Microsomal epoxide hydrolase (mEH) plays a central role in xenobiotic metabolism as well as mediating the sodium-dependent uptake of bile acids into the liver, where these compounds regulate numerous biological processes such as cholesterol metabolism and hepatocyte signaling pathways. Little is known, however, about the factors that control the constitutive and inducible expression of the mEH gene (EPHX1) that is altered during development and in response to numerous xenobiotics. In previous studies we have established that GATA-4 binding to the EPHX1 core promoter is critical for EPHX1 expression. The $-80/+25$ bp core promoter also contained a reversed CCAAT box ($-5/1$ bp), integrity of which was required for maximal basal EPHX1 transcription in HepG2 cells. Transient transfection of CCAAT/enhancer-binding protein $\alpha$ (C/EBP$\alpha$) substantially stimulated EPHX1 promoter activity. Electrophoretic mobility shift assays, however, revealed that nuclear factor $Y$ (NF-Y), but not C/EBP$\alpha$, directly bound to this site although increased expression of NF-Y had no effect on EPHX1 promoter activity. These results suggested that C/EBP$\alpha$ activated EPHX1 expression through its interaction with NF-Y bound to the CCAAT box. The existence of a C/EBP$\alpha$(NF-Y) complex was supported by electrophoretic mobility shift assays using antibodies against NF-Y and C/EBP$\alpha$ as well as by the ability of a dominant-negative NF-Y expression vector to inhibit promoter activity. The interaction between these transcription factors was established by co-immunoprecipitation analysis and glutathione S-transferase pull-down assays, whereas the association of the two factors and the interaction of NF-Y with the CCAAT box in vivo was confirmed by chromatin immunoprecipitation assays. C/EBP$\alpha$-dependent EPHX1 activation was also supported by reconstitution studies in HeLa cells that lack this protein. These results establish that EPHX1 expression is regulated by C/EBP$\alpha$ interacting with DNA-bound NF-Y.

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The abbreviations used are: mEH, microsomal epoxide hydrolase; ChIP, chromatin immunoprecipitation; EPHX1, microsomal epoxide hydrolase gene; EMSA, electrophoretic mobility shift assay; hGH, human growth hormone; CTF, CCAAT transcription factor; WT, wild type; M, mutant; PBS, phosphate-buffered saline; NF-Y, nuclear factor Y; C/EBP$\alpha$, CCAAT/enhancer-binding protein $\alpha$; GST, glutathione S-transferase.
their DNA binding site as observed for USF-1/2 (23), SP1 (24), and NF-Y also interacts with components of the transcriptional machinery such as TFIID (29), as well as other coactivators such as PCAF (30), GCN5 (31), and p300/CBP (32) that play an important role in transcriptional regulation by altering chromatin structure.

Our initial observations demonstrated that EPHX1 core promoter activity was stimulated by C/EBPα despite the absence of a functional DNA binding site for this factor. C/EBPs have been shown to play critical roles in regulating the expression of numerous hepatocyte-specific genes (33). In this study we have demonstrated that, although NF-Y interacts with the EPHX1 promoter, its activity in vivo was unable to stimulate EPHX1 promoter activity. C/EBPα activation of EPHX1 promoter activity was, however, dependent on CCAAT box integrity. Together, these results demonstrate that transcription of the EPHX1 promoter is mediated by the interaction of C/EBPs with NF-Y bound to the CCAAT box element.

EXPERIMENTAL PROCEDURES

Plasmids—The −80/+25 EPHX1 core promoter fragment in the promoterless expression vector pOGH, which contains a human growth hormone (hGH) reporter gene (Nicholas Institute Diagnostics), was prepared as previously described (18). The construct containing a mutated CCAAT box was prepared by polymerase chain reaction using the following oligonucleotides as primers: 5′ primer, 5′-GGCGTTCTGGAAAAATGCATTCTTCGACCGC-3′; 3′ primer, 5′-ACAGACTCCATCTGTCGTTCAATG-3′, containing a HindIII site (underlined); 3′ primer, 5′-ACCGGTATCCATGGAAGATGAGG-3′, containing a BamHI site (underlined). The mutated nucleotides are in boldface type. The PCR products were digested with HindIII and BamHI and inserted into pOGH. The expression vectors for GST-fused NF-YA, NF-YB, and NF-YC in pGEX-4T were provided by Dr. Keiko Funa (Goteborg University, Goteborg, Sweden). The expression vector for full-length mammalian C/EBPα (pSEW-CO1) was provided by Dr. Gary S. Hayward (Johns Hopkins University School of Medicine, Baltimore, MD). The expression vectors for the GST-fused C/EBPα full-length (1–360), N-terminal (1–226), and C-terminal (281–360) portions in pGEX-4T-1 were obtained from Dr. Gretchen Darlington (Baylor College of Medicine, Houston, TX). The cDNA encoding the C/EBPα N-terminal portion (1–226) was isolated from the pGEX-4T-1 vector by BamHI/Xhol digestion and inserted in pcDNA1.1/Amp (Invitrogen) for expression in HepG2 cells. The C-terminal portion of the C/EBPα cDNA was also cloned into pcDNA1 so that the Ago codon at 219 would be used to translate the 219–360 sequence in HepG2 cells. The expression vector C/EBPβ (pcMV-CEBPβ) was provided by Dr. Magnus Nord (Karolinska Institute, Huddinge University Hospital, Huddinge, Sweden). The expression vector for CTF was provided by Dr. Richard Gronostajski (State University of New York, Buffalo, NY). The dominant negative NF-YA expression vector (NF-YAn29) was provided by Dr. Timothy Osborne (University of California, Irvine, CA).

Promoter Reporter Assay—HepG2 and HeLa cells were obtained from the American Type Culture Collection (ATCC) and grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The EPHX1 −80/+25 promoter constructs and transcription factor expression vectors or the NF-YA and C/EBPα and β clones were transiently transfected into HepG2 or HeLa cells using DEAE-dextran transfection methods as previously described (34). A pSV-β-galactosidase control vector (Promega, Madison, WI) was included as an internal control of transfection efficiency. The hGH produced was extracted into the medium and measured by radioimmunoassay (Nicholas Institute Diagnostics, Research Triangle Park, NC). The hGH produced was normalized to the activity of β-galactosidase after subtracting background values of the promoterless pOGH plasmid.

Electrophoresis Mobility Shift Assay (EMSA)—The oligonucleotide probes for the unmodified and mutated (M1–M7) EPHX1 −23/+15 fragment were synthesized, annealed, and radiolabeled with [32P]dATP in fill-in reactions with Klenow large fragment of DNA polymerase. The probes containing the consensus binding sites (Santa Cruz Biotechnology) for NF-Y, C/EBP, and CTF were also prepared with the following sense strands: 5′-AGACCGTACGTGTTGGAATCTCTT-3′ (NF-Y), 5′-TGGCAGTGGCAATCTGGCAG-3′ (C/EBP), and 5′-TGGTCAGGGAAAGATATGAA-3′ (CTF). Expression plasmids for NF-Y subunits A, B, and C (with XhoI sites) were linearized by XhoI and in vitro transcription/translation was carried out by using the T7 quick coupled transcription/translation system (Promega) (50 μl) according to the instructions from the manufacturer. To eliminate the nonspecific effects of the TnT system and the plasmids on C/EBPα DNA binding, the total amount of these two materials were identical in all experiments. Nuclear extracts were extracted from cultured HepG2 cells as previously described (35). A protease inhibitor mixture for mammalian tissues (Sigma) was also included in Buffers A and C during isolation steps at concentrations recommended by the manufacturer. HeLa nuclear extracts were purchased from Promega (Madison, WI). Poly(dI-dC)poly(dI-dC) (1.25 μg) was preincubated with 3–18 μg of nuclear proteins for 10 min at room temperature in a mix of 19 μl containing 20 nm Tris-HCl, pH 7.8, 1 μM MgCl2, 50 nm NaCl, 0.5 μl EDTA, 0.5 μM dithiothreitol, and 10% glycerol. 0.5–1 μl of radiolabeled oligonucleotides (approximately 5–10 fmol/5000–10,000 rpm) were added, and the incubation was continued for another 20 min at 4 °C. Where indicated, unlabeled (cold) oligo (1 μg) was included in the binding mixture prior to the addition of the radiolabeled probes. After mixing with 1 μl of loading buffer (250 mm Tris-HCl, pH 7.8, 0.2% bromophenol blue, 4% glycerol), the mixture was loaded on a 6% non-denaturing acrylamide (acylamide: bisacrylamide 39:1) mini gel (0.5–1.5% TBE buffer prerun at 50 V for 1 h). Gel electrophoresis was carried out at 150 V (approximately 10 V/cm) for 1.5 h. The bands were visualized by autoradiography at −75 °C with an intensifying screen.

Co-immunoprecipitation Assay—A HepG2 nuclear extract (35) (60 μg) was incubated with 40 μl of protein A-agarose beads and 8 μg of antibodies (1 mg/ml) for 2 h in 50 μl of 50 mm NaCl, 50 mm Tris-HCl, 0.5% Nonidet P-40, 1 mm EDTA, 1 mm dithiothreitol, and 0.5 mm phenylmethylsulfonfluor fluoride. As negative controls, the HepG2 nuclear proteins were also incubated with an anti-GATA-3 antibody and with protein A-Sepharose alone. The beads were extensively washed with the same buffer and the eluted proteins separated by SDS-PAGE. The proteins were then transferred to a nitrocellulose filter and Western-blotted with anti-C/EBPα antibody as well as anti-NF-YA antibody. The bands were visualized with alkaline phosphatase-conjugated secondary antibody. The Western analysis was performed with reagents from the WesternBreeze chromogenic Western blot immunodetection kit (Invitrogen) according to the recommendations from the manufacturer. AC/EBPα Antibody Immunoprecipitation—HepG2 cells were cross-linked for 10 min with 1% formaldehyde, incubated with 0.125 μg/ml (5 min), washed with cold PBS supplemented with protease inhibitor mixture (Sigma). Cells were swollen in hypotonic buffer (10 mm Hepes-KOH, pH 7.8, 10 mm KCl, 1.5 mm MgCl2), passed five times through a 26-gauge needle, and nuclei collected at 3000 rpm, suspended in lysis buffer (1% SDS, 50 mm Tris-HCl, pH 8.0, 10% EDTA), and sonicated to produce chromatin fragments with an average length of 500–1000 bp. The chromatin solution (0.01% SDS, 20 mm Tris-HCl, pH 8.0, 1.1% Triton X-100, 167 mm NaCl, 1.2 mm EDTA) was precleared with protein A/G-Sepharose beads (Santa Cruz), preblocked with salmon sperm DNA and bovine serum albumin for 1 h at 4 °C, and then incubated with 5 μl of NF-YA and C/EBPα antibodies for 1 h at 4 °C. The antibody–bead complexes were washed sequentially (5 min) at room temperature with (a) 0.1% SDS, 1% Triton X-100, 2 mm EDTA, 20 mm Tris-HCl, pH 8.0, 150 mm KCl; (b) 0.1% SDS, 1% Triton X-100, 2 mm EDTA, 20 mm Tris-HCl, pH 8.0, 500 mm NaCl; (c) 0.25 μl of LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mm EDTA, 10 mm Tris-HCl; and (d) 10 μg Tris-HCl, 0.5 μg ribonuclease. The complexes were eluted with 1% SDS, 0.1 μg NaIco3, and cross-links reversed by addition of NaCl to a final concentration of 0.3 μm and incubation at 65 °C. The DNA was extracted with phenol/chloroform (1:1) and precipitated with ethanol. PCR was performed with the following primers specific for the EPHX1 −104/−254 region, which includes the core promoter fragment and the exons 1 to 3: 5′-TTTCAGACCTCGTGATTTGCAAGGAGCCTTTGCTTTTCTTGAGGACGACCTGATCTATTCCAGC-3′; 3′ primer, 5′-TGGTCAGGGGTCCTGCGGACAGGATTTACAT-3′ and for the EPHX1 +2199/+2232 linked for 48 h at 37 °C. The DNA was amplified with the following primers specific for the EPHX1 −104/−254 region, which includes the core promoter fragment and the exons 1 to 3: 5′-TTTCAGACCTCGTGATTTGCAAGGAGCCTTTGCTTTTCTTGAGGACGACCTGATCTATTCCAGC-3′; 3′ primer, 5′-TGGTCAGGGGTCCTGCGGACAGGATTTACAT-3′.
ATCGGATCCAGCCAGCTCATGTCACGTAGTT-3'). For input, chromatin (10%) not incubated with antibodies was used for PCR. PCR products were resolved on an agarose gel and visualized with ethidium bromide.

**GST Pull-down Assays**—The expression vectors containing the cDNAs encoding full-length (1–360), N-terminal (1–226), and C-terminal (281–380) parts of C/EBPs or NF-YA, NF-YB, or NF-YC in pGEX-4T (Invitrogen) were transformed into BL21-Gold (DE3) cells (Stratagene), grown to $A_{600} = 0.6$, followed by the addition of isopropyl-$\beta$-1-thiogalactopyranoside to 1 mM and incubation for 2 h at 37 °C. The cells were collected, suspended in PBS, 0.5% Nonidet P-40, and protease inhibitor mixture (Sigma), lysed by sonication, and the cell debris removed by centrifugation at 14,000 rpm for 10 min. Glutathione-Sepharose 4B beads (50 µl) (Amersham Biosciences) was added to the supernatant and the tubes inverted for 1 h at room temperature, washed three times with PBS, and the associated GST fusion proteins quantitated by SDS-PAGE. The GST pull-down assay was performed with 1 µg of each fusion protein in PBS, 0.5% Nonidet P-40, 1 mM dithiothreitol, protease inhibitor mixture, and 200 µg of HepG2 nuclear protein extract in a total of 500 µl by inverting the tubes for 2 h at 4 °C. The beads were washed three times with PBS and the bead-associated proteins separated by SDS-PAGE and analyzed by Western blotting using anti-NF-YA and anti-NF-YB antibodies for the C/EBPα pull-down assay and anti-C/EBPα antibody for the NF-Y pull-down assay.

**RESULTS**

**EPHX1 Promoter Activity Is Dependent on the −5′/−1 bp CCAAT Box and Is Induced by C/EBPα Expression**—Previous studies concerning the regulation of EPHX1 expression by cis elements in the core promoter region have established that the −80/−25 bp fragment affords maximal basal promoter activity in HepG2 cells (18). In addition to two GATA sites that mediated the activation of EPHX1 transcription by GATA-4 (18), sequence analysis also revealed the presence of a reversed CCAAT box at −5′/−1 bp (Fig. 1A). To establish the functional role of this cis element in the transcriptional regulation of EPHX1, the CCAAT box was mutated to CTGAT. Transient transfection of the WT and M reporter constructs into HepG2 cells revealed a 50% loss of promoter activity, demonstrating that the CCAAT box element contributed to the core promoter activity (Fig. 1A). To establish which transcription factor interacts with this element, the −80/−25 bp pOGH-EPHX1 core promoter construct was cotransfected into HepG2 cells with the expression vectors for several putative CCAAT box-binding proteins: NF-Y, C/EBPα, C/EBPβ, and CTF. As shown in Fig. 1B, C/EBPα overexpression resulted in approximately a 64-fold increase in promoter activity, whereas expression of the other factors resulted in little or no effect on EPHX1 promoter activity.

**NF-Y Binds to the EPHX1 CCAAT Box and Interacts with C/EBPα**—To characterize the transcription factor that binds to the −5′/−1 CCAAT box, EMSA was performed with nuclear extracts from HepG2 cells and with radiolabeled consensus probes for NF-Y, C/EBPα, and CTF. As shown in Fig. 2B, all three probes formed DNA-protein complexes (lanes 1, 7, and 13), which could be inhibited by a 50-fold excess of unlabeled probe (WT). To establish which of these factors binds to the CCAAT box or adjacent regions in the −80/−25 bp EPHX1 promoter fragment, four overlapping DNA fragments (a–d) (Fig. 2A) were prepared and utilized as competitors in the EMSA analysis. Fragments a–d did not affect the C/EBPα complex (lanes 9–12), whereas fragment c did not affect the CTF complex (lane 15), establishing that these factors did not directly bind to the CCAAT box and, in addition, that C/EBPα did not bind to adjacent domains in the −80/−25 bp core promoter fragment. In contrast, fragment c (50-fold) was an effective competitor of the NF-Y-DNA complex, indicating that, as opposed to C/EBPα and CTF, NF-Y bound directly to the CCAAT box. However, increased expression of NF-Y had no stimulatory effect on EPHX1 promoter activity as shown in Fig. 1. These results suggest that activation of EPHX1 by C/EBPα may be mediated by its interaction with NF-Y bound to the CCAAT box. To further characterize this mechanism, EMSA was carried out with a radiolabeled EPHX1 –23/+15 bp probe, which contained the CCAAT box. As shown in Fig. 2C, incubation of the probe with a HepG2 nuclear extract produced a DNA-protein complex (lane 1) that was inhibited in the presence of the unlabeled –23/+15 fragment (WT) (lane 2), showing that the binding was specific. Competition with an NF-Y consensus oligonucleotide also blocked the formation of the complex (lane 3), further supporting the notion that NF-Y binds directly to the CCAAT element. Competition with the C/EBPα oligonucleotide, however, had little effect on the complex (lane 4), a result consistent with the conclusion that this factor did not directly bind to the EPHX1 core promoter. To further identify the components of this complex EMSA was carried out in the presence of antibodies against NF-Y, C/EBPα, C/EBPβ, and CTF. As shown in Fig. 2C, an anti-NF-Y antibody supershifted

![Graph A](http://example.com/graphA.png)

**A**

![Graph B](http://example.com/graphB.png)

**B**

**FIG. 1. Analysis of the role of the −5′/−1 CCAAT box on EPHX1 promoter activity by mutagenesis and effects of transcription factors on EPHX1 induction.** A, the effect of mutations in the CCAAT sequence on EPHX1 promoter activity. The WT and M –80/+25 DNA fragments of EPHX1 were connected to the hGH reporter gene in the promoterless expression vector pOGH and the constructs transiently transfected into HepG2 cells and hGH measured by radioimmunoassay 48 h after transfection. Values are the mean ± S.D. of independent experiments (n = 3–4) performed in triplicate. The CCAAT box is represented by a C box with the WT and M sequences in the sense strand shown below. G boxes indicate the location of GATA-4 binding sites. B, the effect of putative CCAAT box binding transcription factors on the induction of EPHX1 promoter activity. HepG2 cells were transfected with only the EPHX1 –80/+25 promoter construct (6 µg) (−) or with expression vectors (4 µg) for NF-Y, C/EBPα, C/EBPβ, CTF, and the promoter activity measured after 48 h as in A.
the whole complex (lane 5), confirming that NF-Y is a component of the complex. In contrast, an anti-C/EBPα antibody abolished the complex (lane 6), whereas antibodies against C/EBPβ and CTF had no effect (lanes 8 and 9), indicating that C/EBPα is also a component of the protein-DNA complex. The inhibitory effect of the anti-C/EBPα antibody may result from the antibody binding close to the NF-Y DNA binding domain or from an induced conformation change resulting in a decreased affinity for the DNA site. A mixture (1:1) of these antibodies resulted in a supershifted band of diminished intensity (lane 7), suggesting that the supershifted band also contained both NF-Y and C/EBPα. These results further support the idea that NF-Y and C/EBPα are components of a complex that activates EPHX1 transcription mediated by the interaction of NF-Y with the CCAAT element, where it serves as an anchor for C/EBPα.

To investigate whether the formation of the C/EBPα/NF-Y complex inhibits C/EBPα DNA binding, EMSA was carried out as shown in Fig. 2B with in vitro translated NF-Y subunits. As shown in Fig. 2D (lane 2), competition with NF-Y(A+B+C) inhibited binding to the C/EBPα oligonucleotide by 79%, indicating that excess NF-Y would compete for C/EBPα binding to DNA. This inhibitory effect was less in the presence of NF-YA (48%) or NF-YB (30%) alone and was not observed with NF-YC, suggesting that subunits A and B are directly involved in the formation of this complex.

**C/EBPα/NF-Y Complex Regulates EPHX1 Expression**

**FIG. 2. Identification of the HepG2 nuclear protein factors that are associated with the EPHX1 −39/+25 promoter region containing the −39/−1 CCAAT box and the four overlapping fragments (a–d) used as competitors in the EMSA analysis.** B, EMSA analysis was performed with a HepG2 nuclear protein extract and radiolabeled probes containing the consensus binding site for NF-Y (lanes 1–6), C/EBPα (lanes 7–12), and CTF (lanes 13–15) in the absence of competitors (−) (lanes 1, 2, and 13) and in the presence of a 50-fold excess of unlabeled oligonucleotide competitor, WT (lanes 2, 8, and 14), oligo a (lanes 3 and 9), oligo b (lanes 4 and 10), oligo c (lanes 5, 11, and 15), and oligo d (lanes 6 and 12). C, EMSA was performed with a radiolabeled EPHX1 −23/+15 oligonucleotide probe containing the CCAAT box, analyzed on 6% polyacrylamide gels, and visualized by autoradiography. Prior to the addition of the probe, the reaction mixture was incubated, when indicated, with unlabeled oligonucleotide competitors or antibodies for 2 h at 4 °C. Binding of the −23/+15 probe to nuclear protein (lane 1) in the presence of a 50-fold excess of unlabeled WT probe (lane 2), NF-Y oligonucleotide (lane 3), and C/EBPα oligonucleotide (lane 4). Binding of the −23/+15 probe in the presence of antibodies against NF-YA (lane 5), C/EBPα (lane 6), NF-YA + C/EBPα [1:1] (lane 7), C/EBPβ (lane 8), and CTF (lane 9). D, EMSA analysis was performed with a radiolabeled probe containing the consensus sequence for C/EBPα in the absence (−) (lane 1) and presence of equivalent amounts of in vitro synthesized NF-YA + B + C (1, 1, 1, each) (lane 2), NF-YA (lane 3), NF-YB (lane 4), and NF-YC (lane 5). Intensity of bands was quantitated with a VersaDoc Image System 1000.
C/EBPα[NF-Y] Complex Regulates EPHX1 Expression

Figure 3. C/EBPα induction of EPHX1 –80/+25 promoter activity. Figure shows the effect of NF-YA-m29, CCAAT box mutations, and requirements for the C/EBPα DNA binding and activation domains. HepG2 cells were transiently transfected with the –80/+25 EPHX1 core promoter construct in pOGH (6 µg) as a control (lane 1), and together with a dominant-negative DNA binding-defective NF-Y mutant (NF-YA-m29) (lane 2) as well as with increasing concentrations of the expression vector for C/EBPα (0.01–0.10 µg) (lanes 3–5) and the promoter activity measured after 48 h. The effect of the addition of the NF-YA-m29 dominant negative vector (lane 6) and mutating the –5/–1 CCAAT box (lane 7) on C/EBPα -fold induction was also measured. The effect of the addition of an expression vector for C/EBPα (1–226) containing the transactivation domains (lane 8) and C/EBPα (219–360) containing the DNA binding domain (lane 9) was also evaluated. Values are the mean ± S.D. of experiments (n = 3–4) performed in triplicate.

mutated EPHX1 –80/+25 promoter construct, where the -fold induction was now greatly reduced (lane 7). The residual activity may result from the induction of other genes, whose products may, in turn, affect EPHX1 activity. To investigate which domain of C/EBPα is involved in the induction of EPHX1 promoter activity, the N-terminal part (1–226), which contains the transactivation domains, and the C-terminal part (219–360), which contains the DNA binding domain were coexpressed with the EPHX1 –80/+25 core promoter in HepG2 cells. As shown in Fig. 3 (lanes 8 and 9), stimulation of EPHX1 was greatly reduced when compared with the full-length C/EBPα (1–360) probe (lane 5), suggesting that both domains of C/EBPα are required for the induction of EPHX1 activity.

C/EBPα Forms a Complex with CCAAT-bound NF-Y and Does Not Bind to Other Contiguous Regions of the –23/+15 bp Probe—Although the above experiments have indicated that C/EBPα interacts directly with CCAAT-bound NF-Y, the possibility cannot be excluded that it might also bind weakly to an adjacent DNA sequence similar to the CCAAT element, where this binding could be stabilized by NF-Y in a fashion similar to that observed for the weak binding of NF-Y to an imperfect site, which is stabilized by Sp1 binding to an adjacent site (36). To examine this possibility, the –5/+1 bp CCAAT box and the flanking sequences in the –23/+15 bp probe were systematically mutated (Fig. 4A) and the effects of these sequence substitutions on NF-Y and C/EBPα binding assessed by EMSA analysis as shown in Fig. 4 (B and C).

Utilizing the WT probe (lanes 8 and 9) resulted in the formation of a complex that contained both NF-Y (Fig. 4C) and C/EBPα (Fig. 4B), as also shown in Fig. 2C. The M4 probe in which the reverse CCAAT box had been mutated was the only probe that resulted in the loss of the DNA-protein complex (Fig. 4, B and C, lane 7), confirming that NF-Y bound to this site. All the other probes still formed the protein-DNA complex, which was sensitive to the anti-C/EBPα and anti-NF-YA antibodies, demonstrating that the complex still contained NF-Y and C/EBPα. The increased intensity of the bands obtained with M3 (Fig. 4, B and C, lane 5) most likely results from the substitutions that result in a superior NF-Y consensus sequence (20), whereas the lighter intensity observed with the M5 probe, which has no change in the NF-Y consensus sequence, may be attributable to the lower specific activity of the probe. If C/EBPα interacted with DNA sequences adjacent to the CCAAT box, one of the introduced mutations would have affected the formation of the complex. Thus, these results establish that C/EBPα only binds to NF-Y and not to adjacent DNA sites and eliminates the possibility that an additional factor binding to an adjacent sequence is necessary for the formation of the NF-Y/C/EBPα complex.

NF-Y Associates with C/EBPα in Vivo—The binding of C/EBPα to NF-Y in vivo was evaluated by immunoprecipitation of HepG2 nuclear extracts with an anti-NF-YA antibody. The precipitated proteins were separated by SDS-PAGE and immunoblotted with anti-C/EBPα and anti-NF-YA antibodies. As shown in Fig. 5, both transcription factors were detected in the anti-NF-YA antibody-immunoprecipitated proteins (lanes 1 and 6). These proteins were also detected in total nuclear extracts (lanes 4 and 5). The ability of anti-NF-YA antibody to immunoprecipitate both NF-Y (lane 6) and C/EBPα (lane 1) demonstrates the physical association of these two factors.

C/EBPα was not detected when protein A-Sepharose alone (lane 2) or an irrelevant antibody (anti-GATA-3) (lane 3) was used.

Interaction of NF-Y and C/EBPα with the EPHX1 –5/+1 bp CCAAT Box in Vivo—ChIP procedures were utilized to establish that NF-Y interacts with the EPHX1 core promoter CCAAT box and forms a complex with C/EBPα on the endogenous EPHX1 promoter. Cross-linked chromatin from HepG2 cells was immunoprecipitated with an anti-NF-YA antibody. The presence of the EPHX1 promoter fragment was established by PCR using primers from –104 to +395 bp. As negative controls, immunoprecipitations were run without antibody or with pre-immune IgG. PCR amplification was also performed with primers (+21,998 to 22,324 bp) that bind to the EPHX1 exon 9 region that does not have a CCAAT box. As shown in Fig. 6, anti-NF-YA antibody immunoprecipitated chromatin that contained the EPHX1 core promoter (Fig. 6A, lane 4). The specificity of this interaction was verified by demonstrating the absence of amplification in the EPHX1 exon 9 region (Fig. 6B, lane 4) or when no antibody (lane 2) or pre-immune IgG (lane 3) were used. Immunoprecipitation of cross-linked chromatin with an anti-C/EBPα antibody also resulted in the formation of an EPHX1 promoter fragment (Fig. 6A, lane 5) that was not observed in the EPHX1 exon 9 region (Fig. 6B, lane 5). These results establish that NF-Y binds the EPHX1 core promoter CCAAT in vivo and forms a complex with C/EBPα associated with the endogenous promoter.

Characterization of the C/EBPα Domains and NF-Y Subunits Involved in the EPHX1 CCAAT Box Complex—To further characterize the NF-Y subunit(s) that interact with C/EBPα, GST-fused subunits of NF-Y were utilized in a GST pull-down assay. As shown in Fig. 7A, only the NF-YB fusion protein (lane 3) was
able to interact with C/EBPα, a result that was supported by the EMSA competition study (Fig. 2D, lane 4). The NF-YA fusion protein, however, was not able to interact with C/EBPα (lane 2), in contrast to the co-immunoprecipitation (Fig. 5) and EMSA competition (Fig. 2D, lane 3) studies, suggesting that the portion of NF-YA involved in the interaction with C/EBPα may be too close to the GST moiety and the interaction is inhibited by steric hindrance. To characterize the C/EBPα domain that interacts with NF-Y, a GST-fused full-length (1–360), a N-terminal portion containing the activation domains (1–226), and a C-terminal portion containing the DNA binding domain (281–360) were utilized in a GST pull-down assay. As shown in Fig. 7B, full-length GST-C/EBPα (lane 2) as well as the C-terminal region (lane 4) interacted with NF-YA in a HepG2 nuclear extract as detected by anti-NF-YA antibody. In contrast, no interaction was observed with the N-terminal region (lane 3) or GST alone (lane 1), thereby establishing that the DNA-binding region of C/EBPα interacted with NF-Y in this complex. Analysis of the pull-down assays with anti-NF-YB antibody, however, failed to detect any proteins interacting with the C/EBPα probes, suggesting that the portion of C/EBPα involved in the interaction with NF-YB may be too close to the GST moiety as described above for GST-NF-YA interaction with anti-C/EBPα antibody. These results demonstrate the importance of using complementary methods in the study of protein-protein interactions.
linked protein–DNA complexes were incubated with anti-NF-YA or anti-C/EBPα antibodies and isolated by immunoprecipitation with protein A-Sepharose beads as described under “Experimental Procedures.” DNA corresponding to the EPHX1 core promoter region (A) and exon 9 region, which does not have a CCAAT box or a C/EBPα binding site (B), was analyzed by PCR with the indicated primers. Figure shows NF-YA GST fusion proteins for NF-YA, NF-YB, NF-YC, and full-length C/EBPα demonstrated abundant NF-Y binding, similar to that observed in HepG2 cells (Fig. 8, lanes 1 and 2); however, binding to a consensus C/EBPα probe was negligible compared with the levels in HepG2 cells (lanes 3 and 4). Commensurate with this observation was the observed absence of promoter activity when the EPHX1 −80/+25 WT constructs in pOGH and basal activity measured by hGH release as in Fig. 1. HeLa cells were also cotransfected with the expression vector for C/EBPα (0.1 and 0.2 μg).

Reconstitution of EPHX1 Promoter Activity in HeLa Cells

Reconstitution of EPHX1 promoter activity in HeLa cells. A, EMSA analysis of NF-Y (lanes 1 and 2) and C/EBPα (lanes 3 and 4) in nuclear extracts from HepG2 and HeLa cells using radiolabeled oligonucleotides containing the consensus binding site for the respective transcription factors. B, HepG2 and HeLa cells were transiently transfected with the EPHX1 −80/+25 WT constructs in pOGH and basal activity measured by hGH release as in Fig. 1. HeLa cells were also cotransfected with the expression vector for C/EBPα (0.1 and 0.2 μg).

**FIG. 6.** NF-Y binds to the EPHX1 promoter and forms a complex with C/EBPα in vivo as assessed by ChIP analysis. Cross-linked protein–DNA complexes were incubated with anti-NF-YA or anti-C/EBPα antibodies and isolated by immunoprecipitation with protein A-Sepharose beads as described under “Experimental Procedures.” DNA corresponding to the EPHX1 core promoter region (A) and exon 9 region, which does not have a CCAAT box or a C/EBPα binding site (B), was analyzed by PCR with the indicated primers. Figure shown NF-YA (lane A4) and C/EBPα (lane A5) binding to the EPHX1 core promoter region. Positive control is chromatin prior to immunoprecipitation (input) (lanes A1 and B1). Negative controls were immunoprecipitated (a) without antibody (lanes A2 and B2), (b) with rabbit pre-immune IgG (lanes A3 and B3), and (c) with anti-NF-YA (lane B4) and anti-C/EBPα (lane B5) antibodies interacting with the EPHX1 exon 9 region.

**FIG. 7.** The DNA binding domain of C/EBPα interacts with NF-Y. GST fusion proteins for NF-YA, NF-YB, NF-YC, and full-length C/EBPα (1–360), C/EBPα(1–226) containing the activation domains, and C/EBPα (281–360) containing the DNA binding domain bound to glutathione-Sepharose 4B beads were incubated with HepG2 nuclear protein extract (200 μg) and the associated proteins separated by SDS-PAGE and analyzed by Western blotting. A, GST pull-down assay analyzed with anti-C/EBPα antibody (α-C/EBPα). Lane 1, GST alone; lane 2, GST-NF-YA; lane 3, GST-NF-YB; lane 4, GST-NF-YC. B, GST pull-down assay analyzed with α-NF-YA. Lane 1, GST alone; lane 2, GST-C/EBPα(1–360); lane 3, GST-C/EBPα(1–226); lane 4, GST-C/EBPα(281–360).

**FIG. 8.** Reconstitution of C/EBPα-inducible EPHX1 promoter activity in HeLa cells. A, EMSA analysis of NF-Y (lanes 1 and 2) and C/EBPα (lanes 3 and 4) in nuclear extracts from HepG2 and HeLa cells using radiolabeled oligonucleotides containing the consensus binding site for the respective transcription factors. B, HepG2 and HeLa cells were transiently transfected with the EPHX1 −80/+25 WT constructs in pOGH and basal activity measured by hGH release as in Fig. 1. HeLa cells were also cotransfected with the expression vector for C/EBPα (0.1 and 0.2 μg).

**DISCUSSION**

In this report we have demonstrated that EPHX1 expression in HepG2 cells is regulated, in part, by a complex composed of C/EBPα and NF-Y bound to a CCAAT box in the EPHX1 core promoter. Results obtained with EMSA in vitro (Figs. 2 and 4), with chromatin immunoprecipitation assays in vivo (Fig. 6), and with transient transfection assays utilizing a NF-YA dominant negative vector (NF-YAm29) (Fig. 3) established that NF-Y bound directly to the CCAAT box but was not sufficient to stimulate EPHX1 promoter activity (Figs. 1B and 8). In contrast, increased expression of C/EBPα, which did not bind directly to the −23/+15 bp oligonucleotide containing the CCAAT box (Fig. 2, B (lane 11) and C (lane 4)), was shown to
stimulate EPHX1 promoter activity in two different cell lines (Figs. 1B and 5B) and to physically interact with NF-Y by co-immunoprecipitation (Fig. 5), ChiP assay (Fig. 6), and GST pull-down assays (Fig. 7). Furthermore, EMSA analyses also supported the notion of a complex formed between these two factors (Figs. 2C and 4). The co-immunoprecipitation of C/EBPs by an anti-NF-Y antibody (Fig. 5) and GST pull-down studies (Fig. 7) demonstrate that C/EBPs can interact with NF-YA and NF-YB subunits. The inability of the activation domains of NF-Y to induce EPHX1 activity when bound to the −5/−1 bp CCAAT box may result from an unfavorable juxtaposition to the transcription initiation complex. The binding of C/EBPs to NF-Y bound to the CCAAT box site, however, is able to activate EPHX1 expression, possibly mediated by the activation domains of C/EBPs, a conclusion based on the requirement of both the activation and DNA binding domains in a functional assay (Fig. 3, lanes 8 and 9) and the observed interaction of the C/EBPa DNA binding domain with NF-Y (Fig. 7B).

The undetectable level of EPHX1 activity and C/EBPs in HeLa cells, which express NF-Y at levels similar to those found in HepG2 cells, also supports the conclusion that C/EBPs is required for EPHX1 promoter activity (Fig. 7). Although expression of C/EBPs in HeLa cells resulted in the stimulation of EPHX1 promoter activity, it was at greatly reduced level from that observed in HepG2 cells, suggesting that additional transcription factors such as GATA-4, which is also not expressed in HepG2 cells (18), are required for a more robust level of expression. Two GATA-4 binding sites in the core promoter are involved in EPHX1 expression (18); however, there is no evidence for an interaction with the CCAAT box described in this report (data not shown). In addition, the EPHX1 core promoter also contains a putative binding site for Nrf2 on the antioxidant response element (ARE) that, based on preliminary evidence, may be involved in the inducible expression of EPHX1 by xenobiotics such as oltipraz.

Although NF-Y has been shown to physically and functionally interact with several transcription factors that are also bound to DNA (23–28), this study appears to be the first report of C/EBPs stimulating promoter activity, not through its DNA binding capacity, but through its interaction with NF-Y bound to the EPHX1 CCAAT box. Although NF-Y and C/EBPs appear to be the components in this complex, we cannot exclude the possibility that other protein(s) may be associated with the C/EBPs/NF-Y complex to promote transcription. Several other genes have been shown to be regulated by transcription factors or coactivators that interact with NF-Y bound to the CCAAT box. The heat shock proteins HSP70 and HSP40 are induced by the coactivator HSP-CBF, which binds to the HSP promoters by forming a complex with CCAAT-bound NF-Y (37). This type of functional complex is similar to those observed for other coactivators that interact with NF-Y such as P/CAP (29) and GCN5 (31). These factors possess histone acetyltransferase activity that affects local chromatin structure (31). The transcriptional regulation of the MDR1 gene by histone acetyltransferase and deacetylase is also mediated by NF-Y (38). The cell cycle-dependent expression of the HSP70 gene has also been shown to be dependent on CCAAT-bound NF-Y where the level of HSP70 expression is regulated by the intracellular concentration of the transcription factor, c-Myc that interacts with NF-Y but not with DNA (39). c-Myc has also been shown to play a critical role in the cell cycle-dependent expression of platelet-derived growth factor β-receptor mRNA (40) by also forming a complex with CCAAT-bound NF-Y. A similar model was proposed for the action of p73 that also represses platelet-derived growth factor β-receptor (41) and the p53 tumor suppressor protein that decreases the surface of CCAAT-bound NF-Y. The surface of CCAAT-bound NF-Y thus permits the subsequent binding of a variety of factors that, in concert with NF-Y, regulate the promoter activity of numerous genes. The observed binding of C/EBPs to NF-Y in this study may inhibit C/EBPa DNA binding activity as suggested in Fig. 2D that could result in the negative regulation of C/EBPs target genes. C/EBPs also forms complexes with DNA-bound factors in addition to NF-Y, as described in liver, where an age-specific C/EBPa-Rb-Brn-E2F4 complex binds to an E2F-dependent (c-Myc) promoter resulting in gene repression and the loss of a proliferative response (43). C/EBPa also autoregulates the human C/EBPs gene by its interaction with DNA-bound USF (44).

C/EBPa, which is an important regulator of hepatocyte proliferation and a variety of liver functions (33), is first expressed at approximately day 16 during rat fetal development when many of these processes are initiated (45) and increases in concentration during the first 2.5 months (46). In previous studies we have also established that rat mEH is first expressed in late fetal development and reaches adult levels in ~2 months (8). The inductive effect of C/EBPa on EPHX1 expression suggests that this factor may play a significant role in regulating the expression of EPHX1 during development. This association is further supported by the observation that C/EBPa mRNA expression is transiently decreased by more than 80% within 24 h after partial hepatectomy (47, 48). A similar response is also observed for the expression of mEH activity during liver regeneration (49).

In conclusion, these studies have demonstrated that C/EBPa plays a critical role in establishing the constitutive expression of hepatic EPHX1 whose gene product (mEH) is involved in carcinogen metabolism and in sodium-dependent transport of bile acids. Furthermore, the results support a model where C/EBPa regulates EPHX1 transcription not by binding to DNA, but through its interaction with NF-Y bound to the −5/−1 bp CCAAT box in the EPHX1 core promoter.

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CCAAT/Enhancer-binding Protein α (C/EBPα) Activates Transcription of the Human Microsomal Epoxide Hydrolase Gene (EPHX1) through the Interaction with DNA-bound NF-Y
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