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The Finnish type of the LDL receptor gene mutation: molecular characterization of the deleted gene and the corresponding mRNA

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In one third of Finnish patients with the heterozygous form of familial hypercholesterolemia the disease is due to a gross deletion at the 3'-end of the LDL receptor gene. The present study demonstrates that an 8-kb deletion completely eliminates exons 16 and 17 and a part of exon 18. Cloning and partial sequencing of a DNA fragment from the mutated allele indicated that the 5'-boundary of the deletion lies within intron 15 while the 3'-breakpoint is located at nucleotide 3390 in exon 18. RNA blot hybridization studies revealed that the mutated allele encodes a truncated 4.2 kb mRNA (normal, 5.3 kb). This type of mutation has not been reported in other ethnic groups.

Hypercholesterolemia; LDL receptor gene; DNA deletion; Southern blotting; Northern blotting; Oligonucleotide probe

1. INTRODUCTION

Familial hypercholesterolemia (FH) is a disease where a defect in the low density lipoprotein (LDL) receptor gene impairs cholesterol homeostasis leading to increased concentration of plasma LDL and premature atherosclerosis [1]. Patients that are heterozygous for a mutated receptor gene have serum LDL cholesterol levels approximately twice as high as normal and suffer, if untreated, from myocardial infarction typically at the age of 40 to 50 years [2]. About 1 in every 500 people carries one mutated allele and one normal gene [3]. The homozygous form of the disease is extremely rare. These patients may have serum LDL cholesterol levels up to six times normal and many of them succumb to ischemic heart disease in childhood [1].

The human LDL receptor is a transmembrane glycoprotein comprising 839 amino acids [4]. The receptor gene extends 45 kb and consists of 18 exons and 17 introns [1]. Exon 1 encodes the hydrophobic signal peptide, and exons 2–16 encode the extracellular, including ligand-binding, areas of the receptor. Portions of exons 16 and 17 encode the membrane spanning region of the receptor, while the 3'-part of exon 17 and the 5'-part of exon 18 encode the cytoplasmic carboxy-terminal tail of the receptor [5].

So far about 17 different mutations of the LDL receptor gene have been characterized [1,6–18]. Some mutations apparently disrupt the biosynthesis or the transport of the receptor from endoplasmic reticulum to Golgi complex, while some affect the binding of LDL to the receptor or the clustering of the receptor-LDL complexes in coated pits. The spectrum of LDL receptor gene mutations in a given population may be narrower, the more homogeneous the population is in genetic terms. We found recently that one type of mutation of the LDL receptor gene affects about one-third of Finnish FH patients [18]. This paper describes the molecular characteristics of the mutant gene and the corresponding mRNA.

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2. MATERIALS AND METHODS

2.1. *Cloning of the deletion point*

Total genomic DNA was prepared [19] from blood leukocytes of a heterozygous FH patient (FH-10) carrying the Finnish allele of the mutant LDL receptor gene [18]. DNA

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(30 μg) was digested with the restriction enzyme *Nco*I (Boehringer Mannheim, FRG) and fractionated by gel electrophoresis on 0.6% agarose. Fractions in the molecular size range of 2 to 4 kb were eluted from the gel using a commercial kit (Geneclean™, La Jolla, USA). The recessed ends created by *Nco*I were filled in by Klenow and *Eco*RI linkers were added by incubating with T_4 DNA ligase [20]. DNA was then ligated with *Eco*RI cleaved, dephosphorylated λgt 10 arms. The ligated DNA was packaged into λ phage particles and plated using *E. coli* Y 1090 cells. The library (10^6 recombinants) was screened using an LDL receptor cDNA probe covering exons 10–17 [18]. One positive clone was found. The inserted DNA was excised by *Eco*RI digestion, isolated by agarose gel electrophoresis, subcloned into the *Eco*RI restriction site of the plasmid pBR322, and used for DNA sequencing and Southern blot analysis. This plasmid was designated pFH-Nco.

2.2. *DNA sequencing*

To determine the nucleotide sequence at the 3′-boundary of the deletion the following strategies were used: (i) the cloned mutated fragment was sequenced starting from its 3′-end using a 16 nucleotide pBR322 primer (5′-GCAATTTAACTGTGAT-3′) close to its *Eco*RI cloning site; (ii) plasmid pFH-Nco was digested with *Bam*HI and religated. This new modified plasmid missing a 926 bp *Bam*HI fragment, consisting of a 551 bp *Bam*HI–*Nco*I fragment of LDL receptor cDNA [4,18] and a 375 bp *Eco*RI–*Bam*HI fragment of pBR322 [21], was sequenced from its 3′-end using another 16 nucleotide pBR322 primer (5′-ATGCGTCCGGCGTAGA-3′) close to its *Bam*HI restriction site. (iii) The complementary strand was sequenced using a 17 nucleotide primer (5′-ACCTTCTCTCACTTGGG-3′) about 200 bp upstream from the *Bam*HI site in exon 18 of the mutant LDL receptor gene. Sequencing was carried out using a commercial kit (Sequenase, Cleveland, USA) and about 400 nucleotides were sequenced at a time. The sequence was compared to that of exon 18 of the normal LDL receptor gene [4].

2.3. *Southern blot analysis*

The plasmid pFH-Nco (see above) was digested with *Eco*RI overnight at 37°C. A plasmid containing the normal LDL receptor cDNA sequence (pLDLR3, kindly provided by Dr D.W. Russell, see [4]) was used as a positive control. This cDNA was first cleaved with *Xho*I to eliminate exon 18 containing a cluster of Alu sequences [4], and then religated. This plasmid containing almost the entire protein coding sequence of the LDL receptor gene, missing only the last 36 coding nucleotides, was then digested with *Xho*I and *Bam*HI. DNA fragments were fractionated by electrophoresis on 0.6% agarose, denatured, neutralized and transferred to nitrocellulose filter. The filters were hybridized in a solution containing 0.9 M NaCl, 0.09 M Tris-HCl, pH 7.6, 1 mM EDTA, 0.5% sodium lauroylsarcosine, 0.1 mg/ml tRNA, 1 × Denhardt's solution and a synthetic 17-mer oligonucleotide probe, either specific for exon 15 (5′-GACAATGTCTCACCAAG-3′) or exon 16 (5′-TCCATTGTCCTCCCCAT-3′) [4,5]. Labelling of the oligonucleotide probes was accomplished using T_4 polynucleotide kinase [20]. After hybridization for 16 h at 42°C, the filters were washed with 2 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M trisodium citrate), 0.1% SDS at 22°C and with 0.2 × SSC, 0.1% SDS at 42°C. Autoradiography of the dried filters was conducted by exposing them to Kodak XAR films for 3–8 h at −70°C.

2.4. *mRNA analysis*

An incisional skin biopsy was taken from the forearm of the patient FH-10 and fibroblasts were grown using standard cell culture techniques. HepG2 (a human hepatoma cell line) cells were obtained from American Type Culture Collection (Rockville, USA). Total RNA from approx. $1–2 \times 10^8$ cells was

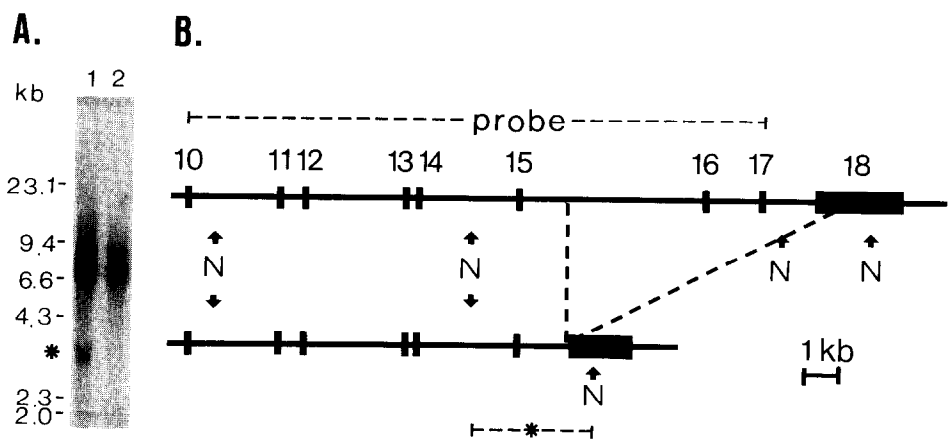

Fig.1. (A) An autoradiogram of *Nco*I-digested DNA samples from a FH-patient (FH-10, lane 1) and a control subject (lane 2) hybridized with a ^{32}P-labelled LDL receptor probe. The abnormal fragment of the patient's DNA is shown by an asterisk. (B) *Nco*I (N) restriction map of the 3′-end of the LDL receptor gene (data from [19,28]). Also shown are the 5′- and 3′-boundaries of the cDNA probe used in Southern blotting (A). The asterisk indicates the abnormal 3 kb *Nco*I fragment that was generated from the mutant allele and subsequently cloned.

isolated by the LiCl-urea method [22] and enriched in poly(A)-containing RNA by oligo(dT)-cellulose affinity chromatography [23]. RNA samples (2 μg) were fractionated on 0.6% agarose gels containing 2.2 M formaldehyde [20] and transferred to nitrocellulose filters. Hybridization of the filter-bound RNA was carried out in a solution containing 6 × SSC, 0.1% SDS, 5 × Denhardt's solution, 100 μg/ml herring sperm DNA, 50% deionized formamide, and 1–2 × 10^6 cpm/ml of ^{32}P-labelled (Multiprime DNA labelling system, Amersham, Buckinghamshire, England) LDL receptor cDNA probe. The probe was a modified version of the plasmid pLDLR3 [4] containing the nucleotides from 1573 to 2544 of the receptor cDNA. After hybridization for 20 h at 42°C the filters were washed with 2 × SSC, 0.1% SDS at 22°C and with 0.2 × SSC, 0.1% SDS at 42°C, and exposed to Kodak XAR films for 1–3 days at −70°C.

3. RESULTS

Our earlier studies demonstrated that about 30% of the Finnish FH patients carry a similar type of deleted LDL receptor gene [18]. In order to precisely define the mutation, a portion of the abnormal gene was isolated from a genomic DNA library derived from an FH patient known to carry the deleted allele. Restriction mapping of the deleted gene [18] indicated that a partial genomic library prepared by digestion with the enzyme *Nco*I and enriched for fragments with a molecular size of about 3 kb would contain the deletion point (fig.1).

The cloned *Nco*I fragment was partially sequenced using primers of plasmid pBR322. Starting from its 3′-end, the upstream sequence was found to be identical with the normal exon 18 sequence up to nucleotide 3390 and to be entirely different beyond that site (fig.2).

In order to determine the 5′-boundary of the deletion, plasmids containing the normal LDL receptor coding sequence and the cloned *Nco*I fragment from the patient FH-10 were analyzed by Southern blotting using exon 15 and exon 16 specific oligonucleotide probes. The normal cDNA sequence hybridized with both oligonucleotide probes, whereas the cloned DNA fragment from the patient hybridized only with the exon 15 specific probe (fig.3).

RNA blot hybridization analysis of poly(A)-containing RNA from a human hepatoma cell line (HepG2) known to express LDL receptors [24,25], and fibroblasts of a normal subject revealed a single 5.3 kb mRNA species (fig.4). RNA from the heterozygous patient FH-10 contained two hybridizable mRNA species, one with an apparently normal size and another smaller but more intensive one with a size of about 4.2 kb (fig.4).

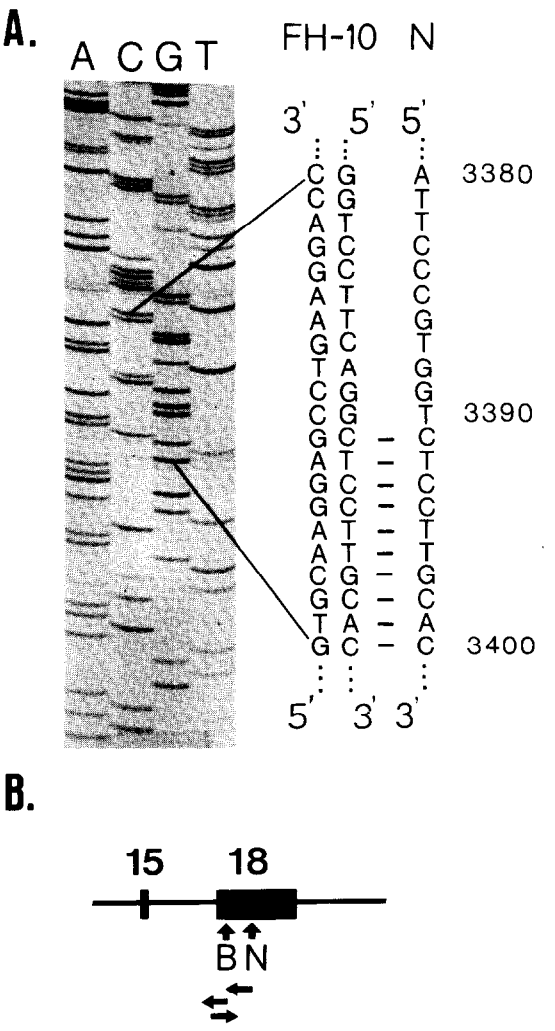

Fig.2. (A) Autoradiogram of a sequencing gel demonstrating the mutation point at exon 18 of the patient FH-10. The DNA sequences of the non-coding and coding strands of the mutant allele as well as the coding strand of the normal allele are shown. Matching DNA sequences are indicated by horizontal bars. Numbering refers to the normal nucleotide sequence of the LDL receptor cDNA [4]. (B) Sequencing strategy for the mutant gene. The arrows show direction and extent of each sequence determination. For detail, see section 2.

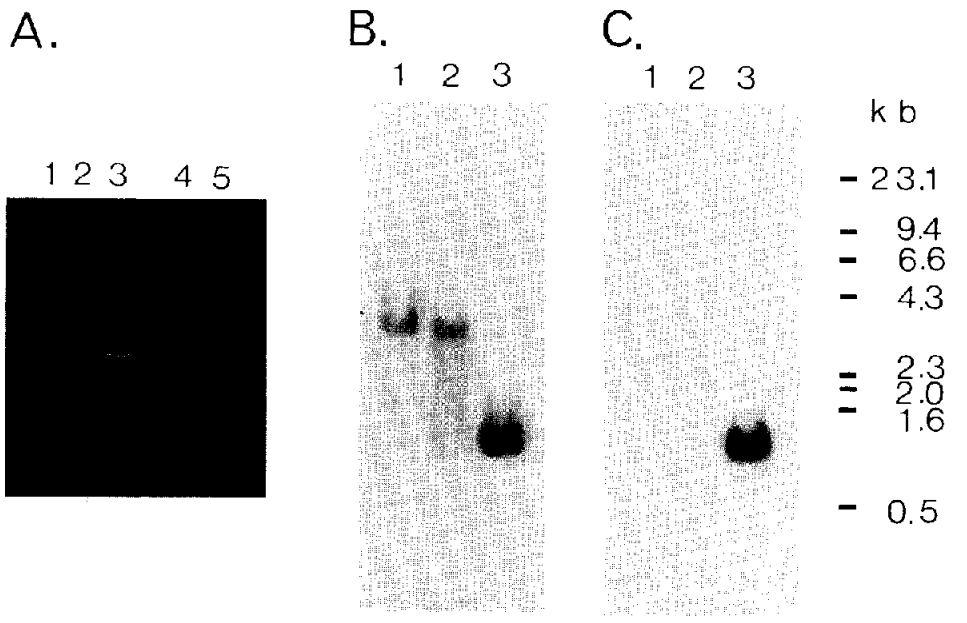

Fig.3. (A) Agarose gel electrophoresis of plasmids containing mutant and normal LDL receptor gene sequences (stained with ethidium bromide). Lanes: 1 and 2, *Eco*RI digested plasmid pFH-Nco (run in duplicate); 3, a plasmid containing a normal LDL receptor cDNA sequence, digested with *Xho*I and *Bam*HI; 4 and 5, *Hin*dIII-digested λ-DNA and *Hin*fI-digested pBR322, respectively, run as molecular size markers. (B and C) Southern blot analysis of nitrocellulose-transferred DNA fragments shown in (A), hybridized with an exon 15 specific oligonucleotide probe (B), or an exon 16 specific oligonucleotide probe (C). Lanes: 1 and 2, *Eco*RI-digested plasmid pFH-Nco (run in duplicate); 3, a plasmid containing a normal LDL receptor cDNA sequence digested with *Xho*I and *Bam*HI.

4. DISCUSSION

We have previously shown that about one third of Finnish patients with FH have an LDL receptor gene defect which is due to a large deletion at the 3′-end of the structural gene [18]. By direct DNA sequencing, the present study demonstrates that the 3′-breakpoint of the mutation lies at exon 18 deleting the 5′-end of this exon up to base pair 3390 and retaining the 3′-end of the exon from base pair 3391 (for nucleotide positions see [4]). The exact boundary of the deletion at its 5′-end cannot be determined. There is circumstantial evidence, however, that the recombination occurs within intron 15. Firstly, the restriction mapping data strongly suggest that this is the case [18]. Secondly, the mutant gene does not hybridize with an exon 16 specific oligonucleotide probe (fig.3). Thirdly, the mutant gene does hybridize with an oligonucleotide probe complementary to a DNA sequence at the very 3′-end of exon 15 (fig.3). Fourthly, the size of the mRNA generated by the mutant gene is in excellent agreement with the suggestion that exon 16 (78 base pairs), exon 17 (158 base pairs) and a portion from the 5′-end of exon 18 (deleted area, 843 base pairs) [5] are deleted. Apparently, a new acceptor site for splicing is formed in exon 18, located 3′ from the mutation point; the exact point of splicing is, however, not known.

Gross deletions of the LDL receptor gene probably represent only 3–6% of the spectrum of mutations [16]. Most of the mutations in the LDL receptor gene so far described contain Alu sequence recombinations [6,9–11,13,14]. Alu sequences are present both in exon 18 and intron 15 [13]. The present data exclude the possibility that the Finnish type of mutation represents an Alu-Alu recombination, the type of mutation found in FH patients from the USA [6] and from Japan [13], since the deletion point in exon 18 is not localized in the Alu region, nucleotides 3704–4465 [4]. This study and another recently published report [17] are in accordance with the assumption of Lehrman

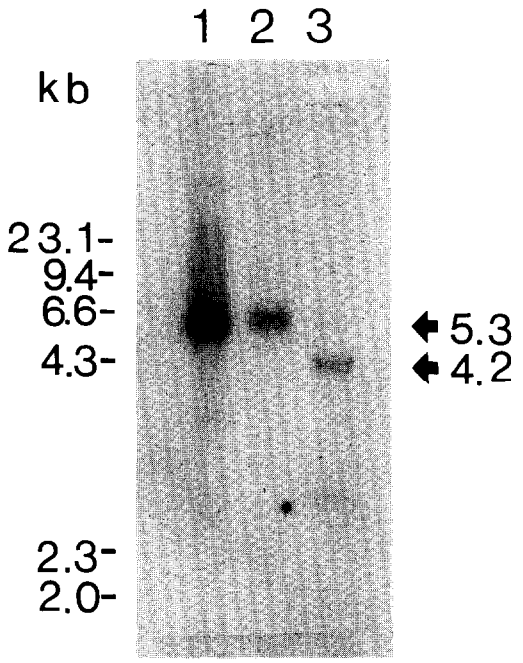

Fig.4. Northern blot hybridization analysis of poly(A)-containing RNA from a human hepatoma cell line, HepG2 (lane 1), fibroblasts of a healthy control subject (lane 2) and a patient with FH (FH-10, lane 3).

et al. [13] that there is some intrinsic instability in intron 15.

Several types of deletions at the 3'-end of the LDL receptor gene have been described in different ethnic groups, including American [6,9], Caucasian [11] and Japanese [13,17] populations. None of them, however, appear to be identical with the Finnish one described in the present paper. Although the exact structure of the receptor encoded by the Finnish type of mutation remains to be investigated, it may be expected to have grossly altered transmembrane and cytoplasmic domains or to be entirely devoid of them. Thus, it is very likely that the Finnish type of mutant allele encodes, like other mutant genes with defects in the same region [6,13], a receptor which displays defective internalization of its bound LDL-ligand.

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