Adrenal hypoplasia congenita with hypogonadotropic hypogonadism: evidence that DAX-1 mutations lead to combined hypothalmic and pituitary defects in gonadotropin production.

R L Habiby, … , W F Crowley Jr, J L Jameson

J Clin Invest. 1996;98(4):1055-1062. https://doi.org/10.1172/JCI118866.

Research Article

Adrenal hypoplasia congenita (AHC) is an X-linked disorder that typically presents with adrenal insufficiency during infancy. Hypogonadotropic hypogonadism (HHG) has been identified as a component of this disorder in affected individuals who survive into childhood. Recently, AHC was shown to be caused by mutations in DAX-1, a protein that is structurally similar in its carboxyterminal region to orphan nuclear receptors. We studied two kindreds with clinical features of AHC and HHG. DAX-1 mutations were identified in both families. In the JW kindred, a single base deletion at nucleotide 1219 was accompanied by an additional base substitution that resulted in a frameshift mutation at codon 329 followed by premature termination. In the MH kindred, a GGAT duplication at codon 418 caused a frameshift that also resulted in truncation of DAX-1. Baseline luteinizing hormone (LIT), follicle-stimulating hormone (FSH), and free-alpha-subunit (FAS) levels were determined during 24 h of frequent (q10 min) venous sampling. In patient MH, baseline LH levels were low, but FAS levels were within the normal range. In contrast, in patient JW, the mean LH and FSH were within the normal range during baseline sampling, but LH secretion was erratic rather than showing typical pulses. FAS was apulsatile for much of the day, but a surge was seen over a 3-4-h period. Pulsatile gonadotropin releasing hormone (GnRH) (25 ng/kg) was […]

Adrenal Hypoplasia Congenita with Hypogonadotropic Hypogonadism

Evidence That DAX-1 Mutations Lead to Combined Hypothalamic and Pituitary Defects in Gonadotropin Production

Reema L. Habiby,* Paul Boepple,‡ Lisa Nachtigall,‡ Patrick M. Sluss,‡ William F. Crowley, Jr.,‡ and J. Larry Jameson*

**Division of Endocrinology, Metabolism, and Molecular Medicine, Northwestern University Medical School, Chicago, Illinois 60611; and ‡Reproductive Endocrine Unit, Massachusetts General Hospital, Boston, Massachusetts 02114*

Abstract

Adrenal hypoplasia congenita (AHC) is an X–linked disorder that typically presents with adrenal insufficiency during infancy. Hypogonadotropic hypogonadism (HHG) has been identified as a component of this disorder in affected individuals who survive into childhood. Recently, AHC was shown to be caused by mutations in DAX-1, a protein that is structurally similar in its carboxyterminal region to orphan nuclear receptors. We studied two kindreds with clinical features of AHC and HHG. DAX-1 mutations were identified in both families. In the JW kindred, a single base deletion at nucleotide 1219 was accompanied by an additional base substitution that resulted in a frameshift mutation at codon 329 followed by premature termination. In the MH kindred, a GGAT duplication at codon 418 caused a frameshift that also resulted in truncation of DAX-1. Baseline luteinizing hormone (LH), follicle-stimulating hormone (FSH), and free-α-subunit (FAS) levels were determined during 24 h of frequent (q10 min) venous sampling. In patient MH, baseline LH levels were low, but FAS levels were within the normal range. In contrast, in patient JW, the mean LH and FSH were within the normal range during baseline sampling, but LH secretion was erratic rather than showing typical pulses. FAS was apulsatile for much of the day, but a surge was seen over a 3–4-h period. Pulsatile gonadotropin releasing hormone (GnRH) (25 ng/kg) was administered every 2 h for 7 d to assess pituitary responsiveness to exogenous GnRH. MH did not exhibit a gonadotropin response to pulsatile GnRH. JW exhibited a normal response to the first pulse of GnRH, but there was no increase in FAS. In contrast to the priming effect of GnRH in GnRH-deficient patients with Kallmann syndrome, GnRH pulses caused minimal secretory responses of LH and no FAS responses in patient JW. The initial LH response in patient JW implies a deficiency in hypothalamic GnRH. On the other hand, the failure to respond to pulsatile GnRH is consistent with a pituitary defect in gonadotropin production. These two cases exemplify the phenotypic heterogeneity of AHC/HHG, and suggest that DAX-1 mutations impair gonadotropin production by acting at both the hypothalamic and pituitary levels. (*J. Clin. Invest.* 1996. 98: 1055–1062.) Key words: adrenal gland • DAX-1 • gene mutation • hypogonadotropic hypogonadism • GnRH • gonadotropins

Introduction

Adrenal hypoplasia congenita (AHC)[1] is an inherited disorder which most often presents in infancy with adrenal insufficiency and severe salt-wasting. AHC occurs in two distinct forms; the X-linked or cytomegalic form (1), and the autosomal recessive miniature adult form (2). The adrenal glands in the X-linked form lack the permanent zone of the adrenal cortex and are characterized by large vacuolated cells resembling fetal adrenocortical cells (3, 4). Although the clinical presentation with cortisol and mineralocorticoid deficiency is similar to that of males with congenital adrenal *hyper*plasia, in AHC there is an absent or blunted response of precursor steroids after stimulation with ACTH, and there is no evidence of androgen excess. AHC is lethal if left untreated with glucocorticoids and mineralocorticoids (1).

Isolated hypogonadotropic hypogonadism (HHG) is also a feature of this syndrome, but it was recognized only after treatment with adrenal steroids allowed survival beyond childhood (5–8). The deficit in pituitary hormones is selective for gonadotropins (LH, FSH) as the production of other hormones (ACTH, TSH, Prl, GH) is normal. Whether the HHG is a result of hypothalamic or pituitary dysfunction, or both, is unclear (9). A primary pituitary defect is supported by several studies in which patients show little or no response to prolonged treatment with pulsatile GnRH (9–12). However, other studies report patients who have responded to pulsatile gonadotropin releasing hormone (GnRH), suggesting a primary defect at the level of the hypothalamus (13, 14).

Until recently, genetic confirmation of the disorder in newborn males, and identification of the carrier state in females, was not possible for families with X-linked AHC. However, the gene responsible for this disorder has now been identified

Address correspondence to J. Larry Jameson, M.D., Ph.D., Division of Endocrinology, Metabolism and Molecular Medicine, Northwestern University Medical School, Tarry 15, 303 E. Chicago Ave., Chicago, IL 60611. Phone: 312-503-0469; FAX: 312-503-0474; E-mail: ljameson@nwu.edu

Received for publication 26 March 1996 and accepted in revised form 7 June 1996.

J. Clin. Invest.

0021-9738/96/08/1055/08 $2.00
Volume 98, Number 4, August 1996, 1055–1062

1. *Abbreviations used in this paper:* AHC, adrenal hypoplasia congenita; DAX-1, dosage-sensitive sex reversal adrenal hypoplasia congenita critical region on the X-chromosome gene-1; FAS, free alpha subunit; FSH, follicle-stimulating hormone; GH, growth hormone; GnRH, gonadotropin-releasing hormone; HHG, hypogonadotropic hypogonadism; LH, luteinizing hormone; Prl, prolactin; SF-1, steroidogenic factor-1; TSH, thyroid-stimulating hormone.

on the X-chromosome, and is referred to as DAX-1 (15, 16). The DAX-1 gene was localized by studying patients with contiguous gene syndromes in which the AHC locus was deleted along with adjacent loci including the Duchenne muscular dystrophy and the glycerol kinase genes (17). Subsequently, point mutations and rearrangements in the AHC locus were found, confirming that DAX-1 is responsible for this disorder (15, 16, 18). Mutations in the DAX-1 gene appear to be sufficient to account for both AHC and HHG phenotypes (16).

DAX-1 encodes an unusual protein that resembles members of the orphan nuclear receptor family in its carboxy terminus, while having a unique amino terminus with several repeated zinc finger motifs that are distinct from those typically found in nuclear receptors (15). DAX-1 has been proposed to serve as a transcription factor that is involved in development of the adrenal cortex and pituitary gonadotropes (15).

In this report, we describe molecular genetic and clinical studies in two kindreds with X-linked AHC and HHG. We identified two previously unreported mutations in the first and second exons of the DAX-1 gene. The etiology of the hypogonadotropic hypogonadism was also investigated by performing frequent gonadotropin sampling and assessing responses to pulsatile GnRH. Based upon these studies, we describe substantial phenotypic variability in gonadotropin production and propose that hypogonadotropic hypogonadism results from dysfunction at both the hypothalamic and pituitary levels.

Methods

Case presentations

Kindred JW. This family of Northern European descent has four living males affected with AHC (Fig. 1 *A*). The proband (patient IV:2) presented at 5 wk of age with vomiting, lethargy, fever, watery diarrhea, severe hyponatremia (Na^+ = 96 meq/liter), and hyperkalemia (K^+ = 6.8 meq/liter). Genitalia were normal without cryptorchidism. He was diagnosed initially with salt-wasting congenital adrenal hyperplasia. ACTH levels were elevated, but adrenal precursor steroids were not elevated (data not shown). Adrenal steroid responses to cosyntropin (Cortrosyn Organon, Durham, NC) were blunted. Family history revealed two male cousins, also carrying a diagnosis of salt-wasting congenital adrenal hyperplasia. Patient IV:3 developed dusky spells, low body temperature, and hypoglycemia in the first 24 h of life, with hyponatremia (Na^+ = 129 meq/liter) and hyperkalemia (K^+ = 6.2 meq/liter) at 14 d. His younger brother, IV:4 developed dehydration and weight loss at 2 wk of age. Neither infant had elevated adrenal steroid precursors. The proband's uncle (III:7) sought medical attention at 8 yr of age when he developed fatigue and generalized hyperpigmentation, and he was diagnosed with Addison's disease. He was reportedly asymptomatic before the age eight, although his sisters recall that he favored salty foods. At age 14, he was diagnosed with hypogonadotropic hypogonadism. On physical examination at age 26, after receiving several years of monthly injections of testosterone enanthate, he was slightly hyperpigmented, Tanner stage V for pubic hair, and testicular volume was 6–8 ml bilaterally. Patient III:8 was the product of a fraternal twin gestation. He had difficulty feeding and gaining weight, and he died at 5 wk of age. An autopsy report revealed bilateral cryptorchidism, hydrocephalus, and large adrenal vacuoles. The description of his adrenal glands was consistent with adrenal hypoplasia congenita, although the diagnosis was not made at the time. The constellation of findings in this family led to the diagnosis of adrenal hypoplasia congenita in the affected males, all of whom are now maintained on hydrocortisone and fludrocortisone acetate. None of the four affected males have cryptorchidism or microphallus, and none have evidence of contiguous gene deletion syndromes (16, 19). Blood was collected from 13 family members for DNA sequence analyses (see below). These same family members also underwent cosyntropin stimulation tests (250 mg cosyntropin IV) to assess adrenal steroid response. With the exception of the affected males, all had normal responses, including the female carriers (data not shown).

Kindred MH. Patient IV:1, also of Northern European descent, presented at 2 wk of age with hyponatremia (Na^+ = 115 meq/liter) and hyperkalemia (K^+ = 10.2 meq/liter) (Fig. 1 *B*). He had a normal phallus and descended testes. He was diagnosed with salt-wasting congenital adrenal hyperplasia and was placed on hydrocortisone and fludrocortisone acetate. Adrenal precursor steroids were never elevated, even in response to stimulation with cosyntropin. At 15 7/12 yr of age, physical exam revealed a thin boy with generalized hyperpigmentation. Testicular volume was 3 ml bilaterally. His penis was 11.5 cm in length, 3.5 cm in breadth, and there was sparse pubic hair. Testicular biopsy revealed sclerosis of the seminiferous tubules and absence of interstitial cells. Bone age was markedly delayed (11 yr). LH, FSH, and testosterone were low and there was no LH or FSH response to a single 100 μg IV GnRH bolus (data not shown). His diagnosis was changed to adrenal hypoplasia congenita with idiopathic hypogonadotropic hypogonadism. No other family members were known to be affected, however, a maternal uncle died in infancy of unknown causes. Subsequently, a nephew (V:1) developed hyponatremia and hyperkalemia at 19 d of age. Physical examination revealed normal genitalia and testes. He, too, was initially diagnosed with salt-wasting congenital adrenal hyperplasia, but this diagnosis was changed to adrenal hypoplasia congenita after learning of his uncle's diagnosis. Neither patient has evidence of contiguous gene deletion syndromes. Blood was obtained for DNA analysis from the in-

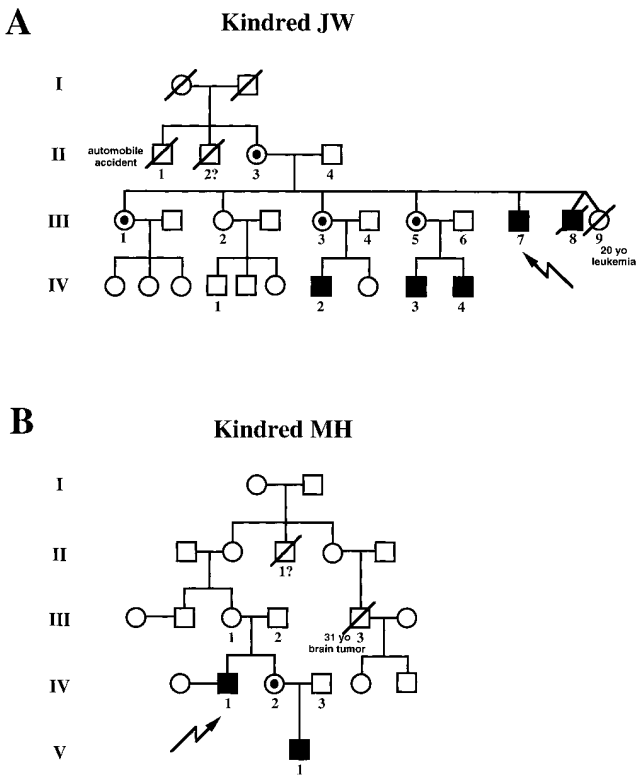

Figure 1. X-linked inheritance of adrenal hypoplasia congenita (AHC) in kindreds JW and MH. Heterozygous carrier females are designated by circles filled with a dot, and hemizygous affected males are indicated by filled squares. Causes of death other than AHC are indicated. Individuals studied for responsiveness to pulsatile GnRH are denoted with arrows. (*A*) Kindred JW; (*B*) Kindred MH.

fant (V:1) and his mother. Blood was unavailable from the patient with HHG at the time of this study.

PCR amplification and sequencing of the DAX-1 gene

After obtaining written informed consent, genomic DNA was extracted from peripheral blood leukocytes. Based on the published DNA sequence of DAX-1 (15), exons 1 and 2 were amplified by the PCR using primers specific for these regions. The primer sequences for exon 1 were: 5′ GCAGAACTGGGCTACGG 3′ (sense) and 5′ GGCCCACATGACTTTTAT 3′ (antisense). PCR of exon 1 was performed in a 50 μl reaction containing 1 μg of genomic DNA, 50 pmoles each primer, 1.5 mM each dinucleoside triphosphate, and 5 U *Taq* DNA polymerase (Promega Biotec, Madison, WI), in a buffer consisting of 6.7 mM Tris (pH 8.8), 6.7 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 16 mM β-mercaptoethanol, and 10% DMSO. After a 3 min hot start at 94°C, the DNA was denatured for 1 min at 94°C, and the primers annealed for 1 min at 60°C. The DNA extension was performed at 72°C for 1.5 min. After 25–30 cycles of PCR, the reactions were completed by a final extension at 72°C for 15 min. The primer sequences for amplification of exon 2 were 5′ TGCAGTGCGTGAAGTACA 3′ (sense) and 5′ GCGCCACATGACTTTAT 3′ (antisense). PCR of exon 2 was performed in a 50 μl reaction containing 200 ng of genomic DNA, 30 pmoles each primer, 0.2 mM each dinucleoside triphosphate, and 2.5 U *Taq* DNA polymerase (Promega Biotec) in a buffer consisting of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 5.5 mM $MgCl_2$, and 0.1% Triton X-100. After a 2 min hot start at 96°C, the DNA was denatured for 1 min at 94°C. Primers were annealed at 54°C (45 s) and extension was performed at 72°C (45 s). After 35 cycles of PCR, reactions were terminated by a final extension at 72°C for 5 min. The purity and abundance of the PCR products were confirmed by analysis of ethidium bromide stained agarose gels.

PCR products were purified from agarose gels using the Mermaid kit (Bio101, Inc., Vista, CA) and subcloned into the pCR II vector using the TA cloning kit (Invitrogen, San Diego, CA). The DNA sequence of both strands was determined by Taq cycle sequencing using a 373A automated sequencer (Applied Biosystems, Foster City, CA). A minimum of four subclones were sequenced in affected patients and eight subclones were analyzed in potential heterozygotes.

Restriction analysis to detect DAX-1 mutations. In the JW kindred, the identified mutation altered the restriction site for the enzyme MspI. PCR of exon 1 was performed on all available JW family members. Subsequently, PCR was repeated in the presence of ^{32}P-labeled dCTP using two new primers (5′ CCCGTGGCACTCCTGTA 3′ and 5′ GCCTCAGCGGGCCTGTT 3′). An aliquot of the PCR product (30 μl) was passed through a Centrisep column (Princeton Separations, Adelphia, NJ), lyophilized to dryness, and resuspended in 15 ml of enzyme buffer, and digested with 10 U of MspI. The digestion products were subjected to electrophoresis through a 6% acrylamide gel and the fragments were visualized using a phosphoimager.

Analysis of gonadotropin secretion. After obtaining written informed consent, a patient from each kindred (patients JWIII:7 and MH IV:1) underwent studies of gonadotropin secretion in the General Clinical Research Center at the Massachusetts General Hospital. Patient JW was studied at 26 yr of age and patient MH was studied at age 21. Testosterone therapy was discontinued for more than 8 wk before testing. Baseline frequent blood sampling (every 10 min) was performed over 24 h for measurement of LH, free α-subunit (FAS), and TSH. Secretory patterns were analyzed for pulses using a modified version (PNADIR) of the Santen and Bardin method (20). FSH was measured in hourly pools, and testosterone was determined from 6 h pools of serum. After completion of baseline sampling, each patient received pulsatile GnRH (25 ng/kg), subcutaneously by infusion pump, every 2 h for 7 d (21, 22). Gonadotropin responses to a single intravenous pulse were monitored each day at 8 AM. LH, FSH, and FAS levels were determined at 15-min intervals for 2 h after the GnRH pulse. Serum LH and FSH concentrations were determined by radioimmunoassay (RIA) using the Second International Reference Preparation of human menopausal gonadotropins as the reference standard (23, 24). FAS was measured by a monoclonal antibody RIA, which uses a highly purified α subunit of hCG as a standard (25, 26). Serum testosterone was also determined using RIA (27). TSH was measured by an ultrasensitive microparticle enzyme immunoassay (28).

Results

Mutational analyses of the DAX-1 gene. In kindred JW, the coding sequence of the DAX-1 gene was analyzed in two affected patients (IV:3, IV:4). Two DNA sequence alterations were found in exon 1 of both patients. A single base deletion at nucleotide 1219 was accompanied by a G to A substitution at nucleotide 1221 (CCGGGGGCCA→CC•GAGGCCA). This combined deletion and substitution changed a glycine to glutamine at amino acid 329 and caused a frameshift alteration that leads to a premature stop codon after translating an additional 41 amino acids. Because the mutation removes an MspI restriction site, a digest of ^{32}P-labeled PCR products was diagnostic for the remaining 11 family members (Fig. 2). This analysis confirmed the presence of the mutation in all affected males and identified carrier females, one of whom (III:1) had not been recognized previously.

In kindred MH, sequence analysis of exon 1 did not reveal a mutation, but a silent T to C polymorphism was detected at nucleotide 348 (Fig. 3). After screening multiple normal DNA samples, the polymorphism at nucleotide 348 (T or C) was con-

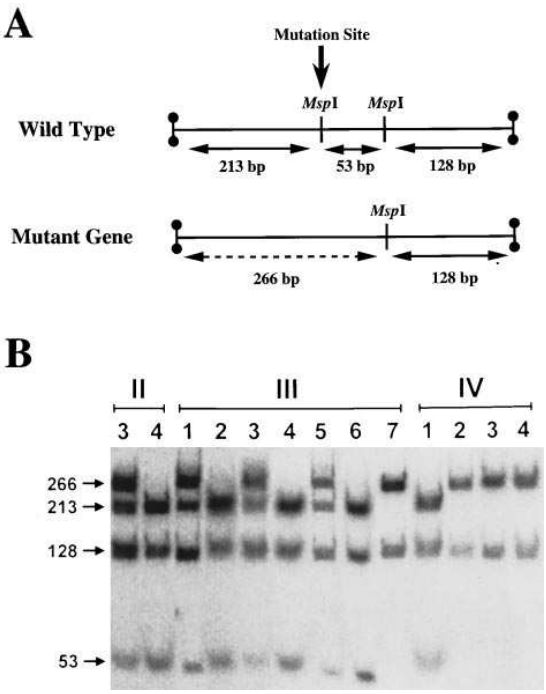

Figure 2. Mutations in the JW kindred detected by alterations in an MspI restriction enzyme site. (*A*) Schematic illustration of the region of exon 1 in the DAX-1 gene that was amplified by PCR. The identified mutation eliminates an MspI restriction site. Unaffected males show 213-, 128-, and 53-bp fragments. Affected males have two fragments of 266 and 128 bp. Heterozygous carrier females exhibit all four fragments (266, 213, 128, 53 bp). (*B*) Phosphoimage of MspI restriction digest, confirming the mutation in all affected subjects. Individual patients are indicated at the tops of the lanes and correspond to the pedigree in Fig. 1.

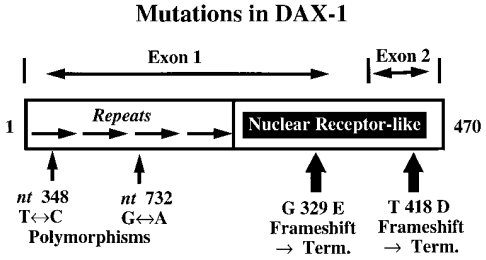

Figure 3. DAX-1 mutations in kindreds JW and MH. DAX-1 is illustrated schematically. The three and one-half repeated motifs in the aminoterminal region are depicted by arrows. The carboxy terminal region is structurally similar to ligand-binding domain of members of the nuclear receptor superfamily. The two new identified mutations each result in a translational frameshift that causes premature termination of DAX-1. The G329E mutation was found in the JW kindred and the T418D mutation was found in the MH kindred. Screening of multiple DNA samples identified two polymorphisms in the aminoterminal domain of DAX-1.

firmed. A second polymorphic variant was identified in other normal individuals at nucleotide 732 (G or A) in the aminoterminal domain of DAX-1 (Fig. 3). In patient V:1, a four nucleotide (nt 1481–1484; GGAT) duplication was identified in exon 2 (ACCAGGATGACG→ACCAGGATGGATGACG). This duplication converts codon 418 from Thr to Asp followed by a six amino frameshift that results in premature termination after codon 424. This mutation does not alter a restriction site, but it was found in all four subclones of the affected patient (V:1) and in four of eight subclones from his mother (IV:2), consistent with her status as an obligate heterozygote.

Baseline gonadotropin secretion. Frequent sampling of baseline gonadotropin (LH, FSH, FAS) levels was performed for 24 h to further delineate the pattern of spontaneous, endogenous gonadotropin secretion in these patients with AHC (Figs. 4 and 5). In patient JW, mean LH and FSH levels from pooled samples were within the normal range (mean LH = 8.9 IU/L; mean FSH = 5.6 IU/L). Although computer algorithms detected several pulses, the LH secretory pattern was erratic, and distinct from the characteristic LH pulsatility that is seen in normal men (22). FAS was below the limits of detection (< 100 ng/liter) for the first half of the study, but there was a surge into the normal range, and a subsequent fall during the second half of the study (Fig. 4). LH and FAS have been shown to be coincident in normal men (26). Of nine detected FAS pulses, eight were coincident with LH pulses. TSH was also assayed in JW to assess whether the increase in FAS might coincide with a diurnal rise in TSH. The pulse algorithm revealed that four of nine FAS subunit pulses were coincident with TSH pulses (44%). His mean testosterone level was 127 ng/dl, which is somewhat lower than expected for the LH level.

Patient MH exhibited apulsatile LH secretion, and none of the LH values reached the normal range (Fig. 5). Although FAS varied in an erratic manner, all of the values were within the normal range. The mean FSH level was 2.7 IU/L and testosterone was less than 10 ng/dl.

Gonadotropin responses to pulsatile GnRH. In patient JW, GnRH administration stimulated a large LH pulse on day 1 (Fig. 6). However, on the subsequent six days, GnRH did not elicit an increase in LH secretion. Of note, FAS also failed to increase in response to pulsatile GnRH. On day 1, FAS levels declined at a time when GnRH stimulated LH secretion, and there was no evidence of FAS responses to GnRH on subsequent days. There was no change in the low level of FSH secretion throughout the time of pulsatile GnRH administration. During the seven days of pulsatile GnRH administration, tes-

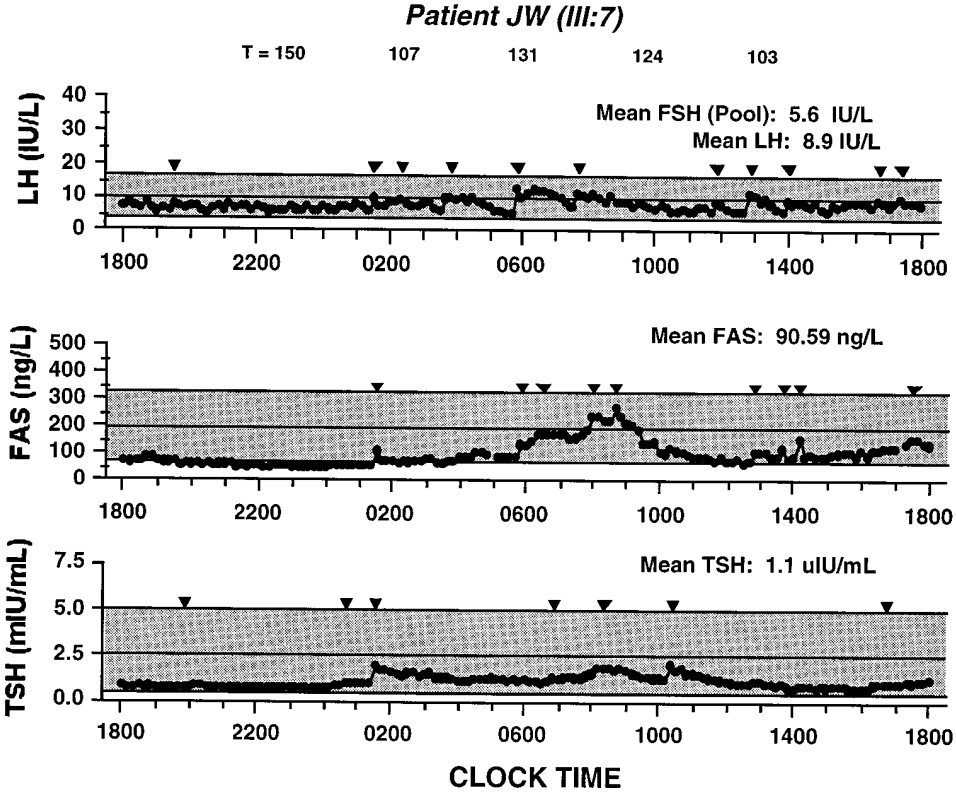

Figure 4. Baseline secretory patterns of LH, free α-subunit (FAS), and TSH in patient JW. Gonadotropin secretion was determined by frequent sampling (q10 min) for 24 h. The shaded areas in each panel represent the mean±2 SD for each hormone determined in 20 normal adult males studied using the same protocol (26, 27). Mean gonadotropin levels (including FSH) and testosterone levels from pools of samples are indicated. Pulses detected by a modified version (PNADIR) of the Santen and Bardin procedure (20) are denoted by arrowheads.

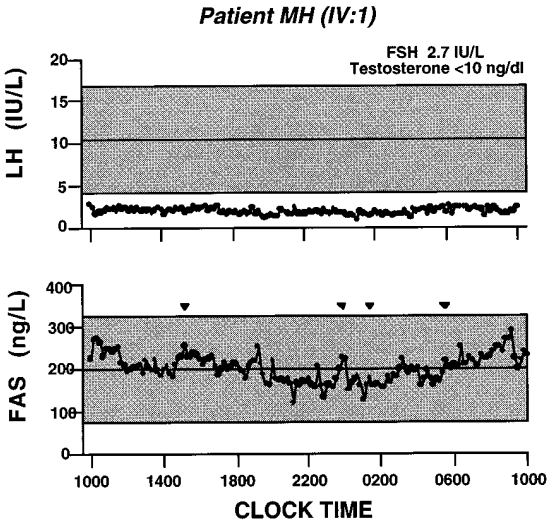

Figure 5. Baseline secretory patterns of LH and free α-subunit (FAS) in patient MH. Gonadotropin secretion was determined by frequent sampling (q10 min) for 24 h as described in the legend to Fig. 4. There was insufficient serum available for measurement of TSH. The shaded areas in each panel represent the mean±2 SD. Mean gonadotropin levels (including FSH) and testosterone levels from pools of samples are indicated. Pulses detected by a modified version (PNADIR) of the Santen and Bardin procedure (20) are denoted by arrowheads.

tosterone levels gradually rose from 103 (day 1) to 241 ng/dl (day 7), despite the absence of detectable LH responses to GnRH. One week after completing pulsatile GnRH, patient JW received 2,000 U of hCG intramuscularly for three consecutive days to evaluate Leydig cell function. Before the first injection, serum testosterone was 70 ng/dl and increased to 679 ng/dl by day 4, confirming normal Leydig cell function.

In patient MH, neither LH nor FAS responded to pulsatile GnRH (Fig. 6). There was a small, but detectable rise in FSH after several days of pulsatile GnRH. Although there was a modest rise in testosterone, the levels remained low (39 ng/dl), even after seven days of treatment.

For comparison, patients with Kallmann syndrome, who are known to be selectively deficient in GnRH (22, 29, 30), were given an identical regimen of pulsatile GnRH. The responses derived from 10 separate patients are shown in Fig. 6. After a clear LH response to the first dose of GnRH on day 1, responses were muted on subsequent days, but then increased between days 4 and 7, reflecting the effect of "GnRH priming." As shown previously (22, 26), FAS provides a particularly sensitive marker of gonadotropin secretion in response to GnRH with robust responses on days 3 to 7. Mean FSH levels increased progressively in response to pulsatile GnRH.

Discussion

The identification of mutations in the DAX-1 gene as the cause of adrenal hypoplasia congenita provides an opportunity to gain new insights into the molecular pathophysiology of this disorder. Although relatively little is known at present about the function of DAX-1, it is notable that it resembles members of the nuclear receptor superfamily (15). Specifically, the carboxy terminal region of DAX-1 is homologous with the ligand binding domain of certain "orphan" nuclear receptors, which are involved in a wide array of developmental events and metabolic functions (31). Of note, DAX-1 lacks the characteristic zinc finger DNA binding domain which is highly conserved among true nuclear receptors. Instead, this domain is replaced by an amino terminal region that contains a series of repeated amino acid motifs, the function of which remains to be defined. Given its relationship to nuclear receptors, DAX-1 may play a role in gene transcription, and one report provides evidence that it can inhibit the action of the retinoic acid receptor (15). Consequently, DAX-1 may be involved in a regulatory cascade that ultimately leads to normal adrenal gland development and to a functional GnRH-gonadotropin axis.

One interesting aspect of AHC is the combination of primary adrenal dysfunction and hypogonadotropic hypogonadism. There are striking similarities between the phenotype of patients with DAX-1 mutations and the characteristics of mice with targeted disruption of the Ftz-F1 gene (32–34). The Ftz-F1 locus encodes an orphan nuclear receptor, referred to as AD4BP (35) or steroidogenic factor-1 (SF-1) (36). The SF-1 gene knockout causes failure of adrenal gland development, selective deficiency of pituitary gonadotropins, and abnormalities in male gonadal differentiation (32, 37, 38). Defects in SF-1, therefore resemble DAX-1 mutations, although males with X-linked AHC appear to have normal gonadal development. Both SF-1 and DAX-1 are expressed in the adrenals, gonads,

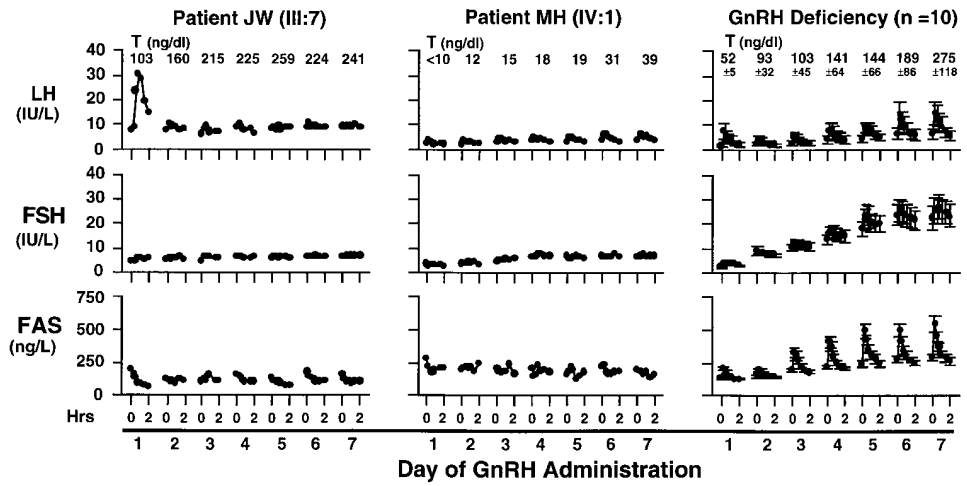

Figure 6. Gonadotropin responses to 7 d of pulsatile GnRH. Following the baseline secretory studies, each patient received pulsatile GnRH at a dose of 25 ng/kg I.V. every 2 h for 7 d. The response to a single GnRH pulse ($t = 0$ to $+ 2$ h) was monitored each day. LH, FSH, and free α-subunit (FAS) responses are depicted. Daily testosterone levels are shown above the graphs. The GnRH responses (mean±SEM) of 10 males with Kallmann Syndrome (GnRH deficiency and anosmia) are also shown, and their testosterone levels are indicated at the top of the figure.

hypothalamus, and pituitary (15, 38–41) (Ito, M., R. Yu, J.L. Jameson, unpublished data). These observations raise the possibility that SF-1 and DAX-1 may function together as transcription factors, or in sequential steps during embryonic development.

In the two families that we studied, the identified mutations are both predicted to truncate the DAX-1 gene product. Although only a limited number of mutations have been reported, it appears that complete gene deletions, or alterations that result in frameshift mutations are relatively common in comparison to the occurrence of missense mutations (15, 16, 18). This may reflect a propensity for recombination at this locus. Alternatively, there may be a bias of ascertainment in which the more severe mutations are found because the phenotypic characteristics are readily identified. The latter explanation has potential importance in that it raises the prospect that milder phenotypes may be more prevalent than previously recognized. The frequency of polymorphisms at this locus remains to be determined. However, we did identify two silent nucleotide substitutions in a relatively small survey of affected and normal alleles. These and other polymorphisms may be useful for linkage studies as some patients with apparent AHC do not have identified mutations in the coding sequence of DAX-1 (16).

As summarized in Table I, there is substantial variability in the expression of adrenal insufficiency, even in patients with the same mutation in DAX-1. Although most patients present with adrenal insufficiency in infancy, patient JWIII:7 did not present until 8 yr of age, consistent with other reports of intrafamilial variability (3, 6, 8, 9). Sills et al. (42) reported a 2-mo-old child with presumed X-linked AHC who was initially mineralocorticoid deficient, but glucocorticoid sufficient. However, adrenal function subsequently declined and he was glucocorticoid insufficient by 3 yr of age. It is possible that our patient might have had subclinical evidence of adrenal insufficiency at a younger age, but this was not investigated. These findings indicate that the abnormalities in adrenal development are variable, and as noted above, more subtle forms of this disorder could result in subclinical adrenal insufficiency or hypogonadism. At present, we are unable to assess variability in the gonadotropin axis within a kindred because only one affected male in each pedigree was postpubertal.

Whether the hypogonadotropic hypogonadism associated with DAX-1 mutations results from hypothalamic or primary pituitary dysfunction remains an unsettled issue. Initial reports documented that in most patients, there was a poor LH response, but variable FSH responses to a single injection of synthetic GnRH (3–6, 8, 43). None of these reports clearly differentiate hypothalamic from pituitary dysfunction as patients with either type of dysfunction typically respond poorly to a single GnRH bolus (44, 45). However, Petersen et al. (46) reported a normal LH response to IV GnRH in a 14 yr old boy with AHC and HHG, consistent with GnRH deficiency in this case. This response may be analogous to the increase in LH that we observed in patient JW upon first exposure to exogenous GnRH.

Prolonged administration of pulsatile GnRH should identify

Table I. Summary of Clinical Features in Patients with DAX-1 Mutations

Patient	Age at presentation	Mode of presentation	HHG	DAX-1 mutation
JW : III 7	8 yr	Fatigue, hyperpigmentation	Yes	G329E w/ frameshift
JW : III 8	birth	Poor feeding; death at 5 weeks; hydrocephalus; vacuolated adrenals	Bilateral cryptorchidism	Not tested
JW : IV 2	5 wk	Vomiting; diarrhea; hyponatremia; hyperkalemia	Unknown; testes descended; nl penis	G329E w/ frameshift
JW : IV 3	birth	Hypothermia; hypogycemia; hyponatremia; hyperkalemia	Unknown; testes descended; nl penis	G329E w/ frameshift
JW : IV 4	2 wk	Dehydration; weight loss; hyponatremia; hyperkalemia	Unknown; testes descended; nl penis	G329E w/frameshift
MH : IV 1	2 wk	Vomiting; hyponatremia; hyperkalemia	Yes	Not tested
MH : V 1	3 wk	Poor feeding; hyponatremia; hyperkalemia	Unknown; testes descended; nl penis	T418D w/ frameshift

nl, normal.

patients with a defect in hypothalamic GnRH, as this physiologic pattern of GnRH stimulation allows "priming" of the pituitary (44, 45). However, the reports of gonadotropin responses to pulsatile GnRH are also conflicting. The GnRH treatment protocols and the overall gonadotropin responses are summarized in Table II. Primary pituitary dysfunction is supported by several reports of deficient gonadotropin responses after prolonged, pulsatile GnRH administration (9–12). Although the regimen of GnRH varies in these studies, LH responses were consistently low. In some cases, there was a gradual rise in FSH even when LH responses were absent (9, 11, 13). Other reports support a primary hypothalamic etiology of the HHG based upon some degree of increase in gonadotropins and testosterone after prolonged administration of pulsatile GnRH (13, 14). In the absence of genetic data, it is possible that some patients may have disorders other than DAX-1 mutations, or that different DAX-1 mutations may vary in phenotypic severity.

To assess pituitary responses to exogenous pulsatile GnRH, we used a regimen which has been shown to induce physiologic levels of LH, FSH, and testosterone in GnRH-deficient males (22). In the Kallmann patients, LH, FAS, and FSH each showed an initial small secretory response followed by progressive increases in response to pulsatile GnRH. It is interesting to contrast the findings in patients MH and JW. Patient MH had very low baseline LH levels without typical pulses of either LH or FAS (Fig. 5). Of note, his FAS levels were in the normal range. In contrast, in patient JW, the baseline FAS levels were low, but LH and FSH levels were in the normal range, indicating that he does not have absolute hypogonadotropic hypogonadism. However, the pattern of LH secretion was erratic and it is unclear whether the variations in measured LH are true GnRH-driven pulses or the consequence variable pituitary secretion or hormone clearance. Despite the normal levels of LH, his testosterone levels are low. We considered the possibility that the DAX-1 mutation might impair Leydig cell function, as it is known to be expressed in the testis (15, 41) (Yu, R., M. Ito, J. L. Jameson, unpublished data). However, like several other reported cases (3, 4, 6–8, 10–14, 43, 46), he had a normal response to exogenous stimulation with hCG. It is possible that his endogenous LH has reduced biological activity. Patients with TRH deficiency have been shown to produce TSH with reduced biological activity, probably because of altered glycosylation (47). Another possibility is that the absence of normal LH pulses alters Leydig cell responsiveness. For example, constant exposure to normal LH levels may lead to down-regulation and desensitization of LH receptors (48).

The response of patient JW to pulsatile GnRH was unusual. He responded normally to the first GnRH pulse, but showed minimal LH secretion in response to pulses on subsequent days. It is also notable that most patients with Kallman syndrome also show an initial response to GnRH (Fig. 6). However, the pattern of decreasing sensitivity to pulsatile GnRH is the opposite of that seen in the Kallmann patients who exhibit dramatic priming in response to pulsatile GnRH (Fig. 6) (22). In patient JW, we suggest that there is a mixed hypothalamic and pituitary defect. In the absence of endogenous GnRH pulses, the initial GnRH response may reflect the release of small amounts of accumulated gonadotropins. However, these stores are rapidly depleted, and in the absence of effective gonadotropin biosynthesis, the gonadotropes are unable to respond to further pulses of GnRH. It is possible that longer periods of GnRH administration, or perhaps different frequencies might result in improved LH responsiveness. Nevertheless, a combined defect of GnRH and gonadotropin production would explain the findings in this patient as well as the variable responses reported in different patients (Table II).

The pattern of free α-subunit (FAS) secretion in JW was also unusual and supports a defect in gonadotropin production (Fig. 4). FAS secretion usually correlates well with GnRH-driven LH pulses (26). However, this pulse pattern was erratic and most pulses were relatively small. Moreover, we know that this patient does not respond well to exogenous pulsatile GnRH, making it unlikely that these are truly GnRH-driven pulses. There was also some correlation between FAS and TSH, and it is likely that some of the circulating FAS corresponds to TSH secretion from thyrotropes.

This study, combined with previous reports, reveals a great deal of phenotypic heterogeneity in this condition, and further detailed studies must be performed in patients with genetically confirmed mutations of DAX-1 to determine the full spectrum of hypothalamic-pituitary-gonadal dysfunction. Future studies of DAX-1 expression and a DAX-1 animal knockout model will provide further insight into the role of DAX-1 in AHC and it will be of great interest to determine the role of DAX-1 as a determinant of gonadotropin biosynthesis.

Acknowledgments

The paper is dedicated to the memory of Sigrid J. Gelber, who initiated this project before her untimely death. We acknowledge the assistance of Kathy Lockhart, Jeff Weiss, and Tom Kotlar.

Conducted as a part of the National Cooperative Program on In-

Table II. Gonadotropin Responses to Pulsatile GnRH in Published Reports of AHC/HHG

Report	Pulstaile GnRH regimen	Response to chronic pulsatile GnRH		
		LH	FSH	FAS
Current study	25 ng/kg q2h × 7 d	↔	↔ or ↑	↔
Kletter et al. (9)	25 ng/kg q2h × 5 d	↔	↑	↔
Gordon et al. (10)	2.8-22 μg/pulse q90min × 16 wk	↔	↔	ND
Kikuchi et al. (11)	5-10 μg q90min × 91 d	↔	↑	ND
Bovet et al. (12)	2.5-10 μg/pulse q90min × 8 wk	↔	↔	ND
Kruse et al. (13)	5 μg/pulse q90min × 26 h	↔	↑	ND
Partsch et al. (14)	50-200 ng/kg q2h × 394 d	↑*	↑*	ND

ND, not done; LH, luteinizing hormone; FSH, follicle-stimulating hormone; FAS, free α-subunit. *Actual hormone values not reported.

fertility Research, supported by grants U54 HD 29164 and core facilities from P30 HD 28138. The Genentech Foundation for Growth and Development provided fellowship support to Reema Habiby.

References

1. Uttley, W.S. 1968. Familial congenital adrenal hypoplasia. *Arch. Dis. Childhood.* 43:724–730.

2. Burke, B.A., M.R. Wick, R. King, T. Thompson, J. Hansen, B.T. Darrae, U. Francke, W.K. Seltzer, E.R. McCabe, and B.W. Scheithauer. 1988. Congenital adrenal hypoplasia and selective absence of pituitary luteinizing hormone: a new autosomal recessive syndrome. *Am. J. Med. Genet.* 31:75–97.

3. Hay, I.D., P.J. Smail, and C.C. Forsyth. 1981. Familial cytomegalic adrenocortical hypoplasia: an X-linked syndrome of pubertal failure. *Arch. Dis. Child.* 56:715–721.

4. Kelch, R.P., R. Virdis, R. Rapaport, F. Greig, L.S. Levine, and M.I. New. 1984. Congenital adrenal hypoplasia. *Pediatr. Adolesc. Endocrinol.* 13:156–161.

5. Prader, A., M. Zachmann, and R. Illig. 1975. Luteinizing hormone deficiency in hereditary congenital adrenal hypoplasia. *J. Pediatr.* 86:421–422.

6. Golden, M.P., B.M. Lippe, and S.A. Kaplan. 1977. Congenital adrenal hypoplasia and hypogonadotropic hypogonadism. *Am. J. Dis. Child.* 131:1117–1118.

7. Zachmann, M., R. Illig, and A. Prader. 1980. Gonadotropin deficiency and cryptorchidism in three prepubertal brothers with congenital adrenal hypoplasia. *J. Pediatr.* 97:255–257.

8. Kelly, W.F., G.F. Joplin, and G.W. Pearson. 1977. Gonadotrophin deficiency and adrenocortical insufficiency in children: a new syndrome. *Br. Med. J.* 2:98.

9. Kletter, G.B., J.L. Gorski, and R.P. Kelch. 1991. Congenital adrenal hypoplasia and isolated gonadotropin deficiency. *Trends Endocrinol. Metab.* 2: 123–128.

10. Gordon, D., H.N. Cohen, G.H. Beastall, I.D. Hay, and J.A. Thomson. 1984. Contrasting effects of subcutaneous pulsatile GnRH therapy in congenital adrenal hypoplasia and Kallmann's syndrome. *Clin. Endocrinol.* 21:597–603.

11. Kikuchi, K., M. Kaji, T. Momoi, H. Mikawa, Y. Shigematsu, and M. Sudo. 1987. Failure to induce puberty in a man with X-linked congenital adrenal hypoplasia and hypogonadotropic hypogonadism by pulsatile administration of low-dose gonadotropin-releasing hormone. *Acta Endocrinol.* 114:153–160.

12. Bovet, P., M.J. Reymond, F. Rey, and F. Gomez. 1988. Lack of gonadotropic response to pulsatile gonadotropin-releasing hormone in isolated hypogonadotropic hypogonadism associated to congenital adrenal hypoplasia. *J. Endocrinol. Invest.* 11:201–204.

13. Kruse, K., W.G. Sippell, and K.V. Schnakenburg. 1984. Hypogonadism in congenital adrenal hypoplasia: evidence for a hypothalamic origin. *J. Clin. Endocrinol. & Metab.* 58:12–17.

14. Partsch, C.J., and W.G. Sippell. 1989. Hypothalamic hypogonadism in congenital adrenal hypoplasia. *Horm. Metab. Res.* 21:623–625.

15. Zanaria, E., F. Muscatelli, B. Bardoni, T.M. Strom, S. Guioli, W. Guo, E. Lalli, C. Moser, A.P. Walker, E.R. McCabe, et al. 1994. An unusual member of the nuclear hormone receptor superfamily responsible for X-linked adrenal hypoplasia congenita. *Nature (Lond.).* 372:635–641.

16. Muscatelli, F., T.M. Strom, A.P. Walker, E. Zanaria, D. Recan, A. Meindl, B. Bardoni, S. Guioli, G. Zehetner, W. Rabl, et al. 1994. Mutations in the DAX-1 gene give rise to both X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism. *Nature (Lond.).* 372:672–676.

17. Bartley, J.A., D.K. Miller, J.T. Hayford, and E.R. McCabe. 1982. Concordance of X-linked glycerol kinase deficiency with X-linked congenital adrenal hypoplasia. *Lancet.* 2:733–736.

18. Guo, W., J.S. Mason, C.G. Stone, Jr., S.A. Morgan, S.I. Madu, A. Baldini, E.A. Lindsay, L.G. Biesecker, K.C. Copeland, M.N. Horlick, et al., 1995. Diagnosis of X-linked adrenal hypoplasia congenita by mutation analysis of the DAX1 gene. *Jama (J. Am. MED/Assoc.).* 274:324–330.

19. Patil, S.R., J.A. Bartley, J.C. Murray, V.V. Ionasescu, and P.L. Pearson. 1985. X-linked glycerol kinase, adrenal hypoplasia and myopathy maps at Xp21. *Cytogenet. Cell Genet.* 40:720–721.

20. Santen, R.J., and C.W. Bardin. 1973. Episodic luteinizing hormone secretion in man. Pulse analysis, clinical interpretation, physiologic mechanisms. *J. Clin. Invest.* 52:2617–2628.

21. Whitcomb, R.W., and W.F. Crowley. 1990. Clinical review 4: diagnosis and treatment of isolated gonadotropin-releasing hormone deficiency in men. *J. Clin. Endocrinol. Metab.* 70:3–7.

22. Crowley, W.F., R.W. Whitcomb, J.L. Jameson, J. Weiss, J.S. Finkelstein, and L.S. O'Dea. 1991. Neuroendocrine control of human reproduction in the male. *Recent Prog. Horm. Res.* 47:27–67.

23. Hall, J.E., D.A. Schoenfeld, K.A. Martin, and W.F. Crowley, Jr. 1992. Hypothalamic gonadotropin-releasing hormone secretion and follicle-stimulating hormone dynamics during the luteal-follicular transition. *J. Clin. Endocrinol. & Metab.* 74:600–607.

24. Taylor, A.E., R.H. Khoury, and W.F. Crowley, Jr. 1994. A comparison of 13 different immunometric assay kits for gonadotropins: implications for clinical investigation. *J. Clin. Endocrinol. & Metab.* 79:240–247.

25. Whitcomb, R.W., J.S. Sangha, A.L. Schneyer, and W.F. Crowley, Jr. 1989. Improved measurement of free alpha subunit of glycoprotein hormones by assay with use of a monoclonal antibody. *Clin. Chem.* 34:2022–2025.

26. Whitcomb, R.W., L.S. O'Dea, J.S. Finkelstein, D.M. Heavern, and W.F. Crowley, Jr. 1990. Utility of free alpha-subunit as an alternative neuroendocrine marker of gonadotropin-releasing hormone (GnRH) stimulation of the gonadotroph in the human: evidence from normal and GnRH-deficient men. *J. Clin. Endocrinol. & Metab.* 70:1654–1661.

27. Spratt, D.I., L.S. O'Dea, D. Schoenfeld, J. Butler, P.N. Rao, and W.F. Crowley, Jr. 1988. Neuroendocrine-gonadal axis in men: frequent sampling of LH, FSH, and testosterone. *Am. J. Physiol.* 254:E658–666.

28. Spencer, C.A., J.S. LoPresti, A. Patel, R.B. Guttler, A. Eigen, D. Shen, D. Gray, and J.T. Nicoloff. 1990. Applications of a new chemiluminometric thyrotropin assay to subnormal measurement. *J. Clin. Endocrinol. & Metab.* 70: 453–460.

29. Franco, B., S. Guioli, A. Pragliola, B. Incerti, B. Bardoni, R. Tonlorenzi, R. Carrozzo, E. Maestrini, M. Pieretti, P. Taillon-Miller, et al. 1991. A gene deleted in Kallmann's syndrome shares homology with neural cell adhesion and axonal path-finding molecules. *Nature (Lond.).* 353:529–536.

30. Legouis, R., J.P. Hardelin, J. Levilliers, J.M. Claverie, S. Compain, V. Wunderle, P. Millasseau, D. Le Paslier, D. Cohen, D. Caterina, et al. 1991. The candidate gene for the X-linked Kallmann syndrome encodes a protein related to adhesion molecules. *Cell.* 67:423–435.

31. Mangelsdorf, D.J., and R.M. Evans. 1995. The RXR heterodimers and orphan receptors. *Cell.* 83:841–850.

32. Luo, X., Y. Ikeda, and K.L. Parker. 1994. A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. *Cell.* 77:481–490.

33. Luo, X., Y. Ikeda, D.A. Schlosser, and K.L. Parker. 1995. Steroidogenic factor 1 is the essential transcript of the mouse *Ftz-F1* gene. *Mol. Endocrinol.* 9: 1233–1239.

34. Sadovsky, Y., P.A. Crawford, K.G. Woodson, J.A. Polish, M.A. Clements, L.M. Tourtellotte, Simburger, and J. Milbrandt. 1995. Mice deficient in the orphan receptor steroidogenic factor 1 lack adrenal glands and gonads but express P450 side-chain-cleavage enzyme in the placenta and have normal embryonic serum levels of corticosteroids. *Proc. Natl. Acad. Sci. USA.* 92:10939–10943.

35. Honda, S., K. Morohashi, M. Nomura, H. Takeya, M. Kitajima, and T. Omura. 1993. Ad4BP regulating steroidogenic P-450 gene is a member of steroid hormone receptor superfamily. *J. Biol. Chem.* 268:7494–7502.

36. Lala, D.S., D.A. Rice, and K.L. Parker. 1992. Steroidogenic factor I, a key regulator of steroidogenic enzyme expression, is the mouse homolog of fushi tarazu-factor I. *Mol. Endocrinol.* 6:1249–1258.

37. Ingraham, H.A., D.S. Lala, Y. Ikeda, X. Luo, W.H. Shen, M.W. Nachtigal, R. Abbud, J.H. Nilson, and K.L. Parker. 1994. The nuclear receptor steroidogenic factor 1 acts at multiple levels of the reproductive axis. *Genes & Dev.* 8:2302–2312.

38. Ikeda, Y., X. Luo, R. Abbud, J.H. Nilson, and K.L. Parker. 1995. The nuclear factor steroidogenic factor 1 is essential for the formation of the ventromedial hypothalamic nucleus. *Mol. Endocrinol.* 9:478–486.

39. Barnhart, K.M., and P.L. Mellon. 1994. The orphan nuclear receptor, steroidogenic factor-1, regulates the glycoprotein hormone alpha-subunit gene in pituitary gonadotropes. *Mol. Endocrinol.* 8:878–885.

40. Morohashi, K., O. Hatano, M. Nomura, K. Takayama, M. Hara, H. Yoshii, A. Takakusu, and T. Omura. 1995. Function and distribution of a steroidogenic cell-specific transcription factor, Ad4BP. *J. Steroid Biochem. Mol. Biol.* 53:81–88.

41. Guo, W., T.P. Burris, and E.R. McCabe. 1995. Expression of DAX-1, the gene responsible for X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism, in the hypothalamic-pituitary-adrenal/gonadal axis. *Biochem. Mol. Med.* 56:8–13.

42. Sills, I.N., M.L. Voorhess, M.H. MacGillivray, and R.E. Peterson. 1983. Prolonged survival without therapy in congenital adrenal hypoplasia. *Am. J. Dis. Child.* 137:1186–1188.

43. Virdis, R., L.S. Levine, D. Levy, S. Pang, R. Rapaport, and M.I. New. 1983. Congenital adrenal hypoplasia: two new cases. *J. Endocrinol. Invest.* 6:51–54.

44. Crowley, W.F., M. Filicori, D.I. Spratt, and N.F. Santoro. 1985. The physiology of gonadotropin-releasing hormone (GnRH) secretion in men and women. *Recent Prog. Horm. Res.* 41:473–531.

45. Marshall, J.C., and R.P. Kelch. 1986. Gonadotropin-releasing hormone: role of pulsatile secretion in the regulation of reproduction. *N. Engl. J. Med.* 315:1459–1468.

46. Petersen, K.E., T. Bille, B.B. Jacobsen, and T. Iversen. 1982. X-linked congenital adrenal hypoplasia. A study of five generations of a Greenlandic Family. *Acta. Paediatr. Scand.* 71:947–951.

47. Beck-Peccoz, P., S. Amr, M.M. Menezes-Ferreira, G. Faglia, and B.D. Weintraub. 1985. Decreased receptor binding of biologically inactive thyrotropin in central hypothyroidism. Effect of treatment with thyrotropin-releasing hormone. *N. Engl. J. Med.* 312:1085–1090.

48. Wang, H., D.L. Segaloff, and M. Ascoli. 1991. Lutropin/choriogonadotropin down-regulates its receptor by both receptor-mediated endocytosis and a cAMP-dependent reduction in receptor mRNA. *J. Biol. Chem.* 266:780–785.