The Secreted Frizzled Related Protein 2 (SFRP2) Gene Is a Target of the Pax2 Transcription Factor*

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Despite their essential role in vertebrate development, the function of Pax proteins in gene regulation is not well understood. To identify potential genes regulated by the Pax2 protein, we screened embryonic kidney cells transformed with Pax2-expressing retroviruses for genes activated in response to Pax2 expression. In this system, the gene encoding the secreted frizzled related protein, Sfrp2, was strongly activated in all Pax2b-expressing cells. This activation of Sfrp2 expression correlated with changes in chromatin structure at the Sfrp2 locus, particularly in and around regions of Pax2 binding. Although the amount of Pax2-dependent transactivation was low in transient assays, the data suggest that local alterations of chromatin structure by Pax proteins can greatly enhance expression when presented in the right cellular context.

The vertebrate Pax genes encode transcription factors that specify correct embryonic patterning and cellular differentiation in a broad array of structures (1). The Pax2 gene is essential for early kidney development, optic stalk fusion, and ear development in both mouse (2) and man (3). The role of Pax2 has been studied extensively in the developing kidney, where it regulates the earliest phase of epithelial cell morphogenesis within the intermediate mesoderm. The Pax2, Pax8, and lim1 transcription factors mark the intermediate mesoderm and the first epithelium, the nephric duct, which is derived from the intermediate mesoderm (4). Pax2 is also found in the metanephric mesenchymal cells adjacent to the posterior nephric duct, where it regulates expression of the gdnf gene to provide a guidance cue for the ureteric bud diverticulum as it invades the mesenchyme (5). Within the mesenchyme, Pax2 is required for cells to respond to inductive signals emanating from the invading ureteric bud (5). These inductive signals promote changes in cell adhesion of the mesenchyme, epithelial polarization, and tubule formation. The mesenchyme-to-epithelial transition characteristic in the developing nephron requires extensive cell and tissue remodeling. These processes are mediated, in part, by secreted signaling molecules such as Wnts (6–8), Bone morphogenetic proteins (9–12), and fibroblast growth factors (13–15). Pax2 continues to be expressed during the epithelial-mesenchymal transition and in a proliferating population of primitive epithelia until the maturation of the nephron nears completion. Although there is little Pax2 expression in mature renal epithelia, reactivation of Pax2 is prominent in a variety of disease states, such as cystic disease (16, 17) and urogenital cancers (18–21), where proliferation or de-differentiation occurs.

Despite its essential role in development and disease, there are few genes known to be directly regulated by Pax2. Early in the metanephric mesenchyme, Pax2 can activate the gdnf (5) and WTI (22, 23) genes by direct interactions with the cis-acting regulatory sequences. The Pax2 protein contains a DNA-binding domain, an octapeptide domain with repressor activity, and a transactivation domain that can be phosphorylated by the c-Jun N-terminal kinase (JNK) (24, 25). Phosphorylation of the trans-activation domain results in increased Pax2 activation potential, yet the mechanism of activation remains to be characterized. Consistent with Pax2 phosphorylation by JNK, phenotypic analyses with JNK1/JNK2 double-mutant embryos suggest that JNK activity modifies a late function of Pax2 in the developing eye and kidney (26). Furthermore, modification of Pax2 activity by JNK is attenuated by the Groucho 4 protein, which is able to completely suppress Pax2 transactivation ability (27). These lines of investigation suggest multiple functions of Pax2 that may be determined in part by different sets of positively and negatively regulated target genes.

Both in the developing kidney and nervous system, Pax2 protein activity plays an essential role in cellular differentiation, cell survival, and tissue remodeling. These events must be mediated by many other proteins, whose activity may be positively or negatively regulated by Pax2. To try and identify potential genes downstream of the Pax2 protein, we developed cell lines from immortalized mouse kidney mesenchyme that were Pax2-negative and introduced the Pax2 protein. These cells were used in a differential screen for genes up-regulated in response to the Pax2 protein. One of the most prominent genes activated in response to Pax2 encodes the secreted frizzled related protein, Sfrp2. The Sfrp proteins can negatively regulate signaling through sequestration of the Wnt ligands in the extracellular space (7, 28, 29). Sfrp2 is expressed in the early polarized epithelial vesicle, in a subset of Pax2-expressing epithelia. In Pax2-expressing cells, the Sfrp2 locus exhibits changes in DNaseI hypersensitivity and association with the nuclear matrix. These data suggest that Sfrp2 is a target of the Pax2 transcription factor, late in epithelial cell polarization.

MATERIALS AND METHODS
Cell Culture—Conditionally immortalized E11 mouse metanephric mesenchyme cells, derived from the ImmortalMouse™, were a gift of L.

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¶ The abbreviations used are: JNK, c-Jun N-terminal kinase; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
The Pax2-paired domain (Pax2-PD), amino acids 1 to 170, was used to fuse to a poly-histidine expression vector (pSET, Invitrogen) and purified by metal affinity chromatography under denaturing conditions. The denatured-paired domain protein was dialyzed stepwise in decreasing amounts of urea and finally into Z buffer (25 mM HEpes, pH 7.8, 20% glycerol, 12.5 mM MgCl2, 0.1 mM EDTA, 1 mM dithiothreitol). Genomic SFRP2 fragments were isolated from the plasmid backbone and digested with either HpaII or TaqI. Approximately 0.1 mg of the total digests were end-labeled with [32P]dCTP and the Klenow fill-in reaction. The binding reaction contained 10,000 dpm of labeled DNA, 5–10 ng of Pax2-PD protein in 25 μl of Z buffer, 1 μg of sonicated salmon sperm DNA, and 0.5 μg of Z buffer. The binding reaction was incubated at room temperature for 20 min, then 20 μl of prewashed nickel-affinity beads were added to the reaction and rotated for an additional 30 min. After binding, the beads were washed 3 times with 0.5 M NaCl, 10 mM Tris, pH 8, and 1% SDS each. The DNA was eluted in 20 μl of 1 M NaCl, 10 mM Tris, pH 8, and 1% SDS, and the DNA was electrophoresed on 4% neutral agarose gels with total input DNA as a marker.

Protein/DNA Pull-down Assay and Electrophoretic Mobility Shift—The Pax2-paired domain (Pax2-PD), amino acids 1–170, was used to fuse to a poly-histidine expression vector (pSET, Invitrogen) and purified by metal affinity chromatography under denaturing conditions. The denatured-paired domain protein was dialyzed stepwise in decreasing amounts of urea and finally into Z buffer (25 mM HEpes, pH 7.8, 20% glycerol, 12.5 mM MgCl2, 0.1 mM EDTA, 1 mM dithiothreitol). Genomic SFRP2 fragments were isolated from the plasmid backbone and digested with either HpaII or TaqI. Approximately 0.1 mg of the total digests were end-labeled with [32P]dCTP and the Klenow fill-in reaction. The binding reaction contained 10,000 dpm of labeled DNA, 5–10 ng of Pax2-PD protein in 25 μl of Z buffer, 1 μg of sonicated salmon sperm DNA, and 0.5 μg of Z buffer. The binding reaction was incubated at room temperature for 20 min, then 20 μl of prewashed nickel-affinity beads were added to the reaction and rotated for an additional 30 min. After binding, the beads were washed 3 times with 0.5 M NaCl, 10 mM Tris, pH 8, and 1% SDS each. The DNA was eluted in 20 μl of 1 M NaCl, 10 mM Tris, pH 8, and 1% SDS, and the DNA was electrophoresed on 4% neutral agarose gels with total input DNA as a marker.

To confirm binding of Pax2 to the candidate fragments, DNA fragments were isolated from a 1.5% agarose gel after digestion with restriction enzymes, subcloned, sequenced, and used for electrophoretic mobility shift experiments, as described. Binding reactions were performed in a total volume of 10 μl for 30 min at room temperature and contained increasing amounts of purified Pax2 PD (1–170 amino acids) protein and 100 ng poly (dI-dC) labeled probe (10,000 dpm). Free DNA and DNA/protein complexes were resolved at room temperature on 4% or 6% polyacrylamide gels in 0.5× Tris-borate-EDTA at 120 V.

**Northern Blotting**—Total RNA from cell lines was prepared using TRIzol reagent (Invitrogen) following the manufacturer's instructions. Cell lines used for analysis included clone 46m Cells and Pax2 retrovirus packaged in 293T cells. Cells were lysed in TRIzol reagent (Invitrogen) following the manufacturer's instructions. Total RNA was isolated from a 1.5% agarose gel after digestion with restriction enzymes, subcloned, sequenced, and used for electrophoretic mobility shift experiments, as described. Binding reactions were performed in a total volume of 10 μl for 30 min at room temperature and contained increasing amounts of purified Pax2 PD (1–170 amino acids) protein and 100 ng poly (dI-dC) labeled probe (10,000 dpm). Free DNA and DNA/protein complexes were resolved at room temperature on 4% or 6% polyacrylamide gels in 0.5× Tris-borate-EDTA at 120 V.

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**Nuclear Matrix Isolation and DNAseq Hypersensitive Site Mapping**—Nuclear Matrix isolation was a modified version of that described by (32). Briefly, nuclei from 46m and 28Pax2 cells were isolated according to the manufacturer's instructions.

**CAT Assays**—NIH 3T3 cells were plated at 500,000 cells per 60-mm dish, cultured in DMEM + 10% fetal calf serum and 1% penicillin/streptomycin at 37 °C, and transfected the following day with FuGENETM (Roche Applied Science), according to the manufacturer's protocol. For each 60-mm dish, 6 μl of FuGENETM were used per 3 μg of DNA, which contained 0.5 μg of reporter plasmid and 0.2 μg of cytomegalovirus-β-galactosidase for standardization. After a 2-h exposure to the FuGENETM/DNA in DMEM (serum free), DMEM + 10% fetal calf serum was added to double the volume. Forty-eight hours after transfection, cells were scraped into PBS, spun down, and resuspended in 0.3 ml of 0.25 M Tris (pH 7.6). Cells were lysed with three freeze/thaw cycles. Debris was pelleted, and 50 μl of lystate was assayed for β-galactosidase activity. Equivalent amounts of β-galactosidase units were then used for the acetylation reactions as described (31). Spots were cut.
out of the thin layer chromatography plate, and scintillation was counted. Counts were standardized for background. Each transfection was performed a minimum of three times.

RESULTS

To identify potential target genes activated by the Pax2 transcription factor, we developed a panel of immortalized cell lines derived from metanephric mesenchyme of E11 Immorto-Mouse™embryos. The cell lines carried a temperature-sensitive SV40 Tag under the control of a γ-interferon-inducible promoter and were cultured at 32°C. Individual clonal lines were screened for the expression of Pax2, WT1, and Wnt4 (data not shown) by Northern blotting. The cells were fibroblastic in morphology and did not express detectable levels of Pax2. The parental cell line 46m was chosen as it expressed very low levels of the metanephric mesenchyme marker WT1, was still mesenchymal in character, but did not express Pax2 or Sfrp2. Both Pax2b and Pax2a were introduced into the 46m cells by retroviral infection, and clonally derived cell lines were selected with G418 and expanded. Individual cells were assayed for the expression of Pax2a or Pax2b by Western blotting (Fig. 1A).

To identify possible Pax2 target genes activated in the Pax2-expressing clones, a suppressive-subtractive hybridization strategy was utilized. Polyadenylated RNA from the parental cells 46m and the Pax2b transformant 28Pax2 were used to make subtracted libraries. The libraries were spotted onto replica grids and screened with probes made from the original forward- (28Pax2 minus 46m) and reverse- (46m minus 28Pax2) subtracted cDNAs. Only clones from the forward-subtracted library that also hybridized with the forward-subtracted probe but not with the reverse probe were analyzed further by Northern blotting to total RNAs from the original panel of Pax2-transformed cell lines. One of the most abundant clones in the forward-subtracted library contained a partial sequence derived from the Sfrp2 gene. Using this sequence tag as a probe, Northern blotting revealed dramatic activation of the Sfrp2 mRNA in mesenchymal-derived cells expressing the Pax2b isoform (Fig. 1B). Strikingly, the parental 46m cell line and cells expressing Pax2a did not show increased levels of Sfrp2 mRNA, whereas all of the Pax2b transformants exhibited increased Sfrp2 mRNA levels, and most of them exhibited it to a very high degree.

The mouse Sfrp2 genomic locus was cloned from a BAC library, and the structure of the gene was determined (Fig. 2A). The mRNA is encoded in three exons spanning about 8 kb. To localize potential regulatory regions within the Sfrp2 genomic locus, we utilized DNase1 hypersensitive site mapping using the parental 46m cells and the Pax2-expressing clonal lines 28Pax2 and 8Pax2 (Fig. 2B). Two hypersensitive sites were mapped, one of which was present in all cell lines (site I) and a second that was prominent only in the Pax2-expressing cells (site II). Site II is located just upstream of the presumed tran-
To further examine the ability of Pax2 to bind directly to and regulate the \textit{Sfrp2} locus, we screened two overlapping \textit{Sfrp2} genomic clones spanning more than 13 kb for Pax2-binding sites by using a novel protein/DNA pull-down assay (Fig. 3A). Total plasmid DNAs from either a 9-kb HindIII or a 12-kb XbaI fragment were digested with frequent cutting restriction endonucleases (HpaII or TaqI) and end-labeled with $^{32}$P. The DNA mixture was incubated with a recombinant Pax2-paired domain fused to a polyhistidine tag and the protein/DNA complexes were pulled down with Ni-affinity agarose. Unlabeled competitor DNAs from the high affinity Pax2-binding sequence H2A were added to test for specificity. In all cases, the pulled down fragments were competed out with an excess of unlabeled H2A Pax2-binding sequence. The Pax2-paired domain specifically bound strongly to two fragments in the HpaII digests and one fragment in the TaqI digest (Fig. 3A). These digests were run on a 2% metaphor agarose gel as follows: lane 1, GAPDH; lane 2, 9.0 HindIII cut with HpaII; lane 3, 9.0 HindIII cut with Sau3A; lane 4, 12.0 Xba cut with HpaII; lane 5, 12.0 Xba cut with Sau3A. Note multiple bands mapping to the \textit{sfrp2} locus hybridize with the 27Pax2 DNAs but not with the 46m DNAs.

To address changes in the regulatory region of the \textit{Sfrp2} locus, we examined the ability of genomic DNA to associate with the nuclear matrix. Changes in nuclear matrix attachment have been correlated with the activation of specific chromatin domains during development (32). Such rearrangements can impact both chromatin replication and transcription of specific loci. To assess rearrangements in matrix attachments, matrix-associated DNA was isolated from 46m and Pax2 cells and used as a probe for Southern blots containing fragments of the \textit{Sfrp2} locus (Fig. 4). Using the 9-kb HindIII \textit{sfrp2} fragment, only the matrix-associated DNA from 28Pax2 cells hybridized (Fig. 4A). Similar results were obtained with 27Pax2 cells, which exhibited hybridization to multiple bands of the genomic \textit{Sfrp2} clones digested with different enzymes (Fig. 4B). At no time did the matrix-associated DNAs from the parental 46m cells hybridize to clones spanning the \textit{sfrp2} locus. As a control for equal labeling, a GAPDH BAC genomic clone or total genomic mouse DNA were used. The GAPDH BAC clone also contained some repetitive elements and was labeled equally well with both 46m and 28Pax2 probes.

Fig. 4. Nuclear matrix-associated DNA from Pax2-expressing cells and control 46m cells. \textit{A}, the left panel shows an ethidium-stained gel of the genomic clones blotted to membranes. A GAPDH BAC was used as a positive control (lane 1). The 9-kb HindIII fragment from the \textit{sfrp2} locus was cut with Sau3A (lane 2). The digests were run on a 1% agarose gel and Southern blotted with subsequent probing by $^{32}$P-labeled matrix-associated DNA. The labeled DNA was purified from either 28Pax2 or 46m cells as indicated. Note the specific bands hybridized by the 28Pax2 probe in the 9.0 HindIII fragment (arrows). The 46m probe did not hybridize to the \textit{sfrp2} locus despite an equal number of counts and equal labeling, as judged by the GAPDH hybridization pattern. \textit{B}, similar experiment as in \textit{A} using nuclear matrix-associated DNAs from the 27Pax2 clonal line. The digests were run on a 2% metaphor agarose gel as follows: lane 1, GAPDH; lane 2, 9.0 HindIII cut with HpaII; lane 3, 9.0 HindIII cut with Sau3A; lane 4, 12.0 Xba cut with HpaII; lane 5, 12.0 Xba cut with Sau3A. Note multiple bands mapping to the \textit{sfrp2} locus hybridize with the 27Pax2 DNAs but not with the 46m DNAs.

Fig. 5. \textit{A}, schematic of the \textit{sfrp2} locus and the reporter construct 2.8 PX \textit{Sfrp2-CAT} is shown. \textit{B}, transactivation assays of the reporter 2.8 PX \textit{Sfrp2-CAT} in transfected 3T3 cells show increased activity with Pax2 co-transfection. Fold activation was calculated relative to the basal level of reporter gene expression. Data were standardized with a $\beta$-galactosidase reporter plasmid. Data is presented as average values for activation. Bars, 1 S.D. from the mean.

\textit{Regulation of SFRP2 by Pax2}

cription start site, based upon available cDNA sequence data. To further examine the ability of Pax2 to bind directly to and regulate the \textit{Sfrp2} locus, we screened two overlapping \textit{Sfrp2} genomic clones spanning more than 13 kb for Pax2-binding sites by using a novel protein/DNA pull-down assay (Fig. 3A). Total plasmid DNAs from either a 9-kb HindIII or a 12-kb XbaI fragment were digested with frequent cutting restriction endonucleases (HpaII or TaqI) and end-labeled with $^{32}$P. The DNA mixture was incubated with a recombinant Pax2-paired domain fused to a polyhistidine tag and the protein/DNA complexes were pulled down with Ni-affinity agarose. Unlabeled competitor DNAs from the high affinity Pax2-binding sequence H2A were added to test for specificity. In all cases, the pulled down fragments were competed out with an excess of unlabeled H2A Pax2-binding sequence. The Pax2-paired domain specifically bound strongly to two fragments in the HpaII digests and one fragment in the TaqI digest (Fig. 3). These fragments were subcloned, sequenced, and analyzed further by electrophoretic mobility shift experiments (Fig. 3B). Two fragments mapped within the potential regulatory region upstream of the \textit{Sfrp2} translation initiation site. Both fragments bound recombinant Pax2 protein in the region spanning –411 to –210, mapping around the DNaseI hypersensitive site II that is prominent only in the 28Pax2 cells. These data suggest that Pax2 binding results in a change in local chromatin structure that alters the sensitivity of the \textit{Sfrp2} locus to DNaseI.
The ability of Pax2 to activate the Sfrp2 promoter region was examined in a heterologous system using transient transfections (Fig. 5). The plasmid p2.8PX-CAT fused a 2.8-kb PstI-Xhol upstream of the CAT reporter gene. Transfection into HEK293 or NIH3T3 cells together with increasing amounts of a Pax2 expression plasmid reveals an ability of Pax2 to transactivate directly this promoter region. Activation of reporter gene expression was 2-fold in the presence of Pax2b protein. Although this was not as robust as other Pax2-responsive elements described, the activation was statistically significant and reproducible.

**DISCUSSION**

The Pax family of transcription factors are essential regulators of embryonic development. Despite the pleiotropic effects of Pax mutations and their association with human disease, relatively few target genes have been described. The data presented in this report suggest that the potential Wnt antagonist, Sfrp2, is a downstream target of the Pax2b protein in embryonic renal cells. Up-regulation of Sfrp2 was observed in clonally derived cells from conditionally transformed metanephric mesenchyme that expressed the Pax2b but not the Pax2a protein. Furthermore, this finding was accompanied by changes in the chromatin organization of the Sfrp2 locus. Pax2 protein can bind to Sfrp2 upstream regulatory elements that map near the DNAseI hypersensitive site that is unique to the Pax2-expressing cells.

Although Pax2 is expressed in metanephric mesenchyme, even prior to induction, high levels persist in mesenchymal aggregates and in polarized epithelial vesicles. Expression of Sfrp2 is found in polarized epithelial vesicles (7, 33, 34), overlapping the Wnt4 and Pax2 expression domains. This would suggest that Pax2 is necessary but not sufficient to activate Sfrp2 in vivo. The cells utilized for the screen seem particularly primed to activate Sfrp2 in response to Pax2 expression. Sfrp2 mRNA is dramatically induced to very high levels from nearly undetectable amounts in the parental cell line. Even transient expression with the Sfrp2 upstream regulatory region that contains two Pax2 binding sites only generates a 2-fold response in NIH3T3 or HEK293 cells, suggesting that the conditionally transformed mesenchymal cells must have other factors that increase the Pax2-dependent response of the endogenous Sfrp2 promoter.

Reciprocal inductive interactions between the mesenchyme and epithelia of the developing kidney promote both branching morphogenesis and polarization of the mesenchyme to form tubular epithelia. Although there is some debate regarding the initial inductive signals that promote mesenchyme aggregation, Wnt4 is essential for propagating these inductive signals, such that the early aggregates form polarized epithelia. Although Sfrp2 expression requires Wnt4 (7), paradoxically the protein can bind to Wnt4 to potentially limit its activity. In the hindbrain and in somitic mesoderm (29), Sfrp2 clearly limits Wnt activity, whereas in the kidney, the role of Sfrp2 may involve derepression of another Wnt antagonist, Sfrp1 (35). How positive and negative regulators of Wnt signaling act to promote epithelial cell polarization and tissue remodeling remains to be determined.

The data presented here indicate that Pax2 may act to alter the local chromatin structure of the Sfrp2 locus such that it is amenable to activation, perhaps by other factors found in the newly polarized renal epithelia. Pax2 is essential for regulating the responses to induction during early kidney development and for specifying the renal epithelial cell lineage. Similar to the role of HNF3 in the liver (36), one potential role for Pax2 in the renal cell lineage may be to alter local chromatin structure so that genes are accessible to other factors, some of which may be expressed at later stages.

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