

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.

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. 1995b, 1996a; 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. 1995a) 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-

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vation in the C-terminal portion of the protein, which is the putative ligand-binding domain (LBD) (Zanaria et al. 1994; Burriss 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. 1995b, 1996b; Burriss 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. 1996a), is conserved in the murine *Ahch* promoter (Guo et al. 1996b; Swain et al. 1996), and binds SF1 in vitro (Burriss 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 RXR α (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. 1996a). 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. 1996a). 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. (1995b)
		TP	Guo et al. (1995b)
9	839delT	TS	Yanase et al. (1996)
10	1267insCC	DT	Guo et al. (1996a)
11	154delGA	JLB	Guo et al. (1996a)
12	935delC	Case 6	Nakae et al. (1996)
13	1376delAT, insG	Case 4	Nakae et al. (1996)
14	Complex deletion/substi- tution: 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. (1996a)
		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

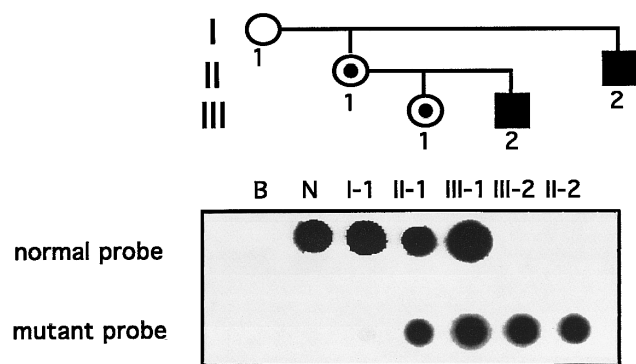


Figure 1 Family with frameshift mutation 16 (table 1) caused by 23-bp deletion, exhibiting gonadal mosaicism. Allele-specific oligonucleotide hybridization confirmed sequence results and showed that individual I-1, the mother of a carrier daughter (II-1) and an affected son (II-2), did not carry the 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 TR α (rTR α) isoform, and the human RXR α (hRXR α) 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 rTR α (Wagner et al. 1995) and hRXR α (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 C α 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 C α ; 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 rTR α and hRXR α . The loop between helices H10 and H11, which assumes different conformations in rTR α and hRXR α , was modeled on that in hRXR α . 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. 1996a), 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.

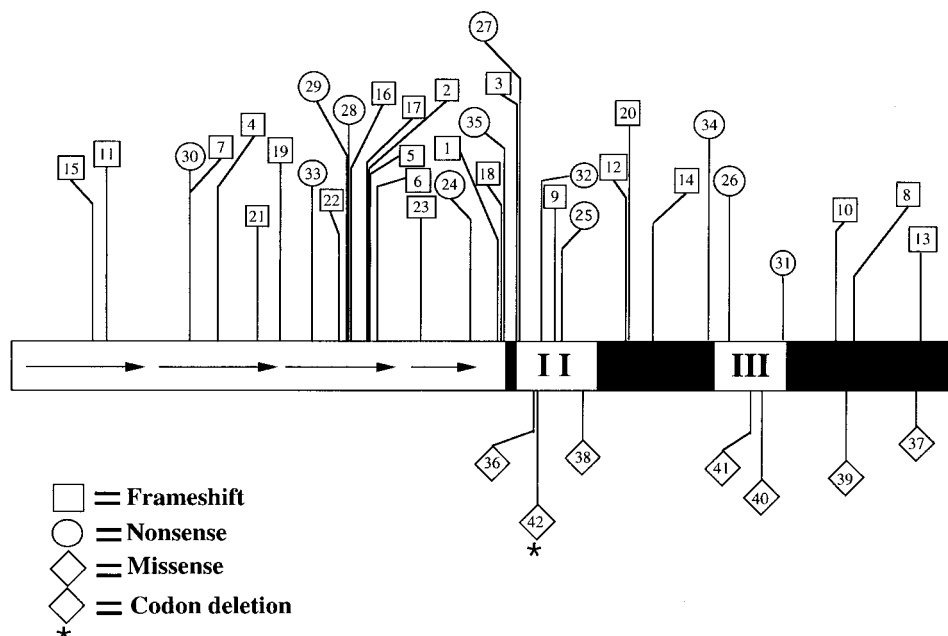


Figure 2 Diagram showing positions of the frameshift, nonsense, and missense mutations and the single-codon deletion, in a model of the DAX1 protein. The frameshift and nonsense mutations are distributed throughout the molecule. The single-amino-acid alterations were found to be located only in the C-terminal 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 rTR α_1 (Wagner et al. 1995) and hRXR α (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. 4B). 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 homologous 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 mu-

tations, 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

Ahch	MAGEDHPWQGSILYNLLMSAKQKHASQEEREVRLGAQWCACCAQPVLG	50
hDAX1	MAGENHQWQGSILYNLLMSAKQTRAAPEAPETRLVDQCWCSCGDEPGVG	50
Ahch	GERLSGGQARSLLYRCCFCGENHPRQGGILYSMLTNRQPSVATQAPRAR	100
hDAX1	REGLLGGRNVALLYRCCFCGKDHPRQGSILYSMLTSAKQTYAAPKAPPEAT	100
Ahch	FGAPWCACGSAEPLVGRGLPAGQAPSLLYRCCFCGEEHPRQGSILYS	150
hDAX1	LG.PCWGCSCGS.DPGVGRAGLPGRPVALLYRCCFCGEDHPRQGSILYS	148
Ahch	LLTSAQQTHVSRAPAHRRGEMWQLSYCTQSVGGPEGLQSTQAMAFLYR	200
hDAX1	LLTSSKQTHVAPAAPEARPGGAWDRSYFARPGGKEALPGGRATALLYR	198
Ahch	SYVCGEEQPQQISVASGTPVSAQDTPATPQEQPRAPWWDASPGVQRLLITL	250
hDAX1	CCFCGEDHPQGGSTLYCVPTSTNQAAPEERPRAPWWDTSAGALRPVAL	248
SHPCQQQRPVRL	52
P Del		
Ahch	KDPQVCEAASAGLLKTLRFVYKLPCEFOILPLDQOVLVLRSCWAPLLMLE	300
hDAX1	KSPQVCEAASAGLLKTLRFVYKLPCEFOVLPLDQOVLVLRNCWASLLMLE	298
SHP	CAPHRTCREALDVLAKTVAFLRNLPSCFCHLPHEdorRLLECCWGPLELLG	92
rTR α	..FSEFTKLTTPATTRVVDFAKKLMPFSELPCEDOIILLKGCCEMIMSLR	262
hRXR α	..VTNICQAAADKOLFVIVWAKRIPHFSELPDQOVLVLRAGWNEILLAS	312
	-----H3-----	-----H5-H6-----
V		
Ahch	LAQDHLHFEMMEIPETNNTQEMLTTRRQETEGPEPAEPQATEQPQMVSAE	350
hDAX1	LAQDRQLQETFEVSEPSMLQKLLTTRRRRETGGNEPLVPVTLQHLAPPAE	348
SHP	LAQDAVTFEVAEAPVPSILKLL.....LEEASSGTQGAQPSD	142
rTR α	AAVRYDPESDTLTSGEMAVK.....REQLKNGGLGVVS.....	296
hRXR α	FSHRSIAVKDGILLATGLHVH.....RNSAHSAGVGAIF.....	346
	---	-----
K G		
Ahch	AGHLLPAAAVQAIKSFFFKCSLNIIDTKEYAYLKGTVLEFNPDLPGLQCVK	400
hDAX1	ARKVPSASQVQAIKCFLSKCSLNIISTKEYAYLKGTVLEFNPDVPGQCVK	398
SHP	.RPQPSLAAVQWLQRCLSEFWSLELGPKEYAYLKGTVLEFNPDVPGLRASC	198
rTR αDAIF.ELGKSLSAFNLDDEVALLOAVLLMSTDRSGLLCVD	336
hRXR αDRVLTELVSQRMDMDKTELGCLRAVLEFNPDKGLSNPA	387
	-----H8-----	-----H9-----
G I		
Ahch	YIEGLQWRTOQILTEHIRMREYQIRSAELNSALFLLRFINSDDVVTFL	450
hDAX1	YIQGLQWGTQQILSEHTRMTHQPHDRFIELNSTLFLRRFINANVIAELF	448
SHP	HIAHLQEAHWALCEVLEPWYPASQGRRLARILLMASTLKNIPCTLLVDLF	242
rTR α	KIEKSQEAAYLLAFEHYVNRHKHNIPIHFWPKLLMKVTDLRMIGACHASRLH	386
hRXR α	EVEALREKVIASLEAYCKHKYPEQPGRFKALLLRAPALRSITGLCKLEHLF	437
	-----H10-----	-----H11-----
Ahch	FRPIIGAVSMDMMLEMLCAKL* 473	
hDAX1	FRPIIGTVSMDMMLEMLCTKI* 471	
SHP	FRPIMGVDVITELLEMLLL* 260	
rTR α	MKVECTELFPPLFLEVF.... 405	
hRXR α	FKLIGDTPIDTFLMTE.... 457	
	-----H12-----	

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, rTR α , and hRXR α . 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. 1996b; 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 mu-

tations 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. 1995b, 1996a; 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. 1995b, 1996a; 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. 1995b, 1996a; 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

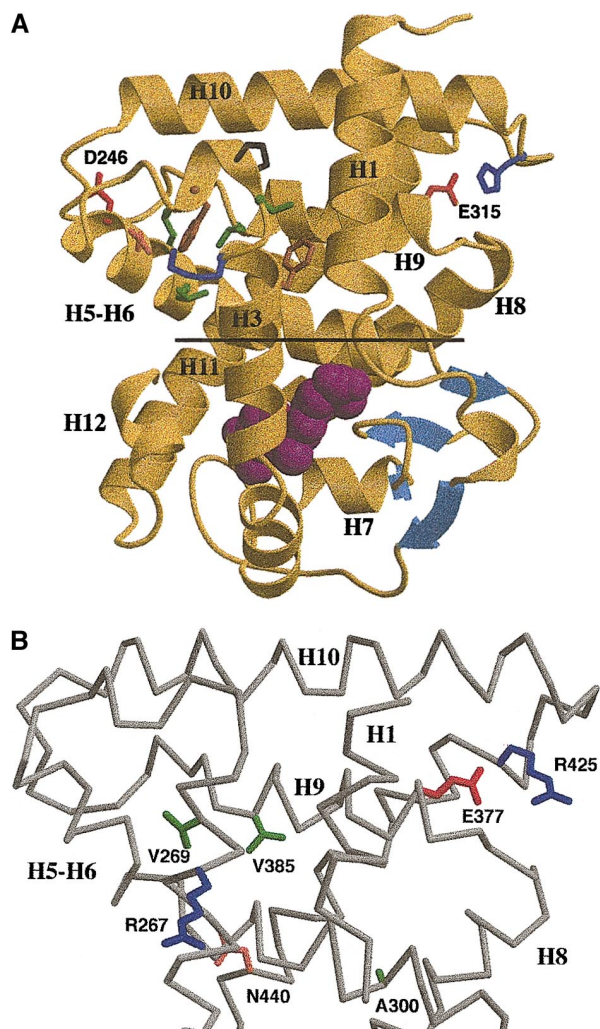


Figure 4 Models of LBDs for rTR α and human DAX1, showing subdomain structure and the impact of DAX1 mutations on structure. *A*, Ribbon diagram of the rTR α isoform, as an example of a nuclear receptor LBD. The black, horizontal line shows the division of the receptor into two subdomains: the structural 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 rTR α , the specific sequence is 232-FAKKLPMFSELPCEIQIILL-251. The conserved charge pair between H9 and H11 also is shown; in rTR α , 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

(Burriss 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 Ahc (Guo et al. 1996b; Swain et al. 1996) and, by homology with the three-dimensional structures of rTR α , (Wagner et al. 1995) and hRXR α (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. 4A). 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. 4B); 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 hTR β 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.

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References

- Bourguet W, Ruff M, Chambon P, Gronemeyer H, Moras D (1995) Crystal structure of the ligand-binding domain of the human nuclear receptor RXR- α . *Nature* 375:377-382
- Bovet P, Reymond MJ, Rey F, Gomez F (1988) Lack of gonadotropic response to pulsatile gonadotropic-releasing hormone in isolated hypogonadotropic hypogonadism associated with congenital adrenal hypoplasia. *J Endocrinol Invest* 11:201-204
- Burris TP, Guo W, Le T, McCabe ERB (1995) Identification of a putative steroidogenic factor-1 response element in the DAX-1 promoter. *Biochem Biophys Res Commun* 214: 576-581
- Burris TP, Guo W, McCabe ERB (1996) The gene responsible for adrenal hypoplasia congenita, *DAX1*, encodes a nuclear hormone receptor that defines a new class within the superfamily. In: Conn PM (ed) Recent progress in hormone research. Endocrine Society, Bethesda, pp 241-260
- Epstein CJ (1995) The new dysmorphology: application of insights from basic developmental biology to the understanding of human birth defects. *Proc Natl Acad Sci USA* 92:8566-8573
- Fawell SE, Lees JA, White R, Parker M (1990) Characterization and colocalization of steroid binding and dimeriza-

- tion activities in the mouse estrogen receptor. *Cell* 60: 953–962
- Golden MP, Lippe BM, Kaplan SA (1977) Congenital adrenal hypoplasia and hypogonadotropic hypogonadism. *Am J Dis Child* 131:1117–1118
- Gordon D, Cohen HN, Beastall GH, Hay ID, Thomson JA (1984) Contrasting effects of subcutaneous pulsatile GnRH therapy in congenital adrenal hypoplasia and Kallmann's syndrome. *Clin Endocrinol (Oxf)* 21:597–603
- Guo W, Burris TP, McCabe ERB (1995a) Expression of DAX-1, the gene responsible for X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism, in the hypothalamic-pituitary-adrenal/gonadal axis. *Biochem Mol Med* 56:8–13
- Guo W, Burris TP, Zhang Y-H, Huang B-L, Mason J, Copeland KC, Kupfer SR, et al (1996a) Genomic sequence of the DAX1 gene: an orphan nuclear receptor responsible for X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism. *J Clin Endocrinol Metab* 81:2481–2486
- Guo W, Lovell RS, Zhang Y-H, Huang B-L, Burris TP, Craigen WJ, McCabe ERB (1996b) Ahch, the mouse homologue of DAX1: cloning, characterization and synteny with GyK, the glycerol kinase locus. *Gene* 178:31–34
- Guo W, Mason JS, Stone CG, Morgan SA, Madu SI, Baldini A, Lindsay EA, et al (1995b) Diagnosis of X-linked adrenal hypoplasia congenita by mutation analysis of the DAX-1 gene. *JAMA* 274:324–330
- Habiby RL, Boepple P, Nachtigall L, Sluss PM, Crowley WF Jr, Jameson JL (1996) Adrenal hypoplasia congenita with hypogonadotropic hypogonadism: evidence that *DAX1* mutations lead to combined hypothalamic and pituitary defects in gonadotropin production. *J Clin Invest* 98:1055–1062
- Hay ID, Smail PJ, Forsyth CC (1981) Familial cytomegalic adrenocortical hypoplasia: an X-linked syndrome of pubertal failure. *Arch Dis Child* 56:715–721
- Ikeda Y, Swain A, Weber TJ, Hentges KE, Zanaria E, Lalli E, Tamai KT, et al (1996) Steroidogenic factor 1 and Dax-1 colocalize in multiple cell lineages: potential links in endocrine development. *Mol Endocrinol* 10:1261–1272
- Ito M, Yu R, Jameson JL (1997) DAX-1 inhibits SF-1-mediated transactivation via a carboxy-terminal domain that is deleted in adrenal hypoplasia congenita. *Mol Cell Biol* 17: 1476–1483
- Kikuchi K, Kaji M, Momoi T, Mikawa H, Shigematsu Y, Sudo M (1987) Failure to induce puberty in a man with X-linked congenital adrenal hypoplasia and hypogonadotropic hypogonadism by pulsatile administration of low-dose gonadotropin-releasing hormone. *Acta Endocrinologica* 114: 153–160
- Kletter GB, Gorski JL, Kelch RP (1991) Congenital adrenal hypoplasia and isolated gonadotropin deficiency. *Trends Endocrinol Metab* 2:123–128
- Kruse K, Sippell WG, Schnakenburg KV (1984) Hypogonadism in congenital adrenal hypoplasia: evidence for a hypothalamic origin. *J Clin Endocrinol Metab* 58:12–17
- Marsden HB, Zakhour HD (1978) Cytomegalic adrenal hypoplasia with pituitary cytomegaly. *Virchows Arch A Pathol Anat Histopathol* 378:105–110
- Martin MM, Martin ALA (1985) The syndrome of congenital hereditary adrenal hypoplasia and hypogonadotropic hypogonadism. *Int J Adolesc Med Health* 1:119–137
- McCabe ERB (1995) Disorders of glycerol metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) *The metabolic and molecular basis of inherited disease*, 7th ed. McGraw-Hill, New York, pp 1631–1652
- McCabe ERB (1996) Sex and the single DAX1: too little is bad, but can we have too much? *J Clin Invest* 98:881–882
- Mumm S, Whyte MP, Thakker RV, Buetow KH, Schlessinger D (1997) mtDNA analysis shows common ancestry in two kindreds with X-linked recessive hypoparathyroidism and reveals a heteroplasmic silent mutation. *Am J Hum Genet* 60:153–159
- Muscattelli F, Strom TM, Walker AP, Zanaria E, Recan D, Meindl A, Bardoni B, et al (1994) Mutations in the DAX-1 gene give rise to both X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism. *Nature* 372: 672–676
- Nakae J, Tajima T, Kusuda S, Kohda N, Okabe T, Shinohara N, Kato M, et al (1996) Truncation at the C-terminus of the DAX-1 protein impairs its biological actions in patients with X-linked adrenal hypoplasia congenita. *J Clin Endocrinol Metab* 81:3680–3685
- O'Donnell AL, Rosen ED, Darling DS, Koenig KJ (1991) Thyroid hormone receptor mutations that interfere with transcriptional activation also interfere with receptor interaction with a nuclear protein. *Mol Endocrinol* 5:94–99
- Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T (1989) Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc Natl Acad Sci USA* 86:2766–2770
- Partsch C-J, Sippell WG (1989) Hypothalamic hypogonadism in congenital adrenal hypoplasia. *Horm Metab Res* 21: 623–625
- Prader A, Zachman M, Illig R (1975) Luteinizing hormone deficiency in hereditary congenital adrenal hypoplasia. *J Pediatr* 86:421–422
- Ribeiro RC, Kushner PJ, Baxter JD (1995) The nuclear hormone receptor gene superfamily. *Annu Rev Med* 46: 443–453
- Rost B, Sander C (1994) Combining evolutionary information and neural networks to predict protein secondary structure. *Proteins* 19:55–72
- Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 74: 5463–5467
- Schwartz M, Blichfeldt S, Muller J (1997) X-linked adrenal hypoplasia in a Greenlandic family: detection of a missense mutation (N440I) in the DAX-1 genes: implication for genetic counseling and carrier diagnosis. *Hum Genet* 99:83–87
- Seltzer WK, Firminger H, Klein J, Pike A, Fennessey P, McCabe ERB (1985) Adrenal dysfunction in glycerol kinase deficiency. *Biochem Med* 33:189–199
- Seol W, Choi H-S, Moore DD (1996) An orphan nuclear hormone receptor that lacks a DNA binding domain and heterodimerizes with other receptors. *Science* 272:1336–1339
- Swain E, Zanaria E, Hacker A, Lovell-Badge R, Camerino G (1996) Mouse *DAX1* expression is consistent with a role in sex determination as well as in adrenal and hypothalamus function. *Nat Genet* 12:404–409

- Takahashi T, Yutaka S, Shoji Y, Haraguchi N, Takahashi I, Takada G (1997) Active hypothalamic-pituitary-gonadal axis in an infant with X-linked adrenal hypoplasia congenita. *J Pediatr* 130:485-488
- Tsai MJ, O'Malley BW (1994) Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu Rev Biochem* 63:451-486
- Vilain E, Guo W, Zhang Y-H, McCabe ERB (1997) DAX1 gene expression upregulated by steroidogenic factor 1 in an adrenocortical carcinoma cell line. *Biochem Mol Med* 61:1-8
- Viridis R, Levine LS, Levy D, Pang S, Rapaport R, New MI (1983) Congenital adrenal hypoplasia: two new cases. *J Endocrinol Invest* 6:51-54
- Wagner RL, Apreletti JW, McGrath ME, West BL, Baxter JD, Fletterick RJ (1995) A structural role for hormone in the thyroid hormone receptor. *Nature* 378:690-697
- Wurtz JM, Bourguet W, Renaud JP, Vivat V, Chambon P, Moras D, Gronemeyer H (1996) A canonical structure for the ligand-binding domain of nuclear receptors. *Nat Struct Biol* 3:87-94
- Yanase T, Takayanagi R, Oba K, Nisiji Y, Ohe K, Nawata H (1996) New mutations of *DAX1* genes in two Japanese patients with X-linked congenital adrenal hypoplasia and hypogonadotropic hypogonadism. *J Clin Endocrinol Metab* 81:530-535
- Yu RN, Ito M, Jameson JL (1996) Transcriptional regulation of the mouse *DAX1* promoter by steroidogenic factor-1. Paper presented at the 10th International Congress on Endocrinology, San Francisco, June 12-15
- Zachmann M, Illig R, Prader A (1980) Gonadotropin deficiency and cryptorchidism in three prepubertal brothers with congenital adrenal hypoplasia. *J Pediatr* 97:255-257
- Zanaria E, Muscatelli F, Bardoni B, Strom TM, Guioli S, Guo W, Lalli E, et al (1994) A novel and unusual member of the nuclear hormone receptor superfamily is responsible for X-linked adrenal hypoplasia congenita. *Nature* 372:635-641