The Wilms’ tumor suppressor gene (wt1) product represses different functional classes of transcriptional activation domains

Tae Ho Lee¹, Peter Moffett¹ and Jerry Pelletier¹,2,*

¹Department of Biochemistry and ²McGill Cancer Center, McGill University, Room 902, 3655 Drummond Street, Montreal, Quebec H3G 1Y6, Canada

Received March 19, 1999; Revised and Accepted May 22, 1999

ABSTRACT

We have studied the ability of the wt1 tumor suppressor gene product to repress different classes of activation domains previously shown to stimulate the initiation and elongation steps of RNA polymerase II transcription in vivo. Repression assays revealed that WT1 represses all three classes of activation domains: Sp1 and CTF, which stimulate initiation (type I), human immunodeficiency virus type 1 Tat fused to a DNA-binding domain, which stimulates predominantly elongation (type IIA), and VP16, p53 and E2F1, which stimulate both initiation and elongation (type IIB). WT1 is capable of exerting its repression effect over a significant distance when positioned ~1700 bp from the core promoter. Deletion analysis of WT1 indicates that the responsible domain resides within the first 180 N-terminal amino acids of the protein. Nuclear run-ons analyzing the effects of WT1 on initiation of transcription demonstrate inhibition of this process. Our observations imply that WT1 can repress activators that stimulate initiation and/or elongation.

INTRODUCTION

Wilms’ tumor (WT) is a malignancy which affects 1 in 10 000 children, usually before the age of 5 years (1). It is thought to arise when multipotential cells of the metanephric blastema fail to differentiate and remain locked in a state of continual proliferation. A tumor suppressor gene, wt1, implicated in predisposition to WT and aberrant differentiation of the urogenital system (7).

The wt1 gene encodes a protein having many characteristics of a transcription factor, including a Gln/Pro-rich N-terminus, nuclear localization and four Cys₂-His₂ zinc finger motifs (reviewed in 2). The three C-terminal-most zinc fingers share 67% identity to the three zinc fingers of the early growth response gene-1 (EGR-1). The mRNA contains two alternative sites of translation initiation (8), two alternatively spliced exons (9,10) and undergoes RNA editing (11), thus potentially encoding 16 different protein isoforms with predicted molecular masses of 49–65 kDa. The function of the alternative translation initiation event, the RNA editing modification and the first alternative splicing event (exon V) have not been well defined, although exon V can repress transcription when fused to a heterologous DNA-binding domain (12). Alternative splicing of exon IX inserts or removes three amino acids (±KTS) between zinc fingers III and IV and changes the DNA-binding specificity of WT1 (13). The WT1 (+KTS) isoforms can bind to two DNA motifs: (i) a GC-rich motif, 5'-G Y GT-GGGCCY-3', similar to the EGR-1-binding site (13); and (ii) a 5'-TCC-3'-containing sequence (14). A role for WT1 in splicing has been postulated based on the subnuclear localization of WT1(+KTS) isoforms and the interaction of WT1 with the U2AF65 splicing factor (15,16). A number of genes involved in growth regulation and cellular differentiation contain WT1-binding sites within their promoters and their expression can be modulated by WT1 in transfection assays (for an example of some of these, see 2).

The wt1 gene product has been shown to mediate both transcriptional repression and activation (2). Initial experiments focusing on transcriptional regulation by WT1 bound to Egr-1 consensus sequences revealed that WT1 was capable of repressing transcription and that this effect is mediated by sequences within the N-terminus of WT1 (17). However, further characterization of the WT1 transcriptional properties and assessment of activity on putative cellular target genes revealed that WT1 can also activate transcription (18). Transcriptional regulation by WT1 appears complex and a number of factors are known to influence whether WT1 acts as a repressor or activator (reviewed in 2). The transcriptional activity of WT1 can be modulated by interaction with three proteins, both interacting with the WT1 zinc fingers: (i) the p53 tumor suppressor gene product (19); (ii) the par-4 (prostate

*To whom correspondence should be addressed: Tel: +1 514 398 2323; Fax: +1 514 398 7384; Email: jerry@med.mcgill.ca
apoptosis response-4) protein (20); and (iii) recently, physical association of WT1 with Hsp70 has suggested that Hsp70 may also play an important role in the regulation of WT1 (21). An understanding of the molecular mechanism(s) by which WT1 exerts its effect on gene expression would shed light on the different transcriptional properties of WT1.

Transcriptional repression can occur by several general mechanisms. These include: (i) occlusion of DNA binding by competition for a DNA-binding site(s) also used by an activator; (ii) interference (or squelching) by sequestration of an activator or an essential co-factor (this phenomenon is not DNA binding dependent); (iii) quenching, in which a repressor assembled at a given site suppresses transactivation by interacting with an adjacent transactivator; and (iv) direct repression by interference with the formation or activity of the basal transcription complex; and (v) alteration of histone acetylase/deacetylase activity to alter the dynamic equilibrium of core histone acetylation. In this report, we have evaluated the ability of WT1 to interfere with different classes of transcriptional activators. Our results indicate that WT1 can inhibit all classes of transcriptional activators (i.e. those that stimulate initiation and/or elongation of transcription).

**MATERIALS AND METHODS**

**Plasmid construction**

Preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, DNA ligation and bacterial transformations were carried out using standard methods (22 and references therein). Subclones of DNA PCR amplifications were always sequenced by the chain termination method using double-stranded DNA templates to ensure the absence of mutations (23). Expression vectors for Gal4 fusion proteins have been previously described (24). The HSP/CAT reporter constructs have been previously described and contain the human hsp70 sequences from –40 (TATA region only) to approximately +160 (25). The c-myc reporter constructs were generated by excising the hsp70 promoter from XhoI/HpaI/CAT and G3HSP/CAT with SalI and SmaI, repairing with T4 DNA polymerase and replacing with a BamHI–SacI (T4 DNA polymerase repaired) fragment of the c-myc promoter obtained from pSP72 Xba-Sac Myc CAT.

The WT1–LexA fusions were generated by overlapping extension PCR. For generating pcDNA3/LexA MTN, a LexA fragment was amplified by PCR using primers LEXH3 (5'-CT-TAAAGCTTACATGAAACGTAAACGGC-3'; HindIII site underlined) and LEXNE (5'-TTCGAATTCATGGTGCGACCAGCAGTGCCGTTG-3'; EcoRI site underlined), digested with HindIII and EcoRI and cloned into the same sites of pKS/LexA. To create a LexA DNA-binding domain that had an epitope tag and would localize to the nucleus, the NeoI fragment of pCS2+MTNLS (containing the SV40 NLS and five Myc epitope tags in tandem) (unpublished data) was ligated into the NeoI site of pKS/LexA. This places the SV40 NLS and five Myc epitope tags downstream of the LexA DNA-binding domain. The HindIII–EcoRI fragment of this construct was then ligated into the same sites of pcDNA3 to create pcDNA3/LexA MTN. pcDNA3/WT1-LexA was generated by transfer of the Ndel–HindIII fragment of WT1 derived from CMV/WT1-Gal4 (26) into the Ndel and HindIII sites of pcDNA3/LexA MTN, pcDNA3/WT1-LexA deletion mutants were generated as follows. (i) WT1–LexA(Δ1–84). The wtI fragment was generated by PCR using primers 5’-th8 (5'-GTGAAGCTTATGTTCCCAATGCGCCC-3') and 3’-th2 (5'-TCCCAAGCTTTTCAGATGCGTTGACGGGCTCG-3'), digested with HindIII (both PCR primers contain HindIII sites) and cloned into the HindIII site of pcDNA3/LexA MTN. This places the wtI gene upstream and in-frame with the LexA DNA-binding domain. The same general strategy was used in fusing all other wtI deletions upstream of the LexA domain. (ii) WT1–LexA(Δ1–126). The wtI fragment was generated by PCR using primers 5’-th8 (5'-GTGAAGCTTATGTTCCCAATGCGCCC-3') and 3’-th2, digested with HindIII and cloned into the HindIII site of pcDNA3/LexA MTN. (iii) WT1–LexA(Δ1–181). The wtI fragment was generated by PCR using primers 5’-th3 (5'-GTTAAGCTTATGCATGTTGTGATGGCG-3') and 3’-th5 (5'-TCCAAAGCTTTTCAGATGCGTTGACGGGGACG-3') from a wtI expression vector containing the exon V alternative splice site, digested with HindIII and cloned into the HindIII site of WT1–LexA, (iv) WT1–LexA(Δn2). The wtI fragment was generated by PCR using primers 5’-th6 (5'-GTGAAGCTTACATGAAACGTAAACGGC-3') and 3’-th2 (5'-TCCCAAGCTTTTCAGATGCGTTGACGGGCTCG-3') from CMV/WT1(−/−), digested with HindIII and cloned into the HindIII site of WT1–LexA. (v) WT1–LexA(Δn2). The wtI fragment was generated by PCR using primers 5’-th4 (5'-GTGAAGCTTACATGAAACGTAAACGGC-3') and 3’-th5 (5'-TCCAAAGCTTTTCAGATGCGTTGACGGGACG-3') from a wtI expression vector containing the exon V alternative splice site, digested with HindIII and cloned into the HindIII site of WT1–LexA, (vi) WT1–LexA(Δn2). The wtI fragment was generated by PCR using primers 5’-th4 (5'-GTGAAGCTTACATGAAACGTAAACGGC-3') and 3’-th5 from pACT/WT2(D201G) (27), digested with HindIII and cloned into the HindIII site of pcDNA3/LexA MTN.

**Cell lines, transfections and CAT assays**

COS-7 and 293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL), penicillin and streptomycin on 100 mm diameter tissue culture plates. C33A cells were maintained in α-modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum with penicillin and streptomycin. The cells were transfected by the calcium phosphate precipitation method (22). Unless stated otherwise, total DNA per plate of cells was 2 µg of pR5V/β-gal vector, 2 µg of reporter plasmid, 4 µg of activator expression plasmid and 4 µg of repressor expression vector. Individual DNA precipitates were adjusted to contain equal amounts of total DNA by the addition of the empty expression vector, pcDNA3. All transfections and subsequent CAT assays were performed at least in duplicate. Cells were washed and refed 16 h post-transfection and harvested ~48 h later. Cells were scraped from the dishes following a phosphate-buffered saline (PBS) wash, centrifuged and resuspended in 150 µl of 250 mM Tris (pH 8.0). They were then subjected to three rounds of freeze–thaw, an aliquot was taken for measurement of β-galactosidase activity and the remainder of the extract heated to 65°C for 10 min and then assayed for CAT activity (28). Following thin layer chromatography, regions containing acetylated [14C]chloramphenicol, as well as unacetylated [14C]chloramphenicol, were quantified by direct analysis on a PhosphorImager (Fujix BAS 2000). All CAT activity values were normalized to β-galactosidase values, which served as internal controls for the transfections.
Nucleic Acids Research, 1999, Vol. 27, No. 14

**Nuclear run-on analysis**

Tissue culture cell nuclei were prepared by lysis in buffer A (0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM HEPES, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.5 mM spermidine, 0.15 mM spermine, 0.1% NP-40) and pelleting at 1500 g. The nuclei (2 x 10^7) were frozen at -70°C until ready for use in 100 µl of storage buffer [50% glycerol, 20 mM Tris, pH 7.9, 75 mM NaCl, 0.5 mM EDTA, 0.85 mM dithiothreitol, 0.125 mM phenylmethylsulfonyl fluoride (PMSF) and 100 U/ml RNasin]. The elongation reaction was carried out in reaction buffer (0.3 M (NH₄)₂SO₄, 100 mM Tris, pH 7.9, 4 mM MgCl₂, 4 mM MnCl₂, 50 mM NaCl, 0.4 mM EDTA, 0.1 mM PMSF,

---

**Figure 1.** Schematic representation of reporter and expression vectors used in this study. For reporter constructs, the filled box denotes the CAT coding region, the speckled box represents the human hsp70 promoter, the diagonal lined box symbolizes the Gal4 DNA-binding sites (two copies are present), boxes with horizontal lines denote LexA DNA-binding sites (six copies are present) and checkered boxes symbolize the c-myc promoter region. The transcription termination region (T2) present within exon 1 of c-myc is represented by a small solid box. Underlined regions below X₆G₂-Myc/CAT represent the regions of this construct against which probes were designed for use in nuclear run-on assays. The DNA-binding domain of the Gal4 fusion constructs is shown as a hatched box. A schematic representation of the WT1–LexA fusions generated for this study is also shown. The LexA DNA-binding domain (including the Myc tags and SV40 nuclear localization signals) is represented by a hatched box. WT1–LexA (+ES) contains the first alternatively spliced exon of WT1 (exon 5, 17 amino acids) and this is represented as a filled box. WT1–LexA (+Zn) contains the four WT1 zinc fingers and these are represented as gray boxes (and labeled I–IV), whereas the upstream non-zinc finger domain of WT1 is represented by an open box. WT1–LexA(D201G) contains a missense mutation at amino acid 201 converting a Gly residue to Asp, a mutation previously documented to convert WT1 from a transcriptional repressor to an activator (5). A schematic representation of the experimental design is shown.
1.2 mM dithiothreitol, 1 mM GTP, ATP and CTP, 10 mM creatine kinase, 20 U/ml RNasin and 150 µCi [3H]UTP for 30 min at 27°C, at which point 100 µg of carrier tRNA was added. The RNA was purified by DNase I and proteinase K digestion, phenol/ chloroform extracted and passed over a Sephadex G-50 spin column. To remove unincorporated [3H]UTP, 10% TCA and 60 mM sodium pyrophosphate were added for 10 min and the precipitate collected by centrifugation. The RNA pellet was resuspended in 250 µl of 20 mM HEPS (pH 7.5), 5 mM EDTA and 62.5 µl of 1 M NaOH was added followed by incubation on ice for 13 min and then the hydrolysis reaction was stopped by adding 125 µl of 1 M HEPS (pH 5.5), followed by ethanol precipitation. Prehybridization of nitrocellulose filters was performed at 42°C overnight and they were then hybridized at 42°C for 4 h. Prehybridization and hybridization solutions consisted of 50 mM HEPS (pH 7.0), 0.75 M NaCl, 50% formamide, 0.5% SDS, 2 mM EDTA, 10× Denhardt’s, 500 µg/ml salmon sperm DNA and 10 µg/ml poly(rA). Five washes with 0.1× SSC, 0.1% SDS were performed at 65°C for 20 min each and filters were exposed to X-ray film (X-OMAT; Kodak) with an intensifying screen.

Single-stranded M13 probes correspond to the following: probe G, 66 base HindIII–SalI fragment of G5/HSP/CAT; probe Myc1, 98 base HindII–AluI fragment of the mouse c-myc promoter; probe Myc2, 170 base AluI–HindII fragment of the myc promoter; probe Myc3, 71 base HpaII–SacI fragment of the myc promoter; probe Myc4, 265 base BglII–EcoRI fragment of the CAT gene; probe Myc5, 418 base EcoRI–ScaI fragment of the CAT gene. As an internal control, a 540 base XbaI–EcoRI fragment of the firefly luciferase gene was used.

RESULTS

WT1 can repress different functional classes of activators

Since it is not known if WT1 inhibits initiation and/or elongation of transcription, we utilized an assay system in which WT1 repression was measured from reporter constructs under the transcriptional influence of well-characterized activation domains (Fig. 1). The activation domains used herein can be classified into three different classes: (i) type I, which stimulate transcription initiation; (ii) type IIA, which stimulate predominantly elongation; and (iii) type IIB, which exert their effects by stimulating both initiation and elongation (24). Thus, by defining the class of activators which could be repressed by WT1, we wished to gain insight into the mechanism of repression of WT1. Reporter constructs contained either the minimal heat shock response promoter (HSP) 70 elements or c-myc regulatory elements (Fig. 1). A series of activation domains fused to the Gal4 DNA-binding domain of Saccharomyces cerevisiae were co-expressed with the various reporter constructs in COS-7, C3A and 293T cells. The activation fusions utilized in this study were: (i) Sp1 [Gln-rich] and CTF [Pro-rich] (type 1 activators); (ii) Tat (a type IIIA activator); and (iii) VP16 [acidic domain], p53 [acidic] and E2F1 [acidic] (type IIB activators) (24). In this experimental design, we are to infer inhibition on activators implicated in initiation, elongation or both (Fig. 1).

When G5/HSP/CAT or X5,G/HSP/CAT was transfected into COS-7 cells either alone or in combination with WT1–LexA, very little CAT activity was observed (T.H.Lee and J.Pellietier, data not shown). These results demonstrate that WT1 does not act as an activator under our experimental conditions. When G5/HSP/CAT or X5,G/HSP/CAT was co-transfected with LexA MTN and Gal4–VP16, Gal4–E2F1 or Gal4–p53, significant levels of activation were observed (Fig. 2A). Co-transfection of G5/HSP/CAT with WT1–LexA in combination with various Gal4 fusions did not appreciably alter the activation status (Fig. 2A). However, similar experiments utilizing the X5,G/HSP/CAT reporter demonstrated that for all type IIB activators tested, WT1–LexA was capable of exerting repression. These results are not cell type-specific since the same general trend was also observed in C3A and 293T cell lines (T.H.Lee and J.Pellietier, data not shown).
Figure 2B illustrates a dose–response experiment in which increasing amounts of WT1 were introduced in the presence of a constant amount of Xg2HSP/CAT and Gal4–VP16 in COS-7 cells. Inhibition of Gal4–VP16-mediated activation was dose responsive and reached a plateau with ~10 µg of WT1–LexA DNA (Fig. 2B). Western blotting of nuclear extracts from the

**Nucleic Acids Research, 1999, Vol. 27, No. 14** 2893
transfected cells demonstrated that increasing amounts of WT1–LexA were synthesized in response to increasing amounts of transfected plasmid (Fig. 2C).

In an attempt to determine if WT1 could inhibit activators that stimulated predominantly initiation or elongation, we tested WT1–LexA in combination with either a type IIA activator (Gal4–Tat) or type I activators (Gal4–Sp1 and Gal4–CTF) (Fig. 2D). In the presence of Gal4–HSP/CAT, WT1–LexA had no effect on Gal4–Tat-mediated activation. However, when LexA DNA-binding sites were present in the reporter, X6G2HSP/CAT, WT1 repressed Gal4–Tat-mediated activation ~3-fold. With Gal4–Sp1 and Gal4–CTF, WT1–LexA also repressed activation mediated on the X6G2HSP/CAT reporter. These results were also reproduced in C33A and 293T cells (T.H. Lee and J.Pelletier, data not shown) and suggest that WT1 is capable of repressing activators that function at the level of transcription initiation or elongation or both.

To ensure that the observed results were not specific for the HSP promoter, we assessed the ability of WT1 to mediate repression of Gal4 activation fusions on a different reporter system, one which employed the c-myc promoter (Fig. 3). Our results indicate that WT1 is also capable of repressing activated transcription mediated by type IIB activators in this system as well (Fig. 3). This data indicates that the observations reported above are not specific to the HSP basal promoter.

To address whether relative distance between WT1 and the activator was an important factor in determining the strength of the observed repression, we used a series of constructs in which the LexA and Gal4 DNA-binding sites were separated from each other by increasing distances. WT1 repression of type IIB-mediated activation on X6G2HSP/CAT was compared to that obtained with reporters where the LexA and Gal4 DNA-binding sites were separated by 294 (X6[300]G2HSP/CAT) or 1671 nt (X6[1700]G2HSP/CAT) (Fig. 4). We note that in the presence of Gal4–E2F1 or Gal4–p53, X6[300]G2HSP/CAT and X6[1700]G2HSP/CAT were less efficiently transactivated than X6G2HSP/CAT. We do not understand the reason for this and this effect was not as pronounced when Gal4–VP16 was used as activator (Fig. 4). Clearly, however, for all three activators tested WT1 is capable of mediating repression over a significant distance (Fig. 4). Despite the observation that X6[1700]G2HSP/CAT is not as strongly activated as X6G2HSP/CAT, for all activators tested, WT1 mediates a 5- to 6.7-fold repression on X6[1700]G2HSP/CAT (Fig. 4). These values are in the same range as the fold repression mediated by WT1 on X6G2HSP/CAT (3.4- to 6.7-fold). Thus, there does not appear to be an apparent decrease in WT1 repression activity when WT1 is separated from the activator and basal promoter by as much as 1671 nt.

The N-terminal domain of WT1 is required for repression of type IIB activators

To define the domain responsible for inhibiting activation by type IIB activators, a series of WT1–LexA deletion constructs were generated (Fig. 1). In this set of experiments, transcription of X6G2HSP/CAT was stimulated by Gal4–VP16 and the effects of different WT1–LexA deletions were tested. As documented above, co-transfection of WT1–LexA and Gal4–VP16 resulted in a 3-fold decrease in the activation potential of Gal4–VP16 (Fig. 5). Deletion of the first 84 amino acid residues from the N-terminus of WT1 resulted in a LexA fusion [WT1–LexA(Δ1–84)] which could no longer suppress activation by Gal4–VP16 (Fig. 5). More pronounced N-terminal truncation deletions, WT1–LexA(Δ1–126) and WT1–LexA(Δ1–181) were also unable to repress VP16-mediated activation (Fig. 5). The presence of the first alternatively spliced exon (exon V) [WT1–LexA(+E5)] or of the four zinc fingers [WT1–LexA(+Zn)], in the context of the complete upstream N-terminal region, produced fusion products that repressed VP16 activation. We also tested the ability of a mutant carrying a D201G missense mutation, and previously reported to convert WT1 from a repressor to an activator (29), to function in this assay. This mutant behaved similarly to WT1–LexA and was capable of repressing VP16-mediated activation.

Nuclear run-on analysis of WT1 inhibition of initiation

We measured the ability of WT1 to inhibit transcription initiation directly by using the nuclear run-on assay. Gal4–VP16 was co-expressed in transiently transfected COS-7 cells with X6G2Myc/CAT and either LexA MTN or WT1–LexA (Fig. 1). After 48 h, nuclei were harvested and allowed to continue transcription in the presence of [α-32P]UTP. RNA was isolated and hybridized to a series of five single-stranded M13 probes spanning the length of the reporter plasmid (Fig. 1). The intensity of the signal on each probe provides a measure of the relative polymerase density on that region of the gene at the time the
nuclei were isolated. Thus, if polymerases are evenly distributed along the length of the gene, then elongation is presumed to be efficient. An inhibitory effect which blocks polymerase elongation should demonstrate more abundant polymerases at the 5'-end than at the 3'-end, indicating that polymerases are stalling or terminating prematurely. To control for non-specific upstream initiation events and transcription which reads all the way around the plasmid, a probe called G, located upstream of the c-myc start site, was included. In addition, M13 probes to the luciferase gene were used as internal controls since all transfections contained RSV/Luc, a luciferase expression vector. M13mp19 was also included on the filters and served as a negative control. So that elongation efficiencies could be easily compared, the autoradiographs in Figure 6 were exposed to give approximately equal signals for the 5'-end G probe and polymerase densities were plotted relative to density at the 5'-end. The c-myc promoter has a well-characterized transcription termination site within its 5'-untranslated region (30). After correction for transfection efficiency, the relative 5' polymerase densities were plotted as a function of distance from the 5'-MYC1 probe.

The VP16 activation domain has been documented to stimulate both initiation and elongation of transcription (24). However, it should be noted that this effect is dependent on the number of activation modules targeted to the promoter. Incremental increases in targeted activation potential results in corresponding increases in initiation, but stimulation of transcription processivity requires a threshold level of activation domain synergy that exceeds the level required for modest amounts of initiation (31). Thus, the use of two Gal4 DNA-binding sites upstream of the myc promoter limits our analysis, since stimulation of elongation by VP16 requires more sites (31). Transcription of G3Myc/CAT in the presence of Gal4(1–147) (i.e. in the absence of an activation domain) is extremely inefficient (Fig. 6) and polymerase densities along the reporter gene are very low. In the presence of Gal4–VP16, a marked increase in polymerase density at Myc1 was observed, relative to what was observed with Gal4(1–147) (Fig. 6). Also, a higher number of polymerase units were found at Myc2 relative to Myc1, consistent with the presence of a termination site within the myc 5'-untranslated region and with the fact that an insufficient number of Gal4–VP16 molecules were binding to be able to stimulate elongation (31). That polymerase units were inefficiently making their way through the T2 site is suggested by the apparent high density of polymerases accumulating before T2, relative to downstream sites Myc3, CAT4 and CAT5 (as judged by the higher intensity of the signal obtained with the Myc2 probe; Fig. 6). Addition of WT1–LexA to this system produced a significant reduction in the number of polymerase units present at the 5'-end, demonstrating inhibition of transcription initiation (Fig. 6).

**DISCUSSION**

Although a number of studies have characterized the transcriptional properties of WT1, none have addressed whether WT1 exerts an effect at the level of initiation and/or elongation of
transcription. In the present study, we demonstrate that WT1 can influence the activity of transcription factors that function by stimulating initiation and/or elongation. Madden et al. (32) were the first to demonstrate a repression activity of WT1. Since they used the Egr-1 promoter, which contained three Egr-1-binding sites and several AP-1- and serum response factor-binding sites, their studies demonstrated that WT1 could repress transcription from a complex, activated promoter (32).

Our results indicate that one mechanism by which WT1 can repress transcription is through inhibition of activators. Although we were unable to determine if inhibition is the outcome of a direct or indirect event, clearly several classes of activators were affected when co-expressed with WT1 (Fig. 2). Taken together with previous data, our results leave us with two non-mutually exclusive mechanisms by which WT1 may repress transcription. In our experimental set-up, WT1 does not inhibit transcription unless the LexA DNA-binding domain is present (T.H.Lee and J.Pelletier, data not shown). Thus, it is unlikely that WT1 inhibits transcription by interference in our system. Consistent with this is the finding by other investigators that WT1 must bind to DNA to mediate its transcriptional effects (for example ref. 32). We suggest that it is unlikely that WT1 mediates its effects by directly interacting with the adjacent activator and quenching its activity. This is based on the lack of conserved structural motifs among the activators used in our assays. VP16, p53 and E2F1 contain acidic activation domains whereas the Sp1 activation domain is Gln-rich and the CTF activation domain is Pro-rich. One mechanism by which WT1 could exert its effect is by direct interference with the basal transcription complex. Whether WT1 utilizes the same mechanism to interfere with both the initiation and elongation steps awaits further experimentation.

We observed that WT1 can exert repression over a large distance (i.e. when bound 1700 nt away from the promoter) (Fig. 4). Thus, WT1 may interact with components of the basal transcription machinery over a long rang, looping out intermediate DNA sequences. Like many other transcription factors, WT1 has a modular structure, with DNA-binding zinc fingers and upstream effector domains. The transcriptional effector domains of WT1 have been mapped by several groups under different conditions. Wang et al. (18) showed that amino acids 84–179 were required for suppression by WT1 through WT1-binding sites, while amino acids 180–294 encoded a transcriptional activation domain that activated through WT1 sites 5′ or 3′ to the promoter start site. Fusion of different regions of the WT1 protein to the Gal4 DNA-binding domain, followed by assessment of activity on a reporter construct containing Gal4 DNA-binding sites, confirmed that amino acids 84–180 were sufficient to confer repression (32). In the context of repressing activated transcription, when we deleted the first 84 amino acids from the N-terminus of WT1 we lost repression (Fig. 5). These results are not at odds with those cited above since Madden et al. (32) reported that deletion of amino acids 21–66 (the Pro- and Gly-rich region), in the context of the full-length protein, abolished repression, suggesting that these two domains may cooperate or contribute to the repression activity. Thus, clearly, deletion of the N-terminus of WT1 disrupts a domain important for active repression by WT1. Contrary to a previous report (29), a WT1 D201G missense mutation did not alter the transcriptional properties of WT1 in our assay, indicating that this mutation does not compromise the repression ability of WT1. An alternative interpretation we cannot exclude is that some of the mutant proteins used in this study have not retained the native conformation. The N-terminal domain required for repression is multifunctional since it has also been implicated in oligomerization of WT1 (27,33).

Our data do not address the ability of WT1 to activate transcription (12,18) since we failed to obtain this effect in our model assay system. WT1 can interact with two proteins, p53 and par-4, that modulate its transcriptional activity (19,20). In cells lacking p53, WT1 activated transcription from the Egr-1 promoter, whereas in cells containing p53, WT1 repressed this promoter (19). On the other hand, par-4 inhibits WT1-mediated activation and augments WT1-mediated repression by contributing an additional repression domain (20). Both p53 and par-4 interact with WT1 through the zinc finger domain (34). Since WT1–LexA does not harbor the zinc finger domain, it is unlikely that the observed inhibition we documented is influenced by p53 or par-4. In fact, the presence of the zinc finger domain in WT1–LexA(+Zn) did not alter the level of repression on Gal4–VP16, compared to the response seen with WT1–LexA. Thus, it is unlikely that the zinc finger domain participates in the response we have documented herein (Fig. 5).

In summary, we have directly demonstrated that WT1 inhibits initiation of transcription (Fig. 6). This was documented by determining the relative number of polymerase units at the 5′-end of the Myc/CAT reporter gene in the presence of the activator...
module Gal4–VP16. The presence of only two Gal4 DNA-binding sites in our reporter precluded us from directly analyzing any putative effects by WT1 on elongation (31). Also, WT1 was capable of exerting its effects on a large number of activation modules that stimulate initiation, predominantly elongation, and both.

ACKNOWLEDGEMENTS

We thank Dr R. E. Kingston for his kind gift of HSP/CAT reporter constructs and Dr D. Bentley for Gal4 activation domain fusion plasmids. T.H.L. is supported by a fellowship from the Medical Research Council of Canada. P.M. is supported by a fellowship for the Cancer Research Society. J.P. is a Medical Research Council of Canada Scientist. This work was supported by a grant from the Medical Research Council of Canada to J.P.

REFERENCES