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DNA Elements Recognizing NF-Y and Sp1 Regulate the Human Multidrug-Resistance Gene Promoter

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SUMMARY

Regulation of the human multidrug resistance gene (*hMDR1*) was studied by mapping DNA elements in the proximal promoter necessary for efficient transcription. Transient transfection analysis in tumor cell lines (HCT116, HepG2, and Saos2) of promoter deletions identified several regulatory domains. These cell lines expressed hMDR1 mRNA. Removal of an element between +25 and +158 reduced promoter activity by 2–3-fold, whereas deletion of sequences from ~−5000 to −138 base pairs gave a ~2-fold increase. The activity of the *hMDR1* promoter (−137 to +25) was comparable in activity to the SV40 early promoter and enhancer combination. Deletion of the *hMDR1* promoter between −86 and −44 reduced activity by 5–10-fold, identifying an important regulatory region. This minimal region (−88 to −37) activated transcription when inserted upstream of a synthetic promoter, suggesting that it acts independently of other regulatory sequences. Two DNA elements within 85 base pairs of the transcriptional start site were required to confer efficient gene expression. A double-point mutation in the Y box (inverted CCAAT box) between −70 and −80 reduced activity of the promoter by 5–10-fold, and a single-point mutation at −52 within a GC-rich element reduced activity by 3-fold. Thus, both the Y-box and GC elements must each remain intact for optimal promoter activity. DNA-binding analyses suggest that the transcription factor NF-Y, but not YB-1 or c/EBP, is most likely responsible for controlling the activity of the Y-box element in these tumor cell lines. DNA-binding analyses also suggest that Sp1, alone or in combination with other nuclear factors, likely controls the activity of the GC element.

Cancer chemotherapy is limited by the existence of inherent forms of multiple drug resistance or by the emergence of multiple drug resistance after chemotherapy. Inherent drug resistance is observed most frequently in solid tumors arising from tissues that normally overexpress the hMDR gene (*hMDR1*) product, notably colon, adrenal, liver, and kidney (1). One embodiment of inherent drug resistance may include regulation of the *hMDR1* gene by tissue-specific factors, but few studies address their identification (2). More critical is the observation that activation of certain oncogenes like *ras* and *raf* (3) or inactivation of the tumor suppressor *p53* (4) may serve to elevate *hMDR1* expression. Acquired resistance develops in most hematological malignancies and breast and ovarian carcinomas after chemotherapy (5). Evaluation of clinical isolates from drug-resistant tumors confirms that expression of the *hMDR1* gene is often elevated and correlates with a poor prognosis (6). Induction of the *hMDR1* gene by a variety of toxic agents, including anticancer drugs, carcinogens, and heavy metals, is proposed as a mechanism important for the acquisition of multidrug resistance (7–10). The *hMDR1* gene is also regulated by heat shock (7, 11) and UV irradiation (12), implying that *hMDR1* promoter activation may be part of a general stress response in many cells.

Several lines of evidence implicate complex mechanisms for transcriptional regulation of *hMDR1* expression in cells. However, the biochemical events responsible for controlling expression of the *hMDR1* gene in human tumors remain to be elucidated. The *hMDR1* promoter has been cloned and sequenced (2, 13, 14). It belongs to a family of housekeeping genes that are without a TATA box but do contain an initiator element at the transcriptional start site, an inverted CCAAT box (Y box) between −70 and −80, and a number of recognition sites for transcription factors, including those for Sp1, NF-Y (CP-1), YB-1, and YY-1. Sequences from +1 to +11 are needed for accurate initiation at the major start site

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ABBREVIATIONS: hMDR, human multidrug resistance; DMEM, Dulbecco's modified Eagle's medium; EGTA, ethylene glycol bis(α-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PCR, polymerase chain reaction; DTT, dithiothreitol; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate; SV, simian virus; LU, light units.

of transcription (13, 15). A few studies begin to characterize the *cis* elements and transcription factors responsible for *hMDR1* promoter activity. One study shows that Sp1 interacts with a GC-rich region at −50 to control hMDR1 expression in a variant of KB cells selected to be drug resistant in culture (16). A phorbol ester-responsive element overlaps this region of the promoter (17). Other data show that deletion or mutation of the Y box also affects promoter activity (12, 18), but the factors responsible for directing transcription through this *cis* element have not previously been identified. The murine *mdr1b* promoter is regulated by NF-Y and C/EBPβ (19). A region in the proximal promoter upstream of the Y-box (−110 to −103) binds a cellular factor and represents a negative element in KB 8–5 cells (16). Finally, deletion of an overlapping region (−137 to −106) in Adriamycin-resistant K562 cells causes a 3-fold increase in promoter activity (20).

To identify regulatory elements of the *hMDR1* gene, we constructed chimeric *hMDR1* promoter-luciferase reporter plasmids and evaluated their activity in three human tumor cell lines that express hMDR1 mRNA without DNA amplification and without prior selection for drug resistance. Deletion analysis of a proximal promoter construct (−137 to +158) revealed three regions important for promoter activity in all three tumor cell lines: two regions lie upstream of the transcription start site, and one lies downstream. Mutational analysis provided evidence that the Y box and a GC box located at −52 with respect to the transcriptional start site may cooperate to regulate hMDR1 expression. DNA-binding studies identified two factors that interact with these elements as NF-Y and Sp1. This study defines two transcription factors that operate in cell lines derived from human tumors having inherent multiple drug resistance and may lead to insights for future design of inhibitors.

Materials and Methods

Plasmid DNA construction. The plasmid −137/+158LUC containing the *hMDR1* proximal promoter was generated by PCR amplification of specific sequences from EMBL MDR-1 phage DNA (22) using primers containing restriction sites (underlined) for *Xho*I (5′-primer) (5′-CGCAGTTTCTCGAGGAATCAGCATTCAGTCAATCC) and *Hin*dIII (3′-primer) (5′-GGCAAGCTTAGTAGCTCCCAGCTTTGCGTGCCCCTAC). The PCR product was digested with restriction enzymes *Xho*I and *Hin*dIII and subcloned into pBS-SK+ (Stratagene, La Jolla, CA). The hMDR1 fragments were directionally subcloned into the luciferase reporter plasmid pGL2 Basic (Promega, Madison, WI) to generate −137/+158LUC. The *hMDR1* promoter deletions were similarly constructed using specific primers to synthesize the indicated *hMDR1* promoter fragments. Point mutations in the *hMDR1* promoter were generated in a two-step PCR amplification protocol. In two PCR procedures, the overlapping primer pairs 5′-CTGTGGTGAGGCTGAcTcGCTGGGCAGGAACAG and 5′-CTGTTCCTGCCCAGCgAgTCAGCCTCACCACAG (mutY1); and 5′-CTGTGGTGAGGCTGATgtGCTGGGCAGGAACAG and 5′-CTGTTCCTGCCCAGCacATCAGCCTCACCACAG (mutY2) containing base changes shown in lowercase (and indicated in Table 1) were paired with primers of wild-type sequence that defined the 5′- and 3′-ends of the final promoter fragment. The overlapping products of the first two reactions were amplified in a second PCR using only the wild-type 5′- and 3′-end primers. These fragments were subcloned into pGL2 Basic. The plasmid pTIluc (TATA-initiator) was constructed by insertion of oligonucleotides with 5′-terminal *Xho*I and 3′-terminal *Hin*dIII ends containing the adenovirus major late promoter TATA box and the terminal deoxynucleotidyl transferase promoter initiator sequence: 5′-TCGACGGGCTATAAAAGGGGGTGGGGGGAGCTCGGCCCTCATTCTGGAGACG (32) into the *Xho*I/*Hin*dIII sites of the plasmid pGL2Basic. Plasmid p52TIluc was generated by insertion of one copy of 5′-TCGAGTGAGGCTGATTGGCTGGGCAGGAACAGCGCCGGGGCGTGGGCTGAGCACAG (hMDR1 sequences −88 to −37), and complement, into the *Xho*I site of pTIluc. The plasmids pLuc, pSVLuc, and pGL2C were obtained from Promega. The relevant DNA sequences of all plasmid constructs were verified.

TABLE 1

Oligonucleotides used for DNA-binding analyses

The oligonucleotides shown were paired with their respective complements and used for investigation of Y- and GC-box binding proteins. The wild-type, mutY1, mutY2, and mutY3 oligonucleotides contain *hMDR1* promoter sequence from −90 to −64. The oligonucleotides spanning Sp1 consensus sites 3 and 4 (34, 3m4, 34m, and 3m4m) contain hMDR1 sequence from −64 to −38, and oligonucleotides spanning only Sp1 consensus site 4 (4 and 4m) contain *hMDR1* promoter sequences from −64 to −38. The mutated bases of all oligonucleotides are shown in lowercase, and the inverted CCAAT box is underlined in the Y box containing probes.

Name	DNA sequence
Wild-type	5′-GTGGTGAGGCTGATTGGCTGGGCAGGA
mutY1	5′-GTGGTGAGGCTGAcTcGCTGGGCAGGA
mutY2	5′-GTGGTGAGGCTGATgtGCTGGGCAGGA
mutY3	5′-GTGGTGAGGCgGAggttCTGGGCAGGA
MHC DRA Y box	5′-AAATATTTTTCTGATTGGCCAAAGAGTAAT
34	5′-ACAGCGCCGGGGCGTGGGCTGAGCACA
3m4	5′-ACAGCGCCGGGGaGTGGGCTGAGCACA
34m	5′-ACAGCGCCGGGGCGTGaaCTGAGCACA
3m4m	5′-ACAGCGCCGGGGaGTGaaCTGAGCACA
4	5′-TCGACATGTGGGCTGAGCACA
4m	5′-TCGACATGTGaaCTGAGCACA

Cell culture and transient transfections. HCT116, HepG2, KB3–1, and Saos2 cells were obtained from American Type Culture Collection (Rockville, MD) and were maintained in DMEM with 10% fetal bovine serum (Biologos, Naperville, IL) and 5% CO_2. Cells were plated at a density of 0.5–1.0 × 10^6/well in six-well plates ~24 hr before transfection. HepG2 cells were seeded onto wells that were precoated with Matrigel (Collaborative Research, Bedford, MA). Plasmid DNAs (0.5 μg of promoter plasmid plus 1.5–2 μg of pUC19 plasmid for a total of 2–2.5 μg of DNA added per well) were transfected into cells by lipofection using either DOTAP (Boehringer-Mannheim, Indianapolis, IN) or Lipofectamine (Life Technologies, Grand Island, NY). The amount of DNA and the cell density required for optimal transfection efficiency were predetermined (data not shown). Plasmid DNAs were diluted into 60 μl of HEPES-buffered saline (20 mM HEPES, pH 7.4, 150 mM NaCl) or 100 μl of DMEM before mixing with either 15 μg of DOTAP in 60 μl of HEPES-buffered saline or 24 μg of Lipofectamine in 100 μl of DMEM, respectively. After a 15–30-min incubation at room temperature, the DNA/lipid mixtures were diluted with DMEM/10% fetal bovine serum (DNA/DOTAP) or with DMEM alone (DNA/Lipofectamine) and then applied to the cells. After a 16–20-hr exposure, the mixture was replaced with medium containing serum. Approximately 40 hr after transfection, the cells were washed twice with cold phosphate-buffered saline and lysed by the addition of buffer containing 25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT and 1% Triton X-100 or 1× Cell Lysis Buffer (Promega), which contains 25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-*N*, *N*,*N*′,N′-tetraacetic acid, 10% glycerol, and 1% Triton X-100. Lysates were spun for 10 min at 4° in a microcentrifuge and assayed immediately for luciferase activity.

Luciferase assay. Luciferase activity was measured in a 200-μl reaction containing 20–40 μl of cell extract, 25 mM glycylglycine, pH 7.8, 15 mM K_2HPO_4, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 0.5 mM DTT, 1 mM ATP, and 80 μM luciferin. Cell extracts and 100 μl of a 2× reaction buffer (without ATP and luciferin) were combined in the wells of a microtiter plate. The luciferase reaction was initiated by

autoinjection of 40 μl of 5 mM ATP and 40 μl of 0.4 mM luciferin using a Dynatech luminometer.

Nuclear extract preparation. HepG2 and HCT116 cells were washed with cold phosphate-buffered saline, and nuclear extracts were prepared (21). Extracts were dialyzed at 4° for 90 min in 20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 2 mM DTT, and 1 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined using BioRad dye reagent.

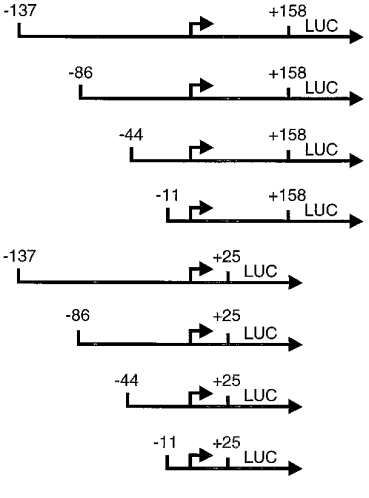
Gel mobility shift assay. The DNA sequence of oligonucleotides used for gel shift analyses are shown in Table 1. Approximately 5–10 fmol of double-stranded oligonucleotide labeled with [γ-^{32}P]ATP was incubated in 10-μl reactions containing nuclear extracts and 10 mM Tris·HCl, pH 7.2, 1 mM EDTA, 0.1% Triton X-100, 5% glycerol, 80 mM NaCl, 3 mM $MgCl_2$, 4 mM DTT, and 2 μg of p(dI-dC) for Y-box binding analysis and 20 mM HEPES pH 7.8, 50 mM KCl, 5 mM $MgCl_2$, 1 μM $ZnCl_2$, 500 ng of p[d(I-C)], 2 mM DTT, and 2% glycerol for GC-box binding analysis. Sp1 binding was analyzed in reactions containing 10 mM HEPES, pH 7.5, 100 mM NaCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 10 ng p[d(I-C)], and Sp1 protein (Promega). Nuclear extracts and antibodies were preincubated on ice for either 3 hr or 20 min for Y- or GC-box DNA-binding analyses, respectively, followed by the addition of radiolabeled DNA probes and incubation at either room temperature (Y-box analyses) or 22–25° (GC-box analyses) for 20–30 min. The reaction products were separated by electrophoresis at 4° (Y-box analyses) or at room temperature (GC-box analyses) on 5% polyacrylamide gels containing 0.5× Tris/borate/EDTA (1× = 89 mM Tris, 89 mM borate, and 1 mM EDTA). Quantification was performed using a PhosphorImager (Molecular Dynamics). The human *YB-1* gene, a generous gift of Dr. Benjamin Schwartz (Monsanto, St. Louis, MO), was cloned into an *Escherichia coli* expression vector, and recombinant YB-1 protein was used to generate anti-YB-1 rabbit polyclonal serum (22). Anti-NF-YA and NF-YB antibodies (38) and anti-YB-1 antibodies were purified by Protein A/G chromatography. Antisera to c/EBPα, c/EBPβ, and c/EBPγ (5) were kindly provided by Dr. Steve McKnight (University of Texas Southwestern Medical Center, Dallas, TX), and Sp1 antibody (PEP2)X was obtained from Santa Cruz Biochemicals (Santa Cruz, CA).

Results

The levels of hMDR1 mRNA and protein vary among normal human tissues (1). In this study, four tumor cell lines (HepG2, a hepatocellular carcinoma; HCT116, a colon carcinoma, KB3–1, an epidermoid carcinoma; and Saos2, an osteosarcoma) were analyzed for hMDR1 mRNA expression. The hMDR1 signal from mRNA of HepG2, HCT116, and Saos2 cells was detectable after 30 cycles of reverse transcription-PCR amplification (data not shown). In contrast, after 50 cycles, the level of hMDR1 cDNA derived from the drug-sensitive cell line KB3–1 was undetectable (data not shown). These results are consistent with previous determinations of very low or undetectable levels of hMDR1 mRNA in KB3–1 cells (1, 23).

The *hMDR1* promoter contains regulatory elements both upstream and downstream of the transcriptional initiation site. The *hMDR1* proximal promoter was analyzed to delineate the regions necessary for efficient transcription. Deletion of DNA sequence from ~−5000 to −138 upstream of −137/+25LUC increased transcription an average of 32% in HepG2 cells and an average of 68% in HCT116 cells (data not shown). Deletion from −86 to −44 resulted in an 8-fold and a 13-fold reduction in activity in HepG2 and HCT116 cells, respectively (Fig. 1). When the promoter was deleted to −11, there was a modest additional decrease in activity in both cell lines. In Saos2 cells, a similar reduction in *hMDR1* promoter activity was observed on deletion from −86 to −44 (~5-fold, data not shown). Deletion of sequences between +25 and +158 reduced the activity of the promoter by ~2-fold in all three cells lines (Fig. 1, and Saos2, data not shown).

The *hMDR1* proximal promoter contains several putative transcription factor binding sites. The *hMDR1* promoter contains no TATA box but contains an initiator element (24) corresponding to the major start site of transcription (2, 13, 14, 25) (Fig. 2). The DNA sequence from −86 to −44 is essential for efficient transcription in HepG2, HCT 116 (Fig. 1), and Saos2 cells. This region of the promoter contains an inverted CCAAT box at −77 (Y-box) with a 13-of-14 match to the consensus binding site for NF-Y and a GC-rich region containing four or five Sp1 consensus binding sites (sites 1–4 are identified). Sp1 sites 1 and 4 match the Sp1 consensus G/T G/A GGC G/T G/A G/A G/T (26), whereas sites 2 and 3 adhere to the consensus at 8 of 9 positions. Downstream of the transcriptional initiation site is a pyrimidine-rich tract containing a recognition site for the transcription factor YY1.



	Relative Light Units	
	HepG2	HCT116
−137/+158LUC	100 (7)	100 (4)
−86/+158LUC	129 ± 28 (5) 89–167	81 ± 31 (4) 60–134
−44/+158LUC	17 ± 5 (4) 10–26	6 ± 2 (4) 5–9
−11/+158LUC	6 ± 2 (4) 4–8	4 ± 3 (4) 2–9
−137/+25LUC	45 ± 19 (3) 25–71	51 ± 14 (4) 38–73
−86/+25LUC	33 ± 11 (5) 19–50	26 ± 15 (4) 14–50
−44/+25LUC	3 ± 2 (4) 0.7–7	3 ± 4 (4) 1–10
−11/+25LUC	1 ± 0.6 (4) 0.3–2	3 ± 3 (4) 0.4–8

Fig. 1. Transient transfection analysis of *hMDR1* promoter activity in HepG2 and HCT116 cells. Cells were transfected with a series of reporter plasmids containing the *hMDR1* promoter sequences driving expression of the luciferase gene. Extracts from transfected cells were assayed for luciferase enzyme activity, and the LU for each construct from several experiments (number of experiments shown in parentheses) were corrected for concentration of protein in the extract and then normalized to the activity from the −137/+158LUC plasmid, which was set at 100. The range of LU/μg for −137/+158LUC was 482–241,068 in HepG2 and 71,307–113,916 in HCT116. The values shown are the average of the relative LU from multiple experiments. Also shown are the standard deviations for the average values and the range of relative LU observed for each plasmid.

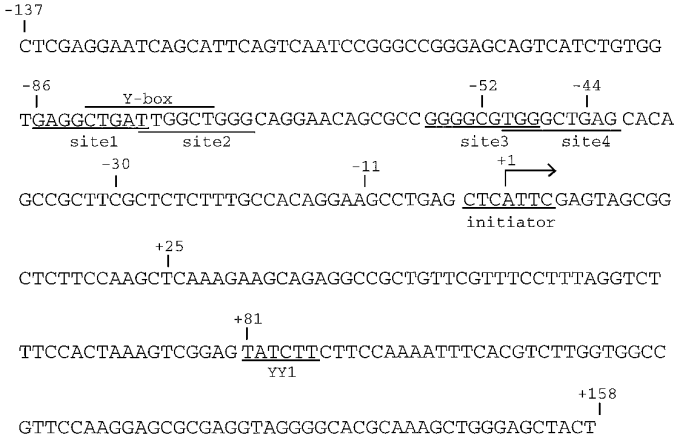


Fig. 2. DNA sequence of the *hMDR1* proximal promoter. The *hMDR1* proximal promoter DNA sequence is shown from −137 to +158 with respect to the major transcriptional start site. *Underlined*, several transcription factor recognition sites, including four consensus sites for Sp1, a YY1 consensus at +81, and an initiator element at +1. *Overlined*, Y box or inverted CCAAT box centered at −77. The Y box of the *hMDR1* promoter is a 13-of-14 match to the consensus sequence for the transcriptional activator NF-Y.

Two elements of the *hMDR1* proximal promoter are necessary for efficient gene expression. We prepared two mutants in the Y box (mutY1 and mutY2) and four mutants of the consensus Sp1 sites (site1m, site3m, site4m, and site1m3m4m) to investigate the functionality of the these sites. MutY1 resulted in a 36% reduction in *hMDR1* promoter function in HepG2 cells and a 45% increase in HCT116 cells (Fig. 3). In contrast, mutY2 caused a consistent and severe reduction in promoter activity in both HepG2 and HCT116 cells.

Three of the four putative Sp1 sites were mutated individually (site1m, site3m, and site4m) and jointly (site1m3m4m). The single point mutation at −52 (site3m) reduced activity an average of 60% in HepG2 cells and 65% in HCT116 cells, whereas the site4m and site1m mutations had little effect. The triple-site mutant (site1m3m4m) reduced the activity to levels comparable with the single-point mutant in site 3. The site3m and mutY2 mutations each had a significant effect on promoter activity, indicating that both the Y box and site 3 GC box must remain intact for optimal activity.

Analysis of the activity of the *hMDR1* promoter element. The 52 nucleotides spanning −88 to −37 were inserted upstream of a heterologous, synthetic promoter to analyze the strength and independence of the *hMDR1* proximal promoter. Transfection of the p52TIluc construct into either HepG2 or HCT116 resulted in luciferase activities that were comparable to the activity of the element in its native context (Fig. 4). In addition, the activities of the *hMDR1* promoter driven constructs were comparable to those of the strong early promoter and enhancer of SV40 (Fig. 4).

NF-Y forms a complex with the *hMDR1* promoter Y box. DNA binding experiments were performed with an hMDR1 Y-box probe (−90 to −64) and nuclear extracts of HepG2 and HCT116 cells. Several protein/DNA complexes of similar migration were detected in both extracts (Fig. 5A, *lanes 1 and 5*). All complexes were specific to the hMDR1 probe, as competition with excess, unlabeled DNA of wild-type sequence dramatically reduced binding of all complexes formed with both extracts (Fig. 5A, *lanes 2 and 6*). Incubation

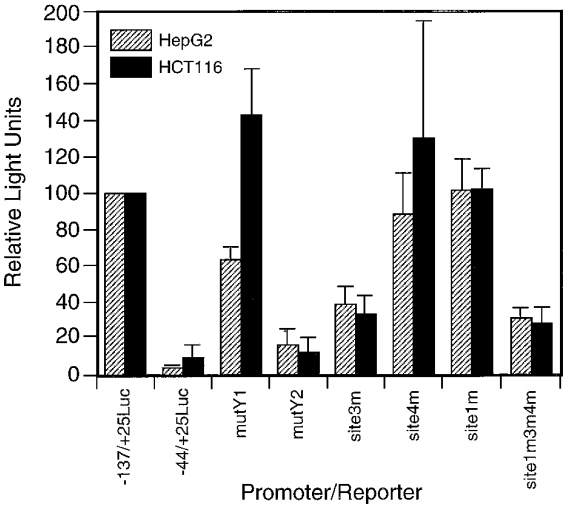


Fig. 3. Transient transfection analysis of *hMDR1* promoter Y- and GC-box point mutants. HepG2 and HCT116 cells were transiently transfected with reporter plasmids containing *hMDR1* promoter sequence driving expression of the luciferase gene. The relative LU expressed from each plasmid in several experiments were averaged. *Error bars*, standard deviation. The mutY1, mutY2, site3m, site4m, site1m, and site1m3m4m mutations were incorporated into the −137/+25LUC parent plasmid. The specific sequence changes in the mutated plasmids (except for the changes in Sp1 consensus site 1) are the same as those made in the oligonucleotides used for DNA binding analyses and are shown in Table 1. The wild-type Y-box in the −137/+25LUC plasmid is ATTGG, in mutY1 it is AcTcG, and in mutY2 it is ATgtG, with mutations shown (*lowercase*). The G residues at −83 and −84 in the Sp1 consensus site 1 (Fig. 2) were both changed to A residues in the site1m and site1m3m4m reporter plasmids. The C residue at position −52 in the Sp1 consensus site 3 was changed to an A residue in the site3m4 and site1m3m4m reporter plasmids. The G residues at −47 and −48 in the Sp1 consensus site 4 were changed to A residues in the site4m and site 1m3m4m reporter plasmids.

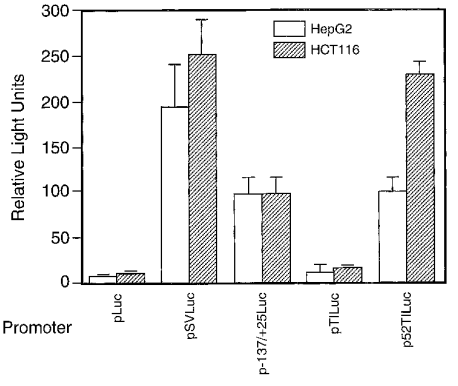


Fig. 4. Transient transfection analysis of SV40 promoter and chimeric *hMDR1* promoter activities in HepG2 and HCT116 cells. Plasmids were transfected into either HepG2 or HCT116 cells, and cell extracts were assayed for luciferase activity. The LU were corrected for the concentration of protein in each extract and normalized to the activity of the p-137/+25 luc construct. Plasmid pTIluc contains a minimal promoter with TATA-box and initiator elements inserted upstream of the luciferase gene, and p52TIluc contains *hMDR1* promoter sequences from −88 to −37 inserted upstream of pTIluc. The luciferase expression plasmid pLuc contains no promoter, and the pSVLuc contains the SV40 early promoter and enhancer.

with an excess of a wild-type fragment from the MHC class II HLA-DRA Y box known to bind NF-Y (27, 28) reduced or eliminated formation of complexes I and II and had minor effects on some of the more rapidly migrating complexes

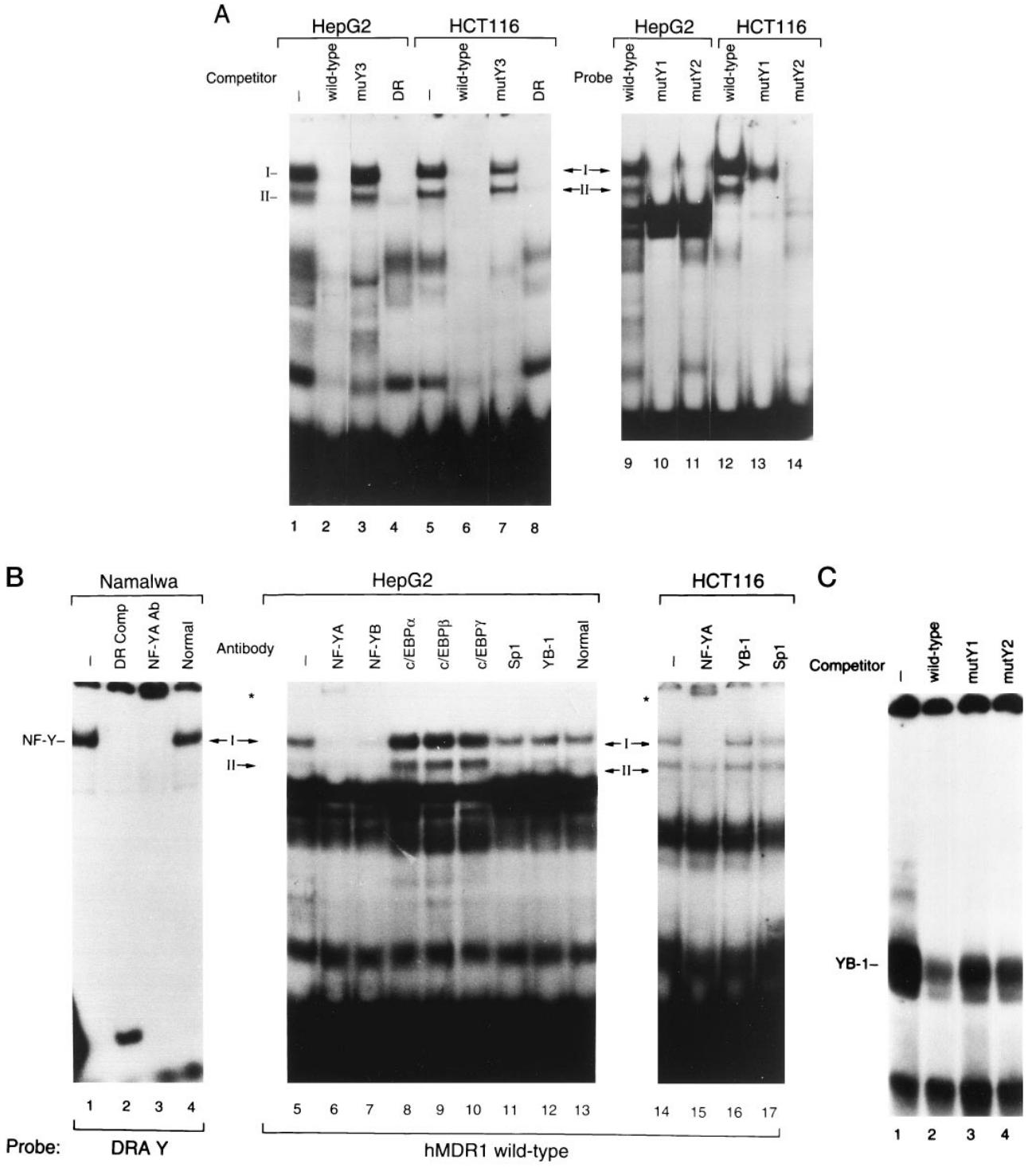


Fig. 5. Gel mobility shift analysis of nuclear extract binding to the *hMDR1* promoter Y box. A, Demonstration of complex specificity. HepG2 (2.8 μg, *lanes 1–4*; 11.6 μg, *lanes 9–11*) or HCT116 (5 μg, *lanes 5–8*; 10 μg, *lanes 12–14*) nuclear extracts were incubated in DNA-binding reactions with a radiolabeled wild-type hMDR1 probe (*lanes 1–8, 9, and 12*) or with DNA probes containing the mutY1 (*lanes 10 and 13*) or mutY2 (*lanes 11 and 14*) mutations. Competitor DNA in 100-fold excess (*lanes 2–4 and 6–8*) was preincubated with extracts for 15 min at 4° before the addition of probe DNA. *DR*, MHC DRA Y-box oligonucleotide listed in Table 1. B, NF-Y binds to the *hMDR1* promoter in complex I. Namalwa nuclear extract (12 μg) was incubated with radiolabeled DNA containing MHC DRA Y-box sequence (*lanes 1–4*) and either no addition (*lane 1*), homologous unlabeled competitor DNA at 100-fold excess (*lane 2*), NF-YA antibody (*lane 3*), or normal serum (*lane 4*). HepG2 (5.8 μg, *lanes 5–13*) and HCT116 (5 μg, *lanes 14–17*) nuclear extracts were incubated with hMDR1 Y-box DNA probe and no additions (*lanes 5 and 14*), NF-YA antibody (*lanes 6 and 15*), NF-YB antibody (*lane 7*), cEBPα antiserum (*lane 8*), c/EBPβ antiserum (*lane 9*), c/EBPγ antiserum (*lane 10*), Sp1 antiserum (*lanes 11 and 17*), YB-1 antibody (*lanes 12 and 16*), or normal serum (*lane 13*). *Lanes 1–4 and lanes 5–13*, separate experiments; however, comigration of the NF-Y complex formed in Namalwa extract on the DRA Y-box probe and complex I formed on the hMDR1 probe was confirmed in other experiments (data not shown). C, YB-1 binds to the *hMDR1* promoter. Recombinant YB-1 (75 ng) was incubated with a DNA probe spanning the *hMDR1* promoter Y box. Competitor DNA fragments were added at 100-fold excess; wild-type DNA homologous to the probe (*lane 2*), mutY1 (*lane 3*), and mutY2 (*lane 4*). *Arrows*, migration of NF-Y (complex I) (A and B). *, Position of antibody supershifted complexes.

(*lanes 4 and 8*). Mutation of the core CCAAT sequence to AACCT (mutY3), previously shown to eliminate NF-Y binding to the Y box in the human *MHC DRA* promoter (29), also eliminated competition of complexes I and II (Fig. 5A, *lanes 3 and 7*). This mutation, however, did not affect competition for formation of the faster migrating complexes in both HCT116 (Fig. 5A, *lane 7*) and in HepG2 extracts when used in greater excess (data not shown). These results suggest that complexes I and II are CCAAT specific and have DNA-binding characteristics of NF-Y.

Binding of nuclear extracts to radiolabeled mutY1, mutY2, and wild-type DNA was compared with correlate Y-box DNA-binding activity with effects on promoter activity presented in Fig. 3 (Fig. 5A, *lanes 9–14*). Minor variability was observed in the formation of the faster migrating complexes (Fig. 5A, compare *lanes 1 and 9*); however, the formation of complexes I and II was observed consistently. Formation of complexes I and II was reduced on the mutY2 probe compared with wild-type probe in both HepG2 and HCT116 nuclear extracts (Fig. 5A, *lanes 11 and 14* versus *lanes 9 and 12*). This effect correlates well with the reduction in promoter activity observed with mutY2. In HepG2 nuclear extracts, sequence changes in mutY1 also eliminated formation of complexes I and II (Fig. 5A, *lane 10*); this, too, is consistent with the decreased luciferase activity observed with the mutY1 promoter in these cells (Fig. 3). In HCT116 extracts, however, a DNA/protein complex formed on the mutY1 probe of unknown identity that nearly matched migration of complex I formed on the wild-type probe (Fig. 5A, *lane 13*), whereas several faster migrating complexes were absent.

A panel of antibodies representing all of the known families of CCAAT-box proteins was used to characterize the complexes formed between the hMDR1 Y-box probe and nuclear extracts. As a control, a Y-box sequence from the MHC DRA promoter was incubated with Namalwa nuclear extracts (Fig. 5B, *lane 1*). The complex formed was identified previously as NF-Y (27, 28) and was competed by excess, homologous unlabeled MHC DRA DNA (Fig. 5B, *lane 2*). NF-Y is composed of at least three subunits: NF-YA, NF-YB, and NF-YC (27). A polyclonal antibody to the A subunit of NF-Y caused a supershift in the migration of complex I to a position near the origin of the gel (Fig. 5B, *lane 3*). The specific complex formed between Namalwa extracts and the MHC DRA Y probe comigrated with complex I formed between the hMDR1 wild-type probe and HepG2 or HCT116 nuclear extracts (Fig. 5B, *lanes 5 and 14*). Antibodies prepared against either the A or B subunits of NF-Y recognized complex I and, to a lesser extent, complex II formed in both HepG2 and HCT116 extracts without affecting other complexes (Fig. 5B, *lanes 6, 7, and 15*). The NF-YA antibody produced a slower migrating, supershifted complex, whereas NF-YB antibodies blocked complex formation. Antibodies reactive against other CCAAT-box binding proteins (c/EBPα, c/EBPβ, c/EBPγ, and YB-1) had no detectable effect on complexes formed with either HepG2 or HCT116 extracts (Fig. 5B, *lanes 8–10, 12, and 16*). The slight increase in intensity of complexes I and II in the presence of the C/EBP antibodies is a nonspecific effect that is also seen with normal whole-serum controls (data not shown). Whole serum alone produced no DNA binding activity on these probes (data not shown). These data are consistent with NF-Y binding to the hMDR1 Y-box probe to form complexes I and II. Neither Sp1 antibody (*lane 11*) nor normal serum (lane 13) affected migration of the complexes.

A single, specific protein/DNA complex was formed when recombinant YB-1 was incubated with a double-stranded hMDR1 Y-box DNA probe (Fig. 5C, *lane 1*). In the presence of excess, wild-type DNA, the YB-1 complex was significantly reduced (*lane 2*), whereas the mutY1 and mutY2 competitors were slightly less effective at reducing the signal (*lanes 3 and 4*, respectively). There was no evidence of a YB-1-specific complex formed in either HepG2 or HCT116 extracts, nor did antibody to YB-1 affect complex formation (Fig. 5B, *lanes 12 and 16*).

A specific complex containing Sp1 forms on an essential element of the *hMDR1* promoter. Gel mobility shift analyses were performed with four DNA probes (Table 1) spanning Sp1 consensus sites 3 and 4 (34, 3m4, 34m, and 3m4m). HCT116 nuclear extract formed two complexes on the wild-type hMDR1 DNA probe spanning both sites 3 and 4 (complexes A and B, Fig. 6A, *lane 2*). These complexes were formed at reduced levels when the probes contained the single-point mutation in site 3 (20% of wild-type) (3m4, *lane 3*) or the triple-point mutation in sites 3 and 4 (5–10% of wild-type) (3m4m, *lane 5*). The double-point mutation in site 4 (34m) did not affect formation of either complex (>100% of wild-type) (*lane 4*), indicating that protein is binding predominantly to site 3. Purified Sp1 protein formed two complexes

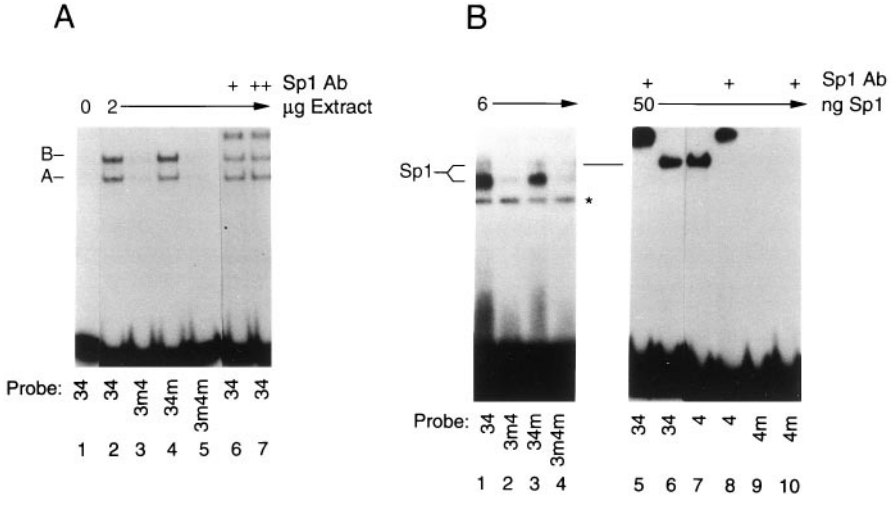


Fig. 6. Gel mobility shift analysis of HCT116 nuclear extract and Sp1 binding to the *hMDR1* promoter. HCT116 nuclear extract or Sp1 was combined in DNA-binding reactions with the indicated radiolabeled DNA probes, and where indicated, Sp1 antibody was preincubated with extract before the addition of probe. A, HCT116 nuclear extract forms two specific complexes (complex A and complex B) with the *hMDR1* promoter probe spanning Sp1 consensus sites 3 and 4. *Lane 1*, DNA probe 34 without added extract. B, Sp1 binds specifically to the *hMDR1* promoter. The Sp1-specific complexes are indicated. *, Migration of a nonspecific band observed in some experiments.

on the wild-type hMDR1 DNA probe spanning sites 3 and 4, having an apparently identical pattern of electrophoretic migration to complexes A and B formed with the HCT116 nuclear extract (Fig. 6B, *lane 1*). These complexes were sensitive to the same point mutations in the Sp1 consensus sites 3 and 4 as were those formed with HCT116 extract (Fig. 6B, *lanes 2–4*). When higher amounts of Sp1 were used, only the upper complex was formed (*lane 6*).

After incubation with antibodies to Sp1, the abundance of complexes A and B formed with nuclear extract was reduced by 25% and 45%, respectively. A new supershifted complex appeared (Fig. 6A, *lanes 6 and 7*), indicating that Sp1 is a component of both complexes A and B. When Sp1 antibody was included in binding reactions with purified Sp1, the migration of the entire complex was shifted (Fig. 6B, *lane 5*).

Sp1 binding to a DNA fragment from the *hMDR1* promoter containing nucleotides −62 to −87 and spanning Sp1 consensus sites 1 and 2 was weak in comparison to its binding to a DNA fragment spanning sites 3 and 4.[2] These data suggest that Sp1 interaction with consensus sites 1 and 2 is poor or absent. Further support was obtained when nuclear extracts failed to form complexes susceptible to supershift by Sp1 antiserum with the hMDR1 Y-box DNA probe that includes these sites (Fig. 5B, *lane 11*).

Discussion

The transcriptional activity of the proximal *hMDR1* promoter was evaluated in three tumor cell lines that express *hMDR1* and do not have amplified forms of the gene. We chose to evaluate *hMDR1* promoter function in human tumor cells, rather than drug resistant lines selected *in vitro*, to evaluate the properties of the promoter in cells that conform more closely to those of clinical isolates. This report provides the first evidence in a single study that both the Y box and the −52 GC box must remain functional for efficient transcription from the *hMDR1* proximal promoter (Fig. 3). This is also the first study to mutate the GC boxes spanning the region between −86 and −44, which functions to regulate the *hMDR1* promoter, and to identify NF-Y and Sp1 as components of the complexes formed on the promoter.

Our data are consistent with those from *hMDR1* promoter/CAT constructs evaluated in SW620 colon carcinoma and 2780 ovarian carcinoma cells (13, 18). A double-point mutation in the Y box was previously reported to ablate promoter activity in the 2780 ovarian cancer cell line (18). Our data on this same mutation (mutY2) in two additional tumor cell lines (Fig. 3) reinforce the importance of the Y box for transcriptional regulation of the *hMDR1* promoter. The mutY1 mutation is reported to block detectable binding of CP1/NF-Y in HeLa extracts to an adenovirus major late promoter CCAAT box (30). Such a mutation in the *hMDR1* promoter decreased promoter function as well as the formation of both DNA/protein complexes I and II in HepG2 cells. This mutation resulted in formation of a complex with slightly faster migration than complex I in HCT116 cell extracts and produced a small but reproducible increase in promoter function, suggesting cell-specific variations.

This report is the first to identify NF-Y as a factor likely to form a functional interaction with the Y box in the *hMDR1* promoter. The murine *mdr1b* promoter requires cooperation of NF-Y and c/EBPβ for optimal activity (19). A number of factors interact with inverted CCAAT boxes, or Y boxes, including NF-Y, YB-1, and c/EBP. Their binding sites are related but do not generally share a common sequence consensus (30). CCAAT elements like the one in the *hMDR1* promoter occupy fixed locations in promoters (−60 to −80) (31). Several pieces of data reported here support the identification of the Y box factor regulating hMDR1 expression as NF-Y. The hMDR1 Y box most closely fits the consensus sequence for NF-Y (13/14 match) (32), and tumor cell nuclear extracts formed complexes with a MHC DRA Y-box promoter probe (Fig. 5) that comigrated with complexes identified here as well as elsewhere to contain NF-Y (27, 28). The two specific complexes formed on the *hMDR1* promoter DNA probe were uniquely competed by a DRA Y-box oligonucleotide and were supershifted by anti-NF-YA and NF-YB antisera. The binding profile of the NF-Y complex to the mutY2 DNA probe correlated well with activity in the functional assay (Fig. 3). Finally, antisera to YB-1 and c/EBP failed to alter migration of any complexes formed in extracts prepared from these three tumor cell lines. An earlier study identified YB-1 binding to an hMDR1 Y-box probe in drug-resistant KB variants (33). In those experiments, YB-1 was believed to respond to environmental stress. Our data in tumor cells expressing *hMDR1* exclude YB-1 as the factor interacting with the Y box by showing that two mutations that effect promoter function do not affect binding of recombinant YB-1.

An earlier study in KB cells identified two GC elements: one between −110 and −103 and one between −59 and −45 (16). The region of the promoter between −86 and −44 is GC rich, having at least four consensus Sp1 sites (Fig. 2). A point mutation at −52 in site 3 reduced promoter activity by 60–70%. This is the first report to show that mutation of other flanking GC sites had little or no effect on promoter strength (Fig. 3). The activities of promoters bearing either the Y-box double-point mutant, mutY2, or the −52 single-point mutant were reduced to a level comparable to that of the −43 deletion construct. Therefore, the Y box and −52 GC box are both necessary for efficient transcription.

The transcription factor Sp1 is ubiquitous and enhances transcription from a number of viral and cellular promoters that contain at least one GC box whose postioning relative to that of upstream regulatory elements can be critical. DNA-binding and mutational analyses provided in this report show that only the single GC box positioned at −52 (site 3) is essential for efficient transcription. Nuclear extracts from HCT116 tumor cells formed specific protein/DNA complexes on an *hMDR1* promoter DNA fragment spanning two Sp1 consensus sites (Fig. 6), and these comigrate with those formed by purified Sp1. Nuclear extracts formed weak interactions with a DNA probe spanning only site 4, and a mutation in site 4 that blocked binding of recombinant Sp1 had no effect on promoter activity.

Sp1 and WT-1 (34) share overlapping DNA-binding consensus with the transcription factor EGR1. Recombinant EGR1 binds to an *hMDR1* promoter probe spanning the −52 GC box (16), and EGR1 participates in the control of hMDR1 expression in certain phorbol ester-sensitive cell lines (17). There are examples in which EGR1 and Sp1 compete for binding to overlapping binding sites (35); therefore, additional layers of regulation of the *hMDR1* promoter through

[2] A. Merritt and A. C. King, unpublished observations.

the Y-box and −52 GC-box elements will form the basis for further investigation.

NF-Y regulates transcription of a variety of genes, including some that are constitutive, tissue specific, or developmentally or cell-cycle regulated (36). The data in this report provide supporting evidence that NF-Y and Sp1 interact with the Y box and −52 GC box, respectively. Cooperative interactions have been observed between NF-Y and Sp1 on the human invariant chain promoter, and there is precedent for formation of protein/protein contacts between NF-Y and other transcription factors located at a distance, such as X box factors on the MHC II promoter (27), p67 serum response factor on the β-actin promoter (36), and c/EBP on the albumin promoter (37). Further experimentation is needed to determine whether NF-Y and Sp1 cooperate to regulate the *hMDR1* promoter.

A number of studies show that an element just upstream of the Y box (−198 to −89) negatively influences *hMDR* promoter activity (13, 16, 20); however, the exact position of the negative element may vary in different cell lines. Deletion from −137 to −86 had opposing effects in HCT116 and HepG2 cells in the current study. These data taken together indicate that a number of factors may interact with this region of the promoter and that there may be tissue-specific variations.

The deletion analyses of the *hMDR1* promoter described in this report and in others (13, 18) differ from those using KB epidermoid variants in two important ways. The most dramatic loss in promoter activity reported in KB8–5 cells is between −73 and −51, which corresponds to a GC box recognized by Sp1 (16). Another study with KB cells reported that the major element required for UV induction corresponds to sequences upstream of the Y box (−136 to −78) (12). Deletion of this element has no detectable effect on uninduced promoter activity in KB cells. Thus, the Y box may be less important in KB cells for constitutive promoter activity than it is in other solid tumor cells lines, like HCT116, HepG2, Saos2 (*vide infra*), and SW620 (18). Instead, the Y box may be part of a stress response in certain cells. An element between −136 and −76 is responsible for heat shock induction in stably transfected KB cells, adding support to this speculation (11). A UV-inducible factor from KB cell extracts binds to a Y-box probe (12) and has a molecular weight similar to that of the NF-YA subunit (40 kDa) (27, 38) and the Y-box binding protein YB-1 (42 kDa) (39). Although speculative, the inducible Y-box factor in KB cells may not be the same as the factor identified here as NF-Y in tumor cell lines having inherently high levels of hMDR1 expression. Part of the regulation of hMDR1 expression in tumors may arise from activation of transcription by constitutive factors like NF-Y and Sp1 or by alternative inductive factors interacting with the Y-box. Our data support this speculation and show that a 52-base pair element spanning the Y- and GC-boxes are sufficient for efficient transcription of the *hMDR1* promoter in three different tumor cell lines expressing this important drug resistance gene.

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