

Opposite Regulation of the Human Apolipoprotein M Gene by Hepatocyte Nuclear Factor 1 and Jun Transcription Factors^{*§}

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HDL is a negative risk factor for atherosclerosis because of its multiple atheroprotective functions. Inflammation converts HDL particles from anti-atherogenic to pro-atherogenic, and this transformation is associated with changes in HDL structure and composition. Apolipoprotein M (apoM) has been recently shown to play a role in the maturation of HDL in plasma and to protect from atherosclerosis. ApoM gene is expressed primarily in the liver and kidney and is down-regulated by pro-inflammatory signals. We now show that the human apoM promoter harbors a dual specificity regulatory element in the proximal region that binds hepatocyte nuclear factor 1 (HNF-1) and members of the AP-1 family of pro-inflammatory transcription factors (c-Jun and JunB). Overexpression of c-Jun or JunB repressed both the basal and the HNF-1-mediated transactivation of the human apoM promoter. Treatment of HepG2 cells with potent inflammation-inducing phorbol esters or overexpression of PKC α was associated with a marked inhibition of apoM gene expression in a c-Jun/JunB-dependent manner. We provide evidence for a novel mechanism of inflammation-induced transcriptional repression that is based on the competition between HNF-1 and Jun proteins for binding to the same regulatory region. A similar mechanism accounts for the down-regulation of the liver-specific apolipoprotein A-II gene by Jun factors. Our studies provide novel insights on the mechanisms that control the expression of liver-specific apolipoprotein genes during inflammation and could affect the maturation and the functionality of HDL particles.

Levels of HDL cholesterol in plasma are inversely related to atherosclerosis susceptibility in humans (1–4). HDL is formed extracellularly by the interaction of apolipoprotein A-I (apoA-I)³ with ATP-binding cassette transporter A1 and is subsequently remodeled by various plasma enzymes to form the

mature spherical α -HDL particles (5–10). HDL has many atheroprotective functions including the removal of excess cholesterol from macrophages of the arterial wall, anti-inflammatory, and anti-thrombotic functions as well as protection of endothelial cells from apoptosis (11–14).

ApoM is a recently identified glycoprotein that belongs to the lipocalin protein superfamily (15–18). ApoM is secreted primarily by the liver and associates with HDL particles through its retained N-terminal signal peptide (19, 20). Lack of apoM expression in hepatocyte nuclear factor 1 α (HNF-1 α) knockout mice or in mice in which the endogenous apoM gene was silenced via small interfering RNA technology was associated with the loss of pre- β -HDL particles and the formation of larger HDL particles, suggesting that apoM may play a role in HDL remodeling (21). The role of apoM in atheroprotection is supported by studies showing that adenoviral overexpression of apoM in LDL receptor (–/–) mice or overexpression of apoM in the liver of transgenic mice reduced atherosclerosis development (21, 22). Furthermore, apoM-containing HDL particles isolated from human plasma or apoM transgenic mice have been shown to be more resistant to oxidation and more efficient in protecting against LDL oxidation as well as stimulating cholesterol efflux from macrophages (22, 23).

ApoM is expressed mainly in the liver and the kidney (24). The regulatory mechanisms that control human apoM gene transcription in the above tissues under healthy or disease states are not well understood. In the liver, apoM gene transcription has been shown to be controlled positively by HNF-1 α , liver receptor homolog 1 (LRH-1), and Forkhead box A2 transcription factors that bind to distinct sites on the proximal apoM promoter (25–27). We have shown recently that specific members of the hormone nuclear receptor superfamily occupy a proximal hormone response element and induce apoM gene transcription in response to retinoic acid, oxysterols, and fibrates, respectively (28). All of the above drugs have been shown to elevate HDL cholesterol levels in human or experimental animals.

ApoM, similar to other apolipoproteins such as apoA-I or apoC-III (29) is a negative acute response protein, and its levels decrease during infection and inflammation. Importantly, inflammation is associated with marked changes in HDL structure and functionality as demonstrated using HDL isolated from plasma of patients with chronic inflammatory diseases (30).

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§ The on-line version of this article (available at <http://www.jbc.org>) contains supplemental text, Tables S1–S4, and Figs. S1–S5.

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³ The abbreviations used are: apo, apolipoprotein; HNF, hepatocyte nuclear factor; LRH, liver receptor homolog; sh, short hairpin; PMA, phorbol 12-myristate 13-acetate; DNAP, DNA affinity precipitation.

Negative Regulation of ApoM by Jun Proteins

Key mediators of inflammatory responses are members of the AP-1 family of transcription factors. AP-1 proteins are dimeric leucine zipper transcription factors that bind as homo- or heterodimers to AP-1-responsive elements (5'-TGA(G/C)TCA-3') on the promoters of many genes (31, 32). Major inducers of AP-1 activity are pro-inflammatory cytokines, phorbol esters, and cAMP (31, 32). Phorbol esters are potent activators of PKC (33), a family of at least 11 isozymes that are involved in many biological processes in different tissues and cell types (34).

We show here that treatment of HepG2 cells with AP-1-inducing agents such as phorbol esters caused a potent down-regulation of apoM gene expression via activation of PKC and Jun (c-Jun and JunB) proteins. ApoM gene repression by Jun requires a novel AP-1 element in the proximal apoM promoter that is localized inside a previously characterized binding site for HNF-1 α (25) and is facilitated by the competition between HNF-1 α and Jun proteins for binding to the same regulatory site. A similar mechanism accounts for the inhibition of the apolipoprotein A-II gene that also plays a role in HDL metabolism and the pathogenesis of atherosclerosis (35).

EXPERIMENTAL PROCEDURES

Plasmid Constructions—The human apoM promoter constructs (–950/+42, –642/+42, –402/+42, –241/+42, –105/+42, and –49/+42)hapoM-luc were generated by PCR amplification of the corresponding fragments using human genomic DNA as template and subsequent cloning into the KpnI-HindIII sites of the pGL3basic vector (Promega). The apoM promoter construct (–20/+8)hapoM-luc was generated by ligation of a double-stranded oligonucleotide corresponding to the –20/+8 region of the human apoM promoter into the KpnI-HindIII sites of the pGL3basic vector. The (–950/+42)mut hapoM-luc, which bears mutations in the AP-1 site, was generated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The mouse apoM promoter construct (–761/–7)mapoM-luc was generated by PCR amplification of the corresponding fragment using mouse genomic DNA as template and subsequent cloning into the KpnI-HindIII sites of the pGL3basic vector. The sh-c-Jun, sh-JunB, sh-HNF-1 α , and sh-control producing vectors were generated by ligation of double-stranded oligonucleotides that contained the siRNA-expressing sequences targeting c-Jun, JunB, HNF1 α , or a scrambled sequence (small interfering control), respectively, into the BglII-HindIII sites of the pSuper.GFP/neo vector (Oligoengine, Seattle, WA). The sequences of all of the oligonucleotides are shown in [supplemental Table S1](#). Expression vectors for human c-Jun and JunB have been described previously (36).

Cell Culture, Transient Transfections, and Reporter Assays—Human hepatoma HepG2 cells, mouse Hep6-16 cells, and human embryonic kidney cells (HEK293T) were cultured in DMEM supplemented with 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin at 37 °C in a 5% CO₂ atmosphere. For the treatment of cells with PMA, the cells were serum-starved for 3 h and then stimulated with 100 or 200 nM PMA for various times. The PKC inhibitor Gö 6976 (1 μ M), the

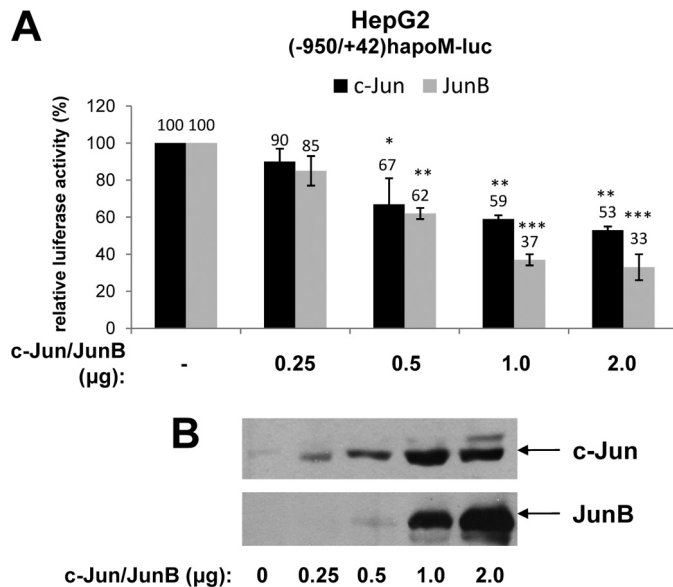


FIGURE 1. c-Jun and JunB are negative regulators of apoM promoter activity in HepG2 cells. *A*, HepG2 cells were transiently transfected with the (–950/+42)human apoM-luc reporter plasmid (1.0 μ g) along with increasing concentrations (0, 0.25, 0.5, 1.0, and 2.0 μ g) of a c-Jun or JunB expression vector as indicated. An expression vector for β -galactosidase (1.0 μ g) was included in each sample for normalization purposes. Luciferase activity was normalized to β -galactosidase activity and presented with histograms. Each value represents the average (\pm S.D.) from at least three independent experiments performed in duplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *B*, monitoring the expression of exogenous c-Jun or JunB in the experiment of *A* by immunoblotting using the corresponding antibodies.

JNK inhibitor SP600125 (10 μ M), or the histone deacetylase inhibitor trichostatin A (1 μ M) was added 1 h before the treatment with PMA. Transient transfections were performed using the Ca₃(PO₄)₂ co-precipitation method. Transient transfections of cells with the sh-RNA producing vectors were performed with the Lipofectamine™ 2000 reagent according to the manufacturer's instructions. Luciferase assays were performed using the luciferase assay kit from Promega according to the manufacturer's instructions. Normalization for transfection efficiency was performed by β -galactosidase assays.

ChIP Assays—Chromatin immunoprecipitation was performed as described previously (37) using chromatin from HepG2 cells and antibodies against JunB, c-Jun, or HNF-1 α . Immunoprecipitated chromatin was analyzed by PCR using primers shown in [supplemental Table S3](#). PCR products were analyzed by agarose gel electrophoresis.

Statistical Analysis—The results are shown as the means \pm S.D. Statistical significance was determined using the Student's *t* test. Differences with $p < 0.05$ were considered to be statistically significant.

RESULTS

Jun Proteins Inhibit ApoM Gene Transcription in Hepatic Cells—To investigate the role of Jun proteins in apoM gene regulation, we overexpressed two members of the Jun family (c-Jun and JunB) in HepG2 cells and monitored the activity of the –950/+42 human apoM promoter (28) by luciferase assays. This experiment showed that overexpression of c-Jun or JunB was associated with a potent and dose-dependent down-regulation of apoM promoter activity (Fig. 1). A similar observation

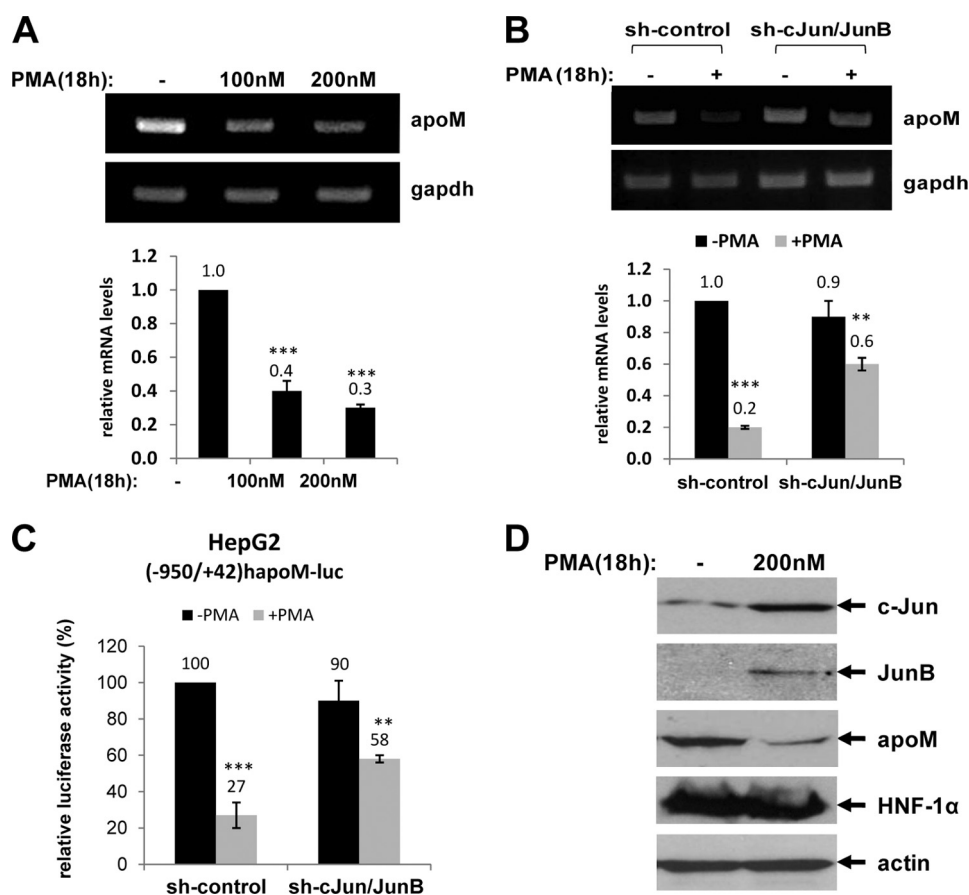


FIGURE 2. Inhibition of apoM gene expression by phorbol esters is mediated by Jun proteins. *A*, HepG2 cells were treated with increasing concentrations of PMA (100 and 200 nM) for 18 h or left untreated. Total RNA was extracted, and apoM mRNA levels were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. The normalized mRNA levels of the apoM gene are shown with a histogram. Each value represents the average from three independent experiments. *B*, HepG2 cells were transiently transfected with the sh-control or sh-c-Jun and sh-JunB producing vectors (2.0 μ g each). Following transfection, the cells were treated with 200 nM PMA for 18 h or left untreated. Total RNA was extracted, and apoM mRNA levels were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. The relative mRNA levels of the apoM gene were quantified and are shown as a histogram. Each value represents the average from three independent experiments. *C*, HepG2 cells were transiently transfected with the (–950/+42)human apoM-luc reporter plasmid (1.0 μ g) along with the sh-control or sh-c-Jun and sh-JunB producing vectors (2.0 μ g each), as indicated, and a β -galactosidase expression vector (1.0 μ g). Following transfection, the cells were treated with 100 nM PMA for 18 h or left untreated. Luciferase activity was normalized to β -galactosidase activity and presented with histograms. Each value represents the average (\pm S.D.) from at least three independent experiments performed in duplicate. *D*, HepG2 cells were treated with 200 nM PMA for 18 h or left untreated, and the protein levels of c-Jun, JunB, apoM, HNF-1 α , and actin (loading control) were determined by immunoblotting using the corresponding antibodies. The experiment was performed three times, and representative images are presented. **, $p < 0.01$; ***, $p < 0.001$.

was made using an additional member of the Jun family (JunD; data not shown).

To activate endogenous Jun proteins in HepG2 cells, we used PMA, a potent inducer of AP-1 gene transcription (38). As shown in Fig. 2*A*, PMA caused a strong and dose-dependent reduction in apoM mRNA levels, and this reduction was counterbalanced by overexpressing shRNAs targeting both c-Jun and JunB but not by a control shRNA (Fig. 2*B*). In agreement with these findings, PMA inhibited the activity of the –950/+42 apoM promoter, and this inhibition was counterbalanced by overexpressing shRNAs targeting both c-Jun and JunB but not by a control shRNA (Fig. 2*C*). The efficiency of c-Jun and JunB gene silencing by the corresponding shRNAs is shown in supplemental Fig. S1.

We also monitored the protein levels of Jun and apoM in HepG2 cells treated with 200 nM PMA for 18 h. As shown in Fig. 2*D*, PMA induced both c-Jun and JunB gene expression, and this was associated with a potent reduction in the protein levels of apoM. In conclusion, the data of Fig. 2 showed that phorbol

esters down-regulate apoM gene expression in HepG2 cells in a Jun-dependent manner.

Protein Kinase C α Inhibits apoM Gene Expression via Jun Proteins—Phorbol esters are potent activators of PKC (33, 34). Treatment of HepG2 cells with PMA caused an early (10 min) activation of PKC α , which was followed by an increase in total and phosphorylated forms of c-Jun at 1 h of treatment (supplemental Fig. 2, *A* and *B*). This activation of c-Jun by PMA was abolished by the JNK-specific inhibitor SP 600125 in agreement with previous studies showing that phorbol esters activate PKC and JNK, and this leads to an increase in c-Jun protein levels via a positive autoregulatory loop (39, 40).

To investigate the potential role of PKC in apoM gene regulation, we treated HepG2 cells with the PKC α/β -specific inhibitor Gö 6976 (41). Gö 6976 abolished the inhibition of apoM gene expression by PMA both at the mRNA (Fig. 3*A*) and the protein (Fig. 3*B*) levels. The PKC α/β inhibitor also abolished the activation of c-Jun gene expression by PMA (supplemental Fig. S2*C*). Furthermore, overexpression of PKC α in HepG2

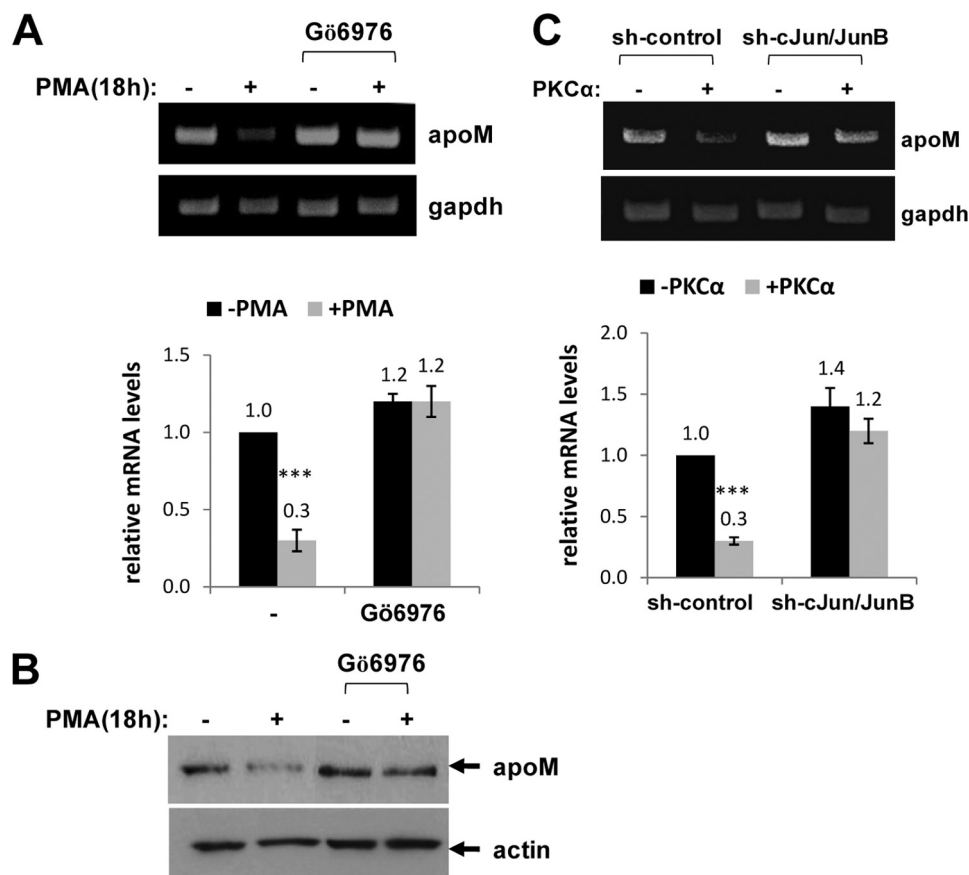


FIGURE 3. Inhibition of PKC abolished the down-regulation of the apoM gene expression by PMA. *A*, HepG2 cells were treated with PMA (200 nM) and/or the PKC α/β -specific inhibitor Gö 6976 (1 μ M) as indicated. 18 h later, total RNA was extracted, and apoM mRNA levels were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. The relative mRNA levels of the apoM gene were quantified and are shown as a histogram. Each value represents the average from three independent experiments. *B*, HepG2 cells were treated with PMA (200 nM) and/or Gö 6976 (1 μ M) for 18 h as indicated, and the protein levels of apoM and actin (loading control) were determined by immunoblotting using the corresponding antibodies. The experiment was performed three times, and representative images are presented. *C*, HepG2 cells were transiently transfected with the sh-control or sh-c-Jun and sh-JunB producing vectors (2.0 μ g each) along with an expression vector for PKC α (2.0 μ g) as indicated. Total RNA was extracted, and apoM mRNA levels were analyzed by RT-PCR. The mRNA levels of the GAPDH gene were determined for normalization purposes. The relative mRNA levels of the apoM gene were quantified and are shown as a histogram. Each value represents the average from three independent experiments. ***, $p < 0.001$.

cells was associated with a 70% reduction in the mRNA levels of the apoM gene, and this reduction was counterbalanced by shRNAs targeting *c-Jun* and *JunB* but not by a control shRNA (Fig. 3C). In conclusion, the combined data of Figs. 2 and 3 establish a PMA/PKC/Jun pathway operating in HepG2 cells that inhibit apoM gene transcription.

The -53/-47 Region of the apoM Promoter Contains an AP-1-responsive Element—To map the region of the apoM promoter that is responsible for the inhibition by Jun proteins, we utilized a panel of apoM promoter deletion mutants shown schematically in Fig. 4A (28). Transient transfections of HepG2 cells with these reporter plasmids in the absence or in the presence of expression vectors for *c-Jun* (Fig. 4B) or *JunB* (Fig. 4C) established that the minimal region of the apoM promoter that is required for Jun-mediated inhibition is defined by nucleotides -105 and -49.

Binding of endogenous *c-Jun* and *JunB* proteins to the apoM promoter *in vivo* was shown by chromatin immunoprecipitation assays in HepG2 cells. As shown in Fig. 5B, both *c-Jun* and *JunB* were recruited to the proximal (-214/-14) apoM promoter, although with different affinities (top row, lanes 3 and 4). In contrast, binding of both proteins to the distal region of the

apoM promoter (-950/-616) was not significant (Fig. 5B, middle row, lanes 3 and 4). As a control, we used primers that amplify the -313/-185 region of the promoter of the human *RhoB* gene (42), and we found no recruitment of Jun proteins to this region (Fig. 5B, bottom row, lanes 3 and 4).

To identify more precisely the putative AP-1 element on the proximal apoM promoter, a series of DNA affinity precipitation (DNAP) assays was performed. As shown in Fig. 5C, both *c-Jun* and *JunB* proteins bound to a biotinylated -105/+42 apoM promoter fragment. In contrast, no binding of *c-Jun* or *JunB* could be observed using a biotinylated -241/-81 apoM promoter fragment or a control biotinylated oligonucleotide corresponding to the -76/-43 region of the human *RhoB* promoter (Fig. 5C).

A close inspection of the DNA sequence of the proximal apoM promoter region between nucleotides -241 and +42 revealed the presence of a putative AP-1 element at position -53/-47 (Fig. 5A). This element contains the sequence 5'-TTACTCA-3' and differs from the consensus AP-1 site (5'-TGACTCA-3') (43) by only one nucleotide at the second position (T instead of a G). Interestingly, this putative AP-1 element resides within a previously characterized binding

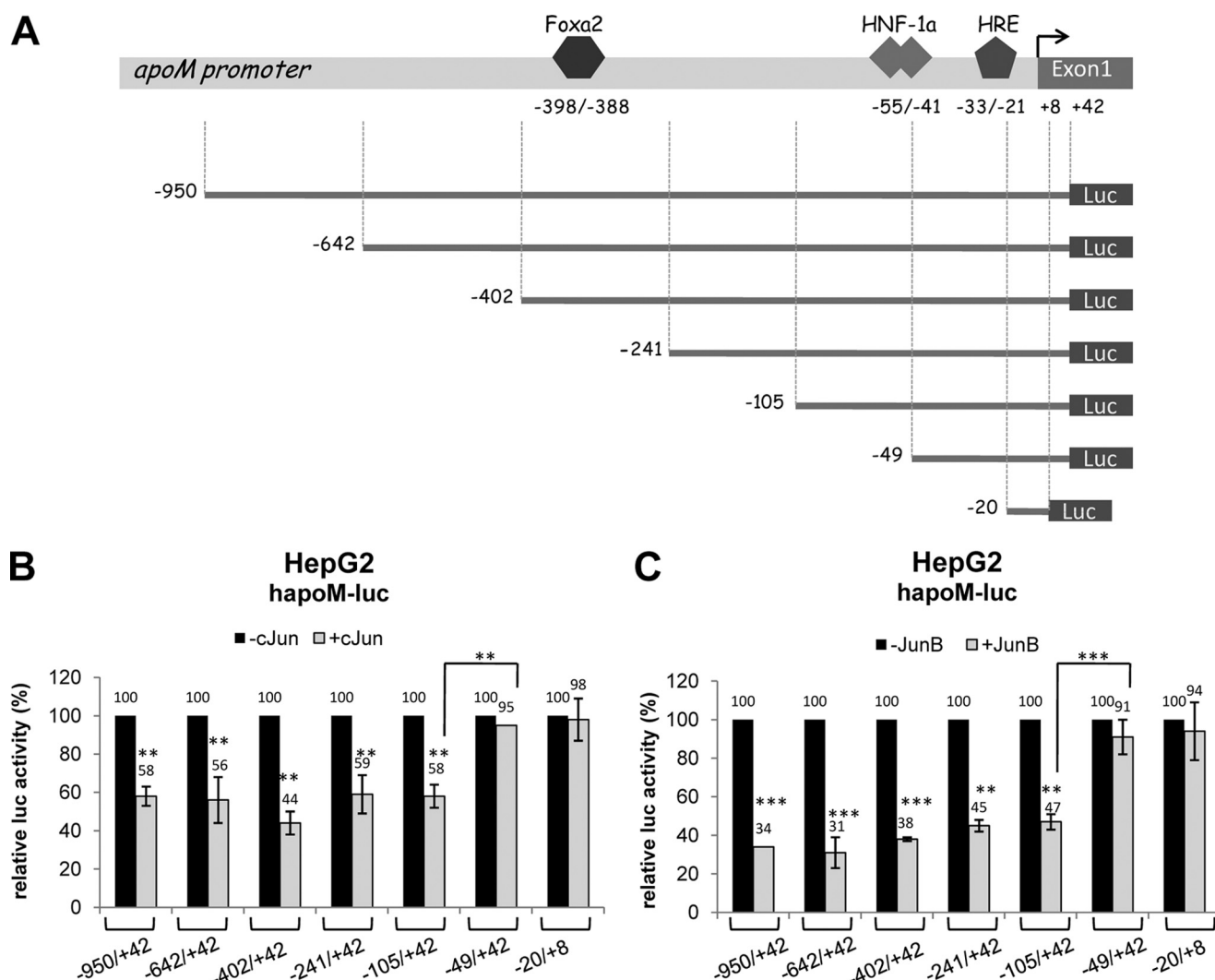


FIGURE 4. The proximal region $-105/-49$ of the human apoM promoter is required for transcriptional repression by c-Jun and JunB. A, schematic representation of the human apoM promoter fragments that were cloned upstream of the luciferase reporter gene and used in the transactivation experiments of B and C. The positions of previously described regulatory elements and factors (Forkhead box A2 (*Foxa2*), HNF-1 α , and hormone response element (*HRE*)) are also shown. B, HepG2 cells were transiently transfected with the luciferase reporter plasmids indicated at the bottom of the graph (1.0 μ g) along with a c-Jun expression vector (1.0 μ g) or an empty vector. C, HepG2 cells were transiently transfected with the luciferase reporter plasmids indicated at the bottom of the graph (1.0 μ g) along with a JunB expression vector (1.0 μ g) or an empty vector (1.0 μ g). In B and C, luciferase activity was normalized to β -galactosidase activity and presented with histograms. Each value represents the average (\pm S.D.) from at least three independent experiments performed in duplicate. **, $p < 0.01$; ***, $p < 0.001$.

site for HNF-1 α , which is located at position $-55/-41$ (Fig. 5A) (25).

Initially, we confirmed the binding of c-Jun and JunB to this site by performing DNAP assays using a biotinylated oligonucleotide corresponding to the $-61/-38$ region of the apoM promoter (Fig. 5D). As anticipated, HNF-1 α bound equally well to the same oligonucleotide as shown in Fig. 5E. To verify that the 5'-TTACTCA-3' element is a true AP-1-binding site, we introduced mutations at positions 5, 6, and 7 of this element (5'-TTACGGG-3') (Fig. 5A). As shown in Fig. 5D, neither c-Jun nor JunB could bind to the mutated oligonucleotide ($-61/-38$)mut. As expected, these mutations also abolished the binding of HNF-1 α (Fig. 5E).

We introduced the same nucleotide substitutions into the corresponding positions of the $-950/+42$ apoM promoter (Fig. 6A) and examined the activity of the mutant *versus* the wild type promoter in HepG2 cells by luciferase assays. At basal

conditions, the activity of the mutant $-950/+42$ apoM promoter was very low (8%) compared with the wild type promoter, confirming that this element is essential for apoM gene expression in hepatic cells (Fig. 6B). Importantly, mutations in the AP-1 element abolished the inhibition of apoM promoter activity by PMA (Fig. 6C) or by PKC α (Fig. 6D), strongly suggesting that this element, in addition to its importance for basal apoM expression, is essential for the regulation of the apoM gene by the PKC/Jun pathway.

We then showed that neither c-Jun nor JunB could repress the activity of the wild type $-950/+42$ apoM promoter in HEK293T cells that lack endogenous HNF-1 α protein (Fig. 7A). HNF-1 α caused a strong (40-fold) transactivation of the wild type apoM promoter but only a minor (4.4-fold) transactivation of the mutant apoM promoter (Fig. 7A). This finding is in agreement with the data of Fig. 5E, which showed that the mutations abolished the binding of HNF-1 α to the apoM promoter. More

Negative Regulation of ApoM by Jun Proteins

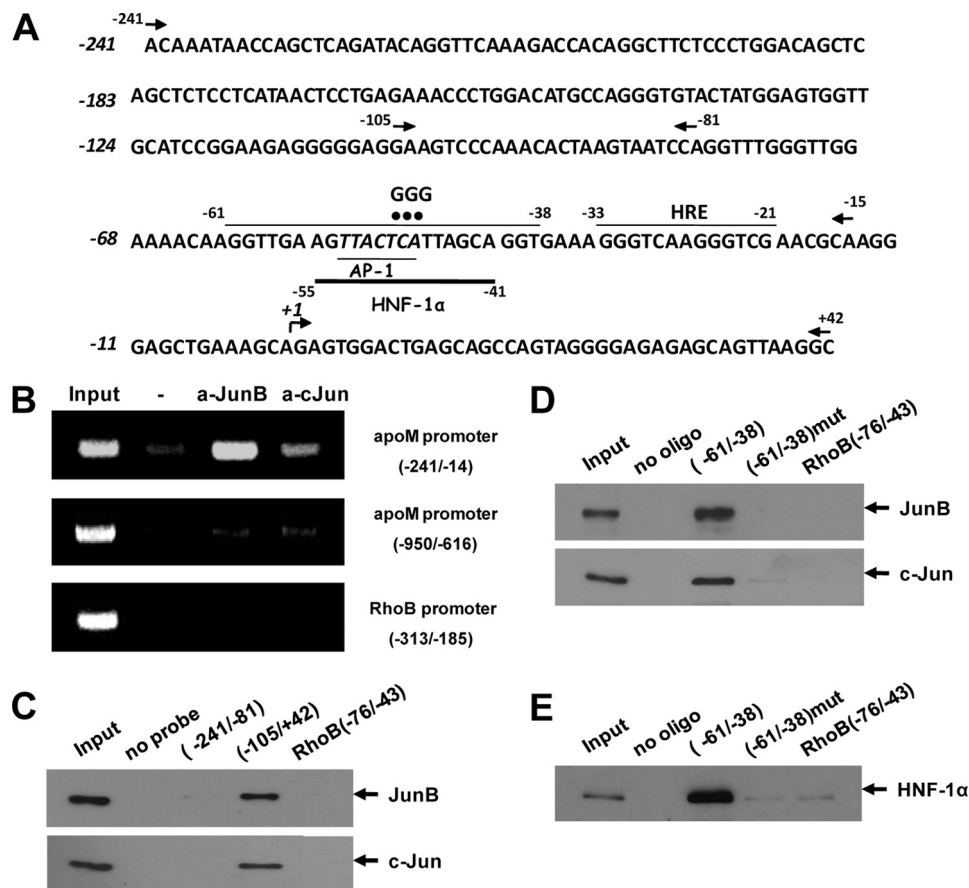


FIGURE 5. c-Jun and JunB bind to a novel AP-1 site located in the $-53/-47$ region of the proximal apoM promoter. *A*, sequence of the proximal human apoM promoter region spanning nucleotides -241 to $+42$, showing the location of the previously characterized regulatory elements that bind HNF-1 α and LRH-1 as well as the location of the novel AP-1 response element. The primer sets used for the amplification of the biotinylated promoter fragments $-241/-81$ and $-105/+42$ are indicated by arrows. Nucleotide substitutions in the AP-1 site of the apoM promoter are indicated with the black dots. *B*, HepG2 cells were subjected to chromatin immunoprecipitation in the absence (*second lane*) or presence of an anti-JunB (*third lane*) or an anti-c-Jun (*fourth lane*) antibody using primers corresponding to the proximal region of the apoM promoter (*top row*), the distal region of the apoM promoter (*middle row*), or the proximal region ($-313/-185$) of the RhoB promoter (*bottom row*, negative control). Nonimmunoprecipitated chromatin was included for comparison (*first lane*, input). The experiment was performed three times, and representative images are presented. *C*, DNA affinity precipitation using nuclear extracts from HEK293T cells transiently transfected with expression vectors for JunB or c-Jun and biotinylated PCR fragments corresponding to the $-241/-81$ or the $-105/+42$ region of the human apoM promoter or no probe. A biotinylated oligonucleotide corresponding to the $-76/-43$ region of the human RhoB promoter was used as a negative control. Oligonucleotide-bound JunB or c-Jun was detected by Western blotting using an anti-JunB or anti-c-Jun antibody, respectively. *D*, DNA affinity precipitation using nuclear extracts from HEK293T cells transiently transfected with expression vectors for JunB or c-Jun and biotinylated oligonucleotides corresponding to the wild type $-61/-38$ region of the human apoM promoter, the $-61/-38$ region bearing mutations in the AP-1 site (*mut*), the $-76/-43$ region of the human RhoB promoter, or no oligonucleotide (*no oligo*). Oligonucleotide-bound JunB or c-Jun was detected by Western blotting using an anti-JunB or anti-c-Jun antibody, respectively. *E*, DNA affinity precipitation using nuclear extracts from HEK293T cells transiently transfected with an expression vector for HNF-1 α and biotinylated oligonucleotides corresponding to the wild type $-61/-38$ region of the human apoM promoter, the $-61/-38$ region bearing mutations in the AP-1 site (*mut*), the $-76/-43$ region of the human RhoB promoter, or no oligonucleotide (*no oligo*). Oligonucleotide-bound HNF-1 α was detected by Western blotting using a polyclonal anti-HNF-1 α antibody. All of the experiments in *B–D* were performed at least three times, and representative images are shown.

importantly, both c-Jun and JunB inhibited the HNF-1 α -mediated transactivation of the wild type but not of the mutated apoM promoter (Fig. 7A), suggesting negative cross-talk between Jun proteins and HNF-1 α .

In HepG2 cells (that express endogenous HNF-1 α), c-Jun and JunB inhibited the basal activity of the proximal $-105/+42$ apoM promoter that contains the dual HNF-1 α /AP-1 element, and this inhibition was compromised by HNF-1 overexpression (Fig. 7B). In contrast, c-Jun and JunB could not suppress the activity of the $-105/+42$ apoM promoter in HepG2 cells in which the endogenous HNF-1 α gene had been silenced by a shRNA targeting HNF-1 α (Fig. 7C and supplemental Fig. S1A).

The Mouse apoM Promoter Does Not Contain a Functional AP-1 Site—The findings of Figs. 5–7 indicated that the $-55/-41$ region of the human apoM promoter contains a dual HNF-

1 α /AP-1-responsive element that mediates activation or repression of apoM gene expression by HNF-1 α and Jun factors, respectively. Interestingly, the corresponding region in the mouse apoM promoter is highly conserved with the exception of a single nucleotide substitution at position 6 of the AP-1 element (5'-TTACTTA-3') (supplemental Fig. S3A). Using DNAP assays, we initially showed that HNF-1 α binds to both the mouse $-109/-86$ and the human $-61/-38$ elements with equal affinity (supplemental Fig. S3B). In contrast, c-Jun and JunB could not bind to the mouse element, possibly because of the nucleotide substitution (supplemental Fig. S3B). In agreement with the DNA binding data of supplemental Fig. S3B, c-Jun and JunB could not inhibit the basal activity of the mouse apoM promoter in HepG2 cells (supplemental Fig. S3C). Furthermore, PMA could not inhibit endogenous apoM gene

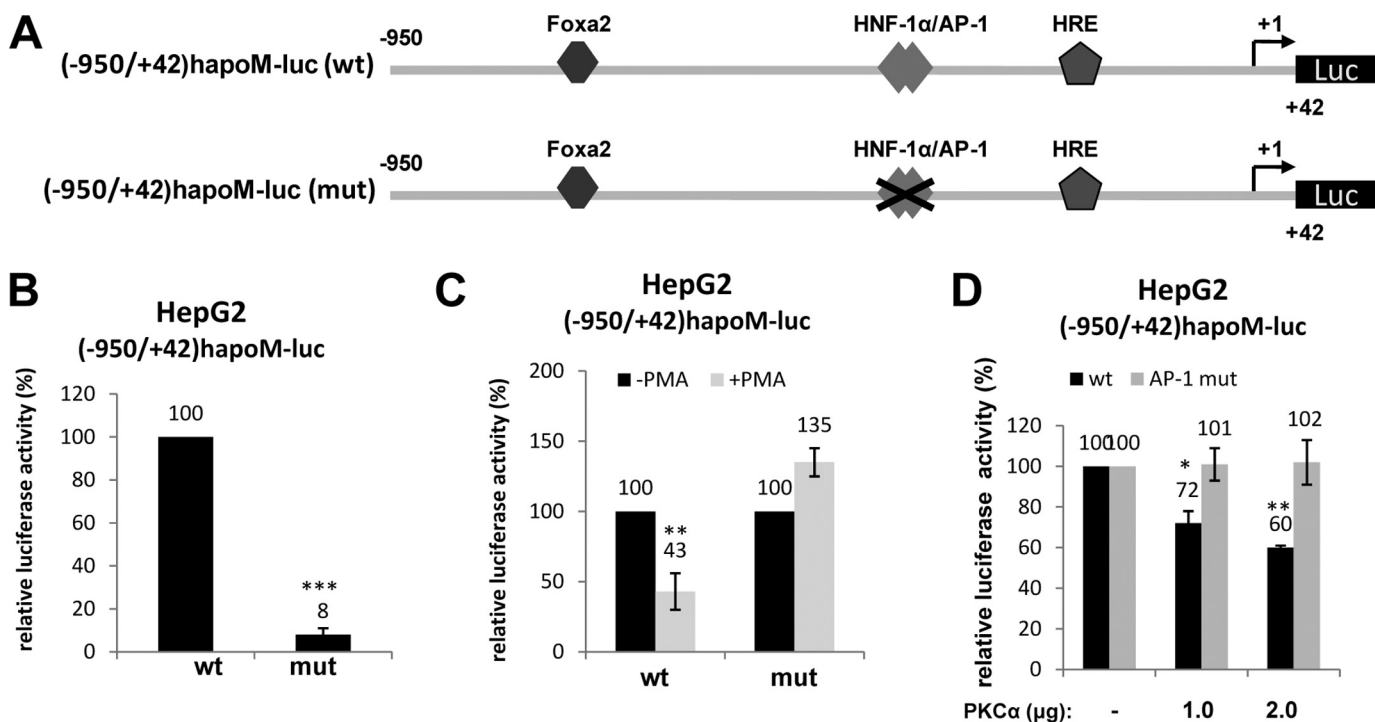


FIGURE 6. Mutations in the dual HNF-1 α /AP-1 site abolished inhibition of apoM gene expression by PMA treatment or by overexpression of PKC α . A, schematic representation of the wild type (-950/+42)hapoM-luc promoter construct and the corresponding construct bearing the mutations in the HNF-1 α /AP-1 site. B, HepG2 cells were transiently transfected with the wild type (wt) or mutated (-950/+42)hapoM-luc reporter plasmid (1.0 μ g) along with a β -galactosidase expression vector (1.0 μ g). C, HepG2 cells were transiently transfected with the wt or mutated (-950/+42)human apoM-luc reporter plasmid (1.0 μ g) along with a β -galactosidase expression vector (1.0 μ g). Following transfection, the cells were treated with PMA (100 nM) for 18 h or left untreated. D, HepG2 cells were transiently transfected with the wt or mutated (-950/+42)hapoM-luc reporter plasmid (1.0 μ g) along with increasing concentrations (1.0 and 2.0 μ g) of an expression vector for PKC α and a β -galactosidase expression vector (1.0 μ g). In B–D, luciferase activity was normalized to β -galactosidase activity and presented with histograms. Each value represents the average (\pm S.D.) from at least three independent experiments performed in duplicate.

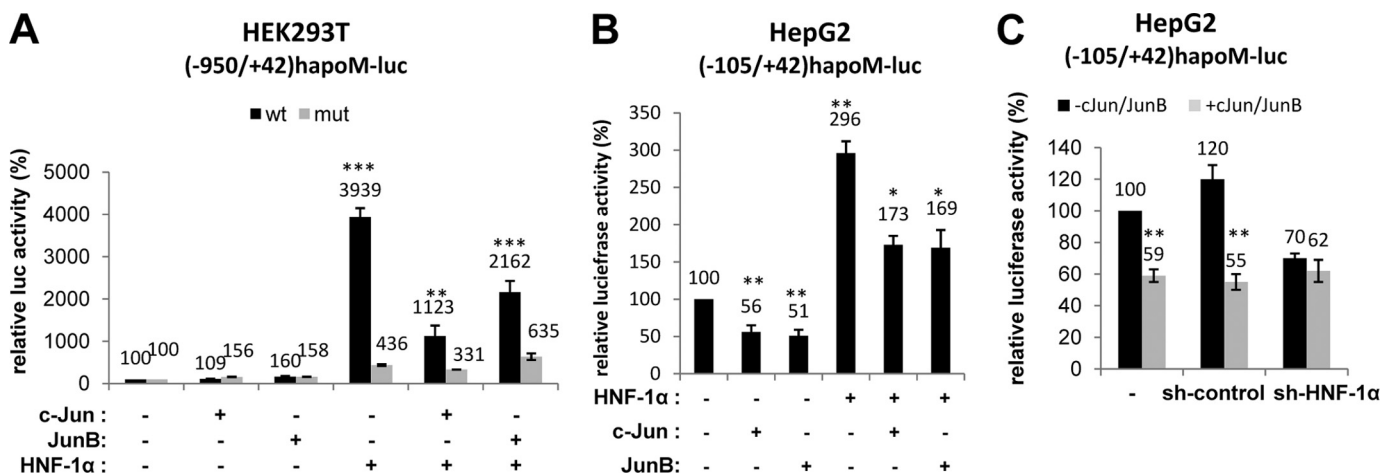


FIGURE 7. HNF-1 α is required for the repression of apoM promoter activity by c-Jun and JunB. A, HEK293T cells were transiently transfected with the wild type (wt) or mutated (mut) (-950/+42)human apoM-luc reporter plasmid (1.0 μ g) along with a β -galactosidase expression vector (1.0 μ g) and expression vectors for HNF-1 α , c-Jun, or JunB (1.0 μ g) as indicated. B, HepG2 cells were transiently transfected with the (-105/+42)hapoM-luc reporter plasmid (1.0 μ g) along with a β -galactosidase expression vector (1.0 μ g) and expression vectors for HNF-1 α , c-Jun, or JunB (1.0 μ g) as indicated. C, HepG2 cells were transiently transfected with the (-105/+42)hapoM-luc reporter plasmid (1.0 μ g) along with a β -galactosidase expression vector (1.0 μ g), the sh-control, or sh-HNF-1 α producing vectors (2.0 μ g) and expression vectors for c-Jun and JunB (1.0 μ g each) as indicated. In all panels, luciferase activity was normalized to β -galactosidase activity and presented with histograms. Each value represents the average (\pm S.D.) from at least three independent experiments performed in duplicate.

expression in mouse hepatocytes (Hep6-16 cells; supplemental Fig. S3D).

Binding of HNF-1 α and AP-1 Factors to the Proximal apoM Promoter Is Mutually Exclusive—Using chromatin immunoprecipitation assays, we sought to examine the recruitment of HNF-1 α and AP-1 factors on the apoM promoter under basal conditions as well as under conditions of AP-1 activation by

PMA. As shown in Fig. 8 (A and B), under basal conditions, HNF-1 α bound strongly to the apoM promoter, whereas binding of either c-Jun or JunB was barely detectable. Upon a 2-h treatment with PMA, binding of HNF-1 α to the apoM promoter was markedly reduced (Fig. 8, A and B, top row, lane 7). In contrast, binding of both c-Jun (Fig. 8A, top row, compare lanes 4 and 8) and JunB (Fig. 8B, top row, compare lanes 4 and 8) to

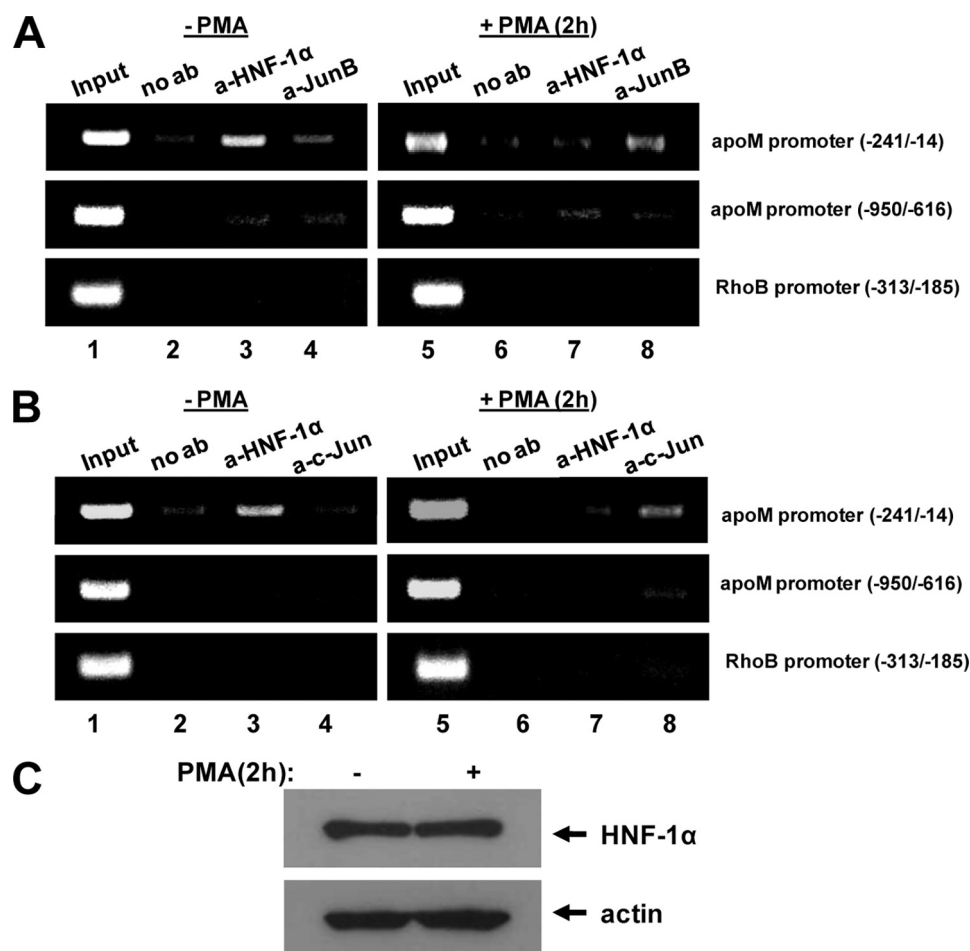


FIGURE 8. Chromatin immunoprecipitation analysis of HNF-1 α , c-Jun, and JunB binding to the apoM promoter following PMA treatment. *A*, HepG2 cells were treated with 200 nM PMA for 2 h and then subjected to chromatin immunoprecipitation in the absence (*second lane*) or presence of an anti-HNF-1 α (*third lane*) or an anti-JunB (*fourth lane*) antibody using primers corresponding to the proximal (-241/-14) or the distal (-950/-616) region of the apoM promoter or the proximal region (-313/-185) of the RhoB promoter. Nonimmunoprecipitated chromatin was included as a positive control (*first lane*, input). The experiment was performed three times, and representative images are presented. *B*, HepG2 cells were treated with 200 nM PMA for 2 h and then subjected to chromatin immunoprecipitation in the absence (*second lane*) or presence of an anti-HNF-1 α (*third lane*) or an anti-c-Jun (*fourth lane*) antibody using primers corresponding to the proximal (-241/-14) or the distal (-950/-616) region of the apoM promoter or the proximal region (-313/-185) of the RhoB promoter. Nonimmunoprecipitated chromatin was included as a positive control (*first lane*, input). The experiment was performed three times, and representative images are presented. *C*, HepG2 cells were treated with 200 nM PMA for 2 h or left untreated, and the protein levels of HNF-1 α and actin (loading control) were determined by immunoblotting using the corresponding antibodies. The experiment was performed three times, and representative images are shown.

the proximal apoM promoter was enhanced upon PMA treatment. No binding of HNF-1 α or Jun proteins was observed on the distal (-950/-616) apoM promoter or to the unrelated RhoB promoter (Fig. 8, *A* and *B*, *middle* and *bottom* row, respectively). By immunoblotting analysis, we showed that the 2-h PMA treatment had no effect on the expression of the endogenous HNF-1 α gene in HepG2 cells (Fig. 8C).

The findings of Fig. 8 indicated that the binding of HNF-1 α and Jun proteins to the dual specificity responsive element on the proximal apoM promoter could be mutually exclusive. To further investigate this mechanism, we performed a series of *in vitro* DNAP assays. These experiments showed that binding of HNF-1 α to the -61/-38 apoM oligonucleotide probe could be competed out by increasing concentrations of c-Jun (Fig. 9A, *top* row, compare *lane 3* with *lanes 6* and *9*) or JunB (Fig. 9B, *top* row, compare *lane 3* with *lanes 6* and *9*). Similarly, binding of c-Jun (Fig. 9C) or JunB (Fig. 9D) to the -61/-38 apoM oligonucleotide could be competed out by increasing concentrations of HNF-1 α (*bottom* row, compare *lane 3* with *lanes 6* and *9*).

These findings provided additional evidence that binding of HNF-1 α and Jun proteins to the -61/-38 region of the apoM promoter is mutually exclusive.

Antagonistic Interactions between HNF-1 α and AP-1 Factors Control the Transcription of the Human Apolipoprotein A-II Gene—In a search for additional genes that could be regulated by a similar mechanism, we identified a putative AP-1 site within a previously identified HNF-1 α -binding site in the promoter of the human apoA-II gene (element H, -573/-554) (*supplemental Fig. S4A*) (44, 45). This element is identical in the mouse apoA-II gene (*supplemental Fig. S4H*).

Initially, we showed that apoA-II mRNA levels in HepG2 cells were decreased following a 18-h PMA treatment, and this decrease was not observed upon overexpression of shRNA targeting c-Jun gene expression or in the presence of the PKC α / β inhibitor Gö 6976 (*supplemental Fig. S4, B and C*, respectively). Using chromatin immunoprecipitation assays, we established that c-Jun, but not JunB, was recruited to the -649/-343 region of the apoA-II promoter that contains the putative AP-1

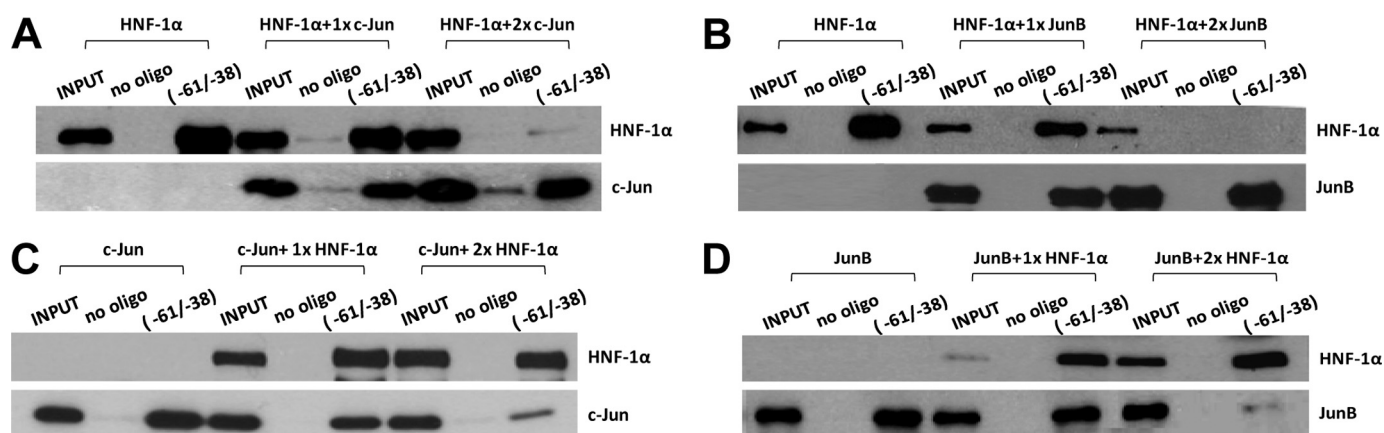


FIGURE 9. Mutually exclusive binding of c-Jun/JunB and HNF-1 α to the $-61/-38$ region of the apoM promoter. *A*, DNA affinity precipitation using nuclear extracts from HEK293T cells transiently transfected with an expression vector for HNF-1 α and increasing amounts of c-Jun and biotinylated oligonucleotides corresponding to the $-61/-38$ region of the human apoM promoter or no oligonucleotide (*no oligo*). Oligonucleotide-bound HNF-1 α and c-Jun were detected by Western blotting using an anti-HNF-1 α or anti-c-Jun antibody, respectively. *B*, DNA affinity precipitation using nuclear extracts from HEK293T cells transiently transfected with an expression vector for HNF-1 α and increasing amounts of JunB and biotinylated oligonucleotides corresponding to the $-61/-38$ region of the human apoM promoter or no oligonucleotide (*no oligo*). Oligonucleotide-bound HNF-1 α and JunB was detected by Western blotting using an anti-HNF-1 α or anti-JunB antibody, respectively. *C*, DNA affinity precipitation using nuclear extracts from HEK293T cells transiently transfected with an expression vector for c-Jun and increasing amounts of HNF-1 α and biotinylated oligonucleotides corresponding to the $-61/-38$ region of the human apoM promoter or no oligonucleotide (*no oligo*). Oligonucleotide-bound HNF-1 α and c-Jun was detected by Western blotting using an anti-HNF-1 α or anti-c-Jun antibody, respectively. *D*, DNA affinity precipitation using nuclear extracts from HEK293T cells transiently transfected with an expression vector for JunB and increasing amounts of HNF-1 α and biotinylated oligonucleotides corresponding to the $-61/-38$ region of the human apoM promoter or no oligonucleotide (*no oligo*). Oligonucleotide-bound HNF-1 α and JunB was detected by Western blotting using an anti-HNF-1 α or anti-JunB antibody, respectively. All of the experiments were performed at least three times, and representative images are shown.

element under basal conditions (supplemental Fig. S4D). Overexpression of c-Jun in HepG2 cells was associated with a 50% reduction in the activity of the $-911/+29$ apoA-II promoter (supplemental Fig. S4E). DNAP assays established that the region of the apoA-II promoter between nucleotides -573 and -554 is a dual HNF-1/AP-1 site that binds both c-Jun and HNF-1 α (supplemental Fig. S4F). Finally, using chromatin immunoprecipitations, we showed that there is competition between c-Jun and HNF-1 α for binding to the apoA-II promoter under conditions of c-Jun activation by PMA (supplemental Fig. S4G).

DISCUSSION

HDL is a heterogeneous population of particles in the plasma comprising larger spherical particles (such as HDL₂ and HDL₃) as well as smaller (pre β -HDL) discoidal particles. HDL particles in plasma are in a dynamic equilibrium that is governed by a continuous remodeling mediated by plasma enzymes, membrane transporter proteins, and receptors (46). HDL undergoes significant changes in structure, protein/lipid composition, antioxidative, and anti-inflammatory activities during the acute phase response (47). Apolipoprotein M is a recently described apolipoprotein that is associated with HDL and has been shown to play an important role in HDL maturation and remodeling (21). The atheroprotective role of apoM was established by studies showing that adenoviral apoM overexpression in LDL receptor-deficient mice (animal model for premature atherosclerosis) or hepatic overexpression of apoM in apoM transgenic mice led to a reduction in atherosclerosis development (21). Thus, it could be anticipated that reduction in apoM synthesis in the liver during inflammation could contribute to the development of atherosclerosis and coronary artery disease.

Hormone nuclear receptors play an important role in the regulation of HDL genes during inflammation. We have shown

recently that the proximal apoM promoter harbors a multifunctional hormone response element that serves as a binding site for a plethora of orphan and ligand-inducible nuclear receptors including HNF-4, homodimers of retinoid X receptor α , and heterodimers of retinoid X receptor α with liver X receptor α , retinoic acid receptor α , peroxisome proliferator-activated receptor α , and thyroid hormone receptor β (28). This site was shown to mediate apoM gene induction by ligands for the above nuclear receptors, whereas mutagenesis of this site severely reduced both the basal and the inducible activity of the apoM promoter in hepatic cells (28). Furthermore, preliminary evidence for a cooperation between HNF-1 and the above hormone nuclear receptors was obtained (28). These findings, combined with our previous studies showing that TNF- α inhibits the transcriptional activity of nuclear receptor HNF-4 α in hepatic cells via NF- κ B activation (48), suggested that regulation of apoM gene transcription during inflammation may be complex and mediated by multiple signaling pathways and molecular cross-talks.

The findings of the present study established PKC as a negative modulator of apoM gene transcription. PKC comprises a family of serine/threonine kinases that play roles in many diseases including atherosclerosis, fibrosis, and cardiac hypertrophy (34). In the case of atherosclerosis, it was shown that inhibition of PKC blocks the production of superoxide anion by activated monocytes and the subsequent oxidation of LDL (49). Both α and β PKCs were shown to be up-regulated upon stimulation of monocytes and translocate to the particulate fraction (50). These findings suggest that specific PKC isozymes may increase the pro-oxidant environment of the atherosclerotic plaque and may lead to plaque instability. Furthermore, by down-regulating apoM gene expression in the liver, activated PKC is expected to influence the maturation of HDL particles in

Negative Regulation of ApoM by Jun Proteins

the plasma, and as a consequence, HDL structure and functions including atheroprotective functions on the arterial wall and the reverse cholesterol transport. Thus, selective regulators of PKC isozymes may be promising candidates for the prevention or even the regression of atherosclerosis via HDL particles (34).

We report here the identification of a dual specificity regulatory element in the proximal apoM promoter that mediates activation or repression of apoM promoter activity by HNF-1 and Jun proteins, respectively, in hepatic cells. The experimental evidence that supports the dual functionality of this element of the apoM promoter is as follows: (a) Jun proteins repress apoM promoter activity only in cells expressing HNF-1 α . Jun-mediated repression was abolished in HepG2 cells in which endogenous HNF-1 α expression had been silenced by specific shRNAs (Fig. 7). (b) Mutations in the AP-1 element abolished the binding of HNF-1 α and the HNF-1 α -mediated transactivation of the apoM promoter (Figs. 5–7). (c) The mouse apoM promoter does not contain a functional AP-1 site because of single nucleotide difference, but it retains a functional HNF-1 α -responsive element. As a consequence, Jun proteins cannot inhibit the mouse apoM promoter activity, and PMA cannot inhibit the expression of the endogenous apoM gene in mouse hepatocytes (supplemental Fig. S3). (d) Competition experiments showed that binding of Jun proteins and HNF-1 α to the apoM promoter is mutually exclusive (Fig. 9), and HNF-1 overexpression in hepatic cells could overcome the inhibitory effect of Jun proteins on the apoM promoter (Fig. 7B). (e) Chromatin immunoprecipitation assays established that AP-1 activation leads to the recruitment of c-Jun and Jun B proteins to the proximal apoM promoter with the simultaneous displacement of HNF-1 (Fig. 8). (f) A similar mechanism of Jun-mediated repression of promoter activity via dual specificity AP-1/HNF-1-responsive element operates on the promoter of the human apolipoprotein A-II gene (supplemental Fig. S4), the expression of which is also under negative regulation during the acute phase response (51). Importantly, this dual specificity element is highly conserved in the mouse apoA-II gene (supplemental Fig. 4H).

A previous study had shown that administration of LPS to mice (model of bacterial infection) was associated with a marked reduction in the expression levels of HNF-1 α in the liver (52). The same group also showed that both apoM and HNF-1 α genes were down-regulated in the kidney of LPS-treated mice (53). We show here that the expression of HNF-1 α in HepG2 cells was not affected by phorbol ester treatment (Fig. 8C), suggesting that a mechanism involving a direct effect of Jun on HNF-1 α gene expression in hepatic cells could be excluded.

The question that remains is why recruitment of Jun proteins to the apoM and apoA-II promoters and the displacement of HNF-1 α is associated with transcriptional repression. We could hypothesize that Jun proteins bind to the AP-1 elements of the apoM and apoA-II promoter without activating transcription. Active repression by Jun could be excluded in light of the findings that both c-Jun and JunB failed to repress the activity of the apoM promoter in HEK293T cells that lack hepatocyte nuclear factors (Fig. 7A), as well as by our finding that a general inhibitor of histone deacetylases (trichostatin A) failed to block apoM gene down-regulation by PMA (supplemental

Fig. S5). The inability of Jun proteins to activate the apoM promoter could be either due to the absence of cooperating factors in the vicinity of the AP-1 element or due to the deviation of the apoM AP-1 element from the consensus sequence (Fig. 5A). Transcriptional repression by Jun and other AP-1 proteins has been reported in many cases including the genes encoding for c-Fos, osteocalcin, adipocyte P2, creatinine kinase, myoD, major histocompatibility class I, chorionic gonadotropin α and β , apoC-III, and insulin (36, 54–61). However, in the majority of the above cases, the molecular mechanisms by which AP-1 proteins inhibit transcription are not understood.

In summary, our model described here provides a mechanistic explanation for the inhibitory effect of pro-inflammatory cytokines or other factors on the expression of acute phase HDL genes such as apoM and apoA-II. HDL is a lipoprotein particle with multiple but still not fully characterized atheroprotective properties. Understanding how the HDL genes are controlled during health and disease will be essential for the development of novel therapeutic and diagnostic tools for diseases such as coronary artery disease and diabetes that affect a large proportion of the population in Western countries.

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