Mutation in the CYP21B gene (Ile-172→Asn) causes steroid 21-hydroxylase deficiency

gene conversion/missense mutation/cytochrome P450/HLA complex/congenital adrenal hyperplasia

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ABSTRACTSteroid 21-hydroxylase deficiency is the most common cause of congenital adrenal hyperplasia. It results from a deficiency in a specific cytochrome P450, P450c21 (P450XXIA). The gene encoding this protein (CYP21B) and a closely linked pseudogene (CYP21A) are located in the HLA complex on chromosome 6p. Many mutant alleles are associated with deletions of CYP21B; we report the cloning and characterization of a nondeletional mutant CYP21B gene. This mutant gene is expressed on transfection into mouse Y1 adrenal cells, producing mRNA levels similar to those seen after transfection of the normal CYP21B gene. In codon 172 of the mutant gene, the normal codon ATC, encoding isoleucine, has been changed to AAC, encoding asparagine. This mutation is normally present in CYP21A pseudogene, so that it may have been transferred to the mutant CYP21B gene by gene conversion. Hybridization of oligonucleotide probes corresponding to this and two other mutations normally present in CYP21A demonstrated that 4 out of 20 patients carried the codon 172 mutation; in one of these patients, the mutation was present as part of a larger gene conversion involving at least exons 3-6. Gene conversion may be a frequent cause of 21-hydroxylase deficiency alleles due to the presence of six chi-like sequences (GCTGGGG) in the CYP21 genes and the close proximity of the CYP21A pseudogene, which has several potentially deleterious mutations.

Congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency is a common autosomal recessive disorder of cortisol biosynthesis that results in disordered sexual differentiation and somatic growth. Two-thirds of the patients with the severe, "classical" form of this disease are also unable to synthesize the mineralocorticoid hormone aldosterone and, if untreated, may die shortly after birth from renal "salt wasting" (1). The affected enzyme is a specific cytochrome P450 (P450c21 or P450XXIA), a heme-containing microsomal protein. The gene CYP21B, encoding this enzyme, and a pseudogene, CYP21A, are located in the HLA major histocompatibility complex on chromosome 6p, respectively adjacent to C4B and C4A, the genes encoding the fourth component of serum complement (Fig. 1A) (2,3) [other names for the 21-hydroxylase genes are 21-OHase A/B, OH21A/B, CA21A/H/B, P450C21A/B, and P450-XXIA1/2 (4)].

CYP21A and CYP21B are each 3.1 kilobases (kb) long and are \( \approx 98\% \) identical in exons (5,6). There are 11 single-base differences in exons between the genes, of which 7 result in amino acid substitutions and 1 results in premature termination of translation. The pseudogene also contains an 8-base-pair (bp) deletion and a 1-bp insertion, each of which shifts the reading frame of translation. Thus, the hypothetical product of the CYP21A gene is completely nonfunctional.

About 5-10% of classical 21-hydroxylase deficiency alleles occur in association with the rare HLA haplotype A3;Bw47;DR7. This haplotype invariably carries a complete deletion of CYP21B (7) and C4B; \( \approx 25\% \) of the remaining classical alleles also carry a deletion of CYP21B (8,9). The mutations causing most other 21-hydroxylase deficiency alleles have not as yet been identified.

We now report that, in one mutant CYP21B gene that is not deleted, codon 172, normally ATC encoding isoleucine, has been changed to AAC encoding asparagine. While this single-base change could have resulted from a point mutation, the identical mutation is normally present in the CYP21A pseudogene. Thus, this mutant allele may represent a gene-conversion event.

MATERIALS AND METHODS

Enzymes and related reagents were purchased from International Biotechnologies (New Haven, CT) and used according to the manufacturer’s directions or as described (10).

Construction of a Partial Genomic Library. High molecular weight DNA was prepared (11) from leukocytes of a patient with 21-hydroxylase deficiency and digested with BamH1. The 14-kb fraction containing the CYP21 genes was isolated by electrophoresis in agarose and by electroelution onto NaA5 paper (Schleicher & Schuell). Fractionated DNA (400 ng) was ligated to 1 \( \mu \)g of BamH1-digested arms of the bacteriophage \( \lambda \) vector EMBL3 (12) and packaged in vitro into phage particles (reagents obtained from Stratagene, San Diego, CA).

Isolation and Analysis of a Mutant CYP21B Gene. The library was screened (13) for the desired insert by hybridization with pc21/3c, which contains a nearly full-length cDNA insert encoding P450c21 (6). Clones carrying CYP21A and -B genes were distinguished by restriction mapping (Fig. 1B). A 7-kb Xho I-BamH1 fragment containing the CYP21B gene was subcloned into the corresponding sites of pBl24. Chain-termination sequencing reactions (14) were performed with supercoiled plasmid DNA as a template (15), adenosine \( 5' [\alpha-\text{32P}] \text{thiotriphosphate (DATP}^{32\text{S}}\)), and 20 specific oligonucleotide primers corresponding to sequences on both strands of the normal CYP21B gene (Fig. 1C).

Transfection into Y1 Cells. Cultured Y1 mouse adrenocortical tumor cells were cotransfected as described (16) with pSV2-neo (2 \( \mu \)g) and plasmids carrying normal or mutant human CYP21B genes (15 \( \mu \)g). After selection with G418 (GIBCO) at 400 \( \mu \)g/ml, resistant clones were pooled and maintained in selective medium.

Analysis of Expression of Transfected CYP21B Genes. The expression of the transfected genes was assessed by RNase protection assays as described (17). The probe consisted of a 316-bp Pst I-EcoRI fragment of the normal human CYP21B gene, containing the third exon (155 bp), the third intron, and 48 bp from the fourth exon, cloned into pBl231. A radioac-

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Patients Dried solution and digested normal activity corresponding cellular RNA for of the diagram bars between sites: B, J, and with TGT TAC 109 Cloning described (D) Sequences of a 172. Characterization of genes. The shorter bars at the ends of the diagram represent 5'- and 3'-untranslated regions. Arrows at the bottom of the diagram indicate positions and orientations of primers used for sequencing. (D) Sequences of the fourth exon from the normal and mutant CYP21B genes. The sequences are identical except for codon 172.

Fig. 1. Cloning and sequencing of a CYP21 gene encoding P450c21. (A) HLA complex on the short arm of chromosome 6. The centromere is indicated by the circle at left. HLA class II, III, and I genes are grouped. Class I and II genes encode transplantation antigens; there are more class I and II genes than are diagrammed here. C2, Bf, C4A, and C4B encode serum complement components; 21A and 21B are the CYP21 genes. (B) Strategy for cloning of the CYP21 genes. The relative sizes and locations of the C4 and CYP21 genes are shown. The genes are all transcribed left to right. Relevant restriction sites: B, BamHI; E, EcoRI; T, Taq I; X, Xho I. (C) Sequencing strategy for the CYP21B gene. Numbered bars represent sequences expressed in mRNA, or exons, whereas the spaces between bars correspond to introns. The shorter bars at the ends of the diagram represent 5'- and 3'-untranslated regions. Arrows at the bottom of the diagram indicate positions and orientations of primers used for sequencing. (D) Sequences of the fourth exon from the normal and mutant CYP21B genes. The sequences are identical except for codon 172.

zymously deleted CYP21B were identified by Southern blot hybridization analysis of genomic DNA digested with various enzymes (Taq I digests are shown, see Fig. 3B) by using a cDNA clone, pC21/3c, encoding human P450c21. In these individuals, any remaining 21-hydroxylase activity must reside in the product of the single nondeleted CYP21B gene [heterozygous carriers of CYP21B deletions who have one normal CYP21B gene do not have 21-hydroxylase deficieny, consistent with the known autosomal recessive mode of inheritance of this disorder (1)]. One such patient (patient H) was studied further. She has the HLA genotype A23;Bw47;DR7/A11;B35;DR5; the first haplotype carries a deletion of CYP21B.

A partial genomic library was prepared from peripheral blood leukocyte DNA of this patient. Six bacteriophage λ clones that contained CYP21 genes were isolated after screening 20,000 plaques by hybridization with pC21/3c, of which two contained CYP21B genes (Fig. 1B).

One of these clones was subjected to DNA sequence analysis. To rule out the possibility of artifacts arising from the use of specific sequencing primers, a characterized (6) normal CYP21B gene was analyzed in parallel with the same primers. [We have corrected several sequence errors in our

RESULTS

Cloning and Characterization of a Mutant CYP21B Gene. Patients with 21-hydroxylase deficiency who carried hetero-
GenBank entry for the CYP21 genes§ based on new and reviewed data and comparison to other published sequences of these genes (5, 6, 21).)

Two single-base substitutions were found in the coding sequences of the mutant CYP21B gene. One (CTC to CTG in codon 248, encoding leucine) is silent, but the other (ATC to AAC in codon 172, exon 4) changes an isoleucine residue to asparagine, a nonconservative substitution (Fig. 1D). No changes were detected affecting intron-exon junctions of the mutant gene.

Expression of Human CYP21B Genes in Transfected Y1 Cells. To establish that this mutant gene did not contain additional mutations in its promoter that altered transcription, plasmids carrying the normal and mutant CYP21B genes were transfected into Y1 mouse adrenocortical tumor cells, and steady-state mRNA levels were assessed by

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Fig. 3. Hybridization analysis of 20 patients with classical 21-hydroxylase deficiency. (A) To the left are sequences of the normal CYP21B gene and CYP21A pseudogene in codons 169–175 (probe 1), codons 104–113 (probe 2), and codons 234–240 (probe 3). To the right are the corresponding oligonucleotides. Differences between each normal (probes B) and mutant (probes A) probe are underlined, and radioactive end-labeling is indicated by an asterisk. (B) Hybridization to Taq I digests of patient DNA samples (letters under each blot identify the patients). Numbers to the right indicate fragment sizes in kb. The top row shows Southern blots with a cDNA probe, pC21/3c. Arrows indicate probable heterozygous deletions of the CYP21B gene. (Based on analysis with other restriction enzymes, patient G may have a gene conversion involving the polymorphic Taq I site rather than a deletion.) Numbers to the left of other rows correspond to the probes listed in A. All patients carry a 3.2-kb Taq I fragment that hybridizes with each probe corresponding to the CYP21A pseudogenes that normally carry these mutations. Four patients (arrows) carry a 3.7-kb Taq I fragment that also hybridizes with the probe corresponding to the codon 172 mutation, presumably signifying a mutant CYP21B gene (row 1). The mutant gene was first isolated from DNA of patient H. Patient I (arrow) carries a 3.7-kb Taq I fragment hybridizing with two additional probes (rows 2 and 3).
RNase protection assays. Pools of transfecant clones were prepared to minimize the effect of clonal variation on 21-hydroxylase (CYP21B) expression. The results (Fig. 2) show that Y1 cells transfected with either the normal or the mutant gene contain CYP21B transcripts, as documented by their protection of a 155-nucleotide fragment that is identical in size to that protected by authentic human adrenal mRNA. As noted (16), parental Y1 cells do not express their own 21-hydroxylase genes. Although this technique is only semiquantitative, the expression of 21-hydroxylase mRNA levels in cells transfected with the codon 172 mutant is roughly equal to that seen with transfection of the normal human CYP21B gene. In addition to the protected fragment of 155 nucleotides, these samples also contained a 48-nucleotide fragment protected by sequences contained in the fourth exon, which was poorly resolved under the gel conditions used (data not shown).

Oligonucleotide Hybridization Studies of Patients. To determine the frequency with which the mutation in codon 172 causes 21-hydroxylase deficiency, two 21-mer oligonucleotides were synthesized (Fig. 3A) corresponding to the normal and mutant CYP21B genes from codon 169 to codon 175. DNA samples from 20 unrelated patients with classical 21-hydroxylase deficiency (Table 1) were digested with Taq I and electrophoresed in agarose gels. The oligonucleotide carrying the mutation was radioactively end-labeled, mixed with a molar excess of the unlabeled normal oligonucleotide, and incubated with the dried gel. The gel was washed under conditions sufficiently stringent that only a perfectly matched probe could yield a hybridization signal (Fig. 3B).

The mutation in codon 172 (as well as the mutation in codon 248) is normally found in the CYP21A pseudogene. Thus, the 3.2-kb Taq I fragment associated with the pseudogene is expected to hybridize with the mutant probe in all individuals, whereas the 3.7-kb Taq I fragment in the CYP21B gene should not hybridize with the probe unless it carries the codon 172 mutation.

In addition to patient H from whose DNA the mutant gene was isolated, DNA samples from three other patients (patients I, O, and S) contained 3.7-kb Taq I fragments that hybridized with the probe. Although 21-hydroxylase deficiency is frequently associated with partial HLA-B antigens, the four patients carrying this mutation had no HLA-B antigen in common. Patients H and O carried heterozygous deletions of CYP21B as determined by Southern blot analysis.

Patient H is a 7-year-old female who, when studied as an inpatient at 1 year of age, could conserve sodium at the expense of a high plasma renin/urinary aldosterone ratio. Thus, her ability to synthesize aldosterone was decreased but not absent. Patients S and O are 12-year-old males who have never demonstrated electrolyte disturbances or aldosterone deficiency ("simple virilizing" 21-hydroxylase deficiency). Patient I is a 26-year-old female who, at birth, had clear salt-wasting symptoms with an aldosterone deficiency that continued until age 12. She subsequently discontinued medication and has been documented to conserve sodium normally.

Because the codon 172 mutation is normally present in the CYP21A pseudogene, it seemed possible that it had been transferred to the CYP21B gene by a gene conversion event. To determine if any of the patients with this mutation carried larger gene conversions, DNA samples were hybridized with probes corresponding to two regions in the CYP21A that flank codon 172 (Fig. 3A). One probe corresponds to an area in exon 3 that contains an 8-bp deletion. The other is complementary to codons 234–240 in exon 6; this area in the CYP21A gene contains four point mutations resulting in three nonconservative amino acid substitutions.

![Fig. 4. Possible gene conversions causing 21-hydroxylase deficiency. The CYP21A and -B genes are drawn schematically as in Fig. 1, with mutations in CYP21A indicated by various shadings of exons. Xs in introns indicate the presence of chi-like sequences, which are postulated to be sites for recombination between homologous regions of CYP21A and CYP21B. The three probes discussed in Fig. 2 are indicated by numbered bars. The solid line encompassing exon 4 and part of exon 7 represents the missense mutation in codon 172 and the silent mutation in codon 246 observed in the sequenced mutant CYP21B gene. The dashed line represents the minimum boundaries of the large gene conversion observed in one patient. The resulting mutant CYP21B genes are also shown.](image)

One of the four patients (patient I) who carried the codon 172 mutation also carried an CYP21B gene that hybridized with both of these additional probes (Fig. 3B). Hybridization analysis of her parents (data not shown) demonstrated that the CYP21B gene donated by her father carried both of these additional mutations, presumably representing a single-gene conversion encompassing at least exons 3–6 (Fig. 4).

**DISCUSSION**

The mutant CYP21B gene was isolated from a patient with classical 21-hydroxylase deficiency, suggesting that the mutation in codon 172 affects the synthesis, enzymatic activity, or both of the encoded P450c21 I) who carried the codon 172 mutation also carried an CYP21B gene that hybridized with both of these additional probes (Fig. 3B). Hybridization analysis of her parents (data not shown) demonstrated that the CYP21B gene donated by her father carried both of these additional mutations, presumably representing a single-gene conversion encompassing at least exons 3–6 (Fig. 4).

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**DISCUSSION**

The mutant CYP21B gene was isolated from a patient with classical 21-hydroxylase deficiency, suggesting that the mutation in codon 172 affects the synthesis, enzymatic activity, or both of the encoded P450c21. However, as the patients with this mutation (two of whom have a heterozygous deletion of CYP21B) retain the ability to make some aldosterone, the mutation probably does not completely abolish enzymatic function. The lower degree of compromise of aldosterone synthesis, as compared to cortisol synthesis, may relate to the much smaller relative amounts of aldosterone normally secreted by the adrenal cortex. The differences in phenotype between patient H (who has decreased but not absent ability to synthesize aldosterone) and patients O and S (who apparently synthesize aldosterone normally) may be due to individual variation in levels of synthesis of the mutant enzyme or to differing rates of catabolism or excretion of various steroid hormones. Similar variations in aldosterone biosynthetic capacity have been documented (22) in HLA-identical siblings with 21-hydroxylase deficiency.

Ile-172 is conserved in the P450c21 (P450XXI) enzymes of mice (23) and cattle (24, 25) and in otherwise poorly homologous enzymes, such as steroid 17-hydroxylase (P450XVII) (26) or P450s induced in the liver by phenobarbital (P450IIB) (27), dioxin (P450I) (28), or steroids (P450III) (29) (Fig. 5). This suggests that Ile-172 participates in a hydrophobic interaction that is important for maintaining the correct conformation of the enzyme. Mutation at this position to a polar residue, such as asparagine, might disrupt such an interaction.

It is unlikely that the mutation in codon 172 or an additional undetected mutation in the promoter region af-
Fig. 5. The predicted amino acid sequences in the region of codon 172 of normal (nl) and abnormal (abn) human P450c21 [XXI, referring to the gene family within the P450 superfamily (4)], are compared with bovine (bov) and murine (mur) P450bc12, with human steroid 17-hydroxylase (XVII), and with cytochromes P450 that are induced in rat liver by phenobarbital (IIB) or by steroids (III) or in human liver by dioxin (I). Functionally conserved residues are indicated by asterisks.

fects transcription of this mutant CYP21B gene, because normal levels of mRNA are detected after transfection of the mutant gene into Y1 cells.

It is notable that the missense mutation in codon 172 and the silent mutation in codon 246 of the mutant CYP21B gene are normally found in the CYP21A pseudogene. Although they might be conventional point mutations, they could have arisen in gene conversion events (Fig. 4) (the silent mutation, of course, could be a polymorphism in the normal population). One patient apparently carries the codon 172 mutation as part of a larger rearrangement involving at least four exons. This large gene conversion includes the 8-bp deletion in exon 3, and, because of the resulting frameshift, the encoded protein is predicted to be truncated and completely nonfunctional. The exact borders of the conversion have not been determined. Because this gene carries a 3.7-kb (not a 3.2-kb) Taq I fragment, the conversion cannot extend to the extra Taq I site near CYP21A, located 211 bp upstream of the initial ATG. The conversion also does not include the nonsense mutation in codon 318 of CYP21A (unpublished observations).

Gene conversion events may be facilitated by the presence of chi-like sequences. Chi is a sequence, usually GCTGG-GG, involved in recombination in bacteriophage λ (30). Similar sequences have been observed near recombinational hot spots in immunoglobulin genes (31) and in human and murine class I and class II genes in the major histocompatibility complex (32). Although a single 7-bp sequence is expected to occur about once every 8000 bp in random DNA, GCTGGGG occurs six times in CYP21B and five times in CYP21A. One such site is present 50 bp away from the mutation in codon 172.

These findings suggest that gene conversion may be a frequent cause of 21-hydroxylase deficiency, a hypothesis that could be tested by hybridizing DNA from patients with additional oligonucleotide probes corresponding to each mutation normally present in the CYP21A pseudogene. In fact, three of the 20 patients carry the nonsense mutation normally found in codon 318 of the CYP21A gene (unpublished observations). If other gene conversions are equally common [however, simple point mutations will certainly account for some mutant alleles (21)], it should be possible to prenatally diagnose 21-hydroxylase deficiency by chorionic villus sampling by using a small number of oligonucleotide probes. This would be of interest in light of the possibility of prenatal therapy of this disorder to prevent abnormal sexual differentiation in female fetuses (33).