Preparation of nuclear extract and immunoblot analysis of HNF1α. Nuclear extract was prepared from HK-2 and HuH-7 cells transfected with the HNF1α expression plasmid or empty plasmid using NE-PER Nuclear and Cytoplasmic extraction reagents (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's protocols. The protein concentration was determined using Bradford protein assay reagent (Bio-Rad Laboratories, Hercules, CA) with γ-globulin as a standard. The nuclear extract (40 μg) was separated by 7.5% SDS-PAGE and transferred to an Immobilon-P transfer membrane (Millipore). The membranes were probed with goat anti-human HNF1α or rabbit anti-human GAPDH antibodies followed by fluorescent dye-conjugated second antibodies. The membranes were then scanned using the Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE).

Statistical analyses. For DNA methylation status, the statistical significance was evaluated by the Mann-Whitney *U*-test or Fisher's exact test using the web-based tool QUMA. For mRNA expression, statistical significance was determined using an unpaired, two-tailed Student's *t* test or one-way analysis of variance followed by Dunnett's test. When the *p* value was less than 0.05, the differences were considered to be statistically significant.

Results

UGT1A1 mRNA expression in human liver and kidney. UGT1A1 mRNA expression in human liver and kidney was determined by real-time RT-PCR. As shown in Fig. 1, UGT1A1 mRNA was detected in the liver, but was negligible in the kidney. The results supported previous studies (Nakamura et al., 2008; Ohno and Nakajin, 2009) that reported the repressed expression of UGT1A1 in the human kidney.

DNA methylation status of the *UGT1A1* promoter region in human liver and kidney. Genomic DNA extracted from the liver and kidney was treated with bisulfite, and the promoter region of UGT1A1 spanning -113 to +111 was amplified by PCR. The PCR product was subcloned into a vector, and 14 clones from each sample were sequenced. The DNA methylation status of the CpG dinucleotides at -85, -54, -12, +12, +36, and +40 of the *UGT1A1* gene is shown in Fig. 2. In the liver, 31 out of 84 CpG sites (37%) were methylated, whereas in the kidney, 70 out of 84 CpGs (83%) were methylated ($p = 0.07$, Mann-Whitney *U*-test). Notably, the methylated CpG sites were biased in five clones in the liver. We surmised that these clones might be from hepatic nonparenchymal cells. Hence, we investigated the DNA methylation status of the *UGT1A1* promoter in human hepatocytes and found that the methylated status was only 24% (20 out of 84 CpG sites). In particular, nucleotide positions -85, -54, and -12 were unmethylated in all hepatocyte clones, but were hypermethylated in the kidney ($p < 0.001$, $p < 0.01$, and $p < 0.0001$, respectively, Fisher's exact test). Thus, the DNA methylation status of the UGT1A1 promoter region is different in the liver and kidney.

Histone H3 acetylation status and recruitment of HNF1α to the *UGT1A1* promoter region. DNA methylation induces chromatin condensation by recruiting chromatin-remodeling factors

such as methyl-CpG-binding protein and histone deacetylase, thus limiting the access of transcription factors (Bird and Wolffe, 1999). We performed ChIP assays to determine the extent of histone H3 acetylation at the *UGT1A1* promoter in the liver and kidney. In addition, the extent of the recruitment of HNF1α to the *UGT1A1* promoter in the liver and kidney was also determined because it has been demonstrated that HNF1α regulates UGT1A1 expression (Bernard et al., 1999). As shown in Fig. 3A, acetylated histone H3 was enriched at the *UGT1A1* promoter in the liver, but not in the kidney. In addition, it was demonstrated that HNF1α was highly recruited to the *UGT1A1* promoter in the liver, but not in the kidney (Fig. 3B). Western blot analysis demonstrated that HNF1α is expressed in kidney and liver equally (Fig. 3C). These results suggest that the DNA hypermethylation in the kidney could be linked to abolished histone H3 acetylation and HNF1α binding.

Effects of the inhibition of DNA methylation and histone deacetylation and the transfection of exogenous HNF1α on UGT1A1 expression. To investigate the significance of the DNA methylation at the promoter region in the repression of UGT1A1 expression, we performed a series of experiments using cell lines. We selected two cell lines, the human kidney-derived HK-2 line and liver-derived HuH-7 cells. We found that the *UGT1A1* promoter region was hypermethylated (98%) in HK-2 cells but was moderately methylated (47%) in HuH-7 cells ($p < 0.0001$, Fig. 4A). UGT1A1 mRNA was marginally expressed in HK-2 cells but was substantially expressed in HuH-7 cells (~4800 fold difference) (Fig. 4B), suggesting that DNA methylation negatively regulates UGT1A1 expression in HK-2 cells. To investigate whether the inhibition of DNA methylation could induce UGT1A1 expression, the cells were treated with 5-Aza-dC, an inhibitor of DNA methylation. Although this treatment increased UGT1A1 mRNA in both cell lines, the induction was higher in HK-2 cells (~400 fold at maximum) than in HuH-7 cells (~6 fold at maximum) (Fig. 4B). We confirmed that 5-Aza-dC

treatment efficiently decreased the methylation status in HK-2 to 33% ($p < 0.001$) and in HuH-7 cells to 7% ($p < 0.001$) (Fig. 4C).

The UGT1A1 mRNA level in HK-2 cells treated with 0.1 μM 5-Aza-dC was still low in comparison to that in HuH-7 cells. We suspected that HNF1α might be lacking in HK-2 cells, thus causing the lower UGT1A1 levels. Western blot analysis demonstrated that HNF1α is expressed at very low levels in HK-2 cells (Fig. 4D). To investigate the significance of the DNA methylation status in the suppression of UGT1A1 expression, we sought to exogenously express HNF1αin HK-2 cells. The HNF1α protein level was dramatically increased by the transfection of the HNF1α expression plasmid into HK-2 cells (Fig. 4D), but UGT1A1 mRNA expression was not increased (Fig. 4E). These results suggested that DNA methylation inhibits the binding of HNF1$\alpha$to the promoter of UGT1A1. However, under 5-Aza-dC treatment, the overexpression of HNF1α resulted in a significant increase of UGT1A1 mRNA expression (4.3 fold) in HK-2 cells. This phenomenon was not observed in HuH-7 cells, implying that endogenous HNF1αexpression levels might be sufficient for UGT1A1 in HuH-7 cells (Fig. 4D).

Finally, we investigated whether histone deacetylation is also involved in the repression of UGT1A1 expression. When the HK-2 and HuH-7 cells were treated with TSA, an inhibitor of histone deacetylation, UGT1A1 mRNA expression was unchanged (Fig. 4E). However, TSA treatment facilitated (by 1.7 fold) the increase of UGT1A1 mRNA by 5-Aza-dC treatment in HK-2 cells in the presence of exogenously expressed HNF1α. This result was not observed in HuH-7 cells. Collectively, these results suggest that DNA methylation status, and to a lesser extent histone deacetylation status, are critical determinants of UGT1A1 expression.

Discussion

Human UGT1A1 is predominantly expressed in the liver and the intestine, but not in the kidney. Previous studies demonstrated that HNF1α and HNF1β are involved in the constitutive (Bernard et al., 1999) and inducible expression of UGT1A1 (Sugatani et al., 2008) by binding to a site approximately 30 bp upstream of the TATA box. The expression of HNF1α and HNF1β is not confined to the liver, as these genes are expressed in various tissues including the kidney, intestine, stomach, and pancreas (Harries et al., 2006). Therefore, the reason for the repressed expression of UGT1A1 in the kidney remained to be clarified. To uncover the underlying mechanism, we conducted studies focusing on epigenetic regulation. HNF1α and HNF1β form homodimers or heterodimers, and equally *trans*-activate the *UGT1A1* gene (Bernard et al., 1999). Therefore, HNF1α was studied as the representative *UGT1A1* activator.

We found that the CpG island at the promoter region of the *UGT1A1* gene in the kidney was hypermethylated, whereas it was hypomethylated in the liver (Fig. 2). Upon DNA methylation, gene silencing occurs by two mechanisms: 1) the methyl group physically interrupts the binding of transcription factors to their recognition sequences, and 2) methyl-CpG-binding proteins bind to the methylated DNA and recruit corepressor molecules including histone deacetylase to induce chromatin structure condensation (Shiota, 2004). Previously, it was demonstrated by gel shift assay that the methylated CpG sites at the UGT1A1 promoter did not prevent the binding of HNF1α (Bélanger et al., 2010). In contrast, the present study demonstrated that DNA hypermethylation of the *UGT1A1* promoter in the kidney was accompanied by increased acetylation of histone H3 and defective recruitment of HNF1α (Fig. 3). Therefore, gene silencing of UGT1A1 in the kidney would be due to the latter mechanism with the abolished binding of HNF1α.

Our cell line based study clearly demonstrated the significance of DNA methylation in the regulation of UGT1A1 as follows: 1) substantial expression of UGT1A1 mRNA is observed in

HuH-7 cells with DNA hypomethylation status, 2) 5-Aza-dC treatment resulted in an increase of UGT1A1 expression that reflected the change in methylation status, and 3) the exogenously expressed HNF1α could increase UGT1A1 expression only in the presence of 5-Aza-dC in HK-2 cells. These findings clearly illustrated that unmethylated DNA is a prerequisite for the transcriptional activation of UGT1A1.

The study using TSA demonstrated that histone acetylation is a supplemental factor for transactivation, supporting the general perception (Cameron et al., 1999). In contrast to our study, a previous study reported a significant increase of UGT1A1 mRNA expression following treatment with 3 mM TSA in HepG2 cells (Mackenzie et al., 2010). When we treated the HK-2 and HuH-7 cells with 1 mM TSA, a prominent decrease of cell viability was observed. Thus, it is possible that there are inter-cell line differences in the response toward TSA. Collectively, DNA methylation at the promoter region of UGT1A1 may evoke the condensed chromatin structure through histone deacetylation, thereby inhibiting the binding of transcription factors such as HNF1α. This theory would explain the defective expression of UGT1A1 in kidney, where HNF1α is substantially expressed.

Although the simultaneous overexpression of HNF1α and inhibition of DNA methylation tremendously induced UGT1A1 mRNA in HK-2 cells, the UGT1A1 level was still lower than the level in HuH-7 cells (Fig. 4). It was surmised that some factors regulating UGT1A1 expression might be insufficient in HK-2 cells. Previous studies have reported that pregnane X receptor (Sugatani et al., 2008), glucocorticoid receptor (Usui et al., 2006), constitutive androstane receptor (Sugatani et al., 2008), peroxisome proliferator-activated receptor α (Senekeo-Effenberger et al., 2007), NF-E2-related factor-2 (Yueh and Tukey, 2007), and aryl hydrocarbon receptor (Yueh et al., 2003) are involved in UGT1A1 regulation. It is possible that such factors may be insufficient in HK-2 cells, although experimental proof is required. As another possibility, differences in histone modifications other than acetylation, namely H3K4

methylation (activating mark), H3K9 methylation (silencing mark), and H3K27 methylation (silencing mark), are feasible. Thus, such factors might also be involved in the regulation of the basal expression of UGT1A1 in cell lines and tissues.

Each member of UGT1A family has a unique promoter. The tissue-specific expression of UGT1As could be attributed to the differences in their promoter activation (Gong et al., 2001). It is reasonable to assume that UGT isoforms other than UGT1A1 showing tissue-specific expression might also be epigenetically regulated. We are currently working on this issue.

In conclusion, we found that the DNA methylation status of the human UGT1A1 promoter is different in the liver and kidney. DNA methylation, hypoacetylation of histone H3, and diminished binding of HNF1α could explain the defective expression of UGT1A1 in the kidney. A remaining future challenge is the elucidation of the effects of factors affecting epigenetic status such as aging, sex, disease, and habits on UGT1A1 expression

Acknowledgements

We acknowledge Drs. Yasuhiro Aoki and Masataka Takamiya in Iwate Medical University for supplying human tissues.

Authorship Contributions

Participated in research design: Oda, Nakajima, Fukami, and Yokoi

Conducted experiments: Oda

Contributed new reagents or analytic tools: none

Performed data analysis: Oda

Wrote or contributed to the writing of the manuscript: Oda, Nakajima, and Yokoi

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Footnotes

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Figure Legends

Fig. 1. UGT1A1 mRNA expression in human kidney and liver. The expression levels of UGT1A1 mRNA were determined by real-time RT-PCR and normalized to GAPDH mRNA levels. Each kidney and liver sample with a given number of donors came from the same donors. The values are expressed as relative to the UGT1A1 levels in the liver from donor 1. Each column represents the mean ± SD of triplicate determinations. K, kidney; L, liver; ND, not detectable.

Fig. 2. DNA methylation status of the *UGT1A1* promoter region in human liver, kidney or hepatocytes. Top, a schematic diagram of the *UGT1A1* 5'-flanking region. The vertical lines and numbers represent the position of the cytosine residues of the CpGs relative to the transcription start site as +1. The HNF1 binding site and TATA box are represented by rectangles. Arrows indicate the positions of the primers used for ChIP analysis. Bottom, DNA methylation status of CpG sites. Bisulfite sequencing analysis was performed using genomic DNAs extracted from human liver (donor 3), kidney (donor 1) or hepatocytes (HH268). Fourteen clones from each sample type were sequenced. The open and closed circles represent unmethylated and methylated cytosines, respectively.

Fig. 3. Histone H3 acetylation and recruitment of HNF1α in the *UGT1A1* promoter region in human kidney and liver. **(A and B)** ChIP assay of acetyl histone H3 and HNF1α in kidney and liver. Human kidney (donor 1) and liver (donor 3) chromatin was precipitated with anti-acetyl histone H3 antibody **(A)** or anti-HNF1α antibody **(B)**. The precipitated DNA was quantified by real-time PCR with a primer pair that amplified the region from -118 to +111 of the *UGT1A1* gene. The results are expressed as the percentage of input. Normal rabbit or goat IgGs (open

columns) were included as negative controls. **(C)** Western blot analysis of HNF1α in kidney and liver. Homogenates (50 μg) from kidney and liver samples were subjected to 10% SDS-PAGE and probed with anti-HNF1α or anti-β-actin antibodies. Each column represents the mean ± SD of triplicate determinations.

Fig. 4. Effects of 5-Aza-dC and/or TSA treatment and transfection of HNF1α on the UGT1A1 expression in HK-2 and HuH-7 cells. **(A)** DNA methylation status of the *UGT1A1* promoter region in HK-2 and HuH-7 cells. Ten clones each were sequenced. The open and closed circles represent unmethylated and methylated cytosines, respectively. **(B)** Effects of 5-Aza-dC on the UGT1A1 expression in HK-2 and HuH-7 cells. UGT1A1 mRNA level was determined by real-time RT-PCR and normalized with the GAPDH mRNA levels. **(C)** Effects of 5-Aza-dC on the DNA methylation status of the *UGT1A1* promoter region in HK-2 and HuH-7 cells. Bisulfite sequencing analysis was performed using genomic DNA extracted from 5-Aza-dC-treated cells. **(D)** Western blot analysis of HNF1α in HK-2 and HuH-7 cells. Nuclear extracts from HK-2 and HuH-7 cells transfected with HNF1α expression plasmid (+) or empty plasmid (-) were analyzed. **(E)** Effects of 5-Aza-dC and/or TSA treatment and transfection of HNF1α on the UGT1A1 mRNA expression in HK-2 and HuH-7 cells. The cells were transiently transfected with HNF1α expression plasmid (+) or empty plasmid (-), followed by treatment with 5-Aza-dC and/or TSA. The expression level of UGT1A1 mRNA was determined by real-time RT-PCR. Data were expressed as relative to UGT1A1 expression compared with non-treated HK-2 cells. Each column represents the mean ± SD of triplicate determinations. $^{**}p < 0.01$, compared with non-treated cells. $^{\dagger\dagger\dagger}p < 0.001$.

Table 1. Oligonucleotides used for the UGT1A1 bisulfite analysis and ChIP assay and for the cloning of HNF1α.

Oligonucleotides	5' to 3' sequence	Position
Bisulfite analysis of UGT1A1		
Forward	TTTGTGGATTGATAGTTTTTTATAG	-113 to -89
Reverse	CAATAACTACCATCCACTAAAATC	+134 to +111
ChIP assay of UGT1A1		
Forward	CTACCTTTGTGGACTGACAGC	-118 to -98
Reverse	CAACAGTATCTTCCCAGCATG	+111 to +91
Cloning of HNF1α		
Forward	GCAGCCGAGCCATGGTTTCT	-11 to +9
Reverse	GGTGCCGTGGTTACTGGGA	+1906 to +1888

Nucleotides are numbered with the transcription start site designated as +1 in the UGT1A1 genomic DNA sequence and base A in the initiation codon ATG designated as +1 in the HNF1α cDNA sequence.

Fig. 1

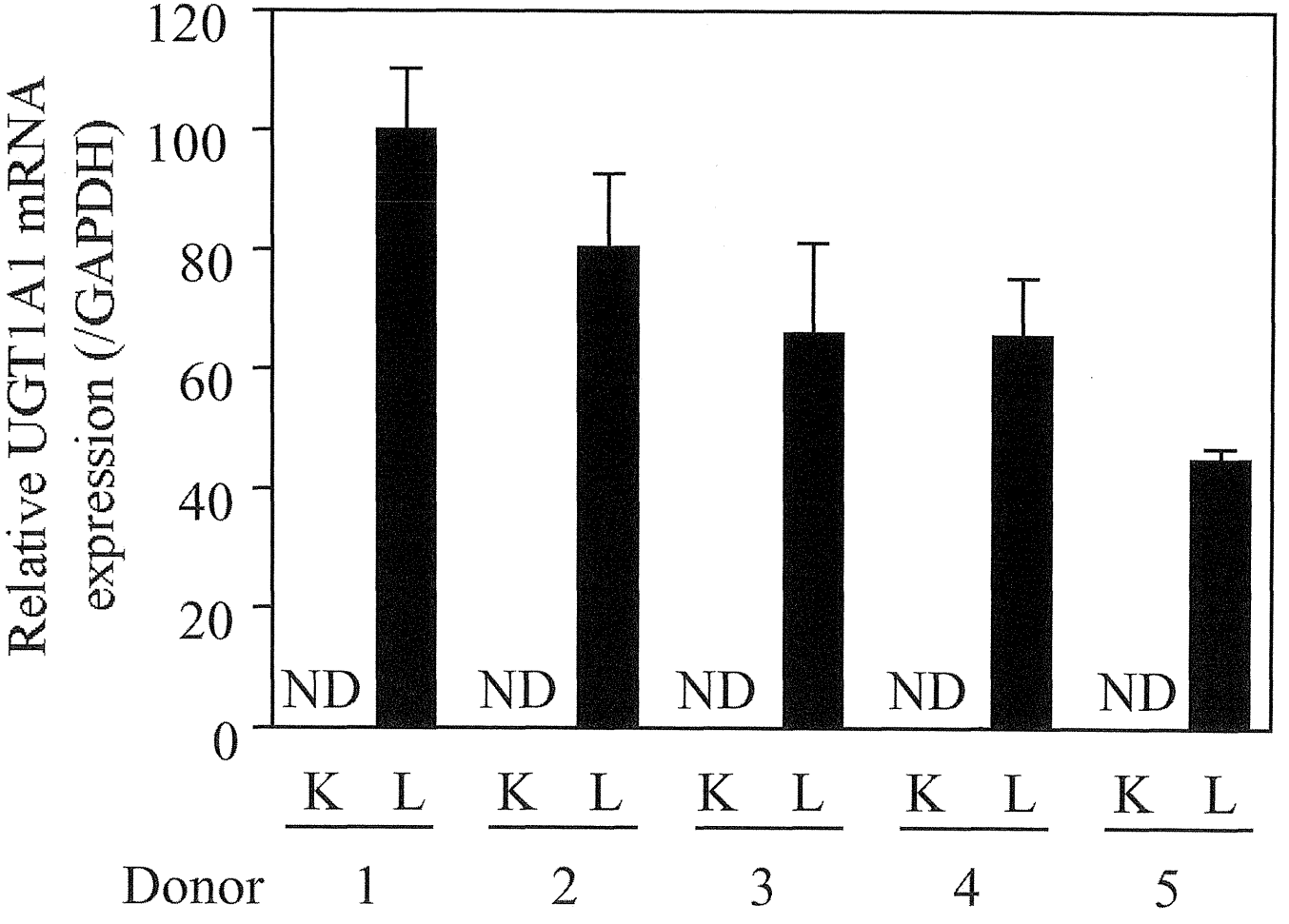

Relative UGT1A1 mRNA expression (/GAPDH)
120
100
80
60
40
20
0
ND
ND
ND
ND
ND
K L
K L
K L
K L
K L
Donor 1 2 3 4 5

Fig. 2

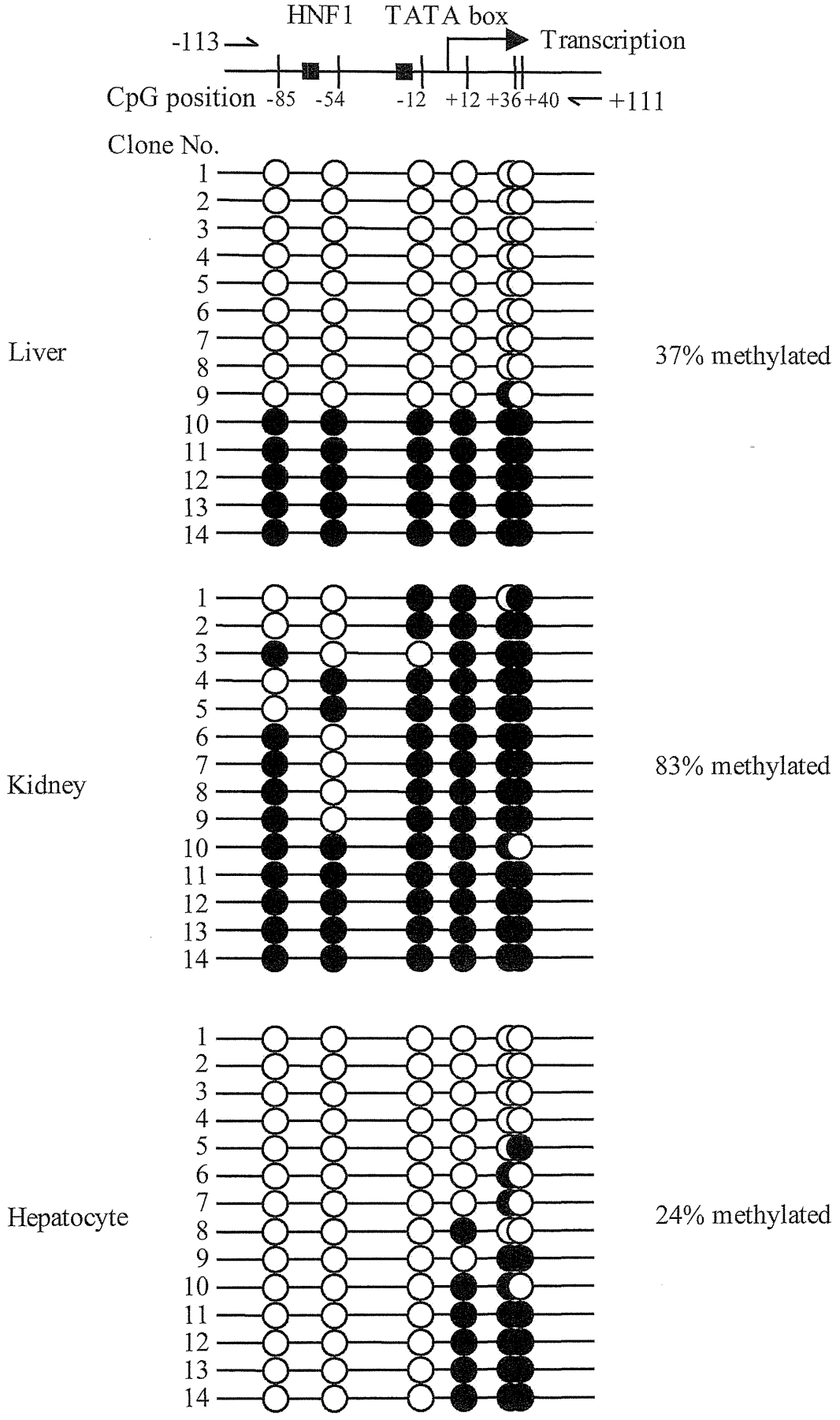
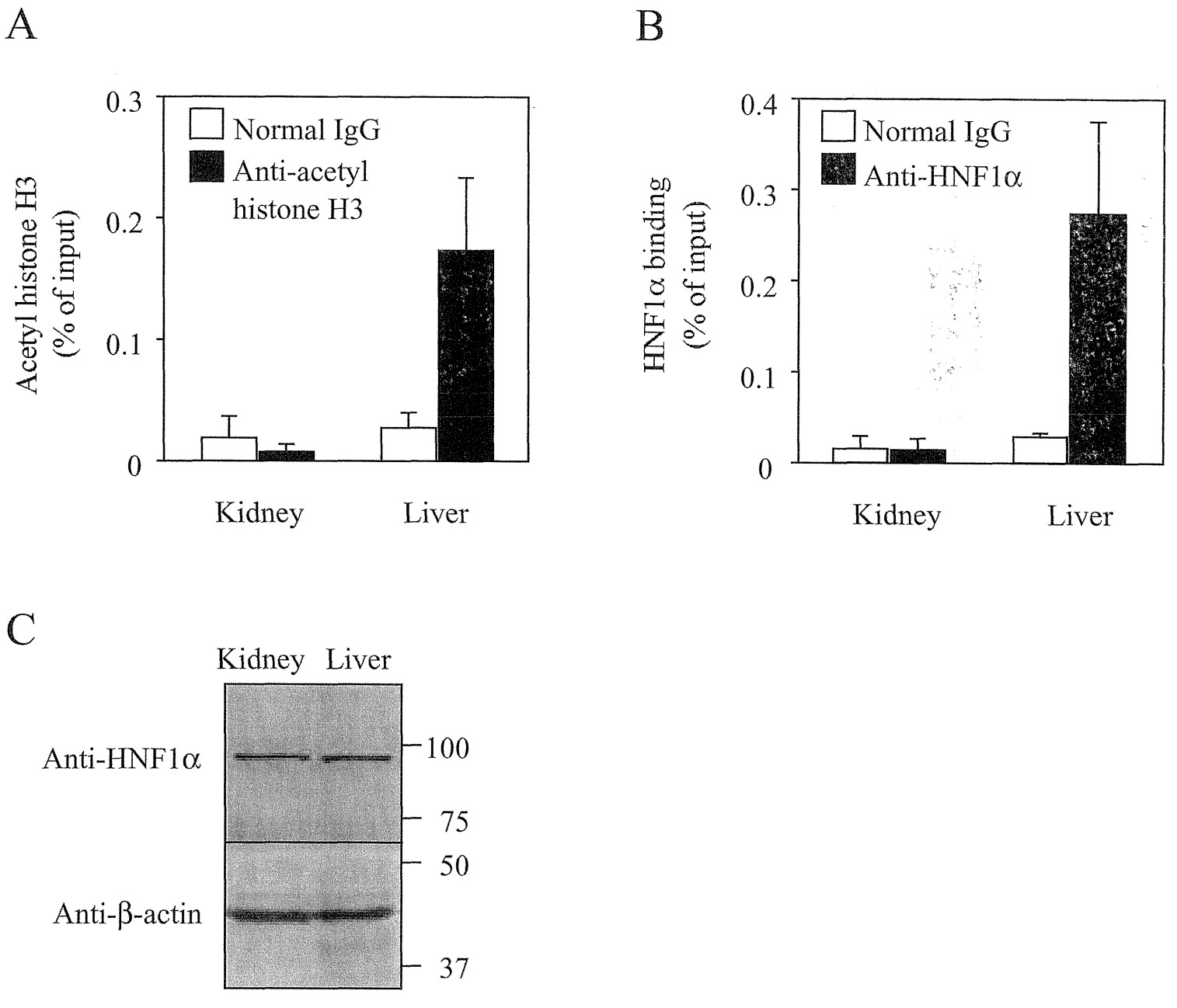

HNF1
TATA box
-113
Transcription
CpG position -85 -54 -12 +12 +36 +40 +111
Clone No.
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Liver
37% methylated
Kidney
83% methylated
Hepatocyte
24% methylated

Fig. 3


A
0.3
0.2
0.1
0
Acetyl histone H3
(% of input)
Normal IgG
Anti-acetyl
histone H3
Kidney
Liver
B
0.4
0.3
0.2
0.1
0
HNF1α binding
(% of input)
Normal IgG
Anti-HNF1α
Kidney
Liver
C
Kidney Liver
Anti-HNF1α
Anti-β-actin
100
75
50
37