The placental form of glutathione S-transferase (GST-P), an isozyme of glutathione S-transferase, is not expressed in normal liver but is highly induced at an early stage of chemical hepatocarcinogenesis and in hepatomas. Recently, we reported that the NF-E2 p45-related factor 2 (Nrf2)/MafK heterodimer binds to GST-P enhancer 1 (GPE1), a strong enhancer of the GST-P gene, and activates this gene in preneoplastic lesions and hepatomas. In addition to the positive regulation during hepatocarcinogenesis, negative regulatory mechanisms might work to repress GST-P in normal liver, but this remains to be clarified. In this work, we identify the CCAAT enhancer-binding protein α (C/EBPα) as a negative regulator that binds to GPE1 and suppresses GST-P expression in normal liver. C/EBPα binds to part of the GPE1 sequence, and the binding of Nrf2/MafK and C/EBPα to GPE1 is mutually exclusive. In a transient-transfection analysis, C/EBPα activated GPE1 in F9 embryonal carcinoma cells but strongly inhibited GPE1 activity in hepatoma cells. The expression of C/EBPα was specifically suppressed in GST-P-positive preneoplastic foci in the livers of carcinogen-treated rats. A chromatin immunoprecipitation analysis showed that C/EBPα bound to GPE1 in the normal liver in vivo but did not bind in preneoplastic hepatocytes. Introduction of the C/EBPα gene fused with the estrogen receptor ligand-binding domain into hepatoma cells, and subsequent activation by β-estradiol led to the suppression of endogenous GST-P expression. These results indicate that C/EBPα is a negative regulator of GST-P gene expression in normal liver.
of this factor with GPE1 in vitro and in vivo and used transient transfection analysis of hepatoma and F9 cells to examine the effect of C/EBPa on GPE1. We also have analyzed endogenous GST-P expression in C/EBPa overexpressing hepatoma cells. From these analyses, we conclude that C/EBPa suppresses the expression of GST-P in the normal liver.

EXPERIMENTAL PROCEDURES

Construction of Plasmids—To construct the luciferase reporter genes, the −2.9-kb/37-bp fragment relative to the cap site of the rat GST-P gene (GST/ELuc) or the −50/37-bp fragment, the minimal promoter region containing the GC and TATA boxes (∆50Luc)(9) was inserted into a promoter-less luciferase plasmid vector, pGVB2 (Nippon Gene, Toyama, Japan). The constructs containing internal deletions or synthetic oligonucleotide elements were made by inserting the following sequences into ∆50Luc: a 0.3-kb SacI-AccI fragment (−2.5/−2.2-kb) containing GPE1 (SA/∆50Loc), GPE1 (5′-GTAGTCAGTCACTATGAA-TTCAGCAACA-3′, GPE1/∆50Luc), or mutated forms of GPE1 (M11/∆50Luc: 5′-GTAGTCAGTCACTATGAA-TTCAGCAACA-3′; M12/∆50Luc: 5′-GTAGTCAGTCACTATGAGTCAGCAACA-3′; and M13/∆50Luc: 5′-GTAGTCAGTCACTATGAGTCAGCAACA-3′; with the mutated positions indicated by lowercase letters). Three copies of the C/EBP-binding consensus sequence (C/EBP-RE) from the mouse transthyretin gene enhancer (5′-TTTCCATCTTACACATCATCTA-3′) were joined in tandem and cloned into ∆50Luc (C/EBP-RE/∆50Luc) (25). The C/EBPa and C/EBPβ expression plasmids were kindly provided by Dr. Takiguchi (University of Chiba). The Nrf2 expression vector was described previously (15).

Cell Culture, Reporter Transfection Analysis, and Retroviral Transduction—H4IIIE and RL34 rat hepatoma and F9 mouse embryonal carcinoma cell lines were maintained in Dulbecco’s modified minimal essential medium (Nissui, Tokyo) with 8% (v/v) fetal bovine serum. For carcinomacell lines were maintained in Dulbecco’s modified minimal essential medium (Nissui, Tokyo) containing 10% fetal bovine serum. For transfection analysis, the cells were plated at a density of 5 × 10³ cells (H4IIIE and RL34) or 2 × 10³ cells (F9)/60-mm plate. A total of 4 μg DNA was transfected into the cells, including 1 μg of the reporter plasmid and 0.5 μg of the β-galactosidase expression plasmid (pSVβgal; Promega, Madison WI) with or without an effector-gene expression plasmid (C/EBPα, C/EBPβ, or Nrf2) and pUC18 DNA, according to the procedure of Chen and Okayama (26). The cells were harvested 45 h after transfection and assayed for luciferase activity using a luciferase assay kit (Nippon Gene) and for β-galactosidase activity (27). Luciferase activities were normalized to β-galactosidase activities. All of the experiments were performed at least twice.

The retroviral construct of C/EBPa fused with the estrogen receptor ligand-binding domain (pBabePuro-C/EBPa-ER) (23, 28) was kindly provided by Dr. Alan D. Friedman (Johns Hopkins University). The BOSC23 packaging cells (29) were cultured in Dulbecco’s modified minimal essential medium with 10% fetal bovine serum and were transfected with pBabePuro-C/EBPa-ER by the calcium phosphate method. The supernatant was collected after 40 h and infected into RL34 hepatoma cells with 4 μg/ml polybrene. Stable transformants were cloned after 2 weeks of selection with 3 μg/ml puromycin. C/EBPa-ER-expressing cells and parent RL34 cells were cultured in phenol red-free minimal essential medium (Nissui, Tokyo) containing 10% fetal bovine serum. C/EBPa-ER was activated by the addition of β-estradiol (1 μM; Sigma) and harvested after 48 h for RNA and protein analyses.

Animals and Chemical Hepatocarcinogenesis—Rats with hyperplastic nodules induced by the Solt-Farber protocol (30, 31) were kindly provided by Dr. K. Satoh (Hirosaki University, Hirosaki, Japan). All of the animal experiments were conducted according to the Guidelines for Animal Experiments of Hokkaido University.

EMSA and DNase I Footprinting Analysis—The cDNA fragment encoding the DNA-binding domain of each transcription factor (amino acids 318–598 for Nrf2, 1–156 for MafK, and 282–396 for C/EBPa) was fused to the Escherichia coli maltose-binding protein (MBP) gene of the pMAL-c2 vector (New England Biolabs). These fusion proteins were expressed in E. coli cells and purified as previously described (15). To prepare probes for the EMSA, synthetic double-stranded DNA elements were cloned into a pBluescript II vector (Stratagene) and fragmented with BamHI (GPE1) or BamHI and Sall (C/EBPβ, mutant GPE1). The overhanging ends were labeled with Klenow DNA polymerase I (Takara, Kyoto) and [α-32P]dCTP. A fragment of the multiple cloning site of the pBluescript II vector (HindIII-XbaI) was labeled as a negative control probe. EMSA was performed as described previously (32). The recombinant MBP-fused proteins were preincubated with 0.5 μg of poly(dI-dC) (Amersham Biosciences) for 10 min in 10 μl of binding buffer (20 mM HEPES, pH 7.9, 20 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 4 mM MgCl₂, 15% glycerol, and 100 μg/ml bovine serum albumin. A 32P-labeled DNA probe (2 × 10⁶ cpm) was added, and the mixture was incubated for another 10 min. Protein-DNA complexes were analyzed by 4% (w/v) polyacrylamide gel electrophoresis in 25 mM Tris borate, 0.5 mM EDTA, pH 8.2.

Nuclear extracts were prepared as described by Dignam et al. (33). The cells were suspended in 5 packed cell volumes of buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol), incubated on ice for 10 min, and briefly centrifuged. The cell pellet was resuspended in 2 packed cell volumes of buffer A with 1 mM phenylmethylsulfonyl fluoride (PMSF), homogenized, and centrifuged. The nuclear pellet was suspended in 1 volume of buffer B (20 mM HEPES, pH 7.9, 25% (v/v) glycerol, 0.42 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF) and gently stirred at 4°C for 30 min. After centrifugation at 35,000 rpm for 30 min, the supernatant was dialyzed against buffer C (20 mM HEPES, pH 7.9, 20% (v/v) glycerol, 0.1 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM PMSF) for 5 h. The nuclear extracts (10 μg/reaction) were incubated with the indicated antibody at 15°C for 30 min prior to EMSA using the same procedures as for recombinant proteins, except that 2.5 μg of poly(dI-dC) was used.

For the DNase I footprinting analysis, the probe (2 × 10⁶ cpm) was incubated with bovine serum albumin or recombinant protein (0.1 μg) in 50 μl of EMSA binding buffer. Fifty μl of DNase I buffer (5 mM MgCl₂, 5 mM CaCl₂, 200 mM KCl, and 4 μg/ml sheared salmon sperm DNA) and 20 ng of DNase I (Takara) were mixed and incubated for 3 min at room temperature. The reaction was terminated by the addition of 100 μl of stop solution (20 mM EDTA, 1% SDS, and 0.2 mM NaCl). DNA was extracted and separated in a 6% (w/v) polyacrylamide gel containing 8 M urea. Guanine and adenine bases of the same probes were modified, digested by the Maxam–Gilbert method, and loaded as a marker ladder.

RNA and Protein Analyses—Total cellular RNAs were isolated from rat tissues and cultured cells using an RNA extraction kit (Isogen, Nippon Gene). Northern blot analysis was performed as described previously (34). The fragments of the C/EBPa, C/EBPβ, and GST-P cDNAs were labeled with a random-primed DNA labeling kit (Takara) using [α-32P]dCTP and used as probes. RNase protection analysis was performed as described previously (34). A DNA fragment of the GST-P gene (~109 to +56-bp, relative to the cap site) was subcloned into pBluescript II and antisense RNA was synthesized by T7 RNA polymerase with [α-32P]UTP for the probe.

For Western blot analysis, the cells were lysed with lysis buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM...
C/EBPα Suppresses the Rat GST-P Gene

PMSF). The proteins were separated by SDS-polyacrylamide gel electrophoresis and blotted on a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was incubated with anti-C/EBPα (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-GST-P antibody (kindly provided from Dr. K. Satoh, Hiroasaki University). After washing, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences), and the immunoreactive bands were detected using an ECL system (Amersham Biosciences). Band intensities in the autoradiograms of Northern and Western blotting were estimated by NIH Image.

Immunohistochemistry—Eight weeks after the Solt-Farber protocol was started, the rats were fixed by an intracardiac perfusion of 4% paraformaldehyde in phosphate-buffered saline. The liver tissues were embedded in paraffin and sectioned at 3 µm, and sections were processed for immunofluorescence staining. The slides were incubated with normal goat serum and then with the anti-C/EBPα antibody. Binding of the primary antibody was localized using a Cy-3-conjugated (GST-P) or FITC-conjugated (C/EBPα) goat anti-rabbit IgG (dilution, 1:200; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The slides were examined using laser scanning confocal microscopy (MRC-1024, Bio-Rad; and LSM 510, Carl Zeiss, Oberkochen, Germany).

Chromatin Immunoprecipitation Analysis—Chromatin immunoprecipitation analysis was carried out as described previously (15, 35). Briefly, the cells were fixed in 1% formaldehyde and washed with phosphate-buffered saline containing protease inhibitors (1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml pepstatin). Harvested cells were sonicated in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) with an ultrasonic transducer (Tomy Seiko, Tokyo, Japan) and centrifuged. The supernatant was diluted 10-fold with immunoprecipitation buffer (0.1% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl) and precleared with 30 µl of protein A-Sepharose (50% protein A slurry (Amersham Biosciences) containing 20 µg/ml sheared salmon sperm DNA and 1 µg/ml bovine serum albumin). The precleared chromatin fraction was incubated with anti-C/EBPα, anti-C/EBPβ (Santa Cruz Biotechnology), anti-Nrf2 (15), or anti-MafK (kindly provided by Dr. K. Igarashi, Hiroshima University) antibody or preimmune serum for 16 h at 4°C. Immune complexes were mixed with 50 µl of protein A-Sepharose and washed sequentially with wash buffer 1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1) containing 150 mM NaCl, wash buffer 2 (0.25% LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.1), and twice with 10 mM Tris-HCl, pH 8.1, 1 mM EDTA. The complexes were eluted in 1% SDS, 0.1 M NaHCO₃, and cross-linking was reversed by heating to 65°C for 4 h. The DNAs were purified, and the GPE1 region of the GST-P gene was amplified by PCR with the primers 5’-TAGTCTGCACTATGATTGCAGCAAC-3’ and 5’-CCAGCTTCTCGGACAAACC-3’. Amplified DNAs were analyzed using 3% agarose gel electrophoresis.

RESULTS

C/EBPα Suppresses GPE1 Enhancer Activity—We found that the 3’-half of GPE1 (5’-TAGTCTGCACTATGATTGCAGCAAC-3’), the C/EBP-binding consensus sequence is underlined) almost completely matches the consensus binding sequence of C/EBP (5’-RTTGGC-GYAAY-3’) (36). Further, C/EBPα is markedly down-regulated in preneoplastic foci of the liver, in which the GST-P gene is strongly induced (24). Therefore, we used transient-transfection analysis to examine whether C/EBP influences GST-P expression. Luciferase reporter constructs containing the –2.9-kb/+37-bp region of the GST-P gene (GST/ELuc), and the GPE1 enhancer region (–2.5/–2.2-kb, SA/Δ50Luc) were transfected into cells of the H4IIE rat hepatoma line, with or without a C/EBPα or C/EBPβ expression vector. Fig. 1A shows that both GST/ELuc and SA/Δ50Luc were highly expressed in H4IIE cells but that the expression of both constructs was strongly inhibited by co-transfection with C/EBPα. C/EBPβ also inhibited transcriptional activity, but to a lesser extent than C/EBPα. C/EBP-RE/Δ50Luc, which contained three tandem C/EBP binding consensus sequences from the transthyretin gene, was activated by both C/EBPα and C/EBPβ.

Previously, we reported that Nrf2/MafK binds to GPE1 and activates GST-P expression during hepatocarcinogenesis. To determine whether C/EBP inhibits the ability of Nrf2/MafK to activate GPE1, we performed a transient-transfection analysis. The GPE1 core element was fused to a luciferase gene with the minimal promoter sequence of the rat GST-P gene (GPE1/Δ50Luc) and transfected into H4IIE cells, with or without co-transfection with the Nrf2, C/EBPα, or C/EBPβ expression vectors (Fig. 1B). In hepatoma cells, Nrf2 stimulated GPE1/Δ50Luc gene expression 30- to 50-fold, as described previously (15), and C/EBPα dramatically down-regulated GPE1 activity. C/EBPβ also suppressed GPE1, but by approximately only one-fifth as much as C/EBPα. The expression of Δ50Luc, which contained only the minimal promoter, did not significantly change with co-transfection by the effector plasmids.

To address why GPE1 was suppressed by C/EBP, we performed a reporter transfection analysis using mouse F9 embryonal carcinoma cells, which do not contain any GPE1-stimulating activity (15). In contrast to the results with hepatoma cells, in F9 cells, C/EBPα stimulated GPE1/Δ50Luc by ~10-fold in a dose-dependent manner (Fig. 1C). However, the stimulation of GPE1/Δ50Luc by Nrf2 was much higher (~300-fold) than that by C/EBPα. The activation of Nrf2 was strongly inhibited by the co-transfection of C/EBPα. These results suggest that C/EBPα interacts with GPE1 and inhibits the binding of the strong positive factor, Nrf2/MafK, in hepatoma cells. C/EBP-RE/Δ50Luc was greatly stimulated by C/EBPα in F9 cells and was not affected by Nrf2.

C/EBPα Binds to GPE1—The nucleotide sequence of GPE1 and the results of the reporter transfection analysis described above suggest that C/EBPα binds directly to GPE1. To test whether GPE1 and C/EBPα interact directly, we performed EMSA using recombinant C/EBPα proteins expressed in E. coli and a GPE1 probe. The multiple cloning site of the pBluescript II vector and the C/EBP-binding consensus sequence (C/EBP-RE) were used as negative and positive control probes, respectively. C/EBPα bound strongly to GPE1 (Fig. 2A, lane 8). The probe with a single C/EBP-RE sequence yielded one shifted band, and the probe with two C/EBP-RE sequences yielded double bands, as expected (Fig. 2A, lanes 4 and 6). The binding specificity was confirmed by a competition analysis with an excess amount of unlabeled GPE1 probe (Fig. 2A, lanes 11–13).

To identify the C/EBPα-binding site in the GPE1 sequence, we performed a DNase I footprinting analysis. The Nrf2/MafK heterodimer completely protected the entire GPE1 core sequence from DNase I digestion (Fig. 2B), as described previously (15). C/EBPα clearly protected the 3’-half of GPE1, the region containing the C/EBP-binding consensus sequence.

The reporter transfection analysis described above suggested that C/EBPα binding interfered with Nrf2/MafK activity on GPE1. We investigated whether these factors bind to GPE1 simultaneously or exclusively, using an EMSA with a fixed amount of C/EBPα and increasing amounts of Nrf2/MafK and vice versa (Fig. 2C). C/EBPα was displaced from GPE1 by increasing amounts of Nrf2/MafK (Fig. 2C, lanes 1–5) and increasing amounts of C/EBPα prevented Nrf2/MafK binding (Fig. 2C, lanes 6–10), indicating that these factors bound to GPE1 in a
mutually exclusive manner. However, the binding reactions of the mixed factors and GPE1 were not exactly stoichiometric, because the intensities of the shifted bands did not increase with increasing amounts of the competing factors (Fig. 2C, lanes 4, 5, 9, and 10). During the course of these experiments, we found that MafK interacts with C/EBP/H9251 and inhibits its binding to GPE1 (data not shown). The MafK homodimer also binds weakly to GPE1 (15). These complicated interactions may explain the nonstoichiometric binding to GPE1.

To further clarify the interaction of C/EBP/H9251 with GPE1 and its activity on the enhancer, we used mutated forms of GPE1. We attempted to mutate GPE1 to disrupt C/EBP binding without affecting Nrf2/MafK binding. Based on a previous report (36), the T and A residues near the ends of the C/EBP-binding consensus sequence (5’-RRTTGCGYAAY-3’) are essential for specific binding by C/EBPα. We therefore prepared three point mutants (GPE1-M11, -M12, and -M13) as shown in Fig. 3C. The binding of C/EBPα to these mutant sequences was determined by EMSA. Fig. 3A shows that the binding of C/EBPα to the GPE1-M11 mutant was almost abolished, although a faint band remained. The binding of C/EBPα to the GPE1-M12 and -M13 mutants was reduced, but significant binding activity remained. To determine the activities of these enhancers, the mutant sequences were joined to Δ50/Luc and analyzed by reporter transfection analysis in H4IIE cells. With wild-type GPE1, C/EBPα inhibited luciferase expression by ~90%. With GPE1-M11, -M12, and -M13, C/EBPα inhibited luciferase expression by ~60, 75, and 88%, respectively (Fig. 3B). The ability of C/EBPα to inhibit the activity of the mutant GPE1s correlated with its binding affinities for the mutants, suggesting that DNA binding is necessary, at least in part, for the inhibition of GPE1. However, the GPE1-M11 mutant, which showed very little binding activity to C/EBPα in the EMSA, was substantially inhibited by C/EBPα in the transfection analysis (Fig. 3). The MBP-fused recombinant C/EBPα may have altered the binding activity of the intact C/EBPα in the cells. Alternatively, a mechanism independent of DNA binding, such as the inhibition of E2F activity (37), may have affected the GPE1 activity.

In contrast to C/EBPα, Nrf2/MafK appeared to bind to all three mutants with higher affinity than to the wild-type GPE1 probe (Fig. 3A). Consistent with the results of the EMSA, the enhancer activities of the mutant GPE1s in H4IIE cells were increased as compared with the wild-type GPE1 in the absence of C/EBPα (Fig. 3B). This suggests that the wild-type GPE1 sequence is not optimal for Nrf2/MafK binding. Indeed,
Nioi et al. (38) recently reported that the sequence TnACnGTnAGnCnnCA is an optimal binding sequence for Nrf2/MafK, as determined by a point mutation analysis of the antioxidant-responsive element sequence of the mouse NAD(P)H:quinone oxidoreductase 1 gene. Most of the bases in the GPE1 sequence (TCACTATGATTCAGCA) match this sequence; however, the underlined nucleotides at the 6th and 10th positions in the optimal sequence are changed from G to A and T in the GPE1 sequence. The 10th position of the antioxidant-responsive element is critical for enhancer activity, and a G-to-T change at this position greatly reduced NAD(P)H:quinone oxidoreductase 1 expression under constitutive and drug-induced conditions (38). In our results, the enhancer activity of the GPE1-M13 mutant, which had a C residue at the 10th position, was ~30 times stronger than that of the wild type (Fig. 3B). This suggests that a C residue, rather than the G of the mouse NAD(P)H:quinone oxidoreductase 1 gene, is the optimal nucleotide at the 10th position of the antioxidant-responsive element sequence.

**Expression of C/EBPα in Normal Liver and Preneoplastic Foci—** Down-regulation of C/EBPα gene expression in preneoplastic foci has
been reported (23, 24). To confirm this, we carried out Northern blotting and immunohistochemical analyses using normal rat livers and livers bearing hyperplastic nodules. C/EBPα mRNA was markedly decreased in the hyperplastic nodule-bearing liver, as well as in hepatoma and embryonal carcinoma cells (Fig. 4A). The preneoplastic focus stained with anti-GST-P antibody. In contrast, C/EBPα protein was down-regulated specifically in the preneoplastic focus but not in the normal liver tissue (Fig. 4B). This result indicated that C/EBPα expression was suppressed specifically in the GST-P-positive focus but not in the whole liver. C/EBPβ mRNA expression also decreased in preneoplastic liver, but substantial amounts were still expressed. C/EBPβ mRNA was highly expressed in hepatoma and F9 cells. GST-P mRNA was specifically expressed in the preneoplastic liver and hepatoma cells, as previously reported (15).

C/EBPα and C/EBPβ to GPE1 in Normal Liver—To investigate the interaction of C/EBP with GPE1 in normal liver and in hepatoma cells, EMSA and supershift analyses were performed using nuclear extracts from normal rat liver and from the H4IIE rat hepatoma cell line and an anti-C/EBPα antibody (Fig. 5A). Strong GPE1-binding complexes were observed with nuclear extracts from both sources. Anti-C/EBPα antibodies supershifted the complexes in nuclear extracts from normal liver. In contrast, hepatoma cell nuclear extracts did not show a supershifted band. These results are consistent with the high expression of C/EBPα in normal liver but not in preneoplastic lesions and hepatomas.

To further examine the binding of C/EBPα to the GPE1 region of the GST-P gene in vivo, we performed a chromatin immunoprecipitation assay using anti-C/EBPα, -C/EBPβ, -Nrf2, and -MafK antibodies (Fig. 5B). We analyzed the chromatin from cells of normal rat livers and livers bearing hyperplastic nodules. The anti-C/EBPα antibody precipitated the GST-P GPE1 from the chromatin of normal liver cells but did not precipitate it from the chromatin of cells from liver with hyperplastic nodules. The anti-C/EBPβ antibody did not precipitate the GST-P GPE1 from the chromatin of normal or hyperplastic cells. In contrast to the anti-C/EBPα antibody, the anti-Nrf2 and anti-MafK antibodies precipitated GPE1 from the cells of hyperplastic nodules but not from the normal liver cells (Fig. 5B) (15). The results of chromatin immunoprecipitation analysis suggest that the replacement of the C/EBPα transcription factor with Nrf2/MafK on GPE1 is a critical event for the induction of the GST-P gene during hepatocarcinogenesis.

Overexpression of C/EBPα Suppressed Endogenous GST-P Expression in Hepatoma Cells—To further define the effects of C/EBPα on the expression of the endogenous GST-P gene, we expressed the C/EBPα-ER fusion protein by retroviral gene transfer into hepatoma cells, as described under “Experimental Procedures.” Because C/EBPα inhibits cellular proliferation, it is difficult to establish permanent overexpressing cell lines. Therefore, C/EBPα fused with the ligand-binding domain of the estrogen receptor such that the fusion protein is activated by β-estradiol has been widely used for analysis of C/EBPα function in cultured cells (23, 28). Four clones (clones 11, 13, 21, and 23) of C/EBPα-ER-expressing RL34 rat hepatoma cells were isolated. Whereas the parental cell line RL34 did not express C/EBPα-ER (75 kDa), it was highly expressed in all of the clones. Endogenous C/EBPα (42 kDa) was not detected in either parental or transformed cells (Fig. 6C). The transcription and trans-suppression activities of C/EBPα-ER in these cells were analyzed using C/EBPα-ER/Δ50Luc and GST/ELuc reporter genes, respectively (Fig. 6A). β-Estradiol treatment activated the C/EBPα-ER/Δ50Luc reporter gene ~2-fold in all transformants. When we used GST/ELuc as a reporter, the activities were suppressed by approximately one-half after treatment with β-estradiol. These results indicate that the C/EBPα-ER system works but that C/EBPα activity against C/EBPα-ER/Δ50Luc and GST/ELuc was much weaker than in that of transient-transfection analysis (Fig. 1A).

GST-P expression was analyzed in C/EBPα-ER-expressing cells treated with or without β-estradiol (Fig. 6, B and C). β-Estradiol treatment of the cells decreased GST-P mRNA to ~25% of the untreated level in clone 13 transformant and to ~50% of the untreated level in the other clones. Western blotting analysis showed that the expression of GST-P was suppressed to ~30% (clone 11), 40% (clone 21), 50% (clone 13), and 70% (clone 23) of the levels in untreated cells. Consistent with reporter transfection analysis (Fig. 6A), GST-P expression was not completely inhibited in the β-estradiol-treated C/EBPα-ER-expressing cells. Although suppression of GST-P expression in the C/EBPα-ER expressing hepatoma cells was not as effective as in the normal liver cells, these results clearly indicated that C/EBPα suppressed GST-P expression in vivo.
C/EBPα Suppresses the Rat GST-P Gene

FIGURE 6. Activation of C/EBPα suppressed GST-P expression in C/EBPα-ER-expressing hepatoma cells. A, trans-suppression of GST-P/luciferase reporter gene in C/EBPα-ER-expressing cells. RL34 and C/EBPα-ER clones (clones 11, 13, 21, and 23) were transfected with the C/EBP-RE/Δ50Luc (left panel) or GST/ELuc reporter genes (right panel) and treated with (closed bars) or without (open bars) β-estradiol (Est, 1 μM, 48 h), as described under “Experimental Procedures.” B, RNase protection analysis of GST-P mRNA in C/EBPα-ER-expressing cells. RL-34 parent cells and C/EBPα-ER clones were treated with (+) or without (−) β-estradiol (1 μM, 48 h) and 10 μM (GST-P) or 2.5 μM (GAPDH) of total RNA were analyzed by RNase protection analysis. C, Western blotting analysis of GST-P and C/EBPα-ER. The indicated cells were treated with (+) or without (−) β-estradiol for 48 h and 10 μg of total protein were analyzed by Western blotting with anti-GST-P antibody (upper panel). C/EBPα-ER was detected by anti-C/EBPα antibody in RL34 and C/EBPα-ER clones (lower panel).

DISCUSSION

The rat GST-P gene is completely silenced in normal liver but is highly and specifically induced during hepatocarcinogenesis. GST-P is the major enhancer element essential for the activation of the GST-P gene in early stage hepatocarcinogenesis. We recently found that the Nrf2/MafK heterodimer binds to GST-P and activates GST-P expression during this process (15). In the present study, we identify C/EBPα as a suppressor of GST-P in normal liver. Reporter transfection analysis indicates that C/EBPα strongly inhibits GST-P expression in hepatoma cells (Fig. 1). In vitro binding analyses shows that C/EBPα strongly binds to the 3′-half of GPE1 and that this region overlaps with the binding site of Nrf2/MafK (Fig. 2). The binding of C/EBPα to mutated forms of GPE1 correlates with its suppression of activity in these mutants, suggesting that the binding of C/EBPα to GPE1 is necessary for suppression (Fig. 3). Furthermore, the expression of C/EBPα is specifically suppressed in GST-P-positive foci (Fig. 4). This result is supported by a chromatin immunoprecipitation analysis that shows that C/EBPα is bound to GPE1 in the chromatin of normal liver but not in carcinogen-treated preneoplastic liver (Fig. 5). Furthermore, the C/EBPα-ER system clearly shows that the activation of C/EBPα activity by β-estradiol suppresses endogenous GST-P expression in hepatoma cells (Fig. 6). All of these results indicate that C/EBPα acts as a negative regulator of GST-P expression in normal liver through the GPE1 enhancer element.

Our data show that C/EBPα inhibits GPE1-mediated gene expression in hepatoma cells but activates it in F9 cells (Fig. 1). These opposite effects suggest that C/EBPα inhibits the binding of the strong transcription factor Nrf2/MafK to GPE1 in hepatoma cells, and we find that the interactions of Nrf2/MafK and C/EBPα with GPE1 are mutually exclusive (Fig. 2). However, this does not fully explain the complete repression of the GST-P gene in normal liver. The negative control region located between –140 and –400 bp of the GST-P gene may also contribute to the silencing of this gene (9). At least three factors bind to this region: silencer factors A, B, and C (39). Osada, Imagawa, and their colleagues identified the NF-1 family as silencer factor A, the C/EBP family as silencer factor B, and the zinc finger proteins (BTEB2, LKLF, TIEG1, MZF1, and TFIIIA) as silencer factor C (24, 39–42). These factors may contribute to the repression of the GST-P gene in normal liver in a cooperative manner, but the details of the physiological mechanisms remain to be elucidated.

Previously, it has been reported that the mechanism of transcriptional inhibition by C/EBPα does not require direct DNA binding but rather is mediated by protein-protein interactions with positive transcription factors, including E2F (21, 22, 37). Our preliminary EMSA results suggest that C/EBPα interacts with MaLF, preventing binding to the C/EBP consensus element (data not shown). Thus, the possibility that these protein-protein interactions results in the transcriptional suppression of the GST-P gene cannot be ruled out.

C/EBPα is a family of transcription factors that plays an important role in the growth and differentiation of many cell types and tissues (19, 43). C/EBPα is expressed at high levels in liver and maintains liver quiescence (20) by direct interaction with cdk2 and cdk4 or by interacting with E2F-DP, which activates several genes required for cell cycle progression (21, 22). These findings are consistent with the induction of GST-P in the preneoplastic foci of the liver. Chemical carcinogens somehow shut down the expression of C/EBPα, releasing C/EBPα from the GST-P region of the GST-P gene and enabling Nrf2/MafK to bind to GST-P and induce GST-P expression. Simultaneously, suppression of C/EBPα leads to progression of the cell cycle. On the other hand, Nrf2 is known to be an essential transcription factor for phase II detoxifying enzymes activated by xenobiotics (44). In the steady state, Nrf2 is retained in the cytoplasm by specific binding with Keap1 (Kelch-like ECH-associated protein 1), a key cytoplasmic mediator of Nrf2 activation. Upon induction by xenobiotics such as chemical carcinogens, Nrf2 is released from Keap1 and translocates into the nucleus, where it induces a group of detoxifying enzyme genes (45, 46). It is known that GST-P is not induced by the majority of chemical carcinogens in normal rat liver but that in hepatoma cells xenobiotic drugs markedly stimulate GST-P expression (15). It is possible that GST-P expression cannot be
activated by the Keap1-Nrf2 pathway when it is completely repressed by C/EBPα and silencer functions in normal liver; however, in hepatoma cells, GST-P expression is derepressed and can be further stimulated by Nrf2 activation. Similarly, in the carcinogen-treated liver foci, the GST-P gene is induced by a dissociation of C/EBPα from GPE1, allowing the Keap1-Nrf2 pathway to reinforce strong GST-P expression. Our findings are very interesting because they show that the expression of a tumor maker gene is directly co-regulated with genes that relate to cellular proliferation and transformation.

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