The Transcriptional Effect of WT1 Is Modulated by Choice of Expression Vector

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The WT1 Wilms' tumor suppressor gene encodes a zinc finger transcription factor which plays a critical role in renal and genitourinary development. The WT1 protein was reported to both activate and repress transcription. We found that the transcriptional effect of WT1 on the Egr1 promoter could be modulated by the use of expression vectors containing different promoters. WT1 activated the Egr1 promoter when expression of WT1 was driven by the Rous sarcoma virus promoter. In contrast, a cytomegalovirus (CMV) promoter-containing WT1 expression vector repressed the Egr1 promoter. However, WT1 activated transcription of a simple test promoter, EGR3 tkCAT, regardless of the expression vector used. Co-transfection of the parental CMV-based vector strongly depressed the basal activity of the Egr1-CAT reporter, suggesting that the CMV promoter competes with the Egr1 promoter for transcription factors or cofactors which may be required for activation by WT1. In support of this hypothesis, WT1 was converted from an activator to a repressor by co-transfection of an excess of the parental CMV-based vector. These results provide an important caveat to the interpretation of co-transfection studies and confirm the bi-functional nature of the WT1 transcription factor.

The Wilms' tumor suppressor gene WT1 was isolated by positional cloning based on the presence of a constitutional deletion of chromosome 11p13 in patients with the WAGR syndrome (Wilms' tumor, aniridia, genitourinary malformations, and mental retardation) (1, 2). The WT1 gene yields four alternatively spliced mRNAs of approximately 3 kilobases which encode zinc finger transcription factors, termed WT1(A–D) (3–5). These isoforms arise from the inclusion of a 17-amino acid segment in the amino terminus of the protein and a 3-amino acid sequence (KTS) between zinc fingers 3 and 4 (7). WT1 can both activate and repress transcription, and several factors have been shown to influence the transcriptional activity of WT1. Studies on the platelet-derived growth factor A-chain (PDGF-A) and other promoters suggest that WT1 represses transcription when it is bound both upstream and downstream of the start site of a promoter (8–12). Conversely, WT1 activates transcription when WT1 binding sites are present only 5' or 3' to the transcription start site of the PDGF-A promoter or when WT1 binding sites are inserted upstream of the HSV-tk or MMTV promoters (10, 13–15). However, WT1 also was reported to repress transcription from upstream binding sites in its native state (16–18) and when expressed as a GAL4-WT1 fusion protein (19). Finally, interaction between WT1 and the p53 tumor suppressor protein can determine whether WT1 acts as a repressor or an activator of transcription (20).

In this study, we identified a new factor influencing the transcriptional effects of WT1 which may help to resolve some of the apparent inconsistencies in this field. We show that WT1 can either activate or repress transcription from the Egr1-CAT reporter, depending on which promoter is used to drive the expression of WT1. In contrast, WT1 activates transcription from the simple EGR3 tkCAT reporter gene regardless of the expression vector used, confirming the role of promoter architecture in determining the transcriptional function of WT1. WT1 repressed transcription of the Egr1 promoter only when expressed by the pCB6+ vector, which contains the strong cytomegalovirus (CMV) promoter. Repression mediated by the pCB6+ vector was not related to the higher levels of protein expressed by this construct. Rather, repression was related to the ability of the pCB6+ vector itself to profoundly depress the basal activity of the Egr1 promoter. We confirmed the importance of this promoter competition effect by demonstrating that WT1 expressed from the RSV vector was converted from an activator to a repressor of the Egr1 promoter by co-transfection of an excess of pCB6+ vector lacking a WT1 cDNA. Taken together, these results suggest that competition between the CMV and Egr1 promoters for transcription factors and/or cofactors results in changes in the basal activity of the Egr1 promoter and that only under conditions of low basal activity is the Egr1 promoter able to be repressed by WT1. These data suggest that WT1 may require one or more cofactors to activate transcription. In the absence of these cofactors the protein acts as a repressor. Our results also provide an important caveat regarding choice of expression vector in transfection experiments.

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The abbreviations used are: CMV, cytomegalovirus; RSV, Rous sarcoma virus; CAT, chloramphenicol acetyltransferase.
**MATERIALS AND METHODS**

Plasmid Construction—The RSV expression vector for the murine WT1(A) protein was described previously (21) (Fig. 1A). An RSV vector lacking the WT1 coding region was constructed by restriction of the RSV-WT1(A) plasmid with BglII to release the WT1 coding region, followed by recircularization. The EGR tkCAT reporter plasmid was described previously (14). The rat β-actin-based WT1 expression vector, pJ6 WT1(A) (Fig. 1B), was constructed by insertion of a 5′SalI/EcoRI fragment of pSP64-WT1(A) (14) into pJ6 WT1(A) (Fig. 1A). pCB6-human (h)WT1(A) and pCB6 murine (m)WT1(A) was constructed by restriction of pl 60-WT1(A) with HindIII and ligating of the resultant fragment, which contains the murine WT1 coding region, into HindIII/BglII-digested pCB6+. The locations of transcription factor binding sites in the target promoters are indicated. RSVLTR, promoter and enhancer sequences derived from the long terminal repeats of the Rous sarcoma virus. MCS, multiple cloning site. SV40 Ori/E, SV40 virus promoter and enhancer sequences, including the viral replication origin. SV40T IVS, pA, intervening sequences and 3′ polyadenylation site of the T antigen gene of the SV40 virus. Neo-R-neomycin resistance gene.

**RESULTS**

Our previous work (14) showed that WT1 was a transcriptional activator of a simple test promoter containing three Egr1/WT1 binding sites upstream of the HSV-tk promoter (EGR tkCAT) (Fig. 1). In contrast, Madden et al. (16) found that WT1 repressed transcription of the Egr1 promoter. The complex Egr1 promoter contains three potential Egr1/WT1 binding sites as well as binding sites for AP-1 and SRF upstream of the start site of transcription (23, 27) (Fig. 1). To investigate the conditions that might affect the ability of WT1 to activate or repress transcription, we co-transfected both NIH 3T3 cells and CV-1 cells with an Egr1 promoter-containing reporter gene and the RSV-WT1 expression vector. To our surprise, we found that expression of murine WT1 activated rather than repressed transcription of this promoter (Fig. 2, A and B). The same RSV-based expression vector also activated the simple EGR tkCAT reporter gene (Fig. 3 and Ref. 14). To further explore this phenomenon, we constructed an expression vector for WT1 containing the β-actin promoter and observed a CMV-based expression vector for WT1 used by many other groups for co-transfection studies (pCB6+ (16); gift of V. Sukhatme). The Egr1 promoter was activated by WT1 expressed from the RSV or β-actin promoters. Repression of the Egr1 promoter only occurred when WT1 was expressed from the pCB6+ vector (Fig. 2, A and B). While only modest repression was observed at 10 μg of input effector plasmid, stronger repression was seen at a dose of 20 μg (Figs. 2, A and B). In contrast, WT1 always activated transcription of the EGR tkCAT reporter, regardless of the expression vector used (Fig. 3).

We then examined the reasons why the choice of expression vector might influence the transcriptional activity of WT1. The effect was not cell type-dependent, since activation and repression were effected by RSV-WT1 and pCB6-WT1, respectively, both in NIH 3T3 cells and CV-1 cells (Fig. 2, A and B). Second, since pCB6-WT1 contains the human WT1 cDNA, whereas RSV-WT1 and β-actin-WT1 contain the murine WT1 cDNA, we wanted to rule out the possibility of a species-specific effect. Therefore, we cloned the murine WT1 cDNA into the pCB6+ vector and found that it repressed the Egr1 promoter in an identical manner to the human cDNA (data not shown).

Next, we determined whether the transcriptional effect of

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**FIG. 1.** Schematic representation of the EGR tkCAT and Egr1-CAT reporter genes and the RSV-WT1, rat β-actin-WT1, and pCB6-WT1 expression vectors used for co-transfection experiments. The locations of transcription factor binding sites in the target promoters are indicated. RSVLTR, promoter and enhancer sequences derived from the long terminal repeats of the Rous sarcoma virus. MCS, multiple cloning site. SV40 Ori/E, SV40 virus promoter and enhancer sequences, including the viral replication origin. SV40T IVS, pA, intervening sequences and 3′ polyadenylation site of the T antigen gene of the SV40 virus. Neo-R-neomycin resistance gene.

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**EXPRESSION VECTORS**

| Plasmid Name | Promoter | MCS | SV40 Ori/E | NeoR | SV40 PA | pTk±6 (Pharmacia) | pUC19-based |
|--------------|----------|-----|------------|------|--------|-----------------|-------------|
| pCB6+        | HCMV Promoter | | | | | | |
| pJ6Ω         | Rat β-actin Promoter | | | | | | |
| RSV          | RSV LTR | | | | | | |

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**SEQUENCE ANALYSIS**

Promoter sequences obtained from GenBank™ were analyzed using the Signal Scan program (25) and the TFD data base (26).
Expression Vector Choice Affects WT1 Function

WT1 might depend on the level of protein expressed from the various expression vectors. We performed immunoblotting analysis on the same transfected cell extracts used in the CAT assays. Transfection of pCB6-WT1 yielded a much higher level of WT1 protein expression than that achieved by either the RSV or β-actin-based expression vectors in both NIH 3T3 and CV-1 cells (Fig. 4A). Transfection of NIH 3T3 cells with 5 or 0.5 μg of each expression vector showed that the pCB6+ expression vector yields approximately 10-fold more WT1 protein than the RSV vector (Fig. 4B). These-dependent transcriptional effects have been reported for the Drosophila Krüppel protein, which activates transcription at low doses but represses transcription at high doses (28). It was therefore possible that WT1 had a similar dose-dependent transcriptional effect. However, we could not confirm this hypothesis, as transfection of even up to 40 μg of RSV-WT1 still activated the Egr1 promoter (data not shown). Conversely, we could not observe activation of the Egr1 promoter by low doses of pCB6-WT1 (data not shown).

Finally, we determined whether the actions of the strong CMV promoter itself might be affecting the results of the trans-
Further co-transfection with the RSV expression vector lacking an insert was still associated with 20% inhibition of the Egr1 promoter. It is possible that some of the WT1 protein expressed from the CMV promoter bound to the RSV promoter instead of the Egr1 target reporter hence diluting the repressive effect of WT1 protein. In addition, less WT1 protein may be produced in the presence of both the RSV and pCB6 expression vectors (Fig. 6B). Nevertheless, WT1 expressed from the CMV promoter was not converted into an activator by the presence of RSV vector. Together from these data we conclude that WT1 is a default activator of the Egr1 promoter and suggest that repression of this promoter by transfection of pCB6-WT1 is facilitated by competition between the CMV and Egr1 promoters.

**DISCUSSION**

While the co-transfection assay is a valuable tool which can provide much information about the regulation of a promoter by a particular protein, there are significant limitations of this method. Removal of a promoter from its endogenous chromosomal context may result in changes in its transcriptional activity and its ability to be regulated by specific transcription factors. For example, the Egr1 promoter fragment used in these experiments is highly active in the absence of co-transfected CMV-based expression vector, yielding 50-fold more CAT activity than the HSV-tk promoter (data not shown). In contrast, the endogenous cellular Egr1 gene is expressed only tran-

![Fig. 4.](image1.png) **Fig. 4.** The pCB6+ expression vector yields a 10-fold higher level of WT1 protein expression than the RSV or β-actin expression vectors. Extracts from transfected NIH 3T3 or CV-1 cells were subjected to immunoblot analysis with affinity-purified polyclonal anti-WT1 C19 antibody (Santa Cruz Biotechnology). A, immunoblot of extracts from NIH 3T3 cells or CV-1 cells transfected with 10 μg of RSV-WT1, β-actin-WT1, or pCB6-WT1. B, immunoblot of extracts from NIH 3T3 cells transfected with the indicated amounts of the RSV-WT1 or pCB6-WT1 expression vectors.

![Fig. 5.](image2.png) **Fig. 5.** The basal activity of the Egr1 promoter is repressed by co-transfection of the pCB6+ expression vector. The Egr1-CAT reporter (0.5 μg) was transfected into NIH 3T3 cells along with the indicated amount of pCB6+ vector lacking the WT1 cDNA insert. The total amount of DNA in the transfection was kept constant by the addition of pBluescript. Transcriptional activity is expressed as percent conversion of chloramphenicol to acetylated chloramphenicol, normalized for the protein concentration of the extracts and the incubation time of the CAT assay. The results are presented as the average of three independent determinations.

![Fig. 6.](image3.png) **Fig. 6.** WT1 is converted from an activator to a repressor of the Egr1 promoter by co-transfection with the pCB6+ expression vector. A, the Egr1-CAT reporter (0.5 μg) was transfected into NIH 3T3 cells along with 10 μg of the RSV expression vector, either lacking or containing the WT1 cDNA, in the absence or presence of pCB6+ lacking the WT1 cDNA as indicated. The total amount of DNA in each transfection was kept constant by the addition of pBluescript. Transcriptional activity is expressed as percent conversion of chloramphenicol to acetylated chloramphenicol, normalized for the protein concentration of the extracts and the incubation time of the CAT assay. The fold activation or repression is indicated. The experiment was repeated several times in duplicate, and the results of a representative experiment are presented as the average of two independent determinations. □, empty expression vector; ■, WT1 expression vector. B, protein extracts from the transfected cells were subjected to immunoblot analysis to determine the level of WT1 protein expressed in the presence or absence of pCB6+. A representative experiment is presented.
in the promoter fragment being studied. As another example, it was found that the MYB protein potently activated a co-transfected mim-1 reporter gene but failed to activate the endogenous mim-1 gene. Only when the MYB protein was co-expressed with C/EBP-β was the endogenous gene activated (30). This indicates that the requirements of reporter genes and endogenous genes for transcriptional regulation may differ.

The transcriptional effects of WT1 depend on the experimental context. Many co-transfection assays using promoters containing GC-rich Egr1/WT1 binding sites showed WT1 to be a repressor of transcription (8, 9, 12, 16, 18, 19, 31–34). WT1 activated transcription of a truncated form of the PDGF-A chain promoter (9), while it repressed the full-length PDGF-A promoter in the same cell type (9, 33). Subsequent reports confirmed the ability of WT1 to activate promoter targets (13, 14). By both deletion analysis and fusion of portions of the WT1 protein to the GAL4 DNA binding domain, the repression domain was mapped to amino acids 85–124 of the protein (10, 15, 19). A potent transactivation domain was mapped between amino acids 181 and 250, adjacent to the repression region (10, 15). These studies indicate that the WT1 transcription factor is intrinsically bi-functional in nature. Similarly, adjacent activation and repression domains were noted in the Drosophila Krüppel and even-skipped proteins as well as the Egr1 protein (35, 36). In particular, the full-length Krüppel protein was shown to both activate and repress transcription (28, 37).

Given that WT1 contains both an activation and repression domain, several experimental factors may determine what transcriptional effect the protein exhibits, including the nature of the target promoter. In particular, WT1 tends to repress promoters containing binding sites both 5’ and 3’ to the start site of transcription, but not truncated promoters containing either 5’ or 3’ sites (8, 9, 12). It is interesting to note that the repression domain of WT1 maps to the same region as a recently identified self-association domain (14). This suggests that WT1 self-association might play a role in repression, perhaps by causing looping of DNA between WT1 molecules bound both 5’ and 3’ to the start site of transcription.

A second factor that may affect the function of WT1 is the cell type in which the protein is expressed. A recent report showed that WT1 activated the insulin-like growth factor-I receptor (IGF1-R) promoter in human embryonic kidney 293 cells and repressed the same promoter in the G401 cell line (38), originally thought to represent a Wilms’ tumor cell line (39), but now thought to be derived from a rhabdoid tumor of the kidney (40). Similarly, Madden et al. (19) found that a GAL4-WT1 fusion protein containing amino acids 84–180 of WT1 repressed transcription in NIH 3T3 cells but not in 293 cells. These findings suggest that there may be cell type-specific co-factors required for activation or repression.

A third potential factor which may influence transcriptional regulation is the level of protein expression. In Drosophila Schneider cells, low doses of Krüppel protein activate transcription, while high doses repress transcription (28). However, this result could not be reproduced in mammalian cells (41), again pointing to cell type-specific functions of transcriptional repressors. We could not find a clear relationship between WT1 expression level and transcriptional function, as both high and low levels of WT1 protein were associated with both transcriptional activation and repression, depending on the target promoter and presence or absence of the pCB6+ expression vector.

We propose a novel factor which influences the transcriptional effect of WT1, namely that the promoter driving protein expression can have a profound effect on the observed action of the WT1 protein. In reviewing the literature, virtually all prior studies which demonstrated repression by WT1 used the pCB6-
uble system (46) WT1 activates rather than represses the Egr1 reporter gene (47). Third, we found that RSV-expressed WT1 can activate a RSV-β-galactosidase reporter (data not shown), suggesting that WT1 can bind to the RSV promoter, probably through GC-rich sites and activate transcription directly rather than by a promoter competition effect. Last, it was demonstrated (48) that WT1 protein binds to pCB6- through the GC-rich sites of the SV40 promoter in this expression vector (Fig. 1B). Together this information supports the more simple model that WT1 directly binds the target gene and may be a default activator. The transcriptional effect of WT1 is then modulated by the presence or absence of co-factors which can be sequestered by the strong CMV promoter.

In further support of the hypothesis that WT1 needs to cooperate with factors in addition to the basal transcriptional machinery to activate transcription, our preliminary data show that WT1 cannot activate transcription through binding sites upstream of a minimal TATA box-containing promoter, whereas WT1 is a potent activator when these same sites are placed upstream of the HSV-tk promoter (Fig. 3 and Ref. 14). It is not yet known which transcription factors and/or co-factors act to augment the activation or repression functions of WT1. These proteins could be other sequence-specific DNA-binding proteins and/or transcriptional co-factors such as the transcription factor IID-associated proteins (reviewed in Ref. 49). One candidate factor might be the p53 protein, which was shown to affect the transcriptional activity of WT1, changing it from an activator to a repressor of transcription (20). Wang et al. (15) recently presented data for the existence of an as yet unidentified nuclear protein that interacts with WT1 to effect transcriptional repression. In vitro transcription studies using fractionated basal factors and transcriptional co-factors will be required to define the biochemical requirements for activation and repression by WT1.

Although all expression vectors co-transfected with the Egr1 reporter gene down-regulated the reporter gene to some extent, the pCB6+ vector was the most potent competitor and the only vector among the ones tested that cooperated with WT1 to facilitate transcriptional repression. The pCB6+ vector contains two promoters: the CMV major immediate-early promoter, which drives the expression of the insert cDNA, and the SV40 late promoter, present in the SV40 origin of replication, which drives expression of a neomycin resistance gene (described in Ref. 48) (Fig. 1). The Egr1-CAT reporter contains nucleotides −957 to +248 of the murine Egr1 promoter and includes binding sites for Egr1, AP-1, CREB, and SRF (27, 50) (Fig. 1). The serum response element is likely responsible for the induction of the Egr1 gene in the G1-G2 transition (29, 50). Since the rat β-actin and RSV promoters contain serum response elements, whereas the CMV promoter does not (25, 51–53), it is unlikely that SRF or other proteins that bind to the serum response element are involved in determining the activation or repression activity of WT1. However, the CMV promoter contains multiple binding sites for Sp1, AP1, AP2, and CREB and might exert its strong negative effect on the Egr1 promoter by competing for binding of these or other sequence-specific DNA binding transcription factors or their respective co-factors (25). pCB6+ also differs from the RSV and β-actin expression vectors as it contains a pUC18 rather than a pBR322 backbone. However, the pUC18 backbone is unlikely to play a role in the unique ability of pCB6-WT1 to convert WT1 into a repressor, as co-transfection of RSV-WT1 along with pBluescript, another pUC18-based plasmid, still leads to activation of the Egr1 reporter gene (Fig. 6 and data not shown).

Our data indicate a relationship between the ability of the target reporter gene to be competed by the pCB6+ promoter and the ability of WT1 to repress the reporter. The pCB6+ plasmid has a significantly smaller effect on the basal activity of EGR3-CAT (8-fold) than on Egr1-CAT (70–100-fold) (Figs. 2 and 5). In turn, WT1 expressed from the pCB6+ vector can still activate transcription from EGR3-CAT (Fig. 3). However, it is important to note that pCB6-WT1 activated the EGR3-CAT promoter less efficiently than either RSV-WT1 or β-actin-WT1, despite the fact that approximately 10 times more WT1 protein is produced by pCB6-WT1 than by the other vectors (Fig. 4B). This latter result suggests that WT1 expressed at these high levels leads to ineffective trans-activation due to transcriptional self-squelching (41, 54). Alternatively, promoter competition by pCB6+ might partially alter the transcriptional state of EGR3-CAT, making it less responsive to trans-activation by WT1.

Our results highlight a major pitfall in the use of co-transfection experiments to assay transcriptional effects. The use of a potent expression vector may have a profound effect on the target gene. While the β-actin and RSV promoters had relatively modest effects on the Egr1 target gene, the CMV promoter led to as much as a 60–100-fold decrease in Egr1 promoter activity (Fig. 2A and data not shown). Therefore the action of the expression vector may overwhelm the effect of the expressed protein, as in this report, where the CMV expression vector changed WT1 from an activator to a repressor. A clear implication of this work is the need to devise more refined procedures for the identification of authentic WT1 target genes. One such method would be to stably express WT1 in a suitable target cell line in an inducible manner and examine the expression pattern of the putative target genes. This approach was recently taken, using the tetracycline-repressor system (46) to tightly induce expression of WT1 in osteosarcoma cells (47). Many of the genes previously identified as WT1 targets by co-transfection assay failed to respond WT1 induction in this endogenous gene assay. A notable exception was the epidermal growth factor receptor, which was down-regulated by the expression of WT1. Of note, transfection of the Egr1 reporter gene into SAOS cells followed by induction of WT1 expression by tetracycline withdrawal was associated with activation of the Egr1 promoter, in accordance with our data. Another group forced the constitutive expression of WT1 in G401 cells leading to the down-regulation of both an IGF1-R–luciferase reporter gene and the endogenous IGF1-R gene (38). This correlated with a reduction of the growth rate and IGF1 responsiveness of the G401-WT1 cells. More studies of this type, focusing on the response of endogenous genes in correlation with a phenotype relevant to tumor suppression or kidney development, will be required to fully understand the direction and magnitude of the transcriptional actions of WT1.

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