

Expression of apolipoprotein C-IV is regulated by Ku antigen/peroxisome proliferator-activated receptor γ complex and correlates with liver steatosis^{☆,☆☆}

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Background/Aims: We previously reported that hepatitis C virus (HCV) core protein up regulated transcription of apolipoprotein C-IV (ApoC-IV, 10.7-fold increase), a member of the apolipoprotein family implicated in liver steatosis. Here, we identified host transcription factors regulating the ApoC-IV gene expression.

Methods: Transcriptional regulators were identified by DNA affinity purification and steatosis was detected by oil red O staining and triglyceride assay.

Results: We defined a 163-bp ApoC-IV promoter as a core protein responsive element, and identified Ku antigen complex (Ku70 and Ku80) as well as nuclear receptors PPAR γ /RXR α as key regulators of ApoC-IV gene expression. Both Ku70 overexpression and PPAR γ agonist significantly increased ApoC-IV promoter activity; conversely, Ku70 silencing or mutation of PPAR γ binding site diminished the ApoC-IV promoter activity. Interestingly, transient transfection of ApoC-IV cDNA into a human hepatoma cell line was able to trigger moderate lipid accumulation. In agreement with this *in vitro* study, ApoC-IV transcript level was increased in HCV infected livers which correlated with triglyceride accumulation.

Conclusions: ApoC-IV overexpression may perturb lipid metabolism leading to lipid accumulation. HCV core protein may modulate ApoC-IV expression through Ku antigen and PPAR γ /RXR α complex.

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Keywords: Apolipoprotein; Gene regulation; Liver steatosis; Viral infection

1. Introduction

Liver steatosis is defined by cytoplasmic accumulation of lipid droplets. It is triggered by a variety of host and

environmental factors including obesity, diabetes, and alcohol consumption. In addition, chronic infection with hepatitis C virus (HCV) is also associated with increased prevalence of liver steatosis from 20% to 30% found in

Received 28 January 2008; received in revised form 1 June 2008; accepted 18 June 2008; available online 7 September 2008

Associate Editor: C.P. Day

* Data Deposition: GeneBank Accession Nos: EF371377 and EF371378. NIH funded study (DK066950, CA109733, CA95490, CA35711, AA08169, and AA20666).

☆☆ The authors who have taken part in the research of this paper declared that they do not have a relationship with the manufacturers of the materials involved either in the past or present and they did not receive funding from the manufacturers to carry out their research.

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Abbreviations: ApoC-IV, apolipoprotein C-IV; PPAR, peroxisome proliferators-activated receptor; RXR, retinoid X receptor; VLDL, very low density lipoprotein; SEAP, secreted alkaline phosphatase; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; RT-PCR, reverse transcription-polymerase chain reaction; Dox, doxycycline; HNF, hepatocyte nuclear factor; PPRE, PPAR responsive element.

the general population to 55% [1,2]. It has been reported that sustained response to antiviral therapy led to disappearance of fat from the liver, whereas a relapse of HCV infection correlated with recurrence of steatosis [1,3], thus highlighting the role of virus replication in the induction of steatosis. The HCV expresses three structural proteins (core, E1, and E2) and seven non-structural proteins (p7, NS2, NS3, NS4A, 4B, NS5A, and 5B) [4]. Steatosis can be induced experimentally in the mouse model by isolated expression of HCV structural proteins [5], or just the core protein [6].

As a major component of the HCV nucleocapsid, the core protein is required for viral genome packaging and virion formation. Interestingly, it is localized to triglyceride-rich lipid droplets in cytoplasm whether in transfected cell lines or in hepatocytes of HCV-infected chimpanzees [7,8]. The core protein has been reported to physically interact with apolipoprotein AII [9] and apolipoprotein B containing lipoproteins [10], both implicated in the storage of triglyceride inside hepatocytes. Indeed, transgenic mice expressing core protein were impaired in the function of microsomal triglyceride transfer protein and secretion of very low density lipoprotein (VLDL); they also manifested intrahepatic triglyceride accumulation [11]. Similarly, transfection of the core protein coding sequence into the Huh-7 hepatoma cell line led to intracellular triglyceride accumulation [12].

Recently, we performed microarray analysis of Huh-7 cells transiently transfected with HCV core gene, and observed transcriptional up regulation of a significant number of genes responsible for fat/lipid metabolism [13]. Of particular interest was a marked up regulation of ApoC-IV, a member of apolipoprotein family that is frequently overexpressed in steatotic livers [14] and HCV-associated HCC samples [15]. In the present study, we identified host transcription factors regulating the ApoC-IV gene expression, evaluated effect of HCV core protein on ApoC-IV promoter activity, and measured ApoC-IV transcripts in HCV infected liver samples. We demonstrate that ApoC-IV was regulated by Ku antigen/PPAR γ and its overexpression could increase lipid accumulation in Huh-7 cells.

2. Materials and methods

2.1. ApoC-IV promoter and expression constructs

A 1.7-kb DNA fragment upstream of the ApoC-IV coding sequence was amplified from genomic DNA of Huh-7 cells using Expand High Fidelity PCR System (Roche Diagnostics, Indianapolis, IN), and cloned to pGL3-Basic luciferase reporter plasmid (Promega, Madison, WI). From this construct, a panel of 5' deletion mutants and a site-directed mutant of PPAR γ /RXR α binding site was generated by PCR. The 384-bp ApoC-IV coding sequence and 1830-bp Ku70 coding region were amplified by RT-PCR from total RNA extracted from Huh-7 cells and cloned into pcDNA3.1/Zeo^{-/+} vector (Invitrogen, Carlsbad, CA). All constructs were verified by DNA sequencing.

2.2. Cell culture and transfection

Culture of Huh-7 and Huh-7.5 cells and transfection of HCV replicons have been described [13]. Huh-7 Tet-on cells, which harbor HCV core coding sequence of 1b genotype under the transcriptional control of tetracycline regulatory elements (Li et al., unpublished), were cultured in tetracycline-free medium. Core protein expression was induced by 1 μ g/ml of doxycycline (Sigma, St. Louis, Missouri). A reporter plasmid for the secreted alkaline phosphatase (SEAP) was co-transfected to evaluate the transfection efficiency. Polyamine (Mirus, Madison, WI) was used as a transfection reagent for DNA constructs [13], and lipofectamine 2000 for siRNA transfection (Invitrogen, Carlsbad, CA). Differences among the groups were examined by Student's *t* test.

2.3. Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP)

EMSA was performed according to User Manual (ProteinOne, Bethesda, MD and Promega, Madison, WI). Chromatin was immunoprecipitated with antibodies against Ku70, Ku80 or irrelevant IgG (Active Motif, Carlsbad, CA). Presence of ApoC-IV promoter sequence in the purified DNA was verified by PCR.

2.4. Western blot and IP-Western blot analyses

Experiments were performed using standard procedures or as recommended by manufacturers. Antibodies: Ku70 and Ku80 (NeoMarkers, Fremont, CA), RNA polymerase II (Active Motif, Carlsbad, CA), actin (Sigma, St. Louis, Missouri), PPAR α , β , γ and histone-1 (Santa Cruz Biotechnology, Santa Cruz, CA), respectively.

2.5. Detection of ApoC-IV mRNA and triglyceride in liver samples

Liver tissues were obtained from the Tissue Bank at University of Minnesota. Total RNA was extracted (Invitrogen, Carlsbad, CA) followed by reverse transcription (Roche Diagnostics, Indianapolis, IN) and real time PCR detection of ApoC-IV mRNA (Qiagen, Valencia, CA) using primers listed in Table 1. The ApoC-IV mRNA in transfected Huh-7 cells was detected by RT-PCR (see Table 1 for primers). Triglyceride was detected by Oil-Red O staining and quantified by enzymatic assay which measures the concentration of glycerol release after hydrolysis of the triglyceride (Zen-Bio, Inc., Research Triangle Park, NC).

3. Results

3.1. Identification of a core protein responsive element in the ApoC-IV promoter

We cloned a 1.7-kb genomic sequence upstream of ApoC-IV gene to examine the molecular mechanism by which HCV core protein up regulates ApoC-IV gene transcription. The promoter was inserted to a luciferase reporter plasmid so as to measure the promoter activity by luciferase assay. Progressive shortening of the 5'-end led to fluctuation of luciferase expression (Fig. 1A) consistent with the presence of multiple positive elements (nt -1672 to -1355, -1223 to -1027, -644 to -472, and -333 to -240, -122 to -52) and negative regulatory sequences (nt -1355 to -1223, -847 to -644, -472 to -333, and -242 to -122). The impact of

Table 1
Primers used for PCR amplification

Gene	Sense	Anti-sense
<i>Real time RT-PCR</i>		
ApoC-IV	CCCCACCAAAGCTAAAGAT	GTTCACTACTGTCTCCAGCA
18S	CCGCAGCTAGGAATAATGGA	CCCTCTTAATCATGGCCTCA
<i>RT-PCR</i>		
ApoC-IV	AGAGGCCAGGAAGGAACCCTG	GCTGTCTTTGGATTTCGAGGAACCA
GAPDH	ATCATCAGCAATGCCTCCTGC	GCCATCAGCCACAGTTTCCCG
<i>ChIP</i>		
ApoC-IV	ATGTACTGTGCCTCCACTTTATG	CCTGTTTCTGAGGAGGGACATTTC
GAPDH	TACTAGCGTTTTACGGGCG	TCGAACAGGAGGAGCAGAGAGCGA

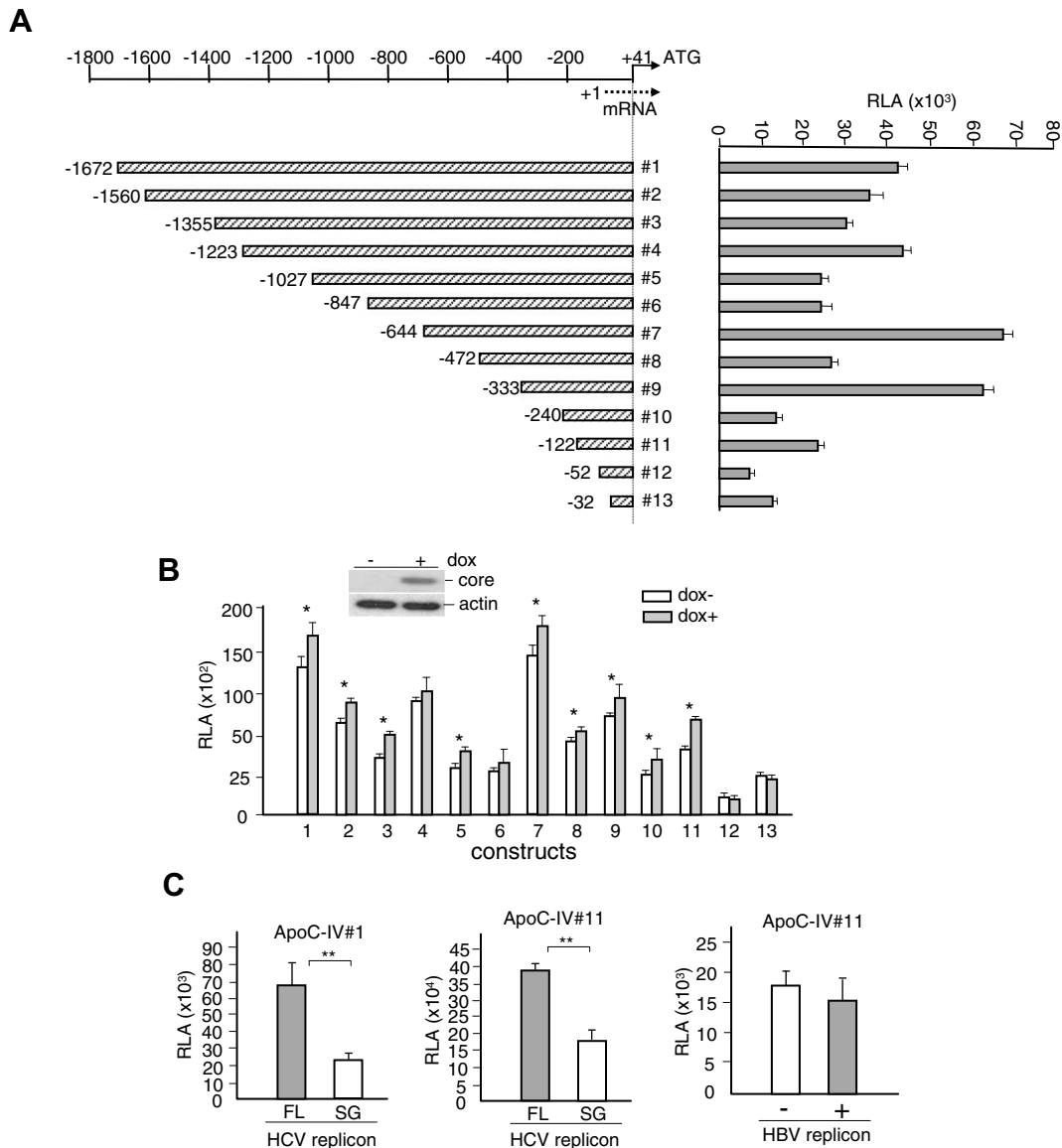


Fig. 1. ApoC-IV promoter activity and impact of core protein expression. (A) Schemes of human ApoC-IV promoter constructs (left panel) and relative luciferase activities (RLA) of transfected Huh-7 cells (right panel). Nucleotide positions are relative to the transcription initiation site. (B) Effect of core protein expression on luciferase activities. Transfected Huh-7 Tet-on cells were treated with doxycycline (dox) to induce core protein expression. (C) Effect of HCV and HBV replication on ApoC-IV promoter activity. *Left and middle panels:* Huh-7.5 cells were electroporated with HCV replicons. After an overnight culture, the cells were further transfected with the ApoC-IV promoter construct #1 or #11, followed by luciferase assay two days later. FL: full-length; SG: subgenomic replicon. *Right panel:* Huh-7 cells co-transfected with HBV and ApoC-IV promoter construct #11. Luciferase activity was measured at day 5 post-transfection (similar results were obtained at day 3). The replication defective mutant (–) contains a frameshift mutation in the core gene. For all the panels, relative luciferase activities are presented as mean ± SD (n = 4) and adjusted by transfection efficiency according to SEAP assay. *P < 0.05, **P < 0.005.

HCV core protein was determined by transfection of the reporter constructs to Huh-7 Tet-on cells with inducible core protein expression (1b genotype) (Fig. 1B). With the exception of constructs #12 and #13, core protein expression increased luciferase expression from all the promoter constructs (the difference did not reach statistical significance for constructs 4 and 6), suggesting its action through the proximal region of the ApoC-IV promoter.

We further examined the ApoC-IV promoter activity in the context of HCV replication. Huh-7.5 cells were electroporated with the full-length replicon of the 1b genotype or the subgenomic replicon that does not express the structural proteins. [13,16]. Indeed, the luciferase expression from both 1.7-kb (#1) and 163-bp (#11) promoter constructs tested were much higher in association with the full-length HCV genome than with the subgenomic fragment of 1b genotype (Fig. 1C, left

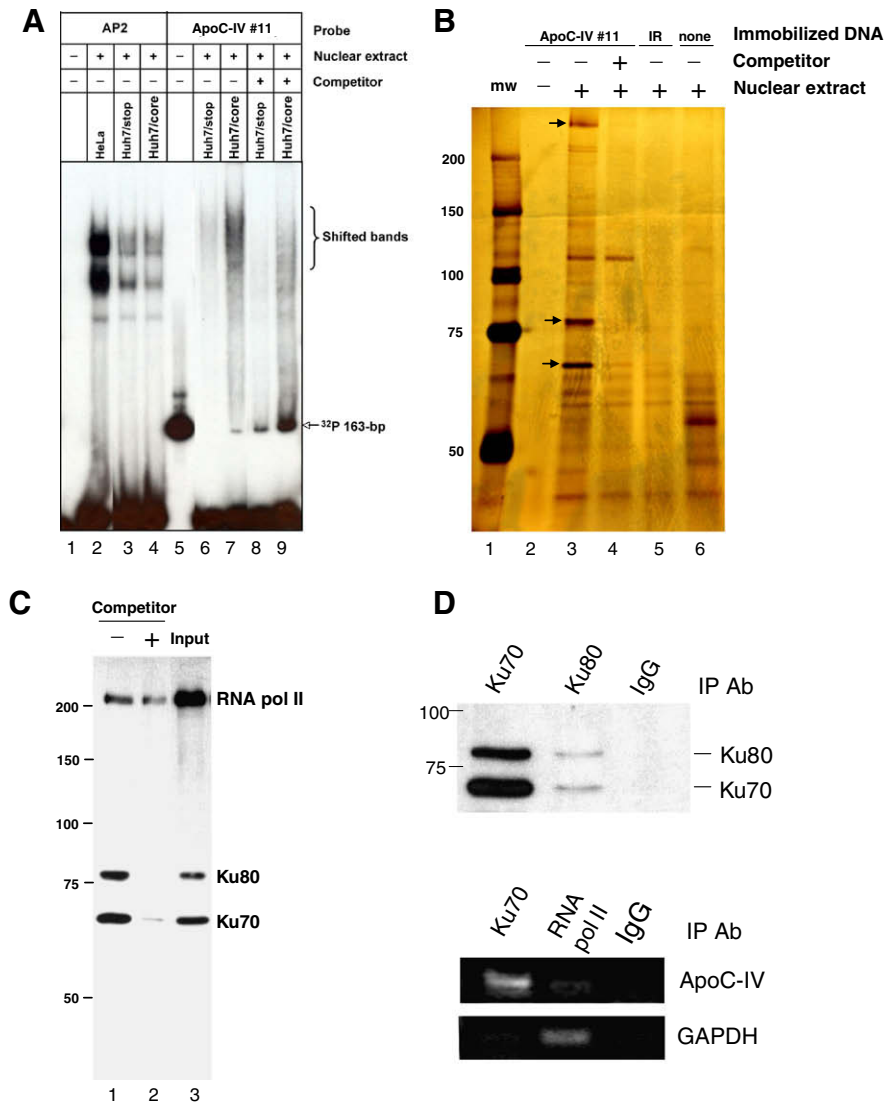


Fig. 2. Interaction of Ku antigen with the 163-bp ApoC-IV promoter (#11). (A) DNA/protein complex revealed by gel shift assay (EMSA). The [γ - 32 P]ATP-labeled 163-bp DNA was incubated with the nuclear extracts from Hela cells, or Huh-7 cells transfected with core gene or its null mutant (Huh7/stop). The [γ - 32 P]ATP-labeled DNA bearing AP2 binding site was assayed in parallel. (B) Nuclear proteins retained to the immobilized ApoC-IV promoter as revealed by SDS-PAGE and silver staining. Arrows indicate the 70-kDa, 80-kDa, and >200-kDa proteins. Control binding experiments were performed on empty beads (none) or with an irrelevant oligonucleotide (IR). Molecular Mass (MW) in kDa is indicated. (C) Western blot to confirm the retained 70 and 80-kDa proteins as Ku70 and Ku80. The blot was incubated with a mixture of antibodies against Ku70, Ku80, and RNA pol II. *Input*: Crude nuclear extracts. For all the three panels, the soluble 163-bp ApoC-IV promoter DNA was used as a competitor. (D) ChIP assay to validate *in vivo* association of Ku antigen with ApoC-IV promoter. Chromatin was immunoprecipitated with antibodies against Ku70, Ku80, or RNA pol II, or irrelevant IgG. Presence of Ku antigen in the precipitate was determined by Western blot analysis using anti-ku70 & ku80 antibodies (upper panel), while presence of ApoC-IV and GAPDH promoters was determined by PCR (lower panel).

Table 2
Peptide sequences of Ku70

Sequence	Location in Ku70
DSLIFLVDASK	36–46
ILELDQFK	116–123
DIISIAEDEDLR	219–230

The nuclear extracts of Huh-7 cells were preincubated with avidin–agarose beads (Pierce, Rockford, IL), followed by incubation with the biotinylated 163-bp ApoC-IV promoter immobilized on avidin–agarose beads. Bound proteins were separated in 0.1% SDS–8% PAGE and revealed by silver staining (Invitrogen, Carlsbad, CA). The protein bands were excised and sequenced at the Proteomic Core Facility of the Rhode Island Hospital.

and middle panels). In addition, the fold increase of the promoter activity observed in the context of the full-length HCV replication (Fig. 1C) was much higher than

those in cells expressing core protein alone (Fig. 1B), which was consistent with the higher expression level of the core protein in replicon transfected cells (data not shown). Similar results were also observed in Huh-7.5 cells infected with HCV of 2a genotype (data not shown). In contrast, replication of hepatitis B virus, another hepatotropic virus that does not induce steatosis, failed to stimulate the ApoC-IV promoter activity (Fig. 1C, right panel). These results are consistent with a role of the core protein in stimulating ApoC-IV promoter activity.

3.2. Core protein expression enhanced binding of Ku antigen to the 163-bp promoter fragment

Considering that construct #11 containing a 163-bp sequence was the minimum ApoC-IV promoter that

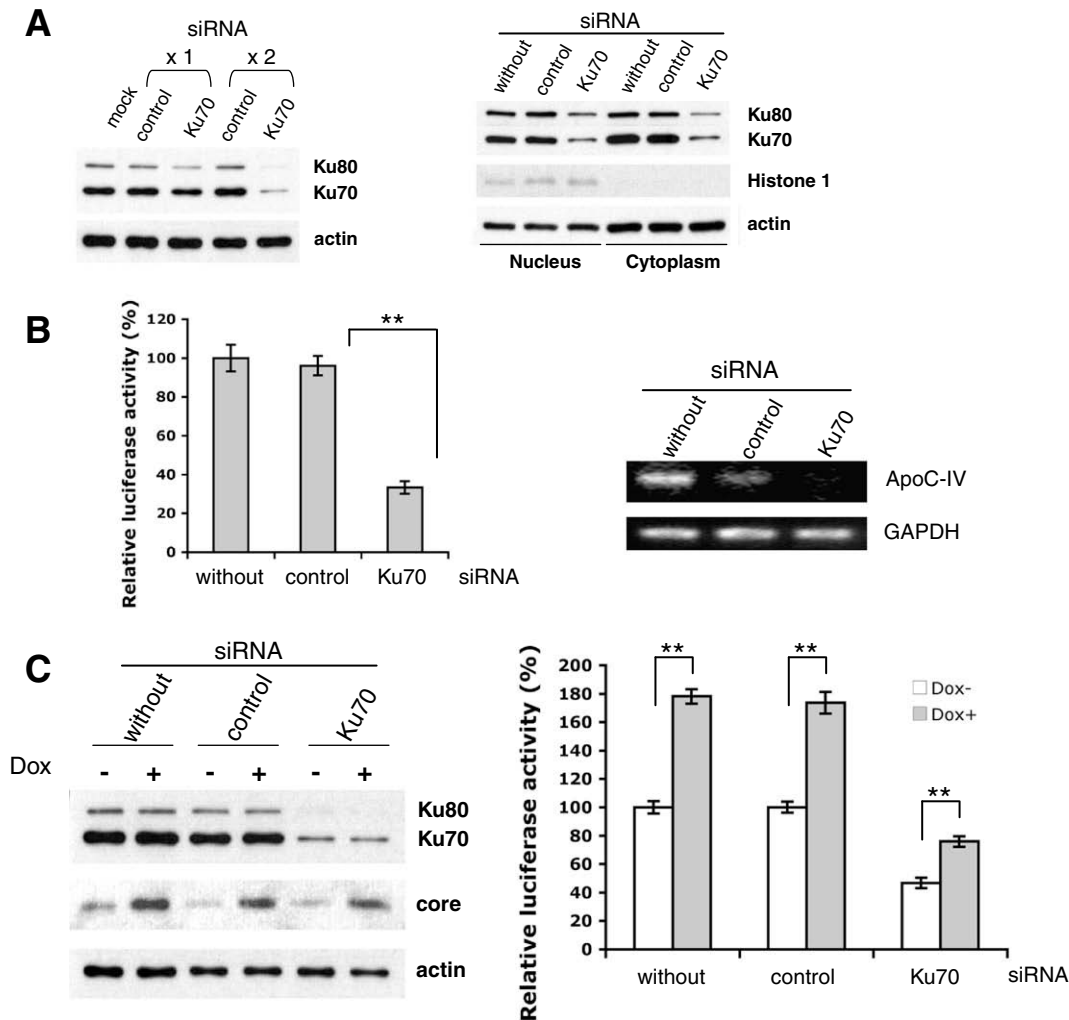


Fig. 3. Impact of Ku70 silencing on ApoC-IV transcription and promoter activity. (A) Western blot analysis of Ku70 and Ku80 in Huh-7 cell lysate. *Left panel:* Total cell lysate following once (1×) or twice (2×) transfection with siRNA. *Right panel:* Nuclear and cytoplasmic fractions after twice transfection with siRNA. The control siRNA targeted duck carboxypeptidase D. Actin served as a marker for loading control, while Histone 1 was a marker of nuclear proteins. (B) luciferase expression from the 163-bp ApoC-IV promoter (*left*) and endogenous ApoC-IV and GAPDH mRNA (*right*) from Huh-7 cells twice transfected with the siRNA. (C) Huh-7 Tet-on cells transfected twice with the Ku70 or control siRNA, with or without doxycycline (Dox) induction of core protein expression. *Left panel:* Western blot analysis. *Right panel:* Luciferase expression from the 163-bp ApoC-IV promoter. ***P* < 0.005.

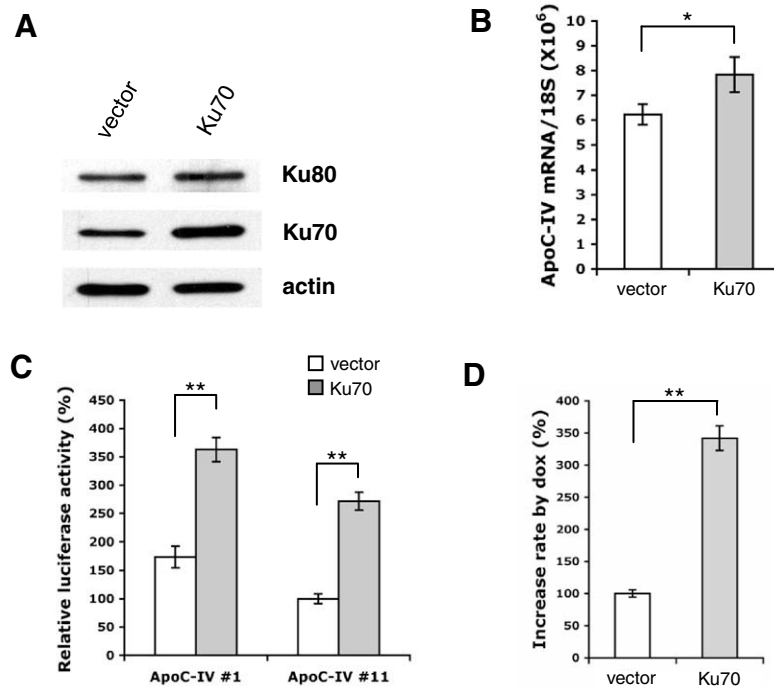


Fig. 4. Effect of Ku70 overexpression on ApoC-IV transcription and promoter activity in Huh-7 cells (A–C) and Huh-7 Tet-on cells (D). Cells were transfected with either pcDNA3.1 vector or Ku70 cloned to this vector (Ku70). (A) Western blot analysis of Ku70, Ku80, and actin. (B) RT-PCR detection of endogenous ApoC-IV mRNA. Data are presented as mean \pm SD ($n = 3$). * $P < 0.05$. (C) Luciferase expression from the 1.7-kb ApoC-IV promoter (#1) or 163-bp promoter (#11). (D) Luciferase expression from the 163-bp ApoC-IV promoter in the absence or presence of doxycycline. Data are presented as fold increase (Dox+ vs. Dox–) (mean \pm SD, $n = 4$). ** $P < 0.005$.

could be activated by HCV core protein, we employed this construct for most of the subsequent experiments. EMSA revealed binding of nuclear proteins, with stronger signals obtained from core expressing cells than from cells transfected with its null mutant (Fig. 2A, compare lanes 7 and 6). Pre-incubation with excess amount of cold 163-bp DNA reduced shifted bands (lanes 9 and 8). To identify the nuclear proteins that were associated with the proximal ApoC-IV promoter, the promoter was biotinylated and immobilized to agarose beads. SDS-PAGE and silver staining of retained proteins revealed three prominent proteins of 70-kDa, 80-kDa, and >200-kDa, which were markedly reduced by an excess of unbiotinylated 163-bp promoter fragment (Fig. 2B). Peptide sequencing revealed the 70-kDa species as Ku70, a protein involved in regulation of DNA replication and transcription (Table 2). Interestingly, Ku70 is known to dimerize with Ku80 to form Ku antigen, the regulatory subunit of a 460-kDa DNA-dependent protein kinase. Thus, the 80-kDa molecule could be Ku80, which was confirmed by Western blot analysis (Fig. 2C). Detection of RNA polymerase II in this complex reinforces the 163-bp fragment as a functional promoter engaged in mRNA transcription.

We next performed ChIP assay to confirm Ku antigen interaction with ApoC-IV promoter as part of chromosomal DNA. DNA was cross-linked to bound

nuclear proteins and sheared to fragments of ~ 500 bp prior to immunoprecipitation. Western blot analysis revealed precipitation of both Ku70 and Ku80 by either Ku70 or Ku80 antibody, but not by irrelevant IgG (Fig. 2D, upper panel). PCR amplification confirmed co-precipitation of ApoC-IV promoter sequence (nt -300 to -21) by the Ku70 antibody, but not the control IgG (Fig. 2D, middle panel). Consistent with active transcription, the ApoC-IV promoter could also be precipitated with an antibody to RNA polymerase II, albeit at reduced efficiency. The GAPDH promoter was immunoprecipitated by an antibody against RNA polymerase II, but not by Ku70 antibody (Fig. 2D, lower panel).

3.3. Ku antigen expression was critical for ApoC-IV gene transcription

The functional significance of Ku antigen in association with the ApoC-IV promoter was evaluated by gene silencing and overexpression. Twice transfection of Ku70 siRNA (30 nM) reduced Ku70 expression by >70%, while the control siRNA targeting duck carboxypeptidase D [17] was ineffective (Fig. 3A, left panel). Both cytoplasmic and nuclear pools of Ku70 were reduced (Fig. 3A, right panel). Consistent with the role of Ku70 in Ku80 stabilization, silencing of Ku70 also reduced Ku80 level. Importantly, the ApoC-IV pro-

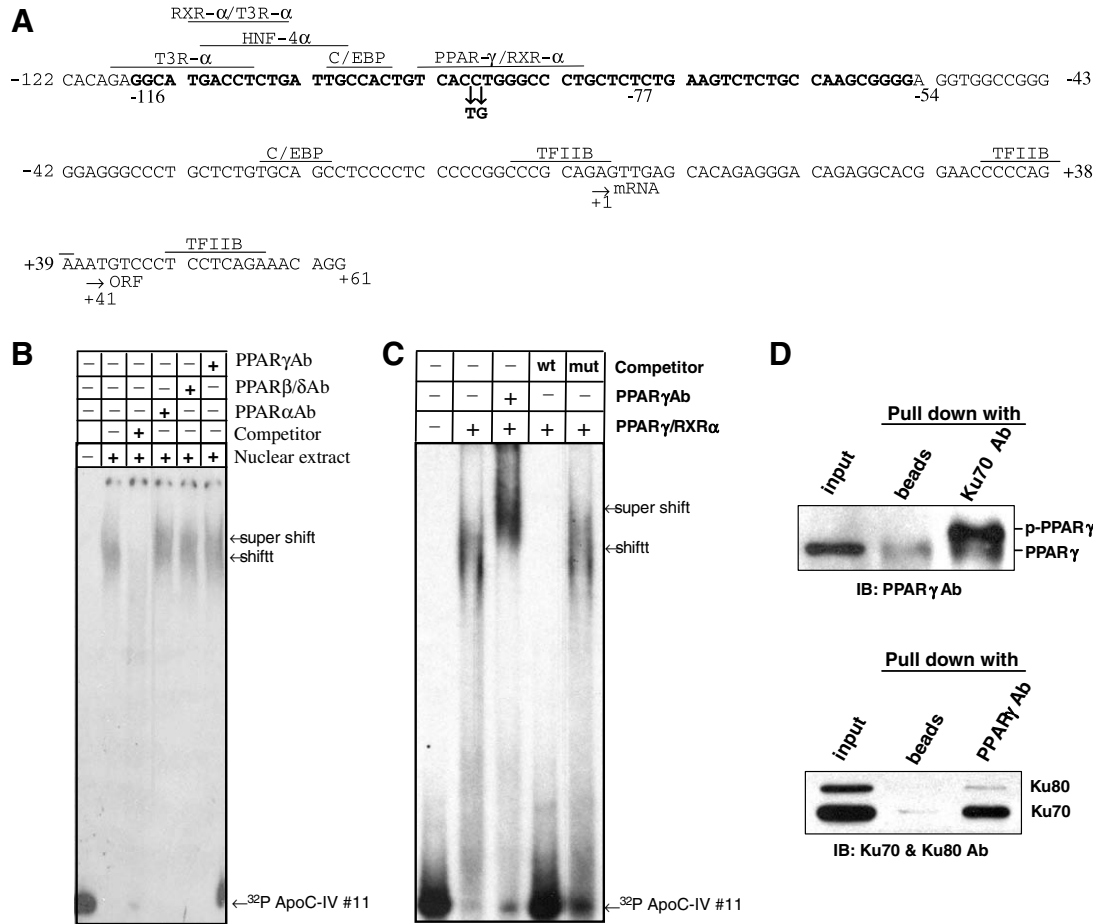


Fig. 5. PPAR γ /RXR α association with the 163-bp ApoC-IV promoter and complex formation with Ku70. (A) Predicted transcription factor binding sites on the 163-bp promoter by TRANSFAC and Genomatix database search. The start sites for transcription (mRNA) and translation (ORF) are indicated by arrows, as well as the two point mutations introduced into the predicted PPAR γ /RXR α binding site. Sequence in boldface (–116 to –54) is required for Ku antigen binding. (B) Binding of PPAR α , β/δ , and γ to the 163-bp ApoC-IV promoter as indicated by supershift assay using specific antibodies. (C) Gel shift assay to demonstrate binding of purified PPAR γ /RXR α to ApoC-IV promoter. Note that PPAR γ antibody supershifted DNA band. (D) IP-Western blot analysis to demonstrate association of Ku70 with phosphorylated PPAR γ (p-PPAR γ). Huh-7 cells were transfected with Ku70 expression plasmid. Cell lysate was immunoprecipitated with Ku70 antibody followed by Western blot detection of PPAR γ (upper panel), or immunoprecipitated with PPAR γ antibody followed by Western blot analysis of Ku70 and Ku80 (lower panel).

motor activity was reduced by 60% in Ku70 silenced cells in comparison to cells transfected with irrelevant siRNA (Fig. 3B, left panel). Similarly, level of the endogenous ApoC-IV mRNA, but not GAPDH mRNA, was reduced as a consequence of Ku70 silencing (Fig. 3B, right panel). In Huh-7 Tet-on cells, induction of core protein expression by Dox increased ApoC-IV promoter activity, while knockdown of Ku70 expression had the opposite effect (Fig. 3C). Although the promoter activity continued to increase after induction of the core protein expression, the fold increase was much reduced. Apparently, the residual Ku70 protein could still respond to core protein and stimulate ApoC-IV transcription.

In a complementary approach, transfection of Ku70 cDNA to Huh-7 cells moderately increased endogenous ApoC-IV mRNA and markedly stimulated luciferase expression from the 1.7-kb or 163-bp promoter construct (Fig. 4A–C). In Huh-7 Tet-on cells, overexpres-

sion of Ku70 greatly induced ApoC-IV promoter activity in the context of core protein expression (>3-fold) (Fig. 4D). Taken together with data from Ku70 knockdown, these findings implicate Ku70 as an important mediator of transcriptional activation by core protein.

3.4. Ku70 formed a complex with nuclear hormone receptors PPAR γ and RXR α

Using TRANSFAC [18,19] and Genomatix database search, we identified other transcription factors/regulators that could potentially bind to the 163-bp ApoC-IV promoter, including PPAR/RXR- α , T3R- α , HNF-4, and C/EBP (Fig. 5A). These factors are important for transcriptional regulation of lipoproteins [20] and their binding sites at –120 to –80 of ApoC-IV promoter overlaps with the tentative Ku antigen binding

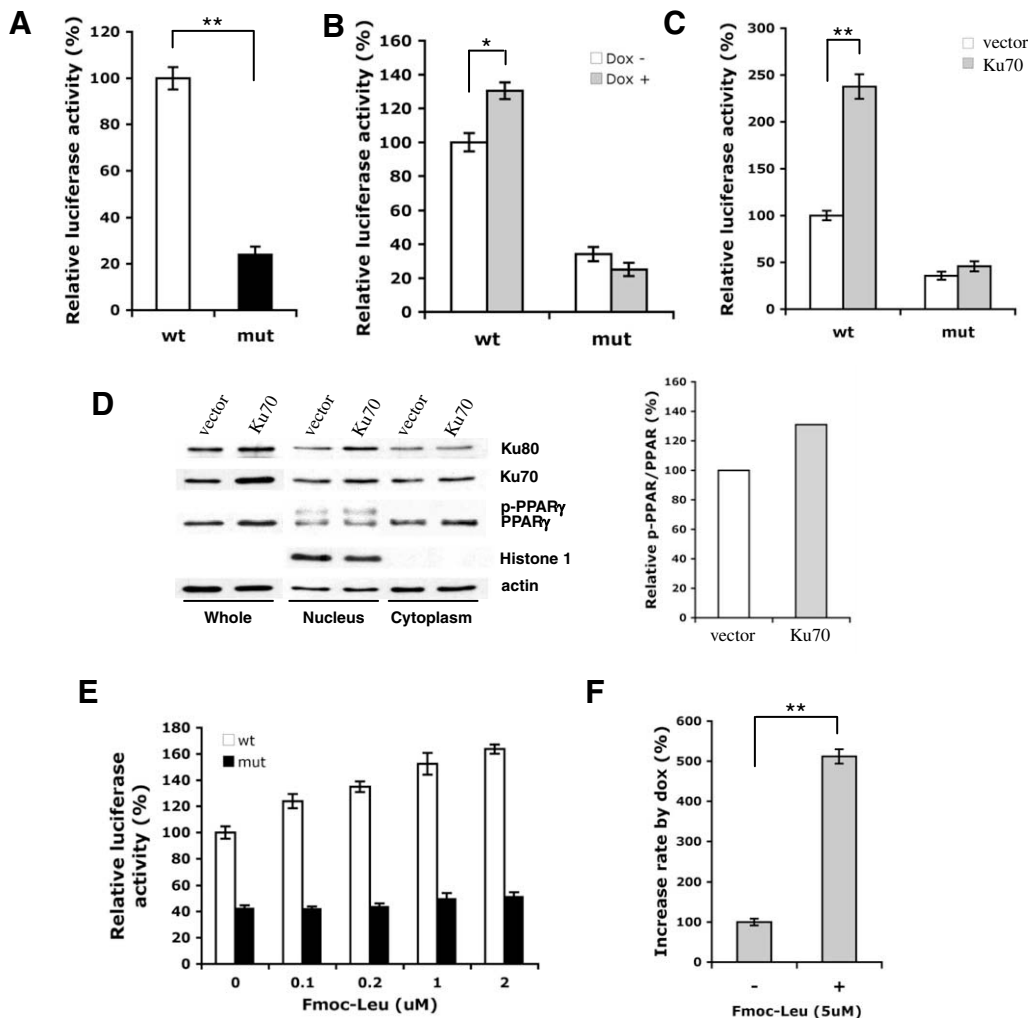


Fig. 6. Requirement of PPAR γ /RXR α for up regulation of ApoC-IV promoter activity by core protein and Ku antigen. (A) Effect of mutating PPAR γ /RXR α binding site on ApoC-IV promoter activity. Huh-7 cells were transfected with #11 ApoC-IV promoter constructs containing the wild-type (wt) sequence or mutated (mut) PPAR γ /RXR α binding motif, and luciferase activity was measured two days later. (B) HCV core protein activated ApoC-IV promoter containing the wild-type but not mutated PPAR γ /RXR α consensus sequence. Core protein was induced in Huh-7 Tet-on cells by doxycycline. (C) Overexpression of Ku70 activated wild-type but not mutated ApoC-IV promoter. (D) Overexpression of Ku70 increased phosphorylated PPAR γ in the nucleus. The relative intensity of the bands of phosphorylated vs. non-phosphorylated PPAR γ is quantified in the right panel using KODAK ID image software. (E) Activation of ApoC-IV transcription by a PPAR γ agonist. Huh-7 cell were transfected with ApoC-IV promoter containing wild-type or mutated PPAR γ /RXR α -binding site, and Fmoc-Leu was added in various concentrations followed by luciferase assay two days later. (F) Fold change of ApoC-IV promoter activity in core protein induced (+) vs. non-induced (-) Huh-7 Tet-on cells in the presence of 5 μ M Fmoc-Leu. For all experiments, luciferase activity is presented as mean \pm SD ($n = 4$). ** $P < 0.005$.

site (our unpublished observation). EMSA revealed interaction of the 163-bp ApoC-IV promoter with all three members of PPAR family (α , β/δ , and γ) present in the nuclear extracts of Huh-7 cells (Fig. 5B), or as purified proteins (ProteinOne PPAR Triad kit, Supplementary Fig. 1). Since PPAR γ has been reported to contribute to hepatic steatosis and triglyceride clearance [21], experiments were performed to examine its relevance in ApoC-IV promoter regulation. We found that binding of PPAR γ to the ApoC-IV promoter was inhibited by an excess of unlabeled promoter DNA but not by a mutated form with two nucleotide substitutions in the consensus sequence of the PPAR γ /RXR α binding site (Fig. 5C, two right lanes). Moreover, immunopre-

cipitation experiments using lysate of Huh-7 cells transfected with Ku70 expression construct revealed that PPAR γ , especially the phosphorylated form involved in transcription [22–24], were efficiently precipitated with the Ku70 antibody (Fig. 5D, upper panel); reciprocal precipitation with an anti-PPAR γ antibody pulled down both Ku70 and Ku80 (Fig. 5D, lower panel).

3.5. Activation of ApoC-IV promoter by HCV core protein and Ku antigen was mediated by PPAR γ /RXR α

Experiments were conducted to determine whether PPAR γ /RXR α binding to the ApoC-IV promoter was required for transcription, and whether the core protein

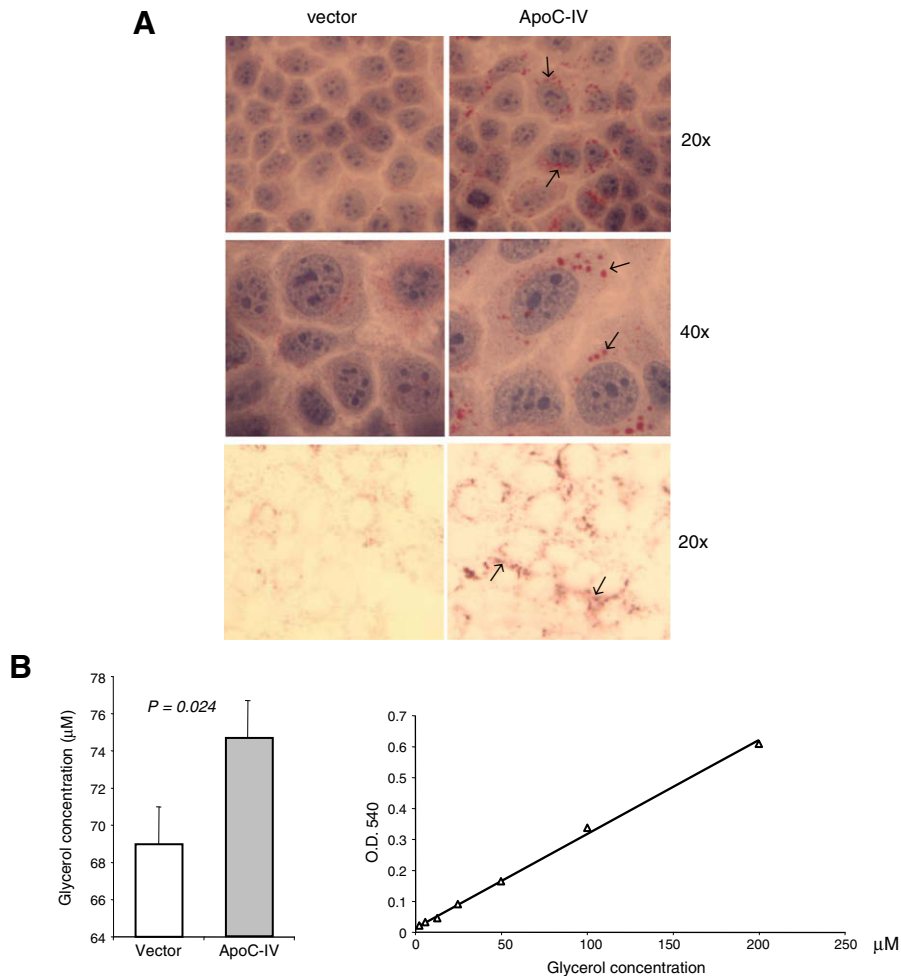


Fig. 7. Triglyceride accumulation in transfected Huh-7 cells. (A) Oil-Red O staining of cells transfected with the ApoC-IV cDNA or empty vector. *Upper and middle panels:* Cells cultured in FBS-free medium with additional H&E staining. *Lower panel:* cells cultured in delipidated medium. Arrows indicate lipid accumulation. (B) Enzymatic assay of triglyceride in transfected cells (*left panel*) and standard curve (*right panel*).

activated ApoC-IV promoter through Ku antigen, which in turn relied on complex formation with PPAR γ /RXR α . We found that double nucleotide substitution in the core sequence of the PPAR γ /RXR α binding site (from ACCT to ATGT, see Fig. 5A) markedly impaired ApoC-IV promoter activity (Fig. 6A), and abrogated stimulatory effect of the core protein (Fig. 6B). Moreover, the mutated promoter was no longer activated by Ku70 overexpression (Fig. 6C). Interestingly, overexpression of Ku70 slightly increased PPAR γ protein level in the cytoplasm and the phosphorylated form in the nucleus, the active form involved in transcription (Fig. 6D) [22–24]. In addition, application of Fmoc-Leu, a PPAR γ activator, increased the ApoC-IV promoter activity in a dose-dependent manner (Fig. 6E). In the presence of Fmoc-Leu, core protein expression increased ApoC-IV promoter activity by five-fold (Fig. 6F). Thus, PPAR γ /RXR α served as a critical transcription factor(s) in ApoC-IV gene expression. It mediated transcriptional up regulation by HCV core protein and Ku antigen.

3.6. Overexpression of ApoC-IV protein in cell culture led to triglyceride accumulation

In order to test the role of ApoC-IV protein in lipid accumulation, we cloned its cDNA from Huh-7 cells. Following a transient transfection of the ApoC-IV cDNA, Huh-7 cells were subsequently cultured in serum-free medium or medium containing delipidated FBS to reduce possible influence of lipids from culture medium. With serum-free medium, cells transfected with the ApoC-IV cDNA displayed lipid droplets of granule-like structure in the cytoplasm and perinuclear space, distinct from the weak staining and homogenous distribution observed in cells transfected with the empty vector (Fig. 7A, upper and middle panels). A similar difference was observed in cells cultured with medium containing delipidated FBS (Fig. 7A, lower panel). The enzymatic assay revealed a moderate increase in triglyceride in cells transfected with ApoC-IV gene as compared to mock transfected cells (Fig. 7B, left, $p = 0.024$). Considering the transfection efficiency of about 40%, the

triglyceride content in ApoC-IV transfected cells was approximately 2.7-fold higher than that in vector transfected cells.

3.7. Elevation of ApoC-IV transcript in HCV infected livers

To determine the relevance of ApoC-IV up regulation to lipid accumulation *in vivo*, we examined ApoC-IV mRNA levels and triglyceride content in nine HCV infected cirrhotic livers and four normal livers. Despite individual variations, the mean ApoC-IV mRNA level was higher in HCV infected livers (6.2 ± 4.2) than in the control group (3.7 ± 2.6) (Fig. 8A, left panel). Importantly, the ApoC-IV transcript levels correlated with the concentration of intracellular triglycerides in the HCV infected livers as revealed by a coefficient of determination ($R^2 = 0.78$) (Fig. 8A, right panel), although no such correlation was found in the normal livers. In the liver tissues with high level of ApoC-IV mRNA, severe steatosis was detected, as indicated by wide intracellular distribution of lipid droplets

(Fig. 8B, right panel). In contrast, accumulation of lipid droplets was mild and sporadic in the liver tissues with low level of ApoC-IV mRNA (Fig. 8B, middle panel). Taken together, these results demonstrate that the ApoC-IV gene expression level is highly associated with liver steatosis *in vivo* in HCV infected livers, and overexpression of this protein alone could trigger triglyceride accumulation (or fusion of lipid droplets) in hepatocytes cultured *in vitro*.

4. Discussion

Hepatitis C virus infection has been recognized as one of the key risk factors for liver steatosis. Accumulating evidence implicates ApoC-IV in the development of liver steatosis. The transcription of ApoC-IV gene has been found up regulated in steatotic liver [14], HCV related HCC samples [15], and human liver cell line expressing HCV core protein [13]. In the present study, we found correlation of ApoC-IV expression with the extent of steatosis in HCV infected liver. We also demonstrated

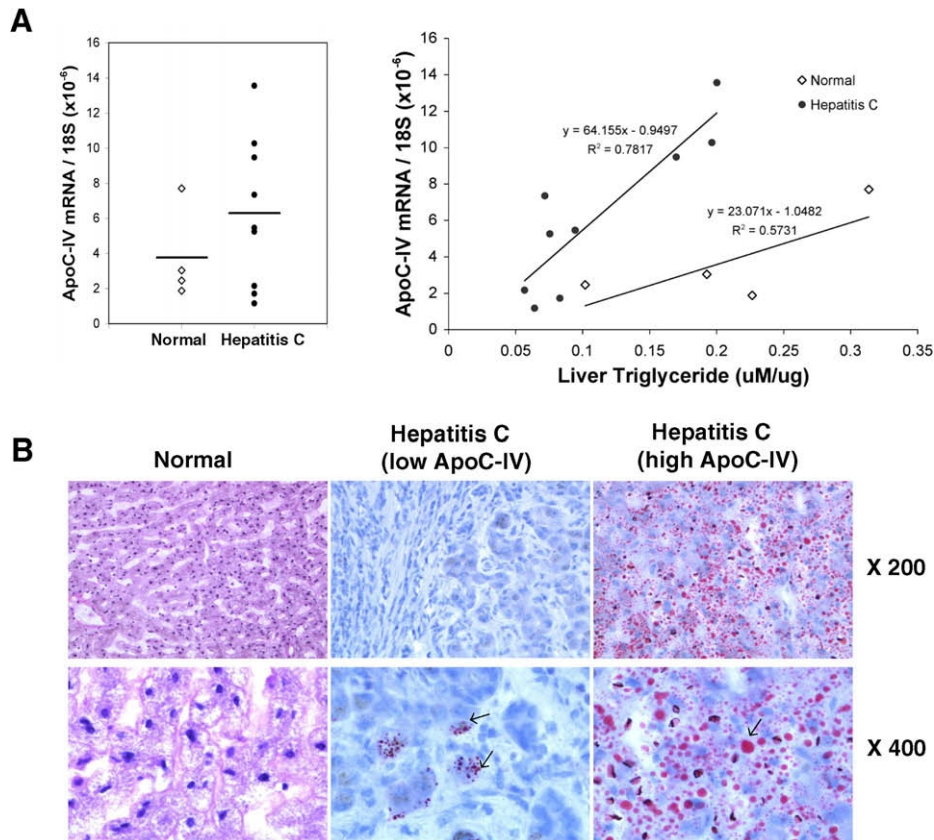


Fig. 8. Correlation of ApoC-IV mRNA level with triglyceride concentration in HCV infected livers. Nine HCV infected liver tissues were collected at the time of liver transplantation (age: 50.1 ± 4.2 ; sex: 8 males, 1 female; HCV viral titer: 2.8×10^3 to 4.9×10^5 i.u./ml; genotype: 4 1a, 2 1b, 1 3a, and 2 ND). Four normal liver tissues were included as controls (age 24.5 ± 13.8 ; sex: 1 male, 3 females). (A) ApoC-IV mRNA/18S ribosomal RNA (*left panel*) and its correlation with triglyceride concentration (*right panel*). The coefficient of determination (R^2) and equation are indicated. Open diamond: normal liver. Dark circle: HCV-infected liver. Horizontal bar: average value. (B) Oil-Red O staining of liver sections. The left, middle, and right panels represent normal liver, HCV infected liver with low and high ApoC-IV mRNA level, respectively. Arrows indicate lipid droplets. Note that the genotype difference in triglyceride accumulation was not observed due to limited samples examined.

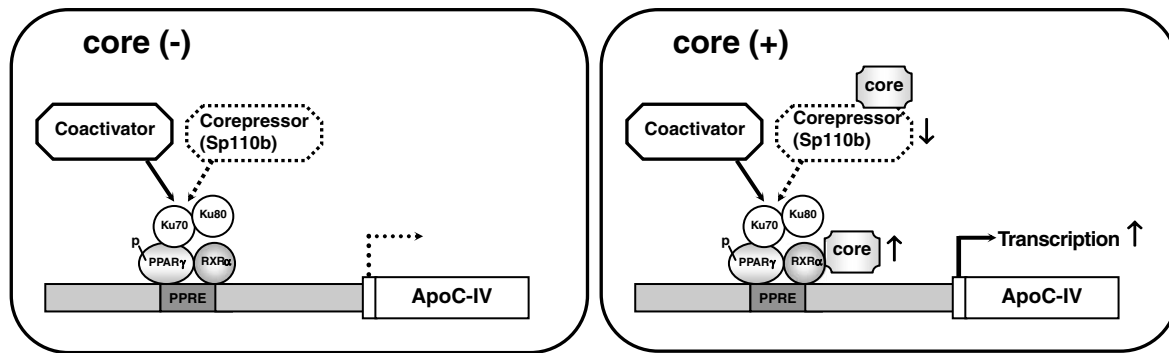


Fig. 9. Proposed model of transcriptional activation of the ApoC-IV gene by the HCV core protein. In the absence of HCV infection, the ApoC-IV gene is tightly controlled by specific transcription factors/regulators. The heterodimer PPAR γ /RXR α binds to the proximal promoter via PPRE site where it recruits other transcriptional regulators including Ku70/Ku80 and coactivator(s), corepressor (e.g., Sp110b). In the presence of HCV infection, viral core protein may enhance Ku70 and PPAR γ /RXR α mediated transcription by its direct binding to RXR α in the nucleus thus activating its activity [26]. Alternatively, the core protein can also bind to Sp110b in the cytoplasm to prevent its nuclear translocation and function as a corepressor [27].

that ApoC-IV overexpression in Huh-7 cells triggered moderate triglyceride accumulation.

The transcriptional control of the human ApoC-IV has not been investigated before. In the present study, we identified both positive and negative elements in the ApoC-IV promoter and established a 163-bp fragment as the minimum promoter. This region also contains a responsive element to HCV core protein. Through EMSA and ChIP technique, we identified Ku antigen (Ku70 and Ku80) as nuclear proteins associated with the minimum ApoC-IV promoter. Ku antigen regulates transcription of a variety of viral and cellular genes such as murine mammary tumor virus, heat shock protein 70, glycophorin B, and NF- κ B1/p50 in a positive or negative manner. We found silencing of Ku70 diminished ApoC-IV transcription, whereas transfection of Ku70 cDNA enhanced ApoC-IV transcription. Thus, the Ku antigen is a positive transcriptional regulator of ApoC-IV gene expression.

Transcriptional regulation of apolipoproteins involves both liver-specific transcription factors such as hepatocyte nuclear factors (HNF-1, 3, and 4) and hormone nuclear receptors (PPAR/RXR α) [20]. PPAR γ plays an essential role in adipogenesis, glucose homeostasis, lipid metabolism as well as development of hepatic steatosis. We found all three members of PPAR in the presence of RXR α could directly bind to the minimum ApoC-IV promoter. Two lines of evidence indicate critical role of PPAR γ /RXR α complex in transcriptional activation of the ApoC-IV promoter. First, mutating the PPAR γ /RXR α binding site in the minimum ApoC-IV promoter impaired reporter gene expression. Secondly, a PPAR γ agonist increased the ApoC-IV promoter activity and augmented core protein's ability to induce ApoC-IV promoter. Importantly, ApoC-IV promoter with mutated PPAR γ /RXR α site was no longer activated by Ku70 overexpression, suggesting the PPAR γ /RXR α complex works downstream of Ku

antigen. In this regard, phosphorylated PPAR γ , the nuclear PPAR γ involved in transcription [22–24], could interact with Ku70. Ku70 overexpression increased total PPAR γ level and phosphorylated PPAR γ in the nucleus (Fig. 6D). It is likely that Ku70 binding to PPAR γ leads to its phosphorylation via the DNA-dependent protein kinase associated with the Ku antigen, which has been found to phosphorylate several transcription factors such as p53, c-fos, and Ap1 [25]. Based on our current findings and work reported by others, a working model of ApoC-IV gene regulation by Ku antigen/PPAR γ complex was proposed (see Fig. 9) [26,27].

Acknowledgements

We are grateful to Dr. Charles Rice, Rockefeller University, New York, for providing replicon constructs and Huh-7.5 cells. This work was supported by grants DK066950, CA109733, CA95490, CA35711, AA08169, and AA20666 from the National Institutes of Health, and RSG 06-059-01-MCB from the American Cancer Society. E. Kim was supported by the Korea Research Foundation Grant from the Korean Government (MOEHRD) (KRF-2005-214-E00052). J. Li was a Liver Scholar of the American Liver Foundation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jhep.2008.06.029](https://doi.org/10.1016/j.jhep.2008.06.029).

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