Transcriptional Repression of the Insulin-like Growth Factor I Receptor (IGF-I-R) Gene by the Tumor Suppressor WT1 Involves Binding to Sequences Both Upstream and Downstream of the IGF-I-R Gene Transcription Start Site*

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The insulin-like growth factor-I receptor (IGF-I-R) has been implicated in the etiology and/or progression of Wilms' tumor, a pediatric malignancy of the kidney that is often associated with deletion or mutation of the WT1 tumor suppressor gene. The expression of the IGF-I-R gene is increased in Wilms' tumor as compared with normal kidney tissue. Furthermore, the levels of IGF-I-R mRNA in individual tumors have been shown to be inversely correlated to the levels of WT1 mRNA, suggesting that the expression of the IGF-I-R gene is under the negative control of WT1. The activity of an IGF-I-R promoter/luciferase construct in Chinese hamster ovary cells was reduced by cotransfection of a WT1 expression vector. An analysis of various reporter constructs containing different portions of the IGF-I-R 5'-flanking and 5'-untranslated regions suggested that the effect of WT1 depends on the number of WT1 binding sites present, with sites located both upstream and downstream of the IGF-I-R transcription start site involved in mediating this effect. Using the purified zinc finger domain of WT1 in gel retardation and DNase I footprinting assays, we mapped five sites in the 5'-flanking and six sites in the 5'-untranslated regions that were involved in WT1 binding. In addition, the initiator element of the IGF-I-R gene contains a sequence that binds WT1. Thus, the repression of IGF-I-R promoter activity by the WT1 tumor suppressor gene product involves multiple interactions of its zinc finger domain with WT1 binding sites located both 5' and 3' of the transcription initiation site.

The insulin-like growth factor-I receptor (IGF-I-R)$^{1}$ is a membrane-bound tyrosine kinase that mediates the trophic, metabolic, and differentiative effects of the insulin-like growth factors, IGF-I and IGF-II (1, 2). Overexpression of the IGF-I-R in Balb/c3T3 cells has been shown to abrogate all requirements for exogenous growth factors, suggesting that this receptor plays a central role during the cell cycle (3). Furthermore, the constitutive expression of the IGF-I-R gene in most tissues is consistent with the putative role of IGF-I as a progression factor (4).

Previous analyses of the IGF-I-R gene promoter indicated that it lacks TATA or CAAT elements and has an extremely high G-C content (5–8). Transcription of the IGF-I-R gene is initiated from a unique site, however, which is contained within an "initiator" motif similar to the one previously described in the terminal deoxynucleotidyl transferase and adenovirus middle late promoters (9). This motif has been shown to direct accurate transcription in the absence of a TATA box and to often act in concert with upstream Sp1 sites (10, 11). The IGF-I-R gene contains several Sp1 binding sites in both the 5'-flanking and 5'-untranslated regions, and we have shown that these sites are active in functional and binding assays, suggesting that Sp1 is involved in IGF-I-R gene regulation (8).

The IGF-I-R gene promoter contains numerous potential binding sites for members of the EGR family of transcription factors. These are encoded by a family of immediate early genes whose transcription is rapidly induced by cell surface stimuli such as growth factor-receptor interaction. This family includes EGR1 (also known as NGFI-A, Zif 268, and Krox 24), EGR2, EGR3, and WT1, the Wilms' tumor suppressor gene product (12). The WT1 gene is located on chromosome 11, band p13, and encodes a DNA-binding protein with a proline- and glutamine-rich N terminus and four zinc finger domains in its C terminus (13). The consensus binding sequence derived for the EGRWT1 gene products is 5'-GGCGGGCG-3' (14). Unlike other members of the EGR family of transcriptional activators, however, WT1 has been shown, in general, to repress the activity of genes whose promoters contain this element, including the IGFI-I, the platelet-derived growth factor A-chain, and the IGF-I-R genes (15–18). Inactivation of the WT1 gene has been postulated to be a key event in the etiology or progression of a subset of Wilms' tumors (19–21).

The primary transcript of the WT1 gene is subject to alternative splicing of two different sequences, a 51-bp sequence encoded by exon 5 and a 9-bp sequence that results from the use of an alternative 5' splice junction in exon 9 (17, 22). This process results in four distinct mRNA species, whose ratio appears to be relatively constant in normal tissues that express this gene as well as in Wilms' tumors (23).

Recent studies in our laboratory have shown that the expression of the IGF-I-R gene is increased in Wilms' tumor (18). These results are consistent with previous studies that demon-
strated high levels of [32P]-labeled IGF-I binding in Wilms' tumor, as compared with normal kidney tissue (24). Furthermore, the involvement of the IGF-I-R in Wilms' tumor etiology was suggested by studies that showed that intraperitoneal administration of a monoclonal antibody directed against the human IGF-I-R (oI93) to nude mice bearing Wilms' tumor heterotransplants prevented tumor growth and resulted in partial tumor remission (25). Since IGF-II mRNA and protein levels are also elevated in Wilms' tumor (26–28), it has been postulated that paracrine activation of the IGF-I-R by IGF-II may result in a mitogenic loop that may contribute to the development of Wilms' tumor (18, 25).

A comparison of the levels of IGF-I-R and WT1 mRNAs in individual Wilms' tumor samples revealed that, in general, tumors with high levels of WT1 mRNA have low levels of IGF-I-R mRNA, whereas tumors with low levels of WT1 mRNA have higher levels of IGF-I-R mRNA. This finding, combined with the presence of multiple potential WT1 binding sites in the IGF-I-R promoter, prompted us to speculate that this promoter is a potential target for the inhibitory action of this tumor suppressor gene product. This hypothesis was supported by experiments that showed that cotransfection of Chinese hamster ovary (CHO) cells with a WT1 expression vector together with a construct containing 2.3 kilobases of 5'-flanking and 640 bp of 5'-untranslated sequences of the IGF-I-R gene cloned upstream of a luciferase reporter gene resulted in a dose-dependent repression of IGF-1 promoter activity (18).

To gain further insight into the molecular basis of IGF-I-R gene regulation by WT1 and in order to define the contribution of specific WT1 sites to this regulation, we have now performed coexpression studies using different fragments of the IGF-I-R promoter as well as alternatively spliced variants of WT1 and have analyzed the DNA-protein interactions between WT1 and the IGF-I-R promoter by means of gel retardation and DNase I footprinting assays using the purified WT1 zinc finger domain (WTZF). The results obtained indicate that WT1 represses transcription of the IGF-1-R promoter via a mechanism that involves multiple interactions of the WT1 zinc finger domain with consensus binding sites in both the 5'-flanking and 5'-untranslated regions of the IGF-I-R gene.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures, Plasmids, and DNA Transfections**—CHO cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in Ham's F-12 nutrient mixture with 10% fetal bovine serum. The following fragments of the rat IGF-I-R gene promoter were fused to a promoterless firefly luciferase reporter gene in the pOUC vector (29) and used in cotransfection experiments: −2350/+640, −476/+640, −416/+232, −455/+30, and −40/+640 (nucleotide 1 corresponds to the transcription initiation site). The construction of some of the above plasmids, as well as their relative basal promoter activities, has been previously described (8).

The following WT1 expression vectors were employed in cotransfection experiments, pCMVhWT and pCMVhWT-17AA-KTS. Both vectors were constructed by inserting a human WT1 cDNA downstream of the CMV promoter in the vector pCB6+ (14). Expression vector pCMVhWT-17AA-KTS contains a 51-bp insert encoding a 17-amino acid fragment following residue 248 and a 9-bp insert encoding a 3-amino acid fragment (Lys-Thr-Ser) following residue 390. The pCMVhWT expression vector encodes a WT1 protein lacking both inserts.

Cells were transfected using 50 μg of lipofectin reagent (Life Technologies, Inc.) in 3 ml of Opti-minimal Eagle's medium reduced-serum medium (Life Technologies, Inc.). Each 60-mm dish received 1 μg of reporter plasmid and variable amounts of expression vector. The total amount of DNA transfected was kept constant using pCB6+ DNA. In addition, 5 μg of a β-galactosidase expression vector (pCMV β, Clontech) was used with each dish.

Twenty-four hours after cotransfection, the DNA-containing medium was changed to serum-containing medium and the plates were incubated for an additional 48 h, at which time the cells were harvested, and luciferase and β-galactosidase activities were measured as previously described (18).

**Gel Retardation Assays**—The following fragments of the proximal 5'-flanking and 5'-untranslated regions of the IGF-I-R gene were employed in gel retardation assays: −494/−331, −331/−135, −135/−26, −29/−185, +185/+115, and +341/+640. All of the fragments other than the −29/+185 fragment were labeled by filling in the 5'-protruding end with [32P]dCTP using the Klenow fragment of DNA polymerase I. The −29/+185 fragment, generated by HaeIII digestion, was end-labeled by exchanging the 3'-terminal phosphate with [γ-32P]ATP using T4 polynucleotide kinase. Labeled probes were separated from unincorporated nucleotides using ELUTiP columns (Schleicher & Schuell), following which probes were loaded on 5% nondenaturing polyacrylamide gels. The wet gels were briefly exposed to a film, and the region of the gel containing the probe bands was excised, macerated, and incubated for 16 h in a buffer containing 500 mM NH₄ acetate, 10 mM Mg acetate, 1 mM EDTA, and 0.1% sodium dodecyl sulfate. Labeled probes were precipitated with ethanol using TRNA as a carrier, and pellets were reconstituted in distilled water to give a concentration of 75,000 dpm/μl.

Binding assays were performed by preincubating 0, 200, and 500 ng of WTZF (14) in 9 μl of 20 mM Hepes pH 7.5, 70 mM KCl, 12% glycerol, 0.05% Nonidet P-40, 100 μM ZnSO₄, 0.5 mM dithiothreitol, 1 mg/ml bovine serum albumin, and 0.1 mg/ml poly(dI-dC), with or without the indicated unlabeled DNA as a competitor, for 15 min at 4 °C. 75,000 dpm (0.2–1 ng) of the labeled fragment were then added, and the reaction was incubated for an additional 10 min. The reaction products were electrophoresed through a 5% polyacrylamide gel that was run at 250 V for 2 h at 4 °C. Gels were fixed in 10% acetic acid and autoradiographed at −70 °C using two intensifying screens.

**DNase I Footprinting**—The following DNA fragments were used in...
TRANSCRIPTIONAL REPRESSION OF THE IGF-I-R GENE BY WT1

The circles represent WT1 sites footprinted in the promoter region of the IGF-I-R gene. The closed circle is a consensus binding site for transcription factors of the EGF-UWT1 family (Fig. 1). Using an RsaI fragment of the IGF-I-R promoter containing ~2.3 kilobases of 5'-flanking and 640 bp of 5'-untranslated sequences, we have recently shown that transcription factor WT1 can repress the activity of this promoter in a dose-dependent manner (18). To study in more detail the molecular mechanisms responsible for the transcriptional regulation of the IGF-I-R gene by WT1, we performed coexpression studies using a reporter plasmid containing only the proximal promoter region. This construct, extending from the HindIII site at -476 to the RsaI site at +640, contains the majority of the putative WT1 binding sites that conform closely to the consensus EGR/WT1 site. The luciferase (LUC) cDNA is not shown to scale.

Table I. The circles represent WT1 sites footprinted in the promoter region of the IGF-I-R gene. The closed circle is a consensus binding site for transcription factors of the EGF-UWT1 family (Fig. 1). Using an RsaI fragment of the IGF-I-R promoter containing ~2.3 kilobases of 5'-flanking and 640 bp of 5'-untranslated sequences, we have recently shown that transcription factor WT1 can repress the activity of this promoter in a dose-dependent manner (18). To study in more detail the molecular mechanisms responsible for the transcriptional regulation of the IGF-I-R gene by WT1, we performed coexpression studies using a reporter plasmid containing only the proximal promoter region. This construct, extending from the HindIII site at -476 to the RsaI site at +640, contains the majority of the putative WT1 binding sites that conform closely to the consensus EGR/WT1 site. The luciferase (LUC) cDNA is not shown to scale.

RESULTS

Functional Studies—The proximal -500 bp of 5'-flanking and -700 bp of 5'-untranslated region of the IGF-I-R gene has an extremely high G-C content (>75%) and contains multiple putative binding sites for transcription factors of the EGR/WT1 family (Fig. 1). Using an RsaI fragment of the IGF-I-R promoter containing ~2.3 kilobases of 5'-flanking and 640 bp of 5'-untranslated sequences, we have recently shown that transcription factor WT1 can repress the activity of this promoter in a dose-dependent manner (18). To study in more detail the molecular mechanisms responsible for the transcriptional regulation of the IGF-I-R gene by WT1, we performed coexpression studies using a reporter plasmid containing only the proximal promoter region. This construct, extending from the HindIII site at -476 to the RsaI site at +640, contains the majority of the putative WT1 binding sites that conform closely to the consensus EGR/WT1 site. The luciferase (LUC) cDNA is not shown to scale.
to the consensus sequence and exhibits a basal promoter activity similar to that of p(-2350/+640)LUC (data not shown). As shown in Fig. 2, expression vector pCMVhWT was able to repress the activity of p(-2350/+640)LUC and p(-476/+640)LUC in a similar fashion. Maximal repression was seen with 20 ng pCMVhWT, at which concentration the activity of both constructs was inhibited by 82–87%.

To determine the involvement of the different WT1 binding sites in the proximal IGF-I-R promoter in transcriptional regulation, coexpression studies were performed using IGF-I-R promoter/reporter plasmids containing different portions of flanking and 5′ untranslated sequences, together with the WT1 expression vector (Fig. 3). The basal promoter activity of some of these constructs was reported (8). Thus, the activities of p(-2350/+640)LUC, p(-416/+232)LUC, and p(-455/+30)LUC in CHO cells were 10% of the activity of the SV40 enhancer/promoter, whereas the basal activity of p(-40/+640) was 50% of the activity of the above constructs. The promoter activity of constructs containing a smaller number of WT1 sites was inhibited to a lower extent, and it appeared that the inhibitory effect of WT1 was proportional to the number of potential sites.

Thus, constructs p(-2350/+640)LUC and p(-476/+640)LUC, containing twelve putative WT1 sites each, were inhibited by 82–87%, as stated above, whereas constructs p(-416/+232)LUC and p(-40/+640)LUC, containing seven putative WT1 sites each, were inhibited by 59 and 64%, respectively. Construct p(-455/+30)LUC, which contains six putative WT1 sites, was inhibited by 46%.

**Interaction of the Zinc Finger Domain of WT1 with the IGF-I-R Promoter**—To study the interactions between WT1 and the IGF-I-R promoter, gel retardation assays were performed using the purified WTZF. For this purpose, we dissected the region extending from -494 to +640 into six fragments, which were individually end-labeled and employed in binding reactions with WTZF (Fig. 4A). Fragments lacking putative WT1 binding sites (-494/-331 and -135/-26) did not generate any retarded bands, consistent with the lack of footprints in these regions (see below). Fragment -331/-135, which contains five putative WT1 sites, generated four retarded bands. It is possible that one of those bands was generated by the two tandem sites located at -262/-254 and -250/-242. Fragment -29/-185, which includes the initiator, and fragment +115/341, containing two sites each, generated two retarded bands, whereas fragment +341/+640, which contains three sites, generated only one retarded band. Formation of DNA-protein complexes was prevented when the binding reactions were performed in the presence of a 14–28-fold molar excess of the unlabeled probe (Fig. 4B).

To more accurately map the positions of the WT1 binding sites, DNase I footprinting assays were performed. For this purpose, four 32P-labeled fragments, which encompassed the region from -331 to +640, were employed. Five footprints were generated in the 5′-flanking region, only one of which is identical to the GCGGGGGCG consensus sequence (Fig. 5). The remaining four sites conform to the consensus sequence at eight of nine nucleotides (Table I). The initiator element itself includes an AGCCCGGAG sequence between nucleotides -7 and +2 which binds WTZF with medium to low affinity. In the 5′-untranslated region, six putative WT1 sites were footprinted by WTZF, although none of these sites was identical to the consensus sequence (Fig. 6).

Regulation of Promoter Activity by Alternatively Spliced Variants of WT1—To study the effect of an alternatively spliced variant of WT1 containing a 17-amino acid insert after residue 248 and a 3-amino acid insert (Lys-Thr-Ser) after residue 390, on IGF-I-R promoter activity, cotransfection experiments were performed using the reporter plasmid p(-2350/+640)LUC and the WT1 expression vector pCMVhWT-17AA-KTS (Fig. 7A). WT1-17AA-KTS inhibited the activity of the IGF-I-R promoter by only 49%, as compared with 87% inhibition achieved with the WT1 gene product lacking both inserts (Fig. 7B).

To determine whether the lower inhibitory activity of WT1-17AA-KTS was associated with a decrease in its binding capacity to the IGF-I-R promoter, DNase I footprinting experiments

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**Table 1**

Sequences in the IGF-I-R gene promoter footprinted by WTZF

| Location | Sequence | Relative binding |
|----------|----------|------------------|
| -262/-254 | GAGGGGGCC | +++ |
| -250/-242 | GAGGGGGCC | ++ |
| -220/-212 | GAGGGGGCC | ++ |
| -196/-188 | GAGGGGGCC | ++ |
| -163/-155 | GAGGGGGCC | + |
| -135/-125 | GAGGGGGCC | + |
| -108/-99 | GAGGGGGCC | ++ |
| -7/-2 | GAGGGGGCC | +++ |
| +78/+86 | GAGGGGGCC | +++ |
| +276/+284 | GAGGGGGCC | +++ |
| +303/+311 | GAGGGGGCC | ++ |
| +412/+420 | GAGGGGGCC | + |
| +453/+461 | GAGGGGGCC | + |
| +590/+598 | GAGGGGGCC | +++ |

* The affinity of WT1 binding sites for the WT zinc finger protein was arbitrarily determined as follows: +++, footprints generated by 60 ng or less of WTZF; ++, footprints generated by 125 ng or more of WTZF; +, footprints generated by 250 ng of WTZF. Nucleotides in **boldface letters** correspond to the consensus sequence.
were performed using a synthetic zinc finger protein containing the Lys-Thr-Ser insert (WTZF + KTS). This approach seems justified in view of previous studies that showed that only the zinc finger domain of WT1 is involved in DNA binding (14). As shown in Fig. 7C, the capacity of WTZF + KTS to generate DNase I footprints was dramatically reduced in comparison with WTZF. A single footprint at the position of the consensus GCGGGGGCG site (-188/-196) was generated with WTZF + KTS, although only at the highest concentration of the protein (125 ng).

**DISCUSSION**

The involvement of the IGF-I-R in the etiology and/or progression of Wilms’ tumor was inferred from studies that showed that passive immunization of nude mice bearing Wilms’ tumor heterotransplants with an IGF-I-R-specific antibody resulted in partial tumor remission (25). Recently, we showed that the expression of the IGF-I-R gene is significantly increased in Wilms’ tumor and that an inverse correlation exists between the levels of WT1 mRNA and IGF-I-R mRNA, suggesting that the expression of the IGF-I-R gene is under the inhibitory control of WT1 (18).

The present coexpression studies, in which both extended and proximal IGF-I-R promoters (containing the same number of WT1 binding sites) were repressed to the same extent by a WT1 expression vector, support this hypothesis. These results suggest the following: (i) the promoter of the IGF-I-R gene constitutes a target for the inhibitory action of WT1; and (ii) only the proximal region of the IGF-I-R promoter is required to mediate this effect. There is, apparently, no involvement of further upstream sequences in WT1-mediated transcriptional repression. Furthermore, cotransfection studies using different fragments of the promoter suggest that the effect of WT1 is somewhat dependent upon the number of active WT1 sites but is less dependent on the location of those sites. Thus, the activity of a fragment extending from -416 to +232 and containing seven WT1 sites was inhibited to the same extent as a fragment with the same number of WT1 sites extending from -40 to +640. These results imply that the specific contribution of WT1 sites in the 5’-flanking region is similar to that of transcribed WT1 sites. These results are similar to those of Drummond et al. (1992) (15) obtained with the P3 promoter of the IGF-II gene; however, they are different from those of Wang et al. (1993) (30) obtained with the promoter of the platelet-derived growth factor A-chain gene. In that case, WT1 repressed transcription of a minimal platelet-derived growth factor A-chain gene promoter only when WT1 sites were present on both sides of the transcription start site, whereas it activated transcription if those sites were either 5’ or 3’ of the start site. Possible explanations for these discrepancies include the much larger number of WT1 sites in the IGF-I-R promoter and/or the presence of the TATA box in the platelet-derived growth factor gene versus an initiator motif in the IGF-I-R gene.

The physical interaction between WT1 and the IGF-I-R promoter was analyzed by gel retardation and DNase I footprinting assays using the purified zinc finger domain of WT1. WTZF generated five footprints in the 5'-flanking region and six footprints in the 5'-untranslated region. In addition, the initiator element itself binds WT1. There was, in general, a very good
correlation between the number of bands seen in gel retardation assays and the number of footprints generated in the same region by WTZF. The only exception was the fragment extending from +341 to +640. In this region, three footprints were generated, although only one retarded band was seen, probably due to the fact that two of those sites had low to medium affinity for WT1. Furthermore, the results obtained indicate that some putative WT1 sites that conformed to the consensus sequence at eight out of nine nucleotides (CGGCGCCGC at position -389 to -381, CGGCGGCGGC at +9 to +17 and +388 to +396, CGGCGCCGC at +184 to +192, and CGC CGGCCGC at +506 to +514) were not bound by WTZF, at least under the conditions studied. This fact, however, does not necessarily preclude them from being functionally active in vivo assays. On the other hand, some of the sequences footprinted conform to the consensus sequence at only six positions (GAGGGGGAA at positions +78 to +86 and +453 to +461). These cases, however, suggest that the core GGGGG sequence appears to be required for binding activity.

The case of the IGF-I-R gene constitutes the first example of an initiator type of promoter shown to be regulated by WT1. Although the proteins involved in binding to the initiator motif have not been fully characterized, it is clear that this element can replace the TATA box in directing transcription initiation from a unique site contained within it (11). Our results suggest that some of the effect of WT1 may be achieved by direct binding of WT1 to the initiator, therefore impairing the ability of this element to assemble a functional transcription complex. Additionally, the effect of WT1 may be mediated by its association with sites located both upstream and downstream of the initiator element. In any event, the multiple interaction sites for WT1 on both sites of the transcription initiation site may constitute the physical basis for a stringent inhibitory control of the IGF-I-R promoter. In view of the fact that the tumor suppressor gene product p53 has been recently shown to interact with WT1, it will be interesting to study the implication of p53 and other tumor suppressors in the regulation of the IGF-I-R promoter.

Finally, we have analyzed the activity of an alternatively spliced variant of WT1, which contains a 17-amino acid insert in the region immediately N-terminal to the zinc finger domain and a 3-amino acid insert between the third and fourth zinc fingers. The presence of the extra 17 amino acids does not affect DNA binding (14) although the presence of the extra three amino acids has been shown to impair DNA binding (14, 22). Consistent with those previous studies, our present results indicate that WT1+ KTS is able to interact only with the consensus sequence within the IGF-I-R promoter and only at high concentrations. No footprints were seen at the other WT1-related sites. Since the 17-amino acid insert does not impair the inhibitory effect of WT1, it is clear that the reduced effect of pCMVhWT-17AA-KTS on promoter activity is due to a diminution in the binding capacity of its zinc finger domain. We cannot exclude the possibility, however, that the DNA binding specificity of WT1 changed as a result of the KTS insertion.

In conclusion, we have characterized the WT1 binding sites in the IGF-I-R gene promoter. Physical interaction of the zinc finger domain of WT1 with consensus binding sites on both sides of the initiator element may constitute the basis for the transcriptional repression of the IGF-I-R gene by WT1. Inability of WT1 in Wilms' tumors to repress transcription from this target gene may result in overexpression of this transcript. Activation of the IGF-I-R by IGF-II may be an important mechanism for the progression of Wilms' tumors.

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Note Added in Proof—After submission of this manuscript, the presence of an alternatively spliced WT1 mRNA lacking the exon 2 sequence was reported to be present in Wilms' tumors but not in normal kidney (Haber, D. A., Park, S., Maheswaran, S., Englert, C., Re, G. G., Hazen-Martin, D. J., Sens, D. A., and Garvin, A. J. (1993) Science 262, 2057–2059).

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