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Characterization of the Promoter Region of the Human Peroxisomal Multifunctional Enzyme Type 2 Gene

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Peroxisomal multifunctional enzyme type 2 (perMFE-2) catalyzes conversion of (24E)-3α,7α, 12α-trihydroxy-5β-cholest-24-enoyl-CoA to (24-keto)-3α,7α,12α-trihydroxy-5β-cholestanoyl-CoA, which are physiological intermediates in cholic acid synthesis. In contrast to long chain fatty acid oxidizing enzymes clofibrate does not induce peroxisomal enzymes metabolizing bile acid intermediates. We proposed the existence of PPAR-independent regulation of cholesterol side chain oxidation in the process of bile acid synthesis. In the present study, we characterized the promoter region of the human perMFE-2 gene. The promoter contains the Sp1/AP2 binding site (−151/−142) within 197 base pairs upstream of the translation start site. Mutation of the Sp1/AP2 binding site decreases the promoter activity. Analysis by the luciferase assay revealed that the activity of the promoter region is strong in HepG2 and HeLa cell lines, although the activity in HepG2 cells was five- to sixfold higher than that in HeLa cells. Transient transfection assays have confirmed that AP2α and AP2γ were able to transactivate the perMFE-2 promoter/luciferase chimeric gene. Cotransfections with Sp1 expression plasmid decreased the promoter activity. We suggest that perMFE-2 promoter activity is the result of both the abundance of AP2 and Sp1 family members and their relative ratios.

Key Words: **peroxisomes; bile acids; AP2; Sp1.**

A number of enzymes, localized to several subcellular compartments, participate in the formation of bile acids from cholesterol. The reactions may be divided into two main stages: (1) oxidation and modification of the ring system; and (2) oxidation and cleavage of the side chain followed by conjugation (1). It is known that peroxisomes are involved in the oxidative cleavage of the steroid side chain in bile acid biosynthesis (2). Oxidation of THCA (3α,7α,12α-trihydroxy-5β-cholestanoic acid) to cholic acid occurs in peroxisomes of human and rat liver through a mechanism analogous to that of fatty acid β-oxidation (3).

Peroxisomes oxidize a variety of substrates including straight chain fatty acids, 2-methyl-branched fatty acids (e.g. pristanic acid), and the side chain of the bile acid intermediates di- and trihydroxycoprostanic acids (3). It has been found that peroxisomes contain two β-oxidation pathways with different substrate specificities. In human liver, for example, straight chain acyl-CoAs are desaturated by palmitoyl-CoA oxidase, and their enoyl-CoAs are then converted to 3-oxoacyl-CoAs by multifunctional enzyme type 1 (perMFE-1), which forms (hydration) and dehydrogenates (3S)-hydroxyacyl-CoAs (3). Straight chain 3-oxoacyl-CoAs are cleaved by 3-ketoacyl-CoA thiolase to acetyl-CoA and shortened acyl-CoA.

In contrast, 2-methyl-branched fatty acids and the CoA esters of the bile acid intermediates di- and trihydroxycoprostanic acids are desaturated by branched chain acyl-CoA oxidase (4), and their enoyl-CoAs are then converted to the corresponding 3-oxoacyl-CoAs by multifunctional enzyme type 2 (perMFE-2) (5). The SCP_x is responsible for the cleavage of the 3-oxoacyl-CoAs of 2-methyl-branched fatty acids and the bile acid intermediates (6).

Another crucial difference between the two peroxisomal β-oxidation pathways is an expressional regulation. Administration of peroxisome proliferators (e.g., fibrates) leads to the rapid and coordinated transcriptional induction of the nuclear genes encoding the enzymes of the first peroxisomal β-oxidation pathway (3). *cis*-acting peroxisome proliferator-responsive elements (PPREs) have been identified in the 5′-flanking regions of the palmitoyl-CoA oxidase, MFE-1 and 3-ketoacyl-CoA thiolase (3, 7) genes. The nuclear hormone receptors called PPARs (peroxisome proliferator-activated receptors α, β and γ) regulate the first peroxisomal β-oxidation pathway (7). Gel retardation and co-

Abbreviations used: perMFE-2, peroxisomal multifunctional enzyme type 2; perMFE-1, peroxisomal multifunctional enzyme type 1; THCA, 3α,7α,12α-trihydroxy-5β-cholestanoic acid; (24E)-Δ^{24}-THCA, (24E)-3α,7α,12α-trihydroxy-5β-cholest-24-enoic acid; 24-OH-THCA, 3α,7α,12α,24ξ-tetrahydroxy-5β-cholestanoic acid.

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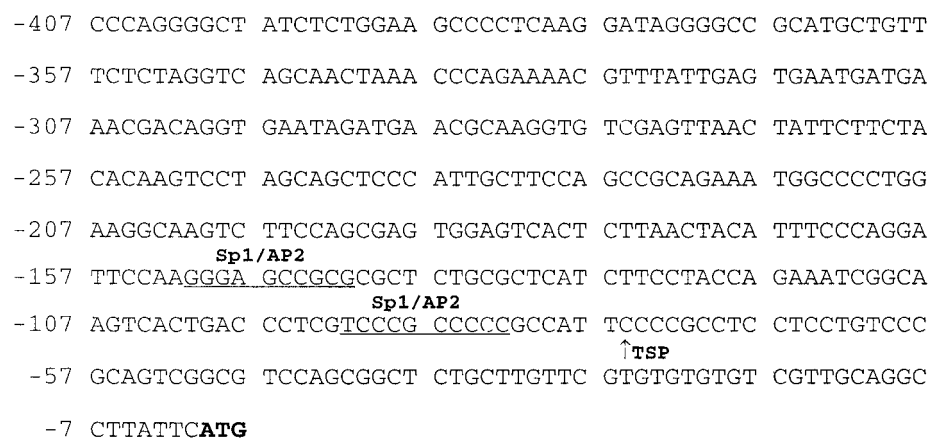

FIG. 1. Nucleotide sequence of the 5′-flanking region of the human perMFE-2 gene. The sequence is numbered relative to the translation start site. Putative Sp1/AP2 binding sites are underlined.

transfection assays revealed that PPAR heterodimerizes with retinoid × receptor β (RXRβ) and that the two receptors cooperate for the activation of the β-oxidation genes. The natural fatty acids, especially polyunsaturated fatty acids, activate PPARs as potently as do the fibrates (clofibrate, Wy 14,643, etc.) (7).

In contrast to the first peroxisomal β-oxidation pathway the second one is not induced by clofibrate (3). We proposed that transcriptional regulation of genes encoding enzymes of the second peroxisomal β-oxidation pathway is modulated by another nuclear receptor(s). Here we isolated the promoter region of the human perMFE-2 gene and investigated its activity in human cell lines by a luciferase reporter system. It turned out that the promoter activity of perMFE-2 gene is mediated by the relative binding of AP2 and Sp1 to Sp1/AP2 recognition sequence.

MATERIALS AND METHODS

Cell lines. HepG2 and HeLa cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% (v/v) fetal bovine serum.

Plasmid construction. The −406/−7 region of human perMFE-2 was obtained from genomic DNA (0.12 μg) by PCR amplification with primers: 5′-atctcgagCCAGGGGCTATCTCTGGAAGCC-3′ and 5′-ataagcttGGCCTGCAACGACACACACACG-3′ (with lowercase sequences indicating mismatches to the region upstream of the first exon of human perMFE-2 gene). The embedded *Xho*I and *Hin*dIII restriction endonuclease sites in the PCR primers allowed the release of a 400 bp insert.

The pGL3-basic vector (Promega), which contains the luciferase gene as a reporter, was used for the generation of all constructs for promoter analysis. Different regions of the human peroxisomal MFE-2 promoter were amplified using PCR and subcloned between the *Xho*I and *Hin*dIII restriction enzyme sites of the pGL3-basic vector. PCR conditions were 2 min at 95°C, followed by 30 cycles of 45 s at 95°C and 45 s at 62°C and extension of 2.5 min at 72°C. The resulting amplified PCR fragments were subcloned into pGL3-basic plasmid and sequenced by BigDye Terminator Cycle Sequence Kit (PE Applied Biosystems, Foster City, CA). Mutations of the putative binding sites were introduced using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. TRANSFAC database on transcriptional regulation was used for searching the sequences of individual regulatory elements within the promoter region (8). The Sp1 expression vector (human Sp1 cDNA subcloned into pEVR2 plasmid) was generously provided by Dr. Suske (Institut fur Molekularbiologie und Tumorforschung, Philipps-Universität Marburg, Marburg, Germany). The AP2α and AP2γ cDNAs subcloned into pcDNA3.1(+) expression vector (Invitrogen) as described previously (9) were generously provided by Dr. Lisa McPherson (Department of Surgery, Stanford University, CA).

Transient transfection assays. Cells were plated at 1×10^5 in ∅60 mm tissue culture plates and incubated for 16–18 h. Transfection was performed using FuGene 6 transfection reagent (Roche Diagnostic Corporation, Indianapolis, IN) according to the manufacturer's instructions. In brief, 0.1 ml of serum free DMEM containing 2.0 μg of plasmid DNA with perMFE-2 promoter region, 0.5 μg of pSV40β-galactosidase vector as internal standard for transfection efficiency and 3.0 μl of FuGene 6 were incubated at room temperature for 30 min. After incubation, the mixture was added to each plate. After 48 h culture, transfected cells were harvested by lysis. Lysis buffer and reagents used in the luciferase assay were supplied as luciferase assay kit (Promega, Madison, WI). Firefly luciferase activities were measured by Labsystems Luminoskan (Labsystems Oy, Helsinki, Finland) and transfection efficiencies were normalized by the activity of β-galactosidase derived from the cotransfected pSV40β-galactosidase plasmid (Promega, Madison, WI). β-Galactosidase activity was measured with β-Gal Reporter Gene Assay Kit (Roche Diagnostics GmbH, Mannheim, Germany).

RESULTS

Recently the gene coding for human perMFE-2 was mapped to chromosome 5q2.3 (5). It has been shown that the region of the 400 bp upstream of the transcription start site is sufficient to activate transcription, but consensus sequences were not identified (10).

To examine the functional activity of the promoter we cloned −406/−7 region of human perMFE-2 promoter DNA into pGL3-basic firefly luciferase reporter plasmid, which contains neither a promoter nor enhancer (Fig. 1). Luciferase activity was measured after

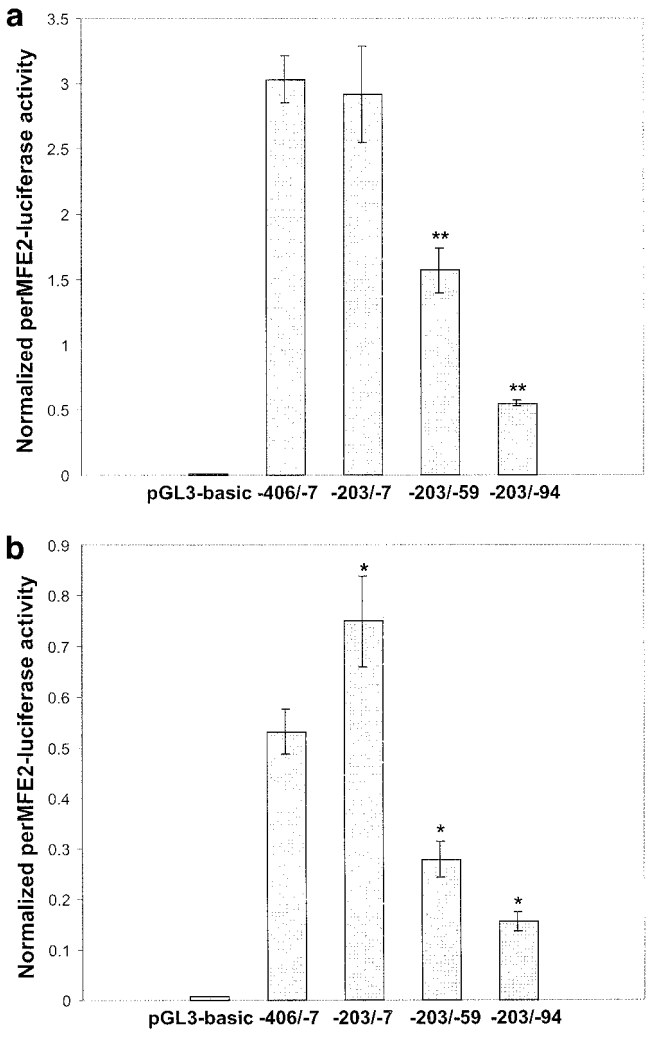

FIG. 2. Deletion analysis of the human perMFE-2 promoter in HepG2 (a) and HeLa (b) cells. HepG2 and HeLa cells were transfected with plasmids containing an perMFE-2 promoter sequence fused to the luciferase reporter gene. All cells were cotransfected with pSV40β-gal to normalize for transfection efficiency. Results are reported as the mean ± SD of three independent transfections. *$P < 0.05$ and **$P < 0.01$ versus −406/−7 construct.

48 h. PerMFE-2 promoter activity was 5.7-fold greater in HepG2 than in HeLa cells (Fig. 2). These findings support previously observed data that perMFE-2 expression is highest in liver (11).

We used deletion analysis to map the basal activities of the promoter. The 197 bp (−203/−7) promoter directed maximal expression of the luciferase reporter gene (Fig. 2). Analysis of the 197 bp sequence by TRANSFAC database (8) has shown GC-rich region (−92/−83) similar to reverse Sp1 binding site (12). Deletion of the Sp1 binding site (construct −203/−94) resulted in decrease of promoter activity by 80% in HepG2 and HeLa cells (Fig. 2). The residual promoter activity should be modulated by another *cis*-element(s). Further analysis of −203/−94 fragment has identified another GC-rich region −151/−142 (Fig. 1) corresponding to Sp1 binding site.

To determine if elevated Sp1 expression is sufficient to increase perMFE-2 promoter activity, HepG2 and HeLa cells were transiently transfected with perMFE-2 promoter luciferase reporter constructs and expression plasmid for Sp1 (Fig. 3). Surprisingly, overexpression of Sp1 decreased perMFE-2 promoter activity of both −203/−7 and −203/−94 fragments in HepG2 and HeLa cells. Statistically significant decrease of perMFE-2 promoter activity was 2-fold greater in HeLa than in HepG-2 cells. Although Sp1 is generally considered as ubiquitous transcription factor, there is considerable evidence that Sp1 participates in cell type-specific gene expression (13–15).

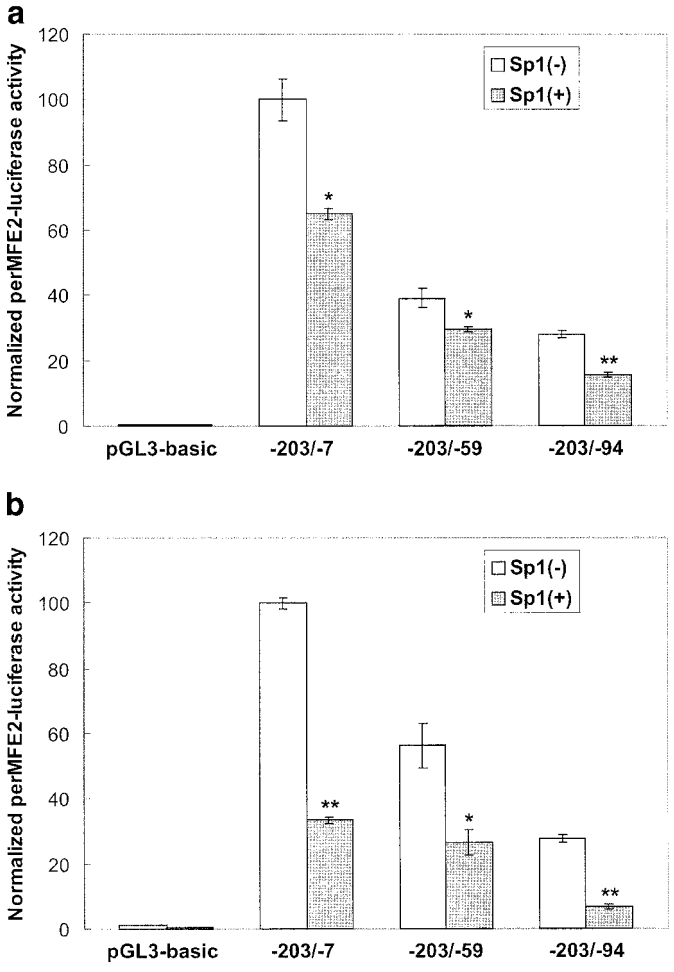

FIG. 3. Effect of cellular expression of Sp1 on perMFE-2 promoter activity in HepG2 (a) and HeLa (b) cell. HepG2 and HeLa cells were transfected with human perMFE-2 promoter luciferase reporter constracts. In addition, cells were cotransfected with either 0.5 μg of Sp1 expression plasmid or 0.5 μg of Sp1(−) control expression plasmid. All cells were also cotransfected with pSV40β-gal to normalize for transfection efficiency. Results are reported as percent of perMFE-2-luciferase activity (mean ± SD of three independent transfections) over that of −203/−7 construct (100%) for each individual cell type. *$P < 0.05$ and **$P < 0.01$ versus Sp1(−) transfection.

The mechanism by which Sp1 preferentially binds to some promoters in specific cell types is not clear. Most studies have not observed differences in the ability of nuclear extracts from different cell type to protect Sp1-binding sites in DNase I footprinting experiments *in vitro*. We proposed that another protein(s) may bind near the Sp1 site, thus preventing Sp1 binding. As soon as application of Dnase I footprinting and EMSA is limited in this particular case we used cotransfections and mutagenesis for identification of specific binding sites.

Computer analysis has revealed that −92/−83 and −151/−142 GC-rich boxes contain AP2 binding sites (Fig. 1). Overexpression of AP2α and AP2γ increased perMFE-2 promoter activity in HepG2 and HeLa from 2.2- to 6.5-fold (Fig. 4). The transactivation by AP2γ was greater than by AP2α factor in both cell types. Transfection of HeLa cells with AP2α and AP2γ increased perMFE-2 promoter activity more significantly than transfection of HepG2 cells. These data show that both GC-rich boxes may act as *cis*-elements for AP2α and AP2γ. To characterize these GC-rich boxes further we substituted key base pairs. The −151/−142 box GGGAGCCGCG was transformed to GG**A**A**TAAA**CG (mutation 1) and −92/−83 box TCCCGCCCCC to TCCC**ATAA**CC (mutation 2). Mutation 2 did not affect the promoter activity (Fig. 5). Mutation 1 decreased the activity by 3.5 times (Fig. 5). These results show that the −151/−142 GC-rich box is a functional one.

DISCUSSION

The oxidative cleavage of THCA, an intermediate in the biosynthetic pathway of cholic acid, is believed to proceed by a mechanism similar to the peroxisomal β-oxidation of fatty acids (3). The first intermediate of this reaction sequence is (24E)-Δ^{24}-THCA-CoA. In human peroxisomes, this reaction is catalysed by branched chain acyl-CoA oxidase (4). The reactions catalysed by perMFE-2 synthesize 24-keto-THCA-CoA from (24E)-Δ^{24}-THCA-CoA. The hydratase component of perMFE-2 converts (24E)-Δ^{24}-THCA-CoA to (24R,25R)-24-OH-THCA-CoA (16), which is converted to 24-keto-THCA-CoA by the dehydrogenase part of the perMFE-2 (5, 11). Then SCP_x cleaves the 24-keto-THCA-CoA to choloyl-CoA and propionyl-CoA (6).

Opposite to the first β-oxidation route the clofibrate treatment does not induce the second peroxisomal β-oxidation pathway (3). Thus we proposed that the second peroxisomal β-oxidation pathway is PPAR-independent and the regulation of its expression has to be investigated.

In this study, we demonstrate that expression of human perMFE-2 is induced by transcription factors AP2α and AP2γ and attenuated by Sp1 binding to recognition sequence located within and −151/−142 region. To our knowledge this is the first demonstration of promoter regulation of peroxisomal enzyme participating in bile acid synthesis. Furthermore, we show PPAR-independent regulation of peroxisomal protein expression, which proofs evolutionary different origin of two peroxisomal β-oxidation routes.

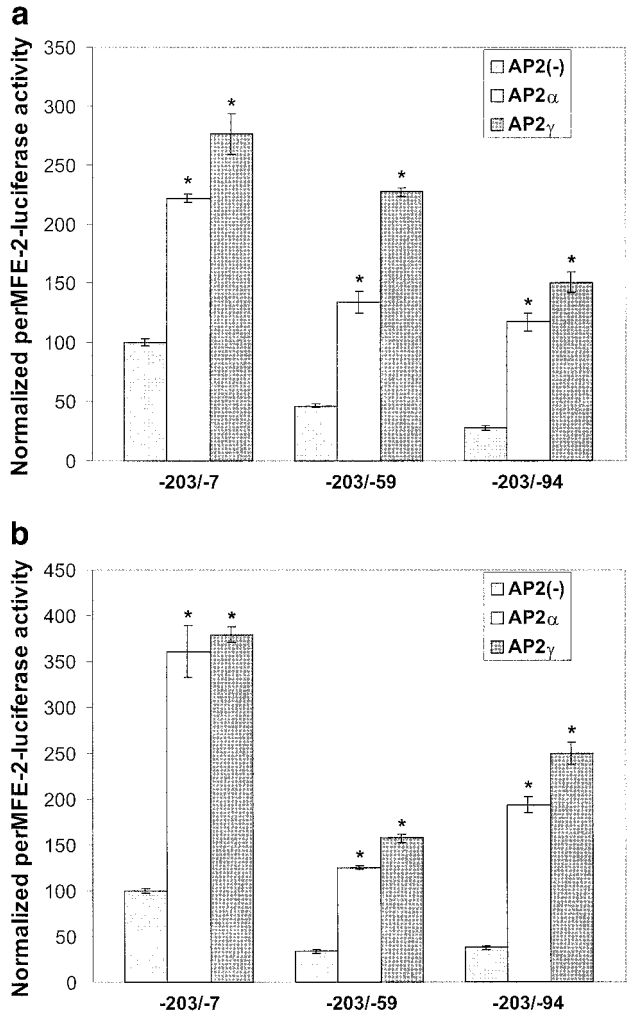

FIG. 4. Effect of cellular expression of AP2α and AP2γ on perMFE-2 promoter activity in HepG2 (a) and HeLa (b) cell. HepG2 and HeLa cells were transfected with human perMFE-2 promoter luciferase reporter constracts. In addition, cells were cotransfected with either 0.5 μg of AP2 expression plasmid or 0.5 μg of AP2(−) control expression plasmid. All cells were also cotransfected with pSV40β-gal to normalize for transfection efficiency. Results are reported as percent of perMFE-2-luciferase activity (mean ± SD of three independent transfections) over that of −203/−7 construct (100%) for each individual cell type. *$P < 0.01$ versus AP2(−) transfection.

Transcription factors play an important role in gene regulation by interacting with specific DNA sequences present in the promoter regions of genes. Often transcription factors are members of larger families that exhibit structural similarities but are expressed in tissue-specific or spatial patterns or recognize different DNA binding sequences. In the past, essential GC-boxes in promoters were often equated with "Sp1-

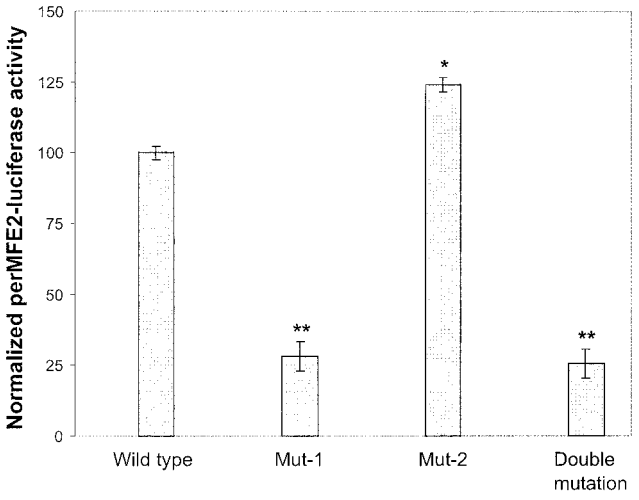

FIG. 5. Mutagenesis study of human perMFE-2 promoter. Mutations of −151/−142 (Mut-1), −92/−83 (Mut-2) or both (double mutation) of GC-rich boxes of −203/−7 region of perMFE-2 promoter were introduced by Stratagene kit. HepG2 cells were transfected with plasmids containing wild type or mutated perMFE-2 promoter sequence fused to the luciferase reporter gene. All cells were cotransfected with pSV40β-gal to normalize for transfection efficiency. Results are reported as the mean ± SD of three independent transfections. $*P < 0.05$ and $**P < 0.01$ versus wild type of −203/−7 construct.

binding sites" thereby overlooking the fact that Sp1 is not the only protein, which recognizes this important element (12). In addition to Sp1 there exist at least three other proteins, BTEB1 (basic transcription element binding protein 1) (17), TIEG1 and TIEG2 (TGFβ-inducible early protein 1 and 2) (18, 19) which have a binding specificity very similar, if not identical, to Sp1. Thus far, little is known how Sp1 act on natural promoters in combination with other transcription factors *in vivo.* Specificity could be obtained also by the interaction with co-activators or co-repressors.

The current study suggests that AP2 may modulate perMFE-2 expression via interaction to the −151/142 GC-rich box. Overexpression of AP2α and AP2γ proteins by transient transfection in both HepG2 and HeLa cells resulted in increase in perMFE-2 promoter activity (Fig. 4). On the other side, overexpression of Sp1 protein resulted in decrease of the perMFE-2 promoter activity (Fig. 3).

AP2 transcription factor family includes AP2α, AP2β and AP2γ (9). Functional AP2 binding sites have been identified in many viral and cellular enhancer elements. AP2 proteins bind DNA as a dimer, which forms through a helix-span-helix domain and the binding of this dimer to DNA is dependent on an adjacent region of net basic charge (20). A methodical study of binding site specificity has shown that both AP2α and AP2γ recognize the same consensus DNA sequence and that transcriptional activation by AP2 proteins is directly related to the affinity of the binding site (9). Overexpression of AP2 proteins can lead to the increased transactivation of promoters containing lower affinity binding sites that would not be activated in the presence of low levels of AP2 expression.

We suggest that the observed AP2 promoter specificity could be the result of both the abundance of AP2 family members and the relative ratios of AP2 and Sp1. On the other side we can not exclude the possibility that the same Sp1/AP2 binding sites in promoter of perMFE-2 are recognized by another nuclear receptors. As has been shown recently the promoter of the rate-limiting enzyme in bile acid synthesis, cholesterol 7α-hydroxylase (*CYP7A*), contains basic transcription element (BTE) and a Sp1 binding site in the −100/−82 region (21). Transient transfection assay have confirmed that BTEB1 was able to transactivate the *CYP7A* promoter/luciferase chimeric gene (21). Subsequently we may suggest that expression of another bile acid producing enzymes can be modulated by BTEB1 also.

The exact mechanism by which perMFE-2 promoter exerts its liver selectivity on the expression of perMFE-2 is still unclear and is the focus of future studies.

ACKNOWLEDGMENTS

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