# THE WT1 WILMS' TUMOR SUPPRESSOR PROTEIN: TRANSCRIPTIONAL ACTIVITY AND MODULATION OF FUNCTION BY TUMOR-ASSOCIATED MUTATIONS

by

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Abstract

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Advisor: Jonathan D. Licht, M.D.

The *WT1* tumor suppressor gene is mutated in a small subset of patients with Wilms' tumor, a childhood kidney cancer. *WT1* encodes four zinc finger DNA-binding transcription factors with both transcriptional activation and repression functions. In this thesis, I characterized the transcriptional effector functions of WT1 and studied the effect of tumor-associated *WT1* mutations on WT1 protein function. WT1 activated a simple test promoter containing three Egr-1/WT1 sites upstream of the HSV-*tk* promoter in transient transfection experiments. The WT1(B) isoform was a slightly stronger transcriptional activator than WT1(A). WT1 also repressed transcription under certain circumstances. A GAL4-WT1 fusion protein repressed transcription through GAL4 binding sites, but failed to regulate transcription through WT1 binding sites, suggesting that the WT1 moiety might be in a non-native conformation. Native WT1 repressed the Egr-1 promoter when it was expressed from a CMV-based expression vector, while WT1 expressed from an RSV-based expression vector activated this same promoter. Co-transfection of this CMV expression vector greatly depressed the basal transcriptional level of the Egr-1 reporter construct, and in this depressed state, WT1 expressed from the RSV vector could repress the promoter. This suggests that the CMV and Egr-1 promoters compete with each other for transcription factors or cofactors which may modulate the transcriptional function

of WT1. Expression of two genetically defined tumor-associated dominant negative *WT1* alleles which yield WT1 proteins unable to bind DNA inhibited transcriptional activation by wild-type WT1. The WT1 protein self-associated and associated with mutant WT1 proteins in an *in vitro* biochemical assay. I therefore propose that these dominant negative WT1 proteins act by binding to wild-type WT1 and inhibiting its transactivation function. In contrast, a tumor-associated point mutation of *WT1* yielded a protein which displayed DNA-binding, transactivation, transrepression, and self-association functions which were indistinguishable from the wild-type protein. This mutation may not be the primary etiologic event in this case of Wilms' tumor, and may be augmented by changes at a second Wilms' tumor locus.

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# CHAPTER I

# INTRODUCTION

## 1. Wilms' tumor - incidence, histology, and associated syndromes.

Wilms' tumor (nephroblastoma) is a childhood tumor of the kidney with an incidence of approximately 1 in 10,000, making it the most common solid tumor of childhood (124). Histopathological examination of Wilms' tumors often reveals a triphasic morpology, in which undifferentiated blastemal elements are present along with stromal and epithelial elements. These tissues recapitulate phases of normal kidney development, suggesting that development of Wilms' tumor may be related to an abnormal differentiation program in the kidney. Wilms' tumors are believed to originate from nephrogenic rests, residual islands of immature kidney tissue which are found in some apparently normal kidneys as well as in kidney tissue surrounding Wilms' tumors (11, 143).

Most cases of Wilms' tumor are unilateral, sporadic, and not associated with other symptoms of systemic disease. However, in rare cases, Wilms' tumor is found as part of one of three syndromes which all include an increased risk for the development of Wilms' tumor. These 3 Wilms' tumor-associated syndromes are believed to arise from a constitutional mutation, either inherited or arising *in utero*, which predisposes the child to the development of nephroblastoma. Characteristic of these syndromes is the more frequent occurrence of bilateral tumors which appear at an earlier age than in sporadic non-syndromic cases. The WAGR syndrome accounts for 1-2% of cases of Wilms' tumor and also includes aniridia (absence of the iris), genitourinary malformations, and mental

retardation. The Denys-Drash syndrome (DDS), which accounts for approximately 2% of all Wilms' tumors, also includes genitourinary malformations and glomerulonephropathy. The Beckwith-Wiedemann syndrome (BWS) includes the clinical findings of hemihypertrophy and macroglossia in addition to an increased risk of developing Wilms' tumor. In addition, a small number of cases of Wilms' tumor (most recently estimated at 5-7% (74)) occur bilaterally, have an early onset, and appear in a familial pattern but are not associated with any of the above-mentioned syndromic findings.

Because of this association between familial inheritance and bilateral, early onset tumors, Knudson *et al.* proposed a "two-hit" hypothesis for the development of Wilms' tumor, similar to the one which they had previously proposed for familial retinoblastoma (94, 95). This model states that both alleles of a putative tumor suppressor gene would have to be mutated in order for the tumor to develop. Familial cases were presumed to be the result of inherited or *in utero* mutations in one allele, which would predispose children to the development of tumors caused by mutations in the remaining wild-type allele. In addition, the model predicts that tumors in familial cases would be more likely to occur bilaterally, and would occur at an earlier age, than in sporadic cases.

However, the relative minority of familial cases of Wilms' tumor (less than 10% compared with 35-40% for retinoblastoma), and the demonstration of linkage between predisposition to Wilms' tumor and least three chromosomal loci suggest that the genetics of Wilms' tumor are more complex than those of retinoblastoma. The WAGR syndrome is associated with cytogenetically detectable deletions of chromosome band 11p13 (46, 74, 166). In addition,

some cases of sporadic Wilms' tumor were also shown to be associated with loss of heterozygosity at 11p13, suggesting an important role for this locus in the development of Wilms' tumor (42, 99, 141, 165). The WAGR-associated deletion was later determined to encompass at least two genes: an aniridia gene (AN2/Pax-6) (195) and a putative Wilms' tumor suppressor gene, WT1, which will be the focus of this review. In addition, two other loci have been linked to predisposition to Wilms' tumor. A locus at 11p15 has been linked to both sporadic cases of Wilms' tumor and cases of Beckwith-Wiedemann syndrome (27, 77, 98), and changes in imprinting of the IGF-II and H19 genes, both located at 11p15, have been implicated as a mechanism of tumorigenesis (136, 137, 158, 190, 215). In addition, a third locus at 16q has been implicated in the development of some cases of familial Wilms' tumor (27, 125).

## 2. Cloning and characterization of *WT1* and its gene product.

**2A. Positional cloning of WT1.** The *WT1* (Wilms' tumor 1) gene was positionally cloned by two groups using different strategies. Call *et al.* (20) constructed a cosmid library spanning the short arm of chromosome 11 and isolated from this library a DNA segment which was deleted in a patient with Wilms' tumor who had a cytogenetically demonstrated abnormality at 11p13. This cosmid was then used to probe several cDNA libraries, and a cDNA clone, WT33, was isolated. By northern blot analysis, this clone detected an mRNA transcript in baboon kidney and spleen as well as in mouse kidney. Gessler *et al.* (56) used chromosome jumping to isolate the *WT1* gene. They began by isolating a DNA fragment near the putative *WT1* locus containing a CpG island, which often denotes the 5' end of a gene, and subsequently probed libraries made with rare-cutting enzymes to obtain sequences both 5' and 3' of this initial CpG island. A DNA fragment was isolated which mapped within the region of

11p13 deleted in WAGR patients, and this fragment was used to isolate a cDNA clone, LK15, from a human fetal kidney cDNA library. LK15 sequences were shown to be deleted in two cases of Wilms' tumor. This clone overlapped with the WT33 clone reported by Call *et al.* (20), and the gene was later termed *WT1*. The *WT1* cDNA was found to encode a putative zinc finger transcriptional regulator (see below).

**2B. Identification of WT1 as a tumor suppressor gene.** Studies of several patients confirmed the nature of *WT1* as a tumor suppressor gene. Shortly after it was cloned, *WT1* was found to be mutated in a patient with a sporadic unilateral Wilms' tumor (64). In this patient, a 25-bp deletion resulted in aberrant splicing of the *WT1* mRNA, yielding an mRNA which lacks coding sequences for the third zinc finger and its associated alternatively spliced sequences (see Figure 2). Interestingly, this mutation was found to be heterozygous, suggesting that this mutant allele acted as a dominant negative mutation; this has been the focus of further study by our group (163). *WT1* transcripts were found to be absent or reduced in some cases of Wilms' tumor (80), and *WT1* was shown to be heterozygously deleted in the germline and homozygously deleted in the tumor of a patient with bilateral nephroblastoma (83). However, the majority of Wilms' tumors studied have shown no apparent WT1 mutations (see below) and many express high levels of *WT1* mRNA. This evidence, in addition to the linkage between development of Wilms' tumor and other genetic loci, suggests that there may be several different mechanisms for the pathogenesis of Wilms' tumor.

Mutations in the *WT1* gene were also found in patients with the Denys-Drash syndrome (147). This syndrome is similar to WAGR, in that it includes a

predisposition to the development of Wilms' tumor. However, in contrast to WAGR, these patients have much more severe genitourinary abnormalities, including pseudohermaphroditism and streak gonads. DDS patients also have the additional finding of glomerulonephropathy. Germline heterozygous point mutations associated with DDS occurred in the zinc finger region and were shown to disrupt DNA binding by WT1. Subsequently, other mutations were isolated which truncated the WT1 protein N-terminal to the zinc fingers (see Table 4). Most of the tumors that developed in these patients were shown to be reduced to homozygosity for the mutant alleles (113, 147). Nevertheless, the profound genitourinary problems of these patients led to the hypothesis that these heterozygous mutations could be acting as dominant negative mutations (147). We have recently described a mechanism by which heterozygous mutations yielding non-DNA-binding forms of WT1 could lead to interference with wild-type WT1 function by physical association between mutant and wild-type WT1 proteins (163).

**2C. Expression pattern of WT1 mRNA and protein and the role of WT1 in normal development.** The positional cloning experiments described above showed that *WT1* was expressed in the kidney and spleen (20, 56). Since then, multiple studies have examined the species-, tissue-, and developmental-specific expression pattern of the *WT1* mRNA and protein. These studies are summarized in Table 1A.

**2C-1. Development of the kidney.** The pattern of *WT1* expression in the kidney suggests roles for *WT1* in multiple stages of renal development. A diagram illustrating the stages of kidney development is presented in Figure 1. Briefly, the ureteric bud (UB), the precursor of the collecting system, and the

metanephric mesenchyme (MM) undergo a series of reciprocal inductive events leading to formation of the mature nephron. Initially, the invading ureteric bud induces condensation of the mesenchyme. This condensing mesenchyme (CM) forms a renal vesicle (RV), in which the cells undergo a mesenchymal-to-epithelial transition. The renal vesicle then matures through the comma-shaped body and S-shaped body stages. Association with the capillaries which will form the glomerulus (G) and fusion with the nascent collecting system occur during the elongation stage, eventually culminating in the genesis of the mature nephron.

In human embryos, *WT1* is expressed in the condensing mesenchyme, renal vesicle, and glomerular epithelium of the developing kidney (152). In the mouse, *WT1* expression in the kidney increases in late gestation and then declines after birth (19). Other studies confirmed these findings (149, 156). The decline in *WT1* expression likely occurs later in the mouse than in humans, as kidney development continues postnatally in mice, while it is finished before birth in humans.

**FIGURE 1** (see next page). A brief summary of kidney development. MM = metanephric mesenchyme; UB = ureteric bud; CM = condensing mesenchyme; RV = renal vesicle; G = glomerulus; PT = proximal tubule. This figure is adapted from (199).

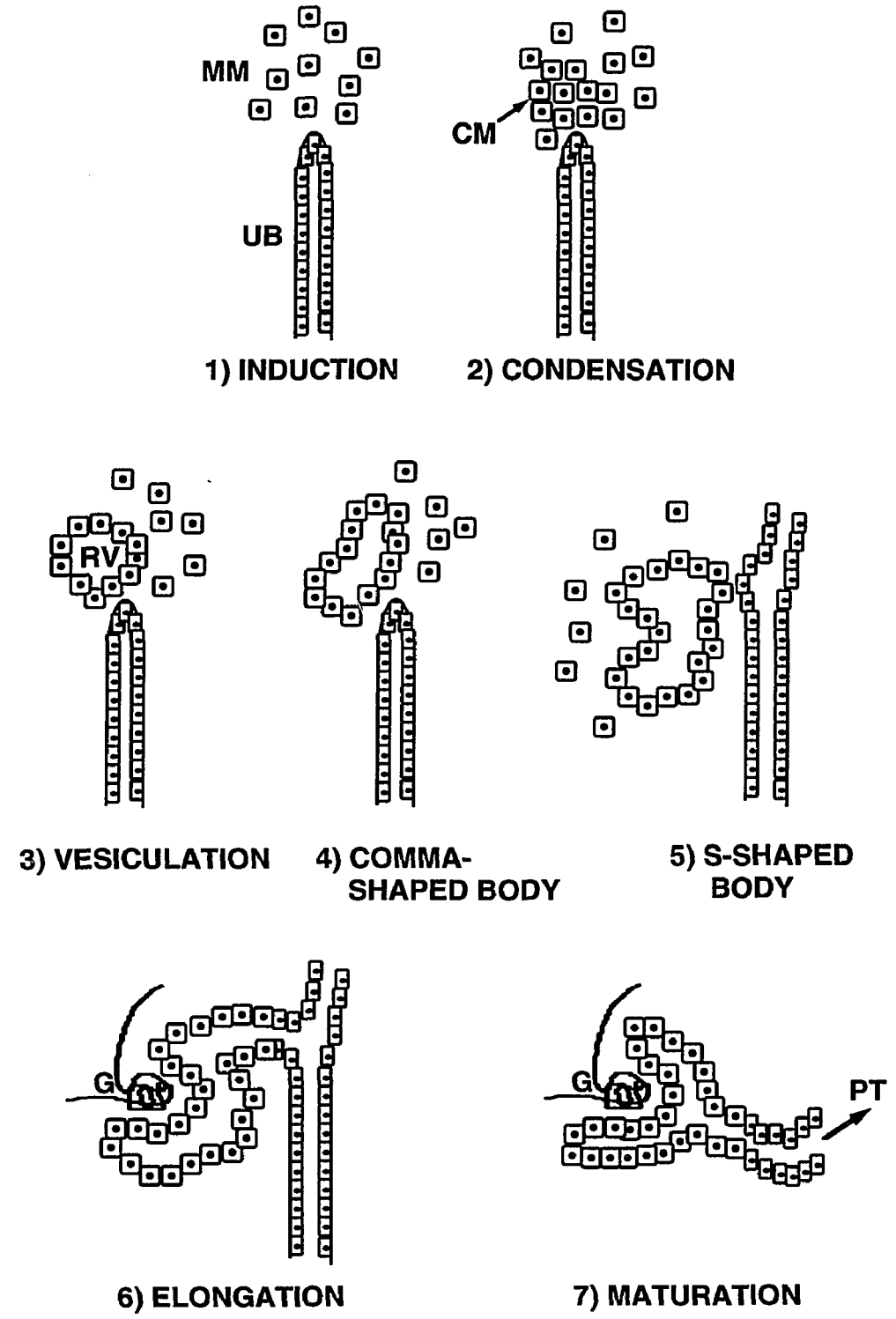



**FIGURE 1.** (see previous page for legend).

# TABLE 1A
## Expression pattern of *WT1* mRNA and protein

Days are indicated as E = embryonic; P = postnatal; p.c. = postcoital; wk = weeks. CM = condensing mesenchyme; RV = renal vesicle.

| LOCATION | STAGE | mRNA/ PROTEIN | SPECIES | REF. |
|---|---|---|---|---|
| **KIDNEY** | | | | |
| Total | adult | mRNA | baboon, mouse | (20) |
| | fetal | mRNA | human | (56) |
| | E13-P15 | mRNA | mouse | (19) |
| CM, RV, glomerular epithelium | 18wk | mRNA | human | (152) |
| Blastemal cells | 10-15wk | protein | human | (62) |
| Podocytes | 7 wk, adult | protein | human | (132) |
| **GONADS** | | | | |
| Genital ridge | 18wk | mRNA | human | (152) |
| Gonad | 18wk | mRNA | human | (152) |
| Granulosa and epithelial cells (ovary) | adult | mRNA | mouse | (149) |
| Sertoli cells (testis) | adult | mRNA | mouse | (149) |
| Uterine wall | adult<br>E10.5 | mRNA | mouse | (149);<br>(156) |
| Uterus (pregnancy) | day 9.5 p.c.<br>day 6 p.c. | mRNA | mouse, rat | (156);<br>(216) |
| **SPLEEN** | adult | mRNA | baboon | (20) |
| Stromal cells | adult | mRNA | mouse | (144) |
| **HEART** | adult | mRNA | baboon | (20) |
| **MESOTHELIUM** | 18wk | mRNA | human | (152) |
| | E18 | mRNA | mouse | (144) |
| **NERVOUS SYSTEM** | | | | |
| Spinal cord | adult | mRNA | rat | (185) |
| Ependymal layer | E13.5 | mRNA | mouse | (156) |
| Motor neurons | E11 | mRNA | mouse | (4) |
| Area postrema | adult | mRNA | rat | (185) |
| Roof of fourth ventricle | E15.5 | mRNA | mouse | (4) |
| **MUSCLES** | | | | |
| Differentiating body wall | E13.5 | mRNA | mouse | (4) |
| **OTHER** | | | | |
| Eye, Tongue | E12.5, adult | mRNA | mouse | (4) |

Recently, studies of the temporal and spatial characteristics of WT1 protein expression have been made possible by the production of antibodies to WT1. Mundlos *et al.* (132) showed that WT1 protein is expressed in the podocyte cells of the glomerulus during kidney development in humans and that this expression continues throughout adulthood. While they detected *WT1* mRNA in developing metanephric blastema and vesicles, they did not detect WT1 protein in these structures in normal tissues, but only in Wilms' tumor specimens. In contrast, a recent study by Grubb *et al.* (62) showed a faint nuclear immunoreactivity in normal blastemal cells. At later times in development, they confirmed the finding by Mundlos *et al.* that nuclear staining was confined to podocytes. The differences between these studies may be attributable to the antibodies used, as Mundlos *et al.* used an antibody specific for the 17 amino acid insertion present only in WT1(B) and (D) (see Figure 2), whereas the specficity of the antibody used by Grubb *et al.* was not stated. In addition, the sensitivity of the two antibodies may differ. The results of Mundlos *et al.* were confirmed in the mouse by Ryan *et al.* and Rackley *et al.* , who used two different antibodies which both recognize the N-terminus of WT1 (156, 170). Rackley *et al.* also observed diffuse WT1 staining in the cytoplasm of mouse podocyte cells at day E15.5, as well as "granular" staining in the nucleus of these cells. In a recent report, Larsson *et al.* also observed expression of WT1 in a speckled pattern in the nucleus of a mesonephric cell line derived from a mouse strain expressing polyoma T antigen, and showed that WT1 colocalized with proteins involved in mRNA splicing, suggesting that WT1 may play a role in post-transcriptional processing of mRNA (105). In human fetal kidney, preliminary data indicates that WT1 protein is expressed exclusively in the cytoplasm of podocyte cells (C. Burrow, personal communication). The significance of this cytoplasmic staining is unknown.

In general, *WT1* expression is low in the developing blastema and rises in the comma and S-shaped bodies, intermediates in the formation of the glomerulus. Expression is then downregulated, except in the podocyte cells of the mature glomerulus. From this pattern of expression, and from the association of *WT1* mutations with some Wilms' tumors, we may infer that *WT1* may be involved in the initiation of renal differentiation and may play a role in maintenance of the glomerulus.

**2C-2. Expression of *WT1* in Wilms' tumors.** The majority of Wilms' tumors are not associated with homozygous deletion of *WT1*, and as such they express the *WT1* mRNA and protein, often at high levels. In Wilms' tumors, *WT1* is expressed only in malignant cell types whose normal counterparts express *WT1* (151). Since expression of *WT1* declines dramatically in the adult human kidney, these authors suggested that expression of *WT1* could be used as a marker for tumor differentiation. Studies comparing the histology of Wilms' tumors with *WT1* expression found that *WT1* is most highly expressed in tumors with predominanly blastemal components (50, 128, 212) and that expression level is inversely correlated with the degree of differentiation (128).

**2C-3. *WT1* in the development of other organ systems.** *WT1* is also expressed in the genital ridge, fetal gonad, and mesothelium in human embryos (152). Expression in mesothelial structures was confirmed in the mouse, and the source of expression in the spleen was traced to stromal cells (144). Others found *WT1* expression in the granulosa and epithelial cells of the ovaries, the Sertoli cells of the testis, and the uterine wall of the adult mouse (149, 156). *WT1* expression is also seen in the decidua of the maternal uterus during

pregnancy in rats and mice (156, 216). Studies of *WT1* mRNA localization in the rat agreed with the findings in mouse and human, and also showed expression of *WT1* in the spinal cord and the area postrema of the brain (185). In the mouse, *WT1* expression was also noted in the ependymal layer of the spinal cord (156), the presumptive motor neurons of the spinal cord, differentiating body-wall musculature, and the roof of the fourth ventricle of the brain (4). Weak expression in the embryonic and adult eye and tongue have also been reported (4). Together these data suggest a role for *WT1* in the development of the kidney and a number of other tissues, though the analysis of WT1(-/-) mice indicates that *WT1* may be most important for the development of the genitourinary system (see below). In addition, *WT1* is expressed in a number of cell types (kidney, gonad, mesothelium) at a time which corresponds to a mesenchymal-to-epithelial transition and thus this gene may play a role in this important developmental step.

WT1 is also expressed in a variety of cell lines, some of which represent normal areas of expression in ontogeny. These include kidney cell lines, Leydig and Sertoli cell lines, and mesothelial cell lines. *WT1* is also expressed in some cell lines for which no normal *WT1*-expressing counterpart exists, such as hematopoeitic cell lines, embryonic carcinoma cells, and melanoma cells. For example, expression of *WT1* in nascent hematopoeitic organs such as blood islands and fetal liver has not been reported; data on *WT1* expression in bone marrow differs and is discussed below. Similarly, *WT1* is expressed in melanoma cell lines but not in normal melanocytes (167). The nature of these patterns of seemingly inappropriate expression and the role of *WT1* in tumorigenesis in these cell types is not known. Some of these cell lines may be

useful in discerning the role of *WT1* in gene expression. A summary of these findings is presented in Table 1B.

# TABLE 1B
## Expression of *WT1* in cell lines

| Name | Cell type and species | mRNA/protein | Reference |
|---|---|---|---|
| **KIDNEY** | | | |
| 293 | human kidney | protein | (156) |
| Cos-1 | monkey kidney | protein | (131) |
| anaplastic WT | human Wilms' tumor | mRNA (RT-PCR) | (75) |
| M15 | mouse mesonephric | mRNA, protein | (105) |
| **TESTIS** | | | |
| TM3 | mouse Leydig cells | protein | (156) |
| 15P-1 | mouse Sertoli cells | mRNA | (159) |
| **HEMATOPOETIC** | | | |
| HEL | human erythro-leukemia | mRNA | (144) |
| MO7 | human mega-karyocytic leukemia | mRNA | (144) |
| K562 | human erythro-leukemia | mRNA<br>protein | (144)<br>(193) |
| HL60 | human myeloid leukemia | mRNA | (179) |
| CCRF-CEM | T cell ALL | mRNA<br>protein | (129)<br>(193) |
| P-ALL 1,2 | Ph'-ALL | mRNA | (129) |
| KG-1 | AML | mRNA | (129) |
| MKPL | AML | mRNA | (129) |
| KCL-22 | CML blast crisis | mRNA<br>protein | (129)<br>(193) |
| EM-3 | CML blast crisis | mRNA | (129) |
| KEN-L-1 | ALL | mRNA | (129) |
| HPB-ALL | ALL | protein | (193) |
| **EMBRYONAL CARCINOMA** | | | |
| F9 | mouse EC | protein | (131) |
| **MESOTHELIAL** | | | |
| LP9 | normal human | mRNA | (144) |
| JMN | mesothelioma | mRNA | (144) |
| various | rat mesothelioma | mRNA | (203) |
| | human mesothelioma | mRNA, protein | (2) |
| **MELANOMA** | | | |
| various | human melanoma | mRNA | (167) |

**2C-4. The role of *WT1* in genitourinary development is confirmed by human mutational studies and a targeted gene disruption in mice.** The role of *WT1* in the development of the kidney and other organ systems was confirmed by a targeted gene disruption of *WT1* in mice (100). Mice lacking *WT1* die before birth and have abnormal development of the heart, lungs, and mesothelium and a complete agenesis of the kidneys. These mice showed a lack of ureteric bud formation and the metanephric blastema (the precursor of the glomerulus) into which the ureteric bud (the nascent collecting system) normally grows, underwent apoptosis. Apoptosis of blastemal cells has been shown to occur during normal kidney development and it has been postulated that a signal must pass between the ureteric bud and the metanephric blastema in order to prevent apoptosis and induce mesenchymal-to-epithelial differentiation of the blastema (26, 97). The blastemal defect in WT1(-/-) embryos was shown to be cell-autonomous, as explanted mutant mesenchyme could not differentiate in response to co-culture with spinal cord, a strong inducer of mesenchymal-to-epithelial differentiation. Kreidberg *et al.* therefore proposed that *WT1* is required for signalling between the metanephric blastema and the ureteric bud, which results in both differentiation of the blastema and growth and branching of the ureteric bud (100).

Interestingly, WT1(-/-) mice did not develop Wilms' tumors (100). This is likely due to the fact that *WT1* is required at an early stage of kidney development, perhaps initiating the kidney differentiation program, as well as at a later stage, such as in the final differentiation of the podocyte. Deletion of *WT1* prior to embryogenesis results in a lack of kidney formation, whereas deletion of *WT1* in a subset of kidney cells after the kidneys have begun to form could lead to tumorigenesis.

The results from the WT1(-/-) mice also suggest a role for *WT1* in the development of the gonads. Specifically, mutant embryos had an abnormally small urogenital ridge which failed to develop after day E11 and did not give rise to any gonadal tissue. However, germ cell migration from the allantois to the urogenital ridge occurred in a similar manner to wild-type embryos. This might be expected since *WT1* is expressed in the supporting cells (Leydig cells and Sertoli cells of the testis, and the granulosa and epithelial cells of the ovary) but not the germ cells of the gonad (see above). In addition, another defect observed in the WT1(-/-) mice, that of incomplete diaphragms, is consistent with the finding that WT1 is expressed in mesothelium and mutated in a mesothelioma (2, 144, 152, 203). However, the WT1(-/-) mice also had small hearts and lungs and appeared to die from hemodynamic disturbances of unknown origin. It is possible that expression of *WT1* in the surrounding mesothelial tissues is required for the proper development and the heart and lungs (100).

Attempts to correlate the nature of developmental defects with the effective gene dosage of *WT1* in humans may also provide clues to the roles of *WT1* in development and adult life. Patients with the WAGR syndrome are hemizygous at the *WT1* locus. These patients have apparently normal kidney development, though they may have unilateral kidneys, horseshoe kidneys, and/or mild genitourinary malformations such as hypospadias or cryptorchidism. This suggests that 50% of the normal *WT1* gene dosage is sufficient for qualitative development of the cells of the nephron and for the entire kidney organ. However, 50% of the normal gene dosage may not be sufficient to allow normal development of the genitourinary system.

Interestingly, mice heterozygously deleted for *WT1* did not develop Wilms' tumors or genitourinary malformations, in contrast to human WAGR patients,. Likewise, mice which carry a deletion of mouse chromosome 2 which is syntenic to the 11p13 deletion of WAGR patients have normal kidney development and also do not develop Wilms' tumors (58). This might reflect differences in exposures to environmental carcinogens between the two species. Alternatively, this difference may be due to the much smaller number of kidney cells in mice compared to humans. Mice have approximately $10^4$ nephrons in each kidney, whereas humans have approximately $10^6$ nephrons (58). If the rate of WT1 mutation in kidney cells were the same in mouse and human, humans would be expected to develop many more WT1 mutations than mice due to a larger population of target cells. Alternatively, human kidney cells may be more sensitive to alterations in *WT1* gene dosage than mouse kidney cells. Mouse and human models of disease are often different. For example, humans carrying a heterozygous deletion of the Rb gene develop retinoblastomas, whereas mice with a similar heterozygous Rb deletion develop pituitary tumors (85).

Patients with dominant negative heterozygous mutations of *WT1*, as in the Denys-Drash syndrome, may have an effective gene dosage that is less than 50% of normal (163). These patients, like WAGR patients, must have enough remaining WT1 function to permit kidney development. However, DDS patients have much more frequent and severe genitourinary malformations than WAGR patients, including the presence of streak gonads and pseudohermaphroditism. Also in contrast to WAGR patients, DDS patients develop progressive glomerulonephropathy after birth, presenting initially with proteinuria and

progressing in most cases to renal failure and death in childhood. Histopathological examination of the kidneys of DDS patients reveals some variability between patients, but in most cases there is an accumulation of fibrillar material in the mesangial matrix (the space between the glomerular caprillaries). Abnormalities of the podocytes, such as effacement of the foot processes or vacuolization and hypertrophy, are also often observed (68, 87). After birth, *WT1* continues to be expressed in the podocytes, whose foot processes ensheath the basal lamina of the capillary endothelium of the glomerulus and are thought to play an important role in filtration of the urine. Therefore continued expression and function of *WT1* in the podocytes may be required for proper maintenance of glomerular function in the postnatal period. Dominant negative *WT1* mutations associated with DDS may inhibit the function of wild-type *WT1* and interfere with this maintenance, leading to progressive decreases in renal function and eventually death. Interestingly, Hastie *et al.* recently reported preliminary results on a mouse model for DDS, where one wild-type *WT1* allele was replaced with a *WT1* allele deleted for the last two zinc fingers (74). They have so far only obtained one mouse heterozygous for this allele, which died of hypertension and nephropathy similar to that of DDS patients, providing further proof that inhibition of wild-type *WT1* function by dominant negative *WT1* alleles is likely the cause of DDS.

Epigenetic factors or the genetic background of the individual may modify the effects of reduction of *WT1* gene dosage, leading to variable penetrance. There has been a report of a patient with Denys-Drash syndrome who inherited his germline heterozygous *WT1* mutation from his father, who was phenotypically unaffected (30). In addition, genetic females with *WT1* mutations associated with Denys-Drash syndrome have a significantly lower frequency of

genitourinary abnormalities than males, suggesting that *WT1* may play an important role in male genitourinary development (28).

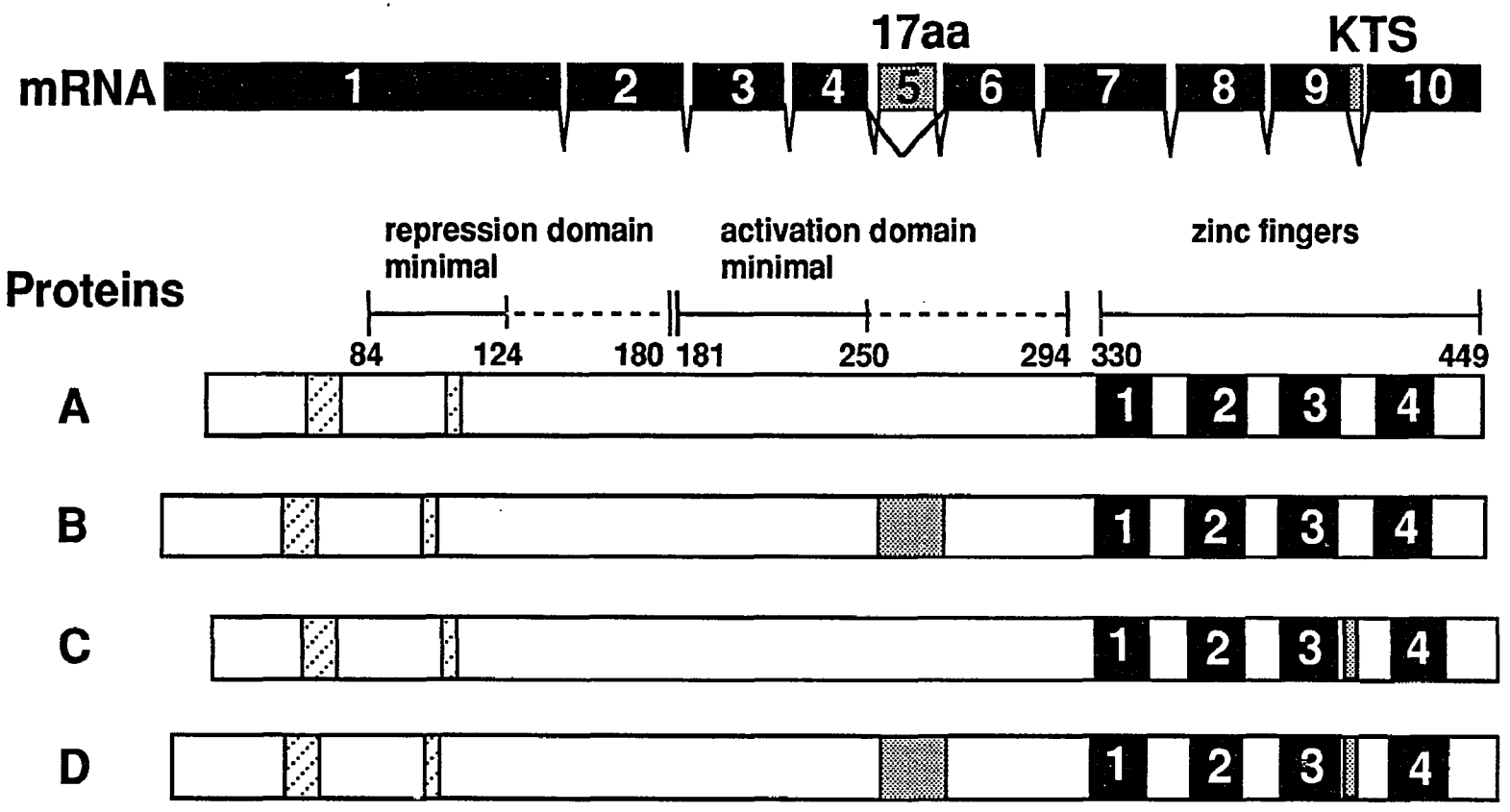
**2D. Regulation of the *WT1* promoter.** In order to understand how the expression of *WT1* is temporally and spatially restricted, a number of groups have characterized the cis-acting regulatory regions of the *WT1* gene. The upstream regulatory region of *WT1* is highly GC-rich and contains neither a CCAAT box nor a TATA box (21, 44, 52, 79). This TATA-less promoter is similar to those of other tumor suppressor genes such as Rb and p53, however, unlike those genes, *WT1* has a highly tissue-specific pattern of expression, suggesting that other regulatory elements are important for restricting expression of *WT1*. DNAse I footprinting analysis showed that the *WT1* promoter was bound by Sp1 at numerous sites (79) as well as by a number of as-yet unidentified nuclear factors, both ubiquitous and cell type-specific (21). In addition, some regions of the promoter were shown to bind a protein consisting of the +KTS zinc fingers of WT1, suggesting that the promoter might be autoregulated (21).

The *WT1* promoter is located in a CpG island which is bidirectionally transcribed. The *Wit-1* gene is located approximately 2 kb upstream from *WT1* and is transcribed in the opposite direction from *WT1* (80). Recent studies indicated that both *WT1* and *Wit-1* have multiple start sites of transcription and that at least some *Wit-1* transcripts include the first exon of WT1 (21, 41). This suggests that heteroduplex formation between *WT1* and *Wit-1* transcripts could potentially regulate WT1 protein expression, and that mutation or disregulation of Wit-1 could result in changes in *WT1* expression in Wilms' tumors without mutations in the *WT1* gene (21, 41).

By transfection of 5' DNA fragments of the *WT1* gene fused to a luciferase reporter gene in COS-7 cells, Hofmann *et al.* mapped the minimal *WT1* promoter to a 650 bp fragment with a high G+C content (79). Transient co-expression of the Sp1 transcription factor transactivated the *WT1* promoter in cell culture experiments. Studies by Fraizer *et al.* localized the minimal *WT1* promoter to a 104 bp GC-rich fragment containing potential binding sites for Egr-1/WT1 and Sp1 (44). This minimal promoter is located just 3' to the start site of transcription of the *WT1* gene. Transcription from this promoter fragment was not cell-type specific, suggesting that other more distant regulatory regions probably acted in concert with the promoter to produce a spatially and temporally regulated pattern of expression. In support of this hypothesis, these authors identified a 350 bp enhancer located approximately 500 bp downstream of exon 10 of *WT1* . This enhancer is estimated to be greater than 50 kb away from the *WT1* promoter in the native genomic configuration. Insertion of this enhancer in a 3' position in the *WT1* minimal promoter reporter construct resulted in enhanced transcription in transiently transfected K562 erythroleukemia cells but not in other cell types (44). Since *WT1* is expressed in K562 cells, this information suggested that ithe enhancer was cell type-specific. Interestingly, this enhancer contains two GATA motifs which can bind the GATA family of transcription factors. Transcriptional activation by GATA-1 or GATA-2 via these motifs might account for expression of *WT1* in early hematopoetic cells or in leukemic cells (see Table IB). In support of this, Wu *et al.* recently reported that endogenously expressed GATA-1 protein binds to this enhancer in K562 human erythroleukemia cells (211). In addition, they showed that transfection of GATA-1 into human embryonic kidney 293 cells results in transactivation mediated by this enhancer. GATA-3, which is expressed in the developing mouse kidney (140), may also regulate *WT1* expression via this cell

type-specific enhancer, and could be one factor determining the normal ontogeny of *WT1* expression. The *WT1* locus may be further controled by a transcriptional silencer which may suppress transcription of the gene in cell types which do not normally express *WT1* (44). Finally, WT1 has recently been shown to repress transcription of its own promoter in transient co-transfection experiments (121, 169), suggesting that a negative feedback loop may play a role in regulating the expression of WT1. These studies, though suggestive of some of the cis-acting sequences and trans-acting factors involved in regulation of *WT1* expression, are not yet complete, as the function of the *WT1* promoter has not been examined in a model kidney cell line, nor have transgenic mouse experiments been performed to map the elements required for proper temporal and spatial expression of *WT1*.

**2E. Structure of the *WT1* mRNA and protein.** A map of the *WT1* mRNA and protein is shown in Figure 2. Conceptual translation of the *WT1* cDNA yielded a protein containing 4 C-terminal zinc fingers of the $Cys_2$-$His_2$ variety, similar to the DNA-binding domain of the *Drosophila* Krüppel protein. The WT1 protein showed a significant degree of homology to the EGR family of zinc-finger transcription factors, which suggested that WT1 was a DNA-binding transcription factor. In addition, the N-terminal part of the protein contains sequences rich in proline and glutamine, similar to those shown to mediate transcriptional activation by CTF-1 (126) and Sp-1 (32), respectively.




## FIGURE 2

## Structure of the WT1 mRNA and proteins

**Stippled boxes represent nucleotides or amino acids included as the result of alternative splicing of the mRNA. Hatched boxes represent proline-rich regions of the protein. The exons of the mRNA and the zinc fingers of the protein are numbered, as are the amino acids comprising the indicated domains. The numbering corresponds to the amino acids in the "D" isoform.**

By Northern blot analysis, the *WT1* cDNA detects a transcript of approximately 3 kb in kidney cells (20, 56, 64, 152), while a shorter transcript of approximately 2.7 kb is detected in testis and mesothelial cells (149, 152, 203). The nature of this shorter *WT1* transcript is not known. The murine and human WT1 genes both consist of 10 exons, spread out over approximately 50 kb of genomic sequence (20), which can be alternatively spliced to yield 4 distinct mRNA species (54, 66) (see Figure 2). The more 5' splice results in the inclusion or exclusion of exon 5, which encodes 17 amino acids. The more 3' splice involves the use of alternative splice donor sites and results in the insertion of three additional amino acids (KTS) between zinc fingers three and four.

Unlike the *IGF-II* and *H19* genes, which are located nearby at chromosome 11p15, *WT1* is not imprinted in normal fetal kidney or in Wilms' tumors, that is, both alleles are expressed regardless of parental origin (111). This is important because if *WT1* were imprinted, a heterozygous null mutation of the active allele could lead to a complete loss of function. Imprinting of WT1 was observed in some, but not all, human placentae and fetal brains, suggesting that WT1 may be imprinted in these tissues in only part of the human population; the significance of this finding is unclear (88). Uniparental disomy (inheritance of both alleles from one parent) of *WT1* was observed in 2 of 49 patients with Beckwith-Wiedemann syndrome. However, it is difficult to assess the contribution of uniparental disomy of *WT1* to the development of disease in these patients, since genetic markers in the patients were uninformative for 11p15 loci (*IGF-II, H19*) for which alterations in imprinting have been previously implicated in the development of BWS and Wilms' tumor (188). These patients might have therefore co-inherited several alleles from one parent and the

individual contribution of each gene to the development of the tumor cannot be assessed.

The nomenclature of the four *WT1* mRNAs and proteins varies in the literature. The unspliced form is referred to as WT1(A), WT1, WT1(-17aa,-KTS), or WT1(-/-); the form containing only the 5' splice is referred to as WT1(B) , WT1(+17aa), or WT1(+/-); the form containing only the 3' splice is referred to as WT1(C), WT1(+KTS), or WT1(-/+); and the form containing both splices is referred to as WT1(D), WT1(+17aa,+KTS), or WT1(+/+). The first two forms are sometimes collectively referred to as the -KTS forms, while the last two are termed the +KTS forms. For the purposes of this review, I will refer to the forms as A, B, C, and D; and I will also use the -KTS and +KTS designations which are particularly useful in discussions of the DNA-binding specificity of WT1 (see below). The ratio of the four alternatively spliced transcripts in developing mouse kidney is 1A : 2.5 B : 3.8 C : 8.3 D; this ratio does not appear to be developmentally regulated (66). Further heterogeneity in *WT1* transcripts and isoforms may be generated by a recently described RNA editing event (183) (see below).

Recently, Hastie *et al.* reported preliminary results on the cloning of the *WT1* gene from chicken and alligator (74). Comparison of these genes with the human, rat, and mouse *WT1* genes shows that the proline-rich N-terminal region and the alternatively spliced exon 5 (see Figure 2) are not conserved in chicken and alligator, suggesting either that these features of the WT1 protein are mammalian-specific or that they do not play a critical role in the function of WT1. The second alternative splice (KTS) is, however, conserved in these lower vertebrates.

The molecular weight of the WT1 proteins has been variously reported as 49-51 kDa (193) or 52-54 kDa (131). In K562 cells, WT1 protein was detected in the nucleus by immunostaining and confocal microscopy (193). WT1 was localized to the nucleus in transfected COS-1 cells (131, 149) and was not found to be phosphorylated (131), however, recent information suggests that the +KTS splice may be a target for phosphorylation ((3), and B.R.G. Williams, personal communication). Immunostaining of endogenously expressed WT1 in podocyte cells showed a "speckled" or "granular" pattern of WT1 protein localization (132, 156). Recently, Larsson *et al.* also observed a speckled pattern of WT1 expression in a mesonephric cell line by immunostaining and confocal microscopy, and found that WT1 colocalized with proteins involved in mRNA splicing (105). By transfecting each of the four WT1 isoforms into COS cells, they determined that the +KTS isoforms colocalize predominantly with the splicing factors while the -KTS isoforms colocalize predominantly with transcription factors. It is not yet known whether WT1(+KTS) plays a role in mRNA splicing.

**2F. DNA-binding specificity of WT1.** Since the conceptual translation of the *WT1* cDNA indicated the presence of four zinc-finger domains, initial studies focused on identifying the DNA-binding site of WT1. The results of all published studies on DNA binding by WT1 are summarized in Table 2A. Rauscher *et al.* (160) used a binding-site selection assay to enrich for WT1-bound DNA sequences from a random pool of oligonucleotides. In this assay the authors used a bacterially synthesized protein containing the four -KTS zinc fingers. The resultant selected sequences were similar, though not identical to, the DNA-binding site of the Egr-1 protein (5'-GCGGGGGCG-3') (22) (Table 2A, row

A). This was not surprising, as zinc fingers 2, 3, and 4 of WT1 show a moderate degree (67%) of amino acid identity to the three zinc fingers of Egr-1, with 6 out of 6 residues proposed to be important for DNA recognition conserved between the two proteins (146). The WT1 protein was then shown to bind to the Egr-1 consensus binding site and to regulate transcription through this sequence in co-transfection experiments. This Egr-1 site was assumed to be a canonical WT1 binding site until recent studies (see below). Several years after the identification of the Egr-1 site as a WT1 recognition sequence, DNAse footprinting analysis of the PDGF A-chain promoter revealed a second type of -KTS binding site which was TC-rich (Table 2A, row C) (206). Similar TC-rich sites were noted to be present in the promoters of other growth-related genes.

Since the +KTS forms of WT1 are more abundant, at least at the mRNA level, than the -KTS forms (66), subsequent studies attempted to identify a binding site and a transcriptional function for WT1(+KTS). Bickmore *et al.* used the technique of whole-genome PCR to isolate genomic DNA sequences which bind to either the +KTS or -KTS zinc finger regions (Table 2A, rows B and G) (12). They isolated primarily GT-rich sequences when the -KTS zinc fingers were used. When the +KTS zinc fingers were used, two extended sequences of approximately 100 bp were obtained, each of which contained an Egr-1-like binding consensus (see Table 2A, row G, underlined). However, the exact nucleotides bound by the +KTS zinc fingers were not localized by DNAse I footprinting or methylation interference analysis. In addition, these extended sequences bound both -KTS and +KTS forms of WT1, suggesting that the two forms might have overlapping DNA-binding specificities.

Recently, several groups performed studies designed to identify higher-affinity binding sites for the -KTS isoforms, and to more clearly identify the binding sites of the more predominant +KTS isoforms. The contribution of each of the four zinc fingers to binding to various DNA sequences was also examined. Footprinting studies on the IGF-II P3 promoter identified an extended 12-bp sequence which can bind both the -KTS and +KTS forms of WT1 (Table 2A, row H) (40). This site contains 3 extra nucleotides adjacent to the previously identified Egr-1 consensus site which binds the -KTS forms. The authors proposed that in the +KTS forms of WT1, all four zinc fingers are involved in DNA binding, whereas in the -KTS forms, only fingers 2, 3, and 4 are involved. In support of this hypothesis, methylation interference analysis of one of these sites showed that WT1(-KTS) contacted the DNA at sites spanning 6 bp while the WT1(+KTS) contacts spanned 12 bp. In addition, this group performed a binding site selection from a pool of random oligomers to attempt to identify the bases best recognized by zinc finger 1. By using a protein containing only zinc fingers 1, 2, and 3, they found that the bases 5'-GTG-3' were preferentially selected as 3' flanking bases for an Egr-1 consensus site, and proposed that these are the bases recognized by zinc finger 1.

Footprinting studies on the WT1 promoter have also identified two sites which could bind both -KTS and +KTS zinc finger proteins (Table 2A, row I) (169). Neither of these sites corresponded to previously identified sequences. However, in contrast to the studies on the IGF-II promoter, methylation interference analysis failed to demonstrate significant differences in the pattern of protection or enhancement due to binding of the -KTS or +KTS zinc finger proteins to these sites in the WT1 promoter.

A similar study on the PDGF-A promoter identified an extended GC-rich site which can bind WT1(+KTS) as well as WT1(-KTS) (Table 2A, line J) (208). This site contains within it two overlapping Egr-1 consensus sequences and a third Egr-1-like sequence. The authors found that WT1(+KTS) binding was severely diminished when two bases were deleted from the 3' end of the GC-rich sequence. This led them to hypothesize the +KTS forms bind to an extended site which includes Egr-1 consensus sequences, rather than having a distinct and separate DNA-binding consensus sequence. However, since they did not analyze the requirement of the 5' end of the GC-rich sequence for WT1(+KTS) binding, and since this sequence contains multiple overlapping Egr-1 and Egr-1-like consensus sequences, the exact location of binding of WT1(+KTS) to this sequence is not clear.

Whole-genome PCR was recently used to attempt to identify authentic high-affinity binding sites for the -KTS forms of WT1. A 10-bp sequence which binds WT1(-KTS) with a 20 to 30-fold greater affinity than the Egr-1 consensus site has been isolated (Table 2A, row D) (133). This site includes an Egr-1-like sequence with an additional T residue at the 3' end. Mutational analysis revealed that zinc fingers 2, 3, and 4 were essential for binding to this site, while finger 1 increased the affinity of WT1 for this site but was not essential for binding. A second group also used the binding site selection assay to identify high-affinity binding sites for WT1(-KTS). They isolated a 12-bp sequence which consisted of an Egr-1-like consensus with three additional bases on the 3' end (Table 2A, row E) (69). These bases were specifically recognized by a protein containing all four zinc fingers but not by a protein containing only fingers 2, 3, and 4. A role for zinc finger 1 in DNA binding by WT1(+KTS) had been previously proposed by several investigators (described above); these

results suggest that finger 1 may also play an important role in DNA binding by the (-KTS) isoforms.

Most recently, Ryan *et al.* identified three sequences in the Pax-2 promoter which bind WT1 in DNAse footprinting assays (Table 2A, rows F and K) (170). All three of these sites contain an "Egr-1-like" binding site. Two of these sites bind the -KTS forms only, while the third site also binds the +KTS forms when these proteins are present at high concentration.

From these data, it appears that the various WT1 isoforms have the capability to bind to numerous, often dissimilar, DNA sequences. There may be some secondary or tertiary DNA-structure requirement for WT1 binding, or perhaps an effect mediated by surrounding chromatin structure, which is not apparent from examination of the primary sequence of these putative binding sites. It also appears that all WT1(+KTS) binding sites identified to date contain within them a site which can also bind WT1(-KTS). The results of all these studies are summarized in Table 2A. The proposed contacts between the zinc fingers of WT1 and the specific bases of the recognition sequences are diagrammed in Figure 2B. This model, based upon the crystal structure of Egr-1/zif268 bound to its cognate recognition site (146), places the c-terminal zinc finger at the 5' end of the binding site. There is good agreement amongst the various groups regarding the putative DNA-protein contacts involving zinc fingers 2, 3, and 4 of WT1. However, there is little agreement as to the role of zinc finger 1, and simple examination of the sequences is probably not sufficient to determine if WT1 will bind to a given site and whether it will regulate transcription through this site.

Upon examination of the physicochemical data regarding DNA binding by WT1, there are significant differences in the order of magnitude in the dissociation constant ($K_d$) of the WT1-DNA complex in these studies, which may be due to the different types of protein used. Specifically, WT1 zinc finger proteins expressed in bacteria may exhibit reduced binding to DNA due to misfolding or insolubility. Modifications such as phosphorylation may occur differently depending upon the type of expression system used. Therefore, the dissociation constants calculated by Nakagama *et al.* using *in vitro* translated full-length protein (133) may not be directly comparable with those obtained with bacterially expressed zinc finger proteins.

It is not apparent why the insertion of the KTS sequence, which is predicted to alter the spacing between zinc fingers 3 and 4, would yield a protein in which the function of zinc fingers 2, 3, and 4 is unaltered, and in which zinc finger 1 provides additional specificity for flanking DNA sequences. Without information concerning the crystal structure of the WT1-DNA complex, it is difficult to predict how such changes in protein sequence might alter DNA-binding specificity.

The heterogeneity of the putative WT1 binding sites also makes it difficult to predict which promoters are actual targets of WT1 *in vivo*. Some of this complexity may be attributable to the fact that some of these binding sites have been isolated from footprinting assays in which a vast (100 to 1000) fold excess of bacterially-produced WT1 zinc finger protein is used. In no case has WT1 isolated from nuclear extracts of kidney tissue or from cells transfected with WT1 expression vector been used to study DNA binding. In some cases, transfection studies have confirmed that putative WT1 binding sequences can function as WT1 binding sites in cell culture. However, the relevance of these sequences to

promoter regulation in the endogenous chromosomal configuration has not been determined. For example, GC-rich sites are very common in cellular and viral promoters (such as SV40, CMV, and RSV), and while we have observed transactivation of these viral promoters by WT1 in transient cotransfection assays (J. C. Morris, J.C.R., and J.D.L., unpublished data), it is unclear whether these promoters would be actual *in vivo* targets for regulation by WT1. In addition, no cellular promoter has been analyzed and found to require WT1 binding sites for tissue-specific expression in cell culture or in transgenic mice. Therefore, a new generation of studies will be required to determine the actual binding sites and genes regulated by WT1. Determination of the definitive functions of each of the WT1 zinc fingers will also require X-ray crystallographic data on the structure of the WT1-DNA complex and comparison of this structure to that of Egr-1/zif268 bound to its cognate binding site (146).

# TABLE 2A

# DNA sequences which bind WT1 proteins

Sequences are presented in the order in which they were published. Egr-1 consensus sequences or "Egr-1-like" sequences (as defined by the authors of the individual papers) are underlined. Bases proposed to contact zinc finger 1 are shown in bold-face type.

## SEQUENCES WHICH BIND WT1(-KTS):

A. Rauscher *et al.*, 1990 (random oligomers)
CCGCCCCTC, CCTCCCCAG, GACCCCCTC, TGCCCCTCG, CACCCACTC
the authors decided on CGCCCCCGC "consensus" based on homology with Egr-1

B. Bickmore *et al.*, 1992 (genomic fragments)
TGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGCATA
TGTATGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTCTCGTGATTTGTACCGGTTA
AAGAGGGTGTGTGTGTGTGTGTGTGTGCGTGTGTGTGTGTGTGTGTGTGT
AAAAATCACTCCAGGATTTTGTTGATCTTTTTAATGATGTGGTTGTGTGGTTGTGTGTGGTGTGTGTGGTT-
GTGTGTTGTGTGTGTGGTATGTGTGATTGTGTGTGTGGTGTGTGGTTGT

C. Wang *et al.*, 1993 (PDGF-A promoter): TCCTCCTCCTCCTCTCC

D. Nakagama *et al.*, 1995 (genomic sequence): GCGTGGGAG**T**

E. Hamilton *et al.*, 1995 (random oligomers): GCGTGGGCG**(T/G) (G/A/T) (T/G)**

F. Ryan *et al.*, 1995 (Pax-2 promoter): TGCCTTTTCCGGGGGCGGGGGCCGGCT,
GGAGAAAAGGAAGGGACGGTGGGGGAGGGAGAGT

## SEQUENCES WHICH BIND BOTH WT1(-KTS) AND WT1(+KTS):

G. Bickmore *et al.*, 1992 (genomic sequences):
ATCTTCATTGTAGTCGGGTGCTGTGGCTTATGTGTAACAATGCCAGCATTTTGTGAGGCTGAGACAGGAGG-
-ATCACTTGAGGCCAGGAGTTCAAGACCAGCCTGGGCAACATAGT
ATATTCTTGGGACTATTCTTGGGACTATCTCCCCTCCCAGGAGAGGGAGGATCAACGGTACTCAAGTCTCT-
-GGTTTTAGGTCTTGTGTTGTAAATCACGAGATATTAGAATTCTAATATCTCAGTTTCTCAGTATTCTAGT-
-GCCCCTCTTGGT

H. Drummond *et al.*, 1994 (IGF-II promoter):
gggttGCGGGGGCG**GGC**cg
gcgggCCGGGGGCG**GGG**cg
aggagGCGGGGGCG**GCC**gg
this group also identified **GTG** from random oligomers as bases best recognized by zinc finger 1

I. Rupprecht *et al.*, 1994 (WT1 promoter): GCAGGGGGAGGC, GAGGAGGGTGTC

J. Wang *et al.*, 1995 (PDGF-A promoter):
GAATCCGGGGAGGCGGGGGGGGGGGGCGGGGGCGGGGGCGGGGGAGGGGCGCGGCGGCGGCGC

K. Ryan et al., 1995 (Pax-2 promoter): (binds -KTS well, binds +KTS at high concentrations)
GCTCAGGGCGGGGGCGGCCCGAGGGCGCCAGGAGGGGAGC

# Table 2B
# Proposed WT1 zinc finger-DNA contacts

| | ZF4 | | | ZF3 | | | ZF2 | | | ZF1 | | | | Protein, Source | $K_d$, Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R | E | R | T | H | R | R | Q | R | M | H | K | | | |
| 5'- | G | C | G | T | G | G | G | A | G | T | ? | ? | -3' | WT1(-KTS) full-length *in vitro* translated | $5 \times 10^{-10}$ (133) |
| 5'- | G | C | G | T | G | G | G | C | G | G<br>T | A<br>G | G<br>T | -3' | ZF(-KTS) bacterial | $8.4 \times 10^{-8}$ (69) |
| 5'- | G | C | G | G | G | G | G | C | G | G | G | C | -3' | ZF(-KTS)<br>ZF(+KTS) bacterial | $1.4 \times 10^{-7}$<br>$2.0 \times 10^{-6}$ (40) |
| 5'- | G | C | G | G | G | G | G | C | G | G | T | G | -3' | ZF 1,2,3 bacterial | N/D (40) |
| | G | C | G | G<br>T | G | G | G | A<br>C | G | G<br>T | A<br>G<br>T | G<br>T<br>C | | consensus | |

The amino acid residues proposed to be important for DNA base recognition in each zinc finger are aligned with the bases which they are proposed to contact in the indicated publications. N/D = not determined.

## 2G. Repression and activation functions of WT1 and putative target genes

### 2G-1. Transcriptional regulation by WT1 in transient transfection studies.

**2G-1a. Transcriptional regulation by WT1(-KTS).** All published results on transcriptional regulation by WT1 are summarized in Tables 3A and B. Initial studies of WT1 protein function focused on transcriptional regulation by WT1 bound to Egr-1 consensus sequences. In co-transfection studies, WT1 expressed from a cytomegalovirus expression vector repressed transcription from a synthetic chloramphenicol acetyltransferase (CAT) reporter construct containing three Egr-1 sites upstream of a minimal *fos* promoter (118). This group also showed that WT1 could repress and Egr-1 could activate the complex Egr-1 promoter, which contains three Egr-1 binding sites as well as several AP-1 and serum response factor binding sites. The N-terminus of WT1 conferred repression activity when fused to the zinc fingers of the Egr-1 protein (118). Therefore, WT1 was proposed to be a repressor of transcription.

The search for authentic cellular target genes for WT1 was initially guided by the finding that insulin-like growth factor II (IGF-II) and platelet-derived growth factor (PDGF) are overexpressed in Wilms' tumors (45, 164, 178). In addition, growth of Wilms' tumor explants is inhibited by antibodies to the IGF-I and IGF-II receptor (IGF1R), suggesting that Wilms' tumors may grow by an autocrine loop mechanism (47). This led to the hypothesis that if WT1 normally transcriptionally repressed these genes, deletion or mutation of WT1 would

result in overexpression of IGF-II and PDGF, leading to neoplasia. However, since most Wilms' tumors are not associated with mutation of WT1, it is apparent that increased expression of these growth factors in Wilms' tumor may occur through other pathways. Nevertheless, in accordance with prediction, WT1 repressed transcription directed by the IGF-II P3 promoter (39). Footprinting analysis indicated that WT1 bound to sites both upstream and downstream of the start site of transcription of this promoter. In transfection assays, maximal repression required the presence of WT1 binding sites both 5' and 3' to the start site of transcription. Elimination of either the 5' or 3' putative WT1 binding sequences resulted in a loss of repression by WT1. Gashler *et al.* examined regulation of the PDGF A-chain (PDGF-A) promoter by WT1 (49). This group defined multiple WT1 binding sites upstream of the start site of transcription of the PDGF-A promoter and showed that promoter fragments truncated from the 5' or 3' end which contained fewer WT1 binding sites were repressed to a lesser extent than those containing more binding sites. In another study, Wang *et al.* (204) identified a GC-rich sequence in the PDGF-A promoter which bound WT1 and showed that WT1 repressed transcription of a reporter construct in which this GC-rich sequence was placed upstream of a TATA box.

Further studies on the PDGF-A promoter demonstrated that WT1 can both activate and repress transcription. Wang *et al.* (205) noted that truncation of the PDGF-A promoter so as to eliminate either either 5' or 3' Egr-1/WT1 binding sites resulted in activation rather than repression of the reporter gene by WT1. In addition, WT1 activated transcription of synthetic reporter genes containing Egr-1 consensus binding sites either 5' or 3' to the start site of transcription, but suppressed transcription from reporter genes with Egr-1 sites on both sides. However, the reporters containing both 5' and 3' Egr-1/WT1 binding sites, which

were repressed by WT1, had significantly higher basal transcriptional activities than those containing binding sites on only one side of the transcription start site, which were activated by WT1. This suggested that the multiple Egr-1 sites were interacting with endogenous cellular transcriptional activators. Therefore, the authors referred to WT1's action on these promoters as "suppression" rather than repression, implying that WT1 may repress transcription by competing for binding sites with endogenous transcriptional activators. While Wang *et al.* reported that expression of the zinc fingers of WT1 alone did not suppress transcription from reporter genes containing both 5' and 3' WT1 binding sites, they did not examine the expression level or DNA-binding activity of the zinc finger-only protein (205). Hence it is not clear from this study whether suppression was merely due to binding site competition or was secondary to an active repression mechanism. The WT1 binding site 3' to the start site of the PDGF-A promoter, whose existence was implied by these studies, was subsequently confirmed by the identification of a TC-rich binding site for WT1 (Table 2, row C) (206).

Like other transcription factors, WT1 has a modular structure, with DNA binding zinc finger motifs which are distinct from its transcriptional effector domain(s). The transcriptional effector domains of WT1 were mapped by several groups, and are diagrammed in Figure 2. As part of the study of the PDGF-A promoter, Wang *et al.* (205) used deletion analysis of the native WT1 protein to show 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. Madden *et al.* (119) fused different regions of the WT1 protein to the GAL4 DNA-binding domain and examined the effect of these fusion proteins on a reporter

gene containing GAL4 binding sites. Confirming the deletion studies of WT1 bound to its own binding site, they found that amino acids 84-180 were sufficient to confer repression activity on the GAL4 DNA-binding domain. This suggested, like the *Drosophila* transcription factors *Krüppel* (108, 109), *even-skipped* (71), and *engrailed* (70), and the mammalian transcription factor YY1 (186), WT1 contained an active repression domain. These studies also showed that, like the transcription factors Egr-1 (48) and *Krüppel* (109), WT1 posessed adjacent transcriptional repression and activation domains. Interestingly, Madden *et al.* showed that the activity of the repression domain was cell type-specific, in that it functioned in NIH 3T3 mouse fibroblast cells but not in 293 human embryonic kidney cells (119). A larger domain consisting of amino acids 1-298 was required for repression of transcription in 293 cells. The cell-type specificity of repression has implications for the mechanism of action of WT1, suggesting that WT1 may interact with cell type-specific co-factors, rather than ubiquitously expressed basal transcription factors, to mediate transcriptional repression. These data also suggest that the transcriptional activation function of amino acids 180-298 may be coopted to aid in repression by amino acids 84-180 in certain contexts.

In addition, Madden *et al.* used deletion and mutational analysis to examine the function of several putative structural motifs in the N-terminal domain of WT1 (119). Deletion of proline- and glycine-rich sequences (amino acids 21-66) in the context of the native full-length WT1 protein abolished repression activity, while mutations designed to disrupt a potential leucine-zipper-like structure between amino acids 226 and 254 resulted in a partial loss of repression activity. Since this leucine zipper-like structure falls within the region defined as the transcriptional activation domain of WT1, it would be of interest to learn

whether deletion of this structure would affect the activation ability of a GAL4-WT1 fusion protein. Though all these mutants were expressed at detectable levels in transfected cells as assayed by western blotting analysis, it is not clear from these studies whether these mutant proteins retained a native conformation and/or the ability to bind DNA. Determination of the binding activity of these proteins could assist in further interpretation of these results, since studies by Golemis *et al.* showed that fusion of various domains to the LexA DNA binding domain can in some cases lead to reduced DNA binding by the hybrid proteins (59). Most recently, Wang *et al.* (207) have used GAL4-fusion mapping to define the minimal repression and activation domains as amino acids 85-124 and 181-250, respectively. This minimal repression domain may cooperate with other sequences, such as the proline and glycine-rich region between amino acids 26 and 66, to effect maximal repression. It appears that when both the repression domain and activation domain of WT1 are fused to GAL4, as in GAL4-WT1(1-298), the repression function dominates (119).

Studies by Maheswaran *et al.* (120) demonstrated that the transcriptional activity of WT1 could be modulated by interaction of WT1 with the p53 tumor suppressor protein. In cells lacking p53, WT1 activated transcription from the Egr-1 promoter, whereas in cells containing p53, WT1 repressed this promoter. Moreover, in a cell line harboring a temperature-sensitive p53 mutation, growth of the cells at the nonpermissive temperature resulted in activation of the Egr-1 promoter, whereas at the permissive temperature the promoter was repressed. The interpretation of these experiments is complicated by the fact that the basal level of transcription of the reporter genes was different at the permissive and non-permissive temperatures, suggesting that the action of p53 on other

sequence-specific transcription factors and/or basal transcription factors, such as TBP (182), may influence the transcriptional environment, and hence the transcriptional function of WT1.

Subsequent studies have examined a number of other growth-related genes whose promoters are repressed by WT1 in transfection assays. These include the insulin-like growth factor-1 receptor gene (IGF1R), which binds the ligand IGF-II and has been shown to be overexpressed in Wilms' tumors (210). The IGF-1R promoter contains multiple putative Egr-1/WT1 binding sites and is repressed by WT1 when co-transfected with a WT1 expression vector (210). Further studies by this group (209) showed that, similarly to the case of the IGF-II promoter, repression of the IGF1R promoter required the presence of WT1 binding sites on both sides of the start site of transcription. The colony-stimulating factor 1 (CSF-1) promoter is also repressed by WT1 in cell culture (73). Interestingly, the putative WT1 binding site identified by this group overlapped with Sp1 and Sp3 binding sites, suggesting that WT1 might act to repress transcription by competitive binding with the Sp1/Sp3 activator proteins. In the transforming growth factor-β (TGF-ß) promoter, Dey *et al.* (35) identified an Egr-1/WT1 binding site upstream of the transcription start site and showed that WT1 repressed transcription of this promoter in transient co-transfection assays. Recently, WT1 was shown to repress the human but not the mouse retinoic acid receptor-α promoter via an Egr-1 consensus sequence upstream of the start site of transcription (61).

Putative WT1 target genes have been identified by their coincident expression with WT1 during renal development. The Pax-2 gene is is expressed in developing but not adult kidney, and it is a putative target for regulation by WT1

since persistent expression of Pax-2 is observed in Wilms' tumors. Ryan *et al.* found that WT1 repressed a Pax-2 promoter reporter construct in transient transfection assays. (170). In addition, these authors showed that WT1 and Pax-2 were expressed in an approximately inversely correlated pattern during kidney development in the mouse, that is, Pax-2 expression decreased in the metanephric blastema at the same time that WT1 expression increased. The authors identified three potential WT1 binding sites 5' to the start site of transcription of the Pax-2 gene, and found that insertion of these sites 3' to the start site of the HSV-*tk* promoter resulted in repression of the reporter gene by WT1. It is not clear what the regulatory action of WT1 would be if these sites had been placed 5' to the start site of transcription of the HSV-*tk* promoter. Interestingly, Pax-2 was not expressed in the metanephric mesenchyme of mice carrying a homozygous deletion of WT1, suggesting that WT1 might activate rather than repress transcription of Pax-2 in these cells (100). In addition, Pax-2 is expressed in the ureteric bud, which does not express WT1, and is subsequently downregulated, indicating the existence of WT1-independent pathways for the positive and negative regulation of the WT1 gene (37, 38).

Recent work from our group showed that WT1 is a default transcriptional activator of a simple test promoter containing three Egr-1/WT1 binding sites upstream of the HSV-*tk* promoter (163). This is consistent with studies on the PDGF-A promoter, which showed that WT1 activated transcription from upstream binding sites (205). Our conclusion that WT1 is a default activator is supported by the recently published work of Hamilton *et al.* (69) who defined a high-affinity binding site for WT1 which consists of an Egr-1-like site with additional 5' flanking bases. When multimerized and placed upstream of a mouse mammary tumor virus promoter, these sites mediated transcriptional

activation by WT1(A). However, these findings are at odds with the original report that WT1 repressed transcription from a promoter containing three Egr-1 sites upstream of a minimal *c-fos* promoter (118). It is possible that interaction with other transcription factors bound to the HSV-*tk* , MMTV, and *c-fos* promoters can determine whether WT1 acts as an activator or a repressor.

In addition, we showed that the (B) isoform of WT1, which contains the 17 amino acid insertion identified as an independent repression domain by Wang *et al.* (208) (see below), was a somewhat stronger transcriptional activator than WT1(A), which lacks this insertion. This implies that the 17 amino acid insertion may have bifunctional properties and that its action may depend on other factors such as promoter architecture or association with other cellular proteins.

**2G-1b. Transcriptional regulation by all four WT1 isoforms.** WT1 can also regulate transcription of its own promoter. Footprinting analysis defined sites upstream of the start site of the WT1 promoter which could be bound by both the (-KTS) and (+KTS) forms of WT1 (169) (see Table 2A). All four isoforms of WT1 repressed the WT1 promoter, with the (D) isoform (which contains both the 17 amino acid and +KTS insertions) having the greatest repression activity. Interestingly, the WT1(D) isoform, which contains the 17 amino acid insertion encoded by the alternatively spliced exon 5, was a much stronger repressor of transcription than the (C) isoform, which lacks this insertion. One of these WT1 binding sites could also mediate transcriptional repression when it was inserted uptream of the herpes simplex virus *tk* (HSV-*tk*) promoter. However, deletion analysis of the WT1 promoter showed that, similar to results with other promoters, sequences both 5' and 3' of the transcription start site were required for maximal repression activity. These results were

supported by data from Malik *et al.* (121), who stably transformed human embryonic kidney 293 cells with a metal ion-inducible metallothionein promoter-WT1 construct and measured the activity of various fragments of the WT1 promoter in induced versus uninduced cells. WT1 was present at fairly high levels in the uninduced state, but upon induction with cadmium, WT1 levels rose, and the WT1 promoter activity declined. These authors also hypothesized that "autosuppression" of the WT1 gene may occur through competition between Sp1 and WT1 for GC-rich binding sites in the WT1 promoter.

All four WT1 isoforms also repressed the PDGF-A promoter (208). These authors defined an extended GC-rich sequence in this promoter which bound both -KTS and +KTS forms of WT1 (see above and Table I-2). The (A), (C), and (D) isoforms of WT1 repressed transcription from a reporter gene containing this extended GC-rich sequence upstream of a TATA box. Interestingly, this group showed that WT1(D) repressed transcription whether GC-rich sites were present upstream, downstream, or on both sides of the transcription start site, whereas WT1(C) activated transcription from upstream or downstream sites but repressed transcription when sites were present on both sides. This implicated the 17 amino acid insertion in WT1(D) relative to (C) as an additional transcriptional repressor domain. This was reminiscent of the finding that WT1(D) was a stronger repressor of the WT1 promoter than WT1(C) (169). The 17 amino acid peptide present in the (B) and (D) isoforms also conferred repression activity when fused to either the WT1 zinc fingers or the GAL4 DNA-binding domain. Furthermore this repression activity was abolished when four consecutive serine residues were deleted from the 17 amino acid peptide,

suggesting that these serines play an important role in repression by the (B) and (D) isoforms of WT1.

# TABLE 3A
# Transcriptional regulation by WT1: Natural promoters

| PROMOTER | 5' WT1 SITES | 3' WT1 SITES | EFFECT OF WT1 | REFERENCE |
|---|---|---|---|---|
| EGR1-CAT | 3 | 0 | repression | (118) |
| IGF-II P3 | 2 | 1 | repression | (39) |
| | 1 | 1 | repression | |
| | 0 | 1 | little effect | |
| PDGF-A | 6 | 0 | repression | (49) |
| | 2 | ? | repression | (204) |
| | 2 | 1 | repression | (205) |
| | 1 | 0 | activation | |
| | 0 | 1 | activation | |
| | 1 +KTS | 1 +KTS | C and D repress | (208) |
| | 1 +KTS | 0 | C activates, D represses | |
| | 0 | 1 +KTS | C activates, D represses | |
| IGF1R | 4 | 6 | repression | (210) |
| | 6 | 6 | repression | (209) |
| | 6 | 1 | less repression | |
| | 6 | 0 | less repression | |
| | 1 | 6 | less repression | |
| CSF-1 | 1 | 0 | repression | (73) |
| TGF-β1 | 1 | 0 | repression | (35) |
| WT1 | 6 | 1 | repression | (169) |
| | 6 | 0 | less repression | |
| | 1 | 0 | less repression | |
| | 5 | 2 | repression | (121) |
| | 5 | 0 | no effect | |
| | 0 | 2 | no effect | |
| RAR-α | 1 | 0 | repression | (61) |
| Pax-2 | 3 | 0 | repression | (170) |

Note: the numbers of sites listed here are in some cases the number of putative or proposed binding sites for WT1.

# TABLE 3B
# Transcriptional regulation by WT1: Synthetic promoters

| TYPE OF SITE | 5' WT1 SITES | 3' WT1 SITES | EFFECT OF WT1 | REFERENCE |
|---|---|---|---|---|
| EGR-1 sites | 3 | 0 | repression | (118) |
| EGR-1 sites or | 3 | 0 | activation | (205, 206) |
| PDGF-A TC-rich sites | 0 | 3 | activation | |
| | 3 | 3 | repression | |
| PDGF-A +KTS sites | 1 | 1 | C and D repress | (208) |
| | 1 | 0 | C activates, D represses | |
| | 0 | 1 | C activates, D represses | |
| GAL4 sites | 1, 3, or 5 | 0 | GAL4-WT1 represses | (119) |
| WT1 promoter site | 1 | 0 | repression | (169) |
| Pax-2 sites | 0 | 3 | repression | (170) |
| EGR-1 sites | 3 | 0 | activation | (163) |
| binding-site selected sites | 1 or 3 | 0 | activation | (69) |

## 2G-2. Mechanism of transcriptional regulation by WT1.

**2G-2a. Factors which influence the transcriptional regulatory function of WT1.** Transcriptional regulation by WT1 appears complex, and may be modified by a number of factors which influence whether WT1 acts as a repressor or an activator of transcription. These variables include differences in WT1 isoforms, promoter architecture, and interaction with other cellular proteins, such as p53. Studies on the PDGF-A promoter suggest that WT1 binding sites both upstream and downstream of the transcription start site are required for repression or "suppression" of transcription by WT1, whereas on many promoters, WT1 activates transcription from either upstream or downstream sites (69, 163, 205). Sites both upstream and downstream are also required for repression of the IGF-II and IGF1R promoters (39, 209), although in these cases activation of truncated promoter fragments has not been observed. In contrast to these results, work on other promoters has shown that WT1 can repress transcription from either upstream or downstream sites, both in its native state and when expressed as a GAL4-WT1 fusion protein (118, 119, 169, 170). To complicate matters further, work from us (163) and others (69) shows that WT1 is a default activator of a simple test promoter containing upstream WT1 binding sites. It is not known whether WT1 represses transcription at the level of initiation or elongation. The requirement for 3' WT1 binding sites for repression of many promoters could be consistent with a mechanism in which bound WT1 blocks elongation of the nascent RNA molecule.

The requirement for WT1 binding sites on both sites of the start site for repression of several promoters (39, 205, 209) suggests that interaction

between WT1 molecules bound upstream and downstream may be required for repression activity. This might proceed via a looping mechanism as has been proposed for repression of the *Ultrabithorax* promoter by the *Drosophila even-skipped* protein (194), resulting in inhibition of transcriptional initiation or elongation. However, an important caveat must be noted with respect to the role of "upstream" and "downstream" sites in repression by WT1. Given the wide variety of WT1 binding sites which have been isolated, it is likely that some of these putative target promoters containing only "upstream" sites may also contain as-yet unidentified "downstream" sites as well.

The provocative finding that WT1 interacts with the p53 tumor suppressor protein (120) and that these two tumor suppressor proteins can influence each others' transcriptional functions suggests that interaction with other cellular proteins can also modulate the transcriptional effector function(s) of WT1. However, it appears that p53 is not required for WT1 to perform all of its developmental functions, as mice with a targeted gene disruption of p53 are morphologically normal but prone to tumor development (36, 86), while mice null for the WT1 gene show prenatal lethality and a variety of developmental defects including complete agenesis of the kidneys (100). The site of interaction of WT1 and p53 has not yet been mapped, and it is not known how mutations in either WT1 or p53 might affect this interaction. As noted, p53 has pleiotropic effects on transcription, activating and repressing many promoters in both binding site-dependent and independent mechanisms (213). These effects may cloud the interpretation of the functional significance of the WT1/p53 interaction.

Lastly, serving as a caveat to the limits of current experimental analysis, the choice of expression vector for WT1 may affect the magnitude or direction of transcriptional regulation observed in transient transfection experiments. The work of Malik *et al.*, in which expression of WT1 was induced in a stably transfected cell line (121), is the only study which has shown repression by WT1 using a WT1 expression vector not driven by the strong CMV promoter. In this case the maximum repression observed was 2-fold (121), compared with up to 25-fold repression observed when a CMV-WT1 expression vector was co-transfected with the PDGF-A promoter (49, 208), suggesting that high levels of WT1 protein may be required for repression. We have seen only activation by WT1, either on a synthetic reporter containing Egr-1 binding sites upstream of the HSV-*tk* promoter, or on the Egr-1 promoter, when using a Rous sarcoma virus (RSV) promoter-driven expression vector, whereas with a CMV-based expression vector we observe repression of the Egr-1 promoter (Chapters III and IV; (162, 163)). We currently do not believe that these differences lie in the amounts of protein produced, but rather that they are due to alteration in the cellular transcriptional milieu secondary to transfection of the strong CMV promoter.

**2G-2b. Modulation of transcriptional regulation by protein-protein interactions.** Recent studies from Wang *et al.* (207) may shed some light on the mechanism of transcriptional regulation by WT1. As noted above, a PDGF-A promoter fragment containing both upstream and downstream WT1 binding sites was repressed by WT1. Upon expression of the repressor domain of WT1 (amino acids 85-179) fused to the GAL4 DNA-binding domain, repression of the PDGF-A promoter was decreased. Conversely, a smaller PDGF-A promoter fragment containing only a 3' WT1 binding site is activated by WT1. Upon

cotransfection of the same GAL4-WT1(85-179) plasmid, activation of this promoter was augmented. Expression of the activation domain of WT1 fused to GAL4 had no effect in either case.

These findings were interpreted as implying the existence of a co-repressor molecule whose activity is titrated out by the GAL4-WT1(85-179) protein. Wang *et al.* (207) also suggest that tumor-associated heterozygous mutations of WT1 which yield mutant WT1 proteins unable to bind DNA could act as dominant negative mutations by titrating out this putative co-repressor. Work from our group (Chapter III and (163)) defined a domain including the repression domain (amino acids 1-182) as a self-association domain of WT1. Expression of this domain, or of two different non-DNA-binding mutant WT1 proteins, resulted in inhibition of transcriptional activation by WT1. These mutant proteins interacted directly with wild-type WT1 as measured by an *in vitro* biochemical assay and by the*in vivo* yeast 2-hybrid system. In light of our finding that WT1 self-associates, and that the self-association domain we defined overlaps with the repression domain, we think it likely that the putative co-repressor proposed by Wang *et al.* (207) may in fact be WT1 itself. In the case of the PDGF-A promoter, expression of the N-terminus of WT1 inhibited repression or augmented activation, whereas in our experiments using a synthetic reporter gene with upstream WT1 binding sites, expression of a similar protein resulted in a decrease in activation (163, 207). This may be due to differences in the structure of these promoters, and the interaction of WT1 with other transcription factors bound to these promoters.

WT1 also appears capable of activating transcription in a non-DNA-binding site dependent manner. Maheswaran *et al.* found that p53-mediated activation of

the muscle creatine kinase (MCK) promoter was augmented by co-expression of WT1, even though the MCK promoter itself did not contain a WT1 binding site and was unaffected by expression of WT1 alone (120). Preliminary results from our group indicate that WT1 can synergize with a chimeric GAL4-Sp1 fusion protein to activate transcription from a test promoter containing 5 GAL4 sites upstream of the HSV-*tk* promoter. Furthermore, this synergistic activation is not dependent upon the presence of WT1 binding sites in the reporter (J.C.R and J.D.L., unpublished results), suggesting that WT1 may be able to interact with other transcription factors even when it is not bound to DNA. Taken together, these results suggest that WT1 may be able to interact with other transcription factors, and with itself, in a non-binding-site dependent fashion, and that these interactions can result in modulation of the regulatory effect of the bound transcription factors.

**2G-2c. Models for the mechanism of transcriptional regulation by WT1.** Despite the large number of studies describing transcriptional regulation by WT1, the mechanisms by which WT1 activates or represses transcription remain obscure. Several possible mechanisms for transcriptional regulation by WT1 are presented in Figure 3. Activation may proceed through interactions with co-activator proteins (including TBP-associated factors or TAFs), or with components of the basal transcriptional machinery. Transcriptional repression can occur via several mechnisms: competition for binding sites with activator proteins (107), "quenching" of the activity of nearby bound activator proteins (107), or "squelching", in which co-factors required for activation are titrated out (154). WT1 may also repress transcription by competing with activator proteins, such as Sp1 or Egr-1, for GC-rich binding sites. As noted above, a looping interaction between WT1 molecules bound upstream and downstream of the

transcription start site may be important for repression by WT1. DNA binding and looping could be cooperative and might depend on the self-association domain of WT1 (163). This might account for the inability of a protein containing the WT1 zinc fingers alone to suppress transcription of the PDGF-A promoter (205). It will also be of great interest to learn whether WT1 interacts with co-factors or basal transcription factors, as these interactions may yield insight into how WT1 exerts its positive and negative transcriptional effects. Preliminary data from our group indicate that the activation domain, but not the repression domain, of WT1 can interact with the basal transcription factors TBP and TFIIB, suggesting a possible mechanism for transactivation by WT1 (S. Hosono, J.C.R, and J.D.L., unpublished data) . The failure of the repression domain of WT1 to interact with basal factors, as well as the cell type-specific nature of repression by amino acids 84-180 (119), suggests a different pathway for repression by WT1, possibly involving cell type-specific co-factors.

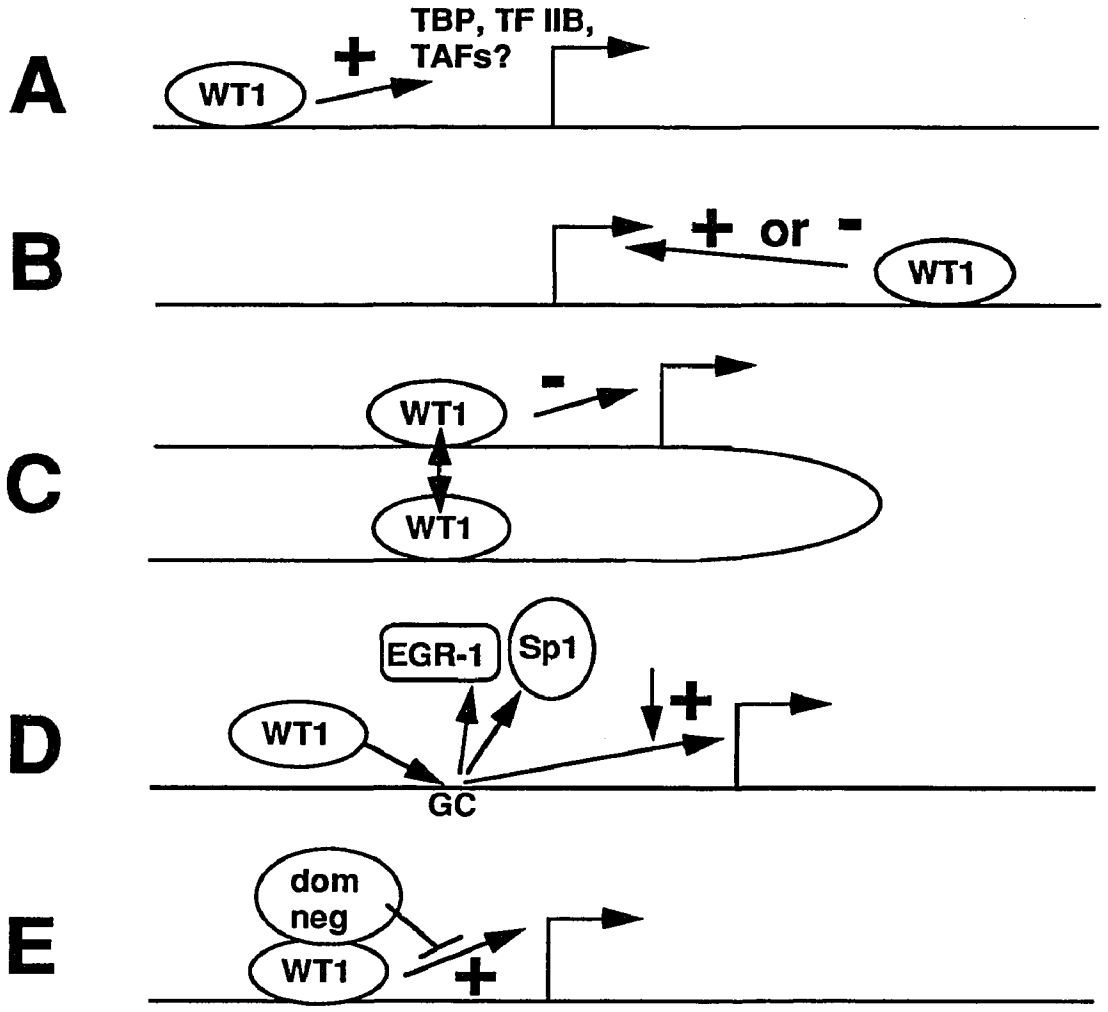



**FIGURE 3**

**Possible mechanisms for transcriptional regulation by WT1**

**A:** WT1 activates transcription when bound to upstream binding sites. This may occur through interactions with basal transcription factors or TAFs.

**B:** WT1 bound downstream of the promoter start site can activate (truncated PDGF-A promoter) or repress transcription (Pax-2 promoter sites fused 3' to the HSV-*tk* promoter).

**C:** WT1 bound both upstream and downstream of the start site represses transcription, possibly via interaction between WT1 molecules and formation of a loop of DNA around the promoter.

**D:** WT1 competes with activators, such as Sp1 and EGR-1, for binding to GC-rich sequences in promoters. If WT1 is a repressor or a weaker activator than these other activators, binding of WT1 results in a decrease in promoter activity.

**E:** Dominant negative WT1 proteins inhibit transcriptional activation by wild-type WT1, possibly by interacting with the wild-type protein and shielding its transactivation domain.

**2G-2d. Potential WT1 target genes.** Studies have suggested that overexpression of *IGF-II*, *PDGF-A*, and *Pax-2* in Wilms' tumors is the result of a loss of repression of these genes by WT1. The data that WT1 can repress these genes in transient transfection experiments is consistent with this hypothesis, but does not address the fact that most Wilms' tumors do not contain detectable WT1 mutations (see below). In addition, it is not clear whether WT1 regulates any of these putative target genes in their endogenous chromosomal configuration. A recent study found no correlation between the levels of expression of the *WT1*, *IGF-II*, and *PDGF-A* genes in mesothelioma cells (104). Zhou *et al.* also failed to observe a correlation between the expression patterns of *WT1*, *IGF-II*, and *IGF-1R* in the decidua of the pregnant rat (216). Examination of the expression pattern of these genes after stable or inducible expression of *WT1* in appropriate cell lines, preferably of kidney origin, or in WT1(-/-) embryonic stem cells, may help to determine which, if any, of these putative target genes are actual regulatory targets of WT1. WT1 has been shown to bind to several kinds of GC-rich sites which are present in many promoters (see Table 2). We found that WT1 can promiscuously activate many such GC-rich promoters, including the Rous sarcoma virus, cytomegalovirus, and simian virus 40 promoters, in transfection experiments (J.C. Morris, J.C.R, and J.D.L., unpublished data). Therefore, transfection studies which examine regulation of GC-rich promoters by WT1 may be complicated by promiscuous binding of and activation by WT1, particularly when it is expressed at high levels.

Examination of gene expression during normal kidney development suggests a number of genes which may be physiological targets for regulation by WT1. Since the metanephric blastema and the ureteric bud undergo reciprocal inductive interactions which are required for normal kidney development (175),

genes which are expressed in one but not the other of these tissues might be targets for either positive or negative regulation by WT1. Hepatocyte growth factor/scatter factor (HGF/SF) is expressed in the metanephric blastema but not in the ureteric bud, while its receptor, c-met, is expressed in the ureteric bud but not in the blastema (189). This suggested that the HGF/c-met interaction might be the signal between the blastema and the ureteric bud that was proposed to be disrupted in the WT1(-/-) mouse, whose blastema fails to undergo a mesenchymal-to-epithelial transition in response to inductive stimuli (100). However, mice homozygously deleted for HGF/SF had apparently normal kidneys (176, 197). Nevertheless, this result may be due to the existance of redundant systems of regulation, and WT1 may still normally coordinately regulate expression of HGF and other cytokines.

The *c-ret* gene is expressed in an opposite pattern to *c-met*, that is, *c-ret* is expressed in the ureteric bud but not the metanephric mesenchyme (142). Homozygous deletion of *c-ret* in mice resulted in partial or complete agenesis of the kidneys (177). *c-ret* encodes a receptor tyrosine kinase whose ligand is as yet unknown; this ligand may be expressed in the metanephric blastema and could be a target for transcriptional regulation by WT1.

Genes encoding extracellular matrix proteins may also be targets for regulation by WT1. Stable expression of WT1 in NIH 3T3 cells results in flattening of cell morphology, suggesting that changes in extracellular matrix and/or intracellular structural proteins may result from expression of WT1 (117). Downmodulation of WT1 function in mature podocyte cells by dominant negative alleles, as is seen in the Denys-Drash syndrome, may lead to disregulation of extracellular matrix genes, leading to nephropathy (147, 163). Finally, WT1 may regulate the

expression of cellular adhesion molecules such as syndecan and uvomorulin, whose expression is upregulated as the metanephric mesenchyme is induced to differentiate (93, 198).

**2H. WT1 as a repressor of cell growth.** In addition to its effects on transcription, WT1 can also inhibit DNA replication, suggesting a direct role for WT1 in control of cellular proliferation. Anant *et al.* (3) reported that expression of any of the four WT1 isoforms resulted in inhibition of replication from SV40 origins of replication. Both the (-KTS) and (+KTS) forms of WT1 were shown to bind to the 21-bp GC-rich repeats in the SV40 origin which also bind Sp1. The regions of WT1 required for inhibition of DNA replication included the N-terminal 180 amino acids (which are also required for transcriptional repression (205, 207) and self-association (163)) as well as the zinc fingers. This implied that the DNA-binding and protein-protein interaction functions of WT1 were required for inhibition of replication. Unlike the p53 tumor suppressor protein, which also inhibits SV40 replication, WT1 did not associate with SV40 T antigen, suggesting that WT1 inhibits SV40 replication via a different mechanism than p53. It is not yet known whether the 21-bp repeats which bind WT1 may be a model for regulatory sequences within the human genome.

Confirming the genetic data which identified WT1 as a tumor suppressor gene, WT1 can repress the transformed phenotype. Re-introduction of wild-type WT1 isoforms along with a neomycin resistance gene by transfection into a Wilms' tumor cell line (RM1) expressing a defectively spliced WT1 mRNA suppressed the ability of these cells to form neomycin-resistant colonies (65). In addition, WT1 can suppress the transformed phenotype of a heterologous tumor type. Stable transfection of WT1(A) into *ras*-transformed NIH 3T3 cells suppressed

colony formation, reduced growth in soft agar, reduced cellular proliferation rates, and inhibited tumor formation in nude mice (117). In addition, WT1 expression in non-transformed cells prevented transformation by activated *ras*.

In support of these data, expression of WT1 or Egr-1 can suppress the transformed phenotype of NIH 3T3 cells transformed with the *v-sis* oncogene, as assayed by focus formation, growth in soft agar, and tumorigenicity in nude mice (81). Conversely, expression of antisense mRNA for Egr-1 decreased the level of endogenous Egr-1 in these cells and augmented the transformed phenotype as measured by these assays. The domain of Egr-1 required for suppression of transformation was mapped to the zinc fingers. This information suggests that WT1 and Egr-1 suppress transcription at the nuclear level in these systems by binding to GC-rich binding sites in the promoters of genes activated by growth factors or by the *ras* signalling pathway. These data suggest that WT1 could interfere with the signal transduction pathways critical for normal and neoplastic growth.

Though Egr-1 is generally associated with positive regulation of growth, Egr-1 also mediates IL-1-dependent growth arrest of melanoma cells (181). Expression of a hybrid protein containing the repression domain of WT1 fused to the DNA-binding domain of Egr-1 resulted in inhibition of IL-1-induced growth arrest in this system. Although it was not examined in this study, it seems likely that WT1 itself would have a similar effect. Coexpression of the WT1/Egr-1 hybrid and Egr-1 (181), or WT1 and Egr-1 (118), has been shown to decrease transactivation of test promoters by Egr-1, further suggesting that WT1 may regulate transcription by competition for binding sites. Taken together, these data suggest that WT1 and other transcription factors, such as Egr-1 or Sp1,

may compete for DNA binding sites in promoters critical for cell growth, possibly explaining how WT1 can inhibit cell proliferation and other neoplastic processes.

Interestingly, while wild-type WT1 has tumor suppression activity, a mutated WT1 allele was shown to cooperate with the adenovirus E1A oncoprotein to transform cells (67). This mutant WT1 protein, in which the third zinc finger is deleted due to a mutation in a splice donor site, cooperated with E1A to transform baby rat kidney cells, while wild-type WT1 neither cooperated with nor suppressed transformation by E1A. The mechanism by which this mutant allele induces transformation has not been elucidated, but may be related to sequestration of p53 or other factors away from promoters by non-DNA-binding forms of WT1.

Finally, recently reported data may shed light on the mechanism whereby *WT1* suppresses cell division. Kudoh *et al.* found that microinjection of a *WT1* cDNA into quiescent NIH 3T3 cells blocked serum-induced entry into S phase (101). The WT1(B) and (D) isoforms exhibited a greater blocking ability than the WT1(A) and (C) isoforms, implying that the 17 amino acid insertion present in WT1(B) and (D) may play a role in this process. Moreover, this blockage of entry into S phase was relieved by overexpression of either cyclin E and CDK2 or cyclin D1 and CDK4. Together, these data suggest a role for *WT1* in cell cycle control.

**2l. A role for WT1 in mRNA splicing?** Most recently, a function for WT1 in mRNA processing as well as transcription was proposed. Using immunostaining and confocal microscopy, as well as immunoprecipitation

techniques, Larsson *et al.* found that WT1 colocalized with proteins involved in mRNA splicing in the nucleus of cells of a mesonephric cell line (105). By transfection of each of the four WT1 isoforms into COS cells, they showed that the +KTS isoforms largely colocalized with these splicing factors, while the -KTS isoforms largely colocalized with the transcription factor Sp1 and the basal transcription factor TFIIB in large "transcription domains". COS cells were used in these studies because they are transformed with simian virus 40 (SV40) and exhibit these large "transcription domains" when they are transfected with expression vectors containing an SV40 origin of replication, such as those used in this study. Since all four isoforms of WT1 have recently been shown to bind to the 21-bp GC-rich repeats in the SV40 origin of replication and inhibit replication (3), it would be of interest to learn whether these "transcription domains" represent sites of transcription of genomic DNA or whether they represent sites of replication and/or transcription of the transfected expression vector. Since Sp1 also binds to these 21-bp repeats, and since the SV40 origin of replication also contains a promoter, Sp1 and TFIIB might also localize to such domains. While these provocative results suggest a possible role for WT1 in mRNA processing, they do not exclude a role for WT1(+KTS) in other cellular processes. Indeed, the +KTS isoforms have been shown to regulate transcription of the PDGF-A (208) and WT1 (169) promoters in transient co-transfection experiments, to suppress growth of a Wilms' tumor cell line which expresses an aberrantly spliced WT1 mRNA (65), and to inhibit DNA replication directed by the SV40 origin of replication (3).

## 3. The role of WT1 in tumorigenesis.

**3A. How do WT1 mutations affect the functions of WT1?** Several categories of mutations in the WT1 gene have been described, inluding gross deletions, small insertions or deletions, point mutations, deletions/insertions associated with di-and tri-nucleotide repeats, and splicing defects. These mutations can be characterized as to whether they present solely in tumors, or in both the tumor and the germline, and whether they are heterozygous or homozygous.

Gross germline deletions are exemplified by WAGR syndrome-associated deletions of 11p13. These mutations delete part or all of the WT1 gene (as well as the adjacent AN2/Pax-6 gene) and are hemizygous. WAGR patients who develop Wilms' tumor always show mutations in the remaining WT1 allele isolated from their tumor tissue (6, 15, 55). By analogy with the mouse model (100), homozygous loss of WT1 function in the human germline is likely to be embryonically lethal. However, a Denys-Drash syndrome patient who carried a homozygous germline WT1 point mutation was recently described (91). This mutation results in the substitution of a tyrosine residue for the a key cysteine residue in the second zinc finger of WT1 and thus would be predicted to interfere with DNA binding. If this is a null mutation, it refutes the hypothesis that WT1 is required for kidney development in the human. However, the protein product of this mutant allele may retain some DNA-binding activity. In addition, this case suggests that genetic events in addition to homozygous alteration of WT1 are required for development of Wilms' tumor (91).

Point mutations and small insertions or deletions account for many of the reported Wilms' tumor-associated WT1 mutations. A listing of such mutations is provided in Tables 4A and 4B. Most of the mutations involve key residues

within the zinc finger region of WT1 and many of these have been shown or have been proposed to disrupt DNA binding by WT1. Other mutations truncate the WT1 protein N-terminal to or within the zinc finger region. Many of these zinc finger point mutations or truncations have been isolated in the heterozygous state from germline DNA of patients with Denys-Drash syndrome (DDS), suggesting that these non-DNA-binding isoforms act as dominant negative mutants, a hypothesis first proposed by Pelletier *et al.* (147). While the Wilms' tumors of many DDS patients have homozygous WT1 mutations, it is believed that the dominant negative allele interferes with WT1 function in non-malignant genitourinary tissues, thus accounting for the genitourinary malformations and nephropathy observed in DDS patients. Based on an analysis of DDS-associated mutations, Bardeesy *et al.* (10) recently proposed that a protein encoded by exons 1, 2, and 3 of WT1 (amino acids 1-222) would define the minimal domain of WT1 required for the dominant negative effect. We confirmed this prediction by showing that a protein containing only amino acids 1-182 acts as a dominant negative mutant in transfection assays by inhibiting transactivation by wild-type WT1 (Chapter III and (163)). Furthermore, our results suggest that this effect is mediated by protein-protein interaction between wild-type and mutant WT1 proteins.

# TABLE 4A

## *WT1* mutations associated with Wilms' tumor:

### Point mutations and frameshifts in the zinc finger region of *WT1*.

* = mutation to stop codon; f=frameshift after the indicated amino acid. Residues important for coordination of the zinc atom (C and H) or recognition of DNA bases (all others) are underlined; the KTS sequence inserted as a result of alternative splicing is indicated in bold type. Mutations which were found to be heterozygous in the germline or tumor are classified as possible dominant negative mutations.

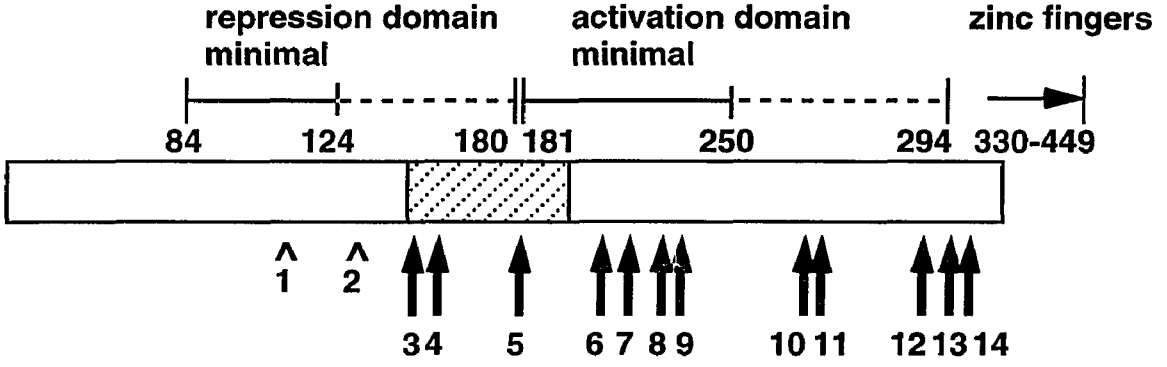
```
                    Y          Y
ZF1  325  CAYPGCNKRYFKLSHLQMHSRKHTGEKPYQ

                    G      C   .   Y    R
          Y         Yf*   FH       Q    Y        f
ZF2  355  CDFKDCERRFSRSDQLKRHQRRHTGVKPFQ

                         P  G
           f       *     W  N
ZF3  385  CKT    CQRKFSRSDHLKTHTRTHTGKTSEKPFS

                                    f
ZF4  416  CRWPSCQKKFARSDELVRHHNMHQRNMTKLQLAL
```

| Mutation | # cases | Dom. Neg.? | References |
|---|---|---|---|
| 330 C -> Y | 1 | Yes | (17) |
| 338 S -> Y | 1 | Yes | (200) |
| 355 C -> Y | 1 | No | (172) |
| 360 C -> Y | 1 | Yes | (25) |
| 360 C -> G | 1 | Yes | (113) |
| 361 frameshift | 1 | Yes | (200) |
| 362 R -> stop | 5 | Yes | (7, 25, 29, 200) |
| 365 S -> F | 1 (rat) | Yes | (184) |
| 366 R -> H | 2 | Yes | (7, 147) |
| 366 R -> C | 1 | Yes | (112) |
| 373 H -> Q | 1 | Yes | (113) |
| 373 H -> Y | 1 (rat) | No | (184) |
| 377 H -> Y | 1 | Yes | (30) |
| 377 H -> R | 1 | Yes | (135) |
| 384 frameshift | 1 | No | (200) |
| 386 frameshift | 1 | Yes | (138) |
| 390 R -> stop | 3 | Yes | (112, 155, 200) |
| 394 R -> W | 18 | Yes | (1, 7, 17, 25, 30, 135, 147, 172) |
| 394 R -> P | 1 | Yes | (17) |
| 396 D -> N | 5 | Yes | (7, 113, 135, 147) |
| 396 D -> G | 1 | Yes | (147) |
| 432 frameshift | 2 | Yes | (29) |

# TABLE 4B

## *WT1* mutations associated with Wilms' tumor:

**Frameshifts and point mutations outside the zinc finger domain of *WT1*.** The hatched box indicates the amino acids coded for by exon 2, which are presumed to be deleted in the Wilms' tumor cell line described in (65). The carets indicate point mutations isolated from carcinogen-induced nephroblastomas in rats; the arrows indicate mutations isolated from Wilms' tumors (except for 273 S->G which was isolated from a mesothelioma). Mutations which were heterozygous in the germline or the tumor are indicated as possible dominant negative mutations.




| # | Mutation | Dom. Neg.? | Reference |
|---|---|---|---|
| 1 | 112 F -> Y | No | (184) |
| 2 | 129 P -> L | Yes | (184) |
| 3 | 154 F -> S | Yes | (143) |
| 4 | 161 frameshift | No | (143) |
| 5 | 181 P -> S | Yes | (53) |
| 6 | 194 frameshift | No | (200) |
| 7 | 201 G -> D | No | (145) |
| 8 | 220 Y -> stop | Yes | (10) |
| 9 | 224 frameshift | Yes | (148) |
| 10 | 273 frameshift | Yes | (7) |
| | 273 S -> G | No | (144) (mesothelioma) |
| 11 | 275 frameshift | Yes | (10) |
| 12 | 291 frameshift | Yes | (148) |
| 13 | 301 frameshift | No | (55) |
| 14 | 312 frameshift | Yes | (7) |

Other point mutations of *WT1* have been isolated which result in a change in transcriptional function of the WT1 protein. A homozygous serine-to-glycine mutation at codon 273 of *WT1* was isolated from a mesothelioma specimen (144). This mutated allele yields a protein which activates rather than represses transcription from the Egr-1 promoter in transfection assays. A glycine-to-aspartate mutation at codon 201 was found in the remaining allele of a hemizygous WAGR patient (145). This mutation also yielded a protein that activated rather than repressed transcription from the Egr-1 promoter. Both of these mutations resulted in single amino acid changes in the region of WT1 mapped by deletion analysis and GAL4-WT1 fusion analysis to be required for activation, but not for repression, by WT1. Hence, it is not apparent why such mutations result in a loss of transcriptional repression activity. Perhaps such mutations result in expression of a misfolded protein which fails to interact with the transcriptional machinery in a normal fashion. An alternative hypothesis would hold that these mutations may increase the potency of the transactivation domain of WT1 without affecting the structure or function of its repression domain. If the transcriptional activity of WT1 were the result of a balance between its repressor and activator functions, an increase in activation potential could mask the repressor function, thereby altering the function of the entire protein.

Deletion and insertion mutations associated with di- and tri-nucleotide repeat sequences in exon 1 of *WT1* were recently detected in 4 Wilms' tumor patients (82). All of these mutations result in frameshifts and premature translation termination of the WT1 protein. Three of these four mutations were found to be homozygous or hemizygous (due to deletion of WT1 on the other chromosome), however, one was apparently heterozygous. As this protein only encodes the

first 26 amino acids of WT1, fused to 61 other amino acids, it is not clear whether this protein could act as a dominant negative in the manner of other heterozygous mutations described above. Further mapping of the minimal self-association domain, currently identified as amino acids 1-182, may help clarify the role of this mutant protein.

Alterations in mRNA splicing of WT1 can also lead to the production of tumor-associated WT1 isoforms. One of the first described tumor-associated WT1 mutations was a heterozygous 25 bp deletion which resulted in the in-frame deletion of the third zinc finger with loss of the alternative splice donor sites that lead to the +KTS insertion (64). In a DDS patient, a heterozygous germline G-to-C mutation in the donor splice site of intron 6 results in skipping of exon 6 during *WT1* mRNA splicing. This mutation leads to a frameshift in exon 7 with a resultant truncation of the WT1 protein N-terminal to the zinc finger domains. In a WAGR patient, a 14-bp insertion was found at the splice donor site of exon 7 in the remaining wild-type allele (173). The nature of the *WT1* mRNA transcript produced in these cells was not examined, but this mutation would be predicted to result in aberrant mRNA processing, yielding a protein deleted for part or all of the zinc finger region. Overexpression of non-DNA-binding mutants can interfere with transactivation by WT1 and could possibly interfere with repression or other functions (163).

Alterations in the ratio of WT1(-KTS) to WT1(+KTS) may also lead to neoplasia and disease. A heterozygous germline WT1 mutation isolated from three different Denys-Drash syndrome patients contains a G-to-A mutation 5 nucleotides 3' to end of exon 9, which results in production of only -KTS transcripts (10, 17, 96). The +KTS forms of WT1 were recently shown to largely

colocalize with components of the mRNA splicing machinery, whereas the -KTS isoforms largely colocalized with other transcription factors (105). An imbalance in mRNA splicing which resulted in production of only WT1(-KTS)-encoding transcripts might interfere with the potential function of WT1(+KTS) in mRNA processing, or alternatively might increase the concentration of WT1(-KTS) in the nucleus, possibly leading to binding of WT1(-KTS) to low-affinity DNA binding sites.

Other splicing mutations affect the transcriptional activation and repression functions of WT1. An aberrantly spliced *WT1* transcript lacking exon 2 was detected in a Wilms' tumor cell line (65). This transcript was the only transcript observed in this cell line, but it was also detected, along with a normal-sized transcript, in RNA from a number of other Wilms' tumors tested. The nature and expression of the protein produced from this transcript is not known, but it would be predicted to lack amino acids 149-189. In addition, the alternatively spliced exon 5, which encodes a 17 amino acid domain which can increase repression or activation by WT1 (208)(163) was not included in WT1 mRNAs from this cell line (65). Re-introduction of any of the four wild-type WT1 cDNAs by transfection suppressed colony formation by this cell line, suggesting that the Δ(exon 2) transcript either results in a loss of WT1 function which can be replaced by any WT1 isoform, or represents a tumorigenic gain-of-function mutation whose effects are suppressed by wild-type WT1. The nature of the splicing defect in this cell line remains to be characterized, as the genomic DNA sequences surrounding exon 2 were found to be normal in these cells. Transfection of the Δ(exon 2) cDNA resulted in activation rather than repression of transcription from the EGR-1 promoter. The exon 2 deletion encompasses a region of the WT1 protein originally predicted to encompass both the repression and

activation domains (119, 205). Recent studies (207) indicate that this region does not include the minimal repression domain of WT1 and encompasses only a small portion of the activation domain of WT1. Therefore, as with the point mutations at codons 201 and 273, it is not clear why a mutation in the activation region would result in a defect in the repression activity of WT1. Perhaps as suggested above, the deletion of exon 2 sequences represents a gain of transcriptional activation function. This could be tested by introducing the exon 2 mutation in to the context of a GAL4-WT1 fusion protein and testing its ability to activate or repress transcription.

RNA editing of the WT1 transcript may also affect the transcriptional effector function of WT1. In rats and humans, WT1 cDNAs have either a T or a C residue at codon 280, while the genomic DNA contains only a T residue (183). An RNA editing mechanism was proposed to explain these changes. The resultant edited mRNA yields a WT1 protein with a proline rather than a leucine residue at codon 280, which is expressed at a similar level to the wild-type protein in transfected cells. This "edited" proline-containing WT1 isoform was a stronger repressor of transcription than the "unedited" leucine-containing protein. Interestingly, the region involved in this putative RNA editing event is not conserved in chicken or alligator mRNA, suggesting a mammalian-specific function for this event (74). Like the point mutations described above, RNA editing of WT1 alters the protein within its previously mapped transcriptional activation region, but effects are seen on its transcriptional repression function. Given the frequency with which this paradox has been noted, the data suggest that deletion mapping of functional domains, or mapping of such domains using hybrid GAL4-WT1 proteins, may yield results different from mutational analysis performed on an intact native protein.

The cause of mutations in the WT1 gene is not known. However, in a recent paper, Sharma *et al.* reported that treatment of rats with N-nitroso-N'-methylurea, an alkylating agent, induced embryonal kidney tumors which histologically resembled Wilms' tumors (184). In 7 of 18 of these tumors, point mutations in the WT1 gene were detected. These mutations resulted in single amino acid changes at codons 111, 128, 364, and 372. In two cases the mutations (codons 364 and 128) were heterozygous while in five other cases (codons 111 and 372) the wild-type allele was lost, rendering the mutation hemizygous. Intriguingly, mutants 111 and 128 fall within the repression and self-association domain of WT1 and may alter one or both of these functions (163, 207). Since this exon has not often been examined for *WT1* mutations in humans because it is highly GC-rich and difficult to amplify by PCR, the frequency of human WT1 mutations in this region is not known. This rat model for Wilms' tumor may provide further insight into how exposure to carcinogens might result in genetic changes at the *WT1* locus and alterations in the function of the WT1 protein.

**3B. Frequency of *WT1* mutation in Wilms' tumors.** Despite intense examination, the percent of Wilms' tumors which have been shown to contain *WT1* mutations is relatively low. Estimates of the frequency of *WT1* mutations are sometimes difficult to interpret because of varying methods of detection. In theory, *WT1* mutations cannot be entirely ruled out without completely sequencing the *WT1* cDNA and its associated genomic regulatory regions. Gross *WT1* deletion was found in only one (homozygous) of 30 Wilms' tumors (168), 12 of 58 tumors (all hemizygous, all WAGR patients) (123), 4 of 55 tumors (one homozygous, three heterozygous) (33), one (homozygous) of 42 tumors

(192), 3 (homozygous) of 25 tumors (92), one (homozygous) of 18 tumors (157), and none of 38 Wilms' tumors examined (202). The total rate of *WT1* mutations detected in these studies is 9.6%. A study of Japanese Wilms' tumor patients showed a significantly higher rate (15/41=36.6%) of abnormalities at 11p13 including two hemizygous deletions of 11p13 (both from WAGR patients) and eleven cases of loss of heterozygosity at 11p13 (90).

Single-stranded conformational polymorphism (SSCP) analysis, which can detect single base-pair mutations, detected only one *WT1* mutation in 20 Wilms' tumors examined (5%) (16). Such studies may underestimate the frequency of point mutations of *WT1*, as they usually examine only the zinc finger region. These results indicate that the rate of *WT1* mutation in Wilms' tumors may be less than 25% and perhaps as little as 5%. Alterations in the *Wit-1* gene, which is adjacent to *WT1* and is transcribed in the opposite direction, may also result in disregulation of *WT1* expression in tumors where the *WT1* gene itself is apparently normal (21, 41).

Mutation of the p53 gene has been suggested as a mechanism for alteration of WT1 function in the absence of mutation of the *WT1* gene, based on the finding that WT1 and p53 interact and modulate each others' transcriptional functions (120). One study showed overexpression of p53 in all of 34 Wilms' tumor specimens as assayed by staining of tissue sections with anti-p53 antibodies (106). High expression of p53 is often, but not always, associated with mutation of the p53 gene (213), however the mutational status of p53 cannot be assessed in this study since DNA sequencing was not performed. In contrast, Waber *et al.* (202) studied the p53 sequences of 38 Wilms' tumors by SSCP analysis but failed to find any mutations. The general chemosensitivity of

Wilms' tumors also supports the idea that p53 is wild-type in most of these tumors (43). Similarly, Malkin *et al.* (122) found only two cases of Wilms' tumors with p53 mutations out of 21 cases studied. These two specimens were both from patients with advanced stages of Wilms' tumor, one of whom had an anaplastic tumor. Consistent with this, it has recently been reported that p53 mutations were found in 8 of 11 anaplastic Wilms' tumors studied (9), a subtype of Wilms' tumor carrying a poor prognosis. The same group subsequently showed that a heterozygous p53 mutation in a non-anaplastic area of a Wilms' tumor was reduced to heterozygosity in an anaplastic area, suggesting that progression to anaplasia is associated with loss of p53 function (8). Furthermore, this group showed that Wilms' tumor cells with mutated p53 exhibit reduced rates of apoptosis, suggesting that reduced cell death may lead to tumor progression.

In summary, though *WT1* is clearly a tumor suppressor gene, *WT1* mutation is observed in only a minority of Wilms' tumors. *WT1* mutation may not be carcinogenic in itself, as DDS patients with germline *WT1* mutations develop Wilms' tumors years after birth. *WT1* mutations may interact with other Wilms' tumor genes, such as the 11p15 and 16q loci, or with other tumor suppressor gene or oncogene loci, to lead to the development of the tumor. In support of this hypothesis, we have characterized the protein product of a *WT1* allele bearing a point mutation at codon 154, which was isolated from a perilobar nephrogenic rest and a Wilms' tumor from the same patient (143). This mutation has no significant effect on the DNA-binding, transactivation, transrepression, or self-association functions of the WT1 protein (Chapter V; (161)). However, this patient exhibited many signs and symptoms of Beckwith-Wiedemann syndrome, suggesting that alterations at 11p15 loci might combine

with a slight effect on *WT1* function to cause neoplasia. Alternatively, this *WT1* mutation might affect functions of the protein which we have not yet examined, such as tumor suppression or inhibition of DNA replication. In addition, there may be many pathways towards the development of Wilms' tumor that may bypass *WT1*. Future clinical studies might contrast the presentation, histology, and response to therapy of tumors with wild-type or mutant *WT1* genes.

**3C. The role of WT1 expression, mutation, or translocation in other tumor types.** While *WT1* is expressed in a limited pattern in normal development, ectopic expression of *WT1* has been reported in a number of tumors other than Wilms'. *WT1* is expressed in some malignant human melanocyte cell lines but not in normal melanocytes, however the sequence of the *WT1* gene in these cell lines was not examined (167). Recent studies revealed a low but detectable level of *WT1* expression in normal bone marrow but not in peripheral blood by RT-PCR analysis, suggesting that WT1 may be expressed in early hematopoetic progenitors (84). However, another group reported that *WT1* expression was undetectable in normal bone marrow or peripheral blood (14). This may be due to differences in technique and in the sensitivity of RT-PCR analyses. *WT1* is expressed in both the K562 erythroleukemia cell line and in the HL60 myeloid leukemia cell line and its expression is downregulated at the post-transcriptional level during differentiation of these cell lines (150, 179). *WT1* mRNA expression has been detected in some cases of acute and chronic leukemia (127) as well as in some myeloid and lymphoid cell lines (129). In addition, expression of *WT1* was shown to be useful in the detection of minimal residual leukemic blast cells in leukemia patients (14, 84), and may be more sensitive than the detection of fusion gene products in cases of leukemia associated with reciprocal

chromosomal translocations. The role of *WT1* mutation in leukemogenesis is unclear, since these cell lines and patient samples were not examined for *WT1* mutations. However, a mutation was detected in the remaining *WT1* allele of a WAGR patient who had been previously treated for Wilms' tumor (153). This mutation affected a cysteine residue in the zinc finger region and would be predicted to abolish DNA binding by WT1. This suggests that complete loss of WT1 function in the myeloid lineage might be associated with the development of leukemia.

Park *et al.* (144) reported expression of *WT1* mRNA in normal and malignant mouse and human mesothelial cells and isolated a homozygous point mutation of *WT1* associated with a mesothelioma, suggesting that mutation of *WT1* might have a causative role in mesothelioma development. Only one *WT1* mutation has been found in 46 mesotheliomas so far examined, suggesting that alteration of *WT1* may be a rare event in mesothelioma (104, 144). *WT1* was also found to be expressed by normal and transformed mesothelial cells of the rat (203), and therefore expression of *WT1* has been proposed as a marker to differentiate mesothelioma from other tumors involving the pleura (2, 203).

*WT1* was recently shown to be involved in a reciprocal translocation with the EWS gene in some cases of desmoplastic small round cell tumor (DSRCT; also known as melanoma of the soft parts) (102). The translocation fuses the first seven exons of EWS, which encode an RNA binding protein (139), to the last three exons of *WT1*, which encode zinc fingers 2, 3, and 4 (51). It has been proposed that this chimeric fusion may be able to bind to WT1 target sequences and may act as a transcriptional activator rather than as a repressor (D. Haber, personal communication).

It is not clear whether mutation of *WT1* is involved in the development of tumors of the urogenital system other than Wilms'. A heterozygous *WT1* point mutation was shown to be reduced to homozygosity in a juvenile granulosa cell tumor as well as in a Wilms' tumor from a DDS patient (147). Bruening *et al.* showed that 75% of ovarian tumors examined expressed *WT1*, however the only abnormalities that were detected were silent intronic mutations (18). Another group also detected *WT1* expression in ovarian tumors but also failed to detect WT1 mutations (201). However, high levels of expression of the p53 tumor suppressor protein were detected in some of the tumor samples, which is suggestive of mutation of the p53 gene. Since p53 and WT1 have been shown to interact and to regulate each other's transcriptional functions (120), these authors proposed that mutation of p53 could affect WT1 function in ovarian tumors, possibly leading to neoplasia.

A polymorphism near the *WT1* gene was shown to be associated with testicular germ cell tumors in Norwegian patients (76), and loss of heterozygosity at 11p13 and 11p15 was frequently associated with such tumors (115). However, another group showed that the *WT1* gene was grossly normal and showed no sequence changes in exons 2 and 6 in testicular cancer cases involving loss of heterozygosity at 11p13 (114). A study of 15 male and female sex cord-stromal tumors also failed to detect *WT1* mutations (31). Loss of heterozygosity at 11p13, including allelic deletions of *WT1*, was also found in some cases of primary bladder carcinoma (187). In a survey of many different types of genitourinary tumors, however, mutations of zinc finger 3 (exon 9) of WT1, a mutational hotspot, were found only in Wilms' tumors and not in any other tumor

type (155). The relationship between *WT1* mutation and genitourinary tumors other than Wilms' tumor, therefore, remains obscure.

**4. Conclusions.** While much has been learned about the structure and function of WT1 proteins, there remain numerous questions about how WT1 exerts its effects on transcription, DNA replication, and cellular transformation. In particular, it has not yet been determined which cellular genes are actual targets for regulation by WT1 in the normal and cancerous states. Transfection experiments using reporter genes are useful tools for identifying protein-DNA interactions required for transcriptional regulation, but it will be of interest to determine whether these potential target genes (PDGF-A and IGF-II, for example) are regulated by WT1 in their endogenous chromosomal conformation. Use of stably transfected cells which overexpress WT1, or of WT1(-/-) ES cells, may help to answer these questions. It is also not known how exactly WT1 activates or represses transcription *in vivo*, and what determines whether WT1 will activate or repress transcription in transient co-transfection experiments. Specifically, what other proteins does WT1 need to interact with to exert its transcriptional effects? Are these proteins components of the basal transcription machinery, transcriptional co-factors or other sequence-specific DNA-binding transcription factors? Are some of these factors cell type-specific? Does DNA looping occur in repression by WT1 bound to sites upstream and downstream of the start site of transcription? The provocative finding that WT1 suppresses SV40 origin-mediated DNA replication raises the question of whether WT1 regulates DNA replication from mammalian origins of replication. Do the +KTS isoforms of WT1 really play a role in mRNA processing? How does expression of WT1 prevent entry into S phase, and is WT1 normally involved in the control of the cell cycle? Finally, it will be of interest to learn how

WT1 either induces (in the mutated state) or suppresses (in the wild-type state) transformation of cells, and whether this occurs in the same way in Wilms' tumors and in other tumor models. Review of the current literature points the way to a new generation of studies required to further define the role of WT1 in normal and neoplastic kidney development.

# CHAPTER II

# Materials and Methods

**Plasmid Construction.** The expression vectors for the murine *WT1*(A) and (B) isoforms and the two *WT(AR)* mutant proteins were described previously (67). The expression vector for WT(PM) was constructed by PCR amplification of sequences encoding amino acids 100-256 using the primers: 5'-GGCCAGTTCACCGGTACA-3' and 5'-GTCGACGTCGACTTATCCACTCTC-GTACCCTATAC-3'. The PCR product was then digested with *Age*I and *Sal*I and inserted into *AgeI/SalI* digested RSV-WT1(A). A RSV vector lacking the *WT1* coding region was constructed by restriction of the RSV-WT1(A) plasmid with *Bgl*II to release the *WT1* coding region, followed by recircularization. The $EGR_3$*tk*CAT reporter plasmid (Figure 1B) was constructed by the insertion of three tandem oligonucleotides containing the Egr-1 binding site (underlined) from the *zif268* promoter:

5'- TCGACCCTCGCCCCCGCGCCGGGC - 3'
3' - GGGAGCGGGGGCGCGGCCCGAGCT - 5'

(24) into the *Sal*I site of pBLCAT2 (116). The tk-growth hormone (*tk*-GH) (Figure 1B) internal control plasmid was described previously (180).

To construct a vector for *in vitro* translation of*WT1* protein, a 1.5 kb *Sau*3AI fragment derived from the RSV-WT1(A), (B), or WT(AR) expression vectors was cloned into the *Bam*HI site of pSP64 (Promega, Madison, WI). The pSP64-WT(PM) vector was constructed by cloning a *Hind*III/*Sal*I fragment of RSV-WT(PM) into *Hind*III/*Sal*I digested pSP64. The pSP64-WT(1-182) vector was

made by digestion of pSP64-WT1(A) with *Nco*I followed by ligation to the duplex oligonucleotide:

5' - CATGTGAGTCGACTCA - 3'
3' - ACTCAGCTGAGTGTAC - 5'

containing a stop codon. To express amino acids 1-182 of WT1 in transfected cells, a *Sau*3AI-*Sal*I fragment of pSP64-WT(1-182) was cloned into *Bgl*II/*Sal*I digested RSV vector. Vectors for translation of the zinc finger moeities of WT1(A) and WT(AR) were prepared by PCR amplification of the WT1 cDNA using an N-terminal primer 5'-GGATCCGGATCCACCATGTGTGCATACCCAGGC-3' and a C-terminal primer 5'-GAATTCGAATTCTCAAAGCGCCACGTGGAGTTT-3'. The resultant fragments were digested with *Bam*HI and *Eco*RI and inserted into pSP64.

To construct a vector for expression of a glutathione-*S*-transferase (GST)-WT1 fusion protein in *E. coli*, the pSP64-WT1A plasmid was digested with *Hinc*II and *Eco*RI with the resulting fragment cloned into *Sma*I/*Eco*RI digested pGEX-2tk (Pharmacia, Uppsala, Sweden) yielding the plasmid pGEX-WT1(A). The resulting fusion protein contains an additional 20 amino acids (GSPTLEDRRSGEPSASEPHL) after the GST moiety. To construct pGEX-WT1(1-183), pGEX-WT1(A) was restricted with *Nco*I, blunted with the Klenow fragment of DNA polymerase I and ligated to a duplex oligonucleotide containing stop codons in all three reading frames (34).

Expression of GAL4(1-147) was driven by the SV40 promoter in the plasmid pBXG1 (gift of M. Ptashne). GAL4-WT1 (gift of Y. Shi) was created by ligation of a blunted 2.1 kb *Cla*I/*Eco*RI fragment of the human *WT1* cDNA from

pGEM7zf(+)WT1 (gift of F. J. Rauscher III) into the blunted EcoRI site of the pSG424 vector (171). $GAL_5$*tk*CAT is described in (186).

pSP64-WT1(154) was constructed by PCR-based mutagenesis. Briefly, one PCR reaction was performed with a wild-type 5' primer 5'-GGCCAGTTCACCGGACA-3' and a mutant 3' primer 5'-GCCCCGTCG**G**AAGTGACCGT-3', using wild-type pSP64-WT1(A) as a template. A second PCR reaction was performed with a mutant 5' primer 5'-ACGGTCACTT**C**CGACGGGGC-3' and a wild-type 3' primer 5'-GAATTCGAATTCTCAAAGCGCCACGTGGGAGTTT-3'. The mutant base in each primer is indicated by bold type. PCR products from these two reactions were purified and annealed to each other, and were then reamplified with the wild-type 5' and 3' primers to produce a DNA fragment encoding amino acids 100-429 of WT1(A) containing the codon 154 mutation. This fragment was digested with *Age*I and *Eco*RI and was inserted into *Age*I/*Eco*RI digested pSP64-WT1 to produce a full-length WT1 coding region containing the codon 154 mutation, which was confirmed by sequencing. The plasmid pGEX-WT1(154) was constructed in an analogous manner. pGEX-WT1(1-182/154) and pSP64-WT1(1-182/154) were constructed from pGEX-WT1(154) and pSP64-WT1(154), respectively, by restriction of the parental construct with *Nco*I, filling in the ends with the Klenow fragment of DNA polymerase I, and ligation to a blunt-ended oligonucleotide containing stop codons in all three reading frames (described above).

pJ6Ω-WT1(A) was the kind gift of S. Hosono and was constructed by insertion of a *Sal*I/*Eco*RI fragment of pSP64-WT1(A) into pJ6Ω (130) which had been restricted with *Sal*I and *Eco*RI. pJ6Ω-154 was constructed by restriction of

pSP64-WT1(154) with *Age*I and *Nco*I and ligation of the resultant fragment into *AgeI/Nco*I digested pJ6Ω-WT1(A).

pCB6+, pCB6-hWT1(A), and EGR1-CAT were the gifts of V. Sukhatme and I. Drummond (131)(57). pCB6-mWT1(A) was constructed by restriction of pJ6Ω-WT1(A) with *Hind*III and *Bam*HI and ligation of the resultant fragment, which contains the murine WT1 coding region, into *Hind*III/*Bgl*II digested pCB6+. pCB6-WT1(154) was constructed in an analogous manner.

GAL4-WT1(1-182) was constructed by a three-way ligation bewteen a *Sma*I/*Nco*I fragment of an antisense version of pSP64-WT1(A), encoding amino acids 1-182, a double-stranded *Nco*I/*Xba*I linker created by annealing the oligonucleotides 5'-CATGGGCTGAGGATCCT-3' and 5'-CTAGAGGATCCTCAGCC-3', and pBXG1 (see above) which had been digested with *Sma*I and *Xba*I. GAL4-WT1(1-182/154) was constructed by restriction of GAL4-WT1(1-182) with *Age*I and *Xba*I and ligation of the resultant backbone to both an *AgeI/Nco*I fragment of pSP64-WT1(154) and the above-mentioned *Nco*I/*Xba*I linker.

**Transfection assays.** CV-1 African green monkey kidney cells were grown in DMEM containing 10% calf serum, while NIH 3T3 mouse fibroblast cells (gift of N. Landau), and 293 human embryonic kidney cells were cultured in 10% fetal calf serum. Cells were transfected with 2 μg of $EGR_3$*tk*CAT or $GAL_5$*tk*CAT reporter plasmid or 0.5 μg of EGR1-CAT reporter plasmid, up to 20 μg of WT1, pJ6Ω, or pCB6+ expression vector or the parental vector lacking the *WT1* insert as indicated, and 1 μg of *tk*GH plasmid as an internal control for transfection efficiency as described (110). Carrier DNA (pBluescript, Stratagene, La Jolla,

CA) was added to a total of 20 to 40 μg DNA. Normalized CAT activity was determined as percent conversion of chloramphenicol divided by the GH value in ng/ml (110), except in the case of experiments using the pCB6+ vectors, which were not corrected for GH values.

**Production of a monoclonal antibody against WT1.** Full-length GST-WT1(A) fusion protein, produced as described in (5), was injected into mice by Dr. Thomas Moran of the Hybridoma and Cell Center Core Facility at Mount Sinai. Dr. Moran provided us with a hybridoma cell line (24B2) which secreted antibodies recognizing GST-WT1 but not GST protein. Antibodies from 800 ml of hybridoma supernatant were purified by perfusion chromatography on a Protein G column using standard methods (72) with help from Lesley Scudder. The domain recognized by this monoclonal antibody was determined to be within the N-terminal 182 amino acids of WT1 by immunoblotting of various truncated WT1 proteins.

**Immunoblotting.** Transfected cells were boiled in 1X SDS sample buffer without ß-mercaptoethanol and extract concentrations were determined with the Bio-Rad DC Protein Assay Kit (Bio-Rad, Hercules, CA) using bovine γ-globulin as a standard. Following this, ß-mercaptoethanol was added to a final concentration of 5% and equal quantities of cellular proteins were separated by electrophoresis through a 10% or 12% SDS-polyacrylamide gel. The gel was then electrophoretically transfered to Immobilon PVDF membrane (Millipore, Bedford, MA) in a buffer containing 192 mM glycine and 25mM Tris base. The membranes were probed as indicated with either 0.1 μg/ml rabbit polyclonal anti-WT1 C19 Antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as directed by the manufacturer, or with 10.0 μg/ml anti-WT1 24B2 mAb (see

above), under similar conditions. Immunoreactive proteins were visualized by chemiluminescence and autoradiography (ECL kit, Amersham, Buckinghamshire, UK).

**Electrophoretic mobility shift assays.** Proteins were translated from pSP64-based vectors using the TNT rabbit reticulocyte lysate system (Promega, Madison, WI). In each case, a reaction containing $^{35}$S-methionine was performed in parallel with each non-labelled reaction. Equal volumes of lysate from the labelled reaction were subjected to SDS-PAGE and quantified on a PhosphorImager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Binding reactions were performed with equimolar quantities of translated WT1 proteins as described previously (147), except that the total volume of the reaction was 20 µl, the amount of probe used was 0.2 ng (approximately $10^6$ cpm/ng), and the electrophoresis was performed for 1.5 hours at 300V at room temperature.

***In vitro* protein binding assays.** GST and GST fusion proteins were purified as described (89) and the yield of each protein was determined by SDS-PAGE analysis and Coomassie blue staining. Volumes of bacterial lysate containing approximately 1 µg of GST or GST fusion protein were incubated with 25 µl of a 50% slurry of glutathione-agarose beads (Sigma, St. Louis, MO) in NET-50 (20 mM Tris pH 8.0, 1 mM EDTA, 50 mM NaCl) for 15 minutes at room temperature with rocking. GST proteins bound to the beads were then collected by brief centrifugation and washed once with 400 ml of NET-50. The beads were rocked for 10 minutes at room temperature in 200 µl of binding buffer (25 mM Hepes pH 7.5, 12.5 mM $MgCl_2$, 20% glycerol, 0.1% NP-40, 150 mM KCl, 1mM dithiothreitol (DTT), 150 µg/ml bovine serum albumin (BSA), 200

µg/ml ethidium bromide (EtBr) as indicated). Coupled *in vitro* transcription-translation reactions containing $^{35}$S-methionine (1175 Ci/mmol) were programmed with the indicated pSP64-WT or control SP6-luciferase plasmid and were diluted 1:10 in binding buffer (without DTT, BSA, or EtBr). Diluted programmed lysate (5 µl) was incubated with immobilized GST or GST-WT proteins for 1 hour at room temperature. The beads were collected by centrifugation and washed 3 times with 1 ml of NETN (20 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) in the presence or absence of 200 µg/ml EtBr as indicated. Bound proteins were eluted from the beads by boiling in 1X loading buffer (62.5 mM Tris pH 6.9, 10% glycerol, 2% SDS, 5% ß-mercaptoethanol) and were separated by electrophoresis through a 10% or 15% SDS-polyacrylamide gel. The gel was fixed in 30% methanol, 10% acetic acid for 1 hour, washed in water for 1/2 hour, soaked in 1M sodium salicylate for 1/2 hour, dried, and exposed to XAR film at -80°C. The percentage of input $^{35}$S-labelled protein bound to GST protein was quantified by exposure of the dried gel on a PhosphorImager and analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**Mapping of WT1 self-association domains using the two-hybrid system in yeast.** This procedure was performed by our collaborators and is described in (163).

**Graphics.** Autoradiographs were imaged using a Silverscan flatbed digital scanner (La Cie, Ltd., Beaverton, OR) or a UMAX UC630 scanner (UMAX, Taiwan, ROC). Image processing was performed on a Power PC (Apple, Cupertino, CA) using Adobe Photoshop (Adobe, Mountain, CA) and Aldus Persuasion (Aldus, Seattle, WA). Densitometry was performed using the NIH

Image 1.56 program for Power PC. Figures were printed using a Tektronix Phaser 400 printer (Tektronix, Wilsonville, OR).

# CHAPTER III

# WT1-Mediated Transcriptional Activation is Inhibited by Dominant Negative Mutant Proteins

## INTRODUCTION

The observation that heterozygous germline mutations of *WT1* were associated with development of the Denys-Drash syndrome led Pelletier to propose that these heterozygous *WT1* mutations were acting in a dominant negative fashion (147). As discussed in Chapter I, WT1 has both transcriptional activation and repression functions whose activity may be dependent upon a number of factors, including promoter architecture. Therefore we set out to characterize the transcriptional effects of WT1 on a simple test promoter and to determine whether these proposed dominant negative alleles of *WT1* could modulate the transcriptional function of wild-type WT1.

We found that WT1 was a potent transcriptional activator of a simple test promoter containing three Egr-1/WT1 binding sites upstream of the herpes simplex virus thymidine kindase (HSV-*tk*) promoter linked to the chloramphenicol acetyltransferase (CAT) gene. WT1(B) was a consistently slightly stronger transcriptional activator than WT1(A), suggesting a role for the 17 additional amino acids in WT1(B) in transcriptional activation. Consonant with Pelletier's hypothesis, we found that two genetically defined dominant negative alleles, which yield proteins unable to bind to the Egr-1/WT1 consensus site, inhibited the transcriptional activation function of wild-type WT1. The domain of the WT1 mutant proteins required for inhibition mapped to the N-

terminal 182 amino acids. This domain was found both by *in vitro* biochemical assays and by the *in vivo* yeast two-hybrid system (163) to mediate self-association of WT1 protein, providing a possible mechanism for the dominant negative effect of these mutant WT1 proteins.

## RESULTS

**Transcriptional activation by *WT1* proteins.** To characterize the transcriptional effects of WT1 proteins, the $EGR_3$*tk*CAT reporter containing three Egr-1 consensus sequences, capable of binding WT1(A) and (B) (160), (Figure 4B) was transfected into CV-1 cells along with increasing amounts of WT1(A) or (B) expression vectors (Figure 4A). Both WT1(A) and (B) acted as dose-dependent transcriptional activators of this simple test promoter. Transfection of 10 μg of the WT1(A) or (B) expression vector produced a 10 to 15-fold increase in reporter gene transcription. Transcriptional activation was binding-site dependent, as neither WT1(A) or (B) affected transcription from the *tk*-hGH plasmid or from a *tk*-CAT reporter gene (data not shown). Similar results were obtained in NIH 3T3 cells and in human embryonic kidney 293 cells (data not shown) indicating that activation was not cell type-specific. In addition, transcriptional activation by WT1(A) was linear and non-saturable at the doses used in these experiments (Figure 5C), implying that any co-factors required for WT1(A) activity are not limiting in CV-1 cells. After six independent determinations using three different preparations of expression vector plasmid, transcriptional activation by WT1(B) was consistently 20 to 50% stronger than activation by WT1(A) (Figure 5A). However, immunoblotting and densitometric analysis indicated that after transfection, WT1(B) was expressed at somewhat lower levels than WT1(A) (Figure 5B). In addition, *in vitro* translated WT1(A)

and (B) bound to the Egr-1/WT1 site to a similar extent (Figure 5D). Therefore, increased DNA binding is unlikely to account for the increased transactivational activity of WT1(B).

**FIGURE 4** (next page): A) Expression vectors for production of *WT1* proteins in transfected cells. Expression of WT1 was directed by the Rous Sarcoma Virus (RSV) promoter. "17" refers to the 17 amino acids inserted in WT1 as the result of alternative splicing of the *WT1* mRNA. The zinc finger domains are indicated by black boxes and are numbered. B) Reporter genes utilized. EGR3*tk*CAT contains 3 Egr-1/WT1 binding sites inserted upstream of the HSV thymidine kinase (*tk*) promoter and the chloramphenicol acetyltransferase (CAT) gene. In *tk*-hGH, the HSV-*tk* promoter is linked 5' to the human growth hormone gene. Secretion of human growth hormone from transfected cells is used as an internal control for transfection efficiency.

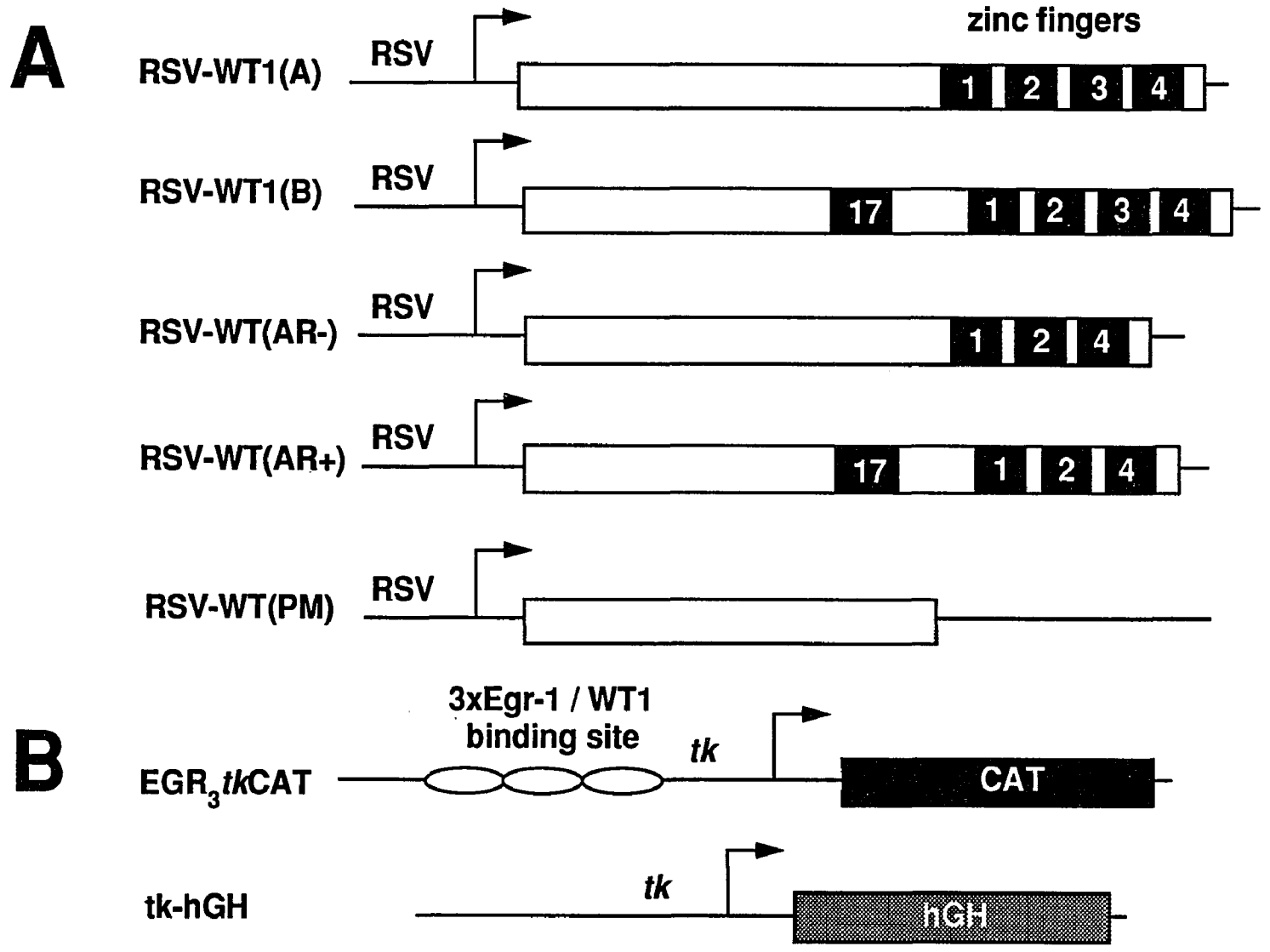



**FIGURE 4** (see previous page for legend)

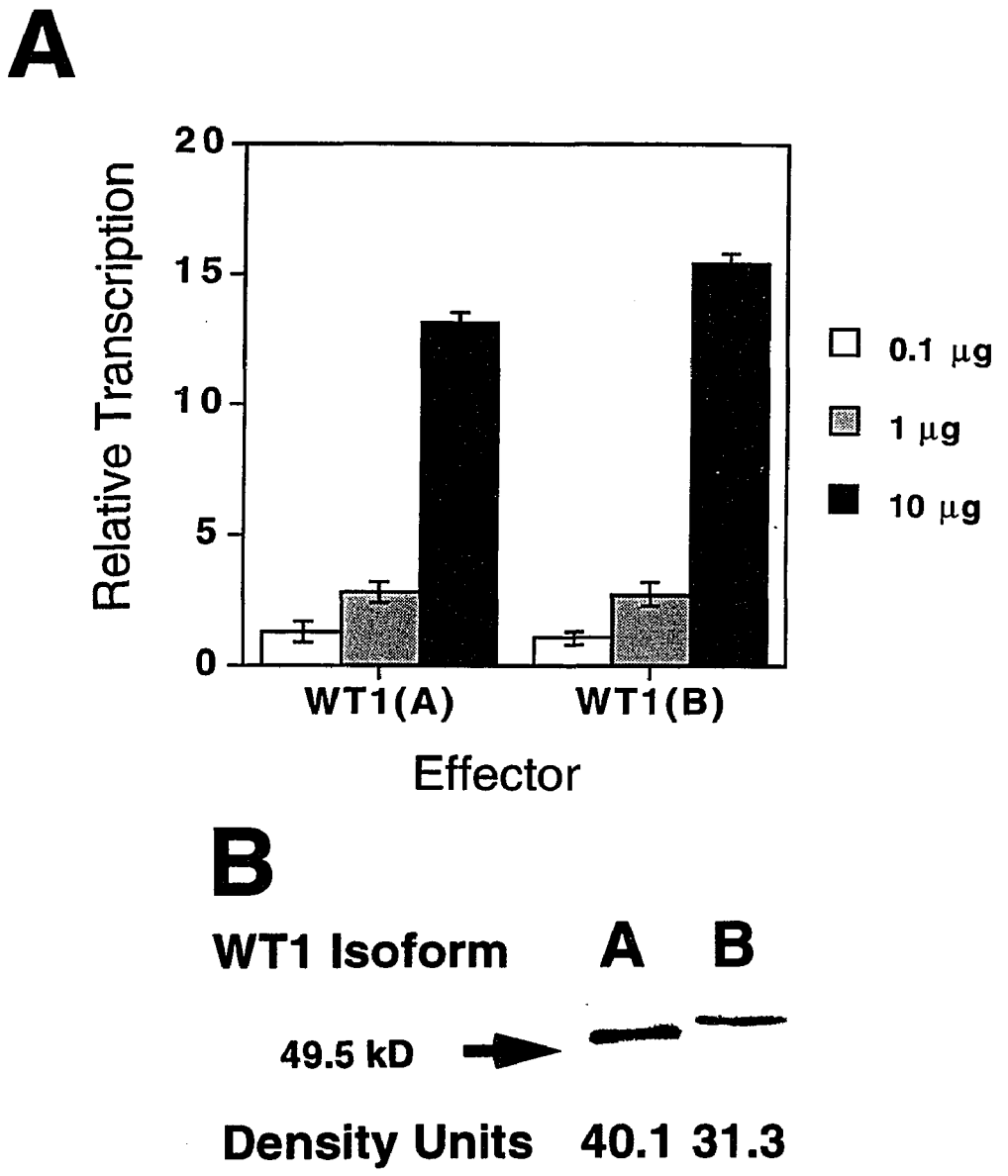



**FIGURE 5**: A) Transcriptional activation of EGR3*tk*CAT by WT1 in CV-1 cells. The average fold induction of transcription (normalized CAT activity) and standard error from six independent experiments is presented. B) Immunoblotting analysis of extracts from CV-1 cells transfected with 5 µg of RSV-WT1(A) or RSV-WT1(B), probed with anti-WT1 C19 antibody (Santa Cruz Biotechnology). The autoradiogram was digitally scanned and densitometrically quantified. The figure is continued on the following page.

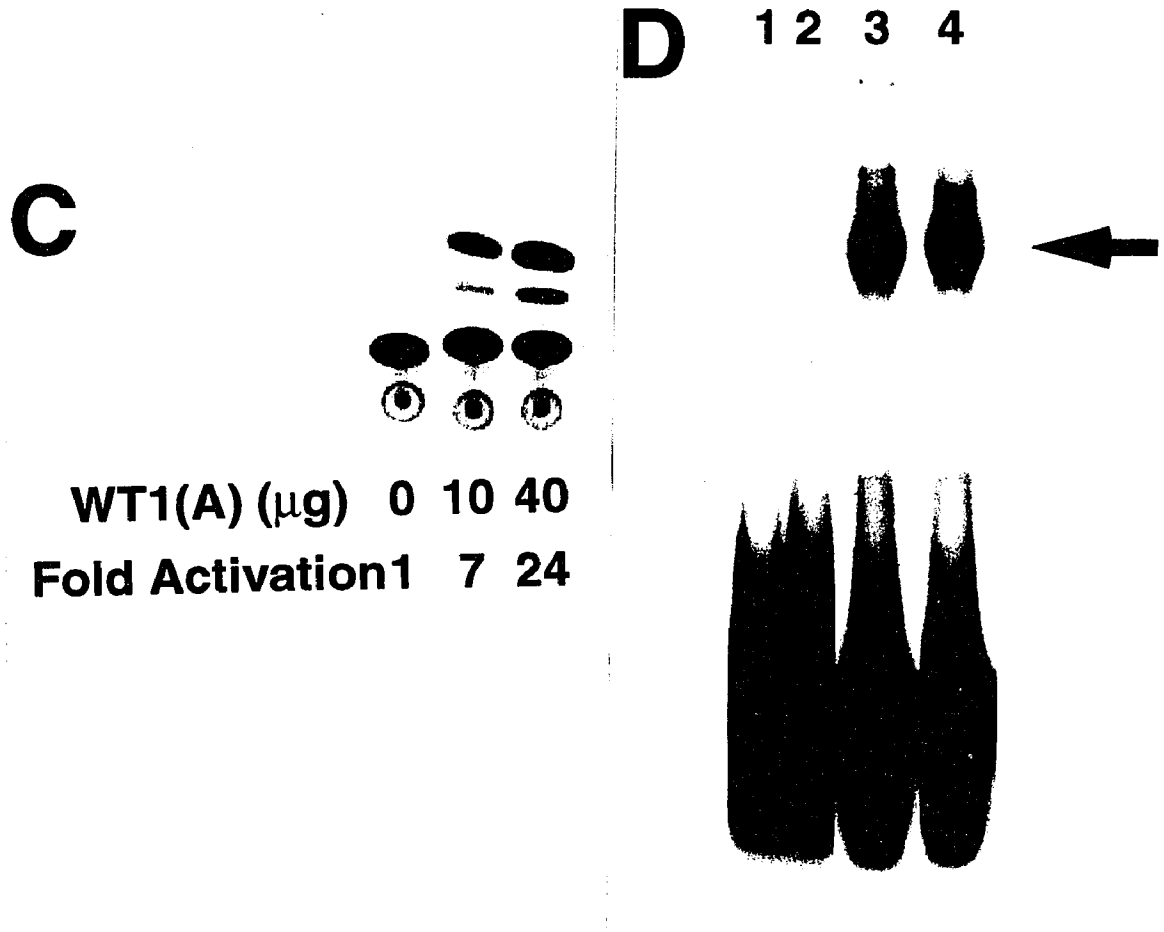



**FIGURE 5** (continued from previous page) C) Transcriptional activation by WT1 increases linearly with the dose of WT1 expression vector. The EGR3*tk*CAT reporter was co-transfected into CV-1 cells with increasing amounts of WT1 expression vector. The fold induction of transcription for a representative experiment is indicated below the chromatogram. D) WT1(A) and (B) bind to the Egr-1 site to the same extent as determined by electrophoretic mobility shift assay (EMSA). 1 = probe only; 2 = unprogrammed reticulocyte lysate; 3 = WT1(A); 4 = WT1(B). The specific WT1-DNA complex is indicated by an arrow.

**Two dominant negative *WT1* alleles inhibit transcriptional activation by WT1(A).** The *WT(AR)* allele of *WT1* was isolated from a patient with a sporadic, unilateral Wilms' tumor. The mutation alters a splice donor site, leading to a deletion of zinc finger 3 of the *WT1* protein (67), and yields a protein which binds poorly to the Egr-1 consensus binding site (160). Since the *WT(AR)* mutation was present in the heterozygous state, it was proposed to be a dominant negative allele, which suppresses the function of the normal allele (67). The protein products of this mutant allele, referred to as WT(AR-) and WT(AR+), differ due to the absence or presence, respectively, of the 17 amino acids coded for by the alternatively spliced exon 5 of *WT1*. The C-terminal splice region is eliminated by the deletion of the third zinc finger (Figure 4).

The *WT(PM)* mutation was isolated in the heterozygous state from germline DNA from a patient with DDS (7). Patients with this syndrome exhibit nephropathy and intersex disorders and occasionally develop Wilms' tumor. Insertion of a single guanine residue in this allele results in the truncation of the WT1 protein at amino acid 256, with an extra amino acid (glycine) added at the C-terminus (7) (Figure 4). This patient presented with nephropathy, ambiguous genitalia, and bilateral Wilms' tumors. While the tumors were most likely homozygous for the mutant WT1 allele (7), its presence in the heterozygous state in germline DNA suggests that it may act as a dominant negative allele resulting in genitourinary malformations and nephropathy.

We first determined whether, consonant with their proposed dominant negative genetic function, the *WT(AR)* proteins could interfere with transcriptional activation by WT1(A). The *WT(AR)* isoforms alone had no effect on transcription from the EGR3*tk*CAT reporter in CV-1 cells at doses of 4 and 8 μg (data not

shown). At doses of 10 μg and above, the WT(AR) proteins produced an approximately 50% increase in transcription from the $EGR_3$*tk*CAT reporter (data not shown), compared with an over 10-fold increase in transcription by WT1(A) (Figure 5A). Although full-length WT(AR) does not bind to the Egr-1 consensus sequence ((160), and see below), it can bind to sequences other than the Egr-1 consensus (40). At high concentrations, WT(AR) could possibly bind to sites in the backbone of the reporter plasmid, resulting in modest activation of transcription.

Transfection of a fixed amount (4 μg) of WT1(A) expression vector with increasing amounts (4, 8, or 16 μg) of WT1(B) further stimulated transcription (Figure 6A). In contrast, transfection of increasing amounts of either WT(AR) expression vector inhibited transcriptional activation by WT1(A) (Figure 6B). At the highest dose (16 μg) of transfected WT(AR) plasmid, inhibition was less marked, perhaps due to the modest stimulatory activity of WT(AR) observed at high inputs.

To determine which domain of the mutant WT1 protein was required for inhibition of transcriptional activation we utilized the *WT(PM)* mutant allele. This protein contains only the first 256 amino acids of WT1 and cannot bind to the Egr-1 site since it lacks zinc finger motifs (see below). Expression of either WT(PM) or of a protein containing the first 182 amino acids of WT1 inhibited transactivation by full-length WT1 in a dose-dependent manner (Figure 6C). Unlike WT(AR), WT(PM) and WT1(1-182) continued to inhibit transactivation at the highest dose of input plasmid. Therefore we conclude that the N-terminal 182 amino acids of WT1 are sufficient to mediate down-modulation of transcriptional activation by WT1(A).

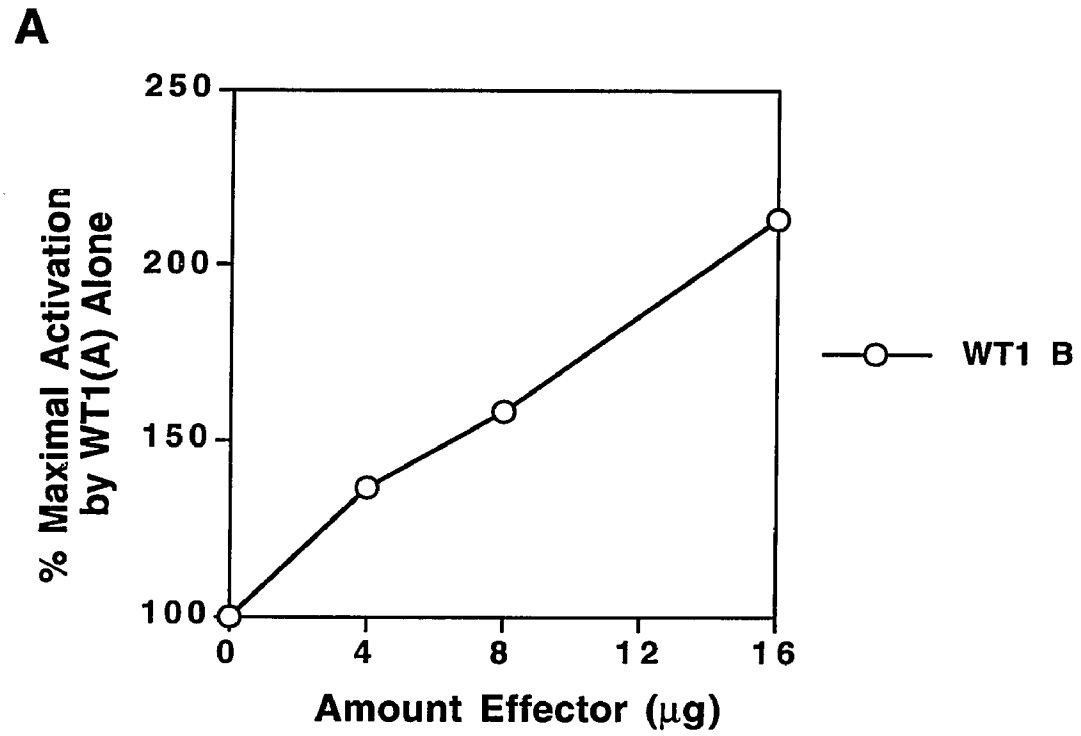
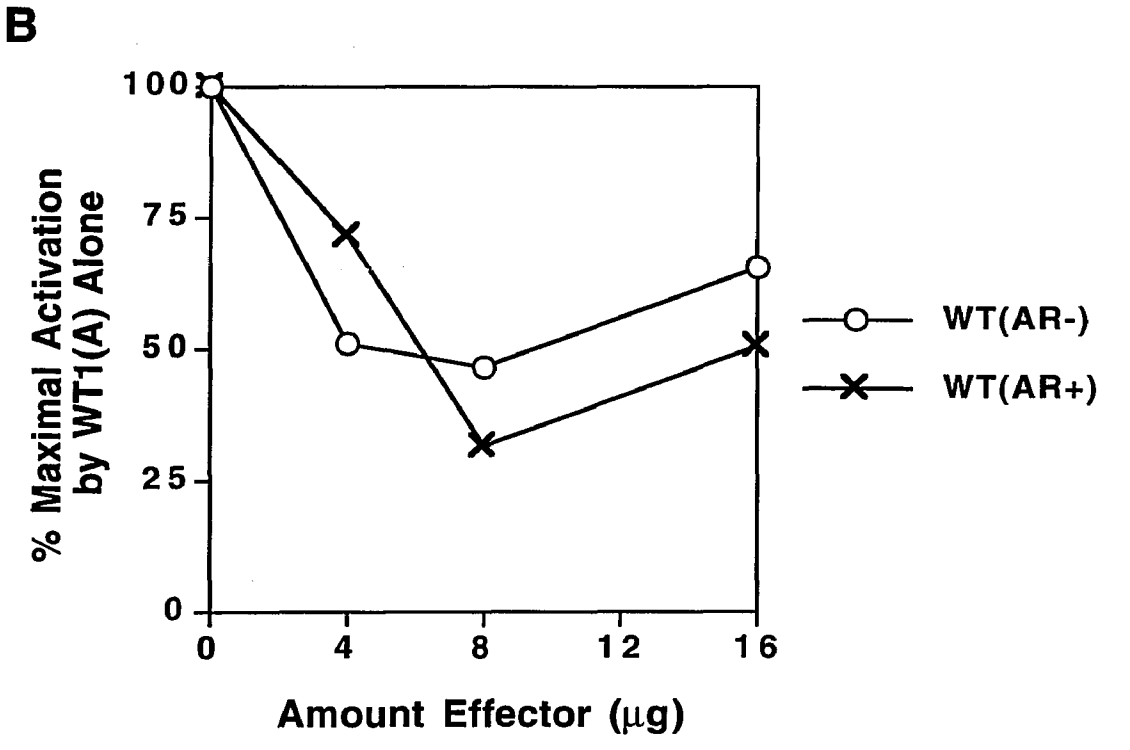
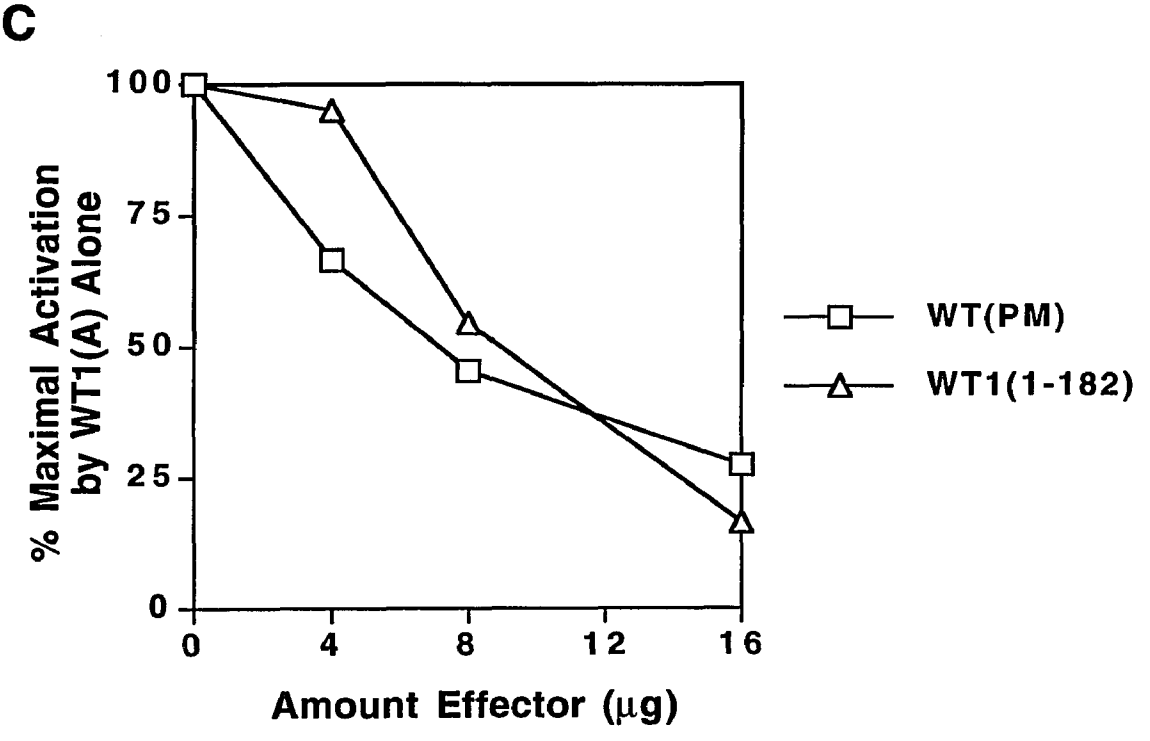



**FIGURE 6:** Two dominant negative *WT1* alleles interfere with transcriptional activation by WT1 A. The EGR3*tk*CAT reporter was co-transfected into CV-1 cells with 4 μg of RSV-WT1(A) and the indicated amounts of (A) RSV-WT1(B), (B) RSV-WT(AR-) or RSV-WT(AR+), and (C) RSV-WT(PM), or RSV-WT1(1-182). The amount of transcription obtained is presented as the percent of activation directed by WT1(A), where the data are averaged from 2 independent experiments for WT1(B), WT(AR-) and WT(AR+), from 5 independent experiments for WT(PM), and from 3 independent experiments for WT1(1-182). Parts B and C are on the following page.




**FIGURES 6B and 6C** (see previous page for legends).

**WT(AR) and WT(PM) do not interfere with DNA binding by WT1**. To determine whether expression of either dominant negative mutant protein might interfere with DNA binding by WT1(A) we performed EMSA with *in vitro* translated WT1(A), WT(AR-), and WT(PM) proteins. As predicted, WT(AR-) and WT(PM) did not bind to the Egr-1 consensus site (Figure 7, lanes 3 and 7) while WT1(A) bound strongly to this site (Figure 7, lanes 2 and 6). This complex was specific as addition of unlabelled Egr-1 consensus site oligonucleotide eliminated the WT1(A)-DNA complex, and addition of an antiserum against WT1, but not preimmune serum, slowed the mobility of the WT1(A)-DNA complex (data not shown). Preincubation of WT1(A) with either an equimolar amount or a two-fold molar excess of WT(AR) or WT(PM) did not interfere with DNA binding by WT1 (Figure 7, lanes 4, 5, 8, and 9).

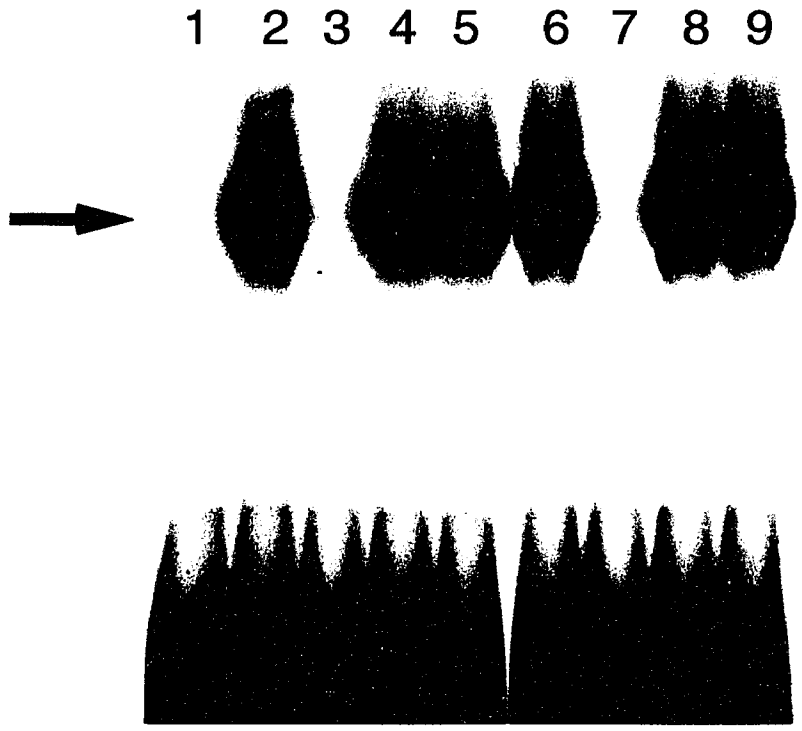



**FIGURE 7**: Dominant negative WT1 proteins do not interfere with DNA binding by wild-type WT1. (A) Lane 1: unprogrammed reticulocyte lysate. Lane 2: WT1(A). Lane 3: WT(AR-). Lane 4: WT1(A) preincubated with an equimolar amount of WT(AR-). Lane 5: WT1(A) preincubated with a two-fold excess of WT(AR-). Lane 6: WT1(A). Lane 7: WT(PM). Lane 8: WT1(A) preincubated with an equimolar amount of WT(PM). Lane 9: WT1(A) preincubated with a two-fold excess of WT(PM). The WT1-DNA complex is indicated by an arrow.

**Mutant and wild-type *WT1* proteins interact *in vitro*.** To further investigate the mechanism of action of the dominant negative WT1 proteins, we determined whether WT1(A) could associate with wild-type *WT1* proteins or with proteins encoded by the *WT(AR)* or *WT(PM)* alleles. GST-WT1(A) fusion protein immobilized on glutathione-agarose beads was incubated with $^{35}$S-methionine-labelled WT1(A), WT1(B), or mutant WT(AR-), WT(AR+), or WT(PM) proteins, produced in reticulocyte lysates. Bound $^{35}$S-labelled proteins were eluted, electrophoretically separated and visualized by fluorography. The two WT(AR) mutant isoforms, as well as wild-type WT1(A) and (B), bound to GST-WT1(A) (Figure 8A, rows 1-4), while a control luciferase protein did not (Figure 8A, row 5). None of the proteins bound to naked glutathione-agarose beads or to GST protein-coated beads (Figure 8A). The interaction between WT1 proteins was unlikely to be due to co-precipitation of these DNA-binding proteins through interaction with DNA fragments in the reaction mixture, since such DNA-protein interactions would be expected to be destabilized by the addition of 200 μg/ml ethidium bromide (EtBr) (103). As seen in Figure 8A, EtBr had no effect on interaction between these full-length WT1 proteins.

We then mapped the domain required for WT1 self-associaton. Full-length WT1(A) and WT(AR) bound both to GST-WT1(A)(1-429) and to a GST-WT1 fusion protein containing only amino acids 1-183 (Figure 8B, Row 1). Interaction between full-length proteins was consistently more efficient than interaction between full-length WT1 and the N-terminus of WT1. Similarly, a greater extent of interaction was observed between $^{35}$S-labelled WT1 containing amino acids 1-182 with GST-WT1(1-183) (35% of input labelled protein retained on the beads) than with the full-length GST-WT1(A)(1-429) fusion protein (6% of input labelled protein retained on the beads) (Figure 8B,

Row 5). This suggests that the full-length protein is folded in such a way that the N-terminal interaction surface is partially inaccessible, and when two full length molecules interact, this blockage is relieved.

The WT(PM) protein, containing amino acids 1-256 of WT1, bound strongly to both GST-WT1(A)(1-429) and GST-WT1(1-183) (Figure 8B, row 4). In addition, a truncated protein containing only wild-type WT1 or WT(AR) zinc finger domains (amino acids 307-429) did not bind to GST-WT1(1-183) but bound weakly to the full-length GST-WT1 protein (Figure 8B, Rows 2 and 3). Intriguingly, the binding of the wild-type WT1 or WT(AR) zinc finger domain (amino acids 307-429) to the full-length protein was intensified upon addition of EtBr (Figure 8B, rows 2 and 3). This suggests that the zinc fingers of WT1 can also mediate a protein-protein interaction *in vitro*, which becomes stronger if DNA binding by the zinc fingers is precluded by EtBr. The presence of two self-association domains within the WT1 protein may also help explain why full-length WT1 molecules interact better with themselves than with N-terminal truncated proteins.

**FIGURE 8** (see following page): Mutant and wild-type WT1 proteins interact *in vitro*. $^{35}$S-labelled proteins (indicated at left) were incubated with either naked glutathione-agarose beads or with GST or GST-WT1(A) proteins bound to the beads (indicated at top), in the presence or absence of 200 µg/ml ethidium bromide (EtBr). After washing and elution, bound proteins were electrophoretically separated and visualized by fluorography. For comparison, the input amount of $^{35}$S-labelled protein for each reaction was loaded in the far left lane. A) Full-length wild-type and mutant $^{35}$S-labelled WT1 proteins and $^{35}$S-labelled luciferase protein were incubated with the indicated GST fusion proteins. B) $^{35}$S-labelled full-length WT1(A) or truncated WT1 proteins containing the indicated amino acids were incubated with the indicated GST fusion proteins.

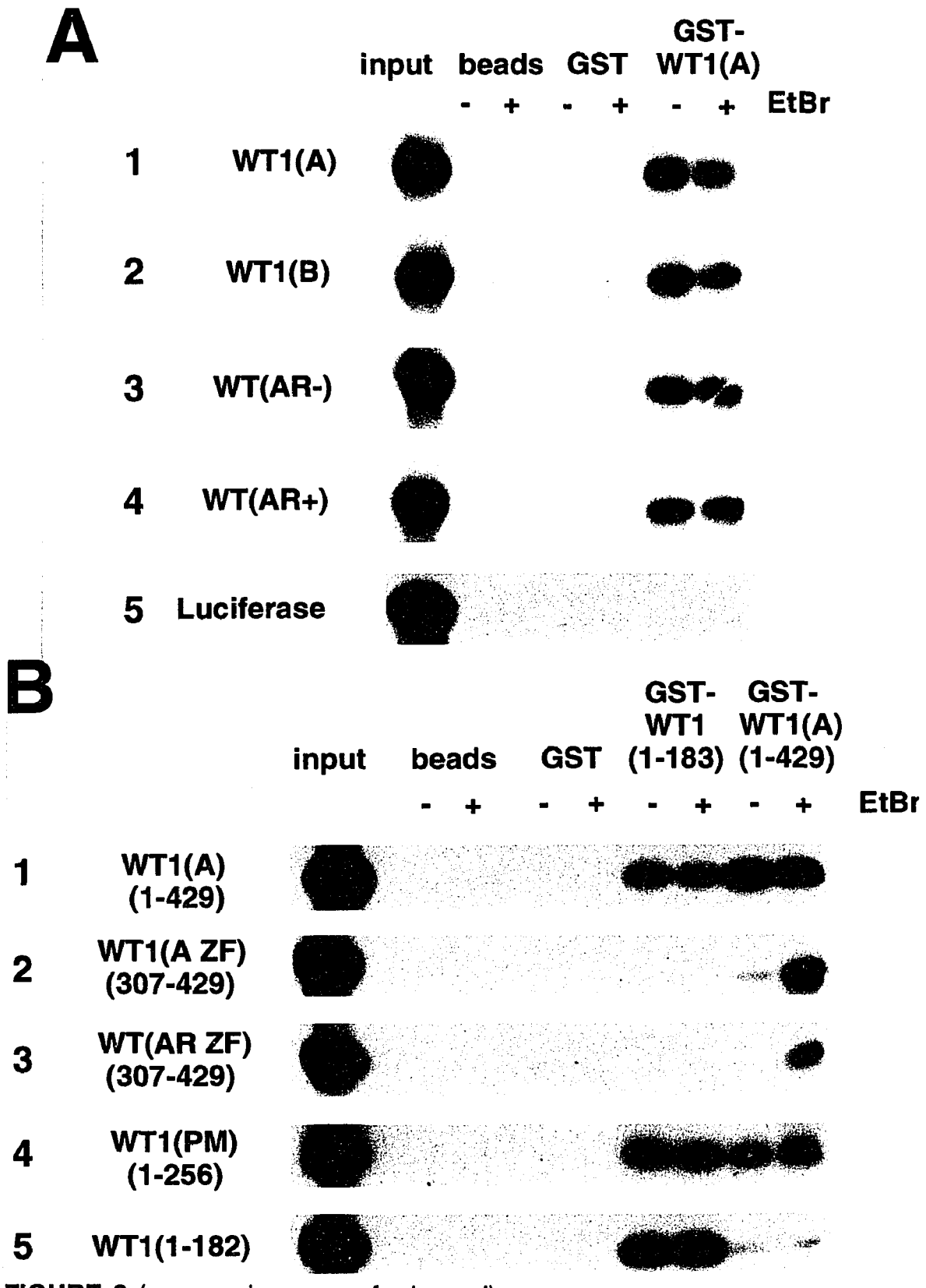



**FIGURE 8** (see previous page for legend).

**Mapping of WT1 self-association domains *in vivo* using the two-hybrid system in yeast.** To determine the ability of WT1 protein to self-associate *in vivo*, our collaborators used a yeast two-hybrid system (63, 214). The full-length WT1 coding region, or smaller fragments of the coding region, were fused in-frame to the DNA-binding domain of the bacterial LexA protein. The second hybrid fused WT1 to the B42 acidic activation domain. Plasmids encoding these hybrids were introduced into yeast strain EGY48, containing the 2μ-based reporter plasmid LexAop-LacZ. This reporter contains 2 Lex operators and the *GAL1* promoter linked to the *E. coli* ß-galactosidase reporter gene. Interaction between WT1 fused to LexA and the WT1/B42 acidic activation domain hybrid will stimulate transcription from the reporter gene. Their work showed that WT1 interacted with itself in this assay as scored by qualitative (blue/white) and quantitative ß-galactosidase assays. The domain of WT1 responsible for self-association mapped to the N-terminal 180 amino acids of the protein (163). These results are consistent with our finding that in transfection studies, WT1(1-182) was a potent inhibitor of transactivation by full-length WT1 (Figure 6C), and that the N-terminal 182 amino acids mediated self-association of WT1 molecules *in vitro* (Figure 8B). In addition, these results suggest that association between the zinc finger domains of WT1 may not contribute to self-association of WT1 *in vivo* .

## DISCUSSION

**Transcriptional activation by WT1.** Consistent with previous work (69, 205, 206), we found that the WT1 protein activated the HSV-*tk* promoter through upstream binding sites. WT1(B) was a stronger transcriptional activator than

WT1(A), however, WT1(A) and (B) bound DNA to similar extents *in vitro* and WT1(B) was expressed at a somewhat lower level than WT1(A) in transfected cells. Together this information suggests that the 17 additional amino acids in WT1(B) augment the transcriptional activation domain of WT1. This insertion (MAAGSSSSVKWTEGQSN) contains a potential protein kinase C phosphorylation site (SVK). Other factors such as the cyclic AMP response element binding protein (CREB) (60) and c-jun (13) can be dynamically regulatd by phosphorylation. While a prior study did not detect phosphorylation of WT1(A) (131), the phosphorylation state of WT1(B) has not been examined. Alternatively, the inserted amino acids in WT1(B) could interact directly with a component of the transcription machinery to stimulate transcription, or could effect a conformational change in the entire *WT1* protein, altering its interaction with the transcriptional machinery. Recently, this 17 amino acid insertion was identified as an independent transcriptional repression domain when fused to a heterologous DNA-binding domain (208). In addition, deletion of the four consecutive serines decreased the repression activity of these fusion proteins. These findings support our conclusion that the 17 amino acid insertion modulates the transcriptional function of WT1 and suggest that this region of the WT1 protein may have bifunctional characteristics.

**Mechanism of action of dominant negative *WT1* alleles**. The ability of the dominant negative alleles *WT(AR)* and *WT(PM)* to inhibit transcriptional activation by WT1 could be explained by one of three mechanisms: competition for binding sites (107), competition for co-factors ("squelching") (154), or physical interaction between wild-type and mutant proteins (78). Competition between wild-type and mutant proteins for binding to the Egr-1 binding site is unlikely to play a role in this effect, since the WT(AR) and WT(PM) mutant

proteins do not bind to this site ((160), and Figure 7). In addition, neither dominant negative protein interfered with DNA binding by WT1(A) *in vitro* (Figure 7). Since co-factors for transcriptional activation by WT1 do not appear to be limiting in our cell lines (Figure 5C), the mutant proteins are also not likely to inhibit transcription by squelching.

By an *in vitro* biochemical assay and by the *in vivo* yeast two hybrid assay WT1 was found to self-associate. We therefore propose that the dominant negative WT(AR) and WT(PM) proteins, which cannot bind to the Egr-1 site, interact with WT1 bound to DNA and inhibit the function of wild-type protein, perhaps by partially shielding its activation domain. WT(PM) was a more potent inhibitor of transactivation by WT1(A) than was WT(AR). This may be due to the fact that WT(PM) is incapable of binding to DNA while WT(AR) can bind to sites other than the Egr-1 site used in this study (40). Some of the WT(AR) protein in the cell may be bound to DNA sequences other than the Egr-1 consensus and would therefore be unavailable for protein-protein association with wild-type WT1 required for inhibition of transactivation. In contrast, WT(PM) cannot bind to any DNA sequence and thus can only function through protein-protein interaction with wild-type WT1. Based on analysis of WT1 mutations isolated from DDS patients, Bardeesy *et al.* (10) predicted that a domain encoded by exons 1, 2, and 3 of WT1 (amino acids 1-222) would mediate a dominant negative effect. We confirm this prediction by mapping the domain responsible for self-association and for the dominant negative effect to amino acids 1-182 of WT1.

WT1 also represses transcription of a variety of growth-related genes, with this effect dependent on promoter architecture and on interaction of WT1 with the

p53 protein (see Chapter I). Studies are now underway to determine whether dominant negative WT1 proteins also inhibit transcriptional repression by WT1. Even if only activation is affected, the resultant imbalance between the repression and activation activities of WT1 might be sufficient to cause neoplasia, developmental abnormalities, or nephropathy. Wang *et al.* (207) have suggested that dominant negative WT1 proteins might act by competing with wild-type WT1 for a putative co-repressor protein. However, our results suggest that transcriptional regulation by WT1 may be modulated by self-association, and suggest that the co-repressor proposed by Wang *et al.* may be WT1 itself.

Heterozygous null mutations of *WT1*, as found in the WAGR syndrome, result in genitourinary malformations and predisposition to Wilms' tumor, but not nephropathy. Patients with such mutations would have a 50% reduction in their effective WT1 activity. Our results suggest that heterozygous dominant negative mutations of *WT1*, such as *WT(AR)* and *WT(PM)*, can result in an inhibition of function of the remaining wild-type *WT1* allele. The maximal inhibition we observed was approximately 3-fold for WT(AR) and 5-fold for WT(PM). In patient AR, this could result in a level of WT1 activity that is less than 50% of normal in the small subset of kidney cells containing the acquired mutation. This might be sufficient to produce a disregulation of growth control of these cells, resulting in tumor formation. Since WT(AR) can bind to specific DNA sequences other than the Egr-1 consensus (40), it could also deregulate growth by binding to novel target genes not normally regulated by WT1.

According to our hypothesis, DDS patients, who have a heterozygous germline *WT1* mutation, would also have a level of effective WT1 activity substantially

lower than hemizygous WAGR patients. The remaining level of WT1 activity in DDS patients must still be sufficient to allow kidney development, since mice with a homozygous disruption of the WT1 locus fail to form kidneys (100). WT1 continues to be expressed in the podocytes of the adult kidney, which may play an important role in glomerular filtration (132). Therefore, inhibition of WT1 activity by a dominant negative mutant protein in the podocytes of DDS patients might contribute to the novel phenotype of progressive glomerulonephropathy.

# CHAPTER IV

# The transcriptional effector function of WT1 is modulated by mode of presentation, promoter architecture, and choice of expression vector

## INTRODUCTION

As stated in Chapter I, there are numerous factors which were shown to influence the transcriptional activity of WT1. Studies on the PDGF-A, IGF-II, and IGF1R promoters suggest that WT1 acts as a repressor of transcription when it is bound both upstream and downstream of the start site of a promoter (39, 204, 205, 209, 210). Conversely, WT1 activates transcription when only 5' or 3' WT1 binding sites are present in the PDGF-A promoter, or when WT1 binding sites are inserted upstream of the HSV-*tk* or MMTV promoters (69, 163, 205, 207)(Chapter III). However, WT1 can also repress transcription from upstream binding sites in its native state (118, 169, 170) and when expressed as a GAL4-WT1 fusion protein (119). Finally, interaction between WT1 and the p53 tumor suppressor protein can determine whether WT1 acts as a repressor or an activator of transcription (120).

In the course of examining transcriptional regulation by WT1, we have made several observations which may help to resolve some of the apparent inconsistencies in this field. We confirm that GAL4-WT1 is a potent transcriptional repressor when bound to GAL4 sites 5' to the transcription start site of a simple test promoter. However, we also show that this GAL4-WT1 fusion protein, which contains the WT1 zinc finger DNA-binding domain, is not

capable of regulating transcription through WT1 binding sites, suggesting that the WT1 moeity of the fusion protein is not in its native conformational state. We also show that WT1 can either activate or repress transcription from the Egr-1 promoter, depending on which promoter is used to drive the expression of WT1, whereas WT1 activates transcription from the simple EGR$_3$*tk*CAT reporter gene regardless of the expression vector used. We only observe repression of the Egr-1 promoter by WT1 when its expression is driven by the strong cytomegalovirus (CMV) promoter, and we show that transfection of vectors containing this promoter profoundly depresses the basal activity of the Egr-1 promoter. Finally, we are able to convert WT1 from an activator to a repressor of the Egr-1 promoter by adding excess CMV-based expression vector lacking a WT1 insert. Taken together, these results suggest that competition between the CMV and Egr-1 promoters for transcription factors or co-factors results in changes in the basal activity of the Egr-1 promoter, and that only under conditions of low basal activity is the Egr-1 promoter able to be repressed by WT1. These results give clues as to the conditions under which WT1 can act as a repressor or an activator and also provide an important caveat regarding choice of expression vector in transfection experiments.

## RESULTS

**Fusion of WT1 to the GAL4 DNA-binding domain results in changes in its transcriptional effector functions.** Work by others (119) showed that fusion of the first 298 amino acids of WT1 to the GAL4 DNA-binding domain yielded a protein which could repress transcription via GAL4 binding sites inserted upstream of the HSV-*tk* promoter. As our initial studies had shown that the native WT1 protein activated transcription from WT1 sites upstream of the

HSV-*tk* promoter, we sought to confirm the GAL4-WT1 result. To that end, we obtained a plasmid which expresses full-length human WT1 fused to the first 147 amino acids of the GAL4 protein, which encode the DNA-binding and dimerization domains of GAL4 (gift of Y. Shi). We transfected this vector, or a vector encoding the GAL4 DNA-binding domain alone, into CV-1 cells along with a reporter which contains 5 GAL4 sites upstream of the HSV-*tk* promoter linked to the CAT gene ($G_5$*tk*CAT; (186)).

As shown in Figure 9, GAL4-WT was a potent repressor of transcription of the $G_5$*tk*CAT reporter, confirming the results of Madden *et al.* (119). This led us to investigate why WT1 activated transcription from upstream sites in its native state but repressed transcription from upstream sites when fused to GAL4. To determine whether fusion of WT1 to GAL4 resulted in a change in WT1 protein conformation, we asked whether this GAL4-WT1 fusion protein, which contains the entire WT1 protein including its DNA-binding domain, was capable of regulating transcription through Egr-1/WT1 binding sites. We observed a slight (2-fold) activation of the $EGR_3$*tk*CAT reporter by GAL4-WT1, however, this activation was also observed on the parental reporter gene lacking Egr-1/WT1 binding sites (*tk*CAT; Figure 9). We therefore conclude that the GAL4-WT1 fusion protein cannot regulate transcription through Egr-1/WT1 binding sites.

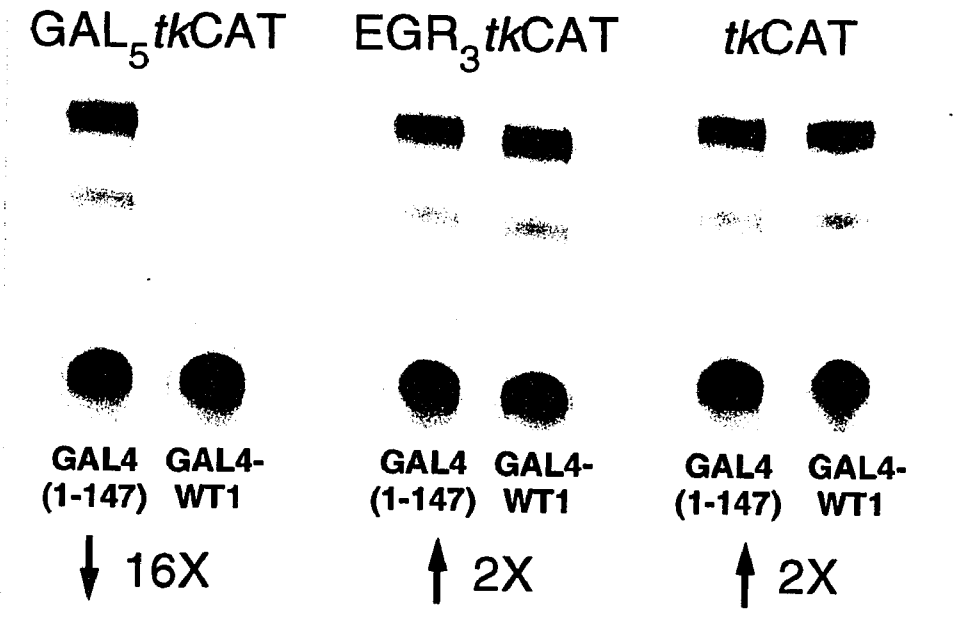



**FIGURE 9:** A GAL4-WT1 fusion protein represses transcription of a reporter gene containing GAL4 binding sites but fails to regulate transcription through Egr-1/WT1 binding sites. 2 μg of reporter and 2 μg of effector were transfected into CV-1 cells as described in Chapter II. Chromatograms and fold activation or repression from a representative experiment are presented.

These results suggest that the WT1 DNA-binding zinc finger domain, as well as possibly other domains, is either misfolded or is somehow shielded in the context of the GAL4-WT1 fusion protein. GAL4(1-147) binds DNA as a dimer (23); this dimerization, or dimerization via the self-association domain of WT1 (see Chapter III) may lead to formation of a GAL4-WT1 dimer in which the WT1 moeity is in a non-native conformation. While we can observe strong expression of the GAL4-WT1 fusion protein in transfected cells by immunoblotting analysis (data not shown), we do not know whether this protein is capable of binding to WT1 binding sites. We conclude that the ability of WT1 to repress transcription from upstream binding sites when expressed as a GAL4-WT1 fusion protein may be a consequence of an alternative conformation of WT1.

**Promoter architecture and choice of expression vector can determine whether WT1 activates or represses transcription.** Our previous work (Chapter III and (163)) showed that WT1 was a default transcriptional activator of a simple test promoter containing 3 Egr-1/WT1 binding sites upstream of the HSV-*tk* promoter linked to CAT (EGR3*tk*CAT). In contrast, WT1 was initially shown to repress transcription of the Egr-1 promoter (118). This complex promoter contains three potential Egr-1 binding sites as well as binding sites for AP-1 and SRF upstream of the start site of transcription (57, 196). To resolve this apparent inconsistency, we sought to confirm the results of Madden *et al.*. To our surprise, we found that expression of WT1 from an RSV-based expression vector activated rather than repressed transcription from the Egr-1 promoter in both NIH 3T3 cells (Figure 10A) and CV-1 cells (data not shown). We have used this RSV-based expression vector, which contains the murine WT1 cDNA, for our previous experiments which show activation of a

simple test promoter containing upstream WT1 binding sites. To further investigate this phenomenon, we obtained expression vectors for WT1 that are driven by the actin (pJ6Ω; gift of S. Hosono) or CMV (pCB6+; gift of V. Sukhatme) promoters. To our surprise, we could only observe repression of the Egr-1 promoter by WT1 when it was expressed from the pCB6+ vector (Figure 10A). While only slight repression was observed at 10 μg of input effector plasmid, stronger repression was seen at a dose of 20 μg (data not shown and Figure 17). pCB6+ was the vector used by Madden *et al.* (118) to show repression of the Egr-1 promoter by WT1. This vector was also used in all other published experiments which have shown repression of promoters by WT1, with the exception of the work of Malik *et al.* (121) which showed 2-fold repression of the *WT1* promoter expressed from an inducible metallothionein promoter. In contrast to the results with the Egr-1 promoter, WT1 always activated transcription of the $EGR_3$*tk*CAT reporter, regardless of the expression vector used (Figure 11A).

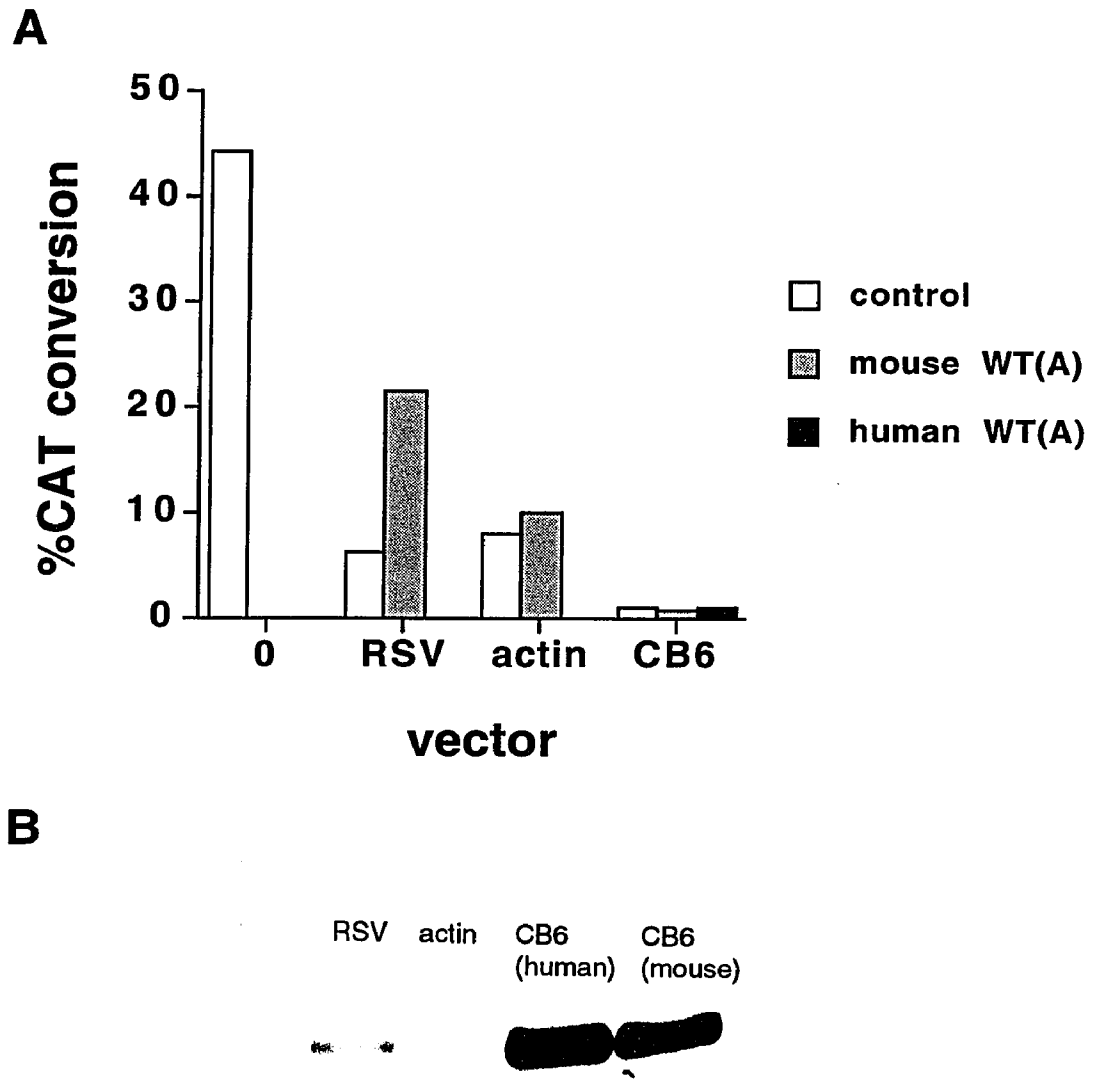



**FIGURE 10**: The choice of expression vector determines the transcriptional regulatory effect of WT1 on the Egr1-CAT reporter gene in NIH 3T3 cells. A. CAT assays: 0.5 μg of reporter and 10 μg of effector were transfected into NIH 3T3 cells as described in Chapter II. The results are presented as the average of four independent determinations. B. Extracts from representative plates of transfected cells used in part A were subjected to immunoblotting analysis with a polyclonal anti-WT1 antibody (Santa Cruz Biotechnology) as described in Chapter II.

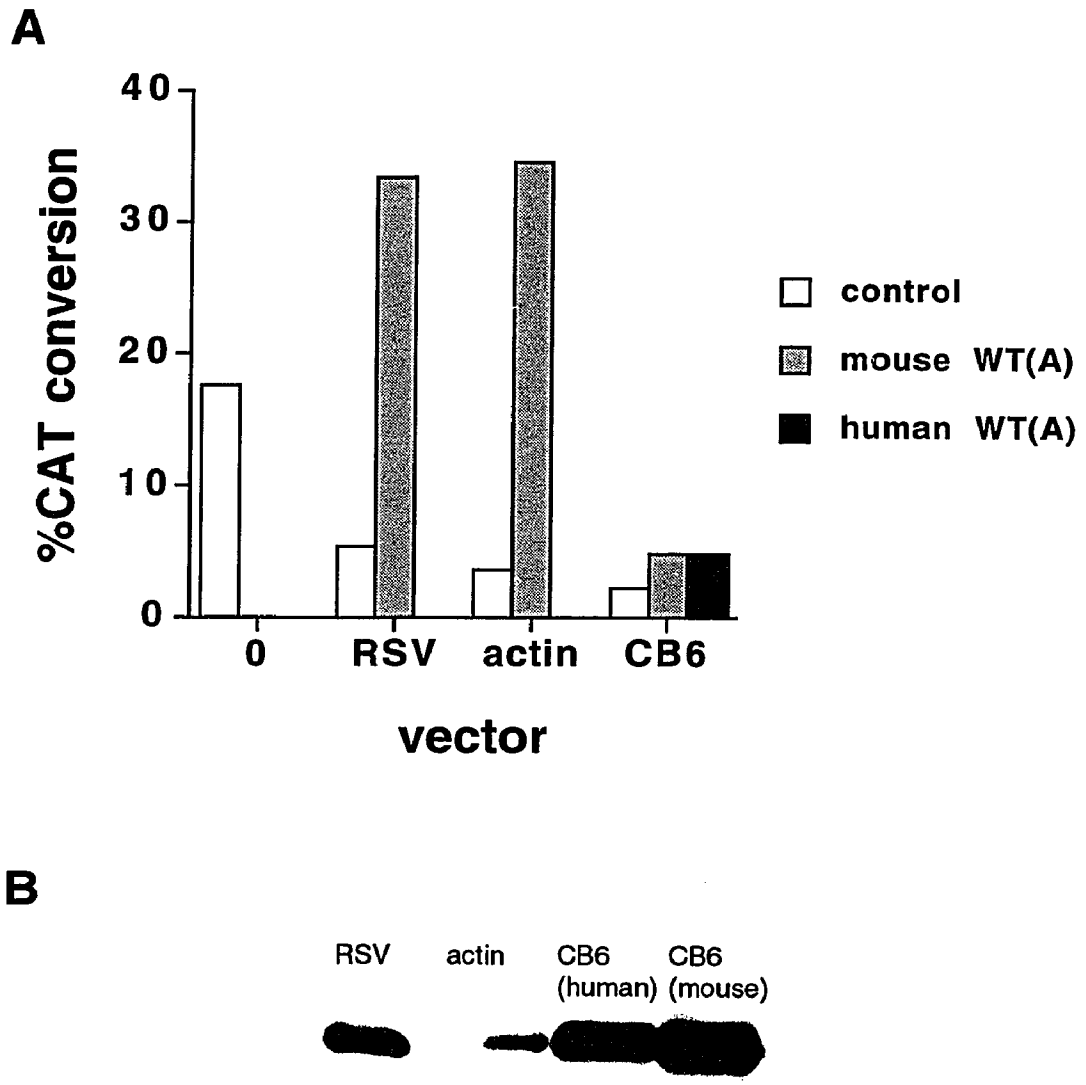



**FIGURE 11:** WT1 activates transcription from the $EGR_3$*tk*CAT reporter in CV-1 cells regardless of the expression vector used. A. CAT assays: 2 μg of reporter and 10 μg of effector were transfected into CV-1 cells as described in Chapter II. The results are presented as the average of four independent determinations. B. Extracts from representative plates of transfected cells used in part A were subjected to immunoblotting analysis with a polyclonal anti-WT1 antibody (Santa Cruz Biotechnology) as described in Chapter II.

We then examined the reasons why the choice of expression vector might influence the transcriptional activity of WT1. Since pCB6-WT1 contains the human WT1 cDNA, whereas RSV-WT1 and pJ6Ω-WT1 contain the murine WT1 cDNA, we wanted to rule out the possibility that a difference in the function of the human and mouse proteins might be responsible for this effect. To that end, we cloned the murine WT1 cDNA into the pCB6+ vector and showed that it had identical effects to the vector containing the human cDNA (Figures 10A and 11A).

Secondly, we wanted to determine whether a difference in the level of WT1 protein expressed from these expression vectors might be the cause of these divergent transcriptional functions. We performed immunoblotting analysis on extracts from the transfected cells used in the CAT assays and showed that pCB6+ drives a level of WT1 protein expression that is much higher than that observed with the RSV or actin-based expression vectors (Figure 10B and 11B). To better quantitate the relative level of WT1 protein expressed from the RSV and pCB6+ expression vectors, we performed an additional immunoblot of extracts from cells transfected with 5 or 0.5 μg of each expression vector. The pCB6+ expression vector yields an approximately 10-fold higher level of WT1 protein than the RSV vector when transfected into NIH 3T3 cells (Figure 12). Dose-dependent transcriptional effects have been reported for the *Drosophila* Krüppel protein, which activates transcription at low doses but represses transcription at high doses (174). It is possible that WT1 has a similar dose-dependent transcriptional effect. However, we felt it more likely that the pCB6+ vector was somehow altering the activity of the Egr-1 promoter such that it became competent for repression by WT1.

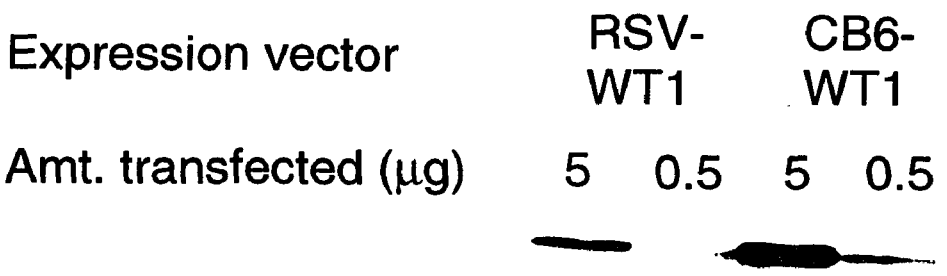



**FIGURE 12:** The pCB6+ expression vector drives an approximately 10-fold higher level of WT1 expression than does the RSV expression vector in NIH 3T3 cells. Extracts from NIH 3T3 cells transfected with the indicated amounts of expression vector were subjected to immunoblotting analysis with anti-WT1 C19 polyclonal antibody (Santa Cruz Biotechnology) as described in Chapter II.

Our supposition that pCB6+ itself was altering the transcriptional effect of WT1 on the Egr-1 promoter was based on several observations. Firstly, all previously reported studies (with the exception noted above) which showed transcriptional repression by WT1 utilized this vector, and in all of those experiments, a total of 20 μg of pCB6+ vector, with or without the *WT1* cDNA insert, has been used per 100 mm dish of cells. In the course of our investigations we noted that addition of increasing amounts of pCB6+ vector lacking the WT1 insert caused a progressive and severe depression of the basal transcriptional activity of the Egr-1 promoter, such that at a dose of 10 μg of pCB6+, the promoter activity was 44-fold depressed compared to cells without co-transfected pCB6+ (Figure 13). This suggested that the pCB6+ vector itself was inducing an alteration in the Egr-1 promoter activity, most likely by competing for transcription factors or co-factors required for activity by both promoters. This also suggested that WT1 might regulate transcription differently on this "depressed" Egr-1 promoter compared to its effects on high-level transcription observed in the absence of pCB6+.

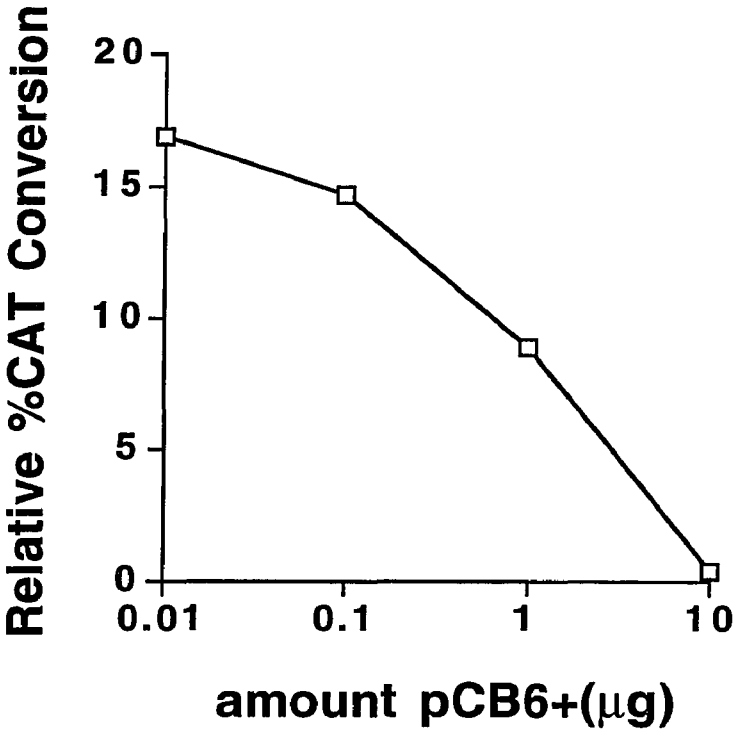



**FIGURE 13:** Co-transfection of pCB6+ vector lacking WT1 coding sequences severely depresses the basal transcriptional activity of the Egr-1 promoter. The results are presented as the average of three independent determinations.

To attempt to dissect apart the effect of WT1 on transcription from the effect of the pCB6+ vector, we activated the Egr-1 promoter by expressing WT1 from the RSV expression vector and then determined the effect of additional co-transfection of pCB6+. As before, addition of the pCB6+ expression vector to the transfection drastically reduced the level of transcription of the Egr-1 promoter. But strikingly, cotransfection of pCB6+ vector converted WT1 from an activator of the Egr-1 promoter to a repressor (Figure 14A). In the absence of pCB6+, WT1 activated transcription approximately 1.7-fold, whereas in the presence of 20 μg of cotransfected pCB6+, WT1 repressed transcription approximately 3.6-fold. Immunoblotting analysis of extracts from these transfected cells showed that there was a somewhat lower level of WT1 expressed in the presence of pCB6+ than in the absence of pCB6+, ruling out a dose-dependent repression effect of WT1 protein on this promoter (Figure 14B). We therefore conclude that, in contrast to previously reported results (118), WT1 is a default activator of the Egr-1 promoter, and suggest that repression of this promoter by transfection of pCB6-WT1 is in part a consequence of promoter competition between the Egr-1 and CMV promoters.

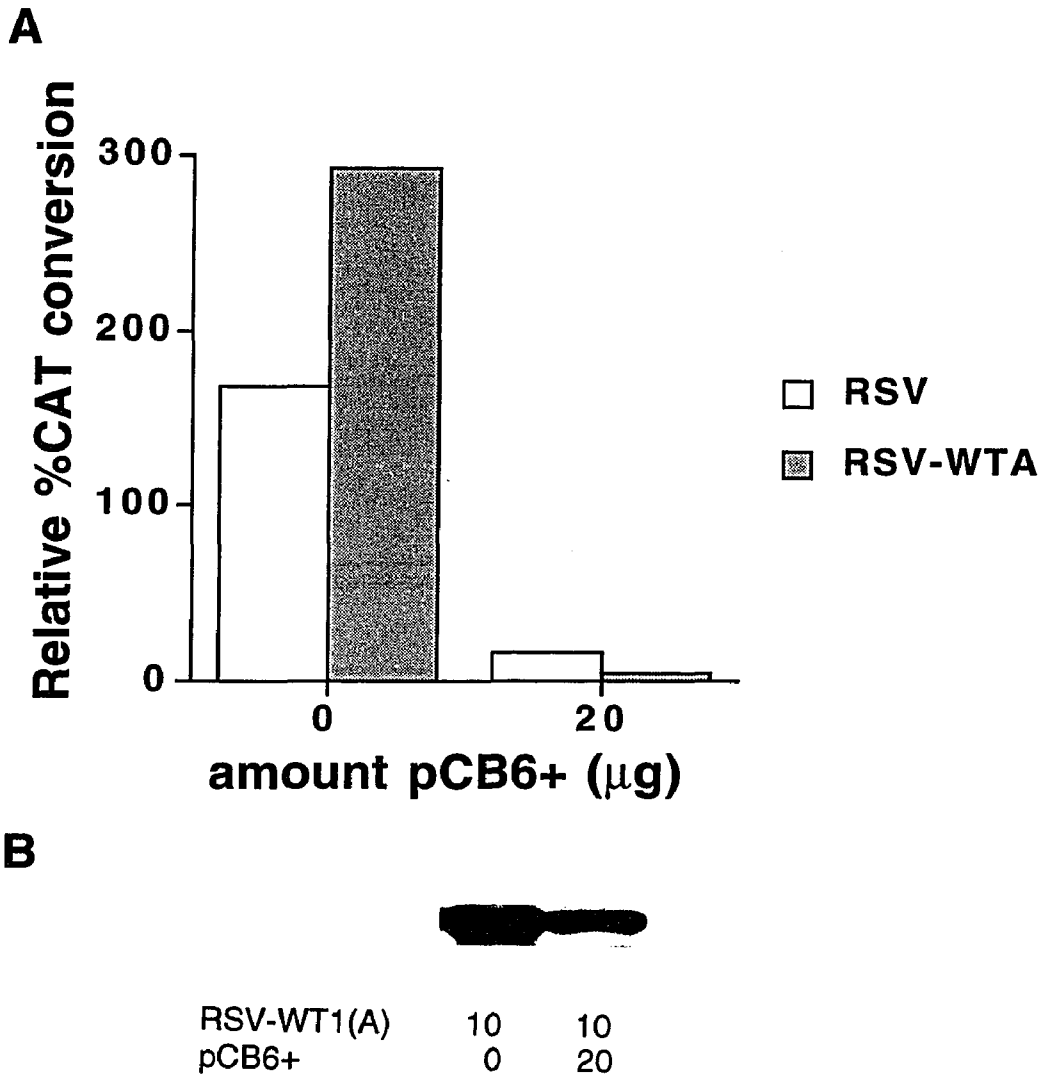



**FIGURE 14**: Cotransfection of pCB6+ vector converts WT1 from an activator to a repressor of transcription. A. CAT assays: 0.5 μg of reporter, 10 μg of RSV-based effector, and the indicated amounts of pCB6+ vector were transfected into NIH 3T3 cells as described in Chapter II. The results are expressed as the average of two independent determinations. B: Extracts from representative plates of transfected cells from part A were subjected to immunoblotting analysis with a polyclonal anti-WT1 antibody (Santa Cruz Biotechnology) as described in Chapter II.

## DISCUSSION

Several well-defined and widely-used experimental systems are used to characterize the protein-DNA interactions which result in the regulation of transcription. The activity of a given promoter is often studied by linkage of a fragment of the promoter to a reporter gene. In many prior reports, WT1 was shown to be a repressor of transcription (see Chapter I). Domains of transcription factors which are important for transcriptional regulation can be mapped by deletion analysis or by fusion of these domains to heterologous DNA-binding domains. Studies of this type on WT1 have mapped adjacent repression and activation domains, suggesting that WT1 has intrinsically bifunctional transcriptional regulatory potential.

While all of these approaches are valuable tools which can provide much information about the regulation of a promoter by a particular protein, significant limitations of these methods exist. 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. As an example, the Egr-1 promoter fragment used in these experiments is highly transcriptionally active (50-fold more than HSV-*tk*) in the absence of co-transfected CMV-based expression vector. In contrast, the endogenous cellular Egr-1 gene is expressed only transiently in response to various types of growth-promoting signals (191). This implies that sequences or chromatin structure required for proper regulation of this promoter are not present in the fragment being studied.

The choice of expression vector for the transcription factor of interest may not only determine the levels of protein produced but also may alter the

transcriptional state of the reporter gene. As shown in these experiments, such alterations in the transcriptional state of the reporter gene may result in changes in regulation by transcription factors. We propose that WT1 can act as a transcriptional repressor only under a limited set of experimental circumstances. The evidence suggests that WT1 represses transcription when fused to the heterologous DNA-binding domain of the GAL4 protein (119), however, our results suggest that this protein may not be presented to the transcriptional machinery in the same manner as the native WT1 protein. Perhaps the activation domain of WT1 is shielded, and the repression domain becomes dominant. It is also possible that the zinc finger domain of the WT1 moiety is misfolded or inaccessible in the GAL4-WT1 fusion protein. Our results also suggest that competition between the Egr-1 promoter and the pCB6+ expression vector result in changes in the transcriptional state of the Egr-1 promoter which make it competent for repression by WT1.

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 (3)). The Egr1-CAT reporter contains nucleotides -957 to +248 of the murine Egr-1 promoter, a region which contains binding sites for Egr-1, AP-1, and SRF (196). All of these promoters contain GC-rich sequences, which could bind factors such as Sp-1, as well as potential binding sites for AP-1 and numerous other transcription factors, which may compete with each other for binding of sequence-specific DNA-binding transcription factors. If WT1 needs to cooperate with other transcription factors to activate or repress transcription, changes in promoter occupancy by specific

transcription factors may result in changes in the transcriptional regulatory effect of WT1.

It will be of interest to determine whether transfection of RSV-WT1 results in activation of transcription from other promoters which have previously been shown to be repressed by pCB6-WT1. In particular, it would be helpful to determine how the basal activities of these various promoters are affected by pCB6+. It is possible that only certain promoters will compete with pCB6+ for common transcription factors or co-factors required for their basal activity, and that particular combinations of these factors or co-factors are required for repression by WT1. This is supported by our findings that pCB6+ does not have nearly as large an effect on the basal activity of $EGR_3$*tk*CAT (8-fold) as it does on Egr1-CAT (40-fold) (Figures 10 and 11), and that WT1 expressed from the pCB6+ vector can still activate transcription from $EGR_3$*tk*CAT (Figure 11A). However, less activation is observed with pCB6-WT1 than with either RSV-WT1 or pJ6W-WT1, despite the fact that approximately 10 times more WT1 protein is produced by pCB6-WT1 than by the other vectors (Figure 11B). This suggests either that WT1 expressed at this high level leads to transcriptional self-squelching (110, 154) in CV-1 cells, or that promoter competition with pCB6+ results in a different transcriptional state of $EGR_3$*tk*CAT that is less responsive to transactivation by WT1. Hamilton *et al.* (69) also observed activation from upstream binding sites by pCB6-WT1, adding more evidence that WT1 does not always repress transcription, even when expressed from pCB6+.

Finally, it will be be of interest to determine which transcription factors and/or co-factors act to augment the activation or repression functions of WT1. *In vitro* transcription studies may help to clarify this point. Our preliminary data suggest

that WT1 is incapable of activating transcription via binding sites upstream of a minimal TATA-box containing promoter, whereas we observe potent transactivation when these same sites are placed upstream of the HSV-*tk* promoter (data not shown). We cannot determine if WT1 represses transcription from this $EGR_3$TATA-CAT reporter due to the very low basal transcriptional activity of the reporter. Taken together, these data imply that WT1 must interact with proteins other than the basal transcriptional machinery in order to activate transcription. It is possible that WT1 represses transcription when these factors are not present. Definition of the circumstances under which WT1 can repress or activate transcription may help to suggest which other transcription factors might interact with WT1.

# CHAPTER V

# The codon 154 mutation of *WT1* yields a protein of apparently normal function

## INTRODUCTION

Identification of tumor-associated mutations in oncogenes and tumor suppressor genes has often yielded insights into the function of the wild-type protein products of these genes. In the case of Wilms' tumor, genetic alterations in the *WT1* gene including deletions and point mutations have been isolated from a minority of tumors (see Chapter I). Some of these mutations result in a null phenotype due to loss of expression of the *WT1* mRNA. Other mutations result in the production of WT1 proteins unable to bind to DNA. When present in the homozygous state, such null mutations result in a complete loss of *WT1* activity. However, when present in the heterozygous state, these mutations may function as dominant negative alleles which inhibit the function of the wild-type *WT1* allele (see Chapter III and (163)).

Several of the characterized tumor-associated *WT1* point mutations result in single amino acid substitutions in the protein. A homozygous serine-to-glycine mutation at codon 273 of *WT1* was isolated from a mesothelioma specimen (144). This mutated allele yields a protein which activates rather than represses transcription from the EGR-1 promoter in transfection assays. A glycine-to-aspartate mutation at codon 201 was found in the remaining allele of a hemizygous WAGR patient (145). This mutation also yielded a protein with transcriptional activation rather than repression activity. Both of these mutations resulted in single amino acid changes in the putative transactivation domain of

WT1. Hence, it is not readily apparent why such mutations result in a loss of transcriptional repression activity. As discussed in Chapter I, it is possible that the transcriptional effector function of WT1 is the result of a balance between the activities of its activator and repressor domains. If these point mutations result in increased potency of the activation domain, the activity of the repression domain might be masked and WT1 would activate rather than repress transcription.

Changes in mRNA splicing in Wilms' tumor cells can also result in the production of aberrant WT1 isoforms. Sequencing of *WT1* cDNA from a Wilms' tumor cell line, RM1, revealed an apparent mRNA splicing defect which results in expression of a *WT1* mRNA lacking exon 2. The predicted protein product of this mRNA would be deleted for amino acids 148-222, though expression of this mutant protein has not been confirmed in this cell line (65). This aberrant mRNA was also detected in a subset of Wilms' tumors but not in normal tissues. Re-introduction of a wild-type *WT1* cDNA into RM1 cells by transfection along with a neomycin resistance gene suppressed the ability of these cells to form neomycin-resistant colonies, consistent with a growth-suppressive effect of *WT1*. In addition, the protein product of this aberrantly spliced mRNA was shown to activate rather than repress transcription from the EGR-1 promoter. This deletion encompasses a region of the protein originally predicted to encompass both the repression and activation domains of WT1 (119)(205). Recent studies (207) indicate that this region encompasses only the activation region of WT1.

RNA editing of the *WT1* transcript may also affect the transcriptional effector function of WT1. In rats and humans, *WT1* cDNAs have either a T or a C

residue at codon 280, while the genomic DNA contains only a T residue (183). An RNA editing mechanism was proposed to explain these changes. The resultant edited mRNA yields a WT1 protein with a proline rather than a leucine residue at codon 280. This "edited" proline-containing WT1 isoform was a stronger repressor of transcription than the "unedited" leucine-containing protein. Similarly to the point mutations described above, this presumed RNA editing event alters the protein within its previously mapped transcriptional activation region, but effects are seen on its transcriptional repression function. As discussed above, this could be the result of an increase in the potency of the activation domain relative to the repression domain.

In a study of *WT1* mutations in Wilms' tumors and associated nephrogenic rests, a single base subsitution (T to C) was found at codon 154, which results in the substitution of serine for phenylalanine in the WT1 protein (143). Nephrogenic rests are small islands of poorly differentiated tissue which are often found in normal kidney tissue in kidneys containing Wilms' tumors. The fact that this mutation was present in the nephrogenic rest and the tumor but not in the surrounding normal tissue led Park *et al.* to propose that nephrogenic rests are genetic precursors to Wilms' tumors, a hypothesis originally proposed by Beckwith (11). Single-strand conformational polymorphism (SSCP) analysis showed the presence of both mutant and wild-type alleles in the tumor and the nephrogenic rest, suggesting that the mutation was heterozygous. However, since SSCP utilizes PCR as a first step, it is possible that the detection of the wild-type allele was due to contamination of the specimen by a small amount of normal tissue. The authors proposed that, similar to the mutant WT1 isoforms described in Chapter III and (163), the protein product of the codon 154 mutant allele could be acting as a dominant negative allele.

To examine this hypothesis, we performed assays to compare the functional properties of the mutant isoform to those of wild-type WT1. DNA-binding activity of the mutant protein *in vitro* was identical to wild-type. The mutant protein activated transcription of the $EGR_3$*tk*CAT reporter in a transient transfection assay in a manner identical to wild-type. Co-expression of mutant and wild-type WT1 did not affect the ability of either protein to activate transcription. This implied that, unlike the mutant WT1 proteins described in Chapter III, it does not act as a dominant negative protein. The mutant protein repressed transcription of the EGR1-CAT reporter when expressed from the pCB6+ expression vector. We did not observe a significant difference between the repression activities of the wild-type and mutant proteins when their N-terminal 182 amino acids were fused to the GAL4 DNA-binding domain. Finally, the mutant protein associated with itself and with wild-type WT1 *in vitro* , and the degree of these interactions was similar to that of self-association of wild-type WT1. In summary, the codon 154 mutation of WT1 yields a protein with apparently normal functional characteristics. If we assume that this mutation is not a coincidental finding in this tumor, this information indicates that standard assays of transcription factor function may not discern all the characteristics of a potentially oncogenic *WT1* mutation.

## RESULTS

**The codon 154 mutation does not interfere with DNA binding by WT1.** Since codon 154 is outside the DNA-binding domain of WT1, mutation of this codon would not be predicted to interfere with DNA binding by WT1. However, it is possible that this mutation could result in a conformational

change in the protein which might result in misfolding or shielding of the DNA-binding domain. Therefore we examined binding of wild-type and mutant WT1 to the Egr-1 consensus oligonucleotide using electrophoretic mobility shift assay (EMSA). PCR-based mutagenesis was used to generate the codon 154 mutation in the background of the A isoform. WT1(A), WT1(B), and WT1(154) were translated *in vitro* and incubated with $^{32}$P-labelled Egr-1 consensus oligonucleotide. As predicted, the codon 154 mutant protein bound to DNA to a similar extent as did the wild-type (A) and (B) isoforms (Figure 15).

**WT1(154) activates transcription to a similar extent as WT1(A).** Codon 154 falls within the region originally defined as the transcriptional repressor region of WT1 (119, 205). Recent studies have defined the minimal repression domain as amino acids 85-124 (207). Therefore, mutation of this codon might not be expected to affect the transcriptional activation ability of WT1. However, results from Park *et al.* (144, 145) showed that point mutations in the presumptive transcriptional activation region of WT1 can convert WT1 from a transcriptional repressor of the EGR-1 promoter to a transcriptional activator, suggesting that there may not be a direct correlation between the location of the mutation and the transcriptional effect. Therefore we examined the transcriptional activation potential of the WT1(154) protein. When transfected into CV-1 cells along with the EGR3tkCAT reporter (see Chapter III), WT1(154) activated transcription to a similar extent as wild-type WT1(A) (Figure 16). In addition, when WT1(A) and WT1(154) were co-expressed in cells, they neither synergized nor interfered with each other's ability to activate transcription of the EGR$_3$*tk*CAT reporter (data not shown). This suggests that WT1(154) is not a dominant negative WT1 mutation.

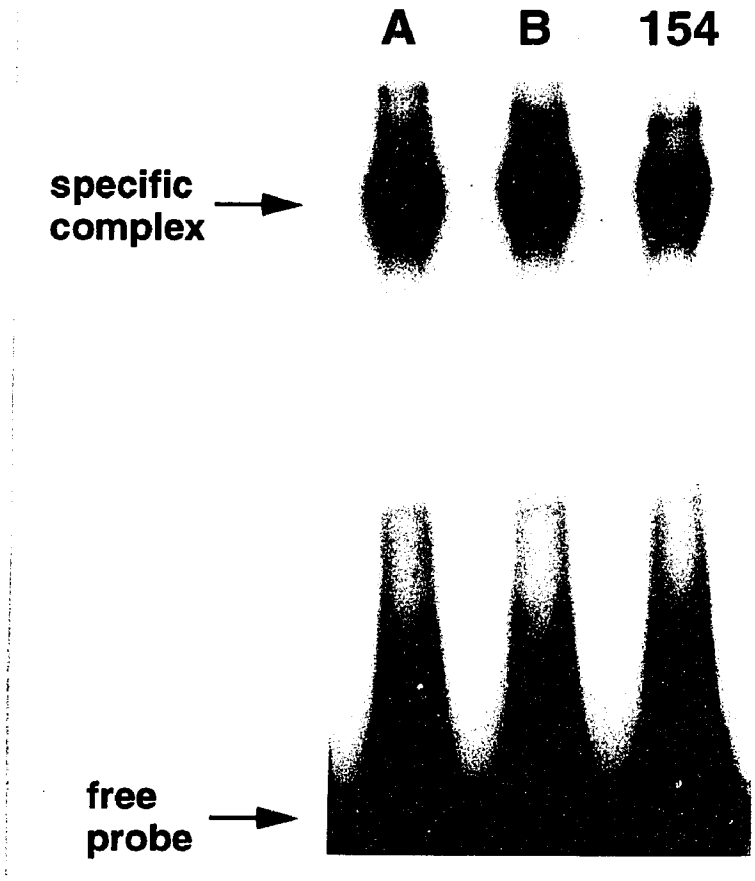



**Figure 15:** The codon 154 mutant WT1 protein binds DNA to a similar extent as the WT1(A) and (B) proteins, as determined by eletrophoretic mobility shift assay. The indicated *in vitro* translated proteins were incubated with the Egr-1/WT1 consensus binding site oligonucleotide and analyzed by nondenaturing polyacrylamide gel electrophoresis, as described in Chapter II. A representative autogradiogram is presented.

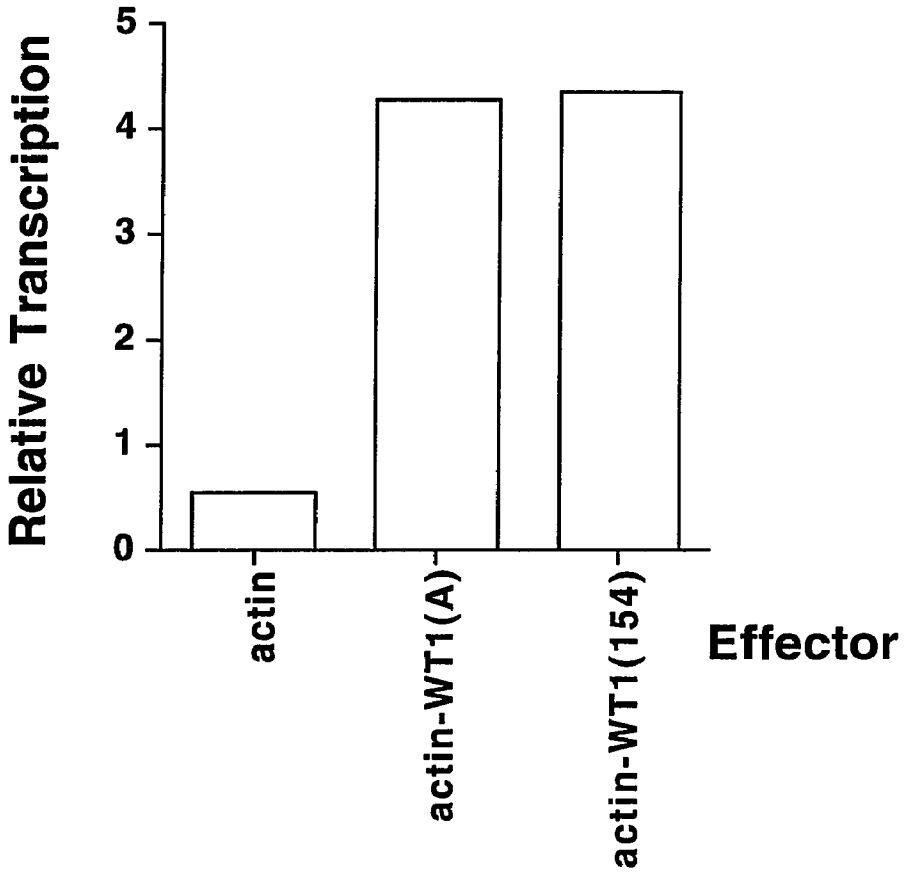



**FIGURE 16**: The codon 154 mutant WT1 protein activates transcription of the $EGR_3$*tk*CAT reporter to a similar extent as the wild-type WT1(A) isoform. 2 μg of reporter and 10 μg of the indicated effector were transfected into CV-1 cells as indicated in Chapter II. The results are presented as the average of four independent determinations.

**WT1(154) represses transcription to a similar extent as WT1(A) when expressed from the pCB6+ expression vector.** As noted above, the codon 154 mutation falls within the region originally defined as the transcriptional repressor domain of WT1 (119, 205), though recently the minimal repression domain was mapped to amino acids 85-124 (207). We therefore examined the transcriptional repressor ability of the mutant protein. As described in Chapter IV, we can only observe transcriptional repression by WT1 on the Egr-1 promoter when WT1 is expressed from the pCB6+ expression vector used by others in the field. We cloned both the murine WT1(A) cDNA and its codon 154 mutant counterpart into the pCB6+ vector. When transfected into NIH 3T3 cells, the wild-type and mutant proteins repressed transcription to a similar extent (Figure 17).

**WT1(154) represses transcription in a manner similar to wild-type WT1 when expressed as a GAL4 fusion protein.** WT1 is a transcriptional repressor of a reporter containing GAL4 DNA-binding sites when either the full-length protein or the N-terminal 182 amino acids are fused to the GAL4 DNA-binding domain (see Chapter IV and (119)). To examine the effect of the codon 154 mutation on transcriptional repression by GAL4-WT1, we fused the N-terminal 182 amino acids of WT1, either mutant or wild-type, to the N-terminal 147 amino acids of GAL4, which encode the DNA-binding and dimerization domains. The wild-type GAL4-WT1(1-182) fusion protein was a potent, dose-dependent transcriptional repressor of the $G_5$*tk*CAT reporter, which contains 5 GAL4 DNA-binding sites upstream of the HSV-*tk* promoter linked to the CAT reporter gene (186) (Figure 18A). The mutant GAL4-WT1(1-182/154) protein also showed strong repression activity which was not significanly different from that of the wild-type GAL4-WT1(1-182) protein. The two proteins

were expressed at similar levels in transfected cells (Figure 18B). These data suggest that the codon 154 mutation does not have an effect on the transcriptional repression activity of WT1 when it is expressed as a GAL4-WT1 fusion.

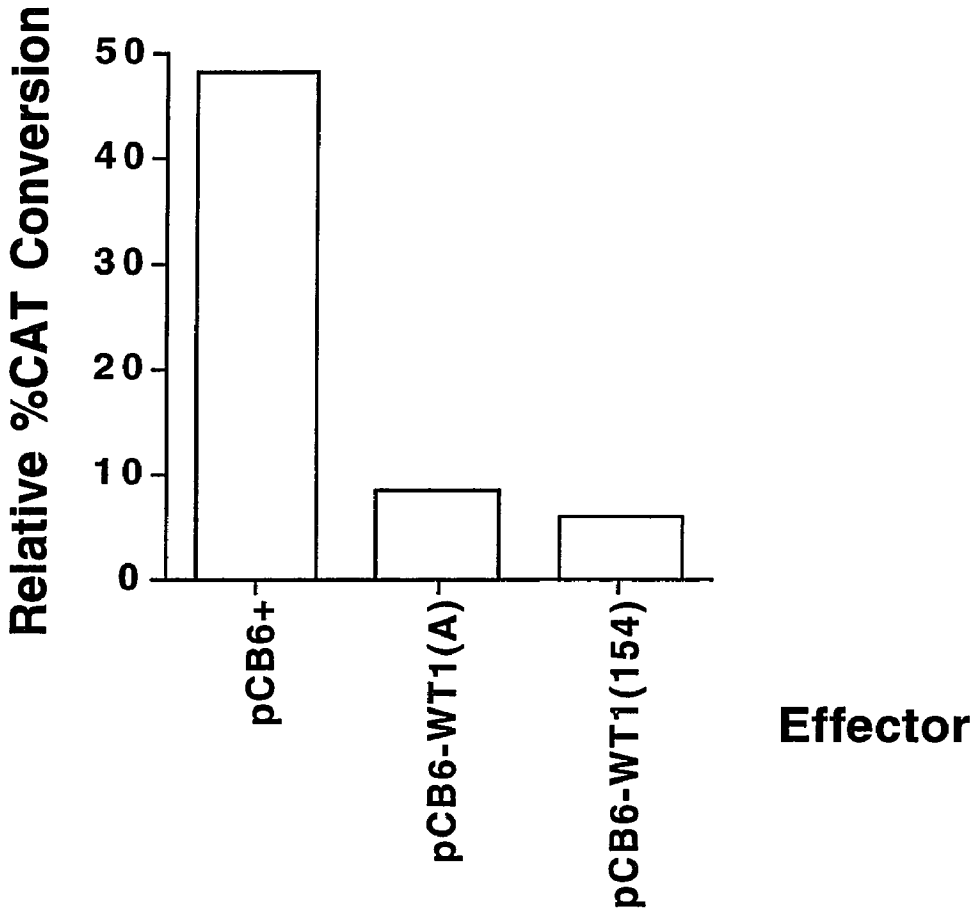



**FIGURE 17:** The codon 154 WT1 mutant protein represses transcription of the Egr1-CAT reporter when expressed from the pCB6 expression vector. 0.5 μg of reporter and 20 μg of the indicated effector were transfected into NIH 3T3 cells as indicated in Chapter II. The results are presented as the average of four independent determinations.

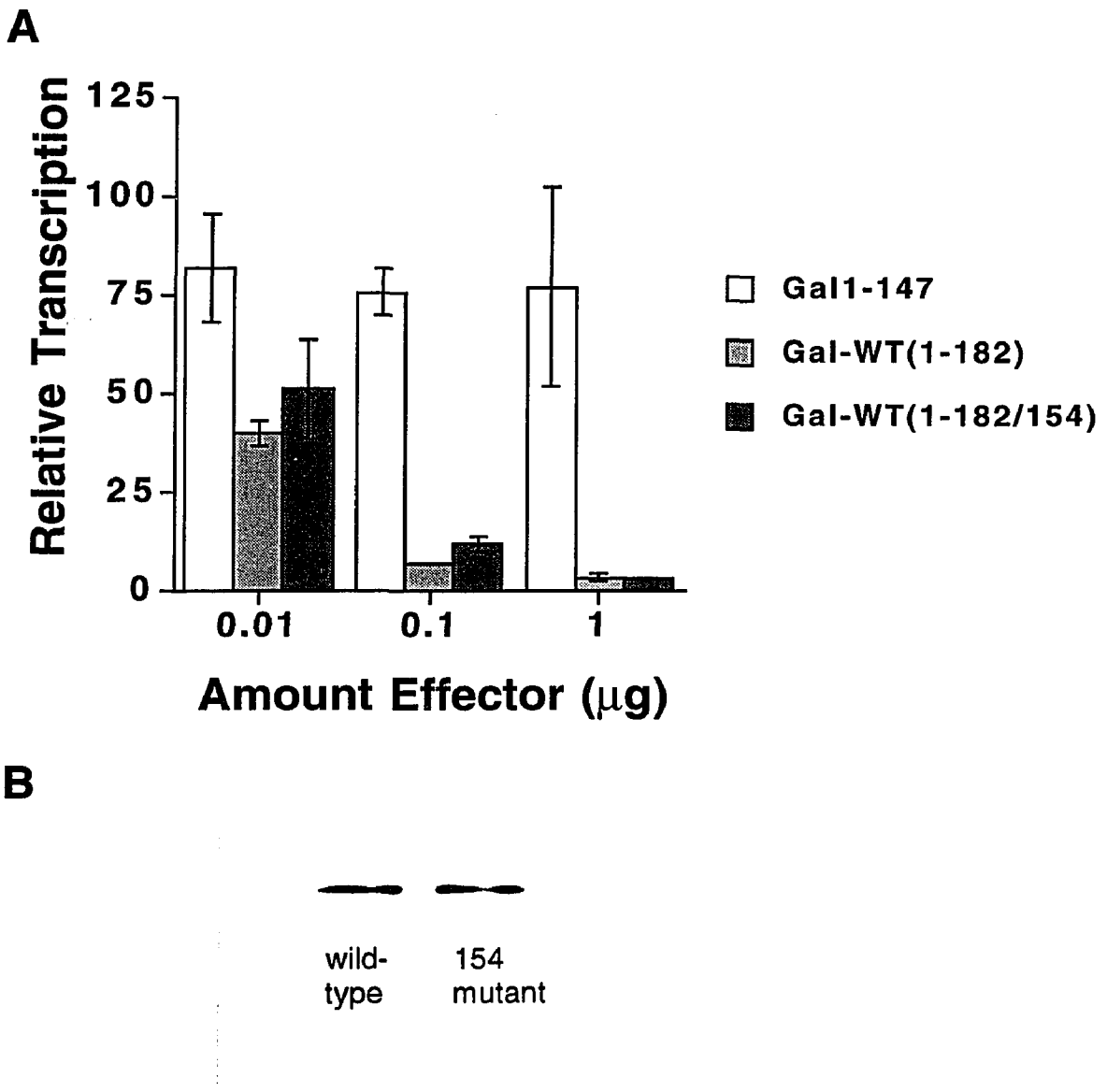



**FIGURE 18**: A GAL4-WT1 fusion protein containing the codon 154 mutation represses the $GAL_5$*tk*CAT reporter in a manner similar to wild-type GAL4-WT1. A: CAT assays: 2 μg of reporter and the indicated amounts of effector were transfected into CV-1 cells as described in Chapter II. The results are presented as the average plus or minus the standard deviation for three independent determinations. B: Extracts from representative plates of transfected cells from part A were subjected to immunoblotting analysis with a monoclonal anti-WT1 antibody as described in Chapter II.

**WT1(154) does not show impairment in association with itself and with wild-type WT1.** Self-association of WT1 has been suggested to be important for regulating the transcriptional activity of WT1 (Chapter III and (163)). In particular, dominant negative mutant WT1 proteins were shown to associate with wild-type WT1 and to inhibit transcriptional activation by the wild-type protein. Self-association may also be important for the transcriptional function of the wild-type protein. We therefore examined the ability of the WT1(154) protein to self-associate and to associate with wild-type WT1.

Since the 154 mutation may have been present in the heterozygous state in the tumor specimen from which it was derived, we investigated both homodimerization of the WT1(154) protein and also heterodimerization between the mutant and wild-type proteins. *In vitro* translated WT1 proteins, labelled with $^{35}$S-methionine, were incubated with GST-WT1 proteins which had been immobilized on glutathione-agarose beads. WT1 proteins containing only the N-terminal 182 amino acids were used in this assay, since this domain was shown to be sufficient for self-association (Chapter III and (163)). The WT1(1-182) and WT1(1-182/154) proteins interacted strongly with themselves and with each other (Figure 19). Upon repetition of this experiment with several different preparations of the mutant and wild-type GST-WT1 and *in vitro* translated WT1 proteins, we could not detect any significant differences in the degree of interaction between these proteins. Therefore the codon 154 mutation does not seem to affect self-association of WT1 proteins.

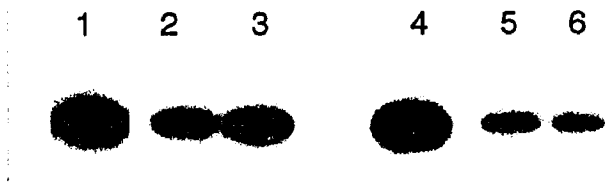



**FIGURE 19:** WT1(154) associates with itself and with wild-type WT1 in a similar manner to wild-type WT1. $^{35}$S-labelled, *in vitro* translated proteins were incubated with bacterially expressed GST-WT1 proteins and the bound protein was analyzed by SDS-PAGE as described in Chapter II. A representative experiment is presented; upon repetition with several different preparations of protein, no significant differences were noted between wild-type and mutant proteins. Lane 1: input amount of $^{35}$S-WT1(1-182). Lane 2: $^{35}$S-WT1(1-182) incubated with GST-WT1(1-182). Lane 3: $^{35}$S-WT1(1-182) incubated with GST-WT1(1-182/154). Lane 4: input amount of $^{35}$S-WT1(1-182/154). Lane 5: Lane 2: $^{35}$S-WT1(1-182/154) incubated with GST-WT1(1-182). Lane 6: $^{35}$S-WT1(1-182/154) incubated with GST-WT1(1-182/154).

## DISCUSSION

Our data suggest that the codon 154 mutation does not have a significant effect on the DNA-binding activity, transcriptional effects, and self-association of WT1 proteins. There are several hypotheses which can be advanced to explain this finding. Firstly, it is possible that this mutation is a coincidental finding which did not contribute to tumor development in this patient. Secondly, the patient's history may offer clues as to the etiology of his Wilms' tumor. This patient had a cleft palate, a protuberant left eye, facial hypoplasia, and a protuberant abdomen. These features are suggestive of Beckwith-Wiedemann syndrome, whose features include hemihypertrophy and a tendency to develop Wilms' tumor. In addition, this patient's tumor was associated with multiple perilobar nephrogenic rests, which are most commonly observed in patients with Beckwith-Wiedemann syndrome (11). The genetic locus for this syndrome has been mapped to chromosome 11p15 (27, 77, 98). It has been proposed that this locus contains a second Wilms' tumor suppressor gene, termed *WT2*, and that mutation of this gene is responsible for some Wilms' tumors where *WT1* is apparently normal. Therefore it is possible that this *WT1* mutation did not play a causative role in the development of this tumor, or that this *WT1* mutation was augmented by a disruption of *WT2* function, leading to tumor formation.

Finally, it is possible that the codon 154 mutation of WT1 disrupts other functions of the wild-type protein which we have not investigated. Within the limits of known assays, we do not observe a difference in function between WT1(154) and wild-type WT1, however, current assays for WT1 function may not be sufficient. It is possible that WT1(154) regulates other promoters which we did not examine, or endogenous genes, differently than wild-type WT1. In

some cases, different results are obtained when the endogenous chromosomally embedded gene is assayed as opposed to a reporter construct. For example, a virally-encoded v-myb protein which has lost its ability to activate the endogenous mim-1 gene still activates a reporter construct containing part of the mim-1 promoter. (134). The activity of WT1(154) may also differ from wild-type WT1 in other types of assays. Wild-type WT1 has been shown to have tumor suppressor activity when transfected into Wilms' tumor cells which express a defectively spliced WT1 mRNA (65). The codon 154 mutant may show a defect in this tumor suppressor activity; this is the subject of further investigation. WT1 has also been shown to suppress DNA replication from an SV40 origin of replication (3). If WT1 has a role in regulating normal cellular DNA replication, it is possible that the codon 154 mutation interferes with this activity. In addition, a role for the +KTS isoforms of WT1 in mRNA processing has recently been proposed (105); the codon 154 mutation might have effects on this function of WT1. A recent report showed that microinjection of a *WT1* cDNA into quiescent cells blocked serum-induced entry into S-phase, suggesting a role for WT1 in cell cycle control (101); this function could potentially be altered by the codon 154 mutation. Finally, WT1(154) may play a role in oncogenesis through alterations in functions of WT1 which have not yet been defined.

# CHAPTER VI

# CONCLUSIONS

As discussed in Chapter I, a large number of studies have attempted to characterize the function of the *WT1* gene product and the relationship between mutations of *WT1* and the development of Wilms' tumor. However, questions remain, particularly with respect to the transcriptional function of WT1. Specifically, what determines whether WT1 activates or represses transcription, and how do tumor-associated *WT1* mutations affect the transcriptional function of WT1?

Work presented in this thesis has helped to clarify several of these issues. With respect to the transcriptional function of WT1, I showed that WT1 activated transcription from a simple test promoter containing 3 WT1/Egr-1 binding sites upstream of the HSV-*tk* promoter linked to the CAT reporter gene (Chapter III). My results are supported by the work of Hamilton *et al.* , who recently showed that WT1 activates transcription through three tandem newly-identified WT1 binding sites upstream of the MMTV promoter (69). In addition, I found that the WT1(B) isoform was a somewhat stronger activator of transcription than the WT1(A) isoform, suggesting a role for the 17 amino acid insertion in WT1(B) in activation. Wang *et al.* have reported that this 17 amino acid peptide can act as a transferable repression domain whose activity is dependent on the presence of four consecutive serine residues (208). Taken together, these results suggest that this 17 amino acid sequence may have roles in both activation and repression.

Experiments reported in Chapter IV have resolved a conflict between our results and those of others in the field. I found that WT1 expressed from an RSV-based expression vector activated transcription driven by the Egr-1 promoter, in contrast to the results of Madden *et al.* who showed that WT1 repressed this promoter construct (118). When I used the expression vector used by this group (and many others), which is driven by the strong CMV promoter, we confirmed the results of Madden *et al.* by showing that WT1 repressed the Egr1-CAT reporter. Furthermore, I showed that WT1 expressed from the RSV-WT1 vector could be converted from an activator to a repressor of the Egr1-CAT reporter by co-transfection of the CMV-based pCB6+ vector lacking any WT1 coding sequences. Cotransfection of the pCB6+ vector alone produced a profound decrease in the level of basal transcription from the normally highly active Egr1-CAT reporter, suggesting that the expression vector and the reporter gene were competing for common sequence-specific DNA-binding transcription factors or non-DNA-binding co-factors. Therefore I propose that WT1 can repress transcription under a limited set of circumstances, which may depend upon the presence of other factors or co-factors bound to the target promoter.

In addition, I presented evidence as to the mechanism of repression of GAL4-WT1 fusion proteins (Chapter IV). These fusion proteins repress transcription from upstream GAL4 binding sites in test promoters, whereas I (Chapter III; (163)) and others (69, 205) observe activation by the native WT1 protein through upstream Egr-1/WT1 binding sites. I showed that a fusion protein containing the GAL4 DNA-binding and dimerization domains fused to the full-length WT1 coding sequence is a potent repressor of transcription from a $GAL_5$*tk*CAT reporter gene. However, I fail to observe any transcriptional effect of this protein on a reporter containing upstream Egr-1/WT1 binding sites,

suggesting that the WT1 moiety of this protein may be present in a non-native conformation.

I also demonstrated how tumor-associated *WT1* mutations can result in changes of transcriptional function. In Chapter III, experiments are described which confirmed the hypothesis that heterozygous germline *WT1* mutations associated with either Wilms' tumor or the Denys-Drash syndrome act in a dominant negative fashion. The protein products of these mutant alleles inhibited transcriptional activation by wild-type WT1, and these mutant proteins interacted with wild-type WT1, both in an *in vitro* biochemical assay and in the *in vivo* yeast 2-hybrid system (in work performed by my collaborators (163)). This data suggests that protein-protein interaction between wild-type and mutant WT1, mediated by an N-terminal self-association domain, may account for the inhibition of wild-type WT1 function we observed, and may explain the dominant negative phenotype of these tumor-associated mutations.

In Chapter V, I investigated the transcriptional effects of a heterozygous *WT1* point mutation that had been proposed to act in a dominant negative fashion in a patient with Wilms' tumor. However, I failed to observe any significant effects on the transcriptional activation or repression functions of this protein compared to wild-type WT1. In addition, I showed that the mutant protein could associate with itself and with wild-type WT1 to approximately the same extent as the wild-type protein. Therefore I suggest three possible hypotheses for the association of this mutation with a case of Wilms' tumor. Firstly, it is possible that this mutation represents a coincidental finding which is not related to the development of the tumor. Secondly, given the clinical history, it is possible that this patient suffered from Beckwith-Wiedemann syndrome, which has been

linked to genetic changes at 11p15 rather than at the *WT1* locus at 11p13. Therefore, alteration of *WT1* may not be the primary etiologic event in this tumor, but rather may have played a secondary role, combining with a putative mutation at 11p15. Finally, it is possible that this mutation affects a function of *WT1* which I did not or cannot study, such as its recently described functions in tumor suppression, DNA replication, or its recently proposed roles in mRNA splicing and cell cycle control. Further studies correlating *WT1* mutations with changes in protein function may yield insight into the relationship between *WT1* mutations and the development of Wilms' tumor. Transcriptional studies alone may not be sufficient to determine the oncogenic mechanisms of a mutant *WT1* allele.

# CHAPTER VII

# REFERENCES

1. **Akasaka, Y., H. Kikuchi, T. Nagai, N. Hiraoka, S. Kato, and J. Hata.** 1993. A point mutation found in the WT1 gene in a sporadic Wilms' tumor without genitourinary abnormalities is identical with the most frequent point mutation in Denys-Drash syndrome. FEBS Lett. **317:**39-43.

2. **Amin, K. M., L. A. Litzky, W. R. Smythe, A. M. Mooney, J. M. Morris, D. J. Mews, H. I. Pass, C. Kari, U. Rodeck, F. J. Rauscher III, L. R. Kaiser, and S. M. Albelda.** 1995. Wilms' tumor 1 susceptibility (WT1) gene products are selectively expressed in malignant mesothelioma. Am. J. Pathol. **146:**344-356.

3. **Anant, S., S. A. Axenovich, S. L. Madden, F. J. Rauscher III, and K. N. Subramanian.** 1994. Novel replication inhibitory function of the developmental regulator/transcription repressor protein WT1 encoded by the Wilms' tumor gene. Oncogene **9:**3113-3126.

4. **Armstrong, J. F., K. Pritchard-Jones, W. A. Bickmore, N. D. Hastie, and J. B. Bard.** 1992. The expression of the Wilms' tumour gene, WT1, in the developing mammalian embryo. Mech. Dev. **40:**85-97.

5. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1989. Current Protocols in Molecular Biology. John Wiley & Sons, New York, NY.

6. **Baird, P. N., N. Groves, D. A. Haber, D. E. Housman, and J. K. Cowell.** 1992. Identification of mutations in the WT1 gene in tumours from patients with the WAGR syndrome. Oncogene **7:**2141-2149.

7. **Baird, P. N., A. Santos, N. Groves, L. Jadresic, and J. K. Cowell.** 1992. Constitutional mutations in the WT1 gene in patients with Denys-Drash syndrome. Hum. Mol. Genet. **1:**301-305.

8. **Bardeesy, N., J. B. Beckwith, and J. Pelletier.** 1995. Clonal expansion and attenuated apoptosis in Wilms' tumors are associated with *p53* gene mutations. Cancer Res. **55:**215-219.

9. **Bardeesy, N., D. Falkoff, M. J. Petruzzi, N. Nowak, B. Zabel, M. Adam, M. C. Aguiar, P. Grundy, T. Shows, and J. Pelletier.** 1994. Anaplastic Wilms' tumour, a subtype displaying poor prognosis, harbours p53 gene mutations. Nat. Genet. **7:**91-97.

10. **Bardeesy, N., B. Zabel, K. Schmitt, and J. Pelletier.** 1994. WT1 mutations associated with incomplete Denys-Drash syndrome define a domain predicted to behave in a dominant-negative fashion. Genomics **21:**663-664.

11. **Beckwith, J. B., N. B. Kiviat, and J. F. Bonadio.** 1990. Nephrogenic rests, nephroblastomatosis, and the pathogenesis of Wilms' tumor. Pediatr. Pathol. **10:**1-36.

12. **Bickmore, W. A., K. Oghene, M. H. Little, A. Seawright, V. van Heyningen, and N. D. Hastie.** 1992. Modulation of DNA binding specificity by alternative splicing of the Wilms' tumor wt1 gene transcript. Science **257:**235-237.

13. **Binetruy, B., T. Smeal, and M. Karin.** 1991. Ha-Ras augments c-Jun activity and stimulates phosphorylation of its activation domain. Nature **351:**122-127.

14. **Brieger, J., E. Wiedmann, K. Fenchel, P. Mitrou, D. Hoelzer, and L. Bergmann.** 1994. The expression of the Wilms' tumor gene in acute myelocytic leukemias as a possible marker for leukemic blast cells. Leukemia **8:**2138-2143.

15. **Brown, K. W., J. E. Watson, V. Poirier, M. G. Mott, P. J. Berry, and N. J. Maitland.** 1992. Inactivation of the remaining allele of the WT1 gene in a Wilms' tumor from a WAGR patient. Oncogene **7:**763-768.

16. **Brown, K. W., H. P. Wilmore, J. E. Watson, M. G. Mott, P. J. Berry, and N. J. Maitland.** 1993. Low frequency of mutations in the WT1 coding region in Wilms' tumor. Genes Chromos. Cancer **8:**74-79.

17. **Bruening, W., N. Bardeesy, B. L. Silverman, R. A. Cohn, G. A. Machin, A. J. Aronson, D. Housman, and J. Pelletier.** 1992. Germline intronic and exonic mutations in the Wilms' tumour gene (WT1) affecting urogenital development. Nat. Genet. **1:**144-148.

18. **Bruening, W., P. Gros, T. Sato, J. Stanimir, Y. Nakamura, D. Housman, and J. Pelletier.** 1993. Analysis of the 11p13 Wilms' tumor suppressor gene (WT1) in ovarian tumors. Cancer Invest. **11:**393-399.

19. **Buckler, A. J., J. Pelletier, D. A. Haber, T. Glaser, and D. E. Housman.** 1991. Isolation, characterization, and expression of the murine Wilms' tumor gene (WT1) during kidney development. Mol. Cell. Biol. **11:**1707-1712.

20. **Call, K. M., T. Glaser, C. Y. Ito, A. J. Buckler, J. Pelletier, D. A. Haber, E. A. Rose, A. Kral, H. Yeger, and W. H. Lewis.** 1990. Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. Cell **60:**509-520.

21. **Campbell, C. E., A. Huang, A. L. Gurney, P. M. Kessler, J. A. Hewitt, and B. R. Williams.** 1994. Antisense transcripts and protein binding motifs within the Wilms tumour (WT1) locus. Oncogene **9:**583-595.

22. **Cao, X., R. A. Koski, A. Gashler, M. McKiernan, C. F. Morris, R. Gaffney, R. V. Hay, and V. P. Sukhatme.** 1990. Identification and characterization of the Egr-1 gene product, a DNA-binding zinc finger protein induced by differentiation and growth signals. Mol. Cell. Biol. **10:**1931-1939.

23. **Carey, M., H. Kakidani, J. Leatherwood, F. Mostashari, and M. Ptashne.** 1989. An amino-terminal fragment of GAL4 binds DNA as a dimer. J. Mol. Biol. **209:**423-432.

24. **Christy, B., and D. Nathans.** 1989. DNA binding site of the growth factor-inducible protein Zif268. Proc. Natl. Acad. Sci. USA **86:**8737-8741.

25. **Clarkson, P. A., H. R. Davies, D. M. Williams, R. Chaudhary, I. A. Hughes, and M. N. Patterson.** 1993. Mutational screening of the Wilms's tumour gene, WT1, in males with genital abnormalities. J. Med. Genet. **30:**767-772.

26. **Coles, H. S. R., J. F. Burne, and M. C. Raff.** 1993. Large-scale normal cell death in the developing rat kidney and its reduction by epidermal growth factor. Development **118:**777-784.

27. **Coppes, M. J., L. Bonetta, A. Huang, P. Hoban, S. Chilton-MacNeill, C. E. Campbell, R. Weksberg, H. Yeger, A. E. Reeve, and B. R. Williams.** 1992. Loss of heterozygosity mapping in Wilms tumor indicates the involvement of three distinct regions and a limited role for nondisjunction or mitotic recombination. Genes Chromos. Cancer **5:**326-334.

28. **Coppes, M. J., C. E. Campbell, and B. R. Williams.** 1993. The role of WT1 in Wilms tumorigenesis. FASEB J. **7:**886-895.

29. **Coppes, M. J., V. Huff, and J. Pelletier.** 1993. Denys-Drash syndrome: relating a clinical disorder to genetic alterations in the tumor suppressor gene WT1. J. Pediatr. **123:**673-678.

30. **Coppes, M. J., G. J. Liefers, M. Higuchi, A. B. Zinn, J. W. Balfe, and B. R. Williams.** 1992. Inherited WT1 mutation in Denys-Drash syndrome. Cancer Res. **52:**6125-6128.

31. **Coppes, M. J., Y. Ye, R. Rackley, X. L. Zhao, G. J. Liefers, G. Casey, and B. R. Williams.** 1993. Analysis of WT1 in granulosa cell and other sex cord-stromal tumors. Cancer Res. **53:**2712-2714.

32. **Courey, A. J., and R. Tjian.** 1988. Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. Cell **55:**887-898.

33. **Cowell, J. K., R. B. Wadey, D. A. Haber, K. M. Call, D. E. Housman, and J. Pritchard.** 1991. Structural rearrangements of the WT1 gene in Wilms' tumour cells. Oncogene **6:**595-599.

34. **DeLuca, N. A., and P. A. Schaffer.** 1987. Activities of herpes simplex virus type I (HSV-1) ICP4 genes specifying nonsense peptides. Nuc. Acids Res. **15:**4491-4511.

35. **Dey, B. R., V. P. Sukhatme, A. B. Roberts, M. B. Sporn, F. J. Rauscher III, and S. J. Kim.** 1994. Repression of the transforming growth factor-ß1 gene by the Wilms' tumor suppressor WT1 gene product. Mol. Endocrinol. **8:**595-602.

36. **Donehower, L. A., M. Harvey, B. L. Slagle, M. J. McArthur, C. A. Montgomery Jr., J. S. Butel, and A. Bradley.** 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumors. Nature **356:**215-221.

37. **Dressler, G. R., U. Deutsch, K. Chowdhury, H. O. Nornes, and P. Gruss.** 1990. *Pax2*, a new murine paired-box-containing gene and its expresion in the developing excretory system. Development **109:**787-795.

38. **Dressler, G. R., and E. C. Douglass.** 1992. Pax-2 is a DNA-binding protein expressed in embryonic kidney and Wilms' tumor. Proc. Natl. Acad. Sci. USA **89:**1179-1183.

39. **Drummond, I. A., S. L. Madden, P. Rohwer-Nutter, G. I. Bell, V. P. Sukhatme, and F. J. Rauscher III.** 1992. Repression of the insulin-like growth factor II gene by the Wilms tumor suppressor WT1. Science **257:**674-678.

40. **Drummond, I. A., H. D. Rupprecht, P. Rohwer-Nutter, J. M. Lopez-Guisa, S. L. Madden, F. J. Rauscher III, and V. P. Sukhatme.** 1994. DNA recognition by splicing variants of the Wilms' tumor suppressor, WT1. Mol. Cell. Biol. **14:**3800-3809.

41. **Eccles, M. R., G. Grubb, O. Ogawa, J. Szeto, and A. E. Reeve.** 1994. Cloning of novel Wilms tumor gene (WT1) cDNAs; evidence for antisense transcription of WT1. Oncogene **9:**2059-2063.

42. **Fearon, E. R., B. Vogelstein, and A. P. Feinberg.** 1984. Somatic deletion and duplication of genes on chromosome 11 in Wilms' tumors. Nature **309:**176-178.

43. **Fisher, D. E.** 1994. Apoptosis in cancer therapy: crossing the threshold. Cell **78:**539-542.

44. **Fraizer, G. C., Y. J. Wu, S. M. Hewitt, T. Maity, C. C. Ton, V. Huff, and G. F. Saunders.** 1994. Transcriptional regulation of the human Wilms' tumor gene (WT1). Cell type-specific enhancer and promiscuous promoter. J. Biol. Chem. **269:**8892-8900.

45. **Fraizer, G. E., D. F. Bowen-Pope, and A. M. Vogel.** 1987. Production of platelet-derived growth factor by cultured Wilms' tumor cells and fetal kidney cells. J. Cell. Physiol. **133:**169-174.

46. **Francke, U., L. B. Holmes, L. Atkins, and V. M. Ricciardi.** 1979. Aniridia-Wilms' tumor association: evidence for specific deletion of 11p13. Cytogenet. Cell Genet. **24:**185-192.

47. **Gansler, T., R. Furlanetto, T. S. Gramling, K. A. Robinson, N. Blocker, M. G. Buse, D. A. Sens, and A. J. Garvin.** 1989. Antibody to type I insulinlike growth factor receptor inhibits growth of Wilms' tumor in culture and in athymic mice. Am. J. Pathol. **135:**961-966.

48. **Gashler, A., S. Swaminathan, and V. P. Sukhatme.** 1993. A novel repression module, an extensive activation domain, and a bipartite nuclear localization signal defined in the immediate-early transcription factor Egr-1. Mol. Cell. Biol. **13:**4556-4571.

49. **Gashler, A. L., D. T. Bonthron, S. L. Madden, F. J. Rauscher III, T. Collins, and V. P. Sukhatme.** 1992. Human platelet-derived growth factor A chain is transcriptionally repressed by the Wilms tumor suppressor WT1. Proc. Natl. Acad. Sci. USA **89:**10984-10988.

50. **Gerald, W. L., T. S. Gramling, D. A. Sens, and A. J. Garvin.** 1992. Expression of the 11p13 Wilms' tumor gene, WT1, correlates with histologic category of Wilms' tumor. Am. J. Pathol. **140:**1031-1037.

51. **Gerald, W. L., J. Rosai, and M. Ladanyi.** 1995. Characterization of the genomic breakpoint and chimeric transcripts in the *EWS-WT1* gene fusion of desmoplastic small round cell tumor. Proc. Natl. Acad. Sci. USA **92:**1028-1032.

52. **Gessler, M., and G. A. Bruns.** 1993. Sequence of the WT1 upstream region including the Wit-1 gene. Genomics **17:**499-501.

53. **Gessler, M., A. Konig, K. Arden, P. Grundy, S. Orkin, S. Sallan, C. Peters, S. Ruyle, J. Mandell, F. Li, W. Cavenee, and G. Bruns.** 1994. Infrequent mutation of the WT1 gene in 77 Wilms' Tumors. Hum. Mut. **3:**212-222.

54. **Gessler, M., A. Konig, and G. A. Bruns.** 1992. The genomic organization and expression of the WT1 gene. Genomics **12:**807-813.

55. **Gessler, M., A. Konig, J. Moore, S. Qualman, K. Arden, W. Cavenee, and G. Bruns.** 1993. Homozygous inactivation of WT1 in a Wilms' tumor associated with the WAGR syndrome. Genes Chromos. Cancer **7:**131-136.

56. **Gessler, M., A. Poustka, W. Cavenee, R. L. Neve, S. H. Orkin, and G. A. P. Bruns.** 1990. Homozygous deletion in Wilms tumours of a gene identified by chromosome jumping. Nature **343:**774-778.

57. **Gius, D., X. M. Cao, F. J. Rauscher III, D. R. Cohen, T. Curran, and V. P. Sukhatme.** 1990. Trancsriptional activation and repression by Fos are independent functions: the C terminus represses immediate-early gene expression via CArG elements. Mol. Cell. Biol. **10:**4243-4255.

58. **Glaser, T., J. Lane, and D. Housman.** 1990. A mouse model of the aniridia-Wilms tumor deletion syndrome. Science **250:**823-827.

59. **Golemis, E. A., and R. Brent.** 1992. Fused protein domains inhibit DNA binding by LexA. Mol. Cell. Biol. **12:**3006-3014.

60. **Gonzales, G. A., and M. R. Montminy.** 1989. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell **59:**675-680.

61. **Goodyer, P., M. Dehbi, E. Torban, W. Bruening, and J. Pelletier.** 1995. Repression of the retinoic acid receptor-α gene by the Wilms' tumor suppressor gene product, wt1. Oncogene **10:**1125-1129.

62. **Grubb, G. R., K. Yun, B. R. Williams, M. R. Eccles, and A. E. Reeve.** 1994. Expression of WT1 protein in fetal kidneys and Wilms tumors. Lab. Invest. **71:**472-479.

63. **Gyuris, J., E. Golemis, H. Chertkov, and R. Brent.** 1993. Cdi, a human G1 and S phase protein phosphatase that associates with Cdk2. Cell **75:**791-803.

64. **Haber, D. A., A. J. Buckler, T. Glaser, K. M. Call, J. Pelletier, R. L. Sohn, E. C. Douglass, and D. E. Housman.** 1990. An internal deletion within an 11p13 zinc finger gene contributes to the development of Wilms' tumor. Cell **61:**1257-1269.

65. **Haber, D. A., S. Park, S. Maheswaran, C. Englert, G. G. Re, D. J. Hazen-Martin, D. A. Sens, and A. J. Garvin.** 1993. WT1-mediated growth suppression of Wilms tumor cells expressing a WT1 splicing variant. Science **262:**2057-2059.

66. **Haber, D. A., R. L. Sohn, A. J. Buckler, J. Pelletier, K. M. Call, and D. E. Housman.** 1991. Alternative splicing and genomic structure of the Wilms' tumor gene WT1. Proc. Natl. Acad. Sci. USA **88:**9618-9622.

67. **Haber, D. A., H. T. Timmers, J. Pelletier, P. A. Sharp, and D. E. Housman.** 1992. A dominant mutation in the Wilms tumor gene WT1 cooperates with the viral oncogene E1A in transformation of primary kidney cells. Proc. Natl. Acad. Sci. USA **89:**6010-6014.

68. **Habib, R., C. Loirat, M. C. Gubler, P. Niaudet, A. Bensman, M. Levy, and M. Broyer.** 1985. The nephropathy associated with male pseudohermaphroditism and Wilms' tumor (Drash syndrome): a distinctive glomerular lesion - report of 10 cases. Clin. Nephr. **24:**269-278.

69. **Hamilton, T. B., K. C. Barilla, and P. J. Romaniuk.** 1995. High affinity binding sites for the Wilms' tumour suppressor protein WT1. Nuc. Acids Res. **23:**277-284.

70. **Han, K., and J. L. Manley.** 1993. Functional domains of the *Drosophila Engrailed* protein. EMBO J. **12:**2723-2733.

71. **Han, K., and J. L. Manley.** 1993. Transcriptional repression by the *Drosophila even-skipped* protein: definition of a minimal repression domain. Genes & Dev. **7:**491-503.

72. **Harlow, E., and D. Lane.** 1988. Antibodies: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

73. **Harrington, M. A., B. Konicek, A. Song, X.-L. Xia, W. J. Fredericks, and F. J. Rauscher III.** 1993. Inhibition of the colony-stimulating factor-1 promoter activity by the product of the Wilms' tumor locus. J. Biol. Chem. **268:**21271-21275.

74. **Hastie, N. D.** 1994. The genetics of Wilms' tumor - A case of disrupted development. Annu. Rev. Genet. **28:**523-558.

75. **Hazen-Martin, D. J., G. G. Re, A. J. Garvin, and D. A. Sens.** 1994. Distinctive properties of an anaplastic Wilms' tumor and its associated epithelial cell line. Am. J. Pathol. **144:**1023-1034.

76. **Heimdal, K. R., R. A. Lothe, S. D. Fossa, and A. L. Borresen.** 1994. Association studies of a polymorphism in the Wilms' tumor 1 locus in Norwegian patients with testicular cancer. Int. J. Cancer **58:**523-526.

77. **Henry, I., S. Grandjouan, P. Couillin, F. Barichard, C. Huerre-Jeanpierre, T. Glaser, T. Philip, G. Lenoir, J. L. Chaussain, and C. Junien.** 1989. Tumor-specific loss of 11p15.5 alleles in del11p13 Wilms tumor and in familial adrenocortical carcinoma. Proc. Natl. Acad. Sci. USA **86:**3247-3251.

78. **Herskowitz, I.** 1987. Functional inactivation of genes by dominant negative mutations. Nature **329:**219-222.

79. **Hofmann, W., H. D. Royer, M. Drechsler, S. Schneider, and B. Royer-Pokora.** 1993. Characterization of the transcriptional regulatory region of the human WT1 gene. Oncogene **8:**3123-3132.

80. **Huang, A., C. E. Campbell, L. Bonetta, M. S. McAndrews-Hill, S. Chilton-MacNeill, M. J. Coppes, D. J. Law, A. P. Feinberg, H. Yeger, and B. R. G. Williams.** 1990. Tissue, developmental, and tumor-specific expression of divergent transcripts in Wilms tumor. Science **250:**991-993.

81. **Huang, R. P., T. Darland, D. Okamura, D. Mercola, and E. D. Adamson.** 1994. Suppression of v-sis-dependent transformation by the transcription factor, Egr-1. Oncogene **9:**1367-1377.

82. **Huff, V., N. Jaffe, G. F. Saunders, L. C. Strong, F. Villalba, and E. C. Ruteshouser.** 1995. WT1 exon 1 deletion/insertion mutations in Wilms tumor patients, associated with di- and trinucleotide repeats and deletion hotspot consensus sequences. Am. J. Hum. Genet. **56:**84-90.

83. **Huff, V., H. Miwa, D. A. Haber, K. M. Call, D. Housman, L. C. Strong, and G. F. Saunders.** 1991. Evidence for WT1 as a Wilms tumor (WT) gene: intragenic germinal deletion in bilateral WT. Am. J. Hum. Genet. **48:**997-1003.

84. **Inoue, K., H. Sugiyama, H. Ogawa, M. Nakagawa, T. Yamagami, H. Miwa, K. Kita, A. Hiraoka, T. Masaoka, K. Nasu, T. Kyo, H. Dohy, H. Nakauchi, T. Ishidate, T. Akiyama, and T. Kishimoto.** 1994. WT1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. Blood **84:**3071-3079.

85. **Jacks, T., A. Fazeli, E. M. Schmitt, R. T. Bronson, M. A. Goodell, and R. A. Weinberg.** 1992. Effects of an Rb mutation in the mouse. Nature **359:**295-300.

86. **Jacks, T., L. Remington, B. O. Williams, E. M. Schmitt, S. Halachmi, R. T. Bronson, and R. A. Weinberg.** 1994. Tumor spectrum analysis in p53-mutant mice. Curr. Biol. **4:**1-7.

87. **Jadresic, L., J. Leake, I. Gordon, M. J. Dillon, D. B. Grant, J. Pritchard, R. A. Risdon, and T. M. Barratt.** 1990. Clinicopathologic review of twelve children with nephropathy, Wilms' tumor, and genital abnormalities (Drash syndrome). J. Pediatr. **117:**717-725.

88. **Jinno, Y., K. Yun, K. Nishiwaki, T. Kubota, O. Ogawa, A. E. Reeve, and N. Niikawa.** 1994. Mosaic and polymorphic imprinting of the WT1 gene in humans. Nat. Genet. **6:**305-309.

89. **Kaelin Jr., W. G., D. C. Pallas, J. A. DeCaprio, F. J. Kaye, and D. M. Livingston.** 1991. Identification of cellular proteins that can interact specifically with the T/E1A-binding region of the retinoblastoma gene product. Cell **64:**521-532.

90. **Kaneko, Y., O. Takeda, C. Homma, N. Maseki, H. Miyoshi, Y. Tsunematsu, B. G. Williams, G. F. Saunders, and M. Sakurai.** 1993. Deletion of WT1 and WIT1 genes and loss of heterozygosity on chromosome 11p in Wilms tumors in Japan. Jpn. J. Cancer Res. **84:**616-624.

91. **Kikuchi, H., Y. Akasaka, Y. Kurosawa, H. Yoneyama, S. Kato, and J. Hata.** 1995. A critical mutation in both *WT1* allels is not sufficient to cause Wilms' tumor. FEBS Lett. **360:**26-28.

92. **Kikuchi, H., Y. Akasaka, T. Nagai, A. Umezawa, H. Iri, S. Kato, and J. Hata.** 1992. Genomic changes in the WT-gene (WT1) in Wilms' tumors and their correlation with histology. Am. J. Pathol. **140:**781-786.

93. **Klein, G., M. Langegger, C. Goridis, and P. Ekblom.** 1988. Neural cell adhesion molecules during embryonic induction and development of the kidney. Development **102:**749-761.

94. **Knudson Jr., A. G.** 1971. Mutation and cancer: Statistical study of retinoblastoma. Proc. Natl. Acad. Sci. USA **68:**820-823.

95. **Knudson Jr., A. G., and L. C. Strong.** 1972. Mutation and cancer: A model for Wilms' tumor of the kidney. JNCI **48:**313-324.

96. **König, A., S. Jakubiczka, P. Wieacker, H. W. Schlosser, and M. Gessler.** 1993. Further evidence that imbalance of WT1 isoforms may be involved in Denys-Drash syndrome. Hum. Molec. Genet. **2:**1967-1968.

97. **Koseki, C., D. Herzlinger, and Q. Al-Awqati.** 1992. Apoptosis in metanephric development. J. Cell Biol. **119:**1327-1333.

98. **Koufos, A., P. Grundy, K. Morgan, K. A. Aleck, T. Hadro, B. C. Lampkin, A. Kalbakji, and W. K. Cavenee.** 1989. Familial Wiedemann-Beckwith syndrome and a second Wilms tumor locus both map to 11p15.5. Am. J. Hum. Genet. **44:**711-719.

99. **Koufos, A., M. F. Hansen, B. C. Lampkin, M. L. Workman, N. G. Copeland, N. A. Jenkins, and W. K. Cavenee.** 1984. Loss of alleles at loci on human chromosome 11 during genesis of Wilms' tumor. Nature **309:**170-172.

100. **Kreidberg, J. A., H. Sariola, J. M. Loring, M. Maeda, J. Pelletier, D. Housman, and R. Jaenisch.** 1993. WT-1 is required for early kidney development. Cell **74:**679-691.

101. **Kudoh, T., T. Ishidate, M. Moriyama, K. Toyoshima, and T. Akiyama.** 1995. G1 phase arrest induced by Wilms tumor protein WT1 is abrogated by cyclin/CDK complexes. Proc. Natl. Acad. Sci. USA **92:**4517-4521.

102. **Ladanyi, M., and W. Gerald.** 1994. Fusion of the EWS and WT1 genes in the desmoplastic small round cell tumor. Cancer Res. **54:**2837-2840.

103. **Lai, J.-S., and W. Herr.** 1992. Ethidium bromide provides a simple tool for identifying genuine DNA-independent protein associations. Proc. Natl. Acad. Sci. USA **89:**6958-6962.

104. **Langerak, A. W., K. A. Williamson, K. Miyagawa, A. Hagemeijer, M. A. Versnel, and N. D. Hastie.** 1995. Expression of the Wilms' tumor gene *WT1* in human malignant mesothelioma cell lines and relationship to platelet-derived growth factor A and insulin-like growth factor 2 expression. Genes, Chromos., & Cancer **12:**87-96.

105. **Larsson, S. H., J.-P. Charlieu, K. Miyagawa, D. Engelkamp, M. Rassoulzadegan, A. Ross, F. Cuzin, V. van Heyningen, and N. D. Hastie.** 1995. Subnuclear localization of WT1 in splicing or transcription factor domains is regulated by alternative splicing. Cell **81:**391-401.

106. **Lemoine, N. R., C. M. Hughes, and J. K. Cowell.** 1992. Aberrant expression of the tumour suppressor gene p53 is very frequent in Wilms' tumours. J. of Path. **168:**237-242.

107. **Levine, M., and J. L. Manley.** 1989. Transcriptional repression of eukaryotic promoters. Cell **59:**405-408.

108. **Licht, J. D., M. J. Grossel, J. Figge, and U. M. Hansen.** 1990. *Drosophila* Krüppel protein is a transcriptional repressor. Science **346:**76-79.

109. **Licht, J. D., W. Hanna-Rose, J. C. Reddy, M. A. English, M. Ro, M. Grossel, R. Shaknovich, and U. Hansen.** 1994. Mapping and mutagenesis of the amino-terminal transcriptional repression domain of the Drosophila Krüppel protein. Mol. Cell. Biol. **14:**4057-4066.

110. **Licht, J. D., M. Ro, M. A. English, M. Grossel, and U. Hansen.** 1993. Selective repression of transcriptional activators at a distance by the *Drosophila Krüppel* protein. Proc. Natl. Acad. Sci. USA **90:**11361-11365.

111. **Little, M. H., R. Dunn, J. A. Byrne, A. Seawright, P. J. Smith, K. Pritchard-Jones, V. van Heyningen, and N. D. Hastie.** 1992. Equivalent expression of paternally and maternally inherited WT1 alleles in normal fetal tissue and Wilms' tumors. Oncogene **7:**635-641.

112. **Little, M. H., J. Prosser, A. Condie, P. J. Smith, V. Van Heyningen, and N. D. Hastie.** 1992. Zinc finger point mutations within the WT1 gene in Wilms tumor patients. Proc. Natl. Acad. Sci. USA **89:**4791-4795.

113. **Little, M. H., K. A. Williamson, M. Mannens, A. Kelsey, C. Gosden, N. D. Hastie, and V. van Heyningen.** 1993. Evidence that WT1 mutations in Denys-Drash syndrome patients may act in a dominant-negative fashion. Hum. Molec. Genet. **2:**259-264.

114. **Looijenga, L. H., M. Abraham, A. J. Gillis, G. F. Saunders, and J. W. Oosterhuis.** 1994. Testicular germ cell tumors of adults show deletions of chromosomal bands 11p13 and 11p15.5, but no abnormalities within the zinc-finger regions and exons 2 and 6 of the Wilms' tumor 1 gene. Genes Chromos. Cancer **9:**153-160.

115. **Lothe, R. A., N. Hastie, K. Heimdal, S. D. Fossa, A. E. Steinwig, and A.-L. Borresen.** 1993. Frequent loss of 11p13 and 11p15 loci in male germ cell tumors. Genes Chromos. Cancer **7:**96-101.

116. **Luckow, B., and G. Schütz.** 1987. CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. Nuc. Acids Res. **15:**5490.

117. **Luo, X.-N., J. C. Reddy, P. L. Yeyati, A. H. Idris, S. Hosono, D. A. Haber, J. D. Licht, and G. F. Atweh.** 1995. The tumor suppressor gene WT1 inhibits *ras*-mediated transformation. Oncogene, in press.

118. **Madden, S. L., D. M. Cook, J. F. Morris, A. Gashler, V. P. Sukhatme, and F. J. Rauscher III.** 1991. Transcriptional repression mediated by the WT1 Wilms tumor gene product. Science **253:**1550-1553.

119. **Madden, S. L., D. M. Cook, and F. J. Rauscher III.** 1993. A structure-function analysis of transcriptional repression mediated by the WT1, Wilms' tumor suppressor protein. Oncogene **8:**1713-1720.

120. **Maheswaran, S., S. Park, A. Bernard, J. F. Morris, F. J. Rauscher III, D. E. Hill, and D. A. Haber.** 1993. Physical and functional interaction between WT1 and p53 proteins. Proc. Natl. Acad. Sci. USA **90:**5100-5104.

121. **Malik, K. T., V. Poirier, S. M. Ivins, and K. W. Brown.** 1994. Autoregulation of the human WT1 gene promoter. FEBS Lett. **349:**75-78.

122. **Malkin, D., E. Sexsmith, H. Yeger, B. R. Williams, and M. J. Coppes.** 1994. Mutations of the p53 tumor suppressor gene occur infrequently in Wilms' tumor. Cancer Res. **54:**2077-2079.

123. **Mannens, M., J. M. Hoovers, E. M. Bleeker-Wagemakers, E. Redeker, J. Bliek, M. Overbeeke-Melkert, G. Saunders, B. Williams, V. van Heyningen, C. Junien, D. Haber, F. Speleman, C. Heyting, R. M. Slater, N. J. Leschot, and A. Westerveld.** 1991. The distal region of 11p13 and associated genetic diseases. Genomics **11:**284-293.

124. **Matsunaga, E.** 1981. Genetics of Wilms' tumor. Hum Genet **57:**231-246.

125. **Maw, M. A., P. E. Grundy, L. J. Millow, M. R. Eccles, R. S. Dunn, P. J. Smith, A. P. Feinberg, D. J. Law, M. C. Paterson, P. E. Telzerow, D. F. Callen, A. D. Thompson, R. I. Richards, and A. E. Reeve.** 1992. A third Wilms' tumor locus on chromosome 16q. Cancer Res. **52:**3094-3098.

126. **Mermod, N., E. A. O'Neill, T. J. Kelley, and R. Tjian.** 1989. The proline-rich transcriptional activator of CTF/NF-1 is distinct from the replication and DNA binding domain. Cell **58:**741-753.

127. **Miwa, H., M. Beran, and G. F. Saunders.** 1992. Expression of the Wilms' tumor gene (WT1) in human leukemias. Leukemia **6:**405-409.

128. **Miwa, H., G. E. Tomlinson, C. F. Timmons, V. Huff, S. L. Cohn, L. C. Strong, and G. F. Saunders.** 1992. RNA expression of the WT1 gene in Wilms' tumors in relation to histology. J. Natl. Cancer Inst. **84:**181-187.

129. **Miyagi, T., H. Ahuja, T. Kubota, I. Kubonishi, H. P. Koeffler, and I. Miyoshi.** 1993. Expression of the candidate Wilm's tumor gene, WT1, in human leukemia cells. Leukemia **7:**970-977.

130. **Morgenstern, J. P., and H. Land.** 1990. A series of mammalian expression vectors and characterization of their expression of a reporter gene in stably and transiently transfected cells. Nuc. Acids Res. **18:**1068.

131. **Morris, J. F., S. L. Madden, O. E. Tournay, D. M. Cook, V. P. Sukhatme, and F. J. Rauscher III.** 1991. Characterization of the zinc finger protein encoded by the WT1 Wilms' tumor locus. Oncogene **6:**2339-2348.

132. **Mundlos, S., J. Pelletier, A. Darveau, M. Bachmann, A. Winterpacht, and B. Zabel.** 1993. Nuclear localization of the protein encoded by the Wilms' tumor gene *WT1* in embryonic and adult tissues. Development **119:**1329-1341.

133. **Nakagama, H., G. Heinrich, J. Pelletier, and D. E. Housman.** 1995. Sequence and structural requirements for high-affinity DNA binding by the WT1 gene product. Mol. Cell. Biol. **15:**1489-1498.

134. **Ness, S. A., A. Marknell, and T. Graf.** 1989. The v-myb oncogene product binds to and activates the promyelocyte-specific mim-1 gene. Cell **59:**1115-1125.

135. **Nordenskjold, A., E. Friedman, and M. Anvret.** 1994. WT1 mutations in patients with Denys-Drash syndrome: a novel mutation in exon 8 and paternal allele origin. Hum. Genet. **93:**115-120.

136. **Ogawa, O., D. M. Becroft, I. M. Morison, M. R. Eccles, J. E. Skeen, D. C. Mauger, and A. E. Reeve.** 1993. Constitutional relaxation of insulin-like growth factor II gene imprinting associated with Wilms' tumour and gigantism. Nat. Genet. **5:**408-412.

137. **Ogawa, O., M. R. Eccles, J. Szeto, L. A. McNoe, K. Yun, M. A. Maw, P. J. Smith, and A. E. Reeve.** 1993. Relaxation of insulin-like growth factor II gene imprinting implicated in Wilms' tumour. Nature **362:**749-751.

138. **Ogawa, O., M. R. Eccles, K. Yun, R. F. Mueller, M. D. Holdaway, and A. E. Reeve.** 1993. A novel insertional mutation at the third zinc finger coding region of the WT1 gene in Denys-Drash syndrome. Hum. Molec. Genet. **2:**203-204.

139. **Ohno, T., M. Ouchida, L. Lee, Z. Gatalica, V. N. Rao, and E. S. Reddy.** 1994. The EWS gene, involved in Ewing family of tumors, malignant melanoma of soft parts and desmoplastic small round cell tumors, codes for an RNA binding protein with novel regulatory domains. Oncogene **9:**3087-3097.

140. **Oosterwegel, M., J. Timmerman, J. Leiden, and H. Clevers.** 1992. Expression of GATA-3 during lymphocyte differentiation and mouse embryogenesis. Dev. Immunol. **3:**1-11.

141. **Orkin, S. H., D. S. Goldman, and S. E. Sallan.** 1984. Development of homozygosity for chromosome 11p markers in Wilms' tumor. Nature **309:**172-174.

142. **Pachnis, V., B. Mankoo, and F. Costantini.** 1993. Expression of the c-ret proto-oncogene during mouse embryogenesis. Development **119:**1005-1017.

143. **Park, S., A. Bernard, K. E. Bove, D. A. Sens, D. J. Hazen-Martin, A. J. Garvin, and D. A. Haber.** 1993. Inactivation of *WT1* in nephrogenic rests, genetic precursors to Wilms' tumour. Nat. Genet. **5:**363-367.

144. **Park, S., M. Schalling, A. Bernard, S. Maheswaran, G. C. Shipley, D. Roberts, J. Fletcher, R. Shipman, J. Rheinwald, G. Demetri, J. Griffin, M. Minden, D. E. Housman, and D. A. Haber.** 1993. The Wilms tumour gene WT1 is expressed in murine mesoderm-derived tissues and mutated in a human mesothelioma. Nat. Genet. **4:**415-420.

145. **Park, S., G. Tomlinson, P. Nisen, and D. A. Haber.** 1993. Altered *trans*-activational properties of a mutated WT1 gene product in a WAGR-associated Wilms' tumor. Cancer Res. **53:**4757-4760.

146. **Pavletich, N. P., and C. O. Pabo.** 1991. Zinc finger-DNA recognition: crystal structure of a zif268-DNA complex at 2.1 Å. Science **252:**809-817.

147. **Pelletier, J., W. Bruening, C. E. Kashtan, S. M. Mauer, J. C. Manivel, J. E. Striegel, D. C. Houghton, C. Junien, R. Habib, L. Fouser, R. N. Fine, B. L. Silverman, D. A. Haber, and D. Housman.** 1991. Germline mutations in the Wilms' tumor suppressor gene are associated with abnormal urogenital development in Denys-Drash syndrome. Cell **67:**437-447.

148. **Pelletier, J., W. Bruening, F. P. Li, D. A. Haber, T. Glaser, and D. E. Housman.** 1991. *WT1* mutations contribute to abnormal genital system development and hereditary Wilms' tumour. Nature **353:**431-434.

149. **Pelletier, J., M. Schalling, A. J. Buckler, A. Rogers, D. A. Haber, and D. Housman.** 1991. Expression of the Wilms' tumor gene WT1 in the murine urogenital system. Genes & Dev. **5:**1345-1356.

150. **Phelan, S. A., C. Lindberg, and K. M. Call.** 1994. Wilms' tumor gene, WT1, mRNA is down-regulated during induction of erythroid and megakaryocytic differentiation of K562 cells. Cell Growth & Diff. **5:**677-686.

151. **Pritchard-Jones, K., and S. Fleming.** 1991. Cell types expressing the Wilms' tumour gene (WT1) in Wilms' tumours: implications for tumour histogenesis. Oncogene **6:**2211-2220.

152. **Pritchard-Jones, K., S. Fleming, D. Davidson, W. Bickmore, D. Porteus, C. Gosden, J. Bard, A. Buckler, J. Pelletier, D. Housman, V. van Heyningen, and N. Hastie.** 1990. The candidate Wilms' tumor gene is involved in genitourinary development. Nature **346:**194-197.

153. **Pritchard-Jones, K., J. Renshaw, and L. King-Underwood.** 1994. The Wilms tumour (WT1) gene is mutated in a secondary leukaemia in a WAGR patient. Hum. Molec. Genet. **3:**1633-1637.

154. **Ptashne, M.** 1988. How eukaryotic transcriptional activators work. Nature **335:**683-689.

155. **Quek, H. H., V. T. Chow, and E. P. Tock.** 1993. The third zinc finger of the WT1 gene is mutated in Wilms' tumour but not in a broad range of other urogenital tumours. Anticancer Res. **13:**157515-80.

156. **Rackley, R. R., A. M. Flenniken, N. P. Kuriyan, P. M. Kessler, M. H. Stoler, and B. R. Williams.** 1993. Expression of the Wilms' tumor suppressor gene WT1 during mouse embryogenesis. Cell Growth & Diff. **4:**1023-1031.

157. **Radice, P., S. Pilotti, V. De Benedetti, P. Mondini, M. Miozzo, R. Luksch, F. Fossati Bellani, G. Della Porta, and M. A. Pierotti.** 1993. Homozygous intragenic loss of the WT1 locus in a sporadic intralobar Wilms' tumor. Int. J. Cancer **55:**174-176.

158. **Rainier, S., L. A. Johnson, C. J. Dobry, A. J. Ping, P. E. Grundy, and A. P. Feinberg.** 1993. Relaxation of imprinted genes in human cancer. Nature **362:**747-749.

159. **Rassoulzadegan, M., V. Paquis-Flucklinger, B. Bertino, J. Sage, M. Jasin, K. Miyagawa, V. van Heyningen, P. Besmer, and F. Cuzin.** 1993. Transmeiotic differentiation of male germ cells in culture. Cell **75:**997-1006.

160. **Rauscher III, F. J., J. F. Morris, O. E. Tournay, D. M. Cook, and T. Curran.** 1990. Binding of the Wilms' tumor locus zinc finger protein to the EGR-1 consensus sequence. Science **250:**1259-1262.

161. **Reddy, J. C., and J. D. Licht.** 1995. The codon 154 mutation of WT1 yields a protein of apparently normal function. Manuscript in preparation.

162. **Reddy, J. C., and J. D. Licht.** 1995. The transcriptional effector function of WT1 is modulated by mode of presentation, promoter architecture, and choice of expression vector. Manuscript in preparation.

163. **Reddy, J. C., J. C. Morris, J. Wang, M. A. English, D. A. Haber, Y. Shi, and J. D. Licht.** 1995. WT1-mediated transcriptional activation is inhibited by dominant negative mutant proteins. J. Biol. Chem. **270:**10878-10884.

164. **Reeve, A. E., M. R. Eccles, R. J. Wilkins, G. I. Bell, and L. J. Millow.** 1985. Expression of insulin-like growth factor-II transcripts in Wilms' tumor. Nature **317:**258-260.

165. **Reeve, A. E., P. J. Housiaux, R. J. M. Gardner, W. E. Chewings, R. M. Grindley, and L. J. Millow.** 1984. Loss of a Harvey *ras* allele in sporadic Wilms' tumor. Nature **309:**174-176.

166. **Ricciardi, V. M., E. Sujansky, A. C. Smith, and U. Francke.** 1978. Chromosomal imbalance in the aniridia-Wilms' tumor association: 11p interstitial deletion. Pediatrics **61:**604-610.

167. **Rodeck, U., A. Bossler, C. Kari, C. W. Humphreys, T. Gyorfi, J. Maurer, E. Thiel, and H. D. Menssen.** 1994. Expression of the wt1 Wilms' tumor gene by normal and malignant human melanocytes. Int. J. Cancer **59:**78-82.

168. **Royer-Pokora, B., S. Ragg, B. Heckl-Ostreicher, M. Held, U. Loos, K. Call, T. Glaser, D. Housman, G. Saunders, B. Zabel, B. Williams, and A. Poustka.** 1991. Direct pulsed field gel electrophoresis of Wilms' tumors shows that DNA deletions in 11p13 are rare. Genes Chromos. Cancer **3:**89-100.

169. **Rupprecht, H. D., I. A. Drummond, S. L. Madden, F. J. Rauscher III, and V. P. Sukhatme.** 1994. The Wilms' tumor suppressor gene WT1 is negatively autoregulated. J. Biol. Chem. **269:**6198-6206.

170. **Ryan, G., V. Steele-Perkins, J. F. Morris, F. J. Rauscher III, and G. R. Dressler.** 1995. Repression of *Pax-2* by *WT1* during normal kidney development. Development **121:**867-875.

171. **Sadowski, I., and M. Ptashne.** 1989. A vector for expressing GAL4(1-147) fusions in mammalian cells. Nuc. Acids Res. **17:**7539.

172. **Sakai, A., K. Tadokoro, H. Yanagisawa, S. Nagafuchi, N. Hoshikawa, T. Suzuki, T. Kohsaka, T. Hasegawa, Y. Nakahori, and M. Yamada.** 1993. A novel mutation of the WT1 gene (a tumor suppressor gene for Wilms' tumor) in a patient with Denys-Drash syndrome. Hum. Molec. Genet. **2:**1969-1970.

173. **Santos, A., L. Osorio-Almeida, P. N. Baird, J. M. Silva, M. G. Boavida, and J. Cowell.** 1993. Insertional inactivation of the WT1 gene in tumour cells from a patient with WAGR syndrome. Hum. Genet. **92:**83-86.

174. **Sauer, F., and H. Jäckle.** 1991. Concentration-dependent transcriptional activation or repression by *Krüppel* from a single binding site. Nature **353:**563-566.

175. **Saxén, L.** 1987. Organogenesis of the kidney. Cambridge University Press, Cambridge.

176. **Schmidt, C., F. Bladt, S. Goedecke, V. Brinkmann, W. Zschiesche, M. Sharpe, E. Gherardi, and C. Birchmeier.** 1995. Scatter factor/hepatocyte growth factor is essential for liver development. Nature **373:**699-702.

177. **Schuchardt, A., V. D'Agati, L. Larsson-Blomberg, F. Costantini, and V. Pachnis.** 1994. Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. Nature **367:**380-383.

178. **Scott, J., J. Cowell, M. E. Robertson, L. M. Priestly, R. Wadey, B. Hopkins, J. Pritchard, G. I. Bell, L. B. Rall, C. F. Graham, and T. J. Knott.** 1985. Insulin-like growth factor-II gene expression in Wilms' tumor and embryonic tissues. Nature **317:**260-262.

179. **Sekiya, M., M. Adachi, Y. Hinoda, K. Imai, and A. Yachi.** 1994. Downregulation of Wilms' tumor gene (wt1) during myelomonocytic differentiation in HL60 cells. Blood **83:**1876-1882.

180. **Selden, R. F., K. Burke-Howie, M. E. Rowe, H. M. Goodman, and D. D. Moore.** 1986. Human growth hormone as a reporter gene in regulation studies employing transient gene expression. Mol. Cell. Biol. **6:**3173-3179.

181. **Sells, S. F., S. Muthukumar, V. P. Sukhatme, S. A. Crist, and V. M. Rangnekar.** 1995. The zinc finger transcription factor EGR-1 impedes interleukin-1-inducible tumor growth arrest. Mol. Cell. Biol. **15:**682-692.

182. **Seto, E., A. Usheva, G. P. Zambetti, J. Momand, N. Horikoshi, R. Weinmann, A. J. Levine, and T. Shenk.** 1992. Wild-type p53 binds to the TATA-binding protein and represses transcription. Proc. Natl. Acad. Sci. USA **89:**12028-12032.

183. **Sharma, P. M., M. Bowman, S. L. Madden, F. J. Rauscher III, and S. Sukumar.** 1994. RNA editing in the Wilms' tumor susceptibility gene, WT1. Genes & Dev. **8:**720-731.

184. **Sharma, P. M., M. Bowman, B. F. Yu, and S. Sukumar.** 1994. A rodent model for Wilms tumors: embryonal kidney neoplasms induced by N-nitroso-N'-methylurea. Proc. Natl. Acad. Sci. USA **91:**9931-9935.

185. **Sharma, P. M., X. Yang, M. Bowman, V. Roberts, and S. Sukumar.** 1992. Molecular cloning of rat Wilms' tumor complementary DNA and a studey of messenger RNA expression in the urogenital system and the brain. Cancer Res. **52:**6407-6412.

186. **Shi, Y., E. Seto, L.-S. Chang, and T. Shenk.** 1991. Transcriptional repression by YY1, a human GLI-Krüppel-related protein, and relief of repression by adenovirus E1A protein. Cell **67:**377-388.

187. **Shipman, R., P. Schraml, M. Colombi, G. Raefle, and C. U. Ludwig.** 1993. Loss of heterozygosity on chromosome 11p13 in primary bladder carcinoma. Hum. Genet. **91:**455-458.

188. **Slatter, R. E., M. Elliott, K. Welham, M. Carrera, P. N. Schofield, D. E. Barton, and E. R. Maher.** 1994. Mosaic uniparental disomy in Beckwith-Wiedemann syndrome. J. Med. Genet. **31:**749-753.

189. **Sonnenberg, E., K. M. Weidner, and C. Birchmeier.** 1993. Expression of the met-receptor and its ligand, HGF-SF during mouse embryogenesis. EXS **65:**381-394.

190. **Steenman, M. J. C., S. Rainier, C. J. Dobry, P. Grundy, I. Horon, and A. P. Feinberg.** 1994. Loss of imprinting of IGF2 is linked to reduced methylation of H19 in Wilms' tumour. Nat. Genet. **7:**433-439.

191. **Sukhatme, V. P., X. M. Cao, L. C. Chang, C. H. Tsai-Morris, D. Stamenkovich, P. C. Ferreira, D. R. Cohen, S. A. Edwards, T. B. Shows, T. Curran, M. M. LeBeau, and E. D. Adamson.** 1988. A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. Cell **53:**37-43.

192. **Tadokoro, K., H. Fujii, A. Ohshima, Y. Kakizawa, K. Shimizu, A. Sakai, K. Sumiyoshi, T. Inoue, Y. Hayashi, and M. Yamada.** 1992. Intragenic homozygous deletion of the WT1 gene in Wilms' tumor. Oncogene **7:**1215-1221.

193. **Telerman, A., H. Dodemont, C. Degraef, P. Galand, S. Bauwens, P. Van Oostveldt, and R. B. Amson.** 1992. Identification of the cellular protein encoded by the human Wilms' tumor (WT1) gene. Oncogene **7:**2545-2548.

194. **TenHarmsel, A., R. J. Austin, N. Savenelli, and M. D. Biggin.** 1993. Cooperative binding at a distance by *even-skipped* protein correlates with repression and suggests a mechanism of silencing. Mol. Cell. Biol. **13:**2742-2752.

195. **Ton, C. C. T., H. Hirvonen, H. Miwa, M. M. Weil, P. Monaghan, T. Jordan, V. van Heyningen, N. D. Hastie, H. Meijers-Heijboer, M. Drechsler, B. Royer-Pokora, F. Collins, A. Swaroop, L. C. Strong, and G. F. Saunders.** 1991. Positional cloning and characterization of a paired box- and homeobox-containing gene from the aniridia region. Cell **67:**1059-1074.

196. **Tsai-Morris, C.-H., X. Cao, and V. P. Sukhatme.** 1988. 5' flanking sequence and genomic structure of Egr-1, a murine mitogen inducible zinc finger encoding gene. Nuc. Acids Res. **16:**8835-8846.

197. **Uehara, Y., O. Minowa, C. Mori, K. Shiota, J. Kuno, T. Noda, and N. Kitamura.** 1995. Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. Nature **373:**702-5.

198. **Vainio, S., M. Jalkanen, M. Bernfield, and L. Saxen.** 1992. Transient expression of syndecan in mesenchymal cell aggregates of the embryonic kidney. Dev. Biol. **152:**221-232.

199. **van Heyningen, V., and N. D. Hastie.** 1992. Wilms' tumour: reconciling genetics and biology. TIGS **8:**16-21.

200. **Varanasi, R., N. Bardeesy, M. Ghahremani, M. J. Petruzzi, N. Nowak, M. A. Adam, P. Grundy, T. B. Shows, and J. Pelletier.** 1994. Fine structure analysis of the WT1 gene in sporadic Wilms tumors. Proc. Natl. Acad. Sci. USA **91:**3554-3558.

201. **Viel, A., F. Giannini, E. Capozzi, V. Canzonieri, C. Scarabelli, A. Gloghini, and M. Boiocchi.** 1994. Molecular mechanisms possibly affecting WT1 function in human ovarian tumors. Int. J. Cancer **57:**515-521.

202. **Waber, P. G., J. Chen, and P. D. Nisen.** 1993. Infrequency of ras, p53, WT1, or RB gene alterations in Wilms tumors. Cancer **72:**3732-3738.

203. **Walker, C., R. Rutten, X. Yuan, H. Pass, D. M. Mew, and J. Everitt.** 1994. Wilms' tumor suppressor gene expression in rat and human mesothelioma. Cancer Res. **54:**3101-3106.

204. **Wang, Z.-Y., S. L. Madden, T. F. Deuel, and F. J. Rauscher III.** 1992. The Wilms' tumor gene product, WT1, represses transcription of the platelet-derived growth factor A-chain gene. J. Biol. Chem. **267:**21999-22002.

205. **Wang, Z.-Y., Q.-Q. Qiu, and T. F. Deuel.** 1993. The Wilms' tumor gene product WT1 activates or suppresses transcription through separate functional domains. J. Biol. Chem. **268:**9172-9175.

206. **Wang, Z.-Y., Q.-Q. Qiu, K. T. Enger, and T. F. Deuel.** 1993. A second transcriptionally active DNA-binding site for the Wilms' tumor gene product, WT1. Proc. Natl. Acad. Sci. USA **90:**8896-8900.

207. **Wang, Z.-Y., Q.-Q. Qiu, M. Gurrieri, J. Huang, and T. F. Deuel.** 1995. WT1, the Wilms' tumor suppressor gene product, represses transcription through an interactive nuclear protein. Oncogene **10:**1243-1247.

208. **Wang, Z. Y., Q. Q. Qiu, J. Huang, M. Gurrieri, and T. F. Deuel.** 1995. Products of alternatively spliced transcripts of the Wilms' tumor suppressor gene, wt1, have altered DNA binding specificity and regulate transcription in different ways. Oncogene **10:**415-422.

209. **Werner, H., F. J. Rauscher III, V. P. Sukhatme, I. A. Drummond, C. T. Roberts Jr., and D. LeRoith.** 1994. Transcriptional repression of the insulin-like growth factor I receptor (IGF-I-R) gene by the tumor suppressor WT1 involves binding to sequences both upstream and downstream of the IGF-I-R gene transcription start site. J. Biol. Chem. **269:**12577-12582.

210. **Werner, H., G. G. Re, I. A. Drummond, V. P. Sukhatme, F. J. Rauscher III, D. A. Sens, A. J. Garvin, D. LeRoith, and C. T. Roberts Jr.** 1993. Increased expression of the insulin-like growth factor I receptor gene, IGF1R, in Wilms tumor is correlated with modulation of IGF1R promoter activity by the WT1 Wilms tumor gene product. Proc. Natl. Acad. Sci. USA **90:**5828-5832.

211. **Wu, Y.-J., G. C. Fraizer, and G. F. Saunders.** 1995. GATA-1 transactivates the WT1 hematopoeitic specific enhancer. J. Biol. Chem. **270:**5944-5949.

212. **Yeger, H., C. Cullinane, A. Flenniken, S. Chilton-MacNeil, C. Campbell, A. Huang, L. Bonetta, M. J. Coppes, P. Thorner, and B. R. Williams.** 1992. Coordinate expression of Wilms' tumor genes correlates with Wilms' tumor phenotypes. Cell Growth & Diff. **3:**855-864.

213. **Zambetti, G. P., and A. J. Levine.** 1993. A comparison of the biological activities of wild-type and mutant p53. FASEB J. **7:**855-865.

214. **Zervos, A. S., J. Gyuris, and R. Brent.** 1993. Mxil, a protein that specifically interacts with Max to bind Myc-Max recognition sites. Cell **72:**223-232.

215. **Zhang, Y., T. Shields, T. Crenshaw, Y. Hao, T. Moulton, and B. Tycko.** 1993. Imprinting of human H19: allele-specific CpG methylation, loss of the active allele in Wilms' tumor, and potential for somatic allele switching. Am. J. Hum. Genet. **53:**113-124.

216. **Zhou, J., F. J. Rauscher III, and C. Bondy.** 1993. Wilms' tumor (WT1) gene expression in rat decidual differentiation. Differentiation **54:**109-114.