J Med Genet 1998;35:1014–1019

Uniparental disomy for chromosome 6 results in steroid 21-hydroxylase deficiency: evidence of different genetic mechanisms involved in the production of the disease

Antonio Ulises López-Gutiérrez, Laura Riba, Maria Luisa Ordoñez-Sánchez, Salvador Ramírez-Jiménez, Mabel Cerrillo-Hinojosa, Maria Teresa Tusié-Luna

Abstract

Congenital adrenal hyperplasia (CAH) is an inherited recessive disorder of adrenal steroidogenesis caused by mutations in the steroid 21-hydroxylase gene (CYP21) in more than 90% of affected patients. The CYP21 gene is located within the HLA complex locus on chromosome 6 (6p21.3). During a molecular characterisation study of a group of 47 Mexican families with 21-hydroxylase deficiency, we identified nine in which the mutation or mutations found in the patient did not appear to originate from one of the parents. Through DNA fingerprinting, paternity was established in all nine families with a probability of non-paternity in the range of 10^{-19} to 10^{-23}. Among these families, we identified one patient with exclusive paternal inheritance of all eight markers tested on chromosome 6p, despite normal maternal and paternal contributions for eight additional markers on three different chromosomes. We did not identify duplication of paternal information for markers in the 6q region, consistent with lack of expression of transient neonatal diabetes owing to genomic imprinting in this patient.

Our results substantiate evidence for the existence of different genetic mechanisms involved in the expression of this recessive condition in a substantial portion (~19%) of affected Mexican families. In addition to the identification of a patient with paternal uniparental disomy, the occurrence of germline mutations may explain the unusual pattern of segregation in the majority of the remaining eight families.

(*J Med Genet* 1998;35:1014–1019)

Keywords: congenital adrenal hyperplasia; germline mutations; isodisomy; non-mendelian inheritance

Departamento de Medicina y Unidad de Genética de la Nutrición del Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Mexico City, Mexico
A U López-Gutiérrez
L Riba
M L Ordoñez-Sánchez
S Ramírez-Jiménez
M T Tusié-Luna

Unidad de Biología Reproductiva, Hospital Angeles del Pedregal, Mexico City, Mexico
M Cerrillo-Hinojosa

Correspondence to:
Dr Tusié-Luna, Instituto Nacional de Pediatría, Apdo Postal 101-48, México DF 04530, Mexico.

Received 3 March 1998
Revised version accepted for publication 28 April 1998

Steroid 21-hydroxylase deficiency is the most common defect leading to congenital adrenal hyperplasia, a recessively inherited disorder that affects adrenal steroidogenesis.[1] The disease is characterised by varying signs of virilisation with or without an inability to retain sodium as a consequence of impaired aldosterone synthesis. The severe "classical form" is present in about 1 in 14 000 liveborns in the general population,[2] whereas the prevalence of the attenuated "non-classical form" is calculated to occur in 1 in 100 people in some populations including eastern European Jews.[3]

Two highly homologous genes located within the HLA class III region on chromosome 6 encode the 21-hydroxylase enzyme: CYP21 is an active gene while CYP21P is a pseudogene that carries several deleterious mutations. The CYP21 gene and its pseudogene share 98% sequence identity.[4–6]

Molecular genetic analysis in patients has shown that most of the mutations found in CYP21 are normally present in the pseudogene,[7–10] implying that mutations are transferred from the pseudogene to the functional gene through recombination.[7 11]

We have performed mutation analysis in a group of 47 Mexican CAH families.[12] Among them, we identified nine families in which one of the parents did not carry the mutation or mutations found in the patient. Through genotyping analysis, we found a case of paternal isodisomy of chromosome 6p, which appears to account for the disease in this patient.

Materials and methods

Forty-seven unrelated 21-hydroxylase deficiency patients were screened for the presence of 13 different reported mutations.[12] Patients were diagnosed based on history, clinical examination, abnormalities of serum and urine electrolytes, and an altered androgen hormonal profile.[9] The study included 21 patients with the salt wasting form, 23 exhibiting simple virilisation, and three diagnosed as having the non-classical phenotype. Nine of the families were selected based on the criteria of an unusual pattern of segregation, where one of the parents did not have the mutation or mutations identified in the patient. The clinical profile of these nine patients is shown in table 1.

Patient 9 is a female with no family history of CAH who had ambiguous genitalia. She developed moderate hyponatraemia four days after birth accompanied by raised levels of DHEA, androstenedione, and testosterone. In addition to corrective surgery the patient has been treated with both glucocorticoids and mineralocorticoids.

DNA was extracted from peripheral blood leucocytes using a proteinase K/SDS digestion protocol followed by phenol extraction as previously described.[9] Analysis of point mutations was carried out by selective amplification of the CYP21 gene by PCR followed by direct

Table 1 Clinical profile of the patients studied. The sex, phenotype, age, signs or symptoms at diagnosis, age of both parents, and number of normal and afected sibs are shown

Patient	Sex	Phenotype	Age at diagnosis	Signs or symptoms at diagnosis	Parents' age		No of sibs	No of affected sibs
					M	F		
1	F	SW	4 d	AG, AC	24	23	1	0
2	F	SW	1 d	HP, AG	ND	ND	ND	ND
3	F	SV	7 d	AG	26	27	1	0
4	M	SV	5 y	PP	18	21	1	0
5	F	SV	8 y	HS, ABE, HP	17	26	3	1
6	F	SW	5 d	AG	17	19	1	0
7	F	SV	5 y	ABE, HP, AG	25	27	4	0
8	M	SV	2 y	ABE, HS, HP	22	ND	0	0
9	F	SW	5 d	AG, HS, HP	30	24	1	0

F = female, M = male, SW = salt waster, SV = simple virilising, y = years, d = days, AG = ambiguous genitalia, AC = adrenal crisis, PP = precocious puberty, HS = hirsutism, ABE = advanced bone age, HP = hyperpigmentation, ND = not determined.

sequencing as previously reported.[12] Primers used for amplification and sequencing are listed in table 2. Four different regions were amplified using primer pairs A/B, C/D, E/G, and H/I, respectively, from table 2. Amplifications were carried out in a mixture containing 0.5-1 μg of genomic DNA, 50 ng of each primer, 2 mmol/l dNTPs, 3 mmol/l $MgCl_2$, and 3% formamide in a reaction volume of 50 μl. A cosmid containing the cloned CYP21P gene was used as a negative amplification control.

Once a point mutation was identified in the patient, confirmation of segregation of each mutation was sought by studying both parents.

Table 2 Primers used for the amplification and sequencing of the CYP21 gene. Location and use of each primer is shown. E = exon, I = intron, s = sense, a = antisense. Underlined bases confer CYP21 specificity to the primer. PCR = primers used for polymerase chain reaction amplification, SEQ = sequencing primers

Primer	Sequence	Location in CYP21	Use
A	5'GTGGGAGGGTACCTGAAG3'	5's	PCR/SEQ
B	3'CCTGCTTTCTCCCCACCA5'	I2a	PCR
C	5'ATCTGGTGGGGAGAAAGC3'	I2s	PCR/SEQ
D	3'AGAGCAGGGAGTAGTCTC5'	E3a	PCR
E	5'CTGTCCTTGGGAGACTAC3'	E3s	PCR
F	3'CCTGAGAAAGGGAATCACGTC	E5a	PCR
G	3'CTGCATCTCCACGATGTGAT	E6a	PCR
H	5'AGGGATCACATCGTGGAGAT3'	E6s	PCR
I	3'AAGCCTCAATCCTCTGCAGCG5'	3'a	PCR
J	5'ATGATCCTCCCACCTCAG3'	I2s	SEQ
K	5'CACTGTTTCTCCACAGCGCAT3'	I3s	SEQ
L	5'CTCCTTTCACCCTCTGCA3'	I6s	SEQ
M	5'TGGGTTGCTGAGGGAGCG3'	I7s	SEQ
N	5'AGCAGGTGACTCCCGAGG3'	I8s	SEQ
O	5'TGAAAATGTGGTGGAGGC3'	I9s	SEQ
P	5'CCACTGCAGTGTCATCCT3'	E10s	SEQ
Q	5'AAGAACTCCAGAGTCCTG3'	E10s	SEQ

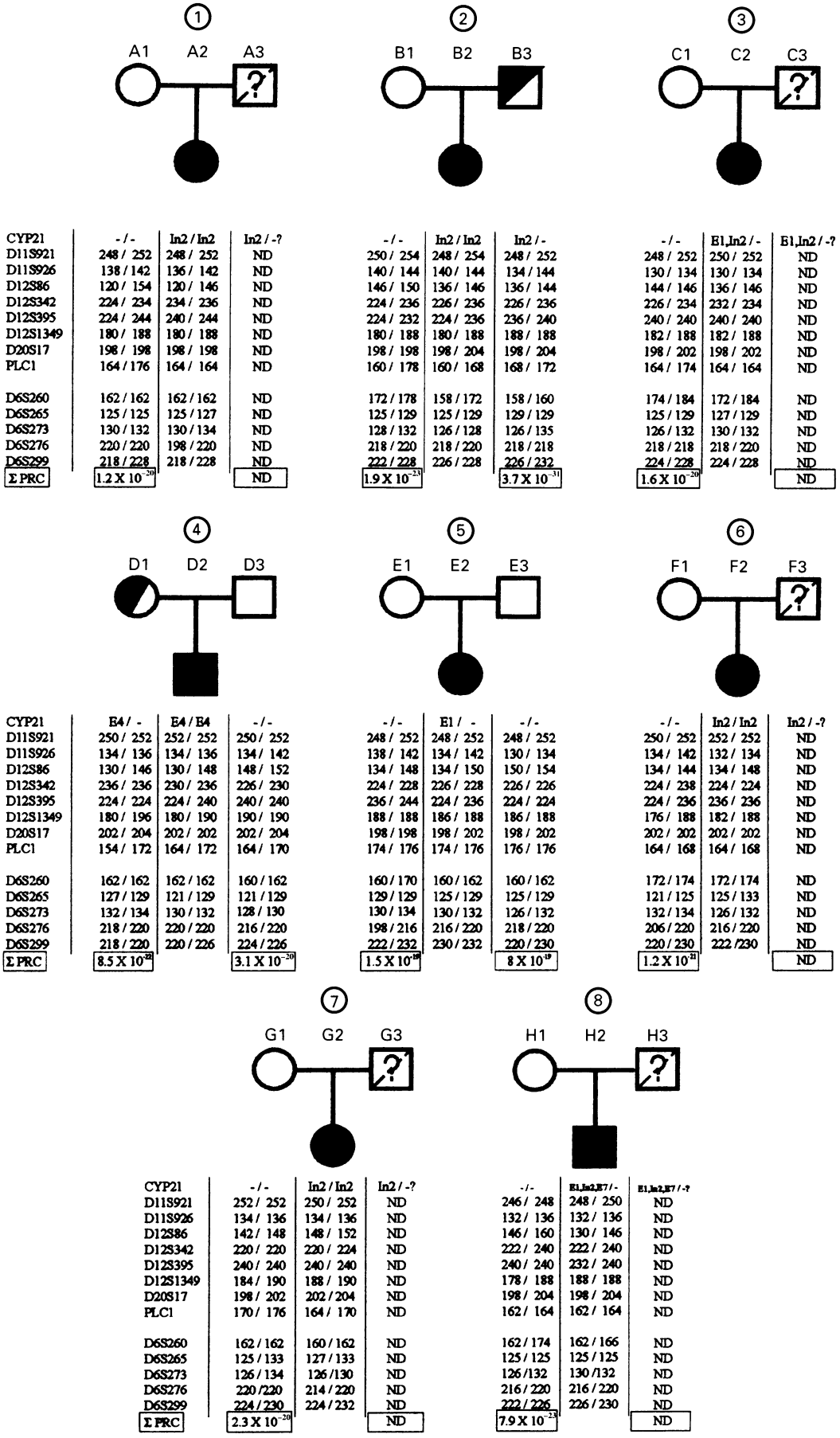
In the nine families where we failed to detect the mutation found in the proband in either parent, a test for paternity was performed by DNA fingerprinting analysis using 13 different microsatellite markers. Five of these markers map to chromosome 6 (D6S260, D6S265, D6S273, D6S276, and D6S299), four to chromosome 12 (D12S86, D12S342, D12S395, and D12S1349), two to chromosome 11 (D11S921 and D11S926), and two more to chromosome 20 (D20S17 and PLC-1). Genotyping of all markers was performed by PCR. The sequence of the amplification primers for all microsatellite markers used was obtained through the Genethon database and are listed in table 3.

For DNA fingerprinting analysis, allelic frequencies for each marker were determined in 15 unrelated Mexican subjects. The probability of random coincidence (PRC) (the probability that two unrelated subjects share the same genotype) was calculated through the product of the relative allelic frequencies of each allele in every subject for each marker according to Berumen *et al.*[13] The summation of PRC reflects the probability that two unrelated subjects share identical genotypes at different loci and therefore represents the probability of non-paternity (Σ PRC values obtained for each parent are shown in fig 1).

To assess erroneous typing owing to selective amplification of mutated alleles when looking for mutations at the intron 2 region, as has

Table 3 Chromosomal location of markers used for DNA fingerprinting analysis and sequence of the corresponding amplification primers. The chromosomal location of microsatellite markers used for DNA fingerprinting is indicated as well as the sequence of the amplification primers

Marker	Sense 5'→3'	Antisense 5'→3'	Chromosomal location
D6S260	TTTTCACTATCAATGGCAGC	TTCATTTTCAGCAGCAATTT	6p23
D6S299	AGGTCATTGTGCCAGG	TGTCTATGTATACTCCTGAATGTCT	6p21.3
D6S265	ACGTTCGTACCCATTAACCT	ATCGAGGTAAACAGCAGAAA	6p21.3
D6S464	TGCTCCATTGCACTCC	CTGATCACCCTCGATATTTTAC	6p21.3
D6S306	TTTACTTCTGTTGCCTTAATG	TGAGAGTTTCAGTGAGCC	6p21.3
D6S273	GCAACTTTTCTGTCAATCCA	ACCAAACTTCAAATTTTCGG	6p21.3
D6S276	TCAATCAAATCATCCCCAGAAG	GGGTGCAACTTGTTCCTCCT	6p21.3
D6S1666	CTGAGTTGGGCAGCATTTG	ACCCAGCATTTTGGAGTTG	6p21.3
D6S300	AGATACATTGACATTCTTCCGC	TCAAAAGCCAAAAGCCTACT	6q14
D6S1671	TTTGGTCAATTTCAATCTGTAG	ATCCTCCAGGGGTGCT	6q14
D6S437	TGTCTTGGTGGAGGCA	GGTACAGTGTTTGACCCTAAGA	6q25.2
D6S473	AGAACTTGGTATTTCCTGCC	GCCACCTTGAGGAGTTTT	6q25.2
D6S1035	ACTTGAACTCAGGCATTCAG	AAAACTCAAGCTCAGAAAGGC	6q25.2
D11S921	TGCATTCAACAAATCAACA	CTTGGACCATTTAATCTAAAGTAAT	11p32.1
D11S926	TGAGTGTGATGTATATGCTCATT	CAAGAGGTCAATTCTGGTGT	11p32.1
D12S86	AGCTAGTCTGGCATGAGCAG	CTATCCCCTGATGATCTCCC	12q24.1
D12S342	CGCTCTCACAGTTCTGGAGG	GCCAAGGCAGAGTTTTGGAC	12q24.1
D12S395	AAACCTCTCTTTGATTGGGG	TCCCCAGTTTGGTTCAGATA	12q24.1
D12S1349	CCCAAGAGTGACATTGGC	GCATGGCTGGAGCATAAG	12q24.1
D20S17	GGCTAAGTATGCAGCAGTTGA	GTACTTTCTCTTTGCAGACCTTG	20q12
PLC 1	AACCAGTCTGCTCTTGTG	CTGCCTTCAACTGATCTCAATGG	20q12

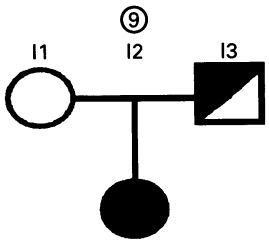


CYP21	-/-	In2/In2	In2/-?	-/-	In2/In2	In2/-	-/-	E1,In2/-	E1,In2/-?
D11S921	248/252	248/252	ND	250/254	248/254	248/252	248/252	250/252	ND
D11S926	138/142	136/142	ND	140/144	140/144	134/144	130/134	130/134	ND
D12S86	120/154	120/146	ND	146/150	136/146	136/144	144/146	136/146	ND
D12S342	224/234	234/236	ND	224/236	226/236	226/236	226/234	232/234	ND
D12S395	224/244	240/244	ND	224/232	224/236	236/240	240/240	240/240	ND
D12S1349	180/188	180/188	ND	180/188	180/188	188/188	182/188	182/188	ND
D20S17	198/198	198/198	ND	198/198	198/204	198/204	198/202	198/202	ND
PLC1	164/176	164/164	ND	160/178	160/168	168/172	164/174	164/164	ND
D6S260	162/162	162/162	ND	172/178	158/172	158/160	174/184	172/184	ND
D6S265	125/125	125/127	ND	125/129	125/129	129/129	125/129	127/129	ND
D6S273	130/132	130/134	ND	128/132	126/128	126/135	126/132	130/132	ND
D6S276	220/220	198/220	ND	218/220	218/220	218/218	218/218	218/220	ND
D6S299	218/228	218/228	ND	222/228	226/228	226/232	224/228	224/228	ND
Σ PRC	1.2×10^{-20}		ND	1.9×10^{-23}		3.7×10^{-31}	1.6×10^{-20}		ND

CYP21	E4/-	E4/E4	-/-	-/-	E1/-	-/-	-/-	In2/In2	In2/-?
D11S921	250/252	252/252	250/252	248/252	248/252	248/252	250/252	252/252	ND
D11S926	134/136	134/136	134/142	138/142	134/142	130/134	134/142	132/134	ND
D12S86	130/146	130/148	148/152	134/148	134/150	150/154	134/144	134/148	ND
D12S342	236/236	230/236	226/230	224/228	226/228	226/226	224/238	224/224	ND
D12S395	224/224	224/240	240/240	236/244	224/236	224/224	224/236	236/236	ND
D12S1349	180/196	180/190	190/190	188/188	186/188	186/188	176/188	182/188	ND
D20S17	202/204	202/202	202/204	198/198	198/202	198/202	202/202	202/202	ND
PLC1	154/172	164/172	164/170	174/176	174/176	176/176	164/168	164/168	ND
D6S260	162/162	162/162	160/162	160/170	160/162	160/162	172/174	172/174	ND
D6S265	127/129	121/129	121/129	129/129	125/129	125/129	121/125	125/133	ND
D6S273	132/134	130/132	128/130	130/134	130/132	126/132	132/134	126/132	ND
D6S276	218/220	220/220	216/220	198/216	216/220	218/220	206/220	216/220	ND
D6S299	218/220	220/226	224/226	222/232	230/232	220/230	220/230	222/230	ND
Σ PRC	8.5×10^{-22}		3.1×10^{-20}	1.5×10^{-19}		8×10^{-19}	1.2×10^{-21}		ND

CYP21	-/-	In2/In2	In2/-?	-/-	E1,In2,E7/-	E1,In2,E7/-?
D11S921	252/252	250/252	ND	246/248	248/250	ND
D11S926	134/136	134/136	ND	132/136	132/136	ND
D12S86	142/148	148/152	ND	146/160	130/146	ND
D12S342	220/220	220/224	ND	222/240	222/240	ND
D12S395	240/240	240/240	ND	240/240	232/240	ND
D12S1349	184/190	188/190	ND	178/188	188/188	ND
D20S17	198/202	202/204	ND	198/204	198/204	ND
PLC1	170/176	164/170	ND	162/164	162/164	ND
D6S260	162/162	160/162	ND	162/174	162/166	ND
D6S265	125/133	127/133	ND	125/125	125/125	ND
D6S273	126/134	126/130	ND	126/132	130/132	ND
D6S276	220/220	214/220	ND	216/220	216/220	ND
D6S299	224/230	224/232	ND	222/226	226/230	ND
Σ PRC	2.3×10^{-20}		ND	7.9×10^{-23}		ND

Figure 1 CYP21 mutation and DNA fingerprinting analysis. Pedigrees 1-8 are shown. Filled symbols indicate affected CAH probands. The mutation or mutations found in the CYP21 gene are indicated. Known mutations of the CYP21 gene are coded as follows: In2 A or C→G in intron 2, E1 CCG→CTG P30L exon 1, E4 ATC→AAC I172N exon 4, E7 GTG→TTG V281L exon 7. -/- absence of the mutation in both alleles, - absence of a mutation in one allele, ND not determined. The question mark and dashed line indicate an untested subject who is assumed to be a heterozygous carrier of the mutation identified in the patient. The corresponding allele haplotypes for all eight markers tested outside chromosome 6 and the five markers from chromosome 6 considered for DNA fingerprinting analysis are shown. Summation of the probability of random coincidence Σ PRC reflects the probability of non-paternity.



CYP21	-/-	In2,E7/In2,E7	In2,E7 / -	Lod score with respect to C4
D11S921	250/252	250/250	248/250	
D11S926	138/140	138/140	140/142	
D11S86	142/150	148/150	142/148	
D12S342	224/238	220/238	220/224	
D12S395	224/240	224/228	228/240	
D12S1349	180/188	180/188	180/184	
D20S17	198/204	202/204	198/202	
PLC1	174/176	164/176	164/176	
Σ PRC	7.9×10^{-13}			
D6S260	162/162	168/168	162/168	3.0
D6S265	125/129	131/131	125/131	4.8
D6S276	218/220	214/214	214/220	9.9
D6S299	230/232	230/230	224/230	6.3
Σ PRC			1.9×10^{-19}	
D6S273	134/136	132/132	132/136	6.4
D6S306	238/238	244/244	238/244	3.1
D6S464	192/196	204/204	192/204	3.5
D6S1666	120/128	120/120	120/122	6.9
D6S1035	136/140	140/140	136/140	Not linked
D6S300	190/194	190/198	188/198	Not linked
D6S1671	258/268	258/260	260/264	Not linked
D6S437	130/134	134/134	134/134	Not linked
D6S473	181/181	181/184	184/184	Not linked

Figure 2 Pedigree of family 9 showing CYP21 mutations, DNA fingerprinting analysis, and genotyping of chromosome 6 markers. The mutations found in the CYP21 gene are indicated and coded as in fig 1. The corresponding alleles for all eight markers tested other than from chromosome 6, as well as the four chromosome 6 markers considered for DNA fingerprinting analysis, are shown below. Σ PRC indicates the probability of non-paternity. The corresponding alleles for nine additional markers tested on chromosome 6 are also indicated at the bottom. The patient carries an exclusive paternal haplotype for all markers tested on chromosome 6p, despite maternal and paternal contributions for all markers tested on chromosome 6q and all other chromosomes analysed. The last column shows the reported lod scores (CEPH database) for the corresponding chromosome 6 markers with respect to the C4 locus located ~30 kb from the CYP21 gene on 6p21.3.

been previously reported,[14] we performed sequence analysis of three different PCR amplifications derived from different sets of amplimers (primer pairs A/B, A/D, C/F from table 2).

To determine the presence of uniparental disomy in the proband from family 9, genotyping of 13 different genetic markers on chromosome 6 was carried out in the patient and both parents (fig 2). The amplification primers used for genotyping are listed in table 3.

Karyotype analysis in the proband, both parents, and one unaffected sib from family 9 was also performed to identify possible chromosomal abnormalities, according to the procedure described by Seabright.[15] Thirty cells were analysed in peripheral blood lymphocytes from each subject.

Results

During the course of the molecular characterisation of a group of 47 Mexican families with 21-hydroxylase deficiency, we identified nine families in which one of the parents did not carry the mutation (or mutations) found in the patient, suggesting that these may represent non-paternity cases. Paternity tests were carried out by DNA fingerprinting analysis using 13 microsatellite markers located in four different chromosomes (chromosomes 6, 11, 12, and 20) for families 1-8 and eight different genetic markers (on chromosomes 11, 12, and 20) for family 9. Paternity was established in all nine families with a probability of non-paternity in the range of 10^{-19} to 10^{-23} (figs 1 and 2) (indicated as Σ PRC). Analysis of eight genetic markers from chromosome 6p in family 9 identified the patient (I.2 in fig 2) as homozygous for one of the paternal alleles for all these markers, suggesting that she inherited two identical copies of one of the father's chromosomes 6 (isodisomy) (fig 2). Testing of five additional markers on 6q (table 3) showed that the duplication did not extend to the long arm of chromosome 6 (fig 2).

Haplotype analysis in families 1-8 using four chromosome 6 markers (D6S260, D6S265, D6S273, D6S276) (fig 1) confirmed inheritance of the 6p21.3 chromosomal region from the apparently non-transmitting parent in all cases.

Mutation analysis of the functional CYP21 gene in family 9 showed that the patient was homozygous for both the A→G mutation in intron 2 and the V281L mutation in exon 7. The father was found to be heterozygous for both mutations, while neither of these mutations was present in the mother. Cytogenetic analysis was also performed in the patient, both parents, and one unaffected sib from family 9. The patient displayed a normal 46,XX karyotype and no cytogenetic abnormalities were identified in any of the tested subjects.

Discussion

Mutation analysis in a large group of Mexican families with 21-hydroxylase deficiency indicated the involvement of different genetic mechanisms in the expression of this recessive condition in approximately 20% of the families studied.[12]

We found an unexpectedly high frequency of obligate carriers in whom no mutation was detected in DNA from blood samples. Through DNA fingerprinting, paternity was established in all nine families in which no corresponding segregation was found (figs 1 and 2). Having confirmed paternity, at least three different genetic mechanisms can be postulated to account for this observation: (1) the presence of mosaicism in the carrier (possibly in the gonad), (2) the occurrence of uniparental disomy (UPD), and (3) the existence of germline mutations.

Among these nine families, we found one patient with a 46,XX normal karyotype (I.2, family 9, fig 2), in whom the disease is apparently the result of paternal uniparental isodisomy of chromosome 6. She displayed homozygosity for one paternal allele for all eight markers tested on chromosome 6p (exclusive paternal haplotype) despite normal paternal and maternal contributions for eight additional markers located on three different chromosomes (chromosomes 11, 12, and 20) (fig 2). In families 1, 3, 6, 7, and 8 where there was only one parent available, uniparental dis-

omy cannot be ruled out owing to the unavailability of the second parent, who is assumed to be heterozygous (fig 1). Hence, UPD could account for at least 2% of the cases of 21-hydroxylase deficiency in our population.

UPD has been described as a genetic mechanism resulting in the expression of other recessive disorders, such as cystic fibrosis,[16] rod monochromacy,[17] and recently in a patient with Herlitz junctional epidermolysis bullosa.[18] Additionally, paternal isodisomy for chromosome 6 has been described in a patient with a complete deficiency of the fourth component of complement,[19] in a newborn with methylmalonic acidaemia, agenesis of pancreatic beta cells, and diabetes,[20] and in three unrelated patients with neonatal transient diabetes.[21 22] It has been proposed that the transient neonatal diabetes (TNDM) in these patients is the result of the expression of an imprinted gene mapped to chromosome 6q22-q23.[21] Genotyping of markers on 6q showed that the duplication in the patient in family 9 did not involve the long arm of chromosome 6, which is consistent with the lack of expression of transient neonatal diabetes in this person. This is the first report of a paternal UPD involving only the short arm of chromosome 6. Since the patient did not have any malformations or other pathological conditions besides 21-hydroxylase deficiency, it is possible that the short arm of chromosome 6 does not carry imprinted genes whose altered expression results in disease.

Spence *et al*[23] proposed three mechanisms leading to UPD. The fact that the patient showed heterozygosity for markers on the long arm of chromosome 6 (fig 2), sharing alleles with the mother for at least three markers (D6S300, D6S1671, and D6S473), suggests that the mechanism by which the UPD occurred was through the fertilisation by the paternal disomic gamete of a maternal normal one, leading to a trisomy that was corrected to disomy by non-disjunction, after recombination occurred betweeen the maternal and paternal chromosomes 6.

It has been postulated that segments around breakpoints of translocations and inversions are prone to duplications, implying that people carrying translocations may be at higher risk of transmitting a duplicated chromosomal region. Translocations of chromosome 15 have been observed in patients and carriers of Angelman and Prader-Willi syndromes.[24 25] However, we did not find alterations at the cytogenetic level in any of the people studied from family 9.

According to the incidence of aneuploidy in spontaneous abortions, it has been estimated that around 1/3000 conceptuses may be diploid as a consequence of aneuploid gamete complementation.[26] Therefore, the frequency of UPD may be underestimated in patients carrying autosomal recessive disorders since recognition of UPD cases requires, in addition to homozygosity, the expression of additional phenotypic features as a result of genomic imprinting and/or molecular analysis in the patient and his family. The identification of UPD in our patient was through investigation for paternity, since she lacked any additional clinical signs that could be attributed to genomic imprinting.

Although the presence of germline mutations cannot be experimentally proven, it has been shown that de novo mutations in the CYP21 gene are generated in a relatively high proportion of cells (in the order of 1 in 10^3-10^6) in sperm derived from normal subjects.[27] Both gene conversion and unequal crossing over events are responsible for the generation of mutated alleles through recombination. It is interesting that in seven of the nine families where no corresponding segregation was found, it was the mother who lacked the mutation found in the patient. Increased recombination frequency has been documented to occur in females at several loci, including the H-2 locus on chromosome 6 in mice,[28] the pericentric region of chromosome 21,[29] and nearly the entire chromosome 3 in humans.[30] This observation is in agreement with the mother being the putative carrier of the germline mutation in the majority of these families.

It is also interesting that in only one of the families where we found this phenomenon is there a second affected child (family 5) (table 1), providing further support for the possibility of germline mutations in the majority of the affected families. In family 5, where only one of the two responsible mutations has been found, the E1 mutation (CCG→CTG P30L) was not detected in either parent (fig 1). The existence of a second affected sib in this family could be the result of a gonadal mosaic cell line in one of the parents.

A similar frequency of apparent germline mutations (20%) has been reported in patients with 21-hydroxylase deficiency in the Japanese population,[10] suggesting that some populations may be at higher risk for the occurrence of these events.

It has been reported that allele drop out can occur during PCR while amplifying the intron 2 region of the CYP21 gene, leading to incorrect genotyping (for example, asymptomatic heterozygous carriers have been typed as homozygous for the intron 2 mutation).[14] Segregation analysis in the affected family using microsatellite markers near the 21-hydroxylase locus is useful in determining both paternal and maternal inheritance of the corresponding alleles. Using this strategy, we proved that patients A2, B2, F2, and G2 (from fig 1), typed as homozygous for the intron 2 mutation (and having only this mutation), inherited one allele from the parent typed as a non-carrier of the mutation (analysis of genetic markers on 6p21.3, fig 1). Moreover, the intron 2 region of the CYP21 gene was analysed using three independent PCR reactions generated from different sets of amplification primers (primer pairs A/B, A/D, and C/F from table 2). This procedure is expected to reduce the possibility of selective allele amplification owing to the presence of sequence polymorphisms located in the primer annealing site.

The identification of families with an unexpected pattern of transmission will undoubtedly contribute to a better understanding of the genetic mechanisms involved in the expression

of this disorder and will lead to better genetic counselling for these affected families, since the risk of having a second affected child is expected to change dramatically depending upon the mechanism by which the mutation is transmitted.

This work was supported by grants 2087-M9302 and 2238-PM from the Consejo Nacional de Ciencia y Tecnología, México.

1 New MI, Crawford C, Wilson RC. Genetic disorders of adrenal steroidogenic enzymes. In: Emery AEH, Rimoin D, eds. *Principles and practice of medical genetics*. 3rd ed. New York: Churchill Livingstone, 1996:1441-76.
2 Pang S, Wallace MA. Worldwide experience in newborn screening for congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Pediatrics* 1988;**81**:866-74.
3 Speiser PW, Dupont B, Rubinstein P, Piazza A, Kastelan A, New MI. High frequency of nonclassical steroid 21-hydroxylase deficiency. *Am J Hum Genet* 1985;**37**:650-67.
4 Higashi Y, Yoshioka H, Yamane M, Osamu G, Fujii-Kuriyama Y. Complete nucleotide sequence of two steroid 21-hydroxylase genes arranged in human chromosomes: a pseudogene and a genuine gene. *Proc Natl Acad Sci USA* 1986;**83**:2841-5.
5 White PC, New MI, Dupont B. Structure of human steroid 21-hydroxylase genes. *Proc Natl Acad Sci USA* 1986;**83**: 5111-15.
6 Rodriguez NR, Dunham I, Yu CY, Carroll MC, Porter RR, Campbell RD. Molecular characterization of the HLA-linked steroid 21-hydroxylase B gene from an individual with congenital adrenal hyperplasia. *EMBO J* 1987;**6**: 1653-61.
7 Higashi Y, Tanae A, Inoue H, Hiromasa T, Fujii-Kuriyama Y. Aberrant splicing and missense mutations cause steroid 21-hydroxylase deficiency P450(c21) deficiency in humans: possible gene conversion products. *Proc Natl Acad Sci USA* 1988;**85**:7486-90.
8 Speiser PW, Dupont J, Zhu DG, *et al.* Disease expression and molecular genotype in congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *J Clin Invest* 1992;**90**: 584-95.
9 Wilson RC, Wei JQ, Cheng KC, Mercado A, New MI. Rapid deoxyribonucleic acid analysis by allele-specific polymerase chain reaction. *J Clin Endocrinol Metab* 1995;**80**:1635-40.
10 Tajima T, Fujieda K, Nakuyama K, Fujii-Kuriyama Y. Molecular analysis of patients and carrier genes with congenital steroid 21-hydroxylase deficiency by using polymerase chain reaction and single strand conformational polymorphism. *J Clin Invest* 1993;**92**:2182-90.
11 White PC, Vitek A, Dupont B, New MI. Characterization of frequent deletions causing steroid 21-hydroxylase deficiency. *Proc Natl Acad Sci USA* 1988;**85**:4436-40.
12 Ordoñez-Sánchez ML, Ramírez-Jimenez S, López-Gutiérrez A, *et al.* Molecular genetic analysis of patients carrying steroid 21-hydroxylase deficiency in the Mexican population: identification of possible new mutations and high prevalence of apparent germ line mutations. *Hum Genet* 1998;**102**:170-7.
13 Berumen J, Casas I, Hernández A, Segura E, Medina R, Larriva J. Diversidad genética de tres sondas de DNA en la huella digital de DNA de una población mexicana. *Rev Invest Clin (Mex)* 1994;**46**:457-64.
14 Day DJ, Speiser PW, Schulze E, *et al.* Identification of non-amplifying CYP21 genes when using PCR-based diagnosis of 21-hydroxylase deficiency in congenital adrenal hyperplasia (CAH) affected pedigrees. *Hum Mol Genet* 1996;**5**: 2039-48.
15 Seabright MA. A rapid banding technique for human chromosomes. *Lancet* 1971;**ii**:971-3.
16 Voss R, Ben-Simon E, Avital A, *et al.* Isodisomy of chromosome 7 in a patient with cystic fibrosis: could uniparental disomy be common in humans ? *Am J Hum Genet* 1989;**45**: 373-80.
17 Pentao L, Lewis RA, Ledbetter DH, Patel P. Maternal uniparental isodisomy of chromosome 14: association with autosomal recessive rod monochromacy. *Am J Hum Genet* 1992;**50**:690-9.
18 Pulkkinen L, Bullrich F, Czarnecki P, Weiss L, Uitto J. Maternal uniparental disomy of chromosome 1 with reduction to homozygosity of the LAMB3 locus in a patient with Herlitz junctional epidermolysis bullosa. *Am J Hum Genet* 1997;**61**:611-19.
19 Welch TR, Beischel LS, Choi E, Balakrishnan K, Bishof NA. Uniparental isodisomy 6 associated with deficiency of the fourth component of complement. *J Clin Invest* 1990;**86**:675-8.
20 Abramowikz MJ, Andrien M, Dupont E. Isodisomy of chromosome 6 in a newborn with methylmalonic acidemia and agenesis of the pancreatic beta cells causing diabetes mellitus. *J Clin Invest* 1996;**94**:418-21.
21 Whiteford ML, Narendra A, White MP, *et al.* Paternal uniparental disomy for chromosome 6 causes transient neonatal diabetes. *J Med Genet* 1997;**34**:167-8.
22 Temple IK, Gardner RJ, Robinson DO, *et al.* Further evidence for an imprinted gene for neonatal diabetes localized to chromosome 6q22-23. *Hum Mol Genet* 1996;**5**: 1117-21.
23 Spence EJ, Perciaccante RG, Greig GM, *et al.* Uniparental disomy as a mechanism for human genetic disease. *Am J Hum Genet* 1988;**42**:217-26.
24 Hultén M, Armstrong S, Challindor P, *et al.* Genomic imprinting in an Angelman and Prader-Willi translocation family. *Lancet* 1991;**338**:638-9.
25 Smeets D, Hamel B, Nelen M, *et al.* Prader-Willi syndrome and Angelman syndrome in cousins from a family with translocation between chromosomes 6 and 15. *N Engl J Med* 1992;**326**:807-10.
26 Engel E, DeLozier-Blanchet D. Uniparental disomy, isodisomy and imprinting: probable effects in man and strategies for their detection. *Am J Med Genet* 1991;**40**:432-9.
27 Tusié-Luna MT, White PC. Gene conversions and unequal crossovers between CYP21 (steroid 21-hydroxylase) and CYP21P involve different mechanisms. *Proc Natl Acad Sci USA* 1995;**92**:10796-800.
28 Shiroishi T, Sagai T, Moriwaki K. Sexual preference of meiotic recombination within the H-2 complex. *Immunogenetics* 1987;**25**:258-62.
29 Tanzi RE, Watkins PC, Stewart GD, Wexler NS, Gusella JF, Haines JL. A recombination linkage map of human chromosome 21: analysis of recombination as a function of sex and age. *Am J Hum Genet* 1992;**50**:551-8.
30 Yamakawa K, Morita R, Takahashi E, Tlathrop MH, Nakamura Y. A genetic linkage map of 41 restriction fragment length polymorphism markers for human chromosome 3. *Genomics* 1991;**11**:565-72.