PAX 8 Regulates Human WT1 Transcription through a Novel DNA Binding Site*

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The Wilms’ tumor gene (WT1) is an essential gene for kidney and gonadal development, although how WT1 expression is induced in these tissues is not known. One kidney transcription factor likely to play a role in this regulation is PAX 8. The co-expression of WT1 and PAX 8 during kidney development and in Wilms’ tumors with an epithelium predominant histology suggested a possible interaction, and indeed, we identified potential core PAX-binding sites in the WT1 promoter. Endogenous PAX 8 plays an important role in the activation of the WT1 promoter, since promoter activity is much stronger in cells with PAX 8 than without. Using binding assays, we searched for evidence of PAX 8-DNA interactions throughout the 652-base pair human WT1 promoter and found only one functional PAX 8 site with DNA binding activity, located 250 base pairs 5’ of the minimal promoter. The responsiveness of the PAX 8 site was confirmed by assessing its ability to function as an enhancer significantly activating the minimal promoter in a position- and orientation-independent manner. Using transfection assays, we demonstrated that either endogenous or exogenously added PAX 8 activated the WT1 promoter and that this promoter up-regulation depended upon the presence of an intact PAX 8-binding site. In contrast, the previously reported core PAX 8-binding sites identified by computer analysis of the WT1 promoter failed to specifically bind in vitro translated PAX 8 protein or activate the minimal promoter. Thus, we identified a novel functional binding site for the transcription factor PAX 8, suggesting that part of its role in kidney development may be as a modulator of WT1 expression in the kidney.

The Wilms’ tumor gene (WT1),1 a tumor suppressor gene, was isolated by positional cloning of the 11p13 region (1–3) and shown to be involved in the etiology of several nephroblastomas (4, 5). WT1 encodes a zinc finger transcription factor (1, 6) that acts in vitro as a transcriptional repressor of several growth factor and growth factor receptor genes (7–18) and functions as a tumor suppressor gene (19). WT1 is essential for the differentiation of mesenchymal blastema to epithelial and stromal structures during kidney development. Kidney development is arrested in transgenic mice containing a homozygous deletion in the WT1 gene (20). Homozygous WT1 mutations result in embryonic lethality and developmental defects in the urogenital system, heart, lungs, and mesothelia. During embryogenesis, WT1 is expressed at high levels in the genitourinary system (21) and mesothelia (22) and at lower levels in fetal hematopoietic organs (1, 3, 23, 24). In the adult, WT1 is expressed in the testes, mesothelia, and immature hematopoietic progenitor cells, e.g. CD34+ bone marrow cells (22, 24, 25).

Differential WT1 expression during development suggests that WT1 expression is tightly regulated by tissue-specific transcription factors. PAX 8 encodes a developmentally important paired box transcription factor that is expressed in the developing kidney, among other tissues (26). The expression of PAX 8 in the developing kidney precedes WT1 expression. Its expression initiates in the induced condensing mesenchyme, peaks in the S-shaped bodies, and finally declines in the epithelial cells of the glomerulus (27). The WT1 expression pattern is similar to the PAX 8 pattern, with its peak occurring after PAX 8 expression begins to decline and WT1 expression persists in the mature podocytes of the glomeruli (27, 28). Like WT1 expression, PAX 8 expression has also been found in the Wilms’ tumors with an epithelial predominant histology2 (27, 29, 30). The presence of PAX 8 mRNA in these tumors with high WT1 expression is consistent with its postulated role as an activator of WT1 expression.

PAX 8 paralogues include PAX 2 and PAX 5. Unlike PAX 2 and PAX 8, PAX 5 is expressed in developing hematopoietic cells (pre-B cells). While in vivo downstream target genes have not been identified for these PAX genes, several genes whose expression is regulated by PAX 5 and PAX 8 in vitro have been identified, and consensus binding sites have been defined by oligonucleotide selection methods. PAX 5 can regulate expression of the B cell-specific surface protein CD19 (31), and PAX 8 can regulate expression of thyropheroxidase and thyroglobulin (32). Recently, engrailed-2 was shown to be a target gene for PAX 2, PAX 5, and PAX 8 (which are co-expressed with Engrailed-2) in mouse embryos during mid-hindbrain development (33). PAX 2, PAX 5, and PAX 8 each have only a partial homeodomain, so DNA binding is mediated by the paired domain, which is composed of three α-helices. The NH2-terminal subdomain of the paired domain is highly conserved and binds a core recognition sequence of the PAX-binding site. However, the COOH-terminal subdomain also influences site-specific binding (34).

While the WT1 promoter coupled to the SV40 enhancer strongly activates transcription in all cell lines tested (35), its basal activity varies among cell lines, suggesting differential transactivation by tissue-specific factors. In constructs lacking

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1 The abbreviations used are: WT1, Wilms’ tumor 1; RT, reverse transcription; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CAT, chloramphenicol acetyl transferase; EMSA, electrophoretic mobility shift assay.

2 S. Hamada and G. F. Saunders, unpublished observations.
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The SV40 enhancer, the transcriptional activity of the WT1 promoter is much lower, as is typical of a TATA-less, CCAAT-less, GC-rich promoter. The ubiquitous transcription factor Sp1 has been shown by footprint analysis to bind at many positions throughout the WT1 promoter and to transactivate the promoter region in cotransfection assays (36, 37). WT1 protein also binds at many positions throughout the WT1 promoter, and the least abundant isoforms lacking the KTS insertion strongly repress the WT1 promoter in cotransfection assays (38, 39). The autoregulatory sites in the WT1 promoter may be essential for the down-regulation of WT1 expression observed during the differentiation of leukemic cells (40, 41). Using deletion analysis, we previously identified the minimal promoter region necessary and sufficient for promoter activity in K562 and HeLa cells (35). This 104-bp minimal promoter is GC-rich (79% G+C); contains seven overlapping potential transcription factor binding sites within a core of 30 bp, including Sp1, AP4, WT1, and CACCC binding sites; and has half the transcriptional activity of the full-length WT1 promoter.

To better understand the mechanism for differential promoter activity in various cell lines, derived from different tissues, we examined the 5′-flanking region of the full-length human WT1 promoter. Sequence analysis identified potential binding sites for the zinc finger GATA factors (42) and two paired box transcription factors, PAX 8 (32) and PAX 2 (43). The latter two are co-expressed with WT1 in the kidney and may contribute to kidney expression of WT1 in vivo. Recently, the murine WT1 promoter was shown to be transactivated by both PAX 2 and PAX 8 (44, 45). We have identified a novel consensus site 250 bp 5′ of the minimal promoter that strongly bound PAX 8 and mediated potent PAX 8 transactivation of the human WT1 promoter. This is in contrast to the potential PAX 8-binding sites previously identified in the human WT1 promoter (35), which were unable to bind PAX 8 or mediate transactivation of WT1.

EXPERIMENTAL PROCEDURES

Cell Culture—Cell lines 293 (derived from adenovirus 5-transformed human embryonic kidney cells) (CB, American Type Culture Collection (ATCC), Rockville, MD), ACHN (human renal carcinoma cells) (CRL1611, ATCC), Madin-Darby canine kidney cells (CCL4, ATCC), and HeLa (a human cervical carcinoma cell line) (CCL2, ATCC) were grown in Eagle’s minimal essential medium. Renal carcinoma cells Caki-1 and Caki-2 (HTB46 and -47, respectively; ATCC) were grown in McCoy’s 5a medium. BHK, a cell line derived from baby hamster kidney cells (CRL10, ATCC) was grown in Dulbecco’s modified Eagle’s medium. TM4, a mouse Sertoli cell line (CCL1715, ATCC) was grown in a 1:1 mixture of Ham’s F-12 and Dulbecco’s modified Eagle’s medium. K562, a cell line derived from a human chronic myelogenous leukemia in blast crisis (ATCC, CCL243), was maintained in RPMI 1640 medium. All cells were maintained in media supplemented with 10% fetal calf serum. Monolayers were seeded, and the suspension culture (K562) was split 48–72 h before transfection. The cells were transfected while in the log phase of growth, i.e., the monolayers were <75% confluent, and the suspension cultures were at <5 × 10^6 cells/ml.

WT1 Promoter and Enhancer Constructs—The 652-bp HindIII-PstI fragment comprising the WT1 promoter contains potential binding sites for the PAX, GATA, E2A, Sp1, and WT1 transcription factors (Fig. 1). To determine the role of the PAX core binding sites, we subchon the 5′-flanking region containing two potential PAX 8 core sites (CTGCCC) for the PAX, GATA, E2A, Sp1, and WT1 transcription factors (Fig. 1). The latter two are co-expressed with WT1 in the kidney and may contribute to kidney expression of WT1 in vivo.

For transactivation assays PAX 8 was co-transfected into HeLa cells as described above except that increasing amounts of the human PAX 8 cDNA expression construct (48), pSVK3PAX8, were added to each transfection of 5μg of reporter pcP7H and 2 μg of pSV40β-Gal. The PAX 8 cDNA expression construct contains a 1.4-kilobase pair DNA fragment including the entire open reading frame of the predominant isoform PAX 8α (32). This cDNA was initially obtained by screening a human fetal kidney cDNA library (CLONTECH, Palo Alto, CA) and was cloned into the expression vector pSVK3 (Pharmacia Biotech Inc.) (48). The mutant clone containing a truncated PAX 8 binding site was phb.P7H, the empty pCAT®-Basic vector (Promega), the minimal promoter phb.1e.1, and the PAX 8 enhancer construct phb.1e.05 were also cotransfected with 5 or 10 μg of the PAX 8 expression construct and 2 μg of pSV40β-Gal.

RT-PCR—Total cellular RNA was extracted from 2-day-old mouse kidneys and all cell lines described above by the method of Chomczynski and Sacchi (49) with STAT-60 (Tel Test, Friendswood, TX) according to the manufacturer’s recommendations. First strand cDNA synthesis and PCR amplification were performed as described previously (50). PCR amplification of cDNA encoding the Pro-Ser-Thr transactivation domain and carboxyl terminus of the PAX 8 gene in human samples was performed by using the primer pairs: forward PAX 8 (5′-TCCACCTCTCCTCTTTATCT-3′) and reverse PAX 8 (5′-AGTCCTGTCCTGTCCTGTA-3′). The forward PCR primer (5′-CCTGCTGAGTGTTCTTCGCTA-3′) (bp 20–39) and the reverse PCR primer (5′-TTTCTCTGGCAGACCCCTGA-3′) were designed using the software program Vector NTI (InforMax, Inc.). The PCR product was subchon to cloning into the pBluescript SK+ vector (Stratagene) and sequenced by the method of Vlcek and Sacchi (51). The resulting sequence was identical to the published sequence (27).

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RESULTS

Identification of Potential PAX 8-binding Sites—The initial sequence analysis of the WT1 promoter identified three potential PAX 8-binding sites containing the paired box core binding site CTGCCC. Two of these sites were located in the 5′-flanking region, 59 and 125 bp 3′ of the HindIII site in the full-length WT1 promoter (Fig. 1). The third site was located in the 5′-untranslated region of the WT1 gene, 445 bp 3′ of the HindIII site (86 bp 3′ of the minimal promoter). However, functional assays revealed no PAX 8 binding at these sites and no transcriptional activation of the minimal promoter by the two 5′-most sites (see “PAX 8 Transactivation of the WT1 Promoter”).

Because the initially identified PAX 8 core binding sites lacked functional activity, we examined additional PAX-binding sites in other genes. An alignment of consensus binding sites for PAX 8, derived from the thyroperoxidase and thyroglobin gene promoters (32); PAX 5, derived from the CD19 gene promoter (55); and PAX 2, derived from oligonucleotide selection (43) revealed a consensus site independent of the CTGCCC core site. Our comparison of all of these paralogous PAX-binding sites indicated that a novel consensus site, CAST-SANGCNK (where S represents G or C and K represents T or G), may mediate PAX 8 activation as well. Two of 11 bases in this consensus site are conserved within all of the binding sites analyzed and are referred to as invariant. This novel consensus site was then identified in the 5′-flanking region of the WT1 promoter (Fig. 1) using the GCC sequence analysis program.

Comparison of WT1 Promoter Activity and Endogenous PAX 8 Expression—The embryonic expression pattern of PAX 8 and
WT1 combined with the potential PAX 8-binding sites within the WT1 promoter suggested that PAX 8 may modulate WT1 expression. To examine the correlation between PAX 8 and WT1 expression, we first obtained RT-PCR evidence of coexpression in various cell lines. Eight of nine cell lines were either positive or negative for both PAX 8 and WT1 expression (Fig. 2), further strengthening the correlation of expression. In one of six kidney cell lines, we observed discordant expression, suggesting that PAX 8 expression is not necessary for WT1 expression in 293, adenovirus 5 transformed human embryonic kidney cells, and the human PAX 8 expression construct pSVK3PAX8. We observed dose-dependent activation of the WT1 promoter by PAX 8 cotransfection: 5 μg of the PAX 8 expression construct greater increased WT1 promoter activity (Fig. 4). Overall, these data showed that PAX 8 increased promoter activity by 30-fold. HeLa cells expressed a very low level of PAX 8 mRNA, as detected by RT-PCR (Fig. 2), and this correlated with their weak basal WT1 promoter activity (Fig. 3). However, the expression of high levels of exogenous PAX 8 greatly increased WT1 promoter activity (Fig. 4). Therefore, the expression of PAX 8 may modulate WT1 expression in the kidney, we examined the promoter regulatory regions by functional and binding assays using CAT cotransfections of HeLa cells and EMSAs. The basal WT1 promoter functioned weakly in HeLa cells (Fig. 3), but the addition of PAX 8 expression construct greatly increased WT1 promoter activity (Fig. 4). The PAX 8 responsiveness of the WT1 promoter was assessed by cotransfection of 5 μg of pch.7PH (the WT1 reporter construct) and 2 μg of pSV40β-Gal and increasing amounts (0–20 μg) of human PAX 8 cDNA expression construct pSVK3PAX8. We observed dose-dependent activation of the WT1 promoter by PAX 8 cotransfection: 5 μg of the PAX 8 expression construct increased WT1 promoter activity 17-fold relative to activation by the pSVK3 empty expression vector control; and 10 μg of PAX 8 increased promoter activity by 30-fold. HeLa cells expressed a very low level of PAX 8 mRNA, as detected by RT-PCR (Fig. 2), and this correlated with their weak basal WT1 promoter activity (Fig. 3). However, the expression of high levels of exogenous PAX 8 greatly increased WT1 promoter activity (Fig. 4). Overall, these data showed that WT1 promoter activity was correlated with PAX 8 expression and that the basal promoter could be strongly transactivated by the addition of a transcription factor (PAX 8) that is normally expressed with WT1 during the development of the kidney (28).

Localization of PAX 8-responsive Sites in the WT1 Promoter—While transactivation of the full-length WT1 promoter demonstrated that PAX 8 up-regulated WT1 expression, it did not determine which site(s) mediated transactivation. To address this question, we compared the activity of two different minimal WT1 promoter constructs containing different potential PAX-binding sites cloned 3’ of the CAT gene (Fig. 5). Initially, we determined whether the WT1 PAX CON site could enhance transcription of the WT1 minimal promoter (Fig. 5A). In the construct pch.1e.05, one copy of the WT1 PAX CON oligonucleotide containing the novel PAX 8-binding site was inserted 3’ of the CAT gene. In K562 cells that express endogenous PAX 8, the CAT activity of the PAX 8 enhancer construct, pch.1e.05, containing one copy of the novel WT1 PAX CON site was 6-fold greater than that of the minimal promoter pch.1 alone. In contrast, in 293 and HeLa cells lacking endog-
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Fig. 4. PAX 8 transactivated the WT1 promoter. This graph depicts the dose-dependent activation of the WT1 promoter by PAX 8 cotransfection of HeLa cells. Five micrograms of the reporter construct pb.7pH was cotransfected with 2 μg of pSV40βGal and increasing amounts (0–20 μg) of pSVK3PAXX or pSVK3 vector control. The values shown are the average percentage of acetylation from three different experiments; the error bars depict S.E. Below the graph CAT activity is depicted relative to the activity of the WT1 promoter cotransfected with the pSVK3 vector control.

To determine whether additional PAX 8 sites were functional, we examined a second construct, pb.1.e.1, containing the 100-bp 5′-flanking region (bp 20–140) of the WT1 full-length promoter cloned 3′ of the CAT gene (Fig. 5B). The 3′-insert in this construct lacks the WT1 PAX CON binding site but includes the two PAX core sites identified by sequence analysis and the Distal and Proximal sites thought to mediate PAX 8 transactivation of the murine wt1 promoter (Fig. 2). In contrast to the significant increase in promoter activity associated with a single copy of the WT1 PAX CON site in the PAX 8 enhancer construct, the construct containing two other potential PAX core binding sites, pb.1.e.1, had no increased activity over the minimal promoter construct, pb.1 (Fig. 5B). This suggests that in K562 cells with endogenous PAX8, the 100-bp region cannot function independently as an enhancer, i.e. these core sites are unable to act in a position- and orientation-independent manner.

Since the activity of the single WT1 PAX CON site is significantly greater than that of the two core sites identified by sequence analysis, we asked whether this novel consensus site could also mediate transactivation by exogenous PAX 8. The responsiveness of the WT1 PAX CON site to endogenous PAX 8 was confirmed by co-transfection of the PAX 8 enhancer construct, pb.1.e.05, with a PAX 8 expression construct in HeLa cells lacking significant PAX 8 expression (Fig. 5C). Five micrograms of each of the reporter constructs, pb.1, pb.1.e.05, and pb.1.e.1, were cotransfected with 10 μg of the pSVK3PAXX expression construct and 2 μg of pSV40βGal in HeLa cells. The CAT activity of the PAX 8 enhancer construct, pb.1.e.05, was 4-fold greater after cotransfection with pSVK3PAX8 than with pSVK3 vector alone. In contrast, the cotransfected PAX 8 expression construct was unable to transactivate pb.1 (the minimal promoter) or pb.1.e.1 (minimal promoter/5′-flanking region), the construct that contained both the Distal and Proximal sites; i.e. the CAT activity of pb.1.e.1 cotransfected with pSVK3PAX8 was only 1.3-fold greater than with pSVK3 vector alone. Overall, a comparison between the transcriptional activity of the WT1 PAX CON site located at the 5′-end of the human WT1 promoter and the potential PAX binding core sites located within the 100-bp 5′-flanking region reveals that the Distal and Proximal sites are nonfunctional but that the novel WT1 PAX CON site mediates PAX 8 activation of the WT1 promoter.

Having identified a PAX 8-responsive site that enhances WT1 promoter activity only in the presence of PAX 8 (either endogenously present or exogenously added PAX 8), we then confirmed that strong WT1 promoter activity in K562 cells depends upon an intact PAX 8-binding site. To assess the importance of two invariant bases (in boldface type) within the WT1 PAX CON site, CASTSANGCNK, we mutagenized the T to A and the G to T and investigated whether this alteration affected CAT activity of the WT1 promoter. The mutagenized binding site resulted in a substantial loss (57%) of WT1 promoter activity in K562 cells containing endogenous PAX 8 (Fig. 6A) and no significant loss of activity in HeLa and 293 cells lacking endogenous PAX 8. Taken together, the data demonstrate that 1) endogenous PAX 8 plays an important role in the activation of the WT1 promoter, since the promoter activity in K562 cells (Fig. 3) is much stronger than that in 293 and HeLa cells and 2) WT1 promoter activity depends upon the presence of an intact PAX 8-binding site. The presence of some residual activity in the mutated promoter, despite the complete absence of PAX 8 binding to the mutated site in EMSA (see below), suggests that additional factors present in K562 cells bind and activate the WT1 promoter. In fact, several functional Sp1- (36, 37) and WT1- (38, 39) binding sites and two GATA-binding sites have been identified and shown to modulate WT1 promoter activity. Thus, while PAX 8 is a significant activator of WT1 expression in vivo, it is not the only factor that can activate the WT1 promoter.

Having identified the PAX8 site sufficient for WT1 promoter activity and two invariant bases essential for strong WT1 promoter activity in K562 cells containing endogenous PAX 8, we then confirmed the necessity of an intact PAX 8 site in HeLa cells co-transfected with the PAX 8 expression construct (Fig. 6B). In HeLa cell co-transfection assays, the PAX 8 expression construct strongly activated the WT1 promoter containing an intact PAX 8 site, but the 2-bp mutation resulted in a substantial loss (50%) of PAX 8 transactivation. Overall, the activities of both the PAX 8 enhancer construct (pb.1.e.05) and the full-length WT1 promoter in K562 cells containing endogenous PAX 8 closely resemble the activities seen in HeLa cells cotransfected with exogenous PAX 8. This demonstrates that the PAX 8 site is functional in vivo and that either endogenous or exogenous PAX 8 regulates WT1 expression. The presence of some residual activity in the mutated promoter despite the complete absence of PAX 8 binding to the mutated site in EMSA suggests that either additional factors present in HeLa cells stabilize PAX 8 binding to the mutated site, weakly activating the WT1 promoter, or alternatively, some weak PAX 8 binding to other cryptic sites may occur in the absence of the preferred binding site. In fact, two alternative PAX-binding sites have been identified in the mouse promoter, which fail to bind in vitro translated PAX 8 protein in EMSA but do contribute to the murine wt1 promoter activity (44). Taken together, these data show that while PAX 8 is a significant activator of WT1 expression in vivo, it is not the only factor that can activate the WT1 promoter, and that while PAX 8 binding at its cognate site is sufficient to strongly activate the WT1 promoter, additional cryptic sites may also be able to partially activate the promoter.

Identification of Functional PAX-binding Sites in the WT1

3 G. Fraizer and G. F. Saunders, unpublished observations.
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To confirm that the activation of WT1 by PAX 8 correlates with DNA binding, we examined potential binding sites within the WT1 promoter by EMSA (Fig. 7). The WT1 PAX CON probe used in the EMSA contains the 11-bp consensus flanked by the corresponding genomic WT1 sequence. Since the consensus sequence is in the first 11 bp of the WT1 promoter cloned into the construct pch.7PH, the oligonucleotide used for EMSA contained 20 bp of the 5'-end of the WT1 promoter cloned into pch.7PH. To position the binding site in the middle of the 30-bp oligonucleotide used for EMSA, we used the adjacent 10-bp sequence from the distal promoter sequence (35), thus allowing analysis of the 30-bp site as it exists in genomic DNA.

Nuclear extracts of Caki-1 cells containing abundant levels of the transcription factor PAX 8 were bound to radiolabeled WT1 PAX CON (WT7 (Fig. 7A). The PAX 8-specific complex is indicated with an arrow. PAX 8 expression in these cells was verified by RT-PCR analysis of Caki-1 cell RNA (Fig. 2). We also prepared in vitro translated PAX 8 protein by using wheat germ extracts programmed with a PAX 8 cDNA expression vector clone (pBSPAX8) to determine whether the DNA-protein complex observed with Caki-1 extracts (Fig. 7A, lane 2) migrated similarly to the complex formed with PAX 8 protein alone (Fig. 7A, lane 3). In vitro translated PAX 8 proteins are able to form an identical appearing complex with the WT1 PAX CON oligonucleotide, suggesting that no additional proteins in Caki-1 extracts are required for PAX 8 complex formation. Negative control lysates from in vitro translated wheat germ extracts generated in the absence of the pBSPAX 8 expression vector were also tested to verify the specificity of PAX 8 binding. As expected, these unprogrammed wheat germ lysates failed to bind the radiolabeled WT1 PAX CON oligonucleotide (Fig. 7A, lane 4). The binding specificity of the WT1 PAX CON oligonucleotide was tested by competition with a control oligonucleotide, CT, containing a known PAX 8-binding site derived from the thyroperoxidase promoter, a target gene for PAX 8. The Caki-1 complex was specifically eliminated by competition with the 20- and 100-fold molar excesses of unlabeled CT (Fig. 7A, lanes 5 and 7). In contrast, the Caki-1 complex was not eliminated by competition with as much as a 100-fold molar excess of unlabeled GATA oligonucleotide (Fig. 7A, lanes 6 and 8). Because the PAX 8-binding site in the WT1 promoter was a

**Fig. 5.** A novel PAX 8 site activates the minimal WT1 promoter in cells with endogenous or exogenously added PAX 8. A, this histogram shows the differential activity of the PAX 8 enhancer construct in cell types that differentially express PAX 8. Five micrograms of the promoter constructs shown below were transfected with 2 μg of pSV40β⁴-Gal either into cells that express PAX 8 protein (K562) or into cells that express little or no PAX 8 (293 and HeLa). The basal promoter construct, pcb.1, contains the minimal WT1 promoter (black box) but lacks any potential PAX 8-binding sites, and the PAX 8 enhancer construct, pcb.1.e.05, includes the minimal promoter and the 30-bp WT1 PAX CON oligonucleotide (shaded box) cloned 3' of the CAT gene. The CAT activity of the PAX 8 enhancer construct is depicted relative to the activity of the minimal promoter construct, pcb.1; the error bars depict S.E. B, this histogram shows the differential activity of the minimal WT1 promoter by either a single copy of the novel WT1 PAX CON site or by the two potential PAX core binding sites in the 100-bp 5'-flanking region. Five micrograms of either the minimal WT1 promoter (pcb.1) or the PAX 8 enhancer construct (pcb.1.e.05) or the flanking region construct (pcb.1.e.1) were transfected with 2 μg of pSV40β⁴-Gal into K562 cells that express endogenous PAX 8 protein. The flanking region construct, pcb.1.e.1, includes the minimal promoter (black box) and the 100-bp 5'-flanking region (hatched box) cloned 3' of the CAT gene. CAT activity is depicted relative to the activity of the minimal promoter construct, pcb.1; the error bars depict S.E. C, this histogram shows the differential response of the minimal WT1 promoter (pcb.1) or the PAX 8 enhancer construct (pcb.1.e.05) or the flanking region construct (pcb.1.e.1) to PAX 8 transactivation in HeLa cells. It demonstrates the ability of a single WT1 PAX CON element to serve as a target for PAX 8 transactivation of the WT1 promoter. Five micrograms of the promoter constructs shown above were cotransfected with 2 μg of pSV40β⁴-Gal and 10 μg of either pSVK3PAX8 or the empty expression construct pSVK3. CAT activity is depicted relative to the activity of the promoter constructs cotransfected with the pSVK3 vector control. The values shown are the average relative activities from three different experiments; the error bars depict S.E.
novel consensus site, we compared the Caki-1 complex formed by binding to radiolabeled CT that formed with radiolabeled WT1 PAX CON (Fig. 7A, lane 10). The Caki-1 complex was specifically eliminated by competition with 20-fold and hundred-fold molar excesses of unlabeled WT1 PAX CON oligonucleotide (Fig. 7A, lanes 11 and 12). We also compared the in vitro translated PAX 8 protein complex formed by binding to radiolabeled CT with radiolabeled WT1 PAX CON (Fig. 6A, lane 13). To confirm that the Caki-1 complex contained PAX 8 protein, we used a cross-reactive antibody prepared by inoculating rabbits with PAX 6 protein (55) to supershift the Caki-1 complex (Fig. 7A, lanes 16 and 17). The PAX antibody dramatically reduced Caki-1 complex formation (Fig. 7A, lane 17). The diminished complex is indicated by a thick arrow on the left, and a portion of the PAX-binding DNA complexes were supershifted (see complex indicated by thin arrow on the right). Although this PAX 6 antibody could also cross-react with PAX 2, Caki-1 cells do not express detectable amounts of PAX 2 (32).
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To determine whether the two invariant bases (see below, in boldface type) contained within the WT1 PAX CON consensus site CASTSANGCNK are essential for binding and activation by PAX 8, the binding site was mutagenized (T to A and G to T). A loss of binding activity was observed by competition EMSA (Fig. 7B). The binding specificity of the WT1 PAX CON oligonucleotide for the in vitro translated PAX 8 protein was tested by competition with the control oligonucleotide CT, which contains a known PAX 8-binding site. As previously shown for Caki-1 nuclear extracts (Fig. 7A), the PAX 8 protein complex was specifically eliminated by competition with 20- and 100-fold molar excesses of unlabeled CT oligonucleotide (Fig. 7B, lanes 4 and 5). In contrast, the PAX 8 complex was not eliminated by competition with as much as a 100-fold molar excess of unlabeled mutant WT1 PAX CON oligonucleotide. Identical results were obtained by using a radiolabeled CT oligonucleotide and an excess of unlabeled mutant WT1 PAX CON oligonucleotide. Radiolabeled mutant WT1 PAX CON oligonucleotide failed to bind either PAX 8 or the Caki-1 extracts (data not shown).

Using EMSA and cotransfection analyses, we examined the rest of the WT1 promoter region and found no additional functional PAX 8-binding sites. Although two potential core binding sites for PAX 8 are located within the 5'-flanking region (35) and one within the 5'-untranslated region of the WT1 gene, we observed DNA binding only at a novel WT1 PAX 8 site, termed WT1 PAX CON, located 250 bp 5' of the minimal promoter. Initially, we demonstrated that these three core PAX 8-binding sites previously identified were not responsible for PAX 8 transactivation, since no specific complexes were observed by using 30-bp oligonucleotides containing these potential binding sites. We confirmed this finding by demonstrating that the Distal and Proximal sites thought to mediate PAX 2 and PAX 8 activation of the murine WT1 promoter (44, 45) were unable to bind specifically to PAX 8 protein (Fig. 8). Again, we observed only nonspecific binding mediated by the unprogrammed wheat germ lysate controls. These results can be explained by the presence of endogenous Sp1 or AP2-like factors in the unprogrammed lysates binding the Sp1 or AP2 sites within the Distal and Proximal site oligonucleotides. Thus, identical com-
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mediated potent PAX 8 transactivation of the promoter. This was shown to be specific for Caki-1 extracts and no additional WT1 PAX CON binding sites were identified by EMSA (data not shown). Thus, a novel functional PAX 8 site, located 250 bp 5' to the minimal promoter, was identified in EMSA but also resulted in a substantial loss of promoter transactivation. Rather, our results demonstrate that the novel PAX-binding sites in the Engrailed-2 promoter (BSII) is nearly identical to WT1 PAX CON (with one mismatch), and the BSII site has been shown to mediate PAX 2, PAX 5, and PAX 8 binding in vitro as well as in vitro.

Our EMSA results strongly argue against the role of the originally reported potential PAX 8-binding sites (CTGCCC) previously identified by sequence analysis, since they are unable to mediate PAX 8 binding and therefore cannot mediate transactivation. Rather, our results demonstrate that the novel PAX consensus site mediated PAX 8 activation of the human WT1 promoter. This site plays a significant role in PAX 8 activation of the WT1 promoter, as demonstrated by the significant increase in promoter activity observed following the addition of a single copy of this PAX 8 site to the minimal promoter construct. A comparison of the activity of the minimal promoter constructs containing either the active WT1 PAX CON or the inactive core PAX sites showed that only the WT1 PAX CON sequence activated the WT1 promoter and mediated PAX 8 transactivation. Additionally, mutagenesis of the two invariant bases in WT1 PAX CON not only eliminated binding in EMSA but also resulted in a substantial loss of promoter activity in cells containing PAX 8 and greatly reduced the transactivation of the WT1 promoter by exogenously added PAX 8. That mutagenesis did not completely eliminate transactivation suggests that additional proteins present in HeLa cells may help stabilize PAX 8 binding to the mutant site and that those stabilizing proteins are absent in Caki extracts. Alternatively, cryptic binding sites may function (in the absence of the predominant site) during overexpression studies.

The human WT1 PAX CON site was not conserved in the mouse wt1 promoter, although there was an analogous potential WT1 PAX CON binding site 350 bp 5' of the minimal promoter. That the WT1 PAX CON site was not conserved in the murine promoter suggests that PAX 8 modulates WT1 expression by using different regulatory elements in the human and mouse systems. While the murine wt1 promoter has recently been shown to be transactivated by both PAX 2 and PAX 8 (44, 45), how this transactivation occurs is unclear. The PAX 2-binding site within the murine promoter was not identified (45). The Distal oligonucleotide containing a potential Pax 2-
binding site failed to form DNA-protein complexes with the in vitro translated PAX 2 protein, and no additional binding sites were identified. Additionally, in our hands, the human Distal and Proximal oligonucleotides containing potential PAX 8 sites both fail to specifically bind in vitro translated PAX 8 protein (Fig. 8). Possibly differences in EMSA conditions could explain the different results; for example, our binding buffer does not include pBlueScript DNA as a nonspecific competitor but relies solely on poly(dI-dC). In contrast to the lack of binding by the PAX core sites, the human WT1 PAX CON oligonucleotide specifically bound both Caki-1 extracts containing endogenous PAX 8 protein and in vitro translated PAX 8 protein (Fig. 7). Formation of the specific PAX 8-DNA complex was blocked by competition with the oligonucleotide CT, which contains a known PAX 8-binding site, and not by competition with the nonspecific oligonucleotide GATA or the mutant WT1 PAX CON oligonucleotide. Also, the formation of the majority of the PAX 8 complexes by the Caki-1 extracts was prevented, and a portion of the complexes were supershifted by PAX-reactive antibodies.

PAX 2 expression in the developing kidney precedes PAX 8 expression; PAX 2 is present in the ureteric bud, which induces the initial lower levels of WT1 expressed in the kidney. These results suggest that the PAX-binding factors expressed in the kidney activate the WT1 promoter so that in kidney, strong positive acting transcription factors induce high levels of WT1 expression. The promoters of many kidney growth-promoting genes contain WT1 binding sites, and WT1 repression from the promoters of many growth-promoting genes contain WT1 binding sites, and WT1 may be sufficient for its function as a repressor of growth factor and growth factor receptor genes.

These results suggest that the PAX-binding factors expressed in the kidney activate the WT1 promoter so that in kidney, strong positive acting transcription factors induce high levels of WT1 expression. The promoters of many kidney growth-promoting genes contain WT1 binding sites, and WT1 repression from the promoters of many growth-promoting genes contains WT1, which suggests that WT1 may be an essential part of the cascade of transcription factors controlling kidney development. We hypothesize that the initial lower levels of WT1 expressed in the kidney may be sufficient for its function as a repressor of growth factor and growth factor receptor genes, but after PAX induction, WT1 expression levels can become very high and are then sufficient to activate the WT1 autorepression mechanism, down-regulating further WT1 expression (38, 39). This suggests that PAX 8 binding and activation of the WT1 promoter is essential for kidney development.

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