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Wilms' Tumor 1 and Dax-1 Modulate the Orphan Nuclear Receptor SF-1 in Sex-Specific Gene Expression

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Summary

Products of steroidogenic factor 1 (SF-1) and Wilms' tumor 1 (*WT1*) genes are essential for mammalian gonadogenesis prior to sexual differentiation. In males, SF-1 participates in sexual development by regulating expression of the polypeptide hormone Müllerian inhibiting substance (MIS). Here, we show that WT1 −KTS isoforms associate and synergize with SF-1 to promote *MIS* expression. In contrast, *WT1* missense mutations, associated with male pseudohermaphroditism in Denys-Drash syndrome, fail to synergize with SF-1. Additionally, the X-linked, candidate dosage-sensitive sex-reversal gene, *Dax-1*, antagonizes synergy between SF-1 and WT1, most likely through a direct interaction with SF-1. We propose that WT1 and Dax-1 functionally oppose each other in testis development by modulating SF-1–mediated transactivation.

Introduction

To date, two related genes, *Sry* and *Sox9* have been identified to direct or participate in testis determination (Koopman et al., 1990; Sinclair et al., 1990; Foster et al., 1994; Wagner et al., 1994; Kent et al., 1996). Both of these putative transcription factors are expressed in the testis, but not the ovary, and must coordinate differentiation of Sertoli cells in the embryonic testes. Subsequently, Sertoli-cell expression of *MIS* regulates secondary sexual development in males by triggering the regression of the Müllerian duct, the anlagen of the female reproductive tract. Female-specific genes that direct ovarian development have yet to be identified, although one candidate is the X-linked gene, *DAX-1*, encoding an orphan nuclear receptor (Zanaria et al., 1994). *DAX-1* is located on the short arm of the X chromosome within the dosage-sensitive sex-reversal (*DSS*) locus. Duplication of the *DSS* locus (Xp21.2-p21.3) in males overrides the male program of development, resulting in XY sex reversal (Muscatelli et al., 1994; Zanaria et al., 1994).

An obligatory gene for mammalian gonadogenesis is the autosomal *Ftz-f1* gene encoding the orphan nuclear receptor SF-1 (for review, Parker and Schimmer, 1997). Mice homozygous for a SF-1 null mutation display a lack of gonadal and adrenal development (Luo et al., 1994; Sadovsky et al., 1995; Shinoda et al., 1995), a loss of pituitary gonadotropins (Ingraham et al., 1994), and altered structural characteristics of the ventromedial hypothalamus (Ikeda et al., 1995). These data demonstrate the essential role of SF-1 in the development of the hypothalamic-pituitary-gonadal axis. The sexually dimorphic expression pattern exhibited by SF-1 (Hatano et al., 1994; Shen et al., 1994) and the role of SF-1 in regulating *MIS* and testosterone synthesis provide strong evidence that this orphan nuclear receptor mediates male-specific gene expression. Indeed, transgenic studies using the proximal *MIS* promoter established that the SF-1–binding site (MIS-RE-1) is required for the sex-dependent onset and maintenance of *MIS* expression in gonadal development. Mutations within the core SF-1–binding site abolish *MIS* promoter activity in vivo and abrogate SF-1 binding in vitro (Shen et al., 1994; Giuili et al., 1997). Because SF-1 is essential for the development of multiple endocrine tissues, mechanisms must exist to restrict expression of SF-1 target genes, such as *MIS*, to the appropriate cell types.

A second gene essential for mammalian gonadal and kidney development is *WT1*, which encodes a zinc-finger transcription factor (Pritchard-Jones et al., 1990; Pelletier et al., 1991a; Kreidberg et al., 1993). Mutations in *WT1* are associated with childhood tumors of the kidney, thus supporting *WT1* as a tumor suppressor gene (Call et al., 1990; Gessler et al., 1990). *WT1* mutations are also associated with three pediatric syndromes: WAGR (**W**ilms' tumor, **A**niridia, **G**enitourinary abnormalities, and mental **R**etardation), Denys-Drash syndrome (DDS), and Fraiser syndrome (Little and Wells, 1997). One prominent feature shared by these distinct syndromes is a high incidence of urogenital defects. Although defects in gonadal and reproductive tract development can vary widely in these patients, most 46,XX females appear normal, while the majority of 46,XY individuals exhibit ambiguous genitalia or male pseudohermaphroditism.

In over sixty percent of DDS patients, de novo germ line *WT1* missense mutations cluster in exon 9, which encodes the third zinc finger (Reddy and Licht, 1996; Little and Wells, 1997). Recreation of DDS WT1 mutations in vitro demonstrates a loss of DNA binding to candidate target elements and an altered subnuclear localization corresponding to the splicing apparatus (Pelletier et al., 1991a; Englert et al., 1995; Larsson et al., 1995). It is proposed that a dominant-negative mechanism accounts for the DDS phenotype due to competition between DDS mutant and wild-type WT1 proteins for homodimerization (Pelletier et al., 1991a). Understanding how the WT1 protein mediates urogenital development has been hampered because clear transcriptional targets have yet to be identified. Although both *SF-1* and *Wt1* are essential for gonadogenesis, the dissimilar phenotypes displayed by mice with *Wt1* versus *SF-1* gene disruptions imply that these two genes have distinct roles in development. Thus far, there has been no evidence to suggest that these two genes are positioned in series within a genetic cascade (Parker and Schimmer, 1997).

We have focused on the molecular mechanisms that

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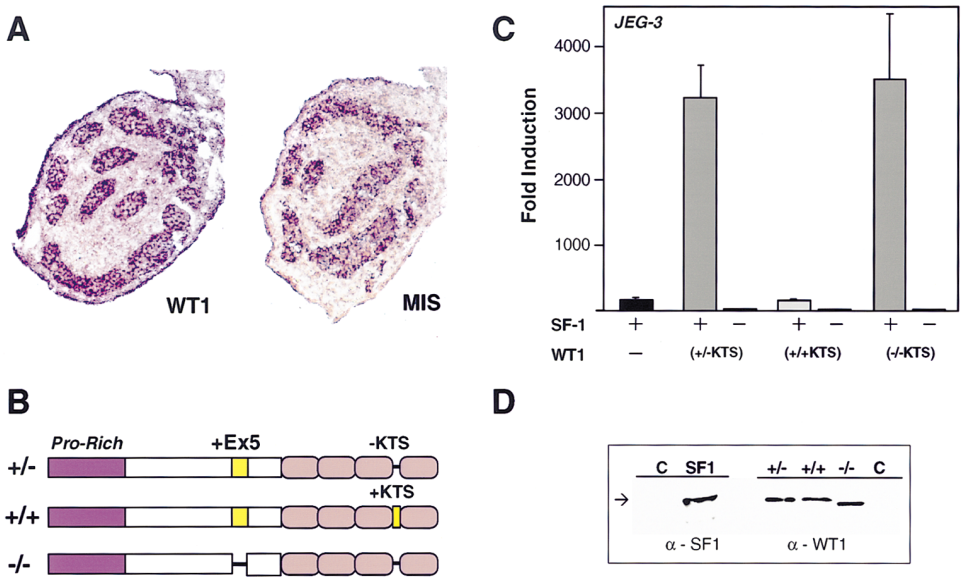

Figure 1. SF-1 and WT1 Act Synergistically To Regulate *MIS*

(A) Nonradioactive in situ hybridization demonstrates colocalization of WT1 and MIS signals to the developing seminiferous tubules in E15.5 rat testes.

(B) WT1 isoforms. WT1 products result from alternative splicing at two sites; + or − indicates inclusion or exclusion of one or both sites, respectively. Ex5: exon 5, KTS: Lys-Thr-Ser.

(C) JEG-3 cells were transiently transfected with the *MIS*-luciferase reporter construct (2X-MIS-RE; 0.3 μg) in the absence or presence of 0.1 μg SF-1, 0.5 μg WT1, or a combination of SF-1 and WT1 isoforms. Luciferase activity was measured in relative light units (RLU) and is reported as fold induction above background activity produced by 2X-MIS-RE alone. The average potentiation of SF-1 activation by WT1 −KTS was determined to be 21 ± 2.3 on the 2X-MIS-RE reporter for at least nine independent experiments.

(D) Western blot analysis of extracts from transfected JEG-3 cells is shown for SF-1, WT1 isoforms (+/−, +/+, −/−), and the pcDNA3 control vector (C) using anti-SF-1 (1:5000 dilution; K. Morohashi), and anti-WT1 (1:4000 dilution; C-19, Santa Cruz Biotechnology).

control male-specific gene expression of *MIS* given its pivotal position downstream of *SRY* in the mammalian sex determination pathway. Because expression of SF-1 is far wider than that of *MIS*, we initiated a search for a cofactor that might act in concert with SF-1 to direct Sertoli cell–specific expression of *MIS*. We predicted that this cofactor may also be present in the postnatal granulosa cell where female expression of *MIS* is observed. Here, we report that the *WT1* gene product interacts with SF-1 and markedly increases expression of *MIS*-reporter constructs. Our results suggest that SF-1 achieves cell-specific gene expression by recruiting a factor (WT1) unrelated to the nuclear receptor superfamily. Because of the potential for DAX-1 to repress male-specific genes, we also investigated the role of Dax-1 to influence the activity of SF-1 and WT1 in our in vitro system. We provide molecular evidence that the dosage of *Dax-1*, as well as *WT1*, are important for male sexual development. Based on our findings, we present a model whereby Dax-1 and WT1 oppose each other to affect SF-1–mediated transactivation of male-specific genes.

Results

Synergistic Activity of Specific WT1 Isoforms with SF-1

In the adult gonad, WT1 expression is restricted to both Sertoli and granulosa cells (Pelletier et al., 1991b) and localizes to the basal epithelium of the seminiferous tubule, suggesting strongly that WT1 is expressed selectively in Sertoli cells (Larsson et al., 1995). WT1 is also expressed in the embryonic Sertoli or pre-Sertoli cell where it colocalizes with MIS transcripts in E15.5 testis (Figure 1A). Four major isoforms of WT1 are generated by alternative splicing, all of which are expressed in WT1-positive tissues. Alternative splicing of exon 5 results in a 17 amino acid insertion, and alternative splicing of exon 9 produces an insertion of Lys-Thr-Ser (+KTS) between the third and fourth zinc finger. The three WT1 isoforms used in this study are depicted in Figure 1B.

Given the Sertoli cell expression of *WT1* in the embryonic testis, we set out to test whether WT1 would influence *MIS* expression by itself or in combination with the orphan nuclear receptor, SF-1. We chose to use the human placental cell line, JEG-3, for our studies because this cell line is null for both SF-1 and WT1 expression (Figure 1D), and SF-1 will activate known target response elements in transient transfection assays (Ito et al., 1997). A *MIS*-reporter construct containing two MIS-RE-1 elements (2X-MIS-RE) was activated robustly following cotransfection with SF-1. By contrast, WT1 was unable to activate the *MIS*-reporter construct (1- to 3-fold above background; Figure 1C). However, cotransfection of SF-1 and the WT1 −KTS isoforms in JEG-3 cells resulted in synergistic *MIS*-reporter expression to levels reaching 4000-fold above background (Figure 1C). Surprisingly, the +KTS isoform failed to synergize with SF-1. The lack

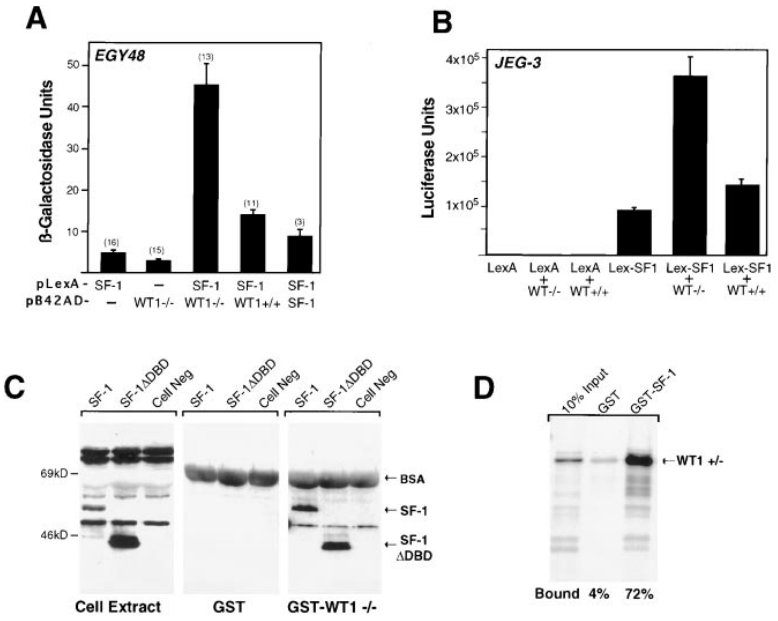

Figure 2. Direct Interaction of SF-1 and WT1

(A) The yeast two-hybrid assay was used to infer a protein–protein interaction between SF-1 and WT1 in strain EGY48. Fusion proteins were generated between the LexA-DNA binding domain and SF-1 (pLexA−), and the B42 activation domain fused with WT1 or SF-1 (pB42AD−). β-galactosidase activity was measured for combinations of experimental or control plasmids (−), as indicated; the standard error of the mean is shown by the error bars. The number of independent transformants measured is indicated in parentheses.

(B) Modified mammalian two-hybrid assay. JEG-3 cells were cotransfected with the 2xLexA-luciferase reporter and either the LexA-DBD or the LexA-SF-1 fusion protein and pcDNA3.WT1 (−/− or +/+).

(C) HA epitope–tagged SF-1 proteins (SF-1, SF-1ΔDBD) were visualized from COS whole-cell extracts by Western analysis (anti-HA IgG, 1:1000 dilution) after transfection of SF-1 expression plasmids. Signals from 6% of the whole-cell extract are shown. Associated proteins bound to GST or GST-WT1 −/− affinity matrix are shown; SF-1 proteins are only observed when incubated with GST-WT1 as indicated. Cross reactivity of the anti-HA antibody with BSA is observed in all samples.

(D) Radiolabeled in vitro translated WT1 +/− protein was incubated with GST alone or a GST-SF-1 fusion protein, as described in Experimental Procedures. Phosphorimager analysis of the data revealed the percent of WT1 +/− bound to the GST or GST-SF-1 fusion protein, respectively. Ten percent of the IVT product added to each reaction is shown (10% Input).

of activity of the WT1 +KTS isoform was not due to differences in the protein expression levels as judged by Western blot analysis (Figure 1D). Thus, our functional data show that specific WT1 −KTS isoforms synergize with SF-1 to activate *MIS*-reporter genes in vitro.

Direct Interaction between SF-1 and WT1

To determine whether direct interaction between SF-1 and WT1 accounted for the functional synergism observed, we looked for evidence of physical association between these two proteins. Indeed, when SF-1 and WT1 −/− were coexpressed in a yeast two-hybrid LexA-B42 system, significant β-galactosidase activity was observed inferring a physical interaction (Figure 2A). By comparison, interaction was reduced if SF-1 was expressed with the WT1 +/+ isoform. Levels of WT1 fusion proteins were similar for all constructs as judged by Western blot analysis (data not shown). Our findings in the yeast two-hybrid system were confirmed using two other independent systems. First, in a modified mammalian two-hybrid experiment, we observed that WT1 −/− could augment SF-1–mediated transactivation, whereas the WT1 +/+ isoform was less active (Figure 2B). Second, SF-1 proteins expressed in COS cells were selectively bound to a GST-WT1 affinity matrix (Figure 2C). Similar results were obtained with in vitro translated (IVT) WT1 and a GST-SF-1 fusion protein (Figure 2D). We note that removal of the SF-1 DNA–binding domain (DBD) diminishes binding of GST-WT1, suggesting that this region may contain crucial residues for a high affinity SF-1/WT1 association.

Synergistic Activation by WT1 is Specific to the Conserved 25 bp MIS-RE1 Element

To determine whether a DNA component was required for the WT1/SF-1 synergy, we tested whether WT1 would bind to the MIS-RE-1 site used in transfection studies. This 25 bp element contains the core SF-1–binding site and additional upstream sequences that are highly conserved across several species. Although this element is GC rich, it does not resemble any of the consensus binding sites defined for recombinant WT1 protein (Reddy and Licht, 1996). While SF-1 was found to bind with high affinity to the MIS-RE-1, a WT1/DNA complex was not observed on this element, nor was the SF-1-DNA complex affected by WT1 protein (Figure 3A). Furthermore, an obvious ternary complex was not observed. On the other hand, IVT WT1 proteins were found capable of binding a known WT1 consensus site, the WTE. In this case, two visible but weak DNA–protein complexes were observed for all −KTS isoforms, including a mutant form of the WT1 protein that was truncated at the amino terminus (ΔN; Figure 3A).

To determine whether WT1 would increase the activation of another SF-1 target gene, we tested the effects of WT1 on wild-type and mutant MIS-RE1 site(s) and the steroidogenic enzyme P450-21-hydroxylase (21-OH) element; all elements were fused to the same minimal promoter. SF-1 is known to bind as a monomer and with high affinity. Only the core hexamer half-ERE consensus site of "AGGTCA" is shared by the core SF-1–binding site of the MIS and 21-OH promoters, (C**C**AAGGTCA and C**A**AAGGTCA, respectively). In contrast to either the MIS-RE-1 or MIS-M1 reporter, altering the sequence

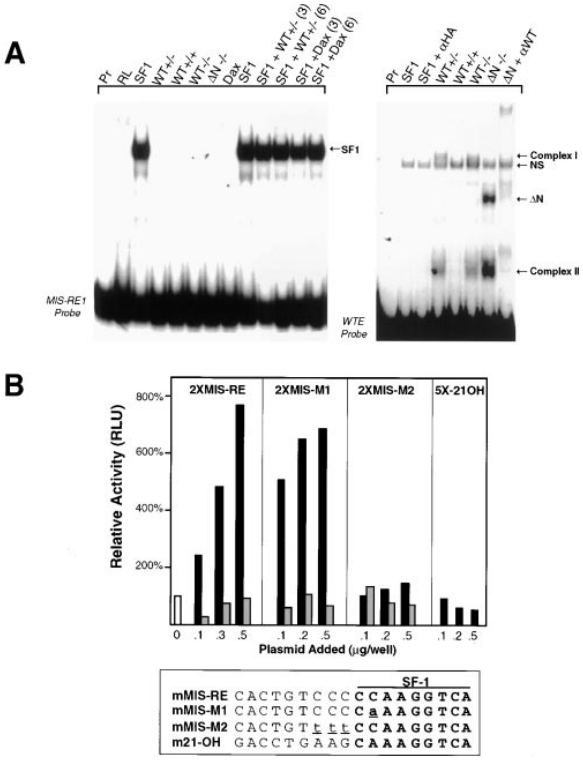

Figure 3. WT1/SF-1 Synergy Requires a DNA Component

(A) Gel mobility shift assays were performed with radiolabeled MIS-RE1 or a WT1 response element (WTE; core WT1 site, GCG TGGGAGT; Nakagama et al. 1995) and incubated with IVT proteins. The SF-1–DNA complex, control reactions with probe alone (Pr), and unreacted reticulocyte lysate (RL) are shown. The amounts of IVT protein used were 1.5 μl of SF-1 and 3 μl of all other proteins, except as indicated (6 μl). Two major WT1 protein–DNA complexes are shown (I and II) for full-length and truncated (ΔN) −KTS isoforms. The WT1/DNA complexes were supershifted using an antibody to WT1 (ΔN + αWT). A nonspecific protein–DNA interaction contributed by protein in the reticulocyte lysate is indicated (NS).

(B) JEG-3 cells were transiently transfected with luciferase reporter constructs containing two wild-type (2X-MIS-RE) or mutant (2X-MIS-M1, M2) MIS-RE-1 sites, or five P450 21-hydroxylase SF-1 response elements (5X-21OH) with 0.3 μg. Increasing amounts of WT1 +/− (black bars), WT1 +/+ (gray bars), or pcDNA3 plasmid (0.1–0.5 μg) were cotransfected with a constant level of pCMV–SF-1 (0.1 μg). The relative luciferase activity is shown as the percent change from SF-1 cotransfected with pcDNA3 (open bar). Sequence comparison of the wild-type MIS-RE1, mutant sites, and the mouse 21-OH is shown with the core SF-1–binding site indicated.

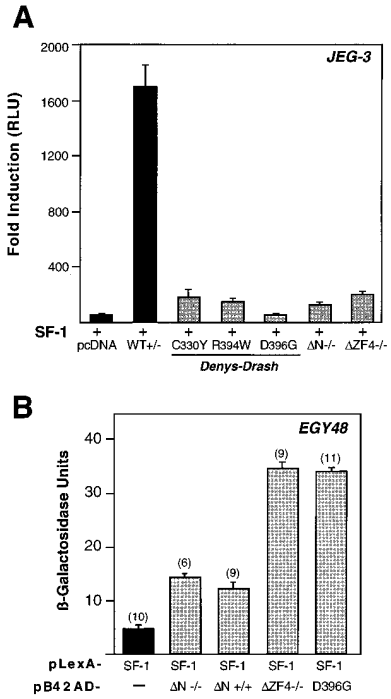

Figure 4. Denys-Drash WT1 Mutants Fail To Synergize with SF-1

(A) JEG-3 cells were transiently transfected with the 2X-MIS-RE reporter, SF-1, and WT1 −KTS isoforms, including point mutants of WT1 frequently occurring in Denys-Drash patients (C330Y: first zinc finger; R394W: third zinc finger; D396G: third zinc finger) and deletions of the N (ΔN: deletion aa 8-180) or C (ΔZF4: L398stop) terminus of WT1. Luciferase activity is reported as fold induction above background reporter activity alone. Expression of mutant proteins was comparable with wild-type WT1 (data not shown).

(B) Interactions between SF-1 and WT1 mutants tested in a yeast two-hybrid assay. β-galactosidase activity is shown for combinations of SF-1 and mutant WT1 fusion proteins (pB42AD−) consisting of an amino terminal truncation (ΔN), deletion of the fourth zinc finger (ΔZF4: L398stop), or a missense mutation in the third zinc finger (D396G). The number of independent transformants measured is indicated in parentheses.

upstream of this core SF-1–binding site abrogates the functional association between SF-1 and WT1 +/−, despite a 10- to 20-fold activation of these reporters by SF-1 (21-OH and MIS-M2, Figure 3B). Thus, a DNA component provided by the MIS-RE1 element is required for WT1 to potentiate SF-1 activation.

DDS WT1 Mutants Fail To Synergize with SF-1

Although the profile of patients diagnosed with DDS is complex and varied, most 46,XY DDS patients exhibit genital abnormalities, some with persistence of the Müllerian duct structures. Therefore, three of these mutations were recreated in the WT1 −KTS background and tested for their ability to potentiate SF-1 activity. In all cases, the mutant WT1 proteins failed to achieve the levels of *MIS* activation observed with the wild-type −KTS isoform (Figure 4A). Truncation of the N terminus (Δ8-180) as well as the fourth zinc finger (ΔZF4 −/−) also abolished activity; the Δ8-180 deletion includes most of the putative proline-glutamine transactivation and dimerization domains but does not eliminate nuclear localization of WT1 (Larsson et al., 1995). It should be noted that the three DDS mutants retained some ability to boost SF-1 activity (2- to 4-fold increase). As proposed for WT1 homodimerization, the proline-glutamine–rich region of WT1 also appears to be critical in mediating a SF-1/WT1 interaction, whereas the WT1 zinc-finger region is less critical (Figure 4B).

Dax-1 Interacts with SF-1 and Represses WT1/SF-1 Synergism

Like SF-1, the nuclear receptor Dax-1 is expressed in interstitial and Sertoli cells of the embryonic testis. The potential interaction between SF-1, Dax-1, and WT1 in the activation of *MIS* is especially relevant given the

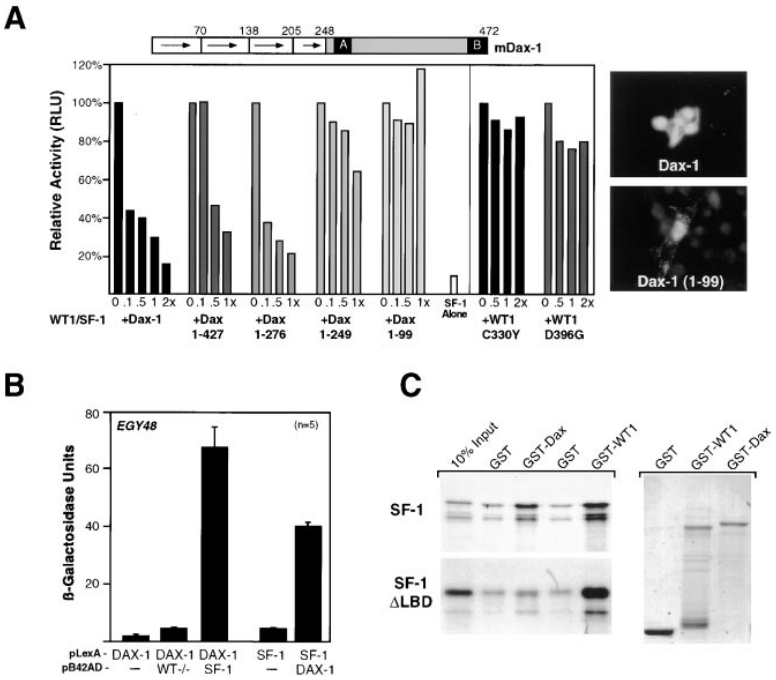

Figure 5. Dax-1 Interacts with SF-1 and Abrogates SF-1/WT1 Synergy

(A) JEG-3 cells were transiently transfected with the 2X-MIS-RE reporter, SF-1 (0.1 μg), WT1 +/− (0.3 μg), and increasing concentrations of full-length and mutant Dax-1 proteins (corresponding to 0.1–2× of SF-1) or WT1 isoforms (corresponding to 0.5–2x of the WT1 +/−). A schematic of Dax-1 depicts the boundaries of the cysteine repeats (arrows) and two putative silencing domains, A and B, as defined by Ito et al. (1997) and Lalli et al. (1997), with numbering of amino acids shown for mouse Dax-1. Luciferase activity is reported as expression relative to levels achieved by SF-1/WT1 synergy. Nuclear localization of FLAG epitope–tagged full-length Dax-1 and the most severely truncated Dax-1 mutant protein (1-99) was detected in transfected JEG-3 cells using an anti-FLAG antibody (KODAK; 1:3000 dilution).

(B) Dax-1 interacts with SF-1 in a yeast two-hybrid system. β-galactosidase activity is shown in yeast cotransformed with Dax-1 and SF-1 or WT1. Five independent transformants were measured for each combination.

(C) Radiolabeled IVT SF-1 or SF-1 lacking the putative ligand binding domain (SF-1ΔLBD) protein was incubated with GST alone, GST-WT1 −/−, or GST-Dax-1 fusion proteins. A Coomassie-stained gel shows the relative amount of each fusion protein used in this assay: right panel.

proposed role of DAX-1 in repressing male-specific genes (Zanaria et al., 1994). We explored the role of Dax-1 in modulating the activation of *MIS* by SF-1 and WT1. Marked reduction of *MIS* activation was observed if Dax-1 was cotransfected simultaneously with SF-1 and WT1 (Figure 5A). Truncating one of the putative inhibitory domains of Dax-1 failed to relieve this repression (Dax 1-276 and 1-427), although at low concentrations the magnitude of inhibition with Dax 1-427 was slightly less when compared to wild-type Dax-1 (Figure 5A). In contrast, removing both inhibitory domains (Dax 1-249) or the entire ligand-binding domain (LBD) and portions of the cysteine repeats (Dax 1-99) relieved repression without altering nuclear localization (Figure 5A). Cotransfection of Dax-1 and SF-1 in the absence of WT1 did not yield a similar inhibitory effect (data not shown). Because DDS *WT1* mutations have been proposed to act in a dominant-negative fashion, we also tested the ability of two DDS mutants to disrupt the WT1 synergistic function with SF-1. In contrast to Dax-1, increasing levels of DDS mutants at ratios of 1:1 or greater than wild type failed to reduce the synergistic activity of SF-1 and WT1 (Figure 5A).

Given this marked effect of Dax-1 on the SF-1/WT1 synergy, we investigated whether Dax-1 could interact with SF-1 and/or WT1. We noted previously that Dax-1 was unable to bind DNA and failed to form a visible Dax-1/SF-1 protein–protein complex on the MIS-RE-1 element (Figure 3A). However, in the yeast two-hybrid assay, a prominent association between SF-1 and Dax-1, but not WT1 and Dax, was detected (Figure 5B). Furthermore, a Dax-1/SF-1 interaction was noted in a GST pull-down experiment consistent with previously published data, although with a lower affinity than observed for a WT1/SF-1 interaction (Figure 5C; Ito et al. 1997). Interestingly, removal of the SF-1 ligand–binding domain abrogated its interaction with GST-Dax-1, but not with GST-WT1, suggesting that the major sites conferring interaction with Dax-1 and WT1 may be mediated by distinct regions on SF-1. Previous studies by others using human DAX-1 and mouse SF-1 have failed to detect a direct interaction in vivo, possibly due to the high degree of protein divergence between human and mouse Dax-1 (65% identity). The use of mouse Dax-1 may underlie our ability to detect such an interaction. In summary, our data suggest that Dax-1 does not bind the MIS-RE1 element, but can heterodimerize with SF-1, and represses the functional interaction between WT1 and SF-1.

Expression of WT1 Isoforms and Dax-1 in Gonadal Development

The dramatic functional difference between the +KTS and −KTS WT1 isoforms in mediating the SF-1/WT1 synergy and the repression of this synergy by Dax-1 led us to examine the relative transcript levels of these nuclear factors in sexual development. Whole-mount in situ hybridization analysis revealed that in contrast to the sexually dimorphic expression pattern of *SF-1*, *WT1* is equivalent in male and female E15.5 gonads (Figure 6A). Furthermore, RNAse protection analysis showed that at all developmental stages examined the relative levels of *WT1* (+KTS or −KTS) in the testis and ovary were not different. Both *WT1* isoforms are present in the embryonic kidney, as expected, and nearly absent

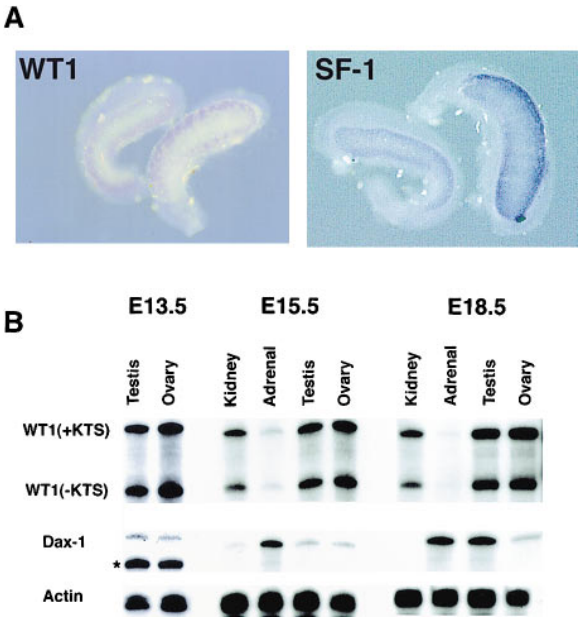

Figure 6. Levels of *SF-1*, *WT1*, and *Dax-1* in the Developing Gonad

(A) Whole-mount nonradioactive in situ hybridization of WT1 and SF-1 in the developing gonad. Genital ridges were harvested from embryonic rats at E15.5, fixed, and hybridized with probe as indicated. Female organs appear on the left and male organs on the right in each panel.

(B) Levels of *WT1* (−KTS and +KTS) and *Dax-1* transcripts from total RNA isolated from the embryonic gonads (minus the mesonephros) were examined by RNase protection analysis at three stages of embryonic development, E13.5, E15.5, and E18.5. Actin serves as an internal control for amount of total RNA. Signals shown for *WT1* and *Dax-1* represent equivalent exposure times at all stages. A longer exposure (10×) is also shown for *Dax-1* at E13.5 (asterisk).

in the adrenal gland (Figure 6B). Consistent with previous in situ analyses at early stages of sexual differentiation (Ikeda et al., 1996), we find that *Dax-1* levels are equivalent in the ovary and testes and are noted to be much lower than WT1 (E13.5 and E15.5, Figure 6B). *Dax-1* is also present transiently in early stages of kidney development (E15.5). Later in gonadal development, *Dax-1* is high in the testis and low in the ovary (E18.5, Figure 6B). Because *Dax-1* is expressed in interstitial Leydig cells (Ikeda et al., 1996) and in Sertoli cells (Tamai et al. 1996; J. N. F. and H. A. I., unpublished data), this increase in testicular *Dax-1* expression may arise from Leydig cell expression. Curiously, our findings differ from another earlier report where *Dax-1* was shown to be down-regulated at later stages of testicular development (Swain et al., 1996).

Discussion

Genetic studies have identified SRY (Sry) and implicated DAX-1 as factors that promote or oppose, respectively, the development of the bipotential gonad into the testis. Two other nuclear proteins, SF-1 and WT1, are also essential for establishing the bipotential gonad in both sexes. Previously, Pelletier and colleagues hypothesized that WT1 may modulate male-specific expression of *MIS* (1991b). Indeed, *WT1* mutations are often associated with persistent Müllerian duct structures, consistent with *MIS* disregulation (Little and Wells, 1997). However, as shown here, WT1 is unable to activate *MIS*-reporter constructs by itself. Our earlier studies proved SF-1 to be an essential but not sole regulator of *MIS* (Shen et al., 1994; Giuili et al., 1997). Based on these data we conclude that neither SF-1 nor WT1 is sufficient to direct cell-specific *MIS* expression. Here, we show that SF-1 and WT1 work in concert to affect expression of a male-specific gene (*MIS*) positioned downstream of *Sry*. Furthermore, Dax-1 serves to antagonize the functional interaction between SF-1 and WT1, suggesting that the interplay between SF-1, WT1, and Dax-1 may account for the activation or repression of male-specific genes.

A Tissue-Specific Cofactor for SF-1

Coactivators and corepressors are now known to modulate ligand-activated nuclear receptors as well as other transcription factors (Perlmann and Evans, 1997). The ubiquitous nature of these cofactors makes it unlikely that they contribute to the tissue-specific expression of target genes activated by nuclear receptors. WT1 may therefore serve as one of the first examples of a tissue-specific cofactor of a nuclear receptor. It is of interest that another WT1-related zinc-finger protein, Egr-1 (Zif268, NGFI-A, Krox-24), functionally synergizes with SF-1 to regulate the β subunit of luteinizing hormone, LHβ (Lee et al., 1996). In this scenario, distinct DNA-binding elements for SF-1 and Egr-1 are present in the LHβ promoter, suggesting that a direct protein association between SF-1 and Egr-1, as postulated here for SF-1 and WT1, may not be required for the observed synergism. Several studies infer that multiple mechanisms can modulate SF-1's activity depending on the cellular and developmental context. SF-1 is influenced by cofactors, such as WT1 and Dax-1 (this study and Ito et al. 1997), by ligands (Lala et al., 1997), and by posttranslational modifications (Zhang and Mellon, 1996). Our data suggest that the subset of monomer-binding nuclear receptors like SF-1 can recruit non–DNA-binding partners, such as WT1 and Dax-1, to affect gene expression. These partners could fulfill a role analogous to the homo- or heterodimeric DNA-binding partners known to exist for the majority of other nuclear receptors (Mangelsdorf et al., 1995).

DDS Mutants: Dosage Sensitivity or Dominant-Negative Proteins?

Intersex/genitourinary defects in the Denys-Drash syndrome are frequently associated with germline heterozygous missense mutations of the *WT1* gene. Phenotypes associated with DDS, and particularly renal nephropathies, are thought to arise via a dominant-negative mechanism rather than simple haploinsufficiency (Pelletier et al., 1991a). DDS mutant proteins are proposed to associate with wild-type WT1, thereby either reducing DNA binding of WT1 −KTS to target sites or subverting the subnuclear localization of the WT1 −KTS isoforms to the splicing apparatus, where WT1 +KTS isoforms are normally distributed (Englert et al., 1995; Larsson et

al., 1995). Both mechanisms effectively lower transcriptionally active WT1 −KTS. We find that DDS WT1 mutants fail to interact functionally with SF-1 but are able to associate in a two-hybrid assay (Figure 4C). Unexpectedly, overexpression of these missense *WT1* mutants did not reduce wild-type WT1 activity as predicted for a dominant-negative protein. These data rekindle the debate as to how *WT1* missense and nonsense mutants lead to genital defects. Self-association of WT1 is relatively weak when compared with SF-1/WT1 association (M. W. N. and H. A. I., unpublished data; Moffett et al., 1995; Reddy et al., 1995). Moreover, severe urogenital disorders are observed in some Wilms' tumor patients predicted to have too little of the N-terminal portion of WT1 to associate with wild-type WT1 (Little and Wells, 1997). Thus, in considering our data and others, we favor a model in which mutant DDS proteins are localized away from the transcriptional machinery, effectively reducing the dosage of wildtype WT1 −KTS protein (Larsson et al., 1995). Additionally, these mislocalized DDS mutant proteins could act as antimorphs to affect the normal functions of the +KTS isoform. Differential localization of the +KTS and −KTS isoforms may also account for the intersex disorders observed in Frasier syndrome. This syndrome is associated with a *WT1* mutant predicted to alter the splice isoform ratio, resulting in lower WT1 +KTS levels (Barbaux et al., 1997). In our assay the WT1 +KTS is inactive, so how might lowering this isoform lead to loss of male-specific gene expression? Decreasing the +KTS isoform may shift the balance of WT1 −KTS associated with the transcriptional machinery; alternatively, WT1 +KTS may participate in the processing of RNAs that encode additional factors required for male urogenital development.

Examination of several DDS mutants revealed that the majority of 46,XY patients exhibit gonadal abnormalities, while most 46,XX patients exhibit normal ovarian development (Little and Wells, 1997). One implication suggested by the phenotypic differences among male and female DDS patients would be that monoallelic expression of wild-type *WT1* is insufficient for testicular maturation but adequate for ovarian development. Thus, male-specific genes may be extremely sensitive to the dosage of *WT1*. Our data raise the possibility that at least for some genes, such as *MIS*, haploinsufficiency, and not gain-of-function mutations, account for the phenotype observed in male DDS and WAGR patients.

Interaction between SF-1, WT1, and Dax-1 and Mammalian Sexual Differentiation

The gene products of *Ftz-f1* (SF-1), *WT1*, and *DAX-1* are all implicated in mammalian sexual development. Colocalization of SF-1 and WT1 is restricted to Sertoli and granulosa cells, consistent with their roles in *MIS* gene regulation. SF-1 and Dax-1 colocalize in multiple endocrine tissues, suggesting that these two nuclear proteins may be linked in function (Ikeda et al., 1996). Human DAX-1 loss-of-function mutants exhibit adrenohypoplasia congenita (AHC), with some disruption of the hypothalamic-pituitary-gonadal axis (Zanaria et al., 1994), implying that like SF-1, DAX-1 is critical for adrenal development and, at least at puberty, for normal testicular function. Paradoxically, in vitro studies show that DAX-1 inhibits SF-1–mediated transactivation by binding DNA directly or by heterodimerization with SF-1 (Ito et al., 1997; Zazopoulos et al., 1997). In our in vitro system, Dax-1 inhibits the SF-1/WT1 synergy; however, Dax-1 inhibition of SF-1–mediated transactivation alone is not robust. Therefore, both the cellular and the promoter context are likely to affect the functional consequence of SF-1 and Dax-1 interactions, just as these factors influence the SF-1/WT1 synergy. The notion that Dax-1 may function solely as a repressor or "anti-testis factor" is difficult to reconcile with the increased Dax-1 expression observed at later stages of testis development. It is plausible that in late embryonic or adult testes, an available ligand converts Dax-1 from a repressor to an activator as proposed (Lalli et al., 1997).

In many respects, WT1 and Dax-1 interact with SF-1 in similar ways, albeit with dramatically different outcomes. WT1 and Dax-1 are unable to bind specifically to a SF-1 response element, neither interferes with SF-1 binding, and the strength of their interactions with SF-1 is relatively weak based on the inability to detect prominent protein–protein complexes on DNA or to coimmunoprecipitate a SF-1/WT1 complex. The nature of SF-1/WT1 and SF-1/Dax-1 interactions promises to be complex given that WT1 or Dax-1 mutants physically associate with SF-1 but are functionally inactive (this study; Ito et al., 1997). WT1 and Dax-1 could simply compete for each other in their interactions with SF-1; however, data shown here suggest that these molecules interact with different regions of SF-1. It is possible that all three molecules form a complex tethered to DNA by SF-1 and that WT1 and Dax-1 functionally oppose each other by altering the balance of ubiquitous coactivators or corepressors associated with SF-1. Further studies are needed to delineate the structural basis for the functional interaction of SF-1, WT1, and Dax-1.

The relative dosage of *SF-1*, *WT1*, and *DAX-1* may now be considered in the context of mammalian sexual development. While SF-1 is high in males and promotes male development by regulating *MIS* and genes involved in testosterone production, DAX-1 has been suggested to antagonize male development because it is associated with the duplicated X-linked dosage-sensitive sex-reversal locus in 46,XY females. We propose that overexpression or a double dose of the *DAX-1* gene in these XY females may impair testis development by interfering with SF-1/WT1 synergy (Figure 7). Indeed, during revision of this manuscipt, gain-of-function studies revealed that high doses of *Dax-1* can counteract Sry in a weakened *Sry* allelic background or in XX sex-reversed male mice (Swain et al., 1998). However, these studies also serve to illustrate that sensitivity to gene dosage during sexual development differs in rodents and humans. Similarly, this difference in sensitivity is noted with the gene dosage of *WT1*, where monoallelic expression is known to affect genital development in humans but not in mice (Kreidberg et al., 1993). In the Denys-Drash syndrome, a 50% reduction of transcriptionally active WT1 −KTS is predicted to shift the balance of WT1 relative to DAX-1, thereby favoring the DAX-1/SF-1 interaction (Figure 7). As a consequence, male-specific genes are suppressed, resulting in impaired male sexual development. It follows

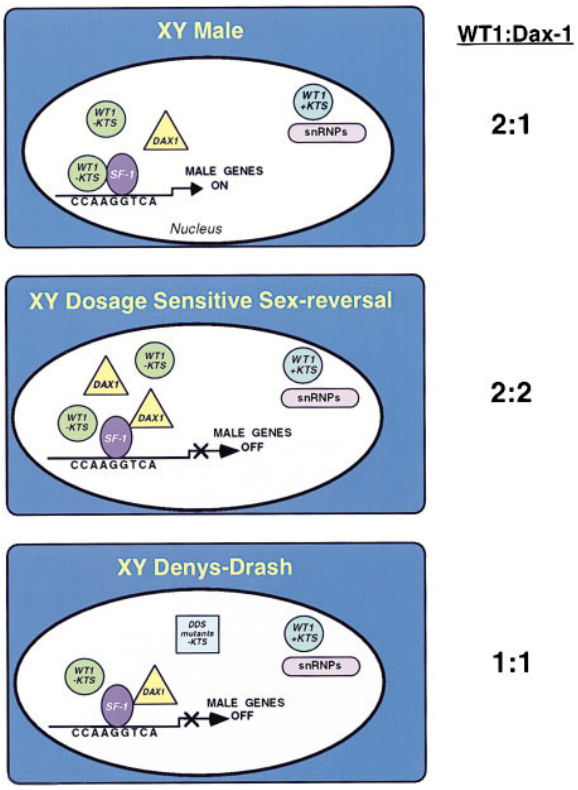

Figure 7. SF-1, WT1, and DAX-1 in Male Sexual Development

A model depicting our results and three genetic conditions in human 46,XY individuals is shown. Because *SF-1* and *WT1* are autosomal genes, whereas *DAX-1* is X-linked, the relative gene dosage of *WT1* −KTS to *DAX-1* would normally be 2:1. Male-specific genes, such as *MIS*, are on because the synergistic interactions between SF-1 and WT1 are favored. In XY dosage–sensitive sex-reversal, male-specific genes are off because a double dose of *DAX-1* competes functionally with WT1 for interaction with SF-1; the ratio of *WT1:DAX-1* is 2:2 in this scenario. In DDS, XY individuals with heterozygous missense *WT1* mutations reduce the levels of wild-type −KTS isoform by half. In these individuals the ratio of *WT1:DAX-1* is 1:1. In all three conditions, the gene dosage of *SF-1* remains at 2. The WT1 +KTS isoform is shown in association with the splicing apparatus, small nuclear ribonucleoproteins (snRNPs).

that *Sry* and/or *Sox9* may function to increase the relative levels of SF-1 in the bipotential gonad to initiate male sexual development. Future genetic manipulations of *Ftz-f1* (SF-1), *Wt1*, and *Dax-1* in vivo should help to delineate how dosage sensitivity of these and other factors operate in mammalian sex determination.

Experimental Procedures

Plasmid Constructions and Transfection Assays

cDNAs encoding the relevant isoforms of mouse WT1 were placed in the eucaryotic expression vector, pCDNA3, using unique EcoRI-XhoI sites from the pWT1 +/−, −/−, and +/+ isoforms (generous gift from J. Pelletier and D. Housman). All DDS WT1 mutants were generated by standard site-directed mutagenesis. Deletion of the N terminus was accomplished by Ppum1 digestion followed by religation. LexA-SF-1 fusion proteins were obtained by fusing the entire coding region of mouse SF-1 minus the DBD (aa 11-124) to the LexA-DBD at a unique BstEII site of pCMV.LexA (Ingraham et al., 1990).

For cell transfection experiments, JEG-3 cells were plated at a density of 1–2 × 10^5 per 22 mm plate (12-well) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics. One day later, cells were cotransfected with expression and reporter plasmids using a standard calcium phosphate precipitation method (Mammalian Transfection Kit; Specialty Media) and harvested 36 hr later. Luciferase activity was measured using the Enhanced Luciferase Assay (Analytical Luminescence Laboratory). Experiments were performed at least three times in triplicate with different preparations of plasmid. β-galactosidase activity was measured as an internal control for transfection. COS cells were plated at a density of 5 × 10^6 per 100 mm plate in DMEM/10% FBS and antibiotics 24 hr prior to transfection. COS cells were transfected using an adenovirus-mediated procedure essentially as described in Allgood et al. (1997).

Gel Shift Binding Studies and GST Pull-Down Assays

Gel mobility shift assays were performed as previously described, except that the buffer for binding to the WTE site was modified (Nakagama et al., 1995). Noncompetitor single-stranded DNA was lowered to detect optimal binding on this site. Binding reactions were carried out (in 20 mM HEPES [pH 7.9], 60 mM KCl, 4 mM Tris-Cl [pH 8.0], 0.6 mM EDTA, 0.6 mM EGTA, 12% glycerol, 5 mM DTT, 50 μg/ml BSA, 5 μg/ml salmon sperm DNA, 5 μg/ml poly dI-dC, 0.25% milk, 10 μM $ZnSO_4$), using in vitro translated proteins and ^{32}P end-labeled annealed MIS-RE-1 oligonucleotides. Prior to binding assays, individual in vitro translated proteins were radiolabeled and checked by 10% SDS-PAGE analysis. Sequences for oligonucleotides were as follows: MIS-RE-1, 5′-GCCAGGCACTG-TCCCCCAAG GTCACCTT-3′ (Shen et al., 1994); WTE, 5′-CGGATCCGCCAGCGT-GGGAGTACCTTAGATCTG-3′ (Nakagama et al., 1995).

GST pull-down assays were conducted using 20 μl of a 50% GST-protein beads slurry suspended in binding buffer; 20 mM HEPES (pH 7.9), 100–500 mM NaCl, 1 mM EDTA, 4 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 0.01% NP40, 1 mg/ml BSA, and protease inhibitor cocktail mixed with 4 μl of [^{35}S]cysteine/methionine radiolabeled IVT reactions in a 150 μl reaction volume for 60 min at 37°C. After washing three times in the same buffer, bound proteins were visualized after separation on a 10% SDS-PAGE by autoradiography. The GST fusion proteins were made using mouse SF-1 corresponding to amino acids 265-462 and full-length mouse WT1 −/− and Dax-1 (Guo et al., 1996) inserted into a pGEX vector (Pharmacia). COS whole-cell extracts were made by lysis in 50 mM HEPES, 250 mM NaCl, 0.5 mM EDTA, 0.1% NP40, and protease inhibitor cocktail. Binding reactions were performed in binding buffer, as described above, in a total volume of 625 μl for 30 min at 37°C. Proteins were visualized by Western analysis and 9% SDS-PAGE.

LexA Yeast Two-Hybrid Assays

Plasmids pEG202, pJG4-5, pSH18-34, and yeast strain EGY48 were obtained from CLONTECH. Plasmid pEG202.SF-1 and pJG4-5.SF-1 were constructed using full-length mouse SF-1 (nt. 1-1389) inserted into BamH1/Xho1 sites. To construct pJG4-5.WT1 −/−, pJG4-5.WT1 +/+, pEG202.Dax-1, and pJG4-5.Dax-1 mouse cDNAs encoding the WT1 isoforms or Dax-1, respectively, were used as template and amplified by PCR, which introduced an EcoRI site at the N terminus and an XhoI site at the C terminus. The WT1 Δ8-180 mutant was created by an internal PpuMI digestion. To examine β-galactosidase activities, strain EGY48 was cotransformed with the *lexAop-lacZ* reporter plasmid pSH18-34 and either pEG202 or a pEG202-derived plasmid and pJG4-5 or a pJG4-5–derived plasmid. At least five independent transformants of each combination were streaked out on Galactose/Raffinose-HIS-TRP-URA/X-Gal plates for whole plate assays. Additionally, β-galactosidase activity was measured from several independent transformants in a liquid culture assay using ONPG as substrate. Activity was normalized to growth (OD 600) and assay time according to the formula provided by CLONTECH.

In Situ Hybridization and RNase Protection Assays

Whole-mount in situ protocols were performed on paraformaldehyde (PFA)-fixed rat genital ridges treated with proteinase K (10 μg/ml; 30–45 min at room temperature) and refixed with 4% PFA. Tissues were incubated with digoxigenin (DIG)-labeled RNA probes,

generated from full-length mouse WT1 cDNA or rat SF-1 cDNA (nt. 47-879), in hybridization buffer (1.3× SSC (pH 5), 50% formamide, 0.2% Tween-20, and 0.5% CHAPS) overnight at 65°C. After extensive washing and blocking (60 min in Boehringer Mannheim blocking reagent), tissues were incubated overnight at 4°C with anti-DIG antibody (1:2000; Boehringer Mannheim). After washing, color development was performed using BM Purple AP Substrate for 30–60 min at RT.

In situ hybridization on cryosections (10 μm) of PFA-fixed rat genital ridges were performed according to the Nonradioactive In Situ Hybridization Application Manual (Boehringer Mannheim) with the following modifications. Sections were hybridized (50% formamide, 20 mM Tris-Cl [pH 8.0], 5 mM EDTA, 300 mM NaCl, 1× Denhardt's, 10 mM NaH_2PO_4 [pH 8.0], 10% dextran sulfate, 0.5 mg/ml tRNA) with DIG-labeled probe overnight at 60°C. After high stringency washes, signals were detected using anti-DIG (1:1000; 60 min at RT). Color development used the NBT-BCIP substrate (Boehringer Mannheim). The WT1 probe is the same as described above; the rat MIS probe is as described (Shen et al., 1994).

RNase protection assays were performed using the Ribonuclease Protection Assay kit (Ambion). RT-PCR was used to isolate cDNA fragments of rat WT1 (372 bp), encompassing the +KTS region, and rat Dax-1 (694 bp; nts 248–942). Both were subcloned into pCRII (InVitrogen). cRNA probes were transcribed from linearized plasmids using a RNA transcription kit (Stratagene). Total RNA (5 μg/sample) was isolated from gonads collected from staged rat embryos (plug date as E0). E13.5 animals were sexed by genomic PCR using the following primers for rat *Sry*: forward, 5′-AAGCGCCCCATGAATGC ATT-3′; reverse, 5′-CGATGAGGCTGATATTTATA-3′. Protected fragments were analyzed on a 5% 1× TBE denaturing polyacrylamide gel.

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