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Characterization of the Wilms' tumor suppressor gene *wt1* and its role in disease

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Doctor of Philosophy

Table of Contents

Abstract

The Wilms' tumor suppressor gene 1 (*wt1*) encodes a transcription factor belonging to the Early Growth Response (EGR) family of proteins. The gene produces an mRNA that is alternatively spliced to yield four different species. In this thesis, it is shown that the *wt1* gene has two sites of translational initiation, an AUG codon and a CUG codon that is upstream of and in frame with the AUG codon. The combination of alternative initiation sites and alternatively spliced exons allows the gene to encode eight different isoforms. It was previously shown that heterozygous mutations of *wt1* , predicted to result in a reduction in dosage of WT1, are associated with a predisposition to Wilms' tumor and mild abnormalities of the genitourinary system. However, other mutations of *wt1*, primarily missense mutations predicted to disrupt the ability of WT1 to bind to DNA, are associated with the severe disease of Denys-Drash syndrome (DDS). To explain the different disease states associated with mutations of *wt1*, it has been suggested that the mutations in DDS individuals must be acting other than as null alleles, i.e. in a dominant or dominant-negative fashion. In this thesis, an individual with DDS is reported to have a splicing mutation of *wt1*, predicted to drastically alter the ratio of WT1 isoforms. Because no altered forms of WT1 are produced in this individual, we were able to eliminate the possibility that DDS is caused by a gain of function of mutated WT1. In this thesis it is also shown that WT1 can form dimers through a domain encoded by its first two exons, and that mutated WT1 proteins are able to antagonize the function of wild-type WT1 by binding to and inactivating the wild-type proteins. This dominant-negative activity is hypothesized to reduce the dosage of functional WT1 to very low levels and to thus cause the severe disease of DDS. The work reported in this thesis also shows that WT1 is imported into the nucleus by a signal contained within its first zinc finger, a region encoded by its seventh exon. It is demonstrated that nuclear localization of mutated WT1 proteins is necessary for dominant-negative activity of mutated WT1, and presumably for development of DDS.

Résumé

Le gène 1 suppresseur de tumeur de Wilm (*wt1*) exprime un facteur de transcription appartenant à la famille de protéines "EARLY GROWTH RESPONSE". Ce gène produit un ARN messager épissé alternativement pour générer quatre transcrits différents. Le travail décrit ci-après démontre qu'il existe deux sites d'initiation de la traduction, soit un codon AUG, et un codon CUG situé en aval et dans le même cadre de lecture que le codon initiateur AUG. L'utilisation de divers sites d'initiation et l'épissage alternatif des exons permet au gène d'exprimer huit isoformes différents. Les mutations hétérozygotes de *wt1* nous permettant d'anticiper une baisse de la quantité de WT1, prédisposent les patients au développement de tumeurs de Wilm et à de légères anomalies du système urogénital. De plus, les mutations de type non-sens de *wt1* qui devrait vraisemblablement perturber l'association entre WT1 et l'ADN sont associées au syndrome plus sévère de Denys-Drash (DDS). Puisque différentes maladies sont liées aux mutations de *wt1*, les mutations responsables du DDS doivent agir de façon dominante ou dominante-négative. Dans cette thèse, nous décrivons le cas d'un patient atteint du DDS et possédant une mutation d'épissage de *wt1* qui devrait grandement affecter la proportion des différentes isoformes de WT1. La maladie ne découle pas d'un gain de fonction de la WT1 mutante puisque la mutation n'altère pas la protéine elle-même. Nous démontrons également que WT1 forme un dimère par l'interaction du domaine codé par les deux premiers exons et que des mutants de WT1 peuvent se lier à la WT1 de type sauvage et en inhiber ainsi le fonctionnement. On suppose que cette activité dominante-négative réduit la quantité de WT1 fonctionnelle à un niveau suffisamment bas pour provoquer le DDS. Le travail qui suit démontre aussi que WT1 est importée dans le noyau par le biais d'un signal contenu dans son premier domaine à doigt de zinc codé par le septième exon. On démontre finalement que la WT1 mutante doit être localisée dans le noyau pour exercer son activité dominante-négative ainsi que le DDS qui semble en en découler.

Preface

Candidates have the option of including, as part of a thesis, the text of one or more papers submitted or to be submitted for publication, or the clearly-duplicated text of one or more published papers. These texts must be bound as an integral part of the thesis.

If this topic is chosen, **connecting texts that provide logical bridges between the different papers are mandatory.** The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include:** A Table of Contents, an Abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.

Acknowledgements

I would like to acknowledge the aid and assistance of my supervisor, Dr. J. Pelletier; the technical assistance of Majid Ghahremani and Lee Lee Chu; the helpful discussions and advice provided by Nabeel Bardeesy and Benoit Giasson; and the aid and assistance of the staff and professors of the Department of Biochemistry.

Chapter 2, A non-AUG translational initiation event generates novel *wt1* isoforms, is entirely my own work.

Chapter 3, Germline intronic and exonic mutations in the Wilms' tumour gene (WT1) affecting urogenital development, is my own work as well, except for: the reverse transcriptase reactions and PCR reactions shown in Fig. 2 were performed by Nabeel Bardeesy, and diagnosis and evaluation of the patients was performed by Bernard L. Silverman, Richard A. Cohn, Geoffrey A. Machin, and Andrew J. Aronson.

Chapter 4, Antagonism of WT1 activity by protein self-association, is only partially my work. I made the initial discovery and performed all of the yeast two-hybrid system work (Figure 3). Figures 1 and 2 are the work of Peter Moffett.

Chapter 5, Identification of nuclear localization signals within the zinc fingers of the WT1 tumor suppressor gene product, is my own work, except for: some of the constructs used were prepared by Peter Moffett and Gunther Heinrich, and the initial observations about the location of the nuclear targetting signal were made by Shea Chia and Gunther Heinrich.

Original Contributions to Knowledge

Chapter 2:
1) Discovered a novel isoform of WT1
2) Discovered the non-AUG initiation event that produces the novel isoform
3) Identified the CUG codon at which the novel isoform initiates translation
4) Confirmed that the CUG-initiated isoform of WT1 is located within the nucleus and is capable of repressing transcription of a reporter gene

Chapter 3:
1) Discovered an intronic mutation of *wt1* in an individual with Denys-Drash syndrome
2) Demonstrated that this intronic mutation affects alternative splice site selection
3) Discovered that an imbalance of WT1 isoforms can result in Denys-Drash syndrome
4) Eliminated the dominant mutation theory of Denys-Drash syndrome

Chapter 4:
1) Discovered that WT1 can form dimers
2) Identified the region within WT1 required for self-association
3) Provided a mechanism for the dominant-negative theory of Denys-Drash syndrome

Chapter 5:
1) Identified the first zinc finger of WT1 as a nuclear targetting signal
2) Discovered that WT1 requires both a nuclear targetting signal and a dimerization domain to be able to act as a dominant-negative
3) Explained the correlation between type of disease and type of WT1 mutation

List of Illustrations

List of Abbreviations

5' UTR	5' untranslated region
bp	base pairs
CAT	chloramphenicol transferase
CSF-1	colony stimulating factor 1
DNA	deoxynucleic acid
DDS	Denys-Drash syndrome
EGF	epidermal growth factor
EGR	Early growth responsive gene
GU	genital-urinary system
IGF II	insulin-like growth factor II
IGF I	insulin-like growth factor I
kbp	kilobase pairs
kDa	kilodaltons
mRNA	messenger ribonucleic acid
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF A	platelet derived growth factor A
RAR α	retinoic acid receptor alpha
RNA	ribonucleic acid
SSCP	single stranded conformational polymorphism
TGF-β1	transforming growth factor beta 1
WAGR	A syndrome characterized by Wilms' tumor, aniridia, GU anomalies, and mental retardation
WT	Wilms' tumor
wt1	the Wilms' tumor suppressor gene 1
WT1	the protein encoded by the *wt1* gene
WT1*	the 60 kDa isoform of WT1

Chapter 1

Chapter 1: Introduction

Part I. Review of the relevant literature

Wilms' tumor, or nephroblastoma, is an embryonic malignancy of the kidney. Nephroblastoma is the second most common solid tumor among children, afflicting one in 10,000, with a peak incidence at 3-4 years of age (Matsunaga 1981). After studying the age of onset of Wilms' tumors, Knudson and Strong (1972) suggested that Wilms' tumors follow the same genetic model previously proposed for retinoblastoma. According to this model, two rate-limiting events are required for tumor formation. The simplest scenario is that these two events consist of mutational inactivation of both copies of a gene, a tumor suppressor gene.

Although most cases of Wilms' tumor appear to be isolated events, some occur in the context of congenital defect syndromes, primarily WAGR syndrome (Wilms' tumor, aniridia, genitourinary defects, and mental retardation; Miller et al, 1964), Denys-Drash syndrome (Wilms' tumor, intersex disorders, and renal nephropathy; Drash et al, 1970), and Beckwith-Wiedemann syndrome (Wilms' tumors, hemihypertrophy, and other overgrowth disorders; Beckwith, 1963). Riccardi et al (1978) found that individuals with WAGR syndrome had deletions of chromosome 11 band p13. Analysis of the extent of the 11p13 deletions from WAGR patients led to the identification of the Wilms' tumor suppressor gene 1 (*wt1*) (Call et al, 1990).

1. The *wt1* gene: characterization

1.1 The *wt1* gene is a member of the *EGR* gene family

The *EGR* (early growth responsive) genes encode a family of transcription factors characterized by three Cys_2-His_2 zinc fingers (Sukhatme 1991). The zinc fingers of each member of the family are almost identical, and in fact all members of the family may bind to the same or very similar DNA sequences within the promoters of the

genes that they regulate (Lemaire et al, 1988). Outside of the zinc finger region, *EGR* genes bear little resemblance to each other.

Five mammalian *EGR* genes (*EGR-1*, *2*, *3*, *4*, and *wt1*) and a yeast version (*MIG-1*) have been cloned and characterized (Call et al, 1990; Joseph et al, 1988; Nehlin et al, 1990; Patwardhan et al, 1991; Sukhatme et al, 1987). Most members (*EGR 1*, *2*, and *3*) of the *EGR* family are immediate-early responsive, i.e. they are rapidly and transiently induced by mitogenic stimulation (Herschman 1989). In tissue culture, EGR-1 (also called Zif268, Krox-24, NGFI-A, and TI S8) and EGR-2 (also called Krox-20) can be induced in practically all cell types, but *in vivo* all *EGR* genes are expressed only in restricted patterns, particularly during fetal development (McMahon et al, 1990; Rackley et al, 1995; Wilkinson et al, 1989). Unlike the majority of immediate-early responsive proteins, EGR proteins do not promote or cause transformation or cell growth; in fact, overexpression of EGR genes inhibits transformation and cell growth (Huang et al, 1994; Huang et al, 1995). It is thought that EGR proteins perform two functions: downregulation of responses to mitogenic stimulation, and maintenance of the differentiated state of certain cell types (Huang et al, 1995).

1.2 The structure and expression pattern of the *wt1* gene

The Wilms' tumor suppressor gene 1 (*wt1*) is an atypical member of the *EGR* gene family. In addition to the defining three zinc fingers of an EGR protein, WT1 has a fourth zinc finger (its first zinc finger; see Figure 1) (Call et al, 1990). The *wt1* gene encompasses 50 kb of genomic DNA at human chromosome 11 band p13. It encodes an mRNA of 3 kb containing 10 coding exons flanked by long (over 1 kb and over 300 bp in length, respectively) 3' and 5' untranslated regions (UTRs). Two alternatively spliced exons, exon 5 and three amino acids (KTS) that are inserted or deleted between the third and fourth zinc fingers, allow the gene to produce four different mRNAs (see Figure 1) (Haber et al, 1991). The gene is highly conserved among vertebrates, with the exception of the alternatively spliced exon 5, which is only present in placental mammals (Kent et al, 1995).

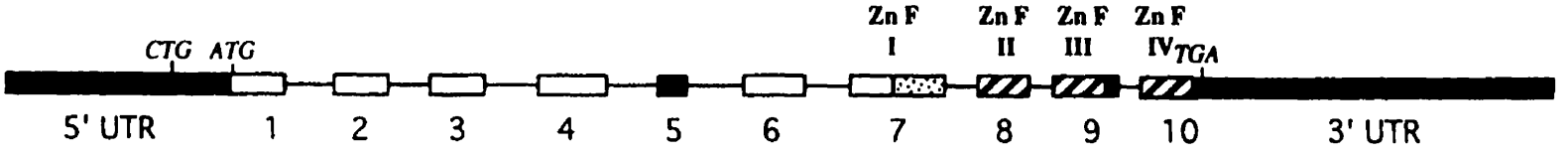


Figure 1: The Wilms' tumor suppressor gene 1 (*wt1*)

The structure of the *wt1* gene. The ten exons are indicated by white boxes; the long 3' and 5' UTRs by gray boxes; the alternatively spliced exons 5 and the KTS between zinc fingers 3 and 4 are indicated by black boxes; the alternatively initiated amino terminal extension is also indicated by a black box; the three characteristic EGR zinc fingers, fingers 2, 3, and 4, are indicated by striped boxes, while the *wt1*-unique zinc finger, finger 1, is indicated by a stippled box. The combination of alternatively spliced exons and alternative initiation allows the gene to produce eight different isoforms.

The *wt1* gene is expressed in the developing gonads, kidneys, lining of the coelomic cavity, and parts of the nervous system. In the adult, WT1 expression is primarily confined to the gonads, uterus, and the decidua of the placenta (Armstrong et al, 1992; Zhou et al, 1993). The *wt1* gene is also expressed in the blastemal component of Wilms' tumors (Pritchard-Jones and Fleming, 1991), in many ovarian tumors (Bruening et al, 1993), and in immature acute leukemias (Miwa et al, 1992).

1.3 WT1 binds to DNA

The zinc finger is a well-characterized and common motif. The classical zinc finger contains the sequence Cys X_{2-4} Cys X_3 Phe X_5 Leu X_2 His X_{3-4} His X_7 and is predicted to recognize a 3 bp sequence of DNA or RNA (for a review, see Pabo and Sauer, 1992). Most zinc finger proteins contain a tandem array of zinc fingers, which are thought to lie in the major groove of DNA and recognize a continuous stretch of nucleotides, 3 bp per finger (see Figure 2) (Pavletich and Pabo, 1991).

Two alternatively spliced isoforms of WT1 (-/- and +/-, where the first symbol denotes the presence or absence of exon 5, and the second symbol indicates the presence or absence of KTS between the third and fourth zinc fingers; see Figure 1) have been shown to be able to bind to the same DNA sequence as EGR-1: GCGGGGGCG (Rauscher et al, 1990). This result is somewhat surprising because a protein with four zinc fingers would be expected to recognize a 12 bp sequence, not a 9 bp sequence (Nardelli et al, 1991). Other groups have identified a $(TCC)_5$ motif to which WT1 can bind (Wang et al, 1993a) as well as a 10 bp sequence GCGTGGGAGT similar to the EGR-1 binding site (Nakagama et al, 1995). The (-/+) and (+/+) WT1 isoforms are able to recognize DNA sequences containing EGR-1 like sites but appear to require additional, as yet undefined, flanking sequences for binding (Wang et al, 1995).

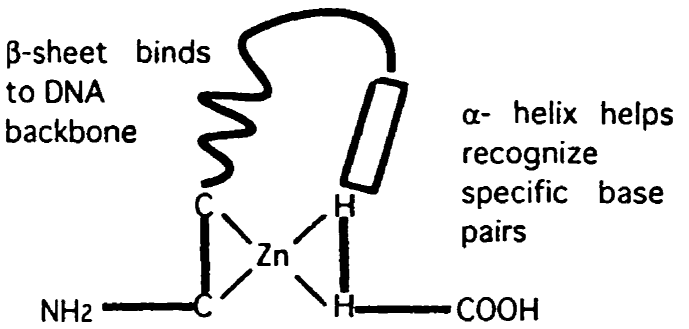


Figure 2. Zinc finger/DNA binding

The zinc finger motif forms a structure that chelates a molecule of zinc between two cystidines and two histidines. Flanking the chelating residues are a β-sheet structure and an alpha helix. When binding to DNA, the α-helices tuck into the major groove of the DNA to contact the base pairs. Most zinc fingers occur in tandem, several fingers wrapping around the DNA helix, each finger contacting 3 bp in sequence.

2. The *wt1* gene: function

2.1 The *wt1* gene regulates transcription

A number of genes have been found to contain WT1 binding sites in their promoters. When tested in co-transfection assays, WT1 is able to repress the transcription of these genes (Table 1). Evidence that any of these genes are really regulated by WT1 during development is scanty at best. Interestingly, most of the proposed downstream targets of WT1 are growth factors, receptors of growth factors, or transcription factors involved in regulation of growth.

Table 1. Genes whose promoters are repressed by WT1

transforming growth factor β1 (TGF-β1)	Dey et al, 1994
retinoic acid receptor α (RAR-α)	Goodyer et al, 1995
insulin-like growth factor II (IGF-II)	Drummond et al, 1992
platelet-derived growth factor A (PDGF-A)	Gashler et al, 1992; Wang et al, 1992
human *wt1*	Malik et al, 1994
insulin-like growth factor I (IGF-I) receptor	Werner et al, 1995
α-inhibin	Hsu et al, 1995
pax-2	Ryan et al, 1995
c-myb	McCann et al, 1995
c-myc	Hewitt et al, 1995
bcl-2	Hewitt et al, 1995
colony stimulating factor 1(CSF1)	Harrington et al, 1993
epidermal growth factor (EGF) receptor	Englert et al, 1995

WT1 has not been found to activate transcription of any native promoters. One group deleted parts of the PDGF-A promoter and

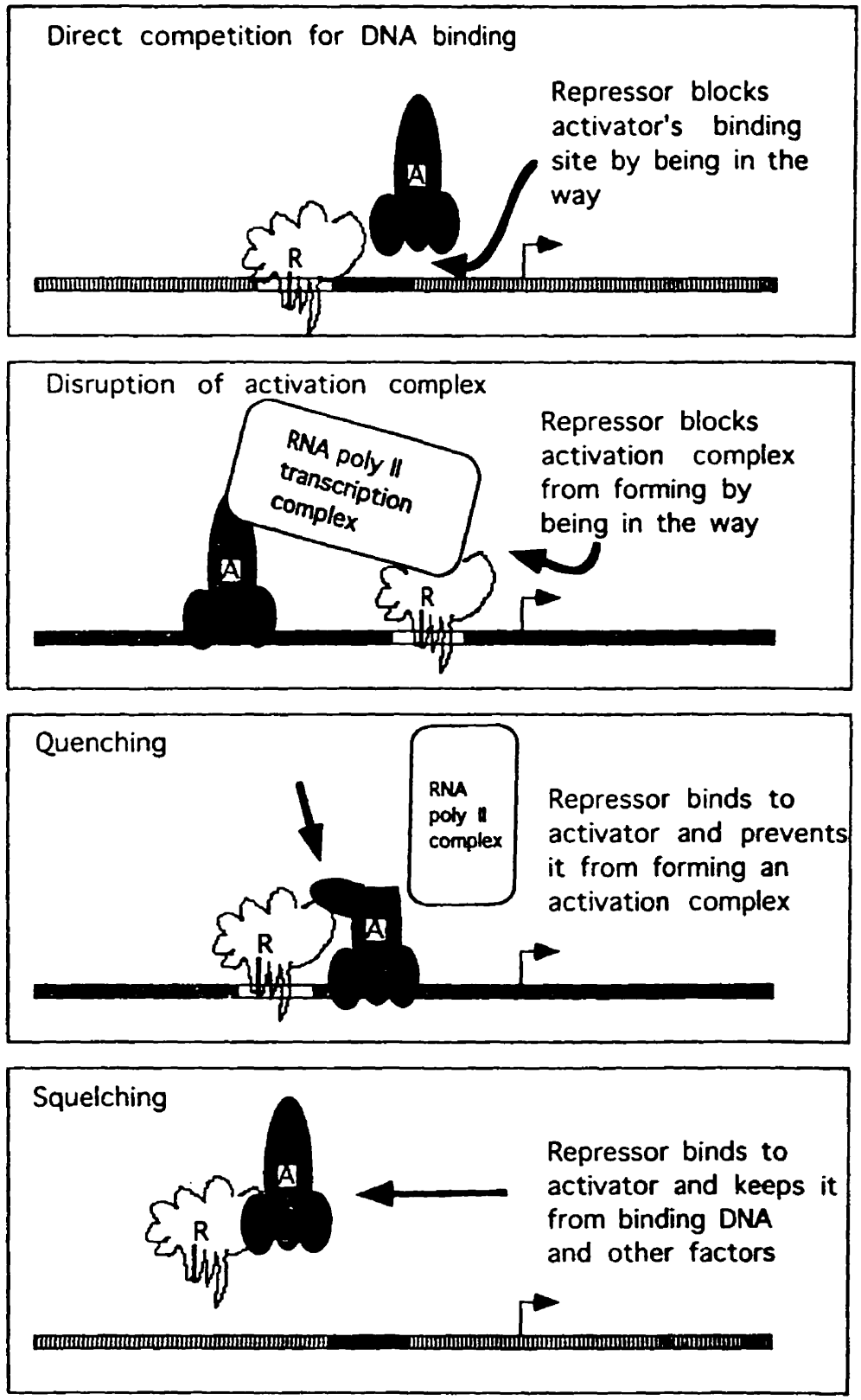
found that WT1 would activate transcription if one binding site was present and repress transcription if two binding sites, one downstream and one upstream of the transcription initiation site, were present (its native state) (Wang et al, 1993b), but this result is applicable only to the PDGF-A promoter. Other promoters, regardless of the placement of WT1 binding sites, are repressed by WT1 (Madden et al, 1991).

Maheswaran et al (1993) reported that a lack of functional p53 converted WT1 into an activator of transcription. This group explained this effect by postulating a direct interaction between p53 and WT1, which they demonstrated by performing co-immunoprecipitations. Maheswaran et al (1995) also reported the curious observation that over-expression of WT1 caused a stabilization of p53. They attributed this effect to WT1's postulated interaction with the p53 protein, but we suspect overexpression of WT1 stresses the cell, causing an induction and stabilization of p53 (see Vogelstein and Kinzler, 1992, for a review of p53).

Repression of transcription is not as well characterized as is activation of transcription. Repression has been proposed to occur by four methods: direct DNA competition, disruption of the transcription complex, quenching, and squelching (see Figure 3) (for a review, see Levine and Manley, 1989). WT1 may repress transcription through several of these mechanisms. Many WT1 binding sites overlap SP1 binding sites, and it is likely that displacement of SP1, a general activator of transcription, would repress transcription (Ackerman et al, 1991; Harrington et al, 1993; Khachigian et al, 1995). Several groups have also proposed that WT1 causes repression by blocking EGR-1 from binding to the same site (Madden et al, 1991; Sukhatme 1992). While this may occur in some cases, as a general mechanism for WT1's repressive ability it has several flaws, primarily by assuming that EGR-1 is always an activator of transcription, which is definitely not correct (Ackerman et al, 1991), and by assuming that EGR-1 is expressed at the same times and locations as WT1, which is also not correct (McMahon et al, 1990; Rackley et al, 1995). The

Figure 3. Methods of transcriptional repression. A number of mechanisms for transcriptional repression by a repressive transcription factor have been described. Here the repressive transcription factor is depicted as white with an "R"; activator transcription factors are depicted as black with an "A"; DNA is indicated as the bar under the transcription factors; binding sites for repressors are white bars; binding sites for activators are black bars; the start site of transcription is indicated with a bent arrow; and the RNA polymerase II complex is shown as a large box. In **direct competition**, the repressor factor physically blocks activators from binding to DNA, either by steric hindrance or by covering up the activator binding site. In **disruption of activation**, the repressor directly blocks the formation of an activation complex, by physically being in the way or by covering up essential DNA sites. In **quenching**, the repressor binds to both DNA and activation factors, and prevents the activation factors from recruiting an activation complex. In **squelching**, the repressor binds to essential factors and titrates them away from their binding sites on the DNA.

Figure 3. Methods of transcriptional repression



amino terminus of WT1 is essential for transcriptional regulation (Wang et al, 1993b), indicating that WT1 may also cause repression by quenching.

2.2 The *wt1* gene can suppress growth

Like all EGR proteins, overexpression of WT1 is able to block cellular division and inhibit transformation (Luo et al, 1995; Kudoh et al, 1995). Although the region of WT1 responsible for this effect has not been identified, it is likely to be mediated by the zinc fingers (Huang et al, 1995). Two groups have reported that different isoforms of WT1 affect growth differently, but their results are not consistent. Menke et al (1996) found that the presence or absence of exon 5 had little effect on WT1's growth suppression, while Kudoh et al (1995) reported that isoforms containing exon 5 suppressed growth to a higher level than did isoforms lacking exon 5. Kudoh et al (1995) found that the presence or absence of the KTS alternatively spliced exon had no effect on WT1's growth suppression, while Menke et al (1996) found that isoforms lacking KTS inhibited tumor growth and isoforms with KTS actually promoted tumor growth. Two other groups reported no isoform-specific differences in growth suppression (Englert et al, 1995; Haber et al, 1993), making it difficult to determine the truth of the matter.

An interesting set of experiments performed by Kudoh et al (1995) has shown that WT1 blocks the cell cycle at the G1 checkpoint. This block was found to be independent of p53 and Rb. If WT1 represses transcription of growth factors and growth factor receptors, one might speculate that WT1 simply prevents cells from responding to mitogenic stimuli, but Kudoh et al (1995) have eliminated this possibility-- they show that WT1-expressing cells can be induced to divide, but are forced to pause before entering S phase due to inactive cyclin/CDK complexes. Overexpression of certain combinations of cyclins and CDKs were able to overcome WT1's blockage of cell division. As shown in Figure 4, the activity of cdk/cyclin complexes is partially regulated by inhibitory proteins, which bind to and inactivate the complexes (Sherr 1994). If WT1 induces expression of such factors, either by direct transcriptional

control or as a downstream effect, cdk/cyclin complexes would be inactivated and the cell cycle would come to a stop. Overexpression of cdk/cyclin complexes would titrate out the inhibitory factors and allow some cdk/cyclin complexes to become active, exactly as is observed. It is, however, important to keep in mind that all of these growth-suppression experiments involve over-expression of WT1, and may be an artifact due to the excess WT1 binding to sites normally bound by EGR-1, 2, or 3, immediate-early responsive genes thought to down-regulate mitogenic responses (Huang et al, 1994; Huang et al, 1995).

3. The *wt1* gene is mutated in several kinds of tumors

3.1 Wilms' tumors

Consistent with *wt1* having a role in regulation of growth is its involvement in tumors. Mutational inactivation of the *wt1* gene is estimated to occur in about 20% of Wilms' tumors, a common childhood malignancy of the kidney (Table 2). However, recent reports suggest that *wt1* alterations may be involved in the majority of Wilms' tumors. One study found that alternative splicing of *wt1* is disrupted in 70% of tumors, causing an excess of the (-/+) and (-/-) isoforms (Simms et al, 1995). The significance of this finding is unclear. Another recent study found that 5% of tumors had small deletions and insertions of di- and trinucleotide repeats within the first exon of *wt1* (Huff et al, 1995), bringing the total to 25% of Wilms' tumors containing mutations of *wt1*, and 70% of Wilms' tumors with splicing abnormalities of *wt1*. It would be interesting to see if these two categories are exclusive or overlapping.

Wilms' tumors are thought to arise from pockets of abnormal, partially differentiated kidney cells called nephrogenic rests (Beckwith 1993). Nephrogenic rests have been found to contain mutationally inactivated *wt1* (Park et al, 1993a), suggesting that inactivation of *wt1* is an early event in Wilms' tumor formation. It also indicates that inactivation of *wt1* does not actively promote tumor growth (most nephrogenic rests are thought to simply regress during childhood); rather, a lack of WT1 prevents kidney cells from

A

cell cycle stage	cyclin/cdk complex	inhibitors
G1	cdk4/cyclin D	p27,p15,p16,p18,p21
G1/S	cdk2/cyclin E	p21,p27
S	cdk2/cyclin A	p21

B

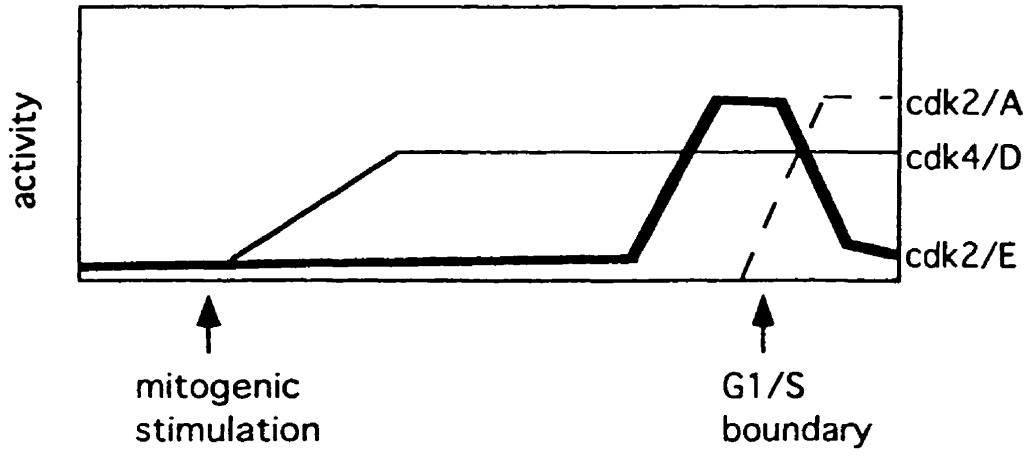


Figure 4. Regulation of the cell cycle

A. A number of proteins, many listed here, have been found to bind to and inactivate cdk/cyclin complexes.

B. Mitogenic stimulation induces the degradation of these inhibitors and allows activation of each cdk/cyclin complex at the appropriate stage in the cell cycle.

fully differentiating, and further mutations are necessary for transformation.

Table 2. Rates and types of mutations of *wt1* in Wilms' tumors.

type of mutation	found in % tumors	study
genomic rearrangement	12% 13%	Kikuchi et al, 1992 Cowell et al, 1991
truncation of all or part of the zinc fingers	6% 5%	Coppes et al, 1993a Varanasi et al, 1994
missense mutations of the zinc fingers	1%	Varanasi et al, 1994
truncation in the amino terminus	1%	Varanasi et al, 1994

3.2 Mesotheliomas, gonadal tumors, and leukemia

Mutations of *wt1* have been found in a few other types of tumors. Tumors derived from the mesothelium, a site of WT1 expression in the fetus, express large amounts of WT1. One mesothelioma was found to contain a homozygous point mutation of *wt1* (Park et al, 1993b), but no large-scale screenings of mesotheliomas for *wt1* abnormalities have been reported, so it is difficult to say if *wt1* is important in these tumors. A juvenile granulosa cell tumor that arose in a Denys-Drash patient was found to have lost the normal *wt1* allele, suggesting that *wt1* may be involved in tumors of gonadal origin (Pelletier et al, 1991a). However, a number of studies have shown that although WT1 is highly expressed in tumors of gonadal origin, mutations of *wt1* have not been found (Bruening et al, 1993; Coppes et al, 1993b; Viel et al, 1994). The *wt1* gene is also highly expressed in acute leukemias, but not in chronic leukemias (Menssen et al, 1995; Miwa et al, 1992).

Mutations of the *wt1* gene are found in 15% of cases of acute myeloid leukemia (King-Underwood et al, 1996). Interestingly, leukemia is a malignancy frequently found in survivors of Wilms' tumor and their relatives, suggesting that a common genetic predisposition underlies both types of cancer (Hartley et al, 1994). The *wt1* gene has also been found to be highly expressed in melanomas, but no mutations have been found (Rodeck et al, 1994).

3.3 Desmoplastic small round cell tumor

The *wt1* gene is modified in the highly aggressive desmoplastic small round cell tumor (DSRCT), a sarcoma that arises in the abdomen, most often in adolescent boys (Gerald et al, 1991). Like many sarcomas, DSRCT is characterized by a specific chromosomal translocation. In the case of DSRCT, this translocation's effect is to fuse the last three zinc fingers of WT1 to part of the EWS gene (for a review, see Ladanyi, 1995). The EWS gene encodes a ubiquitously expressed RNA binding protein of unknown function (Ohno et al, 1994). Fusions of transcription factors and the EWS gene are found in several sarcomas (Ladanyi 1995), and are thought to create powerful, unregulated activators of the genes normally regulated by the transcription factor. Thus, in DSRCT, the EWS/WT1 fusion would be predicted to activate many of the genes normally repressed by WT1.

4. Some isoforms of WT1 are located in the interchromatin particles

4.1 The interchromatin particles

The interchromatin particles are 20-50 structures located throughout the nucleus (for a review, see Spector 1993). These particles are composed of granular clusters connected by fibrils. The interchromatin particles are all interconnected by a latticework of nuclear matrix. Splicing factors, snRNPs, and certain nuclear matrix proteins are all located within interchromatin particles (Nyman et al, 1986; Carcer et al, 1995; Spector et al, 1991), and an arginine/serine rich signal has been found to specifically direct proteins into the

interchromatin particles (Li and Bingham, 1991). Splicing and transcription of RNA polymerase II genes is thought to occur in "transcription domains" located adjacent to but not overlapping interchromatin particles (Xing and Lawrence, 1993). Interchromatin particles are speculated to be sites of spliceosome assembly, active splicing locations, or simply sites of storage (Spector 1993).

4.2 WT1 in the interchromatin particles

A recent report has shown that while the (-/-) and (+/-) isoforms of WT1 are diffuse within the nucleus, the (-/+) and (+/+) isoforms are specifically located within the interchromatin particles (Larsson et al, 1995). As the function of interchromatin particles is not clear, it is difficult to determine the meaning of WT1's presence in them. Mutation or partial deletion of the zinc fingers of WT1 actually increased the proportion of WT1 in the structures, perhaps indicating that WT1 must bind to DNA to remain outside interchromatin particles (Larsson et al, 1995). This theory is consistent with the observation that WT1 isoforms containing the KTS motif, isoforms apparently less able to bind DNA, are found predominantly in the interchromatin particles (Larsson et al, 1995).

Larsson et al (1995) conclude that WT1 must be involved in splicing because it is found in the same place as many splicing factors, but this conclusion is unwarranted. The zinc fingers of WT1 are not involved in localization to the particles, ruling out the possibility that they are binding to certain RNA molecules. The fact that WT1 can be co-immunoprecipitated with splicing factors probably indicates that the entire interchromatin particle structure can be co-immunoprecipitated. Another tumor suppressor protein, the product of the retinoblastoma gene, appears to also be present in the interchromatin particles via direct binding to nuclear matrix proteins (Durfee et al, 1994). The retinoblastoma protein, whose activity is regulated by phosphorylation, is found in the interchromatin particles only when in an active form. Durfee et al (1994) speculate that interchromatin particles are a mechanism for concentrating factors together in a small area to aid interactions; perhaps something of the sort occurs with WT1.

5. The role of WT1 in development

5.1 *wt1*-null mice fail to develop a urogenital system

The expression pattern of *wt1* suggests that it is involved in the development of the urogenital system. Confirmation that this is true was provided when a mouse line was engineered to lack a functional *wt1* gene (Kreidberg et al, 1993). Heterozygous null mice were apparently normal, but homozygotes died before birth in mid-gestation. During normal kidney development, two precursor kidney structures form along the urogenital ridge, the pronephros and the mesonephros. The pronephros degenerates, but an offshoot of it, the Wolffian duct, persists and is important for later development. The mesonephros forms the adult kidney in fishes and amphibians, and may function during embryonic stages in reptiles and birds. The gonads and Mullerian ducts form from offshoots of the mesonephros, which then degenerates. The kidney itself, the metanephros, forms when the uteric bud grows out from the Wolffian duct and induces the surrounding mesenchyme, the metanephric blastemal cells, to differentiate into nephrons (Gilbert 1991).

In the *wt1*-null mice, the pronephros was normal, but the mesonephros was underdeveloped, the uteric bud failed to form, the gonadal ridge degenerated, and the metanephric blastemal cells underwent apoptosis. Further experiments revealed that the mutant metanephric blastemal cells were incapable of responding to artificially supplied induction signals. These *wt1*-null mice indicate that *wt1* is essential for the very earliest stages of kidney and gonad formation.

In addition to renal agenesis, the *wt1*-null mice suffered from abnormalities of the mesothelium, the membrane that lines the abdomenal cavity, a site of high WT1 expression (Armstrong et al, 1992). The lungs and hearts of the mutant mice were abnormal as well, but these defects may be a secondary effect of mesothelial abnormalities (Kreidberg et al, 1993).

5.2 Congenital defects associated with mutations of wt1

Humans with heterozygous mutations of *wt1* suffer from abnormalities of the urogenital system and a predisposition to form Wilms' tumors. Two disease states are recognized: mild abnormalities and the severe disease Denys-Drash syndrome. In the mild disease, the patients frequently develop multiple Wilms' tumors and males suffer from mild genital defects such as hypospadias (penis does not form properly) and cryptorchidism (undescended testicles). Since male genital formation is dependent on hormones released from the gonads but female genital formation is not, the symptoms observed are consistent with a disturbance of gonadal formation; i.e., females appear normal and males have incompletely masculinized genitals, even though in both cases there is presumably an underlying gonadal abnormality (Gilbert 1991). It would be interesting to see if these patients experience puberty normally, and if they are completely fertile, but such data are not available. All other systems in these individuals, including the kidneys, appear to be normal. These children are usually found to have mutations of *wt1* that would eliminate the function of one allele of the gene (Pelletier et al, 1991b; reviewed in Bruening et al, 1995). Thus, a reduction in dosage of WT1 during development in humans has only minor consequences, resulting only in perturbations of gonadal development. In mice, a reduction in dosage of WT1 has no effect at all (Kreidberg et al, 1993), perhaps due to differences in regulation of WT1 expression.

The disease Denys-Drash syndrome (DDS) is a serious congenital syndrome characterized by Wilms' tumors, disruption of gonadal formation, and progressive kidney failure due to glomerulosclerosis (reviewed in Bruening and Pelletier, 1994). These children often develop multiple Wilms' tumors and progress to end-stage renal failure early in life, usually before age 5. Their gonads fail to develop, and as explained above females (XX) appear normal externally but males (XY) either appear to have female genitalia or have ambiguous genitalia. The kidney failure these children experience is due to sclerosis and scarring of the glomeruli, possibly due to aberrant heavy deposition of extracellular matrix (van den

Heuvel et al, 1995). Individuals with DDS have been found to have heterozygous mutations of the *wt1* gene, most often missense mutations of the zinc fingers (Pelletier et al, 1991a; Bruening and Pelletier, 1994). The presence of missense mutations of *wt1* in patients with severe disease and a reduction of dosage of WT1 in patients with mild disease suggests that the mutations found in DDS are acting in a dominant or dominant-negative fashion.

Part II. Rationale and objectives of the presented work

The structure of the WT1 mRNA suggests that the *wt1* gene should encode four polypeptides of about 50 kDa in mass, but this had never been confirmed to be true *in vivo*. In addition, the long (over 350 bp), G/C rich 5' UTR of the *wt1* mRNA suggests that the *wt1* mRNA would be inefficiently translated, leading to lower levels of WT1 protein than would be expected from observations of mRNA levels. Studies undertaken to confirm or reject these predictions are presented in Chapter 2.

The remaining chapters are concerned with clarifying how germline mutations of *wt1* can in some cases cause only mild congenital abnormalities while in other cases the serious Denys-Drash syndrome occurs. Previous work had suggested that Denys-Drash syndrome is the result of dominant or dominant-negative activity of mutated WT1. Which mechanism is at work, how mutated WT1 is able to act in such a way, and why only certain types of *wt1* mutations are able to act in such a way are the questions addressed in Chapters 3, 4, and 5.

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Chapter 2

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A Non-AUG Translational Initiation Event Generates Novel WT1 Isoforms*

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The Wilms' tumor (WT) suppressor gene, *WT1*, is mutated in a small set of WTs and is essential for proper development of the urogenital system. The gene has three sites of transcriptional initiation and produces mRNA transcripts containing 5′-untranslated regions of more than 350 nucleotides. The mRNA, through two alternative splicing events, is predicted to direct the synthesis of four protein isoforms with molecular masses of 47–49 kDa. In this report, we identify and characterize novel WT1 protein isoforms having predicted molecular masses of 54–56 kDa. Mutational analysis of the murine *wt1* mRNA demonstrates that the novel isoforms are the result of translation initiation at a CUG codon 204 bases upstream of and in frame with the initiator AUG. We show that these isoforms are present in both normal murine tissue and in WTs. Like WT1, the larger isoforms localize to the cell nucleus and are capable of mediating transcriptional repression. Our results indicate that regulation of *WT1* gene expression is more complex than previously suspected and have important implications for normal and abnormal urogenital system development.

Wilms' tumor (WT)[1] is a embryonal malignancy of the kidney that afflicts one out of every 10,000 children (Matsunaga, 1981). Approximately 7–15% of sporadic WTs contain detectable mutations in the tumor suppressor gene *WT1* (Coppes *et al.*, 1993; Little *et al.*, 1992; Varanasi *et al.*, 1994). The *WT1* gene encodes a transcription factor belonging to the early growth response family of Cys_2-His_2 zinc finger proteins. The pre-mRNA is alternatively spliced at two exons to produce four WT1 isoforms (Haber *et al.*, 1991). The first alternatively spliced exon inserts or removes 17 residues upstream of the four zinc fingers and is capable of mediating transcriptional repression when fused to a DNA binding domain (Wang *et al.*, 1993a). The second alternatively spliced exon inserts or removes 3 amino acids, KTS, between zinc fingers III and IV and alters the DNA binding specificity of the protein, as well as its subnuclear localization (Drummond *et al.*, 1994; Larrson *et al.*, 1995; Rauscher *et al.*, 1990). The −KTS WT1 isoforms recognize a GC-rich motif (5′-GCGGGGGCG-3′), as well as a $(TCC)_n$ repeat (Rauscher *et al.*, 1990; Wang *et al.*, 1993b), and can affect expression of a number of genes harboring these motifs in their regulatory regions (for a review see Rauscher (1994)). These genes include insulin-like growth factor II (Drummond *et al.*, 1992), insulin-like growth factor 1 receptor (Werner *et al.*, 1993), platelet-derived growth factor A-chain (Gashler *et al.*, 1992; Wang *et al.*, 1992), colony-stimulating factor-1 gene (Harrington *et al.*, 1993), transforming growth factor-β1 (Dey *et al.*, 1994), the retinoic acid receptor-α (Goodyer *et al.*, 1995), and the *wt1* gene itself (Rupprecht *et al.*, 1994). WT1 can mediate both transcriptional repression and activation, depending on the architecture of the promoter under study (Madden *et al.*, 1991; Drummond *et al.*, 1992, 1994; Wang *et al.*, 1992). Activation and repression are mediated by distinct domains within the WT1 protein (Wang *et al.*, 1993a).

In addition to its role as a tumor suppressor gene, *WT1* plays an essential role in the normal development of the urogenital system. The expression pattern of *WT1* is not ubiquitous, being mainly restricted to components of the urogenital system: the gonads, developing glomeruli, and the uterus (Pelletier *et al.*, 1991a). Many children with germline *WT1* mutations suffer from malformations of the urogenital system, ranging in severity from minor genital anomalies to streak gonads and renal nephropathy (for a review see Bruening and Pelletier (1994)). Consistent with a role for *WT1* in the development of the urogenital system is the observation that this system fails to differentiate in *wt1*-null mice (Kreidberg *et al.*, 1993).

Similar to many other genes involved in growth regulation, the *wt1* mRNA transcript contains an AUG-initiated open reading frame (ORF) preceded by a long, GC-rich, 5′-untranslated region (UTR) (Nagpal *et al.*, 1992). There are three sites of transcription initiation within the murine *wt1* promoter, producing mRNA species with 5′-UTRs of 375, 700, or 720 nucleotides. In the course of characterizing the protein isoforms produced from these transcripts, we noted the presence of isoforms having molecular masses greater than expected. In this report, we demonstrate that these isoforms arise from translation initiation at a CUG codon upstream of and in frame with the *wt1* initiator AUG. These novel isoforms are present in normal and malignant tissue and are capable of repressing transcription.

Although AUG codons are essentially exclusively used as initiation codons for eukaryotic mRNAs, there are rare examples of cellular mRNAs where other codons (GUG, ACG, and CUG) are also used for this purpose. These include proto-oncogenes (*MYC*, *INT*-2, *PIM*-1, and *LYL*-1) (Acland *et al.*, 1990; Hann *et al.*, 1988; Saris *et al.*, 1991; Mellentin *et al.*, 1989), as well as the basic fibroblast growth factor gene (Prats *et al.*, 1989), retinoic acid receptor β4 (Nagpal *et al.*, 1992), *krox-24* (also a member of the early growth response family) (Lemaire *et al.*, 1990), and the *ltk* receptor (Bernards and de la Monte, 1990). The nature of the signals that dictate the use of a CUG codon as an initiation codon within the 5′-UTR are not well understood, but immediate downstream sequences can influence the efficiency with which CUG codons are selected (Boeck and Kolakofsky, 1994; Grünert and Jackson, 1994). Our

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[1] The abbreviations used are: WT, Wilms' tumor; ORF, open reading frame; UTR, untranslated region; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

results show that *WT1* gene expression is more complex than previously suspected and that the activity of these novel WT1 isoforms needs to be considered in biological assays involving WT1.

EXPERIMENTAL PROCEDURES

Cell Lines—TM3 and COS-1 cells were obtained from the American Type Culture Collection (ATCC). TM3 cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with 5% horse serum and 2.5% fetal calf serum. COS-1 cells were maintained in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal calf serum.

Northern Blots—Total RNA was isolated from mouse testis and TM3 cells by the $LiCl_2$/urea procedure (Auffray and Rougeon, 1980). 10 μg of RNA was electrophoresed into a 1.2% agarose/37% formaldehyde gel and blotted to nitrocellulose (Schleicher & Schuell). Filters were probed with a ^{32}P-labeled murine *wt1* cDNA as described previously (Pelletier *et al.*, 1991a).

Immunoprecipitations and Western Blots—Crude nuclear extracts were prepared by resuspending washed cells into an equal packed cell volume of 10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% Nonidet P-40, 0.5 mM dithiothreitol, 0.5 μg/ml aprotinin, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride. Following a 10-min incubation on ice, the suspension was passed ten times through a 23 G needle. The nuclei were pelleted and resuspended in three times the packed cell volume of RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate). For Western blots, 30 μg of the nuclear extract was fractionated on a 10% SDS-PAGE gel. Following transfer to Immobilon-P (Millipore), the membranes were probed with the indicated antibodies, and proteins were visualized by chemiluminescence using an ECL kit (Amersham Corp.).

For immunoprecipitations, cells were grown to 80% confluency, washed in PBS, and preincubated in methionine-free medium for 1 h. Cellular protein was labeled with 200 μCi/ml of [^{35}S]methionine for 12 h. Following a wash with PBS, the cells were lysed in RIPA buffer. For each immunoprecipitation, approximately 10^9 cpm of lysate was precleared with protein A-Sepharose (Pharmacia Biotech Inc.) and preimmune serum for 1 h at 4 °C. Incubation with specific antibody and protein A-Sepharose was then performed for 4 h at 4 °C, after which time the immunoprecipitate was washed three times with RIPA buffer and once with PBS. Elution was performed in SDS-PAGE sample buffer. The eluted proteins were fractionated on a 10% SDS-PAGE gel, treated with EN^{3}HANCE (New England Nuclear), and detected by exposure to X-Omat film (Kodak).

Antibodies—The anti-WT1 polyclonal antibody C-19 was purchased from Santa Cruz and was generated against a peptide (LVRHHNMHQRNMTKLQLAL) at the carboxyl terminus of the WT1 protein. The anti-WT1 monoclonal antibodies 13B5 and 8A7 were raised against peptides designed to mimic WT1 isoforms containing or lacking the first alternatively spliced exon. Their characterization has been previously discussed (Mundlos *et al.*, 1993). The rabbit anti-Wt1 polyclonal antibody 605 was raised against a GST-Wt1 fusion protein containing exons 2–9 of the murine *wt1* gene. The rabbit polyclonal antibody S3, which recognizes only the CUG-initiated WT1 isoforms, was raised against the multiple antigenic peptide (Posnett *et al.*, 1988) PASTCVPEPASQHTLR, located within the unique amino-terminal region produced by the CUG-initiated WT1 isoforms, and present in both mouse and human sequences (Fig. 2). (WT1 isoforms containing or lacking the first alternative splice site will be referred to as + or − 17 aa in the text.)

Plasmid Constructs—Restriction enzymes and other DNA-modifying enzymes were purchased from New England Biolabs. CMV-*wt1* and pSP65 constructs lacking the 5′-UTR have been previously described (Goodyer *et al.*, 1995; Pelletier *et al.*, 1991b). To construct SP65-*wt1* plasmids containing various 5′-UTRs, clones isolated from a mouse cDNA library that terminated at various regions within the 5′-UTR were used. The 5′ ends of these clones were excised from pKSII+ with *Eco*RI (which cleaves at the 5′ end of the cDNA) and *Nco*I (which cleaves at position 541 downstream of the initiator AUG) and subcloned into the same sites of SP65-*wt1*. To generate point mutations within the 5′-UTR, site-directed mutagenesis was performed using the polymerase chain reaction and overlapping oligonucleotides harboring the desired change (Pelletier *et al.*, 1991b).

In Vitro Transcriptions and Translations—RNA polymerases were purchased from Promega. For *in vitro* transcriptions, plasmids were linearized with the indicated enzymes, and transcriptions were performed as described previously (Pelletier *et al.*, 1991b). RNA transcripts were quantitated by monitoring incorporation of [^{3}H]CTP (New England Nuclear). *In vitro* translations were performed in rabbit reticulocyte lysate using [^{35}S]methionine as directed by the manufacturer (Promega).

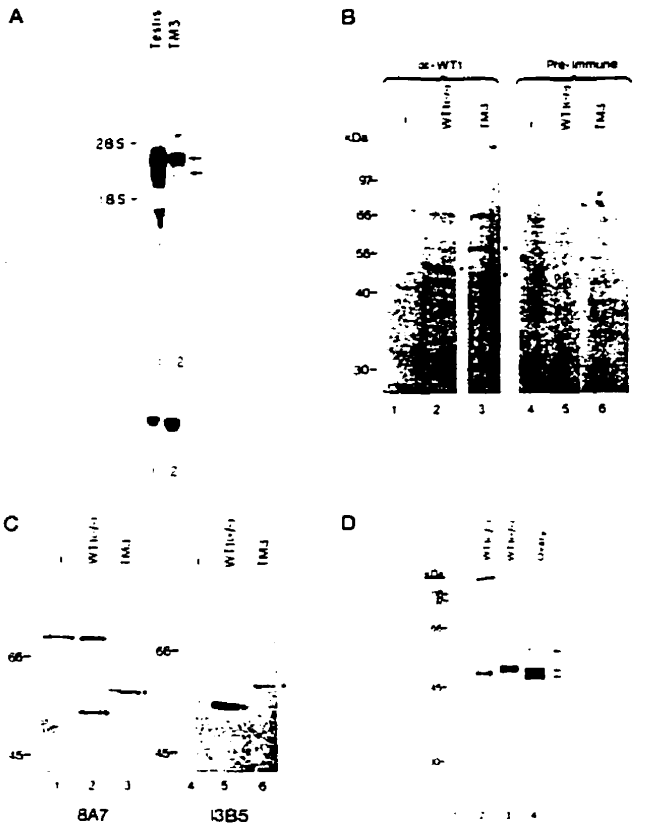

FIG. 1. **Detection of a novel Wt1 isoform *in vivo*.** *A*, Northern blot analysis of RNA isolated from 3-day-old murine testis (*lane 1*) and TM3 cells (*lane 2*). The *arrows* indicate the positions of migration of the *wt1* mRNA species. The position of migration of the 28 S and 18 S rRNA species is indicated to the *right*. The *upper panel* has been hybridized with a murine *wt1* cDNA fragment. The *bottom panel* is the same blot reprobed with ^{32}P-labeled β-actin. *B*, immunoprecipitation from TM3 cells using the polyclonal anti-Wt1 605 antibody. Immunoprecipitations performed on [^{35}S]methionine-labeled cell extracts were resolved on a 10% SDS-PAGE gel. The 47–49-kDa Wt1 isoforms are indicated by a *dot* and are not well resolved on this gel. The ~57-kDa related protein is denoted by a ×. Extracts were prepared from untransfected COS-1 cells (*lanes 1* and *4*), COS-1 cells transfected with CMV/*wt1*(−/−) (*lanes 2* and *5*), or TM3 cells (*lanes 3* and *6*). Samples were immunoprecipitated with the antibody 605 (*lanes 1–3*) or with preimmune serum (*lanes 4–6*). Following electrophoresis, the gel was treated with EN^{3}Hance (New England Nuclear), dried, and exposed to X-Omat (Kodak) film at −70 °C for 2 days. *C*, Western blot analysis of nuclear extracts prepared from COS-1 and TM3 cells. Blots were probed with monoclonal antibodies 8A7 (*lanes 1–3*) and 13B5 (*lanes 4–6*), which recognize WT1 isoforms lacking or containing the first alternatively spliced exon, respectively. Extracts were prepared from untransfected COS-1 cells (*lanes 1* and *4*), COS-1 cells transfected with CMV/*wt1*(−/−) (*lane 2*), CMV/*wt1*(+/−) (*lane 5*), or TM3 cells (*lanes 3* and *6*). The *dot* indicates the position of migration of the 47–49-kDa Wt1 isoforms; whereas the × delineates the position of the ~57-kDa isoforms. Blots were exposed to X-Omat film (Kodak) for 2 min. *D*, expression of Wt1$^-$ in murine ovaries. Extracts were prepared from untransfected COS-1 cells (*lane 1*), COS-1 cells transfected with CMV/*wt1*(−/−) (*lane 2*), COS-1 cells transfected with CMV/*wt1*(+/−) (*lane 3*), and adult murine ovaries (*lane 4*). The *arrows* indicate the positions of migration of the Wt1 isoforms. The blot was probed with the antibody C-19 and exposed to X-Omat (Kodak) film for 5 min.

RESULTS

The wt1 Gene Encodes Novel 54–56 kDa Isoforms—To gain better insight into the regulation of *wt1* gene expression, we screened a number of mouse cell lines for expression of *wt1* mRNA. Of the lines tested, one of these, TM3 (derived from

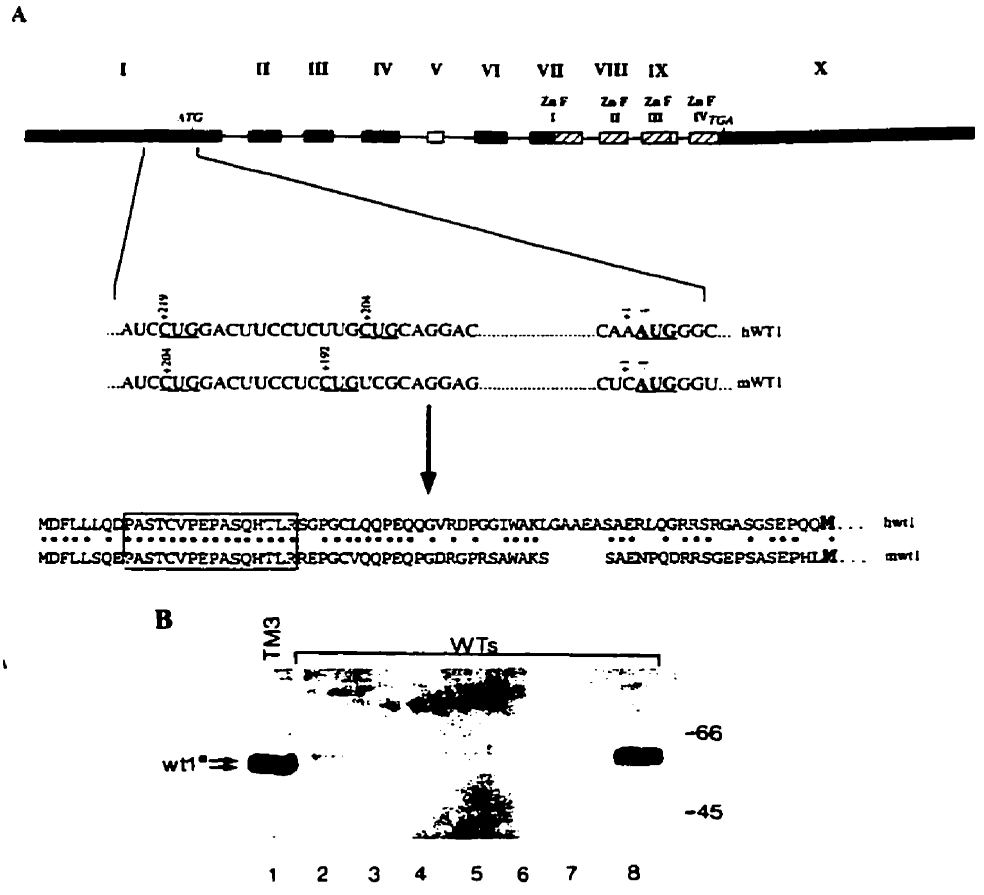

FIG. 2. **Detection of Wt1⁺ in sporadic WTs.** *A*, schematic diagram of the genomic organization of the human *WT1* gene. The nucleotide sequence and predicted polypeptide sequence of both the human and murine genes upstream of the AUG codon is indicated. The *white boxes* represent alternatively spliced exons, whereas *hatched boxes* represent the zinc fingers. Each exon is numbered with a roman numeral. The relative position of two CUG codons present upstream from and in frame with the initiator AUG codon in the human and murine 5′-UTRs is shown. The putative CUG initiation codons are *underlined*, whereas the initiator AUG codon is *underlined* and in *bold*. The peptide sequence used to generated the polyclonal serum S3 is *boxed*. Amino acid homology of the predicted amino-terminal extension from the human and murine cDNAs is presented. *Black circles* signify identity, whereas *open circles* signify conservative changes. The methionine encoded by the initiator AUG is denoted in *bold*. *hWT1*, human *WT1* cDNA sequence; *mWT1*, murine *wt1* cDNA sequence; *hwt1*, human WT1 polypeptide sequence; *mwt1*, murine Wt1 polypeptide sequence. *B*, analysis of WT1⁺ in WTs. A Western blot of nuclear extracts from murine TM3 cells (*lane 1*) or whole cell extracts from WTs (*lanes 2–8*) were probed with anti-WT1⁺ serum, S3. The WT1⁺ isoforms are indicated by two *arrows*. Molecular mass markers (in kDa) are indicated to the *right* of the panel.

mouse testis), revealed significant levels of *wt1* mRNA by Northern blot analysis (Fig. 1*A*, *lane 2*). In addition to the expected 3.1-kilobase *wt1* mRNA, a second mRNA species of ~2.5 kilobases is present in testis (Fig. 1*A*, *lane 1*) and in TM3 cells (*lane 2*). The nature of the 2.5-kilobase cross-hybridizing mRNA species is not well defined but has been previously noted to be testis-specific and may arise from the use of an alternative polyadenylation site within the *wt1* 3′-UTR (Pelletier *et al.*, 1991a).

To detect Wt1 protein from TM3 cells, we made use of the polyclonal anti-Wt1 antibody, 605. This antibody, directed against amino acids 123–299, is capable of immunoprecipitating [^{35}S]methionine-labeled Wt1 from COS-1 cells transfected with a CMV-*wt1* expression vector (denoted by a *dot* in Fig. 1*B*, *lane 2*). This polypeptide is not recognized by preimmune serum (Fig. 1*B*, *lane 5*), nor is it immunoprecipitated from untransfected COS-1 cells (Fig. 1*B*, *lane 1*). In immunoprecipitates from TM3 cells with the 605 antibody, we noted the presence of Wt1 protein (*lane 3*, denoted by a *dot*) as well as a polypeptide of ~57 kDa (indicated by a × in Fig. 1*B*, *lane 3*). Like Wt1, this polypeptide is not visible in immunoprecipitates of TM3 cells with preimmune serum (Fig. 1*B*, *lane 6*).

To extend these results and determine whether the ~57-kDa polypeptide species was related to Wt1, we made use of two anti-WT1 monoclonal antibodies (Mundlos *et al.*, 1993). Antibody 8A7 specifically recognizes WT1 isoforms lacking the 17 amino acids introduced by alternative splicing of exon 5 (−17 aa), whereas 13B5 specifically recognizes WT1 isoforms containing this alternatively spliced exon (+17 aa) (Mundlos *et al.*, 1993). On Western blots of COS-1 cells transfected with CMV/*wt1*(−/−), 8A7 recognizes the Wt1(−/−) protein isoform as expected (denoted by a *dot*, Fig. 1*C*, *lane 2*). This polypeptide is not present in untransfected COS-1 cells (Fig. 1*C*, *lane 1*). In TM3 cells, a ~57-kDa polypeptide is also recognized by this antibody (indicated by a × in Fig. 1*C*, *lane 3*). Antibody 13B5 recognizes the Wt1(+/−) isoform produced in COS-1 cells transfected with CMV/*wt1*(+/−) (indicated by a dot in Fig. 1*C*, *lane 5*). In TM3 cells, a ~57-kDa polypeptide is also detected with this antibody (indicated by a × in Fig. 1*C*, *lane 6*). Upon prolonged exposure of these blots, the 47–49-kDa *wt1* isoforms can also be detected in extracts of TM3 cells (data not shown). These results strongly suggest the existence of novel Wt1 isoforms containing or lacking the Wt1 exon V and having molecular masses ~10 kDa higher than Wt1. In this manuscript, we will refer to these larger isoforms as Wt1⁺ to distinguish them from the 47–49-kDa Wt1 isoforms.

To determine whether Wt1⁺ could be detected in normal tissues, nuclear extracts were prepared from murine ovaries

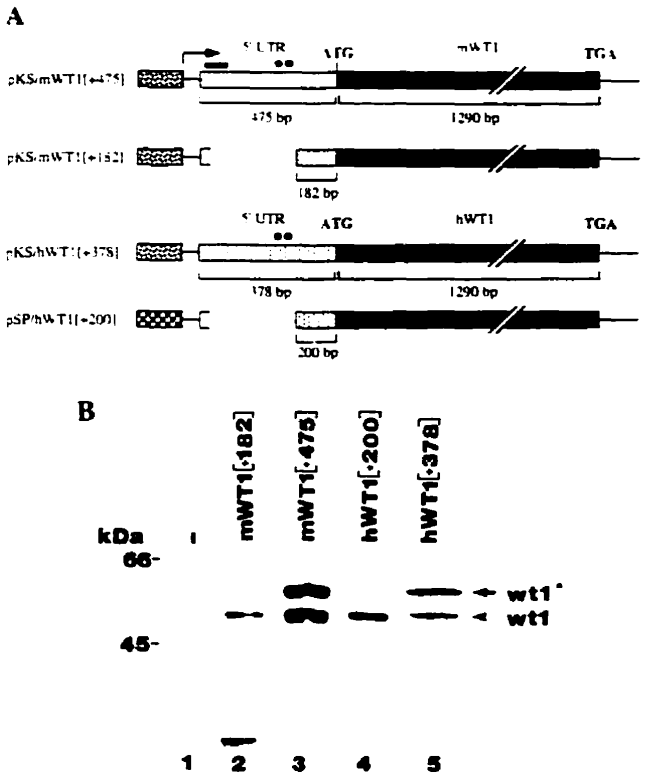

FIG. 3. **In vitro synthesis of Wt1$^+$.** *A*, diagram of *in vitro wt1* expression vectors. The *black boxes* represent the coding regions, whereas the *stippled* and *hatched boxes* represent the murine and human 5'-UTRs, respectively. The *dotted* and *checkered boxes* represent the T7 and SP6 RNA polymerase promoters. The *small black rectangle* above the murine 5'-UTR represents a small ORF present in this portion of the cDNA. The two *black dots* represent the upstream CUG codons highlighted in Fig. 2*A*. *B*, *in vitro* translation products obtained from RNA generated by 5'-UTR deletion mutants. RNA was generated from constructs shown in *A* and translated in rabbit reticulocyte lysate at a mRNA concentration of 16 μg/ml. RNA prepared from the following vectors was used as input template: *lane 1*, no DNA; *lane 2*, pKS/m*wt1*[−182]; *lane 3*, pKS/m*wt1*[+475]; *lane 4*, pSP/h*WT1*[+200]; *lane 5*, pKS/h*WT1*[+378]. 3 μl of the translation reaction were fractionated on a 10% SDS-PAGE gel. Following electrophoresis, the gel was treated with EN^{3}Hance, dried, and exposed to Fuji x-ray film at −70 °C for 12 h.

and probed with the anti-WT1 antibody, C-19, directed against the WT1 carboxyl terminus. Extracts prepared from COS-1 cells transfected with CMV/*wt1*(−/−) (Fig. 1*D*, *lane 2*) or CMV/*wt1*(−/+) (Fig. 1*D*, *lane 3*) produced the appropriate *wt1* isoforms and acted as standards on this blot. No Wt1 protein was present in untransfected COS-1 cells (Fig. 1*D*, *lane 1*). Western blot analysis of nuclear extracts from murine ovaries revealed the presence of the 47–49-kDa Wt1 isoforms, as well as the Wt1$^+$ isoforms (Fig. 1*D*, *lane 4*). A similar result was obtained with nuclear extracts from murine testis (data not shown). These results indicate that Wt1$^+$ is present in normal murine tissue.

Expression of WT1$^+$ in WTs—We felt it unlikely that WT1$^+$ was being generated from an alternatively spliced transcript because the structure of this mRNA has been extensively analyzed by S1 nuclease (Pelletier *et al.*, 1991a) and RNase protection (Haber *et al.*, 1991). We considered the possibility that an alternative translation initiation event was responsible for producing the novel WT1 polypeptides because examination of the murine and human nucleotide sequences upstream of the initiator AUG revealed the presence of two CUG codons ~200 nucleotides upstream from and in frame with the initiator AUG codon (Fig. 2*A*). These CUG codons are conserved between human and mouse cDNAs, although their positions relative to the initiator AUG differ slightly. Because CUG codons are used as translation initiation codons by some eukaryotic mRNAs (see Introduction), we investigated the possibility that WT1$^+$ was generated by alternative translational initiation. We generated a polyclonal antibody against a peptide representing amino acids 9–24 of the predicted amino-terminal extension (*boxed* in Fig. 2*A*). This antibody, called S3, was used to screen a series of WTs for expression of WT1$^+$. Two Wt1$^+$ isoforms (−/− 17 aa) are detected by S3 in murine TM3 cells (Fig. 2*B*, *lane 1*). Two of the tumors examined also showed expression of WT1$^+$ (Fig. 2*B*, *lanes 2* and *8*). In our small sample cohort, there was a direct correlation between the presence of WT1$^+$ and the presence of WT1 protein (data not shown). We hypothesize that WT1$^+$ arises from alternative translation initiation on the *WT1* mRNA template and contains a unique amino-terminal extension not present in WT1.

WT1$^+$ Initiates at a CUG Codon Upstream from and in Frame with the Initiator AUG Codon—To directly demonstrate that the amino-terminal extension of WT1$^+$ is due to alternative translational initiation, a series of deletion mutants was generated from the murine and human cDNAS (Fig. 3*A*). Two of the constructs (pKS/m*wt1*[+182] and pSP/h*WT1*[+200]) contain shortened 5'-UTRs that lack the above described CUG codons, whereas others (pKS/m*wt1*[+475] and pKS/h*WT1*[+378]) had 5'-UTRs that included these CUG codons (Fig. 3*A*). Following *in vitro* transcription and translation, [^{35}S]methionine-labeled protein products were analyzed by SDS-PAGE. In the absence of exogenously added mRNA, no protein product is detectable (Fig. 3*B*, *lane 1*). The truncation mutants, pKS/m*wt1*[+182] and pSP/h*WT1*[−200], produced only one polypeptide species having a molecular mass of ~50 kDa (Fig. 3*B*, *lanes 2* and *4*, indicated by an *arrowhead*). These results are consistent with translational initiation occurring at the predicted AUG codon (*underlined* and *bold* in Fig. 2*A*). Translation of mRNA produced from pKS/m*wt1*[+475] and pKS/h*WT1*[+378] produced two polypeptide species, one having the expected molecular mass for an initiation event occurring at the initiator AUG, and one having a molecular mass identical to that of WT1$^+$ (Fig. 3*B*, *lanes 3* and *5*). Similar results were obtained upon translation of these mRNAs in a wheat germ extract, indicating that the above results are not specific to reticulocyte lysate (data not shown). These results are consistent with our hypothesis that alternative translational initiation is responsible for generating the Wt1$^+$ protein isoforms and demonstrate that both the human and murine mRNAs are competent for producing this protein species.

To define the codon responsible for directing translation initiation of Wt1$^+$, a series of deletion and site-directed mutants were generated within the murine 5'-UTR (Fig. 4*A*). In addition to truncating various portions of the 5'-UTR, stop codons were introduced that flanked the CUG codons identified above (Fig. 2*A*), and mutations affecting the nature of each CUG codon were generated. Deletions within the first 507 nucleotides from the 5'-UTR of the *wt1* mRNA did not abolish production of Wt1$^+$ (Fig. 4*B*, *lanes 2–5*), whereas a deletion removing all but 182 nucleotides of 5'-UTR generated a transcript unable to produce Wt1$^+$ (Fig. 4*B*, *lane 11*). Deletion of the Wt1 initiator AUG codon abolished production of Wt1, instead generating a polypeptide having a molecular mass of ~37 kDa (Fig. 4*B*, *lane 12*, indicated by a *small arrow*). The molecular mass of this polypeptide is similar to that expected from internal initiation of ribosomes at a downstream, in frame AUG codon at position 378 (relative to the A of the initiator AUG codon). Introduction of a UGA codon 229 nucleotides upstream from and in frame with the AUG initiator codon did not affect

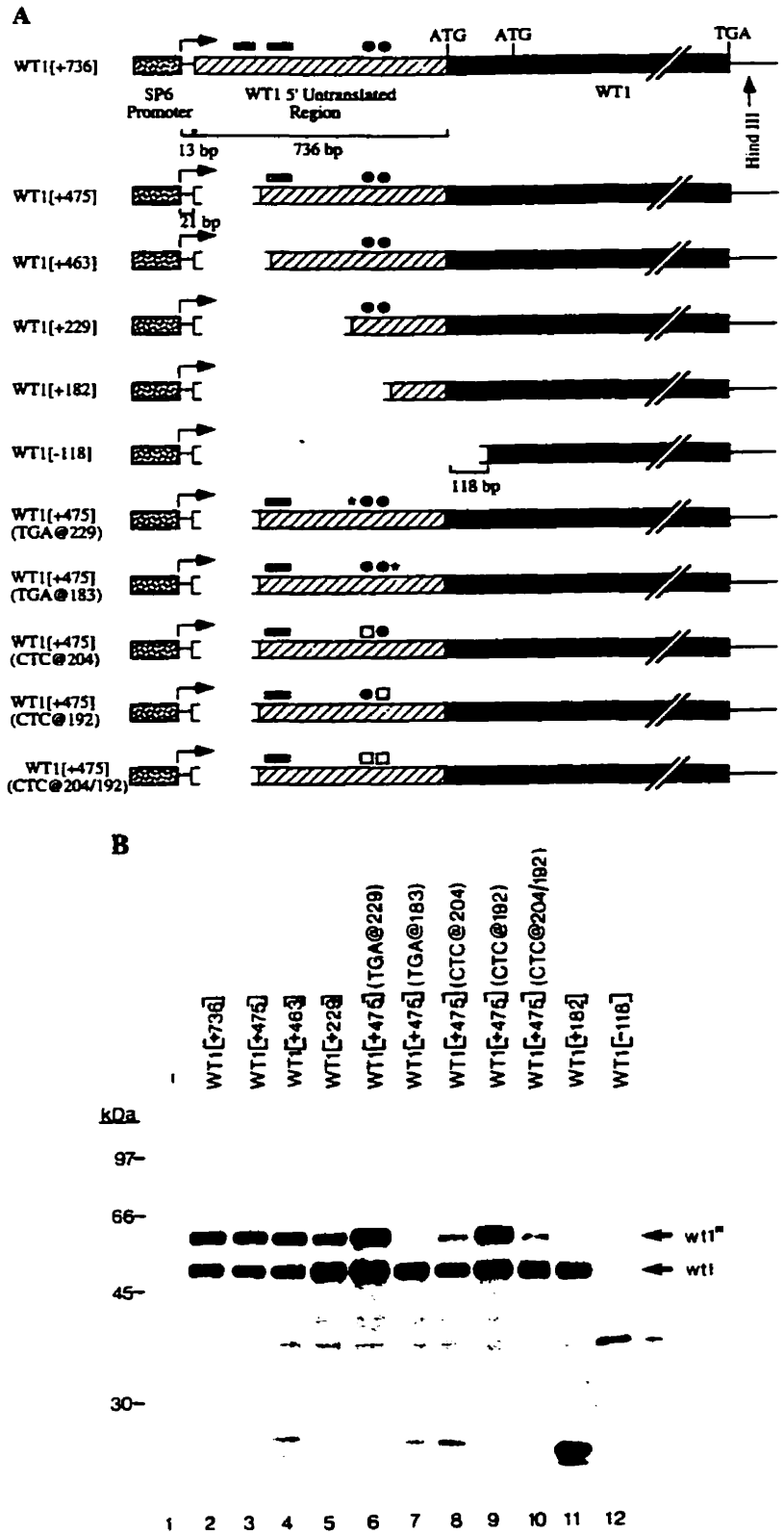

FIG. 4. **Effect of *wt1* 5'-UTR deletion and point mutations on translation initiation.** *A*, diagram of deletion and point mutations generated within the *wt1* 5'-UTR. The *black boxes* indicate the ORFs, the *hatched boxes* represent the 5'-UTRs, and the *stippled boxes* represent the SP6 RNA polymerase promoters. The two upstream CUGs at nucleotides 204 and 192 are represented by *filled circles*, and the *black rectangular boxes* above the 5'-UTRs represent short ORFs. The position of the initiator AUG and stop codons are shown, as well as the position of a downstream, in frame AUG codon. *Stars* represent UGA stop codons, whereas *small open boxes* represent CUG to CUC mutations. *B*, *in vitro* translation of RNA produced from deletion and point mutations within the 5'-UTR. Indicated above each lane is the SP6 construct from which the input RNA was derived. A *dash* represents no input RNA. The position of migration of Wt1 and Wt1* are indicated. The *smaller arrow* indicates the position of migration of the truncated Wt1 polypeptide predicted to arise from translation initiation at the downstream AUG codon. Following electrophoresis in a 10% SDS/polyacrylamide gel, the gel was treated with EN^{3}Hance, dried, and exposed to X-Omat (Kodak) film at −70 °C with an intensifying screen overnight.

production of Wt1* (Fig. 4*B*, *lane 6*), indicating that the Wt1* initiation codon must lie downstream of this site. On the other hand, placing a UGA codon at position 183, in frame, and upstream of the initiator codon generated an mRNA no longer capable of synthesizing Wt1* (Fig. 4*B*, *lane 7*). These data suggest that the signals responsible for Wt1* translation initiation lie between nucleotides +183 and +229. Site-directed mutagenesis was used to abolish the individual CUG codons at position 204 and 192 (see Fig. 2*A*). Converting CUG^{204} to CUC^{204} drastically affected expression of Wt1* while having little effect on production of Wt1 (Fig. 4*B*, *lane 8*). Expression of Wt1* was reduced ~10-fold but not completely abolished. Mutagenesis of CUG^{192} to CUC^{192} had no effect on production of Wt1* (Fig. 4*B*, *lane 9*), and mutagenesis of both CUG codons

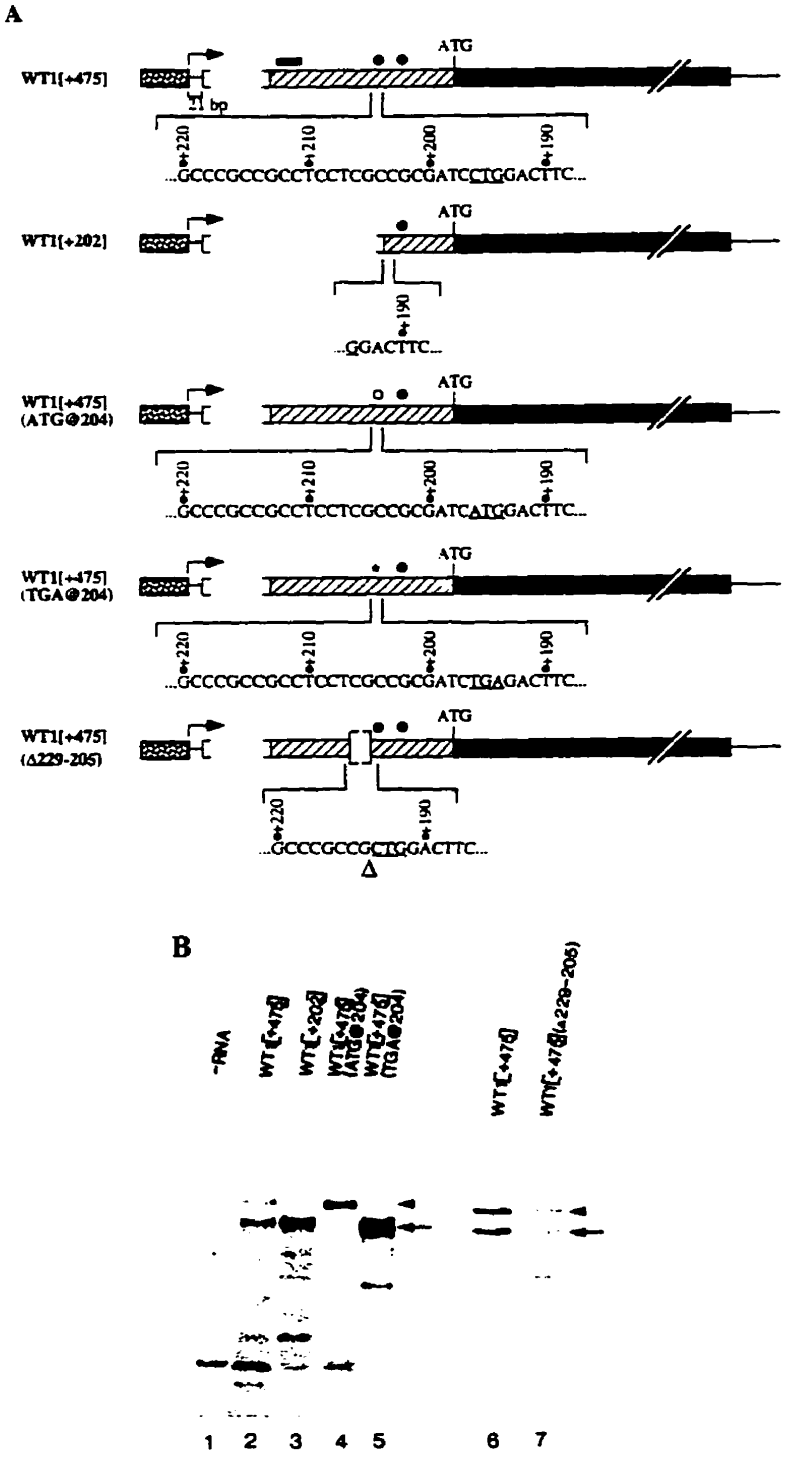

FIG. 5. **Translation analysis of mutations at or upstream from CUG^{204}.** *A*, schematic diagram of mutations generated within the *wt1* 5'-UTR. The *black boxes* indicate the *wt1* ORFs, the *hatched boxes* represent the 5'-UTRs, and the *stippled boxes* represent the SP6 RNA polymerase promoter. The two upstream CUGs at nucleotides 204 and 192 are represented by *filled circles*, and the *filled rectangular boxes* above the 5'-UTRs represent short ORFs. The nucleotide sequence flanking CUG^{204} is shown for each construct. The triplet at position 204 is *underlined*. The *open triangle* signifies a deletion. A *star* represents a stop codon, and an *open circle* represents an AUG codon. *B*, *in vitro* translation of RNA with mutations at or upstream from CUG^{204}. Indicated above each lane is the SP6 construct from which the input RNA was derived. A *dash* represents no input RNA. The position of migration of Wt1 and Wt1* are indicated by an *arrow* and an *arrowhead*, respectively. Translation products were analyzed as described in the legend to Figs. 3 and 4.

had the same effect as altering only CUG^{204} (Fig. 4*B*, *lane 10*). These results strongly indicate that translational initiation at CUG^{204} is responsible for production of Wt1*. We interpret the residual production of Wt1* observed with *wt1*[+475](CTC@204) and *wt1*[+475](CTC@204/192) to indicate that the CUG to CUC mutation is leaky and that nucleotide sequences flanking the CUG codon are influencing translation initiation at this site (Boeck and Kolakofsky, 1994; Grünert and Jackson, 1994).

It is clear from the translation results obtained with *wt1*[+475](TGA@229) and *wt1*[+475](TGA@183) (Fig. 4*B*) that the Wt1* translation codon must lie between nucleotides +183 and +229. To rule out the possibility that the initiator codon for Wt1* was upstream or downstream of CUG^{204} and that the effects on translation initiation of mutating CUG^{204} were not the result of altering a motif that indirectly influenced the efficiency of initiation, four additional constructs were generated (Fig. 5*A*). One of these, *wt1*[+202], retains 202 base pairs of 5'-UTR and terminates at the G residue of CUG^{204}. Should translation of Wt1* commence downstream of CUG^{204}, then this construct should generate RNA competent for Wt1* production. Two other mutants alter the identity of CUG^{204}, converting it either to the more efficient AUG initiation codon, *wt1*[+475](ATG@204), or to a stop codon, *wt1*[+475](TGA@204).

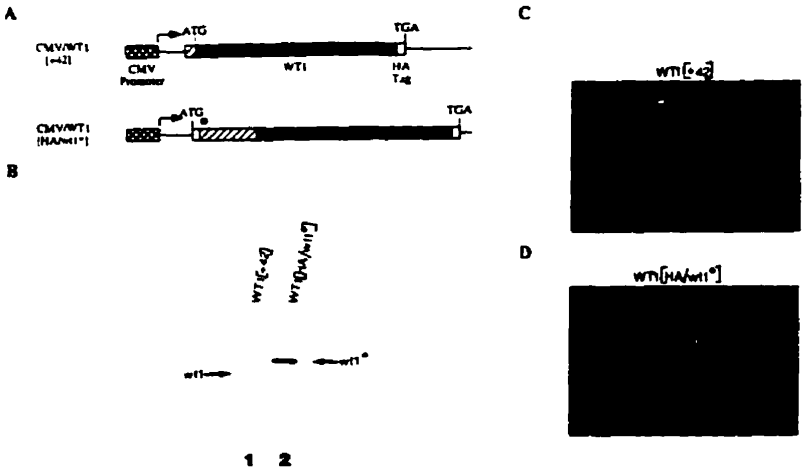

FIG. 6. **Nuclear localization of Wt1*.** *A*, schematic representation of expression vectors used in this study. A *black box* signifies the *wt1* ORF, a *hatched box* represents the 5'-UTR, and a *stippled box* represents the HA epitope tag (Pelletier *et al.*, 1991a), whereas a *checkered box* signifies the CMV promoter. CUG^{204} is represented by a *filled circle*. *B*, Western blot analysis of COS-1 cells transfected with *wt1* expression vectors. Cell extracts were prepared and processed for immunoblotting with the anti-HA antibody 12CA5 as described previously (Goodyer *et al.*, 1995). *C* and *D*, immunofluroescent detection of *wt1* and *wt1** . COS-1 cells were electroporated in PBS + 20 mM Hepes, pH 7.5, at 260 V/960 microfarads with 10 μg of plasmid DNA. The cells were seeded into chamber slides and allowed to recover for 48 h. After washing with PBS, the cells were fixed for 20 min in 3.7% formaldehyde/PBS followed by 5 min in methanol at −20 °C. Slides were blocked with 10% goat serum/0.3% Triton X-100 and probed with the anti-HA antibody 12CA5. Protein was visualized with an fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Jackson Labs). After washing, the cells were mounted in Immunomount (Fisher) and photographed using T-Max 100 black and white film (Kodak). Cells were transfected with CMV/*wt1*[+42] (*C*) or with CMV/*wt1*[HA/*wt1**] (*D*).

wt1[+475](Δ229–205) generates a 24-base pair in-frame deletion immediately upstream of the CUG^{204} codon and will abolish production of Wt1* only if the initiation codon lies upstream of CUG^{204}. Following *in vitro* transcription/translation of these plasmids and their respective mRNAs, the [^{35}S]methionine-labeled protein products were analyzed by SDS-PAGE. Translation of *wt1*[+475] produced both Wt1* and Wt1 as expected (Fig. 5*B*, *lane 2*). Only Wt1 was produced following translation of RNA synthesized from *wt1*[+202], consistent with the notion that the Wt1* initiation codon does not lie downstream of CUG^{204}. Mutation of CUG^{204} to an AUG codon increased the production of Wt1* (Fig. 5*B*, *lane 4*), consistent with the assignment of CUG^{204} as the initiation codon. Conversion of CUG^{204} to a UGA codon abolished production of Wt1*, whereas production of Wt1 was still observed (Fig. 5*B*, *lane 5*). Removal of nucleotides 229–205 affected the overall translational efficiency of the mRNA produced (Fig. 5*B*, compare intensity of products in *lane 7* with those in *lane 6*); however, the production of both Wt1* and Wt1 was still observed (Fig. 5*B*, *lane 7*), indicating that the Wt1* initiator codon does not lie upstream of CUG^{204}. These results indicate that CUG^{204} is the Wt1* initiation codon.

Wt1 Is Present in the Nucleus*—To determine the subcellular localization of Wt1*, an HA tag was placed in frame with and directly upstream from the CUG^{204} initiation codon (Fig. 6*A*). It was necessary to place the HA tag at the amino terminus because translation initiation at CUG^{204} is leaky and allows scanning ribosomes to initiate at the downstream, in frame ATG codon (See Figs. 3, 4, and 5), resulting in the production of both Wt1* and Wt1. The introduction of an HA tag immediately upstream of CUG^{204} was advantageous because the anti-HA monoclonal antibody, 12CA5, would specifically detect expression of only the 57-kDa isoform (Fig. 6*B*, *lane 2*). The construct CMV/*wt1*[+42] (Fig. 6*A*) produced only the 47-kDa isoform (Fig. 6*B*, *lane 1*) and contained an HA epitope at the carboxyl terminus. Immunofluorescence of COS-1 cells transfected with either CMV/*wt1*[+42] or CMV/*wt1*[HA/*wt1**] revealed that both isoforms localize to the nucleus (Fig. 6, *C* and *D*).

*Wt1** Mediates Transcriptional Repression*—In most transient transfection assays, WT1 functions as a repressor of transcription (see Introduction). To determine whether the novel amino-terminal domain of Wt1* altered the transcriptional properties of Wt1, 3 *wt1/GAL4* expression vectors were generated (Fig. 7*A*). The Wt1 zinc fingers were replaced with the DNA binding domain of *GAL4* (Ma and Ptashne, 1987), enabling the scoring of Wt1 activity on a promoter containing *GAL4* binding sites. In such a context, Wt1 produced from CMV/*wt1*[+182/*GAL4*] is able to repress transcription of the *cat* gene under TK promoter control ~10-fold, similar to levels obtained with CMV/*wt1*[+475/*GAL4*], which produces Wt1* and Wt1 (Fig. 7*B*). Western blot analysis indicated that Wt1 and Wt1* were produced from CMV/*wt1*[+475/*GAL4*] to approximately equal levels.[2] Transient transfections with CMV/*wt1*[HA/*wt1**/*GAL4*], which only produces Wt1* (data not shown), represses CAT production ~ 5-fold (Fig. 7*B*). These results demonstrate that in the system we have analyzed, the amino-terminal extension of Wt1* does not significantly change the transcriptional properties of Wt1.

DISCUSSION

The *WT1* gene products are necessary for regulating normal differentiation of the urogenital system. Mutations in the *WT1* gene result in malformations of the urogenital system as well as predispose to WTs. The structure of the *WT1* gene indicates that it should produce four alternatively spliced mRNAs that direct the synthesis of isoforms having molecular masses of 47–49 kDa. In this report, we demonstrate that *WT1* gene expression is more complex than anticipated, with a non-AUG translational initiation event producing additional protein isoforms of 54–56 kDa (WT1*). We have found WT1* to be expressed in a number of tissues and cells including murine TM3 cells (Fig. 1), murine ovaries (Fig. 1), murine testis (data not shown), immortalized rat granulosa cells (data not shown), and human K562 cells (data not shown). We have been unable to accurately establish the relative ratio of WT1 to WT1* in these cells and tissues, because the antibodies we are employing recognize the isoforms with different efficiencies (*e.g.* Fig. 1, compare *C* with *B*), perhaps due to differences in protein con-

[2] W. Bruening, unpublished data.

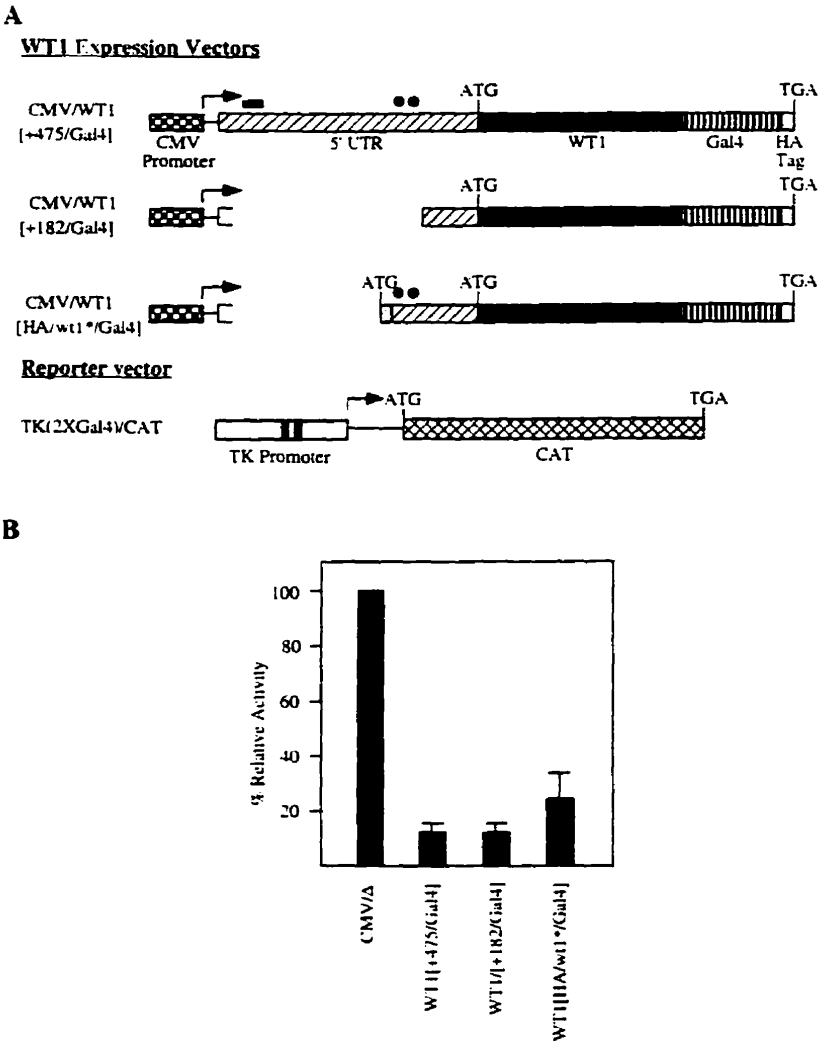

FIG. 7. **Trans-repression by Wt1 and Wt1*.** *A*, schematic representation of vectors used in this study. See legend to Fig. 6 for details. The *cross-hatched box* represents the *cat* gene, whereas the TK promoter is shown by an *open box*. The two *dark rectangles* within the TK promoter illustrate GAL4 binding sites. *B*, CAT assays of transient transfections performed with the expression and reporter vectors in *A*. NIH 3T3 cells were transfected by calcium phosphate and allowed to recover for 48 h. The cells were lysed by freezing and thawing 3 times in 0.25 M Tris, pH 8.0. Following removal of the cellular debris by centrifugation (12,000 × *g* for 5 min), CAT assays were performed on the supernatant as described previously (Gorman, 1985). A vector containing the CMV promoter driving expression of the *β-gal* gene was included in all transfections and used to standardize efficiencies. CMV/Δ is the backbone vector used to generate the expression vectors in *A* but lacking a gene downstream of the CMV promoter (Goodyer *et al.*, 1995).

formation or isoform-specific post-translational modifications. However, our data directly demonstrates that WT1* isoforms containing or lacking the first alternatively spliced exon are generated. Using isoform-specific antibodies, we were able to detect both isoforms in TM3 cells (Fig. 1*C*). In addition, two WT1* isoforms are clearly detectable on Western blots of whole cell extracts of WTs (Fig. 2*B*). Although we have not analyzed the presence or the absence of the second alternative splice site within WT1*, there is no reason not to suspect its presence. Our data, taken together with the documented alternative splicing of the *WT1* gene (Haber *et al.*, 1991) and RNA editing (Sharma *et al.*, 1994), indicate that this gene may produce as many as 16 different protein isoforms. To date, WT1 functional studies have generally been performed using expression vectors capable of producing only the 47–49-kDa isoforms. Given that the *WT1* gene products can oligomerize (Reddy *et al.*, 1995; Moffett *et al.*, 1995) and that the isoform ratio is important for proper urogenital system development (Bruening *et al.*, 1992), functional studies need to take into account the contribution of WT1* isoforms to the overall biological function of the *WT1* gene, including the possible interplay of the various isoforms.

Our data indicate that CUG^{204} is the initiation codon responsible for directing synthesis of Wt1*. A number of mRNAs use non-AUG initiation events to generate amino-terminally extended isoforms (see Introduction). The Wt1 initiator CUG is in a favorable consensus sequence (5′-*XXAXX*CUGG-3′, where the important nucleotide residues are underlined) (Kozak, 1987), and these likely play a role in directing translational initiation. The identity of the codon at this position is important in determining the efficiency with which this site is recognized, because mutagenesis of the initiation codon CUG^{204} to CUC^{204} significantly reduced (~15-fold) the amount of Wt1* produced (Fig. 4). However, it is clear that additional signals influence initiation at this site since the CUG to CUC mutation reduced but did not completely abolish translation initiation (Fig. 4). Surprisingly, deleting 24 nucleotides immediately upstream of CUG^{204} significantly reduced the overall translational efficiency of the template mRNA (Fig. 5*B*, compare *lanes 7* and *6*). Sequences downstream of non-AUG initiation codons have been shown to be important for determining efficiency of recognition of the start codons (Boeck and Kolakofsky, 1994; Grünert and Jackson, 1994). However, in the case of the *wt1* template, the deletion of sequences upstream of CUG^{204} has a global effect on translation initiation, including at the downstream AUG codon. In the case of fibroblast growth factor-2 mRNA, a ribosome landing pad is postulated to directly recruit 43S preinitiation complexes and guide them to a non-AUG codon (Vagner *et al.*, 1995). An internal ribosome binding site near CUG^{204} on the *wt1* mRNA template could account for the effects seen on global initiation by *wt1*[+475](Δ229–205) (Fig. 5*B*). Alternatively, the overall stability of the template may be affected by this 24-nucleotide deletion.

The degree of conservation between the amino-terminal extension of the murine and human WT1* isoforms is not as high as between the WT1 proteins. At the amino acid level, the murine and human WT1 isoforms are 96% identical (Buckler e

al., 1991), but the amino-terminal extensions of the human and murine WT1$^+$ isoforms are only 65% identical. However, the first 35 amino acids of WT1$^+$ are quite conserved (88% identical) and may encode a functional domain. Alternatively, overall charge or conformation may be more important to the function of this domain rather than primary sequence *per se*.

We have analyzed the genomic region encoding the amino-terminal extension of WT1$^+$ for possible mutations in WTs using polymerase chain reaction single stranded conformational polymorphisms (Varanasi *et al.*, 1994).[2] Examination of this region in over 100 WTs failed to detect any mutations. This is not particularly surprising because the majority of *WT1* mutations occur within the DNA binding domain (Bruening *et al.*, 1992; Pelletier *et al.*, 1991b; Varanasi *et al.*, 1994) with very few mutations occurring outside of this region.

Several investigators have noted the presence of a ~54–56-kDa contaminating polypeptide in studies involving immunoprecipitation of WT1. Given our results, these polypeptides likely represent WT1$^+$ isoforms generated by translation initiation at CUG^{204}. The presence of such isoforms is apparent in Fig. 3*F* of Larrson *et al.* (1995), displaying an immunoprecipitation on nuclear extracts with anti-WT1 antibodies from the mesonephric cell line, M15. A protein with similar electrophoretic properties as WT1$^+$ was also detected by Rackley *et al.* (1993) on an immunoblot analysis of nuclear extracts prepared from the *WT1*-expressing kidney cell line, 293 (see Fig. 3*a*; Rackley *et al.* (1993)). The functional significance of producing WT1 isoforms with an amino-terminal extension is not obvious. The amino-terminal domain on its own does not have a strong *trans* effect on transcription because fusion constructs containing this domain fused to the *GAL4* DNA binding domain did not significantly affect transcription of an appropriate reporter in transient transfection experiments.[2] This domain may be involved in a number of other activities such as heterodimer formation, subnuclear localization, or regulation of WT1 intrinsic activity. Our description of a non-AUG translation event at the *WT1* locus indicates that the overall complexity of *WT1* regulation is greater than previously suspected. Biological assays involving all WT1 isoforms should present a better picture of the role of WT1$^+$ in normal and abnormal urogenital system development.

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Chapter 3

Germline intronic and exonic mutations in the Wilms' tumour gene (*WT1*) affecting urogenital development

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Denys-Drash syndrome is a rare human developmental disorder affecting the urogenital system and leading to renal failure, intersex disorders and Wilms' tumour. In this report, four individuals with this syndrome are described carrying germline point mutations in the Wilms' tumour suppressor gene, *WT1*. Three of these mutations were in the zinc finger domains of *WT1*. The fourth occurred within intron 9, preventing splicing at one of the alternatively chosen splice donor sites of exon 9 when assayed *in vitro*. These results provide genetic evidence for distinct functional roles of the *WT1* isoforms in urogenital development.

Wilms' tumour (nephroblastoma) is an embryonic malignancy of the kidney, arising from the metanephric blastema, which affects about one in 10,000 children[1]. A genetic predisposition to Wilms' tumour is observed in two congenital syndromes with slightly overlapping phenotypes. First, the WAGR syndrome, in which Wilms' tumour is associated with aniridia (absence or malformation of the iris), genitourinary anomalies, and mental retardation[2], and second, Denys-Drash syndrome, a constellation of Wilms' tumour, intersex disorders, and renal insufficiency[3,4]. The Wilms' tumour suppressor gene (*WT1*), residing at chromosome 11p13, has recently been cloned and characterized[5,6]. There are four alternatively spliced *WT1* mRNA transcripts, reflecting the presence or absence of two differentially spliced exons in the coding region of the *WT1* mRNA[7,8]. The *WT1* isoforms contain four Cys_2-His_2 zinc fingers and two of the four isoforms differ in DNA binding specificity (the spliced variants with an insertion of 3 amino acids at exon 9 cannot bind the early growth response (EGR) recognition element, whereas those isoforms lacking this insertion can)[9,10]. *WT1* expression during nephrogenesis is limited to the condensed mesenchyme, renal vesicle and glomerular epithelium and likely plays a key part in development of these structures during organogenesis[11]. High levels of *WT1* mRNA are also present in the indifferent gonads[11,12], the Sertoli cells of the testis, the granulosa and epithelial cells of the ovaries, and the uterus[12].

Genotype analysis of the *WT1* gene in two male children with Wilms' tumour and genital system malformations (hypospadias and undescended testis) demonstrated these individuals to have constitutional null mutations within one *WT1* allele[13]. In additon, ten individuals with Denys-Drash syndrome have been identified with germline nucleotide substitutions within the zinc finger domains of one *WT1* allele[14]. These results provide genetic evidence that germline mutations within *WT1* are responsible for Wilms' tumour predisposition and urogenital malformations.

In this report, we characterize the *WT1* gene in four individuals with Denys-Drash syndrome and report on novel mutations associated with this syndrome. One individual was found to contain a mutation affecting alternative splice site selection between the exons encoding zinc fingers III and IV. Assuming a common mechanistic basis for Denys-Drash syndrome, these results provide genetic evidence for a functional difference among the *WT1* isoforms during urogenital development.

Analysis of *WT1* in patient C.S.

Patient C.S. experienced renal failure due to glomerular sclerosis, has female external genitalia and a karyotype of 46, XY (Table 1). Both kidneys and gonads have been removed; no Wilms' tumour was detected. We used single-strand conformational polymorphism (SSCP)[15] to analyse all *WT1* coding exons for alterations within the germline of C.S.. All ten *WT1* exons were amplified by the polymerase chain reaction (PCR) from genomic DNA isolated from EBV-transformed lymphocytes of C.S. and parents. By SSCP analysis, only the PCR product from exon 9 demonstrated a mobility shift different from normal controls (Fig. 1*a*, compare lane 3 with lane 2) and from both parents (Fig. 1*b*, compare lane 3 with lanes 1 and 2). This amplified product, when sequenced, contained a

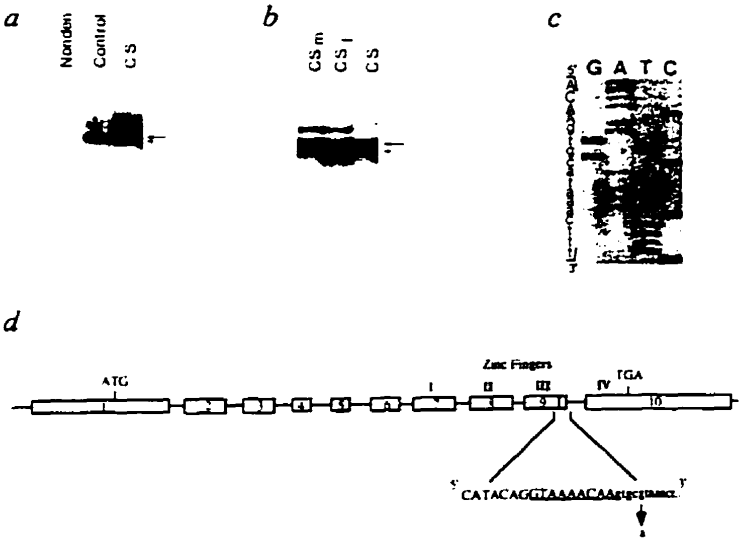

Fig. 1 *WT1* analysis in patient C.S. *a,b*. PCR-SSCP analysis of *WT1* exon 9 and flanking intronic sequences from patient C.S. PCR-SSCP analysis of the *WT1* gene was performed as described in the Methodology using oligonucleotides INT-1 and INT-2. Oligonucleotide INT-1 was kinased for the analysis presented in *a* and *b*. The individuals from which genomic DNA was purified are indicated above each lane. The lane labelled as control indicates amplification of DNA from an unaffected individual, whereas Nonden. refers to the absence of denaturing treatment to the normal sample. This nondenatured sample was used to define the position of migration of double-stranded DNA product (not visible on these photographic mounts). The PCR product from exon 9 of an unaffected individual demonstrates a specific single-stranded conformational species, which is absent if the sample is not denatured (compare lane 2 to lane 1). Note that PCR analysis of exon 9 in >30 normal individuals never demonstrated an altered migrating PCR product. The arrow indicates the position of the fragment with the altered mobility, whereas the arrowhead indicates the position of migration of the PCR product from normal DNA. *c*. Sequence analysis of WT1 exon 9. For sequence analysis, PCR products were gel purified, cloned into the *Eco*RI site of pBluescript KS II+, and individual subclones were sequenced with Sequenase (US Biochemicals) and the T7 primer. A total of ten clones were sequenced, six of which demonstrated the guanine to adenine transition at position +5 of intron 9. *d*. Schematic representation of the point mutation described for patient C.S. The exons of the *WT1* gene are represented as boxes: stippled boxes denote alternatively spliced exons. The four exons which encode the four zinc fingers of WT1 are indicated by roman numerals. The 9 nucleotides of the second alternatively spliced exon are underlined.

guanine to adenine transition at position +5 of the splice donor site within intron 9 (Fig. 2*c*, 2*d*). These results identify a *de novo* germline mutation within one *WT1* allele of patient C.S.

G to A transition affects *WT1* splicing

To examine the possibility that this mutation exerted an effect on alternative splicing at exon 9, complementary DNA was prepared from RNA isolated from snap-frozen gonadal tissue obtained from patient C.S.. Oligonucleotides targeting *WT1* exons 8 and 10 were used to amplify, by PCR, the coding region spanning exon 9. Amplified PCR products of only the two alternatively spliced forms of *WT1* were observed by analysis on 12% nondenaturing polyacrylamide gels (data not shown). No PCR product indicative of a transcript lacking exon 9 was present. These results suggest that the $^{+5}$G to $^{+5}$A transition in the *WT1* gene of patient C.S. does not lead to skipping of exon 9 by the cellular splicing machinery.

We attempted to determine if alternative splice site selection at exon 9 was affected by the $^{+5}$G to $^{+5}$A transition. However, the presence of a normal *WT1* allele in C.S. interfered with such studies. Rather, a mini-gene was constructed containing exon 9, 117 nucleotides of intron 9, and part of exon 10 (Fig. 2*a*). This mini-gene contains the splice donor, splice acceptor, as well as predicted branch point selection site — all required for proper splicing of the mRNA transcript. We introduced the $^{+5}$adenine mutation into the intron of this construct. A CMV-based expression vector was used to drive expression following transfection into COS-1 cells. Complementary DNA from RNA isolated two days after transfection, was amplified by the PCR using oligonucleotides targeting to exons 9 and 10 (Fig. 2*a*). Two products of the expected molecular mass were obtained from RNA produced from the normal mini-gene (Fig. 2*b*, lane 3), whereas analysis of RNA produced from the mutant construct yielded a single product lacking the alternatively spliced nucleotides from exon 9 (Fig. 2*b*, lane 2). These results suggest that the $^{+5}$A mutation at the splice donor site in individual C.S., prevents production of *WT1* isoforms containing the nine alternatively chosen nucleotides of exon 9.

Analysis of *WT1* in Denys-Drash syndrome

Analysis of the *WT1* gene from three other individuals with Denys-Drash syndrome (J.K., L.W. and K.J.) was performed by direct sequencing of amplified products of exons 7, 8, 9 and 10, which respectively encode zinc fingers I, II, III and IV (see Fig. 1*d*). All individuals had glomerular sclerosis, as well as evidence of Wilms' tumour or precursor lesions to Wilms' tumour (Table 1). The results of the PCR-sequencing analysis are summarized in Fig. 3. L.W. contains a point mutation within exon 9 which converts Arg^{394} to Trp (Fig. 3*a*, right panel); L.W.'s mother does not carry this mutation (Fig. 3*a*, left panel). Genomic DNA from J.K. was available only from a Wilms' tumour specimen embedded in paraffin. Analysis of this sample revealed a guanine to cytosine transversion in exon 9, converting Arg^{394} to Pro (Fig. 3*b*). Although untested, we expect that, like other patients with Denys-Drash syndrome[14], J.K. is germline hemizygous for this mutation. Patient K.J. has a point mutation within exon 7 (zinc finger I) converting Cys^{330} to Tyr. (Fig. 3*c*).

Discussion

In this report, we expand the spectrum of *WT1* mutations associated with Denys-Drash syndrome. Three individuals, J.K., L.W. and K.J., contain point mutations within *WT1* which alter amino acids critical for DNA sequence recognition. Both alterations in J.K. and L.W. affect the same amino acid-Arg^{394}. This residue is implicated in hydrogen bonding interactions with a guanine residue in the cognate DNA recognition sequence[16]. Mutations at this amino acid have been previously found in individuals

Table 1 Summary of phenotypes and karyotype analysis associated with Denys-Drash individuals in this study

Patient Karyotype	Nephropathy	Wilms' tumour	Status of reproductive system
C.S.	+	–	Phenotypic female, infantile uterus, streak gonads, Wolffian duct remnants46, XY
L.W.[a]	+	+	Ambiguous genitalia, rudimentary uterus, fimbriated fallopian tubes, streak gonads 46, XY
J.K.	+	+	Phenotypic female 46, XY
K.J.	+	[b]	ND[c] 46, XX

ND, not determined; + present,– not present.
[a]This patient has been described in greater detail in ref. 33.
[b]Extensive intralobar persistent renal blastema was present in both kidneys, no overt Wilms' tumour was present.
[c]The patient had mild clitoromegaly.

with Denys-Drash syndrome and shown to alter DNA sequence recognition[14]. The mutation described for K.J. converts Cys[130] to Tyr in zinc finger I. We predict that this amino acid substitution should also alter *WT1* DNA sequence recognition since it affects a 'cornerstone' residue involved in zinc ion chelation, an event necessary for nucleic acid binding[17,18]. Unlike zinc fingers II, III, and IV, WT1 zinc finger I shows little homology to the zinc finger domains of the EGR genes[5]. Systematic deletions of individual zinc fingers from TFIIIA indicate that clusters of fingers recognize different parts of the binding site[19]. Models for interactions of zinc fingers with DNA have suggested that each finger interacts with three nucleotides in the target sequence[20]. The role of WT1 finger I in sequence recognition is not well defined and mutations within this region may still allow for DNA binding, but with altered sequence specificity.

We also describe a germline mutation predicted to affect splice site selection of the second alternatively chosen exon of *WT1* in an individual with Denys-Drash syndrome. When engineered into a mini-gene and introduced into COS-1 cells, this mutation prevents alternative splice site selection at exon 9 (Fig. 2). The +5 position of the splice donor site is moderately conserved in mammals, generally being a guanine. Several findings of intron mutations at position +5, similar to that found in patient C.S., which affect splicing have been reported[21–27]. These mutations prevent splicing at the normal 5' splice site with activation of cryptic sites[23,28,29] or exon skipping[21]. The ability to suppress such mutations by compensatory base changes in U1 snRNAs[30] suggest that these mutations uncouple 5' splice site selection from U1 snRNA binding.

Germline mutations at the *WT1* locus of individuals with WAGR syndrome are null mutations which inactivate, by gross deletion or small frameshift mutations, one WT1 allele. In males, such mutations are associated with Wilms' tumour predisposition and genital system anomalies (most commonly, hypospadias and cryptorchidism) and thought to be a result of decreased levels of *WT1* gene products during embryogenesis[13]. Germline mutations at the *WT1* locus in Denys-Drash syndrome are associated with more severe urogenital anomalies (intersex disorders and renal nephropathy) and thus likely to exert their effect in a different manner. Two possibilities are that the products of the Denys-Drash alleles behave in a *trans*-dominant suppression or gain-of-function manner. The majority of mutations associated with Denys-Drash syndrome occur in WT1 zinc finger III, although two examples have been described with mutations in zinc fingers II and I (ref. 14

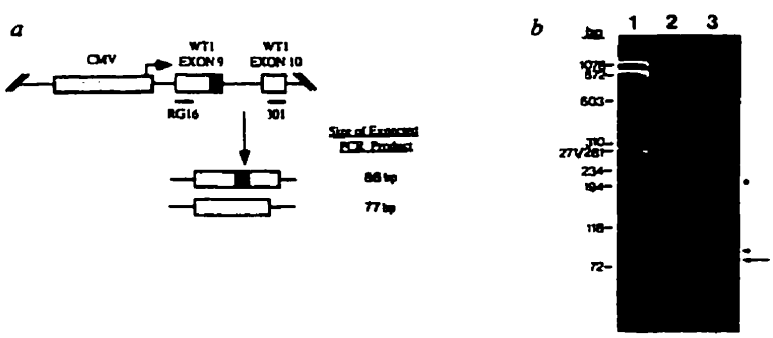

Fig. 2 Transient transfection assay of the *WT1* mini-gene harbouring the $^{+5}$G to $^{+5}$A change in *WT1* Intron 9. *a*, Schematic representation of the *WT1* mini-gene. Alternative splice site selection of *WT1* exon 9 should produce two mRNA species, which, after amplification of cDNA with oligonucleotides 301 and RG16, should yield two PCR products of 78 and 87 bp. *b*, Polyacrylamide gel analysis of the amplified PCR products following transfection of COS-1 cells. RNA, harvested from transfected COS-1 cells, was reverse transcribed using 0.1 μg of oligonucleotide 301: 5'GGCGAATTCGCCACCGACAGCTGAAGGGC3' (complementary to the 5' end of exon 10, except for the *Eco*RI linker at 5' end of the oligonucleotide). A region containing exon 9 and 10 was amplified from the cDNA using oligonucleotides 301 and RG16: 5'CCCGGTCCGACCACCTG3'. The PCR reaction (50 μl) was performed for 35 cycles with an annealing temperature of 58 °C. One fifth of the PCR reaction was analysed on a 12% polyacrylamide gel (30:1 acrylamide:bisacrylamide). Lane 1, ØX179/ *Hae*III; lane 2, PCR amplification of cDNA from COS-1 cells transfected with the $^{+5}$A *WT1* minigene; lane 3, PCR amplification of cDNA from COS-1 cells transfected with the normal *WT1* minigene. The arrow denotes the mobility of the PCR product lacking the alternatively spliced exons, the arrowhead denotes the PCR product containing the 9 alternatively spliced nucleotides. The dot represents amplified product from the input transfected vector. The identity of the PCR products and confirmation of proper splicing between exons 9 and 10 was confirmed by subcloning and sequencing all PCR products present in this Fig. (data not shown).

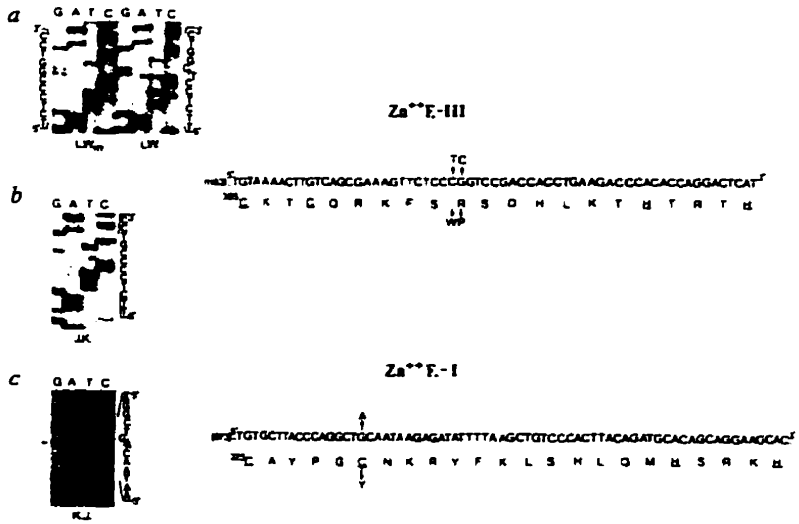

Fig. 3 Direct sequence analysis of mutated *WT1* exons from patients L.W., J.K. and K.J. Each exon encoding WT1 zinc finger I, II, III, and IV was amplified and used directly for asymmetric PCR sequencing as described previously[14]. All mutations described here were confirmed in independent PCR and sequencing studies. *a*, Analysis of L.W. and mother ($L.W._m$) revealed a point mutation within the coding region of exon 9 (cytosine to thymine transition) predicted to convert an Arg to Trp. *b*, Sequence analysis of J.K.'s Wilms' tumour reveals a guanine to cytosine transversion within exon 9. *c*, Sequence analysis of K.J. revealed a point mutation within exon 7 (guanine to adenine transition). These mutations are schematically shown to the right of the sequencing gels. The nucleotide numbering system is based on the coding sequence only and considers the A of the initiator ATG as +1[16]. The Cys and His 'cornerstone' residues of the zinc fingers are underlined.

and this report). Since different point mutations in three of the WT1 zinc fingers have been documented in this disease, they are unlikely to affect *WT1* mRNA half-life or protein stability. The mutation described for C.S. is a splicing defect of the second alternatively chosen exon which also results in Denys-Drash syndrome. Assuming a common mechanistic pathway for these mutations in the aetiology of Denys-Drash syndrome, it is unlikely they behave in a gain-of-function manner, as C.S.'s mutation cannot result in production of a polypeptide with novel function.

The relative abundance of the different *WT1* splice forms is similar among various mouse and human tissues, as well as among different Wilms' tumours[8]. (The various splice forms are referred to as A, which lacks both alternatively spliced exons; B, which contains the first alternatively spliced exon; C, which contains the second alternatively spliced exon; and D, which contains both alternatively chosen exons.) Isoforms A and B have been shown to mediate repression of transcription in transient transfection assays[10]. No functional assay exists for isoforms C and D since they do not bind to the EGR-1 site *in vitro*[9]. The mutation in individual C.S. is expected to result in a decrease of isoforms C and D. A reduction in dosage of these isoforms alone however, cannot explain C.S.'s phenotype since this situation is also observed in the WAGR syndrome; where one *WT1* allele is inactive. It therefore seems likely that disrupting the ratio of *WT1* isoforms is responsible for C.S.'s phenotype. These results provide genetic evidence for distinct functional roles of the *WT1* isoforms in urogenital development.

The absence of tumour lesions in C.S. may indicate that disrupting the relative abundance of *WT1* is insufficient for deregulated cellular proliferation. Our results raise the possibility that cellular control of *WT1* activity may involve modulation of the ratio of *WT1* isoforms. Elucidating the role of the WT1 polypeptides in urogenital development awaits definition of the target genes that they regulate.

Methodology

Preparation of genomic DNA. Lymphocytes were isolated from freshly drawn blood and immortalized with Epstein Barr Virus (EBV) as described previously[11]. Lymphoblastoid cell lines were propagated in Iscove's medium supplemented with 15% fetal calf serum. Genomic DNA was isolated from lymphoblastoid cell lines and blood, as described[14].

Analysis of *WT1* exon 9 from C.S. The PCR was performed with 0.1 μg of genomic DNA in a thermocycler (MJ Research, Inc.) for 35 cycles at 94 °C for 1 min, at 57 °C for 1 min and at 72 °C for 1 min. Exon 9 was amplified using the following oligonucleotides: INT-1: 5'GGAATTCAATTTCATTCCACAATAG3' and INT-2: 5'GGAATTCCTCACTGTGCCCACATTG3'. *Eco*RI sites were engineered into the oligonucleotides to facilitate subcloning of the PCR products. SSCP analysis was performed as described[14]. The mutation described for C.S. has been verified several times in independent PCR and sequencing studies.

Construction of the WT1 mini-genes. WT1 exon 10 was amplified from normal human genomic DNA using the following oligonucleotides, INT-7:5'GGCGAATTCCTGTCTCTTTGTTGC3' and 103:5'GGCGAATTCGTCCCCGAGGGAGACCCC3' which gives rise to a 204 bp DNA product. This PCR product was restricted with *Eco*R1 (which cleaves at the extremeties of the PCR product) and purified on a 3% NuSieve agarose gel (Mandel Scientific)[12]. The purified product was cloned into the *Eco*RI site of pBluescript KS II+, and sequenced to ensure the absence of PCR-generated mutations. A clone was digested with *Hin*dIII (which cleaves within the polylinker of pBluescript KS II+), repaired with the Klenow fragment of DNA polymerase I, and digested with *Msc*I (which cleaves 26 nucleotides downstream of the 3' splice acceptor site of exon 10). This mixture was recircularized by blunt-end ligation using T4 DNA ligase. This clone was named pKS/ex 10. Exon 9 was amplified by the PCR from genomic DNA obtained from both an unaffected individual and patient C.S. The oligonucleotides used for the PCR were INT-1 and INT-2. The PCR products from exon 9 were subcloned into the *Eco*RI site of pKS/ex 10 and sequenced. Normal and mutant mini-genes were subcloned into the *Bam*HI/*Xho*I sites of pgCMV/tat (a CMV-based expression vector)[12].

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Acknowledgements

We dedicate this manuscript to the patients and their families. We are grateful to H. Idikio (Alberta) for biopsy analysis on specimens from K.J.. J.P. thanks the McGill Department of Biochemistry for use of facilities and space. This research was supported by the National Cancer Institute and Medical Research Council of Canada (J.P.).

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Chapter 4

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Genetics

Antagonism of *WT1* activity by protein self-association

(dominant-negative mutations/Wilms tumor/Denys–Drash syndrome/cancer genetics)

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Contributed by David E. Housman, August 15, 1995

ABSTRACT **Germline loss-of-function mutations at the Wilms tumor (WT) suppressor locus *WT1* are associated with a predisposition to WTs and mild genital system anomalies. In contrast, germ-line missense mutations within the *WT1* gene encoding the DNA-binding domain often yield a more severe phenotype consisting of WT, sexual ambiguity, and renal nephropathy. In this report, we demonstrate that the products of mutant alleles that impair DNA recognition can antagonize WT1-mediated transcriptional repression. We demonstrate that WT1 can self-associate *in vitro* and *in vivo* and that the responsible domain maps to the amino-terminal region of the protein. Oligomers of full-length protein form less efficiently or produce less stable complexes than oligomers between truncated polypeptides and full-length protein. Our data suggest a molecular mechanism to explain how *WT1* mutations may act in deregulating cellular proliferation and differentiation.**

Wilms tumor (WT) is a renal malignancy arising from the metanephric blastema affecting ≈1 in 10,000 children (1). This cancer occurs as both a sporadic and hereditary form; the hereditary forms account for ≈5–10% of all cases (1). Associated with a predisposition to WTs are two congenital malformation syndromes in which urogenital-system development is affected: (*i*) WAGR (WTs, aniridia, genitourinary anomalies, and mental retardation) and (*ii*) Denys–Drash syndrome (DDS: WTs, intersex disorders, and nephropathy). Molecular genetics and physical mapping studies of individuals with WAGR syndrome led to the identification of the tumor-suppressor gene *WT1*, residing at 11p13 (2, 3). This gene is mutated in the germ line of individuals with DDS (4), as well as in a small percentage (5–15%) of sporadic WTs (5–7).

The *WT1* gene encodes a putative transcription factor with four carboxyl Cys_2-His_2 zinc fingers and is alternatively spliced to produce four mRNA species (for a review, see ref. 8). The first alternatively spliced exon inserts or removes 17 amino acids from the middle portion of WT1; whereas the second inserts or removes three amino acids (Lys-Thr-Ser) between zinc fingers III and IV, altering the DNA-binding specificity of the molecule (8). The protein isoforms localize to distinct, but overlapping, domains within the nucleus, suggesting possible different functions (9). Three of the four WT1 zinc fingers share a significant degree of homology (≈65%) to the zinc fingers of the early growth response (EGR)-1 gene product and recognize a similar G+C-rich target motif, 5′-GCGGG-GGCG-3′ (8), although with different affinities than EGR-1 (10). A second transcriptionally active DNA-binding site for WT1, $(TCC)_n$, has been identified in the platelet-derived growth factor (PDGF) A-chain promoter (11). A number of growth factor gene promoters contain these motifs and are responsive to WT1 in transient transfection assays (8). The WT1 gene product can mediate both transcriptional repression (8) and activation (11), depending on the architecture of the promoter under study, indicating that the WT1 regulatory network is complex.

The WT1 gene product plays a critical role in differentiation of the urogenital system, as evidenced by failure of this system to develop in WT1-null mice (12). Loss-of-function mutations (e.g., deletions) in humans are associated with WTs and undescended testis and hypospadias (for a review, see ref. 13), whereas more severe developmental disorders, such as those found in DDS, are associated with germ-line *WT1* missense and nonsense lesions (4, 13, 14), indicating that these alterations behave differently than loss-of-function mutations. In this report, we address the issue of whether WT1 mutants can functionally antagonize WT1-mediated transcriptional repression. We demonstrate that the WT1 isoforms can interact *in vivo* and *in vitro* and that the responsible domain maps to the amino terminus. Our results provide a molecular mechanism for the dominant-negative mode of behavior for WT1 mutants impaired for DNA binding.

MATERIALS AND METHODS

Cell Lines and Transfections. COS-7 cells were maintained in Dulbecco's modified Eagles' medium/10% fetal calf serum. For transient transfection assays, cells were plated at a density of 2–5 × 10^5 cells per 10-cm^2 dish 24 hr before transfection. Cells were transfected by calcium phosphate or with lipofectamine (according to the manufacturer's protocol; Life Technologies, Grand Island, NY). Individual DNA precipitates were adjusted to contain equal amounts of cytomegalovirus (CMV)-based expression vector by the addition of control CMV plasmid lacking an insert. Cells extracts were prepared 48 hr later and chloramphenicol acetyltransferase (CAT) assays were done and standardized as described (15).

Yeast Two-Hybrid System. The yeast two-hybrid system used was from Stephen Elledge (Baylor College of Medicine, Houston) (16). pACT/WT1 derivatives (see Fig. 4*A*) were constructed from murine WT1 cDNAs. Transformants containing the indicated plasmids were assayed for the ability to grow on medium lacking histidine and supplemented with 30 mM 3-aminotriazole (Sigma no. A8056), as well as for the presence of β-galactosidase activity (16). Details as to plasmid construction are available from the authors on written request.

***In Vitro* Transcriptions and Translations.** SP64-based plasmids containing the *WT1* gene for *in vitro* transcriptions have been described (4). *In vitro* transcriptions were done on DNA templates that had been linearized with *Hin*dIII (4). RNA templates were translated in a rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine and [^{35}S]cysteine at

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Abbreviations: WT, Wilms tumor; DDS, Denys–Drash syndrome; RAR, retinoic acid receptor; EGR, early growth response; PDGF, platelet-derived growth factor; CMV, cytomegalovirus; CAT, chloramphenicol acetyltransferase; HA, hemagglutinin.

[‡]W.B. and P.M. contributed equally to this work.

final concentrations of 16 μg/ml. Translations were done at 30°C for 1 hr. Differences in specific activities between full-length and truncated WT1 proteins were corrected for when calculating amount of probe to be used for "far-Western" blotting.

RESULTS

Inhibition of WT1-Mediated Repression by a DDS Allele. DDS alleles are thought to behave in a dominant-negative fashion because they are associated with more severe developmental disorders than loss-of-function mutations (4). To address the possibility that such an allele could function in trans to inhibit wild-type WT1 activity, we established a cotransfection assay using the WT1-responsive retinoic acid receptor (RAR)-α promoter (15). Activity of the RAR-α promoter is repressed ≈5-fold by the WT1(−−) isoform (Fig. 1*B*). This repression is mediated through an EGR-1 site in the RAR-α promoter (15) and is not seen with the most common DDS allele (Arg-394 → Trp change) (4) (Fig. 1*B*). However, when this allele is cotransfected with CMV/WT1(−/−), inhibition of RAR-α expression is relieved (Fig. 1*B*), showing that it can behave in trans to prevent WT1-mediated repression. To map the responsible region, we made use of an amino-terminal deletion mutant, CMV/D1(−/+). Both the WT1(−/+) isoform and the CMV/D1(−/+) deletion cannot repress the RAR-α promoter, presumably because they cannot bind to the RAR-α promoter (Fig. 1*B*). However, unlike CMV/WT1(−/−)[R/W], CMV/D1(−/+) does not rescue the RAR-α promoter from WT1-mediated repression (Fig. 1*B*). These results show that a domain within the WT1 amino terminus functions in trans to prevent WT1-mediated repression. This result suggests that under a competitive situation, WT1 mutants can sequester a limiting cofactor required for activity and/or oligomerize with wild-type protein to form nonproductive complexes. The net result is antagonism of wild-type WT1 activity and a dominant-negative mode of action.

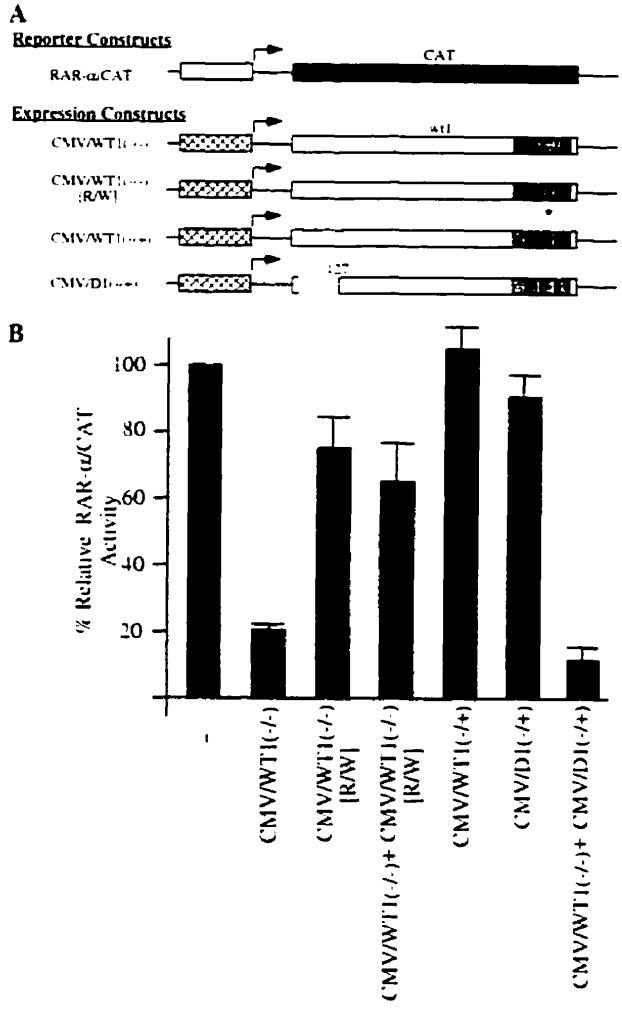


FIG. 1. Rescue of WT1(−/−)-mediated repression of the RAR-α promoter. (*A*) Schematic representation of reporter and expression vectors used. Details on construction of vectors are given in ref. 15. The star represents the Arg-394 → Trp DDS mutation in zinc finger III, whereas the blackened rectangle represents the +Lys-Thr-Ser amino acids between zinc fingers III and IV. (*B*) CAT assays showing trans inhibition of WT1(−/−)-mediated repression. Transfected expression vectors are indicated below. In experiments where a single expression construct was used, 2 μg of reporter plasmid was cotransfected with 4 μg of expression vector and 4 μg of CMV plasmid containing no insert. In cotransfection experiments, 4 μg of CMV/WT1(−/−) was introduced with 4 μg of a second expression vector and 2 μg of reporter. Transfections were done at least three times. Arrow bars indicate SDs.

Oligomerization of Mutant WT1 *in Vitro*. To determine whether mutant WT1 could self-associate *in vitro*, a series of far-Western blots were done. In this assay, recombinant mutant WT1/Gal4 fusion protein was fractionated by SDS/PAGE, transferred to Immobilon-P, and probed with radiolabeled WT1 protein produced by *in vitro* translation. A series of truncated polypeptides were generated to assess specificity of the assay (Fig. 2*A*). In addition, a WT1 mutant harboring a missense substitution, Gly-201 → Asp, was also produced [pSP/WT1-(1–253)*]. This mutation is of interest because it has previously been shown to convert WT1 from a transcriptional repressor to an activator (18). No hybridization was obtained when radiolabeled brome mosaic virus (Fig. 2*B*, lane 1) or luciferase protein (P.M. and J.P., unpublished data) was used as probe on these blots. The translation products from pSP/WT1-(1–217) and pSP/WT1-(1–253) gave a strong hybridization signal (lanes 2 and 3, respectively), whereas no hybridization was observed with the deletion WT1-(127–429), which lacks the amino terminus (Fig. 2*B*, lane 4). These results indicate that mutant WT1 can form oligomers *in vitro* and the responsible domain lies at the amino terminus. Full-length WT1 interacts weakly with the WT1/Gal4 fusion compared to WT1(1–217) (compare lane 6 to 5), suggesting that the interaction domain may be partially hidden due to conformational constraints. Both WT1-(1–253)* and WT1-(1–253) behaved similarly in this assay (compare lane 9 to 8), indicating that changing the transcriptional potential of WT1 had little effect on its oligomerization abilities. Similar results were obtained by using purified full-length WT1 protein as target, indicating that the radiolabeled WT1 protein probes are not interacting nonspecifically with the Gal4 domain (data not shown). In addition, neither WT1 nor WT1-(1–253) were capable of interacting with a WT1 deletion mutant lacking amino acids 8–180 (P.M. and J.P., unpublished data). These results suggest that WT1 oligomerization may be the basis for the observed antagonism of WT1-mediated suppression by alleles incapable of DNA binding (Fig. 1*B*).

Oligomerization of Mutant WT1 *in Vivo*. The possibility that WT1 self-associates *in vivo* was tested by using a yeast two-hybrid system (16). In this assay, the murine WT1 cDNA was fused to the carboxyl terminus of the yeast Gal4 activation domain II (amino acids 768–881) to create pACT/WT(−/−) and pACT/WT(+/−) (Fig. 3*A*). WT1 exons 1–7 were fused to the carboxyl terminus of the DNA-binding domain of Gal4 (amino acids 1–147) to generate pAS1/δWT(−) and pAS1/δWT(+) (Fig. 3*A*). These plasmids were introduced alone or in combination into yeast strain Y190—previously engineered with chromosomally integrated copies of the *Escherichia coli*

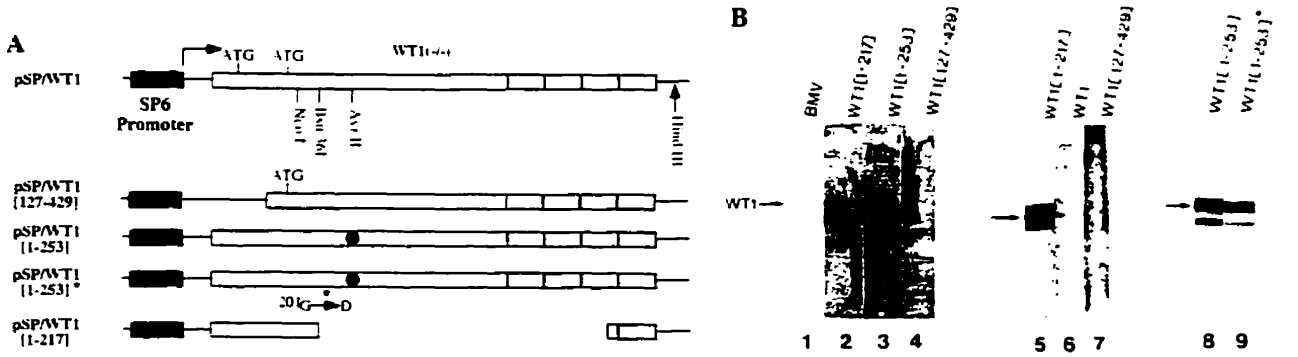


FIG. 2. Self-association of WT1 *in vitro*. (*A*) Transcription vectors used to synthesize wild-type and mutant WT1 protein. The blackened circles indicate the positions of an amber codon. The star denotes the relative position of a missense mutation that changes a glycine to an aspartic acid. (*B*) Far-Western blotting of WT1. A WT1 Gal4 recombinant clone was used as starting material for production of WT1 protein. This pET-3b derivative contains WT1 amino acids 1–324 fused to DNA-binding domain of Gal4 (amino acids 1–94) (J.P., unpublished data). In addition, a hemagglutinin (HA) epitope tag and a $(His)_6$ tail are fused to the Gal4 DNA-binding domain. WT1–Gal4 protein was induced in *E. coli* BL21 with 0.4 mM isopropyl β-D-thiogalactoside and purified by Ni^{2+}-agarose affinity matrix (Qiagen) using the manufacturer's recommended conditions. Far-Western blots were done by resolving 10 μg of recombinant protein on a 10% polyacrylamide (acrylamide/bisacrylamide, 30:1) SDS gel essentially as described (17). Radiolabeled WT1 protein (5×10^6 cpm/μl) was incubated in hybridization buffer [10 mM Hepes, pH 7.9/8% (vol/vol) glycerol, 0.05 M KCl/2.5 mM $MgCl_2$, 0.05 mM $ZnCl_2$, 0.05 mM EDTA, 0.1% Nonidet P-40/1 mM dithiothreitol] with Immobilon strips at room temperature for 10 hr. The filters were washed three times for 20 min in hybridization buffer before exposure to x-ray film. arrow, Specific *WT1–WT1* complex. BMV, brome mosaic virus.

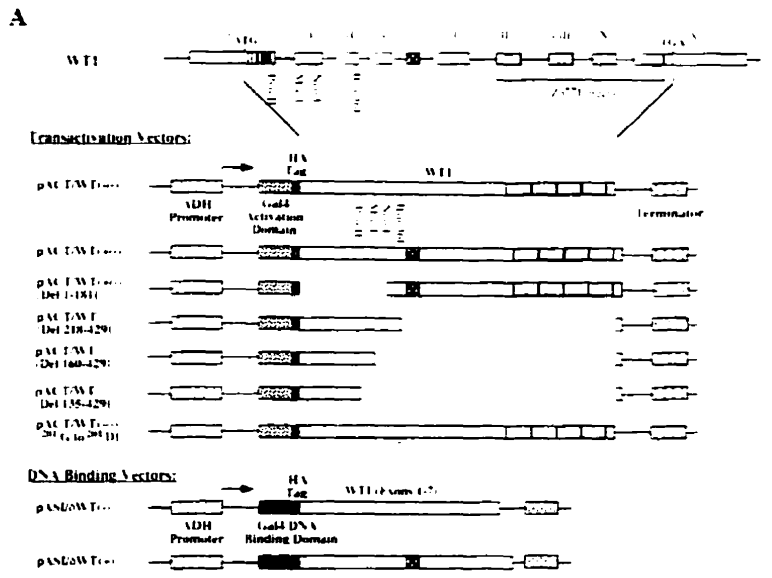
lacZ and the selectable *HIS3* genes, both under Gal4 regulation (16). Introduction of either vector into Y190 cells failed to activate β-galactosidase production or rescue the histidine dependency of these cells (Fig. 3 *B* and *C*). On the other hand, Y190 cells harboring both pAS1/δWT and pACT, WT derivatives can grow in the absence of histidine and synthesize β-galactosidase (Fig. 3 *B* and *C*). These results indicate that WT1 isoforms (containing or lacking exon 5) are capable of self-association *in vivo*.

The region responsible for this association was delineated by using a series of deletion mutants generated from pACT/WT(−/−) (Fig. 3*A*). When introduced into Y190 cells harboring the DNA-binding vectors pAS1/δWT(−) or pAS1/δWT(−), mutants retaining the first 160 amino acids could still interact, whereas those lacking this region failed to do so (Fig. 3 *B* and *C*). The amino-terminal 135 amino acids cannot mediate association (pACT/WT[Del 135–429]), indicating that the carboxyl end of the responsible domain resides between amino acids 135 and 160 (Fig. 3 *B* and *C*). Of note is the presence of a poly(glycine) and several poly(proline) stretches within the amino-terminal domain of WT1 (delineated in Fig. 3*A*). All vectors used in this study produced fusion proteins of the expected molecular mass, as determined by Western blotting of nuclear extracts prepared from the yeast strain under study (Fig. 3*D*). Thus, the failure of several deletion mutants to interact cannot be attributed to the absence of fusion protein. The *WT1* mutant pACT/WT(−/−) (Gly-201 → Asp), is also capable of associating with wild-type WT1 (Fig. 3*B*). These experiments were reproduced in a second yeast two-hybrid system containing the β-galactosidase reporter on an episome plasmid and using WT1–LexA fusions as the DNA-binding vectors, indicating that the above results are not specific to the system used (H.N., D.H., and D.E.H., unpublished data). These results indicate that WT1 self-associates *in vivo* through an amino-terminal domain and are consistent with our far-Western results presented above (Fig. 2).

DISCUSSION

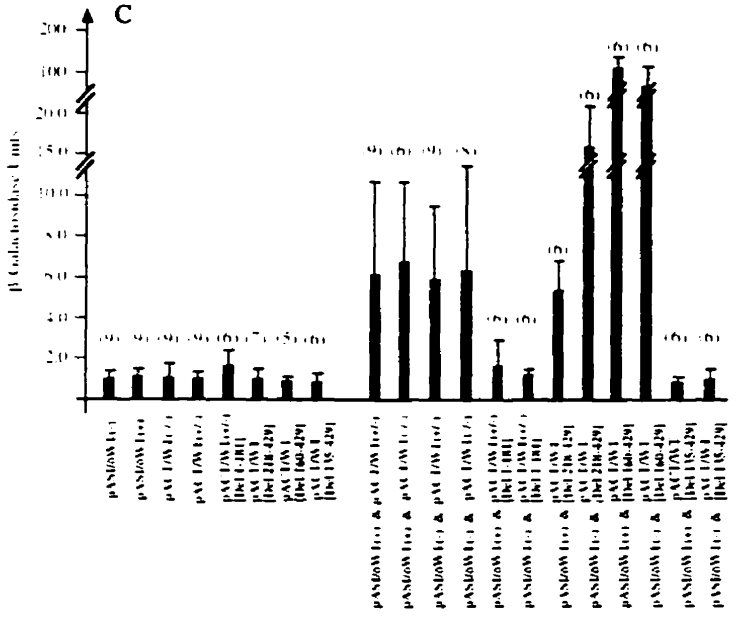
In this report, we demonstrate that WT1(−/−)-mediated transcriptional repression can be antagonized by a DDS allele and that the responsible domain lies within the amino terminus (Fig. 1). These results provide functional evidence that DDS mutations produce molecules that behave in a dominant-negative fashion. Consistent with these mutants mediating their effects by forming oligomers with WT1 is the ability of such molecules to interact *in vitro* and *in vivo* (Figs. 2 and 3). Because wild-type WT1 molecules can also form oligomers, this property is likely important for WT1 transcriptional activity. We have found that some WT1 truncation mutants oligomerize more efficiently or form more stable complexes than full-length WT1 (Fig. 2). This is likely due to amino-terminal conformational differences between truncation mutants and wild-type WT1 protein. Indeed, partial V8 proteolytic digests of truncation mutants of different lengths indicate that conformational differences exist within the amino-terminal region (P.M. and J.P., data not shown). Therefore, some nonsense mutations may produce molecules showing a gain-of-function. Although little phenotypic differences exist between individuals with nonsense versus missense DDS mutations (4, 14), the efficiency by which these mutants usurp activity of the remaining wild-type protein may differ and remains to be elucidated.

Our results are significant in light of experiments demonstrating a difference in the transcriptional properties of WT1, depending on the position and number of WT1 binding sites within the target promoter (11). Whereas WT1 represses transcription of reporter genes containing EGR-1 or TCC$_n$ motifs in transient transfection assays (8), there is a particular situation where WT1 functions as an activator. In the PDGF-A gene, two binding sites (one upstream and one downstream of the transcription start site) are required for transcriptional repression by WT1. Deletion of either site results in transcriptional activation by WT1. In light of our results, we interpret this data to suggest that WT1 dimerizes on the PDGF-A promoter to interfere with assembly of basal transcription factors. As suggested by Wang *et al.* (11), when only one binding site is present, WT1 may function as a transcriptional activator due to unmasking of a usually concealed domain. Consistent with the idea that WT1 has separate functional domains is the finding that amino acid residues 85–124 and 181–250 constitute regions that function independently with a DNA-binding domain to repress or activate transcription, respectively (19). Amino acids 85–124 can enhance activation of a reporter gene by WT1 when cotransfected into NIH 3T3 cells, suggesting that oligomerization may block a repression domain and/or reveal an activation domain (19). Therefore, mutations that change the transcriptional properties of WT1, such as WT1 (Arg-201 → Asp) (18), need not affect oligomer-



B

Vector \ Vector	None	pASI/δWT(-)	pASI/δWT(+)
None	- White	- White	- White
pACTII	- White	- White	- White
pACT/WT(-/-)	- White	++ / Blue	++ / Blue
pACT/WT(+/-)	- White	++ / Blue	++ / Blue
pACT/WT(+/-) [Del 1-181]	- White	n.d.	- White
pACT/WT [Del 218-429]	- White	++ / Blue	++ / Pale Blue
pACT/WT [Del 160-429]	- White	++ /Dk Blue	++ /Dk Blue
pACT/WT [Del 135-429]	- White	- White	White
pACT/WT(-/-) [^{201}G to ^{201}D]	- White	++ / Blue	++ / Blue



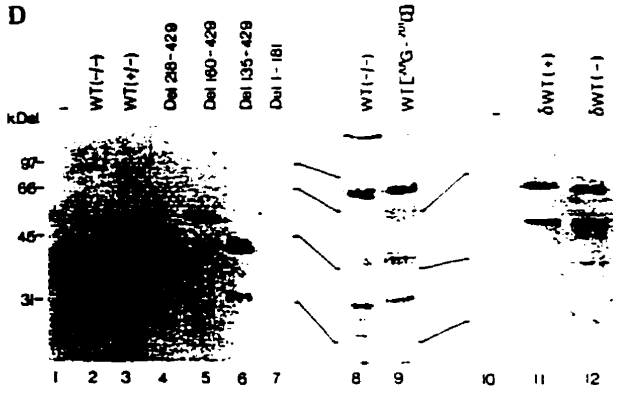


FIG. 3. Self-association of WT1 *in vivo*. (*A*) Schematic representation of yeast transactivation and DNA-binding shuttle plasmids used. Genomic organization of human *WT1* gene is shown at top. Alternatively spliced exons 5 and 9 are represented by cross-hatched boxes. The presence or absence of a WT1 alternative splice site is indicated by a + or − symbol, respectively, after the plasmid name. The four WT1 zinc fingers are represented by dotted boxes. Each exon is numbered with a roman numeral. The poly(glycine) repeat within the first exon is represented by a hatched box, whereas the poly(proline) tracts are represented by darkened boxes. The Gal4 activation and Gal4 DNA-binding domains are represented by stippled boxes and hatched blackened boxes, respectively. The presence of a HA tag in the fusion proteins is indicated. The asterisk represents the approximate location of the amino acid substitution within clone pACT WT(− −)(Gly-201 → Asp). ADH, alcohol dehydrogenase. (*B*) Determination of WT1 interaction dependency and screening. White indicates no β-galactosidase was detected, whereas Blue and Dk (dark) Blue indicate the relative intensity of β-galactosidase. − and + refer to ability to grow on selective plates containing 30 mM 3-aminotriazole and lacking histidine. n.d., Not determined. (*C*) Quantitation of β-galactosidase produced from strain Y190 transformed with yeast expression plasmids of *A*. The number of individual yeast colonies processed for β-galactosidase activity is denoted above each bar in parentheses, and each assay was done in duplicate. Error bars represent the SDs of the experiments. (*D*) Western blot analysis of WT1 fusion proteins produced in strain Y190. One hundred micrograms of protein was fractionated on a 10% polyacrylamide gel and processed for Western blotting with chemiluminescence detection (Amersham) and the anti-HA monoclonal antibody, 12CA5, as recommended by the manufacturer. The WT1 inserts in pACTII are shown above lanes 2–9, and the pASI inserts are shown above lanes 11 and 12. −, Extract prepared from yeast lacking plasmid; a dot denotes the fusion protein of interest.

ization (Figs. 2 and 3) if they alter conformation to expose a previously concealed second, non-overlapping domain.

Mutant *WT1* alleles can inhibit WT1-mediated transcriptional activation in trans (20). Our data indicate that mutant *WT1* alleles can also prevent WT1-mediated transcriptional repression. Reddy *et al.* (20) defined a domain within the first 182 amino acids of WT1 capable of mediating this effect. We have further refined the oligomerization domain. Maheswaran *et al.* (21) have suggested that WT1 can heterodimerize with p53, but we have been unable to demonstrate a direct p53/WT1 interaction *in vivo* using the yeast two-hybrid system (W.B. and J.P., data not shown). The WT1–p53 interaction may thus be mediated by a cofactor not present in yeast cells.

A set of *WT1* nonsense mutations has been previously defined in individuals with WTs and mild genital system anomalies but with no renal nephropathy (13). Although these mutations are also predicted to synthesize truncated WT1 proteins, the affected individuals do not show the more severe phenotypes associated with DDS, and the genetics are more consistent with these being loss-of-function mutations. We speculate that the biochemical properties of these macromolecules differ from those associated with DDS, such that very little or no product results from these alleles (e.g., decreased protein stability, mRNA half-life, impairment of nucleocytoplasmic export). A similar issue regarding structure–phenotype relationships is observed with different alleles of the adenomatous polyposis coli (*APC*) gene in which mutations close to the 5′ end of the gene cause an attenuated form of the disease (22). As well, the variable association of congenital hypertrophy of the retinal pigment epithelium (CHRPE) with adenomatous polyposis coli is defined by the location of mutations with the *APC* gene (23).

Our results are consistent with molecular genetic data in which a small number of nonsense mutations have been associated with DDS—one of which produces a truncated polypeptide of 219 amino acids (14). This mutation, described in an individual with incomplete DDS, defines the smallest known WT1 region predicted to behave in a dominant-negative fashion and encompasses the oligomerization domain we have defined. Our data, as well as results from Reddy *et al.* (20), indicate that the WT1 isoforms cross-regulate each others' activities. Although we did not detect differences in multimerization efficiency among the WT1 isoforms, this event may be regulated *in vivo* either by posttranslational modifications or by subnuclear localization of the isoforms (9). Overexpression of one WT1 isoform could thus function in a dominant-negative manner to deregulate activity of the other isoforms. Experiments using expression vectors to overexpress one WT1 isoform in the context of the endogenous four WT1 isoforms need to be interpreted with caution because the activity of the latter is likely to be perturbed. Clearly the ratio of WT1 isoforms is important for normal embryogenesis, given that mutations that affect splice-site selection at the second alternatively spliced exon (exon 9) deregulate *WT1* gene expression and are associated with DDS (24). The results in this report indicate that WT1 can self-associate and that DDS alleles perturb normal WT1 function by taking advantage of this intrinsic property to sequester functional protein into nonproductive complexes.

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Chapter 5

Identification of Nuclear Localization Signals within the Zinc Fingers of the WT1 Tumor Suppressor Gene Product

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(WT1; Nuclear Localization; Denys-Drash Syndrome; Wilms' Tumor; WT1 gene)

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ABSTRACT

WT1 encodes a zinc finger protein with a key role in urogenital development that is inactivated in a subset of Wilms' tumors. This tumor suppressor gene product contains an amino-terminal dimerization domain required for *trans*-inhibition of wild-type WT1 activity by mutants defective for DNA binding. In the course of characterizing truncation mutants of WT1, we noted that the WT1 zinc fingers contain two functionally independent targeting signals required for nuclear localization of the protein. These novel signals lie within zinc fingers I and within zinc fingers II and III. We demonstrate that nuclear targeting of the WT1 homodimerization domain functionally antagonizes activity of the wild-type protein activity.

1. INTRODUCTION

Wilms' tumor (WT) is an embryonic cancer which presents either sporadically or in the context of genetic susceptibility, generally before the age of 5 years [1]. It accounts for ~5% of pediatric tumors and has been extensively studied to determine how deregulation of the urogenital developmental program leads to cancer initiation. Although most cases of WT are sporadic, ~5-10% show a genetic predisposition and often occur in association with congenital defects [1]. An 11p13 WT suppressor gene, wt1, has been extensively characterized and is implicated by mutational analysis in 10-15% of sporadic WTs [2-4], in some familial WT cases [5], and as playing a role in initiation of this disease [6].

The wt1 gene encodes a protein having many characteristics of a transcription factor: a glutamine/proline-rich amino-terminus [7,8], nuclear localization [9], and four Cys_2-His_2 zinc finger motifs. The three WT1 carboxy-terminal zinc fingers show 64% identity to the three zinc fingers of the early growth response gene-1 (EGR-1) [7,8]. The mRNA contains two alternative sites of translation initiation [10], two alternatively spliced exons [11,12], and undergoes RNA editing [13], thus potentially encoding sixteen different protein isoforms with predicted molecular masses of 52-65 kDa. The function of the alternative translation initiation event, or of the first alternatively spliced exon (exon V), has not been well defined, although exon V can repress transcription when fused to a heterologous DNA binding domain [14]. Splicing of the second alternatively spliced exon (IX) inserts or removes three amino acids (+/-KTS) between zinc fingers III and IV and changes the DNA binding specificity of the protein.

WT1 isoforms can bind to two DNA motifs, albeit with different affinities: i) a GC rich motif with the conserved feature, $^{5'}G^{G}/_{Y}GGGGGA^{G}/_{C}{}^{3'}$, similar to the EGR-1 binding site [15]; and ii) a $(TCC)_n$ containing sequence, $^{5'}$TCCTCCTCCTCCTCTCC$^{3'}$ [16]. A number of genes involved in growth regulation and cellular differentiation contain these binding sites in their promoters and are thought to be regulated by WT1. These include insulin-like growth factor II, insulin-like growth factor 1 receptor, platelet-derived growth factor A-chain, colony stimulating factor-1, transforming growth factor-β1, retinoic acid receptor-α, Pax-2, c-myb, epidermal growth factor receptor, and the wt1 gene itself [for a review, see 17]. The wt1 gene product has the potential to mediate both transcriptional repression and activation [17].

An essential role for WT1 in the development of the urogenital system has been inferred from its normal expression pattern [9, 18], failure of this system to develop in wt1-null mice [19], and aberrant differentiation of this system in children with germline

wt1 mutations [5, 20, for a review see ref. 21]. Male children in which one wt1 allele is deleted as part of the WAGR [WT/ Aniridia/ Genitourinary (GU) anomalies/ Retardation] syndrome often are born with mild genitourinary defects (i.e.- undescended testis and hypospadias). On the other hand, children with Denys-Drash syndrome (DDS) have much more severe developmental disorders of the reproductive system, as well as a characteristic renal nephropathy occurring generally with the first 2 years of life [21]. The majority of DDS individuals harbor wt1 missense mutations within zinc fingers II and III which are thought to behave in a dominant-negative fashion [20, 21]. Consistent with this idea is the recent demonstration that WT1 can oligomerize, that mutant WT1 protein can antagonize activity of wild-type protein [22, 23], and that some WT1 missense mutations in WTs appear to be in a heterozygous configuration [2, 4, 11].

Recently, Larsson et al. [24] demonstrated that different WT1 isoforms localize to distinct compartments of the nucleus - with -KTS isoforms displaying a distribution that parallels that of classical transcription factors such as Sp1 and TFIIB; whereas +KTS isoforms are preferentially associated with interchromatin granules and coiled bodies. These results were recently extended by Englert et al. [25] who demonstrated that overexpression of a WT1 truncation mutant lacking the four zinc finger domains could alter the subnuclear localization of WT1(-KTS). In this report, we set out to define the nuclear localization signal(s) of WT1 and demonstrate that subcellular localization of the WT1 homodimerization domain affects the efficiency by which it can behave in a dominant-negative fashion. Our results identify two nuclear localization domains - one within zinc finger I and one within zinc fingers II and III.

2. MATERIALS AND METHODS

2.1. *Cell culture and transfections*

NIH 3T3 and COS-7 cells were maintained in DMEM containing high glucose plus 10% fetal calf serum (Gibco-BRL). Transfection of NIH-3T3 cells was performed by calcium phosphate precipitation [26], whereas transfection of COS-7 cells was performed by electroporation [9].

2.2. *DNA Constructs*

CMV-based murine WT1 expression vectors were generated by ligating a 1441 bp Sau 3AI WT1 cDNA fragment (containing 42 bp of 5' untranslated region, the coding region, and 109 bp of 3 ' UTR) into the Bam HI site of pgTat-CMV3 [9]. CMV-hWT(+/-) was generated by cleavage of the human WT1 cDNA with Sac II (which cleaves 31 bp upstream of the initiator ATG), repairing with T4 DNA polymerase, followed by ligation to Bgl II linkers ($^{5'}$GGAAGATCTTCC$^{3'}$). The hWT1 cDNA was then digested with Eco RI (which cleaves in the pKS polylinker, 11 bp downstream of the TGA codon), Klenow repaired, and digested with Bgl II. The cDNA insert was then directionally cloned into the Bam HI/Xho I(Klenow repaired) sites of pgTat-CMV3. Expression vectors carrying WT1 mutations previously identified in DDS or hereditary WT patients were generated by PCR-mediated mutagenesis and confirmed by direct sequencing. Details as to the construction of these deletion vectors can be obtained from the author upon request.

2.3. *CAT and β–galactosidase Assays*

Forty eight hours after transfection, cells were harvested and lysed in 0.25 M Tris [pH 8.0]/ 0.1% Nonidet-P40. The cellular debris was pelleted and the lysate assayed for β–galactosidase activity with chlorophenol red β-D-galactopyranoside (Boehringer Mannheim) [26]. Following standardization for β–galactosidase activity, extracts were assayed for CAT activity using ^{14}C-chloramphenicol (Amersham) followed by thin layer chromatography [26]. The position of acetylated and non-acetylated chloramphenicol was identified by autoradiography and CAT activity was calculated by excision of the respective products from the TLC plate followed by scintillation counting.

Subcellular fractions were prepared essentially as described by Dignam et al. (27). Nuclear and cytoplasmic fractions were boiled in 2% SDS for 10 mins. and the insoluble material removed by centrifugation in a microfuge at 4°C for 10 mins. Equivalent cell amounts of each supernatant was fractionated by SDS-PAGE, transferred to Immobilon-P (Millipore Corp.) and probed with the indicated antibodies.

Visualization was performed with Renaissance chemiluminescence reagents (Dupont NEN). The anti-WT1 antibody 180 (directed against the first 180 amino acids of WT1) was purchased from Santa Cruz Biotechnology.

2.4. *Immunofluorescence and β–galactosidase staining*

Cells were seeded onto coverslips and transfected with wt1 expression vectors. Forty eight hours later the cells were washed in cold PBS, fixed for 20 minutes in 3.7% formaldehyde/PBS and 5 minutes at -20°C in methanol. The cells were pre-blocked with 10% goat serum/1% bovine serum albumin/0.3% Triton X-100 for 1 hr., then probed with the indicated antibody for 4 hrs. The antibody was visualized with an anti-rabbit goat antibody conjugated to Texas Red (Jackson Labs). Cover slips were examined under a Zeiss immunofluorescent microscope and photographed with TMax 400 film (Kodak).

For β-galactosidase staining, transfected cells were washed in PBS and fixed in 2% paraformaldehyde/0.2% glutaraldehyde for one minute. The cells were stained at 37°C for 24 hrs with 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) in 5 mM potassium ferrous cyanide, 5 mM potassium ferric cyanide, 2 mM $MgCl_2$. After staining, the cells were fixed with 3.7% formaldehyde for 5 minutes, dehydrated with ethanol, and counterstained with Eosin Y (Sigma). Coverslips were examined under bright light with a Zeiss Axioskop and photographed with TMax 400 film (Kodak).

3. RESULTS

Previous work from our laboratory had demonstrated that WT1 is located exclusively within the nucleus despite the absence of an identifiable nuclear localization signal [9, 28]. Subsequently, Larrson et al. [24] demonstrated that alternative splicing within the WT1 zinc fingers alters the subnuclear distribution profile of the WT1 isoforms. In this report, we undertook to identify the signal(s) responsible for WT1 nuclear targeting.

3.1. The WT1 Nucleic Acid Binding Domain Harbors 2 Nuclear Localization Signals.

To identify the region necessary for nuclear targeting of the WT1 protein, a small number of synthetic deletion mutants, CMV-D3, CMV-D4, and CMV-S1, were generated within the carboxy-terminal domain of WT1 (Fig. 1A). CMV-D3 and CMV-D4 were generated from the murine WT1(-/-) cDNA, whereas CMV-S1 was generated from the human WT1(+/-) cDNA. Since both the human and murine WT1 isoforms localize to the nucleus [9, 24, 28] and the protein product from both species are >96% identical, we do not expect differences in behaviour between the murine and human gene products. Immunofluorescence studies on NIH 3T3 cells transfected with these constructs demonstrated that whereas wild-type WT1 protein concentrated to the nucleus, the protein the product of CMV-D3 was present in both the cytoplasm and the nucleus (Fig 2A). Deletion of zinc fingers III and IV (CMV-D4) or zinc fingers II - IV (CMV-S1) produced mutants still capable of targeting to the nucleus (Fig. 2A). These results indicate that nuclear localization is not a secondary consequence of WT1 binding to DNA.

We and others have previously described a number of missense and nonsense mutations in the WT1 gene occurring in individuals with WT and urogenital system anomalies [21]. Some of these mutations occur in individuals with WT and hypospadias and/or cryptorchidism, but without the renal nephropathy characteristic of DDS, and are thus thought to be loss of function mutations (CMV-P.G., CMV-T.S., CMV-10). Others occur in individuals with renal nephropathy, WT, and genital system anomalies that are much more severe than observed in WAGR syndrome and are thus thought to behave in a dominant-negative fashion (CMV-P.M.) [21]. Still others occur in individuals who have been classified as incomplete DDS, since they do not show the complete triad of malformations generally associated with DDS (CMV-C.N., CMV-S.L.) [21], and the classification of these mutants is not clear. In order to begin the biochemical characterization of these mutants, several were rebuilt into the context of the human WT1(+/-) cDNA (Fig. 1A). When introduced into NIH 3T3 cells, none of

these mutant products were capable of concentrating in the nucleus, consistent with the data presented above. In considering the data obtained with CMV-T.S. and CMV-S1, our results indicate that (a) nuclear localization signal(s) lies between amino acids 291 and 350 of the WT1 polypeptide, possibly within the first zinc finger domain.

This conclusion was substantiated by preparing nuclear and cytoplasmic fractions of COS-7 cells transfected with the various deletion mutants and analyzing the subcellular localization of WT1 by Western blotting (Fig. 2B). Greater than 95% of Sp1 was present in the nuclear fraction of extracts prepared from CMV-WT1(-/-) or CMV-D3 transfected COS cells (Fig. 2B, bottom panel), confirming the integrity of the nuclear preparations. Introduction of CMV-WT1(-/-) into COS-7 cells (Fig. 2B) resulted in the nuclear targeting of >90% of the protein (Fig. 2B, compare lane 2 to 1). Similar results were obtained with CMV-hWT(+/-) (data not shown). However, deletion of amino acids 262 - 364 produced a polypeptide no longer capable of concentrating in the nucleus (compare lanes 3 and 4). Since polypeptides having molecular masses less than 40-50 kDa can passively diffuse into the nucleus [29], we cannot exclude the possibility that nuclear localization of the D3 polypeptide product is due to diffusion. This issue will be experimentally addressed below. All truncation mutants which terminated before the zinc finger domain produced polypeptides which were present in both the cytoplasmic and the nuclear fractions, formally demonstrating that these mutants had lost their ability to concentrate in the nucleus (lanes 5 to 16).

To assess the possibility that (a) nuclear localization domain(s) was present within the WT1 zinc fingers, a series of fusion proteins were generated between the WT1 zinc fingers and β-galactosidase (Fig.3A). Since the molecular mass of the fusion products are greater than 116 kDa and β-galactosidase does not contain a nuclear targeting signal, nuclear targeting should be dependent on the presence of an active signal within the polypeptide molecule. Following transfection of these constructs into COS cells, β-galactosidase localization was determined by direct measurement of enzyme activity (Fig. 3B). Whereas β-galactosidase failed to concentrate in the nucleus, fusion of the four WT1 zinc fingers produced a polypeptide capable of nuclear targeting (p27; Fig. 3B). In addition, fusion of only zinc finger I (p1) or zinc fingers II and III to β-galactosidase (p65) produced polypeptides also capable of nuclear targeting (Fig. 3B), indicating the existence of 2 separable nuclear targeting signals - one in zinc finger I and the other in zinc fingers II and/or III. Interestingly, fusion of zinc fingers III and

IV to β-galactosidase produced a fusion molecule not capable of nuclear targeting (p5; Fig. 3B), suggesting that the presence of a zinc finger *per se* is not sufficient for this behaviour.

3.2. Nuclear targeting is necessary for antagonistic activity by WT1 mutants.

We have previously developed a *trans*-dominant inhibition assay in which DDS alleles, when co-transfected with wild-type WT1 and an appropriate reporter construct, functions *in trans* to prevent transcriptional repression by WT1 (Fig. 4) [23]. Necessary for *trans*-inhibition is the presence of the WT1 multimerization domain which lies within the first 160 amino acids of the protein [22, 23]. WT1 mutants defective for DNA binding, but still retaining the potential for nuclear localization [CMV-WT1/(^{394}R/W) and CMV-S1], are capable of *trans*-dominant inhibition of wild-type WT1 activity in this assay (Fig. 4). The deletion mutant, CMV-P.G., defective for nuclear localization but still capable of multimerization, was unable to effectively *trans*-inhibit wild-type WT1 activity in this assay (Fig. 4B). To directly test whether this was due to decreased efficiency of nuclear targeting, we produced a chimeric fusion whereby the SV40 nuclear localization signal, KKKRKVE, was positioned at the carboxy-terminus of the WT1 dimerization domain (Fig. 4A). This mutant, CMV-ΔBsu36I was targeted to the nucleus (W.B., data not shown) and was capable of partial*trans*-dominant inhibition of wild-type WT1 activity (Fig. 4B). We conclude that deletion mutants which lack nuclear targeting ability cannot inhibit activity of wild-type WT1 protein in*trans* as effectively as missense mutations which disrupt DNA binding but not nuclear targeting.

4. DISCUSSION

In this report, we demonstrate the presence of 2 distinct nuclear localization signals within the DNA binding domain of the WT1 protein (Figs. 2 and 3). Our results indicate that nuclear localization is distinct from DNA binding since mutants impaired for DNA binding can still localize to the nucleus (Fig. 2; CMV-D4). Protein nuclear localization signals (NLS) are generally characterized by one or more clusters of basic amino acids, although no clear consensus sequence has emerged [for a review, see ref. 30]. Although a number of basic residues are present in the WT1 zinc fingers, they can be found in all 4 fingers and no obvious consensus exists with previously identified nuclear targeting signals [30]. We have yet to identify the core sequence motif within finger I and fingers II and III necessary for nuclear targeting of WT1. Recently, a functional receptor for nuclear localization sequences, hSRP1α, has been identified and cloned [31]. This protein can bind both simple and bipartite NLS motifs and is thought to mediate the first step of nuclear import - that is, NLS-dependent docking of the substrate at the nuclear envelope [31]. Using *in vitro* synthesized protein, we failed to detect a protein-protein interaction between WT1 and hSRP1α (S. C., data not shown), suggesting that a second, yet to be identified, receptor may mediate WT1 docking to the nuclear envelope. Consistent with this interpretation is the identification of at least one addition class of NLS receptors believed to mediate import of small nuclear ribonucleoprotein particles [32].

Patients with complete deletions of wt1 (as in the WAGR syndrome) or with mutations that lead to truncation of the WT1 protein have been observed to have mild abnormalities of the genitourinary system, indicating that in humans, loss of one wt1 allele perturbs development of this system. The subsequent discovery of germline missense mutations of wt1 in patients with the severe phenotype of DDS suggested that these mutations act either in a dominant manner or as dominant-negatives. The discovery of a splicing lesion in individuals with DDS supports the dominant-negative model, since the primary sequence of the WT1 isoforms in these individuals are normal and unlikely to exhibit a gain of function [33]. Rather, it is likely that deregulation of the ratio of WT1 isoforms is responsible for the observed phenotype. Further support for this theory was provided when it was shown that WT1 can multimerize and that mutated WT1 can antagonize the function of wild-type WT1 [22, 23]. Thus, in DDS individuals, the mutated WT1 is predicted to reduce the activity of WT1 to levels lower than those found in individuals with deletions of wt1, and thus produce more severe developmental anomalies.

In this report, we demonstrate that WT1 truncation mutants, derived from individuals where the lesions have been postulated to result in a loss of function or to behave as dominant-negatives, failed to concentrate in the nucleus. These results indicate that differences in nuclear targeting cannot account for the differences in phenotypes observed among these individuals. We have measured the half-life of the different truncation mutants in transfected COS-7 cells and have found no significant differences in among them (W. Breuning, data not shown). It is possible that differences in mRNA metabolism between the various mutants may be responsible for whether the mutants behave in a dominant-negative fashion or results in a loss of function, although this hypothesis remains to be tested.

Two reports have documented differences in subnuclear localization among WT1 isoforms [24, 25]. Larsson et al. [24] demonstrated that the -KTS isoforms localized primarily with DNA in transcription factor domains whereas the +KTS isoforms localized primarily with splicing factors. Englert et al. [25] extended these results and demonstrated that deletions of zinc fingers I - IV, I and II, or III and IV, produced a polypeptide which demonstrated predominantly a nuclear speckling pattern. We note one interesting difference between our results and those of Englert et al. [25]. Whereas we find that deletions of zinc fingers I and II produced a polypeptide no longer capable of concentrating in the nucleus (Fig. 2, CMV-D3), Englert et al. [25] have demonstrated that deletions of all four zinc fingers are not required for nuclear localization. Since CMV-D3 also removed amino acids upstream of zinc finger I, we interpret these results to indicate the possible existence of an additional nuclear localization domain immediately amino-terminal of the first zinc finger. We have not tested this region independently as a fusion to β-galactosidase to determine if it is sufficient to impart nuclear localization, although the results of Englert et al. [25] would suggest this to be the case. The presence of several, independently functional, nuclear targeting motifs at the carboxyl-terminus of WT1 may suggests that frameshifts or nonsense mutants with at least one NLS function differently from frameshift or nonsense mutants without an NLS in usurping normal WT1 activity. Our results showing that artificially imparting a NLS to the WT1 dimerization domain improves the efficiency with which *trans*-inhibition of wild-type wt1 activity occurs, is consistent with this hypothesis. Deletion mutants lacking an NLS may function by sequestering wild-type WT1 protein in the cytoplasm.

In summary, our results demonstrate the presence of at least 2 separable NLS within the WT1 zinc fingers (Figs. 2 and 3). Mutants with impaired nuclear targeting

are no longer capable of efficient *trans*-inhibition of wild-type WT1 activity (Fig. 4), and likely affect development of the urogenital system through other mechanisms.

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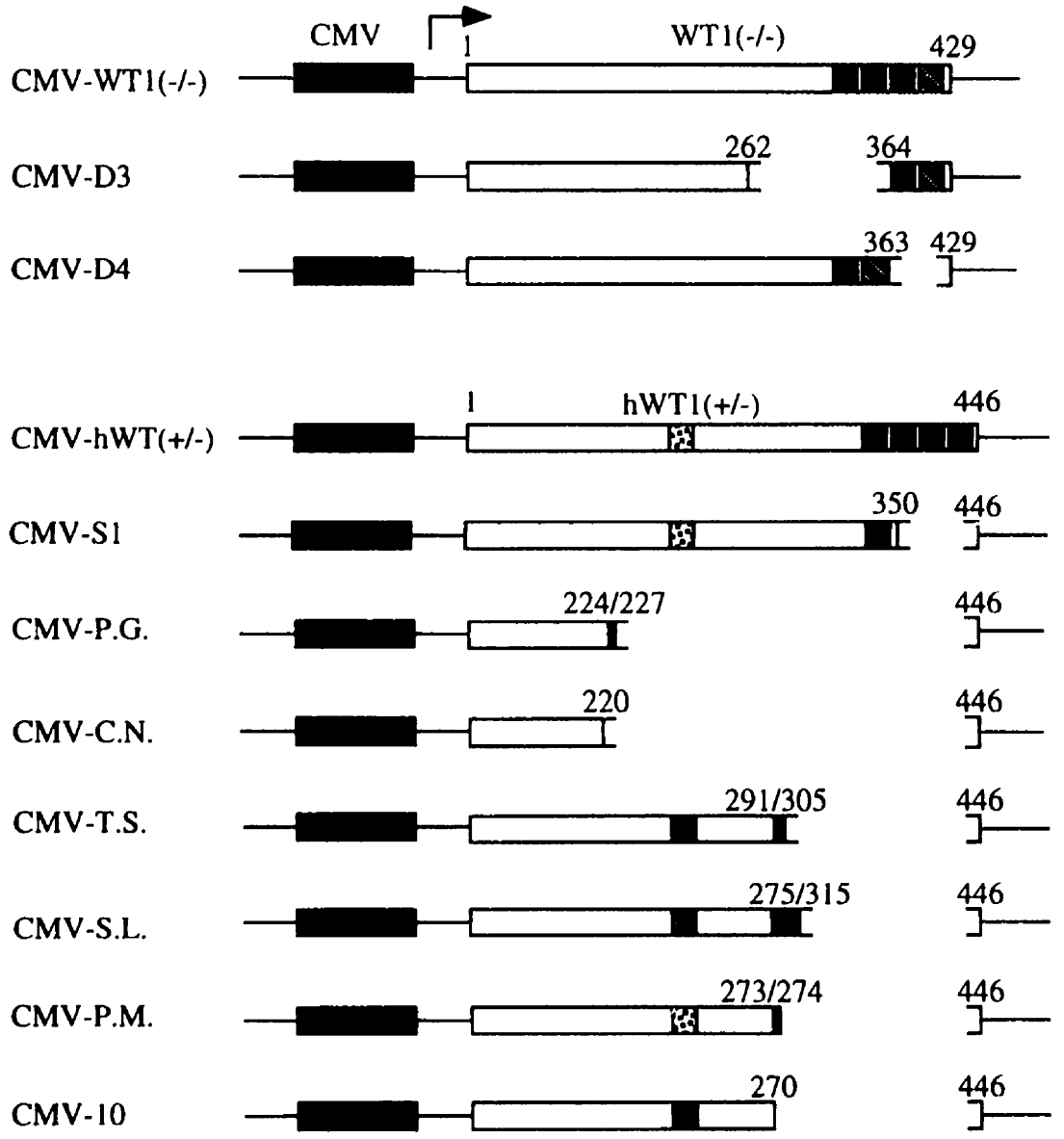
FIGURE LEGENDS

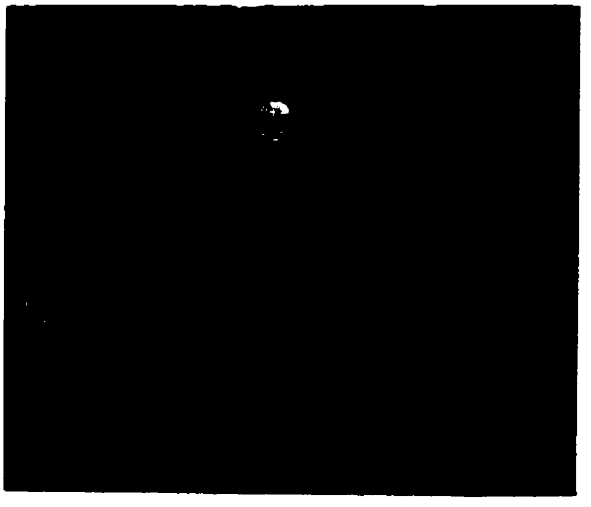
Figure 1. Expression Vectors used for Subcellular Localization of WT1. Schematic diagram of murine and human wt1 expression vectors used in this study. The absence or presence of alternatively spliced exons is indicated by a (-) or (+) sign, respectively. Amino acid positions demarcating the boundary of the deletions are indicated above each construct. The blackened box denotes the CMV promoter, the stippled boxes denote the wt1 zinc fingers, the first alternatively spliced exon is represented by a coarse dotted box. In situations where missense mutations result in a frameshift, the novel open reading frame is denoted by a dotted box. The amino acid position of the predicted frameshift is denoted above the coding region. The start site of transcription is denoted by a right-angled arrow. For those mutations identified in individuals with WTs and genitourinary anomalies, the associated phenotype is presented on the right: N/A, not applicable; WT, Wilms' tumor; WT^b, bilateral WT; Hyp, hypospadias; Cry, cryptorchidism; Cry^b, bilateral cryptorchidism; Nephr., renal nephropathy; XYϕ, XY pseudohermaphroditism. A detailed description of these mutations and the associated phenotypes can be found in the following references: CMV-P.G. [5]; CMV-C.N. [34]; CMV-T.S. [5]; CMV-S.L. [34]; CMV-P.M. [35]; CMV-10 [patient 10 in ref. 36]. The predicted mode of behaviour of each mutation is also indicated: L/F, loss of function; D/N, dominant-negative; ???, unclear. Since patient C.N. has had nephrotic syndrome with minimal change and eventually regained normal renal function [37], and patient S.L. developed renal nephropathy at the age of 15 years, these patients have been classified as incomplete DDS. For the current study, we have not drawn conclusions about the nature of their mutations. Also summarized to the right are the subcellular localization of the deletion mutants from immunofluorescence and cell fractionation studies detailed in Figure 2.

A.

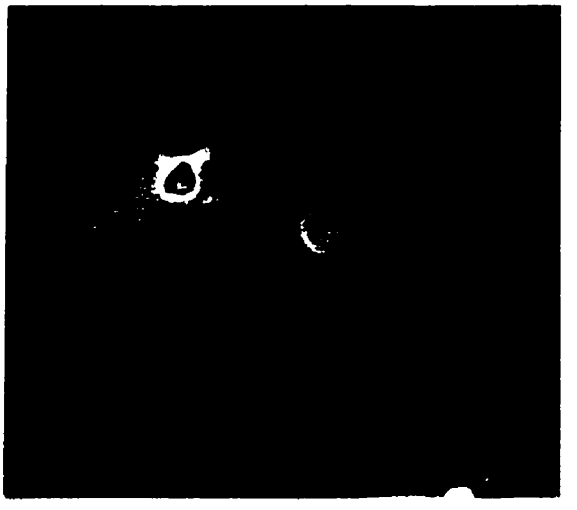


	Associated Phenotype	Predicted Mode of Behaviour of WT1 Mutation	Cellular Localization
CMV-WT1(-/-)	N/A		N
CMV-D3	N/A		N + C
CMV-D4	N/A		N
CMV-hWT(+/-)	N/A		N
CMV-S1	N/A		N
CMV-P.G.	WT^b, Hyp, Cry	L/F	N + C
CMV-C.N.	WT^b, Cry^b	???	N + C
CMV-T.S.	WT, Hyp, Cry^b	L/F	N + C
CMV-S.L.	WT, Nephr.	???	N + C
CMV-P.M.	WT^b,$XY\phi$, Nephr.	D/N	N + C
CMV-10	WT^b, Cry^b	L/F	N + C

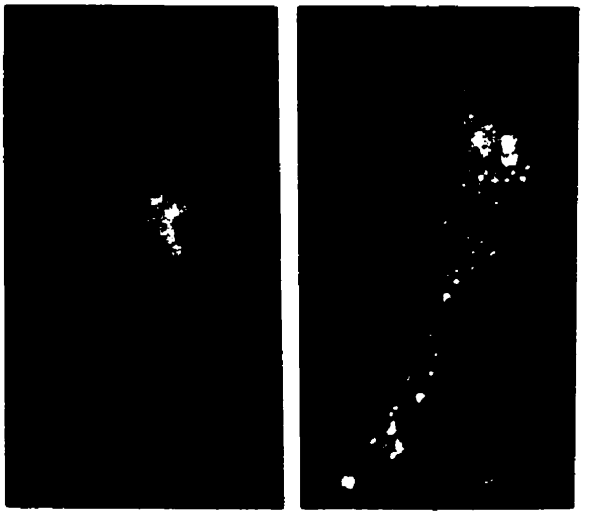
Figure 2. Immunofluorescence and Cell Fractionation Studies of WT1 Deletion Mutants. A) NIH 3T3 cells were transfected with wt1 expression plasmids and the protein products detected with the anti-WT1 antibody 180 by indirect immunofluorescence. The expression vector used in each transfection is indicated below each representative panel. B) Fractionation of cytoplasmic and nuclear extracts prepared from COS-7 cells transfected with wt1 expression vectors. Equivalent cell numbers of cytoplasmic and nuclear extracts were fractionated on a 10% SDS-PAGE, followed by electrophoretic transfer to Immobilon-P. Blots were probed with the anti-WT1 antibody 180 or with the anti-Sp1 antibody PEP2 (Santa Cruz), as previously described [10]. Molecular weight standards are indicated to the left and are New England Biolabs prestained protein markers. Extracts were prepared from COS-7 cells transfected with the following expression vectors: Top Panel - Lanes 1-2, CMV-WT1(-/-); lanes 3-4, CMV-D3; lanes 5-6, CMV-P.G.; lanes 7-8, CMV-C.N.; lanes 9-10, CMV-T.S.; lanes 11-12, CMV-S.L.; lanes 13-14, CMV-P.M.; lanes 15-16, CMV-10. The first four lanes of the bottom panel contain the same protein extracts as analyzed in the first four lanes of the top panel. C, cytoplasmic; N, nuclear.



WT1(-/-)

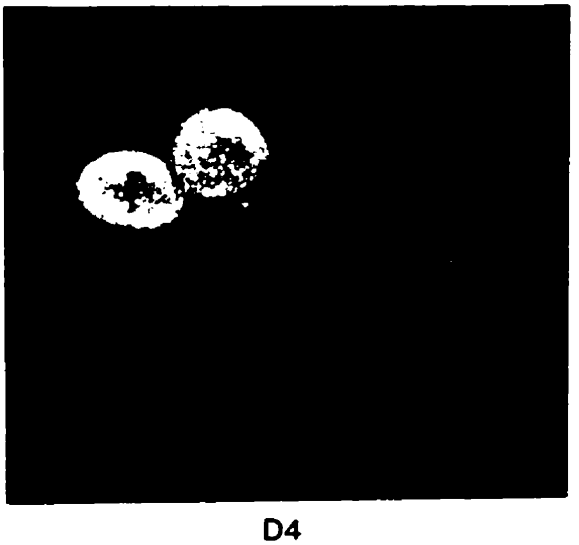


P.G.



D3

T.S. S.L.



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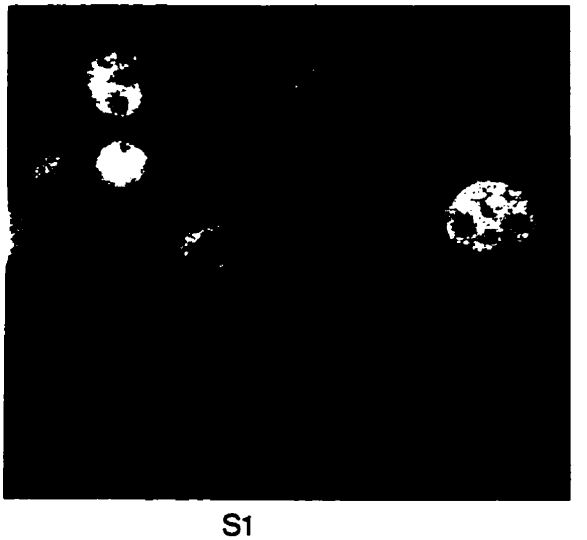


S1

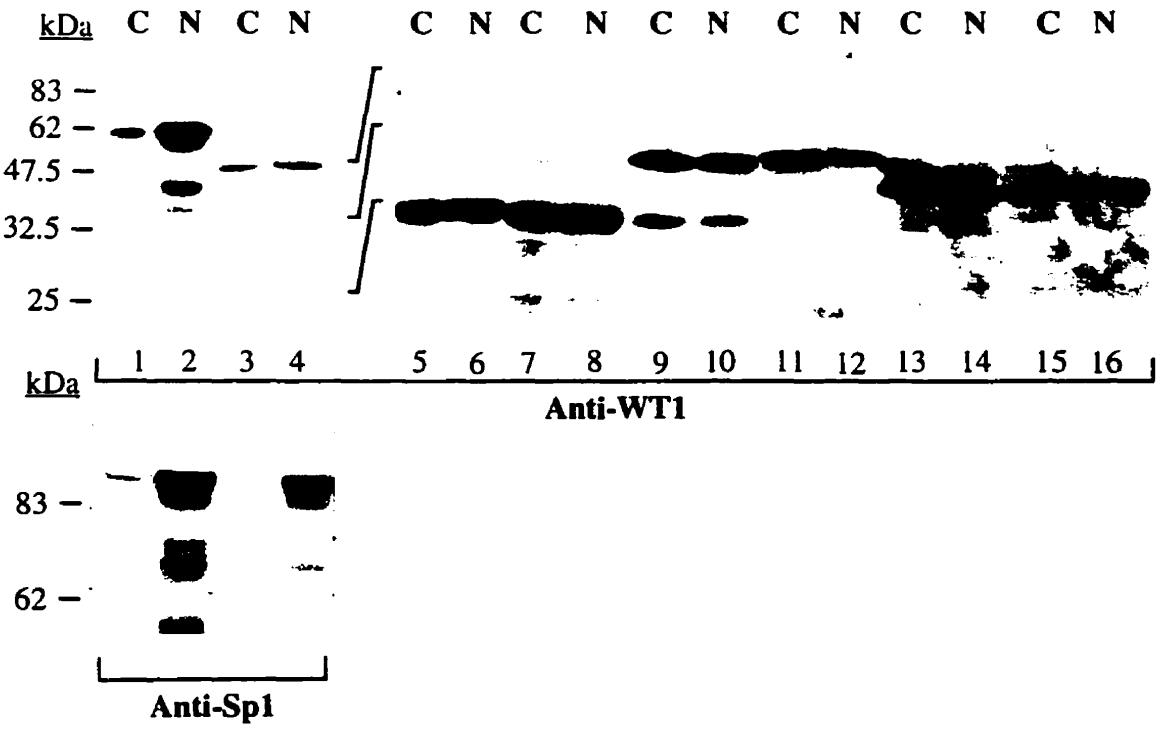
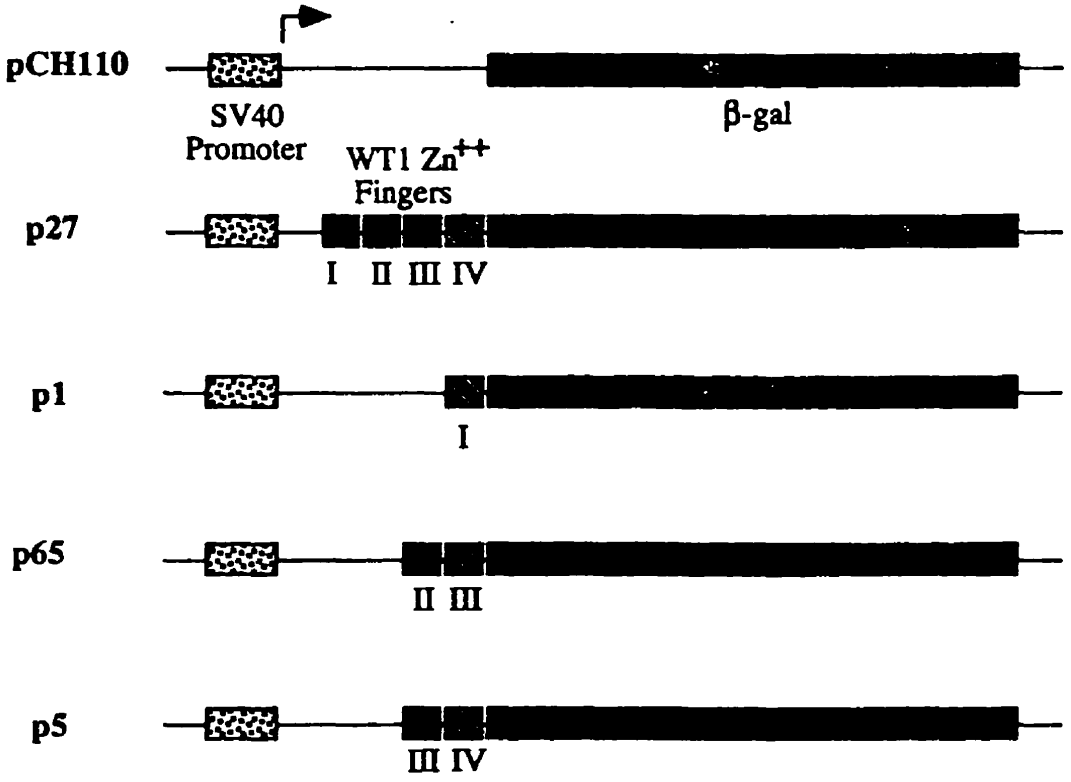

kDa
C N C N C N C N C N C N C N C N
83 –
62 –
47.5 –
32.5 –
25 –
kDa
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Anti-WT1
83 –
62 –
Anti-Sp1

Figure 3. The WT1 Zinc Fingers Harbor 2 Independent Nuclear Transport Signals. A) Expression vectors containing the wt1 zinc fingers fused to the β-galactosidase reporter gene under control of the SV40 promoter. The coarse dotted box represents the SV40 viral promoter, the blackened box symbolizes the β-galactosidase coding region, and the stippled boxes are the wt1 zinc fingers. The identity of the zinc finger(s) fused to β-gal is indicated below each stippled box. Construct names are indicated to the left. B) COS-7 cells were transfected with the expression vectors in A and stained for β-galactosidase activity.

A.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Promoter
β-gal
WT1 Zn++
Fingers
p27
I II III IV
p1
I
p65
II III
p5
III IV

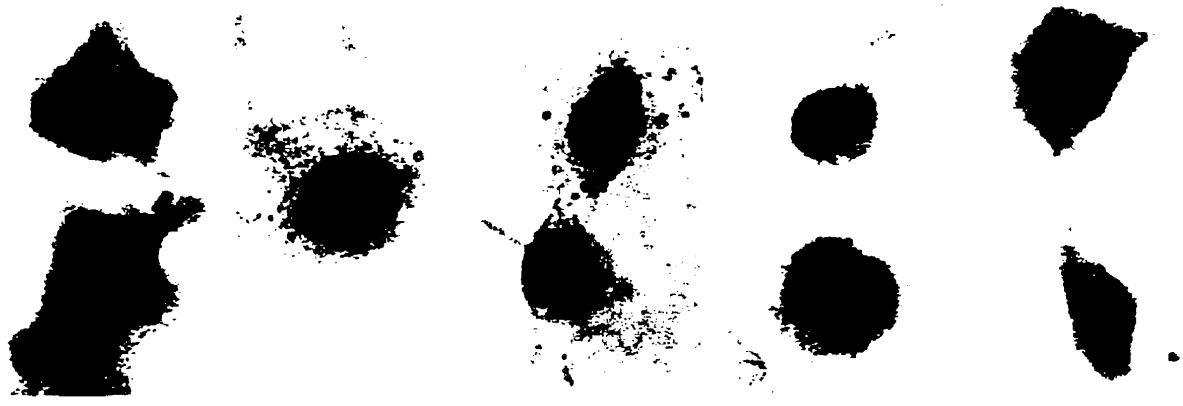
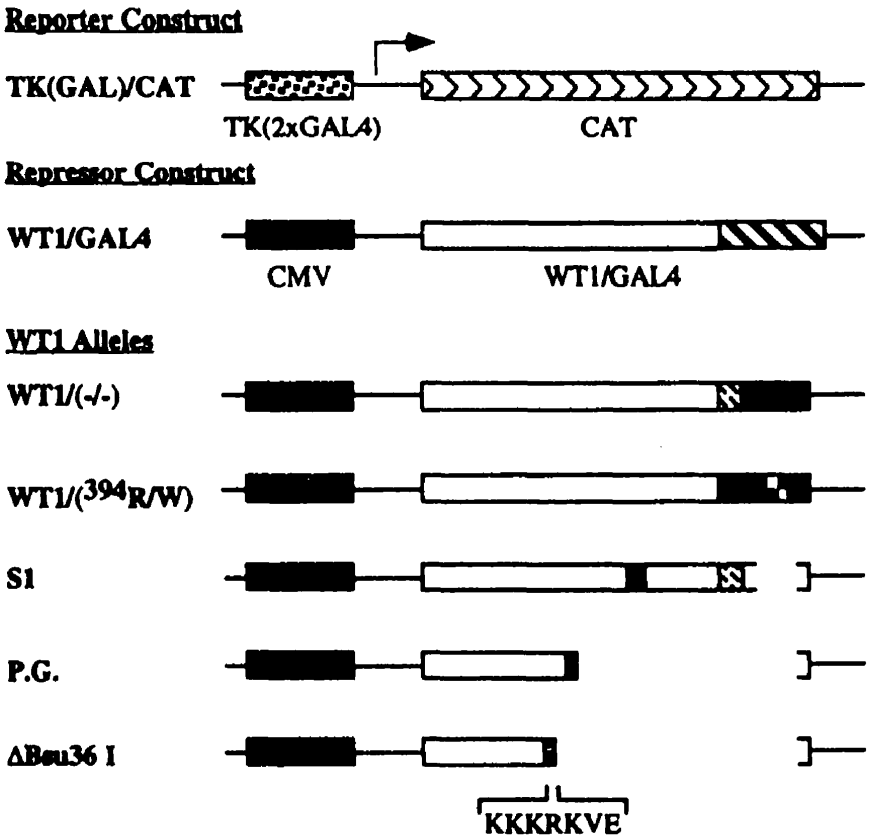
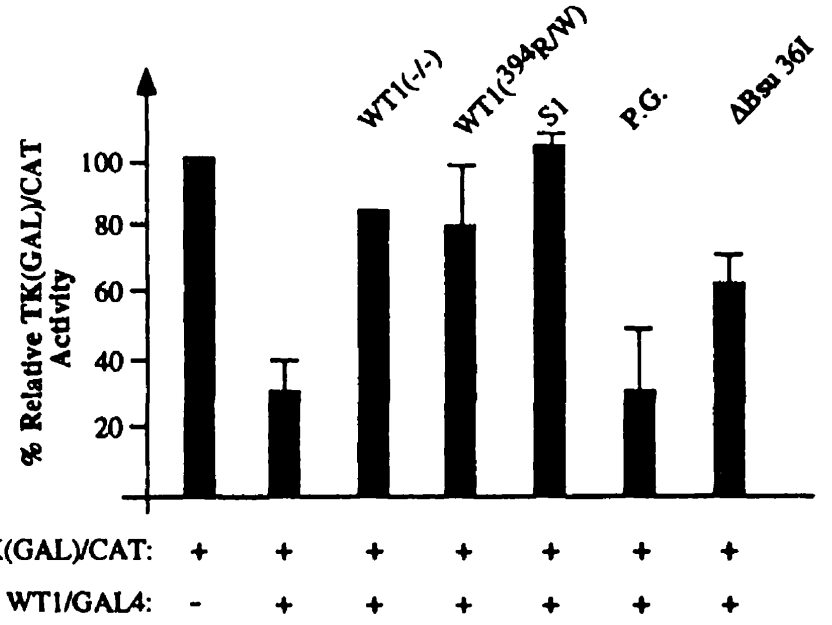

pCH110
p27
p1
p65
p5

Figure 4. Nuclear Localization is Required for *Trans*-dominant Inhibition of Wild-type WT1 Activity. A) Schematic representation of constructs used in the dominant-negative assay. The reporter gene used encodes CAT (zigzag box) under the control of the TK promoter containing two GAL 4 binding sites (coarse dotted box). The WT1 region amino terminal of the zinc fingers is indicated by an open box and the GAL4 binding domain is represented by a hatched box. A variety of wt1 alleles were introduced *in trans* to effect inhibition of WT1/GAL4 activity. CMV-WT1(^{394}R/W) contains the most common DDS mutation - an arginine to tryptophan substitution in zinc finger III (represented by a checkered box) [20]. Construct ΔBsu 36I was generated by introducing an oligonucleotide containing the SV40 nuclear localization signal, followed by a stop codon, into the Bsu 36I/ Avr II sites of CMV/WT1(-/-). B) *Trans*-dominant repression assay of wt1 expression vectors. The CAT activity produced by the reporter alone is defined as 100%. Ten micrograms of WT1/GAL 4 was co-transfected with 5 μg of TK(GAL)/CAT and with 10 μg of the indicated wt1 alleles. Experiments were standardized for transfection efficiency by co-transfection with 2 ug of RSV/β-galactosidase. All experiments were performed at least three times. Standard deviation between experiments is indicated on the graph by error bars. All constructs produced approximately equal amounts of WT1 protein in a dose-dependent fashion as assayed by Western blotting (W. B., data not shown).

A.


Reporter Construct
TK(GAL)/CAT
TK(2xGAL4)
CAT
Repressor Construct
WT1/GAL4
CMV
WT1/GAL4
WT1 Alleles
WT1/(-/-)
WT1/(394R/W)
S1
P.G.
ΔBsu36 I
KKKRKVE

B.


% Relative TK(GAL)/CAT Activity
100
80
60
40
20
WT1(-/-)
WT1(394R/W)
S1
P.G.
ΔBsu 36I
TK(GAL)/CAT: + + + + + + +
WT1/GAL4: - + + + + + +

Chapter 6

Chapter 6: General Discussion

The structure of the *wt1* gene suggests that it should direct the synthesis of four different polypeptides having molecular masses of 47-49 kDa. In Chapter 2 we confirm the presence of these isoforms *in vivo*. In addition, we demonstrate the existence of four additional WT1 isoforms of molecular masses 54-56 kDa. These isoforms were found to be the result of an alternative translational initiation event at a CUG codon upstream of and in frame with the AUG initiation codon. All WT1-expressing tissues and cells were found to express all known isoforms of WT1.

We were unable to identify a new function for the CUG-initiated forms of WT1. The degree of conservation of the amino terminal extension is not as high as for the rest of the WT1 protein. However, the first 35 amino acids of the amino terminal extension are quite conserved (88% identical between mice and humans) and may form a functional domain.

Our discovery of an alternative initiation site brings the total number of different proteins known to be produced from the *wt1* gene to eight. The unique function of each different isoform is not yet known. Efforts to find a function for exon 5 have been inconclusive (Menke et al, 1996; Simms et al, 1995; Wang et al, 1995). The KTS alternatively spliced exon is a different story-- it clearly alters both WT1's DNA binding specificity and its subnuclear localization (Wang et al, 1995; Larsson et al, 1995).

The importance of the KTS motif became especially clear when we found an individual with Denys-Drash syndrome (DDS) carrying a mutation that affected alternative splicing of *wt1* (Chapter 3). This individual's mutation is predicted to dramatically alter the ratio of -KTS/+KTS WT1 isoforms (Table). The discovery of two other individuals with the same lesion confirmed that this particular isoform imbalance can indeed cause DDS. Interestingly, none of these three patients developed Wilms' tumors, although all suffered from severe

sexual ambiguity and kidney failure (Konig et al, 1993; Bardeesy et al, 1994), suggesting that tumor formation requires complete mutational inactivation of *wt1*.

Table. Expected ratios of WT1 isoforms (from Haber et al, 1991).

isoform	normal	DDS splicing mutation
(-/-)	1.0	1.5
(-/+)	3.8	1.0
(+/-)	2.5	3.5
(+/+)	8.3	2.2

Germline mutations at the *wt1* locus which inactivate, by deletion or frameshift, one *wt1* allele, are associated with congenital abnormalities. Such abnormalities usually consist of a predisposition to Wilms' tumors and mild genital system anomalies (such as hypospadias and cryptorchidism). Germline mutations at the *wt1* locus in individuals with Denys-Drash syndrome (DDS) are associated with severe urogenital abnormalities (such as renal failure and intersex disorders) and are thus likely to act in a manner other than as a loss of function. Most mutations associated with DDS are missense mutations of the zinc fingers of *wt1* or mutations that cause truncations of all or part of the zinc finger region (reviewed in Bruening and Pelletier, 1994). The missense mutations have been shown to disrupt WT1's ability to bind to DNA (Little et al, 1995). The mutation described in Chapter 3 alters only the ratio of different WT1 isoforms but still causes DDS. Assuming a common mechanism by which *wt1* mutations cause DDS, it is unlikely that they behave in a dominant, or gain in function fashion, as so many different mutations would be unlikely to have the same effect, and in the individuals with splicing mutations no novel polypeptides are produced.

Another, more likely mechanism for causing DDS is that the mutated WT1 proteins are able to act in a dominant-negative fashion. According to the dominant-negative theory of DDS, mutated WT1 protein binds to and poisons the function of another protein or proteins, causing the severe disease of DDS. Mutations that do not produce mutated WT1 protein, for example, due to deletion of the gene, would only cause a reduction of the amount of WT1 protein available during development, which has only minor consequences for urogenital formation. Our discovery that mutated WT1 could bind to wild-type WT1 and even antagonize wild-type WT1's function strongly supported this theory and suggested that DDS may be caused by a severe depletion of WT1 activity due to dominant-negative activity (Chapter 4).

This theory is able to encompass both missense and splicing types of DDS mutations. If, as our data suggests, the different WT1 isoforms form both homo- and hetero- dimers, and if each type of dimer performs a specific function, one can envisage a situation where the excess of (-/-) and (+/-) isoforms are able to titrate away the (+/+) and (-/+) isoforms from their usual functions and cause serious disruption of WT1 function.

Identification of the region encoded by exons 1 and 2 as the dimerization domain suggested that one can now predict the type of *wt1* mutation a child will be carrying based solely on a description of phenotype. Mutations expected to disrupt the zinc fingers but spare the dimerization domain are predicted to be able to act in a dominant-negative fashion and thus cause DDS. However, examination of all known naturally-occuring germline mutations of *wt1* and associated phenotypes indicates that our theory is not entirely correct. A number of patients with frameshift or nonsense mutations are known (Pelletier et al, 1991). These patients would be predicted to produce a truncated WT1 protein containing a functional dimerization domain, and would thus be expected to develop DDS; however, they do not. It is possible that these patients do

not actually produce a mutated WT1 protein, perhaps due to stability problems or some other mechanism.

Most if not all persons afflicted with DDS carry *wt1* mutations that alter only the zinc finger region (reviewed in Bruening and Pelletier, 1994). Based on the correlation between phenotypes and mutations, it seems an individual must produce a mutated WT1 protein that contains not only the dimerization domain, but also the first zinc finger, in order to develop DDS. Our discovery that the first zinc finger is required for nuclear localization provides an explanation for this correlation-- if the mutated WT1 protein cannot efficiently enter the nucleus, it cannot come into contact with and antagonize the function of wild-type WT1 (and perhaps other transcription factors) (Chapter 5).

The lesions found in DDS individuals can tell us a lot about the function of *wt1*. The gonads are the most obvious site of malformation in children afflicted with DDS, particularly in (XY) children, whose genitals do not masculinize due to the lack of hormonal cues. From the *wt1*-null mouse work, we know that WT1 is required for formation of the gonadal ridge, the earliest stage of gonadal development (Kreidberg et al, 1993). The *wt1*-null mice do not develop kidneys either, but children with DDS develop what appear to be normal kidneys, suggesting that gonadal development is much more sensitive to levels of WT1 than is kidney development. Only later, after birth, do individuals with DDS develop kidney problems. It is unclear whether the lesion in DDS kidneys is some defect in structure that causes damage during kidney function or whether it is due to the lack of some function mediated by WT1 after birth. One group reported that the kidneys of a DDS individual had an excess of mesangial matrix and an aberrant proteoglycan composition of the glomerular basement membrane (van den Heuvel et al, 1995), but these abnormalities may be an effect rather than a cause of the renal disease.

Progressive glomerulosclerosis, the pathology seen in DDS individuals, may be a common pathway of kidney destruction caused by a large number of insults. Animal models of glomerulosclerosis can be generated simply by removing a large portion of the kidney tissue (remnant kidney disease). A model of how progressive glomerulosclerosis gradually destroys kidney function has been developed (Floege et al, 1992). Interestingly, both PDGF and TGF-β are key factors in this disease process; in fact, overexpression of either factor alone can cause glomerulosclerosis to develop (Isaka et al, 1993). WT1 is able to repress the transcription of both of these genes (Gashler et al, 1992; Wang et al, 1992; Dey et al, 1994), suggesting that disruption of *wt1* causes kidney failure by deregulation of these and other growth factors.

The work presented in this thesis shows that the *wt1* locus is more complex than expected, producing a large number of different polypeptides. We have shown that these different isoforms are able to physically interact with each other, suggesting that there are 28 different homo- and hetero- WT1 dimers, each presumably with a different role. We have also shown that the usual ratio of isoforms is vitally important for proper WT1 function; individuals with disrupted ratios develop the severe abnormalities of DDS. This work has also developed and supported a theory for how two different diseases, one mild and one very severe, can both be the result of germline mutations of the same gene, *wt1*.

Final proof of the dominant-negative theory of DDS that is developed and presented in this thesis will require the construction of an animal model of the disease. Mice have proven unsuitable, as they are apparently unaffected by heterozygous mutations of *wt1* (Kreidberg et al, 1993). Perhaps a transgenic rat or pig line could be developed. Such an animal model would provide insight into the function of *wt1* and how its disruption is associated with the development of several human diseases.

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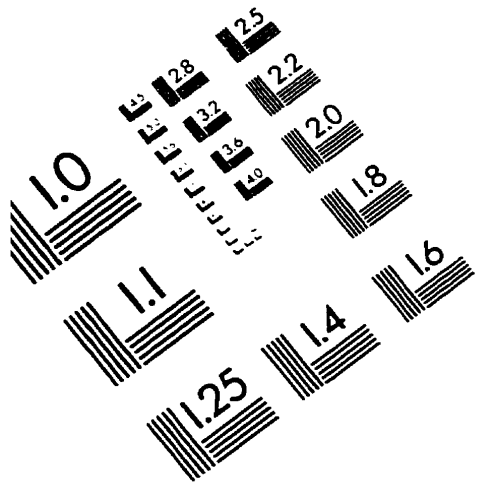
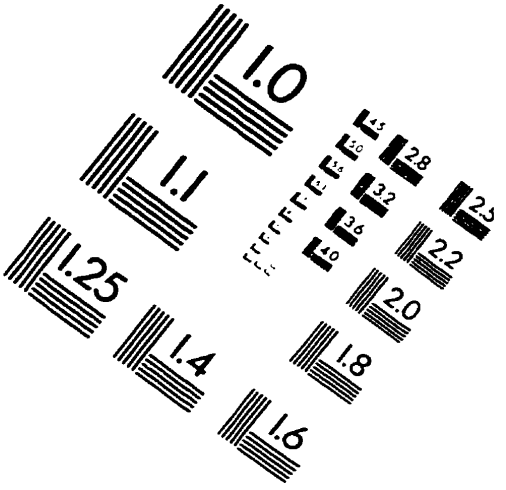
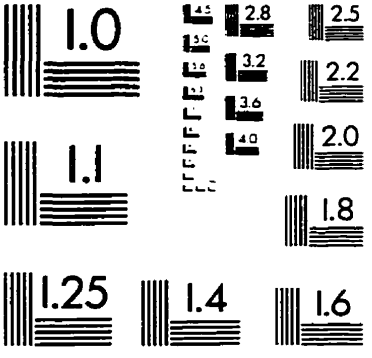
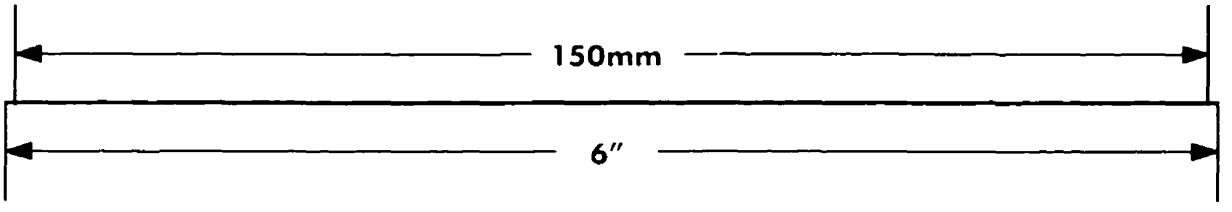
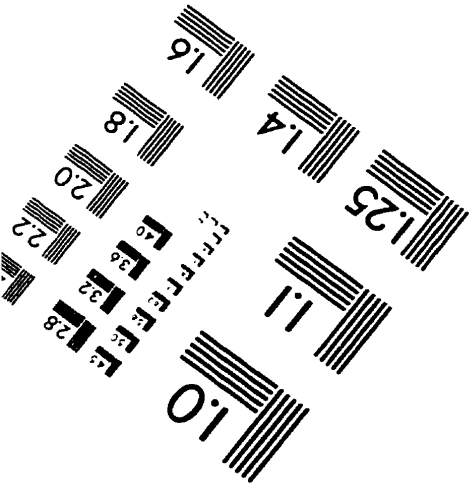
IMAGE EVALUATION TEST TARGET (QA-3)

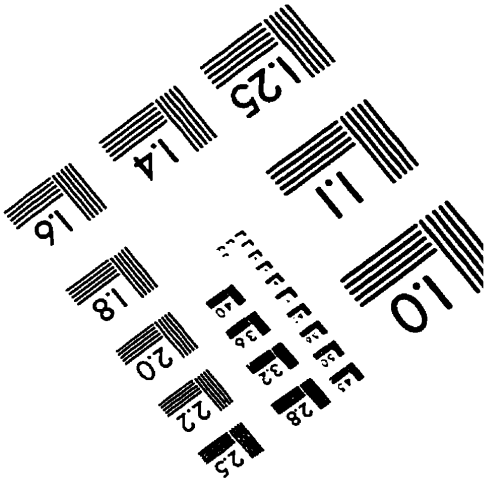












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