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Repression of *Pax-2* by *WT1* during normal kidney development

Gabriella Ryan[1], Victoria Steele-Perkins[1], Jennifer F. Morris[2], Frank J. Rauscher, III[2] and Gregory R. Dressler[3,*]

[1]Laboratory of Mammalian Genes and Development, National Institute of Child Health and Human Development, Bethesda, MD 02982, USA
[2]The Wistar Institute, 3601 Spruce St., Philadelphia, PA 19104, USA
[3]Howard Hughes Medical Institute and Department of Pathology, University of Michigan, Ann Arbor, MI 48109, USA

*Author for correspondence

SUMMARY

The developmental, regulatory gene *Pax-2* is activated during early kidney morphogenesis and repressed in mature renal epithelium. Persistent *Pax-2* expression is also observed in a variety of kidney tumors. Yet, little is known about the signals regulating this transient expression pattern in the developing kidney. We have examined the spatial and temporal expression patterns of Pax-2 and the Wilms' tumor suppresser protein WT1 with specific antibodies in developing mouse kidneys. A marked increase in WT1 protein levels coincided precisely with down-regulation of the *Pax-2* gene in the individual precursor cells of the visceral glomerular epithelium, suggesting a direct effect of the WT1 repressor protein on *Pax-2* regulatory elements. To examine whether WT1 could directly repress *Pax-2* transcription, binding of WT1 to three high affinity sites in the 5′ untranslated *Pax-2* leader sequence was demonstrated by DNAseI footprinting analysis. Furthermore, co-transfection assays using CAT reporter constructs under the control of *Pax-2* regulatory sequences demonstrated WT1-dependent transcriptional repression. These three WT1 binding sites were also able to repress transcription, in a WT1-dependent manner, when inserted between a heterologous promoter and the reporter gene. The data indicate that *Pax-2* is a likely target gene for *WT1* and suggest a direct link, at the level of transcriptional regulation, between a developmental control gene, active in undifferentiated and proliferating cells, and a known tumor suppresser gene.

Key words: *Pax-2*, *WT1*, kidney development, Wilms' tumor, transcription repression, mouse

INTRODUCTION

The interactions among genes that control growth and differentiation of the embryo and potential tumor suppresser genes that may act to limit proliferation by specifying terminal differentiation, are fundamental to understanding the molecular mechanisms of development and oncogenesis. The pediatric nephroblastoma, Wilms' tumor, is a paradigm for how the loss of developmental control processes can result in oncogenic transformation. Wilms' tumor originates in the embryonic kidney through the inactivation of multiple tumor suppresser genes (for review see Van Heyningen and Hastie, 1992; Huff and Saunders, 1993). Genetic analysis has identified a number of candidate tumor suppresser genes mapping to the 11p13 locus (Gessler at al., 1990; Call et al., 1990; Huang et al., 1990) one of which, the *WT1* gene, encodes multiple forms of a DNA binding protein of the zinc-finger type. At least one form of the WT1 protein binds a recognition sequence similar to that of the early growth response gene, *EGR-1*, (Rauscher et al., 1990) and is a potent transcription repressor when assayed in transfected cells (Madden et al., 1991). During normal kidney development, *WT1* is expressed in the mesenchymal cells and in differentiating epithelium, with maximum levels in the podocyte cell of the glomerular epithelium (Pritchard-Jones et al., 1990; Amstrong et al., 1992; Mundlos et al., 1993). Potential targets for WT1 repression include EGR-1 (Madden et al., 1991), insulin-like-growth factor II (Drummond et al., 1992) and its receptor (Werner et al., 1993, 1994) and platelet-derived growth factor (Wang et al., 1993). Mutation of the *WT1* gene by homologous recombination indicates that it is required for the earliest phase of kidney development (Kreidberg et al., 1993).

The adult kidney develops after the ureteric bud induces the metanephric mesenchyme to condense and initiate formation of the renal tubules (for review see Saxen, 1987). Whereas the tubular epithelium of the nephrons are derived from the mesenchyme, the collecting duct epithelium is produced by the branching ureteric bud. Among the genes expressed in early kidney development is *Pax-2*, a member of a multigene family encoding morphogenic transcription factors (Gruss and Walther, 1992). The *Pax-2* gene is expressed in condensing metanephric mesenchyme and in early epithelial structures derived from the mesenchyme, but *Pax-2* mRNA and protein levels are quickly down regulated as the tubular epithelium matures (Dressler et al., 1990; Dressler and Douglas, 1992). In embryonic kidney organ cultures, repression of *Pax-2* by

antisense oligonucleotides inhibits condensation of the mesenchyme and subsequent conversion to epithelium (Rothenpieler and Dressler, 1993). Persistent expression of *Pax-2* in the kidneys of transgenic mice also results in developmental abnormalities, similar to human nephrotic syndromes (Dressler et al., 1993). Thus it appears that properly timed activation and repression of *Pax-2* is essential for normal kidney development.

In the epithelial components of human Wilms' tumors *Pax-2* expression persists (Dressler and Douglas, 1992; Eccles et al., 1992), suggesting that negative regulators of *Pax-2* are inactive. *Pax-2* expression can also be observed in human adult renal cell carcinoma, both in primary tumors and tumor-derived cell lines (J. Gnarra and G. Dressler, unpublished data). Thus, identifying the molecular mechanisms of *Pax-2* down-regulation during normal nephrogenesis and understanding how these mechanisms are lost during the initiation and progression of renal tumors may illuminate the genetic basis of renal oncogenesis. In this work, the ability of the Wilms' tumor suppresser protein WT1 to regulate *Pax-2* transcription was examined. Immunostaining for Pax-2 and WT1 proteins in adjacent sections of developing murine kidneys clearly demonstrates an increase in WT1 expression precisely in those structures that are beginning to down-regulate *Pax-2*. In addition, the 5′ untranslated leader sequence of the *Pax-2* mRNA contains multiple WT1 binding sites, as determined by DNAseI footprinting assays. Furthermore, the WT1 protein can repress transcription of a reporter gene driven by 5′ *Pax-2* sequences or from a heterologous promoter with a *Pax-2* untranslated leader sequence. The functional role of these sequences was confirmed through EGR1 activation. These data suggest that *Pax-2* is a target of *WT1* transcription repression during the course of normal kidney development.

MATERIALS AND METHODS

Immunostaining

FVB/N inbred mice were mated and the day of the appearance of the vaginal plug was designated 0. Embryos were dissected free of extraembryonic tissue and frozen on dry ice. Cryostat sections were cut at 8 μm, collected on gelatinized slides, and air dried for 30-60 minutes. Immunostaining was as described by Harlow and Lane (1988). Sections were fixed in acetone for 10 minutes at –20°C, then washed in PBS, 0.05% Tween-20 twice for 5 minutes. A 10 μg/ml dilution of anti-Pax2 IgG or anti-WT1 IgG in a 1:500 dilution of rat anti-uvomorulin (Sigma) was prepared in 2% goat serum in PBS, and 20 μl were applied to each section. Slides were incubated at room temperature in a humid chamber and then washed twice in PBS/Tween. The second antibodies were diluted 1:32 for the TRITC-conjugated anti-rabbit (Sigma) and 1:20 for the FITC-conjugated anti-rat (Sigma) in 2% goat serum in PBS. After a 30-minutes incubation, slides were washed twice in PBS/Tween and covered with gelvatol. Control sections were incubated with a pre-immune rabbit IgG purified fraction and both second antibodies. No specific staining could be detected with the pre-immune IgG from rabbits immunized with WT1 antigens or Pax-2 antigens (data not shown).

Genomic clones and S1 nuclease protection experiments

The overlapping cosmids, cos-26 and cos-28, were identified by screening a genomic 129 cosmid library (courtesy of B. Hermann) with probes specific for the paired domain. Restriction mapping was done using partial digestion and the λ terminase system (Amersham). For mapping the transcription start sites, DNA probes were isolated from a plasmid containing the 1.7 kb *Not*I-*Pst*I fragment corresponding to the presumptive first exon and upstream genomic sequences. The entire 1.7 kb *Not*I-*Pst*I fragment was sequenced and is available through GenBank, accession number U13975. The Probe 1330 was prepared by digesting with *Not*I, dephosphorylating with calf intestinal phosphatase, and subsequently cutting the DNA with *Kpn*I. Probe 520 was digested with *Pvu*II, dephosphorylated, and then digested with *Apa*I. The fragments were purified on low melting point agarose gels and end-labeled with [γ-^{32}P]ATP and polynucleotide kinase. Approximately 10,000 cpm of probe and 20 μg of total RNA from E18 kidneys or 20 μg of total yeast, control RNA were mixed and precipitated with ethanol. The hybridization conditions and S1 nuclease digestion was as described by Berk and Sharp (1978) with minor modifications. RNA and probes were resuspended in 25 μl of 80% formamide, 400 mM NaCl, 40 mM Pipes, pH 6.5, 2 mM EDTA. Samples were heated to 75°C for 15 minutes and then hybridized overnight at 55°C. Hybridization temperatures were titrated over a range (45-60°C) to maximize RNA/DNA hybrids relative to probe reannealing. Samples were placed on ice and digested with 150 Units of S1 nuclease (Boehringer Mannheim) in 0.5 ml of 33 mM sodium acetate (pH 5.0), 200 mM NaCl, 30 μM $ZnSO_4$, 20 mg/ml denatured salmon sperm DNA. Digestions were done at 37°C for 60 minutes followed by addition of 0.1 ml Stop Buffer (0.5 M Tris, pH 9.5, 0.1 M EDTA), extraction with phenol/chloroform, and ethanol precipitation. Samples were separated on 6% denaturing acrylamide sequencing gels.

DNAseI footprinting

DNAs were digested with restriction enzymes and individual fragments were isolated on low melting point agarose gels. The production of recombinant proteins and purification using nickel chelate affinity chromotography has been described previously (Madden et al., 1991). For DNAseI footprinting assays, DNA fragments were labeled with 2-4 molecules of dCTP and dGTP by the Klenow fill-in reaction. The recombinant proteins were preincubated 15 minutes at room temperature with 1 μg poly dI-dC in 50 μl binding buffer prior to the addition of approximately 20,000 dpm of labeled DNA. After an additional 15 minutes at room temperature, 0.4 units of DNAseI (Boehringer) was added and the reactions were incubated for 60 seconds. The DNAse digestion was stopped by adding 100 μl of Stop buffer (50 mM Tris, pH 8, 100 mM NaCl, 1% SDS, 10 mM EDTA, 1mg/ml pronase, 5 μg salmon sperm DNA). After 60 minutes at 50°C, samples were extracted once with phenol: chloroform (1:1) and precipitated with ethanol. Samples were resuspended in 90% formamide and separated on 6% denaturing acrylamide sequencing gels. The A+G chemical cleavage reactions were performed with piperidine as described by Maxam and Gilbert (1980).

Transfection assays

The CAT/SV40 cassette was made by ligating *Not*I linkers to a 2.2 kb *Hin*dIII/*Pst*I fragment from the plasmid RSV-CAT (Gorman et al., 1982a). This contains the entire CAT coding region and the SV40 early polyadenylation site preceded by a 140 bp intron. For pH1-CAT, the CAT cassette was inserted into the unique *Not*I site of an 11 kb *Hin*dIII fragment from cos-26, cloned into pGem7 (Promega) and containing the first exon of *Pax-2*. The plasmid p4.3CAT contains a 4.3 kb *Bam*HI/*Not*I fragment from cos-26 inserted into the *Bam*HI/*Xho*I site of BLCAT6 (Boshart et al., 1992). The plasmid TK630 was made by cloning a 630 bp *Not*I/*Eag*I (+25 to +675), corresponding to most of the 5′ untranslated leader sequence, and inserting between the thymidine kinase (TK) promoter and the CAT coding region of BLCAT2 (Luckow and Schutz, 1987) with linkers. The expression vectors containing the human WT1 cDNAs (CMV-WT1 and CMV-WT1+KTS) have been described previously (Morris et al., 1991; Madden et al., 1991). The mouse EGR1 coding region was used to construct the expression vector CMV-EGR1 (Sukhatme et al., 1988; Madden et al., 1991).

NIH 3T3 cells were plated at 400,000 cells per 60 mm dish and transfected the following day with lipofectamine (Life technologies) according to the manufacturers protocol. A total of 6 μg plasmid DNA was used with 12 μl of lipofectamine. For reporter plasmids pH1-CAT and p4.3CAT, 1 μg of DNA was used and 0, 2, or 5 μg of CMV-WT1, or CMV-WT1+KTS, or CMV-EGR1 were added. A plasmid, CMV-CB6, was used to bring the total to 6 μg of DNA per sample. For plasmids BLCAT2 and TK630, 0.5 μg of reporter DNA was used. After a 7-hour exposure to the lipofectamine/DNA, the cells were washed twice with DMEM (serum free) and refed with fresh DMEM + 5% fetal calf serum. 72 hours post transfection, cells were scraped, pelleted and resuspended in 0.1 ml of 0.25 M Tris (pH 8.0). Cells were lysed by freeze-thawing 3 times and debris was pelleted. Lysates were assayed for total protein (Bio-rad protein assay) and equivalent amounts of protein were used for acetylation reactions as described (Gorman et al., 1982b). Spots were cut out of a thin layer chromatography plate and counted. Transfections were repeated at least three times in the experiments involving CMV-WT1 and twice in the experiments with CMV-WT1+KTS and CMV-EGR1.

RESULTS

Comparative expression of WT1 and Pax2

In order to localize Pax-2 and WT1 proteins in the developing kidney, specific antibodies were used in adjacent serial sections at various developmental time points. The Pax-2 polyclonal antibodies were produced against a bacterial fusion protein containing the C-terminal Pax-2 domain; the specificity of the Pax-2 antibodies in western blotting and immunohistochemistry has been described (Dressler and Douglas, 1992). The WT1 specific polyclonal antibodies were raised against amino acids 1-179 and detect WT1 in immunoprecipitation and immunohistochemical assays (Morris et al., 1991). As a marker for differentiated tubular and ductal epithelium, each section was double stained with a monoclonal antibody against the epithelial adhesion molecule uvomorulin.

Induction of the mesenchyme by the ureteric bud occurs around E11.5 (embryonic day 11.5) and leads to branching of the ureter and condensation of the mesenchyme. By E12, the ureter has branched four to eight times with mesenchyme condensates found at each tip. At E12, Pax-2 protein is clearly detectable in the condensing mesenchyme and in the ureter epithelium (Fig. 1A), whereas WT1 protein is barely visible (Fig. 1B). The ureter and its branched derivatives stain clearly with the uvomorulin antibodies. By E14, the ureter has branched many times as the collecting duct system is formed and the kidney has assumed its characteristic shape. The mesenchyme continues to proliferate and condense near the perimeter of the growing kidney, whereas nearer the medullary zone, the mesenchyme-derived tubular epithelial structures have undergone a number of morphological changes, including the formation of comma and s-shaped bodies. Pax-2 expression persists in the condensing mesenchyme as well as in the comma-shaped bodies (Fig. 1C), however, protein levels begin to decline in the prospective proximal tubule region of the s-shaped body (Fig. 1E). WT1 protein can now be detected readily in the proximal loop of the s-shaped body (Fig. 1D), where expressing cells are the presumptive precursors of the podocyte epithelium that surrounds the vascularized glomerulus (Fig. 1D,F). Note that structures showing a marked decrease in Pax-2 protein levels are beginning to show increased WT1 expression. These patterns of Pax-2 and WT1 expression are similar throughout later kidney development, showing little overlap and persisting until after birth. In the newborn kidney, Pax-2 expression is still restricted to the periphery where nephrogenesis is ongoing (Fig. 1G). Note that the density of tubules is much greater and the Pax-2 expressing structures are more localized to the immediate perimeter. There are persistent low levels of Pax-2 expression in the ureter derived collecting ducts, as noted previously (Dressler and Douglas, 1992). High levels of WT1 are still localized to the epithelium immediately surrounding the glomerulus and low levels of WT1 expression in the mesenchyme-derived structures, nearer the periphery, are now more evident. However, few, if any, cells express both high levels of WT1 and Pax-2. Note that uvomorulin is expressed in all proximal and distal tubules and in collecting duct epithelium, but is not expressed in the glomerulus. These data show an inverse correlation between the levels of WT1 and Pax-2 proteins in normal developing kidney.

The *Pax2* gene contains WT1 binding sites

The *Pax-2* gene is a complex locus with at least 9 exons spanning more than 50 kb (G. R. D., unpublished data). The first 4 exons were identified in two overlapping genomic cosmid clones (Fig. 2A). In order to determine the start of transcription, S1 nuclease protection assays were designed using genomic probes spanning the first exon (Fig. 2B). Probe 1330 was end labeled at the *Not*I site, 30 nucleotide upstream of the translation start site, and extends approximately 1275 bp to a *Kpn*I site. The double stranded probe was denatured and hybridized with embryonic kidney RNA, or control RNA, under conditions that maximize RNA/DNA hybrids. After digestion with S1 nuclease, probe 1330 protects a fragment of approximately 680 nucleotides in length. This 5′ end was confirmed with a second probe, 520, that was end labeled at a *Pvu*II site and extends 529 nucleotides to an *Apa*I site. Because of the smaller probe size, the improved resolution with probe 520 revealed three major protected fragments, estimated to be 390, 382, and 379 nucleotides in length. The lengths of these closely spaced fragments correspond well to the major fragment seen with probe 1330. Thus, multiple *Pax-2* transcription start sites are clustered from approximately 701-690 nucleotides upstream of the translation start site, with the most 5′ end now designated as +1. It is unlikely that these ends represent a 3′ splice site to an as yet unidentified upstream exon because of their heterogeneity and the lack of splice consensus in the genomic sequence (data not shown).

The entire *Not*I/*Pst*I fragment containing the 5′ leader and approximately 1000 bp upstream of the transcription start site was sequenced. Analysis of the 700 nucleotide 5′ untranslated leader sequence revealed at least two potential WT1 binding sites, as based on the EGR consensus sequence 5′-CGCCCCCGC described previously (Rauscher at al., 1990). To determine if WT1 can indeed bind the 5′ Pax-2 region, DNAseI protection assays were employed using overlapping probes spanning the 5′ region. Both splice forms of the zinc-finger domain of WT1 (WT-ZF and WT-ZF+KTS) were expressed and purified from *E. coli* (Rauscher et al., 1990) and used in the DNA binding assays. Using increasing amounts of WT-ZF protein with probe 1, a major footprint was seen in the region from +140 to +166. No significant footprints were detected with the WT-ZF+KTS protein and probe 1. Probe 2

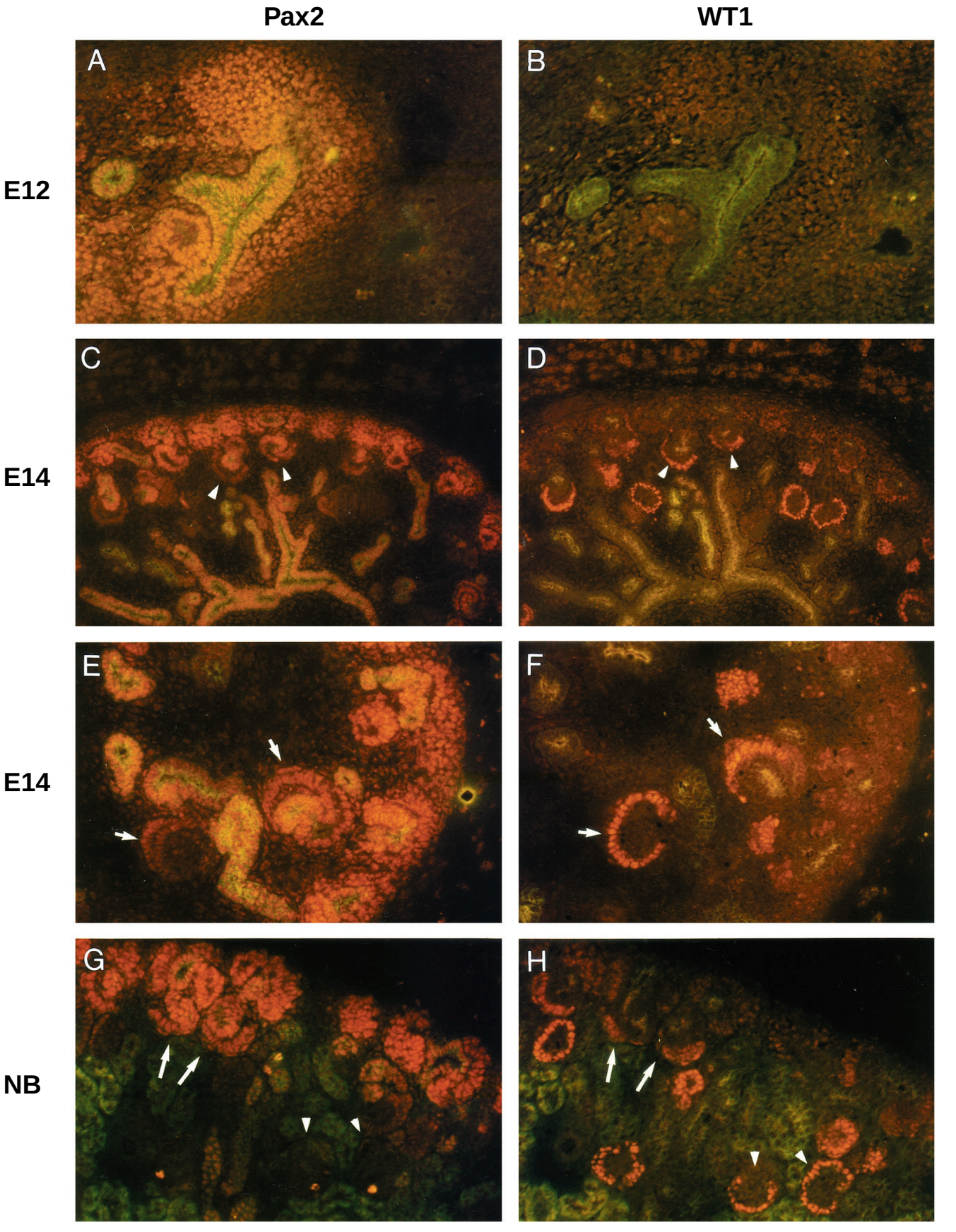

Pax2
WT1
E12
E14
E14
NB
A
B
C
D
E
F
G
H

Fig. 1. Immunostaining of Pax-2 and WT1 proteins in adjacent sections of developing kidneys. Gestation times are as indicated, NB is newborn kidney. Pax-2 is in red/orange in A,C,E and G and WT1 is in red/orange in B,D,F and H. All sections are also stained for uvomorulin (green) an epithelial cell adhesion molecule.
(A,B) Sagittal section through E12 kidney showing high levels of Pax-2 in condensing mesenchyme and little WT1 protein. (C,D) At E14, Pax-2 is repressed in the proximal loop of the s-shaped body (arrowheads) precisely were WT1 levels are increasing. (E,F) Higher magnification of E14 showing decreasing Pax-2 and increasing WT1 in the same structures (arrows). (G,H) In newborns, Pax-2 is still high in the nephrogenic zone, decreasing in proximal loops of the s-shaped body (arrows), and undetectable in the glomerular epithelium (arrowheads). WT1 is now more visible in the nephrogenic zone but also clearly increasing in the proximal loop (arrows) and persists in the glomerular epithelium (arrowheads). Magnification is 200× for A,B,E,F,G and H and 100× for C and D.

exhibited a large footprint with the WT-ZF protein from +263 to +300. Note that the footprint appears first in the region of +276 to +300 and extends 5′ as the protein concentration is increased. A weaker footprint was also seen in the +276 to +300 region with increasing amounts of WT-ZF+KTS protein. Probe 3 exhibits a footprint in the region around +596 to +618 with the WT-ZF protein. At high concentrations the footprint extends 3′ to approximately +628 and may indicate more than one molecule binding this region. The WT-ZF+KTS protein does not protect probe 3 to a significant degree. Probe 4 spans the same region as probe 3 but is labeled on the opposite strand and confirms the WT-ZF footprint seen in the region around +593 to +610.

The sequences of the three major binding regions are also shown in Fig. 3. As was postulated, the WT-ZF protein binds the regions around the CGCCCCCGC consensus sequence beginning at positions +158. A second consensus sequence is found in the reverse orientation beginning at position +294, it is also protected by WT-ZF and binds WT-ZF+KTS weakly. The third binding site, beginning at +596, is generally GC-rich and contains slightly degenerate concensus GTGGGGGAG nanomer sequence. The size of the binding sites, as estimated from the DNAseI protection experiments, generally extend beyond the consensus sequences and are dependent on WT1 concentrations.

Transcriptional regulation is mediated by *Pax-2* 5′ sequences

In order to test the ability of WT1, with and without the KTS region, to regulate transcription a cassette containing the chloramphenicol acetyltransferase gene (CAT) and SV40 polyadenylation signal was introduced into *Pax-2* genomic sequences at the *Not*I site, just upstream of the translation start site. In addition, whether transcriptional modulation would be mediated by EGR1 through these sequences was also tested. The Pax-2 reporter plasmids are outlined in Fig. 4A. The plasmid pH1-CAT contains a 10 kb *Hin*dIII fragment, including approximately 8 kb upstream of the *Not*I site at +675, the CAT cassette, and approximately 2 kb of the first intron. The plasmid p4.3CAT contains a 4.3 kb *Bam*HI-*Not*I fragment, including all of the 5′ leader and 3.6 kb of 5′ sequences, inserted upstream of the CAT cassette. In addition, the WT1 binding sites from the 5′ leader were inserted between the HSV-TK promoter and the CAT cassette such that WT1 regulation could be tested with a heterologous promoter. The WT1 proteins (−KTS and +KTS) and EGR1 were provided in *trans* using a CMV promoter plasmid driving the human *WT1* cDNAs (Morris et al.. 1991; Madden et al., 1991) and the mouse *EGR1* cDNA (Sukhatme et al., 1988).

Transfected NIH3T3 cells were tested for CAT activity using the four reporter plasmids outlined (Fig. 4A). A fixed amount of reporter DNA was mixed with increasing amounts of CMV-WT1, CMV-WT1+KTS or CMV-EGR1 DNA prior to transfection. The experiments were done in triplicate with CMV-WT1 and in duplicate with CMV-WT1+KTS and CMV-EGR1, and the results are shown in Fig. 4B. Quantification of the CAT activities, as determined by acetylation efficiencies as well as representative acetylation reactions are shown. The acetylation efficiencies were normalized to the activity of the reporter plas-

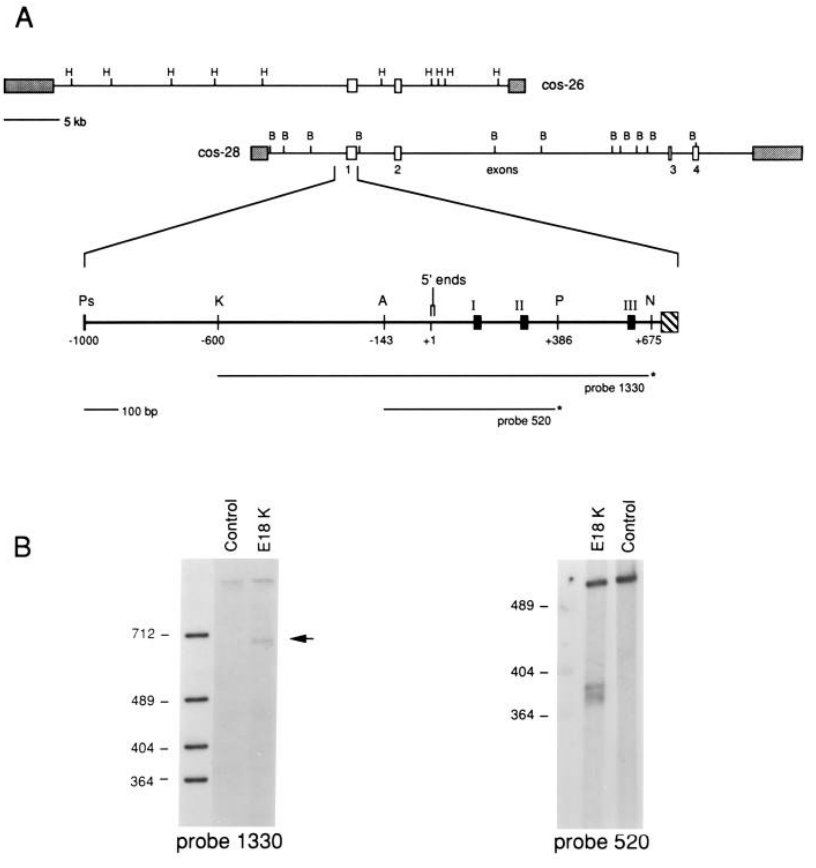


Fig. 2. Mapping *Pax-2* transcription start sites. (A) Schematic of overlapping cosmids containing the 5′ region of the mouse *Pax-2* gene. First four exons are as indicated by open boxes. The solid black boxes correspond to potential WT1 binding sites (I, II, and III); restriction sites are A, *Apa*I; B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; N, *Not*I; P, *Pvu*II; Ps, *Pst*I. The probes 1330 and 520 used for S1 nuclease mapping are also shown. The sequence of the *Not*I-*Pst*I fragment (−1000 to +675) is available from GenBank (accession number U13975). (B) S1 nuclease protection experiments using probes 1330 and 520. End labeled probes were mixed with control RNA and E18 embryonic kidney RNA (E18K).

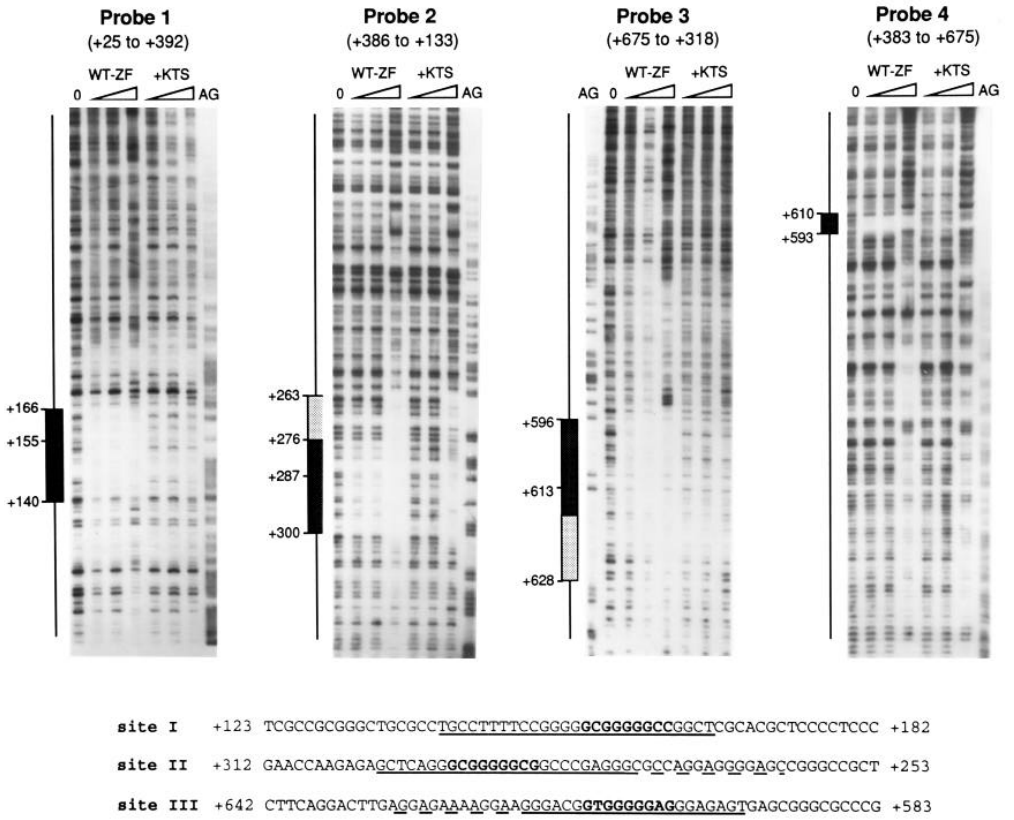
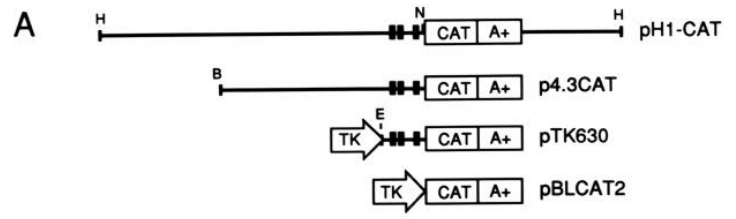


Fig. 3. DNAseI footprinting assays of WT1 binding sites in the Pax-2 5′ leader sequences. Probes spanning nucleotides +25 to +675 are as indicated. Increasing amounts of recombinant WT1 zinc-finger proteins (WT-ZF) and WT1-ZF+KTS zinc finger proteins (+KTS) were used. The dark boxes indicate footprints seen with low concentrations of protein, lighter shading indicates extended footprints seen with higher protein concentrations. The sequences of individual binding regions are shown below with the footprints underlined and the consensus sequence in bold.



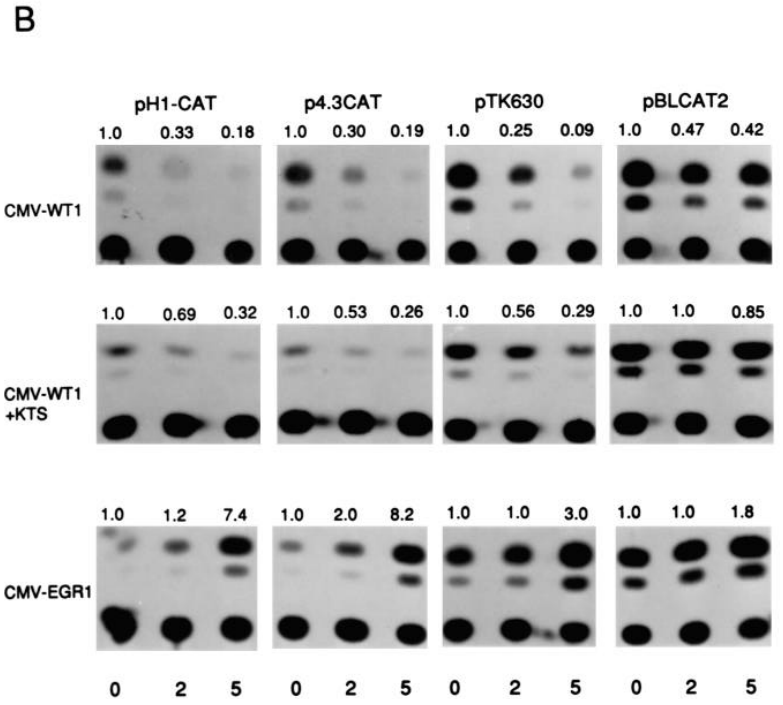


Fig. 4. WT1-dependent repression in transfected cells is mediated by *Pax-2* upstream sequences. (A) Reporter plasmids, as outlined in Materials and methods, are shown schematically. The solid boxes represent the 3 WT1 binding sites in the 5′ Pax-2 leader sequence. B, *Bam*HI; E, *Eag*I; H, *Hin*dIII; N, *Not*I. (B) Thin layer chromatography of acetylation reactions after co-transfection of 1 μg of pH1-CAT or p4.3CAT and 0.5 μg of pBLCAT2 or pTK630. Increasing amounts of CMV-WT1, CMV-WT1+KTS, or CMV-EGR1 plasmids were added as indicated. CAT activities were quantitated relative to the control reactions with no added CMV-WT1, CMV-WT1+KTS, or CMV-EGR1. A representative autoradiogram is shown and the average acetylation efficiencies from three independent experiments for CMV-WT1 and two independent experiments for CMV-WT1+KTS and CMV-EGR1 are indicated above.

mid alone. Using increasing amounts of CMV-WT1, the CAT activity generated by pH1-CAT and p4.3 CAT were reduced by 5 fold at the maximum ratios of CMV-WT1 to reporter plasmids while activities generated in the presence of CMV-WT1+KTS were reduced about 3-4 fold. Repression of TK630 was more than 10 fold, using 5 μg of CMV-WT1 and about 3 fold using CMV-WT1+KTS in the co-transfection. A slight decrease in the TK promoter activity was observed with 2 μg of CMV-WT1 that was not accentuated with increasing amounts of the construct, while CMV-WT1+KTS had a minimal effect. Clearly, the addition of the *Pax-2* 5′ leader sequences in the reporter construct TK630 resulted in an additional level of repression. The response of the *Pax-2* 5′ leader sequence to EGR1 was tested. In the presence of CMV-EGR1, CAT activity generated by pH1-CAT and p4.3 CAT was increased 7-8 fold. The reporter construct TK630 was activated about 3 fold, while CAT activity from the TK promoter in the absence of *Pax-2* sequences was poorly activated. Together, these data indicate that WT1 can repress transcription through the sequences from the 5′ untranslated region of the *Pax-2* gene, and that these same sequences are responsive to activation by EGR1.

DISCUSSION

The direct correlation between increasing WT1 levels and decreasing Pax-2 expression in similar cell types, the presence of multiple WT1 binding sequences, and the ability of WT1 to repress a reporter gene driven by *Pax-2* 5′ sequences is evidence for a direct interaction between the developmental regulatory gene, *Pax-2*, and the Wilms' tumor suppresser gene, *WT1*, during normal kidney organogenesis. Although only a minority of Wilms' tumors have mutations or deletions in the *WT1* gene (for review, see Coppes et al. 1993), persistent expression of *Pax-2* is frequent and correlates with the proliferation of poorly differentiated epithelial cells in these tumors (Dressler and Douglas, 1992; Eccles et al., 1992). The expression of *Pax-2* in adult renal cell carcinoma (J. Gnarra and G. Dressler, unpublished data) also suggests a role in cellular proliferation or maintenance of the dedifferentiated state. Furthermore, inhibition of terminal differentiation is also seen in the podocyte cells of the glomerular epithelium when *Pax-2* is constitutively expressed in transgenic mice (Dressler et al., 1993). These podocyte cells are most sensitive to *Pax-2* deregulation and must have developed suppression mechanisms for normal development to proceed. In fact, it is precisely these cells that show the highest levels of *WT1* expression as they mature. Interestingly, Pax-2 regulatory sequences are also activated by EGR1, an early growth response gene (Sukhatme et al., 1988) that is active in many highly proliferating cell types.

In the mouse, complete loss of *WT1* function results in multiple developmental abnormalitites including renal agenesis (Kreidberg et al., 1993). $WT1^{-/-}$ embryos fail to induce the metanephric mesenchyme as ureter bud outgrowth is inhibited. Even in vitro, the $WT1^{-/-}$ mesenchyme is unable to respond to inductive signals from wild-type tissues. Thus, *WT1* is required to mediate this early response of the mesenchyme to induction by the ureteric bud. The activation of the *Pax-2* gene in the kidney mesenchyme is dependent on induction by the ureteric bud (Phelps and Dressler, 1993). Pax-2 protein is one of the earliest markers for induced mesenchyme and is required for the condensation of the mesenchyme and the subsequent conversion to polarized epithelium (Rothenpieler and Dressler, 1993). There is no expression of *Pax-2* in the mesenchyme of $WT1^{-/-}$ mice, although this may be more the result of a block in induction rather than a lack of direct activation through WT1, as *Pax-2* is expressed in the Wolffian duct and in the mesonephros of $WT1^{-/-}$ mice. Although *WT1* is expressed in the mesenchyme, not the Wolffian duct, the mutant phenotype affects ureter budding from the Wolffian duct epithelium. This would suggest that the mesenchyme can determine the position and direction of ureter bud outgrowth, perhaps through the secretion of factors providing guidance cues.

Normally in the induced mesenchyme, *Pax-2* and low levels of *WT1* are coexpressed at early stages of renal development. This may not be entirely clear from the WT1 antibody staining in the embryonic kidney, although in newborns WT1 antibody staining reveals low levels of protein in the mesenchyme of the nephrogenic zone. However, in situ hybridization analysis has demonstrated WT1 mRNA in uninduced mesenchyme and in derivatives thereof. Clearly, both mRNA (Pritchard-Jones et al., 1990; Amstrong et al., 1992) and protein levels (Mundlos et al., 1993) subsequently increase markedly in the proximal portion of the s-shaped body and its derivatives, the visceral epithelium of the glomerulus including the podocyte cells. These structures are among the first to repress *Pax-2* expression. However, most, if not all, the renal tubular epithelium is derived from progenitor cells that had expressed the *Pax-2* gene earlier. Thus, repression of *Pax-2* in proximal and distal tubules, where increasing levels of WT1 are not observed, is most probably dependent on other factors that have not been identified. Furthermore, it cannot be ruled out that low levels of WT1 are required to activate *Pax-2* in the induced mesenchyme, although these early functions of WT1 may be entirely separate from effects observed at later times in the developing kidney. The ability for EGR1, and perhaps other proteins, to compete with WT1 for binding and activate, rather than supress, transcription further complicates this regulatory pathway. Ultimately, the quantity of individual WT1 splice variants, each with unique DNA binding and transactivation potentials (Haber et al., 1991; Bickmore et al., 1992; Drummond et al., 1994), and their ability to compete for available binding sites may determine the level of expression of target genes.

Coexpression of Pax-2 and WT1 can also be seen in primary Wilms' tumors and in tumor-derived cell lines (Tagge et al., 1994), although it is not clear how many, if any, Wilms' tumors used in that study had mutations in *WT1*. The failure of these tumor cells to suppress *Pax-2* may be related to the amount of WT1 present if the cells never differentiate far enough to exhibit the characteristic up-regulation of WT1. A more quantitative comparison must be made given the competitive nature of the regulatory system. An alternative possibility is that other *Pax-2* suppresser mechanisms are affected. That these WT1-independent mechanisms exists is evident by the repression of *Pax-2* in non-WT1-expressing cells during the course of normal kidney development.

The transcription start site of *Pax-2* has been mapped to approximately 700 bp upstream from the ATG translational start site based on S1 nuclease mapping experiments with two

overlapping probes. Although this method may not detect additional upstream exons, we believe this to be the true site of transcription initiation because of the heterogeneity of 5′ ends and the lack of 3′ splice consensus sequence. Additionally, at least three independently derived cDNA clones terminate in this region, the most 5′ at position +48 (G. R. D., unpublished data). This results in a long untranslated leader sequence with a high GC content that contains 3 WT1 binding sites. However, the possibility of additional sites in upstream regulatory elements cannot be entirely ruled out. Both the 8 kb and 4.3 kb upstream *Pax-2* genomic fragments can drive expression in transiently transfected NIH3T3 cells and are susceptible to repression by WT1 and activation by EGR1. The level of expression in 3T3 cells is lower than that of the HSV-TK minimal promoter. We believe that this transient expression represents a basal levels of transcription and is observed because of the high plasmid copy number peculiar to the transient transfection assay. Because the WT1-dependent repression may be cell type specifc, 3T3 cells were chosen because of their known ability to respond to WT1 transfection (Madden et al., 1991) and their ease of transfectability. The repression requires an excess of WT1- expressing plasmids and may reflect the in vivo correlation between high levels of WT1 and decreasing Pax-2 levels. Of the two alternatively spliced forms of WT1, WT1+KTS is a weaker transcriptional repressor, these results are similar to those shown by Drummond et al. (1994) using IGF II gene sequences. Interestingly, the IGF receptor gene also has WT1 binding sites in the untranslated leader sequence that contribute to the overall repressability of that promoter by WT1 (Werner et al., 1994). That these exonic WT1 binding sites contribute to repression was demonstrated by insertion between the reporter gene and the HSV-TK promoter. These sequences do not destabilize the mRNA as controls and EGR1 transfections have little effect on CAT gene activity. Interestingly, the ability of WT+KTS to repress the pH1-CAT and p4.3CAT was not all that much less that the WT-KTS form of the gene. It is possible that other WT+KTS binding sites may lie further upstream of the transcription start site and remain to be found.

Although the transfection of 3T3 cells can only approximate the in vivo interactions, repression is clearly mediated, at least in part, by the three WT1 binding sites located in the untranslated Pax-2 leader sequences. Other putative target genes of WT1 contain binding sites both upstream and downstream of the transcription start site (Drummond et al., 1992; Wang et al., 1992; Werner et al., 1994). The data presented in this report indicate that *Pax-2* is a potential target of negative regulation by the Wilms' tumor suppresser gene, *WT1*, during normal kidney development. This implies that aberrant *Pax-2* expression in tumor tissue can be, at least in part, due to mutations or deletions at the *WT1* locus. By defining the targets of WT1 repression and the multiple functions of the different WT1 gene products, a clearer understanding of normal developmental events and abnormal growth control will surely be at hand. Certainly the identification of other suppression mechanisms for transiently expressed, developmental regulatory proteins such as Pax-2, can address the underlying causes of oncogenic transformation.

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