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Missense Mutations Cluster within the Carboxyl-Terminal Region of DAX-1 and Impair Transcriptional Repression*

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ABSTRACT

DAX-1 is an orphan nuclear receptor that plays a key role in the development and function of the adrenal gland and hypothalamic-pituitary gonadal axis. Mutations in the gene encoding DAX-1 result in X-linked adrenal hypoplasia congenita (AHC). Affected boys typically present with primary adrenal failure in infancy or childhood and hypogonadotropic hypogonadism at the time of puberty. The majority of *DAX1* mutations described to date are nonsense or frameshift mutations that result in premature truncation of the DAX-1 protein and loss of DAX-1 repressor function. Relatively few missense mutations in *DAX1* have been reported. Here, we describe missense mutations in three additional families with X-linked AHC. When combined with previous reports, the *DAX1* missense mutations appear to cluster within restricted regions of the putative ligand-binding domain of DAX-1 and affect amino acids that are evolutionarily conserved, suggesting that these regions correspond to critical functional domains. Transcription assays, using a variety of artificial and native target genes, were performed to assess the effects of these mutations on the function of DAX-1. All DAX-1 missense mutant constructs showed marked loss of repressor function, with the exception of I439S, a mutation previously shown to be associated with delayed-onset adrenal failure and incomplete hypogonadotropic hypogonadism. These data indicate that most *DAX1* missense mutations associated with classic AHC exhibit marked loss of function. The locations of these mutations thereby identify important functional domains in the carboxyl-terminus of the protein. (*J Clin Endocrinol Metab* **86:** 3171–3175, 2001)

DAX-1 IS A transcription factor that plays a key role in the development and function of the adrenal gland and hypothalamic-pituitary-gonadal axis (1–4). Mutations in the gene encoding DAX-1 (*AHC*) cause X-linked adrenal hypoplasia congenita (AHC; OMIM: 300200) (1, 2). Boys with this condition usually present with primary adrenal failure in early infancy or throughout childhood (5). The associated feature of hypogonadotropic hypogonadism (HHG) becomes apparent at the expected time of puberty, and there is increasing evidence from *Ahch* (*Dax1*) knockout mice (6) and a limited number of patients with AHC (7, 8) that DAX-1 may also influence spermatogenesis directly.

DAX1 encodes a 470-amino acid protein that belongs to the orphan nuclear receptor superfamily (NR0B1). This protein has a carboxyl-terminal region that shares sequence homology with the ligand-binding domain (LBD) of other nuclear receptors (1). However, the amino-terminus of DAX-1 consists of a novel 66- to 67-amino acid repeat motif structure not found in other known transcription factors. No ligand for DAX-1 has been identified.

The majority of functional data reported suggest that DAX-1 represses the transcription of several genes expressed in the adrenal and reproductive axes, either directly or through an interaction with the related orphan nuclear receptor steroidogenic factor-1 (SF-1) (9). Some studies have suggested that this repressor function may involve specific transcriptional silencing domains within the carboxyl-terminus of DAX-1 (10), whereas others have proposed that DAX-1 can bind directly to hairpin loop structures in the promoters of certain target genes (11). Further, direct interactions between DAX-1 and repressors such as nuclear receptor corepressor (NCoR) (12) and Alien (13) have been described. The relative importance of these mechanisms in the regulation of different target genes remains unclear.

Over 60 different mutations in DAX-1 have now been reported in patients and families with X-linked AHC [for review, see Refs. 5 and 14]. The majority of these mutations are nonsense or frameshift mutations that cause premature truncation of the carboxyl-terminus of DAX-1. In fact, loss of as little as 9 amino acids at the carboxyl-terminus of DAX-1 is sufficient to impair DAX-1 function and to produce a clinically severe phenotype (15). In contrast, missense mutations or single codon deletions in DAX-1 are relatively rare; only 15 different mutations have been reported to date in

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published reports or abstracts involving 16 individuals or families with X-linked AHC (2, 8, 16–24). Although the majority of these children present early in life, an I439S missense mutation in DAX-1 was reported recently in a patient who presented first in adulthood with mild adrenal insufficiency and partial HHG (8).

The identification of additional *DAX1* missense mutations may be useful to define critical structural domains involved in DAX-1 function. Here we report *DAX1* missense mutations in three families with X-linked AHC and show that these and other mutations cluster within certain regions of the carboxyl-terminus of DAX-1. The functional effects of all reported DAX-1 missense mutations were assessed in a panel of established and novel gene transcription assays in an attempt to determine whether certain regions of DAX-1 are critical for the regulation of different target genes.

Materials and Methods

DNA sequence analysis

After obtaining institutional review board approval, DNA was extracted from patients' blood leukocytes using standard methods. The two exons, splice sites, and 5′-proximal promoter region of *DAX1* (−234 nucleotides) were amplified by PCR using six primer pairs and conditions described previously (25). Sequencing reactions were performed in forward and reverse directions using the dRhodamine sequencing kit and an ABI377 automated DNA sequencer (PE Applied Biosystems, Foster City, CA).

Construction of DAX1 expression vectors

DAX-1 expression vectors representing all reported naturally occurring missense mutants and single codon deletions were created using standard overlapping PCR strategies and wild-type human *DAX1* complementary DNA (cDNA) as a template. In addition, an artificial *trans*-activating function-2 (AF2) domain missense mutant (C467S) was created that was expected to have wild-type function and served as a negative control. Further, an AF2 deletion mutant (del448–470) was created as a positive control for loss of DAX-1 repressor function, as reported previously (9). Direct sequencing of all plasmids was performed to confirm the presence of the mutations.

Basal transcriptional activity

The effects of DAX-1 and its mutants on basal transcriptional activity were investigated using a modified mammalian two-hybrid system (8, 9). For these studies, cDNA corresponding to the putative LBD of DAX-1 (codons 207–470) was inserted into a pBIND expression vector (Promega Corp., Madison, WI) and linked to a GAL4 DNA-binding domain to allow expression of wild-type and mutant GAL4-DAX-1 fusion proteins. These expression vectors (50 ng) were cotransfected with a UAS-TK109luc reporter (500 ng) containing two GAL4-binding sites (9).

SF-1 mediated trans-activation

The effects of DAX-1 and its mutants on SF-1-mediated *trans*-activation was studied using full-length wild-type or mutant *DAX1* cDNA in a pCMX expression vector. The LBD of human SF-1 (*FTZF1*; codons 133–461) was linked to GAL4DBD in pBIND. These expression vectors (20 ng SF-1, 50 ng DAX-1) were cotransfected with a UAS-TK109luc reporter (500 ng) (9).

SF-1/Egr-1 synergistic activation of LHβ

The effects of DAX-1 and its mutants on SF-1/Egr-1 synergistic activation of the LHβ promoter were studied by cotransfecting full-length human wild-type or mutant pCMXDAX-1 (50 ng), full-length human pCMXSF-1 (20 ng), and full-length rat early growth response-1 (*Egr-1*; 20 ng) with a pA3 luciferase reporter containing nucleotides −154 to +5 of the native rat LHβ promoter (26).

SF-1/cAMP synergistic activation of inhibin α

The effects of DAX-1 and its mutants on SF-1/cAMP synergistic activation of the inhibin α promoter were studied by cotransfecting full-length human wild-type or mutant pCMXDAX-1 (50 ng), full-length human pCMXSF-1 (20 ng), and a protein kinase A expression vector (20 ng) with a pGL3 basic luciferase reporter containing nucleotides −146 to +68 of the native rat inhibin α promoter (27).

Transient transfections

Transient gene expression studies were performed using human embryonic kidney tsa210 cells grown in DMEM supplemented with 10% FBS and 1% streptomycin/penicillin in a 5% CO_2 atmosphere at 37 C. All transfections were performed in triplicate using calcium phosphate precipitation. Each experiment was repeated on six separate occasions. Data for each mutant construct are presented as a percentage of the DAX-1 empty vector control for that study. Results represent the mean ± SEM from the six independent experiments, each consisting of triplicate transfections.

Results

Case reports

Kindred 1 (A300P). The proband, a boy of Ashkenazi descent, was hyponatremic and hyperkalemic at 18 days of age. His serum aldosterone was undetectable, and PRA was markedly elevated. An initial diagnosis of congenital mineralocorticoid deficiency was made, and he was treated with 9α-fludrocortisone. However, he became progressively hyperpigmented, and reevaluation at 2 yr of age revealed hypocortisolemia (<1 μg/dL; 30 nmol/L) and elevated ACTH (>2000 pg/mL; >400 pmol/L), consistent with primary adrenal failure. *DAX1* sequence analysis revealed a hemizygous A300P mutation.

Kindred 2 (A300P). The proband, a male nonidentical twin, was born at 36 weeks gestation by cesarian section. He developed progressive hyponatremia and hyperkalemia in the first 2 weeks of life. Investigations of adrenal function revealed an impaired cortisol response to ACTH stimulation (16 → 19 μg/dL; 450 → 530 nmol/L), low aldosterone (3.1 ng/dL; 86 pmol/L), and elevated ACTH (348 pg/mL; 76 pmol/L) and 11-deoxycortisol (2372 ng/dL; 68.5 nmol/L). Gonadotropins were detectable (FSH, 4.2 mIU/mL; LH, 1.8 mIU/mL), and testosterone at 1 month of age was within the normal range (187 ng/dL; 6.5 nmol/L). A diagnosis of adrenal hypoplasia congenita was considered, and a hemizygous A300P mutation was found in *DAX1*.

Kindred 3 (E377K). The proband presented with a salt-losing crisis at 1 month of age. A diagnosis of 21-hydroxylase deficiency was considered, and he was started on glucocorticoid and mineralocorticoid replacement. However, 17-hydroxyprogesterone remained low despite variable compliance with treatment. Analysis of *DAX1* revealed an E377K mutation.

Clustering of missense mutations in DAX-1

These DAX-1 missense mutations in three kindreds (A300P, A300P, and E377K) and the two we described recently [L381H (22) and I439S (8)] are all located within the

carboxyl-terminal region of the DAX-1 protein. When taken together with other reported DAX-1 mutations, a potential clustering of affected amino acids within three regions of the putative LBD is suggested (Fig. 1). These regions may represent critical domains for DAX-1 function.

Transient gene expression assays

A variety of transient gene expression assays were developed to assess the effects of DAX-1 missense mutants on the transcription of potential target genes. As reported previously, wild-type DAX-1 was a potent repressor of basal transcriptional activity (Fig. 2A) (9) as well as of SF-1-mediated *trans*-activation (Fig. 2B) (9) and SF-1/Egr1 synergistic activation of the native LHβ gene promoter (Fig. 3A) (8, 28). In addition, we show that wild-type DAX-1 represses synergistic activation of the inhibin α gene promoter by SF-1/cAMP (Fig. 3B).

The library of naturally occurring DAX-1 missense mutants showed a loss of repressor activity in all of these assay systems (Figs. 2 and 3). In most cases the loss of function was equivalent to that seen with a mutant construct containing a deletion of the DAX-1 carboxyl-terminal region (del448–470). The effects of these missense mutations on DAX-1 function are consistent with the clinically severe phenotype and early onset of adrenal failure seen in most of these patients (Fig. 4). Of note, the I439S missense mutation, found in a patient who presented first in adulthood, had partial loss of repressor function (8), which seems unique compared with the other missense mutations (Fig. 4).

Discussion

The association between DAX-1 and X-linked AHC is well established, and many frameshift and nonsense mutations in DAX-1 have been described in patients with this condition. In contrast, missense mutations in DAX-1 are relatively rare, but may be important for identifying critical regions or amino acids necessary for DAX-1 function.

To address this issue, we studied the localization and functional effects of three newly identified DAX-1 missense mutations along with 13 others reported in the literature. These missense mutations are located within certain regions of the DAX-1 carboxyl-terminus. This clustering of mutations

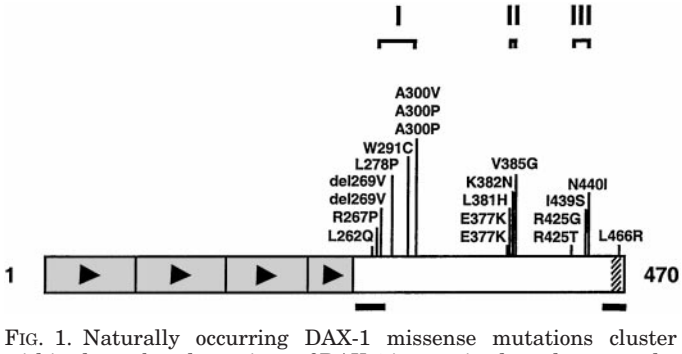

FIG. 1. Naturally occurring DAX-1 missense mutations cluster within the carboxyl-terminus of DAX-1 in a region homologous to the LBD of other nuclear receptors. The repeat motif structure of the amino-terminus of DAX-1 is shown by *arrows*. The carboxyl-terminal putative AF2 domain is *shaded*. Previously reported potential transcriptional silencing domains are shown by *black bars* (10).

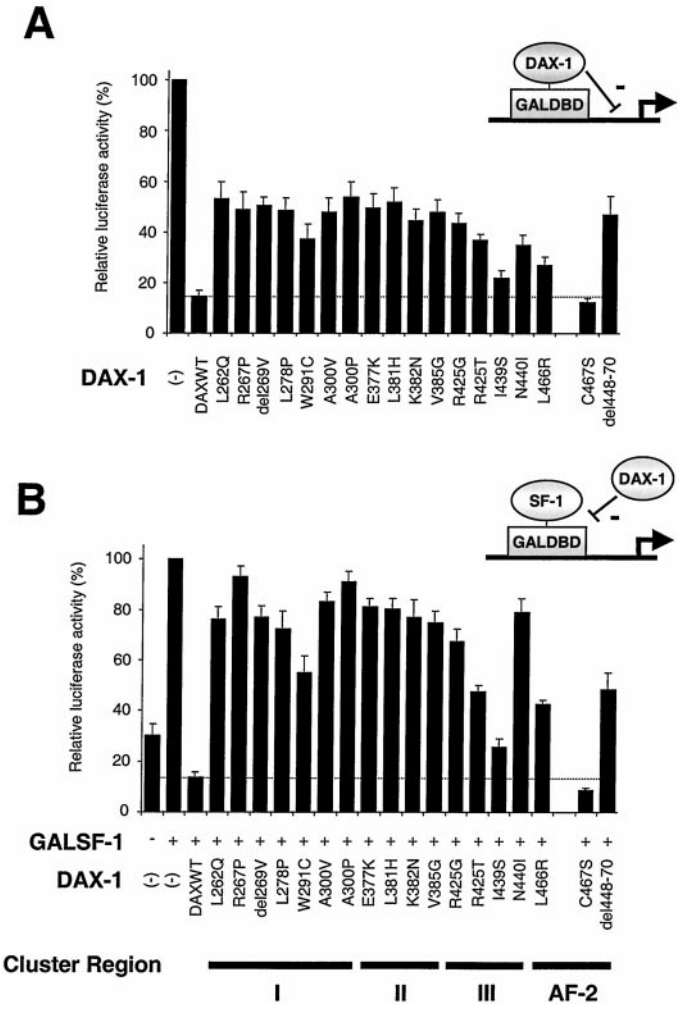

FIG. 2. Effects of naturally occurring missense mutations in DAX-1 on repressor activity in transient gene expression assays. *Upper panel*, The effects of GAL4-DAX-1 and its mutants on basal transcriptional activity. *Lower panel*, The effects of DAX-1 and its mutants on SF-1-mediated transcriptional activation. Cartoons depicting these gene expression assays are shown to the *right* of each panel. Data for each mutant are presented as a percentage ($\pm$SEM) of empty vector activity (100%) for six independent experiments, each performed in triplicate. *Dashed lines* represent the degree of repression obtained using wild-type DAX-1.

is most striking between codons 377 and 385, where four different amino acid mutations have been found, suggesting that this region represents an important functional domain. In addition, the description of different mutations within the same codon (A300V and A300P; R425G and R425T) and the presence of identical missense mutations in DAX-1 in unrelated families with X-linked AHC (del269V; A300P and E377K), suggests that certain amino acids within these regions have a critical function. Indeed, all DAX-1 missense mutations reported to date affect amino acids that are conserved absolutely in all species in which a DAX-1 homologue has been cloned, including the chicken (29) and alligator (30). It is particularly striking that no mutations are located within the amino-terminus of the protein. This finding could be due to chance. It is also possible that the amino-terminus of DAX-1 is not important for its function. More likely, the

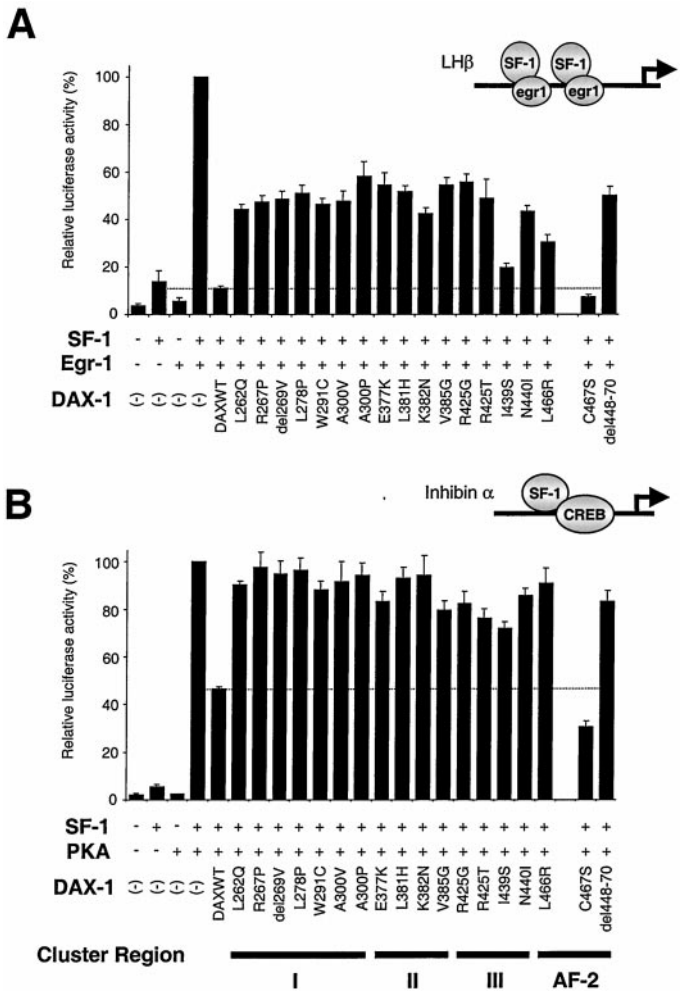

FIG. 3. Effects of naturally occurring missense mutations in DAX-1 on repressor activity in transient gene expression assays. *Upper panel*, The effect of DAX-1 and its mutants on SF-1/Egr-1-mediated synergistic activation of the native LH β promoter. *Lower panel*, The effects of DAX-1 and its mutants on SF-1/cAMP-induced synergistic activation of the native inhibin α promoter. Results are expressed as described in Fig. 2.

repeated nature of the domains within the amino-terminus may serve redundant functions, such that single amino acid substitutions are not sufficient to cause complete loss of function. These findings indicate the value of identifying naturally occurring DAX-1 missense mutations as a means of defining important functional domains.

A similar clustering of missense mutations has been reported previously within the LBD of the thyroid receptor (TRα) in patients with resistance to thyroid hormone (31). Attempts have been made to map DAX-1 missense mutations to the LBD of the TR, as determined by x-ray crystallography (32). Using this approach, it has been proposed that DAX-1 missense mutations affect amino acids that form the hydrophobic core of the DAX-1 LBD (19). These amino acids may affect subdomain structure rather than ligand binding and may be involved in ligand-independent interactions such as dimerization (19). Although our report of additional missense mutations in these regions adds further evidence to support clustering in these regions, some caution must be

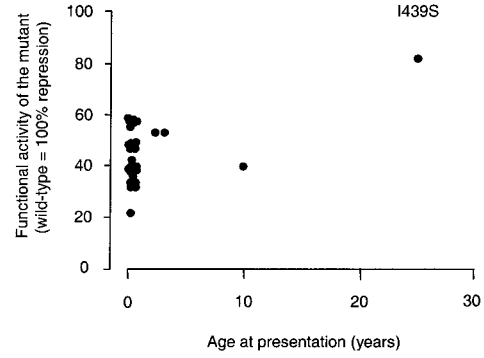

FIG. 4. Functional activity of the DAX-1 missense mutant constructs compared with age at presentation of affected patients. For each missense mutant construct, an index of mean repressor function in relation to wild-type DAX-1 (100%) was calculated using data from all four gene expression studies.

exercised when comparing DAX-1 to TRα, because their sequence homology is limited. Attempts have also been made to map the sequence of DAX-1 to other nuclear receptors, such as the retinoic acid receptor, retinoid X receptor, and peroxisome proliferator-activated receptor ((10, 19, 20, 33). Using this approach, the cluster regions I and II seem to map onto helexes 3, 4, 5 and 8, respectively. Key contact residues that form between these structures may be disrupted by missense mutations. However, the validity of this type of mapping approach will only be known when the crystal structure of the DAX-1 LBD is determined.

An alternative approach has been to consider which DAX-1 domains affect its role as transcriptional repressor. Lalli *et al.* (10) proposed that there are two transcriptional silencing domains in DAX-1, shown by the *black bars* in Fig. 1. Although several of the missense mutants fall within this region (*e.g.* R267P, del269,and L466R), many do not. Additional regions therefore seem to be involved in transcriptional repression. Other studies have proposed that DAX-1 can interact with repressors such as NCoR (12) or Alien (13). Crawford *et al.* (12) showed a weak direct interaction between DAX-1 and NCoR and provided data that DAX-1 repression of SF-1 activation involved NCoR recruitment. This interaction was impaired by introducing the natural missense mutant R267P into DAX-1, by deleting the DAX-1 carboxyl-terminus (from codon 369), or by introducing an artificial point mutation (F449D) into DAX-1 at a position that is conserved in the potent NCoR-interactive factor, RevErb. Altincicek *et al.* (13) showed a direct interaction between DAX-1 and the novel repressor, Alien. This interaction was also impaired with a R267P DAX-1 mutant or by deleting the DAX-1 carboxyl-terminus (del448–470). Finally, some data suggest that DAX-1 may also function as a ribonucleic acid-binding protein (34). Detailed investigation of how DAX-1 missense mutations alter its interactions with these repressors remains to be examined.

The different clusters of missense mutants could potentially have varying effects on different target genes. To investigate this, the library of missense mutants was studied in a variety of transient gene expression assays. In keeping with previous findings (8–10, 12, 28), DAX-1 was shown to function as a repressor of basal transcription, SF-1-mediated

trans-activation, and synergistic activation of the native LHβ promoter by SF-1 and Egr-1. In addition, we provide novel data that DAX-1 represses synergistic activation of the inhibin α promoter by SF-1 and cAMP. This effect may be relevant in the developing adrenal gland and hypothalamic-pituitary-gonadal axis (27, 35). Introducing point mutations into DAX-1 impaired repressor activity with each of these promoters. No difference in loss of repression was found among the different clusters of mutations in different assays. This finding suggests that the mechanism of repression by DAX-1 may be consistent in these different systems.

The only significantly different effect on DAX-1 function occurred with the I439S mutant. This mutation has partial function and was discovered in a patient with delayed-onset adrenal failure and incomplete HHG (8). Further, when repressor activity of these missense mutations was compared with the age at presentation, the I439S mutation once again appeared unique. These data provide evidence that other modifier genes and environmental factors influence the clinical presentation of X-linked AHC significantly, and that a genotype-phenotype correlation cannot be predicted easily.

In summary, these data show how naturally occurring missense mutations can be useful in identifying potentially important domains in DAX-1. Identification of additional missense mutations will help in this regard. In the future, crystallographic studies of the DAX-1 LBD will help determine the structural significance of these regions, and further studies of the interaction between these domains and putative corepressors/coactivators will help elucidate their biological effect in the development and function of the adrenal gland and hypothalamic-pituitary-gonadal axis.

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