Differential Function of Wilms’ Tumor Gene WT1 Splice Isoforms in Transcriptional Regulation*

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The Wilms’ tumor gene WT1, encodes a zinc finger transcription factor that can repress transcription of a number of genes. WT1 mRNA undergoes alternative splicing at two locations, yielding four different mRNA species and protein products. One alternative splice alters the zinc finger region of WT1, resulting in the addition of three amino acids, Lys-Thr-Ser (KTS), between zinc fingers 3 and 4, altering the binding of WT1 to DNA. Here, we show that the WT1 protein with and without the KTS tripeptide can repress transcription from the human full-length WT1 promoter. Repression of transcription by WT1 has been shown to require two WT1 binding sites. We examined WT1 repression of the human minimal WT1 promoter, which contains two potential WT1 binding motifs. WT1 lacking the KTS tripeptide (WT1–KTS) was unable to repress transcription from a minimal WT1 promoter of 304 base pairs, whereas WT1 containing the KTS tripeptide (WT1+KTS) repressed transcription from the minimal promoter. The ability of WT1+KTS to repress transcription where WT1–KTS could not provide a functional assay to define differential WT1 binding motifs based on the presence or the absence of the KTS tripeptides. We present data defining the differential consensus DNA binding motifs for WT1–KTS and WT1+KTS. We demonstrate that WT1 zinc finger 1 plays a role in the differential DNA binding specificity of WT1–KTS and WT1+KTS.

The human Wilms’ tumor gene WT1 is located at 11p13 and encodes a zinc finger transcription factor that functions as a tumor suppressor (Call et al., 1990; Gessler et al., 1990). WT1 has a proline-rich amino terminus that mediates transcriptional repression (Madden et al., 1993) and four Cys-Cys His-His type zinc fingers in the carboxyl terminus that bind DNA (Call et al., 1990; Rauscher et al., 1990). WT1 mRNA undergoes alternative splicing at two sites, resulting in four mRNA species and protein products (Haber et al., 1991; Telemann et al., 1992). The mRNA splice isoforms occur in fixed ratios that are constant in all tissues that express WT1 during development and are conserved between species (Haber et al., 1991). One alternative splice in the WT1 mRNA involves the presence or the absence of exon 5, which is 51 nucleotides long, encodes 17 amino acids (Haber et al., 1991), and has no known homology to sequences in GenBank. Exon 5 is located between the proline-rich amino terminus and the zinc fingers and has no known function.

The second site of alternative splicing is the terminal 9 nucleotides of exon 9, which encodes the tripeptide KTS1 (Haber et al., 1991). This tripeptide is inserted between exons 9 and 10, which encode zinc fingers 3 and 4. The WT1 isoform without the KTS tripeptide (WT1–KTS) represents 23% of the mRNA. WT1–KTS binds a DNA motif similar to the early growth response 1 gene (EGR-1) (Rauscher et al., 1990), the closest homologue to the zinc finger region of WT1 (Call et al., 1990). The WT1 isoform with the KTS tripeptide (WT1+KTS) represents the majority of WT1 transcripts and binds DNA motifs similar to those the WT1–KTS isoform binds, but no consensus motif has been defined (Bickmore et al., 1992; Drummond et al., 1994). WT1–KTS represses transcription from the promoters of the EGR-1 (Madden et al., 1991), platelet-derived growth factor a-chain (Gashler et al., 1992), insulin-like growth factor II (Drummond et al., 1992), transforming growth factor β1 (Dey et al., 1994), colony-stimulating factor 1 (Harrington et al., 1993), retinoic acid receptor-α (Goodyer et al., 1995), PAX2 (Ryan et al., 1995) and the insulin-like growth factor 1 receptor (Werner et al., 1994). WT1+KTS represses the insulin-like growth factor II promoter (Drummond et al., 1994). Recently WT1–KTS and WT1+KTS were shown to autorepress the murine WT1 promoter (Rupprecht et al., 1994), and stable transfectants expressing WT1 show reduction in transcription from the human WT1 promoter (Malik et al., 1994).

Our laboratory has previously characterized the human WT1 promoter (Fraizer et al., 1994). The WT1 promoter is GC-rich and has no CAAT or TATA boxes. Transcription initiates at multiple sites across the 652-bp promoter region (Fig. 1) (Campbell et al., 1994; Fraizer et al., 1994; Hofmann et al., 1993; Phelan et al., 1994). The promoter contains 11 Sp1 binding sites that are believed to be critical in initiating transcription (Hofmann et al., 1993). In addition, the human WT1 promoter contains 10 potential WT1 motifs of the consensus sequence GNGNGGGNG (Fraizer et al., 1994).

Here we report the autoregulation of human WT1, demonstrating that both WT1–KTS and WT1+KTS can repress transcription from the human full-length WT1 promoter. We determined that WT1+KTS but not WT1–KTS could repress transcription from the WT1 minimal promoter.

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†The abbreviations used are: KTS, Lys-Thr-Ser; WT1–KTS, WT1 isoform without the KTS tripeptide; WT1+KTS, WT1 isoform with the KTS tripeptide; EGR-1, early growth response 1 gene; bp, base pair(s); CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay(s).

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transcription from a minimal 104-bp WT1 promoter construct that has half the transcriptional activity of the full-length WT1 promoter. Using the repression of the minimal WT1 promoter as a functional assay of DNA binding, we found that WT1-KTS and WT1-KTS bound similar but not the same sequences in the WT1 promoter. We show that the WT1-KTS and WT1-KTS binding sites differed in the sequences recognized by zinc finger 1. Using the refined WT1 consensus DNA binding motif, we have demonstrated that WT1 represses transcription of the proto-oncogenes c-myc and bcl-2 (Hewitt et al., 1995b).

Experimental Procedures

Cell Culture and Chloramphenical Acetyltransferase Assays—The WT1 promoter constructs containing the 652- and 104-bp promoter fragments have been previously described by Fraizer et al. (1994), and the WT1 cDNA expression plasmids were described by Madden et al. (1991). Transfection experiments were performed in HeLa cells (ATCC CCL2), a cell line derived from a human cervical carcinoma, as described previously (Hewitt et al., 1995a), using 3.75 µg of WT1 promoter CAT reporter, plus 0–10 µg of expression plasmid containing the WT1 cDNA (Madden et al., 1991), and enough pCB6 vector to bring the total amount of DNA to 10 µg. 1.25 µg of CMVβgal (Clontech, Palo Alto, CA) was transfected as an internal control. The CAT assays were performed according to standard methods, and the results were normalized with β-galactosidase activity (Fraizer et al., 1994). The results of all the assays are the average of three experiments.

Electrophoretic Mobility Shift Assays—EMSAs were performed with a protocol modified from Cao et al. (1990). The reaction volume of 10 µl contained a final concentration of 20 mM HEPES, pH 7.5, 70 mM KCl, 5 mM MgCl₂, 100 mM ZnCl₂, 0.5 mM dithiothreitol, 0.05% Nonidet P-40, 2% glycerol, 10 µM 32P-labeled DNA probe, 500 ng of WT1 protein, 1 µg of poly(dI-dC), and 0–150 pmol competitor oligonucleotide. The reaction products were separated on a 4% TBE gel at 4°C or room temperature for 20 min, and the products were electrophoresed on a 5% acrylamide 0.5 x TBE gel at 4°C or room temperature. The minimal promoter region used as a DNA probe in the EMSAs was amplified by polymerase chain reaction with primers previously described (Fraizer et al., 1994). The minimal promoter was labeled by incorporation of [α-32P]dCTP into the amplified fragment during polymerase chain reaction amplification. The double-stranded oligonucleotides used in EMSA are EGR (GGCCCAGCGGGGGGAGGCGC), and WT1-309 (GGCGACCTCCGAGGGCCGCCC). The oligonucleotide probes were labeled with [α-32P]dCTP by using the Klenow fragment to fill in a G overhang. The WT1-KTS and WT1+KTS peptides were previously described by Rauscher et al. (1990). Gels were imaged on a Molecular Dynamics PhosphorImager.

Results

Transcriptional Autorepression of the WT1 Promoter—To determine if WT1 was capable of autorepression of the WT1 promoter, we co-transfected CMV-WT1 cDNA expression plasmids with human WT1 promoter CAT reporter constructs into HeLa cells. We used HeLa cells because they do not express endogenous WT1, but the WT1 promoter does function in this cell line (Fraizer et al., 1994). We used two WT1 promoter reporter doses (Fig. 1). One reporter construct contained the full-length 652-bp WT1 promoter, containing 10 WT1 binding motifs of the consensus sequences GNNGGCGG. The second reporter construct contained only a central promoter region of 104 bp (Fig. 1). This 104-bp minimal promoter region has half the reporter activity of the full-length promoter (Fraizer et al., 1994) and contains two WT1 binding sites of the consensus sequence GNNGGCGG.

Transfection of 10 µg of WT1-KTS cDNA expression plasmid with the full-length WT1 promoter reporter resulted in 65% transcriptional repression (Fig. 2 and 3A). Transfection of WT1-KTS cDNA expression plasmid with the minimal WT1 promoter reporter construct did not repress transcription (Fig. 2). The WT1+KTS cDNA expression vector repressed transcription from both the full-length and the minimal WT1 promoter reporters an average of 47% (Fig. 2 and 3B). WT1+KTS repression of the minimal WT1 promoter suggested that
relative to the 5' end of the full-length WT1 promoter (Fraizer et al., 1994) is the sequence ACCCCGCCCTCACCCCC, which contains the reverse complement of the WT1 consensus binding motif GNGNGGGNG. At position 309 is the sequence GCTGCAGGCGCCC, which also contains the WT1 consensus binding motif. In an EMSA, WT1-KTS was able to bind oligonucleotide WT1-290, which contains the 290 sequence but failed to bind oligonucleotide WT1-309, which contains the 309 sequence (Fig. 5A). The binding of WT1-KTS to WT1-290 was competed with both unlabeled WT1-290 and EGR oligonucleotides (Fig. 5A). The EGR oligonucleotide was a stronger competitor than the WT1-290 oligonucleotide for binding to the WT1-KTS, possibly due to the presence of three potential binding sites within the EGR oligonucleotide. In an EMSA, WT1-KTS formed binding complexes with both oligonucleotides WT1-290 and WT1-309 (Fig. 5, B and C). The specificity of binding was shown by competition with the unlabeled EGR oligonucleotide.

**DISCUSSION**

WT1 can repress the transcription of a number of genes (Werner et al., 1994, Goodyer et al., 1995; Harrington et al., 1993; Dey et al., 1994; Drummond et al., 1992; Madden et al., 1991; Ryan et al. 1995; Hewitt et al. 1995b). Here we demonstrate that WT1 autoregulates the human WT1 promoter, in agreement with the data demonstrating repression of the murine WT1 promoter (Rupprecht et al., 1994). In the study reported here, WT1-KTS repressed transcription of the full-length human WT1 promoter 65% and WT1+KTS repressed transcription approximately 40%. In the process of studying the autoregulation of WT1, we determined that WT1+KTS but not WT1-KTS could repress transcription.
from the minimal promoter region. Drummond et al. (1992) had previously demonstrated that two WT1 binding sites were required for transcriptional repression by WT1. This suggested that the minimal promoter region contains WT1 binding motifs with different specificity and provided a functional assay to determine whether WT1–KTS binds DNA sequences not recognized by WT1–KTS. We found that WT1–KTS bound one site within the 104-bp minimal promoter region, whereas WT1–KTS bound two.

We propose that there are differences in the DNA binding motifs recognized by the WT1 splice isoforms WT1–KTS and WT1–KTS. Comparison of our EMSA results with the consensus motif GNGGNGNGNG does not explain the differences in WT1–KTS and WT1–KTS binding. The consensus motif GNGGNGNGNG was determined by x-ray crystallography studies of Zif268 (mouse EGR-1) (Paveletich and Pabo, 1991). This motif is the DNA binding site of the three zinc fingers of Zif268, which are only 64% identical to three of the four WT1 zinc fingers. Examination of the flanking sequences of the oligonucleotides used in EMSA leads us to propose a new DNA consensus motif that discriminates between WT1–KTS and WT1–KTS binding. We propose that WT1–KTS binds the motif GNGGNGGNG and that WT1–KTS binds GNGGNGNS, where S = G or C. The difference between these two motifs is the 3’ base; WT1–KTS recognizes only G, whereas WT1–KTS recognizes C or G. This motif is present twice in the EGR competitor oligonucleotide used in the EMSA (Table I) and makes the EGR oligonucleotide a stronger competitor in EMSA. This expanded DNA binding motif adds two nucleotides to account for binding of zinc finger 1. Zinc fingers bind DNA as triplets (Paveletich and Pabo, 1991), but the DNA specificity of zinc finger 1 appears to be determined only by the central base of the 3’ triplet, resulting in an 11-bp consensus sequence. The WT1–KTS and WT1–KTS DNA binding motifs differ in the bases recognized by zinc finger 1, not zinc fingers 3 or 4. This unexpected result is probably due to alterations in protein-DNA contact introduced by the insertion of KTS; crystallographic analysis may be required to elucidate the nature of WT1-DNA binding. The spacing between fingers is highly conserved among zinc finger transcription factors; WT1 is the only one described to have alternative splicing of amino acid residues between the zinc fingers.

Analysis of the WT1 promoter region for these new WT1 DNA binding consensus sequences showed that there were six potential WT1–KTS binding sites and eight potential WT1–KTS binding sites (Table II). Review of other previously identified WT1 binding motifs confirmed the presence of these 11-bp expanded WT1 binding motifs in the insulin-like growth factor II, platelet-derived growth factor α-chain, retinoic acid receptor-α, and murine WT1 promoters (Drummond et al., 1992; Gascher, et al., 1992; Goodyer et al., 1995; Rupprecht et al., 1994). We identified two additional target genes, c-myc and bcl-2, which contain this 11-bp WT1 binding motif and are repressed by WT1–KTS and WT1–KTS (Hewitt et al., 1995b).

Our WT1–KTS DNA binding motif differs from that described by Nakagama et al. (1995). However, we used a different assay than Nakagama et al. (1995) to define the WT1–KTS and WT1–KTS binding motifs. We believe that our functional assay is more sensitive, because it is based on in vivo binding whereas Nakagama et al. (1995) used only in vitro methods. EMSA conditions play a key role in the formation of DNA-protein complexes and do not necessarily mimic in vivo conditions. Our data also differ from those of Wang et al. (1993) in that the binding of WT1–KTS to only one WT1 binding motif did not activate transcription of the WT1 promoter, whereas WT1–KTS neither activated nor repressed transcription from the WT1 minimal promoter. This discrepancy may be due to the type of promoter being assayed. The GC-rich WT1 promoter has no TATA box to direct transcriptional initiation from a single site. It should also be noted that other investigators examining TATA-less, GC-rich promoters (Drummond et al., 1992) have also failed to find transcriptional modulation by WT1 when only one WT1 binding site was present.

It has been argued that WT1 binding sites are required both upstream and downstream of the transcriptional start site for transcriptional repression by WT1 (Drummond et al., 1992). Our data do not support this hypothesis; both WT1–KTS binding sites in the minimal promoter were upstream of the major transcriptional start site in the minimal promoter (Fig. 1). In both the EGR1 and transforming growth factor β promoters (Madden et al., 1991; Dey et al., 1994), the only WT1 binding sites are upstream of the transcriptional start site. These promoters are also repressed by WT1–KTS. It appears that multiple WT1 binding motifs are required for transcriptional repression by WT1 but that the multiple sites can be upstream of the transcriptional start site.

It is not clear how WT1 functions as a transcriptional repressor, although experiments by Madden et al. (1993) have demonstrated that the proline-rich exon 1 can mediate repression when fused to Gal4. It appears that two WT1 proteins are required to repress transcription, possibly by interfering with recruitment and/or stabilizing the formation of the initiation complex.

We defined differential consensus DNA binding motifs for WT1 based on a functional assay that can discriminate between WT1–KTS and WT1–KTS binding. By identifying different DNA binding motifs for different splice isoforms of WT1, we have increased the number of potential targets of WT1. Use of multiple WT1 binding sites within a promoter as well as the presence of both WT1 splice isoforms may allow for fine control of transcriptional regulation. Given that WT1–KTS is the predominant splice isoform and binds to sites not recognized by WT1–KTS, identification of the target genes of WT1–KTS may reveal how WT1 regulates cell growth and differentiation.
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