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A New *Apa* LI Restriction Fragment Length Polymorphism in the Low Density Lipoprotein Receptor Gene^{1), 2)}

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Summary: The existence of a new *Apa* LI restriction fragment length polymorphism in the third intron of the low density lipoprotein (LDL) receptor gene was described. As a gene probe we used a newly constructed derivative of pLDLR 3 which did not contain the highly repetitive Alu-sequences in exon 18. This new gene probe detected all exon sequences containing restriction fragments, and enabled us to demonstrate all described polymorphisms, which might be useful for genetic linkage studies. Based on analysis of 72 unrelated normo- and hypercholesterolaemic persons, the frequency of the allele A2, which showed the additional cutting site, was determined to be 0.05. With the simplified gene probe, pLDLR Δ, we also studied other polymorphisms. A clear linkage disequilibrium between the *Pvu* II and *Msp* I polymorphisms was detected. This, and the previously described linkage disequilibrium of the two *Msp* I polymorphisms, demonstrate that the LDL receptor gene is apparently less heterogeneous than expected from the number of described polymorphisms.

Introduction

The low density lipoprotein (LDL) receptor promotes the clearance of cholesterol-rich LDL particles from blood (1). Mutations in the LDL receptor gene cause familial hypercholesterolaemia, an autosomal dominant inherited disease (2). Individuals with one mutant gene for the LDL receptor (heterozygous familial hypercholesterolaemia) have about 2 fold elevated levels of LDL in plasma and an increased risk for myocardial infarction. In individuals who carry two mutant genes (homozygous familial hypercholesterolaemia), the LDL levels are even 3–6 fold higher than normal, and coronary heart disease frequently occurs in childhood. The prevalence of heterozygous

familial hypercholesterolaemia appears to be approximately 1 in 500 in most countries of the world. The homozygous form is about 1 in 1 million (3).

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²⁾ Enzymes:
Restriction endonuclease (EC 3.1.21. ...) *Apa* LI from *Acetobacter pasteurianus*
Bam HI from *Bacillus amyloliquefaciens*
Bsp M 2 from *Bacillus* species M
Dra III from *Deinococcus radiophilus*
Eco RI from *Escherichia coli*
Kpn I from *Klebsiella pneumoniae*
Msp I from *Moraxella* species
Pst I from *Providencia stuartii*
Pvu II from *Proteus vulgaris*
T 4 DNA ligase, EC 6.5.1.1

Recently, the full-length cDNA of the LDL receptor gene has been cloned (4), and a variety of mutations in this gene have been found among patients with familial hypercholesterolaemia (2, 5–9). These experiments were usually carried out by hybridizing genomic DNA or cytoplasmic m-RNA with the cDNA to survey both genomic gene organisation and m-RNA expression. Since highly repetitive Alu sequences were present at the 3' end of the cDNA, direct hybridization experiments using the full-length cDNA clone were impossible. Therefore fragments of the cDNA, which detect only some of the 18 exons, were employed for hybridization (9, 10). The most frequently used gene probe was the *Bam* HI fragment which contained complementary sequences of exon 10 to 18. For this reason, most of the detected polymorphisms have been localized in the 3' region of the gene. In the present work, we prepared a cDNA probe from pLDLR 3 which can detect all exon-containing restriction fragments of the LDL receptor gene without further probe processing.

To date, 11 restriction fragment length polymorphisms have been described in the LDL receptor gene (10–18). For the diagnosis of familial hypercholesterolaemia, the polymorphisms are used to detect alleles which correlated with the disease in pedigree analysis (19). The identification of the new *Apa* LI polymorphism, which was localized in the 5' region of the gene, provided additional information for genetic analysis.

Materials and Methods

Preparation of the gene probe pLDLR Δ

The full-length cDNA clone pLDLR 3 was obtained from the American Type Culture Collection (Rockville, Md., USA, ATCC-Nr. 57005). Cloned pLDLR 3 was cleaved with *Bsp* M2 (Biolabs, Beverly, MA, USA), which cuts at nucleotide position 4580 of the cDNA. The linear molecule was then partially digested with *Dra* III (Biolabs), which cleaves at positions 3115 and 3626. The resulting fragments were separated by preparative agarose gel electrophoresis (6 g/l, Type IIa, Sigma, Deisenhofen, FRG), and the $\sim 7 \times 10^3$ base pairs fragment was eluted. After *Klenow* fragment treatment (Boehringer Mannheim, FRG) to prepare blunt ends, the fragment was recircularized with T4 DNA ligase (BRL, Eggenstein, FRG). *E. coli* cells, strain HB 101, were made competent by the method of Hanahan and transformed with the ligation products (20). One clone, which showed the expected cleavage patterns with *Dra* III, *Hae* III, *Rsa* I or *Msp* I was selected and was designated pLDLR Δ . Plasmid DNA was prepared by the Triton X-100 lysis method and purified by CsCl gradient centrifugation (21).

Other gene probes

Gene probes to detect only certain exons were prepared by digestion of 5 μ g pLDLR Δ DNA with *Pvu* II (Boehringer Mannheim, FRG). The resulting fragments were separated by preparative agarose gel electrophoresis (20 g/l). The elution of the 268 bp *Pvu* II fragment was carried out with the Biotrap® of Schleicher and Schüll (Dassel, FRG).

Labelling of the gene probes

100 ng of pLDLR Δ was radioactively labeled with [32 P]dCTP by the nick translation kit of Boehringer Mannheim (FRG). Purified DNA fragments were radiolabeled by an oligonucleotide priming kit (Amersham, Braunschweig, FRG). The unincorporated nucleotides were separated on a Sephadex G-50 column (Pharmacia, Freiburg, FRG). The specific activity of the probes was greater than 5×10^8 counts/min $\times$ μ g.

DNA isolation

Blood samples were collected in EDTA Monovettes (Sarstedt, Nümbrecht, FRG) and frozen at -70°C . DNA was prepared from leukocytes by the Triton X-100 lysis method (22).

DNA digestion and Southern blot

Ten μ g of human DNA was cleaved with restriction enzymes *Pvu* II, *Msp* I, *Pst* I, *Bam* HI (Boehringer Mannheim, FRG) or *Apa* LI (Biolabs, Beverly, MA, USA), as recommended by the manufacturer, and the fragments were separated on agarose gels (*Pvu* II, *Pst* I, *Apa* LI/*Bam* HI fragments in agarose, 8 g/l; *Msp* I fragments in agarose, 20 g/l). The fragments were transferred to nitrocellulose filters (Ba 85, Schleicher & Schüll, Dassel, FRG) and fixed by baking at 80°C (21).

Hybridization

The conditions for hybridization have been previously described (23). The posthybridization washes consisted of three 10 min washes at room temperature in a solution of $2 \times \text{SSC}$ ($1 \times \text{SSC} = \text{NaCl}, 0.15 \text{ mol/l}$, sodium citrate, 15 mmol/l) and SDS, 1 g/l , and three 15 min washes at 56°C in a solution of $0.1 \times \text{SSC}$ and SDS, 1 g/l . The nitrocellulose filters were then exposed to X-ray films (XAR, Kodak, USA) for 1–4 days at -70°C .

Collective

Patients with familial hypercholesterolaemia (48 individuals), type IIa, were followed up in the outpatient clinic of our department. These patients had a family history of hypercholesterolaemia and in some cases of myocardial infarctions and tendon xanthomata. The LDL-cholesterol ranged between 180 and $240 \text{ mg/dl} \pm 4.7$ and 6.2 mmol/l during a cholesterol-lowering diet and treatment with one or more lipid-lowering drugs. Normolipidaemic subjects (24 individuals) were laboratory personnel and medical students.

Results

Improved LDL receptor gene probe

As expected for hybridization probes which carried Alu repeats, hybridization experiments were impossible with pLDLR 3. Using two restriction sites in close proximity to the Alu repeats (nucleotide position 3717 and 4478, fig. 1) these sequences were deleted in pLDLR 3. The resulting clone was named pLDLR Δ (for details see Materials and Methods). In the analytical restriction experiments, we took into account the modification in the promotor region of pLDLR 3 as mentioned by the American Type Culture Collection. With pLDLR Δ all exon sequences containing restriction fragments could be detected. An example of a genomic *Southern* blot is shown in figure 2.

Fig. 1. Structure of pLDLR Δ

The Alu sequences containing part of pLDLR 3 between the second *Dra* III site (cDNA nucleotide position 3626, numbers as in l.c. (4)) and the *Bsp* M2 site (cDNA nucleotide position 4580) was deleted. The resulting clone was named pLDLR Δ .

— vector part, — cDNA, — deleted part

Fig. 2. *Apa* LI polymorphisms in the LDL receptor gene.

In the upper part, the *Southern* blot analyses of DNA cleaved with *Apa* LI and *Bam* HI are demonstrated. In the left panel, pLDLR Δ was used for hybridization to detect all three polymorphisms. In the right panel the new *Apa* LI polymorphism in intron 3 is shown solely by hybridization with the exon 3- and 4-specific *Pvu* II fragments. In the lower part a rough restriction map of the LDL receptor gene is shown. The relevant *Apa* LI sites (A), *Bam* HI sites (B) and additional cutting sites (○) and the resulting fragments as well as their length (10^3 base pairs) are indicated.

New *Apa* LI polymorphism

DNA from normolipidaemic individuals or from patients with familial hypercholesterolaemia was assayed for the two *Apa* LI polymorphisms previously described (15). Double cleavage with *Apa* LI and *Bam* HI improved the differentiation of fragments (15). The gene probes used contained sequences from exons 16 to 18. After hybridization with the pLDLR Δ gene

probe, which detects 3' and also 5' sequences, a third polymorphic *Apa* LI site became obvious (fig. 2). Two additional fragments with sizes of about 4.8 and 7.2×10^3 base pairs were detectable in some subjects. We designated the allele with the additional cutting site A2, and the allele without this site A1. By double cleavage with *Apa* LI and *Kpn* I, *Eco* RI or *Bam* HI the additional *Apa* LI site in allele A2 was located in intron 3. With the 268 base pairs *Pvu* II fragment from pLDLR Δ , which detects exons 3 and 4, this polymorphism could be selectively visualized (right panel fig. 2).

The allele frequencies of the new *Apa* LI polymorphism were determined in our collective of 72 unrelated individuals. For all investigated persons the frequencies of allele A1 and allele A2 are 0.95 and 0.05, respectively. The normolipidaemic population (24 individuals) showed in 23 cases the genotype A1A1, and in one case the genotype A1A2. In the population of independent patients with familial hypercholesterolaemia (48 individuals), the genotype A1A1 was present in 43 cases, A1A2 in 4 cases, and A2A2 in one case.

Mendelian inheritance of the newly discovered polymorphism was demonstrated in three families. One example is shown in figure 3. In this family the allele A2 was found to be associated with familial hypercholesterolaemia.

Fig. 3. Pedigree of a family with the *Apa* LI polymorphism within intron 3 of the LDL receptor gene.

○ □ normal, female and male
● □ heterozygous familial hypercholesterolaemia
● ■ homozygous familial hypercholesterolaemia
A diagonal stroke indicates that the family member died before onset of the study. The genotype for the polymorphic *Apa* LI site in intron 3 is also indicated.
A1: *Apa* LI site absent, A2: *Apa* LI site present in intron 3.

Linkage disequilibrium between polymorphisms

Including the new *Apa* LI polymorphism, 12 restriction fragment length polymorphisms were described in the LDL receptor gene. Three polymorphisms were detected for *Apa* LI, and two for *Msp* I. In our studies we considered the *Msp* I polymorphism which is located in exon 18 and the *Apa* LI polymorphism in intron 15. The allele frequencies of these and of the *Pvu* II and *Pst* I polymorphisms were determined in our collective. Following a proposal of the Committee on Human Gene Mapping by Recombinant DNA Techniques, the alleles have been designated A1 and A2 according to decreased allelic frequencies (24). A comparison of the homozygous *Pvu* II genotypes A1A1 and A2A2 with the corresponding *Msp* I genotypes of the same patients revealed a striking linkage disequilibrium between the *Pvu* II and the *Msp* I polymorphisms (tab. 1). In 57 out of 61 cases, the *Pvu* II genotypes A1A1 and A2A2 were associated to the corresponding *Msp* I alleles ($\chi^2 = 14.92$, $f = 1$, $p < 0.001$). A similar relationship between the genotypes of other restriction enzymes studied was not found.

Discussion

In order to obtain a gene probe which facilitated detection of all exons in the LDL receptor gene, we deleted the Alu sequences in pLDLR 3. These sequences were located in exon 18 and were involved in some rearrangements detected in patients with familial hypercholesterolaemia (5, 25). Alu repeats occur about 300 000 times in the human genome (26).

All of these repeats hybridized in genomic *Southern* blot experiments when the pLDLR 3 probe was used. With the newly constructed plasmid pLDLR Δ , a gene probe was available to study all exon-containing restriction fragments of the LDL receptor gene in one experiment. All restriction fragment length polymorphisms described so far were detectable with this gene probe, which has thus proved of great practical importance for genetic linkage studies in families with inherited hypercholesterolaemia. A third *Apa* LI polymorphism in the LDL receptor gene has emerged by screening with the gene probe pLDLR Δ . The results of mapping experiments using an exon 3- and 4-specific fragment for hybridization showed the polymorphic site to reside in intron 3. The new polymorphic *Apa* LI site is located closer to the 5' end than any of the other described polymorphisms (fig. 2). The frequency of the rare allele A2 is 0.05 in all studied persons. In the population of patients with familial hypercholesterolaemia the A2 allele occurred at a higher frequency than in normolipidaemic persons. Whether or not a linkage disequilibrium of allele A1 and allele A2 exists between the two populations can only be clarified by the investigation of a larger number of persons. Pedigree analysis to determine whether an association exists between the A2 allele and familial hypercholesterolaemia, as in the analysis shown in figure 3, is in progress.

The three *Apa* LI polymorphisms together with the 9 other described polymorphisms permit the identification of a large number of different alleles. However, the actual number of different alleles observed is smaller than the theoretically possible number. The LDL receptor gene seems to be less heterogeneous than expected from the number of polymorphisms.

Tab. 1. Correlation of the polymorphism-defined genotypes of the LDL receptor gene.

For *Msp* I the polymorphism in exon 18, for *Apa* LI the polymorphism in intron 15 are considered in the analysis. The 19, 9.6, 3.4, 0.44×10^3 base pairs fragment designated the A1 allele of *Pvu* II, *Apa* LI, *Pst* I and *Msp* I. The A2 allele is characterized for *Pvu* II, *Apa* LI, *Pst* I and *Msp* I by the 16, 6.6, 2.8 and 0.47×10^3 base pairs fragment, respectively.

		<i>Msp</i> I			<i>Pst</i> I			<i>Apa</i> LI		
		A1A1	A1A2	A2A2	A1A1	A1A2	A2A2	A1A1	A1A2	A2A2
<i>Pvu</i> II	A1A1	53	3	—	12	29	7	1	8	3
	A1A2	2	27	—	2	13	10	—	6	2
	A2A2	—	1	4	—	3	2	—	—	2
<i>Msp</i> I	A1A1				11	28	7	1	8	3
	A1A2				1	12	8	—	6	2
	A2A2				—	3	3	—	—	2
<i>Pst</i> I	A1A1							5	2	1
	A1A2							—	23	3
	A2A2							—	1	4

Here we report the linkage disequilibrium between the *Pvu* II and *Msp* I genotypes. The linkage disequilibrium of the two *Msp* I polymorphisms was published previously; as a result two haplotypes can now be characterized. In haplotype 1, the *Pvu* II site is absent at the polymorphic position, and the two additional *Msp* I sites are present. In haplotype 2, the additional *Pvu* II site is present, and the two *Msp* I sites are absent.

Since the number of heterozygous individuals for the *Pst* I and *Apa* LI polymorphisms was very large, correlations with other polymorphisms were not possible (tab. 1). In such cases haplotypes can only be established by family analysis. In phenylketonuria, it was shown that polymorphism-defined haplotypes were associated with the disease (27). In South Africa,

such an association was described for familial hypercholesterolaemia (28). This finding and a frequently detected mutation in specific ethnic groups, causing familial hypercholesterolaemia, can presumably be interpreted as a founder gene effect (29, 30). Whether it will be possible to detect distinct haplotypes, which frequently appear in patients with familial hypercholesterolaemia, in a central European population, remains to be elucidated in the future by pedigree analysis.

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References

1. Goldstein, J. L., Brown, M. S., Anderson, R. G. W., Russell, D. W. & Schneider, W. J. (1985) *Annu. Rev. Cell Biol.* **1**, 1–39.
2. Brown, M. S. & Goldstein, J. L. (1986) *Science* **232**, 34–47.
3. Goldstein, J. L. & Brown, M. S. (1983) In: *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., ed.) pp. 672–712, McGraw-Hill, New York.
4. Yamamoto, T., Davis, C. G., Brown, M. S., Schneider, W. J., Casey, M. L., Goldstein, J. L. & Russell, D. W. (1984) *Cell* **39**, 27–38.
5. Lehrmann, M. A., Schneider, W. J., Südhof, T. C., Brown, M. S., Goldstein, J. L. & Russell, D. W. (1985) *Science* **227**, 140–146.
6. Lehrmann, M. A., Goldstein, J. L., Brown, M. S., Russell, D. W. & Schneider, W. J. (1985) *Cell* **41**, 735–743.
7. Hobbs, H. H., Brown, M. S., Goldstein, J. L. & Russell, D. W. (1986) *J. Biol. Chem.* **261**, 13114–13120.
8. Lehrmann, M. A., Goldstein, J. L., Russell, D. W. & Brown, M. S. (1987) *Cell* **48**, 827–835.
9. Horsthemke, B., Dunning, A. & Humphries, S. (1987) *J. Med. Genet.* **24**, 144–147.
10. Humphries, S., Horsthemke, B., Seed, M., Holm, M., Wynn, V., Kessling, A. M., Donald, J. A., Jowett, N., Galton, D. J. & Williamson, R. (1985) *Lancet* **i**, 1003–1005.
11. Funke, H., Klug, J., Frossard, P., Coleman, R. & Assmann, G. (1986) *Nucl. Acids Res.* **14**, 7820.
12. Kotze, M. J., Retief, A. E., Brink, P. A. & Weich, H. F. H. (1986) *S. Afr. Med. J.* **70**, 77–79.
13. Kotze, M. J., Längenhoven, E., Dietzsch, E. & Retief, A. E. (1987) *Nucl. Acids Res.* **15**, 376.
14. Hobbs, H. H., Esser, V. & Russell, D. W. (1987) *Nucl. Acids Res.* **15**, 379.
15. Leitersdorf, E. & Hobbs, H. H. (1987) *Nucl. Acids Res.* **15**, 2782.
16. Geisel, J., Weisshaar, B., Oette, K., Mechtel, M. & Doerfler, W. (1987) *Nucl. Acids Res.* **15**, 3943.
17. Steyn, L. T., Pretorius, A., Brink, P. A. & Bester, A. J. (1987) *Nucl. Acids Res.* **15**, 4702.
18. Yamakawa, K., Okafuji, T., Iwamura, Y., Russell, D. W. & Hamaguchi, H. (1987) *Nucl. Acids Res.* **15**, 7659.
19. White, R., Leppert, M., Bishop, D. T., Barker, D., Berkowitz, J., Brown, C., Callahan, P., Holm, T. & Jerominski, L. (1985) *Nature* **313**, 101–105.
20. Hanahan, D. (1983) *J. Mol. Biol.* **166**, 557–580.
21. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York.
22. Kunkel, L. M., Smith, K. D., Boyer, S. H., Borgaonkar, O. S., Wachtel, S. S., Miller, O. J., Breg, W. R., Jones, H. W. & Rary, J. M. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1245–1249.
23. Stabel, S., Doerfler, W. & Friis, R. R. (1980) *J. Virol.* **36**, 22–40.
24. Skolnick, M. H., Willard, H. F. & Menlove, L. A. (1984) *Cell Genet.* **37**, 210–248.
25. Lehrmann, M. A., Russell, D. W., Goldstein, J. L. & Brown, M. S. (1987) *J. Biol. Chem.* **262**, 3354–3361.
26. Hobbs, H. H., Lehrmann, M. A., Yamamoto, T. & Russell, D. W. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 7651–7655.
27. DiLella, A. G., Marvit, J., Brayton, K. & Woo, S. L. C. (1987) *Nature* **327**, 333–336.
28. Brink, P. A., Steyn, L. T., Coetzee, G. A. & Van der Westhuyen, D. R. (1987) *Hum. Genet.* **77**, 32–55.
29. Hobbs, H. H., Brown, M. S., Russell, D. W., Davignon, J. & Goldstein, J. L. (1987) *N. Engl. J. Med.* **317**, 734–737.
30. Lehrmann, M. A., Schneider, W. J., Brown, M. S., Davis, C. G., Elhammer, A., Russell, D. W. & Goldstein, J. L. (1987) *J. Biol. Chem.* **262**, 401–410.

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