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Increased expression of the insulin-like growth factor I receptor gene, *IGF1R*, in Wilms tumor is correlated with modulation of *IGF1R* promoter activity by the *WT1* Wilms tumor gene product

HAIM WERNER*†, GIAN G. RE‡, IAIN A. DRUMMOND§, VIKAS P. SUKHATME§, FRANK J. RAUSCHER III¶, DONALD A. SENS‡, A. JULIAN GARVIN‡, DEREK LEROITH*, AND CHARLES T. ROBERTS, JR.*

*Section on Molecular and Cellular Physiology, Diabetes Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Building 10, Room 8S-239, Bethesda, MD 20892; ‡Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC 29425; §Howard Hughes Medical Institute and the Departments of Molecular Genetics and Cell Biology and Medicine, University of Chicago, Chicago, IL 60637; and ¶Wistar Institute of Anatomy and Biology, Philadelphia, PA 19104

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ABSTRACT **Wilms tumor is a pediatric neoplasm that arises from the metanephric blastema. The expression of the gene encoding insulin-like growth factor II (IGF-II) is often elevated in these tumors. Since many of the actions of IGF-II are mediated through activation of the IGF-I receptor (IGF-IR), we have measured the levels of IGF-IR mRNA in normal kidney and in Wilms tumor samples using solution hybridization/RNase protection assays. IGF-IR mRNA levels in the tumors were 5.8-fold higher than in adjacent normal kidney tissue. Among the tumors themselves, the levels of IGF-IR mRNA in those containing heterologous stromal elements were 2-fold higher ($P < 0.01$) than in tumors without these elements. IGF-IR gene (designated *IGF1R*) expression in the tumors was inversely correlated with the expression of the Wilms tumor suppressor gene *WT1*, whose inactivation appears to be a key step in the etiology of Wilms tumor. Cotransfection of Chinese hamster ovary cells with rat and human IGF-IR gene promoter constructs driving luciferase reporter genes and with *WT1* expression vectors showed that the active *WT1* gene product represses IGF-IR promoter activity in a dose-dependent manner. These results suggest that underexpression, deletion, or mutation of *WT1* may result in increased expression of the IGF-IR, whose activation by IGF-II may be an important aspect of the biology of Wilms tumor.**

Wilms tumor (WT) is a pediatric kidney neoplasm that arises from multipotential stem cells of the metanephric blastema (1). WT may occur in either sporadic or familial forms, suggesting that several different genetic loci may be involved in WT predisposition and progression (2). Deletion and genetic analyses led to the isolation of a candidate predisposition gene, *WT1*, whose inactivation was postulated to be a key event in the etiology of WT (3–5). *WT1*, located on human chromosome 11, band p13, encodes a DNA-binding protein with a serine- and proline-rich NH_2 terminus and four Zn^{2+}-finger domains. The *WT1* gene product recognizes the sequence GCGGGGGCG, a consensus binding site for members of the early growth response (EGR) family of transcriptional activators (6). Consistent with its putative role as a tumor suppressor, however, the *WT1* gene product has been shown to repress the activity of promoters that contain this motif (7), including the promoter of the insulin-like growth factor II (IGF-II) gene (8), designated *IGF2*.

The IGFs are a family of anabolic hormones that are structurally and functionally related to insulin and are among the most potent mitogenic factors for kidney cells in culture (9, 10). *In vivo*, compensatory hypertrophy subsequent to unilateral nephrectomy leads to an increase in IGF-I in collecting duct cells, suggesting a causative role for this growth factor in kidney hypertrophy (11). Moreover, injection of IGF-I into hypophysectomized rats results in significant increases in kidney weight (12). Most of the biological actions of IGF-I and IGF-II are initiated by their binding to the IGF-I receptor (IGF-IR), a transmembrane tyrosine kinase structurally related to the insulin receptor (13, 14).

Extracts of WT exhibit increased ^{125}I-labeled IGF-I (^{125}I-IGF-I) binding and tyrosine kinase activity as compared with normal kidney tissue (15). Furthermore, a role for IGF-IR action in the etiology of WT is suggested by the observation that an antibody to the human IGF-IR (αIR-3) can inhibit ^{125}I-IGF-I binding and IGF-I-stimulated thymidine incorporation by WT cells in culture (16). Most strikingly, intraperitoneal administration of αIR-3 to nude mice bearing WT heterotransplants can prevent tumor growth and results in partial tumor remission (16).

The promoter regions of the rat (17, 18) and human (19, 20) IGF-IR genes (designated *IGF1R*) contain numerous potential binding sites for the products of the EGR gene family, suggesting that the expression of the IGF-IR gene could be regulated by these factors, including WT1. To assess this possibility, we have measured the expression of the IGF-IR gene in WT and in normal adjacent kidney tissue using specific solution hybridization/RNase protection assays and found that the levels of IGF-IR mRNA in tumor samples were inversely correlated to the levels of WT1 mRNA. Additionally, cotransfection of plasmids containing the IGF-IR gene promoter fused to a luciferase reporter gene with one containing the full-length *WT1* coding sequence resulted in a significant repression of IGF-IR promoter activity in Chinese hamster ovary (CHO) cells. These results suggest that transcription factor WT1 may serve as a negative regulator of IGF-IR gene expression and support the hypothesis that underexpression, deletion, or mutation of the *WT1* gene may result in an increased expression of IGF-IR mRNA and protein in WT. Paracrine activation of IGF-IR by IGF-II, which is thought to be produced in large amounts by the tumor, may result in an increased mitogenic action which may contribute to the etiology and/or progression of the tumor.

MATERIALS AND METHODS

Tissue Samples. Twenty-five samples of tumors and seven samples of normal adjacent kidney tissue were dissected from surgical specimens obtained at the Department of Pathology and Laboratory Medicine of the Medical University of South

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Abbreviations: WT, Wilms tumor; IGF, insulin-like growth factor; IGF-IR, IGF type I receptor; EGR, early growth response.
†To whom reprint requests should be addressed.

Carolina. Samples were immediately frozen in liquid nitrogen and stored at −80°C until RNA preparation. Tumors were divided in two groups: those containing differentiated heterologous elements (striated muscle, cartilage, and bone) and those lacking these elements (i.e., composed primarily of blastema with epithelial differentiation). A more detailed description of the tumors has been presented elsewhere (21).

Measurement of IGF-IR Gene Expression in WT. RNA was prepared by the method of Chomczynski and Sacchi (22) and was quantitated by measuring absorbance at 260 nm. After electrophoresis through a 1.2% agarose/2.2 M formaldehyde gel, RNA integrity and quantitation were confirmed by visualization of the ethidium bromide-stained 28S and 18S ribosomal RNA bands as described (23).

Levels of IGF-IR mRNA were determined by a solution hybridization/RNase protection assay with an antisense RNA probe that was generated by subcloning a 379-base-pair (bp) *Eco*RI–*Xho* I fragment of the human IGF-IR cDNA (13) into pGEM-3 (24). The resulting construct was linearized with *Hin*dIII and transcribed with phage T7 RNA polymerase in the presence of [α-^{32}P]UTP. Ten micrograms of total RNA was hybridized with 2×10^5 dpm of labeled probe at 45°C for 16 hr in a buffer containing 75% formamide. After hybridization, RNA samples were digested with RNases A and T1, and the protected hybrids were extracted with phenol–chloroform, precipitated with ethanol, and electrophoresed on 8% polyacrylamide/8 M urea denaturing gels. Hybridization of this probe to human RNA routinely results in two protected bands (24, 25), which may correspond to alternatively spliced variants of the human IGF-IR mRNA. Both bands in the autoradiograms were scanned by using a model 2202 UltroScan laser densitometer (Pharmacia LKB Biotechnology).

Coexpression Studies. The rat IGF-IR gene promoter–luciferase reporter gene plasmids used in this study have been described (18). Briefly, an ≈3-kilobase (kb) *Rsa* I fragment containing 2.35 kb of 5′ flanking region and 640 bp of 5′ untranslated region was fused to a promoterless firefly luciferase reporter gene (pOLUC). In addition, an ≈0.65-kb *Alu* I fragment containing 416 bp of the 5′ flanking region and 232 bp of 5′-untranslated region was also fused to pOLUC. The basal promoter activity of the resulting constructs, p(−2350/+640)LUC and p(−416/+232)LUC, was recently characterized in CHO and Buffalo rat liver (BRL3A) cells (18). In addition, an ≈0.72-kb *Hin*dIII–*Bam*HI genomic fragment containing 517 bp of the 5′ flanking region and 205 bp of the 5′ untranslated region of the human IGF-IR gene (19) was subcloned into pOLUC. The basal promoter activity of the human construct, ph(−517/+205)LUC, was similar to that of its rat counterpart, p(−416/+232)LUC (data not shown). A WT1 expression vector, pCMVhWT, was constructed by inserting a human WT1 cDNA 3′ of the cytomegalovirus (CMV) promoter–enhancer in the vector pCB6+ (7). As a negative control, we used a WT1 expression vector (pCMVhWT-TTL) in which stop codons were inserted 5′ of the Zn^{2+}-finger coding sequence, thus abolishing the DNA-binding capacity of the expressed protein (7).

CHO cells were grown in Ham's F-12 nutrient mixture containing 10% (vol/vol) fetal bovine serum (FBS). Cells were seeded in 60-mm dishes 4 days before transfection. On the day of the experiment, each dish received 1 μg of reporter plasmid and variable amounts of WT1 expression vector (0, 1, 2, 5, 10, and 20 μg) as well as pCB6+ DNA to bring the total amount of expression vector to 20 μg. In addition, plates received 5 μg of a β-galactosidase expression vector (pCMVβ, Clontech). Cells were transfected by using 50 μg of Lipofectin reagent (GIBCO/BRL Life Technologies) in 3 ml of Opti-MEM reduced serum medium (GIBCO/BRL). Twenty-four hours after transfection, the medium was changed to Ham's F-12 containing 10% FBS and, after an additional 48 hr, the cells were washed three times in ice-cold phosphate-buffered saline and lysed in 0.5 ml of 1% Triton X-100/25 mM glycylglycine, pH 7.8/15 mM $MgSO_4$/4 mM EGTA/1 mM dithiothreitol. Luciferase activity of the extracts was measured by using a Berthold Clini-Lumat luminometer (London Diagnostics, Eden Prairie, MN) as described (18). The values obtained were normalized for β-galactosidase activity.

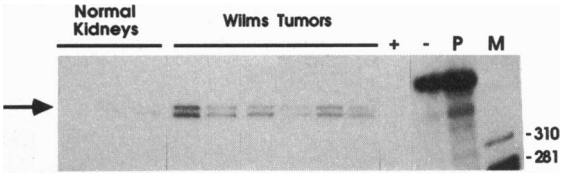

FIG. 1. Expression of the IGF-IR gene in WT and normal adjacent kidney tissue. Levels of IGF-IR mRNA were measured by solution hybridization/RNase protection assays. Ten micrograms of total RNA from six individual WTs and three normal kidneys was hybridized with 2×10^5 dpm of a ^{32}P-labeled human IGF-IR antisense RNA probe, digested with RNases A and T1, and electrophoresed on an 8% polyacrylamide/8 M urea gel. Autoradiographs were exposed for 24 hr. Lanes: +, Probe alone with RNase; −, probe alone without RNase; P, native probe; M, markers (ϕX174 DNA digested with *Hae* III).

Statistical Analysis. Statistical significance was evaluated by Student's *t* test for paired samples. Coefficients of correlation between IGF-IR mRNA and WT1 mRNA levels were estimated by multiple regression analysis. Probability values < 0.05 were considered to be statistically significant.

RESULTS

The level of expression of the IGF-IR gene in WT was determined with a sensitive solution hybridization/RNase protection assay with total RNA obtained from a collection of previously described tumors (21) and a specific ^{32}P-labeled human IGF-IR antisense RNA probe. Scanning densitometry of the two protected probe bands revealed that the levels of IGF-IR mRNA in the tumors were ≈5.8-fold higher than in normal adjacent kidney tissue (Fig. 1 and Table 1). Furthermore, significant differences in the levels of IGF-IR mRNA were seen between tumors that presented with different histologic features. Thus, the levels of IGF-IR mRNA in tumors containing heterologous stromal elements—i.e., striated muscle, cartilage, and bone—were ≈2-fold higher ($P < 0.01$) than in tumors without heterologous elements—i.e., those composed mainly of blastema with epithelial differentiation (Fig. 2 *Upper* and Table 1). When the levels of IGF-IR mRNA in individual tumors were compared to the levels of WT1 mRNA (21), an interesting correlation emerged: with the exception of tumor sample 38, which showed very high levels of both IGF-IR and WT1 mRNAs, multiple regression analysis showed a significant inverse correlation between these two parameters (R, −0.52; $P < 0.05$) (Fig. 3). As

Table 1. Expression of IGF-IR mRNA in WT

Sample	*n*	IGF-IR mRNA*
Normal kidney	7	2.87 ± 0.70
WT†	25	16.77 ± 2.86‡
With heterologous elements	8	23.28 ± 5.43
Without heterologous elements	10	10.83 ± 3.83§
Uncharacterized	7	17.81 ± 5.42

*Levels of IGF-IR mRNA are expressed as arbitrary absorbance units.
†This group includes 8 tumors with heterologous elements, 10 tumors without heterologous elements, and 7 uncharacterized tumors.
‡Significantly different from normal kidney tissue ($P < 0.02$).
§Significantly different from WT with heterologous elements ($P < 0.01$).

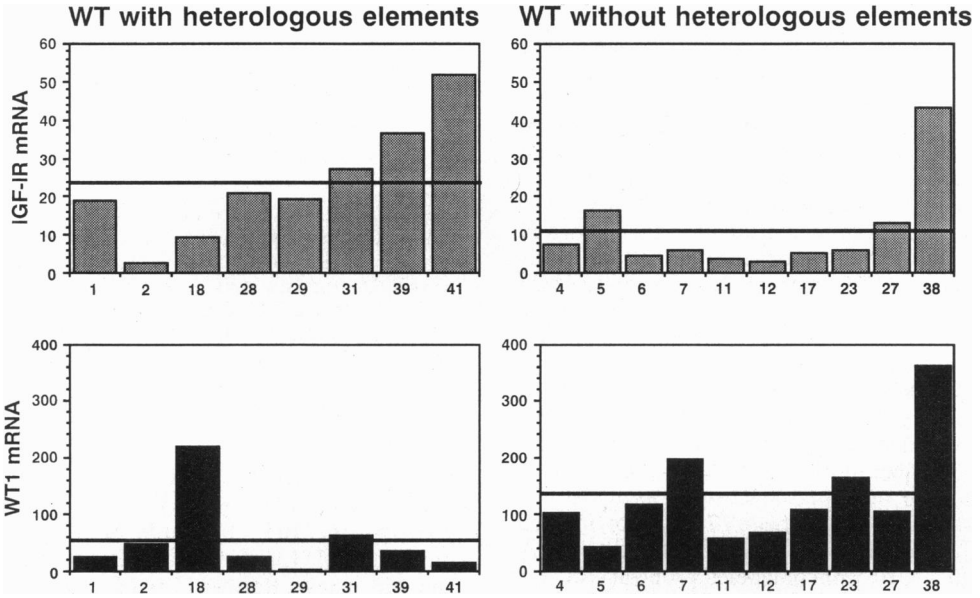

FIG. 2. Expression of IGF-IR and WT1 mRNAs in WT with or without heterologous elements. The numbers on the *x* axis correspond to the designated numbers of the individual tumors reported in ref. 21. Values of WT1 mRNA are from the same study. mRNA values are expressed in arbitrary absorbance units. The thick horizontal line represents the mean for each group. For IGF-IR mRNA mean values, see Table 1. WT1 mRNA level in WT with heterologous elements was 54.3 ± 24.5 (n = 8). WT1 mRNA level in WT without heterologous elements was 132.1 ± 29.6 (n = 10). The difference in WT1 mRNA levels between the two groups was statistically significant ($P < 0.001$).

previously reported, the levels of IGF-II mRNA in the tumors were also significantly increased with respect to normal kidney tissue (21). However, IGF-II mRNA levels did not correlate with the histological status of the tumor or with WT1 or IGF-IR mRNA levels (data not shown).

To determine whether the reciprocal pattern of IGF-IR and *WT1* gene expression in WT was possibly due in part to loss of negative regulation of IGF-IR gene expression by the *WT1* tumor suppressor gene product, coexpression studies were performed by using an IGF-IR gene promoter-containing reporter plasmid [p(−2350/+640)LUC] (Fig. 4*A*) and *WT1* expression vectors (pCMVhWT and pCMVhWT-TTL) encoding full-length and truncated WT1 proteins, respectively. This fragment of the rat IGF-IR gene promoter contains four potential WT1 binding sites in the 5′ flanking region and six sites in the 5′ untranslated region. We performed these studies in CHO cells; we have previously demonstrated that the IGF-IR promoter is very active in this cell line (18), which expresses the endogenous IGF-IR gene as shown by Northern blot analysis and ^{125}I-IGF-I binding studies (H.W., unpublished observations). Results of transient cotransfection experiments showed a dose-dependent repression of IGF-IR promoter activity by the *WT1* gene product. Thus, the luciferase activity in extracts of CHO cells cotransfected with 1 μg of the IGF-IR promoter–reporter plasmid and 20 μg of the active *WT1* expression vector was only 12% of the activity elicited in the absence of *WT1* expression. When the reporter plasmid was cotransfected with 20 μg of a truncated expression vector lacking the Zn^{2+}-finger domain (pCMVhWT-TTL), no reduction in promoter activity was seen (Fig. 4*B*).

Coexpression studies were also performed by using a human IGF-IR promoter–reporter plasmid, ph(−517/+205)LUC, which contains four WT1-like sites in its 5′ flanking region and two sites in its 5′ untranslated region and a rat construct, p(−416/+232)LUC, which also contains six potential WT1 sites, one of which corresponds exactly to the WT1/EGR consensus sequence. In both cases, WT1 repressed promoter activity in a dose-dependent manner (Fig. 4*C*). Thus, 20 μg of the active *WT1* expression vector repressed the activity of the human promoter to 40.2% of its original level and the activity of its rat counterpart to 47.7% of its basal value.

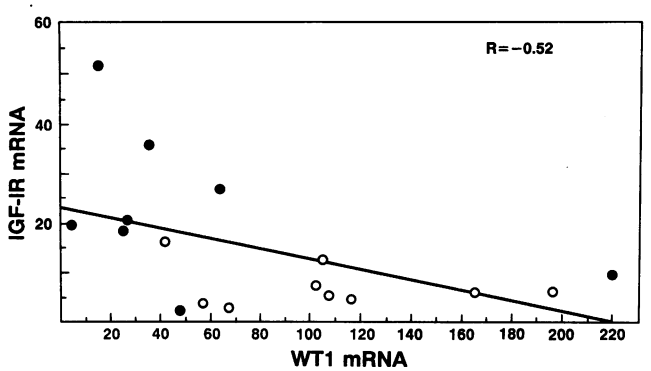

FIG. 3. Multiple regression analysis of IGF-IR mRNA and WT1 mRNA in 17 WT samples. ○, WT samples without heterologous elements; ●, tumor samples that include heterologous elements. Sample 38 (see Fig. 2) is not included.

DISCUSSION

The involvement of the IGF-IR in the progression of WT was suggested by experiments that showed that administration of a monoclonal antibody against this receptor (αIR-3) to athymic mice bearing WT heterotransplants prevented tumor growth for at least 3 weeks and, in some cases, resulted in complete regression of established tumors (16). IGF-II, which is produced by normal kidney (26) and in extremely large amounts by the tumor (27–29), was postulated to be a major mitogenic factor for WT. Most of the effects of IGF-II are thought to involve binding to and activation of the IGF-IR (30).

In the present study, we have shown that the expression of the IGF-IR gene is also increased in WT, and this increment in gene expression probably contributes to the previously reported augmentation in IGF-I binding (15). Unlike IGF-II mRNA, which appears to be present at high levels in all WTs and whose expression does not seem to be a necessary event

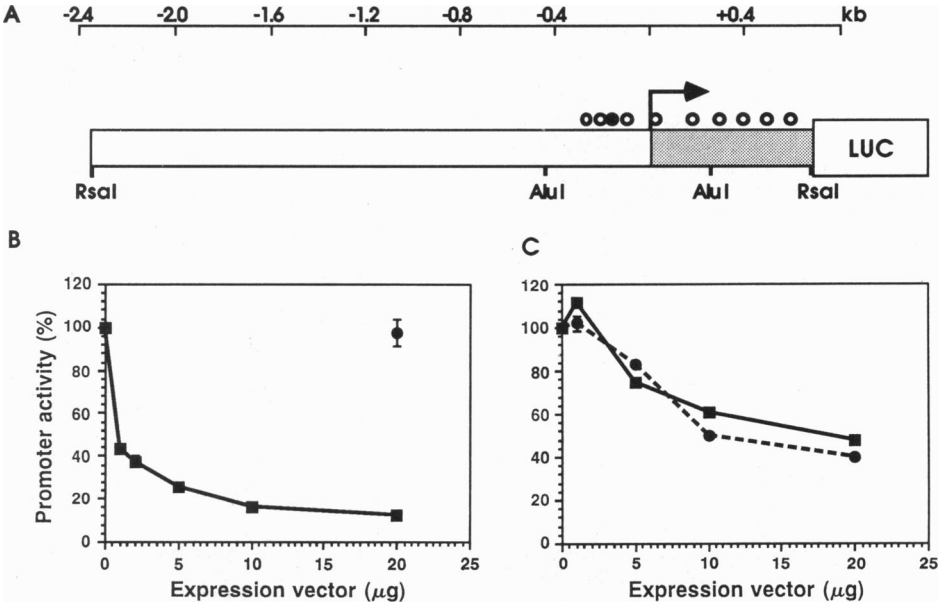

FIG. 4. (*A*) Schematic representation of the rat IGF-IR promoter gene/luciferase reporter gene plasmids. The *Rsa* I fragment contains 2350 bp of 5′ flanking (open bar) and 640 bp of 5′ untranslated (stippled) sequences. The *Alu* I fragment contains 416 bp of 5′ flanking and 232 bp of 5′ untranslated regions. The arrow denotes the unique transcription initiation site. The black circle above the open bar is a consensus EGR/WT1 binding site, and the open dots are putative binding sites that conform to the consensus sequence at eight of nine nucleotides. The luciferase reporter cDNA (LUC) is not shown to scale. (*B*) Repression of IGF-IR gene promoter activity by WT1. One microgram of the reporter plasmid p(−2350/+640)LUC was cotransfected into CHO cells with increasing amounts of the *WT1* expression vector, pCMVhWT (■), or with 20 μg of the mutant *WT1* expression vector, pCMVhWT-TTL (●). The values of luciferase activity shown (normalized per β-galactosidase activity) are means ± SEM ($n = 3$). Where not shown, SEM bars are smaller than the size of the symbol. (*C*) Comparison of WT1 inhibition of human and rat IGF-IR gene promoter activities. One microgram of the human reporter plasmid ph(−517/+205)LUC (●) or of the rat reporter plasmid p(−416/+232)LUC (■) was cotransfected into CHO cells with increasing amounts of the *WT1* expression vector, pCMVhWT.

in WT progression (31), the levels of IGF-IR mRNA appear to correlate with the degree of differentiation of the tumor: tumors with heterologous elements and prominent stromal components are generally associated with higher levels of IGF-IR mRNA. This subset of tumors is mostly seen in the WAGR syndrome (1), a condition in which WT is associated with aniridia, genitourinary abnormalities, and mental retardation. On the other hand, tumors that do not have heterologous elements and are blastema-rich contain lower levels of IGF-IR mRNA. These tumors are generally associated with the Beckwith–Wiedemann, or fetal overgrowth, syndrome.

Furthermore, with the exception of one tumor out of the 18 assayed (tumor 38, Fig. 2), there was a significant negative correlation between the levels of IGF-IR and WT1 mRNAs in individual tumors, a finding consistent with the action of WT1 as a negative regulator of IGF-IR gene expression. This issue is complicated, however, by the fact that normal kidney tissue expresses low levels of both IGF-IR and WT1 mRNAs. It may be likely, then, that changes in the expression of WT1 gene are only one predisposition step in a series of events necessary for tumorigenesis. In fact, evidence suggests that various different genetic loci may be involved in WT predisposition and progression (2).

The IGF-IR gene promoter is a TATA-less, CAAT-less, G·C-rich promoter (17). Transcription from this gene is initiated at a single start site contained within an initiator element that is similar to the motif previously reported by Smale and Baltimore (32). The IGF-IR gene contains a very long 5′-untranslated region of ≈1 kb, which may also regulate translation of the IGF-IR mRNA. The results of transient expression studies indicate that the IGF-IR gene has a high basal promoter activity (≈10–25% of the activity of the simian virus 40 enhancer/promoter used as a positive control) (18). The fact that the IGF-IR gene is expressed at very low levels in most adult tissues (23) may suggest, therefore, that this promoter is predominantly under negative control.

The results of coexpression studies with a *WT1* expression vector and human and rat IGF-IR promoter–reporter genes clearly indicate that the *WT1* gene product can, in fact, repress IGF-IR promoter activity in a dose-dependent manner. The inhibitory effect of WT1 was apparently dependent on the number of functional WT1 sites. Thus, whereas the activity of a promoter fragment containing 10 potential EGR/WT1 binding sites was inhibited by 88%, the activity of fragments containing only six sites was inhibited by 52–60%. Furthermore, the observation that a mutant *WT1* expression vector lacking the Zn^{2+}-finger domain was unable to inhibit promoter activity strongly suggests that this effect was due to binding of WT1 protein to specific recognition sites. Although the shorter rat IGF-IR fragment and the human fragment both contained six potential WT1 binding sites (albeit not in exactly analogous positions in each case), only one of the rat sites conformed to the GCGGGGGCG consensus sequence. Nevertheless, both fragments were repressed by WT1 to the same extent, suggesting that WT1 effects can be mediated through interaction with nonconsensus sites. As such, these data are consistent with the results obtained with the IGF-II promoter (8).

In summary, we have shown that the levels of IGF-IR mRNA in WT are inversely correlated to the levels of WT1 mRNA and that a functional WT1 transcription factor can repress the activity of the IGF-IR promoter *in vivo*. Point mutations that cannot be detected by Northern blot analysis were recently described in the *WT1* gene, resulting in defective WT1 protein (33). Lack of inhibition of the IGF-IR promoter by an inactive WT1 protein may result in increased levels of IGF-IR mRNA and protein. This lack of inhibition may explain the aberrant expression pattern of patient 38, in which IGF-IR gene expression was not repressed in spite of high levels of WT1 mRNA. Paracrine activation of the IGF-IR by locally produced IGF-II may elicit a mitogenic

event, which may be a key step in the etiology and/or progression of WT.

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