

Regulation of Apolipoprotein M Gene Expression by MODY3 Gene Hepatocyte Nuclear Factor-1 α

Haploinsufficiency Is Associated With Reduced Serum Apolipoprotein M Levels

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Hepatocyte nuclear factor-1 α (HNF-1 α) is a transcription factor that plays an important role in regulation of gene expression in pancreatic β -cells, intestine, kidney, and liver. Heterozygous mutations in the HNF-1 α gene are responsible for maturity-onset diabetes of the young (MODY3), which is characterized by pancreatic β -cell-deficient insulin secretion. HNF-1 α is a major transcriptional regulator of many genes expressed in the liver. However, no liver defect has been identified in individuals with HNF-1 α mutations. In this study, we show that Hnf-1 α is a potent transcriptional activator of the gene encoding apolipoprotein M (apoM), a lipoprotein that is associated with the HDL particle. Mutant Hnf-1 α ^{-/-} mice completely lack expression of apoM in the liver and the kidney. Serum apoM levels in Hnf-1 α ^{+/-} mice are reduced ~50% compared with wild-type animals and are absent in the HDL and HDLc fractions of Hnf-1 α ^{-/-}. We analyzed the apoM promoter and identified a conserved HNF-1 binding site. We show that Hnf-1 α is a potent activator of the apoM promoter, that a specific mutation in the HNF-1 binding site abolished transcriptional activation of the apoM gene, and that Hnf-1 α protein can bind to the Hnf-1 binding site of the apoM promoter in vitro. To investigate whether patients with mutations in HNF-1 α (MODY3) have reduced serum apoM levels, we measured apoM levels in the serum of nine HNF-1 α /MODY3 patients, nine normal matched control subjects (HNF-1 α ^{+/+}), and nine HNF-4 α /MODY1 subjects. Serum levels of apoM were decreased in HNF-1 α /MODY3 subjects when compared with control subjects ($P < 0.02$) as well as with HNF-4 α /MODY1 subjects, indicating that HNF-1 α haploinsufficiency rather than hyperglycemia is the primary cause of decreased serum apoM protein concentrations. This study demonstrates that HNF-1 α is required for apoM expression in vivo and that heterozygous HNF-1 α

mutations lead to an HNF-1 α -dependent impairment of apoM expression. ApoM levels may be a useful serum marker for the identification of MODY3 patients. *Diabetes* 52:2989–2995, 2003

Maturity-onset diabetes of the young (MODY), a genetically heterogeneous monogenic disorder responsible for 2–5% of type 2 diabetes, is characterized by an autosomal-dominant inheritance, an early age of onset (usually before 25 years), and abnormal pancreatic β -cell function (1–3). Heterozygous mutations in the glycolytic enzyme glucokinase/MODY2, as well as in five different transcription factors including hepatocyte nuclear factor (HNF)-4 α /MODY1, HNF-1 α /MODY3, insulin promoter factor IPF-1 (PDX-1)/MODY4, HNF-1 β /MODY5, and NeuroD1/MODY6, have been shown to be responsible for MODY (4–9). Recently, it has been suggested that a mutation in the sulfonylurea receptor gene of the ATP-sensitive potassium channel may be associated with a seventh type of MODY (10).

The MODY3 gene is encoded by HNF-1 α , a transcription factor that belongs to the helix-loop-helix homeodomain transcription factor family. It was first identified by its interaction with *cis* regulatory sequences of liver-specific gene promoters (11). HNF-1 α plays a critical role in development, cell differentiation, and metabolism and is essential for the normal functioning of liver, intestine, kidney, and pancreatic β -cells (12).

Mutations in the HNF-1 α gene account for 20–65% of all MODY subtypes. More than 120 different HNF-1 α mutations have been found to cosegregate with diabetes in U.K., German, French, Danish, Italian, Finnish, North American, Japanese, Chinese, and African families (1–3). They include missense, nonsense, deletion, insertion, and frameshift mutations. Most HNF-1 α mutations can be predicted to result in loss of function. However, mutant HNF-1 α proteins with an intact dimerization domain may impair pancreatic β -cell function by forming nonproductive dimers with wild-type protein, thereby exhibiting dominant negative activity. This mechanism has been shown for frameshift mutation HNF1 α -P291fsinsC (13).

Clinical studies have shown that HNF-1 α mutations are associated with impaired pancreatic β -cell function characterized by abnormal insulin secretion (14,15). Because

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apoM, apolipoprotein M; EMSA, electrophoretic mobility shift assay; HNF, hepatocyte nuclear factor; MODY, maturity-onset diabetes of the young.

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HNF-1 α also plays major regulatory roles in other tissues, including the liver and the kidney, mutations in this gene could result in a pleiotropic phenotype such as impairment in cholesterol and lipoprotein metabolism in the liver. We have recently shown that mutant *Hnf-1 α* mice have high plasma cholesterol levels and that cholesterol resides predominantly in large, buoyant, HDL particles as a result of a pleiotropic defect in bile acid metabolism and reduced hepatic lipase activity (16). However, no liver-specific defect has been reported in humans with mutations in HNF-1 α .

In this study, we investigated the role of HNF-1 α on the expression of apolipoprotein M (apoM), a major component of the HDL particle, and studied the effect of HNF-1 α gene dosage reduction on the expression of apoM in vitro and in vivo. Our results indicate that *Hnf-1 α* is required for apoM expression and that decreased HNF-1 α activity in humans leads to low plasma apoM levels.

RESEARCH DESIGN AND METHODS

Animals. *Hnf-1 α* null mice and their heterozygous littermates were kept in a sterile micro isolator and maintained in the Laboratory of Animal Research Center, a pathogen-free animal facility at Rockefeller University (17). The animals were maintained on a 12-h light/dark cycle and fed a standard rodent diet.

Vectors. The apoM cDNA was amplified by PCR and cloned into pBS and expression vector pGEX-4T3 using primers GST apoM1 5'-CGGGATC-CCGAGCGTTCCACCAAGTTGGGAGCTCTG-3' and GST apoM2 5'-CCGCTCGAGCGGGTGAAGTCACTGGTCACTTGC-3'. The reporter plasmid pGL2-papoM was generated by cloning a 1,055-bp fragment of the mouse apoM promoter into pGL2-luciferase. The reporter plasmid pGL2papoMmut is identical to pGL2-papoM except for a mutated HNF-1 binding site at position -103 to -88 (GAAGGGCGTATTAGCA).

Recombinant apoM protein. The mouse apoM cDNA was cloned into the *Nde*/*Bam*HI sites of expression vector PET3a (Novagen), before transformation into *Escherichia coli* BL21(DE3). Protein expression was induced by 0.1 mmol/l isopropyl- β -D-thiogalactoside for 3 h at 37°C. Cells were harvested and washed in buffer A (50 mmol/l Tris, 100 mmol/l NaCl, 5% glycerol, 1 mmol/l DTT, 0.02% Triton X [pH 7.6]) and resuspended in buffer A before sonication. The sonicated cells were centrifuged at 11,000 rpm for 30 min. The pellet was washed three times in buffer A, once with 0.5 mol/l guanidine HCl, and twice with 1 mol/l guanidine HCl. The protein was eluted from the inclusion bodies in 3 mol/l guanidine HCl solution and centrifuged for 2 h at 40,000 rpm. The supernatant containing the recombinant apoM protein was dialyzed against dialysis buffer (0.1 mol/l NaHCO₃, 0.5 mol/l NaCl [pH 8.5]) for 12–16 h.

Antibodies. Two 20-amino acid peptides (apoM113–132: TEGRPDMKTELFSSSCPGGI and apoM140–159: GYQRFLLYNRSPHPPEKCV) were synthesized and processed to >90% purity, conjugated to KLH, and used for immunization of rabbits (Bethyl Laboratories, Montgomery, TX). Antisera were affinity purified and tested by enzyme-linked immunosorbent assay and Western blotting. Affinity-purified antiserum apoM140–159 was used for all studies.

Western blotting. Total protein extracts (10 μ g) of livers and kidney from wild-type, heterozygous, and null *Hnf-1 α* mice were separated by SDS-PAGE (12.5%) and transferred onto a nitrocellulose membrane (Schleicher & Schuell) by electroblotting (30 min, 15 V). ApoM protein was detected with anti-apoM140–159 antiserum (1:1,000) and goat anti-rabbit IgGs conjugated to horseradish peroxidase (1:10,000) in Tris-buffered saline supplemented with 5% nonfat dry milk. For visualization, the Renaissance chemoluminescence substrate (NEN) was used.

RT-PCR. Semiquantitative RT-PCR was performed as described previously (19). PCR synthesis for each primer pair was quantified at 15, 20, 25, and 30 cycles in a test reaction to ensure that the quantitative PCR amplification was in the linear range. The primer sequences apoM-F 5'-GTGCCCCGGAAGTGACATACC-3' and apoM-R 5'-AGCGGGCAGGCCTCTTGATTC-3' amplify a 275-bp fragment and were used for semiquantitative RT-PCR analysis.

Electrophoretic mobility shift analysis. Electrophoretic mobility shift assay (EMSA) analysis was performed as described previously (16). Nuclear cell extracts were incubated with ³²P-labeled ds-stranded oligonucleotide probes containing the wild-type or mutant HNF-1 binding sites in the apoM promoter (sequences: 5'-AGGTTGAAGTTACTTATTAGCAGGT-3' and 5'-AGGTTGAAGGGGCTTATTAGCAGGT-3', respectively). Competition analysis

was performed using a probe with the HNF-1 site in the insulin growth factor binding protein (sequence: 5'-ACTGGTTAATGATTGGCATGC-3') (15). Super-shifts were carried out with an anti-HNF-1 α antibody (Geneka Biotechnology, Montreal, Canada).

Nuclear extract preparation. Livers were dounce homogenized 15 times in 100:1 (vol:vol) of buffer A (10 mmol/l KCl, 1.5 mmol/l MgCl₂, 10 mmol/l HEPES [pH 7.9], 1 mmol/l DTT, 1 mmol/l NaVO₄, 1 \times complete TM protease inhibitor [Boehringer Mannheim]). After centrifugation at 2,000g for 10 min at 4°C, the pellet was resuspended in 4 vol of the same buffer, dounced 10 times, and centrifuged again at 2,000g. The nuclei-containing pellet was resuspended in 2 vol of buffer B (420 mmol/l NaCl, 10 mmol/l KCl, 20 mmol/l HEPES [pH 7.9], 20% glycerol, 1 mmol/l DTT, 1 mmol/l NaVO₄, 1 \times complete TM protease inhibitor) and extracted for 30 min at 4°C on a shaking rotor. After centrifugation at 16,000g, the supernatant was diluted 10-fold in buffer C (10 mmol/l KCl, 20 mmol/l HEPES [pH 7.9], 20% glycerol, 1 mmol/l DTT, 1 mmol/l NaVO₄, 1 \times complete TM protease inhibitor) and centrifuged for 15 min at 16,000g. The supernatant was loaded onto a Biomax-50 filter unit (Millipore) and centrifuged for 2 h at 4,000g at 4°C.

Tissue culture, transient transfections, and luciferase assay. HepG2 cells were grown on Dulbecco's modified Eagle's medium supplemented with 15% FCS. A modified calcium phosphate precipitation procedure was used for transient transfections, as described (18). A total of 0.5 ml of the precipitate, containing 1 μ g of CMV-LacZ, 0.5 μ g of luciferase reporter plasmid, and the indicated amount of expression vectors and carrier DNA (up to 10 μ g), was added per 60-mm dish. Luciferase was normalized for transfection efficiency by the corresponding β -galactosidase activity.

Subjects. Nine diabetic patients with previously identified mutations in the HNF-1 α gene (E129T, P291fsinsC, E132K, R159W, W267R) were identified from the U.K. MODY collection. HNF-4 α Q268X subjects of the RW pedigree were previously identified by sequencing analysis (5). Nine of these diabetic subjects with HNF-4 α mutations were included in the study. Control subjects were spouses of patients who attended the Exeter (U.K.) diabetes clinic. Study groups were matched for sex, age, and BMI, and diabetic groups were matched for HbA_{1c}. The clinical characteristics of the four study groups are shown in Table 1.

Apolipoprotein measurements. Plasma apoM levels were determined by immunoblotting using a standard control serum. Plasmas were diluted 1:100 in PBS, and 10 μ l of diluted plasma was analyzed on 12.5% denaturing acrylamide gels. After immunoblotting, apoM was detected using anti-apoM140–159 antibody (1:1,000) and goat anti-rabbit IgGs conjugated to horseradish peroxidase (1:10,000) in Tris-buffered saline supplemented with 5% nonfat dry milk. Bands were visualized using the Renaissance chemoluminescence substrate (NEN). A standard, consisting of an equal mixture of plasma of all normal subjects, was used in each blot as a reference. All samples were loaded in duplicate, and measurements were repeated twice. Band intensities on autoradiographs were analyzed by densitometry, and the mean value of two measurements was compared with the standard. Concentrations of apoM were determined with immunoturbidimetric assays from Roche Diagnostics using a Hitachi 917 autoanalyzer.

Statistical analysis. Results are given as mean \pm SE. The statistical analysis was performed using Microsoft Excel 98 statistical package. The *t* test was used to assess statistical significance, and the null hypothesis was rejected at the 0.05 level.

RESULTS

ApoM expression is absent in *Hnf-1 α* ^{-/-} mice. To identify genes that are regulated by *Hnf-1 α* , we studied gene expression profiles using oligonucleotide microarrays in livers of *Hnf-1 α* ^{-/-} mice and wild-type littermates. The analysis of this dataset (see www.nature.com/ng/journal/v27/n4/extref/ng0401-375-S1.pdf) revealed that apoM gene expression was ~25-fold increased in *Hnf-1 α* ^{+/+} compared with *Hnf-1 α* ^{-/-} mice. We analyzed the mRNA of apoM by Northern blotting in multiple tissues and found expression to be restricted to kidney and liver, a finding that is consistent with an earlier report (20) (Fig. 1A). Steady-state mRNA levels of apoM were essentially absent in liver and kidney of mutant *Hnf-1 α* mice (Fig. 1B). In contrast, the expression of apoA1 and apoC1, two lipoproteins that are major components of the HDL particle, was similar in mutant and wild-type mice. We also analyzed apoM protein levels in plasma of wild-type,

TABLE 1
Clinical characteristics of the study groups

	Genotype			P	
	HNF-1 α ^{+/+}	HNF-1 α ^{+/-}	HNF-4 α ^{+/-}	1 α ^{+/+} vs. 1 α ^{+/-}	1 α ^{+/-} vs. 4 α ^{+/-}
<i>n</i>	9	9	9	—	—
Sex (F:M)	7:2	7:2	5:4	—	—
Age (years)	49 ± 14	43 ± 15	38 ± 12	0.37	0.4
Height (cm)	169 ± 9	167 ± 10	165 ± 10	0.77	0.6
Weight (kg)	69 ± 11	69 ± 14	65 ± 9	0.99	0.6
BMI (kg/m ²)	24 ± 3	25 ± 3	24 ± 5	0.84	0.9
HbA _{1c}	5.5 ± 0.2	7.2 ± 3.9	7.2 ± 1.8	0.002*	1.0
Cholesterol (mmol/l)	5.7 ± 1.1	5.0 ± 0.9	4.8 ± 1.0	0.09	0.6
HDL cholesterol (mmol/l)	1.8 ± 0.5	1.5 ± 0.3	ND	0.3	ND
LDL cholesterol (mmol/l)	3.4 ± 1.1	2.9 ± 0.7	ND	0.28	ND
Triglycerides (mmol/l)	1.2 ± 0.7	1.6 ± 0.7	0.7 ± 0.2	0.84	0.04*

Data are mean ± SE. ND, not determined.

Hnf-1 α ^{+/-}, and *Hnf-1 α ^{-/-}* mice. Anti-apoM antibodies were generated against a peptide encoding amino acid residues 140–152 of the apoM protein, which are completely conserved between mouse and human. Immunoblot analysis of plasma of wild-type and mutant mice showed that apoM protein was absent in plasma of *Hnf-1 α ^{-/-}* mice and protein levels were reduced ~50% in *Hnf-1 α ^{+/-}* compared with wild-type littermates (133 ± 13 vs. 71 ± 2.6; *P* = 0.009; Fig. 2A). These data indicated that *Hnf-1 α ^{-/-}* mice have a specific defect in apoM gene expression and that gene dosage of *Hnf-1 α* correlates with apoM serum levels in mice.

We have previously shown that *Hnf-1 α ^{-/-}* mice exhibit hypercholesterolemia and that cholesterol accumulates

mainly in large buoyant HDLc particles (16). To show that the reduction in apoM expression is not secondary to hypercholesterolemia, we examined the apoM distribution in plasma lipoproteins of wild-type mice, in animals that lack the LDL receptor (*Ldlr^{-/-}*; *Ldlr^{-/-}* mice accumulate cholesterol predominantly in LDL particles), or in *Hnf-1 α ^{-/-}* (*Hnf-1 α ^{-/-}*) mice (21). Fasting plasma lipoprotein composition was analyzed after fast-protein liquid chromatography fractionation. Figure 2B shows that the cholesterol in the plasma of *Ldlr^{-/-}* mice was mainly carried in LDL particles but also present in VLDL and HDL fractions. In contrast, cholesterol in the plasma of *Hnf-1 α ^{-/-}* mice was almost exclusively carried by the HDL fraction and an abnormal fraction with intermediate buoyancy between HDL and LDL peaks. ApoM expression was only detected in the HDL peak of wild-type and *Ldlr^{-/-}* mice but absent from the HDL and HDLc fraction of *Hnf-1 α ^{-/-}* animals (Fig. 2C; data not shown), demonstrating that apoM is a component of the HDL particle and that apoM expression is independent of plasma cholesterol levels and defects in cholesterol metabolism.

Hnf-1 α is a transcriptional activator of the apoM gene. The in vivo data suggested that apoM expression is significantly reduced in the liver and the kidney of *Hnf-1 α ^{-/-}* mice compared with wild-type littermates, suggesting that *Hnf-1 α* might be a transcriptional activator of the apoM gene. We therefore analyzed the promoter regions of human and mouse apoM and identified a highly conserved putative HNF-1 binding site at position -88 to -103 (Fig. 3A). We cloned a 1,045-bp apoM promoter containing the putative HNF-1 binding site and cloned it upstream of the luciferase gene to generate plasmid pGL2-papoM. Cotransfection of this construct with an HNF-1 α expression vector in Cos7 cells (that do not express HNF-1 α) led to an ~28-fold dose-dependent activation of the apoM promoter (Fig. 3B). Transfection of pGL2-papoM into HepG2 cells that express endogenous HNF-1 α also led to an induction of luciferase expression. To test whether the HNF-1 binding site in the upstream regulatory region was responsible for HNF-1-dependent transactivation, we mutated this site by site-specific mutagenesis. The mutated promoter-construct pGL2-papoMmut was transfected into HepG2 cells, and luciferase activity was compared with cells that were transfected with pGL2-papoM. Luciferase

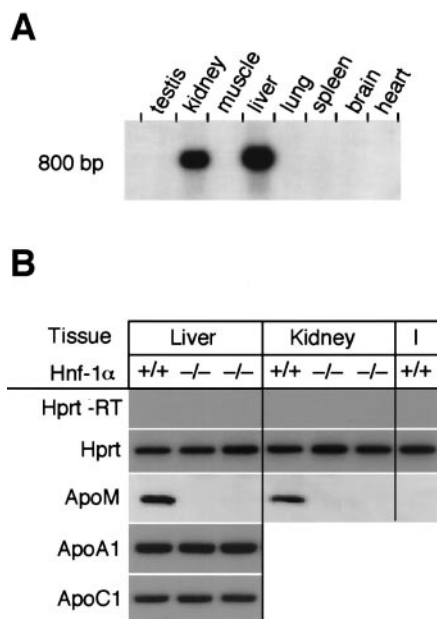
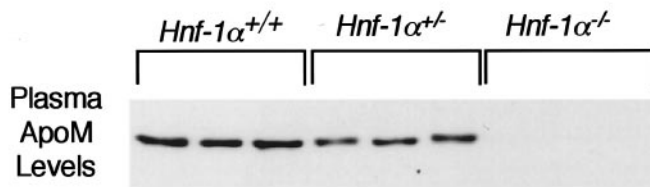
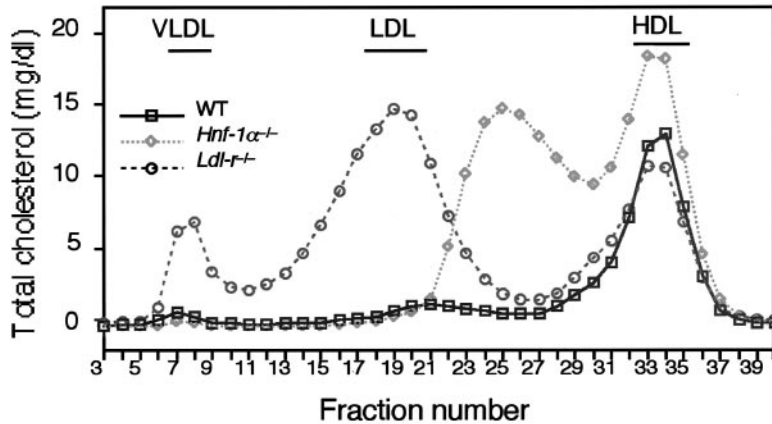


FIG. 1. A: ApoM is expressed in liver and kidney. A multiple-tissue Northern blot was hybridized with a full-length apoM cDNA probe. An ~0.8-kb band was identified in kidney and liver samples only. B: ApoM mRNA is absent in liver and kidney of *Hnf-1 α ^{-/-}* mice. Livers and kidneys of wild-type (+/+) and null (-/-) mice were assayed for hypoxanthine phosphoribosyltransferase (HPRT), apoM, apoA1, and apoC3 mRNA expression by RT-PCR. The HPRT levels show that each sample contained comparable amounts of cDNA. No product was amplified in the absence of reverse transcriptase (data not shown). I, intestine.

A



B



C

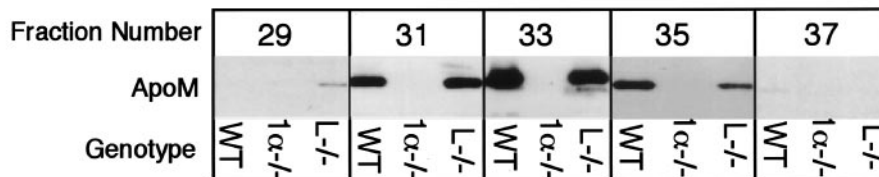


FIG. 2. A: Dose-dependent reduction of plasma apoM levels in wild-type (*Hnf-1 α ^{+/+}*), heterozygous (*Hnf-1 α ^{+/-}*), and null (*Hnf-1 α ^{-/-}*) *Hnf-1 α* mice. A total of 10 μ l of a 1:100 dilution of plasma was electrophoresed on a 12.5% denaturing polyacrylamide gel, transferred onto a nitrocellulose membrane, and hybridized with an anti-apoM antibody. B: Gel permeation of the major lipoprotein fractions from wild-type (black), null (red), and LDL receptor (*Ldlr^{-/-}*; green) mice. Samples (0.5 ml) were applied on a fast-protein liquid chromatography gel filtration column, which was run with PBS as eluent. The fractions (0.5 ml) of the eluate were assayed for total cholesterol. C: ApoM is exclusively located in the HDL particle of wild-type (wt) and *Ldlr*-deficient mice. Measurements of apoM levels in fractions 29–37 of wt, *Hnf-1 α ^{-/-}* (*1 α ^{-/-}*), and *Ldlr^{-/-}* (*L^{-/-}*) mice using immunoblot analysis. No apoM could be detected in fractions 3–28 in plasma of wt, *Hnf-1 α ^{-/-}*, and *Ldlr^{-/-}* mice (data not shown).

gene expression was completely abolished in cells that contained pGL2-papoMmut, indicating that this site is required for transcriptional activation of the apoM gene in vitro (Fig. 3C).

EMSA were performed to investigate whether HNF-1 α can bind to the putative HNF-1 binding site in the apoM promoter. Nuclear extracts were prepared from livers of wild-type mice, incubated with a ³²P-labeled oligonucleotide that contained the HNF-1 binding site, and subjected to electrophoretic shift analysis (EMSA; Fig. 3D). A binding activity could be detected (lane 2) and competed using an unlabeled excess of “cold” HNF-1 α binding oligonucleotide (lane 3) but not with an oligonucleotide containing the mutated HNF-1 binding site (lane 4). Furthermore, a supershift of the complex was observed after preincubation of the liver nuclear extract with a monospecific anti-HNF-1 α antiserum (lane 5). This supershift was not generated with a control (anti-STAT-1) antibody (lane 6). Together, these data suggest that HNF-1 α can bind to a highly conserved HNF-1 binding site in the apoM promoter and activate transcription.

Reduced serum apoM levels in subjects with MODY 3. Because apoM gene expression and serum apoM levels are

critically dependent on Hnf-1 α function in mice, it might be anticipated that loss of function by HNF-1 α mutations in humans could manifest by decreased serum apoM concentrations. To test this hypothesis, we first studied whether apoM could be detected in plasma. The complete coding sequence of apoM was cloned into bacterial expression vector PET3a and expressed as an insoluble protein in inclusion bodies (Fig. 4A). Recombinant protein was refolded as described in RESEARCH DESIGN AND METHODS, and the concentration was determined. We then measured the concentration of apoM protein in plasma by preparing a standard curve of recombinant protein using semiquantitative immunoblotting techniques (Fig. 4B). Protein concentrations of apoM in human plasma were \sim 0.1 g/l. We noticed a small difference in molecular weight of the native and recombinant protein, indicating that apoM may be modified posttranslationally. We next compared the expression levels of apoM protein in plasma of control subjects and HNF-1 α -deficient and HNF-4 α -deficient diabetic subjects (MODY3 and MODY1, respectively). Study groups were composed of nine diabetic patients with HNF-1 α mutation (HNF1 α ^{+/-}), nine diabetic subjects with HNF-4 α mutation (HNF4 α ^{+/-}), and nine normal control

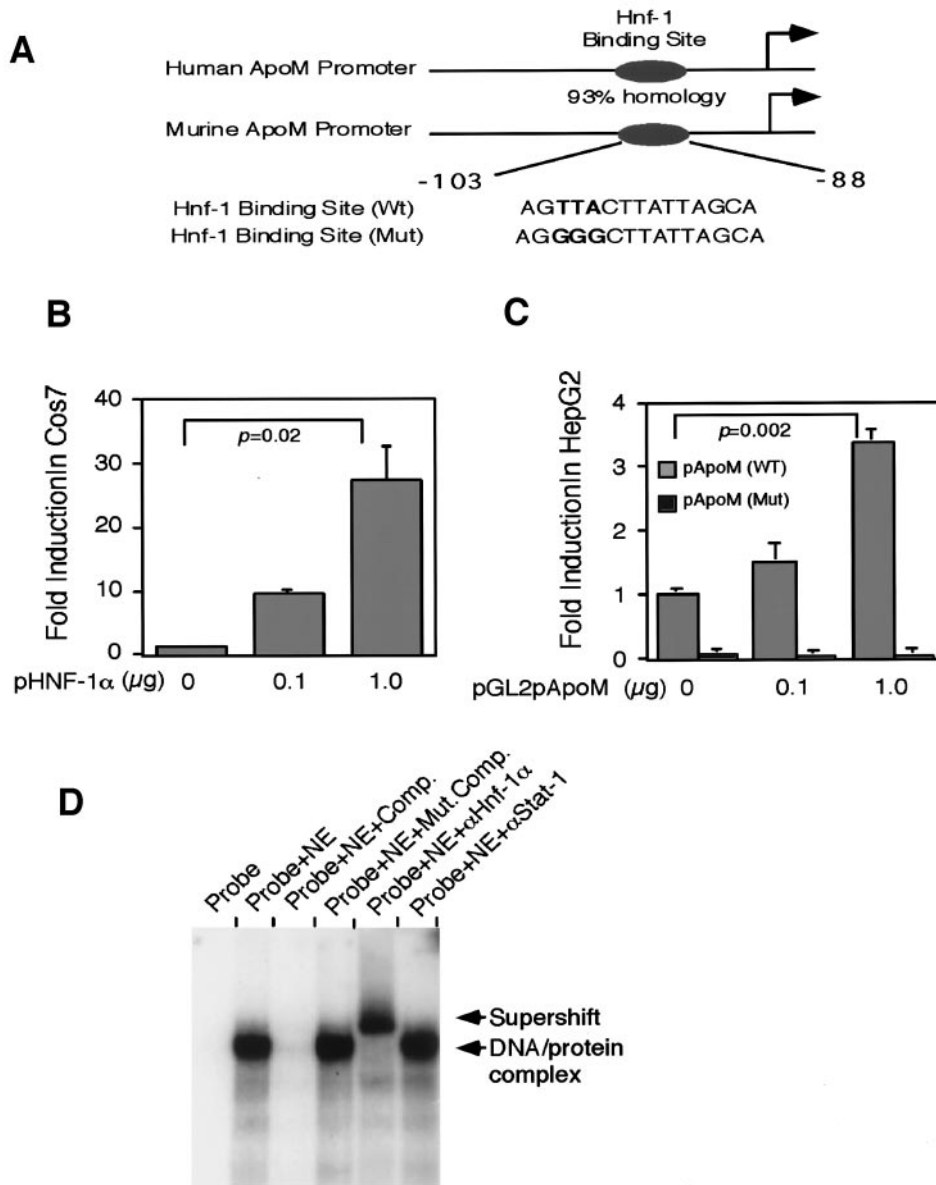


FIG. 3. *A:* Schematic diagram of conserved HNF-1 binding site in the apoM promoter. Nucleotide positions are indicated relative to transcription start site. *B:* Transcriptional activation assay in Cos7 cells that were transfected with pCMV-HNF-1 α , pCMV- β -galactosidase, and the luciferase reporter gene using a 1,045-bp apoM promoter. *C:* HepG2 cells were transfected with either pGL2papoM, containing the wild-type apoM promoter upstream of the luciferase gene, or pGL2papoM mut that has a mutated HNF-1 binding site together with pCMV- β -galactosidase. Luciferase activity was normalized to β -galactosidase activity. Each value represents the mean of six independent experiments \pm SD. *D:* EMSA using oligonucleotides for upstream HNF-1 binding site. NE, nuclear extract of wild-type liver; Comp., unlabeled ("cold") double-stranded HNF-1 binding site used as competitor; Mut. Comp., competitor with mutated HNF-1 binding site. The radioactive probe is at the bottom of the gel and is not shown.

subjects (HNF1 α ^{+/+}). These subjects were matched with respect to ethnicity, sex, age, and BMI. The clinical characteristics of the different study groups are shown in Table 1.

Of the MODY3 patients, four subjects had mutations in the B-domain (HNF-1 α P129T, R159W, E132K) that is responsible for a strong cooperativity in the binding of two monomers to a palindromic HNF-1 binding site. Four subjects had an insertion mutation in the transactivation domain (P291fsinsC) that leads to a truncated protein and exhibits moderate dominant negative activity in vitro (13). One subject had a mutation in a highly conserved residue of helix 3 of the DNA recognition homeodomain (W267R). Subjects with mutations in HNF-4 α (MODY1) were from the RW pedigree. In the RW family, diabetes is caused by Q268X mutation in the HNF-4 α gene (5). This mutation results in a truncated HNF-4 α protein with no transcriptional activity (22). Serum apoM protein levels were measured by immunoblotting and by comparing the data with a standard (control) serum (Fig. 4C). Subjects with HNF-1 α mutations had an \sim 36% reduction in apoM levels

compared with control subjects (1.0 vs. 0.65; $P = 0.02$). In contrast, HDL and serum apoA1 levels were similar in both study groups, suggesting that reduced apoM levels are caused by HNF-1 α haploinsufficiency and are not due to changes in HDL concentrations (Fig. 5). We were concerned that hyperglycemia may contribute to the changes in apoM concentrations because MODY3 subjects had significantly higher HbA_{1c} levels than normal control subjects (7.2 ± 3.9 and $5.5 \pm 0.2\%$, respectively). We therefore measured apoM levels in diabetic patients (HbA_{1c} $7.2 \pm 1.8\%$) with HNF-4 α haploinsufficiency (MODY1). Serum apoM levels of subjects with MODY1 were similar than in the control group and significantly higher than in MODY3 patients (Fig. 5). This result indicated that HNF-1 α haploinsufficiency rather than hyperglycemia is responsible for the decrease in plasma apoM levels.

DISCUSSION

HNF-1 α is a transcription factor that has an important role as a key regulator of pancreatic islet, liver, and kidney

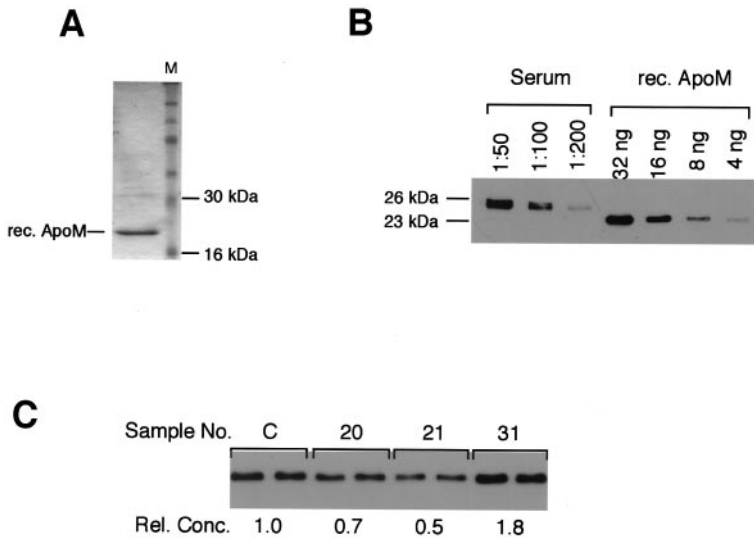


FIG. 4. **A:** Analysis of purified, recombinant apoM protein. Protein was electrophoresed on a 12.5% polyacrylamide gel and stained with Coomassie blue. Recombinant apoM expressed in bacteria has a molecular weight of ~23 kDa. **B:** Analysis of human control plasma and recombinant apoM protein was loaded on a 12.5% SDS-PAGE. The native protein is glycosylated and has a molecular weight of 26 kDa. **C:** Measurements of apoM levels in plasma of subjects by semiquantitative immunoblot analysis. Samples were analyzed in duplicate, and their relative concentration was determined relative to the control plasma. The intensities of the bands on autoradiographies were measured by densitometry, and the means were compared with the control.

gene expression. Genetic studies have shown that mutations in HNF-1 α result in an autosomal-dominant form of type 2 diabetes characterized by defects in pancreatic β -cell function and glucose-stimulated insulin release (14,23,24). In the kidney, HNF-1 α has been shown to be a key regulator of the glucose transporter SGLT2, and haploinsufficiency of HNF-1 α is associated with a low renal threshold for glucose that can lead to early glycosuria, even in the absence of high blood glucose levels (25,26). Liver function has not been thoroughly assessed in patients with HNF-1 α mutations.

We recently performed a genetic screening to identify genes that are regulated by HNF-1 α . We used livers from wild-type and Hnf-1 α -deficient animals and performed a large-scale gene expression analysis using oligonucleotide expression arrays (16). In this study, we identified an expressed sequence tag that encodes apoM and that was highly regulated in livers of mutant animals. We show that Hnf-1 α is required for apoM expression and that Hnf-1 α directly activates apoM transcription by binding to a conserved binding site in the apoM promoter.

ApoM is a novel lipoprotein that is expressed in the liver and the kidneys and is predominantly present in HDL in plasma (20). We have recently shown that Hnf-1 α -defi-

cient mice develop hypercholesterolemia as a result of a defect in bile acid transport, increased bile acid and cholesterol synthesis, and impaired HDL metabolism. The increased plasma cholesterol in Hnf-1 α ^{-/-} mice resides predominantly in large buoyant HDLc that may be caused by reduced activity of hepatic lipase and increased expression of HDL cholesterol esterifying enzyme lecithin: cholesterol acyl transferase (16). The biological function of apoM is currently unknown; however, the absence of apoM in HDL particles of mutant Hnf-1 mice may suggest that it contributes to the defect in HDL cholesterol in these animals. ApoM is distantly related to the lipocalin superfamily. Most members of this family have the ability to bind and transport hydrophobic molecules in the plasma (27). It is possible that apoM serves as a specific function in the metabolism and transfer of cholesterol (and/or other bioactive molecules) in the blood. ApoM is unusual in having its hydrophobic signal peptide retained in the mature protein, where it presumably serves as a phospholipid anchor. The synthesis of apoM therefore may be closely linked to the HDL assembly and secretion from the liver.

ApoM has previously been suggested to be a minor component of the HDL, VLDL, and LDL lipoprotein classes compared with the major apolipoproteins (20). In our studies, we found apoM exclusively associated with HDL particles. Furthermore, to assess the plasma concentration of apoM compared with other lipoproteins, we generated recombinant apoM in bacteria. Using recombinant apoM as a standard, we determined that the range of apoM concentrations in human plasma is 0.05–0.15 g/l. Serum concentrations of other HDL-containing apolipoproteins such as apoCs and apoE range between 0.02 and 0.1 g/l, and apoA1 levels range between 1.1 and 2.2 g/l. This suggests that apoM is a major component of the HDL particle.

Our data demonstrate that HNF-1 α haploinsufficiency is associated with low/normal apoM plasma levels. We have shown previously that MODY1 is characterized by low serum levels of triglycerides (Table 1), lipoprotein(a), apoAII, and apoCIII (28). Thus, concentrations of various lipoproteins in serum may aid in the differential diagnosis between MODY1 and MODY3. Subjects with early-onset

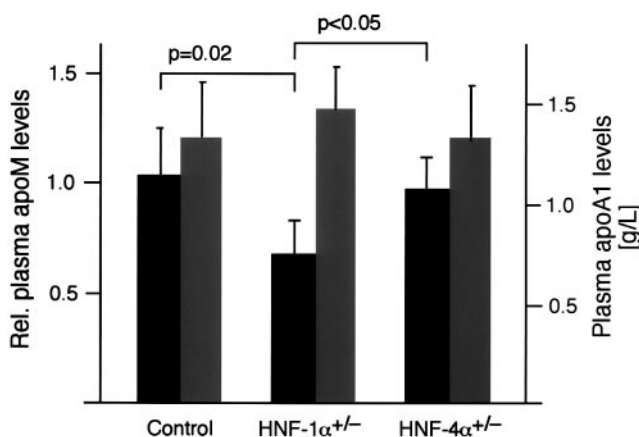


FIG. 5. Plasma concentrations of apoM (black) and apoA1 (red) in nondiabetic control subjects (Control), subjects with MODY3 (HNF-1 α ^{+/-}), and subjects with MODY1 (HNF-4 α ^{+/-}).

type 2 diabetes, positive family history consistent with autosomal-dominant inheritance, negative anti-islet antibodies, early glucosuria, sensitivity to sulfonylureas, and low serum apoM levels should be suspected of having MODY3. Genetic testing for mutations in the HNF-1 α and HNF-4 α genes, as well as other genes, should be carried out for definite identification of various MODY subtypes.

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