Human S mu binding protein-2 binds to the drug response element and transactivates the human apoA-I promoter: role of gemfibrozil

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Abstract Previously, we demonstrated that protein–DNA interactions at the drug response element (DRE) in the human apoA-I promoter were important for the induction of apoA-I gene expression by gemfibrozil. We now report the cloning and characterization of a DRE transactivating factor. The cloned protein is identical to the putative helicase and potential transcription factor human S mu binding protein-2 (HSμBP2). It is also related to glial factor-1 (GF1), an incomplete version of HSμBP2 lacking the first 494 and the last 128 amino acids. Gel mobility shift assays demonstrated that HSμBP2 binds apoA-I DRE oligomers and forms a specific protein–DNA complex. Northern blot analysis showed that HSμBP2 mRNA is expressed at various levels in a wide range of human tissues. Transient cotransfection experiments performed in HepG2 cells demonstrated that overexpression of HSμBP2 or GF1 induced apoA-I proximal promoter activity by 3-fold and that the apoA-I DRE was necessary for transactivation. Additionally, we demonstrated that transactivation was increased a further 2- to 3-fold by exposing the cells to gemfibrozil. Together these observations indicate that HSμBP2 acts as a transcription factor that regulates apoA-I gene expression in hepatoma cells and whose activity may be stimulated by gemfibrozil treatment.—**Mohan, W. S., Z-Q. Chen, X. Zhang, K. Khalili, T. Honjo, R. G. Deeley, and S-P. Tam.** Human S mu binding protein-2 binds to the drug response element and transactivates the human apoA-I promoter: role of gemfibrozil. *J. Lipid Res.* 1998. **39:** 255–267.

Supplementary key words apolipoprotein A-I • gemfibrozil • gene expression • regulatory sequence • transcription factor

Apolipoprotein A-I (apoA-I), the major protein constituent of high density lipoprotein (HDL), is a cofactor for lecithin:cholesterol acyltransferase (LCAT). ApoA-I together with LCAT promotes efflux of cholesterol from peripheral tissues to HDL particles which carry esterified cholesterol to the liver for catabolism; a process that is known as reverse cholesterol transport (1–3). Epidemiological studies indicate that there is a negative correlation between plasma levels of apoA-I and HDL and the incidence of coronary heart disease (4, 5). High levels of apoA-I have been shown to be protective against the development of atherosclerosis. For example, transgenic mice expressing high amounts of human apoA-I were significantly protected against the formation of fatty streak lesions when fed a high fat diet (6). Recently, it has been demonstrated that apoA-I is essential for the selective uptake of HDL-cholesteryl esters in steroidogenic cells and in providing cholesterol substrate for steroidogenesis (7).

Expression of apoA-I in mammals is restricted to specific tissues including liver and intestine (3). Transient transfection analysis of the human apoA-I gene using HepG2 cells and studies with transgenic mice revealed that 256-bp of 5′-flanking sequence is necessary and sufficient for maximal basal liver-specific expression (8–10). DNase I footprinting analysis of the 'liver-specific' regulatory region of the human apo-A gene has revealed three sites designated: A (−214 to −192), B

Abbreviations: bp, base pair(s); CYP, cytochrome P450; DRE, drug response element; DTT, dithiothreitol; EDTA, disodium ethylenediamine tetraacetate; GF1, glial factor-1; HEPES, N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid); HsμBP2, human S mu binding protein-2; kb, kilobase(s); MEM, minimal essential medium; m-apoA-I DRE, mutant apoA-I drug response element; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; XRE, xenobiotic response element.

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(−169 to −142), and C (−134 to −119) (11). Studies by Papazafiri and co-workers (12) have widened the investigated regions and demonstrated a fourth footprint region between nucleotides −22 to +17 of the human apoA-I gene using rat nuclear extracts. These studies demonstrated that liver and HepG2 nuclei contain factors that bind with high affinity and specificity to these sites (11, 12). Several factors that interact with these regions and affect apoA-I promoter activity have been identified. These include ARP-1, RXRα, HNF4, HNF-3β, PPAR, EAR-2, and EAR-3 (11–20). Interactions between these transacting factors and the *cis*-acting elements of the human apoA-I promoter play an important role in basal, as well as, tissue-specific expression of apoA-I.

There are a number of drugs widely used to reduce the progression of coronary heart diseases by altering lipoprotein metabolism. One of the most extensively studied is gemfibrozil. Recently, we demonstrated that exposure of both HepG2 and Hep3B cells to gemfibrozil resulted in a 2-fold increase in apoA-I mRNA (21, 22). We also showed that the induction of apoA-I mRNA by gemfibrozil involved a transcriptional response (22). We have shown that modulation of apoA-I gene expression by gemfibrozil in hepatoma cells is mediated through interaction of drug-induced nuclear factors with a *cis*-acting element, termed the drug response element or DRE (22). The apoA-I DRE is composed of two decanucleotide motifs spaced 10 nucleotides apart in an inverted repeat and is located in the proximal promoter of the human apoA-I gene between nucleotides −77 and −45. A similar element (between nucleotides −3422 to −3406 from the transcription start site (+1) of the rat cytochrome P450 1A1 gene) was first noted by Sogawa and coworkers (23) who proposed that it was involved in induction of cytochrome P450 1A1 (CYP1A1) gene expression by aryl hydrocarbons. This suggestion was subsequently shown to be incorrect.

Recent work in our laboratory has demonstrated that in Hep3B cells two classes of gemfibrozil inducible nuclear factors can bind specifically, and with high affinity, to the DRE of the human apoA-I gene (22). In this paper we describe the cloning and characterization of a DRE transactivating factor that can regulate expression of the human apoA-I gene through interaction at the DRE. This factor corresponds to the previously reported GF1/HSμBP2, a transactivating factor with potential ATPase and helicase activity (24–26). We demonstrate that HSμBP2 mRNA is present in all human tissues and cell lines we examined, although the level of expression varies widely. As well, we show by transient cotransfection assay that, in hepatoma cells, HSμBP2 is a transcription factor that specifically recognizes the DRE and increases apoA-I promoter activity.

MATERIALS AND METHODS

Materials

The following complementary pairs of oligonucleotides were synthesized on a Biosearch model 8600 DNA synthesizer at Queen's University, Department of Biochemistry, Core Facility for Protein/DNA Chemistry. Oligonucleotide apoA-I DRE, 5′-GGTGGCCGGGG CTGGGCTTATCAGCCTCCCAGCCCAGACC-3′ corresponding to the nucleotides between nucleotides −77 and −45 of the human apoA-I gene (11, 12); oligonucleotide xenobiotic response element (XRE), 5′-AGT GCTGTCACGCTAG-3′ corresponding to nucleotides between −1092 and −1078 of the rat CYP1A1 gene (27); oligonucleotide CYP1A1 DRE, 5′-CACAGGG GCTGGGGAG-3′ corresponding to nucleotides between −3422 to −3406 of the rat CYP1A1 gene (23); oligonucleotide AP1, 5′-CCAGTCACAGTGACTCAGCA GAATCT-3′ corresponding to nucleotides between −471 and −446 of the human NAD(P)H:quinone oxidoreductase gene (28); oligonucleotide mutant apoA-I DRE (m-apoA-I DRE), 5′-GGAATTTTAGTTATTACTAGC CTAAATAAAGACCC-3′; in which the consensus DRE sequence (underlined) was replaced (G → T, C → A, T → G, and A → C).

Construction of reporter plasmids and transient cotransfections

The plasmids pAI250-LUC, pAI250m-LUC, pDRE/TK-LUC, and pmDRE/TK-LUC are all derived from pGL2-Basic vector (pLUC) which does not contain any promoter or enhancer sequences (Promega). pSV40-LUC, also obtained from Promega, contains the luciferase reporter gene under the control of the SV40 promoter. pTK-LUC plasmids contain the luciferase reporter gene under the control of the thymidine kinase (TK) minimal promoter. pAI250-LUC contains the apoA-I promoter sequence from base pair −248 to +1 upstream of the luciferase gene. pAI250m-LUC contains the apoA-I promoter sequence from base pair −248 to +1, in which point mutations eliminated the DRE. Plasmids pDRE/TK-LUC and pmDRE/TK-LUC were constructed by cloning a synthetic apoA-I-DRE or a mutated apoA-I-DRE into the SmaI site of pTK-LUC. The construction of the above plasmids has been described in detail previously (22). These plasmids were cotransfected with carrier plasmid pLUC and either a GF1 expression plasmid (pCMV-GF1), or a control vector (pCMV), or a HSμBP2 expression plasmid (pCDL-HSμBP2), or a control vector (pCDL). DNA was transfected into HepG2 or Hep3B cells by the calcium phosphate procedure described by Gorman, Moffat, and Howard (29) and detailed previously (22). Ten

μg of DNA was used per 60-mm plate, including 4 μg of reporter DNA and 1 μg of expression plasmid or empty vector and 5 μg of carrier plasmid. Luciferase assays were performed as described in detail previously (22).

Cell culture and isolation of stably transfected HepG2 cells

Human hepatoma cell lines, Hep3B and HepG2, were obtained from the American Type Culture Collection (Rockville, MD) and maintained as described previously (30). HH02 cells, a long term culture of non-transformed human hepatocytes, were kindly provided by Dr. Eve Roberts (Hospital for Sick Children, Toronto). HepG2 stable transformants were prepared using pEBV7 and pAI250-LUC. The pEBV7 construct is derived from pC7βG which is an eukaryotic expression vector containing an Epstein-Barr virus origin of replication that allows stable transfections of human cell lines (31). The unique features of pEBV7 have been described in detail by Wilson and Deeley (32). pEBV7-apoA-I-250 was constructed by isolating the apoA-I proximal promoter and luciferase gene cassette from pAI250-LUC by digestion with SmaI, SalI, and ScaI. This fragment was ligated, using T4 DNA ligase, into the pEBV7 vector (kindly provided by G. Wilson, Department of Biochemistry, Queen's University) which had been digested first with the restriction endonuclease XbaI and treated with T4 DNA polymerase to generate blunt ends, then digested second with the restriction endonuclease SalI. The fusion plasmid generated, pEBV7-apoA-I-250, maintained the *E. coli* hph gene, encoding hygromycin B phosphotransferase within a herpes simplex virus thymidine kinase expression cassette. The sequence of pEBV7-apoA-I-250 was confirmed by using the Sequenase Version 2.0 sequencing kit from Amersham Canada Limited (Oakville, Ontario). HepG2 cells were split 24 h prior to transfection. Cells were plated into 60-mm dishes and transfected with SacI linearized pEBV7-apoA-I-250 plasmid using a standard calcium phosphate precipitation procedure (29). After overnight incubation with the calcium phosphate–DNA precipitate, the cells were washed twice with phosphate-buffered saline and maintained in minimal essential medium (MEM) plus 10% fetal bovine serum for 48 h. Selection of positive clones was performed by adding hygromycin B (300 μg/ml) obtained from Boehringer-Mannheim (Canada). The culture medium was changed every 2–3 days, in order to remove dead cells. After 10–14 days, colonies of surviving cells were picked and split at low density and exposed to another round of clonal isolation. Two stably transfected cell lines designated HepG2/1A and HepG2/1B were established. The cell line HepG2/1A was used in these experiments and the cells were propagated over 6 months without losing luciferase activity or hygromycin resistance. For gemfibrozil treatment, subconfluent monolayers of hepatoma cells as well as stably transfected HepG2/1A were washed twice with MEM and then incubated with fresh medium for 24 h in the presence of gemfibrozil, dissolved in ethanol, to give a final concentration of 40 μg/ml. Control cells were incubated with an equal volume (40 μl) of ethanol.

RNA isolation and Northern blot analyses

Hep3B nuclei were prepared as described previously (22). Nuclear RNA was isolated from Hep3B cells using the acid phenol method (33). Cytosolic RNA was isolated as described previously (34). Nuclear and cytosolic RNA were enriched for poly A+ RNA by oligo dT cellulose chromatography (34). For Northern blotting, 2 μg of poly A+ RNA was denatured by treatment with glyoxal, subjected to agarose gel electrophoresis, and transferred to Zeta-probe GT membrane (Bio-Rad) as described previously (30). The human multiple tissue poly A+ RNA blots were obtained from Clonetech Laboratories Inc. (Palo Alto, CA). Blots were probed with α^{32}P-dCTP random prime labeled HSμBP2, apoA-I, and β-actin cDNA fragments. In cases where Hep3B RNA was used, the results were normalized using densometric scans of Northern blots probed with radiolabeled glyceraldehyde-3-phosphate dehydrogenase to correct for loading variations (35).

Construction and screening of cDNA library

A λgt11 cDNA expression library prepared from Hep3B cells treated with gemfibrozil was constructed. Briefly, total RNA isolated from gemfibrozil-treated Hep3B cells was enriched for poly A+ RNA by performing oligo dT cellulose chromatography (34). cDNA was synthesized with random hexamers and oligo dT using Moloney murine leukemia virus reverse transcriptase superscript RT (Gibco-BRL). Recombinant phage were constructed and packaged using a Giga pack gold packaging kit following the manufacturer's instructions (Stratagene).

Approximately 1×10^6 independent λgt11 phage plaques from the Hep3B cDNA library were screened essentially according to the method of Vinson and coworkers (36) and Singh, Clerc, and LeBowitz (37). Briefly, bacterial strain Y1090 was infected with recombinant phage, plated, and incubated at 42°C for 4 h. The plates were overlaid with nitrocellulose filters, pretreated with 15 mm isopropyl-1-thio-β-D-galactopyranoside, and incubated at 37°C for 9 h. All subsequent steps were carried out at 4°C with gentle shaking. Filters were lifted and subjected to denaturation in 1× binding buffer (25 mm NaCl, 5 mm $MgCl_2$, 25 mm HEPES, pH 8.0, and 0.5 mm DTT) supplemented with

6 m guanidine HCl for 30 min. Renaturation was accomplished by sequentially diluting the denaturation solution with an equal volume of 1× binding buffer every 5 min. This step was repeated 5 times. The filters were incubated two more times in 1× binding buffer containing 5% Carnation non-fat milk for 1 h. Filters were kept in 1× binding buffer supplemented with 0.25% Carnation non-fat milk until probe was added. DRE probe was prepared as follows. Sense strand DRE oligonucleotides containing nucleotides −77 to −45 of the human apoA-I promoter were end labeled using T4 polynucleotide kinase. The radiolabeled oligonucleotides were annealed to a 10-fold molar excess of unlabeled complementary oligonucleotides by boiling for 10 min and then slowly cooling the mixture to room temperature over a one-hour period. Double-stranded DRE oligonucleotides were ligated into concatemers using T4 DNA ligase for 16 h at 15°C. XRE and CYP1A1 DRE oligonucleotides were prepared in the same way as the apoA-I DRE. An equimolar mixture of radiolabeled, double-stranded, apoA-I DRE and CYP1A1 DRE probes was added to the filters and incubated for 4 h. The filters were then washed 4 times using 1× binding buffer supplemented with 0.25% Carnation non-fat milk for 5 min each time. The filters were then blotted dry and exposed to X-ray film overnight at −78°C.

Electrophoretic gel mobility shift analysis

Gel mobility shift analyses were performed using extracts derived from λS phage infected bacterial strain Y1089 or maltose binding protein (MBP)-GF1 fusion protein expressed from pMAL-CR1-GF1 following manufacturer's instructions (New England BioLabs). Isolation of recombinant phage lysogen and preparation of crude cell extracts were carried out as described by Singh and co-workers (37). For mobility shift assays, 1 μg of phage lysogen or MBP-GR1 fusion protein was incubated with 1 μg of poly (dI-dC) in binding buffer (25 mm HEPES, pH 8.0, 12.5 mm $MgCl_2$, 10% glycerol, 50 mm KC1, and 1 mm DTT) on ice. Then 100 fmol of ^{32}P-labeled double-stranded apoA-I DRE, mutant apoA-I DRE, or XRE oligonucleotides and various amounts of either unlabeled double-stranded apoA-I DRE, AP1, mutant apoA-I, DRE, or XRE oligonucleotide were added as indicated in the figure legend. The mixture was then incubated on ice for 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 borate, and 2.5 mm EDTA, pH 7.8. The gel was pre-run by electrophoresing at 100 volts for 1 h. Samples were then loaded onto the gel and electrophoresed at 100 volts for 3 h at room temperature. Gels were vacuum dried and exposed to X-ray film overnight at −78°C.

DNA sequencing

cDNA inserts from clones K, H, and S were excised with endonuclease EcoR1 and subcloned into the bluescript pKS vector (Stratagene) for sequencing. DNA sequencing was performed by the chain termination method of Sanger, Nicklen, and Coulson (38) using the Sequenase Version 2.0 sequencing kit (Amersham).

RESULTS

Cloning of DRE binding protein

To further characterize the molecular mechanism involved in transcriptional regulation of the apoA-I gene by gemfibrozil, we used a direct screening approach to clone proteins capable of interacting with the DRE. A λgt11 cDNA expression library was constructed using poly A+ RNA isolated from Hep3B cells treated for 18 h with gemfibrozil (40 μg/ml). The library was first probed with a double-stranded, concatenated radiolabeled apoA-I-DRE. As the apoA-I-DRE contained two copies of the consensus sequence in an inverted repeat orientation, we also wanted to examine whether there was a requirement of this type of arrangement for specific protein–DNA interactions. Thus, we also probed the library with a double-stranded, concatenated unidirectional single copy of the consensus DRE sequence derived from CYP1A1. Two clones designated H and S were isolated. Both clones expressed protein that bound to the apoA-I-DRE and CYP1A1-DRE element but not to the unrelated XRE (data not shown). We used clone S in mobility shift analyses to further confirm the binding specificity and to better define the recognition of properties of this protein (**Fig. 1**). Recombinant S fusion protein bound specifically to the apoA-I-DRE, as indicated by the reduction in protein binding when 50- to 100-fold molar excess of unlabeled DRE oligonucleotide was used as a competitor and the lack of competition by 100-fold molar excess of unlabeled AP1 binding site oligonucleotide.

DNA sequencing revealed that the 744 bp clone H and 1649 bp clone S contained overlapping sequences. We used the cDNA insert of clone S to probe a HaCaT λZAP cDNA library (kindly provided by Dr. S. Davey, Cancer Research Labs, Queen's University) to isolate clone K, a 3403 bp full length cDNA clone. Clone K contained an ATG translational start codon at bp 20 and a TAG translational termination codon at bp 2999. The open reading frame encoded a 993 amino acid protein and was flanked by 19 bp of 5′ untranslated sequence and 404 bp of 3′ untranslated sequence. Nucleotide

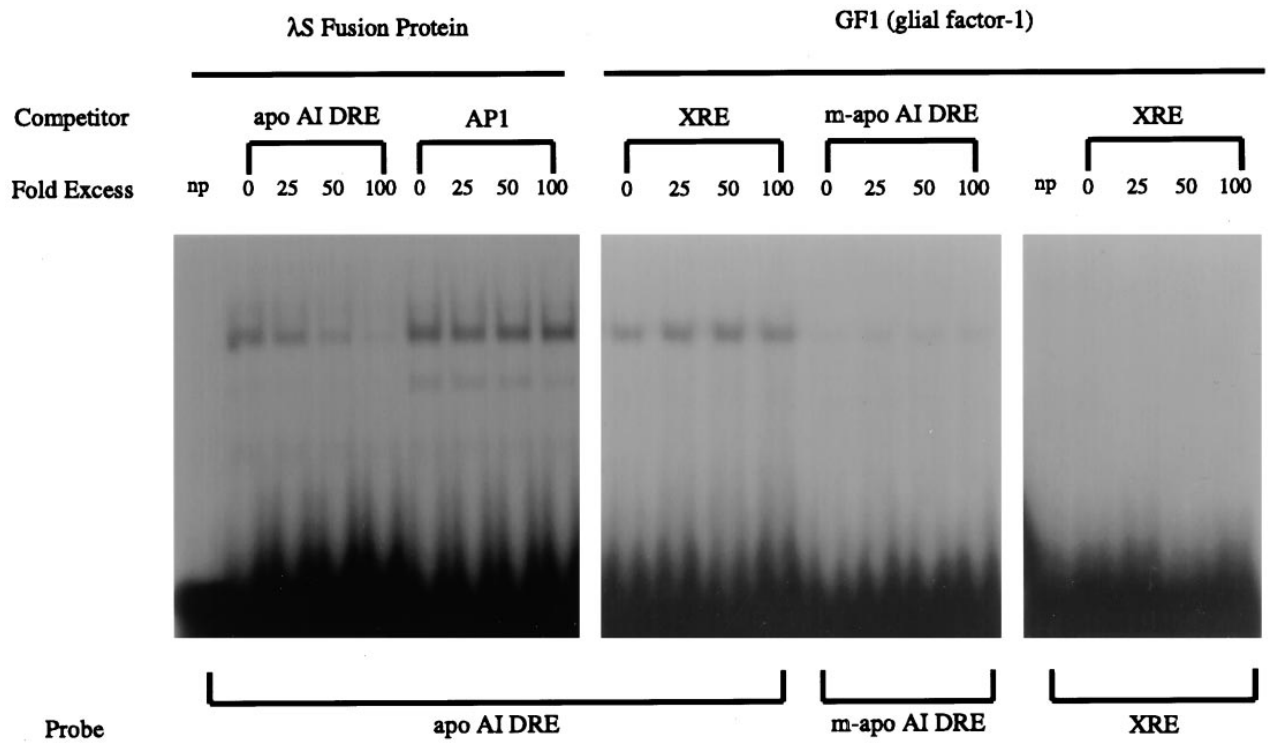

Fig. 1. Electrophoretic mobility shift analysis of λS fusion protein and GF1. One μg of λS lysogen was incubated with 100 fmol of ^{32}P-labeled double-stranded apoA-I DRE oligonucleotide (20,000 cpm) and various amounts (in molar excess) of unlabeled double-stranded apoA-I DRE or double-stranded AP1 binding site oligonucleotide, as indicated, for 30 min at 4°C. Similarly, 1 μg of bacterial expressed GF1 was incubated with the labeled apoA-I DRE probe and various amounts of unlabeled double-stranded XRE oligonucleotide as indicated. One μg of GF1 was also incubated with 100 fmol of ^{32}P-labeled double-stranded m-apoA-I DRE or XRE and various concentrations of unlabeled m-apoA-I DRE or XRE, respectively, as indicated in the figure. These mixtures were separated by electrophoresis through a 5% native polyacrylamide gel for 3 h at 100 V. The bands representing specific protein–DNA complexes were identified by autoradiography; np, represents the mixture containing no protein.

and amino acid sequence analysis of clone K revealed that it was identical to the previously reported human DNA binding protein GF1/HSμBP2 (24, 25). HSμBP2 is encoded by the open reading frame of a 3.9 kb cDNA. It is a DNA binding protein that binds to specific sequences found within immunoglobulin mu switch (Sμ) regions (25). Based on the presence of putative helicase motifs, Fukita and co-workers (25) postulated that HSμBP2 may function as a helicase. However, this activity has not been demonstrated. Other groups have also described related cDNAs. Kerr and Khalili (24) isolated a partial human cDNA clone named GF1 which lacks the first 494 amino acids and the last 128 amino acids of HSμBP2 and proposed that it functions as a transcription factor as it stimulated transcription from the JC viral early and late promoters in human glial cells. Gel mobility shift assays also demonstrated that bacterial-expressed GF1 bound apoA-I-DRE but not to a mutant apoA-I DRE oligonucleotide or to the XRE oligomer (Fig. 1). Homologues of HSμBP2 have been isolated from hamster (RIP-1) (39), mouse (mSμBP2) (26), and rat (CATF1) (40). All of the factors were identified by their ability to bind to similar DNA elements, although a consensus sequence has yet to be established.

Northern analysis

Although the tissue distribution of HSμBP2 mRNA has been reported previously for selected normal tissues in hamster, mouse, and rat, as well as in some human cell lines, its distribution and level of expression in normal human tissues have not been reported. Northern analysis of poly A+ RNA isolated from various human tissues showed that radiolabeled clone S cDNA hybridized preferentially to a 4.3 Kb mRNA, although two other larger mRNA 5.6 and 8.5 Kb in size could also be detected (**Fig. 2**). The highest level of HSμBP2 mRNA expression was found in testis, moderate levels were present in the heart and skeletal muscle, and low levels of expression were detected in the brain, colon, kidney, liver, ovary, pancreas, peripheral blood leucocytes, placenta, prostate, small intestine,

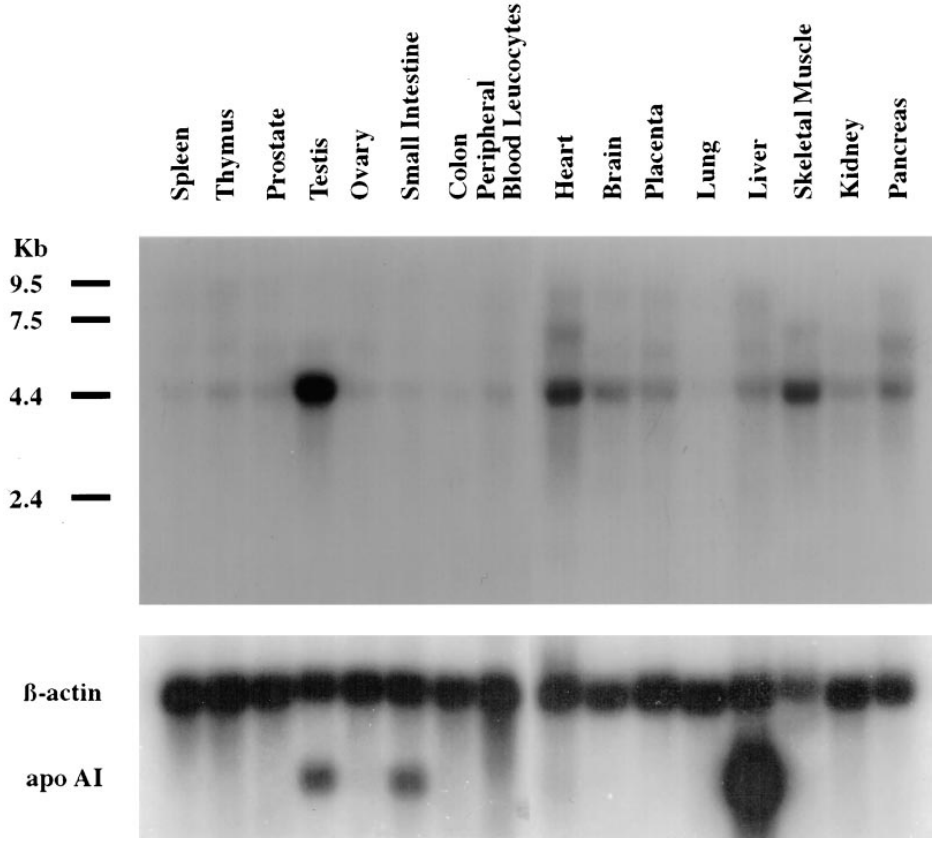

Fig. 2. Northern analysis of DRETF expression in human tissue. Multiple human tissues were examined by Northern blotting using two Multiple Tissue Northern Blot II membranes obtained from Clonetech Laboratories Inc. Each lane contained 2 μg of poly A+ RNA prepared from normal human tissues. Blots were probed with ^{32}P-labeled λS clone or β-actin, and apoA-I cDNA fragments (1×10^7 cpm/ml). RNA molecular weight markers were used to estimate the size of the mRNA.

spleen, and thymus. Barely detectable levels of HSμBP2 mRNA were observed in the lung. Unlike HSμBP2, similar levels of β-actin mRNA were present in all tissues examined while apoA-I mRNA were detected only in the liver, intestine, and testis tissues (Fig. 2). These studies also demonstrated that apoA-I mRNA levels do not correlate with the levels of HSμBP2 mRNA. To investigate the origin of the two larger mRNAs of 5.6 and 8.5 Kb that were evident on the multiple human tissue Northern blot (Fig. 2) we examined cytoplasmic or nuclear poly A+ RNA from control and gemfibrozil-treated Hep3B cells (**Fig. 3**). When the ratios of HSμBP2 mRNA to GADPH mRNA were determined, the results indicated that quantitatively similar levels of HSμBP2 mRNA (cytoplasmic and nuclear) were observed in Hep3B cells cultured in the absence or presence of gemfibrozil (Fig. 3). The presence of the larger species in nuclear poly A+ RNA, but not in cytoplasmic poly A+ RNA, suggests they may be immature, partially spliced HSμBP2 mRNAs. Similar results were observed with RNA isolated from HepG2, and HH02 cells (data not shown).

Overexpression of GF1 or HSμBP2 enhances human apoA-I promoter activity

To determine whether HSμBP2 could enhance apoA-I promoter activity via binding to the apoA-I DRE in hepatoma cells, we performed a series of transient cotransfection experiments. These studies were performed using the expression plasmids, pCDL-HSμBP2 (which contains the full length cDNA) and pCMV-GF1 together with apoA-I-reporter plasmids. Schematic diagrams of the reporter plasmids and expression vectors used in these studies are shown in **Fig. 4A and B**, respectively. Expression of the HSμBP2 protein caused a 2- to 3-fold stimulation of transcriptional activity, as measured by luciferase expression, from a reporter construct containing the proximal apoA-I promoter (pAI250-LUC). However, no increase in luciferase activity was observed in cotransfection experiments using

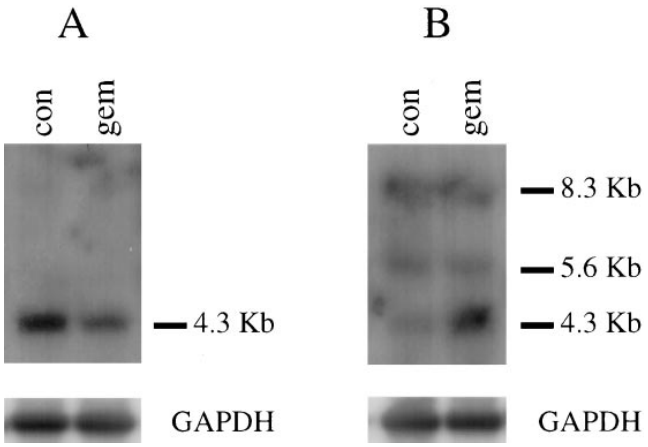

Fig. 3. Northern analysis of DRETF expression in Hep3B cells. Two μg of Poly A+ RNA prepared from Hep3B cells treated with 40 μg/ml gemfibrozil (gem) or ethanol vehicle (con) was denatured by treatment with glyoxal, separated on a 1.25% agarose gel, and transfected onto Zeta-probe GT membrane (as described in Materials and Methods). Blots were probed with ^{32}P-labeled λS clone insert DNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA fragments (1×10^7 cpm/ml). Panel A represents cytoplasmic A+ RNA samples. Panel B represents nuclear poly A+ RNA samples. Similar results were observed in three independent experiments.

pAI250-LUC and the control vectors, pCDL or pCMV. Similar results were obtained using a reporter construct (pAI500-LUC) that contained a larger region of the apoA-I proximal promoter (data not shown). The results indicate that sequences within the −248 to +1 region of the apoA-I promoter are sufficient for HSμBP2 protein transactivation. Stimulation of promoter activity was not observed when HSμBP2 was cotransfected with reporter construct pAI250m-LUC containing the apoA-I proximal promoter with the DRE eliminated by point mutations (**Fig. 5A**). Furthermore, overexpression of HSμBP2 caused a 2- to 3-fold stimulation of promoter activity in reporter constructs containing the DRE fused to the minimal TK promoter, but not in the TK-LUC constructs containing a mutated form of the DRE, indicating the DRE is necessary for HSμBP2 transactivation (Fig. 5B). Cotransfection studies using DRE/TK-LUC reporter plasmids showed that pCMV and pCDL had no effect on the DRE-mediated enhancement of luciferase activity (Fig. 5B).

We wanted to examine whether gemfibrozil treatment had any effect on the ability of HSμBP2 to stimulate apoA-I promoter activity. When transfected alone, pAI250-LUC promoter activity increased 2-fold with gemfibrozil treatment. However, when the HSμBP2 expression plasmid was included in similar experiments a further 2- to 3-fold increase in apoA-I promoter activity was observed with gemfibrozil treatment (Fig. 5A). Similarly, in transfections using pDRE/TK-LUC reporter plasmid, a 2-fold increase in transcriptional activity was observed with gemfibrozil treatment, and an additional

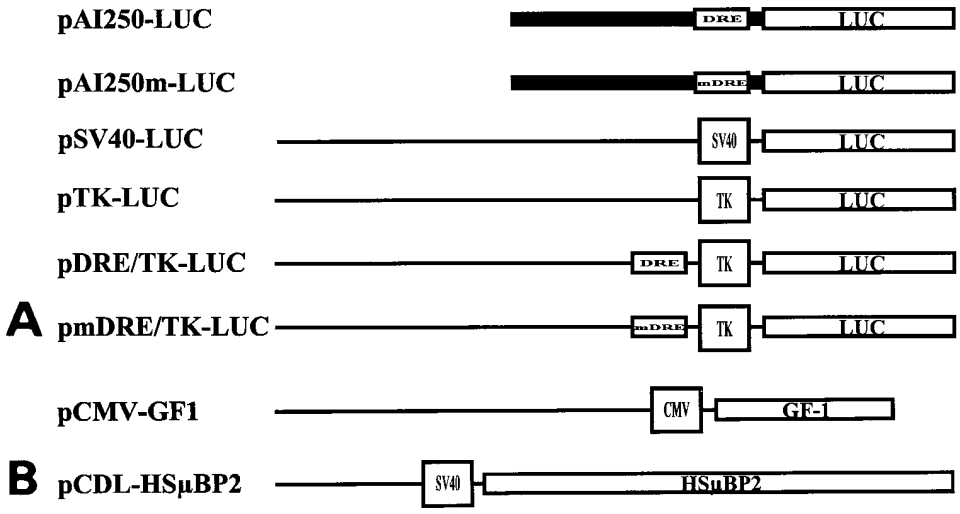

Fig. 4. Schematic diagrams of the reporter and expression plasmids used for transient cotransfections. Panel A shows a schematic representation of plasmids containing the luciferase reporter gene under the control of the human apoA-I proximal promoter nucleotides −248 to +1 (pAI250-LUC), the human apoA-I proximal promoter with the DRE eliminated by point mutations (pAI250m-LUC), the simian virus 40 promoter (pSV40-LUC), the minimal TK promoter (pTK-LUC), the apoA-I DRE fused with the minimal TK promoter (pDRE/TK-LUC), or the mutated apoA-I DRE fused with the minimal TK promoter (pmDRE/TK-LUC). Construction of the above plasmids is described in Materials and Methods. Panel B shows the expression plasmids pCMV-GF1 and pCDL-HSμBP2 used to overexpress GF1 and HSμBP2 protein, respectively.

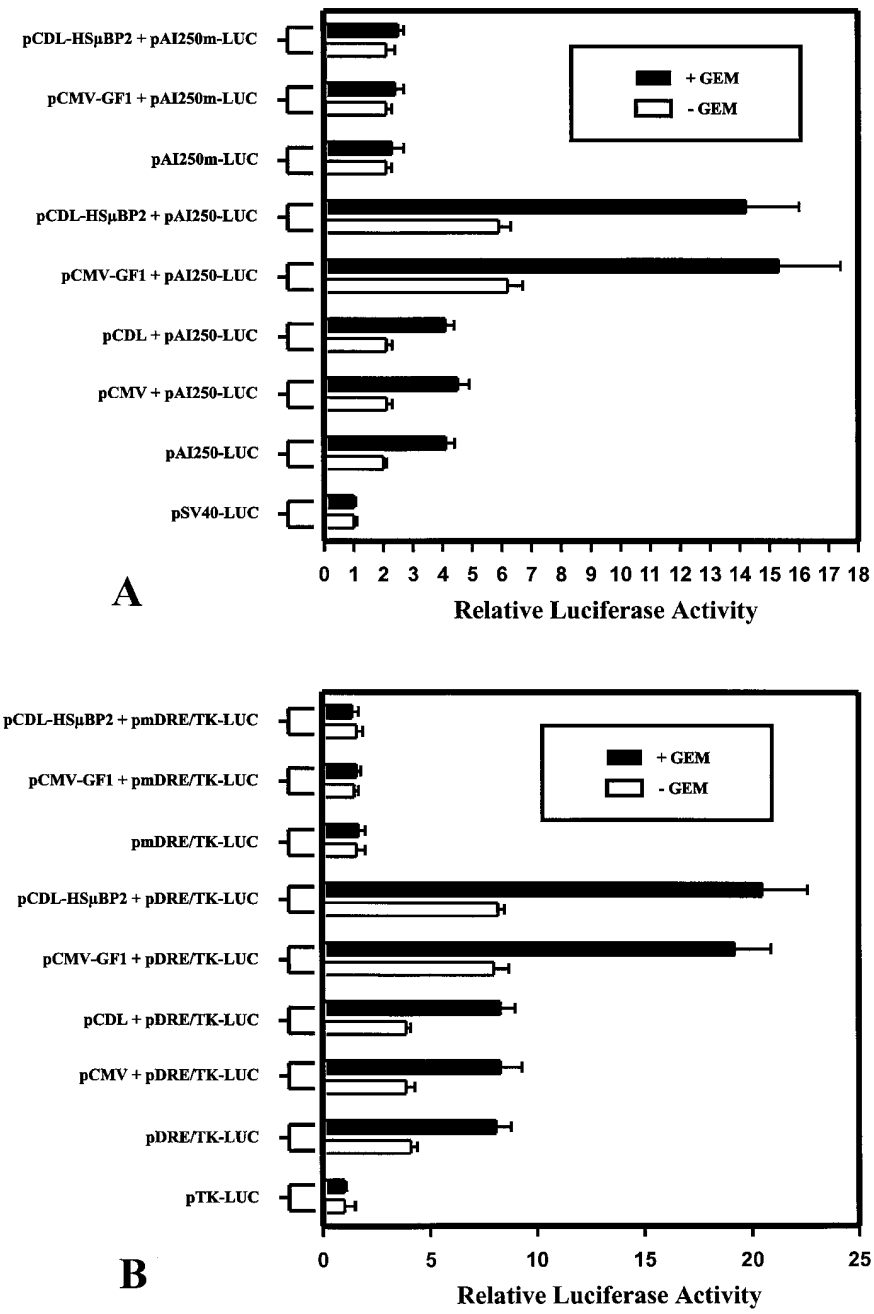

Fig. 5. Transactivation of pAI250 and pDRE/TK luciferase reporter constructs by GF1 and HSμBP2. Freshly seeded Hep3B cells were transfected with the reporter plasmid and expression plasmid. β-Galactosidase plasmid was included in all transfections as an internal control to normalize for differences in transfection efficiency. Cells were then cultured in the absence (open bars) or presence of gemfibrozil (solid bars) for 24 h prior to harvesting. The bar graphs represent relative luciferase activity in Hep3B cells transfected with various plasmids. Panel A: cells were transfected with pSV40-LUC or pAI250-LUC alone; or pAI250-LUC together with pCMV-GF1, pCMV, pCDL-HSμBP2 or pCDL; or pAI250m-LUC alone; or pAI250m-LUC together with pCMV-GF1 or pCDL-HSμBP2. Panel B: cells were transfected with the same type of arrangement as described in panel A except that pDRE/TK-LU and pmDRE/TK-LUC plasmids were used in place of pAI250-LUC and pAI250m-LUC, respectively. Normalized luciferase activities of the reporter constructs are then expressed relative to the pSV40-LUC and pTK-LUC which were arbitrarily set at one. Results represent the mean ± SEM for five independent experiments carried out in duplicate. The above plasmids were also transfected into HepG2 cells and yielded similar results (data not shown).

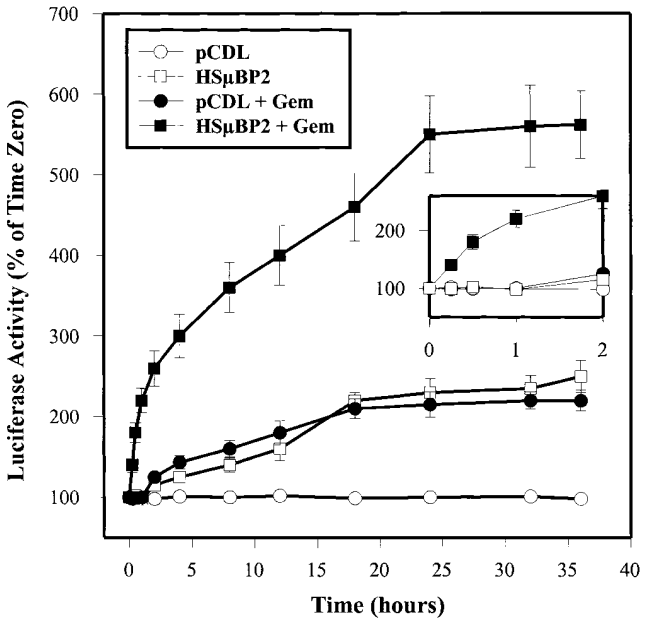

Fig. 6. Time course analysis of the effects of gemfibrozil on luciferase activity in stably transfected HepG2/1A cells transfected with HSμBP2. Freshly seeded HepG2/1A cells were transfected with either an expression plasmid HSμBP2 or pCDL (control vector). After transfection, the cells were allowed to equilibrate for 8 h and time zero began. The cells were then cultured in the absence or in the presence of gemfibrozil (40 μg/ml) and harvested at various times as indicated. Luciferase activities of the cells transfected with HSμBP2 or pCDL at time zero are arbitrarily set at 100%. The inset shows an enlargement of the graphs between time zero and 2 h. Results represent the mean ± SEM for four independent experiments. Similar results were also observed using the expression plasmid pCMV-GF1 (data not shown).

2- to 3-fold increase was observed when HSμBP2 expression plasmid was cotransfected (Fig. 5B). Neither overexpression of HSμBP2 nor gemfibrozil treatment had any effect on the basal luciferase expression in both pAI250m-LUC and pmDRE/TK-LUC transfected cells. In all the above experiments similar results were observed using the GF1 expression plasmid.

Because we demonstrated that gemfibrozil treatment of hepatoma cells resulted in no significant alterations in HSμBP2 mRNA levels, the observations from the above studies could be due to an additive effect of gemfibrozil and HSμBP2 or a post-transcriptional mechanism involving activation of HSμBP2 after gemfibrozil treatment. To distinguish between these two possibilities, HepG2 cells were stably transfected with a vector containing 248 base pairs of the 5′ flanking region of the human apoA-I gene linked to the luciferase gene. These cells, termed HepG2/1A, were subsequently transiently transfected with either HSμBP2 expression vector or pCDL, a control vector. Eight hours after transfection, these two groups of cells were then incubated in the absence or presence of gemfibrozil and assayed at various times for luciferase activity (**Fig. 6**). Strikingly, in cells transfected with HSμBP2 expression plasmid, gemfibrozil caused a 2-fold increase in luciferase activity within 1 h (Fig. 6, inset), reaching a maximal induction of 5.5-fold between 24 and 36 h. In cells cultured in the absence of gemfibrozil, overexpression of HSμBP2 resulted in a 2- to 2.5-fold increase in apoA-I promoter activity between 18 and 36 h. Similar results were also observed when HepG2/1A cells were transfected with the GF1 expression plasmid (data not shown). Cells transfected with the control plasmid and cultured in the presence of gemfibrozil also showed a much lower rate of induction and a much lower overall induction in relative luciferase activity similar to that of HSμBP2 transfected HepG2/1A cells without gemfibrozil treatment. As a control, cells were transfected with pCDL vector. These cells showed no change in luciferase activity over 36 h. Taken together, these data suggest that transactivation by HSμBP2 can be potentiated by gemfibrozil treatment.

DISCUSSION

Recently, many studies have focussed on the use of fibric acid derivatives to treat diet-resistant hyperlipidemic patients. Gemfibrozil is an extensively studied fibtrate that has been shown to effectively lower the risk of coronary heart disease (41). The mechanism of action of gemfibrozil is not fully understood. However, we have demonstrated that treatment of hepatoma cells with gemfibrozil results in a 2-fold induction of apoA-I mRNA and an increase in apoA-I gene transcription (21, 22). Furthermore, we identified a *cis*-acting regulatory element (DRE) in the proximal promoter of the human apoA-I gene that is required for gemfibrozil-mediated induction of apoA-I promoter activity and showed that drug-inducible protein–DNA interactions between the DRE and nuclear factors were associated with modulation of apoA-I gene expression (22). Previously, 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) (42–44). PPAR interacts with a specific response element (PPRE) located upstream of the target genes including human apoA-I gene (18, 45–49). The induction of the genes involved in the peroxisomal β-oxidation system of fatty acids by fibrates has been implicated in a strong proliferation of peroxisomes in rodents (42, 44–46).

PPAR binds to the PPRE located in site A of the human apoA-I promoter through heterodimerization with the 9-*cis* retinoic acid receptor, retinoid X receptor α (18). 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, in humans, this is questionable because, unlike rodents, peroxisome proliferation does not occur in liver biopsy samples from patients on long-term gemfibrozil therapy as evaluated by light and electron microscopy (50). Furthermore, in another peroxisomal proliferator non-responsive species, induction in apoA-I synthesis caused by gemfibrozil was not associated with increased PPARα mRNA levels in primary cultures of cynomologus monkey hepatocytes (51). Taken together, although the experiments do not exclude a role of PPAR in apoA-I transactivation, they do suggest that PPAR activation is not sufficient to explain all the above observed changes in the regulation of apoA-I gene expression in response to gemfibrozil. Recently, we have demonstrated that in vitro translated mouse PPARα synthesized from pSG4-mPPARα expression vector (kindly provided by Dr. S. Green, Alderley Park, Macclesfield, U.K.) using the rabbit reticulocytes in vitro transcription system (Promega Inc.) shows specific DNA binding activity to the PPRE but not to the DRE of the human apoA-I promoter (S-P. Tam and S. Green, unpublished results). In addition, we have performed supershift experiments to determine whether human PPAR interacts with the apoA-I DRE binding site. For supershift assays, the nuclear extracts isolated from gemfibrozil-treated Hep3B cells were preincubated with polyclonal antibodies raised against a PPAR synthetic peptide (Affinity BioReagents, Inc., Golden, CO) at 4°C for 18 h. The experiments showed no effect on the binding activity of DRE oligonucleotide to the gemfibrozil-treated nuclear extracts in the presence of anti-PPAR (data not shown). Consequently, we attempted to isolate and clone the transactivating factor(s) that interact(s) with the apoA-I-DRE.

We cloned and characterized one DRE specific DNA binding protein which appears to be regulated by gemfibrozil. The cloned protein is identical to GF1/HSμBP2, a transcription factor and proposed ATP-dependent helicase. A fusion protein, λS, containing amino acid residues 307 to 857 of HSμBP2, was capable of binding specifically to the apoA-I DRE. We also noted that GF1, which encodes amino acid residues 495 to 865 of HSμBP2, bound specifically to the apoA-I DRE indicating that all of the sequences necessary for DRE specific binding reside within a region of HSμBP2 between amino acids 495 to 857.

Protein encoded by the GF1 has been shown to promote transcription from the JC viral early and late promoters in glial cells by 2- to 3-fold and 6- to 10-fold, respectively (24). Shieh, Stellrecht, and Tsai (39), using the hamster homologue of HSμBP2, RIP-1, demonstrated that it functioned as a transactivator which interacted with the rat insulin promoter. As well, McBride and co-workers (52) showed indirectly that the rat homologue of HSμBP2 (cardiac transcription factor-1) may interact with elements in the cardiac atrial natriuretic factor promoter and affect gene expression. As indicated previously, the suggestion that HSμBP2 may be a DNA helicase remains to be confirmed. However, the roles of transactivator and helicase may not be mutually exclusive since several transcription factors with putative DNA helicase motifs have been characterized (53–55).

Northern analysis showed that HSμBP2 cDNA hybridizes to a mRNA of approximately 4.3 kb in size from a wide range of human tissues and from Hep3B cells. Similar results were also observed with RNA isolated from HepG2 cells of HH02 cells (data not shown). In human tissues, the level of HSμBP2 mRNA varies greatly, with the testis having the highest levels. Heart and skeletal muscle tissue also contained significant levels of HSμBP2 mRNA. In contrast, apoA-I mRNA expression is more restricted. We have apoA-I mRNA in liver, intestine, and testis tissues. Because HSμBP2 mRNA is expressed in many tissues, it appears unlikely that the protein plays a crucial role in determining tissue specificity of apoA-I gene expression. Furthermore, the levels of HSμBP2 and apoA-I mRNA do not correlate; however, at present we do not know whether the levels of HSμBP2 protein correlate with those of its mRNA. Hybridization of HSμBP2 cDNA to mRNA isolated from various human tissues revealed two higher molecular weight mRNAs of 5.8 Kb and 8.3 Kb. However, the fact that they are enriched in nuclear RNA suggests that the larger transcripts of HSμBP2 may represent immature prespliced precursors of the 4.3 kb HSμBP2 mRNA rather than alternatively spliced mRNA or related family members. This conclusion is supported by Southern genomic blots that show a low complexity banding pattern for the HSμBP2 gene (data not shown) as well as chromosomal mapping experiments that localized the HSμBP2 gene to a single locus on chromosome 11 at position q13.2–q13.4 (25).

We confirmed, by transient cotransfection experiments using HepG2 cells, that HSμBP2 could stimulate transcription from the apoA-I promoter and that the DRE was necessary for this response, as mutation of the DRE abolished transactivation and the apoA-I DRE alone conferred HSμBP2 inducibility on a minimal TK promoter. Interestingly, GF1 stimulated apoA-I promoter activity as effectively as full length HSμBP2, despite the fact that it lacked helicase domains I, IA, II,

III, and IV (26). Because GF1 lacks the putative helicase motifs, the results suggest that helicase activity is unnecessary for GF1 to stimulate transcription. Because HepG2 and Hep3B cells are derived from human hepatocarcinomas, they could possess different features than those of human hepatocytes in vivo. Consequently, we have confirmed the results using long term cultures of non-transformed human hepatocytes, HH02, to minimize the possibility that the response observed with HepG2 and Hep3B cells is due to their malignant phenotypes (56). Similar results were observed in cotransfection using HH-2 cells (data not shown).

We have observed that increased expression from the apoA-I promoter after gemfibrozil treatment does not involve changes in the level of HSμBP2 mRNA. Consistent with the transient cotransfection experiments using the expression plasmids HSμBP2 or GF1 and apoA-I promoter constructs or pDRE/TK-LUC, transient transfection studies using the expression plasmids and stably transfected HepG2/1A cells have also demonstrated an additional 3-fold increase in apoA-I promoter activity upon gemfibrozil treatment (Fig. 6). The data suggest that gemfibrozil could influence the level or activity of another transcription factor that acts on the DRE directly or by increasing the level or activity of other proteins which interact and modify the activity of HSμBP2. Alternatively, gemfibrozil may influence the transactivation potential of HSμBP2 directly by inducing post-translational modifications. At present, we cannot distinguish unequivocally between these possibilities. It is noteworthy that gemfibrozil treatment or overexpression of HSμBP2 in HepG2/1A cells increased apoA-I promoter activity approximately 100% over mock transfections; however, when HepG2/1A cells were exposed to both treatments, there was a more than an additive effect in luciferase activity (especially during the first few hours of gemfibrozil treatment, Fig. 6, inset) suggesting that these events may be interdependent. In addition, the rapid rate of induction of apoA-I promoter activity in response to gemfibrozil strongly suggests that HSμBP2 is an important component of the drug response mechanism.

The apoA-I promoter region has been extensively studied and several liver specific and ubiquitous transacting factors have been implicated in regulating apoA-I gene expression (11–20). It has been proposed that interaction of transacting factors with *cis*-elements is essential for maximal expression of the apoA-I gene. The intracellular balance of these transcription factors ultimately determines the levels of apoA-I gene responsiveness to specific signals. We have extended the list of nuclear factors that are known to interact and regulate apoA-I expression in hepatoma cells to include HSμBP2. Although it is unlikely the HSμBP2 plays a role in liver specific basal apoA-I gene expression, it appears that given the appropriate stimulus it can modulate apoA-I gene expression in HepG2 cells. Further experiments will be required to understand the relationship between the structure and function of HSμBP2 in the regulation of human apoA-I expression in response to gemfibrozil treatment.

The authors wish to thank Sandra Caine for her excellent secretarial skills. We wish to express our appreciation to Dr. E. Roberts for providing the HH02 cells and Dr. S. Davey for providing the HaCaT cell λZAP cDNA library. We also wish to thank Mr. G. Wilson for the pEBV7 vector. This work was supported by a grant (11223) from Medical Research Council of Canada. S-P. Tam also holds a Career Investigator Award from the Heart and Stroke Foundation of Ontario.

Manuscript received 7 July 1997 and in revised form 1 October 1997.

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