ELECTRONIC LETTER

Mapping of a de novo unequal crossover causing a deletion of the steroid 21-hydroxylase (*CYP21A2*) gene and a non-functional hybrid tenascin-X (*TNXB*) gene

P F J Koppens, H J M Smeets, I J de Wijs, H J Degenhart

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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.[1 2] Within this region, a section of particular complexity centres around the *C4* genes, which encode the fourth component of complement.[3–7] Centromeric to *C4* lies the *CYP21A2* gene, which encodes steroid 21-hydroxylase, a key enzyme in the biosynthesis of cortisol and aldosterone.[4 8 9] The *TNXB* gene, which encodes the extracellular matrix protein tenascin-X, lies centromeric to *CYP21A2* and is transcribed from the opposite strand.[10–12] Telomeric to *C4* lies the *RP1* gene, encoding a putative serine/threonine kinase.[13–15] A typical chromosome 6 carries a duplication of an area of approximately 30 kb encompassing the entire *C4* and *CYP21* genes[4 8] plus small truncated sections of *RP* and *TNX*.[10–14] This tandem repeat has been named the RCCX module after its four constituent genes.[7 14 16 17] In most white populations, about 70% of all haplotypes have a 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.[7 18–21]

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. As bimodularity is the standard, publications often refer 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. Many studies have used *Taq*I and *Bgl*II restriction analysis of genomic DNA and comparison of the relative intensities of the bands obtained by hybridisation to *C4*, *CYP21A2*, and *TNXB* probes[4 8 22–24] to establish haplotypes in families of patients with congenital adrenal hyperplasia and in controls. This approach is usually sufficient, but in complicated cases, rare cutters such as *Sac*II or *Bss*HII are needed to determine the size of the entire region and, hence, the number of RCCX modules.[25 26]

The tandem repeat structure of the RCCX region promotes the chance of misalignment during meiosis. If a crossover then occurs, it effectively removes one of the modules. Between standard bimodular chromosomes, this process joins a part of the telomeric module to its homologous counterpart in the centromeric module (see fig 5 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 *CYP21A1P* gene occur at a frequency of 5%–20% in the general population.[25 27–29]

An unequal crossover between *CYP21A1P* and *CYP21A2*, on the other hand, usually generates a fusion gene that is *CYP21A1P*-like in its 5′ section and contains several mutations rendering it inactive (a haplotype often referred to as a *CYP21A2* deletion). Absence of a functional *CYP21A2* 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. This is a disorder of adrenocortical steroid biosynthesis which in severe cases causes life threatening salt losing crises in untreated paediatric patients.[18–20]

An unequal crossover between *TNXA* and *TNXB* not only eliminates the *CYP21A2* gene, but may also create a non-functional *TNXB/TNXA* hybrid that contains a 120 bp deletion on an exon-intron boundary normally present in *TNXA* only, and is therefore unable to express the tenascin-X protein. This defect contributes to the Ehlers-Danlos syndrome, a recessively inherited disease of connective tissue.[17 30 31]

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 *CYP21A1P* pseudogene as a premutation has been proposed in a report on a de novo deletion of *CYP21A2* by recombination between a standard bimodular chromosome and a monomodular chromosome.[32] More recently published studies also provide evidence that *TNXB/TNXA* hybrids are the result of a crossover between a bimodular and a monomodular chromosome.[16 17]

Key points

- Defectiveness of the *CYP21A2* gene causes steroid 21-hydroxylase deficiency, a recessively inherited disorder of adrenocortical steroid biosynthesis.
- CYP21A2 is part of a tandemly repeated structure known as the RCCX module, which may misalign during meiosis.
- A paternal de novo deletion of *CYP21A2* contributed to simple virilising steroid 21-hydroxylase deficiency in a patient who inherited the I172N mutation from his mother.
- The de novo deletion was caused by an unequal crossover occurring in a 640 bp region of the *TNXB* gene, adjacent to *CYP21A2*.
- By contrast with earlier observations, this unequal crossover occurred between chromosomes with equal numbers of RCCX modules.

Abbreviations: MHC, major histocompatibility complex; RCCX, RP-C4-CYP21-TNX module; PCR, polymerase chain reaction; TNF, tumour necrosis factor; *TNXB* (also known as *XA*), non-functional hybrid tenascin-X gene

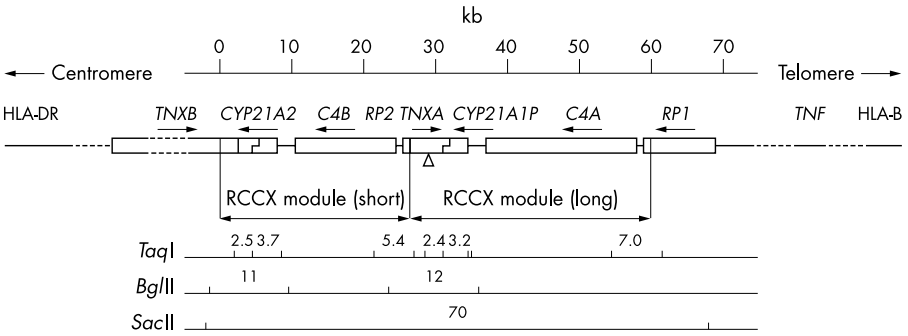

Figure 1 Overview of a typical *C4/CYP21* area within the MHC class III region showing two RCCX modules as found on most chromosomes. *TNXB* is the full size 68 kb gene for tenascin-X. *TNXA* (also known as *XA*) is a truncated pseudogene of 5.7 kb that not only lacks most of the coding sequence of *TNXB* but also has a 120 bp deletion (indicated by the small triangle) spanning an exon-intron boundary.[10 12 17] *CYP21A2* (also known as *CYP21B*) is the active steroid 21-hydroxylase gene; *CYP21A1P* (also known as *CYP21A*) is a full size pseudogene containing several deleterious mutations throughout its sequence, including three in phase stop codons.[9] 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, as 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 *CYP21A2*, and of *TNXA* and *CYP21A1P*, 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 owing to methylation. Top: scale in kb.

We report here a de novo unequal crossover that occurred between two bimodular chromosomes in the father of a patient with congenital adrenal hyperplasia caused by steroid 21-hydroxylase deficiency. The crossover site was mapped to a 640 bp region of the *TNXB* gene that starts at approximately 1.6 kb from the centromeric duplication boundary of the RCCX module. This de novo mutation eliminates the *CYP21A2* gene and also disrupts *TNXB* by replacing its 3′ section by the corresponding part of *TNXA*, conveying the 120 bp deletion.

METHODS

Patient and family members

The patient, a boy, presented to the Sophia Children's Hospital at the age of 7 years 8 months with signs of precocious puberty. The patient was tall for his age (above the 90th centile), and bone age was very advanced (13.5 years). Basal plasma 17α-hydroxyprogesterone was 99 nmol/l, testosterone was 4.3 nmol/l. Sodium and potassium concentrations 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.

CYP21A2/C4 haplotyping

The family participated in our haplotyping study[28] as family 20, and *CYP21A2/C4* haplotypes[33] 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 *CYP21A2* cDNA probe pC21/3c[8] and the 5′ section of the *C4* cDNA probe pAT-A.[3] The resulting autoradiographic bands were quantified by laser densitometry with the exception of the 2.4 and 2.5 kb *Taq*I bands, which produced a weak hybridisation signal and were estimated visually. Long range restriction mapping by *Sac*II digest and pulsed field gel electrophoresis was performed as described elsewhere.[34]

CYP21A2 and *CYP21A1P* mutation analysis

Mutation analysis of all *CYP21A2* and *CYP21A1P* genes in this family was done as described before.[35] Briefly, three sections of either *CYP21A2* or *CYP21A1P* were specifically amplified and hybridised to oligonucleotides detecting the most common mutations: intron2splice; exon3del8bp; I172N; I236N/V237E/M239K or I236K/V237E/M239K; V281L; exon7ins1bp; Q318X; R356W.

Analysis of flanking major histocompatibility complex (MHC) markers

Genomic DNA was digested with *Eco*RI, *Hin*dIII, *Pvu*II, or *Taq*I, separated by electrophoresis and Southern blotted, and hybridised to the HLA-B probe pHLA2[36] and the HLA-DQα probe pDCH-1,[37] resulting in distinctive banding patterns.[38] Length polymorphism of a microsatellite marker[39] near the tumour necrosis factor (TNF) locus was used as an additional marker.

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 *CYP21A2/CYP21A1P* gene (fig 2). A touchdown polymerase chain reaction (PCR) protocol was used to amplify these stretches of approximately 2.7 kb: firstly, eight cycles of 30 seconds at 94°C, 60 seconds at 66°C decreasing 0.5°C per cycle, and three minutes at 72°C; next, 26 cycles of 30 seconds at 94°C, 60 seconds at 62°C, and three minutes at 72°C extending by 30 seconds per cycle. Amplification was done with 0.5 units of Thermoperfect DNA polymerase (Integro, Leuvenheim, The Netherlands) in the presence of 1.5 mmol/l $MgCl_2$ and 1% formamide. The size of the PCR product directly shows the presence or absence of the 120 bp deletion/insertion.

Also, comparison of published *TNX* sequences (DDBJ/EMBL/GenBank accession numbers S38953,[10] L26263,[11 14] X71937,[12] AF077974,[16] AF086641,[17] AL049547,[40] AF019413,[41] and U89337[42]) showed several polymorphic sites throughout the amplified region, most of which can be detected by restriction analysis. Digestion of the PCR product with *Bst*UI

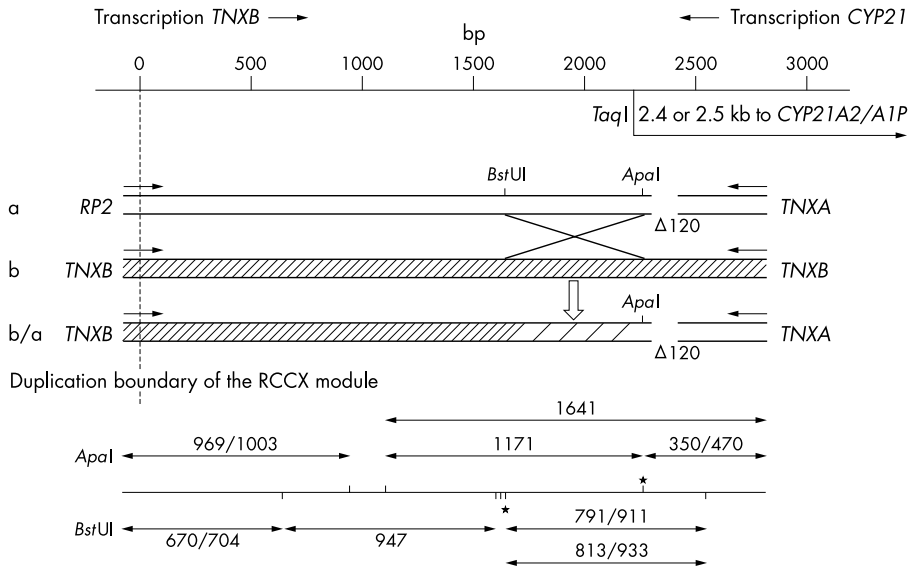

Figure 2 Amplified region of *TNXA* and *TNXB* showing the site of the 120 bp deletion in *TNXA*, the crossover, and the *Apa*I and *Bst*UI restriction sites used to map it to a 640 bp region. The bold arrows represent the primer sites for the *TNX* PCRs; the reverse primer is the same for *TNXA* and *TNXB*, but the forward primers are specific and their starting positions differ by 34 bp, causing an additional size difference between the PCR products. The amplified stretches are: a (2654 bp): the father's first allele in the *TNXA* PCR (open box); b (2808 bp): the father's second allele in the *TNXB* PCR (densely hatched box); b/a (2688 bp): the hybrid found in the patient (densely hatched: from b; open: from a; lightly hatched: recombinant zone). The crossover region lies between the *Bst*UI restriction site shown in allele a and the *Apa*I restriction site shown in allele a and in the b/a recombinant. Top: scale in bp, with underneath it the beginning of the 2.4 or 2.5 kb *Taq*I fragment that partly overlaps the *CYP21A2* or *CYP21A1P* gene (fig 1). Bottom: *Apa*I and *Bst*UI restriction fragments, with * indicating polymorphic sites. The fragment sizes are the same as in fig 4. Variants indicate either the 34 bp difference in the location of the *TNXA* or *TNXB* forward primers (969/1003 and 670/704) or absence or presence of the 120 bp (350/470, 791/911, and 813/933).

(New England Biolabs, Beverly MA, USA) and *Apa*I (Eurogentec, Seraing, Belgium) proved particularly useful in locating the crossover site. *Bst*UI detects a polymorphism at 1626 bp downstream of the duplication boundary of the RCCX module and *Apa*I detects a polymorphism at 2266 bp (in this context, downstream is relative to the transcription of the *TNXB* gene, and sequence AL049547 was used to compute fragment sizes and nucleotide positions).

RESULTS

CYP21/C4 haplotypes

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.[28] 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 duplicated region (fig 1).[26] 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 (fig 3). These results matched the *Taq*I and *Bgl*II band intensities (table 1). As 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 findings.

CYP21A2 mutation analysis

The I172N mutation was found in the mother, the patient, and the healthy daughter. This mutation is typical of the simple virilising form of congenital adrenal hyperplasia,[43] matching the patient's phenotype. None of the common deleterious mutations investigated[35] were found in the *CYP21A2* genes of the patient's father or the twin brothers. Because the patient inherited a pre-existing genetic defect from his mother, the putative de novo deletion had apparently occurred in his father.

Confirmatory analysis of flanking MHC markers

The notion of a paternal de novo recombination was confirmed by analysis of markers centromeric (HLA-DQα) and telomeric (TNF and HLA-B) to the RCCX module (details not shown). Normal Mendelian segregation of all alleles was shown in all healthy family members. The patient, however, carried the father's HLA-DQα markers from one chromosome, together with the TNF and HLA-B markers from the other chromosome, as well as 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 *CYP21A2* gene and the adjacent *C4* gene. The segregation of chromosome 6 in this family is shown in fig 3.

Analysis of the *CYP21A1P* pseudogene

Monomodular chromosomes lacking *CYP21A2* typically retain the *Taq*I 2.5 kb band matching *TNXB* (fig 1) because the single module is a hybrid with the recombination breakpoint located inside the *CYP21* gene.[24 27 33] Surprisingly, in this patient the 2.4 kb band had a higher intensity than the 2.5 kb band (table 1), suggesting a crossover site in the *TNX* gene rather than in *CYP21*; both genes were further analysed to locate the breakpoint.

All *CYP21A1P* genes in this family matched the consensus sequence[9] for all markers tested up to and including the fourth exon. Further downstream, distinctive markers (named after the matching codons in the *CYP21A2* gene) were found on each allele (fig 3). Presence of the L281 marker in the recombinant positioned the putative crossover site downstream of that marker, but the markers Q318 and R356 were not informative.

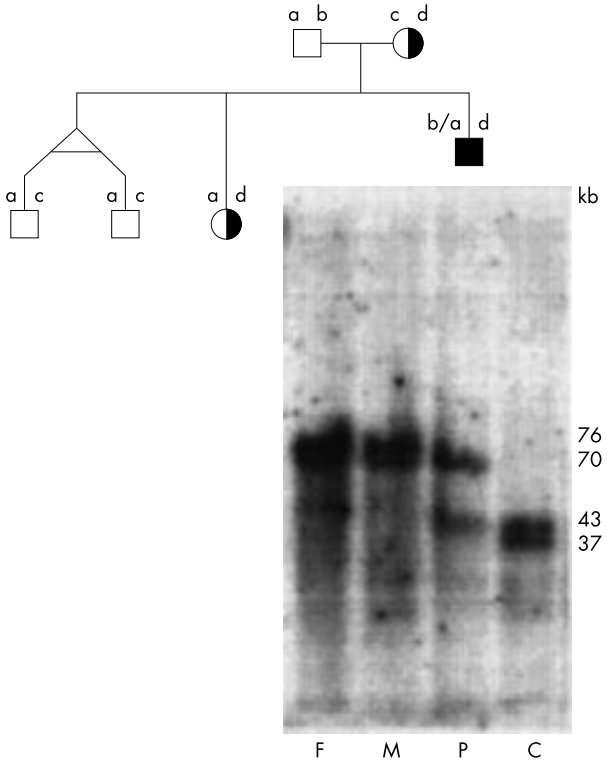

Figure 3 Pedigree of the family and long range restriction mapping. Characteristics of the haplotypes:
a: *CYP21A2*(normal)-*C4*(long)-*CYP21A1P-C4*(long);
b: *CYP21A2*(normal)-*C4*(short)-*CYP21A1P-C4*(long);
c: *CYP21A2*(normal)-*C4*(long)-*CYP21A1P-C4*(long);
d: *CYP21A2*(I172N)-*C4*(short)-*CYP21A1P-C4*(long);
b/a: recombinant: *CYP21A1P-C4*(long). Distinctive markers in exons 7 and 8 of the *CYP21A1P* gene on each haplotype were:
a: L281-G318-R356; b: V281-X318-R356; c: L281-X318-W356;
d: V281-X318-R356; b/a: L281-G318-R356. All *CYP21A2* genes in this family carried the normal codons V281-G318-R356.[35]
Bottom: *Sac*II digested genomic DNA separated by pulsed field gel electrophoresis and hybridised to a mix of a *CYP21A2* 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.

PCR and restriction analysis of the *TNX* genes

The PCR products of *TNXA* and *TNXB*, and restriction patterns after digestion with *Bst*UI and *Apa*I, are shown in figs 2 and 4. The 2688 bp band represents the recombinant *TNXB* gene of the patient, with the 120 bp deletion that has been transferred from *TNXA* (fig 4A). The *TNXB* gene on the patient's other chromosome was apparently intact, as he did not have the Ehlers-Danlos syndrome.

The segregation of the fragments within the family allows assignment of the 120 bp deletion and the *Bst*UI and *Apa*I 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 duplication boundary, and the *TNXA* gene of chromosome a from the *Apa*I polymorphism at 2266 bp. 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).

DISCUSSION

Deletions of approximately 30 kb of DNA containing the *CYP21A2* gene are a major factor in the genetics of steroid 21-hydroxylase deficiency. Such deletions have been attributed to unequal crossover owing to misalignment of homologous chromosomes during meiosis.[16 17 27 32 44] In this report, we present such an unequal crossover as a de novo mutation in the father of a patient with steroid 21-hydroxylase deficiency. The crossover breakpoint was mapped to a 640 bp region between two polymorphic restriction sites in the *TNXB* gene, which lies immediately centromeric to the *CYP21A2* gene (figs 3 and 5). A de novo deletion of the *CYP21A2* gene reported earlier by Sinnott *et al*[32] was caused by an unequal crossover between a chromosome with one RCCX module (monomodular: a single *C4* gene and a *CYP21A2* gene) and a chromosome with two RCCX modules (bimodular: two *C4* genes, a *CYP21A2* gene, and a *CYP21A1P* pseudogene). These authors point out that as the bimodular chromosome has no equally sized homologue to align to during meiosis, misalignment of its *CYP21A1P* and *TNXA* genes to the *CYP21A2* and *TNXB* genes of the monomodular chromosome would have a probability close to 50%. This notion of the common monomodular *CYP21A1P* deletion chromosome serving as a premutation was further strengthened by two more recent reports describing reciprocal *TNXB/TNXA* and *TNXA/TNXB* hybrids in unrelated persons.[16 17] A de novo deletion that eliminated the *CYP21A1P* pseudogene[45] also involved one bimodular and one monomodular chromosome.

The current report shows that de novo unequal crossovers between bimodular chromosomes also contribute to the pathogenesis of steroid 21-hydroxylase deficiency. Fig 5 shows a misalignment that may explain such a rearrangement. It is reasonable to assume that the flanking sequences of the RCCX module, that is, the *TNXB* and *RP1* genes, will align correctly. The crossover could then be explained by two loops of DNA,

Table 1 Relative intensities of *Taq*I and *Bgl*II bands, and size of the *Sac*II bands, after hybridisation to *CYP21A2* and *C4* cDNA probes

	CYP21A2 probe pC21/3c			*C4* probe pAT-A	*CYP21A2* and *C4* probes
	*Taq*I		*Bgl*II	*Taq*I	*Sac*II
	3.7 : 3.2*	2.5 : 2.4†	12 : 11	7.0 : 6.4 : 6.0 : 5.4‡	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

Ratios were measured by laser densitometry; ++, +: estimate, ++ indicating a higher intensity.
*3.7 kb: *CYP21A2* gene; 3.2 kb: *CYP21A1P* pseudogene (fig 1).
†2.5 kb: *TNXB* gene; 2.4 kb: *TNXA* pseudogene (fig 1).
‡7.0 kb: long *C4* gene in the most telomeric RCCX module; 5.4 kb: short *C4* gene in a non-telomeric RCCX module (fig 1); 6.4 kb and 6.0 kb (alternatives not shown in fig 1): short telomeric and long non-telomeric *C4* genes, respectively; the *C4* band sizes are those traditionally given in published reports,[5] although the actual fragments are approximately 0.15 kb larger.

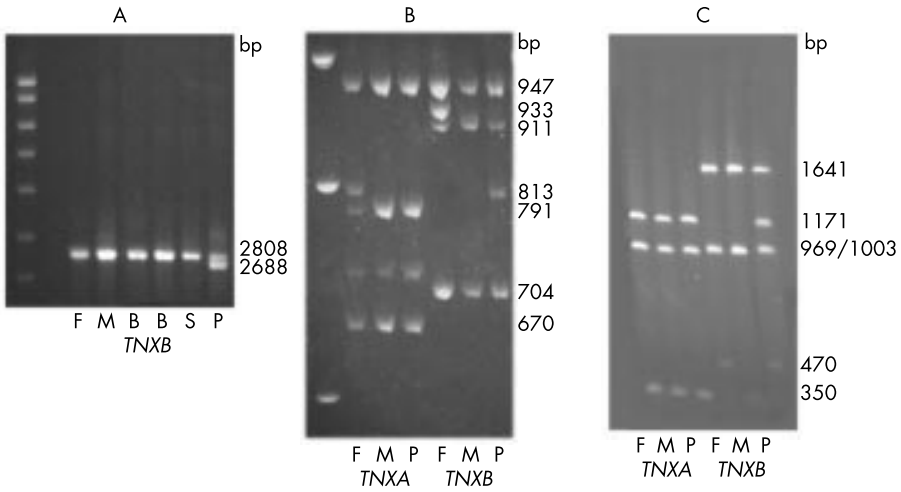

Figure 4 Full size and digested PCR products (fig 2); F, father; M, mother; P, patient; B, twin brother(s); S, sister; fragment sizes in bp. (A) *TNXB* PCR product showing the 120 bp size difference caused by the de novo unequal crossover; left: 5 µl SmartLadder (Eurogentec, Seraing, Belgium); electrophoresis was for 20 hours at 40 V on 1% agarose. (B) *TNXA* and *TNXB* *Bst*UI digest; left: 5 µl SmartLadder; electrophoresis was for 22 hours at 140 V on 3.2% polyacrylamide. (C) *Apa*I digest; electrophoresis was for 10 hours 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 *Apa*I digests (results not shown). Fig 2 shows the size and position of the *Bst*UI and *Apa*I fragments relative to the amplified region.

each comprising a single RCCX module. The size differences between the *C4* genes on the chromosomes involved may contribute to inducing such an arrangement. This difference is the result of the retroviral insert HERV-K(C4),[46 47] and it has been hypothesised that such inserts may have contributed to genetic rearrangement in the MHC during evolution.[48]

The presence of sequences promoting recombination, such as the *E coli* crossover hotspot instigator (χ), and the human minisatellite consensus sequence, has been implicated in genetic rearrangements of the RCCX module.[17 43 49 50] It is possible that such sequences play a part in the generation of small scale gene conversions between the *CYP21A2* and *CYP21A1P* genes, but we do not think 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 causing a *CYP21A2* deletion, despite the fact that over the years, haplotyping studies that could have detected such events have been done in thousands of families by many different research groups around the world. The earlier report[32] was published before the structure of the RCCX module became known in detail,[7 12–17] but the crossover apparently also occurred within the *TNXB* 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[51] and probably arose by a mechanism different from the one discussed here.

Table 2 Presence and absence of polymorphic sites and the 120 bp insertion/deletion

	TNXA PCR			*TNXB* PCR		
Chromosome	*Bst*UI	*Apa*I	120 bp	*Bst*UI	*Apa*I	120 bp
a	+	+	–	+	–	+
b	–	+	–	–	–	+
c	+	+	–	+	–	+
d	+	+	–	+	–	+
b/a recombinant	x	x	x	–	+	–

Presence (+) or absence (–) of the polymorphic *Bst*UI site at 1626 bp from the RCCX duplication boundary, of the *Apa*I 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 3; x: no PCR product: the recombinant cannot be amplified in the *TNXA* PCR)

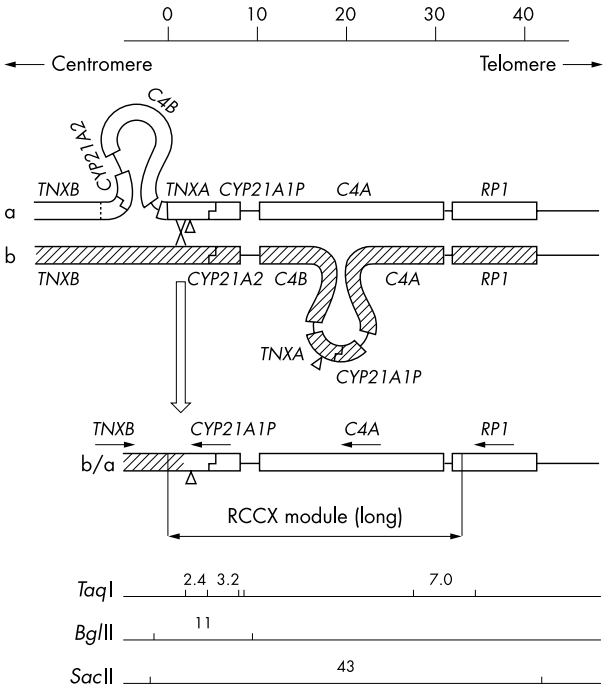

Figure 5 Hypothetical misalignment and loop out of two bimodular chromosomes contributing to an unequal crossover causing deletion of a 30 kb region including the *CYP21A2* gene and the 3′ portion of the *TNXB* gene. The figure shows separate loops of two sections of DNA, but other configurations involved 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. Note that the scale in kb (top) and the restriction map (bottom) apply to the hybrid chromosome shown in the lower part of the figure, not to the looped out parental chromosomes in the upper part (see fig 1 for further details on the graphics).

Despite the small number of de novo deletions in this region described so far, it is remarkable that none of them seems to have its recombination breakpoint within the *CYP21A2* gene. It has been well documented that many apparent *CYP21A2* deletions represent a hybrid gene with a *CYP21A1P*-like 5′ section and a *CYP21A2*-like 3′ section, and that transition zones between these sections are positioned at different locations within the hybrid gene.[27 35 49 52–55] In line with those findings, we did not find the 120 bp deletion in 15 other monomodular deletion haplotypes after testing them with the *TNXB* specific PCR described here (unpublished data). *CYP21A2* gene deletions with the crossover site in the *TNXB* gene have so far been described in a few isolated cases, including this report.[17 30–32] Whether the additional *TNXB* defect adds significantly to selection against this allele compared with a defect in *CYP21A2* alone is unclear, as the frequency of tenascin-X deficiency has not been established. However, steroid 21-hydroxylase deficiency alleles occur at different frequencies in different populations,[29 35 53 55–63] and most reports do not describe putative crossover sites. This monomodular dual deficiency haplotype may therefore play a part in the pathogenesis of congenital adrenal hyperplasia, the Ehlers-Danlos syndrome, or both, in some populations. Curiously, we recently found a relatively high frequency (four out of nine haplotypes) of *TNXB-TNXA* hybrids in bimodular haplotypes with two *CYP21A1P*-like genes.[64] To establish the distribution of different classes of hybrid RCCX modules without a *CYP21A2* gene, studies in different populations could be done by means of PCR methods such as the one described here or elsewhere,[17 30] 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 *CYP21A2* gene,[44] 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. Usage of flanking markers on either side of the recombination site helps to avoid erroneous assignment of carrier status in such cases.

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Authors' affiliations

P F J Koppens, Department of Paediatrics, Erasmus MC Sophia, Rotterdam, The Netherlands
H J M Smeets, Clinical Genetics Centre Maastricht, Maastricht, The Netherlands
I J de Wijs, Department of Human Genetics, University Medical Centre Nijmegen, Nijmegen, The Netherlands
H J Degenhart, Department of Paediatrics, Erasmus MC Sophia, Rotterdam, The Netherlands

Correspondence to: Dr P F J Koppens, Erasmus MC, University Medical Centre, Rotterdam, Laboratory of Paediatrics, Room Ee1502b, PO Box 1738, 3000 DR Rotterdam, The Netherlands; p.koppens@erasmusmc.nl

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