Peroxisome Proliferator-activated Receptor γ-mediated Transcriptional Up-regulation of the Hepatocyte Growth Factor Gene Promoter via a Novel Composite cis-Acting Element

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Hepatocyte growth factor (HGF) is a pleotropic polypeptide that can function as a morphogen, motogen, mitogen, angiogen, carcinogen, and tumor suppressor, depending on the target cell and tissue. Previous studies from our laboratory using transgenic mice have shown that HGF gene expression is tightly regulated at the transcriptional level and that the upstream regulatory elements are crucial for the control of HGF gene transcription. In the present study, we have identified and characterized one of these elements as a peroxisome proliferator-activated receptor γ (PPARγ)-responsive element. This regulatory element was localized at −246 to −233 base pairs upstream from the transcription start site of the HGF gene promoter having the sequence GGGCCAGGTGACCT. Gel mobility shift and supershift assays demonstrated that this cis-acting element strongly binds to the PPARγ isomers as well as to chicken ovalbumin upstream promoter-transcription factor, a member of the orphan nuclear receptor subfamily. Mutational analysis and gel mobility band shift assays indicated that the binding site is an inverted repeat of the AGGTCA motif with two spacers (inverted repeat 2 configuration) and that the two spacers are important for PPARγ binding. This binding site overlaps with functional binding sites for activating protein-2, nuclear factor 1, and upstream stimulatory factor, and together, they constitute a multifunctional composite binding site through which these different transcription factors exert their regulatory effects on HGF promoter activity. Functional assays revealed that PPARγ, with its ligand, 15-deoxy-prostaglandin J2, strongly stimulates HGF promoter activity. On the other hand, nuclear factor 1, activating protein-2, and chicken ovalbumin upstream promoter-transcription factor transcription factors repress the stimulatory action of PPARγ by competing with PPARγ for their overlapping binding sites. Furthermore, for the first time, our studies demonstrate that the PPARγ ligand, 15-deoxy-prostaglandin J2, induces endogenous HGF mRNA and protein expression in fibroblasts in culture.

Received for publication, February 21, 2001, and in revised form, April 4, 2001
Published, JBC Papers in Press, April 5, 2001, DOI 10.1074/jbc.M101611200

The abbreviations used are: HGF, hepatocyte growth factor; bp, base pair(s); NF1, nuclear factor 1; AP2, activating protein-2; USF, upstream stimulatory factor; PPAR, peroxisome proliferator-activated receptor; IR, inverted repeat; COUP-TF, chicken ovalbumin upstream promoter-transcription factor; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; kb, kilobase pair(s); PGJ2, prostaglandin J2; RXR, retinoic X receptor; RA, retinoic acid.
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receptor PPARγ family. Additionally, we found that COUP-TF, an orphan nuclear receptor, also binds to this nuclear hormone binding site and negates the stimulatory effects of PPARγ. Functional studies revealed that PPARγ, with its ligand, 15-deoxy-PGJ2, strongly stimulates HGF promoter activity. On the other hand, NF1 and AP2 transcription factors repress the stimulatory function of PPARγ by competing with PPARγ for their individual overlapping binding sites present within this composite element. Moreover, for the first time, our studies demonstrated that the PPARγ ligand, 15-deoxy-PGJ2, induces the expression of the endogenous HGF mRNA and protein in cultured fibroblasts.

MATERIALS AND METHODS

Plasmids—0.7 mouse HGF-CAT promoter construct (−699 to +29 bp) was described in an earlier work from our laboratory (16). 0.7 HGF-CAT/NF1-M, in which the NF1 binding site was mutated, was created by a sequential PCR mutagenesis method (21). COUP-TFI and AP2 expression vectors were gifts from Dr. M.-J. Tsai, Baylor Medical College, and Dr. R. Buettner at the University of Regensburg, Germany, respectively. COUP-TFI and AP2 expression vectors were gifts from Dr. Xiao-Kan Zhang, University of San Diego, San Diego, CA. Dr. Bin Gao at the Medical College of Virginia generously provided the NF1/X expression vector. Mouse PPARγ1, PPARγ2, and PPARα expression vectors, as well as RXRo, were generous gifts from Dr. Bruce Spiegelman, Dana Farber Cancer Institute, Harvard Medical School, Boston, MA.

Preparation of Nuclear Extracts—Mouse NIH3T3 and 3T3-L1 fibroblasts were originally obtained from the American Type Culture Collection (Manassas, VA) and cultured in the conditions suggested by the American Type Culture Collection. Cells were grown to ~90% confluence, washed twice with cold phosphate-buffered saline, and scraped with a rubber policeman in the same buffer. Nuclear protein extracts were prepared as described previously (20). Rat liver non-parenchymal cells were prepared from collagenase-perfused rat liver followed by centrifugation at 50 × g to remove hepatocytes and were provided by Dr. G. K. Michalopoulos in our department. They were used to prepare nuclear protein extract.

A rat liver stellate cell line (Ito cell line) was kindly provided by Dr. M. Rojkind from the Albert Einstein College of Medicine and cultured as described previously (25, 26). For preparation of mouse liver nuclear protein extracts, livers were removed from mice and homogenized in buffer A containing protease inhibitors (20). The nuclei were collected, and the nuclear extracts were extracted by the same method as described above.

DNA Transfection and CAT Assay—Mouse 3T3-L1 fibroblasts were cultured in 6-well plates for 24 h and then transfected with various mouse HGF promoter-CAT chimerical plasmids using the DNA calcium phosphate method according to the instructions of the CellPhect transfection kit (Amersham Pharmacia Biotech) as described previously (19). The β-galactosidase reference plasmid pCH110 (Amersham Pharmacia Biotech) was used as an internal control for monitoring the transfection efficiency and normalizing the data accordingly. The amount of plasmid used per well in transfection was as follows: 5 μg of one chimerical CAT construct and 1 μg of pCH110. After coprecipitation of the cells with DNA-calcium phosphate for 16 h in serum-containing medium, the cells were washed twice with serum-free medium and kept in serum-containing medium for an additional 24 h before harvesting for determination of CAT activity. CAT activity was determined as described previously (19, 20). Transfections were performed at least three separate times with two independent preparations of purified plasmid DNA.

For cotransfection with COUP-TFI, AP2, NF1/X, RXRα, and PPARγ 1 in 3T3-L1 cells, the amount of plasmid used per well in transfection was as follows: 5 μg of one chimerical CAT construct, 1 μg of pCH110, and different amounts of expression plasmids. The total amount of DNA per well was equilibrated by addition of corresponding empty (no insert) expression plasmid.

Oligonucleotides and Antibodies—The following oligonucleotides and antibodies were purchased from Santa Cruz Biotechnology, Inc. and used for gel-shift and supershift assays: NF1, 5’-TCAAGCCAATAAGTATAA-3’; USF, 5’-CACCCTGTACCGTGCCCTAC-3’; RXR (DR-1), 5’-AGCTCAGCAGCAGGTACAGACT-3’; and anti-PAPγ, anti-PPARα, anti-Sp1, anti-RXRα, anti-thyroid hormone, anti-COUP-TF (which can react with both COUP-TFI and COUP-TFII), anti-nerve growth factor-induced B, and anti-vitamin D receptor antibodies.

Gel Retardation Assays—The double-stranded oligonucleotides used in gel mobility shift assays were labeled with [α-32P]dCTP by end labeling with the Klenow fragment of DNA polymerase. The labeled oligonucleotide probes were then gel-purified and used in gel mobility shift assays as described previously (21). Two μg of poly(dI–dC) (Amersham Pharmacia Biotech) were used as the nonspecific competitor in 10 μl of reaction mixture. When different concentrations of antibodies were used, they (1 μl) were incubated with nuclear extracts at room temperature for 20 min before performing the DNA binding shift assays. The binding reactions were carried out at room temperature for another 20 min before loading onto 5% nondenaturing polyacrylamide (19:1, acrylamide/ bisacrylamide) gels. The amount of the nuclear protein extract used in each reaction was about 4 μg, and that of the labeled probe was between 0.2 and 0.4 ng. For competition experiments, a 100-fold molar excess of unlabeled oligonucleotides was included in reaction mixtures. Gels were run in 0.5 × TBE buffer (0.045 M Tris borate, 0.001 M EDTA) at a constant voltage of 210 V, dried, and autoradiographed with intensifying screens.

RNA Isolation and Analysis—Total RNA was isolated by using RNAzol B solution (Clina/Biotex, Friendswood, TX) according to the manufacturer’s instructions. The RNA concentration was determined by measuring the absorbance at 260 nm. For quantitative RT-PCR, 1 μg of total RNA was reverse-transcribed by using AMV reverse transcriptase (Roche Molecular Biochemicals) in reverse transcriptase reaction and amplified for 25 cycles (the number of cycles were optimized to ensure that quantitative assessment could be performed with Tag DNA polymerase) and then for 40 cycles using primers specific to HGF (sense, 5’-ATCACGACCCACACGCCCAAAAT-3’; antisense, 5’-GAAATGGGGCAATAACCTACGGA-3’) or β-actin (CLONTECH Laboratories). β-actin served as an RNA integrity and normalization control.

In Vitro Transcription/Translation—A transcription and translation-coupled reticulocyte lysate system was used to prepare in vitro translated COUP-TFI, mouse PPARγ1, PPARα, and RXRo proteins from their cognate expression vectors driven by the T7 promoter (27). Transcription/translation reactions were carried out in a 50-μl reaction volume as recommended by the supplier (Promega). The authenticity of translation products was always confirmed in parallel experiments by including radioactive [35S]methionine and radioactive [35S]cysteine in the translation reaction followed by SDS-polyacrylamide gel electrophoresis and autoradiography. Translation reactions using empty vectors were always included as negative controls in these analyses, including the gel shift assays, to exclude potential false signals. Translation products were stored at −80 °C. For each gel mobility shift assay, about 1 μl was used from each translated reaction.

RESULTS

Identification of a Binding Site for PPARγ Transcription Factor in the HGF Gene Promoter—Our recent studies of the HGF promoter revealed that the proximal promoter region at position −260 to −230 is a multifunctional composite site to which different transcription factors such as NF1, USF, and AP2 can bind and regulate HGF gene transcription (21, 22). During those studies, we found that distinct factor(s) also specifically bind to this element that are not related to the NF1, USF, or AP2 families (21). Within this 30-bp region (∼260 to −230) of the promoter, we identified ROGTCA hexanucleotide motifs, which are potential binding sites for the nuclear hormone receptor superfamily (23, 28). To further characterize the composite binding site and its cognate transcription factors, we radiolabeled the HGF promoter composite element (∼260 to −230) with [32P] and used this oligonucleotide as a probe to perform gel mobility band shift assays. In these experiments, nuclear protein extracts from 3T3-L1 and NIH3T3 fibroblast cell lines were used. As shown in Fig. 1, this DNA element forms several specific complexes, of which the major complex(es) (shown by a large arrow head) are efficiently abrogated by an excess amount of binding site for NF1 (compare lane 2 with lane 4 and lane 7 with lane 9, respectively). The remaining two complexes, which we have labeled in the figure as C1 and C2, formed by nuclear protein extract from NIH3T3 cells, are abolished by an excess amount of USF and nuclear hormone binding sites (RXRo), respectively (Fig. 1, lanes 9–11). We have previously shown by super shift assays that the major com-
plex(es) C contain NF1 isoform(s) and that the C1 complex contains USF1 and USF2 isoforms (21). As shown in Fig. 1, 3T3-L1 fibroblasts do not have detectable C1 and only form the C2 complex, which is totally abrogated by RXRE (DR1, direct AGGTCA repeat with one spacing) (Fig. 1, lane 6). Thus, these results extend our previous observation and demonstrate that the complex C2 may contain nuclear hormone receptor(s), because it can be competed by a binding site having an AGGTCA motif (please see Ref. 21). To better define the binding site for complex C2, several mutant versions of the promoter element were synthesized (see Fig. 2E for nucleotide sequences) and used as competitors in electrophoretic mobility band shift assays using NF1-depleted 3T3-L1 nuclear protein extracts. As depicted in Fig. 2A, deletion of up to 11 base pairs from the 5’ region did not have a notable effect on the binding activity (because these oligonucleotides still effectively competed with the radiolabeled wild-type probe; Fig. 2A, lanes 3 and 4, oligonucleotides designated S1 and S2). However, when six nucleotides from the 3’ region were truncated, the resulting oligonucleotide totally lost its competitive ability (Fig. 2A, lane 5). These results revealed that the binding site for C2 should be between −249 and −230. Close examination of the promoter element (−249 to −230) indicated that it harbors a potential RGCTGA inverted repetitive sequence separated by two nucleotides (IR2) (Fig. 2B, shown by arrows). The AGGTCA site is known as the perfect nuclear hormone binding half-site, although in almost every case, it is a variation of this sequence known as a ‘‘supermotif’’ (28). In a strict comparison of the nucleotide sequence of this 31-base pair element among the mouse, rat, and human HGF promoters revealed perfect conservation of this site (Fig. 2B). To define the binding site for the C2 complex in more detail, several mutated oligonucleotides were synthesized and used in gel shift competition assays, as above, using fibroblast nuclear extracts (Fig. 2, C and D). Mutations in the AGGTCA half-site in the 3′ region abolished the binding capability (mutant oligonucleotides named M4 and M5 in which AGGTCA is mutated to AGGGAC and CTCTTA, respectively) (Fig. 2C, lanes 6 and 7). Conservative mutations in the 5′-half-site RGCTGA motif (GGGCCA to AAGCCA or GGGAAA and also mutations in the two spacers (GG to AT) (oligonucleotides named M1, M2, and M3, respectively)) did not dramatically affect binding, because these oligonucleotides still competed with the labeled probe for binding (Fig. 2C, lanes 3–5). However, mutation in the 5′-half-site that totally changes the hexanucleotide from GGGCCA to TTTGGC completely abolished its binding activity (Fig. 2D, lane 8). To define the role of the two spacers in the IR2 configuration, we generated additional mutant oligonucleotides as indicated in Fig. 2E and used them as competitors. As shown in Fig. 2D, deleting of one or both spacer nucleotides (GG) dramatically reduced the binding activity (Fig. 2D, lanes 5 and 6). On the other hand, mutating the two putative spacers from GG to TT did not affect the binding activity (Fig. 2D, lane 7). These results implied that the RGCTGA half-sites and the two-spacer configuration are important for binding of complex C2 to this region. As mentioned above, it is well known that the RGCTGA is the binding half-site for some members of the nuclear receptor superfamily (23, 28). Therefore, several antibodies against the members of the nuclear receptor superfamily were used to identify complex C2 by supershift assays using fibroblast nuclear extract; these antibodies were against retinoic acid receptor, OR-1, thyroid hormone receptor, COUP-TF, vitamin D receptor, and nerve growth factor-induced B. None of these antibodies reacted with the C2 complex (data not shown). Further analysis using additional antibodies against other nuclear receptors revealed that anti-PPARγ antibody reacted with and totally supershifted complex C2 formed by the fibroblast nuclear extracts (Fig. 3, lanes 2 and 6). Neither anti-RXRα antibody nor an unrelated antibody (anti-Sp1; see Fig. 3, lanes 4 and 8) reacted with complex C2. Taken together, the data clearly show that PPARγ binds to the HGF promoter element located at −246 to −233 bp from the transcription start site.

Regulatory Function of PPARγ and Its Ligand 15-Deoxy-PGJ2 on the HGF Gene Promoter Activity—To demonstrate whether the PPARγ binding site and its binding transcription factor PPARγ have regulatory function on the HGF gene promoter, we performed cotransfection experiments with HGF-CAT promoter constructs and PPARγ expression vector. Because the PPARγ binding site overlaps with those of NF1 and USF and because we have shown that NF1 strongly suppresses HGF gene promoter activity (21), we decided to eliminate the interference of NF1 with the PPARγ function. To do this, two different HGF-CAT constructs were generated and used: the wild-type 0.7 HGF-CAT construct and its mutated version, the 0.7 HGF-CAT/NF1-M construct, in which the NF1 site is mutated but the PPARγ binding site remains intact. Another HGF-CAT construct, 0.1 HGF-CAT, which does not have the NF1 and PPARγ binding sites, was also used as a negative control. We selected the 3T3-L1 cell line because the nuclear protein extract from this cell line did not form a USF complex (C1) (see Figs. 1 and 3) with this element that may interfere with the PPARγ function. As shown in Fig. 4, 15-deoxy-PGJ2, the PPARγ ligand, significantly induced the promoter activities of both the 0.7 HGF-CAT and the 0.7 HGF-CAT/NF1-M constructs but not the 0.1 HGF-CAT construct (Fig. 4). Overexpression of PPARγ1 and treatment with its ligand, 15-deoxy-PGJ2, had an even stronger stimulatory effect on both the 0.7 HGF-CAT and the 0.7 HGF-CAT/NF1-M constructs (Fig. 4). It is believed that RXRα is the functional partner of PPARγ through heterodimer formation (28). Even though we did not
FIG. 2. Characterization of the binding motif(s) for the nuclear receptor in the HGF promoter regulatory region. A, truncated (shortened from 5' or 3' ends as indicated in E) oligonucleotides S1, S2, and S3 corresponding to the promoter element were synthesized and used as competitors in gel mobility competition assays using NF1-depleted nuclear protein extract from 3T3-L1 fibroblasts and the wild-type HGF promoter element (−260 to −230) as a probe. B, analysis of the potential RGGTCA motifs within the regulatory region (from −260 to −230 bp) of the mouse HGF gene promoter. RGGTCA inverted repeats separated by two nucleotides (IR2) were identified as indicated by arrows. For comparison, the nucleotide sequences of the rat and human HGF promoter element are also presented. The functional binding sites for NF1, USF, AP2, and an E box (i.e., USF binding site) are also shown. C, mutated oligonucleotides M1–M5 (for sequence information, please see E) were synthesized and used as competitors as described under A. D, the S2 oligonucleotide was labeled and used as a probe with NIH3T3 nuclear extracts to further define the IR2 site. The nuclear extract used in this gel shift assay was not depleted of NF1. The DR1 site (direct AGGTCA motif with one spacing) was also used as competitor. E, the nucleotide sequences of the wild-type and mutant versions of the promoter element used to define the PPARγ binding site are shown. The mutated nucleotides are underlined. C2 denotes complex C2. Self-competitor (wild-type unlabeled probe) was used as a positive control.
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see an obvious supershift complex by anti-RXRα antibody using fibroblast nuclear extracts (see Fig. 3), it is possible that the nuclear protein extracts from NIH3T3 and 3T3-L1 cells do not contain a sufficient amount of RXRs binding activity. In fact, we show that the HGF PPARγ element binds to both PPARγ and RXRα using liver nuclear protein extract, which is presumably rich in RXRα (see below). To find out whether RXRα can regulate the HGF gene promoter through binding to this IR2 element as a partner of PPARγ, RXRα expression vector and its ligand, 9-cis-RA, were used in cotransfection CAT assay experiments using a fibroblast cell line. As shown in Fig. 4, 9-cis-RA itself had a modest effect, but a combination of 9-cis-RA plus its receptor, RXRα, strongly stimulated HGF promoter activity, similar to 15-deoxy-PGJ2 with its receptor, PPARγ. The strongest induction of the promoter was achieved by cotransfection of PPARγ and RXRα expression vectors in the presence of 15-deoxy-PGJ2 (the PPARγ ligand) and 9-cis-RA (the RXRα ligand) (Fig. 4). Because PPARγ/RXRα could induce the promoter activity of the 0.7 HGF-CAT/NF1-M construct, these results indicate that PPARγ/RXRα may not only indirectly (via competing with NF1 binding), but also directly, stimulate the HGF promoter through binding to a functional RGGTCA motif in the HGF proximal promoter.

Binding of the Nuclear Hormone Element with RXRα, PPARγ, and COUP-TF from Liver Tissue and Related Cells—It is well known that the HGF gene is expressed in mesenchymal, but not epithelial, cells of a given tissue. In the liver, HGF is expressed in the non-parenchymal mesenchymal cells (especially stellate cells, also known as the Ito cells or lipocytes because they store lipids and vitamin A). We tested whether the HGF promoter element could bind to PPARγ and possibly other transcription factors present in non-parenchymal liver cells. Nuclear protein extracts from whole liver and cells isolated from liver, such as freshly isolated non-parenchymal cells, as well as an Ito cell line (25, 26), were prepared. We then subjected these extracts to gel mobility shift assay using the HGF promoter composite site as a probe. The probe bound to PPARγ present in all three different nuclear protein extracts, as shown by supershift assays (Fig. 5, lanes 2 and 7, and data not shown for whole liver). A surprising finding in these experiments was that this element could also strongly bind to COUP-TF, a member of the orphan nuclear receptor subfamily, which also binds to the RGGTCA motif (see Fig. 5, lanes 3 and 8) (28). The fact that anti-COUP-TF totally supershifted the C2 complex whereas anti-PPARγ partially did so (Fig. 5, compare lanes 2 and 3) suggests that PPARγ and COUP-TF may form heterodimers on the HGF promoter element. Of course, it is known that COUP-TF also forms homodimers; thus, it is likely that the C2 complex in the nuclear extract of liver cells is mainly composed of COUP-TF homodimer as well as COUP-TF and PPARγ heterodimers. On the other hand, the C2 complex produced by fibroblast nuclear extracts lacks any COUP-TF, because it did not react with anti-COUP-TF (data not shown; also see Fig. 3). Anti-RXRα antibody was also used in these experiments, because RXRα is believed to be the partner of PPARγ, as mentioned above. We did detect weak RXRα binding...
to this element with the nuclear protein extracts from whole liver tissue (the shifted band was very faint and required a very long exposure of the film) (data not shown). Taken together, the results imply that the PPAR\(g\) binding site in the HGF gene promoter also binds to COUP-TF and RXR\(a\), depending on the cell types/tissues analyzed (i.e. fibroblasts versus liver cells).

To confirm that COUP-TF truly binds to the HGF proximal promoter composite element, \textit{in vitro} translated COUP-TFI transcription factor was prepared and used in gel mobility shift assays. Indeed, \textit{in vitro} translated COUP-TF could specifically and avidly bind to this region, as shown in Fig. 6, \textit{lane 1}. Unlabeled excess probe totally competed for this complex (Fig. 6, \textit{lane 2}). Moreover, anti-COUP-TF antibody reacted with and shifted this complex (Fig. 6, \textit{lane 4}). On the other hand, anti-PPAR\(g\) antibody did not react with this complex (Fig. 6, \textit{lane 3}). These results prove that COUP-TF binds specifically to this region. To define the binding motif(s) for COUP-TF in this region, gel shift competition assays were performed using the truncated oligonucleotides S1–S3 and the mutated oligonucleotides M1–M5 (see Fig. 2E for sequences). S1 oligonucleotide retained all of its binding activity. S2 oligonucleotide retained some of its binding ability, but the S3 oligonucleotide totally lost its binding ability for COUP-TF (data not shown). Similarly, the competitive ability of M1, M2, and M3 mutated oligonucleotides reduced only modestly. However, the binding ability for COUP-TF was totally abrogated when the AGGTCA motif in the 3' end of the element was mutated (oligonucleotides M4 and M5; data not shown). Combining all of the above results, we conclude that COUP-TF also binds to the composite site in this regulatory region of the HGF gene promoter (see 6, \textit{lane 2}).

![Figure 6](image-url) **Fig. 6.** Characterization of the COUP-TF binding motif in the HGF promoter composite element. \textit{In vitro} translated COUP-TFI protein was used in these experiments. Supershift assays were performed with antibody against COUP-TF and the wild-type HGF promoter element as a probe. Self-competitor was used to show the specific binding of COUP-TFI with this element. Antibody against PPAR\(g\) was used as a negative control. The COUP-TFI complex is indicated by an arrow. S denotes the supershift complex. Negative controls for the \textit{in vitro} translation product (empty vector control subjected to the \textit{in vitro} translation reaction) and gel shift assays were also carried out to also ensure specificity (data not shown).

![Figure 7](image-url) **Fig. 7.** Repression of the stimulatory function of PPAR\(\gamma\) by COUP-TFI on the HGF gene promoter. Five \(\mu\)g each of 0.3 and 0.1 HGF-CAT reporter plasmids was cotransfected along with 2 \(\mu\)g of PPAR\(\gamma\)1, 2 \(\mu\)g of RXR\(\alpha\), and increasing amounts of COUP-TFI expression vector into the 3T3-L1 fibroblast cell line. The activity of each promoter construct was determined and is expressed as the relative CAT activity normalized by \(\beta\)-galactosidase. The results are from three independent experiments performed in duplicate. Error bars represent the standard error of the mean.
**Fig. 9.** A diagram depicting several functional cis-acting elements and their cognate transcription factors within the murine HGF promoter region. NHR, nuclear hormone receptor; Rep, an unknown repressor; GTF, general transcription factors; C/EBPβ, CCAAT; IL-1, interleukin 1; TNFα, tumor necrosis factor α; EGF, enhancer-binding protein β.

Modulation of PPARγ Function on the HGF Gene Promoter by COUP-TF, NF1, and AP2—Because our data showed that COUP-TF binds to the same binding site as that for PPARγ, it is reasonable to suppose that COUP-TF homodimers could modify the stimulatory function of PPARγ/RXRα by competing with one another for their common binding site. Additionally, published data demonstrate that COUP-TF can form heterodimers with RXRα in solution in the absence of their binding site. In this manner, COUP-TF sequesters RXRα, preventing PPARγ/RXRα heterodimer formation and the stimulatory function of PPARγ/RXRα. Furthermore, the binding sites for COUP-TF and PPARγ/RXRα overlap with the binding sites for the suppressive transcription factors NF1 and AP2. Therefore, the functionality of this unique composite element to regulate HGF gene expression in a given tissue or cell may be dependent on the specific combination, and/or the various concentrations, of transcription factors. To show the individual functions of these transcription factors and how they interact to exert their effects, we used the 0.3 HGF-CAT promoter construct, which has a basal promoter and the proximal region of the HGF promoter containing the NF1, AP2, and IR2 binding motifs. We also used the 0.1 HGF-CAT construct as a negative control, because it only contains the basal promoter. As shown in Fig. 7, COUP-TF dose-dependently repressed the stimulatory function of PPARγ/RXRα on the HGF gene promoter in the 0.3 HGF-CAT construct. It did not have significant repressive function on the 0.1 HGF-CAT construct (Fig. 7). In similar experiments using the expression vectors for the NF1/X isoform and AP2 co-transfected with HGF-CAT constructs we found that they also inhibited the stimulatory function of PPARγ/RXRα on the HGF gene promoter in the 0.3 HGF-CAT construct but not in the 0.1 HGF-CAT construct (data not shown). In these experiments, empty vector controls did not have any effect on the activity of either HGF-CAT construct (data not shown). These experiments indicate that NF1, AP2, and COUP-TFI could individually down-regulate the stimulatory function of PPARγ/RXRα on HGF gene expression and that they may do this through competing with PPARγ/RXRα for the overlapping composite binding site in the HGF proximal promoter region.

### Induction of Endogenous HGF mRNA and Protein Expression by PPARγ Ligand, 15-Deoxy-PGJ2—To determine whether 15-deoxy-PGJ2 can induce endogenous HGF gene expression at the mRNA and protein levels, we treated fibroblasts, which naturally express PPARγ, with 15-deoxy-PGJ2 and determined the level of HGF mRNA by semi-quantitative RT-PCR and the level of HGF protein by a very sensitive sandwich enzyme-linked immunosorbent assay. The results demonstrated that HGF expression is significantly up-regulated after treatment with 15-deoxy-PGJ2 (Fig. 8, A and B, respectively).

**DISCUSSION**

In this study, we demonstrated that the nuclear receptors PPARγ and COUP-TF bind to a composite regulatory element in the HGF upstream promoter region located at −246 to −233 bp from the transcription start site. This binding site also overlaps harbors functional binding sites for NF1, AP2, and the USF family of transcription factors (21, 22), and together, they constitute a multifunctional composite site to which these various transcription factors can bind and regulate the activity of the HGF promoter. Cotransfection assays showed that PPARγ1, with its partner, RXRα, in the presence of their corresponding ligands, 15-deoxy-PGJ2 and 9-cis-RA, respectively, strongly stimulated HGF promoter activity. NF1, AP2, and COUP-TFI suppressed the stimulatory function of PPARγ by competing for their individual binding sites. Moreover, we demonstrated that 15-deoxy-PGJ2, the ligand of PPARγ, induces the expression of the endogenous HGF gene at the mRNA and protein levels in cultured fibroblasts.

PPARγ is a member of the nuclear receptor superfamily that includes receptors for the steroid, thyroid, and retinoid hormones (23, 28). It is known that there are three related but quite distinct PPAR proteins: PPARα, PPARδ, and PPARγ. Two forms of PPARγ, γ1 and γ2, exist as products of alternative promoter usage (29, 30). Like other members of this superfamily, PPARγ contains a central DNA binding domain that binds to a cis-acting element in the promoter of its target genes. Most of the PPARγ response elements described are composed of a directly repeating core site separated by one nucleotide (GGTCA-N-RGGTCA) called DR1 (24, 28). Interestingly, the PPARγ response element in the HGF promoter region is an inverted repeating core site separated by two nucleotides (GGGCA-NN-TGACCT) that we have called IR2. Published data indicate that PPARγ heterodimerizes with RXRα and that
the PARγ/RXRα heterodimer binds to its response element and activates its target genes (23, 31). Our results indicated that the IR2 site in the HGF gene promoter also binds to RXRα, though very weakly. Generally, RXRα is said to be a silent partner. Nonetheless, there are several examples in which PARγ activates its target genes (23, 31). Our results indicated that RXRα binds to the IR2 site in the HGF gene promoter also binds to RXRα, though very weakly. Generally, RXRα is said to be a silent partner. Nonetheless, there are several examples in which PARγ, CCAAT/enhancer-binding protein β binding to the HGF promoter elements occurred and is depicted in Fig. 9. The increase in the binding activity correlated well with an increase in HGF and HGF receptor (Met) expression in these cells, implying that the HGF/Met signaling system may have a role in adipocyte differentiation. A summary of the positive and negative regulators of the HGF gene promoter that we have identified and characterized thus far is presented in Fig. 9.

Acknowledgments—We thank Dr. M.-J. Tsai for providing us with the COUP-TF1 expression vector and the antibody against COUP-TF. Dr. M. Rojkind for the Ito cell line, Dr. Xiao-Kan Zhang for the in vitro transcription/translation vector for COUP-TF, and Dr. B. Spiegelman for PARγ and RXRα expression vectors. We thank Dr. M. C. DeFranco for critical review of this manuscript. We also thank Dr. G. K. Michalopoulos for valuable discussions and continuous enthusiasm during the course of this work.

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