Am. J. Hum. Genet. 62:855–864, 1998

DAX1 Mutations Map to Putative Structural Domains in a Deduced Three-Dimensional Model

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Summary

The DAX1 protein is an orphan nuclear hormone receptor based on sequence similarity in the putative ligand-binding domain (LBD). *DAX1* mutations result in X-linked adrenal hypoplasia congenita (AHC). Our objective was to identify *DAX1* mutations in a series of families, to determine the types of mutations resulting in AHC and to locate single–amino-acid changes in a DAX1 structural model. The 14 new mutations identified among our 17 families with AHC brought the total number of families with AHC to 48 and the number of reported mutations to 42; 1 family showed gonadal mosaicism. These mutations included 23 frameshift, 12 nonsense, and six missense mutations and one single-codon deletion. We mapped the seven single–amino-acid changes to a homology model constructed by use of the three-dimensional crystal structures of the thyroid-hormone receptor and retinoid X receptor α. All single–amino-acid changes mapped to the C-terminal half of the DAX1 protein, in the conserved hydrophobic core of the putative LBD, and none affected residues expected to interact directly with a ligand. We conclude that most genetic alterations in *DAX1* are frameshift or nonsense mutations and speculate that the codon deletion and missense mutations give insight into the structure and function of DAX1.

Received September 18, 1997; accepted for publication February 11, 1998; electronically published March 20, 1998.

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0002-9297/98/6204-0018$02.00

Introduction

The X-linked cytomegalic form of adrenal hypoplasia congenita (AHC; MIM 300200 [http://www.ncbi.nlm.nih.gov/omim]) is caused by loss of *DAX1* (dosage-sensitive sex-reversal adrenal hypoplasia congenita on the X chromosome, gene 1) function, through deletion of the entire gene (McCabe 1995) or intragenic mutations with profound effects (Muscatelli et al. 1994; Zanaria et al. 1994; Guo et al. 1995*b*, 1996*a;* Habiby et al. 1996*;* Nakae et al. 1996; Yanase et al. 1996; Schwartz et al. 1997; Takahashi et al. 1997). AHC is an inborn error in the development (Epstein 1995) of the adrenal gland, characterized by marked underdevelopment or absence of the permanent adult zone of the adrenal cortex and by structural disorganization of the fetal cortex (Marsden and Zakhour 1978; Seltzer et al. 1985). In addition to adrenal insufficiency with low glucocorticoid and mineralocorticoid levels, affected males also have hypogonadotropic hypogonadism (HH) (Prader et al. 1975; Golden et al. 1977; Zachmann et al. 1980; Hay et al. 1981; Virdis et al. 1983; Martin and Martin 1985). Previous clinical investigations into the etiology of HH in patients with AHC indicated mixed results regarding hypothalamic and/or pituitary origin (Gordon et al. 1984; Kruse et al. 1984; Kikuchi et al. 1987; Bovet et al. 1988; Partsch and Sippell 1989; Kletter et al. 1991; McCabe 1996). Recent results have resolved this controversy by showing that DAX1 acts at the levels of both the hypothalamus and the pituitary, to mediate pubertal development (Habiby et al. 1996), consistent with the pituitary and hypothalamic expression of DAX1 in humans (Guo et al. 1995*a*) and mouse (Ikeda et al. 1996; Swain et al. 1996).

DAX1 is an orphan member of the nuclear hormone-receptor superfamily, on the basis of sequence conser-

vation in the C-terminal portion of the protein, which is the putative ligand-binding domain (LBD) (Zanaria et al. 1994; Burris et al. 1996). The orphan receptor with the closest apparent relationship to DAX1 is the small heterodimer partner (SHP) that interacts with and blocks transactivation of the retinoic acid receptor (RAR), the retinoid X receptor (RXR), and the thyroid-hormone receptor (TR), suggesting a role for SHP in negative regulation of receptor-dependent signaling (Seol et al. 1996). The N-terminal portion of DAX1 contains 3.5 repeats of a 65–67-amino-acid motif and forms two putative zinc-finger domains, the cysteines of which are absolutely conserved in the human and murine sequences (Zanaria et al. 1994; Guo et al. 1995*b*, 1996*b;* Burris et al. 1996; Swain et al. 1996). A steroidogenic factor 1 (SF1) response element (SF1-RE) is present in the *DAX1* promoter (Guo et al. 1996*a*), is conserved in the murine *Ahch* promoter (Guo et al. 1996*b;* Swain et al. 1996), and binds SF1 in vitro (Burris et al. 1995). SF1 enhances expression of reporter-gene constructs containing the *DAX1* promoter, in human adrenal cortical carcinoma NCI-H295 cells (Yu et al. 1996*;* Vilain et al. 1997) but not in constructs containing the mouse *Ahch* promoter transfected into Y1 adrenal cortical or MA-10 Leydig cells (Ikeda et al. 1996), and deletion of the SF1-RE region or of the 9-bp SF1-RE in the DAX1 promoter eliminates SF1 enhancement of these reporter-gene constructs (Yu et al. 1996*;* Vilain et al. 1997). Recent data demonstrate direct interaction of DAX1 with SF1, and cotransfection with *DAX1* and *SF1* inhibits SF1-mediated transactivation (Ito et al. 1997). Whereas the DAX1 amino acids involved in the DAX1-SF1 protein-protein interaction appear to reside in the N-terminal half of the protein, the inhibitory activity of DAX1 localizes to the C-terminal portion. Therefore, although limited information is available on functional elements within the promoter and functional domains of the protein, additional structure-function insights are required, to understand the role of the *DAX1* gene in the development and function of the hypothalamic-pituitary-adrenal/gonadal axis.

The purpose of the current investigation was to identify mutations within the *DAX1* gene, in order to determine the types of intragenic mutations responsible for AHC and to identify the positions of single–amino-acid changes in a structural model of DAX1, to attempt to gain a better understanding of the relationship between its structure and function. Of the 17 families reported here, only 3 had missense mutations, and 1 had a single-codon deletion; the others had frameshift or nonsense mutations. These four single–amino-acid changes, plus three others reported elsewhere (Muscatelli et al. 1994; Schwartz et al. 1997; Takahashi et al. 1997), all map within the C-terminal portion of DAX1 and, by homology with the crystal structures of the TR (Wagner et al. 1995) and RXRα (Bourguet et al. 1995), lie within the conserved hydrophobic core of the putative LBD involved in hormone-independent activities such as intermolecular dimerization.

Patients and Methods

Patients

The protocols for sample collection and analysis were approved by the institutional review boards at Baylor College of Medicine and UCLA School of Medicine. Specimens, including whole blood for DNA preparation and/or for lymphoblastoid cell-line generation, were collected from males with clinical features of AHC and from their family members, after informed consent was obtained. The affected males who were old enough also exhibited HH.

SSCP Analysis

SSCP analysis was performed in accordance with the method described by Orita et al. (1989). The coding portion of the *DAX1* gene and the contiguous genomic intron-exon boundaries were divided into 10 overlapping regions, which were amplified, denatured, electrophoresed, and autoradiographed as described elsewhere (Guo et al. 1996*a*). PCR products with anomalous mobility in the gel were sequenced.

DNA Sequence Analysis

For the identification of mutations, after SSCP, sequencing of PCR-amplified DNA fragments was performed by manual Sanger dideoxy-chain termination (Sanger et al. 1977), by use of the Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical) or by automated DNA sequencing with an ABI 377 automated DNA sequencer, with cycle sequencing and dye-terminator chemistry. Direct genomic sequencing without prior SSCP also was used to identify mutations; the primers used for direct sequencing have been reported elsewhere or were derived from the genomic sequence (Guo et al. 1996*a*). Probes used for dot-blot confirmation of mutation 16, 518del23bp (table 1), by allele-specific oligonucleotide hybridization (fig. 1) were the following: normal, 5′-GCT CCT ACT TCG CGC AGA GG-3′; and mutant, 5′-TAC CCC CTG GCC CAC CAC GC-3′. In the family with gonadal mosaicism (mutation 16; table 1 and fig. 1), the matrilineal relationships of the affected males and the carrier females to individual I-1 were confirmed by automated sequencing of the mitochondrial D-loop (Mumm et al. 1997).

Table 1

Mutations in the *DAX1* Gene

Mutation Type and No.	Mutation	Patient	Reference
Frameshift:			
1	749delGTCCACAGGTG	MIN/MEN	Zanaria et al. (1994)
2	547insACCC	2957	Zanaria et al. (1994)
3	785 complex deletion/ insertion	MA	Muscatelli et al. (1994)
4	315insT	LB/LM	Muscatelli et al. (1994)
5	550delAA	2791	Muscatelli et al. (1994)
6	562insCAGG	3741	Muscatelli et al. (1994)
7	273CG-T	2094	Muscatelli et al. (1994)
8	1292delG	RE	Guo et al. (1995*b*)
		TP	Guo et al. (1995*b*)
9	839delT	TS	Yanase et al. (1996)
10	1267insCC	DT	Guo et al. (1996*a*)
11	154delGA	JLB	Guo et al. (1996*a*)
12	935delC	Case 6	Nakae et al. (1996)
13	1376delAT, insG	Case 4	Nakae et al. (1996)
14	Complex deletion/substitution: 985delG, G987A	JW	Habiby et al. (1996)
15	1250insGGAT	MH	Habiby et al. (1996)
16	518del23bp	RR/RD	This study
17	543delA	NW	This study
18	754delC	MKA	This study
19	416insA	KK	This study
20	942insAC	EC	This study
21	375insGCCC	JDW	This study
22	501delA	HD	This study
23	629insG	JWC	This study
Nonsense:			
24	W235X	BF	Zanaria et al. (1994)
		2065	Muscatelli et al. (1994)
		JF	This study
25	Q283X	MT/CB	Muscatelli et al. (1994)
26	W369X	AO/LS	Muscatelli et al. (1994)
27	L263X	BR	Muscatelli et al. (1994)
28	W172X	3743/3744	Muscatelli et al. (1994)
29	W171X	TK	Yanase et al. (1996)
		RM	This study
30	Y91X	PS/RH	Guo et al. (1996*a*)
		Case 2	Nakae et al. (1996)
31	Q395X	Case 1	Nakae et al. (1996)
32	Y271X	Case 5	Nakae et al. (1996)
33	S153X	BA/BB	This study
34	Q357X	GF	This study
35	Q252X	DT/ST	This study
Missense:			
36	R267P	2687/2688	Muscatelli et al. (1994)
37	N440I	From Greenland	Schwartz et al. (1997)
38	A300V	From Akita, Japan	Takahashi et al. (1997)
39	R425G	TC/NC	This study
40	V385G	LRP	This study
41	E377K	EM	This study
Codon deletion:			
42	dV269 (in-frame deletion)	2115	Muscatelli et al. (1994)
		AS	This study

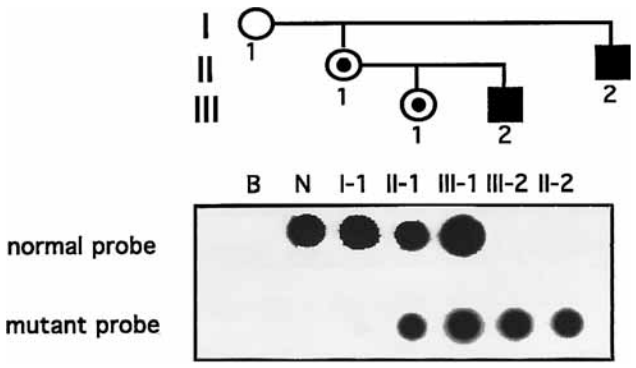

Figure 1 Family with frameshift mutation 16 (table 1) caused by 23-bp deletion, exhibiting gonadal mosaicism. Allele-specific oligonucleotide hybridization confirmed sequence results and showed that individual I-1, the mother of a carrier daughter (II-1) and an affected son (II-2), did not carry the mutant allele and, therefore, exhibited gonadal mosaicism. B = negative control; and N = normal individual.

Sequence Alignment and Computer Modeling

Sequence alignments and secondary-structure prediction for human DAX1, mouse Ahch, mouse SHP, the rat TRα (rTRα) isoform, and the human RXRα (hRXRα) isoform were performed using PHD (European Molecular Biology Laboratory [http://www.embl-heidelberg.de/predictprotein]) and were adjusted outside the conserved helices (Rost and Sander 1994). The Homology module in Insight II (Biosym Technologies) was used to generate a model for the DAX1 LBD, on the basis of the known structures of rTRα (Wagner et al. 1995) and hRXRα (Protein Data Bank entry 1LBD [http://www.nyu.edu/pages/mathmol/pdb.html]) (Bourguet et al. 1995). The rTR and hRXR LBDs were superimposed by use of the Cα atoms of hydrophobic core helices H1 (rTR residues I168–T178 and hRXR residues V232–V242), H3–H5–H6 (rTR residues P254–R266 and hRXR residues K274–R316), H9 (rTR residues D313–V322 and hRXR residues K364–I373), and H10 (rTR residues V335–N354 and hRXR residues P386–K405) (root mean squared deviation of 1.03 Å, for 168 Cα; Wurtz et al. 1996). Coordinates for the DAX1 LBD were generated for structurally conserved regions, assigned by low root mean squared deviation (<1.0 Å), between rTRα and hRXRα. The loop between helices H10 and H11, which assumes different confirmations in rTRα and hRXRα, was modeled on that in hRXRα. Helix H1, which shows weak sequence identity across the nuclear receptors, was tentatively assigned to residues L196–H206. The model for the DAX1 LBD consisted of residues L196–H206 (H1), V253–E307 (H3–H5–H6), and V358–I470 (H8–H9–H10–H11), representing the hydrophobic core of the putative LBD.

Results

SSCP analysis and/or genomic sequencing of the coding portion of the *DAX1* gene, as well as of the boundaries between the intron and exons 1 and 2, identified mutations in 17 new families for which mutations had not been documented previously (table 1). Among these 17 families, 8 had frameshifts, 5 had nonsense mutations, 3 had missense mutations, and 1 exhibited an in-frame triplet deletion resulting in loss of a single codon. Fourteen of these mutations had not been observed previously; but one nonsense mutation, W235X, had been reported twice before (Muscatelli et al. 1994; Zanaria et al. 1994), another nonsense mutation, W171X, had been observed once before (Yanase et al. 1996), and the codon deletion, dV269, had been described by another group (Muscatelli et al. 1994).

Initially, on the basis of previous work from our group (Guo et al. 1996*a*), we screened for mutations, using SSCP analysis. However, the size of the *DAX1* coding sequence, the simplicity of its two-exon genomic organization, and the improved sensitivity of direct sequencing caused us to change our strategy from SSCP screening to direct genomic sequencing as our primary mutation-detection methodology, during the course of these investigations. All mutations were confirmed by at least one other modality, alteration of restriction-enzyme cleavage and/or dot-blot hybridization.

The family with the 23-bp frameshift mutation, 518del23bp (mutation 16; table 1), was evaluated by direct sequencing and dot-blot hybridization (fig. 1). The results from whole blood indicated that individual I-1, whose son and daughter's son had AHC, was not a carrier. Blood samples were redrawn from each of the family members, with extreme care taken during the labeling in the clinic and the laboratory, and the original results were confirmed. The matrilineal relationships of carrier females and affected males within this family to individual I-1 were confirmed by the mitochondrial D-loop sequence (data not shown). Two different buccal brushings were collected from individual I-1, and no evidence of the presence of the mutation was observed in either sample (data not shown). Therefore, the results in this family were consistent with gonadal mosaicism for the DAX1 mutation in individual I-1, with the mutation occurring at a time during her development such that not all cell lineages were affected, but at least a portion of the cells that gave rise to her ova were involved.

The frameshift and nonsense mutations were distributed throughout the DAX1 coding region (fig. 2). However, the alterations resulting in single–amino-acid changes that we observed during these investigations and the other missense mutations—R267P (Muscatelli et al.

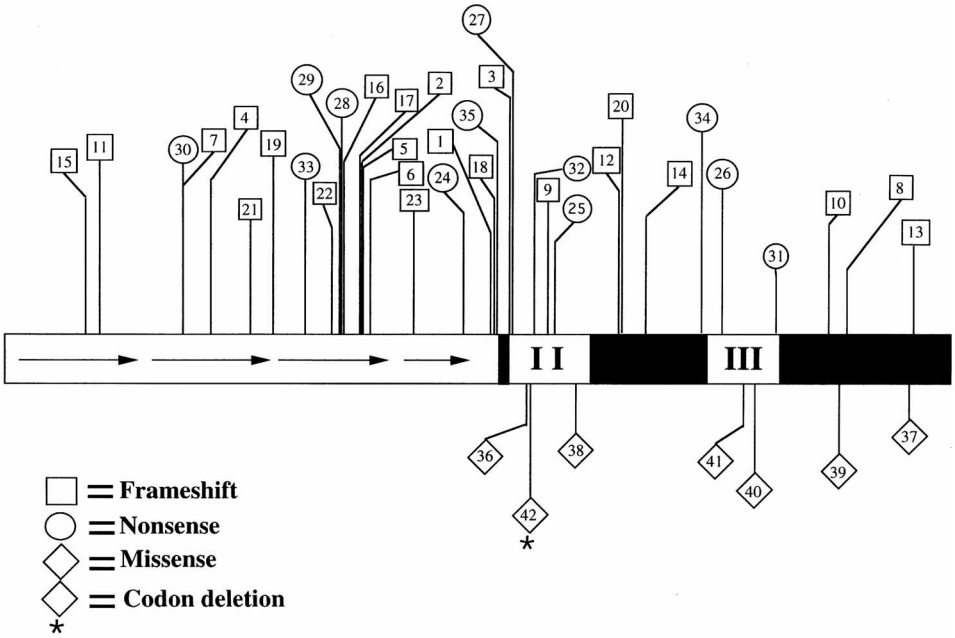

Figure 2 Diagram showing positions of the frameshift, nonsense, and missense mutations and the single-codon deletion, in a model of the DAX1 protein. The frameshift and nonsense mutations are distributed throughout the molecule. The single–amino-acid alterations were found to be located only in the C-terminal half of the protein, representing the putative LBD.

1994), N440I (Schwartz et al. 1997), and A300V (Takahashi et al. 1997)—were found only in the C-terminal portion, which is the region that showed similarity to the LBDs of other nuclear hormone receptors (figs. 2 and 3). We sought to determine whether there was any pattern to single–amino-acid changes in a three-dimensional model of the LBD, on the basis of homology with rTRα_1 (Wagner et al. 1995) and hRXRα (Bourguet et al. 1995) (fig. 4).

In a proposed canonical fold for the nuclear receptors, the hydrophobic core consists of α-helices H1, H3–H5–H6, H9, H10, and H11 (Wurtz et al. 1996). All the single–amino-acid alterations in these naturally occurring DAX1 mutations—R267P, dV269, A300V, E377K, V385G, R425G, and N440I (table 1)—mapped within the hydrophobic core of the putative DAX1 LBD (fig. 4*B*). Certain mutations affected residues predicted to form intramolecular contacts in the DAX1 LBD. For example, at residues E377 and R425, the glutamate and arginine side chains formed a conserved charge pair linking α-helices H9 and H11 (Wagner et al. 1995). Mutations E377K and R425G would disrupt this linkage. Residues homologuous to E377 were conserved absolutely in related members of the nuclear-receptor superfamily (fig. 3), and a charge pair involving those residues was observed in the crystal structures of the rTR, hRXR, and human RAR (hRAR) LBDs (Bourguet et al. 1995; Wagner et al. 1995; Wurtz et al. 1996). Two other mutations, R267P and dV269, would cause structural perturbations in helix H3: mutation R267P inserted a helix-breaking proline, and mutation dV269 altered the phase of the helix. The mutations occurred either immediately preceding or at the beginning of a highly conserved sequence signature for the typical nuclear-receptor LBD, (FW)AKxhxPxFxxLxxxDQxxLL (Wurtz et al. 1996). The motif provided the hydrophobic core for an unusual structure, which is present in the rTR, hRXR, and hRAR LBDs and is adopted by the loop between H3 and H5–H6.

Mutations V385G and A300V affected buried hydrophobic residues. Mutation V385G replaced a buried hydrophobic residue in H9, a buried core helix, with a less bulky residue that increased flexibility at this position. Conversely, mutation A300V replaced a buried hydrophobic residue in helix H6, with a bulkier residue in a tightly packed environment defined by α-helices H8 and H9.

Finally, mutation N440I exchanged a hydrophilic side chain for a hydrophobic side chain in a polar environment. Residue N440 occurred in H11, in a solvent-exposed invagination, near the bend in H5–H6. Replacement of the hydrophilic asparagine side chain for the bulkier, hydrophobic isoleucine might lead to steric clash with H5–H6.

The amino acid deletion and substitutions identified in patients with AHC all involved residues that were

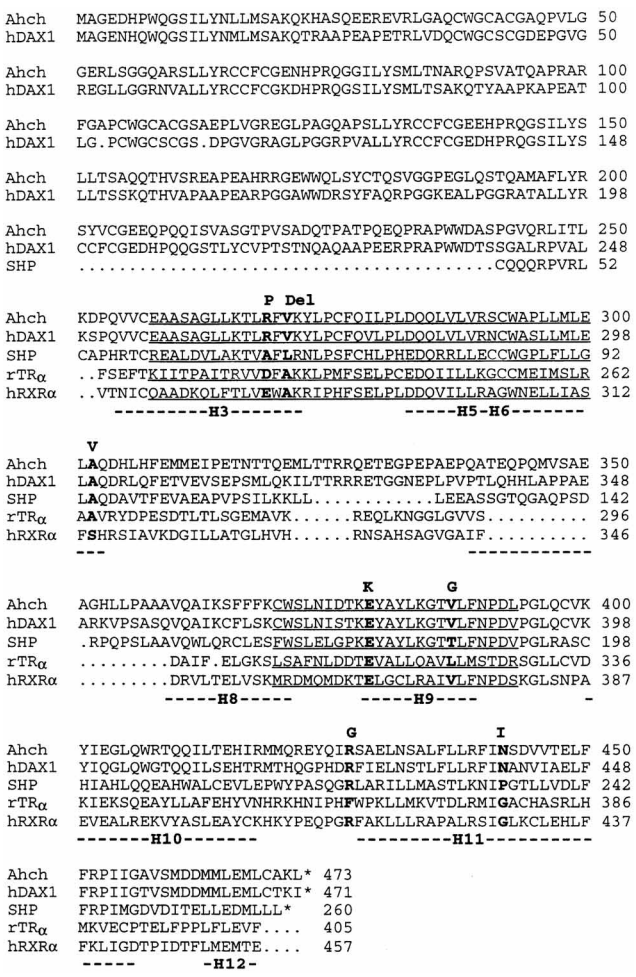

Figure 3 Locations of single–amino-acid alterations, shown in a sequence alignment of Ahch and DAX1, compared with the related nuclear hormone receptors SHP, rTRα, and hRXRα. The missense mutations and single-codon in-frame deletion are shown above the comparison, in boldface, and the corresponding positions in each of the aligned proteins also are given in boldface. The positions of the helices discussed in the text are shown by dashed lines below the comparisons.

conserved in human DAX1 and mouse Ahch (fig. 3) (Guo et al. 1996*b;* Swain et al. 1996), consistent with key structural and functional roles for these residues. In fact, of the seven mutated amino acids, one residue (V385) was conserved in one other, related nuclear hormone receptor, two residues (A300 and R425) were conserved in two others, and a third residue (E377) was conserved in three others (fig. 3).

Discussion

We identified *DAX1* mutations in individuals in 17 previously unreported families with AHC. Each of these families had a different mutation, although three mutations had been reported by others: W235X had been described twice before (Muscatelli et al. 1994; Zanaria et al. 1994), and dV269 and W171X each had been observed by another group (Muscatelli et al. 1994; Yanase et al. 1996). Therefore, these 17 families represent 14 new mutations in the *DAX1* gene, bringing the totals to 48 AHC families and 42 mutations identified in this gene (Muscatelli et al. 1994; Zanaria et al. 1994; Guo et al. 1995*b*, 1996*a;* Habiby et al. 1996*;* Nakae et al. 1996; Yanase et al. 1996; Schwartz et al. 1997; Takahashi et al. 1997; this report); 1 mutation was observed in 3 families, and 4 mutations were observed in 2 families each (table 1) (Muscatelli et al. 1994; Zanaria et al. 1994; Guo et al. 1995*b*, 1996*a;* Nakae et al. 1996; Yanase et al. 1996; this report). For each of these five mutations, for which more than one family is described, our group determined a mutation in one or both of the families (Guo et al. 1995*b*, 1996*a;* this report). To our knowledge, the families in our study are unrelated to the families reported with identical mutations, by others, and ethnicity differed for some families with the same mutation. We had identified previously two unrelated families with the 1292delG frameshift mutation, and evaluation of the mitochondrial D-loop sequence (Mumm et al. 1997) showed substantial differences between the two probands, confirming that their families were not closely related (Y.-H. Zhang, B.-L. Huang, and E. R. B. McCabe, unpublished data).

The majority of the mutations described, to date, involved introduction of a new, premature stop codon in the DAX1 transcript, by frameshift or nonsense mutations, resulting in a shorter deduced protein product (table 1). A total of 23 frameshift mutations have been reported in 24 families, and 12 nonsense mutations have been reported in 16 families. Only seven single–amino-acid alterations have been described: six missense mutations in 6 families and a single-codon deletion in 2 families.

In addition to the observation of identical mutations in apparently unrelated families, one example of gonadal mosaicism was documented. The mother of an affected son and an obligate carrier daughter did not evidence the 23-bp deletion observed in her affected son and grandson and in her carrier daughter and granddaughter. Four different samples, representing two blood specimens drawn at separate times and two independent buccal brushings, were examined, and none showed detectable mosaicism. Documentation of apparent gonadal mosaicism in this family has important implications for genetic counseling of other families, with DAX1 mutations, in which one affected male is the offspring of a noncarrier female. The counselor must acknowledge to the consultand that the existence of gonadal mosaicism in the family reported in this study indicates the possibility that the consultand may have another affected

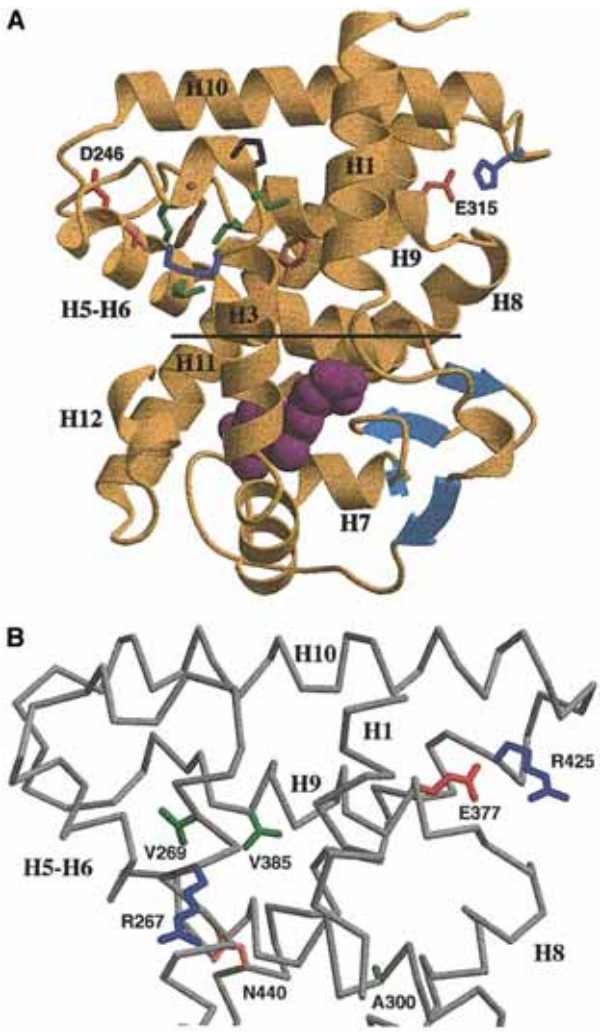

Figure 4 Models of LBDs for rTRα and human DAX1, showing subdomain structure and the impact of DAX1 mutations on structure. *A*, Ribbon diagram of the rTRα isoform, as an example of a nuclear receptor LBD. The black, horizontal line shows the division of the receptor into two subdomains: the structural subdomain, containing the hydrophobic core of the LBD, is above the line, and the ligand-binding subdomain is below the line. Shown within the hydrophobic core are the highly conserved residues discussed in the text. The LBD sequence signature, (FW)AKxhxPxFxxLxxxDQxxLL (Wurtz et al. 1996), runs from H3 to H5–H6; in rTRα, the specific sequence is 232-**F**A**KK**L**P**M**F**SE**L**PCE**DQ**II**LL**-251. The conserved charge pair between H9 and H11 also is shown; in rTRα, the specific residues are E315 and H358. *B*, Model of hydrophobic core of the human DAX1 LBD, consisting of residues V253–E307 and V358–I470. The sites of the single–amino-acid alterations, in the LBD, causing AHC are labeled.

male or a carrier female, despite the absence of the mutation in her genomic DNA.

Although the frameshift and nonsense mutations are distributed throughout the N-terminal and C-terminal portions of DAX1, corresponding to the DNA-binding domain and the LBD in other nuclear hormone receptors (Burris et al. 1996), the single–amino-acid changes reported, to date, are found only in the LBD. Each of the six mutations altering single amino acids (Muscatelli et al. 1994; Schwartz et al. 1997; Takahashi et al. 1997; this report) affects residues that are identical in human DAX1 and mouse Ahch (Guo et al. 1996*b;* Swain et al. 1996) and, by homology with the three-dimensional structures of rTRα_1 (Wagner et al. 1995) and hRXRα (Bourguet et al. 1995), map to the hydrophobic core of the putative DAX1 LBD (fig. 4). The modeling of these missense and single-codon–deletion mutations provides us with the opportunity to compare and contrast these amino acid changes in DAX1 with those in other nuclear hormone receptors.

The LBD of a typical nuclear hormone receptor participates in hormone binding, dimerization, heat-shock protein-complex formation, and transcriptional activation and repression (Tsai and O'Malley 1994; Ribeiro et al. 1995). The three-dimensional structures of the LBDs in three nuclear hormone receptors, hRXR, rTR, and hRAR, recently were determined by x-ray crystallography (Bourguet et al. 1995; Wagner et al. 1995; Wurtz et al. 1996). All three share a common fold consisting of three layers of α-helices, described as an α-helical sandwich. Furthermore, an apparent subdomain structure exists in which the LBD may be divided into two subdomains: a structural subdomain consisting of H1, H3–H5–H6, H9, H10, and H11, representing the hydrophobic core of the LBD, and a ligand-binding subdomain consisting of H3, S1–2, H7, H8, H11, and H12, which recognizes and binds the cognate ligand (fig. 4*A*). Functionally, the subdomains are implicated in distinct activities of the receptor: the structural subdomain mediates hormone-independent activities such as dimerization or unliganded repression, whereas the ligand-binding subdomain mediates hormone-dependent activities such as transcriptional activation. Mutations in the TR that result in generalized resistance to thyroid hormone cluster exclusively in the ligand-binding subdomain, consistent with a primary defect in hormone-binding and hormone-dependent processes (Wagner et al. 1995; R. L. Wagner, J. D. Baxter, and R. J. Fletterick, unpublished data). On the other hand, genetic alterations causing complete–androgen-insensitivity syndrome, the most severe form of the disease, include mutations in the structural subdomain (R. L. Wagner, J. D. Baxter, and R. J. Fletterick, unpublished data). Therefore, there are two classes of LBD mutations associated with disease: (1) those that disturb ligand binding and that are consistent with altered but continued function; and (2) those that disrupt receptor structure and that profoundly reduce function.

The clustering of mutations in the DAX1 LBD hydrophobic core has definite implications for our understanding of the structure and function of this orphan

nuclear receptor. The absence of DAX1 mutations in residues, which, by analogy with the structures of the liganded rTR and hRXR LBDs, would be expected to bind ligand, suggests either that DAX1 may not be regulated by ligand or that such mutations may cause a different phenotype. Alternatively, since DAX1 mutations reside in the structural subdomain, the mutations might disrupt the ability of DAX1 to dimerize, an activity mediated by the structural subdomain. Several lines of evidence are consistent with this proposal. A conserved pattern of hydrophobic residues in H11 is implicated in dimerization, for several receptors (Fawell et al. 1990). Three of the mutations described here are expected to affect the structure of H11. In the model of DAX1, a charge pair between E377 and R425 stabilized H11 (fig. 4*B*); an identical charge pair was observed in the structure of the hRXR LBD, between the homologous residues E366 and R414, and is predicted to occur in other receptors, such as the estrogen receptor (ER) (Bourguet et al. 1995; Wurtz et al. 1996). Mutation of R503 in the ER, the residue homologous to R425 in DAX1, eliminates homodimerization in vitro (Fawell et al. 1990). Both residues in the charge pair are mutated in DAX1 and are likely to have the same effect. Mutation of a buried hydrophobic residue in H11 also may eliminate dimerization, presumably by alteration of the packing of H11 (Fawell et al. 1990). Mutation N440I, leading to a steric clash between H11 and H5–H6, is similar. However, other results suggest that there is unusual repressor activity near the C-terminus of DAX1, and deletions or point mutations in H11 and in other domains in the putative LBD of DAX1 exhibited impaired inhibition of SF1-mediated transactivation (Ito et al. 1997). Since the LBD of DAX1 shares limited sequence identity with other members of the nuclear-receptor superfamily and even differs from its mouse homologue, deletions or point mutations in H11 and in other regions of the putative LBD of DAX1 may cause AHC by mechanisms other than the elimination of dimerization.

Another region implicated in dimerization is the H3–H5 loop; for example, mutation of D300 in H5 in hTRβ eliminates heterodimerization with RXR (O'Donnell et al. 1991). The H3–H5 loop contains a signature sequence motif that is highly conserved in the nuclear-receptor superfamily. DAX1 mutations R267P and dV269 immediately precede the conserved motif in H3 and are comparably disruptive: R267P inserts a helix-breaking proline into H3, and the in-frame deletion dV269 changes the phase of the helix. Finally, mutations V385G and A300V change the packing within the hydrophobic core of the DAX1 LBD, perturbing its structure.

Thus, we can speculate that the DAX1 single–amino-acid mutations may interfere with dimerization or other activities of DAX1. The closely related orphan receptor SHP appears to dimerize with the retinoid receptors and the TR, leading to repression of gene expression (Seol et al. 1996). The six missense mutations and the single-codon deletion all map to the structural subdomain in our model of the DAX1 protein and alter amino acid residues that are highly conserved and that are predicted to disrupt receptor folding, dimerization, and overall DAX1 function in a major way.

In summary, the two-exon structure of the *DAX1* gene facilitates direct sequencing for mutation detection. One family evidenced gonadal mosaicism, which has important genetic-counseling consequences for families with AHC. Although the majority of the DAX1 mutations identified by our group and by others are frameshift or nonsense mutations, seven single–amino-acid changes have been documented and provide valuable structure-function insights. The absence of missense mutations in the N-terminal half of DAX1 and in the putative ligand-binding subdomain suggests that such mutations may be associated with different phenotypes. These other phenotypes may include a variety of possibilities, such as no clinical findings, isolated HH, or even in utero lethality.

Acknowledgments

This work was supported by grant R01 HD-22563 (to E.R.B.M.). E.V. was supported, in part, by a grant from the Institut National de la Santé Étude et de la Recherche Médicale.

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