

Carriership of a defective tenascin-X gene in steroid 21-hydroxylase deficiency patients: *TNXB–TNXA* hybrids in apparent large-scale gene conversions

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Steroid 21-hydroxylase deficiency is caused by a defect in the *CYP21A2* gene. *CYP21A2*, the adjacent complement *C4* gene and parts of the flanking genes *RP1* and *TNXB* constitute a tandemly duplicated arrangement in the central (class III) region of the major histocompatibility complex. The typical number of repeats of the *CYP21/C4* region is two, with one repeat carrying *CYP21A2* and the other carrying the highly homologous pseudogene *CYP21A1P*. By comparison with this standard, three categories of *CYP21A2* defects have traditionally been distinguished: *CYP21A2* deletions, large-scale gene conversions of *CYP21A2* into a structure similar to *CYP21A1P*, and smaller mutations in *CYP21A2* (also derived from *CYP21A1P*, 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 77 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 ~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-scale 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 *CYP21A2* and *CYP21A1P* 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 (1–5). CAH due to 21-hydroxylase deficiency is characterized 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 adrenocorticotrophic hormone (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 virilization.

Steroid 21-hydroxylase deficiency has a wide range of clinical manifestations that are associated with more or less severe defects of the *CYP21A2* gene. Over 15 years ago, it was found that *CYP21A2* and the highly homologous but deficient

pseudogene *CYP21A1P* 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 (6,7). The *C4* genes may be 20.5 or 14.2 kb in size, depending on the presence or absence of an endogenous retroviral sequence in the ninth intron. On the telomeric side, the *CYP21/C4* region is flanked by the *RP1* gene (also named *G11* or *STK19*), which encodes a serine–threonine kinase (8–10). Centromeric to the *CYP21/C4* region lies *TNXB*, which encodes the extracellular matrix protein tenascin-X (11–13). The stretch of DNA that is duplicated encompasses part of *RP1*, all of *C4*, all of *CYP21* and part of *TNXB*. In this report, we will use the shorthand notation ‘RCCX module’ (9,14,15), derived from the names of the above-mentioned genes (*RP–C4–CYP21–TNX*) for the duplicated region. The RCCX module may be ‘long’ or ‘short’ depending on the size of the *C4* gene. Most chromosomes bear two modules, with a *CYP21A2* gene in the centromeric and a *CYP21A1P* gene in the telomeric position (Fig. 1), but

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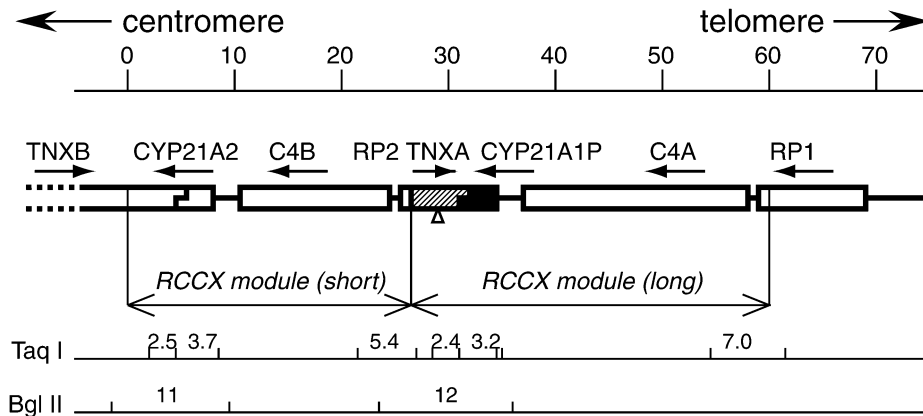


Figure 1. Overview of the *C4/CYP21* area, showing two RCCX modules as found on most chromosomes. *TNXB* is the full-size 68 kb gene for tenascin-X, *TNXA* (shown as a hatched box; also known as *XA*) is a truncated pseudogene of 5.7 kb that lacks most of the coding sequence of *TNXB* and has a deletion of 120 bp (indicated by the small triangle) spanning an exon–intron boundary (11–14). *CYP21A2* (also known as *CYP21B*) is the active steroid 21-hydroxylase gene; *CYP21A1P* (shown in black; also known as *CYP21A*) is a full-size pseudogene containing several deleterious mutations throughout its sequence, including three in-phase stop codons (27). The *C4* genes express variants of the fourth component of complement with different affinities, known as *C4A* and *C4B*. The arrangement with *C4A* in the telomeric and *C4B* in the centromeric module is common, but the specificity of the *C4* genes cannot be determined by means of the restriction sites shown, and many alternative arrangements have been described in the literature. 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 *CYP21A2*, and of *TNXA* and *CYP21A1P*, respectively. Bottom: characteristic *TaqI* and *BglII* restriction fragments. Top: scale in kb, with the centromeric RCCX duplication boundary at 0.

monomodular and trimodular haplotypes are common in most populations studied (2,15), including the Dutch (16). The duplicated sections of *TNXB* and *RPI* are truncated pseudogenes named *TNXA* and *RP2*, respectively.

TaqI and *BglII* restriction analyses of genomic DNA have become tried-and-proven methods to establish the overall genetic arrangement of the *CYP21/C4* region (7,17–22). *TaqI* polymorphisms are especially useful because they provide information about several genes: (i) because of a polymorphism in the 5′ flank, *CYP21A2* is characterized by a 3.7 kb *TaqI* fragment and *CYP21A1P* by a 3.2 kb fragment (6,7); (ii) because of a 120 bp deletion in *TNXA* that does not occur in *TNXB* (11–14), *TNXB* is characterized by a 2.5 kb *TaqI* fragment and *TNXA* by a 2.4 kb fragment (7,21); (iii) the size and the position (but not the *C4A* or *C4B* specificity) of the *C4* genes can be deduced: a telomeric ‘long’ *C4* gene is characterized by a 7.0 kb *TaqI* fragment, a telomeric ‘short’ *C4* gene by a 6.4 kb fragment, a non-telomeric ‘long’ *C4* gene by a 6.0 kb *TaqI* fragment and a non-telomeric ‘short’ *C4* gene by a 5.4 kb fragment (in this context, the term ‘telomeric’ refers to the *C4* gene adjacent to *RPI*, and ‘non-telomeric’ to *C4* genes adjacent to *RP2*) (15,18,22). *BglII* restriction patterns show the number of modules: there is always one 11 kb fragment representing the centromeric module, and a 12 kb fragment for each of the other modules. These fragments and probing strategies used to detect them have been discussed in detail elsewhere (2,18,20–22).

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

- (1) chromosomes with a *CYP21A1P*-like gene and no *CYP21A2* gene (‘*CYP21A2* deletions’);
- (2) chromosomes with two *CYP21A1P*-like genes and no *CYP21A2* gene (‘large-scale gene conversions’);

- (3) chromosomes with at least one (defective) *CYP21A2* gene; here, further analysis demonstrated that most (but not all) defective *CYP21A2* genes carried one or more of a limited set of mutations typically found in *CYP21A1P*, and these defects were therefore designated ‘small-scale gene conversions’.

Although the names of these categories suggest that each has been generated by a specific genetic mechanism, this classification was primarily based on the difference between each category and the typical arrangement shown in Figure 1 (with one *CYP21A2* and one *CYP21A1P* gene). It was soon recognized that the first two categories (‘deletions’ and ‘large-scale conversions’) typically carry a hybrid gene with the 5′ portion of *CYP21A1P* joined onto the 3′ portion of *CYP21A2* (20,23–26). Since the 5′ portion contains the extra *TaqI* site (6,7,27), the hybrid is recognized as a *CYP21A1P*-like gene in restriction analysis of genomic DNA. However, since the neighbouring *TNXB* gene remains unaffected in such hybrids, its characteristic 2.5 kb *TaqI* fragment is retained. Consequently, the ratio between the 12 and 11 kb *BglII* fragments is the same as between the 2.4 and 2.5 kb *TaqI* fragments in these haplotypes (2,21).

Unequal meiotic crossover is believed to be the mechanism causing *CYP21A2* deletions, a notion firmly supported by studies of deletion haplotypes (7,20,23,28–30) and *de novo* events (31; P.F.J. Koppens, H.J.M. Smeets, I.J. de Wijs and H.J. Degenhart, manuscript in preparation). 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 *CYP21A2* has been retained to produce a partially active 21-hydroxylase enzyme (32), 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 (24–26,29,30). Recently, breakpoint locations beyond the 3′ end of *CYP21A2* have been

Table 1. Family studies of bimodular *CYP21/C4/TNX* haplotypes without a *CYP21A2* gene

Person ^a	CYP21A2 probe			C4 probe	<i>Pvu</i> MI digest of <i>TNXB</i> PCR product ^b			Haplotype codes ^c	
	<i>Taq</i> I 3.7:3.2	2.5:2.4	<i>Bgl</i> III 11:12	<i>Taq</i> I 7.0:6.4:6.0:5.4	2808	2688	2344		
3F ^d	2:2	2:2	2:2	2:0:1:1	+	—	—	A	A*
3M ^d	1:3	2:2	2:2	2:0:1:1	+	—	—	A	D*
3P ^d	1:3	2:2	2:2	2:0:1:1	+	—	—	A*	D*
14M ^e	1:3	— ^f	2:2	2:0:1:1	+	—	+	A1	D2x*
14P ^e	0:4	1:3	2:2	2:0:1:1	+	—	+	D1*	D2x*
14S ^e	1:3	— ^f	2:2	2:0:1:1	+	—	+	A1	D2x*
14S ^e	1:3	2:2	2:2	2:0:2:0	+	—	—	D1*	A1
15F	1:2	2:1	2:1	1:1:1:0	+	—	—	B2	D1*
15M	2:2	2:2	2:2	2:0:1:1	+	—	—	A2	A1*
15P	1:3	2:2	2:2	2:0:2:0	+	—	—	D1*	A1*
15S	2:1	2:1	2:1	1:1:0:1	+	—	—	B2	A2
16F	1:2	2:1	2:1	2:0:1:0	+	—	—	A1	E1*
16M	1:3	1:3	2:2	2:0:1:1	+	+	—	A1	D2x*
16P	0:3	1:2	2:1	2:0:0:1	+	+	—	E1*	D2x*
18F	1:3	— ^f	2:2	2:0:1:1	+	+	—	A1	D2x*
18M	2:2	2:2	2:2	2:0:2:0	+	—	—	A1	A1*
18P	1:3	— ^f	2:2	2:0:1:1	+	+	—	D2x*	A1*
19F	2:2	2:2	2:2	2:0:2:0	+	—	—	A1	A1*
19M	1:2	1:2	2:1	2:0:0:1	+	+	—	B1	D2x*
19P	1:3	1:3	2:2	2:0:1:1	+	+	—	A1*	D2x*
19S	2:1	2:1	2:1	2:0:1:0	+	—	—	A1*	B1
28F	1:3	2:2	2:2	2:0:2:0	+	—	—	A1	D1*
28M	2:3	2:3	2:3	2:0:3:0	+	—	—	C1	A1*
28P	1:3	2:2	2:2	2:0:2:0	+	—	—	D1*	A1*
28S	1:4	2:3	2:3	2:0:3:0	+	—	—	D1*	C1
38F	1:3	2:2	2:2	2:0:2:0	+	—	—	A1	D1*
38M	2:2	2:2	2:2	2:0:1:1	+	—	—	A2	A1*
38P	1:3	2:2	2:2	2:0:2:0	+	—	—	D1*	A1*

^aThe number indicates the family: F, father; M, mother; P, patient; S, healthy sib.

^bThe fragments are shown in Figures 3 and 4.

^cSee Table 2 for an explanation of the haplotype codes. *Carries steroid 21-hydroxylase deficiency.

^dThe band ratios can be interpreted as father A1/A2* and mother: A2/D1*, but also as father A2/A1* and mother: A1/D2*.

^eThe father was not available, but the paternal haplotypes could be deduced.

^fThe resolution of the bands was not very good, but the 2.4 kb band was more intense.

found (14,33,34; P.F.J. Koppens, H.J.M. Smeets, I.J. de Wijs, H.J. Degenhart, manuscript in preparation). Such alleles carry an additional genetic defect, because a part of the tenascin-X-producing *TNXB* gene has been replaced by its *TNXA* counterpart, containing a 120 bp deletion on an exon–intron boundary. In these haplotypes, the *TNXB–TNXA* hybrid is characterized by a 2.4 kb rather than a 2.5 kb *Taq*I fragment, and the above-mentioned parity with the *Bgl*III 12 and 11 kb fragment ratio does not apply. A homozygous defect of *TNXB* causes type II Ehlers–Danlos syndrome, a connective-tissue disease (33,34).

Insight into the mechanisms of gene conversions has not progressed at the same pace, however. The ‘large-scale gene conversion’ chromosomes with two *CYP21A1P*-like genes (that is, two genes characterized by a 3.2 kb *Taq*I fragment) indeed carry a stretch of *CYP21A1P*-like DNA encompassing several exons (25,26), although here too an exception where the ‘converted’ region was limited in size and the gene retained some activity has been demonstrated (35). Most ‘small-scale gene conversions’, on the other hand, involve a stretch of *CYP21A1P*-like DNA that is at most a few hundred base pairs in size and contains only one recognizable mutation as a marker of ‘*CYP21A1P*-ness’.

We here report on the extension of the pseudogene-like region in bimodular chromosomes with two *CYP21A1P*-like genes (termed ‘large-scale gene conversions’ in the above-mentioned categorization). Out of nine such haplotypes in a population of steroid 21-hydroxylase deficiency from 39 families studied by us, five had a *CYP21A1P–CYP21A2* transition zone in the *CYP21A2* gene, but the other four were pseudogene-like well into the *TNXB* gene. This implies that ~10% of the patients with classical 21-hydroxylase deficiency in the population that we studied 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.

RESULTS

CYP21/C4 haplotyping

We determined *CYP21/C4* haplotypes in a population of 39 Dutch families of steroid 21-hydroxylase deficiency patients

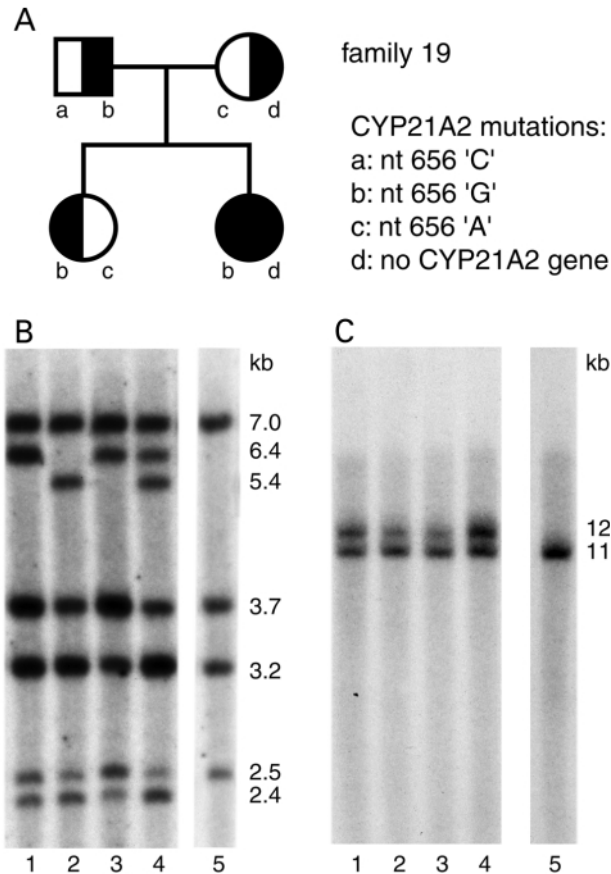


Figure 2. Family study of *TaqI* and *BglII* restriction patterns and *CYP21A2* defects. (A) Pedigree with *CYP21A2* mutations at nucleotide 656 assigned to each haplotype. (B) *TaqI* restriction patterns of genomic DNA hybridized to a mixture of the *CYP21A2* and *C4* cDNA probes; fragment sizes are in kb; the *C4* fragment sizes listed (7.0, 6.0, 5.4) are those traditionally used in the literature (18), although the actual fragments are ~ 0.15 kb larger. (C) *BglII* restriction patterns obtained with the *CYP21A2* probe only. Lanes 1–4: father, mother, healthy sister, patient. Lane 5: unrelated individual with two monomodular chromosomes, one with a *CYP21A2* gene and one with a *CYP21A1P* pseudogene; the *C4* gene on each chromosome is 'long'.

(16,26) using *TaqI* and *BglII* restriction analysis and *CYP21A2* and *C4* cDNA probes. The principal markers for *CYP21A2* and *CYP21A1P* are the 3.7 and 3.2 kb *TaqI* fragments, respectively (Fig. 1). The 3' flanking region of these genes is usually investigated by *BglII* digestion, because the *CYP21A2* cDNA probe overlaps the 2.4 and 2.5 kb *TaqI* fragments in this region by only a few hundred base pairs, often resulting in poor visualization of the bands. Normally, the estimated ratio of the *TaqI* 2.4 and 2.5 kb bands is equal to the ratio of the *BglII* 12 and 11 kb bands. However, we could distinguish the 2.4 and 2.5 kb bands on many autoradiograms (Table 1), and in some patients with an apparent 'large-scale conversion' of *CYP21A2* into *CYP21A1P*, a discrepancy between the *TaqI* and *BglII* patterns clearly existed. An example of a family study is shown in Figure 2; the results were interpreted as follows: lane 1 (father): two bimodular chromosomes with a total of two *CYP21A2* genes, two *CYP21A1P* genes, two long telomeric *C4* genes and two long non-telomeric *C4* genes; lane 2 (mother): one bimodular and one monomodular chromosome with a total of

one *CYP21A2* gene, two *CYP21A1P* genes, two long telomeric *C4* genes, and one short non-telomeric *C4* gene; lane 3 (healthy sister of the patient): one bimodular and one monomodular chromosome with a total of two *CYP21A2* genes, one *CYP21A1P* gene, two long telomeric *C4* genes and one long non-telomeric *C4* gene; lane 4 (patient with salt-losing steroid 21-hydroxylase deficiency): two bimodular chromosomes with a total of one *CYP21A2* gene, three *CYP21A1P* genes, two long telomeric *C4* genes, and one short and one long non-telomeric *C4* gene. The band ratios (listed in Table 1: family 19) were determined by laser densitometry as reported earlier (16). *CYP21A2* mutation analysis (26) showed that the paternal defect was the common splice junction mutation in the second intron (Fig. 2A). The following haplotypes were deduced: a and b (paternal): *CYP21A2*–*C4*long–*CYP21A1P*–*C4*long [haplotype A1 in our earlier report (16)]; c (maternal): *CYP21A2*–*C4*long (haplotype B1); d (maternal): *CYP21A1P*–*C4*short–*CYP21A1P*–*C4*long (haplotype D2). Lane 5 in Figure 2 shows an example of a person (not related to family 19) with two monomodular chromosomes: one with a *CYP21A2* gene and a long *C4* gene and one with a *CYP21A1P* gene and a long *C4* gene.

Surprisingly, the band ratio of the *TaqI* 2.5 and 2.4 kb fragments deviates from the band ratio of the *BglII* 11 and 12 kb fragments in the mother and the patient in family 19 (Figure 2: lanes 2 and 4). This finding, which suggests that the *TNXB* gene on this chromosome is in part *TNXA*-like, triggered the subsequent investigation of the structure of *TNXB* on similar bimodular 'conversion' haplotypes. Table 1 shows the relative band intensities and the deduced haplotypes for all eight families where such a *CYP21A1P*–*CYP21A1P* haplotype was found. Haplotype designations are the same as in our earlier report (16). The families listed in Table 1 represent nine *CYP21A1P*–*CYP21A1P* haplotypes. Even though the 2.4 and 2.5 kb *TaqI* fragments could not always be distinguished clearly, four of these nine haplotypes apparently had two 2.4 kb *TaqI* fragments and no 2.5 kb fragment; these chromosomes all had one short and one long *C4* gene. This suggested that the 120 bp deletion was present not only in *TNXA*, as expected, but also in *TNXB*. The other five chromosomes carried a 2.4 kb and a 2.5 kb *TaqI* fragment, indicating that they did not have the 120 bp deletion in *TNXB*; four of these had two long *C4* genes, while in one case (family 3), two interpretations were possible (Table 1). We found such apparent discrepancies between the *BglII* and *TaqI* restriction fragments on two other chromosomes. One of these carried steroid 21-hydroxylase deficiency and resulted from a *de novo* unequal crossover generating a monomodular chromosome (P.F.J. Koppens, H.J.M. Smeets, I.J. de Wijs and H.J. Degenhart, manuscript in preparation). The other was found in a control on a bimodular chromosome where the *TNXA* gene had partly assumed a *TNXB*-like structure that is probably the same as reported earlier (36). All other chromosomes tested, including 15 monomodular 'CYP21A2 deletion' alleles, had the 120 bp present in *TNXB* and, for multimodular chromosomes, absent in *TNXA*. Table 2 lists the frequencies of the haplotypes carrying steroid 21-hydroxylase in our study group.

Characteristics of normal *TNXA* and *TNXB* genes

The presence of a *TNXA*-like 120 bp deletion in the *TNXB* gene adjacent to the centromeric *CYP21A1P* gene in four of the nine

Table 2. *CYP21/C4/TNX* haplotypes carrying classical steroid 21-hydroxylase deficiency in Dutch patients

Modularity	Genes ^a	Code ^b	Number
Monomodular	C4L; 21A2; XB	B1	6
	C4S; 21A2; XB	B2	3
	C4L; 21A1P-A2; XB	E1	15
	C4L; 21A1P; XA-XB	E1x	1
Bimodular	C4L + C4L; 21A1P + 21A2; XA + XB	A1	26 or 27 ^c
	C4L + C4S; 21A1P + 21A2; XA + XB	A2	11 or 10 ^c
	C4L + C4L; 21A1P + 21A1P-A2; XA + XB	D1	5 or 4 ^c
	C4L + C4S; 21A1P + 21A1P-A2; XA + XB	D2	0 or 1 ^c
	C4L + C4S; 21A1P + 21A1P; XA + XA-XB	D2x	4
Trimodular	C4L + C4L + C4L; 21A1P + 21A1P + 21A2; XA + XA + XB	C1	1
	C4L + C4S + C4S; 21A1P + 21A1P + 21A2; XA + XA + XB	C2	1
	C4L + C4L + C4S; 21A1P + 21A1P + 21A2; XA + XA + XB	C3	1
	C4L + C4S + C4S; 21A1P + 21A2 + 21A2; XA + XA + XB	F1	0
	C4S + C4S + C4S; 21A1P + 21A2 + 21A2; XA + XA + XB	F2	1
Total			77

^aC4L, C4S, long, short; 21A1P-A2, *CYP21* hybrid; XA-XB, *TNX* hybrid.

^bThe haplotype codes are the same as in our earlier report (16); the appended 'x' indicates the haplotypes with a *TNXB-TNXA* hybrid rather than a *CYP21A1P-CYP21A2* hybrid; 'D2x' is the novel dual deficiency haplotype described in this report.

^cExact numbers are not certain, because the results for family 3 could be interpreted in different ways (Table 1).

'large-scale conversion' haplotypes in our population would imply that these steroid 21-hydroxylase deficiency patients would also be carriers of tenascin-X deficiency. To confirm this finding and to further characterize the putative *TNXB-TNXA* hybrid, a 2.8 kb region of either *TNXA* or *TNXB* was specifically amplified. This region was selected because the forward primer lies upstream of the duplication boundary and is specific to *TNXA* or *TNXB*, while the reverse primer lies downstream of the 120 bp deletion (in this context, 'downstream' is relative to the transcription of the *TNXB* gene). Thus, the amplified region includes the *TNXB-TNXA* transition zone in the hybrid (Fig. 3).

To further narrow down the location of the *TNXB-TNXA* breakpoint, we searched for informative differences between normal *TNXA* and *TNXB* genes, other than the 120 bp deletion. Comparison of published *TNX* sequences [EMBL/GenBank/DBJ accession nos S38953 (11), X71937 (13), AF077974 (36), AL049547 (37), AF019413 (38), U89337 (39), AF086641 (14) and L26263 (9,40)] revealed several neutral polymorphisms throughout the amplified region, some of which can be detected by restriction analysis: *Pf*MI at 276 bp downstream of the duplication boundary of the RCCX module, *Sty*I at 719 bp, *Bst*UI at 1626 bp, and *Pvu*II at 2190 bp [Fig. 3; sequence AL049547 (37) was used to compute fragment sizes and nucleotide positions]. In contrast to the 120 bp deletion, these differences do not cause a defect in the *TNX* gene, so they cannot *a priori* be considered pseudogene (*TNXA*)-like, and sequencing of the *TNXA-TNXB* hybrids would not locate a typical transition point. To determine which of the polymorphisms could be used as specific markers, we amplified a large number of *TNXA* and *TNXB* genes from individuals with one to three copies of *TNXA* and two copies of *TNXB*, digested the products with the restriction endonucleases mentioned above, and analysed them on agarose or polyacrylamide gels.

The most informative restriction sites to distinguish *TNXA* from *TNXB* were the *Pf*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 were highly polymorphic in both genes, and therefore could not be used as a reliable marker of either *TNXA* or *TNXB*. All 298 *TNXB* genes tested contained the 120 bp deletion, as opposed to only 1 out of 270 *TNXA* genes.

Characteristics of *TNXB-TNXA* hybrid genes

Since the amplified region contains only a single *Pf*MI site, detection of this polymorphism and of the 120 bp difference could conveniently be done in a single experiment. Figure 3 shows the fragments that can be expected after *Pf*MI restriction of the *TNXA* and *TNXB* PCR products; the restriction fragments found in each family with a 'large-scale conversion' haplotype are listed in Table 1.

Typical *Pf*MI banding patterns are shown in Figure 4. Out of the four bimodular *CYP21A1P-CYP21A1P* chromosomes with the 120 bp deletion in *TNXB*, one carried the *Pf*MI site at bp 276 (Fig. 4: lane 6); the remaining three, and the five *CYP21A1P-CYP21A1P* chromosomes without the 120 bp deletion in *TNXB*, 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 duplication boundary. In the other three cases, the transition probably lies further downstream, because absence of the *Pf*MI site is a *TNXB*-like feature (although it also occurs in ~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

TNXB-TNXA hybrids

Combined defectiveness of the *CYP21A2* and *TNXB* genes has so far been described in a few isolated cases in *CYP21A2* deletions caused by meiotic unequal crossover. We here report

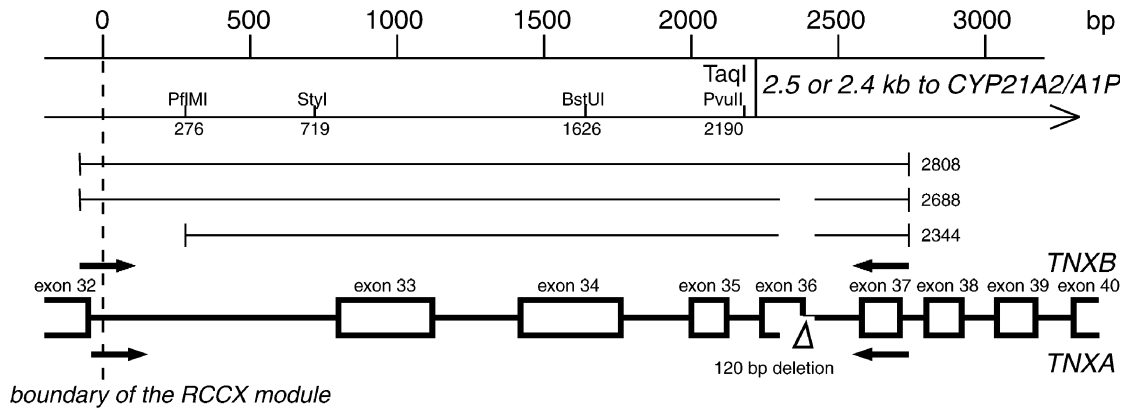


Figure 3. Amplified region of the *TNXB* and *TNXA* genes. Open boxes are exons; the triangle indicates the site of the 120 bp deletion. The bold arrows represent the primer sites for the *TNX* PCRs; the reverse primer is the same for both genes, but the forward primers are specific and their starting positions differ by 36 bp. Polymorphic restriction sites deduced from published *TNXA* and *TNXB* sequences are shown. The centre of the figure indicates the extent of the *Pfl*MI restriction fragments (2344, 2688, 2808) shown in Figure 4.

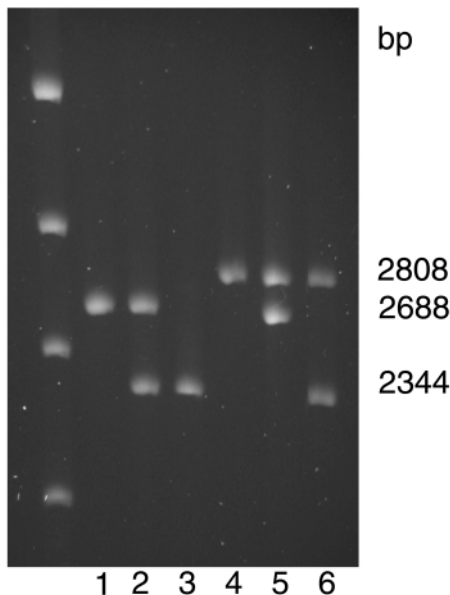


Figure 4. *Pfl*MI restriction analysis of PCR-amplified fragments of the *TNXA* and *TNXB* genes. Lane 1: *TNXA* without the *Pfl*MI site. Lane 2: *TNXA*, heterozygous for the *Pfl*MI site. Lane 3: *TNXA*, homozygous for the *Pfl*MI site. Lane 4: *TNXB*, no *Pfl*MI site. Lane 5: *TNXB*, heterozygous 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 μ l SmartLadder (Eurogentec, Seraing, Belgium); electrophoresis was for 28 h at 40 V on 1% agarose.

that such 'double-deficiency' alleles are indeed rare on monomodular chromosomes, but relatively common (four out of nine cases) on bimodular chromosomes with two *CYP21A1P*-like genes (usually termed 'large-scale conversions' in the literature). This haplotype is characterized 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 *CYP21A2/C4* haplotyping

reports available to date [most of which were recently reviewed by White and Speiser (5)], it is surprising that this haplotype has not been documented before: an apparently similar case was reported only once (41), before the discovery of the *TNX* genes. Poor resolution or poor visualization of the 2.4 and 2.5 kb bands sometimes (but not always) hampers the analysis when a *CYP21A2* cDNA probe is used. However, studies with probes that overlap a large part of these fragments (15,21,42) 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 Results section) are reliable markers of 'TNXA-ness' or 'TNXB-ness'. A characteristic *Pfl*MI site at 276 bp downstream of the RCCX duplication 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: upstream of the *Pfl*MI site in one case (family 14) and downstream in the others (Table 1 and Fig. 5). Previously, we characterized the *CYP21P* pseudogenes in these, and other, haplotypes. The haplotype with the *Pfl*MI site (in family 14; Table 1) also carried different *CYP21P* genes than the other three haplotypes [see Table 5 in our earlier report (26): second haplotype of family 14]. This suggests that these hybrids (Fig. 5C,D) were created by independently occurring recombinations instead of a by a single event followed by secondary mutation that caused the *Pfl*MI difference. In our patient group (26), *TNXB*-*TNXA* hybrids were found on 4 out of 77 chromosomes, a frequency of 0.052 (95% confidence interval 0.018-0.12) (43). Considering a carrier rate for classical steroid 21-hydroxylase deficiency of $\sim 1:50$, we estimate the frequency of such dual-deficiency alleles in the general population in The Netherlands at 1:1000. Since two independent variants exist in the relatively small patient group examined here, it seems likely that a systematic re-evaluation of apparent large-scale conversions in other populations by a suitable PCR method (33; this report) will detect similar haplotypes. Interestingly, *TNXB*-*TNXA* hybrid genes were recently reported in two Dutch patients suffering from Ehlers-Danlos syndrome (34). The number of RCCX modules was not determined in that

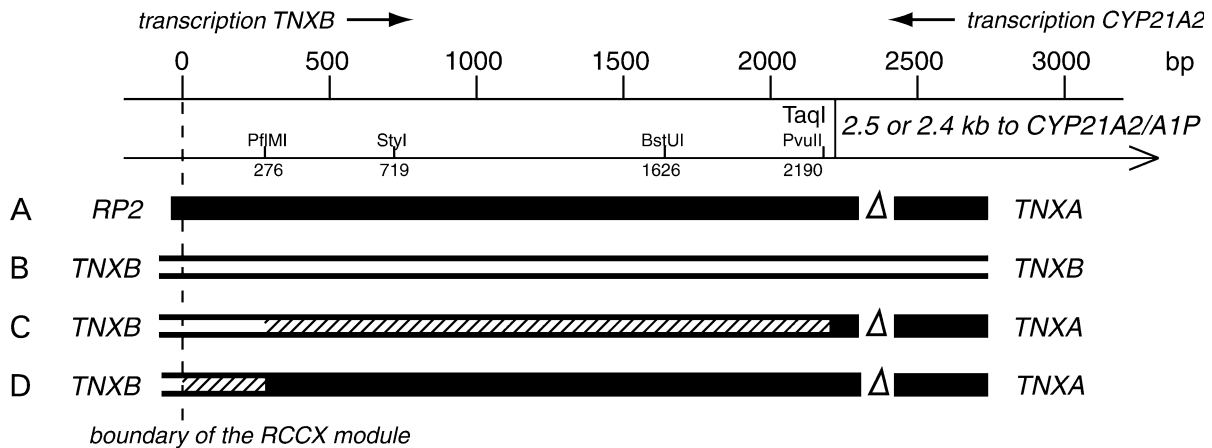


Figure 5. Amplified region of *TNXA* and *TNXB* (bottom) showing the site of the 120 bp deletion (triangle) in *TNXA* and the beginning of the *TaqI* fragment that partly overlaps the *CYP21A2* or *CYP21A1P* gene (Fig. 1). The location of the *TaqI* site in the *TNX* genes is the same relative to the nearby *RCCX* duplication boundary, but the 120 bp size difference determines whether the fragment is 2.4 or 2.5 kb. The polymorphic sites used as markers for *TNXA* or *TNXB* are shown. The four amplified stretches of DNA are (A) regular *TNXA* PCR; (B) regular *TNXB* PCR, which produces similar stretches for a haplotype with a normal *CYP21A2* gene and one with a *CYP21A2-CYP21A1P* 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 and *TNXB*-like sequences in white, and putative transition zones between them are hatched. Top: scale in bp.

study, so it seems possible that these patients have the bimodular structure described here.

Recombinational mechanisms

Chromosomes carrying two *RCCX* modules, each with a *CYP21A1P*-like gene, have been called 'large-scale gene conversions' because, as compared with the typical layout, the *CYP21A2* gene appears to have been converted into a *CYP21A1P* gene. It has, however, become clear that the 'converted' *CYP21A1P* gene on such chromosomes is either a *CYP21A1P-CYP21A2* hybrid (25,26) or a regular *CYP21A1P* gene adjacent to a *TNXA-TNXB* hybrid (this report), and thus structurally indistinguishable from the *CYP21A1P* genes on 'CYP21A2-deletion' chromosomes. Crossing-over between misaligned monomodular and bimodular chromosomes during meiosis was proposed more than a decade ago as a mechanism causing *CYP21A2* deletions (1,7,15,20,28). Two more recent reports (14,36) have described this mechanism in considerable detail for *CYP21A2* deletions where the putative recombination occurred within the *TNX* genes. The recombination breakpoint in the *TNXB-TNXA* hybrid (on a monomodular chromosome) described there (14) was localized between the 120 bp deletion and the centromeric duplication boundary of the *RCCX* module, and this hybrid is therefore quite similar to those described here on bimodular chromosomes. Although definite proof awaits the description of a *de novo* event, the structural similarity makes it highly likely that bimodular *CYP21A1P-CYP21A1P* haplotypes arise by the same mechanism as monomodular *CYP21A1P*-only haplotypes, namely meiotic unequal crossover: in this case involving a trimodular *CYP21A1P-CYP21A1P-CYP21A2* chromosome (1,15). Such a recombination between a bimodular and a monomodular chromosome (1) would only differ from those described elsewhere (14,20,36) by the presence of one additional *RCCX* module on each chromosome. An outline of these putative recombination events is shown in Figure 6. 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 *CYP21A2-CYP21A1P* sequence transfer, as supported by studies of *de novo* mutations (44-46) and sperm cells (47). 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 '*CYP21A2* deletion by unequal crossover' adequately describes all chromosomes with a hybrid *RCCX* module and without a *CYP21A2* gene, irrespective of the number of *CYP21A1P* genes.

MATERIALS AND METHODS

Subjects and haplotyping

The study population of 21-hydroxylase deficiency patients, family members and controls was the same as before (26); a family where a *de novo* *CYP21* deletion occurred was now included into the haplotype count in Table 2. *CYP21/C4* haplotyping was done as described earlier (2,16). Briefly, *TaqI* and *BglII* restriction patterns were obtained with the *CYP21A2* cDNA probe pC21/3c (6) and the 5' section of the *C4* cDNA probe pAT-A (48). Band ratios were measured by laser densitometry, and haplotypes were deduced from the segregation of the patterns (16).

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

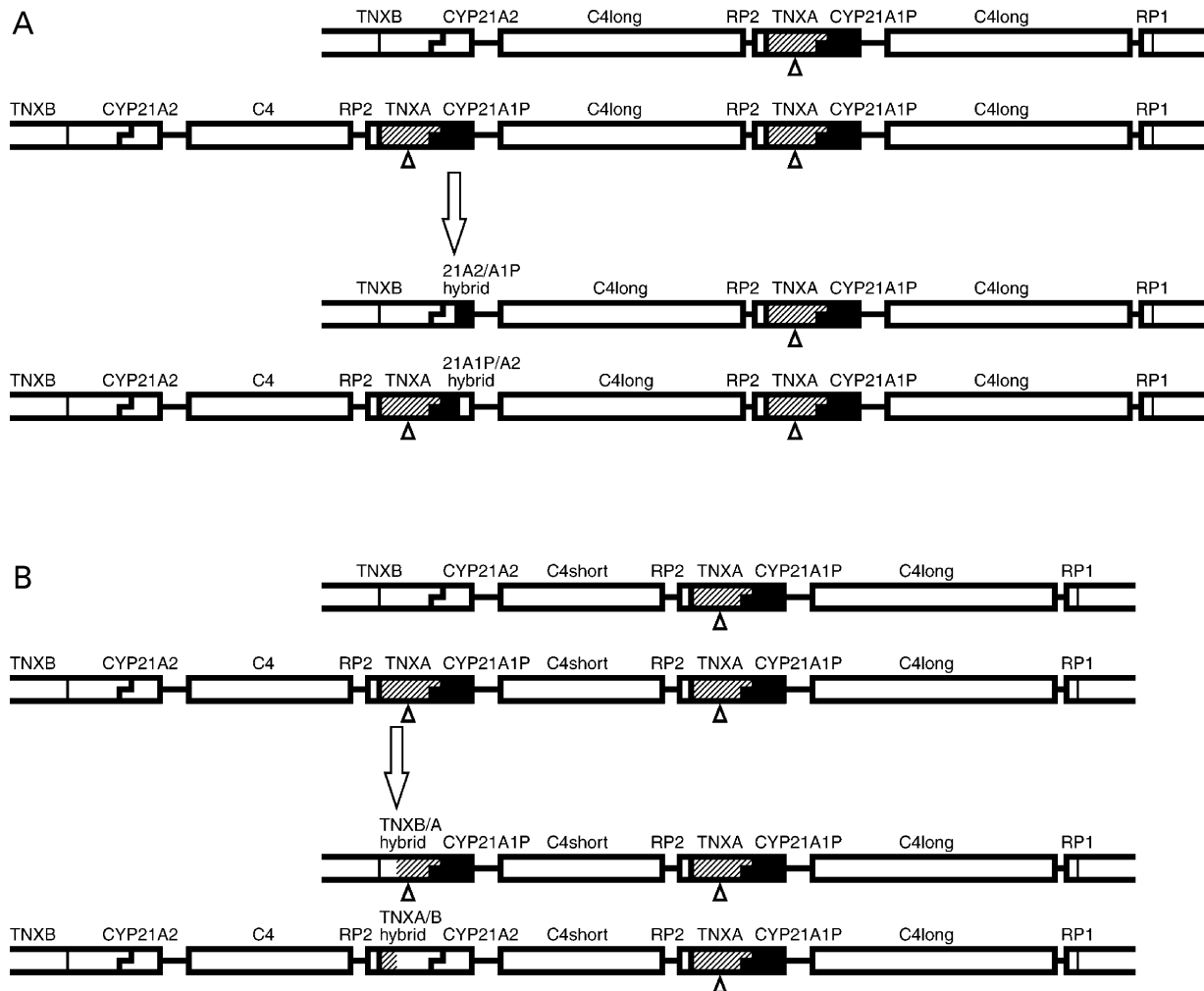


Figure 6. Putative unequal crossovers producing bimodular haplotypes with two *CYP21P*-like genes. (A) Crossover producing a *CYP21A2-CYP21A1P* hybrid. (B) Crossover producing a *TNXB-TNXA* hybrid. Black, *CYP21A1P*; hatched, *TNXA*; the triangle represents the 120 bp deletion in *TNXA*. The exact crossover site may vary within the *TNX* or *CYP21* genes.

of the *TNXB* gene. The forward primer for *TNXA* (CTTGAGCTGCAGATGGGATAC) lies within the *RP2* pseudogene. The reverse primer (CAATCCCCACCCTGAA-CAAGT) was the same for both genes, and lies between the site of the 120 bp deletion and the 3' end of the *CYP21A2/CYP21A1P* gene (Fig. 3). A touchdown PCR protocol was used to amplify these stretches of ~2.8 kb: first, 8 cycles of 30 s at 94°C, 60 s at 66°C decreasing by 0.5°C/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 by 30 s per cycle. Amplification was done with 0.5 units of Thermopperfect 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 the presence or absence of the 120 bp deletion/insertion. For further analysis, the PCR products were digested with *Pvu*II (New England Biolabs, Beverly, MA, USA), *Sst*I (Eurogentec, Seraing, Belgium), *Bst*UI (New England Biolabs, Beverly, MA, USA) or *Pvu*II (Gibco BRL, Gaithersburg, MD, USA). The products were analysed on agarose or polyacrylamide gels.

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