Nuclear Factors AF-1 and C/EBP Bind to the Human ApoB Gene Promoter and Modulate Its Transcriptional Activity in Hepatic Cells*

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ApoB-100, the major protein of low density lipoprotein, is produced primarily in the liver. The apoB promoter region from −86 to −52 binds two hepatic nuclear proteins. The protein that binds to the sequence between −86 to −61, called AF-1, was characterized and also shown to interact with the apoCIII, apoA-I, and apoCII promoters. We now show that the second protein, which by DNase I footprinting binds to the sequence between −60 and −52, is the heat stable hepatic factor C/EBP. The binding of this protein is competed by a known C/EBP-binding sequence in the transthyretin gene enhancer (TRT oligo 3). In addition, the protein protects the same region in DNase I footprinting as purified C/EBP. Mutants that affect AF-1 or C/EBP binding to the apoB promoter were created. These mutants were analyzed by the gel mobility shift assay and transient transfections into HepG2 cells. We have demonstrated that the two factors bind to overlapping sites and that they modulate apoB transcriptional activity.

ApoB-100 is one of 10, well-characterized apolipoproteins (1, 2). In humans, it is synthesized mainly in the liver and is a constituent of very low density lipoproteins, intermediate density lipoproteins, and LDL (3–5). ApoB-100 is virtually the sole protein of LDL and is the ligand for the LDL receptor (4, 5). This interaction mediates the removal of LDL from plasma and mutations in the apoB-100 receptor-binding site (6–9), or the LDL receptor (5) can cause elevated plasma LDL cholesterol levels, which correlate directly with coronary heart disease incidence (10, 11). Alternatively, mutations that decrease apoB production can lead to decreased plasma levels of LDL cholesterol (4, 12–17). Thus, the apoB gene plays a crucial role in determining plasma LDL cholesterol levels.

Because of the central role of apoB-100 in lipoprotein metabolism, we have chosen to study the transcriptional regulation of the gene that codes for this protein. In a previous study (18), we have shown that hepatic specific transcription of apoB is determined by two cis-acting positive elements. The distal element (−128 to −90) has a 4-fold positive effect in HepG2 cells, while the proximal positive element (−86 to −70) has a 13-fold positive effect in HepG2 cells. These results have recently been confirmed by Carlsson et al. (19). We also have shown that HepG2 cell and mouse liver nuclear extracts contain a protein that interacts with sequences between −86 and −70 (designated AF-1, formerly APF-1) and another protein that binds to sequences downstream from −70 (18). AF-1 has recently been shown to interact with several apolipoprotein genes (20) and to be required for high levels of apoCII gene transcription (21).

In the current paper, we demonstrate that this second protein appears to be identical to the hepatic transcription factor C/EBP (22, 23). The binding of this protein is heat stable, can be competed with a known C/EBP-binding sequence, and its DNase I footprint is identical to that of purified C/EBP. In addition, mutants that affect AF-1 or C/EBP binding to the apoB promoter region were created and have been analyzed by gel mobility shift assay and transient expression in HepG2 cells. Elimination of AP-1 binding resulted in a 50-fold reduction of wild-type promoter activity while elimination of C/EBP binding reduced transcriptional activity 2–5-fold. However, this dominant role of AF-1 in driving apoB transcription in HepG2 cells, might be due to low concentrations of C/EBP in hepatoma cell lines relative to liver C/EBP concentrations (24). DNase I footprinting experiments were used to demonstrate that AF-1 and C/EBP can bind to overlapping sequences in the apoB promoter.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—The plasmid pKT261 contains the human apoB gene sequence from nucleotides −261 to +122 (relative to the start site of transcription), inserted upstream of the coding sequence of the bacterial CAT gene (18). To create construction M192, pKT261 was cut at the AaI (nucleotide −142) and Nael (−91) sites, and the AaI-Nael fragment was replaced by a synthetic fragment, containing the unique restriction sites BgII (−128) and Xhol (−55) (described schematically in Fig. 3). To create constructions M192, M321, and M341, the BgII-Xhol fragment was replaced by different synthetic fragments containing specific nucleotide substitutions, as described in Fig. 3.

Cell Culture and Transfection Experiments—HepG2 cells were maintained in minimum essential medium supplemented with 10% fetal calf serum. For DNA transfection experiments, cells were plated in 60-mm culture dishes at approximately 35% confluence and cultured for 24 h prior to transfection. DNA was transfected into cells by the calcium phosphate coprecipitation method (25). Sixteen h post-transfection, cells were shocked with 1 ml of 15% glycerol in phosphate-buffered saline for 3 min. The medium was replaced and the cells harvested at 48 h post-transfection. Cellular protein extracts were prepared from cells disrupted by freeze-thawing. The protein concentration of the extracts was determined with the Bio-Rad reagent. The CAT assay was performed by the method of Gorman et al. (26). CAT activity was quantitated by scintillation counting of spots cut from chromatograms. The percent of [14C]chloramphenicol...
substrate converted to acetylated product was determined. To normalize for equal efficiency of transfection, a reference plasmid containing the bacterial β-galactosidase gene under the control of the SV40 early promoter was cotransfected with the test plasmid. β-Galactosidase activity was determined as described by Miller (28).

**Nuclear Extract Preparation**—Nuclear extract from fresh mouse liver was prepared by the method of Gorski et al. (29), except that the nuclei were extracted in 0.6 M KCl. After removal of the nuclei by centrifugation, the nuclear extract was diluted to a salt concentration of 250 mM and loaded onto a heparin-agarose column. The column was eluted with a linear 250–800 mM gradient of KCl. Fractions containing AF-1 and C/EBP, as analyzed by the gel mobility shift assay, were combined. Purified rat C/EBP was a gift from Dr. Robert Costa (30); purified mouse AF-1 was a gift of Dr. Bertil Ohlsson.

**Gel Mobility Shift Assay**—Protein-DNA binding reactions were carried out in a volume of 100 μl containing 60 mM KCl, 25 mM HEPES, pH 7.9, 3% Ficoll, 0.5 mM dithiothreitol, and 1 mM MgCl₂. Poly(dI)- (dC) (1.6 μg) was added as a nontspecific competitor. A typical reaction contained 10,000–20,000 cpm (approximately 0.5 ng) of 5' labeled DNA with 0.5–5 μg of protein extract. After addition of extract, samples were incubated at room temperature for 15 min and analyzed by electrophoresis on 4% polyacrylamide gels in 0.25 X TBE (2.2 mM Tris-borate, 2.2 mM boric acid, 0.5 mM EDTA). For competition experiments, conditions were as above except that appropriate competitor DNA (as indicated) was added to the reaction mixture prior to addition of extract.

**DNase I Footprinting**—The coding strand of a DNA fragment containing sequences from −176 to +23 with respect to the start site of apoB gene transcription was 5' end-labeled with [γ-32P] ATP and used as a template for footprinting. Ten ng (approximately 25,000 cpm) of the fragment was incubated with 50 μg of nuclear protein for 15 min on ice in a 50-μl reaction containing 20 mM Hepes, 7.5, 60 mM KCl, 4 mM MgCl₂, 1 mM dithiothreitol, 10% glycerol, 2% polyvinyl alcohol, 40 μM ATP, and 1.25 μg of poly(dI)-(dC) (reaction buffer). The mixture was then incubated at room temperature for 3 min. Fifty μg of 5 mM CaCl₂ and 1 mM EDTA solution containing 6 ng of DNase I was added and allowed to digest for 60 s at room temperature. DNase I was inactivated by the addition of 100 μl stop buffer containing 0.6 M sodium acetate, 1% sodium dodecyl sulfate, 20 mM EDTA, and 100 μg/ml glycogen. The DNA was purified by phenol extraction and ethanol precipitation and analyzed on a 6% polyacrylamide, 50% urea DNA sequencing gel. A G+A ladder, generated by sequencing (30) the same end-labeled fragment, was run in the same sequencing gel.

**RESULTS**

A Heat Stable Factor Interacts with the Proximal Positive Region of the ApoB Gene Promoter—In a previous report, a variety of human apoB gene promoter constructions were incubated with HepG2 and mouse liver nuclear extracts and analyzed by gel mobility shift assays. These experiments showed that the region between −66 and −62 binds two factors. The first factor was shown to bind between nucleotides −86 to −70 and to cause the shifted bands called B1 and B2. The second factor required nucleotides −70 to −62 for binding and caused the shifted bands called B3, B4, and B5 (18). In the present study, an apoB gene promoter construction from −176 to +23 was incubated with a mouse liver nuclear extract partially purified by hapiarin-agarse chromatography. When this probe was used in the gel mobility shift assay, six shifted bands were seen (Fig. 1A, lane 1). Five of these bands are comparable to those previously designated B1 to B5. The new band had the slowest mobility and is called B6. A 50- to 100-fold excess of unlabeled DNA fragment from −70 to −6 competed for bands B3 to B5 (Fig. 1A, lanes 3 and 4), whereas a DNA fragment from −66 to −6 competed for all six shifted bands (Fig. 1A, lanes 7 and 8). In addition, a DNA fragment from −152 to +23 containing a −86 to −63 internal deletion (152C) failed to compete for any of the six shifted bands (Fig. 1A, lanes 9 and 10). These results confirm that there are two liver nuclear factors that interact with the apoB promoter in this region. As shown below in DNase I footprinting experiments with purified proteins, it is likely that the multiple bands observed for each binding activity represent multimers or degradation products of single proteins.

The liver nuclear factor that binds to the −86 to −70 region has been identified, partially characterized, and named AF-1 (21). To better characterize the factor that binds between −70 and −62, the DNA fragment from −176 to +23 was incubated with an aliquot of the partially purified mouse liver nuclear extract that had been heated at 90°C for 10 min. When subjected to gel mobility shift assay, this extract revealed only bands B3 to B5. These, therefore, represent the binding of a heat stable factor. Bands B0 to B2 were missing, indicating that AF-1 is a heat labile factor (Fig. 1A, lane 2 compared with lane 1).

Confirmation of these observations was derived by DNase I footprinting analysis. In these experiments the labeled DNA fragment from −176 to +23 was incubated with unheated and heated (90°C for 10 min) partially purified mouse liver nuclear extract and then subjected to DNase I digestion. The unheated extract gave a strong footprint between nucleotides
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The heated extract, however, only protected the region between -69 to -52 (Fig. 2, lane 1 compared with lane 6). The AF-1 footprint between -81 to -69 disappears when the extract is heated.

The Heat Stable Factor That Interacts with the ApoB Promoter Is C/EBP—C/EBP is an abundant heat stable protein present in liver nuclear extracts, that interacts with the SV40 enhancer (22) and the regulatory regions of several liver-expressed genes, such as albumin (31-34), TTR (35), and transferrin (36). C/EBP has been shown to interact with two different sequences. One of these is a sequence motif found in the SV40, murine sarcoma virus, and polyoma virus enhancers, called the enhancer core sequence (Table I). The other is the CCAAT homologous sequence (22). The apoB gene sequence from -69 to -52, that is protected from DNase I by the heat stable factor, in one region -69 to -61 (GACCTTTTG) resembles the enhancer core sequence (TGTTGA(TA/T)(A/T)(A/T)G) and in another region -60 to -52 (CAATCCCTG) contains the CAAT sequence.

To establish that C/EBP binds to the apoB promoter, several experiments were done. A known C/EBP-binding site found in the TTR gene enhancer approximately 1.9 kilobases upstream of the TTR transcription start site (35) (TTR oligo 3, Table I), was used as competitor in the gel mobility shift assay. This fragment was shown to compete very effectively with the apoB template for binding to the heat stable factor (Fig. 1A, lanes 5 and 6). The gel mobility shift assay was also used to show direct interaction of purified rat liver C/EBP with the apoB template (Fig. 1B, lane 3). The mobility of the rat C/EBP-apoB template complex is not identical to the mouse liver heat stable factor-apoB template complex (Fig. 1B, lane 3 compared with lane 2). This may be due to species differences in the molecular weight of C/EBP or partial degradation of rat C/EBP during the purification process.

In the DNase I protection assay, the purified rat C/EBP protected the region of the apoB promoter from -69 to -52 (Fig. 2, lanes 8 and 9). This was identical to the protected region seen with the heated mouse liver extract (Fig. 2, lane 2). These experiments strongly suggest that the heat stable mouse liver nuclear factor that binds to the apoB promoter from -69 to -52 is C/EBP.

The removal of C/EBP binding to the apoB template by competition with the TTR oligo 3 gave surprising results in the DNase I protection assay. The TTR oligo 3 eliminated the footprint in the entire region from -69 to -52 only when heated extract was used (data not shown). With unheated extract the TTR oligo 3 eliminated the footprint only from -61 to -52 (Fig. 2, lanes 3-5). These results indicated that either AF-1 itself or another heat-sensitive protein is also capable of interacting with the region from nucleotides -69 to -61.

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Fig. 3. Description of mutations introduced into the human apoB gene promoter region. The apoB gene fragment from −261 to +122 was inserted into the pKT plasmid (18) to upstream of the structural CAT gene (pKT261). M182 contains two unique restriction sites, BglII at −128 and XhoI at nucleotide −55, that were introduced as described under "Experimental Procedures." Double-stranded synthetic oligonucleotides containing nucleotide substitutions as shown were inserted into M182 to create M192, M321, and M341. M192 contains a three nucleotide substitution in the AF-1-binding site; M321 and M341 contain three nucleotide substitutions in two different regions of the C/EBP-binding site.

Fig. 4. Gel mobility shift assay of the different mutants with purified mouse AF-1 or rat C/EBP. Radiolabeled BglII-XhoI fragments from M182, M192, M321, and M341 were incubated with purified mouse AF-1 (lanes 1–4) or with rat C/EBP (lanes 5–8). M192 contains a mutation in the AF-1-binding site and does not bind AF-1 (lane 2). M321 and M341 contain two different mutations in the C/EBP-binding site and do not bind C/EBP (lanes 7 and 8).

nucleotide substitution (−58 to −56) in the CAAT-binding site also fails to bind C/EBP (Fig. 4, lane 8) but binds AF-1 (Fig. 4, lane 4). These data demonstrate that mutations in the C/EBP-binding site in either the enhancer core motif or in the CAAT box motif can abolish C/EBP binding and that AF-1 binding is independent of C/EBP binding.

The Role of AF-1 and C/EBP in Hepatic ApoB Gene Transcription—Transient transfections of the pKT261 and M182 constructions (Fig. 3) into HepG2 cells resulted in almost equal CAT activity (Fig. 5). The M192 construction, which fails to bind AF-1 but binds C/EBP, is essentially inactive (2%). The M321 and M341 constructions, which fail to bind C/EBP but bind AF-1, both have reduced activity compared with pKT261. However, they do not have the same activity. M321, a mutant in the overlapping region of the AF-1- and C/EBP-binding sites, was at 22% and M341 at 51% of control. These results indicate that in HepG2 cells C/EBP by itself is essentially incapable of activating apoB gene transcription unless AF-1 is present. It has recently been reported that C/EBP is much less abundant in hepatoma cell lines than in the liver (24). In order to determine if the dominant role of AF-1 in HepG2 is due to low concentrations of C/EBP, we cotransfected apoB constructions with expression vectors carrying wild-type and mutated C/EBP cDNA (pMSV-C/EBP-wt, pMSV-C/C/EBP-12V, kindly provided by S. McKnight).

As shown in Fig. 6, over-expression of C/EBP elevates the transcriptional activity of construction M192 (17-fold), which does not bind AF-1 but does contain an intact C/EBP-binding site. Under the same conditions, the transcription driven by construction M321, which contains a mutation in the C/EBP-binding site, is not affected by over-expression of C/EBP.

The cotransfection experiments were performed with three different pMSV-C/EBP-wt plasmid preparations; β-galactosidase activity was noticed. The same results were obtained with three different plasmid preparations of pMSV-C/C/EBP-wt. (The cotransfection experiments were performed with three independent plasmid preparations for each construction. CAT activities were normalized for transfection efficiency using β-galactosidase activity from a cotransfected β-galactosidase plasmid.)

These results suggest that the relatively small effect of C/EBP on apoB gene transcription in HepG2 cells does not necessarily reflect the situation in the liver.

DISCUSSION

C/EBP Binds to the Proximal Positive Region of the ApoB Gene Promoter from −69 to −52—Transfection experiments in HepG2 cells indicate that the apoB gene upstream region from −261 to +122 is sufficient to direct hepatic transcription (18). Two hepatic nuclear factors have been shown to bind to this region. The first protein, named AF-1, binds to the DNA
region between -81 and -69. AF-1 also binds to homologous sequences in the promoters of other liver expressed genes, including three other apolipoprotein genes, apoCII, apoA-I, and apoCIII (20). In the current report, we have demonstrated that the second nuclear factor, which binds to the DNA region between -69 and -52, is a previously characterized hepatic transcription factor C/EBP. The footprint made by this factor observed in the region between -69 and -52 is heat stable and competed for by a known C/EBP-binding sequence (Figs. 1 and 2). Furthermore, this factor shows the same binding specificity as purified C/EBP, when analyzed by DNase I footprinting and gel mobility shift assays (Fig. 2). C/EBP has two known binding sequence preferences, the enhancer core motif, and the CCAAT box motif (22). In the case of the apoB gene these occur adjacent to each other and mutations in either site (mutant M321 or mutant M341) abolish C/EBP binding (Figs. 3 and 4). One interpretation of these results is that C/EBP binds as a dimer requiring two adjacent binding sites.

Recently, it has been shown that another protein, DBP, also binds to the albumin element D (a known C/EBP-binding site) and might be the transcriptional activator in that case (33). It is possible that DBP also recognizes the apoB C/EBP-binding site (homologous to the albumin element D, Table I) and plays a role in the transcriptional activation of the apoB gene.

C/EBP was purified from rat liver nuclear extract and characterized as a 42-kDa protein (22, 23). The rat C/EBP cDNA was cloned and the DNA-binding domain of C/EBP was found to contain a basic region adjacent to a leucine zipper domain (23, 37, 39). Recently, the mouse C/EBP cDNA has also been cloned (39). The partial protein sequence predicted is very homologous to the rat protein. It was shown that liver-specific expression of C/EBP is due to transcriptional regulation of the apoB gene. C/EBP is known to bind to the regulatory region of some liver-expressed genes, where it recognizes either a CCAAT sequence motif (transferrin (36), apoB) or an enhancer core sequence motif (TTP (35), albumin (31-33), α1-antitrypsin (35), apoB). Some of the known C/EBP-binding sites are summarized in Table I.

C/EBP and AF-1 Can Both Bind to the Region of the ApoB Gene Promoter between -69 to -61—Footprinting analysis with a heated hepatic nuclear extract (which inactivates AF-1) or purified C/EBP reveals that C/EBP binds to the apoB promoter from -69 to -52. When C/EBP is removed from an unheated hepatic extract (by competition with TTR oligo 3), a heat labile protein (AF-1) footprints from -81 to -61. The same footprint (-81 to -61) is also observed when C/EBP fails to bind because of mutations in its binding sites (M341, M321) (data not shown) or when purified AF-1 is used (Fig. 2). Thus, the region from -69 to -61 can be protected by either protein. In addition, a mutation in this region (M321) abolishes only C/EBP binding, indicating that it is not required for AF-1 binding. The region from -69 to -61 does contain a partial AF-1 binding sequence which presumably accounts for its ability to bind AF-1 (21). It is interesting in this regard, that the mutation in the -69 to -61 region (M321) decreases transcription by 5-fold, whereas the other mutation that abolishes C/EBP binding, but is outside this region (M341), reduces transcription by only 2-fold. These results suggest that the sequences between -69 and -61 are required for C/EBP binding and affect AF-1 transcriptional activity (at least in the absence of C/EBP) without being required for AF-1 binding.

AF-1 and C/EBP Modulate ApoB Gene Transcription—We have demonstrated that the binding of both AF-1 and C/EBP contributes to the wild-type transcription of the apoB gene. Mutational analysis in HepG2 cells indicates that disruption of the binding of AF-1 almost eliminates transcriptional activity, while elimination of C/EBP binding reduces the transcription level 2-5-fold. The results with a cotransfected plasmid that expressed C/EBP suggest that the strong relative effect of AF-1 on apoB transcription compared with C/EBP in HepG2 cells might be a function of the intracellular C/EBP concentration.

It has been reported that C/EBP is expressed at a much lower level in hepatoma cell lines than in the liver. When the amount of C/EBP is artificially elevated to a saturating level in HepG2 cells by cotransfection with a C/EBP expressing plasmid, strong activation of apoB gene transcription is achieved. However, the cotransfection experiments may produce unphysiologically high amounts of C/EBP in the HepG2 cells and probably exaggerate what is the normal effect of C/EBP on apoB gene transcription in vivo. Nevertheless, in this study we have shown that C/EBP binds to the apoB gene promoter and has the potential to affect its transcription. The developmental expression of C/EBP mRNA and apoB mRNA is very similar in the liver as well as in the intestin (40, 41). These results are consistent with a role for C/EBP as a modulator of apoB gene expression in vivo.

The studies described in this report are helpful in understanding the mechanism controlling hepatic apoB gene transcription. They may also lead to the identification of mutations either in the regulatory regions of the apoB gene or in other genes that produce trans-acting factors interacting with these regions. Such mutations could have a profound influence on the lipoprotein transport system, plasma LDL cholesterol levels, and atherosclerosis susceptibility.

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