Cloning and Characterization of the Human PAX2 Promoter*

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PAX2, a member of the PAX gene family of developmental transcription factors, is expressed at high levels in the developing eyes, ears, central nervous and urogenital systems, as well as in Wilms' tumor and renal cell carcinoma. Expression of PAX2 in the urogenital system is associated with proliferating cells of the ureteric bud and the differentiating nephrogenic mesenchyme. To date, little is known about the molecular mechanisms controlling the regulation of PAX2 expression. This report describes the cloning and characterization of the human PAX2 gene promoter and localization of the transcription start sites in fetal kidney and Wilms' tumor. We identified two transcription start sites in a Wilms' tumor sample, which were found to be different from that in fetal kidney. The activity of a deletion series of the PAX2 promoter was assessed in NIH-3T3, COS-7, 293, and Madin-Darby canine kidney cells. Although some differences were observed in the activity of each promoter construct, the profile of activity for the promoter fragment series was similar in each experiment, regardless of cell type. The WT1 tumor suppressor protein, which has previously been shown to repress murine Pax2 expression in vitro, was shown to also repress expression from the human PAX2 promoter.

PAX2 is one of nine related paired box-containing genes of the PAX gene family (1–3). PAX genes encode a group of transcription factors that have been isolated based on their homology to Drosophila segmentation genes (reviewed in Stuart et al. (2)). The protein products of PAX genes contain a conserved 128-amino acid DNA-binding motif termed the paired-box domain, and are grouped into four classes on the basis of their structural similarity and pattern of expression (2). PAX2 is most similar in structure to PAX5 and PAX8 (reviewed in Stuart and Gruss (4)), and together these three genes constitute a subclass of PAX genes thought to have arisen by duplication and evolutionary divergence of function. Each PAX gene is critically required for development of specific organs or tissues (2). PAX2 is expressed in the developing urogenital tract, spinal cord, midbrain, hindbrain, ear, and optic nerve (5, 6). Indeed, accumulating evidence from mouse mutants and functional studies shows that PAX proteins are an essential part of the developmental process. Mice lacking a functional Pax2 gene fail to develop the urogenital tract and display optic nerve, central nervous system, and inner ear abnormalities (7–9). Heterozygous mutant Pax2 mice exhibit abnormalities of the CNS, optic nerve, and retina and have hypoplastic kidneys (7–10). In humans, PAX2 is mutated in patients with defects of optic nerves and kidneys (renal-coloboma syndrome) (11–14).

As with many developmental genes, the timing and level of expression of PAX2 is critical for normal tissue development. Constitutive expression of Pax2 in transgenic mice results in multicystic and dysplastic kidneys in 18-day gestation fetuses and newborn pups (15). In contrast, repression of Pax2 by antisense oligonucleotides in embryonic kidney organ culture inhibits condensation of mesenchyme cells and their subsequent conversion to epithelium (16). Following differentiation, PAX2 expression is down-regulated in every tissue except gonads (5, 6). Moreover, persistent expression of PAX2 in tissues where PAX2 is normally down-regulated is associated with pathological conditions. For example, PAX2 is expressed in patients with multicystic dysplastic kidneys, a relatively common congenital kidney anomaly in children (17, 18). In addition, PAX2 expression accompanies regeneration of kidney tissue following toxic stress injury (19).

Typically, it has been found that PAX2 expression accompanies high rates of cell division. PAX gene products have the potential for cellular transformation, and can be classified as proto-oncogenes (4). Deregulation and over expression of PAX genes promotes cell focus formation in vitro and tumorigenicity in nude mice (20). PAX2 is expressed in Wilms' tumor (21–23), a childhood renal tumor of embryonic origin, and is also expressed in a proportion of renal cell carcinomas (24), an adult cancer derived from proximal tubules. Antisense oligonucleotides against PAX2 have been shown to specifically inhibit renal cancer cell proliferation (24).

Taken together, the above studies suggest that PAX2 gene expression is tightly controlled during development, and that in certain pathological states PAX2 is abnormally regulated. There is, however, little known about mechanisms of PAX2 regulation. To facilitate the identification of cis- and trans-regulatory factors important for transcriptional regulation of PAX2, we have isolated the minimal human PAX2 promoter. The basic features of this promoter were characterized, including three different transcription start sites, one from fetal kidney and two in Wilms' tumor. These start sites were located 686 bp (fetal kidney), 679 bp, and 804 bp (Wilms' tumor) upstream of the translational start. The human PAX2 promoter is GC-rich, TATA-less and CCAAT-less, with striking homology to the mouse Pax2 promoter. Our results suggest that the minimal PAX2 promoter is an evolutionally conserved constitutively active promoter that is modulated by distally located sequences.

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1 The abbreviations used are: bp, base pair(s); kb, kilobase pair(s); RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; MDCK, Madin-Darby canine kidney.

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EXPERIMENTAL PROCEDURES

Plasmid Constructs and Sequencing

**pRL-TK—pRL-TK (Promega), a plasmid expressing Renilla luciferase, was included in each transfection at a ratio of 1:40 as an internal standard to normalize luciferase assay results within transfection experiments.**

WT1—WT1 expression constructs were gifted by Dr. Ryoishi Miyagawa and contained four isoforms of murine WT1 cDNA, with and without the alternatively spliced exon 5, and the "KTS" amino acids in exon 9 (25). Each cDNA, including the entire coding region, plus 5'- and 3'-flanking sequences, was cloned into the BglI site in the vector RcCMV (Invitrogen). WT1 expressed proteins were detectable from each construct by immunohistochemistry.²

**pGPxp2—The human PAX2 gene promoter was isolated from a genomic bacteriophage lambda clone, α11a, which had previously been shown to contain approximately 10 kb of sequences upstream of PAX2 (26). A 2-kb BamHI DNA fragment was purified from clone α11a, and subcloned into the BglII site of an engineered pGL2-basic vector (Promega), modified so that it contained a novel NcoI restriction site in the cloning cassette between the BglII and HindIII sites. Digestion of the pGL2-basic-PAX2 construct (called pGPxp1) with NcoI removed sequence downstream of the PAX2 ATG translation start site, creating the construct pGPxp2.**

**pGPxp17—Clone pGPxp17, which contains 4.2 kb of the PAX2 promoter and 5'-flanking sequences, was generated with the following steps. A 3-kb Apal fragment was isolated from α11a, and cloned into the Apal site of pBluescript-KS (Stratagene) to create clone pBPpx16. A 2.9-kb KpnI fragment of PAX2 5'-flanking DNA was then removed from clone pBPpx16 and inserted in the 5’ to 3’ orientation into the KpnI site of pGPM3, to generate clone pGPxp17 (now containing 4.2 kb of continuous sequence).**

**Plasmid pGPxp2—Plasmid pGPxp2 was sequenced by constructing EcoRI nested deletion clones (Amersham Pharmacia Biotech). All sequence data were used to analyze the sequence of double stranded EcoRI-ApaI DNA (27) using a Sequenase Version 2.0 kit (U. S. Biochemicals), or an ABI 373 automated sequencer and dye terminators following the manufacturer’s instructions.**

Ribonuclease Protection Assays

Total RNA was isolated from Wilms’ tumors or human fetal kidneys of approximately 12–15 weeks of gestation using the method of Chomczynski and Sacchi (28). Ribonuclease protection assays were carried out with sequences downstream of the PAX2 ATG translation start site, essentially as described previously (23, 28). Hybridization of riboprobes to RNA was done in a 30-μl reaction containing 5 × 10^6 to 1.3 × 10^7 cpm of probe and 15 or 30 μg of total sample RNA. All samples were made up to a 30-μg total RNA concentration with yeast tRNA. Control samples contained 30 μg of yeast tRNA alone.

5' Rapid Amplification of cDNA Ends (RACE)

Transcription start sites were identified by 5'-RACE, using a modification (32) of the protocol from the 5'-AmpliFINDER™ RACE kit (CLONTECH). 5 pmol of a PAX2-specific primer 5R (5'-GCC CCC GCC CCC GGA AAA GCC AG-3') was annealed to 4 μg of total RNA, in a solution containing 300 μM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. The solution was denatured at 70 °C for 5 min, followed by incubation at 65 °C for 20 min. Reverse transcription was carried out at 42 °C for 1 h, followed by 15 min or 30 min in a 40-μl volume containing 1 unit of SuperScript II™ RNaseH− reverse transcriptase (Life Technologies, Inc.). Remaining conditions and reagents were as described previously (30).

Approximately one-fourth of the purified single-stranded cDNA was ligated at 22 °C for 16 h to 4 pmol of AmpliFINDER Anchor (CLONTECH) (5'-C-P-AAC GTA TTT ACC ATC TTT GAA ACC GGA GG-H3'-3') in a solution containing 5 units of T4 DNA ligase, using the single-stranded ligation buffer supplied by CLONTECH. One μl of a 1:10-fold dilution of the ligated cDNA was used in a first round of PCR amplification in reactions containing 1 mM MgCl₂, 82.5 μM dNTPs, 0.06 μM of each primer: anchor primer (5'-CGT GTG CGG CCC ACC TCT GAA GGT TCC AGA ATC GAT AG-3'), and PAX2-specific primer 5R, 1 unit of AmpliTaq Gold™ (Perkin-Elmer), and a final concentration of 10 mM Tris-Cl, pH 8.3, 50 mM KCl. The PCR conditions consisted of one cycle of a 10-min activation at 95 °C, followed by 14 cycles of 20 s at 95 °C, 20 s at 68 °C, and 40 s at 72 °C. A second round of heminested PCR was then carried out with 1 μl from the first PCR reaction in a solution containing 1 mM MgCl₂, 82.5 μM dNTPs, 20 pmol of each primer: anchor primer, and 1R (5'-GAG CCG AGC GGC GCT GGT GCC CGC TCT G-3'), 1 unit of Pwo polymerase (Boehringer Mannheim), and buffer to a final concentration of 10 mM Tris-Cl, pH 8.85, 25 mM KCl, 5 mM (NH₄)₂SO₄. The PCR conditions consisted of one cycle of 1 min at 95 °C, followed by 40 cycles of 20 s at 95 °C, 20 s at 68 °C, and 40 s at 72 °C. A positive control

**Cell Culture and Transient Transfection Assays**

COS-7, NIH-3T3, 293, and MDCK cells were obtained from the American Type Culture Collection (ATCC). MDCK and 293 cells were previously shown to express PAX2 endogenously.³ All four cell lines were maintained at 37 °C in a humidified environment with 5% CO₂. Cells were cultured in either Dulbecco’s modified Eagle’s medium with high glucose (COS-7, 293, and NIH-3T3) or minimal essential medium with Earle’s salts (MDCK), supplemented with either 10% heat-inactivated fetal calf serum (Life Technologies, Inc.) (COS-7, 293, and MDCK) or 10% calf serum (Life Technologies, Inc.) for NIH-3T3 cells and 1% gentamycin sulfate. Transfections were carried out using FuGene 6 reagent (Boehringer Mannheim) in the presence of 1% antibiotic solution, with 1 μg of PAX2 promoter-reporter luciferase plasmid and either 3 μg of pBlue-script DNA (Stratagene) or 3 μg of WT1 expression plasmid, plus 0.05 μg of internal control Renilla luciferase plasmid. Alternately, for co-transfection experiments a total of 4.05 μg of DNA were used. The reactions contained 1 μg of PAX2 promoter-reporter luciferase plasmid and 0.05 μg of pBlue-script DNA (Stratagene) or 3 μg of WT1 expression plasmid, plus 0.05 μg of internal control Renilla luciferase plasmid. The DNA solutions were combined at a 1:3 ratio with FuGene 6 reagent (e.g. 4.05 μg of DNA to 12 μl of FuGene 6) for transfection of each cell line. The cells were overlaid with this mixture, in a final volume of 2 ml of medium, for 24 h prior to lysis. Cell lysates were assayed for luciferase production using firefly and Renilla luciferase dual assay reagents (Promega) following the manufacturer’s instructions. Transfections were done in duplicate or triplicate on a minimum of three separate occasions. Luminometry was measured with a BioOrbit 1253 luminometer.

RESULTS

Cloning and Sequencing of the Human PAX2 Promoter—To identify potential cis-acting regulatory elements in the human PAX2 gene promoter, genomic DNA encompassing PAX2 5'-flanking sequences was isolated from bacteriophage λ clone α11a (26), and used to generate clone pGPxp2 (see Fig. 1A, and “Experimental Procedures”). The sequence from the insert of clone pGPxp2, which contained a 1.7-kb BamHI-NcoI fragment of the PAX2 5'-flanking region (Fig. 1B) revealed a high GC content (68%), and was found to overlap with the longest previously isolated clone of the human PAX2 cDNA. Several inconsistencies in the sequence were identified between the sequence reported here and the previously isolated clone of the human PAX2 cDNA. Several inconsistencies arose as a result of ambiguities in the sequence of the earlier cDNA clone, due to the very high GC content of this region. The ambiguities have now been resolved in this sequence. Consensus binding sites in the PAX2 promoter sequence were identi-

² K. Miyagawa, personal communication.

³ C. K. Stayner, H. E. Cunillie, M. C. French, and M. R. Eccles, unpublished results.
Identification of PAX2 Transcription Start Sites in Wilms' Tumor mRNA by RNase Protection—To localize the position of the PAX2 transcription start site(s), sequences of the PAX2 promoter between −290 and +370 including 5′-leader sequences of PAX2 mRNA, were analyzed by RNase protection analysis. Although human fetal kidneys were available as a source of tissue in which PAX2 transcripts were expressed, the scarcity of available kidneys precluded their use as a source of large quantities of RNA, as would be required for RNase protection experiments. In contrast, Wilms' tumors were thought to be a suitable tissue to provide large quantities of RNA, because Wilms' tumors recapitulate fetal kidney development, and PAX2 is expressed in Wilms' tumor tissue (22). Because of the close relationship between Wilms' tumors and fetal kidney development, it was expected that the PAX2 transcription start site in Wilms' tumor would be identical to that in human fetal kidney.

One Wilms' tumor was chosen that strongly expressed PAX2, as determined by Northern blot (data not shown). A SmalI-PstI fragment of the PAX2 cDNA (refer to “Experimental Procedures”) was fully protected in RNase protection assays (Fig. 2A) of Wilms' tumor RNA, indicating that there are no transcription start sites within this fragment. In contrast, a more distal SmalI fragment of PAX2 (Fig. 2A), including sequences beyond the 5′ end of the longest PAX2 cDNA clone (22), was partially protected in RNase protection assays of Wilms' tumor RNA (Fig. 2A). These experiments suggested that a potential transcription start site in Wilms' tumor RNA was located 124 nucleotides upstream from the start of the longest previous cDNA clone (Fig. 2C, asterisk). A longer exposure of the gel revealed a second protected band at a higher molecular weight, out of range of the sequencing ladder, suggesting the presence of another possible start site in Wilms' tumor RNA (data not shown).

Localization of PAX2 Transcription Start Sites in Wilms’ Tumor mRNA by 5′-RACE—To more precisely map the region of the putative PAX2 transcription start site in Wilms’ tumor RNA, primer extension analysis and 5′-RACE were performed. Primer extension analysis using Wilms’ tumor RNA was unsuccessful, possibly due to the high GC content of this region. 5′-RACE experiments however, yielded two tumor-specific PCR products. Wilms’ tumor RNA, which had been primed with the PAX2-specific primer 5R (see Fig. 2C), was reverse transcribed and amplified using a modified 5′-RACE protocol (see “Experimental Procedures”). Southern blots of the amplified products, primer extension analysis and 5′-RACE experiments however, yielded two tumor-specific PCR products. Wilms’ tumor RNA, which had been primed with the PAX2-specific primer 5R (see Fig. 2C), was reverse transcribed and amplified using a modified 5′-RACE protocol (see “Experimental Procedures”). Southern blots of the amplified products
obtained from six separate PCR amplifications of the reverse transcribed Wilms' tumor RNA were hybridized using a PAX2-specific probe (4R). These studies indicated that several PAX2-related sequences had been amplified. The products of the 5'-RACE PCR reactions, which consistently revealed two bands of approximately 125 and 250 bp by Southern analysis, were subcloned and sequenced. From a total of 15 clones, 8/9 were identified that initiated from an adenosine (T1, +8, Figs. 1B and 2C). This start site lies within a sequence which is a direct match to an Initiator consensus sequence (Py Py A) as reported by Javahery et al. (34), and is at the optimal position of transcription initiation within this motif. Of the remaining six cloned PCR products, five were found to initiate from the distal transcription start site at −118 (T2). Two additional clones were obtained that initiated from intermediate sites between the proximal and distal transcription start sites, although these clones may represent incompletely reverse transcribed sequences.

**RNA from Fetal Kidney Contained Only One PAX2 Transcription Start Site**—We next analyzed PAX2 transcription start sites in human fetal kidney RNA by 5'-RACE to verify whether the transcription start sites of the PAX2 gene in Wilms' tumor RNA corresponded with that in human fetal kidney. In six separate 5'-RACE PCR reactions using fetal kidney total RNA, a single PCR product of approximately 125–130 bp was reproducibly obtained, in comparison to the two

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**Fig. 2. Identification of PAX2 transcription start sites.** A, localization of a tumor-specific PAX2 transcription start site. Riboprobes were prepared as under “Experimental Procedures.” Lanes 1 and 2 contain 15 μg and lanes 3 and 6 contain 30 μg of Wilms' tumor total RNA, which was used as target RNA. Lanes 1–3 represent a control probe (C) which is a Smal-PstI fragment (+190 to +370) present in PAX2 cDNA sequences and gives the expected protected fragment (CP), migrating at 182 bp with respect to the DNA ladder. Lanes 4–6 use a test probe (T), which is a Smal fragment (~290 to +190); this was not fully protected. The protected fragment (TP) corresponded to a size of 176 bp, which places the 5'-end of this protected RNA at a cytosine (position +13) in the PAX2 sequence (see asterisk in panel C). It is not unusual to see shadow bands associated with protected fragments as seen here for CP and TP. This artifact may reflect a shorter in vitro transcribed probe that was co-purified during riboprobe preparation. Lanes A, G, C, and T are a sequencing ladder of pBluescript (SK) primed with KS primer. B, identification of PAX2 transcription start sites by 5'-RACE. i, agarose gel electrophoresis of PCR products from 5'-RACE. RACE reactions were performed as described under “Experimental Procedures.” PCR products from 5'-RACE reactions with Wilms' tumor total RNA (lane 2) and fetal kidney total RNA (lane 4) were electrophoresed on a 2% agarose gel at 80 V for 1 h and then Southern transferred to Hybond N+ membrane (Amersham Pharmacia Biotech). ii, filters were hybridized with 1 pmol of an end-labeled, PAX2-specific oligonucleotide 4R (see panel C) overnight at 42 °C and exposed to film. Specific bands that hybridized were estimated to be 125 and 250 bp from the DNA marker (i, lanes 1 and 3), which contain pUC10 DNA cut with MspI. The lower diffuse band seen in i is a primer dimer and does not hybridize to oligo 4R (data not shown). C, summary of human PAX2 transcription start sites and comparison with corresponding murine sequences. 300 bp of sequence are shown, encompassing the human and mouse PAX2 transcription start sites. FK is the fetal kidney transcription start site identified by sequencing the product shown in panel B, lane 4. T1 and T2 are the two Wilms' tumor start sites identified by sequencing the two products shown in panel B, lane 2. An asterisk denotes the 5'-end of the protected RNA fragment obtained by RNase protection in panel A, lanes 5 and 6. The boxed sequence is a direct match to a eukaryotic initiator sequence. M1 marks the designated +1 transcription start site in the murine PAX2 promoter (35). Nucleotides in boldface indicate the region in the mouse sequence that contained multiple transcription start sites, +1 being the 5' most site. Reverse primers used in the 5'-RACE experiments, 1R, 4R, and 5R are indicated.
bands from Wilms' tumor RNA (Fig. 2B). This band of approximately 125 bp hybridized with a 4R oligonucleotide probe in a Southern blot (Fig. 2B). The identity of the transcription start site was confirmed by subcloning and sequencing multiple clones of the single fetal kidney-specific PCR product. 10/10 clones indicated that the PAX2 transcription start site in human fetal kidney (Fig. 2C, FK, +1) lies within a 5/7-bp match to the mammalian initiator consensus sequence (34).

The Human PAX2 Promoter Shares Very Strong Sequence Conservation with the Murine Pax2 Promoter—The human and murine (35) PAX2 promoter sequences were aligned for sequence comparison. Over the entire 1,690 bp of human PAX2 promoter there was approximately 80% homology with the mouse Pax2 promoter. Less homology (approximately 67%) was observed between −1,010 and −460 in the human sequence relative to the transcription start, but greater homology (approximately 85%) was observed between −460 and +690 (alignment not shown). This high homology may indicate conserved mechanisms of regulation of PAX2 expression. Examples of the degree of sequence similarity between murine and human sequences are illustrated in Figs. 2C and 4.

The sequences immediately surrounding the human transcription start site were compared with the homologous region of the murine Pax2 promoter (Fig. 2C). The human fetal kidney Pax2 transcription start site was located at precisely the 3′ end of a region containing multiple transcription start sites in mouse Pax2, the 5′ end of which is designated as +1 in the murine Pax2 promoter (Fig. 2C, M1).

The Minimal Human PAX2 Promoter Is Active in Different Cell Types and 5′-Flanking Sequences Conferred Enhanced Activity—To define the minimal DNA sequence required for PAX2 promoter activity, and to analyze DNA sequences important for regulation of PAX2 transcription, transient transfections were performed with a series of deleted DNA fragments encompassing the PAX2 promoter, fused to the firefly luciferase reporter gene. The series of deleted promoter fragments used in the transfections is depicted in Fig. 3A. Each promoter-reporter construct was transiently transfected into four different cell types, MDCK, NIH-3T3, 293, and COS-7 cells, in the presence of a constant amount of the internal control plasmid, pRL-TK. The resulting luciferase reporter gene activities were then measured and normalized to Renilla luciferase activity (Fig. 3B). Higher levels of reporter gene expression were detected in COS-7 and 293 cells as compared with NIH-3T3 and MDCK cells (Fig. 3B). In all cell lines, significant basal levels of promoter activity were seen with the construct pExoIII16b, which contains the fetal kidney start site, but not the upstream start site identified in Wilms' tumor. In comparison, a smaller fragment that did not contain the fetal kidney transcription start site had markedly less activity, although activity was not nil (Fig. 3B). When 5′-flanking sequences were progressively added to the minimal PAX2 promoter-reporter constructs, a significant increase in activity was observed (Fig. 3B). With each progressive addition of upstream sequences, a profile of promoter activity of the deletion constructs was obtained that was found to be similar in each cell line transfected (Fig. 3B). When clone pGPxp17, which contained a total of 4.2 kb of continuous 5′-flanking sequences, was transfected into different cell types (see “Experimental Procedures”) there was no increase in activity as compared with pGPxp2, and no cell type specificity was conferred on the regulation of the PAX2 promoter by these sequences.

The Wilms' Tumor Suppressor Gene Product, WT1, Repressed Activity of the Human PAX2 Promoter in NIH-3T3 Cells and MDCK Cells—To identify transcription factors important for regulating PAX2 activity, we reasoned that the binding sites of such transcription factors would be conserved in both human and mouse PAX2 promoter sequences. Consensus binding sites for transcription factors that were conserved between human and mouse sequences included an SP1 site, recognition motifs for NF-IL6, PEA3, and two putative EGR-1-like binding sites (see Figs. 1 and 4). Binding and repression of the mouse Pax2 gene promoter by the Wilms' tumor suppressor gene product, WT1 has previously been reported Ryan et al. (35). The corresponding putative WT1 binding sites in the human PAX2 promoter were highly conserved when compared with the WT1 binding sites in the murine promoter (Fig. 4). To determine whether WT1 was able to repress the human PAX2 promoter, NIH-3T3 cells were co-transfected with WT1 expression constructs (all four isoforms), and two PAX2 promoter-reporter constructs. In NIH-3T3 cells, the PAX2 promoter-reporter plasmid pGPxp2 was repressed 55–70% by co-transfection with a 3-fold molar excess of the +/+ or −/−, and −/− WT1 expression plasmids, and by 30% with +/+ WT1 isoform (Fig. 5). When this experiment was repeated using a 5:1 molar excess of WT1 expression plasmids, the activity of the PAX2 promoter construct was further repressed to a maximum of 80% with the WT1 +/+ isoform and to 65% with WT1 +/+ isoform (data not shown). The reason for this small difference in repression of PAX2 by the + and − KTS isoforms of WT1 is not clear from these experiments. We have not examined whether WT1 binds directly to PAX2 promoter sequences, and cannot rule out the possibility that another WT1-mediated mechanism is involved. In contrast, the smaller promoter construct, pExoIII16d, was repressed only 35–45% by a 3-fold molar excess of all four WT1 expression plasmids (Fig. 5), suggesting that distal PAX2 promoter sequences are important for modulating repression by the WT1 +/+ isoform.
DISCUSSION

PAX2 is expressed in a cell type and temporally restricted pattern in the developing vertebrate urogenital and central nervous systems, eyes, and ears (5, 6). In addition, several tumor types have been shown to express PAX2 (21–24). However, despite obvious regulatory constraints on the expression of PAX2, relatively little is known about the factors required to modulate activity of the PAX2 gene promoter. We report here the isolation and characterization of the human PAX2 gene promoter. The salient features of this promoter are that the entire sequence is GC-rich, it contains only one transcription start site expected that Wilms’ tumors would contain the same features resembling kidney development, and so we expected that Wilms’ tumors would contain the same mor-specific shifts in promoter usage have been identified. A second transcription start site at –118, identified in Wilms’ tumor RNA by 5’-RACE, may correspond to the larger product identified by RNase protection, although the product in RNase protection could not be accurately sized. The distal transcription start site in Wilms’ tumor did not contain an initiator sequence, although a loose consensus site for a CCAAT box was located adjacent to this site. Here we have characterized the products that corresponded to two prominent bands observed in two PCR reactions from only one Wilms’ tumor RNA sample. Whether heterologous transcription start sites might be detectable in a panel of Wilms’ tumor samples is yet to be determined.

Wilms’ tumors arise during kidney organogenesis and exhibit features resembling kidney development, and so we expected that Wilms’ tumors would contain the same PAX2 transcription start sites that would be found in fetal kidney. We have not investigated transcription start sites in other fetal-derived PAX2-expressing tissues, for example the optic stalk or CNS, and it is possible that in tissues other than the kidney, there is alternative usage of transcription start sites of the PAX2 gene. If PAX2 is deregulated in Wilms’ tumor, then expression may occur from alternative transcription start sites normally used in other tissues. In several genes, most notably c-myc (39) and the multidrug resistance gene, mdr3 (40), tumor-specific shifts in promoter usage have been identified. However, in most genes, transcription start sites are the same in tumor and normal samples.

There is significant homology between murine and human PAX2 promoter sequences immediately flanking the transcription start sites (extending upstream). This is not unusual and...
has been observed for other genes, for example, WT1 (36). By comparison, the human PAX2, PAX5, and murine Pax8 promoter sequences do not show significant homology with each other. The PAX5 gene contains two promoters associated with alternative exon ones that are alternatively spliced in different cell types (41). The methods we have used may not have detected distally located alternative exon 1 sequences, although we did not detect any such clones during previous cDNA screening (22). Further studies on the distribution of transcription start sites in Wilms’ tumor and human fetal RNA samples are required.

Several putative binding sites were identified upstream and downstream of the transcription start sites for transcription factors PEA3, NF-κB, NF-IL6, EGR-1, and SP1. By comparison with the murine Pax2 promoter sequences, the NF-IL6 site, an EGR-1 site, an SP1 site, and three PEA3 sites were all conserved. The remaining EGR-1 site, SP1 site, and NF-κB site differed by only 1 base pair to the murine sequence and were all positionally conserved. The functional importance of each of these transcription factors with respect to modulation of PAX2 expression will need to be addressed. Here, we have analyzed the role of one of the transcription factors thought to regulate PAX2; the Wilms’ tumor gene product, WT1, which like PAX2, is critically required for kidney development (16, 42). WT1 binding sites were highly conserved between human and mouse sequences, and expression of the mouse Pax2 gene has previously been shown to be repressed by WT1 (35). We were interested in investigating the effect of WT1 on the human PAX2 promoter, because of potential differences that exist between humans and mice with respect to WT1 regulation. For example, mutations in the WT1 gene have been observed to cause Wilms’ tumor in humans, but not mice.

The PAX2 promoter was found in our experiments to be repressed 55–70% or 65–80% with a 3-fold or 5-fold molar excess of WT1 expression plasmid, respectively. The WT1-mediated repression of PAX2 that we observed was similar to that observed by Ryan et al. (35). Weaker repression of pGIPx2 was consistently observed with the WT1 +/− isofrom although the difference was small. The WT1 +/− isofrom may interact with the PAX2 promoter in a different manner. For example, it is possible that the WT1 +/− isofrom interacts with another protein to modulate repression of PAX2 transcription. In contrast, repression of the shorter promoter construct, pExoIII16d was similar with all four WT1 isoforms, suggesting that repression by WT1 is mediated (at least in part) by binding of WT1 to the 5′-untranslated region of PAX2. Interestingly, the human PAX2 promoter has two exact 9/9 matches, and five 8/9 matches to the EGR-1 consensus binding site, whereas the murine promoter has only one 9/9 and three 8/9 matches. EGR-1 is believed to antagonize the transcriptional repressive effect of WT1, i.e. it has a transactivating function, and may compete with WT1 for binding to at least some EGR-1 consensus sites (43).

The PAX2 promoter had high basal activity in COS-7 and 293 cells, even when most of the 5′-sequences flanking the transcription start site were removed. Activity was markedly less in MDCK cells, and lower again in NIH-3T3 cells; however, the profile of PAX2 promoter activity within a deletion series of promoter constructs was similar between all cell lines. In addition, it was observed that progressive addition of 5′-sequence to the basal PAX2 promoter resulted in only a small relative increase in overall promoter activity. Recently, the 5′-flanking sequences of the human PAX5 and mouse Pax3 and Pax8 gene promoters have been determined (38, 44, 45). Sequences upstream of the PAX5, Pax3, and Pax8 gene transcription start were found to be essential for promoter activity in these genes (38, 44, 45). Our results suggest that the major tissue-specific transcriptional activators of PAX2 expression were either not present in the cell lines used, or could not transactivate the promoter fragments used in this analysis. It is not unusual for tissue-specific regulatory sequences to reside at a long distance from transcription start sites, for example, the Pax2′ homologue sparking from Drosophila, was found to contain an eye-specific enhancer in intron four (46).

The studies described here provide a starting point for the examination of the regulation of the human PAX2 gene in more detail. Further work is required to define factors and elements necessary for PAX2 activation and repression, both in renal development and during malignancy.

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