Protein-DNA Interactions at a Drug-responsive Element of the Human Apolipoprotein A-I Gene*  

(Received for publication, May 2, 1996, and in revised form, July 29, 1996)  

Xia Zhang, Zhang-Qun Chen, Zhiwei Wang, William Mohan, and Shui-Pang Tam‡  
From the Department of Biochemistry, Queen's University, Kingston, Ontario K7L 3N6, Canada  

Previously, we demonstrated that when two human hepatoma cell lines, Hep3B and HepG2, were exposed to gemfibrozil, a hypolipidemic drug, a 2-fold induction in apolipoprotein A-I (apoA-I) mRNA levels resulted. To determine if mRNA stabilization was responsible for the changes in apoA-I mRNA levels, the half-lives for apoA-I mRNA were measured in the presence of actinomycin D with and without gemfibrozil. These experiments revealed no differences in stability. However, nuclear run-on assays indicated that the transcription rate of the apoA-I gene was increased 2-fold in gemfibrozil-treated cells. Transient transfection experiments also indicated that the induction of apoA-I mRNA level in response to gemfibrozil is mediated at the transcriptional level. We have identified two copies of the “drug-responsive element” (DRE) in the apoA-I promoter region that may be responsible for the increase in apoA-I transcriptional activity by gemfibrozil. Using gel mobility shift assays with a synthetic DRE oligonucleotide, we have demonstrated that exposure of Hep3B and HepG2 cells to gemfibrozil resulted in strong induction of a protein-DNA complex. The formation of this complex is highly sequence-specific as indicated by the DNA competition experiments. The drug-inducible nuclear proteins bind to the DRE of the human apoA-I gene with an apparent $K_d$ of 4.1 nM. Methylation interference experiments have localized the contact sites of nuclear factors to the DRE region. Southwestern blot analyses have identified two groups of drug-inducible nuclear proteins with molecular masses of approximately 30 and 15 kDa. When a copy of synthetic DRE oligonucleotide was inserted upstream of the thymidine kinase promoter and luciferase reporter construct, a significant 2-fold induction in luciferase activity was observed in the presence of gemfibrozil following transient transfection of two human hepatoma cell lines, HepG2 and HepB. However, a plasmid containing one copy of mutated apoA-I-DRE oligonucleotide did not confer responsiveness to gemfibrozil treatment. Furthermore, pGL2 (apoA-I –250 mutant DRE), which carried an internal mutation of the DRE in the human apoA-I proximal promoter region, showed no increase in luciferase activity in response to gemfibrozil. These results implicate protein-DNA interactions at the DRE region in the transcriptional induction of human apoA-I gene expression by gemfibrozil.

apoA-I$^-$ is the major constituent of plasma high density lipoprotein (HDL) and participates in cholesterol ester formation as a cofactor for lecithin-cholesterol acyltransferase (1). It has been proposed that apoA-I, together with lecithin-cholesterol acyltransferase, promotes cholesterol efflux from peripheral tissues and carries the excess cholesterol to the liver for catabolism (1–3). This process is known as “reverse cholesterol transport.” Epidemiological and genetic studies have indicated that levels of plasma HDL are inversely correlated with atherosclerotic risk (4–8). Finally, evidence that apoA-I expression is a major determinant of atherosclerosis comes from the work of Rubin et al. (9), which indicated that transgenic mice expressing high amounts of human apoA-I were significantly protected from the development of fatty streak lesions after exposure to high fat diets.

There are a number of drugs widely used for reducing the progression of coronary heart diseases by altering lipoprotein metabolism. One of the most extensively studied is gemfibrozil. The Helsinki Heart Study (10) demonstrated that the elevation of HDL cholesterol resulting from use of this drug had a more protective effect in reducing atherosclerotic risk than the lowering of LDL cholesterol. Recently, we have demonstrated that exposure of two human hepatoma cell lines, HepG2 and Hep3B, to gemfibrozil resulted in a 2-fold induction of apoA-I mRNA (11). The cis- and trans-acting elements involved in apoA-I gene transcription have been extensively studied (12–14). Based on transient transfection studies, it was concluded that the DNA region located between nucleotides –256 and –41 upstream from the transcription start site (+1) of the human apoA-I gene contains regulatory elements that are necessary and sufficient for maximal expression in HepG2 cells (12). This DNA region acts as a powerful liver-specific enhancer (13). The mechanism by which drugs such as gemfibrozil regulate apoA-I mRNA levels has not been determined. Evidence summarized below suggests that expression of the apoA-I gene may be influenced directly by drug-induced transcription factors.

It is well known that the cytochrome P-450 system is responsible for the metabolism of a variety of drugs (15). The induction of the CYP1A1 gene is mediated by the interaction of the aryl hydrocarbon receptor (AhR) together with its ligand to several DNA recognition sites within a dioxin-responsive enhancer upstream of the CYP1A1 gene (16–18). While examining the structure of the human apoA-I gene promoter a sequence match with the 5′-flanking region of the CYP1A1 gene was detected (19). The match involves a decanucleotide motif,

*This work was supported by grants from the Medical Research Council of Canada and by an Ontario Heart and Stroke Foundation Career Investigator Award (to S.-P. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.  
‡To whom correspondence should be addressed. Tel.: 613-545-2826; Fax: 613-545-6830.

1 The abbreviations used are: apoA-I, apolipoprotein A-I; HDL, high density lipoprotein; P-450, cytochrome P-450; AhR, aryl hydrocarbon receptor; MEM, minimal essential medium; FBS, fetal bovine serum; DTT, dithiothreitol; DRE, drug-responsive element; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; bp, base pair(s); PCR, polymerase chain reaction; AP1 and AP2, activator proteins 1 and 2, respectively.
transcription with labeled pGEM SZ control vector. The relative rates of transcription were calculated in terms of parts per million of substitution of control values. Total incorporation into RNA per assay ranged from 1 × 10^5 dpm to 5 × 10^6 dpm, all of which was used for each hybridization selection. Throughout the analysis, 3H sense strand RNAs produced by transcription with Sp6 polymerase were used to normalize for differences in hybridization efficiency. The efficiency ranged from 30 to 35%.

**Expression of Nuclear Extracts—** Hep3B and HepG2 cells were grown as described above, and 3–6 × 10^6 cells were used to prepare nuclear extracts. Nuclear extracts were prepared by a modification of the method of Dignam et al. (27). The modification was as follows: cells were suspended in 2 volumes of buffer (10 mM HEPES (pH 8.0), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 15 min. The cells were then homogenized by a 23-gauge needle five times followed by a 26-gauge needle for another five strokes instead of using a Dounce homogenizer. Nuclear extracts were dialyzed against binding buffer (25 mM HEPES at pH 8.0, 12.5 mM MgCl2, 20% glycerol, and 50 mM KCl) and stored as aliquots at −80°C. Nuclear protein content was determined by the method of Lowry et al. (28).

**Nuclear Mobility Shift Assays—** For mobility shift assays, nuclear extracts were incubated with 100 μg of poly (di-c)-binding buffer containing 5 mM EDTA and 5 μM ZnCl2 on ice for 30 min. Then 2 fmol (10,000 cpm) 5'-end-labeled synthetic oligonucleotide corresponding to the drug response element or other consensus binding elements were added to the reaction mixtures and incubated on ice for another 30 min. Free DNA and protein-DNA complexes were resolved on a 5% polyacrylamide gel in a buffer containing 10 mM Tris, 5 mM sodium acetate and 2.5 mM EDTA, pH 7.2. The gel was preelectrophoresed at 80 V for 1 h. The samples were then loaded onto the gel and electrophoresed at 80 V for 4–6 h at 4°C. Gels were vacuum-dried and exposed to Kodak x-ray film at −80°C with a DuPont Hi-plus intensifying screen. Competition assays were performed by adding the unlabeled competitor DNA 15 min prior to the addition of labeled DNA as indicated in the figure legends. The band representing the protein-DNA complex was identified by autoradiography, excised from the gel, and quantitated by Cerenkov counting.

**Southern Blot Analyses—** Equal volumes of nuclear extract and sample buffer (5% SDS, 5 mM Tris/HC1, pH 6.8, 200 mM DTT, 20% glycerol, and 0.5% pyronin Y) were mixed and subjected to electrophoresis on a 10% SDS-polyacrylamide gel at room temperature. Protein samples were electrophoresed to a Millipore Immobilon P membrane in 25 mM Tris-HCl, 192 mM glycerine, 20% (v/v) methanol, and 0.002% SDS at room temperature for 4 h. Hybridization of 5'-end-labeled oligonucleotides to nuclear proteins immobilized on the membrane was performed according to the method of Singh and Sejnowski (29). For the construction of the 1.4-kb promoter from pGL2 (apoA-I) using S1 nuclease digestion, the cloned region of interest was subcloned into pGL2 plasmid (30). The other set of primers, which contained the human apoA-I promoter between nucleotides −491 and +1 was generated by polymerase chain reaction (PCR) amplification. Two synthetic oligonucleotide primers were made complementary to the apoA-I gene between nucleotides −491 and +460 and between nucleotides −25 and +1, respectively. PCR amplification was carried out by using these two primers together with HepG2 high molecular weight genomic DNA prepared as described (31, 32). The PCR protocol was as follows: 30 cycles of 95°C for 45 s, 52°C for 45 s, and 72°C for 30 s. A 491 bp DNA fragment resulted, and its sequence was confirmed by DNA sequencing. The fragment was inserted into the XhoI site of the pGL2-Basic vector (Promega), upstream of the luciferase gene. This plasmid is hereafter called pGL2 (apoA-I −491 luc). Plasmid pGL2 (apoA-I −250 luc) was constructed by releasing a DNA fragment (−491 to −251 of the apoA-I promoter) from pGL2 (apoA-I −491) using SmaI, and the vector gel-purified and religated. Plasmid pGL2 (apoA-I −250 mutant DR) luc was prepared by a PCR-based protocol for extensive mutagenesis of a target DNA sequence as described by Morrison and Desrosiers (32). Briefly, we selected seven G and three A residues in the DRE region for mutagenesis, since these residues might participate in the interaction with nuclear proteins as determined by methylation interference. To generate this mature DRE plasmid, two sets of primers were used. One set of primers, designated GL and Luc, hybridized to specific regions of the pGL2-Basic vector (Promega), upstream of the luciferase gene. This plasmid is hereafter called pGL2 (apoA-I −491 luc). Plasmid pGL2 (apoA-I −250 luc) was constructed by releasing a DNA fragment (−491 to −251 of the apoA-I promoter) from pGL2 (apoA-I −491) using SmaI, and the vector gel-purified and religated.
complementary to the coding strand of DNA. The plasmid pGL2 (apoA-I TK) was used as the PCR template. Amplification was carried out in an Eppendorf MicroCycler E for 30 cycles (consisting of 1 min of denaturing at 94 °C, 1 min of annealing at 54 °C, and 1 min of extension at 72 °C) followed by a final extension for 10 min at 72 °C. The first round of PCR using GL + A primer pairs in one tube and B + Luc primer pairs in another tube produced fragments of 241 and 153 bp, respectively. These products were purified from agarose gels using the Sepharose Band Prep Kit (Pharmacia Biotech Inc.) combined in equimolar amounts and used as templates for the second round of PCR amplification in the presence of GL and Luc primers to generate a 342-bp product, which contained the DRE mutation. The DNA was then digested with KpnI and HindIII, and the resulting DNA fragment was purified from an agarose gel. The purified DNA fragment was cloned into the pGL2-Basic (Promega) and used to transform competent E. coli cells. DNA was prepared from individual clones by the alkaline lysis miniprep procedure, and the entire DNA insert including the area of mutagenesis was sequenced using the Sequenase version 2.0 system (U.S. Biochemical Corp.).

The vector designated pGL2 TK/luc (kindly provided by Dr. M. Petkovich, Department of Biochemistry, Queen's University) was derived from the pGL2-Basic vector in which the thymidine kinase promoter was inserted upstream of the luciferase gene. Plasmid pGL2 (apoA-I-DRE/TK/luc was constructed by cloning a synthetic oligo-DRE (between nucleotide positions −78 and −43 from the transcriptional start site of the apoA-I gene) into the SmaI site of the pGL2 TK/luc vector. The vector pGL2 (apoA-I-mutant DRE/TK/luc was constructed by cloning a synthetic mutated oligo-DRE 5'-GGCGTTTACTTTTTACTAGCCTAAAGATTAGACC-3' into the SmaI site of pGL2 TK/luc. The inserts in all of the above plasmids have the same 5' → 3' orientation as found in the natural human apoA-I promoter.

Transfections and Luciferase Assay—The human hepatoma cell line, Hep3B, was maintained as monolayers on 100-mm plates in MEM supplemented with 10% FBS. Transient DNA transfections were performed by the calcium phosphate precipitation procedure described by Gorman et al. (33). Ten μg of total DNA was used per plate, including 4 μg of reporter DNA and 6 μg of carrier pGL2 Basic Vector. After glycerol shock, the cells were washed twice with phosphate-buffered saline and once with MEM and cultured in MEM + 10% FBS in the absence or presence of gemfibrozil (40 μg/ml) for 24 h with changes of fresh medium.

Transfected Hep3B cells were harvested by washing three times in phosphate-buffered saline and assayed for luciferase activity as described (34). In all transfections, 5 μg of internal control plasmid (pSG3LacZ) containing the E. coli lacZ gene under the control of the early promoter and enhancer was included in order to correct for differences in transfection and harvesting efficiency. Transfected cells were harvested as described above, and β-galactosidase activities in the cell lysates were determined (34). The pGL2 promoter vector (Promega), which contains an SV40 promoter, is used as a reference for both transfection and luciferase assays. Parallel transfection experiments and luciferase assays were also performed using HepG2 cells by the procedures described above. All luciferase activities are reported as mean ± S.E. Significance of group differences was determined by Student's t test, using two-tailed p values.

Methylation Interference—Single-stranded synthetic oligonucleotides corresponding to the upper or lower strands of the DRE from the human apoA-I gene were radiolabeled at their 5'-ends by [γ-32P] ATP and T4 polynucleotide kinase. Double-stranded oligonucleotides were formed by heating the labeled oligonucleotide with an equimolar amount of the corresponding unlabeled complementary strand at 90 °C for 5 min. In 0.05 M Tris-HCl (pH 8.0), 0.01 M MgCl₂, followed by a slow cooling to room temperature over a period of 2 h. The double-stranded oligonucleotides were partially methylated with dimethyl sulfate as described (35). The methylation experiment was carried out according to the method of Baldwin and Sharp (35). The recovered DNA sample was cleaved with 1M piperidine and subjected to polyacrylamide gel electrophoresis on a 20% acrylamide DNA sequencing gel. An equivalent amount of radioactivity for each sample was loaded in each lane. The gel was exposed to Kodak XAR-5 film at −70 °C. The labeled oligonucleotides were subjected to Maxam and Gilbert sequencing (36), and the G + A reactions serve as markers.

RESULTS

Effect of Actinomycin D on the Induction of Apolipoprotein AI mRNA Levels by Gemfibrozil—Previously, we demonstrated that exposure of HepG2 and Hep3B cells to gemfibrozil resulted in a 2-fold induction in apoA-I mRNA levels (11). To determine whether inhibition of RNA synthesis affects the induction of apoA-I mRNA levels, Hep3B cells were treated both in the absence and presence of increasing amounts of actinomycin D for 30 min prior to the addition of gemfibrozil. Actinomycin D at concentrations greater than or equal to 0.625 μg/ml were able to block the increase in apoA-I mRNA levels that was observed when the cells were treated with gemfibrozil alone. Under the same regimen of drug treatment, similar results were also obtained using HepG2 cells (data not shown).

To examine if mRNA stabilization is responsible for the observed induction of apoA-I mRNA levels by gemfibrozil, the half-lives for apoA-I mRNA were determined in the presence of actinomycin D (1 μg/ml) with and without gemfibrozil. Hep3B cells were pretreated with gemfibrozil (40 μg/ml) or ethanol (drug vehicle) for 24 h. Cells were then cultured for additional periods ranging from 0 to 8 h in the presence of actinomycin D. As shown in Fig. 1, the level of apoA-I mRNA was approximately 2-fold higher in gemfibrozil-treated cells compared with control cells. After the addition of actinomycin D the levels of apoA-I mRNA decreased at the same rate in the absence or presence of gemfibrozil, while levels of actin mRNA remained relatively constant (Fig. 1, top panel). The apparent half-lives obtained for apoA-I mRNAs following the addition of actinomycin D in the absence or presence of gemfibrozil were approxi-
Regulation of Human ApoA-I Gene by Gemfibrozil

...content...

![Fig. 2. Time course of transcription rates of apoA-I genes in Hep3B cells cultured in the absence or presence of gemfibrozil.](image)

...content...

**Fig. 3. Gel mobility shift analysis.** Synthetic double-stranded oligonucleotides corresponding to the DNA sequence between nucleotides –79 and –44 upstream from the transcription start site (+1) of the human apoA-I gene, designated as oligo-apoA-I-DRE, were used as probes to study protein-DNA interactions. In addition, synthetic double-stranded Sp1 and AP1 consensus sequences were also utilized as probes to determine their activities before and after gemfibrozil treatment. 32P-Labeled oligo-apoA-I-DRE, oligo-Sp1, and oligo-AP1 (1 x 10^4 cpm) were incubated with no protein (Blank) or with nuclear extracts from Hep3B cells untreated (Control) or treated with gemfibrozil (40 μg/ml) as described under "Experimental Procedures." Similar results were also observed when nuclear extracts isolated from HepG2 cells were used.

...content...
clear factors that bind to the oligo-DRE, Southwestern analyses were carried out. As indicated by a solid horizontal bar beside lane 2 in Fig. 7, the labeled oligo-DRE bound to a protein band of approximately 28 kDa in size. This band appeared to be inducible by gemfibrozil treatment, since no such band was detectable in the nuclear extracts of control Hep3B cells. The labeled oligo-DRE was also bound to several proteins that were present in both gemfibrozil-treated and untreated samples. Two of these proteins have molecular masses of approximately 68 and 42 kDa, indicated by an open circle and a star, respectively (Fig. 7). These two protein bands gave similar signals on the x-ray autoradiograph. Therefore, they could be used as internal controls for protein loading. In addition, three other protein bands with molecular masses of 30 kDa (open circle), 15 kDa (open triangle), and 14.5 kDa (solid square) were also induced by gemfibrozil treatment.

To study the specificity of these drug-inducible protein bands toward the oligo-DRE, Southwestern blot analyses were performed using 32P-labeled oligo-P-450(I), oligo-P-450(II), and oligo-AhR separately. It was observed that two protein bands with masses of approximately 30 and 15 kDa could bind to oligo-P-450(I) and oligo-P-450(II) (Fig. 6, lanes 3–6). Furthermore, these two bands were highly inducible upon gemfibrozil treatment. It was also noteworthy that no nuclear proteins from control and gemfibrozil-treated cells interacted with oligo-AhR (Fig. 7, lanes 7 and 8). The data demonstrated that the drug-inducible bands bound specifically to the DRE consensus sequence.

**Functional Analysis of ApoA-I-DRE**—To analyze the role of apoA-I-DRE in the regulation of human apoA-I gene expression in response to gemfibrozil, we carried out transient transfection experiments using a series of pGL2-derived luciferase reporter plasmids. The construction of these plasmids is described in detail under “Experimental Procedures.” Plasmids were transfected into Hep3B cells in the absence or presence of gemfibrozil (40 μg/ml), and luciferase activity was measured. As shown in Fig. 8A, both pGL2 (apoA-I–491) and pGL2 (apoA-I–250) constructs showed a significant 2-fold induction of luciferase activity in the presence of gemfibrozil. By contrast, pGL2 (apoA-I–250 mutant DRE) in which the 10 nucleotides (G → T, C → A, T → G, and A → C) in the mutated apoA-I-DRE are indicated by an asterisk (*). The consensus sequences for various elements are underlined.

**TABLE I**

| Designation of oligomer | Features of oligomer | Sequence of oligomer | Competition efficiency |
|-------------------------|----------------------|----------------------|------------------------|
| ApoA-I-DRE              | DRE of the human apoA-I gene | 5′-GGCCGGCGCTGCTTTACTAGCTCCAGGGAGGAAGG-3′ | 100** |
| P-450(I)                | DRE consensus sequence of CYPA1 | 5′-CAACGGGGGTGGGAGGAAG-3′ | 89 ± 4 |
| P-450(II)               | DRE consensus sequence of CYPA1 | 5′-GAACGGCGCTGCTTTACTAGCTCCAGGGAGGAAGG-3′ | 65 ± 5 |
| AP1                     | AP1 consensus binding element | 5′-SCATCGTCAGTGATCGACAGAGATCT-3′ | 75 ± 5 |
| AP2                     | AP2 consensus binding element | 5′-GATCGAACTGAACGGCAGCCCGCCGCGCCTATC-3′ | 82 ± 4 |
| Sp1                     | Sp1 consensus binding element | 5′-CCGGGGCGCGCGCGCCAAG-3′ | 0 |
| Oligo-AhR               | Xenobiotic response element at which the AhR is known to bind | 5′-CGTGGGTGAGGAAGG-3′ | 0** |
| Oligo-M1                | Mutated apoA-I-DRE | 5′-GGCGGTATTTCTTCTCTCTCTTACTACAGAAGG-3′ | 0 **** |

**Fig. 4. Competitive gel mobility shift studies.** The specificity of the drug-inducible nuclear proteins toward the labeled oligo-apoA-I-DRE was determined by comparative competition experiments using various unlabeled synthetic oligomers. The competition efficiency is defined as the amount of competitor required to reduce the protein-DNA complex formation by 50% and arbitrarily set at 100 for unlabeled apoA-I-DRE. Results are the mean ± S.E. of three separate experiments. Mutated nucleotides (G → T, C → A, T → G, and A → C) in the mutated apoA-I-DRE are indicated by an asterisk (*). The consensus sequences for various elements are underlined.
mRNA was 5.6-8-fold higher in HepG2 cells compared with control HepG2 cells (11). In agreement with our previous studies, which measured apoA-I mRNA half-life by pulse-chase methods (25), we have also performed kinetic studies in the presence of actinomycin D to provide a measurement of apoA-I mRNA half-life in HepG2 cells (11). We have demonstrated that exposure of two cultured human hepatoma cell lines, Hep3B and HepG2, to gemfibrozil resulted in a 2-fold induction in apoA-I mRNA level (11). Based on the results of the present report, the increase in apoA-I mRNA level upon gemfibrozil treatment is due primarily to increased rates of transcription of this gene. First, in actinomycin D-treated cells, the half-life of apoA-I mRNAs was not significantly affected by gemfibrozil treatment (Fig. 1). Second, both in vitro nuclear run-off transcription assays (Fig. 2) and transient transfection experiments (Fig. 8) indicated a significant 2-fold increase in apoA-I gene expression in the presence of gemfibrozil.

In the presence of actinomycin D, the half-life of apoA-I mRNA was 5.6 ± 0.5 h in control Hep3B cells, in agreement with our previous studies, which measured apoA-I mRNA half-life by pulse-chase methods (25). By contrast, a recent report by Vandenbrouck and co-workers (39) has shown that the half-life of apoA-I mRNA is about 15 h in control HepG2 cells. Consequently, we also have performed kinetic studies in the presence of actinomycin D to provide a measurement of apoA-I mRNA half-life in HepG2 cells. Our results indicated that in HepG2 cells, apoA-I mRNA degraded with a half-life of 12.0 ± 1.5 h.2 Thus, the data suggest that the rate of degradation of apoA-I mRNA is at least 2-fold faster in control Hep3B cells compared with control HepG2 cells. The difference between the half-lives in these two cell lines might partially explain the 2-fold higher steady state level of apoA-I mRNA in control HepG2 cells compared with control Hep3B cells (11).

Recently, many studies have been focussed on the use of fibric acid derivatives to treat diet-resistant hyperlipidemic patients (40–44). Fibrates are used widely to lower plasma cholesterol and triglyceride levels as a treatment for coronary heart disease. However, these compounds also cause peroxisome proliferation and, in some cases, hepatocarcinogenesis in rodents (45, 46). It has been proposed that fibrates and other peroxisome proliferators activate a member of the steroid hormone receptor superfamily, the peroxisome proliferator-activated receptor (PPAR) (47–51). The first PPAR was isolated from a mouse liver cDNA library by Isselman and Green (47). It appears that one of the physiological roles of PPAR is to regulate fatty acid homeostasis. PPAR binds to a specific response element (PPRE) located upstream of the target genes (48-50). PPARs that function with a response element (PPRE) located upstream of the target genes (49, 52–55), which consists of an almost perfect direct repeat of the element (PPRE) located upstream of the target genes (48, 52–55). In view of these observations, the possibility exists that regulation of human apoA-I gene expression by gemfibrozil might involve the interaction of PPAR at the PPRE of the gene. However,

2 C. Cuthbert, Z. Wang, and S.-P. Tam, unpublished results.

**FIG. 5.** Affinity measurement of gemfibrozil-inducible nuclear proteins for the oligo-apo AI-DRE. The relative DNA binding affinity of oligo-DRE was measured by saturation binding assays using gel mobility shift analysis in which a constant amount (5 μg) of nuclear extract isolated from gemfibrozil-treated Hep3B cells was titrated with increasing amounts (0–500 fmol) of labeled oligo-apoA-I-DRE. The drug-inducible nuclear proteins bound to the oligo-DRE with an apparent Kd of 4.1 nM as determined by Scatchard analysis. Similar results were observed in three separate experiments.

**FIG. 6.** Methylation interference footprinting analysis of the human apoA-I gene DRE. End-labeled, partially methylated synthetic oligo-DRE (−79 to −44, coding or noncoding strand) was incubated with nuclear extracts isolated from gemfibrozil-treated Hep3B cells (Gemfibrozil) or untreated cells (Control). Complexed and free fractions were separated on a nondenaturing low ionic strength mobility shift assay gel. Bound and free DNA fractions were eluted from the 5% acrylamide gel prior to piperidine cleavage at methylated sites. Products were then separated on a 20% sequencing gel. Methylation sites that interfered with protein binding are indicated, and binding is summarized at the bottom. Lane G+A indicates Maxam and Gilbert G + A reaction. *, guanines methylated at the N7 position (major groove); √, adenosines methylated at the N6 position (minor groove). Similar results were observed in three independent experiments.
there is evidence to suggest this may not be the case. First, the consensus sequence of PPRE is different from the DRE described in the present study. Second, the hepatic peroxisome proliferation seen in rodents receiving gemfibrozil does not occur in humans, as evaluated by light and electron microscopy of liver biopsy samples from patients on long term gemfibrozil therapy (65). Third, PPAR has a molecular mass of 52.4 kDa, while the gemfibrozil-induced proteins have sizes of approximately 30 and 15 kDa as observed by Southwestern blot analysis (Fig. 7). Finally, the in vitro translated mouse PPARα synthesized from pSG4-mPPARα expression vector (kindly provided by Dr. S. Green, Alderley Park, Macclesfield, United Kingdom) using the rabbit reticulocyte in vitro transcription system (Promega) shows specific DNA binding activity to a PPRE but not to the DRE of the human apoA-I promoter.¹

Studies from both humans and rodents indicate that gemfibrozil causes an increase in the levels of plasma HDL and/or apoA-I (10, 40, 66–69). Saku et al. (66) observed a 36% increase in HDL cholesterol and a 29% rise in apoA-I levels when their patients with familial hypertriglyceridemia were treated with gemfibrozil. This was accompanied by an increase in the synthetic rate of apoA-I by 27% with no change in the fractional clearance rate. The results from our studies support, in part, Saku’s findings and demonstrate that gemfibrozil may act by selectively increasing expression of the apoA-I gene in human liver. Elucidation of the mechanism by which gemfibrozil modulates apoA-I gene expression requires the identification of promoter elements and transcription factors responsible for mediating the biological response to these agents. We have identified two copies of the DRE in the apoA-I promoter that may be responsible for the increase in apoA-I transcriptional activity by gemfibrozil. These two decanucleotide elements, spaced 10 nucleotides apart in an inverted repeat, are located between nucleotides −77 and −45, relative to the transcriptional start site of the apoA-I gene. We have demonstrated that exposure of Hep3B or HepG2 cells to gemfibrozil results in a strong induction of a protein-DNA complex (Fig. 3). Similar results have been observed with the rat hepatoma cell line H4IIE (obtained from ATCC), indicating that the binding specificity of the factors is conserved across species (data not shown). We observe no difference in the binding specificities of the nucleotides that are involved in the protein-DNA interactions using either control or gemfibrozil-treated nuclear extracts as determined by methylation interference analysis. There exists at least one possible explanation for this observation. If the DRE in the apoA-I promoter is mediating the gemfibrozil action, the activity rather than the abundance or binding affinity of the factor must be regulated by gemfibrozil. Recently, Leighton and Kemper (70) have demonstrated that phenobarbital treatment increases the expression of CYP2C1 and CYP2C2 in rat liver. However, indistinguishable DNase I digestion patterns were obtained using liver nuclear extracts from either control or phenobarbital-treated rats (71). Furthermore, another study has indicated that the DNase I hypersensitivity pattern at the rat liver xenobiotic response elements do not alter when aryl hydrocarbon receptor translocation and DNA bindings are induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin treatment (72).

The competition mobility shift experiments presented in Fig. 4 shows that the nuclear factor(s) that bind(s) apoA-I-DRE is highly sequence-specific. A 25-fold molar excess of a double-stranded oligonucleotide homologous to apoA-I-DRE competed strongly for binding with the ³²P-labeled apoA-I-DRE probe, while an equivalent molar excess of mutated apoA-I-DRE resulted in virtually no competition with the apoA-I-DRE probe. Furthermore, the DRE consensus sequence of CYP1A1 (P-450(I) and P-450(II)) competed efficiently for factor binding to the apoA-I-DRE. However, no competition was observed with 200-fold molar excess of the Sp1 consensus sequence or the xenobiotic consensus sequence for the AhR. It is worth noting that both the AP1 and AP2 consensus sequences compete efficiently for protein binding to the apoA-I-DRE. One possible explanation for this observation is that nuclear factor(s) that recognize(s) the apoA-I-DRE can also bind to the AP1 and AP2 consensus sequences. Alternatively, it is possible that the protein-DNA complex observed in the mobility shift assay consisted of multiple proteins of which AP1 and AP2 binding proteins are two of the potential candidates. Results from the present studies suggest that the former may be the more likely possibility. First, our methylation interference experiments indicated that the contact sites of the nuclear factors with the DRE region did not resemble those of the AP1 (73) or AP2 (74, 75),

¹ S.-P. Tam and S. Green, unpublished results.
Regulation of Human ApoA-I Gene by Gemfibrozil

75) DNA recognition sites. Second, Williams and Tjian (74) have demonstrated that HepG2 cells express neither AP2 mRNA nor protein. Third, the two groups of gemfibrozil-induced nuclear proteins detected by Southern blot analyses have molecular masses of 15 and 30 kDa, whereas AP1 and AP2 proteins have molecular masses between 40 and 75 kDa (75). Finally, we have performed supershift experiments to determine whether c-Jun and c-Fos protein interact with the DRE binding site. For supershift assays, the nuclear extracts isolated from gemfibrozil-treated cells were preincubated with affinity-purified polyclonal antibodies raised against c-Jun and c-Fos (Oncogene Science) at 4 °C for either 2 h or 18 h before the standard gel mobility shift studies. Both sets of experiments showed no effect on the binding activity of DRE oligonucleotide to the gemfibrozil-treated nuclear extract when either anti-c-Jun or anti-c-Fos antibodies were used (data not shown).

The specific protein binding to oligo-DRE was observed with nuclear extracts from gemfibrozil-treated cells but not from cytosolic extracts (data not shown). Consequently, it was not clear whether inducible binding was due to a drug-induced translocation of a preexisting factor from the cytosol to the nucleus or was attributable to its de novo synthesis. The results of inhibiting protein synthesis with cycloheximide suggest the latter is the case. At the moment, we are uncertain whether this involves synthesis of the factors themselves or of ancillary proteins or enzymes required for binding activity.

In order to establish a functional role for the DRE in apoA-I gene transcription in response to gemfibrozil treatment, we have carried out transient transfection experiments using pGL2-derived luciferase reporter plasmids. Constructs that contain nucleotides −491 to +1 and −250 to +1 upstream from the transcription start site (+1) of the human apoA-I gene show a significant 2-fold increase in luciferase activity in the presence of gemfibrozil (Fig. 8A). The involvement of the DRE in gemfibrozil-mediated induction of apoA-I gene expression was established by using the pGL2 (apoA-I −250 mutant DRE) in which the 10 nucleotides involved in possible contact with nuclear factors were substituted (G → T, C → A, T → G, and A → C). The pGL2-TK-luc series are constructed with synthetic DRE or mutated DRE (in which the last eight nucleotides of each DRE consensus sequence were mutated: G → T, C → A, T → G, and A → C) in front of TK promoter (panel B). Construction of these plasmids is described under “Experimental Procedures.” Freshly seeded Hep3B cells were transfected with the reporter plasmid and β-galactosidase as internal control to normalize for differences in transfection efficiency. Cells were then cultured in the absence or presence of gemfibrozil (40 μg/ml) for 24 h prior to harvesting. The graph on the right represents relative luciferase activity in transfected cells cultured in the absence (control) or presence of gemfibrozil. Luciferase activities of the constructs are expressed relative to that of pGL2 promoter vector in panel A and pGL2 TK/luc in panel B, respectively. Results are mean ± S.E. for six independent experiments carried out in triplicate. **, significantly different from control (p < 0.001, two-tail t test). The above noted plasmids were also transfected into HepG2 cells. Similar relative luciferase activities of the constructs were also observed in HepG2 cells compared with Hep3B cells (data not shown).

Acknowledgments—We thank Sandra Caine and Marilyn McCallum for excellent secretarial skills and express our appreciation to Dr. R. G. Deeley for critical comments.

REFERENCES
1. Gotto, A. M., Pownall, H. J., and Havel, R. J. (1986) Methods Enzymol. 128, 3–41
2. Miller, N. E., LaVille, A., and Crook, D. (1985) Nature 314, 109–111
