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The Aromatase Excess Syndrome Is Associated with Feminization of Both Sexes and Autosomal Dominant Transmission of Aberrant P450 Aromatase Gene Transcription*

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ABSTRACT

Increased extraglandular aromatization has been reported as the cause of familial gynecomastia. We studied a kindred with aromatase excess inherited in an autosomal dominant manner, in which affected males had heterosexual precocity and/or gynecomastia, and affected females had isosexual precocity and/or macromastia.

The propositus was a 9-yr-old boy with gynecomastia. His 7.5-yr-old sister had precocious puberty, and their father and paternal grandmother had peripubertal gynecomastia and macromastia, respectively. Serum concentrations of gonadal and adrenal steroid hormones were determined before and after the administration of corticotropin and/or hCG. Aromatase activity was determined by [^{3}H]Δ^4-androstenedione to [^{3}H]estrone conversion by cultured skin fibroblasts and/or Epstein-Barr virus-transformed lymphocytes and was detected by immunohistochemistry and/or Western analysis. Linkage was examined with a polymorphism of the aromatase (P450arom) gene. The P450arom messenger ribonucleic acid was analyzed by rapid amplification of complementary DNA (cDNA) ends, ribonuclease protection assay, and RT-PCR.

hCG testing demonstrated a high rate of conversion of Δ^4-androstenedione to estrone and of testosterone to estradiol in the propositus and his father. Treatment of the propositus and his sister was initiated with an aromatase inhibitor (testolactone) and a GnRH analog, which successfully delayed skeletal and pubertal development in both children. Markedly increased aromatase activity was found in the patients' fibroblasts and Epstein-Barr virus-transformed lymphocytes. The P450arom polymorphism segregated with the disease in the family. A new 5′-splice variant was present in the patients' P450arom messenger ribonucleic acid, thus identifying yet another first exon of this gene, which appears to be aberrantly expressed in this family.

In conclusion, a family with the aromatase excess syndrome is described, in which the condition was inherited in an autosomal dominant manner, led to feminizing manifestations in both sexes, and was associated with the aberrant utilization of a novel transcript of the P450arom gene. (*J Clin Endocrinol Metab* **83:** 1348–1357, 1998)

SOME BREAST development occurs in 30–65% of boys during puberty, but is extremely uncommon in the prepubertal years (1–3). Prepubertal gynecomastia is most frequently caused by exogenously administered estrogens or hormone-secreting tumors (4). Rarely, this condition is inherited as a familial trait (Mendelian inheritance in man 107910), which has been associated with a high rate of conversion of serum C_{19} to C_{18} steroids (5, 6). Increased extraglandular aromatization was first reported in a boy with prepubertal gynecomastia in 1977 (7), then in five members of an African-American kindred in 1985 (8). Interestingly, idiopathic pubertal gynecomastia has been associated with an elevated ratio of serum estrogen to androgen levels during early puberty (9), and excessive aromatase activity in cultured pubic skin fibroblasts from these patients with gynecomastia (10).

Aromatase, also known as estrogen synthetase, is the key enzyme in estrogen biosynthesis. It is an enzymatic complex composed by the aromatase cytochrome P450 (P450arom) and the flavoprotein NADPH-P450 reductase, which catalyzes the conversion of androgens to estrogens (11). Cytochrome P450arom, like the other heme-binding P450 isoforms, is a highly conserved, ancient gene, that is widely expressed in a variety of species in a complex, tissue-specific manner. The human P450arom gene contains 10 exons and is located on chromosome 15q21, an area syntenic to mouse chromosome 9, which harbors a cluster of P450 genes (12–14). It is expressed in the placenta, ovary, testis, brain, skin fibroblasts, adipocytes, normal breast and breast cancer epithelial and stromal cells, and a number of fetal tissues, including liver, brain, and intestine (11). Tissue-specific regulation of the P450arom messenger ribonucleic acid

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(mRNA) is ensured by alternative splicing of exon 1 and part of exon 2, driven by at least five major promoter regions located in the 5′-untranslated end of the gene (15–18). A number of additional minor cDNA species have been identified in various tissues, although their functional significance remains uncertain (11, 15). Both sequences with enhancing and silencing function as well as known responsive elements to a variety of transcription factors have been identified in the regulatory region upstream of the P450arom gene (19–22). Because of its complex regulation of function, the 5′-end of the P450arom gene has long been considered a candidate for disorders in which inappropriate aromatase activity is encountered. Indeed, the 5′-end splicing products of the P450arom gene in mammary neoplastic and human myeloid leukemia cells differ from those in the normal breast or represent new variants, respectively (16, 23, 24). Similarly, the study of uterine, ovarian, and testicular tumors of varying types and grades of malignancy suggested that alternative promoter use was associated with the mechanisms underlying neoplastic transformation (16, 25, 26). Increased aromatization is the mechanism behind the henny feathering trait in an animal model, the Sebright bantam chicken (27, 28).

In the present study, we investigated a kindred with several members expressing clinical and biochemical evidence of aromatase excess. We found increased aromatase activity in cell lines established from the patients and identified a novel exon 1 of the P450arom gene that was uniquely transcribed in the cells from affected individuals.

Subjects and Methods

Subjects

The propositus is a member of a large pedigree (Fig. 1A). He, his sister, father, and mother were seen at the NIH Clinical Center Pediatric Endocrinology clinic under a protocol approved by the institutional review board of the NICHD for the investigation of pediatric endocrine disorders (94-CH-102). The proband, currently a 12-yr-old boy, had presented to one of the authors (D.D.) at the age of 9 yr with gynecomastia and accelerated growth. At that time, his high resolution peripheral blood lymphocyte (PBL) karyotype was 46,XY; imaging studies failed to show any tumors; his bone age was 13.5 yr; he had elevated

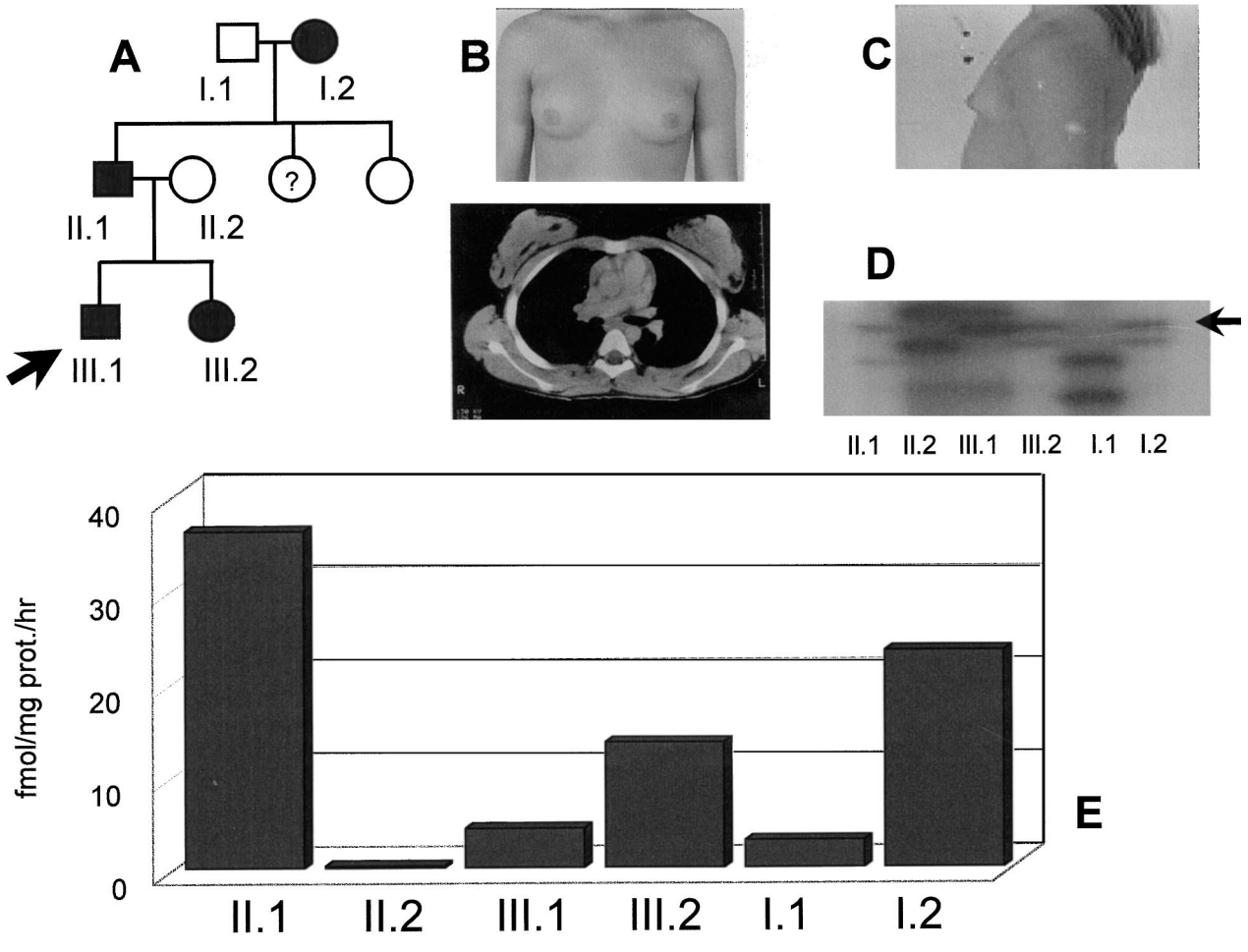

FIG. 1. A, A three-generation kindred with the aromatase excess syndrome; the propositus, a 9-yr-old boy with gynecomastia, is indicated by the *arrow*. His sister had isosexual precocious puberty, his father had peripubertal gynecomastia, and his grandmother had macromastia. B, The breast tissue of the propositus was well formed (*upper panel*) and homogeneous [*lower panel*: magnetic resonance imaging (MRI) of the chest]. C, The breast of the sister of the propositus, a 7.5-yr-old girl, is shown. D, The aromatase excess syndrome segregated with a tetranucleotide repeat polymorphism from the P450arom gene; the 174-bp allele shared by all the affected members of the pedigree is indicated by the *arrow*. E, Aromatase activity measured in EBV-transformed lymphocytes from the patients by [^{3}H]A to [^{3}H]E_1 conversion and expressed as femtomoles per mg protein/h.

estradiol [E_2; 18 pg/mL (66.1 pmol/L)] and estrone [E_1; 69 ng/dL (2546 pmol/L)], prepubertal testosterone [T; 10 ng/dL (0.36 nmol/L)], and undetectable hCG levels. The patient was first seen at the NIH at the age of 10 yr. His examination was remarkable for a height and weight appropriate for a 13-yr-old boy, well formed and homogeneous breasts bilaterally at Tanner pubertal stage IV (TS-IV; Fig. 1B), pubic hair at TS-III, and testicular volume of 4.5 mL bilaterally. The propositus' sister, at age 7.5 yr had height and weight appropriate for 9.5- and 12-yr-old girls, respectively; breast development and pubic hair at TS-III with absence of clitoromegaly; and a normal ano-genital ratio (0.35; Fig. 1C). The father of the two children, a 35-yr-old man, had a height of 165.2 cm and a weight of 86.5 kg (3rd and 90th percentile, respectively). His examination revealed a normally masculinized male with a relatively small testicular volume (15 mL bilaterally). At age 15 yr, he had undergone bilateral mastectomy for severe gynecomastia, first noted at age 11 yr, at which time he entered puberty. He had no impotence or decreased libido; he refused semen evaluation. He and his wife had no other children. The mother of the children was 37 yr old and had a normal physical examination, gynecological evaluation, and history. The paternal grandmother reportedly had macromastia; a paternal aunt was 31 yr old and healthy, whereas another aunt was 23 yr old and had menstrual irregularities. Maternal family history was unremarkable.

Diagnostic evaluation and treatment

Magnetic resonance imaging scans of the head, chest, abdomen, and pelvis were obtained, and bone age was determined in both children. The testes of the propositus and the ovaries of his sister were evaluated by sonography. In the two children, baseline and ACTH (250 μg, iv)-stimulated levels of adrenal steroid hormones and their precursors, and baseline and GnRH (100 μg, iv)-stimulated levels of gonadotropins and gonadal steroids were also determined. The propositus and his father also had a short (1500 U, im, and blood sampling at 48 h) and a long (100 U/kg, im, and blood sampling at 12, 24, 48, 72, and 96 h) hCG stimulation test, as described previously (29–31). Final height prediction was obtained using the Bayley-Pinneau method (32, 33). Both children were started on treatment with an aromatase inhibitor, testolactone (40 mg/kg·day, orally, divided into three daily doses) and a GnRH analog (histrelin acetate, 10–15 μg/kg·dose, as a single sc injection each evening), as previously described (34, 35). For the last 3 yr, the propositus and his sister have been followed with biannual physical examinations, GnRH stimulation tests, bone age evaluations, and determination of serum estrogen levels.

For hormone assays, blood samples were centrifuged immediately after collection, and serum was stored at −20 C until assayed. All hormones were measured by RIA (Hazelton Biotechnologies, Vienna, VA), as previously described (34, 35).

Cell lines, aromatase assay, and immunocytochemistry

PBL were extracted by the Ficoll method from the propositus, his sister, father, mother, and paternal grandparents; 5×10^6 of these cells were used for permanent lymphoblastoid cell line establishment by transformation with the Epstein-Barr virus (EBV), as previously described (36). The remaining cells were frozen in liquid nitrogen and stored at −70 C. A skin biopsy was performed after local anesthesia in the dorsal forearm area, and fibroblasts were grown by standard methods from three individuals: the father, the mother, and their daughter. Aromatase activity was measured by Δ^4-androstenedione (A) to E_1 conversion by primary cultures of skin fibroblasts frozen and regrown only once, PBLs, and EBV-transformed lymphoblasts (EBV-tL), and expressed as femtomols of estrogen per mg protein/h, as previously described (37, 38) The assays were performed twice, and the mean was calculated. Briefly, cell cultures were washed with Hanks' solution before the addition of 1 ml assay medium containing approximately 0.3 μCi 1β-[^{3}H]A and unlabeled substrate. The culture plates were then placed in an incubator (5% CO_2) at 37 C. After 2 h, the medium was transferred to a test tube, and 2 mL chloroform were added. The unconverted substrate and steroid products were extracted into the organic phase. An aliquot of 0.7 mL of the aqueous phase was treated with 2.5% activated, dextran-coated charcoal suspension to remove residual steroids. Tritiated water (^3H_2O) formed during the aromatization reaction was measured by counting the radioactivity in the supernatant.

Unfixed frozen sections of the breast tissue excised from the propositus during reductive mammoplasty were cut into 5-μm thick sections and mounted on slides coated with chrome-alum gel, which were processed and covered with monoclonal mouse antiaromatase antibody prepared against human placental aromatase (provided by E. Simpson, University of Texas Southwestern Medical Center, Dallas, TX), as recently described (39).

DNA, mRNA, and protein studies

Genomic DNA was prepared from the patients and normal controls, as previously described (36, 40, 41). PCR amplification of the P450arom gene was performed with intronic primers previously reported (15, 16, 42, 43) or synthesized from the known untranslated sequences upstream of the P450arom gene (13, 15, 16, 19, 21). The products of these reactions were run on ethidium bromide-stained 1% agarose gels and were found to have the expected size, excluding major gene deletions or rearrangements (data not shown). Single strand conformation polymorphism analysis was performed as previously described (41). Direct and asymmetric PCR sequencing was performed as we previously reported (40, 41), using either the fmol sequencing method (Promega Corp., Madison, MI) or the Sequenase version 2.0 kit (U.S. Biochemical Corp., Cleveland, OH).

cDNA synthesis was accomplished from total RNA extracted from fibroblasts, PBL, EBV-tL of the patients and two normal control cell lines, and the breast tissue of the propositus (methods described in Refs. 40 and 41), with the antisense primer 24 (15) and the RT-PCR kit (Boehringer Mannheim, Mannheim, Germany). The sense primers 1a, 1b, 1c, and 1d and the antisense primer 2d (sequences listed in Ref. 16) were used for the subsequent PCR amplification of the cDNA synthesized from cells from patients and normal controls and from the breast tissue of the propositus. The conditions of the reactions have been previously reported (16).

Rapid amplification of cDNA ends (RACE) was performed after mRNA extraction from EBV-tL (Clontech Laboratories, Palo Alto, CA) using the Marathon cDNA amplification kit (Clontech Laboratories). The sequences of the primers used for this reaction have been published (15, 16). Identification of the cDNA products was performed by cloning into the PCR-II vector, using the TA cloning kit (Invitrogen, San Diego, CA), and subsequent plasmid sequencing, or after a second round of PCR amplification and asymmetric PCR sequencing, using nested primers, as previously described (40, 41). Several PCR products and plasmids were also sequenced with the use of the ABI PRISM dye terminator reaction kit (Perkin-Elmer, Norwalk, CT) in an ABI PRISM 377 automatic DNA sequencer (Perkin-Elmer).

Northern blot analysis was performed after the identification of a novel promoter in affected subjects. The multiple tissue Northern blot (Clontech Laboratories) kit was used for this purpose. A 25-nucleotide-long DNA probe was synthesized and end labeled with the use of T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and [γ-^{32}P]deoxy-ATP, as previously described (36).

Western blot analysis was performed with protein extracted from EBV-tL derived from the patients and normal controls, as previously described (40). Protein lysates were resolved by 8% SDS-PAGE, transferred to nitrocellulose membrane (Novex, San Diego, CA) and incubated with rabbit polyclonal antiserum, which was raised against human placental aromatase, purified, and characterized by Bellino *et al.* (44). This antibody recognizes a single band of 53,000 kDa, the approximate molecular mass of P450arom.

Genetic linkage analysis was performed with a polymorphic marker (tetranucleotide repeat) located within the human P450arom gene on chromosome 15q21.1 (45). Genomic DNA from the propositus, his sister, his parents, and his paternal grandparents was amplified by PCR (conditions of the reaction and primer sequences given in Ref. 45), in which one of the primers was end labeled with [γ-^{32}P]deoxy-ATP, as previously described (36). The reactions were run on an acrylamide denaturing gel, which was then subjected to autoradiography. Linkage analysis was performed with the LINKAGE (version 5.1) package of computer programs (36, 46), assuming a dominant model of inheritance, 100% penetrance in both sexes, and the reported allele frequencies (45).

Ribonuclease protection assay (RPA) was performed with the use of a kit (RPA II, Ambion, Austin, TX), a plasmid constructed in our laboratory following the amplification of normal human genomic DNA

with the 1c and 2d primers of the P450arom gene (sequences given in Ref. 16), unidirectional subcloning into a TA vector (Invitrogen), and total RNA extracted from the proband's EBV-tL cell line and two normal control EBV-tL cell lines. The protected fragments were run in a 6% acrylamide gel and autoradiographed.

Results

Clinical evaluation and treatment

Imaging studies were negative for any tumor in either child. Pelvic ultrasound of the propositus' sister revealed prepubertal ovaries (<0.5 mL bilaterally), but endometrial thickening compatible with estrogen stimulation. Table 1 summarizes the remaining findings. In both children, skeletal age was advanced. Accordingly, the GnRH stimulation test results showed that both children were in central precocious puberty. They also had high serum E_2 and, in particular, E_1 levels. Their father had high E_2 and E_1 levels; his T and dihydrotestosterone levels were in the low normal range at baseline and responded normally to stimulation with hCG. Baseline dehydroepiandrosterone sulfate levels were low for pubertal stage in both children. The high baseline E_2 and E_1 levels did not increase 1 h after ACTH stimulation; hCG stimulation, on the other hand, produced significant increases in both hormones in the propositus and his father. Their response pattern, however, differed from that previously reported for normal males (29, 30, 48), in that there was no immediate increase in the two hormones (data not shown). Thus, E_2 and E_1 increased after the first 24 h, after a brisk elevation of A and T levels. These data and the relatively lower baseline A and dehydroepiandrosterone sulfate levels were consistent with extraglandular aromatization of C_{19} to C_{18} steroids in the propositus, his sister, and their father and excluded other abnormalities of adrenal or gonadal steroidogenesis. Because the children's final height prediction was suboptimal (Table 2), they were both started

TABLE 1. Laboratory evaluation of a kindred with the aromatase excess syndrome

	Propositus		Sister		Father	
Symptom	Prepubertal gynecomastia		Precocious puberty		Peripubertal gynecomastia	
Age (yr)	10		7 ½		35	
Skeletal age (yr)	14		11			
Testosterone (ng/dL)	23		<20		234	
Δ^4-Androstenedione (ng/dL)	9.1		<6.78		26.8	
Estradiol (pg/mL)	8.6[a]		8.9[a]		60[a]	
Estrone (pg/mL)	43.7[b]		62.2[b]		82[b]	
GnRH stimulation test	Basal	Peak	Basal	Peak		
LH (mIU/mL)	<3.7	13.9[c]	<3.7	21.3[c]	NA	
FSH (mIU/mL)	<3.4	6.4	<3.4	14.7	NA	
ACTH stimulation test[d]	0 min	60 min	0 min	60 min		
17-Hydroxyprogesterone (ng/dL)	<77	208	<83	170	NA	
Δ^4-Androstenedione (ng/dL)	<9.2	11.2	<13	28.8	NA	
Estrone (pg/mL)	43.7	84.8	62.2	64.1	NA	
DHEA-S (μg/dL)	119	89[e]	32.4	37.1[e]	NA	
24-h hCG stimulation test	0 min	24 h			0 min	24 h
Testosterone (ng/dL)	23	90.1			234	310
Dihydrotestosterone (ng/dL)	8.5	20.1			40	34.5
Estradiol (pg/mL)	8.6[a]	13.1[a]			60[a]	61[a]
Estrone (pg/mL)	43.7[b]	108[b]			82[b]	126[b]
DHEA-S (μg/dL)	119	NA[e]			175	NA[e]
96-h hCG stimulation test	48 h	96 h			48 h	96 h
Testosterone (ng/dL)	85.2	226			761	716
Dihydrotestosterone (ng/dL)	10	15.6			56.3	58.5
Δ^4-Androstenedione (ng/dL)	28.7	31.6			63	86
Estradiol (pg/mL)	9[a]	29.1[a]			184[a]	132[a]
Estrone (pg/mL)	52.5[b]	61.6[b]			163[b]	153[b]

To convert values of testosterone to nanomoles per L, multiply by 0.0288; of Δ^4-androstenedione to picomoles per L, multiply by 0.0286; of estradiol to picomoles per L, multiply by 0.272; of estrone to picomoles per L, multiply by 0.270; of cortisol to nanomoles per L, multiply by 0.0363; of 17-hydroxypregnenolone to picomoles per L, multiply by 0.0333; of 17-hydroxyprogesterone to picomoles per L, multiply by 0.0317; of dihydrotestosterone to picomoles per L, multiply by 0.0290. DHEA-S, Dehydroepiandiosterone sulfate; NA, not available.

[a] Estradiol values near or slightly exceeding the upper normal range for this age and gender (29–35, 48).

[b] Estrone values significantly above the corresponding normal range for age and gender (29–31, 35, 48).

[c] LH response to GnRH stimulation exceeds that of FSH, indicating that both children at the time of their first evaluation at the NIH were in central precocious puberty according to the criteria set by Oerter *et al.* (49).

[d] The levels of adrenal steroid hormones and their precursors in response to ACTH stimulation are within the expected normal ranges (50).

[e] These are remarkably low DHEA-S levels for the stages of puberty of these children. This may reflect the increased (extragonadal) conversion of this steroid to estrogens. This is further supported by the decrease in DHEA-S levels in response to ACTH, which, in contrast, increased estrone levels in the male propositus. The DHEA-S levels in response to hCG stimulation are not available.

TABLE 2. Aromatase excess syndrome: response to an aromatase inhibitor (testolactone) and GnRH analog (Histrelin) therapy

	Propositus	Sister
Growth SD score[a]		
Before treatment	1.9	1.55
1st yr	1.5	1.5
3rd yr	0.5	0.5
ΔBA/ΔCA[b]		
Before treatment	2	N/A
1st yr	0.5	0
3rd yr	0.3	0.3
Predicted ht (cm)[c]		
Before treatment	165	150
1st yr	167	154
3rd yr	167	160
Estradiol (pg/mL)[d]		
Before treatment	8.6	8.9
1st yr	Undetectable	Undetectable
3rd yr	Undetectable	Undetectable
Estrone (pg/mL)[e]		
Before treatment	43.7	62.2
1st yr	24.4	53.6
3rd yr	NA	25

[a] SD score for age-appropriate height, as previously described (47).
[b] Rate of bone age (BA) advance over chronological age (CA) change, as previously described (35, 47, 49).
[c] Predicted height estimated by the Bayley-Pinneau height prediction method (32, 33).
[d] To convert values of estradiol to picomoles per L, multiply by 0.272; normal ranges for prepubertal ages are 5–11 pg/mL for males and 5–20 pg/mL for females.
[e] To convert values of estrone to picomoles per L, multiply by 0.270; normal ranges for prepubertal ages are 5–17 pg/mL for males and 4–29 pg/mL for females.

on treatment with a GnRH analog (histrelin acetate; 10–15 μg/kg, sc, daily), and 6 months later an aromatase inhibitor (testolactone; 40 mg/kg·day, orally) was added, according to similar regimens used in children with McCune-Albright syndrome and other disorders associated with precocious puberty (34, 35). This treatment was successful in suppressing central precocious puberty and arresting skeletal age as well as decreasing serum estrogen levels in both children, as the most recent (3 yr into the treatment) visit showed (Table 2). Gynecomastia in the propositus was treated by bilateral reductive mammoplasty.

Aromatase activity, immunocytochemistry, and Western analysis

No aromatase activity was detected in nontransformed PBL from the patients and normal controls, although aromatase gene transcripts were detectable (see below) (50a). Increased enzyme activity was, however, found in the cultured skin fibroblasts and EBV-tL derived from clinically affected members of the family. The results are shown in Table 3 and Fig. 1E.

Immunoreactivity was observed in the cytoplasm of epithelial cells lining the glandular ducts of the breast tissue excised from the propositus (Fig. 2, *upper panel*, a and c). Surrounding adipose fibroblasts were also positively stained, but not as strongly. When the antiaromatase antibody was replaced by an irrelevant mouse IgG, no immunoreactivity was seen in the same tissue (Fig. 2, *upper panel*, b and d).

TABLE 3. Aromatase activity in patient-derived cell lines (fentomoles of estrogen per mg protein/h)

	Skin fibroblasts		EBV-transformed lymphocytes
	1st h	2nd h	
Proband[a]	NA	NA	4.22
Sister[a]	84.22	85.8	13.4
Mother	<2	<2	<2[b]
Father[a]	54.93	41.2	53.29
Paternal grandfather	NA	NA	2.96
Paternal grandmother[a]	NA	NA	60.46
Control cell line	<2	<2[b]	2.9

NA, Not available.
[a] These individuals had biochemical or clinical evidence of aromatase excess.
[b] Although there was some aromatization observed, levels below 2 fmol/mgprotein were not measurable.

A polyclonal anti-P450arom antibody was used in Western analysis of protein lysates from EBV-tL derived from the four members of the family with the aromatase excess syndrome. Indeed, the 53K band of the P450arom protein was detected in all four cell lines, but not in four other EBV-tL cell lines established from age- and gender-matched controls (Fig. 2, *lower panel*).

Linkage analysis

To investigate whether the responsible genetic defect in this kindred was located in the P450arom gene, a polymorphic, tetranucleotide [$(TTTA)_n$] repeat marker at nucleotide position 682 of this gene (12, 13, 45) was used. Indeed, the marker cosegregated with aromatase excess in affected members of this family (Fig. 1D). With a model of autosomal dominant inheritance, the allele frequencies previously reported (45), and a 100% penetrance for the biochemical expression of increased aromatase activity, the combined logarithm of odds score was 0.6, with no observed recombinations [recombination fraction (θ) = 0.00]. This represented a chance of approximately 4.6 to 1 that the genetic defect in this family was indeed located in the P450arom gene locus on chromosome 15q21.1.

Sequence analysis of the 5′-end of the P450arom gene

As the genetic defect in this family was likely to be in the P450arom gene, we tested the hypothesis that the former might represent a sequence change in the known regulatory regions of this gene's promoter region, in particular those that have been found to have a silencing function on the expression of the gene (19). Single strand conformation polymorphism analysis of the known genomic sequences of the P450arom gene (15, 16, 19, 42) in the patients showed no pattern alterations that segregated with the disease, or that were not present in normal control DNA specimens (data not shown). Subsequent sequencing of the genomic DNA surrounding the placental (exon 1a or PI.I) and ovarian (exon 1d or PII) promoters (sequences reported in Refs. 13 and 19, respectively) revealed changes that did not segregate with the disease or were present in normal control DNA samples. The most extensive alteration was that at nucleotide 482 of the ovarian promoter region. The reported sequence at this location (19) reads (the numbers refer to cDNA position, the sequences altered in comparison to the published

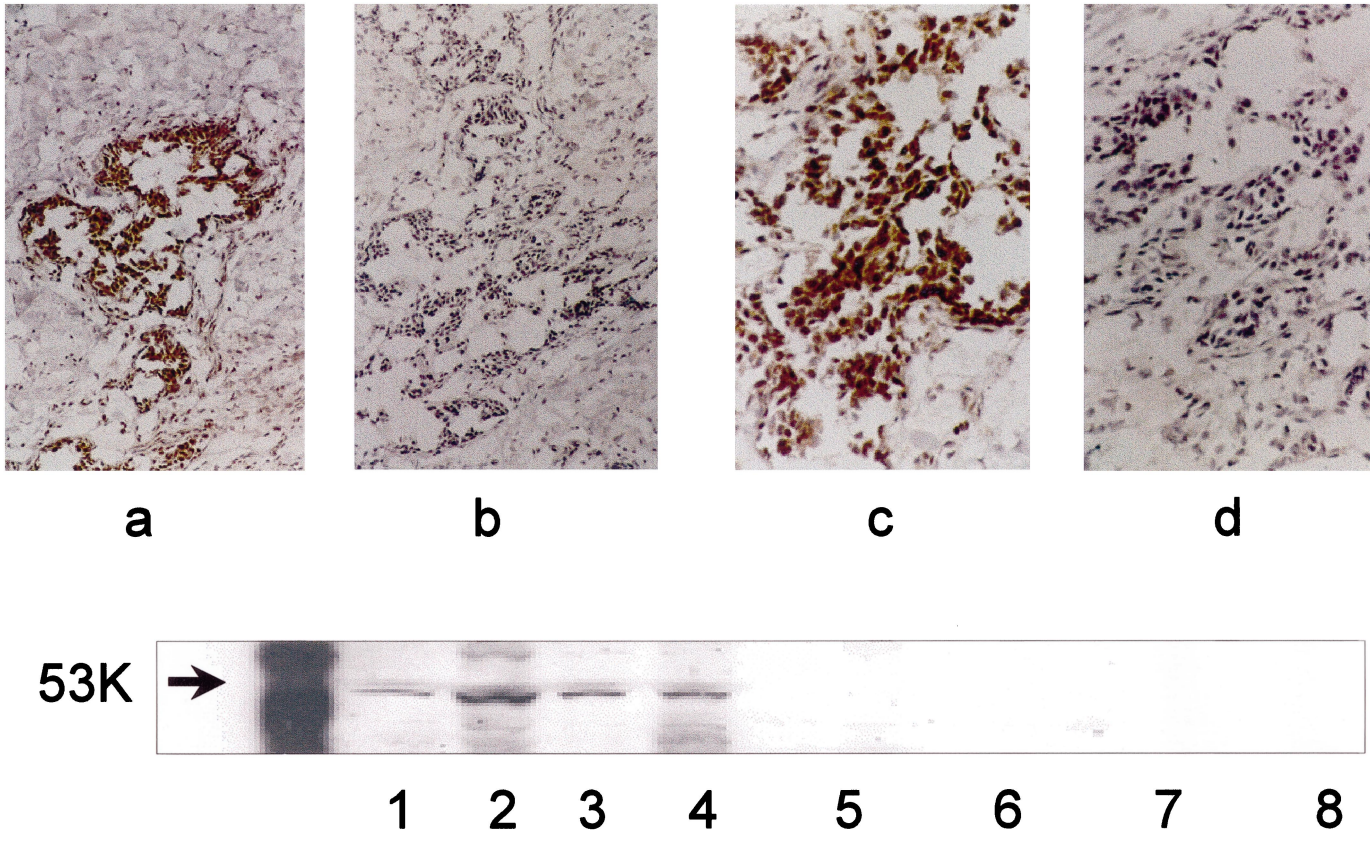

FIG. 2. P450arom protein studies in the aromatase excess syndrome. *Upper panel*, Immunohistochemistry of the propositus' breast tissue using an antiaromatase antibody (a and c) and its negative control (b and d). Magnification: a and b, ×200; c and d, ×400. Immunoreactivity is present in the epithelial cells lining the well formed glandular ducts of the breast; also, there is staining, albeit not as strong, of the surrounding stroma adipose fibroblasts. *Lower panel*, Western blot analysis of protein lysates from EBV-transformed lymphocytes from affected family members (lanes 1–4) and unaffected controls (lanes 5–8).

version are underlined). (478)5′-TTGATCCAATG-3′(488), whereas the following sequence was found in all members of our family and in 20 normal control DNA samples (478)5′-TTGCCTAAATG-3′(488) (data not shown). Similarly, a nucleotide substitution was present 15 bp upstream from the TATA box of the placental promoter in all our patients and in 20 control DNA specimens; the reported sequence (13) was [−45]5′-GACAATAAATG-3′[−35], and the sequence found was [−45]5′-GACAACAAATG-3′[-35] identical to that reported in Ref. 16 (data not shown).

These data showed that there were no disease-causing alterations in the studied promoter regions of the P450arom gene. The latter, however, cover a genomic area that is approximately 10,000 bp long, and the DNA clones containing the various promoters do not, currently, overlap; thus, a gap of unknown size is present in the genomic sequence of the 5′-end of the P450arom gene, which could harbor the defect in this family (11, 16).

Studies of P450arom mRNA: identification of a novel exon 1

To define the type of P450arom mRNA expressed in the cell lines of our patients, we performed RT-PCR analysis with known primers of the various promoters for this gene,[1] which showed that the proximal promoters c and d (14) or PI.3 and PII (11) (Fig. 3A) were present in P450arom cDNA cloned from PBL, EBV-tL, and cultured skin fibroblasts from the patients and breast tissue from the propositus (data not shown). However, the corresponding normal control cells also contained the same cDNA species. The relative amount of P450arom cDNA present in PBL and EBV-tL cell lines from normal controls was lower than that in our patients (data not shown), consistent with their lower aromatase activity, as mentioned above. Nevertheless, these data confirmed earlier evidence that the P450arom gene was weakly expressed in peripheral leukocytes of normal individuals (51) (Vottero, A., L. Kirschner, and C. Stratakis, unpublished data).

As RT-PCR analysis could only identify changes in already known cDNA species of the P450arom gene, we proceeded in cloning novel 5′- transcripts of this gene by RACE from cDNA pools established from EBV-tL derived from our patients. Various new transcripts were identified in both af-

[1] The nomenclature of the various aromatase first exons and corresponding promoters is unsettled. In this manuscript, we use the codes established by Harada *et al.* (16); exons 1a, 1b, Ic, and Id correspond to promoters P1.1 (expressed in placenta), P1.4 (adipose tissue), PI.3 (adipose tissue), and PII (gonads) described by Simpson *et al.* (11), respectively.

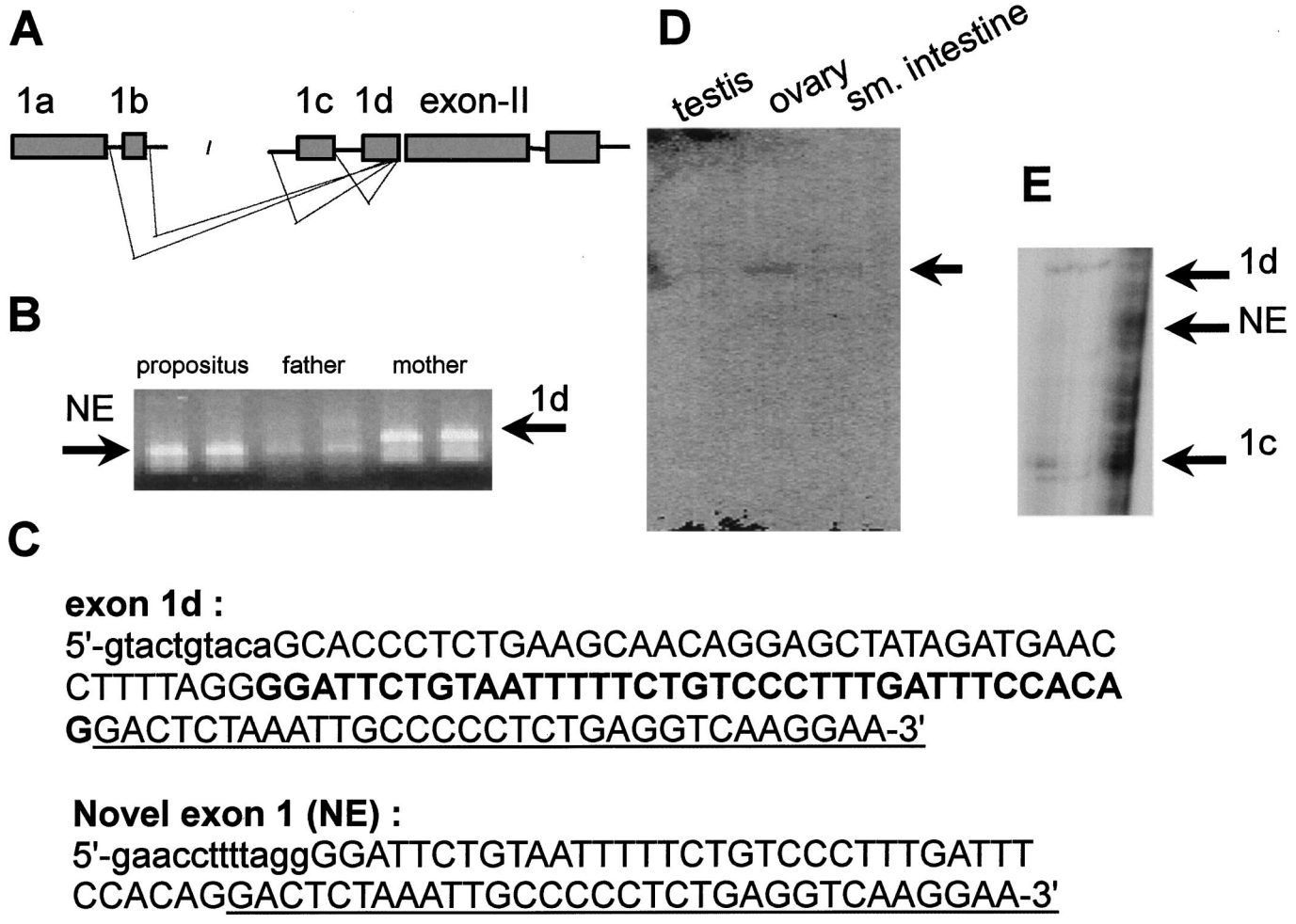

FIG. 3. mRNA studies in the aromatase excess syndrome showing that a novel exon 1 is preferentially expressed in the cell lines from the affected members of our kindred. A, The diagram indicates the main exon 1 sequences, using the terminology established by Harada *et al.* (16); between exon regions 1b and 1c, a large gap of unknown length and sequence exists. B, cDNA products of the P450arom gene cloned by RACE, showing a novel exon 1 (NE) in the propositus and his father, and exon 1d in his unaffected mother. C, Northern blot analysis with a cDNA probe from the novel exon 1 (NE) sequence identified the P450arom mRNA in polyadenylated RNA from the ovary, testis, and small intestine. D, The sequences of exon 1d and NE, cloned, respectively, from the P450arom transcripts in cells derived from the unaffected and affected members of our kindred with the aromatase excess syndrome. The NE sequence, in *bold letters*, is included in exon 1d; the exon 2 sequence is *underlined*. *Uppercase letters* represent translated sequences, and *lowercase letters* represent intronic sequences. E, RPA. The lane to the *right* is RNA from the propositus EBV-tL cell line with the protected NE and Ic exons. The other two lanes contain the same amount of total RNA from normal control EBV-tL cell lines; the latter express exons 1c and 1d. The P450arom gene is significantly overexpressed in the patient's cells.

fected and unaffected members of this kindred (data not shown). One of these cDNA species represented over 90% of the transcripts identified in the propositus and his affected sister and father, but was present in less than 10% of the clones from the unaffected mother (Fig. 3). This novel exon 1 of the P450arom cDNA was present in transcript pools of both PBL and EBV-tL from the affected members of our kindred, but not in those established from normal control PBL or EBV-tL; instead, the d (PII promoter) was cloned from the latter (Fig. 3B). The sequence of the novel exon 1 is presented in Fig. 3C and compared to that of exon 1d. This novel transcript was also present in the fibroblasts of the affected members of the family and the breast tissue of the propositus (data not shown). When a DNA probe was made with the novel sequence, it hybridized with normal control human polyadenylated RNA primarily from the ovary and secondarily from the testis and small intestine, identifying a band of the expected P450arom mRNA size (~3.4 kilobases; Fig. 3D). The RPA identified the new cloned product in mRNA from the cell line of the propositus and excluded the possibility that this was an artifact of the RACE procedure (Fig. 3E); overexpression of the P450arom gene was confirmed by the same assay in this cell line *vs.* control transfer RNA (data not shown). Sequencing of the genomic DNA around the splicing junction of the 5′-end of exon II of the P450arom gene in our patients did not show any mutations that could be responsible for altered splicing on this side (data not shown). However, as the entire sequence of the 5′ end of the P450arom gene is unknown, it is possible that the genetic defect(s) affecting P450arom mRNA splicing in this family is located within the gap that currently separates the known promoter regions of this gene (Fig. 3A).

Discussion

In the present study, a boy with prepubertal gynecomastia and his sister with isosexual precocious puberty had elevated serum estrogen levels. The source appeared to be nongonadal conversion of adrenal androgens (A and T) to estrogens (E_1 and E_2, respectively). Indeed, symptoms were manifested in both children around the time of or shortly after adrenarche. Although increased extraglandular aromatization of C_{19} steroids has been reported as a cause of gynecomastia twice before (7, 8), this is the first time that the aromatase excess syndrome has been associated with clinical manifestations in females. The first patient, reported by Hemsell *et al.* (7), was a 10 7/12-yr-old boy, who presented with a 2-yr history of gynecomastia and was in puberty at the time of his evaluation. Berkovitz *et al.* (8) subsequently reported five affected males, all members of the same family, who developed gynecomastia at an early age (10–11 yr). Because no females were affected in this kindred, and the disorder was present in a maternal uncle, it was speculated, as in yet another family (52), that aromatase excess was inherited in an X-linked manner, although the P450arom gene is on an autosomal and not a sex chromosome (chromosome 15q). Thus, autosomal dominant inheritance with sex-limited expression could not be excluded in these pedigrees. In our kindred, the aromatase excess syndrome was transmitted to the two children from their father, who had peripubertal gynecomastia, elevated serum estrogen levels as an adult, and increased aromatase activity *in vitro*. The male to male transmission and expression of the disease in a female are compatible with autosomal dominant inheritance in this family, suggesting that the aromatase excess syndrome may be a genetically heterogeneous disorder.

Despite increased estrogen levels, males with aromatase excess appear to be fertile (6). Indeed, the father of the propositus in our family was fertile and had normal libido despite a small testicular volume (15 mL bilaterally), and a testosterone level that was in the lower end of the normal range. Conversely, a male patient with aromatase deficiency had a large testicular volume (>25 mL bilaterally) and excessive testosterone levels, probably due to excess gonadotropins (53). In the aromatase excess syndrome, mild suppression of testicular growth and Leydig cell function probably reflect a direct estrogen negative feedback effect on pituitary gonadotropins (54–56). In general, the inhibitory effects of estrogen on reproductive function appear to be milder in males with this syndrome than in patients receiving exogenous estrogens or with estrogen-secreting tumors, probably because serum E_2 and/or E_1 levels in the former are lower than those in the latter.

Increased aromatase activity in females appears to be associated with isosexual precocity at a young age and, very likely, varying degrees of macromastia in adulthood. These findings are consistent with the major functions of estrogens, the growth and maturation of female secondary sexual characteristics, including the breast (56). Sexual function, fertility, and menstrual periods were normal in the paternal grandmother of our patients, who had increased aromatase activity *in vitro*. These findings should be contrasted with the dramatic effects of aromatase deficiency in female patients, which include virilization at birth, followed by hypergonadotropic hypogonadism, and sexual infantilism at puberty (53, 56–59).

Both the boy and the girl with the aromatase excess syndrome had greatly advanced bone age; their degree of skeletal maturity was disproportionate to their height (chronological age < height age < skeletal age) despite accelerated growth. This situation is seen frequently in untreated precocious puberty, when the rate of skeletal maturation overcomes linear growth (56). The short stature of their father was also indicative of the potent effect on estrogens on accelerating premature epiphyseal closure. These findings are in sharp contrast with the tall stature and delayed epiphyseal closure of patients with aromatase deficiency (53, 57–59) or estrogen resistance (60, 61). Because of their predicted final height, our patients were treated with an aromatase inhibitor and a GnRH analog; although the 3-yr evaluation indicated an improvement in their final height prediction, the final outcome of this therapy remains to be seen.

In our kindred, the disorder cosegregated with a polymorphism of the P450arom gene, indicating that it was likely that the responsible genetic defect was located in this gene. Aromatase assays in fibroblast and EBV-tL cell lines established from the patients were in accordance with the clinical and biochemical data of aromatase excess, although they did not indicate whether other tissues were involved in the excessive aromatization. Aromatase activity on EBV-tL appeared to be a useful diagnostic tool in our patients; although P450arom mRNA was present in their peripheral leukocytes, aromatization *in vitro* was measurable only after transformation of the latter. Although the *in vivo* source of estrogen could be determined only with an infusion study, the hCG and ACTH stimulation tests and the 3-yr follow-up evaluation of our patients suggested that most of their aromatization took place in extragonadal tissues, which included the breast and, perhaps, the adrenal glands.

The P450arom protein in these cell lines had the expected mol wt, but was present in increased amounts compared to that in controls. It was likely that the situation in EBV-tL mirrored the pathogenesis of the disorder; an abnormality that led to overexpression of the gene in the tissues of our patients was probably further unmasked in their transformed lymphocytes.

Interestingly, the breast of our patient showed strong P450arom immunostaining in the glandular epithelium; adipose fibroblasts were stained, albeit less strongly, in the surrounding stroma. This pattern is the reverse of that recently observed in a female patient with macromastia and evidence of aromatase excess (62), but is consistent with other findings in cases of male patients with gynecomastia, where little or no stroma immunostaining was observed (63). We speculate that the distribution of P450arom expression in the breast may be gender, pubertal stage, or fat mass dependent. Males, for example, may have a higher expression in the glandular epithelium than in the surrounding stroma. Our patient was a very thin male, early in puberty (TS-II by testicular size), with a firm TS-IV breast. It is possible that the observed pattern of expression would have been different if there was lipomastia or if the breast was examined at a later

stage. Finally, the different pattern of expression of the P450arom gene in our patient may just be due to the disease process, driven by the genetic defect responsible for the disorder in his family.

Due to its complex alternative splicing, the 5′-end of the P450arom gene has been considered an obvious candidate for disorders with increased aromatase activity (11, 16, 23, 24). A preliminary screen of the coding region of the gene and its two known promoter regions did not show any alterations. However, after a search for novel 5′-untranslated ends of the P450 aromatase gene by the RACE method, a novel exon 1 of the P450arom cDNA was cloned from the cell lines that demonstrated aromatase excess. This sequence appeared to be expressed only in gonadal tissues and the small intestine normally. In patients with the aromatase excess syndrome, however, it was present in their cultured skin fibroblasts, lymphocytes, and breast tissue. The cloned sequence is located in the genomic sequence neighboring the 5′-end of exon 2 of the P450arom gene and is a novel product of alternative splicing occurring in this area of the P450arom transcript. It is possible that pathological expression is present in other tissues as well, in particular in the adrenal glands, as symptoms of hyperestrogenemia in our patients appeared at the time of adrenarche. This is also supported by the modest effect that complete gonadal suppression had on E_1 levels in these patients over a period of 3 yr.

It is likely that the genetic defect in the aromatase excess syndrome is a sequence change in the P450arom promoter region. The latter either causes a splicing error or affects the binding and/or activity of a transcription factor that regulates P450arom mRNA synthesis. Under both of these conditions, the tissue-specific selection of the various promoters available for this gene could be altered in favor of the newly identified cDNA, leading to inappropriate expression of the gene. Analogous is the situation in the disorder known as hereditary persistence of fetal hemoglobin, in which nucleotide substitutions in the region immediately upstream from the transcription start site affect the binding of *trans*-acting proteins involved in the normal developmental repression of γ-hemoglobin gene expression (64). Alternatively, a mutation in a regulatory factor of the P450arom gene may be responsible for increased expression of the P450arom gene in our patients. Under both hypotheses, excess aromatization would take place not just in the tissues studied (skin fibroblasts, lymphocytes, and breast), but also, and perhaps predominantly, in other tissues, including the adrenal glands and, to a lesser degree, the gonads.

We conclude that the familial aromatase excess syndrome is a genetically heterogeneous disorder, which can be inherited in an autosomal dominant manner and lead not only to heterosexual precocity and gynecomastia in males, but also to isosexual precocity and macromastia in females. Aromatase expression can be shown in skin fibroblasts and EBV-transformed lymphocytes from affected patients *in vitro* and appears to be associated with the utilization of a novel exon 1 of the P450arom cDNA. The genetic defect responsible for this condition is likely to be located in the 5′-end of the P450arom gene.

References

1. **Carlson HE.** 1980 Gynecomastia. N Engl J Med. 303:795–799.
2. **Braunstein GD.** 1993 Gynecomastia. N Engl J Med. 328:490–495.
3. **Nuttall FQ.** 1979 Gynecomastia as a physical finding in normal men. J Clin Endocrinol Metab. 48:338–340.
4. **August GP, Chandra R, Hung W.** 1972 Prepubertal male gynecomastia. J Pediatr. 80:259–263.
5. **Ljunberg T.** 1960 Hereditary gynecomastia. Acta Med Scand. 168:371–379.
6. **Wallach EE, Garcia C-R.** 1962 Familial gynecomastia without hypogonadism: a report of three cases in one family. J Clin Endocrinol Metab. 22:1201–1206.
7. **Hemsell DL, Edman CD, Marks JF, Siiteri PK, MacDonald PC.** 1977 Massive extraglandular aromatization of plasma androne stedione resulting in feminization of a prepubertal boy. J Clin Invest. 60:455–464.
8. **Berkovitz GD, Guerami A, Brown TR, MacDonald PC, Migeon CJ.** 1985 Familial gynecomastia with increased extraglandular aromatization of plasma carbon-19 steroids. J Clin Invest. 75:1763–1769.
9. **Moore DC, Schlaepfer LV, Paunier L, Sizonenko PC.** 1984 Hormonal changes during puberty V. Transient pubertal gynecomastia: abnormal androgen-estrogen ratios. J Clin Endocrinol Metab. 58:492–499.
10. **Bulard J, Mowszowicz I, Schaison G.** 1987 Increased aromatase activity in pubic skin fibroblasts from patients with isolated gynecomastia. J Clin Endocrinol Metab. 64:618–623.
11. **Simpson ER, Mahendroo MS, Means GD, et al.** 1994 Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr Rev. 15:342–355.
12. **Chen S, Besman MJ, Sparkes RS, et al.** 1988 Human aromatase: cDNA cloning, Southern blot analysis, and assignment of the gene to chromosome 15. DNA. 7:27–38.
13. **Toda K, Terashima M, Kawamoto M, et al.** 1990 Structural and functional characterization of human aromatase P450 gene. Eur J Biochem. 193:559–565.
14. **Youngblood GL, Nesbitt MN, Payne AH.** 1989 The structural genes encoding P450scc and P450arom are closely linked on mouse chromosome 9. Endocrinology 125:2784–2786.
15. **Mahendroo MS, Mendelson CR, Simpson ER.** 1993 Tissue-specific and hormonally controlled alternative promoters regulate aromatase cytochrome p450 gene expression in human adipose tissue. J Biol Chem. 268:19463–19470.
16. **Harada N, Utsumi T, Takagi Y.** 1993 Tissue-specific expression of the human aromatase cytochrome P-450 gene by alternative use of multiple exons 1 and promoters, and switching of tissue-specific exons 1 in carcinogenesis. Proc Natl Acad Sci USA. 90:11312–11316.
17. **Honda S, Harada N, Takagi Y.** 1994 Novel exon 1 of the aromatase gene specific for aromatase transcripts in human brain. Biochem Biophys Res Commun. 198:1153–1160.
18. **Toda K, Simpson ER, Mendelson CR, Shizuta Y, Kilgore MW.** 1994 Expression of the gene encoding aromatase cytochrome P450 (CYP19) in fetal tissues. Mol Endocrinol. 8:210–217.
19. **Wang J, Chen S.** 1992 Identification of a promoter and a silencer at the 3′-end of the first intron of the human aromatase gene. Mol Endocrinol. 6:1479–1488.
20. **Yamada K, Harada N, Honda S, Takagi Y.** 1995 Regulation of placenta-specific expression of the aromatase cytochrome P-450 gene. J Biol Chem. 270:25064–25069.
21. **Zhao Y, Mendelson CR, Simpson ER.** 1995 Characterization of the sequences of the human CYP19 (aromatase) gene that mediate regulation by glucocorticoids in adipose stromal cells and fetal hepatocytes. Mol Endocrinol. 9:340–349.
22. **Michael DM, Kilgore MW, Morohashi K, Simpson ER.** 1995 Ad4BP/SF1 regulates cyclicAMP-induced transcription from the proximal promoter (PII) of the human aromatase P450 (CYP19) gene in the ovary. J Biol Chem. 270:13561–13566.
23. **Jacob F, Homann D, Seufert J, Schneider D, Kohrle J.** 1995 Expression and regulation of aromatase cytochrome P450 in THP 1 human myeloid leukaemia cells. Mol Cell Endocrinol. 110:27–33.
24. **Harada N, Utsumi T, Takagi Y.** 1995 Molecular and epidemiological analyses of abnormal expression of aromatase in breast cancer. Pharmacogenetics 5: S59–S64.
25. **Bulun SE, Rosenthal IM, Brodie AMH et al.** 1993 Use of tissue-specific promoters in the regulation of aromatase cytochrome P450 gene expression in human testicular and ovarian sex cord tumors, as well as in normal fetal and adult gonads. J Clin Endocrinol Metab. 77:1616–1621.
26. **Bulun SE, Economos K, Miller D, Simpson ER.** 1994 CYP19 (aromatase cytochrome P450) gene expression in human malignant endometrial tumors. J Clin Endocrinol Metab. 79:1831–1834.
27. **George FW, Wilson JD.** 1980 Pathogenesis of the henny feathering trait in the Sebright bantam chicken. J Clin Invest. 66:57–65.
28. **Leshin M, Baron J, George FW, Wilson JD.** 1981 Increased estrogen formation and aromatase activity in fibroblasts cultured from the skin of chickens with the henny feathering trait. J Biol Chem. 256:4341–4344.
29. **Saez JM, Forest MG.** 1979 Kinetics of human chorionic gonadotropin-induced steroidogenic response of the human testis. I. Plasma testosterone: implications for human chorionic gonadotropin stimulation test. J Clin Endocrinol Metab. 49:278–283.

30. **Forest MG, Lecoq A, Saez JM.** 1979 Kinetics of human chorionic gonadotropin-induced steroidogenic response of the human testis. II. Plasma 17α-hydroxyprogesterone, Δ^4-andonestedione, estrone, and 17β-estradiol: evidence for the action of human chorionic gonadotropin on intermediate enzymes implicated in steroid biosynthesis. J Clin Endocrinol Metab. 49:284–291.
31. **Forest MG.** 1984 Maturation of human testicular response to hCG. Ann NY Acad Sci. 438:304–328.
32. **Greulich WW, Pyle SI.** 1959 Radiographic atlas of skeletal development of the hand and wrist. Stanford: Stanford University Press.
33. **Zachman M, Sobradillo B, Frank M, et al.** 1978 Height predictions in normal children and in patients with various pathologic conditions. J Pediatr. 93:749–755.
34. **Oerter KE, Manasco P, Barnes KM, Jones J, Hill S, Cutler GB.** 1991 Adult height in precocious puberty after long-term treatment with deslorelin. J Clin Endocrinol Metab. 73:1235–1240.
35. **Feuillan-Philbrick P, Jones J, Cutler GB.** 1993 Long-term testolactone therapy for precocious puberty in girls with the McCune-Albright syndrome. J Clin Endocrinol Metab. 77:647–651.
36. **Stratakis CA, Carney JA, Lin JP, et al.** 1996 Carney complex, a multiple endocrine neoplasia and familial lentiginosis syndrome: analysis of 11 kindreds and linkage to the short arm of chromosome 2 (2p16). J Clin Invest. 97:599–607.
37. **Brodie AMH, Inkster SE.** 1993 Aromatase in the human testis. J Steroid Biochem Mol Biol. 44:549–555.
38. **Inkster S, Yue W, Brodie A.** 1995 Human testicular aromatase: immunocytochemical and biochemical studies. J Clin Endcorinol Metab. 80:1941–1947.
39. **Lu Q, Nakmura J, Savinov A, et al.** 1996 Expression of aromatase protein and messenger ribonucleic acid in tumor epithelial cells and evidence of functional significance of locally produced estrogen in human breast cancers. Endocrinology 137:3061–3068.
40. **Hurley DM, Accili D, Stratakis CA, et al.** 1991 Point mutation causing a single amino acid substitution in the hormone binding domain of the glucocorticoid receptor in familial glucocosticoid resistance. J Clin Invest. 87:680–686.
41. **Stratakis CA, Orban Z, Burns AL, et al.** 1996 Dideoxyfingerprinting (ddF) analysis of the type $\times$ collagen gene (COL10A1) and identification of a novel mutation (S671P) in a kindred with Schmid metaphyseal chondrodysplasia. Biochem Mol Med. 59:112–117.
42. **Sourdaine P, Parker MG, Telford J, Miller WR.** 1994 Analysis of the aromatase cytochrome P450 gene in human breast cancers. J Mol Endocrinol. 133:331–337.
43. **Jenkins C, Michael D, Mahendroo M, Simpson E.** 1993 Exon-specific Northern analysis and rapid amplification of cDNA ends (RACE) reveal that the proximal promoter II (PII) is responsible for aromatase cytochrome P450 (CYP19) expression in human ovary. Mol Cell Endocrinol. 97:R1–R6.
44. **Bellino FL, Tseng L, Lobo JO.** 1987 Antisera against estrogen synthetase from human placental microsomes. Antibody characterization and cross-reactivity studies in other organs. Mol Cell Endocrinol. 52:143–150.
45. **Polymeropoulos MH, Xiao H, Rath DS, Merril CR.** 1991 Tetranucleotide repeat polymorphism at the human aromatase cytochrome P-450 gene (CYP19). Nucleic Acids Res. 19:195.
46. **Lathrop GM, Lalouel JM, Julier C, Ott J.** 1984 Strategies for multilocus linkage analysis in humans. Proc Natl Acad Sci USA. 81:3443–3446.
47. **Magiakou MA, Mastorakos G, Oldfield EH, et al.** 1994 Cushing syndrome in children and adolescents: presentation, diagnosis and therapy. N Engl J Med. 331:629–635.
48. **Forest MG.** 1979 Pattern of the response to testosterone and of its precursors to hCG stimulation in relation to age in infants and children. J Clin Endocrinol Metab. 49:132–137.
49. **Oerter KE, Uriarte M, Rose SR, Barnes KM, Cutler GB.** 1990 Gonadotropin secretory dynamics during puberty in normal girls and boys. J Clin Endocrinol Metab. 71:1251–1258.
50. **Lashansky G, Saenger P, Fishman K, et al.** 1991 Normative data for adrenal steroidogenesis in a healthy pediatric population: age and sex-related changes after adrenocorticotropin stimulation. J Clin Endocrinol Metab. 73:674–686.

50a. **Vottero A, Kirschner LS, Yue W, Brodie A, Stratakis CA.** 1998 Aromatase expression in peripheral blood lymphocytes before and after Epstein-Barr virus (EBV) transformation. J Ster Biochem Mol Biol. In press.

51. **Berstein LM, Santner SJ, Brodie AM, Koos RD, Naftolin F, Santen RJ.** Pseudoaromatase in circulating lymphocytes. J Steroid Biochem Mol Biol. 44:647–649.
52. **Leiberman E, Zachmann M.** 1992 Familial adrenal feminization probably due to increased steroid aromatization. Horm Res. 37:96–102.
53. **Morishima A, Grumbach MM, Smpson ER, Fisher C, Qin K.** 1995 Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. J Clin Endocrinol Metab. 80:3689–3698.
54. **Finkelstein JS, O'Dea LS, Whitcomb RW, Crowley WF.** 1991 Sex steroid control of gonadotropin secretion in the human male. II. Effects of estradiol administration in normal and GnRH-deficient men. J Clin Endocrinol Metab. 74:621–628.
55. **Bagatell CJ, Dahl KD, Bremmer WJ.** 1994 The direct pituitary effect of testosterone to inhibit gonadotropin secretion in men is partially mediated by aromatization to estradiol. J Androl. 15:15–21.
56. **Grumbach MM, Styne D.** 1992 Puberty: ontogeny, neuroendocrinology, physiology, and disorders. In: Wilson JD, Foster DW, eds. Williams' textbook of endocrinology, 8th ed. Philadeplhia: Saunders; 1139–1221.
57. **Conte FA, Grumbach MM, Ito Y, Fisher CR, Simpson ER.** 1994 A syndrome of female pseudohermaphroditism, hypergonadotropic hypogonadism, and multicystic ovaries associated with missense mutations in the gene encoding aromatase (P450arom). J Clin Endocrinol Metab. 78:1287–1292.
58. **Ito Y, Fisher CR, Conte FA, Grumbach MM, Simpson ER.** 1993 Molecular basis of aromatase deficiency in an adult female with sexual infantilism and polycystic ovaries. Proc Natl Acad Sci USA 90:11673–11677.
59. **Harada N.** 1993 Genetic analysis of human placental aromatase deficiency. J Steroid Biochem Mol Biol. 44:331–340.
60. **Smith EP, Boyd J, Frank GR, et al.** 1994 Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Engl J Med. 331:1056–1061.
61. **Carani C, Qin K, Simoni M, et al.** 1997 Effect of testosterone and estradiol in a man with aromatase deficiency. N Engl J Med. 337:91–95.
62. **Agarwal VR, Sasano H, Takayama K, et al.** Excessive levels of aromatase P450arom and its transcripts in breast adipose tissue of a girl with pubertal macromastia [Abstract P1–393]. Proc of the 79th Annual Meet of The Endocrine Soc. 1997.
63. **Sasano H, Kimura M, Shizawa S, Kimura N, Nagura H.** 1997 Aromatase and steroid receptors in gynecomastia and male breast carcinoma: an immunohistochemical study. J Clin Endocrinol Metab. 81:3063–3067.
64. **Gelinas R, Endlich B, Pfeiffer C, Yagi M, Stamatoyannopoulos G.** 1985 G to A substitution in the distal CCAAT box of the γ-globin gene in Greek hereditary persistence of fetal hemoglobin. Nature 313:323–325.