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A Microdeletion in the Ligand Binding Domain of Human Steroidogenic Factor 1 Causes XY Sex Reversal without Adrenal Insufficiency

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Steroidogenic factor 1 (SF-1) is an orphan nuclear receptor that plays key roles in endocrine development and function. Knockout mice lacking SF-1 have adrenal and gonadal agenesis, impaired gonadotropin expression, and structural abnormalities of the ventromedial hypothalamic nucleus. Previous studies have identified three human subjects with mutations in SF-1 causing adrenocortical insufficiency with varying degrees of gonadal dysfunction. We now describe a novel 8-bp microdeletion of SF-1, isolated from a 46, XY patient who presented with gonadal agenesis but normal adrenal function, which causes premature termination upstream of sequences encoding the activation function 2 domain. In cell transfection experiments, the mutated protein possessed no intrinsic transcriptional activity but rather inhibited the function of the wild-type protein in most cell types. To our knowledge, this is the first example of an apparent dominant-negative effect of a SF-1 mutation in humans. These findings, which define a SF-1 mutation that apparently differentially affects its transcriptional activity *in vivo* in the adrenal cortex and the gonads, may be relevant to the cohort of patients who present with 46, XY sex reversal but normal adrenal function. (*J Clin Endocrinol Metab* 89: 1767–1772, 2004)

THE ORPHAN NUCLEAR receptor steroidogenic factor 1 (*SF-1,* officially designated NR5A1) initially was identified because a regulator of the tissue-specific expression of the cytochrome P450 steroid hydroxylases (1, 2). Subsequent studies have shown that SF-1 is a key regulator of endocrine function within the hypothalamic-pituitary-gonadal axis and adrenal cortex (reviewed in Ref. 3). Disruption of the mouse *Nr5a1* gene encoding SF-1 causes adrenal and gonadal agenesis, XY sex reversal, structural abnormalities of the ventromedial hypothalamic nucleus, and altered gonadotropin expression by pituitary gonadotropes.

To date, only three mutations in SF-1 have been described in humans. In each of these patients, the SF-1 mutation caused adrenal insufficiency, supporting a key role of SF-1 in human adrenal development. A 46, XY sex-reversed patient with gonadal dysgenesis was shown to have a heterozygous *de novo* mutation in the first zinc finger domain (G35E) that abolished DNA binding (4). The second mutation, a heterozygous missense mutation resulting from a *de novo* G→T transversion in the hinge region of SF-1 (R255L), also encoded a transcriptionally inactive protein (5). This 46, XX patient had apparently normal ovaries on magnetic resonance imaging, raising the possibility that a single allele of SF-1 is sufficient for ovarian development in humans. Finally, a homozygous R92Q mutation was identified in a 46, XY infant born to consanguineous parents (6). This mutation, located in an accessory DNA binding region of SF-1 called the Ftz-F1 (or A) box, impaired but did not abolish SF-1 transcriptional activity. The partial loss of function caused by this mutation provides a plausible explanation for its autosomal recessive inheritance in this kindred. In this report, we considerably broaden the phenotypic spectrum of SF-1 mutations by describing the first subject in whom a *SF-1* mutation caused embryonic testicular regression syndrome with 46, XY sex-reversal but without adrenal insufficiency.

Patients and Methods

We studied the SF-1 gene of 32 46, XY sex-reversed patients without adrenal insufficiency and found a heterozygous inactivating mutation in one case.

Patient

A 31-yr-old patient was referred due to primary amenorrhea. She underwent pubarche at age 12 yr, and hypertension was diagnosed at 20 yr of age. The patient denied consanguinity and family members were unavailable for genetic studies. She was 161 cm tall and weighed 89 kg, with body mass index of 34.3. Her blood pressure was 170/120 mm Hg. She had a eunuchoid habitus, absence of breast development and ambiguous genitalia characterized by an enlarged clitoris (4.1 cm), a single perineal opening, and no palpable gonads. Cytogenetic analysis revealed a 46, XY karyotype. Laboratory data (Table 1) disclosed normal basal ACTH, cortisol, plasma renin activity, sodium, and potassium levels. Serum cortisol increased normally after acute ACTH stimulation, without an abnormal accumulation of adrenal steroid precursors. Basal gonadotropin levels were elevated, with a predominance of FSH over LH; basal testosterone was at prepubertal levels without any increase after human chorionic gonadotropin (hCG) stimulation. Pelvic ultrasonography showed no uterus, gonads, or prostate. The genitography disclosed a blind vaginal pouch and a urogenital sinus. No gonadal tissue was found at laparoscopic surgery and a bilateral duct resection

Abbreviations: AF-2, Activation function 2; HA, hemagglutinin; hCG, human chorionic gonadotropin; SF-1, steroidogenic factor 1; WT, wild-type.

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TABLE 1. Laboratory and hormonal data of a 46,XY sex-reversed patient with a heterozygous 8-bp microdeletion in SF-1 gene

Laboratory data (SI units)	Patient (SI)	Normal levels (SI)
Basal ACTH pg/ml (pmol/liter)[a]	60 (13)	<60 (<13)
Basal ACTH pg/ml after 6 yr of follow-up (pmol/liter)[a]	53 (12)	<60 (<13)
Basal cortisol μg/dl (nmol/liter) at 0800 h[b]	8.0 (221)	7–31 (193–855)
Basal cortisol μg/dl after 6 yr of follow-up (nmol/liter) at 0800 h[b]	9.0 (248)	7–31 (193–855)
Cortisol after ACTH[c] μg/dl (nmol/liter)[a]	21 (579)	>20 (>551)
DHEA ng/ml (nmol/liter)[d]	2.7 (9.4)	3–5.7 (10–19)
DHEA-S ng/ml (nmol/liter)[e]	1004 (2721)	468–3331 (1270–9040)
Na mEq/liter (mmol/liter)	141 (141)	135–145 (135–145)
K mEq/liter (mmol/liter)	4.0 (4.0)	3.5–5.0 (3–5.0)
PRA ng/ml·h (μg/liter·sec)[f]	3.8 (1.0)	1.5–5.7 (0.4–1.6)
LH IU/liter	18	1–12
FSH IU/liter	57	1.4–9.2
T ng/dl (nmol/liter)[g]	35 (1.2)	240–1030 (8–11)
T after hCG[h] ng/dl (nmol/liter)[g]	38 (1.3)	804–1874 (25–78)

To convert to the SI unit, multiply by the conversion factor: [a] ACTH, 0.2222; [b] cortisol, 27.5862; [d] dehydroepiandrosterone (DHEA), 34.6741; [e] DHEA-sulfate (DHEA-S), 27.2109; [f] plasma renin activity (PRA), 0.2778; [g] testosterone (T), 34.6741.
[c] Cortisol after acute stimulation with 250 μg Cortrosyn IV. [h] Testosterone after 6000 IU hCG im.

was performed. Histopathological analysis showed the absence of gonadal tissue and the presence of bilateral deferent ducts. Estrogen replacement induced normal breast development, and vaginal dilation with vaginal molds was performed successfully, leading to normal sexual activity (7). She was also treated with antihypertensive drugs with good blood pressure control.

Laboratory evaluation

Serum androstenedione, dehydroepiandrosterone, dehydroepiandrosterone-sulfate, and aldosterone were determined by commercial RIAs. Serum LH, FSH, estradiol, testosterone, and cortisol were measured by immunofluorometric assays (Delfia, Wallac, Turku, Finland). Compound S was determined by the method of Abraham (8) without prior chromatography after demonstrating adequate specificity of the antiserum (9). Plasma ACTH was measured with an immunoradiometric kit, and plasma renin activity was measured by RIA. For dynamic testing, a rapid ACTH stimulation test was performed with cosyntropin 250 μg iv, and a GnRH stimulation test was performed with GnRH 100 μg iv. The hCG stimulation test was performed with a single im dose of 6000 IU hCG.

Cytogenetic studies

Chromosome analysis was performed in 50 peripheral blood lymphocyte metaphases using G banding.

DNA sequence analysis

Peripheral blood was obtained and DNA was prepared according to standard procedures (10). The entire coding region and exon-intron boundaries of *SF-1* were amplified by PCR from genomic DNA. Oligonucleotide sequences and PCR protocols are available from the authors on request. The coding region of the SRY gene was also studied in this cohort of patients according to the protocol previously reported (11). The PCR products were pretreated with a combination of shrimp alkaline phosphatase and exonuclease I (United States Biochemical Corp., Cleveland, OH) and directly sequenced using the BigDye TM terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA) in an ABI PRISM 310 automatic sequencer.

Plasmids

pcDNA.hSF1 [pcDNA3.1/Zeo(+), Invitrogen, Carlsbad, CA] containing the human SF-1 cDNA was used as a template for site-directed mutagenesis (QuikChange site-directed mutagenesis kit, Stratagene, La Jolla, CA) to introduce the 8-bp deletion into the SF-1 coding sequence (designated Δ8SF-1) (12). Wild-type (WT) and Δ8SF-1 cDNAs were subcloned into pCMV-HA (Clontech, Palo Alto, CA) to produce N-terminal hemagglutinin (HA)-tagged fusions proteins. The appropriate mutations in all plasmids were verified by DNA sequencing. The human 17α-hydroxylase (CYP17) promoter plasmid contains approximately 1.1 kb of the CYP17 promoter ligated into pGL3 Basic (Promega, Madison, WI) as described (13).

Immunofluorescence

COS-7 cells were grown in DMEM/10% fetal bovine serum (FBS) and plated on Lab-Tek II chamber slides (Nalge Nunc, Rochester, NY) that had been pretreated with poly-L-lysine. Cells were plated at a density of approximately 50,000 cells/well and incubated for 24 h in DMEM/10% FBS. The cells were then transiently transfected with empty vector or HA-tagged WT or Δ8SF-1 expression vector using FuGENE 6 transfection reagent (Roche, Indianapolis, IN) according to the supplier's recommendation. Forty-eight hours after transfection, the cells were fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100. Immunofluorescence analysis was performed using anti-HA primary antibody (HA.11, CRP Inc., Berkeley, CA) at a 1:1000 dilution and fluorescein isothiocyanate-conjugated goat antimouse IgG1 (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:200 dilution. Expression of HA-tagged SF-1 was visualized using an Optiphot microscope (Nikon, Tokyo, Japan) equipped with a UV light source and filters for fluorescein visualization.

EMSAs

Complementary synthetic oligonucleotides (5′-GGCCACAGATTCTCCAAGGCTGAT-3′; 5′-GGCATCAGCCTTGGAGAATCTGT-3′) containing the mouse 21-hydroxylase (Cyp21)-140 SF-1-responsive element were annealed and end labeled with [^{32}P]dCTP using Klenow polymerase (the *underlined* sequence indicates the SF-1 binding site within the oligonucleotide). *In vitro*-translated WT and Δ8SF-1 were produced using the TNT coupled reticulocyte lysate system (Promega). Comparable amounts of WT and mutated SF-1 protein in the *in vitro* translation reactions were confirmed by immunoblotting with an antibody specific for the DNA-binding domain of SF-1 (data not shown). EMSAs were performed as previously described (14) with 3 μg poly-(dI·dC/dI·dC) as nonspecific competitor and 1–8 μl of reticulocyte lysate.

Cell culture and luciferase assays

Transient transfection analyses of transcriptional activity were carried out in steroidogenic and nonsteroidogenic cell lines. Y1 mouse adrenocortical tumor cells were grown in Ham's F-10 supplemented with 15% horse serum and 2.5% FBS. Mouse MA-10 Leydig tumor cells were grown in Waymouth's media supplemented with 15% horse serum. Mouse 3T3 fibroblasts and monkey kidney COS-7 cells were grown in DMEM supplemented with 10% FBS. Human HEK293 embryonic kidney and H295R adrenocortical tumor cells were grown in DMEM/F12 (Life Technologies, Inc., Grand Island, NY) supplemented with 5% NuSerum (Collaborative Research Inc., Bedford, MA) and 10% Cosmic

Calf (Hyclone, Logan, UT), respectively. For luciferase assays, cells were plated on 12-well plates at a density of 100,000–400,000 cells/well. Transfection of H295R cells was performed using Transfast transfection reagent (Promega), whereas transfection of all other cell types was performed using FuGENE 6 transfection reagent (Roche). Cells were cotransfected with constant concentrations of the CYP17 promoter plasmid (100 ng) and WT SF-1 expression vector (50 ng) in the absence or presence of increasing concentrations of Δ8SF-1 expression vector (50–500 ng). Empty expression vector was used to equalize the total DNA concentration in each transfection. Cells were lysed and luciferase assays performed 24–48 h after transfection. Comparable results for each cell line were obtained in at least three separate experiments performed in quadruplicate, and a representative experiment is shown, with data expressed as means ± SDs.

Results

Identification of the mutation

In an effort to identify additional human subjects with SF-1 mutations, we examined the sequence of a cohort of 32 46, XY sex-reversed patients without clinically apparent adrenal insufficiency. No mutations were identified in the *SRY* gene in these patients. As described in more detail in *Patients and Methods*, the index patient was a 31-yr-old phenotypic female who presented with primary amenorrhea, ambiguous genitalia, hypertension, obesity, and normal adrenal function. Analysis of the *NR5A1* gene using genomic DNA obtained from peripheral blood leukocytes revealed a heterozygous deletion of eight consecutive nucleotides beginning at position 2783. This mutation in exon 6 is predicted to cause a frameshift that prematurely terminates translation at position 378, upstream of sequences encoding the activation function 2 (AF-2) transcriptional activation motif (Figs. 1 and 2).

Functional characterization of the mutation

To explore the molecular basis for the endocrine disorder in our patient, we examined the effect of the 8-bp microdeletion on SF-1 function. We first performed gel mobility shift assays with *in vitro*-translated SF-1 or Δ8SF-1 and a probe from the mouse −140 Cyp21 SF-1-responsive element. As shown in Fig. 3A, both WT and Δ8SF-1 bound the probe to form a shifted complex, although the apparent affinity of the Δ8SF-1 for DNA was consistently lower than that of WT SF-1. The molecular basis for this decreased affinity is not immediately apparent because both the zinc finger DNA-binding region and the Ftz-F1 accessory DNA-binding region are intact. Nonetheless, these data indicate that the microdeletion does not abolish the ability of Δ8SF-1 to bind to its cognate response element. Furthermore, adding the Δ8SF-1 to the binding reaction did not impede binding of WT SF-1 to the response element.

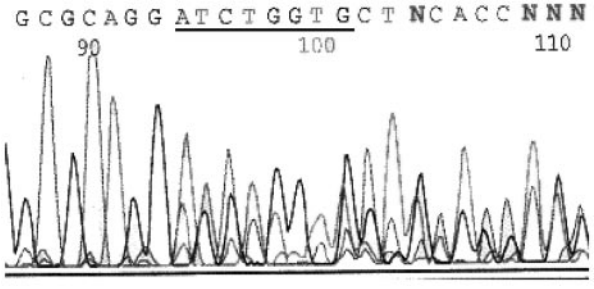

FIG. 1. Direct sequencing identified a heterozygous deletion of eight consecutive nucleotides (1058–1065) in exon 6 of SF-1 causing a frameshift mutation that determined a stop codon at position 378.

To examine the possible effect of the microdeletion on expression and subcellular localization of SF-1, we transiently transfected WT or Δ8SF-1 expression vectors carrying a hemagglutinin epitope tag into COS-7 cells and then examined location and intensity of immunofluorescent staining. The WT and Δ8SF-1 proteins were expressed in comparable amounts and localized exclusively to the nucleus (Fig. 3B). These data suggest that the 8-bp microdeletion does not alter the trafficking or stability of SF-1.

To examine the transcriptional activity of the WT and Δ8SF-1, we performed transient transfection assays in a variety of steroidogenic and nonsteroidogenic cell lines. As shown in Fig. 4, cotransfection of mouse MA-10 Leydig tumor cells with WT SF-1 considerably stimulated reporter gene expression driven by the human 17α-hydroxylase (CYP17) promoter. In contrast, the mutated SF-1 was completely devoid of transcriptional activation. When WT and Δ8SF-1 were mixed, the mutated SF-1 inhibited the transcriptional activity of WT protein in a dose-dependent manner, consistent with a dominant-negative effect. A similar inhibitory effect was observed in HEK293 cells, which do not endogenously express SF-1 and are nonsteroidogenic. In contrast, Δ8SF-1 behaved very differently in H295R human adrenocortical carcinoma cells. Rather than exerting an inhibitory effect in these steroidogenic cells, the mutated SF-1 actually augmented the transcriptional activity of WT SF-1, again in a dose-dependent manner. Thus, the transfection data in MA-10 and H295R cells are congruent with the behavior of the mutated SF-1 *in vivo*, with preserved function in the adrenal cortex but gonadal agenesis. Somewhat inconsistent with these observations, however, is the apparent dominant-negative effect of the Δ8SF-1 in mouse Y1 adrenocortical tumor cells (Fig. 4). Although the precise basis for such cell-specific differences remains to be defined, these differences may reflect differences in the expression of coactivators and/or corepressors in the various cell lines.

Discussion

Gonadal development and sexual differentiation require the interaction of a number of genes encoding transcription factors, including the orphan nuclear receptors *SF-1* and *DAX1*, the zinc finger proteins *WT1* and *GATA4*, and *SOX9* (reviewed in Ref. 15). *SF-1* is essential for gonadal development in both sexes, regulating the expression of hormones that mediate male and female reproduction and also is a key regulator of adrenal development and function (reviewed in Ref. 3). Consistent with these essential roles in adrenal and gonadal development, *sf-1* knockout mice exhibit adrenal and gonadal agenesis (16–18). Adrenal insufficiency is also a common feature in all of the previously reported patients with SF-1 mutations, despite the fact that the G35E, R255L, and R92Q mutations are located at different regions of SF-1 and exhibit some distinct functional effects (4–6). Surprisingly, although the SF-1 microdeletion described here impaired gonadal development and sexual differentiation, as evidenced by the ambiguous external genitalia and the ab-

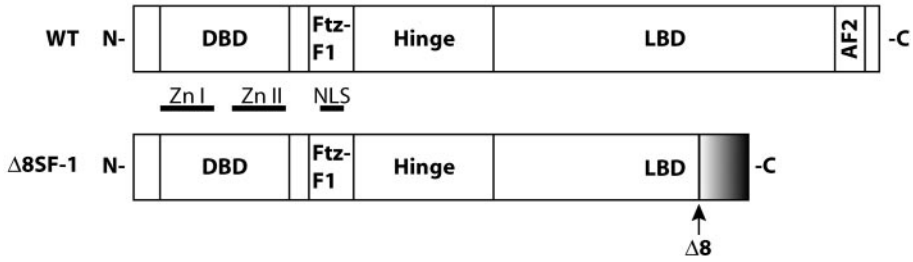

FIG. 2. Location of the 8-bp microdeletion relative to defined functional domains of SF-1. Schematic diagrams are shown of WT and Δ8SF-1 proteins, including the location of regions of known functional significance that include the DNA binding domain (DBD), the first (Zn I) and second (Zn II) zinc finger regions, the Ftz-F1 (or A) box, the nuclear localization signal (NLS), the hinge region, and the ligand binding domain (LBD) that includes the AF-2 activation motif. Note that the Δ8SF-1 microdeletion causes a frameshift upstream of AF-2, thus encoding a protein that lacks this key domain (modified from a figure in Ref. 3; © 2003 Val *et al.*; licensee BioMed Central Ltd. This is an Open Access article; verbatim copying and redistribution of this article are permitted in all media for any purpose, provided this notice is preserved along with the article's original URL, http://www.nuclear-receptor.com/content/1/1/8).

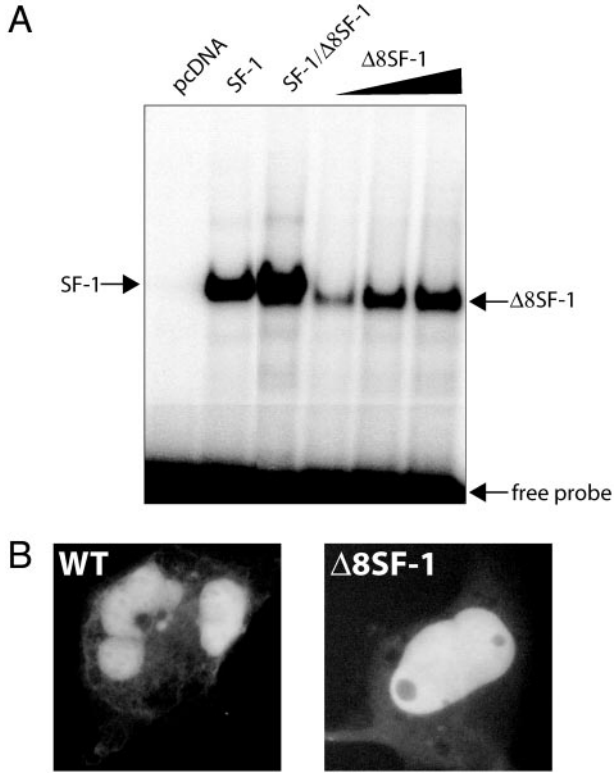

FIG. 3. DNA binding and nuclear localization of WT and mutated SF-1 proteins. A, EMSAs WT and Δ8SF-1 proteins were produced by coupled *in vitro* transcription/translation and used in EMSAs with an oligonucleotide probe comprising the Cyp21 −140 SF-1-responsive element as described in *Patients and Methods*. WT SF-1 (1 μl lysate) formed a prominent shifted complex at the indicated position, whereas the mutated Δ8SF-1 (1, 4, and 8 μl, respectively) formed a complex that migrated slightly more rapidly. The lane labeled SF-1/Δ8SF-1 contained both WT SF-1 (4 μl) and Δ8SF-1 (4 μl). The lane labeled pcDNA contained lysate that was programed only with empty expression vector. B, Nuclear localization of WT and Δ8SF-1. COS-7 cells were transiently transfected with WT or Δ8SF-1 expression plasmids, and SF-1 was detected by immunohistochemistry as described in *Patients and Methods*. Comparable levels of expression and nuclear localization were seen with WT and Δ8SF-1.

sence of detectable gonads, it did not impair adrenocortical function. Thus, despite the dramatic effect of the mutation on SF-1 transcriptional activity in all cell lines examined, the truncated SF-1 protein apparently permits normal adrenal development and function *in vivo*. Moreover, although no gonadal tissue was found in the patient at 31 yr of age, the absence of the uterus suggests that the gonads produced sufficient anti-Mullerian hormone to cause Müllerian duct regression *in utero*, whereas the impaired virilization suggests that the SF-1 mutation affected Leydig cells to a greater extent than it did Sertoli cells.

The molecular basis by which the 8-bp microdeletion inhibits gonadal but not adrenocortical function remains to be determined. We note that the mutated protein exhibited cell-selective effects on the transcriptional activity of WT protein (Fig. 4). In particular, rather than inhibiting WT SF-1 in H295R human adrenocortical tumor cells, the mutated protein actually augmented its activity. This finding suggests that differences in the cohort of transcriptional coactivators and corepressors may affect the function of the mutated SF-1 in different organs *in vivo*. We note that H295R cells express high levels of the orphan nuclear receptor DAX1 (K. Parker, unpublished observation), which is known to inhibit the transcriptional activity of SF-1. Based on previous reports, the DAX1-SF-1 interaction requires at least two domains of SF-1: a carboxy-terminal repressive domain (amino acids 437–447), which is lost in this patient, and a proximal interactive domain (amino acids 226–230) (19). Perhaps the mutated protein, through its proximal interactive domain, is able to sequester DAX1 in H295R cells and diminish its repression of the WT protein. Further studies in cell culture models and transgenic mice will be needed to explore this model.

The molecular mechanisms underlying these differential effects on the expression of known SF-1 target genes remain to be defined. However, it is plausible that SF-1 interacts with distinct repertoires of coactivators and corepressors in the various tissues in which it is active, such that the effect of different mutations *in vivo* may vary in different target tissues. In this regard, it is important to remember that the patient retains one apparently normal *NR5A1* allele. Further characterization of the spectrum of SF-1 coactivators and corepressors in different tissues may provide a molecular explanation for the selective effect of the microdeletion on gonadal but not adrenal development.

The molecular basis for the impaired activity of the SF-1 microdeletion has not been fully defined. The AF-2 motif at the extreme carboxyl terminus of SF-1 has been shown to be

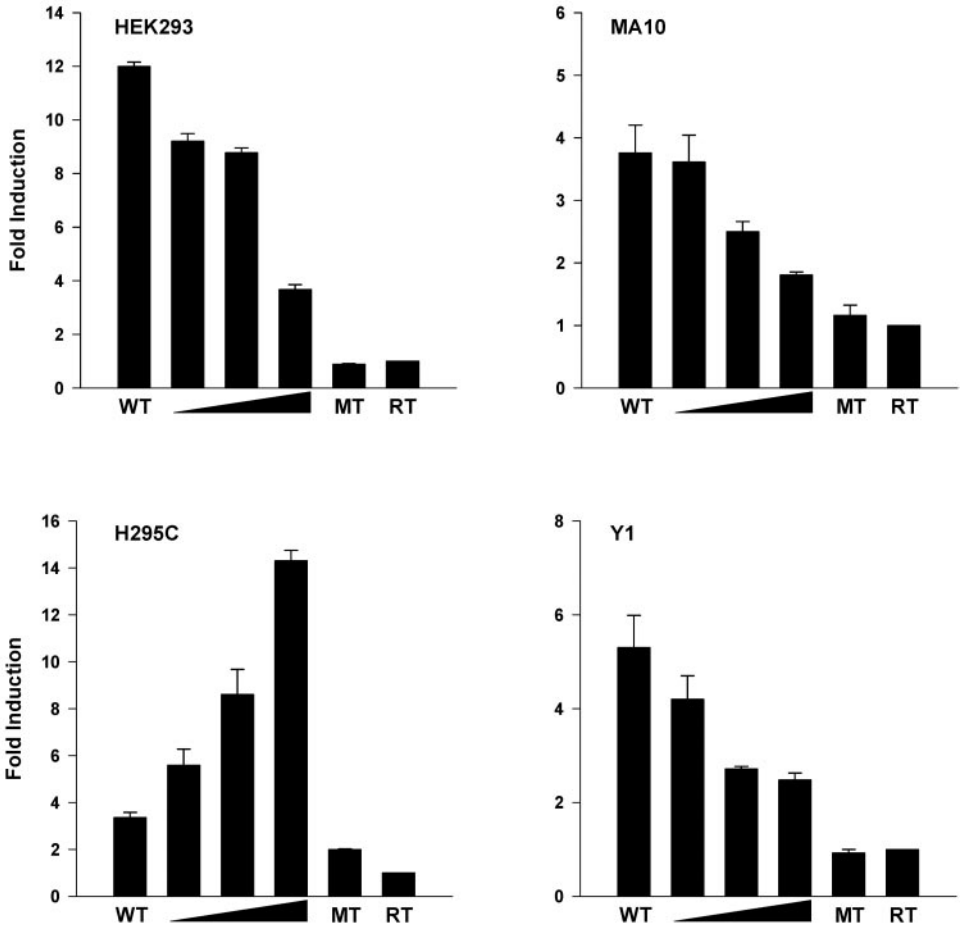

FIG. 4. Effect of the SF-1 mutation on transcriptional activity. Transient transfection assays with the WT and Δ8SF-1 expression plasmids and the human CYP17-luciferase reporter plasmid were performed in the indicated cell lines as described in *Patients and Methods*. The fold inductions over reporter (RT) alone with the WT and mutated (MT) expression vectors are indicated. The *triangle* indicates a mixture of WT plasmid (50 ng) and increasing amounts of MT plasmid (50, 150, or 500 ng, respectively). Data are presented as means ± SDs from a representative experiment performed in quadruplicate.

essential for receptor transactivation in both steroidogenic and nonsteroidogenic cell lines (20, 21). Based on structural analyses of the ligand-binding domains of other nuclear receptors, the AF-2 domain in helix 12 plays a key role in facilitating interactions with coactivators to stimulate transcription (22). The microdeletion reported here prematurely terminates SF-1 by deleting the C-terminal portion of the ligand-binding domain that includes the AF-2 motif (see Fig. 2). Of considerable interest, expression of mutated bovine SF-1 lacking the AF-2 motif impaired the activity of the wild-type protein in transfected Y1 adrenocortical tumor cells (23). This dominant-negative effect strikingly resembles the dose-dependent inhibition seen with the mutated SF-1 allele described here, suggesting that deletion of the AF-2 motif itself accounts for most of the impaired activity in cell transfection experiments.

We describe here the first human subject in whom endocrine disease is associated with a mutation in SF-1 that can inhibit the function of the wild-type protein. This contrasts with the zinc finger transcription factor WT1, which interacts with SF-1 to regulate the expression of target genes such as the AMH (24). Dominant-negative mutations of WT1 that impair DNA binding are observed relatively frequently in Denys-Drash syndrome, an autosomal dominant disorder that includes Wilms tumors, gonadal and urogenital abnormalities, and diffuse mesangial sclerosis (reviewed in Ref. 15). Although we do not fully understand the precise mechanisms by which SF-1, WT1, and other transcription factors cooperate to mediate gonadal development and sexual differentiation, it is noteworthy that the patient described here provides the first example of a SF-1 mutation presenting with 46, XY sex reversal without adrenal insufficiency. Given that isolated XY sex reversal occurs considerably more often than combined adrenal/gonadal deficiency, this study expands the spectrum of patients that may harbor SF-1 mutations. We note, however, that SF-1 mutations are unlikely to be a frequent cause of isolated XY sex reversal because we did not identify other SF-1 mutations in genetic analyses of 31 other patients.

If kept alive with adrenal transplants, the SF-1 knockout mice exhibit late-onset obesity that may reflect its roles within the ventromedial hypothalamic nucleus (25). Interestingly, despite the fact that our patient never received exogenous glucocorticoids, she was obese with a body mass index of 34. Thus, further studies of the role of SF-1 in energy homeostasis in humans are warranted.

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