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12-*O*-Tetradecanoylphorbol-13-Acetate Activation of the *MDR1* Promoter Is Mediated by EGR1

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P-glycoprotein, the product of the *MDR1* gene (multidrug resistance gene 1), is an energy-dependent efflux pump associated with treatment failure in some hematopoietic malignancies. Its expression is regulated during normal hematopoietic differentiation, although its function in normal hematopoietic cells is unknown. To identify cellular factors that regulate the expression of *MDR1* in hematopoietic cells, we characterized the *cis*- and *trans*-acting factors mediating 12-*O*-tetradecanoylphorbol-13-acetate (TPA) activation of the *MDR1* promoter in K562 cells. Transient-transfection assays demonstrated that an *MDR1* promoter construct containing nucleotides −69 to +20 conferred a TPA response equal to that of a construct containing nucleotides −434 to +105. TPA induced EGR1 binding to the −69/+20 promoter sequences over a time course which correlated with increased *MDR1* promoter activity and increased steady-state *MDR1* RNA levels. The −69/+20 promoter region contains an overlapping SP1/EGR site. The TPA-responsive element was localized to the overlapping SP1/EGR site by using a synthetic reporter construct. A mutation in this site that inhibited EGR protein binding blocked the −69/+20 *MDR1* promoter response to TPA. The expression of a dominant negative EGR protein also blocked the TPA response of the −69/+20 promoter construct. Finally, the expression of EGR1 was sufficient to activate a construct containing tandem *MDR1* promoter SP1/EGR sites. These data suggest a role for EGR1 in modulating *MDR1* promoter activity in hematopoietic cells.

The expression of Pgp, the product of the *MDR1* gene, is one cause of resistance to chemotherapy in human tumor cells. Pgp functions, at least in part, as an energy-dependent efflux pump for some hydrophobic chemotherapeutic agents, although its exact functional mechanism is likely to be more complicated (6). *MDR1* gene expression is associated with treatment failure in some hematopoietic malignancies, including acute myelogenous and lymphocytic leukemias (2, 23, 25, 35, 39, 46, 47, 49, 50), the blast crisis phase of chronic myelogenous leukemia (31, 45), and multiple myeloma and non-Hodgkin's lymphoma (14, 15, 20, 41, 48). In these malignancies, *MDR1* expression occurs more frequently after chemotherapeutic treatment, suggesting a selection for $MDR1^+$ cells and/or acquisition of the MDR1 phenotype in response to tumor progression or antineoplastic exposure (6).

MDR1 is also expressed, albeit at lower levels, in some normal hematopoietic subsets (3, 5, 17, 29), although its physiologic role in these cells is unknown. In normal bone marrow, the expression of *MDR1* correlates directly with CD34 expression and inversely with CD33 expression (3, 17). This suggests that *MDR1* is associated with the stem cell phenotype and is regulated during myeloid differentiation. *MDR1* is also expressed in mature CD8 and CD56 lymphoid subsets with lesser expression in CD4- and CD19-positive cells (5, 17, 29) although a role for *MDR1* in immune surveillance or cytotoxicity has not been reported. In mice there exist two *MDR1* homologs, *mdr1a* and *mdr1b*. A knockout mouse model exists for only *mdr1a* (51), and no effect on hematopoietic development or function has been reported in this model.

Because of its association with a multidrug-resistant phenotype and because of its lineage specificity in normal hematopoiesis, the regulation of *MDR1* expression has been of particular interest. In human cells and most tumors, *MDR1* expression is regulated by the more proximal of two promoters (11, 54, 55). In nonhematopoietic cells, this proximal promoter responds to c-Ha-Ras, mutant p53, serum stimulation, v-*raf*, and NF-IL6 (7, 10, 12, 13). In human hematopoietic cells, *MDR1* expression is modulated by agents that induce phenotypic and morphologic differentiation. For instance, the treatment of normal peripheral blood lymphocytes and the hematopoietic cell lines K562, KG1, and H9 with the protein kinase C agonists TPA and diacylglycerol increases steady-state levels of *MDR1* RNA and functional surface Pgp, presumably through protein kinase C-dependent signal transduction (4).

To better understand the regulation of *MDR1* expression in hematopoietic cells, the identification of cellular factors that regulate *MDR1* expression is necessary. To this end, we have characterized the *cis*-acting sequences and *trans*-acting factors regulating *MDR1* expression in K562 cells in response to TPA. Our findings suggest that EGR1 contributes to inducible expression of the *MDR1* gene in hematopoietic cells, thus identifying a novel regulatory role for EGR1.

MATERIALS AND METHODS

Abbreviations. Ab, antibody; β2-m, β2-microglobulin; CDTA, 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, dithiothreitol; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EMSA, electrophoretic mobility shift assay; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; IL-3, interleukin 3; ITT, in vitro transcribed and translated; *MDR1*, multidrug resistance gene 1; PBS, phosphate-buffered saline; Pgp, P-glycoprotein; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

Reagents, Abs, and oligonucleotides. TPA was purchased from Sigma Chemical Co. and stored at −20°C at 10 mg/ml in dimethyl sulfoxide. Before its addition to cultured cells, this TPA solution was diluted further in RPMI 1640 or Iscove's medium so that the dimethyl sulfoxide concentration was less than 0.01%. Luciferin, PMSF, leupeptin, and trypsin inhibitor were purchased from Sigma. Poly(dI-dC) was purchased from Pharmacia. [γ-^{32}P]ATP (6,000 Ci/mmol) was purchased from NEN. Purified SP1 protein was purchased from Promega, and bacterially expressed and column-purified EGR2 was the generous gift of R. Szakaly and P. J. Farnham of the University of Wisconsin. ITT EGR1 was made as follows. An *Eco*RI fragment from CMV-EGR1 (33) containing the full coding

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sequences for EGR1 was subcloned into pGEM3. ITT EGR1 was expressed from this construct by using the TNT coupled in vitro transcription-translation system with T7 RNA polymerase (Promega). Polyclonal Abs specific for human SP1, EGR1, EGR2, and EGR3 were purchased from Santa Cruz Biotechnology as were the blocking peptides for each Ab. All oligonucleotides used in these studies, except the SP1 consensus oligonucleotide (Promega), were made in the Biotechnology Facility at the Fred Hutchinson Cancer Research Center.

Cell culture and preparation of nuclear extracts. The K562 cell line was the kind gift of S. J. Collins of the Fred Hutchinson Cancer Research Center. K562 cells were maintained in RPMI 1640 supplemented with 10% calf serum, 1 mM glutamine, and 50 μg (each) of streptomycin and penicillin per ml in a humidified incubator containing 5% CO_2. Between 2×10^8 and 10^9 K562 cells, treated with 16 nM TPA for various times or left untreated, were harvested by centrifugation at 1,000 × *g* for 10 min at 4°C. After being washed once with PBS, cells were resuspended in buffer A (1 mM potassium acetate, 1.5 mM magnesium acetate, 10 mM Tris-HCl [pH 7.6], 2 mM DTT, 30 mM $NaPP_i$, 100 μM EGTA, 100 μg of PMSF per ml, 2.5 μg of leupeptin per ml, 2.5 μg of trypsin inhibitor per ml) to five times the pellet volume and incubated on ice for 8 min. Cells were then transferred to a Dounce homogenizer, and four strokes with a B pestle were done to release nuclei. The suspension was centrifuged at 15,000 × *g* for 10 min at 4°C, and the pellet, containing the nuclei, was resuspended in 1 ml of buffer C (40 mM Tris [pH 7.9 at 4°C], 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM EDTA, 30 mM $NaPP_i$, 100 μg of PMSF per ml, 2.5 μg of leupeptin per ml, 2.5 μg of trypsin inhibitor per ml) per 2×10^8 cells and transferred to a Dounce homogenizer. After four strokes with a B pestle, the suspension was transferred to a conical tube and stirred on ice for 45 min to lyse nuclei. This suspension was then centrifuged at 15,000 × *g* for 20 min at 4°C to remove nuclear debris. Supernatant (nuclear extract) was dialyzed against a 200× volume of buffer D (40 mM Tris [pH 7.9 at 4°C], 20% glycerol, 0.1 M KCl, 0.5 mM DTT, 2 mM EDTA, 30 mM $NaPP_i$, 100 μg of PMSF per ml, 2.5 μg of leupeptin per ml, 2.5 μg of trypsin inhibitor per ml) for 5 h by using a Spectrapor 7 membrane (Spectrum). The nuclear extract was then centrifuged at 15,000 × *g* for 20 min at 4°C. The supernatant was stored at −70°C in 10- to 20-μl fractions. Protein concentrations were determined by a commercial protein assay (Bio-Rad).

RNA preparation, reverse transcription, and cDNA-PCR. Total cellular RNA was prepared by a standard acid-phenol preparation method (8) except that an additional phenol-chloroform-isoamyl alcohol extraction was added. One microgram of RNA was transcribed in a 20-μl reaction mixture containing 100 ng of random hexadeoxynucleotide primer (Pharmacia) in 50 mM Tris-HCl (pH 8.3)–75 mM KCl–3 mM $MgCl_2$–10 mM DTT–500 μM (each) deoxynucleoside triphosphates–40 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL). After 1 h at 37°C and 10 min at 65°C to inactivate the reverse transcriptase, cDNA was either used immediately in PCR or stored at −70°C.

cDNA-PCR was performed by the method of Noonan et al. (42). The *MDR1* and β2-m primers used span an intron (42) and thus do not amplify genomic DNA sequences that might contaminate RNA preparations. β2-m was used as a control for the integrity of RNA preparations and the accuracy of dilutions (38, 42) as well as to demonstrate that the effect of TPA on *MDR1* expression in K562 cells was not due to nonspecific increases in transcription. The number of cycles producing exponential amplification of β2-m or *MDR1* was determined by performing PCR on fixed amounts of cDNA and varying the cycle number. Simultaneous amplification of β2-m and *MDR1* led to variable amplification of *MDR1*, particularly with cDNA from untreated K562 cells, which express very small amounts of *MDR1*. This phenomenon has been described previously (38, 42). Thus, *MDR1* and β2-m were amplified in separate reaction mixtures containing equal amounts of cDNA. The number of cycles corresponding to the midrange of exponential amplification was determined (22 cycles for β2-m and 29 cycles for *MDR1*), and PCR was performed for 22 (β2-m primers) or 29 (*MDR1* primers) cycles on serial dilutions of cDNA.

All PCRs were performed in duplicate in a 50-μl volume containing 1× Promega PCR buffer (1.5 mM $MgCl_2$, 0.05 mM (each) dATP, dGTP, dCTP, and dTTP), 75 pmol (each) of 5′ and 3′ *MDR1* or β2-m primers, 1.3×10^6 cpm of 3′ primer isotopically labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Promega) by standard methods. The primers used for amplification of β2-m were 5′-ACCCCCACTGAAAAAGATGA-3′ (residues 1544 to 1563; sense strand) and 5′-ATCTTCAAACCTCCATGATG-3′ (residues 2253 to 2262 and 3508 to 3517; antisense strand) (42). PCR with these primers yields a 120-bp product. *MDR1*-specific sequences were amplified by using the sense strand primer 5′-CCCATCATTGCAATAGCAGG-3′ (residues 2596 to 2615) and the antisense strand primer 5′-ATTCAAACTTCTGCTCCTGA-3′ (residues 2733 to 2752) (42), which yield a 167-bp product. PCR mixtures were overlaid with mineral oil, and PCR was carried out in a DNA thermal cycler (Perkin-Elmer/Cetus) in sequential cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 1.5 min, with a 10-min 72°C elongation phase after the last cycle. Amplification was performed for the number of cycles determined to be within the exponential range, as described above. Negative controls containing aliquots of cDNA reaction mixtures prepared without the addition of RNA or water rather than cDNA were included in each experiment.

Fifteen microliters of each PCR product was separated on a 6% polyacrylamide gel in 1× Tris-borate electrophoresis buffer. Gels were dried, and PCR products were visualized by autoradiography. The radiographic signal was measured by using a Molecular Dynamics PhosphorImager and ImageQuant software. Computation of the relative increase in the steady-state *MDR1* RNA level of the 50-ng dilution was performed by the method of Murphy et al. (38) and Noonan et al. (42) as follows: normalized *MDR1* expression was estimated for log-phase and treated K562 cells by dividing the radiographic signal measured from *MDR1* amplification by that measured from β2-m amplification. The relative increase in the *MDR1* steady-state RNA level was estimated by dividing normalized *MDR1* expression in treated K562 cells by that in untreated cells.

Construction of expression vectors and transient transfections. The −434 *MDR1*-luciferase reporter plasmid has previously been described (12). The −69/+20 reporter construct was created by amplifying the −434 plasmid with a −69/+44 sense primer and a −6/+24 antisense primer to create a promoter fragment extending from −69 to +24. PCR amplification was for 1 min at 94°C, 1.5 min at 45°C, and 1 min at 72°C. The PCR product was then treated with T4 polymerase to create blunt ends and was ligated into the *Sma*I site of pGL2-Basic (Promega). Dideoxy sequencing was performed with a Sequenase version 2.0 kit (U.S. Biochemicals) to confirm the specific mutations and endpoints of the construct. The site-specific promoter mutation construct, Δ49/47, was created in a fashion analogous to that of −69/+20 except that the −69/−41 upstream sense primer contained the site-specific mutation underlined in the following sequence: 5′-CAGGAACAGCGCCGGGGCGTTTTCTGAGC-3′.

The control construct TATA-INR is a basal transcription promoter containing a TATA box and the terminal deoxynucleotidyl transferase INR cloned upstream of the luciferase cDNA in pGL2-Basic (12). GC-TI has previously been described (13) and contains *MDR1* promoter sequences cloned upstream of TATA box sequences in TATA-INR. The EGR-TI construct was a kind gift of P. J. Farnham. This construct was made by annealing a sense primer, 5′-CGCGTGACGT CAGCGGGGGGCGAC-3′, and an antisense primer, 5′-TCGAGTCGCCCCCG CTGACGTCA-3′, to create a double-stranded oligonucleotide containing an (underlined) EGR consensus site. The annealed oligonucleotide was ligated into the *Xho*I and *Mlu*I polylinker sites upstream of the TATA sequences in the TATA-INR construct described above. The orientations and integrities of the cloned sequences were confirmed by dideoxy sequencing as described above.

The 3XEGR construct contains three consensus EGR sites cloned upstream of the TATA box in TATA-INR. This construct was made by annealing a sense primer, 5′-TCGAGCGCCCCCGCAAGCTTCGCCCCCGCAAAACGCCCCC GCA-3′, and an antisense primer, 5′-GATCTGCGGGGGCGTTTTGCGGGG GCGAAGCTTGCGGGGGCGC-3′, to create a double-stranded oligonucleotide containing three (underlined) EGR consensus sites. The annealed oligonucleotide was ligated into the *Sma*I and *Bgl*II polylinker sites upstream of the TATA sequences in the TATA-INR construct described above. The orientations and integrities of the cloned sequences were confirmed by dideoxy sequencing as described above.

The 3XMDR1 construct contains *MDR1* promoter sequences from −54 to −41 cloned upstream of the TATA box in TATA-INR. This construct was made by annealing a sense primer, 5′-GATCGGCGTGGGCTGAGCAAGCTTGGC GTGGGCTGAGCTCTAGAGGCGTGGGCTGAGC-3′, and an antisense primer, 5′-GATCGCTCAGCCCACGCCTCTAGAGCTCAGCCCACGCCAAGCT TGCTCAGCCCACGCC-3′, to create a double-stranded oligonucleotide containing three (underlined) tandem copies of the −54-to-−41 sequences from the *MDR1* promoter overlapping the SP1/EGR site. The annealed oligonucleotide was ligated into the unique *Bgl*II site upstream of the TATA sequences in the TATA-INR construct described above. The orientations and integrities of the cloned sequences were confirmed by restriction endonuclease digestion and dideoxy sequencing as described above.

The CMV-EGR1 construct containing the full-length EGR1 cDNA under control of the cytomegalovirus promoter has previously been described (33) and was the kind gift of F. Rauscher III of the Wistar Institute.

The ΔN214 construct was the kind gift of V. Sukhatme of Beth Israel Hospital. This construct has previously been described in detail (22) and contains sequences encoding a truncated EGR1 protein cloned into the pCB6+ vector (22).

Transient transfections were performed by electroporation as follows. A DNA cocktail containing reporter and effector plasmids, 2 to 4 μg of pSV-βgal plasmid as an internal control for transfection efficiency, 5 μg of DEAE-dextran per ml, and Tris-EDTA (pH 8.0) was made to bring the volume to 50 μl per transfection. The amount of reporter construct transfected was 10 μg unless otherwise specified. Each transfection was performed in duplicate. Fifty microliters of DNA cocktail was added to each electroporation cuvette (BTX; Bio-Rad) at room temperature. K562 cells growing in logarithmic phase were collected by brief centrifugation, washed once in HeBS (0.02 M tissue-culture-grade HEPES in PBS), and resuspended at 10^7 cells per ml in HeBS. A 500-μl aliquot of cell suspension was added to each cuvette. The DNA-cell mixture was incubated at room temperature for 5 min and placed in a Gene Pulsor apparatus (Bio-Rad), and current was applied at 300 V and a 500-μF capacitance. Cuvettes were then immediately put on ice, and contents were transferred to a 60-mm-diameter culture dish containing 5 ml of Iscove's medium with 10% calf serum, 1 μM glutamine, and 50 μg (each) of penicillin and streptomycin. After a recovery period of 16 to 24 h, cells were left untreated or TPA was added at a final concentration of 16 nM for the indicated period. For experiments with CMV-EGR1, cells were allowed to recover for 48 h after electroporation. Cells were harvested by centrifugation, washed once in PBS, vortexed in 200 μl of lysis buffer (125 mM Tris-PO_4 [pH 7.8], 10 mM DTT, 10 mM CDTA, 50% glycerol,

5% Triton X-100), and centrifuged briefly to pellet cell debris. An equal volume (20 μl) of lysate was mixed with 100 μl of luciferin substrate mix (0.02 M Tricine, 1 mM magnesium carbonate pentahydrate, 2 mM $MgSO_4$, 0.1 mM EDTA, 33 mM DTT, 0.2 mM coenzyme A, 0.47 mM luciferin, 0.53 mM ATP, 5 mM NaOH) and assayed for luciferase activity in a Berthold Lumat 9501 luminometer. β-Galactosidase activity was determined as previously described (13) to normalize the luciferase activity for the difference in transfection efficiency.

EMSA. The −69/+20 and Δ49/47 double-stranded probes used in EMSAs were made by PCR and end labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase by standard methods. EMSAs were performed by incubating 7.5 μg of nuclear extract with 1.5 ng (0.0263 pmol) of ^{32}P-labelled −69/+20 probe at room temperature for 20 min in the presence of 0.25 μg of poly(dI-dC)–12 mM HEPES [pH 7.5]–42 mM KCl–3 mM $MgCl_2$–60 μM $ZnCl_2$–0.3 mM DTT–0.03% Nonidet P-40–7.2% glycerol. Samples were electrophoresed on a 4% polyacrylamide gel (19:1 bis) in 1× Tris-borate-EDTA. Gels were dried, and bands were visualized by autoradiography. When competitor oligonucleotide was used, the competitor was added in 2-, 20-, or 100-fold molar excess and preincubated in the incubation mix described above for 10 min at room temperature before the addition of the probe. When Ab to SP1 or EGR1 was used, reactions were coincubated with Ab and the incubation mix described above for 20 min. When purified SP1 and EGR2 were used, the purified proteins were incubated with a ^{32}P-labelled −69/+20 or Δ49/47 double-stranded oligonucleotide probe as described above except that 1 mg of bovine serum albumin per ml was added to the incubation mix.

SDS-polyacrylamide gel electrophoresis and Western blotting (immunoblotting). Fifty micrograms of nuclear extract from untreated (logarithmic-phase) or TPA-treated K562 cells or 12 μl of ITT EGR1 was loaded on an SDS-polyacrylamide (4 to 10% gradient) gel (Bio-Rad). After electrophoresis, the proteins were transferred to nitrocellulose membranes (Schleicher and Schuell) and incubated for 2 h with gentle shaking at room temperature in Tris-buffered saline containing 5% blocking agent (nonfat dry milk). Membranes were then incubated for 1 h at room temperature with anti-EGR1 Ab C-19 (Santa Cruz Biotechnology) or neutralized anti-EGR1 at 0.5 μg/ml. Ab was neutralized by preincubation with a 10-fold excess (by weight) of C-19 Ab control peptide (Santa Cruz Biotechnology) for 2 h at room temperature. After being washed for 30 min in Tris-buffered saline–Tween 20, membranes were incubated in a 1:1,000 dilution of horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Amersham International) for 1 h at room temperature, washed for 45 min in Tris-buffered saline–Tween 20, and detected by using enhanced chemiluminescence reagents (Amersham International) and Biomax MR (Kodak) autoradiograph film.

RESULTS

***MDR1* promoter activation correlates with endogenous *MDR1* expression after TPA treatment.** It has previously been demonstrated that the *MDR1* gene is induced in K562 cells by treatment with TPA (4). To confirm the effect of TPA on steady-state *MDR1* RNA levels in K562 cells, cDNA-PCR was performed with serial dilutions of reverse-transcribed RNAs from TPA-treated and log-phase K562 cells. PCR amplification was performed in duplicate on serial dilutions of cDNA with *MDR1*- or β2-m-specific primers at a number of cycles determined to be within the exponential range of amplification as described in Materials and Methods. The *MDR1* and β2-m primers span an intron (42), thus preventing amplification of DNA sequences that might contaminate RNA preparations. β2-m was chosen as a control for the integrity of RNA preparations and the accuracy of serial dilutions. Steady-state β2-m RNA levels were unaffected by TPA treatment (data not shown), so β2-m also served to demonstrate that the effect of TPA on steady-state *MDR1* RNA levels in K562 cells was not due to nonspecific increases in transcription. PCR products were measured by phosphorimage analysis, normalized *MDR1* expression (*MDR1*/β2-m) was calculated for log-phase and TPA-treated samples, and the relative increase was estimated as described in Materials and Methods. The results from two separate experiments are shown (Fig. 1a). An approximately 18-fold increase in the steady-state *MDR1* RNA level was observed after 10 h of treatment with 16 nM TPA, and an approximately 46-fold increase was observed after 24 h.

To determine whether the increase in the steady-state *MDR1* RNA level after TPA addition is a consequence of *MDR1* promoter activation, the −434 *MDR1* promoter-luciferase construct (Fig. 1b) was transiently transfected into K562 cells and luciferase activity was measured at various times after the addition of TPA (Fig. 1c). The minimal promoter construct TATA-INR (12) was used as a control for TPA-induced nonspecific increases in transcription. Minimal luciferase activity was detected in cells transfected with the TATA-INR construct. At 2 h after TPA addition, there was little difference in *MDR1*-promoter driven luciferase activity between TPA-treated and untreated (time zero) cells. By 5 h after TPA addition, however, there was clearly an increase in luciferase activity from the *MDR1* promoter construct, which increased further after 10 and 24 h. In 10 transfections performed with the −434 construct and 10-h TPA stimulation, the increase in promoter activity was 14.98 ± 8.0. In eight transfections performed with 24-hour TPA stimulation, the increase in promoter activity was 38.11 ± 16.1. Thus, as shown for endogenous *MDR1* mRNA, a time-dependent induction of promoter activity was observed after TPA treatment. These data suggest

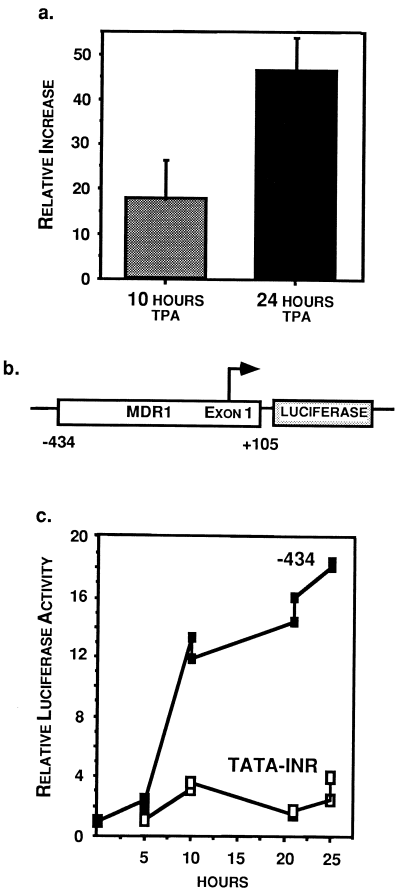

FIG. 1. Comparison of *MDR1* steady-state message levels and *MDR1* promoter activities in K562 cells after TPA treatment. (a) cDNA-PCR was performed as described in Materials and Methods with RNAs from log-phase K562 cells and K562 cells treated with TPA for 10 and 24 h. The results are expressed as relative increases, which were calculated as described in Materials and Methods. Vertical bars show standard deviation. (b) −434, the *MDR1* proximal promoter-luciferase construct, contains 434 bp upstream and 105 bp downstream of the start site. The arrow indicates the transcription start site. (c) The time course of luciferase activity from the −434 promoter construct in K562 cells after TPA treatment. As described in Materials and Methods, K562 cells were transiently transfected with the −434 construct and were either left untreated (time zero) or grown in the presence of 16 nM TPA for the indicated number of hours. A β-galactosidase construct, pSV-βgal, was cotransfected as a control for transfection efficiency. Data were normalized for β-galactosidase activity and the time zero values and are expressed as relative luciferase activities.

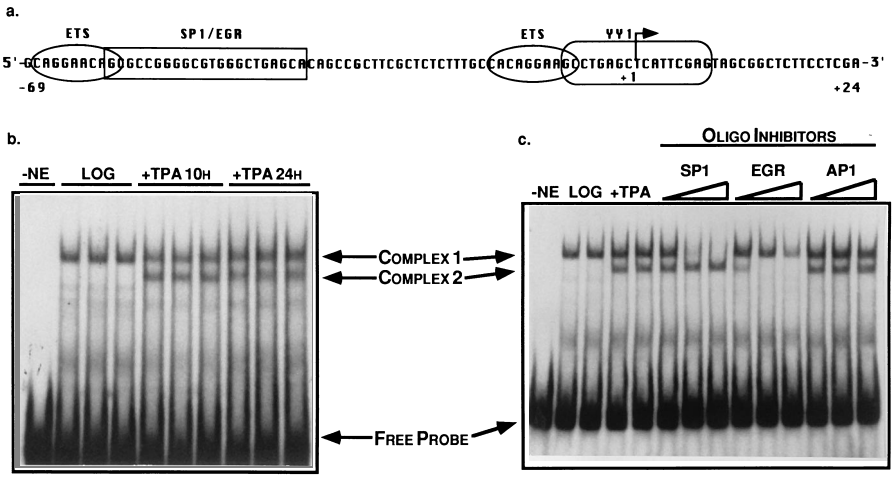

FIG. 2. A new complex binds to the *MDR1* promoter in TPA-treated K562 cells. (a) The −69/+20 *MDR1* promoter construct. Consensus binding sites for transcription factors and transcription factor families are designated. The transcription start site is indicated by an arrow. (b) Nuclear extracts made from K562 cells grown in the absence (log) or presence (+TPA) of 16 nM TPA for 10 or 24 h were incubated (in triplicate) with the labelled −69/+20 oligonucleotide probe as described in Materials and Methods, separated on a 4% polyacrylamide gel, and visualized by autoradiography. −NE, free probe incubated without nuclear extract. (c) EMSAs were performed as described for panel b except that double-stranded oligonucleotide inhibitors (2-, 20-, or 100-fold molar excess) were preincubated with 24-h TPA-treated nuclear extracts. The sense strand of each oligonucleotide is as follows, with the consensus binding sequence underlined: SP1, 5′-ATTC GATCGGGGGCGGGGCGAGC-3′; EGR, 5′-GCCAACGCCCCCGCAACCG-3′; AP1, 5′-GGATGTTATAAAGCATGAGTCAGACACCTCTGGT-3′.

that the TPA-induced increase in *MDR1* expression occurs, at least in part, at the transcriptional level.

TPA induces an EGR site-binding protein. *MDR1* promoter-luciferase constructs containing deletion and point mutations were assayed for TPA inducibility to determine which *cis*-acting sequence(s) of the *MDR1* promoter mediates the TPA response. In several experiments with more than three different plasmid preparations, core promoter sequences from −69 to +20 were induced by TPA to the same extent as was the −434 construct (data not shown). Within the −69/+20 region is a previously characterized overlapping consensus binding site for SP1 and members of the EGR family of transcription factors (13) as well as consensus binding sites for ETS and δ/YY1 (Fig. 2a). EMSAs of a ^{32}P-labelled double-stranded −69/+20 probe were performed to determine if nuclear proteins from TPA-treated and log-phase K562 cells bind to this region. A single slowly migrating complex (complex 1) was observed when extracts from log-phase and TPA-treated cells were incubated with −69/+20 (Fig. 2b). However, a second more rapidly migrating complex (complex 2) was observed in lanes containing extracts from cells treated with TPA for 10 or 24 h.

The specificity of binding was demonstrated by oligonucleotide inhibition (Fig. 2c). The binding of complex 1 in 24-h TPA-treated extracts was inhibited in a dose-dependent manner by a double-stranded oligonucleotide corresponding to an SP1 consensus binding site. The binding of complex 2 was inhibited in a dose-dependent manner by a double-stranded oligonucleotide corresponding to an EGR consensus binding site. Oligonucleotides corresponding to AP1 (Fig. 2c), and C/EBP (data not shown) consensus binding sites did not inhibit the formation of either complex 1 or 2. Both complexes were inhibited in a dose-dependent manner by cold −69/+20 oligonucleotide (data not shown). Because proteins which bind an EGR consensus site are by definition members of the EGR family of transcription factors (34, 53), these data suggest that complex 2 is formed by an EGR family member binding to the −69/+20 oligonucleotide.

Binding to the EGR site is required for the *MDR1* promoter response to TPA. To determine if protein binding to the EGR site is necessary for *MDR1* promoter activation after TPA treatment, an *MDR1* promoter construct containing an EGR binding site mutation was tested in transient-transfection assays for its response to TPA. First, double-stranded oligonucleotides which contained mutations in the overlapping SP1/EGR site were tested for the ability to inhibit the formation of complex 1 or 2 in EMSAs with nuclear extracts from TPA-treated K562 cells. Oligonucleotide Δ49/47, which contains a 3-bp substitution (Fig. 3a), inhibited the formation of complex 1 but did not inhibit the formation of complex 2 (data not shown). This result suggested that Δ49/47 binds SP1 proteins but not EGR proteins. To test directly the ability of Δ49/47 to bind SP1 and EGR proteins, EMSAs were performed with purified SP1 and purified EGR2 (a generous gift of R. Szakaly and P. J. Farnham, University of Wisconsin). The EGR transcription factors (EGR1, EGR2, EGR3, EGR4, and WT1) share highly conserved zinc finger motifs which mediate their identical DNA binding specificity (22, 33, 34). Thus, the ability of an oligonucleotide to bind purified EGR2 reflects its ability to bind EGR family members in general. The wild-type SP1/EGR site oligonucleotide bound both SP1 and EGR2 (Fig. 3a). However, the Δ49/47 oligonucleotide demonstrated SP1 binding equal to that of the wild type but did not bind EGR2 (Fig. 3a).

A luciferase reporter construct which contained the Δ49/47 mutation in a −69/+20 background was made as described in Materials and Methods. This Δ49/47 luciferase construct was tested in transient transfections for its ability to confer a reporter gene response to TPA. As shown in Fig. 3b, activation from this construct was fourfold less than that of the wild-type promoter construct. These data suggest that EGR protein binding is necessary for the *MDR1* promoter response to TPA.

To confirm that binding to the EGR site is necessary for TPA activation, we determined if the expression of a dominant negative EGR, ΔN214 (22), inhibited reporter gene expression in response to TPA. The ΔN214 plasmid construct expresses a truncated EGR1 protein consisting of an N-terminal deletion of 214 amino acids. This modification deletes most of the EGR1 activation domain but does not delete the zinc finger

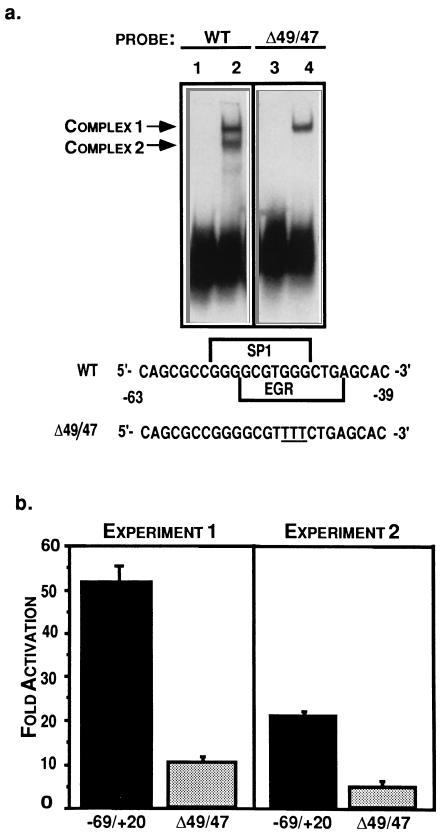

FIG. 3. TPA response of a promoter construct containing an EGR consensus site mutation. (a) Wild-type (WT) or Δ49/47 oligonucleotides were used in EMSAs with purified SP1 and EGR2. Lanes 1 and 3, free probe; lanes 2 and 4, purified SP1 and EGR2. The lanes are from the same gel but were separated on the original gel by additional lanes. The sense strand sequences between nucleotides −69 and −39 of the −69/+20 (WT) and Δ49/47 oligonucleotides are shown. The consensus binding sites for SP1 (26) and EGRs (9) are partially boxed. The mutated oligonucleotides in Δ49/47 are underlined. (b) K562 cells were electroporated with either the −69/+20 or Δ49/47 *MDR1* promoter construct and then treated with TPA or left untreated. Cells were harvested and lysed, and luciferase activities were determined with normalization for β-galactosidase as described in Materials and Methods. Fold activation is defined as normalized luciferase activity (TPA-treated cells)/normalized luciferase activity (untreated cells). Data from two representative experiments are shown. Vertical bars show standard deviations.

(DNA binding) domain or the repression domain (22). To determine whether this construct could be used as a dominant negative inhibitor of EGR proteins in K562 cells, we assessed its ability to inhibit TPA activation of 3XEGR, an artificial promoter construct containing three EGR sites upstream of the luciferase cDNA. K562 cells were cotransfected with 3XEGR and various ratios of ΔN214 or pCB6+ (vector control) so that an equal amount of total DNA was transfected in each sample. Next, transfected cells were left untreated or were treated with TPA, and luciferase activity was assessed 24 h later. As demonstrated in Fig. 4a, the ΔN214 construct inhibited TPA activation of 3XEGR in K562 cells in a dose-dependent manner. This demonstrated the ability of ΔN214 to inhibit TPA activation of a promoter regulated by tandem EGR sites.

Next, we determined whether ΔN214 inhibited TPA activation of the −69/+20 promoter construct. K562 cells were cotransfected with −69/+20 and ΔN214 or pCB6+ vector control at an effector (ΔN214 or pCB6+)-to-reporter (−69/+20) ratio of 10:1. Cells were then left untreated or treated with TPA, and luciferase activity was assessed 24 h later. The results of two representative experiments are shown (Fig. 4b). Reporter gene expression was markedly reduced in response to TPA when ΔN214 was cotransfected compared with expression when the pCB6+ vector control was cotransfected (Fig. 4b). Taken together, these data suggest that EGR protein binding is necessary to confer TPA activation of the *MDR1* promoter in K562 cells.

The *MDR1* promoter overlapping SP1/EGR site is sufficient for the response to TPA. The results discussed above suggest that EGR protein binding is necessary for the *MDR1* promoter response to TPA. If so, the *MDR1* promoter EGR site, which is an overlapping site (13) (Fig. 2), should be sufficient to confer a response to TPA. To test this hypothesis, we transfected K562 cells with either (i) GC-TI, a construct containing *MDR1* promoter sequences −69 to −41 cloned upstream of TATA-INR, or (ii) EGR-TI, which contains an EGR consensus site cloned upstream of TATA-INR. Cells were then treated with TPA, lysed, and harvested as described in Materials and Methods. As shown in Fig. 5, both GC-TI and EGR-TI were activated by TPA. Thus, the overlapping SP1/EGR site is sufficient to confer a response to TPA.

Formation of the inducible complex is inhibited by Ab to EGR1. To determine which EGR protein(s) was bound to the

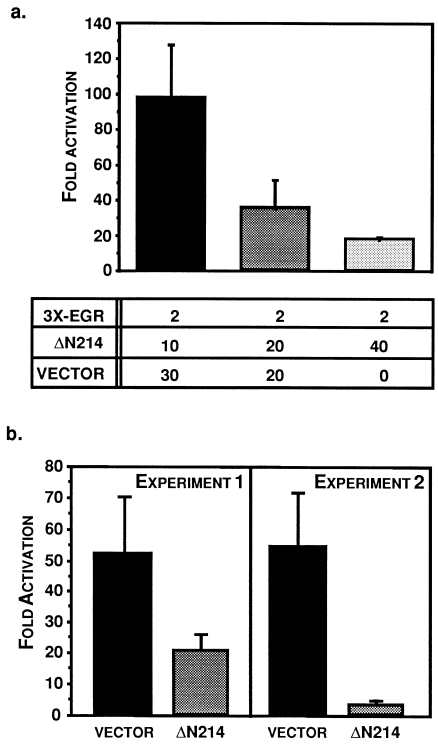

FIG. 4. A dominant negative EGR inhibits *MDR1* promoter activation by TPA. (a) K562 cells were cotransfected with the 3XEGR reporter construct and ΔN214 or pCB6+ vector control. The ratio (in micrograms) of ΔN214 to 3XEGR ranged from 5:1 to 20:1. The plasmid constructs transfected and the amounts transfected are indicated below the bar graph. The cells were treated with TPA or left untreated and were harvested and lysed, and luciferase activities were measured as described in Materials and Methods. The results are expressed as fold activation as described in the legend to Fig. 3. (b) K562 cells were transfected with the −69/+20 luciferase reporter construct and either pCB6+ vector control or the ΔN214 construct and then treated with TPA or left untreated. Cells were harvested and lysed, and luciferase activities measured with normalization for β-galactosidase expression as described in the legend to Fig. 3. The effector (vector or ΔN214)-to-reporter (−69/+20) ratio was 10:1. Data from two representative experiments are presented. Vertical bars show standard deviations.

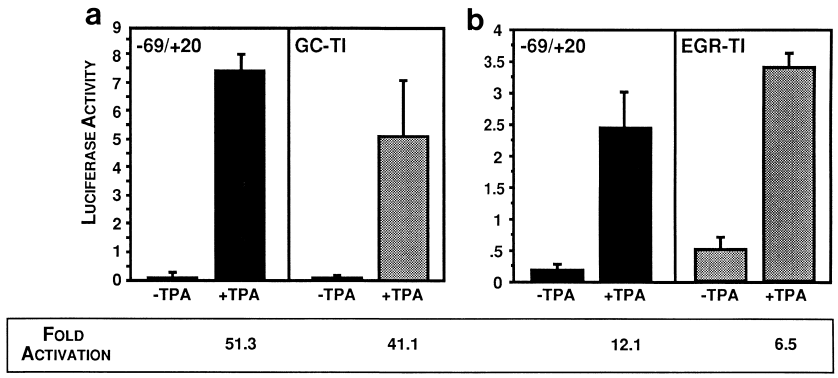

FIG. 5. GC-TI and EGR-TI are activated by TPA. K562 cells were electroporated with the −69/+20, GC-TI, or EGR-TI construct and then either were left untreated (−TPA) or were treated with TPA (+TPA) for 12 (a) or 10 h (b). Cells were harvested and lysed, and luciferase activities were determined with normalization for β-galactosidase as described in Materials and Methods. The average fold activation in each experiment is shown below each panel and is defined as normalized luciferase activity (TPA-treated cells)/normalized luciferase activity (untreated cells). Data from one representative experiment (of two) are shown for each construct. Vertical bars show standard deviations.

−69/+20 probe in complex 2, EMSAs were performed with nuclear extracts incubated in the presence of anti-EGR Abs (Fig. 6). As previously shown in Fig. 2a, only complex 1 was observed in nuclear extracts from log-phase cells (Fig. 6, lane 2). Both complexes 1 and 2 were observed in nuclear extracts from TPA-treated K562 cells (Fig. 6, lane 3). A complex migrating at the same rate as that of complex 2 was observed in lane 8, which contained ITT EGR1 rather than nuclear extract. Ab to SP1 partially inhibited the formation of complex 1 but had no effect on the formation of complex 2 (Fig. 6, lane 4). Likewise, SP1 Ab did not inhibit the formation of the complex formed by incubating ITT EGR1 with −69/+20 (Fig. 6, lane 9). The C-19 and 588 Abs to EGR1 inhibited the formation of complex 2 but had no effect on complex 1 (Fig. 6, lanes 5 and 7, respectively). The same Abs inhibited the formation of the complex formed by incubating ITT EGR1 with −69/+20 (Fig. 6, lanes 10 and 12, respectively). One EGR1 Ab, 675, did not inhibit the formation of either complex 2 (Fig. 6, lane 6) or the ITT EGR1 complex (lane 11). These data suggest that complex 2, observed only with extracts from TPA-treated cells, is formed by EGR1 binding to *MDR1* promoter sequences between −69 and +20.

EGR1 is detected only in extracts from TPA-treated cells. Next, we determined whether expression of the EGR1 protein correlated with *MDR1* expression and complex 2 formation after TPA treatment. Western analysis with the C-19 anti-EGR1 Ab was used to assay for EGR1 expression in extracts from TPA-treated and untreated cells (Fig. 7). This Ab recognizes EGR1 but does not cross-react with EGR2 or EGR3 (Santa Cruz Biotechnology). As shown in Fig. 6, C-19 Ab did not recognize purified EGR2 protein (lane 1) but did recognize ITT EGR1 (lane 2), thus demonstrating the specificity of this Ab for EGR1. No EGR1 protein was detected in nuclear extracts from log-phase K562 cells (Fig. 7, lane 3). However, significant amounts of EGR1 were detected in nuclear extracts from K562 cells treated with 16 nM TPA for 5, 10, or 24 h. No protein was detected when C-19 Ab was preincubated with EGR1 blocking peptide, demonstrating that the bands observed were not due to nonspecific binding (data not shown). Identical results were obtained with two additional nuclear extract preparations (data not shown). Similar experiments were performed with Abs to EGR2, EGR3, and SP1 (data not shown). EGR2 was present at a low level in log-phase cells and

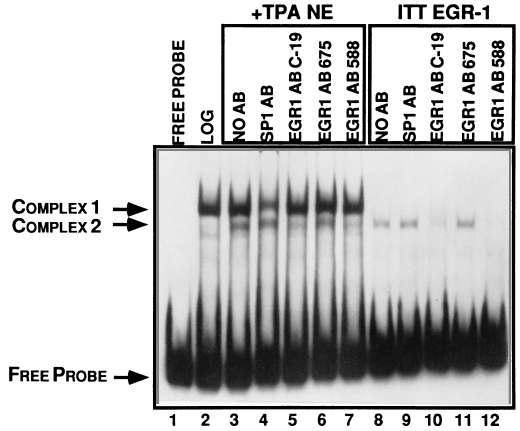

FIG. 6. EGR1 Ab inhibits complex 2 formation. EMSAs were performed with the −69/+20 oligonucleotide probe as described in the legend to Fig. 2 except that reaction mixtures were coincubated with Ab to SP1 or one of three EGR1 Abs. Reaction mixtures contained 7.5 μg of nuclear extracts from untreated or 24-h TPA-treated K562 cells or 4 μl of ITT EGR1. Lane 1, free probe; lane 2, untreated K562 extract (log); lanes 3 through 7, 24-h TPA-treated K562 extract (+TPA NE); lanes 8 through 12, ITT EGR1; lanes 3 and 8, no Ab; lanes 4 and 9, anti-SP1 Ab; lanes 5 and 10, anti-EGR1 Ab C-19; lanes 6 and 11, anti-EGR1 Ab 675; lanes 7 and 12, anti-EGR1 Ab 588.

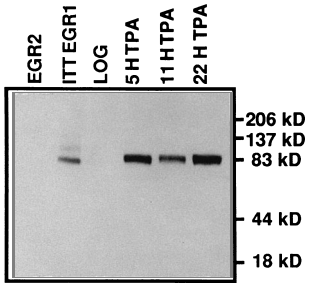

FIG. 7. EGR1 is expressed only in TPA-treated K562 cells. Nuclear extracts from untreated (log) K562 cells; K562 cells treated with TPA for 5, 11, or 22 h; or 15 μl of ITT EGR1 were electrophoresed on an SDS-polyacrylamide (4 to 10% gradient) gel and transferred to nitrocellulose for immunodetection. The filter was incubated with C-19 EGR1 Ab and horseradish peroxidase-conjugated secondary Ab. The signal was detected as described in Materials and Methods. kD, kilodaltons.

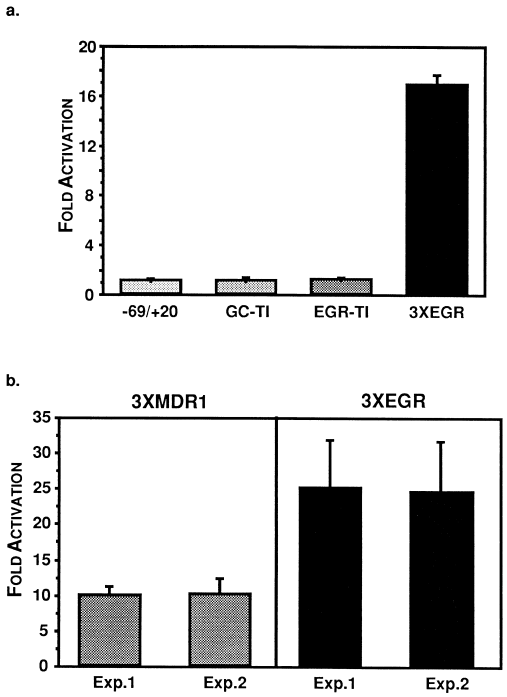

FIG. 8. EGR1 expression in untreated K562 cells induces reporter gene expression from constructs containing tandem, but not single, EGR sites. (a) K562 cells were transfected with 80 μg of pCB6+ vector control or 80 μg of CMV-EGR1 and 8 μg of −69/+20, GC-TI, EGR-TI, or 3XEGR reporter construct. After recovery for 48 h, cells were harvested and lysed; luciferase activities were measured with normalization for β-galactosidase expression as described in Materials and Methods. Fold activation was calculated by dividing the normalized luciferase activity of the samples from CMV-EGR1 transfection by that of the pCB6+ vector transfection. Data from one representative experiment (of at least two) are presented. (b) K562 cells were transfected with 100 μg of CMV-EGR1 or 100 μg of pCB6+ vector control and 10 μg of 3XMDR1 (left panel) or with 10 μg of 3XEGR and 100 μg of vector control or 100 μg of CMV-EGR1 (right panel). After a 48-h recovery, cells were harvested and lysed and luciferase activities were measured. Fold activation was calculated as described for panel a. Data from two experiments are shown for each reporter construct. Vertical bars show standard deviations.

did not change with TPA treatment. EGR3 expression in extracts from log-phase and TPA-treated cells could not be determined because of nonspecific binding by the commercially available Ab. SP1 expression in log-phase and TPA-treated cells was approximately equal. These data suggest that EGR1 expression after TPA treatment parallels *MDR1* promoter activity and steady-state RNA levels.

EGR1 expression in untreated K562 cells induces reporter gene expression from constructs containing tandem, but not single, EGR sites. The studies discussed above strongly suggest that EGR1 binding mediates the *MDR1* promoter response to TPA. If so, coexpression of EGR1 with the TPA-responsive −69/+20 or GC-TI construct might be sufficient to induce reporter gene activity. To test this hypothesis, we measured the activity of −69/+20, GC-TI, and EGR-TI in K562 cells cotransfected with the EGR expression construct CMV-EGR1 (33). The activity of 3XEGR, a construct which contains three tandem EGR consensus sites, was also tested with K562 cells cotransfected with CMV-EGR1. This construct is responsive to TPA (data not shown), and a similar construct has previously been shown to be responsive to CMV-EGR1 (33). As shown in Fig. 8a, cotransfection with CMV-EGR1 induced reporter gene expression from 3XEGR but did not induce reporter gene expression from EGR-TI, which contains only one EGR site. In addition, CMV-EGR1 did not induce reporter gene expression from either −69/+20 or GC-TI, each of which contains only one overlapping SP1/EGR site (Fig. 8a).

The studies discussed above demonstrate that EGR1 expression alone in transfected cells is sufficient to induce reporter gene expression from a construct containing tandem EGR sites, but the activation of a construct containing one EGR or one *MDR1* promoter SP1/EGR site is not detectable. Therefore, we hypothesized that in K562 cells under the conditions used in our assays, a tandem array of EGR binding sites is necessary to detect changes in activity in response to EGR1. If so, then a construct containing tandem *MDR1* promoter SP1/EGR binding sites might be activated by EGR1, whereas a construct containing only one such site (−69/+20 or GC-TI) would not be. To test this hypothesis, we measured the activity of the 3XMDR1 construct in response to EGR1. This construct contains three contiguous repeats of *MDR1* promoter sequences −54 to −41 cloned upstream of the luciferase cDNA. This tandem array of *MDR1* sequences binds both purified SP1 and ITT EGR1 in EMSAs and is responsive to TPA; the TPA response is inhibited by ΔN214 (data not shown). K562 cells were cotransfected with CMV-EGR1 and either 3XMDR1 or 3XEGR. As shown in Fig. 8b, reporter gene activity was induced from both 3XMDR1 and 3XEGR constructs. The average induction from two experiments was approximately 10-fold for 3XMDR1 and 25-fold for 3XEGR.

Thus, the activities of the single-site *MDR1* constructs −69/+20 and GC-TI parallel that of the single-site EGR construct EGR-TI. These constructs are activated by TPA, but activation by EGR1 is undetectable. In addition, the activity of the tandem-site *MDR1* construct 3XMDR1 parallels that of the tandem-site EGR construct 3XEGR in that it is activated by both TPA and EGR1.

DISCUSSION

We have studied the mechanism by which TPA induces the *MDR1* promoter in K562 cells to better understand the factors contributing to Pgp expression in hematopoietic cells. In this study, we demonstrated that (i) EGR1 protein expression and EGR1 binding increased in response to TPA, (ii) a mutation which inhibited EGR binding markedly reduced the *MDR1* promoter response to TPA, (iii) the *MDR1* promoter overlapping SP1/EGR binding region was TPA responsive, and (iv) cotransfection with CMV-EGR1 was sufficient to induce reporter gene expression from tandem overlapping SP1/EGR binding regions. Taken together, these data support a role for EGR1 in inducing the *MDR1* gene in hematopoietic cells and suggest that (i) EGR1 is necessary for *MDR1* promoter activity in response to TPA and (ii) EGR1 is sufficient to induce reporter gene activity by using the *MDR1* promoter overlapping SP1/EGR site. The experiments described here provide the first evidence implicating EGR1 in transcriptional activation of the *MDR1* gene. This work adds *MDR1* to the growing list of genes regulated by EGR1. This list includes the murine thymidine kinase (37), adenosine deaminase (1), and acetylcholinesterase genes (32); the rat cardiac α-myosin heavy-chain (24) and adrenal phenylethanolamine *N*-methyltransferase genes (19); and the human platelet-derived growth factor A (56), transforming growth factor β1 (16), and IL-3 (30) genes. EGR1 can activate (19, 24, 30, 32, 37, 56) or repress (1) these promoters, although its effect may depend on cell type and promoter context (1, 22, 56).

The purpose of this study was to identify the *cis*- and *trans*-acting factors involved in the TPA-induced increase in *MDR1* expression in K562 cells. We observed that inhibition of EGR1

binding markedly reduced the response to TPA of the −69/+20 promoter construct which contains the *MDR1* promoter TPA-responsive element. However, cotransfection with EGR1 did not induce detectable activity from this promoter construct. At least two mechanisms might account for these results. First, the absence of activation of −69/+20 by EGR1 may simply be due to an inability to detect activation in vitro of a single EGR binding site. The observation that cotransfection with CMV-EGR1 did not induce detectable expression from the −69/+20 or GC-TI construct, each of which contains a single SP1/EGR binding site, but did induce expression from 3XMDR, which contains three such sites in tandem, supports this. Likewise, cotransfection with CMV-EGR1 did not induce detectable expression from EGR-TI, which contains a single EGR consensus site, but did induce expression from 3XEGR, which contains three such sites. The observation that tandem sites are synergistic in transient transfection assays is not unique to EGR1 sites. For instance, it has been observed previously for SP1 that activation of a construct containing two SP1 sites is 78-fold higher than that of a construct containing only one site (43).

Alternatively, it is possible that EGR1 is necessary for the binding interactions that mediate the *MDR1* promoter response to TPA but that activation by TPA is also dependent on an array of signals which influence transcription, such as modification of transcriptional coactivators. Such a mechanism may not be limited to the *MDR1* promoter. For instance, TPA induced EGR1 binding to the IL-3 promoter, but cotransfection with EGR1 did not induce IL-3 promoter activity (30). Such a mechanism may not be limited to the TPA response. For example, a single E2F site mediates a serum response but is not activated by cotransfection of E2F family members (20a). These observations and our data indicate that while a factor may be essential for a response, additional signals may be required to elicit a detectable response.

The complex nature of TPA-induced signals may explain why *MDR1* is not induced by TPA treatment in U937 and HL60 cells (4, 36), although TPA induces EGR1 expression in these myeloid cell lines (27, 28, 40, 52). Of note is the fact that unstimulated U937 and HL60 cell lines do not express *MDR1* (4, 36), while K562 cells express detectable levels of *MDR1* (4; this paper). *MDR1* is expressed in unstimulated KG1 cells, and expression increases after TPA treatment (4, 36), suggesting that *MDR1* expression might be regulated differently in cells that constitutively express *MDR1*. For instance, *trans*-acting factors in U937 and HL60 cells may constitutively inhibit *MDR1* expression, even in response to TPA and other stimuli. One possible inhibitory factor is WT1, the product of the Wilms' tumor suppressor gene. WT1 is a member of the EGR transcription factor family. It shares considerable homology with the other EGR transcription factors and binds to the same DNA sequence, albeit with reduced affinity (reviewed in reference 34). Cotransfection of a WT1 cDNA construct inhibits TPA activation of the *MDR1* promoter in K562 cells (data not shown), which is consistent with previously reported repression by WT1 of promoters activated by EGR1 in vitro (16, 18, 21, 33, 56). WT1 expression decreases in K562 cells after TPA treatment (44), so relative transcriptional repression of the *MDR1* promoter in K562 cells might be relieved as WT1 levels fall with TPA treatment. The role of WT1 in regulating *MDR1* promoter activity is currently under investigation.

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