogated (Fig. 4, C, lanes 8 versus 2, and D, lanes 8 versus 2). Similarly, mutant cold oligonucleotides were unable to inhibit complex formation in the same molar range as the unlabeled wild type oligonucleotide competitors (Fig. 4E). Thus, PAX2 recognizes each of the three sites in sequence-specific fashion.

PAX2 Activates Endogenous WNT4 in JTC12 Cells—JTC12 cells are derived from the monkey proximal tubule lineage and express the prox-
imal tubular marker, L. tetragonolobus lectin. The cells express modest levels of endogenous PAX2, identifiable by Western immunoblotting (Fig. 5A). To examine the effect of PAX2 on endogenous WNT4 expression, JTC12 cells were stably transfected with a cytomegalovirus-driven human PAX2 expression vector and selected in neomycin-containing culture medium. Stable transfectants were isolated and re-assessed for PAX2 protein level by Western immunoblotting. As seen in Fig. 5A, the JTC12/PAX2 stable transfectants expressed about five times as much PAX2 protein as the parent cell line.

Endogenous WNT4 mRNA levels were measured in control (parent) JTC12 cells and in the stable JTC12/PAX2 transfectants by real time RT-PCR, normalizing samples for Gapdh mRNA amount. As seen in Fig. 5B, WNT4 mRNA levels were about 7-fold higher in the JTC12/PAX2 transfectants compared with control cells.

WNT4 Expression Is Reduced in the Pax21Neu Mutant Mouse—In previous studies we demonstrated that fetal kidneys from Pax21Neu heterozygotes have reduced PAX2 protein and moderately severe renal hypoplasia, although the architecture of individual nephrons is relatively normal (6). To determine whether the in vitro evidence of WNT4 regulation by PAX2 reflects an important molecular pathway for mammalian kidney development in vivo, we assessed endogenous Wnt4 expression in E15 fetal kidney of heterozygous Pax21Neu mutant mice and their wild type littermates. Heterozygous male Pax21Neu C3H mice were crossed with wild type C3H females. E15 embryos were genotyped as described previously (8), and fetal kidneys were isolated and pro-
cessed for in situ hybridization studies with a Wnt4 riboprobe. As seen in Fig. 6, A and B, Wnt4 mRNA expression is abundant but is highly restricted to the condensing mesenchyme adjacent to ureteric bud branch tips (arrows) in normal fetal kidney. In contrast, the Wnt4 signal is barely detectable in condensing mesenchyme of Pax21Neu fetal kid-
neys (Fig. 6, C and D). Similarly, in situ hybridization studies were performed for Eya1, another transcription factor expressed in early induced renal mesenchyme; Eya1 mRNA expression was comparable in Pax21Neu and wild type E15.5 kidneys (data not shown).

Wnt4 mRNA was quantified in mutant and wild type fetal kidneys by real time RT-PCR and normalized for Gapdh mRNA in the same samples. In keeping with our in situ hybridization studies, relative Wnt4 mRNA in E15.5 Pax21Neu mutant kidney was only 40% of that in kidneys from wild type littermates (Fig. 7).

DISCUSSION

Potential Functions of the PAX2/WNT4 Pathway during Nephrogenesis—PAX2 plays a central role in the caudal descent of the nephric duct, the emergence of ureteric buds, and the subsequent branching morphogenesis of the collecting system. PAX2 mRNA is also abundant in metanephric mesenchymal cells as they cluster at UB tips (2), and its function there is as yet unknown. Our immunohistochemical studies show that PAX2 protein expression is sustained beyond this stage. It appears in distal portions of the developing S-shaped body as
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differentiation proceeds and is still evident in the early fetal proximal tubule. Apparently, Pax2 is involved both in the process whereby metanephric mesenchyme is transformed into polarized epithelium and/or in the maturation of the proximal convoluted tubule as it undergoes terminal differentiation.

Previous reports suggest that these events are also critically dependent on expression of the secreted growth factor, Wnt4. In mice with homozygous inactivation of the Wnt4 gene, the UB successfully contacts metanephric mesenchyme and induces mesenchymal cells to cluster and express Pax2 at its tips (10, 17). However, subsequent progression to the S-shaped body stage does not occur (10, 17). This observation places Pax2 temporally upstream of Wnt4 during this process. Our studies demonstrate reduced Wnt4 mRNA in the Pax2−/− mouse and indicate that Pax2 is functionally upstream of Wnt4 expression in the developmental cascade as well.

There is some evidence that Wnt4 is involved in cell movement during development. Acting through the frizzled3 receptor, Wnt4 guides murine commissural axons as they cross the midline (18). In Drosophila, Wnt4 has been implicated in cell movements requiring disassembly of focal adhesion complexes; Drosophila Wnt4 regulates focal adhesion kinase during ovarian morphogenesis through the non-canonical protein kinase C pathway (19). Conceivably, Wnt4 signaling via this pathway might play a role in the spectacular serpentine growth of the S-shaped body and proximal convoluted tubule.

On the other hand, there is also evidence that Wnt4 may have a set of effects mediated through the canonical β-catenin signaling pathway (13). This intracellular pathway responds to Wnt activation of heterodimeric frizzled/LRP6 receptors at the surface of target cells (20). Activated canonical receptors recruit Dsh and other partners to suppress degradation of β-catenin and facilitate its transfer to the nucleus. There, β-catenin combines with transcription factor partners of the TCF/LEF family to regulate expression of target genes (21). Rod cell production in the developing retina is promoted by canonical Wnt4/Fzd4/Lrp6 signaling (22). Canonical Wnt4 signaling drives synovial joint development (23) and mediates the effects of estrogen on uterine growth (24).

High levels of canonical β-catenin signaling activity have been identified in both the distal S-shaped body and tips of the ureteric bud during mouse kidney development (25, 26). Thus, Pax2-activated Wnt4 expression might drive autocrine canonical signaling during formation of the S-shaped body. There is the interesting possibility that Wnt4 could also provide a paracrine signal to the adjacent ureteric bud tip. Wnt4 is reported to activate the canonical β-catenin signaling pathway in cultured Madin-Darby canine kidney cells derived from the canine UB lineage (13). Wnt4 has been implicated in side-branching of mammary ductal cells during breast development (27). Wnt4 knock-out mouse fail to form S-shaped bodies, but this is accompanied by an abrupt halt to any further UB branching (10).

**PAX2 Binding to Novel Motifs within the Wnt4 Gene Promoter**—We demonstrate that Pax2 directly activates transcriptional activity of the 5′-flanking sequence of the human Wnt4 gene, containing several unique Pax2 recognition motifs. A novel 26-bp sequence (labeled motif A in Fig. 1A) about 0.45 kb upstream of the transcriptional start site showed homology both to the consensus Pax2 recognition motif proposed by Brophy et al. (4) and to the Pax5 motif used in MatInspector software. Pax2−/− and −8 form a subgroup within the Pax gene family based on domain structure; crossover between Pax2 and Pax5 binding sequences has been noted in other gene targets (16). Two other motifs within the 5′-flanking sequence of the human Wnt4 gene (labeled motifs B and C in Fig. 1A) were more similar to the published Pax2 consensus recognition motif (4). Most interestingly, Pax2-binding motifs B1 and C1 were located in close proximity to each other oriented on opposite (sense and antisense) DNA strands and lie within a 0.66-kb cassette (from −5531 to −4672) that was completely duplicated at an upstream site (−7563 to 6904) in the 5′-flanking sequence. Possibly, this duplication event reflects the importance of these Pax2 motifs and/or other elements of the cassette in regulating Wnt4 expression.

It is unclear why the Wnt4 gene should contain several slightly different recognition motifs for Pax2, yet each of the motifs (A, B1/B2, or C1/C2) exhibited specific high affinity binding to Pax2 protein in EMSAs. In each case, the Pax2-probe complex was sensitive to anti-Pax2 antibody, mutation of core Pax2 recognition motif base pairs, and addition of cold-unlabeled motif DNA. Clearly, other transcription factors could be involved in determining the highly restricted expression of Wnt4 in mesenchymally derived portions of the renal tubule; Pax2 is expressed at other sites in the developing kidney (e.g. ureteric bud) where Wnt4 expression is absent. Presumably, additional transcription factors are required to achieve a combinatorial specificity with Pax2 and account for the highly restricted pattern of Wnt4 expression. The variability of Pax2 recognition sequences might reflect the molecular complexity of this regulation.

**PAX2 Activates Endogenous Wnt4 Gene in Vivo**—The in vitro interactions between Pax2 and the Wnt4 promoter are clearly relevant during in vivo renal development. Endogenous Wnt4 mRNA expression was significantly reduced in the mesenchymal condensates/S-shaped bodies of heterozygous mutant Pax2−/+/− mouse fetal kidneys. Interestingly, heterozygous mutant Pax2−/− mouse fetal kidneys are hypoplastic (6), but the reduction in Wnt4 expression is not sufficient to prevent formation of some normal appearing S-shaped bodies during development. This is not surprising, however, because normal appearing S-shaped bodies were also noted in fetal kidneys of heterozygous Wnt4 knock-out mice. Thus, the developmental cascade allowing differentiation of S-shaped bodies seems to require only a modest level of Pax2 and Wnt4. Below some minimal threshold, however (as in homozygous Wnt4 knock-out mice), S-shaped body formation does not occur.

**Conclusion**—During renal development, Pax2 is expressed in the distal portion of the emerging S-shaped body and early proximal tubule. We suggest that it binds directly to novel response elements in the Wnt4 promoter, activating transcription during the mesenchyme to epithelium transition. Wnt4 expression continues in the S-shaped body and early proximal tubule and could influence its serpentine growth and terminal differentiation. Conceivably, Pax2 activation of Wnt4 could initiate a paracrine feedback signal to ureteric bud cells and promote further rounds of branching nephrogenesis.
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