Repression of the Transforming Growth Factor-β1 Gene by the Wilms' Tumor Suppressor WT1 Gene Product

Bhakta R. Dey, Vikas P. Sukhatme, Anita B. Roberts, Michael B. Sporn, Frank J. Rauscher III*, and Seong-Jin Kim

Laboratory of Chemoprevention (B.R.D., A.B.R., M.B.S., S-J.K.)
National Cancer Institute
Bethesda, Maryland 20892

Renal Division (V.P.S.)
Beth Israel Hospital
Boston, Massachusetts 02215

Wistar Institute of Anatomy and Biology (F.J.R.)
Philadelphia, Pennsylvania 19104

The Wilms' tumor suppressor gene (WT1) encodes a zinc finger DNA binding protein which functions as a transcriptional repressor. In this study we investigated whether the human transforming growth factor-β1 (TGF-β1) gene might be a target for transcriptional repression mediated by WT1. Using constructs of the TGF-β1 promoter linked to the chloramphenicol acetyl transferase gene, we have demonstrated that the WT1 protein represses expression of the TGF-β1 gene through a CGCCCCCGC response element spanning nucleotides −111 to −119 of the TGF-β1 promoter. We have also shown in a cotransfection assay that Egr-1, an immediate early growth response gene, activates transcription of the TGF-β1 gene through the same response element and that WT1 represses both the basal and Egr-1-induced TGF-β1 promoter activity in monkey kidney CV-1 cells. Moreover, WT1 and Egr-1 proteins interact directly with the WT1/Egr-1 response element of the TGF-β1 promoter in gel mobility shift assays. These findings provide further definition of transcriptional control of the TGF-β1 gene by showing that the WT1 gene product suppresses TGF-β1 transcription and that the WT1/Egr-1 consensus element of the human TGF-β1 promoter plays a critical role in this repression. (Molecular Endocrinology 8: 595–602, 1994)

INTRODUCTION

Wilms' tumor is a pediatric kidney malignancy that arises from the continued proliferation of embryonic kidney blastemal cells which have lost the property of differentiation (1). Wilms' tumor is observed in both hereditary and sporadic forms (2). In addition to the inheritance pattern, Wilms' tumor is occasionally associated with other disorders, *e.g.* as part of the WAGR syndrome (Wilms' tumor, aniridia, genitourinary abnormalities, and mental retardation) (3, 4). Chromosomal deletion analysis of children with the WAGR syndrome allowed mapping of a potential Wilms' tumor suppressor gene (WT1) to chromosome 11p13 (5–7). This led to the isolation of a candidate WT1 gene encoding a zinc finger protein (8, 9). Some tumors have been demonstrated to have internal deletions and mutations of the WT1 gene (10, 11).

The protein encoded by the WT1 gene contains an amino terminus rich in proline and glutamine residues and a carboxy terminus containing four zinc fingers (12). These structural domains are associated with sequence-specific DNA binding and transcriptional repression, respectively (12, 13). The zinc finger region of WT1 is a sequence-specific DNA binding domain which recognizes the consensus site (5′-CGCCCCCGC-3′) of early growth response gene (Egr-1), a transcription factor also known as NGF1-A (14), Krox 24 (15), TIS-8 (16), and Zif 268 (17). Egr-1 gene induction is noted after mitogenic stimulation in many cell types. However, a variety of differentiation cues also induce this gene (18). The WT1 protein functions as a transcriptional repressor when bound to the Egr-1 consensus sequence (12), and it has been postulated that the antagonistic activities of WT1 and Egr-1 may play a critical role in regulating the balance between growth and differentiation (12).

One potential modulator of kidney glomerular cell function is transforming growth factor-β (TGF-β) (19, 20), the prototypic member of a large family of struc-

0888-8809/94/0595-0602$03.00/0
Molecular Endocrinology

turally related and functionally regulatory proteins referred to as the TGF-β superfamily (21). TGF-βs are multifunctional regulators of cell proliferation and differentiation for a wide variety of cell types, including most normal and transformed epithelial, endothelial, lymphoid, and mesenchymal cells (21). In the kidney, TGF-β significantly increases the production of collagen and fibronectin by glomerular mesangial cells (19), acting through high-affinity receptors that are detected in cultured mouse glomerular endothelial, mesangial, and epithelial cells as well as isolated intact rat glomeruli (19). Anti-TGF-β therapy suppresses experimentally induced glomerulonephritis in an animal model system (22). TGF-βs are present in both cortex and medulla and they have been shown to inhibit DNA synthesis and attenuate the effects of many mitogenic peptides on isolated renal endothelial, epithelial, and mesangial cells *in vitro* (19).

Recent results indicate that human insulin-like growth factor II and the platelet-derived growth factor A chain are transcriptionally repressed by WT1 through a *cis* acting element, CGCCCCCGC, which resides in the promoter of both of these genes (23–25). Since this *cis* acting element is also present in the human TGF-β1 gene and since WT1 protein is expressed in adult kidney podocytes, we examined whether the Wilms' tumor suppressor WT1 might regulate the transcription of the TGF-β1 gene. The studies presented here demonstrate that the human TGF-β1 gene is transcriptionally repressed by Wilms' tumor suppressor WT1 in kidney epithelial cells. This *cis* acting element, which was well characterized earlier as an Egr gene consensus site (12), also acts as a transcriptional activator response element of human TGF-β1 by the Egr-1 gene product.

RESULTS

Mapping of a WT1 Response Element in the TGF-β1 Promoter

To determine whether WT1 regulates the TGF-β1 promoter, a region of the human promoter, −175 to +11 and its 5′-end deletion portions were fused to the bacterial chloramphenicol acetyl transferase (CAT) gene (Fig. 1). Regulation of these TGF-β1-CAT constructs was monitored after transfection into kidney CV-1 cells with vector plasmid DNA (CMV) containing a cytomegalovirus promoter, plasmid containing human WT1 cDNA insert (CMV-WT1), or WT1 deletion expression vectors. Cotransfection of TGF-β1-CAT reporter constructs and the expression vector CMV-WT1 resulted in a 3- to 5-fold repression of activity for constructs phTG7, phTG7-1, phTG7-1-1, phTG7-1-2, and phTG7-1-3. However, the TGF-β1 promoter activity was no longer repressed by CMV-WT1 when the deletion reached −100 (phTG7-2). These results suggest that sequences between −119 and −100, which include the potential Egr-1 binding site 5′-CGCCCCCGC-3′ (Fig. 2), are important for down-regulation of TGF-β1 expression in the presence of the WT1 gene product. Another Egr-1 response element-like sequence is also present in an inverted orientation between −74 and −82, but it did not show significant repression.

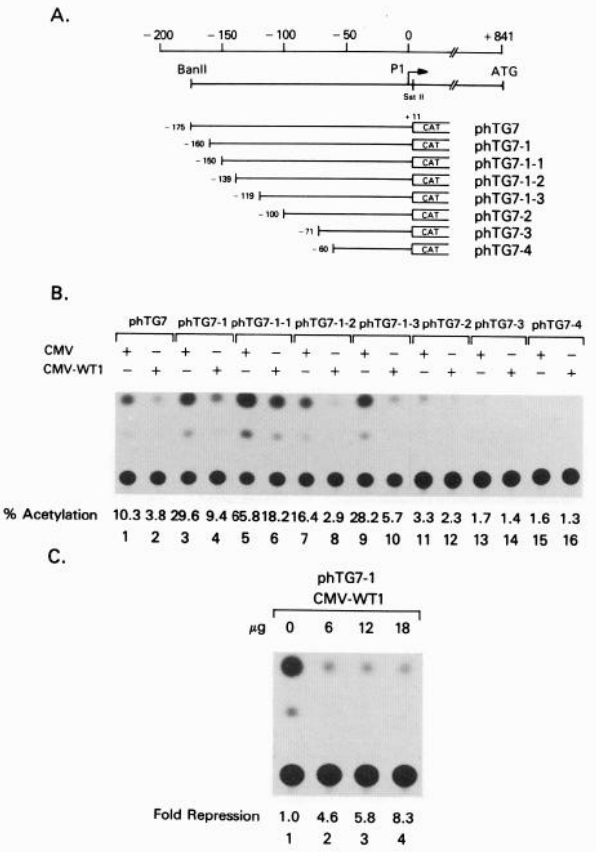

Fig. 1. Identification of Specific Regulatory Regions of the Human TGF-β1 Promoter Required for WT1 Gene Product-Mediated Repression

A, A partial map of the promoter region of the human TGF-β1 gene is shown. Schematic representation of the various TGF-β1 promoter-CAT constructs used to transfect monkey kidney CV-1 cells is presented. B, To analyze the repression of TGF-β1 promoter activity by the WT1 gene product, the TGF-β1-CAT constructs were cotransfected with the WT1 expression construct, CMV-WT1, or a control plasmid (CMV) using calcium phosphate. After transfection, cells were incubated for 48 h and 100 μg cellular protein were analyzed for CAT activity The values (% acetylation) represent an average of three separate experiments. C, The phTG7-1 (6 μg) construct was transfected into monkey CV-1 cells with the indicated amounts of CMV-WT1. The total amount of transfected DNA remained constant at 24 μg by adding the control plasmid CMV in each transfected dish.

We chose plasmid phTG7-1 which spans the region from −160 to +11 for subsequent transient cotransfection assays with the WT1 expression vector (Fig. 1C). When increasing amounts of WT1 expression vector were cotransfected with the phTG7-1 reporter construct, CAT activity decreased progressively (up to 8.3-fold), suggesting that WT1 repressed TGF-β1 gene transcription in a dose-dependent manner.

A

-180 GAGCCCGCCCACGCGAGATGAGGACGGT

GGCCCAGCCCCCCCATGCCCTCCCCCTGGGGGC

CGCCCCCGCTCCCGCCCCGTGCGCTTCCTGGGT

GGGGCCGGGGGCGGCTTCAAAACCCCCTGCCGA

CCCAGCCGGTCCCCGCCGCCGCCGCCCTTCGCG

CCCTGGGCCATCTCCCTCCCA +1

B

hTGF-ß1	-119	CGCCCCCGC -111
hTGF-ß1	-74	CGCCCCCGG -82
hIGF-II	-229	CGCCCCCGC -221
hIGF-II	-124	CGCCCCCGC -132
hIGF-II	+57	CGCCCCCGC +65
hPDGF-A	-56	CGCCCCCGC -64
hPDGF-A	-62	CGCCCCCGC -70
mEgr-1	-275	CGCCCCCGT -283
mEgr-1	-598	CGCCCCCGC -590
mEgr-1	-639	CGCCCACTC -647

Fig. 2. Nucleotide Sequences Containing WT1/Egr-1 Response Element

A, Partial nucleotide sequence of human TGF-β1 promoter (−180/+1) (28). The WT1/Egr-1 consensus element (*top*) and near-consensus element (one nucleotide deviation) are *underlined*. B, The WT1/Egr-1 consensus element and near-consensus elements present in the human TGF-β1, insulin-like growth factor II, and platelet-derived growth factor A chain and mouse Egr-1 promoters are presented. *Underlined* nucleotides are deviations from the consensus element.

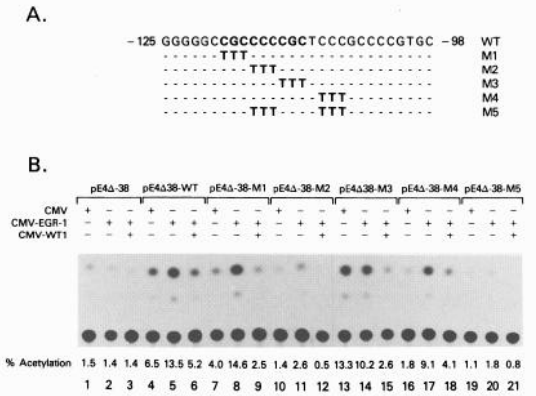

Fig. 3. WT1 Represses Basal and Egr-1 Induced TGF-β1 Promoter Activity Through WT1/Egr-1 Response Element

A, Diagram of the point mutations introduced into the WT1/Egr-1 consensus site of the human TGF-β1 promoter. B, Identification of WT1-responsive element in the human TGF-β1 promoter. The wild type (pE4Δ38-WT) and mutant chimeric TGF-β1 promoter constructs (6 μg) were cotransfected with either the CMV-EGR-1 or CMV-WT1 plasmids (12 μg) or parental CMV plasmid into monkey kidney CV-1 cells. The level of CAT activity is shown. The values represent the average of three individual experiments with comparable results.

Identification of a WT1 Response Element in TGF-β1 Promoter

To characterize the putative Egr-1 response element mapped in Fig. 1B, we generated chimeric constructs containing portions of the TGF-β1 upstream sequence between −125 and −98 linked to an Adenovirus E4-promoter-CAT vector. Multiple nucleotide mutants of the putative Egr-1 responsive element and its 3′ downstream sequence were generated as described in Fig. 3A. In cotransfection assays, the wild type construct (E4Δ38-WT) was induced 2.1-fold by CMV-EGR-1 and this was repressed to the basal level or below by CMV-WT1 (Fig. 3B). A mutation of nucleotides 1 to 3 of the response element of the construct was slightly less inducible by CMV-EGR-1 but down-regulated similarly to the wild type. Mutation of the middle three nucleotides of the response element in construct E4Δ38-M2 resulted in significant reduction in activation and repression activity. Mutation of nucleotides 7 to 9 of the motif in the construct E4Δ38-M3 resulted in a reduction of activation by CMV-EGR-1 but the repression by CMV-WT1 appeared to be unaltered. Mutation of nucleotides 3′ to the response element, by themselves, had little effect on the activation or repression of E4Δ38-M4, but when accompanied by a mutation in the middle three nucleotides (E4Δ38-M5), these mutations completely abolished the induction and repression activity of the response element. Therefore, the sequence CGCCCCCGC located between −119 and −111 appears to be responsible for Egr-1-mediated activation and WT1-mediated repression of the TGF-β1 gene.

To investigate the functional relevance of the CGCCCCCGC sequence for a WT1 response element, we cotransfected the TGF-β1 reporter construct phTG7-1-1 with wild type and mutant WT1 expression vectors (Fig. 4). The wild type WT1 repressed the activity of the reporter construct significantly. In cotransfection assays, CB6ΔBZWT repressed transcription 2- to 3-fold in construct phTG7-1-1. On the other hand, CMV-WT1(1–364), in which the last two zinc fingers have been deleted with resultant destruction of the binding domain, did not repress the activity of phTG7-1-1, suggesting that this truncated WT1 protein did not bind the WT1/Egr-1 response element and therefore did not repress TGF-β1 promoter.

We have also tested the effects of alternatively spliced WT1 proteins for the regulation of the TGF-β1 promoter (Fig. 5). WT1 proteins containing a lysine,

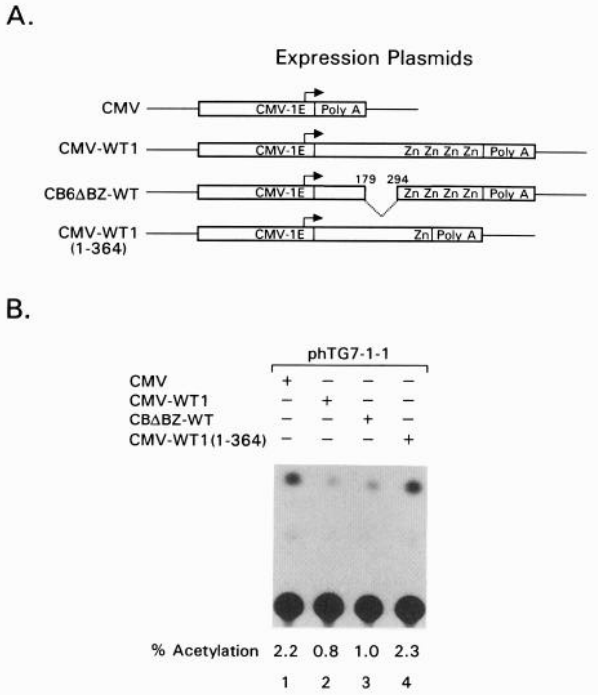

Fig. 4. Effect of WT1 and WT1 Mutant Plasmids on the TGF-β1 Promoter

A, Expression vectors used in cotransfection assays contained the full length WT1 cDNA or zinc finger domain coding regions of human WT1, driven by the cytomegalovirus immediate-early promoter. B, In order to confirm the WT1-responsive element in the human TGF-β1 promoter, the reporter construct phTG7-1-1 (6 μg) was cotransfected with the CMV-WT1 or mutant expression vectors CB6ΔBZ-WT, CMV-WT1(1–364), or CMV plasmid (12 μg) into monkey kidney CV-1 cells. Transfections were repeated three times with comparable results.

threonine, and serine (KTS) insertion did not repress TGF-β1 promoter activity. However, repression was observed in the absence or presence of a 17-amino acid insertion in the N terminus of the WT1 protein.

Identification of Factors that Bind to the WT1 Response Element

To identify factors that interact with the WT1/Egr-1 response element, we have utilized a gel shift assay using annealed oligonucleotides containing the region −125/−98 (Fig. 3A) as a probe. First, we asked whether *in vitro* translated ^{35}S-labeled Egr-1 product (32) can bind to this sequence. As shown in Fig. 6A, lane 1, the Egr-1 protein reacted with the annealed oligonucleotide probe (−125/−98). Addition of increasing amounts of the specific competitor (fragment −125/−98) to the binding reaction gradually competed out the protein-DNA complexes (lanes 2–7). These results suggest that Egr-1 protein can interact with WT1/Egr-1 response element of the human TGF-β1 promoter.

Next, nuclear extracts of kidney CV-1 cells were used in a gel shift assay to determine whether endogenous

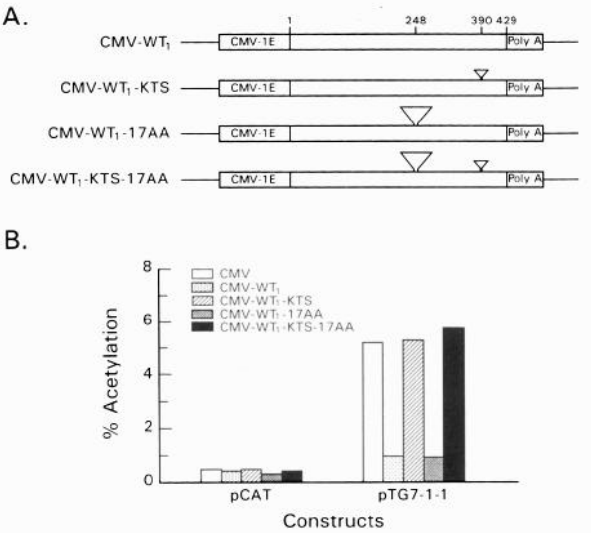

Fig. 5. Effect of the WT1 Alternative Splicing Variants (11) on Human TGF-β1 Promoter Activity

A, A schematic representation of expression constructs containing alternatively spliced WT1 proteins. Insertion of lysine, threonine, and serine (KTS) at position 390 and 17-amino acid insertions at position 248 generated these four WT1 proteins. B, Cotransfection results of TGF-β1 reporter construct phTG7-1-1 and the WT1-expression constructs presented in panel A. Transfections were repeated three times with comparable results.

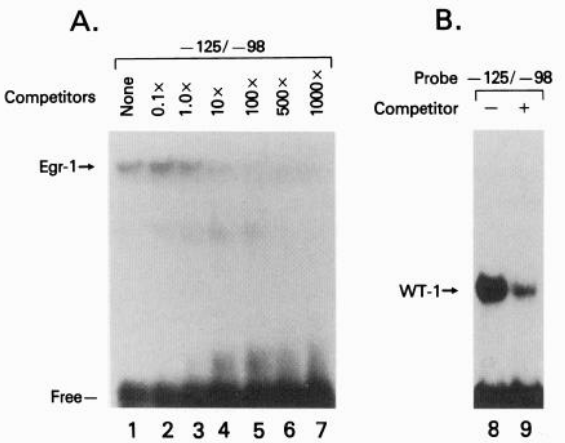

Fig. 6. Egr-1 and WT1 Proteins Bind to the TGF-β1 Promoter

A, *In vitro* translated Egr-1 protein binds specifically to the −125/−98 region of the human TGF-β1 promoter. Radiolabeled wild type oligonucleotide was incubated with *in vitro* translated ^{35}S-labeled Egr-1 protein as described in *Materials and Methods*. In lanes 2 to 7, increasing amounts of specific competitor were added to the reaction mixture. B, Bacterially expressed WT1 protein binds to the TGF-β1 promoter. The WT1 zinc finger protein produced in bacteria (24) and encompassing the WT1 binding site was used in gel shift assays with a wild type oligonucleotide probe. Competition for binding the DNA-protein complex was seen (lane 9) with a 100-fold M excess of specific cold competitor.

nuclear factors interact with the WT1/Egr-1 response element. As indicated in Fig. 7, four complexes could be detected. These specific complexes (designated as a, b, c, and d) were competed out when 10- and 100-fold M excess of unlabeled wild type competitor was added to the binding reaction (lanes 2 and 3, respectively). In order to identify the specific bands possibly representing Egr-1 protein, we used a rabbit antiserum (R5232-2) directed against a bovine GH (bGH)-Egr-1 fusion protein made in *Escherichia coli*. (32). This antiserum (R5232-2) supershifted (*arrow*) multiple bands (b, c, and d of lane 1). Of note, the R5232-2 antiserum does not cross-react with Egr-2 (32), another member of the Egr family of transcription factors (33), and in our hands, supershifting did not occur with antibodies to Egr-2, Egr-3, Egr-4 (data not shown). However, we cannot exclude possible cross-reactivity of R5232-2 with Egr-related proteins. We have identified the specificity of this supershift band as it is competed out in the presence of 100-fold M excess of wild type competitor (lane 5, Fig. 7). Multiple DNA-protein complexes were also detected with the same promoter fragment using extracts of murine myeloid cells (B. R. Dey, unpublished observations).

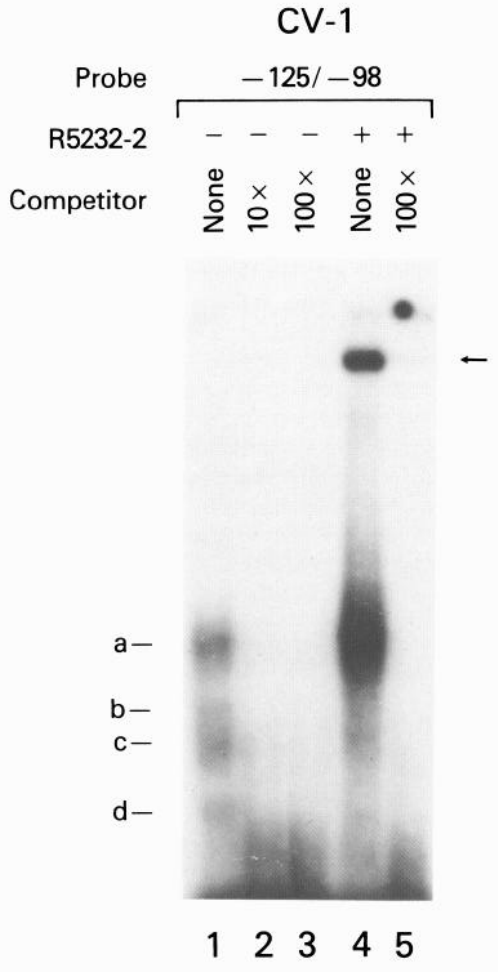

Fig. 7. Identification of Nuclear Factors That Bind to the WT1/Egr-1 Responsive Element in TGF-β1 Promoter

A nuclear extract of CV-1 cells was used in binding reactions with a wild type oligonucleotide (Fig. 3A) probe. The DNA-protein complexes were separated on a nondenaturing 5% polyacrylamide gel and autoradiographed. Specific bands, determined by competition with unlabeled wild type competitor, are marked as a, b, c, and d. Egr-1 polyclonal antibody (R5232-2) supershifted the Egr-1-TGF-β1 complex (*arrow*). Competition for this supershifted band was seen with a specific cold competitor.

Finally, to confirm that WT1 protein binds to the same recognition sequence (*i.e.* CGCCCCCGC) of the TGF-β1 promoter, we performed a binding assay using the WT1 zinc finger protein fragment produced in bacteria (24) and encompassing the WT1 binding site. As shown in Fig. 6B, the WT1 protein binds to the wild type oligonucleotide probe (−125/−98). In the presence of specific competitor at 100-fold M excess, this WT1 DNA-protein complex was markedly competed out. Collectively, these results confirm that WT1 and Egr-1 bind to the CGCCCCCGC sequence element *in vitro*, that Egr-1 protein is present in nuclear extracts of CV-1 cells and can bind to this element in the TGF-β1 promoter, and that Egr-1 is a transcriptional activator whereas WT1 is transcriptional repressor of the TGF-β1 gene.

DISCUSSION

In this study, we have demonstrated that a functional WT1/Egr-1 consensus site, located in the human TGF-β1 promoter, is required for WT1-mediated repression of transcription. Mutation of several nucleotides in the WT1/Egr-1 response element that prevent or partially prevent WT1-mediated transcriptional repression also reduce or partially reduce induction of TGF-β1 promoter activity by Egr-1. In cotransfection assays, in the presence of both WT1 and Egr-1, WT1 was observed consistently to repress the activities of chimeric TGF-β1-promoter constructs. We have also shown that several nuclear proteins (WT1, Egr-1, and proteins related to Egr-1) interact with the WT1/Egr-1 response element. To implicate WT1 directly in repression of TGF-β1, we have demonstrated that WT1 gene products with either a truncated zinc finger or mutant functional domain have significantly reduced ability to repress expression of TGF-β1. Collectively, these results suggest that the TGF-β1 gene may be a target for WT1 and Egr-1-mediated transcriptional regulation.

We have attempted to demonstrate the suppression of expression of endogenous TGF-β1 gene by WT1 in stably transfected CV-1 cells. We observed 50–55% down-regulation of TGF-β1 mRNA and about 38–40% less TGF-β1 protein secretion in stably transfected clones with the WT1 gene as compared to normal CV-1 cells (data not presented). Unfortunately, the levels of WT1 expression in these stably transfected clones were very low and could only be detected by reverse transcription polymerase chain reaction, thus making it difficult to demonstrate our central hypothesis.

WT1 protein has been designated as a transcription factor because it contains two discrete functional domains, one comprising the glutamine- and proline-rich amino-terminal domain responsible for transcriptional repression and the other comprising the four-zinc finger DNA binding domain which is related to similar structures present in the early growth response (Egr) family of proteins (12). WT1 is the only member of this zinc finger transcription factor family that behaves as a transcriptional repressor when it binds to the Egr consensus site (12). Evidence suggesting that the DNA binding domain of WT1 is inactivated in tumors (10) implies its transcriptional repression function is also inactivated. As a result, a target gene may be overexpressed and the cell may lose control of normal cellular proliferation and differentiation. Our results show that the promoter activity of the human TGF-β1 promoter is repressed when cotransfected with wild type WT1 (CMV-WT1), but not with mutant WT1 expression vectors. WT1 has been shown to be modified by alternative splicing (11). One alternative spliced product generates a protein with a 17-amino acid insertion N-terminal to the zinc finger domain; this protein retains its ability to repress the TGF-β1 promoter. A second form of human WT1 protein contains an insertion of three amino acids (Lys-Thr-Ser) between fingers 3 and 4; this protein does not regulate the TGF-β1 promoter and probably binds to a sequence distinct from Egr-1/WT1 (34, 35).

The transcription of the TGF-β1 gene is regulated by other transcription factors and protooncogenes, *e.g.* the AP-1 (*jun-fos*) complex. TGF-β1 has been shown to stimulate the expression of the transcription factor genes *jun-B* and c-*jun* in various cell lines (36, 37). An important contribution to this positive regulation of TGF-β1 is the autoinduction mediated through AP-1 binding sites at positions −371 and −418 (37). Enhanced *jun-B* and c-*jun* gene expression is an early genomic response to TGF-β1 stimulation. c-*fos* mRNA is also rapidly and transiently induced by TGF-β1 (37). However, the level of induction by TGF-β1 of *jun* mRNA far exceeds that of c-*fos* mRNA (37). The TGF-β1 gene is also transactivated by pp60$^{v\text{-}src}$ and the *tax* protein of human T lymphotropic virus type 1 through the AP-1 complex (38, 39). The human and mouse TGF-β1 promoters also contain a number of putative Sp1 binding sites (5′-GGGGCGG) (28, 40). Geiser *et al.* (40) found that the mammalian transcription factor Sp1 both bound to and activated the mouse TGF-β1 and human TGF-β3 promoters. The putative Sp1 binding sites did not contribute equally to activation of the TGF-β1 promoter and Sp1 activation synergy was observed (40). The functional relevance of individual Sp1 binding sites has yet to be determined. The consensus sequence of nuclear factor 1 (at −267) and fat-specific element 2 (at −1241, −1147, and −556) in the TGF-β1 promoter (28) is also unclear.

The mechanism of repression of TGF-β1 expression by WT1 remains unclear. Recently, Maheswaran *et al.* (41) found that WT1 is physically associated with wild type p53 in cells transfected with either protein. In the absence of wild type p53, WT1 functions as a transcriptional activator of Egr-1 sites. Wild type p53 appears to convert WT1 from a transcriptional activator to a transcriptional repressor. In the presence of both the activator (Egr-1) and a repressor (WT1), the dominant effect depends on the amount of each protein (13). This suggests that the competition between an activator and a repressor at a single DNA binding site is important in determining the direction of transcriptional regulation of a gene. This hypothesis is consistent with our transcriptional assays. It is possible that each protein exerts its effect by interacting in a fundamentally different way with the different factors involved in the transcriptional complex since the DNA binding domain of WT1, when expressed alone, is not an effective suppressor. Finally, since WT1 represses transcription of the Egr-1 gene itself, WT1 can also antagonize the action of Egr-1 indirectly (12).

Finally, we would like to speculate on the biological importance of our central finding. Unfortunately, developmental and adult expression of TGF-β1 in kidney is poorly characterized. However, given that WT1 expression persists in the adult kidney podocyte layer (*i.e.* the visceral epithelium that plays a role in extracellular matrix production), loss of podocyte integrity (as occurs in a variety of glomerular disorders) would lead to decreased WT1 expression. In turn, this might derepress TGF-β1 activity, thereby leading to increased TGF-β1 expression and, subsequently, increased collagen production (42). Indeed, the causal role of increased TGF-β1 activity in accumulation of matrix in one model of glomerulonephritis has recently been demonstrated (22). Since, scarring (sclerosis) is a common feature in several glomerular disorders, our findings that WT1 may play a critical role in regulating TGF-β1 expression and indirectly controlling matrix production is of pathogenetic interest.

MATERIALS AND METHODS

Cell Culture and DNA Transfection

The monkey kidney epithelial cell line (CV-1) was obtained from the American Type Culture Collection. Cells were cultured at 37 C in 5% CO_2 in Dulbecco's modified Eagle's media with 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin (GIBCO, Grand Island, NY). These cells were maintained in tissue culture flasks and routinely passaged twice a week. For transfection experiments, cells were plated at a density of 1×10^6/100-mm dish and grown until 60–70% confluent. Cells were cotransfected by the calcium phosphate coprecipitation technique (26) with TGF-β1/CAT constructs (Fig. 1) and different concentrations of expression plasmids containing human WT1 cDNAs along with 1 μg human GH expression plasmid under the control of SV40 promoter (Nichols Institute, San Juan Capistano, CA). For WT1 cotransfection, 10 μg reporter plasmid were cotransfected with either 10 μg expression plasmid or 10 μg pCMV. In the case of Adenovirus E4-promoter-CAT constructs, 6 μg reporter construct and 12 μg expression plasmid were used during cotransfection. After the cells were incubated with the DNA solutions for 8–12 h, cells were exposed to glycerol shock and finally harvested after 48 h. All transfections were repeated at least

three times. Cells were lysed by freeze thawing and 100 μg cell lysates were used for CAT assay (27). In order to control for transfection efficiency, CAT activity was normalized to the amount of GH secreted into the media.

CAT Plasmids and Expression Constructs

The TGF-β1 promoter constructs were generated by polymerase chain reaction amplification of DNA fragments using plasmid phTBG104 which has been described earlier (28). These DNA fragments were cloned into a promoterless basic pCAT vector (Promega, Madison, WI) using *HindIII* and *XbaI* restriction sites. The chimeric constructs were generated by inserting oligonucleotides containing mutants of Egr-1/WT1 response elements and wild type hTGF-β1 promoter sequences (Fig. 3) between positions −125 and −98 into a *HindIII/XbaI* site of an adenovirus E4Δ38-CAT reporter plasmid (29). Each plasmid was purified in two sequential CsCl centrifugation steps for maximum purity. The WT1 and Egr-1 expression vectors are described elsewhere (13). The CMV-WT1 and CMV-EGR-1 contain the full length coding region of WT1 and Egr-1 under the control of the cytomegalovirus immediate early promoter. In the mutant WT1 expression vector CMV-WT1 (1–364), the last two zinc fingers have been deleted and in CB6ΔBZ-WT, the amino acids 179–294 were deleted.

Gel Mobility Shift Assay

Nuclear extracts were made from CV-1 cells essentially as before (30) and gel mobility shift assays were performed as previously described (31). The final protein concentrations were 10.0 mg/ml. Binding reactions were done in a volume of 10 μl by incubating 1.5 μl nuclear extract and 1 μg poly(dI-dC) with 10,000 cpm of end-labeled probe in a buffer containing 50 mM Tris pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 5% glycerol, and 100 μM $ZnCl_2$. The oligonucleotide probes used in the gel shift analysis were prepared by annealing the complementary strands and end labeling using [^{32}P]dATP. The DNA-protein binding complexes were loaded onto 5% polyacrylamide gel (39:1 acrylamide-bisacrylamide) and run in 0.5 × TBE (50 mM Tris, 50 mM boric acid, and 1 mM EDTA pH 8.3) for 2–3 h at 8 V/cm. The gels were dried and autoradiographed using Kodak XAR-5 film.

Acknowledgments

We thank L. Mullen, R. Webbink, J. Choe, and R. Allison for oligonucleotides. We also thank David Romeo for critical reading of the manuscript.

Received November 8, 1993. Rerevision received February 2, 1994. Accepted February 15, 1994.

Address requests for reprints to: Dr. Seong-Jin Kim, Laboratory of Chemoprevention, National Cancer Institute, NIH, Building 41, Room C629, Bethesda, Maryland 20892.

These studies were supported in part by Grants CA-52009 (to F.J.R.), CA-47983 (to F.J.R.), and Core Grant CA-10817 from the National Institutes of Health, and grants from the W. W. Smith Charitable Trust (to F.J.R.), the Hansen Memorial Foundation (to F.J.R.), and the Mary A. M. Rumsey Foundation (to F.J.R.).

*Dr. Rauscher is a Pew Scholar in the Biomedical Sciences.

REFERENCES

1. Bennington JL, Beckwith JB 1975 Atlas of Tumor Pathology, Second Series, Fascicle 12. Armed Forces Institute of Pathology, Washington, DC, pp 31–76
2. Matsunaga E 1981 Genetics of Wilms' tumor. Hum Genet 57:231–246
3. Breslow NE, Beckwith JB 1982 Epidemiological features of Wilms' tumor: results of the National Wilms' tumor study. J Natl Cancer Inst 68:429–436
4. Breslow NE, Norkool PA, Olshan A, Evans A, D'Angio GJ 1988 Second malignant neoplasms in survivors of Wilms' tumor: a report from the National Wilms' tumor study. J Natl Cancer Inst 80:592–595
5. Miller RW, Fraumeni JF, Manning MD 1964 Association of Wilms' tumor with aniridia, hemihypertrophy and other congenital malformations. N Engl J Med 270:922–927
6. Riccardi VM, Sujansky E, Smith AC, Francke U 1978 Chromosomal imbalance in the aniridia-Wilms' tumor association: 11p interstitial deletion. Pediatrics 61:604–610
7. Rose EA, Glaser T, Jones C, Smith CL, Lewis WH, Call KM, Minden M, Champagne E, Bonetta L, Yeger H, Housman DE 1990 Complete physical map of the WAGR region of 11p13 localizes a candidate Wilms' tumor gene. Cell 60:495–508
8. Call KM, Glaser T, Ito CY, Buckler AJ, Pelletier J, Haber DA, Rose EA, Karl A, Yeger, Lewis WH, Jones C, Housman DE 1990 Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. Cell 60:509–520
9. Gessler M, Poustka A, Cavenee W, Neve RL, Orkin SH, Bruns GAP 1990 Homologous deletion in Wilms' tumors of a zinc finger gene identified by chromosomal jumping. Nature 343:774–778
10. Haber DA, Buckler AJ, Glaser T, Call KM, Pelletier J, Sohn RL, Douglass EC, Housman DE 1990 An internal deletion within an 11p13 zinc finger gene contributes to the development of Wilms' tumor. Cell 61:1257–1269
11. Haber DA, Sohn RL, Buckler AJ, Pelletier J, Call KM, Housman DE 1991 Alternative splicing and genomic structure of the Wilms' tumor gene WT1. Proc Natl Acad Sci USA 88:9618–9622
12. Rauscher III FJ, Morris JF, Tournay OE, Cook DM, Curran T 1990 Binding of the Wilms' tumor locus zinc finger protein to the EGR-1 consensus sequence. Science 250:1259–1262
13. Madden SL, Cook DM, Morris JF, Gashler A, Sukhatme VP, Rauscher III FJ 1991 Transcriptional repression mediated by the WT1 Wilms' tumor gene product. Science 253:1550–1553
14. Milbrant J 1987 A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. Science 238:797–799
15. Lemaire P, Revelant O, Bravo R, Charnay P 1988 Two mouse genes encoding potential transcription factors with identical binding domains are activated by growth factors in cultured cells. Proc Natl Acad Sci USA 85:4691–4695
16. Lim RW, Varnum BC, Herschman HR 1987 Cloning of tetradecanoyl phorbol ester-induced "primary response" sequences and their expression in density-arrested Swiss 3T3 cells and a TPA nonproliferative variant. Oncogene 1:263–270
17. Lau LF, Nathans D 1987 Expression of a set of growth-related immediate early genes in BALB/c3T3 cells: Coordinate regulation with c-*fos* and c-*myc*. Proc Natl Acad Sci USA 84:1182–1186
18. Gashler AL, Swaminanathan S, Sukhatme VP 1993 A novel repression module, an extensive activation domain, and a bipartite nuclear localization signal defined in the immediate-early transcription factor Egr-1. Mol Cell Biol 13:4556–4571
19. Mackay K, Striker LJ, Stauffer JW, Doi T, Agodoa LY, Striker GE 1989 Transforming growth factor-β: murine glomerular receptors and responses of isolated glomerular cells. J Clin Invest 83:1661–1666
20. Roberts AB, McCune BK, Sporn MB 1992 Transforming growth factor-β: regulation of extracellular matrix. Kidney Int 41:557–559
21. Roberts AB, Sporn MB 1990 The transforming growth

factor-βs. In: Sporn MB, Roberts AB (eds) Peptide Growth Factors and Their Receptors. Handbook of Experimental Pharmacology. Springer Verlag, Heidelberg, vol. 95/1:419–472
22. Border WA, Okuda S, Languino LR, Sporn MB, Ruoslahti E 1990 Suppression of experimental glomerulonephritis by antiserum against transforming growth factor-β1. Nature 346:371–374
23. Drummond IA, Madden SL, Rohwer-Nutter P, Bell GI, Sukhatme VP, Rauscher III FJ 1992 Repression of insulin-like growth factor II gene by the Wilms' tumor suppressor WT1. Science 257:674–678
24. Gashler AL, Bonthron DT, Madden SL, Rauscher III FJ, Collins T, Sukhatme VP 1992 Human platelet-derived growth factor A chain is transcriptionally repressed by Wilms' tumor suppressor WT1. Proc Natl Acad Sci USA 89:10984–10988
25. Wang JY, Madden SL, Deuel TF, Rauscher III, FJ 1992 The Wilms' tumor gene product, WT1, represses transcription of the platelet-derived growth factor A-chain gene. J Biol Chem 267:21999–22002
26. Luse DS, Roeder RG 1980 Accurate transcription initiation on a purified mouse β-globin DNA fragment in a cell free system. Cell 20:691–699
27. Gorman CM, Moffat LF, Howard BH 1982 Recombinant genomes which express chloramphenicol acetyl-transferase in mammalian cells. Mol Cell Biol 2:1044–1051
28. Kim S-J, Glick A, Sporn MB, Roberts AB 1989 Characterization of the promoter region of the human transforming growth factor-β1 gene. J Biol Chem 264:402–408
29. Gilardi P, Perricaudet M 1984 The E4 transcriptional unit of Ad2: far upstream sequences are required for its transactivation by E1A. Nucleic Acids Res 12:7877–7888
30. Dignam JD, Lebovitz RM, Roeder RG 1983 Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11:1475–1489
31. Kim S-J, Onwuta US, Lee YI, Li R, Botchan MR, Robbins PD 1992 The retinoblastoma gene product regulates Sp1-mediated transcription. Mol Cell Biol 12:2455–2463
32. Cao X, Koski RA, Gashler A, McKiernan M, Morris CF, Gaffney R, Hay RV, Sukhatme VP 1990 Identification and Characterization of the Egr-1 gene product, a DNA binding zinc protein induced by differentiation and growth signals. Mol Cell Biol 5:1931–1939
33. Sukhatme VP 1992 The Egr transcription factor family: from signal transduction to kidney differentiation. Kidney Int 41:550–553
34. Pelletier J, Bruening W, Kashtan CE, Mauer SM, Manivel JC, Striegel JE, Houghton DC, Junien C, Habib R, Fouser L, Fine RN, Silverman BL, Haber DA, Housman D 1991 Germline mutations in the Wilms' tumor suppressor gene are associated with abnormal urogenital development in Denys-Drash Syndrome. Cell 67:437–447
35. Bickmore WA, Oghene K, Little MH, Seawright A, van Heyningen V, Hastie ND 1992 Modulation of DNA binding specificity by alternative splicing of the Wilms' tumor wt1 gene transcript. Science 257:235–237
36. Pertovaara L, Sistonen L, Bos TJ, Vogt PK, Keski-Oja J, Alitalo K 1989 Enhanced *jun* gene expression is an early genomic response to transforming growth factor type β stimulation. Mol Cell Biol 9:1255–1262
37. Kim S-J, Angel P, Lafyatis R, Hattori K, Kim KY, Sporn MB, Karin M, Roberts AB 1990a Autoinduction of transforming growth factor β1 is mediated by the AP-1 complex. Mol Cell Biol 10:1492–1497
38. Birchenall-Roberts MC, Ruscetti FW, Kasper J, Lee H-D, Friedman R, Geiser A, Sporn MB, Roberts AB, Kim S-J 1990 Transcriptional regulation of the transforming growth factor-β1 promoter by *v-src* gene products is mediated through the AP-1 complex. Mol Cell Biol 9:4978–4983
39. Kim S-J, Kehrl JH, Burton J, Tendler CL, Jeang K-T, Danielpour D, Thevenin C, Kim KY, Sporn MB, Roberts AB 1990b Transactivation of the human transforming growth factor-β1 (TGF-β1) gene by human T lymphotropic virus type 1 *Tax:* A potential mechanism for the increased production of TGF-β1 in adult T cell leukemia. J Exp Med 172:121–129
40. Geiser AG, Busam KJ, Kim S-J, Lafyatis R, O'Reilly MA, Webbink R, Roberts AB, Sporn MB 1993 Regulation of the transforming growth factor-β1 and -β3 promoters by the transcription factor Sp1. Gene 129:223–229
41. Maheswaran S, Park S, Bernard A, Morris JF, Rauscher III FJ, Hill DE, Haber DA 1993 Physical and functional interaction between WT1 and p53 proteins. Proc Natl Acad Sci USA 90:5100–5104
42. Nakamura T, Miller D, Ruoslahti E, Border WA 1992 Production of extracellular matrix by glomerular epithelial cells is regulated by transforming growth factor-β1. Kidney Int 41:1213–1221

Pharmacologic Intervention In the Ageing Process

Convenor: Prof A Kligman, University of Pennsylvania, *Venue:* London, Sept 26–27th, 1994. For further details, please contact either Jackie Ford or John Jennings, MAIC Ltd, Croxted Mews, 286A-288 Croxted Road, London SE24 9BY, Tel: 081-671-7521; Fax number: 081-671-7327.