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Subnuclear Localization of WT1 in Splicing or Transcription Factor Domains Is Regulated by Alternative Splicing

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Summary

***WT1* is a tumor suppressor gene with a key role in urogenital development and the pathogenesis of Wilms' tumor. Two alternative splice sites in the *WT1* transcript allow the gene to encode four proteins. These carry four Krüppel-type zinc fingers and to date have primarily been implicated in transcriptional control of genes involved in growth regulation. However, here we demonstrate colocalization of WT1 with splicing factors in the fetal kidney and testis and in expressing cell lines. Using immunoprecipitation, we show that two WT1 isoforms directly associate with one or a limited number of components in the spliceosomes and coiled bodies. Moreover, COS cell expression studies suggest that alternative splicing within the WT1 zinc finger region determines whether the protein localizes mainly with splicing factors or with DNA in transcription factor domains in the nucleus. We propose that WT1 plays roles in posttranscriptional processing of RNA as well as in transcription.**

Introduction

Wilms' tumor (WT) is a childhood kidney tumor, affecting 1 out of 10,000 infants. It is perhaps the most striking example of how loss of developmental control processes can result in oncogenic transformation. WT is frequently associated with congenital urogenital anomalies (for recent reviews see Hastie, 1994; Bruening and Pelletier, 1994). A tumor predisposition gene (*WT1*), implicated by mutation analysis in 10%–15% of cases, has been identified. The predicted WT1 protein has four C-terminal Krüppel-type zinc fingers and a proline/glutamine-rich transregulatory domain (Call et al., 1990; Gessler et al., 1990). Both motifs are found in many transcription factors. Tumor mutation analysis and functional data show that *WT1* is a tumor suppressor gene (Haber et al., 1993).

The *WT1* gene has ten exons that give rise to four protein isoforms through alternative splicing: exon 5 encoding 17 amino acids can be either included or excluded; likewise, lysine, threonine, and serine (KTS) can be either included or excluded between the third and fourth zinc fingers (Figure 1A). WT1 expression is seen in tissues of mesodermal origin in cells that undergo a mesenchymal to epithelial switch, for example, in fetal kidneys and gonads (Hastie, 1994). Immunohistochemical studies have confirmed the predicted nuclear localization of a "coarse speckled pattern" (Mundlos et al., 1993). Analysis of *wt1*-null mice reveals that the gene is required for fetal survival beyond 15 days of gestation and is essential for urogenital development (Kreidberg et al., 1993).

The presence of the zinc fingers in WT1 suggested that its main physiological role would be as a DNA-binding protein. The zinc fingers are closely related to those of the transcription factors EGR1 and Sp1 (Drummond et al., 1994). The direct interaction between EGR1 protein and its consensus target sequence 5′-GCGGGGGCG-3′ has been analyzed by crystallography (Pavletich and Pabo, 1991). WT1 has been shown to bind the same sequence (Rauscher et al., 1990). This G-rich sequence is present in the promoter region of a large number of genes, some of which are involved in growth regulation. In transient transfection assays, the −KTS form of WT1 represses several of these promoters (Hastie, 1994). However, so far no clear in vivo target for transcriptional regulation by WT1 has been documented.

The alternative splicing between the third and fourth zinc fingers of WT1 is probably very important physiologically, since it is conserved from zebrafish to human (Hastie, 1994; Kent, 1994). mRNAs encoding the +KTS isoforms are four times more prevalent than those encoding the −KTS forms (Haber et al., 1991). The +KTS isoforms do not readily bind the EGR1 consensus sequence (Rauscher et al., 1990). Recent studies have suggested that they bind a narrower range of DNA target sequences and with lower affinity than the −KTS forms (Bickmore et al., 1992; Drummond et al., 1994). The biological importance of the zinc finger region and its alternative splice site is clearly demonstrated by the distinct spectrum of constitutional mutations that lead not only to WT but also to severe urogenital abnormalities in Denys–Drash syndrome (DDS) (Bruening and Pelletier, 1994).

WT1 binding to DNA has been studied extensively in vitro, but we still know very little about the role of this protein in vivo. Confocal microscopy has made possible high resolution analysis of nuclear organization in whole cells. Nuclear factors have been shown to be localized in discrete domains, usually according to their particular functional association, such as DNA replication, transcription, or pre-mRNA splicing (for review see Spector, 1993). Nuclear domains containing small nuclear ribonucleoprotein particles (snRNPs) involved in mRNA splicing have been intensively studied. Splicing occurs in spliceosomes, composed of five small nuclear RNAs (snRNAs) (U1, U2, U4/U6, and U5) complexed with numerous protein factors

†The first two authors have contributed equally to this work.

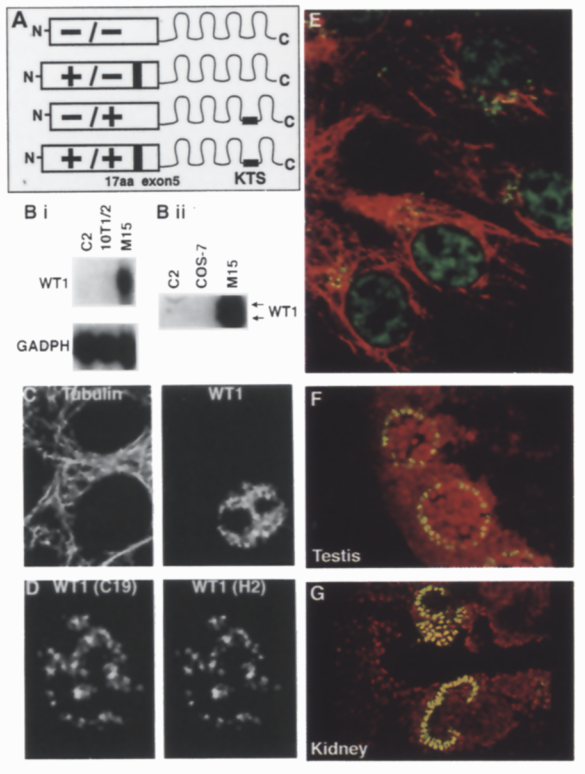

Figure 1. Scheme of WT1 Isoforms and Detection of WT1 in Cultured Cells and Fetal Tissues

(A) Diagram showing the four wild-type WT1 isoforms.
(B) (i) Northern blot from M15 cells and nonexpressing cell lines (C2 and 10T1/2) probed with WT1 and GAPDH cDNA probes. (ii) Western blot with nuclear extracts from M15 cells and two nonexpressing cell lines (C2 and COS7) probed with the H2 anti-WT1 antibody.
(C and D) WT1 (+/+) transfected COS7 cells stained with anti-WT1 antibody C19 or as described.
(E) M15 cells stained for WT1 (C19, green) and tubulin (red).
(F and G) Cryosections of E15.5 mouse testis and kidney stained for WT1 (C19, green/yellow) and nuclear Sm antigen (Y12, red).

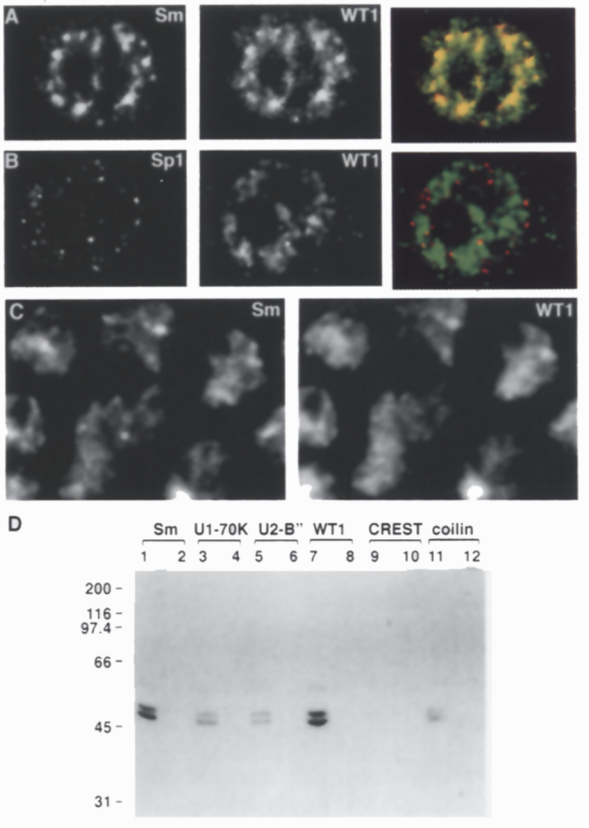

Figure 2. WT1 Colocalization and Coimmunoprecipitation with Splicing Proteins

(A and B) M15 cells stained for Sm antigens (Y12) and WT1 (C19) (A) or Sp1 (IC6) and WT1 (C19) (B). To the right images are merged, red denoting Sm antigen/Sp1, green WT1, and yellow colocalization.
(C) High magnification of kidney section through the proximal end of a S-shaped body, stained as in (A).
(D) IP on nuclear extracts from M15 cells (lanes 1, 3, 5, 7, 9, and 11) or untransfected COS7 cells (lanes 2, 4, 6, 8, 10, and 12). Precipitating antibodies indicated above lanes. The presence of WT1 was analyzed by immunoblotting using the H2 anti-WT1 antibody as a probe.

(for reviews see Moore et al., 1993; Lamm and Lamond, 1993; Newman, 1994). snRNPs have a characteristic localization in two nuclear domains in each nucleus: the interchromatin granules (IGs) seen as 20–50 speckles and the 1–5 coiled bodies (Nyman et al., 1986; Spector et al., 1991; Lamond and Carmo-Fonseca, 1993). Here, we show that the +KTS isoforms of WT1 associate preferentially with these nuclear domains and that WT1 is coprecipitated with specific components of the splicing machinery. In contrast, the −KTS isoforms have a distribution that parallels that of classical transcription factors such as Sp1 and TFIIB, suggesting distinct functions for the WT1 isoforms.

Results

A Mesonephric Cell Line

To be able to study the subnuclear localization of the WT1 protein, we established a mouse mesonephric cell line, M15. These cells have an epitheloid morphology and express high amounts of WT1 mRNA and protein. Two WT1 isoforms (with or without the 17 amino acids encoded by exon 5) of 52 and 54 kDa, respectively, could be detected at approximately equal levels (Figure 1B).

Specificity of Immunohistochemical WT1 Detection

COS7 cells were transfected with the four different splice forms of WT1, stained with a polyclonal antibody raised against the C-terminal 19 amino acids (C19), and analyzed by confocal microscopy. Only transfected cells showed a nuclear stain with the C19 antibody (Figure 1C). No stain was seen in cells transfected with a *WT1* mutant form truncated at zinc finger 3 (T), lacking the C-terminal domain (data not shown). If the transfected COS cells were doubly stained with C19 and any of the four monoclonal WT1 antibodies (WT48, H2, H7, and HC17), the same cells were positive and the subnuclear staining patterns were identical (Figure 1D).

A number of cell lines expressing endogenous *WT1* (as determined by Northern blotting) and nonexpressing controls were stained with antibodies C19, H2, H7, or HC17. Expressing cell lines (M15, PYT45, and AC29) all showed a characteristic nuclear staining pattern, whereas nonex-

pressing cells (10T1/2 and C2) were not stained. The WT1 localization, analyzed by confocal microscopy and serial sections, was characterized by 20–50 distinctly stained irregular foci set against a diffuse nucleoplasmic background generally excluding the nucleoli (data not shown). In some cases, a weak asymmetric cytoplasmic stain was seen immediately adjacent to the nucleus (Figure 1E). Kidneys and testes from embryonic day 15.5 (E15.5) mouse embryos showed very characteristic staining patterns. In the testis, WT1 protein was localized in Sertoli cells lining the early seminiferous tubules (Figure 1F). In the kidney, strong stain was seen in the proximal end of the S-shaped body, corresponding to the cells giving rise to the glomerular podocytes (Figure 1G). In both cases, this is in accordance with previous studies (Pritchard-Jones et al., 1990; Pelletier et al., 1991; Mundlos et al., 1993). Overlapping patterns were seen using C19, H2, and H7 antibodies. High magnification revealed subnuclear staining patterns very similar to those seen in WT1-expressing cell lines (see below). In transfected COS cells, WT1-expressing cell lines, fetal kidney, and testis, no C19 stain could be detected in the presence of excess of immunizing peptide (data not shown).

WT1 Colocalizes with Splicing Factors In Vitro and In Vivo

The subnuclear localization of WT1 is very similar to the "speckled" pattern described for splicing factors (Nyman et al., 1986; Spector et al., 1991). The localization of WT1 and splicing factors was compared by staining WT1-expressing cells for both WT1 (C19) and Sm antigens (Y12 antibody). The latter are motifs common to a number of snRNP proteins (Pettersson et al., 1984). The strong foci, which are most characteristic for both WT1 and snRNP staining, overlap perfectly. The overall staining is not identical, owing to a larger relative contribution of the diffuse background stain for WT1 than for the Sm antigens (Figure 2A).

The colocalization between WT1 and Sm antigens was also studied in fetal testes and kidneys. Figure 2C shows a clear colocalization between WT1 and Sm antigens in a section through the proximal portion of an S-shaped body from an E15.5 fetal kidney.

To investigate whether the localization seen for WT1 is unique or can be demonstrated for other related zinc finger proteins, M15 cells were stained for the transcription factor Sp1. The Sp1 antibody shows a diffuse nuclear stain in the fluorescence microscope, but confocal analysis reveals a large number of small foci. These do not colocalize with the larger foci of WT1; on the contrary, the localization is better characterized as nonoverlapping (Figure 2B). A similar pattern for Sp1 was seen in fetal testis and kidney (data not shown). Excess of the Sp1 immunizing peptide completely abolished staining (data not shown). In immunoprecipitation (IP), a single band was seen at 95 kDa (Briggs et al., 1986; see below).

WT1 Is Directly Associated with Splicing Complexes

To test whether the colocalization of WT1 and splicing factors could be caused by direct association between WT1 and splicing complexes in M15 cells, IPs were performed with a series of antibodies against proteins found in the spliceosomes or coiled bodies. The latter is a highly conserved nuclear organelle of unknown function containing snRNPs involved in splicing (Lamond and Carmo-Fonseca, 1993). The immunoprecipitated proteins were analyzed by Western blotting using the H2 antibody as a probe (Figure 2D). The WT1 protein was found to be coprecipitated with antibodies against the Sm motif (Y12), the 70 kDa polypeptide of the U1 snRNP complex (U1-70K), the B″ protein of the U2 complex (U2-B″), and p80 coilin (a protein unique to the coiled bodies). The monoclonal H2 anti-WT1 antibody was used as a positive control, and autoimmune antibodies against centromere proteins (CREST) were used as a negative control. The specificity of the IPs with antibodies against snRNPs (Y12, U1-70K, and U2-B″) and with the CREST serum was checked by probing Western blots either with the monoclonal Y12 anti-Sm antibody or with the CREST serum, respectively (data not shown).

WT1 Relocalizes in the Nucleus with a Subgroup of Splicing Factors

The localization of splicing factors in the nucleus has been demonstrated to be highly dynamic and influenced by a number of factors such as rate of transcription and heat shock. The changes in localization are to a large extent splice factor dependent (Spector et al., 1991; Carmo-Fonseca et al., 1992; O'Keefe et al., 1994). To test whether WT1 relocalizes with splicing factors and in an attempt to identify a subgroup of splicing factors with which WT1 could interact, M15 cells were exposed to two treatments known to alter nuclear localization of splicing proteins.

Disruption of Pre-mRNA Splicing

The assembly of snRNPs on pre-mRNA requires a number of specific RNA–RNA interactions. Oligonucleotides that interfere with these interactions have been shown to inhibit splicing and to change the nuclear localization of splicing factors in a specific fashion (O'Keefe et al., 1994). To test whether the localization of WT1 in the nucleus depends on adequate assembly of the spliceosomes, M15 cells were injected with either of two oligonucleotides, one complementary to the 5′ end of the U1 snRNA (U1C), the other to nucleotides in U6 snRNA that bind U2 and U4 snRNA (U6C) (Pan and Prives, 1988; O'Keefe et al., 1994). Introduction of either oligonucleotide led to rearrangement of Sm antigens in the nucleus, with a concentration of the protein in enlarged foci and a marked decrease of the diffuse background stain (Figure 3A). WT1 also became concentrated within larger foci and showed a decrease of the diffuse background stain, although more of the latter remained than for Sm antigens (Figure 3C). Two control oligonucleotides (C1 and C2) showed no effect (Figures 3B and 3D).

Actinomycin D

Actinomycin D inhibits cellular transcription and causes a number of splicing factors to relocalize to large foci similar to those seen after oligonucleotide injections. However, a subgroup of splicing factors such as the U1-70K protein, the U2 snRNP auxiliary factor (U2AF), and p80 coilin are

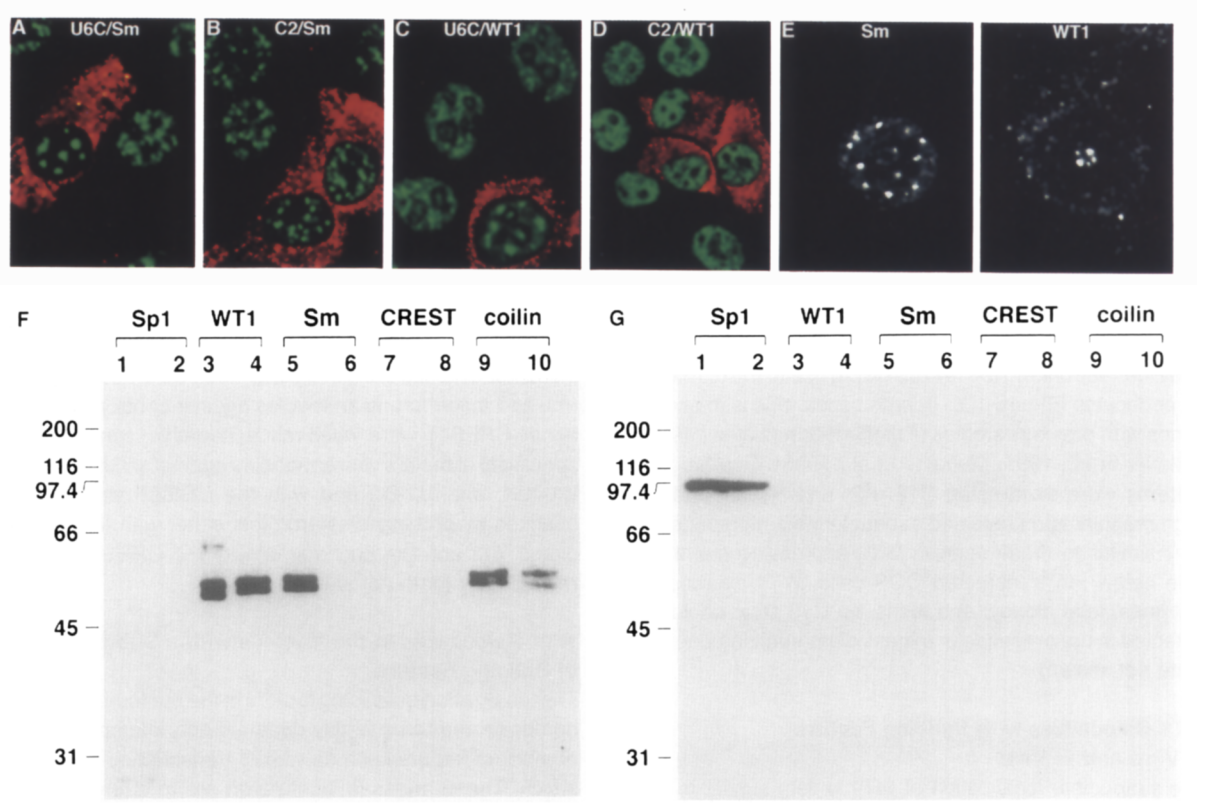

Figure 3. Relocalization of WT1 with Splicing Factors

(A–D) M15 cells injected cytoplasmically with red fluorescent dextran and oligonucleotides U6C (complimentary to U6 snRNP) or C2 (control), and stained for Sm antigens or WT1.

(E) M15 cell after 2 hr of actinomycin D, stained for Sm antigens (Y12) and WT1 (C19).

(F and G) IP on nuclear extract from M15 cells with (lanes 2, 4, 6, 8, and 10) or without actinomycin D (lanes 1, 3, 5, 7, and 9). Precipitating antibodies indicated above lanes. Western analysis was performed in duplicate, either with the anti-WT1 antibody WT48 (F) or the anti-Sp1 antibody PEP2 (G) as probes.

known to concentrate around the remnants of the nucleoli (Carmo-Fonseca et al., 1991, 1992). When M15 and AC29 cells were exposed to actinomycin D, WT1 relocalized to very characteristic circular structures consisting of a small number of linked foci. The stain was found to encircle the nucleolar remnants (as seen with phase microscopy; data not shown). The same pattern was seen with C19, H2, H7, and HC17 antibodies. In contrast, Sm antigen was mainly concentrated to multiple large foci scattered in the nucleoplasm. A weak staining of the perinucleolar structure remains, suggesting that some Sm antigen may relocalize with WT1 (Figure 3E).

The pattern of IP also changed following actinomycin D treatment (Figure 3F). WT1 coprecipitated with snRNP proteins in control cells (Figure 3F, lane 5), but not after treatment with actinomycin D (lane 6) (similarly for U1-70K and U2-B″; data not shown). However, WT1 is associated with a component of the coiled bodies (p80 coilin) both in the absence and in the presence of actinomycin D (Figure 3F, lanes 9 and 10). WT1 was precipitated with the anti-WT1 antibody, before and after actinomycin D treatment (Figure 3F, lanes 3 and 4). Therefore, the IP data closely mimic the findings with immunohistochemistry, namely that WT1 colocalized with snRNPs under control conditions, but after actinomycin D only with one or a small number of splicing proteins, i.e., those found to also encircle the nucleolar remnants. Sp1 was only precipitated with the anti-Sp1 serum and not by any of the antibodies directed against splicing proteins (Figure 3G). WT1 was not precipitated by the anti-Sp1 serum (Figure 3F, lanes 1 and 2).

WT1 Localization Is Splice Form Dependent

Since none of the WT1 antibodies used in this study are splice form specific and assuming that all four messages are translated, the nuclear localization of WT1 described above in vitro and in vivo must be a composite of four proteins. In an attempt to compare the localization of the four different proteins, COS7 cells were transfected with each of the four forms. In situ hybridization and Northern blotting showed that the efficiency of transfection as well as the expression of the vector was the same for all four constructs (data not shown).

The WT1 localization in the nucleus was scored into three categories: "speckled patterns" were characterized by strongly stained irregular foci set against a diffuse nuclear background; "domains" were defined as distinct areas of diffuse stain with little or no background; "diffuse"

Table 1. WT1 Nuclear Staining Pattern in WT1-Transfected COS Cells (Percentage of Transfected Cells)

Splice	Nuclear Pattern Speckles	Domains	Diffuse	n
(−/−)	30	46	24	287
(+/−)	42	11	47	168
(−/+)	62	7	31	212
(+/+)	85	5	10	229

Table 2. Colocalization between WT1 and Sm Antigen in WT1-Transfected COS Cells (Percentage of Transfected Cells)

	−KTS	+KTS	n
−17	24	69	215; 172
+17	32	75	128; 159

stain was defined as nuclei with a general stain, in which neither speckles nor large domains could be clearly distinguished. The analysis of approximately 200 transfected cells, scored blindly from three to six separate experiments, suggested differences in WT1 localization between the four WT1 splice forms: (−/−), (+/−), (−/+), and (+/+), for which + and − denote presence and absence, respectively, of the 17 amino acid insert/KTS insert (see Figure 1A). The (−/−) form is characterized by domains, (+/−) by diffuse stain, and (−/+) and (+/+) by speckled patterns (Table 1). The differences in splice form localization are independent of the method of fixation, since 4% paraformaldehyde gave similar results (data not shown).

IPs from M15 cells showed that WT1 associates with components of the spliceosomes and the coiled bodies (see Figure 2D). To evaluate whether any difference among the WT1 isoforms could be observed in this regard, transfected COS7 cells were counterstained with antibodies against Sm antigen or p80 coilin. When the localization of WT1 and Sm antigens were compared, a high degree of colocalization was found for two of the isoforms, namely (+/+) and (−/+) (Figures 4A and 4B). Table 2 suggests that the most important determinant for colocalization between WT1 and the Sm antigens is the presence of the KTS sequence. In the absence of KTS, the staining patterns were best described as nonoverlapping in 65% (+/−) and 51% (−/−) of the cells (Figures 4C and 4D). For +KTS protein, the frequency of cells with nonoverlapping patterns was only 12% (−/+) and 9% (+/+), respectively.

Besides the three patterns described above, some transfected cells showed strong WT1 stain in the immediate vicinity of, or partially overlapping with the nucleolus (Figures 4E–4G). Cells doubly stained for WT1 and p80 coilin showed colocalization to these structures, suggesting that they are coiled bodies. Besides these larger "paranucleolar" coiled bodies, p80 coilin antibody identified two to five smaller foci in the nucleoplasm (Figure 4G). WT1 speckles were found to colocalize with some

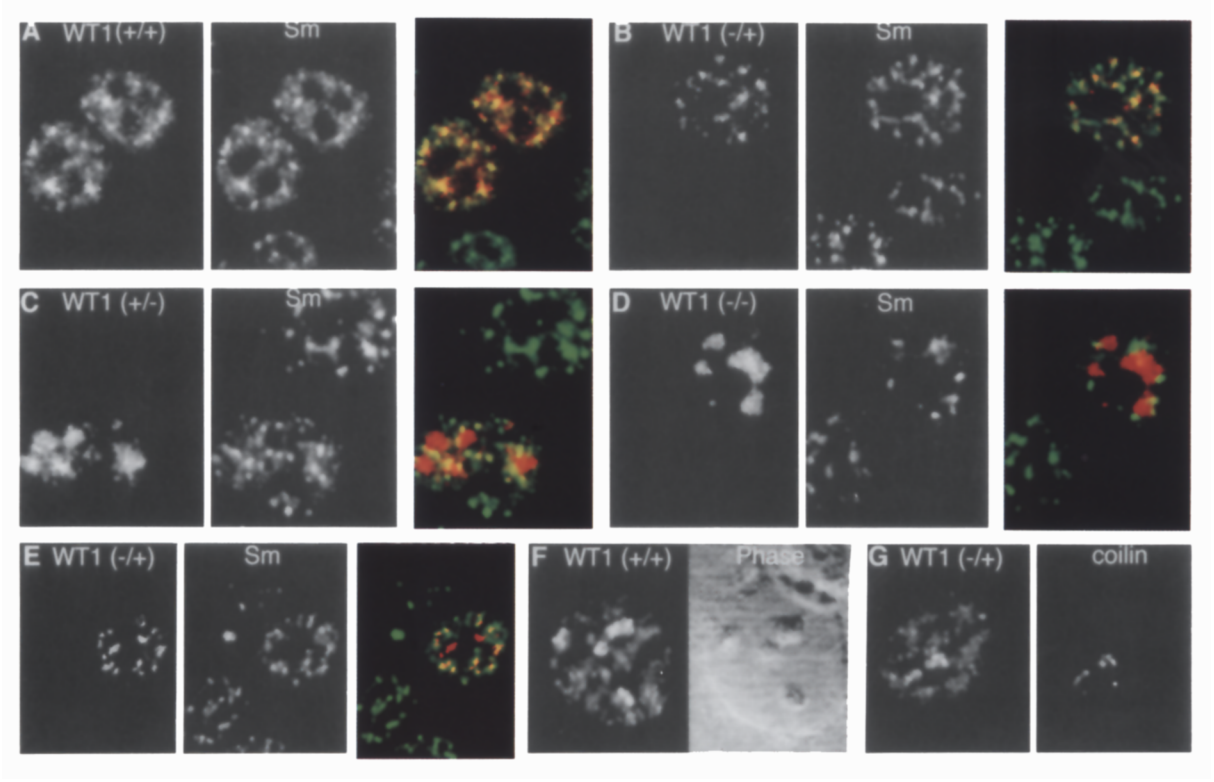

Figure 4. Differential Localization of WT1 Isoforms and Localization of WT1 in Coiled Bodies

COS7 cells transfected with WT1 isoforms as indicated. (A)–(E) stained for WT1 (C19) and Sm antigen (Y12). In merged images, red denotes WT1, green Sm antigen, and yellow colocalization. (E–G) Paranucleolar coiled bodies. WT1 antibodies were C19 (E and F) and H2 (G).

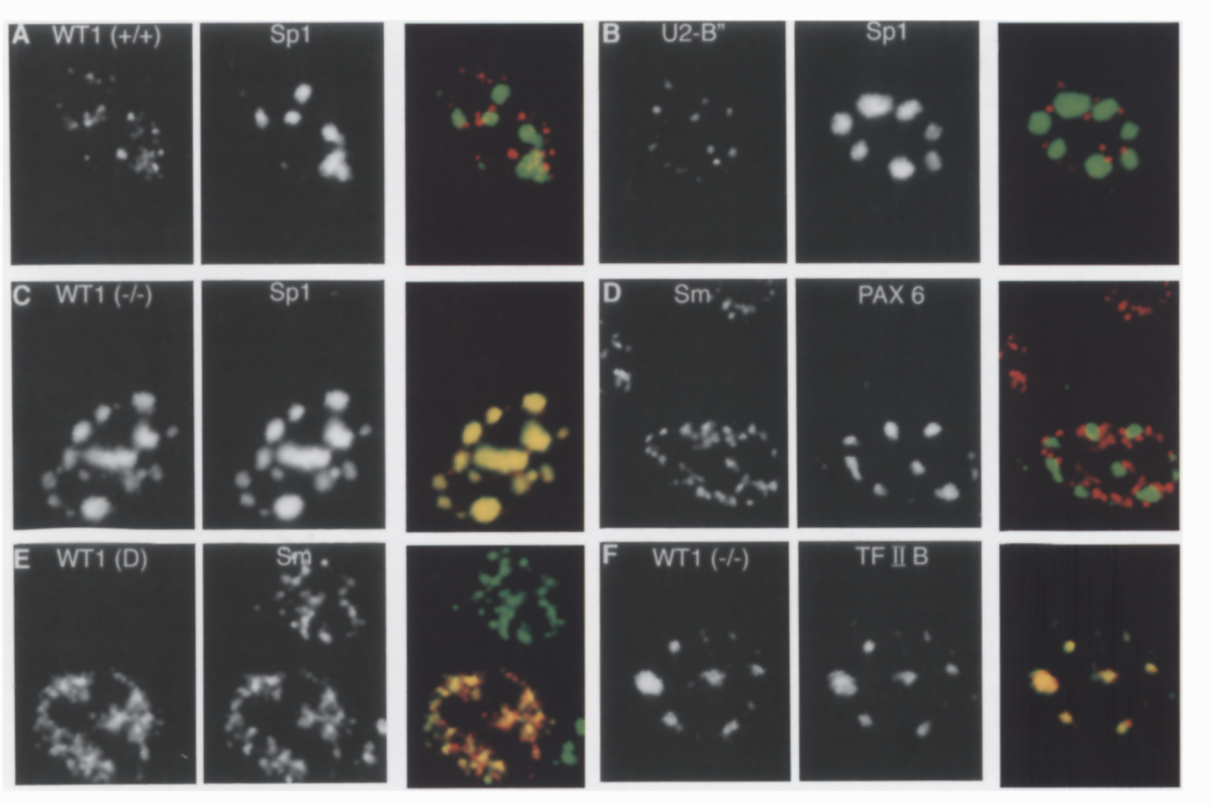

Figure 5. Subnuclear Localization of WT1 Isoforms Relative to Other Nuclear Proteins

COS7 cells transfected with WT1 (A, B, C, E, and F) or PAX6 (D) and stained as indicated: WT1 (H2 in [A], [C], and [F]; C19 in [E]); Sp1 (PEP2). The color image is the left (red) and right (green) images merged, colocalization is seen as yellow.

but not all of these. When the four isoforms of WT1 were compared with regard to their localization in paranucleolar coiled bodies, the KTS insert again was the most important determinant for this localization: 9% (−/−) versus 40% (−/+) and 14% (+/−) versus 32% (+/+) (n = 168–226).

Is WT1 Involved Both in Transcriptional Control and mRNA Processing?

Viral infection often leads to drastic changes in the nuclear organization; the localization of splicing factors changes and large domains for replication and transcription can be identified (Wilcock and Lane, 1991; Bosher et al., 1992; Jiménez-Garcia and Spector, 1993). COS cells are transformed with SV40 virus lacking the viral origin of replication. Transient transfections are carried out using a cloning vector that contains the SV40 origin, so that episomal replication takes place in a process similar to that seen after acute SV40 infection (Gluzman, 1981).

To interpret the subnuclear localization of the −KTS forms of WT1 in transfected COS cells, we set out to test whether the typical diffuse or domain localization could be associated with any functional nuclear domain in the transfected cells. Sp1 is a well-characterized transcription factor with a DNA-binding domain similar to that of WT1 (Kadonaga et al., 1987; Drummond et al., 1994). SV40 infection induces high levels of Sp1 expression (Saffer et al., 1990). Cells transfected with the four forms of *WT1* were stained for WT1 and Sp1. Nontransfected COS cells show a granular staining pattern for Sp1 similar to that of M15 cells (see Figure 2B), whereas transfected cells show a marked increase in Sp1 and a drastic change in its localization. Figures 5A–5C show that overexpressed Sp1 localizes in large domains in the nucleus, which are immediately adjacent to but not overlapping with the U2-B″ splicing factor (Figure 5B). This staining pattern was also seen after transfection with empty vector (containing SV40 origin) and was independent of which WT1 construct was used. The Sp1 domains show poor colocalization with the (+/+) form of WT1 (Figure 5A) but colocalize perfectly with the (−/−) form (Figure 5C). Table 3 shows that the most important determinant for colocalization between WT1 and Sp1 in transfected COS cells is the absence of the KTS sequence.

To test whether the localization in domains after transfection was specific for zinc finger proteins or is also seen for other transcription factors, we studied PAX6, which is a member of a family of transcriptional regulators implicated in developmental control (Strachan and Read, 1994). PAX6 expressed in transfected COS7 cells also localizes in large domains in the nucleus, and as in the case of Sp1, very little overlap is seen between PAX6 and the speckles containing splicing factors (Figure 5D). Double staining for PAX6 and Sp1 shows perfect overlap (data not shown). In COS cells that were cotransfected with PAX6 and WT1, perfect colocalization was found twice as

Table 3. Colocalization between WT1 and Sp1 in WT1-Transfected COS Cells (Percentage of Transfected Cells)

	−KTS	+KTS	n
−17	72	33	64; 40
+17	48	9	40; 69

frequently when the (−/−) form of WT1 was used versus when the (−/+) form was used (data not shown).

In adenovirus-infected HeLa cells, RNA polymerase II relocates to large domains similar to those found to contain WT1 (−KTS), Sp1, and PAX6 in transfected COS cells (Jiménez-Garcia and Spector, 1993). To test whether the shared domains in COS cells could be transcriptional, cells were stained with an antibody to the basal transcription factor TFIIB (SI-1). In untransfected cells, TFIIB is rather diffusely localized in the nucleus; however, following transfection it is concentrated in the same domains as WT1 (−KTS), PAX6, and Sp1 (Figure 5F). If transfected COS cells are treated with DNase I, domains can no longer be identified for Sp1 or for WT1 (−KTS) (data not shown), suggesting that this localization depends on DNA binding. We therefore suggest that the identified domains are areas in which transcription factors are concentrated.

Factors Influencing the Subnuclear Localization of WT1

Our results with transfected COS cells suggest that the presence of the KTS insert between zinc fingers 3 and 4 shifts the protein localization from transcription factor domains to sites where splicing factors are concentrated. To test this hypothesis further, we transfected COS cells with (−/−) and (+/+) forms of WT1. After fixation, the cells were treated with RNase A or DNase I. In (+/+) transfected cells, RNase A treatment decreased the frequency of nuclei with WT1 in speckles to 20% (n = 35) (controls 85%; Table 1), whereas in (−/−) cells the value was unchanged from control (27% [n = 37] versus 30%; Table 1). Instead of localizing in speckles, the (+/+) protein was seen in domains or diffusely in the nucleoplasm (66% and 14%, respectively). Of these RNase A–treated cells, 68% showed good colocalization with Sp1 in domains, but these could not be seen if the cells were treated with both RNase A and DNase I, again suggesting that this localization is dependent on DNA binding.

As stated above, when (−/−) transfected cells were treated with DNase I, the typical domains could no longer be seen. Sp1 was undetectable, suggesting that this protein cannot remain in a cell devoid of DNA. WT1, on the other hand, did remain in DNase I–treated nuclei, but localizing in speckles (86%, n = 65) and colocalizing with Sm antigens (69%, n = 35). DNase I treatment does not change the localization of (+/+) protein, and 89% of the cells show good colocalization between WT1 and Sm antigens (n = 35).

The importance of the zinc fingers for WT1 localization in splicing domains was evaluated by analyzing two mutant WT1 proteins with zinc finger alterations: one point mutation in zinc finger 3 common in DDS patients, Arg-394 to Trp in the (−/+) form (D), and the other a terminal deletion from the middle of zinc finger 3 (T) (Bruening and Pelletier, 1994). When expressed in COS cells, both proteins show localization to nuclear speckles (81% [n = 194] and 97% [n = 35]) and both colocalize very poorly with Sp1 (3% [n = 40] and 4% [n = 27] colocalization). If anything, the mutant forms of WT1 localize better with splicing factors than the wild-type +KTS forms, 93% of D-transfected cells show very good colocalization between WT1 and Sm antigens (n = 154) (Figure 5E). It is therefore highly unlikely that zinc fingers 3 and 4 are directly involved in localizing the +KTS forms to splicing complexes. Likewise, Table 2 suggests that exon 5 (i.e., the 17 amino acid alternative insert) plays no role in localization to splicing complexes.

Discussion

This study shows that the tumor suppressor gene product WT1 associates with components of the cellular splicing machinery in vivo. WT1 and splicing factors colocalize in the two major nuclear domains known to contain snRNPs: speckles and coiled bodies. The subnuclear localization of WT1 is dynamic. Disruption of pre-mRNA splicing and actinomycin D treatment, which are known to relocalize splicing factors, also change WT1 localization similarly. WT1 is coimmunoprecipitated by monoclonal antibodies against a number of well-defined splicing factors, suggesting that it is interacting directly with components of the splicing machinery. In transfected COS cells, the association is most typically seen with the most abundant forms of WT1 (+KTS), whereas the −KTS forms are shown to associate mainly with DNA in transcription factor domains.

Model for the Differential Subnuclear Localization of WT1 Isoforms

The conclusion that the alternatively spliced forms of WT1 (+KTS and −KTS) localize differentially to speckles/coiled bodies and transcription factor domains is based on experiments with transfected COS cells. We believe it reasonable to conclude that WT1 localization in the COS cells will reflect its localization and function in endogenously expressing cells for the following reasons. WT1 localization in large transcription factor domains fits previous data demonstrating DNA binding (Bickmore et al., 1992; Rupprecht et al., 1994; Drummond et al., 1994). Colocalization with splicing factors is seen in fetal tissues, M15 kidney cells, and COS cells (Figures 2 and 4). Furthermore, the sum of the patterns observed in COS cells expressing the four different WT1 isoforms more or less approximates the pattern observed in M15 cells (allowing for the anticipated excess of +KTS over −KTS forms; Figure 1E versus Figures 4A–4D).

We must emphasize that the difference in localization between the +KTS and −KTS forms of WT1, though dramatic, is not absolute (Tables 1–3). This is also underlined by the experiment in which COS cells transfected with the (+/+) form were treated with RNase; this resulted in the localization of most WT1 molecules to transcription factor domains. Conversely, when COS cells transfected with

the (−/−) form of WT1 were treated with DNase, the protein was found to associate with splicing factors in a high proportion of cells. We believe that this represents, in part, relocalization of WT1. In a similar vein, two proteins with zinc finger mutations showed a heightened colocalization with splice factors. Clearly, the domain(s) required for interaction with splice complexes is present in all four WT1 isoforms.

Given all these considerations, why do the +KTS and −KTS forms of WT1 take up (predominantly) different localizations in the nucleus? The model we propose can be summarized as follows: the −KTS forms of WT1 primarily bind DNA, because of broad target site specificity, high DNA affinity, or both. The proteins are able to associate with splicing complexes and will do so if the DNA targets are unavailable. The +KTS forms of WT1, on the other hand, will primarily associate with splicing factors, because of a limited number of DNA target sites, low DNA affinity, or both (Bickmore et al., 1992; Drummond et al., 1994).

What Is the Nature of the Interaction of WT1 with Splicing Complexes?

WT1 appears to be a stably associated component of both speckles and coiled bodies, neither of which appear to contain DNA (Spector et al., 1991; Spector, 1993; Lamond and Carmo-Fonseca, 1993). As deletion of the last two zinc fingers does not appear to disrupt colocalization of WT1 with splice factor complexes, it is unlikely that nucleic acid binding (DNA or RNA) is important for the association. WT1 has no obvious domains characteristic for splicing factors such as RNP or RS motifs (Zahler et al., 1992; Burd and Dreyfuss, 1994). Other possible regions of interaction are under investigation.

Which splicing factors are likely candidates for interaction with WT1? It is unlikely that WT1 directly interacts with all the proteins that contain the motifs that the immunoprecipitating antibodies are known to identify specifically (Figure 2D). The IPs were performed at intermediate ionic strength, and gel electrophoresis of the immunoprecipitated proteins showed 10–15 bands, suggesting that relatively large RNP complexes were precipitated (data not shown) (Behrens and Lührmann, 1991). After disruption of the splicing machinery in M15 cells by oligonucleotide injection, WT1 relocalized in the nucleus in a similar fashion to the snRNPs (Figures 3A and 3C). In contrast, when transcription was inhibited by actinomycin D, WT1 and most of the Sm antigens went separate ways, WT1 showing a localization similar to p80 coilin, U1-70K, and U2AF (Figure 3E). After actinomycin D treatment, only antibodies against p80 coilin immunoprecipitated WT1 (Figure 3F). Although a direct interaction between WT1 and p80 coilin could be envisaged, this is unlikely to be the whole answer, since WT1 in control cells is found in far more nuclear speckles than the few associated with coiled bodies. U2AF is found in both coiled bodies and splicing complexes and could be the common denominator (Lamond and Carmo-Fonseca, 1993). U2AF would be a highly interesting candidate for interaction with WT1, since it has been implicated in control of alternative splicing (Wu and Maniatis, 1993).

RNA Processing, a Role for WT1 in Development and Oncogenesis?

Since the major isoforms of WT1 (+KTS) are associated with splicing factors, it seems likely that they have a role in RNA metabolism. WT1 may be involved in mRNA transport or stability, or it may directly influence splicing.

The functional significance of the speckles (IGs) and coiled bodies remains unclear. Although it now seems that the splicing of most transcripts occurs cotranscriptionally, some specific transcripts are posttranscriptionally spliced in close proximity to the IGs (Mattaj, 1994). It has been proposed that the IGs are sites of splice factor storage, preassembly, or both. Given the close association of WT1 with splicing factors in IGs and coiled bodies, we think it is reasonable to assume that WT1 will also be associated with active splice complexes wherever they are located. Given the recent finding that splicing occurs cotranscriptionally, it should be noted that we cannot distinguish whether WT1 located in transcription factor domains is involved in transcription, splicing, or both. For these reasons, we cannot yet draw conclusions about the respective functions of the +KTS/−KTS forms of WT1. However, our findings raise the intriguing possibility that WT1 may play an important role in linking transcriptional control and RNA processing/splicing.

It is not unprecedented that zinc finger proteins have functions other than DNA binding and transcriptional control. The most carefully studied example so far is TFIIIA. Its nine zinc fingers are involved in transcriptional activation of the 5S RNA promoter, but also bind specifically to the 5S RNA (Clemens et al., 1993). However, in contrast with WT1, one molecule carries out both functions and alternative splicing of the zinc finger domain is not involved. A number of transcription factors have alternatively spliced zinc finger domains (for review see Drummond et al., 1994). However, it is novel that such alternative splicing may completely change the functional role of a protein as may be the case for WT1.

All forms of WT1 have recently been shown to bind RNA via the zinc fingers with high affinity and sequence specificity (A. Caricasole and A. Ward, personal communication). Although the localization of WT1 to the splicing complexes appears to be independent of the zinc fingers, they could still play a very important role in the function of WT1 once it has moved to the splicing domains, for instance by associating with specific mRNA species.

It is interesting to consider the molecular pathology of the DDS in the light of these new findings (Hastie, 1994; Bruening and Pelletier, 1994). Children with DDS have severe abnormalities of the kidneys and gonads and are strongly predisposed to developing Wilms' tumors. DDS arises through constitutional heterozygous mutations of the *WT1* gene, all of which affect the zinc finger region of the protein directly. The majority of the mutations are missense or nonsense mutations that alter or delete resi-

dues critical for DNA binding so that the protein is unable to bind to a range of WT1 DNA targets. Our experiments showed that a high proportion of such forms colocalize with splicing factors. A second class of particularly interesting DDS mutations affects a critical splice site essential for the production of the +KTS form of the molecule. From the studies described here, we would predict that in these DDS patients with a reduced +KTS/−KTS ratio a higher proportion of WT1 would now be able to bind DNA and a reduced proportion would be able to interact with splice factors.

WT1 is a classic tumor suppressor gene. The prevailing view has it that *WT1* mutations cause derepression of a number of genes expressing growth factors or growth factor receptors (Hastie, 1994), allowing uncontrolled growth of the tumor. The present data suggest that the role of *WT1* may be more complex and that mRNA processing may play an important role in WT pathogenesis. Although WT1 is an example of a tumor suppressor gene product directly associated with splicing factors, it was recently demonstrated that the retinoblastoma protein ($p110^{RB}$) binds a nuclear matrix protein that colocalizes with speckles (Durfee et al., 1994).

Regulation of splicing is of fundamental importance in gene expression (for reviews see Horowitz and Krainer, 1994; Parkhurst and Meneely, 1994). Our studies indicate that developmental processes regulated by WT1 may provide an excellent model for studying the regulation of splicing during mammalian development. Furthermore, we can conclude that posttranscriptional regulation may be a key event in kidney and gonad morphogenesis.

Experimental Procedures

Cell Cultures

The WT1-expressing mesonephric cell line M15 was established from mice transgenically expressing the large T protein of polyoma virus under control of the early viral enhancer (Rassoulzadegan et al., 1993). Heterozygous carriers of the transgene on the C57BL/6 × DBA/2 F1 genetic background were mated, and embryos were retrieved 10.5 dpc. Mesonephros was dissected as described (Hogan et al., 1986), and the cells dissociated (0.25% trypsin and 0.02% EDTA in PBS). Cells were seeded (10^5 per 35 mm plate) in Dulbecco modified Eagle's medium (DMEM, Flow Labs) with 10% fetal calf serum (FCS, GIBCO).

Besides M15 cells, two WT1-expressing cell lines were used: PYT45 (mouse Sertoli cells [Rassoulzadegan et al., 1993]) and AC29 (mouse mesothelioma cells [Davis et al., 1992]). Three nonexpressing lines were used: C2 (mouse myoblast), 10T1/2 (mouse fibroblast), and COS7. Cells were cultured in RPMI (AC29) (GIBCO) or DMEM (all other cell lines) with 10% FCS, in 2- or 8-well slides (Chamber Slides) or in plastic flasks (Nunc) (IPs). M15 cells were cultured at 32°C with 7.5% CO_2, the rest at 37°C, all with 5% CO_2 in air.

Transfections

The pRC/CMV vector containing CMV promoter and SV40 replication origin was used (Invitrogen) to express wild-type or mutant isoforms of mouse WT1 and the major splice form of human PAX6 (−exon 5a). COS7 cells were transfected with electroporation (1200 V, 25 μF) (Gene Pulser, Bio-Rad) and analyzed after 40–48 hr. Empty vector or *WT1* in reverse orientation was used for controls.

Antibodies

Antibodies against the following antigens were used in this study.

WT1

C19 (Santa Cruz Biotechnology), H2, H7, HC17 (recognizing different epitopes in the N-terminal 181 amino acids of WT1; J. F. Morris and F. J. Rauscher III, personal communication), and WT48 (Bickmore et al., 1992).

Splicing Factors

Sm/Y12, U1-70K, U2-B″, and p80 coilin (provided by A. I. Lamond).

PAX6

M860 (D. Engelkamp, unpublished data).

Centromere

CREST/CP (provided by P. Jeppesen).

Sp1

IC6 and PEP2 (Santa Cruz Biotechnology)

TFIIB

SI-1 (Santa Cruz Biotechnology)

Tubulin

α-Tubulin (Sigma)

Immunohistochemistry

Fetal kidneys and testes were isolated from Swiss mice at 13.5–15.5 dpc and immediately frozen in liquid N_2. Cryosections (10–14 μm) (Jung CM3000, Leica) were placed on Tespa-coated slides, briefly fixed in acetone:methanol (1:1, −20°C, 5–10 min), rehydrated in PBS with 0.02% sodium azide (Sigma) (4°C), and immediately stained for immunohistochemistry (below). Cultured cells were rinsed in PBS (4°C), fixed as above, rehydrated, and refrigerated until stained (<1 week).

For staining, cells and sections were preincubated for 45 min in blocking solution (2% BSA [Sigma], 0.1% sodium azide, 0.2% Tween 20 [ICN Biochemicals], 6.7% glycerol), incubated with primary antibodies for 1 hr at room temperature, and rinsed. Secondary antibodies conjugated with FITC or Texas red were used against mouse, rabbit, and human IgG (Sigma, Vector, and Jackson Immunoresearch Laboratories). After 30 min at room temperature, cells were rinsed and mounted (nonfluorescent glycerine [Merck]:PBS [1:1] with 2.5% antifading agent 1,4-diazabicyclo-(2.2.2)octane [Sigma]). Antibodies were diluted in blocking solution, and the cells were rinsed in PBS with 0.2% Tween.

Confocal Microscopy

Nuclear localization of antigens was analyzed with an Orholux II microscope (Leitz) equipped for confocal microscopy (MRC 600, Bio-Rad). Labeled cells were excited with an argon laser, and standard filters were used for simultaneous detection of FITC and Texas red. The staining patterns were scored as colocalizing, intermediate, or nonoverlapping. Throughout the study, the sensitivity of the system was set to prevent any contribution of signal between the channels and to allow very thin optical sections. Other control experiments excluded unspecific binding of secondary antibodies.

Northern Blotting

Total RNA was prepared from cells by guanidium thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi, 1987). Of each sample, 15 μg was electrophoresed through a 1% agarose denaturing gel (formaldehyde) and blotted onto a nylon filter (Hybond N, Amersham). Hybridization with random-primed radiolabeled probes was performed using standard methods (Sambrook et al., 1989).

IP and Western Blotting

Nuclei from M15 cells were isolated (Wijnholds et al., 1988), resuspended in ice-cold buffer A (300 mM sucrose, 60 mM KCl, 15 mM NaCl, 60 mM Tris–HCL [pH 8], 0.5 mM spermidine, 0.15 mM spermine, 2 mM EDTA, protease inhibitor cocktail [Boehringer Mannheim]), and lysed by L-lauryl sarcosyl (2%). The DNA was sheared, and the nuclear lysate was cleared with protein A–Sepharose beads (Sigma) for 60 min at 4°C. For IP, antibodies were directly coupled to protein A–Sepharose beads (Harlow and Lane, 1988). IP was performed with protein A–Sepharose antibodies in IPB100 (100 mM NaCl, 10 mM Tris–HCl, 1 mM EDTA [pH 8]) overnight at 4°C and washed in 50× vol for 10 min: once with TE (10 mM Tris–HCl, 1 mM EDTA [pH 8]), three times with IPB100, and three times with RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% Na–deoxycholate, 0.1% SDS, 50 mM Tris–HCl [pH 8]). Immunoprecipitated proteins were eluted with 4% L-lauryl sarcosyl

for 60 min at room temperature and analyzed by Western blotting. The proteins in loading buffer (2% SDS, 10% glycerol, 60 mM Tris–HCL, 100 mM DTT, 0.001% bromophenol blue [pH 8.3]) were boiled for 5 min, separated by SDS–PAGE (10% polyacrylamide), and blotted onto nitrocellulose (Hybond ECL, Amersham). The filter was blocked for 60 min with 1% blocking reagent (Boehringer Mannheim) in TBS-T (150 mM NaCl, 20 mM Tris–HCl, 0.1% Tween 20 [pH 7.6]) and incubated with the primary antibody overnight at 4°C. It was then washed, incubated with the secondary antibody (goat anti-mouse or goat anti-rabbit, HRP conjugated [Sigma]) for 60 min at room temperature, and washed. Antibodies were diluted 1:5,000 to 1:15,000 in 0.5% blocking reagent in TBS-T, and filters were washed in TBS-T. Detection was performed by enhanced chemiluminescence (ECL, Amersham). Hyperfilm ECL films were exposed for 2–60 min.

Manipulations of WT1 Localization

Cell Injections

Four oligonucleotides for injections were synthesized in house (PCR-Mate, Applied Biosystems): U1C, complementary to nucleotides 1–20 of the U1 snRNA; U6C, complementary to nucleotides 49–72 of U6 snRNA; two control oligonucleotides, C1 (Pan and Prives, 1988; O'Keefe et al., 1994) and C2 (5′-GACGTAAGCCGTGTGGAG-3′).

The injection solutions contained oligonucleotides at 150 μM and Texas red–conjugated dextran at 7 mg/ml (lysine fixable, 70 kDa, Molecular Probes) in 70 mM KCl, 10 mM NaH_2PO_4 (pH 7.2) (O'Keefe et al., 1994). The injection solution was filtered (Spin-X, 0.22 μm, Costar) and injected into the cytoplasm of subconfluent M15 cells using Femtotips microinjection needles (Eppendorf) and an Eppendorf Microinjector 5242 mounted on an Axiovert 10 inverted microscope (Zeiss). After injection, the culture medium was changed and the slide returned to an incubator for 2 hr before fixation. Cells were stained for Sm antigen (Y12 antibody) or WT1 (H2 or H7 antibodies), in both cases an FITC-conjugated secondary antibody was used. Texas red–labeled dextran served to identify the injected cells during the analysis.

Actinomycin D

To investigate how WT1 localization was changed by the transcriptional inhibitor actinomycin D, cells were exposed for 2 hr (5 μg/ml, Sigma) and then fixed as above.

RNase A and DNase I Treatment

COS cells were cultured, transfected, and fixed as described above. After rehydration, the cells were incubated in either RNase-free DNase I (400 U/ml, Boehringer Mannheim), in RNase A (200 mg/ml, Boehringer Mannheim), or in a combination of the two. Incubations were performed in PBS (with 3 mM $MgCl_2$, 0.4% Tween 20) for 2 hr at room temperature. The efficiency of respective treatment was verified by the loss of DAPI stain after DNase I and a marked loss of Sm antigen after RNase A.

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