CHAPTER 6

TRANSCRIPTIONAL REGULATION OF THE RAT PHOSPHOLIPID TRANSLOCASE Mdr2 GENE EXPRESSION BY CHOLESTEROL SYNTHESIS INHIBITORS AND STEROL REGULATORY ELEMENT-BINDING PROTEINS

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We have been shown in rats that the hepatic expression of the phosphatidylcholine translocase \textit{Mdr2} is induced by statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase. Statins are known to activate sterol regulatory element-binding proteins (SREBPs), transcription factors that control key enzymes of the cholesterol and fatty acid biosynthetic pathways. Here, we investigated the effects of three different statins on the steady-state mRNA levels of \textit{Mdr2} and of \textit{SREBP} family members in cultured primary rat hepatocytes. In addition, the effects of statins and overexpression of nuclear \textit{SREBP-1a} and \textit{SREBP-2} on \textit{Mdr2} promoter activity were studied in human HepG2 hepatoma cells. Simvastatin, lovastatin as well as atorvastatin equally induced \textit{Mdr2} and \textit{Srebp-2} mRNA levels in cultured hepatocytes, whereas the expression levels of \textit{Srebp-1a} and \textit{–1c} transcripts were reduced. The three statins induced (6 to 8-fold) the activity of a 1572 base pair \textit{Mdr2} promoter fragment of the \textit{Mdr2} promoter in HepG2 cells, as did nuclear \textit{SREBP-1a}. Nuclear \textit{SREBP-2} did not stimulate promoter activity. This does not rule out involvement of \textit{Srebp-2} in \textit{Mdr2} induction but rather indicate that other transcription factors must play a role. Mutations introduced into a 10 bp G+C-rich region, previously identified as potential sterol regulatory element, resulted in dramatically decreased basal activity of the \textit{Mdr2} promoter, but did not abolish the response to nuclear \textit{SREBP-1a}. We conclude that statins increase steady-state \textit{Mdr2} mRNA levels in rat hepatocytes as a result of enhanced gene transcription. \textit{SREBP-1a}, but not \textit{SREBP-2}, stimulates transcription of the \textit{Mdr2} gene in HepG2 cells.
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INTRODUCTION

Active transport across the canalicular membrane of hepatocytes into bile is a critical step in the biliary secretion of both endogenous and exogenous compounds in mammals. For this purpose hepatocytes are equipped with a variety of transport proteins that belong to the ATP-binding cassette (ABC) superfamily, one of the largest superfamilies of proteins in prokaryotes and eukaryotes. These proteins utilize the energy released by ATP hydrolysis to mediate transport of its substrates across membranes. Mammalian P-glycoproteins (Pggs), a subclass of the ABC superfamily, are encoded by a family of highly conserved genes, consisting of four members in rodents, and three members in man. Pggs, encoded by the rodent Mdr1a/1b or human MDR1 genes, were the first ABC transporters shown to be constitutively expressed at the canalicular membrane of hepatocytes and are typically associated with multidrug resistance (mdr) in cancer therapy. Their precise physiological role(s) in bile formation is not clear since mice with a disruption of the Mdr1a/1b genes have unaffected bile formation. The rodent Mdr2 and Bsep and human MDR3 genes encode for Pggs that do not cause mdr and are probably unable to transport drugs at significant rates. In contrast to MDR1 these Pggs are only present at the canalicular membrane of hepatocytes. In hepatobiliary transport, Bsep functions as an ATP-dependent bile salt export pump and Mdr2/MDR3 as a primary active phosphatidylcholine (PC) translocase. Both proteins are crucial for normal liver function. Progressive familial intrahepatic cholestasis (PFIC) subtype 2, a syndrome characterized by extremely low biliary bile salt output, has recently been ascribed to mutations in the BSEP gene, leading to undetectable BSEP protein in liver. Mdr2 gene knockout mice, which do not secrete phospholipids (and cholesterol) into bile, develop progressive liver disease caused by the toxic detergent action of bile salts. Similarly, biliary lipid secretion is absent in humans with mutations in the MDR3 gene. The biliary secretion of PC is tightly coupled to that of cholesterol and bile salts, and appears to be regulated by the activity of Mdr2 as well as Bsep. Recently, several studies have focussed on the control of Mdr2 gene expression. Peroxisome proliferators were found to induce Mdr2 gene expression and protein levels in mice, with concomitant enhanced biliary phospholipid output. Comparable observations were made when mice were fed a cholate-supplemented diet, whereas feeding of ursodeoxycholate, a more hydrophilic bile salt, did not influence the Mdr2 mRNA content or phospholipid output rate. Continuous exposure of rats to simvastatin or pravastatin, inhibitors of the key enzyme in cholesterol biosynthesis (3-hydroxy-3-methylglutaryl coenzyme A reductase [HMGR]), resulted in a coordinated activation of hepatic PC biosynthesis, biliary output and Mdr2 mRNA and protein levels. Taken together, these studies suggest that Mdr2 expression may be controlled by transcription factors that are activated by intermediary metabolic products in fatty acid, bile acid, and/or cholesterol metabolic pathways. However, detailed studies on molecular mechanisms of the regulation of Mdr2 expression have not been reported. Based on our previous study, we hypothesized that transcriptional control of Mdr2 gene expression may, at least partially, be under the control of sterol regulatory element-binding proteins (SREBPs). SREBPs are a family of transcription factors that control key enzymes in cholesterol and fatty acid biosynthesis. Three SREBP isoforms (SREBP-1a, SREBP-1c, and SREBP-2) have been identified. SREBP-1a and SREBP-1c are derived from a single gene through the use of alternative transcription start sites, and SREBP-2 is encoded by a separate gene. When sterol levels fall in cultured cells, e.g. by statin treatment, the SREBPs are released from membranes of the endoplasmic reticulum and nuclear envelope through the action of two sequential proteolytic enzymes. The resulting soluble mature transcription factor enters the
nucleus, where it activates a set of target genes by binding to sterol regulatory elements (SREs) in their promoters, e.g. the gene encoding for HMGR\textsuperscript{32,33}.

In this study we present the first data describing the effects of three statins on expression of \textit{Mdr2} and the different \textit{Srebp} isoforms in cultured primary rat hepatocytes. We also determined whether statins and SREBPs were able to induce rat \textit{Mdr2} promoter activity.

**EXPERIMENTAL PROCEDURES**

**MATERIALS**

Simvastatin and lovastatin were a kind gift of Dr L.H. Cohen (Gaubius Laboratory, TNO Prevention and Health, Leiden, The Netherlands). Atorvastatin was generously provided by Parke-Davis (Hoofddorp, The Netherlands). All other chemicals were of analytical grade. Molecular biology reagents were obtained from Promega (Leiden, The Netherlands) or Roche Biochemicals (Almere, The Netherlands). Oligonucleotide primers and all cell culture media and reagents were from Life Technologies (Breda, The Netherlands).

**PLASMIDS**

The various rat \textit{Mdr2} promoter-\textit{Firefly} luciferase (luc) constructs used in this study have been described\textsuperscript{34}. The \textit{Mdr2} mutant 335mtGC-luc construct was generated by direct site-directed mutagenesis (QuickChange kit, Stratagene, Amsterdam, The Netherlands) using 335WT-luc as template and 5'-GCG CTA GAC GCT TTC TTG AGG CGG GGA C-3' and its complementary sequence as primers (mutated base pairs are underlined). Plasmids pCSA1\textsuperscript{35} and pCSA2, cytomegalovirus early promoter-controlled expression clones encoding either amino acids 1-490 of human SREBP-1a or 1-481 of human SREBP-2, and the empty expression vector pCMV5 were kindly provided by Dr T.F. Osborne (University of California, Irvine, CA). The shortened SREBPs (designated nuclear (n)SREBPs) enter the nucleus directly without a requirement for regulated proteolysis. pRL-CMV plasmid, which contains the CMV promoter fused to the \textit{Renilla} luciferase gene, was purchased from Promega and was used as an internal control for transfection efficiencies in some studies, as indicated.

**CELL CULTURES**

Primary rat hepatocytes were isolated from male Wistar rats using a two-step collagenase perfusion as previously described\textsuperscript{36}. Hepatocytes were suspended in William’s medium E supplemented with 5% fetal bovine serum, 434 mg l\textsuperscript{-1} L-alanyl-L-glutamine (Glutamax), 20 mU ml\textsuperscript{-1} insulin (Novo Nordisk, Bagsvaerd, Denmark), 50 nM dexamethasone, 100 U ml\textsuperscript{-1} penicillin, 100 \mu g ml\textsuperscript{-1} streptomycin, and 250 ng ml\textsuperscript{-1} fungizone. Primary hepatocytes were seeded on 35 mm plastic culture dishes (Costar, Badhoevedorp, The Netherlands) at a density of 125,000 cells per cm\textsuperscript{2}. After 4 hours the complete medium was replaced with the same medium described above without dexamethasone. Twelve hours after medium replacement, cells were incubated with statins, dissolved in dimethyl sulfoxide (DMSO), for the time-points indicated. Final concentrations of DMSO in medium were 0.1% (v/v), controls received DMSO only. Human hepatoma HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum, 4500 mg l\textsuperscript{-1} glucose, 110 mg l\textsuperscript{-1} sodium pyruvate, 862 mg l\textsuperscript{-1} Glutamax, and 100 U ml\textsuperscript{-1} penicillin, 100 \mu g ml\textsuperscript{-1} streptomycin, and 250 ng ml\textsuperscript{-1} fungizone. All cells were maintained in a humidified incubator at 37 °C and 5% CO\textsubscript{2}.

**MRNA ISOLATION AND ANALYSIS BY REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)**

Total RNA was isolated from cultured hepatocytes using TRIzol Reagent (Life Technologies) according to the manufacturer’s instructions. Reverse transcription was performed on 5 \mu g total RNA using random primers, and subjected to relative PCR as described by our laboratory\textsuperscript{37}. For every PCR reaction the level of glyceraldehyde 3-phosphate dehydrogenase (\textit{Gapdh}) mRNA served as internal control. The expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase (\textit{Hmgr})
served as established positive control. The rat Srebp-1a and Srebp-2 cDNAs have presently not been cloned. We therefore used primer pairs designed upon the mouse Srebp-1a and human Srebp-2 sequences to analyze the expression of their corresponding rat orthologues. Primer sequences and resulting PCR products were 5'-GCC GAG ATG TGC GAA CTG GAC A-3' (sense) and 5'-GGG CTG AGC TGC ACA TT-3' (antisense) for Srebp-1a, amplifying a 422 bp product; 5'-CAC GGA GCC ATG GAT TGC ACA TT-3' (sense) and GGG CTG AGC TGC ACA TT-3' (antisense) for Srebp-1c, amplifying a 404 bp product; 5'-CAA TGG CAC GCT GCA GAC CCT TG-3' (sense) and 5'-ATG GCC TTC CTC AGA ACG CCA G-3' (antisense) for Srebp-2, amplifying a 474 bp product; and 5'-TAC GAT GGT GTA GAT GCT GG-3' (sense) and 5'-AGT TCT TCT GTG TT-3' (antisense) for Hmgr, amplifying a 1280 bp product. Primer sequences for the Mdr2 and Gapdh fragments have been described.

Ten microliters of PCR product were loaded on a 2.5% agarose gel and stained with ethidium bromide. Images were taken using a charge-coupled device video camera of the ImageMaster VDS system (Pharmacia, Uppsala, Sweden).

### Transient Transfection Assays

HepG2 cells were plated on day 0 in 35 mm dishes at 300,000 cells per dish. To study the effect of statins on Mdr2 promoter activity, on day 1 the cells were transfected by the calcium phosphate coprecipitation method. The precipitates contained 2 µg of test plasmid and 1 µg pRL-CMV. The total amount of DNA was adjusted to 4 µg with pUC18 DNA. On day 2, 16 h after transfection, cells were washed three times with Hank's Balanced Salt Solution (HBSS) and refed DMEM containing 10 µM of statins, and were incubated for another 24 h. On day 3 cells were harvested in ‘passive lysis buffer’ (Promega) and enzyme assays were performed on cell extracts as described below. When the effect of nuclear SREBP-1a or SREBP-2 expression on Mdr2 promoter activity was studied, on day 1 HepG2 cells were transfected with precipitates containing 2 µg of test plasmid and various amounts of pCSA10, pCSA2 or empty vector pCMV5, whereas pRL-CMV was omitted. Again, the total amount of DNA was adjusted to 4 µg with pUC18 DNA. On day 2, cells were washed with HBSS and refed DMEM. Cells were cultured for an additional 24 h after which they were harvested for luciferase assay. Results are normalized by protein concentration of each sample.

![Figure 1. Steady-state mRNA levels of Mdr2, Srebp-1a, -1c, -2, Hmgr, and Gapdh in primary hepatocytes after statin treatment. Freshly isolated hepatocytes were exposed to: 0.1% DMSO, lanes 1-3 (control); or 10 µM of simvastatin, lanes 4-6; lovastatin, lanes 7-9; or atorvastatin, lanes 9-12. Five µg of total RNA was transcribed into cDNA and subjected to PCR analysis as described in Experimental Procedures. Messenger RNA levels of Hmgr and Gapdh served as positive and internal control, respectively. The number of cycles were 29 for Mdr2, 34 for the Srebps, 26 for Hmgr, and 22 for Gapdh.](image-url)
Firefly and Renilla luciferase activities were measured in a luminometer (Anthos Labtec Instruments GmbH, Salzburg, Austria) using the Dual Luciferase Assay System (Promega). For the statin-treated cells the normalized luciferase values were determined by dividing the Firefly luciferase activity (in RLU) by the Renilla luciferase activity (in RLU). Since SREBP overexpression strongly affected the expression of Renilla luciferase (data not shown), luciferase activities were normalized to the protein concentration of each sample in the SREBP-1a or SREBP-2 overexpression experiments.

**ANALYTICAL PROCEDURES**

Protein concentrations were determined with the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as standard.

**RESULTS**

**ENZYME ASSAYS**

Firefly and Renilla luciferase activities were measured in a luminometer (Anthos Labtec Instruments GmbH, Salzburg, Austria) using the Dual Luciferase Assay System (Promega). For the statin-treated cells the normalized luciferase values were determined by dividing the Firefly luciferase activity (in relative light units) by the Renilla luciferase activity (in RLU). Since SREBP overexpression strongly affected the expression of Renilla luciferase (data not shown), luciferase activities were normalized to the protein concentration of each sample in the SREBP-1a or SREBP-2 overexpression experiments.

**ANALYTICAL PROCEDURES**

Protein concentrations were determined with the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as standard.

**RESULTS**

**EFFECTS OF STATIN TREATMENT ON STEADY-STATE mRNA LEVELS OF MDR2 AND OF SREBPS IN PRIMARY RAT HEPATOCYTES**

Figure 1 shows the results of a typical experiment in which primary rat hepatocytes were treated either with 10 µM of simvastatin, lovastatin, atorvastatin, or vehicle (0.1% DMSO) for 24 hours and analyzed for expression of Mdr2 and various genes involved cholesterol metabolism. Statin treatment caused (as expected) a marked increase in levels of mRNA encoding HMG CoA reductase (Hmgr) and a somewhat smaller increase in the mRNA for Mdr2. Steady-state mRNA levels encoding either isoform of Srebp-1 were reduced, although this was less pronounced for the mRNA encoding Srebp-1c. In contrast, the amount of mRNA encoding Srebp-2 was increased by all three HMG inhibitors. Gapdh expression levels were similar in the four all experimental groups. No distinct differences were observed between the three statin-treated groups.
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EFFECTS OF STATIN TREATMENT ON MDR2 PROMOTER ACTIVITY

The induction of Mdr2 mRNA levels by statins may be the result of enhanced gene transcription. To address this issue, we examined the effect of HMGR inhibitors on the transcriptional activity of the Mdr2 gene promoter. A construct containing a 1572 bp fragment of the Mdr2 promoter34 (Figure 2), fused to a luciferase reporter gene, was transiently transfected into HepG2 cells and assayed for statin-regulated transcription by culturing the transfected cells in the presence of statins. As shown in Figure 3, treatment of transfected cells with 10 µM of the three statins resulted for each drug in an induction of Mdr2 promoter activity, varying between 6 and 8.5-fold compared to vehicle-treated cells. Thus, these experiments demonstrate that the increased Mdr2 mRNA levels found after statin treatment may be the result of enhanced gene transcription.

EFFECTS OF OVEREXPRESSION OF NUCLEAR SREBP-1A AND SREBP-2 ON MDR2 PROMOTER ACTIVITY

To directly evaluate the ability of SREBP-1a and SREBP-2 to activate the Mdr2 promoter, we co-transfected the 1572 bp Mdr2 promoter-reporter construct along with increasing amounts of an expression construct for human nSREBP-1a or nSREBP-2, respectively. Figure 4 shows that nSREBP-1a is able to stimulate Mdr2 promoter activity of the 335 construct in a dose-dependent manner. A maximal, 6.7-fold induction was obtained when 2 µg nSREBP-1a expression plasmid was transfected. A much lower, if any, activation was observed for nSREBP-2.

To identify which region of the Mdr2 promoter is necessary for SREBP-1a-induced transcription, we performed promoter-activation studies in HepG2 cells using a series of promoter-deletion constructs depicted in Figure 2. Figure 5 shows that the basal expression of the 1572 and 335 constructs were similar, whereas the basal luciferase activity of the 219 construct was almost completely lost compared to the other two constructs. Co-transfection of either the 1572 or 335 constructs, together with nSREBP-1a, resulted for both constructs in an increase of luciferase activity. The activity of the 219 construct was slightly induced in the presence of SREBP1-a, although compared to the 1572 and 335 constructs this induction and the maximal level of activity was significantly
less. Taken together, the data presented in Figure 5 suggest that a putative sterol regulatory element may be located between bp 335 and 219.

**THE EFFECT OF A MUTATED G+C-RICH MOTIF ON BASAL AND NUCLEAR SREBP-1A INDUCED PROMOTER ACTIVITY**

In a previous study we hypothesized that the 5'-flanking region of the Mdr2 gene contains elements that are possibly recognized by SREBPs: an inverted SRE-1 half-site (located at position −193 to −188) and an inverted, G+C-rich motif that is homologous to SRE-344 (position −278 to −269). This assumption, combined with the observation that a putative SRE may be located between bp 335 and 219, prompted us to evaluate whether the G+C-rich sequence at position −269 is involved in the activation of the Mdr2 promoter by nSREBP-1a. However, this region is located in close proximity to an established Sp1 binding site. Since Sp1 sites serve as a critical determinant of promoter activity by activating RNA polymerase II, we reasoned that it is conceivable that possible effects on luciferase activity introduced by mutation of the −269 G+C-rich region may be caused by influencing the binding of Sp1 and not of SREBP. Therefore, we also studied the effect of the mutated Sp1 site (335mtSp1-luc) on Mdr2 promoter activity both in absence or presence of nSREBP-1a. In the absence of co-expressed nSREBP-1a, the introduction of 6 base mutations into the −269 G+C-rich region of the 335 promoter construct (335mtGC-luc) greatly diminished the activity compared to control (335wt-luc) (Figure 6). In the presence of co-expressed nSREBP-1a, we observed a similar, about 6-fold, increase in reporter gene activity for both constructs, although the maximal absolute level obtained for 335mtGC-luc was again significantly less than that for 335wt-luc. The basal activity of 335mtSp1-luc was markedly lowered as compared to control, being in accordance with previous observations, and was comparable to that of 335mtGC-luc. nSREBP-1a overexpression resulted in an approximately 5-fold increase of promoter activity, analogous to 335wt-luc and 335-mtGC-luc (Figure 6). Since the relative increase of activity is equal for both wild type and mutant constructs, these studies indicate that the nSREBP-1a-induced specific increase in Mdr2 promoter activity cannot be attributed to

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**Figure 4. Differential activation of the Mdr2 promoter by the transcription factors nSREBP-1A and nSREBP-2.** HepG2 cells were co-transfected with 2 μg of the 335 bp reporter plasmid and various amounts of pCSA10, pCSA2 or pCMV5. Closed circles, pCSA10; open circles, pCSA2. Normalized data are presented as mean ± standard deviation from triplicate plates. For each amount the activity of the 335 bp reporter plasmid co-transfected with pCMV5 was designated 1.
the G+C-rich region located at position -269. The markedly diminished activity of -335mtGC-luc is likely caused by an effect on Sp1 binding present because identical observations were made for the mutant Sp1 construct.

**DISCUSSION**

The molecular regulatory mechanisms involved in Mdr2 gene expression are presently unknown. The current study provides the first (preliminary) data on the regulation of Mdr2 gene expression by statins, cholesterol synthesis inhibitors, and by SREBPs, a family of transcription factors. We started our studies by investigating whether in cultures of primary rat hepatocytes Mdr2 expression could be induced by statin treatment, analogous to previous observations made in vivo. Indeed, incubation of hepatocytes with statins clearly induced steady-state mRNA levels of Mdr2. In addition, analysis of Srebp gene expression revealed the Srebp-1 isoforms and Srebp-2 are regulated inversely in primary hepatocytes after statin treatment. Similar observations have been made in vivo in livers of hamsters and mice during treatment with lovastatin and a bile salt-binding resin. However, with respect to the downregulation of the Srebp-1 isoforms, Shimomura et al. reported that only the mRNA of Srebp-1c was downregulated whereas Srebp-1a mRNA levels were unaffected. A likely explanation for this discrepancy is that in most cultured cells the predominant Srebp-1 isoform is Srebp-1a, whereas in liver the 1c transcript prevails.

Reporter gene assays with a 1572 bp fragment of the 5’-flanking region of the Mdr2 coding sequence in HepG2 cells demonstrated that all three statins used in this study were able to induce Mdr2 gene transcription. This strongly suggests that the increased levels of Mdr2 mRNA observed after statin-treatment are primarily regulated by enhanced transcriptional activity. Obviously, our experiments do not exclude that post-transcriptional events, such as increased stabilization of mRNAs, are involved as well. Surprisingly, co-transfection studies revealed that only nSREBP-1a, but not nSREBP-2, is capable of activating Mdr2 promoter activity in HepG2 cells (Figure 4). Since it is unlikely that the amounts of nSREBP-1a and -2 proteins vary substantially within the cells, these

*Figure 5. Transcriptional activity of MDR2 promoter deletion constructs. HepG2 cells were co-transfected with 2 µg of reporter plasmid and 1 µg of pCSA10 or pCMV5. Normalized data are presented as mean ± standard deviation from triplicate plates and are representative of multiple independent experiments. Various promoter constructs are indicated at the bottom of the figure. Open bars, pCMV5; black bars, pCSA10; pXP1, promoterless luciferase vector.*
results indicate that Mdr2 may be relatively unresponsive to SREBP-2. Differential effects of nSREBP-1a and nSREBP-2 have also been observed previously\(^4\).\(^8\),\(^4\).\(^9\). Using cells that specifically expressed each nSREBP isoform, Pai et al.\(^4\),\(^8\) reported on the differential activation of various genes by the individual nSREBPs. nSREBP-1a and nSREBP-2 both stimulated transcription of the same genes, but nSREBP-2 preferentially stimulated transcription of genes involved in cholesterol biosynthesis. nSREBP-1c displayed little stimulatory activity in their study. Similar observations were made by Guan et al.\(^4\),\(^9\). They demonstrated that both nSREBP-1a and -2 activated a reporter gene driven by the squalene synthase promoter, but they did so by interacting at different sites. nSREBP-2 bound to more sites than did nSREBP-1a, and it continued to stimulate transcription, even when the SRE-like sites had been destroyed by mutagenesis\(^4\),\(^9\). These authors postulate that the multiple sites of action for SREBP-2 explain that nSREBP-2 is more active than nSREBP-1a.

Alternatively, the unresponsiveness of the Mdr2 promoter to nSREBP-2 may also be the result of lack of a specific co-factor(s), required for SREBP-2 mediated regulation in HepG2 cells. In all promoters for SREBP target genes that have been carefully studied thus far, SREBP dependent regulation requires additional co-regulatory DNA binding factor(s) for efficient expression. In the promoter for the low density lipoprotein (LDL) receptor this co-regulator is Sp1\(^3\),\(^5\), the CCAAT-binding factor/nuclear factor-Y (CBF/NF-Y) interacts with SREBP and is a co-regulatory factor for farnesyl-diphosphate synthase promoter\(^5\),\(^0\), while for efficient sterol-regulated transcription of the HMG CoA synthase promoter both CBF/NF-Y and the cAMP response element-binding protein (CREB) are required\(^5\),\(^1\),\(^5\),\(^2\). In addition, not only the identity of the co-regulatory factor(s) differs from promoter to promoter, recent data demonstrate that the SREBP-1a and -1c isoforms also utilize distinct co-regulatory factors to activate the fatty acid synthase promoter\(^5\),\(^3\).

Transfection studies with Mdr2 promoter-deletion constructs indicated that a putative sterol regulatory element is located between base pair –335 and –219. Based upon its homology with SRE-3, we previously identified a G+C-rich motif located at position –278 to –269 as a potential SRE. Mutation of this sequence element reduced the basal activity

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**Figure 6.** TRANSCRIPTIONAL ACTIVITY OF WILD TYPE AND MUTATED MDR2 PROMOTER CONSTRUCTS. HepG2 cells were co-transfected with 2 µg of either 335wt-luc (control), 335mtGC-luc (mutated G+C-rich region) or 335mtSp1-luc (mutated Sp1 binding site), and 1 µg pCSA10 or pCMV5. Normalized data are presented as mean ± standard deviation from triplicate plates and are representative of multiple independent experiments. Various promoter constructs are indicated at the bottom of the figure. Open bars, pCMV5; black bars, pCSA10; pXP1, promoterless luciferase vector.
of the \textit{Mdr2} promoter. However, its activation in response to co-transfected nSREBP-1a was similar, if not identical, to the wild-type \textit{Mdr2} promoter. The activities of the 335mtGC-luc and 335mtSp1-luc constructs were indistinguishable, both in the presence and absence of nSREBP-1a. Consequently, we conclude that this G+C-rich motif is not involved in the nSREBP-1a induced \textit{Mdr2} expression, but rather is of importance for basal expression, probably by influencing the binding of Sp1. Nonetheless, these data do not reject, but instead are in support of the hypothesis that a hitherto unidentified SRE may be present in the \textit{Mdr2} promoter. Yet, our data do not unequivocally demonstrate that nSREBP-1a directly interacts with the \textit{Mdr2} promoter. Therefore we cannot eliminate the possibility that the SREBP-1a related effects on \textit{Mdr2} expression are mediated (indirectly) via other transcription factor(s). In this respect we would like to mention it has been demonstrated that intracellular cholesterol levels and SREBPs modulate the transcriptional activity of the peroxisome proliferator-activated receptor \textit{γ} (PPAR\textit{γ})\textsuperscript{54,55}.

In conclusion, we have shown that statins induce the steady-state \textit{Mdr2} mRNA levels in rat hepatocytes as a result of enhanced gene transcription. More importantly, we have demonstrated SREBP-1a is able to transcriptionally activate the \textit{Mdr2} gene, either directly or indirectly. Further studies are required to characterize in more detail the molecular mechanisms that control \textit{Mdr2} gene expression.

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