Negative Regulation of *MDR*1 Promoter Activity in MCF-7, but Not in Multidrug Resistant MCF-7/Adr, Cells by Cross-Coupled NF-κB/p65 and c-Fos Transcription Factors and Their Interaction with the CAAT Region†

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ABSTRACT: In this study, the possible involvement of repressor protein(s) in suppressing *MDR*1 promoter activity in the sensitive MCF-7 human breast cancer cell line and its drug resistant variant MCF-7/Adr was investigated. RT-PCR revealed that *MDR*1 mRNA is under detectable levels in MCF-7, while it is highly expressed in MCF-7/Adr cells. After treatment of MCF-7 cells with cycloheximide (CHX), *MDR*1 mRNA reached detectable levels, suggesting that *MDR*1 mRNA expression might be controlled by a labile negative regulatory protein(s) in MCF-7 cells. In electrophoretic mobility shift assays (EMSA) using a 5′-end-labeled 241 bp *MDR*1 promoter DNA fragment (residues −198 to +43) as a probe, one protein complex that specifically binds to the CAAT region of the *MDR*1 promoter was detected in MCF-7, but not MCF-7/Adr. In addition, following transient transfections of MCF-7 and MCF-7/Adr cells with a pGL3-Basic plasmid construct containing a CAAT-deleted *MDR*1 promoter DNA fragment, a significant increase in luciferase activity was observed compared to the 241 bp *MDR*1 promoter in MCF-7 but not MCF-7/Adr cells. Moreover, a ds CAAT oligomer, cloned upstream of the SV-40 promoter in the pGL3-Promoter vector, resulted in a 70−80% decrease in luciferase activity in MCF-7 cells. To identify the CAAT binding protein complex, EMSA and SDS−PAGE were performed. Two proteins with molecular masses of about 65 and 60 kDa were detected by silver staining. Western blot analysis revealed that this complex consists of NF-κB/p65 and c-Fos transcription factors. Moreover, incubating MCF-7 nuclear extracts with antibodies specific for NF-κB/p65 or c-Fos in EMSAs almost completely inhibited formation of the complex, supporting the association of NF-κB/p65 and c-Fos. Therefore, this study provides evidence that molecular interplay between the NF-κB/p65 and c-Fos transcription factors exhibits a negative regulatory function on *MDR*1 promoter by interacting with the CAAT region in MCF-7.

One of the major obstacles in cancer chemotherapy is the development of multidrug resistance (MDR)[1] in humans. The MDR phenotype is generally acquired after chemotherapy in some carcinomas, including ovarian and breast, and in adult acute myeloid leukemia (AML). A major factor in the development of MDR is the overexpression of P-glycoprotein (P-gp). P-gp, a 180−190 kDa protein product of the *MDR*1 gene, is an energy-dependent transmembrane efflux pump that binds to and transports various structurally unrelated compounds to maintain drug concentrations below cytotoxic levels in cells (*1*, *2*). Transfection of drug-sensitive human cancer cells with the *MDR*1 gene has been shown to cause MDR (*3*−*5*). In addition, clinical studies have shown that the overexpression of P-gp is a significant prognostic marker for assessing therapeutic efficacy in some human cancers (*6*−*8*). It is known that the *MDR*1 gene is normally overexpressed in the colon, adrenal glands, liver, and kidney; therefore, inherent drug resistance is more frequently observed in solid tumors arising from these tissues (*2*). In addition, the *MDR*1 gene is shown to be upregulated in response to cellular stress triggered by anticancer drugs, carcinogens, heavy metals, ultraviolet light (UV), heat shock, serum starvation, phosphatase inhibitors, and phorbol esters in some cultured human cancer cells (*1*, *9*−*12*).

Many studies have provided evidence implicating complex mechanisms for transcriptional regulation of the *MDR*1 gene in human cancer cells (*2*, *13*). Consistent with this complexity, the human *MDR*1 gene promoter contains a number of recognition sites for SP1, NF-Y, and YB-1 transcription factors (*13*), and these transcription factors have been shown to upregulate *MDR*1 promoter activity (*13*−*15*). It has been shown that the inverted CCAAT box (Y-box) binding protein

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[1] Abbreviations: CHX, cycloheximide; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis; P-gp, P-glycoprotein; MDR, multidrug resistance; AML, acute myeloid leukemia; TCA, tricholoroacetic acid; β-gal, β-galactosidase; ONPG, *o*-nitrophenyl β-D-galactopyranoside; EtBr, ethidium bromide; ER, estrogen receptor; IL-6, interleukin 6; FCS, fetal calf serum.

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YB-1 is directly involved in the stress-induced activation of the *MDR*1 gene in human KB head and neck cancer cells (*15*). Recently, Bargou et al. (*16*) reported that the nuclear localization of YB-1 and *MDR*1 expression are closely associated in several breast cancer cell lines as well as in some primary breast cancers. It was also reported that NF-Y and SP1 are most likely involved in upregulating the *MDR*1 promoter in some tumor cell lines (*14*). Recently, it was reported that *MDR*1 promoter activity might be linked to the cyclic AMP-dependent protein kinase signaling pathway, which plays a role in activating SP1 (*17*). Moreover, other data showed that the nuclear protein NF-R1 specifically binds to two unrelated motifs, ATTCAGTCA and GC-box, and has a negative effect on regulating *MDR*1 gene transcription in doxorubicin-resistant K562 cells (*18*). Taken together, these studies suggest that a number of factors interact with the *MDR*1 promoter, indicating that *MDR*1 promoter activity is controlled by complex mechanisms and that there are tissue-specific variations in its regulation. Most previous studies on the regulation of *MDR*1 gene expression have concentrated on identifying transcription factors involved in the induction of *MDR*1 promoter activity in drug-resistant cancer cell lines. It is well documented that the *MDR*1 gene in MCF-7 breast cancer cells is not transcribed, but it is significantly upregulated in its MDR variant MCF-7/Adr (*4*). The molecular mechanism of suppressing *MDR*1 gene expression has not been explored previously. In this study, we attempted to identify DNA binding proteins involved in repressing *MDR*1 promoter activity in the drug-sensitive MCF-7 and drug-resistant MCF-7/Adr human breast cancer cell lines. We provide evidence for the first time that a protein complex consisting of NF-κB/p65 and c-Fos is involved in the negative regulation of *MDR*1 promoter activity by interacting with the CAAT region of this promoter in MCF-7, but not MCF-7/Adr, cells.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions. The MCF-7 human breast cancer cell line and its multidrug resistant variant MCF-7/Adr cell line were obtained from Dr. Kenneth H. Cowan (National Cancer Institute, Bethesda, MD). Cells were maintained in RPMI-1640 medium with 10% fetal calf serum (FCS) and 100 ng/mL each of penicillin and streptomycin (Life Technologies, Grand Island, NY) at 37 °C in 5% CO_2. MCF-7/Adr cells were grown in the presence of 6−8 μg/mL doxorubicin, which was removed from the media 1 week before each assay.

*Isolation of Total RNA and RT-PCR. MDR*1 mRNA levels were analyzed by RT-PCR as follows: Total RNA from MCF-7 and MCF-7/Adr cells was isolated using a modified SDS−phenol technique as previously described (*19*). One microgram of total RNA was then used in reverse transcription reactions with AMV-reverse transcriptase and random primers (Promega, Madison, WI) as described by the manufacturer. The resulting total cDNA was then used in PCR to measure the *MDR*1 mRNA levels. The intactness of total RNA was confirmed by tight bands of 28S and 18S rRNA separated on denaturing agarose gels and visualized by ethidium bromide (EtBr) staining (*20*). The primers and amplification conditions of PCR were as follows:

*MDR*1 (forward)
5′ CCCATCATTGCAATAGCAGG (2596−2615)

*MDR*1 (reverse)
5′ GTTCAAACTTCTGCTCCTGA (2733−2752)

β-actin (forward)
5′ CAGAGCAAGAGAGGCATCCT (216−235)

β-actin (reverse)
5′ TTGAAGGTCTCAAACATGAT (405−424)

The reactions were performed at 94 °C for denaturation, 60 °C for annealing, and 72 °C for amplification for 30 cycles. The β-actin mRNA levels were used as internal controls in RT-PCR. β-Actin primers were used at a 1:3 ratio to the *MDR*1 primers to achieve linear amplification conditions. The amplified fragments were separated on 2% agarose gels and visualized by EtBr staining.

Inhibition of Protein Synthesis Using Cycloheximide. Protein synthesis inhibition in MCF-7 and MCF-7/Adr cells (grown in 24-well plates) was determined by treating the cells with various concentrations of cycloheximide (CHX) for 4 h and then measuring the total incorporation of [^{35}S]-methionine (4 μCi/mL) into TCA precipitable macromolecules by scintillation counting. The results were normalized to protein concentrations and expressed relative to untreated controls. The cells were then treated with CHX at its optimal concentration that inhibited 95% of protein synthesis for 0, 6, and 24 h, and *MDR*1 mRNA levels were detected by RT-PCR as described above.

Preparation of MDR1 Promoter Fragment by PCR. The 241 and 155 bp *MDR*1 promoter fragments spanning −198 to +43 and −112 to +43 sites, respectively, were produced by PCR using MCF-7/Adr genomic DNA as a template with the following primers:

*MDR*1 promoter (forward)
5′ CTAGAGAGGTGCAACGGA (−198 to −181)

*MDR*1 promoter (forward)
5′ ACCTGTTTCGCAGTTTCTC (−112 to −94)

*MDR*1 promoter (reverse)
5′ GCGGCCTCTGCTTCTTTGA (+25 to +43)

Amplification conditions were as described above except that 5% deionized formamide was added to the reaction, and the annealing temperature was 55 °C. The 241 bp PCR product was then separated on 2% agarose gels and purified using the Qiaex II gel extraction kit (Qiagen, Valencia, CA) as described by the manufacturer prior to end-labeling reactions.

Preparation of Nuclear Extracts. Cells (about 10^9) grown in large flasks were scraped, washed with phosphate-buffered saline (PBS), pH 7.1, and then lysed in lysing buffer containing 0.25 M sucrose, 0.01 M Tris, pH 7.4, 0.01 M NaCl, 0.003 M $MgCl_2$, and 0.5% IGEPAL-40 detergent for 5 min on ice. After centrifugation at 5000*g* for 5 min, they were washed with a 5× volume of buffer A (0.01 M Hepes, pH 7.5, 0.02 M $MgCl_2$, 0.015 M KCl, 0.1 mM EDTA, 0.001 M DTT, and 0.001 M PMSF) and then resuspended

in 5−10 mL of buffer A. The nuclear pellet was then homogenized on ice by 10−20 strokes of an S-pestal and then spun at 13000*g* for 10 min. The proteins were precipitated by adding 80 μL of 0.3 M ammonium sulfate for 30 min on ice. Following centrifugation at 50000*g* at 4 °C for 60 min, the nuclear proteins were resuspended in 4 mL of buffer A and reprecipitated with 0.2 g/mL ammonium sulfate. The pellets were collected by centrifugation at 50000*g* for 15 min and then resuspended in 500 μL of buffer C (0.05 M Hepes, pH 7.5, 0.03 M KCl, 0.1 mM EDTA, 0.001 M DTT, 0.001 M PMSF, and 10% glycerol). After the samples were dialyzed against buffer C (500× volume) at 4 °C for 18−24 h, they were cleared by microcentrifugation at 13000*g* for 10 min and stored at −80 °C. The concentration of nuclear protein was determined by the Bradford assay using the Bio-Rad (Richmond, CA) protein detection kit as described by the manufacturer.

Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts (8−10 μg of protein) were preincubated in 15 μL of binding buffer (0.04 M Hepes, pH 7.5, 0.04 M KCl, 8% glycerol, 0.64 M PMSF, 0.8 mM DTT, and 0.1 mM EDTA) containing 1.5 μg of poly(dI•dC) (Pharmacia, Piscataway, NJ) with or without oligonucleotides used as competitors at 30 or 25 °C for 15 min. Three to five nanograms of 5′-end-labeled 241 bp (−198 to +43) or 155 bp (−112 to +43) *MDR*1 promoter fragments (60000 cpm) was added to the reaction, and the mixture was incubated at 30 or 25 °C for 15 min. End labeling of DNA fragments with [γ-^{32}P]ATP (Amersham, Arlington Heights, IL) was performed using T4 DNA kinase (Promega), and the specific activity of the probes was determined by scintillation counting after TCA precipitation as described by the manufacturer. Competitor double-stranded (ds) oligonucleotides were used at 10−200-fold molar excess. The 5′-end (forward) DNA sequences of ds oligomers used in the gel shift assays were as follows:

CAAT-like motif:
ATCAGCATTCAGTCAATCCGGGCC

CAAT-deleted motif: ATCAGCATTCAGTCCGGGCC

GC-box-like motif:
GGAACAGCGCCGGGGCGTGGGCTGA

nonspecific motif:
GAAGCCTGAGCTCATTCGAGTAGC

NF-κB motif: AGTTGAGGGGACTTTCCCAGGC

SP1 motif: ATTCGATCGGGGCGGGGCGAGC

AP1 motif: CGCTTGATGAGTCAGCCGGAA

The reaction mixtures were separated on 5% native polyacrylamide gels, and autoradiography was performed by exposing dried gels to Kodak X-Omat film. The ds oligonucleotides containing CAAT-like, CAAT-deleted, GC-box, and nonspecific motifs were synthesized and annealed by Midland Certified Reagent Co. (Midland, TX). The ds oligomers containing NF-κB, SP1, and AP1 motifs were purchased from Promega. Supershift experiments were performed by incubating nuclear extracts with supershift reagents of the rabbit polyclonal antibodies to NF-κB/p65 (sc-109-X), c-Fos (sc-7202-X) (Santa Cruz Biotechnology, Santa Cruz, CA), or purified rabbit IgG at 25 °C for about 45 min prior to addition of probes in EMSAs as described above.

Plasmid Construction. The 241 bp *MDR*1 promoter fragment (residues −198 to +43) was amplified by PCR using primers 1 and 4, containing *Nhe*I and *Bgl*II sites (underlined sequences), respectively, as described above. The vectors were digested with NheI and BglII and treated with calf intestinal alkaline phosphatase (Promega) prior to ligation as described by the manufacturer. The gel-purified PCR product (241 bp *MDR*1 promoter fragment) was digested with NheI and BglII and cloned into the pGL3-Basic (Promega) vector using T4 DNA ligase (Promega) as described by the manufacturer. The 237 bp mutant *MDR*1 promoter fragment lacking the CAAT-like motif (residues −116 to −113) was generated by two independent PCRs with primers 1-2 and 3-4, respectively, using the wild-type (wt) 241 bp *MDR*1 promoter cDNA fragment generated by PCR as a template. The overlapping primers 2 and 3 contain a deletion at the −116 to −113 sites (CAAT) of the *MDR*1 promoter. The PCR products above were gel purified separately and then used as templates in a third PCR with primers 1 and 4 to generate the mutated 237 bp *MDR*1 promoter fragment lacking the CAAT motif. The CAAT-deleted 237 bp *MDR*1 promoter fragment was purified from 2% agarose gels and then cloned into the pGL3-Basic vector as described above. The DNA sequences of the wt (241 bp) or mutated (237 bp) *MDR*1 promoter fragments were confirmed by direct sequencing. The sequences of the primers used above are as follows:

primer 1: 5′ GCGCTAGCCTAGAGAGGTGCAACG
(−198 to −182)

primer 2:
5′ CCCGGCCCGGACTGAATGCTGATTCCTC
(−103 to −112 and −117 to −134)

primer 3:
5′ AGGAATCAGTCCGGGCCGGGAGCAGTC
(−133 to −125 and −113 to −96)

primer 4: 5′ GCAGATCTGCGGCCTCTGCTTCTT
(+28 to +43)

The ds oligonucleotides containing the CAAT-like motif, GC-box-like motif, CAAT-deleted motif, and nonspecific motif were synthesized and annealed by Midland Certified Reagent Co. These oligonucleotides are designed to contain 5′ *Nhe*I and 3′ *Bgl*II compatible (sticky) ends (underlined sequences) to clone into the pGL3-Promoter vector (Promega), containing the SV-40 promoter downstream of the cloning sites, as described above. The DNA sequences of the oligonucleotides were as follows:

CAAT-like motif: P-CTAGC-
ATCAGCATTCAGTCAATCCGGGCC
TAGTCGTAAGTCAGTTAGGCCCGGTCTAG-P

CAAT-deleted motif: P-CTAGC-
ATCAGCATTCAGTCCGGGCC
TAGTCGTAAGTCAGGCCCGGTCTAG-P

nonspecific motif: P-CTAGC-
GAAGCCTGAGCTCATTCGAGTAGC
CTTCGGACTCGAGTAAGCTCATCGTCTAG-P

The cloning of these oligonucleotides into the pGL3-Promoter vector was confirmed by restriction enzyme digestion and direct sequencing. All of the resulting plasmids were amplified in *Escherichia coli* JM109 and then isolated using the Qiagen plasmid isolation kit as described by the manufacturer before transfection.

Transient Transfections and Luciferase and β-Galactosidase Assays. Cells [$(2-5) \times 10^5$ cells/well] were seeded into six-well plates and grown in 5 mL of Richter's modified medium (Life Technologies) with 10% FCS for 18 h before transfection. The cells were then cotransfected with 2.5−3 μg/well plasmid constructs and pSV-β-galactosidase control plasmid (Promega) by the calcium phosphate−DNA coprecipitation method using the modified bovine serum (MBS) mammalian transfection kit (Stratagene, La Jolla, CA) in 5 mL of the medium containing 6% MBS for 6−8 h as described by the manufacturer. After the transient transfectants were recovered with Richter's medium with 10% FCS for 18 h, luciferase and β-galactosidase (β-gal) activities were measured using Promega's luciferase and β-gal enzyme assay systems as follows: Cells were washed twice with PBS and then lysed in 400 μL of reporter lysis buffer containing 25 mM Tris−phosphate, pH 7.8, 2 mM DTT, 2 mM diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, and 1% Triton X-100 at room temperature for 15 min. The luciferase reaction was then initiated by autoinjection of 100 μL of luciferase assay reagent containing 20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 mM coenzyme A, 470 mM luciferase, and 530 mM ATP, to 20 μL of lysates using a luminometer (Turner Designs, Sunnyvale, CA). The light intensity of the luciferase reactions measured in the lysates of the transient transfectants was normalized to their β-gal activity, used as an internal control. The β-gal activity of the lysates was determined as follows: 120 μL of lysates in reporter lysis buffer were incubated with 150 μL of assay buffer containing 200 mM sodium phosphate, pH 7.3, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, and 1.33 mg/mL *o*-nitrophenyl β-D-galactopyranoside (ONPG) at 37 °C for 30 min. The reaction was stopped by addition of 1 M sodium carbonate and then read using a spectrophotometer at 420 nm. All of the transfections and assays were performed in triplicate.

Western Blot Analysis. Total cellular proteins (80 μg/lane) were separated on 5−15% SDS−polyacrylamide gels containing 4.5 M urea as described (*21*). After the proteins were transferred to an Immobilon membrane (Millipore, Bedford, MA) using a semidry blotter (Pharmacia) as described by the manufacturer, Western blot analysis was performed using 1 μg/mL NF-κB/p65 (sc-372 and sc-109), NF-κB/p50 (sc-114), c-Fos (sc-52), and c-Jun (sc-822) rabbit polyclonal antibodies (Santa Cruz Biotechnology) and horseradish peroxidase conjugated anti-rabbit secondary antibody (Amersham) at a 1:2500 dilution. Proteins were visualized using the ECL protein detection kit (Amersham) as described by the manufacturer.

Isolation and Analysis of the MDR1 Promoter Binding Protein in MCF-7 Cells. The *MDR*1 promoter binding protein was isolated by gel mobility shift assay coupled with SDS−PAGE as described (*22*). In short, the [^{32}P]ATP-labeled 241 bp wt *MDR*1 promoter fragment was incubated with MCF-7 or MCF-7/Adr nuclear extracts, and the protein/DNA complexes were separated from free probes by electrophoresis in 5% native polyacrylamide gels in Tris−borate buffer as described above. The position of the shifted band in the polyacrylamide gel was determined by autoradiography. To analyze the components of the shifted band, each lane of the gel was then excised and soaked in 50−100 μL of 2× SDS−PAGE sample buffer (0.05 M urea, 0.06 mM DTT, 0.08 M Tris-HCl, pH 6.8, 0.04% bromophenol blue, 4% SDS, and 20% glycerol) for 15 min to dissociate the DNA/protein complexes. The gel slices were then placed horizontally onto a stacking gel of 5−15% SDS−polyacrylamide with 4.5 M urea and run overnight as described. Molecular weight and migration patterns of the *MDR*1 promoter binding protein were determined by silver staining the SDS−polyacrylamide gels using the silver staining kit (Sigma, St. Louis, MO) as described by the manufacturer. The identities of these proteins were determined by immunoblotting as described above.

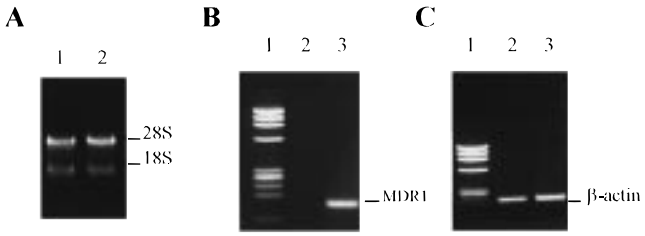

FIGURE 1: Detection of *MDR*1 mRNA levels in MCF-7 and MCF-7/Adr cells. (A) Total cellular RNA from MCF-7 and MCF-7/Adr (lanes 1 and 2, respectively) was isolated using a modified SDS/phenol method, run on 0.8% denaturing agarose gels, and visualized by EtBr staining as described. The 28S and 18S rRNAs are indicated. The *MDR*1 (B) and β-actin (C) mRNA levels in MCF-7 and MCF-7/Adr cells (lanes 2 and 3, respectively) were measured by RT-PCR using 1 μg of total cellular RNA from each cell line. The 167 bp *MDR*1 and 208 bp β-actin cDNA fragments generated by RT-PCR were run on 2% agarose gels and visualized by EtBr staining. Lane 1 in panels B and C contains *Hae*III-cut φX174 DNA fragments used as molecular weight markers. The results shown are representative of three independent experiments.

RESULTS

Effects of Protein Synthesis Inhibition by CHX Treatment on MDR1 mRNA Levels in MCF-7 and MCF-7/Adr Cells. We have previously reported (*21*) the expression of high levels of P-gp, encoded by the *MDR*1 gene, in MCF-7/Adr cells which show about 6000-, 8000-, 1000-, and 1500-fold resistance to doxorubicin, vincristine, taxol, and etoposide (VP-16), respectively, compared to sensitive MCF-7 cells. To uncover the molecular mechanism(s) of negative regulation of the *MDR*1 gene in MCF-7 cells, the mRNA levels of *MDR*1 in MCF-7 and MCF-7/Adr cells were first determined by RT-PCR. Total cellular RNA from MCF-7 and MCF-7/Adr cells was isolated using our modified phenol−SDS method (Figure 1A, lanes 1 and 2, respectively); the intactness of total RNA was determined by tight bands of 28S and 18S rRNAs in EtBr-stained denaturing agarose gels. One microgram of RNA from each cell line was then used in RT-PCR to measure the mRNA levels of *MDR*1 and β-actin. As seen in Figure 1B, a high level of *MDR*1 mRNA was detected in MCF-7/Adr (lane 3), while *MDR*1 mRNA was under detectable levels in MCF-7 cells (lane 2). The β-actin mRNA levels, used as internal controls, were similar in both cell lines (Figure 1C, lanes 2 and 3). In addition, to

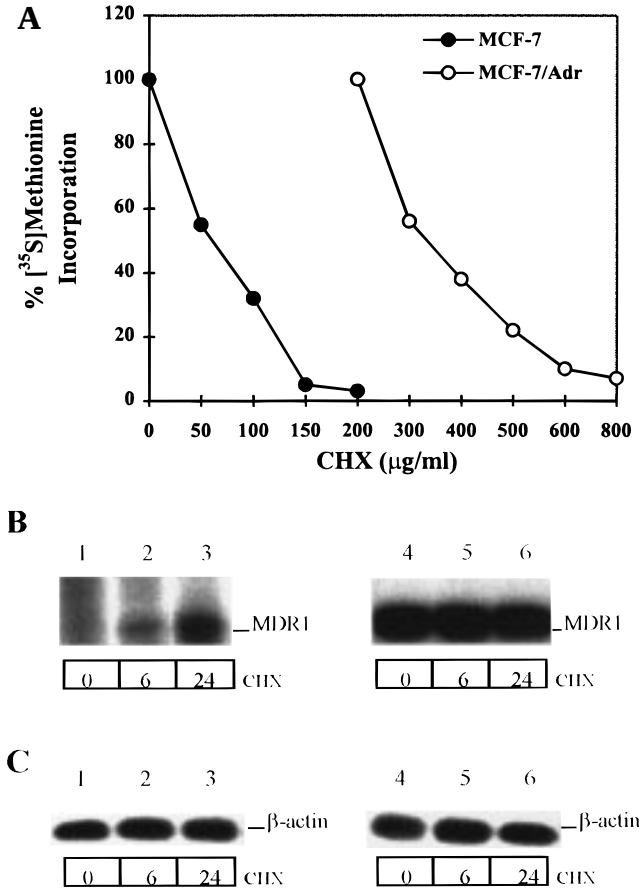

FIGURE 2: Analysis of the effects of CHX treatment on *MDR*1 mRNA levels in MCF-7 and MCF-7/Adr cells. (A) The CHX concentrations that inhibit 95% of protein synthesis in MCF-7 (closed circles) and MCF-7/Adr (open circles) cells were determined as described. Cells were grown in the absence or presence of increasing concentrations of CHX for 2 h and then pulsed with 4 μCi/mL [^{35}S]methionine. The TCA-precipitated labeled macromolecules were then measured by scintillation counting in CHX-treated cells and untreated controls. Duplicate determinants were used for each treatment. The *MDR*1 (B) and β-actin (C) mRNA levels following treatment with the optimal CHX concentrations were determined in MCF-7 (lanes 1−3) and MCF-7/Adr (lanes 4 and 5) cells at various times (0, 6, and 24 h) by RT-PCR using [α-^{32}P]-CTP. The labeled 167 bp *MDR*1 and 208 bp β-actin cDNA fragments were run on 2% agarose gels and visualized by autoradiography. Incubation time is indicated at the bottom of the gels. The results are representative of two independent experiments.

determine whether inhibiting protein synthesis has any impact on the *MDR*1 mRNA levels in MCF-7 and MCF-7/Adr cells, CHX treatments were performed as described in Experimental Procedures. First, the optimum concentrations of CHX that inhibit 95% of protein synthesis in these cells were determined by measuring the total [^{35}S]methionine incorporation into TCA-precipitable macromolecules and determined to be 150 and 800 μg/mL for MCF-7 and MCF-7/Adr, respectively (Figure 2A). The effect of protein synthesis inhibition by CHX treatment at various time points on *MDR*1 mRNA levels in MCF-7 and MCF-7/Adr cells was then determined by RT-PCR as described in Experimental Procedures. As shown in Figure 2B, a low level of [^{32}P]CTP-incorporated 167 bp *MDR*1 cDNA fragment was detected in MCF-7 cells after 6 h of CHX treatment (lane 2) and at significantly higher levels following 24 h treatment (lane 3), while *MDR*1 mRNA expression was not detectable in untreated MCF-7 cells (lane 1). CHX treatment did not have any significant effect on the *MDR*1 mRNA level in MCF-7/Adr cells (Figure 2B, lanes 4−6). The specificity of the 167 bp RT-PCR product of *MDR*1 was confirmed by direct sequencing of the gel-purified cDNA fragments in both cell lines after each treatment. The 167 bp *MDR*1 cDNA fragment has been shown to be specific for *MDR*1, but not *MDR2*, and has been used in many studies to detect *MDR*1 mRNA expression levels (*23*). β-Actin mRNA levels remained unchanged following CHX treatment in both MCF-7 and MCF-7/Adr cells (Figure 2C, lanes 1−3 and 4−6, respectively). These results suggest that *MDR*1 mRNA expression might be controlled by a labile negative regulatory factor(s) in MCF-7 cells, but not in MCF-7/Adr cells.

Detection of DNA Binding Protein(s) Interacting with the Proximal MDR1 Promoter in MCF-7 Cells. The human *MDR*1 promoter contains several transcription factor recognition sites including SP1, Y-box, AP1-like, and CAAT-box-like motifs between 198 bp upstream and 43 bp downstream of the major start site of transcription (+1), as shown in Figure 3. It should be noted that two of these recognition sites on the *MDR*1 promoter, AP1-like (TCAGTCA) and CAAT-box-like (GTCAAT), are different from their original motifs (TGAGTCA for AP1 and GCCAAT for CAAT-box) by one nucleotide. In an attempt to detect DNA binding proteins interacting with the *MDR*1 promoter, electrophoretic mobility shift assay (EMSA) was performed using a 5′-end-labeled 241 bp DNA probe, spanning the −198 to +43 region of the *MDR*1 promoter, with nuclear extracts from MCF-7 and MCF-7/Adr as described in Experimental Procedures. Interestingly, two major protein complexes (C1 and C2) that interact with the *MDR*1 promoter probe were detected using MCF-7 extracts (Figure 4, lane 2). The C1 complex seemed to be highly specific for the *MDR*1 promoter probe in MCF-7 cells, as revealed by its complete competition with 200-fold molar excess of unlabeled 241 bp *MDR*1 DNA fragment (cold probe) as seen in Figure 4, lane 3. However, the C2 complex binding to the *MDR*1 probe was not significantly affected by excess cold probe (Figure 4, lane 3), showing that C2 interacts with the probe with very low affinity or nonspecifically. To determine the specific recognition site of C1 on the *MDR*1 promoter probe, several unlabeled ds oligomers containing CAAT-like, CAAT-deleted, GC-box, nonspecific, NF-κB, SP1, and AP1 motifs were used at 200-fold molar excess for competition of C1 in MCF-7 extracts as described in Experimental Procedures. As shown in Figure 4, the incubation of excess CAAT-like oligomer dramatically reduced the binding of C1 to the *MDR*1 promoter probe (lane 4), whereas the CAAT-deleted oligomer did not have any effect on C1/probe binding (lane 5), similar to GC-box, nonspecific, NF-κB, SP1, and AP1 oligomers which did not compete with C1 (lanes 6−10, respectively) in MCF-7 nuclear extracts. In addition, no specific *MDR*1 promoter binding protein was detectable in MCF-7/Adr nuclear extracts (Figure 4, lane 11). These results show that C1 specifically binds to the CAAT region of the *MDR*1 promoter in MCF-7 cells, but not in MCF-7/Adr cells. These data and the CHX experiments (Figure 2) further suggest that C1 might be involved in suppressing *MDR*1 gene expression in MCF-7 cells. To assess the affinity of C1 in MCF-7 extracts for the CAAT segment of the *MDR*1 promoter, competition experiments were performed using increasing concentrations of CAAT-like and CAAT-deleted

-198
CTAGAGAGGTGCAACGGAAGCCAGAACATTCCTCCTGGAAATTCAACCTGTTTCGCA

-141 AP1-like motif
GTTTCTCGAGGAATCAGCATTCAGTCAATCCGGGCCGGGAGCAGTCATCTGTGGTGA
CAAT-like motif SP1
-84 Y-box SP1 (GC-box)
GGCTGATTGGCTGGGCAGGAACAGCGCCGGGGCGTGGGCTGAGCACAGCCGCTTCG
SP1 SP1
-28
CTCTCTTTGCCACAGGAAGCCTGAGCTCATTCGAGTAGCGGCTCTTCCAAGCTCAAA
+1
+30
GAAGCAGAGGCCGC

FIGURE 3: DNA sequence of the *MDR*1 proximal promoter. The human *MDR*1 proximal promoter DNA sequence, spanning residues −198 to +43 with respect to the major transcriptional start site (+1), is shown. Several transcription factor recognition sites are underlined (SP1 and CAAT-box-like motif) or overlined (AP1-like and Y-box).

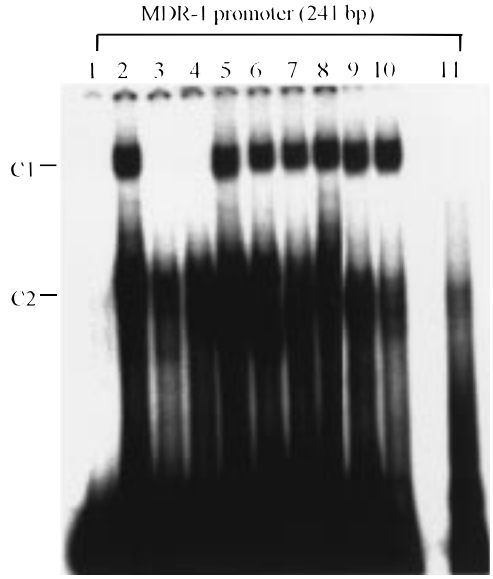

FIGURE 4: Detection of DNA binding proteins by EMSA in MCF-7 and MCF-7/Adr cells. The 5′-end-labeled 241 bp *MDR*1 promoter DNA fragment, used as a probe, was incubated with MCF-7 nuclear extracts in the absence (lane 2) or presence of competitor cold probe (lane 3) or oligomers containing CAAT-like, CAAT-deleted, GC-box, nonspecific, NF-κB, SP1, and AP1 (lanes 4−10, respectively) at 200-fold molar excess in EMSA as described. Nuclear extracts of MCF-7/Adr cells were incubated with the probe in the absence of any competitors (lane 11) for EMSA. Lane 1 contains unbound probe. Protein/DNA complexes were separated on 5% native polyacrylamide gels and visualized by autoradiography. Results shown are representative of three independent experiments.

oligomers in EMSAs. As Figure 5A shows, CAAT-like oligomer at 10-, 50-, 100-, and 200-fold molar excess significantly reduced (about 80%, 90%, 95%, and 98%, respectively) the binding of C1 to the *MDR*1 promoter probe (lanes 3−6, respectively) compared to controls without competitor oligomers (lane 2). However, competition experiments with the CAAT-deleted oligomer at 10−200-fold molar excess did not have any significant effect on C1/*MDR*1 promoter probe binding in MCF-7 extracts (Figure 5A, lanes 7−11). The same protein complex (C1) with high affinity to CAAT on the *MDR*1 promoter was detected when a 155 bp 5′-end-labeled *MDR*1 promoter DNA (spanning residues −112 to +43) was used with MCF-7, but not MCF-7/Adr, nuclear extracts (data not shown). Similarly, using the 5′-end-labeled ds CAAT-like oligomer as a probe in EMSA with nuclear extracts from MCF-7, one specific protein complex (C1) interacting with the probe (Figure 5B, lane 2) was detected and completely competed with excess unlabeled ds CAAT-like oligomer (cold probe), but not with the CAAT-deleted or GC-box oligomers (Figure 5B, lanes 3−5, respectively). No protein/DNA complex was observed using MCF-7/Adr extracts with the CAAT-like probe (Figure 5B, lanes 6−9). These results confirm that MCF-7 nuclear extracts contain a protein complex (C1) which specifically binds to the CAAT region of the *MDR*1 promoter. In control experiments, ds 5′-end-labeled SP1 and AP1 oligomers were used as probes in EMSAs using nuclear extracts from MCF-7 and MCF-7/Adr cells (Figure 6, lanes 2−4 and 8−10 and lanes 5−7 and 11−13, respectively) and detected SP1 and AP1 binding proteins in both cell lines. Interestingly, four SP1 complexes were detected in nuclear extracts of MCF-7, and the levels of SP1 binding proteins in MCF-7/Adr nuclear extracts were significantly lower than in MCF-7 (compare lanes 5−7 to 2−4 in Figure 6). Multiple SP1 complexes may be present due to the interaction of different spliced variants of this protein with the SP1 oligonucleotide. Moreover, EMSA revealed that AP1 binding activity levels in both MCF-7 and MCF-7/Adr nuclear extracts were similar (Figure 6, lanes 11−13 and 8−10, respectively).

Analysis of MDR1 Promoter Activity in MCF-7 and MCF-7/Adr Cells. To determine whether the CAAT segment of the *MDR*1 promoter is involved in negative control of promoter activity, wild-type (wt) 241 bp *MDR*1 promoter (residues −198 to +43) and its mutated (CAAT-deleted) 237 bp DNA fragments were cloned into the luciferase-expressing pGL3-Basic vector upstream of the luciferase gene (Figure 7A) as described in Experimental Procedures. The activity of the *MDR*1 promoter was measured as a function of luciferase activity, normalized to β-galactosidase (β-gal) levels which reflect equal transfection efficiency, following cotransfections of pGL3-Basic plasmid constructs with the pSV-β-gal control plasmid into MCF-7 and MCF-7/Adr cells as described in Experimental Procedures. As seen in Figure 7B, a high level of promoter activity of the wt 241 bp *MDR*1 promoter DNA fragment was detected in MCF-7/Adr, about a 15-fold increase in luciferase activity compared to that of MCF-7 cells, in which luciferase activity was similar to that of the pGL3-Basic control vector. However, when MCF-7 cells were transfected with the pGL3-Basic plasmid containing the CAAT-deleted 237 bp *MDR*1 promoter DNA

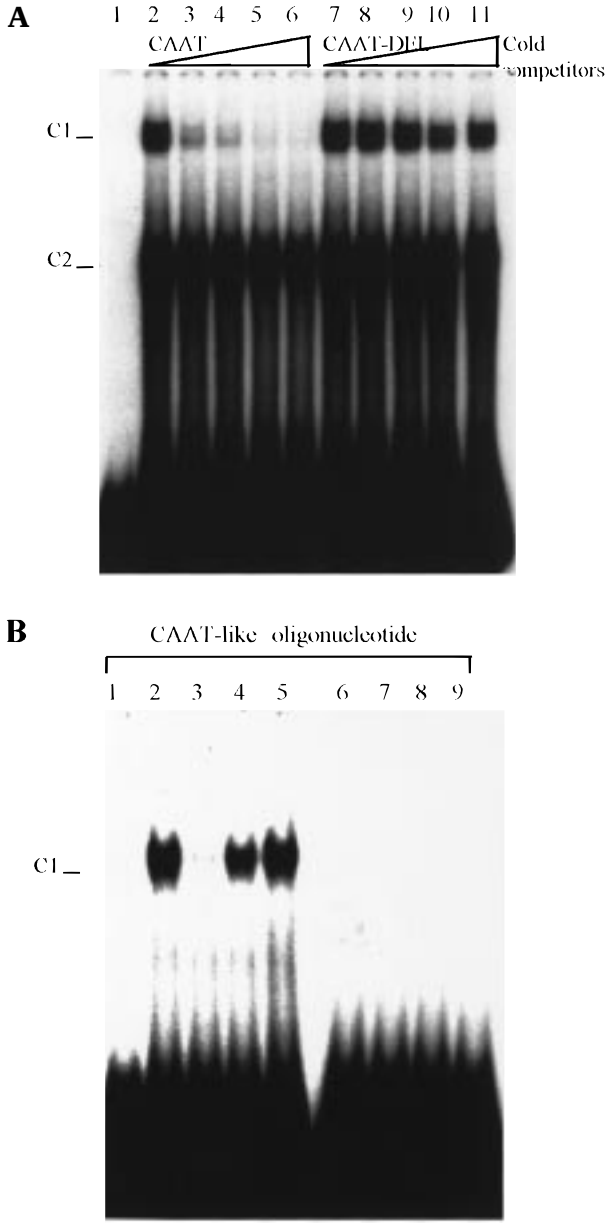

FIGURE 5: Analysis of the *MDR*1 promoter binding complex (C1) and CAAT interaction by EMSA. (A) The affinity of the C1 complex to the CAAT segment on the promoter is tested by incubating MCF-7 nuclear extracts with 5′-end-labeled 241 bp *MDR*1 promoter probe in the absence (lanes 2 and 7) or presence of increasing concentrations (10-, 50-, 100-, and 200-fold molar excess) of ds oligomers containing CAAT-like (lanes 3−6, respectively) and CAAT-deleted motifs as competitors in EMSAs. (B) Nuclear extracts from MCF-7 (lanes 2−5) and MCF-7/Adr (lanes 6−9) cells were incubated with the 5′-end-labeled ds CAAT-like oligomer, used as a probe, in the absence (lanes 2 and 6, respectively) or presence of excess (200-fold) unlabeled oligomers containing CAAT-like (lanes 3 and 7, respectively), CAAT-deleted (lanes 4 and 8, respectively), and GC-box (lanes 5 and 9, respectively) motifs as competitors. Lane 1 in panels A and B contains unbound probes. Protein/DNA complexes were run on 5% native polyacrylamide gels and visualized by autoradiography. The results shown are representative of three independent experiments.

fragment, luciferase activity increased dramatically (about 12-fold) compared to MCF-7 cells transfected with the plasmid containing the wt 241 bp promoter DNA fragment (Figure 7B).

The deletion of CAAT did not have any significant effect on *MDR*1 promoter activity in MCF-7/Adr cells, causing a

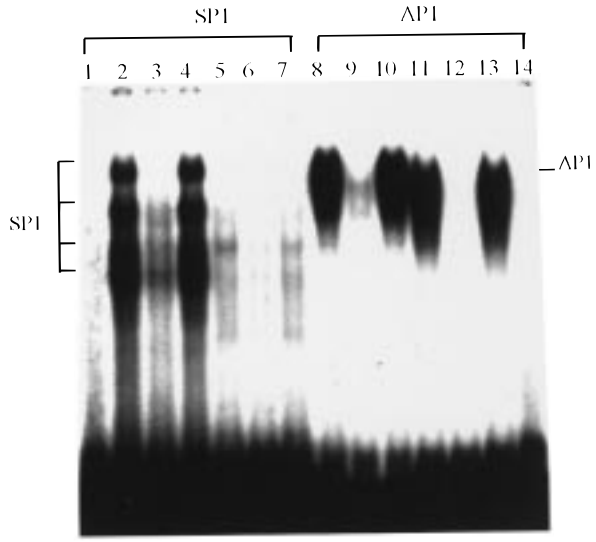

FIGURE 6: Detection of SP1 and AP1 binding proteins in MCF-7 and MCF-7/Adr cells by EMSA. DNA binding proteins interacting with SP1 or AP1 recognition sequences were detected by incubating nuclear extracts from MCF-7 (lanes 2−4 and 8−10, respectively) and MCF-7/Adr cells (lanes 5−7 and 11−13, respectively) with 5′-end-labeled ds oligomers containing SP1 or AP1 motifs in EMSAs. Unlabeled SP1 or AP1 oligomers (cold probes) (lanes 3 and 6 and lanes 9 and 12, respectively) were used as specific competitors for the SP1 and AP1 probes, respectively. The unlabeled AP1 oligomer for the SP1 probe (lanes 4 and 7) and unlabeled SP1 oligomer for the AP1 probe (lanes 10 and 13) were used as nonspecific competitors at 200-fold molar excess in the above EMSAs. Lanes 1 and 14 contain unbound SP1 and AP1 probes, respectively. Protein/DNA complexes were run on 5% polyacrylamide gels and visualized by autoradiography. The results shown are representative of two independent experiments.

slight increase (about 1.3-fold) in luciferase activity compared to the wt CAAT insert (Figure 7B). These results are somewhat consistent with the EMSA results, suggesting that CAAT is involved in the downregulation of *MDR*1 promoter activity, possibly by interacting with the C1 complex in MCF-7, but not MCF-7/Adr, cells. To test whether the CAAT region of the *MDR*1 promoter is also involved in downregulating the SV-40 promoter in MCF-7 cells, luciferase-expressing pGL3-Promoter plasmids containing CAAT-box-like, CAAT-deleted, and nonspecific oligomers upstream of the SV-40 promoter and luciferase genes were constructed (Figure 8A). The effects of the inserts on the SV-40 promoter activity in MCF-7 and MCF-7/Adr cells were determined after transient transfections with the pGL3-Promoter constructs and the pSV-β-gal plasmid as described above. As seen in Figure 8B, the CAAT-box-like oligomer, but not the CAAT-deleted or nonspecific oligomers, caused a significant reduction in luciferase activity (about 80%) compared to that of the pGL3-Promoter in MCF-7 cells. These oligomers did not have any significant effect on luciferase activity in MCF-7/Adr cells (Figure 8B). These results further suggest that CAAT interacts with a negative regulatory factor(s) involved in the downregulation of *MDR*1 and SV-40 promoter activities in MCF-7 cells.

Characterization and Identification of the MDR1 Promoter Binding Protein Interacting with CAAT. To characterize and identify the DNA binding protein (C1) interacting with CAAT on the *MDR*1 promoter probe in MCF-7 nuclear extracts, EMSA coupled with SDS−PAGE was performed as described in Experimental Procedures. After MCF-7

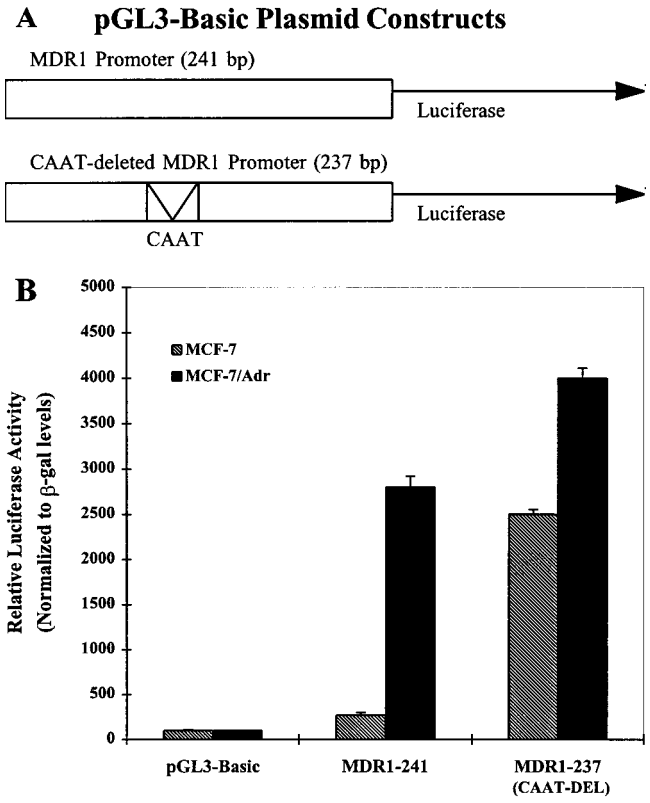

FIGURE 7: Analysis of wt and CAAT-deleted *MDR*1 promoter activity by luciferase assay in MCF-7 and MCF-7/Adr cells. (A) The 241 bp wt *MDR*1 promoter DNA and its mutated derivative (237 bp CAAT-deleted DNA fragment) were cloned into the pGL3-Basic vector upstream of the luciferase gene as described in Experimental Procedures. The figures are not drawn to scale. (B) After transient transfections, the luciferase activity levels were measured in MCF-7 and MCF-7/Adr cells containing the pGL3-Basic vector (controls) or plasmid constructs with wt or mutated *MDR*1 promoter cDNA fragments as described in Experimental Procedures. Cells were cotransfected with the pSV-β-gal control plasmid, and the luciferase levels were normalized to the levels of β-gal in each transfectant. Triplicate samples were used for each point in three independent experiments. Error bars represent standard deviations.

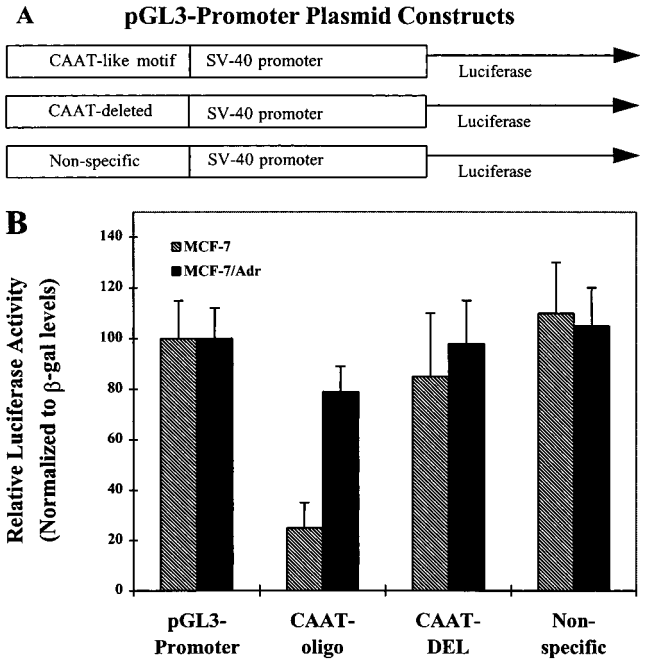

FIGURE 8: Analysis of the effects of oligomers containing CAAT-like, CAAT-deleted, and nonspecific motifs on SV-40 promoter activity in MCF-7 and MCF-7/Adr cells. (A) The ds oligomers containing CAAT-like, CAAT-deleted, and nonspecific motifs were cloned into the pGL3-Promoter vector upstream of SV-40 promoter and luciferase gene as described in Experimental Procedures. The figures are not drawn to scale. (B) The effects of CAAT-like, CAAT-deleted, and nonspecific oligomers on the SV-40 promoter activity were analyzed after transient transfections of MCF-7 and MCF-7/Adr cells with the pGL3-Promoter vector (controls) and plasmid constructs above by luciferase assay as described in Experimental Procedures. Cells were cotransfected with the pSV-β-gal control vector, and luciferase activity levels were normalized to β-gal levels in each transfectant. Triplicate samples were used for each point in three independent experiments. Error bars represent standard deviations.

nuclear extracts were incubated with the 5′-end-labeled 241 bp *MDR*1 promoter probe, protein/DNA complexes were run on 5% native polyacrylamide gels in the absence or presence of excess CAAT and CAAT-deleted oligomers used as competitors (Figure 9A, lanes 2−4, respectively). Gel fragments containing the C1 complex were excised and subjected to SDS−PAGE for silver staining or Western blotting as described. Figure 9B, panel 1, shows a silver-stained SDS−polyacrylamide gel containing C1 of MCF-7, demonstrating that C1 is a heterodimer protein with two peptides of about 65 and 60 kDa molecular mass. It is known in the literature (*24*) that NF-κB and AP1 transcription factors are heterodimer proteins containing p65/50 and c-Fos(p62)/c-Jun(p39), respectively. In light of this, we tested whether p65/p60 of C1 is recognized by rabbit polyclonal antibodies raised against NF-κB/p65, p50, c-Fos, and c-Jun by Western blotting following EMSA coupled with SDS−PAGE as described above. Interestingly, the slower migrating protein of C1 (about 65 kDa) was recognized by two different NF-κB p65 antibodies specific to its COOH- and NH_2-terminal peptide sequences (Figure 9B, panels 2 and 4, respectively), but not by the NF-κB/p50 antibody (Figure 9B, panel 3). In addition, the antibody specific for c-Fos reacted with the faster migrating peptide (about 60 kDa) of C1 in Western blots (Figure 9B, panel 5). However, c-Jun antibody did not recognize either of the C1 proteins (Figure 9B, panel 6). These results suggest that the *MDR*1 promoter binding complex (C1) which recognizes CAAT in MCF-7 nuclear extracts is a product of the interaction of two distinct transcription factors, NF-κB/p65 and c-Fos, termed cross-coupling (*25*). To confirm these results, MCF-7 nuclear extracts were incubated with NF-κB/p65 or c-Fos rabbit polyclonal antibodies and purified rabbit IgG, used as a control, prior to addition of the 241 bp 5′-end-labeled *MDR*1 promoter probe in EMSAs as described in Experimental Procedures. As shown in Figure 10, NF-κB/p65-c-Fos binding to the *MDR*1 promoter probe was significantly inhibited in the presence of NF-κB/p65 and c-Fos antibodies in EMSA (lanes 4 and 5, respectively) compared to the rabbit IgG controls (lane 6). Lanes 1−3 in Figure 10 demonstrate C1 binding reactions in which the labeled *MDR*1 promoter probe was incubated with MCF-7 nuclear extracts in the absence or presence of excess CAAT- and GC-box oligomers as competitors. Similarly, NF-κB/p65 and c-Fos proteins were detected in the C1 complex isolated from EMSAs using the 5′-end-labeled 155 bp *MDR*1 promoter DNA and ds CAAT oligomer as probes with MCF-7 nuclear extracts (data not shown).

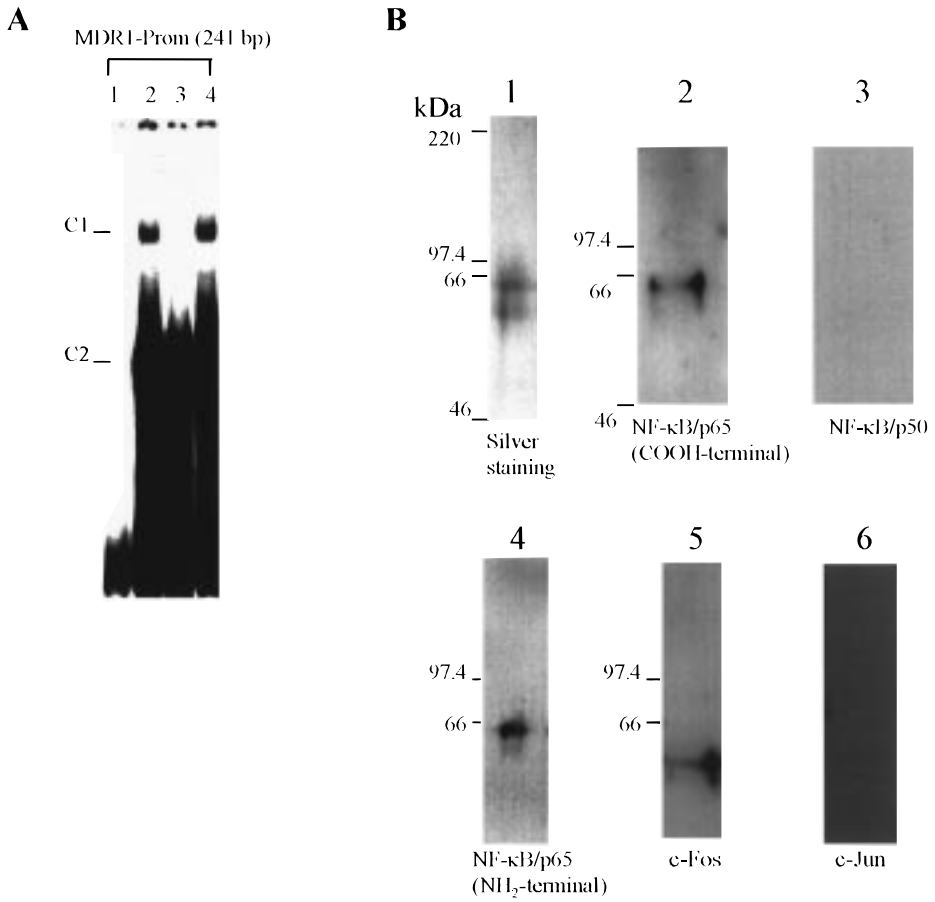

FIGURE 9: Characterization and identification of the *MDR*1 promoter binding complex (C1) in MCF-7 nuclear extracts. The characterization and identification of the *MDR*1 promoter binding complex (C1) in MCF-7 extracts were performed by EMSA coupled with SDS−PAGE followed by silver staining or Western blotting as described in Experimental Procedures. (A) Nuclear extracts from MCF-7 cells were incubated with the 5′-end-labeled *MDR*1 promoter probe in the absence (lane 2) or presence of excess CAAT-like or CAAT-deleted oligomers (lanes 3 and 4, respectively) in EMSAs. Protein/DNA complexes were run in polyacrylamide gels and visualized by autoradiography. Lane 1 contains unbound probe. (B) Following EMSAs, the C1 complex in polyacrylamide gels (several lanes of the undried portion of the gels) was excised and subjected to SDS−PAGE, and then silver staining was performed as described in Experimental Procedures (panel 1). The C1 complex was then analyzed by Western blotting using several different rabbit polyclonal antibodies specific for NF-κB/p65 at its C-terminus (panel 2) or N-terminus (panel 4), NF-κB/p50 (panel 3), c-Fos (panel 5), and c-Jun (panel 6), as described in Experimental Procedures. Molecular weights are indicated on the left. Antibodies used in Western blots are indicated at the bottom of the figures. Results shown are representative of three independent experiments.

DISCUSSION

The data presented in this paper show that the *MDR*1 gene is highly transcribed in multidrug resistant MCF-7/Adr cells, while its mRNA is below detectable levels in its sensitive parental MCF-7 cell line. While upregulation of *MDR*1 has been shown in cells exposed to a variety of stress-inducing factors, the mechanisms involved in suppressing *MDR*1 gene expression in cells not exposed to such factors are not known. Therefore, in this study we investigated whether the *MDR*1 promoter is regulated by a possible repressor protein(s) in MCF-7 cells, a way in which control of the *MDR*1 promoter had not been explored previously in sensitive MCF-7 cells. To detect DNA binding factors which specifically interact with *MDR*1 proximal promoter DNA, spanning residues −198 to +43 where several transcription factor recognition sites are located, EMSAs were performed with MCF-7 and MCF-7/Adr nuclear extracts. The inhibition of *MDR*1 promoter activity in these cells was studied by luciferase assay following transient transfections of these cells with different *MDR*1 promoter plasmid constructs. In addition, the *MDR*1 promoter binding proteins were identified by EMSAs coupled with immunodetection. This study provides the first evidence that cross-coupling between NF-κB/p65 and c-Fos transcription factors is involved in the negative regulation of *MDR*1 promoter activity in the sensitive MCF-7 human breast cancer cell line, but not in its drug resistant variant MCF-7/Adr. In addition, NF-κB/p65-c-Fos is shown to exert its negative regulatory effect by interacting with the CAAT region (residues −116 to −113) of the *MDR*1 promoter in MCF-7 cells. Cross-coupling of NF-κB/p65 and Fos or Jun has been previously shown to activate κB and AP1 enhancer-dependent promoters (*26*). It has also been reported that the functional and physical interaction of these two unrelated transcription factors requires the Rel homology domain of NF-κB/p65 and the bZIP region of Fos or Jun (*26*).

Interestingly, Stein et al. (*26*) have shown that cross-coupling between NF-κB/p65 and Fos or Jun is restricted to the nucleus in vivo. The classical NF-κB is a heterodimer transcription factor composed of 50 kDa (p50) and 65 kDa (p65 or RelA) proteins (*27*). NF-κB/Rel proteins are usually sequestered as inactive forms in the cytoplasm by interacting with inhibitory proteins termed IκB (*27*). Activation and nuclear translocation of NF-κB/Rel proteins can be stimulated

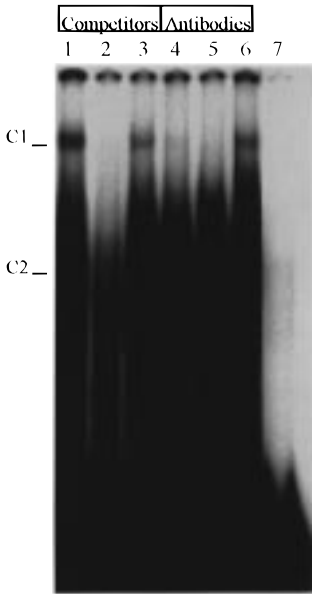

FIGURE 10: Identification of *MDR*1 promoter binding proteins in the C1 complex by EMSA using rabbit polyclonal antibodies. The nuclear extracts from MCF-7 cells were incubated with the 5′-end-labeled *MDR*1 promoter probe in the absence (lane 1) or presence of 200-fold excess ds CAAT-like and CAAT-deleted oligomers (lanes 2 and 3, respectively) in EMSAs. In addition, MCF-7 nuclear extracts were preincubated with rabbit polyclonal antibodies specific for the NH_2 region of NF-κB/p65 (sc-109) and c-Fos transcription factors (lanes 4 and 5, respectively) or rabbit IgG (lane 6) used as a control, prior to addition of the *MDR*1 promoter probe in EMSAs. Protein/DNA complexes were run on polyacrylamide gels and visualized by autoradiography. Results shown are representative of two independent experiments.

by many signals including immune and inflammatory responses, cellular proliferation, apoptosis, and adhesion molecules (*28*, *29*) that are involved in IκB degradation. However, Sovak et al. (*30*) have recently reported constitutive expression of active NF-κB/p65 in MCF-7 nuclei in the absence of any activating signals (*30*).

The mechanisms of interplay between NF-κB/p65 and c-Fos proteins and their molecular complex configuration in MCF-7 cells are not known. However, our EMSA data, in which neither NF-κB/p50 nor c-Jun alone bound to the *MDR*1 promoter, support the formation of monomeric association between NF-κB/p65 and c-Fos as suggested previously by Stein et al. (*26*). Interestingly, our results show that the NF-κB/p65-c-Fos complex is involved in the negative regulation of the *MDR*1 promoter, in contrast to early studies in which the complex of NF-κB/p65 and Jun or Fos is shown to activate the 5′ long terminal repeat of human immunodeficiency virus type 1 by enhanced DNA binding activity via both the κB and AP1 response elements (*26*). Similarly, NF-κB and AP1 are known to be independently involved in activating several different genes in human cancer cells (*24*). However, it is also known that NF-κB acts as a negative regulator for the rat androgen receptor gene (*31*). In addition, it was reported that physical interaction between the estrogen receptor (ER), NF-κB, and C/EBPβ results in reduced activity of promoters with ER binding sites, including interleukin 6 (IL-6) in human osteoblasts (*32*), supporting a complex tissue-specific functional regulation of transcription factors. Interestingly, the EMSA results and analysis of *MDR*1 promoter activity by luciferase assay show that NF-κB/p65-c-Fos is involved in the trans-regulation of *MDR*1 promoter activity by interacting with the CAAT segment in MCF-7, but not in MCF-7/Adr, cells. Recognition of CAAT by this complex has not previously been reported. However, NF-κB recognition of different promoter sequences interacting with HAP1, C/EBP, COUP, TEF-1, Oct2, and TSAP transcription factors has been previously reported (*33*−*38*). Ogura et al. (*18*) have also shown the presence of a possible negative regulatory protein, NF-R1, that interacts with two unrelated sequences of the *MDR*1 promoter. Interestingly, the p65 homodimer of NF-κB is reported to recognize the 5′-GAAAATTTCC-3′ sequence of the human IL-4 gene and result in IL-4 repression (*39*). Recently, it was reported that NF-κB/p65 dimers can bind to target sequences with only one cognate half-site, which allows many more sites to be recognized by this transcription factor and thus expands the number of genes controlled by NF-κB family proteins (*40*). In general, a single DNA binding domain of a protein is mainly responsible for recognition of divergent sequences, and many transcription factors could potentially interact with distinct DNA sequences. In fact, it was shown that a single base pair change resulted in a large degree of conformational change in NF-κB/p65 (*39*). Therefore, it is possible that the association of NF-κB/p65 and c-Fos might create the particular conformation of a putative DNA binding domain, which in turn can interact with the CAAT region of the *MDR*1 promoter. This possibility, however, needs further investigation. Furthermore, we have shown here that preincubation of MCF-7 nuclear extracts with antibodies specific for the NH_2-terminus of NF-κB/p65 and c-Fos completely inhibited binding of the complex to the *MDR*1 promoter, suggesting a cooperative interaction of NF-κB/p65 and c-Fos with the *MDR*1 promoter. Moreover, our data are consistent with previous reports which show the presence of a negative regulatory element at positions −198 to −89 in the *MDR*1 promoter in various human cancer cells (*13*, *18*, *41*). However, the deletion of residues −137 to −86 had the opposite effect on the *MDR*1 promoter in HCT116 and HepG2 cells (*14*), supporting the idea that there might be tissue- or cell line-specific variations in the mechanisms controlling *MDR*1 promoter activity. This was evident in our previous study, in which no protein/DNA complex formation was observed when the ^{32}P-labeled 241 bp *MDR*1 promoter probe was incubated with nuclear extracts from sensitive HL-60 human acute myeloblastic leukemia (AML) cells (data not shown). Nevertheless, the mechanism of association of the NF-κB/p65 and c-Fos complex at the molecular level and its involvement in the negative regulation of the *MDR*1 promoter in other human cancer cell lines, especially those of breast origin, need to be examined in future studies. Moreover, the undetectable levels of NF-κB/p65-c-Fos interacting with the CAAT region of the *MDR*1 promoter in MCF-7/Adr cells, as shown by EMSAs and luciferase experiments, might play a role in the increased *MDR*1 promoter activity in MCF-7/Adr cells. However, these results do not eliminate the possibility that other cis-acting factors activated in response to cellular stress might also contribute to the upregulation of *MDR*1 gene expression in MCF-7/Adr cells in vivo.

Consequently, this study shows an alternative mechanism of gene regulation involving the association of two unrelated transcription factors, NF-κB/p65 and c-Fos, which form a protein complex that exhibits a negative regulatory function

on the *MDR*1 promoter by interacting with the CAAT segment in MCF-7, but not MCF-7/Adr, cells. Identifying the molecular mechanisms involved in regulating the *MDR*1 promoter in different human cancer cell lines should lead to better understanding of *MDR*1 gene expression and may potentially provide novel therapeutic strategies to circumvent or prevent *MDR*1-related drug resistance.

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