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In vivo role of the HNF4α AF-1 activation domain revealed by exon swapping

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The gene encoding the nuclear receptor hepatocyte nuclear factor 4α (HNF4α) generates isoforms HNF4α1 and HNF4α7 from usage of alternative promoters. In particular, HNF4α7 is expressed in the pancreas whereas HNF4α1 is found in liver, and mutations affecting HNF4α function cause impaired insulin secretion and/or hepatic defects in humans and in tissue-specific 'knockout' mice. HNF4α1 and α7 isoforms differ exclusively by amino acids encoded by the first exon which, in HNF4α1 but not in HNF4α7, includes the activating function (AF)-1 transactivation domain. To investigate the roles of HNF4α1 and HNF4α7 *in vivo*, we generated mice expressing only one isoform under control of both promoters, via reciprocal swapping of the isoform-specific first exons. Unlike *Hnf4α* gene disruption which causes embryonic lethality, these 'α7-only' and 'α1-only' mice are viable, indicating functional redundancy of the isoforms. However, the former show dyslipidemia and preliminary results indicate impaired glucose tolerance for the latter, revealing functional specificities of the isoforms. These 'knock-in' mice provide the first test *in vivo* of the HNF4α AF-1 function and have permitted identification of AF-1-dependent target genes.

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Introduction

Hepatocyte nuclear factor 4α (HNF4α), a member of the nuclear receptor superfamily, is essential for metabolic processes, as revealed by analyses of *Hnf4α* gene disruptions in the mouse (see below). Originally purified from rat liver nuclear extracts (Sladek *et al*, 1990), HNF4α is also present in the intestine, pancreas, kidney and stomach, as well as in the visceral endoderm of the yolk sac during mouse embryogenesis (Duncan *et al*, 1994; Taraviras *et al*, 1994; Nakhei *et al*, 1998). Absence of HNF4α is embryonic lethal (Chen *et al*, 1994; Duncan *et al*, 1997; Parviz *et al*, 2003) and disruption of the gene, specifically in adult liver, leads to death, due to severe dyslipidemia, high-serum bile acid levels and ureagenesis defects (Hayhurst *et al*, 2001; Inoue *et al*, 2002). In addition, HNF4α is involved in glucose homeostasis, especially in neoglucogenesis (Rhee *et al*, 2003), and disruption of the gene in pancreatic β-cells leads to impaired secretagogue-stimulated insulin secretion (Gupta *et al*, 2005), as reported for *Hnf4α*-associated non-insulin-dependent diabetes mellitus MODY1 in humans (Yamagata *et al*, 1996). Extra-pancreatic abnormalities have been reported in MODY1 patients showing low plasmatic triglyceride, apoAII and apoCIII levels, most probably due to hepatocyte defects (for reviews, see Ryffel, 2001; Sladek and Seidel, 2001). Thus, HNF4α is necessary for metabolic functions in liver and for proper insulin secretion in the pancreas. The central role of HNF4α is also highlighted by the huge number of putative HNF4α target genes, as reported in analysis combining chromatin immunoprecipitation (ChIP) from hepatocytes and pancreatic islets with promoter microarrays (Odom *et al*, 2004).

HNF4α can be divided into six domains (A–F), the A/B and F domains being the most variable among nuclear receptors. The *Hnf4α* gene gives rise to several isoforms differing within these domains, via internal splicing and transcription from two alternative promoters. HNF4α1 and HNF4α7 are the prototypes of the isoforms derived from the P1 and P2 promoters, respectively, and differ only by their first exons (Nakhei *et al*, 1998). Just like many other nuclear receptors (Warnmark *et al*, 2003), the HNF4α1 protein contains two activating functions (AF): the conserved AF-2 motif in the C-terminal ligand-binding domain and an AF-1 motif contained at the N-terminus, encoded by the α1-specific first exon (Hadzopoulou-Cladaras *et al*, 1997; Sladek *et al*, 1999). These motifs are essential for HNF4α transactivation capacity *in vitro* and act via recruitment of cofactors (e.g., Wang *et al*, 1998; Sladek *et al*, 1999; Yoon *et al*, 2001). In contrast with HNF4α1, no AF-1 activity was found for the HNF4α7 A/B domain using one-hybrid assays. Hence, HNF4α1 and HNFα7 display distinct capacities to interact with cofactors (Torres-Padilla *et al*, 2002; Eeckhoute *et al*, 2003). In addition, the two promoters show different tissue-specific activities: P1-derived HNF4α1 is almost exclusive in the liver, whereas P2-derived HNF4α7 may be the predominant form in the pancreas (Nakhei *et al*, 1998; Boj *et al*, 2001; Eeckhoute *et al*, 2003).

Since HNF4α1 and HNF4α7 differ only by the presence or not of a functional AF-1 motif, these isoforms should present both redundancy and specificity, depending on the cell type and the target gene. The structure of the *Hnf4α* gene permitted us to design the first direct test *in vivo* of HNF4α AF-1 function, making use of a naturally occurring AF-1-deficient variant rather than targeted deletion, as reported for retinoid X receptor α (RXRα) (Mascrez *et al*, 2001). We created two 'knock-in' mouse lines expressing only HNF4α1 or HNF4α7

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(and their splice-derived variants) under control of both promoters, replacing by homologous recombination either the HNF4α1 first exon-coding sequence by that specific for HNF4α7 or the reciprocal. The resulting mice present hepatic and possible pancreatic defects, revealing the physiological role of the HNF4α1 AF-1 motif. Furthermore, analysis of the gene expression pattern in livers of these mice has permitted identification of target genes whose expression is AF-1 dependent.

Results and discussion

HNF4α1 and HNF4α7 transcript accumulation in HNF4α-expressing tissues

To investigate the roles of P1 and P2 promoter-derived isoforms in the mouse (Figure 1A and B), it was essential to know which isoform is present in each HNF4α-expressing tissue. By replacing the α1 isoform by α7 in a tissue expressing predominantly or exclusively α1 in the wild-type (WT) mouse, we expected to diminish those functions for which the α1-specific AF-1 motif is required. In reciprocal fashion, the expression of target genes in tissues expressing mainly HNF4α7 could be affected by ectopic addition of the AF-1 motif.

The liver contains only traces of HNF4α7 transcripts and kidney expresses exclusively HNF4α1, whereas both isoforms are expressed in the intestine (Figure 1C; Nakhei *et al*, 1998; Briancon *et al*, 2004). While information in the literature concerning HNF4α isoforms in the pancreas and islets is contradictory (Boj *et al*, 2001; Eeckhoute *et al*, 2003), we found essentially HNF4α7 transcripts, while those for HNF4α1 were hardly detectable in either whole mouse pancreas or isolated islets (Figure 1C). Similarly, only HNF4α7 was detected for the stomach, contradicting earlier results (Nakhei *et al*, 1998).

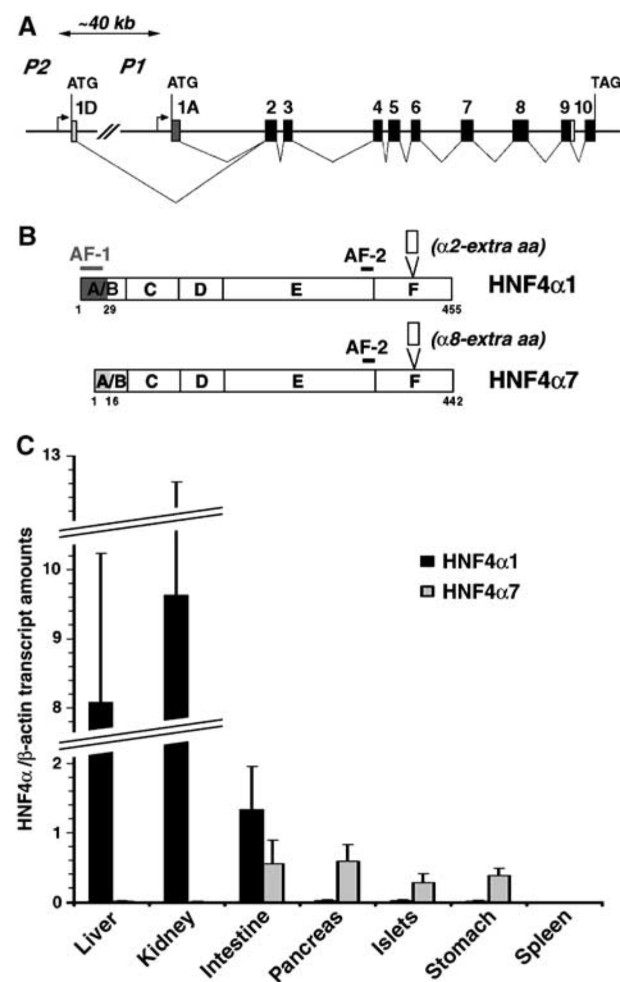

Figure 1 Structure of the mouse *Hnf4α* gene, its isoforms and expression of transcripts. Scheme of the *Hnf4α* gene (**A**) and isoforms (**B**). HNF4α1 and HNF4α7 are transcribed from P1 and P2 promoters, respectively, and differ only by the amino acids encoded by their first exon (A and D, respectively). Exon 1A carries the AF-1 motif. In (A), exon-coding sequences are shown as boxes. Arrows, transcription start sites. Other isoforms (α2 and α8; see Figure 2) present a 10-amino-acid insertion in the F domain due to usage of an alternative splice donor site 3′ to exon 9 (white box). (**C**) Quantitative real-time PCR assays performed on cDNA from 11–16-week-old mouse tissues ($n \geqslant 3$). In the intestine (duodenum and part of the small intestine), the ratio of HNF4α1 mRNA to that of HNF4α7 is about × 2.4. The spleen was used as a negative control for HNF4α expression.

Hnf4α1/α7 reciprocal replacement

The most direct approach to determine the roles of the α1 and α7 isoforms would be to generate isoform-specific knockouts. However, since P1 and P2 promoter usage is tissue specific, in order to maintain HNF4α levels constant, we expressed either the α1 or the α7 isoform from both promoters. Targeting constructs contained the coding sequence (CDS) of the alternative first exon flanked by promoting/5′untranslated regions (5′UTR) and intronic sequences from the endogenous locus (Figure 2A and B). In one line, the exon 1D CDS was deleted and replaced by that of exon 1A, and the reciprocal for the other targeting event. This minimal intervention strategy permits conservation of the ratio among the internal splicing isoforms (see below). Recombinant ES cells were obtained (Supplementary Figure S1) and injected into blastocysts to obtain founder chimeras. Mice that are homozygous for exon 1D and exon 1A replacements are referred to as 'α1-only' and 'α7-only' mice, respectively.

It is worth noting that insertion of the floxed phosphoribosyltransferase gene (*neo*) in the HNF4α7-specific first intron (Figure 2A) was not lethal in the homozygous mice, contrary to the same insertion downstream of the HNF4α1-specific first exon (Figure 2B): this suggests that the knockout of P2-derived transcripts would be viable (see Results section in Supplementary Figure S1). *Neo* cassettes were deleted by mating with pgk-cre mice.

The α1-only and α7-only mouse livers express the expected isoform under control of both P1 and P2 promoters

The α1-only and α7-only mice are viable, fertile and do not present any obvious phenotype. We investigated whether the profile of the HNF4α isoforms was as expected in the isoform replacement mice, focusing on the liver. In the adult α7-only mouse liver, HNF4α7 is strongly expressed from the P1 promoter and HNF4α1 transcripts are not detectable (Figure 2C). No HNF4α7 transcripts could be detected in the α1-only mouse liver. We also verified that the ratio between the C-terminal splicing-derived isoforms, α2 and α8, compared to α1 and α7, was maintained in the mutant mice (Figure 2D). This confirms that the exon replacement does not affect downstream splicing events.

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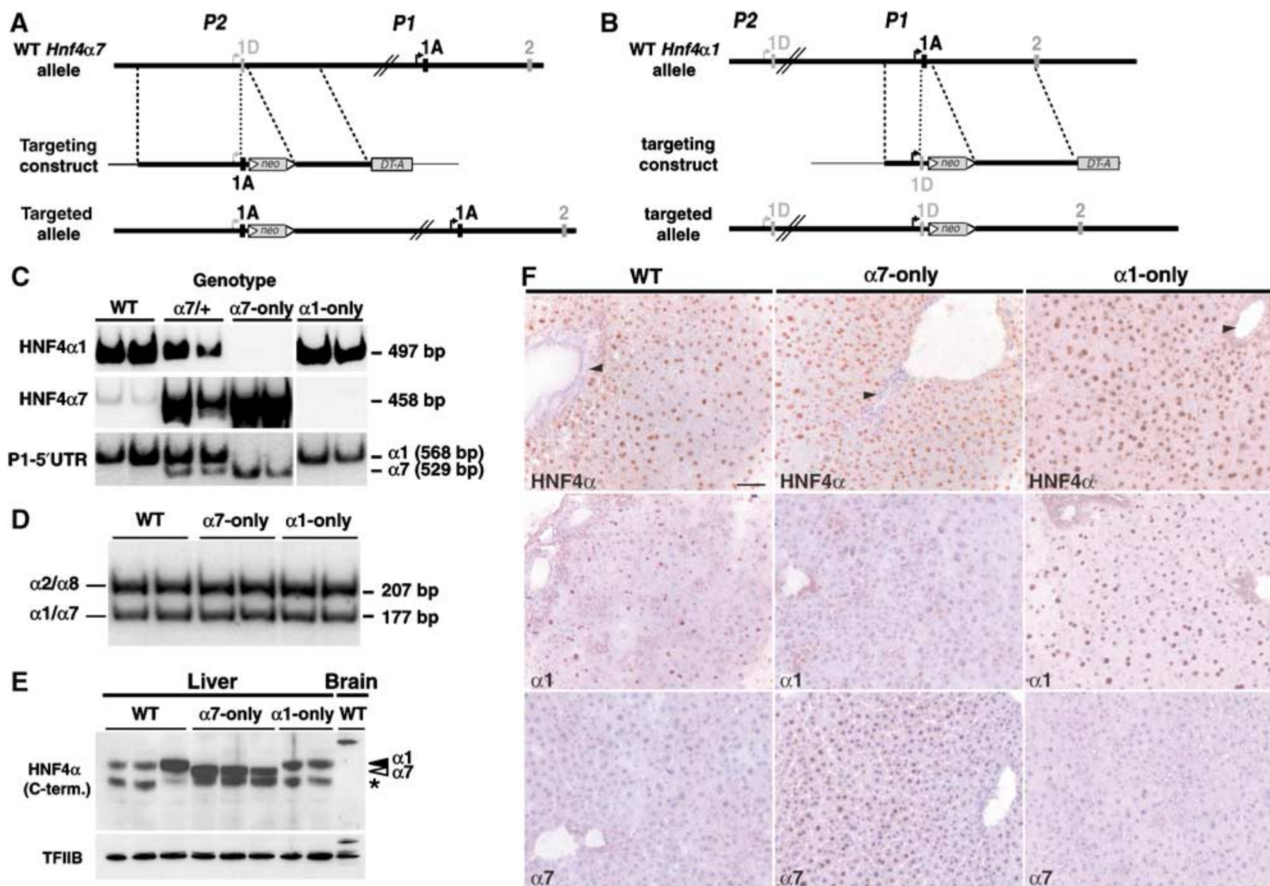

Figure 2 *Hnf4α1/α7* reciprocal knock-in replacement. (**A, B**) Scheme of the plasmid constructs and the genomic loci before and after homologous recombination in ES cells. In (A), replacement of the HNF4α7 exon 1D CDS by the HNF4α1 exon 1A CDS, and the reciprocal in (B). Neo, G418-resistance cassette; DT-A, diphtheria toxin A (see Supplementary Figure S1). (**C**) Semiquantitative radioactive RT–PCR was performed with liver total RNA using specific forward primers (left) designed in the HNF4α1 or HNF4α7 exon1 CDS or in the 5′UTR at the P1 promoter. A reverse primer common to both isoforms was used. The HNF4α7 first exon is 39 bp shorter than that of HNF4α1, giving rise to a smaller band in the α7-only and heterozygous (α7/+) mouse livers while using the P1-5′UTR primer. Enhanced expression from the P2 promoter in α7-only mice has already been described and interpreted (Briancon *et al*, 2004). (**D**) Semiquantitative RT–PCR showing normal ratios of the C-terminal splicing-derived isoforms in the mutant mouse livers (α2 and α8). The PCR primers frame the 30-nucleotide insertion (see Figure 1; (Torres-Padilla *et al*, 2001)). (**E**) Western blot performed with liver nuclear extracts of each genotype using an antibody that recognizes all HNF4α isoforms. The HNF4α7 protein migrates faster than the HNF4α1 isoform (arrowheads). *HNF4α protein degradation product or nonspecific signal. Brain nuclear extracts were used as a negative control for the presence of HNF4α proteins and TFIIB as a loading control. (**F**) Detection of HNF4α isoforms on α7-only liver cryosections (middle) compared to WT and α1-only livers. Immunohistochemistry was performed with the C-terminal antibody used in Western blotting (HNF4α), the α1-specific N1-14 antibody (α1) and an α7-specific antiserum (α7). Arrowhead, bile duct cells. Scale bar, 250 μm.

In Western blots (Figure 2E), HNF4α proteins from liver extracts of the α7-only mice migrate faster than those in WT and α1-only samples, consistent with the expected smaller protein. In addition, total amounts of HNF4α proteins appear grossly equivalent among the different genotypes. On α7-only and WT liver sections, using adequate antibodies, the isoform replacement was validated at the protein level, HNF4α being restricted to hepatocytes and excluded from bile duct cells (Figure 2F).

The α7-only mice present a nonlethal dyslipidemia

To reveal potential subtle defects in the mutant mice, a panel of electrolytes, metabolites, nutrients, enzymes and hormones was investigated (Figure 3A). As the adult-liver specific *Hnf4α* disruption causes ureagenesis and lipid and bile acid metabolism defects (Hayhurst *et al*, 2001; Inoue *et al*, 2002), we anticipated that the α7-only mice would present an attenuated form of these metabolic dysfunctions. Indeed, serum cholesterol and triglyceride contents were lower in these mice compared to WT (41% and 53% decrease, respectively), whereas FFA levels were slightly diminished (by 24%) and ketone body contents were increased (by 82%). The cholesterol content in all lipoprotein fractions was diminished in the α7-only mice, as shown on fast-performance liquid chromatography (FPLC) lipoprotein profiles (Figure 3B). These results reveal that the HNF4α AF-1 domain is implicated in lipid metabolism, since its absence in α7-only mice leads to dyslipidemia. However, blood bile acid and urea levels were not altered.

In addition to lipid metabolism defects, serum bilirubin levels were higher in α7-only females. This could reflect a hepatic defect, and is consistent with low levels of constitutive androstane receptor (CAR), a main regulator of bilirubin clearance (see below and Huang *et al*, 2003). Iron amounts were reduced in the α7-only mouse serum and this might be due to intestinal absorption and/or hepatic defects (excluding a possible alteration of hepatic transferrin expression; Figure 5D). Other serological parameters associated with

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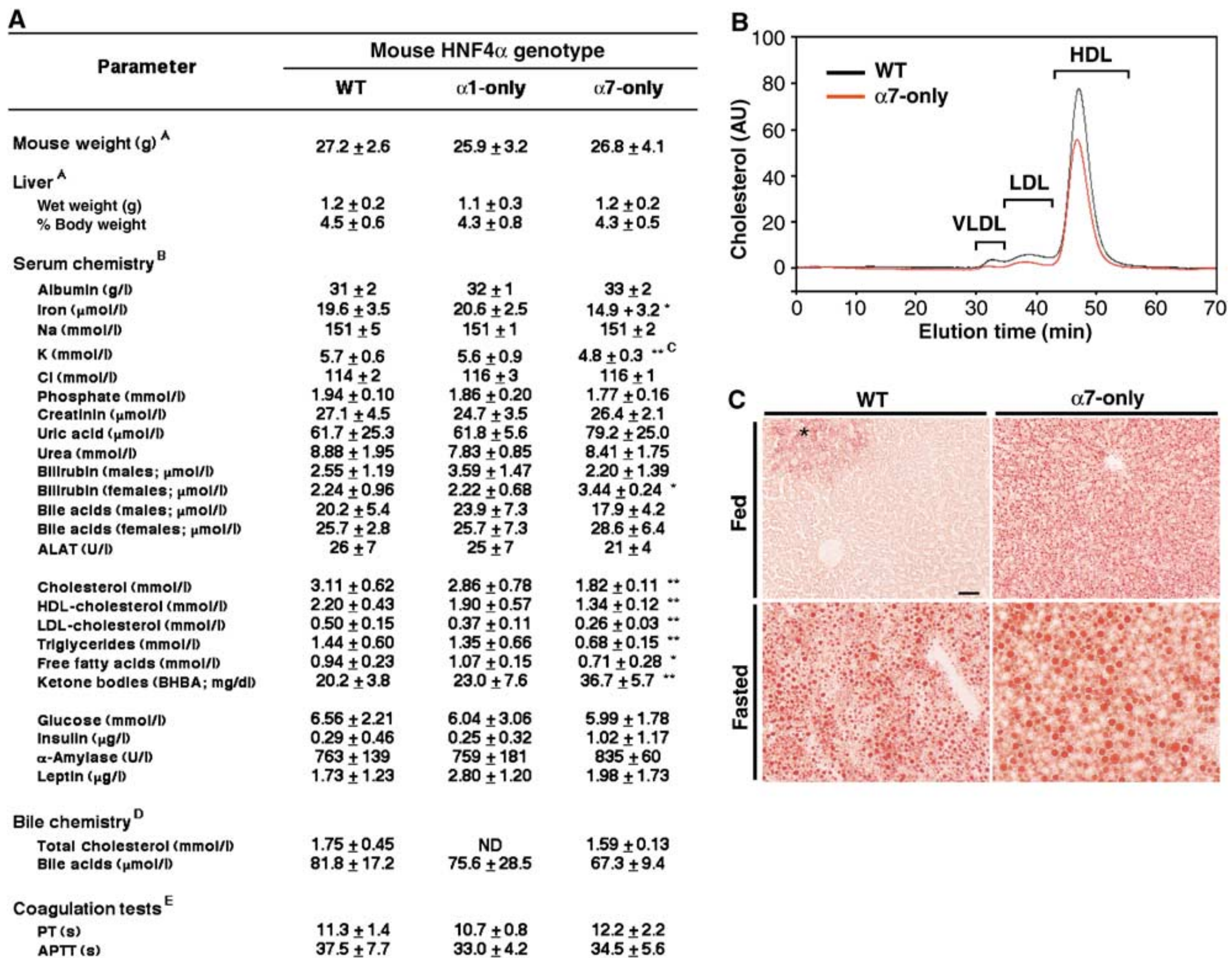

A

Parameter	Mouse HNF4α genotype		
	WT	α1-only	α7-only
Mouse weight (g) [A]	27.2 ± 2.6	25.9 ± 3.2	26.8 ± 4.1
Liver [A]			
Wet weight (g)	1.2 ± 0.2	1.1 ± 0.3	1.2 ± 0.2
% Body weight	4.5 ± 0.6	4.3 ± 0.8	4.3 ± 0.5
Serum chemistry [B]			
Albumin (g/l)	31 ± 2	32 ± 1	33 ± 2
Iron (μmol/l)	19.6 ± 3.5	20.6 ± 2.5	14.9 + 3.2 *
Na (mmol/l)	151 ± 5	151 ± 1	151 ± 2
K (mmol/l)	5.7 ± 0.6	5.6 ± 0.9	4.8 ± 0.3 ** [C]
Cl (mmol/l)	114 ± 2	116 ± 3	116 ± 1
Phosphate (mmol/l)	1.94 ± 0.10	1.86 ± 0.20	1.77 ± 0.16
Creatinin (μmol/l)	27.1 ± 4.5	24.7 ± 3.5	26.4 ± 2.1
Uric acid (μmol/l)	61.7 ± 25.3	61.8 ± 5.6	79.2 ± 25.0
Urea (mmol/l)	8.88 ± 1.95	7.83 ± 0.85	8.41 ± 1.75
Bilirubin (males; μmol/l)	2.55 ± 1.19	3.59 ± 1.47	2.20 ± 1.39
Bilirubin (females; μmol/l)	2.24 ± 0.96	2.22 ± 0.68	3.44 ± 0.24 *
Bile acids (males; μmol/l)	20.2 ± 5.4	23.9 ± 7.3	17.9 ± 4.2
Bile acids (females; μmol/l)	25.7 ± 2.8	25.7 ± 7.3	28.6 ± 6.4
ALAT (U/l)	26 ± 7	25 ± 7	21 ± 4
Cholesterol (mmol/l)	3.11 ± 0.62	2.86 ± 0.78	1.82 ± 0.11 **
HDL-cholesterol (mmol/l)	2.20 ± 0.43	1.90 ± 0.57	1.34 ± 0.12 **
LDL-cholesterol (mmol/l)	0.50 ± 0.15	0.37 ± 0.11	0.26 ± 0.03 **
Triglycerides (mmol/l)	1.44 ± 0.60	1.35 ± 0.66	0.68 ± 0.15 **
Free fatty acids (mmol/l)	0.94 ± 0.23	1.07 ± 0.15	0.71 ± 0.28 *
Ketone bodies (BHBA; mg/dl)	20.2 ± 3.8	23.0 ± 7.6	36.7 ± 5.7 **
Glucose (mmol/l)	6.56 ± 2.21	6.04 ± 3.06	5.99 ± 1.78
Insulin (μg/l)	0.29 ± 0.46	0.25 ± 0.32	1.02 ± 1.17
α-Amylase (U/l)	763 ± 139	759 ± 181	835 ± 60
Leptin (μg/l)	1.73 ± 1.23	2.80 ± 1.20	1.98 ± 1.73
Bile chemistry [D]			
Total cholesterol (mmol/l)	1.75 ± 0.45	ND	1.59 ± 0.13
Bile acids (μmol/l)	81.8 ± 17.2	75.6 ± 28.5	67.3 ± 9.4
Coagulation tests [E]			
PT (s)	11.3 ± 1.4	10.7 ± 0.8	12.2 ± 2.2
APTT (s)	37.5 ± 7.7	33.0 ± 4.2	34.5 ± 5.6

Figure 3 Weights, serum and bile chemistry, and coagulation tests. (**A**) [A] Values obtained (±s.d.) on at least 6 mice (9–13 weeks old), not separated by sex but using equal numbers of males and females. [B] Assays were performed on male and female groups of 9–16-week-old mice ($4 \leqslant n \leqslant 19$). When gender-specific differences were obtained while comparing mutant versus WT mice, results from both sexes are shown or specified. [C] Values obtained with female mice; α7-only males were not significantly affected. [D] Assays performed on 2–10 males of 14–16 weeks old. [E] Prothrombin time (PT) and activated partial thromboplastin time (APTT) tests performed on 11–12-week-old mice not separated by sex ($n \geqslant 8$). Serum and bile chemistry were performed with mice that were fasted overnight. Statistical analyses were performed with GraphPad Instat® software using a one-way analysis of variance test, followed when applicable by the multiple-comparison Dunnett's post-test (*$P<0.05$ and **$P<0.01$). BHBA, β-hydroxy butyrate; ALAT, alanine aminotransaminase. (**B**) Cholesterol in HDL, LDL and VLDL fractions was determined by FPLC on at least three pools of serum from 2–4 males each that were fasted overnight. Results obtained with one representative pool for WT and α7-only mice are shown. Profiles of α1-only mice were not significantly different from WT (not shown). (**C**) Mice, 5 months old, were either fed *ad libitum* or fasted for 24 h before killing. Liver cryosections were stained with Oil red O. *Slight lipid accumulation on the WT section, near a centrolobular vein (not shown). All images are at the same magnification (scale bar, 100 μm).

hepatic functions (albumin, ALAT and coagulation tests) were unchanged.

Since kidney expresses only HNF4α1, α7-only mice could present renal defects, but serum creatinin and urea levels were not affected. However, since HNF4α is expressed in renal proximal tubules and is absent from glomeruli (Chabardes-Garonne *et al*, 2003), renal re-absorption defects might occur. No glucosuria was detected ($n = 8$) but potassium concentrations were reduced by 16% in α7-only females, indicating possible gender-dependent defects in renal re-absorption. Alternatively, this hypokaliemia may be due to intestinal absorption defects.

Lipid accumulation in the α7-only mouse liver

Focusing on dyslipidemia, we investigated whether α7-only mice accumulated lipids in liver as in the liver-specific *Hnf4α* null mice. Indeed, the α7-only mice present slight hepatic steatosis. Small lipid droplets, homogeneously distributed within the parenchyma, were revealed by Oil Red O staining of liver sections from 5-month-old α7-only mice (Figure 3C). Interestingly, this steatosis was very subtle in 9-week-old mice (not shown), indicating a chronic amplification of metabolic defects with age. To exacerbate phenotypic differences, animals were fasted for 24 h to stimulate release of fatty acids from adipose tissue and their accumulation/catabolism in hepatocytes. As expected, fat droplets, much larger than in controls, were revealed in α7-only livers.

Expression of some key genes of lipid metabolism/transport is affected in α7-only mouse liver

To understand the basis for the dyslipidemia presented by the α7-only mice, we investigated expression of a panel of

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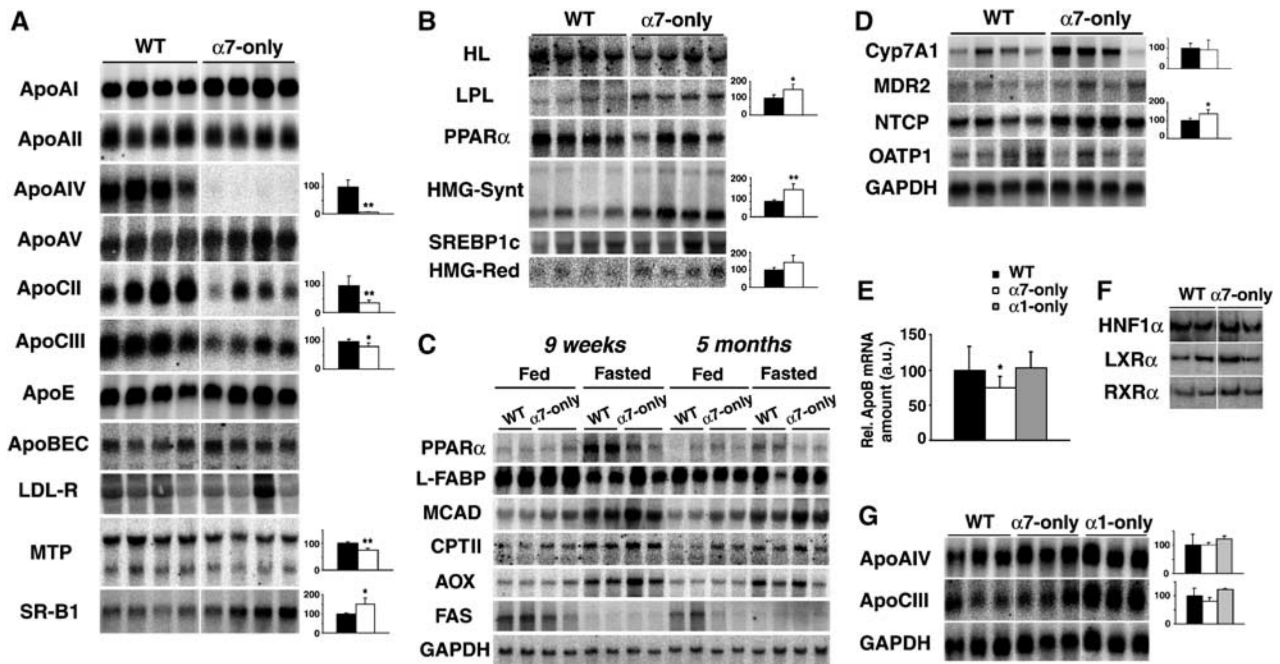

Figure 4 Expression profiles of genes implicated in lipid transport/metabolism in α7-only mouse livers (**A–F**) and intestine (**G**) compared to WT tissues. (A–D) Northern blots performed with liver RNA of 9–12-week-old α7-only and WT mice. Quantification of the transcript levels of some genes is shown to the right of the corresponding blot (black and white bars, WT and α7-only livers, respectively). Values were normalized to GAPDH (see (C, D)) and are represented as percentage of the WT samples ($n = 4$–6). GAPDH transcript levels are known to remain stable in the absence of HNF4α (Wiwi *et al*, 2004). (A–C) Genes implicated in blood triglyceride and cholesterol transport (apolipoproteins), VLDL secretion (apoBEC, MTP), lipoprotein uptake (LDL-R, SR-B1) (A) and fatty acid/cholesterol metabolism (B, C). For (C), expression analysis from 9-week- or 5-month-old mice that were fasted or not (see Figure 3C). ApoBEC, apoB mRNA editing catalytic subunit; LDL-R, LDL-receptor; MTP, microsomal tryglyceride transfer protein; SR-B1, scavenger receptor class B type I receptor; HL, hepatic lipase; LPL, lipoprotein lipase; HMG-Synt/Red, 3-hydroxy-3-methylglutaryl-coenzymeA synthase/reductase; SREBP1c, sterol receptor element binding protein 1c; L-FABP, liver-fatty acid binding protein; MCAD, medium-chain acyl-coA dehydrogenase; CPT II, carnitoyl-palmitoyl transferase II; AOX, acyl-coA oxidase; FAS, fatty acid synthase. (D) Genes involved in bile acid synthesis, excretion and re-uptake from the blood. Cyp7A1, cholesterol 7α hydroxylase; MDR2, multidrug resistance protein 2; NTCP, sodium taurocholate cotransporter protein; OATP1, organic anion transporter protein 1. (E) Quantitative real-time PCR analysis of hepatic apoB transcript amounts (shown as percentage of WT values; $n \geq 6$). (F) Semiquantitative RT–PCR. Hepatic expression of the HNF4α target gene HNF1α and of transcription factors (LXRα/RXRα) known to play an essential role in cholesterol metabolism is shown. No obvious changes in these transcript amounts could be observed in α7-only versus WT livers. LXRα, oxysterol receptor α. (G) Northern blot analysis performed with total RNA from adult mouse intestines. Quantification as for (A–D) (gray bars, α1-only mice; $n \geq 3$).

hepatic genes, mostly known to be transactivated by HNF4α and/or for which expression was altered in the liver-specific HNF4α KO (Hayhurst *et al*, 2001). Strikingly, very few genes involved in lipid transport/metabolism were strongly deregulated in the α7-only livers (Figure 4A–F). α1-only mice were checked for some of these deregulated genes and presented normal transcript levels (Supplementary Figure S2).

Hepato-specific downregulation of apoAIV and apoCIII expression. In the α7-only livers, transcripts of the HDL component apoAIV were nearly undetectable, and components apoCII and apoCIII of VLDL were reduced by 64 and 19%, respectively (Figure 4A). This suggests that the α1-specific AF-1 domain is required for full expression of these putative HNF4α direct targets (Hayhurst *et al*, 2001; Odom *et al*, 2004 and references therein).

Disruption of the *apoAIV* gene in the mouse triggered a decrease in VLDL/HDL-cholesterol levels and also in triglyceride levels due to altered expression of the *apoCIII* gene within the same gene cluster (Maeda *et al*, 1994; Weinstock *et al*, 1997). Hence, in the α7-only mice, combined diminished hepatic expression of the *apoAIV* and *apoCIII* genes could contribute to low serum cholesterol and triglyceride levels. However, since apoAIV and apoCIII are expressed both in the liver and the intestine, with apoAIV being mainly expressed in the intestine (Elshourbagy *et al*, 1985), we investigated expression of both genes in this tissue. Their downregulation in the α7-only mice is hepato-specific: no decline in transcript levels was revealed in the α7-only mouse intestine (Figure 4G). Thus, expression of the *apoAIV* gene in liver and intestine is regulated by HNF4α, but only in the former is AF-1 necessary (Ktistaki *et al*, 1994; Sauvaget *et al*, 2002).

VLDL secretion may be altered. The two genes essential for VLDL secretion MTP and apoB (Figure 4A and E) were both downregulated by 25% in α7-only liver compared to WT. In the endoplasmic reticulum, the abetalipoproteinemia-associated gene product MTP is absolutely required for lipid assembly with apoB and, thus, for VLDL secretion (Leung *et al*, 2000). In addition, mice heterozygous for a disrupted apoB allele presented reduced cholesterol levels in all lipoprotein fractions (Farese *et al*, 1995; Huang *et al*, 1995), and in another study (Leung *et al*, 2000) reduced triglyceride levels and higher hepatic triglyceride accumulation. Thus, the combined small decreases in apoB and MTP transcript levels in the α7-only mouse livers may contribute to low-serum triglyceride and cholesterol levels and to steatosis.

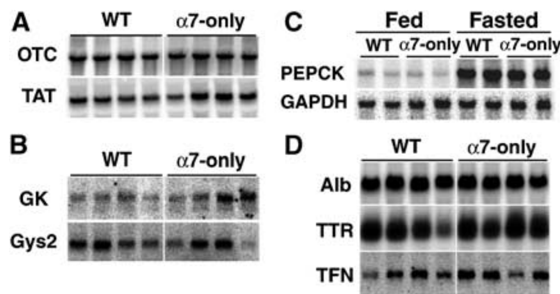

Figure 5 Expression profile of genes implicated in amino-acid (**A**) and glucose metabolism (**B, C**), and of serum protein carriers (**D**). These genes are known or putative direct targets for the HNF4α or HNF1α transcription factors (Odom *et al*, 2004 and references therein). TAT, tyrosine aminotransferase; GK, glucokinase; Gys2, glycogen synthase; PEPCK, phosphoenolpyruvate carboxykinase; Alb, albumin; TTR, transthyretin; TFN, transferrin.

Lipid uptake from blood may be increased. While the rate-limiting enzyme for hydrolysis of VLDL and chylomicron triglyceride, LPL, is barely detectable in WT liver, its expression was weakly induced in α7-only liver (Figure 4B). Hepatic LPL gene expression has been reported to be induced by cytokines (Merkel *et al*, 1998 and references therein), suggesting that inflammatory signals could be present in α7-only liver. Transgenic mice overexpressing LPL specifically in the liver on a *LPL* null background presented hepatic steatosis and increased circulating ketone body levels (Merkel *et al*, 1998), as is the case for the α7-only mice. Moreover, the major HDL receptor gene *SR-B1* seemed induced in the α7-only livers and may participate in steatosis and in low HDL-cholesterol (Figure 4A).

Fatty acid/cholesterol synthesis and catabolism are not likely to trigger α7-only dyslipidemia. We queried whether lipid synthesis and catabolism were disturbed in α7-only mouse liver. Transcript levels of two enzymes involved in the rate-limiting steps of mitochondrial and peroxisomal β-oxidation pathways (MCAD and AOX, respectively) were increased or unchanged compared to WT, both in fed and fasted mice, and those of CPTII, allowing the incorporation of long-chain fatty acids into mitochondria for β-oxidation, were increased (Figure 4C). In addition, transcripts of HMG-coA-synthase, the rate-limiting enzyme in ketogenesis, were increased (Figure 4B), consistent with higher serum levels of ketone bodies. These genes are targets for a key player in lipid metabolism, PPARα (Gulick *et al*, 1994; Rodriguez *et al*, 1994; Hashimoto *et al*, 1999; Barrero *et al*, 2003), whose variation in expression levels was not correlated with the induction of some of its target genes (Figure 4B and C). In contrast, transcript level of the key enzyme for fatty acid synthesis (FAS) is decreased in the α7-only liver, and the difference between the genotypes is accentuated with aging (Figure 4C). This indicates an inverse correlation between lipid and FAS transcript levels and suggests a negative feedback loop. These observations, although fragmentary, suggest that increased FAS or defects in β-oxidation/ketogenesis are not involved in the observed hepatic steatosis.

Transcript levels of HMG-coA-reductase, the enzyme responsible for the committed step in cholesterol synthesis, were not significantly affected in the α7-only livers (Figure 4B) as for those of a few genes involved in bile acid synthesis (cyp7A1, but also cyp8B1 and cyp7B1; not shown) and transport (Figure 4D; except for NTCP, which was mildly elevated). Thus, these preliminary observations did not reveal defects in cholesterol synthesis and transformation (via bile acid synthesis) to account for dyslipidemia. Further investigations are required to decipher the molecular aspects of the α7-only mouse phenotype.

Expression of some key genes of glucose and amino-acid metabolism is not affected

HNF4α is also a well-known regulator of carbohydrate and amino-acid metabolism. Transcripts of enzymes involved in these metabolic pathways (Figure 5A–C) were not altered in the α7-only livers, including ornithine transcarbamylase (OTC), a known target gene of HNF4α whose deregulation is responsible for the ureagenesis defects reported in the *Hnf4*α null livers (Inoue *et al*, 2002). Since, in the absence of HNF4α, the induction of neoglucogenic genes by a fast was impaired (Rhee *et al*, 2003), we investigated whether PEPCK induction was altered in the fasted α7-only mouse livers. This induction was normal (Figure 5C), as expected since neoglucogenic genes are reported to be induced through coactivation with PGC1α, which interacts with the AF-2 domain common to both HNF4α isoforms (Yoon *et al*, 2001).

CAR expression is drastically reduced in the α7-only mouse livers

Expression of cytochrome P450 genes, central in xenobiotic detoxification, is induced by several nuclear receptors, including the CAR and the pregnane-X receptor (PXR). In the absence of the HNF4α1 AF-1 motif, the expression profile of both factors (Figure 6A) was reminiscent of that of the liver-specific HNF4α KO (Hayhurst *et al*, 2001; Tirona *et al*, 2003). Whereas PXR was not affected, CAR transcripts in the α7-only mice were drastically reduced compared to WT (Figures 6B and 7B). This was associated with a lack of induction by the CAR-specific ligand TCPOBOP of the CAR inducible gene, cyp2b10, in the α7-only mice (Figure 6C).

Since no HNF4α-binding sites have been described in the mouse CAR promoter, we scanned for sites with the MatInspector program. In the 10 kb region upstream of the ATG, eight sites were identified, three of which strongly bound HNF4α in electrophoretic mobility shift assay (EMSA) (oligos −1341, −3624 and −7598; Figure 6D). However, these sites seemed to bind α1 and α7 homodimers at equivalent levels, as expected from previous *in vitro* work performed with HNF4α1 and a deletion construct lacking the A/B domain (Sladek *et al*, 1999). *In vivo*, only the most proximal site (−1341) bound HNF4α, and both α1 and α7 isoforms were found at this site, as shown in ChIP assays using α7-only versus WT and α1-only livers (Figure 7). Thus, the drastic reduction of CAR expression in the α7-only livers is likely mediated by differences other than differences in the affinity of the HNF4α isoforms for this site. We propose that the diminished capacity of HNF4α7 to regulate CAR gene expression is caused by differences in cofactor recruitment capacity of the α7 isoform (Torres-Padilla *et al*, 2002).

From a physiological perspective, loss of CAR in the mouse increases sensitivity to zoxazolamine-induced paralysis, while decreasing sensitivity to acetaminophen or cocaine-induced acute hepatic response (Wei *et al*, 2000; Zhang *et al*,

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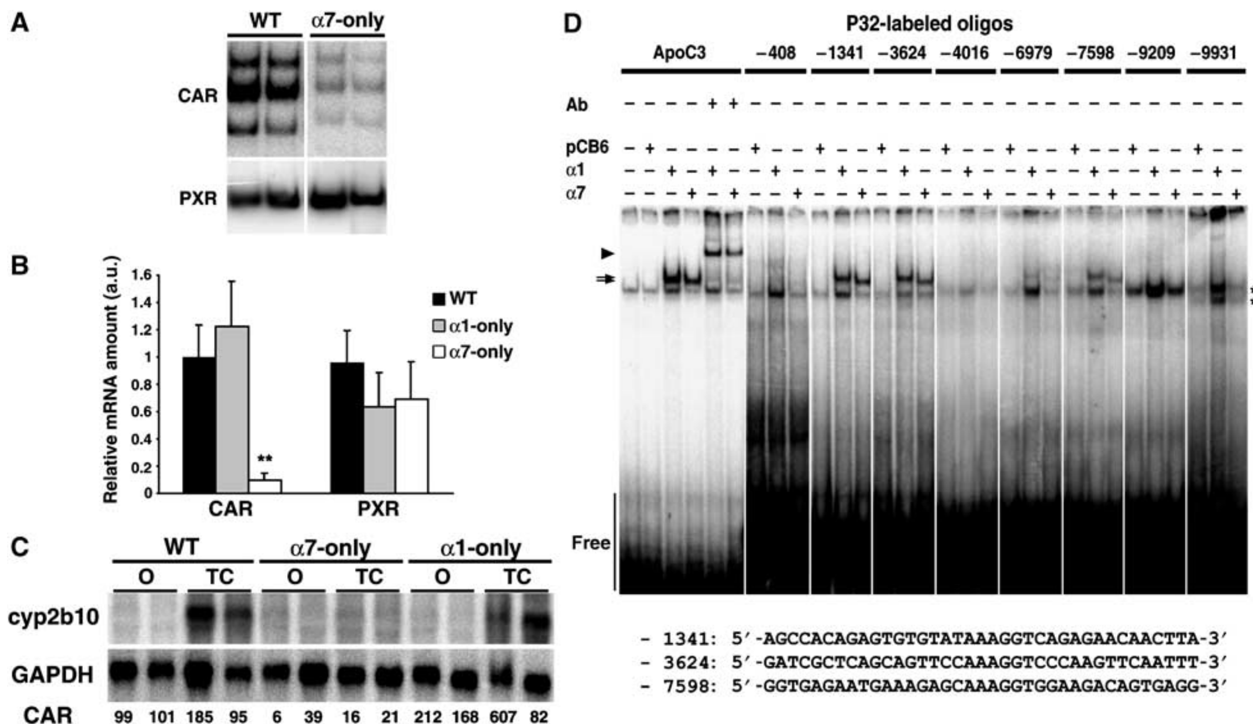

Figure 6 CAR expression is strongly diminished in the liver of the α7-only mice. (**A**) Semiquantitative RT–PCR revealing a decrease in CAR transcript amounts (three isoforms) in α7-only livers compared to WT, whereas expression of PXR is not altered. (**B**) Quantitative real-time PCR. CAR and PXR transcript amounts are represented relative to the WT values ($n \geqslant 3$). (**C**) Northern blot performed with liver RNA of mice that were injected either with TCPOBOP (TC) or vehicle (O). Absence of induction of cyp2b10 was observed in three out of four α7-only mice; the fourth mouse did express cyp2b10 despite very low levels of CAR transcripts (not shown). CAR expression was quantified by real-time PCR, and normalized values are given below the gel for each sample (relative to the non-induced WT mice). (**D**) EMSA. Cos7 cells were transfected with expression vectors for HNF4α1 or HNF4α7. Amounts of whole-cell extracts were adjusted for HNF4α1 and HNFα7 protein amounts, as deduced from titrations in Western blots (not shown). Oligonucleotides are named according to their 5′ ends relative to the mouse CAR start codon (GenBank contig NT_078306.1) and the sequences of those which clearly bind HNF4α proteins in EMSA are given below the gel. ApoCIII is a well-known HNF4α-binding oligonucleotide. Arrows, HNF4α-DNA complex. Arrowhead, HNF4α1 and α7 supershifts obtained with the antibody-recognizing part of the HNF4α C-terminus domain (Ab). *Nonspecific bands.

2002). Thus, it can be predicted that the α7-only mice will show interesting behavior towards some pharmaceuticals.

The α1-only mice present a slight glucose intolerance

In humans, mutations affecting HNF4α activity are known to be associated with MODY1 (Yamagata *et al*, 1996). In addition, mice deleted for HNF4α in β-cells display a weak hyperinsulinemia and paradoxically an impaired response to a glucose tolerance test associated with an insulin secretion defect that was correlated with low transcript levels of the ATP-dependent potassium channel subunit Kir6.2 (Gupta *et al*, 2005). Since normal pancreatic cells express only HNF4α7 (Figure 1), we investigated whether the α1-only mice could also present insulin secretion defects.

While the α1-only mice did not display abnormal insulin and/or glucose levels (Figure 3A), they showed a weak but significant glucose intolerance to glucose injection (Supplementary Figure S3A). In order to distinguish between an insulin secretion defect and insulin resistance from peripheral tissues, we performed insulin sensitivity tests. The α1-only mice did not show significant variations in glycemia following insulin injection (Supplementary Figure S3B), as expected for an islet defect in the absence of peripheral resistance.

We evaluated Kir6.2 transcripts in pancreas and isolated islets of α1-only mice, as well as other genes susceptible to account for the phenotype. However, preliminary results (Supplementary Figure S3C–D) did not permit to highlight genes whose expression was clearly disturbed in α1-only islets, even if Kir6.2 transcript levels tended to be lower.

Concluding remarks

In this study, we have reported HNF4α knockin mice expressing only one type of HNF4α isoform (HNF4α1 or HNF4α7 and their splice-derived variants) under control of the two promoters, P1 and P2. The α1-only and α7-only mice do not present any evident phenotype, contrasting with the embryonic lethality due to visceral endoderm defects of the constitutive HNF4α deletion (Chen *et al*, 1994; Duncan *et al*, 1997): this indicates functional redundancy of the isoforms in the yolk sac. In addition, contrasting with the severe architecture defects reported in the fetal-liver-specific HNF4α KO (Parviz *et al*, 2003), α7-only fetal livers appear indistinguishable from WT (see epithelial markers in Supplementary Figure S4). In the adult, the α7-only mice exhibit a nonlethal dyslipidemia associated with slight hepatic steatosis, reminiscent, in more subtle form, of the phenotype of the liver-specific *Hnf4α* null mice. This indicates not only that HNF4α7 is sufficiently redundant functionally with HNF4α1—the main isoform in the WT liver—to enable long-term survival of the α7-only mice, but also that there are specificities inherent to each isoform due to the presence or absence of the AF-1 domain. Although the molecular mechanisms supporting the α7-only mouse phenotype were not fully elucidated in this study,

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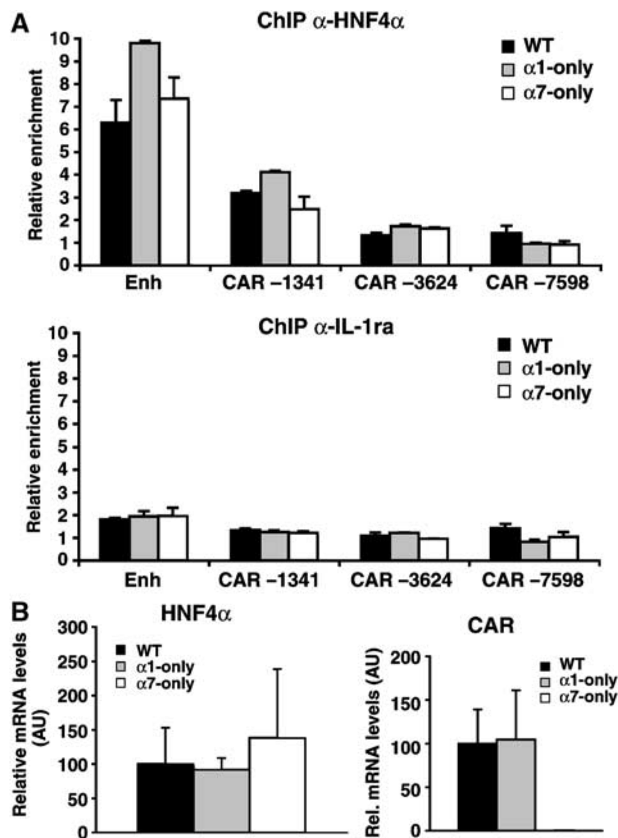

Figure 7 Decreased CAR gene expression in α7-only liver is not due to differences in DNA-binding affinity between HNF4α1 and HNFα7 isoforms. (**A**) ChIP experiments were performed with livers from WT, α1-only and α7-only female mice ($n = 2$), using the antibody-recognizing part of the HNF4α C-terminus domain (upper panel) or a nonrelevant antibody as a negative control (IL-1ra, lower panel). Similar results were obtained with male mice (not shown). HNF4α binding at the three putative binding sites within the CAR gene 5′ region (Figure 6D) was measured by real-time PCR. Oligonucleotides framing an *Hnf4α* gene enhancer element (enh) known to bind HNF4α *in vivo* (A Bailly, personal communication) were used as a positive control for HNF4α binding. (**B**) Real-time PCR confirming a drastic drop in CAR expression in the liver of α7-only mice used in ChIP assays compared to WT and α1-only livers. *Hnf4α* expression is constant among genotypes.

alterations of the expression of *apoAIV, apoCIII, apoB, MTP* and *LPL* genes are likely to be involved.

To our mind, the most important finding of this study is the ability to discriminate between genes whose expression is strictly or mainly dependent on the presence of a functional AF-1 domain in the HNF4α protein (CAR, apoAIV, apoCII), and genes whose expression is independent of this motif, although dependent upon HNF4α (i.e., OTC and apoAII).

The cases of differential regulation by HNF4α isoforms imply that different cofactors are recruited *in vivo* on some promoters by HNF4α1 versus HNF4α7. Indeed, the AF-1 domain has been shown to interact *in vitro* with general factors associated with the basal RNA polymerase II machinery, elements of the mediator complex TRAP220/DRIP205 and TRAP170, and coactivators such as SRC-1, GRIP1 and CBP/p300 (Green *et al*, 1998; Sladek *et al*, 1999; Malik *et al*, 2002; Torres-Padilla *et al*, 2002). The affinity and coactivation efficiency of CBP/p300 and GRIP1 for HNF4α1 has been shown to be higher than for HNF4α7 since these cofactors interact with both AF-1 and AF-2 motifs (Eeckhoute *et al*, 2003). It will be a challenge to define which interactions are disturbed in hepatocytes in the absence of the AF-1 domain on the regulatory regions of the *apoAIV* or *CAR* genes, whose expression levels are most affected in α7-only livers. In addition, for *apoAIV* expression, we showed that the presence of a functional AF-1 domain is required in liver but apparently not in intestine, suggesting that recruitment of different cofactors by HNF4α may occur in these tissues.

It is perhaps surprising to observe enhanced expression of some HNF4α target genes in the 'loss of function' α7-only mice. This could be due to indirect physiological effects. However, it could also reflect the differential capacities of HNF4α isoforms to act as competitors for more robust activators at regulatory sites or to repress expression of target genes via recruitment of corepressors. Indeed, the gene for HMG-coA synthase was repressed by HNF4α1 in transfection assays, HNF4α1 competing with PPARα for a binding site in the promoter (Rodriguez *et al*, 1998). Thus, HNF4α7 may not compete efficiently with PPARα, which may be a more potent activator for the regulation of the HMG-coA synthase gene, leading to gene de-repression. In addition, histone deacetylases (HDAC) are constitutively associated with nuclear receptors and inhibition of HDAC activity in transfection assays for HNF4α targets has been shown to result in enhanced target gene expression (Ruse *et al*, 2002; Torres-Padilla *et al*, 2002). HNF4α7 activity was shown to be less potently repressed *in vitro* than HNF4α1 by the corepressor SMRT, and the α7 isoform may act as a less potent repressor than α1 on some target genes (see Briancon *et al*, 2004).

The mice described here will be useful to define the ensemble of the HNF4α target genes which require AF-1 for expression in the 'loss of function' tissues, as well as those that are misregulated by a 'gain of function' like the expression of HNF4α1 in the pancreas. Array analyses of HNF4α-expressing tissues from mutant and WT mice should permit identification of the full spectrum of AF-1-sensitive target genes. This knowledge will permit 'clustering' of the targets and identification of common regulatory elements, paving the way for identification of cofactors mediating AF-1 dependency.

Materials and methods

Plasmid constructs for homologous recombination at the Hnf4α locus

To replace the coding sequence of the α7-specific exon 1D by that of the α1-specific exon 1A, a plasmid construct was prepared carrying exon 1A CDS cloned 3′ of promoter sequences/5′UTR of exon 1D, and 5′ of G418-resistance cassette, intron 1D sequences and DT-A cassette. The reciprocal construct was created for exon 1A replacement. Details are given in Supplementary Methods.

ES cell screening and mouse breeding

CK35 ES cells were a gift of C Kress. Transfectants were selected in 300 μg/ml G418 (Calbiochem-Novabiochem Corp.). Two ES clones of each construct were microinjected into C57Bl/6 blastocysts and founder male chimeras obtained. The floxed *neo* cassette was deleted following mating with pgk-Cre mice (Lallemand *et al*, 1998), and a subsequent cross with C57Bl/6 mice eliminated the Cre transgene. Since no differences in phenotype were observed between Cre^{+} and Cre^{-} or mice from different ES clones, we used mice of all genotypes (not shown). PCR genotyping of mice is detailed in Supplementary Methods. Homozygous α1-only and α7-only mice were born in mendelian proportions (Supplementary Methods). WT littermates served as controls.

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Northern blots and RT–PCR/-quantitative real-time PCR

See Supplementary Methods for RNA extraction and Northern blot. Conditions for RT–PCR and sequences of the primers specific for the α1 and α7 exon 1 CDS have been described (Briancon *et al*, 2004). Quantitative real-time PCR assays were performed with SYBR Green Master Mix (Applied Biosystems) and analyzed following the standard curve method to take into account the amplification efficiency of primers (for HNF4α1/α7, see details in Briancon *et al*, 2004). All real-time PCR results are normalized to β-actin (±s.d.). Primer sequences can be obtained upon request.

Western blot analysis and HNF4α immunodetection on histological sections

Nuclear protein extracts were prepared and Western blots performed as described in Supplementary Methods. Membrane was probed with a HNF4α C-terminal peptide antibody (sc-6556; Santa Cruz Biotechnology), and reprobed with a TFIIB antibody (sc-225). Bound antibody was revealed by peroxidase-conjugated secondary antibody (Caltag, DakoCytomation) detected with the ECL Plus reagent (Amersham Biosciences). For immunohistochemistry, the HNF4α1 (N1–14) and α7-specific primary antisera were provided by Sladek *et al* (1999) (and unpublished data). Immune complexes were detected with 3,3′-diaminobenzidine (DakoCytomation) and sections counterstained with Mayer's hematoxylin.

Serum and bile chemistry

Blood collection and assays were performed on overnight fasted cohorts of male and female adult mice, mainly at the Mouse Clinical Institute, Illkirch, France. Details are given in Supplementary Methods.

Ligand-induced activity of CAR

5-Month-old mice were injected intraperitoneally with the CAR agonist 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) at a dose of 3 mg/kg body weight, or vehicle (5% DMSO in sunflower oil), and killed 6 h later (Wei *et al*, 2000).

Electrophoretic mobility shift assays

Details and oligonucleotide sequences are given in Supplementary Methods/Tables.

Chromatin immunoprecipitation

Nuclei were prepared from livers and chromatin crosslinking, sonication and immunoprecipitation were performed as described in Gresh *et al* (2004), except that nuclei from different animals were not pooled. After sonication, the soluble chromatin was precleared and subjected to immunoprecipitation using α-HNF4α (sc-6556) or α-IL-1ra (sc-8482) antibody in the presence of 1 μg/ml salmon sperm DNA and 1 mg/ml BSA. Immune complexes were collected by adsorption to protein G-Sepharose (Sigma). Relative enrichments at HNF4α-binding sites were determined by real-time PCR using the standard curve method and were normalized to CAR gene 3′UTR, which is devoid of HNF4α-binding sites. The same enrichments were obtained relative to a nonrelevant sequence within the β-actin promoter (not shown). Primer sequences are given in Supplementary Table.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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