The promoter of the rat pgp2/mdr1b gene has a GC-rich region (pgp2GC) that is highly conserved in mdr genes and contains an consensus Sp1 site. Sp1’s role in transactivation of the pgp2/mdr1b promoter was tested in Drosophila Schneider cells. The pgp2/mdr1b promoter was strongly activated by co-transfected wild type Sp1 but not mutant Sp1 and mutation of the Sp1 site abrogated Sp1-dependent transactivation. In gel shift assays, the same mutations abolished Sp1-DNA complex formation. Moreover, basal activity of the pgp2/mdr1b Sp1 mutant promoter was dramatically lowered. Enforced ectopic overexpression of Sp1 in H35 rat hepatoma cells revealed that cell lines overexpressing Sp1 had increased endogenous pgp2/mdr1b mRNA, demonstrating that Sp1 activates the endogenous pgp2/mdr1b gene. Pgp2GC oligonucleotide also bound Egr-1 in gel shift assays and Egr-1 competitively displaced bound Sp1. In transient transfections of H35 cells (and human LS180 and HepG2 cells) Egr-1 potently and specifically suppressed pgp2/mdr1b promoter activity and mutations in the Egr-1 site decreased Egr-1 binding and correlated with pgp2/mdr1b up-regulation. Ectopic overexpression of Egr-1 in H35 cells decreased Pgp expression and selectively increased vinblastine sensitivity. In conclusion, Sp1 positively regulates while Egr-1 negatively regulates the rat pgp2/mdr1b gene. Moreover, competitive interactions between Sp1 and Egr-1 in all likelihood determine the constitutive expression of the pgp2/mdr1b gene in H35 cells.

Overexpression of the multidrug resistance gene (MDR1) product P-glycoprotein (Pgp) is often correlated with chemotherapeutic failure (1, 2). Pgp is a member of the large ATP-binding cassette transporter gene family (3). In humans Pgp-mediated efflux of cytotoxic drugs is linked to only one human gene (MDR1), whereas in rodents drug efflux is shared between two mdr1 genes (pgp1/mdr1a and pgp2/mdr1b). Whether the two rodent mdr1 genes are functionally redundant with respect to the efflux of cytotoxic chemotherapeutic agents remains controversial (4). There is evidence that the two rodent mdr1 genes are differentially regulated. For example, pgp1/mdr1a and pgp2/mdr1b show divergent tissue regulation with some organs, such as murine intestine and brain, expressing exclusively mdr1a while adrenal and placenta express predominantly mdr1b (5, 6). In addition, the rat pgp2/mdr1b and pgp1/mdr1a are divergently regulated during hepatocarcinogenesis in rats (7). In order to extrapolate rodent studies to humans it is critical to determine the molecular controls regulating the rat and human MDR1 genes.

Previous studies have suggested that human and rodent MDR1 genes are also distinctly regulated. For instance, in many cell lineages from rodents (liver, kidney, intestine, etc.) short exposures to chemotherapeutic agents lead to dramatic increases in mdr1 expression, an effect that appears secondary to specific increases in mdr1 transcription (8). In contrast, transient exposure to these chemotherapeutic agents had no effect on the expression of MDR1 in human cells (8). Other studies, using primary cultured hepatocytes also showed a divergence in rodent and human MDR1 expression after exposure to polycyclic aromatic hydrocarbons (9–11). These studies suggest that fundamental differences in MDR1 regulation exist between rodent and humans.

Recent studies suggest that events linked to transcription are important in controlling MDR1 expression. For instance, Scotto and co-workers (12) suggested that a conserved element and binding proteins may be important in hamster Pgp1 transcriptional initiation in Pgpp expressing cells. A different conserved sequence in the mouse mdr1b gene and human MDR1 gene, functionally important for both promoters (13–15), was found to be activated in the MDR1 promoter by a member of the C/EBP family (15). The human and rodent MDR1 promoters also contained a common stretch of GC-rich nucleotides. The conservation of this GC region across species suggests that it is important for regulation of the MDR1 genes. In the human MDR1 promoter, Sp1 binds to a portion of the GC element and activates the MDR1 promoter (16). This MDR1 GC element also binds the transcription factor Egr-1 (17) and Egr-1 mediates 12-O-tetradecanoylphorbol-13-acetate activation of the MDR1 promoter in K562 hematopoietic cells. More recent studies have suggested that the human MDR1 promoter has multiple Sp1 sites that may not be functionally equivalent (18).

However, the importance in general of Sp1 and Egr1 to regulation of the endogenous rat mdr1b gene and to its expression in tissues that highly express this gene, such as liver, have not been examined. These studies were undertaken to evaluate the functional importance of Sp1 and Egr-1 to the regulation and expression of the rat mdr1b gene in the rat H35 hepatoma cell line. In this report we identify a Sp1/Egr-1 binding site in the promoter of pgp2/mdr1b that is similar to the Sp1/Egr-1 site in human MDR1 (16). Using transfection and DNA binding
assays of the pgp2/mdr1b promoter and ectopic overexpression of the Egr-1 and Sp1 transcription factors these studies revealed regulation of pgp2/mdr1b expression by Egr-1 and Sp1 that is unique from what has been reported for regulation of mammalian MDR1.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes, T4 polynucleotide kinase, and T4 DNA ligase were from Life Technologies (Gaithersburg, MD), Promega (Madison, WI), and New England Biolabs (Beverly, MA). [γ-32P]ATP or [α-32P]ATP were from EN Life Science Products (Wilmington, DE). PCR reagents were from Perkin-Elmer (Norwalk, CT). Cell culture medium and fetal bovine serum were from BioWhittaker (Piscataway, NJ). G418 sulfate (gentamicin) was from Life Technologies (Gaithersburg, MD). Prime-It II kits were from Stratagene (La Jolla, CA). Affinity-purified Sp1 was from Promega (Madison, WI). Polyclonal antibodies to Egr-1 and Sp1 were purchased from Santa Cruz Biotech, and the mdr1 (Ab-1) and actin antibodies were purchased from Calbiochem (La Jolla, CA).

**Construction of Vectors**—250WT was generated by PCR amplification using the sense oligonucleotide (−250 to −228) 5′-gaagatctATGCACCCGTGTCATCAGGCCTG-3′ and the antisense oligonucleotide (+137 to +150) 5′-gaagatctCTCACGTAACACCTCCGGGTTTACCATC-3′. The promoter region of rat mdr1b/mdr1a gene and represents nucleotides −250 to +150 of the rat pgp2/mdr1b gene. The PCR amplified product was digested with BglII and subcloned into pG2 Basic (Promega, Madison, WI). Orientation was confirmed by restriction mapping and DNA sequence analysis.

The Sp1 mutation was introduced into the −36 to −60 region of the Pg2/mdr1b promoter by the overlap-extension method used previously (19) to generate −250MT. Briefly, two independent PCR products spanning nucleotides −250 to −36 and −60 to +150 were made that overlap the mutation region. The 5′-half was made using the sense oligonucleotide −250 to −228 (above) and the antisense oligonucleotide (−60 to −36) 5′-CCCGCGGCGCCAACTGTTGCCTAGC-3′ with the underlined base substitutions. The 3′-half was made using the sense oligonucleotide (−60 to −36) 5′-CTGAGGCAACCAGTTGGCAAGGCGGAG-3′ and antisense oligonucleotide (+137 to +150) (above). The two PCR products were combined, denatured, and reannealed at their common overlapping 24-bp sequence. Extension of this overlap followed by PCR amplification using the two outer primers gave a recombinant molecule with four specific base changes in the GC region. This fragment was digested with BglII, ligated into pG2 Basic, and the mutational DNA sequence was confirmed previously (26). The sp1′/Egr-1 expression plasmid was developed by digestion of the −250MT plasmid with NcoI followed by treatment with mung bean nuclease, gel purification, and re-ligation.

The mammalian Sp1 expression vector was constructed by subcloning a 4.4-kilobase XbaI fragment encoding the full-length Sp1 cDNA from pBS-Sp1-I (a gift of Dr. R. Tjian, University of California, Berkeley, CA) into the unique site of the pcDNA3 vector (Invitrogen, San Diego, CA) and orientation confirmed by restriction mapping and DNA sequence analysis. The Drosophila Sp1 expression construct (pPACPsp1) and COOH-terminal deletion (pPACPsp1N539) (from R. Tjian) were subcloned downstream of the Drosophila actin promoter in pPAC0 (20).

**Transient Transfections**—Reuber H35 hepatoma cells (American Type Culture Collection) were maintained as described previously (19) and were transfected by calcium phosphate co-precipitation with 10 μg of QIAGEN (Qiagen Corp., Chatsworth, CA) purified plasmid DNA for 16–18 h. Cultures were then washed with serum-free medium, refed with fresh medium, harvested after 24 h, and assayed for luciferase.

**Luciferase Assays**—After 24 h, cells were harvested and assayed for luciferase activity. Cells were harvested and assayed for luciferase activity.

**Stable Transfections**—H35 cells were transfected with Sp1-pcDNA3, Egr-1-pcDNA3, or pcDNA3 as the calcium phosphate for 16 h, washed, and refed, and after the 24-h expression period, the cells were expanded and selected in medium containing 400 μg/ml G418. Individual G418-resistant colonies were isolated after 3–4 weeks drug selection. To obtain growth curves of the pcDNA3, Sp1, and Egr-1 clones, cells were plated in 60-mm dishes with 10,000 cells per plate, washed with phosphate-buffered saline, scraped, and counted with a hemocytometer on consecutive days. Population doubling times were calculated as described previously (23, 24).

**RNA Isolation and Northern Blot Analysis**—Total RNA was isolated from H35 cells plated on 100-mm tissue culture dish (19) and analyzed by Northern Blot analysis. Briefly, cells were washed with phosphate-buffered saline, and then incubated and washed with phosphate-buffered saline, scraped, and counted at a hemocytometer on consecutive days. Population doubling times were calculated as described previously (23, 24).

**Western Immunoblot Analysis of Egr in Cell Lysates and Egr-1 and Sp1 in Nuclear Extracts**—For analysis of Pgp, cells lysates (35 μg) were analyzed by immunoblot with an anti-Mdr polyclonal antibody (Ab-1) and developed as described previously (19). Intensity of bands was quantified by densitometry and values were expressed relative to untreated controls. For Egr-1 or Sp1, nuclear extracts from H35 cell lines (prepared as below) were analyzed by immunoblot using specific polyclonal antibodies directed against the carboxyl-terminal 14 amino acids of Egr-1 or anti-Sp1 polyclonal PEP2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

**Oligonucleotides and Electrophoretic Mobility Shift Assays (EMSA)**—The following double-stranded oligonucleotides were used in EMSAs: pgp2GC, 5′-gaagatctGGGGGGCCAAAGCGCCCGCggagtcgt-3′; mtpgp2GC, 5′-gaagatctCTCAGCAAGCCTGGCCGGG-3′; Sp1/wtEgr-1, 5′-gaagatctGCGGGCGCCACACTTGTTCGCGGCG-3′; Sp1′/Egr-1, 5′-gaagatctGGGGCGCCACACTTGTTCGCGGCG-3′; Sp1′/Egr-2, 5′-gaagatctGGGGCCACACTTGTTCGCGGCG-3′; Sp1′/Egr-3, 5′-gaagatctGGGGCCACACTTGTTCGCGGCG-3′; EgrMT, 5′-ggATCCAGTGACCGGACGTAAGCGGCGGAG-3′; Sp1′-AACTGTCAGCAGGGCGGATGCGGCAGCCG-3′. The Xs in Sp1′/Egr-2 indicate deletion of the corresponding nucleotides. The pgp2GC, mtpgp2GC, and the Sp1′/Egr-2 oligonucleotides were obtained from Biosynthesis Inc. (Lewisville, TX). The consensus and mutant Egr oligonucleotides were obtained from Santa Cruz Biotechnology Inc. and the consensus Sp1 oligonucleotide was obtained from Promega.

All oligonucleotides were purified by agarose electrophoresis, annealed with the complementary oligonucleotide, and then end labeled with [γ-32P]ATP and T4 polynucleotide kinase. The labeled oligos were purified by Nuc-trap (Stratagene, CA). EMSAs were performed as described previously (26). Protein (either H35 nuclear extract or affinity purified Sp1 or the recombinant Egr-1-GST fusion protein) was incubated with 1 μg of poly(dI-dC) in 20 μl Tris-HCl, 80 mM NaCl, and 1 mM dithiothreitol. Unlabeled specific competitor was then added and specific radiolabeled oligonucleotide was added last. Incubation was carried out at room temperature for 20 min. The reaction mixture was electrophoresed on 4% polyacrylamide gels with 0.25% TBE as gel running buffer. Gels were dried and bands visualized by autoradiography. Competition oligo was added at 2-, 50-, or 100-fold excess and concurrent with the radiolabeled probe. When antibody to Egr-1 was used, the nuclear extract and antibody were preincubated at 4 °C for 25 min before the radiolabeled probe was added.

**Nuclear Extracts and Expression of Egr-1-GST Fusion Protein—** Nuclear Extracts were prepared from H35 cells essentially as described by Latchman (27). Briefly, cells grown on 60-mm dishes were washed 1× with phosphate-buffered saline, and then harvested in 1 ml of chilled phosphate-buffered saline, spun down, and resuspended in 100 μl of nuclear harvest buffer (20 mM Hepes, 450 mM NaCl, 0.5 mM dithiothreitol, and 25% glycerol, 2 mM phenylmethylsulfonyl fluoride). The suspension was then frozen and thawed three times in a dry-ice ethanol bath, spun down at 10,000 × g at 4 °C for 10 min and the supernatant used in gel-shift assays.

Recombinant Egr-1 was prepared using the Egr-1-GST fusion vector according to the manufacturer's instructions (Pharmacia, Milwaukee, WI). Briefly, an overnight culture of Escherichia coli transformed with the expression vector was diluted 1:10 into fresh LB medium containing glucose and grown until an OD of 1–2 was reached. Expression of the fusion protein was induced with isopropyl-1-thio-β-d-galactoside to a final concentration of 0.1 mM. After growing for an additional 2–6 h, the bacteria were pelleted, and resuspended in 1× phosphate-buffered saline. Phenylmethylsulfonyl fluoride was added to 2 mM and the suspension sonicated on ice with six 30-s bursts. Triton X-100 was then added to a final concentration of 10%. The suspension mixed, allowed to sit on ice for another 30 min, spun down, and the
supernatant used in gel-shift assays.

**In Vitro Transcription-Translation**—Egr1-pcDNA3, the plasmid used in *in vitro* transcription-translation contains Egr1 downstream of the T7 promoter in pcDNA3 (Invitrogen). Egr-pcDNA3 was translated using a TNT T7 quick coupled transcription-translation system (Promega) under conditions recommended by the manufacturer. Preliminary studies determined that 1/10 to 1/50 of the *in vitro* translation mixture could be used in EMSA with a consensus Egr probe.

**Short-term Drug Cytotoxicity Assays**—The proportion of total cellular lactate dehydrogenase released into the culture medium was used to measure cytotoxicity of various antitumor drugs and has been previously described (28). 3–5 × 10^5 cells of each of the H35 clones stably expressing Egr-1 or neo vector were plated onto 60-mm dishes. Cells were then cultured for 48 h in the presence of either 100 nM vinblastine or 100 μM fluorodeoxyuridine. After this interval aliquots of medium were sampled. The medium was then aspirated and the remaining attached cells lysed. Lactate dehydrogenase activity was measured in the medium and cell lysate by using a Cytotoxicity Assay kit as described previously (29). The proportion of lactate dehydrogenase released into the medium as a fraction of the total lactate dehydrogenase was then determined.

**RESULTS**

**A Phylogenetically Conserved GC Box in MDR1 Genes**—Sequence comparison of the 5′ promoter regions of the rat, hamster, human, and murine MDR1 genes revealed within each of the promoters a cluster of GC bases containing a consensus site for Sp1 (Fig. 1). Because of the phylogenetic conservation of the GC region in the MDR1 genes and the fact that a number of transcription factors bind to GC-rich regions (e.g. Sp1, Egr-1, and Ap-2) we hypothesized that the interactions between these factors and Sp1 in this GC region are important in basal regulation of the MDR1 promoters.

**Sp1 Binds to and Is a Positive Transcriptional Regulator of the pgp2/mdr1b Promoter**—We first tested whether the GC region in the pgp2/mdr1b promoter could bind Sp1 by preparing a wild-type oligonucleotide (pgp2GC) and a mutant oligonucleotide (pgp2GC) that contained a 2-base pair mutation in the GC box that we predicted would disrupt Sp1 binding. EMSA demonstrated that wild type oligonucleotide was effectively shifted by purified Sp1 and that the mutations in the pgp2/mdr1b Sp1 site resulted in a complete loss of Sp1 binding (Fig. 2A).

The functional significance of Sp1 mutation upon pgp2/mdr1b constitutive promoter activity were assessed *in vivo* by introducing the identical mutations of the mtgp2GC oligonucleotide into the pgp2/mdr1b reporter (−250MT). H35 hepatoma cells were chosen for transfection because they express pgp2/mdr1b (19), and maintain normal liver characteristics such as production of bile and expression of cytochromes P450 (30). The introduction of two point mutations in the pgp2/mdr1b promoter decreased basal promoter activity of −250MT to approximately 40% of the activity of −250WT in transfected H35 cells (Fig. 2B).

The finding that Sp1 mutations in the mdr1b promoter decreased its basal activity suggested Sp1 might play a role in positively regulating pgp2/mdr1b. This possibility was evaluated by transient transfection of −250WT or −250MT into Drosophila Schneider cells (that are Sp1 negative) along with various amounts of either a wild-type Sp1 expression vector or an inactive COOH-terminal Sp1 mutant (N539) (20) (Fig. 2C). Sp1 (200–300 ng) readily increased pgp2/mdr1b promoter activity from 7–25-fold. In contrast, cells co-transfected with N539, a zinc-finger deletion mutant showed no dose-related increase in the activity of the pgp2/mdr1b promoter. Finally, the pgp2/mdr1b promoter with the mutated Sp1 site was not appreciably activated by Sp1 (Fig. 2C) thus demonstrating that an intact Sp1 site is required for Sp1 dependent transactivation.

**Induction of the Endogenous pgp2/mdr1b Gene by Enforced Overexpression of Sp1 in H35 Rat Hepatoma Cells**—Although the pgp2/mdr1b promoter requires Sp1 for full basal activity (Fig. 2) it is unknown if ectopic overexpression of Sp1 can affect basal Pgp expression. In preliminary studies, we found that co-transfection of the cytomegalovirus-driven Sp1 expression vector (see “Methods”) and the −250WT plasmid into H35 cells lead to an Sp1-dependent transactivation in H35 cells (not shown). This Sp1 expression vector was then transfected into H35 cells and G418-resistant colonies were isolated. Several independent clones were selected and evaluated for immunoreactive Sp1, the amount of Sp1 DNA binding activity and the amount of immunoreactive Pgp. Compared with the Neo (pc2) clone, immunoreactive Sp1 expression increased from 1.8-fold to almost 5-fold in Sp1 clones 20 and 9, respectively (Fig. 3A). Further analysis of representative Sp1 clones revealed increased Sp1 DNA binding complexes compared with the Neo cell line (Fig. 3B). The expression of Pgp in Sp1 cell lines 9, 18, and 20 increased 3–6-fold above the Neo control line (Fig. 3C). Finally, we have shown that the transcriptional activity of the pgp2/mdr1b promoter increased 4.3-fold in the Sp18 cell line, compared with its activity in the Neo control line (not shown), a value comparable to the increase in Sp1 expression. These studies are the first to forge a direct link between enforced overexpression of Sp1 in cells and increased Pgp expression.

**Egr-1 Binds the pgp2GC Site but Negatively Regulates the pgp2/mdr1b Promoter**—To first test whether the pgp2GC region interacts with Egr1, H35 cell extracts were prepared. EMSA analysis demonstrated a specific DNA-protein complex formed with pgp2GC that was specifically reduced by incubation with either unlabeled consensus pgp2GC or Egr consensus and not by unrelated oligonucleotides such as Oct-1 and Sp1 (Fig. 4A).

To evaluate whether pgp2GC bound Egr-1, we *in vitro* translated Egr-1 and then performed EMSA analysis utilizing either radiolabeled pgp2GC or Egr as probes. Egr-1 readily bound the pgp2GC and Egr probes. The nonspecific competitor Oct-1 did not displace bound Egr-1 from either pgp2GC or Egr probes. In contrast, pgp2GC readily displaced Egr-1 as did the 100-fold excess wild-type Egr competitor. A small displacement was seen with 200-fold excess mutant Egr competitor.

To evaluate the biological importance of Egr-1 in regulating the pgp2/mdr1b promoter, we co-transfected H35 cells with −250WT and either a wild-type Egr-1 expression plasmid (wtEgr-1) or a mutant Egr-1 that has a zinc-finger deleted and
is incapable of binding DNA (mtEgr-1) (Fig. 5). Egr-1 suppressed the −250WT promoter in a dose-dependent manner reaching nearly complete suppression at 5 μg of Egr-1. Egr-1 suppression of the pgp2/mdr1b promoter was specific and not secondary to squelching because Egr-1 did not repress thymidine kinase promoter activity (not shown). Thus, the potent suppression of pgp2/mdr1b by wtEgr-1 but not mtEgr-1 indicates that an intact Egr-1 DNA-binding domain is required.

**Fig. 2.** Sp1 activates and is essential for full pgp2/mdr1b promoter activity. A, radiolabeled pgp2GC or mtpgp2GC oligonucleotide was incubated with (+) and without (−) affinity-purified Sp1 (one footprinting unit) and analyzed by EMSA. The last lane with mtpgp2GC contains 5 footprinting units of Sp1. B, the activity of −250WT promoter was compared with the −250MT pgp2/mdr1b mutant promoter that contained the identical mutations as were found in the mtpgp2GC oligonucleotide. 10 μg of each of these plasmids were transiently transfected into H35 cells and luciferase activity measured. Results are expressed as activity relative to the −250WT promoter (100%). C, Drosophila Schneider cells were transiently transfected with 1 μg of the −250WT or −250MT plasmid along with indicated amounts of pPacSp1 (wild-type Sp1 expression plasmid) or N539 (a COOH-terminal Sp1 deletion mutant) and luciferase activity measured. Values in B and C are the average of three separate transfection experiments each performed in duplicate to quadruplicate. The error bars represent the standard error of the mean. Results are expressed as fold-activation relative to −250WT (1.0) or −250MT (1.0) co-transfected with empty vector.

**Fig. 3.** Pgp and Sp1 levels and Sp1 DNA binding in H35 clones stably expressing Sp1 (Sp1–9, 10, 18, or 20) or Neo vector (pc2). A, analysis of Sp1: 50 μg of nuclear extract protein from the indicated cell lines was analyzed by immunoblot with anti-Sp1 IgG. B, a radiolabeled consensus Sp1 oligonucleotide was incubated with nuclear extracts prepared from H35 clones stably expressing Sp1 or pcDNA vector and analyzed by EMSA. C, analysis of Pgp: 35 μg of total cell lysate protein from H35 clones was analyzed by immunoblot.
Furthermore, Egr-1 suppressed the pgp2/mdr1b promoter in both human colon carcinoma cells (LS180) and in a human hepatoblastoma cell line (HepG2) (not shown). Thus, Egr-1 potently transrepressed the pgp2/mdr1b promoter regardless of cell context.

Egr-1 Displaces Sp1 from Its Site on the GC-rich Promoter Element in the Rat pgp2/mdr1b Gene—Because Egr-1 and Sp1 have opposing effects on the pgp2/mdr1b promoter activity (Figs. 2 and 5) we assessed by EMSA whether Egr-1 and Sp1 might competitively interact at the pgp2GC site (Fig. 6). The Egr-1/pgp2GC complex migrates between Sp1 and the free probe. As a control for the specificity of the complexes, the Egr-1 DNA complex was competed with excess pgp2GC and the Sp1 DNA complex was competed with a consensus Sp1 oligonucleotide (not shown). In preliminary EMSA studies using limiting amounts of radiolabeled pgp2GC, we determined that recombinant Egr1 and Sp1 do not simultaneously bind to pgp2GC because a third slower migrating complex was not observed when the two proteins were added together and then compared with separate Sp1 or Egr1 complexes individually (not shown). To test whether Egr1 and Sp1 share an overlapping site, the pgp2GC probe was incubated with increasing concentrations of recombinant Egr1 in the presence of a fixed amount of Sp1. As the concentration of Egr1 was increased, the amount of Egr1 and pgp2GC complex increased while the Sp1/infusion complex decreased (Fig. 6). However, when the amount of recombinant Egr1 was held constant and Sp1 changed incrementally, the Egr1/pgp2GC complex showed only minimal displacement. Clearly, the inability to displace Egr1 is not due to excess probe because in the adjacent Egr1 versus Sp1 competition experiment (left panel) the free probe is at a slightly higher amount (Fig. 6). These data indicate that Sp1 and Egr1 do not simultaneously bind to pgp2GC and that these two transcription factors competitively interact at this site. It is also obvious under these conditions that Sp1 is not very effective in displacing Egr1, a finding that likely depends upon the relative binding affinity of these two factors for the site.

Diminished Egr-1 Binding Up-regulates the Basal Pgp2/mdr1b Promoter in H35 Cells—To address the role of Egr-1 in pgp2/mdr1b promoter activity, a 4-bp deletion was introduced in the Sp1 mutant oligonucleotide (mtpgp2GC) to generate Sp1mt/Egrmt. The Sp1 mutant, mtpgp2GC oligonucleotide, readily bound the recombinant Egr-1 (Fig. 7).

**Fig. 4.** Pgp2/mdr1b binds authentic Egr-1 and Egr-1 in H35 Cells. A, 32P end-labeled pgp2GC oligonucleotide was incubated with H35 nuclear extract in the presence or absence (NC, no competitor) of excess unlabeled pgp2GC, Sp1, Egr, or Oct-1 oligonucleotide (see “Experimental Procedures” for amounts) and analyzed by EMSA. B, 32P-labeled pgp2GC or Egr consensus oligonucleotides were incubated with in vitro translated Egr1 in the presence or absence (NC, no competitor) of various competitor oligonucleotides. The molar excess of competitors was as follows: Oct-1 (200-fold), pgp2GC (100-fold), EgrWT (100-fold), and EgrMT (200-fold).

**Fig. 5.** Egr-1 specifically represses the Pgp2/mdr1b promoter in H35 cells. −250WT (5 µg) was co-transfected into H35 cells with the indicated amounts of a wild-type (wtEgr-1) or mutant (mtEgr-1) cytomegalovirus-Egr-1 expression vector. The amount of cytomegalovirus promoter was kept constant by co-transfecting in empty parent vector pCB6+. Cells were harvested and luciferase activities were measured. Values represent the mean ± S.D. of two different experiments from a total of four independent determinations per experiment.

**Fig. 6.** Competitive displacement of Sp1 binding to the −36 GC-rich region in the Pgp2/mdr1b promoter by Egr-1. EMSA was performed on 32P end-labeled pgp2GC oligonucleotide (left panel) incubated in the presence of a constant amount of Sp1 (1 footprinting unit/incubation) and increasing amounts of bacterially expressed Egr-1-GST fusion protein, or (right panel) incubated with a fixed amount of recombinant Egr-1-GST (2 µl/reaction) and increasing amounts of recombinant Sp1 (1–10 footprinting units).
Sp1 sites. However, equivalent amounts of Egr-1 and even 2.5 times this amount of recombinant Egr-1 protein showed significantly diminished Egr-1 binding to the Sp1mt/Egrmt oligonucleotide (Fig. 7A).

In order to evaluate the effect of Egr-1 on the pgp2/mdr1b promoter independent of Sp1, we engineered the same 4-bp deletion (bp -39 to -36) into the putative Egr-1 site using as template the -250MT construct that contains disruptions of the Sp1 binding motif to generate -250 Sp1mt/Egrmt. Like the experimental results depicted in Fig. 2B, disruption of the Sp1 binding motif alone (-250MT) decreased pgp2/mdr1b transcriptional activity (Fig. 7B) in H35 cells. Despite the loss in Sp1 binding, the additional disruption of the Egr-1-binding site (-250 Sp1mt/Egrmt) led to a greater than 4-fold increase in basal promoter activity compared with -250MT (Fig. 7B). Moreover, this promoter was more active than the plasmid containing the unaltered wild type pgp2/mdr1b promoter (-250WT). These studies show that in the absence of an Sp1 site, the pgp2/mdr1b promoter activity is increased by disruption of the Egr-1 DNA-binding site and that the decreased Egr-1 binding correlates with an up-regulation of pgp2/mdr1b promoter activity. Therefore, it is most likely that the loss of activity of -250MT promoter (Fig. 2C) is due to an overall negative effect of Egr-1 in these cells. To additionally prove that Egr-1 repressed the pgp2/mdr1b promoter through the Egr1 site alone, we attempted to selectively disrupt the Egr-1 site but were unable to successfully generate this deletion.

Enforced Expression of Egr-1 in H35 Hepatoma Cells Leads to Decreased Pgp Levels—Although H35 cells express Egr-1 (31) to assess the possible dominant role of Egr-1 upon Pgp expression, H35 cells were stably transfected with an Egr-1 expression vector and clonal cell lines selected (Fig. 8). We selected this approach over chemical treatments known to affect Egr-1 (e.g. 12-O-tetradecanoylphorbol-13-acetate or thapsigargin) (17) because these agents might affect other signaling pathways in H35 cells. Western blot analysis of these lysates revealed that Egr-1 expression in Egr-Neo clones increased significantly compared with the Neo cell line (Fig. 8). It is interesting to note the level of immunoreactive Sp1 also increased, but not in direct proportion to Egr-1 expression. The steady-state levels of pgp2/mdr1b mRNA among these same clones was inversely related to Egr-1 expression and decreased an average of 3-fold in the Egr-1 overexpressing cell lines compared with either the Neo2 or a cell line that contained Egr-1 in the reverse orientation (not shown).

To determine how pgp2/mdr1b promoter activity was affected by Egr1 overexpression, -250WT and -250MT were transiently transfected into Neo and Egr-Neo#6 cells and the ratio of -250WT: -250MT promoter activity was determined. For the Neo cells the ratio was 2.2, a value consistent with the findings in Figs. 2B and 7B showing decreased basal activity of -250MT. In contrast, in Egr-Neo#6 cells the ratio was only 1.2, a value reflecting suppression of the -250WT promoter in the Egr1 overexpressing cells (not shown).

Increased Levels of Egr-1 Correlates with Reduced Drug Resistance—The functional consequence of increased Egr-1 expression on drug sensitivity was measured by 5-fluorodeoxyuridine and vinblastine sensitivity. Because in some cell types, Egr-1 overexpression is linked to an increased susceptibility to apoptosis (32), we also evaluated the Egr-1 cell lines (three additional Egr-1 expressing cells were also analyzed) for altered sensitivity to 5-fluorodeoxyuridine, a fluorinated nucleoside analog and a non-Pgp substrate. Enhanced expression of Egr-1 correlated with decreased Pgp protein (Fig. 9A) and a selective increase in vinblastine sensitivity (Fig. 9B). In contrast, the 5-fluorodeoxyuridine sensitivity was almost identical among the cell lines (Fig. 9C). Furthermore, the enforced expression of Egr-1 had no gross affect upon the doubling time of the Egr-1 clones (Egr-1 = 27.4 ± 3.0 and neo 24.9 ± 4.3), thus indicating that the selective difference in vinblastine sensitiv-
pressed as a percent of total cellular lactate dehydrogenase. The toxicity of Pgp levels and leads to a selective increase in vinblastine cytotoxicity. A, total cell lysates (35 μg) from Egr-1 H35 clones or vector Neo H35 clones (Neo2) were analyzed by immunoblot for Pgp or actin expression. B and C, 3–5 × 10^6 cells of each of the indicated Egr-1 and Neo H35 clones were plated on 60-mm dishes, 24 h later the dishes were washed and treated with either (A) vinblastine (100 nM) or (B) 5'-fluorodeoxyuridine (100 μM). After a 48-h treatment, lactate dehydrogenase released into the medium and remaining in attached cells were independently measured and lactate dehydrogenase in the media expressed as a percent of total cellular lactate dehydrogenase.

**FIG. 9. Egr-1 overexpression in H35 hepatoma cells decreases Pgp levels and leads to a selective increase in vinblastine cytotoxicity.** A, total cell lysates (35 μg) from Egr-1 H35 clones or vector Neo H35 clones (Neo2) were analyzed by immunoblot for Pgp or actin expression. B and C, 3–5 × 10^6 cells of each of the indicated Egr-1 and Neo H35 clones were plated on 60-mm dishes, 24 h later the dishes were washed and treated with either (A) vinblastine (100 nM) or (B) 5'-fluorodeoxyuridine (100 μM). After a 48-h treatment, lactate dehydrogenase released into the medium and remaining in attached cells were independently measured and lactate dehydrogenase in the media expressed as a percent of total cellular lactate dehydrogenase.

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**DISCUSSION**

We have identified a GC-rich region (pgp2GC) in the rat pgp2/mdr1b promoter that is highly conserved among the human and rodent MDR1 promoters (Fig. 1). This region has a consensus Sp1-binding site. An intact Sp1 site is required for full activity of the pgp2/mdr1b promoter as defined by the following criteria. 1) Transient transfection of an Sp1 expression vector results in the activation of the pgp2/mdr1b promoter in Sp1 negative cells, and 2) mutation of the Sp1-binding site abrogates Sp1 binding and produces a dramatic reduction in promoter activity. The in vitro relevance of Sp1 was shown by increased Pgp expression in H35 cells stably overexpressing Sp1. Further studies of pgp2GC revealed that this 13-bp GC region in pgp2/mdr1b promoter bound recombinant Egr-1, however, unlike Sp1, co-transfected Egr-1 suppressed the pgp2/mdr1b promoter. Moreover, a 4-bp mutation in the Egr-1 site decreased Egr-1 binding and correlated with the up-regulation of the pgp2/mdr1b promoter in the absence of a functional Sp1 site. Ectopic overexpression of Egr-1 decreased Pgp expression in H35 cells and was correlated with a selective increase in vinblastine sensitivity, thus identifying Pgp as a downstream target of Egr-1. Finally, EMSA revealed that Sp1 and Egr-1 compete and cannot simultaneously bind to the pgp2GC element in the pgp2/mdr1b promoter. In total, the concordance between the effects of ectopic expression of Sp1 and Egr1, transient co-transfection with these factors and EMSA with Sp1 and Egr1 on pgp2/mdr1b suggest that the interactions between Sp1 and Egr1 at the pgp2/mdr1b promoter defined here are functionally significant in vitro.

These studies were undertaken to determine whether in liver cells a conserved GC region in the pgp2/mdr1b promoter, similar to a corresponding GC region in human MDR1, influences pgp2/mdr1b transcriptional activity. Our studies revealed that loss of the Sp1 site leads to a dramatic reduction in pgp2/mdr1b basal activity in H35 cells. Moreover, the Sp1 site is required for Sp1-dependent transcriptional activation of the pgp2/mdr1b promoter. These findings underscore the significance of Sp1 and the conserved GC region for MDR1 (16, 18) and mdr1b expression (this study) and extend previous findings by determining that ectopic overexpression of Sp1 affects endogenous mdr1 expression. While a previous study with a drug-selected cell line showed a concurrent increase in MDR1 and Sp1 (33), undoubtedly, cellular changes during drug selection (e.g., Fos (34), Ras (35), or p53 (29)) could have affected MDR1 expression in that study. Enforced overexpression of Sp1 in a defined cell system provides more direct evidence that increased Sp1 expression correlates with increased Pgp levels in H35 cells. Although the cells lines with increased Sp1 have corresponding changes in Pgp expression, we cannot completely exclude an indirect mechanism, i.e., Sp1 may activate other genes that affect Pgp expression. Regardless, the current studies predict that factors that either increased Sp1 or Sp1 function (e.g., viral infection increases the amount of Sp1 (36)) would lead to increased Pgp expression and correlate with the outgrowth of multidrug-resistant cells. Conversely, diminishing either Sp1 DNA binding or amount (e.g., phosphorylation by casein kinase II (37)) might be expected to decrease Pgp expression and lead to increased drug sensitivity.

The mdr1b/pgp2 promoter Egr1 site is not typical, however, it contains a single consensus Sp1 site. The interaction described by EMSA with recombinant Egr1 and Sp1 depicts Egr1 displacing Sp1 leading to only Egr1 binding. This is recapitulated in H35 nuclear extracts because Egr1 seems to be the predominant protein binding to pgp2GC. However, EMSA studies, alone, do not indicate how this interaction is relevant to the pgp2/mdr1b promoter activity in cells. In some reports genes that contain overlapping Egr1 and Sp1 sites are positively regulated by both factors. For instance, despite Egr1 and Sp1 competition for an overlapping site in the adrenal phenylethanolamine N-methyltransferase gene, both transcription factors activate this promoter (38). Similar findings were reported for the human MDR1 promoter (16). Based upon these precedents we might have predicted that both Sp1 and Egr1 would activate the pgp2/mdr1b promoter. Unexpectedly, Egr1 repressed the pgp2/mdr1b promoter, while Sp1 activated it. Moreover, the up-regulation of the pgp2/mdr1b promoter by decreased Egr1 binding is consistent with its role as a transrepressor of pgp2/mdr1b. Thus, the opposing functional interaction between Sp1 and Egr1 at the pgp2/mdr1b promoter in H35 cells could not have been predicted from the DNA binding assays in this system. Furthermore, only in cases where Sp1 either dramatically increased or Egr1 decreased would pgp2/mdr1b transcription be expected to increase.

Currently it is unknown why Egr1 activates the human
MDR1 promoter and not the rat pgp2/mdr1b promoter. We do not believe these differences are due to position effects since the Egr-1/Spi site in pgp2/mdr1b is essentially in equal position with respect to transcriptional initiation of MDR1. Nor do we believe that Egr-1 transpression of pgp2/mdr1b is due to cell context because Egr-1 repressed the pgp2/mdr1b promoter in human colon (LS180) and hepatoblastoma (HepG2) cells. A more likely explanation is that the human MDR1 promoter is simply transactivated by Egr-1. If support of this possibility we have obtained preliminary evidence that the human MDR1 promoter (between -137 and +30) is transactivated by co-transfected Egr-1 to HepG2 cells and that enforced expression of Egr-1 leads to up-regulation of endogenous MDR1 (not shown).

The decrease in pgp2/mdr1b expression in H35 cells ectopically overexpressing Egr-1 identifies pgp2/mdr1b as a downstream target of Egr-1 in H35 cells. While it cannot be ruled out that Egr-1 could affect other transcription factors that act in stream target of Egr-1 in H35 cells. While it cannot be ruled out that Egr-1 repressed the pgp2/mdr1b promoter specifically bound by Egr-1. 2) The pgp2/mdr1b promoter is readily and specifically repressed by Egr-1. 3) The pgp2/mdr1b promoter is down-regulated in cells that ectopically overexpress Egr1. Current studies are evaluating whether chemical treatments (12-O-tetradecanoylphorbol-13-acetate, thapsigargin) or other stresses (e.g., ionizing radiation) reported to produce alterations in Egr-1 result in similar changes in Egr-1 in our system. Nevertheless, it is clear in H35 cells that Sp1 and Egr-1 have distinct biological roles in regulating pgp2/mdr1b as Sp1 can activate while Egr-1 suppresses the pgp2/mdr1b promoter.

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