

Clinical and Functional Effects of Mutations in the *DAX-1* Gene in Patients with Adrenal Hypoplasia Congenita*

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ABSTRACT

Adrenal hypoplasia congenita (AHC) is an X-linked disorder caused by mutations in a gene referred to as *DAX-1*. AHC is characterized by adrenal insufficiency and failure to undergo puberty because of hypogonadotropic hypogonadism. The *DAX-1* protein is structurally related to orphan nuclear receptors, although it lacks the characteristic zinc finger DNA-binding domain that is highly conserved in other members of this family. In this report, we describe the clinical features and genetic alterations in six families with AHC. These patients reveal the variable clinical presentation of adrenal insufficiency in AHC and underscore the importance of considering this diagnosis. Nonsense mutations that introduce a stop codon were

found in three cases (W171X, W171X, Y399X). Frameshift mutations (405delT, 501delA, and 702delC), each of which resulted in a premature stop codon at amino acid 263, were found in the other three families. Three of these mutations (Y399X, 405delT, 702delC) are novel. Using transient gene expression assays to assess *DAX-1* function, these mutations were shown to eliminate the ability of *DAX-1* to repress the transcription of genes that are stimulated by a related nuclear receptor, steroidogenic factor-1. These studies reveal the variable clinical presentation of *DAX-1* mutations and emphasize the value of genetic testing in boys with primary adrenal insufficiency and suspected X-linked AHC. (*J Clin Endocrinol Metab* 84: 504–511, 1999)

ADRENAL hypoplasia congenita (AHC) is a rare inherited disorder that occurs in two distinct forms: X-linked and autosomal recessive (1, 2). In X-linked AHC, primary adrenocortical failure occurs because the adrenal glands lack the permanent adult cortical zone. The remaining cells are termed “cytomegalic” because they are larger than typical fetal adrenal cells and contain characteristic nuclear inclusions from cytoplasmic invaginations (3). Patients with X-linked AHC frequently develop severe salt-wasting with glucocorticoid and mineralocorticoid insufficiency in infancy (4). The disorder is lethal unless appropriate steroid

hormone replacement is provided. Hypogonadotropic hypogonadism is also commonly associated with X-linked AHC (5). It is usually detected because of the absence of pubertal development, and gonadotropin deficiency is caused by abnormalities in both hypothalamic and pituitary control of gonadotropin secretion (6).

The genetic locus for X-linked AHC was mapped to Xp21 through studies of male patients with contiguous gene deletion syndromes (glycerol kinase deficiency, Duchenne muscular dystrophy, ornithine transcarbamylase deficiency, and mental retardation) (7–9). This region of the X chromosome is also the location of the dosage-sensitive sex reversal (DSS) locus important in sex determination (10, 11). In 1994, the human *DAX-1* (DSS–AHC critical region on the X chromosome, gene 1) gene was cloned and mutations in *DAX-1* were identified as causing both AHC and the associated hypogonadotropic hypogonadism (12, 13). This gene encodes a 470 amino acid protein, with approximately 50% homology between the carboxy-terminal region of *DAX-1* and the ligand-binding domain (E domain) of the nuclear hormone receptor superfamily (12, 14, 15). *DAX-1* is classified as an orphan nuclear receptor because no specific ligand has been identified to date (16). However, unlike other members of this family, *DAX-1* lacks a typical zinc-finger DNA-binding domain. The unique amino-terminal portion of *DAX-1* contains 3½ repeats of a 65–67 amino acid motif (12) that may bind to hairpin loop structures in DNA (17).

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DAX-1 plays a key role in the development of the adrenal gland and the hypothalamo-pituitary-gonadal axis. It is expressed in the developing urogenital ridge, ovary, testis, adrenal cortex, hypothalamus, and anterior pituitary gland (18, 19), and it colocalizes with another nuclear receptor protein, steroidogenic factor-1 (SF-1) (20). SF-1 regulates the expression of steroidogenic enzymes (21–23). Targeted disruption of the gene in mice leads to complete adrenal and gonadal agenesis, persistence of Müllerian structures in male mice (24), impaired expression of gonadotrope-specific markers in the anterior pituitary (25), and disruption of the hypothalamic ventromedial nucleus (26). Recently, *in vitro* studies showed that DAX-1 and SF-1 bind to one another through protein–protein interactions (27–29). DAX-1 has been shown to repress SF-1-mediated transactivation (27–30). A repression domain has been localized to the carboxy-terminus of DAX-1 (17, 27), a region that is deleted in many patients with AHC. In this report, we describe the clinical features and genetic analyses in six racially and geographically diverse families with DAX-1 mutations who had early- and late-onset adrenal insufficiency. The functional effects of these mutations were examined using transient expression assays of SF-1-mediated transcription.

Materials and Methods

PCR and direct sequencing of DAX-1 gene

After obtaining Institutional Review Board approval and informed consent from patients and family members, DNA was extracted from blood leukocytes. The 3 kb DAX-1 intron was amplified by PCR using a GeneAmp XL kit (Perkin Elmer, Foster City, CA) to allow the design of intronic oligonucleotide primers. Exons 1 and 2 of the DAX-1 gene were amplified from genomic DNA using the M13-tagged primers shown below (M13 sequence is not shown):

DAX 1.1 For:	5'-GCT CCC ACG CTG CTG TTC TTC-3'
DAX 1.1 Rev:	5'-CCG CCC ACC CGG AAG CCC CGC-3'
DAX 1.2 For:	5'-CGA AGG CGC CCG AGG CGA CGC-3'
DAX 1.2 Rev:	5'-GGA CGC CCA GCA GTT GCG CAC-3'
DAX 1.3 For:	5'-CGC TTC GTC AAG TAC TTG CCC-3'
DAX 1 Splice Rev:	5'-GTG TAG AGA GCC AAG TAC-3'
DAX 2 Splice For:	5'-TCC ACA CGT GTG CAT AGA AAC-3'
DAX 2 Splice Rev:	5'-TGT ACA GAG CTA TGC TAC CTG-3'

PCR was performed in a 100- μ L reaction containing 100 ng genomic DNA, 50 pmol primers, 50 μ M dNTPs, 1.1 mM MgCl₂, and 5 U *Taq* polymerase (Promega, Madison, WI) in a buffer containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 5% dimethylsulfoxide, and 0.1% Triton X-100. PCR conditions were 1 min predenaturation at 96 C, nine cycles of 1 min at 94 C, annealing for 1 min at 60 C, extension for 1 min at 72 C, 26 cycles of 1 min at 94 C, annealing (55–57.5 C) for 1 min, extension for 1 min at 72 C, and 15 min elongation at 72 C. Direct DNA sequencing was performed with Dye Primer Cycle Sequencing kits (Perkin Elmer) using an automated sequencer (Applied Biosystems Model 373A DNA Sequencer, Foster City, CA). For each exon, products from three different PCR reactions were sequenced in both directions. Sequences obtained from members of each kindred were compared with those from two unrelated normal control subjects. In potential heterozygotes, results of direct DNA sequencing were confirmed by subcloning purified PCR products into the pCR II vector (TA cloning kit, Invitrogen, San Diego, CA), and *Taq* cycle sequencing was performed on both strands as described above. At least eight subclones were sequenced.

Plasmid construction and transient expression assays

Eukaryotic expression vectors for the DAX-1 mutants were constructed from the pBKCMV (*-lacZ* promoter) human DAX-1 cDNA vector as described previously (27). The mutations were created by the

overlapping PCR technique using primers containing the appropriate nucleotide substitutions. PCR-amplified mutant fragments were digested with restriction enzymes and inserted into the wild-type cDNA sequence. Construction of the expression vector for the GAL4-SF-1 fusion protein and the reporter construct UAS-TK109luc has been described (27).

Human choriocarcinoma JEG-3 cells (American Type Culture Collection HTB 36) were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin in a 5% CO₂ atmosphere at 37 C. Cells were transfected with 500 ng UAS-TK109luc, 50 ng pSG424-GAL4 or GAL4-SF-1, and 20 ng pBKCMV expression vector (empty vector, DAX-1 wild type or DAX-1 mutant) using calcium phosphate precipitation as described previously (27). Each individual transfection reaction was performed in triplicate. Cell extracts were prepared 24 h after transfection, and luciferase assays were performed. The mean luciferase activity of each triplicate reaction was expressed as a percentage of GAL4-SF-1 to allow comparison of data from different experiments. The results represent the mean \pm SEM from four different experiments, each consisting of triplicate transfections.

Results

Case reports

Individuals from six families with suspected X-linked AHC were studied. The pedigrees of four families are shown in Fig. 1. Clinical details of individual presentations are summarized in Table 1, and investigations of adrenal and gonadal function are shown in Tables 1 and 2, respectively.

The majority of our patients presented in the neonatal period with salt-losing states and adrenal insufficiency. Patient RG (III-1) is of Irish Caucasian origin. He developed progressive failure to thrive, vomiting and dehydration, and had biochemical evidence of hyponatremia and hyperkalemia.

Half-brothers in kindred DK (III-1, III-3) were of American Caucasian origin and had a similar early clinical presentation. In patient DK:III-1, a diagnosis of 21-hydroxylase deficiency was made initially but was revised when he failed to enter puberty. His brother (DK:III-3) had normal electrolytes and cortisol at birth but developed hyperkalemia at 1 week of age.

Both BK brothers (IV-2, IV-3) presented in the first 2 weeks of life. They were also from an American Caucasian family. BK:IV-2 presented in a salt-losing crisis at 2 weeks of age but responded well to steroid replacement. His younger brother (IV-3) had normal electrolytes and cortisol (30 μ g/dl) 24 h after birth but presented 2 weeks later with meningitis, seizures, and a salt-losing crisis. Of note, an older brother (IV-1) had died of congenital adrenal insufficiency and had hypoplastic adrenal glands at autopsy. Two maternal uncles (III-3, III-4) died in the first 2 days of life and had similar findings, and a cousin of their mother (III-1) was diagnosed with Addison's disease at the age of 7 yr. BK:IV-2 failed to develop puberty. He showed little response to GnRH stimulation and remains on testosterone replacement (Table 2). BK:IV-3 has severe learning difficulties after a head injury in childhood, and no formal investigations have been performed; at 24 yr of age he remains prepubertal.

Kindred LS are of Mexican descent and were older at the time of diagnosis. The eldest son (IV-1) presented at the age of 2.5 yr with vomiting, dehydration, and shock, and a diagnosis of primary adrenal insufficiency was made. Cortisol was undetectable and ACTH was elevated. His maternal uncle (III-2) had been given a diagnosis of Addison's disease when 2 yr old, and his great uncle (II-1) had died unexpect-

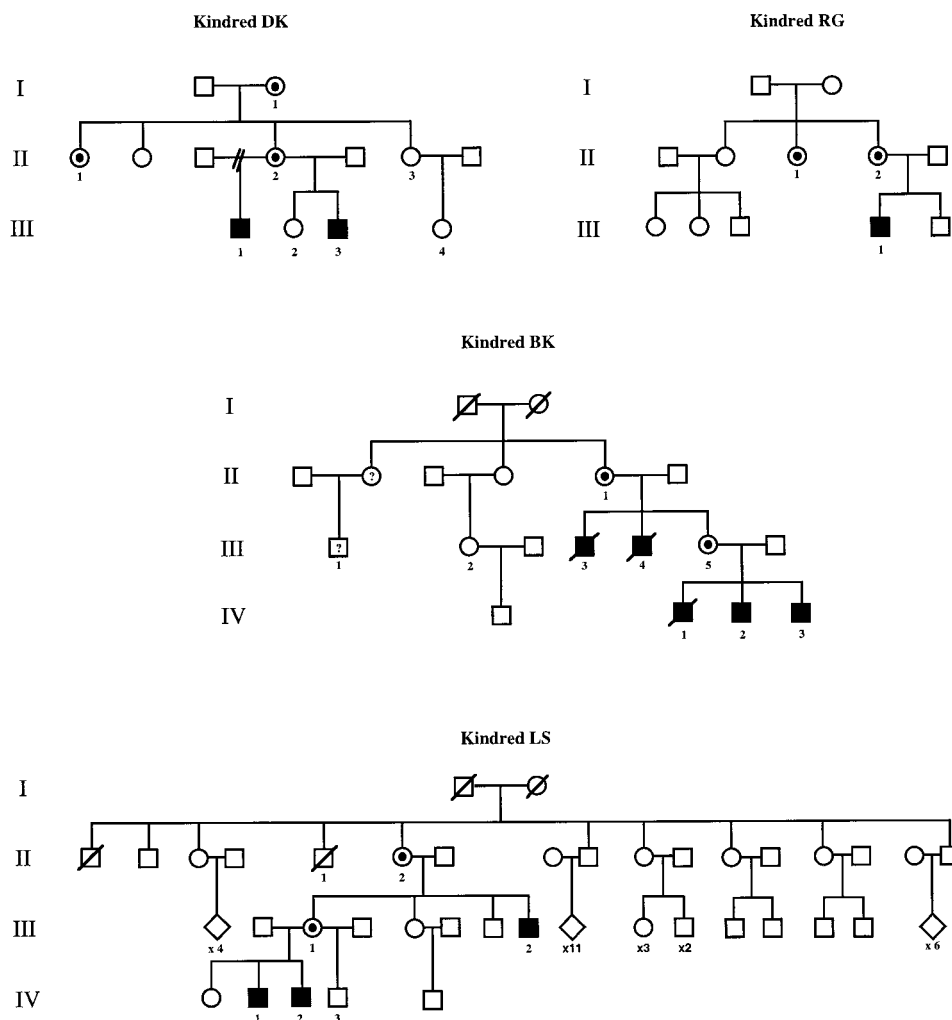


FIG. 1. The pedigrees of four kindreds with X-linked AHC. Hemizygous affected males are indicated by *filled squares*, and heterozygous carrier females are designated by a *circle filled with a dot*. Carrier status was determined by direct sequencing or, in the case of kindred DK, by restriction fragment analysis. This was performed in all numbered individuals still alive, other than BK:III-1. Patient SS was likely to have a *de novo* mutation after investigation of his nuclear family. The family members of patient LP have not yet been studied.

edly at 2 months of age from gastroenteritis. When his younger brother (IV-2) became hyperpigmented at 1 yr of age, the diagnosis of adrenal insufficiency was made, although he was otherwise asymptomatic at the time.

Patient LP was born in Scotland. He was diagnosed with craniosynostosis and developed cyanosis while under sedation for a cranial computed tomographic scan. He had repeated vomiting, cyanotic episodes, and epileptic seizures thereafter, but improved with glucocorticoid, mineralocorticoid, and salt replacement and underwent corrective surgery uneventfully.

SS, the eldest son in a family of Asian Indian descent, also had a delayed presentation. He was diagnosed with adrenal insufficiency at the age of 7 yr after a hypotensive episode and hyponatremia during an acute asthma attack. Peak cortisol response to ACTH stimulation was 3.2 ng/dl, and he was started on glucocorticoid and mineralocorticoid replacement. Although hyponatremia had been noted during an episode of *Escherichia coli* septicemia at 6 days of age, he had been well in early childhood but had developed progressive malaise before presentation. He improved on treatment but grew poorly and was reinvestigated at the age of 10 yr. Steroid replacement was temporarily withdrawn, and primary adrenal insufficiency was confirmed (Table 1). On steroid replacement, an unprimed insulin tolerance test pro-

duced a suboptimal peak GH response of 5.1 mU/L (1 ng/ml = 2.6 mU/L) (Hybritech Tandem-R, Liege, Belgium), and growth hormone treatment was started. However, his failure to enter puberty led to the diagnosis of X-linked AHC. He was shown to have a poor gonadotropin response to GnRH and a moderate testosterone response to three doses of hCG (Table 2). Virilization was induced with testosterone, and reevaluation at 18 yr of age confirmed hypogonadotropic hypogonadism and revealed a borderline GH response to stimulation (11.8 mU/L). At the age of 22 yr, he reached a predicted height of 170.6 cm and remains on testosterone replacement.

Mutational analyses

The *DAX-1* gene was sequenced in each of the probands and in available family members. The sequence findings are summarized in Table 1. Nonsense mutations that introduce a stop codon were found in three cases, and frameshift mutations were found in three cases. These mutations, along with others reported in the literature, are summarized in Fig. 2. The recommendations of Antonarakis (31) were adopted to provide a consistent approach to nucleotide numbering, because the designation of *DAX-1* mutations differs in var-

TABLE 1. Clinical features and age at diagnosis of boys with *DAX-1* mutations

Mutation	Kindred	Patient	Age at diagnosis	Mode of presentation	Cortisol ($\mu\text{g/dl}$)	ACTH (pg/ml)
Nonsense W171X	SS	<i>de novo</i>	7 yr	Hyperpigmentation; progressive malaise; hypotensive during an asthma attack, hyponatremic with sepsis, at age 6 days	3.2 ^a /1.0 ^b	1100 ^b
W171X	DK	III-1	2 weeks	Salt-wasting crisis; pubertal failure suggested X-linked AHC		
Y399X	LP ^e	III-3	1 week 6 weeks	Salt-wasting crisis Craniosynostosis diagnosed at birth; collapsed under sedation for a cranial CT scan; repeated vomiting, cyanotic episodes, and seizures ensued	3.2 ^c 0.6	253 ^d 3587
Frameshift 405delT; codon 135 (stop codon 263)	RG	III-1	2 weeks	Poor feeding; poor weight gain; dehydration	2.4	
501delA; codon 167 (stop codon 263)	BK ^f	IV-2	2 weeks	Salt-wasting crisis	10.7 ^g / <2.0 ^h	
		IV-3	2 weeks	Salt-wasting crisis; meningitis and seizures	30.6 ⁱ	
702delC; codon 233/4 (stop codon 263)	LS ^j	III-2	2 yr	Progressive malaise; shock	<1.0 ^k	937
		IV-1	2.5 yr	Vomiting; dehydration; shock	2.0 ^k	640
		IV-2	1 yr	Hyperpigmented; asymptomatic	3.8 ^k	802

^a Peak cortisol response to standard synacthen test: 3.2 $\mu\text{g/dl}$.

^b Age 10 yr; peak cortisol response to prolonged synacthen test: 1.4 $\mu\text{g/dl}$ (day 3) (off hydrocortisone treatment).

^c Peak cortisol response to standard synacthen test: 18 $\mu\text{g/dl}$ (basal) to 28 $\mu\text{g/dl}$ (peak).

^d ACTH concentration age 19 days on glucocorticoid and mineralocorticoid replacement.

^e Kindred LP: other family members have not been screened for *DAX-1* mutations.

^f Kindred BK: eldest brother (IV-1) and two maternal uncles (III-3 and III-4) died in the first week of life from "congenital adrenal insufficiency" with hypoplastic adrenal glands; mother's cousin (III-1) diagnosed as having Addison's disease.

^g Subnormal 17-ketosteroids, even after ACTH stimulation.

^h Steroid replacement started once cortisol concentrations had declined.

ⁱ Presented 2 weeks later with meningitis, seizures, and a salt-losing crisis.

^j Kindred LS: great uncle (II-1) died from gastroenteritis at 2 months of age.

^k Measurements at various ages, reflecting the variable compliance with treatment in this family.

ious original reports. In this system, the A of the ATG translational initiation codon is designated as nucleotide +1.

In SS, three nucleotide changes were present in exon 1. Two of these resulted in silent polymorphisms: cysteine at codon 38 was encoded by TGC rather than TGT (change at nucleotide 114), and CGA replaced CGG encoding arginine at codon 166 (change at nucleotide 498). The latter substitution was also found in the patient's father. The third change caused a mutation in *DAX-1*. A TGG \rightarrow TAG conversion at nucleotide 512 resulted in a premature stop codon at codon 171 in place of tryptophan. This mutation was not found in the patient's mother or brother, suggesting that she was not a carrier, and that it may have arisen *de novo* in the affected patient. However, the demonstration of gonadal mosaicism for this disease by Zhang et al. (32) indicates that females who are shown not to be carriers for *DAX-1* mutations are potentially at risk of having affected sons, and such families should be counseled appropriately. In the DK kindred, a nonsense mutation was also found in codon 171. However, in this case, the substitution was TGG \rightarrow TGA at nucleotide 513. This eliminated an *Xcm* I site and allowed the screening of additional family members by restriction enzyme analysis. The proband's mother (II-2), grandmother (I-1), and a maternal aunt (II-1) were found to be carriers (Fig. 1).

In LP, a nucleotide transversion at position 1197 (C \rightarrow A) resulted in a novel mutation, introducing a stop codon at

TABLE 2. Investigations of gonadal function in boys (>14 yr) with *DAX-1* mutations

Mutation	Patient	Age (yr)	Basal/Peak _{LHRH}				Basal/post-hCG	
			LH (IU/L)		FSH (IU/L)		Testosterone (ng/dl)	
Nonsense W171X	SS	10.8	1.9	2.4	0.9	1.3	<23	310
		16.8	<1.0	1.6	1.3	1.3		
		18.7	<1.0	<1.0	1.4	1.5		
W171X	DK: III-1	14.0	11.7		4.4		7	
		15.1	<0.5	<0.5	0.6	0.9	u/d	
Frameshift 501delA	BK: IV-2	15.7 ^a	7.3	8.1	1.4	3.1	3	<10
		18.8 ^b	10.3	13.6	3.0	3.5		
		19.6 ^c	<3.0	<3.0	2.4	4.6		
		19.7 ^c	<3.0	8.8	<2.0	<2.0		
		19.9 ^c	<3.0	8.2	<2.0	3.9		
BK:IV-3 ^d								

u/d, Undetectable.

^a BK: IV-2 had received six monthly injections of testosterone enanthate (200 mg im) before this test.

^b Off testosterone (100 mg im monthly) for 2 months.

^c LHRH tests performed at the start of and during 8 months treatment with leuprolide, 1 $\mu\text{g/kg}$ sc qd. His testicular volume increased from 2.5 to 2.9 cm.

^d BK: IV-3 has severe learning difficulties after a head injury at age 3 yr. He has not had any tests of gonadal function and remains Tanner stage 1 at 24 yr of age.

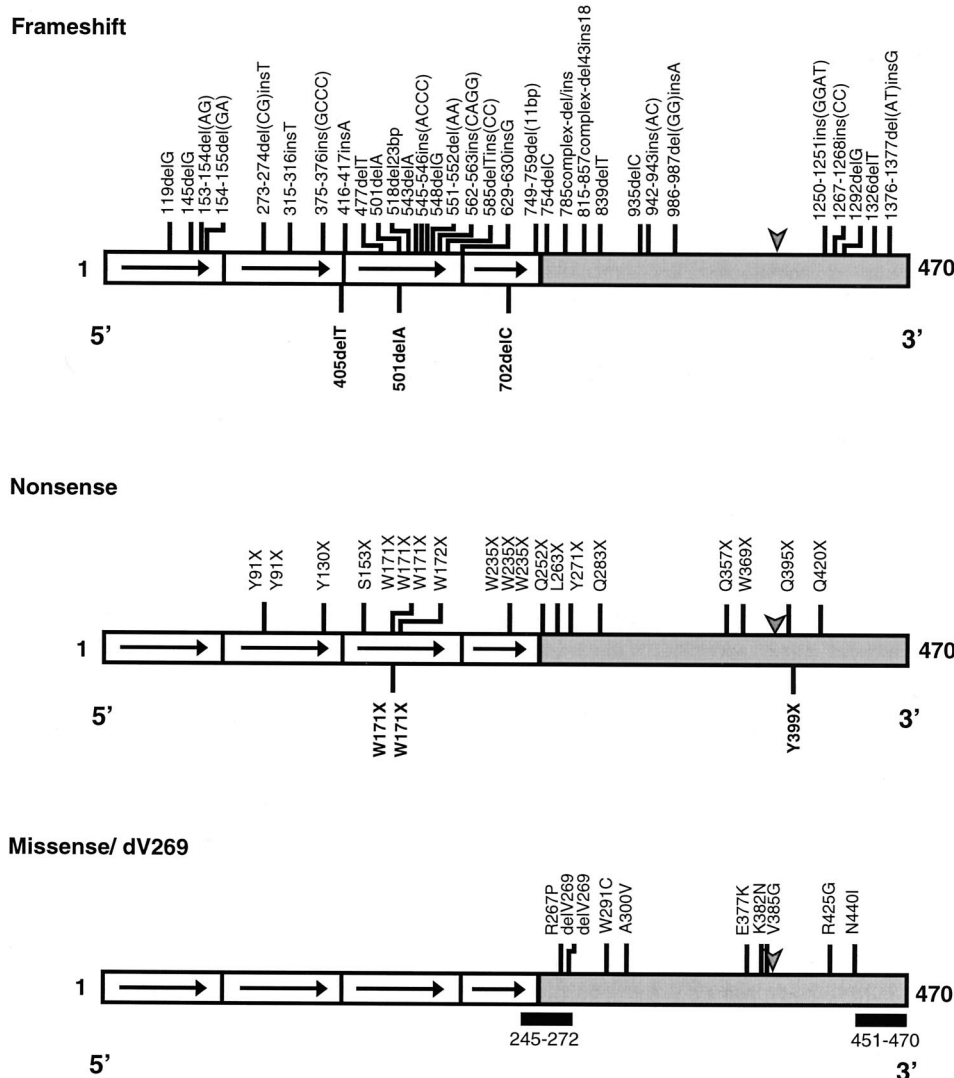


FIG. 2. Summary of naturally occurring *DAX-1* mutations. The locations of *DAX-1* mutations are depicted relative to their domain structure. The nuclear receptor-like domain is shaded, and the aminoterminal repeats are depicted by arrows. The junction of exons 1 and 2 is denoted by an arrowhead. The black bar represents the reported transcriptional silencing domain (17). Because the numbering system and designation of mutations differ in various original reports, all mutations are indicated as described below (31). The A of the ATG translational initiation codon is designated nucleotide +1. The locations of frameshift mutations are shown at the position of the actual mutation rather than at the location of the resultant premature stop codon. For mutations that occur within nucleotide repeats, the mutation (either insertion or deletion) is depicted as the 3'-most nucleotide within the repeat. Frameshift and nonsense mutations that lead to premature truncation of the protein are shown in the *top two panels*. Nucleotide insertions or deletions are illustrated in the *top figure*, and the locations of mutations that create stop codons are shown in the *middle figure*. Missense mutations and the single codon deletion (delV269) are shown in the *bottom panel*. Mutations described in this report are shown in *bold* below the schematic diagrams. Note the broad distribution of mutations and the relatively high frequency of frameshift and nonsense mutations (data derived from this report and mutations reported in references 6, 12–14, 32, 34–42, 49).

amino acid 399 within the putative ligand-binding domain of *DAX-1*. Other family members were not available for screening.

RG had a novel deletion of thymidine at nucleotide 405, causing a frameshift and premature stop codon at amino acid 263. His mother was heterozygous for this mutation, and a maternal aunt, pregnant at the time of the study, was also found to be a carrier of the mutation. His father was hemizygous and his mother heterozygous for the T → C polymorphism at nucleotide 114.

In the BK kindred, a deletion of adenosine at nucleotide 501 resulted in a frameshift and premature stop codon at

amino acid 263. Affected males (IV-2, IV-3) were hemizygous for this deletion, and carrier females (II-1, III-5) were heterozygous. A cousin of their mother (III-2), pregnant at the time of the study, was not a carrier of this mutation. She gave birth to a healthy baby boy. Silent polymorphisms were found in one brother (IV-3, G → A at nucleotide 498) and in his mother (III-5, T → C at nucleotide 114).

In the LS kindred, all three affected males (III-2, IV-1, IV-2) were hemizygous for a previously unreported deletion of cytosine at nucleotide 702, causing a frameshift and premature stop codon, again at amino acid 263. As predicted from X-linked inheritance, both the mother and grandmother were

heterozygous for this frameshift deletion, but it was not found in the unaffected baby boy (IV-3). Two silent polymorphisms were also detected in this kindred; a T → C transversion at nucleotide 114 was identified in all members, and III-1 and II-2 were homozygous for this polymorphism. A G → A transversion at nucleotide 498 was present in all members apart from the unaffected baby. The mother and grandmother were heterozygous for this polymorphism.

Function of DAX-1 mutations

DAX-1 has been shown to inhibit the transcription of reporter genes that are driven by SF-1 (17, 27–29). Therefore, we tested whether the mutations in these patients altered this property of DAX-1 (Fig. 3). The W171X (kindreds SS and DK), Y399X (kindred LP), and 702delC (patient LS, truncation at codon 263) mutations were inserted into a DAX-1 expression plasmid. GAL4-SF-1-driven expression of the UAS-TKLuc reporter gene was used to assay for SF-1-mediated transactivation. GAL4-SF-1 alone stimulated the reporter gene 18-fold when compared with the GAL4 DNA-binding domain construct. Coexpression of wild-type DAX-1 greatly repressed SF-1 activity (relative luciferase activity, 6%). This inhibition was markedly reduced with each of the DAX-1 mutant vectors tested (W171X, 44%; 702delC, 90%; Y399X, 132%).

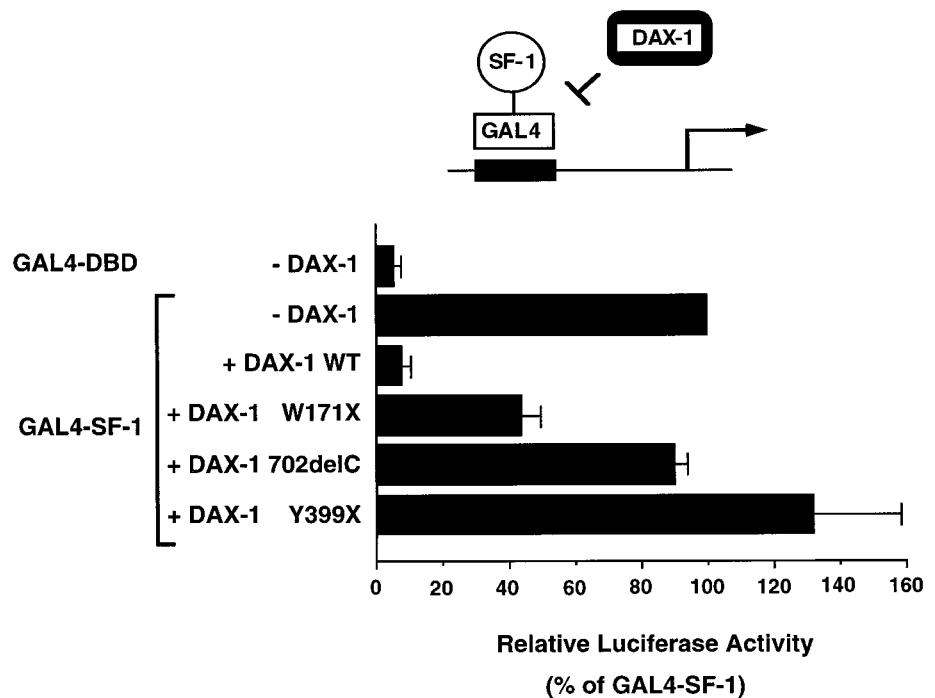
Discussion

In this study, we describe the clinical features and functional effects of DAX-1 mutations in nine patients from six families with adrenal hypoplasia congenita. In keeping with other reports, disparity in the age of diagnosis was seen. Several boys presented in neonatal life with salt-losing states, whereas others had a more insidious presentation with adrenal failure later in childhood.

The clinical diagnosis of AHC is not always easily recognized. Boys who present in the neonatal period with salt-wasting and adrenal insufficiency are sometimes misdiagnosed with the more common disorder, 21-hydroxylase deficiency (congenital adrenal hyperplasia), although the adrenal steroid profiles of these conditions are quite different. In AHC, 17-hydroxyprogesterone levels are low, whereas they are increased in congenital adrenal hyperplasia. Distinguishing these two disorders is important because they differ in their clinical course, steroid management, and genetic counseling. The recessive form of AHC should also be considered as a cause of primary adrenal insufficiency in infancy. It has a distinct miniature adult adrenal morphology, characterized by small glands with a permanent cortical zone but a diminished fetal zone (2). The genetic basis of the recessive form of AHC is unknown. Finally, adrenal gland hypoplasia may also occur in neonates with congenital defects of the hypothalamus or pituitary, leading to ACTH deficiency. This can be differentiated from the primary adrenal failure seen in X-linked AHC by relevant measurements of electrolytes, mineralocorticoids, glucocorticoids, and ACTH, and by the phenotypic features (e.g. anencephaly) present in a subset of these children.

Some of our patients who presented in childhood were diagnosed with Addison's disease. However, the failure to enter puberty, and the family history of adrenal insufficiency, prompted reconsideration of the initial diagnosis and genetic investigation for X-linked AHC. In fact, when a strong family history suggests X-linked adrenal insufficiency, adrenoleukodystrophy should also be excluded, because it can occasionally occur as adrenal insufficiency without associated neurological features (33). Serum very long-chain fatty acids will be elevated in adrenoleukodystrophy but normal in AHC.

FIG. 3. Effect of DAX-1 mutations on SF-1-mediated transcription. A schematic depiction of the format of the transcription assay is shown at the top. Transcription by GAL4-SF-1 is blocked by DAX-1. The GAL4 reporter gene UAS-TK109luc (500 ng) was transfected into JEG-3 cells with GAL4-SF-1 (50 ng) and full-length DAX-1 vector (20 ng) or with expression vectors for the indicated DAX-1 mutants (20 ng). Transfections were performed in triplicate on four occasions. The relative luciferase activity was obtained by comparing the mean of each set of triplicates with the luciferase response of the GAL4-SF-1/pBCKMV empty vector in that study. The activity of the GAL4 DNA-binding domain alone (GAL4-DBD) and GAL4-SF-1 are shown in the top two bars. The results are the mean ± SEM of four different experiments, each consisting of triplicate transfections.



Variability in the presentation of patients with AHC raises the issue of whether the type of *DAX-1* mutation predicts the severity of the disorder. That is, is there a relationship between genotype and phenotype? In the absence of detailed or standardized biochemical and physiological data in many cases, age at diagnosis remains the most accessible surrogate marker for assessing the severity of adrenal dysfunction. When the age of diagnosis of AHC is summarized for patients reported here and in other studies (6, 13, 14, 32, 34–42), an apparent bimodal distribution emerges (Fig. 4). The majority of patients are diagnosed within the first 2 months of life. Subsequently, few patients are diagnosed until later in childhood, with similar numbers of patients presenting between the ages of 2 to 9 yr.

Based on the information available to date, there is no obvious correlation between the type of mutation in *DAX-1* and the age of presentation (13, 34). However, several factors may confound this analysis. For example, symptoms may be nonspecific and may be present for some time before the diagnosis is made, particularly in childhood. Clinical presentation can be precipitated by environmental stresses (e.g. infection, operative procedures) that may occur independent of the degree of adrenal insufficiency. The diagnosis of AHC is made sooner in boys with a previous family history of X-linked adrenal failure in their brothers or uncles. Access to medical care also varies in different regions of the world. Finally, other genetic factors (modifier genes) may also influence the severity of adrenal failure. Studying individuals within a single family may reduce some of these variables, although disparate presentations of AHC within the same kindred can be seen.

It is possible that the neonatal period is a particularly vulnerable time for adrenal insufficiency because, in reported cases, many boys present in severe salt-losing crises at this time (Fig. 4). For those who do not, a more delayed and insidious presentation in childhood seems to occur. There are

several factors that could explain this. The normal aldosterone secretion rate, although fairly constant throughout life, is much higher in infancy when related to body surface area (43, 44). This finding suggests that there is normally a greater requirement for mineralocorticoids in early life. Increased mineralocorticoid requirements could reflect relatively low sodium intake and limited access to fluids at this age, the tendency to urinary sodium loss caused by higher concentrations of atrial natriuretic peptide (45), and the relative insensitivity of the immature kidney to mineralocorticoid action (46). Certain other clinical conditions associated with salt loss (such as 21-hydroxylase deficiency and aldosterone synthase deficiency) show an improvement in salt retention as patients get older (46–48). Thus, if patients with AHC survive the neonatal period, they may become less susceptible to adrenal crisis until faced with severe illness or another environmental stress later in life.

The *DAX-1* mutations described in our study resulted in abnormal *DAX-1* proteins that either completely lacked or had truncated ligand-binding domains. Most reported *DAX-1* mutations reported so far have arisen from gene deletions or premature stop codons that cause a loss of the carboxy-terminal region (6, 13, 14, 32, 34–36, 38, 39, 41, 42, 49, 50). The exceptions include nine different missense mutations (Fig. 2), located at amino acids 267 and 269 (13, 32), 291 (39), 300 (37), 377 (32), 382 (39), 385, 425 (32), and 440 (40). These missense mutations are particularly useful for identifying important functional domains in *DAX-1*. In a proposed three-dimensional model of *DAX-1*, residue 382 was suggested to maintain helix-to-helix contact through a buried salt bridge (17). The location of a missense mutation in the extreme carboxy-terminus of *DAX-1* (e.g. N440I) is consistent with the observation that various mutations that truncate this region of *DAX-1* are sufficient to cause AHC.

Wild-type *DAX-1* was shown recently to inhibit the transcriptional effects of SF-1 (27). *DAX-1* has also been shown to suppress expression of the SF-1-regulated steroidogenic acute regulatory protein promoter (17). Deletion of the carboxy-terminal end of *DAX-1* reduces its ability to silence gene expression (17, 27, 29). Therefore, AHC appears correlated with loss of *DAX-1* transcriptional repression by disruption of its silencing domain function. We used these features of *DAX-1* to test whether the W171X, 702delC, and Y399X mutations reported here altered *DAX-1* function. Each one of these mutations was found to eliminate the ability of *DAX-1* to inhibit SF-1-mediated transcription. These types of reporter gene assays may be useful to assess the functional effects of *DAX-1* mutations.

In summary, we identified six mutations in the *DAX-1* gene causing AHC with a spectrum of clinical presentations. Mutational analysis of the *DAX-1* gene was useful for definitive diagnosis of the patient as well as for genetic counseling in families. These mutations were shown to eliminate the ability of *DAX-1* to inhibit SF-1-mediated transcription. Animal models of *Dax-1* mutations and overexpression (11), in conjunction with longitudinal studies in humans, will be useful to further define the functional role of *DAX-1*.

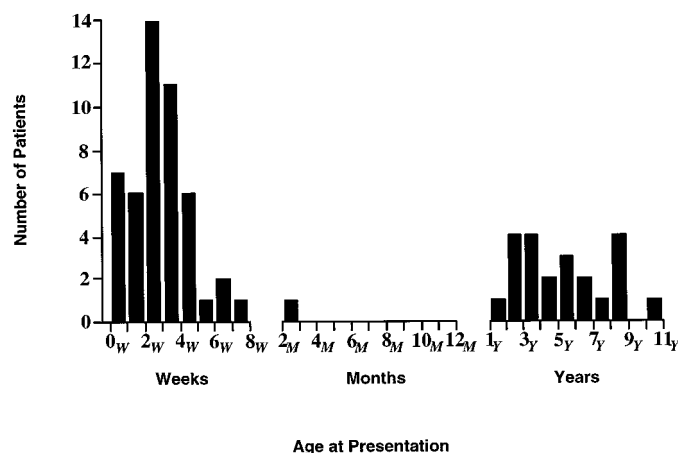


FIG. 4. Age at presentation of boys with *DAX-1* mutations described in the literature to date. The majority of patients presented in the first 2 months of life, or at various times after infancy. The patients described in this report are included. Note the nonlinear division of the age into weeks, months, and years (data derived from this study and cases reported in references 6, 13, 14, 32, 34–42).

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