American Journal of Pathology, Vol. 146, No. 1, January 1995

Short Communication

Comparative *in Situ* Hybridization Analysis of *PAX2*, *PAX8*, and *WT1* Gene Transcription in Human Fetal Kidney and Wilms' Tumors

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Wilms' tumor (WT) is a childhood renal neoplasm with histological features resembling fetal kidney development. Two members of the paired box family of genes,* PAX2 *and* PAX8*, are expressed in WT and are potentially involved in its induction. A zinc finger gene,* WT1*, which is involved in WT induction, encodes a DNA binding protein, and like PAX2 and PAX8 proteins is a transcription factor with an important role in kidney development. We have compared the expression patterns of* PAX2*,* PAX8*, and* WT1 *in fetal kidney and WTs by* in situ *hybridization. The* PAX2*,* PAX8*, and* WT1 *genes were transcribed in the condensed mesenchyme and early stages of epithelial differentiation in fetal kidney.* WT1 *gene transcription was observed in the glomeruli of fetal kidney until a later stage in development than* PAX *genes. In WTs all three genes were expressed in the condensed blastema, but WT1 expression was not detectable in the epithelial structures in two WTs. No evidence of attenuation of* PAX *gene expression was found in WT. These results suggest that in some WTs the expression of WT1 is attenuated in structures that continued to express* PAX *genes. It is unlikely that both* PAX2 *and* PAX8 *genes would be mutated in WT. However, failure of* PAX *gene expression to attenuate in WTs may result from mutations involved in the onset of the tumor. (Am J Pathol 1995, 146:40–45)

Mammalian kidney development involves a mutual inductive interaction between two cell types that have developmentally distinct histories and fates.[1] Epithelial cells, derived from the ureteric bud, induce the metanephrogenic mesenchyme to differentiate into several cell types of the mature kidney, including podocytes, Bowman's capsule, proximal and distal tubules, and stroma. Although little is known about the genetic events regulating renal and urinary tract development, failure of this process to occur properly may be associated with conditions such as renal agenesis, congenital kidney malformations, and renal malignancies.[2] Wilms' tumor (WT) is a solid renal tumor of childhood, which recapitulates fetal kidney development.[3] Three cell types are found in classical WT, blastema, epithelia, and stroma, which are thought to correspond to metanephrogenic mesenchyme, glomeruli and renal tubules, and stroma, respectively, in fetal kidney.[3]

The genetic mutations contributing to WT are believed to occur in genes involved in kidney development. A recently cloned tumor suppressor gene, mutated in 5 to 10% of WTs, is the *WT1* gene.[4–6] *WT1* is expressed in the induced metanephrogenic mesenchyme and in differentiating epithelial structures, including S-shaped bodies and the podocytes of developing glomeruli. The *WT1* gene is not transcribed in the ureteric bud or in the collecting tubule derivatives of the ureteric bud.[7,8] The protein product of *WT1* contains four zinc finger domains, enabling it to bind to specific DNA sequences and to function as a transcription factor.[4,5] Although the genes mutated in the remaining 90 to 95% of WTs are not known, two members of the paired box family of genes, *PAX2* and *PAX8*, have recently been shown to be expressed in

Supported by the Cancer Society of New Zealand.

Accepted for publication September 16, 1994.

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WT and in fetal kidney in a pattern consistent with a role in kidney development.[8–10] The proteins encoded by *PAX* genes have DNA-binding activity associated with the paired box domain[11] and are believed to function as transcriptional regulators, participating in a hierarchical network of gene regulation during kidney embryogenesis.

In the present study we have compared the expression patterns of *PAX2*, *PAX8*, and *WT1* in fetal kidney and WT by *in situ* hybridization. We show that the genes are coordinately expressed with the morphological events that are involved in kidney cell differentiation.[7–10] In human fetal kidney *PAX2* is expressed firstly in the ureteric bud, which is an outgrowth of the Wolffian duct, and then *WT1*, *PAX2*, and *PAX8* are transcribed concurrently in the induced mesenchyme surrounding the ureteric bud. After the early stages of epithelial differentiation the expression of *PAX2* and *PAX8* decline, but *WT1* expression becomes stronger. *WT1* expression peaks in the podocyte cells of the glomeruli in the fetal kidney and then declines. This sequence of induction, followed by expression of *WT1*, *PAX2*, and *PAX8* in the induced mesenchyme occurs repeatedly until the end of gestation when the kidney is fully formed. In two WTs no *WT1* gene transcription was detected in epithelial structures, even though the epithelial structures in these two WTs expressed both *PAX* genes. There was no evidence of attenuation of *PAX* gene expression in any of the WTs. These results suggest that *WT1* expression attenuated in the epithelial structures, whereas *PAX2* and *PAX8* expression continued at high levels. This observation is consistent with the notion that persistent *PAX* gene expression in WTs is associated with events leading to WT onset.

Materials and Methods

WT and fetal kidney sections were cut from frozen tissue onto 3-aminopropyl triethoxysilane-treated or gelatin-coated slides. After brief fixing in 4% paraformaldehyde/phosphate-buffered saline (PBS) the slides were dehydrated in ethanol and stored at −20 C. Murine and human *PAX2* and *PAX8* cDNA sequences were cloned into pGem3 (Promega Corp., Madison, WI). The murine *Pax2* probe was a 540-bp *Bam*HI-*Eco*RI fragment from clone c31A.[12] The human *PAX2* probe was a 450-bp *Hin*dIII-*Pst*I fragment from clone λJ, as previously described,[9] and the *PAX8* probe was a 251-bp *Pvu*II-*Stu*I fragment from H26PS3.[10] The *WT1* (31E1) probe was the full length cDNA, as described,[13] and was cloned into pGem3Z.

Sense and antisense RNA probes were transcribed from linearized templates with SP6 or T7 RNA polymerases (Promega), in the presence of [^{35}S]UTP and [^{35}S]CTP (Amersham, Buckinghamshire, England). The probes (40,000 cpm/μl) were hybridized to tissue sections that had been pretreated with proteinase K (fetal kidney, 2 μg/ml; Wilms tumor, 0.2 μg/ml) for 15 minutes at 37 C and then acetylated (0.1 mol/L triethanolamine/0.25% acetic anhydride). Hybridizations were carried out in 0.3 mol/L NaCl, 10 mmol/L Tris-Cl, pH 6.8, 10 mmol/L sodium phosphate, 5 mmol/L EDTA, 10% dextran sulfate, 50 mmol/L dithiothreitol, 0.02% Ficoll, 0.02% polyvinylpyrollidine, 0.02% bovine serum albumin, 1 mg/ml tRNA, and 50% formamide at 48 C for 16 hours. After hybridization the slides were treated with RNAse A (100 μg/ml) and RNAse T1 (5 ng/ml, Sigma Chemical Co., St. Louis, MO) at 48 C and washed in 2X SSC. Slides were coated in LM-1 emulsion (Amersham) and exposed for 10 to 30 days at 4 C.

Results

Comparison of the Transcription Patterns of PAX2, PAX8, and WT1 Genes in Human Fetal Kidney

Using human-specific *PAX2*, *PAX8*, and *WT1* gene sequences to make RNA probes, we analyzed the expression of each gene in sections of 18-week gestation human fetal kidney (Figure 1). Unlike adult kidneys, the outer cortical region (nephrogenic zone) of kidneys from 18-week gestation human fetuses contain all stages of epithelial differentiation of the metanephrogenic mesenchyme. Hybridization was observed with the antisense RNA probes in the nephrogenic zone near the edge of the growing kidney. In this zone the branching ureteric epithelium grows into and induces the loose metanephrogenic mesenchyme to proliferate. During proliferation the loose nephrogenic mesenchyme aggregates to become regions of condensing mesenchyme. These cells then differentiate to form comma-shaped and S-shaped bodies. In Figure 1b, d, and f hybridization was observed with the *PAX2*, *PAX8*, and *WT1* probes, respectively, on the ureteric epithelium and the condensed mesenchyme and its immediate derivatives.

Using the *PAX2* probe, we observed specific hybridization over the ureteric epithelium and condensed mesenchyme. The expression level of *PAX2* over the ureteric epithelium was equivalent to the level of *PAX2* over the condensing mesenchyme (Figure

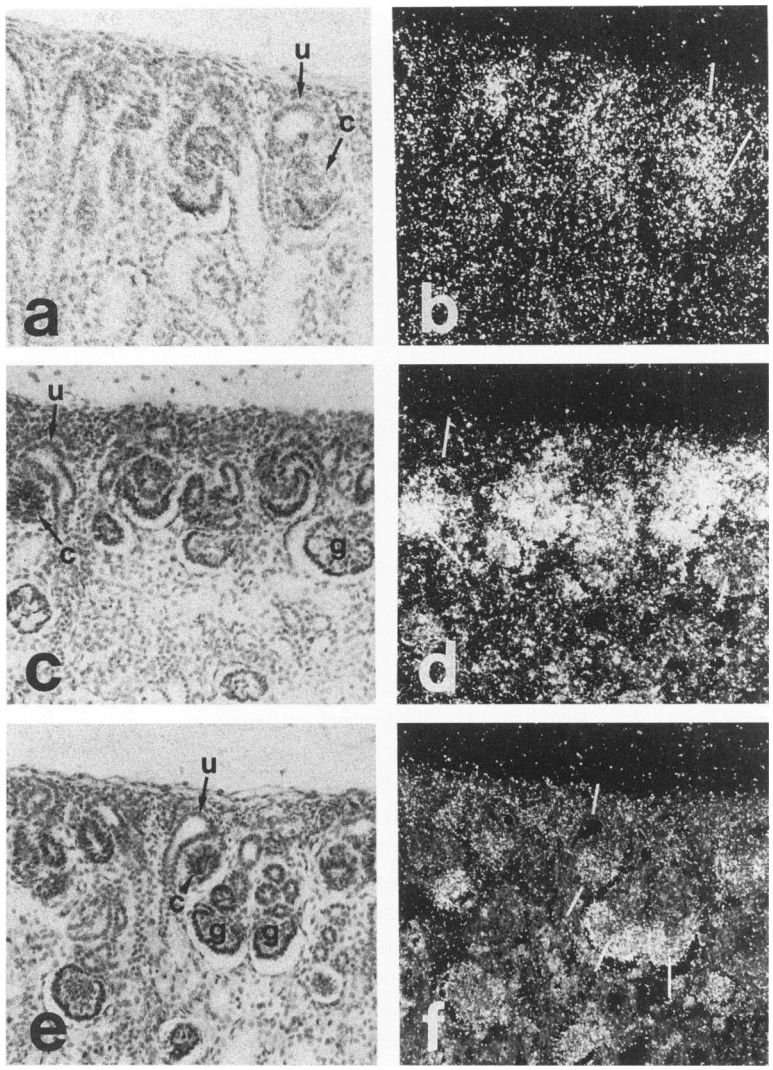

Figure 1. In situ *hybridization analysis of* PAX2, PAX8, *and* WT1 *gene expression in human fetal kidney.* ^{35}*S-radiolabeled RNA riboprobes were hybridized to tissue sections of human fetal kidney. Bright-field photomicrographs are shown at the left, and dark-field photomicrographs at the right. Silver grains, appearing as white spots in the dark-field photomicrographs, indicate hybridization of the probe to mRNA transcripts in the tissue section. Hybridization of* PAX2, **a** *and* **b**; *hybridization of* PAX8, **c** *and* **d**; *and hybridization of* WT1, **e** *and* **f**. *Hybridization was detected with each probe on condensed mesenchyme (c). Hybridization was detected with* PAX2 *on ureteric bud epithelium (u), but not with* PAX8 *or* WT1. *Hybridization was detected with WT1 on glomeruli (g), but not with* PAX2 *or* PAX8. *Magnification, ×400.*

1a, b). *PAX2* transcription was not observed over mature glomeruli (not shown).

The transcription of *PAX8* was largely confined to the edge of the fetal kidney in the condensing mesenchyme of the nephrogenic zone. Like *PAX2*, the transcription of *PAX8* attenuated in differentiating structures and was at background levels before the formation of glomeruli (Figure 1c, d).

The *WT1* gene was transcribed in the condensing mesenchymal cells and also in the immature and mature glomeruli, particularly in the presumptive podocyte cells of the glomerulus (Figure 1e, f). Transcription of *WT1* was not restricted to the nephrogenic zone near the edge of the growing kidney and continued in the glomeruli deep into the kidney cortex. Transcription levels of *WT1* were much higher in the podocyte cells than in the condensed mesenchyme.

Although caution should be taken in comparing levels of gene expression from *in situ* hybridizations done on different tissue sections, expression levels within the same slide may be compared, and patterns of expression between slides may be compared. Comparing the level of *PAX8* hybridization between structures in Figure 1c and d shows that *PAX8* was weakly transcribed in the ureteric epithelium, as hybridization was not totally absent over the ureteric epithelium as previously reported.[9] The level of *PAX8* in the ureteric epithelium was, however, very much lower than in condensed mesenchyme. In contrast, *WT1* transcription was not detected in the ureteric epithelium (Figure 1e, f) but was strongly transcribed in the podocyte cells of the glomerulus. The glomeruli showed no transcription of *PAX2* or *PAX8*, indicating that *PAX* gene transcription was rapidly downregu-

lated compared with *WT1* transcription as the nephron structures differentiated.

Comparison of the Transcription Patterns of PAX2, PAX8, *and* WT1 *Genes in WTs*

The probes used for this analysis were synthesized from human-specific gene sequences, except for *PAX2*, which was a murine probe. Specific patterns of transcription were observed only with the antisense riboprobes of *PAX2*, *PAX8*, and *WT1* in WT. A brief description of the histology of each WT is given in Table 1. The transcription of *PAX2*, *PAX8*, and *WT1* genes was detected in condensed blastema in each WT analyzed (Table 1 and Figures 2 and 3). The transcription of *PAX2* and *WT1* was detected at high levels in condensed blastema irrespective of the size of the group of cells, whereas *PAX8* transcription was detected at low levels in small groups of condensed blastemal cells. Within the same sections, however, *PAX8* transcription was detected at high levels in larger groups of condensed blastemal cells (Table 1).

WT1 gene transcription was not detected in epithelial structures in two WTs (for example, Figure 2a), whereas *PAX2* and *PAX8* transcription was detected in epithelial structures in these tumors (Fig 2b, c). This pattern contrasts with the results of other WTs, in which *WT1*, *PAX2*, and *PAX8* gene expression was always detected in the epithelial structures (for example, Figure 3a–c). The results of the analysis are summarized in Table 1.

Discussion

WT arises in the metanephrogenic mesenchyme of the developing kidney and has features resembling kidney development.[3] Candidate genes that cause WT are likely to be involved in controlling the differentiation of metanephrogenic mesenchyme. The WT gene, *WT1*, is a tumor suppressor gene involved in WT, that is located on chromosome 11p13[4,5] and is essential for kidney development.[14] This gene has been shown to incur deletions and mutations in 5 to 10% of sporadic WTs.[6] Recently, two additional genes involved in kidney development have been cloned, *PAX2* and *PAX8*.[9,10] In this study we examined the transcription patterns of the *PAX2*, *PAX8*, and *WT1* genes in fetal kidney and WTs by *in situ* hybridization. Although the transcription patterns of these genes have been individually reported in fetal kidney and WT,[7–10] their expression patterns have not been compared, except by Northern blot analysis.[15] We detected *PAX2*, *PAX8*, and *WT1* gene transcription in fetal kidney in patterns that were very similar to previous reports.[7–10] In addition, transcription of the three genes was observed in epithelial structures and condensed blastema in WTs as previously described.[7–10] When the expression patterns of the three genes in WTs were compared, significant differences were observed between *WT1* and the two *PAX* genes. High levels of *PAX2* and *PAX8* transcription were detected in the epithelial structures in all WTs examined, whereas in some WTs transcription of *WT1* was not detectable in epithelial structures.

The differences in transcription between *PAX2*, *PAX8*, and *WT1* genes that we have observed in WTs cannot be explained by the expression patterns observed within the equivalent structures in fetal kidney. In fetal kidney the *WT1*, *PAX2*, and *PAX8* genes were coexpressed in the condensed mesenchyme and its derivatives. After differentiation of the metanephrogenic mesenchyme in fetal kidney, transcription of *PAX2* and *PAX8* attenuated rapidly relative to *WT1*.

Table 1. *Comparison of PAX2, PAX8, and WT1 Expression in Wilms' Tumors*

Tumor	Anaplasia	Predominant cell type	Cell type	*PAX2* expression	*PAX8* expression	*WT1* expression
Mich9	No	Blastema	CB	+	+ (larger)†	++
			Ep	+	+	++
			Stroma	–	–	–
55	No	Triphasic	CB	++	+++ (larger)	+++
			Ep	+	+	+
			Stroma	–	–	–
65	No	Triphasic	CB	++	+++ (larger)	+++
			Ep	+++	+++	–
			Stroma	–	–	–
77	Yes	Blastema	CB	++	++ (larger)	++
			Ep	++	++	–
			Stroma	–	–	–

CB, condensed blastema; Ep, epithelia.
*–, not transcribed; +, low transcription, ++, moderate transcription; +++, high transcription.
†*PAX8* transcription was detected in larger groups of cells.

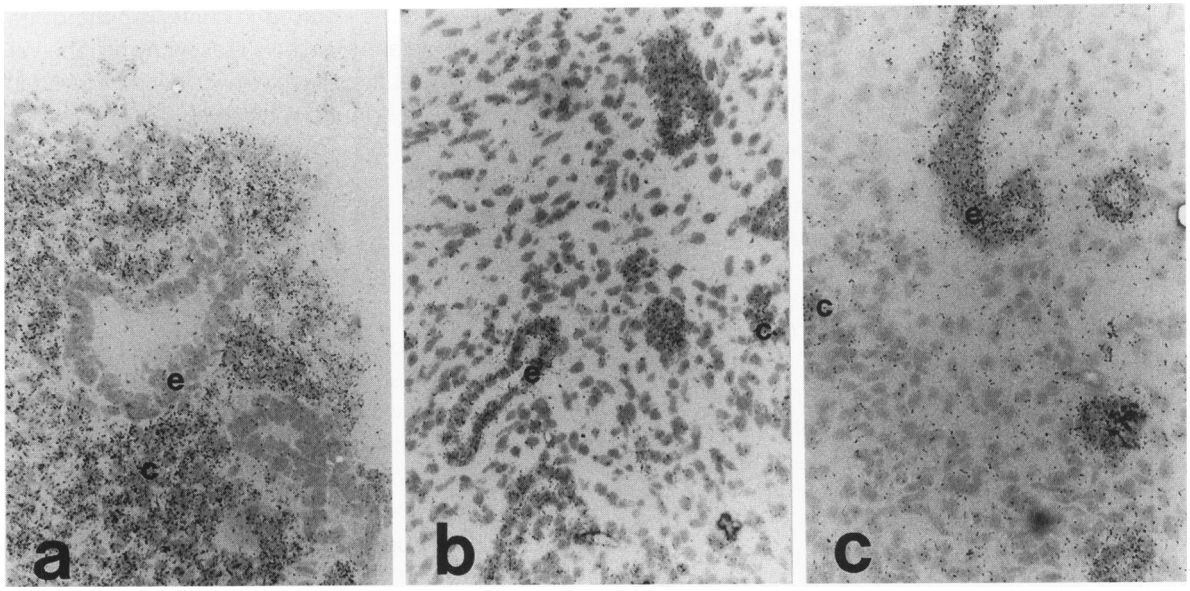

Figure 2. In situ *hybridization analysis of* PAX2, PAX8, *and* WT1 *gene expression in WT 65. Bright-field photomicrographs are shown in which silver grains above the tissue indicate hybridization of the probe to mRNA in the tissue section. The* WT1 *probe was used in* panel a, *the* PAX2 *probe in* panel b, *and the* PAX8 *probe in* panel c. *Hybridization was observed to condensed blastema (c) with each probe and to epithelial structures (e) with* PAX2 *and* PAX8, *but not with WT1. Magnification, ×400.*

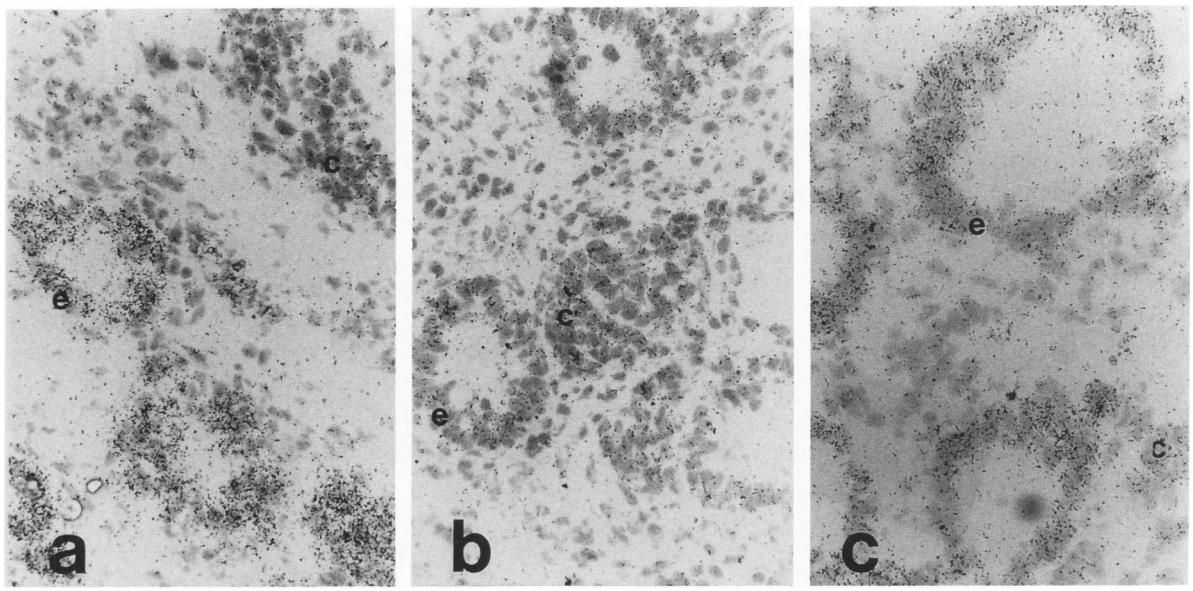

Figure 3. In situ *hybridization analysis of* PAX2, PAX8, *and* WT1 *gene expression in WT 55. Bright-field photomicrographs are shown in which silver grains above the tissue indicate hybridization of the probe to mRNA transcripts in the tissue section. The* WT1 *probe was used in* panel a, *the* PAX2 *probe in* panel b, *and the* PAX8 *probe in* panel c. *Hybridization was observed to condensed blastema (c) and epithelial structures (e) with each probe. Magnification, ×400.*

WT1 was not expressed in the ureteric epithelium or its derivatives in fetal kidney, and *PAX8* was expressed at very much reduced levels in the ureteric epithelium. We have observed that the epithelial structures in WTs lacking *WT1* expression continued to express high levels of *PAX8* as well as *PAX2*. The most likely explanation is that the epithelial structures in the tumors were derived from the condensed blastema of the tumor and that the *WT1* expression attenuated in the epithelial structures, whereas *PAX2* and *PAX8* expression continued at high levels.

The exact role of *PAX2*, *PAX8*, and *WT1* in kidney development is not known, although several studies suggest a role in differentiation.[14,16,17] Each of the three genes are believed to encode DNA-binding proteins.[11,18] It has been suggested that downregulation of *PAX2* expression is a necessary event for the terminal differentiation of kidney cells.[16] Constitutive

PAX2 expression in transgenic mice resulted in severe kidney abnormalities in 18-day gestation and newborn pups, namely, multifocal microcystic tubular dilation.[16] In contrast, the complete absence of the WT gene product (*WT1*) in mice resulted in failure of the kidneys to develop,[14] suggesting that *WT1* is important in the development of kidneys. In mice lacking *WT1* the *PAX2* gene was not expressed in the mesenchymal cells, although it was expressed in the ureteric bud.[14] This suggests that *WT1* is required for both the induction of the mesenchymal cells and for *PAX2* expression in the mesenchyme. The function of *PAX2* in the induced mesenchyme must be at a later stage in development than that of *WT1*.

It is possible that the failure of *PAX2* and *PAX8* expression to attenuate in WTs is associated with WT onset. However, it is unlikely that both *PAX2* and *PAX8* genes would be directly involved in WT through mechanisms involving gene mutation. A more likely possibility is that a gene regulating transcription of the *PAX* genes would be implicated in sporadic WTs. A gene that transcriptionally represses *PAX2* and *PAX8* could be a tumor suppressor gene. Interestingly, the 5′ sequence of the *PAX2* cDNA contains EGR-1 consensus sequences.[9] WT1 has been shown to bind to and repress transcription from promoters that contain EGR-1 consensus sequences,[18,19] and therefore the possibility exists that WT1 may repress *PAX2* transcription. Determination of the roles and interactions of the products of *PAX2*, *PAX8*, and *WT1* will improve our understanding of WT and abnormalities in renal development.

Acknowledgments

We thank Drs. G. Dressler for the murine *PAX2* cDNA clones, D. Plachov for the human *PAX8* cDNA clone, and B. Williams for the *WT1* plasmid p31E1. We also thank K. Bove and D. Becroft for tissue samples and for histological analyses and W. Gillet for fetal kidney tissue. Ethical approval was granted by the Otago Hospital Board.

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