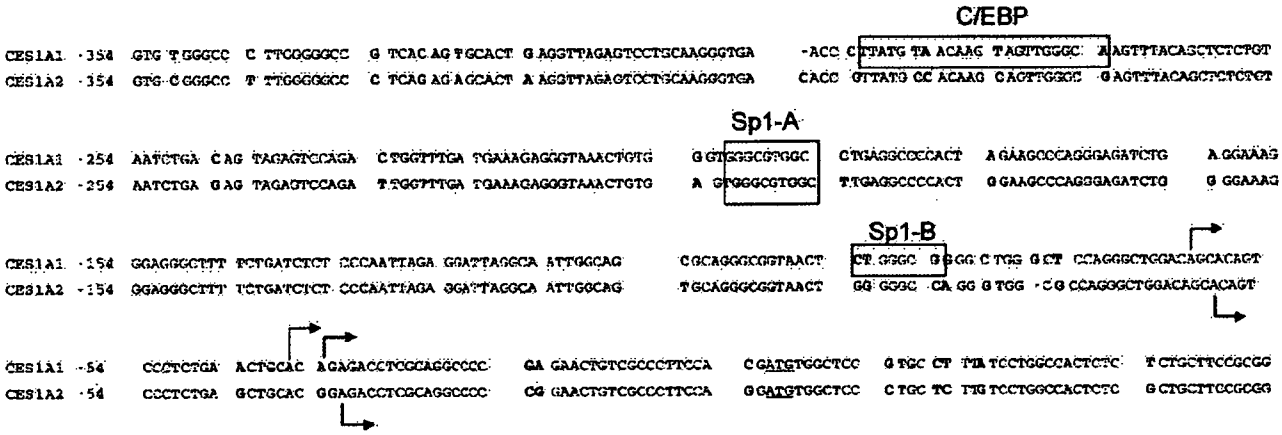

Fig. 2. Alignment of nucleotide sequences of the two *CES1A* gene promoter regions
The predicted binding sites for Sp1 or C/EBP are shown in boxes. Bold letters indicate nucleotide differences. Arrows indicate transcription start sites. Underlines indicate translation start sites.

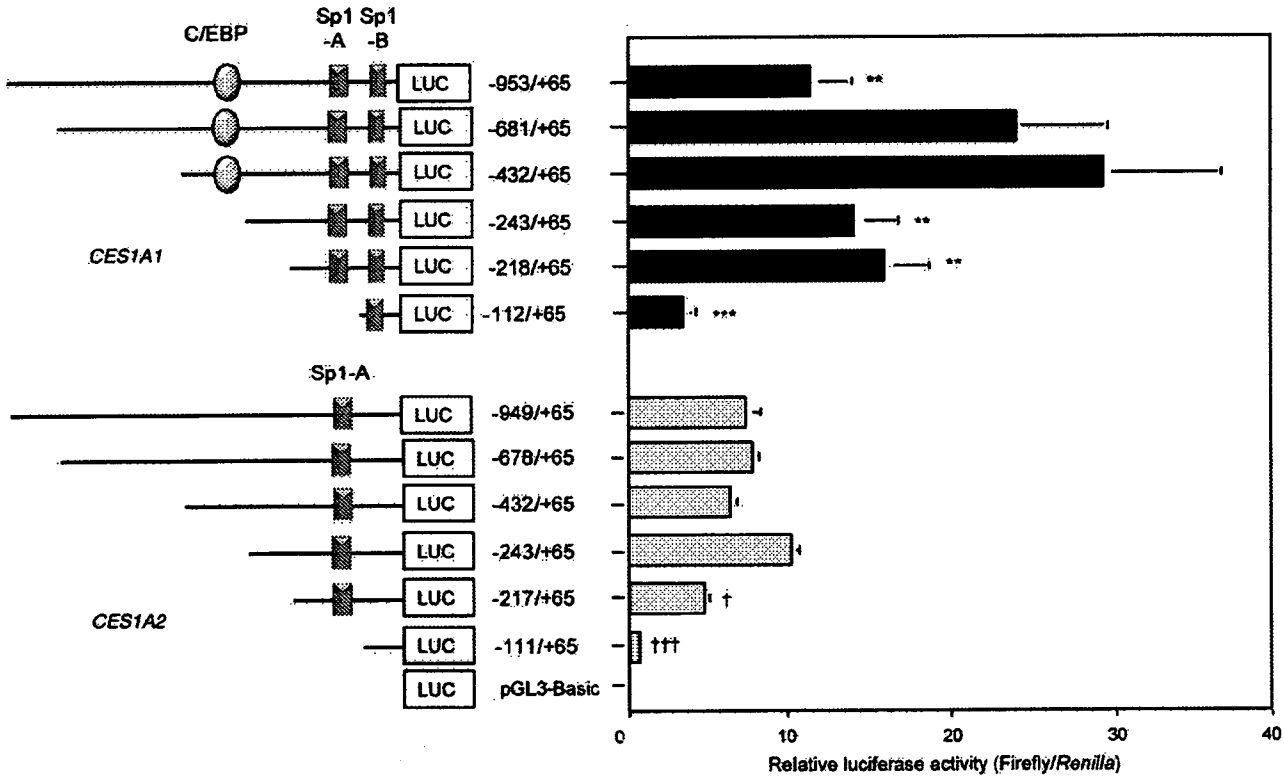

Fig. 3. Characterization of the promoter regions of the two *CES1A* genes
Either one of the deletion constructs or pGL3-Basic (200 ng) and a phRL-TK plasmid (4 ng) were transiently transfected into FLC7 cells. The names of the deletion constructs are shown on the left side of each bar. An oval indicates a C/EBP-binding site, and boxes indicate Sp1-binding sites. Each value is the mean ± SD of relative activities (Firefly/*renilla*) for three separate experiments, each performed in triplicate. *, ** and *** indicate statistically significant differences compared with CES1A1 −432/+65 ($p<0.05$, $p<0.01$ and $p<0.001$, respectively). † and ††† indicates statistically difference compared with CES1A2 −243/+65 ($p<0.05$ and $p<0.001$ respectively).

quences of *CES1A1* and *CES1A2* genes revealed about 98% homology in 30216 nucleotides from the translation start site (ATG) to before the site of poly-A addition. There are only six nucleotide differences resulting in four amino acid differences in the open reading flame, and all of the difference exist in exon1.

On the other hand, the 5′- flanking regions of *CES1A1* and *CES1A2* were also cloned from human liver genome DNA us-

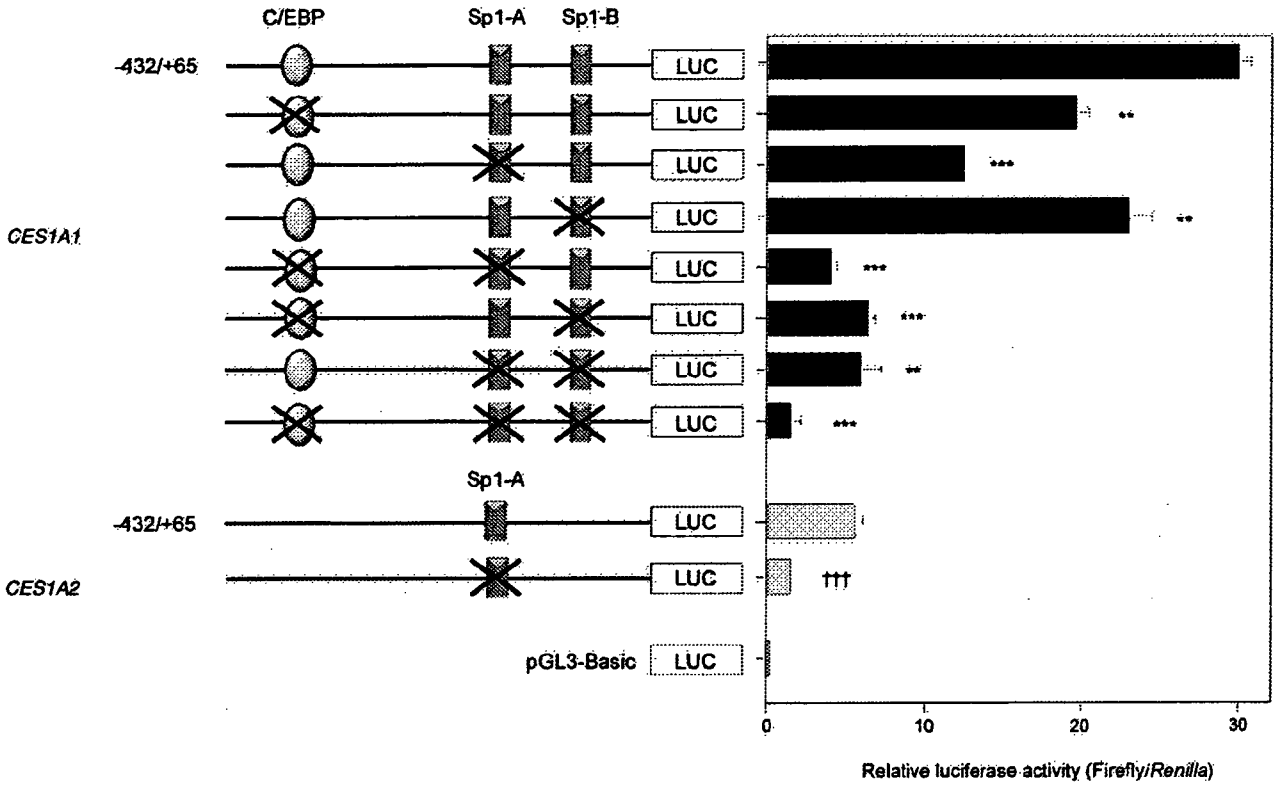

Fig. 4. Mutation analysis of the two *CES1A* gene promoters
Either a deletion construct (CES1A1 −432/+65 or CES1A2 −432/+65) or pGL3-Basic (200 ng) and a phRL-TK plasmid (4 ng) were transiently transfected into FLC7 cells. Symbols are explained in the legend of Fig. 3. Crosses indicate mutations introduced into each binding site. Each value is the mean ± SD of relative activities (Firefly/renilla) for three separate experiments, each performed in triplicate. ** and *** indicate statistically significant differences compared with CES1A1 −432/+65 ($p<0.01$ and $p<0.001$, respectively). ††† indicates statistically difference compared with CES1A2 −432/+65 ($p<0.001$).

ing PCR amplification and sequenced. The difference in the nucleotide sequences of the two genes was 91% in the 1-Kbp 5′-flanking region.

Determination of the transcription start sites of *CES1A1* and *CES1A2* genes

The RLM 5′-RACE method was used to determine the transcription start sites of *CES1A1* and *CES1A2* genes. DNA sequencing analysis of a PCR product showed that it contained a partial nucleotide sequence of the *CES1A1* or *CES1A2* gene ligating to the adopter sequence. The transcription initiation sites of the *CES1A1* promoter were localized 61, 41 and 39 upstream and these of the *CES1A2* promoter were localized 59 and 37 upstream from the translation initiation site (**Fig. 2**).

Identification of the major cis elements responsible for transcriptional activity of the 5′-flanking regions of the *CES1A1* and *CES1A2* genes

Regions homologous to known cis elements were identified using searching programs for transcription factor-binding sites (http://www.gene-regulation.com/ and http://www.drkazu.com/KENKYUU.htm/). In the 5′-flanking regions of *CES1A1* and *CES1A2* sequence, several possible binding sites for transcription factors have been identified, but no TATA-box was present in either promoters. A putative SP 1 transcription factor-binding site of the *CES1A1* promoter were located at position −196(Sp1-A) and −84(Sp1-B). A putative SP1 transcription factor-binding site of the *CES1A2* promoter was only located at position −195(Sp1-A) from the translation initiation site. A putative C/EBP binding site of the *CES1A1* promoter was located at position −290 from the translation initiation site, but the *CES1A2* promoter has no C/EBP binding site in this region.

Deletion analysis and mutation analysis were performed to examine the transcriptional activity of the 5′-flanking regions of *CES1A1* and *CES1A2* genes and to identify the elements that contribute to the transcriptional activity. Six deletion constructs were transiently transfected into FLC cells, human hepatoma cell lines. Deletion analysis showed that the highest level of transcriptional activity was in −432/+65 of the *CES1A1* promoter (**Fig. 3**). Deletion of the region from −432 to −243, which contained the C/EBP-binding site of the *CES1A1* promoter, caused a 2.0-fold decrease in activity ($p<0.01$) and further deletion to −218 resulted in no significant effect on activity. Finally, deletion of the region from −218 to −112, which contained the GC box (Sp1-A), caused a 9.0-fold decrease in activity of the highest level observed in

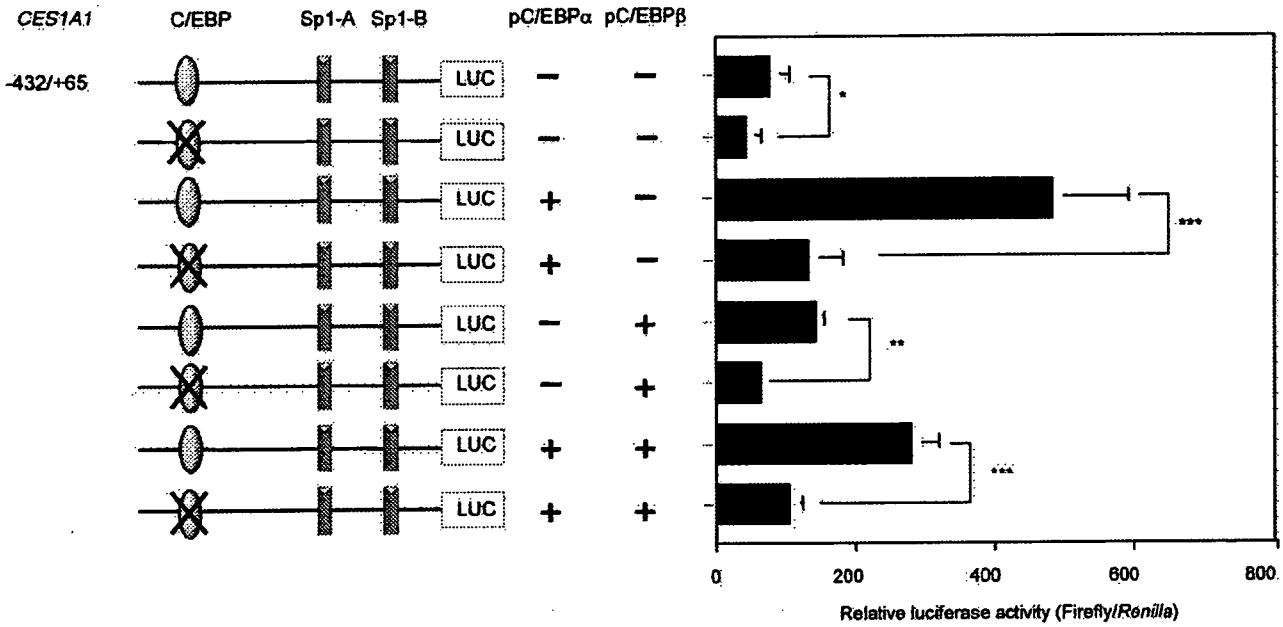

Fig. 5. Effects of C/EBP proteins on promoter activity of the *CES1A1* gene
The construct CES1A1 −432/+65 (200 ng) and a phRL-TK plasmid (4 ng) were transiently transfected into FLC7 cells together with 50 ng of C/EBPα expression plasmid and/or C/EBPβ expression plasmid. Total DNA amount was adjusted to 304 ng by using an empty pTARGET vector. Crosses indicate mutation introduced into C/EBP binding site. Symbols are explained in the legend of Figs. 3 and 4. Each value is the mean ± SD of relative activities (Firefly/*renilla*) for three separate experiments, each performed in triplicate. *, ** and *** indicate statistically significant differences between control and mutation introduced into C/EBP binding site ($p<0.05$, $p<0.01$ and $p<0.001$, respectively).

−432/+65 ($p<0.001$). In contrast, deletion analysis showed that the level of transcriptional activity of the *CES1A2* promoter was lower than that of the *CES1A1* promoter. Deletion of the region from −217 to −111, which contained the GC box (Sp1-A), resulted in the level of activity to 5% of the highest level observed in −243/+65 ($p<0.001$).

The results of mutation analysis were similar to those obtained from deletion analysis of the 5′-flanking regions of the human *CES1A1* and *CES1A2* genes (**Fig. 4**). All of the mutational constructs showed significant reduction of transcriptional activity when compared with that of the intact −432/+65. The introduction of mutation into C/EBP, SP1-A and the Sp1-B reduced activity to 66% ($P<0.01$), 42% ($P<0.001$) and 77% ($P<0.01$) of the wild type of *CES1A1* promoter activity, respectively. In the case of the *CES1A2* promoter, the introduction of mutation into SP1-A caused 73% ($P<0.001$) reduction of the activity relative to that of −432/+65. The effects of double mutations on the transcriptional activity were different among the constructs. Additional mutation in the C/EBP element to Sp1-A was effective for 80% reduction of the transcriptional activity of wild type *CES1A1* promoter ($P<0.001$). The double C/EBP and Sp1-B element mutant and double Sp1-A and Sp1-B mutant also caused 79% ($P<0.001$) and 81% ($P<0.01$) reduction of the activity relative to that of the wild type of *CES1A1* promoter, respectively. Interestingly, in the case of double C/EBP and Sp1-B mutation of the *CES1A1* promoter, transcriptional activity was the same as that of the wild type of the *CES1A2* promoter. Triple mutation of C/EBP, SP1-A and SP1-B elements also caused 95% ($P<0.001$) reduction of transcriptional activity relative to that of the wild type of the *CES1A1* promoter, and this reduction of activity was the same as that in the case of mutation in the SP1-A element of the *CES1A2* promoter.

To clarify whether C/EBPα or C/EBPβ was effects of the transcriptional activity of C/EBP-binding site of the *CES1A1* promoter, cotransfection analyses were performed by using the human hepatoma cell line FLC7. The *CES1A1* −432/+65 promoter was cotransfected with pC/EBPα and/or pC/EBPβ. **Figure 5** shows that the transcriptional activity level of the *CES1A1* promoter cotransfected with pC/EBPα was 3-times higher than that of the promoter cotransfected with pC/EBPβ. Introduction of mutation into the C/EBP-binding site of the *CES1A1* promoter caused 73% reduction of the activity relative to that of the wild type *CES1A1* promoter cotransfected with pC/EBPα. When the CES1A1 −432/+65 promoter was cotransfected with pC/EBPα and pC/EBPβ, the transcriptional activity was reduced compare to that in the case of cotransfection of pC/EBPα alone (**Fig. 5**).

Identification of nuclear proteins interacting with C/EBP and Sp1 of the *CES1A1* and *CES1A2* gene promoters

The results of deletion and mutation analysis showed that the 5′ flanking region of −432 to +65 consists of a minimal promoter of the *CES1A1* and *CES1A2* genes and that three *cis*

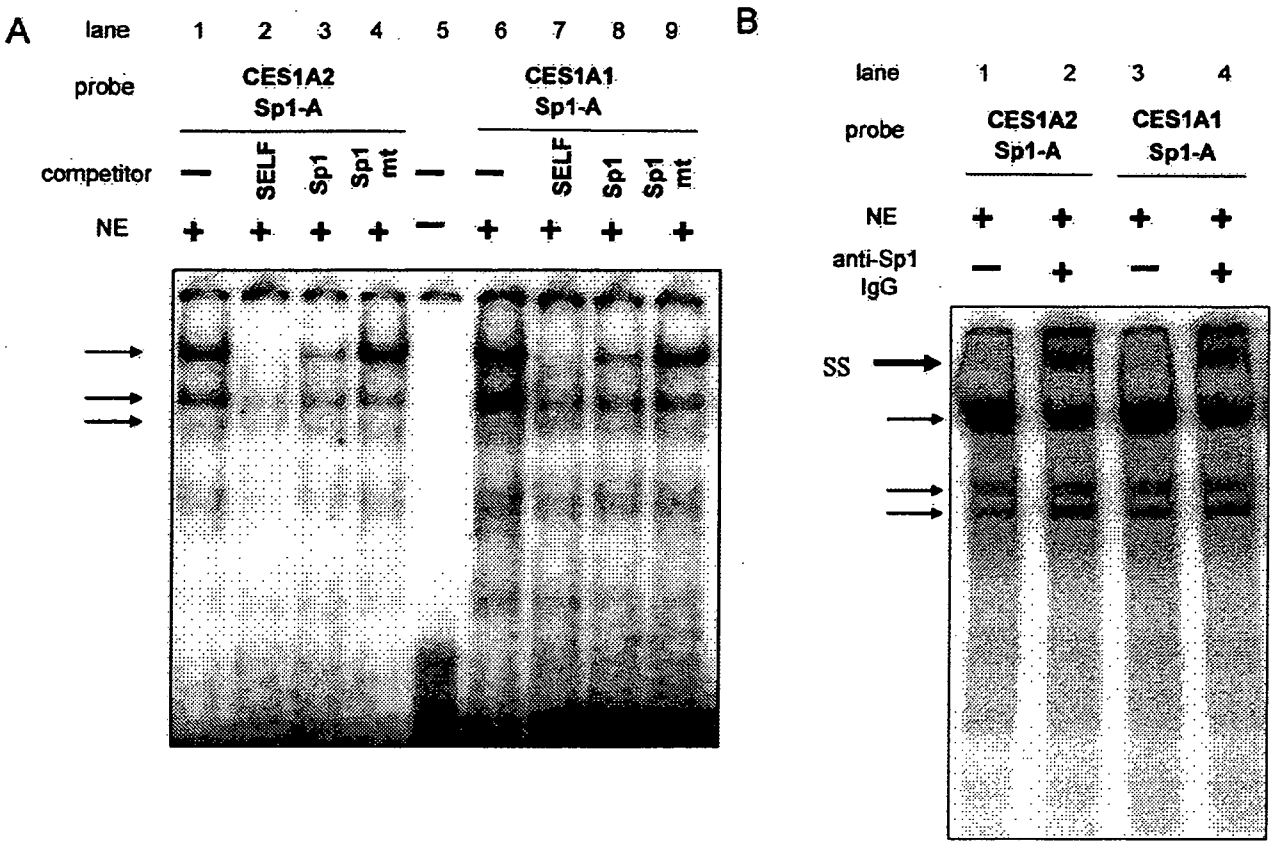

Fig. 6. Binding of Sp1 to the promoter region of the two *CES1A* genes
A: GMSA was performed using probes, CES1A2-Sp1 and CES1A1-Sp1, that contain an Sp1-binding site (Sp1-A). NE, FLC7 cells nuclear extracts. SELF, cold probes (50-fold excess amounts of the probe, lanes 2 and 7). Sp1 and Sp1mt, oligonucleotides containing a consensus or mutated Sp1-binding site (50-fold excess amounts of the probe, lanes 3 and 8 and lanes 4 and 9, respectively). A shifted band is indicated by an arrow.
B: Supershift asssay was performed using the same probes, Anti-Sp1 IgG (4 μg) was added to the reaction mixture (lanes 2 and 4). SS, a supershifted band.

elements, a C/EBP-binding site and two Sp1-binding site, (Sp1-A and Sp1-B) located within the region were important for transactivation of the promoter. To determine the nuclear proteins that could interact with these elements, electrophoretic mobility shift assays (EMSAs) were carried out. Probes used in EMSAs are listed in **Table 1**.

Figure 6 shows the results of EMSAs and supershift assays for identification of proteins interacting with the Sp1-A site (GC box). The probes, CES1A1-Sp1-A and CES1A2-Sp1-A, were incubated with FLC7 nuclear extracts, resulting in three DNA-protein complexes (**Fig. 6A**, lanes 1 and 6). All of the complexes disappeared when an 50-fold excess amount of CES1A1-Sp1-A, CES1A2-Sp1-A (**Fig. 6A**, lanes 2 and 7) or Sp1 consensus oligonucleotide (**Fig. 6A**, lane 3 and 8) was added as a competitor but not in the case of addition of Sp1mt, a mutated Sp1 consensus oligonucleotide (lane 4 and 9). To identify the proteins composing these complexes, supershift assays were performed using anti-Sp1 antibodies. The addition of anti-Sp1 antibodies generated a supershifted band together with a slight decrease in the amount of the upper complex formed (Fig. 6B, lanes 2 and 4). These findings indicate that Sp1 protein is bound to Sp1-A (GC box) of both the *CES1A1* and *CES1A2* gene promoters.

Figure 7A shows the results of EMSAs and supershift assays for identification of proteins interacting with the other GC box (Sp1-B) as described above. The probe, CES1A1-Sp1-B, was incubated with FLC7 nuclear extracts, resulting in DNA-protein complexes (**Fig. 7A**, lanes 6 and 9). All of the complexes disappeared when a 50-fold excess amount of Sp1, an Sp1 consensus oligonucleotide, was added as a competitor (**Fig. 7A**, lane 7) but not in the case of addition of Sp1mt, a mutated Sp1 consensus oligonucleotide (**Fig. 7A**, lane 8). To identify the proteins composing the three complexes, supershift assays were performed using anti-Sp1 antibodies. The addition of anti-Sp1 antibodies generated a supershifted band together with a slight decrease in the amount of the upper complex formed (**Fig. 7A**, lane 10). On the other hand, CES1A2-Sp1-B could not bind to the nuclear protein forming a complex with FLC7

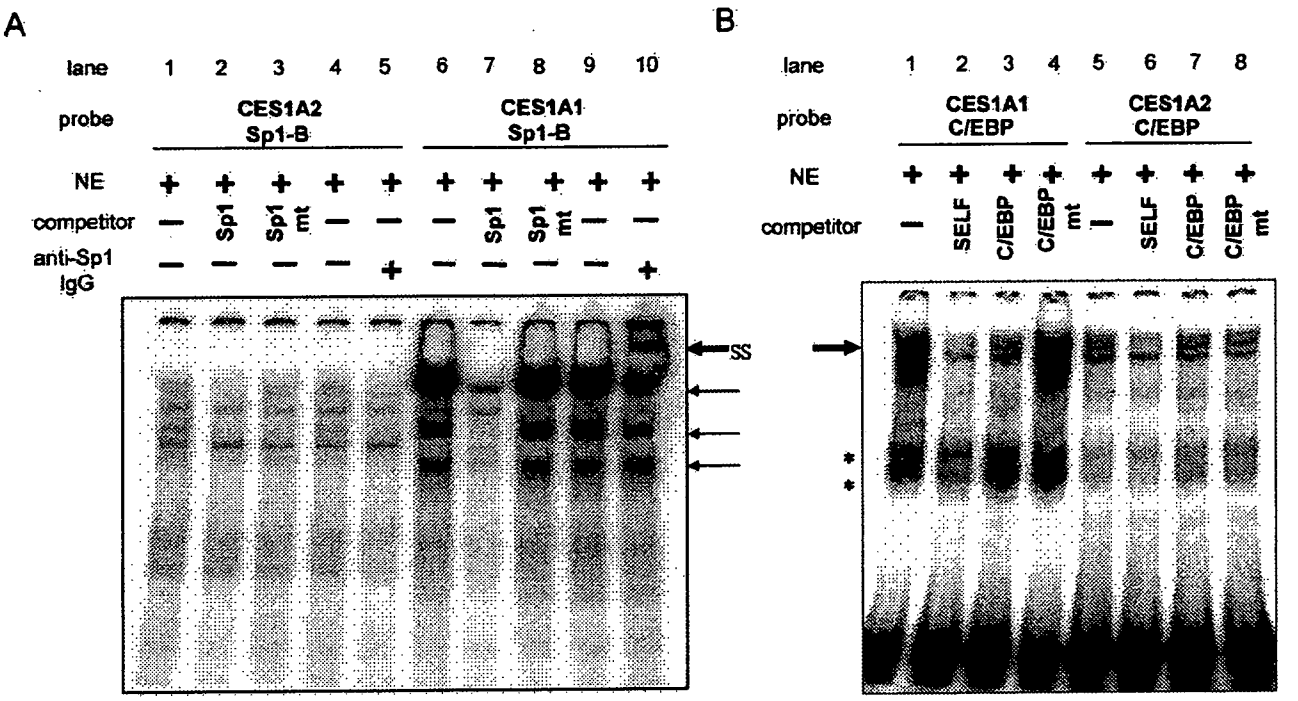

Fig. 7. Binding of Sp1 and C/EBP to the promoter region of the two *CES1A* genes
A: GMSA was performed using the probes CES1A2-Sp1-B and CES1A1-Sp1-B. CES1A1-Sp1-B contains an Sp1-binding site (Sp1-B). NE, FLC7 cells nuclear extracts. Sp1 and Sp1mt, oligonucleotides containing a consensus or mutated Sp1-binding site (50-fold excess amounts of the probe, lanes 2 and 7 and lanes 3 and 8, respectively). Anti-Sp1 IgG (4 μg) was added to the reaction mixture (lanes 5 and 10). SS, a super-shifted band.
B: GMSA was performed using the probes CES1A2-C/EBP and CES1A1-C/EBP. CES1A1-C/EBP contains a C/EBP-binding site. SELF, cold probes (50-fold excess amounts of the probe, lanes 2 and 6). C/EBP and C/EBPmt, oligonucleotides containing a consensus or mutated C/EBP-binding site (50-fold excess amounts of the probe, lanes 3 and 7 and lanes 4 and 8, respectively). An arrowhead indicates a shifted band.

nuclear extracts (**Fig. 7A**, lanes 1–4). These findings indicate that Sp1 protein is bound to SP1-B (GC box) of only the *CES1A1* gene promoter.

Next, we examined nuclear proteins that interacted with the C/EBP-binding site of the *CES1A1* promoter (**Fig. 7B**). Incubation of the labeled probe CES1A1-C/EBP with the nuclear extracts resulted in the formation of a complex together with an unknown band indicated by asterisks (**Fig. 7B**, lane 1). The complex disappeared when a 50-fold excess amount of CES1A1-C/EBP or C/EBP, a C/EBP consensus oligonucleotide, was added as a competitor (**Fig. 7B**, lanes 2 and 3) but not in the case of addition of C/EBPmt, a mutated C/EBP consensus oligonucleotide (lane 4). On the other hand, CES1A2-C/EBP could not specifically bind to the nuclear protein forming a complex with FLC7 nuclear extracts (**Fig. 7B**, lanes 5–8). These finding indicate that C/EBP protein is bound to the element of only the *CES1A1* gene promoter.

Transactivation of the *CES1A* promoter by Sp1 and Sp3 in Drosophila SL2 cells

We analyzed functional capabilities of Sp1 and Sp3 for transactivation of the *CES1A1* and *CES1A2* gene promoters. CES1A1-432/+65/pGEL3 or CES1A2-432/+65/pGEL3 was cotransfected with Sp1 and/or Sp3 in Drosophila SL2 cells, which possess a null background for these transcription factors,[22] and luciferase activities were compared (**Fig. 8**). Sp1 was shown to be a strong transactivation of the *CES1A1* promoter (2.8-fold). Sp3 also strong transactivation of the *CES1A1* promoter (3.6-fold). However, in the case of a combination of Sp1 with Sp3, the promoter activity level was greatly increased by 4.3 fold ($P < 0.001$). Sp1 or Sp3 was not shown a transactivation of *CES1A2* promoter (**Fig. 8**), but in the case of a combination of Sp1 with Sp3, the promoter activity was slightly but significantly increased (1.3-fold, $p < 0.01$).

Comparison of *CES1A1* and *CES1A2* mRNA expression levels in human liver samples and human tissues

Table 2 shows the mRNA expression levels of CES 1A1 and CES1A2. In most samples, CES1A1 mRNA expression level was much higher than that of CES1A2. CES1A2 mRNA was not expressed in some human liver samples.

Table 3 shows a comparison of CES1A1 and CES1A2 mRNA expression levels in human tissues. When the amounts of CES1A1 and CES1A2 mRNA in human adult liver, lungs, testis, small intestine, brain (cerebellum) and heart were measured by real-time PCR, CES1A1 mRNA was detected in all or-

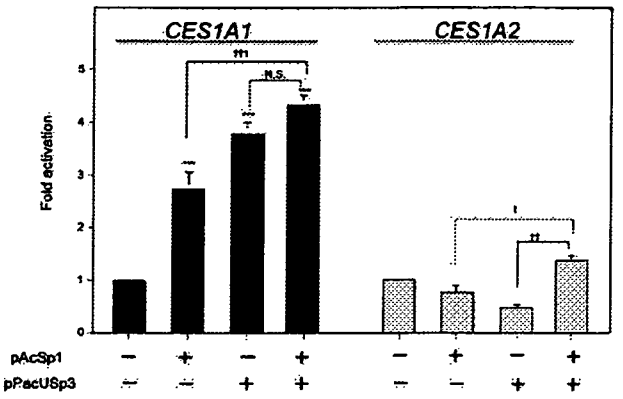

Fig. 8. Effects of Sp family proteins on promoter activity of the two *CES1A* genes
Either 200 ng of CES1A1 or CES1A2 was transiently transfected into *Drosophila* SL2 cells together with 50 ng of pAcSp1 and/or pPacUSp3. Total DNA amount was adjusted to 300 ng by using an empty pAc5.1V5/His vector. Luciferase activity was determined as relative activity (firefly/total cellular protein). Each value is the mean ± SD of fold induction towards the activity obtained from each CES1A promoter construct alone for three separate experiments, each performed in duplicate. *** indicate statistically significant differences compared with control ($p<0.001$). †, †† and ††† statistically significant difference between two transfectants indicated in the figure ($P<0.05$, $P<0.01$, $p<0.005$, respectively).

Table 3. Quantitation of the CES1A1 and CES1A2 mRNA expression levels in human tissues

Sample	CES1A1	CES1A2
	($\times 10^3$ copies/μg total RNA)	($\times 10^3$ copies/μg total RNA)
liver	254	86.1
kidney	2.71	1.18
testis	6.41	1.04
lung	80.4	0.0135
small intestine	9.09	ND
brain	0.514	0.0342
heart	27.6	0.00579

ND, Not detected.

gans. The expression levels of mRNA in the liver was the highest, and high appearance was comparatively shown next in lungs and heart. The mRNA expression levels of CES1A1 in small intestine and testis were low, and the level was very low in the brain. On the other hand, the expression levels of CES1A2 mRNA in the liver was lower than CES1A1 mRNA. The expression levels of CES1A2 mRNA in other organs were very low, and CES1A2 mRNA was not detected in small intestines.

Discussion

We isolated and characterized two genes encoding the human *CES1A1a* (AB119997) and *CES1A2* (AB119998), and we also cloned and sequenced the 5′ flanking region of each gene in order to elucidate the structure of the promoter.

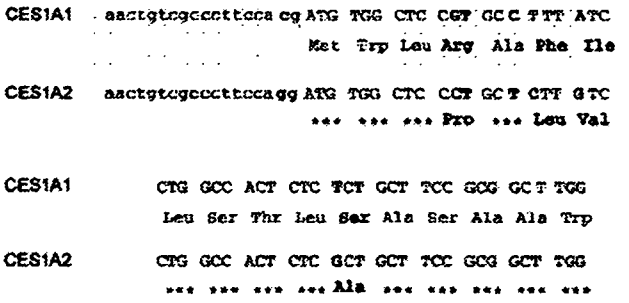

Fig. 9. Comparison of exon 1 nucleotide and N-terminal amino acid sequences between two *CES1A* genes
Bold letters indicate nucleotide or amino acid differences. A shadowed box indicates the change that eliminates positive charge from the signal peptides.

It was clarified that both the *CES1A1* and *CES1A2* genes existed in all human genome DNA of 20 specimens. We also found that complete CES1A1 and CES1A2 cDNA were able to be obtained from the same liver (data not shown) of 20 specimens. These results suggested that the *CES1A* gene is caused as a result of the gene duplication. They share a common intergenic region spanning about 9 Kbp. There are only six nucleotide differences resulting in four amino acid differences in the open reading frame, and all of the differences existed in exon 1 (**Fig. 9**), and the difference in the nucleotide sequences of the two genes was 91% in the 1-Kbp 5′-flanking region. Our result suggested that the two genes evolved independently after gene duplication and that the nucleotides changed within exon 1 and the 5′-flanking region. Gene duplication has generally been viewed as a necessary source of material for the origin of evolutionary novelties, and duplicate genes evolve new functions.[16,17] The majority of gene duplicates are silenced within a few million years, with the small number of survivors are subsequently subjected to strong purifying selection. Although duplicate genes may only rarely evolve new functions, the stochastic silencing of such genes may play a significant role in the passive origin of new species.[16,17] Therefore, it is thought that *CES1A1* and *CES1A2* genes have different roles in human.

We examined the difference in the mechanisms of transcriptional regulation of the two gene promoters. We cloned an approximately 1.0-kbp fragment of the 5′-flanking region of each of the *CES1A1* and *CES1A2* genes (**Fig. 2**). This region shows characteristics of a TATA-less promoter, such as the lack of a TATA box but the presence of a GC box in precedence to the transcription start site. A TATA-less promoter seems to be shared by other members of the CES family, since two previously reported *CES* promoters also do not have a functional TATA-box.[18,19] The next issue was how this 5′-flanking region can regulate transcription of the two genes.

The *CES1A1* promoter activity gradually increased with progressive 5′-flanking deletions from −953 to −432 (**Fig. 3**). This pattern of activity suggests that the promoter sequence between −953 and −432 may harbor several negative element. The results of deletion and mutation analyses showed

that *CES1A1* transcription resulted from the independent action of three cis acting elements, two GC box (Sp1-A and Sp1-B) and a C/EBP responsive element (**Figs. 3** and **4**), but the degrees of contribution of these elements to the promoter activity were not equivalent. Interestingly, one of the GC box (Sp1-B) and the C/EBP responsive element do not exist in the *CES1A2* promoter (**Fig. 2**).

Sp1 can bind to the GC box and is known to be important for transactivation of TATA-less promoters.[20] The Sp family consists of four proteins designated Sp1, Sp2, Sp3, and Sp4.[20] Sp2 is structurally different from other Sp members, and accordingly it does not bind to the GC box, which Sp1, Sp3, and Sp4 recognize with identical affinity.[21-23] Sp4 is predominantly expressed in the brain,[21,24] and Sp3 is expressed in all mammalian cells that express Sp1.[21-23] Thus, Sp1 was our first choice as a candidate for nuclear proteins that interact with the GC box (Sp1-A) of the *CES1A* promoter, and accordingly we were able to determine the involvement of Sp1 in complex formation (**Fig. 3**). We also determined that Sp1 could bind to the other GC box (Sp1-B). Since the *CES1A2* promoter does not have a GC box consensus sequence, the Sp1-B site of the *CES1A2* promoter can not bind to nuclear proteins (**Fig. 7A**, lanes 1–4). These findings suggested that Sp1 protein can bind to Sp1-B (GC box) of the *CES1A1* promoter but can not bind to that of the *CES1A2* promoter.

Since Sp1 and Sp3 endogenously expressed in FLC cells (data not shown), the functional role of the Sp family in transactivation of the *CES1* promoter was demonstrated by cotransfection assays in *Drosophila* SL2 cells, which possess a null background for Sp1 family.[22] Sp1 and Sp3 were shown to be transactivate the *CES1A1* promoter (**Fig. 8**). Recently we have shown that Sp1 could interact with Sp3 to synergistically increase mouse *mCES2* promoter activity rather than compete with Sp1.[15] The interaction between Sp1 and Sp3 was already reported that the transcriptional regulation of the mouse *hepatocyte growth factor* gene[25] or the human *extracellular matrix metalloproteinase inducer* gene.[26] Together with these data, our result may suggest that Sp1 could interact with Sp3 to synergistically increase *CES1A1* and *CES1A2* gene.

The C/EBP transcription factors form a family within the basic region-leucine zipper (bZIP) class of transcription factors.[27,28] C/EBPs are expressed in several organs and are involved in controlling differentiation-dependent gene expression. C/EBP factors belong to the bZIP class of basic domain transcription factors. Six members constitute the mammalian C/EBP family. Of these, C/EBPα was first identified and is the founding member. The basic region of C/EBP factors is a highly positively charged domain that directly interacts with the DNA. All members of the C/EBP family have similar basic region DNA-binding motifs. As a consequence of the high similarity in the basic region, C/EBPα, C/EBPβ and C/EBPδ have been shown to interact with virtually identical DNA sequences. The leucine zipper region is also conserved between the different family members, whereas the amino-terminal transactivation domain is more diverse. Although C/EBPα and C/EBPβ are expressed at highest levels in the liver and fat, C/EBPδ is expressed at highest levels in the lung.[27,28] Therefore, we chose C/EBPα and C/EBPβ as candidates for nuclear proteins that bind to the *CES1A1* promoter. The results of deletion and mutation analyses showed that *CES1A1* transcription resulted from the independent action of the C/EBP responsive element (**Figs. 3** and **5**). The transactivation of C/EBPα for the *CES1A1* promoter is much stronger than that of C/EBPβ. In addition, when C/EBPβ was cotransfected with C/EBPα, there was decreased activity, suggesting a negative cooperation between these transcription factors (**Fig. 5**). Recently, cytochrome P450 2D6 (*CYP2D6*) promoter activity has also been shown to be negatively influenced by cotransfection with C/EBPβ.[29] These findings suggested that C/EBPα/β protein can bind to C/EBP binding site of the *CES1A1* promoter, and increase *CES1A1* promoter activity.

We also observed inter-individual difference of the CES1A1 and CES1A2 mRNA expression levels in human liver samples by real-time RCR analysis with a specific TaqMan probe (**Table 2**). When mRNA expression levels of CES1A1 and 1A2 were measured in 20 human adult liver specimens, the appearance of CES1A1 mRNA was detected in all of the samples (**Table 2**). These findings suggested that CES1A1 is constitutively expressed in the adult human liver. On the other hand, CES1A2 mRNA was not expressed in some samples, and mRNA expression level was much lower than that of CES1A1. These results are in good agreement with the structure and function of the 5′-flanking region of the *CES1A2* gene promoter. Transactivation of the *CES1A1* promoter was much stronger than that of the *CES1A2* promoter, which lacks SP1-B and C/EBP binding element. **Table 3** shows a comparison of CES1A1 and CES1A2 mRNA expression levels in human tissues. When amounts of CES1A1 and CES1A2 mRNA in human adult liver, lungs, testis, small intestine, brain (cerebellum) and the heart were measured by the real-time PCR, CES1A1 mRNA was detected on all organs. Then the expression levels of CES1A1 mRNA were compared, and the expression level of mRNA was found to be highest. As for the tissue expression profiles of CES1A1 mRNA expression, our results showed constitutive expression of CES1A1 in many tissues in this experiment. In contrast, the highest CES1A2 mRNA expression level was seen in the liver, but the CES1A2 mRNA expression levels in other tissues were very low, and CES1A2 mRNA was not detected in the small intestines. These findings suggest that CES1A2 is mainly expressed in the adult liver and the CES 1A1 is mainly expressed in liver and lung.

In conclusion, we isolated two CES genes encoding human carboxylesterase CES1A, which were designated as the *CES1A1* and *CES1A2* genes. These genes were completely identical except for exon 1 and the 5′ regulatory element. We investigated the transcriptional regulation of these two CES genes. Reporter gene assays and electrophoretic mobility shift assays demonstrated that Sp1 and C/EBPα could bind to each responsive element of the *CES1A1* promoter but that Sp1 and C/EBP could not bind responsive element of the *CES1A2* promoter. Thus,

CES1A1 mRNA expression level is much higher than that of CES1A2 mRNA in the liver and lung. It is thought that these results provide information on individual variation of human carboxylesterase isozymes.

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Regular Article

Involvement of Hepatocyte Nuclear Factor 4alpha in Transcriptional Regulation of the Human Pregnane X Receptor Gene in the Human Liver

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Summary: Pregnane X receptor (PXR; NR1I2), a key transcriptional factor that regulates genes encoding drug-metabolizing enzymes and drug transporters, is abundantly expressed in the human liver. However, studies on the molecular mechanism of human *PXR* gene regulation are limited. In this study, we examined the involvement of hepatocyte nuclear factor 4alpha (HNF4α; NR2A1) in the transcriptional regulation of the human *PXR* gene in the human liver. The activities of the human *PXR* promoter containing the direct repeat 1 (DR1) element located at −88/−76 of the promoter were significantly increased by co-expression of HNF4α in the human hepatocellular carcinoma cell line. In addition, introduction of mutation into the DR1 element abolished the transcriptional activation of the human *PXR* promoter by exogenous HNF4α. The results of gel mobility shift assays and chromatin immunoprecipitation assays showed that HNF4α was bound to the promoter region containing the DR1 element. A knock-down of HNF4α by siRNA significantly decreased expression levels of endogenous PXR mRNA in HepG2 cells. Furthermore, expression levels of PXR mRNA positively correlated with those of HNF4α mRNA in 18 human liver samples. These results suggested that HNF4α transactivated the human *PXR* gene by binding to the DR1 element located at −88/−76 of the promoter and was involved in the expression of PXR in the human liver.

Keywords: pregnane X receptor (PXR); hepatocyte nuclear factor 4alpha (HNF4α); nuclear receptor; transcriptional regulation; human liver

Pregnane X receptor (PXR; NR1I2) is a member of the orphan nuclear receptor family of ligand-activated transcriptional factors and is expressed abundantly in the liver.[1–3] Human PXR is a key transcriptional regulator of a number of drug-metabolizing enzyme genes, including *CYP3A4*, *CYP2B6*, *CYP2C9* and *UGT1A1*, and of several important drug transporter genes, including *MDR1* and *MRP2*.[1,3–9]

While human PXR is known to activate many genes at the transcriptional level, studies on the molecular mechanism of human *PXR* gene regulation are limited. Pascussi *et al.*[10] reported that dexamethasone increased the PXR mRNA but did not influence the stability of PXR mRNA in human primary hepatocytes, suggesting that PXR is regulated at the transcriptional level by the glucocorticoid receptor. Recently, Aouabdi *et al.*[11] identified several putative protein/DNA interaction sites by an *in silico* analysis of the human *PXR* proximal promoter and showed that peroxisome proliferators-activated receptor α was involved in transcriptional activation of the human *PXR* gene. However, functional contributions of other factors to transcriptional regulation of the human *PXR* gene remain unclear.

Hepatocyte nuclear factor 4alpha (HNF4α; NR2A1), a member of the nuclear receptor family, has been reported to play a significant role in the regulation of many genes expressed in the liver.[12,13] It has been reported that mouse *Pxr* promoter has an HNF4α binding site and that HNF4α is required for expression of PXR in fetal hepatocytes.[14,15] However, the role of HNF4α in transcriptional regulation of

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Abbreviations: PXR, pregnane X receptor; DR1, direct repeat 1; HNF4α, hepatocyte nuclear factor 4alpha; CYP, cytochrome P450; PCR, polymerase chain reaction; WT, wild-type; MT, mutant-type.

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the human *PXR* gene has not been clarified.

Therefore, we examined the involvement of HNF4α in transcriptional regulation for the human *PXR* gene. Human PXR has several transcripts.[1-3,16,17] In this study, we focused on the upstream sequences from the transcriptional start site of PXR mRNA identified as the major transcript in the human liver.[18]

Materials and Methods

Cloning and plasmids construction: A search for direct repeat 1 (DR1) elements within the sequences (3.6 kb) of the human *PXR* proximal promoter (GenBank accession no. **AF364606**) as putative HNF4α binding sites was carried out by using NUBIScan[19] (http://www.nubiscan.unibas.ch/) with a threshold raw score setting of 0.7. Based on the results of the search, cloning of promoter sequences of the human *PXR* gene (3.6 kb) and construction of different deletion reporter vectors were performed. The *PXR* promoter region (−3568/+54) was amplified by PCR with specific primers, 5′-ACCTTTTCCCC-TCAATCCCTTTAT-3′ (forward) and 5′-AAGGCAGTGCTT-CCTCTTCCCGTCCT-3′ (reverse), and human genomic DNA extracted from human whole blood (Promega, Madison, WI, USA) as a template. The DNA fragment was subcloned into the pCR-Blunt II-TOPO vector (Invitrogen, Carlsbad, CA, USA). Subsequently, the region (−3568/+54) was re-amplified by PCR using the pCR-Blunt II-TOPO vector containing the −3568/+54 region as a template. The forward primer described above and a reverse primer including *Hind*III recognition sites as indicated by small letters, 5′-TCaagcttAAGG CAGTGCTTCCTCTTCC-3′, were used. After *Nhe*I and *Hind*III digestion, the fragment was ligated into the pGL3-Basic vector (Promega), resulting in PXR-3297/+54. Six deletion constructs were generated by nested PCR of the primary clone using the following forward primers: 5′-CCgctagcGGCAACA-TAATGAGACCTCGTCT-3′ (PXR-2428/+54), 5′-CAgctagc-CCTGGGTGACACAGCAAAAC-3′ (PXR-2258/+54), 5′-CC-gctagcGGCAGTAAGTCCCCAGCAGT-3′ (PXR-1194/+54), 5′-ACgctagcTAACACTATCCAGGGAGGTGGTT-3′(PXR-346/+54), 5′-CTgctagcTTGCTAGTTCAAGTGCTGGAC-3′ (PXR-104/+54) and 5′-GAgctagcATGGAGCCGCTTAGTGCCTA-3′ (PXR-64/+54). These forward primers contain *Nhe*I recognition sites as indicated by small letters. The reverse primer used was the same as that used in re-amplification of the −3568/+54 region. After *Nhe*I and *Hind*III digestion, the obtained 5′-deletion fragments were transferred into the pGL3-Basic vectors as described above. All deletion constructs are named as shown in parentheses.

The coding region of human HNF4α2 (Genbank accession no. **NM_000457**) was amplified from cDNA prepared from human liver (Human and Animal Bridging Research Organization, Chiba, Japan) by using specific primers, 5′-CGTGGA-GGCAGGGAGAATGCGACT-3′ (forward) and 5′-CAAGGGT-GGCAGTGGGATGTGGC-3′ (reverse). The PCR fragment was cloned into the pCR-Blunt II TOPO vector. After digestion with *Bam*HI and *Not*I, the fragment was inserted into the pTARGET mammalian expression vector (Promega), resulting in hHNF4α/pT.

The DNA sequences of all constructs were determined by using a Dye Terminator Cycle Sequencing-Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) and a CEQ 2000 DNA Analysis System (Beckman Coulter).

PCR mutagenesis of DR1 element at −88/−76 in *PXR* promoter: The wild-type DR1 element (TGGACTT-GGGACT) at −88/−76 in the *PXR* promoter was mutated to TGGACAGGACT by site-directed mutagenesis as described elsewhere.[20] Primers, 5′-AGTTCAAGTGCTGGACAGGACTTA-GGAGGGGC-3′ (forward) and 5′-GCCCCTCCTAAGTCCT-GTCCAGCACTTGAACT-3′ (reverse), were designed for introduction of mutation into the reporter plasmids, PXR-3297/+54 and PXR-104/+54, resulting in PXR-3297/+54 mt and PXR-104/+54 mt, respectively.

Cell cultures: The human hepatocellular carcinoma cell line HuH-7 was obtained from Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). HuH-7 cells were cultured in RPMI 1640 medium (Invitrogen) with 5% CO_2 at 37°C. The medium was supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sanko Junyaku, Tokyo, Japan) and 100 units/mL penicillin G and 100 μg/mL streptomycin (Invitrogen).

Transient transfection and dual luciferase assays: HuH-7 cells were plated on a 24-well dish one day before transfection. The reporter plasmids (200 ng/well), phRL-TK vector (4 ng/well, Promega) and human HNF4α expression vector (hHNF4α/pT, 100 ng/well) were transfected into cells by using a Trans IT (Mirus, Madison, WI) according to the manufacturer's protocol. At 24 h after transfection, luciferase reporter activities were measured by using a Dual-Luciferase reporter assay system (Promega) according to the manufacturer's protocol. The *Renilla* luciferase activities of the control plasmid phRL-TK were used to normalize the results of the firefly luciferase activities of the reporter plasmids.

Gel mobility shift assays: Gel mobility shift assays were performed by using double-stranded DNA labeled with [γ-^{32}P]ATP (GE Healthcare, Piscataway, NJ, USA) and 10 μg of the nuclear extracts prepared from HepG2 cells or 40 μg of in vitro transcribed/translated human HNF4α protein synthesized using TNT T7 Quick Coupled Transcribed/Translation Systems (Promega) following the manufacturer's protocol as previously described.[20] Oligonucleotide sequences containing wild-type DR1 element of the human *PXR* promoter used as a probe were designed as follows: 5′-AAGTGCTGGACTT-GGGACTTAGGAG-3′ (hPXRDR1WT). For competition experiments, the mutated sequences of hPXRDR1WT and the HNF4α-binding sequences located in apolipoprotein CIII promoter and the mutated sequences were designed as follows: 5′-AAGTGCTGGACAGGACTTAGGAG-3′ (hPXRDR1MT), 5′-TCGAGCGCTGGGCAAAGGTCACCTGC-3′ (APF1WT) and 5′-TCGAGCGCTAGGCACCGGTCACCTGC-3′ (APF1MT).[12] Only the sequences of the sense strands are displayed above, and mutated nucleotides are underlined. Unlabeled competitive double-stranded DNA was added to the binding reaction

mixture at 10-, 25- and 50-fold excess of the probe amount before addition of the probe. For supershift assays, either 2 μg of IgG against HNF4α (2ZK9218H, Perseus Proteomics, Tokyo, Japan) or control mouse IgG (sc-2025, Santa Cruz Biotech, Santa Cruz, CA, USA) was added to the binding reaction mixture at room temperature for 30 min before addition of the probe.

Chromatin immunoprecipitation (ChIP) assays: ChIP assays were performed by using a ChIP-IT kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocol as previously described.[21] Human liver (from a 34-year-old black female) was supplied by the National Disease Research Interchange (Philadelphia, PA, USA) through HAB Research Organization (Tokyo, Japan), and this study was approved by the Ethics Committee of Chiba University (Chiba, Japan). The human liver tissue (*ca.* 3.2 g) was isolated and chopped on ice and then cross-linked by 1% formaldehyde for 12 min. Cross-linking was stopped by the addition of glycine solution. The chromatin was sheared by using an Ultrasonic disruptor UD-201 (TOMY, Tokyo, Japan) at 25% power with 9 pulses. The sheared chromatin (*ca.* 40 μg) was immunoprecipitated with either control mouse IgG or anti-HNF4α IgG. After incubating for 4 h at 4°C with gentle rotation, salmon sperm DNA/protein G agarose was added to the mixture and it was further incubated for 1.5 h under the same conditions. The DNA fragment was purified and used as a template for PCR. The DNA sequences around the DR1 (−195/+46) and a non-DR1 (+25495/+25729, located in intron 1) elements of the *PXR* genes were amplified by using primers, 5′-TCTTCCCC-TTTTCCTGTGTTTTTG-3′ (forward) and 5′-GCTTCCTCTT-CCCGTCCTAGTCATAG-3′ (reverse) for DR1 and 5′-CTCA-TTTCTTTCCATTTTTCTCTTC-3′ (forward) and 5′-ATACCT-GCTGCTCTTTGCTAGTGAC-3′ (reverse) for non-DR1, respectively. PCR conditions were as follows: 94°C for 2 min, followed by 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, 40 cycles. The amplicons were visualized by ethdiumbromide staining, and the sequence of each amplicon was confirmed by direct DNA sequencing.

Knock-down of HNF4α by siRNA: Two pre-designed siRNAs for human HNF4α (Genome Wide siRNA, Hs_HNF4A_1 and Hs_HNF4A_9) and a negative control siRNA (AllStar Negative Control siRNA) were purchased from Qiagen (Hilden, Germany). The siRNAs were transfected into HepG2 cells by a reverse transfection method using siPORT NeoFX (Applied Biosystems, Foster City, CA, USA) according to the manufacture's protocol except that siPORT NeoFX (2.5 μl/well) and Opti-MEM (50 μl/well) were used. HepG2 cells (0.9×10^5 cells/well) were transferred to the 12-well culture plate containing the siRNA/transfection agent complexes. At 48 h after transfection, the cells were harvested by trypsinization. Subsequently, cell homogenates and total RNA were prepared for Western blotting and analyses of mRNA expressions, respectively.

Cell homogenates were prepared by sonicating siRNA-transfected HepG2 cells in SET buffer (pH 7.4) containing a protease inhibitor cocktail (EMD Biosciences, San Diego, CA, USA). Proteins (6 μg/lane) was separated on an 8% SDS-polyacrylamidegel and transferred to nitrocellulose membranes. The membranes were incubated for overnight at 4°C with primary mouse anti-human HNF4α IgG (1:1000 dilution, Perseus Proteomics) or mouse anti-human β-actin IgG (1:4000 dilution, Sigma-Aldrich, St. Louis, MO, USA) in 1% bovine serum albumin in phosphate buffered saline and were incubated with hourseradish peroxidase-conjugated anti-mouse IgG (1:6000 dilution, Sigma-Aldrich). Protein bands were visualized by using an ECL Western Blotting Analyses System (GE Healthcare) and LAS-1000 plus (Fujifilm, Tokyo, Japan).

To measure the mRNA levels of HNF4α and PXR, cDNAs prepared from total RNA of siRNA-transfected cells were subjected to quantitative real-time PCR with ABI Prism 7000 Sequence Detection System (Applied Biosystems). The mRNA levels of HNF4α and PXR were determined by using Gene Expression Assays (Applied Biosystems), gene expression products for human HNF4α (Hs00230853_m1) and human PXR (Hs00243666_m1), respectively. The expression levels were normalized against that of 18S rRNA determined by Eukaryotic 18S rRNA endogenous control (Applied Biosystems).

Analyses of mRNA expressions in human liver tissues: Human liver tissues ($n=18$) were obtained from Japanese patients undergoing partial hepatectomy for treatment of metastatic liver tumors at the Division of Oncology, Department of Medicine, National Cancer Center Hospital East (Chiba, Japan). This study was approved by the ethics committee of Chiba University. Liver tissues were rapidly frozen in liquid nitrogen immediately after excision and were stored in liquid nitrogen until use. The mRNA levels of HNF4α and PXR were measured as described above except that four housekeeping genes, β-glucronidase, cyclophilin, acidic ribosomal protein and glyceraldehydes-phosphate dehydrogenase, were selected as internal standards based on the previously reported methods in which the levels of expression of target genes were normalized by the averaged value of multiple internal standard genes.[22,23] The expression levels were expressed as arbitrary unit, with the lowest expression levels normalized to 1.

Statistical analyses: Data are presented as means ± S.D. Comparison of two groups was made with Student's *t* test. Comparison of multiple groups was made with ANOVA followed by Post-hoc test of Scheffe's F. $P<0.05$ was considered statistically significant.

Results

Identification of DR1-type binding sites in the 5′-flanking region of human *PXR* gene: As shown in **Table 1**, nine potential DR1-type binding sites were found in the sequences of the human *PXR* gene proximal promoter (3.6 kb). Among them, only the −88/−76 position was detected as a DR1-type binding site in both positive and negative strands.

Effect of HNF4α on transcriptional activity of the human *PXR* promoter in HuH-7 cells: Co-transfection analyses were performed by using HuH-7 cells to examine the ef-

Table 1. Putative DR1 elements identified in the 5′ flanking region of the *PXR* gene

Position	Sequence (strand)	Raw score	Z score
−88/−76	TGGACTtGGGACT (+)	0.723293	5.840614
	AGTCCCaAGTCCA (−)	0.800265	6.559396
−263/−251	AGGACAgAGACCC (+)	0.776475	6.337245
−467/−455	GGGGCTgAGCACA (−)	0.733091	5.932116
−811/−799	GGGGCAtGGGCCC (−)	0.804334	6.597399
−1214/−1202	GGGTCCcAGTCCC (+)	0.739553	5.992457
−1235/−1223	AGGACAgAGCTCT (+)	0.778838	6.359314
−1449/−1437	AGCACAcTGTTCA (+)	0.739478	5.991762
−2287/−2275	TGGTCAtGGCTCA (−)	0.748861	6.079374
−2737/−2725	GGCACTtAGGGCA (−)	0.712435	5.739223

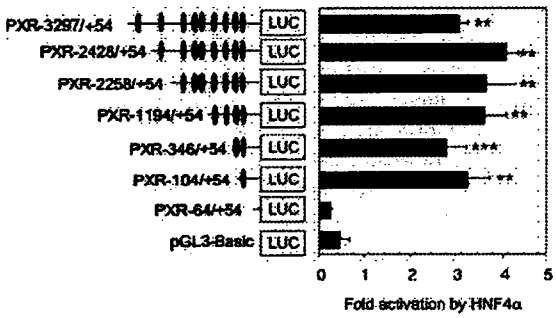

Fig. 1. Effect of exogenous HNF4α on transcriptional activity of the *PXR* promoter in HuH-7 cells
An expression vector for HNF4α or an empty vector was co-transfected with seven different constructs of PXR/pGL3-Basic Vector or empty pGL3-Basic Vector into HuH-7 cells. Firefly luciferase activity was normalized with the *Renilla* luciferase activity and was represented by the ratio of normalized luciferase activity in the presence of HNF4α to this activity in the absence of HNF4α. $^{**}p<0.01$ and $^{***}p<0.001$ compared with the value in cells co-transfected with an empty vector. Data are expressed as means ± S.D. of three separate experiments, each performed in triplicate.

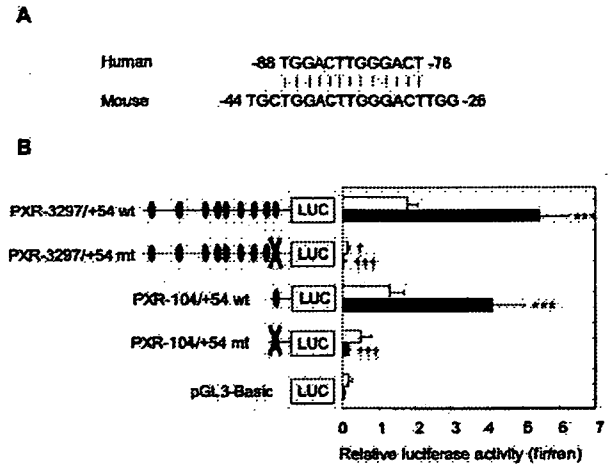

Fig. 2. Mutational analyses for the DR1 element of the *PXR* promoter in HuH-7 cells
A: The HNF4α response element is conserved between the mouse and the human *PXR* promoters.
B: HuH-7 cells were transiently transfected with four different constructs of PXR/pGL3-Basic Vector (PXR-3297/+54 wt, PXR-3297/+54 mt, PXR-104/+54 wt or PXR-104/+54 mt) or an empty pGL3-Basic Vector. An expression plasmid for HNF4α (closed columns) or an empty vector (open columns) was co-transfected into the cells. Relative luciferase activity was expressed as the firefly luciferase activity normalized with the *Renilla* luciferase activity. Data are expressed as means ± S.D. of three separate experiments, each performed in triplicate. $^{***}p<0.001$ compared with the value in cells co-transfected with an empty vector. †$p<0.05$ and †††$p<0.001$ compared with the value in cells co-transfected with wild-type constructs.

fect of HNF4α on promoter activity of the human *PXR* gene. HuH-7 cells were used since this cell line expresses endogenous HNF4 at the greatly lower level than HepG2 cells at protein levels (data not shown). Seven constructs containing various numbers of DR1-type binding sites of the human *PXR* promoter region and HNF4α expression vector were co-transfected into HuH7-cells. As shown in **Figure 1**, deletion of the most proximal binding site completely abolished the transcriptional activation of the human *PXR* promoter by HNF4α whereas deletion of any other sites could not affect the activation.

Effects of introduction of mutation to the DR1 element on the transcriptional activation of the human *PXR* promoter by HNF4α in HuH-7 cells: As shown in **Figure 1**, transcriptional activation of the human *PXR* promoter by HNF4α in HuH-7 cells was abolished by deletion of the region −104/−65, which contains a DR1 element (**Table 1**). In addition, the sequence of the DR1 element is completely consistent with that of HNF4α-response element in the mouse *Pxr* gene (**Fig. 2A**). Therefore, mutation analyses were performed to examine whether HNF4α required the DR1 element for its transactivation ability. As shown in **Figure 2B**, HNF4α enhanced the promoter activities of wild-type constructs (PXR-3297/+54 wt and PXR-104/+54 wt) by about 3 fold, but introduction of the mutation to this element located at the −88/-76 region completely disrupted the transcriptional activation of the mutated human *PXR* promoter (PXR-3297/+54 mt and PXR-104/+54 mt) by HNF4α.

Binding of HNF4α to the DR1 element of the human *PXR* promoter: Gel mobility shift assays were performed to examine whether HNF4α could bind to the region −88/−76 of the *PXR* promoter *in vitro*. Because it has been reported that HepG2 cells endogenously express HNF4α,[24,25] nuclear extracts of HepG2 cells were used for the assays. As shown in **Figure 3A**, when nuclear extracts were incubated with the labeled probe, two retarded bands were observed (lane 2). The lower band was efficiently competed out by an unlabeled probe (lane 3), but not by an unlabeled mutated probe (lane 4). An unlabeled APF1WT probe derived from the HNF4α-binding sequence located in apolipoprotein CIII promoter also competed with the binding (lane 5), whereas the mutated probe APF1MT did not (lane 6). Moreover, a supershifted band

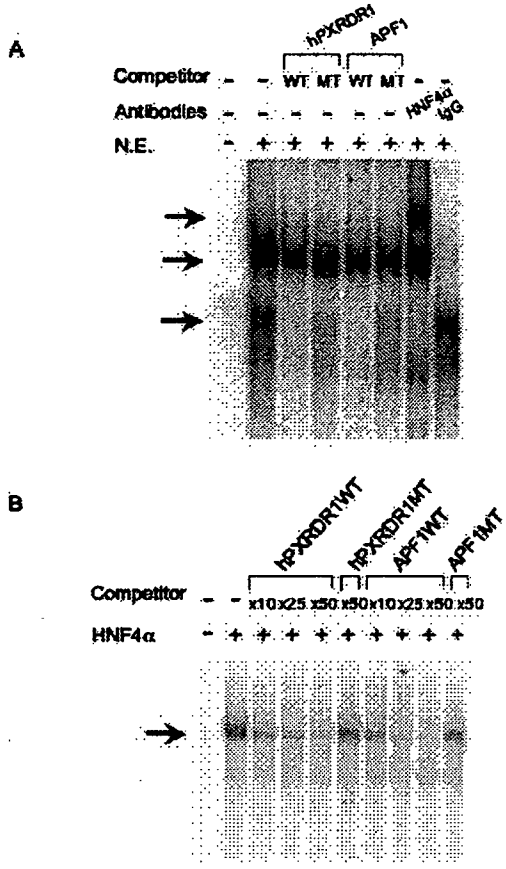

Fig. 3. Specific binding of HNF4α to the DR1 element of the human *PXR* promoter *in vitro*
A: Nuclear extracts (N.E.) prepared from HepG2 cells were subjected to gel mobility shift assays. A probe (PXR-94/-70) was labeled with ^{32}P and incubated with the nuclear extracts. For competition assay, nuclear extracts were incubated with a 50-fold excess of unlabeled DNA before addition of the probe. The lower arrow indicates the position of the HNF4α-dependent shifted band, the middle arrow indicates non-specific band and the upper arrow indicates the supershifted HNF4α complexes.
B: *In vitro* transcribed/translated HNF4α proteins were subjected to gel mobility shift assays. For competition assay, HNF4α proteins were incubated with 10-, 25- and 50-fold excess of unlabeled DNA before addition of the probe. Arrow indicates the position of the HNF4α-dependent shifted band.

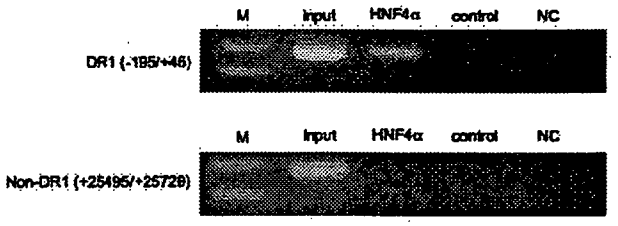

Fig. 4. Binding of HNF4α to the DR1 element of the human *PXR* promoter *in vivo*
Chromatin immunoprecipitation (ChIP) assays were performed using the sheared genomic DNA extracted from human liver with control mouse IgG and anti-HNF4α IgG, respectively. A region (−195/+46, upper panel) that contains the DR1 element and a region (+25495/+25729, lower panel) that does not contain DR1 element were amplified by PCR, respectively. M, DNA size marker; Input, control sheared genomic DNA; HNF4α, sheared genomic DNA immunoprecipitated with anti-HNF4α IgG; Control, sheared genomic DNA immunoprecipitated with control mouse IgG; NC, non-template control.

was observed by addition of anti-HNF4α antibodies (lane 7). Since non-specific binding derived from nuclear extracts of HepG2 cells was observed in **Figure 3A**, the assays using in vitro transcribed/translated HNF4α were also performed (**Fig. 3B**). When HNF4α was incubated with the labeled probe, a specific band was observed (lane 2), which was competed out by unlabeled probes (lanes 3–5 and lanes 7–9) but not by unlabeled mutated probes (lanes 6 and 10).

ChIP assays were performed using human liver to examine whether HNF4α could bind to the *PXR* gene promoter *in vivo* (**Fig. 4**). Following DNA extraction of the immunoprecipitated chromatin, PCR was performed to detect the occupancy of DR1 element of the *PXR* gene by HNF4α. The DR1 element was much more abundant in DNA extracted from chromatin immunoprecipitated with anti-HNF4α IgG than in that with control mouse IgG. On the other hand, no DNA fragment around the non-DR1 elements of the *PXR* gene was detected in both extracted DNA samples.

Involvement of HNF4α on the expression of endogenous PXR in HepG2 cells: Since HepG2 cells endogenously express HNF4α and PXR,[24–26] we studied an involvement of HNF4α in the expression of endogenous PXR in HepG2 cells using two different siRNAs to knock down endogenous HNF4α. Western blot analyses showed 5- and 2-fold reductions of HNF4α protein in HepG2 cells by transfection of siHNF4α#9 and siHNF4α#1, respectively (**Fig. 5A**). In HepG2 cells transfected with a negative control siRNA, expression level of HNF4α protein was not reduced (**Fig. 5A**, siControl). In addition, β-actin protein levels, which were measured as a control, were not modified after siRNA transfection. As shown in **Figure 5B**, expression levels of HNF4α mRNA in HepG2 cells were reduced to less than 23% and 42% of control, when two different siRNAs for HNF4α (siHNF4α# 9 and siHNF4α#1) were transfected respectively at the concentration-range of 3 to 30 nM. Expression levels of PXR mRNA in the HepG2 cells transfected with siHNF4α#9 and siHNF4α#1 were reduced to less than 45% and 50% of control, respectively.

Correlation between expression levels of PXR and HNF4α in human liver tissues: To examine whether HNF4α contributes to the expression of PXR mRNA in the human liver, the expression levels of PXR and HNF4α mRNAs were measured in each of the 18 Japanese liver samples by using quantitative real-time PCR. As shown in **Figure 6**, large interindividual differences were observed in both PXR and HNF4α mRNA expression levels (7.5- and 16.4-folds, respec-

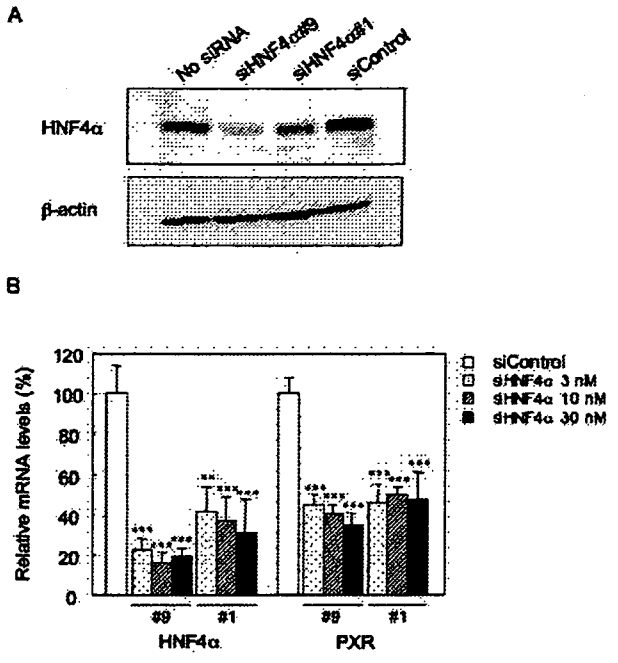

Fig. 5. Effects of HNF4α knock-down on PXR mRNA expression in HepG2 cells
A: Western blot analyses of HNF4α and β-actin expression in cell homogenates from HepG2 cells at 48 h after siRNA transfection. B: HepG2 cells were transfected with 3, 10, and 30 nM of siRNA for HNF4α (each of siHNF4α #9 and siHNF4α #1) or 10 nM of negative control siRNA (siControl). At 48 h after transfection, total RNA was extracted for analysis by quantitative real-time PCR as described in *Materials and Methods*. Expression levels of mRNA normalized by 18S rRNA are expressed as percentages relative to control set for each gene. Each value is the mean ± S.D. of thee independent experiments. **, $p<0.01$ and *** $p<0.001$ compared with the value in cells transfected with siControl.

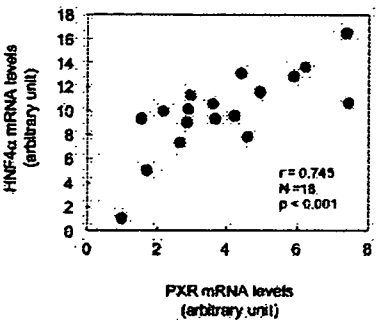

Fig. 6. Correlation of the expression levels of PXR and HNF4α mRNAs in 18 human liver tissues
Expression levels of PXR and HNF4α mRNAs in Japanese liver tissues were determined by quantitative real-time PCR as described in *Materials and Methods*. Expression levels of mRNA normalized by 4 different endogenous controls are expressed as arbitrary unit, with the lowest mRNA expression level normalized to 1. The correlation coefficient (r) was calculated by the least-squares regression method.

tively). The highly positive and statistically significant correlation ($r=0.745$, $p<0.001$) was obtained between PXR and HNF4α mRNA expression levels.

Discussion

The present study showed that the transcriptional activities of human *PXR* promoter were increased by exogenous HNF4α in HuH-7 cells (**Fig. 1**). Deletion of the −104/−65 region from the *PXR* promoter and introduction of mutation to the DR1 element located in the −88/−76 region completely disrupted transactivation of the *PXR* promoter by HNF4α (**Figs. 1 and 2**). In addition, HNF4α was bound to the region containing the DR1 element located in the −88/−76 region in the *PXR* promoter *in vitro* and *in vivo* (**Figs. 3 and 4**). These results suggest that HNF4α increases the transcriptional activity of the human *PXR* promoter through binding to the DR1 element located in the −88/−76 region of the human *PXR* promoter.

Although nine putative HNF4α binding sites were identified in computer analyses of the human *PXR* promoter sequences by using NUBIScan (**Table 1**), no large difference was observed among the levels of transactivation of the deletion constructs except for PXR-64/+54 by HNF4α in HuH-7 cells (**Fig. 1**). In addition, the introduction of mutation to the DR1 element located at −88/−76 in construct PXR-3297/+54 wt completely disrupted the transactivation of the *PXR* promoter by HNF4α (**Fig. 2**), despite the fact that the other putative DR1 elements located in the −3297/+54 region of the *PXR* promoter were not mutated. Moreover, binding of HNF4α to two other putative DR1 elements (−263/−251 and −1214/−1202) was not observed in gel mobility shift assays (data not shown). These results suggest that the DR1 element located in the −88/−76 region of the human *PXR* promoter is functional to increase the transcriptional activity of the human *PXR* promoter but the other putative DR1 elements are not. It has been reported that HNF4α could interact with transcription factor TFIIB and recruit it to the promoter complex, leading to the formation of a preinitiation complex acting downstream of TFIIB.[27] Furthermore, functional HNF4α binding sites have been identified in proximal regions of *CYP2A6*, *CYP2C9* and *CYP2D6* promoters.[21,28-31] Therefore, these findings suggest that a proximal region of the *PXR* promoter (−88/−76) is important for transactivation of the human *PXR* gene by HNF4α, although it remains unclear whether regions other than −3297/+54 of human *PXR* gene relate to activate the human *PXR* gene by HNF4α.

It has been reported that human PXR shows a tissue-specific expression and that PXR mRNA is abundantly expressed in the human liver.[1-3] In general, tissue-specific gene expression is often mediated by the presence of binding sites for tissue-enriched transcription factors in the promoters of the genes. HNF4α is abundant in the liver and plays a significant role in the regulation of many genes expressed in the liver.[13,32] The results shown in the present study suggests that HNF4α is involved in transcriptional activation of the human *PXR* gene by

cell-based reporter gene assays (**Figs. 1 and 2**), gel mobility shift assays (**Fig. 3**) and ChIP assays using human liver tissue (**Fig. 4**). In addition, expression levels of endogenous PXR mRNA in HepG2 cells were reduced by knock-down of HNF4α by siRNA (**Fig. 5**). Furthermore, a highly positive and statistically significant correlation was observed between expression levels of PXR and HNF4α mRNA in 18 human liver samples (**Fig. 6**). Therefore, HNF4α is thought to be one of important transcriptional factors responsible for liver-enriched expression of human PXR.

Pxr expression was absent from fetal livers of HNF4α-null mice, suggesting expression of Pxr in fetal mouse liver is highly dependent on HNF4α.[15] However, no differences in Pxr expression were shown between mature HNF4α-null mice and control mice. This suggests that the mechanism of *Pxr* gene regulation by HNF4α differs between the adult and fetal livers in mice. On the other hand, the expression levels of PXR in human adult liver samples were positively correlated with those of HNF4α (**Fig. 6**). The present results of ChIP assay using human adult liver tissues showed that HNF4α was bound to the DR1 element in *PXR* promoter (**Fig. 4**). Furthermore, the expression levels of PXR in human primary hepatocytes were repressed by siRNA for HNF4α.[33] Therefore, it is likely that the expression of PXR is highly dependent on HNF4α in adult liver at least in humans.

In conclusion, the present study suggested that HNF4α increased transcriptional activity of the human *PXR* promoter through binding to the DR1 element located in the −88/−76 region of the human *PXR* promoter. Since the mRNA expression levels of PXR in human liver samples were positively correlated with those of HNF4α, which plays a significant role in the regulation of many genes expressed in the liver, HNF4α is thought to be one of important transcriptional factors responsible for liver-enriched expression of human PXR.

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1. 代謝特性，クリアランス，相互作用の予測
8) 代謝酵素およびトランスポーターの誘導機構と予測（転写因子による制御）

小林カオル・千葉　寛

薬物代謝酵素および薬物トランスポーターの誘導の多くは遺伝子の転写活性化によるものである。転写活性化は，主に標的遺伝子の5'上流領域に核内レセプターを含む転写因子（AhR, PXR, CAR など）が結合することに起因する。また，HNF4により転写活性化作用が相乗的に増強することも報告されている。これらの転写活性化には，転写因子に結合し，遺伝子のクロマチン構造を変化させるコアクチベーターも必須である。本稿では，薬物代謝酵素および薬物トランスポーターの誘導機構と誘導の予測実験系について概説する。

はじめに

薬物代謝酵素および薬物トランスポーターの誘導は，医薬品の体内動態および薬理効果を変動させ，薬物間相互作用を引き起こす要因となる。近年，誘導現象を観察できる初代培養肝細胞系の確立と核内レセプター[用解1]研究の急激な進歩により，チトクロムP450（P450あるいはCYP）をはじめとした種々の薬物代謝酵素および薬物トランスポーターの誘導機構が解明されつつある。タンパクの発現における誘導機構は，転写活性化[用解2]，mRNAの安定化，タンパクの安定化の3つに分類できるが，現在までに明らかにされている薬物代謝酵素の誘導機構の多くは，転写活性化によるものである。本稿では，転写活性化による薬物代謝酵素と薬物トランスポーターの誘導機構と予測実験系について最新の知見を含めて概説する。

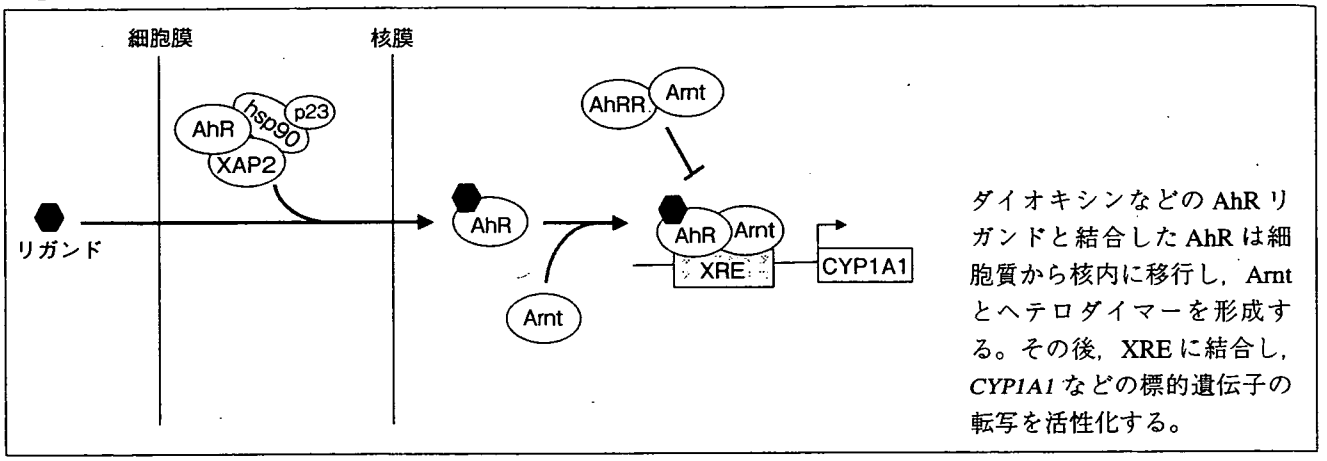
Ⅰ．代謝酵素の誘導機構

1. CYP1A1およびCYP1A2

CYP1A1およびCYP1A2は喫煙により誘導される。その機構として，タバコに含まれる多環芳香族炭化水素が，転写因子の1つであるarylhydrocarbon receptor（AhR）を活性化することにより*CYP1A1*遺伝子を誘導するAhRを介した誘導機構がよく知られている。図❶に示したように，リガンド非存在下において，AhRは細胞質中にhsp90, XAP2, p23と複合体を形成して存在しているが，AhRにリガンド（ダイオキシン，β-ナフトフラボン，3-メチルコラントレンなど）が結合するとAhRは核へ移行する。核移行したAhRは核内に存在するAhR nuclear translocator（Arnt）とヘテロダイマーを形成し，*CYP1A1*遺伝子上のxenobiotic responsive element

key words
AhR, CAR, PXR, HNF4, GR, 核内レセプター，核移行，レポータージーンアッセイ，コアクチベーター，TR-FRET

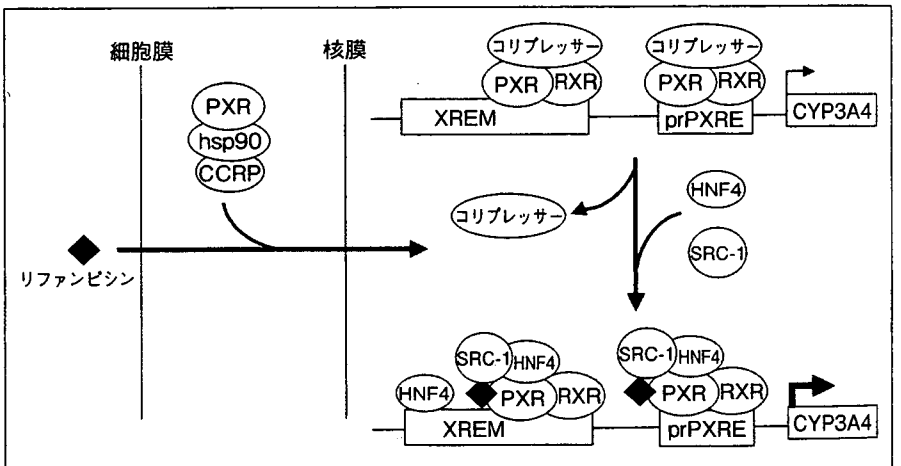
図❶　AhR を介した CYP1A1 の誘導機構



ダイオキシンなどの AhR リガンドと結合した AhR は細胞質から核内に移行し，Arnt とヘテロダイマーを形成する。その後，XRE に結合し，*CYP1A1* などの標的遺伝子の転写を活性化する。

（XRE）に結合する。AhR を介した *CYP1A1* 遺伝子の誘導には，AhR と類似した構造をもつ AhR repressor（AhRR）によるネガティブフィードバック機構も存在している。*CYP1A1* 遺伝子の上流には 5'-TNGCGTG-3' というコンセンサス配列からなる XRE が複数個存在し，AhR による誘導に関与している。一方，*CYP1A2* 遺伝子の誘導に関しては，AhR の関与は示唆されているものの，*CYP1A1* 遺伝子にみられるような XRE は存在せず，誘導に関連する領域は明らかではない。*CYP1A1* 遺伝子と *CYP1A2* 遺伝子は，染色体 DNA 上において向かいあって存在しており，*CYP1A1* 遺伝子の 5' 上流域に存在する XRE を *CYP1A2* 遺伝子の転写においても利用していることが示唆されている[1]。また，*CYP1A2* 遺伝子の 5' 上流域に存在する XRE とは異なる配列に結合した何らかの因子に AhR と Arnt が結合することにより転写活性化されるとの報告もある[2]。

図❷　リファンピシンによる CYP3A4 の誘導機構



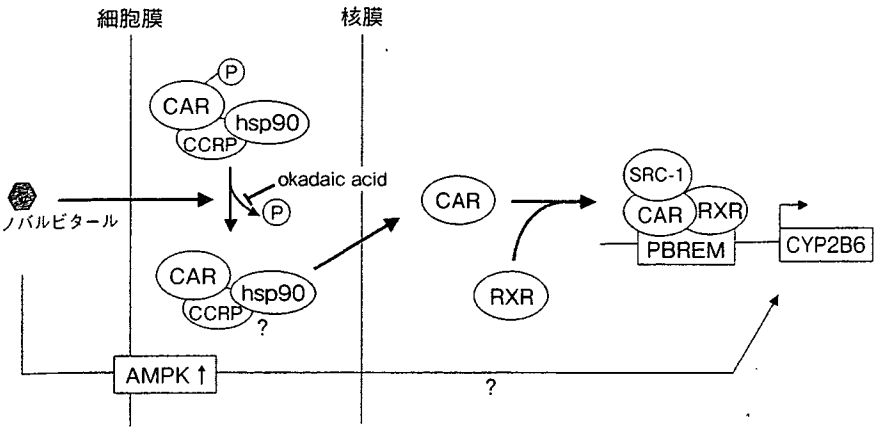
PXR は *CYP3A4* 遺伝子の上流に存在する XREM および prPXRE に RXR とのヘテロダイマーとして結合する。リガンド非存在下で，PXR はコリプレッサー[用解3]と結合しているが，リガンドであるリファンピシンの結合によりコリプレッサーを解離する。その後，HNF4 やコアクチベーターである SRC-1 をリクルートし，*CYP3A4* の転写を活性化する。PXR は CAR と同様，リガンド非存在下で CCRP および hsp90 と複合体を形成し，細胞質に局在するという報告もある。

2. CYP3A4

CYP3A4 は多くの医薬品代謝に関わる CYP 分子種であり，抗結核薬であるリファンピシン，抗てんかん薬であるフェニトインやカルバマゼピン，健康食品であるセントジョーンズワートなどによって誘導される。

CYP3A4 の誘導は，**図❷**に示したように，*CYP3A4* 遺伝子の 5' 上流領域の -160 bp 付近に ER6 モチーフを含んだ proximal PXR response element（prPXRE）および上流 -7700 bp 付近に direct repeat 3（DR3）モチーフを含んだ distal xenobiotic-responsive enhancer module（XREM）が存在し，これらのモチーフに核内レセプターであ

図❸ CARを介したCYP2B6の誘導機構



CARはCCRPおよびhsp90と複合体を形成し，細胞質に局在する。フェノバルビタール刺激によりCARは脱リン酸化され，核内に移行する。核内でCARはRXRとヘテロダイマーを形成し，*CYP2B6*遺伝子の上流に存在するPBREMにコアクチベーターとともに結合し転写活性化する。AMPKの活性化がCYP2B6を誘導するという報告があるが，CARの関与は明らかではない。

るpregnane X receptor（PXR）とretinoid X receptor（RXR）のヘテロダイマーが結合[用解4]することにより調節されている。PXRを介したリファンピシンによるCYP3A4の誘導は別の核内レセプターであるhepatocyte nuclear factor 4（HNF4）により相乗的に増大することが報告されている[3]。その機構として，XREM内のDR3モチーフの上流にHNF4が結合するためであると考えられていたが，最近，リファンピシンによりPXRへのHNF4とコアクチベーター[用解5]であるsteroid receptor coactivator -1（SRC-1）の結合が増強するためであることを示唆する知見も報告された[4]。

3. CYP2B6

CYP2B6は，P450を誘導する代表的な薬物であるフェノバルビタールによって強く誘導される。*CYP2B6*遺伝子の5'上流配列内には，phenobarbital-responsive enhancer module（PBREM）と呼ばれるフェノバルビタール誘導性エンハンサー領域が同定されている。PBREM配列はマウス，ラット，ヒトの*CYP2B*遺伝子で保存されており，2つのDR4モチーフが含まれている。

図❸に示したようにPBREM配列内のDR4モチーフには，constitutive androstane receptor（CAR）とRXRのヘテロダイマーが結合し，*CYP2B6*遺伝子の転写を活性化させる。CARは非誘導下では肝の細胞質に存在し，フェノバルビタール刺激により核へ移行する。CARの細胞質への局在には，hsp90およびcytoplasmic CAR retention protein（CCRP）が関与することが示唆されている[5]。タンパク脱リン酸化酵素阻害薬であるokadaic acidが，フェノバルビタール刺激によるCARの核移行を阻害することは以前から明らかとなっていたが，最近，マウスCARの202番目のセリンの脱リン酸化がCARの核移行の制御に関与していることを示唆する知見が報告された[6]。さらに，フェノバルビタールによるCYP2Bの誘導にAMP-activated protein kinase（AMPK）が関与することを示唆する知見も報告されている[7]。

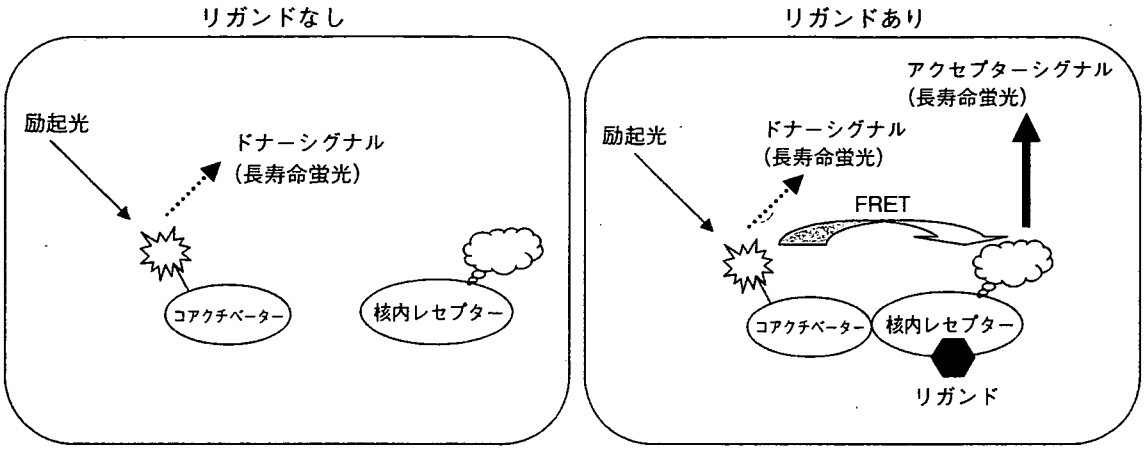
4. CYP2C8およびCYP2C9

CYP2C8はパクリタキセルやピオグリタゾンの代謝，CYP2C9はワルファリンや非ステロイド性酸性抗炎症薬の代謝に関与する。これらは，リファンピシンやフェノバルビタールにより誘導される。

*CYP2C9*遺伝子の5'上流領域には，DR4モチーフ（-1800 bp付近）およびDR5モチーフ（-2700 bp付近）が存在し，PXRあるいはCARを介して発現が調節されている[8)9)]。また，-1670 bp付近に存在するglucocorticoid-responsive element（GRE）には，glucocorticoid receptor（GR）が結合し，GRリガンドであるデキサメタゾンによるCYP2C9の誘導を制御している[8]。vitamine D receptor（VDR）はDR3モチーフを認識するが，*CYP2C9*遺伝子のDR4にも結合し，VDRリガンドはCYP2C9 mRNAを増加させる[10]。

*CYP2C8*遺伝子の5'上流領域には，-8800 bp付

図❹　TR-FRETによるリガンド結合アッセイの原理



コアクチベーターに標識したドナーフルオロフォアに励起光を照射すると蛍光を生じる。リガンド結合により核内レセプターとコアクチベーターが結合するとドナーフルオロフォアからアクセプターフルオロフォアへの蛍光共鳴エネルギー転移（FRET）が起こり，長寿命蛍光を生じる。蛍光物質にエネルギー照射し，一定時間後に蛍光測定を行う時間分解測定法では，試料中の他の化合物やフルオロフォアの直接励起に由来する非特異的短寿命蛍光が消失した後の特異的長寿命蛍光のみを測定できる。ドナーフルオロフォアの代わりにランタニド錯体を使用することでドナーシグナルの長寿命蛍光が期待できる。

近のDR4モチーフにCARあるいはPXRが結合し，PXRおよびCARを介したCYP2C8の誘導に重要であることが示唆されている[11]。また，-1900 bp付近に存在するDR4モチーフには，GRが結合しデキサメタゾンによるCYP2C8の誘導を制御している。

Ⅱ．トランスポーターの誘導機構

1. MDR1（multidrug resistant protein 1）

ABC（ATP binding cassette）トランスポーターに分類される*MDR1*遺伝子は，小腸における薬物吸収の制御に重要な役割を果たしているP-糖タンパクをコードしており，リファンピシンにより誘導されることが知られている。リファンピシンは上述したように，PXRを介してCYP3A4を誘導する。*MDR1*遺伝子の5'上流領域には，-8000 bp付近にDR4モチーフが存在し，PXRとRXRのヘテロダイマーが結合することにより転写活性化が引き起こされる[12]。

2. MRP（multidrug resistance-related protein）

MDR1と同じABCトランスポーターに分類されるMRPは主に肝臓に発現しており，各種抱合体（グルタチオン抱合体，グルクロン酸抱合体，硫酸抱合体）を基質とし，それらを胆汁中へ排泄する重要な役割を担っている。マウスに関しては，ほとんどのMRP（Mrp2, 3, 5-7）がAhR，CARおよび塩基性ロイシンジッパー型転写因子であるnuclear factor- erythroid 2（NF-E2）-related factor 2（Nrf2）のリガンドあるいはアクチベーターにより誘導される[13]。MRP2およびMRP3はPXRリガンドによっても誘導される。

Ⅲ．誘導の予測系

1. 転写活性化

転写レベルでの誘導を予測するための実験系として最も一般的な手法は培養細胞を用いたレポータージーンアッセイである。転写因子結合領域をルシフェラーゼ遺伝子の上流に組み込んだものをレポーターベクターとし，転写因子発現ベクターとともにHepG2細胞やCV-1細胞などの哺乳動物細胞に発現させ，被験化合物の曝露後，細胞抽出液を用いてルシフェラーゼ活性を測定する。この方法により，被験化合物が転写因子を介して転写活性化を引き起こすかどうかを調べることができる。転写因子の相互作用や，コレギュレーター