A Far Upstream Cis-element Is Required for Wilms’ Tumor-1 (WT1) Gene Expression in Renal Cell Culture*  

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The Wilms’ tumor-1 (WT1) gene plays a critical role in genitourinary development and in the pathogenesis of Wilms’ tumors. Wilms’ tumor (nephroblastoma) is a childhood malignancy of the kidney that affects 1 in 10,000 children. Wilms’ tumors arise when the metanephric mesenchyme fails to differentiate into developing glomeruli and tubules. Histomorphologically the tumors often have a triphasic appearance consisting of blastemal, stromal, and epithelial elements (1). These tissue components mimic, although incompletely, normal kidney development suggesting that Wilms’ tumors result from an abnormal differentiation program of embryonic kidney cells. Most cases of Wilms’ tumors are sporadic and unilateral. Occasionally, tumors develop in both kidneys, in a familial pattern, and are associated with a more complex malformation syndrome. A single WT1 allele loss resulting from a deletion on chromosome 11p13 is found in patients with WAGR syndrome which includes Wilms’ tumor, aniridia, genitourinary malformations, and mental retardation (2, 3). The Denys-Drash syndrome is caused by a single allele point mutation in the WT1 gene and is characterized by Wilms’ tumors, male genital ambiguity, and progressive nephropathy (4–6). WT1 gene defects are found in up to 20% of Wilms’ tumors suggesting that the WT1 gene encodes a tumor suppressor.

The WT1 gene product is a zinc finger protein of the Cys2-His2 type with significant homology to the DNA binding domain of the EGR family of zinc finger transcription factors (7–9). Alternative splicing of exon 5, which encodes 17 amino acids, and insertion of three additional amino acids (KTS) between zinc fingers three and four results in four different isoforms of WT1 proteins (10, 11). These four WT1 isoforms, conserved among mammals both in their structure and relative abundance, differ in their DNA binding affinities and specificities (12, 13). RNA editing of WT1 transcripts may contribute to even more heterogeneous WT1 proteins (14). In most contexts, the WT1 protein binds to GC- and TC-rich consensus sequences and acts as a transcriptional repressor (12, 15, 16). Putative WT1 target genes include the genes for insulin-like growth factor-2 (17), platelet-derived growth factor A-chain (18, 19), epidermal growth factor receptor (20), insulin-like growth factor-1 receptor (21), PAX2 (22), and WT1 itself (23, 24), among others.

Major sites of WT1 gene expression in the body are the genitourinary tract and the mesothelial cells of heart, lung, and abdomen (25–27). WT1 is tightly regulated during kidney development. Low levels of WT1 mRNA are detectable in the undifferentiated mesenchyme of the metanephric kidney (27–29). WT1 message dramatically increases upon induction of mesenchymal cells by the ureteric bud and persists in the renal vesicle, comma-, and S-shaped bodies, where it is restricted to the podocytes of the developing glomerulus (25). WT1 is an essential gene as its homozygous disruption in mice caused agenesis of the kidneys probably resulting from apoptosis of the metanephric blastema (30). In addition, the wt1 knock-out mice also showed hypoplasia of the heart and lungs likely due to defects of the mesothelium (30). The characteristic expression pattern and the results obtained from homozygous germ line
disruption studies suggest a critical role for WT1 in mesenchymo-
mal-epithelial differentiation.

Little is known about the molecular mechanisms of WT1 gene regulation. The human WT1 gene contains a GC-rich, TATA- and CCAAT-less promoter (31, 32) which has several potential consensus sequences for EGR/WT1 (33), Pax-8 (34), and other transcription factors. Recent studies from our laboratory indicate that a substantial part of WT1 gene expression is regulated at the level of transcription (35). We have recently identified DNA elements (35). A plasmid containing a 1900 to 2200 bp of the human WT1 promoter region linked to a lacZ reporter gene, however, did not mimic the characteristic pattern of the endogenous wt1 gene in transgenic mice. These findings suggested that additional cis-elements might be required for the tissue-restricted WT1 expression. A 350-base pair enhancer which contains two GATA motifs (33, 36) and a transactivator which directs (35). A plasmid containing a 350-base pair enhancer which contains two GATA motifs (33, 36) and a transactivator which directs gene expression in renal cells but failed to direct expression of WT1 in cells of renal origin. Our efforts have therefore focused on the identification of novel cis-regulatory elements responsible for WT1 gene expression in renal cells.

**EXPERIMENTAL PROCEDURES**

**DNase I-hypersensitive Site Mapping**

**Prote Preparation**—A set of unique probes were obtained for the entire WT1 locus. Briefly, cosmids L109, L156, and L159 (gift of Dr. Daniel Haber, described in Ref. 7) were digested with a variety of restriction enzymes, separated by electrophoresis through agarose, blotted onto nylon membranes, and hybridized with probes corresponding to the matrix remained in the supernatant. Halos were photographed on man 3MM paper, and autoradiographed. Fragments associating with the nuclear matrix appeared in the pellet fraction, and those not binding to the matrix remained in the supernatant.

**Nuclei Preparation**—Nuclei were prepared according to the method of Shapiro et al. (38). 200 μl of nuclear stabilization buffer (5 mM Tris-HCl, pH 7, 0.05 mM spermine, 0.125 mM spermidine, 0.5 mM KCl, 0.1% digitonin) and incubated at 4°C for 5 min. After centrifugation, the DNA was precipitated by the addition of 1 ml of sterile digestion buffer and resuspended in 150 μl of digestion buffer. Supernatants and pellets were subjected to DNase I digestion followed by deproteinization and isolation of DNA. The amount of DNase I chosen for each digest was determined empirically for each cell type. A series of DNase I digests were performed on each cell type to obtain a gradual digestion of the genomic DNA by DNase I. Following the recovery of the DNase I-digested DNA, it was subjected to genomic Southern blotting using the non-replicative probes identified above. By scanning the lanes from low to high concentration of DNase I, the appearance of sub-bands on the autoradiogram signified the identification of DNase I-hypersensitive sites (Fig. IA).

**Matrix Attachment Region Assays**

Nuclear matrix was prepared according to the method of Bode and Maass (39). Nuclei from approximately 5 × 10^7 cells were prepared as follows. Cells were washed with 2 × 50 ml of isolation buffer (3.75 mM Tris-HCl, pH 7.4, 0.05 mM spermine, 0.125 mM spermidine, 0.5 mM K-EDTA, 20 mM KCl, and 1% Triton X-100) and scraped off into 15 ml of isolation buffer containing 0.1% digitonin. Nuclei were released with 15 strokes in a tightly fitting Dounce homogenizer. Following centrifugation (900 × g, 5 min at 4°C) they were washed twice in the same medium. Nuclei were resuspended in 100 μl of nuclear stabilization buffer (5 mM Tris-HCl, pH 7.4, 0.05 mM spermine, 0.125 mM spermi-
0.1% SDS and exposed to x-ray film with intensifying screens at −80 °C. After the films were developed, the membranes were washed sequentially to increase stringency (55 °C and then 65 °C) and re-exposed each time. Random primed probes (Primet, Stratagene, La Jolla, CA) were generated from human regions 56IR1.5 and 56IR12 (Life Technologies). The DNA targets were detectable by autoradiography after a high stringency wash (65 °C) were subcloned into pBluescript II KS+ for further analysis.

**YAC Clones**

A ~620-kb YAC clone (clone address, YAC-901A) containing the mouse wt1 gene in the pYAC4 vector (Fig. 5) was obtained by PCR screening of a mouse super pool YAC library (Genome Systems, St. Louis, MO). A 213-base pair sequence (~586 to ~356 bp relative to the major transcription start site) from the mouse wt1 proximal promoter was amplified with the following PCR primers: 5’-CAATTTCACTT-GAATCTCAAC-3’ (forward primer) and 5’-TTTTAATCAGAAGGGTG-GGGG-3’ (reverse primer). The YAC was designated Y620mWT1 and was maintained in *Saccharomyces cerevisiae* strain AB1380.

**Growth of the YAC Clones**—Yeast cells were grown at 30 °C in liquid medium (per liter) 1.7 g of yeast nitrogen base (Difco), 5 g of ammonium sulfate, 20 g of dextrose (Sigma), 100 mg of adenine hemisulfate, 0.72 g of complete supplement mixture lacking l-tryptophan (BBL Microbiology, Cockeysville, MD), 101.2 ml of La Jolla (pH 8) with 100 mM EDTA, 10 mM Tris-HCl, pH 8. The agarose plugs were incubated overnight at 37 °C, washed with 1% (w/v) lithium dodecyl sulfate, 100 mM EDTA, 10 mM Tris-HCl, pH 8, and 100 μg/ml of semicarbazide, and were equilibrated in 50 ml of 0.5 M sorbitol, 20 mM EDTA, 10 mM 2-mercaptoethanol (Life Technologies, Inc.). G418 selection was performed for at least 2 weeks. Colonies on SC Trp minus 2% agarose (Sea Plaque GTG), 0.5 × TBE buffer, 30–60 s switching time, 6 V/cm, 110° field angle, 24 h running time. The YAC band was excised from the gel and dialyzed overnight at 4 °C in 20 mM Tris-HCl, 1 mM EDTA, 100 μM spermine (Sigma), pH 7.6. On the day of the transfection, the agarose slice was divided into four 1.5-ml pieces and transferred to a 15-ml polystyrene tube. Poly-i-Lysine (Sigma) was added to each slice at a final concentration of 4 μg/ml. The gel slices were melted at 65 °C and incubated with 10 μl of β-agarase (New England Biolabs, Beverly, MA) each at 40 °C for 90 min. After the β-agarase treatment, 50 μl (100 μg) of LipofectAMINE reagent (Life Technologies, Inc.) was added to the DNA, gently mixed, and incubated at room temperature for 30 min. The lipid-DNA complex was supplemented with 500 μl of 10 × Dulbecco’s modified Eagle’s medium in a final volume of 5 μl using Opti-MEM (Life Technologies, Inc.) and applied to a 10-cm dish with 90% confluent HFK 293 cells. The cells were incubated with the transfection complex for 8 h at 37 °C. Transfection was stopped by washing the cells twice with Opti-MEM and changing to fresh Dulbecco’s medium containing 10% fetal calf serum. The cells were grown for 48 h and then split into selective medium containing 300 μg/ml G418 (Life Technologies, Inc.). G418 selection was performed for at least 2 weeks before the colonies of approx 5 mm diameter were transferred into 96-well plates using cloning cylinders. Cells were expanded in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 150 μg/ml G418. To test for chromosomal integration of the YAC, the cell lines were grown in the absence of G418 for approximately 30 cell cycles. Those lines that continued to grow after readdition of G418 (300 μg/ml) were found to have at least one copy of the YAC stably integrated.

**Expression of the Mouse wt1 Gene in Stably Transfected HFK 293 Cells—**RT-PCR and RNase protection assays were used to measure WT1 gene expression in HFK 293 cells stably transfected with the YACs. Total RNA was isolated from the G418-resistant colonies with Trizol reagent (Life Technologies, Inc.). After a 15-min incubation with trizol, 2 μg of total RNA was reverse-transcribed using oligo(dT) as a primer (Superscript II, Life Technologies, Inc.) and hybridized to the DNA probes. **Fluorescent in Situ Hybridization Analysis—**Fluorescent in situ hybridization analysis was performed by Genome Systems (St. Louis, MO) according to their standard procedure.

**Retrofitting the YAC with a Dominant Selectable Marker—**A neomycin resistance gene was targeted into the URA3 site of the centric YAC vector arm using homologous recombination in yeast. The retrofitted vector arm contained a gene and a lacZ gene both driven by the pgk promoter, as well as a LYS2 marker for positive selection of yeast transformants (42). YAC containing yeast cells were transformed by the lithium acetate method (43). Yeast cultures were grown in 100 ml of dropout medium (SC Ura- Trp-) to a density of approximately 107 cells/ml. After pelleting (5 min at 1000 × g) and washing in sterile H2O, the cells were incubated at 30 °C for 30 min in 15 ml of a solution containing 100 mM lithium acetate, pH 7.5, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. A 200-μl aliquot of the yeast suspension was carefully mixed with 5 μl of the DNA to be transformed and 15 μl (150 μg) of denatured salmon sperm DNA as a carrier (CLONTECH, Palo Alto, CA), 1.2 ml of polymerase reaction buffer (200 mM KCl, 10 μm deoxyribonucleotide triphophates) of 10 × TE buffer, pH 7.5, 1 volume 1 mM lithium acetate, pH 7.5) was added, and the samples were shaken for 30 min at 30 °C. Heat shock was performed at 42 °C for 15 min. The cells were collected and resuspended in 300 μl of TE buffer. A 100-μl aliquot of the suspension was plated on a 10-cm Petri dish and grown for 3 days on an SC Trp minus solid dropout plate. The clones were replica-plated on SC Trp-/Lys-/Ura- medium, and colonies that grew on the SC Trp-/Lys- plates but did not replicate on SC Trp-/Lys-/Ura- plates were picked and grown to saturation in liquid medium.

**Transfection of Human Fetal Kidney 293 Cells with the YACs—**Human fetal kidney (HFK) 293 cells (ATCC CRL 1573) were transfected by lipofection as described (42). Yeast DNA was prepared at high density in agarose plugs (~4 × 109 yeast cells/ml), and the chromosomes were separated by pulsed-field gel electrophoresis according to the following protocol: 0.8% low-melting agarose gel (Sea Plaque GTG), 0.5 × TBE buffer, 30–60 s switching time, 6 V/cm, 110° field angle, 24 h running time. The YAC was excised from the gel and dialyzed overnight at 4 °C in 20 mM Tris-HCl, 1 mM EDTA, 100 μM spermine (Sigma), pH 7.6. On the day of the transfection, the agarose slice was divided into four 1.5-ml pieces and transferred to a 15-ml polystyrene tube. Poly-I-Lysine (Sigma) was added to each slice at a final concentration of 4 μg/ml. The gel slices were melted at 65 °C and incubated with 10 μl of β-agarase (New England Biolabs, Beverly, MA) each at 40 °C for 90 min. After the β-agarase treatment, 50 μl (100 μg) of LipofectAMINE reagent (Life Technologies, Inc.) was added to the DNA, gently mixed, and incubated at room temperature for 30 min. The lipid-DNA complex was supplemented with 500 μl of 10 × Dulbecco’s modified Eagle’s medium in a final volume of 5 μl using Opti-MEM (Life Technologies, Inc.) and applied to a 10-cm dish with 90% confluent HFK 293 cells. The cells were incubated with the transfection complex for 8 h at 37 °C. Transfection was stopped by washing the cells twice with Opti-MEM and changing to fresh Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. The cells were grown for 48 h and then split into selective medium containing 300 μg/ml G418 (Life Technologies, Inc.). G418 selection was performed for at least 2 weeks before the colonies of approx 5 mm diameter were transferred into 96-well plates using cloning cylinders. Cells were expanded in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 150 μg/ml G418. To test for chromosomal integration of the YAC, the cell lines were grown in the absence of G418 for approximately 30 cell cycles. Those lines that continued to grow after readdition of G418 (300 μg/ml) were found to have at least one copy of the YAC stably integrated.

**Expression of the Mouse wt1 Gene in Stably Transfected HFK 293 Cells—**RT-PCR and RNase protection assays were used to measure WT1 gene expression in HFK 293 cells stably transfected with the YACs. Total RNA was isolated from the G418-resistant colonies with Trizol reagent (Life Technologies, Inc.). After a 15-min incubation with trizol, 2 μg of total RNA was reverse-transcribed using oligo(dT) as a primer (Superscript II, Life Technologies, Inc.). PCR amplification of the cDNA was performed with the following primer pairs: mouse wt1 gene-specific primers from the untranslated region of exon 10 (forward primer: 5’-TTCAAGGAGGACAGCTTGG-GATC-3’; reverse primer: 5’-CAGCGCCAGCTCATGTTACGACT-3’) and Exm5 (exon 10) primer pair specific for the human WT1 gene (forward primer: 5’-TCTGAACTCCGGAGTTCGCC-3’; reverse primer: 5’-ATCCTCCCTTTGCGTCAAG-3’). As an internal control co-amplification of human β-actin transcripts was performed. Twenty nine PCR cycles were done on a 480 Thermal Cycler (Perkin Elmer) using 60-s denaturation at 94 °C, 120-s primer annealing at 58 °C, and 120-s primer extension at 72 °C. The PCR products were analyzed on a 1.5% agarose gel.
RNase protection assay (HybSpeed RPA kit, Ambion, Austin, TX) was performed with 30 μg of total RNA isolated from stably transfected HFK 293 cell lines using Trizol reagent (Life Technologies, Inc.). Anti-sense riboprobes with a specific activity of 5 x 10^6 cpm/μg were obtained by in vitro transcription with T7 RNA polymerase (MaxScript T7, Promega, Madison, WI) from linearized template DNA. The template for the mouse utl riboprobe consisted of a 429-bp PCR product from the untranslated region of exon 10 (1918–2347 bp of the published mouse utl cDNA sequence) subcloned into the pCR2.1 vector (Invitrogen, San Diego). The human WT1 riboprobe was transcribed from a 299-bp PCR product of exon 10 (1867–2166 bp of the published cDNA sequence) in pCR2.1. RNase protection assays were done according to the protocol supplied by the manufacturer. The protected fragments of 299 nucleotides (human WT1) and 211 nucleotides (mouse utl) were separated on a 8% denaturing polyacrylamide gel. The gel was dried on a gel drier (Bio-Rad) and autoradiographed. The relative intensities of the hybridization signals were determined by densitometry scanning of the autoradiographs (Molecular Dynamics).

Introducing Deletions into the YAC—Deletions were targeted into the YAC (Yac 620mWT1neoR) by homologous recombination in yeast. The 1.4-kb EcoRI fragment that cross-hybridized at high stringency to a DNase I-hypersensitive site and matrix attachment region in the human WT1 gene (56R13-5) was deleted from the YAC by a transplacement technique (44). A 5-kb XbaI fragment encompassing the 1.4-kb EcoRI piece was first subcloned from the mouse P1 clone 1407 (Genome Systems, St. Louis, MO) into the yeast integration plasmid pRS406 (Stratagene, La Jolla, CA). The 1.4-kb EcoRI fragment was replaced by the insert that was blunt-ended religated thus leaving at least 1.5 kb of flanking sequence on either side of the 1.4-kb deletion. Lithium acetate transformation of the YAC containing yeast cells was performed with 3 μg of linearized (SacI) and gel-purified plasmid as described above. “Pop-in” transformants were selected on SC Trp/Lys/URA plates, picked, and grown to saturation in liquid medium. Genomic DNA was prepared from these cultures and screened by PCR for integration of the fragment. PCR was done with a primer pair which flanked the 1.4-kb EcoRI deletion. Transformants that had incorporated the deletion vector were grown for “pop-out” in SC Trp/LYS liquid medium and plated onto SC Trp/LYS plates supplemented with 1 mg/ml fluororotic acid (Sigma). Colonies growing on these plates were replica-plated onto SC Trp/LYS and SC Trp/LYS/URA plates, respectively. Transformants that grew on SC Trp/LYS plates but that did not grow on SC Trp/LYS/URA plates were further analyzed. Genomic DNA was prepared from these colonies, run on a pulsed-field gel, and transferred to nylon membranes (Boehringer Mannheim) using 10 × SSC as a transfer buffer. After UV cross-linking and a 30-min pre-hybridization at 65 °C, the DNA was probed with the 1.4-kb EcoRI fragment. Membranes were washed for 40 min at 65°C with 0.1% SDS and exposed on film at −80 °C with intensifying screens for 24 h.

PCR Analysis of Stably Transfected HFK 293 Cell Lines—A PCR-based strategy was chosen to assay the structural integrity of the transfected YAC clones. Multiple PCR reactions on different sites of known DNA sequence were performed with 2 μg each of genomic DNA prepared from the stable cell lines. The following PCR primer pairs were used to amplify sequences distributed along the entire length of the YAC; exon YAC4 vector arm: 5'-GCTGTGTCGGCTATGAGGCTG-3' (forward primer) and 5'-ATGCGGTAGTTTATCACAGTTAA-3' (reverse primer); acentric pYAC4 vector arm: 5'-GGAAAGGACACGACTGTGGATC-3' (forward primer) and 5'-AACAAGATGGATTGCACGAGCAG-3' (reverse primer) and 5'-TGCCAGTCTACGTAGGGCACC-3' (reverse primer).

RESULTS

The long term goal of our experiments is to identify cis-regulatory elements residing in the WT1 locus that faithfully reproduce the tissue and developmental pattern of WT1 gene expression. We began by utilizing DNase I-hypersensitive site mapping as an unbiased approach to uncover such elements. This technique allows for the identification of regions of “naked” chromatin, existing within the highly ordered nucleosome structure. These regions have been shown to bind tissue-specific transcription factors (45, 46) and contain locus control regions (47, 48) and nuclear matrix attachment sites (49, 50). It is thought that a gene has to be accessible for binding by numerous protein factors to become transcriptionally active. DNase I-hypersensitive site mapping allows one to quickly scan a gene segment for regions of open chromatin, thus identifying potential functional sites.

As a prerequisite to mapping the entire human WT1 locus for DNase I-hypersensitive sites, a series of probes unique to the WT1 gene and devoid of repetitive sequences had to be identified. Three cosmid clones, L156, L109 and L159 (7), which spanned the entire human WT1 locus of approximately 85 kilobases (kb) of DNA were obtained from Dr. Daniel Haber. To identify DNA fragments to use as probes for DNase I-hypersensitive site mapping, a simple method was devised. Three regions were identified in this manner suitable for DNase I-hypersensitive site mapping of the first intron and the 5'-flanking region of the WT1 gene (Fig. 1B).

DNase I-hypersensitive Sites—A total of 11 hypersensitive sites were identified, some of which were tissue-restricted in their appearance (Fig. 1 and Table I). Most notably, the promoter region of those cell lines that expressed WT1 mRNA (HFK 293, CEM, and K562) yielded bands corresponding to DNase I-hypersensitive regions, whereas those cell lines negative for WT1 expression (HeLa, TK10) failed to show DNase I hypersensitivity (Fig. 1 and Table I). HeLa cell nuclei exhibited five DNase I-hypersensitive sites in the far upstream region (HS I to HS V in Fig. 1). Although negative for WT1 mRNA by Northern blotting, HeLa cells showed low levels of WT1 expression after 40 cycles of RT-PCR (data not shown). For comparison, no DNase I-hypersensitive sites were detectable in TK10 cells that did not show WT1 transcripts even after 40 PCR cycles (data not shown). We also identified two regions of DNase I super hypersensitivity in the far upstream region (HS I and HS III in Fig. 1) most easily detected in nuclei from CEM cells. These sites were evident with the minimal amount of DNase I added, and surprisingly, in some experiments, they were strongly present even in untreated nuclei, presumably due to activation of endogenous DNases by the addition of reaction buffer.

The 56R13-5 region also contained a DNase I-hypersensitive site (HS II in Fig. 1) and may overlap with the region of DNase I super hypersensitivity (HS III in Fig. 1). Due to the resolution of the technique of DNase I-hypersensitive site mapping and the size of the WT1 locus, individual hypersensitive sites were “placed” plus or minus approximately 500 bp. Another interesting feature of this analysis was the observation that 56R13-12 bound to nuclear matrix prepared from 293 cells but not to matrix obtained from HeLa cells, which do not express the wt1 locus, individual hypersensitive sites were “placed” plus or minus approximately 500 bp. Another interesting feature of this analysis was the observation that 56R13-12 bound to nuclear matrix prepared from 293 cells but not to matrix obtained from HeLa cells, which do not express the wt1 locus. This raises the question as to how such elements were placed at specific sites on the nuclear matrix. Some of these sites were strongly present even in untreated nuclei, presumably due to activation of endogenous DNases by the addition of reaction buffer.
This region may be a target for binding of a negative regulatory molecule after the fragment is made accessible and transcriptionally competent. The absence of HS VI from HeLa and TK10 cells may reflect the fact that both cell lines are negative for WT1 expression, and therefore the chromatin at this region is in a closed configuration.

We next examined the possible role of these DNase I-hyper-sensitive sites in WT1 transcriptional regulation. A series of constructs were made to test whether these sites conferred the tissue-restricted expression pattern of the WT1 gene to a heterologous promoter assayed in tissue culture. Serial deletions of a WT1-reporter construct containing up to 24 kb of 5'-flanking sequence had significant effects on gene expression suggesting enhancer function for at least some of the DNase I-hyper-sensitive sites (35). None of the elements, however, tested both alone and in combination, proved to be specific for renal

| Cell line | WT1 mRNA | HSI | HSI | HSI | HSI | HSI | HSI | HSI | HSI | HSI | HSI | HSI |
|-----------|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| HFK 293   | ++       | -   | -   | X   | x   | x   | x   | x   | x   | x   | x   | x   |
| CEM       | ++       | x   | x   | x   | x   | x   | x   | x   | x   | x   | x   | x   |
| K562      | ++       | x   | x   | x   | x   | x   | x   | x   | x   | x   | x   | x   |
| TK10      | -        | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -   |
| HeLa      | -        | x   | x   | x   | x   | x   | x   | x   | x   | x   | x   | x   |

**TABLE I**

Summary of DNase I-hypersensitive site data in various WT1 expressing (+) and non-expressing (−) cell lines

A5' -Element Is Required for Wilms' Tumor-1 Gene Expression
cells. In addition to transcriptional control, DNase I-hypersensitive sites may mediate other functions such as insulators (51, 52), locus control regions (47, 48), or nuclear matrix attachment sites (49, 50). We therefore asked whether these elements could mediate attachment of DNA in the WT1 locus to nuclear matrix. To this end a matrix attachment assay was performed using as probes regions surrounding the DNase I-hypersensitive sites identified above.

Matrix Attachment Regions—Nearly the entire WT1 locus was scanned for its ability to bind nuclear matrix. Nuclear matrix was prepared from 293 and HeLa cells and probed with end-labeled WT1 genomic fragments. Reactions were separated into pellet and supernatant fractions and run on analytical agarose gels. Several sites proved to bind to the nuclear matrix (Fig. 2). Two fragments were used as positive controls, SAR800 (39) and MAR-μH (40), obtained from the interferon-β and immunoglobulin μ heavy chain enhancer and the scaffold attachment region (SARopo) from the interferon-β gene. The Bluescript plasmid (pBS) served as a negative control. P indicates the pellet fractions, i.e. DNA that has attached to the pellet, and S refers to the supernatant. The size of the fragments is not of relevance but depends on the enzyme used for the preparation of the nuclear matrix and probes (see “Experimental Procedures”).

WT1 probe hybridized to the more upstream P1 clones (P 1405 to P 1408) only but not to the downstream clones (P 1409 to P 1411, see “Experimental Procedures”). The ~6 kb SacI fragment from P1 clone 1407 detected with the human probe 56R13-5 (Fig. 3) was subcloned into pBluescriptIIKS" for further analysis. This plasmid was digested with a variety of restriction enzymes and reprobed at high stringency with 56R13-5. As a result a 1.4 kb EcoRI fragment was obtained which was compared with the full-length sequence of human 56R13-5.

**Fig. 2. Identification of two MARs contained within 56R13-5 and 56R13-12 (Fig. 1) in HFK 293 cells.** Note that the Bg 4.1-kb fragment that was obtained from the far upstream region of the human WT1 gene (see Fig. 4) is essentially negative in this assay. The positive controls are the matrix attachment regions (MAR-μH) from the immunoglobulin μ heavy chain enhancer and the scaffold attachment region (SARopo) from the interferon-β gene. The Bluescript plasmid (pBS) served as a negative control. P indicates the pellet fractions, i.e. DNA that has attached to the pellet, and S refers to the supernatant. The size of the fragments is not of relevance but depends on the enzyme used for the preparation of the nuclear matrix and probes (see “Experimental Procedures”).

**Fig. 3. Cross-hybridization at high stringency between human and mouse WT1 genomic DNA sequence.** The mouse P1 clone 1407 (Fig. 5) containing wt1 upstream sequence was digested to completion with several restriction enzymes and hybridized by the Southern technique with 56R13-5 (Fig. 1) as a human probe. The membrane was washed at 65 °C in 0.1 × SSC, 0.1% SDS and exposed to x-ray film with intensifying screens for 5 days. A single band of similar intensity was detectable in each lane. The ~6 kb SacI fragment indicated by asterisks was subcloned into pBluescript and re-hybridized with 56R13-5. As a result a 1.4 kb EcoRI fragment was obtained which was compared with the full-length sequence of human 56R13-5.

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*H. T. Cohen, S. A. Bossone, and V. P. Sukhatme, unpublished observations.*
A 5′-Element Is Required for Wilms’ Tumor-1 Gene Expression

To determine whether wt1 expression of the YAC was cell type-specific, we also transfected HeLa cells that are negative for WT1 mRNA by Northern blotting. In three independent transfection experiments, we could not establish a single stable cell line possibly due to the very low level expression of the neomycin resistance gene in HeLa cells (Fig. 7B). As an alternative approach, we performed transient transfections simultaneously of HFK 293 and HeLa cells. Using YAC DNA from the same preparation for the transfection of both cell lines, HFK 293 but not HeLa cells were found by RT-PCR (34 cycles) to express the mouse wt1 gene (Fig. 7A). Low amounts of neomycin resistance gene transcripts could be detected in HeLa cells after 34 PCR cycles indicating transcriptional activity of the transfected Y620mWT1neoR (Fig. 7B). Although the YAC could not be tested in the chromosomal context of a non-WT1 expressing cell line, the results obtained with transiently transfected 293 and HeLa cells strongly suggest a cell type specific regulation of the mouse wt1 (trans)gene.

To test whether the 1.4-kb EcoRI mouse fragment that cross-hybridized to the human 56R13-5 (Figs. 3 and 4) contained cis-regulatory elements required for wt1 gene expression, we deleted this region from Y620mWT1neoR using homologous recombination in yeast. The deletion construct consisted of a pRS406 vector (Stratagene, La Jolla, CA) which had ~1.5 kb of flanking sequence on either side of the targeted 1.4-kb EcoRI piece subcloned into the XbaI site. Yeast transformants were screened by PCR and Southern hybridization of genomic DNA. Using the 1.4-kb EcoRI fragment from the mouse wt1 gene as a probe, one out of 6 clones was found to have the expected deletion (data not shown). A single band of the same size (~620 kb) as the original (undeleted) Y620mWT1neoR was observed when the DNA blot was re-hybridized with a specific probe for the centric vector arm suggesting that the YAC was largely intact and no fragmentation had occurred during the transformation procedure (data not shown). Genomic DNA from this YAC clone, designated Y620mWT1neoRΔEcoRI, was prepared in agarose plugs and separated on a pulsed-field gel. The YAC band was excised from the gel and transfected into HFK 293 cells. Three transfection experiments using YAC DNA from two different preparations provided a total of 11 stable cell lines. RT-PCR was performed with 2 μg of total RNA from stably transfected cells using human and mouse WT1 gene-specific primer pairs. Transcripts of the endogenous human WT1 gene were detectable in all cell lines after 34 PCR cycles (Fig. 8). However, we could not detect significant levels of mouse wt1 mRNA in any of the 11 stable lines that had the 1.4-kb upstream element missing on the YAC (Fig. 8). To confirm this result, we performed another transfection experiment with Y620mWT1neoRΔEcoRI in which we measured WT1 mRNA in pools of stable 293 cell lines. Again, after 34 cycles of RT-PCR, significant amounts of mouse wt1 mRNA could not be detected, whereas the endogenous human WT1 gene was normally expressed (Fig. 8). To ensure that the transfected YAC DNA was largely intact and no major fragmentation and/or deletion had occurred during the lipofection procedure, we performed repeated PCR on genomic DNA from the transfected cell lines. Specific PCR primer pairs were used to amplify 90–350 base pair stretches of DNA from both YAC vector arms and from exon 10, exon 6, and a 15-kb upstream region in the mouse wt1 gene. The expected PCR products were obtained with at least 8 out of 11 lines transfected with Y620mWT1neoRΔEcoRI suggesting that the transfected YAC was largely intact.

**DISCUSSION**

Studies on the regulation of WT1 are important for several reasons. First, since WT1 is expressed in induced blastema and

**Fig. 4. Sequence of the human region 56R13-5 that contained both a matrix attachment region and a DNase-hypersensitive site and that cross-hybridized at high stringency with mouse wt1 upstream sequence.** Regions of 100% homology between human and mouse are indicated in bold, and the AT-rich (~70% AT) sequence in the human gene is underlined. Note the 20–30-base pair stretches of >80% sequence conservation in the human and mouse WT1 gene. Both the human and the mouse sequences are located approximately 15 kb upstream of the respective transcription start sites in the WT1 gene. The location of DNase I-hypersensitive sites (HS) is indicated by downward arrows.
Note that the level of mouse wt1 gene were obtained by PCR screening of a containing different segments of the genomic DNA clones (P 1405 to P 1412) the pYAC4 centric vector arm. Eight nated as Y620mWT1neoR, was performed mapping of the retrofitted YAC, desig- nated WT1. This was achieved by PCR screening of a mouse YAC and P1 clones used. The original YAC clone (Y620mWT1) has been used in podocytes, these studies may provide new insights into some of the earliest transcriptional signals mediating kidney development and into podocyte-specific gene expression. Second, since WT1 has been suggested to play a role in mesenchymal-epithelial conversion (25, 30), regulators of WT1 might be involved in epithelial cell differentiation as well. Finally, WT1 regulation studies may reveal molecular events leading to the formation of Wilms’ tumors. In an effort to study the transcriptional control of WT1, we and others (34–37) have previously localized several regulatory elements on the gene. None of the cis-elements identified so far, however, have mimicked the tissue-specific expression pattern of WT1. Likewise, transgenic mice harboring a ∼1.9 to +0.2 kb WT1-lacZ construct did not express the transgene in a tissue-restricted fashion. We therefore reasoned that additional, possibly far upstream and/or downstream elements, might be important for WT1 gene reg- ulation. In this study we used human fetal kidney 293 cells and various other WT1 expressing and non-expressing cell lines to map DNase I-hypersensitive sites and matrix attachment regions in the WT1 gene locus. We report the following novel findings. 1) A total of 11 DNase I-hypersensitive sites were found in WT1 expressing cells only suggesting a role in cell type-specific gene regulation. 2) A 1.4-kb fragment of the human WT1 gene residing approximately 15 kb upstream of the transcription start site, which contained DNase I-hypersensitive sites and also bound to the nuclear matrix in vitro, showed significant sequence conservation with an upstream fragment of similar size from the mouse wt1 gene. 3) Stable transfection of a ∼620-kb YAC clone carrying the mouse wt1 locus into human fetal kidney cells resulted in (trans)gene expression at a level comparable to the endogenous WT1 gene. 4) Deletion of this 1.4-kb 5’-element from the YAC caused a loss of wt1 gene expression in human fetal kidney cells. We have therefore identified a novel cis-regulatory region in the mouse wt1 gene critical for expression in renal cells.

DNase I-hypersensitive sites occur in the chromatin of transcriptionally competent genes probably due to a local relaxation of chromatin organization which allows the binding of protein factors, a subset of which may be tissue-specific transcription factors (53, 54). Occasionally DNase I-hypersensitive sites are located within AT-rich matrix attachment regions (MARs). MARs are thought to anchor the DNA to the nuclear matrix, and they may also insulate the DNase I-hypersensitive sites from the influence of outside sequences (49, 55). MARs have also been found to be responsible for the tissue-restricted expression of immunoglobulin μ chains in a transgenic setting (40). Moreover, several transcription factors have been shown to be localized to the nuclear matrix (56, 57).

None of the DNase I-hypersensitive sites and/or MARs that we have identified in the first intron and 5’-flanking region of the WT1 gene had a tissue-specific effect on the transcriptional activity of transiently and stably transfected reporter constructs both in the context of the natural WT1 minimal promoter and an SV40 promoter. These initial results were not completely unexpected. Although DNase I-hypersensitive regions have been found to correlate very closely with transcription in a number of genes studied so far, the complicated pattern of tissue-specific expression of the WT1 gene may require additional elements that were missing from these smaller constructs. For example, the developmental regulation of the human β-globin gene relies on a locus control region at the 5’-end of the β-globin cluster. The locus control region consists of four subdomains that exhibit exquisite hypersensitivity to DNase I in erythroid but not in non-erythroid cells (47, 58, 59). An upstream DNase I-hypersensitive site corresponding to a distal enhancer element was also required for the tissue-spe-
Specific primers for the neomycin resistance gene were used for the PCR reaction (see “Experimental Procedures”). pSV2neo was used as a positive control. Initial work focused on showing that an intact YAC gives rise to WT1 transcripts de novo. We reasoned that a far upstream and/or downstream sequence is likely required for WT1 gene expression in a renal cell line expressing endogenous WT1. Then we deleted a small (1.4 kb) region of DNase I hypersensitivity and assessed sequence conservation between the human and mouse WT1 gene to ask whether it was necessary for WT1 expression. For these purposes we used an approximately 620-kb YAC carrying the entire mouse WT1 locus. Compared with reporter constructs the use of a YAC clone offered the following advantages. First, YACs allow one to introduce genes into tissue culture cells and animals in a more natural chromosomal context. Second, gene expression from YACs in transgenic animals and transfected cells normally occurs in a copy number-dependent and integration site-independent way (64). Third, YACs are relatively easy to manipulate (introducing deletions, insertions, etc.) by homologous recombination in yeast. We have chosen a mouse YAC (instead of a human), because most WT1 expressing cell lines are derived from human tissue. Transfection of the mouse WT1 gene into human cells allowed us to quantitate “transgene” expression directly by an RNase protection assay with a mouse wt1-specific riboprobe. The use of YACs in gene regulation studies is limited by transfection efficiency into mammalian cells which is significantly lower than for regular plasmid DNA (65). We have therefore taken highly transfecable HFK 293 cells in which we introduced a YAC carrying the mouse wt1 gene by the lipofection method (42). Twelve of the 16 stable cell lines expressed the mouse wt1 gene at a level of at least 30% of the endogenous human gene (Fig. 6). Cell lines in which we could not detect mouse wt1 mRNA had portions of the structural gene missing likely due to DNA shearing during the transfection (data not shown). These results encouraged us to utilize stably transfected human fetal kidney cells as a model to narrow down cis-regulatory elements on the mouse wt1 gene.

To this end we examined whether one of the DNase I-hypersensitive sites, although not sufficient, might still be required for wt1 gene expression in HFK 293 cells. We focused our initial studies on a 1.4-kb EcoRI fragment located approximately 15 kb upstream of the transcription start site in the mouse wt1 gene. The reasons for this were as follows. First, the corresponding sequence in the human gene was found to be hypersensitive to DNase I suggesting a transcriptionally active site (Fig. 1). Second, this human analogue bound to the nuclear matrix in vitro (Fig. 2). Third, a sequence comparison of this region revealed significant homology between the human and mouse gene. In particular, we found 20–30 base pair stretches of genomic DNA flanking the site of DNase I hypersensitivity.
of >50% sequence conservation which were roughly co-linear in the WT1 gene of human and mouse (Fig. 4). Most interestingly, deletion of this 1.4-kb upstream fragment from the YAC resulted in complete loss of mouse wt1 gene expression in stably transduced HFK 293 cells (Fig. 8). These results strongly suggested that a cis-regulatory element in the 1.4-kb EcoRI region was required for transcription of the wt1 gene. There was still a minor chance, however, that accidental fragmentation of the YAC in addition to the targeted deletion had occurred and was responsible for the loss of wt1 gene expression. We addressed this issue by showing that sequence-tagged sites along the entire length of the YAC could be amplified by PCR in at least 8 out of 11 stable cell lines (data not shown). These findings suggested that more than 70% of the transfectants had an intact copy of the YAC stably integrated into their genome. For comparison, a similar percentage of cell lines that had been stably transfected with the original (undeleted) YAC expressed the mouse wt1 (trans)gene at a significant level. Moreover, the 1.4 kb-deleted YAC ran on a pulsed-field gel at roughly the same molecular size as the original YAC indicating absence of major deletions or fragmentation (data not shown).

Notably, the 1.4-kb EcoRI fragment that had no homology to any known sequence was not sufficient for the tissue-specific expression of the wt1 gene but was functioning only in the context of a YAC carrying the entire mouse wt1 gene. These findings together with the observation that the human sequence analogue contained a matrix attachment region are consistent with a role for chromatin organization in WT1 gene regulation. A novel class of gene regulatory elements, termed "facilitators," which have been linked to chromatin unfolding has recently been described. Facilitators, although having no effect on reporter gene expression in transient assays, were essential for copy number proportional and integration site-independent expression in transgenic animals (66). A lack of facilitator function prevented DNase I hypersensitivity and significantly reduced transcriptional activating function of a T cell-specific enhancer in the human adenosine deaminase gene (66). To date, we have no direct experimental evidence to indicate that the identified upstream element meets the criteria for a facilitator. It is also possible that the critical region we have identified in some way interacts with other cis-elements to activate WT1 gene expression. Cooperative interaction between distant regulatory regions has previously been found to be important for gene transcription. For example, a 3-kb upstream DNase I-hypersensitive site in the gene for the murine stem cell antigen CD34 conferred position-independent gene expression in stably transfected hematopoietic progenitor cells only after addition of 3'-downstream sequence (67). A model of mutual interaction between distant enhancer/locus control regions and proximal promoter sequence has also been suggested for the mechanism of chromatin opening in the chicken βA globin gene (68).

In conclusion, we have identified a novel cis-element in the mouse wt1 gene required for expression in human fetal kidney cells. This regulatory region is located approximately 15-kb upstream of the transcription start site and contains a sequence which is highly conserved between the human and mouse. The human region is also located ~15-kb upstream of the human WT1 transcription start site, shows DNase I hypersensitivity, and contains a site for attachment of nuclear matrix. Future investigations will further narrow down the 1.4-kb region, identify putative transactors binding to it, explore its mechanistic role, and assay its importance in transgenic mice in tissue-restricted WT1 expression, both in the kidney and outside.

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