Regulation of ureteric bud outgrowth by Pax2-dependent activation of the glial derived neurotrophic factor gene

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INTRODUCTION

The development of the mammalian kidney, or metanephros, occurs in a region of posterior intermediate mesoderm by inductive interactions between the metanephric mesenchyme and the ureteric bud epithelium (Kuure et al., 2000; Lechner and Dressler, 1997; Schedl and Hastie, 2000). Induction of the mesenchyme by signals emanating from the bud initiates the aggregation of the mesenchyme and the conversion of these aggregates to the tubular epithelial cells of the nephron. Reciprocally, signals from the mesenchyme induce the ureteric bud to proliferate and undergo branching morphogenesis of the ureteric bud epithelium. We demonstrate that Pax2 expression in the metanephric mesenchyme is independent of induction by the ureteric bud. Pax2 mutants are deficient in ureteric bud outgrowth and do not express GDNF in the uninduced metanephric mesenchyme. Furthermore, Pax2 mutant mesenchyme is unresponsive to induction by wild-type heterologous inducers. In normal embryos, GDNF is sufficient to induce ectopic ureter buds in the posterior nephric duct, a process inhibited by bone morphogenetic protein 4. However, GDNF replacement in organ culture is not sufficient to stimulate ureteric bud outgrowth from Pax2 mutant nephric ducts, indicating additional defects in the nephric duct epithelium of Pax2 mutants. Pax2 can activate expression of GDNF in cell lines derived from embryonic metanephoi. Furthermore, Pax2 protein can bind to upstream regulatory elements within the GDNF promoter region and can transactivate expression of reporter genes. Thus, activation of GDNF by Pax2 coordinates the position and outgrowth of the ureteric bud such that kidney development can begin.

Key words: Pax2, GDNF, Kidney Development, Ureteric Bud, RET signaling, BMP4, Mouse
The metanephric mesenchyme is specified before induction by the ureteric bud. By E11, the mesenchyme appears as a morphologically distinct aggregate of cells that express several unique molecular markers. The Wilms’ tumor suppressor gene Wil1 is expressed in the mesenchyme before induction and is necessary for cell survival and the ability of the mesenchyme to respond to inductive signals (Kreiberg et al., 1993). The Pax2 gene is expressed in the nephric duct and in the epithelial tubules of the mesonephros (Dressler et al., 1990; Dressler and Douglass, 1992). In the developing metanephros, Pax2 is expressed in the mesenchymal cells directly adjacent to the ureteric bud and in the early epithelial derivatives of these mesenchymal cells. Homozygous embryos carrying a Pax2 null allele exhibit nephric duct growth and extension but fail to form mesonephric tubules (Torres et al., 1995), derived from the more anterior periductal mesenchyme. Pax2 mutants also do not have a ureteric bud, although the metanephrine mesenchyme can be observed morphologically. Pax2 encodes a nuclear protein that binds DNA through a conserved paired-domain and activates transcription through a C-terminal domain rich in proline, serine and threonine (Lechner and Dressler, 1992). In the developing metanephros, Pax2 is expressed in the nephric duct and in the epithelial derivatives of these mesenchymal cells. Homozygous embryos carrying a Pax2 null allele exhibit nephric duct growth and extension but fail to form mesonephric tubules (Torres et al., 1995), derived from the more anterior periductal mesenchyme. Pax2 mutants also do not have a ureteric bud, although the metanephrine mesenchyme can be observed morphologically. Pax2 encodes a nuclear protein that binds DNA through a conserved paired-domain and activates transcription through a C-terminal domain rich in proline, serine and threonine (Lechner and Dressler, 1992). Pax2 can activate expression of heterologous promoters that consist of multiple engineered Pax2-binding sites. However, genes expressed in the developing kidney that are under the control of Pax2 have remained elusive.

In this report, we examine the relationship between Pax2 and GDNF. Through the use of mutants in RET, we demonstrate that Pax2 is expressed in the metanephric mesenchyme before induction by the ureteric bud. Experiments with Pax2 mutants demonstrate that within the uninduced mesenchyme, Pax2 is necessary for expression of GDNF. Yet, failure to express GDNF is not the sole cause for the ureteric bud defects in Pax2 mutant embryos. Pax2 can activate GDNF in cultured mesenchymal cells derived from the embryonic kidney. Furthermore, GDNF promoter sequences upstream of the translation start site contain a Pax2-binding site that confers Pax2-dependent activation upon a reporter gene in transfected cells. Thus, Pax2 regulation of GDNF expression establishes a link between the control of mesenchymal patterning and signaling to the nephric duct epithelium in the developing kidney.

MATERIALS AND METHODS

Embryos and organ culture
The Pax2 mutant and the Ret mutant mice (kindly provided by F. Costantini) were kept in a C3H genetic background. Timed matings were set up with the day of the vaginal plug designated as E0.5. Organ cultures were carried out in six-well plates using 0.4 μm transwell inserts and DMEM supplemented with 10% fetal calf serum. For the bead experiments, heparin acrylamide beads (Sigma, St Louis, MO) were incubated in 10 ng/μl of recombinant GDNF (Promega) or a combination of bone morphogenetic protein 4 (BMP4; 10 ng/ml, R&D Systems) and GDNF for 1 hour on ice and washed briefly in phosphate-buffered saline (PBS) before placing on organ cultures.

Whole-mount antibody staining of E11.5 kidneys and organ cultures was as described previously (Cho et al., 1998). Briefly, tissues were fixed in methanol for 10 minutes. and washed twice for 10 minutes. in PBS, 0.1% Tween-20 (PBST). Anti-Pax2 (0.5 mg/ml) and anti-Pan-cytokeratin (Sigma, 1:50) antibodies were diluted in PBST, 2% goat serum and incubated for 3-4 hours at 4°C. Tissues were washed in PBST four times for 1 hour each with one wash overnight at 4°C. The second antibodies, FITC-anti-mouse (Sigma) and TRITC-anti-rabbit (Sigma), were used at 1:100 dilution in PBST, 2% goat serum and incubated for 3 hours. After extensive washing, the samples were placed on slides and covered with gelvatol.

For genotyping: DNA was extracted from embryo yolk sacs by overnight proteinase K digestion in 100 mM NaCl, 10 mM Tris (pH 8.0), 1% SDS at 56°C. Tissues were then treated with RNase at 37°C for 1 hour, extracted with phenol:chloroform, isopropyl alcohol precipitated, washed with 75% ethanol and dissolved in water. Pax2 genotyping was performed by EcoR1 digestion and southern blot analysis using a 1.7 kb NotI/ProtI fragment, which spanned the 5' regulatory sequences and exon 1. PCR was used for Ret genotyping using the primers: Ret P1, TGGGAGAAGGGAGTTGGGAAA; RetP2, TTCAAGAGACTGCTTACATG; NeoP3, AGAGGCTATCTGCCTATCTAGC; and Neo P4, CCTGATCGACAAGAGCCGGTCTC.

Expression analysis of GDNF
Whole-mount in situ hybridization was performed as described (Wilkinson, 1992). To allow better penetration of the GDNF probe the nephrogenic tubules, duct and mesonephric mesenchyme were dissected from E11.5 Pax2+/− null mutants and wild-type embryos for whole-mount analysis. The tissues were fixed in 4% paraformaldehyde (PFA) overnight at 4°C, dehydrated through a PB-TX/methanol series, and stored at −20°C until required.

The tissues were rehydrated, treated with protease K, re-fixed with 0.2% gluteraldehyde/4% PFA and hybridized overnight at 65°C. The following day the tissues were washed and preblocked with 10% lamb serum, 2% BSA in TBTX. The preabsorbed anti-digoxigenin (DIG) antibody was incubated overnight at 4°C. The samples were then washed for 2 days and color was developed with NBT (4-nitro blue tetrazolium chloride) and BCIP (X-phosphate/5-Bromo-4-chloro-3-indolyl-phosphate) in NTMT (pH9.5). Pictures were taken through a Nikon Eclipse E800 with a Diagnostic Instruments SPOT digital camera.

For RT-PCR, metanephric mesenchymes were dissected from E11.5 wild-type, Pax2+/−, Pax2+/−, and Ret null mutants. The RNA was isolated with Trizol (Life Technologies, Bethesda, MA). The Titan one step RT-PCR kit (Roche) was used with dilutions of input RNA. To detect GDNF mRNA, the forward primer was: 5′ GGTATGGGATGCTCGTGCCTGTC and the reverse primer used was: 3′ CCGTTTATGCGGAATGCTTTCCATTAG.

Cell culture
Conditionally immortalized E11 mouse metanephric mesenchyme cells, derived from the Imorto-mouse, were a gift of L. Holzman (University of Michigan). The cell lines contain a temperature-sensitive SV40 T-antigen gene under the control of the interferon response element and were cultured at 32°C with 100 U/ml of interferon in Dulbecco’s modified Eagles media (DMEM) with 10% inactivated fetal calf serum plus 1% penicillin/streptomycin. The clonally derived 46m cell line expresses low levels of WT1, but tested negative for Pax2 expression. Line 46m was subsequently transformed with a Pax2b- and Pax2a-expressing retrovirus containing the neomycin resistance gene and clonally derived cell lines were established. These cell lines were examined for Pax2 expression by western and northern blotting. The 46m cell line also served as the host for Pax2-adenoviral infection. Briefly 2×10⁵ 46m cells/well were seeded out onto a six-well plate and incubated for 24 hours. The cells were washed with 1×PBS and 1 ml of serum free media (DMEM) was added for the infection. Cells were incubated, with serial dilutions of Pax2-adenoviral vector (stock 6.6×10⁹ PFU/ml) ranging from 2×10⁵ to 2×10⁶ PFU/ml for 2 hours and then 3 ml of complete DMEM was added. Infection success was analyzed at 24 hours using the inherent GFP expression by the Pax2-adenoviral vector. At 2×10⁹ PFU/ml,
nearly 100% of the cells were infected as judged by expression of green fluorescent protein. For GDNF activation analyses, cells were infected with 2×10^5 PFU/ml and harvested for RNA at 8, 24 and 48 hours after infection.

**Northern blotting**

Total RNA from cell lines was prepared using the TRIZOL reagent (GIBCO) following the manufacturer’s instructions. Cell lines used for analysis included clone 46 cells, Pax2 retrovirally transformed cells (designated clones 5,8,12,13,24,26,27,28) and Pax2-adenviral vector infected (2×10^6 PFU/10 ml media) clone 46 cells at 8, 24 and 48 hours post infection. Each 100 mm plate contained 1×10^7 cells. Once RNA was isolated and resuspended in RNAase free water, an OD260 was obtained for each sample to determine yield. An aliquot of 10 μg total RNA was electrophoresed in 1% agarose gel containing formaldehyde, blotted on a Hybond-N membrane (overnight) and probed with an exon 2/3 fragment from the GDNF cDNA. The probe was labeled with [32P]dCTP via the Klenow fill in reaction. Binding reactions were performed in a total volume of 10 μl of binding buffer for 15 minutes at room temperature. Subsequently, the DNA probe was added (20,000 dpm) and incubated on ice for 10 minutes followed by a 5 minute room temperature incubation. The DNA was digested with 0.2 units of RNase free DNaseI for 60 seconds and the reaction was terminated with 100 μl stop buffer (50 mM Tris pH8, 100 mM NaCl, 1% SDS, 10 mM EDTA, 1 mg/ml proteinase K, 1 mg/ml sonicated herring sperm). Samples were then incubated at 50°C overnight and extracted once with phenol:chloroform (1:1) and precipitated with ethanol. Samples were resuspended in DNA dye mix (90% formamide) and separated on a 6% denaturing acrylamide sequencing gel. Adenosine + guanine chemical cleavage reactions were performed with piperidine as described (Maxam and Gilbert, 1980).

**GDNF genomic clones and expression plasmids**

A murine GDNF bac clone was identified by screening filter arrays (Research Genetics) with a probe for exon 1. The 4.2 kb BamHI fragment was identified, that contained approximately 1.0 kb of 5’ UTR from exon 1 and 3.2 kb of upstream sequence. The sequence for exon 1 and the potential 5’ regulatory sequences were identical to the published GDNF promoter region (Matsushita et al., 1997; Tanaka et al., 2000) (GenBank Accession Number, D88351). Two reporter vectors were constructed by cloning the 4.2 kb BamHI fragment, containing the 5’ promoter region and a portion of exon 1, into the BamHI multiple cloning site of the BLCAT 6 vector (Luckow and Shuetz, 1987). The plasmid p2.4-CA T contains a 2.4 kb HindIII/BamHI fragment spanning position -1260 to +1052 inserted into the BamHI/HindIII sites of BLCAT 6. The vector p2.4-CA T was digested with Aphi, which cuts in the 5’ UTR at position +713 and +963 [all numbers are from previously published work (Tanaka et al., 2000)], and re-ligated to make the plasmid pΔAphi-CA T.

**Electrophoretic mobility shift assays and DNasel footprinting**

The Pax2 pairwise binding pair (Pax2-PD), amino acids 1-170, was fused to the demonstrated Pax2-binding sites on the GDNF promoter and 5’ UTR. Total digests were labeled with [32P]dCTP by Klenow fill in reaction. Increasing amounts of recombinant Pax2-PD, were pre-incubated with 1 μg poly(dI-dC) in 50 μl of binding buffer for 15 minutes at room temperature. Subsequently, the DNA probe was added (20,000 dpm) and incubated on ice for 10 minutes followed by a 5 minute room temperature incubation. The DNA was digested with 0.2 units of RNase free DNaseI for 60 seconds and the reaction was terminated with 100 μl stop buffer (50 mM Tris pH8, 100 mM NaCl, 1% SDS, 10 mM EDTA, 1 mg/ml proteinase K, 1 mg/ml sonicated herring sperm). Spots were then incubated at 50°C overnight and extracted once with phenol:chloroform (1:1) and precipitated with ethanol. Samples were resuspended in DNA dye mix (90% formamide) and separated on a 6% denaturing acrylamide sequencing gel. Adenosine + guanine chemical cleavage reactions were performed with piperidine as described (Maxam and Gilbert, 1980).

**CAT assays**

NIH 3T3 cells were plated at 500,000 cells per 60 mm dish, cultured in DMEM + 10% FCS and 1% penicillin/streptomycin at 37°C, and transfected the following day with FUGENE (Roche), according to the manufacturer’s protocol. For each 60 mm dish, 6 μl of FUGENE was used per 3 μg of DNA, which contained 0.5 μg of reporter plasmid and 0.2 μg CMV-β-gal for standardization. After a 2 hour exposure to the FUGENE/DNA in DMEM (serum free), DMEM+10%FCS and 1% PS was added to double the volume. Forty-eight hours post-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 lysate was assayed for β-gal activity. Equivalent amounts of β-gal units were then used for the acetylation reactions as described (Gorman et al., 1982). Spots were cut out of the thin layer chromatography plate and scintillation counted. Counts were standardized for background. Each transfection was performed a minimum of three times.

**Site-directed mutagenesis**

Two deletions were made in the p2.4-CA T plasmid using the Quickchange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s directions. The two sites chosen corresponded to the demonstrated Pax2-binding sites on the GDNF promoter and 5’ UTR. Specifically, a 10 bp sequence corresponding to an area within PBS1 was deleted using an HPLC purified set of primers, 5’ GCAAGAACCCTCTGCAGATATTTGGAGCG 3’ and 5’ CGTCTCC-AAAAATTCTGCAAGGCTTTGC 3’. A new PstI restriction site was introduced. A 34 bp deletion was introduced into the 5’UTR which encompassed the Pax2-binding site PBS2. This was accomplished using the primer pair: 5’ CCAAGGCAGGGGCGCCCGGTTGTGG- CGAAGGTG 3’ and 5’ CACCTTCTGACACCGGCGGCCTGTGC- TGG 3’, which introduced an Erei restriction site. Sequencing analysis confirmed that the deletions had been introduced. The parental 2.4-CA T plasmid had the PBS2 site initially deleted (pΔ2.4- CA T) and subsequently the PBS1 was also deleted.

**RESULTS**

**Expression of Pax2 and GDNF in wild-type and mutant embryos**

The expression of Pax2 in the mesenchyme of the developing metanephros was thought to be dependent on induction of the mesenchyme by the ureteric bud. This hypothesis was based upon expression analysis of Pax2 mRNA and protein in wild
type and mutant embryos. At E11, the ureteric bud has grown out of the nephric duct and invaded the metanephric mesenchyme. High levels of Pax2 protein are observed in mesenchyme cells adjacent to the ureteric bud and in the epithelium of the ureteric bud itself. After repeated branching of the ureteric bud, Pax2-positive mesenchymal cells continue to be tightly associated with the ureteric bud tips. In the mutant *Danforth's Short tail* (*Sd*), the ureteric bud grows out but does not induce the mesenchyme and there is no Pax2 expression observed in any posterior mesenchyme from the region of the intermediate mesoderm (Phelps and Dressler, 1993). However, *Sd* embryos lack a posterior notochord and potentially have multiple, posterior patterning defects.

In order to examine the dependence of Pax2 expression on ureteric bud induction, we stained wild-type and *Ret* mutant kidneys for Pax2 expression (Fig. 1). Mice homozygous for a null mutation in *Ret* exhibit a high frequency of renal agenesis, owing to inhibition of ureteric bud outgrowth (Schuchardt et al., 1994; Schuchardt et al., 1996). Because Ret is expressed only in the nephric duct and ureteric bud of the kidney, the metanephric mesenchyme in *Ret* mutants remains competent to respond to inductive signals and are essentially wild type in nature. Surprisingly in E11.5 kidneys, Pax2 expression is still detected in the uninduced mesenchyme of *Ret* mutants and clearly demarcates the metanephric anlagen within the posterior intermediate mesoderm (Fig. 1). Thus, activation of Pax2 expression is independent of induction by the ureteric bud. Despite expression of Pax2, in in vitro cultures of isolated *Ret* mutant, uninduced mesenchyme undergo apoptosis within 24-48 hours of explantation and quickly lose Pax2 expression (data not shown).

Although the nephric duct initially forms in Pax2 mutants, ureteric bud outgrowth is not observed in Pax2 homozygous null embryos (Torres et al., 1995), similar to the *Ret* and *Gdnf* mutant phenotypes. Given the expression of Pax2 in the uninduced mesenchyme, we examined GDNF expression to determine if lack of GDNF may underlie the failure of ureteric bud growth in Pax2 mutants (Fig. 2). Whole-mount in situ hybridization of normal and Pax2 mutant kidneys demonstrated a significant reduction of GDNF expression in Pax2 mutants. At E10.5, GDNF is detected in the posterior intermediate mesoderm in an aggregate of cells adjacent to the nephric duct (Fig. 2A). This region corresponds to the metanephric mesenchyme, although it is otherwise morphologically indistinguishable from surrounding mesoderm. In Pax2 mutants, GDNF expression is not detected significantly above background levels, either at E10.5 or E11.5 (Fig. 2A). However, the levels of GDNF mRNA are low and require extended development times in order for signals to be realized. To confirm this whole-mount data, RT-PCR was performed on isolated E11.5 metanephric mesenchyme from wild-type and Pax2 null mutants using GDNF-specific primers and tenfold serial dilutions of reverse transcribed cDNAs (Fig. 2B). Both wild-type and Pax2 heterozygous kidneys exhibited

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**Fig. 1.** Expression of Pax2 in E11.5 metanephric mesenchyme. Whole-mount antibody staining with antibodies against Pax2 (red) and pan-cytokeratin (green) in wild-type (+/+ ) and *Ret* null mutants as indicated. Pax2 protein is localized to the nephric duct (ND), ureteric bud (UB) and metanephric mesenchyme (MM) in wild-type E11.5 kidneys. Anti-pan-cytokeratin stains the epithelium of the nephric duct and ureteric bud only. Note the expression of Pax2 in the mesenchyme of *Ret* mutants despite the absence of any ureteric bud. Scale bars: 80 μm.

**Fig. 2.** Expression of GDNF in *Pax2* mutants. (A) Whole-mount in situ hybridization of GDNF probe to E10.5 and E11.5 nephric duct (nd) and metanephric mesenchyme (m). In wild-type embryos, GDNF localizes to the posterior mesenchyme of the E10.5 urogenital region. Note the lack of GDNF staining in *Pax2* mutants at both E10.5 and E11.5. (B) RT-PCR from RNAs isolated from E11.5 metanephric mesenchyme; genotypes are as indicated. Serial dilutions of total RNA were used for RT-PCR with GDNF specific primer pairs. Control RT-PCR reactions used primers for the *Gapdh* gene (GP3DH). Note lack of GDNF-specific PCR product in *Pax2* null mutants, but not in *Ret* null mutants.
GDNF expression, as evidenced by the correct sized RT-PCR products, whereas no GDNF specific products were observed in the Pax2 homozygous null cDNAs. The absence of GDNF in Pax2 null mutants is not due to a failure of induction, as mesenchyme from Ret mutants still express GDNF despite the absence of a ureteric bud.

**GDNF responsiveness in wild-type and mutant nephric ducts**

Given the absence of GDNF expression in Pax2 mutants, we examined the ability of exogenously added GDNF to induce ureteric bud outgrowth in Pax2 mutant nephric duct. The entire mesonephros and metanephros regions were dissected out of E10.5 embryos and laid flat onto transwell filters for in vitro culture. A heparin agarose bead, soaked in GDNF was placed near the midline, between the left and right nephric ducts and the cultures allowed to develop for 24 hours. The cultures were then stained for Pax2 and cytokeratin using whole-mount immunostaining. Wild-type cultures exhibited multiple ectopic ureteric buds, emanating from the nephric ducts and growing medially towards the GDNF beads (Fig. 3A). The normal ureteric buds were also observed growing laterally at the posterior end of the nephric ducts. However, Pax2 mutant nephric ducts were unable to respond to GDNF beads, exhibiting no evidence of ureteric bud outgrowth (Fig. 3B), despite a relatively normal epithelial character, as judged by cytokeratin staining and E-cadherin staining (not shown). In wild-type cultures, ectopic ureteric buds were observed only in the posterior region of the nephric duct, in response to GDNF beads (Fig. 3A), whereas the more anterior half of the cultures, including the mesonephric region, were unable to respond to GDNF. The anterior bead appeared further from the nephric duct, but that is due to squashing the organ culture with a glass slide after whole-mount staining. During the culture stage, the anterior and posterior beads are equidistant from the nephric duct. Analysis of Bmp4 heterozygous mouse mutants suggested that BMP4 acts to suppress ureteric bud outgrowth in the more anterior portion of the nephric duct (Miyazaki et al., 2000). Thus, we tested beads soaked in a mixture of GDNF and BMP4 for the ability to promote ureteric bud outgrowth (Fig. 3C). Strikingly, BMP4 was able to suppress the effects of GDNF in the posterior region of the nephric duct. These experiments demonstrate that GDNF is sufficient to direct ureteric bud outgrowth in the posterior nephric duct, that Pax2 mutants are unable to respond to GDNF if supplied exogenously, and that BMP4 can suppress the ability of GDNF to promote ureteric bud outgrowth.

The inability of Pax2 mutant nephric duct to respond to GDNF may be due to lack of RET expression. Thus, we examined wild-type, Pax2 heterozygote and Pax2 homozygous null embryos by whole-mount in situ hybridization using RET-specific probes (Fig. 3D-G). In dissected intermediate mesoderm from E10.5 embryos, wild types and heterozygotes showed RET expression along the entire nephric duct, with particularly strong expression in the developing ureteric bud (Fig. 3D, E). In Pax2 null embryos at E10.5, RET expression was evident in the more anterior nephric ducts and in a few isolated segments of the more posterior duct, albeit overall levels were clearly reduced (Fig. 3F). However, at E9.5, RET expression was not significantly reduced in the Pax2-null embryos (Fig. 3G). The loss of RET expression may be related to a general failure of the mutant nephric duct epithelial to thrive over time. Thus, it is most likely that the Pax2 mutant nephric duct cannot respond to GDNF because of its inability to maintain high levels of RET expression at the time of ureteric bud outgrowth.

**Pax2 mutant mesenchyme is unable to respond to inductive signals**

The lack of GDNF expression and ureteric bud outgrowth...
could explain the complete renal agenesis phenotype in the Pax2 mutants. Though the clear absence of mesonephric tubules in Pax2 null embryos would not have been examined directly. Thus, we cultured Pax2 mutant metanephric mesenchyme with wild-type spinal cord to examine if Pax2 mutant mesenchyme could respond to inductive signals (Fig. 4). As a positive control, uninduced metanephric mesenchyme from homozygous RET embryos were also co-cultured with dorsal spinal cord. After 24 hours in culture, some RET mesenchyme already showed evidence of tubule formation (Fig. 4A). By 72 hours, 100% (3/3) of the RET mesenchymal cultures exhibited characteristic tubules that stained with anti-E-cadherin antibodies (Fig. 4B). By contrast, none of the Pax2 mutant mesenchymes (0/4) exhibited any sign of tubule formation. After 24 hours, the Pax2 mutant tissue was still discernible (Fig. 4A). However, after 72 hours there was little recognizable Pax2 mesenchyme left in the cultures and no expression of E-cadherin (Fig. 4B). Pax2 expression was detected in the spinal cord, which was used as a heterologous inducer. Thus, Pax2 mutant mesenchyme was neither viable for more than 24 hours, nor was it responsive to strong inductive signals.

**Pax2-dependent activation of GDNF**

The phenotypic and genetic analysis suggest that GDNF expression is regulated by Pax2. However, this could be an indirect effect due to compromised viability of the mutant mesenchyme. To demonstrate whether Pax2 was sufficient to activate GDNF, we used an immortalized cell line derived from the metanephric mesenchyme of E11 kidneys. The parental cell line 46m is mesenchymal in character and does not express Pax2 or GDNF. Both Pax2b and Pax2a were introduced by retroviral infection and clonally derived cell lines were selected. The nature of the Pax2-expressing cell lines will be described in more detail in a subsequent report. As determined by Northern blotting, GDNF mRNA was activated in the 46m derived cell lines that also expressed Pax2b (Fig. 5A) with low levels of GDNF mRNA observed in some cell lines expressing Pax2a. As these cells are clonally selected, it was possible that activation of GDNF may have been due to the selection of small populations of GDNF-positive cells from within the larger parental pool. Thus, we used a Pax2-expressing adenovirus to transiently express Pax2 and assay the total cell population. Activation of GDNF mRNA was thus observed in 46m cells within 8 hours of infection with a Pax2-expressing adenoviral vector (Fig. 5B).
Thus, endogenous GDNF can be activated upon Pax2 expression in cell culture.

In order to determine if Pax2 can bind and transactivate the GDNF gene directly, we examined potential GDNF regulatory sequences for Pax2-binding activity and Pax2-dependent transcription activation potential. The mouse (Matsushita et al., 1997) and human GDNF (Grimm et al., 1998) genes consists of three exons, of which exon 1 is entirely non-coding. A mouse BAC library was used to identify exon 1-containing fragments that were then fused to the reporter gene, chloramphenicol acetyltransferase (CAT) (Fig. 6A). Sequences spanning 2.4 or 4.2 kb upstream from a unique BamHI site in exon 1 [+1052, according to Tanaka et al. (Tanaka et al., 2000)] were indistinguishable in their ability to activate reporter gene expression in a Pax2-dependent manner. We thus focused on the smaller construct, p2.4-CAT. Using increasing amounts of Pax2 expression vectors, p2.4-CAT was activated 20- to 30-fold in response to Pax2b and approximately 10- to 15-fold in response to Pax2a (Fig. 6B).

A combination of electrophoretic mobility shift and DNaseI footprint analyses was used to map potential Pax2-binding sites within the 2.4 kb region that demonstrated Pax2-dependent activation of the reporter. Fragments spanning the entire region were isolated and used in gel shift experiments with increasing amounts of recombinant Pax2-PD. In addition, total digests of the 2.4 kb region were subject to gel shift analysis to ascertain if specific fragments could be identified that showed an increase in electrophoretic mobility. From these initial digests at least two binding sites were identified (Fig. 7A). Pax2-binding site 1 (PBS1) was found in a fragment spanning position –237 to –38, upstream of the GDNF mRNA transcription start site. A second, more prominent site (PBS2) was found within the 5' UTR of exon 1 between positions +697 and +968 (Fig. 7A). To map the Pax2-PD binding sequences more precisely, DNaseI footprinting was carried out using the corresponding fragments for PBS1 and PBS2. However, only the fragment corresponding to PBS2 gave a recognizable DNaseI footprint (Fig. 7D), most probably owing to better binding efficiency. The Pax2-PD protected a region spanning +783 to +794 under low concentrations of Pax2-PD, with an extended footprint down to approximately +770 with higher concentrations of Pax2-PD. A double-stranded oligonucleotide corresponding to the DNaseI footprint of PBS2 was used in gel shift experiments to confirm binding specificity. Within the fragment spanning PBS1, a closely related sequence to PBS2 was identified and oligonucleotides made against this region also bound Pax2 (Fig. 7B). Binding to the PBS1 and PBS2 oligonucleotides could be inhibited with increasing amounts of unlabeled competitor DNA (Fig. 7C).

The DNaseI footprinted region of PBS2 and its related sequence in PBS1 were deleted in the 2.4-CAT reporter construct. The plasmid pΔPBS2-CAT has a small 34 bp deletion of PBS2, the stronger of the two Pax2-PD binding sites. Pax2-dependent activation of the CAT gene is reduced approximately threefold using the pΔPBS2-CAT reporter, relative to the p2.4-CAT wild-type construct (Fig. 6B). The vector pΔApa-CAT has a 250 bp ApaI fragment deleted, which encompasses all of the Pax2-binding region and approximately 60 bp 5' and 150 bp 3'. The ability of pApa-CAT to respond to Pax2 was reduced tenfold relative to the full-length insert. However, an additional deletion of PBS1 did not significantly reduce Pax2-dependent activation of the reporter (data not shown). Thus, the significance of the weaker Pax2 binding site, PBS1, is questionable. However, PBS2 is a strong Pax2-binding site that is required for full activation of CAT in this reporter system. The sequence of PBS2 was compared with other known binding sites for Pax2 using the ClustalW alignment program (MacVector, IBI) (Fig. 8). From several published Pax2-binding sites, a consensus sequence emerged. This sequence corresponds to the same region of PBS2 first evident on the DNaseI footprint, with lower concentrations of Pax2 protein.

**DISCUSSION**

Pax2 is essential for the development of the renal epithelia (Torres et al., 1995). Despite its apparent DNA-binding capacity, few genes regulated by Pax2 have been identified. In this report, we demonstrate that activation of GDNF, which is
essential for ureteric bud outgrowth, depends on Pax2. Pax2 is able to bind and transactivate the Gdnf promoter in transfected cells, through interactions with at least one high-affinity binding site, and is able to activate the endogenous Gdnf gene in cells derived from the metanephric mesenchyme. These results indicate a critical early function for Pax2 in the uninduced metanephric mesenchyme that had not been previously appreciated.

The expression analyses of Pax2 in the Ret mutants have led to a re-evaluation of the role of Pax2 in the metanephric mesenchyme. In the mouse, Pax2 expression in the intermediate mesoderm is first detected at around E8.5, before nephric duct formation. Expression in the chick embryo is similar and marks the region destined to form the nephric duct (Obara-Ishihara et al., 1999). At the ureteric bud stage, Pax2 localizes to the epithelium and the mesenchymal cells surrounding the ureteric bud (Dressler and Douglass, 1992; Ryan et al., 1995). Previous analyses in wild type and Sd mutants have suggested that Pax2 expression in the mesenchyme is coincident with induction (Phelps and Dressler, 1993). This interpretation was based on the following observations. In normal embryonic kidneys, Pax2-expressing cells are tightly associated with the ureteric bud tips. In Sd mutants, which fail to induce the mesenchyme, Pax2 is localized to the ureteric bud but not the metanephric mesenchyme. Finally, in vitro cultures of metanephric mesenchyme do not express Pax2 in the absence of inducing tissues. However, the present report clearly demonstrates that the E11.5 Ret mutants express Pax2 protein in the uninduced mesenchyme. As Ret is expressed only in the nephric duct of the developing kidney, the mesenchyme remains essentially wild type in character. Indeed, expression of Pax2 demarcates the metanephric mesenchyme in the absence of any ureteric bud outgrowth. Pax2 expression in the posterior intermediate mesoderm most probably depends on environmental cues, independent of ureteric bud growth. As demonstrated in the chick embryo, such cues may emanate from paraxial mesoderm (Mauch et al., 2000). In vitro culture of dissected metanephric mesenchyme, from either wild type or Ret mutants, results in the rapid decline of Pax2 expression, presumably because these positional cues are lost. Thus, the potential for Pax2 to regulate genes expressed in the uninduced mesenchyme, as well as the induced mesenchyme and the newly differentiating epithelia, must be considered.

GDNF is expressed in the early metanephric mesenchyme and is essential for activating the RET receptor to promote ureteric bud growth (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Vega et al., 1996). We have confirmed that GDNF is sufficient to stimulate ureteric bud outgrowth from wild-type nephric duct, similar to previous reports (Sainio et al., 1997). However, the ability of GDNF to stimulate secondary ureteric bud outgrowth is limited to the posterior half of the nephric duct. Based on the analysis of heterozygous mutants, the secreted signaling peptide BMP4, which is expressed in the surrounding mesenchyme, may limit the effect of GDNF to the more posterior nephric cord.

![Fig. 7. Identification of Pax2-binding sites on the 2.4 kb GDNF promoter region.](image-url)

(A) Electrophoretic mobility shift experiments using isolated fragments or pools of fragments corresponding to the position of the published sequence as indicated. Increasing amounts (0, 1, 5 µl) of diluted Pax2-PD protein were used. The arrows indicate the shifted species in the fragment –237/+38 (PBS1) and +697/+968 (PBS2). PBS2 is also seen in the pooled fragments +500/+1054 (arrow). (B) Electrophoretic mobility shift experiments using increasing amounts of Pax2 protein and oligonucleotides corresponding to the predicted binding sites, based on DNaseI footprinting experiments. (C) Competition experiment using oligonucleotides for PBS1 and PBS2 and increasing amounts of excess (50x, 500x) unlabeled competitor oligonucleotide. (D) DNaseI footprint of PBS2 using increasing amounts of Pax2-PD bound to the fragment corresponding to the region of +697 to +968. Unbroken black bar indicates position of major protected region, with broken line indicating the extended footprint observed at higher concentrations of Pax2-PD.
Regulation of GDNF by Pax2

Fig. 8. Comparison of Pax2-binding sites. The PBS2 site from the GDNF 5’ UTR was
compared with known Pax2 DNA-binding sites.
The previously described sites include a consensus sequence derived by PCR (Epstein et al., 1994); the consensus sequence from chromatin immunoprecipitation studies (Phelps and Dressler, 1996); the Pax2-binding site from the Pax5 enhancer (Pfeffer et al., 2000); and a Pax2 binding site from the WT1 promoter (McConnell et al., 1997). The alignment was made with the ClustalW feature from the MacVector DNA analysis software (Oxford Molecular Group). The PBS2 sequence corresponds to the position +768 to +807 of the published mouse 5’ UTR (Tanaka et al., 2000). The region protected by the Pax2 paired domain is underlined. The gray boxes mark the consensus nucleotides for the set of sequences.

(Miyazaki et al., 2000). Our results with organ culture experiments are consistent with these observations, as BMP4 can suppress the effects of exogenous GDNF. RET signaling activates the mitogen-activated protein kinase pathways (Durick et al., 1998; Marshall et al., 1997) and the phosphatidylinositol 3-kinase pathway (Besset et al., 2000; Murakami et al., 1999; Segouffin-Cariou and Billaud, 2000) in different cell types. At present, it is unclear how BMP4 signaling, presumably through activation of Smad proteins, can suppresses these potential RET signaling pathways.

Despite the early expression of Pax2 in the nephric duct, Pax2 null mutants are able to initiate nephric duct epithelium formation and exhibit nephric duct extension towards the cloaca. Yet the failure to express GDNF in the mesenchyme is not the only defect that suppresses ureteric bud outgrowth in Pax2-null embryos, as GDNF replacement in organ culture cannot rescue the Pax2-null phenotype. Thus, the Pax2 mutant nephric duct is unable to respond to GDNF, demonstrating a cell autonomous defect in the epithelium. Although RET expression in Pax2 mutant nephric duct is normal at early stages (Torres et al., 1995), by E11, RET expression levels may be insufficient to generate bud outgrowth when exposed to ectopic GDNF.

The Pax2 gene is essential for regulating GDNF expression in the posterior intermediate mesoderm. Pax2-null mutants have little to no detectable expression of GDNF mRNA, as determined by whole-mount in situ hybridization and RT-PCR. The Pax2-dependent activation of GDNF is mediated, at least in part, by a high-affinity binding site (PBS2) located within the 5’ UTR of exon 1. These sites were identified by screening isolated fragments, or pools of fragments, by electrophoretic mobility shift experiments. The importance of PBS2 is underscored by a clear reduction in Pax2-dependent transactivation of reporter gene expression when this site is deleted. However, PBS1, which appeared much weaker in the screen did not significantly reduce reporter gene expression when it was deleted from the pA2.4-CAT vector. Thus, the possibility remains that there are additional Pax2-binding sites within the 2.4 kb GDNF sequences that were not identified in our screen. GDNF expression is also lost in mutants for the eyes absent homolog Eya1 (Xu et al., 1999), a gene associated with branchio-oto-renal syndrome (Abdelhak et al., 1997), although this may be indirect because of suppression of Pax2 in the metanephric mesenchyme. A negative regulator of GDNF expression is the forkhead transcription factor Foxc1 (Kume et al., 2000), which acts to restrict the GDNF expression domain to the more posterior metanephric region. Thus, Pax2 is the first direct positive regulator of GDNF that has been identified to date. Other potential targets of Pax2 in the uninduced mesenchyme include WT1, which can be activated by Pax2 in cell culture (Dehbi et al., 1996; McConnell et al., 1997). In the absence of WT1, the metanephric mesenchyme is unable to respond to inductive signals and undergoes apoptosis (Kreidberg et al., 1993). Indeed, the inability of Pax2 mutant mesenchyme to thrive also may be due to the loss of WT1 expression. This would place Pax2 upstream of Wt1 in the genetic hierarchy of mesenchyme specification. Consistent with this model, the expression of Pax2 and GDNF mRNA has been reported in Wt1 mutants (Donovan et al., 1999).

Once mesenchymal cells are induced and form aggregates around the tips of the ureteric bud, GDNF expression begins to decrease. Little GDNF mRNA is detected in the newly polarized renal vesicles (Hellmich et al., 1996; Sainio et al., 1997), despite high levels of Pax2 protein present at this stage and in subsequent stages of epithelial differentiation. Thus, suppression of GDNF in more differentiated mesenchyme must be mediated by some mechanism other than just loss of Pax2 expression. The identification and specification of Pax2 target genes along with their binding sites remains an important issue in elucidating the underpinnings of the molecular mechanisms during urogenital development. This report demonstrates that the Gdnf gene is an early target for Pax2, and that this regulatory axis is essential for controlling ureteric bud outgrowth. While previous mutant analyses have demonstrated a clear need for Pax2 in mesenchymal-to-epithelial conversion (Rothenpieler and Dressler, 1993; Torres et al., 1995), this report establishes an early function for Pax2 in the uninduced mesenchyme that is essential for patterning the posterior kidney region.

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