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CLINICAL CASE SEMINAR

Progressive Onset of Adrenal Insufficiency and Hypogonadism of Pituitary Origin Caused by a Complex Genetic Rearrangement within DAX-1

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DAX-1 [dosage-sensitive sex reversal, adrenal hypoplasia congenital (AHC) critical region on the X chromosome, gene 1] is a transcription factor expressed in the adrenal gland and at all levels of the gonadotrope axis. Inactivating mutations of *DAX1* result in the X-linked form of AHC with associated hypogonadotropic hypogonadism. AHC usually reveals itself as adrenal failure in early infancy, although a wide range of phenotypic expression has been reported. We describe a patient who was diagnosed with adrenal failure at 6 wk of age, but who experienced recovery of adrenal function of several months' duration later in infancy. He subsequently failed to undergo puberty because of hypogonadotropic hypogonadism of pituitary origin, and he was also diagnosed with schizophrenia in early adulthood. Molecular genetic analyses revealed a complex rearrangement in *DAX1*, including a 2.2-kb deletion spanning the entire second exon and a small 27-bp insertion. The putative protein encoded by this mutated gene is 429 amino acids long. The initial 389 residues probably correspond to the wild-type DAX-1 sequence, whereas the last 40 amino acids are presumably completely unrelated, being transcribed from the intronic sequence adjacent to exon 1. *In vitro* functional analyses confirm the absence of repressor activity exerted by such mutant protein. These studies expand the genotypic and phenotypic spectrum of DAX-1 insufficiency in humans. (*J Clin Endocrinol Metab* 87: 4094–4100, 2002)

DAX-1 [DOSAGE-SENSITIVE SEX reversal, adrenal hypoplasia congenital (AHC) critical region on the X chromosome, gene 1] is a member of the orphan nuclear receptor superfamily (1, 2). It is expressed in the adrenal glands and at all levels of the hypothalamo-pituitary-gonadal axis (3, 4). This pattern of expression is consistent with a role for DAX-1 in the development and function of the adrenal gland, as well as in reproduction. It is also reminiscent of the expression pattern of steroidogenic factor 1 (SF-1), a transcription factor involved in the regulation of steroidogenic enzymes as well as that of gonadotropic hormones (5, 6). Duplication of DAX-1 is associated with male-to-female sex reversal (7, 8). On the other hand, inactivating mutations of this gene cause the X-linked form, so called cytomegalic variant, of AHC (1, 2). This rare syndrome results in adrenal failure and is usually lethal in early infancy unless recognized and treated. Affected individuals commonly fail to undergo puberty later in life (9) because of an association with hypogonadotropic hypogonadism (10).

In vitro data suggest that one function of DAX-1 is to act as a repressor of SF-1-stimulated transcription (11). However, the phenotype of the *Ahch* (mouse equivalent of *DAX1*) knockout mouse (12), similar to a recently described human case of DAX-1 mutation (13), also implicates DAX-1 in the control of spermatogenesis. To date, a number of frameshift and nonsense mutations of DAX-1 have been described (summarized in Refs. 14 and 15). They all result in a truncated protein lacking its carboxy-terminal end. This pattern is fully consistent with the localization of a repression domain in that part of the DAX-1 protein (11, 16). Fewer missense mutations have been described, also localized at the carboxy-terminal end of the molecule.

We report here the clinical features of a patient who presented an unusual evolution of adrenal failure in infancy, and complete hypogonadotropic hypogonadism of pituitary origin later in life. This patient harbors a complex mutation of DAX-1, combining a large deletion including the entire second exon of the gene with a small insertion of 27 bp. *In vitro* studies confirmed the complete lack of functional repression of SF-1-mediated transcription resulting from this mutation. The phenotype of this patient, who presented with a gradual progression of adrenal insufficiency early in childhood, confirms the existence of a wide spectrum of clinical presentations of AHC (17, 18) and is therefore important for the appropriate clinical management of the syndrome.

Abbreviations: AHC, Adrenal hypoplasia congenita; DAX-1, dosage-sensitive sex reversal, AHC critical region on the X chromosome, gene 1; DBD, DNA binding domain; SF-1, steroidogenic factor 1; StAR, steroidogenic acute regulatory protein.

Subjects and Methods

Case report

The proband was a male infant born after an uneventful full-term pregnancy, second son of two. There is no family history of somatic

disorder, and in particular no history of endocrinopathy of any sort. However, family history is remarkable for the presence in the patient's mother of an unspecified psychiatric illness, which required several hospital stays during the patient's childhood. The mother has now been stable for several years on haloperidol decanoate treatment.

In wk 6 of life, the proband presented with vomiting, dehydration, hyponatremia, and hyperkalemia with normal renal function. The work-up showed elevated plasma levels of ACTH at 172 ng/liter, decreased levels of plasma aldosterone and markedly decreased levels of urinary 17-ketosteroids, tetrahydrocortisone, and tetrahydrocortisol. There was no response of plasma aldosterone or of the urinary metabolites to cosynthropin administration (250 mg iv). A diagnosis of adrenal insufficiency was made. He subsequently did well on cortisone and deoxycorticosterone acetate replacement therapy associated with salt supplementation, his plasma ACTH levels decreasing to 73 ng/liter.

At age 2 yr and 6 months, he was evaluated for failure to thrive possibly due to exogenous glucocorticoid excess. Under no hormonal therapy, baseline ACTH levels were 1340 ng/liter in the face of a cortisol value of 480 nmol/liter (normal range: 200–700 nmol/liter). Stimulation with cosynthropin, 250 mg iv, induced only a marginal rise in cortisol to 504 nmol/liter. The patient was then considered to have relative primary adrenal insufficiency. Hormonal replacement was withheld, and the patient performed well clinically. However, after more than 6 months on no hormone supplementation, he experienced recurrent vomiting accompanied by dehydration, hyponatremia, and hyperkalemia. Therefore, hormonal replacement therapy was resumed. The patient, now 25 yr old, has been under this medication ever since, lately as hydrocortisone and fludrocortisone.

His medical history remained uneventful until age 16 yr, when he was evaluated for delayed puberty. His testicular volume was 4 ml bilaterally. Biochemical work-up showed low gonadotropin levels: LH was 0.6 IU/liter (normal adult range: 3.0–13 IU/liter) and FSH 1.0 IU/liter (normal adult range: 2.0–12 IU/liter), in the face of a low circulating testosterone concentration of 2.1 nmol/liter (normal adult range: 12.0–42.0 nmol/liter). Anatomy of the hypothalamo-pituitary region was normal on magnetic resonance imaging exam, and formal testing disclosed a normal sense of smell, all findings consistent with isolated GnRH deficiency. Together with the primary adrenal insufficiency occurring early in childhood, it allowed to make the diagnosis of DAX-1 deficiency (2, 9). The patient has been subsequently treated with monthly testosterone ester injections (250 mg, im).

Further assessment of the hypogonadism was performed by testing with pulsatile GnRH therapy at age 24 yr (5, then 10 mg/pulse every 2 h sc for a total of 9 consecutive days), when the patient had reached his target height of 177 cm and a body weight of 69 kg. This testing was done 7 wk after the last injection of testosterone. Pituitary gonadotropin secretion was stimulated neither by low dose pulsatile GnRH, nor by the acute administration of 100 mg GnRH iv bolus (Table 1), demonstrating a pituitary participation to the defect.

Of note, the patient also presented an initial episode of catatonic schizophrenia at age 19, when he started to work as a professional cook. This episode required a brief stay in a psychiatric hospital at that time, but the patient was discharged on no medication. He was subsequently unable to cope with a regular job, and more recently suffered from recurrent attacks of catatonia. The diagnosis of schizophrenic disorder was made, and the patient is now being treated with haloperidol decanoate. His social adaptation remains very poor: he does not have a regular employment, and still lives with his parents.

TABLE 1. Lack of stimulation of gonadotropin secretion by acute administration of GnRH (100 μg iv), either at baseline or after several days of administration of pulsatile GnRH

	Baseline		Day 4 GnRH 5 μg q 2 h		Day 7 GnRH 10 μg q 2 h	
	0 min	60 min	0 min	60 min	0 min	60 min
LH (IU/liter)	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
FSH (IU/liter)	<0.4	<0.4	0.4	0.4	0.5	0.6
FAS (IU/liter)	0.17	0.18	0.16	0.13	0.16	0.14
T (nmol/liter)	2.0		1.7		1.1	
E2 (nmol/liter)	<0.09		<0.09		<0.09	

Testing was performed 7 wk after withdrawal of testosterone ester supplementation.

PCR and direct DNA sequencing of DAX-1 gene

The following studies were approved by the ethical committee of our institution, and formal informed consent was obtained from the patient and all members of his family. Control DNA was obtained from two normal volunteers. Genomic DNA was extracted from blood leukocytes, using commercially available reagents (Nucleon BACC2, Amersham Pharmacia Biotech, Little Chalfont, UK). Overlapping genomic DNA fragments encompassing the two exons of the DAX-1 gene as well as 4 kb of the 3′untranslated region were obtained by PCR, using the following sets of forward and reverse primers (each primer is identified by a number corresponding to the nucleotide coordinates of the GenBank accession no. U31929, and a letter that indicates the strand orientation, either F for forward or R for reverse):

1523F: 5′TAAATAGGTCCCAGGAGGCAG
1869F: 5′CCGAGGCGACGCTGGGTCCGTG
4499F: 5′GCCTCTCTTTGCTGTTTATCC
6074F: 5′AGCTAGCAAAGGACTCTGTGG
7904F: 5′GAAAGCTTCCAGAAGCAGTTC
2133R: 5′TCTTTACCCCCTGGCCTCTGC
2316R: 5′GAGACCACGCGACGCCGGCCA
2799R: 5′CCGATGCTTTTGTGAGCTGGG
6419R: 5′CATGGTGAACTGCACTACT
8847R: 5′TCACAGATCCTAATTCCCTGG

PCRs were performed in a final volume of 50 μl, using: 200 ng of genomic DNA, 5 pmol of each primer, 50 pmol of each deoxy-NTP, 60 mM Tris-SO_4, 18 mM $(NH_4)_2SO_4$, 1.5 mM $MgSO_4$, and 1 μl of the Elongase enzyme mix (Life Technologies, Inc., Gaithersburg, MD). Reactions were run for 30–35 cycles in a Perkin-Elmer (Foster City, CA) 9700 apparatus. Length of extension times and annealing temperatures varied and were optimized according to, respectively, the size of the specific PCR-product and the set of primer pair used.

All sequencing reactions, including direct DNA sequencing of PCR products, were performed by Microsynth (Balgach, Switzerland) using an automated sequencer. For improved accuracy and precision, sequences of PCR fragment products were determined in both directions. Alignments and comparisons between the wild-type DAX-1 gene and mutated DAX-1 gene were done using the programs BestFit and PileUp from the GCG sequence analysis package (Genetics Computer Group, Madison, WI).

Southern blot analysis

Genomic DNA (10 μg) from the proband and normal controls was digested with *Eco*RI, then separated through a 1% agarose gel. The DNA was transferred onto a nylon membrane (Hybond-N^+, Amersham Pharmacia Biotech), fixed by UV cross-linking and subjected to hybridization to a ^{32}P-labeled probe homologous to the last 944 nucleotides of the DAX-1 gene. This probe was generated by PCR, employing the 7904F and 8847R primer pairs and ^{32}P-labeled by using a random priming kit (Roche, Rotkreuz, Switzerland), under conditions suggested by the manufacturer.

Construction of plasmids

The expression vector coding for the new putative form of DAX-1 protein of the proband, was constructed by cloning a PCR-generated genomic DNA fragment into the pcDNA3 mammalian expression plasmid. This fragment starts 30 nucleotides upstream of the starting ATG and extends into the intronic sequence that follows exon 1, terminating at the first intronic *Eco*RI site of the gene (position 3428). The amplification primers used were respectively: 5′AGGAGGATCCACTGGGCAGAACTGGGC (forward sequence, coordinates 1546–1562) and 5′AGGAGAATTCTAAAATACAGTGCTCCTGC (reverse sequence, coordinates 3428–3404). *Bam*HI and *Eco*RI sites used for the directional cloning are *underlined*.

All the other plasmids used in this study have already been described elsewhere (11, 19). Briefly, the *firefly* luciferase reporter construct UAS-TK109luc contains two yeast transcription factor GAL4 upstream activating sequence (UAS) sites upstream of the −109 TK promoter, and the

hStAR-luc construct contains the human promoter of the steroidogenic acute regulatory protein (StAR). GAL-SF-1 contains the first 147 amino acids of GAL4 [representing the DNA binding domain (DBD)] fused to the mouse SF-1 devoid of its own DBD (residues 133–463). The control vector GAL4-DBD contains only the DBD. The full-length mouse SF-1 cDNA was cloned into the pBKCMV expression plasmid (Stratagene, La Jolla, CA) as well as the wild-type full-length human DAX-1. Δ347–470 is a carboxy-terminal deletion mutant of human DAX-1, also cloned into the pBKCMV backbone.

Cell culture, transient transfections, and luciferase assays

The human choriocarcinoma cell line JEG-3 was used for all transfection experiments. These cells were grown in DMEM supplemented with 7.5% fetal bovine serum (Life Technologies, Inc.) in a 5% CO_2 atmosphere at 37 C. JEG-3 cells were seeded on 12-well plates at a density of about 2×10^5 cells/well and transiently transfected by the calcium phosphate technique employing the ProFection mammalian transfection system kit (Promega Corp., Madison, WI), according to the manufacturer's instructions. Each well was cotransfected with 1 μg of the specific *firefly* luciferase plasmid reporter, 5 ng of the *Renilla* luciferase reporter pRL-TK (Promega Corp.), and varying amounts of expression plasmids as specified in the figure legends. The use of the second plasmid reporter that encodes for the *Renilla* luciferase under the control of the thymidine kinase promoter conveniently allows for the normalization of all results according to transfection efficiency of each individual sample within the same luciferase assay. Total amount of DNA was kept constant by complementing each transfection with empty backbone plasmid when needed (either pBKCMV or pcDNA3). Luciferase assays were performed 48 h after transfection using the Dual Luciferase Reporter Assay System (Promega Corp.), according to the manufacturer's instructions. Luminescence was measured with a Turner Designs Luminometer Model TD-20/20 (Promega Corp.). Results are reported as a percentage of luciferase activity of either the GAL4-SF-1 or SF-1 expression vectors to allow comparison of data from different experiments (means ± SE of at least three separate experiments, each consisting of triplicate transfections).

Western blot analysis

Aliquots of the protein extracts prepared from transfected JEG-3 cells were assayed by Western blot analysis employing a monoclonal antibody specific for DAX-1. This antibody (2F4) was raised against a peptide corresponding to amino acids 135–166 of the human DAX-1 protein (20). Equal amounts of proteins were separated onto 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Antibody complexes were visualized by the ECL system (Amersham Pharmacia Biotech, Arlington Heights, IL).

Results

Mutation analysis

To search for mutations in *DAX1*, genomic DNA from the proband was subjected to PCR amplification followed by direct sequencing of the PCR products. A panel of primer pairs was designed to span the two exons of the DAX-1 gene (see Fig. 2 and below). Failure to obtain the expected PCR product in the proband with a primer pair designed to amplify exon 2 (6074F and 6419R) suggested the presence of a deletion in this region. This hypothesis was further confirmed by Southern blot analysis, as displayed in Fig. 2A: using as a probe a 944 bp-long DNA fragment corresponding to the terminal part of the gene (coordinates 7904 to 8847, see *Subjects and Methods*), we could detect a correctly sized 4022 bp-long band in the *Eco*RI restricted DNA prepared from normal controls. Instead, the *Eco*RI digested DNA prepared from the proband yielded a shorter band, slightly larger than 3 kb in size. These results were interpreted as consistent with the presence of a deletion eliminating the *Eco*RI site located at position 4825 of the DAX-1 gene.

We were then able, using the 4499F and 8847R set of primers, to amplify a 2108-bp fragment from the genomic DNA of the proband whereas in normal controls, the same set of primers amplified a 4349-bp fragment (Fig. 1B). This result confirmed the suspected presence of a deletion of about 2.2 kb in the DAX-1 gene of the proband. His mother was heterozygous for the deletion (Fig. 1C), whereas his unaffected brother and father both carried a normal allele (Fig. 1C). Direct sequencing of the amplified fragments revealed that the deletion extends from position 4561 through position 6801, completely eliminating exon 2 of the gene. In addition, an insertion of a short stretch of 27 bp at the site of deletion was present (Fig. 2).

Next, we tried to verify the possibility that the 2.2-kb deleted fragment could be present in other regions of the chromosomes due to a translocation event, as suggested by homology of the 27-bp insertions with a region on chromosome 5. However, fluorescent *in situ* hybridization and direct PCR amplification of genomic DNA with primers located inside of the deletion failed to yield positive results (data not shown).

Of note, during the course of sequencing, we found that the 10th nucleotide of the first exon of the proband did not correspond to the published GenBank sequence (U31929): a C was replaced by a G (data not shown). If correct, this C → G substitution in the first nucleotide of the fourth codon would result in a Q4E missense mutation. However, this could represent a mistake in the published sequence rather than a real missense mutation, as the same substitution was also found in all controls tested (data not shown).

Functional analysis of the new mutated form of DAX-1 protein produced by the deleted gene

As described above, mutation analysis showed the complete lack of exon 2 of DAX-1 in the proband. Thus, the transcription of this deleted form of DAX-1 would generate a truncated protein lacking the last 81 amino acids, which are encoded by exon 2. However, because the natural splice acceptor site is eliminated by the deletion as well, it can be predicted that the 119 bp-long intronic sequence that happens to be in frame with exon 1 could be translated. This would result in a protein that has a new, 40 amino acid-long carboxy-terminal end replacing the wild-type sequence encoded by exon 2. To analyze the functionality of this new putative form of mutated DAX-1, we cloned a stretch of genomic sequence containing exon 1 plus the first 681 nucleotides of intronic sequence terminating at an *Eco*RI site into the pcDNA3 mammalian expression vector, and tested it in transient transfection experiments.

Transfection assays were based on the well characterized transcriptional silencing activity of wild-type DAX-1 on the function of the transcriptional activator orphan nuclear receptor SF-1. As controls, we used expression vectors coding for wild-type human DAX-1 and the Δ347–470 DAX-1 carboxy-terminal deletion mutant, lacking the last 124 amino acids (11). In the first assay format, we used a native pro-

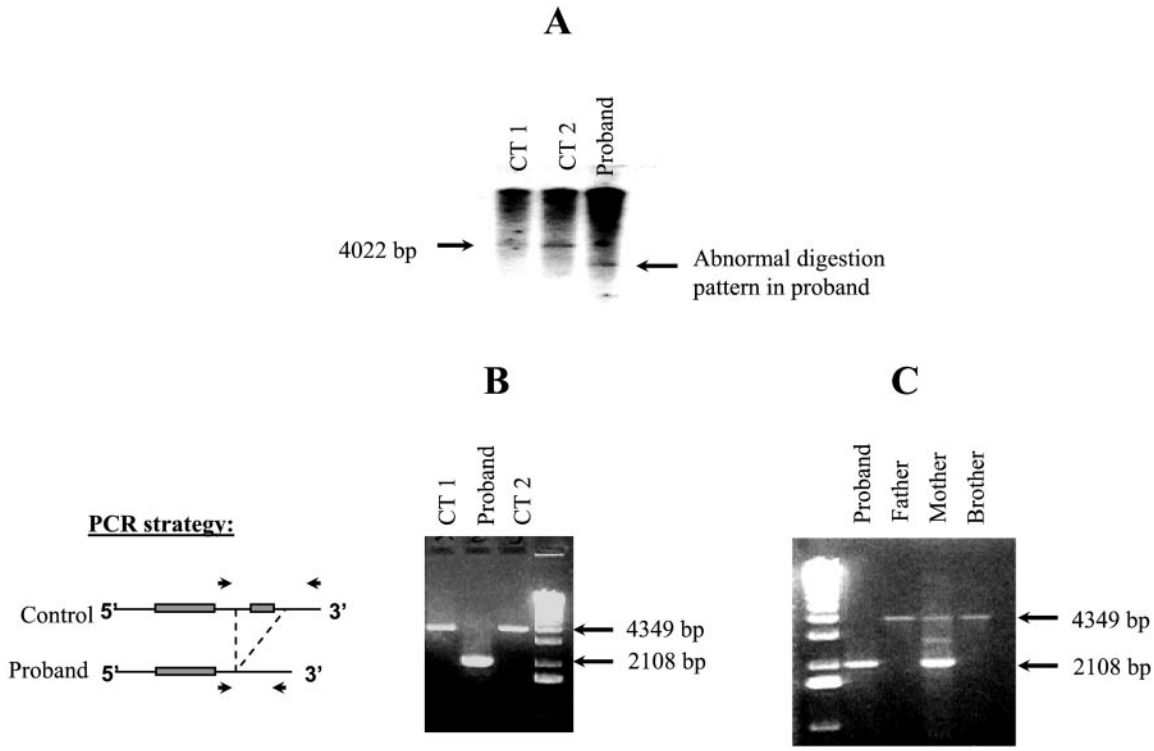

FIG. 1. A, Southern blot analysis of an *Eco*RI digestion of genomic DNA, demonstrating an abnormal digestion pattern in the proband. *Left arrow* indicates the position of the wild-type band as in control 1 (CT1) and 2 (CT2). *Right arrow* indicates the presence, in the DNA prepared from the proband, of a shorter fragment hybridizing with the same probe. B, PCR analysis of the genomic DNA from the proband and two unrelated controls (CT1 and CT2), demonstrating the existence of a 2.2-kb deletion in the proband. A schematic depiction of the PCR assay is shown on the left side. C, Genotyping of the rest of the family by the same PCR analysis as above. This gel shows that the mother is heterozygote, whereas the brother and father carry a wild-type DAX-1 allele.

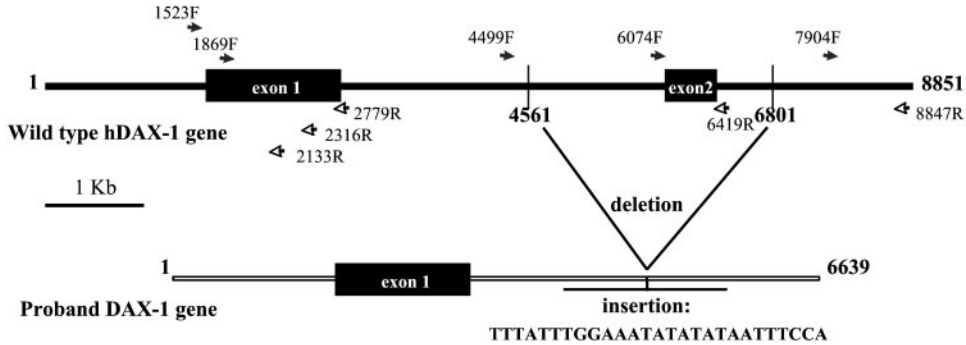

FIG. 2. Schematic representation of the genetic rearrangement of DAX-1 in the proband. Direct sequencing of genomic DNA allowed to identify a 2240-bp deletion combined with a 27-bp insertion at the site of deletion. Position of the various primer pairs used for PCR amplification of genomic DNA is shown on the wild-type DAX-1 gene.

moter. In this experiment, the expression of the luciferase reporter gene was driven by the human StAR promoter. This promoter contains three putative SF-1 sites, which are functionally responsive to SF-1. In addition, DAX-1 can inhibit the activity of this promoter through an alternative mechanism that appears to be independent of SF-1 (21). As shown in Fig. 2, this promoter is robustly stimulated (20-fold stimulation with respect to the reporter/empty vector) by an SF-1 expression construct (mouse SF-1 cDNA downstream of the CMV promoter). Cotransfection of increasing amounts of wild-type DAX-1 (5, 10, and 20 ng) greatly reduced the stimulatory activity of SF-1, reaching 95% of repression of SF-1-mediated transactivation at the highest DNA dose. As expected, the Δ347–470 DAX-1 deletion control was capable of only partially inhibiting SF-1 activity, with a repression of 37%, 50%, and 62%, respectively, at the increasing doses of DNA. In contrast, the construct expressing the mutant DAX-1 of the proband produced absolutely no repression at the 5 ng dose, 10 ng of DNA caused only 11% inhibition, and a 53% inhibition of SF-1-mediated transcription was reached at the highest dose of DNA. Therefore, repressor activity of the mutated DAX-1 protein described here was lower than the previously described Δ347–470 deletion mutant (11).

Western blot analysis of the protein extracts from JEG-3 transfected cells and using a DAX-1 specific monoclonal antibody demonstrated the production of mutant DAX-1 proteins of the expected size (shown as *inset* in Fig. 3).

We also assayed the function of proband DAX-1 on the UAS-TK109luc reporter (data not shown), which is an artificial promoter containing two GAL4 sites upstream of the

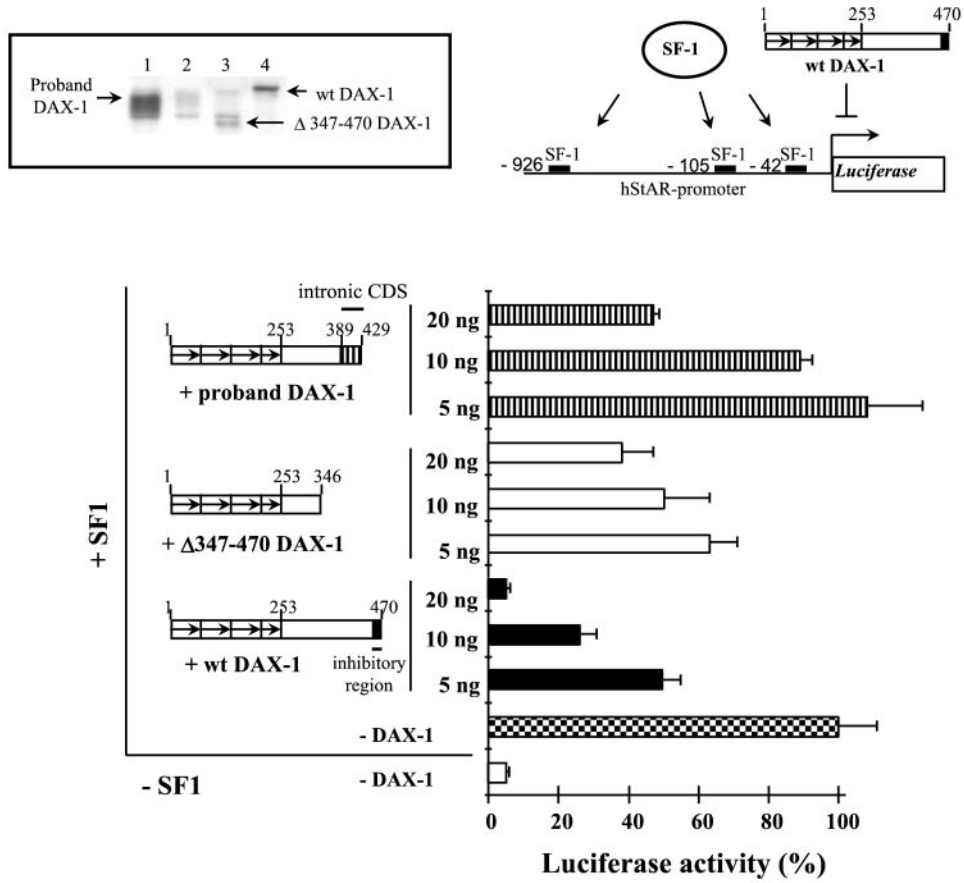

FIG. 3. Effect of the mutated protein on SF-1-mediated transcription. A schematic depiction of the transient cotransfection assay format is shown at the *top, right corner*: an SF-1 expression vector is able to transactivate the StAR-luc reporter, which contains three *SF-1* sites of the native promoter. As expected, wild-type (wt) DAX-1 dramatically decreased SF-1-mediated transcription (*bottom*) in a dose-dependent fashion. Proband DAX-1 (*top*), instead, showed a loss of transcriptional silencing activity. DAX-1 deletion mutant Δ347–470, used as control (*middle*, see Ref. 11), exhibits intermediate silencing activity. Results (means ± SE of at least three separate experiments, each consisting of triplicate transfections) are reported as a percentage of the luciferase activity due to SF-1 in the absence of any DAX-1. The activity of the StAR-luc reporter alone is shown in the *last bottom bar*. *Inset* in the *top left corner* shows the DAX-1 protein forms present in the cells transfected with the respective expression vectors, as detected by Western blot using a DAX-1 monoclonal antibody.

luciferase gene. The results obtained with this assay format basically mirrored the results obtained with the assay employing the native promoter. Cotransfection of SF-1 produced a 20-fold increase in activation over the empty vector. Coexpression of wild-type DAX-1 was able to repress this activation in a dose-dependent manner, with complete repression of SF-1-mediated stimulation at a 20-ng dose. Similar doses of the Δ347–470 construct and the proband mutant construct inhibited transactivation by only 62% and 53%, respectively.

Discussion

In the present study, we describe an unusual case of adrenal insufficiency caused by a complex mutation of DAX-1, associated later in life with hypogonadotropic hypogonadism of pituitary origin. Similar to previously published patients (14), adrenal insufficiency was diagnosed early after birth on the occasion of the work-up of a severe salt-losing state. The clinical history of this patient is remarkable for the subsequent recovery of a sufficient adrenal reserve to allow full withdrawal of all hormonal supplementation for over 6 months. Heterogeneity both in the age at diagnosis as well as in the severity of the adrenal syndrome is a well recognized feature of DAX-1 deficient patients (13, 14, 22, 23). In the patient presented here, persistence of residual adrenal function more than 2 yr after birth suggests that a milder phenotype was associated with that particular mutation, at least with respect to the onset of the disease. That adrenal insufficiency of DAX-1 patients can spontaneously evolve toward a transiently compensated state has already been described (17, 18). Our similar finding suggests that such clinical course might be more frequent than initially envisioned. This possibility should therefore be kept in mind by physicians taking care of these patients, as it could bear significant implications for their clinical management.

Variability in the hypogonadic syndrome that classically accompanies adrenal insufficiency has also been reported (24). There is evidence in the literature for a purely hypothalamic origin of the defect (25), for a pituitary origin (26, 27) or for both (28). Therefore, the effects of pulsatile GnRH administered at physiological doses were assessed in this patient. In addition, the response to stimulation with pharmacological concentrations of the decapeptide was also evaluated. Neither physiological, nor pharmacological stimulation with GnRH was found to stimulate gonadotropin secretion, demonstrating the participation of pituitary gonadotrope cells to the hypogonadism. It should be noted, however, that these results do not exclude a hypothalamic component.

This patient was also recently diagnosed with schizophrenia, requiring multiple hospitalizations in a psychiatric setting over the last year. Such phenotypic trait has never been reported previously in DAX-1 patients. Interestingly, his mother is affected by a milder psychotic disorder. However, his brother who carries a wild-type DAX-1 allele also suffers from mild psychosis, suggesting that the mental disease may

not necessarily be related to the DAX-1 genotype in this family.

Recent reports underline the importance of DAX-1 for normal human spermatogenesis (13, 24). This finding is consistent with the phenotype of the mouse model of DAX-1 deficiency (12). In this context, a testicular biopsy is desirable in all DAX-1 deficient patients, and even probably indicated in the work-up of their infertility. The psychiatric disorder affecting the patient under discussion precluded any attempt to obtain a testicular biopsy.

The human mutations affecting DAX-1 have been summarized recently (14, 15). They have in common to cause a loss of the carboxy-terminal end of the DAX-1 protein. The vast majority of them are single base pair insertions or deletions, resulting in frameshift and premature stop codons (14, 15). This is consistent with *in vitro* data attributing the transcription repressor activity of DAX-1 to its carboxy-terminal part (11). In addition, some of the reported mutations are missense (15). These have been particularly useful in structure-function analyses, thus helping to identify important residues in the protein. However, when considering all available information regarding the clinical presentation of the adrenal insufficiency or of the hypogonadism, there is no clear correlation between the type of mutation and the phenotype except in the rare late-onset cases due to missense mutants.

We report here for the first time a complex genetic rearrangement within DAX-1, combining a 2.2-kb deletion encompassing part of the intron as well as the entire second exon with a small insertion of a 27-bp fragment. Because of the presence of a small insertion at the site of deletion, we initially hypothesized that this rearrangement could result from an imbalanced translocation. And indeed, the Xp2.21 region of the X chromosome where DAX-1 is located seems to be particularly prone to such events. Consistent with this hypothesis, BLAST analysis of the 27-bp fragment inserted at the site of the deletion indicated the existence of a highly homologous region on human chromosome 5. Thus, we attempted to demonstrate that the missing 2.2-kb fragment was still present in the genome of the patient. This was done with two different experimental approaches: fluorescent *in situ* hybridization and direct PCR amplification of genomic DNA. However, both methods yielded negative results, suggesting that the 2.2-kb fragment has indeed been deleted from the genome of this patient.

Wild-type DAX-1 encodes for a 470-amino acid protein. The putative protein synthesized by the mutated DAX-1 gene described here will lack all the residues encoded by exon 2. These correspond to the last 81 amino acids located at the C-terminal extremity of the wild-type DAX-1 protein. In addition, because of the deletion of the entire exon 2, the mutated gene will also lack the splice acceptor site of that exon. It could therefore be hypothesized that transcription will continue after the end of exon 1 and into the intron, until the next stop codon is reached. Sequence analysis of the intronic sequence immediately adjacent to exon 1 demonstrates the existence of a stop codon located 120 bp downstream of the 3′ end of the exon. Therefore, the putative protein produced by this mutated DAX-1 gene should be 429 amino acids long: the initial 389 residues at the N-terminal extremity, encoded by the first exon, would correspond to the wild-type sequence, whereas the last 40 amino acids should be completely unrelated to the wild-type sequence, being transcribed from the intronic sequence immediately adjacent to the 3′ end of exon 1. Western blot experiments seem to support this hypothesis, by showing the production of mutant DAX-1 proteins of the expected size. *In vitro* functional analyses confirm the greatly reduced repressor activity exerted by such mutant protein. Therefore, our findings are entirely consistent with previous data, showing that the repressor domain of DAX-1 is located at its carboxy-terminal end (11, 16). With respect to previously published inactivating mutations of DAX-1, the mutation reported here has the most dramatic effect on the function of the protein, resulting in a complete loss of any measurable biological activity.

In conclusion, we describe here a male patient harboring a novel and complex rearrangement of *DAX1*, who presented an unusual evolution of adrenal insufficiency during infancy. Therefore, this case expands the spectrum of clinical presentation of adrenal insufficiency in AHC patients.

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