Published in final edited form as:

Science. 1985 January 11; 227(4683): 140–146.

Mutation in LDL Receptor: Alu-Alu Recombination Deletes Exons Encoding Transmembrane and Cytoplasmic Domains

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Abstract

The molecular size of the plasma LDL (low density lipoprotein) receptor synthesized by cultured fibroblasts from a patient with the internalization-defective form of familial hypercholesterolemia (FH 274) was smaller by 10,000 daltons than the size of the normal LDL receptor. The segment of the gene encoding the truncated portion of the FH 274 receptor was cloned into bacteriophage lambda. Comparison of the nucleotide sequences of the normal and FH 274 genes revealed a 5-kilobase deletion, which eliminated the exons encoding the membrane-spanning region and the carboxyl terminal cytoplasmic domain of the receptor. The deletion appeared to be caused by a novel intrastrand recombination between two repetitive sequences of the Alu family that were oriented in opposite directions. The truncated receptors lack membrane-spanning regions and cytoplasmic domains; they are largely secreted into the culture medium, but a small fraction remains adherent to the cell surface. The surface-adherent receptors bind LDL, but they are unable to cluster in coated pits, thus explaining the internalization-defective phenotype.

Approximately one person in every 500 inherits one mutant gene for the low density lipoprotein (LDL) receptor, a cell surface protein that carries the cholesterol transport protein LDL from plasma into cells by receptor-mediated endocytosis in coated pits (1). When the receptors are defective, LDL cannot be removed normally from plasma, it accumulates to high levels, and premature atherosclerosis ensues. Mutations in the gene for the receptor are important clinically because a single copy produces heterozygous familial hypercholesterolemia (FH), a common cause of heart attacks in middle-aged people (1). About one in 1 million persons inherits two copies of a mutant LDL receptor gene. In such individuals, called FH homozygotes, plasma levels of LDL-cholesterol are even higher than in heterozygotes, and heart attacks occur before 20 years of age (1).

Mutations in the LDL receptor are important subjects of study because they provide insights into the general mechanism of receptor-mediated endocytosis (2). These mutations were originally defined through functional assays of LDL receptor activity in cultured fibroblasts and freshly isolated lymphocytes from FH patients (3). Subsequently, the LDL receptor was purified, and antibodies were generated. These advances led to the demonstration that some mutant alleles produce reduced amounts of receptor protein, whereas others encode receptor proteins with a demonstrably altered structure (4).

Among the most informative mutations have been those in which the LDL receptor reaches the cell surface but does not cluster in coated pits, a defect that prevents the receptors from carrying LDL into cells (5–7). Study of these mutations demonstrated the essential role of coated pits in receptor-mediated endocytosis (2, 5). As a result of kinetic and genetic complementation studies, we proposed that mutations that block internalization reside in the gene for the LDL receptor, and not in some other gene necessary for receptors to be incorporated into coated pits (5, 7).

Recently it has become possible to study the molecular genetics of these mutations as a result of the cloning of a full-length complementary DNA (cDNA) for the human LDL receptor (8, 9) and the subsequent isolation of genomic clones covering most of the LDL receptor gene, which spans at least 60 kilo-bases (kb) of DNA (10). The amino acid sequence of the LDL receptor was deduced from the nucleotide sequence of its cDNA (9). This sequence, coupled with protein chemistry studies (8), revealed that the normal receptor consists of at least five structural and functional domains. Listed in order from the amino terminus, the domains are (i) a cysteine-rich region of 322 amino acids that contains the binding site for LDL; (ii) a region of ~350 amino acids that is homologous to the precursor for epidermal growth factor; (iii) a serine- and threo-nine-rich region of 48 amino acids that is the site for *O*-linked glycosylation; (iv) a stretch of 22 hydrophobic amino acids that spans the plasma membrane; and (v) a carboxyl terminal region of 50 amino acids that projects into the cytoplasm (9). The mature messenger RNA (mRNA) for the receptor is unusual in that its 3′ untranslated region contains several copies of a repetitive DNA sequence of the Alu family (9).

We now report the use of our cDNA and genomic clones to characterize a mutation in the structural gene for the LDL receptor in a family with FH. The index case is a young man (B.H.), here-after designated FH 274, who has all of the clinical features of homozygous FH (7). Previous functional studies revealed that cultured fibroblasts from FH 274 bound about one-third of the normal amount of ^{125}I-labeled LDL. However, the receptors in FH 274 did not cluster in coated pits and hence did not transport their bound LDL into the cell (7). Thus, FH 274 was categorized functionally as an “internalization-defective” mutation. Studies of fibroblasts from the relatives of FH 274 revealed that he had inherited two different mutant alleles; the allele encoding the internalization-defective receptor was inherited from his mother and a null (or silent) allele that produced no functional receptor protein was inherited from his father (7).

Preliminary biosynthetic studies of cultured fibroblasts from FH 274 revealed that the LDL receptor protein encoded by the internalization-defective allele is about 10,000 daltons smaller than the normal receptor. Accordingly, we initiated a study of this mutation by analyzing the genomic DNA from FH 274 and his family members. As described below, we found that the mutant gene has undergone a large deletion that eliminates two exons completely and one exon partially. The deletion results from a recombination between two repetitive DNA elements: an Alu element in the intervening sequence (IVS) that precedes the exon encoding the membrane-spanning region of the receptor and an Alu element in the exon encoding the 3′-untranslated region of the gene. The resulting mutant gene produces a truncated LDL receptor that lacks a membrane-spanning region and a cytoplasmic domain.

Most of these truncated receptors are secreted, but some of them remain associated with the outer surface of the cell. In this position they can bind LDL, but the lack of a cytoplasmic domain renders them incapable of clustering into coated pits.

Abnormal restriction fragment

Southern blots of genomic DNA from normal cells and from FH 274 after digestion with Xba I and hybridization with several cDNA probes are shown in Fig. 1. Probe 1 was a mixture of cDNA fragments that spanned most of the translated region of the LDL receptor mRNA (Fig. 1A). When this probe was hybridized to the Xba I-digested genomic DNA from the normal subject, we observed three bands of 23-, 10-, and 7-kb, designated A, C, and D, respectively (Fig. 1B). All of these normal bands plus one additional band of 13 kb, designated B, were present in the genomic DNA of FH 274. These findings suggest that one of the mutant alleles in FH 274 has a normal restriction pattern, whereas the other allele gives rise to band B.

To localize the DNA segment that gives rise to band B, we probed the Xba I digest with short probes that corresponded to discrete regions of the LDL receptor cDNA (probes 2 to 9, Fig. 1A). Probes 2 to 5 (10) hybridized to identical bands in normal and FH 274 DNA. Probes 6, 8, and 9 (but not probe 7) hybridized to the abnormal 13-kb band B in the FH 274 DNA. Inasmuch as band B does not hybridize with probe 7 but does hybridize with probes on either side of probe 7 (that is, probes 6, 8, and 9), this fragment appears to result from a deletion of DNA that includes the region encoding the mRNA encompassed by probe 7. This deletion would presumably involve the removal of at least one Xba I site with fusion of the adjacent DNA sequences into a single Xba I fragment of 13 kb, namely, band B.

To determine whether band B originated from the maternal (internalization-defective) or paternal (null) allele, we performed Xba I digests of genomic DNA from the two parents. Figure 2 shows that band B was present in the DNA from the mother, but not the father, indicating that the deletion was present on the internalization-defective allele.

Characterization of the gene deletion

Genomic clones representing the normal LDL receptor gene were isolated from a bacteriophage λ library (10). The exon organization of the 3′ end of the normal receptor gene was determined by restriction mapping and sequencing of exon-IVS junctions of cloned genomic DNA. This 3′ region consists of six exons, designated A to F, which are separated by five IVS's, designated a to e (Fig. 3). Exon C encodes the *O*-linked sugar domain of the receptor protein. Exon D and the 5′ end of exon E encode the membrane-spanning domain. The 3′ end of exon E encodes 39 of the 50 amino acids in the cytoplasmic domain. Exon F encodes the 11 amino acids at the carboxyl terminus of the cytoplasmic domain and all of the 3′ untranslated region of the mRNA (10).

The deletion-bearing fragment from FH 274 DNA was cloned by Xba I digestion of genomic DNA, size fractionation, and insertion of the isolated 13-kb fragment (band B) into a bacteriophage λ vector (Fig. 3). This cloned fragment is designated λFH 274-10. The Pvu II site in IVSc and the Sst I site in exon F were separated by ~5.9 kb in the cloned fragments

of the normal gene but only by ~0.6 kb in the cloned fragment of the FH 274 gene (Fig. 3). Moreover, several restriction enzyme sites between the Pvu II site and the Sst I site were missing from the cloned FH 274 gene (Fig. 3). These data suggested that ~5 kb of DNA was deleted from the FH 274 gene. The deletion included the 3′ end of IVSc, all of exons D and E and the IVS's separating them, and the 5′ end of exon F.

To locate precisely the 5′ and 3′ breakpoints and the structure at the deletion joint in FH 274, we determined the nucleotide sequences of the cloned portions of the genes delimited by the bars in Fig. 3. These sequences revealed that the deletion joint occurred between two repetitive elements of the Alu family that were oriented in opposite directions (Fig. 4). The 5′ side of the deletion joint was derived from an Alu sequence in IVSc. The 3′ side of the deletion joint was derived from an oppositely oriented Alu sequence in exon F. The DNA sequence of the entire Alu element in exon F has been reported (9).

The nucleotide sequence of ~400 bases surrounding the deletion joint in FH 274 and the corresponding sequences in the normal gene are shown in Fig. 5. The regions identical to the consensus Alu sequence are indicated by solid lines above or below the normal sequences. The consensus Alu sequence (Fig. 4) consists of a left arm containing ~132 nucleotides and a right arm containing ~166 nucleotides; the right arm is a tandem repeat of the left arm with an insertion of ~30 additional nucleotides (11, 12). The Alu sequence is repeated ~3×10^5 times in the human genome and occurs, on average, every 6 kb. The Alu repeats are generally located in IVS's and in the flanking region between genes (11, 12). The human LDL receptor gene is unusual in that Alu sequences are present in the 3′ untranslated region of the mature mRNA (9).

The DNA sequences in Fig. 5 indicate that the 5′ breakpoint in FH 274 is located at nucleotide consensus position −116 in the left arm of an Alu sequence in IVSc and that this has been joined to consensus position −7 in the oppositely oriented right arm of an Alu sequence in exon F. A single additional nucleotide (thymidine, T) has been inserted at the point where the two pieces of DNA are joined.

Although recombination between repetitive DNA sequences has been postulated to be a cause of deletions, to our knowledge such rearrangements have not previously been reported in eukaryotic cells. In the most well characterized set of mammalian deletion mutations, that is, those that occur in the human α-and β-globin genes, one of the deletion breakpoints frequently occurs within an Alu sequence but the other breakpoint thus far has always occurred in a nonrepetitive sequence of DNA (13).

In considering the potential mechanism by which the deletion in FH 274 occurred, we must explain three phenomena: (i) the occurrence of the deletion between two Alu sequences that are oriented in opposite directions and which are ~5 kb apart; (ii) the presence of a staggered break point involving the first Alu sequence at nucleotide position −116 and the second Alu sequence at position −7; and (iii) insertion of a single new residue (T) at the deletion joint.

One mechanism for generating the observed deletion joint in FH 274 could involve mispairing and unequal crossing-over between the Alu sequence from IVSc on one DNA strand and the oppositely oriented Alu sequence from exon F on another DNA strand.

However, computer-assisted comparisons of the Alu sequences from IVSc and exon F (in opposite orientation) did not reveal sufficient nucleotide pairing to support this model. On the other hand, the sequence did suggest an alternative model in which the deletion could arise as a result of a homologous recombination involving the two Alu sequences on a single strand of DNA (Fig. 6). In formulating this model, we assume that, at some point during DNA replication when the DNA strands were separated, a stem-loop structure formed between the Alu sequence in IVSc and the oppositely oriented Alu sequence in exon F (Fig. 6A). A staggered break was then introduced by an unknown mechanism such that the first Alu sequence was cleaved at position −116, and the second Alu sequence was cleaved at position −7. The 5 kb of DNA between these cleavages dissociated or were destroyed by nucleases (Fig. 6B). These events generated a single-stranded segment of 106 nucleotides at the end of the upstream Alu sequence (Fig. 6C). If this single-stranded segment folded back on itself, the terminal five residues would pair with five complementary residues at the base of the loop structure (Fig. 6D and inset). This would bring the 3′ end of the upstream segment (residue −116 of the Alu in IVSc) into proximity with the 5′ end of the downstream segment (residue −7 of the Alu in exon F). It would leave a gap of a single nucleotide that would be opposite an A in the upstream Alu of IVSc. Repair enzymes would fill this gap with a T, and the DNA would be ligated to form a continuous strand from which ~5 kb of DNA had been deleted and a single T had been inserted, as observed in the deletion joint of FH 274 (Fig. 4).

Consequences of the gene deletion

The deletion in FH 274 occurs in IVSc and removes the normal 3′ acceptor splice site in this IVS (Fig. 7A). We do not know where the new 3′ acceptor site is located, and therefore we cannot be certain about the structure of the 3′ end of the mRNA (Fig. 7B).

The uncertainty about the exact 3′ end of the mRNA also creates an ambiguity concerning the carboxyl terminal end of the mutant receptor protein. The last normal exon that is retained in FH 274 (exon C, Fig. 3) codes for the amino acids of the serine- and threonine-rich region to which *O*-linked sugars are added (Fig. 7C) (10). The 3′ boundary of this exon encodes amino acid residue 749, which is located 18 amino acids upstream from the membrane-spanning domain (9). These findings suggest that the truncated receptor protein encoded by the deleted FH 274 gene lacks the last 90 amino acids at the carboxyl terminus of the normal receptor (Fig. 7C) (9, 10). If we assume that a new 3′ splice site is used in generating the FH 274 mRNA and that this creates a random sequence of nucleotides, then it is statistically likely that a termination codon would occur within about 20 codons. This would yield a protein that should be about 10 kD shorter than the normal receptor and thus would migrate on sodium dodecyl sulfate (SDS) polyacrylamide gels as a species with an apparent molecular size of approximately 150 kD.

Secretion of truncated LDL receptor

When initially synthesized, the normal LDL receptor migrates with an apparent molecular size of 120 kD on SDS polyacrylamide gels. About 30 minutes after synthesis, its apparent size increases to 160 kD as its *O*-linked carbohydrate chains are processed in the Golgi

apparatus (4, 14). About 30 minutes later, the receptor appears on the surface as a mature protein with an apparent size of 160 kD (4, 15). We performed experiments in which cultured fibroblasts were incubated for 2 hours with [^{35}S]methionine (pulse) and the LDL receptors were immunoprecipitated either immediately (Fig. 8A) or 2 hours after the addition (chase) of unlabeled methionine (Fig. 8B). In normal cells after the pulse, about half of the receptors were in the immature 120-kD form and about half were in the mature 160-kD form. After the 2-hour chase, the vast majority of the normal receptors were in the 160-kD form. In FH 274 cells, the precursor was clearly smaller than that of the normal cells, corresponding to an apparent molecular size of about 110,000. The mature form of the receptor in FH 274 was also about 10 kD smaller than the normal mature form. The observation that the apparent size of the receptor in FH 274 cells increased by 40 kD after synthesis is consistent with the conclusions drawn from the DNA studies, which indicated that the exon encoding the serine- and threonine-rich, *O*-linked sugar domain (exon C, Fig. 3) is conserved in the truncated protein.

Proband FH 274 inherited the deleted receptor gene from his mother, who has one copy of the normal gene and one copy of the deleted gene (Fig. 2). Consistent with this formulation, the receptor precursor in the mother's cells showed a broad band that appeared to contain both the 110-kD and 120-kD precursors (Fig. 8A). After the chase period, the mother's cells also showed a relatively broad band, but it was not possible to clearly discern the separation between the expected 150-kD and 160-kD bands. Cells from the father, who is heterozygous for a null allele at the receptor locus (7), showed evidence for only the normal 120-kD precursor and the 160-kD mature form of the receptor. In addition to the known precursor and mature forms of the receptor, the SDS polyacrylamide gels (Fig. 8) contained several minor bands that we believe to be proteolytic fragments of the receptor (4, 14); the most prominent of these is designated x in Fig. 8.

The deletion in the receptor gene in FH 274 removes the genomic segments encoding the membrane-spanning region and the entire cytoplasmic domain of the protein. When similar deletions have been created experimentally in genes encoding viral membrane proteins such as the influenza hemagglutinin (15) and the vesicular stomatitis virus protein (16), the truncated proteins pass entirely across the endoplasmic reticulum membrane during synthesis and reside in the lumen of this organelle. Eventually, such proteins are secreted from the cell. These truncated proteins have been termed "anchor-minus" proteins (15).

To determine whether the truncated LDL receptor in FH 274 was secreted, we incubated the cells for 18 hours with [^{35}S]methionine and subjected the medium and cells to separate immunoprecipitations (Fig. 9a). In the normal cells, more than 98 percent of the LDL receptor was associated with the cell membrane (lane A), and less than 2 percent was found in the medium (lane B). On the other hand, in the FH 274 cells the majority of the synthesized receptors were found in the culture medium (lane D), and only a relatively small amount was associated with the cell (lane C). By densitometric scanning of the gels from this and other experiments, we determined that the total amount of receptor synthesized by the internalization-defective allele in the FH 274 cells (cells plus medium) was about twice that synthesized by a single normal allele in the normal cells. In the FH 274 cells there was

about nine times as much LDL receptor in the medium as there was attached to the cell surface.

Analysis of receptor biosynthesis in the fibroblasts of the parents of FH 274 (Fig. 9b) showed the expected genetic result. The mother's cells contained two populations of receptors, a normal-sized receptor associated with the cells and a truncated receptor that was secreted into the medium. In contrast, the father's cells contained only normal-sized receptors that were cell bound.

During the course of these studies, we noted that the secreted form of the receptor in FH 274 cells always migrated faster than the cell-associated receptor when studied on SDS polyacrylamide gels (Fig. 9a). At least three explanations are possible. (i) The secreted receptor may be cleaved proteolytically; (ii) the carbohydrate chains of the secreted receptor may differ from those of the cell-bound form; or (iii) two different forms of the receptor may be synthesized, owing to differential splicing at the 3′ end of the deleted gene. The latter event might create a situation in which one of the 3′ splice sites leads to an open reading frame that contains a hydrophobic stretch of amino acids, such that the carboxyl terminal end of the truncated receptor adheres to the membrane. Another 3′ splice site might lead to a shorter open reading frame before a terminator codon is reached. The receptor produced by this spliced mRNA would lack hydrophobic amino acids at the carboxyl terminus, and therefore it would be secreted like the "anchor-minus" viral proteins discussed above. These possibilities could be resolved by cloning and sequencing a series of cDNA's for the receptor from the FH 274 cells, a technically difficult task.

In previous experiments we found that the FH 274 cells bound about one-third the normal amount of ^{125}I-labeled LDL, but failed to internalize the lipoprotein when the cells were warmed to 37°C (7). We have repeated these experiments and obtained similar results (17). These data, together with the protein biosynthetic experiment of Fig. 9, suggest that in FH 274 cells the surface-adherent receptors are able to bind LDL but they cannot carry the LDL into coated pits.

Implications for other receptor mutations

Inasmuch as FH 274 is the only receptor mutation that has been elucidated at a molecular level, the current results may have general implications. First, the observation that the truncated protein is secreted supports the general model for the hydrophobic anchoring of transmembrane proteins that has emerged over the past 15 years (18). Second, these findings are consistent with previous conclusions about the location of the single membrane-spanning domain in the LDL receptor (8, 9). Third, the findings indicate that one form of receptor internalization defect can result from synthesis of a receptor that lacks a membrane-spanning domain and thus can no longer come into contact with clathrin or clathrin-associated proteins in the cytoplasm.

In another FH family with the internalization defect, the index case J.D. [the original internalization-defective mutation described (5)] does not have any large deletion of DNA, as determined by restriction enzyme digestion of genomic DNA (19). Unlike the cells of FH 274, the cells from J.D. produce LDL receptors that appear to have the same molecular size

as the normal receptor; none of J.D.'s receptor is secreted into the culture medium. In order to further elucidate the spectrum of mutations that may give rise to LDL receptor internalization defects, it is necessary to clone and sequence the gene segments corresponding to the cytoplasmic domain of J.D.'s receptors. When the various internalization mutations have been delineated, they should provide additional insights into the general features of cell surface receptors that direct their incorporation into coated pits.

References and Notes

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26. We thank Edith Womack, Daphne Davis, and Mark Woelfle for technical assistance. Supported by NIH research grants HL 20948 and HL 31346; a fellowship from the Jane Coffin Childs Memorial Fund for Medical Research (M.A.L.); a fellowship from the Deutsche Forschungsgemeinschaft (T.C.S.); and a Research Career Development Award from the NIH (HL 01287) (D.W.R.). W.J.S. is an Established Investigator of the American Heart Association.

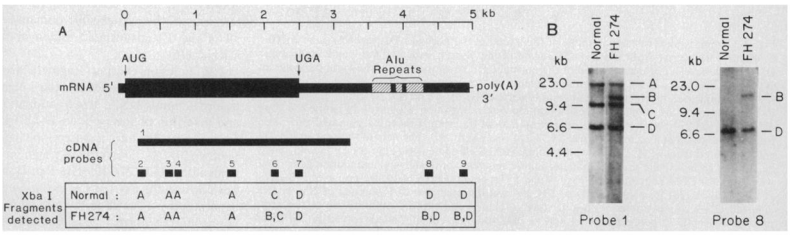

Fig. 1.

Analysis of Xba I restriction digests of genomic DNA from FH 274 with probes from different regions of the LDL receptor cDNA. (A) A diagram of the mRNA for the human LDL receptor (9) is shown with AUG and UGA (A, adenine; U, uracil; G, guanine) indicating the beginning and end of the translated region. The hatched areas in the 3′ untranslated region represent Alu repetitive sequences. The sizes and locations of ^{32}P-labeled cDNA probes used to map the LDL receptor gene are shown by the closed bars and are numbered 1 to 9. Probe 1 (double-stranded DNA) was a mixture of a 2.1-kb Eco RI-Sma I fragment and three different 0.9-kb Bam HI-Xho I fragments from p101 (9), which together spanned most of the translated region; the fragments were purified by polyaerylamide gel electrophoresis and electrocution (20) and then labeled with ^{32}P by random hexanucleotide priming (21). Probes 2 to 9 were prepared from M13 subclones of pLDLR-2 (9) as single-stranded, uniformly ^{32}P-labeled DNA, ~100 nucleotides (nt) in length, by the method of Church and Gilbert (22). All probes had a specific radioactivity of at least 5×10^8 cpm/μg. (B) Genomic DNA (5 μg) was isolated from cultured fibroblasts as indicated (20), digested with Xba I (New England Biolabs), subjected to electrophoresis in 1 percent agarose containing buffer A (40 m*M* tris-acetate, 3 m*M* disodium EDTA, 20 m*M* sodium acetate, 18 m*M* NaCl, pH 8.15), and transferred to nitrocellulose paper (20). The paper was incubated for 16 hours at 42°C with the indicated ^{32}P-labeled cDNA probe (2×10^6 to 4×10^6 cpm/ml) in 50 percent formamide, 1 percent SDS, 5x Denhardt's solution, 5x (saline, sodium phosphate, and EDTA) (SSPE), and *E. coli* DNA at 100 μg/ml after preliminary hybridization for 1 hour at 42°C in the same solution without the ^{32}P-labeled probe. After hybridization, the paper was washed in 1 percent SDS plus 2x SSC (saline and sodium citrate) for 15 minutes at 23°C and then in 1 percent SDS plus 0.1 × SSC (probe 1) or in 1 percent SDS plus 0.5 × SSC (probes 2 to 9) for 4 hours at 68°C. Denhardt's solution, SSPE, and SSC were prepared as described (20). Filters were exposed to x-ray film with an intensifying screen for 24 hours at −70°C. Two representative blot hybridizations (with probes 1 and 8) are shown. The four Xba I restriction fragments are designated A to D along the right side of each blot. Molecular size standards were generated by Hind III cleavage of bacteriophage λ DNA. The Xba I fragments detected by probes 2 to 9 in the normal subject and FH 274 are indicated at the bottom of (A).

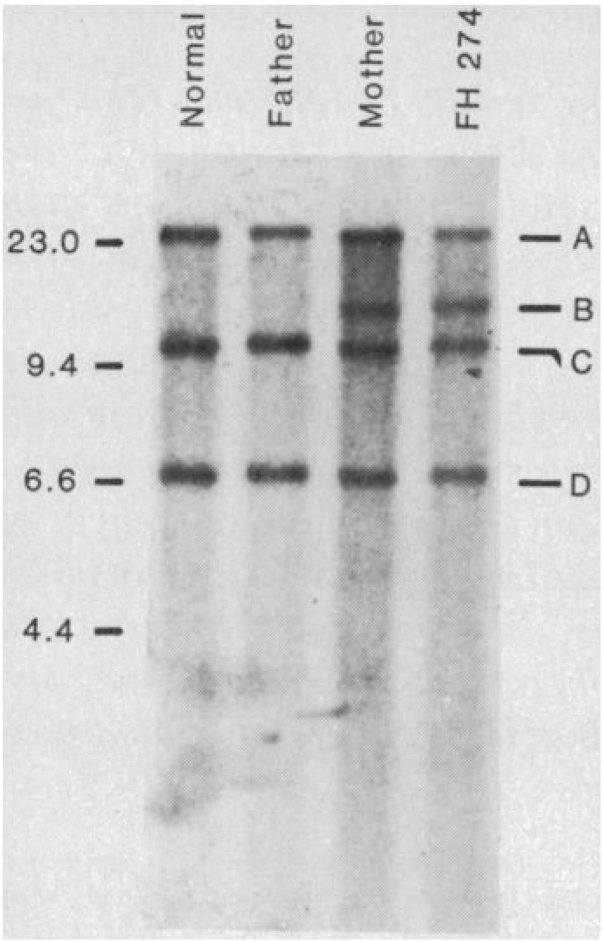

Fig. 2.

Blot hybridization of Xba I-cleaved genomic DNA from FH 274 and his parents. Genomic DNA (5 μg) isolated from cultured fibroblasts from the indicated subject was digested with Xba I, subjected to electrophoresis, transferred to nitrocellulose, and hybridized with ^{32}P-labeled probe 1 (see legend to Fig. 1). The four relevant Xba I restriction fragments are designated A to D along the right side of the blot. Molecular size standards were generated by Hind III cleavage of bacteriophage λ DNA.

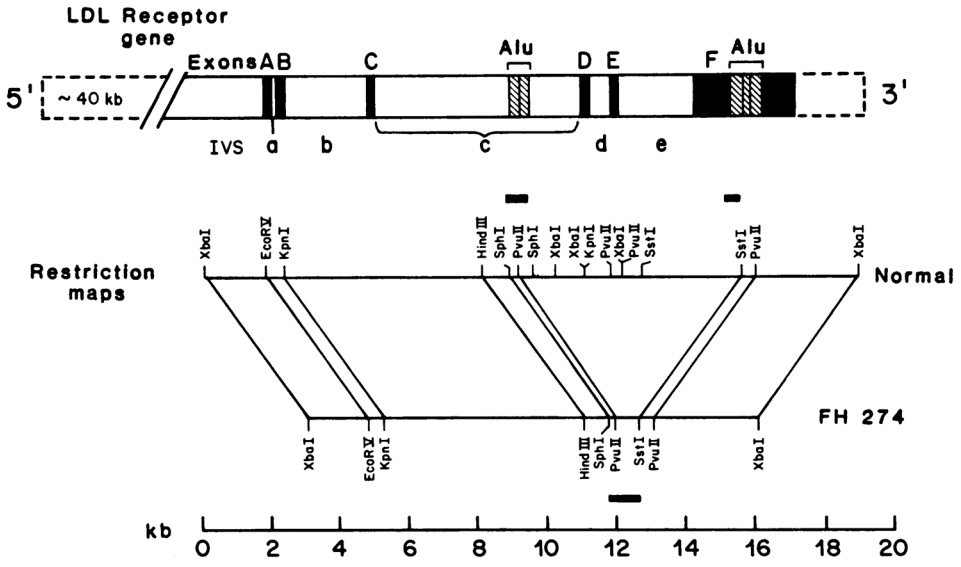

Fig. 3.

Comparison of restriction maps of the 3′ end of the normal LDL receptor gene and the deletion-bearing gene from FH 274. The scale at the bottom indicates the length of genomic DNA in kilobases. The organization of the normal LDL receptor gene is shown in the diagram at the top. Exons are indicated by solid segments and upper case letters; intervening sequences (IVS) are indicated by open segments and lower case letters. The Alu repetitive sequences in IVSc and exon F are indicated. Restriction enzyme recognition sites used to define the gene deletion in FH 274 are shown. The restriction map of the normal receptor gene was generated from studies of three λ genomic clones (λ 33-2, λ 33-1, λ hl) (10). The map of the gene in FH 274 was generated from λFH 274-10, which contains Xba I fragment B (Fig. 2) (see below). Solid bars above or below the restriction maps denote segments of the normal and mutant genes that were used for DNA sequencing in Figs. 4 and 5. To obtain λFH 274-10, we prepared 570 μg of genomic DNA (20) from fibroblasts of FH 274 and digested it with 1700 units of Xba I. The digested DNA was extracted with a mixture of phenol and chloroform and then chloroform, precipitated with 70 percent ethanol and 86 m*M* sodium acetate, and dissolved in 200 μl of buffer B (10 m*M* tris-chloride and 1 m*M* disodium EDTA at *pH* 7.5) The DNA (80 μg) was redigested with 100 units of Xba I, and subjected to electrophoresis on a 1 percent “low-gelling-temperature” agarose gel (Bethesda Research Laboratories) containing buffer A (40 V, 72 hours, 4°C). After electrophoresis, ten 2-mm slices of the gel containing DNA fragments, 9 to 23 kb in size, were extracted and concentrated (20), and dissolved in 20 μl of buffer B. One portion (4 μl) of each DNA fraction, 5 μg of Xba I-digested genomic DNA from FH 274, and Hind III-digested λ marker fragments were placed onto individual lanes of a 0.8 percent agarose gel containing buffer A and subjected to electrophoresis (35 V, 16 hours, 23°C). The DNA was transferred to nitrocellulose paper and hybridized with ^{32}P-labeled probe 1 as described in Fig. 1. The resulting autoradiogram identified the fraction that contained the abnormal 13-kb Xba I fragment (fragment B, Fig. 1). The remaining DNA from this fraction (100 ng) was mixed with 500 ng of Xba I-digested arms of λ Charon 35 (23) and incubated (72 hours, 14°C) with 490 units of T4 DNA ligase (New England Biolabs). The ligated material was

packaged into λ phage particles in vitro (Amersham) to yield a total of 6.7×10^3 plaque-forming units. This library was screened with ^{32}P-labeled probe 8 (Fig. 1), which was expected to detect only the abnormal fragment (13 kb) since the corresponding normal fragment (~7 kb) was too small to generate viable recombinant phage. One recombinant clone was identified (λFH 274-10) and isolated after an additional cycle of plaque purification. The 13-kb insert in λFH 274-10 was isolated from purified λ DNA, subcloned into pSP65 (Promega Biotec), and used for restriction endonuclease mapping.

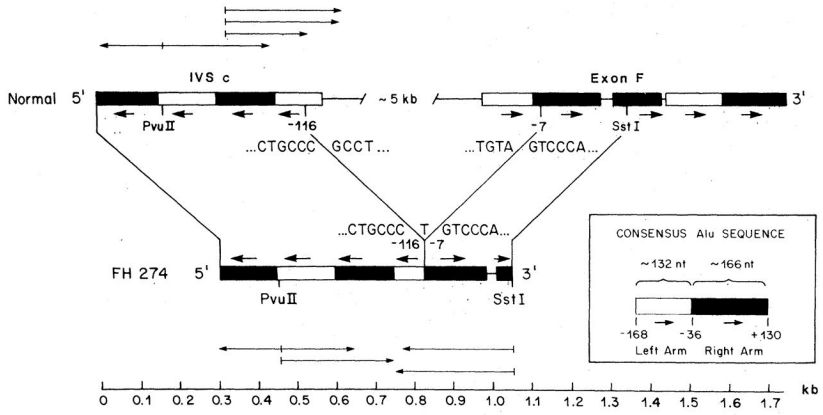

Fig. 4.

Strategy for sequencing and orientation of the relevant Alu repetitive sequences in the normal LDL receptor gene and the corresponding region from the deletion-bearing gene from FH 274. The upper and lower parts of the figure show the relevant regions of the normal and FH 274 genes used for DNA sequencing. These sequences (corresponding to the regions delimited by the solid bars in Fig. 3) were determined from the following clones: IVSc from λ33-1 (10), exon F from the previously reported sequence of pLDLR-2 (9), and the FH 274 deletion joint from λFH 274-10. DNA sequencing was performed by the dideoxy-chain termination method (24). The direction and extent of sequence established in a given experiment are indicated by the long arrows above or below the relevant DNA segments. Those sequences not coinciding with a restriction endonuclease site were determined with synthetic oligonucleotides as primers on M13 subclones (25). The boxed area shows a consensus Alu sequence with the left and right tandem repeats (open and closed, respectively). The nucleotide numbering scheme of the consensus Alu is the same as that described (11); position +1 refers to the C of the Alu I endonuclease recognition site, AGCT. Alu sequences in the normal and FH 274 genes are illustrated according to the same numbering scheme. Orientations of the left and right tandem Alu repeats are indicated by the short arrows. Segments of the normal and FH 274 genes that correspond to each other are connected by the diagonal lines. The exact DNA sequences around nucleotide positions −116 and −7 in the Alu repeats of IVSc and exon F, respectively, and of the deletion joint in FH 274 are shown.

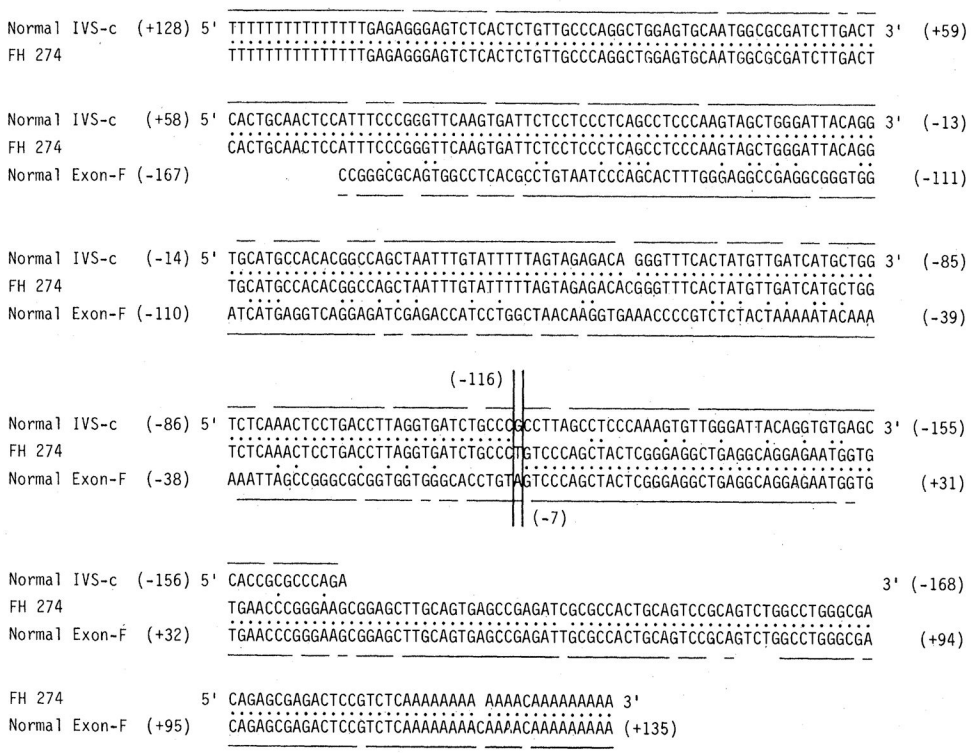

Fig. 5.

Nucleotide sequences across the deletion joint in FH 274 and for the corresponding normal 5′ and 3′ DNA's. The normal and FH 274 sequence data were obtained as described in Fig. 4 and are numbered according to the scheme used for the consensus Alu sequence (11). The normal 5′ sequence (top line), the sequence across the deletion joint in FH 274 (middle line), and normal 3′ sequence (bottom line) are aligned with only two gaps of one base each. Dots between sequences indicate positions at which the sequences are identical. Horizontal lines above or below the normal sequence indicate positions at which the normal sequence is identical to the consensus Alu sequence (11). The two vertical lines at positions −116 and −7 denote the position of the deletion joint.

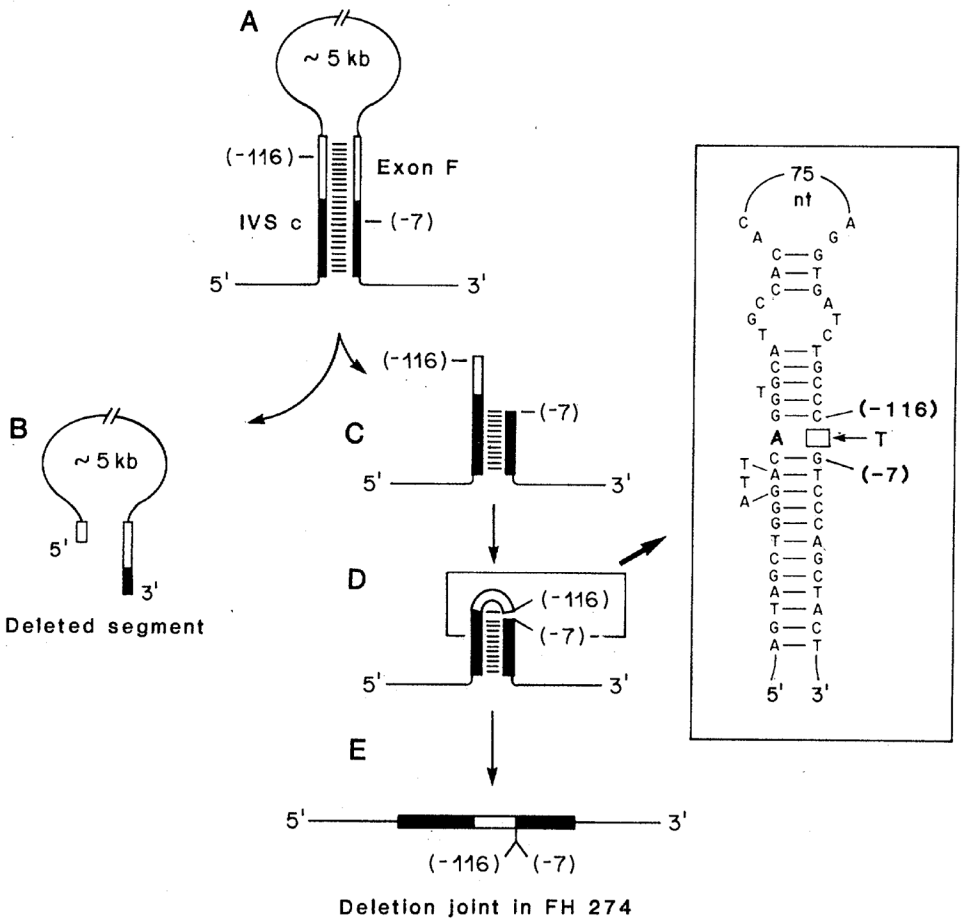

Fig. 6.

Potential mechanism for the gene deletion in FH 274. The deletion joint is shown to result from a homologous recombination between the Alu sequences in IVSc and exon F occurring on a single strand of the DNA molecule (steps A–E). The left and right arms of an Alu sequence are indicated by the open and closed bars, respectively. The boxed area at the right shows the actual base pairing that is postulated to occur at step D.

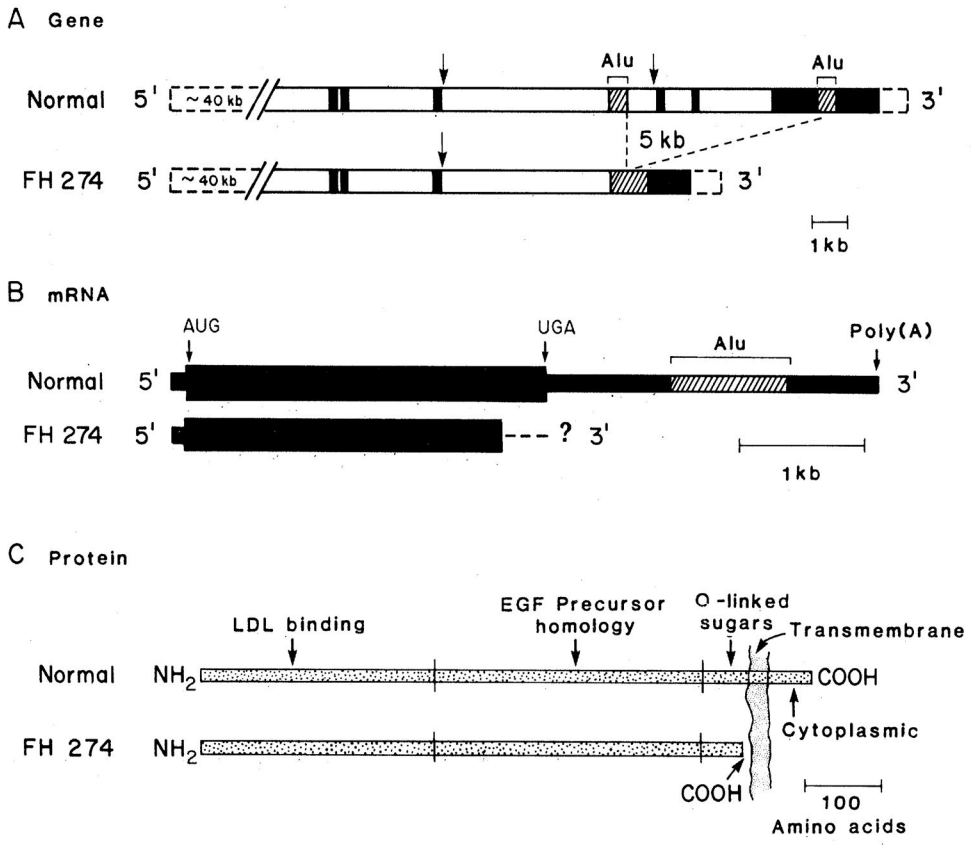

Fig. 7.

Consequences of the 5-kb gene deletion for the structures of the LDL receptor mRNA and truncated protein in FH 274. (A) The location of exons (closed bars) and intervening sequences (IVS, open bars) in the 3′ end of the normal gene and the corresponding segments of the deleted gene in FH 274 are marked. Alu sequences are cross-hatched. The arrows indicate splice sites for IVSc. (B) The translated and untranslated regions (thick and thin closed bars, respectively) of the normal mRNA and corresponding sections of the mutant mRNA are indicated. The location of the 3′ end of the mutant mRNA is unknown (“?”). (C) The structural domains of the normal receptor protein are shown in comparison with those of the mutant receptor protein, which lacks the transmembrane and cytoplasmic domains.

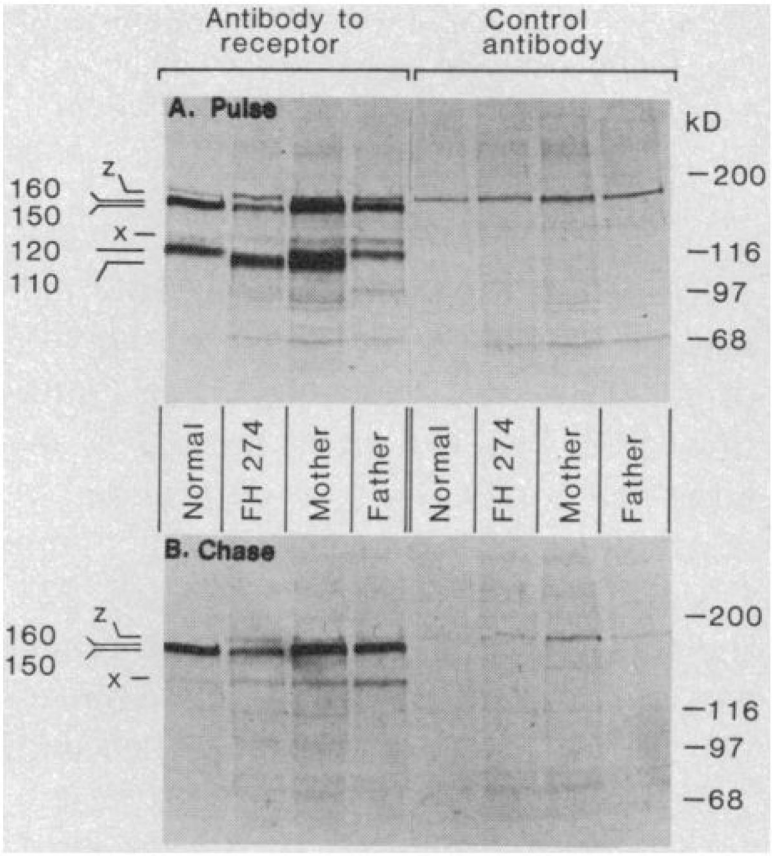

Fig. 8.

Electrophoresis of LDL receptors immunoprecipitated from ^{35}S-labeled fibroblasts from FH 274 and his parents. Fibroblasts were cultured for 6 days and induced for synthesis of LDL receptors by incubation in lipoprotein-deficient serum for 16 hours (4). Cells were pulse-labeled with [^{35}S]methionine (140 μCi/ml) (4) for 2 hours at 37°C, and isotopically labeled LDL receptors were solubilized either immediately (A) or 2 hours after the addition (chase) of unlabeled methionine (B). Cell monolayers were washed once with phosphate-buffered saline at 4°C, and detergent extracts were prepared and incubated with immune complexes (4) containing either monoclonal antibody to LDL receptor (IgG-C7) or a control monoclonal antibody (IgG-2001) (4). The incubation mixtures were layered onto multistep sucrose gradients, and the immunoprecipitates were collected by centrifugation and washed (4). The precipitates were dissolved by heating to 90°C for 5 minutes in buffer containing 10 percent (by volume) glycerol, 0.2*M* dithiothreitol, 2.3 percent (weight to volume) SDS, and 75 m*M* tris-chloride at *p*H 6.8 and subjected to SDS electrophoresis on 7 percent polyacrylamide gels, followed by autoradiography (4). The gels were exposed to x-ray film for 2 days. Apparent molecular sizes of the ^{35}S-labeled proteins were calculated from the position of migration of the following standards as determined by Coomassie blue staining: myosin (200 kD), β-galactosidase (116 kD), phosphorylase b (97 kD), and bovine serum albumin (68 kD). Band z is a protein that appears in control immunoprecipitations and is not related to the LDL receptor; band x is a proteolytic fragment of the receptor.

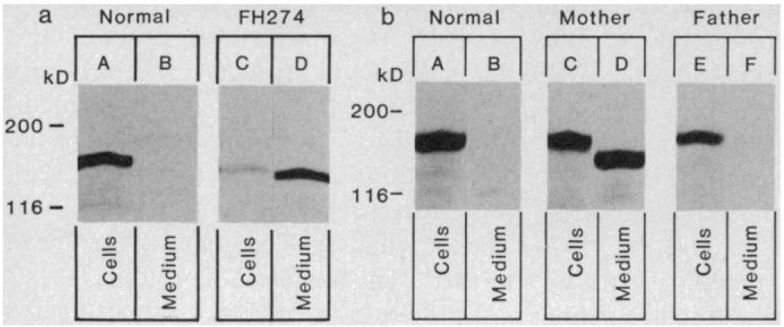

Fig. 9.

Recovery in the culture medium of newly synthesized LDL receptor from fibroblasts of FH 274 (a) and his mother and father (b). Fibroblasts from the indicated subject were induced for LDL receptor synthesis as described in Fig. 8. Cells were labeled with [^{35}S]methionine (215 μCi/ml for top panel and 275 μCi/ml for bottom panel) for 18 hours at 37°C in medium containing 5 μ*M* unlabeled methionine. The medium (1.1 ml per 60-mm dish) was removed and saved, and each monolayer was washed rapidly twice with a total of 2 ml of phosphate-buffered saline at room temperature. The combined medium and washings from three dishes were pooled and centrifuged at 5000*g* for 5 minutes at 4°C, and the resulting supernatant was saved (designated medium). Each monolayer was washed an additional four times as above, the washings were discarded, and detergent-extracts from three dishes of each cell strain were prepared as described in Fig. 8. Cell extracts and medium were immunoprecipitated with monoclonal antibody to the receptor (IgG-C7) and analyzed by SDS gel electrophoresis and autoradiography as described in Fig. 8. The gel was exposed to x-ray film for 20 hours.