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Human platelet-derived growth factor A chain is transcriptionally repressed by the Wilms tumor suppressor WT1

(DNA-binding protein/zinc finger/repressor/kidney development)

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ABSTRACT **Wilms tumor, an embryonic kidney malignancy, accounts for ≈6% of all pediatric neoplasms. A gene implicated in the genesis of this tumor, the Wilms tumor suppressor gene (*WT1*), encodes a zinc-finger DNA-binding protein (WT1) that functions as a transcriptional repressor. In certain Wilms tumors, the platelet-derived growth factor A chain (PDGF-A) is overexpressed; it has therefore been suggested that it may play an autocrine role in development of these neoplasms. Since the PDGF-A promoter contains putative binding sites for WT1, we explored the role of WT1 in regulating A-chain expression. The major PDGF-A promoter activity was localized in transient transfection assays to a region spanning from −643 to +8 relative to the transcription start site. WT1 bound to several sites in this region of the promoter, as demonstrated by gel-shift analysis and DNase I footprinting, and functioned as a powerful repressor of PDGF-A transcription *in vivo*. Maximal repression (>50-fold) of the PDGF-A promoter was dependent on the presence of multiple WT1 binding sites in transient transfection assays. Our observations suggest a mechanism for normal downregulation of a growth factor gene and of an autocrine growth process of import in kidney development and other biological systems.**

Wilms tumors result from the continued proliferation of embryonic kidney blastemal cells, which are insensitive to the normal signals to differentiate (1). From the pattern of familial and sporadic inheritance, the early age of onset of bilateral tumors, and the frequency of associated chromosomal deletions, it has been proposed that the primary defect causing Wilms tumors is the loss of a recessive, tumor suppressor gene product. In hereditary tumors, a germ-line predisposing mutation is later followed by a second somatic mutation or by loss of heterozygosity at the same locus (2). Comparison of overlapping chromosomal deletions in patients with Wilms tumor has allowed identification of a putative tumor suppressor gene *WT1* at locus 11p13 (3–5).

The Wilms tumor gene (*WT1*) encodes a DNA-binding protein containing four zinc fingers, which bind to the target sequence GCGGGGGCG (6), also recognized by the zinc-finger transcription factors EGR1, EGR2, and EGR3 (7, 8). However, in contrast to the latter group, WT1 functions as a transcriptional repressor of synthetic promoter constructs through this sequence (9). The biological importance of the DNA-binding domain is highlighted by naturally occurring mutations in the zinc-finger domain of WT1 from Wilms tumor patients (10–12). In addition, the Pro+Gln-rich N-terminal domain of WT1 is required for transcriptional repression (9). Although the genes subject to WT1 regulation are not known, the targets of a transcriptional repressor, which was defined genetically as a tumor suppressor, are expected to include positive regulators of cell growth, such as growth factors.

One mechanism responsible for the abnormal proliferative abilities of cancer cells may be overproduction of specific growth factors. Several small peptide growth factors such as platelet-derived growth factor (PDGF) (13–15), transforming growth factors α and β (16–19), and insulin-like growth factors (IGFs) (20) are in fact secreted by malignant tumors. Specifically, some Wilms tumors and osteogenic sarcomas are known to overproduce PDGF (21, 22).

PDGF is a disulfide-linked homo- or heterodimer consisting of two related subunits, the A and B chains, which are encoded by different genes on chromosomes 7p22 and 22q12.3–q13.1, respectively. The three isoforms of PDGF (AA, AB, and BB) stimulate biological responses by binding to two cell-surface receptors, which function as noncovalent dimers: the α receptor can bind all three isoforms of PDGF with equal affinity, whereas the β subunit can bind only the B chain with high affinity (23, 24).

Physiological roles for PDGF have been postulated in various biological processes including wound healing, atherosclerosis, differentiation, and embryogenesis (25). PDGF A chain (PDGF-A) transcripts of 1.8, 2.3, and 2.8 kilobases (kb) [due to differential splicing and polyadenylylation (26–28)] have been detected in a variety of cultured normal cells (23). Levels of PDGF-A mRNA are increased in normal cultured cells stimulated with growth factors (29) or cytokines (30). Moreover, high levels of PDGF-A transcripts are found in a number of transformed cell lines (23); simultaneous expression of high levels of PDGF and the corresponding receptors has been implicated in maintenance of the transformed phenotype. In particular, PDGF-A has also been shown to be overexpressed in both epithelial and stromal tissue culture outgrowths from various Wilms tumors (22); it has therefore been suggested that it may play an autocrine role in genesis of these embryonic kidney neoplasms. Given the data on overexpression of PDGF-A in Wilms tumors, we asked whether the PDGF-A gene might be a target for transcriptional repression by WT1 and, specifically, whether WT1 might interact directly with its G+C-rich 5′ flanking sequence.

MATERIALS AND METHODS

Construction of Expression Vectors. pCMVhWT1 contains the full-length human WT1 cDNA driven by the cytomega-

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Abbreviations: PDGF, platelet-derived growth factor; IGF, insulin-like growth factor; CMV, cytomegalovirus; CAT, chloramphenicol acetyltransferase.

lovirus (CMV) early promoter/enhancer as described (31). In pCMVhWT1-TTL, a synthetic oligonucleotide containing stop codons in all three reading frames has been inserted at a unique *Bam*HI site (amino acid 179), terminating translation N-terminal to the zinc fingers.

Construction of Reporter Plasmids. The starting point for all subsequent constructs, pACCAT12, was made by attaching *Hin*dIII linkers to a 900-base-pair (bp) *Bss*HII fragment of the PDGF-A gene and inserting this fragment into the *Hin*dIII site of the promoterless chloramphenicol acetyltransferase (CAT) plasmid pSPCAT3 (32). This promoter fragment contains only 8 bp of PDGF-A sequence downstream of the cap site (+1) and 900 bp upstream.

Several 5′ deletions of pACCAT12 were made by digesting the plasmid with *Sac* I (5′ flanking polylinker site), *Sac* I and *Xho* I, or *Sac* I and *Sma* I followed by exonuclease III/S1 nuclease digestion (33). This yielded the constructs pACCATΔSacI, pACCATΔXhoI, and pACCATe30, which was further treated with *Eco*RI, BAL-31, and, finally, *Hin*dIII to release the desired promoter fragments. Treatment of the fragments with Klenow and subcloning into the *Sma* I site of pSPCAT3 produced the derivatives pACCAT.89 and pACCATf27. The deletion endpoints of all CAT plasmids were determined by sequencing (34).

Transfections and CAT Assays. NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium with 10% calf serum. Cells were seeded at 8×10^5 cells per 100-mm plate the day before transfection. The medium was replaced 2–6 hr before transfection by calcium phosphate-mediated precipitation with a total of 31 μg of DNA. Each precipitate contained 10 μg of reporter plasmid, 20 μg of pCMVhWT1 or pCB6+ vector, and 1 μg of the internal reference pON260, a CMV driven β-galactosidase plasmid (35), and was left on the cells for 16–20 hr.

Forty-eight hours after transfection, cell extracts were prepared and CAT assays were performed according to Gorman *et al.* (36) with equal amounts of β-galactosidase activity assayed to normalize for any variation in transfection efficiency. Results shown are the averages of two separate experiments with each sample transfected in duplicate.

Gel-Retardation Assays. The zinc-finger domain of WT1 with the addition of six histidine residues and an initiator methionine, expressed in bacteria and purified by nickel chelate chromatography (WTZF), has been described (6). As a negative control for specific DNA binding, a WT1 variant was expressed that lacks fingers three and four (WTZF-1β_2).

Probes for the gel-retardation analyses were prepared by gel purification of the desired fragments from 3% NuSieve/1% Seaplaque with Mermaid (Bio 101, La Jolla, CA). Fragments were labeled with the Klenow fragment of DNA polymerase and [α-^{32}P]dCTP and unincorporated nucleotides were removed with a Stratagene Nuctrap column. The 114-bp *Bam*HI fragment of the PDGF-A promoter contains nucleotides −584 to −470. The 87-bp *Apa* I/*Eag* I fragment consists of nucleotides −451 to −364, and the 143-bp *Apa* I/*Eag* I fragment includes nucleotides −154 to −11 (ref. 27; D.T.B. and T.C., unpublished data).

Gel-shift complexes were formed at room temperature under conditions used previously for Egr-1 binding (37) with up to 200 ng of WTZF protein. Complexes were resolved on 5% polyacrylamide/0.5× TBE (1× TBE = 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3) gels at 4°C before fixing with 10% acetic acid and drying down for autoradiography.

DNase I Footprinting. The probe spanning nucleotides −642 to −452 was prepared by PCR amplification with the primer 5′-GCT CGC TCC CAA GTG GAA ATG-3′ (labeled with T4 polynucleotide kinase) and the unlabeled primer 5′-CTC TCC ACG GTT CGG ATC CAG-3′. Probes spanning −643 to −262 and −262 to +8 were prepared by Klenow labeling *Xho* I-digested pACCAT12 with [α-^{32}P]dCTP and [α-^{32}P]dATP, followed by restriction with either *Sac* I or *Hin*dIII and gel purification of the 381-bp *Sac* I/*Xho* I and 266-bp *Hin*dIII/*Xho* I fragments. DNase I reactions were performed essentially as described (38) with protein–DNA complexes formed in Egr-1 binding buffer at room temperature.

RNA Preparation and Northern Blot Analysis. Total RNA was prepared using guanidium isothiocyanate (39). The RNA samples (10 μg) were separated on a 1% formaldehyde/agarose gel, transferred to nitrocellulose, and hybridized with the *Sac* II/*Hin*dIII fragment of the PDGF-A coding region (40), labeled by random priming with [α-^{32}P]dCTP. The blot was washed with 0.5× standard saline citrate at 65°C before exposing overnight.

RESULTS AND DISCUSSION

PDGF-A Promoter Activity. Definition of the functional elements of the A-chain promoter may provide the basis for activation of the gene in normal settings as well as for altered expression of the gene in transformed cells. Toward this end, the architecture of the human PDGF-A gene has been defined (27, 41). Previous analysis of the 5′ end of the A-chain gene revealed a major transcriptional start site downstream of a consensus TATAA box (27). We have used deletion analysis to define the segment of the promoter region required for constitutive basal activity. Reporter constructs containing promoter fragments were cloned upstream of the bacterial CAT gene and transiently transfected into NIH 3T3 cells, which express high levels of PDGF-A transcripts. Deletion analysis revealed that maximal promoter activity was obtained with construct pACCATΔSacI containing ≈600 bp of upstream sequence, but significant basal promoter activity remained with 90 bp of upstream sequence in pACCAT.89 (Fig. 1 and Table 1). Very similar data were noted in the osteosarcoma cell line U2-OS, which also expresses high levels of PDGF-A mRNA (D.T.B., unpublished data).

WT1 Repression of PDGF-A. Sequence analysis of the PDGF-A promoter reveals several potential high-affinity sites for WT1, suggesting that the A-chain gene might be subject to transcriptional repression mediated by the Wilms tumor gene product (Fig. 2). To test the functional importance of these elements, the pACCATΔSacI reporter construct, which includes multiple consensus sites (GNGGGGGNG), was chosen for subsequent cotransfection assays with the WT1 expression vector. The human WT1 cDNA was inserted downstream of the CMV early promoter/enhancer in the vector pCB6+ and the resulting plasmid was designated pCMVhWT1. When increasing amounts of WT1 expression vector were transfected into NIH 3T3 cells with the PDGF-A promoter construct, CAT activity fell progressively to <2% of control levels (Fig. 2*C*); as little as 1 μg repressed transcription >5-fold. Furthermore, pCMVhWT1 had little effect on the minimal PDGF-A promoter construct pACCATf27. Others have shown that WT1 is incapable of repression in the absence of WT1 binding sites; for example, no effect is seen on the herpes simplex virus thymidine kinase

GGTCCGCACGAACCCCGAGCGCTTCCGAGGTGCGGGTCCCAGGCCCGGAATC

.89 .f27

CGGGGGAGGCGGGGGGGGGGGGGCGGGGGCGGGGGCGGGGGAGGGGCGC

*

GGCGGCGGCGC**TATAA**CCCTCTCCCCGCCGCCGGCCGGCTCCACACGCGCG

FIG. 1. Characterization of the PDGF-A promoter. Sequence of the minimal PDGF-A promoter. The 5′-most nucleotide present in each promoter construct is indicated by a dot. Transcriptional start site is depicted by *. TATAA element is shown in boldface type, and tandem copies of the WT1 consensus element GNGGGGGNG in the promoter proximal region are underlined.

Table 1. Relative basal levels of PDGF-A promoter constructs in transient expression assays in NIH 3T3 cells

Construct	5′ endpoint of deletion	Relative CAT activity, % of pACCAT12
pACCAT12	−890	100
pACCATΔSacI	−643	147
pACCATΔXhoI	−262	37.0
pACCAT.89	−92	62.1
pACCATf27	−60	4.2

Each of the PDGF-A promoter constructs contains sequence from the 5′ endpoint indicated to +8 relative to the transcription start site. Relative CAT activity is the CAT activity of a given construct with 20 μg of vector pCB6+ relative to the CAT activity of the most extensive promoter construct pACCAT12. NIH 3T3 cells were transfected with 10 μg of reporter, 20 μg of vector pCB6+, and 1 μg of β-galactosidase internal reference plasmid. Equal amounts of β-galactosidase activity have been assayed to normalize for any variation in transfection efficiency. CAT activity represents the average of two experiments with each sample transfected in duplicate.

promoter construct $pBLCAT_2$ (31). These data indicate that WT1 is indeed a potent repressor of A-chain transcription.

WT1 Binding to the PDGF-A Promoter. To examine whether WT1 protein interacts directly with the PDGF-A promoter, electrophoretic mobility-shift assays were performed with a WT1 zinc-finger protein fragment (WTZF) produced in bacteria. Three probes, encompassing the putative WT1 binding sites, were tested for their ability to form DNA–protein complexes (Fig. 3*A*). When incubated with WT1 zinc-finger protein, the PDGF-A promoter fragment spanning nucleotides −584 to −470 formed a single complex at 10–50 ng and, at higher amounts of protein, a second predominant slower-migrating species, suggesting that two high-affinity sites are present within this fragment. We do not believe this second species represents multimers of WT1 associating at a single binding site because even 200 ng of WTZF protein does not form more than a single complex with a synthetic probe encoding one WT1 binding site (6). As a negative control, a WT1 zinc-finger mutant lacking fingers three and four was purified from bacteria; this variant WTZF-$1\beta_2$ does not form the complexes seen with the wild-type WT1 zinc finger domain.

The PDGF-A promoter fragment consisting of nucleotides −451 to −364 formed a single complex with WTZF. The promoter-proximal fragment consisting of nucleotides −154 to −11 contains two high-affinity binding sites, as evidenced by the two predominant complexes formed at high protein concentrations. Thus, gel-shift analyses suggest that multiple high-affinity WT1 binding sites are present in the PDGF-A promoter.

Consistent with these results, DNase I footprinting (Fig. 3*B*) demonstrated that multiple sequences in the promoter were protected from cleavage by the WT1 zinc-finger domain. Each of the regions footprinted, −578 to −560, −537 to −496, −440 to −420, and an extensive protection between −98 and −49, contains one or more sequences homologous to the previously defined WT1 consensus. Thus, *in vitro* binding of WTZF to the PDGF-A promoter, as demonstrated by these gel-shift and DNase I analyses, suggests a direct regulatory role for WT1 *in vivo*.

Elements Required for WT1 Repression. The functional relevance of the WT1 binding sites was investigated by cotransfecting deletion derivatives of the PDGF-A promoter and pCMVhWT1 or pCMVhWT1-TTL into NIH 3T3 cells. Our results show that the PDGF-A promoter derivative pACCATΔSacI is repressed ≈50-fold when cotransfected with 20 μg of pCMVhWT1 (Fig. 4*A*). Deletion of the three distal Wilms tumor binding sites yields constructs that are repressed significantly but to a lesser extent; pACCATΔXhoI

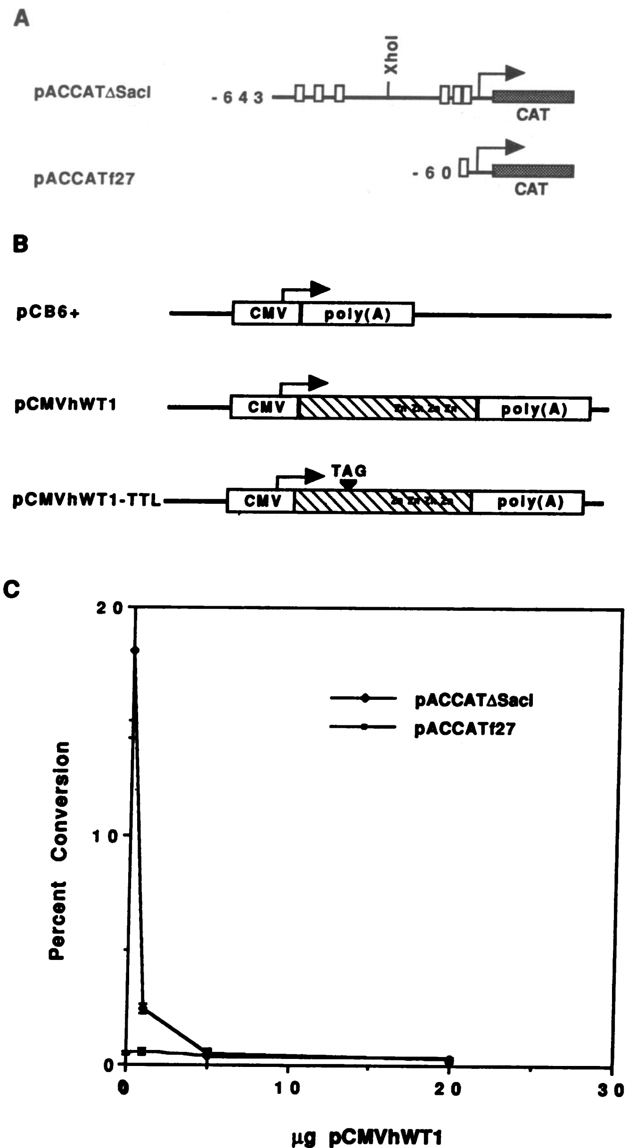

FIG. 2. Effect of WT1 on transcription from the PDGF-A promoter. (*A*) PDGF-A promoter–CAT constructs used in cotransfection assays. (*B*) Expression vectors used in cotransfection assays. The CMV early promoter/enhancer of pCB6+ drives expression of the full-length human WT1 cDNA (pCMVhWT1). As a negative control, pCMVhWT1-TTL contains a linker with stop codons in all three reading frames inserted at amino acid 179. (*C*) Repression of pACCATΔSacI but not pACCATf27 by WT1. NIH 3T3 cells have been transfected with 10 μg of the indicated reporter; 0, 1, 5, or 20 μg of pCMVhWT1 expression vector; and 1 μg of the internal β-galactosidase reference plasmid pON260. Percentage conversion is of equal amounts of β-galactosidase activity to control for variation in transfection efficiency. Each data point represents the average of two separate experiments with each sample transfected in duplicate.

and pACCAT.89 are both repressed ≈14-fold. In contrast, the construct pACCATf27, with low basal activity and a single Wilms tumor binding site, is affected <2-fold by the addition of 20 μg of WT1 expression vector. A control expression vector, pCMVhWT1-TTL, containing a translation termination linker N-terminal to the zinc fingers is not able to repress transcription from any of the PDGF-A reporters (Fig. 4*B*). Collectively, these data and our DNA binding studies suggest that WT1 transcriptionally represses the A chain of PDGF through interactions with multiple sites.

PDGF-A Expression. The early expression of WT1 in the developing kidney and its ability to function as a repressor of transcription implicate the downregulation of WT1 target

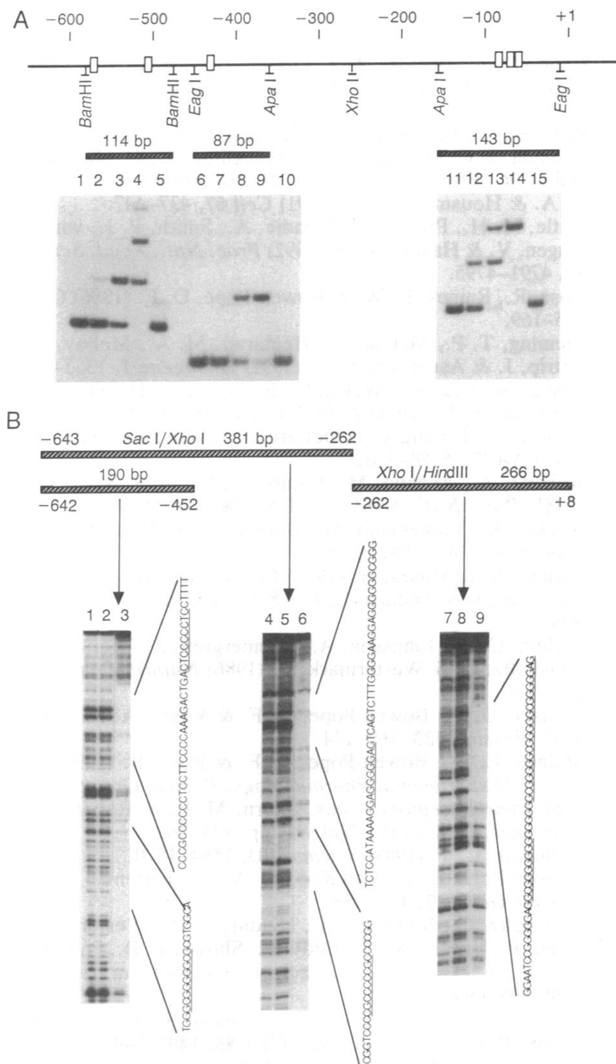

FIG. 3. Interaction of WT1 with the PDGF-A promoter. (A) PDGF-A promoter gel-shift analysis with purified WT1 zinc-finger protein. No protein or 10, 50, or 200 ng of purified WT1 zinc-finger protein WTZF was incubated with the PDGF-A promoter fragment shown. As a negative control, a WT1 zinc-finger domain deleted for fingers three and four (WTZF-1β_2) was tested for DNA-binding activity. Lanes: 1, 6, and 11, no protein; 2, 7, and 12, 10 ng of WTZF; 3, 8, and 13, 50 ng of WTZF; 4, 9, and 14, 200 ng of WTZF; 5, 10, and 15, 200 ng of WTZF-1β_2. (B) DNase I footprinting of the putative WT1 binding sites in the PDGF-A promoter. Each probe was exposed to DNase I in the presence of no protein (lanes 1, 4, and 7), 1000 ng of bovine serum albumin (lanes 2, 5, and 8), or 1000 ng of purified WTZF (lanes 3, 6, and 9). Shown is the footprint obtained with the top strand of the 190- and 266-bp Xho I/HindIII probes and the bottom strand of the 381-bp Xho I/Sac I probe.

genes in arresting blastemal cell proliferation and instituting a program of epithelial differentiation. We asked whether PDGF-A expression in kidney is consistent with its being a physiologic target for WT1 repression. The detailed temporal and spatial pattern of PDGF-A expression in the developing kidney (and in Wilms tumors) is not known. Northern blot analysis revealed high levels of the three major PDGF-A transcripts in 18- to 20-week human fetal kidney as compared to adult kidney (Fig. 5). In addition, in several Wilms tumors PDGF-A is somewhat overexpressed as compared to surrounding normal tissue (compare lanes 2 and 4 to lanes 1 and 3), extending previous data derived from cell cultures of Wilms tumors (22). More detailed characterization is needed to determine whether WT1 and PDGF-A are indeed ex-

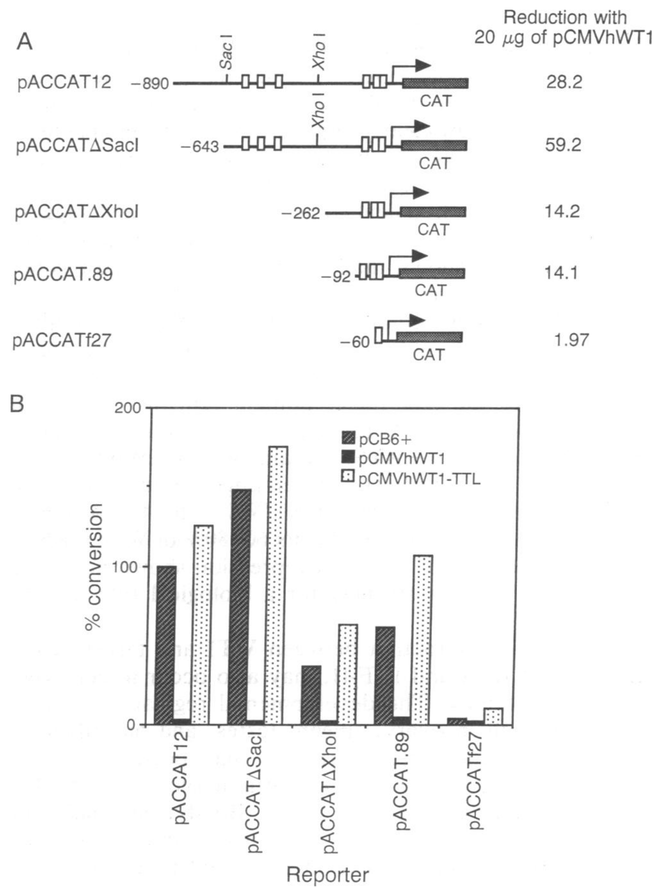

FIG. 4. Effect of WT1 on deletion derivatives of the PDGF-A promoter. (A) PDGF-A promoter–CAT constructs. Boxes show positions of putative WT1 binding sites. Reduction (-fold) is ratio of the percentage conversion of a given promoter construct with 20 µg of vector pCB6+ to the percentage conversion with 20 µg of WT1 expression vector. (B) pCMVhWT1 represses PDGF-A promoter derivatives, while pCMVhWT1-TTL has little effect. Each PDGF-A reporter was assayed for basal activity (with pCB6+ vector alone) or for activity with either pCMVhWT1 or the translationally terminated negative control pCMVhWT1-TTL. NIH 3T3 cells were transfected with 10 µg of reporter, 20 µg of vector or expression vector, and 1 µg of β-galactosidase internal reference plasmid. CAT assays were normalized as described in Fig. 2.

pressed in the same cell type in the developing kidney and, moreover, whether there is an inverse correlation between the expression of the WT1 repressor and PDGF-A as our data imply. Further experiments are also needed to assess whether the expression levels of WT1 protein in these transient transfection studies and others (9, 31) are comparable to the expression of WT1 in the developing kidney.

Sustained expression of one or more positive regulators of cellular proliferation has been proposed to contribute to the

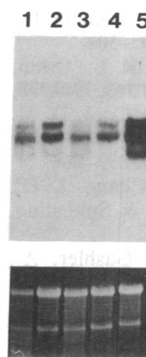

FIG. 5. PDGF-A expression. (Upper) Northern blot analysis of normal kidney and Wilms tumor RNAs. Total RNA was probed with a Sac II/HindIII fragment of the PDGF-A coding region. Bars indicate 1.8-, 2.3-, and 2.8-kb PDGF-A transcripts. Lanes: 1, adjacent normal kidney tissue (patient 1); 2, Wilms tumor sample (patient 1); 3, adjacent normal kidney tissue (patient 2); 4, Wilms tumor sample (patient 2); 5, 18- to 20-week human fetal kidney sample. (Lower) Ethidium bromide-stained RNA gel.

development of Wilms tumor. Included in this group are IGF-II (overexpressed in >95% of Wilms tumors), the *NMYC* gene, and the PDGF-A gene. Based on the occurrence of mutations in the *WT1* gene in cases of Wilms tumor, the *WT1* gene is now clearly implicated in the development of this malignancy. The data in this study demonstrate that the PDGF-A promoter is a direct target for the WT1 transcriptional repressor in transient transfection assays and suggest that the endogenous gene may be regulated similarly. Recently, we have shown that a second growth factor gene, IGF-II, is also a target for WT1 repression (31).

It has lately been demonstrated that the WT1 transcript is the subject of alternative splicing. One alternative splice product generating a protein with a 17-amino acid insertion N-terminal to the zinc-finger domain remains capable of binding to the EGR1/WT1 site (9). A second alternative splice product, WT1(+KTS) containing an insertion of three amino acids (Lys-Thr-Ser) between fingers 3 and 4, binds to a sequence distinct from the EGR1/WT1 site (42). Although the +KTS form of WT1 represents 60–80% of WT1 mRNA, the striking extent of PDGF-A repression shown here with WT1(−KTS) argues strongly for a biological role for this species.

Finally, the interaction between WT1 and target genes, such as PDGF-A and IGF-II, may also occur in cell types other than kidney. The developmental regulation of WT1 occurs as well in ovary, spleen, testes, and mesothelium. Furthermore, inducible expression may occur in diverse situations; for example, in the central nervous system after ischemic injury (W.-H. Lee and C. Bondy, personal communication). Thus, PDGF-A–WT1 protein interactions may be functionally important in multiple cell types in various biological settings.

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