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Analysis of DAX1 (*NR0B1*) and Steroidogenic Factor-1 (*NR5A1*) in Children and Adults with Primary Adrenal Failure: Ten Years' Experience

Lin Lin, Wen-Xia Gu, Gokhan Ozisik, Wing S. To, Catherine J. Owen, J. Larry Jameson, and John C. Achermann

UCL Institute of Child Health and Department of Medicine (L.L., W.S.T., J.C.A.), University College London, London WC1N 1EH, United Kingdom; Department of Medicine (W.-X.G., J.L.J.), Northwestern University Feinberg School of Medicine, Northwestern Memorial Hospital, Chicago, Illinois 60611; Division of Endocrinology and Metabolism (G.O.), Gulhane Askeri Tip Akademisi Medical School, Haydarpasa Hospital, Istanbul 34670, Turkey; and Institute of Human Genetics (C.J.O.), School of Clinical Medical Sciences, University of Newcastle, Newcastle upon Tyne NE1 3BZ, United Kingdom

Context: Primary adrenal failure is a life-threatening condition that can be caused by a range of etiologies, including autoimmune, metabolic, and developmental disorders. The nuclear receptors DAX1 (*NR0B1*) and steroidogenic factor-1 (SF1/Ad4BP, *NR5A1*) play an important role in adrenal development and function, and mutations in these transcription factors have been found in patients with adrenal hypoplasia.

Objective: Our objective was to investigate the prevalence of DAX1 and SF1 mutations in children and adults with primary adrenal failure of unknown etiology (*i.e.* not caused by congenital adrenal hyperplasia, adrenoleukodystrophy, or autoimmune disease).

Patients: One hundred seventeen patients were included. Eighty-eight individuals presented in infancy or childhood with adrenal hypoplasia or primary adrenal failure of unknown etiology (n = 64 46,XY phenotypic males; n = 17 46,XY gonadal dysgenesis/impaired androgenization; n = 7 46,XX females). Twenty-nine individuals presented in adulthood with Addison's disease of unknown etiology.

Methods: Mutational analysis of DAX1 (*NR0B1*) (including exon 2α/1A) and SF1 (*NR5A1*) was done by direct sequencing.

Results: DAX1 mutations were found in 58% (37 of 64) of 46,XY phenotypic boys referred with adrenal hypoplasia and in all boys (eight of eight) with hypogonadotropic hypogonadism and a family history suggestive of adrenal failure in males. SF1 mutations causing adrenal failure were found in only two patients with 46,XY gonadal dysgenesis. No DAX1 or SF1 mutations were identified in the adult-onset group.

Conclusions: DAX1 mutations are a relatively frequent cause of adrenal failure in this group of boys. SF1 mutations causing adrenal failure in humans are rare and are more likely to be associated with significant underandrogenization and gonadal dysfunction in 46,XY individuals. **(*J Clin Endocrinol Metab* 91: 3048–3054, 2006)**

PRIMARY ADRENAL FAILURE is a potentially life-threatening disorder that can present with a salt-losing crisis or profound hypoglycemia in infancy or childhood and requires urgent resuscitation and appropriate steroid replacement. Determining the exact cause of this condition can be challenging once the child has started treatment, but defining a precise etiology has important implications for long-term management, for identifying associated features, and for appropriate counseling regarding inheritance and the risks of other family members being affected (1, 2).

Two related transcription factors that have emerged as key regulators of adrenal development are the nuclear receptors DAX1 [dosage-sensitive sex reversal, adrenal hypoplasia congenita (AHC), critical region on the X chromosome, gene-1, NR0B1/*AHC*] and steroidogenic factor-1 (SF1, *NR5A1*, also known as Ad4BP) (3).

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Abbreviations: AHC, Adrenal hypoplasia congenita; HH, hypogonadotropic hypogonadism; SF1, steroidogenic factor-1.

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Deletions or mutations of DAX1 (*NR0B1*, Xp21) [Mendelian Inheritance in Man (MIM) no. 300473] cause the X-linked form of primary adrenal hypoplasia (AHC) (MIM no. 300200) (4, 5). Boys with this condition usually present with salt-losing primary adrenal failure in early infancy or in childhood (6). Absent or arrested puberty occurs in adolescence because of hypogonadotropic hypogonadism (HH), and intrinsic abnormalities in spermatogenesis contribute to impaired fertility (4–10). Although more than 100 individuals or kindred with X-linked AHC have been reported, the prevalence of DAX1 mutations in individuals with different adrenal phenotypes or associated features is not clear (11–18).

Recently, a late-onset form of X-linked AHC has been described in several individuals who first presented with adrenal failure and partial hypogonadism in adulthood (19–21). These reports raise the possibility that milder or even adrenal-only forms of AHC resulting from DAX1 mutations could exist. Furthermore, as gonadal (testicular) dysgenesis has now been reported in *Dax1* (*Ahch*) deleted mice when bred onto certain genetic backgrounds (22), we hypothesize that DAX1 mutations might be identified in a subset of 46,XY individuals with primary adrenal failure and impaired testicular development.

The related nuclear receptor SF1 (*NR5A1*) (9q33) regulates transcription of many genes involved in adrenal and gonadal development, steroidogenesis, and reproduction (23, 24). Targeted deletion of *Sf1* in the mouse causes adrenal and gonadal agenesis, retained Müllerian structures, and impaired androgenization in males, abnormal gonadotropin release, and late-onset obesity (25, 26).

Human mutations in SF1 have been described in three patients with primary adrenal failure to date (MIM no. 184757). Two individuals with a 46,XY genotype, female phenotype, and Müllerian structures harbor missense mutations that affect DNA binding (27–29), whereas a 46,XX girl with a SF1 mutation has primary adrenal failure and apparently normal ovarian development (30). In addition, it is now emerging that heterozygous nonsense or frameshift mutations associated with haploinsufficiency of SF1 can cause 46,XY gonadal dysgenesis in patients with normal adrenal function (31–33). Thus, it is possible that a range of different endocrine phenotypes could be associated with mutations in different domains of SF1.

Here, we report our experience over the past 10 yr of analyzing DAX1 and SF1 in 117 children and adults with primary adrenal failure that is not caused by disorders that are commonly recognized (*i.e.* congenital adrenal hyperplasia, adrenoleukodystrophy, or autoimmune disease).

Subjects and Methods

Subjects

A total of 117 individuals with primary adrenal failure were considered for this study (Table 1). This cohort represents referrals to our centers over a 10-yr period. For the purposes of this analysis, we have included 15 mutations identified and reported in the literature by our groups (6–8, 27, 28, 34–36). We have omitted cases where a molecular diagnosis had been made elsewhere and we undertook functional work (*e.g.* 19–21). Thus, our aim was to obtain an estimate of the relative prevalence of DAX1 and SF1 mutations in individuals with adrenal insufficiency that was not readily attributed to disorders that are commonly diagnosed in practice, such as autoimmune conditions or classic steroidogenic defects.

Subjects were divided into infancy/childhood-onset and adult-onset groups (infancy, <1 yr; childhood, 1–15 yr; adult, >15 yr). Steroid biosynthetic defects (*e.g.* 21-hydroxylase deficiency), metabolic disorders (*e.g.* adrenoleukodystrophy), and autoimmune disorders (*e.g.* autoimmune Addison's disease, polyglandular autoimmune endocrinopathy) had been excluded, where relevant. No patients had features of a syndrome causing adrenal insufficiency where the genetic etiology is known (*e.g.* triple A syndrome, *AAAS*).

The infancy/childhood-onset group consisted of 88 individuals who presented with salt-losing adrenal failure early in life. Most of this group were 46,XY phenotypic males (n = 64), with or without a family history of primary adrenal failure and/or unexplained death in infancy/childhood or a personal history of abnormal puberty/HH (Tables 1 and 2). Other clinical features (*e.g.* skeletal abnormalities or intrauterine growth retardation) were present in 12 patients. Of these, six individuals had potential variants on the IMAGe syndrome (intrauterine growth retardation, metaphyseal dysplasia, adrenal hypoplasia, genitourinary abnormalities), but no children had features of the Antley-Bixler syndrome (37). Transient or borderline adrenal failure was present in five cases (Table 2). This phenomenon was defined as a cortisol response to synacthen less than 15 μg/dl (420 nmol/liter) when investigated for hypoglycemia in the neonatal period and requiring steroid replacement for several months or years, which was eventually weaned and stopped (Table 2). These boys were included because transient forms of AHC have been described in patients who present in early infancy, improve in childhood, but develop significant adrenal failure in adolescence (38).

In addition to the 46,XY phenotypic males, we considered a cohort of underandrogenized 46,XY patients (ambiguous genitalia or female phenotype) with primary adrenal failure (n = 17), with (n = 4) or without (n = 13) Müllerian structures, as well as a small group of 46,XX girls with isolated primary adrenal failure of unknown etiology (n = 7) (Table 1). Three of the underandrogenized 46,XY children had low birth weight or mild dysmorphic features. Two of these 46,XX girls had evidence of low birth weight and short stature/skeletal abnormalities.

The adult-onset group (n = 29) consisted of 46,XY males (n = 14) and 46,XX females (n = 15) who presented with primary adrenal failure of unknown etiology after puberty (median age, 33 yr; range, 15–70 yr) (Table 1). These patients were thought initially to have autoimmune Addison's disease but were autoantibody negative and had no other autoimmune endocrinopathies or infective causes and no remarkable family history. One male had partial HH, and all patients were receiving mineralocorticoid replacement.

Mutational analysis of DAX1 (NR0B1) and SF1 (NR5A1)

After institutional board approval and with informed consent, genomic DNA was extracted from peripheral blood lymphocytes and the entire coding regions and splice sites of SF1 (*NR5A1*, exons 2–7, 10 primer pairs) and DAX1 (*NR0B1,* exons 1–2, six primer pairs) were amplified by PCR using variations on conditions reported previously. Exon 2α/1A of DAX1α/*NR0B1A* was amplified by the following primers: forward, 5′-CTCTGTGATGATTGGCATGGTG-3′; reverse, 5′-GCAGTATAGTGTGATACCGAAG-3′ (39, 40). PCR products were purified by gel extraction (QIAGEN, Valencia, CA) or by using exonuclease I (New England Biolabs, Ipswich, MA)/shrimp alkaline phosphatase (USB, Columbus, OH) and then subjected to direct sequencing using dye terminator sequencing kits (dRhodamine/BigDyev1.1 from PE Applied Biosystems Inc., Foster City, CA) and automated gel or capillary-based sequencers (373A/377 from PE Applied Biosystems; MegaBACE1000 from Amersham Biosciences Inc., Piscataway, NJ). Sequence Navigator (PE Applied Biosystems), Sequence Analyser version 3.0 (Amersham Biosciences), and Sequencher version 4.1 (Genecodes Corp., Ann Arbor, MI) were used to analyze the data.

Results

Analysis of DAX1

Mutation analysis revealed a total of 37 DAX1 mutations in the cohort studied. All of these changes were identified in

TABLE 1. Mutational analysis of DAX1 (*NR0B1*) and SF1 (*NR5A1*) in children referred with potential adrenal hypoplasia and in adults with Addison's disease of unknown etiology

	Infancy/childhood onset (n = 88)				Adult onset (n = 29)	
	46,XY male	46,XY underandrogenized		46,XX female	46,XY male	46,XX female
		No Müllerian structures	Müllerian structures			
No. of patients	64	13	4	7	14	15
Median age (range)	10 d (birth to 13 yr)	7 d (birth to 2.5 yr)	10 d (birth to 3 wk)	2 yr (6 d to 6 yr)	29 yr (15–67 yr)	38 yr (28–70 yr)
Mutations						
DAX1	37	0	0	0	0	0
SF1	0	0	2	0	0	0

Age refers to median age (range) at presentation.

TABLE 2. Characteristics of boys (46,XY) with and without DAX1 mutations/deletions

	Total cohort (46,XY boys)	DAX1 mutation detected [n (%)]	No mutation detected [n (%)]
Total no.	64	37 (58)	27 (42)
Age at presentation			
Early infancy	51	30 (59)	21 (41)
Childhood	13	7 (54)	6 (46)
Puberty/FH			
Prepubertal/no FH	44	20 (45)	24 (55)
Prepubertal/FH	9	6 (67)	3 (33)
HH/no FH	3	3 (100)	0 (0)
HH/FH	8	8 (100)	0 (0)
Additional features	12	1 (8)	11 (92)
Transient/mild adrenal dysfunction	5	0 (0)	5 (100)

The data are focused on age at presentation, presence or absence of HH and/or family history (FH) of adrenal failure or unexplained death, presence of additional clinical features (*e.g.* skeletal abnormalities and intrauterine growth retardation), and severity of adrenal dysfunction.

46,XY phenotypic males who had presented with primary adrenal failure in infancy or childhood (37 of 64, 58%) (Table 1). No DAX1 mutations were found in the underandrogenized 46,XY subjects, in 46,XX girls with adrenal failure, or in any of the adult-onset group. No mutations were identified in exon 2α/1A of *DAX1/NR0B1*.

Most boys with DAX1 mutations presented in early infancy compared with childhood (30 of 37, 81%). A typical bimodal distribution pattern of ages was observed (early infancy n = 30, 5–60 d; childhood n = 7, 2–13 yr) (6). Although age at presentation was similar in the group where no DAX1 mutations were found (21 of 27, 78%, presenting in infancy) (Table 2), boys who were found to harbor a DAX1 mutation were more likely to have a positive family history of adrenal failure or unexplained death in males or a personal history of abnormal puberty/HH (Table 2). Indeed, all (eight of eight, 100%) individuals with a positive family history and abnormal puberty had a DAX1 mutation.

Despite the importance of family history and HH as potential indicators of X-linked AHC, the prevalence of DAX1 mutations was still 45% (20 of 44) in boys with no family history who were preadolescent (<14 yr) at the time of referral. Furthermore, milder or transient forms of adrenal insufficiency or associated features such as skeletal abnormalities were more likely to be found in the group in whom no DAX1 mutations were identified (Table 2). If these individuals with transient adrenal insufficiency or additional features were excluded from analysis, the percentage of preadolescent boys with no family history who were found to harbor DAX1 mutations rose from 45% (20 of 44) to 68% (19 of 28).

The proportions of different DAX1 mutations identified compared with those reported in the literature are shown in Fig. 1. Deletion of the locus containing DAX1/*NR0B1* (Xp21) was found in 10 patients (27%). Eight of these deletions involved only the *NR0B1* gene, whereas two patients had a contiguous gene deletion syndrome including glycerol kinase deficiency (*GKD*) and Duchenne muscular dystrophy (*DMD*). In both of these cases, the molecular diagnosis was made in the first months of life before the onset of neuromuscular or developmental symptoms/signs. The remainder (n = 27) of DAX1 mutations were nonsense (n = 7),

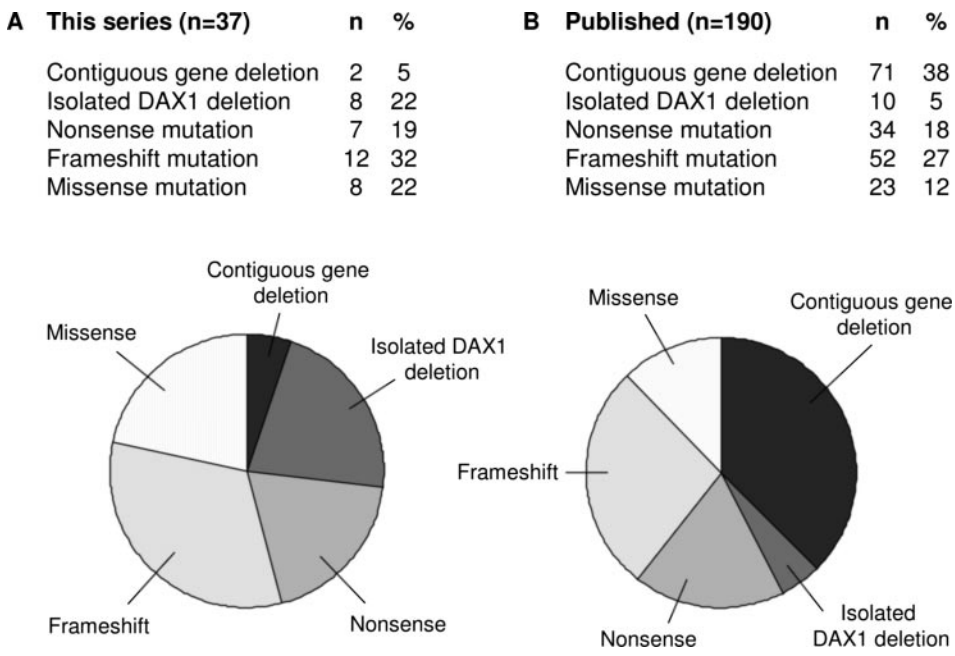

FIG. 1. Relative prevalence of different types of DAX1 mutations in this series (A) compared with the published literature (excluding abstracts) (B). Data shown represent the total number of different individuals or kindred with mutations. The historical literature has a bias toward cases of contiguous gene deletion syndrome where X-linked AHC has been reported together with glycerol kinase deficiency, Duchenne muscular dystrophy, and/or X-linked mental retardation. Our series focused on children with primary adrenal failure. In the two cases of contiguous gene deletion found, the diagnosis of a deletion of the DAX1 (*AHC*) locus was made before the clinical or biochemical diagnosis of glycerol kinase deficiency/Duchenne muscular dystrophy.

frameshift (n = 12), or missense changes (n = 8). These nonsense and frameshift changes are located throughout the *NR0B1* gene with a similar distribution to previous reports (Fig. 2A). Missense mutations, once thought to be relatively rare, were found in 22% of cases and involved highly conserved amino acids in the putative ligand-binding domain of DAX1 (Fig. 2B).

Analysis of SF1

Mutational analysis of SF1 in this cohort of individuals with primary adrenal failure revealed mutations in two patients (Table 1) (Fig. 3). These cases have been reported previously (27, 28). Both these individuals were found to have a 46,XY genotype, female phenotype, and Müllerian structures in addition to adrenal failure and harbored missense mutations that affected DNA binding (*de novo* heterozygous G35E; recessively inherited homozygous R92Q). No SF1 mutations were identified in 46,XY males with adrenal hypoplasia, in underandrogenized 46,XY females without Müllerian structures, in 46,XX girls, or in the adult-onset adrenal failure group.

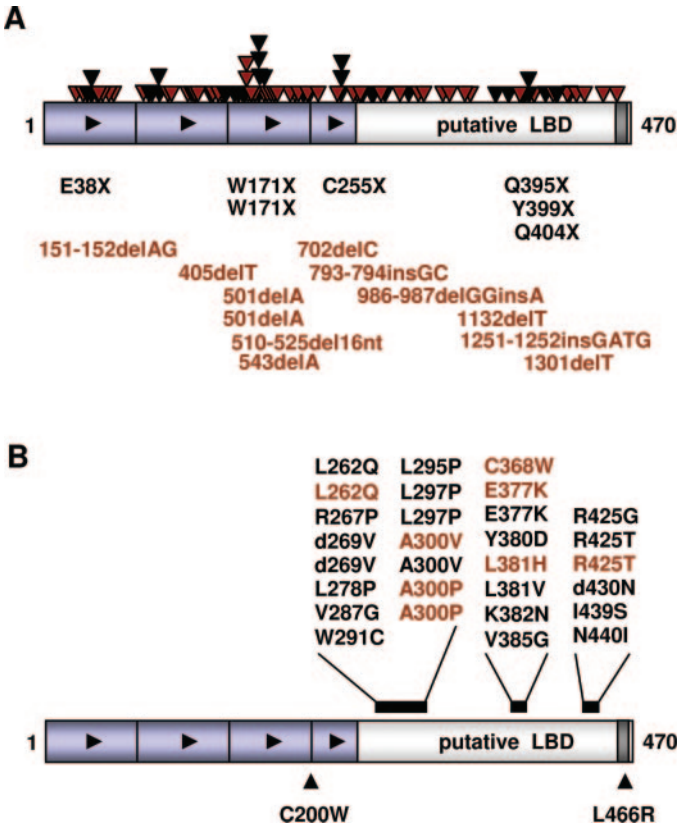

FIG. 2. A, Overview of nonsense (*black*) and frameshift (*red*) mutations in DAX1. Specific mutations identified by our centers are shown below the DAX1 model. Previously reported changes in the literature are shown above the DAX1 model by *arrowheads*. B, Naturally occurring missense mutations in DAX1 cluster within the carboxy terminus of DAX1 in a region that is homologous to the ligand-binding domain (LBD) of nuclear receptors. Mutations identified by our centers are shown in *red*, whereas mutations reported in the literature are shown in *black*. The three major cluster regions are indicated by *black bars*. Single amino acid deletions (d269V, d430N) are included.

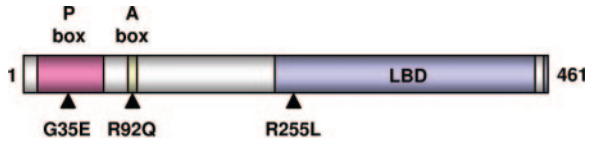

FIG. 3. Mutations in SF1 (Ad4BP) associated with primary adrenal failure and 46,XY gonadal dysgenesis affect the P-box and A-box regions of the DNA-binding domain (G35E, R92Q). A heterozygous mutation has also been reported in a 46,XX girl with primary adrenal failure and apparently normal ovarian differentiation (R255L) (30). LBD, Ligand-binding domain.

Discussion

The past 15 yr have seen significant progress in our understanding of the molecular basis of childhood adrenal disorders. More than 20 single-gene disorders have now been reported that can affect adrenal function in infancy or childhood, and a genetic diagnosis should be attainable in well over 50% of individuals with these conditions (2). Obtaining a precise biochemical and genetic diagnosis can have important consequences for treatment, for predicting prognosis, for investigating possible associated features, and for counseling the individual and family so that the risk of other family members being affected can be assessed accurately.

The identification and characterization of DAX1 as the cause of X-linked primary AHC in 1994 has had significant implications for diagnosis of individuals and families with this condition. An association with disordered puberty means that the majority of boys with X-linked AHC will need pubertal induction and long-term sex steroid replacement. It also seems likely that an intrinsic defect in spermatogenesis, which may be present in humans as well as mice, results in a worse fertility prognosis for individuals with X-linked AHC compared with young men with isolated idiopathic HH (8, 19, 20).

The true population prevalence of DAX1 mutations is not currently known. The prevalence of congenital adrenal hypoplasia is often quoted as being 1:12,500, following the 13-yr study of infant autopsies at the Royal Women's Hospital, Melbourne, Australia (1959–1971) by Laverty *et al.* (41). However, there was no sex bias in cases (six of 11 male), and only one male infant had cytomegalic histological changes consistent with the X-linked form of AHC. Furthermore, in a 20-yr review of primary adrenal insufficiency in children (0–18 yr) presenting to Sainte-Justine Hospital, Montreal (1981–2001), and reported by Perry *et al.* (42), X-linked AHC resulting from a DAX1 mutation was found in only one boy (one of 103). Congenital adrenal hyperplasia was diagnosed in 74 of 103 children (71.8% of the population) and has an estimated occurrence of 1:16,630 (42). Taken together, these studies suggest that X-linked AHC might occur in anywhere between 1:140,000 and 1:1,200,000 children (or between 1:70,000 and 1:600,000 males). However, extreme caution is needed in interpreting these data, as the number of boys with X-linked AHC in each cohort was extremely small (n = 1).

Here, we have focused on children and adults with primary adrenal insufficiency of unknown etiology, where common causes of adrenal failure such as steroidogenic defects (*e.g.* 21-hydroxylase deficiency) and metabolic disorders (*e.g.* X-linked adrenoleukodystrophy) had been excluded. We

show that DAX1 mutations are a relatively frequent cause of adrenal failure in phenotypic boys (46,XY) referred to us with potential primary adrenal hypoplasia. DAX1 mutations were found in over half of individuals studied (37 of 64, 58%), and in all eight (100%) cases where there was a family history of adrenal failure or unexpected death in males (consistent with an X-linked inheritance pattern) together with a history of arrested or absent puberty. Thus, detailed questioning about family history that could reveal any insight into possible adrenal disease is important. Furthermore, when no such family history was obtained, and when the individual was prepubertal at the time of assessment, it was still possible to detect DAX1 mutations in a substantial proportion of cases (20 of 44, 45%). If boys with additional features (*e.g.* low birth weight or skeletal abnormalities) and those with transient forms of adrenal failure were omitted from analysis, the proportion of boys with DAX1 changes found rose to 68% (19 of 28). Thus, mutational analysis of DAX1 may be worthwhile in any male infant presenting with salt-losing adrenal failure, where steroidogenic disorders (*e.g.* 21-hydroxylase deficiency, P450 oxidoreductase deficiency), metabolic conditions (*e.g.* Wolman syndrome and Zellweger syndrome), multisystem syndromes (*e.g.* IMAGe), and adrenal hemorrhage have been excluded, and in the older child where, in addition, autoimmune endocrine disorders (*e.g.* autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome, autoimmune polyglandular syndrome 2, and isolated autoimmune Addison's disease), syndromic ACTH resistance syndromes (*e.g.* triple A), infection, or iatrogenic causes of adrenal failure are not present.

The 37 DAX1 mutations and deletions detected had a similar distribution to those reported in the literature to date, although we did identify relatively few contiguous gene deletion syndromes compared with isolated deletions of *NR0B1* and a significant proportion of missense mutations (Fig. 1) (3, 43). The contiguous gene deletion syndromes were diagnosed before the onset of signs and symptoms of muscular dystrophy in both the cases shown. Although none of our cohort has evidence of deletion of the *IL1RAPL1* gene telomeric to *NR0B1*, which is associated with developmental delay, it is important to be aware of this potential association when a child with a Xp21 deletion seems to be failing to reach developmental milestones (44). The nonsense and frameshift mutations in our cohort were located throughout the *NR0B1* gene, with relatively few hotspots that could help to focus sequencing strategies. Missense mutations in DAX1 do tend to cluster in certain regions of the ligand-like binding domain, in highly conserved amino acids (35).

No SF1 mutations were found in the cohort of boys (n = 27) who did not have abnormalities in DAX1. These findings suggest that mutations in SF1 are unlikely to be a frequent cause of an adrenal-only phenotype, with normal male sex development. Other candidate genes for this group include potential regulators of adrenal development that are emerging from studies of gene expression or transgenic mice (*e.g. ACD*, *CITED2*, and *PBX1*). Several of our patients had phenotypic features consistent with a variant of the IMAGe syndrome, but the molecular basis of this condition is at present unknown (37, 45, 46). None of these children were found to harbor changes in DAX1 or SF1.

Analysis of our cohort of 46,XY individuals with adrenal failure and gonadal dysgenesis/impaired androgenization failed to reveal any DAX1 mutations, although there is evidence from studies of transgenic mice that Dax1 may function to support testis development at early stages of embryogenesis (9, 22, 47). SF1 mutations were found in only two patients, both of whom had 46,XY complete gonadal dysgenesis and persistent Müllerian structures (27, 28). These findings are consistent with the hypothesis that gene dosage effects of SF1 are important and that gonadal (testicular) development is more sensitive to loss of SF1 function than adrenal development in humans (48). Thus, if severe adrenal failure is present because of an SF1 mutation, it is likely that there will be significant underandrogenization, whereas less severe disruption of SF1 can result in partial gonadal dysgenesis/impaired androgenization, and normal adrenal function (31–33). It is possible that mutations in the SF1 promoter or noncoding sequences, or in related target genes or cofactors, might be identified in those individuals where no SF1 mutations were found. Furthermore, abnormalities in the early stages of steroidogenesis (*e.g.* steroidogenic acute regulatory protein, CYP11A) might present as complete adrenal failure and impaired androgenization, but without Müllerian structures. The adrenal glands in children with these conditions may not always be enlarged.

Our reports of a late-onset form of X-linked AHC presenting with primary adrenal failure in young adulthood in three men (19–21) as well as potential female phenotypic expression of DAX1-related phenotypes (8) led to studies of DAX1 and SF1 in a cohort of 29 men and women who had Addison's disease of unknown etiology. Thus, we hypothesized that milder forms of AHC might account for a subset of patients with these phenotypes. However, no mutations were found. Although the number of patients was small, these findings suggest that mild forms of adrenal hypoplasia are unlikely to be found in patients with adult-onset Addison's disease of unknown etiology in the absence of at least partial HH in males (DAX1) or impaired androgenization (SF1).

Taken together, this study shows that mutations in DAX1 are a relatively frequent cause of primary adrenal hypoplasia, even in the absence of a positive family history of adrenal failure or unexpected death in males or a personal history of abnormal puberty. In contrast, although SF1 mutations are emerging as a cause of 46,XY gonadal dysgenesis in patients with normal adrenal function, SF1 mutations causing adrenal failure are rare and are likely to be associated with significant underandrogenization and gonadal dysfunction in 46,XY individuals. Genetic analysis of DAX1 is now offered by a number of clinical laboratories worldwide. The prevalence of DAX1 changes identified in our series might warrant having a low threshold to undertake this analysis. Although pursuing a commercial approach can be relatively expensive, it may well be worthwhile if such an approach was able to prevent recurrent hypoglycemia, a severe salt-losing crisis, or even death in a presymptomatic brother or male relative or after a future pregnancy (34). However, it is also important that appropriate counseling is available for the individual and family throughout their interactions with health care services over the years, that appropriate and timely trans-

lation of care from pediatric to adult services is established, and that the clinical and research communities work together to determine the molecular basis of disorders of adrenal development when no changes in DAX1, SF1, or other candidate genes are found.

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Address all correspondence and requests for reprints to: Dr. John C. Achermann, Biochemistry, Endocrinology & Metabolism, University College London Institute of Child Health, 30 Guilford Street, London WC1N 1EH, United Kingdom. E-mail: j.achermann@ich.ucl.ac.uk.

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