Identification of Connective Tissue Growth Factor as a Target of WT1 Transcriptional Regulation*

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The Wilms tumor suppressor WT1 has transcription-activating and -suppressing capabilities. WT1-responsive promoters have been described; however, in large part, it remains unclear which potential downstream genes are physiologically relevant and mediate the function of WT1 in tumorigenesis and development. To identify genes regulated by WT1 in vivo, we used a dominant-negative version of WT1 to modulate WT1 activity in a Wilms tumor cell line. Screening oligonucleotide arrays with RNA from these cells uncovered a number of genes whose expression was altered by abrogation of WT1 function. Several of the genes encode members of the CCN family of growth regulators. The promoter of one of these genes, connective tissue growth factor (CTGF), is suppressed by WT1 both in its endogenous location and in reporter constructs. WT1 regulation of CTGF expression is not mediated by previously identified WT1 recognition elements and may therefore involve a novel mechanism. Our results indicate that CTGF is a bona fide target of WT1 transcriptional suppression and likely plays a role in Wilms tumorigenesis and associated disease syndromes.

Wilms tumor (pediatric nephroblastoma) is one of the most common solid tumors found in children. A subset (5–10%) of Wilms tumors is caused by mutations in the tumor suppressor gene, WT1 (reviewed in Refs. 1 and 2). In addition to its role in tumorigenesis, WT1 is also required for normal kidney and urogenital development. WT1 is expressed in a temporally and spatially restricted pattern in the developing kidney and urogenital structures, and WT1 knockout mice die before birth with both kidney and urogenital development blocked at an early stage (3). Based upon these findings, it has been suggested that WT1 is required for the expression of signaling molecules and receptors involved in the reciprocal inductive events of early kidney differentiation (reviewed in Ref. 4). Involvement of WT1 in normal development is further substantiated by the presence of constitutional heterozygous WT1 mutations in patients with congenital syndromes associated with Wilms tumor (reviewed in Ref. 1). Both the WAGR syndrome and the Denys-Drash syndrome (DDS)† are characterized in part by genitourinary malformations as well as predisposition to development of Wilms tumors.

Although it is clear that WT1 plays an important role in both development and tumorigenesis, the mechanisms through which it functions in these processes remain poorly understood. WT1 has been implicated in such diverse pathways as RNA splicing, DNA replication, and apoptosis (reviewed in Refs. 2 and 4). The most well characterized function of WT1, however, is that of a transcription factor. The amino terminus of WT1 is rich in proline and glutamine, a feature characteristic of transactivation domains, and the carboxyl terminus contains four C2H2 zinc finger DNA-binding motifs and a nuclear localization signal sequence. In vivo, there are four major isoforms of WT1 generated by alternative splicing at two sites. Splicing of exon 5 removes 17 amino acids from the middle of the protein, and a second alternative splicing event removes three amino acids (KTS) from between the third and fourth zinc fingers of the protein. The four isoforms of WT1 are present in a constant ratio that is conserved among species, suggesting that they have non-overlapping functions.

WT1 binds to DNA via its zinc finger motifs, but this binding is isoform-dependent, with the −KTS and +KTS isoforms binding with distinct affinities to somewhat different sets of sequences (reviewed in Ref. 2). Furthermore, the binding of each isoform is only relatively sequence-specific. For example, the −KTS isoforms of WT1 bind sequences resembling the EGR1 consensus (5’-CGC-GC-3’) as well as TC-rich motifs. Upon binding to DNA, WT1 has the capacity to act as either a transcriptional activator or repressor. Most of the experiments done to assess these functions have involved transient transfection with engineered reporter constructs into cells that do not normally express WT1. The results from these experiments vary significantly depending upon experimental conditions such as the cell type and isoform of WT1 used and the exact sequence and arrangement of WT1-binding sites within the reporter.

The function of WT1 as a transcriptional regulator in vivo is likely affected by post-translational modification and interaction with other proteins. Serine phosphorylation within the zinc finger domain of WT1 has been shown to interfere with nuclear localization and DNA binding (5, 6). WT1 physically associates with other cellular proteins, including SF1 (7), p53 (8), and PAR4 (9). These interactions affect the ability of both partners to regulate transcription. For example, it has been shown that WT1 functions as a transcriptional repressor in the presence of wild-type p53, but functions as an activator in its absence (8). However, these conclusions were drawn from experiments performed with cell lines.

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1 The abbreviations used are: DDS, Denys-Drash syndrome; EGR1, early growth response-1; IGF-2, insulin-like growth factor-2; CTGF, connective tissue growth factor; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; IGF-1R, insulin-like growth factor-1 receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGFBP, insulin-like growth factor-binding protein; bp, base pair(s).
Regulation of CTGF Expression by WT1

Fig. 1. A, expression of endogenous and transfected WT1 proteins in clones of WT49. Whole cell lysates from equivalent numbers of cells from control clone N1 and DDS-WT1-transfected clone D5 were analyzed by Western blotting. The membrane was probed with an anti-hemagglutinin antibody (α-HA; upper panel), stripped, and reprobed with an anti-WT1 antibody (α-WT1; lower panel). Bands corresponding to the endogenous wild-type protein and the transfected mutant protein are indicated by arrows. B, DDS-WT1 acts as a dominant-negative protein to alleviate transcriptional repression of a WT1-responsive reporter construct. The WT1-responsive luciferase reporter construct pZ4luc was transiently transfected into WiT49 cells. Expression constructs for various forms of WT1 were cotransfected along with the reporter and included wild-type WT1 with (+/+), or without (-/-) both alternative splices and DDS-WT1 (DDS) as indicated below each bar. 1 μg of each WT1 expression construct was included in the transfection unless otherwise indicated. The fifth and seventh bars represent transfections that included 1 μg of DDS-WT1 in addition to the indicated amount of wild-type +/+ WT1. The eighth bar represents a transfection that included 1.8 μg of DDS-WT1 together with wild-type WT1. Luciferase activity is shown normalized for transfection efficiency. C, the steady-state activity of a WT1-responsive reporter is higher in DDS-WT1-transfected clones than in control clones. The pZ4luc reporter construct was transiently transfected into stable transfected clones N1, D5, and D15. Luciferase activity is shown normalized for transfection efficiency.

Experiments performed in cells that do not express endogenous WT1, and WT1 has also been shown to have transcriptional regulatory capabilities that are independent of p53 (10).

Fig. 2. DDS-WT1 overexpression alters transcription of the endogenous IGF-1R gene. A, total RNA was reverse-transcribed to first-strand cDNA and amplified by PCR (30 cycles) using primers specific for the IGF-1R gene (upper panel) or the GAPDH gene (lower panel). Samples included cDNA from untransfected WT49 cells (lane 1), untransfected WT49 clone W3 (lane 2), three control stable transfected clones (N1–N3; lanes 3–5), and five DDS-WT1 stable transfected clones (D1, D5, D8, D20, and D24; lanes 6–10). B, RT-PCR analysis of IGF-1R (upper panel) and GAPDH (lower panel) was performed on cDNA from WT49 cells transiently transfected with an empty expression vector (lane 2), a DDS-WT1 expression construct (lane 3), or a wild-type WT1 expression construct (lane 4). Lane 1 contains molecular mass standards (M).

Given the heterogeneity of sequences that can be bound by WT1 in different circumstances, it is difficult to predict which genes may be relevant targets of WT1 in vivo. Nevertheless, a number of potential WT1 targets have been identified in model systems (reviewed in Ref. 2). Many of these genes encode products that are involved in the regulation of cell growth and/or differentiation such as growth factors and their receptors. However, with the possible exception of IGF-2 (11, 12), most of these putative target genes have only been indirectly implicated in Wilms tumorigenesis through the ability of WT1 to regulate their promoters in transient transfection/reporter construct assays.

To identify physiologically relevant transcriptional targets of WT1, we used a dominant-negative mutant version of WT1 to inhibit the function of the endogenous wild-type protein expressed in a Wilms tumor cell line, WT49.Dominant-negative WT1 mutations are found in patients with DDS, which is characterized by genitourinary malformations, progressive renal failure, and predisposition to development of Wilms tumor (reviewed in Ref. 1). In contrast to what is observed for sporadic Wilms tumors, nearly 100% of DDS-associated Wilms tumors can be accounted for by mutations in WT1. Furthermore, the spectrum of WT1 mutations seen in DDS is unique in that they are nearly all point mutations within the exons encoding the zinc fingers of the protein. Interestingly, the phenotype associated with DDS point mutations is much more severe than that associated with larger deletions encompassing the region of the point mutation. This suggests that production of DDS mutant protein may be more deleterious than production of no WT1 at all. DDS point mutations abrogate the ability of WT1 to bind to DNA and function as a transcriptional regulator (13, 14). Furthermore, since WT1 self-associates, the mutant protein is capable of blocking the activity of the wild-type protein and acting as a dominant-negative protein (15–17). Further evidence that DDS mutant forms of WT1 act as dominant-negative proteins in vivo is provided by the fact that DDS mutations are heterozygous in the germ line of patients and are reduced to homozygosity only in the Wilms tumor itself. Therefore, the genitourinary malformations and progressive nephropathy seen in DDS patients must be due to deleterious actions of the point mutant form of WT1 (reviewed in Ref. 18).

To identify WT1-regulated transcripts that might play a role in DDS or Wilms tumorigenesis, we generated an expression profile for WT1-expressing Wilms tumor cells and for the same cells stably expressing DDS-WT1 by probing GeneChip high-density oligonucleotide arrays (19, 20). Seventy-one genes were
identified whose expression was altered by 2.6-fold or more upon expression of DDS-WT1. These genes represent potential in vivo targets of WT1 and may therefore play important roles in Wilms tumorigenesis and/or normal kidney or urogenital development. One of the differentially expressed genes, connective tissue growth factor (CTGF), was analyzed in detail and found to be transcriptionally repressed by WT1.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—The WiT49 cell line was derived from a primary lung metastasis of an aggressive Wilms tumor. WiT49 cells were maintained in 1:1 high-glucose Dulbecco's modified Eagle's medium/nutrient mixture F-12, 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Transfections were done using LipofectAMINE Plus (Life Technologies, Inc.) according to the manufacturer's protocol. Stably transfected cells were selected with 1 mg/ml G418 (Life Technologies, Inc.) and maintained in 0.6 mg/ml G418. Reporter constructs containing various portions of the CTGF promoter were generated by cloning PCR-generated fragments into either the pGL2-basic or pGL2-promoter vector (Promega). Mutation of 4 base pairs of the promoter for construct 69–561mut was accomplished using PCR with a mismatched oligonucleotide as described (21).

Transient transfections with luciferase reporter constructs shown in Figs. 1 and 7 also included pRLTK (Promega) as a control for transfection efficiency. Normalized luciferase activity was determined by firefly luciferase/galactosidase enzyme assay system.

**Western Analysis**—Whole cell lysates were prepared by scraping cells from culture dishes and washing them once with phosphate-buffered saline. The cell pellet was resuspended in 40 µl of SDS-polyacrylamide gel electrophoresis sample buffer, vortexed, and incubated at room temperature for 10 min. The lysate was boiled for 10 min, and 40 µl of sample buffer supplemented with bromphenol blue and β-mercaptoethanol was added. Lysates were centrifuged for 5 min at 14,000 rpm, and the supernatants were run on 10% SDS-polyacrylamide gels. The proteins were then transferred to Immobilon-P membrane (Millipore Corp.), probed with anti-hemagglutinin antibody (Y11, Santa Cruz Biotechnology), stripped, and probed with anti-WT1 antibody (C19, Santa Cruz Biotechnology).

**Proliferation Analysis**—Cells were plated in 96-well flat-bottomed tissue culture plates at 3000 cells/well in 100 µl of WiT49 medium containing either 2 or 10% fetal calf serum and 0.6 mg/ml G418. Viable cells were quantitated using the Promega Cell Titer 96 AQuieux non-radioactive cell proliferation assay system according to the manufacturer's protocol.

**GeneChip Expression Analysis**—cRNA samples to be hybridized to GeneChips were prepared according to the protocol provided by Affymetrix. Briefly, total RNA was prepared from subconfluent plates of N1 and D5 cells using Trizol (Life Technologies, Inc.). Poly(A)+ mRNA was isolated from 100 µg of total RNA using the Qiagen Oligotex matrix. Approximately 1 µg of poly(A)+ mRNA was reverse-transcribed to double-stranded cDNA using an oligo(dT) primer containing a T7 promoter. The cDNA was then amplified and labeled by in vitro transcription with T7 RNA polymerase in the presence of biotinylated CTP and UTP (Enzo). The resulting cRNA was fragmented and hybridized to the Hu6800 GeneChip set exactly according to the Affymetrix protocol. Chips were stained with streptavidin-phycocerythin, followed by a biotinylated antibody/streptavidin-phycocerythin amplification step. Data were analyzed using Affymetrix GeneChip Version 3.1 software with scaling of all genes to 2500, and the AB mask was applied to eliminate probes derived from intronic sequences. Data were sorted based on fold change, and genes that had a difference call of “no change” despite having a fold change of >1.0 were eliminated from further consideration.

**RT-PCR Analysis**—First-strand cDNA was prepared using Superscript II reverse transcriptase (Life Technologies, Inc.) and amplified using platinum Taq DNA polymerase. RT-PCR products were separated on 1.1% agarose gels and stained with ethidium bromide for visualization. Primers for RT-PCR analysis of IGF-1R, MAGE-3, and MAC25 transcripts were designed to span introns.

**TaqMan Quantitative RT-PCR Analysis**—First-strand cDNA prepared using Superscript II reverse transcriptase and treated with DNase I (Ambion DNA-Free) was amplified in an ABI Prism 7700 sequence detection system. Reactions contained CTGF-specific primers and a 6-carboxy-fluorescein-labeled internal probe (designed using the Primer Express program according to PerkinElmer Life Sciences) and PerkinElmer Life Sciences TaqMan Universal PCR Master Mix. Each cDNA was amplified in parallel with the PerkinElmer Life Sciences 18 S rRNA primer/probe set as an internal control. Data were analyzed according to the comparative Ct method (58) and displayed normalized for 18 S rRNA and relative to a particular sample. Each bar in the figures represents the average of four independent experiments. Quantitative analysis of WT1 expression was performed similarly, except that PerkinElmer Life Sciences SYBR Green Universal PCR Master Mix was used, and a gene-specific probe was not included.

**Northern Analysis**—20 µg of total RNA was separated on a 1% formaldehyde-agarose gel and transferred to an uncharged nylon membrane (Duralon UV, Stratagene) in 1× NH4 acetate. The membrane was cross-linked (Stratalinker, Stratagene) and hybridized in 0.5× NaPO4 (pH 7), 7% SDS, 1× EDTA, and 5 mg/ml bovine serum albumin at 65°C. Probes were generated by PCR amplification of

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2. H. Yeger and B. R. G. Williams, unpublished data.

3. Details of the DDS-WT1 expression construct used in generation of stable transfectants and of the wild-type WT1 expression constructs and reporter constructs used in transient transfections are available upon request.

4. The sequences of the IGF-1R, MAGE-3, and MAC25 transcripts are available upon request.
Genes showing at least a 2.6-fold increase or decrease in clone D5 as compared with the base line of clone N1 are listed in order of decreasing fold change. Genes that had a fold change of 2.6 or more but were nevertheless identified as “no change” by the GeneChip software were excluded.

| Accession No. | N1 intensity | N1 call | D5 intensity | D5 call | Difference call | Change | Gene definition |
|---------------|--------------|---------|--------------|---------|----------------|--------|----------------|
| X70218        | 328          | P       | 4957         | P       | I              | 7.1    | Human mRNA for protein phosphatase X |
| X03656        | 1065         | A       | 7236         | A       | MI             | 6.8    | Human gene for granulocyte colony-stimulating factor |
| M16364        | -1422        | A       | -547         | P       | MI             | 6.3    | Human creatine kinase-B |
| HG2743–HT2846 | 688          | A       | 5046         | P       | I              | 6.3    | Caldesmon-1 alternative splice “4”, non-muscle |
| L07564        | -1811        | A       | 1103         | P       | I              | 4.8    | Human MXI |
| U52446        | 240          | A       | 3617         | P       | I              | 4.6    | Human mRNA for uridine phosphorylase |
| X90858        | -821         | A       | 2693         | A       | I              | 4.4    | Ovarian cancer down-regulated myosin homolog (DOCl) |
| U04285        | -144         | A       | 1914         | P       | MI             | 4.5    | Lyasosomal acid “lipase,” cholesteryl ester hydrolase (LIPA) gene |
| L77701        | -268         | A       | 1418         | A       | MI             | 4.0    | Human COX17 |
| A10001047     | -239         | A       | 1123         | A       | MI             | 3.5    | Human mRNA for matrixin-3 |
| M15517        | -1459        | A       | -61          | A       | MI             | 3.5    | TTR gene (prealbumin) linked to familial amyloidotic polyneuropathy |
| X55740        | 326          | A       | 2408         | P       | I              | 3.5    | Human placental cDNA coding for 5′-nucleotidase |
| U37426        | 701          | A       | 2484         | P       | I              | 3.5    | Human kinesin-like spindle protein |
| HG1400–HT1400 | 515          | A       | 1920         | P       | I              | 3.4    | Carboxyl “methyltransferase,” “aspartate,” alternative splice 1 |
| J03060        | 141          | A       | 141          | A       | MI             | 3.3    | Human glucocerebrosidase |
| D890805       | -403         | A       | 1474         | P       | I              | 3.3    | Human mRNA for KIAA0183 “gene” |
| M26679        | -1256        | A       | 869          | A       | I              | 3.3    | Human homeobox protein (HOX-1.3) |
| M30135        | -1089        | A       | 909          | A       | I              | 3.2    | Human P40 T-cell and mast cell growth factor |
| HG4322–HT4592 | 2477         | P       | 7916         | P       | I              | 3.2    | β-Tubulin |
| U24183        | 1982         | A       | 5586         | P       | I              | 3.2    | Human phosphofructokinase (PFKM) |
| HG2846–HT2983 | -600         | A       | 1059         | A       | MI             | 3.1    | Dihydrofolate "reductase," alternative splice 6 |
| D21090        | 936          | P       | 2922         | P       | I              | 3.0    | Human interleukin-8 receptor type B variant “IL8RB9” |
| M92934        | 25639        | P       | 61679        | P       | I              | 3.0    | XP-C repair complementing protein "p58/HHR23B" |
| X15187        | 3231         | P       | 9976         | P       | I              | 3.0    | Human TRAF homolog of murine tumor rejection antigen gp96 |
| X04145        | -2280        | A       | -766         | A       | I              | 3.0    | Human mRNA for T-cell receptor T3 γ-polypeptide |
| D90279        | 903          | A       | 1474         | P       | I              | 3.0    | Human mRNA for collagen α1(V) chain |
| U08087        | 3521         | P       | 3521         | P       | I              | 3.0    | Tnn gene (survival motor neuron protein SMN) |
| L36847        | -757         | A       | 310          | A       | MI             | 2.9    | Human rearranged iduronate-2-sulfatase homolog gene |
| D32002        | -881         | A       | 216          | A       | I              | 2.9    | Human mRNA for nuclear cap-binding protein |
| HG2255–HT2844 | -394         | A       | 215          | A       | I              | 2.9    | Phosphoribosyl pyrophosphate synthetase, subunit II |
| L13923        | 284          | A       | 1313         | P       | I              | 2.9    | Human fibrillin |
| U09099        | 434          | M       | 1852         | P       | I              | 2.8    | Human clone 23722 mRNA sequence |
| J04449        | -2175        | A       | -781         | A       | MI             | 2.8    | Human cytchrome P-450-nifedipine oxidase |
| U42408        | -3350        | A       | 2119         | A       | MI             | 2.8    | Human ladinin (LAD) |
| S3929         | -535         | A       | 1130         | A       | I              | 2.8    | Glandular kallikrein-1 |
| Y09636        | -959         | A       | 389          | A       | I              | 2.8    | Lymphocyte function-associated antigen-3 |
| HG1317–HT2344 | -1394        | A       | -94          | A       | I              | 2.7    | Mps1 (Gβ:L20314) |
| M19154        | 72           | A       | 1022         | P       | I              | 2.7    | Human transforming growth factor β2 |
| U07804        | -114         | A       | 774          | A       | I              | 2.7    | Human DNA topoisomerase I |
| U19316        | -90          | A       | 1229         | A       | I              | 2.7    | Human acyl-CoA: thiester hydrolase |
| X62429        | -376         | A       | 453          | A       | I              | 2.7    | Human mRNA for transcription factor Pit-1 |
| X90780        | -1482        | A       | -186         | A       | I              | 2.7    | Human cardiac trophinin 1 "gene,” exons 1–5 |
| M64936        | -1423        | A       | -316         | A       | I              | 2.6    | Human retinoic acid-inducible endogenous retroviral DNA |
| M93119        | -438         | A       | 1324         | A       | I              | 2.6    | Human zinc finger DNA-binding motif (IA-1) |
| U15128        | 474          | A       | 1894         | A       | I              | 2.6    | Human "β-1,2-N-acetylglucosaminyltransferase" II (MGAT2) |
| U77949        | -559         | A       | 959          | P       | I              | 2.6    | Human CDC6-related protein (HsCDC6) |
| X76059        | -1873        | A       | -678         | A       | I              | 2.6    | Human mRNA for YRRM1 |
| D85313        | 1610         | M       | 4205         | P       | I              | 2.6    | Mhc-associated zinc finger protein of human “islet” |
| U17077        | -152         | A       | 816          | A       | MD             | 2.6    | Human BENV "mRNA" |
| L18877        | 3977         | P       | 1893         | A       | MD             | 2.6    | Human MAGE-12 |
| D86425        | 935          | P       | -13          | A       | D              | 2.6    | Human osteoblast mRNA for "osteoidogen" |
| Z75190        | 346          | A       | 467          | A       | D              | 2.6    | Human mRNA for apolipoprotein E receptor-2 |
| D87119        | 686          | A       | -327         | A       | MD             | 2.6    | Human cancellous bone osteoblast mRNA for "GS3955" |
| M55905        | 941          | A       | -722         | A       | D              | 2.5    | Human mitochondrial NAD(P)−dependent malic enzyme |
| L07590        | 1903         | P       | 657          | P       | D              | 2.9    | Human protein phosphatase 2A 130-kDa regulatory subunit |
genomic DNA and labeled with \( ^{32}P \) dCTP by random priming. Northern blots were washed in several changes of 2× to 0.2× SSC, 0.1% SDS, and 0.005% sodium pyrophosphate at room temperature and 65 °C.

**RESULTS**

**Stable Expression of DDS-WT1 in WiT49 Wilms Tumor Cells**—To probe the function of WT1 in a Wilms tumor environment, we used expression of a dominant-negative mutant version of the protein, DDS-WT1, to abrogate the activity of the endogenous wild-type protein in a Wilms tumor cell line, WiT49. The expression construct for the dominant-negative protein contained the full-length WT1 cDNA with both the exon 5 and KTS alternative splices and a single point mutation (DDSS) under the control of the strong constitutive elongation factor-1α promoter (22). A tag consisting of three copies of the hemagglutinin epitope was engineered onto the amino terminus of the DDS-WT1 protein. The DDSS point mutation results in a single amino acid change of Arg to Trp at position 394 of the DDS-WT1 protein. The DDS5 point mutation results in a single amino acid change of Arg to Trp at position 394.

**DDS-WT1 Functions as a Dominant-negative Protein to Inhibit the Transactivating Capabilities of Wild-type WT1**—To determine whether stable expression of DDS-WT1 affected the activity of endogenous wild-type WT1 in the clones, we transiently transfected the WT1-responsive reporter into clones N1 and D5 as well as a second DDS-WT1-expressing clone, D15 (Fig. 1C). Luciferase activity was consistently higher (~5-fold) in D clones as compared with N clones, suggesting that DDS-WT1 expression interfered with the ability of the endogenous WT1 protein to repress transcription.

Based upon these differences in reporter activity, it seemed likely that expression of endogenous WT1-responsive genes would be altered by DDS-WT1 expression. Accordingly, the expression levels of a variety of genes previously suggested to be WT1-regulated were analyzed using RT-PCR and Northern blotting. No significant difference in expression between control and DDS-WT1-expressing clones was detected for several genes, including the epidermal growth factor receptor and IG-2 (data not shown); however, we did observe modest overexpression of IG-1R in a subset of stable D clones (Fig. 2A). Furthermore, transient transfection of WT49 cells with DDS-WT1 led to increased IG-1R expression, whereas transfection with wild-type WT1 did not (Fig. 2B). These results are cons-

**TABLE I—continued**

| Accession No. | N1 intensity | N1 call | D5 intensity | D5 call | Difference call | Change | Gene definition |
|---------------|--------------|---------|--------------|---------|-----------------|--------|-----------------|
| U66832        | 2034         | P       | 505          | A       | MD              | −3    | Human cyclin A1 |
| M21056        | −4542        | A       | −5681        | A       | D               | −3.1  | Human pancreatic phospholipase A-2 |
| U34044        | 3578         | P       | 509          | A       | D               | −3.3  | Human selenoprotein (selD) |
| U19594        | 5351         | P       | 1633         | P       | MD              | −3.3  | Human AF1q |
| XT8879        | 1762         | P       | 162          | A       | A               | −3.4  | Human 14A2AK DNA sequence |
| U23070        | 3692         | P       | 1057         | A       | D               | −3.4  | Human putative transmembrane protein (NMA) |
| D50683        | 1105         | P       | 607          | A       | D               | −3.5  | Human mRNA for transforming growth factor-β IIR-α |
| M83667        | 1030         | P       | 89           | A       | D               | −3.5  | Human NF-IL-6-β |
| L11424        | −417         | A       | −2597        | A       | D               | −3.6  | Cb-binding protein β-chain |
| U67934        | 2229         | A       | −2597        | A       | D               | −3.7  | Human 44.9-kDa protein C18B11 homolog |
| U67949        | 2592         | A       | 139          | A       | D               | −3.8  | Human β-galactoside α-2,6-sialyltransferase (SIAT1) |
| L18920        | 2097         | P       | 66           | D       | A               | −4.5  | Human MAGE-2 gene exons 1–4 |
| U52969        | 3858         | P       | 547          | A       | D               | −4.6  | Human PEP19 (PCP4) |
| HG987         | 4312         | P       | 599          | A       | D               | −6.1  | MAC25 |
| HT987         | 7152         | A       | −609         | A       | D               | −18.5 | Human MAGE-3 antigen |

**Fig. 4.** RT-PCR analysis of the MAGE-3 transcript confirms that its levels are reduced in DDS-WT1-expressing clones. cDNA was generated from poly(A)+ RNA for control clone N1 (lane 1) and DDS-WT1-expressing clones D5 (lane 3), D6 (lane 3), and D20 (lane 4). cDNAs were amplified (30 cycles) with primers specific for the MAGE-3 gene (upper panel). Amplification of GAPDH (lower panel) was used to confirm that the four cDNA samples were of equivalent concentration and integrity.
sistent with previous studies showing that the IGF-1R promoter is repressed by WT1 and that IGF-1R is frequently overexpressed in Wilms tumors (23, 24). Moreover, the altered transcription of an endogenous gene upon DDS-WT1 expression in WiT49 cells indicates the potential of this system to identify other WT1 target genes.

DDS-WT1-expressing clones did not show any striking differences in morphology in comparison with control clones, and the clones proliferated with identical kinetics when cultured in medium containing 10% fetal calf serum (Fig. 3A). However, under reduced serum conditions (2% fetal calf serum), DDS-WT1-expressing clones had a growth advantage (Fig. 3B). These findings suggest that the introduced DDS-WT1 protein interfered with the growth suppressor activity of endogenous wild-type WT1, presumably by blocking its ability to regulate the expression of other genes.

**GeneChip Expression Analysis of Control and DDS-WT1-expressing Clones of WiT49 Cells**—To identify additional genes whose expression was altered by DDS-WT1 expression, we hybridized cRNA from two WiT49 clones, N1 and D5, to Affymetrix GeneChip oligonucleotide arrays. The arrays contain probes for ~6800 cloned human genes on a set of four chips, A–D. In addition to unique sequences, each chip also contains control probes (such as those for GAPDH) that control for the quality of each cRNA preparation and hybridization. Based upon hybridization to the control probes, all of the eight chips used (A–D for both clones N1 and D5) were of comparable quality (data not shown).

Of the ~6800 genes assayed, only a relatively small number showed significant differences in expression between clones N1 and D5. Table I lists the 71 genes that showed changes of 2.6-fold or greater when the expression profile of clone D5 was compared with the base-line profile of clone N1. These changes ranged from a 7.1-fold increase to an 18.5-fold decrease. None of the genes that showed differential expression between clones N1 and D5 had been previously identified as WT1-regulated. This experiment was done twice using the same cDNA preparation on two independent sets of chips with essentially identical results (data not shown).

**Confirmation of GeneChip Results**—To confirm that the GeneChip accurately identified expression differences between clones N1 and D5, we followed up several genes using RT-PCR and Northern blot analyses. MAGE-3 showed the largest decrease in clone D5 as compared with clone N1 on the GeneChip (18.5-fold reduced) and three other members of the MAGE gene family (MAGE-1, MAGE-2, and MAGE-12) were also identified as decreases (Table I and data not shown). MAGE genes encode tumor antigens and are frequently overexpressed in human cancers (25–27). RT-PCR analysis of MAGE-3 using cDNA from multiple control and DDS-WT1-expressing clones confirmed that DDS-WT1 expression in WiT49 cells resulted in a striking decrease in MAGE-3 gene expression (Fig. 4 and data not shown).

The MAC25 gene, which encodes an insulin-like growth factor-binding protein (IGFBP) (29–31), also showed a large decrease (6.1-fold) in expression in clone D5 as compared with clone N1 on the GeneChip (Table I). Semiquantitative RT-PCR analysis of MAC25 transcript levels indicated that MAC25 expression was reduced ~3-fold in clone D5 as compared with clone N1 (data not shown).

We also examined the expression of several genes that were identified as increased in clone D5 as compared with clone N1 on the GeneChip, such as DOC-1 (4.8-fold increased) (Table I). The DOC-1 gene encodes a myosin heavy chain homolog that is down-regulated in ovarian cancer (32). Northern blot analysis of RNA from multiple control and DDS-WT1-expressing clones confirmed that this gene is up-regulated in D clones as compared with N clones (data not shown).

We performed a similar Northern analysis for the CTGF gene, which was identified on the GeneChip as being up-regulated 3.1-fold in clone D5 as compared with clone N1 (Fig. 5A). We were intrigued by the presence of CTGF in the list of differentially expressed genes since it is a member of the same gene family as MAC25 and encodes a protein with IGFBP and growth regulatory functions (29, 33). Northern analysis revealed some variability in the level of CTGF expression between clones; however, for several D clones, including D5, CTGF expression was strikingly increased in comparison with control clones. Expression differences between N and D clones were quantitated using TaqMan real-time RT-PCR (Fig. 5B). In this experiment, three out of four DDS-WT1-expressing clones showed increases in CTGF mRNA levels as compared with the average level in control N clones. CTGF expression in clone D5 was 5-fold greater than the average level in a panel of N clones and 11-fold greater than the level in clone N1.

An inverse correlation between WT1 activity and CTGF expression was also supported by quantitative RT-PCR analysis of a second Wilms tumor cell line, WT13. This cell line was derived from a tumor that contained a homozygous deletion of chromosome 11p13 spanning the WT1 gene and thus does not express WT1 (34). As shown in Fig. 5C, CTGF expression was much higher in WT13 cells, which lack WT1, than in WiT49 cells, which express WT1 at a relatively high level (equivalent to fetal kidney) (data not shown).

**The CTGF Promoter Is Suppressed by WT1**—To confirm that the CTGF gene is WT1-regulated, we analyzed a 887-bp genomic sequence (GenBank™/EMBL Data Bank accession number X92511) containing the translation start site of CTGF and two upstream TATA boxes. We identified two sites between the translation start site and the most downstream TATA box that match the EGR1-binding site consensus (5′-GCCG(CG-3′)) at eight out of nine positions (Fig. 6). As expected based upon the 67% amino acid identity between WT1 zinc fingers 2–4 and the three zinc fingers of EGR1, WT1 has been shown to bind to GC-rich sequences approximating the EGR1 consensus (35). To test whether the EGR1-like sites confer WT1 responsiveness upon the CTGF promoter, we amplified a portion of the promoter (bp 69–561) and cloned it upstream of the luciferase gene. The CTGF promoter directed transcription of the luciferase gene in WiT49 cells, and this transcription was significantly repressed by cotransfection of either the +/+ or −/− isoform of wild-type WT1 (Fig. 7A). The DDS mutant form of WT1, however, did not have any effect on luciferase expression. These results indicate that the CTGF promoter is WT1-responsive.

The CTGF promoter/luciferase reporter construct was also transiently transfected into clones N1 and D5 (Fig. 7B). As in the parental WiT49 cells, cotransfection of wild-type WT1 into either clone N1 or D5 together with the reporter led to a suppression of luciferase activity. However, in the absence of cotransfected WT1, expression of luciferase from the CTGF promoter was significantly higher in clone D5 than in clone N1. This mirrors the increased expression of the endogenous CTGF gene in clone D5 as compared with clone N1 that was observed in the GeneChip experiment and supports our conclusion that CTGF is repressed by wild-type WT1 in vivo.

**WT1 Regulation of the CTGF Promoter Occurs through Novel Recognition Elements**—Given the presence of two EGR1-like motifs in the CTGF promoter and the known interaction of WT1 with such sequences in other contexts, it seemed likely that they would mediate regulation of the CTGF promoter by WT1. To test this, we generated reporter constructs in which
luciferase expression is driven by bp 69–627 of the CTGF promoter (which contains both EGR1-like motifs), bp 69–561 (which contains only one EGR1-like motif), or bp 69–561 with the single EGR1-like motif mutated. For the mutant construct, we changed four GC base pairs to AT base pairs as shown in Fig. 8B. Mutation of these positions in EGR1-like binding sites has been shown to completely abrogate WT1 binding to DNA (35). Upon cotransfection of these three reporters into WiT49 cells with various isoforms of WT1, we observed no significant differences in the ability of WT1 to repress luciferase expression (Fig. 8A). These results demonstrate that the EGR1-like motifs within the CTGF promoter are completely dispensable for repression of transcription by WT1.

To clarify the mechanism by which WT1 represses the CTGF promoter, we were interested in identifying sequences within the promoter that are recognized by WT1. To do this, we used truncated portions of the CTGF promoter to drive luciferase expression in transient transfection assays. Of particular interest in this analysis was a region of 142 base pairs that shows 100% conservation between the human CTGF promoter and its mouse homolog, Fisp12 (GenBank™/EBI Data Bank accession number M70641). However, we found that deletion of the conserved region of the promoter had only a minimal effect on the ability of WT1 to repress transcription. As shown in Fig. 9, a construct that lacked the conserved region (bp 383–561) was repressed by WT1 to a similar extent as one that contained the conserved region (bp 238–561). In contrast, deletion of bp 455–561 resulted in a loss of WT1 repression. This was most striking for the 2/2 isoform of WT1, but was significant for the 1/1 isoform as well. Together with the results shown in Fig. 8, these data indicate that WT1 interacts with sequence elements within bp 455–561 of the promoter that are distinct from the EGR1-like motif.

To further examine whether the conserved region of the CTGF promoter plays any role in transcriptional repression by WT1, we tested additional reporter constructs containing fragments of the promoter from bp 69 to 375 (Fig. 10). Since these promoter fragments did not include the downstream TATA box, we inserted them upstream of the minimal SV40 promoter in the pGL2-promoter vector. Previous experiments (shown in Figs. 7–9) used reporters constructed in pGL2-basic, which contains no promoter. The portions of the CTGF promoter that were cloned into pGL2-promoter are indicated in Fig. 10. Luciferase assays using the resulting constructs showed that there are indeed sequences within the conserved region of the promoter that can be recognized by WT1. It is apparent that sequences between bp 261 and 321 are essential for repression by the −/− isoform of WT1 in this experimental context. Inter-

FIG. 5. Decreased WT1 activity is correlated with elevated CTGF expression. A, Northern analysis confirmed the GeneChip identification of differential expression of CTGF in clones D5 and N1. Total RNA from three control transfectant clones (N1, N2, and N3; lanes 1–3 and 12), six DDS-WT1 transfectant clones (D1, D5, D8, D15, D20, and D24; lanes 6–11), and the control (Neo) and DDS transfectant pools (lanes 4 and 5) was analyzed by Northern blotting using a CTGF-specific probe (upper panel). All D clones except for D8 express DDS-WT1. The membrane was stripped and rehybridized with a GAPDH probe to control for variability in loading and RNA integrity (lower panel). B, TaqMan quantitative RT-PCR analysis was performed on total RNA from three N clones and five D clones. CTGF expression was normalized for 18 S rRNA expression and is shown relative to the average amount of CTGF mRNA in the N clones (set at 1). C, TaqMan quantitative RT-PCR analysis was performed on cDNA from WT13 and WT49 cells using primers specific for CTGF or WT1. Transcript levels in the two cell lines were normalized for 18 S rRNA expression and are shown relative to one another, with the lowest expressing cell line set at 1. WT1 expression was >64-fold higher in WT49 cells than in WT13 cells, off the scale of the figure. kb, kilobases.
esting, sequences within this same region appear to prevent repression by the /+/- isoform of WT1.

We have shown that endogenous CTGF expression is dependent at least in part upon WT1 activity. Furthermore, wild-type (but not DDS) WT1 in both the /-/- and /+/- isoforms represses CTGF promoter/reporter constructs in transient transfection assays. Contrary to our expectations, the EGR1-like sequences within the CTGF promoter are not the essential elements directing WT1-mediated repression. Deletion analyses have pointed to two other regions of the promoter, bp 261–321 and 455–561, as playing a role in WT1 interaction with the promoter. Previously identified WT1-binding sites are not present within these regions, suggesting that a novel binding site(s) may be utilized. Future studies will be aimed at defining the WT1 regulatory elements in the CTGF promoter and gaining an understanding of how they function in vivo during kidney development and tumorigenesis.

**DISCUSSION**

To address the function of WT1 in its native setting, we established a Wilms tumor cell line, WiT49, from a xenograft of an aggressive tumor metastasis. Immunohistochemical analysis of various markers showed that the cell line contains both blastemal and epithelial components, features characteristic of primary Wilms tumors. WiT49A cells express high levels of mutant p53 and levels of wild-type WT1 comparable to those present in fetal kidney. Thus, WiT49 cells represent the subset of Wilms tumors that do not contain WT1 mutations (~90% of the cases) and actually express WT1 at a relatively high level. The ability to propagate WiT49 cells in culture is likely dependent upon their p53 mutation. However, it has been reported that p53 physically interacts with WT1 and can modulate its transcriptional activity in some experimental situations (8). Consequently, it will be interesting to assess the gene expression profile of WiT49 cells conditionally expressing wild-type WT1.

Since WiT49 represents cell types that normally express WT1, we used a naturally occurring dominant-negative mutant, DDS-WT1, to modulate WT1 function and to uncover WT1 target genes. DDS-WT1 expression was sufficient to inhibit the function of the wild-type protein as evidenced by altered expression of a WT1-responsive reporter gene as well as the endogenous IGF-1R gene. Furthermore, DDS-WT1 transfectants displayed a growth advantage under certain conditions in comparison with control cells. For these reasons, we believe that this system has the potential to reveal differences in gene expression that are dependent upon WT1 activity. Screening of oligonucleotide arrays allowed analysis of the expression of ~6800 known human genes. Seventy-one (~1%) genes showed significant changes of >2.6-fold upon inhibition of WT1 function. This is a relatively small number of changes, as might be expected since clones N1 and D5 are presumably identical to those present in fetal kidney. Thus, WT1 functions as downstream effectors of WT1 since there is no functional information available for the gene product (32). Likewise, the positive regulation of MAGE-3 (and MAGE-1, MAGE-2, and MAGE-12) gene expression by WT1 that is suggested by our results has unclear functional implications. The genes of the MAGE family encode tumor-specific antigens that are expressed in many different tumor types, but not in normal tissues except for testes (25–27). Since many human cancers have inappropriate expression of one or more MAGE genes, the altered MAGE gene expression identified here may not be a direct result of WT1 activity, but rather a secondary reflection of the transformed phenotype of WT49 cells.

In contrast to the genes described above, CTGF is likely to be a bona fide transcriptional target of WT1. Two independent lines of evidence support this. First, as revealed by GeneChip analysis and confirmed by Northern blotting, the CTGF gene is up-regulated in DDS-WT1-expressing WiT49 clones as compared with control clones. Second, the CTGF promoter is repressed by wild-type WT1 in cotransfection experiments and is constitutively more active in DDS-WT1-expressing clones than in control clones. These results indicate that WT1 negatively regulates CTGF expression in vivo. The placement of CTGF downstream of WT1 suggests that it may mediate the essential functions of WT1 in development and/or tumorigenesis. Several features of CTGF make it a good candidate to fill such a role. CTGF was originally identified as a mitogen secreted by vascular endothelial cells with homology to the v-src-induced immediate-early gene product CEF-10 (33). Subsequently, a family of genes, including CTGF, was delineated based upon homology to immediate-early genes and to genes encoding the immediate-early gene product (Ref. 29; review in Ref. 37). This family, termed CCN after three of its members (CTGF, CEF-10/ Cyr61; NovH), also includes the MAC25 (30), ELM1 (38), and WISP1–3 genes (39). The proteins encoded by members of this gene family are all secreted and have similar primary structures including an N-terminal cysteine-rich IGBPBP domain.

As predicted by the presence of the IGBPBP domain, several CCN proteins, including CTGF and MAC25, have been shown to bind IGFBPs (29, 30). However, in contrast to the classical

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**FIG. 6.** The CTGF promoter contains potential WT1-binding sites. A 687-bp genomic sequence corresponding to the CTGF promoter is shown with the translation start site boxed and two upstream TATA boxes in boldface. Two motifs that fit the EGR1-binding site consensus at eight out of nine positions are boxed and shaded. A stretch of 142 bp that is 100% identical between the CTGF promoter and its mouse homolog, Fisp12, is underlined.
IGFBP-1–6, members of the CCN family have only low affinity for IGFs (29), and some members are also capable of binding insulin (40). These differences are presumably due to the presence of a second cysteine-rich IGFBP domain in IGFBP-1–6 that is lacking in CCN proteins. IGFBPs are critical components of IGF signaling pathways, serving to protect the growth factors from degradation, but also limiting their binding to cell-surface receptors (reviewed in Ref. 41). Thus, both IGFBP-1–6 and the CCN family of low-affinity IGFBPs likely function at least in part to modulate the actions of IGFs in the regulation of cell growth.

IGF signaling is thought to play a critical role in the development and progression of Wilms tumorigenesis (reviewed in Ref. 2). Overexpression of the imprinted IGF-2 gene, which resides at 11p15, is one of the most consistent features of Wilms tumors. This overexpression may arise from loss of heterozygosity, loss of imprinting, or mutation of WT1, which can repress the IGF-2 promoter. The cognate receptor for IGF-2, IGF-1R, is also a target of WT1-mediated transcriptional suppression and is frequently overexpressed in Wilms tumors. Overexpression of both the growth factor and its receptor is thought to result in activation of an autocrine loop that leads to dysregulated proliferation. Given the important role of IGFs in Wilms tumorigenesis as well as normal embryonic development, it is conceivable that modulation of IGF signaling by IGFBPs such as CTGF may be critical to both processes.

CTGF was originally identified as a mitogen (35), and other members of the CCN family such as CYR61 (42) have also been shown to promote cell growth. In contrast, some members of the family such as MAC25 (43, 44) and ELM1 (38) appear to be negative growth regulators. We have found that abrogation of WT1 activity leads to both elevated CTGF expression and enhanced growth under certain conditions. Although these results are consistent with the definition of CTGF as a mitogen, it remains unclear whether CTGF is directly responsible for the growth phenotype of DDS-WT1-expressing WT49 cells.

The IGFBP function of CCN proteins likely accounts for their ability to regulate growth at least in part; however, there is also evidence that these proteins may act in IGF-independent pathways as well. For example, the murine ortholog of CTGF, FISP12, has been shown to promote adhesion, migration, and survival of vascular endothelial cells as well as neovascularization in vivo (45). Several of the CCN proteins have been shown to associate with the extracellular matrix, and CTGF also stimulates extracellular matrix production and deposition (46, 47). Transforming growth factor-β-induced CTGF has been shown to be responsible for extracellular matrix production by fibroblasts during wound healing and in fibrotic disorders (48). Furthermore, hyperglycemia has also been shown to induce CTGF expression, which results in an accumulation of the extracellular matrix (49). This has been suggested to be a mechanism behind the nephropathy that leads to renal failure in diabetic patients. Similarly, it is possible that overexpression of CTGF due to WT1 mutation accounts for the renal fibrosis and ultimate failure that is observed in patients with DDS (50).

Our interest in CTGF as a potential downstream effector of WT1 is bolstered by the fact that expression of another member of the CCN family of genes was also altered by inhibition of WT1 function in clone D5. The MAC25 gene was identified as being down-regulated 6.1-fold in clone D5 as compared with clone N1 in the GeneChip experiment. This suggests that WT1 normally activates MAC25 expression, whereas it suppresses CTGF expression. The opposite effects of WT1 on expression of these two genes is interesting given that they have been reported to have opposite effects on cellular growth. As described above, MAC25 inhibits proliferation (43, 44), whereas CTGF stimulates growth (39). Thus, both the induction of MAC25 and the suppression of CTGF are consistent with WT1 functioning as a growth suppressor.

Involvement of CTGF in Wilms tumorigenesis is also supported by its homology to NovH (51). The NovH gene was not identified in our GeneChip experiment as being WT1-regulated; however, previous studies have shown that the chicken Nov gene is overexpressed in avian nephroblastoma, a model for Wilms tumor (52). Furthermore, expression of the human

**Fig. 7. WT1 represses transcription from the CTGF promoter.** A promoterless luciferase reporter construct (b) or a luciferase reporter construct containing the CTGF promoter (CTGF) was transiently transfected into WT49 cells (A) or clones N1 and D5 (B). Expression constructs for various forms of WT1 were included as indicated beneath each bar. These included the wild-type isoform containing both alternative splices (+/+ ) and that lacking both splices (−/−) as well as the DDS mutant. Transfections that did not include cotransfected WT1 assayed the activity of the endogenous WT1 protein (endog.). Luciferase activity is shown normalized for transfection efficiency.
NovH gene is elevated in some primary Wilms tumors, with the levels of NovH and WT1 inversely correlated (53), and the NovH promoter is repressed by WT1 in cotransfection assays (54). Together with our evidence for WT1 regulation of CTGF expression, the relation of CTGF to other genes and functions implicated in kidney development and tumorigenesis suggests that the CCN family of proteins are likely downstream effectors of WT1 function in vivo.

In theory, downstream targets of WT1 may be oncogenes or tumor suppressor genes themselves. Since all Wilms tumors share certain defining characteristics, but only a small fraction of them harbor WT1 mutations, it is reasonable to expect that some tumors will show dysregulation or mutation of other genes that function in the same pathways as WT1. We have initiated a study to look at whether altered CTGF expression may be a causative event in primary Wilms tumors using TaqMan quantitative RT-PCR. An inverse correlation between WT1 and CTGF expression was observed for the majority of the tumor samples analyzed to date (16 out of 21). Furthermore, 7 out of 21 tumors showed increased expression of CTGF (ranging from 2.5 to 33-fold) in comparison with matched normal kidney tissue. Thus, overexpression of CTGF may be physiologically significant in the development of a subset of Wilms tumors.

During the course of this study, another group reported the use of oligonucleotide array screening for identification of WT1 transcriptional targets (55). The results of the two studies are quite different in terms of both general patterns of gene expression and particular targets. The study reported by Lee et al. (55) analyzed gene expression in an osteosarcoma cell line conditionally expressing particular isoforms of wild-type WT1. In contrast to our findings, they did not observe repression of any genes by WT1, but identified several genes specifically activated by the 2/2 isoform. The gene most potently activated by the 2/2 isoform of WT1 in this scenario was amphiregulin, a member of the epidermal growth factor family. Using quantitative RT-PCR, we have shown that amphiregulin is expressed in WT49 cells, but is not altered by inhibition of WT1 function by DDS-WT1 (data not shown). Similarly, CTGF was not identified by Lee et al. as being regulated by WT1 in their system. These discrepancies likely reflect differences in the two experimental systems used since the transcriptional function of WT1 is certain to be dependent upon the intracellular environment, including the presence of cooperating transcription fac-

![Fig. 8. The GC-rich EGR1-like motifs within the CTGF promoter are not involved in WT1-mediated repression of the promoter.](image_url)

A, WiT49 cells were transiently transfected with an empty expression vector or various isoforms of WT1 as indicated below each bar. Each transfection also included a luciferase reporter construct containing bp 69–627 of the CTGF promoter (first through fourth bars), bp 69–561 (fifth through eighth bars), or bp 69–561 with four point mutations (ninth through twelfth bars) in the EGR1-like site. Luciferase activity was normalized for transfection efficiency and is displayed relative to the vector control for each reporter (set at 100). B, the point mutations made in the EGR1-like site within bp 69–561 of the CTGF promoter (yielding reporter construct 69–561mut) are indicated by asterisks.

![Fig. 9. Sequences within bp 455–561 of the CTGF promoter are required for repression of transcription by WT1.](image_url)

A, WiT49 cells were transiently transfected with an empty expression vector or the +/+ or −/− isoform of WT1 as indicated below each bar. Luciferase reporter constructs containing bp 69–561 (first through third bars), bp 238–561 (fourth through sixth bars), bp 383–561 (seventh through ninth bars), or bp 383–455 (tenth through twelfth bars) of the CTGF promoter were cotransfected. Luciferase activity was normalized for transfection efficiency and is displayed relative to the vector control for each reporter (set at 100). B, shown is a schematic representation of the CTGF promoter with TATA boxes, EGR1-like GC-rich sequences (GC), the translation start site (ATG), and a region of complete conservation between the mouse and human sequences indicated. The portions of the promoter included in each reporter construct used are shown (not to scale).
Regulation of CTGF Expression by WT1

Interestingly, analysis of the amphiregulin promoter revealed a novel WT1-binding motif (55). This adds to the complex array of sequences that have been shown to be specifically recognized by WT1 (reviewed in Ref. 2). Our preliminary study of the CTGF promoter suggests that it may also harbor a previously unidentified WT1-binding motif. On the other hand, it is also possible that WT1 interacts with the CTGF promoter in a complicated indirect manner through interaction with other DNA-binding factors and multiple sequence elements. Evidence for WT1 transcriptional effects independent of its binding to DNA has recently been provided for the E-cadherin gene (56).

Together with the recent publications by Harkin et al. (57) and Lee et al. (55), this is one of the first studies to illustrate the utility of oligonucleotide array technology for the analysis of expression changes within a complex genome that are due to alterations in the function of a particular transcription factor. Examination of gene expression patterns in Wilms tumor cells expressing endogenous wild-type WT1 and a naturally occurring WT1 dominant-negative mutant has revealed new potential downstream targets for WT1. Further study of these target genes, including CTGF, will allow new insight into WT1-regulated growth and differentiation pathways and the mechanism of WT1 transcriptional regulation.

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Fig. 10. Sequences within bp 261–321 are involved in WT1 interaction with the CTGF promoter. A, WT49 cells were transiently transfected (if) with an empty expression vector or the $+1$ or $+1$-isoform of WT1 as indicated below each bar. Cotransfected luciferase reporter constructs included pGL2-promoter (first through third bars) or pGL2-promoter containing various portions of the CTGF promoter upstream of the minimal SV40 promoter. These constructs included bp 69–261 (fourth through sixth bars), bp 92–261 (seventh through ninth bars), bp 238–375 (tenth through twelfth bars), or bp 321–317 (thirteenth through fifteenth bars) of the CTGF promoter. Luciferase activity was normalized for transfection efficiency and is displayed relative to the vector control for each reporter (set to 100). B, shown is a schematic representation of the CTGF promoter with TATA boxes, EGR1-like GC-rich sequences (GC), the translation start site (ATG), and a region of complete conservation between the mouse and human sequences indicated. The portions of the promoter included in each reporter construct are shown (not to scale).
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