Am. J. Hum. Genet. 43:060–068, 1988

Characterization of Six Partial Deletions in the Low-Density-Lipoprotein (LDL) Receptor Gene Causing Familial Hypercholesterolemia (FH)

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Summary

Two hundred thirty-four unrelated heterozygotes for familial hypercholesterolemia (FH) were screened to detect major rearrangements in the low-density-lipoprotein (LDL) receptor gene. Total genomic DNA was analyzed by Southern blot hybridization to probes encompassing exons 1–18 of the LDL receptor gene. Six different mutations were detected and characterized by the use of exon-specific probes and detailed restriction mapping. Each mutation is unique and suggests that molecular heterogeneity underlies the molecular pathology of FH. There appear to be preferential sites within the LDL receptor gene for major rearrangements resulting in deletions.

Introduction

Famililal hypercholesterolemia (FH) is a common autosomal dominant disorder caused by a defect in the low-density-lipoprotein receptor (LDL receptor), disrupting the normal control of cholesterol metabolism (Brown and Goldstein 1986). One of 500 individuals is heterozygous for FH and presents with elevated LDL cholesterol and has an increased risk for premature atherosclerotic disease.

Over the past few years, work done by Brown, Goldstein, and co-workers has provided major insights concerning the pathogenesis of this disorder. In 1981 the bovine LDL receptor was purified (Schneider et al. 1982). From this, a partial amino acid sequence was obtained that allowed the isolation of a partial cDNA clone that encodes the COOH-terminal one-third of the bovine LDL receptor (Russell et al. 1983). Subsequently, the full-length 5.3-kb cDNA for the human LDL receptor was isolated (Yamamoto et al. 1984). The amino acid sequence was then deduced, and subsequent studies have allowed the delineation of six structural domains of the protein: the signal sequence domain, the LDL-binding domain, the EGF precursor homology domain, the O-linked sugar domain, the membrane-spanning domain, and the cytoplasmic domain (Brown and Goldstein 1986).

Progress in the understanding of the molecular pathology of disorders inherited in an autosomal dominant manner has been slow. Further insights into the molecular pathology of FH may serve as an important model for other autosomal dominant disorders.

In this study we have screened 234 unrelated heterozygotes for FH by Southern blot hybridization using LDL-receptor cDNA probes. Our aim has been to assess the frequency and nature of major structural rearrangements at the LDL-receptor locus in patients with FH.

We have identified and characterized, by using a combination of exon-specific probes and detailed restriction-endonuclease mapping, six previously undescribed mutations. Segregation analysis in all six families has confirmed the relationship between the described mutation and the FH phenotype.

Received February 12, 1988.

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0002-9297/88/4301-0009$02.00

Subjects and Methods

Subjects

All patients were assessed at the Vancouver Lipid Clinic between January 1984 and July 1987. A diagnosis of FH was made based on the following criteria: (1) total and LDL cholesterol above the 95th percentile for age and sex, (2) a positive family history of hypercholesterolemia and/or premature atherosclerosis in a first-degree relative (at <55 years of age in a male and <60 years of age in a female), (3) physical signs of FH, including an arcus cornealis and tendon xanthomas. All 234 unrelated individuals had to have met at least two of the three criteria for the diagnosis to be made. Full clinical details concerning these patients will be reported separately elsewhere.

Biochemical Analysis

Levels of triglyceride and of both total and high-density-lipoprotein (HDL) cholesterol were determined using methods described elsewhere (Bucolo and David 1973; Allain et al. 1974; Warnick and Albers 1978). LDL cholesterol was calculated using the Friedewald formula (Friedewald et al. 1972). Apoprotein B determination was done by radioimmunodiffusion assay (TAGO Inc., Burlington, CA).

DNA Analysis

DNA from 234 unrelated FH heterozygotes and five normolipidemic controls was prepared from fresh EDTA blood by using a technique described elsewhere (Hayden et al. 1987). Five-microgram aliquots of DNA were digested with the restriction endonuclease *Bgl*II (Pharmacia Ltd., Dorval, Quebec). DNA fragments were electrophoresed through a 0.7% agarose gel and transferred to nylon filters by means of Southern blotting (Southern 1975). The filters were prehybridized in 5 × SSPE (pH 7.5), 10 × Denhardt's (0.2% BSA, 0.2% PVP360, and 0.2% Ficoll), 0.05 μg sheared denatured salmon-sperm DNA/ml, 0.3% SDS and incubated at 65 C for several hours. Filters were hybridized overnight at 65 C in 5 × SSPE, 5 × Denhardt's, 0.05 μg sheared denatured salmon-sperm DNA/ml, and 0.1% SDS. Two cDNA probes were used for hybridization, a 1.8-kb fragment representing exons 1–11 isolated by *Hin*dIII/*Bgl*II digestion of the plasmid pLDL-R3 and a 1.9-kb fragment representing exons 11–18 isolated by *Bam*HI digestion of the plasmid pLDLR2-HH1. These two plasmids were a gift from Dr. D. W. Russell of Dallas. Approximately 20 ng of each probe was labeled with P^{32} (Amersham, Canada) to $\geq 10^8$ cpm by using a random oligonucleotide priming method (Feinberg and Vogelstein 1983, 1984). Filters were washed in 0.5 × SSPE, 0.1% SDS for 30 min twice at 65 C and then once in 0.1 × SSPE, 0.1% SDS for 20 min at 65 C and were then exposed to Kodak XAR-5 films with intensifying screens at −70 C for 2–5 days. Before rehybridization the filters were washed in 0.4 N NaOH at 42 C for 30 min and then in 0.1 × SSC, 0.5% SDS, 0.2 M Tris (pH 7.5) at 42 C for 30 min.

Restriction mapping and exon-specific cDNA probes were used for detailed mapping. Probes for exons 2, 4–6, 7, and 13–15 were prepared from M13 subclones of pLDLR-2 in Dr. Helen H. Hobbs's laboratory in Dallas. These single-stranded probes ~100 nucleotides in length were uniformly P^{32} labeled by the method of Church and Gilbert (1984). Exon 16–specific, exon 17–specific, and exon 18–specific probes were made in our laboratory. The exon 17 probe was made by *Pvu*II digestion of the plasmid pDD1 and by isolation of the 236-bp insert; a probe encompassing exon 16 and the 5′ end of exon 17 was made by *Alu*I digestion of the plasmid pLDLR-2HH1 and by isolation of the 226-bp fragment; finally, a 683-bp fragment isolated by *Sma*I digestion of the same plasmid was used as a probe corresponding to the 5′ end of exon 18.

Results

Subjects

After a screening of the genomic DNA of 234 FH heterozygotes, six individuals were found to have a major structural rearrangement of one LDL-receptor gene. Table 1 describes the biochemical findings of these six FH heterozygotes. The ranges and percentiles of total cholesterol, LDL cholesterol, HDL cholesterol, and triglycerides have been published elsewhere (Hayden 1986). FH 17 and FH 49 were the only two patients to present with symptoms of early atherosclerosis. They, as well as FH 131, had physical signs of FH, including an arcus cornealis and tendon xanthomas. FH 15, FH 245, and FH 254 were asymptomatic and free of physical findings, but all had (1) cholesterol levels significantly above the 95th percentile for age and sex and (2) a positive family history of hypercholesterolemia and premature atherosclerosis. Three of our probands are of English

Table 1

Biochemical Findings for the FH Heterozygotes with Deletions in Their LDL Receptor Gene

Mutant (Age in Years)	Total Cholesterol	LDL Cholesterol	HDL Cholesterol	Apo B	Triglyceride
FH 15 (49)	383	314	32	181	185
FH 17 (36)	374	315	32	162	134
FH 131 (43)	350	271	46	178	165
FH 49 (35)	492	376	52	183	201
FH 145 (15)	338	273	49	224	79
FH 154 (27)	314	236	49	188	146

NOTE.—All data are in mg/dl.

descent, two are Irish, and one has his ancestral origins in Ecuador.

DNA Analysis

Hybridization of the *Bam*HI insert of pLDLR-2HH1 (exons 11–18) to *Bgl*II digests of control DNA reveals a 21-kb band. FH 49, FH 131, FH 145, and FH 154 only show a 21-kb fragment similar to the control, whereas FH 15 and FH 17 have the normal band as well as a smaller, mutant band of ~19.5 kb in FH 17 and of ~16.5 kb in FH 15 (fig. 1*A*).

Hybridization of the *Hin*dIII/*Bgl*II insert of pLDL-R3 (exons 1–11) probe to a *Bgl*II digest of control DNA reveals bands of 13 and 8.75 kb. The same pattern is seen in FH 15 and FH 17. FH 49, FH 131, FH 145, and FH 154 all have a mutant band in addition to the normal two bands (fig. 1*B*). These results suggested that FH 15 and FH 17 have a mutation involving the 3′ end of the gene and that FH 49, FH 131, FH 145, and FH 154 have a major structural rearrangement involving the 5′ end of the gene. Further mapping was done by restriction-endonuclease analysis and the use of smaller cDNA exon-specific probes.

FH 15 (fig. 2)

*Bgl*II digests were hybridized with an exon 13 and 14 cDNA probe, and only the normal 21-kb band was visualized. When the same filter was hybridized with an exon 15–specific probe, both the normal 21-kb band and a 16.5-kb band were seen (fig. 2). This implies that FH 15 has a 4.5-kb deletion that does not involve the *Bgl*II restriction site found in exon 12. On the basis of these results, it would seem that the deletion involves in all likelihood only exons 13 and 14.

FH 17 (fig. 3A, 3B)

*Pvu*II digests were hybridized with the 1.9-kb cDNA insert of pLDLR-2HH1 (exons 11–18), and the normal 16.5-kb band as well as a 19-kb band were seen. There are two *Pvu*II restriction sites located on either side of exon 17 (Hobbs et al. 1985).

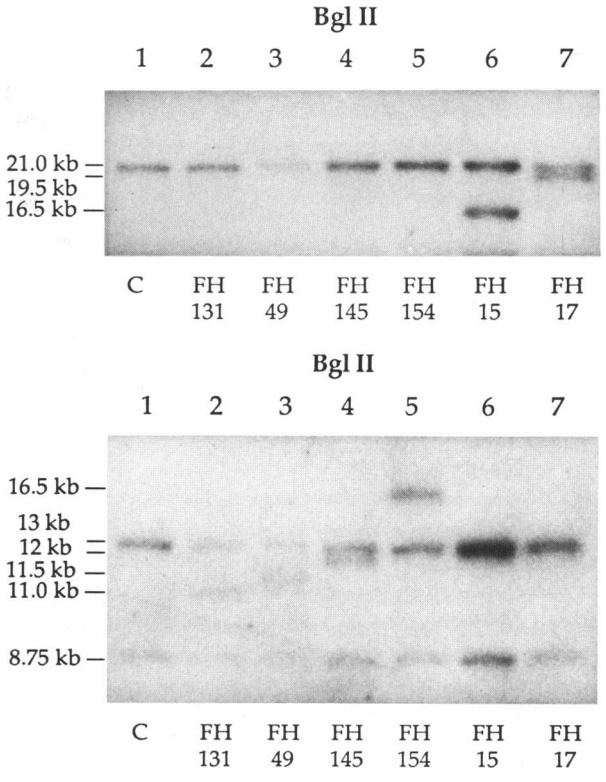


Figure 1 *A*, Southern blot hybridization, with exons 11–18 of pLDLR-2HH1, of *Bgl*II digests of control DNA (lane 1) and DNA from six FH heterozygotes (lanes 2–7). *B*, Southern blot hybridization, with exons 1–11 of pLDL-R3, of *Bgl*II digests as in fig. 1*A*.

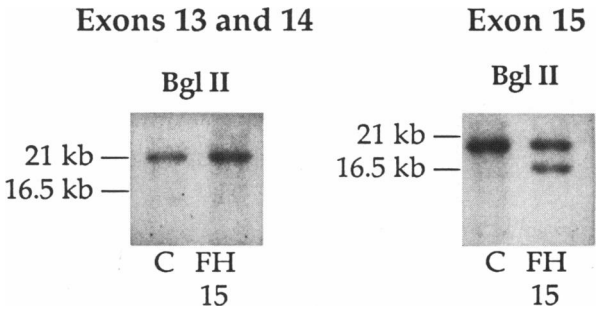


Figure 2 Southern blot hybridization, with an exon 13 and 14–specific cDNA probe and subsequently with an exon 15–specific probe, of *Bgl*II digests of control (C) DNA and FH 15 DNA.

The 19-kb band could be explained by a deletion involving these two *Pvu*II sites, suggesting that exon 17 had been deleted. To confirm this hypothesis, *Stu*I-digested DNA was hybridized with the probe encompassing exons 11–18 (fig. 3*A*). After stripping, the filter was rehybridized with exon 17, thereafter rehybridized with exons 16 and 17, and finally rehybridized with a probe encompassing the 5′ end of exon 18 (fig. 3*B*). The normal 12-kb and 7.2-kb fragments and a mutant 5.8-kb fragment were seen after each hybridization except when exon 17 was used as a probe, in which case the mutant band was absent (fig. 3*A*, 3*B*). This proved that FH 17 has a 1.4-kb deletion involving only exon 17.

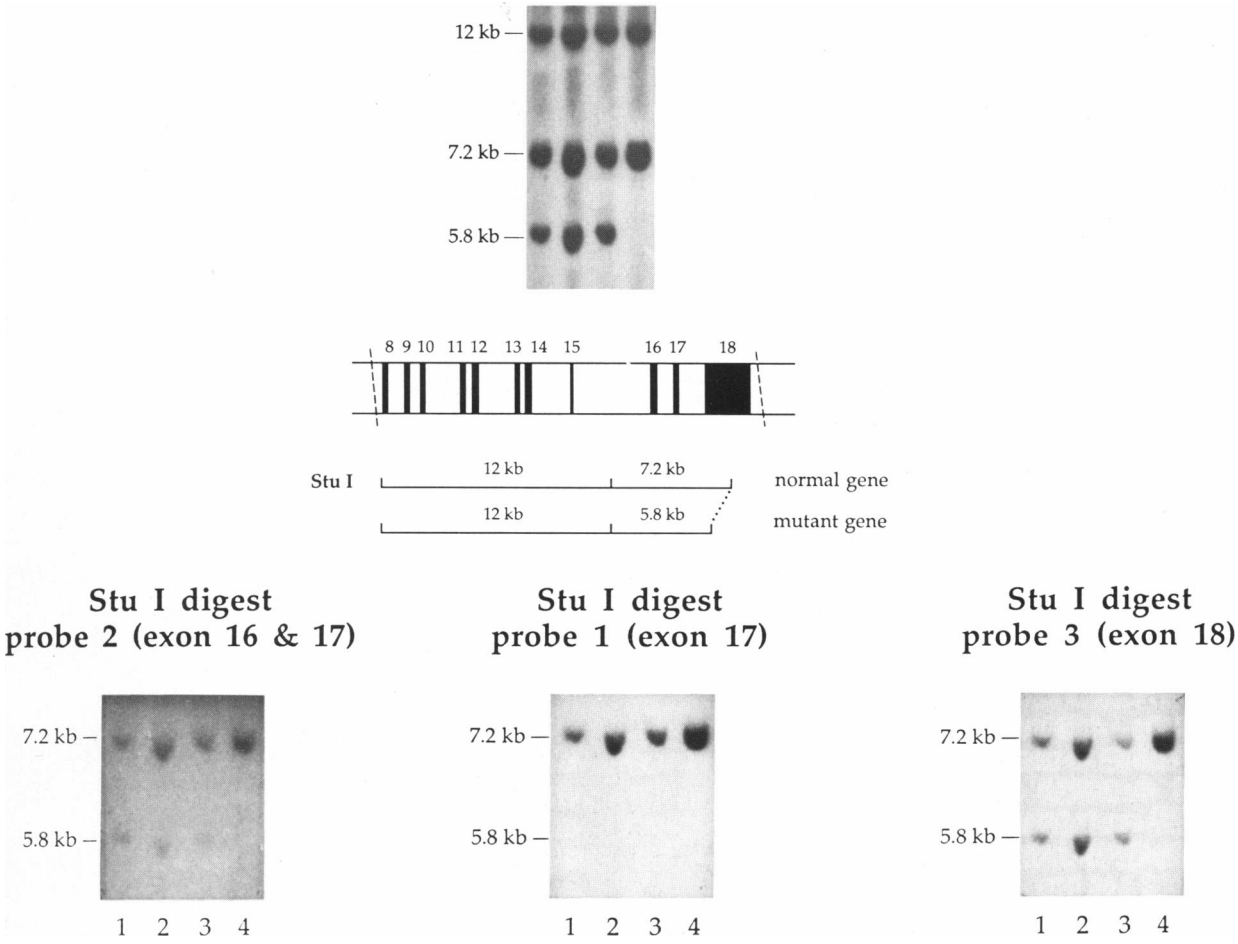


Figure 3 *A*, Southern blot hybridization, with exons 11–18 of pLDLR-2HH1, of *Stu*I-digested DNA from three affected family members (lanes 1–3) and control DNA (lane 4). *B*, Same filter as in fig. 3*A*, rehybridized with exon-specific probes.

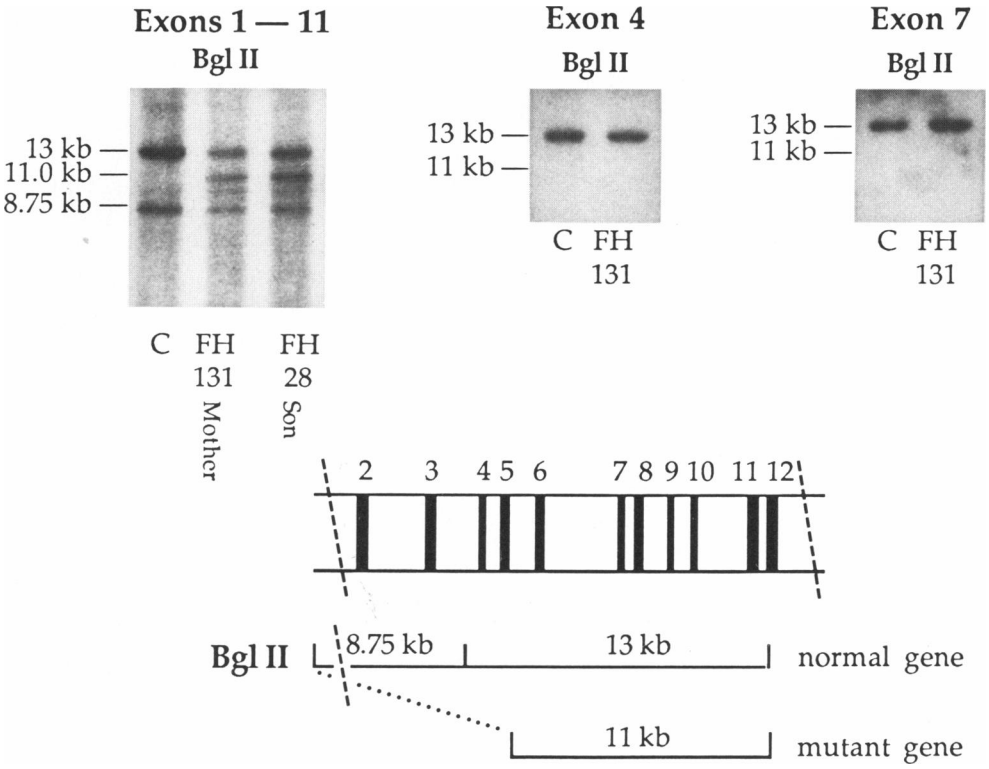


Figure 4 Southern blot hybridization, with exons 1–11 of pLDL-R3 and with exon 4– and exon 7–specific probes, of *Bgl*II-digested DNA from a control (C), FH 131, and from her son, FH 28.

FH 131 (fig. 4)

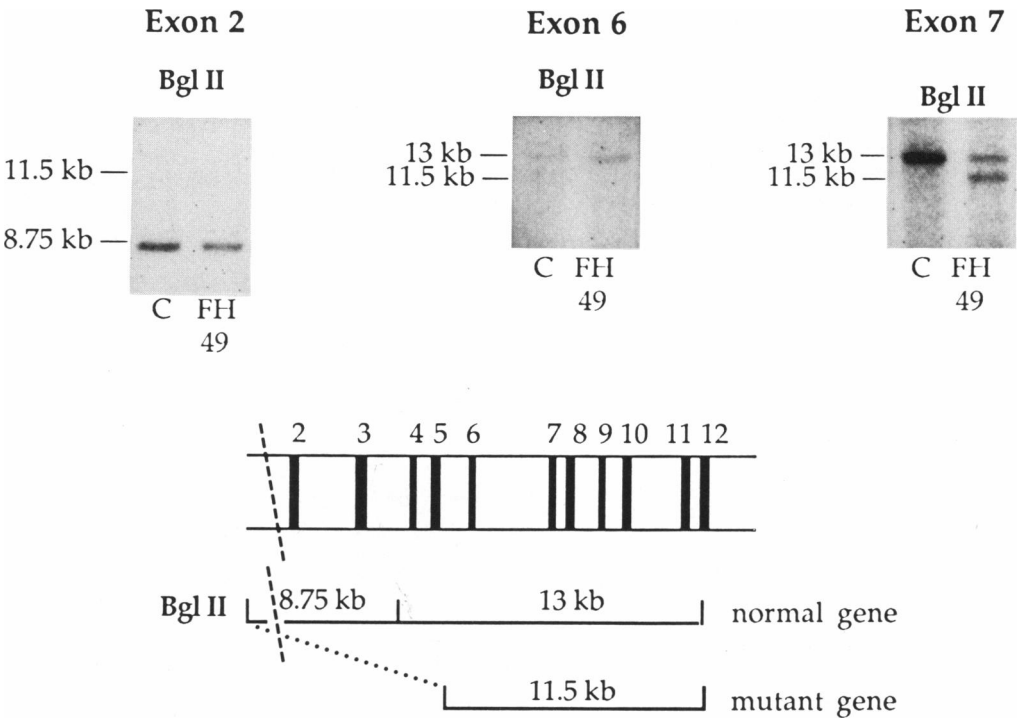
*Bgl*II-digested DNA from FH 131 was hybridized with exons 1–11 and revealed the normal 13-kb and 8.75-kb bands as well as an 11-kb band (fig. 1*B*). Three explanations are possible: there may be (1) a small deletion of ~2 kb involving the 13-kb fragment, (2) an insertion in the region of the 8.75-kb fragment, or (3) a larger deletion involving both of the fragments and loss of a *Bgl*II restriction site. Hybridization of *Bgl*II-digested DNA with exon-specific probes for exons 4 and 7 showed hybridization to the normal bands but not to the mutant 11-kb band, implying a deletion involving at least exons 4–7 (fig. 4). Further restriction mapping using *Eco*RV and *Kpn*I restriction endonucleases revealed that the *Eco*RV restriction site found in exon 8 was lost but not the *Kpn*I restriction site found in intron 2. Therefore FH 131 has an 11-kb deletion involving exons 4–8 and, in all likelihood, on the basis of estimates of the size of the deletion and the known restriction map, also exon 3.

FH 49 (fig. 5)

*Bgl*II-digested DNA from FH 49 hybridized with exons 1–11 revealed the normal 13-kb and 8.75-kb bands as well as an 11.5-kb band (fig. 1*B*). The same three hypotheses proposed for FH 131 apply in this situation. Hybridization of the *Bgl*II-digested DNA with exon-specific probes for exons 2 and 5–7 showed that only the exon 7 probe hybridized to the mutant fragment (fig. 5). Therefore, FH 49 has a 10-kb deletion involving exons 2–6 inclusively.

FH 145 (fig. 6)

As shown in figure 1*B*, FH 145's additional band is larger than the mutant band of FH 49. This was also found when *Xba*I-digested DNA from FH 49 and FH 145 were run on the same gel (fig. 6). This implies that FH 145 has a deletion of ~9.5 kb. Restriction mapping using *Kpn*I digestion and hybridization to exons 1–11 revealed the normal 20-kb band as well as a slightly smaller, mutant band, creating a doublet which implies the loss of the *Kpn*I re-



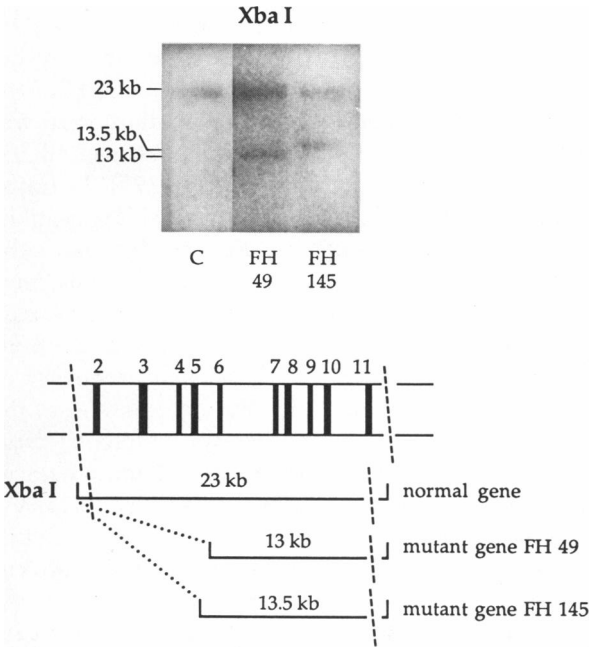


Figure 6 Southern blot hybridization, with exons 1–11 of pLDL-R3, of *Xba*I-digested DNA from a control (C), FH 49, and from FH 145.

striction site normally found in intron 2. These results and the mapping of FH 49 described previously lead us to conclude that, in all likelihood, FH 145 has a 9.5-kb deletion involving exons 2–6.

FH 154 (fig. 7)

Hybridization of *Bgl*II-digested DNA from FH 154 showed a 16.5-kb band in addition to the normal 13-kb and 8.75-kb bands (fig. 1*B*). This implies either an insertion of ~3.5 kb or a deletion with loss of the *Bgl*II restriction site found in exon 3, a loss resulting in a larger restriction fragment. Hybridization of *Xho*I-digested DNA from FH 154 revealed the normal 11.5-kb, 9.5-kb, and 5.5-kb bands as well as a mutant 10-kb band. This implies that the *Xho*I site in intron 6 is deleted (fig. 7). Therefore, FH 154 has a 5-kb deletion involving exons 4–6.

Discussion

This study represents the largest number of heterozygotes for FH to be screened by Southern blot hybridization for major structural rearrangements.

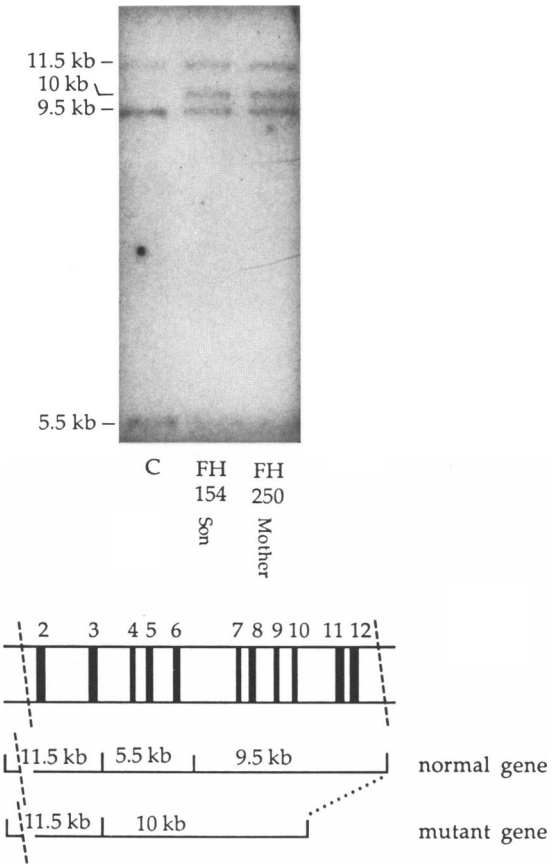


Figure 7 Southern blot hybridization, with exons 1–11 of pLDL-R3, of *Xho*I-digested DNA from a control (C), FH 154, and from his mother, FH 250.

Six new deletions have been detected in this population of 234 patients (frequency 2.5%) and have been precisely mapped by using the published restriction map and exon-specific probes. Elsewhere Humphries and co-workers have described four deletions in 70 FH heterozygotes screened (Horsthemke et al. 1987). From these studies, we conclude that major structural rearrangements account for 2%–6% of the mutations causing FH. Although a case of duplication has been detected by Southern blot hybridization (Lehrman et al. 1987*a*), deletions remain the most common major structural rearrangement in the LDL-receptor gene.

At least six specific domains in the LDL-receptor protein have been recognized. These include a signal sequence coded for by exon 1, a ligand-binding domain coded for by exons 2–6, an epidermal growth factor (EGF)–precursor homologous region coded for by exons 7–14, a clustered O-linked sugar domain coded for by exon 15, a transmembrane segment coded for by exon 16 and part of exon 17, and a cytoplasmic domain coded for by parts of exons 17 and 18. There is a strong correlation between the intron-exon structure of the LDL-receptor gene and the predicted protein domains. Three of the six mutants described here have deletions involving the exons coding for amino acids that bind the LDL particle. FH 49 and FH 145 have a deletion of exons 2–6; FH 154, exons 4–6.

FH 131 has a deletion involving both the binding domain and the EGF-precursor domain (exons 3–8). Exons 13 and 14 of the EGF-precursor domain are deleted in FH 15. Finally, FH 17 has a deletion involving only exon 17, which has been implicated in both the transmembrane and cytoplasmic domains.

Together with the mutants described here, a total of 16 deletions have now been reported (Horsthemke et al. 1985, 1987; Lehrman et al. 1985, 1986, 1987*b*; Hobbs et al. 1986, 1987). These results are summarized in figure 8. It is noteworthy that within the LDL-receptor gene there appear to be preferential sites for major rearrangements resulting in deletions. These involve exons 1–8 (10 subjects) and exons 13–18 (six subjects). Exons 9–12 have not yet been implicated in a major rearrangement event in the LDL receptor. Of the 16 deletions, five have been defined at the molecular level. All five are the result of recombination between repetitive elements found at the deletion breakpoints (FH 381, FH 274, TD, FH 781, and FH 626). An Alu-Alu recombination event was also responsible for the duplication of exons 2–8 that was reported by Lehrman et al. (FH 295) (Lehrman et al. 1987*a*). It is therefore likely that this is the most common mechanism for major rearrangements in the LDL-receptor gene. The clustering of major rearrangements in the 5′ end and 3′ end of the gene may be related to the high number of Alu repeats in introns 1–8 and 12–17 and to a lower frequency of such repeats in exons 9–13. Further analysis, at the molecular level, of the mechanism underlying these deletions, together with complete sequence data of introns 8–13, will help answer this question.

Rouyer et al. (1987) have recently described, in a human XX male, a sex-chromosome rearrangement caused by Alu-Alu recombinations. The implied role of Alu repeats as recombinational hot spots will gain further credibility with the increased knowledge of the molecular mechanisms underlying rearrangements causing other genetic disorders. Shapiro and co-workers have shown that deletions involving the

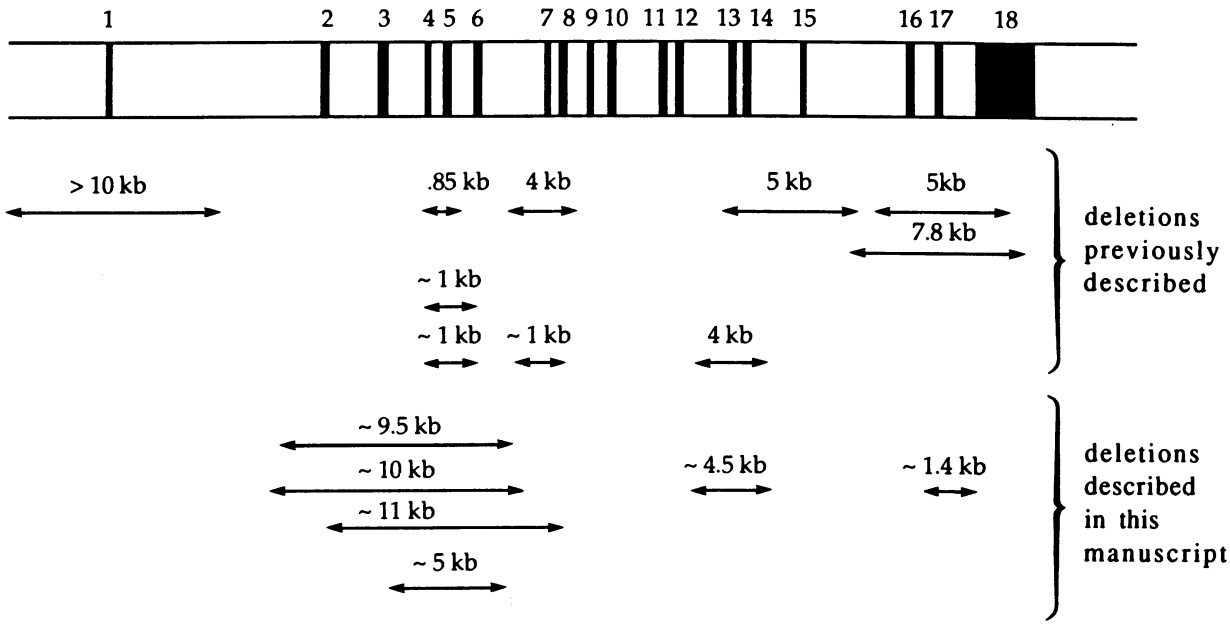


Figure 8 Summary of deletions previously mapped elsewhere and of those described in the present paper

steroid sulfatase gene are responsible for X-linked ichthyosis in 24 of 25 affected males (Shapiro et al. 1987). Van Ommen and co-workers have detected major deletions in the Duchenne muscular dystrophy gene in 50% of boys that have Duchenne muscular dystrophy (den Dunnen et al. 1987). Analysis of the deletion breakpoints is needed to determine whether repetitive elements of the Alu family are involved in these unusually frequent recombinational events. This will help to determine whether recombination between repetitive elements is a common mechanism for major rearrangements of genes throughout the human genome.

It is of interest that most mutations found in the LDL-receptor gene have been unique. The only common mutations reported have been in persons of French-Canadian (Hobbs et al. 1987) and Lebanese descent (Lehrman et al. 1987*c*), in whom it can be accounted for by a founder effect. It is apparent that in the absence of a founder effect molecular heterogeneity underlies the molecular pathology of FH.

FH is a common disorder with a high heterozygote frequency of 1/500, despite no known selective advantage for the heterozygote carrier. The high frequency of FH in the general population may be due, at least in part, to a higher mutation rate in the LDL-receptor gene than previously has been suspected. The molecular heterogeneity as documented in the present paper is in keeping with this hypothesis. The higher mutation rate may be due to the high frequency of repetitive elements in the LDL-receptor gene. It is possible that the lower population frequency with lower mutation rates that occurs in other late-onset autosomal dominant disorders such as Huntington disease or polycystic kidney disease will be reflected in significantly different organization of these genes.

We therefore may expect that patients presenting with clinical features suggesting FH but with either a negative family history or parents with normal serum cholesterol represent new mutations. Another possibility is that the gene is nonpenetrant or extremely mild in its expression in one of the parents.

The finding of six gross alterations in the LDL-receptor gene that are detectable by Southern blot hybridization, coupled with the segregation analysis of the mutant genes and the clinical phenotype in these six families, will allow us to address the question of penetrance and variability of expression of this mutant gene.

Acknowledgments

We would like to acknowledge Dr. Helen H. Hobbs of Dallas for making the exon-specific probes available to us and for her support and helpful discussions. Gail James and Hannelore Vannetta helped in collation of the clinical details. We thank Drs. J. Frohlich and S. W. Rabkin for mak-

ing their patients available for this study. This work was supported by grants from the British Columbia Children's Hospital Research Foundation, the British Columbia Heart Foundation, and Merck-Frosst Canada.

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