

# Molecular characterisation of a low-frequency mutation in exon 8 of the human low-density lipoprotein receptor gene

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## Summary

The prevalence of familial hypercholesterolaemia (FH), an autosomal dominant disease characterised by raised low-density lipoprotein (LDL) cholesterol levels, is at least five times higher in the white Afrikaner population than in most other population groups in the world. A founder gene effect has been suggested to explain this abnormally high frequency. Detection of a polymorphic *Stu* I site in the 5' region of the LDL receptor gene and association of both restriction fragment length polymorphism alleles with FH in Afrikaners, indicated the existence of at least two founder members for the disease in this population. DNA from a hetero-allelic FH homozygote from this South African group has been analysed through genomic cloning and sequencing. The DNA polymorphic site is caused by a single guanine to adenine transition within exon 8 of the LDL receptor gene and can be used in the determination of haplotype-associated defects.

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Familial hypercholesterolaemia (FH) is a common autosomal dominant disease caused by DNA mutations in the low-density lipoprotein (LDL) receptor gene locus. The prevalence of heterozygous FH appears to be approximately 1 in 500 among the general population of most countries in the world, but in Afrikaners FH occurs at a 5 - 10 times higher frequency. A founder gene effect has been suggested to explain this abnormally high frequency.<sup>1</sup> Clinical studies have demonstrated that tendon xanthomas and premature coronary artery disease are encountered in middle-aged FH heterozygotes, while in the homozygous condition both cutaneous and tendon xanthomas as well as aortic and coronary atherosclerosis appear before the age of 10 years.<sup>2</sup> It is therefore advantageous to identify affected subjects at a young age before they develop symptomatic disease, in order to counsel on diet and drug treatment to reduce their future risk of myocardial infarction. It has been shown that measurement of total or LDL cholesterol levels does not always allow unequivocal diagnosis of FH, especially in childhood,<sup>3,4</sup> since these values may fall in the upper range of normal.

The cloning of the LDL receptor gene<sup>5</sup> has made it possible to analyse mutations causing FH at the DNA level. A number of different mutations affecting the LDL receptor, including deletions, insertions, missense and nonsense mutations, have

been described.<sup>6</sup> Langlois *et al.*<sup>7</sup> have recently shown that about 6% of all mutations in the UK population represent gene deletions, which can be detected directly using Southern blotting techniques. The majority of defects are, however, the result of point mutations and will require other methods of diagnosis.

The mutational heterogeneity of FH creates a problem in the application of recombinant DNA methods for diagnosis at the genomic level. It is usually not feasible to screen genomic DNA from a patient who has the defined clinical syndrome for all the mutations that are known to have occurred in the suspected gene. Detection by methods that specifically screen for the mutational defect by restriction analysis or by differential oligonucleotide hybridisation may, on the other hand, be successful in populations that carry only a small number of mutations. This was shown in the Lebanese,<sup>8</sup> French Canadian<sup>9</sup> and Finnish<sup>10</sup> FH patients, where the spectrum of LDL receptor gene mutations was narrower, since the populations are more homogenous in genetic terms. This strategy may also be applicable in the efficient characterisation of the mutations causing FH in the Afrikaner population, which has remained isolated through religious belief and cultural bonds.

LDL receptor studies of South African FH homozygotes have shown a predominance of a receptor-defective type of abnormality.<sup>11</sup> This is in accordance with the founder gene hypothesis for Afrikaner FH, implying that the majority of patients will manifest the same mutation in the LDL receptor gene. Since the nature and number of molecular gene defects in this population have not yet been defined and our Southern blot analyses of Afrikaner FH patients have not shown gross structural alterations, we used restriction fragment length polymorphism (RFLP) studies to investigate the founder hypothesis. Ten useful two-allele RFLPs of the LDL receptor gene were used for haplotype studies in the Afrikaner population.<sup>12</sup> Pedigree analysis has shown the segregation of at least 17 haplotypes in the normal population compared with a predominant association of two of these haplotypes with the disease in the FH subjects. In 70% of FH families studied the defective gene co-segregated with the rare allele of a *Nco* I RFLP<sup>13</sup> (haplotype 2), while the rare allele of a *Stu* I RFLP<sup>14</sup> (haplotype 6) segregated with FH in 20% of these families. This association was further confirmed in 27 unrelated FH homozygotes; 24 were homozygous for haplotype 2, while 3 showed compound heterozygosity for haplotypes 2 and 6. Since specific mutations may occur in different populations in close association with single RFLPs or RFLP haplotype patterns, haplotype analysis can serve as an important means of genetic characterisation of a population with regard to a particular mutation causing the disease.

Association of both alleles of the rare *Stu* I RFLP with FH in some of the Afrikaner families studied, first indicated the existence of at least two 'founder' members for the disease in South Africa. Südhof *et al.*<sup>15</sup> published data on the genomic map of the human LDL receptor gene indicating the cleavage sites for selected restriction endonucleases. We used these known fragment sizes and DNA double digests with sets of different restriction enzymes to determine the exact sizes generated by *Stu* I. Comparing the fragment sizes found with

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those described by Südhof *et al.*,<sup>15</sup> we mapped the variable *Stu* I site within exon 8 of the gene and an extra recognition site for the enzyme was found between exons 15 and 16 to produce the common 7,2 kb fragment.<sup>14</sup> Accurate mapping of this variable RFLP site is very important, since it can be used to determine haplotype-associated defects in cDNA from hetero-allelic FH homozygotes. To date, the *Stu* I RFLP is the only variable site found within an exon of the 5' region of the LDL receptor gene and true FH homozygotes for this rare haplotype have not been detected for cloning purposes. In this study, using genomic cloning and DNA sequencing techniques, we confirm that the *Stu* I RFLP is located within exon 8 of the gene and is caused by a single base substitution in this region.

## Materials and methods

### Genomic cloning

High-molecular-weight DNA was prepared from a blood sample of FH homozygote 8 by a Triton X-100 lysis method.<sup>16</sup> This DNA (300 µg) was digested overnight at 37°C with *Bgl* II using conditions recommended by the manufacturers (Boehringer Mannheim). The digested DNA was subjected to electrophoresis in a 0,6% agarose gel together with DNA size markers. Two DNA fractions were eluted from the gel,<sup>17</sup> after which Southern blotting with a 1,05 kb *Pst* I probe, subcloned into pBR 322 from pLDLR-3, was used to identify the fraction containing the desired 15 kb *Bgl* II fragment. pLDLR-3 contains a full-length LDL receptor cDNA and was obtained from the American Type Culture Collection (ATCC). The human DNA (100 ng) was mixed with 0,5 µg *Bam* HI-digested arms of lambda L47.1<sup>18</sup> and incubated overnight at 14°C with T4 DNA ligase (Amersham International). The ligated material was packaged in lambda phage particles *in vitro* to yield a total of  $1 \times 10^6$  plaque-forming units. Lambda DNA packaging extracts (Gigapack Plus) were purchased from Vector Cloning Systems.

Approximately  $5 \times 10^5$  plaque-forming units were screened after plating on *E. coli* strain WL 95. One recombinant clone was identified with the 1,05 kb *Pst* I clone, encompassing exons 2 - 8 of the normal LDL receptor gene. This clone, designated lambda FH 8 - 30, was isolated after two additional cycles of plaque purification. Restriction endonuclease mapping and Southern blotting indicated that this clone contained the 15 kb *Bgl* II LDL receptor gene fragment comprising exons 4 - 11. The genomic human DNA insert in this phage lacked a polymorphic *Stu* I site found in exon 8.<sup>14</sup> The DNA from clone lambda FH 8 - 30 was digested with *Eco* RI and a purified 9 kb human DNA insert (Fig. 1) was subcloned into *Eco* RI-digested pBR 328 DNA.

### DNA sequencing

Exon 8 of the LDL receptor gene was sequenced by the dideoxy chain termination method using an oligonucleotide primer specific for the 5' end of exon 8 (5' TTCTCTCTCTTCCAGATA3'). A double-stranded plasmid, derived from pBR 328 and containing the cloned insert of lambda FH 8 - 30, was denatured with sodium hydroxide and then annealed with the exon 8 primer.<sup>19</sup> Sequencing was carried out using a commercial kit (Sequenase). The sequence was compared with that of exon 8 of the normal LDL receptor gene.<sup>5</sup>

## Results

### Molecular cloning

In the gene for the normal LDL receptor, exon 8 is

contained within a 15 kb *Bgl* II fragment.<sup>15</sup> To clone this fragment, genomic DNA from a hetero-allelic FH homozygote (FH 8) was restricted with *Bgl* II, size-fractionated in agarose gels and fragments of approximately 15 kb were used to construct a genomic DNA library in the *Bam* HI sites of bacteriophage lambda L47.1. The library was screened as described under 'Materials and methods'. This procedure yielded a single recombinant bacteriophage that harboured a human DNA fragment of approximately 15 kb. Restriction mapping revealed that this fragment lacked the *Stu* I site normally found in exon 8 of the LDL receptor gene and thus represented the FH 8 - haplotype 6 allele.<sup>12</sup>

### DNA sequence analysis of exon 8

A 9 kb *Eco* RI fragment from the recombinant bacteriophage was subcloned into a pBR 328 plasmid vector. A restriction map of the resulting human DNA insert in the plasmid is shown in Fig. 1. Sequencing of the denatured double-stranded plasmid DNA using an oligonucleotide primer specific for the 5' end of exon 8 revealed that allele 6 differed by a single base in the 6-base pair sequence recognised and cleaved by *Stu* I. Fig. 2 shows this portion of the exon 8 sequence, where an

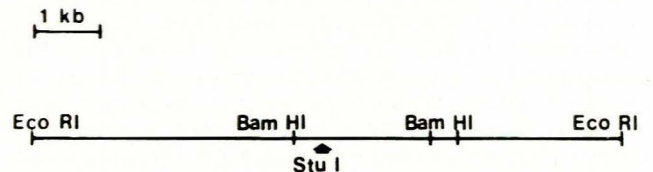


Fig. 1. Restriction map of the 9 kb *Eco* RI fragment of the 5' end of the LDL receptor gene analysed in FH 8. The *Eco* RI sites used for construction of the pBR 328 recombinant clone are shown, together with the *Bam* HI and *Stu* I sites in this region. The absence of a *Stu* I site within the DNA fragment is denoted by an arrow.

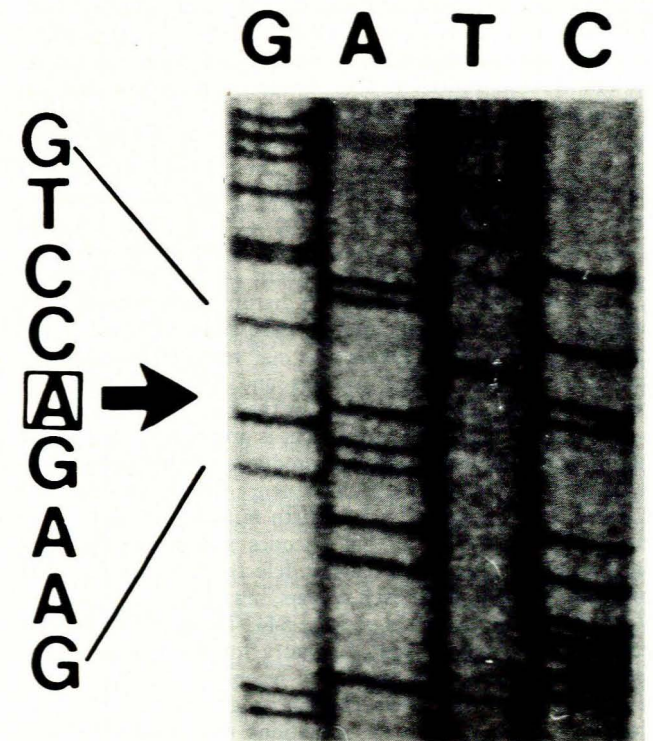


Fig. 2. Autoradiograph of a sequencing gel demonstrating the mutation point in exon 8 of the LDL receptor gene. The DNA sequence in the region surrounding alanine 370 is shown, with the single base substitution indicated (arrow) at nucleotide position 1171.



adenine replaced a guanine at nucleotide position 1171, changing amino acid 370 from alanine to threonine. The remaining exon 8 sequence from the cloned genomic fragment is similar to that of the normal gene.

## Discussion

The gene for the human LDL encompasses 45 kb of DNA divided into 18 exons on the short arm of chromosome 19.<sup>15</sup> In this paper we characterised a mutation in exon 8 of the LDL receptor gene, giving rise to a *Stu* I RFLP. The presence or absence of this site produces two allelic fragments of 15 and 17 kb respectively, in genomic Southern blots using cloned cDNA probes. Analysis of DNA isolated from 60 normal, unrelated Afrikaner individuals revealed that the frequency of the common allele (*S1*) is 0,93 and that of the rare allele (*S2*) is 0,07. This did not differ significantly from that observed in our FH population (90 alleles)<sup>20</sup> or in the UK population.<sup>21</sup> Cloning and sequencing data from DNA of a hetero-allelic FH homozygote indicated that the *Stu* I polymorphism was caused by a guanine to adenine transition in exon 8 of the LDL receptor gene. This RFLP, the only one so far detected in the 5' coding sequences of the LDL receptor gene, can be used to distinguish the RFLP alleles for determination of the haplotype-associated defects in cDNAs from hetero-allelic FH homozygotes. To date, a true FH homozygote for the rare *Stu* I allele has not been found for this purpose. Studies are under way to determine the DNA sequence of the remaining LDL receptor gene exons cloned in lambda FH 8 - 30, using exon-specific oligonucleotide primers. This may permit the molecular analysis of the genetic lesion which causes FH among Afrikaners carrying the haplotype 6 allele of the LDL receptor gene.

At least four different classes of LDL receptor gene mutations can be distinguished based on measurements of LDL receptor activity found on the surface of cultured fibroblasts. These disrupt synthesis, intracellular transport, LDL-binding ability or internalisation of the LDL receptor.<sup>22</sup> A variant of the class 2 mutation that produces receptors that are converted to the mature form at an abnormally slow rate has been identified in WHHL rabbits and in several individuals with homozygous FH,<sup>23</sup> including three South African FH homozygotes.<sup>11,24</sup> Previous studies have suggested that alterations in cysteine-rich repeats of the LDL receptor gene are likely to disrupt the disulfide pattern of the protein in these regions, causing abnormal movement to the cell surface.<sup>25</sup> Three different FH mutations that disrupt the transport of the protein to the plasma membrane have been localised to the cysteine-rich repeats. Yamamoto *et al.*<sup>25</sup> reported that the gene mutations in the WHHL rabbits and a patient with FH involve small in-frame deletions in the fourth exons of their LDL receptor genes. A third defect is attributable to a single base substitution in exon 14 that produces a termination codon in the middle of a cysteine-rich sequence.<sup>8</sup> Recently Esser and Russell<sup>26</sup> have characterised a fourth mutation in exon 11 of the LDL receptor gene that encodes a transport-deficient protein. In contrast to the earlier defects, this mutation does not reside in the cysteine-rich repeats, but causes a substitution of a valine for a glycine at residue 544. These results suggest that free cysteines are not obligatory for the blocked intracellular movement of mutant LDL receptors and that regions of a polypeptide backbone containing glycine residues may also be sensitive targets for mutations that disrupt folding. The characterisation of additional transport-defective LDL receptor mutations in FH may serve to identify folding hot spots involving glycine.<sup>26</sup>

The receptor-defective mutation of the 'slow maturation' type was also reported in a family in which there is unusual

longevity and in which FH heterozygotes did not express constant or statistically significant hypercholesterolaemia.<sup>27,28</sup> The fact that obligate FH heterozygotes can have normal or near-normal cholesterol levels called our attention to the fact that a mutant FH allele may be expressed in a background of factors that fail to produce substantial hypercholesterolaemia. With this background, we decided to have a closer look at the base pair changes creating the *Stu* I RFLP within exon 8 of the LDL receptor gene.<sup>14</sup> The frequency of the RFLP is very low in both our normal and FH populations, and the recognition sequence for *Stu* I involves a cysteine codon in the LDL receptor gene. We described the possible role of alterations in cysteine-rich regions of the LDL receptor gene in the production of transport-deficient proteins above. Our sequencing data have revealed that the mutation in exon 8 causes a substitution of a threonine for an alanine at residue 370, which does not cause a gross alteration in protein structure. It is thus highly unlikely that this mutation affects the processing of the receptor protein in FH patients. Biochemical studies of receptor proteins of cultured fibroblasts from FH patients homozygous for the exon 8 mutation and site-directed mutagenesis will be necessary to detect possible abnormalities in protein behaviour.

To date, more than 20 different mutations, which abolish the function of the LDL receptor protein, have been defined at the molecular level. It is likely that, as the number of such mutations analysed is increased, the application of recombinant DNA technology in the diagnosis of FH will become more precise and more universally applicable. This is of particular importance in South Africa, where the high FH frequency is prevalent as a result of a founder effect.

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## Uroporphyrinogen decarboxylase and protoporphyrinogen oxidase in dual porphyria

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### Summary

The urinary and faecal porphyrin excretory profiles of dual porphyria are said to represent the superimposition of those found in porphyria cutanea tarda on those seen in variegate porphyria. To test this hypothesis the enzymes responsible for these conditions, protoporphyrinogen oxidase (variegate porphyria) and uroporphyrinogen decarboxylase (porphyria cutanea tarda) were measured *in vitro* in haemolysates and lymphoblasts of 10 subjects with dual porphyria in order to clarify the enzymatic defects. Mean protoporphyrinogen oxidase activity in lymphoblasts from subjects with dual porphyria was decreased by 45% from  $0,82 \pm 0,10$  to  $0,45 \pm 0,09$  nmol protoporphyrin/mg protein/h ( $P < 0,001$ ). Uroporphyrinogen decarboxylase activity was also significantly reduced from  $0,12 \pm 0,05$  nmol 7-, 6-, 5- and 4-carboxyl porphyrin/mg protein/h in lymphoblasts from normal subjects to  $0,08 \pm 0,02$  nmol in lymphoblasts of subjects with dual porphyria ( $P < 0,01$ ). There was a similar 27% decrease in mean uroporphyrinogen decarboxylase activity of haemolysates from the same dual porphyria group ( $P < 0,01$ ). Mean activity of this enzyme in 5 patients with variegate porphyria did not differ significantly from that in normal subjects. These findings may well provide a rational basis for the abnormal porphyrin excretory profiles found in subjects with dual porphyria.

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Dual porphyria is a disorder of porphyrin metabolism present in up to 25% of subjects with variegate porphyria. Subjects with dual porphyria exhibit haem precursor overproduction characteristic of both porphyria cutanea tarda and variegate porphyria.<sup>1</sup> This relates particularly to the raised faecal excretion of isocoproporphyrin and the elevated urinary uroporphyrin and 7-carboxyl porphyrin. The clinical features of dual porphyria are similar to those of variegate porphyria. They include cutaneous fragility and photosensitivity as well as a propensity to develop acute attacks consisting of varying combinations of abdominal pain, vomiting, constipation, pain in the back and limbs, abnormal behaviour, tachycardia, hypertension and a predominantly motor neuropathy.<sup>2</sup> The cutaneous involvement in porphyria cutanea tarda is identical to that of variegate porphyria, but acute attacks do not occur.<sup>1</sup> Variegate porphyria is inherited as an autosomal dominant disorder.<sup>3</sup> The enzyme primarily affected is protoporphyrinogen oxidase.<sup>4-6</sup> There are thought to be two forms of porphyria cutanea tarda: a familial form inherited in an autosomal dominant manner and a sporadic form, the hereditary characteristics of which, if any, remain unclear.<sup>7-9</sup> In familial porphyria cutanea tarda uroporphyrinogen decarboxylase activity is decreased in all tissues.<sup>9,10</sup> Although it is generally accepted that only hepatic uroporphyrinogen decarboxylase is decreased in sporadic porphyria cutanea tarda,<sup>7,8,11-13</sup> some workers have shown a decrease in uroporphyrinogen decarboxylase activity in erythrocytes of patients with sporadic porphyria cutanea tarda.<sup>14</sup> We measured the activities of both protoporphyrinogen oxidase and uroporphyrinogen decarboxylase in subjects with dual porphyria in an attempt to elucidate the mechanism responsible for the porphyrin excretory profiles associated with this condition.

### Material and methods

We studied 10 patients with dual porphyria. Two of the