Cloning and Characterization of the Murine and Rat mrp1 Promoter Regions

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ABSTRACT

The ATP-binding cassette transporter multidrug resistance protein 1 (MRP1) confers resistance to a number of clinically important chemotherapeutic agents. The proximal promoter region of MRP1 is GC-rich and contains binding sites for members of the Sp1 family of trans-acting factors that seem to be important for basal expression. As an approach to searching for other elements that may contribute to expression, we have sequenced and functionally compared the promoters of the murine and rat mrp1 genes with that of the human gene. All three promoters are GC-rich, TATA-less, and CAAT-less. Conservation of sequence between rodent and human promoters is limited to a proximal region of 100 nucleotides containing binding sites for members of the Sp1 family and a putative activator protein-1 element. The 5′-untranslated region (UTR) of human MRP1 contains an insertion of approximately 160 nucleotides comprising a GCC-triplet repeat and a GC-rich tandem repeat that is absent from the rodent sequences. Transient transfection analyses demonstrated that the conserved GC-boxes of all three genes are the major determinants of basal activity. Based on electrophoretic mobility shift assays, each GC-box can be bound by Sp1 or Sp3. Unlike the rodent genes, the human MRP1 5′UTR also binds Sp1 but not Sp3, and the human promoter retains substantial activity even in the absence of the conserved GC-boxes. Finally, we show that the tumor suppressor protein p53 can repress the human and rodent promoters by a mechanism that is independent of the Sp1 elements.

The human multidrug resistance protein 1 (MRP1), which is encoded by the ABCC1/MRP1 gene, is a 190-kDa transmembrane glycoprotein that confers cellular resistance to a broad range of structurally and functionally unrelated chemotherapeutic agents. The cDNA encoding MRP1 was initially cloned in 1992 from the doxorubicin-selected, multidrug-resistant (MDR) human lung cancer cell line H69AR (Cole et al., 1992). MRP1 mRNA can be detected in many normal human tissues, with highest levels in skeletal muscle, testis, and lung (Cole et al., 1992; Deeley and Cole, 2002). MRP1 mRNA or protein has also been detected in a broad range of malignancies and numerous cell lines that display the MDR phenotype (Campling et al., 1997; Bates, 2002; Deeley and Cole, 2002). In certain types of cancer, including some forms of breast cancer, neuroblastoma, prostate cancer, and non–small-cell lung cancer, the protein has been found to be a negative prognostic indicator of outcome (Bates, 2002; Deeley and Cole, 2002).

At present, relatively little is known of the mechanisms that regulate either tissue-specific or short-term regulation of MRP1. In some multidrug-resistant cell lines that over-express MRP1, such as H69AR, the MRP1 gene is clearly amplified (Cole et al., 1992). In others, no evidence of gene amplification has been detected; in some cases, the increased levels of MRP1 mRNA have been shown to be the result of increased transcription (Slovak et al., 1993; Eijndens et al., 1995). In addition, increased levels of MRP1 mRNA or protein have been observed under conditions of chemically induced oxidative stress (Yamane et al., 1998), in response to gamma radiation (Harvie et al., 1997; Hennes et al., 2002), and in various proliferating cells. For example, MRP1 protein has been detected on the lateral membranes of only proliferating HepG2 hepatocarcinoma cells (Roeoifsen et al., 1997). Similarly, hyperplastic type II pneumocytes in archival human lung specimens have been shown to be immunoreactive against MRP1 antibo-

ABBREVIATIONS: MRP, multidrug resistance protein; MDR, multidrug-resistant; AP-1, activator protein 1; kb, kilobase(s); UTR, untranslated region; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; FCS, fetal calf serum; EMSA, electrophoretic mobility shift assay; RLM, RNA ligase mediated; TDN, trans-dominant negative; bp, base pair(s).
ies, whereas normal resting type II cells are not (Wright et al., 1998).

The promoter region of the human MRPI gene was initially cloned in 1994 by Zhu and Center from the MDR cell line HL60/ADR (Zhu and Center, 1994). The proximal promoter region is TATA-less, GC-rich, and was shown to contain at least one Sp1-type binding site that made a major contribution to basal expression of the promoter (Zhu and Center, 1994, 1996). Subsequent DNasel footprinting analyses of the promoter region using nuclear extracts from H69AR and parental drug-sensitive H69 cells revealed numerous protein-DNA interactions, including a footprint encompassing a potential AP-1-like antioxidant response element (Kurz et al., 2001). Although this element behaved as a classic enhancer when introduced into the upstream region of a minimal promoter and was able to bind several members of the c-Jun family of transcription factors, it failed to mediate an oxidative response in the context of the MRPI promoter (Kurz et al., 2001).

Subsequent mutagenesis studies have also failed to confirm the importance of this element in regulating expression of MRPI (E. U. Kurz, R. A. Burtch-Wright, S. P. C. Cole, and Roger G. Deeley, unpublished observations). Recent clinical studies and in vitro analyses of the human MRPI promoter have implicated p53 as a negative regulator of the gene (Wang and Beck, 1998; Sullivan et al., 2000). However, the promoter does not contain any identifiable potential p53 binding sites, and it has been suggested that suppression may operate through an Sp1-mediated mechanism (Wang and Beck, 1998).

Sequence analyses of the region upstream of the MRPI proximal promoter have identified numerous potential binding sites for trans-acting factors. However, current data obtained from short-term transfection assays have not been useful in identifying a functional role for any of these elements (E. U. Kurz, R. A. Burtch-Wright, S. P. C. Cole, and Roger G. Deeley, unpublished observations). Consequently, we have searched for evidence of evolutionarily conserved elements that may be involved in regulating expression of the gene by isolating and characterizing the MRPI promoter from two commonly used animal models, the mouse and the rat.

We have established previously that the tissue distribution of MRPI mRNA in both the mouse and the rat is similar to that observed in humans, although both mouse and rat proteins display some striking differences in substrate specificity compared with the human protein (Stride et al., 1996; Nunoya et al., 2003). In this study, we have isolated the 5′ ends of the rat and mouse genes, together with 4.1 and 3.3 kb of flanking sequence from each gene, respectively, and have defined the transcriptional initiation sites of the rodent and human mRNAs. These studies reveal that there is little conservation of sequence outside of a GC-rich, TATA-less proximal promoter region common to all three species and that this region is adequate to sustain full basal levels of expression. In addition, we have defined three conserved Sp1 sites that contribute to transcription from all three promoters, as well as human-specific sequences in the 5′-untranslated region (UTR) of MRPI mRNA that are able to bind Sp1 and to compensate partially for mutation of the conserved Sp1 sites. Finally, we show that all three promoters are suppressed by wild-type p53 and, contrary to previous speculation, that this suppression is independent of the presence of functional Sp1 sites.

### Materials and Methods

#### Library Screening and Cloning of Human, Murine, and Rat Promoters

The 5′-flanking sequence of the human MRPI gene was identified by screening a genomic library from the human multidrug-resistant small cell lung cancer cell line H69AR as described previously (Grant et al., 1997). The 5′-flanking sequence of the murine mrp1 gene was identified by screening a murine genomic bacterial artificial chromosome library with a PCR-generated probe corresponding to the first 130 nucleotides of the mrp1 coding region (Centre for Applied Genomics, Hospital for Sick Children, Toronto, ON, Canada). A 3.3-kb BamHI fragment containing the first 41 nucleotides of the mrp1 coding region was subcloned into pBlueScript II KS+ (Stratagene, LaJolla, CA) cloning vector. The 5′-flanking sequence of the rat mrp1 gene was identified by screening a rat genomic DNA library (BD Biosciences Clontech, Palo Alto, CA) with oligonucleotide corresponding to the first 41 nucleotides of rat mrp1 coding sequence.

#### Purification of poly(A)+ RNA

Total RNA was extracted from human H69AR and A549 cells, murine Sol8 cells, and rat skeletal muscle tissue using TRIzol reagent (Invitrogen, Burlington, ON, Canada) as recommended by the manufacturer. Polyadenylated RNA from each source was selected from 1 mg of total RNA using the Poly(A) tract mRNA isolation kit (Promega, Madison, WI). The integrity of the purified poly(A)+ RNA was determined by formaldehyde agarose gel electrophoresis and Northern blotting using standard procedures (Ausubel et al., 2003).

#### 5′-Rapid Amplification of cDNA Ends

Initial 5′-RACE experiments were performed on 250 ng of poly(A)+ RNA from murine Sol8 skeletal myoblasts using the Smart RACE cDNA amplification kit (BD Biosciences Clontech), as recommended by the manufacturer. Subsequently, 5′-RACE was performed on 250 ng of human, murine, and rat poly(A)+ RNA isolated from the sources identified under Results using the 5′cap-dependent RLM-RACE kit (Ambion, Austin, TX), as recommended by the manufacturer. Gene-specific reverse primers used in the primary PCR amplifications of RACE-ready RNAs were as follows: primer 1 (human), 5′-ATCTGAAACAAGGACAGACGAGGC-3′, complementary to nucleotides 579 to 554 of MRPI; primer 2 (murine), 5′-CGAGCTGAGAAGGACAGACGAC-3′, complementary to nucleotides 582 to 560 of murine mrp1; and primer 3 (rat), 5′-AAGAGTAAAGGCTTGGTGTGC-3′, complementary to nucleotides 275 to 254 of rat mrp1. The primary PCR reactions were performed using Advantage II polymerase (BD Biosciences Clontech) using a two-step cycling protocol, as recommended by the manufacturer, with the addition of 5% dimethyl sulfoxide to each reaction. Nested reverse primers used in the secondary PCR amplifications were as follows: primer 4 (human), 5′-TGTTGAGGCTGC-3′, complementary to nucleotides 213 to 187 of MRPI; primer 5 (murine), 5′-TTTGAATCCCTCCTCCTCGCAGGC-3′, complementary to nucleotides 398 to 373 of murine mrp1; primer 6 (rat), 5′-TTGGTGAAGGCTGC-3′, complementary to nucleotides 215 to 201 of rat mrp1, and primer 7 (human), 5′-AGCCATCGCGCTGAGACGACGAC-3′, complementary to nucleotides 35 to 7 of human MRPI. The secondary PCR reactions were performed under conditions identical to those used in the primary PCR amplifications. The products of each secondary PCR reaction were cloned into the pBlueScript II KS+ vector, and the resulting clones were differentially screened by colony hybridization using oligonucleotide probes complementary to either the first or the second coding exons of the human, murine, or rat genes. Approximately 180 clones from each RACE reaction were screened by this method. cDNAs isolated from clones that gave strong positive signals in colony Southern blot hybridizations (8 to 10 clones per RACE reaction) were sequenced using standard vector-specific oligonucleotide primers. 5′-RACE experiments were also performed on HepG2 cells transiently transfected with rodent promoter/luciferase reporter constructs. Briefly, three 150-mm dishes of subconfluent HepG2 cells were transfected with rodent promoter/luciferase reporter constructs.
with 10 μg of either the murine ~3300 or the rat ~4100 mrp1 luciferase reporter constructs (see next section) using Fugene 6 reagent according to the manufacturer's recommendations. Cells were harvested 48 h after transfection, and total RNA was isolated using TRIzol reagent as described in "Purification of poly(A)+ RNA. 5′-RLM-RACE was performed as described above using 10 μg of total RNA from each source as a template and the following nested primers: luciferase downstream primer (luc nucleotides 85–63), 5′-CCTCATTGCAGTTCCTCAGC-3′ and luciferase upstream primer (luc nucleotides 34–10), 5′-CCGGGCTTTTCTTATGTTTGGC-3′. Products from the secondary PCR amplifications were ligated into the EcoRV sites of pBlueScript KS− and individual clones from each amplification were differentially screened by Southern blot hybridization using oligonucleotide probes corresponding to either the first 20 nucleotides of the luc gene or to the 5′ UTRs of the murine and rat mrp1 mRNAs. Positive clones identified by differential screening were sequenced using standard vector-specific primers.

Construction of Luciferase Reporter Vectors and Dual Luciferase Assays of Promoter Activity. Luciferase reporter vectors containing varying lengths of the human, murine, and rat MRP1/mrp1 5′UTR for 10 min and slow cooling to room temperature over 1 h. The specific activity of each double-stranded probe was estimated by spotting each probe on DE81 ion exchange filters followed by scintillation counting. For standard EMSAs, 50,000 cpm of each oligonucleotide probe or 25,000 cpm of gel-purified restriction fragments were spotted on DE81 filters to determine efficiency. In EMSA binding buffer [1 mM HEPES, pH 7.9, 1% glycerol, 10 μM EDTA, 5 mM KCl, 5 mM dithiothreitol, 8 mM MgCl2, 1 mg/ml bovine serum albumin, and 0.1 mg/ml poly(dI-dC)] with 50 μg of purified Sp1 or Sp3 maltose-binding protein fusion proteins (a generous gift from Dr. C. Mueller, Queen’s University) for 30 min on ice. Complexes were resolved by electrophoresis on native 5% polyacrylamide gels in 1× Tris-borate/EDTA buffer at 40 mA/gel. Gels were dried and exposed to phosphor screens for 12 h.

Results

Cloning of Murine and Rat mrp1 5′-Flanking Regions and Comparison with the Human MRP1 5′-Flanking Region. To identify conserved elements within the promoters and 5′ flanking regions of the human and rodent MRP1/mrp1 genes, we cloned sequences 3.3 and 4.1 kb upstream from the translational initiation codons in exon 1 of the murine and rat genes, respectively. Comparison with the previously cloned 5′-flanking region of the human MRP1 gene revealed a proximal GC-rich (>70%) region of approximately 100 nucleotides that was relatively conserved among all three species (Fig. 1A). This region of the human sequence has been previously identified as the core promoter of the MRP1 gene (Zhu and Center, 1994, 1996). Upstream from the conserved 100 nucleotides, conservation between the rodent and human sequences was very low. The rat and mouse flanking regions, however, retained more than 70% sequence identity over the 3.5 kb of sequence compared. The 100-nucleotide region conserved in all three species contained several overlapping GC-boxes that were potential binding sites for members of the Sp1 family of transcription factors. None of the sequences contained a TATA box or an identifiably potential transcription initiation sequence. Alignment of the human and rodent sequences revealed that of the 13 ‘footprinted’ elements we identified in a previous study, only the elements encompassed by the GC-box were conserved in all three species (Kurz et al., 2001). Comparative analyses of the proximal 3500 nucleotides of all three promoters using the TRANSFAC transcription factor database (http://transfac.gbf.de/TRANSFAC/) failed to identify any additional conserved regions that might serve as potential binding sites for transcription factors.

Identification of The Transcription Initiation Sites of the Rodent mrp1 Genes. Given the limited sequence conservation between the putative rodent promoters and the human promoter, we sought to confirm their functionality by identifying the transcriptional initiation sites of the rat and mouse genes. To date, the transcriptional initiation site(s) of the human MRP1 gene has not been defined precisely. Previous primer extension studies and sequencing of 5′ CDNA fragments predicted a 5′-UTR ranging from 175 to 196 nucleotides for the human MRP1 mRNA (Zhu and Center, 1994; Grant et al., 1997). To directly compare the initiation sites of all three genes, we used a ligated-mediation 5′-RACE method (RLM-RACE) that requires the presence of a capped 5′ end on the original RNA template. For comparative purposes, we also performed 5′-RACE on murine poly(A)+ RNA from the same source using a 5′-RACE method that uses a unique terminal transferase-dependent selection of the 5′ ends of mRNA transcripts. Sequencing of multiple clones isolated by either 5′-RACE method using poly(A)+ RNA from the murine Sol8 skeletal muscle cell line, which expresses relatively high levels of mrf1, identified 5′-UTRs of 26 and 34 nucleotides (Fig. 1B, labeled as +8 and +1, respectively). Seven 5′-RLM-RACE clones generated from rat skeletal muscle poly(A)+ RNA shared a single transcriptional initiation site 18 nucleotides upstream from the translational start codon (Fig. 1B, designated +1). Using the RLM-RACE approach, we also
identified a single transcriptional initiation site for the human MRP1 gene, using poly (A)+ RNA from H69AR cells as a template, that was coincident with the site we predicted previously by extensive screening of MRP1 cDNA clones isolated from the same cell line. This initiation site results in a 5'-UTR of 196 nucleotides and is located 23 nucleotides downstream from that predicted by primer extension studies (Zhu and Center, 1994). The rodent 5'-UTRs can be aligned with the 5' end of human MRP1 mRNA, but the UTRs of the rodent mRNAs lack an apparent insertion of approximately 160 nucleotides that begins with a GCC triplet repeat and includes a 57-base pair direct repeat. The entire 5'-UTR is highly GC-rich and contains several potential Sp1-binding elements, including Sp1 site 5 (Fig. 1B). The transcriptional initiation sites identified by 5'-RACE in all three species lie within 50 nucleotides of the conserved GC-box identified by sequence alignment. As an additional check on the location of transcriptional initiation sites of the rat and mouse genes, we also carried out 5'-RACE using mRNA isolated from HepG2 cells transiently transfected with either the −3300 murine mrp1 or −4100 rat mrp1 luciferase reporter constructs. Sequence analysis of several clones isolated from RLM-RACE identifies a series of conserved elements within the proximal 5'-flanking regions identified 5'-ends for the murine and rat reporter constructs that mapped to −34 and −18, respectively (Fig. 1A). These sites are coincident with the transcription initiation sites we identified using endogenous mouse and rat mRNAs as templates for RLM-RACE. Interestingly, this approach also identified an additional site for both the murine and rat reporter constructs that mapped to −61 (Fig. 1A, *).

**Short-Term Transfection Studies of the Human and Rodent MRP1/mrp1 Promoters.** To confirm the functionality of the putative rodent promoters, the 5'-flanking fragments of 3.3 and 4.1 kb of the murine and rat genes, respectively, were cloned upstream of the firefly luciferase reporter gene in the pGL3B plasmid and transiently cotransfected into the human HepG2 cell line with the internal control vector pRL-TK. Both the −3300 murine mrp1 and −4100 rat mrp1 luciferase reporter genes demonstrated significant promoter activity (>10- and >20-fold, respectively) compared with a promoter-less luciferase gene under the same conditions (Fig. 2).

To determine which regions of the cloned 3.3- and 4.1-kb fragments of the murine and rat promoters contained positive to a luciferase reporter gene. Analysis of the luciferase activities of the rodent deletion mutants revealed that the regions between −104 and +1 within the murine mrp1 promoter and −107 and +1 within the rat mrp1 promoter contained regulatory elements required for full basal transcriptional activity in HepG2 cells. Truncation of the murine mrp1 promoter to −30, or the rat promoter to −42, which eliminated the conserved GC-boxes, reduced the reporter gene activities to less than 10% of those observed with the full-length mouse and rat reporter genes, respectively (Fig. 2, A and B). Interestingly, truncations of the sequence 5' of −64 in the rat promoter, which eliminates Sp1 sites 1 and 2 but retains Sp1 sites 3 and 4 within the conserved GC-box, reduced reporter gene activity by 50% compared with the full-length −4100 rat promoter construct (Fig. 2B), suggesting that multiple
Sp1 sites are functional. For comparative purposes, the relative luciferase activities of the reporter gene deletion mutants of the human MRP1 promoter were also determined. Truncation of the human MRP1 promoter to −22, which eliminates Sp1 sites 1, 2, and 3 in the conserved GC-box, reduced the luciferase activity of the promoter to less than 25% of the activity demonstrated by the −824 reporter gene (Fig. 2C). However, this construct retained considerable luciferase activity compared with the promoterless pGL2B reporter (Fig. 2C). To determine whether the relative reporter gene activities observed were cell type dependent, we also conducted similar transfection studies using several other cell lines of human and murine origin that are known to express the endogenous MRP1/mrp1 genes. Reporter gene activities similar to those in the human HepG2 cell line were observed when we transiently transfected the human and murine deletion mutants into human A549 lung adenocarcinoma cells, and murine Sol8 skeletal myoblasts or NIH3T3 fibroblasts (data not shown). We also observed similar reporter gene activities when we transiently transfected the deletion mutants into the human embryonic kidney 293 cell line, which expresses relatively low levels of MRP1 (data not shown).

Sp1-Dependent Transcriptional Activation of the Human and Rodent MRP1/mrp1 Promoters. Previous studies in HL60/ADR cells suggested a role for transcription factor Sp1 in the transcriptional activation of the human MRP1 promoter (Zhu and Center, 1996). To confirm that Sp1 was capable of activating the human and rodent promoters, we examined their activity in the Sp1-deficient Drosophila melanogaster SL-2 cell line, in the presence of exogenously supplied Sp1 cDNA. Neither the human MRP1 promoter nor the rodent mrp1 promoters demonstrated detectable reporter gene activity when transiently transfected into SL-2 cells alone (data not shown). However, cotransfection of the full-length human and murine promoter/reporter genes with an Sp1 expression vector resulted in a strong, dose-dependent activation of both promoters (Fig. 4A). To confirm the role of the multiple Sp1 sites in transactivation of the human and rodent promoters, we constructed a series of luciferase reporter genes in which we mutated conserved residues within the putative Sp1 binding elements. Mutation of the three Sp1 sites within the GC-box in the murine reporter gene resulted in a >5-fold reduction in the luciferase activity in HepG2 cells (Fig. 3, A). The observed luciferase activity of the murine triple Sp1 mutant reporter construct was comparable with that of a reporter gene that was truncated to −30 and lacked the conserved GC-box (Fig. 3A). Mutation of Sp1 sites within the conserved GC-box in the rat mrp1 promoter also caused a reduction in basal promoter activity (Fig. 3B). Mutation of Sp1 sites 1 and 2 alone or sites 1, 2, 3, and 4 in the rat mrp1 promoter resulted in a 2- to 2.5-fold reduction of the basal activity compared with the activity of the wild-type −435 reporter construct in HepG2 cells (Fig. 3B). Surprisingly, the luciferase activity of the rat −435/Sp1(1,2,3,4) reporter construct was considerably higher than the activity of the rat −42 deletion mutant, which lacks the conserved GC-box (Fig. 3B). Mutation of Sp1 sites 1, 2, and 3 (which reside in the conserved GC-box) alone or in combination with Sp1 site 5 (which resides in the 5′-UTR) in the human MRP1 promoter caused a 5-fold reduction in the luciferase activity observed in HepG2 cells (Fig. 3C). The luciferase activities we observed in cells transfected with either of the human Sp1-mutant reporter genes were comparable with the activity of the −22 deletion mutant, which physically lacks the conserved GC-box, but retains Sp1 site 5 within the 5′-UTR (Fig. 3C). Unexpectedly, mutation of only Sp1 site 5 within

Fig. 2. The basal activity of the human and rodent MRP1/mrp1 promoters is mediated by elements located within the 100 nucleotides proximal to the transcription initiation sites of each gene. HepG2 cells were transfected with 480 ng of serial 5′ deletion mutants of the murine (A), rat (B), or human (C) luciferase reporter vectors and 20 ng of the internal control vector pRL-TK. Luciferase activities were measured 48 h after transfection. The numbering of the 5′-terminal nucleotide of each reporter gene is relative to the transcription initiation sites identified within each promoter. Data represent the means of normalized triplicate analyses of each construct in a typical experiment ± S.D. Mean R. reniformis luciferase values used for normalizations were within ±20% between reporter constructs.
the 5′-UTR caused a significant stimulation of the basal promoter activity of the human reporter gene in HepG2 cells.

To further clarify the role of Sp1 and the conserved GC-box/Sp1 elements in the transcriptional activity of the human and rodent promoters, we transfected D. melanogaster SL-2 cells with the various human and rodent Sp1-mutant reporter genes in the presence or absence of an Sp1 expression vector. Both the murine and rat mrp1 promoters were trans-activated 3- to 5-fold by Sp1 (Fig. 4, B and C). Mutation of all of the Sp1 sites within the conserved GC-boxes of the rodent promoters resulted in the elimination of Sp1-dependent trans-activation of these reporter constructs. However, mutation of only Sp1 sites 1 and 2 in the rat promoter had little effect on inducibility. Mutation of all three putative Sp1-binding elements within the GC-box of the human reporter construct (sites 1, 2, and 3) did not significantly diminish the Sp1-dependent induction of the human reporter gene (Fig. 4D), whereas mutation of site 5 alone increased inducibility (Fig. 4D). The latter observation is consistent with results obtained with HepG2 cells, in which mutation of Sp1 site 5 alone resulted in a modest stimulation of luciferase activity.

**Binding of Transcription Factors Sp1 and Sp3 to Wild-Type and Mutant Human and Rodent MRP1/mrp1 Promoters.** To investigate the binding of Sp1 to the GC-boxes in all three promoters, and to confirm that mutation of these sites abolishes binding, we performed EMSAs using radiolabeled oligonucleotides corresponding to the wild-type sequence from each promoter, with unlabeled wild-type or mutant oligonucleotides as competitors. Incubation of radiolabeled oligonucleotide probes corresponding to the conserved GC-boxes from the human and rodent promoters with purified Sp1 in the absence of competitor resulted in the formation of three retardation complexes, the profile of which was similar for all three GC-boxes (Fig. 5, lanes 1 and 6). To ensure that the low mobility complexes we observed were sequence-specific, we also carried out EMSAs in the presence of a 25-fold molar excess of unlabeled competitor oligonucleotides corresponding to the wild-type or Sp1-mutant GC-boxes or a random sequence. The unlabeled wild-type oligonucleotides markedly diminished formation of all three retardation complexes, whereas the random and mutant oligonucleotides were ineffective competitors (Fig. 5A, lanes 2, 4, 7, 9, and 11–14).

Several reports in the literature have described the differential regulation of tissue-selective promoters by competition between Sp1 and Sp3 proteins (for review, see Suske, 1999). Both proteins bind with similar affinities to conserved consensus sites. The two proteins differ, however, in that the binding of Sp3 is generally associated with repression of transcription, whereas the binding of Sp1 is associated with trans-activation. In light of these observations, we determined whether the conserved GC-box present in the MRP1 promoter was capable of binding transcription factor Sp3. We found that an oligonucleotide containing the conserved GC-box from the human MRP1 promoter efficiently bound purified Sp3 protein in EMSA (Fig. 5B). The binding we observed was specific and was not diminished in the presence of a 25-fold molar excess of an unlabeled competitor oligonucleotide corresponding to the wild-type or Sp1-mutant GC-boxes or a random sequence. The unlabeled wild-type oligonucleotides markedly diminished formation of all three retardation complexes, whereas the random and mutant oligonucleotides were ineffective competitors (Fig. 5A, lanes 2, 4, 7, 9, and 11–14).

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Sp1 site 5 mutant 5'-UTRs (encompassing nucleotides −22 to +196) of the MRP1 gene. We found that both the wild-type and Sp1-mutant 5'-UTR probes were capable of binding transcription factor Sp1 (Fig. 5C, lanes 2 and 5). However, we did not observe binding of transcription factor Sp3 to either of these fragments (Fig. 5C, lanes 3 and 6).

Repression of the Human and Murine MRP1/mrp1 Promoters by p53 Is Independent of the GC-Box/Sp1 Element. Previous studies have described the transcriptional suppression of the human MRP1 gene by wild-type p53 (Wang and Beck, 1998; Sullivan et al., 2000). These studies suggested that repression of the MRP1 promoter by p53 was probably the result of interactions between p53 and the Sp1 elements or proteins bound to these elements within the GC-box. We tested this hypothesis by cotransfecting p53-null Calu-6 cells with wild-type and Sp1-mutant human and murine MRP1/mrp1 luciferase reporter genes and expression vectors encoding either a wild-type or trans-dominant-negative (TDN) p53 cDNA. We found that both the human and murine full-length MRP1 promoters were repressed by WT p53 in a dose-dependent fashion (Fig. 6A). However, transcriptional repression mediated by p53 was independent of the Sp1 elements within the conserved GC-boxes, as well as Sp1 site 5 located in the 5' UTR of the human MRP1 gene (Fig. 6B, □). The repression observed with wild-type p53 was reversible by coexpression of a molar excess of the p53 TDN cDNA (Fig. 6B, □). Cotransfection of the p53 TDN cDNA alone with either of the human or murine MRP1/mrp1 promoters did not affect the luciferase activities of these reporter constructs (data not shown).

**Fig. 4.** The rodent mrp1 promoters are trans-activated by transcription factor Sp1 through the conserved GC-box/Sp1 element in D. melanogaster SL-2 cells. SL-2 cells were transfected with either wild-type reporter genes or reporters containing mutations within the conserved Sp1-binding sites. A, 430 ng of DNA from each of the −557hMRP1Luc3B (human promoter, ■) and −516CkmrplLuc3B (murine promoter, □) reporters was cotransfected into SL-2 cells with 20 ng of pRL-TK and 0 to 50 ng of pPacSp1 or 0 to 50 ng pPac. Luciferase activities were measured 48 h after transfection. The data represent the means of triplicate analyses of each reporter gene normalized to total protein content in a typical experiment ± S.D. B, cells were cotransfected with 460 ng each of either the murine wild-type −516mmrp1Luc3B (−516) reporter gene or the Sp1 triple mutant −516/−Sp1(1,2,3)mmrp1Luc3B [−516/−Sp1(1,2,3)] reporter with 20 ng each of either pPAC alone or pPacSp1 and pRL-TK in triplicate. C, SL-2 cells were cotransfected with 460 ng each of either the rat wild-type −435rmrp1Luc3B (−435) reporter gene, the double Sp1 mutant −435/−Sp1(1,2)rmrp1Luc3B [−435/−Sp1(1,2)] reporter gene, or the quadruple Sp1 mutant −435/−Sp1(1,2,3,4)mrp1Luc3B (−435/−Sp1(1,2,3,4)) reporter gene and 20 ng each of either pPAC alone or pPacSp1 and pRL-TK as an internal control. D, SL-2 cells were cotransfected with 460 ng each of either the human wild-type −557hMRP1Luc3B (−435) reporter gene, the double Sp1 mutant −435/−Sp1(1,2)hMRP1Luc3B [−435/−Sp1(1,2)] reporter gene, or the triple Sp1 mutant −435/−Sp1(1,2,3)hMRP1Luc3B [−435/−Sp1(1,2,3)] reporter and 20 ng each of either pPAC alone or pPacSp1 and pRL-TK as an internal control.
Discussion

So far, little is known of the mechanisms involved in transcriptional regulation of the MRP1/mrp1 gene in humans or animal models. Attempts to identify functional regulatory elements outside of a GC-box in the proximal promoter region of the human gene have been unsuccessful, although a number of protein binding sites have been identified by DNaseI footprinting studies (Kurz et al., 2001). The promoter regions of genes encoding MRPI orthologs in other species have not been identified. Based on the premise that functionally important regulatory sites may be conserved between species, we have cloned the 5'-flanking regions of the genes encoding murine and rat *mrp1* and compared them with the corresponding region of the human gene. The murine and rat *mrp1* genes share significant sequence identity within the entire 3500 nucleotides of sequence 5' to the start of the open reading frame in exon 1. In contrast, we found little sequence conservation with the flanking region of the human gene with the exception of a GC-rich region of approximately 100 nucleotides. The region contains a GC-box consisting of three (in the human and murine promoters) or four (in the rat promoter) potential consensus Sp1 sites and has been identified previously as the proximal promoter region of the human gene (Zhu and Center, 1994, 1996). The GC-rich regions in the rodent genes were approximately 160 nucleotides closer to the start of the open reading frame in exon 1 than in the human sequence. Consequently, when aligned according to the start of the open reading frames, the GC-rich regions of the rodent genes align with the GC-rich 5'-UTR encoded by the human gene. Given this limited sequence conservation, we confirmed that the GC-rich regions identified in the rodent sequences were functional promoters using a 'cap' dependent 5'-RACE protocol to identify precisely the transcriptional initiation sites of both rodent and human genes. We used endogenous mRNAs as templates for 5'-RACE, and each of the transcription initiation sites we identified mapped to within 50 nucleotides downstream of the conserved GC-boxes, resulting in 5'-UTRs of 34 and 18 nucleotides for the rat and murine mRNAs, respectively. Consistent with our previous estimate, the 5'-UTR of the human *MRP1* gene was 196 nucleotides long. 5'-RACE analysis of mRNA from HepG2 cells transiently transfected with rodent promoter/luciferase reporter constructs confirmed the location of the 5' ends of the rodent mRNAs. However, these analyses identified additional transcription initiation sites for each of the murine and rat luciferase reporter constructs, located 27 and 43 nucleotides upstream of the sites we identified using endogenous mRNAs as templates for 5'-RACE. Despite extensive screening of 5'-RACE clones generated from endogenous murine

![Fig. 5](https://mol.pharm.aspetjournals.org/)

**Fig. 5.** The conserved GC-box/Sp1 element in all three MRP1/mrp1 genes binds Sp1 and Sp3 in EMSAs. Purified, recombinant Sp1 or Sp3 proteins were incubated with 32P-labeled probes, and complexes were resolved on native 5% polyacrylamide gels. A, oligonucleotide probes containing either the human (lanes 1–5), murine (lanes 6–10), or rat (lanes 11–15) conserved GC-box/Sp1 element were incubated with purified, recombinant Sp1 protein in an EMSA experiment. Reactions were incubated in the presence or absence of unlabeled excess random (lanes 2, 7, and 11), self (lanes 3, 8, and 15), or Sp1-mutated (lanes 4, 9, 12–14) oligonucleotides. Control reactions that lacked Sp1 protein are shown in lanes 5 and 10. Note that oligonucleotide competitors containing mutations within all of the Sp1 elements were unable to diminish the interaction observed between Sp1 and the wild-type oligonucleotide binding sites. B, an oligonucleotide probe containing the human GC-box/Sp1 element was incubated with purified Sp3 protein in an EMSA experiment. Unlabeled, excess random, self, or Sp1-mutant oligonucleotides were added to reactions as depicted. The GC-box is capable of binding Sp3 in vitro specifically, and the interaction is not disturbed by the presence of excess unlabeled mutated binding site. C, restriction fragment probes encompassing the −22/+196 region of the wild-type and Sp1 site 5 mutant *MRP1* promoter were incubated in the presence or absence of Sp1 and Sp3 proteins as depicted. Both probes bind to Sp1 but not to Sp3.
and rat mRNAs, we have not succeeded in identifying transcrip-
tion initiation sites that map farther upstream than
−34 and −18, respectively. Consequently, we are unable to
confirm whether the upstream site is used during transcrip-
tion of the endogeneous gene or is peculiar to the short-term
transfection assay. Based on the results of 5′-RACE of en-
dogenous mRNAs, the 5′-UTR of the human sequence con-
tains a GC-rich region of approximately 160 nucleotides,
including a GCC triplet repeat and a 57-bp direct repeat, that
is absent from both rodent genes. Given the lack of identifi-
able TATA, CAAT, or Inr elements within the 5′-UTRs of the
three MRP1 genes, these results are consistent with a role for
the conserved GC-boxes in specifying the transcription initia-
tion sites of each gene.

Previously, we identified a number of protein-DNA inter-
actions within the 5′-proximal 1000 nucleotides of the human
gene (Kurz et al., 2001). Of the 13 potential regulatory ele-
ments identified, only the Sp1 sites within the conserved
GC-boxes were also present in the murine and rat promoters,
and computer-assisted analyses of the 5′-flanking 2 kb of the
rat, mouse, and human genes failed to identify any additional
potential regulatory motifs that were conserved in all three
species. Reporter gene analysis of 5′ truncation constructs of
the promoters in HepG2 cells demonstrated that each pro-
moter retained essentially full basal activity provided that
the conserved region encompassing the GC-box was retained.
However, unlike the rodent promoters, a response to exoge-
nous Sp1 in SL-2 cells was retained in the human promoter,
even when the GC-box was deleted. Consequently, we exam-
ined the possibility that the GC-rich 5′-UTR of the human
gene might function as an Sp1-response element in the ab-
ence of the conserved upstream GC-box. Computer-assisted
sequence analysis of the GC-rich repeat region of the 5′-UTR
using the TRANSFAC transcription factor database identi-
fied several putative Sp1 binding elements, in addition to
Sp1 site 5, that may contribute to the residual Sp1 response
we observed. In support of this possibility, we found that
binding of Sp1 protein to the 5′-UTR of human MRP1 mRNA
assessed by EMSAs occurred even in the absence of the
putative Sp1 element located at site 5. We have not suc-
cceeded in identifying the precise locations of the Sp1-re-
spoon element(s) within this region, partly because of the
extremely GC-rich nature of the repeat region of the 5′-UTR.
The insertion of a GC-rich sequence capable of binding Sp1
within the 5′-UTR of the human gene suggests that it may
contribute to basal expression of the gene.

Several reports have described chromosomal rearrange-
ments, duplications, and sequence repeats that map to re-
gions of chromosome 16 in the vicinity of the MRP1 locus,
raising the possibility that either the GCC triplet repeat or
the 57-bp direct repeat within the MRP1 5′-UTR may be
present in multiple copies on chromosome 16 (O’Neill et al.,
1998; Cai et al., 2000; Ringpfeil et al., 2001). This locus has
also clearly undergone a duplication (as evidenced by the
presence of the adjacent gene for the MRP1 homolog, MRP6,
in an inverted orientation) and is a known area of low abun-
dance repeats (Cai et al., 2000). However, computer-assisted
(BLAST) sequence analysis of the human genome using the
57-bp direct repeat within the human MRP1 5′-UTR as a
query failed to identify any regions of chromosome 16 with
significant sequence similarity. This sequence was also ab-
sent from the 5′ end of the MRP6 gene. Interestingly, the
5′-UTR of MRP1 beginning with the triplet repeat has been
reported in an mRNA initially thought to encode a novel drug
resistance protein, ARA (O’Neill et al., 1998). This mRNA
contains, in addition to the 5′-UTR of MRP1 and the first 24
nucleotides of coding sequence, copies of eight 3′-proximal
exons from MRP6. The ARA transcript was first identified in
a multidrug-resistant leukemia cell line after an amplifica-
tion of the MRP1/MPR6 locus. Thus, it seems likely that it
was generated by a recombinational event at this locus dur-
ing the amplification process.

The studies described above indicate that although se-
quence homology upstream from the human and rodent
MRP1/mrp1 genes is very limited, the core promoters of the
genes are structurally and apparently functionally conserved
with respect to basal regulatory mechanisms. This contrasts
with the human and murine MDR1/mdr1 genes encoding
type I P-glycoproteins. In the case of the type I P-glycoprotein genes, the two murine mdr1 promoters contain both TATA and CAAT boxes, whereas the human MDR1 promoter, like the MRPI/mrp1 promoters, lacks a consensus TATA box (Ueda et al., 1987; Hsu et al., 1990; Cohen et al., 1991; Labialle et al., 2002). The MRPI-related MRP2 and MRP3 promoters are TATA-less and each of the genes encodes a relatively short 5'-UTR. Comparative analyses of the human and rat MRPI/mrp2 promoters suggest that although the promoters share >50% sequence identity, their basal activities may depend on functionally unique sites within the 5'-flanking regions of the genes (Kauffman et al., 2001). Conversely, the human and rodent MRPI genes display little sequence identity within their 5'-flanking regions, but both require positionally-conserved Sp1 elements for basal activity (Fromm et al., 1999; Takada et al., 2000). Our analysis of the human and rodent MRPI promoters suggests that, like the MRPI promoters, they share a series of conserved Sp1 elements, within a putative GC-box, that are required for basal activity. Both mutation studies, and the similar pattern of retardation complexes observed in EMSA with Sp1 and the GC-boxes from all three MRPI/mrp1 promoters, suggest that the three conserved binding sites within the GC-box contribute to basal activity. Despite the presence of a fourth potential site in the rat sequence, no differences were detected in the pattern of retardation products observed in EMSA, suggesting that in the intact GC box, only three sites may be capable of simultaneously binding Sp1. However, in contrast to both human MRPI and MRP3 genes, the insertion of a GC-rich repeat in the 5'-UTR encoded in exon 1 of the human MRPI gene has introduced an additional potential regulatory region that may be unique to the human gene.

Several reports have correlated expression of human MRPI with p53 status in various human cancer cell lines, including lines derived from human prostate carcinoma, melanoma, and non–small-cell lung cancer (Wang and Beck, 1998; Fukushima et al., 1999; Sullivan et al., 2000; Bahr et al., 2001). One of these studies demonstrated transcriptional suppression of the MRPI gene in human H1299 cells by wild-type p53, and the authors suggested that the Sp1 elements within the conserved GC-box in the human promoter may play a role in the observed repression (Wang and Beck, 1998). We also found that both the human and rodent promoters are negatively regulated by wild-type p53 in transient transfection assays and that repression was abrogated by cotransfection with a vector encoding a dominant-negative p53 mutant. The extent of repression was also unaffected by a series of truncations that eliminated 5'-flanking sequences upstream from the proximal promoter region, consistent with the possible involvement of the Sp1 elements. However, despite the fact that the level of basal activity was much reduced when GC boxes in the proximal promoter regions of the rodent genes were mutated, the -fold repression observed with wild-type p53 remained unchanged, suggesting that it may not be attributable to an Sp1-mediated mechanism. The region involved is also devoid of any known p53-binding elements or half-elements, and we have been unable to detect direct binding of p53 to this region by EMSA (M. Muredda and P. G. Deley, unpublished observations). Three distinct classes of p53-repressed genes have been described in the literature. In one class, repression by p53 involves interaction with promoter-bound transcriptional activators, such as Sp1 (Kanaya et al., 2000) and AP-1 (Sun et al., 1999). In another class, which includes the human MDR1 promoter, p53-response elements mediate transcriptional repression by direct protein-DNA interactions (Johnson et al., 2001). Finally, several early reports described interactions between p53 and protein components of the basal transcription machinery, such as the TATA-binding protein and TATA-binding protein-associated factors (Seto et al., 1992; Farmer et al., 1996a,b). p53-dependent modulation of promoters within this class is believed to be caused by disruption of the transcription initiation complex. The lack of identifiable p53 binding elements and the apparent lack of requirement for functional GC-boxes strongly suggest that the human and murine MRPI/mrp1 genes may belong to the third class of p53-repressed genes.

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References


Evidence for Regulation by Transcription Factors Sp1 and Sp3


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