ABSTRACT

The importance of ornithine decarboxylase (ODC) to cell proliferation is underscored by the complex array of cell-specific mechanisms invoked to regulate its synthesis and activity. Misregulation of ODC has severe negative consequences on normal cell function, including the acquisition of tumorigenic growth properties by cells overexpressing ODC. We hypothesize that ODC gene expression is a candidate target for the anti-proliferative function of certain tumor suppressors. Here we show that the Wilms’ tumor suppressor WT1 binds to multiple sites within the human ODC promoter, as determined by DNase I protection and methylation interference assays. The expression of WT1 in transfected HCT 116, NIH/3T3 and HepG2 cells represses activity of the ODC promoter controlling expression of a luciferase reporter gene. In contrast, WT1 expression enhances ODC promoter activity in SV40-transfected HepG2 cells. Both the extent of modulation of ODC gene expression and the mediating WT1 binding elements are cell specific. Constructs expressing WT1 deletion mutants implicate two regions required for repressor function, as well as an intrinsic activation domain. Understanding the regulation of ODC gene expression by WT1 may provide valuable insights into the roles of both WT1 and ODC in development and tumorigenesis.

INTRODUCTION

Ornithine decarboxylase (ODC, EC 4.1.1.17), a key regulatory enzyme in polyamine biosynthesis, is exquisitely responsive to a wide range of mitogenic agents, including growth factors, hormones, regenerative stimuli and tumor promoters (reviewed in 1–3). This proliferation-associated enzyme is subject to a complex array of cell-specific regulatory mechanisms that govern transcription, mRNA stability and translation and enzyme turnover (4–7). Misregulation of ODC expression has severe negative consequences on cell growth and differentiation. The disruption of ODC function by pharmacological agents or mutagenesis results in arrest of cell proliferation (8,9) and aberrant embryonic development (10). Conversely, ODC overexpression is intimately associated with cell transformation and carcinogenesis. Levels of ODC mRNA and enzyme activity are constitutively elevated in transformed cell lines and virtually all animal tumors (11,12). The induction of ODC is also essential for tumor promotion in a variety of experimental models of skin, breast and colon carcinogenesis (13). Lastly, it has been recently demonstrated that overexpression of human ODC confers transformation-related growth advantages, such as loss of contact inhibition, anchorage-independent growth and increased tumorigenicity, on ODC-transfected NIH/3T3 cells (14–16). Since ODC overexpression disrupts growth control, cells must be able to down-regulate ODC gene expression for normal differentiation and development to occur. The mechanisms responsible for ODC repression are not known, but we hypothesize that ODC gene expression is a candidate target for the anti-proliferative function of at least some types of tumor suppressor proteins.

One such tumor suppressor, WT1, has been implicated in the etiology of Wilms’ nephroblastoma, an embryonic renal malignancy (17,18). Wilms’ tumor is thought to arise from aberrant differentiation and, ultimately, malignant transformation of abnormally persistent renal stem cells. WT1 is a member of the early growth response (EGR) family of transcription factors based on its binding to the EGR consensus sequence GCGGGGGCG (19) via highly conserved zinc finger motifs (20,21). Recently WT1 was shown to contain transcriptional activating as well as repressing domains (22–24). These characteristics are consistent with the proposed role for WT1 in normal kidney development of repressing genes driving blastemal cell proliferation and/or activating genes involved in blastemal differentiation (27). To date several genes involved in mesenchymal cell proliferation have been reported to be transcriptionally repressed by WT1, including the genes encoding insulin-like growth factor II (28), the A-chain of platelet derived growth factor (PDGF) (23), colony stimulating factor-1 (29), bcl-2 and c-myc (30).
We have cloned and characterized the human ODC gene to elucidate the molecular mechanisms controlling ODC gene expression in normal and neoplastic cells (31,32). In this report we show that the human ODC gene promoter is a target for both negative and positive transcriptional modulation by WT1. Both the extent of modulation and the mediating WT1 binding elements are cell specific. Understanding WT1 regulation of ODC gene expression may provide valuable insight into the role of both ODC and WT1 in genitorinary development and tumorigenesis.

MATERIALS AND METHODS

DNA constructs

The human ODC–luciferase reporter constructs phODC-luc(–1491) and phODCluc(–378) and the parental plasmid pAA lucA (32) were kindly provided by Peggy Farnham. (University of Wisconsin). Deletion mutant constructs phODC-luc(–1215), phODCluc(–880) and phODCluc(–206) were made by digesting phODCluc(–1491) with SpeI, which cuts vector sequences immediately 5′ of the ODC promoter insert, and either BglII, BSH2 or MilI respectively, which cut within the promoter sequence, and religating plasmid termini. Promoter constructs containing a 38 bp deletion including binding site WT1A were generated from parental construct phODC-luc(–206Δ) WT1A. This plasmid was made by digesting phODCluc(–206) with restriction enzymes XmalIII and BglII, making the ends blunt and religating the large fragment. WT1 expression vector CMV-WT1 and Egr1 expression vector CMV-Egr1 were as described previously (33). The Sp1 expression vector pSvSp1-F (34) and parental plasmid pSV2A101 were the generous gifts of Dr Jeffery D.Saffer (Batelle Pacific Northwest Laboratories). Mutant WT1 expression constructs (25) were generously provided by Dr Zhao-Yi Wang (Princeton University, Princeton, NJ).

Cell culture and DNA transfections

Human colon carcinoma cell line HCT 116 and NIH/3T3 murine fibroblasts (American Type Culture Collection, Rockville, MD) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with high glucose and 10% fetal bovine serum (FBS). HepG2 (35), Na2 (derived from a 2.2.15-induced tumor) (36) and G2P9T2 (37) cell lines obtained from Dr Judith Christman were cultured in minimal essential medium (MEM) supplemented with Earle’s salts, l-glutamine and 10% FBS. Plasmid DNA, purified either by two successive cesium chloride density gradient centrifugations or by Qiagen-tip chromatography (Qiagen Inc., Chatsworth, CA), was transfected into cells by the calcium phosphate protocol (38) using CellPhect™ transfection kits. Briefly, cells seeded in 60 mm² plates were transfected with 2 μg of the appropriate phODCluc DNA and 2 μg pCH110 DNA (Pharmacia Biotech, Piscataway, NJ) encoding the enzyme β-galactosidase under transcriptional control of the SV40 early promoter. Expression constructs and the corresponding control vectors were co-transfected as described for each experiment so that the total DNA transfected was 20 μg. After 6 h incubation with calcium phosphate-precipitated cells were shocked for 30 s with 15% glycerol in HEPES-buffered saline, pH 7.12, at room temperature. The cells were then cultured for 40 h, washed three times with phosphate-buffered saline and lysed on the plate by adding 200 μl 1× lysis reagent (Luciferase Assay System; Promega Corp., Madison, WI) containing 25 mM Tris–phosphate, pH 7.8, 2 mM dithiothreitol (DTT), 10 mM 1,2-cyclohexanedi-amine tetraacetic acid, 10% glycerol and 5% Triton X-100 and incubating at 25°C for 15 min. The lysates were harvested, microcentrifuged briefly, and the supernatants collected for enzyme assays.

Enzyme assays

Luciferase activities were determined by injecting 100 μl luciferin reagent (Promega Corp., Madison, WI) into 20 μl cell extract and measuring the luminescence for 10 s in a Berthold Lumat LB9501 luminometer (Wallac Inc., Gaithersburg, MD). β-Galactosidase activity was assayed as described (39) except cell extracts were diluted with 0.25 mM Tris–HCl, pH 7.8, instead of 1× lysis reagent to avoid precipitate formation. Transfection efficiencies were normalized with respect to β-galactosidase activity. The experiments shown represent a minimum of three independent transfections performed with at least two different plasmid preparations.

DNase I protection assay

Footprinting assays were done essentially as published (40) using the Hotfoot DNase I Footprinting kit (Stratagene, La Jolla, CA). Overlapping restriction fragments from either phODCluc(–1491) or phODCluc(–378) were radiolabeled on one strand with 200 μCi [α- 32P]dNTPs (3000 Ci/mmol) catalyzed by Klenow DNA polymerase I (Bethesda Research Laboratories, Gaithersburg, MD). The fragments were gel purified using Prep-A-Gene DNA purification matrix (BioRad). For each reaction 80 000 c.p.m. DNA were incubated on ice for 15 min with 25–500 ng purified WT-ZF, WT-ZF(ins KTS) or Sp1 (Promega Corp.) protein in 50 μl binding buffer containing 10 mM HEPES–KOH, pH 7.5, 50 mM KCl, 10 mM ZnSO4, 10% glycerol, 0.1% NP-40, 1 mM DTT, 4% polyvinyl alcohol and 1 μg poly(dI·dC). Samples were incubated at room temperature for 2 min, 50 μl DNA 1 buffer (Stratagene) were added and then the samples were digested with empirically determined amounts of DNase I for 2 min. The reaction was terminated by addition of 100 μl stop buffer (Stratagene) and extraction with an equal volume of phenol/chloroform (1:1). Samples were precipitated with 2.5 vol. ethanol in the presence of 0.3 M NaOAc, washed with 70% ethanol and resuspended in formamide dye (Stratagene), then run on a 8% acrylamide–7 M urea gel. Sequencing reactions were run as size markers. The gels were dried and exposed to X-Omat film.

Methylation interference assay

The binding of WT-ZF to ODC promoter regions WT1-A and WT1-F was analyzed by the methylation interference assay as described (41). Briefly, ODC promoter DNA fragments BamHI–BglI (∼1346 to ∼1222) and MilI–NcoI (∼250 to ∼50) were 32P-radiolabeled at one end, BamHI and MilI respectively, gel purified and methylated according to the protocol. Each DNA was then subjected to a preparative electrophoretic mobility shift assay (EMSA) to isolate WT-ZF bound and unbound probe for binding site analysis. Approximately 2 × 105 c.p.m. probe DNA was incubated with 250 ng WT-ZF protein in 25 mM HEPES–KOH, pH 7.5, 50 mM KCl, 10 mM ZnSO4, 10% glycerol, 0.1% NP-40, 0.2% bovine serum albumin, 1 mM DTT, 2 μg...
WT1 binds to multiple sites on the ODC promoter

WT1 binding elements in the ODC promoter were identified by DNase I protection footprinting. $^{32}$P-Radiolabeled DNA restriction fragments extending from nt +77 in exon 1 to nt −1491 in the ODC promoter were incubated with varying concentrations of WT-ZF (35) or WT-ZF(ins KTS) proteins (24). WT-ZF contains the DNA binding zinc finger region of wild-type WT1 synthesized from an Escherichia coli expression vector and purified by nickel chelate affinity chromatography. WT-ZF(ins KTS) protein, also synthesized in vitro, contains the naturally occurring insertion of lysine, threonine and serine within the zinc finger domain of WT1 which interrupts binding to the consensus element GCGGGGCG. DNase I digestion resulted in protection of four sites with relatively high binding affinities for WT-ZF (WT1-A, -D, -E and -F) and several sites with lower affinities (Fig. 2). WT-ZF(ins-KTS) does not bind to sequences in the ODC promoter. Two WT-ZF protected regions, WT1-A and WT1-F, are GC-rich, each with two possible WT1 binding sites and overlapping Sp1 consensus sequences (Fig. 3). We performed methylation interference studies to determine more precisely the guanine bases contacting WT-ZF within footprints WT1-A and WT1-F (Fig. 4). The ODC promoter sequences protected by WT-ZF, the nucleotide positions of each element relative to the transcription start site and the methylated bases in WT1-A and WT1-F that most severely disrupt WT-ZF binding are shown in Figure 3.

WT1 repression of 5′-deleted ODC promoter constructs

5′-Terminal deletions were generated from phODCluc(−1491) to produce a panel of subclones each containing 77 bp of exon 1 and extending 1490, 1215, 880, 378 and 206 bp respectively into the ODC promoter. These constructs were transfected into HCT 116 cells to ascertain the relative abilities of the major WT1 binding elements to mediate WT1 repression of the ODC promoter (Fig. 5A). WT1-A and WT1-F mediate inhibition of ODC promoter activity by 62 and ~15% respectively. There is no significant statistical difference in promoter function of constructs extending ~206, ~378, ~880 and ~1215 nt into the ODC promoter (P > 0.15 for each pair). The difference between constructs extending ~1490 and ~1215 nt is statistically significant (P = 0.012). Based on these studies it appears that binding sites WT1-A (~94 to ~116) and WT1-F (~1231 to ~1247) are responsible for most of the inhibition of ODC promoter activity by WT1 in HCT 116 cells.

To confirm a direct interaction between WT1 and the putative WT1A binding site we examined the impact of mutating WT1A on WT1-mediated inhibition of the ODC promoter. A 38 bp deletion (~91 to ~129) including the entire WT1A binding site was generated in three reporter constructs with varying lengths of the ODC promoter (Fig. 5B). The removal of WT1A from phODCluc(−206) resulted in mutant construct phODCluc(−206Δ WT1A), containing no WT1 consensus binding sites and shown to be incapable of binding to purified WT1-ZF protein by EMSA (data not shown). Compared with the promoter activity of phODCluc(−206), which was inhibited to 62% of the control by WT1 (Fig. 5A), there was no statistically significant inhibition of phODCluc(−206Δ WT1A) by WT1 (P > 0.05). This result supports the conclusion that binding to WT1A is required for at least part of the observed inhibition of ODC promoter activity by WT1. In addition, we have observed that expression of a WT1 mutant lacking the zinc finger DNA binding domain (25) has no effect on promoter activity of the largest ODC reporter construct.
Figure 2. DNase I protection analyses of WT-ZF binding sites in the ODC promoter. $^32$P-Radiolabeled DNA fragments spanning 1491 bp of the ODC promoter were incubated with WT-ZF and mutant WT-ZF(ins KTS) proteins and digested with empirically determined amounts of DNase I. The figure shows protected sequences in restriction fragments KpnI–Tsp509I, BamHI–BglI and NruI–NcoI which were generated with increasing concentrations of each protein. The starred (*) restriction sites indicate radiolabeled termini. The positions of the protected sites were determined from sequencing ladders co-electrophoresed in parallel lanes (not shown).

**Table:**

<table>
<thead>
<tr>
<th>WT1 SITES</th>
<th>POS.</th>
<th>ODC SEQUENCES</th>
<th>POS.</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT1-A</td>
<td>-116</td>
<td>3'-GAGGCTCTCAAGGGGCGCCGACA-5'</td>
<td>-94</td>
</tr>
<tr>
<td>WT1-B</td>
<td>-221</td>
<td>5'-CGGGGGCGG-3'</td>
<td>-214</td>
</tr>
<tr>
<td>WT1-C</td>
<td>-611</td>
<td>3'-GAAGCCGAGGGCGCCGACG-5'</td>
<td>-555</td>
</tr>
<tr>
<td>WT1-D</td>
<td>-676</td>
<td>5'-ACCGGCGCCGTGGCA-3'</td>
<td>-661</td>
</tr>
<tr>
<td>WT1-E</td>
<td>-1101</td>
<td>3'-GAGGATCGGGGATCCTGCA-5'</td>
<td>-1161</td>
</tr>
<tr>
<td>WT1-F</td>
<td>-1247</td>
<td>5'-GGGGGGGGGTGGGTTG-5'</td>
<td>-1231</td>
</tr>
</tbody>
</table>

Figure 3. ODC promoter nucleotide sequences protected by WT1. The nucleotide sequences of the regions protected from DNase I digestion by WT1-ZF protein (Fig. 2) are listed, together with their sequence location. The G-rich strand is shown for each protected footprint. Highlighted sequences in WT1-A and WT1-F represent guanine bases most closely contacting WT-ZF, as determined by methylation interference studies (Fig. 4). Boxed sequences indicate elements with perfect homology to Sp1 binding sites.

Figure 4. Methylation interference analyses of WT-ZF binding to WT1-A and WT1-F ODC promoter sites. DNA fragments from the ODC promoter were $^32$P-labeled, methylated and incubated with WT-ZF in EMSA as described in Materials and Methods. Free (F) and WT-ZF-bound (B) DNA from EMSA were chemically cleaved, fractionated on DNA sequencing gels and exposed to film. The restriction fragments used, the radiolabeled termini (*) and the sequence of WT1-A and WT1-F are shown. Bases most strongly contacting WT-ZF are marked in the WT1 protected nucleotide sequence (+).

phODCluc(–1491) (data not shown). Thus the entire effect of WT1 on ODC promoter activity in HCT 116 cells is dependent on WT1 binding to the promoter.

In deletion constructs with larger portions of the ODC promoter the loss of WT1A revealed that binding sites other than WT1A and WT1F also mediate WT1 repression. For example, binding site WT1-B did not appear to be important for WT1 repression in the 5'-deletion series represented in Figure 5A. However, in the internal deletion construct phODCluc(–485Δ WT1A) WT1B alone appears to mediate a 2-fold inhibition (Fig. 5B). In addition, the loss of WT1A from the longest ODC promoter construct phODCluc(–1491Δ WT1A) resulted in the same relative level of
Figure 5. The effect of WT1 co-expression on the relative activities of 5′-deleted ODC promoter mutants. The schematic shows the relative positions of the WT-1 binding sites (open boxes) within the human ODC promoter. Bold letters identify those sites with the strongest binding affinities as determined by DNase I protection. ODC promoter sequences are depicted by the thick black line, ODC exon 1 sequences by the hatched line and luciferase reporter gene sequences by Luc. The nucleotide positions of each protected sequence are shown below the line. (A) 5′-Deleted ODC promoter/luciferase constructs containing sequences extending 206–1491 nt upstream of the ODC gene transcription start site. (B) ODC promoter/luciferase constructs containing a deletion spanning the WT1-A binding site were constructed as described in Materials and Methods. The position of the last promoter sequence in each construct is shown. These plasmids were transiently transfected into HCT 116 cells, as described in Figure 1, in the presence and absence of expression vector CMV-WT1 to assay the relative strengths of the major WT1 binding sites on ODC promoter function. The percent inhibition of ODC promoter activity is the level of luciferase activity in CMV-WT1 transfected cells compared with levels in control cells transfected with the same pHODCluc construct and CMV vector.

ODC promoter activity as the intact promoter (Fig. 5A and B). Thus the loss, or unavailability, of one WT1 binding site may be compensated for by other sites in the ODC promoter. We conclude that the ODC promoter contains multiple binding sites that interact directly with WT1 protein to mediate repression in HCT 116 cells.

Sp1 and the regulatory specificity of WT1

Each WT1 footprint mapped to the ODC promoter includes a putative Sp1 binding site which exactly or nearly so matches the consensus sequence GGGCGG (Fig. 3). Interestingly, there are multiple Sp1 elements within the proximal ODC promoter that are not protected from DNase I digestion by WT1 (data not shown). Overlapping WT1 and Sp1 binding sites suggested that competition for binding by transactivating factor Sp1 could regulate the effect of WT1 on promoter function. Using the functional WT1A binding site with a perfect Sp1 consensus element we examined the possibility that Sp1 and WT1 competition influences transcription from the proximal ODC promoter. We first determined whether Sp1 does, in fact, bind to
Figure 6. DNase I protection analysis of Sp1 and WT-ZF binding to overlapping sequences within WT1-A in the ODC promoter. $^{32}$P-Radiolabeled DNA fragment NruI–NcoI* containing site WT1-A was incubated with various concentrations of WT-ZF and Sp1 transcription factors, digested with empirically determined amounts of DNase I, fractionated on a sequencing gel and exposed to autoradiography. The starred (*) restriction site indicates the radiolabeled termini. The proteins used for protection of each sample were: lanes 1 and 8, no protein; lanes 2 and 7, 50 ng WT-ZF; lane 3, 50 ng Sp1; lanes 4, 5 and 6, 50 ng WT-ZF and 25, 50 and 250 ng Sp1 respectively. The positions of the protected sites determined from sequencing ladders co-electrophoresed in parallel lanes are indicated.

sequences within WT1-A. DNase I protection footprinting shows that purified Sp1 protein binds and protects a subset of WT1-A sequences (–98 to –116) (Fig. 6). AP-2, which also binds GC-rich sequences, does not bind to WT1-A (data not shown). Because the patterns of protected sequences generated by these two proteins are distinguishable, we were able to perform competitive footprinting assays to examine the relative binding affinities of WT1 and Sp1 for WT1-A. The simultaneous incubation of Sp1 and WT1-ZF with ODC promoter DNA produced an Sp1 protection pattern with Sp1:WT-ZF ratios of from 0.5 to 5 (Fig. 6). Thus the binding of WT1 and Sp1 to WT1-A is mutually exclusive and Sp1 competes more efficiently than WT-ZF for binding to WT1-A in vitro.

The effect of Sp1 on WT1 repression of ODC promoter activity was examined by transiently transfecting both HCT 116 and NIH/3T3 cells with phODCluc(–206) containing sequence WT1-A and combinations of Sp1 and WT1 expression vectors (Fig. 7A). First, expression of Sp1 alone had a slightly inhibitory effect on phODCluc(–206) promoter activity in both cell lines. That this result does not reflect a lack of Sp1 expression is supported by our observation that every phODCluc reporter construct containing larger portions of the ODC promoter is stimulated 2- to 3-fold when co-transfected with the Sp1 expression vector under the same conditions. Second, WT1 repression of phODCluc(–206) promoter activity was augmented in an additive manner in every experiment involving co-transfec-

Figure 7. Effect of Sp1, Egr1 and WT1 co-expression on ODC promoter function. (A) HCT 116 and NIH/3T3 cells were co-transfected with phODC-luc(–206) and either Sp1 expression vector pSVSp1-F (open columns), CMV-WT1 (gray columns) or both pSVSp1-F and CMV-WT1 (hatched columns). (B) HCT 116 and NIH/3T3 cells were co-transfected with phODCluc(–206) and either expression vector CMV-Egr1 (hatched columns) or CMV-WT1 (gray columns). The ratio of expression construct DNA to reporter DNA was 5:1 for the experiments represented in the figure. ODC promoter activities are expressed as a percentage of the luciferase activities of control cells transfected with phODCluc(–206) and control vectors (black columns). Error bars represent SE.

Effect of Egr1 on ODC expression

Since WT1 and Egr1 bind the same consensus sequence, we examined the effect of Egr1 expression on WT1-A regulation of ODC promoter activity. HCT 116 and NIH/3T3 cells were co-transfected with phODCluc(–206) and CMV-Egr1 expression vector (Fig. 7B). Egr1 expression had contrasting effects on ODC promoter activity in these two cell lines. In HCT 116 cells ODC promoter activity from phODCluc(–206) was repressed to 64% of controls not transfected with CMV-Egr1. In comparison, Egr1 expression enhanced ODC promoter activity from this same construct by 50%. Thus, as seen in HCT 116 cells, both WT1 and Egr1 are capable of down-regulating ODC promoter activity via the same proximal binding element. It is also clear from the
disparate results with WT1 and Egr1 in NIH/3T3 cells that the two factors are differentially regulated and have opposing effects on ODC promoter activity within a given cell type.

**Enhancement of ODC promoter activity by WT1**

We transfected parental HepG2 hepatoblastoma cells and HepG2 cells stably transfected with genomic DNA from either hepatitis B virus (designated Nu2 cells) or SV40 DNA tumor virus (designated G2P9T2 cells) with phODCluc(-1491) and phODC-luc(-378) and CMV-WT1 to examine the generality of ODC gene repression by WT1. WT1 expression represses phODC-luc(-1491) promoter activity by 50 and 37% in HepG2 and Nu2 cells respectively (Fig. 8). Distal elements contribute to down-regulation of the ODC promoter in these two cells to varying extents, but based on the repression of phODC-luc(-378) it appears that most of the effect of WT1 is mediated by a proximal cis-element(s). In contrast to HepG2 and Nu2 cells, ODC promoter activity was elevated 200% in G2P9T2 cells by WT1 co-expression. This represents a 4-fold change in ODC promoter activity relative to the ODC promoter response to WT1 expression in parental HepG2 cells. The activation of ODC promoter activity was mediated primarily by a proximal WT1 binding element(s).

HepG2 and G2P9T2 cells were co-transfected with reporter phODCluc(-1491) and DNA constructs expressing mutated WT1 to identify potential repression and activation domains within WT1. N-Terminal truncated WT1 mutant WT1Δ1–84 functions as wild-type WT1 (Fig. 9); it repressed and activated the ODC promoter in HepG2 and G2P9T2 cells respectively. Removal of another 13 amino acids in WT1Δ1–97, however, resulted in the loss of WT1 repressor activity in HepG2 cells. On the other hand, WT1 activation of ODC promoter function was maximum for mutant WT1Δ179–294 in both cell lines. Finally, mutant WT1Δ1–294, which contains the DNA binding zinc finger domains of WT1 and has no significant effect on ODC promoter activity in HepG2 cells (P > 0.05), appears to stimulate the promoter in G2P9T2 cells. Thus, although WT1 contains repressor and activator activities, some of the effect of WT1 on the ODC promoter, in G2P9T2 cells at least, may be due to WT1 blocking access of other factors to the promoter.

**DISCUSSION**

WT1 loss of function has been strongly implicated in a variety of genitourinary developmental anomalies, as well as in the etiology of Wilms’ nephroblastoma (42). However, the exact role WT1 plays in normal development and tumorigenesis is not well understood. The identification of target genes regulated by WT1 is crucial to understanding the consequences of WT1 function at the molecular and cellular levels. We have shown that the human ODC gene promoter is a target for both negative and positive transcriptional modulation by WT1 in transient co-transfections. WT1 binds to multiple DNA cis-elements within the ODC promoter and represses ODC transcription in HCT 116 colon carcinoma, HepG2 hepatoblastoma and NIH/3T3 fibroblast cell
In contrast, ODC promoter activity is enhanced by WT1 expression in SV40-transfected HepG2 cells. Our results do not demonstrate that WT1 regulates the ODC promoter in vivo. However, the importance of ODC and polyamines to cell proliferation is widely reported and is consistent with the hypothesis that WT1 may act during normal development to suppress the expression of genes controlling cell proliferation (27). Failure to repress ODC due to loss of WT1 function may block blastemal cell differentiation by interfering with the cessation of proliferation. However, WT1 regulation of ODC may have a more direct impact on blastemal cell differentiation. There is limited but convincing evidence that polyamine metabolism is critical for some aspects of differentiation and embryogenesis (10). However, the cellular effects of polyamines are complex, as both increases and reductions in ODC and polyamine levels have been shown to induce cell differentiation, depending on the experimental system. At present the role of ODC in organogenesis and, specifically, in the mesenchymal–epithelial transition common to genital and urinary tract formation is unknown. Focusing on the regulation of ODC by WT1, which is specifically expressed in embryonic kidney, gonad and mesothelium, may help define the relationship of ODC to genitourinary development.

The ODC gene is only weakly expressed in most adult tissues, as reflected in the extremely low abundance of ODC mRNA and protein in non-proliferating tissues. Furthermore, the ODC promoter appears to be repressed in differentiated or quiescent cells, as suggested by studies of transgenic mice carrying the human ODC gene (43) and mammalian cells transiently transfected with ODC promoter/reporter fusions containing various lengths of the ODC gene promoter (44,45; M.Flanagan, personal communication). We hypothesize that the ODC promoter in most terminally differentiated cells exists in a repressed state. In support of this several studies show that stimulation of ODC expression in quiescent cells can occur without prior protein synthesis, suggesting involvement of short-lived repressor proteins in ODC gene regulation (46,47; M.Flanagan, personal communication). Different cell types may utilize a variety of repressors which function similarly to WT1 but which are themselves regulated in a tissue-specific manner to control ODC expression. From this study WT1, Egr1 and, to a limited extent, Sp1 each affect down-regulation of ODC promoter activity from the WT1A element. In addition, Li et al. (47) have reported that multiple nuclear proteins, including Sp1 and a repressor termed NF-ODC1, bind to sequences in the ODC promoter corresponding to the WT1A element. Similarly to WT1 and EGR1 in this study, Li et al. have shown that the response of the ODC promoter to Sp1 and NF-ODC1 varies with cell type. These observations, coupled with growing evidence that the multiple members of the EGR/WT1 (24) and Sp1 (48) families of transcription factors are subject to different cell-specific and growth-specific regulation, suggest that WT1-A may serve as a ‘hot-spot’ for mediating ODC repression.

WT1 has been shown to repress transcription via a functional repressor domain (22,25) or by competing for either Sp1 (31) or Egr1 binding (23,28). We examined the possibility that WT1 regulation of ODC gene expression involved competition for binding to DNA element WT1-A between WT1 and either Sp1 or Egr1 transcription factors. Sp1 exhibited a higher affinity for binding to WT1-A than WT-ZF in vitro, but the results of co-transfection of Sp1 and WT1 expression vectors in two cell lines indicated that Sp1 did not compete efficiently with WT1 in vivo. The difference in the results of the two assays may simply reflect the fact that the in vitro binding studies utilized truncated WT-ZF protein, whereas entire WT1 was expressed in the transfection studies. Alternatively, competition between ectopically expressed Sp1 and WT1 for binding to this element may not be responsible for the differential response of the ODC promoter to WT1 in NIH/3T3 and HCT 116 cell lines. That multiple endogenous factors bind to the region including WT1A and modulate ODC promoter function in a cell-specific manner (47) suggests the possibility that any one or more of these unidentified proteins may interact differentially with WT1. With regard to Egr1, the inhibition in HCT 116 cells and activation in NIH/3T3 cells of ODC promoter function by Egr1 expression suggests that competition by Egr1 and WT1 for binding to WT1A could play a part in the differential response of the ODC promoter to WT1.

The enhancement of ODC promoter activity by WT1 expression in G2P9T2 cells suggests that other factors may disrupt or alter WT1 repressor function. Mutations in cellular factors that modulate WT1 function could result in blastemal cell transformation despite the presence of wild-type WT1 and may explain why more WT1 mutants have not been identified in Wilms’ tumors. The positive and negative modulation of ODC expression by both WT1 and Egr1 underscore the importance of understanding the interactions of WT1 with putative regulatory sequences. Our studies of mutant WT1 in HepG2 and G2P9T2 cells suggest the presence of a candidate regulatory domain between amino acid residues 85 and 98 of WT1 that may be responsive to factors capable of modulating repressor function. The loss of N-terminal residues 1–84 had no effect on either WT1 repression or activation of ODC promoter activity in HepG2 and G2P9T2 cells respectively. However, removal of the next 13 residues resulted in the loss of WT1 repression in HepG2 cells. That residues 85–98 are important, but not sufficient, for WT1 repressor activity is indicated by the results obtained with mutant WT1Δ179–294. This mutant exhibits the highest levels of WT1 activating function in both cell lines, despite containing an intact region surrounding and including residues 85–98. These findings suggest that the deleted region 179–294 in this construct must also function in conjunction with region 85–98 for maximum WT1 repressor activity.

The mapping of WT1 domains with the ODC promoter in HepG2 and G2P9T2 cells agrees to a certain extent with a similar study using the PDGF-A promoter in NIH/3T3 cells (25). Both studies show that the N-terminal 84 amino acids do not contribute to transcriptional modulation by WT1. In addition, Wang et al. (25) conclude that the WT1 repressor domain is located between amino acid residues 84 and 179. Our studies show an essential and modulatable repressor domain between amino acids 85 and 97. The differences in assigning the boundaries of this domain are probably due to the different set of WT1 mutants utilized in the two studies. The two studies differ significantly, however, with regard to defining the boundaries of the WT1 activation domain. Wang et al. showed that residues 180–294 are required for activation of the PDGF-A promoter, whereas this study implicates residues 98–179 in activation of the ODC promoter. The discrepancy may be partially explained by differences in the broadly mutated WT1 constructs used. It is more likely, however, that these differences reflect cell-specific and/or promoter-specific differences in transcription factors that modulate WT1 activity. In any case, activation of the ODC promoter by wild-type WT1 in G2P9T2 cells is matched by expression of mutant WT1Δ1–294 containing
only the DNA binding domain. Thus the intrinsic activation processes leading to both normal and abnormal genitourinary development.

ACKNOWLEDGEMENTS

This research was supported by grant 6-FY94-0278 from The March of Dimes Foundation for Birth Defects, The Department of Internal Medicine, Funds for Medical Research and Education, Wayne State University School of Medicine and by grants CA52009 and CA47983, core grant CA10815 from the National Institutes of Health and grants from the W.W. Smith Charitable Trust, the Hansen Memorial Foundation and the Mary A.H. Rumsey Foundation. FJR is a Pew Scholar in the Biomedical Sciences.

REFERENCES