Occurrence of multiple aberrantly spliced mRNAs of the LDL-receptor gene upon a donor splice site mutation that causes familial hypercholesterolemia ($FH_{Benevento}$)

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Abstract A novel point mutation of the LDL-receptor gene was found in an Italian patient with homozygous familial hypercholesterolemia. The SSCP analysis of the promoter and of 16 out of the 18 exons of the LDL-receptor gene was negative, suggesting that the mutation might be located in the region of the gene encompassing exons 14 and 15, a region that had not been amenable to polymerase chain reaction (PCR) amplification from genomic DNA. This region was amplified from cDNA by reverse transcription PCR (RT-PCR). RT-PCR of proband cDNA generated three fragments of 800, 600, and 550 bp, respectively, as opposed to a single 720 bp fragment obtained from control cDNA. The sequence of these fragments showed that: *i)* in the 800-bp fragment exon 14 continued with the 5' end of intron 15 (90 nucleotides), which in turn was followed by exon 16; *ii)* in the 600-bp fragment exon 14 was followed by the 5' end of exon 15 (50 nucleotides), which continued with exon 16; *iii)* in the 550-bp fragment exon 14 joined directly to exon 16. These abnormally spliced mRNAs resulted from a G→A transition at the +1 nucleotide of intron 15, which changed the invariant GT dinucleotide of the 5' donor splice site. That was associated with the activation of two cryptic donor splice sites in intron 15 and exon 15, respectively, and the use of an alternative splicing leading to the skipping of exon 15. Northern blot analysis showed that the overall content of these aberrantly spliced mRNAs in proband fibroblasts was one-fourth that found in control cells. These abnormally spliced mRNAs are predicted to encode three abnormal receptor proteins: the first would contain an insertion of 30 novel amino acids; the second would be a truncated protein of 709 amino acids; the third would be devoid of the 57 amino acids of the O-linked sugar domain. Ligand blot experiments indicated that the amount of LDL-receptor present in proband's fibroblasts was approximately one-tenth that found in control cells.—**Lelli, N., R. Garuti, M. Ghisellini, R. Tiozzo, M. Rolleri, V. Aimale, E. Ginocchio, A. Naselli, S. Bertolini, and S. Calandra.** Occurrence of multiple aberrantly spliced mRNAs of the LDL-receptor gene upon a donor splice site mutation that causes familial hypercholesterolemia ($FH_{Benevento}$). *J. Lipid Res.* 1995. **36:** 1315–1324.

Supplementary key words reverse transcription • polymerase chain reaction • fibroblasts

Familial hypercholesterolemia (FH) is an autosomal dominant disorder characterized by an increased level of plasma low density lipoprotein (LDL), which is frequently associated with the development of premature coronary artery disease (1). The classical FH is caused by mutations of the gene encoding the LDL-receptor (LDL-R) (1, 2). A large number of mutations of the LDL-R gene, both gross rearrangements and minute mutations, have been reported (3, 4). In genetically heterogeneous populations more than 90% of all mutations are point mutations (5, 6). While gross gene rearrangements are easily detected by Southern blot analysis, the detection of point mutations is more difficult and time-consuming, as it requires the sequence of *1)* all the 18 exons; *2)* the exon/intron boundaries; and *3)* the promoter of the LDL-R gene. Recently, however, several methods [chemical cleavage of mismatches, single strand conformation polymorphism (SSCP), and denaturing gradient gel electrophoresis (DGGE)] have been successfully adopted for the rapid localization of point mutations of LDL-R gene (7–14). Most of the point mutations reported so far are located in the exons where they produce missense, nonsense, and frameshift mutations (see ref. 2 for review). By adopting the SSCP method, we have been able to identify several point mutations in Italian FH patients (S. Calandra and S. Bertolini, unpublished results). During this study we

Abbreviations: LDL, low density lipoprotein; LDL-R, LDL-receptor; PCR, polymerase chain reaction; RT, reverse transcription; FH, familial hypercholesterolemia; SSCP, single strand conformation polymorphism; DGGE, denaturing gradient gel electrophoresis; LPDS, lipoprotein-deficient serum.

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found a patient who was homozygous for a G→A transition at the first nucleotide of intron 15, a mutation that converts the invariant GT motif of the 5'-donor splice site into AT. As only one of the few mutations found in the introns of the LDL-R gene (4, 15, 16) has been fully characterized so far (16), we thought that the description of our proband provided an interesting example of the disruption of the splicing process of LDL-R pre-mRNA, which may occur upon the elimination of a normal 5' donor splice site.

MATERIALS AND METHODS

Subjects

The proband (G.R.) was a 10-year-old boy, who on physical examination had all the clinical features of classical homozygous FH (**Table 1**). Although coronary arteriography was not performed, B-mode ultrasonography of the carotid arteries revealed the presence of a large atherosclerotic lesion in the left internal carotid artery. The proband's parents, who were apparently unrelated, were found to have elevated serum cholesterol and apolipoprotein B. The proband's brother was found to have serum lipids within the normal range. All subjects gave their informed consent for this study. The proband's family has been living for several generations in southern Italy, close to the city of Benevento.

Biochemical analysis

Plasma lipoproteins were separated by preparative ultracentrifugation in a Beckman 50.4 Ti rotor (17) and cholesterol and triglyceride were assayed by enzymatic methods (Boehringer, Mannheim, Germany) (17). Plasma apoA-I and apoB were measured by immunonephelometry (Behringwerke AG, Marburg, Germany) Plasma Lp[a] was measured by a commercial ELISA kit (Macra, Terumo Corp., Elkton, MD) (18, 19).

Fibroblast culture and LDL-receptor activity

A skin biopsy was taken from the proband. Explants were cultured in 25-cm² flasks in DMEM (Dulbecco's modification of Eagle's medium), 100 IU/ml of penicillin, and 50 μg/ml of streptomycin, 2 mM glutamine, 15% fetal calf serum, and 95% air–5% CO_2. The assay of ^{125}I-labeled LDL binding, internalization, and degradation by cultured skin fibroblasts was performed as previously described (20).

Ligand blotting

For ligand blotting, cell monolayers that had been incubated for 48 h in LPDS were washed three times with phosphate-buffered saline, scraped from the dishes with a cell scraper, and centrifuged at 2000 *g* for 5 min. The cell pellet was solubilized with 200 mM Tris-maleate, pH 6.5, 2 mM $CaCl_2$, 0.5 mM PMSF (phenylmethylsulfonylfluoride), 25 μm leupeptin, and 40 mM β-octyl-D-glucoside. The cell extracts were centrifuged at 300,000 *g* (Beckman TLA 100.1 rotor) at 4°C for 40 min and the pellet was discarded. Protein concentration in the supernatant was measured by the method of Lowry et al. (21). For blotting experiments the samples did not contain reducing agents and were not heated prior to application to polyacrylamide gel. Proteins were separated by 7% SDS-PAGE (22). Electrophoretic transfer of proteins to nitrocellulose filters was performed in a buffer containing 10 mM $NaHCO_3$ and 3 mM Na_2CO_3 at pH 9.9 in 20% (v/v) methanol (23). Ligand blotting with ^{125}I-labeled LDL (sp act 350 cpm/μg of protein) and ^{125}I-labeled rabbit β-VLDL (90 cpm/μg of protein) was performed as described by Daniel et al. (24). Human LDL were isolated from healthy blood donors (20). Rabbit β-VLDL were isolated from the plasma of cholesterol-fed rabbits (25).

Southern blot analysis

Genomic DNA was extracted from peripheral blood leukocytes by a standard procedure (26). DNA was digested using 5–10 U/μg of several restriction enzymes, separated by agarose gel electrophoresis, transferred to nylon membranes, and hybridized with LDL-R cDNA probes as previously specified (27).

Northern blot analysis

Total cellular RNA was isolated by extraction in guanidine-thiocyanate (28) from cultured skin fibroblasts

TABLE 1. $FH_{Benevento}$ family: clinical and biochemical features

Subject	Age	Cholesterol	LDL Cholesterol	HDL Cholesterol	Triglyceride	ApoA-I	ApoB	Lp[a]	Clinical Data[a]
	yr		*mmol/l*				*mg/dl*		
Proband	10	17.53	16.49	0.54	1.09	75.5	458	90	Tx, Tux, Px, Xa, ATS
Father	39	6.80	4.88	1.66	0.52	142	175	84	
Mother	36	7.52	6.08	1.03	0.92	128	205	8.7	
Brother	12	4.45	2.87	1.33	0.52	135	78	97	

[a]Tx, tendon xanthomas; Tux, tuberous xanthomas; Px, planar xanthomas; Xa, xanthelasma; ATS (B-mode ultrasonography), atherosclerotic lesion (stenosis 75%) at the origin of the left internal carotid artery.

that had been maintained in a lipoprotein-deficient serum (LPDS) for 15 h. RNA (15 μg) was denatured in 50 μl of 50% formamide, and separated by electrophoresis in 1.2% agarose gels and transferred to Hybond-N membranes, which were then hybridized with the full size cDNA probe (27). The cDNA clone pHFβA-1 of human β-actin was used to normalize the RNA filters. Prehybridization and hybridization were performed as previously described (27).

Single strand conformation polymorphism (SSCP)

SSCP was performed according to Orita et al. (29). The promoter region as well as all the exons of the LDL-R gene (with the exception of exons 14 and 15; see below) were amplified by PCR from genomic DNA using the primers reported previously by Leitersdorf et al. (30). PCR products were labeled by adding 5 μCi [α^{32}P]dCTP to the PCR mix. Three μl of reaction product was mixed with 20 μl 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol in formamide. The reaction mixture was heated at 95°C for 3–5 min, placed immediately on ice, and then loaded (2 μl) onto the gel. Gels were run on a conventional sequencing apparatus (40 × 20 × 0.4 mm). The gels consisted of 5.5% acrylamide, 2 × TBE (0.18 M Tris-borate, 4 mM EDTA, pH 8) and 10% glycerol. The samples were electrophoresed at 250 volts for 21 h at room temperature. Gels were dried at 80°C for 1–2 h and then were exposed to X-ray film (Hyperfilm-MP, Amersham International, UK). This SSCP method was successfully validated by the detection of the HincII RFLP in exon 12 (31) and of the previously characterized point mutations (Cys_{358}→Arg; Gly_{528}→Asp) found in two Italian FH homozygotes (4).

Reverse transcription and PCR amplification

The amplification and sequence of exons 14 and 15 were performed starting from the cDNA (RT-PCR). RNA (1 μg) from cultured fibroblasts of the proband G.R. and a normal subject were reverse-transcribed in a 20 μl reaction mixture containing 5 mM $MgCl_2$, 1 mM of each dNTP, 1 unit of RNAsin, 100 pmol random hexamers, and 12 units of AMV reverse transcriptase in 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9) (30). After heating the sample at 95°C for 5 min, 80 μl of 1 × PCR buffer containing 20 pmol of each primer was added as well as 2.5 units of Taq polymerase. $MgCl_2$ concentration in the reaction mixture ranged from 1.5 to 3.5 mM. The following primers were used: 5' TTG TTG GCT GAA AAC CTA CTG TCC C 3' (forward primer in exon 13); 5' CAA GGC CGG CGA GGT CTC AGG A 3' (reverse primer in exon 18). The conditions were: 95°C for 5 min, 70°C for 1.30 min for the first time, and subsequently 94°C for 1.30 min, 70°C for 1.30 min for 28 cycles. PCR products were separated from each other and from the unincorporated primers by electrophoresis on 1.5% agarose gel. After staining with ethidium bromide, the bands were excised and DNA was extracted from the gel using Quiaex (Diagen, GmbH, Germany). DNA fragments were sequenced directly using the fmol Sequencing System (Promega Co., Madison, WI). Primers used in the sequencing reaction were: 5' AGT ATC TGT GCC TCC CTG CCC CG 3' (forward primer in exon 14); 5' CGT AAG GAC ACA GCA CAC AAC 3' (forward primer in exon 15) (32, 33).

PCR amplification of genomic DNA

To amplify the region of LDL-R gene encompassing the point mutation at the 5' end of intron 15, 1 μg of genomic DNA was amplified in a 100 μl mixture containing 0.2 mM of each dNTP, 50 pmol of each primer, 2.5 units of Taq DNA polymerase in 1 × PCR buffer (see above), and 1.5–5 mM $MgCl_2$. Exon 15 forward primer (see above) and a reverse primer on intron 15 (5' GGA CCT GGC CCC TCA TAT GAT 3') (31) were used. The conditions were: 95°C for 5 min, 58°C for 1.30 min, 72°C for 1.30 min for the first time and subsequently 95°C for 1.30 min, 58°C for 1.30 min, 72°C for 1 min for 28 cycles. PCR products were separated and purified (see above) and then either sequenced directly using exon 15 forward primer (see above) or subjected to digestion with StyI restriction enzyme (see below).

Restriction analysis of the G→A mutation at the donor splice site of intron 15

Because the G→A transition at the donor splice site of intron 15 found in proband G.R. eliminates an StyI site, aliquots of the PCR products obtained by the amplification of exon 15–intron 15 junction (see above) were digested with 5 U of StyI. The digested products were electrophoresed on 10% polyacrylamide gel and stained with ethidium bromide.

RESULTS

Southern blot and SSCP analysis

The clinical and biochemical features of proband G.R. were consistent with the diagnosis of homozygous FH (Table 1). This was confirmed by the assay of LDL-R activity in cultured skin fibroblasts, which showed that the degradation of human ^{125}I-labeled LDL in the proband was 11% of that observed in control cells. To exclude the presence of major structural defects of LDL-R gene, genomic DNA from the proband, from his relatives, and from a normal control subject was digested with the following restriction enzymes: PvuII, EcoRI, XbaI, BglII, KpnI, BamHI, NcoI, ApaLI, and StuI. After Southern blotting and hybridization with the LDL-R cDNA no major rearrangements of the LDL-R gene were detected (data not shown).

SSCP analysis of the promoter region and all the exons (with the exception of exons 14 and 15; see below) of LDL-R gene amplified from genomic DNA showed no abnormal features in proband G.R. These findings restricted the localization of a putative point mutation to the region encompassing exons 14–15.

Northern blot analysis and reverse transcription PCR (RT-PCR)

Northern blot analysis of total RNA from cultured skin fibroblasts revealed a strikingly decreased level of LDL-R mRNA in the proband, although the level of β-actin mRNA was identical to that found in two lines of control cells (**Fig. 1**). Densitometric scanning of the film indicated that in the proband the intensity of the LDL-R mRNA band was 1/4–1/5 of that of the normal counterparts. Despite its decreased content, the size of LDL-R mRNA in proband cells was similar to that found in normal cells.

In order to define whether some point mutation was located in exons 14 and 15 (which we had been unable to amplify from genomic DNA) the LDL-R mRNA was reverse transcribed and a cDNA region spanning from exon 13 to exon 18 was amplified by PCR. While the amplification of control cDNA generated a single fragment of 720 bp, that of proband cDNA resulted in three fragments of 800, 600, and 550 bp, respectively (**Fig. 2**). The 800- and 600-bp fragments had a similar intensity, whereas the 550-bp fragment was barely visible on the ethidium bromide-stained gel. Thus, this finding indicated that three LDL-R mRNA species were present in proband fibroblasts.

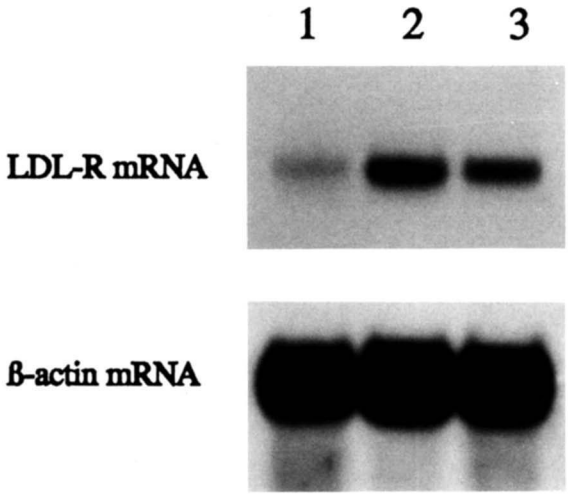

Fig. 1. Northern blot analysis of LDL-R mRNA. Total RNA extracted from cultured skin fibroblasts of proband G.R. (lane 1) and two control subjects (lanes 2 and 3) was hybridized with a full size LDL-R cDNA. The membranes were subsequently hybridized with human β-actin cDNA.

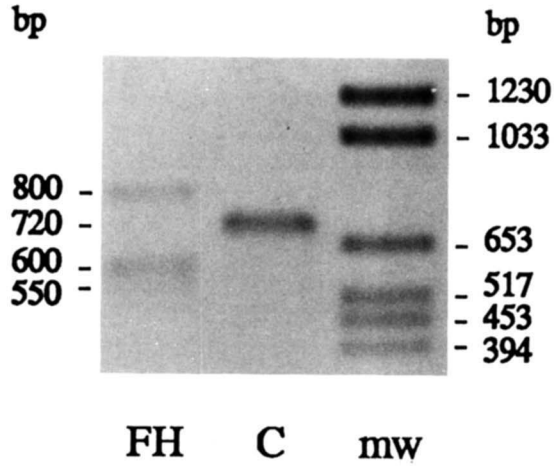

Fig. 2. Reverse transcription and PCR amplification (RT-PCR) of LDL-R mRNA. Total RNA extracted from cultured skin fibroblasts of proband G.R. (FH) and a control subject (C) was reverse-transcribed and amplified by PCR using primers complementary to exon 13 and exon 18, respectively (see Methods for details). A single fragment of 720 bp was obtained in the control subject, whereas three fragments of 800, 600, and 550 bp, respectively, were obtained in proband G.R. Size markers are shown on the right.

Sequence of the RT-PCR fragments

The results of the nucleotide sequences of the RT-PCR fragments are shown in part in **Fig. 3** and **Fig. 4** and are summarized in **Fig. 5.** In the 800-bp fragment (Fig. 2) a normal exon 15 continues with intron 15 (34) (Fig. 3); the latter however is interrupted after 90 nucleotides and continues with a normal exon 16 (Fig. 4). In the 600-bp fragment (Fig. 2) a normal exon 14 is followed by a short sequence (50 nucleotides) of the 5' end of exon 15, which in turn joins to a normal exon 16 (Fig. 5). Finally, in the 550-bp fragment a normal exon 14 joins to a normal exon 16 with the complete skipping of exon 15 (Fig. 5).

The sequence of the 5' end of the 800-bp fragment (Fig. 3) also reveals the presence of a G→A transition at the first nucleotide of intron 15. This transition, which converts the invariant GT of the donor splice site into AT, suggested that the disruption of the normal splicing of LDL-R mRNA in proband G.R. (as illustrated in Fig. 5) was due to the elimination of the 5' donor splice site of intron 15. Figure 5 also shows that the presence of a cryptic donor splicing site in intron 15 (AG/GT at nucleotides 89–93) allows the partial elimination of intron 15 from the mature mRNA. On the other hand, the presence of a cryptic donor splice site in exon 15 (AG/GT nucleotides 49–52) allows an alternative abnormal splicing to occur. This leads to the elimination of 121 nt of the 3' end of exon 15 in the mature mRNA. The third mRNA species found in proband G.R. (Fig. 2) derives from the activation of an alternative splicing that occurs at the authentic splice sites and leads to the skipping of exon 15.

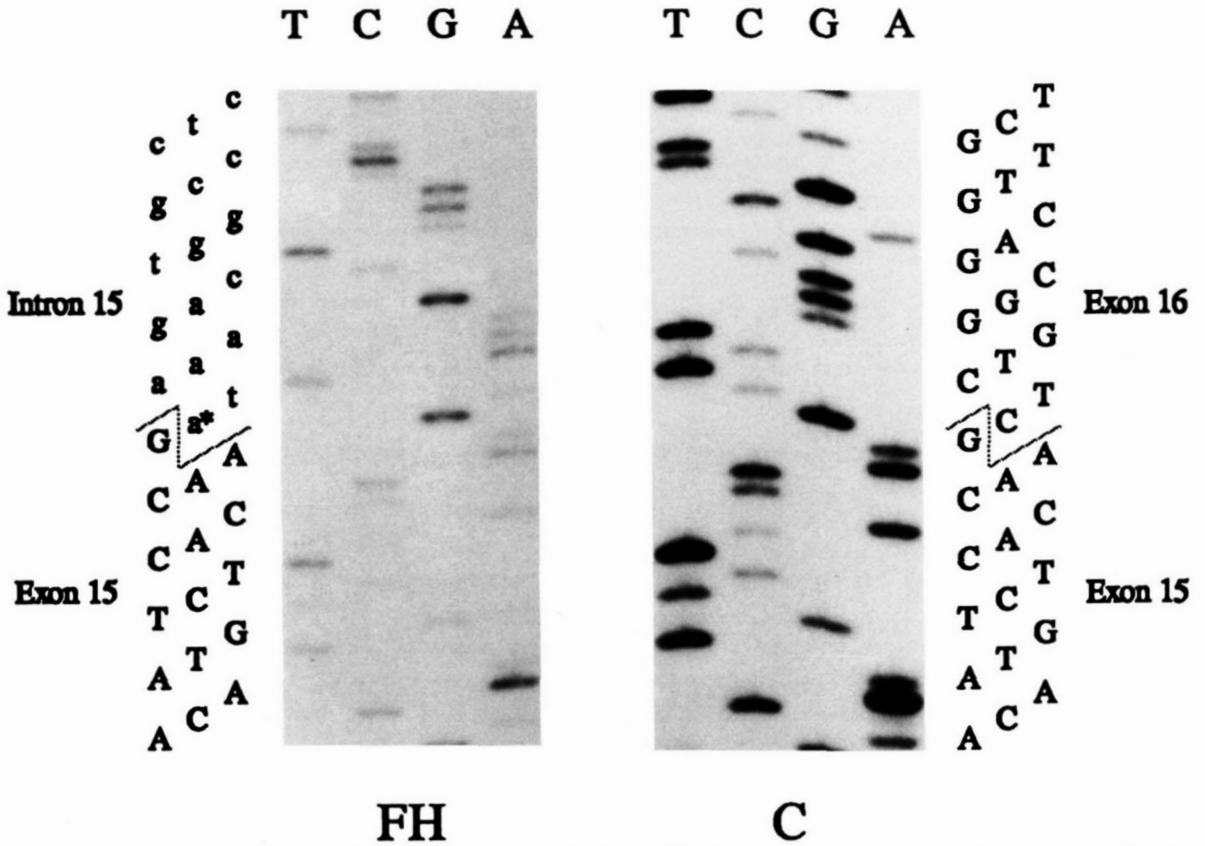

Fig. 3. Nucleotide sequence of the largest RT-PCR fragments shown in Fig. 2. The right panel shows the junction between exons 15 and 16 in the 720-bp RT-PCR fragment of the control subject (C). The left panel shows the same region in the 800-bp RT-PCR fragment of proband G.R. (FH). In this case exon 15 is followed by the 5' end of intron 15 (ref. 34). The presence of a g→a transition in the first nucleotide of intron 15 (*) abolishes the 5' donor splice site. As a consequence, the 5' end of intron 15 is retained in the cDNA (see also the schematic representation in Fig. 5).

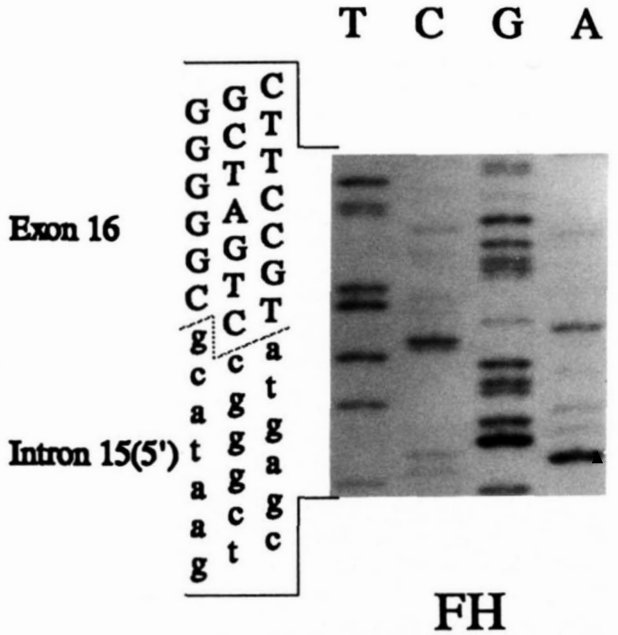

Fig. 4. Nucleotide sequence of the largest RT-PCR fragment shown in Fig. 2. The figure shows the junction between the 5' end of intron 15 (see Fig. 3) and exon 16 in the 800 RT-PCR fragment of proband G.R. (FH). The 5' end of intron 15 (90 nucleotides) retained in the cDNA continues with a normal exon 16. This feature results from the activation of a cryptic 5' donor splice site in intron 15 (see also Fig. 5).

PCR amplification and sequence of the exon 15/intron 15 boundary

To confirm the presence of the G→A transition at DNA level, the exon 15/intron 15 boundary was amplified by PCR from the genomic DNA of the proband and his father. The sequence of this fragment (data not shown) confirmed that proband G.R. was indeed homozygous for a G→A transition at the first nucleotide of intron 15. As expected, proband's father was heterozygote for the same mutation.

The G→A transition affecting the first nucleotide of intron 15 disrupts a StyI restriction enzyme recognition sequence (C/CAAGG) at the exon 15/intron 15 boundary. We investigated the restriction digestion pattern of this region in the proband and his relatives. When the exon 15/intron 15 boundary was amplified by PCR using an exon 15 forward primer and an intron 15 reverse primer (see Methods for details), a 334-bp band was produced (**Fig. 6**). In a normal subject the digestion with StyI generated three fragments of 217, 105, and 22 bp, respectively. In the proband the 105-bp fragment was replaced by a larger fragment (127 bp); in his parents both the normal 105-bp and the abnormal 127-bp fragments were present (Fig. 6). The StyI restriction pattern found in the

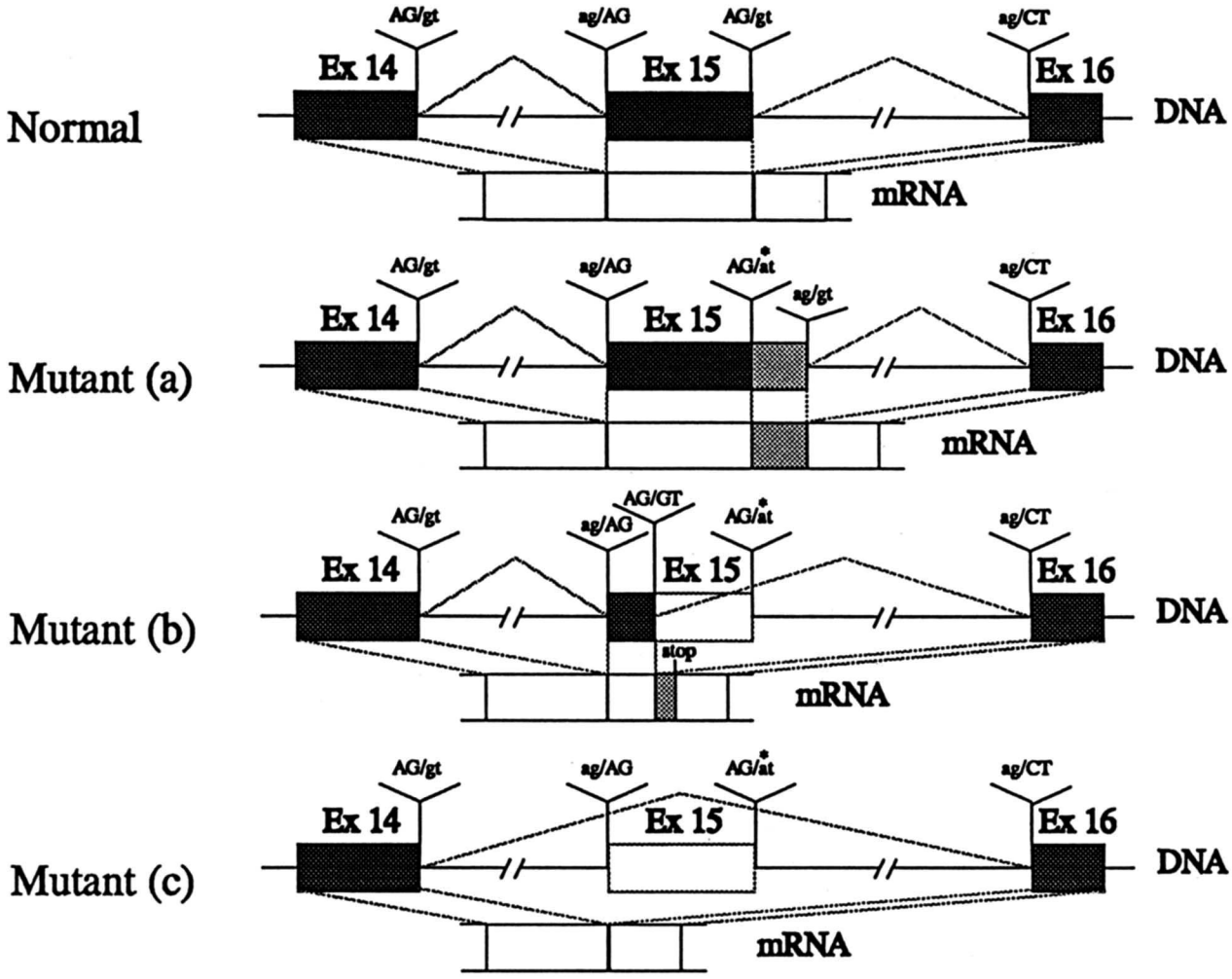

Fig. 5. Schematic representation of the abnormal splicing of LDL-R mRNA in proband G.R. The g→a transition (*) eliminates the 5' donor splice site in intron 15 and allows the activation of two cryptic donor splice sites in intron 15 and exon 15, respectively, (a, b) and the formation of two abnormal mRNAs. Another mRNA species, present in minute amount, results from the skipping of the whole exon 15 (c).

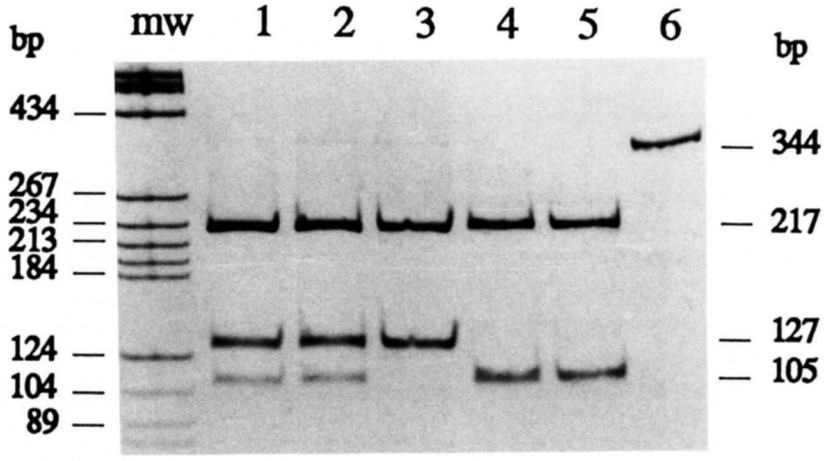

Fig. 6. Digestion of the DNA fragment containing the exon 15/intron 15 boundary with the restriction enzyme StyI. The figure shows the electrophoretic analysis of the amplified DNA from the proband's parents (lanes 1 and 2), proband G.R. (lane 3), the proband's healthy brother (lane 4), and a control subject (lane 5); lane 6, undigested sample. Size markers are shown on the left.

TABLE 2. Exon/intron junctions in mutant LDL-receptor mRNAs

Normal mRNA	Ex 15 | In 15 | Ex 16 ... CAA G|gu aaa ..... |CU CUG ... ... Gln_{749} Ala_{750}Leu ...
FH-Benevento mRNA (a)	Ex 15 | In 15 | Ex 16 ... CAA G|au aaa ..... cca g|CU CUG ... ...Gln_{749} *Asp_1 Lys ..... Pro_{30}* Ala_{750}Leu ...
FH-Benevento mRNA (b) * nt50 Ex 15	Ex 15 | Ex 16 ... CUA AAG* | CUC ..... GCG UGA ... Leu Lys$_{709}$ *Leu $_1$ ..... Ala_{16} Stop*
FH-Benevento mRNA (c)	Ex 14 | Ex 16 ... ACA G|CU CUG ....Thr_{692} $Ala_{750}$$Leu_{751}$ .....

proband's healthy brother was identical to that found in a normal control subject.

Predicted translation products of LDL-R mRNAs

Table 2 shows the predicted proteins produced by the three LDL-R mRNA species found in proband G.R. fibroblasts. The longest mRNA (corresponding to the 800-bp fragment shown in Fig. 2) would produce a receptor protein with an in-frame insertion of 30 novel amino acids (shown in italics in Table 2) downstream from Gln_{749}. In the other main mRNA species (corresponding to the 600-bp fragment shown in Fig. 2) the shift in the reading frame would produce the insertion of 16 novel amino acids preceding a stop codon, downstream from Lys_{709}. Finally, the shortest mRNA species would generate a receptor protein with an in-frame deletion of 57 amino acids.

Ligand blotting experiments

In order to define whether a receptor protein was present in proband fibroblasts, ligand blotting experiments were carried out using ^{125}I-labeled human LDL and ^{125}I-labeled rabbit β-VLDL and detergent-solubilized fibroblast proteins. A faint band migrating in 7% SDS-PAGE at approximately the same position as that corresponding to normal LDL-R was detected with both ligands (**Fig. 7**). Densitometric scanning of the autoradiographic films of ^{125}I-labeled LDL binding indicated that the intensity of this band was approximately 1/10 of its normal counterpart. Figure 7 allows the comparison of the intensity of the band found in proband G.R. with that found in two other Italian FH-homozygous individuals, one with the Gly_{528}→Asp substitution (4) (with less than 2% LDL-R activity and no band detectable in ligand blotting) and the other with a Pro_{664}→Leu substitution (4) (with a 20% residual LDL receptor activity and a band with an intensity approximately twofold higher than that found in proband G.R.). From the intensity of the bands shown in Fig. 7, it emerges that rabbit ^{125}I-labeled β-VLDL bind less strongly to the receptor than human ^{125}I-labeled LDL. It should be taken into account, however, that the specific activity of rabbit ^{125}I-labeled β VLDL was much lower than that of human ^{125}I-labeled LDL (see Methods for details).

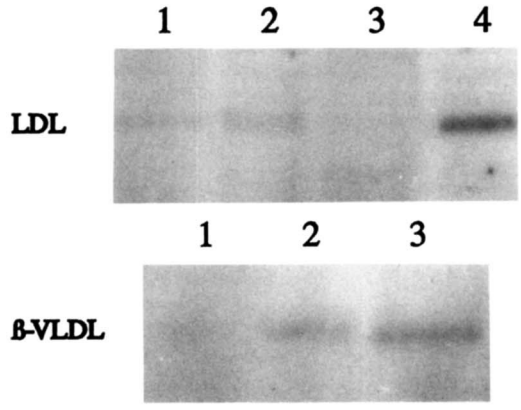

Fig. 7. Ligand blotting of human LDL and rabbit β-VLDL to solubilized LDL receptor. Detergent-solubilized cell extracts (150 μg/protein) were subjected to electrophoresis on 7% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were then overlayered with ^{125}I-labeled human LDL (upper panel) and ^{125}I-labeled rabbit β-VLDL (lower panel). In the upper panel: lane 1, proband G.R.; lane 2, FH-homozygous for the Pro_{664}→Leu mutation (ref. 4); lane 3, FH-homozygous for Gly_{528}→Asp mutation (ref. 4); lane 4, control subject. In the lower panel: lane 1, proband G.R.; lane 2, FH-homozygous for the Pro_{664}→Leu mutation (ref. 4); lane 3, control subject.

DISCUSSION

In this study we report a novel point mutation of the LDL-R gene in an Italian patient with homozygous familial hypercholesterolemia ($FH_{Benevento}$). By using a combination of methods (SSCP analysis, RT-PCR amplification, and sequencing of a cDNA region encompassing exons 14–15 and StyI digestion of the exon 15/intron 15 boundary amplified from genomic DNA) we reached the conclusion that the proband was homozygous for a G→A transition at position + 1 of intron 15 of the LDL-R gene.

It is firmly established that the GT dinucleotide is highly conserved at the 5' end of introns of most eukaryotic genes (35–37). More specifically, the first nucleotide of a normal intron is never an A (36) and mutations of the strictly conserved G at this position prevent normal splicing (38). This guanine at position +1 is involved in binding U1 small ribonucleoprotein particles (U1 snRPNs) and is the residue that undergoes cleavage in the spliceosome and lariat formation (39, 40). Although G→A mutation in the conserved splice donor site has been reported previously in many other human genetic diseases, the resulting changes in the sequence and the content of the mRNA transcripts vary considerably (41–45). Examples of mutations in the guanine residue of the GT dinucleotide of donor splice sites have been reported in the field of lipid research (46–50) and, more specifically, in the LDL-R gene (4, 15). Some of these mutations of the LDL-R gene are characterized by an extremely low LDL-receptor activity (<5%) and the presence of an LDL-R mRNA larger than its normal counterpart (4). Top et al. (15) found that the G→A transition in the first nucleotide of intron 9 of the LDL-R gene resulted in the formation of a premature stop codon in the triplet adjacent to the mutant GT dinucleotide.

In the present study we have demonstrated that the G→A transition at the 5' donor splice site of intron 15 of the LDL-R gene disrupts the normal splicing in that it causes: *a)* a markedly reduced content of LDL-R mRNA; *b)* the activation of two cryptic splice sites, both upstream (in exon 15) and downstream (in intron 15) from the normal splice site; and *c)* the use of an alternative normal splice site with the skipping of exon 15. We have been unable to demonstrate the accumulation of abnormally spliced mRNA precursor (i.e., high molecular pre-mRNA) as reported in other FH patients with 5' donor splice mutations (4).

The activation of two cryptic splice sites, in intron 15 and exon 15, respectively, which allows the formation of approximately equal amounts of two mRNAs, is not surprising as it has been reported in other genetic diseases (41–45). Previous in vitro studies have shown that a mutation in the invariant GT dinucleotide of introns reduces, to a variable extent, the affinity of the mutant sites for the U1 snRNPs and promotes the interaction of the latter with adjacent cryptic sites (51). The selection of the cryptic site(s) to be activated depends on several factors, such as the proximity with respect to the authentic splicing site (51), the degree of homology with the 5' splice sites of other genes (37), or a favorable free energy value (52) for binding to U1 snRNP or other factors involved in splicing (40). In our case the fact that the two activated cryptic sites were approximately at the same distance (90 and 121 nucleotides, respectively) from the authentic 5' donor site might have rendered them equally accessible to the 1U snRNPs and thus have favored the formation of approximately equal amounts of aberrantly spliced mRNAs (Fig. 2).

Our results also indicate that the activation of cryptic sites was a preferential mechanism of splicing as compared to the use of an alternative normal splice site (exon skipping), as the amount of the LDL-R mRNA devoid of exon 15 was much less than that of the two other mRNA species (Fig. 2).

Of the two major mRNAs species found in fibroblasts of proband G.R. the one that derives from the activation of a cryptic splice site in exon 15 contains a premature stop codon (Table 2). The translation product of this mRNA is expected to be a truncated receptor without part of the O-linked sugar, the whole transmembrane, and cytoplasmic domains. The product of the other major mRNA species could resemble a normal receptor, apart from the insertion of 30 novel amino acids downstream from the O-linked sugar domain (Table 2). The translation product of the minor form of the abnormal mRNAs (the one without exon 15) (Table 2) would be a protein devoid of 57 amino acids, including 18 serine and threonine residues, which form the O-linked sugar domain of the receptor. This shortened receptor is likely to maintain a partial function as the sole deletion of exon 15 produces a mild form of familial hypercholesterolemia (53, 54). However, the cellular content of this shortened receptor is expected to be negligible in view of the minute amount of its encoding mRNA (Fig. 2). Ligand blot experiments showed that a protein of approximately the same size as the normal receptor was present in cell extracts, and bound both human LDL and rabbit β-VLDL. It is most unlikely that this band corresponds to the receptor devoid of the O-linked sugar domain. Apart from the reduction in protein mass (due to the deletion of 57 amino acids), the removal of the site for the addition of the O-linked sugar would result in a substantial reduction of the apparent molecular weight of this shortened receptor on SDS-PAGE as reported by Davis et al. (55). It is conceivable, therefore, that the band detected on the ligand blot corresponds to that abnormal receptor which has the in-frame insertion of 30 novel amino acids (Table 2).

We also show in this study that G→A mutation in +1 nucleotide of intron 15 eliminates a restriction site for the

enzyme StyI. By taking advantage of this observation, we set up a simple screening for this mutation based on the PCR amplification of the exon 15/intron 15 boundary followed by digestion with StyI. This procedure is now being used for the screening of the $FH_{Benevento}$ mutation in Italy.

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