A 3-basepair deletion in repeat 1 of the LDL receptor promoter reduces transcriptional activity in a South African Pedi

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Abstract **We have examined a naturally occurring mutation in the promoter region of the low density lipoprotein receptor (LDLR) gene of a South African Black patient with a clinical diagnosis of familial hypercholesterolemia (FH). The mutation constitutes a 3-bp deletion at nucleotide position −92 (FH Pedi-2) in the distal Sp1 binding site in repeat 1 of the LDLR promoter. The patient carries a second mutant LDLR allele containing a 1-bp deletion in exon 2 (FH Pedi-1) that gives rise to a frameshift mutation. Consistent with low receptor activity previously observed in cultured fibroblasts from the patient (5–15%), the rate of LDL receptor synthesis was markedly reduced to less than 20% of normal. DNase I footprint analysis indicated that the −92 mutation abolished binding of Sp1 to repeat 1 in the LDLR promoter. Transcription studies in transfected cells using normal and mutant promoter fragments linked to a luciferase reporter gene demonstrated that the promoter fragment containing the −92 mutation had approximately 10% of normal promoter activity. These findings indicate that the distal Sp1 binding site is essential for maximal activity of the normal intact LDLR promoter.—**Peeters, A. V., M. J. Kotze, C. L. Scholtz, L. F. De Waal, D. C. Rubinsztein, G. A. Coetzee, G. Zuliani, R. Streiff, J. Liu, and D. R. van der Westhuyzen. **A 3-basepair deletion in repeat 1 of the LDL receptor promoter reduces transcriptional activity in a South African Pedi. *J. Lipid Res.* 1998.** 39: **1021–1024.**

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Familial hypercholesterolemia (FH) is a dominantly inherited disease of lipid metabolism that is recognized clinically by elevated serum low density lipoprotein cholesterol (LDLC) levels, tendon xanthomata and premature coronary heart disease (CHD) (1). The FH phenotype results from a defective catabolism of LDL, which is caused by mutations in the LDLR gene. Most of the FH mutations identified to date affect the structure or function of the LDLR and are located within the coding region, including the splice junctions, of the LDLR gene (2). Mutations in the 5′-untranslated region that impair transcription of the LDLR gene could, however, also lead to FH.

The *cis*-acting elements responsible for promoter activity of the LDLR gene consist of three imperfect direct repeats located within 177 basepairs upstream of the LDLR transcription start site (3). Basal transcription is promoted by the interaction of the universal positive transcription factor, Sp1, with binding sites within repeats 1 and 3. High level expression additionally requires repeat 2 which contains a sterol regulatory element (SRE) (3, 4). The SRE contains a binding site for a family of proteins, the sterol regulatory element binding proteins (SREBPs) that are required for maximal promoter activity (5). Regulation is via a novel mechanism in which the generation of an active SREBP occurs by a proteolytic cleavage that is regulated by cellular sterol levels (6).

Abbreviations: LDLR, low-density lipoprotein receptor gene; FH, familial hypercholesterolemia; LDLC, low-density lipoprotein cholesterol; CHD, coronary heart disease; SRE, sterol regulatory element; SREBP, sterol regulatory element binding protein.

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To date, four different point mutations have been reported in repeat 3 of the LDLR promoter (2, 7–9). All of these were shown to markedly reduce transcriptional activity, causing FH. Alterations of repeat 1 in CAT constructs resulted in considerably decreased promoter activity although not always to the same degree as alterations in repeat 2 or 3, which virtually abolished activity (3). To further investigate the role of repeat 1 in the etiology of FH, we analyzed the effect of a 3-bp deletion found in a compound heterozygote (2) and demonstrate that the distal Sp1 binding site is essential for maximal activity of the normal intact LDLR promoter.

METHODS

Subject

The index patient, JL, is a Pedi from Lebowa and is the first Black FH homozygote identified in South Africa (10). The severity of the clinical features in this patient was typical for homozygous FH, although his serum total cholesterol level ranged between 5.64 and 8.87 mmol/l. JL presented with extensive xanthomata, corneal arci and signs of arteriosclerosis.

DNase I footprinting

DNase I footprinting was carried out as described previously (11) with certain modifications (4). DNA fragments corresponding to nucleotides −130 to +59 were 3′ end-labeled with ^{32}P on the coding strand. Five fmol of the end-labeled DNA fragment was incubated for 20 min at 0°C in 25 mm Tris chloride at pH 7.9, 2.5 mm $MgCl_2$, 0.5 mm EDTA, 0.5 mm dithiothreitol, 50 mm KCl, 10% glycerol, 2% (v/v) polyvinyl alcohol, with the indicated amount of purified Sp1 and treated for 60 s with DNase I (5 ng of DNase I for sample with no protein, 7.5 ng of DNase I for samples with Sp1). After DNase I digestion, each reaction mixture was subjected to electrophoresis on a denaturing gel. The gel was exposed to X-ray film for 16 h at −70°C.

Transient transfection assays

Reporter plasmids were constructed by directional cloning of normal and mutant LDLR promoter fragments into the BglII/HindIII site of a promoterless luciferase reporter vector (pGL3-Basic; Promega). Inserts extending from position –244 to +55 of the LDLR gene were obtained by genomic DNA amplification of JL with primers PB (5′-CCAATTTTGAGGGGGCGTCAGATCT TCACC-3′) and PH (5′-CACGACCTGCTGTGTCCAAGCTTGA AACCC-3′) which were modified to contain a BglII and a HindIII enzyme cutting site, respectively.

HepG2 cells were transfected with plasmid DNA by the method of calcium phosphate co-precipitation. Briefly, cells were plated 1 day before transfection in 48-well culture plates at a density of 1.25×10^5 cells per well in medium A (EMEM medium containing 10% FBS, 100 units/ml of penicillin G, and 100 μg/ml streptomycin). One hour before transfection, fresh medium was added. Calcium phosphate precipitates containing 330 ng of LDLR reporter plasmid and 110 ng of pRSV-βgal (to normalize transfection efficiency) were prepared for each well. The DNA/calcium phosphate precipitates were incubated with the cells at 37°C for 3 h, at which time the cells were washed once with PBS, incubated with 15% glycerol/1× HBS for 1 min, washed twice more with PBS and re-fed with either medium A or medium B (same as medium A except with 10% LPDS instead of FBS). Where sterol regulation of LDLR was measured, cells received medium B with or without cholesterol (10 μg/ml plus 1 μg/ml 25-OHC). Twenty four hours after replacement of media, cells were washed twice with 1× PBS and lysed with 150 μl of 1× reporter lysis buffer (Promega Corporation, Madison, WI). Luciferase activity (20 μl of lysate/sample) was measured in a Berthold Autolumat luminometer model LB953 (Aliquippa, PA) using substrate prepared in accordance with Promega's luciferase assay system. β-galactosidase activity (50 μl of lysate) was measured according to standard methods. Absolute luciferase activity was normalized against β-galactosidase activity to correct for transfection efficiency. Triplicate wells were assayed for each transfection condition and at least three independent transfection assays were performed for each luciferase construct.

RESULTS

Two LDLR gene mutations, a deletion of a single base at codon 37 (FH Pedi-1) and a 3-bp deletion at base −92 in the LDLR promoter (FH Pedi-2), were identified in a South African black patient (2). To further characterize the consequence of the promoter mutation, this region was amplified in genomic DNA using the polymerase chain reaction (PCR). Three PCR fragments were generated from the DNA of patient JL whereas only a single band was identified in the control DNA. The three bands in the case of JL were generated by the formation of homoduplexes and heteroduplexes in the PCR reaction (data not shown) and provide a basis for a rapid and convenient assay for the −92 mutation.

The PCR product corresponding to the homoduplex DNA fragment was cloned into a promoterless luciferase plasmid vector pGL3 and sequenced (**Fig. 1**). The −92 mutation corresponded to a 3-bp deletion involving 2 C's and a single T. The sequence was confirmed in more than one cloned product and also by direct sequencing of the PCR product (data not shown). Given the repetitive type sequence of the normal repeat 1, it is not possible to state exactly which of three nucleotides from the normal sequence had been deleted. The mutant sequence differed from that reported previously for this patient (del CTT) (2).

We have previously reported that fibroblasts from JL exhibited very low LDLR activity (5–15% of normal) as assessed by ligand and antibody binding and uptake studies (12). The 1-bp deletion in exon 2 gives rise to a frameshift mutation that would abolish the synthesis of functional LDLR from this mutant allele. To assess whether the −92 mutation also affects LDLR synthesis and thereby contributes to reduced LDLR expression, the rate of LDLR synthesis was measured in fibroblasts from JL. LDL receptor synthesis was decreased in JL fibroblasts to a level less than 20% (D. C. Rubinsztein, G. A. Coetzee, and D. R. van der Westhuyzen, unpublished results). This strongly suggested that the −92 mutation affects LDLR synthesis by interfering with transcription of this gene.

DNase footprint analysis was performed to determine the effect of the −92 mutation on the ability of Sp1 to bind to the repeat 1 sequence of the LDLR. (**Fig. 2**). As expected, purified Sp1 was shown to give clear footprints

Fig. 1. Partial nucleotide sequence of a cloned fragment of the normal (N) and mutant (JL) promoter region of the LDL receptor gene. Sequencing of the sense strands is shown.

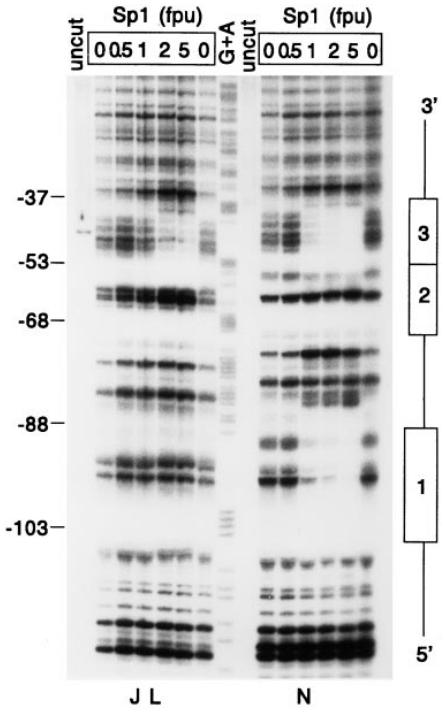

Fig. 2. DNase I footprint of normal (N) and mutant (JL) LDL receptor promoter fragments protected by Sp1. The locations of repeats 1–3 are indicated to the right. (fpu, footprint units)

over repeats 1 and 3 of the normal promoter sequence. In the case of the mutant JL (−92) mutation only repeat 3 bound Sp1, while repeat 1 showed no evidence of Sp1 binding and protection.

PCR-amplified fragments encompassing region −244 to +55 of the wild-type and mutated LDLR promoters were inserted into a luciferase reporter construct. Promoter function was assessed by luciferase assays in transiently transfected HepG2 cells. The wild-type LDLR promoter was capable of directing high levels of luciferase activity, whereas the mutant promoter supported luciferase activity of only approximately 8% of the wild-type level (**Fig. 3**). When cells were grown in medium supplemented with sterols instead of LPDS, luciferase activities were reduced by approximately 70–80% for both the wild-type and mutant promoters.

DISCUSSION

In the present study we have examined the functional consequences of a 3-bp deletion mutation (FH Pedi-2) in the LDLR promoter. This mutation occurred in a patient whose fibroblasts demonstrated markedly reduced LDLR activity (12). The FH Pedi-2 mutation alters the distal Sp1 binding site in repeat 1 of the LDLR promoter and severely decreases LDLR gene expression in fibroblasts of the patient as well as the activity of promoter fragments that were assayed in reporter constructs in transfected cells. These findings therefore confirm the previous conclusions, based on the activities of isolated promoter constructs, that the distal Sp1-binding sequence in repeat 1 plays an important role in supporting LDLR gene transcription (3).

Four other promoter mutations have been described and these all affect the proximal Sp1 binding site in repeat 3. A C-43T mutation has been identified in two patients and shown to severely reduce receptor expression to approximately 5% of the normal level (7, 9). A C-42G and a C-44T mutation both reduced promoter activity to 5–15% of normal levels as assessed by cellular LDLR activity (2). However, a T-45C mutation affecting a base that is not strongly conserved in the consensus sequence for Sp1 binding was shown to reduce promoter activity to a lesser extent (8). In the case of the FH Pedi-2 mutation, the effect on transcription activity can be explained by a gross alteration of the Sp1 binding sequence and a consequent abolishment of Sp1 binding, resulting in a significant but not complete loss of promoter activity due to a residual activity exhibited by repeats 2 and 3.

The finding that the distal Sp1 site is important for transcription is consistent with the recent findings based on DNase I footprinting studies in vivo that demonstrated that each of repeats 1 to 3 are protected by protein binding (13, 14). Futhermore, in the case of the two Sp1 sites little change was observed in protein binding between up- and down-regulated cells. Changes in promoter activity were limited to changes in the protection pattern observed for the SRE regulatory element. The fact that the SRE element cannot function solely as a positive transcriptional element, even when in multiple copies, suggests a possible interaction between the different transcription factors binding to the neighboring repeats. In fact, recent evidence indicates that SREBP increases the binding of Sp1 to the adjacent repeat 3 binding site and that these two proteins act synergistically to activate LDLR transcription (15, 16). The possible interactions between Sp1 bound to the first repeat

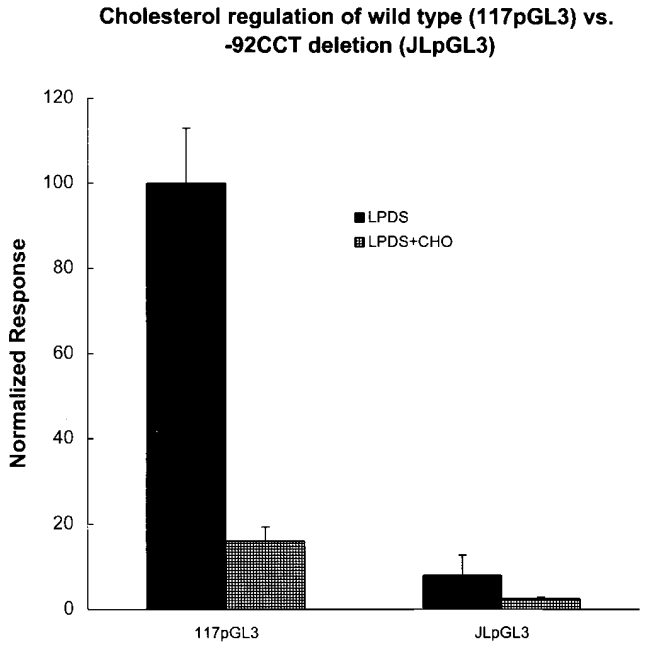

Fig. 3. Analysis of the wild-type (N) and mutated (JL) LDL promoter activity under transient transfection conditions. Luciferase activities were normalized over β-galactosidase activity.

and those proteins bound to the other two repeats are not known.

The FH patient had an additional LDLR mutation, a 1-bp deletion in exon 2 that caused a frame-shift (FH Pedi-1) (2). The patient was therefore classified as a compound heterozygote, being heterozygous for each of two LDLR mutations. This would explain the original clinical diagnosis of homozygous FH. It is noteworthy that the patient had a total plasma cholesterol value at presentation (6.5–8.9 mmol/l) that was particularly low for an FH homozygote and that was similar to the values for FH heterozygotes in more affluent societies. The severe clinical outcome for this patient, who died suddenly at the age of 24, possibly illustrates an important clinical lesson: in spite of only moderately elevated cholesterol levels, this patient still manifested early xanthomata, an aortic stenosis murmur, and premature sudden death probably from a myocardial infarct. This may imply that cholesterol levels in Black FH homozygotes, even if partially normalized by a prudent diet and possibly by other genetic factors (17), are not necessarily a reliable predictive factor for the critical pathology of FH homozygotes, namely, coronary artery disease.

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