Transactivation of the ApoCIII Promoter by ATF-2 and Repression by Members of the Jun Family†

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ABSTRACT: It was shown previously that cytokines such as tumor necrosis factor-α that stimulate signal transduction pathways involving transcription factors ATF-2 and Jun repress apoCIII promoter activity in HepG2 cells. In the present study, DNase I footprinting analysis established that ATF-2 protected three regions in the apoCIII promoter. One region (−747/−726) present in the apoCIII enhancer is within the previously identified footprint I and has overlapping boundaries with the binding sites of Sp1 (−764/−742) and HNF-4 (−736/−714). The other two regions represent new footprints and have been designated D/E (−219/−199) and B/C (−102/−75). The B/C region overlaps with the previously identified footprint B which contains an HNF-4 binding site (−87/−63). Cotransfection experiments in HepG2 cells showed that ATF-2 transactivated the −890/+24 apoCIII promoter 1.6-fold. In addition, mutations in the proximal D/E (−219/−199) and distal I (−747/−726) ATF-2-binding sites reduced the apoCIII promoter strength to 33 and 9% of control, respectively, indicating that ATF-2 is a positive regulator of apoCIII gene transcription. Cotransfections with ATF-2 and HNF-4 expression plasmids resulted in additive transactivation of the apoCIII promoter. Furthermore, apoCIII promoter constructs bearing mutations in the D/E and I ATF-2 binding sites were efficiently transactivated by HNF-4, suggesting that these two factors contribute independently to the apoCIII promoter strength. Members of the Jun family (c-Jun, JunB, and JunD) caused a dose-dependent inhibition of the −890/+24 apoCIII promoter activity. A synthetic promoter containing the apoCIII enhancer in front of the minimal AdML promoter was also repressed by Jun. In contrast, apoCIII promoter segments lacking the enhancer region were transactivated by Jun. The findings suggest that homodimers of Jun or heterodimers of Jun with other AP-1 subunits could be responsible for the observed repression by interfering with the function(s) of the apoCIII enhancer. Repression by Jun could be reversed in the presence of ATF-2 and HNF-4, suggesting that ATF2 and possibly Jun/ATF-2 heterodimers exert a positive effect on apoCIII gene transcription, as opposed to Jun homodimers or heterodimers with other AP-1 members. These findings suggest a role for members of the Jun family and ATF-2 that participate in signal transduction pathways in basal or induced apoCIII promoter activity in cells of hepatic origin.

Plasma apoCIII[1] is a 79 amino acid protein of known primary structure (*1*) and known gene sequence (*2*). ApoCIII is a major component of very low-density lipoprotein and a minor component of high-density lipoprotein (*3*). ApoCIII has been implicated in modulating the binding of lipoproteins to cell receptors and subsequently in the catabolism of triglyceride-rich lipoproteins (*4*−*6*) and, thus, in the development of hypertriglyceridemia. This concept is further supported by recent findings showing that overexpression of the apoCIII gene in transgenic mice is associated with severe hypertriglyceridemia and accumulation of apoB-48 containing lipoprotein remnants in plasma (*7, 8*).

ApoCIII gene expression is tissue-specific (*9*) and developmentally regulated (*10*). The apoCIII promoter contains four proximal (A to D) and six distal (E to J) regulatory elements. The distal elements F to J (located between nucleotides −592 to −792) comprise a general enhancer that potentiates the strength of the proximal apoCIII as well as the apoA-I and apoA-IV promoters (*11*−*13*). This region is also essential for intestinal apoA-I gene expression in vivo (*14*). Other elements important in apoCIII gene regulation are two hormone response elements (HREs), located in the

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[1] Abbreviations: ATF-2, activating transcription factor-2; CRE, cAMP-responsive element; CREB, cAMP-responsive element-binding protein; AP-1, activating protein-1; HRE, hormone response element; apoA-I, apolipoprotein A-I; apoA-IV, apolipoprotein A-IV; apoCIII, apolipoprotein CIII; HNF-4, hepatocyte nuclear factor-4; CAT, chloramphenicol acetyl transferase; wt, wild-type; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; CMV, cytomegalovirus; DTT, dithiothreitol; AdML, adenovirus major late promoter; TAFs, TATA box binding protein associated factors; TIFs, transcriptional mediators/intermediary factors; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; RLNE, rat liver nuclear extracts; JNK, c-Jun N-terminal kinase; TNF-α, tumor necrosis factor-α; SEM, standard error of mean; NF-kB, nuclear factor kB; cdc42, cell division cycle 42; MEK, mitogen activated extracellular response kinase; SEK, stress-activated protein kinase activator; MEKK1; MEK kinase-1; PAK, p21 activated kinase.

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proximal promoter and enhancer regions, as well as multiple Sp1 binding sites located in the apoCIII enhancer (*12, 13, 15*).

Treatment of HepG2 cells with proinflammatory cytokines such as TNF-α and interleukin-1 (IL-1) caused a considerable drop in apoCIII mRNA levels and promoter activity (*16, 17*). It was proposed, based on cotransfection experiments and DNA-binding assays, that this effect is due to the binding of transcription factors NF-kB and C/EBPδ to regulatory element CIIID located in the proximal apoCIII promoter (*16, 17*).

Proinflammatory cytokines stimulate signal transduction pathways that lead to the activation of transcription, mainly by phosphorylation of specific transcription factors. The three most thoroughly characterized factors are activating transcription factor-2 (ATF-2), nuclear factor-kB (NF-kB), and the proto-oncogene c-Jun. ATF-2, also called CRE-BP1, and c-Jun are members of the AP-1 family of transcription factors (*18*). Previous studies have established that ATF-2 can form heterodimers with c-Jun that bind to "AP-1-like" target sites and this interaction leads to transcriptional activation (*19*−*26*). In addition to c-Jun, ATF-2 has also been shown to interact with other regulatory proteins, including adenovirus protein E1A, the tumor suppressor pRb, the high-mobility group protein HMG-1(Y), and the transcription factor NF-kB (*27*−*34*). These interactions lead to transcriptional activation of the target genes. Cytokine stimulation leads to the phosphorylation of ATF-2 by the proline-directed kinase p38 (*35, 36*). ATF-2, as well as Jun, is also phosphorylated at threonine 69 and 71 by the c-Jun N-terminal kinase JNK, suggesting that the physiologically relevant target of the cytokine signal transduction is the c-Jun/ATF-2 heterodimer.

In the present study, we have investigated the potential role of ATF-2/c-Jun in the transcriptional regulation of the apoCIII promoter. The results indicate that ATF-2 binds to three novel sites on the apoCIII promoter and is a positive regulator of apoCIII gene transcription. In addition, c-Jun, JunB, and JunD repress the apoCIII promoter activity. Repression by Jun is mediated by elements present in the distal enhancer region and can be reversed by cotransfection with either ATF-2 or HNF-4. The findings provide evidence for positive and negative regulation of apoCIII gene expression by different cytokine and stress-activated kinases present in HepG2 cells and suggest that modulation of apoCIII promoter activity may occur in response to extracellular signals.

EXPERIMENTAL PROCEDURES

Materials. Reagents were purchased from the following commercial sources. Restriction enzymes and modifying enzymes (T4 DNA ligase, T4 polynucleotide kinase, Klenow, and calf intestinal alkaline phosphatase) were from Minotech, New England Biolabs or Gibco-BRL. Vent DNA polymerase was from New England Biolabs. The Sequenase V2 kit was from Amersham/USB. Poly dI/dC, acetyl CoA, and dNTPs were from Pharmacia. DNase I was from Worthington. ATP[γ-^{32}P] and dCTP[α-^{32}P] were from Amersham or New England Nuclear. Cell culture reagents (DMEM, fetal bovine serum, trypsin-EDTA, and PBS) were from Gibco-BRL. *O*-Nitrophenyl-β-D-galactopyranoside (ONPG) and PMSF were from Sigma.

Plasmid Constructions. The apoCIII reporter constructs −890/+24, −686/+24, −163/+24, and −790/−500 AdML CAT have been described (*11, 13*). The mammalian expression vectors encoding HNF-4, ATF-2, c-Jun, JunB, and JunD have been previously described (*37*−*39*). Plasmids pRSV JunB and pRSV JunD were the generous gift of Dr. Nikolakaki, Laboratory of Biochemistry, Department of Chemistry, University of Thessaloniki. Bacterial expression vectors for HNF-4 and ATF-2 have been described (*13, 40*).

Expression and Purification of HNF-4 and ATF-2 Proteins in Bacteria and Footprinting Analysis of the ApoCIII Promoter. HNF-4 and ATF-2 cDNAs were cloned in the bacterial pET15b vector under the control of the T7 promoter and were expressed in *Escherichia* coli BL21(DE3) strain. Expression of the His-tagged proteins was induced by the addition of 1 mM IPTG for 3 h at 30 °C. Cells were lysed by sonication, and the His-tagged proteins were purified by incubation with Ni-NTA-agarose beads in a buffer containing 8 M urea, 0.1 M sodium phosphate, 0.01 M Tris-HCl, pH 8.0, 10 mM immidazole, and 10 mM β-mercaptoethanol. HNF-4-bound protein was eluted using a pH 6.5 to 4.0 gradient in 8 M urea, 0.1M sodium phosphate, and 0.01 M Tris-HCl. ATF-2 bound protein was eluted with 25, 50, 150, and 500 mM and 1 M immidazole. Purified proteins were renatured by dialysis in buffer containing 6 M urea, 0.5 M NaCl, 20 mM HEPES, pH 7.8, 5 mM $MgCl_2$, 2 mM DTT, 0.1% NP-40, 10% glycerol, and 0.5 mM PMSF. The urea concentration was gradually decreased to 0 M by the addition of urea-free buffer, and a final dialysis was made in a buffer containing 100 mM NaCl, 20 mM HEPES, pH 7.8, 5 mM $MgCl_2$, 2 mM DTT, 0.1% NP-40, 10% glycerol, and 0.5 mM PMSF. The purified proteins were used for DNase I footprinting analysis of apoCIII promoter fragments −890 to +24 and −283 to +24, labeled either at nucleotide −890 or −283 with [γ-^{32}P]ATP by T4 polynucleotide kinase as described previously (*41*).

Cell Culture and Transfections. Cultures of HepG2 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum. The day prior to transfection, cells were seeded at 50−60% confluency into 30 mm diameter plates. Transient transfections were performed by the Ca−PO_4 coprecipitation method as described previously (*42*). The plasmids and the concentrations used in the transfections are indicated in the corresponding figure legends. In each transfection vector DNA was added as necessary to achieve a constant amount of transfected DNA. Forty hours later, the cells were harvested and subjected to three consecutive freeze−thaw cycles. The CAT activity of the cell extracts was determined as described previously (*11*). The activity of β-galactosidase enzyme was measured in order to normalize for transfection efficiency.

RESULTS

The Human ApoCIII Promoter Contains Multiple Binding Sites for ATF-2 in the Proximal Promoter and Distal Enhancer Region that Contribute to the Overall Promoter Strength. Previous studies have established that activation of the apoCIII promoter requires synergistic interactions between HNF-4 bound to the proximal promoter and distal enhancer sites and Sp1 and other factors bound to the apoCIII enhancer (*12, 13*). It was also shown that cytokines may

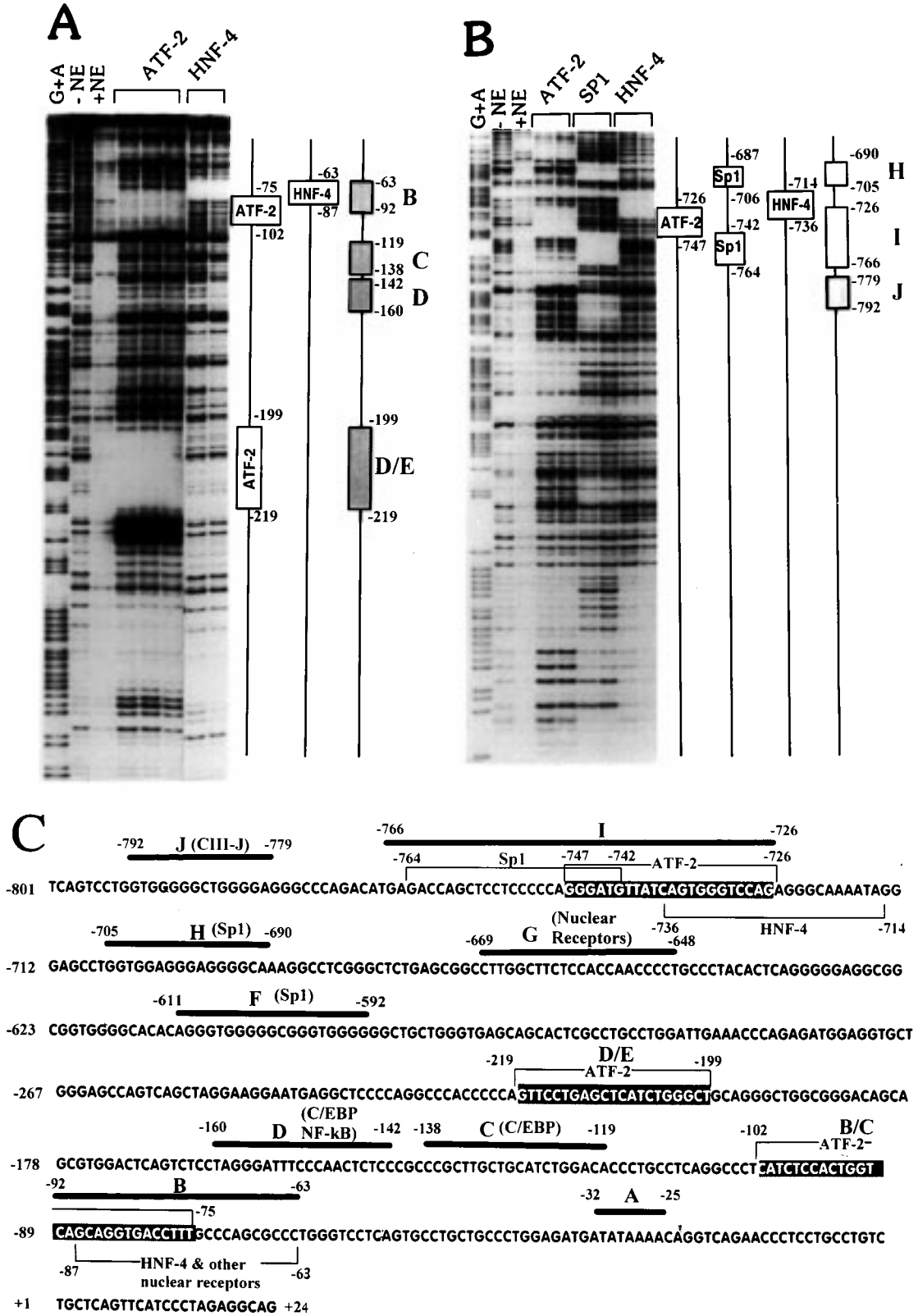

FIGURE 1: Definition of the binding sites of ATF-2, HNF-4, and Sp1 on the apoCIII promoter by DNase I footprinting. (A) DNase I footprinting analysis of the apoCIII promoter fragment −283/+24 labeled with [γ-^{32}P]ATP at the nucleotide −283, performed with purified bacterially expressed ATF-2 and HNF-4 proteins. (B) DNase I footprinting analysis of the apoCIII promoter fragment −890 to +24, labeled with [γ-^{32}P]ATP at the −890 nucleotide, performed with purified Sp1 and bacterially expressed ATF-2 and HNF-4 proteins. Factors Sp1 was purchased from Promega. In both panels, footprinting was performed with 100 ng of the factors expressed in bacteria as indicated at the top of the figure. G+A is a Maxam and Gilbert sequencing ladder of the same DNA fragment used in the footprinting analysis. (−NE) Footprinting reaction performed in the absence of nuclear extracts using 25 ng of DNase I. (+NE) Footprinting reaction performed in the presence of 40 μg of rat liver nuclear extracts and 100 ng of DNase I. Boxes show areas protected from DNase I digestion and numbers refer to their positions relative to the transcription initiation site (+1). (C) Schematic representation of apoCIII promoter showing binding sites of ATF-2 in relationship to binding sites of Sp1, HNF-4, and other known factors which recognize the human apoCIII promoter (*12*, *13*). Regulatory elements A to J are symbolized by thick lines. Brackets indicate boundaries of newly identified footprints.

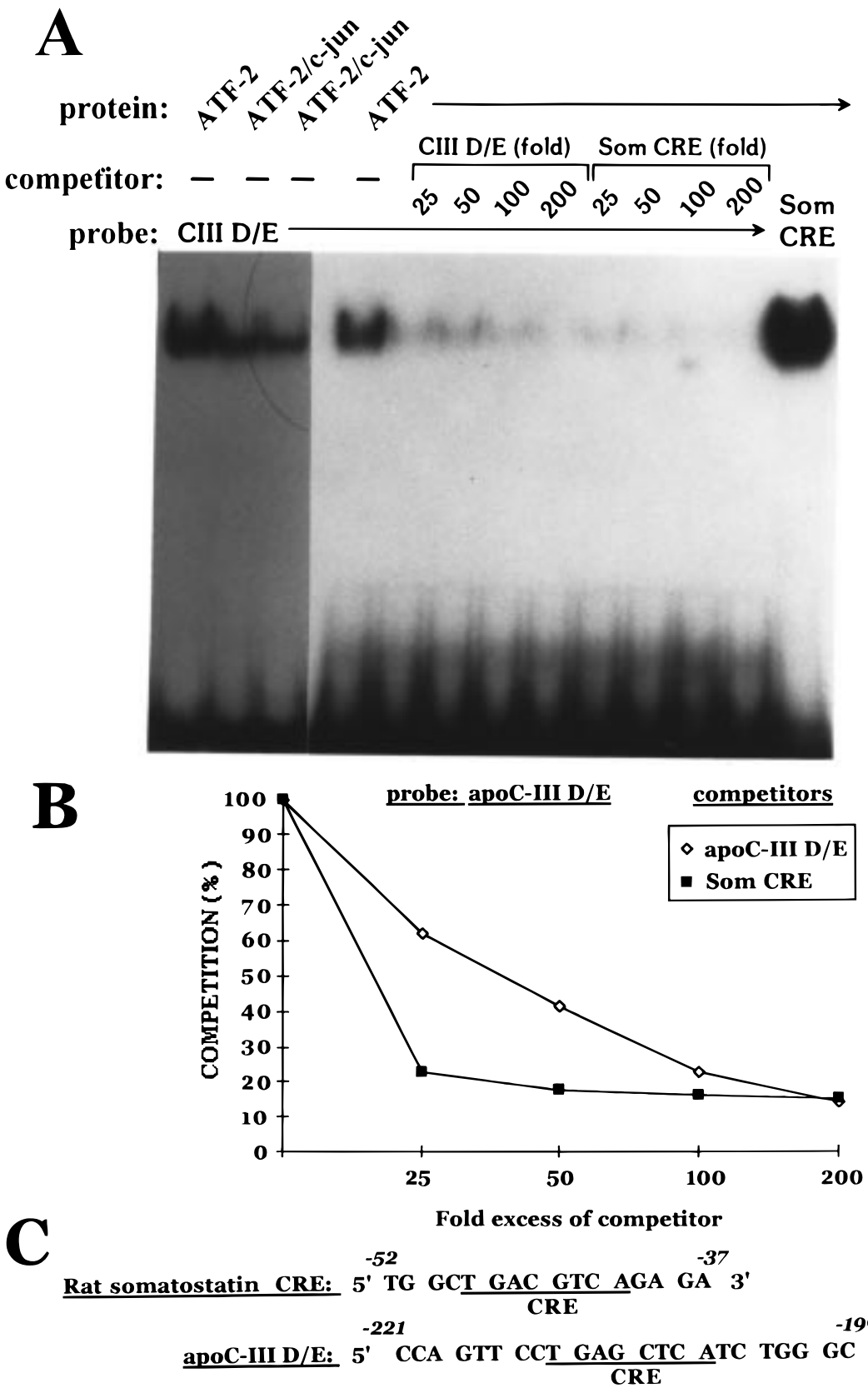

FIGURE 2: (A) DNA binding gel electrophoresis assays of ATF-2 and ATF-2/c-Jun to the regulatory element D/E (−219/−199) and the somatostatin CRE and competition experiments using excess of the two probes. (B) Competition of binding of ATF-2 to the element D/E by excess of the unlabeled somatostatin CRE probe and the DE (−219/−199) apoCIII promoter probe. (C) Sequence of the somatostatin CRE and apoCIII D/E probes utilized in panels A and B.

affect the overall promoter strength via signal transduction pathways that lead to the activation of specific transcription factors (*16*, *17*).

To identify the binding sites for cytokine-inducible factors in relationship to known binding sites, we performed DNase I footprinting analysis with factors HNF-4 and SP-1 previ-

ously shown to bind and to activate the apoCIII promoter as well as with the cytokine-inducible factor ATF-2. HNF-4 and ATF-2 were obtained by expression of the corresponding cDNAs in bacteria and partial purification as described in the Experimental Procedures. SP1 was purchased from Promega.

The results of the DNase I footprinting analysis showed that ATF-2 protects the −747 to −726 apoCIII enhancer region and has overlapping boundaries with factors Sp1 and HNF-4, which protect the −764/−742 and −736/−714 region within element I (−766/−726) (*11*, *13*) (Figure 1B). A second binding site for ATF-2 (−219/−199) was identified within a new footprinting region designated D/E (−219/−199). Interestingly, the sequence between nucleotides −212 to −205 contains a palindromic repeat TGAGCTCA that is similar to the cAMP response element (CRE) TGACGTCA. ATF-2 also protected the region −102 to −75, which is partially overlapping with the proximal HNF-4 (−87/−63) binding site (Figure 1A). This region contains an asymmetric TGACCAGTG site that may serve as a recognition sequence for ATF-2. The location of the ATF-2-binding sites relative to the binding sites for SP1 and HNF-4 is shown in Figure 1C.

These observations prompted us to investigate the role of ATF-2 and its heterodimeric partner c-Jun on the transcriptional regulation of the human apoCIII promoter.

The DNA-binding and competition experiments showed that the binding of ATF-2 to the regulatory element D/E (−219/−199) was competed out by excess of the homologous probe as well as by the CRE of the somatostatin promoter (*43*). Binding to this element was also observed when ATF-2 was mixed with c-Jun (Figure 2A−C).

To investigate the contribution of ATF-2 in the apoCIII promoter strength, HepG2 cells were transiently transfected with reporter genes bearing either the wild-type −890/+24 apoCIII promoter (Figure 3A) or promoters with point mutations in the ATF-2-binding sites (Figure 3, panels B and C). Cotransfection of the wild-type −890/+24 apoCIII promoter with ATF-2 resulted in a moderate increase of 1.6-fold in the promoter activity in a dose-dependent manner (Figure 3A). However, when mutations were introduced in the ATF-2-binding sites present in the proximal promoter D/E (−219/−199) region or in the distal enhancer I (−747/−726), the level of apoCIII promoter activity was reduced to 33 and 9% of the control, respectively (Figure 3, panels B and C). These mutations abolished the binding of ATF-2 to mutated oligonucleotides corresponding to these regions (data not shown). Cotransfection experiments also showed that the −890/+24 apoCIII promoter mutated in the proximal D/E (−219/−199) ATF-2-binding site was transactivated 4-fold by ATF-2, whereas the promoter mutated in the distal (−747/−726) ATF-2-binding site could no longer be transactivated by ATF-2 (Figure 3, panels B and C). The combined data of Figure 3, panels A and B, suggest that ATF-2 or related factors present in HepG2 cells contribute to the overall activity of the human apoCIII promoter by binding to the AP-1-binding sites as homodimers or possibly as heterodimers with other AP-1 family members and that activation of these factors by extracellular stimuli leads to transactivation of the apoCIII promoter.

Previous studies have established that binding of HNF-4 to elements B and I of the apoCIII promoter results in an

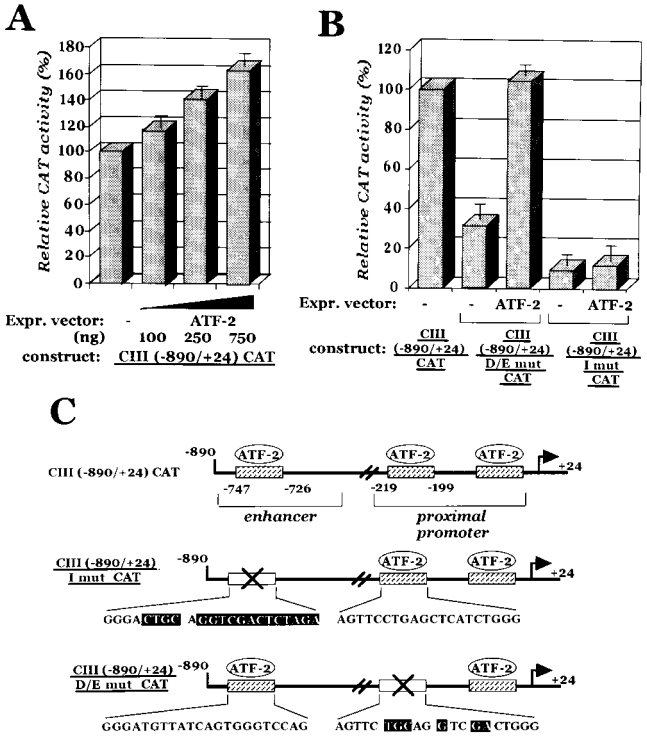

FIGURE 3: Transactivation of the apoCIII promoter by ATF-2. (A) HepG2 cells were transiently transfected with 3 μg of the wild-type apoCIII (−890/+24) CAT reporter plasmid shown in panel C along with 1 μg of CMV β-gal plasmid and increasing concentrations (100−750 ng) of pcDNAI/ATF-2 expression plasmid. Extracts from the transfected cells were assayed for β-gal and CAT activity as described in the Experimental Procedures. Mean values (±SEM) of CAT activity from at least two independent normalized experiments performed in duplicate are presented in the form of a bar graph. (B) HepG2 cells were cotransfected transiently with 3 μg of the wild-type and mutated apoCIII (−890/+24) CAT reporter plasmid, shown schematically in panel C along with 1 μg of CMV-β-gal plasmid and 1 μg of pcDNAI/ATF-2 expression plasmid as indicated. Extracts from transfected cells were assayed for β-gal and CAT activities as described in the Experimental Procedures. Mean values (±SEM) of CAT activity from at least two independent normalized experiments performed in duplicate are presented in the form of a bar graph. The activity of the wild-type apoCIII (−890/+24) CAT plasmid was set at 100%. (C) Schematic representation of the apoCIII promoter plasmids utilized for the experiments presented in panels A and B. Mutated sequences in elements I and DE are highlighted.

8-12-fold transactivation of apoCIII promoter (*12*, *13*, *15*, *39*). It was also shown that this activation of transcription reflects synergistic interactions of HNF-4 with factor Sp1 and other factors that bind to distal regulatory elements H, I, and J (*12*, *13*, *15*). To investigate whether ATF-2 can act in synergy with HNF-4, we carried out cotransfection experiments in HepG2 cells with both ATF-2 and HNF-4. As shown in Figure 4A, cotransfection with ATF-2 and HNF-4 resulted in additive transactivation of the apoCIII promoter. Furthermore, apoCIII promoter plasmids bearing mutations in the proximal (−219/−199) or distal (−747/−726) ATF-2-binding sites (Figure 3C) were transactivated by HNF-4 to the same level as the wild-type promoter, suggesting that ATF-2 and HNF-4 contribute independently to the apoCIII promoter strength (Figure 4B).

ApoCIII Promoter Activity Is Downregulated by Members of the Jun Family of Transcription Factors. Repression by Jun Requires the Presence of the ApoCIII Enhancer Region. To explore the role of c-Jun in apoCIII gene expression, we

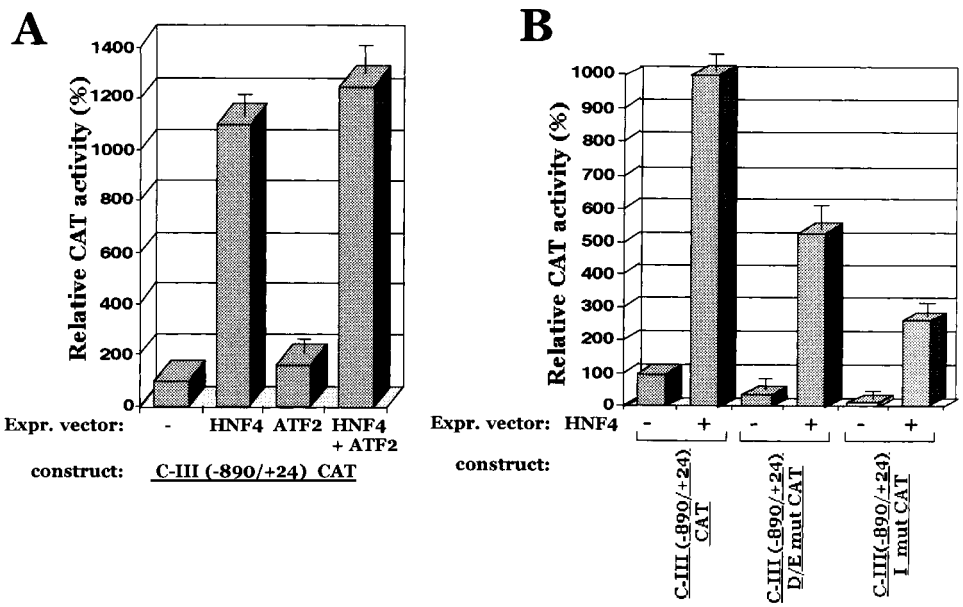

FIGURE 4: Additive transactivation of the human apoCIII promoter by transcription factors HNF-4 and ATF-2. (A) HepG2 cells were cotransfected transiently with 3 μg of apoCIII (−890/+24) CAT reporter plasmid shown in Figure 3C, along with 1 μg of CMV-β-gal plasmid and 1 μg of either the pCDNA-1/HNF-4 or pCDNA-1/ATF-2 plasmids or both. Extracts from transfected cells were analyzed for β-galactosidase and CAT activities as described in (C). The results (±SEM) from at least two independent experiments performed in duplicate are presented in the form of a bar graph. (B) HepG2 cells were cotransfected with the wild-type apoCIII (−890/+24) CAT reporter plasmid or plasmids containing mutations in either of the two ATF-2 binding sites, shown in Figure 3C, in the absence or presence of 1 μg of the pCDNA-1/HNF-4 expression plasmid. Extracts from transfected cells were analyzed for β-galactosidase and CAT activities as described in the Experimental Procedures. The normalized CAT activity values (±SEM) of at least two independent experiments performed in duplicate is presented in the form of a bar graph. The difference in the transactivation of the of apoCIII (−890/+24) promoter in HepG2 cells by pCDNA/HNF-4 or by pcDNAI/HNF-4 and pcDNAI/ATF-2 is statistically significant ($p < 0.05$).

performed cotransfection experiments in HepG2 cells with different apoCIII promoter segments and expression vectors for different members of the Jun family. This analysis showed that c-Jun, JunB, and JunD caused a dose-dependent repression of the −890/+24 apoCIII promoter strength by 70, 78, and 85% for c-Jun, JunB, and JunD, respectively (Figure 5A). The findings suggest that homodimers of Jun (*44*−*46*) or heterodimers of Jun with other partners (*19*−*27*) can repress apoCIII promoter activity. In contrast, cotransfections of apoCIII promoter fragments (−163/+24) or (−686/+24), which lack the enhancer region, with c-Jun and JunB exhibited a 2- and 8-fold transactivation, respectively (Figure 5B). These findings are consistent with the hypothesis that repression by Jun requires an intact apoCIII enhancer region and that homodimers of Jun and/or heterodimers of Jun with other partners could be responsible for the observed repression by interfering with the function(s) of the apoCIII enhancer. The involvement of the apoCIII enhancer in the Jun-mediated transcriptional repression was further supported by experiments using chimeric reporter constructs that carry the apoCIII enhancer region (−790/−500) in front of the minimal AdML promoter (Figure 5D). This analysis showed that c-Jun and JunB repressed the activity of the chimeric promoter by approximately 35% $p > 0.01$ (Figure 5B). The smaller repression of the chimeric promoter as compared to the intact −890/+24 apoCIII promoter probably resulted from different protein−protein interactions of the proteins bound to the enhancer and those bound either to the proximal apoCIII or the minimal AdML promoter. Cotransfection experiments showed that the activity of the −890/+24 apoCIII promoter mutated in the proximal D/E (−219/−199) ATF-2-binding site was repressed to 30% by c-Jun as compared to the wild-type promoter. In contrast, the promoter mutated in the distal I (−747/−726) ATF-2-binding site was not affected by c-Jun (Figure 5C). These results support the finding that repression by c-Jun is mediated by the distal I (−747/−726) ATF-2-binding site.

The repression in apoCIII promoter activity caused by c-Jun could be reversed by cotransfecting increasing amounts of ATF-2 and HNF-4 (Figure 6, panels A and B), thus reinforcing the initial observation that homodimers of ATF-2 and possibly heterodimers of ATF-2/c-Jun act as positive activators (Figures 6 and 7A). In contrast, homodimers of Jun or heterodimers of Jun with other partners act as repressors, possibly by interfering with the transactivation of the apoCIII promoter by HNF-4, Sp1, and/or other factors bound to proximal and distal sites (Figures 5A and 7B). These findings indicate that the level of expression of the human apoCIII gene in hepatic cells can be modulated by the relative concentration or state of activation of these two factors in the cell. Potential physical interactions of Jun with HNF-4 and Sp1 which may impede transcription is the topic of ongoing research. Putative positive and negative interactions of ATF-2 and Jun dimers with other proteins of the apoCIII promoter/enhancer complex and the basal transcriptional apparatus are illustrated in Figure 7.

DISCUSSION

Background. Recent transgenic mouse experiments suggest that the plasma apoCIII levels are correlated with plasma triglyceride levels (*47*). The plasma apoCIII levels and production rates are also increased in some patients with hypertriglyceridemia (*48, 49*). ApoCIII inhibits in vitro the function of lipoprotein lipase, the enzyme that hydrolyzes

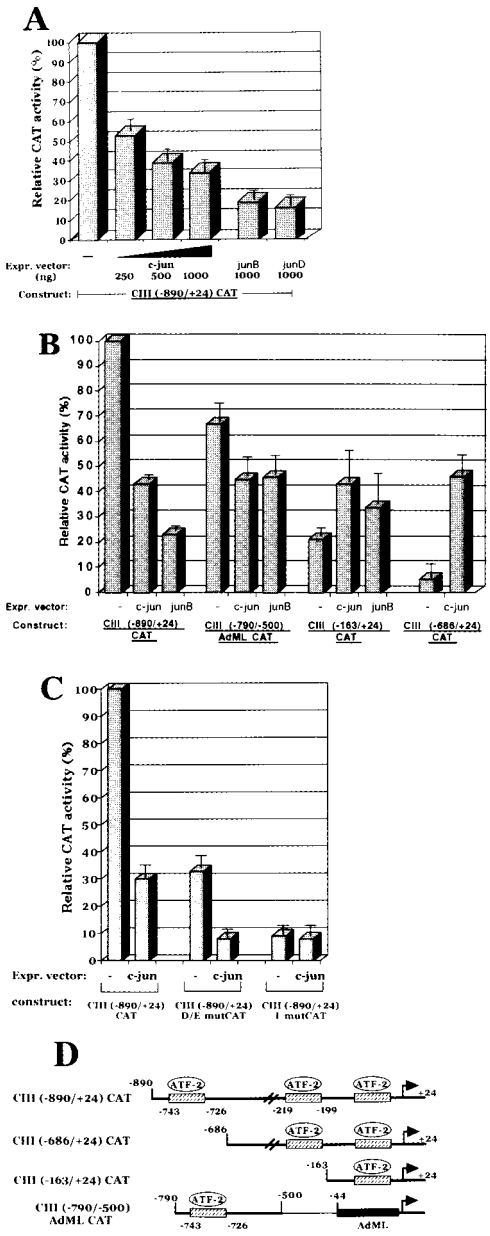

FIGURE 5: Repression of the apoCIII (−890/+24) and apoCIII (−790/−500) adML CAT promoter activity and activation of the apoCIII (−686/+24) CAT and apoCIII (−163/+24) CAT promoter activity by members of the Jun family (c-Jun, JunB, and JunD). (A) HepG2 cells were transiently transfected with 3 μg of the apoCIII (−890/+24) CAT reporter plasmid along with the indicated amounts (250−1000 ng) of the pCDNA-1/c-Jun expression plasmid or 1000 ng of pRSV-JunB, or pRSV-JunD expression plasmids. Extracts from transfected cells were utilized for determination of β-galactosidase and CAT activities as described in the Experimental Procedures. Normalized CAT values (±SEM) of at least two independent experiments performed in duplicate are presented in the form of a bar graph. The activity of the apoCIII (−890/+24) CAT plasmid transfected in the absence of Jun was set at 100%. (B) HepG2 cells were transiently transfected with 3 μg of one of the reporter constructs shown in panel D along with 1 μg of the pCMV-β-gal plasmid and 1 μg of pCDNA-1/c-Jun or pRSV-JunB expression vectors. Extracts from transfected cells were utilized for determination of β-galactosidase and CAT activities as described in the Experimental Procedures. Normalized values (±SEM) of CAT activity are shown in the form of a bar graph. The activity of the apoCIII (−890/+24) CAT plasmid was set at 100%. (C) HepG2 cells were transiently transfected with 3 μg of one of the reporter constructs shown in panel D along with 1 μg of the pCMV-β-gal plasmid and 1 μg of pCDNA-1/c-Jun expression vectors. Extracts from transfected cells were utilized for determination of β-galactoside and CAT activities as described in the Materials and Methods. Normalized values (±SEM) of CAT activity are shown in the form of a bar graph. The activity of the apoCIII (−890/+24) CAT plasmid was set at 100%. (D) Schematic representation of the apoCIII promoter plasmids used in the experiments presented in panels A−C.

the triglyceride moieties of chylomicrons and VLDL (*5*), and also the binding of apoE-containing lipoproteins to the LDL receptor, but not to the LDL receptor-related protein (LRP) (*50*). Other studies have shown that the receptor-mediated binding and catabolism of lipoproteins is enhanced by apoE and is inhibited by apoCIII (*6, 7*).

On the basis of these in vivo and in vitro findings, it has been suggested that apoCIII inhibits the catabolism and subsequent clearance of triglyceride-rich lipoprotein remnants (*48, 51*). It has also been shown that apoCIII gene expression is negatively regulated in response to the proinflammatory cytokines such as TNF-α and IL-1, and it was suggested that these two cytokines function by a transcriptional repression mechanism involving nuclear factors C/EBPδ and NF-kB bound to the proximal regulatory element CIIID (*16, 17*).

Exposure of cells to cytokines triggers a series of phosphorylation events that ultimately lead to the activation of specific transcription factors which bind and regulate the transcription of target genes. One of the most extensively studied systems of signal transduction by phosphorylation is the c-Jun N-terminal kinase (JNK) pathway (*52*). This cascade of events involves small GTPases (rac, cdc42) (*53*) and protein kinases such as PAK (*54*), MEKK1 (*55*), MEK and/or SEK (*56*), and ultimately JNK (*57*), which phosphorylates and activates c-Jun and ATF-2. ATF proteins can form heterodimers with Jun that recognize palindromic AP-1 sites TGANNTCA separated by two spacer nucleotides (*19*−*21, 23*−*26, 58, 59*) whereas Jun/Fos heterodimers recognize TGANTCA repeats with one spacer nucleotide. The DNA motif and adjacent nucleotides may also influence dimer selection and conformation (*18*).

ATF-2 Binds and Transactivates the Human ApoCIII Promoter. Inspection of the apoCIII promoter regions that are protected by DNase I footprinting either with liver nuclear extracts or with purified ATF-2 identified a perfect TGANNTCA repeat on the sense strand of element D/E between nucleotides −212 to −205 separated by two spacer nucleotides. Such motifs are the preferred binding site of ATF/CREB heterodimers (*19, 22, 58, 59*). In addition, an imperfect TGGNTCC repeat was identified on the sense strand of the footprint of element I (−747/−726) between nucleotides −733 to −727. No obvious AP-1 binding motif is evident in the proximal ATF-2 footprinting region (−102/−75), although this region is characterized by the presence of an asymmetric TGACCAGT site in the antisense strand between nucleotides −88 to −95 that may serve as a recognition sequence for ATF-2.

The binding of ATF-2 on element D/E (−219/−199) has been confirmed by gel electrophoretic mobility shift assays. ATF-2/c-Jun heterodimers and possibly other AP-1 family members may also bind to this site. These observations suggest that ATF/CREB family members may contribute to the apoCIII promoter activity in hepatic cells under physiological conditions as well as following stimulation by cytokines and other stress-inducing stimuli.

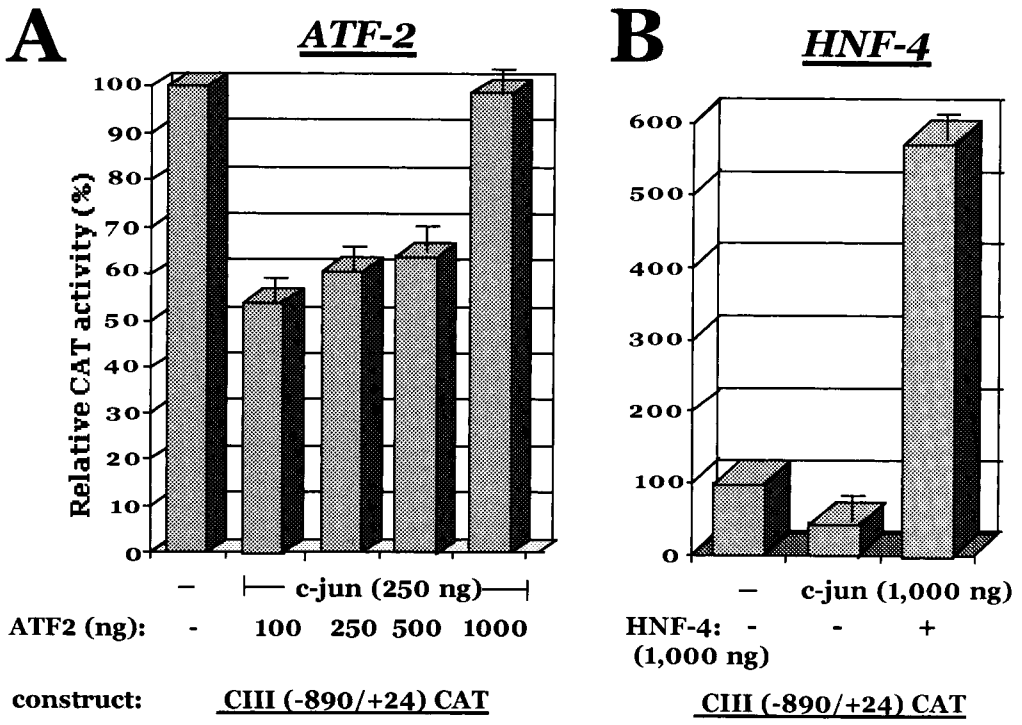

FIGURE 6: Repression of apoCIII promoter activity by c-Jun can be reversed by ATF-2 and HNF-4. (A) HepG2 cells were transiently transfected with 3 μg of the apoCIII (−890/+24) CAT plasmid in the absence or presence of 250 ng of pCDNA-1/c-Jun and the indicated amounts (100−1000 ng) of the expression plasmid pCDNA-1/ATF-2. Extracts from transfected cells were utilized for determination of β-galactosidase and CAT activities as described in the Materials and Methods. The mean values (±SEM) of at least two independent experiments performed in duplicate is presented in the form of a bar graph. (B) HepG2 cells were transiently transfected with 3 μg of the apoCIII (−890/+24) CAT plasmid in the absence or presence of 1 μg of pCDNA-1/HNF-4 or pCDNA-1/c-Jun expression vectors as indicated at the bottom of the figure. Extracts from transfected cells were utilized for determination of β-galactosidase and CAT activities as described in the Materials and Methods. Mean values (±SEM) of at least two independent experiments performed in duplicate is shown in the form of a bar graph.

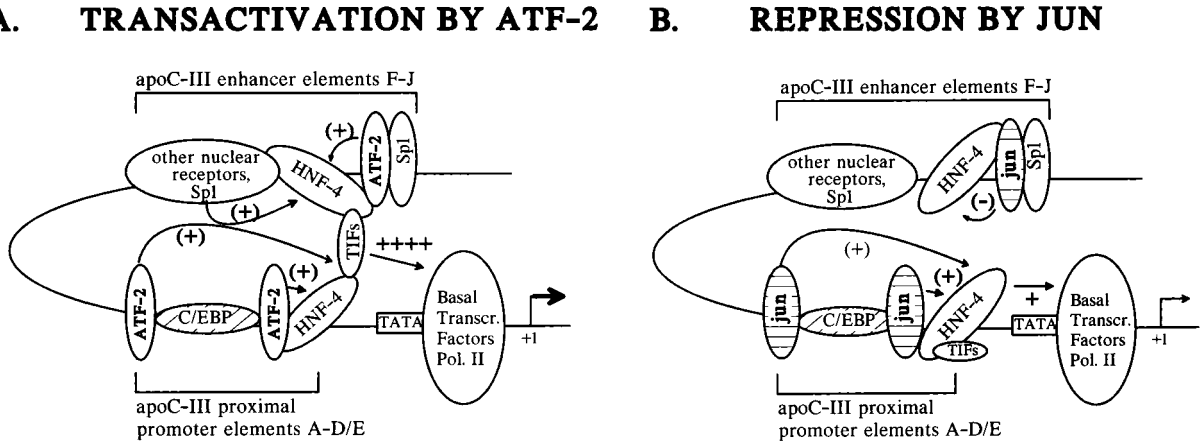

FIGURE 7: Schematic presentation showing the role of ATF-2 and Jun on the transcriptional activation or repression of the human apoCIII promoter. The model assumes participation of transcriptional mediators/intermediary factors (TIFs) and putative protein−protein interactions that facilitate the HNF-4 mediated transactivation of the apoCIII promoter which may either lead to further transactivation by ATF-2 (A) or repression by Jun family members (B).

The potential participation of ATF-2 in apoCIII gene regulation was assessed by cotransfection experiments as well as by mutagenesis of the ATF-2-binding sites. Both sets of experiments indicated that ATF-2 acts as a positive regulator that influences apoCIII promoter strength. ATF-2 could transactivate the apoCIII promoter up to 1.6-fold in a dose-dependent manner. The same level of transactivation was observed by cotransfection of HepG2 cells with the apoCIII promoter along with expression vectors for MEK4 and JNK1 that phosphorylate ATF-2 (D.K. and V.I.Z., unpublished data). This observation indicates that apoCIII gene expression can be modulated by the JNK pathway, following cytokine stimulation. In addition, mutations in the proximal D/E (−219/−199) and the distal enhancer I (−747/−726) ATF-2-binding sites reduced the promoter strength by approximately 70 and 90%, respectively, establishing the importance of ATF-2 in normal apoCIII gene expression.

The ATF-2-binding site on element I overlaps partially with the binding sites of Sp1 and HNF-4. Thus, it is possible that this mutation may affect protein−protein interactions of the factors bound on element I as well as their interactions with the basal transcription factors.

Previous studies in HepG2 cells have shown that transactivation of the apoCIII promoter requires synergistic interactions between HNF-4 bound to the proximal promoter and distal enhancer sites and Sp1 or other factors which bind to the enhancer and proximal promoter regions (*11*−*13*).

The present study established that transactivation of the apoCIII promoter by combination of HNF-4 and ATF-2 is additive. In addition, the mutations in either the proximal (−219/−199) or the distal (−747/−726) ATF-2-binding sites did not affect the HNF-4-mediated transactivation of the apoCIII promoter, indicating that HNF-4 and ATF-2 contribute independently to the apoCIII promoter strength.

Repression of the ApoCIII Promoter Activity by Jun Family Members Is Mediated by the ApoCIII Enhancer Region. A surprising finding of the current study was that Jun family members repressed the activity of apoCIII promoter segments and synthetic promoters which contained the apoCIII enhancer region. This region was shown recently to act as a general enhancer that potentiates the activity of proximal promoters which contain nuclear hormone receptor binding sites (HREs) (*13*, *14*, *60*). The enhancer itself contains three binding sites for transcription factor Sp1, three hormone response elements, and a site for a still unidentified factor (Figure 1C). Mutation analysis showed that the enhancer activity was severely compromised by mutations in the HRE of element I, by mutations in element G, and by mutations in the Sp1-binding sites (*12*, *13*). It has been shown previously that c-Jun down regulates the rat-α-fetoprotein (AFP) promoter in HepG2 cells. Repression by c-Jun did not involve binding to DNA but rather it was proposed that protein−protein interactions between c-Jun and another transactivator acting on the AFP promoter are involved in the mechanism of repression (*61*).

In the present study, the activity of the apoCIII promoter could be restored by a combination of Jun and ATF-2, thus, reinforcing the finding that ATF-2 acts as a positive regulator of the apoCIII promoter. Combination of HNF-4 and Jun reversed the repression caused by Jun alone, and resulted in a 5-fold transactivation. This level of transactivation is lower than the 10-fold transactivation obtained with HNF-4 alone (Figure 4A). Therefore, Jun may interfere with the functions of factor(s) bound to the apoCIII enhancer including HNF-4, Sp1, and ATF-2.

The concentration of c-Jun is very low in HepG2 cells but it increases dramatically and transiently following treatment of HepG2 cells with phorbol ester (PMA) compounds that strongly repress apoCIII promoter activity (data not shown) (*62*). Another possibility could be interference of Jun with proteins of the basal transcriptional apparatus or squelching of positive intermediary factors which are required for transactivation (*63*). However, the fact that promoter segments lacking the apoCIII enhancer are activated by high concentrations of Jun argues against a squelching mechanism as a cause for the observed repression.

According to previously proposed models, it is assumed that HNF-4, which binds to the proximal and distal HREs as well as other factors that have overlapping boundaries with HNF-4 on the apoCIII enhancer, forms a stereospecific DNA−protein complex (*64*). This complex may interact directly or indirectly through TAFs or TIFs (*64*−*66*) with the basal machinery. These putative interactions could be potentially reinforced when the ATF-2-binding sites are occupied by AP-1 dimers containing ATF-2 (Figure 7A). On the other hand, such interactions may be impeded when the AP-1 sites are occupied by Jun homodimers or heterodimers of Jun with other members of the Fos/ATF/CREB family besides ATF-2 (Figure 7B).

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