DNA Recognition by Splicing Variants of the Wilms' Tumor Suppressor, WT1

IAIN A. DRUMMOND,¹ HARALD D. RUPPRECHT,¹ PATRICIA ROHWER-NUTTER,^{2†}
JESUS M. LOPEZ-GUISA,³ STEPHEN L. MADDEN,³ FRANK J. RAUSCHER III,³
AND VIKAS P. SUKHATME^{1*}

Harvard Medical School and Renal Division, Department of Medicine, Beth Israel Hospital, Boston, Massachusetts 02215¹; Howard Hughes Medical Institute and Departments of Molecular Genetics and Cell Biology and Medicine, University of Chicago, Chicago Illinois 60637²; and The Wistar Institute of Anatomy and Biology, Philadelphia Pennsylvania 19104³

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The Wilms' tumor suppressor, WT1, is a zinc finger transcriptional regulator which exists as multiple forms owing to alternative mRNA splicing. The most abundant splicing variants contain a nine-nucleotide insertion encoding lysine, threonine, and serine (KTS) in the H-C link region between the third and fourth WT1 zinc fingers which disrupts binding to a previously defined WT1-EGR1 binding site. We have identified WT1[+KTS] binding sites in the insulin-like growth factor II gene and show that WT1[+KTS] represses transcription from the insulin-like growth factor II P3 promoter. The highest affinity WT1[+KTS] DNA binding sites included nucleotide contacts involving all four WT1 zinc fingers. We also found that different subsets of three WT1 zinc fingers could bind to distinct DNA recognition elements. A tumor-associated, WT1 finger 3 deletion mutant was shown to bind to juxtaposed nucleotide triplets for the remaining zinc fingers 1, 2, and 4. The characterization of novel WT1 DNA recognition elements adds a new level of complexity to the potential gene regulatory activity of WT1. The results also present the possibility that altered DNA recognition by the dominant WT1 zinc finger 3 deletion mutant may contribute to tumorigenesis.

The long-standing proposition that Wilms' tumors can arise from the loss or inactivation of one or more tumor suppressor genes has recently been substantiated by the cloning and characterization of a Wilms' tumor suppressor gene, *WT1*, from the chromosomal locus 11p13 (6, 10, 24). WT1 is a DNA-binding protein which contains four zinc fingers and functions primarily as a transcriptional repressor, although recently, transcriptional activation by WT1 has also been described (19, 23, 39). WT1 is expressed at a critical juncture in kidney development when proliferating kidney blastemal cells are induced to differentiate into a polarized epithelium (9, 46, 47). Targeted disruption of the *WT1* gene in mice results in embryonic lethality and a complete block in the mesenchymal-epithelial inductive interactions which give rise to the metanephric kidney (36). In humans, tumor-associated mutations in *WT1* include deletions of zinc finger coding sequences, frame-shift mutations which result in truncated proteins lacking one or more zinc fingers, and point mutations in the WT1 zinc fingers (1, 2, 14, 15, 27, 28, 30, 38, 45). This genetic evidence strongly suggests that DNA binding is essential for normal WT1 function.

WT1 belongs to the C₂-H₂ class of zinc finger proteins and exhibits a high degree of homology to the EGR family of transcriptional regulators (10, 11, 24). X-ray crystallographic analysis of the zif268 (EGR1) zinc finger-DNA recognition element complex has demonstrated that each zinc finger interacts with a defined nucleotide triplet subsite within the

9-bp sequence GCG GGG GCG (43). Although it has been shown that a WT1 zinc finger polypeptide can bind this 9-bp sequence (48), the nucleotide-protein interactions involved are not precisely known. In addition, the interaction of WT1 with DNA is likely to be more complex owing to the presence of an additional zinc finger in WT1 and also to the existence of multiple WT1 proteins which result from differential splicing of the *WT1* transcript (10, 29). Alternate *WT1* mRNAs result from the inclusion of exon 5, a 51-nucleotide sequence encoding 17 amino acids N terminal to the zinc fingers, or the use of an alternate splice donor sequence at the end of exon 9 (29). Four mRNAs are possible: mRNAs having no insertions, one insertion, or both insertions. Notably, alternative splicing at the end of exon 9 results in the insertion of nine nucleotides encoding lysine, threonine, and serine (KTS) in the H-C link region between the third and fourth zinc fingers of the WT1 protein (WT1[+KTS]). Transcripts which include the nine-nucleotide insertion account for 67 to 77% of *WT1* mRNA, suggesting that WT1[+KTS] is the most abundant form of the zinc finger protein (8, 29). Currently, very little is known about the interaction of this WT1 zinc finger splice variant with DNA. Compared with the WT1 zinc finger lacking the KTS splice, the WT1[+KTS] protein binds poorly to the 9-bp EGR binding site, presumably because of an altered zinc finger alignment caused by the KTS insertion (48). A recent report described the selection of genomic DNA fragments which bound to both the +KTS and -KTS splicing variants of WT1 (5). However, it remains unclear how either form of the WT1 protein interacts with DNA and what constitutes a WT1[+KTS] binding site. The definition of this sequence and identification of target gene promoters in which it functions as a regulatory *cis* element are essential steps in elucidating the gene regulatory network in which WT1 exerts its antiprolifera-

* Corresponding author. Mailing address: Harvard Medical School and Beth Israel Hospital, Department of Medicine, Renal Division, Dana 517, 330 Brookline Ave., Boston MA 02215. Phone: (617) 735-2105. Fax: (617) 735-7843.

† Present address: McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI 53706.

tive effect and participates in the differentiation of kidney epithelia.

MATERIALS AND METHODS

Cell culture and transfection assays. HepG2 cells were grown and transfected with WT1 expression vectors as previously described (19, 39). The insulin-like growth factor II (IGF-II) P3 reporter plasmid was pP3[−295,+135] (19); the reporter plasmid pP4[−677,+206] consisted of an 883-bp *Pst*I fragment of the λ phage clone λ hIGF2-1 (4) containing 677 bp of promoter P4 upstream sequence cloned between the *Pst*I and *Hind*III sites of pGCAT-C (22).

Electrophoretic mobility shift assays. Polypeptides containing different sets of the WT1 zinc fingers (either with or without the KTS insertion between fingers 3 and 4) as well as eight flanking amino acids on either side were produced as 6 $\times$ histidine fusion proteins in *Escherichia coli* as previously described (48). Gel shift assays were performed on ice in a final volume of 10 μ l containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.5), 70 mM KCl, 12% glycerol, 0.05% Nonidet P-40, 100 μ M ZnSO₄, 0.5 mM dithiothreitol, 100 μ g of poly(dI-dC) per ml, and 1 mg of bovine serum albumin (BSA) per ml with 0.2 to 0.5 ng of probe and various amounts of WT1 proteins. IGF-II P3 promoter probes consisted of the 198-bp *Eco*RI-*Sac*II fragment spanning nucleotides −295 to −98 and the 177-bp *Sac*II-*Sal*I fragment spanning −42 to +135 (19). The sequences of the oligonucleotides used were as follows: EBS, 5'-CCCGGCGCGGGGCGGAGGGCG-3'; P3 E site, 5'-CCCCCCTTCCGGCCGC CCGCCTCTGCGCCAC-3'; P3 B1 site, 5'-GGCTCG GGTGCGGGGGCGGGCCG-3'; and P3 B1 site nucleotide triplet probes (Fig. 4), 5'-GATAAGCTTTAGCGGGGCG GGCTCTAGACT-3', with modifications noted in Fig. 4. Oligonucleotides were annealed to their complementary strands and cloned in pSKII+. Probes were prepared from purified plasmid inserts and used at 0.2 to 1 ng per assay. In experiments in which an unlabeled competitor was used, binding reaction mixtures containing various amounts of an unlabeled double-stranded competitor oligonucleotide and all other components except probe were preincubated on ice for 10 min and then processed. Binding and also competition by unlabeled competitor oligonucleotides were dependent on positioning the binding sites in the middle of the DNA fragment with adequate (nonspecific) flanking nucleotides.

Equilibrium dissociation constants. Equilibrium dissociation constants (K_d s) were determined by incubating a constant amount of DNA probe (10^{-10} M) with increasing amounts of WT1 zinc finger polypeptides (3×10^{-8} to 1×10^{-6} M for WT[−KTS] and 3×10^{-7} to 1×10^{-5} M for WT[+KTS]) in 10- μ l binding reaction mixtures. To ensure equivalent renaturation of the polypeptides, assays were performed with protein preparations which had been dialyzed together against 4 liters of binding buffer overnight at 4°C. Binding reactions were performed in 20 mM HEPES (pH 7.5)–70 mM KCl–12% glycerol–0.05% Nonidet P-40–100 μ M ZnSO₄–0.5 mM dithiothreitol–100 μ g of poly(dI-dC) per ml–1 mg of BSA per ml. After a 15-min incubation on ice, the reactions were analyzed on a 0.022 M Tris-borate–4% polyacrylamide gel run at 250 V at 4°C for 2 h. Gels were fixed, dried, and exposed to X-ray film. Levels of bound and free probe were quantitated by densitometric scanning of the resulting autoradiographs. The K_d was calculated with the equation $K_d = [\text{DNA}_{\text{free}}]/[\text{protein}_{\text{free}}]/[\text{DNA} \cdot \text{protein}]$. Because $[\text{protein}] \gg [\text{DNA}]$ in these reactions, $[\text{protein}_{\text{free}}]$ was approximated to equal $[\text{protein}_{\text{total}}]$. The inclusion of relatively high amounts of

poly(dI-dC) and BSA was required to prevent the formation of aggregates which otherwise would not enter the gel. Within these constraints, the dissociation constants determined by this method provide a relative measure of the DNA binding affinity of WT[−KTS] and WT[+KTS] for various binding sites under uniform conditions.

DNase I footprinting assays. Binding reactions for DNase I footprinting assays were scaled up twofold but otherwise were performed as described for gel shift assays. After a 15-min incubation with asymmetrically labeled probes, the reactions were diluted 1:1 with an ice-cold solution of 1 mM MgCl₂–0.2 mM CaCl₂, 1 μ l of a freshly diluted 50- μ g/ml DNase I solution was added, and the mixtures were incubated on ice for 2 min. Reactions were stopped with the addition of an equal volume of room temperature 20 mM EDTA–1% sodium dodecyl sulfate–0.2 M NaCl–100 μ g of yeast tRNA per ml. After phenol-chloroform extraction and ethanol precipitation, pellets were resuspended in sequencing gel buffer and denatured at 95°C, and equal counts were run on an 8% polyacrylamide–8 M urea sequencing gel.

DMS methylation interference assays. Double-stranded oligonucleotides were cloned in pSKII+ and single end labeled, using the *Sal*I or *Sma*I polylinker site. Probes were purified on 3% NuSieve–1% SeaPlaque (FMC) agarose gels and dimethyl sulfate (DMS) treated as described previously (3); 100,000 to 300,000 cpm was used in binding assays with 500 ng of WT1 proteins. Bound and free probe were purified from a 4% polyacrylamide–0.5 $\times$ Tris-borate-EDTA gel, cleaved with piperidine, and resolved on 8% polyacrylamide–8 M urea sequencing gels.

F1 binding site selection. A partially degenerate 61-bp oligonucleotide was synthesized with the central sequence 5'-CTTGATGCGGGGCGNNNNATTGTAGA-3' flanked by the PCR primer sequences 5'-TGACTGAATTCAT ATG-3' (PA) and 5'-GTACATCTAGATTAGAC-3' (PB). Following an initial PCR, the 61-bp product was gel purified, end labeled with T4 kinase, and used in a gel shift assay with 200 ng of the WT1[Δ F4] protein. Bound DNA was eluted from the dried polyacrylamide gel, precipitated with 5 μ g of glycogen as carrier, and PCR amplified to generate probe for the subsequent round of selection. Three rounds of selection were performed with decreasing amounts of protein (200, 100, and 50 ng); 100 ng of the WT1[Δ F4] protein was used in a final fourth round of selection. Products of the final selection were cloned blunt in the *Eco*RV site of pSKII+, and double-stranded plasmid miniprep DNA from individual clones was sequenced by the dideoxy method (52). Only PCR products with correct sequence flanking the four degenerate positions were included in the analysis.

RESULTS

Transcriptional regulation of the IGF-II gene by WT1 splicing variants. We have previously shown that the IGF-II P3 fetal promoter is markedly repressed in transient cotransfection assays by a WT1 expression vector because of the presence of multiple WT1[−KTS] binding sites (19). The present work was undertaken to determine whether the most abundant WT1 zinc finger splicing variant, WT1[+KTS], could also repress IGF-II transcription and, if so, exploit this interaction to define a WT1[+KTS] DNA binding site. Therefore, we tested WT1 expression vectors containing cDNAs encoding different WT1 splice variants for the ability to repress transcription from cotransfected IGF-II promoter reporter plasmids. HepG2 cells were used in these experiments since they express a similar amount and pattern of IGF-II transcripts as

TABLE 1. Repression of the IGF-II gene by WT1 expression vectors^a

Reporter plasmid	Repression by WT1 (mean % of basal activity $\pm$ SE)	
	WT1[−KTS]	WT1[+KTS/17 aa]
pP3[−295,+135]	20.4 $\pm$ 2.6	18.0 $\pm$ 0.1
pP4[−675,+206]	7.8 $\pm$ 1.5	3.8 $\pm$ 0.1

^a HepG2 cells were transiently transfected with 2 μ g of an IGF-II promoter reporter vector, 16 μ g of either WT1 splice variant expression vector, and 2 μ g of a cytomegalovirus- β -galactosidase internal control vector for transfection efficiency. The chloramphenicol acetyltransferase conversion for each reporter vector cotransfected with the pCB6+ expression vector lacking an insert was normalized to 100% (basal activity). Values are means for at least three independent experiments. aa, amino acids.

fetal kidney (19) and because kidney cell lines that express IGF-II are not available. IGF-II P3 promoter activity was markedly repressed by WT1 expression vectors. The results for vectors containing either no insertions or both the 9-nucleotide insertion encoding KTS between the third and fourth zinc fingers and the 51-nucleotide N-terminal insertion are shown in Table 1. In addition, the IGF-II P4 promoter, which is the most active IGF-II fetal promoter in rodents (20), was repressed to an even greater extent by both zinc finger splice variants of the WT1 gene (Table 1). As the amount of expression vector plasmid was increased in the transfection assays, the degree of repression increased to similar extents for the two WT1 expression vectors (data not shown). These results suggest that binding sites for the WT1[+KTS] variant zinc finger exist in both the IGF-II P3 and P4 fetal promoters.

Analysis of WT1 binding sites. Binding of WT1[+KTS] and WT1[−KTS] to the IGF-II P3 promoter, which is the most active fetal IGF-II promoter in human kidney (19), was analyzed with DNase I footprinting assays. As we have previously shown (19), the WT1[−KTS] zinc finger polypeptide bound to several sites in a fragment spanning −295 to −98 of the P3 promoter (Fig. 1A) and predominantly to one site in a fragment spanning −42 to +135 (Fig. 1B). Three of these sites contained a perfect EGR1 consensus sequence, GCG GGGGCG (A1, B1, and E). Previously unrecognized, and possibly lower-affinity, WT1[−KTS] sites (A2, A3, and B2) contained one or several mismatches to the 9-bp site. The A3 site, which showed the least homology to the EGR1 site (Fig. 1C), was the best fit found within the constraints of the protected sequence. The WT1[+KTS] polypeptide, on the other hand, bound well to only a subset of these sites (Fig. 1, sites B1, B2, and E). No binding of WT1[+KTS] was observed to a probe spanning nucleotides −98 to −42 (data not shown). The relatively small B2 footprint was confirmed by gel shift assays. WT1[−KTS] and WT1[+KTS] each formed two complexes with an 86-bp probe fragment spanning the site B1/B2 region (nucleotides −186 to −98; data not shown), indicating the presence of two binding sites (B1 and B2) for each protein. Additional experiments (not shown) have indicated that the DNase I-hypersensitive site at the bottom of the B2 footprint may be occurring within a binding site for WT1. It is significant that a comparison of two sites containing a perfect EGR consensus sequence (A1 and B1; Fig. 1C) revealed differential binding of the WT1 zinc finger proteins. WT1[−KTS] bound to the sites designated A1 and B1 (Fig. 1A), while WT1[+KTS] bound selectively to site B1.

The specificity of the interaction between sequences in the IGF-II promoter and the two WT1 zinc finger splice variants

FIG. 1. DNase I footprinting of the IGF-II P3 promoter. End-labeled restriction fragments of the IGF-II P3 promoter were incubated with increasing amounts of either WT1[+KTS] or WT1[−KTS] protein and treated with DNase. (A) Top strand of 5' P3 promoter sequences. Lane 1, G+A ladder; lane 2, no protein; lanes 3 to 5, WT1[+KTS] protein at 30, 100, and 300 ng, respectively; lane 6, no protein; lanes 7 to 9, WT1[−KTS] protein at 30, 100, and 300 ng, respectively. (B) Top strand of P3 sequences −42 to +135. Lane 10, G+A ladder; lane 11, no protein; lane 12, 1,000 ng of WT1[+KTS]; lane 13, 500 ng of WT1[+KTS]; lane 14, 1,000 ng of WT1[−KTS]; lane 15, 500 ng of WT1[−KTS]. (C) P3 sequences underlying regions of DNase I protection. Boxed sequences are the best fit to previously described WT1/EGR1 recognition elements (see text).

was examined further in gel shift competition assays. Unlabeled double-stranded oligonucleotides were added to binding reactions containing either WT1[−KTS] or WT1[+KTS] and the 200-bp 5' fragment of the IGF-II promoter (identical to the probe used in footprinting assays). Consistent with our footprinting results, WT1[−KTS] formed multiple complexes with this probe, while WT1[+KTS] formed predominantly two complexes (Fig. 2A and B). Addition of an EGR1 binding site oligonucleotide (EBS-1) to the binding reactions resulted in a progressive loss of WT1[−KTS] complexes, while WT1[+KTS] complexes were not significantly affected (Fig. 2A and B). Binding of WT1[−KTS] to the IGF-II promoter was apparently due to the presence of multiple GC

FIG. 2. Competition gel shift assays of WT1 zinc finger splice variants. Probe consisting of sequences -295 to -96 in the IGF-II P3 promoter were incubated with WT1 proteins and increasing amounts of unlabeled competitor oligonucleotides. Leftmost lane, probe alone. (A) WT1[-KTS] protein (250 ng) with 0, 10, 30, 100, and 300 ng of unlabeled EBS-1 oligonucleotide as noted. (B) WT1[+KTS] protein (250 ng) with 0, 10, 30, 100, and 300 ng of unlabeled EBS-1 oligonucleotide as noted. (C) WT1[+KTS] protein (250 ng) with 0, 10, 30, 100, and 300 ng of unlabeled IGF-II P3 E site oligonucleotide as noted.

GGGGGCG/EGR1 sites. However, these 9-bp sequences were not sufficient to account for the observed WT1[+KTS] binding. A competitor oligonucleotide containing the IGF-II P3 E site (Fig. 2C) sequence to the binding reactions did interfere with complex formation by WT1[+KTS] (Fig. 2C),

indicating that the interaction of the WT1[+KTS] protein with the IGF-II P3 probe constitutes specific binding. Taken together with the DNase footprinting data, these results suggested that WT1[-KTS] and WT1[+KTS] could bind overlapping sites and that WT1[+KTS] binding required additional flanking sequences which may be similar in sites B1, B2, and E but absent from site A1 (Fig. 1).

Methylation interference analysis of DNA binding by WT1 splice variants. The identity of the flanking sequence required for WT1[+KTS] binding was sought by using DMS methylation interference assays. Methylated probe containing the sequence at site B1 of the IGF-II P3 promoter (P3 B1) was used in binding assays with both splice variants of the WT1 zinc finger domain (Fig. 3). This site was chosen since it was protected from DNase I cleavage with the lowest amounts of WT1 proteins (Fig. 1) and thus may represent the highest-affinity binding site. Significant differences in the methylation interference patterns for WT1[+KTS] and WT1[-KTS] were found on both strands of this binding site. Binding interference was observed for WT1[-KTS] when either of four of the five central G residues on the top strand of the binding site was methylated (Fig. 3A). On the bottom strand, methylation of only one G residue significantly interfered with binding of WT1[-KTS] (Fig. 3A). On the other hand, WT1[+KTS] binding was impaired when any of six top-strand G residues were methylated (Fig. 3A). Bottom-strand contacts were also more extensive for WT1[+KTS]; methylation of three G residues was found to interfere with binding. Significantly, WT1[+KTS] contacts were found to span a 12-bp binding site

FIG. 3. Methylation interference analysis of DNA binding by WT1 splice variants. Methylation events which interfered with binding are summarized at the bottom of each panel. ■, WT1[-KTS] interference; ●, WT1[+KTS] interference. Open symbols indicate an intermediate level of interference. (A) IGF-II P3 B1 site. Arrowheads indicate methylated guanines which interfered with binding by either WT1[+KTS] or WT1[-KTS] on top or bottom strands of the site. (B) IGF-II P3 B1 site with mutated first three nucleotides (GCG to TTA). (C) WT1 binding site mutated at both the 5' (GCG to ATA) and 3' (GGC to GCA) ends. The results are representative of two or more independent experiments with identical results. U, unbound probe; F, free probe; B, protein-bound probe.

as opposed to a minimum of 6 bp observed here for WT1[−KTS] (Fig. 3A) or a 9-bp site for the related zinc finger protein EGR1 (12). This result suggested that perhaps all four WT1 zinc fingers in WT1[+KTS] were involved in DNA binding and that WT1[−KTS] binding required only three of the four fingers. In light of the more extensive WT1[+KTS] binding site, the observation that similar sequences are present at the 3' end (relative to the G-rich strand) of WT1[+KTS] binding sites B1 and E (GGC and GCC, respectively; Fig. 1C) but not in EBS-1 or site A1 (AGG in both), neither of which binds WT1[+KTS], supports the idea that binding selectivity may be determined by nucleotides 3' to the 9-bp GCGGGGGCG sequence. A clear pattern of interference was observed when bottom-strand G residues at the 3' end of the binding site were methylated in a probe lacking the first three nucleotides (GCG) of binding site B1 or a similarly truncated probe with a modified 3' sequence (Fig. 3B and C). These oligonucleotides were used so as to limit the number of possible protein-nucleotide interactions and make binding more strongly dependent on remaining G residues. Both proteins bound at a lower affinity to these sites (see below). It is noteworthy that unlike the differences observed in binding to the B1 site, the pattern of interference for the two WT1 splice variants was essentially identical on the truncated sites (Fig. 3B and C). This might be expected if the set of WT1 zinc fingers responsible for binding, i.e., fingers 1, 2, and 3, were structurally similar in both proteins. Also, an additional bottom-strand contact was observed for both proteins in one of these truncated sites (Fig. 3B). This observation and the appearance of new contacts for the WT1[−KTS] protein in both truncated sites (Fig. 3B and C) suggests that as G residues which provide a favorable binding interaction are removed from the binding site, other protein-nucleotide interactions become important contributors to binding stability.

Modular WT1 zinc finger-nucleotide triplet interactions. DNA binding by members of the C₂-H₂ class of zinc finger proteins, in particular the EGR1/WT1/Sp1 family of zinc finger proteins, has been characterized in terms of individual zinc finger-nucleotide triplet interactions where amino acids in defined positions in the alpha-helical portion of the zinc finger make characteristic contacts with three nucleotides within a DNA recognition element (16–18, 41–43). Thus, the three-finger protein EGR1 has a 9-bp recognition element (12, 43), and the four-finger protein WT1 might be expected to bind to a 12-bp site. To test whether this paradigm could provide a framework for understanding the WT1 splicing variant-DNA interactions that we have observed, electrophoretic mobility shift assays were performed, allowing WT1 zinc finger proteins to interact with different sets of nucleotide triplets derived from the IGF-II P3 B1 site (GCG GGG GCG GGC, Fig. 4). WT1 zinc finger proteins containing all four fingers (WT1[−KTS] and WT1[+KTS]), three contiguous fingers (WT1[ΔF4] and WT1[ΔF1]), or an in-frame deletion of finger three (WT1[ΔF3]) were incubated with oligonucleotide binding sites containing different groups of nucleotide triplet sequences (Fig. 4). Several important results emerged from this analysis. First, WT1 binding to DNA occurs in an antiparallel orientation relative to the G-rich strand of the binding site, similar to what has been observed for the related zinc finger protein EGR1 (43). A WT1 protein containing zinc fingers 1 to 3 (WT1[ΔF4]) bound best to a probe spanning the 3'-most nucleotide triplets (GGG GCG GGC; Fig. 4B) and failed to bind to a probe spanning the 5'-most set of triplets (GCG GGG GCG; Fig. 4C). The opposite was true for a WT1 protein containing zinc fingers 2 to 4 (WT1[ΔF1]). This protein bound best to probe spanning the 5'-most set of nucleotide

FIG. 4. Nucleotide triplet recognition by alternate sets of the WT1 zinc fingers. Wild-type and various zinc finger deletion proteins (200 ng of each) were incubated with four different probes containing different combinations of nucleotide triplet recognition subsites as indicated above each finger. Leftmost lanes in all panels, no added protein. (A) Binding of WT1 proteins to a 12-bp IGF-II P3 B1 site. (B) Binding of WT1 proteins to a IGF-II P3 B1 site missing triplet I (GCG-to-TTA mutation). (C) Binding of WT1 proteins to a IGF-II P3 B1 site missing triplet IV (GGC-to-TCT mutation). (D) Binding of WT1 proteins to a IGF-II P3 B1 site with triplet II deleted, fusing triplets I, III, and IV.

triplets (GCG GGG GCG; Fig. 4C) and bound poorly to the probe spanning the 3' set of triplets (Fig. 4B). Second, the data in Fig. 4 confirmed that 3' sequences adjacent to the previously defined WT1[−KTS] 9-bp GCG GGG GCG site are important determinants of WT1[+KTS] binding (compare Fig. 4A and C) and most likely represent a nucleotide recognition triplet for WT1 zinc finger 1. Finally, DNA binding by a protein with an in-frame deletion of finger 3 (WT1[ΔF3]) displayed an entirely new binding specificity (Fig. 4D; see below).

The interaction of WT1[−KTS] and WT1[+KTS] with different binding sites was characterized further by determining their relative equilibrium dissociation constants as described in Materials and Methods. The K_d s that we measured are comparable to K_d s reported for the interaction of structurally similar zinc finger polypeptides with DNA under roughly

TABLE 2. Equilibrium dissociation constants for WT1 zinc finger variants^a

Protein	K_d (M)		
	5'-GCG GGG GCG GGC-3'	5'-TTA GGG GCG GGC-3'	5'-GCG GGG GCG TCT-3'
WT1[−KTS]	1.4×10^{-7}	1.1×10^{-5}	2.2×10^{-7}
WT1[+KTS]	2.0×10^{-6}	7.7×10^{-6}	5.8×10^{-6}

^a A fixed amount of labeled DNA probe was incubated with increasing amounts of WT1 zinc finger proteins with or without the KTS splicing insertion (WT1[−KTS] and WT1[+KTS]), and the K_d was calculated over a range of protein concentrations as described in Materials and Methods. Quantitation of gel shift assays yielded equilibrium binding isotherms, demonstrating that the relative binding affinity for different probe-protein combinations was maintained at various protein concentrations. Three different probes which bound (Fig. 4) to WT1 zinc finger splicing variants were tested, and a relative measure of binding affinities was derived.

similar binding conditions (18). Highest-affinity binding was observed to occur between WT1[−KTS] and the binding site containing all four nucleotide triplets (Table 2). A small but consistent reduction in WT1[−KTS] affinity was observed at all protein concentrations when the 3′ end of the binding site was mutated, disrupting putative contacts with zinc finger 1. Disrupting DNA-protein interactions at the 5′ end of the binding site, presumably involving zinc finger 4, was significantly more deleterious, lowering the overall WT1[−KTS] binding affinity by 2 orders of magnitude. WT1[+KTS] bound at highest affinity to the complete 12-bp binding site, although this affinity was roughly 10-fold lower than that observed for WT1[−KTS]. Mutating DNA contacts for finger 1 or 4 had significant effects on WT1[+KTS] binding, lowering the affinity three- to fourfold. Consistent with the fact that WT1[−KTS] and WT1[+KTS] have the same primary amino acid sequence for zinc fingers 1, 2, and 3, these two proteins bound with roughly equivalent affinity to a site containing nucleotide contacts known to interact with this set of zinc fingers (Table 2; Fig. 4B).

A novel recognition element for a tumor-associated, WT1 zinc finger deletion mutant. A variety of naturally occurring WT1 deletion mutants have been isolated from Wilms' tumor patients, and in most cases, both copies of the WT1 gene are mutated in tumor DNA, consistent with the designation of WT1 as a recessive, tumor suppressor gene (1, 2, 14, 15, 27, 28, 30, 38, 45). A particularly interesting mutation consisting of an in-frame deletion of the third WT1 zinc finger has been isolated and shown to be heterozygous in tumor tissue, suggesting that this particular mutation may act in a dominant fashion (28, 30). To gain insight into a potentially novel mechanism of tumorigenesis and to further demonstrate modular WT1 zinc finger binding specificity, an in-frame zinc finger 3 deletion protein (WT1[ΔF3]) was tested for binding to a site containing juxtaposed nucleotide triplets GCG GCG GGC, which, assuming an antiparallel DNA-WT1 interaction, would be expected to interact with zinc fingers 1, 2, and 4. This probe interacted specifically with WT1[ΔF3]; no other form of the WT1 protein bound to this site (Fig. 4D). WT1[ΔF3] also bound to other probes containing the sequence GCG GGG GCG (Fig. 4A and C) which were found to bind WT1[−KTS] and WT1[ΔF1]. These results suggest that the WT1[ΔF3] protein may recognize an expanded spectrum of target genes and, in addition, may interact with WT1 binding binding sites in physiologically relevant genes. WT1[ΔF3] did not bind a probe containing the sequence GGG GCG GGC (Fig. 4B), indicating that certain base substitutions could be tolerated (a C to G in the middle or 3′-most triplet), while others (a C to G in the 5′-most triplet) were not.

Binding site selection for the WT1 finger 1 recognition subsite. To define the interaction of WT1 zinc finger 1 with DNA, we have taken advantage of newly discovered WT1[+KTS] binding sites in the IGF-II P3 promoter. These sites contain either GGC (site B1) or GCC (site E) as the first finger recognition subsite. To further define the optimal triplet sequence for finger 1 binding, a low-degeneracy oligonucleotide containing the sequence GCG GGG GCG NNNN flanked by PCR primer sequence was used in a reiterative binding site selection assay. The oligonucleotide sequence was designed such that the presence of recognition triplets for fingers 2, 3, and 4 would direct an interaction between finger 1 and the degenerate portion of the binding site. Also, since at least three zinc fingers appeared to be necessary for significant binding under our conditions (Fig. 4), and since previous selection assays using the WT1[−KTS] or WT1[+KTS] protein did not yield a clear result (unpublished observations), the WT1[ΔF4] protein (containing fingers 1 to 3) was used to

FIG. 5. Binding site selection for the WT1 finger one recognition subsite. A low-degeneracy probe with the central sequence GCG GGG GCG NNNN was subjected to four rounds of binding and selection with WT1[ΔF4], after which 33 cloned binding sites were analyzed for their sequence in the starting degenerate positions (numbered 10 to 13 relative to the fixed portion of the binding site, positions 1 to 9). (A) Frequency of individual nucleotides at the designated starting degenerate positions, i.e., the number of times each nucleotide was observed at the position. (B) Gel shift analysis of an individual cloned binding site. A probe containing the GTGG sequence at positions 10 to 13 was incubated with or without 200 ng of WT1[+KTS] protein and analyzed on a polyacrylamide gel. Probe NNNN is the PCR product of a starting 61-bp degenerate probe mixture.

provide maximum discrimination among potential binding sites. Following four rounds of binding selection, individual PCR products were cloned and sequenced. Sequences were aligned by the nonvariant portion of the binding site. The frequencies of the four possible nucleotides at the starting degenerate positions (10 to 13) are shown in Fig. 5A. A clear preference emerged for GTG at nucleotide positions 10, 11, and 12, while little discrimination was observed at position 13. Triplets of the sequence GGC and GCC, similar to sites IGF-II B1 and E, were also selected in this experiment although at lower frequency than GTG. Gel shift assays using a probe prepared from an individual selected clone confirmed that a GTGG sequence in this position significantly enhanced WT1[+KTS] binding (Fig. 5B). WT1[+KTS] binding to this site was comparable to binding to the IGF-II E site (data not shown). The selected sites share two important features with the WT1[+KTS] bindings sites derived from the IGF-II P3 promoter. First, a G residue is invariantly present at position 10, and second, bottom-strand purines (A or G) are present at position 11 or 12 (or both) in the sites with the highest affinity. As noted above, methylation of G residues at these bottom-strand positions was found to interfere with WT1[+KTS] binding. In long exposures of DMS methylation experiments on a binding site containing a GTG finger 1 triplet, binding interference was observed when bottom-strand A residues were methylated (data not shown). In previous work, we have shown that the B1 site in the IGF-II P3 promoter plays an important role in repression by WT1 and is a highly conserved

sequence element among different species (19). In mice and humans, the sequences are identical (26, 50), while in the rat gene, the GGC finger 1 recognition triplet is changed to GTC (53). Our binding site selection results suggest that this is a conservative change and that for each of these species, the B1 site represents a recognition element for both splicing variants of the WT1 zinc finger domain.

DISCUSSION

The existence of multiple mRNA splicing variants of the Wilms' tumor suppressor gene *WT1*, and in particular, splicing variants of the zinc finger DNA binding domain, has suggested that multiple WT1 proteins might serve different functions during development or in the control of cell proliferation (29). The interaction of one WT1 zinc finger splicing variant, WT1[−KTS], with the EGR1 recognition element has facilitated studies of its transcriptional regulatory functions and allowed the identification of several *WT1* target genes which are repressed during kidney development or overexpressed in Wilms' tumors (19, 23, 39, 40, 48, 56, 58). However, the major WT1 zinc finger splicing variant, WT1[+KTS], has been less well characterized, leaving open the question of whether it also can function as a transcriptional regulator and, if so, what target genes it may regulate. Our work here describing the interaction of WT1[+KTS] with the P3 IGF-II promoter demonstrates that WT1[+KTS] binds specifically to a 12-bp recognition element and can act as a transcriptional repressor. We have recently shown that the WT1 promoter itself is also subject to regulation by WT1[+KTS] owing to the presence of similar 12-bp binding sites (51). The presence of multiple binding sites for both WT1 splicing variants in the IGF-II P3 promoter and the finding that reporter constructs of the IGF-II P4 promoter are also repressed by both WT1 variants significantly strengthens the hypothesis that WT1 plays a major role in the repression of the IGF-II gene during nephrogenesis (19). Although further studies will be required to demonstrate that the endogenous IGF-II gene is repressed by WT1 *in vivo*, this analysis of DNA binding *in vitro* has provided important insights into how the most abundant form of the WT1 zinc finger interacts with DNA.

Binding site discrimination by WT1 zinc finger splicing variants. Differential splicing of transcription factor mRNAs has been shown in several instances to alter the binding specificity or function of the variant proteins (5, 7, 25, 31, 35, 49, 55). Our results demonstrate that alternative splicing of the WT1 zinc finger domain results in proteins have altered binding specificities. Initially we hypothesized that the insertion of three amino acids in the H-C link between WT1 fingers 3 and 4 would alter the relative position of nucleotides in the recognition subsites for these two zinc fingers, perhaps creating a corresponding gap in the WT1[+KTS] DNA binding site. Our DMS interference data show that this is clearly not the case, since the methylation of nucleotides in the recognition sequence for fingers 4 and 3 (GCG and GGG, respectively) interfered with WT1[+KTS] binding to an equal or greater degree than with WT1[−KTS] binding. Our working hypothesis is that finger 4 is not displaced from the binding site but rather that its alignment to DNA may be altered by the KTS insertion, reducing the stability of nucleotide-amino acid hydrogen bonds. The affinity of WT1[+KTS] for a binding site with contacts for zinc fingers 4, 3, and 2 (GCG, GGG, GCG, respectively/an EGR1 site) is correspondingly reduced (Fig. 4C; Table 2) relative to WT1[−KTS]. Furthermore we suggest that the loss in binding energy may be made up by additional protein-DNA contacts and that this requirement for additional

contacts is reflected in an optimal 12-bp WT1[+KTS] binding site involving all four WT1 zinc fingers. The end result in this case is an increase in specificity whereby WT1[+KTS] binds with highest affinity to a subset of WT1[−KTS] binding sites which have the correct nucleotides in contact positions for finger 1. The amounts of the different *WT1* mRNAs have been found to be relatively invariant during development and between species (29). At present it is unclear why the more highly sequence specific transcriptional regulator WT1[+KTS] should be consistently coexpressed with the potentially more broadly active splicing variant WT1[−KTS]. It is possible that in addition to affecting DNA binding, the KTS insertion in the DNA binding domain serves other functions, perhaps in directing protein-protein interactions on certain promoters. Our results are consistent with binding site selection assays reported by Bickmore et al. (5), in which genomic DNA fragments purified on the basis of binding to WT1[+KTS] also bound WT1[−KTS] while the reverse was not observed. It was also recently reported that a novel TCC element could function as a WT1[−KTS] binding site (57). The G-rich strand of this binding site contains the sequence GAG GAG GAG GAG, which presents G residues at five of the six critical contact positions that we have determined for WT1[−KTS] binding and therefore may interact with WT1[−KTS] in a fashion similar to what we have described. However, in related work, we have found functional WT1 binding sites with GAG motifs in the promoter of the *WT1* gene which exhibit a slightly different pattern of methylation interference (51), implying a certain amount of flexibility in the WT1-DNA interaction.

Alternate binding site recognition by subsets of the WT1 zinc fingers. Experiments using truncated binding sites revealed that the WT1 proteins could bind to different recognition elements by employing different subsets of three zinc fingers, although these interactions were of lower affinity than DNA binding by all four WT1 zinc fingers. Similar observations have been made by others using a chimeric, four-zinc-finger protein derived from the PRDI-BP1 and PRDII-BP1 proteins and also in studies of nucleic acid binding by the original member of the C₂-H₂ zinc finger protein family, TFIIIA (13, 35). In the case of TFIIIA, different sets of zinc fingers have been found to be responsible for DNA versus RNA binding (13). Our results imply that WT1, using overlapping sets of three zinc fingers, may regulate a broader spectrum of genes than previously thought. The presence of either of two 9-bp recognition elements in gene promoter regions may confer regulation by WT1. It is interesting that the recognition element for WT1 fingers 1, 2, and 3 (GGGGCGGGC) is very similar to an Sp1 recognition element (GGGGCGGGGC) (34). This observation is in line with the fact that the primary amino acid sequence for WT1 fingers 1, 2, and 3 is similar to the amino acid sequence of the three Sp1 fingers (33), particularly in the positions of putative DNA-contacting amino acids (see below). It may be that in addition to antagonizing the activity of the EGR transcriptional activators (39), WT1 also interacts with genes dependent on Sp1 activity.

The common structural features of zinc finger proteins which have emerged from X-ray crystallographic studies of zif/268 (EGR1) and the more distantly related C₂-H₂ zinc finger proteins Tramtrack and GLI are that (i) zinc fingers bind in the major groove of DNA, with successive fingers wrapping around the double helix, and (ii) critical nucleotide contacts are most commonly made by defined amino acid positions in the alpha-helical portion of the finger (21, 43, 44). Our observations fit within the paradigm that for WT1 and its close relatives EGR1 and Sp1, each zinc finger can function in a modular fashion, recognizing its own nucleotide triplet recog-

nition sequence. X-ray crystallographic and site-specific mutagenesis studies of DNA recognition by EGR1 and Krox 20 (EGR2) have focused attention on the positions of three critical DNA-contacting amino acids in the zinc finger (41–43). On the basis of this work and the frequency of amino acids at analogous positions in a variety of zinc fingers, synthetic zinc finger structures with predictable DNA binding sites have been designed (16–18). The amino acids occupying the putative DNA contact positions in the four WT1 zinc fingers in the carboxyl-to-amino direction are RER THR RQR MHK (zinc fingers 4 to 1) (10). The fact that the amino acids in contact positions in EGR1, RER THR RER (zinc fingers 3, 2, and 1 [54]), are identical to the analogous amino acids in WT1 fingers 4, 3, and 2 (with the exception of a glutamine-for-glutamic acid substitution) most likely accounts for the binding of a WT1 zinc finger protein containing fingers 2, 3, and 4 (WT1[ΔF1]) to the EGR1 recognition element. The amino acid contacts in WT1 fingers 3 to 1, THR RQR MHK, more closely resemble the fingers of Sp1, KHR RER AHK (33), and, correspondingly, bind a similar recognition element. The similarity to Sp1 extends further to the observation that similar bottom-strand G contacts are made by the first zinc finger of WT1 and presumably the first finger of Sp1 (32, 59). In our binding site selection assays, we observed that some degree of sequence diversity could be tolerated at the WT1 finger 1 recognition triplet. A relatively high degree of sequence diversity is also observed at the 3' end of the Sp1 recognition element (finger 1 contacts), leading to the suggestion that a greater degree of conformational flexibility for amino acid side chains in an AHK versus RER zinc finger may tolerate a greater range of nucleotide contacts and contact positions (37). It is also relevant that in a systematic analysis of zinc finger amino acid-nucleotide contacts, histidine has been observed to interact favorably with adenine residues (17). The triplets GTG and GTC, which we selected as WT1 finger 1 contacts, have an adenine in the bottom strand at what may be an appropriate position for interacting with the histidine of WT1 finger 1. Methylation of this adenine was also observed to interfere with WT1[+KTS] binding. A question remains as to why a guanine residue in the first position of a finger 1 triplet for both Sp1 and WT1 should be so highly conserved (34) when our results show that methylation of this residue did not interfere with WT1 binding and the predicted contact amino acids (alanine and methionine for Sp1 and WT1, respectively) are not known to have stable interactions with this base. This finding reveals a limitation of the current attempts to predict DNA binding sites from knowledge of zinc finger primary amino acid sequence alone.

A ΔF3 WT1 mutant with altered DNA recognition. Our observation that a ΔF3 WT1 protein with fingers 1, 2, and 4 juxtaposed was the only WT1 protein to bind to a recognition element consisting of predicted corresponding nucleotide triplet subsites illustrates the potential for aberrant gene regulation when zinc fingers are assembled in an unintended order. New DNA binding specificities can emerge and potentially result in regulation of a new spectrum of target genes. A similar alteration of binding specificity by differential splicing of zinc fingers has been documented for two *Drosophila* zinc finger proteins, the chorion transcription factor CP2 and the Tramtrack zinc finger protein (25, 31, 49). The proto-oncogene *Evi-1* is also subject to alternative splicing in its zinc finger domain, although the effect on DNA binding is not known (7). The existence of WT1[ΔF3] as a heterozygous mutation in Wilms' tumor tissue and its ability to cooperate with the E1A gene in malignant transformation of cultured kidney cells has led to the proposition that the WT1[ΔF3] mutation may act as

a dominant-negative mutation (28, 30). Whether or not the altered DNA binding specificity of WT1[ΔF3] contributes to tumorigenesis could potentially be examined in stable transformants expressing the WT1[ΔF3] protein.

Although previous structural data and mutagenesis studies of closely related zinc fingers provided a useful framework for analyzing WT1 DNA binding, it should be noted that neither our data nor the X-ray crystallographic structural determinations of three different C₂-H₂ zinc finger proteins (21, 43, 44) allow predictions of (i) how many amino acid-nucleotide contacts will be made by a single zinc finger, (ii) which strand of the double helix will be contacted by amino acid side chains, (iii) what the optimal spacing of nucleotide subsites for each zinc finger will be, or (iv) what negative amino acid-nucleotide interactions may occur and predominate over an otherwise optimal array of DNA-protein contacts. For these reasons, we believe that no generalizable code for DNA recognition by zinc finger proteins exists except perhaps within very closely related families of proteins.

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