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Role of Wilms Tumor 1 (WT1) in the Transcriptional Regulation of the Mullerian-Inhibiting Substance Promoter[1]

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ABSTRACT

The Wilms tumor 1 (*WT1*) gene product may regulate the mullerian-inhibiting substance (*MIS*) gene, because mutations in *WT1* can cause persistence of the mullerian duct in men. In the present study, we show by gel shift and chromatin immunoprecipitation assays that WT1 bound to a GC-rich sequence in the murine *Mis* promoter. Mutation in this site abolished WT1-mediated activation of the *Mis* promoter. The WT1, SRY box protein 9, and steroidogenic factor 1 could synergistically activate the *Mis* promoter, and at least two factors were necessary for minimal activation. The WT1 is an essential factor for activation of the *Mis* promoter; therefore, the persistence of the mullerian duct in patients with Denys-Drash syndrome may result from deregulation of the *MIS* gene.

gene regulation, male reproductive tract, mullerian ducts

INTRODUCTION

An essential stage of male development is the differentiation of the wolffian ducts by androgen and regression of the mullerian ducts that normally give rise to the oviducts, uterus, and fallopian tubes in females [1]. Sertoli cells secrete mullerian-inhibiting substance (MIS), a member of the transforming growth factor β superfamily and also known as antimullerian hormone [2]. The Mis type II receptor is expressed in the mesenchymal cells surrounding the mullerian ducts [3]. Interaction between Mis and its receptor causes regression of the mullerian ducts by apoptosis through a paracrine mechanism [4]. Mutations in *MIS* or the gene for its type II receptor cause male pseudohermaphroditism in humans [5], and deletion of *Mis* or the *Mis* type II receptor leads to pseudohermaphroditism in mice [6, 7]. The spatial and temporal regulation of *MIS* is very important in its biological action [8]. *Mis* begins to be expressed in a sexually dimorphic pattern during embryo development. In mice, *Mis* is expressed in male embryonic Sertoli cells from Embryonic Day 13 until birth [9]. SRY box protein 9 (SOX9), steroidogenic factor 1 (SF1), Wilms tumor gene 1 (*WT1*), and GATA-binding factor 4 (GATA-4) have been implicated in regulation of the *Mis* promoter [10–12]. Arango et al. [8] showed the importance of SOX9- and SF1-binding sites in positive regulation of the *Mis* promoter in vivo. Earlier, Nachtigal et al. [12] reported that SF1 and WT1 synergistically activate the *Mis* promoter in vitro.

A zinc finger containing DNA-binding protein, WT1 acts as a transcriptional activator or repressor depending on the cellular or chromosomal context [13, 14]. It has four major isoforms because of the insertion of three amino acids (KTS) between zinc fingers 3 and 4 and the insertion of an alternatively spliced, 17-amino acid segment encoded by exon 5 in the middle of the protein [15]. These four isoforms are conserved among mammals. Recent reports have described specific in vivo function of the different isoforms of WT1 in embryonic development. Severe defects in kidney and gonads are found in mice lacking the WT1(−KTS) isoforms, and mice lacking the WT1(+KTS) isoform show defects of kidney and male-to-female sex reversal [16]. Natoli et al. [17] reported that mice carrying a deletion of exon 5 have no developmental defects and are fertile.

WT1 binds to the highly GC-rich canonical early growth response gene-1 DNA-binding motif [13]. Many genes are proposed to be regulated by WT1. Specific mutations in *WT1* cause two different types of genitourinary abnormalities, Denys-Drash syndrome (DDS) [18] and Frasier syndrome [19]. Besides kidney abnormalities, patients often showed male-to-female sex reversal, male pseudohermaphroditism, and cryptorchidism. Most of the missense mutations in the *WT1* gene found in patients with DDS are in DNA-binding zinc finger domains of WT1 [20, 21]. If pseudohermaphroditism in patients with DDS is caused by the deregulation of *MIS* gene activation by WT1, then most likely WT1 needs to bind DNA to regulate the *MIS* promoter. To understand the pathogenesis of DDS, it is important to know whether WT1 regulates the *Mis* promoter. The role of SOX9 with WT1 in this process is not clear. In the present report, we provide evidence that WT1 can activate the murine *Mis* gene in the presence of SOX9 as well as SF1 and that WT1 must bind DNA to do that.

MATERIALS AND METHODS

Plasmids

Expression vectors, pcDNA3WT1 (−KTS) and pcDNA3WT1 (+KTS), were made by polymerase chain reaction (PCR) amplification of wild-type WT1 from pCB6WT1$^{-/-}$ and pCB6WT1$^{+/+}$, respectively. The resulting PCR products were subsequently subcloned into a modified pcDNA3 vector [22] harboring the 5′ untranslated region of the herpes simplex virus-thymidine kinase gene. All WT1 mutants were created from this vector, pcDNAWT1 (−KTS), by site-directed mutagenesis, and the mutations were verified by sequencing.

The 203-base pair (bp) *Mis* upstream sequence that drove transcription of the luciferase gene was amplified by PCR from mouse *Mis* (nucleotides −180 to +23) and cloned in front of the luciferase gene in the vector pGL3 basic (Promega, Madison, WI). The construct was designated

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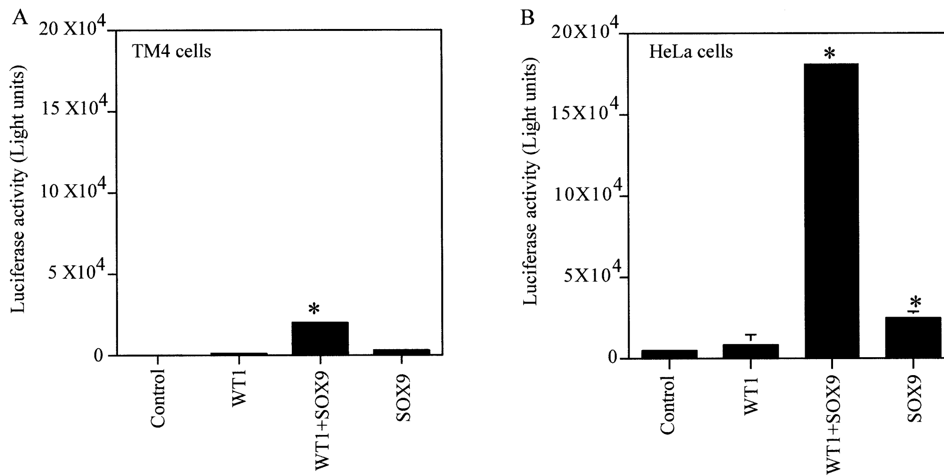

FIG. 1. WT1 synergistically activated the *Mis* promoter. TM4 cells (**A**) and HeLa cells (**B**) were transfected with 0.2 μg of the Mis promoter driving a luciferase reporter construct and 0.1 μg of pCB6WT1−KTS (WT1), pcDNASOX9 (SOX9), or the empty expression vector, pCB6+ (control). Ten nanograms of CMV promoter-driven β-galactosidase construct were cotransfected with each sample to control for differences in transfection efficiencies. Empty pcDNA3 was added to keep the plasmid amounts equal in each transfection. The assay was performed 36 h after transfection. Luciferase values were normalized with β-galactosidase activity. All results are expressed as the mean ± SD of at least three experiments. Asterisks indicate values significantly different from control.

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−180MISP. All site-directed mutagenesis of the promoter constructs was performed with the Quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA). All constructs were confirmed by sequencing from both directions.

Cell Culture and Transfection

HeLa and TM4 cells were grown at 37°C in Dulbecco modified Eagle medium-Ham F-12 supplemented with 10% fetal calf serum in 5% CO_2. The cells were seeded at a density of 50 000–70 000 cells/well in 12-well plates 16–18 h before transfection. The cells were cotransfected with expression and reporter plasmids as indicated in the figure legends. The plasmidCMV-β-galactosidase (10 ng) was cotransfected as an internal control to normalize for differences in transfection efficiency. The transfections were performed with Lipofectamine-Plus reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations, and the cells were harvested after 40–48 h. Luciferase activity was measured with a luciferase assay kit (Tropix) and a Lumat LB9507 luminometer (EG&G Berthold). β-Galactosidase was measured with the Galacto-Light plus kit (Tropix).

Statistical analyses performed on transfection data included a two-way, paired analysis of variance followed by Student-Newman-Keuls analysis with a 95% confidence score. These analyses were performed using STATISTICA 6 program (Stat Soft, Tulsa, OK).

Gel Shift Assay

Gel shift reactions were performed in a total volume of 20 μl on ice. Radiolabeled probes were prepared by end labeling with [γ-^{32}P]ATP, and 100 pmol of each labeled probe and 2.5 μl of in vitro-translated (IVT) protein were used for each reaction. For competition of wild-type and mutant oligonucleotides, a 50- or 100-fold excess of unlabeled oligonucleotides was added to the reaction mixture before addition of the labeled probe. Thirty minutes later, the reaction mixture was loaded onto a 5% polyacrylamide gel in Tris-glycine buffer, and electrophoresis was performed at 150 V for 3 h. In the supershift with antibody assay, reaction mixture without labeled probe was incubated with 2.0 μg of rabbit anti-WT1 antibody (C-19; Santa Cruz Biotechnology, Santa Cruz, CA) for 15 min at room temperature, and labeled probe was added with further incubation on ice for 30 min.

Western Blot Analysis

Whole-cell extracts were prepared with cell lysis buffer consisting of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1.0% NP-40, 1 mM dithiothreitol, 1 mM PMSF, and 5 μg/ml each of aprotinin, leupeptin, and benzamidine. Western blots were developed by enhanced chemiluminescence (Amersham, Piscataway, NJ). The primary and secondary antibodies used were N-terminal-specific rabbit anti-WT1 at a 1:100 dilution (N180; Santa Cruz Biotechnology), anti-rabbit immunoglobulin (Ig) G conjugated with peroxidase (Amersham).

Chromatin Immunoprecipitation Assay

Formaldehyde was added to TM4 cells (1 × 10^7) at a final concentration of 1%. Fixation was allowed to proceed at room temperature for 15 min and was stopped by addition of glycine to a final concentration of 0.125 M. The cells were then washed with PBS and collected by centrifugation. Next, the cells were incubated with buffer A (10 mM potassium acetate, 15 mM magnesium acetate, and 0.1 mM Tris [pH 7.4] with Roche protease inhibitor cocktail; Roche Applied Science, Indianapolis, IN) on ice for 20 min and homogenized with a dounce homogenizer. The nuclei were collected by centrifugation, resuspended in sonication buffer, and incubated on ice for 15 min. The samples were sonicated on ice with an sonicator (Fisher model 100 Sonic Dismembrator; Fisher Scientific, Inc.,

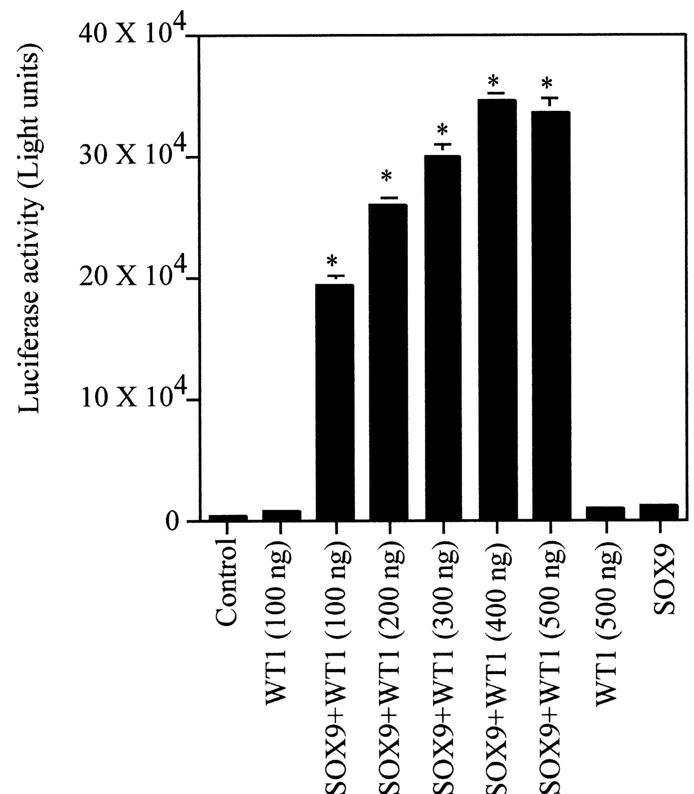

FIG. 2. Dose-dependent increase of WT1-mediated synergistic activation of the *Mis* promoter with SOX9 in HeLa cells. HeLa cells were transfected with 0.2 μg of the Mis promoter driving the luciferase reporter construct and 0.1 μg of pcDNASOX9 (SOX9) with or without the increasing amount of pCB6WT1−KTS (WT1) or the empty expression vector, pCB6+ (control). The CMV promoter-driven β-galactosidase construct (CMV-βgal) was cotransfected with each sample to standardize the transfection efficiency. Empty pcDNA3 was added to keep the plasmid amounts equal in each well. The assay was performed 36 h after transfection. All results are expressed as the mean ± SD of at least three experiments. Asterisks indicate values significantly different from control.

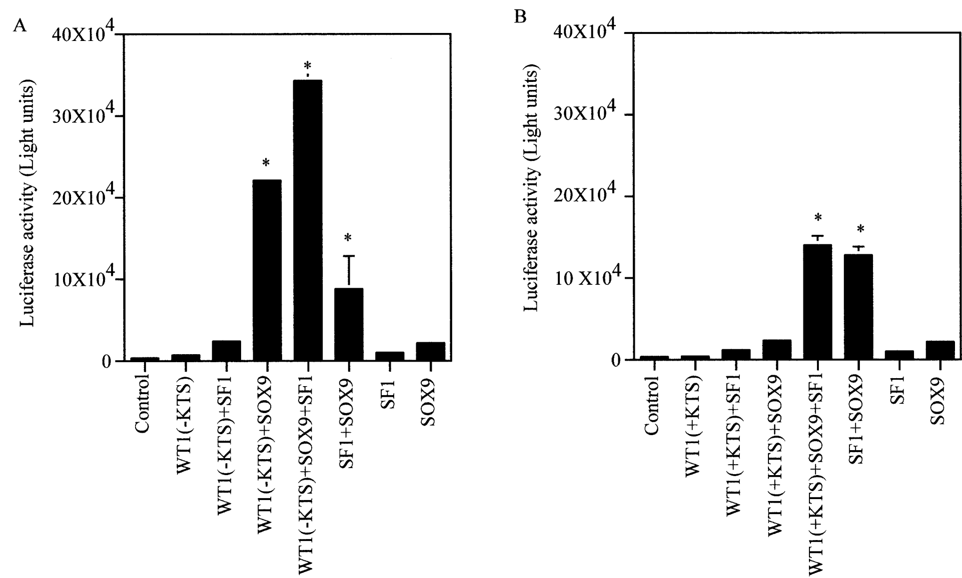

FIG. 3. Synergistic activation of the Mis promoter by WT1 and SF1 in HeLa cells. HeLa cells were transfected with 0.2 μg the *Mis* promoter driving a luciferase reporter construct and different combinations of 0.1 μg each of pCB6WT1−KTS (**A**) and pCB6WT1+KTS (**B**), pcDNASOX9 (SOX9), and pCMV5SF1 (SF1) or the empty expression vector, pCB6+ (control). Ten nanograms of CMV promoter-driven β-galactosidase construct were cotransfected with each sample to control for differences in transfection efficiencies. The assay was performed 36 h after transfection. All results are expressed as the mean ± SD of at least three experiments. Asterisks indicate values significantly different from control.

Pittsburgh, PA) at a setting of 10 for six 20-sec pulses to an average length of approximately 1000 bp (confirmed by electrophoresis) and then microcentrifuged. The chromatin solution was precleared with protein A sepharose for 15 min at 4°C. Immunoprecipitations (IP) were performed overnight at 4°C using 1 μg of anti-WT1 antibody (C-19). After the final ethanol precipitation, each IP sample was resuspended in 30 μl of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (TE). Total input chromatin samples were resuspended in 30 μl of TE and further diluted to 1:100. Each 50 μl of PCR reaction mixture contained 5 μl of IP sample; 1.5 mM $MgCl_2$; 50 ng of each primer; 300 μM each of ATP, dGTP, dCTP, and dTTP; 1× PCR buffer (PerkinElmer, Wellesley, MA); and 1.25 U of *Taq* DNA polymerase (PerkinElmer). After 35 cycles of amplification, 5 μl of the PCR products were electrophoresed on a 1.5% agarose gel, and the DNA was stained with ethidium bromide and visualized under ultraviolet light. The sequences and positions (parentheses) of primers used for PCR were as follows: MisPF, (−182) CAG GCC TCT GCA GTT ATG; MisPR, CAT GGT GGT ACA GCA AGG (+10).

RESULTS

To determine whether WT1 can act as cofactor with SOX9 to activate the *Mis* promoter, we cotransfected a luciferase reporter construct containing the *Mis* promoter with SOX9, WT1, or both into the mouse Sertoli cell line TM4 and measured the luciferase activity after 36 h. As expected, SOX9 activated reporter gene expression approximately 10-fold, and WT1 alone can activate it 3-fold. The two factors synergistically activated the luciferase reporter gene approximately 50-fold (Fig. 1A). This synergistic activity was dose dependent for both WT1 and SOX9 (Fig. 2 and data not shown). To elucidate the roles of SOX9 and WT1, we examined HeLa cells, which do not express SOX9 or WT1. Both SOX9 and WT1 alone could not activate significantly the *Mis* promoter in HeLa cells (Fig. 1B). This indicated that both SOX9 and WT1(−KTS) were required to activate the *Mis* promoter.

We next examined the contribution of SF1 to WT1 and SOX9 activation of the *Mis* promoter. The SF1 was cotransfected with different combinations of WT1 and SOX9 and the presence of a reporter gene into HeLa cells. Alone, SF1 also could not activate the reporter gene in HeLa cells; however, SF1 could activate the reporter gene in the presence of either WT1 or SOX9. The WT1-SOX9 combination

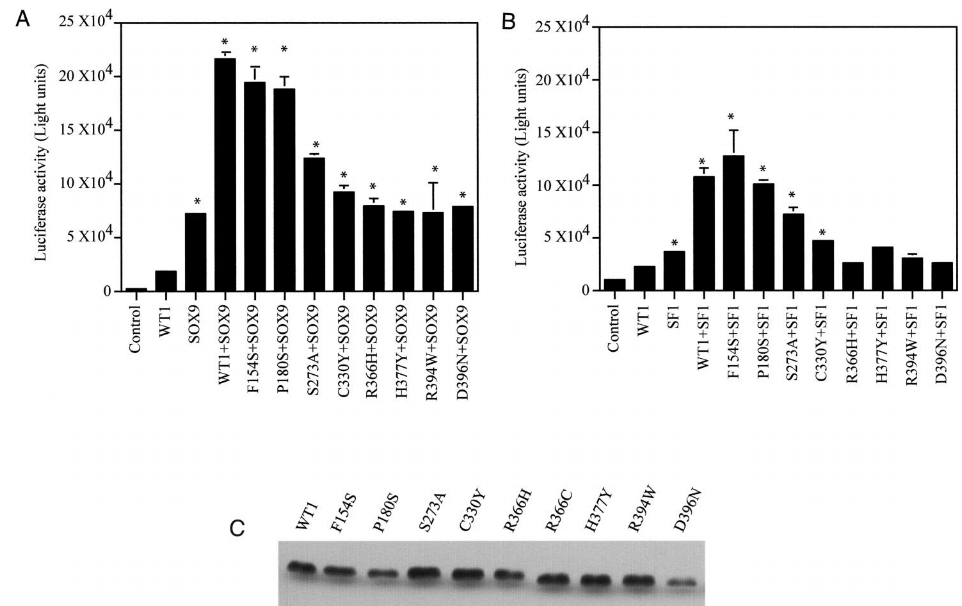

FIG. 4. Activation of the *Mis* promoter in HeLa cells expressing WT1 mutants. **A** and **B**) Cells were transfected with luciferase reporter vector (0.2 μg) containing different point mutants of the WT1 and with SOX9 (**A**) and SF1 (**B**) expression vectors. Empty pcDNA3 was added to keep the plasmid amounts equal in each transfection. The assays were performed 36 h after transfection. All results are expressed as the mean ± SD of at least three experiments. Asterisks indicate values significantly different from corresponding wild-type values. **C**) Western blot using anti-WT1 antibody (N-180) and showing equal expression of all mutants and wild-type WT1.

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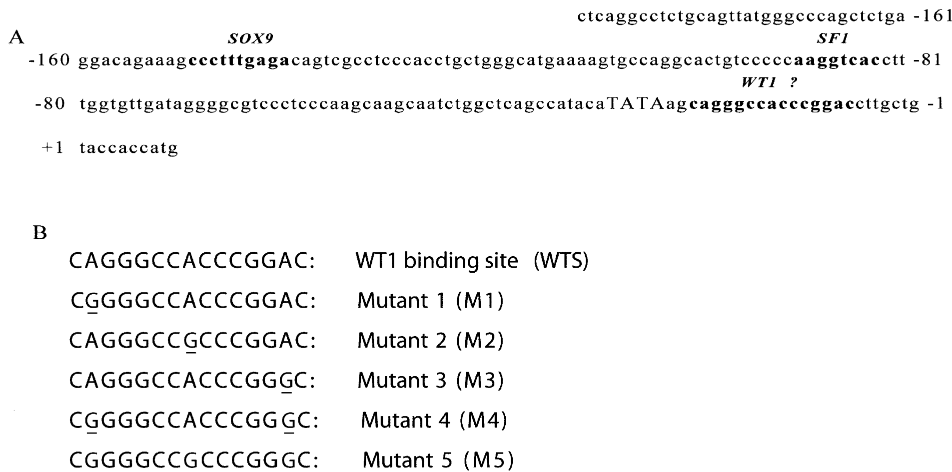

FIG. 5. Promoter sequence of the murine *Mis* gene and potential WT1-binding site mutants. **A**) The SOX9- and SF1-binding sites are in bold. The putative WT1-binding site is also shown. The potential TATA sequence is in uppercase letters. The transcription start site is indicated by +1. **B**) Sequences of the WT1-binding site and mutations within it (underlined).

was the most potent activator, followed by WT1-SF1 and SOX9-SF1. Maximum luciferase reporter gene expression was achieved by all three of these factors together (Fig. 3A). These results indicate that neither factor alone is sufficient to activate the *Mis* promoter. At least two factors are needed, and maximal activation is achieved with all three factors. The synergistic activation of the Mis promoter by WT1 in combination with either SOX9 or SF1 was found to be statistically significant, with a confidence value of 0.05. The activation of the *Mis* promoter by WT1, SF1, and SOX9 when transfected alone was variable in many independent experiments, and the differences were not always statistically significant (Figs. 1–3 and data not shown). From these results, we concluded that WT1 can act as a cofactor with SOX9 as well as with SF1, at least in activation of the *Mis* promoter. However, WT1(+KTS) isoforms, either alone or in the presence of SOX9 or SF1, could not activate the *Mis* promoter (Fig. 3B). These results are consistent with the hypothesis that −KTS isoforms are involved in transcriptional activity, whereas +KTS isoforms are associated with the spliceosome and are involved in the regulation of certain genes at the posttranscriptional level (i.e., Sry [23]).

The DDS phenotype often arises from the alteration of one allele by a missense point mutation, usually in the zinc finger DNA-binding domain of WT1 [20]. Most *WT1* mutations found in patients with DDS cause loss of the DNA-binding ability of WT1 [20]. We cotransfected several mutant WT1s with either SOX9 or SF1 and reporter constructs containing the *Mis* promoter. One group of mutants (C330Y, R366H, D396N, H377Y, and R394W) contained changes in the zinc finger region, and other mutants (S273A, F154S, and P180S) had changes outside that region. The most common DDS mutants (D396N, R366H, H377Y, and R394W) failed to synergistically activate the *Mis* promoter with SOX9 or SF1 (Fig. 4, A and B). In contrast, WT1 with a mutation outside the zinc finger region (S273A, P180S, and F154S) had a cotransactivational potential similar to that of wild-type WT1. Another mutant (C330Y) retained some transactivation ability even though its mutation was in the zinc finger region. This mutant behaves similarly in the activation of the SRY promoter [21]. Western blot data showed that all the mutant proteins were expressed at similar levels (Fig. 4C).

The SOX9-binding site is required for *Mis* gene activation in vivo [8]. Although the SF1-binding site is essential for the activation of the *Mis* promoter in vitro [12], mutation in this binding site decreased expression of the MIS, but mullerian ducts regressed normally [8]. Nachtigal et al. [12] have shown that WT1 can synergistically activate the *Mis* promoter with SF1. They also reported that WT1 DNA binding is not required for the activation of this promoter [12]. However, most patients with DDS are pseudohermaphrodites because of a mutation in the DNA-binding domain of WT1 [20], and mutations within the zinc finger DNA-binding region abrogate transactivation of the *Mis* promoter by WT1, indicating that at least some WT1 DNA binding is required to activate the *Mis* promoter (Fig. 4, A and B).

To resolve this apparent contradiction, we re-examined whether DNA binding of WT1 is essential. We identified the region responsible for WT1-mediated activation of this promoter. Because the *Mis* promoter is quite small, we used a site-directed mutagenesis approach to identify the WT1 DNA-binding site in the *Mis* promoter. No consensus WT1 DNA-binding site was found in the 180-bp *Mis* promoter (Fig. 5A). However, one GC-rich region (caggcccagggccac) was similar to the WT1 consensus site near the transcription start site of the *Mis* gene. To test the functional significance of this site, we introduced mutations at several positions (Fig. 5B), and these constructs were assayed for their responsiveness to WT1 in HeLa cells and compared with the wild-type construct (−180MISP). A single A-to-G substitution in the second or 14th position did not significantly reduce reporter gene activity. An A-to-G substitution at both those positions reduced reporter gene activity by 50%, and replacement of the A at the eighth position with a G reduced reporter gene activity by 40%. Substitution of all three As with Gs almost completely blocked transactivation by WT1. Because this putative WT1-binding site was very close to the transcription initiation site, it is possible that it interfered with the basal promoter activity. However, that was not the case, because these base substitutions did not affect the activation by SOX9 and SF1 (Fig. 6).

The WT1-responsive sequence was further characterized by gel shift assays. The gel shift assay in Figure 6 shows that WT1 synthesized in vitro bound to a ^{32}P-labeled, 16-bp, WT1-binding site oligonucleotide. Binding was competed by unlabeled wild-type probe (Fig. 7, lanes 4 and 5). The mutants, which showed complete (M5), partial (M2 and M4), or no reduction (M1 and M3) in reporter gene activity, also competed accordingly with the wild-type probe (Fig. 7, lanes 6–10). A WT1 N-terminal-specific antibody (Fig. 7, lane 11) substantially reduced binding of the

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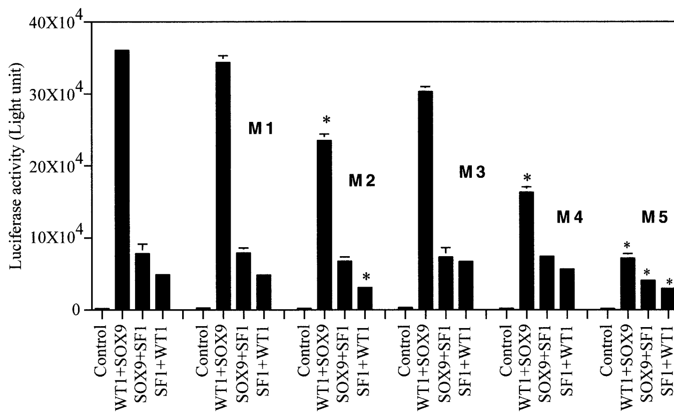

FIG. 6. WT1-binding site in the −180-bp Mis promoter. HeLa cells were transfected with 0.1 μg of reporter gene and different combinations of WT1, SOX9, and SF1 expression vectors or 0.2 μg of pCB6+ as a control. The CMV promoter-driven β-galactosidase construct (10 ng) was cotransfected to control for differences in the transfection efficiencies. All results are expressed as the mean ± SD of at least three experiments. Asterisks indicate values significantly different from corresponding wild-type values.

specific WT1-DNA complex. This binding site was further characterized by gel shift assays (Fig. 8). Two overlapping binding sites are found in the *Mis* promoter. The WT1 can bind to the half-binding site (cagggccac) in the *Mis* promoter (Fig. 8, lane 2) but can compete with wild-type probe (Fig. 8, lane 4). The IVT WT1 did not bind to mutant binding sites (M4 and M5) and also showed loss of cotransactivational properties in reporter gene assays (Fig. 6). However, the mutants M1, M2, and M3 showed some degree of to full reporter gene activity by WT1 in the presence of SF1 or SOX9, and WT1 was also able to bind in these DNA sequences (Fig. 8). These data suggest that the WT1-binding sequence we identified in the *Mis* promoter is required for binding of WT1 to the *Mis* promoter and for WT1-mediated transactivation of the *Mis* promoter.

To explore the mechanism of DDS mutants, the DNA-binding abilities of DDS mutants were assessed by gel shift assays. Mutations in the DNA-binding region of WT1 caused loss of DNA-binding ability (Fig. 9, lanes 5–9 and 11) and concomitant loss of transactivational properties. Although mutant D396N retained some of its DNA-binding ability (Fig. 9, lane 11), it showed loss of transactivational activity (Fig. 4, A and B). However, the mutant C330Y, which has a mutation in the zinc finger DNA-binding region, retained some cotransactivational ability as well as DNA-binding ability (Fig. 9, lane 5). This implies that the cysteine in codon 330 in WT1 is not so crucial for DNA binding, although the mutant did lose some of its activity. On the other hand, mutations outside the DNA-binding region did not have major effects on DNA binding to the *Mis* promoter (Fig. 9, lanes 4, 9, and 10). Interestingly, mutations outside the zinc finger region of WT1 are rarely seen in patients with DDS. These data clearly indicate that some of the DDS phenotype arises from the loss of DNA-binding ability of WT1 because of the mutations in the zinc finger region.

To address more fully the possibility of WT1-mediated regulation of *Mis* gene expression, we used the chromatin immunoprecipitation assay to determine whether WT1 bound the endogenous *Mis* promoter. We choose the TM4 cell line, because it expresses a significant amount of endogenous WT1 (Fig. 10A) and Mis (data not shown). Anal-

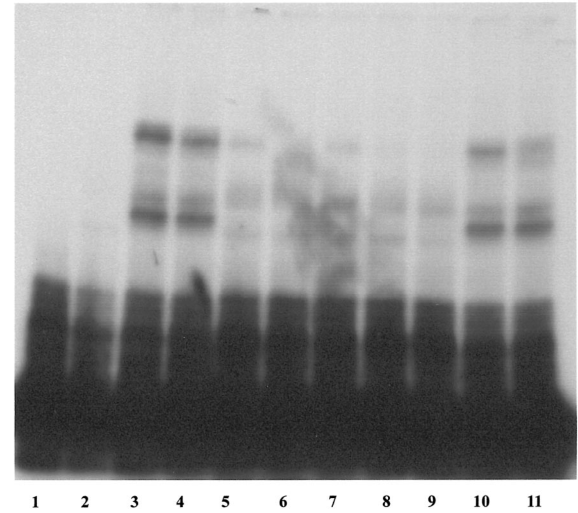

FIG. 7. WT1 binding to WT1-binding site (WTS). Gel mobility shift assays were performed with the radiolabeled WT1-binding site from the *Mis* promoter, WTS (caggcccagggccac), and incubated with 5 μl of IVT WT1. The WT1-DNA complexes were competed with a 50- or 100-fold excess of wild-type unlabeled probe and a 100-fold excess of mutant probe. Anti-WT1 antibody (2.0 μg) was used to supershift the specific WT1-DNA complex. The arrow indicates the specific WT1-DNA complexes.

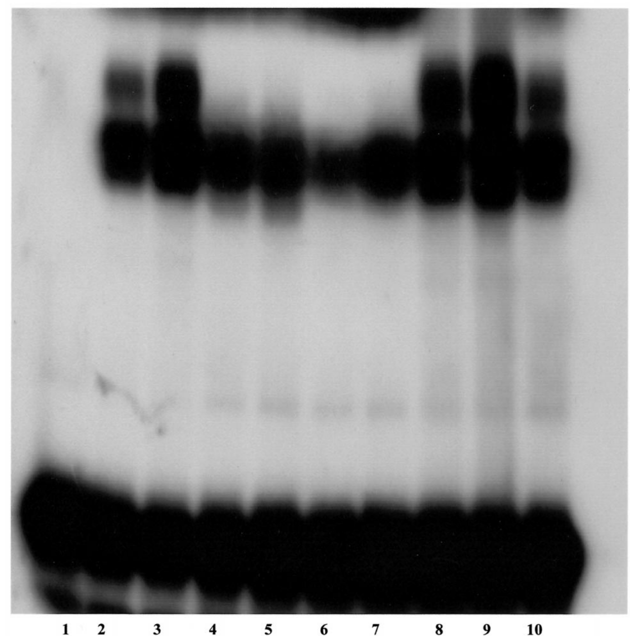

FIG. 8. Characterization of the WT1-binding site (WTS) in the *Mis* promoter by gel shift assays. Gel mobility shift assays were performed with radiolabeled WT1-binding elements: WTS (caggcccagggccac), half-site WTS1/2 (cagggccac), and its mutant derivatives and incubated with IVT WT1. The amount of IVT WT1 proteins used was 5 μl. The arrow indicates the WT1-DNA complexes.

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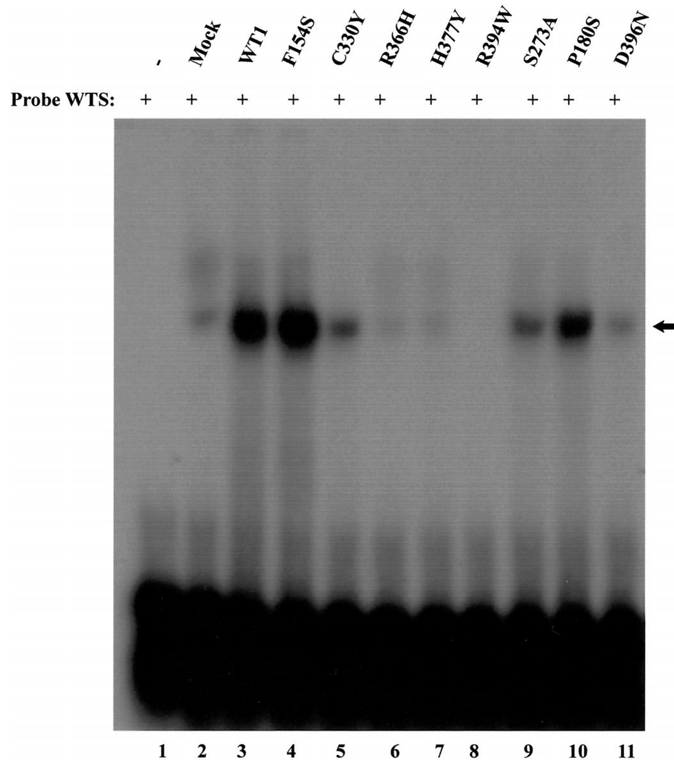

FIG. 9. Mutations in the zinc finger region abolished the DNA binding of WT1. Gel mobility shift assays were performed with radiolabeled WT1-binding sequence in the *Mis* promoter, WTS (caggcccagggccac), and incubated with IVT WT1 and its point mutants. The amount of IVT WT1 proteins used was 5 μl. The arrow indicates specific WT1-DNA complexes.

ysis by PCR of formaldehyde cross-linked chromatin from TM4 cells immunoprecipitated with antibodies specific to the C-terminal region of WT1 revealed that WT1 bound to the *Mis* promoter sequences (220-bp band) (Fig. 10B), whereas rabbit IgG antibody controls did not. As a negative control, PCR was done using the Pax6-specific primers to show that WT1 antibody specifically immunoprecipitated only the Mis promoter-bound Wt1-DNA complex and not the nonspecific genomic DNA (Fig. 10B).

DISCUSSION

WT1 plays an important role in sex determination and differentiation. One of the early steps in sex differentiation is the up-regulation of MIS expression in Sertoli cells, which results in regression of the mullerian duct. All the regulatory elements required for MIS expression during sex differentiation are located within a relatively small, 180-nucleotide promoter fragment [24]. At least four factors (SOX9, SF1, WT1, and GATA-4) have been implicated in regulation of the *Mis* promoter [10–12]. In the present study, we show by gel shift and chromatin immunoprecipitation assays that WT1 bound to a GC-rich sequence in the murine Mis promoter. Mutation in this site abolished WT1-mediated activation of the Mis promoter. The WT1, SOX9, and SF1 could synergistically activate the Mis promoter, and at least two factors were necessary for minimal activation.

We were unable to detect an interaction between WT1 and SOX9 by coimmunoprecipitation and the yeast two-hybrid system. It is possible that WT1 and SOX9 and SF1 do not interact directly, although they bind in very close

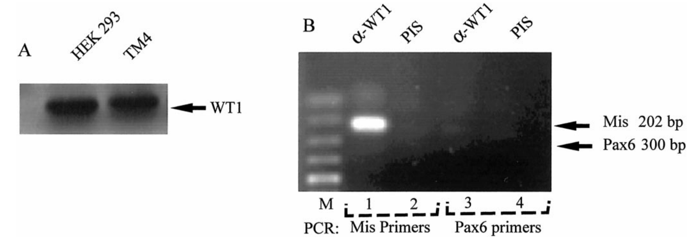

FIG. 10. WT1 bound the *Mis* promoter in vivo in TM4 cells. **A**) Detection of endogenous Wt1 in TM4 cell line by Western blot analysis using N-terminal-specific anti-WT1 antibody. We used HEK 293 cell extracts as a positive control. **B**) Cross-linked chromatin from TM4 cells was incubated with antibodies to the N-terminal region of WT1. The immunoprecipitated DNA was analyzed by PCR for the primers specific to *Mis* promoter sequences: 220-bp band (lanes 1 and 2). As a negative control, PCR was run with Pax6-specific primers using the same DNA obtained from immunoprecipitation with WT1-specific antibody and with rabbit IgG (PIS; lanes 3 and 4, respectively). No Pax6-specific band is expected at 300 bp. The first lane contains a 100-bp DNA ladder (M).

proximity on the *Mis* promoter. Alternatively, WT1 may bind to another, yet-to-be-identified cofactor, and only that complex may associate with SOX9 and regulate the *Mis* promoter. The WT1-binding site in the *Mis* promoter is between the TATA box and the transcription start site. The WT1 may help to form the transcription initiation complex in the *Mis* promoter, and SOX9 may just allow WT1 to bind to its cognate binding site. Further investigation is needed to determine which of these hypotheses is correct.

The role of WT1 in sex determination and differentiation is multiple in both mice and humans. The WT1 plays many roles in each step of this process. Different isoforms of WT1 also have very different roles in the process. It has been shown recently that WT1-KTS isoforms also activate the SF1 promoter [25]. Different isoforms of WT1 are also involved in regulation of SRY, DAX1, and MIS [12, 16, 21, 26]. These observations indicate the importance of isoforms of WT1 in different stages of mammalian sex determination and differentiation. Involvement of the WT1(−KTS) isoforms in up-regulation of the SRY promoter [21] and of the WT1(+KTS) isoform in stabilization of the SRY transcript is linked to sex reversal in patients with DDS [16]. This syndrome has variable phenotypes, occurs only in men, and is most likely caused by the haploinsufficiency of WT1. The MIS as well as androgen receptors may be deregulated for this phenotype in patients with DDS. The MIS gene is a good candidate gene for this phenotype in patients with this syndrome, both because WT1 is expressed in Sertoli cells during the production of the MIS and because its promoter is regulated by WT1 [12, 27]. We propose here that *Mis* meets all the criteria of a true WT1 target gene. Expression of the Mis in Sertoli cells during mullerian duct regression is closely correlated with WT1 expression [27]. The *Mis* promoter is directly regulated by WT1 with SOX9 and SF1 [12; present study]. The WT1 must bind to DNA to regulate the Mis promoter. Our chromatin immunoprecipitation studies showed that WT1 directly bound to the *Mis* promoter in vivo. Therefore, we conclude that deregulated *MIS* expression because of the mutation in WT1 may cause the pseudohermaphroditism in patients with DDS.

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REFERENCES

1. Josso N, Picard JY, Tran D. The anti-mullerian hormone. Birth Defects Orig Artic Ser 1977; 13:59–84.
2. Cate RL, Mattaliano RJ, Hession C, Tizard R, Farber NM, Cheung A, Ninfa EG, Frey AZ, Gash DJ, Chow EP, Fisher RA, Bertonis JM, Torres G, Wallner BP, Ramachandran KL, Ragin RC, Manganaro TF, MacLaughlin DT, Donahoe PK. Isolation of the bovine and human genes for mullerian-inhibiting substance and expression of the human gene in animal cells. Cell 1986; 45:685–698.
3. Teixeira J, Donahoe PK. Molecular biology of MIS and its receptors. J Androl 1996; 17:336–341.
4. Roberts LM, Hirokawa Y, Nachtigal MW, Ingraham HA. Paracrine-mediated apoptosis in reproductive tract development. Dev Biol 1999; 208:110–122.
5. Imbeaud S, Faure E, Lamarre I, Mattei MG, di Clemente N, Tizard R, Carre-Eusebe D, Belville C, Tragethon L, Tonkin C, Nelson J, McAuliffe M, Bidart J, Lababidi A, Josso N, Cate RL, Picard JY. Insensitivity to antimullerian hormone due to a mutation in the human anti-mullerian hormone receptor. Nat Genet 1995; 11:382–388.
6. Mishina Y, Rey R, Finegold MJ, Matzuk MM, Josso N, Cate RL, Behringer RR. Genetic analysis of the mullerian-inhibiting substance signal transduction pathway in mammalian sexual differentiation. Genes Dev 1996; 10:2577–2587.
7. Behringer RR, Finegold MJ, Cate RL. Mullerian-inhibiting substance function during mammalian sexual development. Cell 1994; 79:415–425.
8. Arango NA, Lovell-Badge R, Behringer RR. Targeted mutagenesis of the endogenous mouse Mis gene promoter: in vivo definition of genetic pathways of vertebrate sexual development. Cell 1999; 99:409–419.
9. Munsterberg A, Lovell-Badge R. Expression of the mouse anti-mullerian hormone gene suggests a role in both male and female sexual differentiation. Development 1991; 113:613–624.
10. Tremblay JJ, Viger RS. Transcription factor GATA-4 enhances mullerian inhibiting substance gene transcription through a direct interaction with the nuclear receptor SF-1. Mol Endocrinol 1999; 13:1388–1401.
11. De Santa Barbara P, Bonneaud N, Boizet B, Desclozeaux M, Moniot B, Sudbeck P, Scherer G, Poulat F, Berta P. Direct interaction of SRY-related protein SOX9 and steroidogenic factor 1 regulates transcription of the human anti-mullerian hormone gene. Mol Cell Biol 1998; 18: 6653–6665.
12. Nachtigal MW, Hirokawa Y, Enyeart-Van Houten DL, Flanagan JN, Hammer GD, Ingraham HA. Wilms' tumor 1 and Dax-1 modulate the orphan nuclear receptor SF-1 in sex-specific gene expression. Cell 1998; 93:445–454.
13. Rauscher FJ III, Morris JF, Tournay OE, Cook DM, Curran T. Binding of the Wilms' tumor locus zinc finger protein to the EGR-1 consensus sequence. Science 1990; 250:1259–1262.
14. Wang ZY, Qiu QQ, Deuel TF. The Wilms' tumor gene product WT1 activates or suppresses transcription through separate functional domains. J Biol Chem 1993; 268:9172–9175.
15. Haber DA, Sohn RL, Buckler AJ, Pelletier J, Call KM, Housman DE. Alternative splicing and genomic structure of the Wilms tumor gene WT1. Proc Natl Acad Sci U S A 1991; 88:9618–9622.
16. Hammes A, Guo JK, Lutsch G, Leheste JR, Landrock D, Ziegler U, Gubler MC, Schedl A. Two splice variants of the Wilms' tumor 1 gene have distinct functions during sex determination and nephron formation. Cell 2001; 106:319–329.
17. Natoli TA, McDonald A, Alberta JA, Taglienti ME, Housman DE, Kreidberg JA. A mammal-specific exon of WT1 is not required for development or fertility. Mol Cell Biol 2002; 22:4433–4438.
18. Pelletier J, Bruening W, Kashtan CE, Mauer SM, Manivel JC, Striegel JE, Houghton DC, Junien C, Habib R, Fouser L, Fine RN, Silverman JL, Haber DA, Housman D. Germline mutations in the Wilms' tumor suppressor gene are associated with abnormal urogenital development in Denys-Drash syndrome. Cell 1991; 67:437–447.
19. Barbaux S, Niaudet P, Gubler MC, Grunfeld JP, Jaubert F, Kuttenn F, Fekete CN, Souleyreau-Therville N, Thibaud E, Fellous M, McElreavey K. Donor splice-site mutations in WT1 are responsible for Frasier syndrome. Nat Genet 1997; 17:467–470.
20. Little M, Wells C. A clinical overview of WT1 gene mutations. Hum Mutat 1997; 9:209–225.
21. Hossain A, Saunders GF. The human sex-determining gene SRY is a direct target of WT1. J Biol Chem 2001; 276:16817–16823.
22. Lefebvre V, Huang W, Harley VR, Goodfellow PN, de Crombrugghe B. SOX9 is a potent activator of the chondrocyte-specific enhancer of the pro alpha1(II) collagen gene. Mol Cell Biol 1997; 17:2336–2346.
23. Davies RC, Calvio C, Bratt E, Larsson SH, Lamond AI, Hastie ND. WT1 interacts with the splicing factor U2AF65 in an isoform-dependent manner and can be incorporated into spliceosomes. Genes Dev 1998; 12:3217–3225.
24. Shen WH, Moore CC, Ikeda Y, Parker KL, Ingraham HA. Nuclear receptor steroidogenic factor 1 regulates the mullerian inhibiting substance gene: a link to the sex determination cascade. Cell 1994; 77: 651–661.
25. Wilhelm D, Englert C. The Wilms tumor suppressor WT1 regulates early gonad development by activation of Sf1. Genes Dev 2002; 16: 1839–1851.
26. Kim J, Prawitt D, Bardeesy N, Torban E, Vicaner C, Goodyer P, Zabel B, Pelletier J. The Wilms' tumor suppressor gene (wt1) product regulates Dax-1 gene expression during gonadal differentiation. Mol Cell Biol 1999; 19:2289–2299.
27. De Santa Barbara P, Moniot B, Poulat F, Berta P. Expression and subcellular localization of SF-1, SOX9, WT1, and AMH proteins during early human testicular development. Dev Dyn 2000; 217:293–298.

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