Ten Novel Mutations in the *NR5A1* Gene Cause Disordered Sex Development in 46,XY and Ovarian Insufficiency in 46,XX Individuals

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Context: Steroidogenic factor-1 (SF-1/*NR5A1*) is a nuclear receptor that regulates adrenal and reproductive development and function. *NR5A1* mutations have been detected in 46,XY individuals with disorders of sexual development (DSD) but apparently normal adrenal function and in 46,XX women with normal sexual development yet primary ovarian insufficiency (POI).

Objective: A group of 100 46,XY DSD and two POI was studied for *NR5A1* mutations and their impact.

Design: Clinical, biochemical, histological, genetic, and functional characteristics of the patients with *NR5A1* mutations are reported.

Setting: Patients were referred from different centers in Spain, Switzerland, and Turkey. Histological and genetic studies were performed in Barcelona, Spain. *In vitro* studies were performed in Bern, Switzerland.

Patients: A total of 65 Spanish and 35 Turkish patients with 46,XY DSD and two Swiss 46,XX patients with POI were investigated.

Main Outcome: Ten novel heterozygote *NR5A1* mutations were detected and characterized (five missense, one nonsense, three frameshift mutations, and one duplication).

Results: The novel *NR5A1* mutations were tested *in vitro* by promoter transactivation assays showing grossly reduced activity for mutations in the DNA binding domain and variably reduced activity for other mutations. Dominant negative effect of the mutations was excluded. We found high variability and thus no apparent genotype-structure-function-phenotype correlation. Histological studies of testes revealed vacuolization of Leydig cells due to fat accumulation.

Conclusions: SF-1/NR5A1 mutations are frequently found in 46,XY DSD individuals (9%) and manifest with a broad phenotype. Testes histology is characteristic for fat accumulation and degeneration over time, similar to findings observed in patients with lipoid congenital adrenal hyperplasia (due to *StAR* mutations). Genotype-structure-function-phenotype correlation remains elusive. ***(J Clin Endocrinol Metab* 97: E1294–E1306, 2012)**

Steroidogenic factor-1 (SF-1; also called Ad4BP) is a nuclear receptor that regulates adrenal and reproductive development and function (1, 2). It is considered a master transcriptional regulator of genes involved in the hypothalamic-pituitary-gonadal and adrenal axes. SF-1 regulates the transcription of many genes involved in steroidogenesis, sexual development, and reproduction (2). However, detailed regulation of SF-1 expression itself and its downstream ef-

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Abbreviations: *AR*, Androgen receptor gene; DBD, DNA binding domain; DSD, disorders of sexual development; hCG, human chorionic gonadotropin; LBD, ligand binding domain; POI, primary ovarian insufficiency; SF-1, steroidogenic factor-1; StAR, steroidogenic acute regulatory protein; WT, wild-type.

TABLE 1. Clinical, biochemical, anatomic, and genetic characteristics of the 10 patients harboring novel mutations in the *NR5A1* gene

Patient no.	Origin, YOB	Karyotype/assigned sex	Genital anatomy	Testes histology (age)
1	Spanish, 2008	46,XY/male	Scrotal hypospadia. Bilateral cryptorchidism. No Müllerian ducts	ND
2	Spanish, 1981	47,XYY/female	Female external genitalia. Bilateral inguinal hernia. No Müllerian ducts	Abnormal (3.5 yr), Fig. 3A
3	Spanish, 1971	46,XY/female to male at 18 yr	Ambiguous external genitalia at birth. Progressive virilization at puberty with nonpalpable gonads. No Müllerian ducts	Cryptorchidic, dysgenetic testes (19 yr)
4	Turkish, 1997	46,XY/female	Ambiguous external genitalia. Bilateral inguinal hernia. No Müllerian ducts	Seminiferous tubules devoid of spermatogonia
5	Spanish, 1998	46,XY/female	Female external genitalia. Bilateral inguinal hernia. No Müllerian ducts	No histology upon gonadectomy (1 yr)
6	Spanish, 2006	46,XY/female	Ambiguous external genitalia. Gonads palpable in labia majora. No Müllerian ducts	Testis biopsy (4 yr): seminiferous tubules with variable diameters; some without germ cells. Few Leydig cells in interstitium
7	Spanish, 2003	46,XY/female	Female external genitalia. Gonads in labia majora. No Müllerian ducts	Abnormal (2 yr), Fig. 3, B and C
8	Spanish, 2003	46,XY/female to male at 8 months	Ambiguous genitalia (Prader 3). Palpable gonads. No Müllerian ducts	Abnormal (2 yr), Fig. 3D
9	Swiss, 1994	46,XX/female	Normal female at birth. Pelvic US (16 yr), prepubertal uterus, nondetectable ovaries	ND
10	Turkish, 2009	46,XY/male	Ambiguous genitalia (Prader 3). Palpable gonads. No Müllerian ducts	ND

YOB, Year of birth; F, father; M, mother; (N), normal sequence; *SRD5A2*, 5α-reductase type 2 gene; *LHCGR*, LH receptor gene; *CYP17A1*, cytochrome P450 17α-hydroxylase/17,20-lyase gene; US, ultrasound; ND, not done; T, testosterone; E2, estradiol; AMH, anti-Müllerian hormone

fects remain to be elucidated (2). The human SF-1 protein consists of 461 amino acids and is encoded by the *NR5A1* gene (3). It is expressed in the fetal and adult adrenal cortex as well as in fetal and adult testes and ovaries (2).

Human *NR5A1* mutations were first described in 46,XY individuals with combined adrenal failure and disorders of sexual development (DSD) (4, 5). To date, only one case with apparently isolated primary adrenal failure has been reported in a 2-yr-old 46,XX girl (6). By contrast, many *NR5A1* mutations have been found in a large number of 46,XY DSD individuals with apparently normal adrenal function (7–16), as well as in several 46,XX individuals with normal female sexual development but premature ovarian failure (17, 18). Some cases were familial (5, 10–12, 17–20), and some were sporadic (12, 17). Besides the aforementioned phenotypes, *NR5A1* mutations are associated with a very wide range of phenotypes related to reproductive function and development (21), such as hypospadias (13, 22), anorchia (10), isolated micropenis (23), male infertility (24), and primary ovarian insufficiency (POI) (2, 10, 17, 18, 21).

Since the first description of *NR5A1* mutations in 1999 (4), over 50 mutations have been reported. Most subjects carry mutations in a heterozygote state (4, 6, 7, 9, 10, 12–18, 20, 22–26), some are compound heterozygous (8, 11, 12, 17, 24), and only a few are homozygous (5, 27). Although many patients with *NR5A1* mutations have been studied extensively, no clear genotype-phenotype and SF-1 protein structure-function correlation has been found. The search for modulating factors explaining the broad range of clinical manifestations of *NR5A1* mutations continues.

In this study, 10 novel *NR5A1* mutations are described. Nine mutations were identified in 46,XY DSD patients and one in a 46,XX POI patient. Clinical, biochemical, histological, and genetic data of the patients are presented. Functional studies and protein structure analyses of the novel *NR5A1* mutations were performed to study phenotype-genotype correlations.

Patients and Methods

Patients and ethical approval

A total of 102 patients of Spanish, Turkish, and Swiss origin were analyzed for mutations in the *NR5A1* gene. The 65 Spanish

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TABLE 1. Continued

Gonadal function (age)	Adrenal function (age)	*NR5A1* gene mutation	Family	Previous genetic studies
Normal baseline (1 d and 1 yr)	Normal baseline (1 d and 1 yr)	c.58G>C; Val20Leu	F = carrier, M = WT	*AR* (N), *SRD5A2* (N)
Abnormal (T slightly decreased and normal precursor response to hCG) (3 yr)	Normal baseline (30 yr)	c.70C>T; His24Tyr	Brother and sister = WT	*AR* (N), *SRD5A2* (N)
Abnormal (normal T and precursors, high LH and FSH) (18 yr)	Normal baseline (18 yr)	c.70delC; His24ThrfsX51	Sister = carrier (infertility). (History of early menopause in M and of ambiguous genitalia in maternal great aunt)	Patient *AR* (N), *SRD5A2* (N). Patient and sister, *LHCGR* (c.51_52insTGCAGC polymorphism)
Abnormal (no T response to hCG) (1 yr)	ND	c.90T>G; Cys30Trp		*AR* (N), *SRD5A2* (N)
ND	Normal (22 months)	c.268G>C; Gly90Arg	M = carrier. (History of ambiguous genitalia in maternal great uncle and of early menopause in M and grandmother)	*AR* (N), *SRD5A2* (N)
Abnormal (low T and normal precursor response to hCG) (7 months)	ND	c.389delC; Pro130ArgfsX165	F = WT, M = WT	*AR* (N), *SRD5A2* (N)
Abnormal (no T response to hCG) (14 d)	Abnormal? (slightly increased ACTH and decreased precursor response to Synacthen) (6 yr)	c.614_615insC; Gln206ThrfsX20		*AR* (N), *CYP17A1* (N)
Abnormal (no T response to hCG) (1 month)	ND	c.690_691dupCTGCAGCTG; Leu231_Leu233dup	F = WT, M = WT	*AR* (N), *SRD5A2* (N)
Abnormal (increased LH and FSH and undetectable E2 and AMH) (15 yr)	Normal (15 yr)	c.704C>T; Pro235Leu		
Normal baseline (2 months)	ND	c.905G>A; Trp302Stop		*AR* (N), *SRD5A2* (N)

patients included 61 subjects with 46,XY DSD, one with 47,XYY DSD, and three 46,XY patients with anorchia. *AR* (androgen receptor) and *SRD5A2* (5α-reductase type 2) genes had been previously analyzed in these patients and were normal in all except one carrying the P378R *AR* mutation (28). In some, the *CYP17A1*, *HSD17B3*, and *LHCGR* genes were analyzed for mutations, but none were found (Table 1). Similarly, a group of 35 Turkish patients with 46,XY DSD and two Swiss POI 46,XX patients were evaluated. *AR* and *SRD5A2* genes were analyzed in all 35 Turkish patients, but no mutations were detected. The Swiss patients were only analyzed for *NR5A1* mutations.

All studied subjects and/or their legal guardians gave written informed consent for the biochemical and molecular studies, which were approved by the respective ethical committees of the three study centers.

Genetic analyses

Genetic analyses of the *NR5A1* gene were performed as explained in Supplemental Data and Supplemental Table 1 (published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

In vitro functional studies

In vitro functional studies were performed in human steroidogenic NCI-H295R and nonsteroidogenic HEK293 cells (see Supplemental Data and Supplemental Table 1).

In silico protein structure analysis

For bioinformatic studies, the mouse SF-1 DNA binding domain (DBD) NMR structure (PDB no. 2FF0; N terminus, AA 10-111, 100% similar to human SF-1) and human SF-1 ligand binding domain (LBD) x-ray crystal structure (PDB no. 1ZDT; C terminus, AA 221-461) were used (see Supplemental Data).

Results

Identification of 10 novel *NR5A1* gene mutations in patients presenting with 46,XY DSD or 46,XX POI

We found 10 novel, disease-causing mutations in the *NR5A1* gene in a combined group of 102 patients presenting with 46,XY DSD (eight of 99; 8%), 47,XYY DSD (one of one), or 46,XX POI (one of two). All details of the patients' characteristics are summarized in Table 1, and individual case reports are available as Supplemental Data.

Six of nine 46,XY subjects harboring *NR5A1* mutations presented with ambiguous genitalia at birth and were assigned either male (two of six) or female (four of six) sex of rearing. Patients 3 and 8, who were initially assigned to female sex were assigned to male sex at age 18 yr and 8 months. Three 46,XY subjects presented with normal external female genitalia but were noted to have either inguinal hernia at the age of 2–3 yr or palpable gonads in labia majora at age 2 wk. Gonadal function was tested in nine subjects and found to be abnormal in seven of the 46,X(Y)Y. Six subjects were gonadectomized at ages 2–19 yr, and a testis biopsy was performed in one aged 4 yr. The single 46,XX female patient presented with absent pubertal signs at age 15 yr due to POI/hypergonadotropic hypogonadism. None of the patients had a history of adrenal insufficiency, but adrenal function was only evaluated in six patients, suggesting normal function in five (using variable assessments, *e.g.* basal or stimulated tests) and minor abnormalities such as slightly increased ACTH and decreased cortisol precursor secretion in subject 7. Family history and/or genetic testing of relatives revealed other affected family members in at least two subjects (one healthy 46,XY carrier excluded), but it was only available from six families.

The 10 novel mutations in the *NR5A1* gene were scattered throughout exons 2 to 5 (Figs. 1 and 2). Nine mutations were single nucleotide changes that led to six point mutations (five missense and one nonsense) and three frameshift mutations (two deletions and one insertion). A duplication of nine nucleotides (in-frame) was also detected, leading to an insertion of three amino acids and a prolongation of the protein sequence.

Three mutations were found in other family members (cases 1, 3, and 5), whereas the mutations Pro130ArgfsX165 and Leu231_Leu233dup appeared to be *de novo* mutations

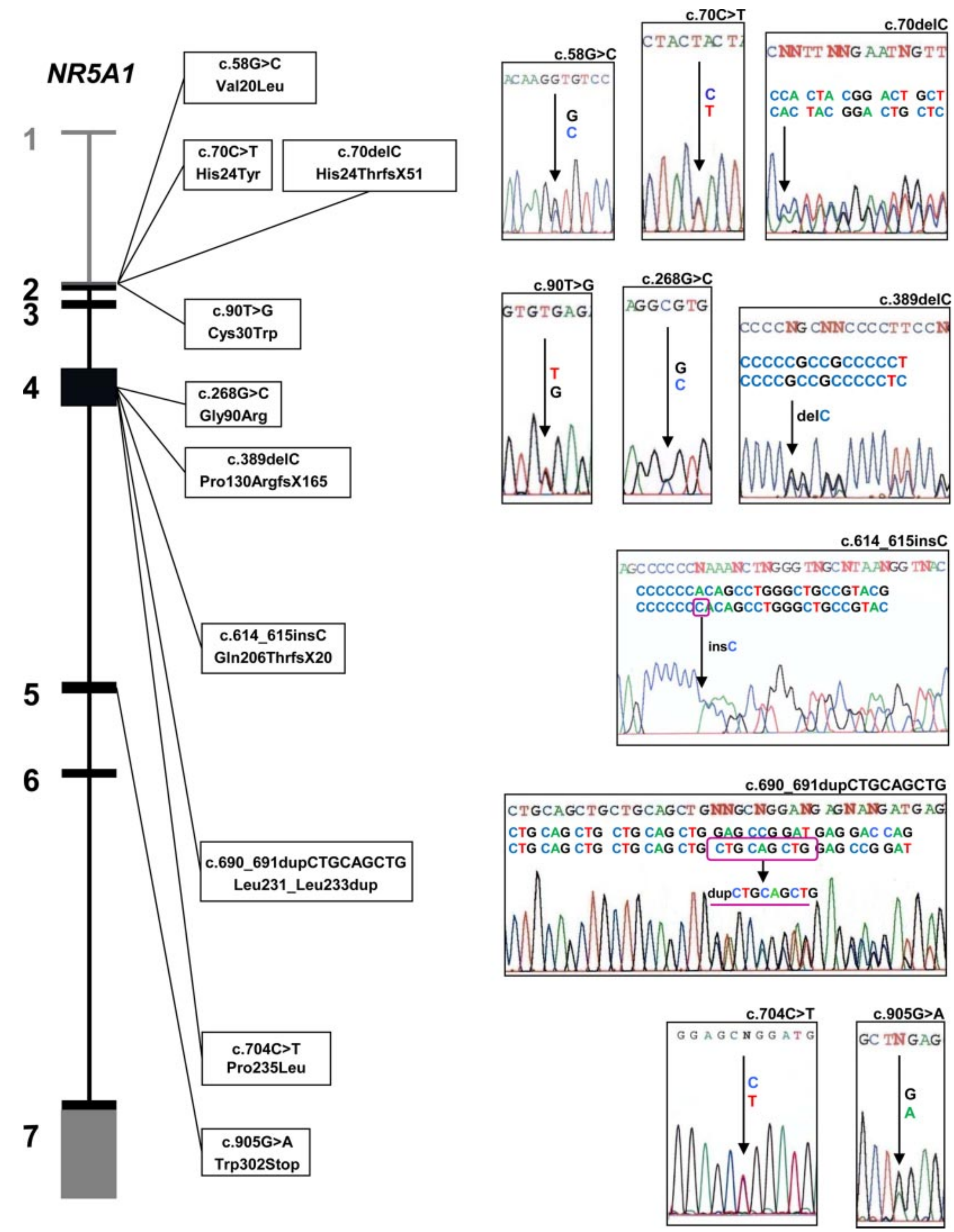

FIG. 1. Diagram of the *NR5A1* gene and chromatograms of the 10 novel mutations. The mutations identified in patients and their exact location in the *NR5A1* gene sequence are shown. In the *NR5A1* gene diagram, *numbers* refer to exons 1 to 7 and noncoding sequences are shown in *gray*.

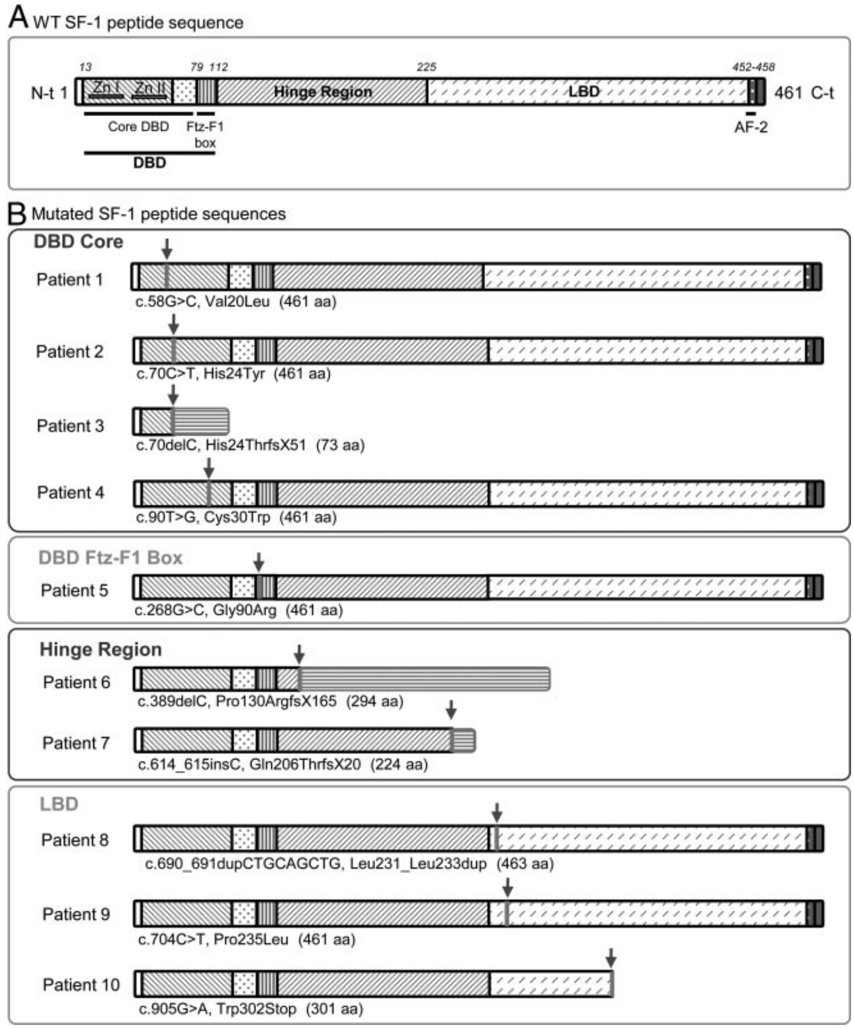

FIG. 2. Diagrams of the WT and mutated SF-1 peptide sequences. A, Diagram of the WT SF-1 protein; B, predicted peptide sequences of the 10 SF-1 mutations. Four mutations are located in the core domain and one is located in the Ftz-F1 box domain of the DBD. The hinge region contains two mutations, and the LBD contains the remaining three. All mutations were found to be heterozygous. In the diagrams, point mutations are shown as *vertical bars* (indicated by *arrows*), and sequence changes are given as *horizontal lines*, shown in *red*. In panel A, *numbers above the diagram* indicate the amino acid at the start of the protein domain or those contained in the domain (for AF-2) (19, 34, 35). N-t, N terminal; C-t, C terminal; Zn I, first zinc finger; Zn II, second zinc finger; AF-2, activation function domain 2.

in patients 6 and 8 because both of their parents were noncarriers (Table 1).

In addition, the polymorphism Gly146Ala (c.437G>C) (rs1110061) was identified in seven of 102 (6.8%) 46,XY DSD subjects (Table 2). Six were heterozygous, whereas one was homozygous (subject 17). Subject 17 also carried two other sequence variations: Pro125Pro (rs1110062) in the *NR5A1* gene, and Pro378Arg in the *AR* gene (28).

Histopathology findings of testes in patients with heterozygote *NR5A1* mutations

Testis tissue for histopathological studies was available from five patients aged 2 to 4 yr (patients 2, 4, 6, 7, and 8; Fig. 3 and Table 1). Gonadal function in those subjects appeared to be abnormal (Table 1). Tissue sections were compared with normal control tissue and testis tissue originating from a patient with lipoid congenital adrenal hyperplasia owing to *StAR* mutations (Fig. 3). Morphology of testis tissues obtained before puberty from patients harboring heterozygote *NR5A1* mutations revealed diminished tubular diameter and absent or decreased spermatogonia. Interstitium was remarkable for cell nests with vacuolized, xanthomatous, or adipoblast-like Leydig cells (Fig. 3, A, B, and D). Osmium staining revealed osmium-positive lipid deposits (Fig. 3C), not present in control testis (Fig. 3F), and which resembled the Leydig cells typically observed in prepubertal testes of patients with lipoid congenital adrenal hyperplasia (Fig. 3E).

In vitro functional studies of the novel *NR5A1* mutations

Functional activity was tested by studying transactivation of wild-type (WT) and mutant NR5A1 on promoters of genes for steroidogenic enzymes regulated by SF-1 in steroidogenic and nonsteroidogenic cell models (Fig. 4).

Transfection of human adrenal NCI-H295R cells with WT and NR5A1 mutants together with a CYP17A1 promoter reporter construct revealed that all 10 *NR5A1* mutations had impaired ability to stimulate the CYP17A1 promoter (Fig. 4A). Among the 10 mutants, Pro235Leu (identified in a 46,XX POI patient; Table 1) was most active, having 80% of WT activity. Overall, mutations located in the DBD (cases 1–5) appeared to be functionally less active than mutations in the LBD (cases 8–10). Studies of SF-1 transactivating the CYP11A1 and HSD3B2 promoters were not possible in NCI-H295R cells because background activity of endogenous SF-1 was too high and promoter stimulation using WT overexpression too low (data not shown). Therefore, we performed further studies in nonsteroidogenic HEK293 cells, which do not express SF-1. Similar to NCI-H295R cells, all 10 NR5A1 mutants had impaired activity on the CYP17A1 promoter (Fig. 4B). Again, the Pro235Leu mutant displayed highest activity (80% of WT), and mutations in the LBD seemed slightly more active than mutations in the DBD.

TABLE 2. Clinical, biochemical, and anatomic characteristics of seven 46,XY DSD patients harboring the Gly146Ala *NR5A1* sequence variation

Patient no.	Origin, YOB	Karyotype/assigned sex	Genital anatomy	Testes histology (age)
11	Spanish, 1996	46,XY/female to male (1 yr)	Scrotal hypospadias. Bilateral cryptorchidism. No Müllerian ducts	ND
12	Spanish, 2001	46,XY/male	Scrotal hypospadias. Bilateral cryptorchidism	ND
13	Turkish, 2001	46,XY/male	Scrotal hypospadias. Bilateral palpable gonads	ND
14	Turkish, 2002	46,XY/male	Scrotal hypospadias. Bilateral palpable gonads	ND
15	Turkish, 2005	46,XY/male	Scrotal hypospadias. Bilateral palpable gonads	ND
16	North African, 2008	46,XY/male	Ambiguous external genitalia. Gonads palpable in labia majora. Müllerian duct remnants present	ND
17	Sub-Saharan, 1998	46,XY/male	Scrotal hypospadias. Intraabdominal testes. Müllerian duct remnants present	Normal prepubertal interstitium. Sertoli cell hyperplasia and lack of spermatogonia (4 yr)

YOB, Year of birth; (N), normal sequence; *SRD5A2*, 5α-reductase type 2 gene; *AMH*, anti-Müllerian hormone gene; *AMHRII*, *AMH* receptor type 2; ND, not done; T, testosterone.

However, studies of SF-1 activating the CYP11A1 and HSD3B2 promoters in HEK293 cells confirmed these results only partially (Fig. 4, C and D). *NR5A1* mutations located in the DBD (1–4) showed a profound loss of CYP11A1 and HSD3B2 promoter stimulation similar to the CYP17A1 promoter. But in contrast to the CYP17A1 promoter, all NR5A1 mutants located in the LBD (8–10) had either unchanged activity in stimulating the CYP11A1 or HSD3B2 promoters or even higher activity (Trp302X on HSD3B2). Promoter studies of the NR5A1 mutants located in the Ftz-F1 box (case 5) and hinge region (cases 6 and 7) showed mixed results (Fig. 4, C and D).

Because all our patients harbor heterozygote *NR5A1* mutations, the dominant negative effect of mutants over the WT NR5A1 was assessed in our cell model. Similar to other investigators testing various *NR5A1* mutations (6–8, 11, 12, 14, 20, 29), our reporter assays did not reveal a dominant negative effect for any mutant (Fig. 4E). Thus, the mechanism of heterozygote *NR5A1* mutations causing the phenotype of DSD in our patients remains a conundrum. To assess the expression of *NR5A1* variants in our cell model, we performed Western blots (Fig. 4F). These studies showed that transiently transfected cells expressed all WT and mutant SF-1 proteins. Only His24ThrfxX51 seemed to be too small to be detected on our blot. On our Western blots, no significant differences were seen in protein expression of mutant SF-1 when compared with WT. Yet, some mutants rather showed a higher level of expression (Gly90Arg, Pro235Leu).

Studies of the Gly146Ala variant on promoter activity of *CYP17A1* in HEK293 cells revealed no effect overall and also no dominant negative effect in particular (Supplemental Fig. 1).

In silico protein structure analysis

Human SF-1 protein has two distinctive domains. The DBD contains a core with two Cys4-zinc-finger motifs and a highly conserved Ftz-F1 box that includes a highly conserved RGGR motif potentially involved in interaction with DNA. Mutants Val20Leu, His24Tyr, and Cys30Trp are present in the DBD core, and Gly90Arg is located in the RGGR motif of the Ftz-F1 box (Fig. 5A). The Pro235 and Leu-Gln-Leu repeats are located at the N terminus of the LBD (Fig. 5B).

In the three-dimensional structure, the four DBD mutations are located in a critical hotspot that comprises the C terminus of the DNA-recognition helix, the β-hairpin loop, and the RGGR motif in the loop before the critical C-terminus helix of DBD required for stability of the SF-1/DNA complex (Fig. 5A). All these mutations are in the region that directly interacts with DNA and may alter the pattern/recognition of specific DNA sequences. A change from Val20 to Leu causes alterations in the side chain packing environment of the N-terminal loop due to interaction of altered Leu20 with Asp10 and Glu11 (Fig. 5C). This may slightly alter the DBD structure and influence the interaction with DNA molecule. However, a major conformational change that could have a major influence on DNA binding was not expected. The His24 residue is crucial for DNA binding, and its substitution with Tyr shifts the Tyr side chain away from the DNA binding site, breaking the hydrogen bond (Fig. 5D). The Cys30 residue is essential for zinc binding and stability of the core DBD. *In silico* mutagenesis and molecular dynamics simulations were used to change the Cys30 to Trp, and resulting structure shows the disruption of a Cys-zinc contact resulting in an unstable core structure (Fig. 5E). The mutation Gly90Arg is located in the RGGR motif formed by R89-R92 in human SF-1 (in the loop before the C-terminus helix in Ftz-F1 box). The mutation of Gly90 to Arg results in a major change at the DNA binding site due to the large positively charged Arg side chain (Fig. 5F). The change from Gly to Arg will alter the binding pattern and may have variable effects depending on sequence of DNA interaction partner. Additional interactions with DNA will be created due to Arg side chain at the binding site that may change binding selectivity and result in activation of dif-

TABLE 2. Continued

Gonadal function (age)	Adrenal function (age)	Family	*NR5A1* gene	Other genes studied
Abnormal (subnormal T response to hCG) (2 yr). (T decreased and LH + FSH increased) (13 yr)	Normal (1 month)		Hetero c.437G>C; Gly146Ala	*AR* (N)
Normal (T response to hCG) (1.5 yr)	Normal (3 yr)		Hetero c.437G>C; Gly146Ala	*AR* (N)
Abnormal (subnormal T response to hCG) (1 yr)	Normal (1 yr)		Hetero c.437G>C; Gly146Ala	*AR* (N), *SRD5A2* (N)
Abnormal (decreased T response to hCG) (1 yr)	Normal (1 yr)	Consanguinity	Hetero c.437G>C; Gly146Ala	*AR* (N), *SRD5A2* (N)
Normal (T response to hCG) (2 yr)	Normal (2 yr)		Hetero c.437G>C; Gly146Ala	*AR* (N), *SRD5A2* (N)
Normal baseline (10 d). Abnormal (T slightly decreased and normal precursor response to hCG) (3 months)	Normal (10 d)		Hetero c.437G>C; Gly146Ala	*AR* (N)
Normal baseline (5 d)	Normal baseline (5 d, 4 months)	Parents first cousins	Homo c.437G>C; Gly146Ala. Homo c.375G>A; Pro125Pro	*AR* (c.1139C>G; Pro378Arg) (Ref. 28), *AMH* (N), *AMHRII* (N)

ferent promoters and may have variable effects on individual promoters.

The Pro235 separates the two N-terminus helices of the LBD of SF-1 (Fig. 5G). The mutation Pro235Leu may abolish the break in between two helices and creates a larger single helix. Such a change will not result in the alteration of the core structure but may cause subtle changes in the neighboring residues near the ligand binding site. A likely effect would be modification of ligand binding and recognition and may have variable effects depending on specific ligands. The dual repeat units of Leu-Gln-Leu located at the N terminus of LBD form the N-terminal helix. The insertion of another Leu-Gln-Leu repeat unit changes the composition of this helix and results in a longer loop structure located in between the two N-terminal helices (Fig. 5H). The addition of extra Leu side chain from Leu-Gln-Leu insertion creates changes in the environment of neighboring residues to accommodate the extra Leu side chain pointing toward the core of the LBD. The specific effect of such a change is unpredictable, and a variable effect depending on specific ligand is expected. Both the Pro235Leu and Leu-Gln-Leu insertion do not appear to alter the ligand-binding properties, and their effects may therefore be related to relatively minor changes caused by altered flexibility of the two N-terminus helixes.

Discussion

We found 10 new mutations in the *NR5A1* gene in 102 patients studied for either 46,XY DSD or 46,XX POI (9.8%). These mutations were all heterozygous and were predominantly single-nucleotide changes.

Individuals with heterozygous *NR5A1* mutations present with a broad range of phenotypes covering completely normal phenotype to severe forms of 46,XY DSD or POI in 46,XX subjects (2, 16, 17, 21, 24). Even identical mutations within families may lead to differing phenotypes (10, 17–19). Similarly, our patients presented with a wide clinical spectrum. External genitalia in 46,XY individuals varied from completely female to ambiguous with cryptorchid testes; none presented with Müllerian duct remnants. Gonadal steroid production studied at prepubertal age ranged from apparent lack of testosterone secretion to only slightly diminished testosterone response upon human chorionic gonadotropin (hCG) stimulation with normal steroid precursors. Only one patient developed spontaneous puberty and reached a normal male adult serum testosterone level but had grossly elevated gonadotropin levels. Adrenal function appeared to be normal in most patients but was not assessed in all. However, adrenal function should be followed in patients with *NR5A1* mutations because adrenal insufficiency has been described with *NR5A1* mutations and may only develop over time.

Family history of two patients (cases 3 and 5) revealed ambiguous genitalia and premature menopause in several relatives, of whom some were also found to have heterozygous *NR5A1* mutations. By contrast, the father of a moderately affected 46,XY DSD patient (case 1) was phenotypically normal but was a carrier of Val20Leu. Similarly, normal carriers with heterozygous *NR5A1* mutations have been reported (10, 16, 22). Arg281Pro located in the LBD was found in a 46,XY DSD patient who had hypospadias and micropenis and a phenotypically normal carrier father (16). Val355Met was detected in a boy with micropenis and testicular regression syndrome and a normal carrier brother (10). Thus, the wide range of phenotypes of individuals with mutations in the *NR5A1* gene includes normal sexual development, or at least normal enough to guarantee fertility.

Overall, we analyzed testis tissue of five heterozygous *NR5A1* patients (Table 1 and Fig. 3) and found similar alterations as seen in patients with mutations in the steroidogenic acute regulatory protein (StAR). StAR mediates cholesterol transfer into the mitochondrion toward the P450ssc enzyme cleavage system (30, 31). Therefore,

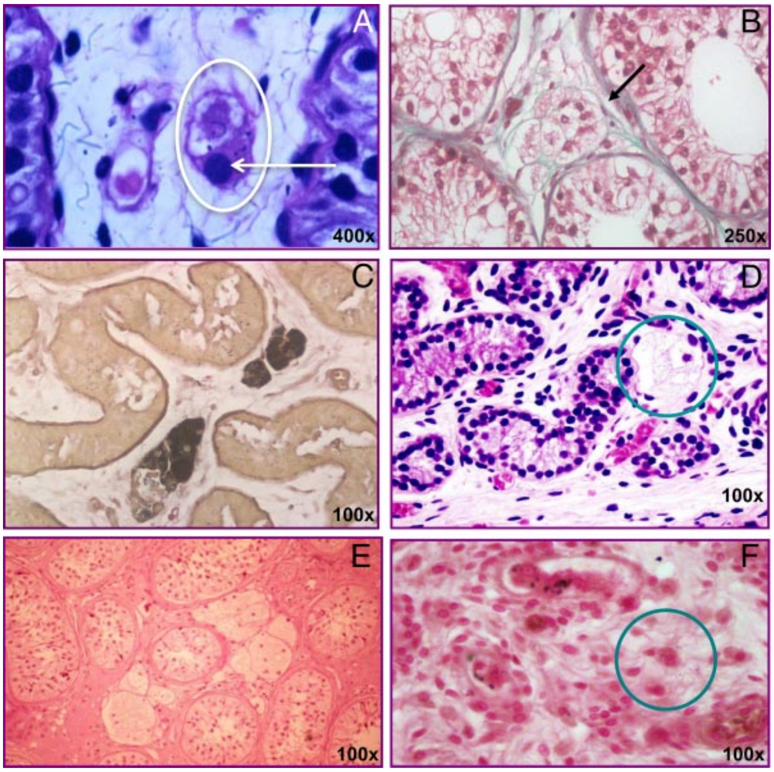

FIG. 3. Histology findings of testes of patients 2, 7, and 8 with heterozygous *NR5A1* mutations. A, Hematoxylin eosin stain (HE) Image originates from patient 2 (47,XYY DSD) at the age of 3.5 yr and shows interstitial tissue (between two seminiferous tubules) with some Leydig cells (one is *circled*) with cytoplasmic vacuoles (*arrow*). B and C, Images belong to patient 7 (46,XY DSD) at the age of 2 yr. B, Trichrome stain image shows interstitium surrounded by different seminiferous tubules. In these tubules, with a thin tunica propria, there are predominantly Sertoli cells but no spermatogonia; only a few nests of xanthomized Leydig cells (*arrow*) can be seen in the interstitium. C, Osmium stain image reveals that the cytoplasmic vacuoles of Leydig cells contain lipids (*dark brown* staining indicates osmium deposits). D, Image originates from patient 8 (46,XY DSD) at the age of 2 yr and shows seminiferous tubules full of pre-Sertoli cells but without germ cells. As in patient 7, the interstitium contains some nests of Leydig cells (*circled*) with a xanthomatous appearance. None of these structures can be seen in normal testes. E, Image depicts testis histology (HE) of a patient with *StAR* mutations at age 3 yr showing similar histological findings as seen in our *NR5A1* patients. F, Image corresponds to an osmium histochemical staining of normal testis tissue originating from a 2-yr-old subject without any positivity in Leydig cell cytoplasm (*circle*).

severe StAR deficiency causes intracellular accumulation of lipids and disrupts steroidogenesis. Leydig cells of patients with SF-1 mutations may also be defective in the conversion of cholesterol to pregnenolone (mediated by CYP11A1/P450ssc). In addition, SF-1 deficiency affects further enzymes of steroidogenesis. Thus, similar mechanisms may cause vacuolization of Leydig cells due to accumulation of lipid droplets not processed by steroidogenesis. In our tissue samples, osmium staining confirmed this for the first time, although other reports have presented histological data for testis of subjects with *NR5A1* mutations without describing lipid accumulation (4, 7–11, 14, 17–20, 22, 24, 26).

Overall, data suggest a progressive degeneration of the testicular tissue in individuals with mutations in *NR5A1*. Relatively preserved testicular architecture progresses to dysgenesis in babies and to replacement with connective tissue causing loss of seminiferous tubules and leading to absence of gonadal tissue in adults. Regarding the cellular profile, children of young ages up to puberty have rare germ cells (9, 11, 14, 17, 20, 22, 26) and nests of (vacuolized) Leydig cells (11, 14, 17–20, 26). 46,XY DSD patients carrying heterozygous SF-1 mutations (with undervirilization and diminished fertility index) demonstrate that testosterone secretion is variably affected in fetal life and may progress, probably due to interstitial cell degeneration, similar to what occurs in patients with *StAR* mutations. Similarly, but at puberty or even later, 46,XX carrier females present with ovarian insufficiency (10, 17, 18). Because this process appears to affect steroidogenesis of only the gonads and not the adrenals, the evolution appears reverse to nonclassic congenital lipoid adrenal hyperplasia due to mild *StAR* mutations, where gonadal function may be preserved into adulthood whereas adrenal insufficiency usually manifests at a young age (32, 33).

The 10 novel *NR5A1* mutations described in this paper are located in exons 2 to 5 (Figs. 1 and 2). Generally, mutations in the DBD region that is crucial for transcription factor binding to promoters (2) are more severe. The first four mutations (Val20Leu, His24Tyr, His24ThrfsX51, and Cys30Trp) are located in the first zinc-finger domain of the DBD but not in the P box. Thus, they are involved in the binding of the major groove of DNA but not in the specific recognition of the DNA target sequence (hormone response element) (2). His24ThrfsX51 will result in a truncated protein with total loss of function. Gly90Arg is located in the Ftz-F1 box, which is considered a stabilizing region for the binding of SF-1 to DNA (2, 34, 35). Pro130ArgfsX165 and Gln206ThrfsX20 in the hinge region result in a truncated protein with no LBD, which will lead to an inactive SF-1. The remaining mutations (Leu231_233dup, Pro235Leu, and Trp302Stop) are located in the LBD. This region modulates SF-1 activity owing to its C-terminal AF-2 domain, which binds phospholipid ligands (2, 20, 36, 37) and other cofactors (2). Thus, mutations in the DBD are expected to lead to markedly affected transactivation assays, whereas LBD mutations may have varying effects depending on their location and alterations in the ligand specificity/recognition.

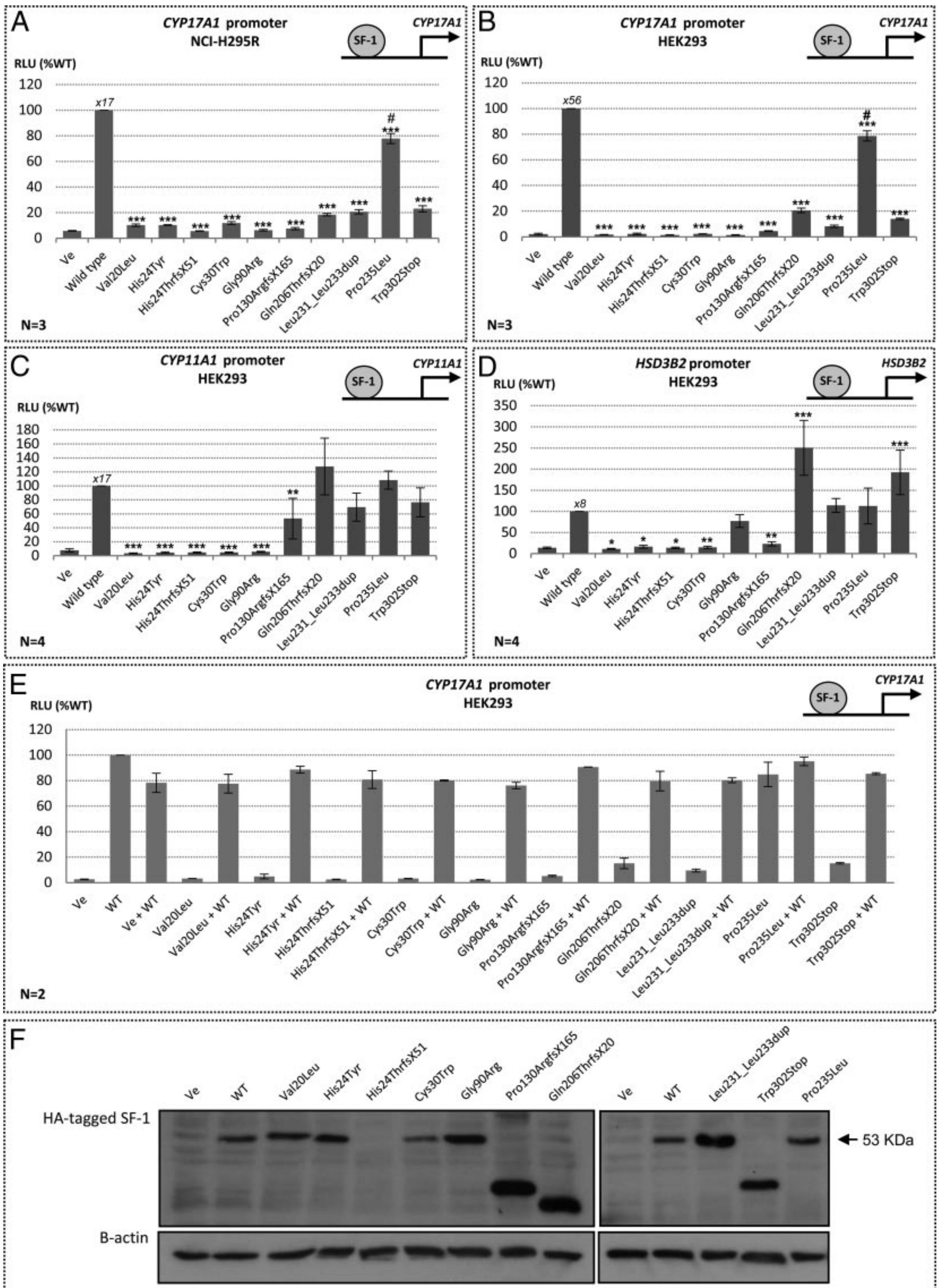

FIG. 4. Functional and expression studies of the 10 novel *NR5A1* mutations. A–D, The ability of WT and mutant NR5A1 to activate steroidogenic enzyme promoter luciferase reporter constructs was tested in adrenal NCI-H295R cells (A) and nonsteroidogenic HEK293 cells (B–D). A–C, Cells were transiently transfected with NR5A1 expression vectors and CYP17A1 (A and B), CYP11A1 (C), and HSD3B2 (D) promoter reporter constructs. E, Similarly, the impact of the mutants on the activity of the WT SF-1 was checked by transfecting the 10 different mutants with or without WT NR5A1 expression vector together with the CYP17A1 promoter reporter construct in HEK293 cells. No dominant negative effect was observed. A–E, Luciferase activity was measured with the Promega Dual Luciferase assay system. Results are expressed as percentage of WT activity, which was set at 100% RLU, and represent the mean and SEM of two to four independent experiments performed in duplicate. F, Western blot showing expression of WT and mutant SF-1 proteins. Hemagglutinin-tagged SF-1 was recognized by HA antibody in the Western blot (band at 53 kD). B-actin was used as a control. Note that His24ThrfsX51 was not detected for its small weight/size. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; #, p.Pro235Leu is statistically different from all other mutants ($P < 0.001$); °, Numbers indicate average fold increase of WT over Ve. RLU, relative light units; Ve, empty vector control; HA, hemagglutinin.

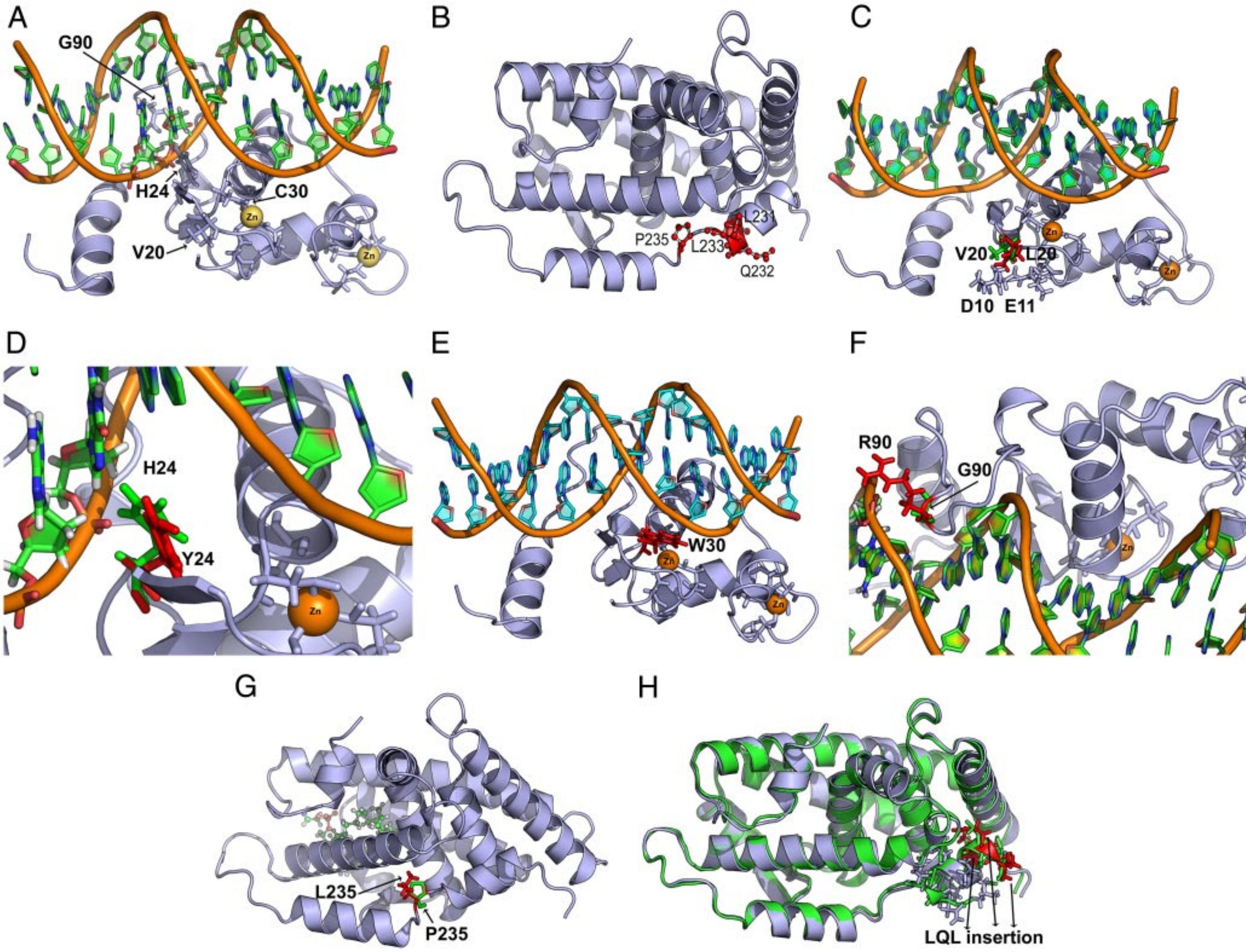

FIG. 5. Structural analysis of SF-1 mutations. In the figures showing the mutated amino acids, the mutant and WT structures have been superimposed; mutant residues are shown in *red*, whereas WT residues are shown in *green*. The structures are drawn as *ribbons*; model and location of mutated amino acids described in this study are shown as stick models. A, Location of mutations in the DBD of the SF-1 protein. B, Structure of SF-1 LBD showing the location of mutations. C, Effect of Val20Leu on SF-1 DBD structure. A change from Val (shown as *green sticks*) to Leu causes alterations in the side chain packing environment of the N-terminal loop due to interaction of altered Leu residue (shown as *red sticks*) with the Asp10 and Glu11. This may modify the core DBD structure and alter the interaction with DNA molecule. D, Effect of His24Tyr mutation of SF-1 DNA interaction. The His24 residue is crucial for DNA binding, and its substitution with Tyr alters the interaction due to a shift of Tyr side chain away from the DNA binding site, breaking the hydrogen bond interactions. E, The Cys30Trp mutation on zinc binding in the human SF-1 structure. The structure of DBD of SF-1 in complex with DNA is shown as in panel A, the zinc ion is shown as an *orange sphere*, and Cys residues involved in zinc binding are shown as *stick models*, with Trp30 change shown in *red*. *In silico* mutagenesis and molecular dynamics simulations were used to change the Cys30 to Trp, and the resulting structure was superimposed on normal structure for analyzing the changes. The change of Cys30 to Trp results in disruption of a Cys-zinc contact, resulting in an unstable structure. F, Effect of Gly90Arg mutation. The Gly90 residue is located near the DNA binding site, and its mutation to Arg results in a major change at the binding site due to the large positively charged Arg side chain. The change from Gly to Arg will alter the binding pattern and may have variable effects, depending on the sequence of DNA interaction partner. Additional interactions with DNA will be created due to the addition of Arg side chain at the binding site. G, Effect of Pro235Leu mutation in the LBD of SF-1. The Pro235 separates the two N-terminus helices of the LBD of SF-1. The mutation from Pro to Leu may abolish the break between two helices and create a larger single helix. Such a change will not result in the alteration of the core structure but causes subtle changes in the neighboring residues near the ligand binding site. A likely effect would be modification of ligand binding and recognition and may have variable effects depending on specific ligands. H, Effect of Leu-Gln-Leu insertion on LBD domain of SF-1. The dual repeat units of Leu-Gln-Leu located at the N terminus of LBD form the N-terminal helix, and insertion of another Leu-Gln-Leu repeat unit changes the composition of this helix and results in a longer loop structure located between the two N-terminal helices. The addition of an extra Leu side chain from Leu-Gln-Leu insertion creates changes in the environment of neighboring residues to accommodate the extra Leu side chain pointing toward the core of the LBD. Specific effects of such a change are hard to predict, and a variable effect depending on specific ligand is expected.

In vitro promoter transactivation assays have been used previously to assess the functional impact of sequence variations (4, 6). Therefore, we tested the 10 novel SF-1 variants for their ability to transactivate different promoters of steroidogenic enzymes in steroidogenic adrenal NCI-H295R and nonsteroidogenic human embryonic kidney HEK293 cells. We found that SF-1 mutations in the DBD consistently showed impaired transactivation activity when studied on different promoters and in different cell systems. By contrast, promoter studies of mutations located outside the DBD showed a more variable picture with different promoters or cell systems. For *NR5A1*, Pro235Leu minor changes in activity were found leaving the question open whether this mutation may be disease causing. The observation that functional tests correlate poorly with phenotype was also made by several other investigators (6, 7, 10, 20, 38) indicating that results from these studies may be of limited value and that testing of several promoters in different cell systems may be necessary to assess the functional impact of *NR5A1* sequence variations. Because most patients harbor heterozygote *NR5A1* mutations, dominant negative effect may be considered and was therefore tested. However, we and others (6–8, 11, 12, 14, 20, 29) did not find experimental confirmation for such a mechanism of mutant SF-1 over WT protein in cell models. *In vitro* protein structure studies have not been able to give a clear picture on the structure-function correlation of sequence variations in LBD.

In addition to the diversity of *NR5A1* mutations in the *in vitro* studies, phenotype variability of *NR5A1* mutations in patients make a genotype-phenotype correlation (so far) impossible. Because SF-1 is a transcription factor, (co-)modulators may play a pivotal role, and their number may have been underestimated in the past. In a recent publication, ChIP-on-chip analysis revealed a large number of new SF-1 targets that are currently under further investigation (39). Thus, the conundrum of variability in SF-1 mutations may be explained by additional variations in cofactors.

In our group of 46,XY DSD patients, we found the *NR5A1* polymorphism Gly146Ala (rs1110061) in 6.8% (Table 2). It has been detected in other 46,XY DSD patients (8, 11, 12) and 46,XX subjects with sporadic POI (17). Its allele frequency varies depending on the control population studied (http://www.ncbi.nlm.nih.gov/) and occurs frequently in Japanese patients with adrenal disorders (40). It has been associated with micropenis (23, 41) and cryptorchidism (42). Gly146Ala leaves SF-1 transactivation activity unaltered (Supplemental Fig. 1) and does not affect cofactor interaction and cellular localization (11, 40). Therefore, it has been considered a polymorphism only. However, after finding this polymorphism in so many 46,XY DSD subjects, and knowing that *in vitro* functional tests are poor predictors, its causal role remains elusive.

In conclusion, the detection of 10 novel *NR5A1* mutations in nine of 100 46,XY DSD patients and in one of two 46,XX patients with ovarian insufficiency highlights the crucial role of SF-1 in sexual development and function. Clinical and biochemical phenotypes of these 46,XY DSD patients are variable and genotype-phenotype correlation remains elusive. Although functional studies help in defining the impact of *NR5A1* sequence variations, results differ depending on the *in vitro* systems employed. Overall the SF-1 system is very complex and appears to involve as yet unknown modulators, whereas dominant negative effects may be excluded.

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