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An Alternate Translation Initiation Site Circumvents an Amino-Terminal DAX1 Nonsense Mutation Leading to a Mild Form of X-Linked Adrenal Hypoplasia Congenita

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Mutations in *DAX1* [dosage-sensitive sex reversal-adrenal hypoplasia congenita (AHC) critical region on the X chromosome gene 1; *NR0B1*] cause X-linked AHC, a disease characterized by primary adrenal failure in infancy or childhood and reproductive abnormalities later in life. Most of these patients have nonsense or frameshift mutations that cause premature truncation of the DAX1 protein, thereby eliminating its transcriptional silencing activity. We evaluated a patient with an unusual form of AHC manifest as late-onset adrenal insufficiency and gonadal failure. DNA sequence analysis revealed a novel amino-terminal DAX1 nonsense mutation (Q37X), predicted to cause a severe truncation of the protein. Using a combination of *in vitro* translation assays and studies of DAX1 expression and function in transfected cells, we demonstrate that, in contrast to more distal mutations leading to a nonfunctional protein, this mutation is associated with a milder phenotype due to the expression of a partially functional, amino-truncated DAX1 protein generated from an alternate in-frame translation start site (methionine, codon 83). The production of this amino-truncated isoform appears to rescue the classical AHC phenotype, thereby delaying the onset of clinically significant adrenal dysfunction until early adulthood. Thus, this case demonstrates a relatively rare phenomenon by which the clinical severity of an inherited human disease is reduced after alternate translation from a site downstream of a premature stop codon. (*J Clin Endocrinol Metab* 88: 417–423, 2003)

DAX1 (also known as adrenal hypoplasia congenita (*AHC*), *NR0B1*) encodes an orphan nuclear receptor that regulates the development and function of the adrenal gland and hypothalamic-pituitary gonadal axis (1, 2). The carboxyl-terminal region of DAX1 resembles the ligand-binding domain (LBD) of other nuclear receptors, whereas the amino-terminus consists of a unique repeat structure that contains several LXXLL-like motifs implicated in protein-protein interactions (3). Functional studies suggest that DAX1 is a repressor of gene transcription, acting in part by inhibiting the activity of another nuclear receptor, steroidogenic factor-1 (SF1, NR5A1) (4).

Mutations or deletions of *DAX1* cause X-linked AHC (AHC OMIM, 300200) (1). AHC is an inherited disorder of adrenal gland development, characterized by lack of the permanent zone of the adrenal cortex. Boys with this condition usually present with severe primary adrenal failure in infancy or childhood. Hypogonadotropic hypogonadism becomes apparent at puberty, and infertility results from gonadotropin deficiency in combination with a primary defect in spermatogenesis (5, 6). Over 80 different mutations in *DAX1* have been described (7), most of which are nonsense or frameshift mutations that cause premature truncation of the protein. Deletion of as few as the last nine amino acids of DAX1, which constitute a putative activation function-2 (AF2) domain, is associated with a severe clinical phenotype (8).

Recently, an adult-onset form of AHC has been described in two patients who have missense mutations in the putative LBD of DAX1 (9, 10). These mutations (I439S, Y380D) were shown to have intermediate levels of repressor activity in transient gene expression studies, consistent with the mild phenotype of the affected individuals. Other variant phenotypes include isolated hypogonadotropic hypogonadism in a female homozygous for a *DAX1* mutation through gene conversion and extreme pubertal delay in heterozygous female carriers (6, 11). Here we report a novel mechanism of mild AHC; namely, reduced production of a shorter DAX1 isoform resulting from alternate translation downstream to an amino-terminal stop codon. Our observations provide a rare example of the phenotypic rescue of an inherited disease by the circumvention of an otherwise severe mutation at the level of translation.

Abbreviations: AF2, Activation function-2; AHC, adrenal hypoplasia congenita; LBD, ligand-binding domain; SF1, steroidogenic factor-1; WT, wild type.

Materials and Methods

DNA sequencing and mutational analysis

After obtaining written consent, genomic DNA was extracted from peripheral blood leukocytes using standard procedures. Both exons of *DAX1* were amplified by PCR using specific oligonucleotide primer pairs and conditions described previously (12). Direct sequencing of PCR products was performed using a *Taq* Big Dye Terminator Sequencing Kit and ABI 3100 automated sequencer (PE Applied Biosystems, Foster City, CA).

Construction of human DAX1 expression vectors

DAX1 expression vectors (pCMX) containing the Q37X, W39X, F448X, M83K, and Q37X/M83K mutations were created by overlapping PCR using methods described previously (9, 13). Expression vectors containing cDNA for wild-type (WT) *DAX1*, the naturally occurring Y399X nonsense mutant (13), and an artificial F448X mutant were used as positive and negative controls for both DAX1 function and *in vitro* translation.

The construction of the amino-terminally deleted expression vectors lacking the first 40, 80, and 85 amino acids was made by insertion of the PCR-generated fragments (see below) after digestion with *EcoR*I and *Spl*I (d40), and *EcoR*I and *BspE*I (d80, d85), respectively.

The following primer pairs were used to amplify the three fragments, respectively: 1) forward, 5′-GATCGAATTCTGTTCGTGCGGCGATGAG-3′; and reverse, 5′-CCTCTGCGCGAAG TAGGAGC-3′; 2) forward, 5′-GATCGAATTCTACAGCATGCTGACG AGCG-3′; and reverse, 5′-G GACGCCCAGCAGTTGCGCA-3′; and 3) forward, 5′-G ATCGAATTCAGCGCAAAGCAAACG TAC-3′; and reverse, 5′-GGA CGCCCAGCAGTTGCGCA-3′.

To allow antibody-mediated detection of recombinant DAX1 proteins, *DAX1* cDNAs for WT and Q37X were cloned into the pcDNA 6/V5-HisA expression vector (Invitrogen, Carlsbad, CA). Briefly, cDNAs were amplified using primers that introduce *Nhe*I and *EcoR*I sites (forward, 5′-GATCGCTAGCCAGTGGGCAGAAC TGGGCTAC-3′; reverse, 5′-GATCGAATTCTATC TTTGTACAGAGCATTTC-3′), and PCR-generated fragments were cloned into the expression vector after digestion with the corresponding endonucleases.

The presence of the desired mutations/deletions and the integrity of the constructs were confirmed by direct sequencing before studies of protein expression and function.

In vitro translation of WT and mutant DAX1

WT and mutant *DAX1* cDNAs were *in vitro* transcribed and translated in the presence of [^{35}S]methionine using a TNT-coupled reticulocyte lysate system (Promega Corp., Madison, WI). A total of 250 ng of each dsDNA template were incubated at 30 C for 90 min in a reaction mixture of 12.5 μl. Denatured protein products were resolved with SDS-PAGE and detected by autoradiography after overnight exposure.

Western blotting

Human embryonic kidney tsa201 cells were transfected with 10 μg pcDNA6/V5-HisA *DAX1* WT or Q37X. Equivalent amounts of protein lysates from transfections were resolved with SDS-PAGE and transferred to polyvinylidene difluoride membranes using standard methods. Blots were blocked in Tris-buffered saline containing 0.1% Tween 20 and 5% skim milk powder; incubations with antibodies were performed in the same buffer but without the skim milk powder. Recombinant DAX1 was probed with a 1:5,000 dilution of the primary antibody toward the V5 epitope and a 1:10,000 dilution of the secondary antimouse antibody. Reactive bands were detected using a chemiluminescence kit (NEN Life Science Products, Boston, MA) with Kodak MS x-ray film (Rochester, NY).

Functional analysis of WT and mutant DAX1

Transient gene expression studies were performed using human embryonic kidney tsa201 cells grown in DMEM supplemented with 10% fetal bovine serum and 1% streptomycin/penicillin in a 5% CO_2 atmosphere at 37 C. A luciferase reporter construct (500 ng) containing the native rat *LH*β promoter (−154 to +5) was cotransfected with expression vectors containing full-length human *SF1* (*NR5A1*; 20 ng), full-length rat early growth response-1 (*Egr1*; 20 ng), and full-length human WT or mutant *DAX1* (50 ng), as described previously (10, 14). Luciferase assays were performed 48 h later. The results of triplicate transfections are expressed as the mean ± SEM.

Quantitation of DAX1 mRNA

DAX1 mRNA levels were measured by RT-PCR using real-time fluorescent detection. Enzymes were purchased fromPE Applied Biosys-

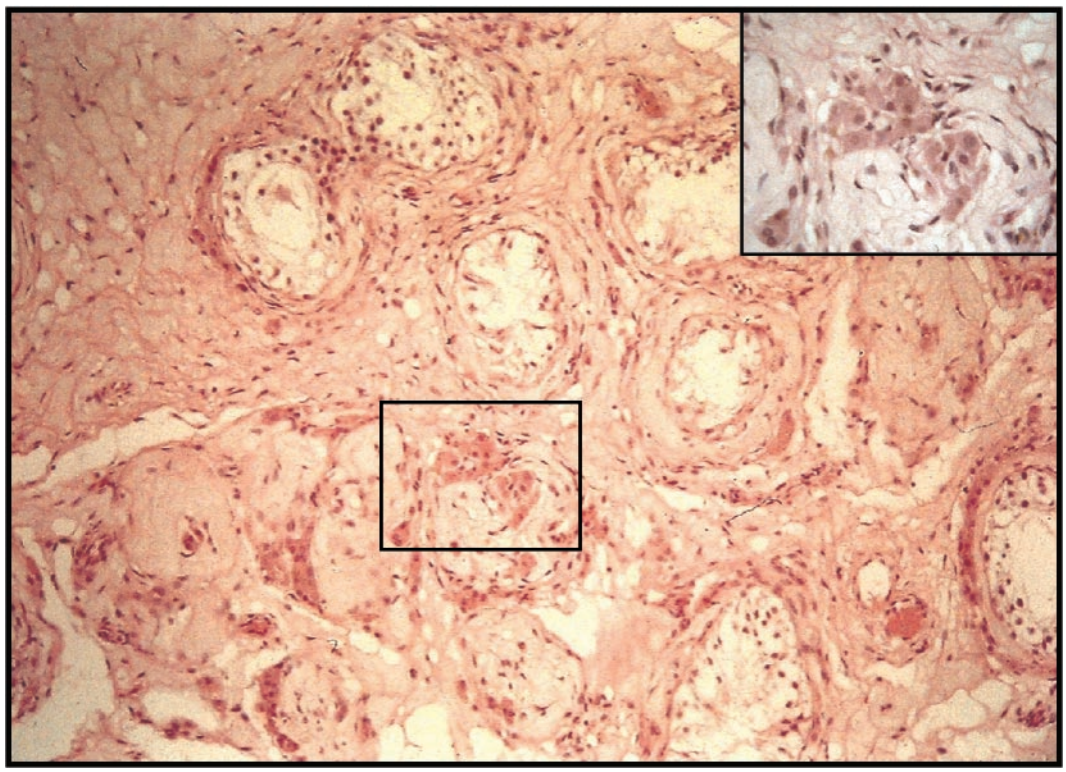


FIG. 1. Photomicrograph (hematoxylin and eosin staining; magnification, ×50) of the testicular biopsy. Disorganization of the normal tubular structure and abnormal proliferation of the interstitial tissue. *Inset*, moderate Leydig cell hyperplasia (magnification, ×200). Ectasia of the rete testis was also noted by Doppler ultrasound (data not shown).

tems, and assays were carried out according to the manufacturer's protocol. Detection was performed on an ABI 7700 sequence detector. Sequences were: 5′ primer, CTGCAGCACCATTTGGCAC; 3′ primer, GGT ACTGATGTTCAGACTCCAGCAT; probe, FAM-CCTCCCAG-GTCCAAGCCATCAAGTG-BlackHoleQuench (Quiagen Operon, Alameda, CA). To permit quantitation of copy number, a 259-bp fragment encompassing the RT-PCR amplicon was subcloned into the pGEM-3z plasmid and transcribed using T7 RNA polymerase. A linear dilution of the transcribed RNA was included in each assay as a standard curve.

Results

Clinical presentation of late-onset AHC

A 20-yr-old male presented with a history of fatigue, nausea, and hyperpigmentation. Clinical laboratory investigations revealed hypocortisolemia (25 nmol/liter; normal range, 140–700), hypoaldosteronism, and elevated ACTH (226 pg/ml; normal, 10–60), consistent with primary adrenal failure. He was hypogonadal (4-ml testes bilaterally) and had subnormal testosterone (7.5 nmol/liter; normal, 10–35), normal LH (4.8 IU/liter; normal, 1–5), and elevated FSH (20 IU/liter; normal, 1–5) with low inhibin B levels (43.5 pg/ml; normal, 100–400). The Sertoli cell aromatase bioassay, which evaluates the FSH-dependent aromatase activity (conversion of androgen substrate to estradiol) in cultured Sertoli cells from 7- to 10-d-old rats, revealed a normal bioactive/immunoreactive ratio (0.49; normal, 0.3–1.5), indicating the presence of biologically active FSH in the patient's serum. Azoospermia was found on semen analysis and did not improve after 6 months of treatment with exogenous gonadotropins. Testicular biopsy revealed disorganization of the normal seminiferous tubular structure, and there was moderate Leydig cell hyperplasia (Fig. 1), confirming an intrinsic defect in spermatogenesis similar to that found in *Ahch* (also known as *Dax1*) knockout mice (15, 16). There was no family history of affected males. The patient's mother was not available for genetic testing.

Diagnosis of X-linked AHC and characterization of the molecular mechanism leading to the unexpected mild phenotype

Direct DNA sequencing revealed a novel nonsense mutation (Q37X, CAG→TAG) in the amino-terminal region of DAX1 (Fig. 2A), which is predicted to result in a severely truncated protein devoid of repressor activity. However, consistent with the mild clinical phenotype, transient gene expression studies unexpectedly showed that this mutation causes only partial loss of DAX1 function (Fig. 2B). Similar results were obtained after the introduction of a W39X mutation into DAX1, a change reported recently in a patient with transient neonatal hypoaldosteronism due to AHC who did not develop significant adrenal dysfunction until adolescence (17).

We hypothesized that the mild clinical phenotype seen in patients with these amino-terminal nonsense mutations might be accounted for by alternate translation from a site downstream of the mutation, yielding an amino-truncated, yet partially functional, form of the protein. Compared with WT DAX1 (51-kDa), *in vitro* proteins translated from cDNA constructs containing the Q37X or W39X mutations generated a shorter protein product of 43 kDa (Fig. 1B). This

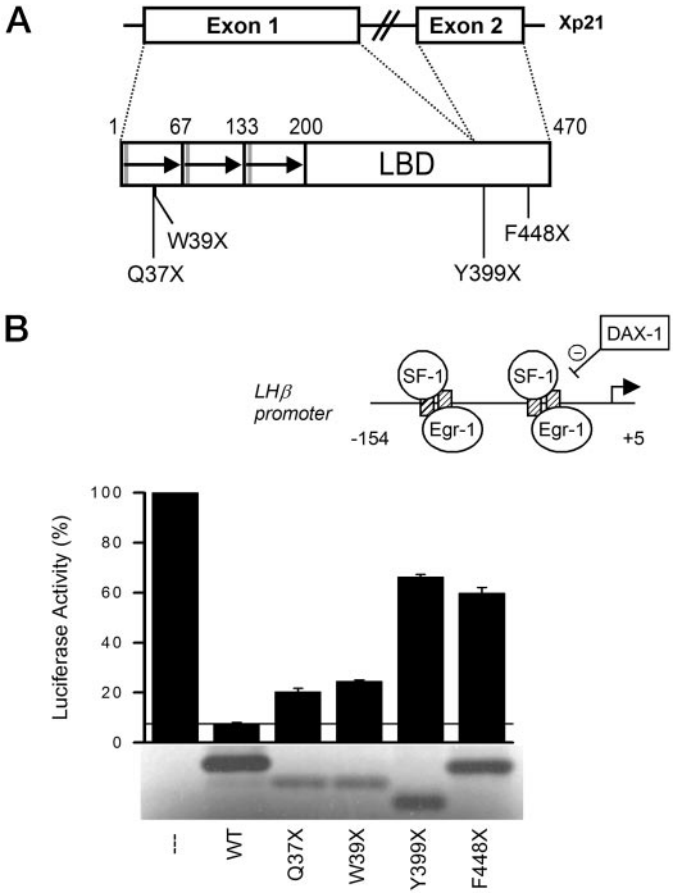
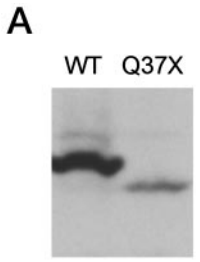


FIG. 2. A, Schematic representation of the human *DAX1* (*NR0B1*) gene. The amino-terminal repeat motif is shown. The LxxLL-type domains are indicated by *gray boxes* and begin at amino acids 13 (LYNML), 80 (LYSML), and 146 (LYSLL). The position of the Q37X nonsense mutation described in this report is also shown along with those of W39X, Y399X (another naturally occurring mutation that is associated with a severe AHC phenotype), and an artificial F448X mutant that removes the putative AF2 domain. B, Transcriptional activity of mutant DAX1. A luciferase reporter construct (500 ng) containing the native rat *LHβ* promoter (−154 to +5) was cotransfected into tsa201 cells with expression vectors containing full-length human *SF1* (*NR5A1*; 20 ng), full-length rat early growth response-1 (*Egr1*) (20 ng), and full-length human WT or mutant *DAX1* (50 ng). Both Q37X and W39X mutants showed only partial loss of DAX1 wild-type repressor function compared with the more profound loss of repression activity seen with the C-terminal deletion mutants (Y399X and F448X). Data are presented as a percentage (±SEM) of empty vector activity (100%) for triplicate experiments. Partial loss of repressor function was similarly observed with both native rat aromatase (*Cyp19*) and *inhibin α* promoters (data not shown). WT and mutant DAX1 cDNAs were *in vitro* transcribed and translated using a reticulocyte lysate system in the presence of [^{35}S]methionine. As expected, WT *DAX1* cDNA produced a 51-kDa protein. The Q37X cDNA as well as W39X generated a shorter 43-kDa product, which was weakly detectable in the WT lane.

43-kDa band is also faintly visible in the WT lane, suggesting that this protein may be normally expressed as a minor protein variant.

The translation of this smaller protein product in the presence of the Q37X mutation was confirmed by Western blot analysis (Fig. 3A). Levels of mutant protein were lower than WT levels in the experiments. This observation appears to



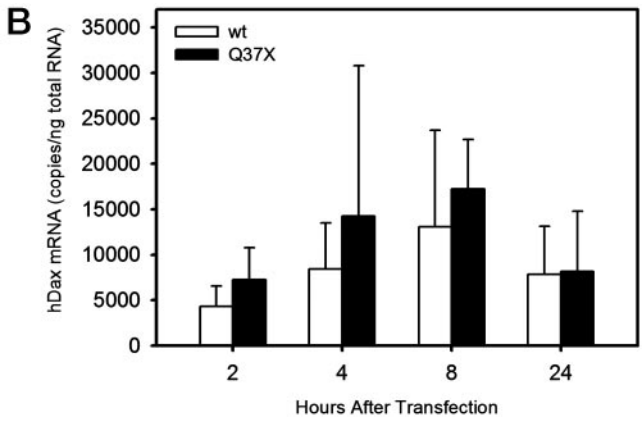


FIG. 3. A, Detection of the recombinant DAX1 proteins containing the carboxyl-terminal V5 epitope and a polyhistidine tag. Whole cell extracts were probed with the anti-V5 antibody by Western blotting. Reactive bands represent 56- and 48-kDa products for the WT and Q37X mutants, respectively (the epitope and the tag add approximately 5 kDa to the molecular mass of the corresponding proteins). B, WT and Q37X transcript levels measured after 2, 4, 8, and 24 h of transfection. The *bars* represent the copy number of mRNA detected by real-time fluorescent RT-PCR (the results of triplicate transfections are expressed as the mean ± SEM).

reflect a lower translation efficiency from the alternate start site, as mRNA levels were not different for the two constructs (Fig. 3B).

Examination of the *DAX1* nucleotide sequence identified a putative internal in-frame translation start site, with a partially conserved Kozak consensus sequence flanking the methionine at codon 83 (**A**GC**ATGC**) (18). This methionine residue lies within the second LXXLL-like motif in DAX1 and is highly conserved among several species (Fig. 4A) (19). Serial amino-terminal deletions of DAX1 were made to investigate the putative role of M83 in translation initiation. A loss of function occurred with progressive deletion from codon 80 to codon 85 (Fig. 4B). Further, introduction of an M83K missense mutation (ATG→AAG) at the putative translation start site did not affect WT DAX1 repressor activity, but abolished synthesis of the 43-kDa product. As expected, introduction of this M83K missense mutation into the background of the Q37X mutant sequence impaired DAX1 function and prevented translation of the shorter isoform (Fig. 4C). These findings suggest that methionine 83 serves as an alternative internal translation initiation codon.

Discussion

The presentation of X-linked AHC in an adult male is unusual and prompted us to investigate the functional properties of the Q37X mutant. The premature termination of the DAX1 protein at amino acid residue 37 was predicted to generate a small peptide devoid of repressor activity. The partially preserved repressor function of this mutant in transient gene expression studies was surprising and led us to consider the possibility that translation might occur at an alternate site downstream from this codon, thereby leading to an amino-truncated, yet partially functional, protein. Several independent lines of evidence support this idea, including 1) the expression of shorter 43-kDa protein in an *in vitro* translation assay; 2) the loss of the 43-kDa product after deletion or introduction of a missense mutation into methionine 83; 3) detection of the shorter protein in transfected cells; and 4) the correlation of transcriptional repression with expression of the truncated protein.

The amino-terminus of DAX1 is unusual among nuclear receptors because it consists of a repeat domain structure containing three LXXLL-like motifs implicated in protein-protein interactions instead of a characteristic zinc finger DNA-binding domain. The alternatively translated Q37X mutant DAX1 predicted for this patient would lack 82 amino acids at the N terminus, thereby disrupting the first two LXXLL-like motifs. The partial loss of function observed in this amino-terminally truncated protein and the mild phenotype of the patient suggest that the first 82 amino acids are partly dispensable, and that conservation of one motif is sufficient for residual DAX1 function.

As noted previously, the 43-kDa band is also faintly visible when the WT protein is translated, suggesting that the ribosomes can bypass the optimal M1 site and initiate translation at the downstream M83 site. However, these *in vitro* experiments do not allow us to determine whether translation initiation at this position occurs at low levels in normal subjects or whether this alternate site is preferentially used in the presence of an upstream premature stop codon.

The levels of the alternately translated Q37X and W39X isoforms are less than that of the WT protein. There are at least three possible mechanisms for this: 1) although Kozak sequence flanking the ATG at position 83 suggests that it can be used as an initiator methionine (18), translation from internal methionines may be less efficient; 2) eukaryotic cells employ proofreading systems to identify and degrade mRNAs that harbor a premature stop codon or lack a termination codon (*i.e.* nonsense-mediated mRNA decay and nonstop mRNA decay, respectively) (20, 21). We did not observe such a decrease in transcript levels in transiently transfected cells, but interpret this finding with caution because mRNA stability may differ *in vivo*; or 3) the truncated DAX1 protein itself may be unstable.

In humans, milder phenotypes of inherited disorders can arise by various mechanisms. For example, exon skipping has been reported as a mechanism to restore an open reading frame in cases in which an mRNA transcript otherwise contains nonsense or frameshift mutations [Becker muscular dystrophy (OMIM 310200), *etc.*] (22–24). Recently, alternative translation using an open reading frame generated by a 5-bp deletion in *NBS1* has been suggested to produce a protein that diminishes the severity of the Nijmegen breakage syndrome (OMIM 602667) phenotype in humans (25). This finding provides an explanation for why *Nbs1*-null mice are not viable, but humans who are homozygous for the 657del5 allele survive, albeit with a predisposition to develop cancer later in life. Of note, an unexpectedly mild (and late-onset)

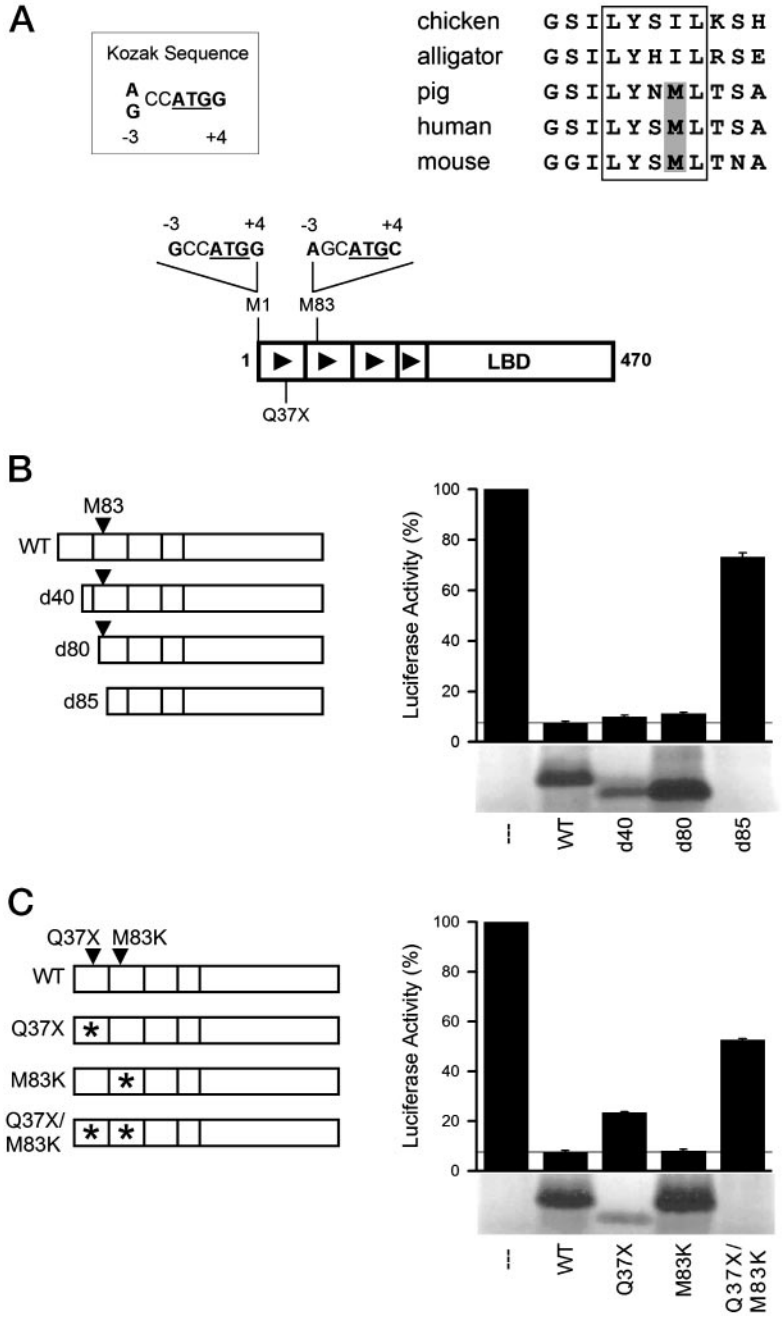


FIG. 4. A, Kozak sequences flanking methionines at codon 1 (M1) and 83 (M83); M1 displays a consensus Kozak sequence, whereas M83 lacks a guanosine at position +4. Conservation of the methionine within the LXXLL-like motif among human, mouse, and pig, but not chicken and alligator, is also shown. B, Mapping of the alternate translation start site. Three expression vectors containing different amino-terminal deletions were generated lacking the first 40, 80, and 85 amino acids (d40, d80, and d85), respectively. The two most amino-terminal deletion mutants showed conserved DAX1 repressor activity in functional studies, whereas d85, which lacks the predicted alternative initiation site (M83), showed a complete loss of DAX1 function. When these three amino-terminal deletion mutants were *in vitro* translated, the 43-kDa protein was present in those containing M83. In contrast, its translation was abolished in the d85 mutant. C, Replacement of methionine at codon 83 with lysine (M83K) did not affect DAX1 function, but prevented *in vitro* synthesis of the shorter DAX1 protein. When both Q37X and M83K mutations were introduced into DAX1, no bands were detected by *in vitro* translation. In functional assays, a more profound loss of repression was observed than in the construct containing the Q37X mutation alone.

variant of peroxisome biogenesis disorder (OMIM 601539) was identified in a patient compound heterozygous for two seemingly severe mutations in the *PEX12* gene. Molecular studies suggest that the transcript of the allele harboring a 2-bp deletion in codon 9 allows translation of a shorter protein from a downstream in-frame methionine (26). It appears that internal ribosome entry is the mechanism underlying this mild phenotype. Finally, although alternative protein isoforms resulting from different translation start sites are recognized to have different biological roles (*e.g.* glucocorticoid and progesterone receptor A and B isoforms) (27–29), the importance of these isoforms in human disease has yet to be defined.

There are other rare instances where amino-terminally truncated proteins are generated by downstream initiation after premature termination in the extreme amino terminus. However, in all of these cases the amino-truncated protein does not possess sufficient function to reduce the severity of the clinical phenotype (30–32). DAX1 is different from these other proteins because it contains a repeat motif structure in the amino terminus with presumed functional redundancy among the motifs. In contrast, loss of the carboxyl terminus of DAX1 (including the AF2 domain) produces a severe clinical phenotype, whereas partial or less severe disease phenotypes have been reported when a premature stop codon leads to a carboxyl truncation of other proteins (33).

Obviously, the functional effects of these truncations in various proteins depend on whether deletion of the carboxyl terminus affects domains that are important for protein stability or function. Finally, reduced translational efficiency due to point mutations that alter the consensus context of the authentic ATG initiator represents another mechanism underlying less severe disease phenotypes (34).

The data presented here suggest that internal in-frame translation of a shorter DAX1 protein partially rescues the clinical phenotype of a patient with a nonsense mutation in the extreme amino terminus of DAX1 and delays the onset of adrenal dysfunction. Interestingly, except for the Q37X and W39X variants, other naturally occurring nonsense or frameshift mutations that cause a premature termination codon 5′ to the putative alternative start site (codon 83) are associated with the classical AHC phenotype (35–37). In one of these cases (35), it is possible that the relative proximity of the nonsense mutation (codon 81) interferes with recognition of the juxtaposed alternate start site by ribosomes. Similarly, deletion or insertion of nucleotides (36, 37) may disrupt the sequence required for internal ribosomal entry, thereby impairing alternate translation start.

Of note, all missense mutations in DAX1 reported to date are located within the putative carboxyl-terminal LBD (13). Functional redundancy may exist within the amino-terminal repeat motif structure, which varies in number in different species (19). The presence of at least one repeat in the translated protein appears to be sufficient to preserve partial DAX1 function, particularly in view of the fact that the amount of expressed protein is likely to be reduced.

Here, we show that translation from an internal in-frame start site downstream of a nonsense mutation can reduce the clinical severity of AHC. On the other hand, in the case of DAX1, gene dosage appears to play a critical role in protein function (38). Thus, it is possible that the clinical features reflect reduced expression from a hypomorphic allele in addition to effects on the protein itself. Our findings also demonstrate that residual DAX1 function is sufficient to delay the onset of overt adrenal failure, but is not capable of supporting normal testis development and function.

One might predict from this observation with DAX1 that the physiological impact of polymorphic variants or mutations in other genes might also be modified when functional protein products can be produced from alternative translation initiation sites.

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