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Isolation, Characterization, and Expression of the Murine Wilms' Tumor Gene (WT1) During Kidney Development

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The human Wilms' tumor predisposition gene, WT1, is a Cys-His zinc finger polypeptide which appears to be a transcription factor controlling gene expression during embryonic kidney development. In order to analyze the role of the WT1 gene in nephroblast differentiation, we have isolated the murine homolog of human WT1. An extremely high level of amino acid sequence conservation (>95%) extends throughout all regions of the predicted mouse and human WT1 polypeptides. Two alternative splices within the WT1 transcript have been conserved between mice and humans, suggesting that these have functional significance. Expression of the mouse WT1 mRNA in fetal kidney increases during late gestation, peaks just prior to or shortly after birth, and declines dramatically by 15 days postpartum. Developmental regulation of WT1 expression appears to be selective for the kidney. The restriction of WT1 expression to a limited number of tissues is in contrast to previously described tumor suppressor genes. In addition, the narrow window of time during which WT1 is expressed at high levels in the kidney is consistent with the origin of Wilms' tumor from primitive nephroblasts and the postulated role of this gene as a negative regulator of growth.

Wilms' tumor is an embryonal renal neoplasm which develops in 1 in 10,000 children, occurring in both sporadic and familial forms (22). Wilms' tumor is thought to arise in multipotent cells of the metanephric blastema, which give rise to the various structures of the mature nephron (26). Genetic data indicate that inactivation of a negative regulator of cell growth, or tumor suppressor gene, is a rate-limiting step in the development of Wilms' tumor, in a fashion analogous to what occurs in retinoblastoma (17, 19). The contribution of a tumor suppressor locus on chromosome 11 band p13 to the etiology of Wilms' tumor was originally identified by constitutional deletions of 11p13 in patients with WAGR syndrome (a symptom complex of Wilms' tumor, aniridia, genitourinary anomalies, and mental retardation) (9, 24, 29). Subsequent analysis of sporadic Wilms' tumors led to the identification of smaller deletions of genetic material within 11p13 in some Wilms' tumors (21). Recently, we and others (5, 12) have isolated a human gene, WT1, which fits the profile for the 11p13 Wilms' tumor suppressor gene. WT1 maps precisely to the 11p13 region defined by deletions in WAGR patients and is uniquely inactivated by deletion in a number of sporadic Wilms' tumors. WT1 spans approximately 50 kbp of genomic DNA and is predicted to encode a polypeptide with four Cys-His zinc fingers. This structural relationship between the predicted WT1 polypeptide and DNA-binding proteins known to control transcription suggests that WT1 may be a regulator of transcription.

Preliminary studies indicate that expression of the WT1 gene is restricted to a limited set of tissues (5). Highest expression levels were observed in the developing kidney (16, 27). In situ hybridization showed that WT1 expression is limited primarily to condensing cells of the metanephric blastema and the inner face of the developing Bowman's capsule, both of which give rise to structures of the mature nephron (27). These observations suggest that WT1 may regulate transcription during nephroblast development.

An animal model such as the mouse would clearly contribute to an understanding of the role of WT1 in kidney development. In addition to the obvious technical advantages which the mouse presents for studies of differentiation and development, we have recently described a deletion encompassing the *Sey* locus in the mouse which resembles human 11p13 WAGR deletions (14). The isolation and characterization of a mouse homolog to the WT1 gene will permit a number of important issues regarding the role of WT1 in differentiation and development to be addressed. We report here on the properties of the mouse homolog to WT1.

MATERIALS AND METHODS

cDNA libraries, screening, and sequencing. A cDNA library was constructed in lambda ZAPII (Stratagene) from FVB/N 17-day embryonic kidney mRNA by using Lambda Librarian cDNA synthesis reagents (Invitrogen). Plaques (5×10^5) were screened with a low-stringency hybridization solution (35% formamide, 10% dextran sulfate, 1 M NaCl, 1× Denhardt's solution [0.02% each bovine serum albumin, Ficoll, and polyvinylpyrrolidone], 50 mM Tris [pH 7.5], 0.5% sodium dodecyl sulfate [SDS], 100 μg of sheared denatured salmon sperm DNA per ml) at 42°C. The probe used was a 1.7-kbp *Eco*RI-*Bgl*II fragment of the human cDNA WT33, labeled with [α-^{32}P]dCTP to high specific activity by the random priming method (8). Testis cDNA clones were derived from a library constructed in λgt10 from FVB/N adult testis, kindly provided by the laboratory of D. Page. This library was screened at high stringency (50% formamide) with the mouse cDNA clone pMEK91.

Sequencing of cDNAs was performed by the dideoxynucleotide chain termination method (31).

Southern analysis. Genomic DNA was isolated from mouse tissues by the method of Gros-Bellard et al. (15).

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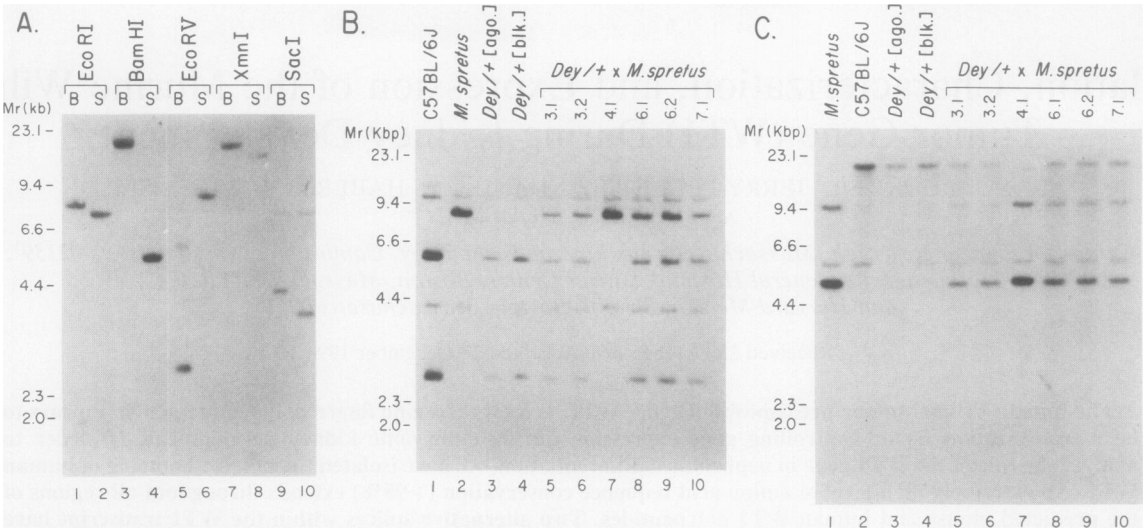


FIG. 1. Localization of murine WT1 cDNAs homologous to the Sey^{Dey} deletion. (A) Identification of interspecific RFLPs with the cDNA pMEK91. Genomic DNA from C57BL6/J (B) and *M. spretus* (S) tissues was digested with various restriction endonucleases and analyzed by Southern hybridization. Representative RFLPs defining the specific alleles are shown. (B and C) Analysis of the Sey^{Dey} deletion for sequences which encompass pMEK91. Progeny from interspecific crosses between Sey^{Dey}/+ *M. domesticus* females and *M. spretus* males have been described before (14). Genomic DNAs from these animals, their parents, and B6 mice were digested with *Eco*RV (B) or *Bam*HI (C) and analyzed for the presence of *M. domesticus*-specific alleles by using pMEK91 insert DNA as the probe. DNAs from two *Dey*/+ females were analyzed. These animals differed in coat color, one agouti (ago.) and one black (blk.), due to the alternative crossing of the Sey^{Dey} allele between C57BL6/J and C3H/HeJ genetic backgrounds in order to maintain strain vigor. The Dey^{Sey} allele arose in a C3H/HeJ mouse. C3H/HeJ and C57BL6/J mice do not show RFLPs with *Bam*HI or *Eco*RV with pMEK91 as the probe (not shown). The six progeny mice are offspring of the black female. The higher signal intensities of panels B and C (relative to A) were used to emphasize the complete absence of *M. domesticus*-specific alleles in the affected offspring 4.1.

DNA (10 μg) was then digested with the indicated restriction endonuclease, electrophoresed through an 0.8% agarose gel, and blotted onto a Zetabind membrane filter (AMF-Cuno). The filter was then washed in 0.1% standard saline citrate (SSC; 0.15 M NaCl, 0.015 M sodium citrate)–1% SDS at 65°C. Prehybridization and hybridization were performed in 50% formamide–10% dextran sulfate–1 M NaCl–1× Denhardt's solution–50 mM Tris (pH 7.5)–0.5% SDS–100 μg of sheared denatured salmon sperm DNA per ml at 42°C. DNA probes were labeled by the random priming method. Filters were washed to a stringency of 0.2× SSC–0.1% SDS at 65°C and exposed to autoradiographic film.

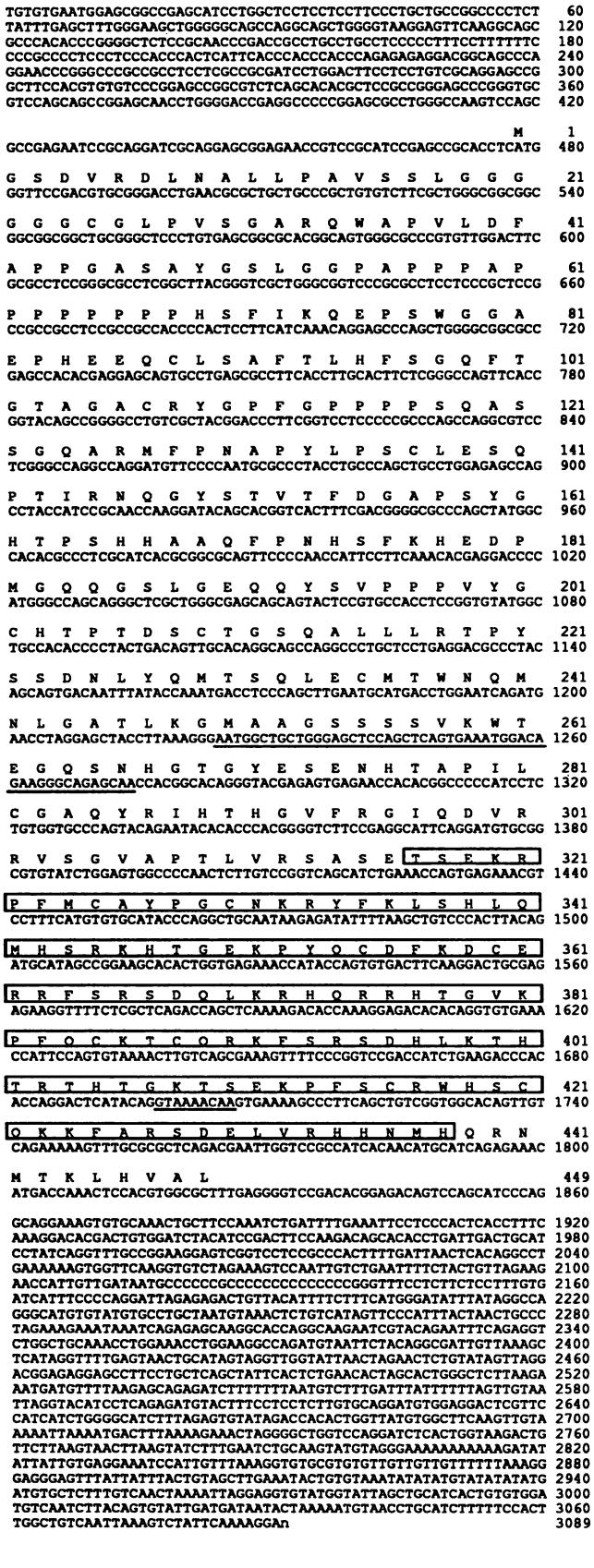
RNA isolation and Northern (RNA blot) analysis. Total RNA was extracted from mouse tissues by a modification of the method of Auffray and Rougeon (1). Briefly, tissues were dissected, snap-frozen in liquid nitrogen, and homogenized in ice-cold 3 M LiCl–6 M urea. RNA was precipitated twice in LiCl-urea solution, and the resulting pellet was resuspended, extracted once with phenol-chloroform (1:1) and once with chloroform, ethanol precipitated, and resuspended in sterile H_2O. RNA samples were then electrophoresed through a 1% agarose–6% formaldehyde gel and blotted onto either nitrocellulose (Millipore) or Gene-Screen Plus (New England Nuclear) membrane filters. Filters were prehybridized and hybridized in 50% formamide–10% dextran sulfate–0.8 M NaCl–5× Denhardt's solution–0.1% sodium PP_i–50 mM Tris (pH 7.5)–100 μg of sheared denatured salmon sperm DNA per ml–0.5% SDS. Filters were washed to a stringency of 1× SSC–0.1% SDS at 65°C and exposed to autoradiographic film.

Nucleotide sequence accession number. The sequence discussed here has been given GenBank accession number M55512.

RESULTS

Our initial studies with the human cDNA WT33 indicated that WT1 mRNA is expressed in mouse fetal kidney (data not shown). A cDNA library was thus constructed from mRNA derived from the kidneys of 17-day mouse embryos and screened at low stringency with a 1.7-kbp *Eco*RI-*Bgl*II fragment of WT33. Five clones were isolated, the longest of which was approximately 1.9 kbp in length.

To confirm that clones from this series were indeed derived from the murine homolog of WT1 rather than other zinc finger genes detected by the low-stringency screening, we determined the chromosomal location of the gene represented by these clones. The Sey^{Dey} allele is the deletion which we have recently shown to be genetically homologous to WAGR deletions of human chromosome 11p13 (14). To determine whether the DNA sequences represented by our cDNA clones were indeed deleted on one allele in $+/Sey^{Dey}$ heterozygotes, we made use of interspecific matings between *Mus domesticus* mice carrying the Sey^{Dey} allele and *M. spretus* (14). The genomes of these two mouse species vary considerably, allowing allelic losses to be identified easily (2, 30). A number of restriction fragment length polymorphisms (RFLPs) defining the relevant *M. domesticus* (C57BL6/J [B6] and C3H/HeJ) and *M. spretus* alleles were identified by using the 1.1-kbp embryonic kidney cDNA clone pMEK91 (Fig. 1A). Offspring from B6 (+/Sey^{Dey}) × *M. spretus* crosses were analyzed for the presence of each of the two alleles. F_1 offspring 4.1 had the Sey^{Dey} phenotype. As indicated in Fig. 1B and C, analysis of *Eco*RV and *Bam*HI RFLPs showed the absence of all restriction fragments unique to the *M. domesticus* allele in the affected offspring. Additionally, the *Bam*HI RFLP detected here has



previously been detected by using the human WT1 cDNA k13 (14). Thus, these cDNAs were indeed derived from a gene located within the deleted region, consistent with the derivation of these clones from transcripts of the murine WT1 gene.

Sequence analysis of the embryonic kidney cDNA clones indicated that none of these clones contained the 5'-terminal region of the known human cDNAs (5, 12). To obtain longer clones, we screened a cDNA library derived from adult mouse testis, a tissue also found to express significant levels of WT1 mRNA (25a). Fifteen cDNAs were purified and sequenced, the longest of which was pMWT21.

The complete nucleotide sequence of the murine WT1 cDNAs (3,089 bp) is shown in Fig. 2. A long open reading frame (ORF) was observed, extending from nucleotide 106 to a stop codon at position 1825. An ATG at position 478 predicts a polypeptide product of 449 amino acids, having a molecular mass of 49,202 Da. A small ORF exists upstream of this translation start site (nucleotides 8 to 67). Sequence extension (at the 5' end) by RNA-based polymerase chain reaction reveals termination codons in all three reading frames upstream of this small ORF (25a). The site of the initiator methionine for the human WT1 has not been established. However, an ATG codon is present in the human cDNA at the site corresponding to the mouse initiator methionine (12). We suggest that this ATG is the translational initation site for the human WT1 mRNA. The predicted polypeptides of the human and mouse cDNAs show striking amino acid sequence identity (>96%; Fig. 3). The amino acid differences between the species occur primarily in the non-zinc finger regions of the polypeptide and, interestingly, are not conservative changes.

Two alternative splices recently identified in humans (16) were also observed in the mouse and were almost completely conserved. The first results in the addition of 17 amino acids after amino acid 249, within the amino-terminal domain, while the second introduces three amino acids between the third and fourth zinc fingers. Interestingly, cDNAs were isolated that contained neither or one of the alternative splice additions, but no clone containing both insertions was isolated. The nearly complete conservation of the alternative splices between mice and humans suggests that these domains have important functional properties.

The overall nucleotide sequence homology between the murine and human cDNAs is approximately 81%. An extremely high degree of homology exists within the protein-encoding region (91%) and is most pronounced in the region comprising the zinc finger domains (95%). Significant homology also exists within the 5' and 3' untranslated regions (79 and 73%, respectively), suggesting that conserved sequences in these areas also may be functionally important.

During normal kidney development, nephroblasts progressively lose proliferative capacity and concomitantly terminally differentiate into glomeruli and proximal convoluted tubules (4). Since loss of WT1 function apparently results in uncontrolled growth of these stem cells, expression of the

FIG. 2. Complete nucleotide sequence of WT1 cDNAs and the predicted amino acid sequence. The nucleotide sequence presented represents a composite sequence of cDNAs derived from alternatively spliced mRNAs. The sequences produced by alternative splicing are underlined and are either present or absent in the various cDNAs. The region of the predicted polypeptide spanning the zinc finger domains is boxed.

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Human  MGSDVRDLNALLPAVPSL-GGGGGCALPVSGAAQWAPVLDFAPPGASAYGSLGGPAPPPA  59
       ||||||||||||||| || |||||| |||||| ||||||||||||||||||||||||||||
Mouse  MGSDVRDLNALLPAVSSLGGGGGGCGLPVSGARQWAPVLDFAPPGASAYGSLGGPAPPPA  60

       PPPPPPPPPPHSFIKQEPSWGGAEPHEEQCLSAFTVHFSGQFTGTAGACRYGPFGPPPPSQ 119
        |||||||||||||||||||||||||||||||| |||||||||||||||||||||||||
       -PPPPPPPPPHSFIKQEPSWGGAEPHEEQCLSAFTLHFSGQFTGTAGACRYGPFGPPPPSQ 119

       ASSGQARMFPNAPYLPSCLESQPAIRNQGYSTVTFDGTPSYGHTPSHHAAQFPNHSFKHE 179
       |||||||||||||||||||| |||||||||||| ||||||||||||||||||||||||||
       ASSGQARMFPNAPYLPSCLESQPTIRNQGYSTVTFDGAPSYGHTPSHHAAQFPNHSFKHE 179

       DPMGQQGSLGEQQYSVPPPVYGCHTPTDSCTGSQALLLRTPYSSDNLYQMTSQLECMTWN 239
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
       DPMGQQGSLGEQQYSVPPPVYGCHTPTDSCTGSQALLLRTPYSSDNLYQMTSQLECMTWN 239

       QMNLGATLKG VAAGSSSSVKWTEGQSN HSTGYESDNHTTPILCGAQYRIHTHGVFRGIQ 298
       |||||||||| |||||||||||||||| |||| ||| ||||||||||||||||||||||
       QMNLGATLKG MAAGSSSSVKWTEGQSN HGIGYESENHTAPILCGAQYRIHTHGVFRGIQ 298

       DVRRVPGVAPTLVRSASETSEKRPFMCAYPGCNKRYFKLSHLQMHSRKHTGEKPYQCDFK 358
       ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
       DVRRVSGVAPTLVRSASETSEKRPFMCAYPGCNKRYFKLSHLQMHSRKHTGEKPYQCDFK 358

       DCERRFSRSDQLKRHQRRHTGVKPFQCKTCQRKFSRSDHLKTHTRTHTG KTS EKPFSCR 417
       |||||||||||||||||||||||||||||||||||||||||||||||| ||| |||||||
       DCERRFSRSDQLKRHQRRHTGVKPFQCKTCQRKFSRSDHLKTHTRTHTG KTS EKPFSCR 417

       WPSCQKKFARSDELVRHHNMHQRNMTKLQLAL 449
       | ||||||||||||||||||||||||||  ||
       WHSCQKKFARSDELVRHHNMHQRNMTKLHVAL 449
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FIG. 3. Amino acid comparison of the predicted WT1 polypeptides of humans and mice. Amino acid identity between the two proteins is indicated by a vertical bar. The alternative splices compared are boxed, and their positions are indicated by a vertical bar.

gene might be developmentally regulated. We analyzed WT1 expression in the mouse as a function of kidney development. Comparison of RNA isolated from embryonic and adult kidneys revealed a substantially higher level in that derived from fetal tissue (Fig. 4A). Expression of WT1 was readily detectable at day 13 of gestation and increased with time, reaching a peak between day 17 of gestation and the third day after birth (approximately 12-fold higher than in adult). At 8 days postpartum, the level of WT1 mRNA began to decline, nearing that of adult kidney by day 15 (Fig. 4B).

In addition to the kidney, WT1 mRNA was present in a number of other tissues. In the adult mouse, kidney and spleen showed the highest expression. However, heart, lung, and thymus also showed detectable levels of WT1 mRNA (Fig. 5) upon longer exposure of Northern blots, as well as ovary and testis (not shown). Northern analysis of cytoplasmic RNA from two mouse kidney cell lines showed a low level of expression in 293 cells, but none was evident in TCMK cells (not shown). No expression was detected

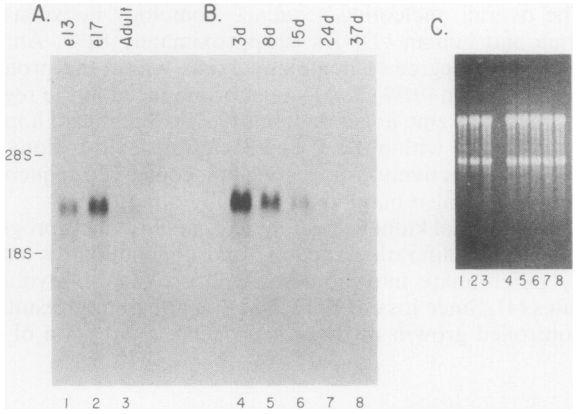

FIG. 4. Developmental expression of WT1 RNA in the kidney. Total RNA was isolated from kidneys and analyzed by Northern blotting with pMEK91 insert DNA as the probe. Exposure time for the autoradiograph shown was 2 days. (A) RNA (10 μg) from kidneys of 13-day (e13) and 17-day (e17) FVB/N embryos as well as those of an adult mouse. (B) RNA (15 μg) from kidneys of BALB/c mice. Ages (in days) are indicated. (C) Ethidium bromide staining of samples blotted in panels A and B is shown as a control for loading.

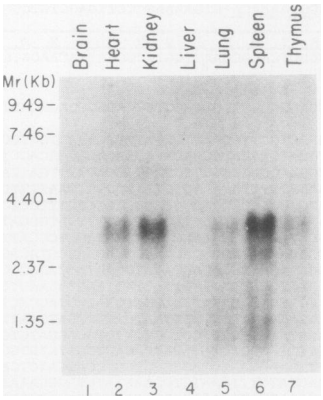

FIG. 5. Expression of WT1 RNA in various mouse tissues. Total RNA was isolated from the indicated BALB/c mouse tissues, and 10 μg of each sample was analyzed by Northern blotting with pMEK91 insert DNA as the probe. Exposure time for the autoradiograph shown was 5 days.

when RNAs from two mouse lung-derived cell lines (LA-4 and RAG) were similarly analyzed. A comparison of WT1 expression in heart and lung tissues from 17-day embryos and adults showed no differences in mRNA levels between the two ages (not shown). Developmental regulation of WT1 expression therefore appears to be selective for the kidney.

DISCUSSION

In order to study in detail the developmental expression of WT1, we have isolated the mouse homolog of the human gene. WT1 is highly conserved across species, with greater than 95% amino acid homology between the mouse and human genes. In addition, the two alternatively spliced sequences of WT1 transcripts are also conserved, consistent with their functional significance. Analysis of the relative abundance of these various forms of WT1 requires RNase protection analysis and is currently under investigation. Our mouse cDNA clones also extend the 5′ sequence of WT1 beyond the human sequences described previously (5, 12) and allow identification of the likely initiator methionine codon. The WT1 polypeptide can therefore be predicted to be between 47 and 49 kDa, reflecting the presence or absence of spliced sequences.

Our analysis of WT1 expression clearly identifies this gene as one which is regulated as a function of kidney development, suggesting a role in nephroblast differentiation. The time course of WT1 expression in the mouse kidney is also consistent with its high expression in 18- to 20-week human fetal kidney (16, 27). The striking modulation of WT1 expression observed during kidney development together with its localization to the developing nephric structures suggests that this gene plays an important role in regulating growth and/or differentiation processes in the mammalian kidney. These findings should facilitate characterization of genes which interact with this potential growth-regulatory gene. Sequence analysis of the predicted polypeptide suggests that it serves as a transcriptional regulator and thus may interact with other genes expressed at specific intervals in nephron development. In this context, it is of interest that expression of the dominant-acting oncogene N-*myc* is also regulated during kidney development (32, 35). Since N-*myc* is also expressed at high levels in most Wilms' tumors (25), it is tempting to speculate about a possible interaction between these two oncogenes, either in the regulation of gene expression or in gene product interactions, as has been demonstrated for other oncoproteins (6, 7, 33, 34).

The WT1 gene differs from other known tumor suppressor genes in that its expression is limited to certain tissues. Indeed, while RB1 expression is widespread, germ line inactivation of RB1 predisposes an individual only to development of retinoblastomas and osteosarcomas (3, 10, 11). The mechanism of this apparent specificity is unclear. While WT1 may yet prove to be involved in the development of tumors other than nephroblastoma, its apparent role in kidney differentiation is likely to explain its association with Wilms' tumor.

The identification of WT1 as a gene affected by a deletion syndrome in the mouse (Sey^{Dey}) (14) which is homologous to the human WAGR syndrome (9, 24, 29) raises important questions regarding Wilms' tumorigenesis. While WAGR patients, who are constitutionally heterozygous for an 11p13 deletion including WT1, have a high frequency of Wilms' tumor (22), such is not the case for $Sey^{Dey}/+$ mice. These mice also possess only one WT1 allele, yet development of nephroblastoma has not been observed (14). One possible explanation for this discrepancy is that the significantly smaller population of nephroblasts or their more rapid maturation makes the effects of a second mutation less likely.

Alternatively, the absence of nephroblastoma in mice expected to have an increased susceptibility may reflect the genetic complexities of Wilms' tumor. A second Wilms' tumor locus has been localized near WT1 at human chromosome 11 band p15 (17, 20, 28). In humans, a single chromosome nondisjunctional or recombinational event could thus affect both of these putative Wilms' tumor genes. In the mouse, however, WT1 is located on chromosome 2 (13), whereas the chromosomal region syntenic to human 11p15 is located on chromosome 7 (23). Since these loci are present on different chromosomes, two separate genetic events would be required to affect these genes.

An understanding of the steps required for the development of Wilms' tumor awaits both identification of other genes which contribute to the occurrence of Wilms' tumor in humans and controlled genetic manipulations of WT1 and these genes in the mouse. Knowledge of the site of action and the developmental window of WT1 function in the kidney is critical to these studies. The mouse provides an excellent system with which to analyze the contribution of WT1 to normal kidney development and tumorigenesis.

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