0026-895X/04/6504-906–916$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 65:906–916, 2004

Vol. 65, No. 4
2853/1136427
Printed in U.S.A.

CCAAT/Enhancer-Binding Protein β (Nuclear Factor for Interleukin 6) Transactivates the Human *MDR*1 Gene by Interaction with an Inverted CCAAT Box in Human Cancer Cells

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Received July 30, 2003; accepted December 22, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

We investigated the mechanisms of *MDR*1 gene activation by CCAAT/enhancer binding protein β (C/EBPβ, or nuclear factor for interleukin 6) in human cancer cells. Transfection of the breast cancer cell line MCF-7 and its doxorubicin-selected variant MCF-7/ADR by either C/EBPβ or C/EBPβ-LIP (a dominant-negative form of C/EBPβ) confirmed their roles in the activation or repression of the endogenous, chromosomally embedded *MDR*1 gene. Cotransfection experiments with promoter constructs revealed a C/EBPβ interaction on the *MDR*1 promoter via the region within −128 to −75. Deletions within the putative AP-1 box (−123 to −111) increased *MDR*1 promoter activity when stimulated by C/EBPβ, suggesting that the AP-1 site negatively regulates *MDR*1 activation by C/EBPβ. Mutations within the inverted CCAAT box (Y box) (−82 to −73) abolished the C/EBPβ-stimulated *MDR*1 promoter activity, indicating that the Y box is required for *MDR*1 activation by C/EBPβ. Chromatin immunoprecipitation (ChIP) revealed that C/EBPβ precipitates a transcription complex containing C/EBPβ, the *MDR*1 promoter sequences (−250 to +54), and the hBrm protein. In conclusion, alteration of expression or function of C/EBPβ plays an important role in *MDR*1 gene regulation. C/EBPβ activates the endogenous *MDR*1 gene of MCF-7 cells, and this activation was associated with a novel C/EBPβ interaction region within the proximal *MDR*1 promoter (−128 to −75). The mechanisms of *MDR*1 activation by C/EBPβ include C/EBPβ binding of the chromatin of the *MDR*1 gene and interactions of C/EBPβ with the Y box and Y box-associated proteins.

The multidrug transporter P-glycoprotein (P-gp), encoded by the *MDR*1 gene, is expressed in many normal tissues and cancers and has been associated with clinical multidrug resistance (MDR) and poor prognosis in some cancers (Arceci, 1993; Gottesman, 1993; Sikic, 1993). Activation of *MDR*1 in tumors that do not constitutively express the gene may be a relatively frequent and adverse event in many types of cancers (Arceci, 1993; Sikic, 1993). Thus, the acquisition of or increase in *MDR*1 expression may be of particular interest for understanding clinical drug resistance.

The regulation of *MDR*1 transcription has involved tissue-specific and induced expression in cells in which the molecular mechanisms are not well elucidated. Analysis of the human *MDR*1 promoter reveals that the gene does not have a TATA box. However, structural and functional studies revealed that an initiator sequence (Inr) surrounding the major transcription start site (+1) governs accurate initiation of *MDR*1 transcription (Ueda et al., 1987; Madden et al., 1993). It has been shown that several *cis*-acting elements are involved in regulating *MDR*1 promoter activity. These motifs include a distinct GC box (Cornwell and Smith, 1993b), an inverted CCAAT box (Y box) (Goldsmith et al., 1993; Bargou et al., 1997; Sundseth et al., 1997; Jin and Scotto, 1998; Ohga et al., 1998), a putative AP-1 site that overlaps with a CAAT-like box (Ogretmen and Safa, 1999), a binding motif for CCAAT/enhancer-binding protein β (C/EBPβ) (Combates et al., 1994), and a novel head-to-tail site within the *MDR*1

This investigation was supported by National Institutes of Health grant R01-CA92474 (to B.I.S.) and National Cancer Institute grant CA09302 (to G.K.C.).

ABBREVIATIONS: P-gp, P-glycoprotein; CAT, chloramphenicol acetyltransferase; C/EBPβ, CCAAT/enhancer binding protein β; NF-IL6, nuclear factor for interleukin-6; LUC, luciferase; ChIP, chromatin immunoprecipitation; MDR, multidrug resistance; PCR, polymerase chain reaction; Y box, an inverted CCAAT box; WT, wild-type; IL, interleukin; DC-1018, human *MDR*1-CAT construct pBLCAT3/DC-1018; B145, human *MDR*1-CAT construct pBLCAT3/B145; DC-190, human *MDR*1-CAT construct pBLCAT3/DC-190; DC-128, human *MDR*1-CAT construct pBLCAT3/DC-128; bp, base pair; p*MDR*1-1202, the wild-type Y box *MDR*1-LUC construct; MUTC1, the mutant form of the wild-type Y box *MDR*1-LUC construct; EMSA, electrophoretic mobility shift assay.

promoter for the tumor suppressor protein p53 (Johnson et al., 2001). Thus, several corresponding transcription factors of these sites such as Sp1, NF-Y, YB-1, c-fos, c-jun, p53, and C/EBPβ have been implicated in the regulation of *MDR*1.

C/EBPβ, also known as nuclear factor for interleukin (IL)-6 expression (NF-IL6) in humans, is expressed in many tissues such as spleen, liver, and kidney and is involved in acute phase reactions, inflammation, and hematopoiesis (Akira et al., 1990). C/EBPβ can be induced by cytokines (e.g., IL-1 and IL-6) and lipopolysaccharide (Akira et al., 1990) and is regulated by p21 (ras)-dependent mitogen-activated protein kinases and protein kinase C (Nakajima et al., 1993; Trautwein et al., 1993). The leucine-zipper motif of C/EBPβ may interact with many other leucine-zipper family members (e.g., c-fos and c-jun), and these interactions may have positive or negative effects on gene expression (Hsu et al., 1994).

Conze et al. (2001) have shown that transfection of the IL-6 gene into MCF-7 breast cancer cells activates *MDR*1 gene expression, and demonstrated increased binding activity of C/EBPβ in nuclear extracts of these cells to a canonical C/EBPβ consensus (Conze et al., 2001). The *MDR*1 gene promoter contains a similar but not identical C/EBPβ consensus (−148 to −140) that has been shown to be involved in transactivating an *MDR*1 promoter construct in HepG2 cells (Combates et al., 1994). We sought to provide direct evidence for the role of C/EBPβ in the activation of *MDR*1 in MCF-7 cells and to further investigate the mechanism of this activation. We therefore performed functional analyses of the *MDR*1 promoter and examined the effect of C/EBPβ on endogenous *MDR*1 gene expression. Our data revealed that C/EBPβ activates the endogenous *MDR*1 gene of MCF-7 cells, and this activation was associated with a novel C/EBPβ interaction region within the proximal *MDR*1 promoter (−128 to −75), in which it exerts its functional role by interacting mainly with the Y box and, to a lesser extent, with the AP-1 binding sequence.

Materials and Methods

Cell Culture and Chemicals. Both the human breast cancer line MCF-7 and ovarian cancer cell line OVCA 433 were obtained from the American Type Culture Collection (Manassas, VA). MCF-7/ADR (a doxorubicin-selected MCF-7 cell line) was originally derived from Dr. K. Cowan's laboratory (National Cancer Institute, Bethesda, MD) (Fairchild et al., 1987) and obtained from Dr. J. Ford (Stanford University, Stanford, CA). All cell lines were maintained in MEM (Minimum Essential Medium) (Life Technologies, Rockville, MD) and incubated at 37°C in a humidified atmosphere incubator containing 5% CO_2. Doxorubicin was obtained from Adria Laboratories (Columbus, OH), and all other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Plasmid Constructs. Both *MDR*1-chloramphenicol acetyltransferase (*MDR*1-CAT) and *MDR*1-luciferase (*MDR*1-LUC) reporter constructs were used in this study. The human *MDR*1-CAT constructs pBLCAT3/DC-1018 (DC-1018), pBLCAT3/B145 (B145), pBLCAT3/DC-190 (DC-190), and pBLCAT3/DC-128 (DC-128) were gifts from Dr. D. Cohen (Combates et al., 1994). DC-1018, DC-190, and DC-128 contain 1201 bp (−1018 to +83), 273 bp (−190 to +83), and 211 bp (−128 to +83) of *MDR*1 promoter sequences, respectively (Fig. 2A). B145 is a mutant form of DC-1018, which possesses point mutations at the C/EBPβ (NF-IL6) binding motif (Fig. 2A).

The *MDR*1-LUC constructs containing the wild-type AP-1–like site and its deletion mutant constructed in the pGL3 vector (Promega, Madison, WI) were gifts from Dr. A. Safa (Indiana University, Bloomington, IN) (Ogretmen and Safa, 1999). The wild-type Y box *MDR*1-LUC (p*MDR*1-1202), its mutant form (MUTC1), and p*MDR*1-75 (containing the *MDR*1 promoter sequences from −75 to +144) in the pGL2-basic vector (Promega) were gifts from Dr. K. Scotto (Fox Chase Cancer Center, Philadelphia, PA) (Jin and Scotto, 1998).

The pCMV/C/EBPβ (containing the mouse C/EBPβ cDNA) and pCMV/C/EBPβ-LIP (containing a truncated form of C/EBPβ that lacks the transactivation domain) expression plasmids were obtained from Dr. S. Chen-Kiang (Cornell University, Ithaca, NY). The pEF/NF-IL6 expression vector (containing the human C/EBPβ isoform in pEF-Bos plasmids) was provided by Dr. S. Akira (Osaka, Japan) (Akira et al., 1990; Mizushima and Nagata, 1990). The pCG/hBrm expression vector was provided by Dr. G. Crabtree (Stanford University).

Transient Transfection Experiments. The plasmids used for transfection experiments were extracted by the QIAGEN protocol (QIAGEN, Valencia, CA). A modified electroporation protocol (Baum et al., 1994) using Gene Pulser II (Bio-Rad Laboratories, Hercules, CA) and the calcium phosphate precipitation method (Invitrogen, Carlsbad, CA) were used for transient transfection of MCF-7, MCF-7/ADR, and OVCA 433 cells. The pSV-β-galactosidase plasmid (pSV-β-gal) (Promega) was used as an internal control for normalization of transfection efficiency. Cells were harvested either 48 or 72 h after electroporation or transfection. The cell lysates were obtained by freezing and thawing. Protein concentrations were determined by the BCA protein assay (Pierce Chemical, Rockford, IL).

Polymerase Chain Reaction. Total RNA and genomic DNA were isolated, and polymerase chain reaction (PCR) was performed according to the method described previously (Chen et al., 1994; Strauss, 1998). The PCR products were analyzed by agarose gel electrophoresis and stained by ethidium bromide.

CAT Assays. CAT enzyme-linked immunosorbent assays using a colorimetric enzyme immunoassay kit (Roche Diagnostics, Indianapolis, IN) were used for the quantitative determination of CAT expression in transfected cells. CAT activity for each sample was normalized to β-galactosidase activity to account for differences in electroporation or transfection efficiency.

Luciferase Reporter Assays. Luciferase assays were performed with total cell lysates using the Luciferase Reporter Assay System (Promega). Total light production was measured with a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). When a single luciferase assay (i.e., firefly luciferase) was used, β-galactosidase activity was also determined to normalize the luciferase activity.

Chromatin Immunoprecipitation. The formaldehyde cross-linking and immunoprecipitation protocols were performed as described previously (Boyd and Farnham, 1999; Orlando, 2000), with the following modifications. Cells were grown overnight, harvested, and incubated in 1% formaldehyde solution (Mallinckrodt Laboratory Chemicals, Phillipsburg, NJ) at room temperature for 10 min. The cross-linking reaction was terminated by adding 125 mM glycine. The cells were then disrupted by repeated passage through a 21-gauge needle and transferred to a 15-ml centrifuge tube. Samples were sonicated with an ultrasonicator (Kimble Kontes, Vineland, NJ) at full power for three 45-s pulses on ice to an average DNA fragment length of 200 to 500 bp and then microcentrifuged at 14,000 rpm. Whole-cell extracts were precleared with an isotypic IgG control (1 μg of affinity-purified rabbit polyclonal antibody sc-2027; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 30 min with gentle mixing, followed by incubation with 10 μl of Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology). Immunoprecipitates were pelleted by centrifugation at 2500 rpm at 4°C for 5 min and the supernatant was divided into two equal aliquots. The samples were then precipitated by addition of the rabbit polyclonal antibody against C/EBPβ (sc-150) or normal rabbit IgG with 10 μl of Protein A/G PLUS-Agarose beads at 4°C with gentle mixing over-

night. The precipitates were collected by the centrifugation at 2500 rpm for 5 min at 4°C and washed four times in radioimmunoprecipitation assay buffer. Half of each precipitate was subjected to Western blotting analysis using antisera against C/EBPβ and hBrm (Santa Cruz Biotechnology).

The remaining half of each precipitate was used for PCR analysis of the presence or absence of *MDR*1 promoter sequences. A 5-μl sample of the total C/EBPβ precipitates was quantitated by ethidium bromide staining. An equal amount of DNA was analyzed by PCR using specific primers. The sequences of the *MDR*1 −250 forward and the *MDR*1 +54 reverse primers, respectively, are the following: 5′-ATC GAT CGG TCG ACG TTG AAA TGT CCC CAA TGA TTC AGC TGA TG-3′ and 5′-ATC GAT CGG TCG ACG GAA ACG AAC AGC GGC CTC TGC TTC TT-3′. The underlined bases represent the enzyme restriction site for facilitating PCR subcloning.

Preparation of Cytoplasmic and Nuclear Extracts. Nuclear extracts of the MCF-7 cell line were isolated according to the method described by Dignam et al. (1983). Both cytoplasmic and nuclear extracts of the MCF-7 and MCF-7/ADR cell lines were isolated according to the method published previously (Andrews and Faller, 1991). Nuclear extracts prepared from both methods produced the same result.

Electrophoretic Mobility Shift Assays. Electrophoretic mobility shift assays (EMSAs) were performed according to the method described previously (Xu et al., 2001). The sequences of the oligonucleotides used in these experiment are listed in Fig. 5A. These oligonucleotides were either synthesized by Operon Technologies Inc. (Alameda, CA) or obtained from Santa Cruz Biotechnology, end-labeled with [γ-^{32}P]ATP using T4 nucleotide kinase, and purified. The nuclear extracts (10 μg) of MCF-7 cells were incubated for 40 min on ice in the presence of the binding buffer (20 mM HEPES, pH 7.9, 1 mM $MgCl_2$, 4% Ficoll, 0.5 mM dithiothreitol, and 50 mM KCl), 1 μg poly(dI-dC), and 20 fmol ^{32}P-labeled DNA probe in a final volume of 10 μl. For competition NF-Y EMSAs, both the unlabeled typical NF-Y (Santa Cruz Biotechnology) and the *MDR*1 Y-box oligonucleotides were used (Fig. 5A). For gel mobility supershift assays, the nuclear extracts were incubated with the following antibodies before the addition of the labeled probe: a polyclonal antibody against NF-YB (1 μg), a monoclonal antibody against C/EBPβ (1 μg) (Santa Cruz Biotechnology), and Pab421 (0.5 μg) monoclonal antibody against p53 (Oncogene Research Products, San Diego, CA). The reaction mixtures were then resolved by electrophoresis on a 4% nondenaturing polyacrylamide gel (in 0.25× Tris-borate-EDTA buffer) and quantified by a phosphorimager (Bio-Rad).

Western Blotting. The light-enhanced chemiluminescence Western blot protocol (Amersham Biosciences Inc., Arlington Heights, IL) was used for the detection of protein expression. Both cytoplasmic and nuclear extracts from the exponentially growing cells were used for immunoblotting with the monoclonal antibody C219 (Alexis Biochemicals, San Diego, CA) and a specific goat polyclonal or a monoclonal antibody against C/EBPβ (NF-IL6) (sc-150 and sc-7962) (Santa Cruz Biotechnology). Actin expression was probed with a goat polyclonal antibody (Actin I-19, sc-1616; Santa Cruz Biotechnology). Protein expression was normalized to actin content in these cells.

Statistical Analysis. Both paired and unpaired *t* tests were performed with the StatView software program (version 4.51) from Abacus Concepts (Berkeley, CA) on a Macintosh Power G3 computer (Apple Computers, Cupertino, CA). Comparisons between two groups used an unpaired *t* test unless indicated otherwise.

Results

C/EBPβ Transactivates the Endogenous MDR1 Gene in Both MCF-7 and OVCA 433 Cells. To determine whether the induced-C/EBPβ expression by cytokines such as IL-6 in MCF-7 cells possesses the capacity to activate the endogenous *MDR*1 gene, we transiently transfected either the human form of C/EBPβ (pEF/NF-IL6) or the mouse form of C/EBPβ (pCMV/C/EBPβ) expression plasmids into MCF-7 cells that do not express *MDR*1 mRNAs. C/EBPβ species are extremely conserved proteins throughout evolution. Both the murine and human proteins shared identical structural domains (Fig. 1A) and display an identical function with respect to the regulation of *MDR*1 promoter activity and the effect on expression of endogenous *MDR*1 (data not shown). C/EBPβ-LIP is a truncated form of C/EBPβ, which lacks the transactivation domain. Sequence analysis reveals that mouse and human C/EBPβ-LIPs are identical except for a few amino acid differences. Figure 1B shows that mouse C/EBPβ activates the *MDR*1 gene in MCF-7 cells (lane 3), whereas mouse C/EBPβ-LIP fails to induce *MDR*1 expression in MCF-7 cells (lane 4). Moreover, C/EBPβ-LIP dramatically decreases the *MDR*1 mRNA level (by 68%) in MCF-7/ADR cells (lane 7). These data demonstrate the positive and negative roles of C/EBPβ and C/EBPβ-LIP, respectively, in regulating the endogenous *MDR*1 gene in these breast cancer cells. Further experiments confirmed the role of C/EBPβ in transactivating the silent *MDR*1 gene in another cellular model, the ovarian cancer cell line OVCA 433 (Fig. 1, C1 and C2).

C/EBPβ Transactivates Both the Wild-Type and Mutant MDR1-C/EBPβ-CAT Constructs. To elucidate the mechanism of *MDR*1 activation by C/EBPβ, we analyzed the effect of C/EBPβ on *MDR*1 promoter activity using an *MDR*1 promoter-CAT system. It has been reported that C/EBPβ (NF-IL6) transactivates the *MDR*1-CAT construct (DC-1018) via a C/EBPβ-like motif at the region from −148 to −140 (TTTCGCAGT, with conserved bases underlined) in HepG2 hepatoma cells (Combates et al., 1994). To test whether *MDR*1 activation by C/EBPβ in human MCF-7 breast cancer cells could also use this C/EBPβ site, we coelectroporated C/EBPβ expression plasmids (pEF/NF-IL6) with either the wild-type *MDR*1-C/EBPβ−CAT construct (DC-1018) or its mutant form B145 (mutations generated at the putative C/EBPβ binding site) into MCF-7 cells (Fig. 2A). Our data revealed that the basal *MDR*1 promoter activity observed in both DC-1018 and B145 constructs was significantly higher than that of the pBLCAT3-basic vector control (Fig. 2B, $P < 0.0001$ and 0.03, respectively). The mutations within the C/EBPβ site resulted in a minimal (1.7-fold or 40%) decrease in basal promoter activity compared with the wild-type control DC-1018 (Fig. 2B, columns 2 and 3; $P = 0.06$). As expected, there was a major (16-fold) increase in *MDR*1 promoter activity in MCF-7 cells transfected with the wild-type *MDR*1-CAT construct (DC-1018) in the presence of C/EBPβ (Fig. 2D, columns 3 and 4; $P = 0.002$) compared with that of the pBLCAT3 control (Fig. 2D, columns 1 and 2). However, C/EBPβ also significantly modulated the mutant *MDR*1 promoter activity when cells were transfected with the mutant *MDR*1-C/EBPβ–CAT construct (B145) and compared with that of the pBLCAT3 control (Fig. 2D, columns 2 and 6; $P < 0.0001$). Moreover, the stimulated *MDR*1 promoter activity by C/EBPβ in the mutant construct was comparable with that of the wild-type control construct DC-1018 (Fig. 2D, columns 4 and 6; $P = 0.22$). Thus, these data suggest that the intact *MDR*1 C/EBPβ (NF-IL6) motif (−148 to −140) is not essential for *MDR*1 promoter activation by C/EBPβ in MCF-7 breast cancer cells.

Identification of a Novel Region Responsible for the C/EBPβ Transactivation in the MDR1 Promoter. We reasoned that other site(s) would be critical for *MDR1* activation by C/EBPβ. To map this C/EBPβ interaction site(s), we transfected MCF-7 cells with the *MDR*1-CAT deletion constructs (DC-190 and DC-128) in the presence or absence of C/EBPβ expression plasmids. Our data localized the core *MDR*1 promoter in MCF-7 to a region within −128 to +83 (Fig. 2C, column 4). Of note, deletions between both −1018 to −190 and −190 to −128 resulted in an increased basal *MDR*1 promoter activity (P = 0.04 and 0.07, respectively), suggesting that there might be several weak repressive elements on the distal region of the *MDR*1 proximal promoter (Fig. 2C). Further cotransfection experiments revealed a repressor element (within −1018 to −190) that also suppressed *MDR*1 activation by C/EBPβ by 63% (2.7-fold, P = 0.002) (Fig. 2E, columns 4 and 6). Of note, both DC-190 and DC-128 constructs demonstrated a greater *MDR*1 promoter activity than DC-1018 when cells were treated with C/EBPβ (P = 0.0002 and 0.0009, respectively) (Fig. 2E, columns 4, 6, and 8). However, there was little difference in *MDR*1 promoter activity in the DC-190 and DC-128 constructs when stimulated by C/EBPβ (Fig. 2E, columns 6 and 8). Taken together, these data suggest that the major C/EBPβ interaction site on the *MDR*1 promoter in these breast cancer cells lies in the region from −128 to +83.

C/EBPβ Does Not Interact with the Sp1 Site within the *MDR1* Promoter. Several *cis*-acting elements are located on the *MDR*1 proximal promoter (the AP-1–like site, the Y box, and the GC box), potentially implicating c-fos, NF-Y or YB-1, and Sp1 in the regulation of *MDR*1 promoter activity. Moreover, these transcription factors have been reported to interact with C/EBPβ in other cellular models (Hsu et al., 1994; Yu et al., 1995; Lee et al., 1997). To investigate a potential C/EBPβ-Sp1 interaction at the GC box (−62 to −43) on the *MDR*1 promoter, we coelectroporated C/EBPβ expression plasmids with the *MDR*1-LUC deletion construct (p*MDR*1-75, containing the *MDR*1 promoter sequence from −75 to +118) that lacks both the AP-1–like site and Y box. Our data revealed that there was no increase in *MDR*1 promoter activity in the deletion construct when stimulated by C/EBPβ. The positive control p*MDR1*-1202 demonstrated 2-fold stimulation. Hence, our data eliminated a C/EBPβ-Sp1 interaction within the region −75 to +83 that contains a major Sp1 interaction site. Therefore, the interaction site of C/EBPβ within the *MDR*1 promoter was localized at the region from −128 to −75. Two sites (the putative AP-1 site and Y box) in this region are of particular interest, because both of the corresponding transcription factors for these sites (i.e., c-fos and NF-Y) have a structural motif that enables them to dimerize with or bind to C/EBPβ (Hsu et al., 1994; Yu et al., 1995).

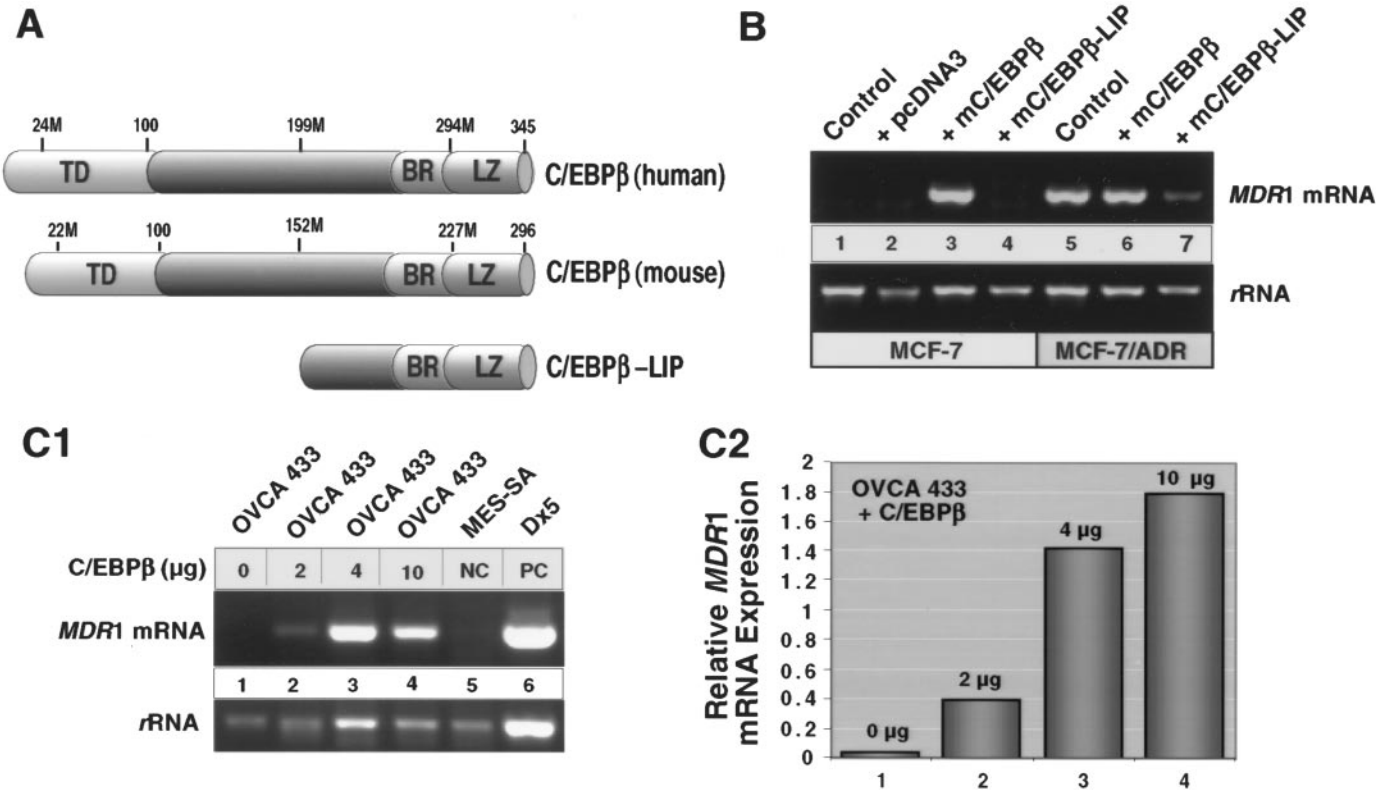

Fig. 1. C/EBPβ transactivates the chromatin-embedded *MDR*1 gene in both MCF-7 and OVCA433 cancer cells. A, structures of C/EBPβ (NF-IL6) family members in the expression plasmids. B, both MCF-7 and MCF-7/ADR cells were electroporated with either pcDNA3 (control) or pCMV/C/EBPβ or pCMV/C/EBPβ-LIP. The cell pellets were collected 48 h after electroporation. C1, ovarian cancer cells (OVCA 433) were seeded in 6-well plates (2×10^5 cells/well) and transfected with the human C/EBPβ expression vector (pEF/NF-IL6) by the calcium phosphate precipitation method. The cell pellets were harvested 48 h after transfection. *MDR*1 RT-PCRs (35 cycles) were performed as described previously (Chen et al., 1994). The ribosomal RNA PCR products were used as loading controls. PCR products were analyzed by a 2% agarose gel and stained with ethidium bromide (B and C1). C2, semiquantitative analysis of *MDR*1 mRNA expression from the result of Fig. C1. The results were normalized to ribosomal cDNA controls. BR, basic region; LZ, leucine zipper; M, methionine; mC/EBPβ, the mouse isoform of C/EBPβ; NC and PC, the negative and positive controls, respectively, for *MDR*1 reverse transcriptase PCR; TD, transactivation domain.

The AP-1/c-fos Site Moderately Represses MDR1 Activation by C/EBPβ. To test whether C/EBPβ could interact with the AP-1/c-fos site (12), we cotransfected C/EBPβ with either p*MDR*1-240 or its deletion mutant (p*MDR*1-236, deletion of the CAAT sequences) (Fig. 3A) into MCF-7 cells. Our data confirm a previous study showing that the AP-1–like site negatively regulates *MDR*1 promoter activity in these breast cancer cells; deletion gives rise to an increased basal *MDR*1 promoter activity (Fig. 3B, columns 3 and 5; $P < 0.0001$). However, C/EBPβ contributes to an even higher luciferase activity in MCF-7 cells cotransfected with the pGL-3/*MDR*1 promoter construct (p*MDR*1-240) compared with the pGL3 control (Fig. 3B, columns 2 and 4; $P = 0.002$). Moreover, our data indicate that C/EBPβ modulates not only the wild-type promoter activity, it also modulates that of the AP-1 mutant promoter (Fig. 3B, columns 5 and 6; $P = 0.001$). Deletion of the CAAT sequences resulted in a 2-fold elevation in *MDR*1 promoter activity by C/EBPβ (Fig. 3B, columns 4 and 6; $P = 0.008$), indicating that this site also represents a repressive element for *MDR*1 activation by C/EBPβ.

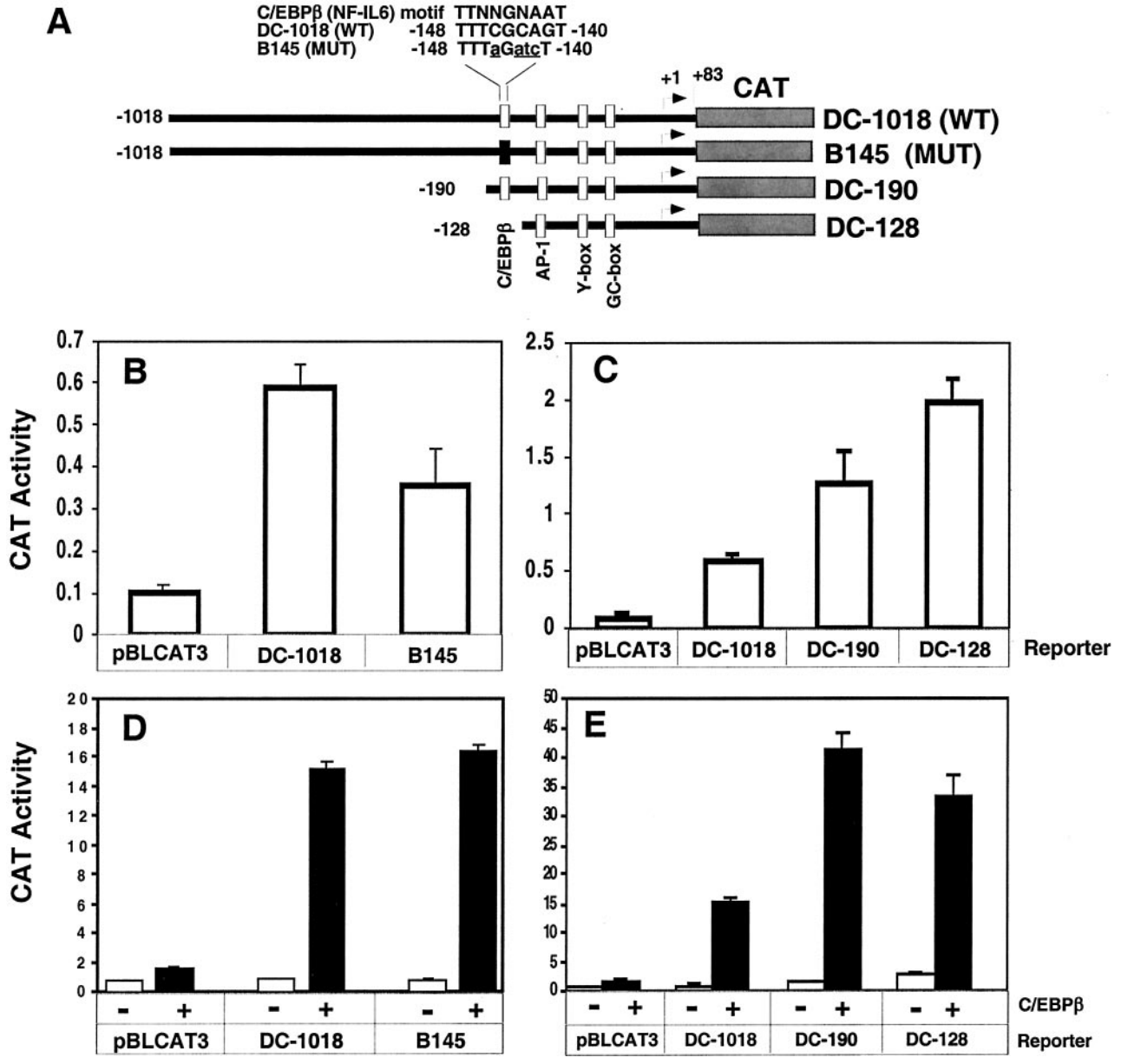

Fig. 2. C/EBPβ transactivates the mutant *MDR*1-C/EBPβ promoter reporter in MCF-7 cells. A, structures of the *MDR*1-CAT constructs: DC-1018, the wild-type (WT) *MDR*1 C/EBPβ-CAT reporter; B145, the mutant (MUT) *MDR*1 C/EBPβ-CAT reporter (point mutations generated at the C/EBPβ site and indicated in lowercase and underlined bases); DC-190 and DC-128, the deletion constructs of DC-1018 as indicated. The previously identified *cis*-acting elements (C/EBPβ, AP-1, Y box, and GC box) are labeled. B and C, basal *MDR*1 promoter activity assays: *MDR*1-CAT plasmids (DC-1018, B145, DC-190, and DC-128; 10 μg each) or the pBLCAT3 control (10 μg) were introduced into MCF-7 cells (2×10^7 cells/450 μl of complete medium/reaction) with pSV-β-gal expression plasmids (5 μg) by electroporation twice (voltage = 0. 26 kV, $\mu F = 975$). D and E, C/EBPβ-stimulated *MDR*1 promoter activity assays: *MDR*1-CAT plasmids (DC-1018, B145, DC-190, and DC-128; 5 μg each) or the pBLCAT3 control (5 μg) were introduced into MCF-7 cells with (8 μg pEF/NF-IL6, ■) or without (8 μg pEF, □) C/EBPβ by the calcium phosphate precipitation method. pSV-β-gal expression plasmids (2 μg) were included in every transfection. CAT activity was assessed in total cell lysates (~70 μg, in 0.25 M Tris-HCl buffer) by the CAT–enzyme-linked immunosorbent assay method and normalized to β-gal activity. The mean of three independent experiments with standard errors is shown.

The Y Box Represents a Major C/EBPβ Interacting Site in MCF-7 Cells. To determine whether C/EBPβ could also interact with the Y box, we cotransfected a C/EBPβ expression plasmid with either the wild-type *MDR*1 Y box (p*MDR*1–1202) or a mutant construct (pGL2/MUTC1) into MCF-7 cells (Fig. 4A). The mutations of the Y box did not decrease basal *MDR*1 promoter activity under these transfection conditions (Fig. 4B, columns 2 and 3; $P = 0.8$). C/EBPβ significantly increased luciferase activity in the pGL-2B vector (Fig. 4C, columns 1 and 2; $P < 0.0001$). However, the C/EBPβ-stimulated luciferase activity was 5-fold elevated in MCF-7 cells cotransfected with p*MDR*1-1202 reporter plasmids relative to that of pGL-2B under the same treatment (Fig. 4C, columns 2 and 4; $P < 0.001$). The mutations in the Y-box consensus sequences (MUTC1) resulted in a 77% decrease in the stimulated-*MDR*1 promoter activity by C/EBPβ compared with the wild-type promoter (Fig. 4C, columns 4 and 6; $P = 0.0002$). Moreover, the C/EBPβ-stimulated promoter activity in the Y box mutant was comparable with that of the pGL2B vector (Fig. 4C, columns 2 and 6; $P = 0.2$), suggesting that the Y-box consensus is a major C/EBPβ interaction site that may be responsible for the transactivation of the *MDR*1 gene by C/EBPβ.

NF-Y Does Not Bind to the Y Box of the *MDR1* Promoter. NF-Y is the predominant protein that binds to CCAAT sequences (Mantovani, 1998). To determine whether NF-Y also binds to the Y box in the *MDR*1 promoter, we performed EMSAs. Our results showed that a binding protein on the *MDR*1 Y-box DNA probe (25 mer) was competed either by a well-characterized wild-type NF-Y oligonucleotide or its mutant form (Fig. 5B), indicating that the binding protein was not NF-Y. This was further confirmed by using a known binding site for NF-Y in MCF-7 cells, the CCAAT element in the SHP-1 (the SH2 domain-containing protein tyrosine phosphatase) promoter (Fig. 5A) (Xu et al., 2001). Figure 5C reveals that the binding protein on the wild-type SHP-1 NF-Y probe was competed away only by the wild-type SHP-1 NF-Y oligonucleotide but not by the mutant (Fig. 5C, lanes 2–4). The identity of NF-Y binding on the SHP-1 site was verified by the supershift assay with an anti–NF-YB polyclonal antibody (Fig. 5C, lane 8). Other unrelated antibodies (such as anti-C/EBPβ and anti-p53) did not result in supershifted bands in this assay (Fig. 5C, lanes 7 and 9). Neither the wild-type nor the mutant *MDR*1 Y-box oligonucleotides were able to compete with the labeled SHP-1 NF-Y

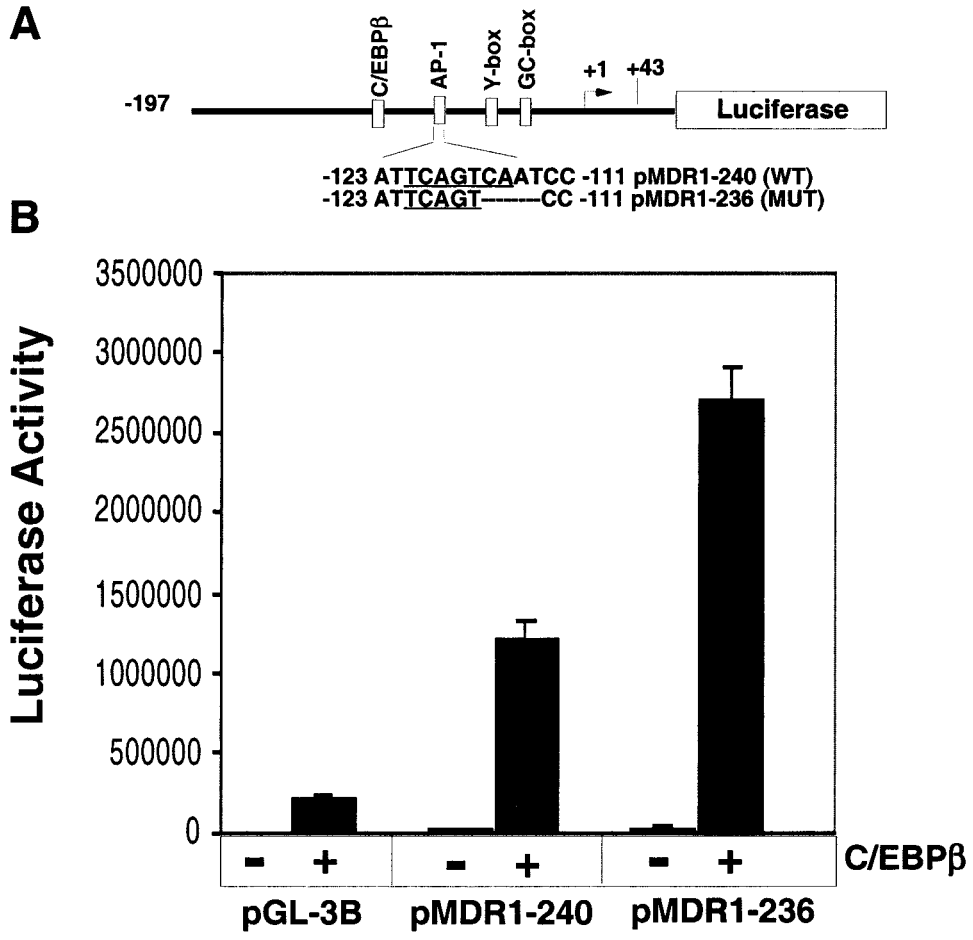

Fig. 3. C/EBPβ interacts with the AP-1/c-fos site on the *MDR*1 proximal promoter in MCF-7 cells. A, structures of the WT (p*MDR*1-240) and mutant (p*MDR*1-236) *MDR*1-AP-1-LUC constructs with the AP-1/c-fos site indicated. B, functional analysis of *MDR*1 promoter activity by luciferase assays: MCF-7 cells were seeded in 6-well plates (2×10^5 cells/well). Both the *MDR*1-LUC plasmids (p*MDR*1–240 and p*MDR*1–236; 1 μg each) and the pGL-3B control (1 μg) were introduced into MCF-7 cells with (8 μg pEF/NF-IL6) or without (8 μg pEF) C/EBPβ by the calcium phosphate precipitation method. pSV-β-gal expression plasmids (2 μg) were included in every transfection. Luciferase activity was assessed in total cell lysates and normalized to β-gal activity. The mean of four independent experiments with standard errors is depicted.

DNA probe (Fig. 5C, lanes 5 and 6), further suggesting that the *MDR*1 Y box is not bound by NF-Y.

C/EBPβ Binds to the Chromatin of the *MDR1* Proximal Promoter. We used ChIP to test whether C/EBPβ could preferentially bind to the chromatin of the proximal *MDR*1 promoter of MDR cells to facilitate transcription. The ChIP experiments demonstrated that the anti-C/EBPβ antiserum precipitated a transcriptional complex containing a similar amount of the C/EBPβ protein in both MCF-7 and MCF-7/ADR cells (Fig. 6A, lanes 1 and 2 at the top). The *MDR*1 promoter sequences (−250 to +54) were significantly coimmunoprecipitated with the anti-C/EBPβ antiserum in the MCF-7/ADR cell line compared with that of rabbit IgG, which only produced a faint band (Fig. 6A, lanes 1 to 4 at the bottom). In addition, the anti-C/EBPβ antiserum precipitated the hBrm protein, a major subunit of the human chromatin-remodeling complex (hSWI/SNF) (Fig. 6B). These data demonstrated that C/EBPβ might interact with other transcription factors on the chromatin of the *MDR*1 proximal promoter in vivo to participate in transcription of the *MDR*1 gene.

***MDR1*/P-gp Expression Correlates with the Accumulation of C/EBPβ (NF-IL6) on the Chromatin of the MDR1 Promoter.** The correlation between *MDR1* and C/EBPβ expression was assessed in experiments presented in Fig. 6, C and E. We examined the protein level of C/EBPβ in both cytoplasmic and nuclear extracts derived from both MCF-7 and its MDR subline MCF-7/ADR by immunoblotting

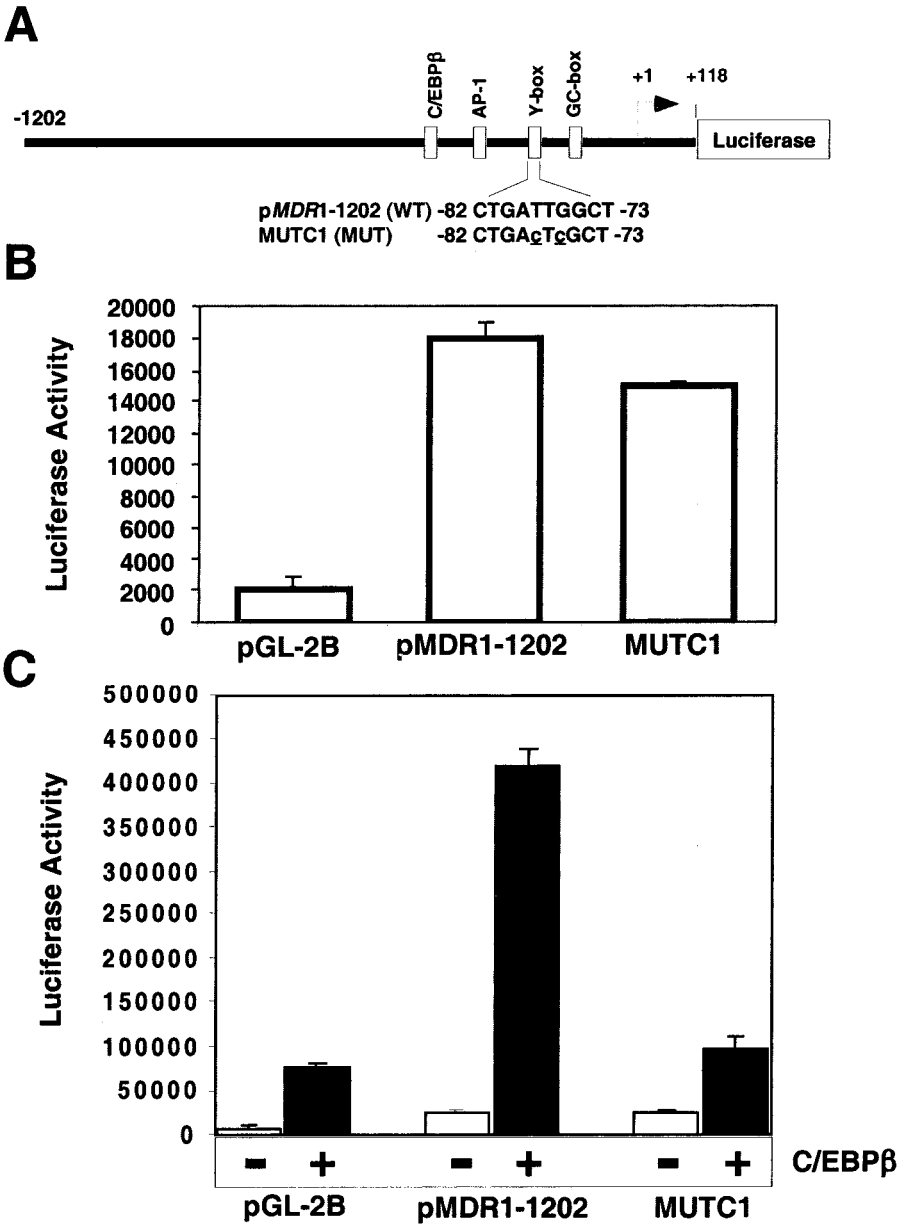

Fig. 4. The Y-box element is required for *MDR*1 activation by C/EBPβ on the *MDR*1 proximal promoter in MCF-7 cells. A, structures of the WT (p*MDR1*-1202) and mutant (MUTC1) *MDR*1-Y-box-LUC constructs with the mutation sites indicated. B, luciferase assays were performed by following the same procedures described in Fig. 3. The *MDR*1-LUC plasmids (1 μg each) or the pGL-2B control (1 μg) were introduced into MCF-7 cells (2×10^5 cells/well) with (8 μg pEF/NF-IL6, ■) or without (8 μg pEF, □) C/EBPβ by the calcium phosphate precipitation method. Luciferase activity was assayed and normalized to β-gal activity. The mean of three independent experiments with standard errors is shown.

with a specific monoclonal antibody against C/EBPβ. Our data showed a 6-fold increase in nuclear C/EBPβ in MCF-7 cells compared with their cytoplasmic compartmentation (Fig. 6C, lanes 1 and 3). No significant increase in the total nuclear C/EBPβ content was found in MCF-7/ADR compared with parental MCF-7 cells (Fig. 6C, lanes 3 and 4).

Of note, a previous report showed that there was a 60-fold *MDR*1 gene amplification in MCF-7/ADR cells that had undergone selection with high doxorubicin concentrations (Fairchild et al., 1987). However, we observed that the *MDR*1 gene copy numbers in MCF-7/ADR were comparable with the parental MCF-7 cells or to a normal liver control (Fig. 6D), indicating that the batch of MCF-7/ADR cells used in this study had lost its amplified *MDR*1 copies during the course of drug-free growth or reselection with a lower doxorubicin concentration (100–500 nM) (Fig. 6D). However, these cells retained overexpressed *MDR*1 mRNA and P-glycoprotein (Fig. 6E). Thus, these data ruled out that the enrichment of C/EBPβ on the *MDR*1 promoter was caused by the recruitment of C/EBPβ by increased *MDR*1 gene copy numbers.

The Y Box also Represents a Major hBrm Interacting Site in MCF-7 Cells. The above data link the chromatin-remodeling factor hBrm to the interaction of C/EBPβ with the Y box. To determine whether hBrm could also interact with the Y box, we cotransfected hBrm expression plasmids with either the p*MDR*1-1202 or MUTC1 into MCF-7 cells. Our data demonstrated that the hBrm-stimulated *MDR*1-luciferase activity was approximately 3-fold elevated in MCF-7 cells (Fig. 7, columns 3 and 4). The mutations in the Y-box consensus sequences (MUTC1) completely abolished the effect of hBrm on *MDR*1 promoter activity (Fig. 7, columns 5 and 6).

Discussion

The mechanisms responsible for *MDR*1 gene activation are complex and include acquired, constitutive, or induced expression of *MDR*1 in normal tissues or tumors. *MDR*1 has been believed to be regulated by a variety of tissue-specific transcription factors. However, several transcription factors implicated in the regulation of the *MDR*1 gene belong to general transcription factors that also regulate many other genes. This led us to hypothesize that the regulation of the *MDR*1 gene may be via the formation of a distinct transcription complex among these general transcription factors. These transcription factors include Sp1, NF-Y, YB-1, AP-1, p53, and C/EBPβ (NF-IL6). Of note, these transcription factors-associated DNA binding sites are near each other or overlapping, which would probably provide a structural platform for protein interactions among these transcription factors. For example, an overlapping site for Sp1 and *EGR*-1 (−69 to +20) is further overlapped with a newly identified

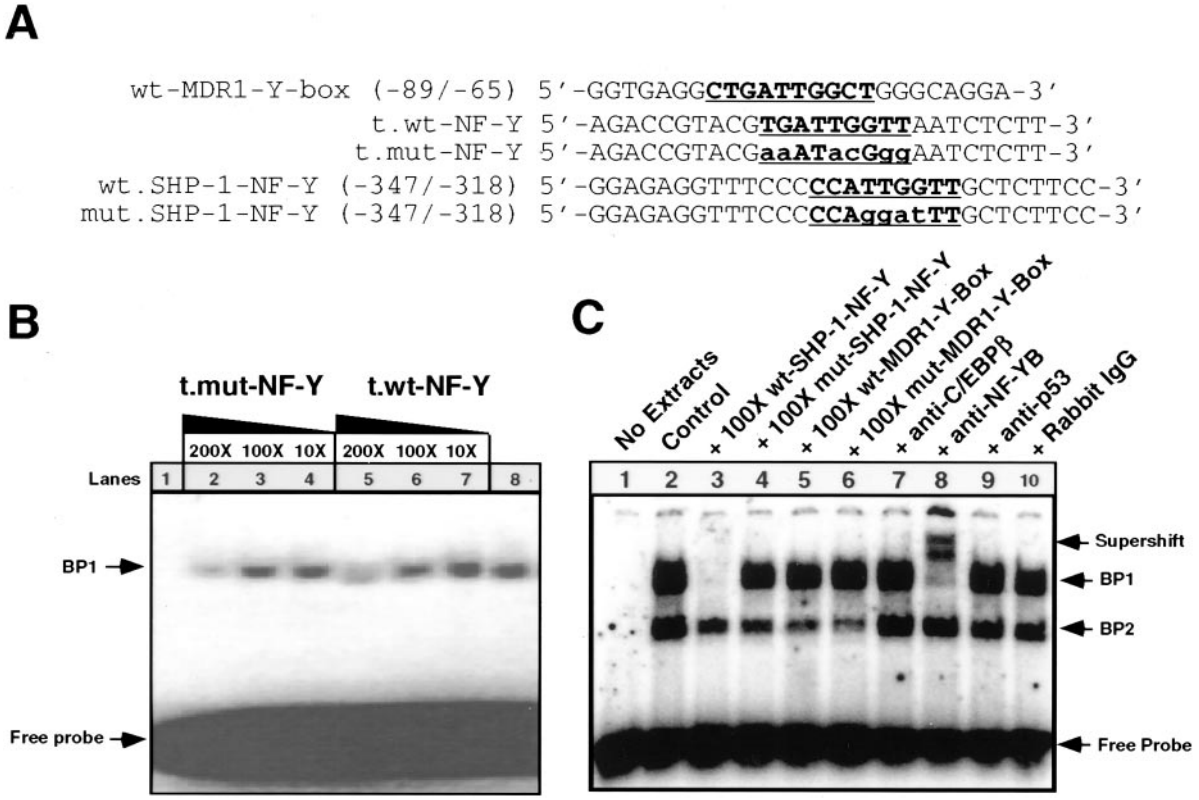

Fig. 5. EMSAs of NF-Y binding on the Y box of the *MDR*1 promoter. EMSAs were performed according to the method described under *Materials and Methods* (Xu et al., 2001). The sequences of the oligonucleotides used in these experiments are listed in Fig. 5A. wt-*MDR*1-Y-box, the wild-type *MDR*1 Y-box oligonucleotides; t.wt-NF-Y and t.mut-NF-Y, typical wild-type and mutant NF-Y oligonucleotides, respectively; wt.SHP-1-NF-Y and mut.SHP-1-NF-Y, the wild-type and mutant SHP-1 NF-Y oligonucleotides, respectively. B, for competition NF-Y EMSAs, the wt-*MDR*1-Y-box oligonucleotides (double-stranded DNAs) were [γ-^{32}P]ATP-labeled and incubated with either an excess of unlabeled t.wt-NF-Y or t.mut-NF-Y competitors (10-fold, 100-fold, and 200-fold as indicated) in this experiment. C, the wt-SHP-1-Y-box oligonucleotides (double-stranded DNAs) were [γ-^{32}P]ATP-labeled and incubated with either 100-fold excess of unlabeled wt-SHP-1-NF-Y (lane 3), mut-SHP-1 NF-Y (lane 4), wt-*MDR*1-Y-box (lane 5), mut-*MDR*1-Y-box competitor (lane 6). For gel mobility supershift assay, the nuclear extracts were incubated with the indicated antibodies before the addition of the labeled probe. The reaction mixtures were analyzed on a 4% nondenaturing 0.25× Tris/borate/EDTA polyacrylamide gel.

p53 site within the *MDR*1 promoter (−72 to −40) (McCoy et al., 1995; Johnson et al., 2001). Moreover, both the NF-Y and Sp1 binding sites are needed for *MDR*1 promoter activation by UV irradiation (Hu et al., 2000), suggesting a coordinate regulation of the *MDR*1 gene by multiple transcription factors. Nonetheless, more protein interactions and assembly at the *MDR*1 promoter and the mechanistic details on either *MDR*1 activation or repression remain an area to be actively investigated.

It has been shown that increased C/EBPβ activity is associated with *MDR*1 activation in MCF-7 breast cancer cells (Conze et al., 2001), consistent with the capacity of C/EBPβ to transactivate the *MDR*1 promoter constructs in HepG2 hepatoma cells (Combates et al., 1994). However, the transcriptional mechanism of *MDR*1 expression mediated by the IL-6–C/EBPβ pathway remains obscure in these breast cancer cells. Thus, we examined both the effect of C/EBPβ on endogenous *MDR*1 expression and the role of C/EBPβ in the regulation of the *MDR*1 promoter activity in MCF-7 cells. Our results revealed several novel mechanisms with respect to the C/EBPβ-mediated activation of the *MDR*1 gene.

Transactivation of the Chromatin-Embedded *MDR1* Gene by C/EBPβ in MCF-7 Cells. It has been documented that C/EBPβ acts as either an activator or a repressor for gene transcription, whereas C/EBPβ-LIP is generally believed to be a dominant-negative regulator (Akira et al., 1990; Descombes and Schibler, 1991; Buck et al., 1994; Hsu et al., 1994). In the case of the *MDR*1 gene, we observed that C/EBPβ is indeed an activator in both MCF-7 and OVCA 433 cells (Figs. 1–4), and C/EBPβ-LIP is a negative regulator for both the endogenous *MDR*1 gene (Fig. 1B) and *MDR*1 promoter-reporter constructs (data not shown). The interpretation of these results could be 3-fold. First, an increased expression or overexpression of C/EBPβ is a critical initial step to transactivate the endogenous *MDR*1 gene (Fig. 1). Second, *MDR*1 activation by C/EBPβ represents an example that the alteration of a single transcription factor can result in activation of the silent *MDR*1 gene in some cancer cells (such as MCF-7 breast cancer cells or OVCA433 ovarian carcinoma cells). Last, these results also reinforce the importance of induction mechanisms for *MDR*1 expression by chemotherapeutic drugs.

A Novel Mechanism of Acquired *MDR1* Gene Expression via C/EBPβ. Our data eliminated a potential interaction of C/EBPβ with the previously identified site (−148 to −140) described in HepG2 cells (Combates et al., 1994), suggesting that the C/EBPβ interaction with the *MDR*1 promoter may be cell type-dependent. In MCF-7 cells, the major

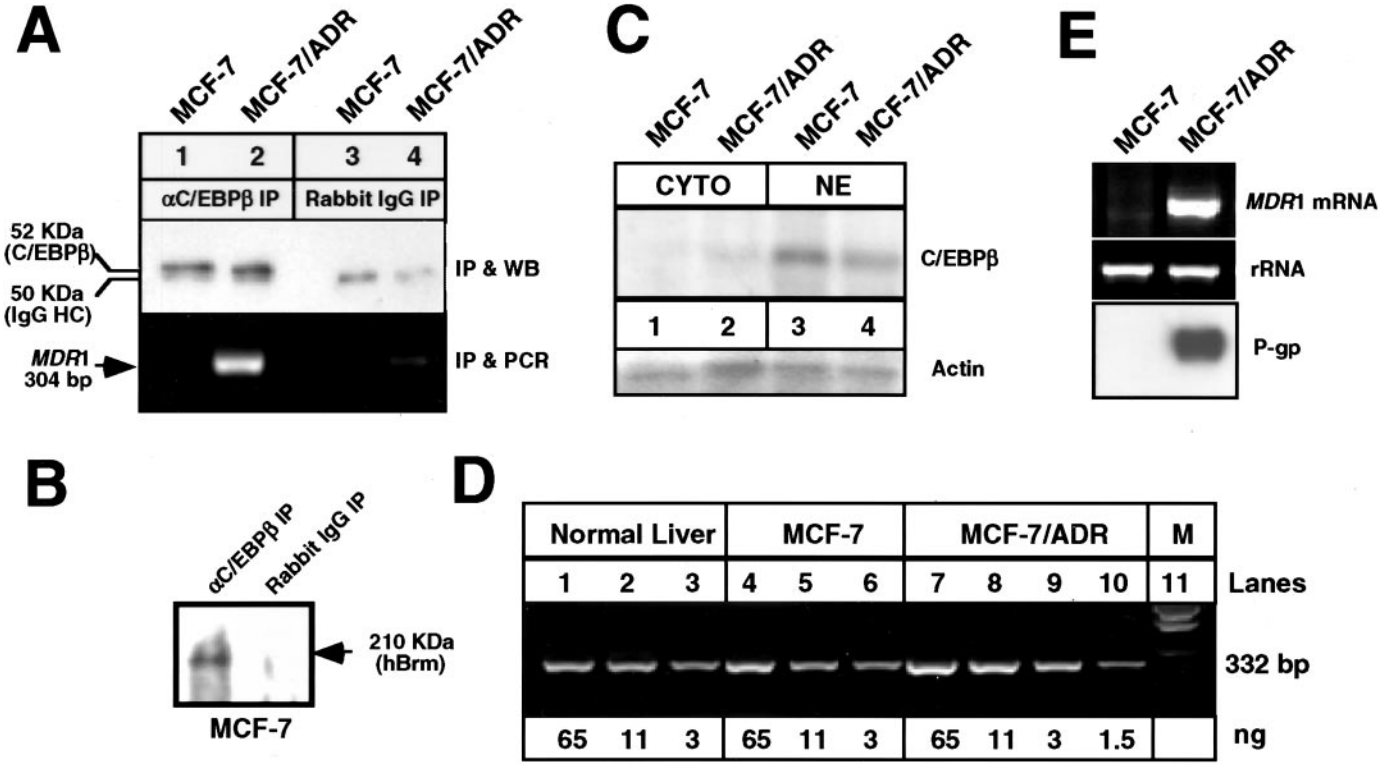

Fig. 6. Correlation between *MDR*1 expression and the localization of C/EBPβ on *MDR*1 chromatin in MCF-7 cells. A, the detailed procedures for the ChIP experiments are described under *Materials and Methods*. Briefly, the anti-C/EBPβ antiserum (C-19) was used to precipitate the complex associated with C/EBPβ. Western blotting (WB) analysis (top) of C/EBPβ with the anti-C/EBPβ antiserum (C-19) was performed. PCR analysis (bottom) of the *MDR*1 proximal promoter sequences (−250 to +54) in the genomic fragments from the immunoprecipitates was carried out. Normal rabbit IgGs were used as a control for the immunoprecipitation (IP). IgG HC, IgG heavy chain. B, Western analysis of the C/EBPβ-hBrm association on the chromatin. The chromatin immunoprecipitates of both anti-C/EBPβ and normal rabbit IgG antisera in MCF-7 cells were analyzed by a 4 to 20% Tris-Glycine gradient gel and probed with the anti-hBrm antiserum. C, analysis of C/EBPβ (NF-IL6) expression was performed in both cytoplasmic (CYTO) and nuclear extracts (NE) (12 μg/lane) in both MCF-7 and MCF-7/ADR cells that were maintained in doxorubicin-free medium. The results were normalized to actin expression. D, genomic DNA PCR. Genomic DNAs (1.4–65 ng) from normal liver, MCF-7, and MCF-7/ADR cells were subjected to PCR (35 cycles) using the same set of *MDR*1 promoter specific primers as described in Fig. 7A. M, DNA mass ladder (Invitrogen). E top, *MDR*1 mRNA expression was determined by 35 cycles of PCR amplification as described previously (Chen et al., 1994). The ribosomal cDNA PCR products were used as loading controls. Bottom, 20 μg of cytoplasmic extracts were used for Western analysis of P-gp expression by the monoclonal antibody C219.

C/EBPβ interaction site(s) was localized to the region within −128 to −75. The mechanism of *MDR*1 activation by C/EBPβ was mainly associated with the C/EBPβ interaction at the Y box, possibly with other Y box-associated proteins (Figs. 4 and 5). The putative AP-1/c-fos site decreases the interaction between C/EBPβ and the Y box, possibly because of its structural proximity to the Y-box site. The c-fos protein has been shown to bind to the AP-1–like region (−121/−115, 5′-TCAGTCA-3′) of the *MDR*1 promoter (Ogretmen and Safa, 1999). It is likely that an increased *MDR*1 promoter activity by C/EBPβ could be achieved also by reducing the expression of inhibitory factors such as c-fos and NF-R1 (also shown to bind to ATTCAGTCA in K562 leukemic cells) (Ogura et al., 1992). Thus, the AP-1–like motif may represent a minor site for the regulation of *MDR*1 promoter activity by C/EBPβ.

The Y Box is Essential for *MDR1* Activation by C/EBPβ. Mutations within the Y box eliminated the response to C/EBPβ modulation, clearly establishing that the major site for the regulation of *MDR*1 by C/EBPβ is at the Y-box site (Fig. 4). C/EBP is a CCAAT box-binding family member. However, C/EBPβ possesses a distinct binding consensus (TKNNGNAAK) that is different from both its C/EBP relatives and an inverted CCAAT-box (Y-box) consensus. Previous studies have established that the predominant binding species of the human Y box is NF-Y (Sundseth et al., 1997; Jin and Scotto, 1998; Mantovani 1998). YB-1 also may be an important binding factor of the Y box on the *MDR*1 promoter in breast cancer cells (Bargou et al., 1997). C/EBP has not been shown to directly bind to the Y box or Y box-associated proteins in the human *MDR*1 promoter of SW620 colon cancer cells (Jin and Scotto, 1998). It seems that C/EBPβ activates the *MDR*1 promoter via protein-protein interactions at the Y-box site of the *MDR*1 chromatin but not via the NF-Y protein (Figs. 5 and 6). Our data further confirmed that C/EBPβ binds to the chromatin of the proximal *MDR*1 promoter (−250 to +82) that overlaps with the region −128 to −75, whereby C/EBPβ interacts with both the Y box- and the AP-1 box-associated proteins within the proximal *MDR*1 promoter (Figs. 3–6).

Involvement of the Chromatin-Remodeling Factor in *MDR1* Activation. Because the *MDR*1 gene is silent in parental MCF-7 cells, an initial step to activate this gene should include a chromatin-remodeling process. It is known that C/EBPβ possesses such a capacity to recruit the remodeling-complex (i.e., the SWI/SNF complex) to a chromatin-embedded promoter by its N-terminal transactivation domain (Kowenz-Leutz and Leutz, 1999). Moreover, the hBrm protein is a major component of the human SWI/SNF complex expressed in the nucleus of MCF-7 cells (data not shown). Our data revealed that the Y-box consensus is also a major hBrm interaction site on the *MDR*1 promoter, which links the remodeling complex hSWI/SNF to C/EBPβ (Fig. 7). We further confirmed a C/EBPβ-hBrm association in vivo on the chromatin in MCF-7 cells (Fig. 6B). Thus, *MDR*1 activation by C/EBPβ is probably mediated by the C/EBP-hBrm interaction on chromatin containing the Y box.

Furthermore, the *MDR*1 Y box has been shown to interact with p300/CREB binding protein-associated factor that has an intrinsic histone acetyltransferase activity. This activity is also important for restructuring the *MDR*1 chromatin (Jin and Scotto, 1998). In addition, Hassan et al. (2001) demonstrated that histone acetyltransferase complexes could stabilize the SWI/SNF complex binding to promoter nucleosomes with specificity. Taken together, these data illustrate potential functional links among C/EBPβ, Y box-binding proteins, the hSWI/SNF complex, and histone acetyltransferase complexes, which are absolutely crucial for the initiation of a transcription process. Thus, this study opens avenues for future investigations on the interactions among these complexes.

A Central Role for C/EBPβ (NF-IL6) in the Regulation of *MDR1* Gene Expression. C/EBPβ may represent one of the central components of the *MDR*1 transcription complex, because our and others' data may account for the mechanisms of *MDR*1 activation in the majority of MDR cellular models. For example, it has been reported that several cytokines (e.g., IL-2 and tumor necrosis factor α) that regulate C/EBPβ also down-regulate *MDR*1 gene expression and sensitize colon cancer cells to anticancer drugs (Stein et al., 1996; Buck et al., 2001). It is also conceivable that an increase in the C/EBPβ level in cells induced by IL-6, a cytokine with pleiotropic biological activities of the host defense, can also lead to *MDR*1 gene activation (Conze et al., 2001). Moreover, the kinases that phosphorylate C/EBPβ include mitogen-activated protein kinases (Nakajima et al., 1993) and protein kinase C (Trautwein et al., 1993), all of which are also regulated by a variety of MDR-related cytotoxins, oncogenes, and other factors (Yu et al., 1991; Blobe et al., 1993; Cornwell and Smith, 1993a; Osborn et al., 1999; Buck et al., 2001). This evidence provides the link between MDR-related signal transduction and C/EBPβ phosphorylation, which facilitates the translocation of C/EBPβ into the nucleus and the binding of chromatin, thus activating the *MDR*1 gene.

In conclusion, an increased expression or overexpression of C/EBPβ can activate the endogenous *MDR*1 gene. C/EBPβ interacts with the Y box on the chromatin of *MDR*1. Thus, signals such as cytotoxic insults and cytokine stimulation that are mediated by C/EBPβ may provide potential molecular targets for modulation of drug sensitivity in human cancer cells.

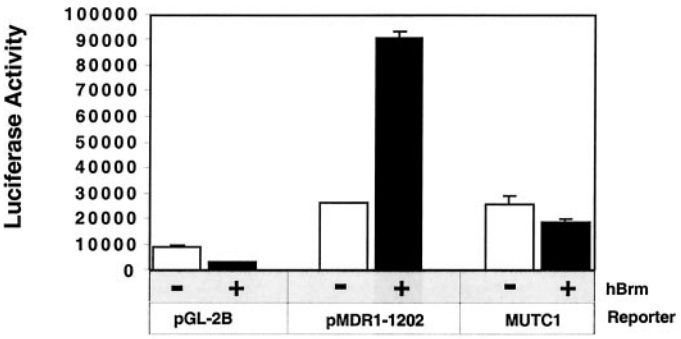

Fig. 7. The Y-box element is required for *MDR*1 activation by hBrm on the *MDR*1 proximal promoter in MCF-7 cells. Both the *MDR*1-LUC plasmids (1 μg/each) and the pGL-2B control (1 μg) were introduced into MCF-7 cells (2×10^5 cells/well) with (8 μg pCG/hBrm, ■) or without (8 μg pCG, □) hBrm by the calcium phosphate precipitation method. Luciferase activity was assessed and normalized to β-gal activity. The mean of three independent experiments with standard errors is depicted.

Acknowledgments

We are grateful to Drs. Dalia Cohen (Novartis Inc.), Kathleen W. Scotto (Fox Chase Cancer Center), Ahmad R. Safa (Indiana Univer-

sity), Selina Chen-Kiang (Cornell University), and Gerald Crabtree (Stanford University) for providing plasmids as described under *Materials and Methods*. We thank Beverly Kennon-Kelley for her excellent assistance in preparing this manuscript.

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