Molecular genetics and epidemiology of steroid 21-hydroxylase deficiency
Origin of disease-causing mutations

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Moleculaire genetica en epidemiologie van steroïd 21-hydroxylase deficiëntie
Herkomst van klinisch relevante mutaties

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Contents

Abbreviations

3βHSD	3β-hydroxysteroid dehydrogenase.
ACTH	adrenocorticotropin
AGS	adrenogenital syndrome
bp	basepair(s)
C4	fourth component of complement (A or B)
CAH	congenital adrenal hyperplasia
CYP	cytochrome P450
CYP	any human cytochrome P450 gene
Cyp	any non-human cytochrome P450 gene
CYP11A	cytochrome P450 11A: cholesterol desmolase; side chain cleaving enzyme
CYP11B1	cytochrome P450 11B1: steroid 11β-hydroxylase
CYP11B2	cytochrome P450 11B2: aldosterone synthase
CYP17	cytochrome P450 17: steroid 17α-hydroxylase / 17,20-lyase
CYP21	cytochrome P450 21: steroid 21-hydroxylase
CYP21	the human steroid 21-hydroxylase gene
CYP21P	the human steroid 21-hydroxylase pseudogene
Cyp21	a non-human steroid 21-hydroxylase gene
HERV	human endogenous retrovirus
HLA	human leukocyte antigen
kb	kilobasepair(s)
Mb	megabasepair(s)
MHC	major histocompatibility complex
NC	nonclassical
PCR	polymerase chain reaction
PRA	plasma renin activity
psi	pounds per square inch
RCCX	*RP1-C4-CYP21-XB*
RFLP	restriction fragment length polymorphism
RP1	serine/threonine protein kinase gene (named after Rodney Porter)
RP2	the truncated pseudogene of *RP1*
SEM	standard error of the mean
SL	salt loss / salt-losing
SLE	systemic lupus erythematosus
StAR	steroidogenic acute regulatory protein
SV	simple virilising
TNX	tenascin X
TNXA (also: *XA*)	the truncated tenascin-X pseudogene
TNXB (also: *XB*)	the tenascin-X gene

Scope of this thesis

Steroid 21-hydroxylase deficiency is an inherited defect of the synthesis of aldosterone and cortisol by the adrenal cortex. It is the cause of more than 90 % of all cases of congenital adrenal hyperplasia and has a worldwide incidence of 1:10,000 to 1:15,000. Lack of cortisol induces enhanced secretion of ACTH by the pituitary gland, causing hyperplasia of the adrenal cortex. The hyperplastic adrenal produces excessive amounts of androgens which are converted peripherally to testosterone, causing pre- and postnatal virilisation. Severe steroid 21-hydroxylase deficiency is a potentially life-threatening condition because lack of aldosterone causes massive urinary salt loss with severe dehydration in untreated paediatric patients.

Steroid 21-hydroxylase is encoded by the *CYP21* gene in the central region of the human Major Histocompatibility Complex (MHC), a highly variable and gene-dense part of the human genome.

This thesis is aimed at understanding the relationship between the genetic defects that cause steroid 21-hydroxylase deficiency and the clinical phenotype, and the molecular mechanisms that create these mutations in the *CYP21* gene, in a patient population from the Netherlands.

Soon after the discovery of the *CYP21* gene and the nearby *CYP21P* pseudogene, a picture emerged of great variability in the arrangement of these genes on chromosome 6p21.31. Some of these variants constitute genetic defects that cause steroid 21-hydroxylase deficiency. The study's initial objective was therefore to obtain an image of that variability by charting the layout of the genetic environment of the *CYP21* gene in steroid 21-hydroxylase deficiency patients and in the general population. Two genetic mechanisms that are fundamental to the evolution of the genome, and especially the MHC, are also instrumental in generating the variability in this region:

- simultaneous deletion and duplication of genes by unequal crossover, and:
- non-reciprocal sequence transfer between genes by gene conversion.

A major point of attention in this thesis is how these mechanisms create alleles that carry steroid 21-hydroxylase deficiency.

The allele frequency of genes in the MHC varies among human populations, and the association between the different categories of steroid 21-hydroxylase deficiency and specific HLA serotypes has been noted even before the advent of molecular genetics. The thesis therefore addresses the relationship between *CYP21* defects and the clinical severity of steroid 21-hydroxylase deficiency, and compares the mutational spectrum in the Dutch population to the variability found elsewhere. Ancient mutations in conserved haplotypes are likely to have spread among human populations, whereas recent mutations may be concentrated in a single group due to a founder effect, contributing to differences between populations. On the other hand, defects that already exist in the population may again arise independently as *de novo* mutations, as illustrated in this thesis.

CYP21 mutation analysis is a valuable diagnostic tool in individual steroid 21-hydroxylase patients and their family members. Knowledge of *CYP21* mutations and their phenotypical severity has already led to a genotype-based definition of patient categories. Since the overall relationship with the phenotype is good in most patients, the danger of overtreatment can be reduced by determining the genetic defect to confirm the clinical diagnosis. Still, there may be discrepancies between clinical manifestation, biochemical data and genetic defects - two such cases are described in detail in this thesis.

Mutation detection in family members of a patient provides information for genetic counselling and is especially relevant if the patient has *CYP21* defects of differing severity. Genetic analysis may also identify previously undiagnosed patients with mild symptoms. Finally, since over 90 % of all *CYP21* defects are attributable to a limited set of about 10 mutations, it is possible to identify most unrelated heterozygotes, which is of relevance to the spouses of known carriers.

Chapter 1

General Introduction

1.1. Perspective

The traditional route to understanding a genetically controlled disease leads from phenotype to genotype - schematically, from medicine to biochemistry to molecular genetics, with knowledge from each of these fields adding to progress in all of them.
Congenital adrenal hyperplasia (CAH) is no exception. Nowadays, the statement that this common inherited metabolic disease is mostly due to steroid 21-hydroxylase deficiency caused by a genetic defect in the *CYP21* gene is routinely made in most reports on this subject, including the chapters of this thesis - but decades of research were needed to gain such knowledge.

Early medical reports

The most frequently quoted early report on CAH was written in 1865 by de Crecchio (summarised by Bongiovanni and Root [1963]). However, for a scientist living in the Netherlands, it is difficult to forego an opportunity to claim the primacy of the famous Dutch physician Reinier de Graaf by quoting a 1672 case report, translated by Jocelyn and Setchell 300 years later [Jocelyn and Setchell 1972; Wallace 1995]:

> 'On 27th June 1670, there was born at Delft an infant, whom, because of malformation of its genital parts, its parents did not want distinguished with either male or female name until they consulted experts in the matter. The experts judged it to be a male because of an apparently perfect male member and swellings hanging like testicles underneath. The infant was, therefore, called Cornelius. Struck by a serious illness, Cornelius departed this life on 19th July.'

Prenatal virilisation of female patients (at autopsy, Cornelius turned out to be a girl) remains a hallmark of CAH caused by steroid 21-hydroxylase deficiency. In addition, severe urinary salt loss becomes manifest in many male and female patients within the first weeks of life. Untreated salt-losing CAH in paediatric patients is a very dangerous condition: before the advent of steroid replacement therapy [Wilkins *et al.* 1950; Crigler *et al.* 1952], the disease was fatal in most cases [Knudson 1951; Iversen 1955]. The term 'congenital adrenal hyperplasia' was not ubiquitously used during that period: 'adrenogenital syndrome' was more popular and used in early reports [Broster 1934], but the nomenclature often depended on the nationality of the author [Iversen 1955]. However, it later became evident that the 'adrenogenital syndrome' also included unrelated adrenocortical abnormalities [Bongiovanni and Root 1963], and the term was eventually considered too generic [Stempfel and Tomkins 1966].

Steroids and enzymes

By the mid-1960's, considerable knowledge had been acquired on the biosynthesis of steroid hormones in the adrenal cortex and steroid 21-hydroxylase deficiency had been recognised as the cause of more than 90 % of all cases of CAH [Wilkins 1962; Stempfel and Tomkins 1966]. The finding that adrenocortical steroid synthesis is controlled by ACTH led to the establishment of the ACTH stimulation test as a diagnostic tool in suspected 21-hydroxylase deficiency. These developments were complemented by improvements in the measurement of (first) urinary steroid metabolites and (later) steroids in blood serum (notably, 17α-hydroxyprogesterone). Radioimmunoassay of 17α-hydroxyprogesterone in dried blood spots [Pang *et al.* 1977] is the basis for present-day neonatal screening programmes [Brosnan and Brosnan 2000; Therrell 2001]. A choice of steroid analysis techniques for basic research and clinical diagnosis is now available [Wallace 1995; Andrew 2001].

Parallel to recent advances in molecular genetics, progress in the field of enzymology has been ongoing. Steroid 21-hydroxylase was the first cytochrome P450 for which an enzymatic function was demonstrated [Estabrook *et al.* 1963], but its isolation and its reconstitution in an active form proved difficult due to its hydrophobicity and strong association with the microsomal membrane [Kominami *et al.* 1980; Hiwatashi and Ichikawa 1981]. By 1980, the adrenocortical steroidogenic pathways and the enzymatic activities involved were understood in considerable detail [Grant 1978; Degenhart 1979; Mitani 1979; Fevold 1983], but it was not until the 1990's that the mapping of those activities to individual cytochrome P450 proteins was completed [White 1994; Pascoe and Curnow 1995].

Classical genetics

The hereditary nature of CAH was first perceived in the 1930's [Broster 1934; Jacobziner and Gorfinkel 1936] and discussed in detail by Knudson [1951]. Differences in aldosterone secretion between salt-losing and non-salt-losing patients were attributed to differences in enzymatic defect [Degenhart *et al.* 1965], a hypothesis later confirmed by mutation analysis [Amor *et al.* 1988]. A major breakthrough in the genetics of steroid 21-hydroxylase deficiency was achieved in 1977, when Dupont *et al.* found that the disease-carrying allele co-segregated with the HLA type in family studies of CAH patients [Dupont *et al.* 1977]. No genetic linkage to HLA was found for 11β-hydroxylase deficiency, which is the next most common defect in CAH (although it accounts for only about 5 % of all cases in most populations) [New *et al.* 1981]. Association with a specific HLA allele (B47) was demonstrated for salt-losing steroid 21-hydroxylase deficiency [Klouda *et al.* 1980], but not for the non-salt-losing ('simple virilising') form. A mild 'late onset' variant of 21-hydroxylase deficiency frequently found in combination with HLA-B14, was recognised as an allelic variant [Kohn *et al.* 1982]. Association of steroid 21-hydroxylase deficiency with different alleles of the genes encoding the fourth component of complement (*C4A* and *C4B*) was established at the same time [O'Neill *et al.* 1982]. By the early 1980's, differences in hormonal response to ACTH stimulation between severely and mildly affected patients,

heterozygotes, and controls, had been firmly established [New *et al.* 1983], although a substantial overlap between some of these categories was found.

Molecular genetics

The availability of a reliable procedure to purify steroid 21-hydroxylase from bovine adrenal cortex [Kominami *et al.* 1980; Hiwatashi and Ichikawa 1981] paved the road to cDNA cloning [White *et al.* 1984b] and soon afterwards, to the identification in man of the gene now known as *CYP21* by White and co-workers in 1984 [White *et al.* 1984c]. *CYP21* and its closely linked pseudogene *CYP21P*, each approximately 3.4 kb in size, were mapped to the class III region of the Major Histocompatibility Complex (MHC) on chromosome 6p21.3, adjacent to the complement *C4* genes. Absence or reduced gene dosage of *CYP21* was detected in some, but not all, steroid 21-hydroxylase deficiency patients [White *et al.* 1985; Carroll *et al.* 1985a; Carroll *et al.* 1985b].

After *CYP21* and *CYP21P* had been sequenced [Higashi *et al.* 1986], it soon became evident that a limited number of mutations typically found in *CYP21P* [Amor *et al.* 1988; Globerman *et al.* 1988; Higashi *et al.* 1988a; Higashi *et al.* 1988b; Speiser *et al.* 1988; Chiou *et al.* 1990; Tusié-Luna *et al.* 1991] accounted for the majority of the defects causing steroid 21-hydroxylase [Higashi *et al.* 1991; Mornet *et al.* 1991; Speiser *et al.* 1992; Wedell *et al.* 1994]. Today, molecular genetic analysis is a valuable resource in understanding the aetiology of steroid 21-hydroxylase deficiency [Nordenström *et al.* 1999; Ritzén *et al.* 2000] and it is indispensable in the management of prenatal treatment (see Chapter 8 for a more elaborate discussion and references).

Twenty years of molecular genetic research of the *CYP21*/*C4* region have provided us with a picture of great detail, but also of amazing complexity. Haplotyping studies [Harada *et al.* 1987; White *et al.* 1988; Collier *et al.* 1989; Morel *et al.* 1989a; Koppens *et al.* 1992a; Lobato *et al.* 1998; Blanchong *et al.* 2000] show great differences between individuals and between populations in the size and composition of this region, now known as the RCCX module [Blanchong *et al.* 2001]. This variability reflects ancient genetic rearrangements and contributes to new ones: recombinational events involving *CYP21* and *CYP21P* followed by unequal crossover [Sinnott *et al.* 1990] or gene conversion [Collier *et al.* 1993] were among the first examples of *de novo* mutations causing a genetic defect in humans.

Insight into the structure and function of the adjacent genes that are also duplicated or deleted by these unequal crossovers has progressed at a similar pace. The difference between C4A and C4B and their variants was shown at a molecular level [Yu *et al.* 1988] and the first genomic sequence of a *C4* gene was published [Yu 1991]. The size variation between *C4* genes (16 or 23 kb) was attributed to an endogenous retroviral sequence present in the long variant only [Dangel *et al.* 1994]. Another surprising feature was the presence of a gene transcribed from the opposite strand of *CYP21*, partly overlapping its 3' flank [Morel *et al.* 1989b]. This gene was later shown to encode a tenascin-like protein [Bristow *et al.* 1993b] and turned out to be the opposite of *CYP21* in more than one way: discovery of the gene came

first, and a molecular defect with an associated clinical phenotype came later [Burch *et al.* 1997].

A crossroads of genetic research

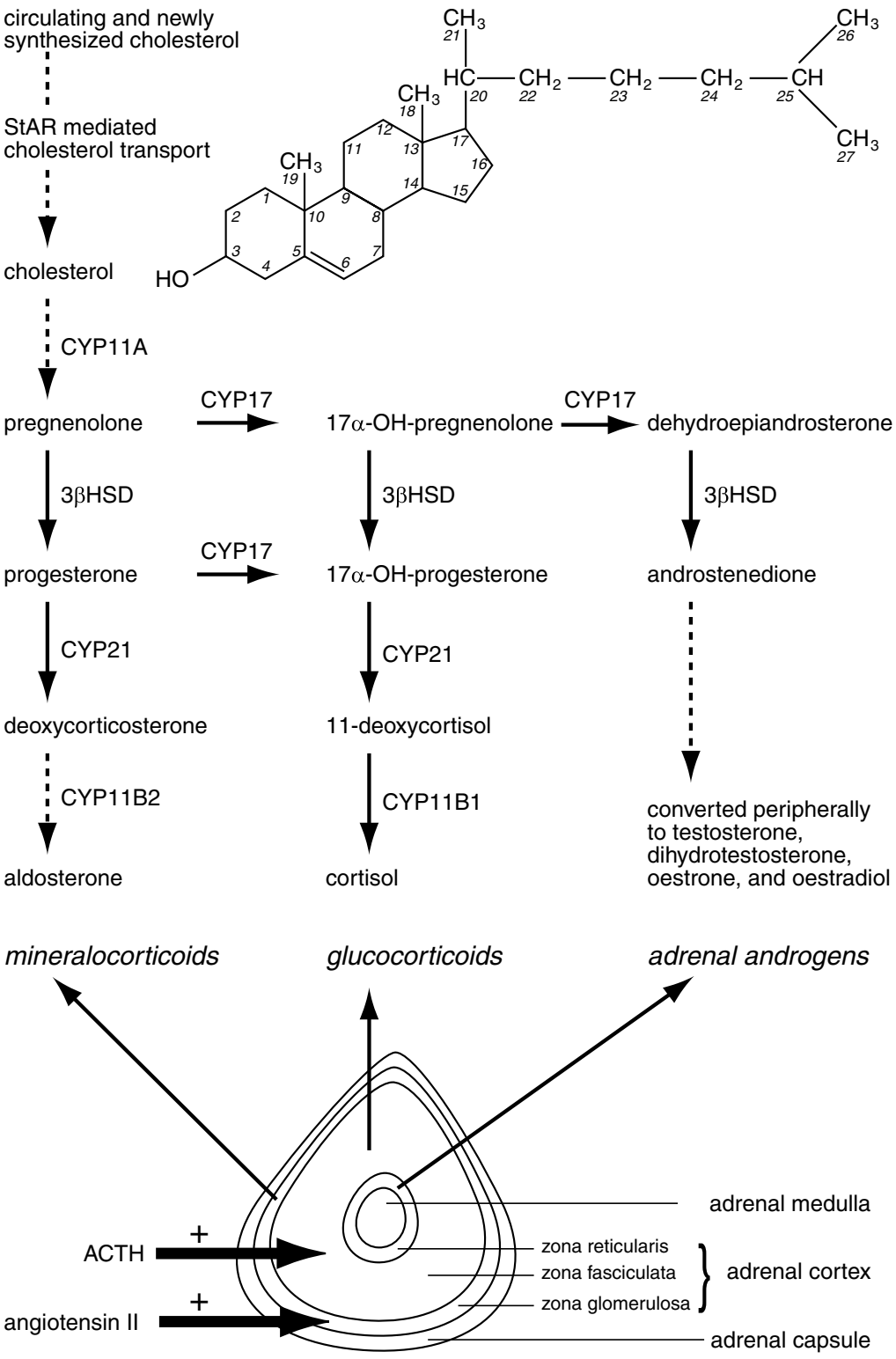
Though research on the *CYP21* gene has been initiated, and intensively pursued, by research groups interested in the causes of steroid 21-hydroxylase deficiency, it is not surprising that many others were also involved: to name but a few fields, the study of complement genetics, HLA alleles, autoimmunity, evolution of the MHC, genetic recombination, and recently the availability of complete genomic sequences of the MHC class III region [MHC Sequencing Consortium 1999; International Human Genome Sequencing Consortium 2001], have each provided invaluable contributions to our current knowledge of *CYP21* genetics and will continue to do so in the future. In addition, questions regarding the discrepancies between phenotype and genotype observed in some patients [Wilson *et al.* 1995b; Krone *et al.* 2000] and the highly complex regulation of the *CYP21* gene [Zanger *et al.* 1992; Wijesuriya *et al.* 1999; Bobba *et al.* 2000] warrants an ongoing interest from the field of molecular endocrinology itself.

1.2. Adrenocortical steroid biosynthesis

The human adrenal

The adult human adrenal is an inconspicuous organ situated on top of each kidney. In adults, each adrenal weighs no more than 4 to 5 g and consists of the small adrenal medulla in the centre, covered by the adrenal cortex that makes up more than 80 % of the total mass of the adrenal. The adrenal cortex consists of three histologically distinct and metabolically specialised zones: the outer *zona glomerulosa*, the intermediate *zona fasciculata* (about 80 % of the adrenal cortex) and the narrow *zona reticularis* on the inside (see fig. 1 for a schematic overview).

Fig. 1. Overview of human adrenocortical steroid biosynthesis. Top: structure of cholesterol with the carbon atoms numbered in italics. Middle: principal steroidogenic steps; dashed arrows indicate multiple steps, sometimes performed by the same enzyme. StAR: steroidogenic acute regulatory protein; CYP11A: cholesterol desmolase / side chain cleaving enzyme; CYP11B1: steroid 11β-hydroxylase; CYP11B2: aldosterone synthase; CYP17: steroid 17α-hydroxylase / 17,20-lyase; CYP21: steroid 21-hydroxylase; 3βHSD: 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase. Bottom: diagram of the adrenal. Each cortical zone produces a different class of steroid hormones. Minor pathways (such as 17α-hydroxyprogesterone -> androstenedione) and intermediate products of single-enzyme conversions (notably, deoxycorticosterone->corticosterone->18-OH-corticosterone->aldosterone) are not shown; for details and references, see the text.


circulating and newly synthesized cholesterol
StAR mediated cholesterol transport
cholesterol
CYP11A
pregnenolone
CYP17
17α-OH-pregnenolone
CYP17
dehydroepiandrosterone
3βHSD
3βHSD
3βHSD
progesterone
CYP17
17α-OH-progesterone
androstenedione
CYP21
CYP21
deoxycorticosterone
11-deoxycortisol
CYP11B2
CYP11B1
converted peripherally to testosterone, dihydrotestosterone, oestrone, and oestradiol
aldosterone
cortisol
mineralocorticoids
glucocorticoids
adrenal androgens
ACTH
angiotensin II
adrenal medulla
zona reticularis
zona fasciculata
zona glomerulosa
adrenal cortex
adrenal capsule

Since ACTH exerts its influence on the adrenal cortex , it is this part of the adrenal that becomes hyperplastic in steroid 21-hydroxylase deficiency patients. However, additional adverse effects on the adrenal medulla have recently been reported [Merke *et al.* 2000].
Surprisingly, the adrenal cortex is at least as large in newborns as in adults (and thus, much larger relative to the body mass). The foetal adrenal consists of a large inner 'foetal' zone surrounded by a narrow outer 'definitive' zone and reaches its maximum size in the fourth month of gestation. Gradual replacement by the definitive adrenal starts thereafter and speeds up considerably after birth. The layered structure mentioned above is complete by the third year of life [Mesiano and Jaffe 1997; Stratakis and Rennert 1999; White and Speiser 2000]. Enhanced transcription of genes involved in growth and development [Rainey *et al.* 2001] probably accounts for the large size of the foetal adrenal.

Enzymatic conversion of steroids

In the adrenal cortex, three distinct biosynthetic pathways convert cholesterol into the main mineralocorticoid aldosterone, the main glucocorticoid cortisol and the (pre-)androgen androstenedione, respectively (see fig. 1). Androstenedione is converted to the primary androgen testosterone by the testis, or to the oestrogens oestrol and oestradiol by the ovary. These three steroids are the end products of the three pathways, but they are by no means the only products secreted by the adrenal cortex. The intermediates shown in fig. 1 also circulate: some of them act as hormones in their own right (although their biological activity is usually limited), others reach characteristic high levels in deficiencies (see below) and can be monitored for diagnostic purposes. After performing their function in metabolism, steroids are inactivated by the liver and excreted. Since steroid biosynthesis and metabolism have been thoroughly reviewed elsewhere [Degenhart 1979; Mitani 1979; Fevold 1983; New *et al.* 1989; Morel and Miller 1991; White 1994; Simpson and Waterman 1995; Andrew 2001], a concise description should suffice here.
Most adrenocortical steroidogenic enzymes are cytochrome P450s. Cytochrome P450s are haem-containing catalysts of oxidative reactions present in many tissues and in a wide range of organisms ranging from mammals to plants and bacteria. They were named after the characteristic 450 nm spectrophotometric absorption maximum obtained by saturation of the reduced form with carbon monoxide [Estabrook *et al.* 1963; Omura and Sato 1964a; Omura and Sato 1964b; Tétreau *et al.* 1997]. Nowadays, hundreds of cytochrome P450s make up the CYP superfamily [Nebert and Gonzalez 1987; Graham-Lorence and Peterson 1996; Negishi *et al.* 1996; Honkakoski and Negishi 2000], and the number of available sequences and alleles is ever-expanding [Cytochrome P450 Nomenclature Committee 2002]. The basic scheme of a cytochrome P450 catalysed hydroxylation in mammals is:

$$\text{substrate-H} + \text{NADPH} + \text{H}^+ + \text{O}_2 \longrightarrow \text{substrate-OH} + \text{NADP}^+ + \text{H}_2\text{O}$$

This reaction requires electron transfer mediated by either one or two 'redox partners', a distinction which has been used to categorise cytochrome P450s: somewhat inconsistently,

class I P450s have two redox partners (generically termed ferredoxin and ferredoxin reductase; the adrenocortical enzymes are adrenodoxin and adrenodoxin reductase), while class II P450s have one partner (cytochrome P450 reductase) [Lambeth *et al.* 1982; Takemori and Kominami 1984; Guengerich 1988; Hall 1989; Graham-Lorence and Peterson 1996; Lewis and Lee-Robichaud 1998].

With a few exceptions, cytochrome P450s are tightly bound to membranes: in mammalian cells, typically to mitochondria or to microsomes. Three out of the five adrenocortical cytochrome P450s are mitochondrial: CYP11A (cholesterol desmolase, the side-chain-cleaving enzyme, also known as $P450_{scc}$), CYP11B1 (steroid 11β-hydroxylase, $P450_{c11}$) and CYP11B2 (aldosterone synthase, $P450_{aldo}$). Two are microsomal: CYP17 (steroid 17α-hydroxylase / 17,20-lyase, $P450_{c17}$) and CYP21 (steroid 21-hydroxylase, $P450_{c21}$). The mitochondrial enzymes are class I P450s, the microsomal enzymes are class II P450s. The adrenal has one steroidogenic enzyme that is not a P450: 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase [Miller 1988; Morel and Miller 1991; White 1994; Lewis and Lee-Robichaud 1998].

Differences in the distribution of cytochrome P450s between the zones of the adrenal cortex (*glomerulosa*, *fasciculata* and *reticularis*) were demonstrated early [Ichikawa *et al.* 1970] and functional studies confirmed that each zone plays its own role in steroidogenesis [Kater and Biglieri 1983; Yagi *et al.* 1983; Stoner *et al.* 1986; Biglieri and Kater 1991]. The overview presented in fig. 1 is a simplification, outlining the major pathways of adrenocortical steroid biosynthesis. Reactions that are of minor importance under normal conditions can become prominent when changed physiological conditions alter their stoichiometry: for example, concentrations of 17α-hydroxyprogesterone in steroid 21-hydroxylase deficiency patients can be so high that this steroid becomes an important substrate for the 17,20-lyase activity of CYP17 and for 11β-hydroxylation by CYP11B1. Also, differences in steroid synthesis and function between humans and other mammals limit the usefulness of animal models.

Steroid function and regulation

Quantitatively, cortisol is by far the most prominent adrenocortical steroid hormone: throughout life, its secretion surpasses that of aldosterone and the adrenal androgens by several orders of magnitude [Sippell *et al.* 1980; Kuhnle *et al.* 1986; Eldar-Geva *et al.* 1990; Glass *et al.* 1994; Witchel and Lee 1998]. Cortisol is the primary glucocorticoid, and its action are manifold: to name but a few, increased cortisol levels mobilise bodily energy resources by glycogenolysis and gluconeogenesis, increases the heart rate, alters brain physiology and has an anti-inflammatory effect by mediating the immune response. Thus, an adequate cortisol response is of particular importance in stressful conditions [Munck and Náray-Feyes-Tóth 1995; Miesfeld 1995; Sapolsky *et al.* 2000].

Adrenocortical steroid biosynthesis is primarily under the control of ACTH [Simpson and Waterman 1995; Waterman and Keeney 1996; Honkakoski and Negishi 2000]. ACTH is produced by the anterior lobe of the pituitary gland as a part of the precursor molecule pro-

opiomelanocortin, and its effect on the adrenal is enhanced by other peptides that are also parts of this precursor [Szalay 1993; Tóth and Hinson 1995; Bicknell *et al.* 1996]. ACTH production is stimulated by hypothalamic neuropeptides such as corticotropin releasing factor, arginine vasopressin and oxytocin. Cortisol decreases these activities by the hypothalamus and pituitary, thus regulating its own production [Engler *et al.* 1999]. This negative feedback mechanism is known as the hypothalamic-pituitary-adrenal (HPA) axis.
The adrenal responds to ACTH stimulation in several ways: within minutes, the production of steroids increases; within hours to days, the synthesis of steroid-producing enzymes increases. Eventually, long-term ACTH stimulation leads to morphological changes and hyperplasia of the adrenal cortex [Knudson 1951; Bongiovanni and Root 1963; Mitani *et al.* 1996; Hernanz-Schulman *et al.* 2002], from birth in untreated steroid 21-hydroxylase patients, or later in life when an ACTH-secreting tumour causes Cushing's disease. Zonation becomes irregular and may be completely lost [Bongiovanni and Root 1963; Robbins 1974; Merke *et al.* 2000].
Aldosterone is the main mineralocorticoid in man, and its function is to promote sodium resorption and potassium excretion in the distal tubuli of the kidney [Crabbé 1978; Mortensen *et al.* 1995; Funder and Marver 1995; White 1996]. Synthesis of aldosterone is primarily under the control of the renin-angiotensin system: low serum sodium levels increase renin secretion by the kidney, resulting in an enhanced conversion of angiotensinogen by renin and angiotensin converting enzyme. The resulting angiotensin II is a potent stimulator of aldosterone secretion, which is also enhanced by high levels of potassium [Aguilera 1993; Clark and Brown 1995; Ehrlich *et al.* 1995].
The adrenal androgens, dehydroepiandrosterone(-sulphate) and androstenedione, do not have a high biological activity, but they are converted peripherally to the potent androgen testosterone. During foetal development, high levels of testosterone produced by Leydig cells, combined with anti-Müllerian hormone from Sertoli cells, result in the development of male internal urogenital structures - in the absence of these factors, female structures are formed. Dihydrotestosterone is formed from testosterone by steroid 5α-reductase and causes development of the prostate and virilisation of the external genitalia [New and Wilson 1999; White and Speiser 2000; American Academy of Pediatrics 2000]. The ovaries express CYP19 (steroid aromatase), which converts androstenedione to oestrone and testosterone to oestradiol. Since fluctuations of the serum levels of adrenal androgens are not recognised by the organism as an abnormal condition, there is no regulatory feedback mechanism such as for cortisol or aldosterone.

1.3. Steroid 21-hydroxylase

The adrenocortical enzyme

Steroid 21-hydroxylase (CYP21; $P450_{c21}$; E.C. 1.14.99.10) is a microsomal cytochrome P450 enzyme that converts progesterone to deoxycorticosterone and 17α-hydroxyprogesterone to 11-deoxycortisol (see fig. 2). It is tightly bound to the microsomal membrane: after re-

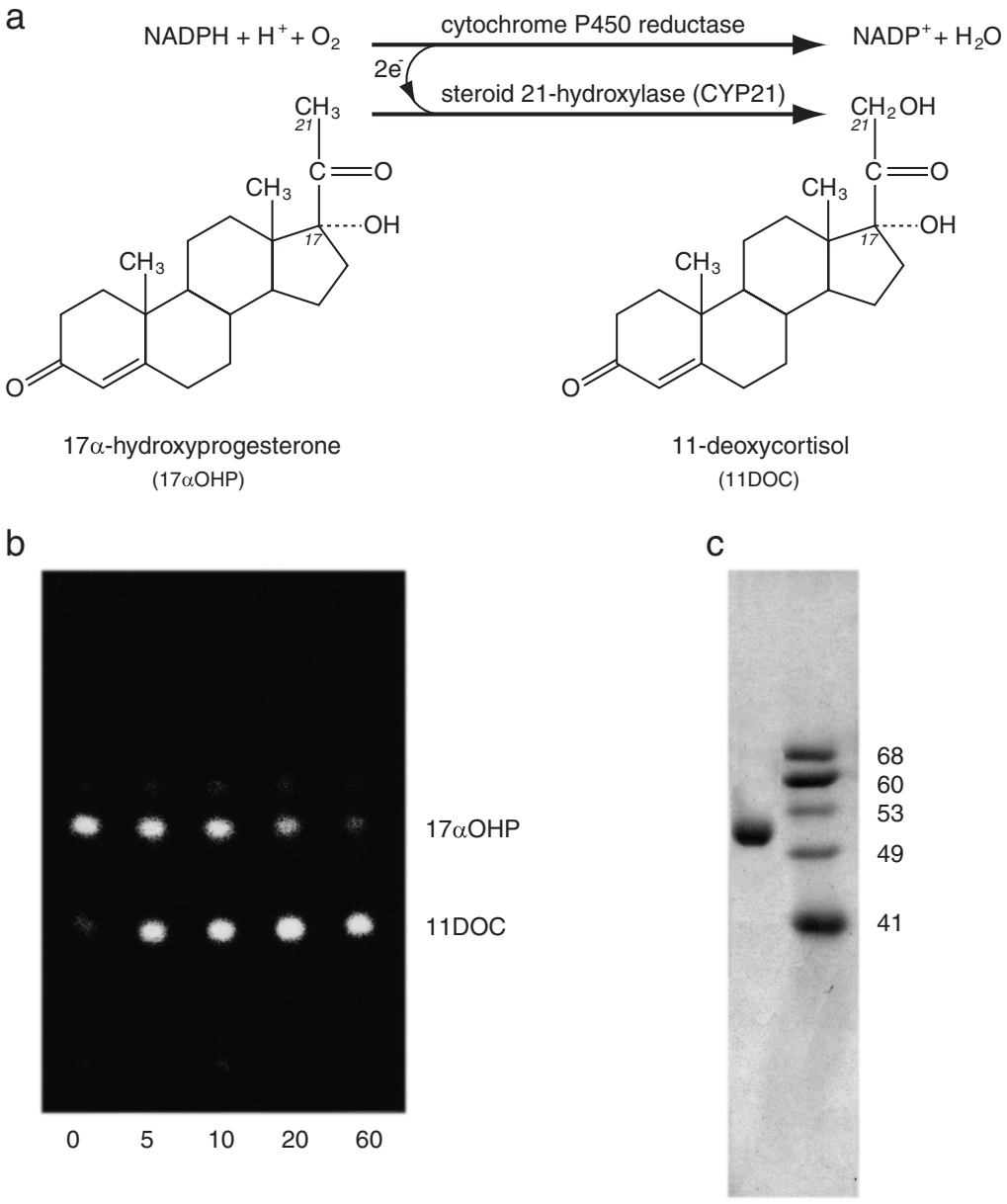


Fig. 2.a. Conversion of 17α-hydroxyprogesterone to 11-deoxycortisol by steroid 21-hydroxylase. The conversion of progesterone to deoxycorticosterone is exactly the same, but without the hydroxyl group in position 17. The reaction mechanism and the electron transport as shown here is a simplification of a highly complex interaction; the electrons are actually transferred one at a time [Guengerich 1988; Sevrioukova *et al.* 1999; Kominami *et al.* 2001]. b. Progress of this reaction in a reconstituted enzyme system [Kominami *et al.* 1980]; separation by thin-layer chromatography; bottom: incubation time in minutes. c. Bovine adrenocortical steroid 21-hydroxylase purified to homogeneity [Kominami *et al.* 1986], as shown on a 15 % SDS-polyacrylamide gel (leftmost lane; right: reference proteins with their molecular mass in kD) . Figures 2b and 2c courtesy of Mrs. S. Zwakhals and Mr. J.W.M. van der Maarel.

incorporation into vesicle membranes, the purified enzyme retains its catalytic activity and other physicochemical properties even after tryptic digest [Kominami *et al.* 1993]. Contrary to mitochondrial adrenocortical P450s, which lose an N-terminal 5 kD 'signal sequence' before membrane integration, steroid 21-hydroxylase is synthesised as a full-size enzyme of approximately 54 kD [DuBois *et al.* 1981; Ogishima *et al.* 1983; Ogishima *et al.* 1985]. Reliable protocols to isolate and assay bovine adrenocortical steroid 21-hydroxylase were established in the early 1980's [Kominami *et al.* 1980; Hiwatashi and Ichikawa 1981; Bumpus and Dus 1982]. NADPH-cytochrome P450 reductase (E.C. 1.6.2.4) receives two electrons from NADPH and transfers them, one at a time, to P450's such as steroid 21-hydroxylase. Cytochrome P-450 reductase contains one molecule of FAD and one molecule of FMN as cofactors in the electron transfer. A reconstituted system containing the isolated enzymes, NADPH, a substrate, and an energy source, performs the steroidogenic reaction *in vitro*. Steroid 21-hydroxylase converts 17α-hydroxyprogesterone more efficiently than it does progesterone. It was previously assumed that transfer of the second electron from the reductase to the cytochrome P450 was the rate-limiting step, but a recent study showed that the rate-limiting step is the actual hydroxylation in the case of 17α-hydroxyprogesterone and the dissociation of the substrate in the case of progesterone [Kominami *et al.* 2001]. Expression studies have shown that common mutations in steroid 21-hydroxylase deficiency affect functions such as substrate binding, membrane integration and reductase interaction in different ways (see table 3 in paragraph 1.7). Molecular modelling studies have already been performed for the normal enzyme and the reductase [Lewis and Lee-Robichaud 1998; Sevrioukova *et al.* 1999] and similar efforts may soon provide additional insight into the effects of disease-causing mutations at protein level.

Peripheral 21-hydroxylase activity

The *zona fasciculata* and the *zona glomerulosa* of the adrenal cortex are the primary sites of *CYP21* gene expression. However, numerous reports have shown that steroid 21-hydroxylation also occurs elsewhere. Deoxycorticosterone [Casey and MacDonald 1982; Winkel *et al.* 1983; Antonipillai *et al.* 1983; Agdere *et al.* 1989] and 21-hydroxypregnenolone [Shackleton *et al.* 1987] were found in steroid 21-hydroxylase deficiency patients, and urinary aldosterone metabolites [Speiser *et al.* 1991] and serum aldosterone [Koppens *et al.* 1998] even in patients where a complete genetic defect of the *CYP21* gene could be demonstrated. Substantial peripheral conversion of progesterone to deoxycorticosterone has been demonstrated in microsomes from several human foetal tissues [Casey *et al.* 1983].

Early molecular studies based on Northern blotting did not find *CYP21* mRNA outside the adrenal cortex [Voutilainen and Miller 1986; Mellon and Miller 1989], but recent use of the more sensitive technique of RT-PCR demonstrated *CYP21* expression in the human skin [Slominski *et al.* 1996], lymphocytes [Zhou *et al.* 1997], heart [Kayes-Wandover and White 2000] and hippocampus [Beyenburg *et al.* 2001], and a putative unrelated 21-hydroxylase activity in skin keratinocytes [Rogoff *et al.* 2001]. Despite the low levels of expression, the

large size of these organs as compared to the adrenal cortex could make them important contributors to peripheral steroid 21-hydroxylation.
Other candidate enzymes to perform extra-adrenal steroid 21-hydroxylation may be hepatic cytochrome P450s. The liver contains many P450s with a wide spectrum of metabolic activities, and 21-hydroxylation of progesterone is one of them in the rabbit [Dieter *et al.* 1982; Sethumadhavan and Senciall 1987], the cow [Ichikawa *et al.* 1984] and the rat [Endoh *et al.* 1995].
Obviously, the extra-adrenal 21-hydroxylation of steroids by CYP21 and by (possibly multiple) unidentified other enzymes, may complicate the diagnosis of defects in the 'regular' adrenocortical steroid biosynthesis and possibly alter the phenotype of patients affected by such diseases.

1.4. Congenital adrenal hyperplasia

Classical steroid 21-hydroxylase deficiency

Steroid 21-hydroxylase deficiency (MIM: 201910) accounts for at least 90 % of all cases of classical congenital adrenal hyperplasia. The next most frequent type of CAH, representing 5-8 % of all cases, is steroid 11β-hydroxylase deficiency; other deficiencies of adrenocortical steroid biosynthesis are relatively rare [White 1994; Pascoe and Curnow 1995; Pang 1997; Stratakis and Rennert 1999; White 2001]
As normal adrenocortical steroidogenesis (see fig. 1) is impaired, the concentrations of steroid hormones change, alternative biosynthetic pathways gain importance and attempts by the organism to counteract these effects further complicate matters. Complete steroid 21-hydroxylase deficiency results in the inability to synthesise aldosterone and cortisol. Lack of aldosterone incapacitates renal resorption of Na^+ and excretion of K^+ and H^+, with a characteristic massive urinary salt loss. Salt-losing (also termed salt-wasting) steroid 21-hydroxylase deficiency is often fatal in untreated paediatric patients [Knudson 1951; Iversen 1955]. Typical symptoms occur within a few weeks after birth and include weight loss and lack of appetite, dehydration, vomiting, diarrhea, acidosis and a general failure to thrive. Lack of cortisol impairs cardiac output and the ability to cope with stress. Patients may succumb to an 'adrenal crisis' involving shock and circulatory collapse [New *et al.* 1989; Pang 1997; New 1998; Speiser 2001; White 2001]. Continual ACTH stimulation in response to absent or reduced cortisol causes hyperplasia of the adrenal cortex and accumulation of precursor steroids such as progesterone and 17α-hydroxyprogesterone which may serve as mineralocorticoid receptor antagonists [Jacobs *et al.* 1961; Holcombe *et al.* 1981; Kuhnle *et al.* 1986] in salt-losing steroid 21-hydroxylase deficiency even in patients where aldosterone is unexpectedly present [Koppens *et al.* 1998]. Curiously, there have been several reports of non-persistence of salt loss later in life [Horner *et al.* 1979; Stoner *et al.* 1986; Sinnott *et al.* 1989; Speiser *et al.* 1991; Hoffman *et al.* 1996] and a patient with a homozygous deletion of the *CYP21* gene who never experienced a salt-losing crisis has been mentioned [Bachega *et al.*

1999]. Also, steroid 21-hydroxylase deficient mice needed exogenous corticosteroids only during a few weeks after birth and survived without treatment after that period [Gotoh *et al.* 1994]. Such seemingly contradictory phenomena may be due to improved renal function later in life [Rösler 1984], to extra-adrenal production of aldosterone, or to both.

Physiological aldosterone levels are typically two or three orders of magnitude smaller than cortisol levels. About 25 % of all patients with classical steroid 21-hydroxylase deficiency retain the capacity to synthesise sufficient amounts of aldosterone to avert the acute crises outlined above. However, the adrenal cortex still turns hyperplastic and produces excess amounts of steroids that do not need 21-hydroxylation (see fig. 1). Although the most abundant of these, 17α-hydroxyprogesterone, is a poor substrate for the 17,20-lyase activity of CYP17 [Auchus *et al.* 1998], peripheral conversion of excess adrenal androgens still leads to high testosterone levels. Testosterone itself does not cause major abnormalities of the internal genitalia in female steroid 21-hydroxylase patients [New *et al.* 1989], but it is converted to dihydrotestosterone in the skin of the external genitalia, causing pre- and postnatal virilisation [Speiser 1999b; White and Speiser 2000; American Academy of Pediatrics 2000]. Virilisation is prominent in all patients with classical steroid 21-hydroxylase deficiency; as opposed to salt losers, patients without salt loss constitute a separate category termed 'simple virilisers'. Since virilisation is obviously more perceptible in girls, steroid 21-hydroxylase deficiency was often not recognised in boys in previous decades and many of them died of an unsuspected adrenal crisis soon after birth.

Nonclassical steroid 21-hydroxylase deficiency

A further category of patients has 'nonclassical' (also termed 'late onset') steroid 21-hydroxylase deficiency, with no salt-losing crises or prenatal virilisation. Here, signs of androgen excess are typically noticed during childhood or later in life [Kohn *et al.* 1982; New *et al.* 1989; Dewailly *et al.* 1993; Morán *et al.* 1998; Morán *et al.* 2000; Speiser 2001]. It should be emphasised here that the distinction between the three categories described above is not very sharp: the severity of salt loss and the degree of virilisation may vary between patients with the same genetic defect, and designations such as 'mild salt loss' or 'some degree of virilisation' are common in the literature.

Deviant growth patterns and precocious puberty are hallmarks of all forms of steroid 21-hydroxylase deficiency, and a frequent reason for medical examination of patients not previously admitted with the more severe symptoms described above. While early growth appears to be relatively normal [Thilén *et al.* 1995], bone age often advances beyond chronological age by several years during childhood (see New [1995] for a striking example), but adult height is below average due to early epiphyseal closure [Grumbach *et al.* 1978; Kohn *et al.* 1982; Kandemir and Yordam 1997; Hagenfeldt 2000; Eugster 2001; New 2001b; Speiser 2001].

Several other signs of androgen excess, especially in women, in steroid 21-hydroxylase deficiency may become manifest later in life [Eldar-Geva *et al.* 1990; Holmes-Walker *et al.*

1995; Rumsby *et al.* 1998; Morán *et al.* 2000]. Reduced fertility has been reported in women and men [Morán *et al.* 1998; Meyer-Bahlburg 1999; Jääskelainen *et al.* 2000; Cabrera *et al.* 2001; Krone *et al.* 2001].

Several types of tumour-like tissue have been found in the adrenals of CAH patients [Takayama *et al.* 1988; Ogo *et al.* 1991; Ravichandran *et al.* 1996]. Also, so-called 'incidentalomas' [Ogo *et al.* 1992; Sasano 1994; Seppel and Schlaghecke 1994; Kloos *et al.* 1995; Abo *et al.* 1999; Sadoul *et al.* 1999; Morioka *et al.* 2000; Grossrubatscher *et al.* 2001] are frequently discovered in the adrenal by computed tomography scans performed for unrelated purposes. Some of these produce steroids and cause clinical phenomena that may resemble various hereditary or acquired defects of adrenocortical steroidogenesis [Rao and Melby 1997; Reincke *et al.* 1997; Forsbach *et al.* 2000; Phillips *et al.* 2000]. Adrenal rest tissue and steroidogenic Leydig cell tumours have been found in the testis [Bongiovanni and Root 1963; Solish *et al.* 1989; Namiki *et al.* 1991; Avila *et al.* 1996; Rich *et al.* 1998]; a recent systematic study found these at high frequency even in well controlled steroid 21-hydroxylase patients [Stikkelbroeck *et al.* 2001]. Adrenal-like tissue has also been detected in the ovary [Russo *et al.* 1998; Al-Ahmadie *et al.* 2001].

Diagnosis

Patients with classical congenital adrenal hyperplasia typically present with one or several of the symptoms summarised above. Deviant serum electrolytes and the resulting increase in plasma renin activity (PRA) are clinical-chemical characteristics of salt loss. Plasma sodium concentrations may drop to 100 mM (normal reference range: 135-145 mM [Mitruka and Rawnsley 1981]). A defining marker of steroid 21-hydroxylase deficiency is a serum level of 17α-hydroxyprogesterone [Lippe *et al.* 1974; Hughes and Winter 1978; Petersen and Christensen 1979] that is well above the upper limit of the normal reference range of 6 nmol/l. Basal levels of more than 1,000 nmol/l have been observed in some patients with classical steroid 21-hydroxylase deficiency [Koppens *et al.* 1998]. An ACTH stimulation test [New *et al.* 1983; Höller *et al.* 1985] allows a distinction between the different categories of the disease, although there is some overlap. Androstenedione offers similar information, but the differences are less pronounced [New *et al.* 1983]. The alternative of 21-deoxycortisol, which is synthesised from 17α-hydroxyprogesterone by CYP11B1 in steroid 21-hydroxylase deficiency and thus provides a convenient distinction with 11β-hydroxylase deficiency, has been advocated more recently [Gueux *et al.* 1988; Fiet *et al.* 1989; Fiet *et al.* 1994].

Radioimmunoassay of steroids purified by conventional chromatography purification is a reliable method, but cross-reactivity of antibodies has been reported [Makela and Ellis 1988; Hashimoto *et al.* 1989; Koshida *et al.* 1989; Brotherton and Rothbart 1990; Al Saedi *et al.* 1996; Andrew 2001]. HPLC separation prior to RIA avoids this problem [Imaizumi *et al.* 1987; Koshida *et al.* 1989; Senciall *et al.* 1990; Volin 1995; Koppens *et al.* 1998]. Other steroid analysis methods have been described [Wallace 1995; Andrew 2001], the most

prominent of which is gas chromatography-mass spectrometry of urinary or serum steroids [Miyazaki *et al.* 1985; Blau *et al.* 1987; Wudy *et al.* 1995; Wudy *et al.* 2000].
Prenatal diagnosis of congenital adrenal hyperplasia in new pregnancies in families with a known index case was first based on steroid measurements in amniotic fluid. The discovery of the genetic linkage of steroid 21-hydroxylase deficiency to the MHC [Dupont *et al.* 1977] soon led to the introduction of HLA serotyping and segregation analysis as a supplementary technique [Price *et al.* 1978; Levine *et al.* 1979; Pollack *et al.* 1979; New *et al.* 1981; Pang *et al.* 1985; Grosse-Wilde *et al.* 1988]. Later, DNA analysis of the *CYP21* gene itself and closely linked MHC markers [Mornet *et al.* 1986; Strachan *et al.* 1987; Killeen *et al.* 1988; Olerup *et al.* 1989; New 1990] allowed earlier diagnosis by chorionic villi sampling. Nowadays, direct detection of *CYP21* mutations [Wedell 1998b; New *et al.* 2001] or microsatellite analysis [Ezquieta *et al.* 1997; Lako *et al.* 1999] are the methods of choice.
Newborn screening to detect unsuspected cases of steroid 21-hydroxylase deficiency is based on assay of steroids (notably, 17α-hydroxyprogesterone) in blood spots on filter paper [Pang *et al.* 1977; Broessler *et al.* 1986]. Screening programmes have been introduced in many countries [Wallace *et al.* 1986; Pang *et al.* 1988; Cicognani 1992; Cutfield and Webster 1995; Lange *et al.* 1995; Balsamo *et al.* 1996; Allen *et al.* 1997; Sack *et al.* 1997; Tajima *et al.* 1997; Witchel *et al.* 1997; Therrell *et al.* 1998; Brosnan *et al.* 1999; Cartigny-Maciejewski *et al.* 1999; Brosnan and Brosnan 2000], including the Netherlands [van der Kamp *et al.* 2001].

Treatment

Steroid replacement therapy has been the universally accepted treatment of CAH patients for more than half a century now. Lacking aldosterone in salt-losing patients is replaced with 9α-fluorocortisol (usually termed fludrocortisone in this context), a strong mineralocorticoid. The glucocorticoid replacement in children is cortisol itself (termed hydrocortisone). Glucocorticoid replacement therapy is aimed at suppressing the hypothalamic-pituitary-adrenal axis in order to avoid excessive ACTH-driven androgen production [Miller and Levine 1987; New 1995; Lopes *et al.* 1998; Charmandari *et al.* 2001; Speiser 2001; White 2001]. Several alternative approaches to the treatment of steroid 21-hydroxylase deficiency have been discussed in recent years [Van Wyk *et al.* 1996; Speiser 1999a; Stratakis and Rennert 1999; White and Speiser 2000].

Incidence of different types of steroid 21-hydroxylase deficiency

The incidence of classical steroid 21-hydroxylase deficiency is often reported as 1:10,000 to 1:15,000. Several factors may influence that figure. Before the advent of neonatal screening, estimates were based on case survey: the number of reported CAH patients who were born within a certain period of time divided by the total number of newborns during that period. A few decades ago, many cases escaped detection, and salt-losing patients often died before the diagnosis of CAH was made. The survival of newborn boys with SL CAH, where virilisation

(which could serve as a warning sign in girls) was not apparent, was especially problematic. This is reflected in the ratio between male and female patients, and between salt losers and simple virilisers (table 1).

Table 1. Percentages of male patients and salt losers in historic and recent reports on classical congenital adrenal hyperplasia

time period[a]	region	patients	% male	% SL	reference
1924-1955	worldwide	135	42.2	--	Iversen [1955][b]
before 1950	Switzerland	49	34.7	20.4	Prader *et al.* [1962][c]
1950-1962	Switzerland	42	47.6	64.3	Prader *et al.* [1962][c]
before 1963	worldwide	--	--	30	Bongiovanni and Root [1963][d]
before 1950	USA (Mass.)	57	--	7.0	Fife and Rappaport[1983]
1950-1959	USA (Mass.)	76	--	47.4	Fife and Rappaport[1983]
1960-1979	USA (Mass.)	57	--	61.8	Fife and Rappaport[1983]
1958-1985	Birmingham, UK	117	40.2	60.2	Virdi *et al.* [1987][e]
1959-1988	Netherlands	48	54.2	70.8	Koppens *et al.* [1992a][f]
1960-1969	USA (Texas)	40	35.0	65.0	Marks and Fink [1969]
1963-1979	France	472	41.9	72.7	Bois *et al.* [1985][g]
1969-1986	Sweden	143	44.1	65.0	Thilén and Larsson [1990][h]
1969-1998	Central Europe	484	43.0	64.7	Kovács *et al.* [2001]
1971-1995	Turkey	219	17.4	38.8	Kandemir and Yordam [1997][i]
1980-1988	worldwide	77	--	75.3	Pang *et al.* [1988][j]
1980-1991	Northern Italy	29	58.6	69.0	Balsamo *et al.* [2000][j]
1986-1994	Sweden	66	45.5	83.6	Thilén *et al.* [1998][j,k]
1989-1995	USA (Texas)	121	54.5	72.7	Therrell *et al.* [1998][j]

--: not reported

a: approximate period during which the patients were born (not always mentioned in the cited reports)

b: report includes only salt losers

c: calculated from table 4

d: percentage salt losers as stated in this review

e: percentage salt losers based on 113 patients

f: supplemented with data from Chapter 2.2

g: compiled from table II and the text

h: calculated from table 2

i: compiled from table 1

j: neonatal screening programme; the other studies are case surveys

k: compiled from table 3 and the text

Earlier diagnosis during recent years [Fife and Rappaport 1983; Virdi *et al.* 1987; Thilén and Larsson 1990; Kovács *et al.* 2001] has probably contributed to the improvement in the detection of salt-losing patients, but screening programmes are necessary to avoid missing SL patients, since they find a higher incidence of steroid 21-hydroxylase deficiency than case survey in the same region [Pang *et al.* 1988; Hofman and Pang 1993; Therrell 2001]. Clearly,

reliable estimates of disease incidence and allele frequency in each population can only be made by systematic searches in large groups of individuals. Table 2 summarises such data from several countries.

Table 2. Approximate incidence of classical congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency in several countries.

country	incidence	reference
Belgium	1 : 25000	F. Eyskens[a]
Brazil	1 : 7500	b
France	1 : 11337	Cartigny *et al.* [1999][c]
Germany	1 : 8000	A.A. Roscher[d]
Israel (Arabs)	1 : 7979	Sack *et al.* [1997]
Israel (Jews)	1 : 29589	Sack *et al.* [1997]
Italy (Emilia)	1 : 25090	Balsamo *et al.* [2000]
Italy (Romagna)	1 : 7437	Balsamo *et al.* [2000]
Japan	1 : 18000	Tajima *et al.* [1997]
Netherlands	1 : 11779	van der Kamp *et al.* [2001]
New Zealand	1 : 23344	Cutfield and Webster [1995]
Phillipines	1 : 7000	C.D. Padilla[b]
Portugal	1 : 14285	e
Sweden	1 : 9800	Thilén *et al.* [1998]
Switzerland	1 : 12730	Lange *et al.* [1995]
Taiwan	1 : 28000	Lee *et al.* [2000b][f]
UK (Scotland)	1 : 18410	Wallace *et al.* [1986]
USA (Texas)	1 : 16008	Therrell *et al.* [1998]
USA (Wisconsin)	1 : 10692	Allen *et al.* [1997]
USA (Alaska, Yupik)	1 : 282	Pang *et al.* [1982][g]

a: quoted by van der Kamp *et al.* [2001]
b: quoted by Therrell [2001]
c: including three suspected patients who died before recall
d: quoted by Krone *et al.* [2000]
e: quoted by Hofman and Pang [1993]
f: no neonatal screening; calculated from a mutation analysis of 1000 controls
g: salt-losers only

Clearly, classical steroid 21-hydroxylase deficiency is a common inherited metabolic disorder, with an estimated worldwide allele frequency of 1:60 [Pang *et al.* 1988], but considerably higher in some populations. Remarkable differences in incidence may exist over a relatively small distance between apparently related population groups, such as between two adjacent regions in Italy [Balsamo *et al.* 2000].

Similar data are not available for nonclassical 21-hydroxylase deficiency, because patients with this mild form of the disease often escape detection by neonatal screening. Reported estimates based on ACTH testing in selected groups are much higher: 0.1 to 1 % of the

general Caucasian population (which implies a carrier frequency of 1:5 to 1:16) and 3 to 5 % in Ashkenazi Jews [Speiser *et al.* 1985; Sherman *et al.* 1988; Speiser *et al.* 1989; Dumic *et al.* 1990; Zerah *et al.* 1990]. However, searches for the most common mutations in this disease in several countries [Dacou-Voutetakis and Dracopoulou 1999; Fitness *et al.* 1999; Dolzan *et al.* 1999; Chapter 3.2] did not confirm this high carrier frequency in other Caucasian populations. Since androgen excess is a feature of congenital adrenal hyperplasia, several authors have searched for unrecognised cases of (nonclassical) steroid 21-hydroxylase deficiency in hyperandrogenic women. Here too, recent molecular data [Blanché *et al.* 1997; Ostlere *et al.* 1998; Dolzan *et al.* 1999; Escobar-Morreale *et al.* 1999] did not confirm earlier findings based on hormonal data [Morán *et al.* 1998].

1.5. The *CYP21* gene in the Major Histocompatibility Complex

The significance of the Major Histocompatibility Complex (MHC) to the study of steroid 21-hydroxylase deficiency is one of genetic linkage. The co-segregation of the deficient allele with the HLA type in families of 21-hydroxylase patients was first described by Dupont *et al.* in 1977 [Dupont *et al.* 1977], a discovery foreshadowed five years earlier by the finding that testosterone production in mice was linked to the H-2 system (the murine MHC) [Iványi *et al.* 1972]. Additional linkage studies allowed the construction of an early version of an MHC map that included the steroid 21-hydroxylase locus [Levine *et al.* 1978].

The associations of salt-losing steroid 21-hydroxylase deficiency with the HLA-B47 serotype and of the nonclassical (then termed 'late-onset') form with HLA-B14 were described soon after [Klouda *et al.* 1978; Pucholt *et al.* 1978; Laron *et al.* 1980]. HLA serotyping was soon used for segregation studies and prenatal diagnosis of steroid 21-hydroxylase deficiency [Levine *et al.* 1979; New *et al.* 1981]. These insights led the way to the association of the 21-hydroxylase gene with the gene encoding the fourth component of complement [O'Neill *et al.* 1982] and to the identification of the gene later known as *CYP21* [White *et al.* 1984c]. Closely linked markers remain an important tool to follow the segregation of steroid 21-hydroxylase deficiency, although HLA serotyping has given way to microsatellite marker analysis [Ezquieta *et al.* 1997; Lako *et al.* 1999; White and Speiser 2000; Matsuzaka *et al.* 2001].

The human MHC

The human MHC is a segment of the short arm of chromosome 6, located at the chromosomal band 6p21.31 (see fig. 3). The size of the conventional MHC is at least 3.3 Mb and inclusion of peripheral areas that have been characterised in recent years extends it to nearly 8 Mb [Beck and Trowsdale 2000]. Understandably, there has been some dispute as to where the MHC should end [Klein and Sato 1998].

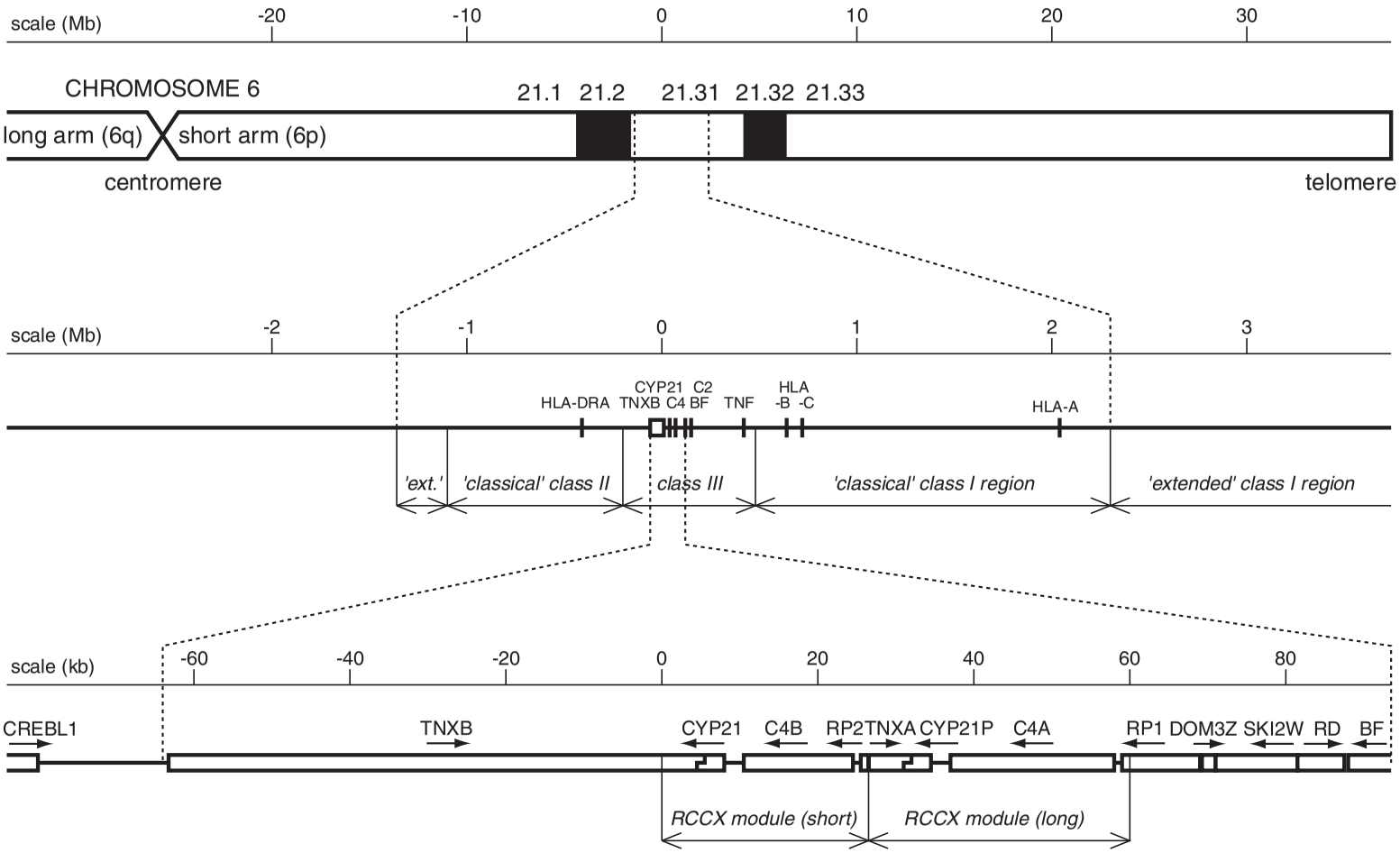
The MHC derives its name from the term 'histocompatibility', referring to the early studies - on rejection or acceptance of transplanted tumours in mice - that defined this area of research

[Gorer *et al.* 1948]. The proteins involved in this host-versus-graft reaction were termed H-2 in mice and HLA (Human Leukocyte Antigen) in humans. Insight into the crucial role of the MHC in immunity came later, initiated by the Nobel-Prize-winning research of Zinkernagel and Doherty [1974].

Class I HLA molecules are on the outside of most cells, presenting cytosolic peptides to patrolling cytotoxic T lymphocytes. Class II HLA molecules are expressed on B lymphocytes and macrophages (and on some other cells), presenting extracellular peptides (such as the remains of bacteria that have been eliminated by macrophages) to helper T cells [Abbas *et al.* 1997].

The classical HLA class I genes, *HLA-A*, *-B* and *-C*, are highly polymorphic; several more recently discovered class I-like genes show a smaller degree of polymorphism [Marsh *et al.* 2001]. The designation HLA-D was originally used to define a serologically identifiable B lymphocyte antigen that was soon found to be identical to another antigen first named 'DR' (D-related). The name *HLA-DR* was assigned to the corresponding class II locus and by a peculiar twist of genetic etymology, other HLA class II loci were baptised *HLA-DP*, *-DQ*, *-DM*, etc.

Fig. 3. Overview of the human MHC. Top: short arm of chromosome 6 with band 6p21.31 (not all chromosome bands are shown). Centre: Major Histocompatibility Complex with the 'classical' and 'extended' ('ext.') class II and class I regions and the central region known as 'class III'. Only a few 'well-known' genes are shown; many more genes and pseudogenes exist in each region [Beck and Trowsdale 2000; Milner and Campbell 2001]. Bottom: the RCCX region and its surroundings. A bimodular arrangement is shown; the Human Genome Project sequence of the area [Rowen *et al.* 1999a] features a monomodular sequence and uses the designation *'CYP21A2'* for the *CYP21* gene. The origin of the scale is the centromeric boundary of the RCCX region. The dotted lines do not delineate either the MHC in the central part of the figure or the RCCX region in the bottom part, but merely serve as an indicator of the magnification. The arrows indicate the direction in which each gene is transcribed. Proteins encoded are: *HLA*-A, -B, -C and -DRA: HLA antigens; *TNF*: tumour necrosis factor; *BF*: complement factor B (properdin); *C2*: second component of complement; *RP1*, *C4, CYP21, TNXB*: see text; the exact function of the ubiquitously expressed genes *DOM3Z*, *SKI2W* and *RD* [Yang *et al.* 1998] remains to be elucidated.


scale (Mb)
-20
-10
0
10
20
30
CHROMOSOME 6
21.1
21.2
21.31
21.32
21.33
long arm (6q)
short arm (6p)
centromere
telomere
scale (Mb)
-2
-1
0
1
2
3
HLA-DRA
CYP21
TNXB
C4
C2
BF
TNF
HLA
-B
-C
HLA-A
'ext.'
'classical' class II
class III
'classical' class I region
'extended' class I region
scale (kb)
-60
-40
-20
0
20
40
60
80
CREBL1
TNXB
CYP21
C4B
RP2
TNXA
CYP21P
C4A
RP1
DOM3Z
SKI2W
RD
BF
RCCX module (short)
RCCX module (long)

The 0.7 Mb of DNA that separates the MHC class I and class II areas has been termed 'class III', although the genes it contains are structurally and functionally unrelated to the HLA antigens. Quite a few class III genes are somehow related to immune function, but many others are not, and many non-HLA genes have been found in the class I and II regions as well. A recent gain in subdividing the MHC has been the 'class IV region' [Gruen and Weissman 2001], a proposed split of class III to distinguish genes involved in inflammation from the complement area. However, others have questioned the wisdom of any concept of the entire MHC as a immunological 'superstructure' and indeed there seems no reason to assume that unrelated genes residing in the MHC owe their physical closeness to anything but chance - although a case can be made for co-ordination of expression [Kelly and Trowsdale 1994]. It has been rightfully pointed out that while 'class I' and 'class II' are acceptable designations for the respective HLA genes and proteins, the term 'class III' has no functional significance [Klein and Sato 1998; Dawkins *et al.* 1999]. Summarising, the question: 'What is the MHC?' [Powis and Geraghty 1995] is a matter of ongoing research and debate.

Polymorphism and evolution of the MHC

The classical class I HLA genes (*HLA-A*, *-B* and *-C*) and most of the class II genes (notably, *HLA-DRB1* and *-DPB1*) are highly polymorphic [Marsh *et al.* 2001]. Several types of natural selection are believed to be responsible for maintaining this high degree of polymorphism in different populations [Forsdyke 1991; Kaufman *et al.* 1993; Parham and Ohta 1996; Meyer and Thomson 2001]: in the eloquent wording of Potts and Wakeland [1993], it is 'a tale of incest, pestilence and sexual preference'. The complexity and the organisation of the mammalian MHC are not typical of other vertebrates: for example, the chicken MHC is only 0.1 Mb in size and has the regions in a II-I-III order [Kaufman *et al.* 1999] and the class I and class II region are not linked in many teleostic fish [Klein and Sato 1998]. How the MHC evolved is a matter of controversy in evolution biology [Abi Rached *et al.* 1999; Kasahara 1999; Yeager and Hughes 1999; Hughes and Pontarotti 2000]. However, consensus seems to exist about the following issues:

- the genes now referred to as 'class III' existed before the class I and class II genes [Klein and Sato 1998];
- duplication and translocation of chromosomal segments have generated the tandem arrangement of structurally similar class I and II genes [Trowsdale *et al.* 1995; Dawkins *et al.* 1999];
- the layout of each region varies dramatically between species [Kaufman *et al.* 1993; Kronenberg *et al.* 1994; Trowsdale 1995; Klein and Sato 1998];
- the class I and class II regions contain many pseudogenes [Beck and Trowsdale 2000], but the class III region does not (the 21-hydroxylase pseudogene *CYP21P* is a rare exception) [Milner and Campbell 2001];
- recombination, gene conversion and point mutation have each contributed to the variability of the MHC genes and alleles in various ways [Kuhner *et al.* 1991; Ohta 1995;

Ohta 1997; Martinsohn *et al.* 1999; Högstrand and Böhme 1999; Carrington 1999], which may differ per species, per region and per locus [Cadavid *et al.* 1999; Matsui *et al.* 1999; Yeager and Hughes 1999];
- recombination occurs more often in specific small areas ('hotspots') than in the relatively stable large regions ('frozen blocks') that separate them [Carrington 1999; Dawkins *et al.* 1999; Jeffreys *et al.* 2001].

The genetic mechanisms that drive the evolution of the MHC also play a key role in generating diversity at the *CYP21* locus, thus creating the inherited defects that cause steroid 21-hydroxylase deficiency (see below).

Ancestral haplotypes and MHC disease association

An ancestral haplotype is a conserved set of (MHC) loci that occurs in a specific population at a higher frequency than might be expected from the frequencies of each individual marker that is characteristic of the haplotype. The concept of ancestral haplotypes (previously: extended haplotypes, or supratypes) [Dawkins *et al.* 1983; Degli-Esposti *et al.* 1992] has proved its value to define the association between certain diseases and the MHC: although some diseases (such as steroid 21-hydroxylase deficiency) are clearly caused by a defect in a single gene, many others remain elusive and are more clearly associated with an extended haplotype than with an allelic variant at any individual MHC locus. Ancestral haplotypes consist of 'polymorphic frozen blocks' that vary in size and composition [Tokunaga *et al.* 1988; Zhang *et al.* 1990], and a growing number of characteristic alleles is assigned to each haplotype [Dawkins *et al.* 1999]. Although such areas show reduced combination [O'hUigin *et al.* 2000], the 'freezing' should not be taken too literally, as the study of the *CYP21/C4* loci has clearly shown: for example, differences in size [Donohoue *et al.* 1987] and composition [Chu *et al.* 1992; Levo and Partanen 1997a] have been found in the HLA-B47 haplotype that is strongly associated with steroid 21-hydroxylase deficiency, and unequal crossing-over has been observed in the well-characterised 8.1 ancestral haplotype [Sinnott *et al.* 1990; Yang *et al.* 1999], as well as other variants of the RCCX module [Fasano *et al.* 1992; Koppens *et al.* 2002a].

Not surprisingly, many MHC-associated diseases have a (putative or confirmed) immunological component. To name but a few: ankylosing spondylitis [Szöts *et al.* 1986; Dawkins *et al.* 1999], common variable immunodeficiency and IgA deficiency [Volanakis *et al.* 1992; Price *et al.* 1999], insulin-dependent diabetes mellitus [Cohen-Haguenauer *et al.* 1985; Cheong *et al.* 2001], multiple sclerosis [Hillert and Olerup 1993; Ramachandran and Bell 1995; Haines *et al.* 1998], myasthenia gravis [Leelayuwat *et al.* 1994; Franciotta *et al.* 2001], myocardial infarction [Kramer *et al.* 1994; Lefvert *et al.* 1995; Nityanand *et al.* 1999]; psoriasis vulgaris [Dawkins *et al.* 1999; Gruen and Weissman 2001], rheumatoid arthritis [Hillarby *et al.* 1990; Mimori *et al.* 1990; Park *et al.* 1996; Rupert *et al.* 1999; Ota *et al.* 2001], spontaneous abortions [Laitinen *et al.* 1992; Lokki and Laitinen 2001], sudden infant death [Schneider *et al.* 1989; Opdal *et al.* 1999], systemic sclerosis [Briggs *et al.* 1993; Takeuchi *et*

al. 1998; Venneker *et al.* 1998], systemic lupus erythematosus [Schur *et al.* 1990; Takeuchi *et al.* 1996; Würzner and Dierich 1997; Carroll 1998; Reveille *et al.* 1998; Veggeberg 1998; Takeda *et al.* 1999; Einav *et al.* 2002] and susceptibility to several infectious diseases [Hill *et al.* 1991; De Messias *et al.* 1993; Leelayuwat *et al.* 1993; Jaatinen *et al.* 1999; Price *et al.* 1999; Meyer and Thomson 2001; Seppänen *et al.* 2001]. The HLA- and complement C4-association of the diseases on this far-from-complete list were often identified early, but recent research has expanded to defects in other genes as well [Gruen and Weissman 2001]. Steroid 21-hydroxylase deficiency was the first HLA-linked disease that was obviously not related to the immune system [Dupont *et al.* 1977]. Although the completion of the MHC genomic sequence [MHC Sequencing Consortium 1999] has greatly facilitated the analysis of monogenic diseases, finding the genetic basis of many clinically diverse and probably polygenic disorders that are associated with specific ancestral haplotypes, remains a challenging task for years to come [Price *et al.* 1999; Alper and Awdeh 2000; Gruen and Weissman 2001].

Genes of the central MHC (the class III region)

As explained above, the genes of the central MHC share no common characteristics and the term 'class III region' is merely a designation of a 730 kb stretch of DNA that lies between the class II and class I regions without harbouring any of the typical HLA genes (see fig. 3). The class III area contains at least 62 genes, with *NOTCH4* near the centromeric and *BAT1* near the telomeric end [Beck and Trowsdale 2000; Milner and Campbell 2001]. Insight in to the structure and function of the individual genes is growing rapidly [Cheng *et al.* 1993; Iris *et al.* 1993; Marshall *et al.* 1993; Dangel *et al.* 1995b; Albertella *et al.* 1996; Nalabolu *et al.* 1996; Yang *et al.* 1998; Yu 1998; Dawkins *et al.* 1999; Allcock *et al.* 2001; Gruen and Weissman 2001; Milner and Campbell 2001].

The area surrounding the *CYP21* gene lies some 200 kb from the centromeric end of the class III region. This stretch of DNA has the most complicated structure of the MHC and probably of the entire human genome. The four key constituent genes (starting from the telomeric side) are:

- *RP1* (also known as *G11* or *STK19*), 11.4 kb in size and ubiquitously expressed [Sargent *et al.* 1994; Shen *et al.* 1994; Yang *et al.* 1998], encoding a nuclear serine/threonine protein kinase [Gomez-Escobar *et al.* 1998];
- *C4*, either 20.5 or 14.2 kb in size and primarily expressed in the liver, encoding the fourth component of complement [Carroll and Porter 1983; Belt *et al.* 1984; Carroll *et al.* 1984; Yu *et al.* 1988; Yu 1991; Dangel *et al.* 1994];
- *CYP21* (also known as *CYP21B*, *21OHB*, or several variant designations), 3.4 kb in size and primarily expressed in the adrenal cortex, encoding steroid 21-hydroxylase [White *et al.* 1984c; White *et al.* 1985; Carroll *et al.* 1985b; Higashi *et al.* 1986; White *et al.* 1986; Rodrigues *et al.* 1987];

- *TNXB* (also known as *TNX* or *XB*), 68.2 kb in size and expressed in several tissues including skin, tendons and blood vessels, encoding an extracellular matrix protein [Morel *et al.* 1989b; Gitelman *et al.* 1992; Matsumoto *et al.* 1992; Bristow *et al.* 1993b].

These four genes constitute the RCCX module, a stretch of DNA that is usually present in a tandemly duplicated arrangement (see below). As a consequence of this duplication, most chromosomes carry two complement *C4* genes (*C4A* and *C4B*) and one *CYP21* gene plus one *CYP21P* pseudogene.

The complement system is aimed at destroying the cell walls of invading bacteria, or generally, any cell to which an antibody has attached. The fourth component (C4) is required for the classical pathway of complement activation [Abbas *et al.* 1997]. C4 has two isoforms, C4A ('acidic') and C4B ('basic') that differ from each other by a few amino acids in a protein region known as C4d. Activated C4 binds covalently to the cell surface; C4A has a higher affinity towards amino groups, C4B towards hydroxyl groups [Campbell *et al.* 1986; Williamson and Turner 1987; Law and Dodds 1990; Dodds and Law 1990; Yu 1998; Martinez *et al.* 2001]. The C4 proteins are highly polymorphic: the C4d region also contains the Chido and Rodgers blood cell antigens, each of which have multiple subtypes [Carroll and Alper 1987; Yu *et al.* 1988; Yu 1998; Blanchong *et al.* 2001; Martinez *et al.* 2001].

Non-expression of either *C4A* or *C4B* is relatively common in the general population, with reported frequencies of *C4A**Q0 and *C4B**Q0 of 5-20 % [Howard *et al.* 1986; Wang *et al.* 1989; Hartung *et al.* 1992; Briggs *et al.* 1993; Franciotta *et al.* 1995; Nityanand *et al.* 1995; Lhotta *et al.* 1996]. Nevertheless, an increased frequency of C4 null alleles has been observed in many patients reporting with diverse immunological problems, including most of the HLA-associated diseases listed in the previous paragraph. Complete C4 deficiency is a rare disease, associated with severe forms of systemic lupus erythematosus in most cases [Reveille *et al.* 1985; Fredrikson *et al.* 1991; Fasano *et al.* 1992; Lokki *et al.* 1999].

Tenascin-X is the name assigned to the product of the *TNX* gene [Bristow *et al.* 1993b]. Tenascins are large extracellular matrix proteins, expressed in specific embryonal and adult tissues. At present, five tenascins have been described [Jones and Jones 2000; Joester and Faissner 2001]. The *TNXB* gene was first discovered by cDNA cloning of a gene expressed on the opposite strand of *CYP21* [Morel *et al.* 1989b; Gitelman *et al.* 1992]. The biological function of tenascin-X was explained by the finding that patients with *TNX* deficiency suffer from a variant of the Ehlers-Danlos syndrome [Burch *et al.* 1997; Mao and Bristow 2001; Schalkwijk *et al.* 2001], a term that covers a spectrum of skin and connective tissue disorders, some of which have been characterised at the molecular level [Burrows 1999]. Tenascin-X deficiency patients manifest characteristics such as hyperextensible skin, poor wound healing, joint laxity and vascular fragility.

The RCCX module

The RCCX module was named after the four genes listed in the previous paragraph (*RP1-C4-CYP21-XB*) [Shen *et al.* 1994; Yu 1998]. Its modular character has significance at the DNA

level only, but exerts a powerful influence on the structure and expression of three of these genes. The RCCX module has its centromeric boundary in intron 32 of *TNXB* and its telomeric boundary in exon 5 of *RP1*. There are two size variants: a 'short' RCCX module is 26,483 bp and a 'long' RCCX module is 32,856 bp [Rowen *et al.* 1999a]. The difference is determined by the presence or absence of a 6373 bp insert in the ninth intron of the *C4* gene, which is an inserted human endogenous retrovirus [Patience *et al.* 1997] known as HERV-K(C4) [Schneider *et al.* 1986; Palsdottir *et al.* 1987; Yu and Campbell 1987; Dangel *et al.* 1994; Tassabehji *et al.* 1994; Chu *et al.* 1995; Dangel *et al.* 1995a]. HERV-K(C4) is oriented in an opposite direction to *C4* and it may provide an antisense-based protection mechanism against exogenous retroviruses [Schneider *et al.* 2001].

Most human chromosomes have two RCCX modules (see figs. 3 and 4; the term 'RCCX *region*' will be used to designate all contiguous RCCX *modules* on the same chromosome). This is apparently due to a duplication of a primordial single RCCX module that happened more than 23 million years ago, well before the speciation of the great apes [Kawaguchi *et al.* 1990; Bontrop *et al.* 1991; Kawaguchi *et al.* 1992; Horiuchi *et al.* 1993]. In an evolutionary step typical of the expansion of the MHC, this duplication created two separate *C4* genes. The *CYP21* gene was copied at the same time. A mechanism can be envisioned by which *C4* and *CYP21* developed in opposing directions from this situation of two identical tandem RCCX modules. Variants of *C4* with different affinities probably already existed, and a crossover or gene conversion involving such a variant could well have generated the first bimodular chromosome with two different *C4* genes, conferring a selectional advantage due to a greater flexibility of attachment to different antigens. This notion is supported by the observation that *C4* duplicated independently in many other species [Horiuchi *et al.* 1993]; for example, a region of 55 kb (again including the *Cyp21* gene) has been duplicated in the mouse [Chaplin *et al.* 1983; White *et al.* 1984a]. In contrast to *C4*, two active *CYP21* genes offered no benefit and may have been a liability considering the sensitivity of regulatory mechanisms (despite the variability of the RCCX region found in modern humans, no chromosome with two active *CYP21* genes has yet been found). At the very least, the second *CYP21* gene was not needed, and analysis of *CYP21P* variability [Horiuchi *et al.* 1993; Koppens *et al.* 2000] suggests that it gradually accrued several deleterious mutations in a specific order.

The *TNXB* and *RP1* genes were duplicated in part, giving rise to truncated remainders known as *TNXA* (*XA*) and *RP2*. While the 1 kb duplicated part of *RP2* is identical to the corresponding part of *RP1*, the 5.7 kb *TNXA* pseudogene has a 120 bp deletion on an intron-exon boundary as compared to *TNXB*. This is inconsequential in the normal situation, but can play a role in the generation of genetic defects (see below).

As mentioned above, the *TNX* genes are transcribed in the opposite direction of the *RP*, *C4* and *CYP21* genes. The 3' ends of *TNXB* (which is also expressed in the adrenal cortex) and *CYP21* overlap, and their mRNAs need protection by nuclear proteins to avoid hybridisation *in vivo* [Speek and Miller 1995]. The transcriptional complexity of the RCCX region is remarkable: in this extremely gene-dense area, no fewer than fifteen RNA transcripts have so far been found (see fig. 4). By analogy to the designation 'gene X' which was initially used for

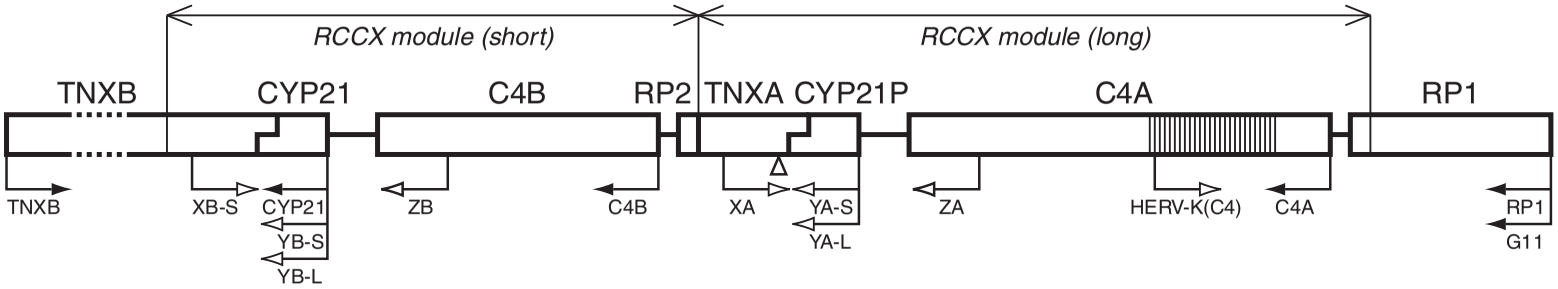


Fig. 4. Transcriptional complexity of the RCCX module. The arrows indicate the orientation, but not the size, of RNAs produced in the RCCX module. When multiple transcripts use the same promoter, the arrows start in the same position. The figure is an example of the common bimodular arrangement of the RCCX region; many other possibilities exist (see fig. 1 in Chapter 2.2) and the RNAs shown change accordingly. The hatched area in the *C4A* gene represents the HERV-K(C4) retroviral insert. Closed arrowheads represent RNAs that are translated into protein: *RP1*, *C4A*, *C4B*, *CYP21* and *TNXB* (for references, see the text). *G11* [Sargent *et al.* 1994] is a shorter form of *RP1* [Shen *et al.* 1994]. Open arrowheads represent RNAs that are not translated or where no protein has been described: *TNXA*(*XA*) [Gitelman *et al.* 1992], *XB-S* [Tee *et al.* 1995a], *YA-S* (short), *YA-L* (long), *YB-S*, *YB-L* [Bristow *et al.* 1993a; Speek and Miller 1995; Endoh *et al.* 1998]; *ZA*, *ZB* [Tee *et al.* 1995b]; *HERV-K(C4)* [Dangel *et al.* 1994]; [Schneider *et al.* 2001]. Upstream regulatory elements for *CYP21* expression are near *ZB* [Tee *et al.* 1995b]; [Wijesuriya *et al.* 1999]. No *CYP21P* mRNA equal in size to the *CYP21* mRNA has been found [Al-Othman *et al.* 1988; Endoh *et al.* 1998].

TNX, some of these RNA's have been assigned to 'gene Y' and 'gene Z', each of which is also duplicated (hence *YA*, *YB*, *ZA* and *ZB*). However, the mRNA's of 'only' five genes in the RCCX region (*C4A*, *C4B*, *CYP21*, *TNXB* and *RP1/G11*) are actually translated into protein.

CYP21 and *CYP21P* gene structure

The *CYP21* gene, encoding steroid 21-hydroxylase and its pseudogene *CYP21P*, are highly similar, sharing about 98 % sequence homology [Higashi *et al.* 1986; White *et al.* 1986; Rodrigues *et al.* 1987]. The most significant differences are shown in fig. 8 in paragraph 1.7: several mutations cause *CYP21P* to be inactive, and the same mutations are the most frequent cause of CAH caused by 21-hydroxylase deficiency (see below). There is nothing very remarkable about the *CYP21* gene itself: it owes its complex genetics exclusively to the turbulent genetic environment of the RCCX region.

Steroid 21-hydroxylase is either 494 or 495 amino acids in size (the difference is due to an 3 bp insert in the first exon that has no influence on the enzyme's function [Higashi *et al.* 1991]). It has domains for integration into the membrane, interaction with cytochrome P450 reductase, haem binding, substrate binding and oxygen/water binding [Lewis and Lee-Robichaud 1998; White and Speiser 2000]. Studies of artificial and naturally occurring mutations have identified the amino acids that define the functioning of these domains [Sakaki *et al.* 1990; Wu *et al.* 1991; Hsu *et al.* 1993; Hsu *et al.* 1996; Hu *et al.* 1996; Nikoshkov *et al.* 1998].

Several elements in the promoter region of *CYP21* influence its expression in different ways [Rice *et al.* 1990; Schimmer and Parker 1992; Parissenti *et al.* 1993; Wilson *et al.* 1993; Morley *et al.* 1996; Chin and Chang 1998]. It has been found that the promoter region of *CYP21P*, although structurally similar to the *CYP21* promoter, has at most 10-20 % of its activity [Bristow *et al.* 1993a; Chang and Chung 1995; Kyllo *et al.* 1995; Endoh *et al.* 1998]. Some regulatory elements of *CYP21* lie as far as 3 to 5 kb upstream, inside the *C4* gene [Milstone *et al.* 1992; Watanabe *et al.* 1993; Tee *et al.* 1995b; Wijesuriya *et al.* 1999].

1.6. Variability of the RCCX region

Approximately two-thirds to three-quarters of all copies of chromosome 6 have two RCCX modules, and a large majority of these have the (centromere-to-telomere) layout *TNXB-CYP21-C4B-RP2-TNXA-CYP21P-C4A-RP1*, as described above and illustrated in figs. 3 and 4. However, there are many variants with regard to the number of modules and to the composition of each gene within those modules. The overall layout of the RCCX region, which can vary from monomodular to quadrimodular, is described in detail in Chapter 2, and Chapters 4, 5 and 8 provide a discussion about the genetic mechanisms that account for this variability. The most prominent disease-causing mutations of *CYP21* and the consequences of the variability of *CYP21P* are discussed in Chapter 3.1. A concise overview of RCCX variability should therefore suffice here.

Historically and technically, the dual complementary approach to analysing RCCX variability is:

- 'haplotyping' by restriction analysis, Southern blotting and hybridisation;
- a gamma of other methods, often based on PCR amplification of sequences of interest.

Of course, this approach is based on the insights gained from earlier biochemical and genetic research, and a wealth of other techniques from molecular biology have been successfully applied to answer many questions regarding the structure, regulation and expression of the genes in the RCCX module - but the approach outlined above covers a vast area of research in this field and is as viable today [White and Speiser 2000; Baumgartner-Parzer *et al.* 2001; Blanchong *et al.* 2001] as it was more than a decade ago [White *et al.* 1988; Morel *et al.* 1989a; Owerbach *et al.* 1990].

Conceptually, analysing RCCX variability revolves around two questions:

- How many RCCX modules are aligned on each chromosome 6 and what is their composition?
- What defines the structure and function of the genes in each module?

The answers to these questions allow us to understand the origin of disease-causing mutations in this intriguing part of the human genome. As explained below, the dual experimental approach mentioned above roughly parallel these questions.

CYP21/*C4* haplotyping

A haplotype can simply be described as a chromosomal segment inherited from one parent [Dawkins *et al.* 1983]. In terms of the RCCX region, a haplotype is usually defined in terms of certain characteristics of the *CYP21* (and *CYP21P*) and *C4* genes. There is no generally accepted standard that defines which characteristics make up an RCCX haplotype, so depending on the focus of their research, different authors have described haplotypes in terms of *CYP21* genes [Werkmeister *et al.* 1986; Dawkins *et al.* 1987; White *et al.* 1988; Morel *et al.* 1989a; Strumberg *et al.* 1992], *C4* genes [Palsdottir *et al.* 1987; Partanen 1987; Teisberg *et al.* 1988; Blanchong *et al.* 2000], or both [Carroll *et al.* 1985a; Rumsby *et al.* 1988; Collier *et al.* 1989; Partanen *et al.* 1989; Hillarby *et al.* 1990; Schneider 1990; Haglund-Stengler *et al.* 1991; Koppens *et al.* 1992a; Weg-Remers *et al.* 1997; Lobato *et al.* 1998]. In this thesis, each haplotype is assigned a capital letter to represent the configuration of *CYP21*/*CYP21P*, plus a number to distinguish between the different sizes of the *C4* genes [Koppens *et al.* 1992a], but several other systems have been used by other groups [Hillarby *et al.* 1990; Schneider 1990; Haglund-Stengler *et al.* 1991; Weg-Remers *et al.* 1997; Blanchong *et al.* 2000]. A nomenclature of that is both concise and systematic has not been proposed so far.

Technically, *CYP21*/*C4* haplotyping has greatly benefited from the fortuitous circumstance that *Taq*I restriction patterns proved to be an extremely reliable indicator of the composition of the RCCX region. The *Taq*I sites and cDNA probes used for haplotyping are shown in fig. 5 (the method is discussed in detail in paragraph 1.8 and Chapter 2).

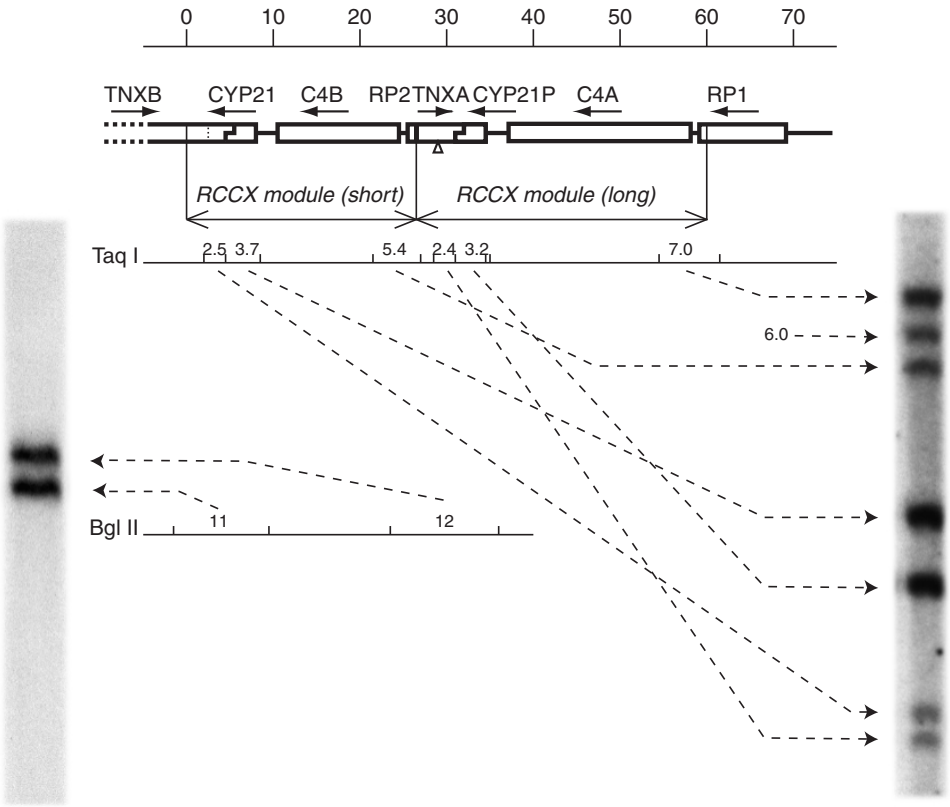


Fig. 5. *Taq*I and *Bgl*II restriction sites in a bimodular RCCX module, and typical banding patterns. This example shows two RCCX modules, a long one and a short one; the 5.4 and 7.0 kb *C4* bands are characteristic of this haplotype. The *Taq*I banding pattern shown indicates heterozygosity: the other haplotype of this individual has two long RCCX modules, as shown by the 6.0 kb *Taq*I band. The small triangle underneath *TNXA* represents a 120 bp deletion; the corresponding site in *TNXB* (which has no deletion) is indicated by a dashed line. The *Bgl*II patterns are the same for all bimodular chromosomes: the 11 kb band represents the most centromeric module, while all other modules have a 12 kb band.

The *CYP21* probe allows a distinction between *CYP21* and *CYP21P* and between *TNXA* and *TNXB*:

- 3.7 kb: *CYP21*;
- 3.2 kb: *CYP21P*;
- 2.5 kb: *TNXB*;
- 2.4 kb: *TNXA*.

The 3.7 kb versus 3.2 kb difference is due to an additional *Taq*I site in the 5' flank of *CYP21P* only (see figs. 5 and 6). The nucleotide change involved lies close to, but not inside, a region of importance to the activity of the *CYP21* promoter [Chang and Chung 1995]. A completely functional *CYP21* gene that has this extra *Taq*I site has never been found, but partly active hybrid genes have been described [Killeen *et al.* 1991; l'Allemand *et al.* 2000]. Although rare, these exceptions may confound the interpretation of *CYP21* haplotypes. The 2.5 kb versus 2.4 kb difference is due a 120 bp deletion in *TNXA*.

The *C4* probe allows a distinction between 'long' (20.5 kb) and 'short' (14.2 kb) *C4* genes and it shows whether *C4* is adjacent to an *RP1* gene or to a truncated *RP2* pseudogene. The following fragments can be found:

- 7.0 kb: long *C4* gene adjacent to *RP1*;
- 6.4 kb: short *C4* gene adjacent to *RP1*;
- 6.0 kb: long *C4* gene adjacent to *RP2*;
- 5.4 kb: short *C4* gene adjacent to *RP2*.

These differences are due to the position of the most telomeric *Taq*I site, which lies outside of the RCCX region, and to a *Taq*I site in the 6.3 kb HERV-K(*C4*) insert. The sizes listed above are those traditionally mentioned in the literature [Schneider *et al.* 1986], but the fragments are actually about 0.2 kb larger [Koppens *et al.* 1992b; Rowen *et al.* 1999a].

Other *Taq*I bands than those listed above have been described in a few isolated cases [Higashi *et al.* 1988a; Goldstein *et al.* 1991; Koppens *et al.* 1992b; Lim and Chan 1996; Bachega *et al.* 1999].

*Taq*I digestion of genomic DNA and hybridisation to *CYP21* and *C4* probes is usually sufficient by itself, especially when a segregation study can be done. *Bgl*II restriction analysis is a useful complementary method, especially when no *C4* probe is used, to distinguish between monomodular and bimodular in CAH patients with an apparent deletion of the *CYP21* gene. In complicated cases, long-range *Sac*II restriction mapping can provide additional information. Haplotyping is discussed in detail in Chapters 2 and 6 (see fig. 1 in Chapter 2.2 for an overview of monomodular, bimodular, trimodular and quadrimodular *CYP21/C4* haplotypes). A comparison of different populations shows considerable differences between the haplotype frequencies found in each group (see Chapter 2); such differences probably influence the frequency of *de novo* mutations that cause 21-hydroxylase deficiency [Sinnott *et al.* 1990; Yang *et al.* 1999] or *C4* [Fasano *et al.* 1992] deficiency.

CYP21/*C4* haplotyping has a dual significance in understanding the genetic defects that cause steroid 21-hydroxylase deficiency. First, the relative intensity of the 3.7 kb *Taq*I band on autoradiograms is a reliable indicator of the number of *CYP21* genes a person has. Absence of *CYP21* is the molecular defect in approximately one-third of the 21-hydroxylase deficiency alleles in Western European populations. Second, determining the overall arrangement of the RCCX region is the first step in understanding how unequal crossover and gene conversion create genetic defects in the RCCX region (see below).

Haplotyping was the only available approach to study *CYP21* defects until the advent of PCR-based mutation analysis about ten years ago. Recent results underline the continued

importance of determining the composition of the RCCX module by means of haplotyping, especially in 'non-standard' cases [Krone *et al.* 1998; Wedell 1998a; Baumgartner-Parzer *et al.* 2001; Koppens *et al.* 2002c].
An elegant direct demonstration of the arrangement of the RCCX module by fluorescent in situ hybridisation has been published a few years ago [Suto *et al.* 1996], but this approach has not been widely adopted.

Variation not detected by haplotyping

The duplicated structure of the 'standard' RCCX region features genes that are, by their very nature, highly similar but slightly different. Many methods to characterise the DNA sequence alterations that define the functionality of each gene have been applied. Before the advent of the PCR, these methods were rather laborious, for example:

- complement *C4* serotyping [O'Neill *et al.* 1978; Marcus and Alper 1986; Mauff *et al.* 1993];
- RFLP analysis of genomic DNA with restriction enzymes that have been selected to recognise functional sequence differences [Yu and Campbell 1987; Partanen and Campbell 1989; Urabe *et al.* 1990];
- hybridisation of allele-specific oligonucleotides to digested genomic DNA in dried agarose gels [Amor *et al.* 1988; Globerman *et al.* 1988; Speiser *et al.* 1988] or on Southern blots [Higashi *et al.* 1988a];
- sequencing of patient genes to detect defects directly [Harada *et al.* 1987; Rodrigues *et al.* 1987; Higashi *et al.* 1988b; Barba *et al.* 1993].

Nowadays, mutation detection is usually based on (diverse variants of) the PCR; a few popular methods are:

- hybridisation of allele-specific oligonucleotides to membrane-fixed PCR products [Braun *et al.* 1990; Owerbach *et al.* 1992a; Rumsby *et al.* 1992; Speiser *et al.* 1992; Dondi *et al.* 1994; Ezquieta *et al.* 1996; Koppens *et al.* 2000; Weintrob *et al.* 2000];
- allele-specific PCR with oligonucleotides that have a single-base mismatch at the 3' end [Wedell and Luthman 1993b; Wilson *et al.* 1995a; Carrera *et al.* 1997];
- electrophoresis of digested and undigested amplified DNA fragments [Barba *et al.* 1993; Shevtsov *et al.* 1994; Killeen *et al.* 1998; Grant *et al.* 2000; Lee *et al.* 2000a; Yokoyama *et al.* 2000; Koppens *et al.* 2002a; Koppens *et al.* 2002b];
- amplification-created restriction sites, based on the creation of a restriction site that matches part of an oligonucleotide an part of either the wild type or the mutated sequence [Lee *et al.* 1996; Oriola *et al.* 1997; Tajima *et al.* 1997; Ko *et al.* 1998];
- direct sequencing of PCR products by various methods [Berg *et al.* 1990; Rumsby *et al.* 1992; Ohlsson and Schwartz 1997; Chin *et al.* 1998; Lokki *et al.* 1999];
- single strand conformational polymorphism analysis [Barba *et al.* 1993; Siegel *et al.* 1994; Vaishnaw *et al.* 1995; Bobba *et al.* 1997; Hayashi *et al.* 1997];
- ligase detection of amplified fragments reaction [Day *et al.* 1995];

- cleavase fragment length polymorphism analysis [Wei and Killeen 1998];
- reverse dot-blot hybridisation [Yang *et al.* 2001].

The merits of most of these methods have been compared in two recent reviews [White and Speiser 2000; Lee 2001]. In the author's opinion, allele-specific PCR is currently the method of choice for rapid detection of the most common mutations, especially when the number of samples is small. Allele-specific oligonucleotide hybridisation is still a good alternative for large numbers of samples and offers the advantage that blots can be re-hybridised many times with different probes. Microarray technology has so far not been used in the routine detection of *CYP21* mutations, but is likely to be implemented in the near future [Lee 2001].

Defective *CYP21* genes usually contain one or more of nine defects typically found in *CYP21P*. Other mutations are relatively rare: in classical 21-hydroxylase deficiency they represent fewer that 5 % of all deficiency alleles in most populations (see tables 3 and 5), but they may be more common in NC 21-hydroxylase deficiency. The *CYP21P* pseudogene shows a high variability in the general population, a factor which complicates the analysis of hybrid *CYP21* genes [Helmberg *et al.* 1992a; Wedell and Luthman 1993a; Koppens *et al.* 2000]. Gene conversion-like processes are believed to be responsible for sequence exchange between *CYP21* and *CYP21P*. With respect to the layout of the RCCX module, one element seems to be constant: all *CYP21*-like genes that are transcribed and translated into an enzyme are part of the most centromeric RCCX module. No chromosomes have ever been found where an active *CYP21* gene lies telomeric to an inactive *CYP21P* pseudogene, or even to another *CYP21* gene.

Sequence variability has a rather different impact at the complement *C4* locus. Defects may lead to non-expression of the *C4* gene (*C4A**Q0 or *C4B**Q0), but a small number of differences, primarily in the *C4*d region, determines whether the gene is *C4A* or *C4B* gene and what its Chido or Rodgers antigen is. In a bimodular RCCX region, the telomeric *C4* gene usually expresses C4A and the centromeric usually expresses C4B, but all kinds of variants to this theme have been described in the literature [Yu *et al.* 1986; Carroll and Alper 1987; Yu and Campbell 1987; Braun *et al.* 1990; Barba *et al.* 1993; Barba *et al.* 1994; Paz-Artal *et al.* 1994; Moulds *et al.* 1996; Schneider *et al.* 1996; Yu 1998; Lokki *et al.* 1999; Blanchong *et al.* 2001; Martinez *et al.* 2001].

Considerable polymorphism exists at the *TNX* locus. The most important difference between *TNXB* and the truncated *TNXA* pseudogene is the 120 bp deletion in *TNXA*. This difference can be detected by haplotyping (see the previous paragraph), but PCR-based methods [Burch *et al.* 1997; Koppens *et al.* 2002a; Koppens *et al.* 2002b] are more convenient. Presence of the deletion (which lies on an exon-intron boundary) in *TNXB* causes a frameshift and contributes to tenascin-X deficiency [Burch *et al.* 1997; Schalkwijk *et al.* 2001]. *TNXA* pseudogenes without the deletion have also been reported [Rupert *et al.* 2001; Koppens *et al.* 2002b], but this change is phenotypically inconsequential. Apart from this 120 bp difference, several other (neutral) polymorphisms exist in this region [Weissensteiner and Lanchbury 1997; Koppens *et al.* 2002a; Koppens *et al.* 2002b].

The 1 kb section of the *RP* gene that lies within the RCCX module is also polymorphic [Ulgiati and Abraham 1996].

1.7. Genetic mechanisms creating *CYP21* defects

Misalignment and genetic defects

Mutational mechanisms have been thoroughly studied in lower eukaryotes such as yeasts, where generation times are short, chemicals or radiation can be used to induce specific mutations, and the products of *de novo* events are readily available. In studies of human patients suffering from an inherited disease however, genetic mechanisms can often only be inferred by comparing consensus 'wildtype' sequences with sequences that somehow deviate from that consensus - and assuming that a certain type of mutation has caused the difference. *De novo* mutations are relatively rare (see table 4 for examples involving *CYP21* and *CYP21P*) and chance determines which of the recombined chromosomes will be available for analysis. Nevertheless, recent studies on single mammalian cells [Högstrand and Böhme 1997; Carrington 1999; Högstrand and Böhme 1999; Jeffreys *et al.* 1999; Martinsohn *et al.* 1999; Johnson and Jasin 2000; Jeffreys *et al.* 2001] have greatly contributed to our knowledge of two mutational mechanisms:

- recombination, a reciprocal exchange of a large part of homologous chromosomes aligning during meiosis;
- gene conversion, a non-reciprocal copying of a small part of one sister chromatid to the other in somatic cells.

Actually, an important lesson from these (and other) recent reports is that this grouping of mechanisms into two categories constitutes a simplification in several respects. Recombination also occurs during mitosis [Tischfield *et al.* 1997; Holt *et al.* 1999; Wijnhoven *et al.* 2001], gene conversion in the mouse MHC appears to be limited to germline cells [Högstrand and Böhme 1997; Högstrand and Böhme 1999], and several lines of evidence indicate that complex rearrangements including but not limited to combinations of gene conversion and recombination occur during mitosis, meiosis and DNA repair [Jeffreys *et al.* 1999; Martinsohn *et al.* 1999; Johnson and Jasin 2000; Wijnhoven *et al.* 2001]. The preference for the sister chromatid in gene conversion is probably simply a matter of proximity [Johnson and Jasin 2000].

In the light of this recent evidence, it is clear that routine assignment of terms such as 'gene conversion' or 'gene deletion' to specific *CYP21* defects should be seen as a categorisation of mutations rather than a concise description of the exact genetic mechanism that caused these mutations. Still, careful analysis of *de novo* mutations (see table 4 and Chapter 4) and hybrid genes (see Chapters 3 and 5) does provide considerable insight into the 'origin of disease-causing mutations'.

The high degree of sequence similarity between tandemly repeated RCCX modules increases the propensity of chromatids to misalign in this region. Difference in the number of RCCX

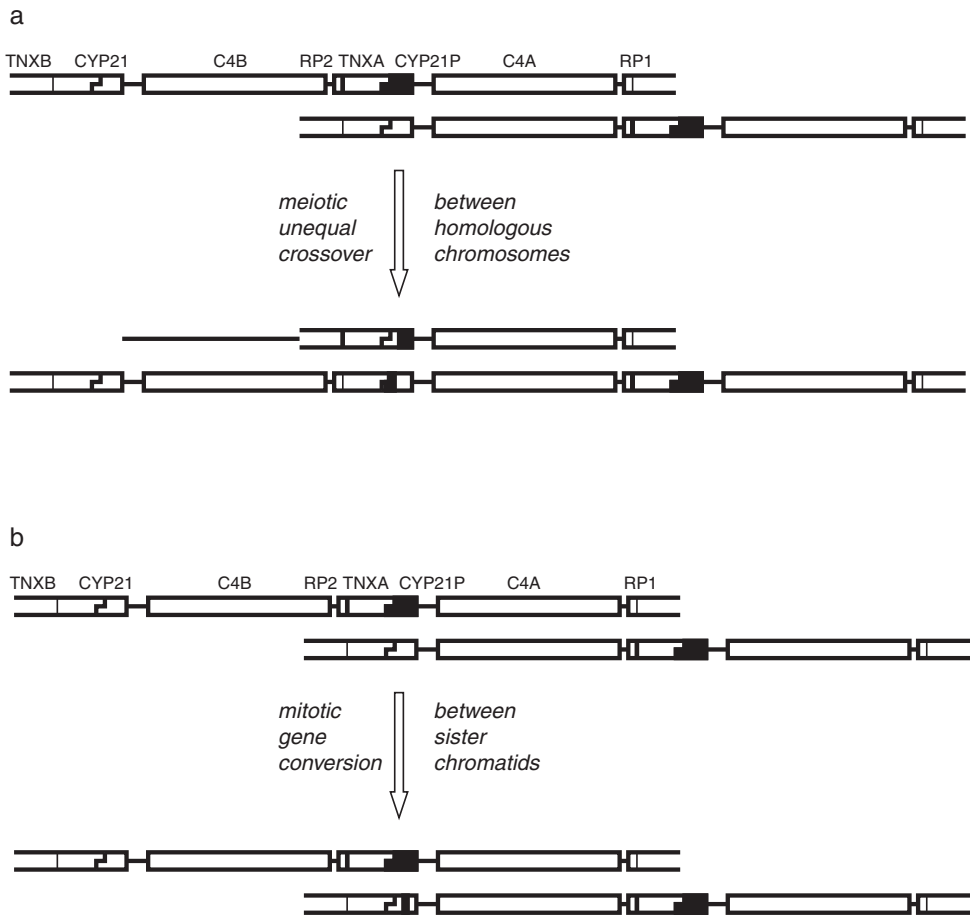


Fig. 6. Hypothetical unequal crossover and gene conversion between two bimodular chromosomes. The misalignment requires additional loops to correctly align neighbouring areas of the chromosomes that lie outside the RCCX module; for an example, see fig. 6 in Chapter 4. 6a: Unequal crossover after misalignment of homologous chromosomes during meiosis leads to the transfer of an RCCX module from one chromosome to the other, creating a defective *CYP21P/CYP21* hybrid. 6b: Non-reciprocal gene conversion in somatic cells copies a part of one sister chromatid to the other, introducing a *CYP21P*-like defect into *CYP21* if these genes misalign.

modules necessarily causes misalignment, but two bimodular chromatids may also misalign [Koppens *et al.* 2002a]. Within the limitations outlined above, a simple model of unequal crossover and gene conversion in the RCCX region can be envisioned (see fig. 6). The only direct study of these mechanisms in the RCCX region in single cells [Tusié-Luna and White 1995] showed that unequal crossovers were exclusively meiotic, and gene conversions primarily mitotic.

Large-scale gene deletions by unequal crossover

As illustrated in fig. 6a, meiotic unequal crossover effectively results in the transfer of an RCCX module from one chromosome to the other. The location of the actual crossover site determines the composition of the hybrid module that is formed by this process: fig. 7 shows how different breakpoint locations between a misaligned bimodular and monomodular chromosome result in various hybrids. Depending on the analysis method, such hybrids may - by comparison to the standard bimodular layout - be classified as, for example, *'CYP21* and *C4B* deletion' (fig. 7a, 7b and 7c), *'CYP21P* and *C4B* deletion' (fig. 7d), or *'CYP21P* and *C4A* deletion' (fig. 7e).

The existence of specific zones in the RCCX module where the crossover occurs preferentially or even exclusively, has been suggested [Donohoue *et al.* 1989]. However, *CYP21P* variability [Helmberg *et al.* 1992a; Wedell and Luthman 1993a; Koppens *et al.* 2000] has blurred the borders of such putative crossover zones, and the identification of several additional crossover sites in the *CYP21* [Killeen *et al.* 1991; Tusié-Luna and White 1995; l'Allemand *et al.* 2000; Baumgartner-Parzer *et al.* 2001] and *TNX* genes [Burch *et al.* 1997; Rupert *et al.* 1999; Yang *et al.* 1999; Koppens *et al.* 2002a; Koppens *et al.* 2002b] suggests that crossovers may take place anywhere in the RCCX module. However, the high degree of DNA sequence similarity limits the possibility to pinpoint the exact location.

Again by comparison to the standard layout, bimodular RCCX regions with two *CYP21P* genes (as judged by *Taq*I restriction analysis - see fig. 1 in Chapter 2.2) are usually called 'large-scale conversions of *CYP21* to *CYP21P*'. However, mutation analysis of the hybrid RCCX modules found in such haplotypes showed that they are highly similar to the above-mentioned monomodular 'deletion' haplotypes [Koppens *et al.* 2000; Koppens *et al.* 2002a; Koppens *et al.* 2002b]. Since there seems to be no reason to postulate a different mechanism for the generation of monomodular and bimodular chromosomes with one hybrid RCCX module, the term 'large-scale gene conversion' should be avoided in this context: '*CYP21* gene deletion by unequal crossover' is more consistent [Koppens *et al.* 2002b].

Small-scale gene conversions and other mutations

CYP21 defects that are characteristics of the consensus sequence of the *CYP21P* pseudogene [Higashi *et al.* 1986; White *et al.* 1986; Rodrigues *et al.* 1987] are attributed to gene conversion (see fig. 6b). Although gene conversion can cover several kb [Johnson and Jasin 2000], a gene conversion-like event that transfers a part of *CYP21P* to *CYP21*, or vice versa, typically involves a few hundred bp at most - a phenomenon that has also been observed elsewhere in the MHC [Martinsohn *et al.* 1999]. Theoretically, no distinction can be made between a double crossover (or two consecutive single crossovers) and a gene conversion, since the non-reciprocity of the conversion cannot be demonstrated - however, in a short stretch of DNA, gene conversion is far more likely.

Nine or ten small-scale gene conversions (see below; Pro453→Ser may or may not be a conversion) and combinations of these, account for the majority of the genetic defects that

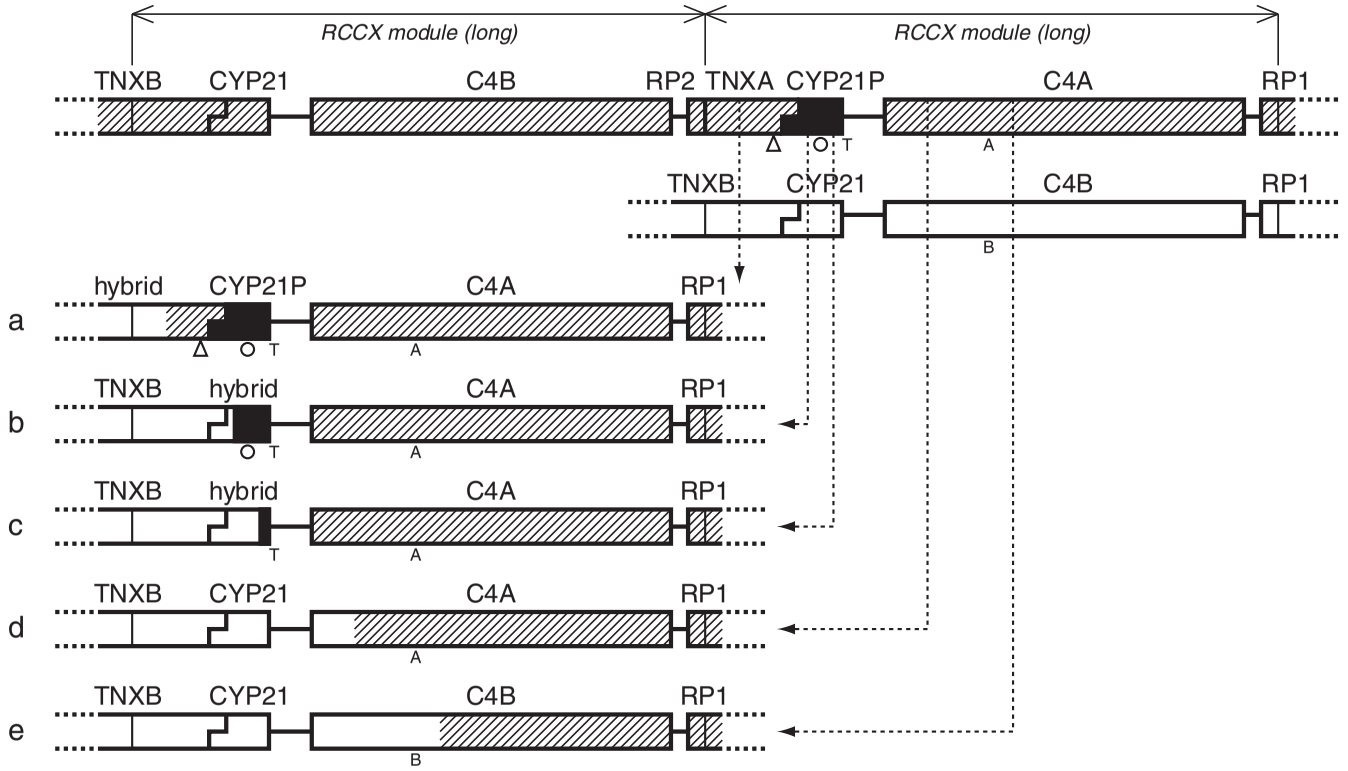


Fig. 7. Examples of different crossover sites generating different hybrid RCCX modules. Some characteristics that may or may not be transferred are: triangle: 120 bp deletion in the *TNXA* pseudogene; circle: approximate location of several deleterious defects in the *CYP21P* pseudogene; T: *Taq*I site in the 5' flank of *CYP21*; A, B: sequence difference that distinguishes *C4A* from *C4B*. Results of the crossover: 7a: *CYP21* deleted and *TNXB* defective; 7b: *CYP21P*/*CYP21* hybrid carrying severe defects; 7c: *CYP21P*/*CYP21* hybrid carrying milder defects; 7d: *CYP21P* deleted, with a *C4A*-like hybrid complement gene left; 7e: *CYP21P* deleted, with a *C4B*-like hybrid gene left. See text for further details; note that any *CYP21P*/*CYP21* hybrid is recognised as *CYP21P* in *Taq*I-based haplotyping studies. Usually, the exact crossover site cannot be determined because the sequences of both RCCX modules are highly similar.

cause steroid 21-hydroxylase deficiency in all populations studied to date (see table 5). Many other mutations have been identified (see table 3), but they have usually been found in isolated cases and are not present in *CYP21P*.

A small-scale gene conversion that transfers the *Taq*I restriction site in the 5' flanking sequence of *CYP21P* to *CYP21* will be recognised as a larger defect in haplotyping studies. No conversion limited to this single marker has so far been reported; it could be distinguished from a 'genuine' hybrid RCCX module by examining sequences that lie telomeric to the *Taq*I site.

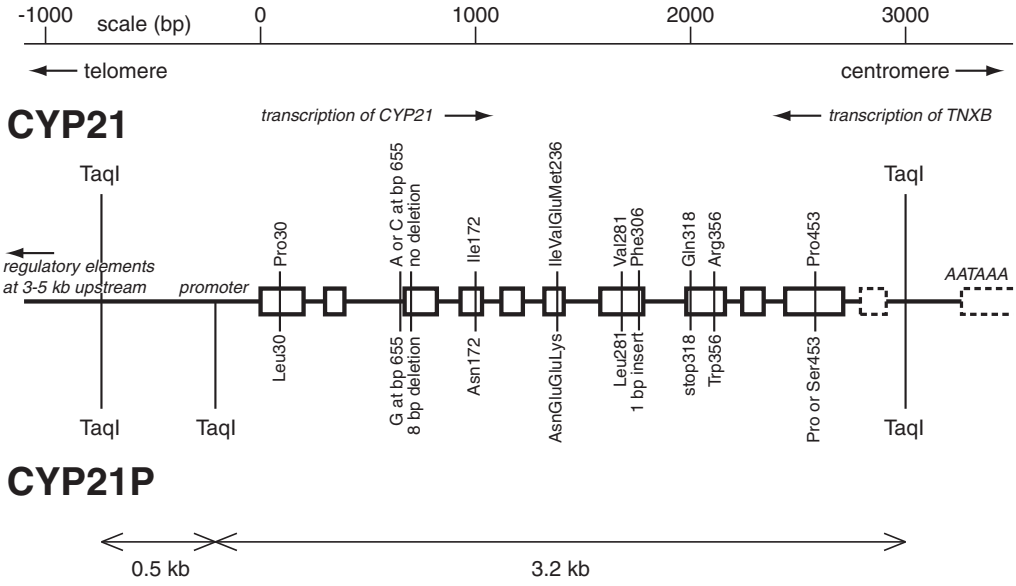


Fig. 8. Principal differences between the *CYP21* gene and the *CYP21P* pseudogene. The orientation of the figure is opposite to fig. 3: the transcription of *CYP21* is from left to right, so the centromere is on the right. Open boxes represent *CYP21* exons [Higashi *et al.* 1986]; the open boxes with the dashed outline represent the two final exons of the *TNXB* gene [Bristow *et al.* 1993b], which is transcribed in the opposite direction. The *CYP21* polyadenylation signal lies at the leftmost A of AATAAA. The differences in the coding area also represent the most common *CYP21* defects found in steroid 21-hydroxylase deficiency (see the text). The amino acids shown for *CYP21P* are an indication of substitution mutations; *CYP21P* is not translated. G versus A or C at bp 655 is the intron 2 splice junction mutation. Ser453 has been reported in some *CYP21P* pseudogenes but not in the consensus sequence. The characteristic *Taq*I sites are shown, with the 3.2 kb *CYP21P* fragment and the extra 0.5 kb for *CYP21*.

Phenotype and genotype

The relationship between genotype and clinical phenotype is rather straightforward in most steroid 21-hydroxylase deficiency patients: the more severe the genetic defects as predicted by the DNA sequence or demonstrated by expression studies, the more severe the manifestation of CAH. However, there are quite a few deviations from this pattern. As explained above, the clinical manifestations of steroid 21-hydroxylase deficiency are manifold and may change as patients age [Horner *et al.* 1979; Stoner *et al.* 1986; Sinnott *et al.* 1989; Speiser *et al.* 1991; Hoffman *et al.* 1996]; also, aldosterone production in patients with a homozygous *CYP21* deletion [Speiser *et al.* 1991; Koppens *et al.* 1998] constitutes a paradox. Phenotypic variation between patients with the same genetic defect [Speiser *et al.* 1992; Wilson *et al.* 1995b; Witchel *et al.* 1996; Krone *et al.* 2000] is another peculiar phenomenon, especially when the patients are sibs [Sinnott *et al.* 1989; Bormann *et al.* 1992; Chin *et al.* 1998] (many of the mutation analysis studies listed in table 5 also report on clinical differences between patients where the same mutations were found).

To address phenotype-genotype relationship in a systematic way, the concept of 'mutation groups' has been introduced [Speiser *et al.* 1992; Wilson *et al.* 1995b]: patients are categorised on the basis of the *CYP21* defects found on both chromosomes and this genotype is matched to a set of clinical and biochemical parameters. Thematically, this is a refinement of an earlier notion: patients should theoretically have the phenotype that matches their 'least defective' allele [New *et al.* 1989]. Phenotypic variation has recently been reported in 23 out of 85 mutation groups [New 2001a].

Expression studies of mutated steroid 21-hydroxylase have provided a wealth of information on the enzyme's structure and function [Higashi *et al.* 1988b; Tusié-Luna *et al.* 1990; Higashi *et al.* 1991; Wu *et al.* 1991; Chung *et al.* 1995; Hu *et al.* 1996; Lajic *et al.* 1997; Nikoshkov *et al.* 1997; Nikoshkov *et al.* 1998; Lajic *et al.* 1999; Ohlsson *et al.* 1999]. However, these *in vitro* experiments do not necessarily predict the *in vivo* enzymatic activity or the clinical severity of CAH [White and Speiser 2000].

An overview of naturally occurring *CYP21* mutations together with their reported clinical association is presented in table 3 (amino acid and nucleotide numbering is according to Higashi *et al.* [1986]). A brief discussion of the most common defects (those attributed to recombination or gene conversion), in descending order of severity, is warranted:

- deletion of *CYP21* by unequal crossover: no enzyme is produced;
- nonsense and frameshift mutations (8 bp deletion in exon 3; 1 bp insertion in exon 7; Gln318→stop): truncation of the protein before the haem binding site, due to premature termination of translation [Globerman *et al.* 1988; Higashi *et al.* 1988a];
- cluster of three mutations (Ile236→Asn, Val237→Glu, Met239→Lys) in exon 6: no enzymatic activity, possibly due to a defect in substrate binding [Higashi *et al.* 1988b];
- Arg356→Trp: usually reported as complete deficiency due to impaired reductase interaction [Chiou *et al.* 1990; Lajic *et al.* 1997]; unlike the defects listed above, this mutation is sometimes associated with SV 21-hydroxylase deficiency (see table 5);

- additional splice acceptor site in intron 2: the most frequent mutation in SL 21-hydroxylase deficiency, but not consistently associated with that form because a small amount of RNA is still spliced correctly [Higashi *et al.* 1988b; Higashi *et al.* 1991; Witchel *et al.* 1996];
- Ile172→Asn: results in a conformationally changed enzyme with an altered cellular location, but retains about 1 % of the wildtype enzymatic activity, sufficient to prevent salt loss; this is the most common defect in SV 21-hydroxylase deficiency [Amor *et al.* 1988; Hsu *et al.* 1996];
- Pro30→Leu: reduces enzyme stability and activity, causing NC 21-hydroxylase deficiency but occasionally a more severe form [Tusié-Luna *et al.* 1990; Hu *et al.* 1996; Frisch *et al.* 2001];
- Val281→Leu: probably reduces haem group binding, causing NC 21-hydroxylase deficiency; this mutation is associated with HLA-B14 DR1 [Speiser *et al.* 1988; Wu *et al.* 1991];
- Pro453→Ser: conformational effect unknown, but contributes to a subtle form of NC 21-hydroxylase deficiency that may remain largely asymptomatic; reports differ as to whether this mutation represents a *CYP21P* to *CYP21* gene conversion or an independent mutation [Helmberg *et al.* 1992b; Owerbach *et al.* 1992b; Wedell *et al.* 1992; Nikoshkov *et al.* 1997; Chapter 3.2].

The location of each of these mutations is shown in fig. 8.

De novo mutations

In the study of steroid 21-hydroxylase deficiency, several reasons have been described why a patient can have a mutation that was not present in the previous generation:
non-paternity (or even non-maternity); this can be checked with independent genetic markers (microsatellite analysis is the method of choice);
a new meiotic mutation; in this case, one of the parents is not a carrier;
a new somatic mutation in a parent's germline; in this case, the parent is a carrier, but the mutation is not found in leukocyte DNA (which is routinely used for genetic analysis);
uniparental isodisomy, when the patient receives two identical copies of chromosome 6 from a parent who is a steroid 21-hydroxylase deficiency carrier.
Reports on *de novo* mutations are summarised in table 4. The mechanics of such mutations are not always easy to visualise: because most authors did not analyse putative template genes in gene conversions, the actual extent of the converted area often remains uncertain.

Table 3. Naturally occurring mutations and polymorphisms in the *CYP21* gene
(top: common mutations, shown in fig. 8; bottom: rare mutations - see table 5 for frequencies).

basepair(s)	location	designation	(putative) effect of the mutation	severity	references
-209	5' flank	T→C	neutral polymorphism, *Taq*I restriction site	normal	Higashi *et al.* [1986]; Chin and Chang [1998]
89	exon 1	Pro 30→Leu	reduced enzyme stability	SV/NC	Tusié-Luna *et al.* [1991]; Hu *et al.* [1996]
655	intron 2	extra splice acceptor site: A/C →G	incorrect splicing	SL	Higashi *et al.* [1988b]
707 - 714	exon 3	8bp deletion: GAGACTAC	frameshift mutation	SL	Higashi *et al.* [1988a]
999	exon 4	Ile172→Asn	conformational change; cellular location	SV	Amor *et al.* [1988]; Higashi *et al.* [1991]; Hsu *et al.* [1996]
1380 – 1389	exon 6	IleValGluMet→AsnGluGluLys	substrate binding?	SL	Higashi *et al.* [1988b]; Higashi *et al.* [1991]
1683	exon 7	Val281→Leu	haem binding	NC	Speiser *et al.* [1988]; Wu *et al.* [1991]
1760	exon 7	1 bp insertion: T (Phe306)	frameshift mutation	SL	Higashi *et al.* [1988a]
1994	exon 8	Gln318→stop	nonsense mutation	SL	Globerman *et al.* [1988]
2108	exon 8	Arg356→Trp	interaction with P450 reductase?	SL	Chiou *et al.* [1990]; Higashi *et al.* [1991]
2577	exon 10	Pro453→Ser	unknown	NC	Owerbach *et al.* [1992b]; Nikoshkov *et al.* [1997]

basepair(s)	location	designation	(putative) effect of the mutation	severity	references
-353	5' flank	T→G	neutral polymorphism	normal	Bobba *et al.* [2000]
-350	5' flank	1 bp insertion: G	neutral polymorphism	normal	Bobba *et al.* [2000]
-331	5' flank	1 bp insertion: G	neutral polymorphism	normal	Bobba *et al.* [2000]
-295 - -281	5' flank	4 bp changes	unknown	unknown	Bobba *et al.* [2000]
-279	5' flank	1 bp insertion: T	neutral polymorphism	normal	Bobba *et al.* [2000]
-73	5' flank	C→T	unknown	unknown	Bobba *et al.* [2000]

basepair(s)	location	designation	(putative) effect of the mutation	severity	references
-4	5' flank	C→T	neutral polymorphism	normal	Wedell *et al.* [1992]; Nikoshkov *et al.* [1997]
27	exon 1	3 bp insertion: CTG (Leu9)	neutral polymorphism	normal	Rodrigues *et al.* [1987]; Higashi *et al.* [1991]
64	exon 1	1 bp insertion: T (Trp22)	frameshift mutation	SL	Ezquieta *et al.* [1999]
66	exon 1	Trp 22→stop	nonsense mutation	SL	Lajic and Wedell [1996]
89	exon 1	Pro 30→Gln	unknown	SL	Lajic *et al.* [1999]
141	exon 1	1 bp deletion: T (Tyr47)	frameshift mutation	SL	Krone *et al.* [1999]
185	exon 1	His 62→Leu	unknown	unknown	Lobato *et al.* [1999]
193	exon 1	Gly 64→Glu	protein structure?	SL	Ohlsson *et al.* [1999]
296	intron 1	defective splice acceptor site: A→G	incorrect splicing	SL	Lajic and Wedell [1996]
317	exon 2	Lys 74→stop	nonsense mutation	SL	Lobato *et al.* [1999]
362	exon 2	Ala89→Ser	unknown	unknown	Lobato *et al.* [1999]
366	exon 2	Gly 90→Val	substrate binding?	SL	Lobato *et al.* [1999]; Nunez *et al.* [1999]
387	intron 2	defective splice donor site: G→A	incorrect splicing	SL	Lee *et al.* [1998]
669	exon 3	Tyr 97→stop	nonsense mutation	SL	Krone *et al.* [1998]
671	exon 3	Lys 98→Arg	neutral polymorphism	normal	Ordoñez-Sánchez *et al.* [1998]
683	exon 3	Lys102→Arg	neutral polymorphism	normal	Rodrigues *et al.* [1987]
683	exon 3	Arg102→Lys	neutral polymorphism	normal	Rodrigues *et al.* [1987]
692	exon 3	Pro105→Leu	unknown	NC	Wedell *et al.* [1992]; Nikoshkov *et al.* [1997]
989 - 990	exon 4	1 bp deletion: TG→A (Cys169)	frameshift mutation	SL	Witchel *et al.* [1998]
996	exon 4	Ile171→Asn	conformational change	SV/NC	Hsu *et al.* [1996]; Balsamo *et al.* [2000]
1017	exon 4	Gly178→Ala	unknown	SV	Lobato *et al.* [1999]; Nunez *et al.* [1999]
1021	exon 4	Asp179→Glu	unknown	unknown	Lobato *et al.* [1999]
1121	exon 5	Asp183→Glu	neutral polymorphism	normal	Higashi *et al.* [1991]
1128	exon 5	Leu186→Val	unknown	unknown	Lobato *et al.* [1999]

basepair(s)	location	designation	(putative) effect of the mutation	severity	references
1158 - 1160	exon 5	3 bp deletion: GAG (Glu196)	moderately reduced enzyme stability	SV	Nikoshkov *et al.* [1998]
1203	exon 5	Val211→Leu	neutral polymorphism	normal	Speiser *et al.* [1988]
1340	exon 6	Leu223→Val	unknown	unknown	Lobato *et al.* [1999]
1621	exon 7	Met260→Arg	unknown	unknown	Lobato *et al.* [1999]
1628	exon 7	Gln262→stop	nonsense mutation	SL	Ohlsson *et al.* [1999]
1640	exon 7	Gln266→His	unknown	unknown	Lobato *et al.* [1999]
1645	exon 7	Ser268→Thr	neutral polymorphism	normal	Rodrigues *et al.* [1987]; Wu *et al.* [1991]
1683	exon 7	Val281→Gly	substrate and haem binding	SV	Krone *et al.* [2000]; Lajic *et al.* [2001]
1695	exon 7	Ala285→Ser	unknown	unknown	Lobato *et al.* [1999]
1713	exon 7	Gly291→Cys	proton transfer? reduced stability?	SL	Lobato *et al.* [1999]; Nunez *et al.* [1999]
1713	exon 7	Gly291→Ser	proton transfer? reduced stability?	SL	Wedell *et al.* [1992]; Nikoshkov *et al.* [1998]
1740	exon 7	Leu300→Phe	conformational change; reduced stability	SV	Krone *et al.* [2000]; Lajic *et al.* [2001]
1748	exon 7	Trp302→stop	nonsense mutation	SL	Levo and Partanen [1997b]
1770	exon 7	His310→Asn	unknown	unknown	Lobato *et al.* [1999]
1779	intron 7	defective splice donor site: G→C	incorrect splicing	SL	Wedell and Luthman [1993b]
1780	intron 7	defective splice donor site: T→G	incorrect splicing	unknown	Ordoñez-Sánchez *et al.* [1998]
1988	exon 8	Arg316→stop	nonsense mutation	SL	Lee *et al.* [1998]
2030 - 2039	exon 8	10 bp deletion: TCCAGCTCCC	frameshift mutation	SL	Lee *et al.* [1998]
2058	exon 8	Arg339→His	interaction with P450 reductase?	NC	Helmberg *et al.* [1992b]; Nikoshkov *et al.* [1997]
2065	exon 8	Arg341→Trp	interaction with P450 reductase?	NC	Gunn *et al.* [1993]
2102	exon 8	Arg354→Cys	interaction with P450 reductase?	SL	Krone *et al.* [2000]
2103	exon 8	Arg354→His	interaction with P450 reductase?	SL	Lobato *et al.* [1999]; Nunez *et al.* [1999]
2109	exon 8	Arg356→Gln	interaction with P450 reductase?	SV/NC	Lajic *et al.* [1997]
2109	exon 8	Arg356→Pro	interaction with P450 reductase?	SL	Lajic *et al.* [1997]
2129	exon 8	Ala362→Val	substrate binding?	SL	Ohlsson *et al.* [1999]
2130	exon 8	Leu363→Trp	unknown	SV/NC	Jääskelainen *et al.* [1997]

basepair(s)	location	designation	(putative) effect of the mutation	severity	references
2153	exon 8	Ser371→Cys	unknown	unknown	Lobato *et al.* [1999]
2254	exon 9	Asp377→Tyr	unknown	unknown	Lobato *et al.* [1999]
2265	exon 9	Glu380→Asp	haem group binding	unknown	Kirby-Keyser *et al.* [1997]; Hsu *et al.* [1999]
2292	exon 9	Gln389→His	unknown	unknown	Lobato *et al.* [1999]
2319	exon 9	16 bp duplication	frameshift mutation	SL	Lee *et al.* [1998]
2339	exon 9	Trp405→stop	nonsense mutation	SL	Wedell and Luthman [1993b]
2491	exon 10	Gly424→Ser	unknown	SV	Billerbeck *et al.* [1999]
2498	exon 10	Arg426→His	unknown	SV	Baumgartner-Parzer *et al.* [2001]
2498	exon 10	Arg426→Pro	neutral polymorphism	normal	Higashi *et al.* [1986]; White *et al.* [1986]
2498	exon 10	Pro426→Arg	neutral polymorphism	normal	Higashi *et al.* [1986]; White *et al.* [1986]
2498	exon 10	Pro426→His	unknown	SV	Baumgartner-Parzer *et al.* [2001]
2516	exon 10	Pro432→Arg	unknown	unknown	Lobato *et al.* [1999]
2533	exon 10	Glu437→Asp	neutral polymorphism	normal	Higashi *et al.* [1986]; Ko *et al.* [1998]
2589	exon 10	Asp456→Glu	unknown	unknown	Lobato *et al.* [1999]
2596	exon 10	Pro459→Ser	unknown	unknown	Lobato *et al.* [1999]
2646	exon 10	1 bp deletion: T (Pro475)	frameshift mutation	SL	Ordoñez-Sánchez *et al.* [1998]
2662	exon 10	Gln481→Lys	unknown	unknown	Lobato *et al.* [1999]
2665	exon 10	Pro482→Thr	unknown	unknown	Lobato *et al.* [1999]
2665	exon 10	Pro482→Ser	unknown	unknown	Balsamo *et al.* [2000]
2669	exon 10	Arg483→Pro	severely reduced enzyme stability	SV	Wedell and Luthman [1993a]; Nikoshkov *et al.* [1998]
2669 - 2670	exon 10	1 bp deletion: GG→C (Arg483)	frameshift mutation	SL	Wedell *et al.* [1992]
2699	exon 10	Asn493→Ser	neutral polymorphism	normal	Rodrigues *et al.* [1987]; Chiou *et al.* [1990]

The reports cited above are usually the first description of the mutation and an *in vitro* expression study of the altered enzyme. The (putative) functional significance of the change is an assumption or a speculation (indicated by '?') of the authors of (one of) these reports. Base pairs are numbered according to Higashi *et al.* [1986].

Table 4. Reported *de novo* mutations of the *CYP21* and *CYP21P* genes

CYP21(P) mutation	frequency[a]	mode[a]	most likely mechanism	reference
CYP21 deletion	1 / 30	M	unequal crossover within the *TNX* gene, between a monomodular and a bimodular chromosome	Sinnott *et al.* [1990]; Sinnott *et al.* [1991]
CYP21 deletion	unknown	M	unusual deletion including the HLA genes	Hejtmancik *et al.* [1992]
CYP21 deletion	1 / 85	M	unequal crossover within the *TNX* gene, between two bimodular chromosomes	Koppens *et al.* [2002a]
CYP21P deletion	unknown	M	unequal crossover within the *C4* gene, between a monomodular and a bimodular chromosome	Fasano *et al.* [1992]
Pro30→Leu	2 / 94	G	*CYP21P* to *CYP21* conversion of unknown extent	Ordoñez-Sánchez *et al.* [1998]
Pro30→Leu and intron 2 splice	1 / 94	G	*CYP21P* to *CYP21* conversion of unknown extent	Ordoñez-Sánchez *et al.* [1998]
intron 2 splice	2 / 154	G	*CYP21P* to *CYP21* conversion of unknown extent	Levo and Partanen [2001]
intron 2 splice	1 / 184	G	*CYP21P* to *CYP21* conversion of unknown extent	Wedell *et al.* [1994]
intron 2 splice	2 / 176	G	*CYP21P* to *CYP21* conversion of unknown extent	Speiser *et al.* [1992]
intron 2 splice	2 / 46	G	*CYP21P* to *CYP21* conversion of unknown extent	Tajima *et al.* [1993]
intron 2 splice	4 / 94	G	*CYP21P* to *CYP21* conversion of unknown extent	Ordoñez-Sánchez *et al.* [1998]
Ile172→Asn	unknown	G	*CYP21P* to *CYP21* conversion within a 390 bp region	Collier *et al.* [1993]
Ile172→Asn	1 / 310	G	*CYP21P* to *CYP21* conversion of unknown extent	Krone *et al.* [2000]
Ile172→Asn	2 / 134	G	*CYP21P* to *CYP21* conversion of unknown extent	Ohlsson *et al.* [1999]
Ile172→Asn	1 / 94	G	*CYP21P* to *CYP21* conversion of unknown extent	Ordoñez-Sánchez *et al.* [1998]
Arg356→Trp	2 / 46	G	*CYP21P* to *CYP21* conversion of unknown extent	Tajima *et al.* [1993]
not reported	1 / 116	G	*CYP21P* to *CYP21* conversion of unknown extent, either the intron 2 splice mutation or Arg356→Trp	Balsamo *et al.* [2000]
Leu300→Phe	1 / 310	G	novel mutation, not a *CYP21P* to *CYP21* conversion	Krone *et al.* [2000]
AsnGluGluLys236 →IleValGluMet	1 / 85	G	*CYP21* to *CYP21P* conversion within a 683 bp region	Koppens *et al.* [2000]
intron 2 splice and Val281→Leu	1 / 94	D	paternal uniparental disomy for chromosome 6	Ordoñez-Sánchez *et al.* [1998]; López-Gutiérrez *et al.* [1998]
Ile172→Asn	unknown	D	maternal uniparental disomy for chromosome 6	Spiro *et al.* [1999]

The mutation frequencies here apply to each individual mutation listed in the leftmost column. To calculate the compound mutation rate reported by a specific research group, the number of mutations should be summed, but the number of chromosomes should not; for example, the total number of *de novo* mutations reported by Tajima *et al.* [1993] is 4, in 46 chromosomes.

a: number of *de novo* events / number of haplotypes in the study (unknown: report on a single case)

b: presumed mode of inheritance: M=meiotic *de novo* mutation; G=parental germline mosaicism; D=uniparental disomy

Table 5. Frequency of *CYP21* defects in steroid 21-hydroxylase deficiency patients in different populations

population	reference(s)[a]	type[b]	number of alleles	no *CYP21* gene[c]	Pro30 → Leu	intron 2 splice	exon 3 del 8 bp	Ile172 → Asn	cluster exon 6	Val281→ Leu	exon 7 ins 1 bp	Gln318 → stop	Arg356 → Trp	Pro453 → Ser	combination[d]	other mutation[e]	not found[f]
Argentina	1	SL	48	10.4+14.6	nd	22.9	2.1	0	0	nd	nd	18.8	2.1	nd	2.1	nd	27.1
		SV	24	0+4.2	nd	4.2	0	45.8	0	nd	nd	4.2	12.5	nd	0	nd	29.2
Austria	2		158	31.0	3.2	22.8	0	15.8	1.9	12.0	nd	2.5	3.2	1.3	4.4	1.3	0.6
Brazil	3	SL	51	5.9+11.8	nd	27.5	2.0	9.8	0	2.0	3.9	7.8	0	nd	7.8	nd	19.6
		SV	24	12.5+16.7	nd	12.5	0	37.5	0	0	4.2	0	8.3	nd	8.3	nd	4.2
Brazil	4		228	4.4+6.6	--	20.6	--	14.0	--	18.0	--	--	--	--	6.3	--	20.0
Brazil	5	SL	102	18.6	0	26.5	6.9	2.9	nd	2.0	1.0	9.8	10.8	nd	13.7	nd	7.8
		SV	32	18.8	0	28.1	0	25.0	nd	3.1	0	9.4	0	nd	15.6	nd	0
Chile	6	SL	96	28.1	nd	18.8	nd	9.4	2.1	nd	0	9.4	14.6	nd	2.1	nd	15.6
Chile	7	SV	30	10.0	0	6.7	nd	26.7	0	13.3	3.3	6.7	0	0	0	nd	33.3
Denmark	8		134	36.6	2.2	32.8	0	9.7	0.7	2.2	0.7	7.5	0.7	nd	2.2	4.5	0
Finland	9		100	35.0+6.0	0	12.0	0	30.0	0	3.0	0	2.0	1.0	0	8.0	1.0	2.0
France	10	SL	84	33.3	nd	22.6	4.8	8.3	4.8	1.2	1.2	4.8	nd	0	3.6	7.1	8.3
		SV	44	11.4	nd	27.3	4.5	27.3	6.8	9.1	0	2.3	nd	0	6.8	2.3	2.3
		CL	22	27.3	nd	45.5	0	9.1	4.5	0	4.5	0	nd	0	9.1	0	0
		NC	108	9.3	nd	11.1	0.9	1.9	4.6	35.2	0.9	4.6	nd	0	1.9	8.3	19.4
Germany	11	SL	184	25.5+7.6	0	37.0	2.2	7.6	1.6	0	0.5	7.6	6.0	0	2.2	1.6	0.5
		SV	104	12.5+5.8	4.8	21.2	1.0	44.2	0	1.0	0	1.0	2.9	0	1.0	1.9	2.9
		NC	22	13.6+9.1	13.6	18.2	0	4.5	0	36.4	0	0	0	4.5	0	0	0
Greece	12	SL	98	24.5	0	42.9	5.1	2.0	0	0	0	14.3	1.0	0	1.0	0	9.2
		SV	68	5.9	19.1	29.4	0	35.3	0	1.5	0	5.9	0	0	0	0	2.9
		NC	56	3.6	21.4	5.4	0	7.1	0	41.1	0	1.8	0	14.3	0	0	5.4

population	reference(s)[a]	type[b]	number of alleles	no *CYP21* gene[c]	Pro30 → Leu	intron 2 splice	exon 3 del 8 bp	Ile172 → Asn	cluster exon 6	Val281→ Leu	exon 7 ins 1 bp	Gln318 → stop	Arg356 → Trp	Pro453 → Ser	combination[d]	other mutation[e]	not found[f]
India	13	SL	33	12.1	3.0	27.3	nd	12.1	nd	nd	nd	27.3	0	nd	0	nd	15.2
		SV	20	10.0	0	10.0	nd	50.0	nd	nd	nd	0	0	nd	0	nd	30.0
Israel	14	NC	90	0	2.2	6.7	0	2.2	0	80.0	nd	4.4	nd	nd	0	nd	4.4
Italy	15	SL	90	23.3+12.2	2.2	26.7	0	0	0	1.1	0	8.9	0	nd	13.3	nd	24.4
		SV	30	3.3+3.3	0	10.0	0	23.3	0	3.3	0	6.7	0	nd	16.7	nd	33.3
		NC	26	0+0	7.7	7.7	0	7.7	0	53.8	0	7.7	0	nd	3.8	nd	11.5
Italy	16	SL	34	8.8	0	64.7	0	0	0	0	0	2.9	8.8	0	14.7	nd	0
		SV	16	6.3	0	50.0	0	25.0	0	0	0	6.3	0	0	6.3	nd	6.3
		NC	6	0	0	16.7	16.7	0	0	50.0	0	0	0	0	16.7	nd	0
Italy	17	SL	38	34.2+5.3	2.6	31.6	2.6	2.6	2.6	0	0	2.6	7.9	0	2.6	5.3	0
		SV	16	25.0	6.3	18.8	0	12.5	0	12.5	0	12.5	0	0	0	0	12.5
		NC	62	1.6+1.6	1.6	14.5	1.6	9.7	1.6	43.5	0	4.8	0	4.8	3.2	3.2	8.1
Japan	18	SL	60	15.0+1.7	0	33.3	0	0	1.7	0	0	0	15.0	nd	10.0	nd	40.0
		SV	28	25.0+0	0	21.4	0	35.7	0	0	0	0	0	nd	3.6	nd	14.3
Japan	19	SL	22	0	nd	36.4	nd	4.5	nd	nd	nd	22.7	0	nd	13.6	nd	22.7
		SV	14	7.1	nd	50.0	nd	42.9	nd	nd	nd	0	0	nd	0	nd	0
Japan	20	NC	14	0	50.0	14.3	0	7.1	0	0	0	0	0	0	28.6	0	0
Japan	21	SL	46	g	10.9	37.0	g	nd	4.3	2.2	nd	2.2	4.3	nd	0	nd	4.3
Lebanon	22	SL	28	14.2	nd	42.9	10.7	0	0	0	nd	10.7	nd	nd	21.4	nd	0
		SV	8	12.5	nd	25.0	12.5	25.0	0	0	nd	0	nd	nd	12.5	nd	12.5
		NC	14	0	nd	0	0	0	0	85.7	nd	7.1	nd	nd	0	nd	7.1
Mexico	23	SL	42	0	0	40.5	4.8	0	0	2.4	0	4.8	4.8	0	23.8	0	19.0
		SV	46	2.2	6.5	30.4	0	17.4	nd	2.2	0	0	0	0	10.9	0	23.9
		LO	6	0	50.0	0	0	0	0	0	0	0	0	0	0	16.7	33.3

population	reference(s)[a]	type[b]	number of alleles	no *CYP21* gene[c]	Pro30 → Leu	intron 2 splice	exon 3 del 8 bp	Ile172 → Asn	cluster exon 6	Val281→ Leu	exon 7 ins 1 bp	Gln318 → stop	Arg356 → Trp	Pro453 → Ser	combination[d]	other mutation[e]	not found[f]
Netherlands	24	SL	59	22.0+13.6	1.7	37.3	1.7	1.7	8.5	0	0	1.7	3.4	0	5.1	nd	5.1
		SV	20	15.0+5.0	0	5.0	0	35.0	0	0	0	0	5.0	0	5.0	nd	25.0
		LO	6	0	0	16.7	0	33.3	0	50.0	0	0	0	0	0	nd	0
Netherlands	25		>100	35	< 1	29	5	11	2	< 1	1	5	11	nd	0	nd	0
Russia	26	SL	34	39	0	23.5	5	3	0	0	nd	6	0	nd	0	nd	23.5
		SV	20	16.5	0	16.5	0	22	0	0	nd	0	5	nd	0	nd	40
		NC	78	1.3	0	0	0	0	0	0	nd	0	0	nd	0	nd	98.7
Spain	27	CL	46	19.6+13.0	0	30.4	6.5	2.2	0	2.2	2.2	4.3	4.3	nd	4.3	nd	10.9
		NC	30	0+0	6.7	10.0	0	0	0	36.7	0	3.3	3.3	nd	0	nd	40.0
Spain	28	SL	104	19.2+15.4	0	21.2	1.9	0	1.0	1.0	1.9	3.8	5.8	0	21.2	3.8	3.8
		SV	10	0+0	0	20.0	0	30.0	0	0	0	0	0	0	30.0	0	20.0
Sweden	29		400	32.2	1.6	26.6	1.1	19.8	1.1	5.7	< 1	2.4	3.0	< 1	< 3	< 3	--
Taiwan	30	SL	16	25.0	0	37.5	0	0	6.3	0	6.3	0	12.5	nd	12.5	0	0
		SV	22	18.2	0	18.2	0	45.5	0	0	0	4.5	0	nd	4.5	4.5	4.5
Taiwan	31	CL	130	10.8	0	41.5	1.5	22.3	0	0	1.5	0.8	15.4	nd	0	5.4	0.8
United Kingdom	32	CL	190	25	1.1	34	nd	15	--	2.6	nd	nd	nd	nd	--	--	20.0
		NC	30	6.7	10.0	13.3	nd	6.7	3.3	36.7	nd	nd	nd	nd	10.0	0	13.3
United Kingdom	33	CL	284	45.0	0	30.3	nd	7.0	0	0	0	0	9.8	0	0	0	7.8
United States	34	SL	254	30.3+8.3	0.8	35.0	3.9	2.8	0	1.6	0	4.3	3.5	nd	3.9	nd	5.9
		SV	78	15.4+0	5.1	23.1	1.3	38.5	0	1.3	0	6.4	7.7	nd	0	nd	0
		NC	62	18.3+1.7	6.7	20.0	0	1.7	0	51.7	0	0	0	nd	0	nd	1.7

Notes to table 3

nd: not determined; --: not reported, which may cause the sum of the percentages to be lower than 100 % in a few cases

a: see reference table below

b: SL: salt-losing; SV: simple virilising; CL: unspecified classical; NC: nonclassical; none: no specification

c: when two figures are given: (*CYP21* only) + (*CYP21P-CYP21P*), both apparently created by an unequal crossover; when one figure is given, the study made no distinction between these haplotypes

d: nearly always including at least one severe mutation consistent with classical 21-hydroxylase deficiency; only single mutations have been listed separately; see the original report for details

e: rare and often novel mutations, specified in the original report

f: not detectable by the methods used

g: no distinction made between absence of *CYP21* and 8 bp deletion in exon 3: together, they make up 34.8 % of the defects

References to table 3

population		reference	note
Argentina	1	Dardis *et al.* [1997]	compiled from table 2
Austria	2	Baumgartner-Parzer *et al.* [2001]	copied from table 1
Brazil	3	Paulino *et al.* [1999]	compiled from tables 2 and 5
Brazil	4	Bachega *et al.* [2000]	130 patients: 29 SL, 51 SV, 50 LO; 228 independent alleles; only limited mutation data was presented in this report
Brazil	5	Witchel *et al.* [2000]	compiled from table 2
Chile	6	Fardella *et al.* [1998]	compiled from table 2, not counting 8 patients where no family study was done
Chile	7	Fardella *et al.* [2000]	compiled from table 2, not counting 4 patients where no family study was done
Denmark	8	Ohlsson *et al.* [1999]	compiled from table 2
Finland	9	Levo and Partanen [1997a]	compiled from table 3, counting the 3' conversion as a combination and ignoring 2 patients where the family study was not informative
France		Mornet *et al.* [1991]	included in a more recent report by the same group [Barbat *et al.* 1995]
France	10	Barbat *et al.* [1995]	includes data from an earlier report by the same group [Mornet *et al.* 1991]
Germany	11	Krone *et al.* [2000]	copied from table 1
Greece	12	Dracopoulou-Vabouli *et al.* [2001]	copied from table 1
India	13	Mathur *et al.* [2001]	compiled from table 1, not counting three of the haplotypes because of consanguinity

population		reference	note
Israel	14	Weintrob *et al.* [2000]	compiled from figure 1
Italy		Carrera *et al.* [1993]	included in a more recent report by the same group [Carrera *et al.* 1996]
Italy	15	Carrera *et al.* [1996]	calculated from table 2 and table 1 in the earlier report [Carrera *et al.* 1993]
Italy	16	Bobba *et al.* [1999]	compiled from table 2
Italy	17	Balsamo *et al.* [2000]	compiled from table 2
Japan	18	Higashi *et al.* [1991]	compiled from table 1, not counting one patient where multiple interpretations are possible
Japan	19	Tajima *et al.* [1993]	compiled from table 1, not counting patients where no family study was done
Japan	20	Tajima *et al.* [1998]	compiled from table 1
Japan	21	Yokoyama *et al.* [2000]	copied from table III
Lebanon	22	Delague *et al.* [2000]	calculated from table 3
Mexico	23	Ordoñez-Sánchez *et al.* [1998]	compiled from table 3; there is some uncertainty about several combinations of mutations on the same chromosome
Netherlands	24	Koppens *et al.* [2000]	with a few recent additions from Chapter 3.2
Netherlands	25	Lases *et al.* [2001]	copied from table 2
Russia	26	Osinovskaya *et al.* [2000]	copied from the table, assuming that 'R350G' should read 'R356W'
Spain	27	Ezquieta *et al.* [1995]	calculated from table 1
Spain	28	Lobato *et al.* [1999]	compiled from table 1
Sweden		Wedell *et al.* [1994]	included in a more recent report by the same group [Wedell 1998a]
Sweden	29	Wedell [1998a]	copied from table 1; the number of alleles is an approximation (see text); includes data from an earlier report by the same group [Wedell *et al.* 1994]
Taiwan	30	Ko *et al.* [1998]	compiled from table IV, not counting a single patient with nonclassical steroid 21-hydroxylase deficiency
Taiwan	31	Lee *et al.* [1998]	calculated from table 2; classical steroid 21-hydroxylase deficiency assumed from the text
United Kingdom	32	Rumsby *et al.* [1998]	compiled from table 1 and the text; no further details on the classical patients were provided
United Kingdom	33	Lako *et al.* [1999]	copied from table 3
United States		Speiser *et al.* [1992]	included in a more recent report by the same group [Wilson *et al.* 1995b]
United States	34	Wilson *et al.* [1995b]	compiled from tables 1 and 2; includes data from an earlier report by the same group [Speiser *et al.* 1992]

1.8. Methods used to study *CYP21* and the RCCX module

The dual approach of RCCX variability analysis (see paragraph 1.6) is reflected in the methods used during the project described in this thesis. This paragraph contains a brief outline of these methods and a discussion of some of their technical complications. Comprehensive descriptions and details such as hybridisation circumstances, primer composition and PCR conditions, can be found in Chapters 2 to 8 of this thesis.

Restriction analysis of genomic DNA

Basic molecular genetic techniques suffice for *CYP21*/*C4* haplotyping [Ausubel *et al.* 1989; Sambrook *et al.* 1989]. Briefly, the following protocol was applied:

- human genomic DNA was isolated from leukocytes [Wyman and White 1980];
- 10 µg of DNA was digested with an appropriate restriction enzyme;
- the DNA was separated by electrophoresis on an agarose gel;
- the DNA was transferred to a nitrocellulose membrane by capillary blotting [Southern 1975];
- the DNA was fixed to the membrane by baking it at 80°C in a vacuum oven;
- the membrane was hybridised to a mixture of ^{32}P labelled cDNA probes;
- after washing, the membrane was exposed to an X-ray film to detect the hybridisation signal by fluorography [Amersham International 1992];
- the intensity ratios of the bands were quantitated by laser densitometry;
- haplotypes were deduced by segregation analysis in families of 21-hydroxylase deficiency patients;
- some complicated cases required further analysis by long-range restriction mapping using pulsed-field gel electrophoresis [van Ommen and Verkerk 1986; Southern *et al.* 1987].

These techniques have been available for many years and have been applied by many investigators, but their technical intricacies should not be underestimated. In many studies, banding patterns are used to establish segregation of haplotypes identifiable by unique bands. However, due to the high variability of the RCCX module (see paragraph 1.6 and Chapter 2), *CYP21/C4* haplotyping depends on the *ratio* of specific bands: for example, it should be possible to distinguish a 2:1 ratio of the *CYP21* 3.2 and 3.7 kb bands from a 3:2 ratio. There is no need to quote here several reports showing autoradiographs that are obviously unsuitable for this purpose, but considering that the average scientist probably does not select the worst available results for publication, it is clear that applying the techniques listed above requires some expertise. Initially, our own results were by no means satisfactory either. So, while the detailed protocols published elsewhere (see the references above) provide adequate general instructions, it seems suitable to address a few crucial technical issues here.

Human genomic DNA (10 µg) was digested overnight with 40-60 units of the appropriate restriction enzyme, usually according to the manufacturer's instructions. *Taq*I digest (at 65° C) typically produced good results without additional measures. For most other enzymes,

addition of spermidine at a final concentration of 1-2 mM and addition of half the amount of enzyme initially followed by the other half after two hours of incubation, proved beneficial.
Overnight electrophoresis was done on 0.7 - 1.5 % agarose gels, in 1xTBE with 0.5 µg/ml ethidium bromide, at 50-90 volts (depending on the expected fragment size), in a Pharmacia GNA-200 submarine electrophoresis unit, using its cooling facility when running at more than 75 volts. To obtain sharp bands, a 10 mm slot was used for 10 µg of DNA and ethidium bromide was added to the sample at a final concentration of 0.5 µg/µl.
After electrophoresis, the DNA was partly hydrolysed by soaking the gel in 1 litre of 0.25 M HCl for exactly 15 minutes, followed by denaturation and neutralisation steps. The hydrolysis step markedly improved the transfer of DNA. Traditional overnight capillary Southern blotting yielded a more complete transfer of DNA than the use of a vacuum blotting apparatus. Accurate band ratios require complete transfer of DNA, which can be checked by re-staining the gel with ethidium bromide after blotting.
Hybridisation was most often done in a plastic box, with 10 % dextran sulphate (later replaced with 8 % polyethylene glycol 6000 for economic reasons) in the buffer to improve the hybridisation rate. The *CYP21* probe used was pC21/3c [White *et al.* 1985]; since this cDNA probe only partly overlaps the adjacent *TNX* genes and primarily hybridises to the 3.2 and 3.7 kb *CYP21P* and *CYP21* bands, we sometimes used a mixture of 0.3 kb *Kpn*I-*Bam*HI fragments covering the 3' end of the insert in pC21/3c to obtain a better signal from the 2.4 and 2.5 kb bands. The *C4* DNA probe was pAT-A [Belt *et al.* 1984]; a 0.9 kb *Pst*I fragment produced somewhat better results than the 0.6 kb *Bam*HI-*Bgl*II fragment used initially [Koppens *et al.* 1992a]. Quantitation by laser densitometry is discussed in detail in Chapter 2.1. For interpretation, measured band ratios need to be rounded to integers.
The procedure outlined here includes several overnight steps and may seem relatively slow, but the logistics of processing a few hundred samples (a number typical of this study) are very different from those of rapid tests aimed at DNA analysis of one or a few persons.
Translating band ratios into two separate haplotypes per individual is not always easy and straigthforward, as illustrated by a controversy that arose between two prominent research groups during the early years of *CYP21* haplotyping [Matteson *et al.* 1987; Miller 1987; White *et al.* 1987; White *et al.* 1988]. The key to understanding these haplotypes is the notion that deletions and insertions involve entire RCCX modules and not individual genes. Anecdotal reports of deletions of *CYP21* [Nakura *et al.* 1987; Rumsby *et al.* 1988] or *CYP21P* [Lim and Chan 1996] without concomitant deletion of an adjacent *C4* gene, probably represent misinterpretations of non-standard bimodular RCCX configurations where only the *C4* gene yields distinct banding patterns. *Bgl*II patterns can be used to facilitate the interpretation of *Taq*I-based haplotypes, because pC21/3c hybridises to a 12 kb *Bgl*II fragment, the copy number of which is one fewer than the number of RCCX modules. Several authors have therefore emphasised the importance of *Bgl*II digests [Donohoue *et al.* 1986; White *et al.* 1988; Morel *et al.* 1989a]. Although we did use *Bgl*II patterns for the interpretation of all haplotypes reported in this thesis, simultaneous hybridisation of the *CYP21* and *C4* cDNA probes to Southern blots of *Taq*I digested DNA, combined with segregation studies, was

nearly always sufficient to deduce the haplotypes. Since this co-hybridisation provides valuable additional information without requiring significant additional effort, it is difficult to understand why some investigators omit one of the probes, or perform the hybridisations separately.

A final point in the interpretation of *CYP21/C4* haplotypes, discussed in more detail in Chapter 2, is the fact that some combinations of haplotypes are indistinguishable and can be correctly assigned only by segregation study or by long-range restriction mapping. In population studies, it is usually safe to exclude combinations of very rare haplotypes in cases where more common haplotypes provide an obvious explanation, if at least the Hardy-Weinberg equilibrium is observed. However, this strategy sometimes fails: for example, in an isolated individual with a total of three long and two short *C4* genes, there is no compelling reason to prefer a distribution of long-short-short plus long-long to the alternative of long-long-short plus long-short. Surprisingly, this point has been missed even in a recent authoritative report from one of the leading groups in complement *C4* genetics [Blanchong *et al.* 2000].

*Sac*II restriction mapping was used to assess the total length of the RCCX module in complicated cases. We devised a method to use DNA that had been isolated by a conventional protocol [Wyman and White 1980] in pulsed-field gel electrophoresis; the results suggest that prior to *Sac*II digestion, most DNA was present as fragments of at least 200 Mb in size (see Chapter 7 for technical details).

Specific amplification of *CYP21*, *CYP21P* and the *TNX* genes

The analysis of the RCCX region by specific amplification of sequences of interest by means of the polymerase chain reaction [Saiki *et al.* 1985; Mullis and Faloona 1987] is hampered by the high similarity of the genes and pseudogenes in this region. *CYP21* and *CYP21P* are 98 % similar [Higashi *et al.* 1986], so the prospect of specific amplification depends on whether the small sequence differences allow the design of primer sets that are structurally sound, adequately specific and have the most important mutation sites between them. Small-scale gene conversion creates the majority of the mutations that cause 21-hydroxylase deficiency, but it may likewise alter the site of the primer on which the detection of such mutations depends. Also, amplification of sequences more than 1 kb in length was not feasible when *CYP21* mutation analysis was first undertaken in the early 1990's [Owerbach *et al.* 1990; Mornet *et al.* 1991].

We therefore initially designed a comprehensive PCR strategy [Koppens *et al.* 2000] to make sure that the genes amplified with *CYP21*- and *CYP21P*-specific primer sets were indeed the same genes that were previously identified on the basis of *Taq*I restriction patterns. First, genomic *Taq*I/*Eco*RI double digestion was used to confirm that an *Eco*RI site in the second intron of *CYP21P* was a reliable marker [Koppens *et al.* 1998]. Since this site was indeed present in all *CYP21P* genes and none of the *CYP21* genes, *Eco*RI digestion could be used to confirm the specificity of a PCR that covered the second intron and third exon (section 1 in

fig. 1 in Chapter 3.1). The section 1 PCR product includes the site of an 8 bp deletion in exon 3 of *CYP21P* but not *CYP21*. This difference was then used to specifically amplify section 2. Section 2 includes the site of 4 base pair changes in a small area of exon 6; this difference was in turn used to amplify section 3. This cautious approach proved its value, because patients with mutations at the section 2 and section 3 primer sites were indeed found. Because the sections 1 to 3 covered all conversion sites associated with classical steroid 21-hydroxylase deficiency, this strategy was used to detect those mutations in the patients' *CYP21* genes and to chart the variability of the *CYP21P* pseudogene [Koppens *et al.* 2000].

The high performance of currently available thermostable polymerases allowed a more convenient strategy in later studies (see Chapter 3.2). Since the 8 bp deletion in exon 3 was found in all *CYP21P* genes but in only one patient's *CYP21* gene, specific primers based on that difference could be used, and the amplified section continued into the 5' and 3' flanking areas (see Chapter 3.2 for details).

Amplification of a 2.7-2.8 kb section of *TNXA* or *TNXB* was based on one common primer and one specific primer positioned beyond the boundary of the RCCX module [Koppens *et al.* 2002a; Koppens *et al.* 2002b].

Optimal primer design was not always possible due to the limited differences in sequence. Design of the first primer sets were based on algorithms available at the time [Thein and Wallace 1986]; later, freely accessible web sites (see paragraph 1.9) were used. The addition of 1 % formamide to the reaction buffer [Sarkar *et al.* 1990] greatly improved the specificity of several PCR's. DNA samples that were not amplified efficiently were adjusted to 2.5 M ammonium acetate, precipitated with 2 volumes of ethanol and re-dissolved - this procedure very often solved the problem.

Non-amplification of one allele has been reported in the analysis of *CYP21* mutations [Day *et al.* 1996; Ozturk *et al.* 2000], but this was not observed in the PCR methods applied here (see Chapter 3).

Mutation detection with allele-specific oligonucleotides

The detection method used in this study to detect *CYP21* mutations was hybridisation of ^{32}P-labelled DNA oligonucleotides to membrane-bound denatured PCR products (commonly known as allele-specific oligonucleotide hybridisation) [Thein and Wallace 1986; Owerbach *et al.* 1990; Mornet *et al.* 1990]. It is a convenient and reliable method for processing a large number of samples and it offers the important advantage that blots can be stripped and re-probed many times to screen for different mutations that lie within the amplified region. The main technical complication is oligonucleotide design [Thein and Wallace 1986; http://eatworms.swmed.edu/~tim/primerfinder/; http://www.idtdna.com/program/bioinformatics/Sequence_Analysis_Tools.asp]: the environment of the mutation to be detected may be an obstacle (for example, no well-designed oligonucleotide exists for the rare *CYP21* mutation Arg483→Pro). Also, some oligonucleotides require careful post-hybridisation washing steps. Ideally, previously known positive and negative controls should be available, but for

mutations that have not been checked before this is not feasible. The purpose of the washing steps should not be to reduce the radioactivity of a negative control to background levels, but rather to obtain an optimal ratio between a positive and a negative control and to visualise the results by a carefully timed fluorographic exposure.

1.9. Reference section

Patients, family members, and controls

The study group included 47 families of steroid 21-hydroxylase deficiency patients and 149 controls. Single patients were not included for genotyping because segregation studies were necessary. The hospitals participating in this study were the Sophia Children's Hospital in Rotterdam (33 families), the Leiden University Medical Centre (10 families), the Catharina Hospital in Eindhoven (2 families), the Oosterschelde Hospital in Goes (1 family) and the Maria Hospital in Tilburg (1 family). Families of Dutch ethnic origin were included into the haplotyping and mutation analysis studies (see Chapters 2 and 3), to assess allele frequencies in the Dutch population. Three other families were also genotyped to check for unusual mutations, but their numbers were to small for epidemiological purposes.
The 44 Dutch families included 51 steroid 21-hydroxylase deficiency patients and 123 healthy family members.
At the onset of the study, most patients were regularly seen at the outpatient department of these hospitals; most older patients responded positively to a recall request. All families involved gave their informed consent. The diagnosis of SL 21-hydroxylase deficiency CAH was based on characteristic phenomena that occurred during the first weeks of life, such as hyponatraemia, hyperkalaemia, dehydration, vomiting, shock and highly elevated plasma levels of 17α-hydroxyprogesterone. Patients with SV 21-hydroxylase deficiency had no history of salt-losing crises and presented during childhood, usually with ambiguity of the *genitalia externa* in females and precocious puberty in males, as well as accelerated growth in most patients.
The clinical diagnosis was SL steroid 21-hydroxylase deficiency in 36 patients from 30 families, SV in 12 patients from 11 families and NC in 3 patients from 3 families.

Nomenclature

In this thesis, the designation *CYP21* (in italics) is used to indicate the steroid 21-hydroxylase gene; *CYP21P* is the pseudogene. The non-italicised variant *CYP21* is sometimes used for the enzyme, although the full 'steroid 21-hydroxylase' is preferred. This is in agreement with most of the current literature; alternatively, some authors use *CYP21A1P* and *CYP21A2* for the pseudogene and the gene, respectively. The latter is in agreement with the recommended nomenclature [Nebert *et al.* 1991], but seems a bit cumbersome, and recently the

nomenclature committee has again moved away from these designations [Cytochrome P450 Allele Nomenclature Committee 2002].
In the older literature, *CYP21A* is used for the pseudogene and *CYP21B* for the active steroid 21-hydroxylase gene; this matches the designation of the adjacent *C4* genes. Note that this positional notation has no functional implications: in the mouse, the active gene lies between the *C4A* and *C4B* genes. Mouse genes are distinguished from human genes by capitalising the first letter only; for example, *Cyp21*. Several variants, such as '21OHB', have also been in used in the past.
TNXB and *TNXA* are used for the *TNX* gene and the pseudogene, to underline their similarity; several authors prefer just *XA* for the *TNX* pseudogene. Earlier literature used designations such as 'gene *X*' and '*OSG*' (opposite strand gene) for *TNXB*.
RP1 is used here for the most telomeric gene in the RCCX module (it was named after the late Nobel Prize winner Rodney Porter). Alternative names are *STK19* and *G11*.
CYP21 amino acid and nucleotide numbering throughout this thesis is according to Higashi *et al.* [1986]. This implies that the last amino acid is Gln494 and that the neutral polymorphism in exon 1, a 3 bp insertion encoding an extra leucine residue, is not counted. Some authors include this polymorphism, which may cause some confusion as common mutations get a different codon number (for example: Val282→Leu instead of Val281→Leu). Although the single letter amino acid code has been recommended, the more descriptive (and, according to the current nomenclature [den Dunnen and Antonarakis 2000], 'acceptable') three letter code has been used in this thesis, considering that no long protein sequences are listed anywhere.

Reviews

To readers who require additional information, the author recommends the following reviews:

On steroid 21-hydroxylase deficiency and CAH

Ritzén EM, Lajic S, Wedell A (2000)
How can molecular biology contribute to the management of congenital adrenal hyperplasia?
Hormone Res 53 suppl. 1: 34-37

Speiser PW, Guest Editor (2001)
Congenital Adrenal Hyperplasia
Endocr Metab Clin North Am 30

White PC, Speiser PW (2000)
Congenital adrenal hyperplasia due to 21-hydroxylase deficiency
Endocr Rev 21: 245-291

On adrenocortical steroidogenesis and its regulation

Andrew R (2001)
Clinical measurement of steroid metabolism
Best Practice Res Clin Endocrinol Metab 15: 1-16

Pestell RG, Jameson JL (1995)
Transcriptional regulation of endocrine genes by second-messenger signaling pathways
In: Weintraub BD, ed.: Molecular endocrinology. Basic concepts and clinical correlations, pp. 59-76. New York, Raven Press

White PC (1994)
Genetic disease of steroid metabolism
Vitam Horm 49: 131-195

Waterman MR, Keeney DS (1996)
Signal transduction pathways combining peptide hormones and steroidogenesis
Vitam Horm 52: 129-148

On cytochromes P450 and their genes

Estabrook RW, Masters BSS, Prough RA, Waterman M, eds. (1996-1997)
Cytochromes P450 serial review (12 parts)
Faseb J 10: 202-227; 428-434; 552-558; 683-689; 809-818; 1112-1117; 1241-1248; 1369-1377; 1456-1463; 1569-1577, and 11: 29-36; 419-427.

Honkakoski P, Negishi M (2000)
Regulation of cytochrome P450 (CYP) genes by nuclear receptors
Biochem J 347: 321-337

On the genetics of the MHC

Dawkins R, Leelayuwat C, Gaudieri S, Hui J, Cattley S, Martinez P, Kulski J (1999)
Genomics of the major histocompatibility complex: haplotypes, duplication, retroviruses and disease
Immunol Rev 167: 275-304

Milner CM, Campbell RD (2001)
Genetic organization of the human MHC class III region
Front Biosc 6: d914-d926

On genetic mechanisms and evolution

Carrington M (1999)
Recombination within the human MHC
Immunol Rev 167: 245-256

Jeffreys AJ, Barber R, Bois P, Buard J, Dubrova YE, Grant G, Hollies CRH, May CA, Neumann R, Panayi M, Ritchie AE, Shone AC, Signer E, Stead JDH, Tamaki K (1999)
Human minisatellites, repeat DNA instability and meiotic recombination
Electrophoresis 20: 1665-1675

Klein J, Sato A (1998)
Birth of the major histocompatibility complex
Scand J Immunol 47: 199-209
Martinsohn JT, Sousa AB, Guethlein LA, Howard JC (1999)
The gene conversion hypothesis of MHC evolution: a review
Immunogenet 50: 168-200
Meyer D, Thomson G (2001)
How selection shapes variation of the human major histocompatibility complex: a review
Ann Hum Genet 65: 1-26

On the RCCX module

Blanchong CA, Chung EK, Rupert KL, Yang Y, Yang Z, Zhou B, Moulds JM, Yu CY (2001)
Genetic, structural and functional diversities of human complement components C4A and C4B and their mouse homologues, Slp and C4
Int Immunopharmacol 1: 365-392
Yu CY (1998)
Molecular genetics of the human MHC complement gene cluster
Exp Clin Immunogenet 15: 213-220

Databases and web sites

A growing number of databases and other resources relevant to the issues discussed in this thesis is available through the world wide web. Below is a short list of freely accessible web sites per February 18th, 2002. Doubtlessly, it is far from complete, outdated by the time this thesis is printed and inadequate in listing some of the reader's cherished favourites – but some of these sites proved useful to the author.

Table 6. Web sites relevant to the subject of this thesis per February 18th, 2002.

EMBL Search Page	http://srs.ebi.ac.uk/srs6bin/cgi-bin/wgetz?-page+top+-id+10EIW1DOHxp
Offers access to various databases	
MHC Sequence	http://www.sanger.ac.uk/HGP/Chr6/MHC.shtml
Sequence of the MHC, from the Sanger Institute	
Ensembl	http://www.ensembl.org/Homo_sapiens/mapview?chr=6
Chromosome 6 gene browser	
NCBI BLAST	http://www.ncbi.nlm.nih.gov/BLAST/
Sequence database and sequence analysis tools	
NHGRI	http://www.nhgri.nih.gov/
The human genome project	
HGMDB	http://archive.uwcm.ac.uk/uwcm/mg/search/120605.html
Human Gene Mutation Database, steroid 21-hydroxylase mutations	
SwissProt	http://www.expasy.org/cgi-bin/niceprot.pl?P08686
SwissProt entry for steroid 21-hydroxylase	
OMIM	http://www.ncbi.nlm.nih.gov/htbin-post/Omim/dispmim?201910
Online Mendelian Inheritance in Man entry for steroid 21-hydroxylase deficiency	
Diseases Database	http://www.diseasesdatabase.com/sieve/item1.asp?glngUserChoice=1854
Diseases Database entry for steroid 21-hydroxylase deficiency	
FIDD	http://archive.uwcm.ac.uk/uwcm/mg/fidd/
Frequency of Inherited Disorders Database	
IHGW	http://www.ihwg.org/contents.htm
International Histocompatibility Working Group	
IMGT	http://www.ebi.ac.uk/imgt/hla/
HLA sequence database	
P450 Nomenclature	http://www.imm.ki.se/cypalleles/
Cytochrome P450 nomenclature and mutations	
Cytochrome P450	http://www.icgeb.trieste.it/~p450srv/450.html
Cytochrome P450 database	
WebCutter 2.0	http://www.firstmarket.com/cutter/cut2.html
Restriction site analysis	
Primerfinder	http://eatworms.swmed.edu/~tim/primerfinder/
The oligonucleotide analysis tool most frequently used for this study	
Oligo Analyzer	http://207.32.43.248/
Another useful oligonucleotide analysis tool	
JaMBW 1.1	http://members.aol.com/_ht_a/lucatoldo/myhomepage/JaMBW/
Various sequence analysis tools	
IDT	http://www.idtdna.com/program/bioinformatics/Sequence_Analysis_Tools.asp
Various sequence analysis tools	
EBI Catalogue	http://corba.ebi.ac.uk/Biocatalog/Sequence_analysis.html
A catalogue of molecular biology software	
Molecular ToolBox	http://www.pitt.edu/~rsup/molectoolbox.html
A set of useful molecular biology bookmarks	

Sequences

A list of sequences that were used for this thesis appears in table 7. These sequences were available from the EMBL web site http://srs.ebi.ac.uk/srs6bin/cgi-bin/wgetz?-page+top+-id+10EIW1DOHxp on February 18th, 2002.

Table 7. Accession numbers of sequences used in this thesis.

M12792	*CYP21*, 5.1 kb	Higashi *et al.* [1986]
M12793	*CYP21P*, 5.1 kb	Higashi *et al.* [1986]
M13936	*CYP21*, 3.4 kb	White *et al.* [1986]
M13935	*CYP21P*, 3.2 kb	White *et al.* [1986]
HSCP2113	*CYP21*, 4.0 kb	Rodrigues *et al.* [1987]
M26857	*CYP21P*, 4.0 kb	Rodrigues *et al.* [1987]
M28548	mutated *CYP21*, 4.0 kb	Rodrigues *et al.* [1987]
L26263	*C4A*, 5.0 kb	Yu [1991; Shen *et al.* [1994]
M94068	*C4B-CYP21* intergenic region, 1.8 kb	Donohoue and Collins [1992]
M94069	*C4B-CYP21* intergenic region with *CYP21P* deletion, 1.8 kb	Donohoue and Collins [1992]
M94070	*C4A-CYP21P* intergenic region with *CYP21* deletion, 1.8 kb	Donohoue and Collins [1992]
S38953	*TNXA*, 6.9 kb	Gitelman *et al.* [1992]
X71937	*TNXB*, 12.7 kb	Bristow *et al.* [1993b]
AF077974	RP2-TNXA-CYP21P*, 7.5 kb*	Rupert *et al.* [1999]
AF086641	*TNXB-TNXA* recombinant, 2.5 kb	Yang *et al.* [1999]
AF019413	class III region, 109.6 kb	Rowen *et al.* [1999a]
U89337	class III region, 100.3 kb	Rowen *et al.* [1999b]
AL049547	class III region, 129.8 kb	Barlow 2000]

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Chapter 2

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2.1. Family studies of the steroid 21-hydroxylase and complement *C4* genes define eleven haplotypes in classical congenital adrenal hyperplasia in the Netherlands

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Abstract

Two steroid 21-hydroxylase genes are normally present within the human major histocompatibility complex near the genes encoding the fourth component of complement (C4A and C4B). Steroid 21-hydroxylase is encoded by the *CYP21* gene, while the highly homologous *CYP21P* gene is a pseudogene. We studied steroid 21-hydroxylase and complement *C4* haplotypes in 33 Dutch patients (29 families) suffering from classical congenital adrenal hyperplasia (CAH) and in their 80 family members, and also in 55 unrelated healthy controls, using 21-hydroxylase and complement *C4* cDNA probes. Eleven different haplotypes, defined in terms of gene deletions, gene duplications, conversions of *CYP21* to *CYP21P*, and 'long' and 'short' *C4* genes, were found. In 23 % of the patients' haplotypes, the *CYP21* gene was deleted; in 12 %, it was converted into a *CYP21P* pseudogene. In the remaining 65 %, the defect was apparently caused by a mutation not detectable by this method. The most common haplotype (with one *CYP21* and one *CYP21P* gene) was significantly more often observed in patients with simple virilising CAH than in those with salt-losing CAH. Comparison of the 21-hydroxylase haplotypes found in CAH patients from several countries shows evidence for considerable genetic variation between the groups studied.

Introduction

Congenital adrenal hyperplasia (CAH; also known as the adrenogenital syndrome (AGS)) is caused by an inborn error of adrenocortical steroidogenesis. In about 95 % of all cases, a

defect in steroid 21-hydroxylation is the cause of this disorder [Degenhart 1979; Miller and Levine 1987; White *et al.* 1987a; White *et al.* 1987b]. Steroid 21-hydroxylase deficiency is one of the most common inborn errors of metabolism, affecting about one in every 10,000-15,000 newborns in Caucasian populations. In the most severely afflicted patients, both cortisol and aldosterone synthesis are impaired, resulting in insufficient sodium retention as well as virilisation of the external genitalia. These patients usually suffer from severe salt-losing crises shortly after birth ('salt-losing' CAH). If the cortisol synthesis is blocked but the aldosterone production is impaired to a lesser degree, virilisation will occur but the patients do not suffer from salt-losing crises ('simple virilising CAH'). Salt-losing and simple virilising CAH are manifestations of the so-called classical 21-hydroxylase deficiency. Non-classical 21-hydroxylase deficiency is a milder form of the same disorder; in this form, clinical symptoms do not become manifest until late childhood ('late onset' CAH) or remain absent ('cryptic' CAH).

The enzyme steroid 21-hydroxylase (cytochrome P-450_{C21}) is encoded by the *CYP21* gene in the class III region of the human major histocompatibility complex (MHC) on the short arm of chromosome 6. It is the most centromeric of a cluster of two 21-hydroxylase genes (3.4 kb each), alternating with two genes encoding the fourth component of complement (16-22.5 kb). Usually, complement *C4* locus I (the most telomeric) is occupied by a *C4*A gene and locus II by a *C4B* gene. The other 21-hydroxylase locus contains the *CYP21P* pseudogene, which is highly homologous to *CYP21*, but inactive due to several deleterious mutations [Higashi *et al.* 1986; White *et al.* 1986].

Deletions and duplications of a 30 kb area encompassing either *CYP21* or *CYP21P* and one adjacent *C4* gene and (partial) conversions of *CYP21* into a *CYP21P*-like pseudogene have been reported to be very common in the 21-hydroxylase/complement *C4* region [White *et al.* 1985; Carroll *et al.* 1985; Harada *et al.* 1987; Matteson *et al.* 1987; Rumsby *et al.* 1988; White *et al.* 1988; Collier *et al.* 1989; Miller and Morel 1989; Morel *et al.* 1989; Partanen *et al.* 1989; White 1989]. In addition, a size difference of the *C4* genes depending on the presence or absence of a 6.5 kb intron has been described [Schneider *et al.* 1986; Palsdottir *et al.* 1987; Yu and Campbell 1987]. *C4*A genes are (almost) always 'long' (22.5 kb), while *C4B* genes may be 'long' or 'short' (16 kb). While most of these variations are inconsequential, there are several mutations that affect the *CYP21* gene and cause steroid 21-hydroxylase deficiency. These mutations of *CYP21* can be roughly categorised into three groups: *CYP21* deletions, conversions into a *CYP21P*-like gene, and smaller mutations inside the *CYP21* gene.

In humans, use of 21-hydroxylase and complement *C4* cDNA probes and several restriction enzymes (especially, *Taq*I and *Bgl*II) provides useful information about the configuration of the 21-hydroxylase / complement *C4* section of the MHC class III region. In this paper we describe the prevalence of gene deletions, duplications, conversions, and smaller mutations (by exclusion) in 29 Dutch CAH families. The genetic variation between these families, healthy controls, and CAH populations from several other countries is discussed.

Patients and methods

Patients, families and controls

We studied 33 patients and 80 healthy family members from 29 families (all of Dutch ethnic origin). The patients are regularly seen at the outpatient department of the Sophia Children's Hospital; most older patients responded positively to a recall request. All families involved gave their informed consent. At least three members of each family (including the patient) were available for haplotyping. Four families each included two patients. In 25 families, blood samples from both parents were obtained; in the other four families, blood samples from the fathers were not available. Samples were also obtained from 26 clinically healthy brothers and sisters of the patients. In all, 22 patients from 19 families were classified as having salt-losing (SL) CAH. These patients were usually admitted to the hospital in the first month of life with characteristic phenomena such as hyponatraemia, hyperkalaemia, dehydration, vomiting, shock, and highly elevated plasma levels of 17α-hydroxyprogesterone. The other 11 patients, from 10 families, had no history of salt-losing crises. They presented during childhood, usually with ambiguity of the external genitalia in girls or precocious puberty in boys, and accelerated growth in most patients. These patients were characterised as simple virilisers (SV).

DNA samples from 55 healthy controls without a reported family history of CAH were obtained and investigated in a similar manner, though no corresponding family studies were performed.

Biochemical data

The clinical and biochemical data is summarised in table 1. Serum electrolytes are the lowest (Na) and the highest (K) measured. In SL patients, these values were generally found in the first weeks of life during hospitalisation; in some patients, the values presented in table 1 were obtained during a clinical re-evaluation when they were following a low-sodium diet and not receiving steroid therapy. 17α-hydroxyprogesterone levels are the highest measured using a standard radioimmunoassay. If no such data was available, patients were categorised as SL or SV on the basis of their clinical history.

Southern blotting and hybridisation

Genomic DNA was prepared from peripheral leukocytes essentially as described elsewhere [Wyman and White 1980]; 10 µg was digested overnight with 40 units *Taq*I, *Bgl*II or *Kpn*I as advised by the manufacturers' (Boehringer, Mannheim, FRG, or Pharmacia, Uppsala, Sweden) instructions. After agarose gel electrophoresis and Southern blotting, hybridisation was performed with the 2.1 kb 21-hydroxylase cDNA probe pC21/3c [White *et al.* 1985], or the 0.6 kb *Bam*HI-*Bgl*II fragment of the complement *C4* cDNA probe pAT-A [Belt *et al.* 1984]. The probes were obtained from the American Type Culture Collection. To estimate the amount of DNA in each lane and deduce the number of *CYP21* and *CYP21P* genes in each individual, the *Taq*I blots were rehybridised with the 0.7 kb genomic probe pJ3.11 [Cooper *et al.* 1985], which was kindly provided by Dr. J. Schmidtke (University of Berlin, FRG). As a reference, we used DNA from a healthy individual with two *CYP21*, *CYP21P*, and (long) *C4A* genes, and a long and a short *C4B* gene (haplotypes A1/A2 in fig. 1).

Laser densitometry

The optical densities of the autoradiographic bands were determined by densitometry with an LKB 2222-020 Ultroscan XL laser densitometer. The relative quantity of the fragments was calculated as the ratio of the area under the respective curves. For a number (n) of different individuals with equal quantities of each band as determined by family studies, the mean ratios of the bands (all from different autoradiograms) were, respectively: *CYP21* and *CYP21P*: *Taq*I 3.2:3.7 kb = 0.99 ± 0.023 (SEM) (n=18); *Bgl*II 12:10 kb = 1.15 ± 0.028 (SEM) (n=16); complement *C4*: *Taq*I 7.2:6.6 kb = 1.01 ± 0.012 (SEM) (n=18); *Taq*I 7.2:5.6 kb = 0.98 ± 0.021 (SEM) (n=15). The coefficient of variation (CV) was ≤ 10 %. Virtually identical results were obtained upon repeating the entire procedure 17 times for a single individual. The contribution of the densitometry itself to the CV was less than 1 %; the blackening of the film used (Kodak X-Omat AR) was linear in proportion to the radioactivity present within an absorption range of 0.5-2.5 (results not shown). The band ratios presented in table 1 have been rounded to whole numbers.

Interpretation of the haplotypes

Haplotyping of steroid 21-hydroxylase genes in CAH patients and healthy individuals using *Taq*I is based on the characterisation of the genes as *CYP21*-like or *CYP21P*-like because of the presence or absence of a *Taq*I site normally found in the 5' flanking sequence of the pseudogene only. This extra site causes the *CYP21* gene to be characterised by a 3.7 kb *Taq*I fragment, and the *CYP21P* gene by a 3.2 kb fragment [Higashi *et al.* 1986; White *et al.* 1986]. Deletion of a 21-hydroxylase gene results in absence of the corresponding *Taq*I fragment. Normally, a 12 kb *Bgl*II fragment contains the *CYP21P* gene, and a 10 kb fragment the *CYP21* gene; the difference is caused by a *Bgl*II site present in the 3' flanking sequence of *CYP21* only. The 12 kb *Bgl*II fragment is absent in case of a deletion of either *CYP21P* or *CYP21*, because the 30 kb deletion resulting in loss of *CYP21* actually extends from the 3' region of *CYP21P* to the corresponding part of *CYP21*, so that the 3' flanking sequence of *CYP21* is retained [White *et al.* 1988; Donohoue *et al.* 1989].

Duplication of a 21-hydroxylase gene can be deduced from an increased intensity of the respective *Taq*I band, and of the 12 kb *Bgl*II band. In case of a conversion of *CYP21* to *CYP21P*, the *Bgl*II pattern is normal, but the *Taq*I 3.7 kb fragment is absent.

*Taq*I banding patterns also distinguish between long and short *C4* genes and between their position at either locus I or locus II [Schneider *et al.* 1986; Yu and Campbell 1987; Partanen *et al.* 1989]. Four different fragments (usually described as 7, 6.4, 6, and 5.4 kb, but probably 0.2 kb longer [Koppens *et al.* 1992]) are characteristic of, respectively: a long gene at locus I, a short gene at locus I, a long gene at locus II, and a short gene at locus II. This approach allows the definition of several haplotypes, which are summarised in fig. 1 and table 2.

Table 1. Summary of clinical and biochemical patient data and CAH-bearing haplotypes

patient	sex	CAH type	electrolytes (mmol/l)		17α-OH prog.[b] (nmol/l)	relative band intensities			CAH-bearing haplotypes[c]
			Na[a]	K[a]		21-hydroxylase		complement *C4*	
						*Taq*I 3.2:3.7	*Bgl*II 12 : 10	*Taq*I 7.2:6.6:6.2:5.6	
1	F	SL	110	8.6	n.a.	2 : 1	1 : 2	2 : 0 : 1 : 0	A1 / E1[d]
2	F	SL	104	9.4	> 48	2 : 1	1 : 2	2 : 0 : 1 : 0	A1 / E1[d]
3	M	SL	n.a.	n.a.	> 48	3 : 1	2 : 2	2 : 0 : 1 : 1	A / D [e]
4a	M	SL	121	6.4	24	1 : 2	1 : 2	2 : 0 : 1 : 0	A1 / B1
4b	F	SL	125	11.2	> 48	1 : 2	1 : 2	2 : 0 : 1 : 0	A1 / B1
5a	F	SL	105	8.4	92	2 : 2	2 : 2	1 : 1 : 0 : 2	F2 / E1
5b	M	SL	126	8.2	>120	2 : 2	2 : 2	1 : 1 : 0 : 2	F2 / E1
6	F	SL	123	6.9	> 48	2 : 1	1 : 2	2 : 0 : 1 : 0	A1 / E1
7	F	SL	125	9.5	> 48	1 : 1	0 : 2	2 : 0 : 0 : 0	B1 / E1
8	F	SL	117	8.5	> 48	1 : 1	0 : 2	1 : 1 : 0 : 0	E1 / B2
9	M	SL	126	6.9	> 48	2 : 1	1 : 2	2 : 0 : 1 : 0	E1 / A1
10	F	SL	low	high	> 48	1 : 2	1 : 2	1 : 1 : 0 : 1	B2 / A2
11a	F	SL	122	5.3	n.a.	3 : 2	3 : 2	2 : 0 : 1 : 2	C2 / A1[d]
11b	M	SL	n.a.	n.a.	> 48	3 : 2	3 : 2	2 : 0 : 1 : 2	C2 / A1[d]
12	F	SL	111	11.6	> 48	1 : 2	1 : 2	2 : 0 : 1 : 0	B1 / A1[d]
13	F	SL	126	10.5	> 48	2 : 1	1 : 2	2 : 0 : 0 : 1	E1 / A2
14	F	SL	85	15.0	> 48	4 : 0	2 : 2	2 : 0 : 1 : 1	D1 / D2
15	M	SL	113	7.8	>150	3 : 1	2 : 2	2 : 0 : 2 : 0	D1 / A1
16	M	SL	110	8.7	1990	3 : 0	1 : 2	2 : 0 : 0 : 1	E1 / D2
17	M	SL	124	5.7	159	2 : 1	1 : 2	2 : 0 : 1 : 0	A1 / E1
18	M	SL	112	8.2	> 48	3 : 1	2 : 2	2 : 0 : 1 : 1	D2 / A1[d]
19	F	SL	103	11.6	> 48	3 : 1	2 : 2	2 : 0 : 1 : 1	.../...[f]
20	M	SV	134	5.1	99	2 : 1	1 : 2	2 : 0 : 0 : 1	.../...[f]
21	M	SV	138	4.2	240	3 : 1	2 : 2	2 : 0 : 1 : 1	.../...[f]
22	F	SV	138	5.6	> 48	2 : 2	2 : 2	2 : 0 : 1 : 1	A1 / A2
23a	F	SV	normal	normal	n.a.	2 : 1	1 : 2	2 : 0 : 1 : 0	E1 / A1[d]
23b	F	SV	normal	normal	n.a.	2 : 1	1 : 2	2 : 0 : 1 : 0	E1 / A1[d]
24	M	SV	127	4.6	> 48	2 : 2	2 : 2	2 : 0 : 2 : 0	A1 / A1[d]
25	F	SV	138	5.0	96	2 : 2	2 : 2	2 : 0 : 1 : 1	A2 / A1
26	M	SV	normal	normal	154	2 : 1	1 : 2	2 : 0 : 0 : 1	E1 / A2
27	M	SV	n.a.	n.a.	> 48	1 : 2	1 : 2	1 : 1 : 1 : 0	B2 / A1
28	F	SV	147	5.0	> 48	3 : 1	2 : 2	2 : 0 : 2 : 0	D1 / A1
29	F	SV	138	6.6	> 48	2 : 2	2 : 2	2 : 0 : 0 : 2	A2 / A2

a: exact data not always available; norm.: normal

b: upper limit of the reference range of 17α-hydroxyprogesterone is 6 nmol/l

c: the paternal haplotype is shown first

d: alternative scheme possible, but unlikely (see text)

e: only the 21-hydroxylase haplotypes could be deduced

f: no CAH-bearing haplotypes could be deduced

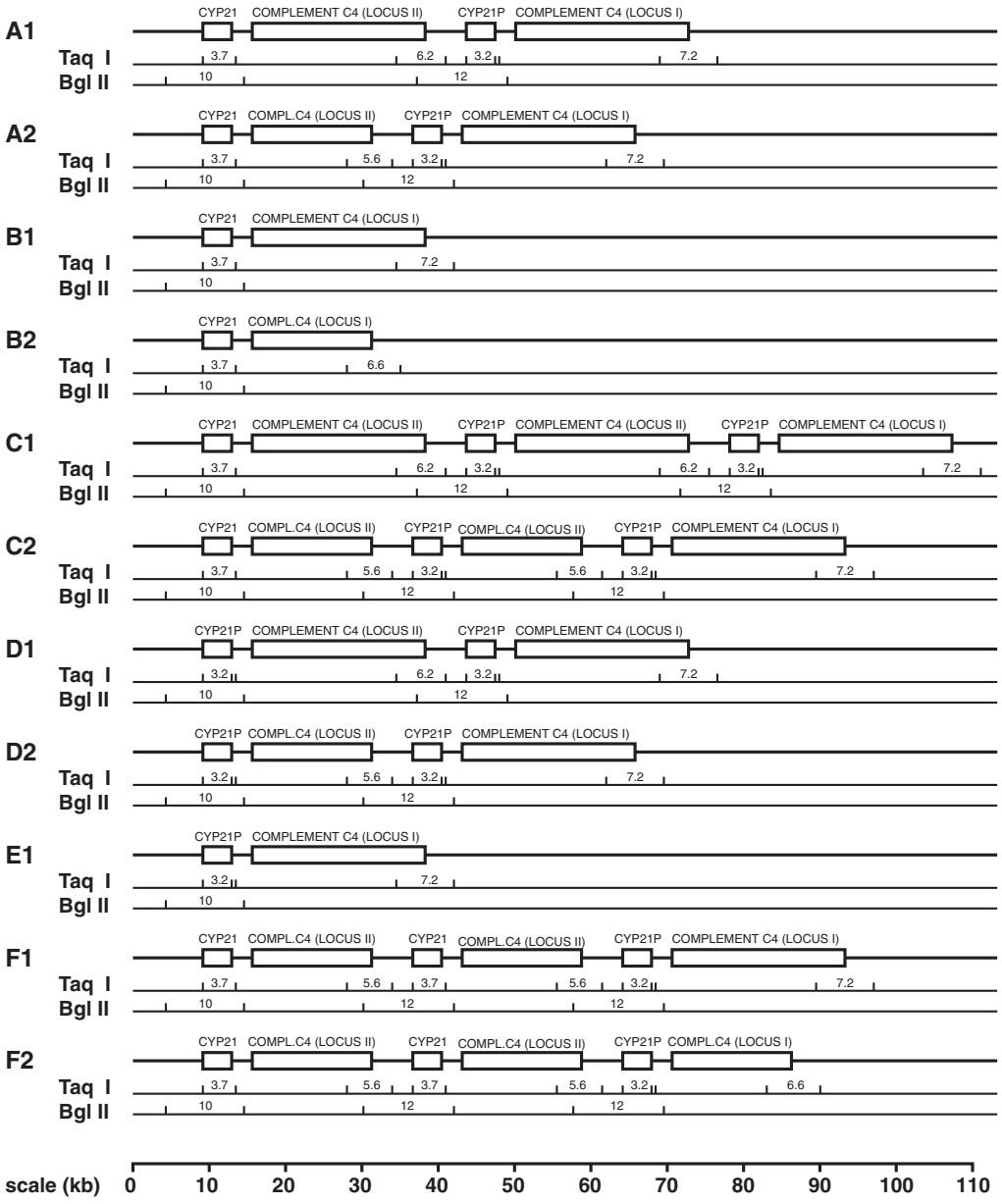

Fig. 1. 21-hydroxylase / complement *C4* haplotypes and associated restriction sites

In a number of cases we also used *Kpn*I, because an extra site in the 5' flanking sequence of *CYP21* results in a characteristic 2.9 kb fragment as opposed to 4.0 kb for *CYP21P* [Higashi *et al.* 1986; White *et al.* 1986].

Following the recommendations of Sokal and Rohlf [1981], statistical analyses were performed using the G-test of independence for two-way tables with Williams' correction.

Results and discussion

Gene rearrangements and haplotypes

We have studied 29 families from the Netherlands in order to obtain 21-hydroxylase / complement *C4* haplotypes. These haplotypes are defined in terms of the *Taq*I and *Bgl*II restriction fragments (see fig. 1 and table 2). Eleven different haplotypes were found, most of which have also been described by other authors [Carroll *et al.* 1985; Donohoue *et al.* 1986; Garlepp *et al.* 1986; White *et al.* 1988; Collier *et al.* 1989; Morel *et al.* 1989; Partanen *et al.* 1989; White 1989; Schneider 1990]; we will refer to the combined 21-hydroxylase / complement *C4* haplotypes as A1, A2, etc., and to the 21-hydroxylase haplotypes independent of the *C4* genes as A, B, C, etc (so that, in effect, A1+A2=A).

Table 2. Ratios of restriction fragments characterizing 21-hydroxylase / complement *C4* haplotypes

haplotype	21-hydroxylase				complement *C4*			
	*Taq*I		*Bgl*II		*Taq*I			
	3.2 : 3.7		12 : 10		7.2 : 6.6 : 6.2 : 5.6			
A1[a]	+	+	+	+	+	-	+	-
A2[a]	+	+	+	+	+	-	-	+
B1[a]	-	+	-	+	+	-	-	-
B2[a]	-	+	-	+	-	+	-	-
C1[a]	++	+	++	+	+	-	++	-
C2[a]	++	+	++	+	+	-	-	++
C3[ab]	++	+	++	+	+	-	+	+
D1	++	-	+	+	+	-	+	-
D2	++	-	+	+	+	-	-	+
E1	+	-	-	+	+	-	-	-
F1[a]	+	++	++	+	+	-	-	++
F2[a]	+	++	++	+	-	+	-	++

+ : normal quantity
++: double quantity
- : absent
a : apparently bearing a defective *CYP21* gene on CAH haplotypes
b : the C3 haplotype has been included since it is mentioned in the text as an alternative possibility

The haplotypes reflect gene rearrangements within the 21-hydroxylase / complement *C4* region of the MHC. They are thought to have originated from haplotype A through unequal crossovers, resulting in the simultaneous duplication and deletion of either *CYP21* and *C4B*, or *CYP21P* and *C4B*, or *CYP21P* and *C4A*, or through gene conversion-like events. These processes have been discussed in more detail elsewhere [Donohoue *et al.* 1986; Garlepp *et al.* 1986; White *et al.* 1988; Collier *et al.* 1989; Donohoue *et al.* 1989; Miller *et al.* 1989; White 1989]. Unequal crossover events are thought to have simultaneously generated gene deletions and duplications, resulting in haplotypes B and C, as well as in haplotypes E and F. Haplotype D is generated from A by conversion of (a part of) *CYP21* into a *CYP21P*-like structure. When we hybridised the 21-hydroxylase probe to *Kpn*I blots, the ratio of the 4 kb and 2.9 kb fragment was found to be equal to the ratio of the *Taq*I 3.2 and 3.7 kb fragments in all cases. These results indicate that all 21-hydroxylase genes studied have either a *Taq*I site (characteristic of *CYP21P*) or a *Kpn*I site (characteristic of *CYP21*) in their 5' flanking sequence, but not both. Though these sites lie outside the coding sequence, maintenance of a *CYP21*-like structure in the 5' flanking sequence may be necessary for gene expression. In

mouse steroid 21-hydroxylase genes, several 5' regulatory elements have been described [Rice *et al.* 1990]. Of course, the current approach presumes a gene conversion on the basis of an extra *Taq*I site (reducing the original 3.7 kb fragment to 3.2 kb), and the absence of a *Kpn*I site (resulting in a 4 kb fragment instead of 2.9 kb). However, there are no reports of normal biological activity of a *CYP21* gene characterised by a 3.2 kb *Taq*I fragment or a 4 kb *Kpn*I fragment (it should be noted that patients 14 and 16 in this study, both of whom completely lack the 3.7 kb *Taq*I and 2.9 kb *Kpn*I fragments, have SL CAH). Nevertheless, the presence of the extra *Taq*I site and the absence of the *Kpn*I site do not necessarily imply that the entire 'converted' gene is *CYP21P*-like.

Family studies

A problem rarely mentioned in the literature is that combinations of some of the haplotypes described above yield the same patterns. In single individuals, no distinction can be made between: A1/A1 and B1/C1; A1/A2 and B1/C3; A1/C2 and A2/C3 and D1/F1; A1/C3 and A2/C1; A1/D1 and C1/E1; A1/D2 and A2/D1 and C3/E1; A1/E1 and B1/D1; A2/A2 and B1/C2 and E1/F1; A2/C2 and D2/F1; A2/D2 and C2/E1; A2/E1 and B1/D2; B2/C3 and E1/F2; C1/C2 and C3/C3. Though family studies greatly reduce this problem, such complications limit the number of families where the haplotypes can be fitted unambiguously into a Mendelian scheme. To be certain, we took account of some unusual haplotypes comprising two *CYP21* genes and no *CYP21P* genes; three *CYP21P* genes; one *CYP21* gene and three *CYP21P* genes; and four *CYP21P* genes (the last possibility has been described by Collier *et al.* [1989]). In addition, alternative sizes of the *C4* genes were taken into consideration. In 18 families, no other CAH-bearing haplotypes than those presented in table 1 were possible. In 7 families, other possibilities than those presented in table 1 existed but were highly unlikely (these alternatives implied replacement of at least two, and usually three, haplotypes A by other, far less frequent haplotypes). In one family, only the 21-hydroxylase haplotypes (A-F) could be deduced for the CAH-bearing chromosomes; there was no certainty about the *C4* genes. In the remaining three families, no unambiguous Mendelian scheme could be drawn up. Thus, a total of 52 haplotypes carrying 21-hydroxylase deficiency and 52 normal haplotypes were finally established. The relative intensities of the *Taq*I and *Bgl*II bands (rounded; patients only) and the 21-hydroxylase / complement *C4* haplotypes deduced are presented in table 1. The frequencies of the CAH and non-CAH haplotypes which were finally established are shown in table 3.

Our approach was primarily aimed at the establishment of the haplotypes described. Nevertheless carrier status for steroid 21-hydroxylase deficiency could often be deduced: a distinction between carriers and non-carriers was possible in 16 families. However, for the purpose of carrier detection and antenatal diagnosis, a combination of probes, including HLA probes, is better suited [Strachan *et al.* 1987; Speiser *et al.* 1990].

Table 3. Frequencies of 21 hydroxylase / complement *C4* haplotypes in classical congenital adrenal hyperplasia in the Netherlands

haplotype	SL	SV	total	%	fam.	contr.	total	%
A1[a]	10	7	17	32.7	31	52	83	51.6
A2[a]	2	5	7	13.5	9	25	34	21.0
A[ab]	1	0	1	1.9	1	7	8	4.9
B1[a]	3	0	3	5.8	2	8	10	6.2
B2[a]	2	1	3	5.8	7	10	17	10.5
C1[a]	0	0	0	0.0	2	0	2	1.2
C2[a]	1	0	1	1.9	0	0	0	0.0
C[ab]	0	0	0	0.0	0	7	7	4.3
D1	2	1	3	5.8	0	0	0	0.0
D2	3	0	3	5.8	0	0	0	0.0
D[ab]	1	0	1	1.9	0	0	0	0.0
E1	10	2	12	23.1	0	0	0	0.0
F1[a]	0	0	0	0.0	0	1	1	0.6
F2[a]	1	0	1	1.9	0	0	0	0.0
total	36	16	52	100	52	110	162	100

SL: patients with salt-losing CAH
SV: patients with simple virilising CAH
fam.: non-CAH haplotypes in CAH families
contr.: unaffected controls
total: totals for classical CAH and unaffected haplotypes, respectively
a : apparently bearing a defective *CYP21* gene on CAH haplotypes
b : only the 21-hydroxylase haplotype could be deduced (see text)

Control haplotypes

Almost all controls had two *CYP21* genes; one had three. We assumed presence of haplotype A on both chromosomes when the *Taq*I and *Bgl*II bands representing the 21-hydroxylase genes were of equal intensity. However, considering the substantial number of haplotypes B and C, it seems likely that one or two controls considered to have A/A, in fact have B/C (E/F, combining CAH carrier status on one chromosome with a rare haplotype on the other, seems very improbable). Seven controls had three *CYP21P* genes, two *CYP21* genes, and two long *C4* genes at locus I; six of them had two long and one short *C4* gene on the loci II, one had one long and two short genes. In such cases, no distinction can be made between the combinations A1/C3 and A2/C1, and A1/C2 and A2/C3, respectively. Therefore, these haplotypes have simply been designated 'A' and 'C' in table 3.

CAH and non-CAH haplotypes

Haplotypes D and E lack a functional *CYP21* gene and are always associated with 21-hydroxylase deficiency. Haplotypes A, B, C, and F are found in the general population and are only associated with 21-hydroxylase deficiency if the *CYP21* genes present carry deleterious mutations. A statistically significant difference between SL and SV patients was only found for haplotype A (13/36 *vs* 12/16; $P<0.01$), which is more prominent in the latter. The difference in the number of gene deletions and conversions is not statistically significant.

Taking into account a carrier frequency of CAH of 1:50 to 1:60, the contribution of carriers to the haplotypes of the control group can be assumed to be very limited. The chance that more than three control subjects from a group of 55 are CAH heterozygotes is < 3 %; also, controls carrying *CYP21* deletions or gene conversions are easily detectable from the *Taq*I and *Bgl*II banding patterns in most cases (*ie*, when combined with haplotypes A, B, or C).

As expected, the frequencies of all haplotypes are similar in controls and in the non-CAH-carrying haplotypes in the CAH families. Haplotypes D and E, which carry CAH because of the absence of a normal *CYP21* gene, are necessarily absent among the non-CAH haplotypes of the (unaffected) parents of CAH patients, and obviously rare in controls. When D and E are excluded from the comparison, the frequencies of the other haplotypes are similar in CAH-bearing and non-CAH-bearing chromosomes.

Comparison of different populations

In the last few years, a number of reports on 21-hydroxylase haplotypes and their occurrence in different populations have been published. These include CAH patients from centres in the United States, Japan and some European countries. Most of this data has been reviewed by Miller and Morel [1989]. Unfortunately, comparisons between a number of these studies are hampered by differences in approach, which sometimes make it actually impossible to arrive at definite conclusions. For purposes of comparison, we used data from a limited number of recent studies from several countries, involving a sufficiently large number of patients and presenting established haplotypes defined in terms of gene rearrangements. We compared only the 21-hydroxylase haplotypes (A-F), since most authors have not established combined 21-hydroxylase / complement *C4* haplotypes.

Table 4. Comparison of 21-hydroxylase haplotypes on CAH-bearing chromosomes in several countries

haplotype	Rotterdam, the Netherlands[a]	France[b]	France[c]	Helsinki, Finland[d]	Japan[e]	London, UK[f]	Manchester, UK[g]	New York, USA[h]
A	25	68	81	18	14	32	24	45
B	6	4		0	0	2	5	4
C	1	5		1	0	0	3	0
D	7	13	11	3	4	3	5	2
E	12	13	24	12	0	11	19	13
F	1	0	0	0	0	0	1	0
total	52	103	116	34	18	48	57	64

a: this study; see tables 1 and 3

b: Morel *et al.* [1989]; not including two chromosomes with uninterpretable haplotypes

c: Mornet *et al.* [1991]; the number of haplotypes A, B and C is not specified separately

d: Partanen *et al.* [1989]

e: Harada *et al.* [1987]; not including three patients heterozygous for either haplotype D or haplotype E

f: Rumsby *et al.* [1988]; not including three patients with possible loss of a single restriction site

g: Collier *et al.* [1989]; not including one chromosome bearing four *CYP21P* genes

h: White *et al.* [1988]

Several authors give data on SL and SV patients; the number of SL patients always exceeds the number of SV patients, but its ratio relative to the total number of patients is variable (*eg*, 22/33 in our study *vs* 24/27 in a report from the United Kingdom [Rumsby *et al.* 1988]; $P<0.05$). While such variations may be real, they may also reflect differences in interpretation of clinical phenomena. Also, some authors do not specify the haplotypes of each category of patients. We therefore combined the data presented for SL and SV patients to obtain a total for classical 21-hydroxylase deficiency. Patients with non-classical 21-hydroxylase deficiency were excluded; they are few in number in most haplotyping studies. A compilation of data from several reports is presented in table 4. Comparison of our results with those of other authors shows similarities and dissimilarities between different populations. Haplotype A is less prominent in the Dutch patients than in the patients in the American study [White *et al.* 1988] ($P<0.025$) and one of the two French studies [Morel *et al.* 1989] ($P<0.05$).

Several reports show significantly more *CYP21* deletions (haplotype E) than conversions to the *CYP21P* pseudogene (haplotype D) [Rumsby *et al.* 1988; White *et al.* 1988; Collier *et al.* 1989; Partanen *et al.* 1989; Mornet *et al.* 1991] ($P<0.025$ in all cases). We also found more deletions, but the difference is not statistically significant; the (French) group of Morel *et al.* [1989] found the number of deletions and conversions to be equal, whereas in the Japanese study no deletions were reported [Harada *et al.* 1987]. Apart from difficulties in interpretation, it is clear that ethnical differences have contributed to the controversy about the number of *CYP21* deletions in CAH families in recent years.

Non-CAH haplotypes

A remarkable feature in the non-CAH haplotypes studied is the high incidence of deletions of the *CYP21P* pseudogene in comparison with duplications of this gene (haplotype B *vs* C; $P<0.005$ (this study); $P<0.001$ ([Morel *et al.* 1989]; 12 *vs* 1 / 84 haplotypes); $P<0.01$ ([Partanen *et al.* 1989]; 5 *vs* 0 / 31 haplotypes)). As haplotypes B (deletion) and C (duplication) are thought to have resulted simultaneously from an unequal crossover event, an equal frequency would be expected. Attempts to explain the difference are necessarily speculative; possibly, the mechanisms of the (apparently frequent) unequal crossovers in this region play a role. Sinnott *et al.* [1990] describe a crossover between a chromosome with both 21-hydroxylase and complement *C4* genes present and a chromosome with a *CYP21P*/*C4A* deletion; in such an event, about 30 kb of the longest chromosome is thought to loop out. A similar phenomenon could be expected in an unequal pairing between a normal chromosome and one with a duplication. Such an extended 'bulge' during meiosis may be a disadvantageous configuration compared with the 'straight' arrangement of the other chromosome. As such loops are necessarily on the longest chromosome, there would be a tendency to favour the shorter ones.

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2.2. *CYP21/C4* haplotypes in the Netherlands: an update

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Introduction

The genetic map of the central (class III) region of the human MHC contains one to four tandem copies of a repeat known as the RCCX module after its main constituent genes: *RP-C4-CYP21-TNX*. These repeats vary in size (either 27 or 33 kb, depending on the *C4* gene), position (centromeric: adjacent to the full-size *TNXB* gene; telomeric: adjacent to the full-size *RP1* gene; intermediate: between two other RCCX modules), and composition (*C4A* or *C4B*, encoding variants of the fourth component of complement with different activities and specificities; *CYP21* encoding steroid 21-hydroxylase or the pseudogene *CYP21P*; a truncated part of either *TNXB* or the *TNXA* pseudogene). *Taq*I/*Bgl*II restriction analysis and hybridisation to *C4* and *CYP21* cDNA probes allows characterisation of the overall structure of the RCCX region in individuals and families [Carroll *et al.* 1985; Higashi *et al.* 1988; White *et al.* 1988; Morel *et al.* 1989]. We previously studied *CYP21*/*C4* haplotypes in a group of steroid 21-hydroxylase deficiency patients and controls from the Netherlands [Koppens *et al.* 1992a]. This article is an update to our earlier study: the studied population was increased and novel haplotypes, some of which have been described in more detail elsewhere in this thesis [Koppens *et al.* 2002a; Koppens *et al.* 2002b; Koppens *et al.* 2002c], were found.

Methods

The original study group [Koppens *et al.* 1992a] was expanded to 44 families and 141 controls. Patients were from several hospitals in the Netherlands (see table 1). A few families with late-onset 21-hydroxylase deficiency were added; otherwise, inclusion criteria and separation between clinical categories (salt-losing, simple virilising) were as before.

Haplotyping was done as before; several unusual haplotypes were also characterised by *Sac*II digest and pulsed field gel electrophoresis [Koppens *et al.* 2002c]. Two smaller *Taq*I fragments (2.4 and 2.5 kb) that were visible on many autoradiograms, were also considered. These fragments correspond to a *TNXA*-like (2.4 kb) or *TNXB*-like (2.5 kb) sequence downstream of *CYP21* or *CYP21P*, and we developed a PCR-based method [Koppens *et al.* 2002a; Koppens *et al.* 2002b] to further characterise this region. The relative intensity of the 2.4 and 2.5 kb fragments normally matches the 12 and 11 kb *Bgl*II fragments, respectively, but there are deviations in some haplotypes (see table 2).

Table 1. CAH-bearing haplotypes (continued from p. 109)

patient	hospital[a]	sex[b]	CAH type[c]	relative band intensities			CAH-bearing haplotypes[d]
				21-hydroxylase		complement *C4*	
				*Taq*I	*Bgl*II	*Taq*I	
				3.2:3.7	12 : 10	7:6.4:6:5.4	
19	S	F	SL	3 : 1	2 : 2	2 : 0 : 1 : 1	A1 / D2
20	S	M	SV	2 : 1	1 : 2	2 : 0 : 0 : 1	E1 / A2[e]
30	L	F	SL	2 : 1	1 : 2	2 : 0 : 0 : 1	E1 / A2
31	L	M	SL	2 : 1	1 : 2	2 : 0 : 0 : 1	E1 / A2
32	M	F + M	SL	1 : 1	0 : 2	2 : 0 : 0 : 0	B1 / E1
33	C	M + M	SL	2 : 2	2 : 2	2 : 0 : 2 : 0	A1 / A1
34	L	F	SL	2 : 2	2 : 2	2 : 0 : 1 : 1	C3 / B1
35	O	M	SV	2 : 2	2 : 2	2 : 0 : 2 : 0	A1 / A1
36	L	F	SL	2 : 2	2 : 2	2 : 0 : 1 : 1	A1 / A2[f]
37	L	M	SL	3 : 2	3 : 2	2 : 0 : 3 : 0	A1 / C1
38	L	M	SL	3 : 1	2 : 2	2 : 0 : 2 : 0	D1 /A1
39	L	M + M	SL	2 : 2	2 : 2	2 : 0 : 2 : 0	A1 / A1
40	L	M	SL	1 : 2	1 : 2	2 : 0 : 0 : 1	B1 / A2
41	S	M	SL	1 : 2	1 : 2	2 : 0 : 1 : 0	B1 / A1
44	L	F	LO	3 : 2	3 : 2	2 : 0 : 1 : 2	C2 / A1
45	S	F	LO	3 : 2	3 : 2	2 : 0 : 1 : 2	C2 / A1
46	L	M	LO	3 : 2	3 : 2	2 : 0 : 0 : 3	C2 / A2

a: S=Sophia Children's Hospital, Rotterdam; L=Leiden University Medical Centre; C=Catharina Hospital, Eindhoven; M=Maria Hospital, Tilburg; O=Oosterschelde Hospital, Goes

b: two patients in some families

c: SL=salt-losing 21-hydroxylase deficiency; SV=simple virilising; LO=late onset

d: the paternal haplotype is shown first

e: originated from a *de novo* unequal crossover [Koppens *et al.* 2002a]

f: maternal haplotype not counted in table 3 due to known consanguinity

In this update, we use 7-6.4-6-5.4 kb for the sizes of the *C4 Taq*I fragments to keep in line with the large body of literature that uses these designations, despite the fact that the sizes first mentioned by us [Koppens *et al.* 1992a, Koppens *et al.* 1992b] are more accurate, as confirmed by recent sequencing data [Rowen *et al.* 1999]. We here use 11 kb instead of 10 kb for the size of the smallest *Bgl*II fragment - the exact size is 10,758 bp [Rowen *et al.* 1999].

Table 2. Ratios of restriction fragments and *Sac*II fragment sizes for each haplotype

haplotype	21-hydroxylase				complement *C4*				both[f]
	*Taq*I		*Bgl*II		*Taq*I				*Sac*II
	3.2	3.7	12	10	7	6.4	6	5.4	size (kb)
A1[a]	+	+	+	+	+	-	+	-	76
A2[a]	+	+	+	+	+	-	-	+	70
A3[b]	+	+	+	+	-	+	+	-	64
B1	-	+	-	+	+	-	-	-	43
B2	-	+	-	+	-	+	-	-	37
C1	++	+	++	+	+	-	++	-	109
C2	++	+	++	+	+	-	-	++	96
C3	++	+	++	+	+	-	+	+	103
D1	++	-	+	+	+	-	+	-	76
D2[c]	++	-	+	+	+	-	-	+	70
E1[d]	+	-	-	+	+	-	-	-	43
F1	+	++	++	+	+	-	-	++	96
F2	+	++	++	+	-	+	-	++	90
G1	-	++	+	+	+	-	+	-	76
H1	+++	+	+++	+	+	-	+++	-	142
I1[e]	++++	-	+++	+	+	-	+++	-	142

a: one control who carried A1 / A2 had a *Taq*I 2.4:2.5 kb band ratio of 1:3, indicating a *TNXB*-like structure in both RCCX modules in one of these haplotypes

b: Hillarby *et al.* [1990]; Schneider [1990]; Weg-Remers *et al.* [1997]

c: all of these haplotypes had a double quantity of the *Taq*I 2.4 kb band and no 2.5 kb, indicating a *TNXA*-like structure in both RCCX modules [Koppens *et al.* 2002b]

d: these haplotypes had a 2.5 kb *Taq*I band as expected, except for the *de novo* deletion in family 20, where the 2.4 kb band was found ([Koppens *et al.* 2002a]

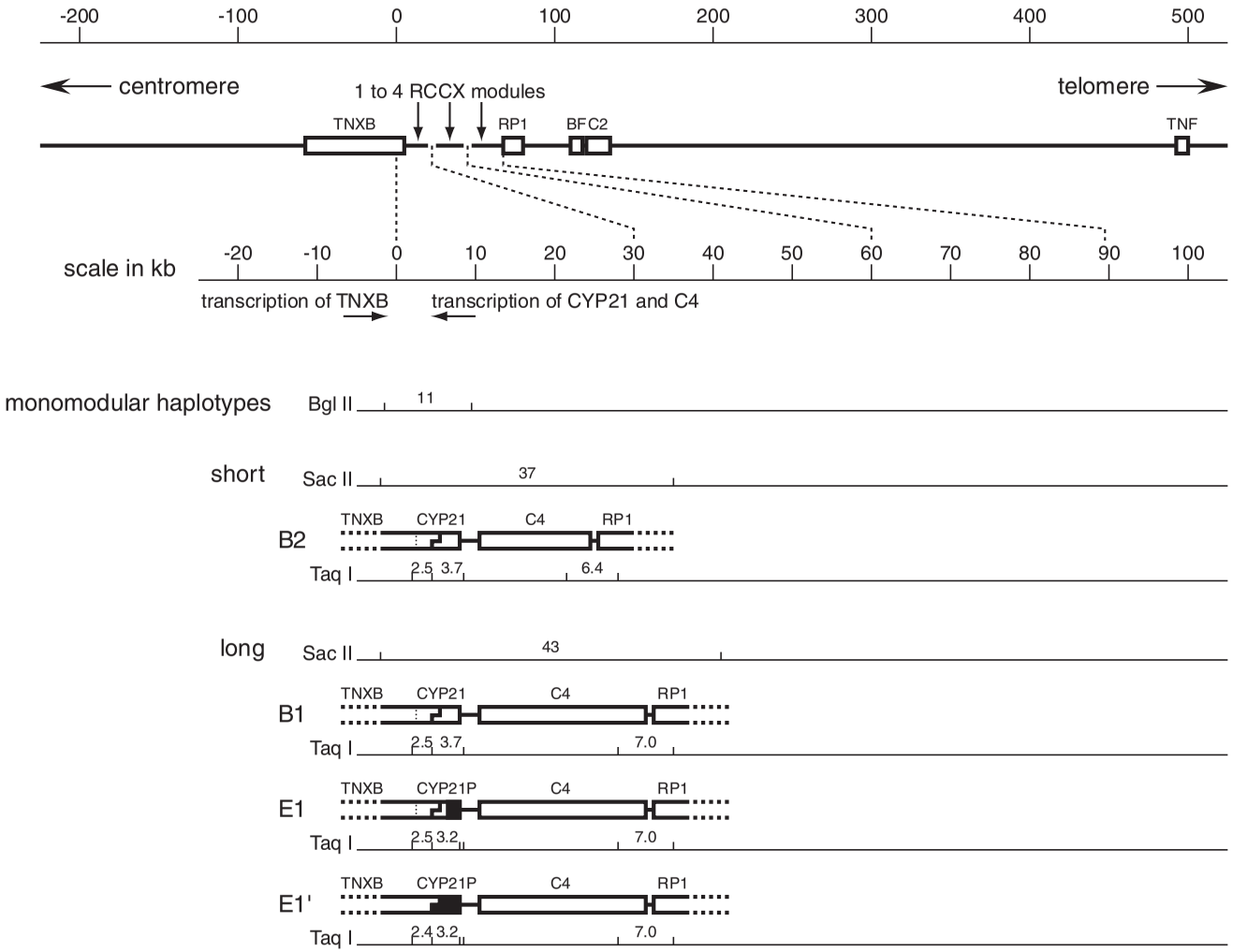
e: Collier *et al.* [1989]

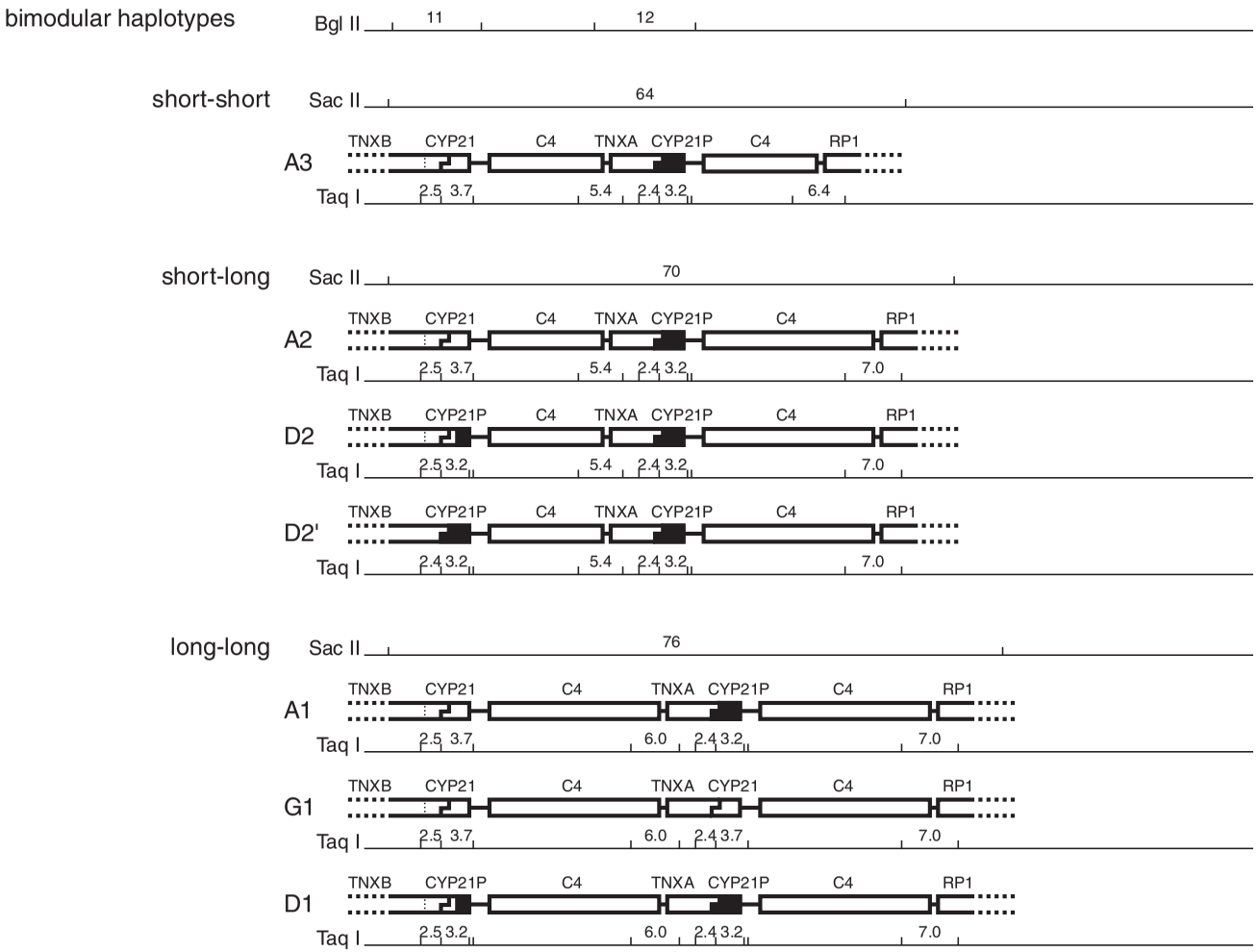
f: covers both genes; most of these fragments are shown elsewhere in this thesis [Koppens *et al.* 2002b; Koppens *et al.* 2002c], some are deduced

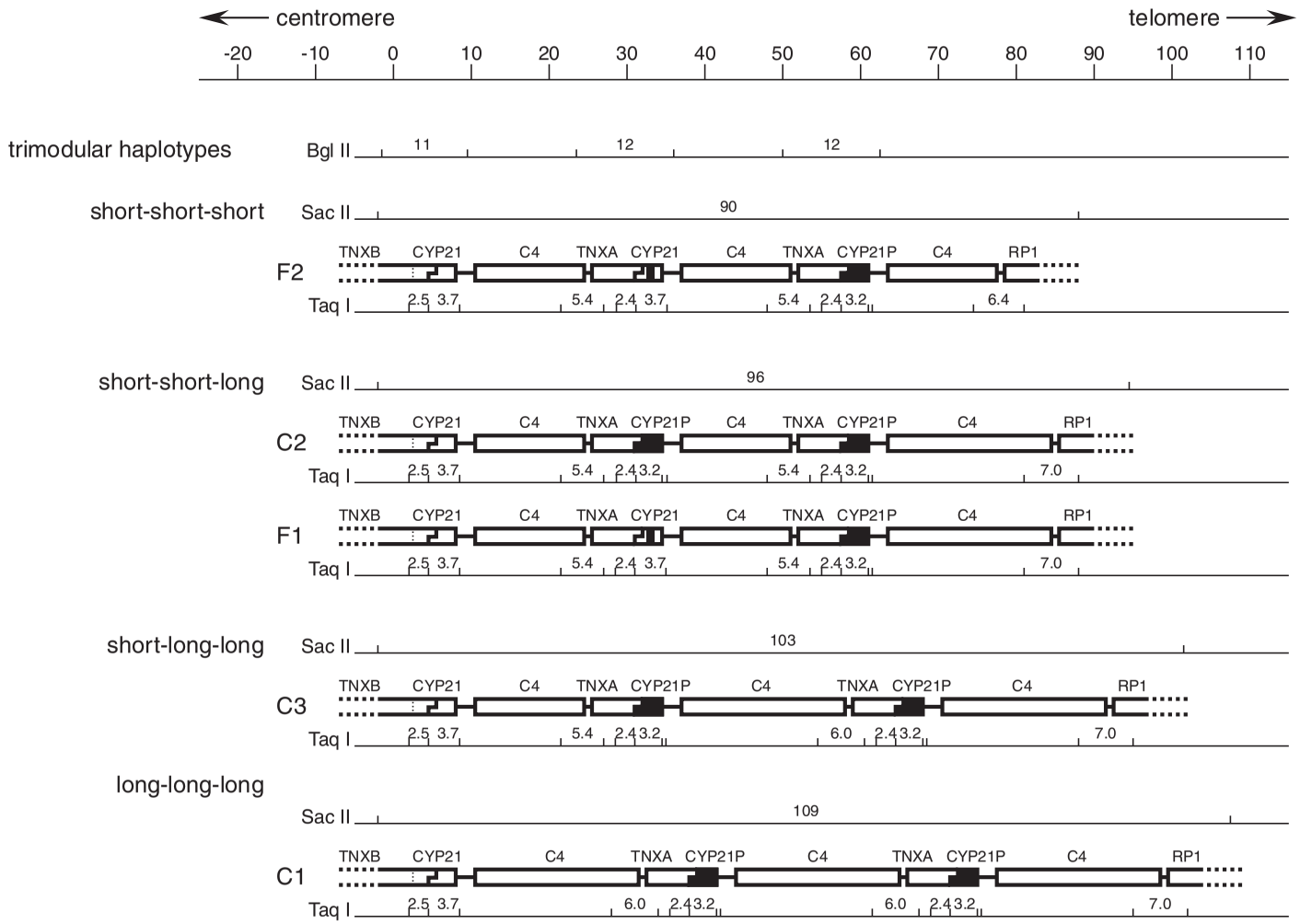
Results and discussion

CYP21/*C4* haplotypes

Table 1 is an overview of the steroid 21-hydroxylase deficiency patients added since our original study [Koppens *et al.* 1992a] and contains a few updates of the earlier data. The number of haplotypes found in this population has increased from 11 to 14; fig. 1 and table 2 also include a few additional rare haplotypes described in the literature. The rendering of haplotypes in fig. 1 is partly based on data from other chapters of this thesis or from the general literature: the designation of each haplotype follows the original definition, but details such as *TNX* variants and pseudogene-like regions have been added. Long-range restriction mapping is more informative than *Bgl*II digests (see fig. 1) and the combination of *Sac*II and *Taq*I provides all information necessary to accurately characterise the RCCX region. However, *Bgl*II mapping is technically easier and suffices in most cases, especially when family studies can be done.


-200
-100
0
100
200
300
400
500
centromere
1 to 4 RCCX modules
telomere
TNXB
RP1
BFC2
TNF
scale in kb
-20
-10
0
10
20
30
40
50
60
70
80
90
100
transcription of TNXB
transcription of CYP21 and C4
monomodular haplotypes
Bgl II
11
short
Sac II
37
TNXB
CYP21
C4
RP1
B2
Taq I
2.5
3.7
6.4
long
Sac II
43
TNXB
CYP21
C4
RP1
B1
Taq I
2.5
3.7
7.0
TNXB
CYP21P
C4
RP1
E1
Taq I
2.5
3.2
7.0
TNXB
CYP21P
C4
RP1
E1'
Taq I
2.4
3.2
7.0


bimodular haplotypes
Bgl II
11
12
short-short
Sac II
64
TNXB CYP21 C4 TNXA CYP21P C4 RP1
A3
Taq I
2.5 3.7 5.4 2.4 3.2 6.4
short-long
Sac II
70
TNXB CYP21 C4 TNXA CYP21P C4 RP1
A2
Taq I
2.5 3.7 5.4 2.4 3.2 7.0
TNXB CYP21P C4 TNXA CYP21P C4 RP1
D2
Taq I
2.5 3.2 5.4 2.4 3.2 7.0
TNXB CYP21P C4 TNXA CYP21P C4 RP1
D2'
Taq I
2.4 3.2 5.4 2.4 3.2 7.0
long-long
Sac II
76
TNXB CYP21 C4 TNXA CYP21P C4 RP1
A1
Taq I
2.5 3.7 6.0 2.4 3.2 7.0
TNXB CYP21 C4 TNXA CYP21 C4 RP1
G1
Taq I
2.5 3.7 6.0 2.4 3.7 7.0
TNXB CYP21P C4 TNXA CYP21P C4 RP1
D1
Taq I
2.5 3.2 6.0 2.4 3.2 7.0


centromere
telomere
-20
-10
0
10
20
30
40
50
60
70
80
90
100
110
trimodular haplotypes
Bgl II
11
12
12
short-short-short
Sac II
90
TNXB
CYP21
C4
TNXA
CYP21
C4
TNXA
CYP21P
C4
RP1
F2
Taq I
2.5
3.7
5.4
2.4
3.7
5.4
2.4
3.2
6.4
short-short-long
Sac II
96
TNXB
CYP21
C4
TNXA
CYP21P
C4
TNXA
CYP21P
C4
RP1
C2
Taq I
2.5
3.7
5.4
2.4
3.2
5.4
2.4
3.2
7.0
TNXB
CYP21
C4
TNXA
CYP21
C4
TNXA
CYP21P
C4
RP1
F1
Taq I
2.5
3.7
5.4
2.4
3.7
5.4
2.4
3.2
7.0
short-long-long
Sac II
103
TNXB
CYP21
C4
TNXA
CYP21P
C4
TNXA
CYP21P
C4
RP1
C3
Taq I
2.5
3.7
5.4
2.4
3.2
6.0
2.4
3.2
7.0
long-long-long
Sac II
109
TNXB
CYP21
C4
TNXA
CYP21P
C4
TNXA
CYP21P
C4
RP1
C1
Taq I
2.5
3.7
6.0
2.4
3.2
6.0
2.4
3.2
7.0

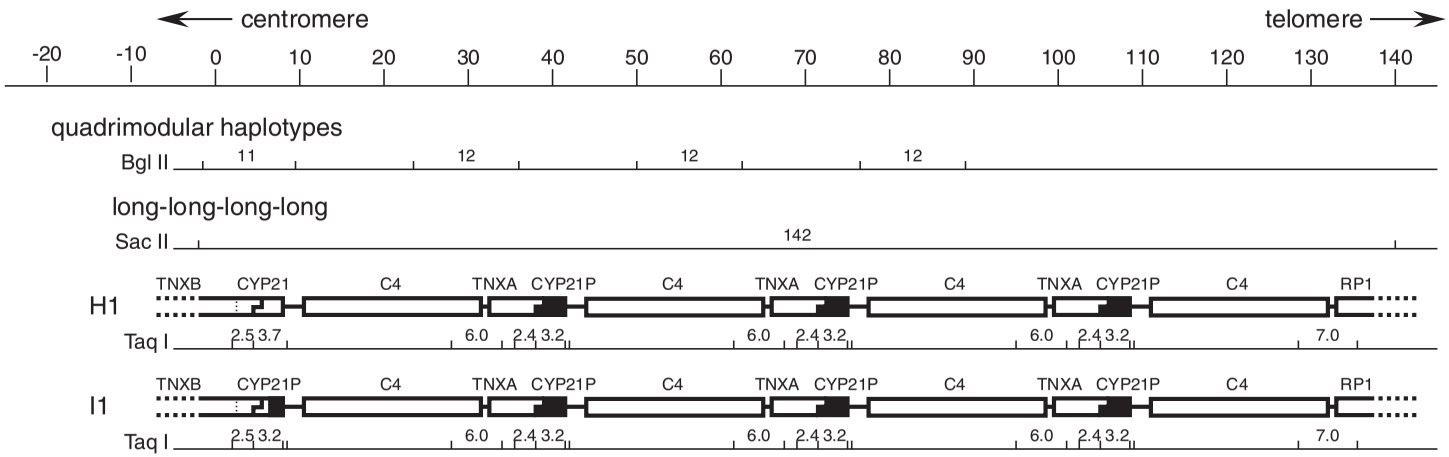


Fig. 1. *CYP21*/*C4* haplotypes and defining restriction sites. Top: MHC class III region with the location of the variable number of tandemly repeated RCCX modules shown in the remainder of the figure; *BF* = factor B gene; *C2* = complement C2; *TNF*: tumour necrosis factor; many other genes exist in this region [Beck and Trowsdale 2000; Milner and Campbell 2001]. Details: haplotypes ordered by increasing modularity and size. *Bgl*II digestion creates a single 11 kb fragment representing the centromeric RCCX module and a 12 kb fragment for each non-centromeric module. *Sac*II digestion creates a single fragment containing the entire RCCX area. *Taq*I digestion provides details about the composition of the individual modules. *CYP21P* and *CYP21P*-like stretches are shown in black; the haplotyping method used here recognises a gene as *CYP21P* if it contains the additional *Taq*I site shown, even though mutation analysis shows that some *CYP21P*-like genes are hybrids. *TNXB* contains 120 bp extra as compared to *TNXA* (indicated by a little dashed line); in the haplotypes E1' and D2' (described in detail elsewhere [Koppens *et al.* 2002a; Koppens *et al.* 2002b], these 120 bp are missing, resulting in a different *Taq*I pattern. A variant of haplotype A in which the 120 bp was present in *TNXA* (producing a 2.5 kb *Taq*I fragment there; see Koppens *et al.* 2002b for details) is not shown here, because it couldn't be determined whether this was an A1 or an A2 variant. The rare haplotypes A3 [Hillarby *et al.* 1990; Schneider 1990] and I1 [Collier *et al.* 1990] are included for illustration but were not actually found in the population studied here. Additional unusual trimodular haplotypes have been described [Weg-Remers *et al.* 1997], and others are conceivable, such as a D1' variant with a mutated *TNXB* gene as in D2' and E1'. Finally, independent *Taq*I polymorphisms [Higashi *et al.* 1988; Goldstein *et al.* 1991; Koppens *et al.* 1992b; Bachega *et al.* 1999] may produce deviant restriction patterns in rare cases.

We no longer endorse the concept of gene conversion as the most likely mechanism to create the 21-hydroxylase deficiency haplotypes D1 and D2. Recombination by unequal crossover provides an adequate explanation for the origin of all chromosomes without a *CYP21* gene [Koppens *et al.* 2002a; Koppens *et al.* 2002b]. Also, the assumption that these haplotypes have evolved from haplotype A during evolution needs to be amended since monomodular chromosomes (matching haplotype B) necessarily predate the primordial duplication of the RCCX module that generated haplotype A.

Haplotype frequencies

Band ratios do not provide direct information about single haplotypes, but rather about the combination of two haplotypes in an individual. As mentioned before [Koppens *et al.* 1992a], some combinations cannot be distinguished from other combinations that yield the same banding pattern unless a family study or long-range restriction mapping is performed. With one exception (family 3), complete *CYP21*/*C4* haplotypes could be assigned to all chromosomes bearing 21-hydroxylase deficiency and all but two of the non-carrier chromosomes in all families; known consanguinity between two of the families reduced the total to 83 and 85 chromosomes, respectively. Table 3 shows the band ratios found in the controls and the haplotypes that can be deduced from these ratios. To count the haplotypes, we assumed that the banding patterns that matched the standard 'A' haplotype on both chromosomes indeed represented A1/A1, A1/A2 and A2/A2 rather than B1/C1, B1/C3 and B1/C2 (or even E1/F1), respectively; also, A2/B2 was preferred over A3/B1. This assumption is based on the low frequency of the 'B' and 'C' haplotypes as compared to 'A', which makes a combination of them stochastically unlikely as compared to A/A. As a single exception, one of the 40 banding patterns that could represent A1/A2 was counted as B1/C3. This arbitrary correction matches the expected genotype frequency of B/C versus A/A as calculated from the family studies and results in a closer approximation of the Hardy-Weinberg equilibrium. No similar assumption was made for undistinguishable combinations of 'A' and 'C' in order not to arrive at unreliable frequencies of each individual 'C' haplotype. In each case it was assumed that in controls, combinations that did not involve carriership of steroid 21-hydroxylase deficiency were far more likely than those that did. Table 3 also lists the combinations of the non-carrier haplotypes in the 21-hydroxylase deficiency families; here, the family study eliminated the interpretation problems just mentioned. Some families actually have a son or daughter with this combination of haplotypes, most do not. Table 4 shows the frequencies of all haplotypes thus obtained.

Table 3. Band ratios and deduced genotypes in parents and controls

21-hydroxylase		complement *C4*	assigned haplotypes[a]	alternative haplotypes[a]	number of genotypes	
*Taq*I	*Bgl*II	*Taq*I			parents[b]	controls
3.2:3.7	12 : 10	7 : 6.4 : 6 : 5.4				
2 : 2	2 : 2	2 : 0 : 2 : 0	A1 / A1[c]	B1 / C1[c]	13	31
2 : 2	2 : 2	2 : 0 : 1 : 1	A1 / A2[d]	B1 / C3[d]	8	40
1 : 2	1 : 2	2 : 0 : 1 : 0	A1 / B1		2	11
1 : 2	1 : 2	1 : 1 : 1 : 0	A1 / B2		3	18
3 : 2	3 : 2	2 : 0 : 3 : 0	A1 / C1		1	0
3 : 2	3 : 2	2 : 0 : 1 : 2	A1 / C2[e]	A2 / C3[e]	1	2
3 : 2	3 : 2	2 : 0 : 2 : 1	A1 / C3[e]	A2 / C1[e]	2	7
1 : 3	2 : 2	2 : 0 : 2 : 0	A1 / G1[f]		0	1
2 : 2	2 : 2	2 : 0 : 0 : 2	A2 / A2[c]	B1 / C2[c]	4	6
1 : 2	1 : 2	2 : 0 : 0 : 1	A2 / B1		1	2
1 : 2	1 : 2	1 : 1 : 0 : 1	A2 / B2[c]	A3 / B1[c]	2	8
3 : 2	3 : 2	2 : 0 : 0 : 3	A2 / C2		1	0
2 : 1	1 : 2	1 : 1 : 0 : 1	A2 / E1[c,g]	B1 / D2[c,g]	0	1
2 : 3	3 : 2	2 : 0 : 0 : 3	A2 / F1[e]		0	1
1 : 3	2 : 2	2 : 0 : 1 : 1	A2 / G1[h]		0	1
0 : 2	0 : 2	2 : 0 : 0 : 0	B1 / B1		0	1
0 : 2	0 : 2	1 : 1 : 0 : 0	B1 / B2		0	2
1 : 3	2 : 2	2 : 0 : 0 : 2	B1 / F1[f]		0	1
0 : 2	0 : 2	0 : 2 : 0 : 0	B2 / B2		1	3
2 : 2	2 : 2	1 : 1 : 2 : 0	B2 / C1		0	1
1 : 3	2 : 2	0 : 2 : 0 : 2	B2 / F2[f]		0	1
3 : 2	3 : 2	1 : 1 : 3 : 0	B2 / H2		1	0
0 : 3	1 : 2	1 : 1 : 1 : 0	B2 / G1		0	1
4 : 2	4 : 2	2 : 0 : 4 : 0	C1 / C1		0	1
4 : 2	4 : 2	2 : 0 : 1 : 3	C2 / C3[i]		0	1

a: the most likely alternative is mentioned first, excluding haplotypes D and E which are rare in controls; the rare haplotypes F1, F2, G1 and H1 were confirmed by *Sac*II restriction mapping [Koppens *et al.* 2002c]

b: combinations of the father's and the mother's non-carrier haplotypes; totals do not completely add up to those in table 4 because three families where only one non-carrier haplotype could be deduced were not included here

c: all counted as the most likely alternative in table 4

d: 39 of these were counted as 'A1/A2', one as 'B1/C3' in table 4 (see text for rationale)

e: counted as 'A1 or A2', 'C1 or C3' and 'C2 or C3' in table 4, but assuming 1 x A1/C2, 1 x A2/C3, 5 x A1/C3 and 2 x A2/C1 for the comparison in table 6 (see text for rationale)

f: alternatives excluded by *Sac*II long-range restriction mapping [Koppens *et al.* 2002c]

g: a carrier of classical steroid 21-hydroxylase deficiency identified among the controls

h: the alternative of A1 combined with a novel 'G' haplotype was ruled out by a family study

i: no *Sac*II results were available, but alternatives such as a combination of A1 and a novel 'H' haplotype are unlikely since mutation analysis showed that this control is a carrier of Val281→Leu, which we found on all chromosomes with haplotype C2 (see Chapter 3.2)

Table 4. Frequencies of 21-hydroxylase/complement *C4* haplotypes in the Netherlands

haplotype	SL	SV	LO	SL + SV	%	fam.	contr.	fam. + contr.	%
A1	19	9	2	28	35.4	45	131[a]	176	48.2
A2	5	6	1	11	13.9	20	64[a]	84	23.0
A1 or A2[b]	1	0	0	1	1.3	0	9	9	2.5
B1	7	0	0	7	8.9	4	19[a]	23	6.3
B2	2	1	0	3	3.8	8	37	45	12.3
C1	1	0	0	1	1.3	1	3	4	1.1
C2	1	0	3	1	1.3	2	1	3	0.8
C3	1	0	0	1	1.3	2	2[a]	4	1.1
C1 or C3[b]	0	0	0	0	0.0	0	7	7	1.9
C2 or C3[b]	0	0	0	0	0.0	0	2	2	0.5
D1	3	1	0	4	5.1	0	0	0	0.0
D2	4	0	0	4	5.1	0	0	0	0.0
D1 or D2[b]	1	0	0	1	1.3	0	0	0	0.0
E1	13	3	0	16	20.3	0	1	1	0.3
F1	0	0	0	0	0.0	0	2	2	0.5
F2	1	0	0	1	1.3	0	1	1	0.3
G1	0	0	0	0	0.0	0	3	3	0.8
H1	0	0	0	0	0.0	1	0	1	0.3
total	59	20	6	79	100	83	282	365	100

SL: patients with salt-losing CAH
SV: patients with simple virilising CAH
fam.: non-CAH haplotypes in CAH families
contr.: unaffected controls
a: assuming B1/C3 rather than A1/A2 in one of the controls with similar patterns (see text for rationale)
b: for a few haplotypes, no distinction could be made between two possibilities (see text).

Population differences

A compilation of representative data from haplotyping studies done in different countries is presented in table 5 (more such studies are listed in our previous report [Koppens *et al.* 1992a]). Information on the configuration of the *C4* genes is often not available from *CYP21* haplotyping reports, but has been provided by several groups focusing on diseases that may be complement-associated. A comparison of *C4* haplotypes in healthy controls from several countries is presented in table 6. The haplotypes are grouped by the number and size of the *C4* genes here, without taking the number of *CYP21* and *CYP21P* genes into account. In terms of the *CYP21*/*C4* haplotypes used in fig. 1, this implies that the following haplotypes are merged: A1, D1 and G1; A2 and D2; B1 and E1; C2 and F1. For clarity, a nomenclature that is entirely *C4*-based [Blanchong *et al.* 2000] is also listed in table 6.

Interpretation of *CYP21*/*C4* haplotypes is as difficult today as it was ten years ago: not all authors avoid the 'haplotypes combination' pitfall mentioned above and the necessity to do family studies is not always appreciated either. For example, one recent report [Blanchong *et*

al. 2000] provides details on the frequencies of each individual 'C' haplotype and also on the frequency of haplotype combinations in a way somewhat similar to table 3 (figure 4C in that report). However, they interpret some combinations as A1/C2 and A2/C1 rather than A2/C3 and A1/C3, respectively and thus arrive at a very low frequency of haplotype C3 (LSS in their report). Since no mention is made of either family studies or long-range restriction mapping, this interpretation seems arbitrary. Such issues keep limiting the number of studies suitable for a comparison of haplotype frequencies; in addition, many recent reports focus on *CYP21* mutation analysis and no longer provide details on the layout of the RCCX module.

Reliable and extensive information about *C4*/*CYP21* haplotypes and their frequencies remains valuable today, because it can provide insight into questions regarding evolution, population genetics and mutational mechanisms. For example, it has been suggested that monomodular haplotypes (notably, B2 associated with HLA-B8,DR1) can serve as a 'premutation', promoting unequal crossovers between RCCX modules and contributing to *de novo* loss of the *CYP21* gene and to the relatively high frequency of the E1 haplotype in Western European populations [Sinnott *et al.* 1990]. Frequencies found in some, but not all, haplotyping studies support that notion.

Table 5. Comparison of *CYP21* haplotypes on chromosomes bearing classical 21-hydroxylase deficiency in different populations

haplotype	the Netherlands[a]	Essen, Germany[b]	Munich, Germany[c]	Sweden[d]	Ireland[e]	Italy[f]	Spain[g]	Columbus, Ohio, USA[h]	Campinas, Brazil[i]	Sao Paulo, Brazil[j]
A	40	18	24	46	16	47	56	18	27	94
B	10	1	6	1	3	1	13	2	1	19
C	3	1	0	2	0	7	14	4	3	9
D	9	1	11	6	0	2	17	11	7	10
E	16	15	11	12	10	7	20	9	3	9
F	1	0	0	3	0	0	0	0	0	1
other	0	0	0	4	1	2	0	0	0	0
total	79	36	52	74	30	66	120	44	41	142

a: this study, including the previous results [Koppens *et al.* 1992a]

b: compiled from table 2 in the report of Keller *et al.* [1991]

c: addition of the numbers for SL and SW from table 1 in the report of Strumberg *et al.* [1992]

d: compiled from several tables in the report of Haglund-Stengler *et al.* [1990]

e: compiled from table 1 and the text in the report of Sinnott *et al.* [1991]

f: compiled from table 1 in the report of Sinnott *et al.* [1992]

g: calculated from the percentages in fig. 2 in the report of Lobato *et al.* [1998]; contains 6 non-independent alleles due to consanguinity

h: compiled from table V in the report of Blanchong *et al.* [*et al.* 2000]

i: addition of the numbers for SL and SW from table 2 in the report of de-Araujo *et al.* [1996]

j: calculated from the percentages for SL and SW from table 1 in the report of Bachega *et al.* [1999]

Table 6. Comparison of *C4* haplotypes on chromosomes of healthy controls, or not carrying 21-hydroxylase deficiency, in different populations

haplotypes as in fig. 1	*C4* haplotype[a]	the Netherlands[b]	Essen, Germany[c]	Norway[d]	Italy[e]	Finland[f]	Northern Europe[g]	Columbus, Ohio, USA[h]	Brazil (Guarani)[i]	Brazil (Kaingang)[j]
A1, D1, G1	LL	185	18	45	39	35	50	138	18	92
A2, D2	LS	87	11	12	15	21	19	69	30	57
B1, E1	L	24	1	2	2	12	14	18	0	4
B2	S	45	3	10	3	7	22	33	0	0
C1	LLL	6	3	1	0	0	0	22	56	104
C3	LLS	10	1	2	0	3	1	1	0	2
C2, F1	LSS	6	1	3	1	0	0	19	0	0
other		2	0	1	1	0	0	0	7	77
total		365	38	76	60	78	106	300	111	336

a: L=long *C4* gene, S=short *C4* gene [Blanchong *et al.* 2000]

b: this study, including the previous results [Koppens *et al.* 1992a], and interpreting the control haplotypes as described under table 3

c: compiled from table 2 in the report of Keller *et al.* [1991]

d: from table 1 in the report of Teisberg *et al.* [1988], interpreting a-d as 'long' and e as 'short'

e: from table 2 in the report of Sinnott *et al.* [1992]

f: compiled from table 1 in the report of Partanen [1987]

g: from table 1 in the report of Palsdottir *et al.* [1987]; probably mixed British, Icelandic and Norwegian

h: calculated from the percentages in fig. 3 in the report of Blanchong *et al.* [2000]

i: from tables 3 and 5 in the report of Weg-Remers *et al.* [1997] (the numbers are not quite consistent in these tables); Guarani Amerindian tribe

j: as i; summation of two genetically very similar groups of the Kaingang Amerindian tribe

An issue discussed in our earlier report [Koppens *et al.* 1992a] is the balance between monomodular and trimodular haplotypes, which are expected to be generated in equal amounts by crossing-over events between two bimodular chromosomes [Koppens *et al.* 2002a]. One reason why trimodular chromosomes are not widespread may be the contribution of pre-existing monomodular chromosomes just mentioned: HLA-B8,DR1 may be an ancient conserved haplotype that escaped the RCCX module duplication. There seems to be no disadvantage to having a trimodular RCCX area, as demonstrated by the high frequency in two South American Indian tribes [Weg-Remers *et al.* 1997].

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2.3. The *Pvu*II restriction site in the second intron of the human steroid 21-hydroxylase gene *CYP21* is polymorphic

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Candidate gene
CYP21 encoding steroid 21-hydroxylase, involved in adrenocortical steroidogenesis; deficiency leads to congenital adrenal hyperplasia. The *CYP21* cDNA probe used also hybridises to the 98 % homologous pseudogene *CYP21P* [White *et al.* 1985].

Source/description of probe
We analysed *Taq*I/*Pvu*II patterns using the 0.6 kb *Bam*HI-*Eco*RI fragment of pC21/3c as a probe. Probe availability: pC21/3c is available from the ATCC.

Technical comments
10 µg of human genomic DNA was first digested with *Taq*I and then with *Pvu*II. The fragments were separated on a 1.2 % agarose gel and transferred to nitrocellulose membranes by Southern blotting. After hybridisation, the final washing step was performed at 60 °C, 0.3 x SSC.

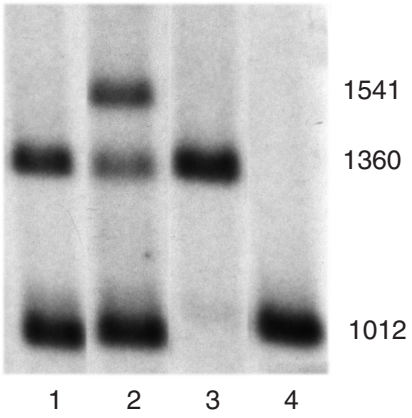
Polymorphism
*Taq*I/*Pvu*II double digestion results in 3 fragments: a constant band at 1012 bp (representing *CYP21P*) and polymorphic bands at 1541 and 1360 bp (representing *CYP21*). The fragment sizes have been deduced from published sequencing data [Higashi *et al.* 1986]. Lane 1: allele B / allele B; lane 2: allele A / allele B; lane 3: allele B / allele B, with a homozygous deletion of *CYP21P*; lane 4, homozygous deletion of *CYP21*.

Frequencies
The polymorphism was studied in 44 families with patients affected with steroid 21-hydroxylase deficiency and 78 unrelated healthy controls. Most of the patients and their family members have been described in a previous report [Koppens *et al.* 1992]; the others

have been classified and studied in a similar way, allowing a distinction between chromosomes carrying and not carrying steroid 21-hydroxylase deficiency. The controls were individuals randomly selected from the Dutch population and had no reported family history of steroid 21-hydroxylase deficiency. The frequencies of allele A (the 1541 bp fragment) and allele B (the 1360 bp fragment) did not differ significantly between chromosomes carrying or not carrying steroid 21-hydroxylase deficiency (table 1).

Table 1. Frequencies of allele A (1541 bp) and allele B (1360 bp) in steroid 21-hydroxylase deficient and normal chromosomes

	allele A	allele B	total
deficient (patients)	1	87	88
normal (family members)	1	87	88
normal (controls)	3	153	156
total	5	327	332
frequency	0.02	0.98	



Chromosomal localisation
CYP21 has been mapped to the human MHC on chromosome 6p21.3.

Mendelian inheritance
Codominant segregation was observed in two informative families.

Other comments
Haplotyping studies performed as described [Koppens *et al.* 1992] showed that the variation in fragment size described here could not be attributed to a *Taq*I polymorphism. Absence of the *Pvu*II site involved is normally a feature of the *CYP21P* pseudogene. This was confirmed in this study, since no *Taq*I/*Pvu*II fragments smaller than the 1012 bp fragment expected for *CYP21P* were found. Gene conversion has been proposed as a mechanism causing deficiency of *CYP21* genes by transfer of parts of *CYP21P*. Presence of both deleterious and apparently harmless *CYP21P*-like sequences has been observed [Higashi *et al.* 1988]. It therefore seems possible that the polymorphism described here has also resulted for a gene conversion-like event.
Care should be taken when interpreting *Pvu*II restriction patterns in haplotyping studies. Presence of this polymorphism in *CYP21* could lead to identification of *CYP21* genes as *CYP21P*-like and hence to erroneous assignment of carrier status.

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2.4. A rare *Taq*I polymorphism in a human complement *C4* gene is caused by an additional restriction site in the first intron

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Abstract

We studied the configuration of the complement *C4*/*CYP21* (steroid 21-hydroxylase) region of the human major histocompatibility complex in patients suffering from congenital adrenal hyperplasia (CAH) and in the general population in the Netherlands, using *C4* and *CYP21* probes and the restriction enzymes *Taq*I and *Bgl*II. We found a rare *Taq*I 3.9 kb restriction fragment in the mother of a CAH patient and present evidence that this polymorphism is caused by an additional restriction site in the first intron of a complement *C4* gene.

Introduction

The human major histocompatibility complex class III genes usually include two genes encoding the fourth component of complement, *C4A* and *C4B*, which are about 10 kb apart. About 3 kb centromeric of *C4B* lies a gene (*CYP21*) encoding the adrenocortical enzyme steroid 21-hydroxylase; in a similar arrangement, *C4A* is accompanied by the *CYP21P* pseudogene, which is inactive due to several deleterious mutations [Carroll *et al.* 1985; White *et al.* 1985; Higashi *et al.* 1986; White *et al.* 1986].

Deletions and duplications of the complement *C4* and 21-hydroxylase genes are common; mutations affecting *CYP21* lead to congenital adrenal hyperplasia (CAH), an inherited metabolic disease afflicting about 1:15,000 newborns.

In recent years, the rearrangements in this region have been extensively studied using *C4* and 21-hydroxylase DNA probes. The restriction enzyme *Taq*I is especially useful, providing information about both the *C4* and 21-hydroxylase genes.

In a study of *C4* and 21-hydroxylase haplotypes in the Netherlands, we found a rare *Taq*I polymorphism in the mother of a CAH patient, on the chromosome not carrying CAH. We

here present evidence that this polymorphism is caused by an additional restriction site in the first intron of a *C4* gene.

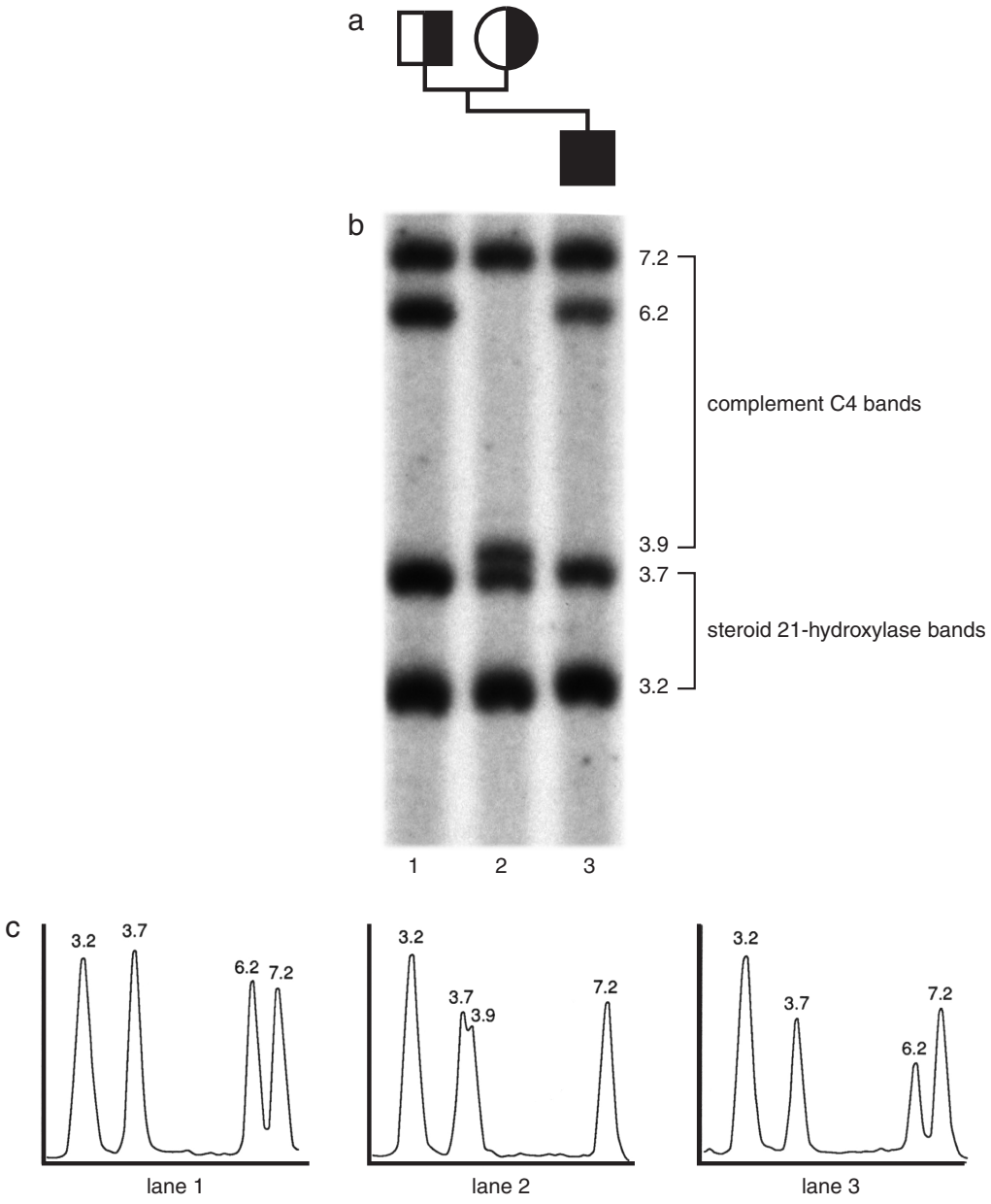

Fig. 1. a. Pedigree of family W. b. *Taq*I restriction patterns in family W. Hybridisation was performed with the 0.6 kb *Bam*HI-*Kpn*I fragment of pAT-A and with pC21/3c in a single experiment. Lane 1, father W; lane 2, mother W; lane 3, son W (CAH patient). c. Densitometric scans corresponding to fig. 1b, lanes 1-3.

Materials and methods

CAH patients and controls

We characterised the configuration of the complement *C4*/*CYP21* region in 44 unrelated families with CAH patients and 147 unrelated healthy controls, all of Dutch ethnic origin (results to be published elsewhere). *Taq*I and *Bgl*II restriction patterns were established using complement *C4* and steroid 21-hydroxylase probes. In one CAH family (family W), an unusual *Taq*I restriction pattern was found (see fig. 1). The son is a CAH patient, who presented with the salt-losing form of the disease in the first month of life.

Southern blotting, hybridisation and laser densitometry

Genomic DNA was prepared from peripheral leukocytes essentially as described [Wyman and White 1980]; 10 µg was digested overnight with 40 units *Bam*HI, *Bgl*II, HindIII, *Kpn*I, *Pst*I, *Pvu*II or *Taq*I, according to the manufacturers' (New England Biolabs, Beverly MA, USA, or Pharmacia, Uppsala, Sweden) instructions. After agarose gel electrophoresis and Southern blotting, hybridisation was performed with the 1 kb *Pst*I fragment of the complement *C4* cDNA probe pAT-A [Belt *et al.* 1984] or the 2.1 kb 21-hydroxylase cDNA probe pC21/3c [White *et al.* 1985]; the probes were obtained from the American Type Culture Collection. The optical densities of the autoradiographic bands were determined by densitometry with an LKB 2222-020 Ultroscan XL laser densitometer. The relative quantities of the fragments were calculated as the ratio of the areas under the respective curves.

Results and discussion

In the most common arrangement, two complement *C4* loci are present in the human MHC class III region, with *C4A* positioned at locus I and *C4B* at locus II. *Taq*I polymorphisms are used to distinguish between these loci, as well as between 'long' and 'short' complement *C4* genes (22 *vs* 16 kb, depending on the presence or absence of a 6-7 kb intronic sequence) [Schneider *et al.* 1986; Palsdottir *et al.* 1987; Yu and Campbell 1987].

Fig. 1 shows the *Taq*I restriction patterns obtained with the complement *C4* and 21-hydroxylase cDNA probes in family W. The 7.2 kb and 6.2 kb bands shown represent long *C4* genes at locus I and II, respectively; the 3.7 and 3.2 kb bands represent the steroid 21-hydroxylase genes *CYP21* and *CYP21P* [Carroll *et al.* 1985; White *et al.* 1985; Higashi *et al.* 1986; White *et al.* 1986; Schneider *et al.* 1986; Palsdottir *et al.* 1987; Yu and Campbell 1987]. In mother W (lane 2 in fig. 1), a band was observed at 3.9 kb; when the probes were used separately, the 3.9 kb band hybridised to the *C4* probe only. For father W, the intensity of the *Taq*I 3.2 and 3.7 kb fragments is equal; the 3.7 kb fragment of mother and son is only half as intense as the 3.2 fragment (fig. 1c), which means the number of *CYP21* and *CYP21P* genes is equal in father W, while in mother and son there are twice as many *CYP21P* genes as *CYP21* genes. Using *Bgl*II with the 21-hydroxylase probe, we found an equal intensity of the 12 and 10 kb bands in father, and a diminished 12 kb fragment in mother and son (results not shown). The 10 kb *Bgl*II band is constant; the relative intensity of the 12 kb band varies with the number of *C4*/*CYP21(P)* repeats present, the bands being equal for individuals with four

such units. These results indicate that father W has two 22 kb *C4* genes, a *CYP21* gene and a *CYP21P* pseudogene on each chromosome. Mother W has a single 22 kb *C4* gene and a *CYP21P* pseudogene on one chromosome. On the other chromosome she has a 22 kb *C4* gene and a *C4* gene characterised by the 3.9 kb fragment, and a *CYP21* gene and a *CYP21P* pseudogene. Deletions in this region usually encompass a *C4* gene as well as one of the adjacent 21-hydroxylase genes [Carroll *et al.* 1985; White *et al.* 1985; Schneider *et al.* 1986; Palsdottir *et al.* 1987; Uring-Lambert *et al.* 1987; Yu and Campbell 1987]. The son, a CAH patient, apparently inherited the chromosome carrying a deletion of *CYP21* and one *C4* gene from his mother. The rare 3.9 kb *Taq*I fragment is therefore not associated with CAH in this family. This data strongly suggests that this fragment represents a *C4* gene at locus II on the mother's chromosome not carrying a deletion.

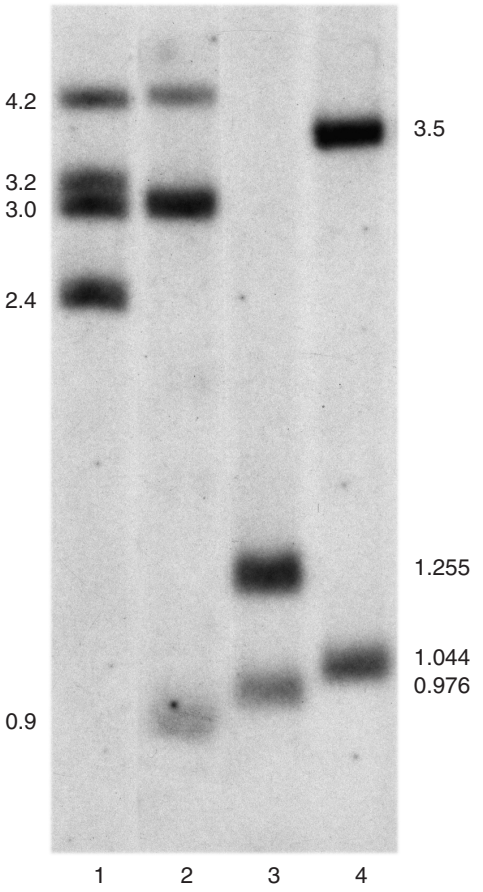

Fig. 2. Restriction patterns of mother W and a control. Hybridisation was performed with the 1 kb *Pst*I fragment of pAT-A. Lane 1, *Taq*I/*Kpn*I pattern of a control with long *C4* genes at locus I and short *C4* genes at locus II, showing bands characteristic of locus I (4.2 kb), locus II (3.2 kb), long *C4* genes (3.0 kb) and short *C4* genes (2.4 kb). Lane 2-4, mother W: *Taq*I/*Kpn*I, *Pst*I/*Kpn*I and *Bam*HI/*Kpn*I patterns, respectively. Double digests of DNA from controls with long *C4* genes showed the same *Pst*I/*Kpn*I and *Bam*HI/*Kpn*I patterns as in lanes 3 and 4. Mother W's bands correspond to restriction fragments shown in fig. 3. The numbers indicate the sizes of the *Taq*I/*Kpn*I fragments (left) and of the *Pst*I/*Kpn*I and *Bam*HI/*Kpn*I fragments (right).

We attempted to further characterise this polymorphism using several other restriction enzymes. First, mother W's DNA was digested with *Kpn*I, HindIII and *Bam*HI. The complement *C4* probe hybridised to the following restriction fragments: *Kpn*I: 12 kb, 7.5 kb and 3.7 kb; HindIII: 32 kb and 15 kb; *Bam*HI: 4.5 kb. These fragments have been described in

the literature [Carroll *et al.* 1985; Palsdottir *et al.* 1987; Uring-Lambert *et al.* 1987; Yu and Campbell 1987]. From these data, it can be concluded that all complement *C4* genes in mother W are 'long' (characterised by the 7.5 kb *Kpn*I and 4.5 kb *Bam*HI fragments), and that both locus I and II are present (characterised by the 12 and 3.7 kb *Kpn*I fragments, respectively). This was confirmed by the HindIII pattern (the 15 and 32 kb bands are characteristic of long genes at locus I and II, respectively). Also, the relative intensity of the 7.5 and 3.7 kb *Kpn*I bands and the 32 kb HindIII band was diminished relative to the same bands in individuals with two long *C4* genes on each chromosome, confirming a deletion of one *C4* (and one 21-hydroxylase) gene on one of mother W's chromosomes.

These findings suggest that the 3.9 kb *Taq*I fragment is the result of an additional restriction site rather than a major rearrangement or a deletion. Normally, a 'long' *C4* gene at locus II would be characterised by a 6.2 kb *Taq*I fragment; an extra *Taq*I site within this fragment could reduce its size to 3.9 kb. We determined the location of this putative additional *Taq*I site using double digests with several restriction enzymes.

Fig. 2 shows that in a *Taq*I/*Kpn*I double digest, mother W (lane 2) lacks the 3.2 kb band typically seen in controls having *C4* genes at locus II (lane 1), but has an additional band at 0.9 kb. Recently, the nucleotide sequence of a *C4* gene was published [Yu 1991]; within about 1 kb of the 5' terminus are a *Bam*HI site and a *Pst*I site (both in the first exon) and a *Pvu*II and a *Kpn*I site (see fig. 3). Lane 2-4 of fig. 2 show mother W's additional 0.9 kb *Taq*I/*Kpn*I band in comparison with the 1044 bp *Bam*HI-*Kpn*I fragment and the 976 bp *Pst*I-*Kpn*I fragment predicted by the sequence. The *Taq*I/*Kpn*I fragment is about 70 bp smaller than the *Pst*I/*Kpn*I fragment, and thus approximately 900 bp in size. These findings were in agreement with the size differences of the fragments observed after *Taq*I/*Pvu*II, *Bam*HI/*Pvu*II and *Pst*I/*Pvu*II double digests (results not shown).

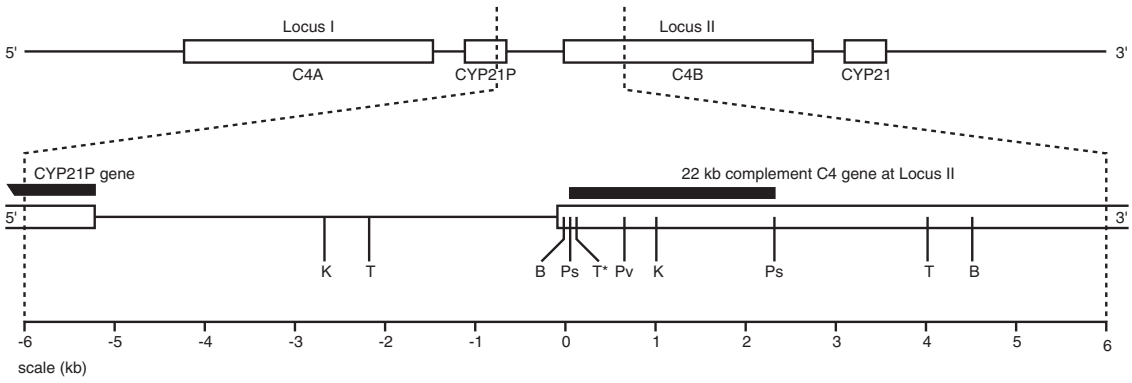

Fig. 3. Restriction map of the 5' end and flanking sequence of mother W's *C4* gene at locus II; the 3' end of the adjacent *CYP21P* gene is shown. B-*Bam*HI; K-*Kpn*I; Ps-*Pst*I; Pv-*Pvu*II; T-*Taq*I; T*: additional restriction site described here. The beginning of the *C4* gene (0 on the kb scale) and the relative position of several restriction sites are according to the sequence published by Yu [1991]. The black bars indicate the sections covered by the *C4* cDNA probe (right) and the 21-hydroxylase cDNA probe (left), respectively.

These results can only be explained by an additional *Taq*I site in the first intron of the *C4* gene, located about 70 bp downstream of the *Pst*I site in the first exon (see fig. 3). In a *Taq*I digest (fig. 1), no 2.7 kb fragment (the other part of the original 6.6 kb) is seen, because this would hybridise to only about 30 bp of the cDNA probe. It is highly unlikely that the sequence alteration described here could in any way adversely affect the functioning of the *C4* gene. The data presented here shows that it is located well outside the coding sequences. Also, assuming a point mutation has occurred, generation of a consensus splice junction sequence seems improbable, because this would require a sequence TCGNG to be changed into TCGAG, and no such sequence is present in the first intron of the published *C4* sequence [Yu 1991] (apart from the fact that GAG is rarely seen at splice junctions).

Recently, Goldstein *et al.* [1991] reported a polymorphism in a human complement *C4* gene characterised by a 3.5 kb *Taq*I fragment in two patients with systemic lupus erythematosus. In their study as well as in ours, a 21-hydroxylase probe was used, which hybridises to a 3.73 kb *Taq*I band fragment (the size of which has been determined by sequence analysis [Higashi *et al.* 1986; White *et al.* 1986]). A clear distinction between 3.5 and 3.9 kb should therefore be possible; when the *C4* and 21-hydroxylase probes are used in a single hybridisation experiment, the 3.9 kb *C4* band is located above the 3.73 kb *CYP21* band (see fig. 1). Therefore, the 3.5 and 3.9 kb bands appear to represent different polymorphisms as well as two new classes when the combined *C4* / *CYP21* haplotypes defined in terms of *Taq*I restriction patterns are considered.

Clearly, these polymorphisms are rare: Goldstein *et al.* found the 3.5 kb band twice in 79 SLE patients and 60 controls (278 chromosomes), and we found the 3.9 kb band once in 44 CAH families and 147 controls (470 chromosomes); neither has been previously reported in the literature.

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Chapter 3

CYP21 mutation analysis

3.1. *CYP21* and *CYP21P* variability in steroid 21-hydroxylase deficiency patients and the general population in the Netherlands
Eur J Hum Genet 2000; 8: 827-836
3.2. Distribution of CYP21 mutations in a Dutch population

3.1. *CYP21* and *CYP21P* variability in steroid 21-hydroxylase deficiency patients and in the general population in the Netherlands

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Eur J Hum Genet 2000; 8: 827-836

Abstract

Steroid 21-hydroxylase deficiency is caused by defectiveness of the *CYP21* gene. Such defects have presumably originated from interactions with the nearby *CYP21P* pseudogene during evolution. We studied these mechanisms by comparing the genetic variability of *CYP21*, *CYP21P* and *CYP21P*/*CYP21* hybrids (resulting from large-scale rearrangements) at eight mutation sites in a group of Dutch steroid 21-hydroxylase deficiency patients, their family members, and controls. The most common *CYP21* defect in patients with salt-losing steroid 21-hydroxylase deficiency was a splice junction mutation in intron 2. The most common defect in the simple virilising form of the disease was Ile172→Asn. *CYP21P* showed considerable sequence variation in its central and 3' sections; the 5' section was constant. A single nucleotide (T) insert in exon 7 was found in all *CYP21P* genes. During the course of evolution, this was probably the third defect introduced into *CYP21P* after the splice junction mutation in intron 2 and the 8 bp deletion in exon 3. Gene conversions introducing *CYP21*-like sequences contribute to *CYP21P* variability. Such an event has occurred *de novo* in one family. A comparison of *CYP21* and *CYP21P* mutations on the same chromosome shows that at least some of the small-scale gene conversions that supposedly transfer defects to *CYP21* involve interaction between homologous chromosomes. The majority of the putative *CYP21P*-*CYP21* transitions in hybrid genes appears to occur in a distinct zone that lies 5' of nucleotide 2108, which is further downstream than previously hypothesised. The other transitions lie upstream of nucleotide 999. Apparent 'large-scale' *CYP21*-*CYP21P* gene conversions lead to hybrid genes that are very similar to those found in *CYP21* deletions, so these haplotypes have probably resulted from a meiotic double unequal crossover.

Introduction

The human *CYP21* gene, located on chromosome 6 (6p21.3), encodes the adrenocortical enzyme steroid 21-hydroxylase [White *et al.* 1985]. *CYP21P* is a pseudogene sharing 98 % sequence homology with *CYP21* but containing several alterations rendering it inactive [Higashi *et al.* 1986; White *et al.* 1986]. *CYP21* and *CYP21P* are about 30 kb apart and are part of a cluster of duplicated genes that includes genes encoding the fourth component of complement (C4) and tenascin-X [White *et al.* 1985; Gitelman *et al.* 1992; Miller *et al.* 1992]. Defectiveness of *CYP21* leads to steroid 21-hydroxylase deficiency, which is the cause of over 90 % of all cases of congenital adrenal hyperplasia. Complete deficiency results in severe salt-loss crises in untreated paediatric patients due to lack of aldosterone ('salt-losing' 21-hydroxylase deficiency), while increased androgen levels cause virilisation even in patients with less severe defects ('simple virilising' 21-hydroxylase deficiency) [White 1994; Pang 1997; Wedell 1998].

Genetic analysis of steroid 21-hydroxylase deficiency is complicated by the existence of many different arrangements of *CYP21* and *CYP21P*, including gene deletions, duplications and large-scale gene conversions. Analysis of *Taq*I and *Bgl*II restriction patterns is commonly used to determine the gross arrangement of the *CYP21*/*C4* region in steroid 21-hydroxylase deficiency patients and their family members [Carroll *et al.* 1985; White *et al.* 1985; Donohoue *et al.* 1986; Schneider *et al.* 1986; Matteson *et al.* 1987; Higashi *et al.* 1988; White *et al.* 1988; Morel *et al.* 1989]. This approach allows the definition of *CYP21*/*C4* haplotypes, some of which are associated with steroid 21-hydroxylase deficiency [White *et al.* 1988; Collier *et al.* 1989; Morel *et al.* 1989; Mornet *et al.* 1991; Helmberg *et al.* 1992; Koppens *et al.* 1992; Strumberg *et al.* 1992; Lobato *et al.* 1998; Paulino *et al.* 1999]. Three categories of defects can be distinguished:

(a) Haplotypes with a single gene that features a *CYP21P*-like *Taq*I restriction pattern ('*CYP21* deletions'). These have resulted from a meiotic unequal crossover between *CYP21* and *CYP21P*, creating a gene consisting of the 5' section of *CYP21P* and the 3' section of *CYP21* [Carroll *et al.* 1985; White *et al.* 1985; Donohoue *et al.* 1989; Collier *et al.* 1993; Tusié-Luna and White 1995].

(b) Haplotypes with two genes, both with a *CYP21P*-like restriction pattern ('large-scale gene conversions'). Here, a section of *CYP21* that includes at least the *Taq*I site in the 5' flank has been replaced by a *CYP21P*-like sequence [Donohoue *et al.* 1986; Matteson *et al.* 1987; Higashi *et al.* 1988].

(c) Haplotypes where a gene with a *CYP21*-like restriction pattern is present (by exclusion).

On chromosomes bearing steroid 21-hydroxylase deficiency, the *CYP21* gene carries one or more deleterious mutations [Higashi *et al.* 1991; Mornet *et al.* 1991; Owerbach *et al.* 1992; Speiser *et al.* 1992; Wedell 1998]. With a few exceptions, these mutations are found in the consensus sequence of *CYP21P* [Higashi *et al.* 1986; White *et al.* 1986] and have supposedly been transferred to *CYP21* during evolution ('small-scale gene conversions').

The nature and the frequencies of the mutations in *CYP21* have been well studied. They vary among different populations [Higashi *et al.* 1991; Mornet *et al.* 1991; Helmberg *et al.* 1992; Owerbach *et al.* 1992; Speiser *et al.* 1992; Barbat *et al.* 1995; Evgrafov *et al.* 1995; Ezquieta *et al.* 1995; Wilson et al, 1995; Wedell 1998; Paulino *et al.* 1999]. However, information on the variability of the *CYP21P* pseudogene, especially in association with the *Taq*I/*Bgl*II haplotypes that define the overall structure of the region, is rather limited [Helmberg *et al.* 1992; Wedell and Luthman 1993]. Such information is highly relevant to the hypotheses describing the origin of the different categories of disease-causing mutations in *CYP21*: the location of crossover sites and the extent of conversion zones depend on the composition of the *CYP21P* gene involved. We compared *CYP21* and *CYP21P* in a single population, to assess the consequences of *CYP21P* variability to the hypothetical concepts of 'hybrid genes' and 'gene conversions'. The distribution of the ten most common *CYP21* defects (gene deletion, large-scale conversion, and eight mutations) was determined in 38 steroid 21-hydroxylase deficiency patients from the Netherlands, a population which has not been studied before. The sites corresponding to these mutations were also studied in *CYP21P* in these families, and in 46 controls having only one *CYP21P* gene.

Methods

Patients, family members, and controls

Steroid 21-hydroxylase deficiency patients, their parents and any available sibs from 38 families were included; in six families, only one of the parents was available. The number of independent chromosomes was 150 (due to consanguinity between two of the families), 75 of which carried a steroid 21-hydroxylase deficiency allele. Single patients and two families with apparent *de novo* gene rearrangements were not included. There were 29 families with salt-losing (SL) patients and 9 families with simple virilising (SV) patients. From our group of 143 healthy controls from the general Dutch population, 46 had a heterozygous deletion of *CYP21P* and were selected to study *CYP21P* separately. All subjects were informed about the purpose of the study and gave their consent.

Haplotyping and mutation analysis

*Taq*I and *Bgl*II restriction analysis [Carroll *et al.* 1985; White *et al.* 1985; Donohoue *et al.* 1986; Schneider *et al.* 1986; Matteson *et al.* 1987; Higashi *et al.* 1988; White *et al.* 1988; Morel *et al.* 1989] was used as described earlier [Koppens *et al.* 1992] to establish *CYP21*/*C4* haplotypes in all families and controls.

Polymerase chain reaction (PCR) amplification of *CYP21* and *CYP21P* followed by oligonucleotide hybridisation [Mornet *et al.* 1991; Helmberg *et al.* 1992; Owerbach *et al.* 1992; Speiser *et al.* 1992; Barbat *et al.* 1995] was used to determine sequence variability.

Table 1. Oligonucleotides used as primers and for mutation detection

sequence	description	final wash[a]
CAGACCTGAGCCACTTACCT	nt 367: forward primer *CYP21* and *CYP21P*, section 1	
ATCCCCAATCCAGGTCCCT	nt 530: forward primer *CYP21*, section 1	
TTTGTTAGAGATGGGGTCTTG	nt 430: forward primer *CYP21P*, section 1[b]	
CAGGAGGAGTTGGGGGCTG	A at nt 655: splice junction intron 2 (normal)	52ºC, 0.5xSSC
CAGGAGGAGGTGGGGGCTG	C at nt 655: splice junction intron 2 (normal)	52ºC, 0.5xSSC
CAGGAGGAGCTGGGGGCTG	G at nt 655: splice junction intron 2 (mutant)	52ºC, 0.5xSSC
TCCTTGGGAGACTACTCCCT	nt 700: absence of 8 bp deletion in exon 3 (normal); also forward primer *CYP21*, section 2	48ºC, 0.4xSSC
TGTCGTTGGTCTCTGCTCTG	nt 698: presence of 8 bp deletion in exon 3 (mutant); also forward primer *CYP21P*, section 2	52ºC, 0.4xSSC
TTCTTGTGGGCTTTCCAGAG	nt 721: reverse primer *CYP21* and *CYP21P*, section 1	
GCGGCATCATCTGTTACCT	T at nt 999: Ile172 (normal)	48ºC, 0.5xSSC
GCGGCATCAACTGTTACCT	A at nt 999: Asn172 (mutant)	48ºC, 0.5xSSC
CACATCGTGGAGATGCAGCTG	TCGTGGAGAT at nt 1380-1389: IleValGluMet236 (normal), also forward primer *CYP21*, section 3	52ºC, 0.3xSSC
CACAACGAGGAGAAGCAGCTG	ACGAGGAGAA at nt 1380-1389: AsnGluGluLys236 (mutant), also forward primer *CYP21P*, section 3[c]	52ºC, 0.3xSSC
CACAAGGAGGAGAAGCAGCTG	AGGAGGAGAA at nt 1380-1389: LysGluGluLys236 (mutant)	52ºC, 0.1xSSC
AGTCCCCACCTTGTGCTGCCT	nt 1397: reverse primer *CYP21* and *CYP21P*, section 2	
CCATGTGCACGTGCCCTTC	G at nt 1683: Val281 (normal)	48ºC, 0.5xSSC
CCATGTGCAAGTGCCCTTC	T at nt 1683: Leu281 (mutant)	48ºC, 0.5xSSC
GTGAAGCAAAAAAACCACGG	nt 1760: absence of T insertion in exon 7 (normal)	48ºC, 0.4xSSC
GTGAAGCAAAAAAAACCACG	nt 1760: presence of T insertion in exon 7 (mutant)	48ºC, 0.4xSSC
GCTCCTCCTGCAGTCGCTG	C at nt 1994: Gln318 (normal)	48ºC, 0.7xSSC
GCTCCTCCTACAGTCGCTG	T at nt 1994: stop318 (mutant)	48ºC, 0.7xSSC
CTGCGCCTGCGGCCCGTTG	C at nt 2108: Arg356 (normal)	52ºC, 0.2xSSC
CTGCGCCTGTGGCCCGTTG	T at nt 2108: Trp356 (mutant)	52ºC, 0.2xSSC
AACCCTCGGGAGTCACCTGCT	nt 2153: reverse primer *CYP21* and *CYP21P*, section 3	

nt = nucleotide number according to Higashi *et al.* [1986]

a: the final wash in the oligonucleotide hybridisation was done for 20 minutes at these conditions

b: primer site chosen about 100 bp upstream of the *CYP21* primer, to include the *Eco*RI site in exon 2

c: section 3 of genes carrying LysGluGluLys236 was amplified normally using this primer, but it was easily washed off in the hybridisation experiments of the section 2 PCR product

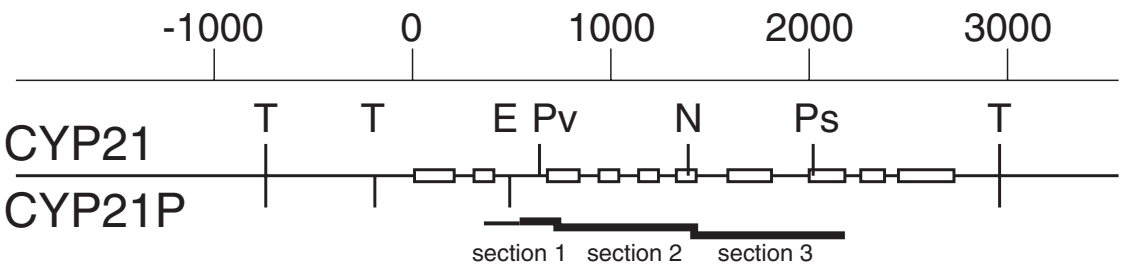

Fig. 1. Map of the consensus *CYP21* and *CYP21P* genes; open boxes are exons. Restriction sites used for identification (see text) are shown: E=*Eco*RI site, N=*Nde*II site, Ps=*Pst*I site, Pv=*Pvu*II site, T=*Taq*I site. The bold black lines below represent the three amplified sections. The amplification of *CYP21* and *CYP21P* in a single reaction (see text) matched section 1, extended further to the 5' side as indicated by the thinner black line to the left.

Three overlapping parts of either *CYP21* or *CYP21P* (see following sections 1, 2 and 3; fig. 1) were specifically amplified using the primers listed in table 1. PCR conditions (annealing temperature, number of cycles, $MgCl_2$ concentration) were: *CYP21*, section 1: 54 °C, 30 cycles, 2.5 mM; *CYP21P*, section 1: 55 °C, 32 cycles, 2.5 mM; *CYP21*, section 2: 55 °C, 33 cycles, 2.5 mM; *CYP21P*, section 2: 57 °C, 32 cycles, 2.5 mM; *CYP21*, section 3: 56 °C, 32 cycles, 2.4 mM; *CYP21P*, section 3: 56 °C, 32 cycles, 2.4 mM. All reactions were done with 1 unit of GoldStar DNA polymerase (Eurogentec, Seraing, Belgium) in a final volume of 50 µl in the presence of 2 % formamide. In each case, the reverse primer was non-specific (not distinguishing between *CYP21* and *CYP21P*) and downstream of the forward primer of the next section. The specificity of each reaction was checked by digesting 20 % of the amplified product with *Pvu*II (section 1), *Nde*II (section 2), or *Pst*I (section 3), followed by electrophoresis in 2-2.5 % agarose gels. These restriction sites are normally present in *CYP21* and absent from *CYP21P*. Section 1 was also digested with *Eco*RI; the *Eco*RI site is specific for *CYP21P* (see fig. 1). The *Pvu*II and *Eco*RI restriction patterns of the section 1 PCR were matched to the bands shown by *Taq*I/*Pvu*II and *Taq*I/*Eco*RI double digests of genomic DNA [Koppens *et al.* 1995; Koppens *et al.* 1998]. Control samples containing or not containing these sites were included in each reaction. The results for section 1 were confirmed by amplification of *CYP21* and *CYP21P* in a single reaction using the same (non-specific) reverse primer and a non-specific forward primer (see fig. 1; 53 °C, 32 cycles, 3 mM $MgCl_2$) followed by *Eco*RI digestion to distinguish between *CYP21* and *CYP21P*, Southern blotting and hybridisation as described elsewhere [Koppens *et al.* 1998]. The remainder of the product was slot-blotted onto Hybond-N^+ (Amersham Pharmacia, Little Chalfont, UK), and mutations were detected by hybridisation to allele-specific oligonucleotides, some of which were also used for amplification (see table 1 and fig. 2).

Results

Arrangement of *CYP21*/*CYP21P* and complement *C4* genes

An overview of the *CYP21*/*CYP21P* arrangements in the 38 families and 143 controls investigated is presented in table 2. Several *CYP21*/*C4* haplotypes not described in our earlier report [Koppens *et al.* 1992] were detected in the larger population now studied. Three controls had two *CYP21* genes and no *CYP21P* genes on one chromosome, suggesting a 'large-scale' conversion of *CYP21P* to *CYP21*. Both *C4* genes were 'long' (22 kb) in this haplotype. One control carried one *CYP21* gene, three *CYP21P* genes, one long *C4* gene and three short (16 kb) *C4* genes on one chromosome. The mother of one patient had one *CYP21* gene, three *CYP21P* genes and four 'long' *C4* genes on one chromosome. These unusual haplotypes were confirmed by *Sac*II digestion and pulsed field gel electrophoresis (unpublished observations).

Table 2. *CYP21*/*CYP21P* haplotypes in the Dutch population

	families, normal haplotypes[a]	families, defective haplotypes[b]	controls all haplotypes
haplotype			
CYP21-CYP21P (normal)	58	38	212
CYP21 (*CYP21P* deletion)	11	9	55[c]
CYP21-CYP21P-CYP21P (*CYP21P* duplication)	2	3	11
CYP21-CYP21-CYP21P (*CYP21* duplication)	0	1	3
CYP21-CYP21P-CYP21P-CYP21P (*CYP21P* triplication)	1	0	1
CYP21-CYP21 (*CYP21P* to *CYP21* conversion)	0	0	3
CYP21P[d] (*CYP21* deletion)	0	15	1
CYP21P-CYP21P[d] (*CYP21* to *CYP21P* conversion)	0	9	0
total	72	75	286

a: haplotypes not carrying steroid 21-hydroxylase deficiency; in three families, these could not be deduced because one of the parents was missing

b: haplotypes carrying steroid 21-hydroxylase deficiency

c: the genotypes were: four persons with *CYP21*/*CYP21*, one person with *CYP21-CYP21*/*CYP21* and 46 persons with *CYP21-CYP21P*/*CYP21*; these 46 were used as controls in the *CYP21P* mutation analysis

d: these haplotypes carry steroid 21-hydroxylase deficiency due to absence of the *CYP21* gene

Polymerase chain reaction and oligonucleotide hybridisation

The *Pvu*II and *Eco*RI restriction patterns for section 1 matched the genomic *Taq*I/*Pvu*II and *Taq*I/*Eco*RI results in each case. The specificity of the amplification of section 2 could then be deduced from the mutation analysis of section 1, which contains its forward primer sites. Similarly, section 2 contains the forward primer site of section 3. This strategy allowed assignment of each sequence variant to either *CYP21* or *CYP21P* in all cases.

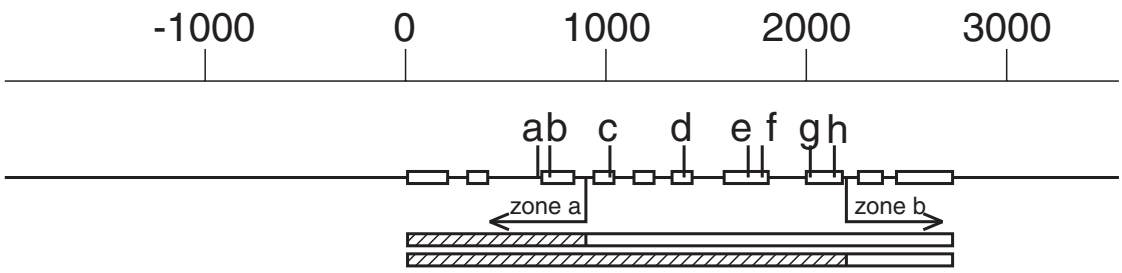

Fig. 2. *CYP21* intron-exon structure (open boxes are exons) and the mutations studied: a = intron 2 splice junction mutation (A/C/G) (nt 655); b = 8 bp deletion (present or absent) (nt 700); c = Ile172/Asn172 (nt 999); d = IleValGluMet236/AsnGluGluLys236/LysGluGluLys236 (nt 1380); e = Val281/Leu281 (nt 1683); f = 1 bp insertion between codons 306 and 307 (present or absent) (nt 1760); g = Gln318/stop318 (nt 1994); h = Arg356/Trp356 (nt 2108). The characteristics of the consensus *CYP21* gene at each mutation site are, respectively: A or C at the splice junction mutation; no 8 bp deletion; Ile172; IleValGluMet236; Val281; no 1 bp insertion; Gln318; Arg356. The characteristics of the consensus *CYP21P* gene are: G; 8 bp deletion; Asn172; AsnGluGluLys236; Leu281; 1 bp insertion; stop318; Trp356. The limits of the putative *CYP21P-CYP21* transition zones have been drawn at arbitrary places relative to Ile172/Asn172 (zone a, 5' limit) and Arg356/Trp356 (zone b, 3' limit), respectively, since no further distinction is possible because the sequences of *CYP21* and *CYP21P* are equal there. The arrows indicate that the actual transition site within these zones may vary for each hybrid. Bottom: hybrid genes with transition zone a and b, respectively; the hatched section is *CYP21P*-like.

Mutation detection by oligonucleotide hybridisation to slot blots or Southern blots usually produced straightforward results, with the exception of the (non-defective) 'C' variant at nucleotide 655 (the site of the splice junction mutation in intron 2; see fig. 2), which hybridised poorly and required careful washing steps. Similar weak signals with the 'C' variant were found by Rumsby *et al.* [1993]. The 'C' variant was readily detected in individuals homozygous for it, but the hybridisation signal was much weaker when the other chromosome carried either 'A' or 'G'. The (normal) 'A' and (defective) 'G' variants performed equally well in this analysis. In each parent carrying the 'G' allele on one chromosome, a non-defective ('A' or 'C') allele could also be detected, indicating that dropout of one allele in the PCR [Day *et al.* 1996] did not occur here. On the other hand, the amplification of both genes in a single reaction often produced a higher amount of *CYP21P* (containing 'G') than *CYP21*, especially when one or both of the *CYP21* genes carried a 'C' at nucleotide 655.

***CYP21* mutations**

In 89 % of the patients' *CYP21* genes, the defect could be attributed to one of the deleterious mutations investigated here (see table 3). In general, there was a good match between phenotype and genotype: a severe defect (deletion, large conversion, intron 2 splice mutation, 8 base pair deletion, AsnGluGluLys236, 1 base pair (T) insertion, stop318, Trp356 - mutation group A in the classification of Speiser *et al.* [1992]) was found on both chromosomes in 25 out of 29 families with the salt-losing variety of the disease. None of the SV patients had one of the more severe defects on both chromosomes. The *Eco*RI site in intron 2 was not found in

any *CYP21* gene. The *Pvu*II site in intron 2 was missing from one *CYP21* gene that also carried the splice junction mutation, suggestive of a small-scale conversion transferring both alterations.

Table 3. *CYP21* defects in Dutch patients with classical steroid 21-hydroxylase deficiency

defect	SL	SV
CYP21 deletion (5' *CYP21P* / 3' *CYP21* hybrid)	13	2
CYP21 large conversion (5' *CYP21P* / 3' *CYP21* hybrid and *CYP21P* gene)	8	1
splice junction mutation in intron 2[a]	21	1
8 bp deletion in exon 3	1	-
Ile172 → Asn	1	7
IleValGluMet236 → AsnGluGluLys	4	-
IleValGluMet236 → LysGluGluLys	-	-
Val281 → Leu	-	-
1 bp insertion in exon 7 (between codon 306 and 307)	-	-
Gln318 → stop	1	-
Arg356 → Trp	2	1
1 bp insertion in exon 7 and Gln318→stop	1	-
Val281 → Leu and Gln318 → stop and Arg356 → Trp	1	1
splice junction mutation and Gln318 → stop (2 *CYP21* genes on one allele)	1	-
no defect found	3[b]	5
total	57[c]	18

The numbers refer to the independent chromosomes carrying the steroid 21-hydroxylase deficiency allele (see text)

a: a G at position 655; of the remaining genes, 83 had an A and 18 a C (equally distributed between SL and SV patients)

b: in one of these genes, the milder defect Pro30 → Leu was detected in a separate experiment (results not shown)

c: one fewer than expected for 29 families due to known consanguinity (see text)

One patient had both the 1 bp insertion in exon 7 and stop318 in exon 8, which have probably been transferred from *CYP21P* in a single event during evolution. Two patients had Val281→Leu, the 1 bp insertion (codon 306-307), Gln318→stop and Arg356→Trp in the *CYP21* gene, suggesting that a single conversion event has occurred in the past to introduce these defects into *CYP21*.

One patient had a defective allele with two *CYP21* genes. This haplotype carried the intron 2 splice junction mutation and Gln318→stop, and is probably the same as a haplotype described in the Swedish population [Wedell *et al.* 1994].

***CYP21P* mutations**

CYP21P mutation analysis was complicated by the lack of clinical association and by the relatively frequent occurrence of chromosomes with more than one *CYP21P* gene. Only mutations that could be assigned to a specific haplotype with certainty were included. The distribution of *CYP21P* mutations in the most common haplotypes is shown in table 4.

Table 4. Distribution of *CYP21P* variability per haplotype

mutation	*CYP21-CYP21P* (normal) families[a]	*CYP21-CYP21P* (normal) controls[b]	*CYP21P* alone (*CYP21* del.) patients[c]	*CYP21P-CYP21P* (large conv.) patients[d]	*CYP21-CYP21P-CYP21P* (*CYP21P* dupl.) families[e]
	(n=96)	(n=46)	(n=15)	(n=18)	(n=10)
presence of *Eco*RI site in intron 2	100	100	100	100	100
absence of *Eco*RI site in intron 2	0	0	0	0	0
absence of *Pvu*II site in intron 2	100	100	100	100	100
presence of *Pvu*II site in intron 2	0	0	0	0	0
splice junction in intron 2, 'G'	100	100	100	100	100
splice junction in intron 2, 'A'	0	0	0	0	0
splice junction in intron 2, 'C'	0	0	0	0	0
presence of 8 bp deletion in exon 3	100	100	100	100	100
absence of 8 bp deletion in exon 3	0	0	0	0	0
Asn172	98	100	67	82	100
Ile172	2	0	33	18	0
AsnGluGluLys236	86	81	33	82	100
LysGluGluLys236	13	19	33	18	0
IleValGluMet236	1	0	33	0	0
Leu281	72	79	25	64	87
Val281	28	21	75	36	13
presence of 1 bp insertion in exon 7	100	100	73	82	100
absence of 1 bp insertion in exon 7	0	0	27	18	0
stop318	89	88	27	31	70
Gln318	11	12	73	69	30
Trp356	60	52	21	67	50
Arg356	40	48	79	33	50

The mutations are grouped per site; the variant matching the consensus sequence is mentioned first. The frequency of each mutation per haplotype is shown in percentages. The numbers in each column heading refer to the *CYP21P* genes, not to the chromosomes: for the haplotypes *CYP21-CYP21P-CYP21P* and *CYP21P-CYP21P*, the number of chromosomes is half the number of *CYP21P* genes. Mutations were only counted when they could be assigned to a haplotype with certainty.

a: steroid 21-hydroxylase deficiency families; numbers for chromosomes carrying and not carrying steroid 21-hydroxylase deficiency were added; 'standard' *CYP21-CYP21P* haplotypes; n=90 for Val/Leu281; n=93 for Gln/stop318 and for Arg/Trp356

b: controls with *CYP21-CYP21P* on one chromosome and a *CYP21P* deletion on the other; n=46 for mutations upstream of and including the 8 bp deletion, n=42 for the remaining mutations

c: steroid 21-hydroxylase deficiency patients; hybrid genes due to a 30 kb deletion; n=12 for Val/Leu281, n=14 for Arg/Trp356

d: patients; hybrid genes due to a large-scale gene conversion (two *CYP21P* genes were counted per chromosome); n=14 for Val/Leu281, n=16 for Gln/stop318, n=12 for Arg/Trp356

e: families; numbers for chromosomes carrying and not carrying steroid 21-hydroxylase deficiency were added; *CYP21P* duplication (two *CYP21P* genes were counted per chromosome); n=8 for Val/Leu281 and for Arg/Trp356

Table 5. Combined mutations on chromosomes carrying *CYP21P* alone ('deletion') and *CYP21P-CYP21P* ('large-scale conversion')

family	haplotype	172	236[a]	281	306-307[b]	318	356
	consensus *CYP21*	Ile	Ile	Val	no ins	Gln	Arg
	consensus *CYP21P*	Asn	Asn	Leu	ins	stop	Trp
1	*CYP21P*	Asn	Asn	Leu	ins	stop	Arg
2	*CYP21P*	Asn	Asn	...	ins	Gln	...
5	*CYP21P*	Asn	Lys	Leu	ins	Gln	Arg
6	*CYP21P*	Asn	Lys	Val	ins	Gln	Trp
7	*CYP21P*	Asn	Lys	Val	ins	Gln	Trp
8	*CYP21P*	Ile	Ile	Val	no ins	Gln	Arg
9	*CYP21P*	Ile	Ile	Val	no ins	Gln	Arg
13	*CYP21P*	Asn	Lys	Val	ins	Gln	Arg
16	*CYP21P*	Asn	Asn	...	ins	Gln	Trp
17	*CYP21P*	Ile	Ile	Val	no ins	Gln	Arg
23	*CYP21P*	Ile	Ile	Leu	ins	stop	Arg
26	*CYP21P*	Asn	Asn	Val	ins	stop	Arg
30	*CYP21P*	Asn	Asn	Val	ins	stop	Arg
31	*CYP21P*	Asn	Asn	...	ins	stop	Arg
32	*CYP21P*	Asn	Lys	Val	ins	Gln	Arg
3	*CYP21P-CYP21P*	Asn+Ile	Asn+Ile	Leu+Val	ins+no ins	stop+Gln	Trp+Arg
14	*CYP21P-CYP21P*	Ile+...	Ile+...	Val+...	no ins+...	Gln+...	Arg+...
14	*CYP21P-CYP21P*	Asn+Asn	Asn+Asn	Leu+Leu	ins+ins	...+...	Trp+Trp
15	*CYP21P-CYP21P*	Asn+Asn	Asn+Asn	Leu+Leu	ins+ins	stop+stop	Trp+Trp
16	*CYP21P-CYP21P*	Asn+Asn	Asn+Asn	...+...	ins+ins	Gln+Gln	Trp+Trp
18	*CYP21P-CYP21P*	Asn+Asn	Asn+Asn	Val+...	ins+ins	Gln+...	...+...
19	*CYP21P-CYP21P*	Asn+Asn	Asn+Asn	Leu+Val	ins+ins	Gln+Gln	Trp+Trp
28	*CYP21P-CYP21P*	Asn+Ile	Asn+Ile	Leu+Val	ins+no ins	stop+Gln	Trp+Arg
38	*CYP21P-CYP21P*	Asn+Asn	Asn+Asn	Leu+Leu	ins+ins	Gln+...	Arg+Arg

Consensus sequences (top) are according to Higashi *et al.* [1986]. The sequence upstream of codon 172 matched the consensus *CYP21P* sequence in all cases (*Taq*I site in 5' flank at nucleotide -211; *Eco*RI site in intron 2; no *Pvu*II site in intron 2; 'G' at nucleotide 655 (splice junction mutation site); 8 bp deletion in exon 3); ...: both alternatives mentioned are possible; not counted in table 4. Middle section: *CYP21* deletion, bottom: *CYP21* to *CYP21P* conversion.

a: mutation cluster in exon 6; Asn: AsnGluGlyLys; Ile: IleValGluMet; Lys: LysGluGluLys; ...: AsnGluGluLys or IleValGluMet

b: 306-307: location of the 1 bp insert (T) in exon 7

On 36 chromosomes where the *CYP21* defect was found, at least one *CYP21P* gene was present. On 32 of these chromosomes, there was a match between the *CYP21* defect and the *CYP21P* sequence at the corresponding site - in these cases, *CYP21P* matched the consensus sequence [Higashi *et al.* 1986]. Four *CYP21* genes had a defect not found in the accompanying *CYP21P* gene. These were: Leu281 and stop318 and Trp356 on *CYP21 vs* Val281 on *CYP21P*; Leu281 and stop318 and Trp356 on *CYP21 vs* Arg356 on *CYP21P*; Trp356 on *CYP21 vs* LysGluGluLys236 and Val281 and Arg356 on *CYP21P*; and Trp356 on *CYP21 vs* Val281 and Arg356 on *CYP21P*. Seven chromosomes carried a defective *CYP21* gene but no *CYP21P* gene. The *CYP21* defects were: all four AsnGluGluLys236 mutations; two of the intron 2 splice junction mutations; and one stop318 mutation.

The variation in the central and 3' sections of 'normal' *CYP21P* genes (*ie*, *CYP21P* genes on the *CYP21*-*CYP21P* haplotype) was considerable. The most frequent deviations from the consensus *CYP21P* sequences were LysGluGluLys236, Val281, Gln318 and Arg356. On the other hand, the T insert between codons 306 and 307 in exon 7 was present in all pseudogenes on the *CYP21*-*CYP21P* haplotype.

Many *CYP21P* genes carry more than one mutation as compared with the consensus sequences [Higashi *et al.* 1986; White *et al.* 1986]. The following combinations were found in the control group of individuals with a single *CYP21P* gene, where they could be unambiguously linked: AsnGluGluLys236-Leu281-stop318-Trp356 (20x); AsnGluGluLys236-Leu281-stop318-Arg356 (8x); LysGluGluLys236-Val281-stop318-Arg356 (8x); AsnGluGluLys236-Leu281-Gln318-Arg356 (4x); AsnGluGluLys236-Leu281-Gln318-Trp356 (1x); AsnGluGluLys236-Val281-stop318-Arg356 (1x).

Additional combinations were found in the family studies: of the 16 possible combinations of AsnGluGluLys/LysGluGluLys236, Leu/Val281, stop/Gln318 and Trp/Arg356, only LysGluGluLys-Leu-Gln-Trp, LysGluGluLys-Val-Gln-Trp, LysGluGluLys-Leu-Gln-Arg and LysGluGluLys-Leu-stop-Arg were not found. One *CYP21P* gene carried both Ile172 and Arg356; another carried both Ile172 and IleValGluMet236. There were no statistically significant differences in the frequencies of the *CYP21P* mutations between 'standard' (*CYP21*-*CYP21P*) chromosomes with functional or non-functional *CYP21* genes, or between 21-hydroxylase deficiency families and controls.

Table 5 shows all combined mutations on haplotypes carrying a single *CYP21P*-like gene (*CYP21* 'deletions') or two *CYP21P*-like genes ('large-scale' *CYP21* conversions). All these genes are *CYP21P*-like from their 5' flank to, at least, the 8 bp deletion in exon 3, and therefore defective.

De novo *CYP21P* mutation in one family

In a family with two patients each having two *CYP21P* genes, one patient carried IleValGluMet236 on one chromosome and AsnGluGluLys236 on the other, whereas all other family members only had AsnGluGluLys236 on their *CYP21P* genes. Paternity was confirmed by testing independent genetic markers (results not shown). All other investigated mutations and the *CYP21*/*C4* haplotypes were inherited in a normal Mendelian fashion in this

family. A *de novo* conversion event locally changing *CYP21P* to a *CYP21*-like sequence is the most obvious explanation. All *CYP21* genes in this family carried IleValGluMet236.

Discussion

Mutations in the *CYP21* gene

The *CYP21* mutations causing salt-losing 21-hydroxylase deficiency occurred at frequencies similar to those observed in other populations, the intron 2 splice mutation (a 'G' at nucleotide 655) being the most common. The frequency distribution is different for simple virilising 21-hydroxylase deficiency, where Ile172→Asn is clearly more abundant. This finding is in agreement with some studies [Helmberg *et al.* 1992; Speiser *et al.* 1992; Carrera *et al.* 1996; Dardis *et al.* 1997]; others found the intron 2 splice mutation more often in simple virilising patients as well [Higashi *et al.* 1991; Mornet *et al.* 1991; Ordoñez-Sánchez *et al.* 1998; Paulino *et al.* 1999]. The chance that a randomly selected Dutch individual who is a carrier of salt-losing steroid 21-hydroxylase deficiency can be detected by analysis of the mutations described here, is about 95 % (table 3). This degree of certainty is unlikely to become much higher by checking additional mutations because *de novo* events are relatively frequent (in our population, twice in 40 families). In all patients described here, a genetic defect was found on at least one of both *CYP21* alleles. Non-detection of both mutations in a single patient is unlikely, so it may indicate clinical misdiagnosis of steroid 21-hydroxylase deficiency.

Variability and evolution of *CYP21P*

CYP21P is constant in its 5' section up to and including the 8 bp deletion in exon 3, but shows considerable variation in its central and 3' parts even on 'standard' chromosomes carrying one copy of *CYP21* and *CYP21P* each. The evolutionary origin of *CYP21P* variability is uncertain. The concept of 'back conversions' has been proposed to introduce *CYP21*-like sequences into *CYP21P* [Day *et al.* 1995]. A *de novo* mutation introducing IleValGluMet236 into *CYP21P* (not previously described in the literature) was observed in one 21-hydroxylase deficient patient, so 'back conversions' evidently play a role in maintaining *CYP21P* variability.

It is assumed that the duplicated arrangement of the *CYP21-C4-TNX* gene cluster has resulted from unequal crossover events that occurred independently in primates and other mammals during evolution [Gitelman *et al.* 1992; Kawaguchi *et al.* 1992; Miller *et al.* 1992; Horiuchi *et al.* 1993]. Therefore, *CYP21*-like sequences in the pseudogene may also be a leftover predating such a duplication. Comparison of primate *CYP21P* genes [Kawaguchi *et al.* 1992] suggests that after the duplication, deleterious mutations gradually accumulated in the gene now known as *CYP21P*. The splice junction mutation in intron 2 (found in the gorilla) was first introduced into the pseudogene, the 8 bp deletion in exon 3 (found in the chimpanzee) came next, and other mutations (only found in humans) followed later. The T insertion in exon 7 seems to be the next in line, because it is found in all *CYP21P* genes on normal (*CYP21-CYP21P*) chromosomes. Two previous studies [Helmberg *et al.* 1992; Wedell and Luthman 1993] found no variation at this site either on the *CYP21-CYP21P* haplotype. The

evolution of *CYP21P* may be further clarified by determining whether the T insert is ubiquitous in other human (especially, non-Western) populations as well.

CYP21P variability may influence the outcome of small-scale gene conversions, because the physical conversion zone may extend beyond the region of similarity between a mutated gene and the *CYP21P* consensus sequence. In addition, *CYP21P* variability may contribute to the creation of 'mosaic' genes with multiple apparent *CYP21*/*CYP21P* transitions.

Mechanisms of small-scale gene conversions

By analogy to fungal genetics, the term gene conversion is used in higher eukaryotes to describe a non-reciprocal transfer of genetic information. The presence of *CYP21P*-like defects in *CYP21* is usually attributed to a gene-conversion-like mechanism, although donor and recipient can only be studied directly in case of a *de novo* event. Collier *et al.* [1993] described a *de novo* Ile172→Asn conversion from *CYP21P* to *CYP21*; all *CYP21P* genes involved carried Asn172. The opposite, a *de novo* conversion from *CYP21* to *CYP21P* described in this report, was also non-reciprocal: AsnGluGluLys236 was changed to IleValGluMet, while all *CYP21* genes retained IleValGluMet. The necessary interaction between *CYP21P* and *CYP21* may occur either between sister chromatids or between homologous chromosomes, but the distinction could not be made in the two *de novo* conversions mentioned above, because all potential donor sequences were identical. Since small-scale gene conversions are primarily mitotic processes [Tusié-Luna and White 1995], sister chromatid interaction might be expected to provide the donor sequence. Four of the chromosomes studied here carried a *CYP21* defect not found in the *CYP21P* gene on the same chromosome. In these cases, *CYP21P* matched the consensus non-mutated *CYP21* sequence at the sites involved, and cannot have provided the donor sequence for the *CYP21* conversion. This implies that at least some of the small-scale gene conversions involve interactions between homologous chromosomes rather than between identical sister chromatids.

***CYP21P-CYP21* transition zones in hybrid genes**

Absence of the *CYP21* gene (as judged by *Taq*I/*Bgl*II restriction analysis) is one of the major causes of steroid 21-hydroxylase deficiency. Allowing for rare exceptions [Collier *et al.* 1989], there are two main categories of chromosomes without a *CYP21* gene: those with one and those with two *CYP21P* genes. Chromosomes with one *CYP21P* gene (*CYP21* deletion; table 5, middle) carry a single hybrid gene that has a *CYP21P*-like 5' section and a *CYP21*-like 3' section. Differences between the *CYP21* and *CYP21P* consensus sequences (table 5, top) have frequently been used to define a putative *CYP21P-CYP21* transition zone, and thus to determine the breakpoint of the unequal crossover that created these hybrids during the course of evolution. However, several of such landmarks (Val281, Gln318, Arg356, and to some degree even Ile172 and IleValGluMet236) are not necessarily specific for *CYP21* on normal chromosomes, but appear in *CYP21P* as well (table 4). This causes ambiguity in locating the 5' limit of *CYP21P-CYP21* transition areas (unless a *de novo* mutation is available for analysis).

Two crossover regions have been proposed for hybrid genes: (a) between the 8 bp deletion in exon 3 and the Ile172→Asn site in exon 4, and (b) between the end of exon 7 and the

Gln318→stop site in exon 8 [Donohoue *et al.* 1989; Chu *et al.* 1992; Levo and Partanen 1997]. In our patient group, the breakpoint lies in zone (a) in the families 8, 9 and 17 (see table 5). Zone (a) extends further upstream, because it has been shown that *de novo* recombinations can also occur upstream of the 8 bp deletion in exon 3 [Tusié-Luna and White 1995]. In the other twelve families with the 'deletion' haplotype, the breakpoint cannot lie immediately downstream of exon 7, because several of the hybrid genes carry either stop-318 or Trp-356 instead of the expected *CYP21*-like sequence. We therefore hypothesise that the second putative conversion zone (b) lies downstream of the Arg356→Trp site (see fig. 2), and that the differences between these haplotypes can be explained by variation between the *CYP21P* genes involved in the crossover. This model also explains the variability at the other sites listed in table 5, and the differences at the Val281→Leu and Gln318→stop sites previously found in the analysis of several HLA-B47 haplotypes [Chu *et al.* 1992; Levo and Partanen 1997]. However, the alternative of later introduction of these mutations in a pre-existing HLA-B47 associated hybrid gene cannot be excluded. A reliable marker for distinguishing between conversion zone (a) and conversion zone (b) in hybrid genes, is the absence (zone a) or presence (zone b) of the T insert in exon 7.

Chromosomes with two *CYP21P* genes ('large-scale conversions'; table 5, bottom) carry one regular *CYP21P* gene and one *CYP21P-CYP21* hybrid. The transition zones in the hybrid gene are the same as for the 'single' hybrid genes described above: zone (a) in the families 3, 14 (one of the two chromosomes) and 28, and zone (b) in the other cases.

Tusié-Luna and White [1995] have found that *de novo* unequal crossovers leading to *CYP21* deletions occur during meiosis only. However, this also applies to apparent 'large-scale conversions', because the hybrid genes found in *CYP21* deletions and in large-scale conversions are so similar (see table 5) that the PCR amplification used in that study doesn't distinguish between them.

Since *CYP21* 'deletions' and 'large-scale' conversions are both meiotic and result in a very similar layout of the *CYP21P-CYP21* hybrid gene, they are probably created by similar processes. We therefore hypothesise that large-scale gene conversions of *CYP21* to a *CYP21-CYP21P* hybrid are actually meiotic double unequal crossovers. One putative crossover site lies within region (a) or (b) as shown in fig. 2, the other one lies upstream of the *Taq*I restriction site at nucleotide -211 in the 5' flank. Interestingly, the other product expected from such an event, a chromosome with two *CYP21*-like genes and no *CYP21P*-like genes, was found in three healthy controls. Contrary to the single unequal crossovers that lead to deletions and duplications of *CYP21* or *CYP21P* [Carroll *et al.* 1985; White *et al.* 1985; Donohoue *et al.* 1989; Miller *et al.* 1992; White 1994], the proposed double unequal crossover does not alter the number of *C4-CYP21-TNX* units or the overall size of the region.

Conclusions

Our mutation analysis detects 95 % of all defects causing salt-losing steroid 21-hydroxylase deficiency in the Netherlands and hence provides a guideline for the diagnosis of the most severe form of this disease in the Dutch population.

CYP21P shows a high variability, and non-consensus *CYP21P* genes are widespread in the general population. This necessitates a revised definition of *CYP21P*-*CYP21* transition zones in gene conversions and unequal crossovers generating *CYP21* defects.

At least some of the small-scale gene conversions between *CYP21* and *CYP21P* involve interaction between homologous chromosomes. Large-scale gene conversions appear to be the result of a completely different genetic mechanism: these rearrangements are probably generated by a meiotic double unequal crossover.

Future research on the genetic mechanisms underlying the variability in the *CYP21* / complement *C4* region should take account of the high variability of the *CYP21P* pseudogene. This notably applies to the study of *de novo* mutations, where information on donor and recipient DNA can be obtained more easily than in population studies.

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3.2. Distribution of *CYP21* mutations in a Dutch population

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Introduction

Steroid 21-hydroxylase deficiency is an inherited metabolic disease characterised by partial or complete impairment of the adrenocortical conversion of progesterone to 11-deoxycorticosterone and 17α-hydroxyprogesterone to 11-deoxycortisol. These reactions are necessary intermediate steps in the biosynthesis of aldosterone and cortisol, respectively. Steroid 21-hydroxylase deficiency is the cause of more than 90 % of all cases of congenital adrenal hyperplasia and has an incidence of approximately 1 in 10,000 to 1 in 15,000 in most populations [New *et al.* 1989]. The severity of the disease varies and the spectrum of its clinical manifestations is manifold [New *et al.* 1989; Pang 1997; White and Speiser 2000]. In classical 21-hydroxylase deficiency, androgen excess causes pre- and postnatal virilisation in untreated patients and about three-quarters of all patients are to some degree unable to synthesise aldosterone, a defect that may result in a life-threatening salt-losing crisis in the first weeks of life. Nonclassical (NC) steroid 21-hydroxylase deficiency is an attenuated form, often diagnosed at a later age ('late onset'). Patients typically present with manifestations of mild androgen excess, such as accelerated growth or precocious puberty in children and hirsutism, acne, or infertility in adults [New *et al.* 1989; Pang 1997; Morán *et al.* 1998; White and Speiser 2000]. A high frequency of NC 21-hydroxylase deficiency alleles has been observed in some populations: for example, 0.223 in Ashkenazi Jews [Sherman *et al.* 1988] and 0.092 in Croatians [Dumic *et al.* 1990].

Steroid 21-hydroxylase is encoded by the *CYP21* gene in the central region of the human Major Histocompatibility Complex on the short arm of chromosome 6 (6p21.3). Over the past decade, many *CYP21* defects have been characterised at a molecular level (recently reviewed by [White and Speiser 2000; Lee 2001]). We previously investigated the most common *CYP21* defects in a group of Dutch patients with classical steroid 21-hydroxylase deficiency [Koppens *et al.* 2000]. Here, we report on an exhaustive search of NC mutations in the same group, which was expanded with three families with NC 21-hydroxylase deficiency. To assess the frequency of 21-hydroxylase deficiency alleles in the general population in the Netherlands, we also scanned for these mutations and those previously reported [Koppens *et al.* 2000], in a group of healthy controls.

Non-amplification of specific alleles in PCR-based mutation analysis of *CYP21* ('allele dropout') has been reported by several authors [Day *et al.* 1996; Ozturk *et al.* 2000]. This notably affects the analysis of the 'intron 2 splice mutation', the most common genetic defect in classical steroid 21-hydroxylase deficiency. Instead of the normal 'A' or 'C' at nucleotide 655, this defective allele has a 'G', creating a spurious splice acceptor site [Higashi *et al.* 1988]. To check for non-amplification, we determined the number of heterozygotes and homozygotes for this mutation and for a neutral polymorphism in the same PCR fragment.

Methods

Nearly all steroid 21-hydroxylase deficiency patients, their family members, and the controls, had participated in our earlier haplotyping [Koppens *et al.* 1992] and mutation analysis [Koppens *et al.* 2000] studies (new data on three patients with NC steroid 21-hydroxylase deficiency has not been published before). A small number of persons with more than two *CYP21* genes as found by Southern blotting analysis [Koppens *et al.* 1992] were not included. We adapted our earlier PCR strategy [Koppens *et al.* 2000] to include the sites of several additional mutations. The entire *CYP21* gene was specifically amplified in two separate reactions: a section from the 5' flank to exon 3 using the forward primer CCAGGCAAACCAGGCTTAAAC and the reverse primer AGGGAGTAGTCTCCCAAGGA (basepair -496 to 719 [Higashi *et al.* 1986]) and a section from exon 3 to the 3' flank using the

Notes to table 1.

a: 141 persons were included, but the totals for each mutation add up to 281 chromosomes, because one control carried a heterozygous *CYP21* deletion

b: referred to as 'non-carrier' because these were the chromosomes not inherited by the index case in each family, although some of these chromosomes were now found to carry a *CYP21* gene with a NC mutation (see text)

c: chromosomes of all patients with classical steroid 21-hydroxylase deficiency with a *CYP21* gene present

d: chromosomes of three patients with NC steroid 21-hydroxylase deficiency, each having Val281→Leu on one chromosome and a more severe defect on the other

e: these conditions produce a good signal-to-background ratio, but the exposure times needed vary from one hour to overnight

f: not including *CYP21* genes that also carry other, more severe defects

g: represents one *CYP21* deletion and one *CYP21* gene carrying the contiguous mutations Ile172→Asn, IleValGluMet236→AsnGluGluLys and Val281→Leu

h: represents all previously reported defects (including deletions) and a few new cases, but excludes *CYP21* genes where no defect has been found yet

Table 1. Nonclassical steroid 21-hydroxylase deficiency mutations and neutral polymorphisms in *CYP21* genes in Dutch steroid 21-hydroxylase deficiency patients, family members and in the general population

mutation or polymorphism	general population[a]	families, non-carrier[b]	patients, classical[c]	patients, nonclassical[d]	allele-specific oligonucleotide probe	final wash step[e]
	(n=282)	(n=88)	(n=56)	(n=6)		
insertion Leu10 (normal)	183	53	29	4	GCCTGCTGCTGCTGCTGCCC	0.2x SSC, 52°C
no insertion Leu 10 (normal)	98	35	27	2	GCCTGCTGCTGCTGCCCCTG	0.1x SSC, 52 °C
Pro30 (normal)	281	88	55	6	TCCACCTCCCGCCTCTTGC	0.1 x SSC, 48 °C
Leu30 (NC 21-OH deficiency)	0	0	1	0	TCCACCTCCTGCCTCTTGC	0.1 x SSC, 48 °C
nucleotide 655 A (normal)	166	52	20	5	CAGGAGGAGTTGGGGGCTG	0.3 x SSC, 48 °C
nucleotide 655 C (normal)	115	36	12	0	CAGGAGGAGGTGGGGGCTG	0.2 x SSC, 48 °C
nucleotide 655 G (classical 21-OH deficiency)	0	0	24	1	CAGGAGGAGCTGGGGGCTG	0.3 x SSC, 48 °C
Pro105 (normal, CCG)	273	84	55	6	GACAGGTCCGGGTAGTTC	0.2 x SSC, 42 °C
Pro105 (normal variant, CCC)	8	4	1	0	GACAGGTCGGGGTAGTTC	0.1 x SSC, 42 °C
Leu105 (NC 21-OH deficiency)	0	0	0	0	GACAGGTCCAGGTAGTTC	0.2 x SSC, 42 °C
Glu196 (normal)	281	88	56	6	AAATGTATCCAGGAGGTGTTAAAA	1 x SSC, 48 °C
deletion 196 (NC 21-OH deficiency)	0	0	0	0	AAATGTATCCAGGTGTTAAAAACC	1 x SSC, 48 °C
Val281 (normal)	279	86	56	3	CCATGTGCACGTGCCCTTC	0.3 x SSC, 48 °C
Leu281 (NC 21-OH deficiency)[f]	2	2	0	3	CCATGTGCAAGTGCCCTTC	0.2 x SSC, 48 °C
Arg339 (normal)	281	88	56	6	GTCCCCTACAAGGACCGTGCAC	1 x SSC, 50 °C
His339 (NC 21-OH deficiency)	0	0	0	0	GTCCCCTACAAGGACCATGCAC	1 x SSC, 50 °C
Glu380 (normal)	281	88	56	6	CATCCCTGAGGGCACAGTC	1 x SSC, 48 °C
Asp380 (NC 21-OH deficiency)	0	0	0	0	CATCCCTGACGGCACAGTC	1 x SSC, 48 °C
Pro453 (normal)	279	86	56	6	CCCGGAGGGCAGCAGCGT	0.3 x SSC, 54 °C
Ser453 (NC 21-OH deficiency)	2	2	0	0	CCCGGAGGACAGCAGCGT	0.3 x SSC, 54 °C
other mutations typical of classical 21-OH def.	2[g]	0	25[h]	2	see Koppens *et al.* [2000]	

The numbers refer to chromosomes and, at the same time, to *CYP21* genes.

forward primer ACCTGTCCTTGGGAGACTACTC and the reverse primer AAGAGCCAGGGTCCTTCACCA (basepair 695 to 2857). A touchdown PCR protocol was used: for the 5' section: first, 8 cycles of 30 s at 94°C, 60 s at 60 °C decreasing 0.5 °C per cycle and 3 min at 72 °C; next, 28 cycles of 30 s at 94°C, 60 s at 56 °C and 3 min at 72 °C extending 20 s per cycle; and for the 3 min section: first, 10 cycles of 30 s at 94°C, 60 s at 63 °C decreasing 0.5 °C per cycle and 5 min at 72 °C; next, 30 cycles of 30 s at 94°C, 60 s at 58 °C and 5 min at 72 °C. Amplification was done with 0.5 units of Thermoperfect DNA polymerase (Integro, Leuvenheim, The Netherlands) in the presence of 1.5 mM $MgCl_2$ and, for the 3 min section, 1 % formamide. The reaction was checked by electrophoresis of 20 % of the product on agarose gels; the remainder of the product was slot-blotted onto Hybond-N+ (Amersham Pharmacia, Little Chalfont, UK) and mutations were detected by hybridisation to ^{32}P-labelled allele-specific oligonucleotide probes, in the presence of a 50-fold excess of the unlabeled complementary probe. Oligonucleotides for NC mutations are listed in table 1, the others are the same as in our earlier study [Koppens *et al.* 2000]. The DYEnamic ET terminator cycle sequencing kit (Amersham Pharmacia, Little Chalfont, UK) was used in the detection of a novel polymorphism at Pro105.
Codon and nucleotide numbering is according to Higashi *et al.* [1986].

Results and discussion

The results of the mutation analysis are presented in table 1. Mutation detection with the allele-specific oligonucleotides usually produced straightforward results; notably, the distinction between the normal 'A' and 'C' variants at nucleotide 655 was much clearer than in our earlier study [Koppens *et al.* 2000]. All classical mutations detected earlier, including the splice junction mutations ('G' at nucleotide 655), were confirmed using the current protocol. In a few cases, hybridisation with the oligonucleotides detecting Pro105 and Leu105 both failed; sequencing analysis (details not shown) revealed a novel neutral polymorphism, with Pro105 encoded by CCC instead of the usual CCG.
To check for allele dropout in the PCR, the distribution of two neutral polymorphisms in the 5' section was established in persons with two *CYP21* genes (see table 2). The genotype frequencies found in the control group are in agreement with the Hardy-Weinberg equilibrium. The same applies to the distribution in a group of parents of the patients, taking into account a selection bias against homozygous defects. We conclude that non-amplification of specific alleles is not a problem in our studies.
The neutral polymorphisms (insertion/deletion of Leu10 and A or C at nucleotide 655) occurred at frequencies similar to those reported elsewhere [Lobato *et al.* 1999; Ozturk *et al.* 2000]. The high variability at these sites makes them attractive markers for tracking the segregation of individual alleles in 21-hydroxylase deficiency families.
The three patients with NC steroid 21-hydroxylase each carried Val281→Leu on one chromosome and a classical mutation (the intron 2 splice mutation, once; and Ile172→Asn, twice) on the other. All Val281→Leu mutations identified in the families studied were

associated with a duplication of the *CYP21P* pseudogene and one 'long' (32 kb) plus two 'short' (26 kb) *C4* genes on the same chromosome. Both controls with the Leu281 mutation also carried this duplication and so did two parents of 21-hydroxylase patients where Leu281 was detected on the chromosome not transmitted to the patient. Other characteristics of this haplotype were the presence of Leu10, 'A' at nucleotide 655 and the absence of any of the other defects investigated here or in our earlier study [Koppens *et al.* 2000].

The two parents with Ser453 on their 'non-carrier' chromosome (see table 1) had this mutation on a chromosome with one *CYP21* gene, one *CYP21P* pseudogene and one long plus one short *C4* gene. Without the instrument of family studies, the same cannot be said with certainty for the controls carrying Ser453, but the band intensities found there [Koppens *et al.* 1992] were in agreement with the configuration mentioned. Other characteristics of this haplotype were the presence of Leu10, 'C' at nucleotide 655 and the absence of any other defect. Curiously, these characteristics differ from those found on a Ser453-carrying chromosome found in Sweden [Wedell *et al.* 1992], which had a *CYP21* deletion and carried the additional mutation Pro105→Leu. Another *CYP21* gene with Ser453 carried the additional mutation Arg339→His [Helmberg *et al.* 1992]. These differences suggest that Pro453→Ser is evolutionary older than Pro105→Leu and Arg339→His, and may provide an explanation for its higher frequency. The other mutations we checked were not found in the control group. With the exception of Pro30→Leu and, to some extent, Arg339→His, these defects appear to be quite rare.

In conclusion, we found four NC 21-hydroxylase deficiency alleles in 370 chromosomes tested (141 controls and the non-carrier chromosomes in 44 families). Thus, the total frequency of NC steroid 21-hydroxylase deficiency alleles in the general population in the Netherlands is 0.011 (95 % confidence limits: 0.004 to 0.024) [Linder 1960].

Table 2. Genotype distribution of two neutral polymorphisms (insertion of Leu 10 and A or C at nucleotide 655) in the 5' part of the *CYP21* genes

genotype	general population (141 persons)	parents of patients (88 persons)
Leu10 / Leu10	60	17
Leu10 / no Leu10	62	32
Leu10 / deletion	1	17
no Leu10 / no Leu10	18	7
no Leu10 / deletion	0	15
nucleotide 655 A / A	46	16
nucleotide 655 A / C	73	10
nucleotide 655 A / G	0	13
nucleotide 655 A / deletion	1	20
nucleotide 655 C / C	21	7
nucleotide 655 C / G	0	10
nucleotide 655 C / deletion	0	12
nucleotide 655 G / G	0	0
nucleotide 655 G / deletion	0	0
deletion / deletion	0	0

The allele frequencies in the general population are: Leu10, 0.649; no Leu10, 0.348; deletion, 0.004; and 655A, 0.589; 655C, 0.408; deletion, 0.004. In the parents of steroid 21-hydroxylase deficiency patients, the frequencies are: Leu10, 0.472; no Leu10, 0.347; deletion, 0.182; and 655A, 0.426; 655C, 0.261; 655G, 0.131; deletion, 0.182. The term 'deletion' also includes haplotypes previously termed 'large-scale gene conversion'.

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Chapter 4

A *de novo* unequal crossover generates a dual deficiency allele carrying no steroid 21-hydroxylase (*CYP21*) gene and a non-functional hybrid tenascin-X (*TNX*) gene: mapping of the recombination site between chromosomes each carrying two RCCX modules

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Abstract

The RCCX module is a tandemly repeated set of (parts of) four genes (*RP, C4, CYP21, TNX*) in the human Major Histocompatibility Complex on chromosome 6p21.3. Most chromosomes are bimodular (carrying two RCCX modules). Meiotic unequal crossover within the RCCX module potentially causes deletion of the *CYP21* gene, contributing to congenital adrenal hyperplasia (CAH) caused by steroid 21-hydroxylase deficiency. Misalignment of parental chromosomes that differ in RCCX modularity probably increases the likeliness of such unequal crossovers. We here describe a CAH patient with a *CYP21* gene deletion that was not present in his parents. Using a combination of long-range and short-range restriction analysis, we found that a *de novo* unequal crossover had occurred in the patient's father. The crossover site was mapped to a 640 bp region of the *TNX* gene, adjacent to *CYP21*. This type of crossover does not create the *CYP21P/CYP21* hybrid gene found in most CAH 'deletion' haplotypes. Instead, it eliminates *CYP21* in its entirety and also confers a defect to *TNX*, which has been implicated in the pathology of the Ehlers-Danlos syndrome. In contrast with earlier observations, this disease-causing unequal crossover occurred between chromosomes with equal numbers of RCCX modules.

Introduction

In the human genome, the Major Histocompatibility Complex class III region on chromosome 6p21.3 stands out as an area of remarkably high gene density [MHC Sequencing Consortium 1999; International Human Genome Sequencing Consortium 2001]. Within this region, a section of particular complexity centres around the *C4* genes, which encode the fourth component of complement [Belt *et al.* 1984; Carroll *et al.* 1985; Schneider *et al.* 1986; Yu 1991; Blanchong *et al.* 2001]. Centromeric to *C4* lies the *CYP21* gene, which encodes steroid 21-hydroxylase, a key enzyme in the biosynthesis of cortisol and aldosterone [White *et al.* 1985; Carroll *et al.* 1985; Higashi *et al.* 1986]. The *TNX* gene, which encodes the extracellular matrix protein tenascin-X, lies centromeric to *CYP21* and is transcribed from the opposite strand [Gitelman *et al.* 1992; Matsumoto *et al.* 1992; Bristow *et al.* 1993]. Telomeric to *C4* lies the *RP* gene, encoding a putative serine/threonine kinase [Sargent *et al.* 1994; Shen *et al.* 1994; Gomez-Escobar *et al.* 1998]. Some 70 % of all chromosomes 6 carry a duplication of an area comprising the entire *C4* and *CYP21* genes and small truncated sections of *RP* and *TNX*. This stretch of DNA is known as the RCCX module [Shen *et al.* 1994; Rupert *et al.* 1999; Yang *et al.* 1999; Blanchong *et al.* 2001], and most haplotypes have a characteristic bimodular arrangement similar to the one shown in fig. 1. The complex genetics of this region and the activities and clinical significance of the proteins encoded here have been the subject of several recent reviews [Wedell 1998; White and Speiser 2000; Blanchong *et al.* 2001].

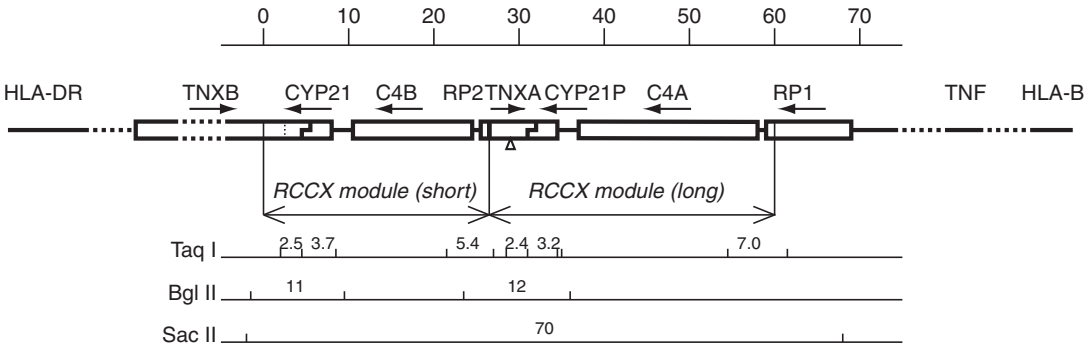

Fig. 1. Overview of a typical *C4*/*CYP21* area within the MHC class III region, showing two RCCX modules as found on most chromosomes. *TNXB* is the full-size 68 kb gene for tenascin-X, *TNXA* (also known as *XA*) is a truncated pseudogene of 5.7 kb that not only lacks most of the coding sequence of *TNXB* but also has a 120 bp deletion (indicated by the small triangle) spanning an exon-intron boundary [Gitelman *et al.* 1992; Bristow *et al.* 1993]. *CYP21* (also known as *CYP21B*) is the active steroid 21-hydroxylase gene; *CYP21P* (also known as *CYP21A*) is a full-size pseudogene containing several deleterious mutations throughout its sequence, including three in-phase stop codons [Higashi *et al.* 1986]. The *C4* genes express variants of the fourth component of complement with different affinities, known as *C4A* and *C4B*. About three quarters of the *C4* genes, including nearly all *C4A* genes, are 'long' (20.6 kb in size); the others are 'short' (14.2 kb). This difference is reflected in the size of the entire RCCX module, since the other components total approximately 12 kb. Assignment of *C4A* and *C4B* is tentative: the arrangement shown is the most common, but the specificity of the *C4* genes cannot be determined by means of the restriction sites shown. The arrows show the orientation of transcription; there is an overlap between the 3' sections of the oppositely transcribed genes *TNXB* and *CYP21*, and of *TNXA* and *CYP21P*, respectively. Several short transcripts encoded in the same region are not shown. Bottom: characteristic *Taq*I, *Bgl*II and *Sac*II restriction fragments. Only fragments mentioned in the text are shown; a *Sac*II site internal to the RCCX module is not cut in genomic DNA, possibly due to methylation. Top: scale in kb.

Each chromosome 6 has at least one RCCX module, most have two as described above, some have three, and in rare cases, as many as four contiguous RCCX modules have been found on a single chromosome. Since bimodularity is the standard, the literature often refers to haplotypes with one module as 'deletions' and to haplotypes with three modules as 'duplications', especially when focusing on the *C4* or *CYP21* genes. The overall layout of the RCCX region can be determined by short-range and long-range restriction mapping. *Taq*I/*Bgl*II restriction analysis of genomic DNA and comparison of the relative intensities of the bands obtained by hybridisation to *C4*, *CYP21* and *TNX* probes [White *et al.* 1985; Carroll *et al.* 1985; Harada *et al.* 1987; Higashi *et al.* 1988; Morel *et al.* 1989], has been used in many studies to establish haplotypes in families of CAH patients and controls. This approach is usually sufficient, but in complicated cases, large fragments from rare-cutters such as *Sac*II or *Bss*HII can be used to determine the size of the entire region and hence, the number of RCCX modules [Collier *et al.* 1989; Dunham *et al.* 1989].

The tandem-repeat structure of several lined-up RCCX modules promotes the chances of misalignment during meiosis. If a crossover then occurs, it effectively removes one of the RCCX modules. On a 'standard' bimodular chromosome, this process joins a part of the telomeric module to its homologous counterpart in the centromeric module (see fig. 6 in the Discussion for a typical example). The site of such a crossover determines whether or not the remaining monomodular chromosome carries a genetic disorder. An unequal crossover between *C4A* and *C4B* is relatively harmless, because both genes express a functional C4 protein and so does their fusion gene. Such monomodular haplotypes lacking one of the *C4* genes and the *CYP21P* gene occur at a frequency of 5 to 20 % in the general population [White *et al.* 1988; Collier *et al.* 1989; Koppens *et al.* 1992; Ezquieta *et al.* 1995].

An unequal crossover between *CYP21P* and *CYP21* on the other hand, usually generates a fusion gene that is *CYP21P*-like in its 5' section and contains several mutations rendering it inactive (a haplotype often referred to as a '*CYP21* deletion'). Absence of a functional *CYP21* gene is one of the defects that contributes to steroid 21-hydroxylase deficiency, the cause of over 90 % of all cases of congenital adrenal hyperplasia (CAH). CAH is a disorder of adrenocortical steroid biosynthesis which in severe cases causes life-threatening salt-losing crises in untreated paediatric patients [Wedell 1998; White and Speiser 2000].

An unequal crossover between *TNXA* and *TNXB* not only eliminates the *CYP21* gene, but may also result in a defective *TNXB*/*TNXA* pseudogene containing the 120 bp deletion normally found in *TNXA* only and unable to express the tenascin protein. This defect contributes to the Ehlers-Danlos syndrome, a disease of the connective tissue [Burch *et al.* 1997; Yang *et al.* 1999; Schalkwijk *et al.* 2001].

The mechanisms of these crossovers are difficult to understand, because usually only the recombinational end product is available for analysis. The concept of a deletion of the *CYP21P* pseudogene as a 'premutation' has been advanced in a report on a *de novo* deletion of *CYP21* by recombination between a 'standard' bimodular chromosome and a monomodular chromosome [Sinnott *et al.* 1990]. More recently published studies also provide evidence that *TNXB*/*TNXA* hybrids are the result of a crossover between a bimodular and a monomodular chromosome [Rupert *et al.* 1999; Yang *et al.* 1999].

We here present a *de novo* unequal crossover that occurred between two bimodular chromosomes in the father of a patient suffering from CAH caused by steroid 21-hydroxylase deficiency. The crossover site was mapped to a 640 bp region of the *TNX* gene that starts at approximately 1.6 kb from the centromeric boundary of the RCCX module. This *de novo* mutation eliminates the *CYP21* gene and also disrupts *TNXB* by replacing its 3' end by a homologous section from *TNXA*, conveying the 120 bp deletion.

Methods

Patient and family members

The patient, a boy, presented to the Sophia Children's Hospital at age 7 years 8 months with signs of precocious puberty. The patient was tall for his age (above the 90th percentile) and

bone age was strongly advanced (13½ years). Basal plasma 17α-hydroxyprogesterone was 99 nmol/l, testosterone was 4.3 nmol/l. Sodium and potassium levels were normal. The patient was diagnosed with simple virilising congenital adrenal hyperplasia caused by steroid 21-hydroxylase deficiency, and hydrocortisone replacement therapy, initially supplemented with cyproterone acetate, was installed. The family consists of both parents, the patient, two healthy brothers who are monozygotic twins and a healthy sister. They were informed about the purpose of the study and gave their consent.

***CYP21*/*C4* haplotyping**

The family participated in our haplotyping study [Koppens *et al.* 1992] as family 20, and *CYP21*/*C4* haplotypes were established as described there. Briefly, genomic DNA was digested with *Taq*I or with *Bgl*II, separated by electrophoresis on agarose gels, Southern-blotted onto nitrocellulose and hybridised to the *CYP21* cDNA probe pC21/3c [White *et al.* 1985] and the 5' section of the *C4* cDNA probe pAT-A [Belt *et al.* 1984]; the resulting autoradiographic bands were quantitated by laser densitometry.

Long-range restriction mapping

Genomic DNA had been prepared earlier for *CYP21*/*C4* haplotyping [Koppens *et al.* 1992], using a conventional method [Wyman and White 1980] not aimed at conserving large DNA fragments. Nevertheless, careful handling of the available DNA (stored at -20 °C in 10 µg aliquots of varying volume) by using wide-bore tips and avoiding repeated freeze-thaw cycles, permitted its use in long-range restriction mapping. 10 µg of DNA was digested overnight with 50 units of *Sac*II (Eurogentec, Seraing, Belgium) in a total volume of 250 µl at 37 °C. The next day, the volume was reduced to approximately 90 µl by evaporation at 65 °C, after which 30 µl of molten 2 % low-melting agarose (FMC Bioproducts, Rockland ME, USA) was added. This mixture was allowed to solidify in the slots of a 10-slot 1 % agarose gel in a CHEF-DR III electrophoresis system (Bio-Rad, Hercules CA, USA). The gel was equilibrated in the electrophoresis buffer (0.5 x TBE with 0.2 µg/ml ethidium bromide) for one hour, after which pulsed-field gel electrophoresis was performed for 18 hours at 6 V/cm with an included angle of 120 ° and a switch time linearly increasing from 2 to 17 seconds, at 14 °C. Southern blotting and hybridisation were the same as for short-range mapping (see above).

Analysis of flanking MHC markers

Genomic DNA was digested with *Eco*RI, *Hind*III, *Pvu*II and *Taq*I, separated by electrophoresis and Southern-blotted and hybridised to the *HLA-B* probe pHLA2 [Sood *et al.* 1981] and the *HLA-DQA* probe pDCH-1 [Auffray *et al.* 1982], resulting in distinctive banding patterns [Mornet *et al.* 1986]. Length polymorphism of a microsatellite marker near the tumour necrosis factor (*TNF*) locus was used as an additional marker [Nedospasov *et al.* 1991].

***CYP21* and *CYP21P* mutation analysis**

Mutation analysis of all *CYP21* and *CYP21P* genes in this family was done as described before [Koppens *et al.* 2000]. Briefly, three sections of either *CYP21* or *CYP21P* were specifically amplified and hybridised to oligonucleotides detecting the most common mutations: intron2splice; exon3del8bp; Ile172→Asn; IleValGluMet236→ AsnGluGluLys/

LysGluGluLys; Val281→Leu; exon7ins1bp; Gln318→stop and Arg356→Trp. We since altered our PCR strategy to include the mutations Pro30→Leu and Pro453→Ser (details to be published elsewhere).

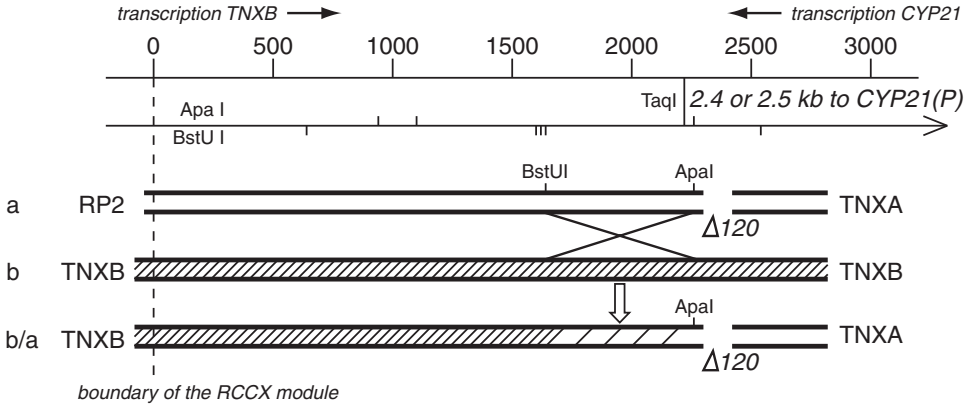

Fig. 2. Amplified region of *TNXA* and *TNXB* showing the site of the 120 bp deletion in *TNXA*, the crossover, and the ApaI and *Bst*UI restriction sites used to map it to a 640 bp region. The amplified stretches are : a: the father's first allele in the *TNXA* PCR (open box); b: the father's second allele in the *TNXB* PCR (densely hatched box); b/a: the hybrid found in the patient (densely hatched: from b; open: from a; lightly hatched: recombinant zone). Note that the specific *TNXA* and *TNXB* forward primer sites differ by 34 bp, causing an additional size difference between the PCR products. crossover region lies between the *Bst*UI and ApaI restriction sites shown. Top: scale in bp, with underneath it the beginning of the 2.4 or 2.5 kb *Taq*I fragment that partly overlaps the *CYP21* or *CYP21P* gene (see fig. 1).

Amplification and restriction analysis of *TNXA* and *TNXB*

Parts of *TNXA* and *TNXB* that encompass the site of the 120 bp deletion normally found in *TNXA* only, were specifically amplified. The forward primer for *TNXB* (TCTCTGCCCTGGGAATGACAG) lies beyond the duplication boundary of the RCCX module, in the large non-duplicated part of the *TNXB* gene. The forward primer for *TNXA* (CTTGAGCTGCAGATGGGATAC) lies within the *RP*2 pseudogene. The reverse primer (CAATCCCCACCCTGAACAAGT) was the same for both genes and lies between the site of the 120 bp deletion and the 3' end of the *CYP21*/*CYP21P* gene (see fig. 2). A touchdown PCR protocol was used to amplify these stretches of approximately 2.7 kb: first, 8 cycles of 30 s at 94°C, 60 s at 66 °C decreasing 0.5 °C per cycle and 3 min at 72 °C; next, 26 cycles of 30 s at 94°C, 60 s at 62 °C and 3 min at 72 °C extending 30 s per cycle. Amplification was done with 0.5 units of Thermoperfect DNA polymerase (Integro, Leuvenheim, The Netherlands) in the presence of 1.5 mM $MgCl_2$ and 1 % formamide. The size of the PCR product directly shows presence or absence of the 120 bp deletion/insertion. In addition, comparison of published *TNX* sequences (EMBL/GenBank/DDBJ accession numbers S38953 [Gitelman *et al.* 1992], X71937 [Bristow *et al.* 1993], AL049547 [Barlow 2000], AF019413 [Rowen *et al.* 1999a],

U89337 [Rowen *et al.* 1999b], AF077974 [Rupert *et al.* 1999], AF086641 [Yang *et al.* 1999] and L26263 [Yu 1991; Shen *et al.* 1994]) revealed several polymorphic sites throughout the amplified region, most of which can be detected by restriction analysis. Digestion of the PCR product with *Bst*UI (New England Biolabs, Beverly MA, USA) and ApaI (Eurogentec, Seraing, Belgium) proved particularly useful in locating the crossover site. *Bst*UI detects a polymorphism at 1626 bp downstream of the border of the RCCX module, ApaI detects a polymorphism at 2266 bp (in this context, 'downstream' is relative to the transcription of the *TNX* gene and sequence AL049547 [Barlow 2000] has been used to compute fragment sizes and nucleotide positions).

Results

CYP21/*C4* haplotypes and flanking MHC markers

The relative intensities of the *Taq*I and *Bgl*II restriction bands in this family are listed in table 1. The results, notably the diminished intensity of the *Taq*I 3.7 kb and the *Bgl*II 12 kb fragments in the patient, could not be explained by normal segregation of regular *CYP21*/*C4* haplotypes [Koppens *et al.* 1992]. Long-range restriction mapping by *Sac*II digestion and pulsed field gel electrophoresis provides a size estimate of the entire contiguous array of RCCX modules, because the *Sac*II sites lie just outside the module (see fig. 1). In this family, both parents had 70 and 76 kb bands, typical of a bimodular arrangement with two long *C4* genes on one chromosome and one long and one short gene on the other chromosome. The patient, on the other hand, had one bimodular chromosome, but also showed a 43 kb band indicating the presence of a single RCCX module with a long *C4* gene on the other chromosome (see fig. 3). These results match the *Taq*I/*Bgl*II band intensities (see table 1). Since testing of several independent genetic markers on chromosomes 1, 7, 16 and 21 confirmed paternity (results not shown), a *de novo* mutation seemed the most obvious explanation for these observations.

This notion was confirmed by analysis of markers centromeric (*HLA-DQA*) and telomeric (*TNF* and *HLA-B*) to the RCCX module (details not shown). Normal Mendelian segregation of all alleles was demonstrated in all healthy family members. The patient however, carried the father's *HLA-DQA* markers from one chromosome, together with the *TNF* and *HLA-B* markers from the other chromosome, in addition to a normal maternal chromosome.

Combining these findings, it was concluded that an unequal crossover had occurred *de novo* between the father's chromosomes, eliminating the *CYP21* gene and the adjacent *C4* gene. The segregation of chromosome 6 in this family is shown in fig. 4.

Table 1. Relative intensities of *Taq*I and *Bgl*II bands and size of the *Sac*II bands, after hybridisation to *CYP21* and *C4* cDNA probes.

	CYP21 probe pC21/3c		*C4* probe pAT-A	*CYP21* and *C4* probes
	*Taq*I	*Bgl*II	*Taq*I	
	3.7 : 3.2	12 : 11	7.0 : 6.4 : 6.0 : 5.4	*Sac*II band sizes
father	2 : 2	2 : 2	2 : 0 : 1 : 1	76 and 70 kb
mother	2 : 2	2 : 2	2 : 0 : 1 : 1	76 and 70 kb
patient	1 : 2	1 : 2	2 : 0 : 0 : 1	43 and 70 kb
twin brothers	2 : 2	2 : 2	2 : 0 : 2 : 0	not determined
sister	2 : 2	2 : 2	2 : 0 : 1 : 1	not determined

C4 band sizes are those traditionally given in the literature [Schneider *et al.* 1986], although the actual fragments are approximately 0.15 kb larger.

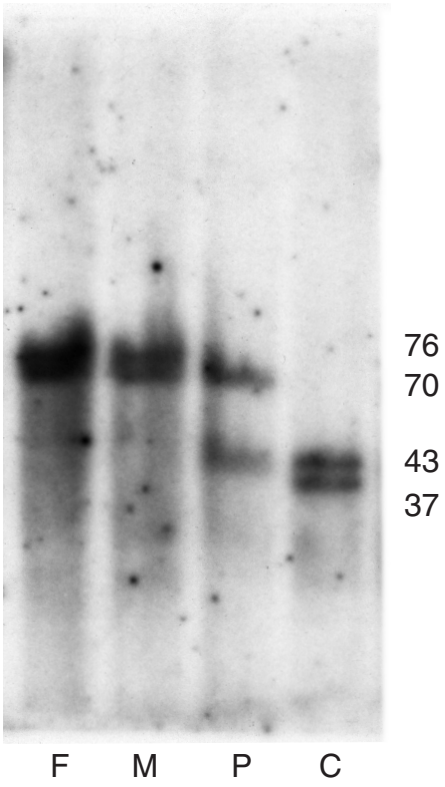

Fig. 3. *Sac*II digested genomic DNA separated by pulsed field gel electrophoresis and hybridised to a mix of a *CYP21* and a *C4* cDNA probe. F: father; M: mother; P: patient with a *de novo* deletion; C: control with two monomodular chromosomes (one with a long and one with a short *C4* gene). Right: fragment sizes in kb.

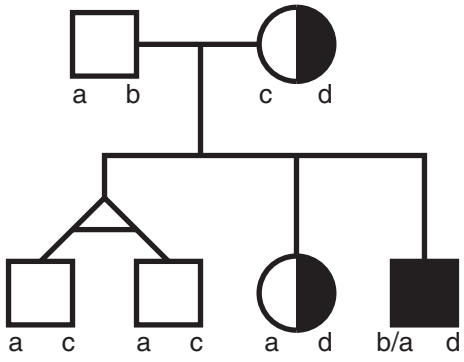

Fig. 4. Pedigree of the family. Characteristics of the haplotypes: a: *CYP21*(normal)-*C4*(long)-*CYP21P*-*C4*(long); b: *CYP21*(normal)-*C4*(short)-*CYP21P*-*C4*(long); c: *CYP21*(normal)-*C4*(long)-*CYP21P*-*C4*(long); d: *CYP21*(Ile172→Asn)-*C4*(short)-*CYP21P*-*C4*(long); b/a: recombinant: *CYP21P*-*C4*(long).

***CYP21* and *CYP21P* mutation analysis**

The Ile172→Asn mutation was found in the mother, the patient and the healthy daughter. This mutation is typical of the simple virilising form of CAH [Amor *et al.* 1988], matching the patient's phenotype. Consistent with the segregation of the haplotypes, no deleterious mutations were found in the *CYP21* genes of the patient's father or the twin brothers.

All *CYP21P* genes in this family matched the consensus sequence [Higashi *et al.* 1986] for all markers tested up to and including the fourth exon. Further downstream, distinctive markers (named after the matching codons in the *CYP21* gene) were found on each allele (see fig. 4). Presence of the Leu281 marker in the recombinant positioned the putative crossover site downstream of that marker, but the markers Gln318, Arg356 and Pro453 were not informative.

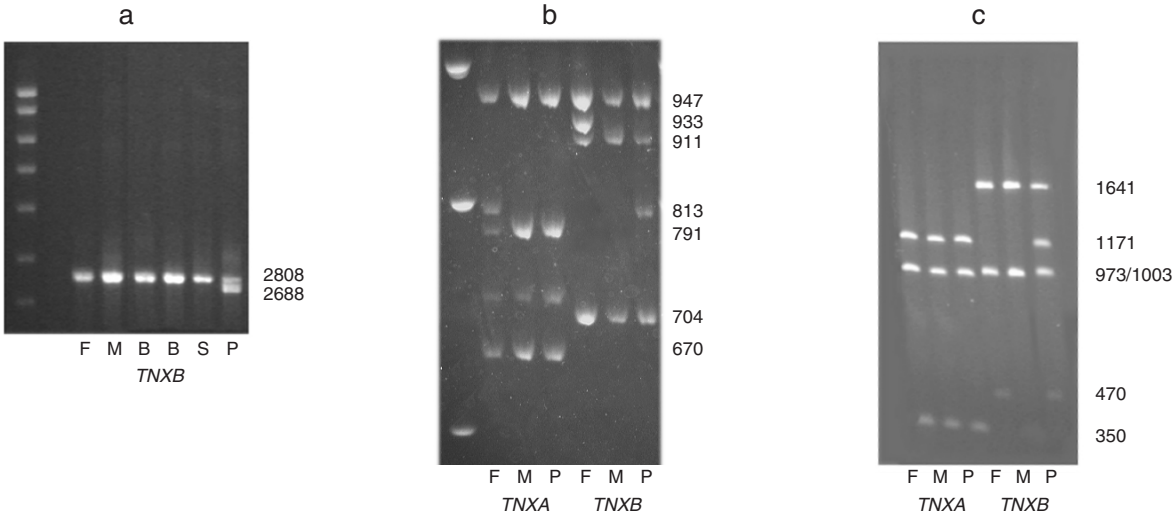

Fig. 5. Full-size and digested PCR products (see fig. 2); F: father; M: mother; P: patient(s); B: twin brother(s); S: sister; fragment sizes in bp. 5a: *TNXB* PCR product showing the 120 bp size difference caused by the *de novo* unequal crossover; left: 5 µl SmartLadder (Eurogentec, Seraing, Belgium); electrophoresis was for 20 h at 40 V on 1 % agarose. 5b: *TNXA* and *TNXB* *Bst*UI digest; left: 5µl SmartLadder; electrophoresis was for 22 h at 140 V on 3.2 % polyacrylamide. 5c: ApaI digest; electrophoresis was for 10 h at 50 V on 1.2 % agarose. The twin brothers and the sister showed the same pattern as the mother in the *Bst*UI and ApaI digests (results not shown).

PCR and restriction analysis of the *TNX* genes.

PCR products of *TNXA* and *TNXB* and restriction patterns after digestion with *Bst*UI and ApaI, are shown in fig. 5. The 2688 bp band represents the recombinant *TNXB* gene of the patient, with the 120 bp deletion that has been transferred from *TNXA* (fig. 5a). The segregation of the fragments within the family allows assignment of the 120 bp deletion and the *Bst*UI and ApaI polymorphisms to each allele as listed in table 2. The recombinant matches the *TNXB* gene of

chromosome b up to and including the *Bst*UI polymorphism 1626 bp downstream of the RCCX boundary and the *TNXA* gene of chromosome a from the ApaI polymorphism at bp 2266. This implies that the crossover occurred in the 640 bp stretch that separates these sites, as shown in fig. 2. A *Bse*RI polymorphism at 1951 bp and a *Pvu*II polymorphism at 2191 bp were not informative in this family (results not shown).

Table 2. Presence (+) or absence (-) of the polymorphic *Bst*UI site at 1626 bp from the RCCX boundary, of the ApaI site at 2266 bp and of the 120 bp (+: present; -: deleted) in the *TNXA* and *TNXB* genes on each chromosome (the letters match the alleles shown in fig. 4; x: no PCR product: the recombinant cannot be amplified in the *TNXA* PCR).

	TNXA			*TNXB*		
chromosome	*Bst*UI	ApaI	120 bp	*Bst*UI	ApaI	120 bp
a	+	+	-	+	-	+
b	-	+	-	-	-	+
c	+	+	-	+	-	+
d	+	+	-	+	-	+
b/a recombinant	x	x	x	-	+	-

Discussion

Deletions of approximately 30 kb of DNA containing the *CYP21* gene are a major factor in the genetics of steroid 21-hydroxylkase deficiency. Such deletions have been attributed to unequal crossover due to misalignment of homologous chromosomes during meiosis [White *et al.* 1988; Sinnott *et al.* 1990; Tusié-Luna and White 1995; Rupert *et al.* 1999; Yang *et al.* 1999]. In this report, we present such an unequal crossover as a *de novo* mutation in the father of a steroid 21-hydroxylase deficiency patient. The crossover breakpoint was mapped to a 640 bp region between two polymorphic restriction sites in the *TNX* gene, which lies immediately centromeric to the *CYP21* gene (see figs. 2 and 6). A *de novo* deletion of the *CYP21* gene reported earlier [Sinnott *et al.* 1990] was caused by an unequal crossover between a chromosome with one RCCX module (monomodular: a single *C4* gene and a *CYP21* gene) and a chromosome with two RCCX modules (bimodular: two *C4* genes, a *CYP21* gene and a *CYP21P* pseudogene). The authors point out that since the bimodular chromosome has no equally sized homologue to align to during meiosis, misalignment of its *CYP21P* and *TNXA* genes to the *CYP21* and *TNXB* genes of the monomodular chromosome would have a probability close to 50 %. This notion of the common monomodular *CYP21P* deletion chromosome serving as a premutation was further strengthened by two more recent reports describing reciprocal *TNXB*/*TNXA* and *TNXA*/*TNXB* hybrids in unrelated individuals [Rupert *et al.* 1999; Yang *et al.* 1999]. A *de novo* deletion that eliminated the *CYP21P* pseudogene [Fasano *et al.* 1992] also involved one bimodular and one monomodular chromosome.

The current report provides a clear illustration of the fact that *de novo* unequal crossovers between bimodular chromosomes also contribute to the pathogenesis of steroid 21-hydroxylase deficiency. Fig. 6 shows a misalignment that may explain such a rearrangement.

It is reasonable to assume that the flanking sequences of the RCCX module (*ie*, the *TNXB* and *RP*1 genes) will align correctly. The crossover could then be explained by two looping-out stretches of DNA, each comprising a single RCCX module. The size differences between the *C4* genes on the chromosomes involved may contribute to inducing such an arrangement. It has been hypothesised that retroviral inserts such as HERV-K(C4), which makes up this size difference, have contributed to genetic rearrangement in the MHC during evolution [Dawkins *et al.* 1999].

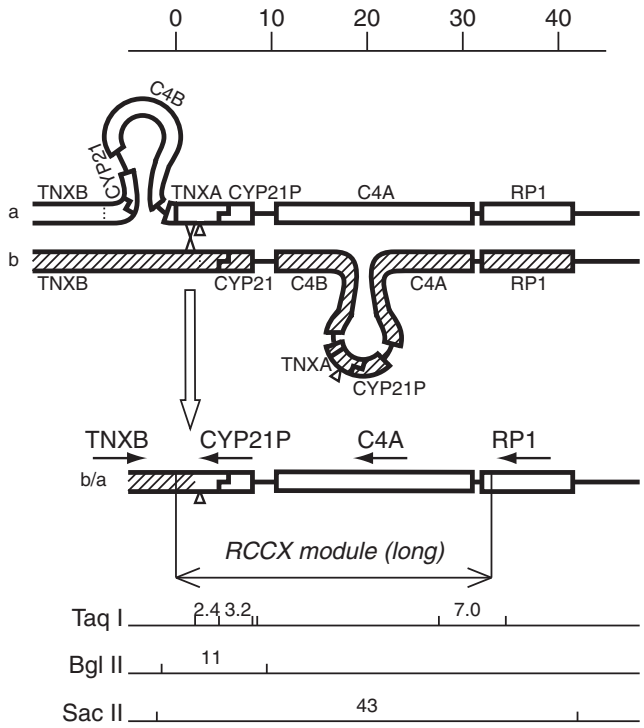

Fig. 6. Hypothetical misalignment and loop-out of two bimodular chromosomes contributing to an unequal crossover causing deletion of a 30 kb region including the *CYP21* gene and the 5' portion of the *TNXB* gene. The figure shows separate loops of two sections of DNA, but other configurations involved in this crossover events can also be envisioned. The open and hatched boxes match the father's chromosomes a and b and the sections of the patient's recombinant chromosome derived from them. See fig. 1 for details on the graphics.

The presence of sequences promoting recombination, such as the *E. coli* crossover hotspot instigator (chi) and the human minisatellite consensus sequence, has been implicated in genetic rearrangement of the RCCX module [Amor *et al.* 1988; Chu *et al.* 1992; Martínez-Quiles *et al.* 1998; Yang *et al.* 1999]. It is possible that such sequences play a role in the generation of small-scale gene conversions between the *CYP21* and *CYP21P* genes, but we do

not believe that at present, there is any reason to assume that the RCCX module is a region of increased levels of crossover. Instead, the high degree of sequence homology between genes in this area combined with the frequent size differences and variability in the number of modules, seems to promote misalignment, so that when a crossover does occur, it has an increased chance of producing a genetic rearrangement and potentially an inherited disease. Consistent with this notion, the present case is only the second clearly documented instance of a *de novo* unequal crossover in this region, despite the fact that over the years, haplotyping studies that could have detected such events have been done in several thousands of families by many different research groups around the world. The earlier report [Sinnott *et al.* 1990] was published before the structure of the RCCX module became known in detail [Bristow *et al.* 1993; Shen *et al.* 1994; Sargent *et al.* 1994; Rupert *et al.* 1999; Yang *et al.* 1999; Blanchong *et al.* 2001], but the crossover apparently also occurred within the *TNX* gene, because the 2.4 kb *Taq*I fragment was retained. Another case involved a very large area including HLA-D in the MHC class II region [Hejtmancik *et al.* 1992] and probably arose by a mechanism very different from the one discussed here. Despite the small number of *de novo* deletions in this region described so far, it is remarkable that none of them appears to have its recombination breakpoint within the *CYP21* gene. It has been well documented that many apparent *CYP21* deletions represent a hybrid gene with a *CYP21P*-like 5' section and a *CYP21*-like 3' section and that transition zones between these sections are positioned at different locations within the hybrid gene [White *et al.* 1988; Chu *et al.* 1992; Donohoue *et al.* 1995; Levo and Partanen 1997; Koppens *et al.* 2000; l'Allemand *et al.* 2000; Baumgartner-Parzer *et al.* 2001]. In line with those findings, we did not find the 120 bp deletion in 15 other deletion haplotypes after testing them with the *TNXB*-specific PCR described here (unpublished observations). *CYP21* gene deletions with the crossover site in the *TNX* gene have so far been described in a few isolated cases [Sinnott *et al.* 1990; Burch *et al.* 1997; Yang *et al.* 1999; Schalkwijk *et al.* 2001; this report]. Whether the additional *TNX* defect adds significantly to selection against this allele as compared to a defect in *CYP21* alone is unclear, since the frequency of tenascin-X deficiency has not been established. However, steroid 21-hydroxylase deficiency alleles occur at different frequencies in different populations [Mornet *et al.* 1991; Owerbach *et al.* 1992; Wedell *et al.* 1994; Ezquieta *et al.* 1995; Wilson *et al.* 1995; Levo and Partanen 1997; Ordoñez-Sánchez *et al.* 1998; Lako *et al.* 1999; Balsamo *et al.* 2000; Koppens *et al.* 2000; Krone *et al.* 2000; Baumgartner-Parzer *et al.* 2001], and most reports do not describe putative crossover sites. This dual-deficiency allele may therefore play a role in the pathogenesis of congenital adrenal hyperplasia, the Ehlers-Danlos syndrome, or both, in some populations. Future studies could address this question by means of PCR methods such as the one described here or elsewhere [Burch *et al.* 1997; Yang *et al.* 1999], or by checking the intensity ratio of the 2.4 and 2.5 kb *Taq*I bands on autoradiograms of genomic DNA. Analysis of *de novo* mutations in the male germline, which has previously been done for a small area within the *CYP21* gene [Tusié-Luna and White 1995] can determine whether there are differences in the frequency of (unequal) crossover between different sections of the RCCX module.

Finally, the current report clearly illustrates that a *de novo* recombination may be a pitfall in understanding RCCX haplotypes, emphasising the importance of studying entire families rather than isolated patients and of using flanking MHC markers on either side of the *CYP21* locus to avoid erroneous assignment of carrier status.

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Chapter 5

Carriership of a defective tenascin-X (*TNXB*) gene in steroid 21-hydroxylase deficiency patients: consequences of breakpoint location in bimodular RCCX modules that lack the *CYP21* gene

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Abstract

Steroid 21-hydroxylase deficiency is caused by a defect of the *CYP21* gene. *CYP21*, the adjacent complement *C4* gene and parts of the flanking genes *RP1* and *TNX* make up the RCCX module, a tandemly duplicated arrangement in the central Major Histocompatibility Complex (MHC). The standard layout is bimodular, with one RCCX module carrying *CYP21* and the other one carrying the highly homologous pseudogene *CYP21P*. By comparison to this standard, *CYP21* defects have traditionally been categorised into deletions of the RCCX module that contains *CYP21*, large-scale gene conversions of *CYP21* into a structure similar to *CYP21P* and smaller mutations in *CYP21* (also derived from *CYP21P* by means of small-scale gene conversions). The genetic mechanisms suggested by these designations have originally been inferred from the layout of the haplotypes involved and were later confirmed by observation of deletions and small mutations, but not large-scale conversions, as *de novo* events. Apparent large-scale conversions account for the defect in 9 out of 75 chromosomes in our patient group. We here demonstrate that 4 out of these 9 'conversions' extend into the flanking *TNXB* gene, which encodes tenascin-X. This implies that approximately 1 in every 10 steroid 21-hydroxylase deficiency patients is a carrier of tenascin-X deficiency, which is associated with a recessive form of the Ehlers-Danlos syndrome. Currently available data on the structure of 'deletion' and 'large conversion' chromosomes strongly suggests that both are the result of the same mechanism, namely unequal meiotic crossover. Since it is unlikely that the term 'large-scale gene conversion' describes a mechanism that actually occurs between the *CYP21* and *CYP21P* genes, we propose the discontinuation of that terminology.

Introduction

Steroid 21-hydroxylase deficiency is the foremost cause of congenital adrenal hyperplasia (CAH), an inborn error of metabolism with an incidence ranging between 1:10,000 and 1:15,000 in most populations [Strachan 1990; New 1998; Wedell 1998; White and Speiser 2000]. CAH due to 21-hydroxylase deficiency is characterised by an impaired adrenocortical synthesis of cortisol and aldosterone. Lack of aldosterone often results in severe salt loss in untreated paediatric patients, a potentially life-threatening condition. Also, the adrenal which has increased in size due to continuous ACTH stimulation induced by lack of cortisol, shunts excess precursor steroids into the androgen synthesis pathway. The elevated androgen levels then cause pre- and postnatal virilisation.

Steroid 21-hydroxylase deficiency has a wide range of clinical manifestations which are associated with more or less severe defects of the *CYP21* gene. Over 15 years ago, it was found that *CYP21* and the highly homologous but deficient pseudogene *CYP21P* lie in the central region of the human Major Histocompatibility Complex (MHC) near the two genes encoding the fourth component of complement (*C4A* and *C4B*) in a tandemly duplicated arrangement [White *et al.* 1985; Carroll *et al.* 1985].

Later studies have shown that the *CYP21* and *C4* genes are part of a repeated stretch of approximately 30 kb of DNA that also includes parts of the *RP1* gene (also named *G11* or *STK19* and encoding a serine-threonine kinase [Sargent *et al.* 1994; Shen *et al.* 1994; Gomez-Escobar *et al.* 1998]) and of the *TNXB* gene (encoding the extracellular matrix protein tenascin-X [Gitelman *et al.* 1992; Matsumoto *et al.* 1992; Bristow *et al.* 1993]). This unit, which comes in two sizes variants ('long' or 'short') depending on the presence or absence of an endogenous retroviral sequence in the *C4* gene, has been named the RCCX module after the principal genes it encompasses [Shen *et al.* 1994; Yang *et al.* 1999; Blanchong *et al.* 2000]. *Taq*I/*Bgl*II restriction analysis of genomic DNA has become a tried-and-proven method to establish the overall genetic arrangement of the *CYP21*/*C4* region [Carroll *et al.* 1985; Donohoue *et al.* 1986; Schneider *et al.* 1986; Higashi *et al.* 1988; White *et al.* 1988; Morel *et al.* 1989]. A 'standard' chromosome bears two modules, containing a *CYP21* gene characterised by a 3.7 kb *Taq*I fragment and a *CYP21P* gene characterised by a 3.2 kb *Taq*I fragment (see fig. 1).

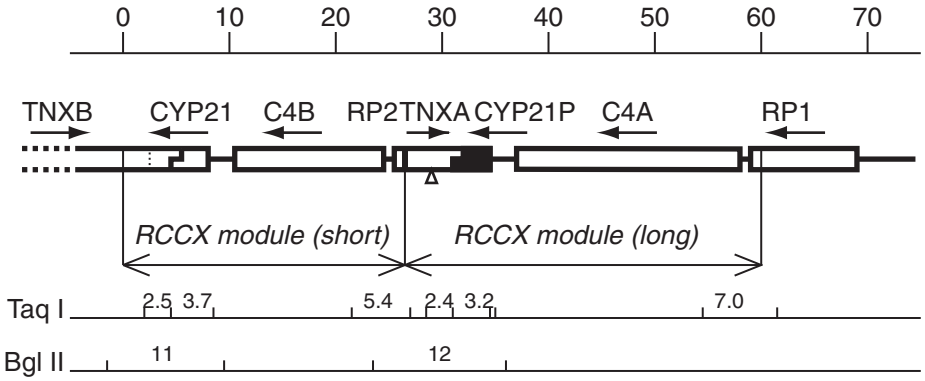

Fig. 1. a. Overview of the *C4*/*CYP21* area, showing two RCCX modules as found on most chromosomes. *TNXB* is the full-size 68 kb gene for tenascin-X, *TNXA* (also known as XA) is a truncated pseudogene of 5.7 kb that lacks most of the coding sequence of *TNXB* and has a deletion of 120bp (indicated by the small triangle) spanning an exon-intron boundary [Gitelman *et al.* 1992; Bristow *et al.* 1993]. *CYP21* (also known as *CYP21*B) is the active steroid 21-hydroxylase gene; *CYP21P* (shown in black; also known as *CYP21*A) is a full-size pseudogene containing several deleterious mutations throughout its sequence, including three in-phase stop codons [Higashi *et al.* 1986]. The *C4* genes express variants of the fourth component of complement with different affinities, known as C4A and C4B. Assignment of C4A and C4B is tentative: the arrangement shown is the most common, but the specificity of the *C4* genes cannot be determined by means of the restriction sites shown. About three quarters of all RCCX modules are 'long' (33 kb in size); the others are 'short' (27 kb). The difference depends on the presence or absence of an endogenous retroviral sequence in one of the introns of the *C4* gene. The arrows show the orientation of transcription; there is an overlap between the 3' sections of the oppositely transcribed genes *TNXB* and *CYP21*, and of *TNXA* and *CYP21P*, respectively. Bottom: characteristic *Taq*I and *Bgl*II restriction fragments; top: scale in kb, with the centromeric RCCX duplication boundary at 0.

Studies of fragment ratios of 21-hydroxylase deficiency alleles early on led to their classification into three main categories of defects:

(a) chromosomes with a single RCCX module having a *CYP21P*-like gene and no *CYP21* gene ('*CYP21* deletions');
(b) chromosomes with two RCCX modules where the *CYP21* gene has apparently been replaced by an additional *CYP21P*-like gene ('large-scale gene conversions');
(c) chromosomes with varying numbers of RCCX modules (mostly, one to three), at least one of which contains a *CYP21* gene.

Further analysis of the latter category demonstrated that most (but not all) defective *CYP21* genes carried one or more of a limited set of mutations typically found in *CYP21P*. These defects were therefore designated 'small-scale gene conversions'.

Unequal meiotic crossover is believed to be the mechanism causing *CYP21* deletions, a notion firmly supported by studies of deletion haplotypes [Carrroll *et al.* 1985; Rumsby *et al.* 1986; White *et al.* 1988; Chu *et al.* 1992; Donohoue *et al.* 1995; Lee *et al.* 2000] and *de novo* events [Sinnott *et al.* 1990; Koppens *et al.* 2002]. These chromosomes usually carry a hybrid gene, where the crossover joined the 5' portion of *CYP21P* onto the 3' portion of *CYP21*. Since the 5' portion contains the extra *Taq*I site [White *et al.* 1985; Higashi *et al.* 1986], the hybrid is recognised as a *CYP21P*-like gene in restriction analysis of genomic DNA. The location of the recombination breakpoint determines the size of the pseudogene-like portion and hence the genetic defects carried by these deletion chromosomes. While in rare cases, enough of *CYP21* has been retained to produce a partly active 21-hydroxylase enzyme [l'Allemand *et al.* 2000], nearly all of the hybrid genes studied so far include at least an 8 bp deletion in the third exon leading to premature termination of translation [Chu *et al.* 1992; Helmberg *et al.* 1992; Levo and Partanen 1997; Koppens *et al.* 2000; Lee *et al.* 2000]. Recently, breakpoint locations beyond the 3' end of *CYP21* have been found [Burch *et al.* 1997; Yang *et al.* 1999; Schalkwijk *et al.* 2001; Koppens *et al.* 2002]. Such alleles carry an additional genetic defect, because a part of the tenascin-X-producing *TNXB* gene has been replaced by its *TNXA* counterpart, a truncated pseudogene carrying a 120 bp deletion on an exon-intron boundary. A homozygous defect of *TNXB* causes type II Ehlers-Danlos syndrome, a connective-tissue disease [Burch *et al.* 1997; Schalkwijk *et al.* 2001].

Insight into the mechanisms of gene conversions has not progressed at the same pace, however. The 'large-scale gene conversion' chromosomes with two *CYP21P*-like genes (that is, two genes characterised by a 3.2 kb *Taq*I fragment) indeed carry a stretch of *CYP21P*-like DNA encompassing several exons [Levo and Partanen 1997; Koppens *et al.* 2000] although here too, an exception where the 'converted' region was limited in size and the gene retained some activity, has been demonstrated [Killeen *et al.* 1991]. Most 'small-scale gene conversions', on the other hand, involve a stretch of *CYP21P*-like DNA that is at most a few hundred base pairs in size and contains only one recognisable mutation as a marker of '*CYP21P*-ness'.

We here report on the extension of the pseudogene-like region in bimodular chromosomes with two *CYP21P*-like genes. Out of nine such haplotypes in a population of steroid 21-

hydroxylase deficiency from 38 families studied by us [Koppens *et al.* 2000], five have a *CYP21P*-*CYP21* transition zone in the *CYP21* gene, but the other four are pseudogene-like well into the *TNXB*-gene. This implies that approximately 10 % of the patients with classical 21-hydroxylase deficiency are also carriers of tenascin-X deficiency. Thus, defectiveness of the *TNXB* gene due to the 120 bp deletion normally found in the *TNXA* pseudogene appears to be much more common than previously reported, and also much more common in bimodular 'conversion' haplotypes than in monomodular 'deletion' haplotypes.

Methods

Subjects and haplotyping

The study population of 21-hydroxylase deficiency patients, family members, and controls was the same as before [Koppens *et al.* 2000], and *CYP21*/*C4* haplotyping was done as described earlier [Koppens *et al.* 1992]. Briefly, *Taq*I and *Bgl*II restriction patterns were obtained with the *CYP21* cDNA probe pC21/3c [White *et al.* 1985] and the 5' section of the *C4* cDNA probe pAT-A [Belt *et al.* 1984]. The principal markers for *CYP21* and *CYP21P* are the 3.7 and 3.2 kb *Taq*I fragments, respectively (see figs. 1 and 3). The 3' flanking region of these genes is usually investigated by *Bgl*II digestion, because the *CYP21* cDNA probe overlaps the 2.4 and 2.5 kb *Taq*I fragments in this region by only a few hundred bp, often resulting in poor visualisation of the bands. Normally, the estimated ratio of the *Taq*I 2.4 and 2.5 kb bands is equal to the ratio of the *Bgl*II 12 and 11 kb bands. However, we could distinguish the 2.4 and 2.5 kb bands on many autoradiograms, and in some patients with an apparent 'large-scale conversion' of *CYP21* into *CYP21P*, a discrepancy between the *Taq*I and *Bgl*II patterns clearly existed (see fig. 3).

Amplification and restriction analysis of *TNXA* and *TNXB*

Parts of *TNXA* and *TNXB* that encompass the site of the 120 bp deletion normally found in *TNXA* only, were specifically amplified. The forward primer for *TNXB* (TCTCTGCCCTGGGAATGACAG) lies beyond the duplication boundary of the RCCX module, in the large non-duplicated part of the *TNXB* gene. The forward primer for *TNXA* (CTTGAGCTGCAGATGGGATAC) lies within the *RP2* pseudogene. The reverse primer (CAATCCCCACCCTGAACAAGT) was the same for both genes and lies between the site of the 120 bp deletion and the 3' end of the *CYP21*/*CYP21P* gene (see fig. 2). A touchdown PCR protocol was used to amplify these stretches of approximately 2.7 kb: first, 8 cycles of 30 s at 94°C, 60 s at 66 °C decreasing 0.5 °C per cycle and 3 min at 72 °C; next, 26 cycles of 30 s at 94°C, 60 s at 62 °C and 3 min at 72 °C extending 30 s per cycle. Amplification was done with 0.5 units of Thermoperfect DNA polymerase (Integro, Leuvenheim, The Netherlands) in the presence of 1.5 mM MgCl2 and 1 % formamide. The size of the PCR product directly shows presence or absence of the 120 bp deletion/insertion. In addition, comparison of published *TNX* sequences (EMBL/GenBank/DDBJ accession numbers S38953 [Gitelman *et al.* 1992], X71937 [Bristow *et al.* 1993], AL049547 [Barlow 2000], AF019413 [Rowen *et al.* 1999a], U89337 [Rowen *et al.* 1999b], AF077974 [Rupert *et al.* 1999], AF086641 [Yang *et al.* 1999]

and L26263 [Yu 1991; Shen *et al.* 1994]) revealed several polymorphisms throughout the amplified region, most of which can be detected by restriction analysis: *Pfl*MI at 276 bp downstream of the border of the RCCX module, *Sty*I at 719 bp, *Bst*UI at 1626 bp and *Pvu*II at 2190 bp (in this context, 'downstream' is relative to the transcription of the *TNXB* gene and sequence AL049547 [Barlow 2000] has been used to compute fragment sizes and nucleotide positions). To determine whether these polymorphisms could be used as reliable markers of a typical *TNXA* or *TNXB* gene, we amplified a large number of 'standard' *TNXA* and *TNXB* genes from individuals with one to three copies of *TNXA* and two copies of *TNXB*, digested the products with these enzymes and analysed them on agarose or polyacrylamide gels.

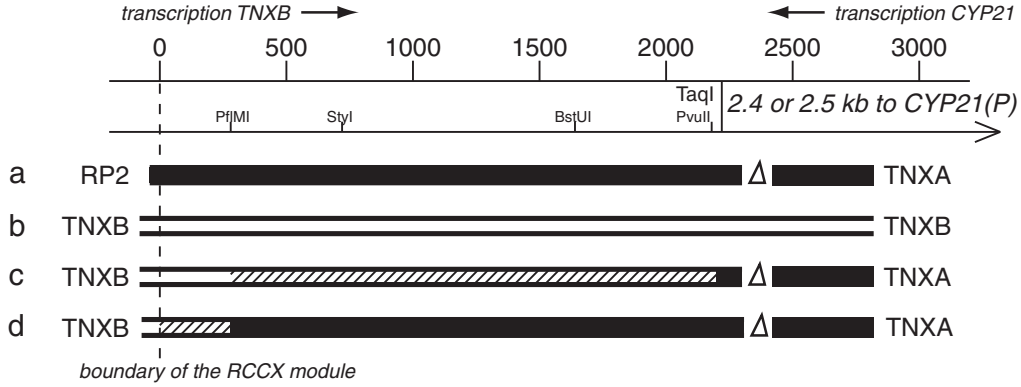

Fig. 2. Amplified region of *TNXA* and *TNXB* (bottom) showing the site of the 120 bp deletion (shown as a triangle) in *TNXA* and the beginning of the *Taq*I fragment that partly overlaps the *CYP21* or *CYP21P* gene (see fig. 1). The location of the *Taq*I site in the *TNX* genes is the same relative to the nearby RCCX boundary, but the 120 bp size difference determines whether the fragment is 2.4 or 2.5 kb. The polymorphic sites used as a marker for *TNXA* or *TNXB* are shown. The four amplified stretches of DNA are, from top to bottom: a. regular *TNXA* PCR; b. regular *TNXB* PCR, which produces similar stretches for a haplotype with a normal *CYP21* gene and one with a *CYP21*/*CYP21P* hybrid; c and d. *TNXB* PCR of a *TNXB*/*TNXA* hybrid with two different conversion zones, typical of the novel haplotype described here. *TNXA*-like sequences are shown in black, *TNXB*-like sequences in white, and putative transition zones between them are hatched. Top: scale in bp.

Results

*Taq*I/*Bgl*II restriction analysis

Typical *Taq*I and *Bgl*II restriction patterns are shown in fig. 3. Some steroid 21-hydroxylase deficiency patients have two bimodular chromosomes and a total of three *CYP21P*-like genes as indicated by the 3.2 and 3.7 kb *Taq*I bands. These persons all have equal density ratios of the *Bgl*II 11 and 12 kb bands (fig. 3b, lane 5), but while some of them also have equal *Taq*I 2.4 and 2.5 kb fragments (fig. 3a, lane 3), others have a diminished intensity of the 2.5 kb fragment and an increased 2.4 kb fragment (fig. 3a, lane 4). The difference depends on the

presence or absence of a 120 bp stretch of DNA on the boundary of exon 36 and intron 36 of the *TNXB* gene [Gitelman *et al.* 1992; Bristow *et al.* 1993]. In the population studied, there were nine bimodular chromosomes with two *CYP21P* genes. Four of these had two 2.4 kb *Taq*I fragments and hence, the 120 bp deletion in *TNXB*; these chromosomes all had one short and one long *C4* gene. The other five did not have the 120 bp deletion in *TNXB*, and all of these had two long *C4* genes. Such apparent discrepancies between the *Bgl*II and *Taq*I restriction fragments were found on two other chromosomes in our study population: once as a result of a *de novo* unequal crossover on a monomodular chromosome [Koppens *et al.* 2002] and once on a bimodular chromosome where the *TNXA* gene had partly assumed a *TNXB*-like structure that is probably the same as reported earlier [Rupert *et al.* 1999]. All other chromosomes tested, including 15 monomodular '*CYP21* deletion' alleles, had the 120 bp present in *TNXB* and absent in *TNXA*.

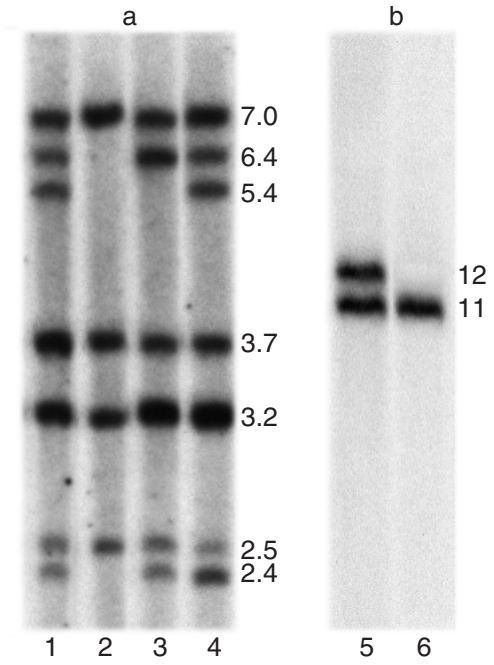

Fig. 3. a. *Taq*I restriction patterns of genomic DNA hybridised to a mixture of the *CYP21* and *C4* cDNA probes. Genotypes: lane 1: normal pattern, two bimodular RCCX modules, one with a 'short' and one with a 'long' *C4B* gene; lane 2: heterozygous deletion of *CYP21* on one chromosome and *CYP21P* on the other, two monomodular chromosomes, both with 'long' *C4* genes; lane 3: heterozygous 'large conversion', two bimodular RCCX modules, one with *CYP21-CYP21P* and one with *CYP21P-CYP21P*; 'long' *C4* genes only and equal intensity of the 2.4 and 2.5 kb bands, so no 120 bp deletion in *TNXB*; lane 4: as lane 3, but one short and one long *C4* gene and increased intensity of the 2.4 kb band due to a 120 bp deletion in *TNXB*. Fragment sizes in kb; the *C4* fragment sizes listed (7.0, 6.4, 6.6) are those traditionally used in the literature [Schneider *et al.* 1986] although the actual fragments are approximately 0.15 kb larger.

b. *Bgl*II restriction patterns obtained with the *CYP21* probe only. Genotypes: lane 5: pattern of two bimodular chromosomes, matching the *Taq*I patterns in lanes 1, 3 and 4; lane 6: two monomodular chromosomes, matching the *Taq*I pattern in lane 2.

Informative markers in the *TNX* genes

The most informative restriction sites to distinguish *TNXA* from *TNXB* were the *Pfl*MI site (present in 193 out of 241 *TNXA* genes and 0 out of 294 *TNXB* genes) and the *Pvu*II site (present in 5 out of 131 *TNXA* genes and 85 out of 129 *TNXB* genes). The *Sty*I and *Bst*UI sites provided no distinction between *TNXA* and *TNXB*. All 298 *TNXB* genes tested contained the 120 bp, as opposed to only 1 out of 270 *TNXA* genes.

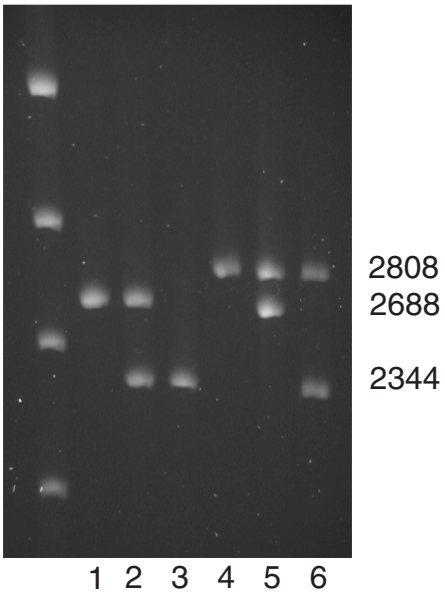

Fig. 4. *Pfl*MI restriction analysis. Lane 1: *TNXA* without the *Pfl*MI site; lane 2: *TNXA*, heterozygous for the *Pfl*MI site; lane 3: *TNXA*, homozygous for the *Pfl*MI site; lane 4: *TNXB*, no *Pfl*MI site; lane 5: *TNXB*, heterozygous for the 120 bp deletion without the *Pfl*MI site; lane 6: *TNXB*, heterozygous for the 120 bp deletion with the *Pfl*MI site. Left: 5 μl SmartLadder (Eurogentec, Seraing, Belgium); electrophoresis was for 28 h at 40 V on 1 % agarose.

Restriction analysis of the *TNX* genes

Typical *Pfl*MI restriction patterns of the *TNXA* and *TNXB* PCR products are shown in fig. 4. Out of the four bimodular *CYP21P-CYP21P* chromosomes with the 120 bp deletion in *TNXB*, one carried the *Pfl*MI site at bp 276 (fig. 4, lane 4); the other three, and the other five *CYP21P-CYP21P* chromosomes, did not have this site (fig. 4, lane 5). This shows that in one case, the transition between a *TNXA*-like sequence and a *TNXB*-like sequence lies within 276 bp of the RCCX boundary. In the other three cases, the transition probably lies further downstream because absence of the *Pfl*MI site is a *TNXB*-like feature (although it also occurs in about 20 % of the *TNXA* genes). All four genes are, of course, *TNXA*-like at the site of the 120 bp insertion (bp 2290) and possibly already at bp 2190 since they did not carry a *Pvu*II site found in most *TNXB* genes (results not shown).

Discussion

Combined defectiveness of the *CYP21* and *TNX* genes has so far been described in a few isolated cases in *CYP21* deletions caused by meiotic unequal crossover. We here report that such 'double deficiency' alleles are indeed rare on monomodular chromosomes, but relatively common (four out of nine cases) on bimodular chromosomes with two *CYP21P*-like genes (usually termed 'large-scale conversions' in the literature). This haplotype is characterised by two 2.4 kb *Taq*I fragments matching the 120 bp deletion in both *TNX* genes and distinguishing it from previously described 'large-scale conversions' which have one 2.4 and one 2.5 kb fragment. Given the large number of *CYP21*/*C4* haplotyping reports available to date (most of which were recently reviewed by White and Speiser [2000]), it is surprising that this haplotype has not been documented before: an apparently similar case was reported only once [Dawkins *et al.* 1987], before the discovery of the *TNX* genes. Poor resolution or poor visualisation of the 2.4 and 2.5 kb bands sometimes (but not always) hampers the analysis when a *CYP21* cDNA probe is used. However, studies with probes that overlap a large part of these fragments [Morel *et al.* 1989; Bachega *et al.* 1999; Blanchong *et al.* 2000] did not detect this haplotype either. To find the *TNXB*-*TNXA* transition zone in this type of hybrid, we checked which of the polymorphisms in published *TNX* sequences (for references, see the Methods section) are reliable markers of '*TNXA*-ness' or '*TNXB*-ness'. A characteristic *Pfl*MI site at 276 bp downstream of the RCCX boundary was present in one of the four *TNXB*-*TNXA* hybrids, but absent in the others, indicating that at least two distinct transition zones exist. In the other three cases, the transition apparently occurred further downstream. The *TNXB*-*TNXA* transition zone in a *de novo* unequal crossover was also found in this downstream area, beyond the *Bst*UI site at bp 1626 [Koppens *et al.* 2002].

In our patient group [Koppens *et al.* 2000], this *TNXB*/*TNXA* hybrid was found on four out of 75 chromosomes, a frequency of 0.053 (95 % confidence limits 0.018 to 0.12) [Linder 1960]. Considering a carrier rate for classical steroid 21-hydroxylase deficiency of approximately 1:50, we estimate the frequency of this dual deficiency allele in the general population in the Netherlands at 1:1,000. If the high frequency of this allele in 21-hydroxylase deficiency patients is unique to the Dutch population, a founder effect would be a logical explanation, but one would then have to postulate an additional mutation to explain the *Pfl*MI difference. This issue could be resolved by systematic re-evaluation of apparent large-scale conversions in other populations by a suitable PCR method [Burch *et al.* 1997; this report]. Interestingly, *TNXB*-*TNXA* hybrid genes were recently reported in two Dutch patients suffering from the Ehlers-Danlos syndrome [Schalkwijk *et al.* 2001]. The number of RCCX modules was not determined in that study, so it seems possible that these patients have the bimodular structure described here.

Chromosomes carrying two RCCX modules, each with a *CYP21P*-like gene, have been called 'large-scale gene conversions' because as compared to the 'standard' layout, the *CYP21* gene appears to have been converted into a *CYP21P* gene. It has, however, become clear that the 'converted' *CYP21P* gene on such chromosomes is either a *CYP21P*/*CYP21* hybrid [Levo and Partanen 1997; Koppens *et al.* 2000] or a regular *CYP21P* gene adjacent to a *TNXA*/*TNXB*

hybrid (this report), and thus structurally indistinguishable from the *CYP21P* genes on '*CYP21* deletion' chromosomes. Although definite proof awaits the description of a *de novo* event, the structural similarity makes it highly likely that bimodular *CYP21P-CYP21P* haplotypes arise by the same mechanism as monomodular *CYP21P*-only haplotypes, namely meiotic unequal crossover: in this case, involving a trimodular *CYP21P-CYP21P-CYP21* chromosome [Strachan 1990, Blanchong *et al.* 2000]. We therefore propose that in this context, the term 'gene conversion' be reserved for small-scale events only, ideally with demonstrable non-converted regions on either side of the converted region. Gene conversion is indeed a reasonable explanation for small-scale *CYP21-CYP21P* sequence transfer, as supported by studies of *de novo* mutations [Collier *et al.* 1993; Tajima *et al.* 1993; Ordoñez-Sánchez *et al.* 1998] and sperm cells [Tusié-Luna and White 1995]. Although historically understandable, the term 'large-scale gene conversion' in its present sense suggests a mechanism that probably never occurs between RCCX modules. Instead, the term '*CYP21* deletion by unequal crossover' adequately describes all chromosomes with a hybrid RCCX module and without a *CYP21* gene, irrespective of the number of *CYP21P* genes.

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Chapter 6

Aldosterone production despite absence or defectiveness of the *CYP21* genes in two patients with salt-losing congenital adrenal hyperplasia caused by steroid 21-hydroxylase deficiency

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Abstract

Aldosterone and cortisol were found in plasma samples from two patients with salt-losing congenital adrenal hyperplasia caused by steroid 21-hydroxylase deficiency. One patient had a *CYP21* gene deletion on one chromosome and a mutation causing erroneous mRNA splicing on the other. The other patient had a *CYP21* gene deletion on one chromosome and a large-scale conversion of *CYP21* to *CYP21P* on the other. All *CYP21P*-like genes in these patients were defective, as they carried a deleterious 8 bp deletion in the third exon. After HPLC purification of the patients' plasma samples, cortisol was no longer detectable in the radioimmunoassay, but aldosterone levels were still within or slightly above the normal reference range. Aldosterone dropped to very low levels after steroid replacement therapy had taken effect. In at least one of these patients, the genetic defect rules out normal functioning of the adrenocortical steroid 21-hydroxylase, which implies involvement of an alternative enzyme system.

Introduction

In the adrenal cortex, multiple enzymatic steps are required for the synthesis of steroid hormones. The main mineralocorticoid, aldosterone, is produced in the *zona glomerulosa* under control of the renin-angiotensin system. The larger *zona fasciculata*, which is under control of ACTH, produces the main glucocorticoid, cortisol, as well as the androgen precursor androstenedione. Steroid 21-hydroxylase (E.C. 1.14.99.10) is involved in two of these pathways: it converts both progesterone to 11-deoxycorticosterone and 17α-hydroxyprogesterone to 11-deoxycortisol. A deficiency of this enzyme occurs in about one in every 15,000 newborns, causing over 90 % of all cases of congenital adrenal hyperplasia (CAH). The most severely afflicted patients suffer from urinary salt loss due to lack of aldosterone, frequently resulting in a life-threatening condition within several weeks after birth ('salt-losing' CAH). In others, the enzyme defect is milder, and aldosterone production is sufficient to preclude salt loss ('simple virilising' CAH). Abnormally high levels of precursor steroids (notably, 17α-hydroxyprogesterone) and androgens (which do not require 21-hydroxylation) are characteristic of steroid 21-hydroxylase deficiency.

Steroid 21-hydroxylase is encoded by the *CYP21* gene in the class III region of the major histocompatibility complex on the short arm of chromosome 6. On most chromosomes, a single *CYP21* gene and the highly homologous pseudogene *CYP21P* lie interspersed between the genes encoding the fourth component of complement (*C4A* and *C4B*). However, many alternative configurations exist, including some which lead to steroid 21-hydroxylase deficiency: *CYP21* gene deletions and conversions of (parts of) *CYP21* into a *CYP21P*-like pseudogene [White 1994].

Although molecular genetic analysis is often in good agreement with clinical phenomena, phenotypical variation exists between genotypically identical sibs [Stoner *et al.* 1986; Sinnott *et al.* 1989; Bormann *et al.* 1992], between patients carrying the same mutation [Higashi *et al.*

1991; Mornet *et al.* 1991; Speiser *et al.* 1992; Kohn *et al.* 1995; Schulze *et al.* 1995; Wilson *et al.* 1995], and in the same patient over time [Stoner *et al.* 1986; Speiser *et al.* 1991; Hoffman *et al.* 1996]. This variation remains unexplained. Biochemically, the presence of 21-hydroxylated steroids such as (plasma) aldosterone [Horner *et al.* 1979; Stoner *et al.* 1986], (urinary) aldosterone metabolites [Stoner *et al.* 1986; Speiser *et al.* 1991; Speiser *et al.* 1992], deoxycorticosterone [Antonipillai *et al.* 1983] and 21-hydroxypregnenolone [Shackleton *et al.* 1987], even in patients with severe salt loss and apparently complete enzyme deficiency, is remarkable. Most case reports deal with aldosterone production by adult or adolescent patients. We here present data on two paediatric patients with salt-losing steroid 21-hydroxylase deficiency. One of these patients lacked the *CYP21* gene altogether. The other patient had one single *CYP21* gene, which carried a splice junction mutation in intron 2, seriously impairing gene expression. Nevertheless, their plasma aldosterone levels were equivalent to those of healthy controls. We also present accurate measurement of plasma steroid levels in these patients using HPLC.

Patients and methods

Patients

The patients (A and B), both boys born at term, were admitted to the Sophia Children's Hospital within the first 10 days of life because of dehydration, vomiting and weight loss. Low sodium levels and extremely high levels of 17α-hydroxyprogesterone led to the diagnosis of salt-losing steroid 21-hydroxylase deficiency. Electrolytes were measured upon admission to the hospital, as were plasma steroid levels and plasma renin activity (PRA) in patient A; in patient B, steroids and PRA were measured during clinical observation at age 7 months, when no replacement steroids were given. Patient A was number 16 and patient B was number 18 in a previously published haplotyping study [Koppens *et al.* 1992]. Treatment with cortisol and 9α-fluorocortisone was started, and both patients are regularly seen at the outpatient department.

Biochemical assays and HPLC

Aldosterone, cortisol, 17α-hydroxyprogesterone and androstenedione were purified from plasma samples by extraction with dichloromethane using Extrelut columns (Merck no. 15371). Cortisol was measured with a Baxter-Travenol GammaCoat RIA kit. 17α-hydroxyprogesterone was measured with a Medgenix RIA kit. Aldosterone was measured according to Van Vliet *et al.* [1992]. The sensitivity of the aldosterone assay was 0.07 nmol/l; the intra- and inter-assay coefficient of variation were 5 % and 9 %, respectively (data collected over a number of years; W. Hackeng, personal communication). Androstenedione was measured using an antibody obtained from Dr. J.J. Pratt [De Lange *et al.* 1980]. Initial measurements had shown extremely high levels of 17α-hydroxyprogesterone, but normal to elevated cortisol and aldosterone concentrations (see table 1). Because these findings appeared to contradict the diagnosis of 21-hydroxylase deficiency, the aldosterone and cortisol extracts were purified by HPLC. A Waters HPLC instrument with a 15 cm 5 µm

silica column (Merck no. 15655) was used. The peaks were detected by a Waters 441 absorbance detector equipped with a 214 nm optical filter. Cortisol was eluted with 25 % isooctane, 71 % chloroform, 3.75 % methanol and 0.25 % water; aldosterone was eluted with 56.5 % isooctane, 40.5 % chloroform, 2.9 % methanol and 0.12 % water. The flow rate was 1 ml/min at 1250 psi. A standard addition method [Willard *et al.* 1965] was used to establish the base level and the recovery of these steroids. Briefly, commercially available standards were added to the plasma samples, and the recovery after HPLC was determined.
At the time of these measurements, no urine was available for steroid assays (the limited amounts that had been collected were used for electrolyte production measurements).

Table 1. Biochemical parameters and therapeutical effects in patients A and B.

without steroid replacement therapy[a]	patient A	patient B	
Na (mmol/l)	110	112	
K (mmol/l)	8.7	8.2	
PRA (ng/ml/hr)	130	150	
17α-hydroxyprogesterone (nmol/l)	1990	1200	
androstenedione (nmol/l)	24	14.5	
aldosterone before HPLC (nmol/l)	0.63	1.4	
aldosterone after HPLC (nmol/l)	0.46	0.47	
cortisol before HPLC (μmol/l)	0.37	0.36	
cortisol after HPLC (μmol/l)	< 0.03	< 0.03	
after therapy had taken effect[b]			
daily intake of cortisol (mg)	1+1+2	3+3+3	3+3+6
daily intake of 9α-fluorocortisone (μg)	50+50	30+30	30+30
age	5 months	1 year	3 y 8 m
Na (mmol/l)	141	134	141
K (mmol/l)	5.1	5.5	4.4
PRA (ng/ml/hr)	0.6	10	3.9
17α-hydroxyprogesterone (nmol/l)	4.3	> 48	43
androstenedione (nmol/l)	< 0.03	2.1	2
aldosterone (no HPLC) (nmol/l)	< 0.05	0.86	< 0.05

Reference ranges: 17α-hydroxyprogesterone 0.5-6 nmol/l; androstenedione 0.35-1.2 nmol/l; aldosterone 0.03-0.35 nmol/l (recumbent); cortisol 0.15-0.7 μmol/l (in the morning); PRA 8-17 ng/ml/hr (0 - 3 months of age); 3-10 ng/ml/hr (3 - 12 months); 3-8 ng/ml/hr (12 months - 4 years).

a: upon admission or during later clinical observation (see text)

b: measurements at age 5 months (patient A) and at ages 1 year and 3 years 8 months (patient B)

HLA typing and *CYP21*/*C4* haplotyping

In both families, blood samples from the father, the mother and the patient were available for HLA typing and DNA studies. HLA-A, -B, -DR, and -DQ typing was performed at the Department of Immunohaematology of the Leiden University Hospital using a standard microlymphocytotoxicity assay. The haplotypes found previously indicated complete absence

of the *CYP21* gene in patient A. Patient B had a single *CYP21* gene on one chromosome and a conversion of *CYP21* into *CYP21P* on the other [Koppens *et al.* 1992].

Mutation analysis of the *CYP21* gene of patient B

A part of the second intron and third exon of the single *CYP21* gene of patient B was amplified using the oligonucleotide primers NORMin2F and BOTHex3R. PCR was performed at 3 mM $MgCl_2$ and 2 % formamide, for 32 cycles consisting of 30 s at 95 °C, 45 s at 53 °C and 2 min at 72 °C. The identity of the amplified product was confirmed by *Pvu*II digestion (see fig. 1). The *Pvu*II site distinguishes *CYP21* from *CYP21P* in these patients, and matches the *Taq*I restriction patterns [Koppens *et al.* 1995]. The remainder of the PCR product was slot blotted onto Hybond-N^+ (Amersham) and hybridised to the oligonucleotides in2GsplR, in2CsplR and in2AsplR. The final washing steps were at 52 °C in 0.5 x SSC.

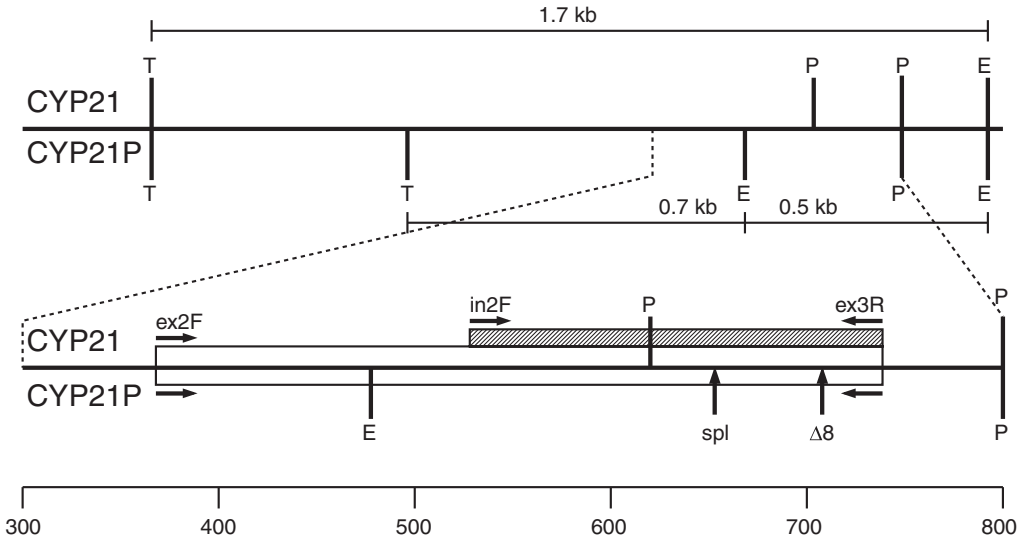

Fig. 1. Top: restriction sites (E=*Eco*RI, P=*Pvu*II, T=*Taq*I) and *Taq*I/*Eco*RI fragments in the 5' section and flanking area of *CYP21* and *CYP21P*. Bottom: PCR fragments and primers; open box: amplified with BOTHex2F (forward; CAGACCTGAGCCACTTACCT) and BOTHex3R (reverse; TTCTTGTGGGCTTTCCAGAG); hatched box: amplified with NORMin2F (forward; ATCCCCAATCCAGGTCCCT) and BOTHex3R. Hybridisation site spl: splice junction mutation in intron 2; oligonucleotides in2GsplR (defective; CAGGAGGAGCTGGGGGCTG), in2CsplR (normal; CAGGAGGAGGTGGGGGCTG) and in2AsplR (normal: CAGGAGGAGTTGGGGGCTG). Hybridisation site Δ8: 8 base pair deletion in exon 3; oligonucleotides ex3del8F (defective; TGTCGTTGGTCTCTGCTCTG) and ex3normF (normal; TCCTTGGGAGACTACTCCCT). Scale below in bp; nucleotide numbering matches Higashi *et al.* (1986).

Mutation analysis of the *CYP21P* pseudogenes

In haplotyping studies, it is usually assumed that steroid 21-hydroxylase genes corresponding to a 3.7 kb *Taq*I restriction fragment are genuine *CYP21* genes, while genes corresponding to a

3.2 kb fragment are non-functional *CYP21P* pseudogenes. Because in the normal arrangement one *CYP21* gene and one *CYP21P* pseudogene are present, haplotypes with two 3.2 kb fragments and no 3.7 kb fragments are supposed to represent 'large-scale' gene conversions of *CYP21* to *CYP21P*. However, the size of these *Taq*I fragments merely reflects the presence or absence of a restriction site in the 5' flanking sequence of the gene. Killeen *et al.* [1991] described a haplotype with two steroid 21-hydroxylase genes both characterised by a 3.2 kb fragment, one of which encoded a partially active enzyme. The unexpected presence of aldosterone in our patients and the fact that both carry a 'converted' gene on one chromosome called for an investigation of their *CYP21P* genes. We checked the presence of an 8 bp deletion usually present in *CYP21P*, rendering this gene inactive. *CYP21* and *CYP21P* were amplified in a single PCR, which was performed at 2.5 mM $MgCl_2$ and 2 % formamide, for 32 cycles consisting of 30 s at 95 °C, 45 s at 58 °C and 3 min at 72 °C. The product was digested with *Eco*RI, resulting in a distinct banding pattern for each gene. The Southern blots thus obtained were hybridised to the oligonucleotides ex3del8F and ex3normF, detecting the presence or absence of an 8 bp deletion found in virtually all *CYP21P* genes. The final washing steps were at 52 °C in 0.4 x SSC (ex3del8F) and 48 °C in 0.4 x SSC (ex3normF). Concordance between *Taq*I and *Eco*RI patterns was established by *Taq*I/*Eco*RI double digestion of genomic DNA and hybridisation to the 0.6 kb *Bam*HI-*Eco*RI fragment of pC21/3c [White *et al.* 1985]. DNA samples from healthy individuals and, when appropriate, other CAH patients from the previous study, were used as controls.

Results

Steroid measurements and HPLC

Electrolytes, plasma steroid levels and PRA with and without treatment are presented in table 1. Since steroid replacement therapy initially did not result in normalisation of all biochemical parameters in patient B, measurements from a later date are also given. The HPLC protocol used to purify the aldosterone and cortisol fractions before RIA was tested by addition of known amounts of each compound. Aldosterone was virtually 100 % recovered; the recovery of cortisol was about 70 %. After HPLC purification of the patient samples, cortisol was below the detection limit (< 0.03 µmol/l), but aldosterone remained slightly above the reference range. Apparently, the steroid measured before HPLC was not cortisol but an interfering compound, while the aldosterone measured was authentic. If the compound measured was not aldosterone, the assumption would have to be made that it cross-reacted in the RIA as well as co-eluted in the HPLC. None of over 150 other steroids tested was even close to fulfilling both conditions (results not shown). A similar procedure (HPLC followed by RIA) was not deemed necessary for 17α-hydroxyprogesterone, since this steroid was present in amounts directly measurable by HPLC. The location of the 17α-hydroxyprogesterone HPLC peak matched the standard, and the amount was in agreement with the RIA results.

HLA types and *CYP21*/*C4* haplotypes

In family A, the father's haplotypes were: (a): HLA-A1,B71,DR13,DQ1; *CYP21*/*C4*: A1; (b): HLA-A3,B47,DR7,DQ2; *CYP21*/*C4*: E1. The mother's haplotypes were: (c): HLA-A28,B27,DR4,DQ3; *CYP21*/*C4*: A1; (d): HLA-A30,B49,DR13,DQ1; *CYP21*/*C4*: D2. In family B, the father's haplotypes were: (a): HLA-A2,B51,DR11,DQ3; *CYP21*/*C4*: A1; (b): HLA-A30,B49,DR13,DQ1; *CYP21*/*C4*: D2. The mother's haplotypes were: (c): HLA-A2,B62,DR1,DQ1; *CYP21*/*C4*: A1; (d): HLA-A3,B44,DR13,DQ1; *CYP21*/*C4*: A1. The patients inherited the (CAH-bearing) haplotypes (b) and (d). The *CYP21*/*C4* haplotypes are as defined previously [Koppens *et al.* 1992]; A1: the *CYP21* and *CYP21P* genes are both present; D2: large-scale conversion of the *CYP21* gene to a *CYP21P*-like pseudogene; E1: deletion of the *CYP21* gene.

Defects in the *CYP21* gene of patient B

Hybridisation of the *CYP21* specific PCR fragment (the first PCR described in the Methods section) of patient B to oligonucleotide in2GsplR showed that a mutation leading to alternative mRNA splicing was present in the second intron of the patient's single *CYP21* gene. This *CYP21* gene was also checked for the presence of several other severe defects: the 8 bp deletion in exon 3 (see fig. 2), a change of 3 codons in exon 6, a frameshift mutation in exon 7, a termination codon in exon 8 and the Arg→Trp mutation in exon 8 (results not shown), but none of these were detected. As expected, the same results were found in the mother of patient B.

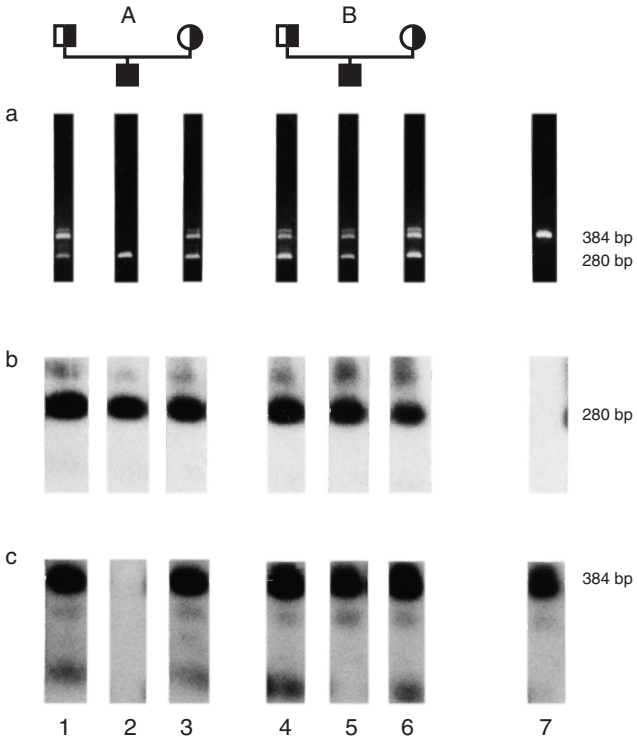

Fig. 2. PCR and hybridisation of (parts of) *CYP21* and *CYP21P*. Lanes 1-3: family A (father, patient, mother); lanes 4-6: family B (father, patient, mother); lane 7: control having only *CYP21* genes. Fig. 2a: *Eco*RI digested PCR products; fig. 2b: hybridisation to ex3del8F (detecting the 8 bp deletion in exon 3); fig. 2c: hybridisation to ex3normF (detecting the normal sequence). The 384 bp fragment represents *CYP21*; the 280 bp fragment represents *CYP21P*.

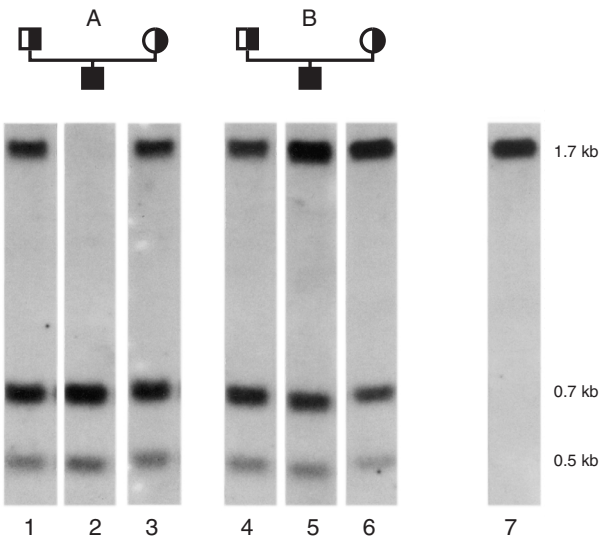

Fig. 3. Hybridisation patterns of the 0.6 kb *Bam*HI - *Eco*RI fragment of pC21/3c to genomic DNA double digested with *Taq*I and *Eco*RI. Lanes 1-3: family A (father, patient, mother); lanes 4-6: family B (father, patient, mother); lane 7: control having only *CYP21* genes. The 1.7 kb bp fragment represents *CYP21*; the 0.7 kb and the (weaker) 0.5 kb fragment represent *CYP21P* (see fig. 1).

Defects in the *CYP21P* genes of both patients

Fig. 2 shows restriction (2a) and hybridisation (2b and 2c) patterns of PCR products obtained after amplification of both *CYP21* and *CYP21P* in a single reaction. The results show that all *CYP21P* genes in both families are indeed defective. Fig. 3 shows typical patterns of *Taq*I/*Eco*RI digested genomic DNA hybridised to the 0.6 kb *Bam*HI-*Eco*RI fragment of pC21/3c. The 0.5 kb and 0.7 kb fragments characteristic of *CYP21P* and the 1.7 kb fragment characteristic of *CYP21*, respectively, corresponded to the *Taq*I patterns in all members of both families, indicating that presence of the *Eco*RI site in intron 2 accurately identified *CYP21P*. In contrast with the nearby *Pvu*II site [Koppens *et al.* 1995], this apparently is a general feature, since similar results with *Taq*I/*Eco*RI were obtained in 162 unrelated controls, including CAH patients and healthy individuals.

Discussion

Steroid measurements

We studied two patients suffering from salt-losing steroid 21-hydroxylase deficiency with severe defects in their *CYP21* genes, who nevertheless were able to produce significant amounts of aldosterone. In steroid 21-hydroxylase deficient children aldosterone does not preclude salt loss, because high 17α-hydroxyprogesterone levels counteract aldosterone action [Jacobs *et al.* 1961]. In addition, increased amounts of 11-deoxycorticosterone function as a competitor at receptor level [Land and Ulick 1987].

Cortisol was also found initially, but the levels fell below the detection limit after HPLC purification. Several compounds may interfere with cortisol RIAs, most notably 21-deoxycortisol [Brotherton and Rothbart 1990]. Aldosterone measurements were also lower after HPLC, but amounts typical of normal individuals were still found.

Because aldosterone is one of the (quantitatively) minor plasma steroids, antisera used in its assay must be highly specific *a priori*. The only interfering compounds described in the literature after 1980 are aldosterone metabolites, glucuronic acid conjugates and a few drugs. All such compounds are easily removed by HPLC. Schöneshofer and Weber [1983] used HPLC purification to ascertain the analytical aspects of 15 steroids, including aldosterone. Egfjord and Olgaard [1992] showed convincing HPLC separation of labelled aldosterone and aldosterone metabolites, some of which showed cross-reactivity in the RIA they used.

***CYP21*/*CYP21P* haplotypes and mutations**

Patient A and patient B each had one chromosome with two *CYP21P* genes and no *CYP21* genes. Patient A had a *CYP21* deletion on the other. Thus, the genetic defect precluded all activity of the normal adrenocortical steroid 21-hydroxylase enzyme in this patient. All *CYP21P*-like genes in both patients were genuine pseudogenes carrying an 8 bp deletion causing a frameshift (see fig. 2).

The single *CYP21* gene in patient B had a mutation in intron 2 creating an alternative splice site. Patients homozygous or 'hemizygous' (having a *CYP21* deletion on the other chromosome) for this common mutation usually have salt-losing steroid 21-hydroxylase deficiency. However, in some cases the symptoms were milder [Mornet *et al.* 1991; Speiser *et al.* 1992, Wilson *et al.* 1995] or delayed [Kohn *et al.* 1995], and homozygosity for this mutation has even been reported in asymptomatic relatives of patients [Schulze *et al.* 1995]. The presence of a small amount of correctly spliced mRNA cannot be excluded, as *in vitro* studies have demonstrated some remaining enzyme activity [Higashi *et al.* 1991]. Remaining adrenocortical steroid 21-hydroxylase activity may be the source of aldosterone in this patient.

Aldosterone in CAH patients

Factors influencing measured aldosterone levels in CAH patients are the genetic defect, the age of the patient, the renin-angiotensin system, and the use of different aldosterone assays. Patient A has no *CYP21* genes at all and was nevertheless able to produce aldosterone in early childhood. The only similar case described in the literature concerns a patient of approximately the same age when urinary aldosterone 18-glucuronide was detected [Speiser *et al.* 1992]. Although this patient (no. 21) had two *CYP21* genes, each carried a change of 3 codons in exon 6, a mutation expected to completely abolish gene function. An earlier study [Speiser *et al.* 1991] described aldosterone synthesis in a 19-year-old patient who carried a homozygous deletion of the *CYP21* genes and had no detectable aldosterone production in early childhood.

The influence of the renin-angiotensin system on aldosterone synthesis in 21-hydroxylase deficient patients is difficult to assess. The ratio of PRA to urinary aldosterone 18-glucuronide ranges widely and shows a relatively poor correlation with the *CYP21* genotype [Speiser *et al.* 1992]. Plasma aldosterone levels are more directly influenced by PRA, as a close synchronisation between the two exists [James *et al.* 1978]. Horner *et al.* [1979] described seven older patients (10-19 years of age) with salt-losing CAH having elevated plasma aldosterone levels when they were off treatment. Aldosterone reached normal levels when reinstitution of mineralocorticoid therapy resulted in suppression of PRA, which

suggested a functional renin-angiotensin-aldosterone system. In our (much younger) patients, plasma aldosterone was not found at all when therapy had taken effect (table 1). Stoner *et al.* [1986] measured significant plasma aldosterone levels in patients 7-33 years of age, in some of whom an aldosterone synthesis defect existed in childhood. Although methodological aspects in aldosterone measurements play a role, ageing is evidently instrumental in aldosterone synthesis in many 21-hydroxylase deficient patients.

Alternative enzymatic pathways

Alternative steroid 21-hydroxylases either inside or outside the adrenal and alternative synthetic pathways towards aldosterone may account for the presence of aldosterone in the blood of patients not having a functional *CYP21* gene, such as patient A. The existence of an alternative adrenal 21-hydroxylase system has been postulated [Franklin *et al.* 1987]. Although no mRNA for such an enzyme has so far been found, conditions of adrenal hyperplasia and high levels of precursor steroids might induce the expression of hitherto unknown enzymes *in vivo*.

Extra-adrenal steroid 21-hydroxylation, notably conversion of progesterone to deoxycorticosterone, has been demonstrated in human adults [Winkel *et al.* 1980] and in foetal tissue [Casey *et al.* 1983]. High amounts of 21-hydroxypregnenolone have been found in newborns with a deficiency of the adrenal steroid 21-hydroxylase [Shackleton *et al.* 1987]. Although steroid 21-hydroxylase activity in the rat and mouse kidney has recently been associated with the presence of genuine *CYP21* mRNA [Lajic *et al.* 1995], several human tissues capable of steroid 21-hydroxylation do not contain *CYP21* mRNA [Voutilainen and Miller 1986; Mellon and Miller 1989]. Peripheral conversion of progesterone to deoxycorticosterone is only between 0.2 % and 3 % [Winkel *et al.* 1980; Antonipillai *et al.* 1983; Speiser *et al.* 1991], which makes entry of circulating deoxycorticosterone into the (relatively small) adrenal cortex followed by resumption of the normal biosynthetic pathway most unlikely.

The involvement of other organs than the adrenal in aldosterone formation provides a more plausible hypothesis. Lewicka *et al.* [1987] have demonstrated enhanced production of 21-deoxyaldosterone by patients with steroid 21-hydroxylase deficiency. Aldosterone can be synthesised from this steroid by a single enzymatic step, which may be mediated by a non-adrenal enzyme. Low sodium concentrations, comparable to those initially present in our patients, enhance 21-deoxyaldosterone synthesis in rat adrenal tissue [Lewicka *et al.* 1990]. Angiotensin II also stimulates 21-deoxyaldosterone synthesis [Abdelhamid *et al.* 1994]. This is in agreement with our finding that when therapy had resulted in normalisation of sodium levels and reduction of PRA, aldosterone could no longer be detected (table 1).

In this model, the renin-angiotensin system influences peripheral aldosterone secretion in an indirect way, by controlling adrenal precursor synthesis. This might contribute to the poor correlation of PRA to urinary aldosterone metabolites [Speiser *et al.* 1992].

Synthesis of significant amounts of cortisol, normally required in amounts 100 - 1,000 times larger than aldosterone, from circulating 21-deoxycortisol is improbable. The normal pathway involves adrenal 17α-hydroxylase and 21-hydroxylase, both located in the microsomes of the

zona fasciculata. It is unlikely that a peripheral mechanism could be equally efficient. Nevertheless, amounts equal to the aldosterone measured may be present but will go undetected by assays aimed at the much higher 'normal' levels.

In conclusion, aldosterone but not cortisol is found in blood samples of some untreated paediatric salt-losing steroid 21-hydroxylase deficiency patients. HPLC is valuable in analysing apparently controversial steroid profiles in such patients. Although in some cases (patient B) there may be remaining activity of the defective *CYP21* gene, in others (patient A) alternative mechanisms or enzymes must be considered.

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Chapter 7

Duplication of the CYP21 gene may complicate mutation analysis of steroid 21-hydroxylase deficiency: characteristics of three unusual haplotypes

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Abstract

Steroid 21-hydroxylase deficiency, the primary cause of congenital adrenal hyperplasia, is caused by defects of the *CYP21* gene. Mutation analysis of *CYP21* plays a key role in the diagnosis of steroid 21-hydroxylase deficiency and in fundamental research of phenotype-genotype relationship in this common inherited metabolic disease. Contemporary PCR-based mutation detection protocols often depend on the assumption that no more than one *CYP21* gene is present on each chromosome 6. We here describe three haplotypes with two *CYP21* genes on the same chromosome, with defects typical of salt-losing steroid 21-hydroxylase deficiency in one of those genes, but not necessarily in the other. The frequency of these haplotypes in the general population is 6/365 (1.6 %), so they are not less frequent than other haplotypes that indeed carry steroid 21-hydroxylase deficiency. Chromosomes that carry two *CYP21* genes therefore represent a significant pitfall in the molecular diagnosis of steroid 21-hydroxylase deficiency. We recommend that whenever *CYP21* mutation analysis of an individual who is not a known carrier of steroid 21-hydroxylase deficiency is performed, the overall structure of the *CYP21*/*C4* region (RCCX area) is determined to avoid erroneous assignment of carrier status.

Introduction

The *CYP21* gene, which encodes the adrenocortical enzyme steroid 21-hydroxylase, lies in the central (class III) region of the human Major Histocompatibility Complex (MHC) on the short arm of chromosome 6 (6p21.3). Together with the adjacent complement *C4* gene and truncated parts of the RP1 and *TNXB* genes, *CYP21* constitutes a highly variable stretch of DNA known as the RCCX module [Blanchong *et al.* 2000]. All chromosomes 6 so far studied carry one to four RCCX modules, each of which may be either 27 or 33 kb in size depending on the presence or absence of an intronic sequence in the *C4* gene [Dangel *et al.* 1994]. The active *CYP21* gene lies on the centromeric module next to the full-size *TNXB* (tenascin-X) gene, while any other RCCX modules on the same chromosome usually carry the pseudogene *CYP21P* which shares 98 % sequence homology with *CYP21* [Higashi *et al.* 1986] but contains several deleterious mutations.

Defectiveness of *CYP21* results in steroid 21-hydroxylase deficiency, the foremost cause of congenital adrenal hyperplasia (CAH), an inborn error of metabolism affecting 1:10,000 to 1:15,000 newborns in most populations. In steroid 21-hydroxylase deficiency patients, impaired cortisol synthesis leads to ACTH-induced hyperplasia of the adrenal cortex, while accumulating cortisol precursors are converted to androgens. If the enzyme block is complete, synthesis of aldosterone (which is required in far lower quantities than cortisol) also fails. Untreated patients with a severe defect may therefore succumb to a neonatal salt-losing crisis, and all patients with this 'classical' form of steroid 21-hydroxylase deficiency have signs of virilisation and abnormal growth patterns due to the androgen excess [Morel and Miller 1991; Wedell 1998; White and Speiser 2000].

Molecular analysis of *CYP21* defects is the key to understanding the aetiology of steroid 21-hydroxylase deficiency, both in basic science and in clinical diagnosis. In most Caucasian populations, about one-third of the chromosomes carrying 21-hydroxylase deficiency have a *CYP21P*-like pseudogene in place of the regular *CYP21* gene in the centromeric RCCX module. Such configurations are often referred to as '*CYP21* deletions' on monomodular chromosomes and 'large-scale gene conversions of *CYP21* to *CYP21P*' on bimodular chromosomes, although the molecular mechanisms causing these defects appear to be similar. The remaining two-thirds of all *CYP21* defects is caused by one or more small changes in *CYP21* that are associated with varying degrees of enzyme deficiency and clinical defects [Speiser *et al.* 1992; Wedell 1998; White and Speiser 2000].
The overall layout of the RCCX area can be established by restriction enzyme mapping, a method which has often been used to distinguish the above-mentioned categories of *CYP21* defects in steroid 21-hydroxylase deficiency patients [White *et al.* 1988; Collier *et al.* 1989; Dunham *et al.* 1989; Morel *et al.* 1989]. If a *CYP21* gene is present, further mutation analysis is usually done by any one of a steadily growing number of PCR-based approaches that rely on sequence differences between *CYP21* and *CYP21P* [White and Speiser 2000]. Since the restriction mapping method, which requires Southern blotting and hybridisation, is rather laborious, it has gradually gone out of fashion for the detection of presence or absence of the *CYP21* gene, and recent studies rely on PCR for this purpose as well. Thus, mutation analysis is often based on the assumption that each chromosome 6 carries at most one *CYP21* gene. While that assumption holds true for the large majority of all 21-hydroxylase deficiency patients and controls investigated to date, exceptions have been reported [Schneider 1990; Haglund-Stengler *et al.* 1991; Koppens *et al.* 1992; Wedell *et al.* 1994; Bachega *et al.* 1999].
We here describe four unusual *CYP21*/*C4* haplotypes, three of which carry two *CYP21*-like genes. Because the second *CYP21*-like gene on these chromosomes carries one or more defects, non-carrier individuals from the general population who have such a haplotype would have been mistyped as steroid 21-hydroxylase deficiency heterozygotes had an entirely PCR-base approach been used. This *CYP21* duplication was found in six out of 365 independent haplotypes. This phenomenon may complicate mutation analysis in family members of CAH patients and it is a definite pitfall in the detection of unrelated heterozygotes because in the general population, the haplotypes described here are not less common than the mutations associated with classical congenital adrenal hyperplasia.

Methods

Haplotyping

Common *CYP21/CYP21P* haplotypes in the general population are: *CYP21-CYP21P* ('standard'), *CYP21* ('*CYP21P* deletion') and *CYP21-CYP21P-CYP21P* ('*CYP21P* duplication'), with *CYP21P* ('*CYP21* deletion') and *CYP21P-CYP21P* ('*CYP21* large-scale gene conversion') as additional possibilities in carriers of steroid 21-hydroxylase deficiency [Morel *et al.* 1989]. In a previous study, we used *Taq*I and *Bgl*II restriction analysis [White *et*

al. 1988] and *CYP21* [White *et al.* 1985] and *C4* [Belt *et al.* 1984] cDNA probes to analyse the haplotypes in a group of families with 21-hydroxylase deficiency patients and healthy controls in the Netherlands [Koppens *et al.* 1992]. We since expanded this group to 43 families and 141 controls, found several banding patterns that could not be explained in terms of the five haplotypes mentioned above and selected these for further analysis.

Long-range restriction mapping

Genomic DNA had been prepared earlier for *CYP21*/*C4* haplotyping [Koppens *et al.* 1992], using a conventional method [Wyman and White 1980] not aimed at conserving large DNA fragments. Nevertheless, careful handling of the available DNA (stored at -20 °C in 10 µg aliquots of varying volume) by using wide-bore tips and avoiding repeated freeze-thaw cycles, permitted its use in long-range restriction mapping. 10 µg of DNA was digested overnight with 50 units of *Sac*II (Eurogentec, Seraing, Belgium) in a total volume of 250 µl at 37 °C. The next day, the volume was reduced to approximately 90 µl by evaporation at 65 °C, after which 30 µl of molten 2 % low-melting agarose (FMC Bioproducts, Rockland ME, USA) was added. This mixture was allowed to solidify in the slots of a 10-slot 1 % agarose gel in a CHEF-DR III electrophoresis system (Bio-Rad, Hercules CA, USA). The gel was equilibrated in the electrophoresis buffer (0.5 x TBE with 0.2 µg/ml ethidium bromide) for one hour, after which pulsed-field gel electrophoresis was performed for 18 hours at 6 V/cm with an included angle of 120 ° and a switch time linearly increasing from 2 to 17 seconds, at 14 °C. Southern blotting and hybridisation were the same as described before [Koppens *et al.* 1992].

***CYP21* mutation analysis**

CYP21/*CYP21P* mutation analysis by specific amplification of three sections of each gene was performed as described earlier [Koppens *et al.* 2000]. In addition, a smaller part of *CYP21* (from the third to the sixth exon) was amplified to check whether the mutations Ile172→Asn and IleValGluMet-236→AsnGluGluLys, which were found on the same chromosome by segregation analysis, were in the same *CYP21* gene. This was done with the forward primer TCCTTGGGAGACTACTCCCT (*CYP21*, exon 3) and the reverse primers TGCCTCAGCTGCATCTCCAC (IleValGluMet-236) and TGCCTCAGCTGCTTCTCCTC (AsnGluGluLys-236); 30 cycles of PCR at an annealing temperature of 57 °C using 1 unit of GoldStar DNA polymerase (Eurogentec, Seraing, Belgium) in a final volume of 40 µl in the presence of 1 % formamide and 1.25 mM $MgCl_2$. Nucleotide and amino acid numbering is according to Higashi *et al.* [1986].

***TNXB* and *XA* analysis**

The tenascin-X (*TNXB*) gene differs from the truncated *XA* pseudogene by a 120 bp deletion [Gitelman *et al.* 1992]. To determine the extent of any unequal crossover or gene conversion that may have created the unusual haplotypes described here, we amplified an approximately 2.7 kb section of either gene using specific primers that lie beyond the RCCX module's duplication boundary combined with a non-specific primer between the 3' part of *CYP21* or *CYP21P* and the location of the 120 bp deletion (details to be published elsewhere).

Results

Restriction mapping

Characteristic *Taq*I restriction patterns are shown in fig. 1. Since the band intensities obtained after hybridisation to the *CYP21* and *C4* probes did not match the common haplotypes, we used *Sac*II digestion and pulsed field gel electrophoresis [Carroll *et al.* 1989; Dunham *et al.* 1989] to determine the size of the entire RCCX area on each chromosome (see fig. 2). Combining these banding patterns and sizes with family study data where available (details not shown), the results were interpreted as follows:

- lane 1: four RCCX modules on one chromosome, with one *CYP21* gene and three *CYP21P* pseudogenes and four long *C4* genes (haplotype H1 in fig. 3); on the other chromosome, a standard bimodular arrangement (haplotype A2 [Koppens *et al.* 1992]);
- lane 2: three RCCX modules on one chromosome, with two *CYP21* genes and one *CYP21P* pseudogene and three short *C4* genes (haplotype F2); on the other chromosome, a monomodular arrangement with a *CYP21* gene and a short *C4* gene (haplotype B2);
- lane 3 and 4: as lane 2, but one long and two short *C4* genes on the trimodular chromosome (haplotype F1); on the other chromosome, a bimodular (haplotype A1) and a monomodular (haplotype B1) arrangement, respectively;
- lane 5: a bimodular chromosome with two *CYP21* genes and no *CYP21P* gene (haplotype G1); on the other chromosome, a bimodular (haplotype A1) arrangement.

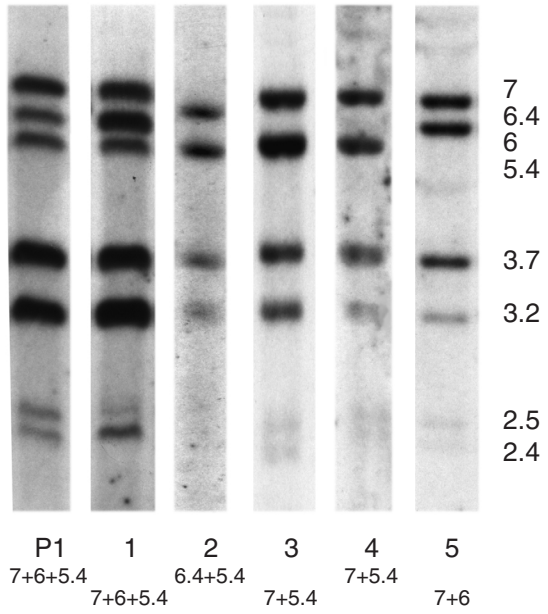

Fig. 1. *Taq*I digested genomic DNA separated by agarose gel electrophoresis and hybridised to a mix of a *CYP21* and *C4* cDNA probe. P1: person with two bimodular chromosomes, one with two long C4 genes and one with a short and a long C4 gene; 1-5: persons with an unusual RCCX arrangement on one chromosome (see text for interpretation). Right: CYP21 (3.7/3.2) and TNX (2.5/2.4) fragments; the C4 fragment sizes are at the bottom of each lane.

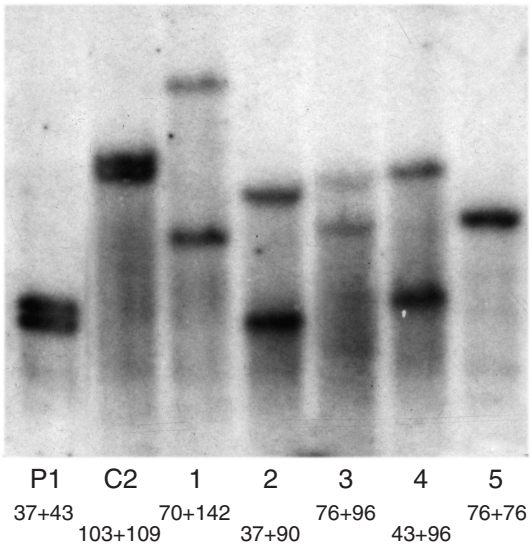

Fig. 2. *Sac*II digested genomic DNA separated by pulsed field gel electrophoresis and hybridised to a mix of a *CYP21* and *C4* cDNA probe. P2: person with two monomodular chromosomes; P3: person with two trimodular chromosomes; 1-5: persons with an unusual RCCX arrangement on one chromosome (see text for interpretation). The fragment sizes in kb are at the bottom of each lane.

***CYP21* mutation analysis**

The haplotypes with two *CYP21* genes and one *CYP21P* gene were found in three controls (haplotype F1, twice; F2, once) and in one 21-hydroxylase deficiency patient. They all carried the Gln318→stop mutation in one of the two *CYP21* genes. No other mutations were found in the controls (all of them also had Gln318). The 21-hydroxylase deficiency haplotype (haplotype F2) had the intron 2 splice mutation in addition.

The haplotypes with two *CYP21* genes and no *CYP21P* genes (haplotype G1) were found in three controls. All these chromosomes carried Ile172→Asn and IleValGluMet→ AsnGluGluLys. A separate PCR from exon 3 to exon 6 confirmed that the two mutations were in the same *CYP21* gene: the allele-specific oligonucleotide detecting Asn172 hybridised only to the product of the PCR that used the AsnGluGluLys oligonucleotide as a reverse primer. Still, these haplotypes were not completely identical in all three persons: one person had only the 'A' variant at nucleotide 655, one had 'C' only, and one had both 'A' and 'C'.

The number of independent haplotypes not carrying steroid 21-hydroxylase deficiency in our study was 365; the total for haplotypes with two *CYP21* genes (F1, F2 and G1) among these was 6, or 1.6 %; 95 % confidence interval 0.7-3.3 % [Linder 1960].

The chromosome with four RCCX modules was found in the mother of a CAH patient and does not carry 21-hydroxylase deficiency. As expected, none of the common mutations were found in the *CYP21* gene on this chromosome.

***TNXB* / *XA* characteristics**

The 120 bp deletion was found in all RCCX modules except in the most centromeric one in each haplotype, so only the genuine full-size *TNXB* gene contained the extra 120 bp.

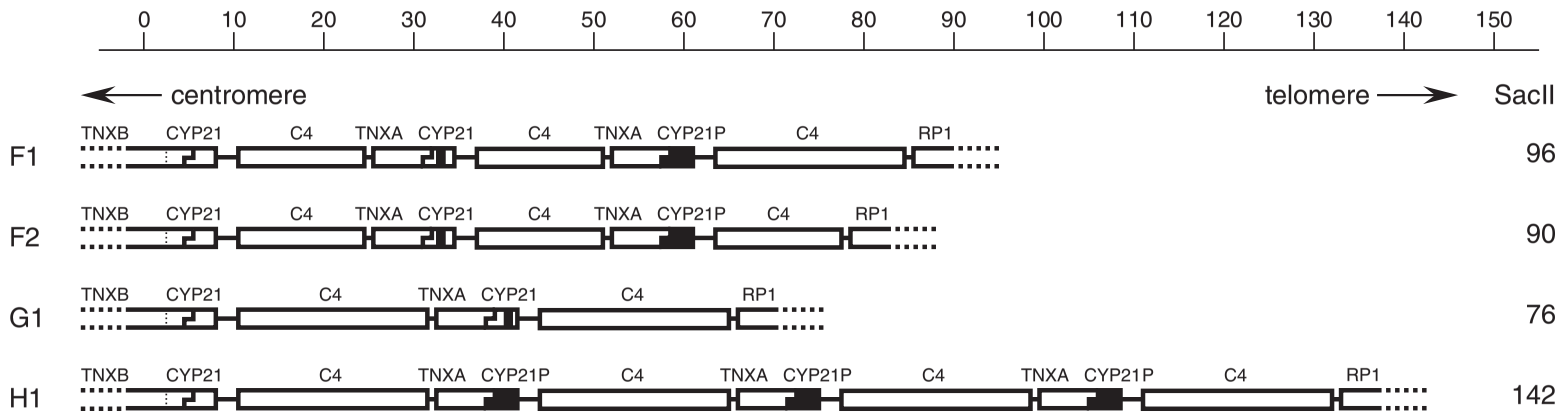

Fig. 3. Four unusual CYP21/C4 haplotypes. CYP21P-like regions are shown in black, with the small black areas within the duplicated CYP21 genes representing defects in those genes that match the normal sequence of CYP21P. A 120 bp sequence present in TNXB but not in TNXA, is indicated by a little dashed line. Right: size of the corresponding *Sac*II restriction fragments in fig. 2. Assignment of the defects to the telomeric CYP21 in each haplotype is tentative: it has not been determined which CYP21 gene actually carries the defect, but the active CYP21 gene is normally in the centromeric module and borders on TNXB. Top: scale in kb, with the centromeric RCCX duplication boundary at 0.

Discussion

We used a combination of restriction mapping and PCR-based mutation analysis to characterise four uncommon variants of the RCCX region found in a population of steroid 21-hydroxylase deficiency patients and controls from the Netherlands (see fig. 3).

Two haplotypes (F1 and F2 in our earlier report [Koppens *et al.* 1992]) each carry three RCCX modules and two *CYP21* genes. Haplotype F1 is the same as haplotype H in the report of Schneider [1990] and haplotype 9 in the report of Haglund-Stengler *et al.* [1991]. Each of the haplotypes F1 and F2 has the Gln318→stop mutation in one of the *CYP21* genes, but not all of them carry a defect in the other *CYP21* gene. Haplotype F1 has been found in three Swedish patients who also carried the codon 318 nonsense mutation, plus the intron 2 splice mutation in the other *CYP21* gene [Wedell *et al.* 1994]. We also found the intron 2 splice mutation as an additional defect in one patient, but in haplotype F2 rather than F1. Haplotype F2, which has not been described before in the literature, has the rare feature of lacking a long *C4* gene despite being multimodular. Bimodular [Hillarby *et al.* 1990; Schneider 1990] and trimodular [Weg-Remers *et al.* 1997] haplotypes without a long *C4* gene have been found before, but their frequency is very low in Caucasians.

Haplotype G1 has two *CYP21* genes and no *CYP21P* genes and is the same as haplotype 10 in the report of Haglund-Stengler *et al.* [1991]; it has also been mentioned by Sinnott *et al.* [1991]. We here demonstrate that one of the *CYP21* genes on such chromosomes has two contiguous mutations (Ile172→Asn and IleValGluMet236→AsnGluGluLys) and that not all haplotypes G1 are the same.

Chromosomes with more than three RCCX module are quite rare. Haplotype H1 is characterised by three *CYP21P* pseudogenes and one functional *CYP21* gene. The only previously described chromosome with four RCCX modules carried only *CYP21P* genes and was found in a steroid 21-hydroxylase patient who was homozygous for it due to consanguinity [Collier *et al.* 1989].

In total, we found six chromosomes carrying two *CYP21* genes each in our control group of (currently) 145 persons and three more such chromosomes in a group of 45 families with steroid 21-hydroxylase deficiency patients. All these chromosomes carry one *CYP21* gene with a genetic defect that would have caused classical steroid 21-hydroxylase deficiency in the absence of a second, functional, *CYP21* gene. This has significant implications for PCR-based carrier detection in the general population: without knowledge of the overall structure of their RCCX regions, these individuals could easily have been mistaken for carriers of salt-losing steroid 21-hydroxylase deficiency. This diagnostic pitfall is likely to play a role in other populations too, since *CYP21* duplications have been found in several countries, sometimes with a frequency equal to or above the normal carrier rate of 21-hydroxylase deficiency [Haglund-Stengler *et al.* 1991; Koppens *et al.* 1992; Wedell *et al.* 1994; Bachega *et al.* 1999]. Mutation analysis of spouses of known carriers for purposes of genetic counselling should take special account of this possibility.

In conclusion, we have characterised four unusual variants of the RCCX module. Three of these variants carry two *CYP21* genes, and they are not less frequent than steroid 21-hydroxylase deficiency alleles. We therefore recommend that whenever a *CYP21* defect is found in an individual not otherwise known as a carrier of steroid 21-hydroxylase deficiency, the overall structure of the RCCX module is ascertained to avoid erroneous assignment of carrier status that could otherwise result from PCR-based mutation analysis.

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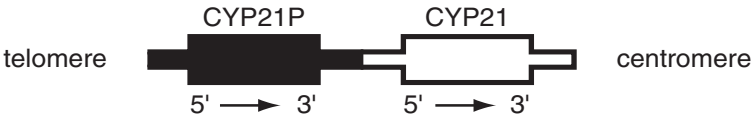
Chapter 8

General Discussion

8.1. Mutational mechanisms causing *CYP21* defects

What has mutated?

Mutation means change, so the study of disease-causing mutations finds its basis in the assumption that certain states are 'normal', and that mutations may change those states to 'defective'. Understanding the mutational mechanisms that ultimately lead to steroid 21-hydroxylase deficiency therefore starts with charting the differences between normal and defective *CYP21* genes and haplotypes. For clarity, mutations will be described here relative to a 'standard' haplotype with a repetitive layout consisting of two tandemly arranged RCCX modules (schematically shown below in a 5' to 3' orientation relative to the transcription of *CYP21*; *C4*, *TNX* and *RP1* have been omitted):



CYP21P differs from *CYP21* by a number of deleterious mutations rendering it a pseudogene and by several neutral polymorphisms, the most characteristic of which associates *CYP21P* with a 3.7 kb *Taq*I fragment as opposed to 3.2 kb for *CYP21* [Higashi *et al.* 1986; White *et al.* 1986; Rodrigues *et al.* 1987]. With a frequency of 40-70 %, this haplotype is the most common in all populations studied (see table 4 in Chapter 2.1 and table 5 in Chapter 2.2).

Categories of defects

Early haplotyping studies that predate *CYP21* mutation analysis [White *et al.* 1985; Carroll *et al.* 1985; Donohoue *et al.* 1986a; Donohoue *et al.* 1986b; Werkmeister *et al.* 1986] found three categories of defects on the chromosomes of steroid 21-hydroxylase deficiency patients, which by comparison to the standard haplotype were named:

- *CYP21* gene deletion:

- *CYP21* gene conversion:

- and, by exclusion, *CYP21* 'small' mutation:

Various other configurations were also found (*CYP21P* deletion, *CYP21P* duplication), but these entailed only differences in the copy number of the *CYP21P* pseudogene and the size of the *C4* genes (not shown) and were categorised '*CYP21* small mutation' if they carried steroid 21-hydroxylase deficiency.

This approach was based on the presence or absence of a *Taq*I restriction site normally found in the 5' flanking sequence of *CYP21P* only. This marker has since proved its value as a very reliable identifier of pseudogene-like structures and hence, defectiveness. The above-mentioned categorisation still persists and is used even by modern mutation analysis studies, although the distinction between the categories is often made by a PCR-based method. While there is nothing wrong with this categorisation from the viewpoint of establishment of mutation frequencies in different populations (see table 5 in Chapter 1), later studies have shown that the names originally assigned to the three categories do not completely match the genetic mechanisms that created them [White *et al.* 1988; Higashi *et al.* 1988; Donohoue *et al.* 1989; Sinnott *et al.* 1990; Donohoue *et al.* 1995; Yang *et al.* 1999; Chapters 3-5]. This has resulted in a somewhat confusing terminology to describe different configurations and defects. As discussed in the following three paragraphs, there are indeed three categories, but they do not necessarily match the three possibilities mentioned above on a 1-to-1 basis.

Sequence transfer by unequal crossover

Meiotic unequal crossover between homologous chromosomes occurs when similar sequences that are part of different genes align and a crossover transfers the stretch of DNA separating the misaligned gene from one chromosome to the other. Differences in RCCX modularity apparently enhance the propensity to misalign [Sinnott *et al.* 1990, Yang *et al.* 1999], but unequal crossover can also occur between two 'standard' chromosomes [Chapter 4]. Depending on the original RCCX arrangement on each chromosome, the crossover may give rise to new configurations with the same or a different number of modules than the original chromosomes. As shown in fig. 1, this process of combined expansion and contraction can hypothetically generate all known *CYP21* haplotypes. Of course, many other crossover events than the examples shown are conceivable: fig. 1 is a simplification, providing a hypothetical origin for *CYP21* haplotypes that have been defined on the basis of *Taq*I restriction analysis. In reality, the site of the crossover may vary and the high degree of similarity between RCCX modules makes it uncertain whether crossovers are limited to specific regions [Donohoue *et al.* 1989; Chu *et al.* 1992; Donohoue *et al.* 1995; Burch *et al.* 1997; Levo and Partanen 1997; Rupert *et al.* 1999; Yang *et al.* 1999; l'Allemand *et al.* 2000; Chapters 3-5] or whether they can occur anywhere within the approximately 30 kb region involved.

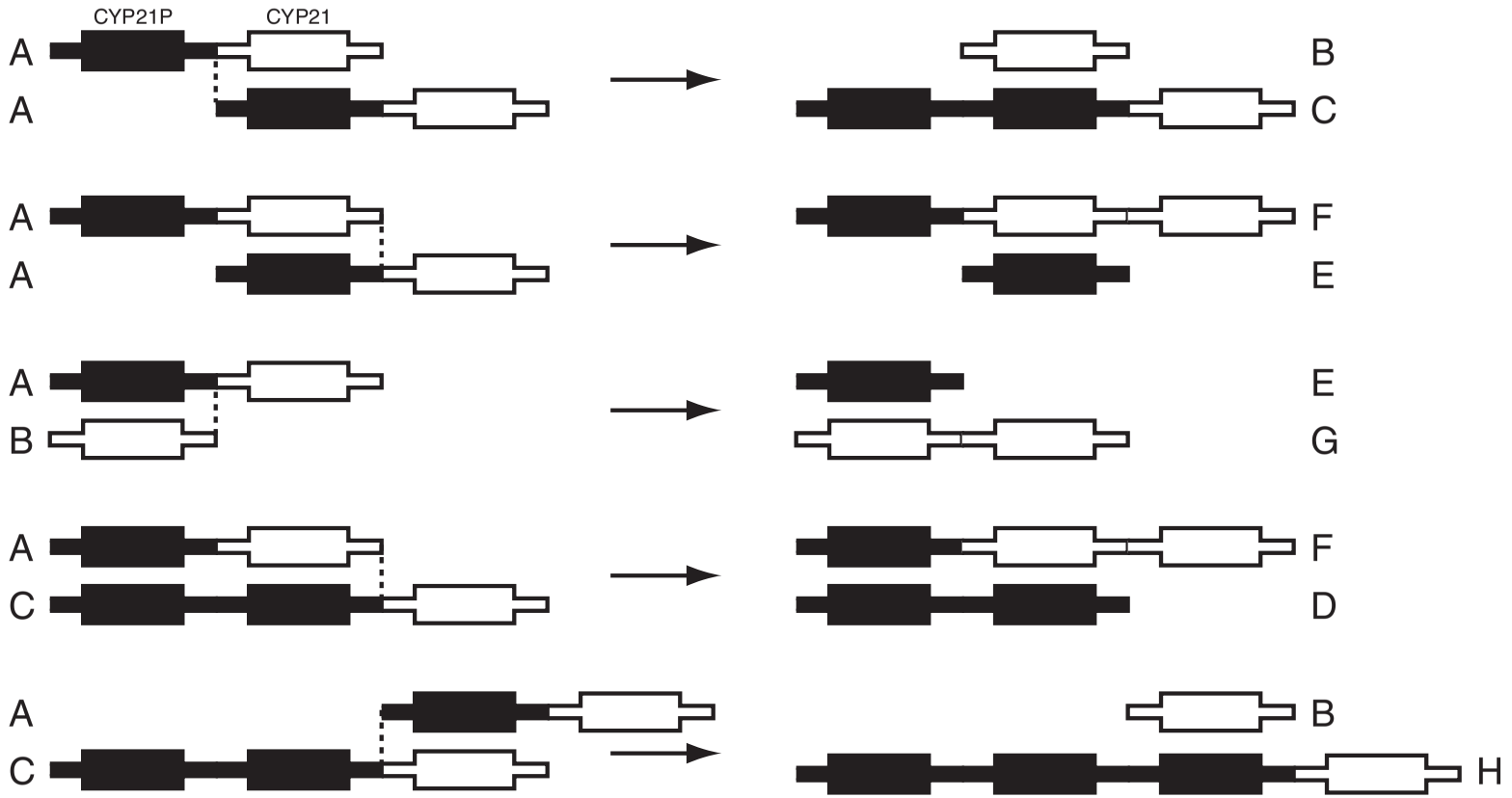


Fig. 1. Putative crossover events generating variable modularity of the RCCX module and hence, different *CYP21* haplotypes. Some of these possibilities have actually been observed as *de novo* mutations in steroid 21-hydroxylase deficiency patients [Sinnott *et al.* 1990; Chapter 4], the others are hypothetical. The haplotype designations (A-H) are defined in Chapter 2. The dashed lines connecting each set of chromosomes shown on the left hand side of the figure indicate the crossover site.

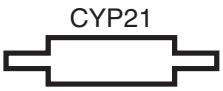
Examples of hybrid RCCX modules generated by crossovers occurring at different sites are given in fig. 7 in Chapter 1. Focusing on *CYP21* and *CYP21P*, some typical outcomes are:

or:

or:

or:

or:



Clearly, not each unequal crossover necessarily contributes to steroid 21-hydroxylase deficiency. This concept provides an adequate explanation for the '*CYP21* gene deletions' and '*CYP21* gene conversions' mentioned above. The notion that these configurations represent different outcomes of essentially the same process, effectively unites the first two of the original categories. Unequal crossover was early recognised as the mechanism for 30 kb deletion in the *C4*/*CYP21* region [Carroll *et al.* 1985], and detailed comparison of the centromeric RCCX modules of haplotypes previously designated 'deletion' and 'conversion' later showed a high degree of similarity [Chapters 3 and 5]. Since there is no reason to propose different mechanisms for equal outcomes, the term 'conversion' should be avoided in this context [Chapter 5], especially since it may cause confusion with the mechanism that causes 'smaller' mutations of *CYP21*.

Defects attributed to past meiotic unequal crossover account for 20-40 % of the steroid 21-hydroxylase deficiency alleles in most populations studied.

Sequence copying by gene conversion

Gene conversion is a non-reciprocal transfer of sequences of near-identity and is believed to be responsible for the large majority of *CYP21* defects other than those caused by unequal crossover. Introduction of *CYP21P*-like defects into *CYP21* by putative gene conversion occurs primarily during mitosis [Tusié-Luna and White 1995]. This would seem to imply copying of a section of the *CYP21P* gene that lies on the same chromosome by means of sister chromatid interaction, but a comparison of homologous sequences in *CYP21* and *CYP21P* suggests that the other (homologous) chromosome is involved in at least some of these conversions [Chapter 3.1]. Consistent with this finding, the preference for sister chromatid interaction in mitotic repair processes has been attributed to its proximity rather than to any mechanism excluding the other chromosome from conversion-like events [Johnson and Jasin 2000].

Gene conversions in the MHC typically involve an area not larger than a few hundred bp [Kuhner *et al.* 1991; Bergström *et al.* 1998; Högstrand and Böhme 1999; Martinsohn *et al.* 1999], a much smaller area than the entire 30 kb RCCX module exchanged by the large-scale unequal crossovers described above. These small-scale gene conversions may therefore transfer individual defects of varying severity (as well as neutral polymorphisms) between *CYP21P* and *CYP21*:

or:

or:

or:

A gene conversion is assumed whenever comparison to the consensus sequences [Higashi *et al.* 1986; White *et al.* 1986; Rodrigues *et al.* 1987] shows that a *CYP21P*-like change is present in an otherwise (especially, *Taq*I restriction pattern-wise) *CYP21*-like gene, or vice

versa. Small-scale gene conversions transferring a mutation from *CYP21P* to *CYP21* have attracted attention as the most common cause of *CYP21* defects, but they may also involve neutral polymorphisms [Chapter 2.3], provide transfers in the opposite direction [Chapter 3.1], or change the flanking *C4* or *TNX* genes.

Old and new mutations

Recombination has been extensively studied in yeast, where the possibilities to examine all products of crossing over or gene conversion and to artificially induce mutagenic DNA repair mechanisms provide excellent research opportunities. Advances in PCR-based analysis methods have greatly contributed to our understanding of analogous processes in the mammalian MHC [Carrington 1999; Martinsohn *et al.* 1999; Jeffreys *et al.* 2001], but so far only one study directly comparing meiotic crossovers and mitotic conversions between *CYP21* and *CYP21P* has been done [Tusié-Luna and White 1995]. Therefore, assumptions about the causes of *CYP21* defects are typically based on comparison of the mutated sequence to the *CYP21* and *CYP21P* consensus sequence [Higashi *et al.* 1986; White *et al.* 1986; Rodrigues *et al.* 1987] or on relatively rare *de novo* events that have occurred in patients suffering from 21-hydroxylase or complement C4 deficiency [Fasano *et al.* 1992; Chapter 4]. While firm evidence now supports *de novo* unequal crossover, gene conversion is still a more difficult process to understand. It has been demonstrated that gene conversion can introduce *CYP21P*-like sequences into *CYP21* [Tusié-Luna and White 1995], but authors reporting on *de novo* small-scale mutations in 21-hydroxylase deficiency patients have been quick to attribute similar events to gene conversion without analysing the *CYP21P* genes that supposedly provided these altered sequences (see table 4 in Chapter 1). Since *CYP21P* genes present in the general population are highly variable, especially in the 3' region [Helmberg *et al.* 1992a; Wedell and Luthman 1993a; Chapter 3.1], a complete analysis is necessary to distinguish gene conversion from unequal crossover. Vice versa, a conversion involving the *Taq*I site in the 5' flank of *CYP21P* may resemble an unequal crossover with the crossover site in or near the first exon of *CYP21*; the difference can often be inferred from the composition of the adjacent *C4* gene.

Deducing mutational mechanisms from the analysis of existing defects is equally difficult: for example, 'mosaic genes' with multiple *CYP21-CYP21P* transitions can be explained by multiple independent conversion events, by a single complex 'patchwork' conversion [Jeffreys *et al.* 1999], or by a crossover with a non-standard *CYP21P* gene. While the RCCX module provides an interesting model to study recombinational events in humans, a full understanding the intricacies of misalignment and sequence transfer in this region is still beyond the scope of present-day knowledge.

Mutations not related to *CYP21P*

A small number (under 5 % in most populations) of all steroid 21-hydroxylase deficiency alleles are attributed to 'other' mutations: *CYP21* defects that are not found in the consensus *CYP21P* sequence:

The only mutation of this category found at a somewhat higher frequency is Pro453→Ser, typical of late-onset 21-hydroxylase deficiency [Helmberg *et al.* 1992b; Owerbach *et al.* 1992; Wedell *et al.* 1992; Chapter 3.2]. An overview of all *CYP21* defects known to date is presented in table 3 in Chapter 1.

8.2. Steroid 21-hydroxylase deficiency alleles in the population

Classical congenital adrenal hyperplasia conveys a severe selective disadvantage

Today's high standard of medical care allows patients with severe steroid 21-hydroxylase deficiency to live and thrive. However, only a few decades ago the picture was quite different: salt-losing congenital adrenal hyperplasia was nearly always fatal [Iversen 1955]. In addition, reduced fertility is an issue even today in classical [Meyer-Bahlburg 1999; Jääskelainen *et al.* 2000; Cabrera *et al.* 2001; Krone *et al.* 2001] and, to a lesser degree, in nonclassical patients [Morán *et al.* 2000]. This raises the question how steroid 21-hydroxylase deficiency alleles have persisted at the relatively high frequency of (approximately) 1 %.

Ancestral haplotypes and heterozygosity

Specific combinations of MHC alleles appear to be evolutionary conserved in certain populations [Degli-Esposti *et al.* 1992; Dawkins *et al.* 1999]. Although it is not known whether the HLA specificities carried by these 'ancestral' haplotypes confer an immunological advantage, association of steroid 21-hydroxylase deficiency alleles with such haplotypes may contribute to maintaining these alleles in the population. It has been suggested that HLA specificities associated with the 21-hydroxylase deficiency allele found at high frequency in Yupik-speaking Inuit population of Alaska offer protection against *Haemophilus influenzae* type b infections [Petersen *et al.* 1984]. If the total number of MHC alleles in a population is limited, the benefit of HLA heterozygosity in pathogen resistance [Meyer and Thomson 2001] may counteract the disappearance of specific MHC alleles and at the same time maintain recessive defects associated with them. In addition, a more vigorous cortisol response to ACTH in carriers of 21-hydroxylase deficiency than in controls has been observed [Witchel *et al.* 1997a]. The authors suggest that this constitutes a state of heightened resistance to

environmental stress and thus offers a selectional advantage to heterozygotes, but this interpretation is open to debate [Wilckens 1997; Witchel *et al.* 1997b].

Founder effects and *de novo* mutations

The frequency of steroid 21-hydroxylase deficiency alleles varies between populations (see table 5 in Chapter 1). Two well-known examples are the high frequency of classical 21-hydroxylase deficiency in Yupik-speaking Inuits [Speiser *et al.* 1992] and of nonclassical 21-hydroxylase deficiency in Ashkenazi Jews [Speiser *et al.* 1985, Zerah *et al.* 1990], each caused by a specific mutation and associated with specific HLA serotypes. These phenomena are attributed to founder effects.

The contribution of *de novo* mutations to the genetic defects in classical steroid 21-hydroxylase deficiency has been estimated at 1-2 % [Speiser *et al.* 1992; Levo and Partanen 2001]. Assuming a carrier rate of 1:55 for classical 21-hydroxylase deficiency, this corresponds to a mutation frequency of 1:5500 to 1:2750. *De novo* unequal crossovers and gene conversions have been tested in a 100 bp area of *CYP21* and were found in frequencies of 1 in 10^5 to 10^6 and 1 in 10^3 to 10^5, respectively [Tusié-Luna and White 1995]. For the crossovers, this corresponds to a mutation frequency of 1:2,000 to 1:20,000 in the approximately 5 kb area where they can occur. The only gene conversion tested was the common intron 2 splice mutation; a similar contribution may be expected from other mutations.

Thus, *de novo* mutations appear to play a significant role in maintaining the relatively high frequency of steroid 21-hydroxylase deficiency alleles. Improved medical care may tilt the balance between *de novo* mutations and alleles lost to selection that apparently existed throughout history, but the above numbers clearly indicate that no significant addition to the current carrier rate is to be expected from *de novo* mutations within the foreseeable future.

Allele frequencies and genetic mechanisms

It is an appealing thought that population genetics, mutational mechanisms and disease severity may each have contributed to the frequencies of different steroid 21-hydroxylase deficiency alleles in different ways. Apart from founder effects and selectional advantages conferred by linked genes see above), *de novo* mutations may contribute to the relatively high allele frequencies of some severe defects that constitute a large selectional disadvantage as compared to milder defects.

Chromosomes without a *CYP21* gene have been observed at high frequencies (30-45 %) in steroid 21-hydroxylase patients from Northern Europe (for references, see table 5 in Chapter 1, table 4 in Chapter 2.1 and table 5 in Chapter 2.2). *De novo* unequal crossovers involving chromosomes with a single RCCX module [Sinnott *et al.* 1990; Yang *et al.* 1999] may have contributed to this high frequency because they necessarily misalign to bimodular chromosomes during meiosis (see fig. 7 in Chapter 1). The ancestral haplotype HLA-

A1,B8,DR3 [Tokunaga *et al.* 1988; Zhang *et al.* 1990; Price *et al.* 1999] accounts for many of these monomodular chromosomes, since its frequency in the general population is as high as 13 % in Great Britain [Davidson *et al.* 1988] and 24 % in Ireland [T. Barnes, quoted by Sinnott *et al.* 1990]. For the Netherlands, the phenotype frequency of HLA-B8 is 0.228 [Lagaay *et al.* 1991], matching an allele frequency of 0.12; the data from Great Britain show that the large majority of HLA-B8 haplotypes also carries DR3 [Davidson *et al.* 1988]. In contrast, the lower frequency of chromosomes lacking *CYP21* in 21-hydroxylase deficiency patients from Japan is paralleled by a low frequency of HLA-A1,B8,DR3 in that country [Tokunaga *et al.* 1997].

Another example is the 'intron 2 splice' mutation, the most common defect in many populations (see table 5 in Chapter 1). Intron 2 is a region of high sequence variability in both *CYP21* and *CYP21P* [Killeen *et al.* 1998; Jiddou *et al.* 1999] and a neutral intron 2 polymorphism typical of *CYP21P* was found at a low frequency in *CYP21* genes [Chapter 2.3]. This suggests a relatively high frequency of small-scale gene conversion in or around intron 2, and since the splice junction defect is found in all *CYP21P* genes [Chapter 3.1], a small-scale *CYP21P* to *CYP21* gene conversion involving this site will always transfer this defect.

Other severe defects (IleValGluMet-236→AsnGluGluLys, Gln318→stop and Arg356→Trp), are not as common as the two mentioned above (see table 5 in Chapter 1). *De novo* mutations more often involve the region from intron 2 to intron 3 than the region from exon 6 to exon 8, which is the location of these mutations (see table 4 in Chapter 1). This region may be less prone to gene conversion; also, many conversions at these sites would remain without effect since *CYP21P* does not always carry these defects [Helmberg *et al.* 1992a; Wedell and Luthman 1993a; Chapter 3.1].

The less severe defect Val281→Leu lies in the same area as the three severe mutations just mentioned. Here, the contribution of *de novo* mutations to the allele frequency is probably less than in the case of the intron 2 splice mutation, but the selectional disadvantage of this nonclassical mutation is also smaller. It has even been suggested that virilisation and early puberty caused by nonclassical steroid 21-hydroxylase deficiency may be a selectional advantage in dire circumstances [Hochberg 1995].

Defects that are not normally found in *CYP21P* typically have very low frequencies in *CYP21*, because their numbers are not augmented by gene conversion. The only exception is Pro453→Ser, again a very mild mutation.

Summarising, a picture emerges of severe *CYP21* defects that confer a definite selectional disadvantage, but are more likely to arise as *de novo* mutations, versus mild *CYP21* defects that spread more freely through the population. This is in line with the rather similar worldwide mutational spectrum and incidence of classical steroid 21-hydroxylase deficiency (ranging between 1 in 10,000 to 1 in 20,000 in most populations) versus the much larger differences for nonclassical 21-hydroxylase deficiency [Speiser *et al.* 1985; Zerah *et al.* 1990; Chapter 3.2].

8.3. Implications and perspectives of *CYP21* mutation analysis

Phenotype-genotype relationship

Whereas 'genotype' of course refers to DNA sequence variability, 'phenotype' may be treated as a biochemical or a clinical entity, or both. The possibility of different genetic defects in salt-losing and simple virilising steroid 21-hydroxylase deficiency was recognised early [Degenhart *et al.* 1965] and combinations of 'severe defect', 'mild defect' and 'normal' were used to categorise patients and heterozygotes [New *et al.* 1988] even before these defects could be identified at a molecular level. Mutation analysis has since allowed definition of 26 'mutation groups': combinations of two specific mutations in a patient. Many of these groups show some degree of discordance between the observed phenotype and the phenotype predicted from the mildest of the two defects [Wilson *et al.* 1995]; a recent review [New 2001] mentions 85 mutation groups. The authors of these reports emphasise the importance of this 'mutation group' concept especially for directing prenatal treatment.

In most patients, there is a good correlation between genotype and clinical phenotype [Speiser *et al.* 1992; Wilson *et al.* 1995; Krone *et al.* 2000]. Nevertheless, the severity of steroid 21-hydroxylase deficiency shows a remarkable variation between individuals within the same mutation group [Wilson *et al.* 1995; Witchel *et al.* 1996b; Chapter 3.1], even between sibs [Bormann *et al.* 1992; Speiser *et al.* 1992; Wilson *et al.* 1995; Chin *et al.* 1998] and in the same patient over an extended period of time [Speiser *et al.* 1991; Kohn *et al.* 1995; Hoffman *et al.* 1996; Morán *et al.* 2000]. The most remarkable cases are those where mutation analysis indicates complete steroid 21-hydroxylase deficiency, but steroids normally requiring 21-hydroxylation were still found [Speiser *et al.* 1991; Chapter 6]. Identification of alternative enzymes that may explain such findings has already been described as 'the next major advance in our understanding of the clinical biology of congenital adrenal hyperplasia due to 21-hydroxylase deficiency' [Miller 1997]. The versatility of cytochrome P-450 enzymes [Negishi *et al.* 1996] makes them prime candidates to be involved in alternative pathways, notably in the unusual physiological circumstances provided by accumulating adrenocortical precursor steroids. A further indication of the involvement of alternative synthetic routes are the genotype-phenotype discrepancies found in other defects of adrenocortical steroid biosynthesis [Zachmann 1995].

Mutation analysis in patients and their family members

The complications in genotype-phenotype relationship notwithstanding, mutation analysis holds definite benefits to steroid 21-hydroxylase deficiency patients and their family members [Nordenström *et al.* 1999, Ritzén *et al.* 2000]. Checking for the most common mutations can conveniently be done by rapid detection methods such as allele-specific PCR [Wedell and Luthman 1993b], and these mutations account for approximately 90 % of all defects. Mutation analysis can take away any doubts by confirming a diagnosis based on 17α-hydroxyprogesterone measurements, notably in cases where higher levels may be

attributable to other factors (such as stress, or an adenoma), or in premature infants where they are intrinsically high. In patients where steroid replacement therapy has already been installed, a discrepancy between the established diagnosis and the *CYP21* defects found would justify a re-evaluation of clinical chemical data that may result in alteration of the diagnosis and the treatment protocol.

A lot of experience has been gained with prenatal treatment of 21-hydroxylase deficient patients [Forest *et al.* 1998; New 2001], although this remains a controversial issue [Miller 1998]. Prenatal treatment is installed before the diagnosis can be made, and mutation analysis provides the earliest possibility to determine whether the foetus is affected and thus, whether treatment should be continued. It has been advised that only centres able to perform rapid genotyping should be involved in prenatal treatment [Ritzén 1998].

In members of a patient's family, direct analysis of *CYP21* defects provides the most reliable identification of heterozygote carriers for purposes of genetic counselling, since it avoids interpretation problems due to recombination that may occur when flanking markers are used. Knowledge of the carried mutation is especially relevant to family members of patients with milder forms of steroid 21-hydroxylase deficiency. These patients are often compound heterozygotes with mutations of different severity, so sibs may be carriers of either a mild or a severe defect.

Finally, since the clinical phenotype may vary considerably, mutation analysis may identify sibs (notably, older brothers) who carry the same defects as the index case, or even an unsuspected different defect [Witchel *et al.* 1996a; Oriola and Pavia 1997], but have so far not presented with clinical signs of congenital adrenal hyperplasia. This may justify a clinical (re-)examination of such sibs.

Mutation analysis in the general population

Mutation detection in unrelated individuals is more difficult due to the variable nature of the RCCX module. Routine PCR-based analysis typically produces false-negatives in carriers with a heterozygous *CYP21* deletion [Ritzén *et al.* 2000] and false-positives in case of a heterozygous *CYP21* duplication [Chapter 7]. To avoid such pitfalls, the overall genetic structure of the RCCX module should be charted by means of, for example, restriction analysis of genomic DNA.

When common *CYP21* defects have been reliably ruled out in a person without a family history of steroid 21-hydroxylase deficiency, the availability of allele frequencies in a typical patient group and in the general population [Chapter 3] allows an estimate of the remaining chance that the person in question is still a carrier. This falls short of 'absolute' certainty, but in view of the frequent occurrence of *de novo* mutations (see above), the possibility of transmitting steroid 21-hydroxylase deficiency to one's children cannot be ruled out even by exhaustive mutation searching.

Molecular genetic research may yet provide an answer to the question whether some persons are especially susceptible to *de novo* mutations in this region. As argued before, misalignment

between *CYP21* and *CYP21P* is more likely to occur when the modularity of the RCCX area differs on each chromosome. In addition, subtle sequence variations that influence the RCCX mutation rate may be identified, by analogy to the small changes that have been found to exert a profound influence on minisatellite instability [Jeffreys *et al.* 1999].

Mass screening of *CYP21* mutations in the general population is not feasible at present. Therefore, case survey and neonatal screening programmes will remain the key to early detection of at-risk children. Nevertheless, ongoing advances in DNA technology may make mutation analysis replace 17α-hydroxyprogesterone measurement as the method of choice for this purpose within the foreseeable future. The authors of a recent large genotyping study [Fitness *et al.* 1999] discussed several methods to make routine DNA analysis in population screening programmes more rapid and cost-effective. *CYP21* genotyping has already been used to confirm screening results in preterm infants [Nordenström *et al.* 2001]. Mutation analysis will also improve the detection of patients with nonclassical steroid 21-hydroxylase deficiency, who may escape detection by current screening programmes [Tajima *et al.* 1997]. Inevitably, implementing mutation analysis will lead to the identification of not only steroid 21-hydroxylase deficiency patients, but also many heterozygous carriers of the disease, so the ethical implications of such a change need to be carefully considered.

Future developments: a speculation

For all the benefits that DNA analysis already offers to basic science and clinical diagnosis, we only begin to understand the impact of mutations on the functioning of enzymes such as steroid 21-hydroxylase *in vivo*. Nobody knows the treatment protocols of the future, but since the *CYP21* defects hitherto discovered affect such various aspects of enzymatic function as membrane attachment, haem group integration, cytochrome P-450 reductase interaction and enzyme stability, it may one day seem less than obvious to continue the current practice of treating these different defects in the same way.

Gene therapy of steroid 21-hydroxylase deficiency has met with success in mouse models [Gotoh *et al.* 1994; Tajima *et al.* 1999], but considering the difficulties of mimicking the subtle regulatory mechanism of the hypothalamic-pituitary-adrenal axis, its application in humans does not seem imminent [White 2001].

Finally, basic genomic research in this field is also far from completed: the intricacies of the genetic mechanisms causing *CYP21* and RCCX variability are still far from clear. The complexity of this highly variable section of the central MHC makes it as challenging a subject as ever to investigators of human evolution.

A quote from a 1987 Lancet editorial reads: 'Nothing in genetics is as straightforward as it seems at first sight, and this is certainly true of 21-hydroxylase deficiency' [Lancet 1987; ii: 663]. This seems every bit as true today as it was 15 years ago.

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Chapter 9

Summary / Samenvatting

9.1. Summary

The human adrenal cortex synthesises three main classes of steroids from cholesterol: mineralocorticoids, glucocorticoids, and (pre-)androgens. Aldosterone, the main mineralocorticoid, contributes to the regulation of plasma sodium levels, primarily by stimulating renal salt resorption. Cortisol, the main glucocorticoid, plays a key role in the body's response to stress: it influences the metabolism of both lipids and carbohydrates, has an anti-inflammatory effect and functions in many other physiological processes. Pre-androgens secreted by the adrenal have a limited biological activity, but they are converted peripherally to the powerful androgen testosterone.

Three biosynthetic pathways are active in distinct parts of the adrenal cortex to produce these steroids. Two of these pathways, those producing aldosterone and cortisol, require an enzymatic conversion step catalysed by steroid 21-hydroxylase. Deficiency of steroid 21-hydroxylase occurs in approximately 1 in 10,000 to 1 in 15,000 newborn children around the world. In its severe (classical) form, lack of cortisol continually stimulates the hypothalamic-pituitary-adrenal axis, and the resulting high levels of plasma ACTH cause the symptom that gives this disease its name: congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency (other adrenocortical enzyme deficiencies cause under 10 % of all cases of congenital adrenal hyperplasia). The hyperplastic adrenal produces excess amounts of pre-androgens, which cause pre- and postnatal virilisation and initially, rapid growth with advanced bone age, but results in below-average final height. If the defect is complete, lack of aldosterone causes urinary salt loss. Untreated patients may succumb to dehydration combined with loss of sodium and electrolyte imbalance within the first weeks of life.

Steroid 21-hydroxylase is encoded by the *CYP21* gene, which lies near the gene encoding the fourth component of complement (C4) in the central (class III) region of the human major histocompatibility complex (MHC) on chromosome 6p21.31. *CYP21* is part of a 30 kb stretch of DNA known as the RCCX module after its constituent genes: (part of) *RP*-*C4*-*CYP21*-(part of) *TNX*. Most chromosomes have two RCCX modules arranged in tandem, and the counterpart of *CYP21* in the other module is *CYP21P*, a pseudogene which is 98 % homologous to *CYP21* but inactive due to several deleterious mutations. Steroid 21-hydroxylase deficiency is caused by a defect in *CYP21*, and nearly all such defects are the result of some form of interaction that occurred between *CYP21* and *CYP21P* during the course of evolution.

Many alternatives to the 'standard' arrangement with two RCCX modules exist, and the first step towards understanding the origin of disease-causing mutations in this region is mapping these so-called 'haplotypes'. Haplotyping, which is described in Chapter 2, is based on the analysis of characteristic restriction patterns that match various arrays of *CYP21*, *CYP21P*, *C4*, and *TNX*. A combination of short-range and long-range mapping and segregation analysis in families of 21-hydroxylase deficiency patients, allowed the definition of about 15 distinct haplotypes, the frequencies of which vary between populations.

Haplotyping also provides a categorisation of *CYP21* defects: as judged by haplotype analysis, about one-third of all steroid 21-hydroxylase deficiency alleles results from absence of the *CYP21* gene. Such chromosomes carry one or two RCCX modules with only *CYP21P*-like genes. Unequal crossover during meiosis is the genetic mechanism that causes deletions of *CYP21*. The high degree of similarity between RCCX modules promotes misalignment, as illustrated in Chapter 4: a *de novo* deletion had occurred in a patient's father, who thus transmitted this genetic defect without being a carrier himself. The crossover site was mapped to the *TNX* gene, centromeric to *CYP21*.

By comparison to the standard layout (one *CYP21* gene and one *CYP21P* pseudogene), bimodular haplotypes with two *CYP21P* genes (carrying 21-hydroxylase deficiency) have been labelled 'large-scale gene conversion of *CYP21* to *CYP21P*' (as opposed to monomodular '*CYP21* deletion' chromosomes with one *CYP21P* gene). However, the identification of each gene as either *CYP21* or *CYP21P* by means of haplotyping is based on a very reliable but functionally indifferent marker: a single *Taq*I restriction site in the 5' flanking sequence of *CYP21P* only. A detailed comparison of haplotypes without *CYP21* genes (Chapters 3 and 5) showed that two categories exist: *CYP21P*/*CYP21* hybrids, with the putative crossover site in *CYP21*, and *TNX* hybrids, where *CYP21* is completely missing and the crossover site lies within the *TNX* gene. The hybrid *TNX* gene in the latter category carries an additional genetic defect: tenascin-X deficiency, which in its homozygous state causes a variant of the Ehlers-Danlos syndrome, a group of connective-tissue diseases. The two categories are identical in monomodular and in bimodular chromosomes without a *CYP21* gene, so we hypothesised that these hybrids were generated by similar genetic mechanism (unequal crossover) regardless the presence or absence of an additional RCCX module, and argued against the usage of the term 'large-scale gene conversion' in this context (Chapter 5).

Gene conversion does play a role in the origin of *CYP21* defects, however. In two-thirds of all steroid 21-hydroxylase deficiency alleles, *CYP21* is present. Chapter 3 contains a comparison of the disease-causing mutations in defective *CYP21* genes to the variability of *CYP21P* pseudogenes. *CYP21* defects can be transferred from *CYP21P* by small-scale gene conversions covering at most a few hundred base pairs. However, *CYP21P* is so variable (especially in its 3' section) that often, conclusions about the extension of small-scale gene conversions cannot be inferred from the mutations found in *CYP21*. This notably applies to the 'mosaic' conversions with multiple apparent *CYP21*-*CYP21P* transitions that have been reported in the literature.

As shown in Chapters 2 and 3, about 90 % of all deficiency alleles in salt-losing 21-hydroxylase deficiency are the result of either a *CYP21* deletion or a small-scale conversion that causes a *CYP21P*-like mutation in *CYP21*.

Some steroid 21-hydroxylase defects are relatively mild and do not become manifest until puberty or adolescence. The allele frequency of this 'nonclassical' form of 21-hydroxylase deficiency is very high (up to 20 %) in some populations. However, a mutation analysis in a group of healthy controls (Chapter 3) demonstrated that the frequency of nonclassical 21-hydroxylase deficiency alleles in the Netherlands is approximately 1 %.

In steroid 21-hydroxylase deficiency, the correlation between genotype and phenotype is good in most patients: a severe defect on both chromosomes nearly always causes salt loss. The most severe defects are those that preclude any transcription of *CYP21*, or cause a structural distortion of steroid 21-hydroxylase that abolishes its enzymatic activity. Surprisingly, two patients with such severe defects (described in Chapter 6) were able to synthesise significant amounts of aldosterone. Presence of aldosterone in these patients' serum was confirmed by combining two analytical methods (HPLC and radioimmunoassay). Peripheral aldosterone synthesis by other enzymes may be the cause of this peculiar phenomenon.

For genetic analysis of *CYP21* defects, it is often assumed that a chromosome either carries a *CYP21* gene, or has a *CYP21* deletion. Chapter 7 demonstrates that this assumption is not without risk: chromosomes with two *CYP21* genes in tandem are found in the general population at a frequency equal to or higher than steroid 21-hydroxylase deficiency alleles. Since one of the two *CYP21* genes is defective on such chromosomes, PCR-based mutation analysis without haplotyping can lead to erroneous assignment of carrier status.

9.2. Samenvatting

De bijnierschors van de mens zet cholesterol om in drie hoofdgroepen van steroïden: mineralocorticoïden, glucocorticoïden en (pre-)androgenen. Aldosteron is het belangrijkste mineralocorticoïde: het draagt bij tot de regulatie van de natriumspiegels in het bloed, o.a. door de zoutresorptie door de nieren te stimuleren. Cortisol is het belangrijkste glucocorticoïde en speelt een belangrijke rol bij de reactie van het lichaam op stress: het beïnvloedt het koolhydraat- en het vetmetabolisme, onderdrukt ontstekingsreacties en heeft functies in vele andere fysiologische processen. De pre-androgenen die door de bijnier geproduceerd worden hebben zelf een beperkte biologische activiteit, maar ze worden perifeer omgezet in het krachtige androgeen testosteron.

Deze steroïden worden in drie verschillende compartimenten van de bijnier gesynthetiseerd via afzonderlijke routes. Een enzymatische omzetting die door steroïd 21-hydroxylase gekatalyseerd wordt, maakt deel uit van twee van deze routes: de produktie van aldosterone en van cortisol. Wereldwijd worden ongeveer 1:10000 à 1:12000 kinderen geboren met steroïd 21-hydroxylase deficiëntie. In de ernstige (klassieke) vorm van deze ziekte induceert de lage cortisolspiegel via de hypothalamus-hypofyse-as afscheiding van extra ACTH. De permanent verhoogde ACTH-spiegel leidt weer tot het verschijnsel waar de ziekte haar naam aan ontleent: congenitale bijnierschorshyperplasie. Steroïd 21-hydroxylase deficiëntie komt verreweg het meeste voor: deficiënties van andere bijnierschors-enzymen veroorzaken minder dan 10 % van de gevallen van congenitale bijnierschorshyperplasie. De sterk in omvang toegenomen bijnier produceert dan grote hoeveelheden pre-androgenen, wat tot prenatale en postnatale virilisatie leidt, en aanvankelijk snelle groei met sterk voorlopende botleeftijd veroorzaakt, maar in relatief geringe eindlengte resulteert. Bij volledige deficiëntie is ook geen aldosteron aanwezig, waardoor zoutverlies via de urine optreedt. Onbehandelde patiënten kunnen hierdoor binnen enkele weken na de geboorte overlijden door ernstig natrium- en vochtverlies, gepaard gaande met andere electrolyt-afwijkingen.

Het steroïd 21-hydroxylase-gen heet *CYP21*. Dit gen ligt naast het gen voor de vierde component van het complement-systeem (*C4*) in het ‘major histocompatibility complex’(MHC) op chromosoom 6p21.31. *CYP21* maakt deel uit van een stuk DNA van 30 kb dat bekend staat als de RCCX-module. Vier genen geven de RCCX-module haar naam: (een deel van) *RP1*-*C4*-*CYP21*-(een deel van) *TNX*. Op de meeste chromosomen liggen twee RCCX-modules naast elkaar. Het equivalent van *CYP21* in de ander module heet *CYP21P*, een pseudogen dat voor 98 % overeenkomt met *CYP21* maar niet functioneert omdat het verschillende mutaties bevat. Steroïd 21-hydroxylase deficiëntie wordt veroorzaakt door een defect in *CYP21*. Zulke defecten zijn bijna altijd het gevolg van tijdens de evolutie opgetreden interactie tussen *CYP21* en *CYP21P*.

Er bestaan veel andere mogelijkheden dan de standaardconfiguratie met twee RCCX-modules. Om te begrijpen hoe de mutaties die tot 21-hydroxylase deficiëntie leiden ontstaan zijn, moeten eerst deze zogenaamde 'haplotypen' in kaart gebracht worden. Haplotypering (zie

hoofdstuk 2) is gebaseerd op analyse van restrictiepatronen die karakteristiek zijn voor diverse configuraties van *CYP21*, *CYP21P*, *C4* en *TNX*. Ongeveer 15 verschillende haplotypen konden gedefinieerd worden door in families van patiënten met 21-hydroxylase deficiëntie een segregatieanalyse van restrictiepatronen uit te voeren.
Haplotypering levert ook een indeling van defecten in *CYP21* op: deze methode laat zien dat ongeveer een derde van alle deficiëntie-allelen veroorzaakt wordt door afwezigheid van het *CYP21*-gen. Op zulke chromosomen zijn één of twee RCCX-modules aanwezig, steeds met alleen *CYP21P*-achtige genen. Dergelijke deleties van *CYP21* worden veroorzaakt door ongelijke crossing-over tijdens de meiose: omdat de RCCX-modules sterk op elkaar lijken kunnen ze 'verkeerd' naast elkaar gaan liggen. Hoofdstuk 4 geeft een voorbeeld hiervan: een deletie van *CYP21* was *de novo* opgetreden in de vader van een patiënt, die zodoende een defect erfde terwijl de vader zelf geen drager was. Deze crossing-over was opgetreden in het *TNX*-gen, dat aan de centromere kant van *CYP21* ligt.
In vergelijking met de standaardconfiguratie (één *CYP21*-gen en één *CYP21P*-pseudogen) worden haplotypen met twee RCCX modules die allebei een *CYP21P*-gen bevatten (en dus 21-hydroxylase deficiëntie dragen) vaak 'grootschalige genconversies van *CYP21* naar *CYP21P*' genoemd (tegenover 'deleties van *CYP21*' voor haplotypen met één RCCX-module met een *CYP21P*-gen). Maar de genen worden alleen als *CYP21* of als *CYP21P* geïdentificeerd op grond van één betrouwbare, maar functioneel neutrale, marker: een *Taq*I restrictiesite die alleen voorkomt in de flankerende sequentie aan de 5' kant van CYP21P. Een gedetailleerde vergelijking van haplotypen zonder *CYP21*-genen (hoofdstukken 3 en 5) toonde aan dat er twee categorieën zijn: *CYP21/CYP21P*-hybriden, waarbij de plaats van de veronderstelde crossing-over in *CYP21* ligt, en *TNX*-hybriden, waarbij *CYP21* volledig ontbreekt en de plaats van de crossing-over in *TNX* ligt. In deze laatste categorie is er een extra defect door het hybride *TNX*-gen: tenascine-X deficiëntie. Als dit defect homozygoot aanwezig is, leidt het tot een vorm van het syndroom van Ehlers-Danlos, een groep van bindweefselziekten. De beide categorieën zijn identiek op monomodulaire en bimodulaire chromosomen zonder *CYP21*-gen. We gaan er daarom van uit dat, ongeacht de aan- of afwezigheid van nog een extra RCCX-module, hetzelfde genetische mechanisme (ongelijke crossing-over) ten grondslag ligt aan deze hybriden. Dit vormt een argument tegen het gebruik van de term 'grootschalige genconversie' in dit verband (zie hoofdstuk 5).
Toch speelt genconversie een rol bij het ontstaan van defecten in *CYP21*. Bij tweederde van de steroïd 21-hydroxylase deficiëntie-allelen is *CYP21* aanwezig. In hoofdstuk 3 worden de *CYP21*-mutaties die steroïd 21-hydroxylase deficiëntie veroorzaken, vergeleken met de variabiliteit van het *CYP21P* pseudogen. Kleinschalige genconversie die hoogstens een paar honderd baseparen omvatten kunnen defecten overbrengen van *CYP21P* naar *CYP21*. Omdat *CYP21P* echter zo variabel is, vooral aan de 3'-kant, kunnen conclusies over de grootte van deze conversies niet afgeleid worden uit de mutaties in *CYP21*. Dit speelt in het bijzonder bij de in de literatuur beschreven 'mozaiek-conversies' die meerdere *CYP21-CYP21P*-overgangen lijken te hebben.

Uit de hoofdstukken 2 en 3 blijkt dat ongeveer 90 % van de deficiëntie-allelen in steroïd 21-hydroxylase met zoutverlies, het resultaat is van hetzij een deletie van *CYP21*, hetzij een kleinschalige conversie die een *CYP21P*-achtig defect in *CYP21* veroorzaakt heeft.
Bepaalde steroïd 21-hydroxylase-defecten zijn relatief mild en komen pas tegen de puberteit of later naar voren. De allelfrequentie van deze 'niet-klassieke' vorm van 21-hydroxylase deficiëntie is zeer hoog (tot 20 %) in sommige bevolkingsgroepen. Een mutatie-analyse in een groep controlepersonen (hoofdstuk 3) toonde echter aan dat de allelen voor niet-klassieke 21-hydroxylase deficiëntie in Nederland een frequentie hebben van ongeveer 1 %.
Er bestaat bij steroïd 21-hydroxylase deficiëntie een goed verband tussen fenotype en genotype bij de meeste patiënten: een ernstige mutatie op beide chromosomen leidt bijna altijd tot zoutverlies. De ernstigste defecten verstoren de transcriptie van *CYP21* of veroorzaken een structurele verandering van steroïd 21-hydroxylase die enzymatische activiteit onmogelijk maakt. Het is dan ook verrassend dat twee patiënten met dergelijke ernstige defecten toch in staat waren, aldosteron te synthetiseren (zie hoofdstuk 6). Een combinatie van twee analytische methoden (HPLC en radioimmunoassay) werd gebruikt om de aanwezigheid van aldosteron in het serum van deze patiënten te bevestigen. Dit eigenaardige verschijnsel wordt wellicht veroorzaakt door perifere vorming van aldosteron door andere enzymen.
Bij de genetische analyse van *CYP21*-defecten wordt vaak aangenomen dat een chromosoom een *CYP21*-gen heeft, of dat er een *CYP21*-deletie is. In hoofdstuk 7 wordt aangetoond dat deze aanname niet zonder risico is: chromosomen met twee *CYP21* genen komen in de bevolking minstens zo vaak voor als steroïd 21-hydroxylase deficiëntie-allelen. Omdat één van deze twee *CYP21*-genen op dergelijke chromosomen defect is, zou mutatieanalyse uitsluitend op basis van de polymerase-kettingreactie en zonder haplotypering tot verkeerde conclusies over dragerschap leiden.

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Curriculum vitae

I, Paul Koppens, was born on February 16, 1958 in Brecht in the North of Belgium. My parents are Frans Koppens and Mieke Koppens-de Koning. I grew up in and around The Hague in the Netherlands, and finished Gymnasium B at the local Aloysius College in 1976. I read biology at Leiden University and graduated in 1983, specialising in biochemistry and also qualifying as a teacher.

From 1984 onwards, I worked as a research scientist on two projects at the Laboratory of Paediatrics, Erasmus University / Sophia Children's Hospital, Rotterdam (supervisor: Prof. Dr. H.J. Degenhart). This thesis is the final result of the second project, which was started in late 1988 but was gradually discontinued as a full-time activity during 1991 due to lack of funding, and pursued when circumstances allowed it for many more years than could be foreseen at that time.

In 1992, I switched to informatics as a professional career, and was employed by several companies and institutions, most of the time by Datastream/SQL Systems, a global provider of asset lifecycle management software and services. Fortunately, it turned out that software development suited my abilities better than did biochemistry – from a viewpoint of creativity as well as economy. At present, I am happily combining my interests and experience in both fields by designing a research database at the Laboratory of Paediatrics of the Erasmus University, subdivision Paediatric Oncology/Haematology.

In the mean time, the research project had been lingering on, typically during off-hours and at a highly irregular pace. After carefully considering whether continuing the project would be a worthwhile effort, I took some time off (in 2000-2001) to finish the job.

I have no clue what to do next. I'll consider that an opportunity.

Ik, Paul Koppens, ben geboren op 16 februari 1958 in Brecht in het noorden van België. Mijn ouders zijn Frans Koppens and Mieke Koppens-de Koning. Ik ben opgegroeid in de buurt van Den Haag en behaalde het Gymnasium-B-diploma aan het Aloysius College in 1976. In 1983 studeerde ik in Leiden af in de biologie met als specialisatie biochemie. Tevens verkreeg ik een eerstegraads onderwijsbevoegdheid.

Vanaf 1984 werkte ik als wetenschappelijk onderzoeker aan twee projecten bij het Laboratorium Kindergeneeskunde, Erasmus Universiteit Rotterdam / Sophia Kinderziekenhuis (onder supervisie van Prof. Dr. H.J. Degenhart). Dit proefschrift is het eindresultaat van het tweede project, dat eind 1988 van start ging maar door gebrek aan geldmiddelen in de loop van 1991 niet meer op voltijd-basis voort te zetten was. Voor zover de omstandigheden het toelieten werd het project daarna nog lange tijd vervolgd – veel langer dan we toen dachten.

In 1992 stapte ik over naar de automatisering en werkte bij verschillende bedrijven en instanties, de langste tijd bij Datastream/SQL Systems, een bedrijf dat wereldwijd software en diensten aanbiedt voor het levenscyclusbeheer van bedrijfsmiddelen. Gelukkig bleek software-ontwikkeling in creatief en economisch opzicht beter bij mijn talenten te passen dan biochemie. Momenteel kan ik tot mijn voldoening mijn belangstelling en ervaring in deze gebieden combineren door een onderzoeksdatabase te ontwerpen op het Laboratorium Kindergeneeskunde van de Erasmus Universiteit, subafdeling Kinderoncologie/Haematologie.

Intussen liep het onderzoeksproject nog steeds door, zij het op vreemde tijdstippen en in onregelmatig tempo. Na zorgvuldig overwogen te hebben of het verstandig was er nog meer energie in te steken heb ik (in 2000-2001) een tijd vrijaf genomen om dit karwei af te ronden.

Ik heb geen idee wat ik nu ga doen. Dat biedt kansen.

Nawoord

Nou, het was nog een hele klus, dat proefschrift. Gelukkig heb ik het niet helemaal alleen hoeven doen. Veel mensen hebben geholpen: sommigen door actief bij te dragen, anderen door mogelijkheden te bieden, weer anderen door er gewoon te zijn - en menigeen op al deze manieren.

Prof. Dr. Herman Degenhart, mijn eerste promotor. Beste Herman, sinds ik me ergens diep in het vorig millennium op het Laboratorium Kindergeneeskunde meldde, heb je mij vele malen aangezet tot kritisch nadenken, en daarna tot heroverweging van het resultaat van dat gepieker. Zelfs deze passage in het nawoord is geen uitzondering....het is niet eenvoudig om de merkwaardige onderneming die we samen ten einde hebben gevoerd in enkele rake bewoordingen te schetsen. Het was een bijzondere tijd, en een bijzondere ervaring om met een waar geleerde te mogen samenwerken. Ik geloof niet dat er ooit een zinnig onderwerp ter tafel is gekomen waarover je niet een interessant gezichtspunt kon bieden. Dank voor al je kennis en vriendschap. En dank voor de gastvrijheid die Syt en jij in Ruinen geboden hebben.

Theo Hoogenboezem, steun en toeverlaat gedurende vele jaren. Beste Theo, je bent een onderzoeker in de ware zin des woords: iemand die nieuwsgierig in het leven staat en (schijnbaar) argeloos zijn vragen stelt. Vaststellen van haplotypen vereist natuurlijk een experiment - maar vaststellen van het vocabulaire of de irritatiedrempel van een nieuwe medewerker eveneens. De inventiviteit waarmee je menig praktisch probleem oploste, heeft altijd diepe indruk op me gemaakt. Een betere paranymf kan ik me niet wensen. Bedankt voor al je inzet en vriendschap, voor de gezellige avonden bij jou en Hanna thuis, en vooral ook voor de licht-absurdistische conversatie die we zo vaak voerden. Wees niet teleurgesteld als de mensen je soms lijken te begrijpen: ze kijken stiekem in een woordenboek.

Dr. Dicky Halley, bron van kennis van begin tot einde. Beste Dicky, ook al ben je er zelf nog zo bescheiden over: ik geloof nooit dat al ons geploeter zonder jouw nuchtere en deskundige inbreng veel had opgeleverd. Geweldig bedankt voor al je hulp aan een groepje lieden dat zomaar op een willekeurige dag, nu al meer dan tien jaar geleden, binnen kwam vallen. En ook voor de vele 'valuable suggestions about the manuscript' - al die veel te lange verhalen van mij heb je toch steeds weer doorgespit. Helaas, 'kort en bondig' is nog altijd niet mijn sterkste punt....op welke bladzijde zitten we hier eigenlijk?

Prof. Dr. Hans Büller, mijn tweede promotor. Beste Hans, hartelijk dank dat je zo vlot bereid was om deze taak op je te nemen - ik ben uiteindelijk bepaald niet de enige die op zo'n manier een beroep op je doet. Je vriendelijke en bemoedigende woorden over het manuscript waren een welkome opsteker voor mij. Ook wil ik je hartelijk danken voor de gastvrijheid die ik als 'overjarige' promovendus binnen de afdeling Kindergeneeskunde genoten heb.

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André, Anne-Paulet, Annette, Bas, Bas, Bert, Bob, Bob, Bram, Chen, David, David, Debra, Diny, Dirk, Dorine, Edzard, Ella, Elly, Eric, Floris, Fred, Geoffrey, Gerard, Guus, Hans, Henk, Henk, Henk, Henk, Henk-Jan, Herman, Ineke, Inge, Jaap, Jack, Jacques, Jan, Jan, Jan, Jan, Jan-Erik, Jan-Willem, Jelco, Jochen, Joost, Jos, Josée, Kareem, Liesbeth, Luc, Marcel, Marcel, Marcel, Marian, Marion, Marion, Marja, Marja, Maryam, Michael, Mu, Murat, Nathalie, Onno, Patricia, Paul, Peter, Peter, Pieter-Dirk, Rembert, Rene, René, Rob, Roel, Ronald, Ruud, Sagita, Sam, Sam, Suzanne, Tineke, Tom, Vincent, Wilbert, Willem, Wim, Wim, Wistaria, Wouter: bedankt voor een onvergetelijke tijd.
Mijn lieve zus Hanneke, je hebt er 'even' op moeten wachten, maar ik vind het fantastisch dat je nu toch mijn paranymf wilt zijn. Frans en Mieke, ik weet hoe jullie over 'dankbare kinderen' denken, maar mag ik na een paar honderd bladzijden taaie tekst een halve regel gebruiken om te zeggen dat ik van jullie hou?
Tenslotte een woord van dank aan de Sophia Stichting Wetenschappelijk Onderzoek, die dit project gedurende een aantal jaren heeft gefinancierd, en aan de Stichting 'Irene Kinderziekenhuis', die ons een financiële bijdrage verschafte op een moment dat die hard nodig was.

Een nawoord schrijven is leuk, maar ook lastig. Het is het laatst geschreven en eerst gelezen deel van een proefschrift, dus het vergt enige oplettendheid. Het duiden van de formuleringen in de dankjewel-paragraaf en het afleiden van verborgen betekenissen daaruit zou een nieuwe categorie schriftgeleerden in het leven kunnen roepen. De verleiding is groot om er een soort 'disclaimer' in te zetten, zoiets als: "wie niet expliciet als 'gezellig' omschreven wordt, moet daaruit niet opmaken dat...." - maar ja, dat snappen jullie wel.
Beste Herman, één kleine revanche moet je me maar gunnen: ik laat het uitgaan 'als een nachtkaars'.

26 juni 2002 Paul Koppens

'There was a point to this story, but it has temporarily escaped the chronicler's mind.'

Douglas Adams