A single nucleotide substitution in the promoter region of the apolipoprotein C-II gene identified in individuals with chylomicronemia

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Abstract Apolipoprotein (apo) C-II plays a major role as a cofactor for lipoprotein lipase, the enzyme involved in the hydrolysis of triglyceride-rich particles. We identified in two relatives of a family (mother and son) massive hypertriglyceridemia with chylomicronemia. In these individuals apoC-II was not measurable in plasma by radial immunodiffusion. On isoelectric focusing of very low density apolipoproteins, trace amounts of apoC-II became obvious in the regular position. By sequencing, no abnormalities in the exons or neighboring intron sequences were detected. However, three alterations in the DNA sequence were found upstream from the transcription initiation site. Two variations could be explained by differences in previously published DNA sequences. The third variation (A→G; position −86; Das et al. 1987. *J. Biol. Chem.* **262:** 4787–4793) was present only in the homozygous form in the two hypertriglyceridemic probands. In 46 hypertriglyceridemic individuals outside the family, this mutation was not found. In electrophoretic mobility shift experiments with nuclear extracts from HepG2 cells, the 31 bp DNA fragment carrying the A→G substitution resulted in a markedly diminished protein binding compared with the wildtype DNA fragment. In promoter reporter gene assays, the activity of the basal promoter was reduced in the case of the A→G substitution and the deletion of the bases −91 to −58. The pedigree analysis and the experimental results are evidence that this is the first mutation in the apolipoprotein C-II gene where a single nucleotide substitution diminishes the binding of a transcription factor to a positive *cis*-acting element in the promoter resulting in a depletion of apolipoprotein C-II in plasma.—**Streicher, R., J. Geisel, C. Weisshaar, H. Avci, K. Oette, D. Müller-Wieland, and W. Krone.** A single nucleotide substitution in the promoter region of the apolipoprotein C-II gene identified in individuals with chylomicronemia. *J. Lipid Res.* 1996. **37:** 2599–2607.

Supplementary key words hypertriglyceridemia • lipolysis • transcription factor • BsaJI RFLP

Human plasma apolipoprotein (apo) C-II is required for activation of lipoprotein lipase, the major enzyme involved in the hydrolysis of triglycerides of chylomicrons and very low density lipoproteins (VLDL) (1, 2). Molecular defects in the coding regions of the genes for lipoprotein lipase and apoC-II result in a markedly decreased catabolism of chylomicrons and VLDL particles (3). The biochemical findings in homozygous affected individuals include severe fasting hypertriglyceridemia and chylomicronemia. Clinically these patients are often detected by recurrent episodes of abdominal pain and pancreatitis. Heterozygous carriers are in most cases normolipidemic. The diagnosis of the lipoprotein lipase deficiency can be confirmed by measuring the postheparin enzyme activity of plasma in vitro (3, 4). The apoC-II deficiency can be diagnosed by quantification of the protein mass, by isoelectric focusing to detect functional inactive variants, or by functional assays in which the capacity of apoC-II to restore lipoprotein lipase activity to normal is measured (3, 4). Patients with significantly reduced activity of lipoprotein lipase or apoC-II must follow a strict low fat diet to prevent further clinical events, especially attacks of pancreatitis.

The human apoC-II gene is approximately 3,4 kbp in size with four exons that encode a mature protein of 79 amino acids (5–7). Several mutations in the apoC-II gene have been described that alter the transcription in such a way that a premature stop codon was found, an abnormal splicing site was created, or the amino acid sequence was changed by single nucleotide substitu-

Abbreviations: apoC-II, apolipoprotein C-II; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; PCR, polymerase chain reaction.

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TABLE 1. Biochemical and clinical characteristics of the six subjects of the P.S. family

Subject	Age	Sex	Chylo	TG	TC	VLDL	LDL	HDL	Apo E Genotype	Comments
	yr					*mg/dl*				
P.S.	42	f	+++	1670	198	174	13	11	3/3	pancreatitis
P.A.	47	m		67	163	8	109	46	3/4	
P.V.	22	f		150	148	24	87	37	3/4	
P.C.	24	f		151	195	12	116	67	3/4	
P.M.	16	m	++	2995	204	190	3	11	3/3	
G.V.	44	m		229	202	37	135	30	3/4	

Chylo, chylomicrons; TG, triglycerides; TC, total cholesterol; VLDL, very low density lipoprotein cholesterol plus chylomicron cholesterol in the case of P.S. and P.M.; LDL, low density lipoprotein cholesterol; HDL, high density lipoprotein cholesterol; f, female; m, male.

tions or small deletions. The mutations resulted in most cases in a deficiency of plasma apoC-II (8–15) or, in two cases, in a functionally inactive protein (16, 17). In the present study, we describe two related individuals with massive hypertriglyceridemia and chylomicronemia. The marked decrease of apoC-II in plasma indicates that the hypertriglyceridemia was related to an apoC-II deficiency. Sequence analysis revealed a single nucleotide substitution upstream of the transcription initiation site. Gel electrophoretic mobility shift assays with nuclear proteins and promoter reporter gene assays with wildtype and mutated promoter fragments showed that the efficiency of the apoC-II promoter was reduced by the described point mutation. This is the first case of dramatically reduced quantities of apoC-II in plasma without a mutation in the transcribed part of the gene.

PATIENTS AND METHODS

Patients

The proband was a 42-year-old Greek female who was found to have massive hypertriglyceridemia with chylomicronemia. Two episodes of acute pancreatitis were reported by the patient at ages 39 and 42. In addition to the proband, the spouse, three children, and her brother were investigated. Massive hypertriglyceridemia with chylomicronemia was also present in one of the children in whom abdominal pain and pancreatitis were absent until age 16. From the family history we know that the proband and the spouse are related. Their grandparents were brother and sister (see Fig. 1). The family of the proband and her spouse originated from Baluri, a small village 120 km from Alexandroupolis in the area of the triangle formed by Greece, Bulgaria, and Turkey. The biochemical data of the persons investigated are given in detail in **Table 1.** In addition, 46 unrelated patients with hypertriglyceridemia living in the Cologne area were investigated.

Plasma lipids, lipoproteins, and apolipoproteins

Blood samples were obtained after an overnight fast. Cholesterol and triglycerides were determined in plasma and lipoproteins by enzymatic assays (Boehringer, Mannheim, Germany). VLDL was separated by preparative ultracentrifugation (18). HDL cholesterol was determined in the VLDL-free infranatant after precipitation of LDL by phosphotungstic acid–magnesium chloride (Boehringer, Mannheim, Germany). The apolipoproteins A-I and B were measured by nephelometric methods (Behring, Marburg, Germany). ApoC-II was quantified by radial immunodiffusion (Daiichi, Tokyo, Japan).

Isoelectric focusing of apo-VLDL and immunoblotting

For analytical isoelectric focusing, VLDL was isolated by ultracentrifugation and delipidated with ethanol–diethylether 3:1 (v/v). The screening for apoCs was performed in an immobilized pH gradient, 4.5–5.5, that contained 7 M urea, corresponding to a pH gradient 4.0–5.0 in water. After isoelectric focusing the gel was stained with Coomassie Brilliant Blue R-250. Electroblotting onto a nylon membrane was performed after electrophoresis for immuno detection. To characterize the apoC-II protein, a goat anti-apoC-II antibody (Paesel & Lorei, Frankfurt, Germany) was used as the first antibody in a dilution of 1:25000. For the detection a biotin-labeled anti-goat sheep antibody and the enzyme peroxidase were used. The procedure of isoelectric focusing and the immunoblotting were previously described in detail (19, 20).

DNA sequencing

The sequencing reaction was carried out with the T7 DNA polymerase (Pharmacia, Freiburg, Germany) and 3′-(α-^{35}S)-dATP. Each exon was sequenced separately. The primers annealed to intron sequences including the splicing sites. For the exons 1, 2, and 3, single-stranded DNA was produced by asymmetric PCR. For

exon 4, the downstream was 5′ biotinylated and the separation of the single-stranded templates was achieved with a magnetic system (Dynal, Hamburg, Germany). The sequences of the primers used were as follows: exon 1: 5′CGGAGGCGAATTCTCAGAGTG 3′ and 5′TACAG TTCTAAATTCTGTGATTTG 3′; exon 2: 5′CAAGTC CATGCATGGGAAACTTGAC 3′ and 5′GAGAGTGTG TCAGGAGAGCCCGTGG 3′; exon 3: 5′ TCCAAGCA TCTTCCCAGCCCAGGCC 3′ and 5′ CTGGGTCCTG GATGCAGTCGGTG 3′, exon 4: 5′ CTGGATCCAGG ACCCAGAAGTTC 3′ and 5′ TAAATGTTGGCTGGAC TGAGTCAG 3′. The PCR was subjected to 35 cycles of a denaturing step for 1 min at 96°C, different annealing temperatures for 1 min (exon 1: 55°C, exon 2,3 and 4: 62°C) and an extension step at 72°C (exon 1 + 2: 1 min; exon 3 + 4: 2 min). In the 100 μl PCR reaction mixture, different primers quantities were used (exon 1: 2,5 pmol/25 pmol; exon 2: 3 pmol/30 pmol; exon 3: 1 pmol/10 pmol; and exon 4: 20 pmol). In addition to the buffer recommended by the manufacturer of the DNA polymerase (Amersham, Braunschweig, Germany), different volumes of formamide (exon 1 and 4: 3% (v/v), exon 3: 5% (v/v)) were used.

Restriction enzyme analysis for the detection of the mutation

The A→G mutation in position −86 introduced an additional BsaJI restriction site. The polymorphism was determined by digestion of amplified DNA. The upstream primer was identical to the primer used for sequencing; the downstream primer has the following sequence: 5′ TAAAATTCCTGCTTTATCGATC 3′. The amplified DNA was incubated with 5 units of BsaJI at 37°C in buffer recommended by the manufacturer (Boehringer, Mannheim, Germany). DNA fragments were separated on a 6 g/dl agarose gel and stained with ethidium bromide.

Gel electrophoretic mobility shift assays

For nuclear protein–DNA binding reactions, two double-stranded oligonucleotides comprising positions −101 to −71 from the position of transcription start were used. The two oligonucleotides differed in the position of the mutation (wildtype allele: A in position −86; mutant allele: G in position −86). Nuclear protein extracts were prepared from HepG2 cells (21). The reactions were carried out in a volume of 20 μl containing 15 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM DTT, 10% (w/v) glycerol, 0.5 μg Poly-dIdC, and 10 fmol of end-labeled DNA with 5 μg protein extract. The samples were incubated at 20°C for 30 min and immediately electrophoresed on a native 5% (w/v) polyacrylamide gel. For competition experiments, conditions were as above except that appropriate competitor DNA (as indicated in Fig. 3) was included in the reaction mixture prior to addition of the extract.

Promoter reporter gene constructs

DNA fragments corresponding to the promoter region of the apoC-II gene (position −170 to −1 relative to the transcription start) were amplified by PCR from human genomic DNA of the proband (P.S.) and a normolipidemic individual without the A→G mutation using the primers 5′ CCGGACGGGCACAGAGAGGATT TAT 3′ and 5′ AGAAGGTTCCCTGTGACGTGACCTT 3′. The PCR product was subcloned as a blunt ended fragment into the SmaI site of pUC20 (pUC/c2w and pUC/ps) with the 5′ end of the insert adjacent to the SacI site and sequenced. The deletion construct (position −91 to −58) of the apoC-II promoter was achieved by amplification of pUC/c2w with the apoC-II specific primers ($^{-92}$CCAACAGAACTGCGGAGGGGGT^{-113}) and ($^{-57}$TGGCTGTGGAGCGGAAGTGGG^{-37}) and religating of the resulting fragments. The new construct was named pUC/c2-Del. The inserts from these parental clones were subcloned into the pGL2Basic vector by HindIII and SacI digestion to generate pC2-W, pC2-PS, and pC2-Del.

Cell cultures and transfection

HepG2 cells were grown in RPMI medium (Gibco, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS, Gibco). For transfection experiments 2×10^5 cells were seeded in 35-mm dishes 1 day before. Transfections were performed in triplicate with DNA constructs and pSVβGal (Promega, Heidelberg, Germany), 0.8 μg each and 5 μl of lipid (Transfectam, Promega) in medium without serum. After 4 h the transfection medium was changed to normal medium. The cells were cultured for a further 24 h and then harvested. Luciferase activity was measured by the luciferase assay system with reporter lysis buffer (Promega) following the manufacturer's instructions. Cotransfection with the pSVβGal vector allowed correction for transfection efficiency. The β-galactosidase activity was determined by a colorimetric assay (22).

RESULTS

Figure 1 presents the pedigree of a family in which the proposita (P.S.) and her son (P.M.) are affected by massive hypertriglyceridemia with chylomicronemia. Upon exploring the ancient pedigree it became obvious that the proposita and her spouse had a common ancestry. Their grandparents were brother and sister from a family that originated in Greece. At present the propos-

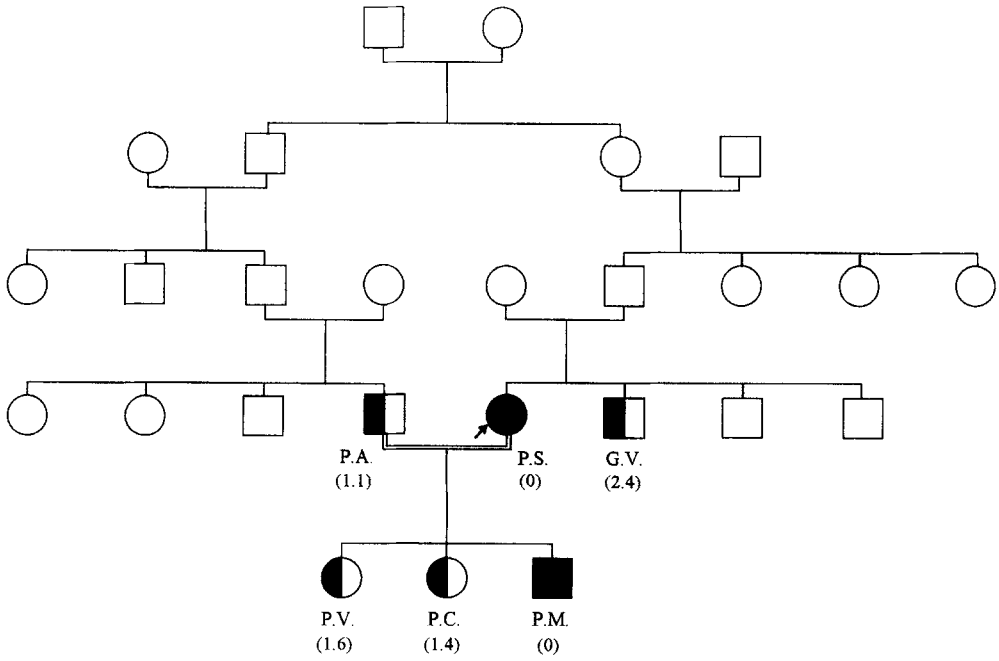

Fig. 1. Pedigree of the P.S. family. The arrow indicates the proposita; ● ■ indicate massive hypertriglyceridemia with chylomicronemia and the homozygous presence of the A→G transition in position −86; ◐ ◧ indicate the heterozygous presence of the A→G transition in position −86. The apoC-II levels in plasma (in parentheses) are shown under the symbols (reference value 2.2–4.7 mg/dl). Data were available only from members of the Greek kindred who are now living in the Cologne area.

ita lives with her family and her brother in the Cologne area. The levels of plasma lipids, lipoproteins, and apolipoprotein E genotype were analyzed in these individuals and are listed in Table 1. Radial immunodiffusion was carried out to clarify whether there exists an apoC-II depletion in plasma. In neither the proposita nor her son were measurable amounts of apoC-II detected (Fig. 1). Although apoC-II was not found in the plasma on radial immunodiffusion, the apolipoproteins of the VLDL fraction were separated by isoelectric focusing. After staining with Coomassie Brilliant Blue R-250, trace amounts of a protein in the position of apoC-II were detected in the proposita and her son, while the other members of the family had a distinct protein band **(Fig. 2).** By immunoblotting it could be confirmed that in all cases apoC-II had focused in the regular position. To quantify the relative amount of apoC-II in the VLDL fraction, the ratio of apoC-II to apoC-III was analyzed. For this purpose the apolipoproteins C-$II_{1/2}$ and C-$III_{0/1/2}$ were stained with Coomassie Brilliant Blue R-250 and quantified by densitometry (20). The proposita and her son had the expected marked decrease in the ratio (Fig. 2). The other members of the family showed, in comparison to a control, a lower apoC-II/C-III ratio. This suggests that these individuals are heterozygous carriers of a deficient allele.

To clarify the cause of the apoC-II deficiency, DNA from the proposita and her son was sequenced. For DNA sequencing each exon was amplified separately. However, no differences were observed in any of the exons or the neighboring intron sequences from the published sequence of Das et al. (5). This raises questions to the cause of the observed apoC-II deficiency. As a possible location of the mutation we sequenced the promoter region upstream from exon 1. There an A to G transition was found 86 bp upstream from the major site of transcription initiation (according to the sequence of Das et al., 5). Both the proposita and her son were homozygous for this mutation. The mutation can easily be detected because the A→G change creates a site for the enzyme BsaJI at position −91. A PCR-based method was developed to show the additional BsaJI site. The undigested DNA fragment had a length of 315 bp. A second invariant BsaJI site is present at position −149. In the case of the mutation, the resulting fragments were 63 bp, 58 bp, and 194 bp in length (wildtype gene: 63 bp and 252 bp). In addition to these two BsaJI restriction sites, a second variable BsaJI site was found in this fragment. Das et al. (5) and Fojo et al. (7) reported a C at position −105. On the other hand, Wei et al. (6) reported that G was present at that position. If G was present, the second variable BsaJI site would be created.

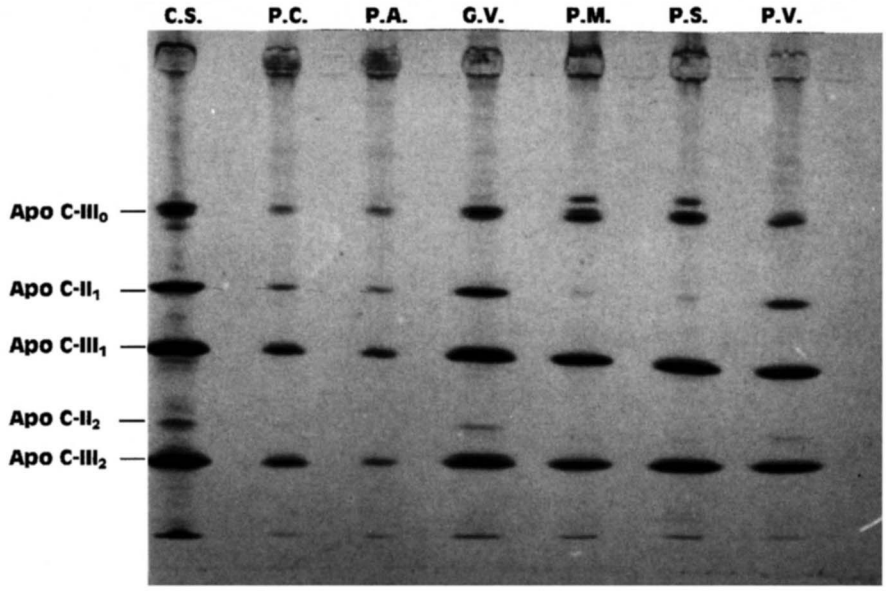

Fig. 2. Isoelectric focusing of apoCs from delipidated VLDL. After electrophoresis, pH gradient 4.5–5.5 in 7 M urea, the gel was stained with Coomassie Brilliant Blue R-250 and the apolipoproteins C-$II_{1/2}$ and C-$III_{0/1/2}$ were determined by densitometry. The total area under the curve was equivalent to the 100% value. The percentage of apoC-II_1 and the ratios apolipoprotein C-$II_{1/2}$/C-$III_{1/2/3}$ were calculated in all members of the family: P.S.: 0.8% (0.011); P.M.: 1.0% (0.012); P.A.: 10.7% (0.135); P.V.: 10.3% (0.137); P.C.: 8.8% (0.102); G.V.: 21.4% (0.287); control subject (C.S.): 29.5% (0.439).

The 58 bp fragment, in the case of the A→G mutation in position −86, and the 252 bp fragment, in the case of the wildtype allele, were shortened by 40 bp, respectively. The proposita and her son were homozygous for the mutation in position −86, while the other family members were heterozygous. In addition to the family studied, 46 unrelated patients with type IV hyperlipoproteinemia were examined for the presence of this mutation by restriction fragment length analysis; however, outside the family this mutation was not found. In the family two different alleles were found. The G in position −86 was associated with the C in position −105, while the A in position −86 was combined with the G in position −105. The frequency of the second variable BsaJI site was determined in the 46 unrelated individuals with type IV hyperlipoproteinemia. For the second variable BsaJI site, a high heterozygosity was found: 21 of the 46 patients were heterozygous, 14 were homozygous for the C, and 11 patients showed only the G. Beside these nucleotides substitutions creating additional BsaJI sites, a third nucleotide exchange according to the sequence of ref. 5 was found for which the proposita and her son were homozygous. In position −20 the T was changed to G. This nucleotide substitution was previously described in the DNA sequence of Wei et al. (6).

To clarify whether the mutation in position −86 had consequences in binding of a transcription factor, gel electrophoretic mobility shift assays were performed. Radiolabeled oligonucleotides comprising the positions −101 to −71 were incubated with nuclear extracts from HepG2 cells. In the case of the wildtype allele a single retarded DNA–protein complex with high intensity was detected (**Fig. 3**). In the case of the mutant oligonucleotide, the signal of the DNA–protein complex was markedly decreased. In competition experiments, unlabeled wildtype oligonucleotides or mutant oligonucleotides were incubated with radiolabeled wildtype DNA. Using the wildtype oligonucleotide as competitor in an excess of 50-fold and 100-fold, the radioactive signal was clearly diminished, whereas in these concentrations the mutant oligonucleotide failed to compete with the radiolabeled wildtype DNA (Fig. 3). The marked differences in protein binding of the wildtype and mutant allele and the results of the competition experiments indicate that the substitution of one nucleotide in the promoter region clearly diminished the binding of a protein.

A functional promoter reporter gene assay was performed to investigate the relevance of the point mutation at −86 and the changed nuclear protein binding pattern for reduced apoC-II promoter activity. Wildtype and mutated fragments representing the basal apoC-II promoter region from −1 to −170 were cloned to the luciferase gene as a reporter. The different ability of these fragments to direct transcription of the reporter gene was analyzed in HepG2 cells by measuring lucifer-

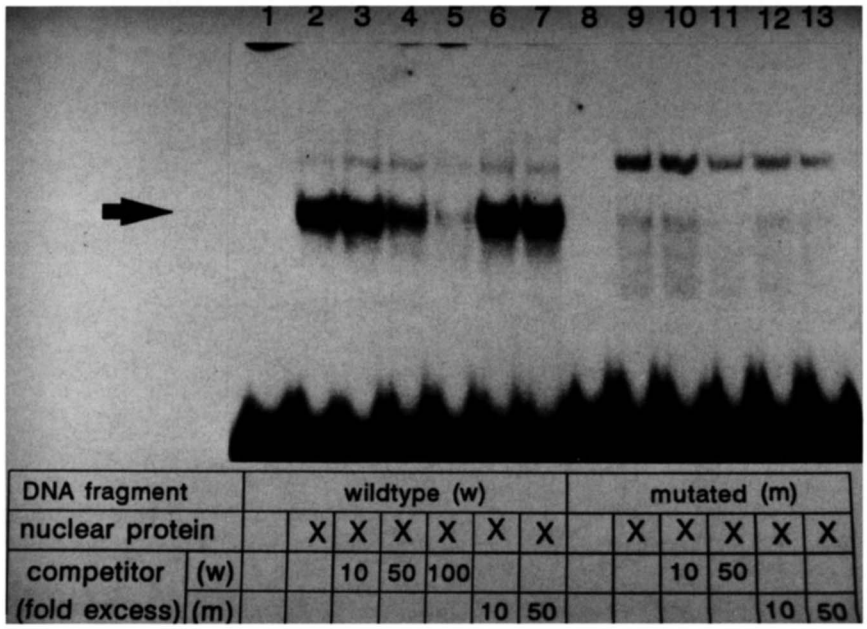

Fig. 3. Electrophoretic mobility shift assay analysis of HepG2 nuclear protein binding to the mutated and wildtype promoter region of apoC-II. Radiolabeled wildtype (w) or mutated (m) double-stranded oligonucleotides (corresponding to nucleotides −101 to −71) enclosed the mutation at position −86. The oligonucleotides were incubated in the absence (lanes 1 and 8) and in the presence of nuclear extracts (lanes 2 and 9). Additionally, competition experiments with a 10-, 50-, and 100-fold excess of nonradiolabeled oligonucleotides were performed. Each radiolabeled oligonucleotide competed with the unlabeled wildtype and mutated oligonucleotide (lanes 3–7 and 10–12).

ase activity after transient transfection with these constructs (**Fig. 4**). Transfection of HepG2 cells with construct pC2-W (wildtype) resulted in a 30-fold increase in luciferase activity compared to cells transfected with the vector without the promoter as a control. Introduction of the point mutation at position −86 (pc2-PS) and a deletion mutation from bases −91 to −58 reduces reporter gene expression to 46% and 25%, respectively. The promoter activity seems to be significantly reduced by both mutations implicating the location of an important *cis*-regulatory element in this region. Data base analyses to identify this element and its binding protein

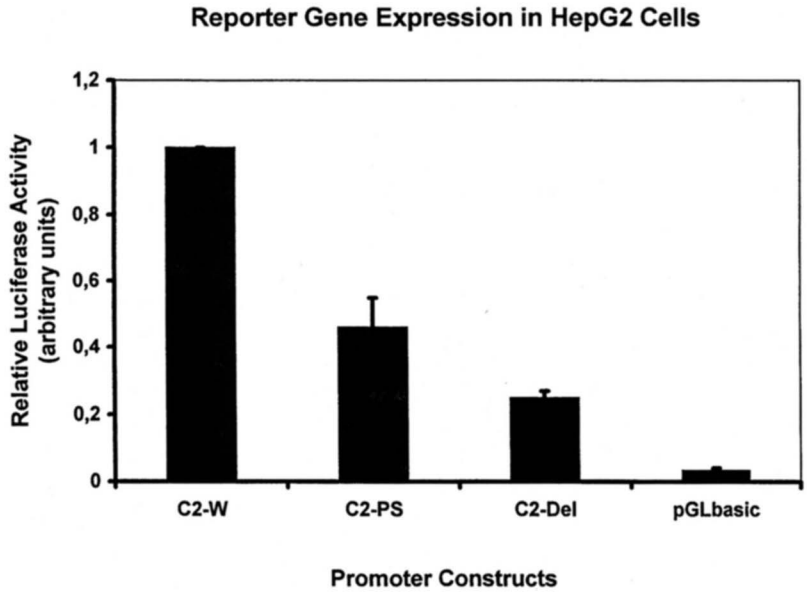

Fig. 4. ApoC-II promoter reporter gene expression in HepG2 cells. HepG2 cells were transiently transfected with the promoter reporter gene constructs. Expression of the luciferase reporter gene was measured under control of the wildtype basal apoC-II promoter (pC2-W), the promoter with the mutation at position −86 (pC2-PS), a deletion promoter between position −91 to −58 (pC2-Del), and without promoter (pGL2Basic). Bars represent means of luciferase activities relative to construct pC2-W ± S.D. of 4–6 separate experiments, each performed in triplicate. Cotransfection with the pSVβGal vector allowed standardization of transfection efficiency.

remain unsuccessful because we found no homology to any known element. The gel shift analyses and the promoter reporter gene assays are functional experiments that strengthen the hypothesis that the mutation at −86 causes a reduced apoC-II expression.

DISCUSSION

In the present study we describe a family in which the mother and her son had massive hypertriglyceridemia with chylomicronemia. The mother suffered recurrent pancreatitis. The quantification of apoC-II by radial immunodiffusion revealed no measurable levels. The analysis of the apoCs by isoelectric focusing showed trace amounts of apoC-II in the regular position. The level in plasma and the position on isoelectric focusing probably reflect the basis of the molecular defect. The data are more consistent with a DNA mutation that interferes with the normal transcription and/or processing of the mRNA in the liver than with a mutation that alters the protein structure. To date, most of the apoC-II mutations identified have been single point mutations resulting in premature stop codons (11, 12, 14) or frameshift mutations (8, 13, 16, 17). In these cases no detectable apoC-II was found in plasma. The only example of a mutation with markedly reduced levels of an intact apoC-II is the apoC-II$_{Hamburg}$ (9) in which a single base substitution at the first base of intron 2 resulted in a defective splicing, whereby a small amount of correctly spliced RNA was detected. The apoC-II$_{Hamburg}$ mutation can easily be identified by gene amplification and restriction enzyme digestion with HphI (9). Using this restriction fragment length polymorphism the apoC-II$_{Hamburg}$ mutation could be excluded in the family studied.

In order to find the molecular basis of the markedly reduced apoC-II level, the exons and the neighboring intron sequences of the proposita's DNA were sequenced. The DNA sequence was compared with those of Das et al. (5). However, neither in the coding region nor in the neighboring intron sequences was any difference to the published sequence found. This raises questions to the pathophysiological cause of the apoC-II reduction. The transcription of genes is controlled by the interaction of promoter regulatory elements with specific nuclear proteins. Possibly this interaction may be disturbed which resulted in a markedly reduced amount of apoC-II in plasma. However, until now, positive or negative regulatory factors are not described. To clarify whether there exists a mutation with regulatory consequences, the DNA upstream of the transcription initiation site was sequenced.

Three single nucleotide substitutions upstream of the transcription initiation site were detected according to the sequence of Das et al. (5). The first nucleotide exchange (C→G) was localized 105 bp upstream of the transcription initiation site. The G has already been described in this position in a previous publication (6). The nucleotide substitution resulted in the creation of a new BsaJI restriction site. By this restriction fragment length polymorphism the nucleotide exchange could easily be detected. To evaluate the clinical consequence of this nucleotide substitution, additional individuals with hypertriglyceridemia were examined. The investigation of a further 46 unrelated individuals revealed a high heterozygosity in this position. Therefore, a functional consequence of this variable nucleotide is improbable. But, in addition, the observed heterozygosity of the BsaJI polymorphism makes it useful in cosegregation analysis.

The second nucleotide exchange (A→G) was localized 86 bp upstream of the transcription initiation site. The proposita was homozygous for the G in this position. According to other authors this nucleotide exchange has never been previously described. The nucleotide substitution created a new BsaJI site that permitted the rapid identification of this mutation in individuals. In the family studied, the proposita and her son were homozygous for the A to G substitution, while the other family members were heterozygous carriers of the mutation. The additional screening of 46 unrelated individuals with type IV hyperlipoproteinemia revealed no evidence of this single nucleotide substitution. The homozygous presence of this mutation in the two family members with massive hypertriglyceridemia and chylomicronemia, the heterozygous status of the other family members, and the absence of the mutation outside the family suggest that the A to G exchange in position −86 is not a common polymorphism, but is more likely to be a rare mutation causing hypertriglyceridemia with chylomicronemia. The family lives now in the Cologne area, but the proposita, her brother, and husband immigrated from Greece where they all lived in the small village of Baluri, 120 km from Alexandroupolis. The presence of the mutation in the proposita and her husband indicates that they have a common ancestry. On exploring the ancient pedigree it became obvious that the grandparents of the proposita and her husband were brother and sister.

The third nucleotide exchange (T→G) was localized 20 bp upstream of the transcription initiation site. In a previous publication the G in this position has been described (6). Therefore it is probable that this variable site is more a polymorphism than functionally relevant. In the family studied, the proposita and her son were homozygous for the T in this position; but, in addition, the brother of the proposita had the same genotype.

The homozygous presence of this nucleotide substitution in individuals without massive hypertriglyceridemia is a further hint that this mutation has no functional influence.

To determine how the A→G nucleotide exchange in position −86 contributes to the diminished expression of the apoC-II gene, we characterized the binding of nuclear extracts to radiolabeled oligonucleotides enclosing the variable site. The mobility shift assays demonstrated the formation of a specific protein–DNA complex with the wildtype oligonucleotide. The oligonucleotide containing the mutation showed a marked decrease in protein binding. In competition experiments the mutated oligonucleotide was ineffective in competing with the wildtype allele. These experiments suggest that the part of the gene enclosing position −86 represents a positive regulatory *cis*-element, and that a single nucleotide substitution dramatically affects transcription factor binding and gene transcription. Our suggestion was confirmed by the result of promoter reporter gene assays. The basal apoC-II promoter region from positions −1 to −170 is sufficient to drive transcription of the luciferase gene as a reporter in HepG2 cells. Transfection experiments with the basal promoter region containing the point mutation at −86 or a deletion of bases −91 to −58 showed a significant reduction of reporter gene expression. This provides functional evidence that a positive regulatory *cis*-element is affected by this point mutation leading to reduced promoter function. We failed to identify the affected *cis*-element by sequence homology analysis, indicating that we found an unknown regulatory element. Further experiments are necessary to characterize this part of the gene in more detail and to investigate the effect of nucleotide substitutions independent of the identified mutation.

In summary, we have described a family in which massive hypertriglyceridemia with chylomicronemia is associated with markedly reduced levels of apoC-II. Sequencing of the exons and neighboring intron sequences revealed a nucleotide substitution 86 bp upstream of the transcription initiation site. Mobility shift experiments with nuclear extracts and promoter reporter gene assays suggest that this part of the gene is necessary for maximal transcription of the apoC-II gene. This is the first case of an apoC-II mutation in a relevant promoter region which might contribute to the pathophysiological state observed in the patient.

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