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

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(Received for publication, November 1, 1995)

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 an embryonal malignancy of the kidney that affects one out of every 10,000 children (Matsumaga, 1981). Approximately 7–15% of sporadic WTs contain detectable mutations in the tumor suppressor gene WT1 (Coppe 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 and is predicted to direct the synthesis of a GC-rich motif (5’-CCGGGGCCG-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 protooncogenes (MYC, INT-2, PIM-1, and LYL-1) (Adland 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 (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 (Bock and Kolakofsky, 1994; Grünert and Jackson, 1994). Our
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/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 32P-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 MgCl2, 10 mM KCl, 0.1% Nonidet P-40, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride. Following a 10-min incubation on ice, the suspension was passed ten times through a 23-g needle. The nuclear extract pellets and resuspended in three times the packed cell volume of RIPAp 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 (Amerham 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 [35S]methionine for 12 h. Following a wash with PBS, the cells were lysed in RIPAp buffer. For each immunoprecipitation, approximately 108 cpm of lystate was pre-cleaned with protein A-Sepharose (Phamacia Biotech Inc.) and preimmune serum for 1 h at 4°C. Immune serum for 1 h at 4°C. Incubation with specific antibody and protein A-Sepharose was then performed for 4 h at 4°C. Following electrophoresis, the gel was treated with ENHance (New England Nuclear), and detected by exposure to X-Omat film (Kodak).

**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. 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 32P-labeled β-actin. B, immunoprecipitation from TM3 cells using the polyclonal anti-WT1 605 antibody. Immunoprecipitations performed on [35S]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 57–58 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) and preimmune serum (lanes 4–6). Following electrophoresis, the gel was treated with ENHance (New England Nuclear), dried, and exposed to X-Omat (Kodak) film at −70°C for 2 days. C, Western blot analysis of nucleic extracts prepared from COS-1 and TM3 cells. Blots were probed with monoclonal antibodies B87 (lanes 1–3) and 13B5 (lanes 4–6), which recognize WT1 isoforms lacking or containing the first alternatively spliced exons.

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 [3H]CTP (New Eng-
mouse testis), revealed significant levels of wt1 mRNA by Northern blot analysis (Fig. 1A, 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. 1A, 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 [35S]methionine-labeled Wt1 from COS-1 cells transfected with a CMV-wt1 expression vector (denoted by a dot in Fig. 1B, lane 2). This polypeptide is not recognized by preimmune serum (Fig. 1B, lane 5), nor is it immunoprecipitated from untransfected COS-1 cells (Fig. 1B, 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. 1B, lane 3). Like Wt1, this polypeptide is not visible in immunoprecipitates of TM3 cells with preimmune serum (Fig. 1B, 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. 1C, lane 2). This polypeptide is not present in untransfected COS-1 cells (Fig. 1C, lane 1). In TM3 cells, a ~57-kDa polypeptide is also recognized by this antibody (indicated by a × in Fig. 1C, lane 3). Antibody 13B5 recognizes the Wt1(+/−) isoform produced in COS-1 cells transfected with CMV/wt1(+/−). 8A7 recognizes the Wt1(+/−) protein isoform as expected (denoted by a dot, Fig. 1C, lane 5). In TM3 cells, a ~57-kDa polypeptide is also detected with this antibody (indicated by a × in Fig. 1C, 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
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. 2A). 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. 2B, lane 1). Two of the tumors examined also showed expression of WT1* (Fig. 2B, 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. 3A). Two of the constructs {pKS/mwt1[+182] and pSP/hWT1[+200]} contain shortened 5'-UTRs that lack the above described CUG codons, whereas others {pKS/mwt1[+475] and pKS/hWT1[+378]} had 5'-UTRs that included these CUG codons (Fig. 3A). Following in vitro transcription and translation, [35S]methionine-labeled protein products were analyzed by SDS-PAGE. In the absence of exogenously added mRNA, no protein product is detectable (Fig. 3B, lane 1). The truncation mutants, pKS/mwt1[+182] and pSP/hWT1[+200], produced only one polypeptide species having a molecular mass of ~50 kDa (Fig. 3B, 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. 2A). Translation of mRNA produced from pKS/mwt1[+475] and pKS/hWT1[+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. 3B, 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. 4A). In addition to truncating various portions of the 5'-UTR, stop codons were introduced that flanked the CUG codons identified above (Fig. 2A), 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. 4B, lanes 2-5), whereas a deletion removing all but 182 nucleotides of 5'-UTR generated a transcript unable to produce WT1* (Fig. 4B, 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. 4B, 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
production of Wt1* (Fig. 4B, 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. 4B, 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. 2A). Converting CUG<sup>204</sup> to CUC<sup>204</sup> drastically affected expression of Wt1* while having little effect on production of Wt1 (Fig. 4B, lane 8). Expression of Wt1* was reduced ~10-fold but not completely abolished. Mutagenesis of CUG<sup>192</sup> to CUC<sup>192</sup> had no effect on production of Wt1* (Fig. 4B, lane 9), and mutagenesis of both CUG codons...
had the same effect as altering only CUG\textsuperscript{204} (Fig. 4B, lane 10). These results strongly indicate that translational initiation at CUG\textsuperscript{204} is responsible for production of Wt1\textsuperscript{*}. We interpret the residual production of Wt1\textsuperscript{*} observed with wt1\textsuperscript{1475}(CTC\textsuperscript{204}) and wt1\textsuperscript{1475}(CTC\textsuperscript{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\textsuperscript{1475}(TGA\textsuperscript{229}) and wt1\textsuperscript{1475}(TGA\textsuperscript{183}) (Fig. 4B) that the Wt1\textsuperscript{*} translation codon must lie between nucleotides +183 and +229. To rule out the possibility that the initiator codon for Wt1\textsuperscript{*} was upstream or downstream of CUG\textsuperscript{204} and that the effects on translation initiation of mutating CUG\textsuperscript{204} were not the result of altering a motif that indirectly influenced the efficiency of initiation, four additional constructs were generated (Fig. 5A). One of these, wt1\textsuperscript{1475}(ATG\textsuperscript{204}), retains 202 base pairs of 5'-UTR and terminates at the G residue of CUG\textsuperscript{204}. Should translation of Wt1\textsuperscript{*} commence downstream of CUG\textsuperscript{204}, then this construct should generate RNA competent for Wt1\textsuperscript{*} production. Two other mutants alter the identity of CUG\textsuperscript{204}, converting it either to the more efficient AUG initiation codon, wt1\textsuperscript{1475}(ATG\textsuperscript{204}), or to a stop codon, wt1\textsuperscript{1475}(TGA\textsuperscript{204}).
wt1[+475](Δ229–205) generates a 24-base pair in-frame deletion immediately upstream of the CUG204 codon and will abolish production of Wt1* only if the initiator codon lies upstream of CUG204. Following in vitro transcription/translation of these plasmids and their respective mRNAs, the [35S]methionine-labeled protein products were analyzed by SDS-PAGE. Translation of wt1[+475] produced both Wt1* and Wt1 as expected (Fig. 5B, 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 CUG204. Mutation of CUG204 to an AUG codon increased the production of Wt1* (Fig. 5B, lane 4), consistent with the assignment of CUG204 as the initiation codon. Conversion of CUG204 to a UGA codon abolished production of Wt1*, whereas production of Wt1 was still observed (Fig. 5B, lane 5). Removal of nucleotides 229–205 affected the overall translational efficiency of the mRNA produced (Fig. 5B, compare intensity of products in lane 7 with those in lane 6); however, the production of both Wt1* and Wt1 was still observed (Fig. 5B, lane 7), indicating that the Wt1* initiator codon does not lie upstream of CUG204. These results indicate that CUG204 is the Wt1* initiation codon.

Wt1* Is Present in the Nucleus—To determine whether the novel amino-terminal domain of Wt1* alters the transcriptional properties of Wt1, 3 wt1/GAL4 expression vectors were generated (Fig. 7A). 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. 7B). 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. 7B). 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.
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. 1C). In addition, two WT1* isoforms are clearly detectable on Western blots of whole cell extracts of WTs (Fig. 2B). 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), indicates 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 CUG204 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’-XXAXXCUGG-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 CUG204 to CUC204 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 CUG204 significantly reduced the overall translation efficiency of the template mRNA (Fig. 5B, 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 CUG204 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 CUG204 on the wt1 mRNA template could account for the effects seen on global initiation by wt1(+475)[Δ229–205] (Fig. 5B). 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 et
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 WT1 isoforms with an amino-terminal extension is not obvious. The amino-terminal domain on its own does not have a strong 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. 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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