# Alternative splicing of mutant LDL-receptor mRNA in an Italian patient with familial hypercholesterolemia due to a partial deletion of LDL-receptor gene ($FH_{Potenza}$)

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**Abstract** An analysis of LDL-receptor gene was performed on an Italian patient with heterozygous familial hypercholesterolemia. Restriction enzyme analysis showed that the proband was heterozygous for a deletion of 4.5 kb spanning the 5' end of exon 13 (45 nucleotide residues) to intron 15. Amplification of genomic DNA, using polymerase chain reaction (PCR), followed by direct sequencing, showed that this deletion was identical to the one reported by Lehrman et al. (1986. *Proc. Natl. Acad. Sci.* **83:** 3679–3683). As only the normal LDL-receptor mRNA was detectable in proband fibroblasts by Northern blot, we used reverse transcription-PCR to amplify the mutant mRNA using primers complementary to exon 6 (sense) and exon 18 (antisense). The amplification of control cDNA resulted in a single fragment of 1725 nucleotides containing the normal sequence. The amplification of cDNA from the proband produced the 1725-nucleotide fragment (as in the control) and three additional fragments (F1, F2, and F3) of smaller size. The direct sequence showed that in the F1 fragment exon 12 was joined to exon 16; in the F2 fragment exon 12 was joined to exon 17; and in the F3 fragment exon 11 was joined to exon 16. Thus, the deletion-bearing allele generated three mRNAs, two of which resulted from alternative splicings leading to the skipping of exons 16 and 12, respectively. It is expected that the translation of these mutant mRNAs will generate three aberrant proteins, the synthesis of which should be negligible in view of the very low content of the corresponding mRNAs.—**Lelli, N., R. Garuti, F. Zambelli, S. Cassanelli, R. Tiozzo, A. Corsini, S. Bertolini, E. Riva, M. T. Ortisi, R. Bellù, and S. Calandra.** Alternative splicing of mutant LDL-receptor mRNA in an Italian patient with familial hypercholesterolemia due to a partial deletion of LDL-receptor gene ($FH_{Potenza}$). *J. Lipid Res.* 1993. **34:** 1347–1354.

**Supplementary key words** LDL-receptor gene • polymerase chain reaction

Familial hypercholesterolemia (FH) is an autosomal dominant disorder caused by mutations of the LDL-receptor gene (1). FH is genetically heterogeneous as a large array of mutations (deletions, insertions, missense and nonsense mutations) has been found in various countries (1, 2). Although it is generally accepted that most of the known LDL-receptor mutant alleles are sporadic (private alleles), certain mutations are common in some defined populations (3–8) or they can be observed more frequently in some geographical areas (9, 10).

In this study we report the characterization of a partial deletion of LDL-receptor gene that has been accomplished by using Southern blot analysis and PCR-based methods. This deletion (which includes a considerable part of exon 13 as well as exons 14 and 15) was observed in an Italian FH heterozygote and it appeared to be identical to the one described by Lehrman et al. (11) in an American heterozygous FH patient of Italian descent ($FH_{Potenza}$) (2). In their report Lehrman et al. (11) did not characterize the mRNA derived from the deletion-bearing allele. In the present report we show that the mutant allele of $FH_{Potenza}$ is expressed in three mRNAs that were undetectable by Northern blot but they were revealed by the use of reverse transcription- PCR (RT-PCR). It is worth noting that two mutant mRNAs derive from a missplicing of the primary transcript which causes the skipping of exons 16 and 12, respectively.

## METHODS

### Subjects

Proband FH 86, from a family living in Northern Italy, was a 13-month-old girl with primary hypercholesterole-

Abbreviations: FH, familial hypercholesterolemia; LDL, low density lipoprotein; PCR, polymerase chain reaction; RT, reverse transcription.

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mia, who was referred to our laboratory from the V Pediatric Clinic of the University of Milan.

### Fibroblast culture and LDL-receptor activity

Skin biopsies were taken from proband FH 86 and from a control subject representative of our series of normolipidemic control individuals. Explants were cultured in 25-$cm^2$ flasks in Dulbecco's modification of Eagle's medium (DMEM), 100 IU/ml of penicillin, and 50 $\mu$g/ml of streptomycin, 2 mM glutamine, 15% fetal calf serum, under 95% air and 5% $CO_2$. The assay of $^{125}$I-labeled LDL binding, internalization, and degradation by cultured skin fibroblasts was performed as previously described (12).

### Southern blot analysis

DNA was extracted from blood leukocytes by a standard procedure (13). DNA (5–15 $\mu$g) was digested using 5–10 U/$\mu$g of several restriction enzymes (PvuII, StuI, NcoI, EcoRI, BglII, HindIII, BamHI, KpnI, EcoRV, XbaI, AvaII, ApaLI, SacI, PstI, SphI) (Boehringer Mannheim, Italy) and transferred to nylon membranes (Hybond-N, Amersham International, UK) (13). The two cDNA clones of the human LDL-receptor gene that was used were a kind gift from Dr. D. W. Russel (Dallas, TX): pLDLR-2HH1 is a plasmid that contains a BamHI insert of 1.9 kb corresponding to the last 8 exons of the gene; pTZ1 is a plasmid that contains the whole coding sequence of the gene in a HindIII insert of 2.6 kb. Exon-specific cDNA probes were obtained by digesting both inserts with restriction enzymes as previously specified (12). Pre-hybridization and hybridization of the filters were carried out as reported previously (12). Filters were subjected to autoradiography on Hyperfilm-MP (Amersham International UK) for 24–48 h at −80°C.

### Northern blot analysis

Total cellular RNA was extracted in guanidine–thiocyanate (14) from cultured skin fibroblasts that had been maintained in lipoprotein-deficient serum for 15 h (12). Total RNA (15 $\mu$g) was denatured in 50 $\mu$l 50% formamide, 2.2 M formaldehyde, and 1 × MOPS buffer [20 mM 3-(N-morpholino)-propane-sulfonic acid, 5 mM sodium acetate, and 1 mM $Na_2EDTA$] and was separated on 1.2% agarose in 1 × MOPS buffer. After separation, RNA was transferred to Hybond-N membranes and hybridized with LDL-receptor cDNA probe complementary to exons 1–11. The same membranes were stripped and rehybridized with the cDNA clone pHF$\beta$A-1 of human $\beta$-actin (12). Prehybridization and hybridization were performed according to a procedure described elsewhere (12).

### Reverse transcription and PCR amplification

Cellular RNA (1 $\mu$g) was reverse-transcribed in a 20-$\mu$l reaction mixture containing 4 mM $MgCl_2$, 1 mM of each dNTP, 1 unit of RNAsin, 100 pmol random hexamers, and 10 units of AMV reverse transcriptase in 1 × PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9, at 25°C, 0.1% Triton X-100) (15). After heating the sample at 95°C for 5 min, 80 $\mu$l of 1 × PCR buffer containing 20 pmol each of upstream primer and downstream primer were added as well as 2.5 units of Taq DNA polymerase. $MgCl_2$ concentration in the reaction mixture ranged from 1 to 5 mM. The following primers were used: *1)* 5' AAT GCA TCA CCC TGG ACA AAG TCT G 3' (forward primer in exon 6); *2)* 5' GAT GTT CAC GCC ACG TCA TCC 3' (reverse primer in exon 18). The conditions were: 94°C for 5 min, 50°C for 2 min, and 72°C for 3 min the first time, and subsequently 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min for 30 cycles (15).

PCR products were separated from each other and from the unincorporated primers by electrophoresis through a 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 in both strands using the fmol Sequencing System (Promega Co., Madison, WI). Primers used in the sequencing reactions were: *1)* 5' ATG ACA CCG TCA TCA GCA GGG A 3' (forward primer in exon 10); *2)* 5' ACC GGA AGA CCA TCT TGG AGG ATG A 3' (forward primer in exon 12); and *3)* 5' GAT GTT GAC GCC ACG TCA TCC 3' (reverse primer in exon 18, the same used in the RT-PCR).

### Amplification of the deletion joint

To amplify the deletion joint, the following primers were used: *1)* 5' TCA TCT TCC TTG CTG CCT GTT TAG 3' (forward primer in intron 12, at the intron 12/exon 13 boundary); *2)* 5' TAG GGT CTT GCT ATG TTG CCC 3' (reverse primer in intron 15) (11). Genomic DNA (1 $\mu$g) from patient FH86 and a control subject was added to a final volume of 100 $\mu$l of 1 × PCR buffer containing 0.2 mM of each dNTP, 50 pmol of each primer, 1–5 mM $MgCl_2$, and 2.5 units of Taq DNA polymerase. The PCR conditions were the same as those used for RT-PCR as specified above.

An aliquot of the reaction mixture was subjected to agarose gel electrophoresis and the PCR fragment of expected size (163 nucleotides) was detected only in FH86. This fragment was purified using a Chroma Spin-30 column (Clontech, Palo Alto, CA) and the sense strand was sequenced directly with the same forward primer in intron 12 (see above).

## RESULTS

### Proband FH86

Proband FH86 was a 13-month-old girl whose plasma cholesterol had been found to be persistently elevated

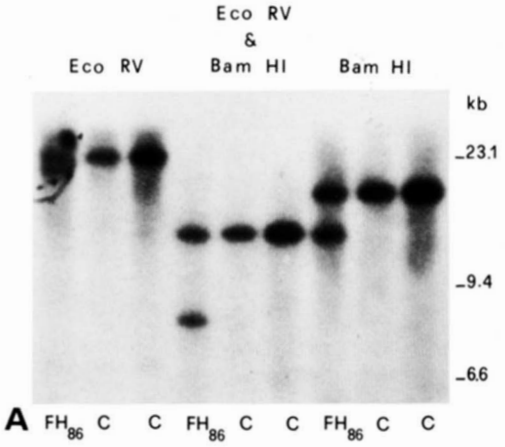
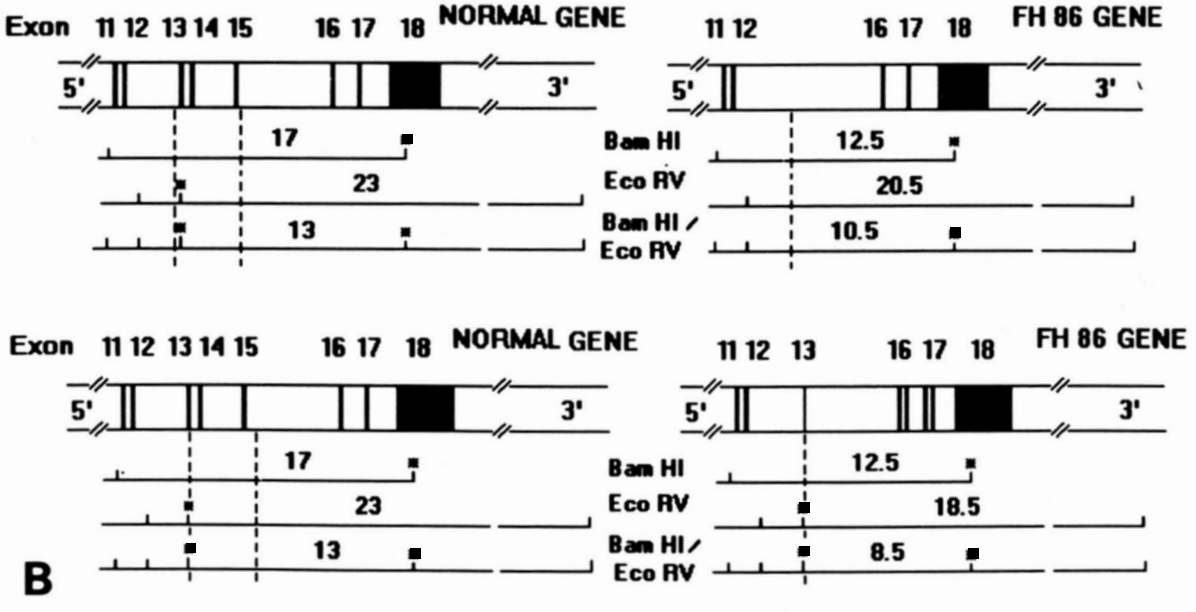



**Fig. 1.** Southern blot analysis of LDL-receptor gene in proband FH86. DNA was subjected to single and double digestion with EcoRV and BamHI and hybridized with E12-E18 cDNA probe; FH86, proband; C, control. The diagrams show the 3' end of LDL-receptor gene and the restriction patterns that could be expected if the 5' end of the deletion was either in intron 12 (i.e., loss of EcoRV in exon 13) (A) or in exon 13 (i.e., presence of EcoRV in exon 13) (B). The digestion pattern we observed was consistent with the idea that the EcoRV site in exon 13 was not involved in the deletion. Had this site been deleted, the EcoRV/BamHI fragment would have been 10.5 kb instead of 8.5 kb. Molecular weight markers are indicated on the right. Restriction sites located in exons are indicated by asterisks. Vertical lines indicate the putative boundaries of the deletion.




since the neonatal period. Her father and several members of her family (paternal line) had primary hypercholesterolemia. From 2 to 13 months of age her plasma cholesterol values ranged from 279 mg/dl (LDL-cholesterol 193 mg/dl) to 480 mg/dl (LDL-cholesterol 426 mg/dl).

The assay of LDL-receptor activity on cultured skin fibroblasts demonstrated that binding, internalization, and degradation of $^{125}$I-labeled LDL were reduced to approximately 40% of the corresponding values found in a control cell line. These values suggested that the proband might be an FH heterozygote.

### Southern blot analysis

The first indication that proband FH 86 carried a major rearrangement of the LDL-receptor gene emerged from the hybridization of DNA with a cDNA probe complementary to the 3' end of the gene. Several single digestions with restriction enzymes, that have cutting sites in the 3' half of LDL-receptor gene, indicated that FH 86 was heterozygous for a deletion of approximately 4.5 kb involving exons 13–15 of LDL receptor gene (data not shown). As the 5' boundary of the deletion appeared to be very close to the beginning of exon 13, we performed EcoRV/BamHI double digestion to ascertain whether the Eco RV site located at the 5' site of exon 13 was maintained. EcoRV/BamHI digestion produced a single 13 kb fragment in control DNA but two fragments of 13 kb (normal) and 8.5 kb (abnormal), respectively, in FH86 DNA (**Fig. 1**). As illustrated in the diagrams of Fig. 1, the size of the abnormal fragment was consistent with the idea that the EcoRV site in exon 13 was not eliminated by the deletion.

### Sequence of the deletion joint

By comparing our data with the known deletions of LDL-R gene we realized that the mutation found in FH

86 resembled the one initially reported by Lehrman et al. (11) in patient FH 381 and subsequently named $FH_{Potenza}$ (2). To test this hypothesis we amplified the deletion joint by using primers complementary to intron 12 and intron 15 (see Methods for details and diagram of Fig. 1). In FH86 we obtained an amplification product of 163 nucleotides as expected on the assumption that the abnormal allele of our proband was the same as that described by Lehrman et al. (11). (**Fig. 2**). The direct sequence of the amplification product showed that the first 15 codons of exon 13 were followed by a sequence of intron 15 that starts with the triplet CTC and continues with two identical 11 nucleotide sequences (**Fig. 3**). The sequence of the deletion joint shown in Fig. 3 is identical to that reported in $FH_{Potenza}$ by Lehrman et al. (11).

### Northern blot analysis and reverse-transcription PCR

Northern blot analysis (**Fig. 4**) demonstrated that only the normal LDL-receptor mRNA (5.3 kb) was detectable in patient FH 86. Even in an overloaded gel we failed to clearly detect an mRNA of smaller size (approximately 4.9 kb), as was expected on the basis of deletion size (Fig. 1). To ascertain whether minute amounts of mutant mRNA were present in FH 86 fibroblasts, we reverse-transcribed FH 86 and control mRNA and amplified the region of interest using PCR (RT-PCR). The primers used in the reaction were complementary to exon 6 (sense) and exon 18 (antisense), respectively (see Methods for details). From control cDNA we obtained a single amplification product (WT), which migrated close to the 1766 nucleotide marker (**Fig. 5**). From FH 86 cDNA we obtained four amplification products: one comigrating with the normal fragment (WT), and the others (F1, F2, and F3) migrating in the 1230–1033 nucleotide range (Fig. 5). The visual inspection of ethidium bromide-stained gels revealed that fragment WT largely exceeded that of fragments F1–F3, which were present in approximately the same amount.

The direct nucleotide sequence of the sense strand of each fragment demonstrated that: *a)* the WT fragment (1725 nucleotides) corresponded to the mRNA transcribed from the normal allele (exon 13 was followed by exon 14) (**Fig. 6**); *b)* F1 fragment (1259 nucleotides) corresponded to a mutant mRNA in which exon 12 was followed by exon 16) (Fig. 6); *c)* F2 fragment (1181 nucleotides) corresponded to a mutant mRNA in which exon 12 was followed by exon 17) (**Fig. 7**); *c)* F3 fragment (1119 nucleotides) corresponded to a mutant mRNA in which exon 11 was followed by exon 16 (Fig. 7).

## DISCUSSION

In this study we report the characterization of a 4.5 kb deletion of LDL-receptor gene that was accomplished using a combination of restriction enzyme analysis and PCR-based methods. Southern blot analysis allowed us to

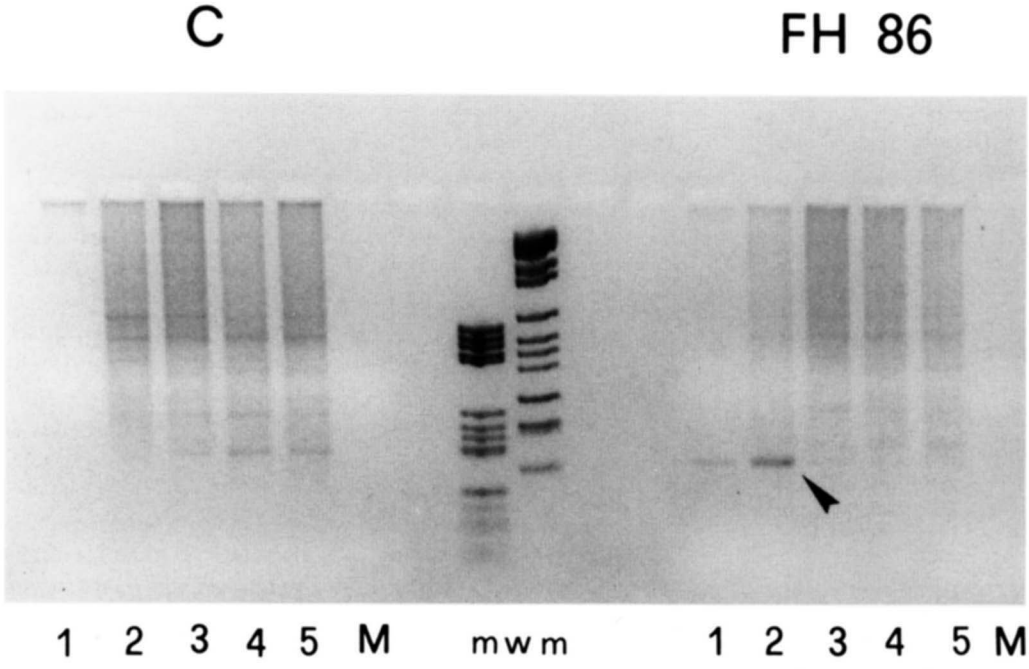



**Fig. 2.** Agarose gel electrophoresis of the products of PCR amplification of genomic DNA from a control subject (C) and proband FH 86. Primers complementary to intron 12 and intron 15 of LDL-receptor gene were used (see Methods). Numbers below each lane indicate $MgCl_2$ concentration in each test tube. M, mock PCR amplification; mwm, molecular weight markers.

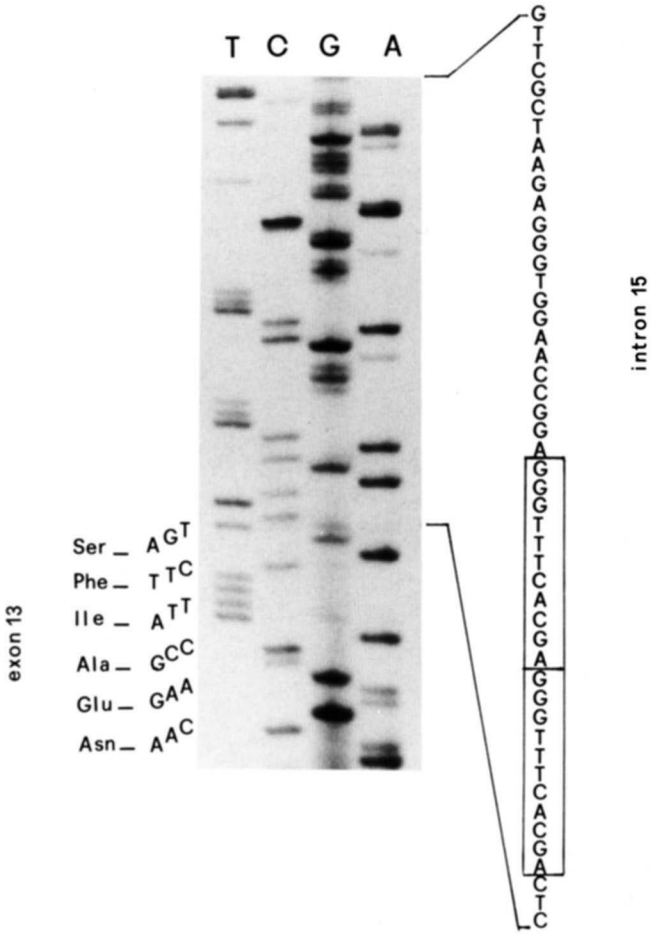



**Fig. 3.** Nucleotide sequence across the deletion joint in proband FH 86. The 163 nucleotide fragment obtained after PCR amplification of genomic DNA of proband FH 86 (Fig. 2) was sequenced directly. The figure shows that the sequence of exon 13 (corresponding to codons 10–15) continues with that of intron 15 starting with the triplet CTC (ref. 11). An 11-nucleotide sequence, which is present once in normal intron 15 (ref. 11) and is duplicated at the deletion joint, is boxed.

map the boundaries of the deletion and to demonstrate that the 5' end of exon 13 was not included in the deletion. This observation suggested that the deletion found in patient FH86 could be the same as the one described by Lehrman et al. (11) in an American FH heterozygote of Italian descent (FH381); this mutation was subsequently designated $FH_{Potenza}$ (2) after the name of the city that patient FH 381's ancestors came from. The report by Lehrman et al. (11) gave us the essential information for the synthesis of suitable oligonucleotide primers to be used for the amplification of the deletion joint at the DNA level. The sequence of the amplification product demonstrated the complete identity between the deletion found in proband FH 86 and the one named $FH_{Potenza}$ (11). The discovery of an identical mutation in two apparently unrelated subjects strongly suggests that they have a common ancestor (10). When a complete genealogical analysis

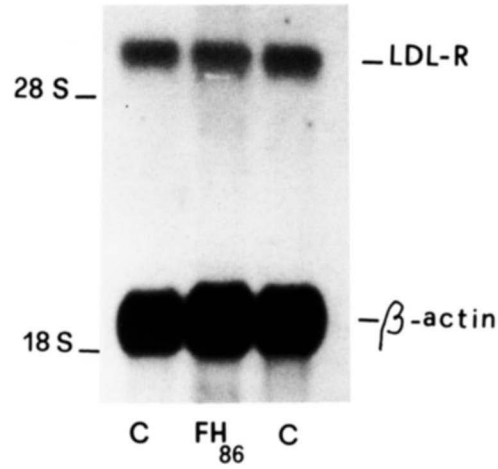



**Fig. 4.** Northern blot analysis of LDL-receptor mRNA. RNA was extracted from cultured skin fibroblasts incubated for 15 h in lipoprotein-deficient serum and hybridized with a cDNA probe complementary to exons 1–11 (E1–E11). Filter was re-hybridized with human β-actin cDNA.

is not feasible, as in this case, indirect ways of supporting the existence of a common ancestor might be the finding that both subjects: *a)* share the same haplotype in linkage with the mutation, and *b)* come from the same geographical area.

The haplotype of the proband described by Lehrman et al. (11) was BsmI (+), SphI(+), NcoI(+), PstI(+) and ApaLI-3' flanking region (FR) (+ ' presence of restriction site) (H. Hobbs, personal communication). In proband FH 86 we investigated six informative RFLPs (SphI, StuI,

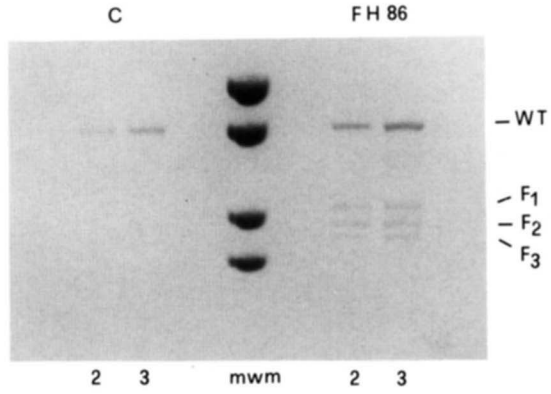



**Fig. 5.** Agarose gel electrophoresis of the fragments obtained after reverse transcription of LDL-receptor mRNA and PCR amplification of the region of interest. Lanes 2 and 3 indicate the concentration of $MgCl_2$ (mM) used in the amplification reaction; mwm, molecular weight markers (from top to bottom: 2176, 1766, 1230, 1033 nucleotides). C, RT-PCR product obtained from control mRNA; FH86, RT-PCR products obtained from FH 86 mRNA; WT, amplification product from normal mRNA; F1, F2, and F3, amplification products obtained from mutant mRNAs (see also Figs. 6 and 7).

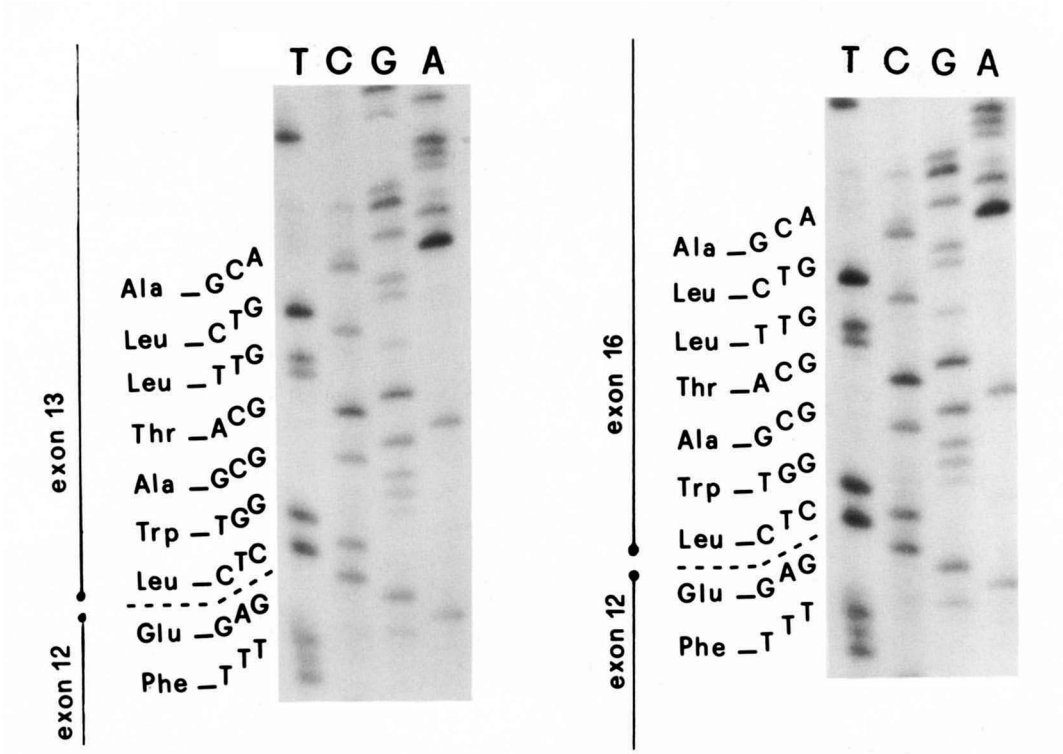



**Fig. 6.** Nucleotide sequence of the sense strands of fragments WT and F1 obtained by RT-PCR from LDL-receptor mRNA of patient FH86 (Fig. 5). Left panel: boundary between exons 12 and 13 in fragment WT; right panel: boundary between exons 12 and 16 in fragment F1.

PvuII, Apa LI in intron 15, Pst, and ApaLI -3'RF) and found that the haplotype in linkage with the deletion was ApaLI in intron 15 (+), ApaLI - 3'FR (+) PvuI(+), StuI(+), SphI(+), and PstI(+). As three RFLPs are in common, it is most likely the two probands share the same haplotype.

With regard to the geographical origin of proband FH 86, we know that *a)* FH 86's family on her father's side

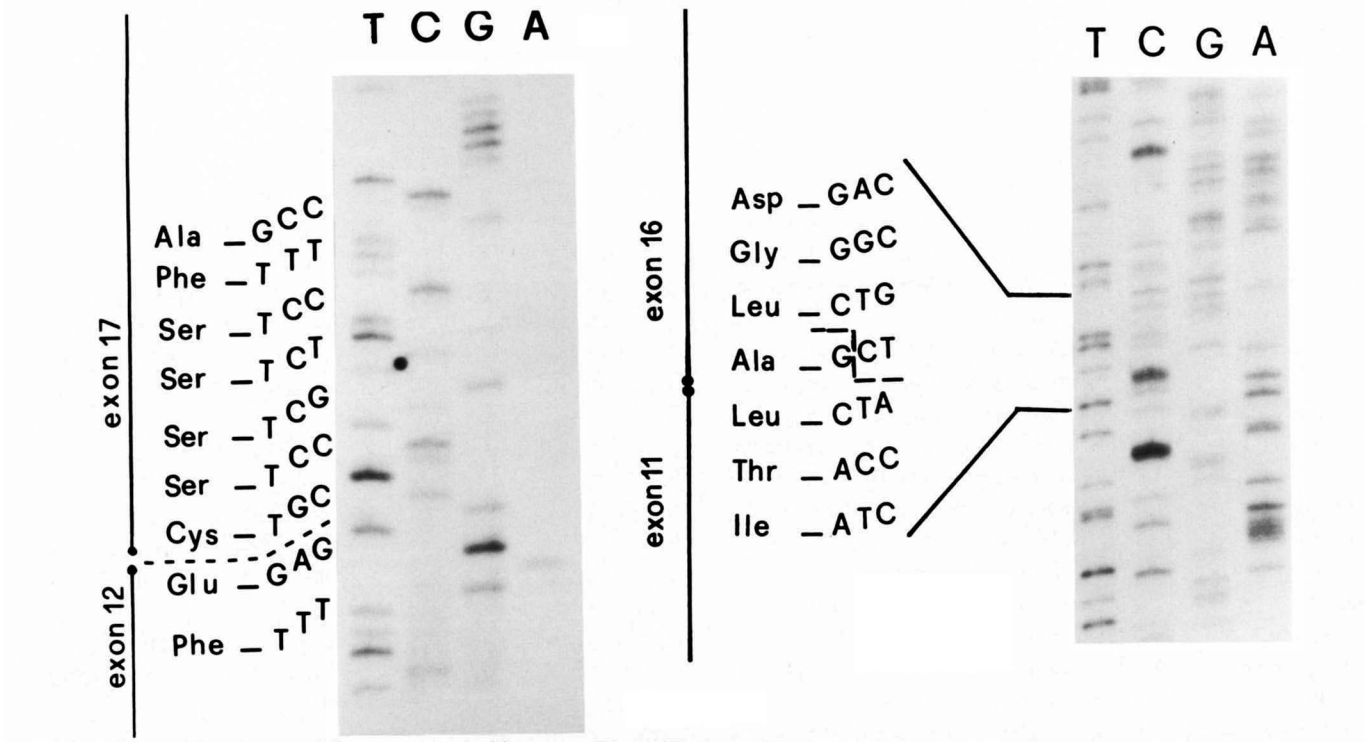



**Fig. 7.** Nucleotide sequence of the sense strand of fragments F2 and F3 obtained by RT-PCR of FH 86 mRNA (Fig. 5). Left panel: boundary between exon 12 and exon 17 in fragment F2; right panel: boundary between exon 11 and exon 16 in fragment F3.

comes from Southern Italy, and *b)* a search in the Italian telephone directory indicated that FH 86's surname is widespread only in the district of Potenza in Southern Italy (16). The common geographical origin of the two probands makes it most likely that they might have had a common ancestor and that a cluster of $FH_{Potenza}$ mutations might be present in that district of Southern Italy.

The PCR procedure we adopted to amplify the deletion joint in FH 86 genomic DNA (Fig. 2) appears to be a relatively simple, non-radioactive method for quickly detecting $FH_{Potenza}$ mutation in other Italian FH patients. A survey of 320 Italian FH subjects, mostly living in Northern and Central Italy, failed to identify other $FH_{Potenza}$ mutations. Thus, it is likely that this mutation is confined to a small geographical area around the city of Potenza in Southern Italy.

The novel finding of the present study was the identification of three mRNA species that derived from the same deletion-bearing allele. As the mutant mRNA was undetectable by Northern blot, we used RT-PCR to amplify the region of interest of both normal and mutant mRNA. In proband FH 86, in addition to the fragment corresponding to the normal mRNA, we found three shorter fragments designated F1, F2, and F3. A common feature of these mutant mRNAs (F1–F3) is that they do not contain the sequence corresponding to the residue of exon 13 (i.e., the first 15 codons of exon 13 are missing from mature mRNAs). In F1 mRNA, exon 12 is joined to exon 16, as was expected on the basis of the size of the deletion (Fig. 1); in F2 mRNA, exon 12 joins exon 17, with a complete skipping of exon 16; and in F3 mRNA, exon 11 joins exon 16 with the complete skipping of exon 12. As far as we know this is the first example of an "alternative splicing" leading to exon skipping in a mutation of LDL-receptor gene. It is conceivable that a systematic use of RT-PCR in the study of mRNA generated by mutant LDL-receptor alleles will show that alternative splicing is more common than previously believed. As a matter of fact, by the use of RT-PCR it has recently been shown that "alternative" mRNAs (derived from alternative splicing of mutant or even normal alleles) are present in a variety of genetic diseases (17–19).

Our findings raise two general questions. First, why is the 5' end of exon 13 (which is not involved in the deletion) missing from mutant mRNAs? Second, which is the mechanism underlying the skipping of exon 11 or 16?

With regard to the first question, one can assume that the deletion of the 5' donor site at the exon 13/intron 13 junction is the cause of the "skipping" of exon 13 residue. Several reports indicate that even a point mutation located in an exon at position −1 of a 5' donor splice site is sufficient to abolish or greatly reduce the utilization of that splicing site (17, 20, 21). This produces a missplicing of the pre-mRNA leading to either the skipping of the preceding exon (where the mutation is located) or the activation of a cryptic 5' splicing site, usually in the downstream intron. We considered the possibility that the loss of the 5' donor site of intron 13 had activated a cryptic 5' splicing site in intron 15. Had this been the case, we would have been able to amplify a mature mRNA containing the residue of exon 13 plus a sequence of intron 15. Since we failed to detect this mRNA, we concluded that either this mRNA was not produced or its intracellular content was negligible as compared to that of the other mutant mRNA species.

The answer to the second question is much more difficult, especially because the mechanisms regulating the alternative splicing are numerous and not fully elucidated (22–24). For example, it is likely that the elimination of 4.5 kb from the primary transcript perturbs the sequential chain of events of splicing, which renders the 3' site of intron 15 less efficient than the corresponding site of intron 16. In keeping with this view, the correct splicing of exons 13–15 would be a prerequisite for the correct splicing of exon 16. It is also possible that, as a consequence of the deletion, the secondary structure of pre-mRNA changes in such a way that the intron 15/exon 16 boundary is sequestered in a hairpin loop that renders the 3' site of intron 15 less accessible to the U2 and U5 snRPN (23).

Northern blot analysis showed that the only LDL-receptor mRNA species present in detectable amounts in FH 86 fibroblasts was the one derived from the normal allele. A similar, though less dramatic, imbalance between normal and mutant mRNA had previously been reported by Lehrman et al. (11) in proband FH 381. It is likely that deletions of three or four exons (as in the case of exon 16 or exon 12 skipping) alter mRNA secondary structure thus rendering the molecule more susceptible to cleavage. A similar reduction of mutant mRNA content had previously been observed by us in two FH heterozygotes with a deletion of exons 13–14 (12).

Finally, one can speculate on the features of the mutant LDL-receptor proteins derived from the translation of the three mutant mRNAs. The joining of exon 12 to exon 16 ("normal splicing") causes a shift in the reading frame thus producing a stretch of 16 novel amino acids before the occurrence of a premature stop codon. The joining of exon 12 to exon 17 ("first alternative splicing") causes a nucleotide shift that allows the reading frame to remain open for 133 novel codons through exon 17 and 18, before the occurrence of a stop codon. In the joining of exon 11 to exon 16 ("second alternative splicing") no change in the reading frame occurs; however, the receptor protein encoded by this mRNA is devoid of 202 amino acids corresponding to the protein region encoded by exons 12–15. It is reasonable to assume that these abnormal receptors would be completely nonfunctional as they lack several important domains (part of EGF precursor homology, O-linked sugars, and two of them, also the transmembrane

and cytoplasmic domains). In any case, as the cellular content of mutant mRNAs is extremely low, the number of abnormal receptor molecules, if synthesized, would be negligible in proband FH 86. For this reason it is appropriate to include $FH_{Potenza}$ mutation among the class I mutations (null alleles) (2, 25).

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