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New Insights into the Regulation of CYP2C9 Gene Expression: The Role of the Transcription Factor GATA-4

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ABSTRACT:

CYP2C9 is an important drug-metabolizing enzyme that metabolizes, e.g., warfarin, antidiabetics, and antiphlogistics. However, the endogenous regulation of this enzyme is largely unknown. In this study, we examined the role of GATA transcription factors in the gene expression of *CYP2C9*. We investigated four putative GATA binding sites within the first 200 base pairs of *CYP2C9* promoter at the positions I: −173/−170, II: −167/−164, III: −118/−115, and IV: −106/−103. Luciferase activity driven by a wild-type *CYP2C9* promoter construct was strongly up-regulated in Huh-7 cells upon cotransfection with expression plasmids for GATA-2 and GATA-4, whereas mutations introduced into GATA binding site III or I and II reduced this induction to a significant extent. Electrophoretic mobility shift assays revealed specific binding of GATA-4 and GATA-6 to the oligonucleotides containing GATA binding sites I and II. Furthermore, the association of GATA-4 with *CYP2C9* promoter was confirmed by chromatin immunoprecipitation assays in HepG2 cells. Taken together, these data strongly suggest an involvement of liver-specific transcription factor GATA-4 in the transcriptional regulation of *CYP2C9*.

CYP2C9 is an important enzyme involved in the metabolism of a large number of different drugs. It is the second most abundant cytochrome P450 enzyme in human liver (Miners and Birkett, 1998), and it is responsible for the transformation of approximately 16% of all used therapeutics including drugs like warfarin, losartan, phenytoin, tolbutamide, and different antiphlogistics (Urquhart et al., 2007). CYP2C9 is polymorphically expressed. The most common allelic variants in whites are *CYP2C9*2* and *CYP2C9*3*, which occur at a frequency of approximately 7 and 11%, respectively. Carriers of these variants show a slower metabolism toward CYP2C9 substrates and a considerably higher risk for adverse drug reactions (Kirchheiner and Brockmoller, 2005). An important example is the occurrence of bleeding complications upon treatment of CYP2C9 slow metabolizers with warfarin (Flockhart et al., 2008).

The CYP2C9 activity varies significantly within wild-type carriers (Yasar et al., 2001; Scordo et al., 2002; Sandberg et al., 2004). Possible reasons for this phenomenon are the inducibility of CYP2C9 by different substrates, interindividual differences in the constitutive CYP2C9 expression (Peyvandi et al., 2004; Kirchheiner and Brockmoller, 2005), as well as polymorphic variations of the regulatory region (Kramer et al., 2008). Thus, it was demonstrated that CYP2C9 expression can be regulated by hepatocyte nuclear factor (HNF)4α and its coregulators peroxisome proliferator-activated receptor-γ coactivator and steroid receptor coactivator 1 via direct repeat 1 promoter elements (Kawashima et al., 2006; Martinez-Jimenez et al., 2006). *CYP2C9* promoter activity is moreover influenced by HNF3γ (Bort et al., 2004) and by a cross-talk between constitutive androstane receptor and pregnane X receptor with HNF4α upon induction by rifampicin (Chen et al., 2005).

In this study, we focus on the zinc finger transcription factor family GATA, which is an important group of transcriptional regulators. The GATA family comprises six different members, GATA-1 to GATA-6, which recognize the consensus sequence (A/T)GATA(A/G). GATA-1, -2, and -3 regulate the expression of genes involved in the development of blood cells (Harigae, 2006; Wu et al., 2007). GATA-4, -5, and -6 are specifically expressed in cardiac tissue (Peterkin et al., 2005) and play a key role in the transcriptional regulation of different genes involved in cardiac development and cardiomyocyte differentiation (Reiter et al., 1999; Crispino et al., 2001). In addition, GATA-4 is also expressed in liver and regulates here the expression of different liver detoxifying enzymes and transporters (Zhu et al., 2004; Kwintkiewicz et al., 2007; Sumi et al., 2007).

In silico analysis of the proximal *CYP2C9* promoter indicates the presence of multiple GATA binding motifs, which prompted us to

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J.M. and J.N. contributed equally to this work.

S.M. and M.I.-S. should be regarded as co-last authors.

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ABBREVIATIONS: HNF, hepatocyte nuclear factor; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; FOG-2, Friend of GATA-2; FOG-2; ds, double-stranded; bp, base pair; PCR, polymerase chain reaction.

study the possible involvement of these factors in the regulation of CYP2C9 expression. In this study, we show by luciferase gene reporter assay, electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) analysis that *CYP2C9* can be regulated by the transcription factor GATA-4. Furthermore, GATA-4-dependent activation of *CYP2C9* is down-regulated by an important coregulator of GATA-4, Friend of GATA-2 (FOG-2).

Materials and Methods

Plasmid Constructs. Fragments of different length of *CYP2C9* promoter were subcloned into the MluI/XhoI cloning sites of pGL3-Basic vector (Promega, Madison, WI) upstream of the luciferase gene (constructs 2C9_−735, 2C9_−421, and 2C9wt_ −331) (Table 1; Fig. 1B). Constructs with destructive mutations at all detected hypothetical GATA binding sites [positions (−173/−170, site I), (−167/−164, site II), (−118/−115, site III), and (−106/−103, site IV)] alone (constructs 2C9_−331_m1, 2C9_−331_m2, 2C9_−331_mut3, and 2C9_−331 _m4) or in combination (site I and site II, construct 2C19_−331_mut1 + 2) were generated using GeneTailor kit (Invitrogen, Carlsbad, CA) (Table 1; Fig. 1B).

The human pCMV-FLAG2-GATA2 and mouse pcDNA1.1-GATA4 expression plasmids were kind gifts from Prof. Gokhan Hotamisliglil (Harvard University, Boston, MA) and Prof. Jeffery Molkentin (Children's Hospital Medical Center, Cincinnati, OH), respectively. The human pcDNA3-FOG-2 wild-type construct as well as human pcDNA3-FOG-2_1-247 (expresses truncated protein missing the GATA-4 binding zinc finger domain) were kind gifts from Prof. Erik Svensson (University of Chicago, Chicago, IL) (Svensson et al., 2000).

Transient Transfections. Huh-7 human hepatoma cells were grown at 37°C in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, and 1% penicillin/streptomycin (Invitrogen). One day before transient transfection, 2×10^5 Huh-7 cells were plated into 12-well plates. Two micrograms of the different pGL3-Basic expression vectors carrying *CYP2C9* promoter fragments of different lengths as well as the constructs mutated at the different GATA sites were cotransfected with 0.5 μg of mouse pcDNA1.1-GATA4, human pCMV-FLAG2-GATA2, or 0.5 μg of pcDNA3.1 empty vector (negative control). To investigate possible interactions between GATA and FOG proteins, GATA-2 or GATA-4 constructs were cotransfected with different amounts of a wild-type (pcDNA3-FOG2) or 1 μg of a mutated FOG construct (pcDNA3_FOG-2_1-247). Transient transfections were performed by using Lipofectamine 2000 (Invitrogen) according to manufacturer's recommendations. Cells were harvested and analyzed for luciferase activity at 24 (GATA cotransfections; Fig. 2, A and B) or 48 h (cotransfections with GATA and FOG; Fig. 5) after cotransfection. In each transfection mixture, 2 ng of the plasmid harboring *Renilla* luciferase gene (pRL SV40; Promega) was included as an internal control for the transfection efficiency. Luciferase activity is therefore expressed as a ratio of firefly luciferase activity (in arbitrary units) to the corresponding activity of the *Renilla* luciferase. All experiments were performed in triplicates and repeated three times.

Electrophoretic Mobility Shift Assay. Nuclear protein extracts from Huh-7 cells were prepared according to the protocols of Dignam et al. (1983) and Nakabayashi et al. (1991) with slight modifications. Eight different double-stranded (ds) oligonucleotides comprising 50 base pairs (bp) of the *CYP2C9* promoter were generated by annealing sense and antisense oligonucleotides (Table 1). The oligonucleotides were carrying the hypothetical GATA binding sites in wild-type or in mutated form. Oligonucleotide labeling was carried out using ^{32}P (PerkinElmer Life and Analytical Sciences, Waltham, MA) and T4 DNA polynucleotide kinase system (Invitrogen) in a final reaction volume of 25 μl. The mixes were incubated at 37°C for 15 min. The reaction was stopped with 5 μl of Na_2EDTA (0.2 M). For binding reactions, 4% glycerol, 8 mM HEPES (Sigma-Aldrich, St. Louis, MO) (pH-7.9), 0.6 mM $MgCl_2$, 50 mM NaCl, 1 μg of poly(dI-dC), 12.8 fmol of ^{32}P-labeled ds oligonucleotide probe (approximately 20,000 cpm), and 4 μg of nuclear protein were mixed together in a total end volume of 25 μl. After 15 min of preincubation at 37°C, the different labeled ds oligonucleotides were added to the mixes and the complete mixture was again incubated at 37°C for

TABLE 1

Oligonucleotides used for cloning, EMSA, and ChIP experiments

Primers for cloning were designed based on the *CYP2C9* sequence (GenBank accession number NT_030059). Fragments for reporter constructs were PCR amplified from a CYP2C9 construct encompassing the first 1.8 kb of *CYP2C9* 5-flanking region [a gift from Dr. Mia Sandberg Lundblad (Lundbeck Inc., Copenhagen, Denmark), construct slightly modified]. Nonsense mutations in GATA sites I to IV were introduced into construct 2C9_−331_wt, resulting in the plasmids 2C9_−331_m1, 2C9_−331_m2, 2C9_−331_m3, 2C9_−331_m4, and 2C9_−331_m1+2, respectively. 2C9wt1+2 and 2C9wt3+4: EMSA oligonucleotides containing wild-type GATA binding site I and II or GATA binding site III and IV. 2C9mut1, 2C9m2, 2C9m3, 2C9m4, 2C9m1+2: oligonucleotides with destructive mutations in GATA binding site I, II, III, IV or I and II together. GATA binding sites are underlined. Mutations that destroy any GATA motifs are highlighted in bold. ChIP Primer set was used in ChIP analysis leading to a PCR product of 225 bp of length. The PCR product includes all four possible GATA binding sites.

Primer Name	Sequence
Cloning primers	Wild type
CYP2C9_−735 fw	5′-CAGACGCGTGCTATGAGCTGTGTGGC ACC
CYP2C9_−421 fw	5′-CAGACGCGTAATATACAAGG CATAGAATAT GG
CYP2C9_−331 fw	5′-CAGACGCGTCA GATTATTTACTTCAGTGCT
CYP2C9 rev	5′-CAGCTCGAGTGAAGCCTTCTCTTCTTGTTAA
	Mutant
CYP2C9_mut1_fw	5′-CAAAGGACATTTTATTTTTTTTTGTATCAGTG
CYP2C9_mut1_rev	5′-AAA AATAAAATGTCCTTTGGTCTTGTTCT
CYP2C9_mut2_fw	5′-GACATTTTATTTTTATCTGTTTTAGTGGGTCAA
CYP2C9_mut2_rev	5′-ACAGATAAAAATAAAATGTCCTTTGGTCTT
CYP2C9_mut3_fw	5′-ATATAGTGGACCTAGGTTTTTGGTCAATTT
CYP2C9_mut3_rev	5′-ACCTAGGTCCACTATATGCTCCTTCTGAAA
CYP2C9_mut4_fw	5′-GGTGATTGGTCAATTAAAACATCAAAGAGG
CYP2C9_mut4_rev	5′-AATTGACCAATCACCTAGGTCCACTATATG
CYP2C9_mut1+2_fw	5′-CAAAGGACATTTTATTTTTTTTGTTTTTAGTG
CYP2C9_mut1+2_rev	5′-AAA AATAAAATGTCCTTTGGTCTTGTTCT
EMSA oligonucleotides (forward)	
2C19wt1+2	5′-ACCAAAGGACATTTTATTTTTATCTGTATCAGTGGGTCAAAGTCCTTTCA
2C9wt3+4	5′-ATATAGTGGACCTAGGTGATTGGTCAATTTATCCATCAAAGAGGCACACA
2C9mut1	5′-ACCAAAGGACATTTTATTTTTTTTTGTATCAGTGGGTCAAAGTCCTTTC
2C9mut2	5′-ACCAAAGGACATTTTATTTTTATCTGTTTTAGTGGGTCAAAGTCCTTTCA
2C9mut3	5′-ATATAGTGGACCTAGGTTTTTGGTCAATTTATCCATCAAAGAGGCACACAC
2C9mut4	5′-ATATAGTGGACCTAGGTGATTGGTCA ATTAAAACATCAAAGAGGCACACAC
2C9mut1+2	5′-ACCA AAGGACATTT TATTTTTTTT TGTTTTAGTG GGTCAAAGTC CTTTCA
Oligonucleotides used for ChIP	
Primer set	5′-CCAA CCAAGTACAG TGAAACT
	5′-TTAAGACAACCATGAGCTTGCACT

fw, forward; rev, reverse primers.

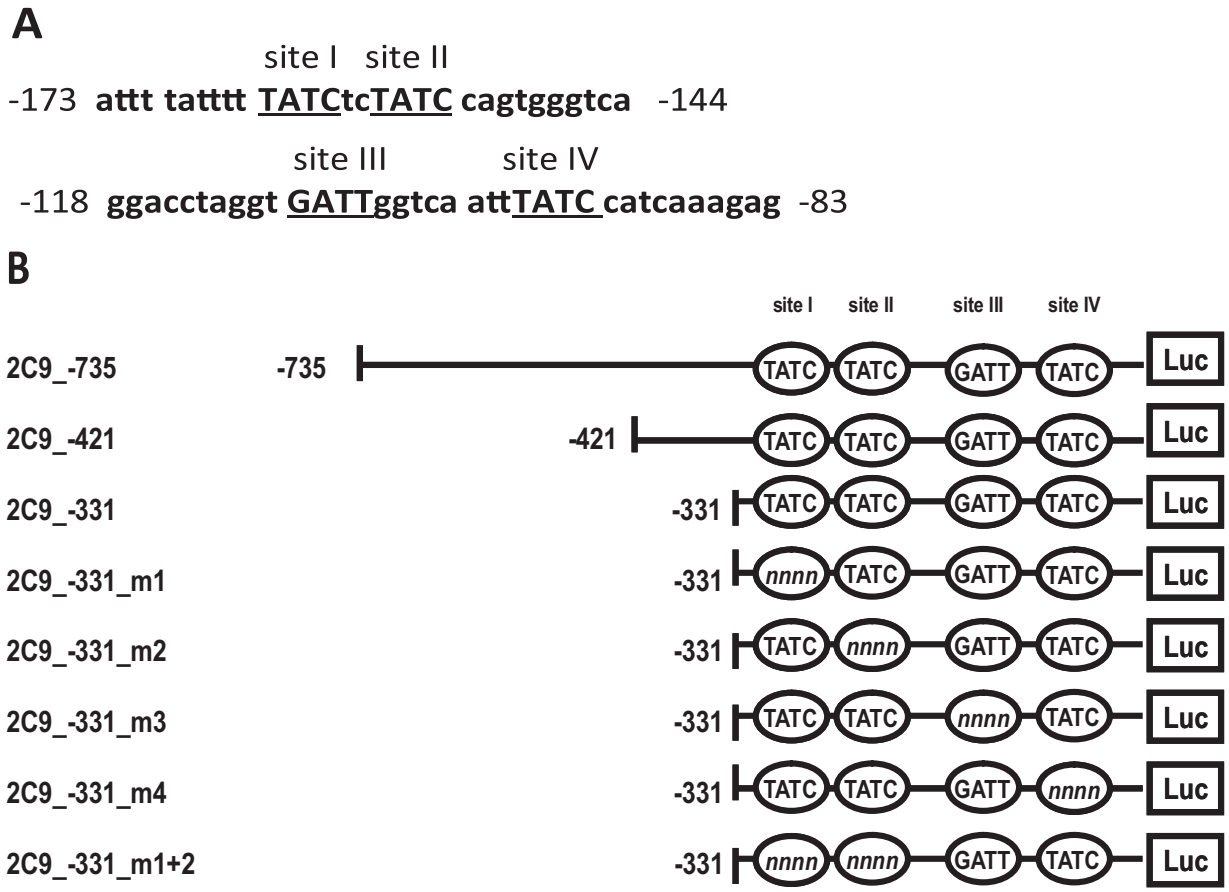


FIG. 1. Detected GATA motifs in *CYP2C9* promoter and design of luciferase reporter constructs encompassing four possible GATA binding sites. A, the DNA sequence surrounding four possible GATA binding sites of *CYP2C9* gene promoter. The A of the first codon ATG is numbered as +1. GATA motifs I to IV are shown in upper case and highlighted in bold. B, schematic representation of 5′-truncated *CYP2C9* promoter fragments cloned upstream to luciferase reporter gene. Numbers refer to fragment length. Mutated GATA binding sites are indicated in italic.

15 min. For competition experiments, 50- and 100-fold excess of the respective unlabeled ds oligonucleotide was added to the probe before the addition of ^{32}P-labeled ds oligonucleotides. Supershift experiments were carried out by adding 6 μg of GATA-2, -3, -4, or GATA-6 antibody (sc-1235X, sc-268X, sc-1237X, and sc-9055X; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) to the respective binding reaction before the addition of labeled oligonucleotides, and samples were incubated on ice for 45 min. Finally, 5 μl of loading buffer were added to each sample. Protein-bound and unbound DNA were resolved on a 4% none denaturing polyacrylamide gel. Dried gels were subjected to autoradiography by using phosphorimager (Fujifilm BAS-1800; Fujifilm, Tokyo, Japan).

Chromatin Immunoprecipitation. ChIP assay kit (Millipore Corporation, Billerica, MA) was used according to the manufacturer's protocol. HepG2 cells were grown at 37°C in small dishes in minimal essential medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (Invitrogen), as well as 1% sodium pyruvate and 1% none essential amino acids. DNA-bound proteins were cross-linked to chromatin by adding 1% formaldehyde and incubated at 37°C. Harvested and lysed cells were sonicated to shear the DNA to fragments of approximately 500 bp. DNA was precleared with salmon sperm DNA/protein A agarose slurry and afterward incubated overnight with 2 μg of GATA-4 antibody (sc-1237X; Santa Cruz Biotechnology, Inc.) or control IgG (rabbit normal IgG, sc-2027; Santa Cruz Biotechnology, Inc.) at 4°C. The antibody/histone complexes were collected with salmon sperm DNA/protein A agarose slurry rotating the mixes for 2 h at 4°C. The immunoprecipitate was pelleted and washed with low and high salt containing buffers and with TE buffer (10 mM Tris-HCl and 1 mM EDTA). The histone complex was eluted from the antibody, and histone-DNA cross-links were reversed at 65°C overnight. After treatment with proteinase K (QIAGEN, Valencia, CA) and purification using QIAamp DNA Mini Kit (QIAGEN), samples, including the input/sonicated DNA sample (positive control), were subjected to touch-down polymerase chain reaction (PCR) using a primer pair, which generates a 225-bp fragment including all four GATA sites of interest.

Statistical Analysis. Statistical differences in reporter gene activity among *CYP2C9* promoter constructs were determined by one-way analysis of variance followed by a Turkey's post hoc test using GraphPad Prism version 5.00 for Windows (GraphPad Software Inc., San Diego, CA). A *p*-value threshold of <0.05 was considered as statistically significant in all analyses.

Results

GATA Factors Activate the *CYP2C9* Promoter. Analysis of the *CYP2C9* promoter revealed four putative GATA binding sites at the positions (−173/−170), (−167/−164), (−118/−115), and (−106/−103) (Fig. 1A). To investigate the influence of GATA transcription factors on CYP2C9 expression, 5′-deletion fragments of *CYP2C9* promoter were cloned upstream to firefly luciferase gene into the pGL3-Basic vector (Fig. 1B) and transiently transfected into Huh-7 cells together with GATA-2, -4, and -6 expression vectors. The strongest effect was observed for GATA-2 and -4 transcription factors (GATA-6 results are not shown). As demonstrated in Fig. 2, A and B, both factors strongly up-regulate 331-bp long 5′-deletion construct of the wild-type *CYP2C9* promoter. Identical up-regulation was also observed for two other 5′-deletion constructs, 435- and 735-bp long (results not shown), which would suggest localization of the potential GATA-responsive site(s) in the proximal CYP2C9 promoter region.

GATA-Dependent Up-Regulation of *CYP2C9* Promoter Is Driven by GATA Binding Site I+II. To investigate to which extent the in silico-detected putative GATA binding sites are important for *CYP2C9* regulation, the 331-bp-long promoter constructs containing

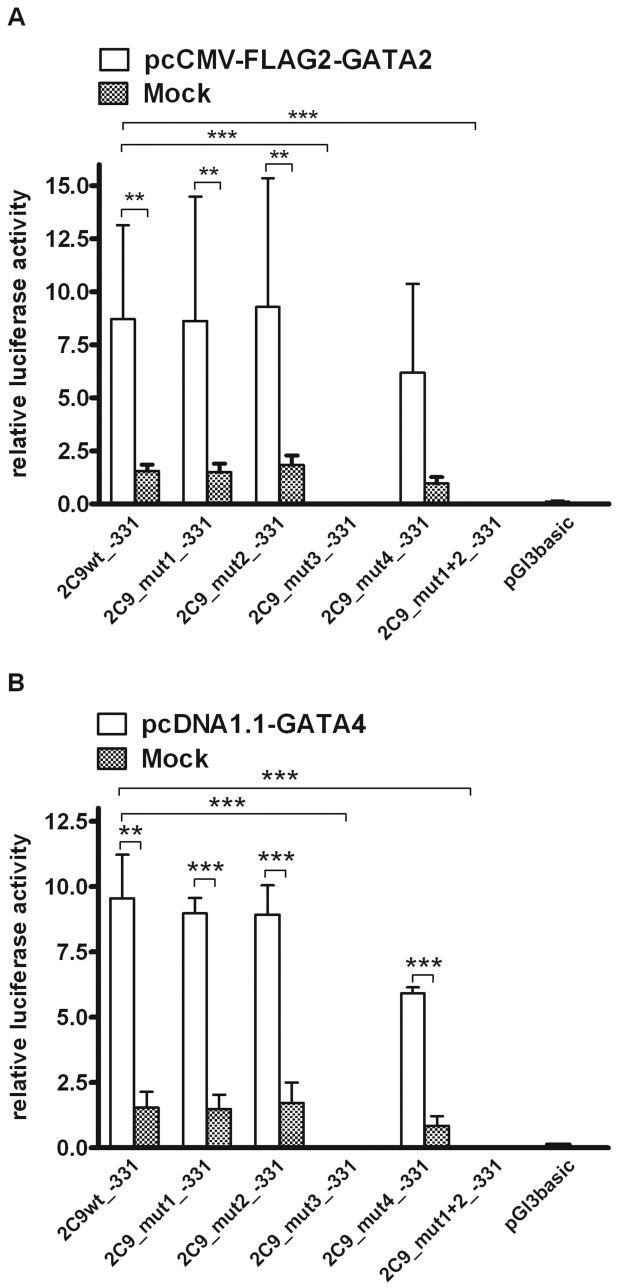


FIG. 2. GATA-2 and GATA-4 up-regulate *CYP2C9* promoter in gene reporter assay. Relative luciferase activities of *CYP2C9* promoter fragments subcloned into pGL3-Basic vector (Fig. 1B) and of pGL3-Basic control vector (negative control) after cotransfection with pGATA2-CMV-FLAG2 (GATA-2) or pcDNA3.1 empty vector (mock) (A) and pGATA4-pcDNA1.1 (GATA-4) or pcDNA3.1 empty vector (mock) (B) in Huh-7 cells. All deletion constructs were in general highly up-regulated upon cotransfection with GATA-2 or GATA-4. **, $p < 0.01$ for 2C9wt_−331, 2C9_mut1_−331, 2C9_mut2_−331 (A), and 2C9wt_−331 (B) co-transfected with pcDNA3.1 against cotransfection with GATA2 (A) or GATA-4 (B). ***, $p < 0.001$ for 2C9_mut1_−331, 2C9_mut2_−331, and 2C9_mut4_−331 (B) cotransfected with pcDNA3.1 against cotransfection with GATA-4 (B). ***, $p < 0.001$ for 2C9_−331_mut1+2 or 2C9_−331_mut3 versus 2C9_−331_wt (A), and ***, $p < 0.001$ for 2C9_−331_mut1+2 or 2C9_−331_mut3 (B) versus 2C9_−331_wt. Data are presented as mean values ± S.D. of three independent experiments. Each experiment was performed in triplicate.

wild-type or mutant GATA binding motifs (Fig. 1B) were cotransfected with GATA-2 or GATA-4 expression vectors into Huh-7 cells. Artificial disruption of GATA sites I or II did not affect GATA-2- and GATA-4-dependent up-regulation of luciferase activity (Fig. 2, A and B). By contrast, disruption of the putative GATA site III caused a drastic drop of GATA-2 and GATA-4 effects down to pGL3-Basic level. We were surprised to find that an equally strong loss in luciferase activity was observed for the *CYP2C9* promoter construct carrying combined GATA I+II mutations (Fig. 2, A and B).

The luciferase activity pattern in mock-transfected cells always mimicked (although at a significantly lower level) the activity pattern observed with GATA-2 and GATA-4 overexpression. This phenomenon might be explained by the endogenous expression of GATA factors in the Huh-7 cell line (data not shown).

GATA-4 Binds to Two Different GATA Binding Sites in Proximal *CYP2C9* Promoter. Next, we investigated whether the putative GATA binding sites interact with GATA transcription factor(s) using electrophoretic mobility shift assay. Different wild-type and mutant ^{32}P-labeled oligonucleotides comprising different combinations of wild-type or disrupted forms of GATA binding sites (Fig. 3, A and B) were incubated with nuclear extracts from Huh-7 cells. Protein-DNA complexes were formed with both 2C9wt1+2 (comprises GATA binding sites I and II) and 2C9wt3+4 (comprises GATA binding sites III and IV) oligonucleotides (Fig. 3, A and B, lane 1). Mutation of site I and II (Fig. 3A, lanes 2 and 3) did not affect the binding, whereas the oligonucleotide containing both mutations (mut1+2; Fig. 3A, lane 4) is completely devoid of any binding activity. Likewise, disruption of the GATA site III (but not site IV) led to a significant loss of protein binding activity (Fig. 3B, lanes 2 and 3). Taken together, these results suggest binding of the nuclear factor to GATA site I+II as well as to the GATA site III, which is consistent with the gene reporter data.

Next, we attempted to identify the protein that binds to these sites using antibodies against different members of the GATA family (GATA-2, GATA-3, GATA-4, and GATA-6). Among all antibodies used, anti-GATA-4 and anti-GATA-6 were able to successfully supershift the formation of the protein/oligonucleotide complex observed with oligonucleotides 2C9wt1 and 2C9wt1+2 (Fig. 3A, lanes 10 and 11). Neither of these antibodies was found to shift the 2C9wt3 complex (Fig. 3B). These findings suggest that GATA-4 and GATA-6 can interact with CYP2C9 promoter.

GATA-4 Is Associated with *CYP2C9* Promoter. To investigate whether GATA-4 is indeed endogenously associated with the hypothetical GATA binding sites within *CYP2C9* promoter, we performed a chromatin immunoprecipitation assay by using genomic DNA from HepG2 cells and antibodies against GATA-4. Promoter fragments were amplified by PCR using a primer set encompassing the putative GATA binding sites (Table 1). As shown on Fig. 4 (lane 1), the primer set was able to generate a PCR product that matches exactly with the predicted promoter fragment containing the GATA sites in question. No PCR product was observed with immune complexes formed by control IgGs, demonstrating the specificity of the assay (Fig. 4, lane 2). This finding confirms that GATA-4 is associated with *CYP2C9* promoter even in intact cells.

FOG-2 Counters the GATA-4-Dependent *CYP2C9* Promoter Activation. GATA proteins are known to be often coregulated by the transcription factor family FOG, including the members FOG-1 and FOG-2. In particular, FOG-2 interacts with GATA-4 and influences a GATA-4-dependent transcriptional regulation of different target genes. Several studies showed that a disruption of FOG-2/GATA-4 interaction can lead to pathological forms of heart development during organogenesis (Tevosian et al., 1999; Svensson et al., 2000; Crispino et al., 2001). To investigate whether FOG-2 is also involved in GATA-4-dependent *CYP2C9* promoter regulation, we used the luciferase gene reporter approach cotransfecting GATA-4 and FOG-2 expression plasmids with the *CYP2C9* promoter constructs used in previous experiments. Figure 5 shows the cotransfection results for promoter construct 2C19_−331_wt, GATA-4, and FOG-2. Whereas

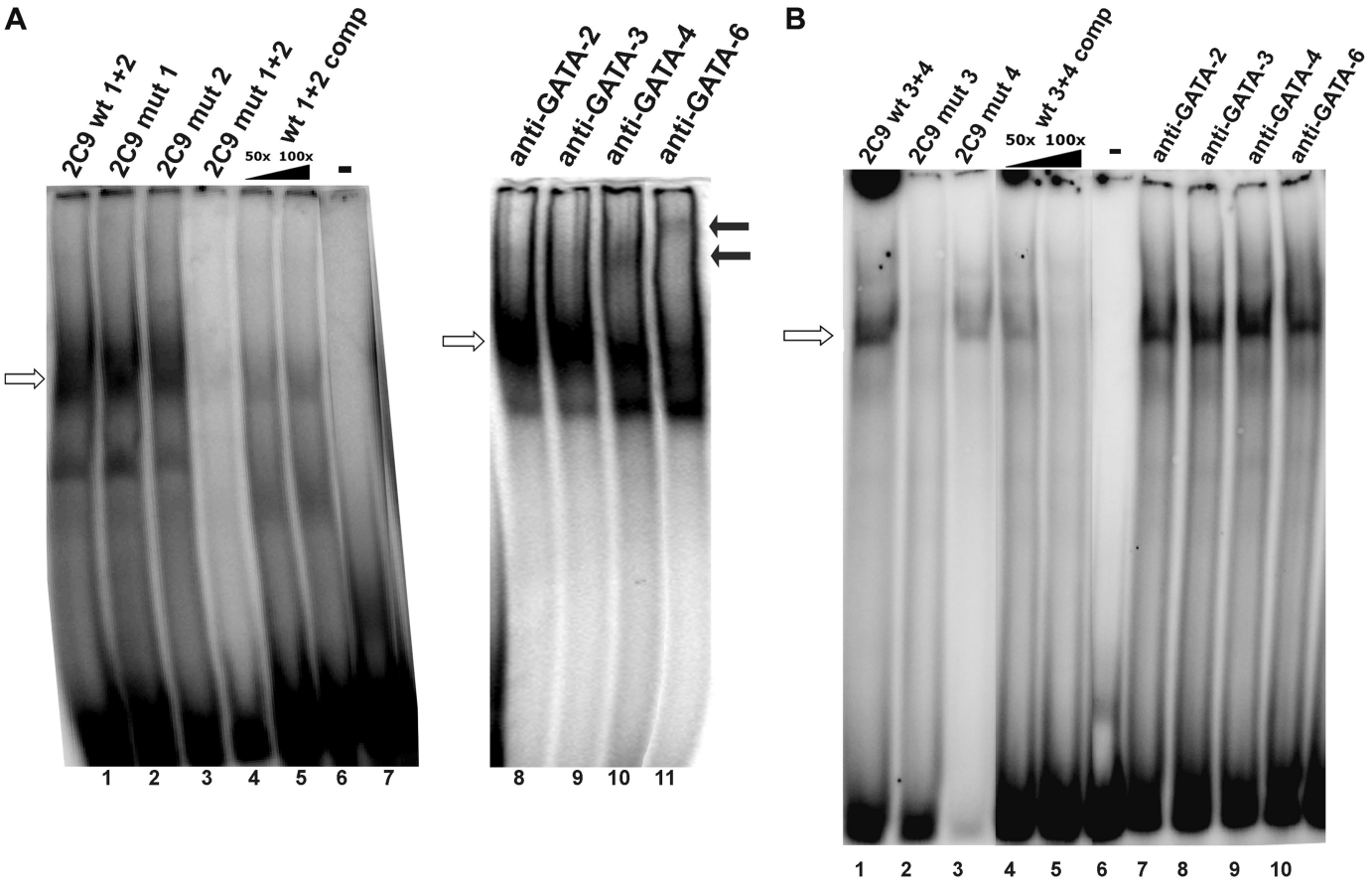


FIG. 3. GATA-4 binds to the oligonucleotides containing GATA binding motif I and II in EMSA analysis using nuclear extracts from Huh-7 cells. EMSA was performed using double-stranded oligonucleotides (Table 1) comprising GATA binding site I and II (A) or GATA binding site III and IV (B), respectively, in wild-type (2C9 wt 1+2 or 2C9 wt 3+4) or mutated (2C9 mut 1, 2C9 mut 2, 2C9 mut 1+2, 2C9 mut3, 2C9 mut 4) forms. Nuclear extracts were prepared from Huh-7 cells. The binding complexes are indicated by white arrows. Supershift experiments were performed by using antibodies against GATA-2, -3, -4, and -6. A successful competition was observed with antibodies against GATA-4 and GATA-6 using oligonucleotide 2C19 wt 1+2 (Fig. 3A, lanes 10 and 11, indicated by black arrows). comp, competition reactions with cold 2C9 wt 1+2 (A) and 2C9 wt 3+4 (B). Additional competition controls were performed by using cold mutant oligonucleotides in combination with labeled wild-types oligonucleotides (results not shown).

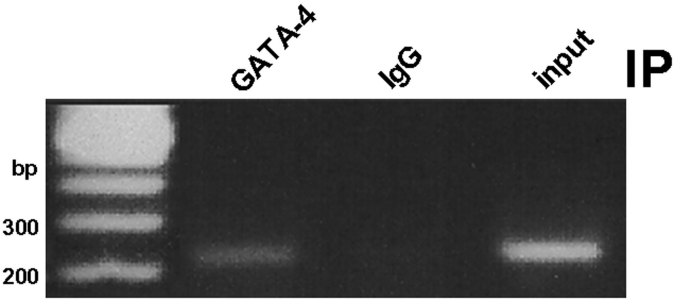


FIG. 4. GATA-4 associates with *CYP2C9* promoter in HepG2 cells. GATA-4 binding to *CYP2C9* promoter was analyzed by using ChIP assay. Immune complexes precipitated with GATA-4 antibody and control IgG antibody were PCR amplified using a primer set encompassing a fragment around all four putative GATA binding sites. Successful amplification was seen in the agarose gel electrophoresis after immunoprecipitation with GATA-4 antibody. Additional negative control was performed with a primer set generating an amplicon not containing any GATA site (results not shown). Input is a positive PCR control, i.e., sonicated DNA (starting material before immunoprecipitation).

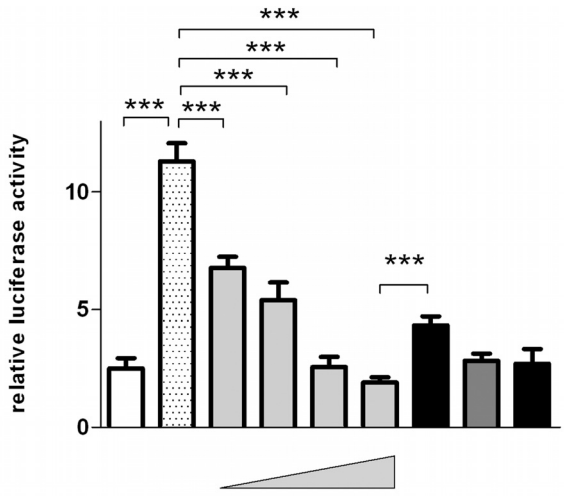
the cotransfection with only GATA-4 led to an expected up-regulation of *CYP2C9* promoter (Fig. 5, bar 2), the addition of FOG-2 attenuated this effect. The extent of inhibition correlated with the amount of transfected FOG-2 plasmid (Fig. 5, bars 3–6). In line with this result, the inhibitory effect was clearly reduced when cotransfecting the mutant construct pcDNA3-FOG-2_1-247 that lacks the fragment coding for the GATA-binding zinc finger domain (Fig. 5, bar 7). These data strengthen the hypothesis that *CYP2C9* is regulated by the GATA-4 transcription factor.

Discussion

For the first time, we provide experimental evidence that GATA transcription factors are involved in the regulation of CYP2C9 expression. This assumption is supported by the following facts: 1) GATA-2 and -4 significantly increase the activity of CYP2C9 promoter fragments containing potential GATA binding sites; 2) FOG-2, a known regulator of GATA proteins, was found to modulate these effects; and 3) physical interaction of GATA factors (GATA-4 in particular) with the promoter was shown by ChIP and EMSA experiments, indicating that such binding may occur both in vivo and in vitro.

Which of the GATA family transcription factors are actually important for CYP2C9 regulation, and which of the four potential GATA *cis*-elements in the promoter are their immediate targets? Whereas the comprehensive answers to these questions are still not available, our data would support the following hypotheses:

1. GATA-2, -4, and -6 displayed CYP2C9 promoter-regulating activity in various experimental approaches shown in this study. This result is not surprising provided the high degree of conservation between the DNA binding domains of different family members and



FOG-2 wt			0.05	0.1	0.5	1		1	
FOG-2_1-247							1		1
GATA-4		0.5	0.5	0.5	0.5	0.5	0.5		
MOCK	0.55	0.05						0.5	0.5

FIG. 5. FOG-2 attenuates GATA-4 effects on *CYP2C9* promoter. Relative luciferase activities of promoter construct 2C9_−331_wt cotransfected with 0.5 μg of GATA-4 and varying amounts of wild-type FOG-2 or FOG-2_1-247 in Huh-7 cells. pcDNA3.1 empty vector (mock) was used as a negative control and to adjust the total amount of transfected plasmid to a minimal level of 0.55 μg or a maximum of 1.5 μg. ***, $p < 0.001$. Data are presented as mean values ± S.E.M. of three independent experiments.

their ability to bind to the conserved GATA response element. Therefore, it can be speculated that the selection of CYP2C9-specific GATA factor should be resolved by the tissue-specific expression of the particular GATA factor. It has previously been shown that some liver-expressed cytochromes P450 and drug transporters are regulated by GATA-4. These include CYP19 (Cai et al., 2007), epoxide hydroxylase (Zhu et al., 2004), and the ATP-binding cassette transporters ABCG5 and ABCG8 (Sumi et al., 2007). These data suggest that GATA-4, which has previously been associated mainly with the regulation of genes involved in heart development, also seems to be important for the expression of genes implicated in the drug metabolism or drug transport in the liver. Our findings are further supported by the fact that liver cells predominantly express only two members of GATA family, GATA-4 and to a much lesser extent GATA-6, two important regulatory factors of liver-specific gene expression (Molkentin, 2000).

2. To answer the question of which of the potential GATA binding sites is actually more involved in the interaction with GATA factors, a comparison of all results obtained for both putative double binding sites is necessary. The ChIP assay gives ambiguous information because the minimal length of the GATA site carrying promoter fragment, which is a target of PCR amplification, is approximately 200 bp and it comprises all four predicted GATA binding elements.

The gene reporter assay indicates site I+II and III as binding candidates. Both sites form specific complexes in EMSA; however, only the site I+II complex was supershifted by GATA-4 and -6. In addition, this sequence is strongly conserved in the proximal promoter of the closely related *CYP2C19*. Therefore, this site can be suggested as a most likely candidate for the interaction with GATA factor(s).

CYP2C9 has also been shown to be regulated by other transcription factors including, e.g., HNF4α and HNF3γ (Bort et al., 2004; Kawashima et al., 2006). Binding sites for these transcription factors are located in direct neighborhood of the newly detected GATA sites within the *CYP2C9* promoter. It remains to be investigated to which extent GATA factors are able to interact with these regulatory proteins.

Transcriptional regulation of CYP2C9 by the GATA transcription factor family might be partly responsible for interindividual differences in CYP2C9 activity seen in wild-type carriers of CYP2C9. Further understanding of this variation is of importance because CYP2C9, a principal-metabolizing enzyme of coumarins together with VKORC1, is strongly involved in the development of drug side effects seen in patients.

In addition to hepatocytes, GATA-mediated regulation of CYP2C9 also might be of physiological relevance in other tissues, in particular in endothelial cells. CYP2C9 is expressed in these cells where it metabolizes arachidonic acid into epoxyeicosatrienoic acids with a concomitant generation of reactive oxygen species (Chehal and Granville, 2006). Both factors are important vasoreactive regulators and are also involved in the pathogenesis of cardiovascular diseases. GATA-2 is the most abundantly expressed GATA factor in endothelial cells (Lee et al., 1991), and it is the key regulator of endothelial-specific genes (Lugus et al., 2007). Further exploration of the GATA-2 involvement in the regulation of CYP2C9 in the cardiovascular system will certainly shed more light on the role of CYP2C9 in vascular biology and cardiovascular diseases.

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