Identification of nuclear localization signals within the zinc fingers of the WT1 tumor suppressor gene product

Wendy Bruening[a], Peter Moffett[a], Shea Chia[a], Gunther Heinrich[c], Jerry Pelletier[a,b,*]

[a]*Department of Biochemistry, McGill University, 3655 Drummond Street, Montreal, Quebec H3G 1Y6, Canada*
[b]*Department of Oncology, McGill University, 3655 Drummond Street, Montreal, Quebec H3G 1Y6, Canada*
[c]*KAO Technology, 1 Kendall Square, Cambridge, MA, USA*

Received 7 June 1996

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.

Key words: WT1; Nuclear localization; Denys-Drash syndrome; Wilms' tumor; *WT1* gene

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 16 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: (1) a GC-rich motif with the conserved feature, 5′-$G^G/_YGGGGGA^G/_C$-3′, similar to the EGR-1 binding site [15], and (2) 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 [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 homo-

*Corresponding author. McIntyre Medical Sciences Building, McGill University, Room 902, 3655 Drummond St., Montreal, Quebec H3G 1Y6, Canada. Fax: (514) 398-7384.
E-mail: Jerry@Medcor.McGill.CA

PII S0014-5793(96)00853-8

dimerization 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-HCl (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 min and the insoluble material removed by centrifugation in a microfuge at 4°C for 10 min. 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 min in 3.7% formaldehyde/PBS and 5 min 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 h, then probed with the indicated antibody for 4 h. 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 1 min. The cells were stained at 37°C for 24 h 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 min, 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 two 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 more than 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

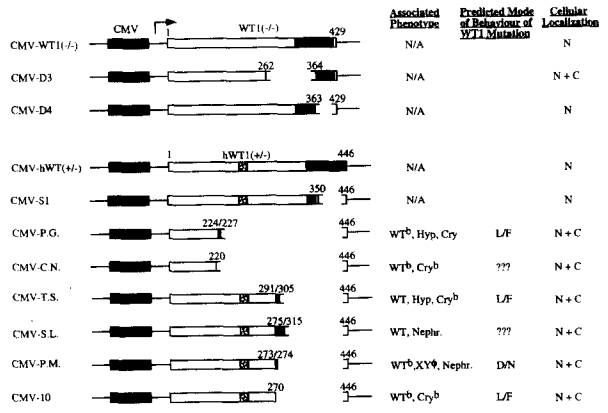


Fig. 1. Expression vectors used for subcellular localization of WT1. Schematic diagram of murine and human *wt1* expression vectors used in this study. The absence or presence of alternatively spliced exons is indicated by a (−) or (+) sign, respectively. Amino acid positions demarcating the boundary 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 shift, 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; $XY\phi$, 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 [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 Fig. 2.

(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 more than 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 5–16).

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 is 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 two 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.

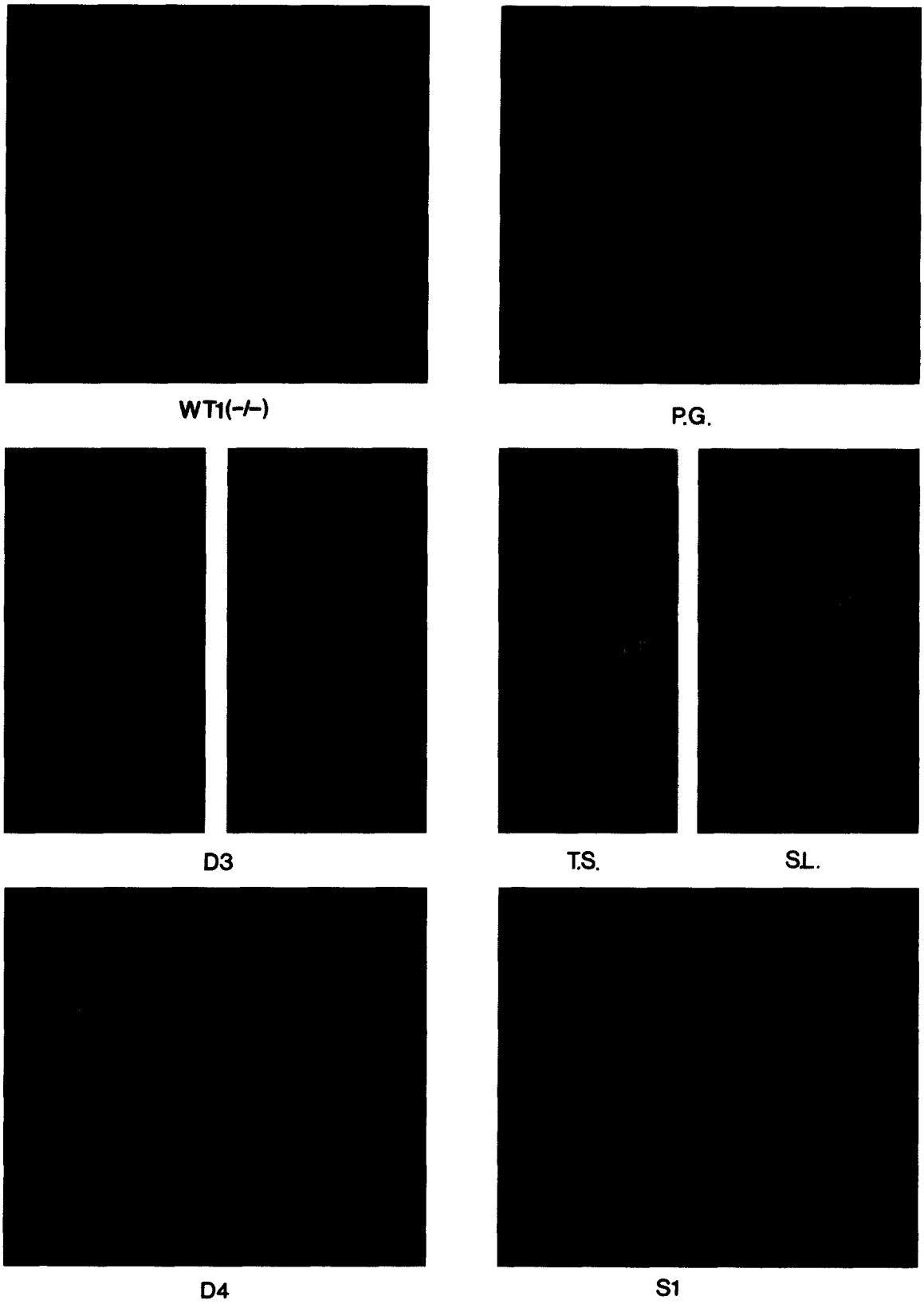
3.2. *Nuclear targeting is necessary for antagonistic activity by WT1 mutants*

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 two 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 four 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

A



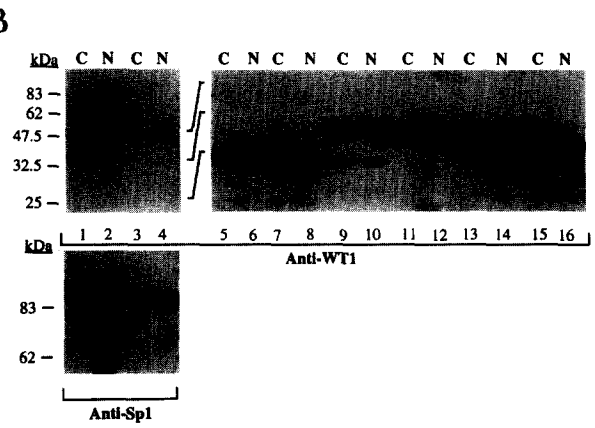


Fig. 2. EImmunofluorescence and cell fractionation studies of WT1 deletion mutants. (A) NIH 3T3 cells were transfected with *wt1* expression plasmids and the protein products detected with the anti-WT1 antibody 180 by indirect immunofluorescence. The expression vector used in each transfection is indicated 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.

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 (Fig. 4). 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. Bruening, 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, pro-

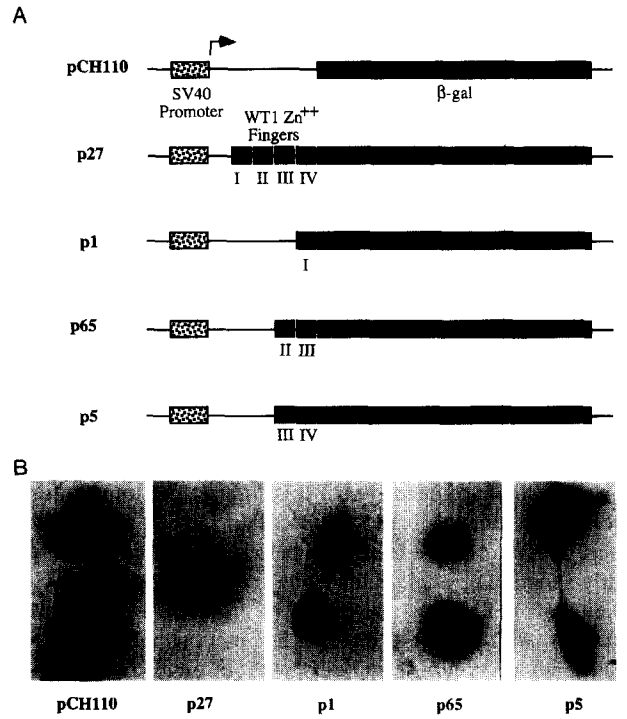


Fig. 3. The WT1 zinc fingers harbor two independent nuclear transport signals. (A) Expression vectors containing the wt1 zinc fingers fused to the β-galactosidas reporter gene under control of the SV40 promoter. The coarse dotted box represents the SV40 viral promoter, the blackened box symbolizes the 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 iindicated to the left. (B) COS-7 cells were transfected with the expression vectors in A and stained for β-galactosidase activity.

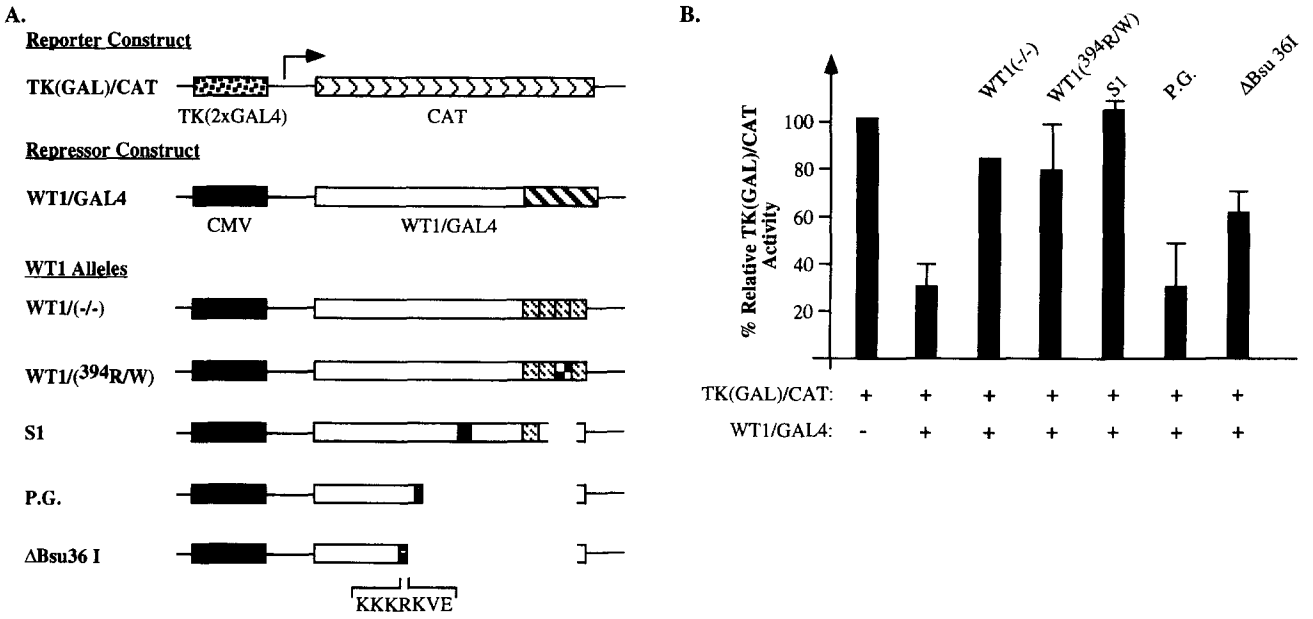


Fig. 4. Nuclear localization is required for *trans*-dominant inhibition of wild-type WT1 activity. (A) Schematic representation of constructs used in the dominant-negative assay. The reporter gene used encodes CAT (zigzag box) under the control of the TK promoter containing two GAL4 binding sites (coarse 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/GAL4 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 μg of RSV/β-galactosidase. All experiments were performed at least 3 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).

duced 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 two 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.

Acknowledgements: W.B. is supported by a fellowship from the Medical Research Council of Canada. J.P. is a Medical Research Council Scientist and this work was supported by a grant from the Medical Research Council of Canada to J.P.

References

[1] Matsunaga, E. (1981) Hum. Genet. 57, 231–246.
[2] Little, M.H., Prosser, J., Condie, A., Smith, P.J., van Heyningen, V. and Hastie, N.D. (1992) Proc. Natl. Acad. Sci. USA 89, 4791–4795.
[3] Coppes, M.J., Liefers, G.J., Paul, P., Yeger, H. and Williams, B.R.G. (1993) Proc. Natl. Acad. Sci. USA 90, 1416–1419.
[4] Varanasi, R., Bardeesy, N., Ghahremani, M., Petruzzi, M.-J., Nowak, N., Adam, M.A., Grundy, P., Shows, T. and Pelletier, J. (1994) Proc. Natl. Acad. Sci. USA 91, 3554–3558.
[5] Pelletier, J., Bruening, W., Li, F.P., Haber, D.A., Glaser, T. and Housman, D.E. (1991) Nature 353, 431–434.
[6] Park, S., Bernard, A., Bove, K., Sens, D.A., Hazen-Martin, D.J., Julian Garvin, A. and Haber, D.A. (1993) Nature Genet. 5, 363–367.
[7] Call, K.M., Glaser, T., Ito, C.Y., Buckler, A.J., Pelletier, J., Haber, D.A., Rose, E.A., Kral, A., Yeger, H., Lewis, W.H., Jones, C. and Housman, D.E. (1990) Cell 60, 509–520.
[8] Gessler, M., Poutska, A., Cavenee, W., Neve, R.L., Orkin, S.H. and Bruns, G.A.P. (1990) Nature 343, 774–778.
[9] Pelletier, J., Schalling, M., Buckler, A.J., Rogers, A., Haber, D.A. and Housman, D. (1991) Genes Dev. 5, 1345–1356.
[10] Bruening, W. and Pelletier, J. (1996) J. Biol. Chem. 271, 8646–8654.

[11] Haber, D.A., Buckler, A., Glaser, T., Call, K., Pelletier, J., Sohn, R., Douglass, E. and Housman, D. (1990) Cell 61, 1257–1269.
[12] Haber, D.A., Sohn, R.L., Buckler, A.J., Pelletier, J., Call, K.M. and Housman, D.E. (1991) Proc. Natl. Acad. Sci. USA 88, 9618–9622.
[13] Sharma, P.M., Bowman, M., Madden, S.L., Rauscher III, F.J. and Sukumar, S. (1994) Genes Dev. 8, 720–731.
[14] Wang, Z.-Y., Qiu, Q.-Q., Huang, J., Gurrieri, M. and Deuel, T.F. (1995) Oncogene 10, 415–422.
[15] Rauscher, F.J. III, Morris, J.F., Tournay, O.E., Cook, D.M. and Curran, T. (1990) Science 250, 1259–1262.
[16] Wang, Z.Y., Qiu, Q.-Q., Enger, K.T. and Deuel, T.F. (1993) Proc. Natl. Acad. Sci. USA 90, 8896–8900.
[17] Rauscher, III, F.J. (1993) FASEB J., 7, 896–903.
[18] Pritchard-Jones, K., Fleming, S., Davidson, D., Bickmore, W., Porteous, D., Gosden, C., Bard, J., Buckler, A., Pelletier, J., Housman, D., van Heyningen, V. and Hastie, N. (1990) Nature 346, 194–197.
[19] Kreidberg, J.A., Sariola, H., Loring, J.M., Maeda, M., Pelletier, J., Housman, D. and Jaensich, R. (1993) Cell 74, 679–691.
[20] Pelletier, J., Bruening, W., Kashtan, C.E., Mauer, S.M., Manivel, J.C., Striegel, J.L., Houghton, D.C., Junien, C., Habib, R., Fouser, L., Fine, R.N., Silverman, B.L., Haber, D.A. and Housman, D.E. (1991) Cell 67, 437–447.
[21] Bruening, W. and Pelletier, J. (1994) Sem. Dev. Biol. 5, 333–343.
[22] Reddy, J.C., Morris, C., Wang, J., English, M.A., Haber, D.S., Shi, Y. and Licht, J.D. (1995) J. Biol. Chem. 270, 10878–10884.
[23] Moffett, P., Bruening, W., Nakagama, H., Bardeesy, N., Housman, D., Housman, D.E. and Pelletier, J. (1995) Proc. Natl. Acad. Sci. USA 92, 11105–11109.
[24] Larsson, S.H., Charlieu, J.-P., Miyagawa, K., Engelkamp, D., Rassoulzadegan, M., Ross, A., Cuzin, F., van Heyningen, V. and Hastie, N.D. (1995) Cell 81, 391–401.
[25] Englert, C., Vidal, M., Maheswaran, S., Ge, Y., Ezzell, R.M., Isselbacher, K.J. and Haber, D.A. (1995) Proc. Natl. Acad. Sci. USA 92, 11960–11964.
[26] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (eds.) (1995) Current Protocols in Molecular Biology. John Wiley.
[27] Dignam, J.D. (1990) Meth. Enzymol. 182, 194–203.
[28] Mundlos, S., Pelletier, J., Darveau, A., Bachmann, M., Winterpacht, A. and Zabel, B. (1993) Development 119, 1329–1341.
[29] Breeuwer, M. and Goldfarb, D. (1990) Cell 60, 999–1008.
[30] Görlich, D. and Mattaj, I.W. (1996) Science 271, 1513–1518.
[31] Weis, K., Mattaj, I.W. and Lamond, A.I. (1995) Science 268, 1049–1053.
[32] Michaud, N. and Goldfarb, D.S. (1991) J. Cell. Biol. 112, 215–223.
[33] Bruening, W., Bardeesy, N., Silverman, B.L., Cohn, R.A., Machin, G.A., Aronson, J., Housman, D. and Pelletier, J. (1992) Nature Genet. 1, 144–148.
[34] Bardeesy, N., Zabel, B., Schmitt, K. and Pelletier, J. (1994) Genomics 21, 663–665.
[35] Baird, P.N., Santos, A., Groves, N., Jadresic, L. and Cowell, J.K. (1992) Hum. Mol. Genet. 1, 301–305.
[36] Nordenskjöld, A., Friedman, E., Sandstedt, B., Söderhöll, S. and Anvret, M. (1995) Int. J. Cancer 63, 516–522.
[37] Schmitt, K., Zabel, B., Tulzer, G., Eitelberger, F. and Pelletier, J. (1995) Eur. J. Pediat. 154, 577–581.