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Characterization of the rat *mdr2* promoter and its regulation by the transcription factor Sp1

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ABSTRACT

The *mdr2* gene encodes a P-glycoprotein that transports phospholipids across the canalicular membrane in hepatocytes. In this report we describe the isolation, sequencing and first functional characterization of the promoter of *mdr2*. Analysis of 1.6 kb of DNA upstream of the initiation of translation revealed that this sequence has a high GC content, lacks a TATA element and contains a number of putative transcription factor binding sites. We observed that transcription initiates at several sites between –290 and –463 and that this region was critical for promoter activity. Gel mobility shift assays indicated that Sp1 protein binds to a Sp1 consensus site located at –263. Co-expression of Sp1 protein with a reporter construct containing the –263 GC box demonstrated that Sp1 regulates transcription of this promoter. Expression of a non-functional Sp1 protein did not increase transcription from the *mdr2* promoter. Mutation of the –263 GC box diminished the response of the promoter to Sp1 protein. Mutation of this site also decreased expression of this promoter in cells which normally express this gene. These data show that Sp1 has a role in the regulation of *mdr2* expression.

INTRODUCTION

Members of the multidrug resistance gene family can be functionally divided into class I and class II genes. Class I comprises the human MDR1 and the rodent *mdr1a* and *mdr1b* genes, while class II comprises the human MDR2 and rodent *mdr2* (1,2). Recent work has provided strong evidence that the *mdr2* P-glycoprotein is a phosphatidylcholine transporter (3,4). Mice with a homozygous disruption of the *mdr2* gene are entirely deficient in the transport of phosphatidylcholine into the bile. As a result these animals develop pronounced hepatic disease consisting of non-suppurative inflammatory cholangitis (3,5). These mice also develop liver tumors at 4–6 months of age. Phosphatidylcholine labeled with the fluorescent group 7-nitro-2,1,3-benzoxadiazol-4-yl is transported across the lipid membrane bilayer of vesicles isolated from yeast overexpressing the murine *mdr2* gene (4). As with the *mdr1* P-glycoproteins, transport mediated by the *mdr2* P-glycoprotein is ATP and Mg^{2+} dependent and verapamil inhibitable. These data suggest that alterations in the expression of MDR2, the human homolog of mouse *mdr2*, could contribute to the development of human diseases with pathologies similar to those observed in the knockout mice (6).

Although the class I and class II *mdr* genes have a high degree of sequence identity, the functions of the corresponding P-glycoproteins are significantly different, since class II genes do not contribute to drug resistance. Considerable effort has focused on understanding class I genes, since they play a role in clinical manifestations of drug resistance. The P-glycoprotein products of class I genes are ATP-dependent transmembrane pumps which decrease the concentration of drugs in cells. When transfected into drug-sensitive cells the class I genes confer resistance to a wide variety of drugs (7,8). The ability of the class I P-glycoproteins to transport drugs across the cell membrane has led to the proposal that these proteins normally act to help protect organisms from exposure to harmful environmental compounds. Mice with a homozygous knockout of the *mdr1a* gene appear normal until challenged with drugs (9). These mice then show an increased accumulation of drugs in the brain, suggesting that *mdr1a* plays a role in maintaining the blood–brain barrier.

mdr2 mRNA is expressed predominantly in the liver and spleen, to a lower level in the skeletal muscle, heart, lung and brain and is undetectable in the kidney and small intestine (10–12). In addition, unlike *mdr1b*, *mdr2* is not regulated in cells in response to treatment with xenobiotics. *Mdr2* expression remains steady after treatment of cells with 2- acetylaminofluorene, aflatoxin B_1, cycloheximide and other chemicals which cause increased expression of *mdr1b* (13,14; P.C.B., unpublished observations). Moderate increases in *mdr2* expression are seen after treatments which induce a proliferative response in the liver, such as in rats treated with carbon tetrachloride or subjected to partial hepatectomy (10,15). These data suggest that *mdr2* expression is not increased directly by xenobiotics but does respond to some growth- and cell type-specific stimuli.

We have characterized the rat *mdr2* cDNA and shown with primer extension analysis that transcription of this gene is initiated at more than one start site (10). However, little else is known about the regulation of this gene. We present here the first functional characterization of the *mdr2* promoter and identify one transcription factor which can regulate *mdr2* expression. To better understand the regulation of *mdr2* expression we cloned and sequenced 1.6 kb of the 5′ flanking region of the rat *mdr2* gene. Promoter activity of this DNA has been examined through the use of a series of deletion mutations. Putative transcription factor binding sites were identified and electrophoretic mobility shift

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assays were carried out to investigate interaction of the Sp1 transcription factor with this promoter. In addition, we have shown with co-transfection experiments that Sp1 can regulate transcription from this promoter.

MATERIALS AND METHODS

Cloning of the 5′ region of rat *mdr2*

A genomic library constructed from *Bam*HI-digested Fischer rat liver DNA cloned into the λDASH vector (Stratagene, La Jolla, CA) was screened with a 59 bp 5′ fragment of the rat *mdr2* cDNA labeled with [γ-^{32}P]dCTP (10). Bacteriophage were grown and transferred to Magnagraph nylon membranes (MSI, Westboro, MA) as previously described (16). Following UV crosslinking, the filters were prehybridized in 6× SSC 5× Denhardt's solution, 1% SDS, 40% formamide at 42°C for a minimum of 2 h. Filters were hybridized with 10^6 c.p.m./ml probe for 24–48 h. The filters were washed to a final stringency of 0.1× SSC, 0.1% SDS at 50°C for 1 h then exposed to XAR film (Kodak, Rochester, NY) at –70°C with intensifying screens. Positively hybridizing phage were rescreened until pure. Clones were further characterized by Southern blot analysis. A 3.5 kb *Eco*RI fragment and a 1.2 kb *Pvu*II fragment were subcloned into pGem7zf(+) (Promega, Madison, WI) and sequenced by dideoxy sequencing using Sequenase 2.0 (US Biochemical, Cleveland, OH) and [α-^{35}S]dATP. Nucleotide sequences were analyzed with the PC/Gene software package (Intelligenetics, Mountain View, CA).

Construction of luciferase–*mdr* plasmids

Deletion fragments of the 1572 bases of the *mdr2* 5′ non-coding sequence were obtained from the *Eco*RI or *Pvu*II fragments by PCR or restriction enzyme digestion and were subcloned into the luciferase gene-containing vectors pXP1 and pXP2 (17) in forward and reverse orientations respectively. Dideoxy sequencing confirmed that the sequences of all PCR-generated fragments were correct.

To obtain the –335 construct with a mutated Sp1 site three PCR were executed. First, a fragment from –385 to –254 was amplified using as primer for the 3′-end 5′-CGG CTC GTC CTC CAC TCC CAC A-3′, in which three mutations were introduced into the Sp1 consensus site (underlined bases). Second a fragment from –275 to +6 was amplified using a 3′ primer containing a *Hin*dIII site to facilitate cloning into the pXP1 vector and as primer for the 5′-end 5′-TGT GGG AGT GGA GGA CGA GCC G-3′, in which the same three bases were mutated as in the first PCR. The products were then combined, heated to 95°C, allowed to anneal at 55°C and extended with *Taq* DNA polymerase. Primers at either end were added and PCR was performed. The resulting fragment was cut with restriction enzymes *Sac*I and *Hin*dIII and ligated into pXP1. The presence of the mutations was confirmed by dideoxy sequencing.

Measurement of promoter activity

COS cells or H4-II-E cells were transiently transfected with 10 µg luciferase constructs plus 2 µg CMV β-gal by calcium phosphate precipitation (18). Cells were harvested 48 h after transfection and lysed by freeze–thawing three times in 0.1 M sodium phosphate, pH 7.4, 1 mM dithiothreitol. Luciferase activity was measured in a Berthold Lumat LB9501 luminometer. One hundred microliters of 1 mM luciferin and 350 µl 25 mM glycylglycine, pH 7.8, 5 mM ATP, 15 mM $MgSO_4$ were mixed with an aliquot of sample and luminescence was measured for 30 s. β-Galactosidase activity was measured in an aliquot of extract after reacting for 15 min with 3 mM *o*-nitrophenyl-β-D-galactopyranoside in 0.1 M sodium phosphate, pH 7.4, 1 mM $MgCl_2$, 45 mM β-mercaptoethanol. Absorbance at 420 nm was measured in a Beckman DU65 spectrophotometer. Luciferase activity was corrected for transfection efficiency by dividing by the corresponding β-galactosidase activity.

Primer extension analysis

Primer extension analysis was used to map the distance of the transcription start point (*tsp*) from the start of translation (19). A primer corresponding to the non-coding strand at positions +10 to +27 was end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. This primer was annealed to 50 µg rat liver total RNA at 45°C for 1 h after heating briefly to 70°C in buffer containing 550 mM Tris–HCl, pH 8.3, 450 mM KCl. Avian myeloblastosis virus reverse transcriptase was used to extend the hybrid in a buffer containing 0.1 mM dithiothreitol, 25 µM deoxynucleotides, 1 mM $MgCl_2$. The extension products were electrophoresed through 8% denaturing polyacrylamide gels and then visualized by autoradiography. A sequence ladder was run on these gels to permit sizing of the extension products.

Preparation of nuclear extracts and electrophoretic mobility shift assays

Nuclear extracts were prepared according to the method of Rathmell and Chu (20). Briefly, 1×10^7 cells were washed with phosphate-buffered saline (PBS) and resuspended in 20 µl buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol) with 0.1% NP-40. The cells were incubated on ice for 10 min and the nuclei were then pelleted by centrifugation for 10 min at 12 000 *g* at 4°C. The pellet was resuspended in 15 µl buffer C [20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM Pefabloc (Boehringer Mannheim Corp., Indianapolis, IN), 0.5 mM dithiothreitol] and pelleted by centrifugation for 10 min at 12 000 *g* at 4°C. The supernatant was recovered as the nuclear extract and protein concentrations were determined by a modified Bradford method (21) (BioRad, Richmond, CA).

A 53 bp fragment of the *mdr2* promoter (–279 to –227) was obtained by cutting a larger PCR fragment with the restriction enzyme *Mlu*I. This fragment was radiolabeled with [α-^{32}P]dCTP using the Klenow fragment of DNA polymerase I (22). The probe was purified on a 12% polyacrylamide gel and eluted into 10 mM Tris–HCl, pH 7.4, 1 mM EDTA. Purified Sp1 protein (0.5 footprinting units; Promega Corp., Madison, WI) or 2 µg nuclear extract protein was incubated in 10 µl binding buffer (12 mM HEPES, 5 mM $MgCl_2$, 4 mM Tris, pH 7.9, 100 mM KCl, 0.6 mM EDTA, 0.6 mM dithiothreitol, 12% glycerol) (20) with 0.5 µg poly(dA·dT)·poly(dA·dT), 0.5 µg bovine serum albumin and with or without competitor for 5 min and then 0.015 pmol probe were added and the incubation continued for 20 min. In supershift experiments, Sp1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added at this time and the incubations were continued for an additional 60 min on ice. Two different 22 bp oligonucleotide competitors were used: one contained a wild-type Sp1 consensus sequence, the other was identical to the first except

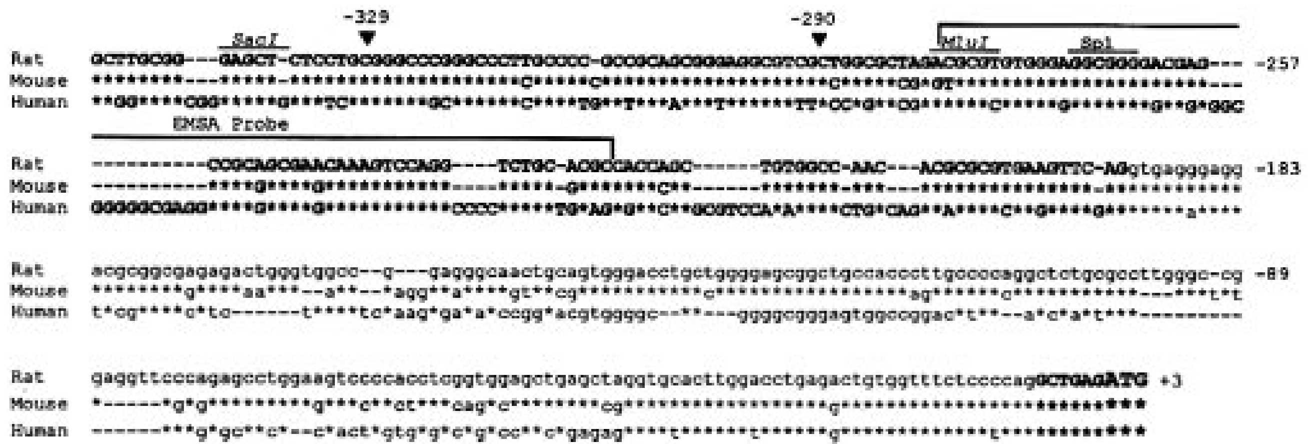

Figure 1. Comparison of proximal regions of rat, mouse and human *mdr2*/MDR2 promoters. Intron sequence is presented in lower case. Numbering is relative to the start of translation. The *Mlu*I restriction enzyme site used in creating the electrophoretic mobility shift probe and the *Sac*I site used to construct the –335 bp deletion are indicated. The bracketed region corresponds to the 53 bp probe from bases –227 to –279 used in electrophoretic mobility shift assays. Transcription start sites of the rat gene are identified by triangles over the sequence. The conserved Sp1 site at –263 is also marked.

for a GG→TT substitution in the Sp1 consensus site (Santa Cruz Biotechnology, Santa Cruz, CA). Probe and probe–protein complex were resolved on a Tris–glycine 4% polyacrylamide gel containing 2.5% glycerol. Gels were dried and analyzed by phosphorimager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Sp1 transfection experiments

Schneider's *Drosophila* line 2 cells were maintained in Schneider's *Drosophila* medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 50 µg/ml Gentamicin. The cells were plated at 1×10^6/100 mm dish and transfected by calcium phosphate precipitation (18) 16 h later. Cells were transfected with 10 µg luciferase constructs and 2 µg either pP_{ac}Sp1, pP_{ac}Sp1N539 or the empty vector pP_{ac}U+Nde. The pP_{ac} vectors are expression vectors which contain the *Drosophila* actin 5C promoter and were kindly provided by Dr Robert Tjian (23). The pP_{ac}Sp1 vector expresses a protein of 696 amino acids which exhibits wild-type activity. The pP_{ac}Sp1N539 vector expresses a Sp1 protein which has 157 amino acids of the C-terminus of the protein deleted. This deleted region contains three zinc finger DNA binding domains essential for Sp1-mediated transcriptional activation. Co-transfections were also performed with the Sp1 constructs and either the empty luciferase vector pXP1 or the positive control pGL2-Control (Promega Corp., Madison, WI). Transfected cells were harvested 48 h after transfection by washing in cold PBS and then lysing with 0.1 M sodium phosphate, pH 7.4, 1 mM dithiothreitol, 1% Triton X-100. Luciferase activity and protein concentrations were measured as described above.

RESULTS

Screening of a rat genomic library with a 5′ fragment of the rat *mdr2* cDNA identified several bacteriophage clones which contained 5′ genomic regions of this gene. Following further characterization by Southern blot analysis, a 1572 bp fragment was subcloned and sequenced (Fig. 1). This sequence was unambiguously identified as *mdr2* since it contained a large region of nucleotides which overlap with the previously cloned cDNA. The sequence of the 1572 bases of 5′ non-coding region of the rat *mdr2* gene has been submitted to GenBank and has been assigned the accession number U37694. The numbering scheme is based on designating the start of translation +1. The nucleotides from –7 to –192 were identified as intron, since they were not present in the cDNA (10). The sequence contains a number of putative transcription factor binding sites. In particular, the sequence is rich in guanine and cytosine residues and has four Sp1 transcription factor consensus elements. Other putative consensus binding sites were also identified, such as those for the AP-1 and AP-4 transcription factors. Large portions of the sequence exhibit a high degree of sequence identity with the mouse and human *mdr2*/MDR2 promoters (Fig. 1).

Previously, we identified multiple transcription start sites of the *mdr2* gene (10). With the promoter of the gene now sequenced, we could assign these start sites to specific bases. Primer extension experiments were conducted with a primer just inside the coding region of the cDNA (Fig. 2). No significant extension products were seen shorter than the –290 product. We obtained extension products which correspond to start sites at –290, –329, –349, –375, –411 and –463. The same start sites were identified when extension products were obtained using several other primers located further 5′ of the one used for the extensions shown in Figure 2. From this data we conclude that this promoter has multiple start sites, none of which are clearly associated with any TATA-like elements. These start sites are indicated on the sequence in Figure 1 by the small triangles over the sequence. Thus these data are consistent with our previous observation and indicate that transcription of *mdr2* initiates at several distinct start sites between –290 and –463.

A series of fragments of the 5′ genomic DNA was functionally tested for promoter activity by transient transfection of luciferase constructs of these fragments into several cell lines (Fig. 3). The relative basal transcriptional activity of the different promoter constructs was similar in both COS and H4-II-E cell lines (Fig. 3), as well as HepG2 and NIH 3T3 cell lines (data not shown). Deletion of the 5′-most 259 bases had little effect on promoter activity. Each subsequent deletion decreased the activity of the

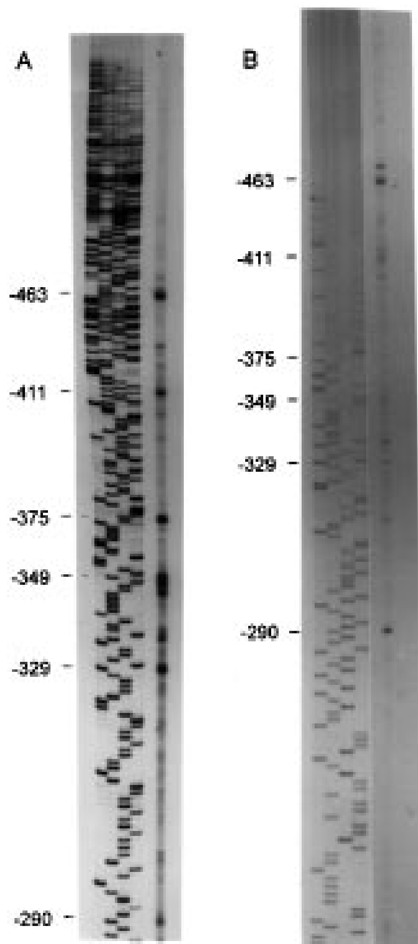

Figure 2. Determination of transcription start site by primer extension analysis. The numbers correspond to the number of bases 5′ of the start of translation. Fifty micrograms of rat liver total RNA was hybridized to a [γ-^{32}P]ATP end-labeled oligonucleotide probe and transcribed with avian myeloblastosis virus reverse transcriptase. Extension products were resolved on an 8% polyacrylamide–urea gel. A dideoxy sequencing ladder is shown next to the extension products for size determination. Gel (A) was run longer than gel (B) to better resolve the area from –290 to –463.

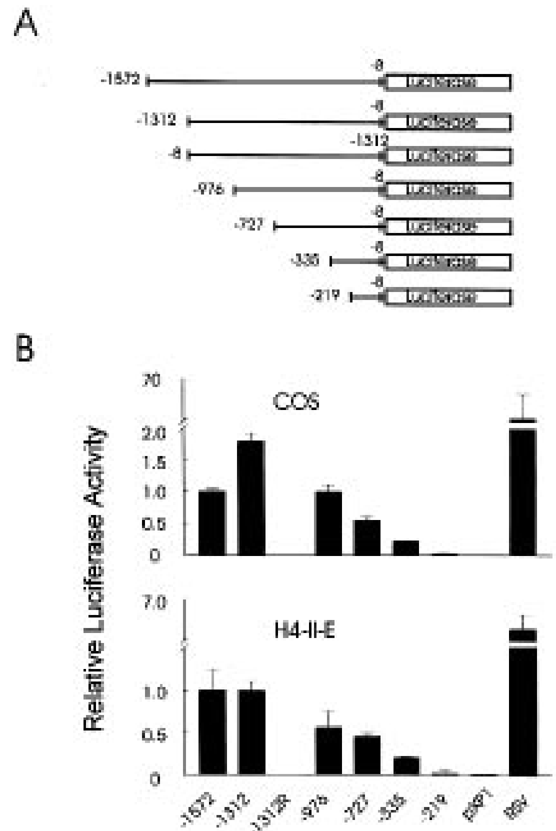

Figure 3. Transcriptional activity of *mdr2* promoter deletions. (**A**) *mdr2* promoter deletion constructs. The size and orientation of each construct is indicated by the numbers, which are relative to the start of translation, designated +1. Fragments of untranslated DNA 5′ of the rat *mdr2* gene were cloned upstream of the luciferase gene in the pXP1 or pXP2 vector (17). (**B**) *mdr2* deletion construct promoter activity expressed as luciferase activity in H4-II-E and COS cells The activity of the 1572 bp construct was designated 1. Constructs correspond to those described in (A). In order to control for transfection efficiency a CMV-β-galactosidase vector was co-transfected with each deletion construct and luciferase activity was divided by the resulting β-galactosidase activity. The luciferase activity obtained from transfection of the empty pXP1 vector or the positive control RSV construct is also shown. The data presented are the means ± SD from triplicate plates and are representative of multiple independent experiments.

promoter until the –219 construct, which had the same luciferase activity as the empty luciferase reporter vector pXP1. The lack of transcription from the –219 construct is consistent with the primer extension data, since no transcription start site was found shorter than –290. A construct with the reverse orientation of bases –1312 to –8 was also without activity, therefore, the transcriptional activity of this fragment is orientation dependent. The activity of a luciferase vector under the control of the Rous sarcoma virus (RSV) promoter was used as a positive control in these experiments.

The smallest construct with activity was GC rich and contained two perfect match consensus sequences for the Sp1 transcription factor,therefore, we investigated whether this factor might play a role in regulation of transcription of this gene. Electrophoretic mobility shift experiments were done to determine whether this region of the promoter could interact with Sp1 protein. A 53 bp fragment from –279 to –227 which contains an Sp1 consensus site was recognized by purified Sp1 and resulted in a slower migrating band in an electrophoretic mobility shift assay (Fig. 4A). Unlabeled Sp1 consensus oligonucleotide successfully competed with the probe for Sp1 binding. However, an Sp1 consensus oligonucleotide with two base changes rendered this oligonucleotide unable to compete for Sp1 binding. These experiments show that Sp1 can bind to the *mdr2* promoter.

The ability of Sp1 binding to functionally regulate *mdr2* was tested by expression of Sp1 in cells which were also transfected with *mdr2* promoter constructs. Schneider's *Drosophila* line 2 cells, which lack Sp1 (23), were transfected with a Sp1 expression vector under the control of the *Drosophila* actin 5C promoter and an *mdr2* reporter construct. Co-transfection of the –335 *mdr2* promoter construct with the Sp1 expression vector showed that activity of the promoter was greatly increased in the presence of Sp1 protein (Fig. 5). However, co-transfection of the *mdr2* promoter with a non-functional Sp1 deletion mutant, N539, did not increase activity of the promoter. Introduction of three base mutations into the –263 Sp1 site of the –335 promoter construct

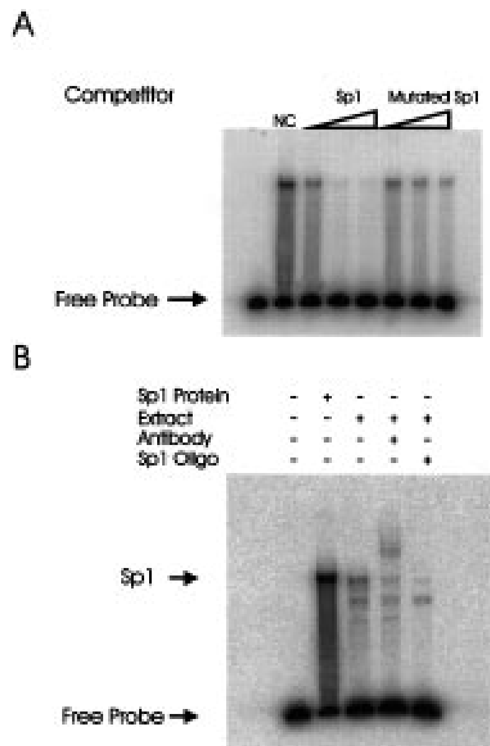

Figure 4. Electrophoretic mobility shift assay of Sp1 protein using bases –279 to –227 of the *mdr2* promoter as probe. (**A**) The shifted band is competed with a 22 bp Sp1 consensus oligonucleotide or the same oligonucleotide with a GG→TT substitution in the Sp1 binding site. The competitors were present at 20-, 100- and 200-fold molar excess. (**B**) Electrophoretic mobility shift assay of Sp1 protein from nuclear extract of H4-II-E cells. The probe was hybridized to 0.5 footprinting units of purified Sp1 (lane 2) or 2 µg nuclear extract (lanes 3–5). Lane 4 contains probe–protein complexes which were further incubated with Sp1 antibody prior to electrophoresis. The hybridization reaction was performed in the presence of a 500-fold molar excess of an Sp1 consensus oligonucleotide in the sample resolved in lane 5. The band specifically supershifted by Sp1 antibody and competed by the Sp1 consensus site oligonucleotide is marked with an arrow. Gels were analyzed by phosphorimager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

(see Materials and Methods) caused the response to Sp1 to be greatly diminished. Transcriptional activity of the –219 bp construct of the *mdr2* promoter was not increased by co-transfection with the Sp1 expression vector. This construct contains no perfect match Sp1 consensus sites and, as shown in Figure 4, has essentially no activity in transfection experiments with mammalian cells. Sp1 expression did not increase the activity of the empty pXP1 luciferase vector, indicating that the increase observed with the *mdr2* construct is specifically due to the presence of the promoter sequence. As a positive control, expression of the pGL2-Control plasmid was greatly increased by Sp1, but not mutant Sp1. The pGL2-Control vector contains the SV40 promoter and enhancer regions, which are known to contain multiple Sp1 consensus sites. These experiments demonstrate that binding of Sp1 to the *mdr2* promoter in cells results in a specific increase in transcription.

In order to confirm the relevance of the regulation of *mdr2* by Sp1, we used the rat hepatoma cell line H4-II-E, which constitutively expresses *mdr2* (14). When an electrophoretic mobility shift was performed using nuclear extract from these cells several reduced mobility bands were observed, one of which

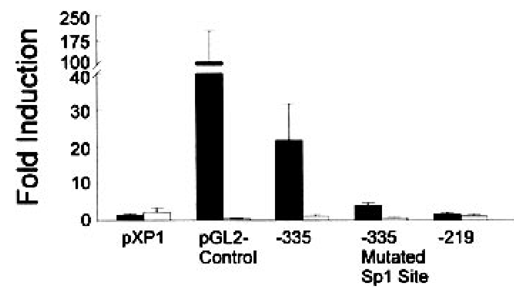

Figure 5. Activation of the *mdr2* promoter by Sp1 expression in *Drosophila* cells. Activity of the luciferase constructs in cells co-transfected with either $pP_{ac}Sp1$, which encodes functional Sp1 (filled bars), or $pP_{ac}Sp1N539$, which encodes a non-functional Sp1 (open bars). Results are expressed as fold induction over cells co-transfected with the test plasmid and the empty $pP_{ac}U$+Nde vector. The pXP1 vector is the empty vector in which the *mdr2* promoter fragments were cloned. The pGL2-control vector is a vector in which luciferase expression is driven by the SV40 promoter and enhancer. The –335 and –219 constructs are the same as those described in Figure 3. The –335 construct with the mutated Sp1 site has three base changes in the Sp1 consensus site as described in Materials and Methods. The data presented are the means ± SD from triplicate plates and are representative of multiple independent experiments.

coincided with the band observed when purified Sp1 was shifted (Fig. 4B). When cold Sp1 consensus DNA was used as competitor this band disappeared, suggesting that this band represents the Sp1 protein present in the extract. In addition, the mobility of this band was further retarded, 'supershifted', when the probe and bound Sp1 were incubated with an anti-Sp1 antibody. These data show that Sp1 protein, which is capable of binding to the *mdr2* promoter, is present in cells which normally express this gene. Therefore, we tested whether mutation of the Sp1 site identified as important for expression in *Drosophila* cells was also important in these cells. Transfection of the wild-type and mutated forms of the –335 *mdr2* promoter construct into H4-II-E cells shows that mutation of the –263 Sp1 site decreases expression from this promoter fragment by ~70% (Fig. 6). This suggests that this Sp1 site is important for promoter activity in cells which normally express the rat *mdr2* gene.

DISCUSSION

Recent experiments identifying the *mdr2* P-glycoprotein as a transmembrane phospholipid transporter have emphasized the importance of understanding regulation of expression of this gene. The experiments presented here represent the first functional characterization of the rat *mdr2* promoter and demonstrate that the transcription factor Sp1 is important for transcriptional activity of this gene. Sequence analysis of the rat *mdr2* gene did not identify any strong TATA element consensus sites, however, the promoter does have a high GC content. Similarly, the sequence of the mouse and human promoters have recently been determined (24). These promoters are also GC-rich and contain no TATA box, however, no detailed analysis of these promoters has yet been reported. Deletion analysis of the rat *mdr2* promoter revealed an incremental decrease in activity as the promoter was reduced in size from the 5′-end. This is consistent with initiation of transcription occurring at a variety of sites along the length of the promoter. Stepwise deletion of the promoter would sequentially eliminate these transcription start points, thus providing fewer opportunities to initiate new transcripts and therefore leading to

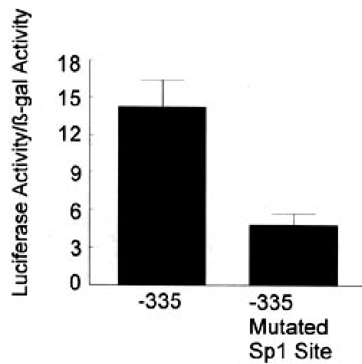

Figure 6. Activity of the wild-type and mutated –335–luciferase construct in H4-II-E cells. The *mdr2* promoter constructs were transfected into H4-II-E cells and harvested 48 h later. Luciferase activity was measured and corrected for transfection efficiency by dividing by β-galactosidase activity expressed from a co-transfected plasmid. The mutated –335 construct contains three base pair changes in the Sp1 consensus site as described in Materials and Methods. The data presented are the means ± SD from triplicate plates and are representative of multiple independent experiments.

an overall decrease in expression from the promoter. A number of genes which lack TATA elements have also been shown to have multiple start sites, including the rat TGFα gene (25), the human MDR2 gene (24), the human multidrug resistance-associated protein (26) and the hamster *mdr1a*(*pgp1*) gene (27).

The presence of several Sp1 consensus sites in the *mdr2* promoter suggested that this transcription factor might play a role in regulation of expression of this gene. We have shown that the most 3′ of these sites binds Sp1 protein and that expression of Sp1 in cells normally lacking this transcription factor causes an increase in expression from a promoter construct containing this region. Furthermore, mutation of this site prevents it from competing with the wild-type sequence for Sp1 binding and reduces the activity and inducibility of this site. Mutation of this site also reduces transcription from the basal *mdr2* promoter in cells which normally express this gene, thereby strengthening the physiological relevance of our findings. This site is well conserved in the rat, mouse and human *mdr2*/MDR2 promoters and falls close to likely transcription start points (see Fig. 1). In the case of the human promoter several transcription start points have been identified 30–80 bases downstream of this particular Sp1 site (24). Sp1 has been shown to play an important role in establishing accurate transcription initiation in other TATA-less promoters. For example, Sp1 consensus sites play a major role in the selection of the start site of the hamster CAD gene (28), the rat TGFα gene (29), the rat insulin-like growth factor binding protein-2 gene (30) and the human adenosine deaminase gene (31).

Regulation of the rat *mdr2* gene is unchanged by exposure to drugs. This is in sharp contrast to the rat *mdr1b* gene, which increases expression in response to a variety of xenobiotic stimuli (32–36). The rat *mdr1b* promoter differs from the *mdr2* promoter in that it contains a TATA consensus element and employs a single transcription start point both under basal and induced expression conditions (32). Although the coding regions of the *mdr1b* and *mdr2* genes share a high degree of sequence identity, their functions appear to be different and so the divergence in their regulation is not surprising. *mdr2* expression in the rat increases after partial hepatectomy or carbon tetrachloride-induced hepatic damage (10,15). This suggests that *mdr2* expression increases during times of cell growth and proliferation. It has recently been shown that Sp1 plays a role in mediating the activation of growth-responsive genes, including human MDR1 (37). Sp1 is hypophosphorylated during liver regeneration and this leads to greater DNA binding and transactivation activities (38). Since we have shown that *mdr2* is regulated by Sp1, it is possible that the increased DNA binding activity of Sp1 during liver regeneration increases expression of *mdr2*.

In conclusion, we have identified the basal promoter of the rat *mdr2* gene. We have shown that transcription is initiated at several sites on the promoter and that the transcription factor Sp1 regulates transcription of the minimal *mdr2* promoter. Several other Sp1 consensus sites can be identified on the promoter and future work will establish what role these play in regulation of this gene. A number of other transcription factor consensus sites can be identified in the promoter sequence and it is likely that some of these play a role in controlling expression of the *mdr2* gene. We are investigating which of these additional transcription factors regulate the basal, growth-stimulated and tissue-specific expression of *mdr2*.

REFERENCES

1 Gottesman,M.M. and Pastan,I. (1993) *Annu. Rev. Biochem.*, **62**, 385–427.
2 Chin,K.-V., Pastan,I. and Gottesman,M.M. (1993) *Adv. Cancer Res.*, **60**, 157–180.
3 Smit,J.J.M., Schinkel,A.H., Oude Elferink,R.P.J., Groen,A.K., Wagenaar,E., van Deemter,L., Mol,C.A.A.M., Ottenhof,R., van der Lugt,N.M.T., van Roon,M.A., van der Valk,M.A., Offerhaus,G.J.A., Berns,A.J.M. and Borst,P. (1993) *Cell*, **75**, 451–462.
4 Ruetz,S. and Gros,P. (1994) *Cell*, **77**, 1071–1081.
5 Mauad,T.H., van Nieuwkerk,C.M.J., Dingemans,K.P., Smit,J.J.M., Schinkel,A.H., Notenboom,R.G.E., Weerman,M.van den B., Verkruisen,R.P., Groen,A.K., Elferink,R.P.J.O., van der Valk,M.A., Borst,P. and Offerhaus,G.J.A. (1994) *Am. J. Pathol.*, **145**, 1237–1245.
6 Ludwig,J., Czaja,A.J., Dickson,E.R., LaRusso,N.F. and Wiesner,R.H. (1984) *Liver*, **4**, 105–116.
7 Gros,P., Ben-Neriah,Y., Croop,J.M. and Housman,D.E. (1986) *Nature*, **323**, 728–731.
8 Guild,B.C., Mulligan,R.C., Gros,P. and Housman,D.E. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 1595–1599.
9 Schinkel,A.H., Smit,J.J.M., van Tellingen,O., Beijnen,J.H., Wagenaar,E., van Deemter,L., Mol,C.A.A.M., van der Valk,M.A., Robanus-Maandag,E.C., te Riele,H.P.J., Berns,A.J.M. and Borst,P. (1994) *Cell*, **77**, 491–502.
10 Brown,P.C., Thorgeirsson,S.S. and Silverman,J.A. (1993) *Nucleic Acids Res.*, **21**, 3885–3891.
11 Borst,P., Schinkel,A.H., Smit,J.J.M., Wagenaar,E., van Deemter,L., Smith,A.J., Eijdems,W.H.M., Baas,F. and Zaman,G.J.R. (1993) *Pharmacol. Ther.*, **60**, 289–299.
12 Silverman,J.A. and Thorgeirsson,S.S. (1995) In Boyer,J.L. and Ockner,R.K. (eds), *Progress in Liver Diseases.* W.B.Saunders, Philadelphia, PA, Vol. 13, pp. 101–124.
13 Gant,T.W., Silverman,J.A. and Thorgeirsson,S.S. (1992) *Nucleic Acids Res.*, **20**, 2841–2846.
14 Hill,B.A., Brown,P.C., Preisegger,Karl-H. and Silverman,J.A. (1996) *Carcinogenesis*, **17**, 451–457.
15 Nakatsukasa,H., Silverman,J.A., Gant,T.W., Evarts,R.P. and Thorgeirsson,S.S. (1993) *Hepatology*, **18**, 1202–1207.
16 Silverman,J.A., Raunio,H., Gant,T.W. and Thorgeirsson,S.S. (1991) *Gene*, **106**, 229–236.
17 Nordeen,S.K. (1988) *Biotechniques*, **6**, 454–457.
18 Gorman,C., Padmanabhan,R. and Howard,B.H. (1983) *Science*, **221**, 551–553.
19 Archer,T.K., Tam,S.-P., Deugau,K.,V and Deeley,R.G. (1985) *J. Biol. Chem.*, **260**, 1676–1681.
20 Rathmell,W.K. and Chu,G. (1994) *Genome Res.*, **14**, 4741–4748.
21 Bradford,M.M. (1976) *Anal. Biochem.*, **72**, 248–254.

22 Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
23 Courey,A.J. and Tjian,R. (1988) *Cell*, **55**, 887–898.
24 Smit,J.J.M., Mol,C.A.A.M., van Deemter,L., Wagenaar,E., Schinkel,A.H. and Borst,P. (1995) *Biochim. Biophys. Acta*, **1261**, 44–56.
25 Blasband,A.J., Rogers,K.T., Chen,X., Azizkhan,J.C. and Lee,D.C. (1990) *Mol. Cell. Biol.*, **10**, 2111–2121.
26 Zhu,Q. and Center,M.S. (1994) *Cancer Res.*, **54**, 4488–4492.
27 Ince,T.A. and Scotto,K.W. (1995) *Gene*, **156**, 287–290.
28 Kollmar,R., Sukow,K.A., Sponagle,S.K. and Farnham,P.J. (1994) *J. Biol. Chem.*, **269**, 2252–2257.
29 Chen,X., Wright,K.L., Berkowitz,E.A., Azizkhan,J.C., Ting,J.P.-Y. and Lee,D.C. (1994) *Oncogene*, **9**, 3179–3187.
30 Boisclair,Y.R., Brown,A.L., Casola,S. and Rechler,M.M. (1993) *J. Biol. Chem.*, **268**, 24892–24901.
31 Dusing,M.R. and Wiginton,D.A. (1994) *Nucleic Acids Res.*, **22**, 669–677.
32 Gant,T.W., Silverman,J.A., Bisgaard,H.C., Burt,R.K., Marino,P.A. and Thorgeirsson,S.S. (1991) *Mol. Carcinogen.*, **4**, 499–509.
33 Thorgeirsson,S.S., Silverman,J.A., Gant,T.W. and Marino,P.A. (1991) *Pharmacol. Ther.*, **49**, 283–292.
34 Silverman,J.A. and Hill,B.A. (1995) *Mol. Carcinogen.*, **13**, 50–59.
35 Gant,T.W., Schrenk,D., Silverman,J.A. and Thorgeirsson,S.S. (1994) *Environ. Hlth Perspect.*, **102**, 209–212.
36 Schrenk,D., Gant,T.W., Michalke,A., Orzechowski,A., Silverman,J.A., Battula,N. and Thorgeirsson,S.S. (1994) *Carcinogenesis*, **15**, 2541–2546.
37 Miltenberger,R.J., Farnham,P.J., Smith,D.E., Stommel,J.M. and Cornwell,M.M. (1995) *Cell Growth Differentiat.*, **6**, 549–556.
38 Leggett,R.W., Armstrong,S.A., Barry,D. and Mueller,C.R. (1995) *J. Biol. Chem.*, **270**, 25879–25884.