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RNA binding by the Wilms tumor suppressor zinc finger proteins

(early growth response factor 1/gene expression/insulin-like growth factor II/posttranscriptional regulation)

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ABSTRACT **The Wilms tumor suppressor gene *WT1* is implicated in the ontogeny of genito-urinary abnormalities, including Denys–Drash syndrome and Wilms tumor of the kidney. *WT1* encodes *Krüppel*-type zinc finger proteins that can regulate the expression of several growth-related genes, apparently by binding to specific DNA sites located within 5′ untranslated leader regions as well as 5′ promoter sequences. Both WT1 and a closely related early growth response factor, EGR1, can bind the same DNA sequences from the mouse gene encoding insulin-like growth factor 2 (*Igf-2*). We report that WT1, but not EGR1, can bind specific *Igf-2* exonic RNA sequences, and that the zinc fingers are required for this interaction. WT1 zinc finger 1, which is not represented in EGR1, plays a more significant role in RNA binding than zinc finger 4, which does have a counterpart in EGR1. Furthermore, the normal subnuclear localization of WT1 proteins is shown to be RNase, but not DNase, sensitive. Therefore, WT1 might, like the *Krüppel*-type zinc finger protein TFIIIA, regulate gene expression by both transcriptional and posttranscriptional mechanisms.**

The tumor suppressor gene, *WT1*, was identified by positional cloning at chromosome 11p13 on the basis of predisposition to Wilms tumor of the kidney (1, 2). Mutation of *WT1* has been associated with abnormalities of the genito-urinary tract, in both humans (reviewed in refs. 3 and 4) and rodents (5, 6), establishing a clear developmental role for the *Krüppel*-type zinc finger proteins it encodes. Alternative splicing of *WT1* results in the production of four variant WT1 proteins that differ by the presence or absence of 17 amino acids, encoded by exon 5, and 3 amino acids (lysine, threonine, and serine; KTS), encoded at the 3′ terminus of exon 9 (7). All of the WT1 proteins contain four zinc fingers, which mediate binding to specific DNA sequences, and zinc fingers 2, 3, and 4 are highly homologous with the three zinc fingers of the early growth response factor EGR1 (1, 2, 8, 9). The KTS insertion occurs in the conserved linker region between zinc fingers 3 and 4, such that WT1 variants lacking these three amino acids (WT1-KTS) resemble EGR1 more closely than those in which they are present (WT1+KTS). Consistent with this is the observation that WT1-KTS binds DNA sequences that resemble the EGR1 consensus-binding site (5′-GCGGGGGCG-3′), whereas WT1+KTS binds more disparate sequences (8–13). In transient transfection assays WT1 can regulate the expression of several growth-related genes containing these motifs (e.g., refs. 14–20), and usually acts as a repressor of these genes. WT1 has thus been described as a transcription factor.

We were prompted to examine the possibility of a posttranscriptional regulatory role for WT1 by a number of observations. For maximum effect on at least some of its target genes, WT1-binding sites must be present both upstream and downstream of transcript initiation sites (14, 17, 18, 21, 22). Functional WT1-binding sites are present within 5′ untranslated leader sequences of several candidate target genes studied so far (14, 15, 17, 18, 20, 21). This is exemplified by exon 2 of the insulin-like growth factor-2-encoding gene (*Igf-2*; refs. 14 and 21), which is one of three alternative 5′ untranslated leader exons attached to *Igf-2* transcripts (23). It is known that translational discrimination occurs between *Igf-2* transcripts during mouse embryogenesis, with those bearing exon 2 sequences being underrepresented on polysomes at certain developmental stages (24, 25). We have shown that expression from *Igf-2* reporter gene constructs containing promoter 2 and exon 2 sequences is down-regulated when WT1-binding sites are present within 5′ untranslated leader sequences (21), and nuclear run-on assays indicate that this down-regulation is mediated by posttranscriptional events (A.W., A.D., and A.C., unpublished data). Finally, it was recently reported that WT1 protein in the nucleus is portioned into regions in which it associates intimately with splicing factors, or with transcription factor domains (26).

Here we demonstrate that both WT1-KTS and WT1+KTS can bind, in a sequence-specific manner, to RNA derived from exon 2 sequences. Mutational analysis reveals that the WT1 zinc fingers are required for RNA binding. Furthermore, the nuclear localization of WT1 is shown to be dramatically affected by treatment with RNase, but not DNase. Collectively, these findings indicate that, in addition to its role as a transcription factor, WT1 might regulate gene expression at the posttranscriptional level.

MATERIALS AND METHODS

Cell Culture. Buffalo rat BRL-3A cells were obtained from the collection housed at the Sir William Dunn School of Pathology (University of Oxford, UK), and the mouse mesothelioma cell line AC29 was that used by Larsson *et al.* (26). Cells were cultured as monolayers at 37°C, 5% CO_2/95% air in α-Ham's medium (GIBCO/BRL) supplemented with 10% fetal calf serum, 75 mg/liter ampicillin, 50 mg/liter streptomycin, and 50 mM 2-mercaptoethanol.

Plasmids. All glutathione *S*-transferase- (GST-) fusion constructs were formed using the pGEX-3X vector (Pharmacia) and cDNA sequences encoding either WT1 or EGR1 zinc finger domains. Details of both wild-type (9) and mutant (12) WT1 constructs were published elsewhere. A comparable

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Abbreviations: EGR1, early growth response factor 1; GST, glutathione *S*-transferase; IGF-II, insulin-like growth factor II; WT1, Wilms tumor suppressor protein 1.

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EGR1 fusion construct was produced following excison of the zinc finger encoding region, as a 1-kb *Rsa*I fragment, from the mouse cDNA clone pMexNeo (R. Bravo, unpublished data) and its ligation into the *Sma*I site of pBluescript SK+ (Stratagene). The insert was recovered from this subclone utilizing *Bam*HI and *Eco*RI sites from the pBluescript SK+ polylinker (now located 5′ and 3′ of the *Egr*-1 cDNA sequences, respectively) and ligated into the pGEX3X vector, which had been cleaved at the same sites. Binding specificity of the EGR1 fusion protein was shown in gel electrophoretic mobility-shift assays using oligonucleotides containing a consensus EGR1-binding site (5′-CGCCCCCGC) and a mutated derivative (5′-CGCCCCATA), as described (ref. 8; data not shown).

Protein–Nucleic Acid Interaction Analysis. GST-fusion proteins used in gel electrophoretic mobility-shift assays and RNase CV1 footprinting were bacterially expressed and recovered by sonication, as stated (12), then affinity purified as follows: Extracted soluble proteins, in phosphate-buffered saline (PBS) supplemented with 3 μg/ml each of aprotinin, leupeptin, and pepstatin (Sigma), were combined with one-third volume of glutathione-Sepharose (Pharmacia) pre-equilibrated with PBS. This was mixed by gentle rotation for 30 min at 4°C then washed successively with 20× volumes of ice-cold PBS containing 1% Triton X-100 (twice) and PBS alone (three times). GST-fusion proteins were eluted after incubation for 5 min at 37°C with 200 μl of a prewarmed solution containing 50 mM Tris (pH 8.0), 20 mM glutathione, and supplemented with protease inhibitors. To ensure equivalent loadings in electrophoretic gel mobility-shift and RNase CV1 footprinting assays, aliquots of purified proteins were assessed on SDS/10% polyacrylamide gels together, essentially as described (12), and the integrity of fusion proteins was checked by Western blotting and staining with an anti-GST antibody (Molecular Probes).

DNA probes were derived from an exon 2 fragment spanning nucleotides +39 to +307 (23), which was excised from the *Igf-2* subclone pP2P3 (27) following *Nae*I digestion. The same fragment was ligated into the *Hin*cII site of pGEM-4Z (Promega) such that a sense-strand exon 2 RNA could be transcribed *in vitro* using bacteriophage SP6 RNA polymerase (28), following linearization of the plasmid with *Hin*dIII. Probes were prepared by γ-[^{32}P] end-labeling approximately 100 ng of either the DNA fragment or the *in vitro* transcribed RNA to specific activities of 5–10 × 10^7 cpm/μg. Between 1 and 5% of the labeled nucleic acid was used in each reaction. Unlabeled RNAs, of similar length to the exon 2 RNA, were derived by *in vitro* transcription of plasmids containing sequences from either the coding region of the glyceraldehyde 3-phosphate dehydrogenase gene (*Gapdh*) or the simian virus 40 polyadenylylation signal and t intron region. In the case of *Gapdh*, a sense RNA was synthezised using Sp6 RNA polymerase following linearization of the pGAPDH plasmid (29) with *Bam*HI. An RNA representing anti-sense simian virus 40 sequences was derived using a 1.2-kb *Dra*I fragment isolated from pLUC (30), which was transcribed with T7 RNA polymerase.

Binding reactions were carried out for 20–30 min on ice, in 20 μl volumes of binding buffer (50 mM Hepes, pH 7.5/50 mM KCl/5 mM $MgCl_2$/10 μM $ZnSO_4$/10 mM DTT/20% glycerol/0.2 mg/ml bovine serum albumin), equivalent amounts of GST-fusion proteins (as indicated), approximately 1 ng of labeled probe, and either 0.1 mg/ml poly(dI·dC) (DNA probes) or 100 ng yeast tRNA (RNA probes). Where unlabeled competitor RNAs were used they were added 10 min prior to, and as a 30- or 100-fold molar excess over, labeled exon 2 RNA. Gel electrophoretic mobility-shifts were assayed directly on nondenaturing 6% polyacrylamide gels. Footprinting reactions were initiated by addition of 0.1 unit of RNase CV1 (Pharmacia) and, following an incubation of 1 min at 37°C, the reaction was terminated by extraction with phenol:chloroform:isoamyl alcohol (25:24:1). Samples were ethanol precipitated, resuspended in 12 μl of loading buffer (48% formamide/10 mM EDTA/0.025% xylene cyanol FF) and separated on 6% denaturing polyacrylamide gels, using dideoxynucleotide sequence reactions to provide molecular weight markers.

Cell Extractions and Immunofluorescence Microscopy. Detergent extractions were carried out *in situ*, on cells grown on coverslips, as described in Todorov *et al.* (31). Briefly, cells were washed in PBS, followed by cytoskeleton buffer (CSK: 10 mM Pipes, pH 7.0/100 mM NaCl/300 mM $MgCl_2$/300 mM sucrose), and then extracted for 5 min at 20°C with CSK containing 0.5% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg aprotinin, and 10 μg leupeptin. Where appropriate, 100 μg/ml of either DNase I or RNase A was also included in the extraction buffer. Cells were washed in CSK, fixed for 30 min on ice in CSK containing 4% paraformaldehyde and processed for immunofluorescence microscopy using standard techniques (31). The C-19 polyclonal (Santa Cruz Biotechnology) and WT48 monoclonal (9) antibodies, raised against WT1, or an affinity-purified anti-BM28 polyclonal antibody (32) were visualized using fluorescein isothiocyanate or Texas red conjugated with either horse anti-mouse or goat anti-rabbit IgG (Vector Laboratories). DNA staining was achieved using 0.2 μg/ml of 4d′,6-diamino-2-phenylindole in the final washing steps.

RESULTS

WT1 Can Bind to RNA Derived from *Igf-2* Exon 2 in a Sequence-Specific Manner. Gel electrophoretic mobility-shift assays demonstrated that GST-fusion proteins representing both WT1-KTS and WT1+KTS strongly bound a [^{32}P]-labeled RNA probe derived from exon 2 sequences, which encompassed the WT1-binding sites previously identified in human and mouse *Igf-2* DNA (11, 14, 21), whereas a comparable EGR1 fusion protein bound poorly, if at all (Fig. 1*a*). In contrast, both WT1-KTS and EGR1 efficiently bound the analogous DNA probe (Fig. 1*b*), and DNase I footprinting of exon 2 sequences has shown that WT1-KTS binds to a subset of sites to which EGR1 binds (A.D., A.C., and A.W., unpublished data). Weaker binding of WT1+KTS to the DNA probe is consistent with studies showing that this WT1 isoform does not readily recognize sequences related to the EGR1 consensus-binding site (8, 9, 11–13). Interestingly, WT1+KTS consistently bound the RNA probe with greater affinity than WT1-KTS (Fig. 1*a*). WT1 reverse protein (REV), used as a control in all experiments, was derived from a construct containing WT1 zinc finger coding sequences inserted in the anti-sense orientation in the pGEX-3X vector (9). REV protein was unable to bind either the exon 2 DNA or RNA probes.

The specificity of the interaction between WT1 protein and *Igf-2* exon 2 RNA was demonstrated by competition assays using unlabeled RNA species (Fig. 1*c*). Binding of both WT1-KTS and WT1+KTS to labeled exon 2 RNA was diminished in the presence a 30-fold excess of unlabeled exon 2 RNA, whereas binding was unaffected in the presence of up to a 100-fold excess of similar sized RNAs derived from either the *Gapdh* or simian virus 40 T-antigen genes. Specificity of binding was also investigated using RNase CV1 footprinting (Fig. 2). A discrete region of *Igf-2* exon 2 RNA, mapping between nucleotides +47 to +72 (which overlaps the WT1-binding site in exon 2 DNA), was protected from RNase degradation by both WT1 isoforms, but not by EGR1 or reverse protein (not shown). We estimate that the mapping of this footprinted region is accurate to within two nucleotides, given that molecular weight markers were provided by a DNA sequence ladder.

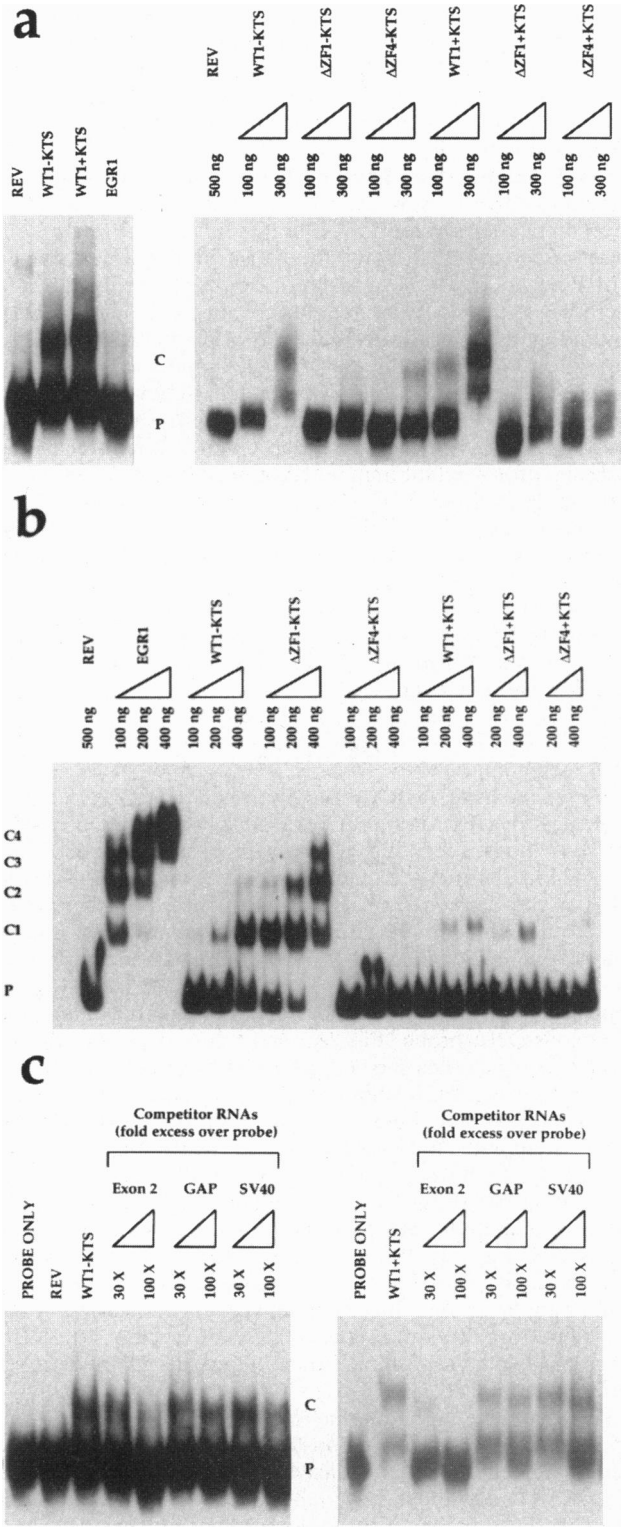

FIG. 1. Gel electrophoretic mobility-shift analysis of WT1 and EGR1 binding to *Igf-2* exon 2 RNA and DNA (P, unbound probe; C, nucleic acid–protein complex; REV, reverse control GST-fusion protein). (*a*) Binding of wild-type WT1 isoforms (WT1-KTS and WT1+KTS) of mutant WT1 isoforms lacking either zinc finger 1 (ΔZF1-KTS and ΔZF1+KTS) or zinc finger 4 (ΔZF4-KTS and ΔZF4+KTS), and of EGR1, to *Igf-2* exon 2 RNA. (*b*) Binding of WT1 isoforms and EGR1 to *Igf-2* exon 2 DNA. (*c*) Competition assays showing binding specificity of WT1 isoforms to *Igf-2* exon 2 RNA. Unlabeled RNAs containing *Igf-2* (Exon 2), *Gapdh* (GAP), and simian virus 40 sequences (SV40) were added as indicated at a 30-fold (30×) and 100-fold (100×) molar excess over labeled exon 2 RNA, in the presence of 200 ng WT1-KTS (*left*) or WT1+KTS (*right*).

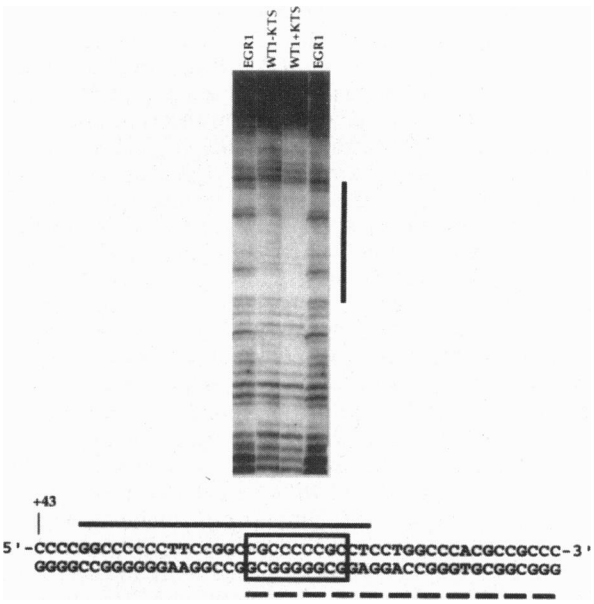

FIG. 2. Localization of WT1-binding site on *Igf-2* exon 2 RNA. Autoradiograph of labeled exon 2 RNA incubated with WT1-KTS, WT1+KTS, or EGR1, subjected to controlled RNase CV1 digestion and with resulting fragments separated on a 12% denaturing polyacrylamide gel. The region protected from RNase CV1 degradation (solid line) has been mapped to the *Igf-2* exon 2 sequence (23), part of which is given below. The footprint encompasses an EGR1 consensus DNA-binding site (boxed) and overlaps with the region of exon 2 DNA footprinted by WT1-KTS (broken line; ref. 21).

Zinc Fingers Are Required for the Interaction of WT1 with RNA. The contribution of individual zinc fingers to nucleic acid binding was assessed using additional GST fusion proteins representing mutant forms of WT1 in which either zinc finger 1 (WT1ΔZF1-KTS and WT1ΔZF1+KTS) or zinc finger 4 (WT1ΔZF4-KTS and WT1ΔZF4+KTS) was deleted. In gel electrophoretic mobility-shift assays RNA binding by either WT1 isoform was abolished following removal of zinc finger 1 (Fig. 1*a*), whereas there was no deleterious effect on DNA binding (Fig. 1*b*). In fact, there was a marked increase in binding of WT1ΔZF1-KTS to the DNA probe, compared with WT1-KTS, such that the affinity of the mutant WT1 protein was almost equal to that of EGR1 for this DNA target. We have recently obtained similar results using other DNA probes containing sequences related to the EGR1 consensus-binding site (A.D., A.C., and A.W., unpublished data). In contrast, mutant WT1 proteins lacking zinc finger 4 retained some binding to RNA (Fig. 1*a*; seen more clearly in the case of WT1ΔZF4-KTS than WT1ΔZF4+KTS) and binding to DNA was considerably diminished (Fig. 1*b*). These data suggest that zinc finger 1, which has no counterpart in EGR1, has a more significant role in RNA binding than zinc finger 4.

Intact RNA Is Required for the Normal Subcellular Localization of WT1. We next sought evidence that the observed interaction between WT1 and RNA might be significant *in vivo*. For this, a monoclonal antibody specific for WT1 protein was used to stain cell lines naturally expressing *WT1* transcripts. These immunocytochemical studies confirmed that WT1 is largely confined to the nucleus, where there is a speckled distribution of antibody staining (Fig. 3*a*; refs. 26, 33, and 34). Regions of brightest staining with WT1 antibodies were consistently found to correspond to areas containing lower concentrations of DNA. Nuclear staining was unaltered following extraction of cells with the detergent Triton X-100, although the diffuse staining in the cytoplasm was lost (Fig. 3*b*). Extracted cells treated with DNase also showed little, if any, change in nuclear staining (Fig. 3*c*). However, following

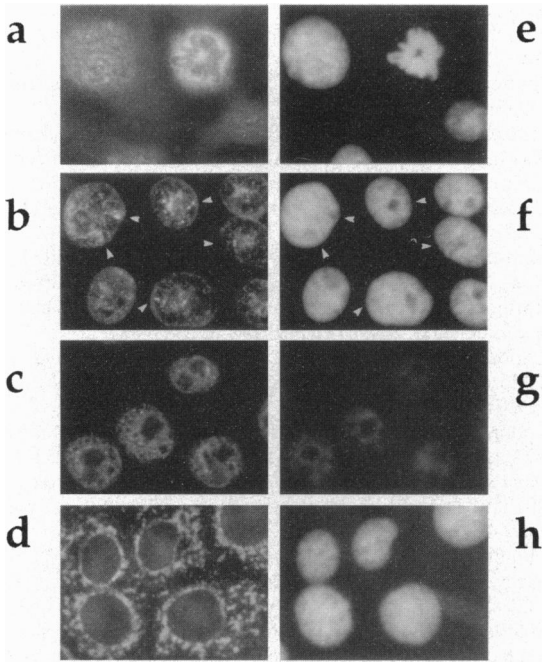

FIG. 3. Immunolocalization of WT1 in rat liver-derived BRL-3A cells using the C-19 anti-WT1 antibody. (*a*) Nonextracted cells. (*b*) Cells extracted with detergent (Triton X-100). (*c*) Extracted cells treated with DNase I. (*d*) Extracted cells treated with RNase A. (*e–h*) DAPI staining of cells in *a–d* to reveal DNA. Arrows highlight areas of intense antibody staining, corresponding with areas of lower DNA concentration.

detergent-extraction and treatment with RNase, nuclear staining characteristic of the anti-WT1 antibody was no longer observed, instead there was intense staining of the perinuclear margin (Fig. 3*d*). In contrast, we note that when BRL-3A cells were double-stained with an antibody raised against the nuclear antigen BM28 its distribution was affected by treatment with DNase and not RNase (not shown), as reported previously using HeLa cells (31). Similar data were obtained both with BRL-3A cells (Fig. 3) and the human mesothelioma cell line AC29 (not shown), and results were essentially reproduced using a polyclonal anti-WT1 antibody (not shown). These experiments suggest that WT1 associates, either directly or indirectly, with cellular RNA, which is in accord with evidence that a proportion of WT1 in the nucleus colocalizes with small nuclear ribonucleoproteins (26).

DISCUSSION

Our studies of interactions between WT1 and the *Igf-2* gene lead us to propose a posttranscriptional regulatory function for WT1. In common with several other genes that have been considered as targets for WT1 action, *Igf-2* contains WT1-binding sites within a transcribed region as well as 5′ promoter sequences (11, 14, 21). We have shown that WT1 can bind specific sequences in RNA derived from *Igf-2* exon 2. The closely related zinc finger protein EGR1, is unable to bind exon 2 RNA although it readily occupies the binding site in exon 2 DNA, which contains a consensus EGR1-binding motif. Without direct evidence of an interaction of WT1 with endogenous *Igf-2* transcripts it was important to establish that part of the WT1 activity *in vivo* might involve an association with RNA. This was achieved by nuclease treatment of cells, which revealed the distribution of WT1 in the nucleus to be sensitive to RNase, but not DNase treatment.

WT1 has characteristics typical of many transcription factors, including a proline- and glutamine-rich amino-terminal effector region and domains that facilitate either binding to DNA or protein–protein interactions (22, 35–39). It is also clearly capable of regulating the expression of a variety of target genes that contain WT1-binding sites within 5′ promoter sequences (11, 14–17, 19, 40–43) and a proportion of WT1 protein (predominantly the WT1-KTS isoforms) is associated with transcription factor domains in the nucleus (26). These observations indicate a role for WT1 as a regulator of gene transcription. However, a large amount of WT1 in the nucleus is found outside of transcription factor domains, with most of the WT1+KTS protein colocalizing with splicing factors (25). Together with the evidence from the present study, this suggests WT1 also acts to regulate gene expression posttranscriptionally. Indeed, the findings of Larsson *et al.* (26) suggest distinct regulatory functions for WT1 proteins, with WT1-KTS isoforms acting on transcription and WT1+KTS isoforms influencing posttranscriptional processes. In keeping with this idea, WT1-KTS isoforms bind a variety of DNA sequences with greater affinity than does WT1+KTS (refs. 9, 11–13, 21 and this study), whereas WT1+KTS has the greater affinity for *Igf-2* exon 2 RNA. As the subcellular localization of WT1 is primarily nuclear, the posttranscriptional action of WT1 is likely to be at the level of nuclear transcript processing and/or compartmentalization, rather than at the level of message translation.

TFIIIA represents the only other cellular factor known to regulate gene expression through binding to both DNA and RNA and it is intriguing that it, like WT1, contains *Krüppel*-type zinc fingers. All of the nine zinc fingers in TFIIIA may participate in binding both types of nucleic acid; however, they can be grouped into distinct subsets of fingers that are the most critical for binding to either 5S ribosomal RNA or DNA (reviewed in ref. 44). An analogous situation is suggested by our experiments with WT1 mutant fusion proteins, since the absence of zinc finger 1 is more deleterious for RNA binding (and can enhance binding to DNA), whereas the absence of zinc finger 4 is more deleterious for DNA binding. This difference in the relative contribution of the two terminal zinc fingers of WT1 is underlined by comparison with EGR1, which did not bind *Igf-2* exon 2 RNA and has only three zinc fingers that are homologous to WT1 zinc fingers 2, 3, and 4.

The spectrum of RNA sequences to which WT1 can bind is clearly now in need of investigation. The overlap in *Igf-2* exon 2 RNA and DNA sequences bound by WT1 is again reminiscent of the binding of TFIIIA to the corresponding sites in 5S RNA and 5S DNA. However, the binding specificity of TFIIIA is determined by the primary sequence in the case of DNA, but by the secondary/tertiary structure of RNA (reviewed in ref. 44). Should this prove to be the case for WT1, then there may not be a straightforward relationship of sequences among RNAs to which it binds. The identification of binding sites in RNA might be complicated further, as they could occur outside of exonic sequences if WT1 interacts with primary transcripts in the nucleus.

In conclusion, our findings are consistent with a role for WT1 in the regulation of gene expression at the posttranscriptional level, mediated by sequence-specific binding of RNA molecules. Although many other RNA-binding factors are known, this represents a novel function for a tumor suppressor gene product and must be taken into account for a full understanding of the mechanism of WT1 action.

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