The tumor suppressor gene WT1 encodes a zinc finger protein, which consists of four C-terminal C2-H2 zinc fingers of the Krüppel type, and at the N terminus a Q/P-rich trans-regulatory domain, both characteristic of transcription factors. However, recent findings suggest that WT1 may also be involved in a post-transcriptional process. Specifically, WT1 isoforms containing the alternatively spliced exon 9 (+lysine-threonine-serine (KTS)) preferentially associate with nuclear speckles and co-immunoprecipitate splicing antigens (Larsson, S. H., Charlier, J.-P., Miyagawa, K., Engelkamp, D., Bassoula-degan, M., Ross, A., Cuzin, F., van Heyningen, V., and Hastie, N. D. (1995) Cell 81, 391–401); furthermore, WT1 has been shown to interact with the ubiquitous splicing factor U2AF65 (Davies, R. C., Calvo, C., Larsson, S. H., Lamond, A. I., and Hastie, N. D. (1998) Genes Dev. 12, 3217–3225) and binds to RNA in vitro (Caricasole, A., Duarte, A., Larsson, S. H., Hastie, N. D., Little, M., Holmes, G., Todorov, I., and Ward, A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7562–7566; Bardee, N., and Pelleter, J. (1998) Nucleic Acids Res. 26, 1794–1792). To extend these findings, we have fractionated nuclear extracts to see if particles containing WT1 have the properties of ribonucleoprotein (RNP). In summary, WT1 is enriched by oligo(dT) chromatography, as are U2AF65, the US small nuclear RNP-associated protein p116 and hnRNP A1. Gel filtration and sedimentation profiles suggest that WT1 is present in RNA-sensitive particles, >2 MDa in size, peaking at ~60 S, and ~1.27 g/cm³ on Nycodenz. Similar results were obtained from two cell lines expressing WT1, fetal kidneys (day E17), and transiently transfected cells, suggesting that the presence of WT1 protein in nuclear poly(A)* RNP is a general aspect of WT1 function.

Wilm’s tumor is one of the most common childhood malignancies, affecting 1/10,000 children. In the search for genes involved in this disease, a candidate tumor suppressor gene, named WT1, was identified and cloned, and shown by mutational analysis to be involved in 10–15% of Wilm’s tumors (5, 6). Although described as a tumor suppressor gene, it should be noted that the growth suppressor effects of WT1 are context-dependent (7). Mutations in the WT1 gene also result in urogenital abnormalities; WT1 therefore provides an excellent opportunity to study the relationship between cancer and development (8–10). Given the expression pattern of WT1, the phenotypes associated with a number of genetic syndromes in which it is implicated, and a mouse knockout model (11), it is thought that WT1 plays an essential role in the transition from proliferative mesenchyme to differentiated epithelium (12).

The WT1 gene encodes a protein which includes, at its C terminus, four C2-H2 zinc fingers of the Krüppel-type, with close structural homology to zinc fingers in the early growth response family of transcription factors. At the N terminus, WT1 possesses a proline/glutamine-rich putative transactivation domain. Molecular modeling also suggests the presence of an RNA recognition motif at the N terminus (13). Murine WT1 is >95% identical at the amino acid level to its human counterpart (14). The structure of mammalian WT1 protein is complicated by two alternative splicing events as follows: inclusion of exon 5, which inserts 17 amino acids in the middle of the protein, and exon 9, which inserts three amino acids lysine-threonine-serine (KTS) between the third and fourth zinc fingers (15); an RNA editing event (16); and an alternative upstream translation start site (17) giving a total of at least 16 possible isoforms.

Given its salient features, the immediate assumption was that WT1 protein is a transcription factor. Numerous studies have investigated the effects of WT1 on the expression of candidate target genes, for example IGF2, IGF2R, EGF, CSF1, TGF-β, PDGFA, Pax-2, Nov, and ODC (7). These studies are generally based on the expression of reporter constructs in cells co-transfected with WT1; in general, data suggest that WT1 acts as a transcriptional repressor of these growth-associated genes. WT1 binds to a G-rich DNA sequence, similar to the consensus binding site for the early growth response family of transcription factors (18). In addition, recent evidence suggests that WT1 can also act as a transcriptional activator, up-regulating the anti-apoptotic gene bcl-2 (19), and as a transcriptional co-factor, modulating SF-1-mediated transactivation in testis development (20).

Was WT1 therefore a typical transcription factor whose target genes needed to be defined? This picture was complicated when Larsson et al. (1) began to look in detail at the subcellular distribution of WT1 protein. They showed that a proportion of WT1 is concentrated in nuclear “speckles,” which contain splicing factors, and co-immunoprecipitated WT1 with Sm (snRNP) antigens, the splicing factors U170, U2-B*, and p80 coilin. Co-immunoprecipitation of WT1 with Sm antigens was abolished by actinomycin D treatment. Both actinomycin D and injection of antisense snRNAs resulted in the relocation of WT1 to larger nuclear structures. Similarly, in HeLa cells, the miRNA target genes were down-regulated, and Sm antigens were displaced from the speckles. The immediate assumption was that WT1 was involved in RNA binding and processing, either by directly binding to RNA or by recruiting proteins that could process RNA. This assumption suggested that WT1 could control the expression of genes by regulating their processing, rather than by controlling their transcription.

1 The abbreviations used are: snRNP, small nuclear ribonucleoprotein; RNP, ribonucleoprotein; PCRs, polymerase chain reaction; TMV, tobacco mosaic virus; snRNA, small nuclear RNA; hnRNP, human RNP; FITC, fluorescein isothiocyanate; DEPC, diethyl pyrocarbonate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; TMV, tobacco mosaic virus; PCNA, proliferating cell nuclear antigen.

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croinjection of oligonucleotides and antibodies that disrupt splicing produced rounder and larger interchromat granule clusters (21). Larsson et al. (1) also showed that isoforms of WT1 that included exon 9 (+KTS) preferentially associated with nuclear speckles, whereas (−KTS) isoforms were enriched in areas where the transcription factor Sp1 was more abundant. Thus, the possibility arose that WT1, and in particular the +KTS isoforms, may be involved in post-transcriptional events, specifically splicing.

To extend these findings, Davies et al. (2) subsequently described an interaction between WT1 and the ubiquitous splicing factor U2AF65. This interaction was defined and analyzed using the yeast two-hybrid approach, coupled to in vitro binding and in vivo co-immunoprecipitation. Moreover, both WT1 and the splicing factor U2-B incorporated in vitro into large molecular weight complexes associated with the sense, but not the antisense strand, of a biotinylated adenosine pre-mRNA.

In the same study, +KTS isoforms preferentially associated with U2AF65. Significantly, the presence of +/−KTS isoforms, and their correct ratio, is evolutionarily conserved throughout vertebrates (22). It has recently been shown that Frasier syndrome, which is characterized by slow progressive nephropathy and streak gonads, can arise when this alternative splicing event is perturbed (23–25). In addition, WT1 was shown to bind RNA in vitro. In one study, the WT1 zinc fingers, particularly the first out of the four, bound an RNA sequence encoded by exon 2 of the IGF2 gene (3), which overlaps with a putative DNA target sequence. A more recent paper describes a SELEX (in vitro ligand selection) experiment that used the zinc fingers of WT1 to define three candidate RNA target sequences (4). The significance of these findings remains to be investigated.

Almost a decade after its discovery, the molecular function of WT1, in particular at the post-transcriptional level, is still unclear. Understanding the biochemistry of WT1 is an urgent priority in the field. In particular, its association with the splicing machinery is still controversial, lacking functional data. The aim of this study was to use established fractionation techniques to see if WT1 is present in nuclear RNP particles. It is hoped that these fractionation techniques can, in the near future, help determine the molecular function of WT1 and identify its RNA targets in a physiological context.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Media—**Extracts were obtained from two expressing mouse cell lines as follows: M15, derived from mesonephros (26), and AC29, derived from an asbestos-induced mesothelioma (27); also from whole embryonal E17 kidneys; and COS7 cells transfected with constructs expressing WT1. AC29 mouse mesothelioma cells were cultured in RPMI 1640 (Life Technologies, Inc.) with 10% fetal calf serum (Life Technologies, Inc.) and 100 units/ml of penicillin, 100 units/ml of streptomycin, and 200 µg/ml of geneticin. M15 cells, established from mouse mesonephros, were cultured in Dulbecco's modified Eagles medium (Life Technologies, Inc.) and 100 units/ml of penicillin, 100 units/ml of streptomycin, and 200 µg/ml of geneticin. Cell culture was performed in a humidified atmosphere of 5% CO2 in air.

**Preparation of Nuclear Extracts—**Nuclei from AC29 and M15 cells were isolated according to the method of Lee and Green (68). 17-Day fetal kidneys were dissected and immediately frozen in liquid nitrogen. When required, fetal kidneys, dissected manually, were thawed on ice and homogenized in 2× 1-ml volumes of phosphate-buffered saline (PBS) in a Dounce homogenizer, 10 strokes with a loose fitting pestle. The resulting cell suspension was pelleted by centrifugation at 3000 rpm for 10 min. Briefly, cells were suspended in Buffer A (10 mM KCl, 1.5 mM MgCl2, 10 mM Hepes, pH 8.0) for 15 min on ice. Following 10 passages through a 25-gauge needle, greater than 90% of cells were lysed. Nuclei were lysed in Buffer C (1.5 mM MgCl2, 20 mM Hepes, pH 8.0, 420 mM NaCl, 0.2 mM EDTA pH 8.0, 25% (v/v) glycerol). The resulting lysate was clarified by centrifugation. Both the cell lysate Buffer A and nuclear lysate Buffer C contained protease inhibitor mixture (Roche Molecular Biochemicals). Buffer A contained DNase I, 1000 units/ml, and RNase inhibitor, 400 units/ml. Buffer C contained DNase I, 200 units/ml, and RNase inhibitor, 80 units/ml (Roche Molecular Biochemical). Extracts to be treated with RNase A or T1 were prepared in the absence of RNase inhibitor. For subsequent fractionation, nuclear extracts were dialyzed against the appropriate buffer in a Microdialyzer System 100 using a 50,000 molecular weight cut-off dialysis membrane (Fisher).

**Oligo(dT) Chromatography—**500 µg of oligo(dT)-cellulose (Amersham Pharmacia Biotech) was pre-swollen in DEPC-treated distilled H2O and then applied to a 10-ml syringe in column binding buffer (CB: 10 mM Tris-HCl, pH 7.5, 0.3 mM KCl, 1.5 mM MgCl2, 1 mM dithiothreitol, 0.2% Nonidet P-40). Total cell extracts were prepared as follows: cells were washed once in low salt buffer as above, and nuclei were pelleted at 3,000 rpm for 5 min. Nuclei were lysed in 2× CB, debris spun out 2× 20 min at 13,000 rpm, and the supernatant combined either with an equal volume of cytoplasmic supernatant to make a total cell extract or with DEPC-treated water, when only nuclear extract was desired. Up to 5 ml of extract was applied to the column and left to cycle for 4 h at 4 °C via a peristaltic pump. The poly(A)+ fraction (flow-through) was collected, and the column was washed in 2× CB. Optional 5-ml salt washes were then applied (0.6 M and 1.2 M KCl in CB), and finally the poly(A)+ fraction eluted in 5 ml of 10 mM Tris-HCl, pH 7.5, in warm DEPC-treated distilled water, with 0.001% (w/v) xylene cyanol as an elution marker.

**Cell Fractionation—**Nuclear extracts were dialyzed into sucrose gradient buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1.5 mM MgCl2, 0.2% (v/v) Nonidet P-40). 300-µl samples containing 1.5 mg of total protein, Buffer C, was aliquoted and distilled water to half the NaCl concentration to 210 mM and loaded onto a 10-ml Sephacryl S-500 (Amersham Pharmacia Biotech) column, previously equilibrated with a 2-fold dilution of the same Buffer C at room temperature. Up to 80 fractions of 250 µl were collected by gravity flow for subsequent analysis.

**Sucrose Gradients—**Nuclear extracts were dialyzed into sucrose gradient buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1.5 mM MgCl2, 0.2% (v/v) Nonidet P-40). 300-µl samples containing 1.5 mg of total protein were loaded onto pre-formed 5-ml sucrose gradients, 15–30% sucrose (w/v), and spun at 12,000 rpm or 18,000 rpm for 18 h at 0 °C in a Sorvall AH650 rotor. Samples were collected manually into 18 fractions of 250 µl. To load the samples directly onto SDS-PAGE, samples were kept hot after boiling to prevent precipitation of sucrose. RNA was extracted with the RNeasy kit (Qiagen) and RNA visualized by running samples onto a standard denaturing agarose gel. 20% sucrose cushions were used to pellet poly(A)+ RNP by ultracentrifugation at 40,000 rpm for 3 h using the same rotor.

**Analysis of RNA by Reverse Transcriptase-PCR—**RNA samples extracted from gradients were reverse-transcribed using a cDNA kit (Roche Molecular Biochemical). 5-µl RNA samples were heated to 95 °C for 5 min, cooled on ice, and then combined with 100 pM of random hexamer, 1 µM each dNTP, 1 unit of ribonuclease inhibitor, and 20 units of M-MuLV reverse transcriptase in a total volume of 30 µl, and incubated for 10 min at room temperature, 60 min at 42 °C, and then 10 min at 95 °C. 1 µl of each sample was used in PCR. Each 50-µl PCR reaction contained 10 µl of each primer and was run for 30 cycles (1 min 95 °C, 1 min 58 °C, and 3 min 72 °C). Product sizes were compared against DNA markers on 2% agarose gels. The following primers were obtained (Genosys): mouse U1 snRNA, based on data base entry X01623, forward 5'-GCATACTTACCTGGCAGGGGAG-3', reverse 5'-AGGGGACCGGCAAGGAGCTC-3', yielding a 192-base pair product. R27 snRNA, based on data base entry X007913, forward 5'-GGTATCGCTTCTCGGCCTTTTGGC-3', reverse 5'-GGGGGTGGGACCCGTTCCTGGGAG-3', yielding a 192-base pair product.

**Western Blotting and Antibodies—**Protein fractions in SDS-PAGE loading buffer (2% SDS, 10% glycerol, 60 mM Tris-HCl, pH 6.9, 100 mM dithiothreitol, 0.001% bромphenol blue, pH 8.3) were boiled for 5 min, separated by SDS-PAGE (10% polyacrylamide, NBL Gene Sciences)
Presence of WT1 in Nuclear Poly(A)⁺ RNP

In Vitro Translation—A cDNA encoding the +/+ isoform of WT1 (including exons 5 and 9) with a thymidine kinase 5’ leader sequence was subcloned into a pSTBlue-1 plasmid vector (Novagen), as per manufacturer’s specifications, and verified by automated ABI sequencing (Perkin Elmer Applied Biosystems). In vitro translated WT1 was prepared using the TNT-coupled transcription/translation system (Promega), as per manufacturer’s instructions. Proteins remain bound in high salt and are finally eluted, whereas the Y box “mRNA masking” can be eluted in 0.6M KCl, whereas the Y box “mRNA masking” proteins remain bound in high salt and are finally eluted, bound to mRNA, in distilled water (37). Similarly, whereas specific poly(A)⁺ proteins eluted in the 0.6 M KCl or 1.2 M KCl salt washes, others eluted in distilled water. In this experiment, we estimate that approximately 5% of total extract proteins eluted in the 0.6 M KCl wash, 5% in the 1.2 M KCl wash, and 4% in the final distilled water elution. The bulk of WT1, U2AF65, and p116 remained bound after the 1.2M salt wash fractions from M15 total extract comparing the elution of WT1, U2AF65, p116, and hnRNP A1. D, controls. First, WT1 (in M15 extract) bound to oligo(dT) in the presence, but not in the absence, of 0.3 M KCl (Western blots, top and middle panels). Second, in vitro translated WT1, in RNase-treated reticulocyte lysate diluted 20-fold in column binding buffer, did not bind (autoradiograph; bottom panel).

RESULTS

Oligo(dT) Chromatography—This technique is typically used to prepare nuclear or cytoplasmic poly(A)⁺ RNP from a variety of sources, both animal and plant (for examples see Refs. 28–34). We carried out oligo(dT) chromatography on both total and nuclear extracts (Fig. 1A). Total extracts were prepared by combining soluble nuclear extract with the cytosolic supernatant (see “Experimental Procedures”). Poly(A)⁺ fractions obtained from total extracts contained a number of abundant proteins; of these, a 46-kDa protein was particularly prominent. Abundant proteins were also apparent in the poly(A)⁺ fraction derived from nuclear extract alone. We found WT1 to be highly enriched in total extract poly(A)⁺ fractions, as was U2AF65, U2 snRNP-associated splicing factor that recognizes the polypyrimidine tract at the 3’ splice site (35; p116, a U5 snRNP-associated GTPase structurally related to the ribosomal translocase EF2 (36); and hnRNPA1, a core hnRNP particle component (Fig. 1B). Similar WT1 enrichment was seen in nuclear poly(A)⁺ fractions (not shown). Based on Western blots, we estimate up to 100-fold enrichment of WT1 in poly(A)⁺ fractions. In contrast, PCNA (proliferating cell nuclear antigen, involved in DNA replication), Sp1 (transcription factor, containing zinc fingers structurally related to WT1), and TBP (TATA-binding protein, involved in basal transcription) were not detected in the poly(A)⁺ fraction.

Before elution in distilled water, it is possible to wash off bound proteins with increasing salt concentrations. For example, the Xenopus oocyte mRNAP-associated RNA helicase, Xpo4, can be eluted in 0.6 M KCl, whereas the Y box “mRNA masking” proteins remain bound in high salt and are finally eluted, bound to mRNA, in distilled water (37). Similarly, whereas specific poly(A)⁺ proteins eluted in the 0.6 M KCl or 1.2 M KCl salt washes, others eluted in distilled water. In this experiment, we estimate that approximately 5% of total extract protein eluted in the 0.6 M KCl wash, 5% in the 1.2 M KCl wash, and 4% in the final distilled water elution. The bulk of WT1, U2AF65, and p116 remained bound after the 1.2 M salt wash (Fig. 1C). In contrast, hnRNPA1 eluted mostly in the 1.2 M salt wash, consistent with the reported salt sensitivity of core hnRNPA1 particles (38). Given the possibility that WT1 may have a direct affinity for oligo(dT), two controls were performed (Fig. 1D). First, binding of WT1 in M15 extract to oligo(dT) did not occur in the absence of 0.3 M KCl, salt being required for hybridization of poly(A) sequences to oligo(dT). Second, in vitro translated WT1, present in RNase-treated reticulocyte lysate diluted 20-fold with binding buffer (including 0.3 M KCl), also did not bind.

Gel Filtration—To determine the size of macromolecular complexes containing WT1, nuclear extracts were applied to a 10-ml Sephacyr-500 column, and up to 80 fractions were collected by gravity flow. The elution profiles of WT1, U2AF65, and p116 were compared (Fig. 2). WT1 in AC29 extract eluted...
WT1 derived from fetal kidney (day E17) nuclear extract eluted in a similar fashion. Particles eluting in these fractions would be expected to include single, or multiple spliceosomes associated with nascent pre-mRNA. In contrast, U2AF65 eluted in a much broader range, up to fraction 48, approaching the elution peak for a monomer (bovine serum albumin, 66 kDa), whereas the U5 snRNP-associated protein p116 was mainly present in fractions 30–40. As a further marker, we used TMV (tobacco mosaic virus), a well-characterized plant virus. TMV virions consist of genomic RNA packaged by the coat protein p17 into large particles (>200S); as expected, TMV eluted in early fractions.

Sucrose Gradient Centrifugation—AC29 nuclear extract was applied to a 15–30% sucrose gradient and initially spun at 12,000 rpm (Fig. 3A). Cytoplasmic extract was run in parallel on a similar gradient as a marker. The bulk of WT1 overlapped in fractions 5–7 with p116 but not hnRNP A1 and U2AF65; however, a significant proportion of WT1 and hnRNP A1 also pelleted in fraction 18. When extracts were run at 24,000 rpm, all of the WT1 signal was now in the pellet (not shown). To investigate further the sedimentation of WT1 relative to p116, another sucrose gradient was run, this time at an intermediate speed of 18,000 rpm (Fig. 3B). Cytoplasmic extract was again run in parallel to act as a sedimentation marker, but now in low magnesium buffer to promote the dissociation of ribosomal subunits. This time, both WT1 and p116 sedimented at ~60 S in fractions 14–16; also apparent in these fractions were a number of abundant, low molecular weight proteins (not shown). A significant proportion of p116, but not WT1, was also present at the top of the gradient, consistent with gel filtration results.

Density Gradient Centrifugation on Nycodenz—This technique can be used to separate macromolecules, even organelles and cell types, on the basis of their buoyant density. For the purposes of this study, the key property is that the density of RNP species differs from free RNA and protein. We preferred Nycodenz over cesium gradients, because they do not require prior fixation of the extract (39, 40). Cytoplasmic messenger RNA particles have recently been fractionated on Nycodenz, in which they peak at 1.21 g/cm^3 (37, 41, 42). Previously, mRNP were fractionated on metrizamide, an earlier version of Nycodenz (43). In general, density values obtained on metrizamide (44) are very close to those obtained on Nycodenz (40), the two compounds being closely related in structure. In an early study, metabolically labeled nuclear pre-mRNP peaked at two densities in metrizamide as follows: 1.31 and 1.18 g/cm^3; in particular, the 1.31 peak contained a higher proportion of poly(A)^+ RNA relative to the 1.18 peak (45).

On Nycodenz gradients, particles containing WT1 peaked, along with U1 and U2 snRNA, in a density range comparable to the 1.31 g/cm^3 metrizamide pre-mRNP peak (Fig. 4A). Treatment of whole nuclear extracts with RNase A resulted in the formation of precipitate at 1.19 g/cm^3; similar results were obtained with RNase T1 (not shown). WT1 was greatly enriched in this precipitate, which also contained residual histones; 1.19 g/cm^3 is close to the reported density value for chromatin on Nycodenz, 1.184 g/cm^3 (40). Similar results were obtained with fetal kidney (E17) extracts; furthermore, the density of particles containing WT1 was shifted by both EDTA and actinomycin D treatment (not shown). EDTA treatment is known to disrupt ribosomes and also ~200 S large nuclear RNP particles, whose structural integrity requires divalent cations (46).

For reference, the transcription factor Sp1 peaked at 1.14 g/cm^3, in a lower density range compared with WT1, and the position of the Sp1 peak was not shifted by RNase A (not shown). Gradients were also blotted for the splicing factors U2AF65, p116, and the pre-mRNA binding protein hnRNP A1. Like WT1, p116 and hnRNP A1 peaked in the higher density fractions and were shifted to lower density fractions after RNase A treatment (Fig. 4B); however, this did not apply to U2AF65 (see “Discussion”).

Next, we considered whether the concentration of WT1 and other proteins at 1.19 g/cm^3 after RNase A treatment was an artifact involving precipitation with residual chromatin in the total nuclear extract, rather than disruption of bona fide RNP containing WT1. Devoid of residual chromatin, poly(A)^+ RNP was first concentrated by pelleting through a 20% sucrose cushion and then run on Nycodenz gradients (Fig. 4C). Now WT1 peaked sharply in fraction 14 (1.27 g/cm^3). Again, as in whole nuclear extract, RNase treatment reduced density significantly. Numerous abundant poly(A)^+ proteins also peaked in the same fractions that contained WT1 (not shown). For comparison, in vitro translated WT1 was also applied to Nycodenz gradients (Fig. 4D). When preincubated with excess AC29 nuclear extract in the presence of ribonuclease inhibitor, in vitro translated WT1 precipitated in a density range consistent with native particles. In contrast, when combined with nuclear extract pretreated with RNase A, WT1 peaked in a lower density range, similar to RNase-treated native particles.

Transient Transfection of Epitope-tagged Constructs—Constructs expressing epitope-tagged WT1 were prepared, in order to test whether full-length WT1 or truncated protein exhibits the properties of native protein both in terms of intracellular
localization and biochemical fractionation. The 11 amino acid prokaryotic tag T7 was chosen because of its small size and recognition by a highly specific antibody. T7 epitope-tagged constructs expressing full-length murine WT1 (1 exon 5, and KTS), and two deletions (“N terminus,” amino acids 1–235; “C terminus,” amino acids 236–449, KTS) were transiently transfected into COS7 cells (African green monkey kidney cells). Expression of the correct size T7-tagged protein was verified by Western blotting (not shown), and intracellular localization was determined by immunofluorescence. 20–50 transfected cells were examined for each transfection; examples are shown (Fig. 5A).

Full-length WT1 accumulated in the nucleus, as did the C terminus. Consistent with previous findings, full-length WT1 signal was speckled, over a diffuse background; and nucleoli were excluded. On the other hand, the N terminus was predominantly cytoplasmic. The above constructs were also expressed in AC29 (mouse mesothelioma) and HeLa (human cervical carcinoma cells), giving comparable results (not shown). To see which of the three proteins might associate with poly(A)+ RNP, COS7 cells were re-transfected with the same constructs, and nuclear extracts were applied to oligo(dT)-cellulose. In summary, both the full-length and the C terminus were strongly enriched in the poly(A)+ fraction but not the N terminus, which lacks the zinc fingers (Fig. 5B). Finally, since p116 co-fractionates with native WT1 on both sucrose and Nycodenz gradients, a double-labeling experiment was performed to ascertain the degree of co-localization be-

**Fig. 4.** Nycodenz density gradients. Nuclear extracts, containing 1.5 mg of protein, from AC29 and M15 cells were applied to 5-ml 20–60% (w/v) gradients and spun at 36,000 rpm for 18 h at 4 °C. A, density profile of WT1 in total nuclear extracts from AC29 and M15 cells, with or without RNase A pretreatment (Western blots), compared with U1 and U2 snRNA (reverse transcriptase-PCR); 1, AC29 WT1 – RNase A; 2, AC29 WT1 + RNase A; 3, M15 WT1 – RNase A; 4, M15 WT1 + RNase A; 5, M15 U1 snRNA (–RNase A); and 6, M15 U2-snRNA (–RNase A). B, M15 extract gradients blotted for J; U2AF65 – RNase A; 2, U2AF65 + RNase A; 3, p116 – RNase A; 4, p116 + RNase A; 5, hnRNP A1 – RNase A; and 6 hnRNP A1 + RNase A. C, total poly(A)+ RNP was first concentrated by pelleting through a 20% sucrose cushion and then applied to a Nycodenz gradient. I, WT1 – RNase A; 2, WT1 + RNase A. D, in vitro translated WT1 was applied to similar Nycodenz gradients (autoradiographs). I, WT1, combined with excess AC29 nuclear extract in the presence of ribonuclease inhibitor. 2, as I but the nuclear extract was pretreated with RNase A. 3, WT1 in RNase-treated reticulocyte lysate alone; no added nuclear extract.

**Fig. 5.** Cells were transfected with constructs expressing T7 epitope tagged full-length WT1 (+ exon 5, + KTS), the C terminus (amino acids 236–449, + KTS), and the N terminus (amino acids 1–235). A, the intracellular localization of the three proteins in COS7 cells was determined by immunofluorescence; blue (left panels) is 4,6-diamidino-2-phenylindole (DAPI) staining showing the position of nuclei, and green (right panels) is the corresponding FITC signal. B, oligo(dT) chromatography. Total COS7 poly(A)+ and poly(A)+ proteins are shown on the left (Coomassie Blue stain). Poly(A)+ and poly(A)+ fractions obtained with extracts from transfected cells were blotted with anti-T7 antibody. C, double-labeling experiment: COS7 and HeLa cells were transfected with full-length WT1 (+ exon 5, + KTS). Gray scale images are shown (left and middle), and merged in color (right; green (FITC) represents full-length WT1 (+/+); red (Texas Red) represents the splicing factor p116, and the merged signal is represented in yellow.
between WT1 and p116 (Fig. 5C). Full-length WT1 (+/+) was transfected into COS7 and HeLa cells. Again, WT1 concentrated in the nucleus; overall, the T7-tagged WT1 signal was more diffuse compared with p116, which was more clearly speckled. Significant co-localization was observed between WT1 (+/+) and p116 in nuclear speckles in both COS7 and HeLa cells (Fig. 5C).

**DISCUSSION**

The first indication that WT1 might have a post-transcriptional role came from the observation that WT1 protein, preferentially +KTS isoforms, are associated with nuclear speckles (1). A later study expanded these findings showing an interaction between WT1 (again, preferentially +KTS isoforms) with the ubiquitous splicing factor U2AF65; in the same study, WT1 was incorporated into large molecular weight complexes associated with the sense, but not the antisense strand, of a biotinylated adenosine pre-mRNA (2). To support these findings, the aim of this study was to determine the size and density of macromolecular complexes containing WT1, to ascertain whether or not they have the properties of RNP. To this end, we used established fractionation techniques as follows: oligo(dT) chromatography, gel filtration, sedimentation on sucrose, and equilibrium density gradient centrifugation on Nycodenz.

**Presence of WT1 in Poly(A)⁺ Fractions**—We found WT1 to be highly enriched in poly(A)⁺ fractions, along with a number of highly abundant and other less abundant proteins. Along with WT1, the splicing factors U2AF65 and p116 and the core hnRNP protein hnRNP A1 were also enriched, whereas PCNA, Sp1, and TBP, which are not involved in pre-mRNA processing, were not. The poly(A)⁺ fraction contains pre-mRNA processing machinery in the form of RNP complexes. It should be noted that under these conditions, oligo(dT)-cellulose will not necessarily hybridize exclusively to poly(A) at the 3' end of transcripts but may hybridize to other A-rich stretches, whether in pre-mRNA or other RNA species. Consequently, the proportion of WT1 associated with polyadenylated pre-mRNA, as opposed to other RNP species, is not yet known.

**Size of Particles Containing WT1**—Next, we examined the size of particles containing WT1 by gel filtration and sucrose gradient centrifugation. Gel filtration suggested that WT1 is present in large particles, >2 MDa. Consistent with gel filtration, sedimentation on 15–30% sucrose gradients showed WT1 sedimenting at ~60 S; in addition, WT1 could also be detected in heavier particles. In general, the distinct impression was of better co-sedimentation of WT1 with p116, as opposed to U2AF65 or hnRNP A1. For reference, core hnRNP particle sedimentation at 30 S (47). The salt concentration in the sucrose gradients was kept deliberately low (100 mM), due to the reported salt sensitivity of hnRNP particles (38). Fully assembled spliceosomes sediment at 40–60 S (48). The U2 snRNP particle sediments at 12–17 S, depending on the association of the auxiliary factors SF3a and SF3b (49, 50); the U5 snRNP particle sediments at 20 S, whereas the U4/U6.U5 tri-snurposome sediments at 25 S (36); and a more recently defined particle, the large nuclear RNP particle, thought to include four spliceosomes in association with pre-mRNPs, sediments at 200 S (51–53). In summary, it seems unlikely that WT1 is a component of core hnRNP particles; on the other hand, WT1 sediments in the single spliceosome range (40–60 S), along with p116.

**Density of Particles Containing WT1**—Density gradient centrifugation showed that poly(A)⁺ RNP particles containing WT1 peaked at 1.27 g/cm³ on Nycodenz, comparable to values previously reported for both nuclear pre-mRNPs and polysomes (42, 45). In contrast, cytoplasmic mRNPs peak at 1.21 g/cm³ on Nycodenz; the lower density of mRNPs compared with other RNP species is due to their higher protein to RNA ratio (37, 41, 42). The density of particles containing WT1 was significantly reduced by RNase treatment. Together with WT1, both p116 and hnRNP A1 also peaked in the higher density range, as did U1 and U2 snRNA; however, U2AF65 did not. This was surprising, because, as discussed, U2AF65 has been shown to interact with WT1 (2). Like WT1, U2AF65 was enriched by oligo(dT) chromatography (Fig. 1) but did not co-sediment on sucrose gradients with WT1 (Fig. 2). It is possible that only a small fraction of WT1 and U2AF65 interact at any given time. Significantly, recent work suggests that a large fraction of U2AF65 appears not to be associated with spliceosomes (54). Alternatively, it is also possible that the buffers used disrupt biochemical associations involving U2AF65.

**T7-tagged WT1 Is Incorporated into Poly(A)⁺ RNP**—Next, we sought to test the properties of epitope-tagged WT1 in transiently transfected cells that do not express WT1. In COS7 cells, full-length WT1 accumulated in the nucleus, as did the C-terminus (amino acids 233–449), whereas the N-terminus did not (amino acids 1–235); comparable results were obtained in AC29 and HeLa cells. Furthermore, the C-terminal but not the N-terminal half of WT1, like the full-length protein, was enriched in poly(A)⁺ fractions. By implication, the putative RNA recognition motif at the N terminus of WT1 does not appear to be required for nuclear localization and incorporation into RNP. Finally, in our hands the T7 epitope appears to be particularly well suited for immunoprecipitation. It is hoped that this epitope can now be used to establish which subset of poly(A)⁺ proteins and RNA species WT1 is associated.

**Presence of WT1 in Nuclear Speckles**—Double-labeling experiments suggested a significant degree of co-localization of the +KTS isoform of WT1 with p116 in both COS7 and HeLa cells, consistent with previous results associating the +KTS isoform with the splicing machinery (1, 2). In one report, the lack of co-localization of WT1 (+KTS) with the essential splicing factor SC35 suggested that WT1 is not involved in splicing (55). On the other hand, in this study, the co-localization of WT1 with another essential splicing factor (p116) in nuclear speckles, correlates with co-sedimentation on sucrose and similar density peaks on Nycodenz. These results do not necessarily imply a direct interaction between WT1 and p116, nor do they demonstrate that WT1 is involved in splicing; further experimentation is required to resolve these issues.

Nuclear speckles contain poly(A)⁺ RNA, detected with oligo(dT) probes (56, 57). It has been suggested that RNA present in speckles includes non-coding sequences that may have a structural role (58). The precise composition and organization of nuclear speckles is not yet known, both in terms of their protein and RNA content. It has been suggested that speckles act as storage sites for splicing factors (59–61). For example, U2AF65 was shown to co-localize with other “SR” proteins in speckles; this localization was independent of splicing activity, as it persisted after the inhibition of transcription (62). Similarly, at any given time, a significant proportion of WT1 may be stored in speckles and not be actively involved in pre-mRNA processing.

**Concluding Remarks**—In summary, results indicate that WT1 is present in nuclear poly(A)⁺ RNP particles, further implicating WT1 in a post-transcriptional process. In contrast, the transcription factor Sp1, which contains zinc fingers similar to those in WT1, behaved differently in our assays; it was not enriched by oligo(dT) chromatography and peaked in a distinctly lower density range. There are some immediate caveats. First, in the preparation of extracts, a significant amount of WT1 was pelleted in the insoluble, chromatin-rich fraction. It is therefore unclear what proportion of total WT1 is present in RNP. Second, both the +/−17 amino acids (insertion
of exon 5) and isoforms due to the upstream translation start site, all of which produce proteins with distinct mobilities on SDS-PAGE, are all present in RNP particles; however, the presence of +KTS over −KTS isoforms in RNP has not yet been compared. As previously discussed, it is the +KTS isoforms that appear to associate preferentially with the pre-mRNA processing machinery (1, 2). These issues ought to be addressed in the near future. For the purposes of this study, the bulk of WT1 in the soluble extract appears to be present in RNP particles and is associated with the splicing machinery, as opposed to core hnRNP particles. It is now hoped that combining the above fractionation techniques with immunoprecipitation can prove useful in determining the role of WT1 in pre-mRNA processing, while looking for physiological RNA targets. A subsequent question would be in what tissues and in what phases of development does WT1 become associated with the RNA processing machinery and is this a general or a specialized aspect of WT1 function? The impression in this study is that WT1 is present in RNP complexes in all extracts tested (two expressing murine cell lines: M15, from mesonephros; AC29, from mesothelioma; transfected COS7 and HeLa cells, and mouse fetal kidneys).

WT1 would not be the first example of a multifunctional protein that binds to both DNA and RNA; numerous examples have emerged (reviewed in Refs. 63–65). A particularly intriguing example is TRA-1, a transcription factor necessary for normal sexual development in Caenorhabditis elegans. Recently, TRA-1 has also been shown to affect gene activity at the post-transcriptional level by regulating the nuclear export of tra-2 messenger RNA; like WT1, its nucleic acid binding is mediated by zinc fingers (66). Thus in addition to its more well-established role in transcriptional regulation, evidence is accumulating that WT1 also has a post-transcriptional role; the priority is to define what process and what RNA targets are involved. The biochemistry of WT1 would appear to be even more complex; recent data describe an interaction between WT1 and the chaperone hsp70 (67). WT1 is therefore one example in an increasingly large list of “biochemically promiscuous” proteins, affecting gene expression at multiple levels.

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