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Proximal HNF1 element is essential for the induction of human UDP-glucuronosyltransferase 1A1 by glucocorticoid receptor

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Abbreviations:
UGT, UDP-glucuronosyltransferase
PB, phenobarbital
PBREM, PB response enhancer module
DE, distal element
DEX, dexamethasone
GR, human glucocorticoid receptor
GRE, glucocorticoid responsive element
CAR, constitutive androstane receptor
PXR, human pregnenolone xenobiotic receptor

ABSTRACT

Previous study showed noinduction of the reporter gene (−3174/+14) of UGT1A1 in HepG2 by bilirubin, but induction by dexamethasone (DEX). This induction was enhanced seven-fold by the co-expression of human glucocorticoid receptor (GR) and was inhibited by a GR antagonist, RU486, indicating stimulation by DEX-GR. Meanwhile, we could not detect stimulation by β-estradiol, phenobarbital or rifampicin (RIF) in the presence of GR. We investigated the position playing a role in this induction by GR in the promoter region of UGT1A1 using deletion mutants, and clarified the essential sequence (−75/−63) for the binding site of hepatocyte nuclear factor 1 (HNF1). However, GR did not bind directly to this sequence, because UGT-PE2 did not compete for binding to a glucocorticoid responsive element (GRE) probe in an electrophoretic mobility shift assay (EMSA) method. Labeled [^{32}P]DNA probe of HNF1 binds with nuclear extracts as shown by the EMSA. This shift of the complex of probe–protein was not inhibited by unlabeled GRE but was inhibited by unlabeled HNF1 element. This shift was not influenced by the addition of anti-GR, but was supershifted by the addition of anti-HNF1. GR did not stimulate the induction of HNF1, because we detected no-elevation of the mRNA level of HNF1 by reverse transcription-polymerase chain reaction (RT-PCR). Therefore, the induction of UGT1A1 by DEX-GR did not depend on the elevation of HNF1 but on the interaction of GR with HNF1 or the activation of HNF1 through the transcription of other proteins. Also given the lack of evidence of binding of DEX-GR to HNF1 in the EMSA, the data suggest that the mechanism of DEX-GRE effect on HNF1 is indirect by whatever mechanisms.

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HNF, hepatocyte nuclear factor
BIL, bilirubin
RIF, rifampicin
NE, nuclear extracts
PE, proximal element
PCN, pregnenolone 16α-carbonitrile
RT-PCR, reverse transcription-polymerase chain reaction

1. Introduction

During the metabolism of most xenobiotics, such as drugs, non-nutrient chemicals in foods, and pollutants, phase I metabolizing enzymes functionalize lipophilic drugs by adding active residues such as OH to the drugs, and then phase II metabolizing enzymes conjugate with water-soluble substances, such as UDP-glucuronic acid (UDPGA) for UDP-glucuronosyltransferase (UGT), sulfuric acid for sulfotransferase, and glutathione for glutathione S-transferase, to further functionalize the drugs [1]. These soluble conjugates are excreted in bile and urine by transporters. UGT is the most functional of the phase II enzymes. UGT transfers the glucuronic acid in UDPGA to ligands, such as steroid hormones, catecholamines, and bilirubin (BIL), to make water-soluble glucuronides [2]. The C-terminal portion of UGT also plays a role, such as binding with UDPGA and with membrane. Meanwhile, the N-terminal half of UGT plays roles in ligand recognition, providing substrate specificity and oligomerization [3]. UGT is a membrane-bound enzyme; UGT in microsomal preparations is activated by the addition of certain detergents, such as Triton X-100, suggesting that it internalized to inner microsomal vessels [4–6]. UGT has also species specificity and organ specificity. UGT is mostly found in the liver [7], but is also found in the kidney, gastric duct, lung, and brain [8].

There are many UGT species and they are classified into UGT1 and UGT2 families, with a molecular mass of 50–57 kDa [9]. The human UGT1A subfamily gene is found on chromosome 2, and nine kinds of UGT1 can be made by alternative splicing between several exon 1 and a constant exon 2–5 [10]. Also, the UGT2 family gene is found on chromosome 4 [11]. From a genetic map of the UGT1A subfamily, four pseudo genes of exon 1 have been declared [12]. UGT1A1 is a main enzyme in the conjugation of BIL and a defect in UGT1A1 causes hyperbilirubinemia and jaundice, such as Crigler–Najjar syndrome type II and Gilbert's syndrome. We previously analyzed the promoter region (−3174/+14) of the human UGT1A1 gene in relation to Gilbert's syndrome [13].

Regarding the enhancer region of UGT1A1, which is activated by phenobarbital (PB), a drug for the treatment of jaundice, PB response enhancer element (PBREM) was found at position −3483/−3194 as a functional site [14,15]. This transcriptional stimulation by PB was involved a mechanism like that found in CYP2B [16,17]. In this region of UGT1A1, three elements for binding nuclear transcriptional factors, NR1 (−3472/−3457), gtNR1 (−3366/−3351) and NR3 (−3272/−3257), have been reported and gtNR1 showed strong binding in a gel-shift mobility assay. This PBREM region also contains the xenobiotic response element (XRE) (−3319/−3300) [18], the element for binding pregnenolone xenobiotic receptor (PXR) (−3430/−3285) [19], which is equivalent to SXR (−3424/−3410) [18]. PBREM is activated by nuclear orphan receptor human constitutive androstane receptor (CAR) [20]. The XRE in PBREM reflects the stimulation of the expression of UGT1A1 by 2,3,7,8-tetrachlordibenzo-*p*-dioxin and β-naphthoflavone binding with arylhydrocarbon receptor [18]. Such stimulation of the expression of UGT1A6 by polycyclic aromatic hydrocarbon, 2,3,7,8-tetrachlordibenzo-*p*-dioxin and oltpraz has also been reported [21,22]. It has been suggested that the nuclear receptor CAR/RXR expresses the hepatic effects of PB and is a regulator of BIL clearance [23]. Meanwhile, Sugatani et al. [24] reported that double defects among the three mutations of T-3263G in PBREM $(TA)_7TAA$, and G71R in the exon are associated with mild hyperbilirubinemia (Gilbert's syndrome).

We found two transcriptional regulatory elements in the promoter region (up to −3174) by conducting transient transfection assays; one was the distal element (DE, −1346/−1204) and the other was the proximal element (PE, −97/−54). PE consisted of two regions, an E-box (−88/−79) and an HNF-1 site (−75/−63) [13]. Bosma et al. [25] and Monaghan et al. [26] reported that one-third of patients with Gilbert's syndrome had a TATA box mutation, i.e. $(TA)_7TAA$ instead of $(TA)_6TAA$. Sato et al. [27] compared the promoter activity of the two TATA boxes, did but not find a substantial difference. These results suggest that the TATA box mutation itself is not the major cause of the syndrome Some patients with Gilbert's syndrome had simultaneous mutations in the TATA box and in the coding region (G71R) [27]. Koiwai et al. [28] considered that the high frequency of the syndrome, compared to the frequency of the gene, might depend on the dominant negative phenomenon.

In this report, we show the induction of UGT1A1 expression by GR with dexamethasone.

2. Materials and methods

2.1. Materials

The drugs used were dexamethasone (DEX) and phenobarbital (PB) from Wako Chemicals, rifampisin (RIF) and β-estradiol (β-Est) from Sigma Chemicals, and bilirubin (BIL), pregnenolone 16α-carbonitrile (PCN) and RU486 [29] (mifeprostone) from

CaHK, Japan. These were dissolved in DMSO and added to culture medium at appropriate times for transfection assays. Fetal bovine serum (FBS) (Trace) treated with charcoal was used for cell culture in the stimulation with drugs. This treatment was carried out to remove endogenous stimulators, such as steroids, as follows. The charcoal solution, made of 0.25% Norit A SX-II (Wako Chemicals) and 0.025% Dextran T70 (Amersham) in 0.25 M sucrose, 1.5 mM $MgCl_2$, and 10 mM Hepes (pH 7.4), was mixed overnight at 4 °C. The charcoal was collected by centrifugation at 500 × *g* for 10 min and was mixed with FBS. The mixture was centrifuged at 500 × *g* for 10 min, and then the supernatant containing FBS was treated once more with charcoal and centrifuged. The supernatant after centrifugation was treated at 56 °C for 45 min and sterilized by filtration through a 0.45-μM membrane (Becton–Dickinson). FBS not treated with charcoal was used in the general cell culture medium.

2.2. Plasmid

The plasmids used were pSV-β-galactosidase vector (Promega), pGV-B (Wako), pGV-C (Wako), pSG5 basic vector (Stratagene), pSG5-hGR vector (a gift from Dr. Pierre Chambon of IGBMC, France)[30], and pSG5-hPXR vector (a gift from Dr. Steven A. Kliewer of Dallas Southwestern Medical Center, USA) [31]. Deletion mutants containing the 5′-upstream promoter region of human UGT1A1 in the luciferase reporter plasmid pGV-B (Wako) were prepared as described previously [13]. DNA from these constructs for transfection was prepared on a large scale by ultra-centrifugation in CsCl–ethidium bromide [13] and the DNA concentration was adjusted to 1 μg/μl.

2.3. Culture of HepG2

The human cell line used in this study was the hepatoma-derived HepG2. HepG2 cells were maintained at 37 °C in 95% air and 5% CO_2 in sterilized Dulbecco's modified Eagle's medium (DMEM, phenol red-free) supplemented with 10% FBS. The transplantation of cells was carried out at 70–80% confluence. The dish was washed with PBS (−, Mg-free) and then the cells were collected by treatment with PBS (−) containing 0.02% EDTA and 0.05% trypsin. The solution of cells was diluted to one-third and transplanted to dishes.

2.4. Transfection assay

Transfection of the deletion mutants in the promoter region of UGT1A1 in the luciferase reporter plasmid was done by the standard calcium phosphate method [13]. HepG2 cells were plated in a 12-well culture plate at 30–40% confluence and transfected after 18 h. In general, the transfection mixture contained 2 μg of hUGT1A1 deletion mutants, 400 ng of β-galactosidase expression vector (pSV-β-galactosidase), and either 400 ng of pSG5, pSG5-hGR or pSG5-hPXR in 0.2 ml of Hepes-buffered saline and 0.125 M $CaCl_2$ per well. After 6 h, the medium was replaced with DMEM supplemented with 10% FBS, treated with charcoal, and the cell were treated with various chemicals for 48 h. Chemicals were dissolved in DMSO and diluted in DMEM–FBS medium. The HepG2 cells in the dish were washed with PBS. The cells were lysed, and analyzed for luciferase and β-galactosidase activities [13]. Luciferase activity was measured with a luminometer (ARVO 1420) as follows. HepG2 cells were harvested in 100 μl of lysis buffer (25 mM Tris–phosphate (pH 7.8), 2 mM DTT, 2 mM *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, and 1% TritonX-100). Cell lysates (25 μl) were mixed with 50 μl of luciferin reaction mixture (20 mM Tricine, 1.07 mM $(MgCO_3)_4$ $Mg(OH)_2 5H_2O$, 2.67 mM $MgSO_4$, 0.10 mM EDTA, 0.53 mM ATP, 33.3 mM DTT, 0.27 mM coenzyme A, and 0.47 mM luciferin). Light output was measured for 5 s, and luciferase activity was expressed as relative light units. β-galactosidase activity was determined using a standard o-nitro-phenyl-β-D-galactopyranoside assay.

2.5. Electrophoretic mobility shift assay (EMSA)

A complementary pair of synthetic oligonucleotides, 5′-CACAGTCAAACATTAACTTGG-3′, encoding the consensus core sequence (underlined) of the UGT1A1-PE2 (HNF1 site) element, was synthesized.

The competitors for EMSA were as follows:

UGT1A1-PE2 mut: 5′-CACAGTGGCACATCGACTTGG-3′, ALB-HNF: 5′-TCTAGTTAATAATCTTACAATT-3′ [human albumin (ALB) gene], TAT-GRE: 5′-GACCCTAGAGGATCTGTACAGGATGTTCTAGAT-3′ [tyrosine aminotransferase gene], and TAT-GRE mut: 5′-GACCCTAGAGGATCTCAACAGGATCATCTAGAT-3′ double-stranded DNA was prepared by annealing and then labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase (TaKaRa). The labeled oligonucleotide was separated from ATP on a Sephadex G50 (Pharmacia) column [32]. Binding assays were carried out on ice in 15 μl of solution composed of 12 mM Hepes (pH 7.6), 12% glycerol, 150 mM NaCl, 1 mM $MgCl_2$, 0.2 mM EDTA (pH 8.0), 2 mM DTT, 0.05% NP-40, 10,000 cpm of the above labeled oligonucleotides, 15–20 μg of nuclear extract from HepG2 cells (described later) or recombinant GR (PANVERA), and 1 μg of poly(dI-dC). This mixture was incubated at 0 °C for 15 min. In order to examine the specificity of HNF1 or GR binding, 200 ng of anti-HNF1 or anti-GR antibody (Santa Cruz) was incubated in the binding mixture. The mixture was electrophoresed on a 4% polyacrylamide gel in 0.25× TBE buffer as a running buffer. After electrophoresis, the gel was then dried, and the [^{32}P]DNA pattern on the gel was analyzed with a BAS 2500 (Fuji Film).

Nuclear extracts from HepG2 cells were prepared as follows. The isolating procedures were carried out at 4 °C. After the transfection of pSG5-hGR into HepG2 cells, the cells were treated with 100 μM DEX or other drugs for 48 h, then washed twice with PBS (−), and collected by scraping into EMSA-A buffer made of 20 mM Hepes at pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA at pH 8.0, 1 mM DTT, 0.1% NP-40, and protease inhibitor cocktail (1 tablet/50 ml) (Roche). The solution was incubated for 10 min and cells were collected by centrifugation at 600 × *g* for 5 min. The precipitate was lysed in EMSA-B buffer made of 20 mM Hepes at pH 7.6, 20% glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA at pH 8.0, 1 mM DTT, 0.1% NP-40, and protease inhibitor cocktail (Roche) for 30 min. After the incubation, the lysates were centrifuged at 12,000 × *g* for 20 min, and the supernatant was designated as nuclear extract (NE) and used for the above EMSA assay.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA for RT-PCR was prepared with the mRNA extraction kit Aurum Total RNA Mini (BIO-RAD). cDNA was synthesized from mRNA with the kit iScript cDNA synthesis (BIO-RAD), and DNA primers for the preparation of cDNA were as follows:

human GAPDH (product 623 bp)
forward: 5′-ACCATCTTCCAGGAGCGAGA-3′
reverse: 5′-ACCACCTGGTGCTCAGTGTA-3′
human HNF1α (product 131 bp)
forward: 5′-TACACCTGGTACGTCCGCAA-3′
reverse: 5′-CACTTGAAACGGTTCCTCCG-3′
human HNF1β (product 675 bp)
forward: 5′-ATGGTGTCCAAGCTCACGTCG-3′
reverse: 5′-CTCAGAGCAGGCATCATCGGA-3′
human GR (product 478 bp)
forward: 5′-CCTAAGGACGGTCTGAAGAGC-3′
reverse: 5′-GCCAAGTCTTGGCCCTCTAT-3′

2.7. Statistical analysis

The values indicate the mean ± S.D. of three determinants (three dishes). Statistical significance $^{*}P < 0.05$; $^{**}P < 0.01$.

3. Results

Previously, we analyzed the promoter region of human UGT1A1 with deletion mutants, in relation to the expression [13]. The deletion of DE element (−1346/−1204) resulted in a decrease in activity to about half and the mutant missing PE element (−97/−56) showed no expression. In order to clarify the regulation of UGT1A1 expression by BIL, we investigated the expression level of the luciferase reporter gene containing the promoter region (−3174/+14) of UGT1A1 in HepG2 on stimulation with various concentrations of BIL. However, the levels of the induction of UGT1A1 by BIL were low at 1.3–1.5-fold (data not shown). Thus, we could not provide evidence of significant induction by BIL at 0.01–100 μM.

Fig. 1A shows the dose-dependent induction of UGT1A1 expression with dexamethasone. DEX at 1–100 μM induced the expression of reporter gene in the absence of GR at approximately 1.5–2.3 times the control level, the same result as in a previous report [33]. The levels of relative luciferase activity were greater in the presence of GR (2–3-fold) than in the absence of GR. At 1 μM DEX, the level of induction of the luciferase reporter gene was 2.3-fold that in the absence of GR and seven-fold that in the presence of GR. Fig. 1B shows the expression of GR with the plasmid pSG5-hGR measured by RT-PCR. DEX at 100 μM did not induce the expression of GR. This result shows that expression of UGT1A1 is stimulated by DEX with GR, and GR is necessary for the effective induction of UGT1A1 by DEX. In Fig. 1C, the increase continued for 48 h. Fig. 2A shows the influence of the induction of UGT1A1 by certain drugs, such as DEX, β-Est, BIL, PB and RIF, in the presence or absence of GR. The induction of the luciferase reporter gene by DEX is low in the absence and high in the presence of GR. We could not detect any induction by β-Est, BIL, PB or RIF in the presence or

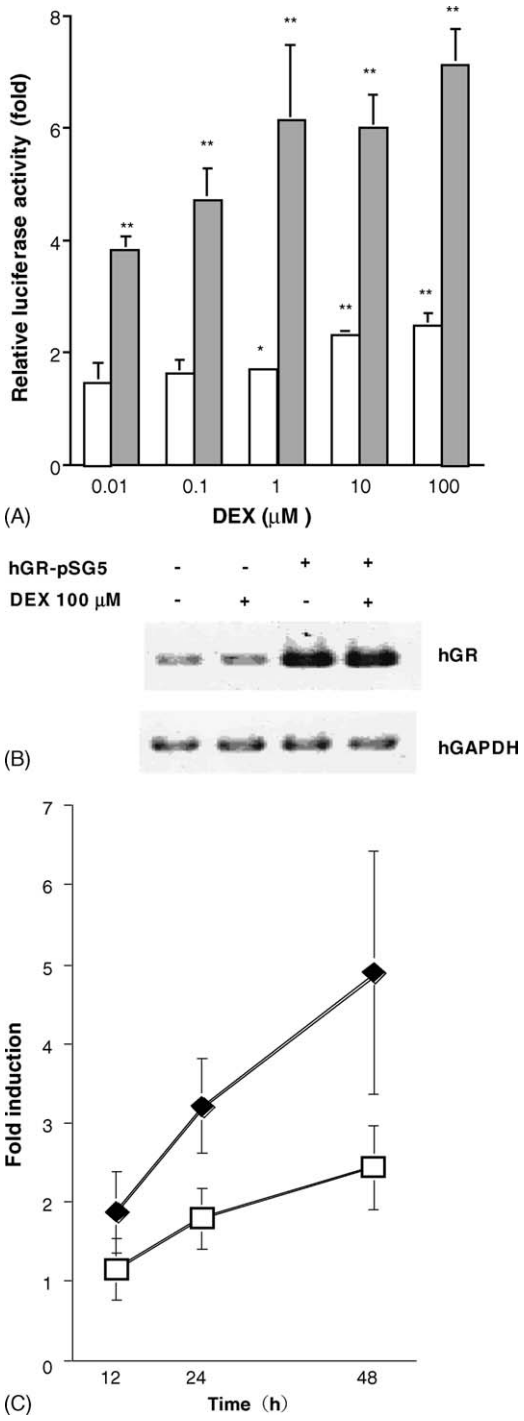

Fig. 1 – The expression of the reporter gene of UGT1A1 induced by dexamethasone in the presence of GR. (A) The expression of UGT1A1 induced by DEX in a dose-dependent manner in the presence (black) or absence (white) of GR. These values were calculated from the value of DMSO (minus DEX-GR) = 1. They are the mean ± S.D. of three determinants (three dishes). Statistical significance $^{*}P < 0.05$; $^{**}P < 0.01$. (B) The expression level of GR in the presence or absence of 100 μM DEX as determined by RT-PCR. (C) Time courses of the induction of the UGT1A1 reporter gene by DEX in the presence (black) or absence (white) of GR. Values are the mean ± S.D. of three determinants (three dishes).

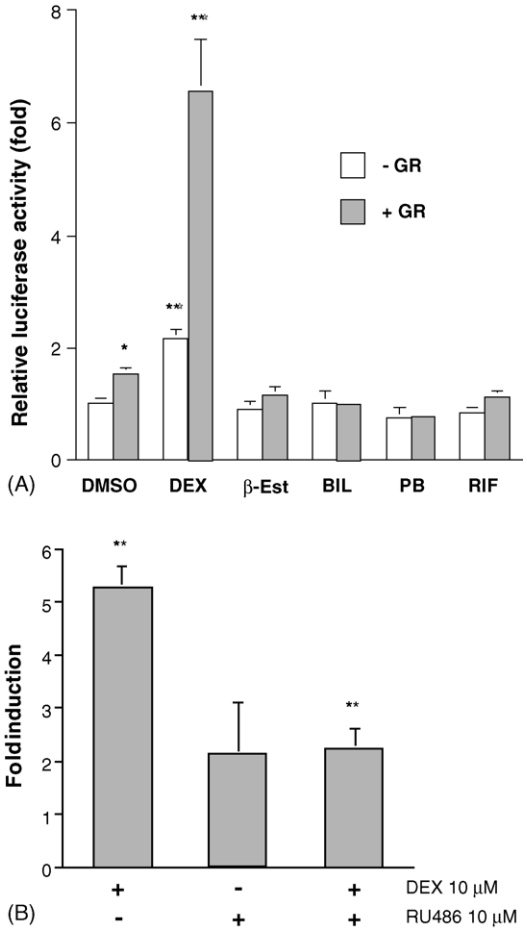

Fig. 2 – The influence of drugs on the expression of UGT1A1 in the presence (black) or absence (white) of GR. (A) Influence of drugs (100 μM DEX, 1 μM β-estradiol (β-Est), 5 μM bilirubin (BIL), 1 mM phenobarbital (PB), or 10 μM rifampicin (RIF)) on the expression of the reporter gene. DMSO is a dimethylsulfoxide used for the control experiment. (B) Influence of RU486, a typical GR antagonist, on the expression. Values are the mean ± S.D. of three determinants (three dishes).

absence of GR. The lack of induction by RIF suggests that the luciferase reporter gene (−3174/+14) of UGT1A1 did not contain the element for PXR/RXR. Of course, the region (−3174/+14) did not contain the element responding to PB, PBREM (−3483/−3194) [14].

Fig. 2A also shows that GR itself (DMSO) did not induce the expression, and the expression of UGT1A1 was induced through the cooperation of DEX and GR. Fig. 2B shows the effect of the GR antagonist RU486 on the induction by DEX in the presence of GR. RU486 reduced the induction by DEX at 10 μM from 5.3- to 2.3-fold. These results indicate that the induction by GR depends on the ligand-binding of GR. We ascertain that this induction is caused by the UGT reporter gene itself, given that there was noinduction with the control vector pGL-2 in the presence of DEX-GR.

Fig. 3 shows the position responsible for the induction of the reporter gene by DEX in the presence or absence of GR. We found clear induction with plasmids #1–#15 but not with plasmid #16. Plasmid #16 did not contain the site (−97/−53) responsible for the induction by GR. We have previously shown two functional positions, DE (−1344/−1204) and PE (−97/−54), in the 5′-upstream promoter region of UGT1A1 [13]. In this study, DE was not responsible for the induction by GR. We further searched for the region responsible for the induction by GR with deletion mutants, as shown in Fig. 4. Plasmid #15δ, which does not contain the region −75/−63, was not induced by the stimulation with GR, and region −75/−63 contains the element (PE2) for HNF1. The level of induction with plasmid #15-7 was marginal, and not only PE2 (HNF1 site) but also PE1 may be better for induction by GR.

Next, we studied the interaction of GR with synthetic [^{32}P]DNA elements containing the HNF1 element, using the EMSA method. Fig. 5A shows that GR specifically interacted with the authentic radiolabeled GR element in tyrosine aminotransferase (TAT) (lane 1), and that this shift was inhibited by un-labeled TAT-GRE (lanes 2 and 3) and not inhibited by un-labeled mutated TAT-GRE (lane 4) (see the mutated position in Section 2) or UGT-PE2 (−75/−63) (lane 5). The band was super-shifted by the addition of anti-GR (lane 6). Fig. 5B shows the results of the EMSA with [^{32}P]radiolabeled PE2 probe (−75/−63) in UGT1A1. PE did not interact with GR (lane 1) but did interact with NE (lane 2). This interaction was not inhibited by non-labeled TAT-GRE (lane 3) but was inhibited by un-labeled PE2 DNA of UGT1A1 (lane 4) and ALB-HNF1 DNA (lane 6). The mutated PE2 did not inhibit the shift (lane 5), indicating that the sequence in PE2 is essential for the binding. This mobility of [^{32}P]PE2 and NE was not influenced by anti-GR (lane 7), showing that GR is not contained in the complex of PE2 and NE. The band was further shifted (super-shifted) by the addition of anti-HNF1 (lane 8), indicating that HNF1 is involved in the complex of [^{32}P]PE2 and NE. NE from HepG2 cells stimulated with DEX-GR was used in lane 10, while NE from un-treated HepG2 cells was used in lane 9. The band in lane 10 was stronger than that in lane 9. This indicates that HNF1 in NE from the stimulated cells showed stronger binding to the probe than that from un-treated cells. This may not depend on the amount of HNF1 (Fig. 6) but the higher affinity of HNF1 in the stimulated NE. The binding of HNF1 to the probe might be promoted by the support of unknown associated proteins, or activation by phosphorylation of HNF1, as discussed later.

Next, we studied the influence of the stimulation with GR on the level of HNF1α and HNF1β mRNA using the RT-PCR method, as shown in Fig. 6. The levels of HNF1α and HNF1β mRNA did not change with the induction by GR, and GR did not influence the expression of the reporter gene in the presence or absence of 100 μM DEX. Therefore, the induction of UGT1A1 expression by GR is not dependent on the increase in the level of the HNF1. We also studied the influence of co-expression of PXR but could not detect the induction of the luciferase reporter gene of UGT1A1 in the presence of RIF and PCN, good ligands for PXR (data not shown). Meanwhile, the presence of the element for PXR has been reported at position −3430/−3285 [18,19] in PBREM upstream of UGT1A1.

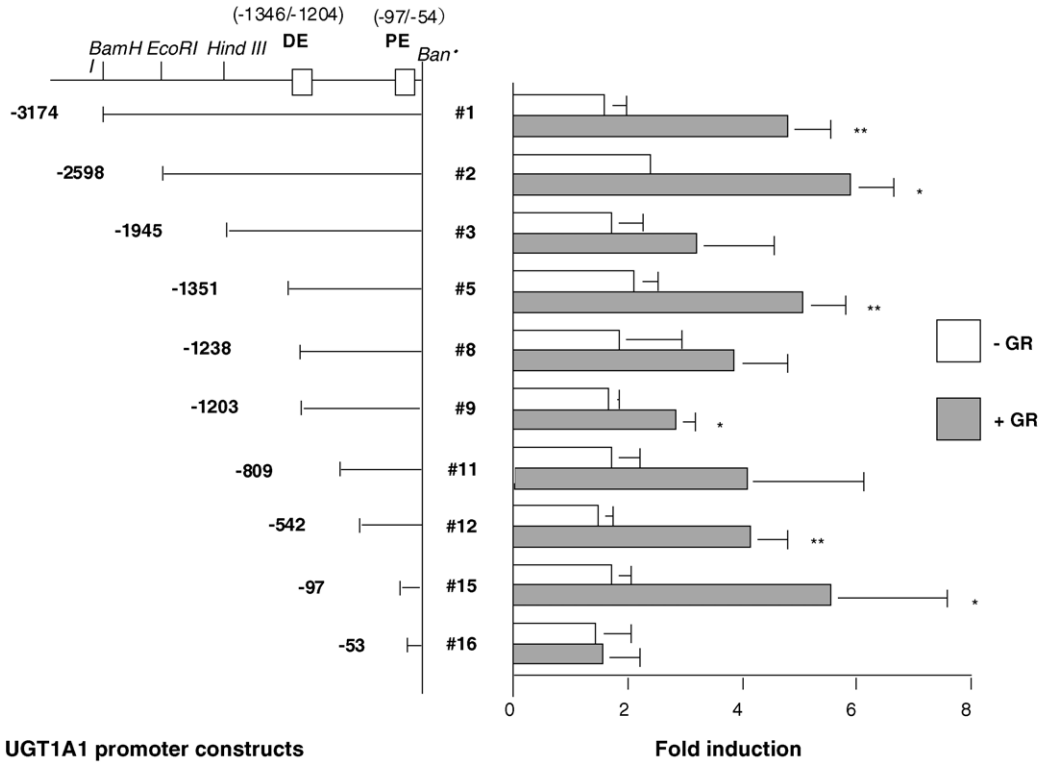

Fig. 3 – Identification of the region responsive to DEX-GR. The left side shows the length of the gene promoter region, and the positions of DE and PE. The right side shows the expression levels in the presence (black) or absence (white) of GR. Values are the mean ± S.D. of three determinants (three dishes).

4. Discussion

We could not detect by induction of UGT1A1 expression by BIL at 50 μM, near the concentration of human hyperbilirubinemia. One possible explanation for this is that BIL does not play a role in the stimulation of UGT1A1, or that the expression level of UGT1A1 on stimulation with BIL is low. Alternatively, BIL may act with CAR, which is not constitutively expressed in HepG2 cells. Huang et al. [23] reported that BIL indirectly activates CAR, promotes the transfer of CAR to the nucleus, and induces the transcription of UGT1A1 and the transporter, which conjugate BIL and excrete the conjugate. CAR-responsive element (−3366/−3351) is found in PBREM (−3483/−3194) of UGT1A1 [18]. However, our gene (−3174/+14) did not contain PBREM and we could not detect the induction of UGT1A1 expression by BIL.

We found a marked expression of human UGT1A1 induced by DEX in the presence of GR. It has been reported that there are two patterns of DEX stimulation at 0.1 or 10 μM. At 0.1 μM, DEX binds with GR, and the DEX-GR dimer binds to GRE and induces the expression of PXR, CAR or RXR mRNA. These CAR/RXR or PXR/RXR complexes play roles in the stimulation of expression of many proteins. At 10 μM, DEX binds to PXR or CAR, and DEX-PXR/RXR or DEX-CAR/RXR binds with ER and DR

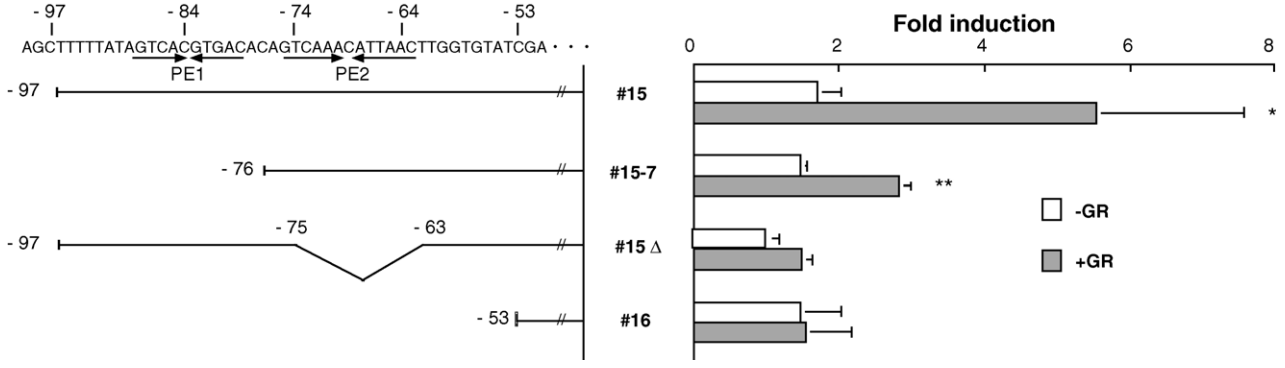

Fig. 4 – The assignment of the functional site in the PE region for the stimulation by DEX-GR. The left side shows the sequence of the PE gene promoter region, and the positions of PE1 and PE2 (HNF1). The right side shows the induction by DEX in the presence (black) or absence (white) of GR. Values are the mean ± S.D. of three determinants (three dishes).

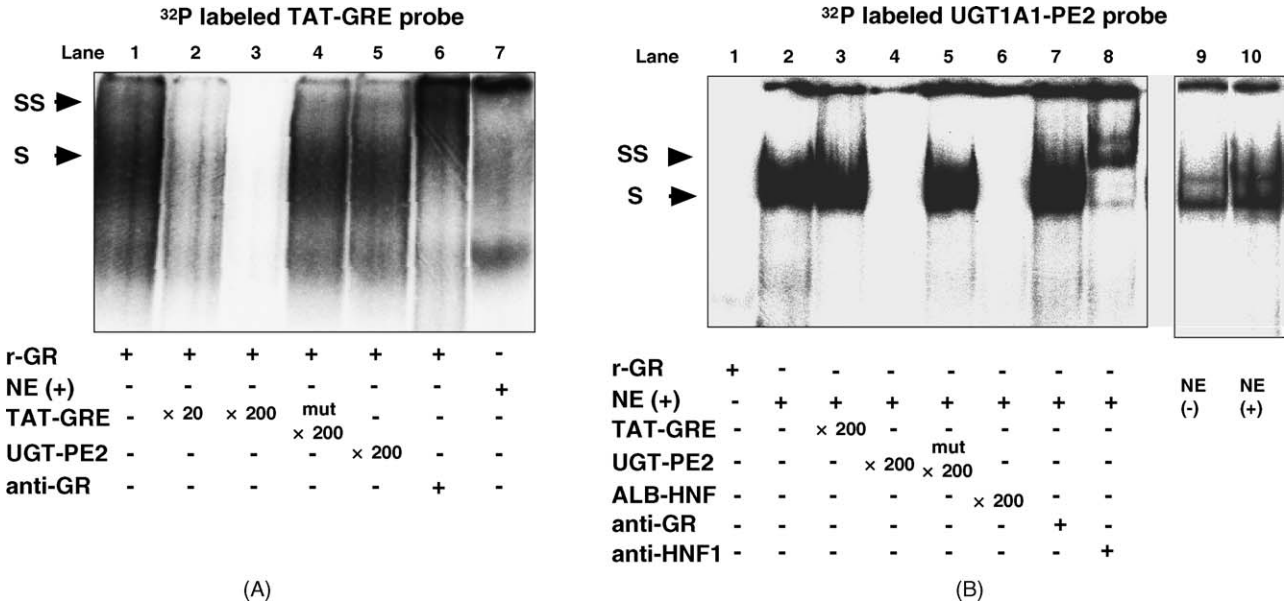

Fig. 5 – The gel-shift patterns obtained by EMSA. The left side (A) shows the results obtained with the probe [^{32}P]GRE in tyrosine aminotransferase (TAT) (sequence is shown in Section 2). The probe used for the right side (B) was [^{32}P]PE2 (−75/−63) (as shown in Fig. 4) in UGT1A1. The symbol + indicate the presence of those factors, DNA elements or antibody and the symbol −, their absence. Abbreviations used are; r-GR, recombinant GR; NE(+), nuclear extract from HepG2 cells stimulated with DEX-GR; NE(−), nuclear extract from un-treated cells; TAT-GRE and UGT-PE2, un-labeled DNAs; ALB-HNF, HNF element in the human albumin gene; anti-GR and anti-HNF1, antisera against GR and HNF1, respectively; S, shifted band; SS, super-shifted band; mut, mutated DNA elements shown in Section 2. Lanes 9 and 10 indicate control experiments with NE from un-stimulated cells and HepG2 cells stimulated with DEX-GR, respectively.

in the promoter enhancer regions of many genes [20,34]. The level of constitutive GR in HepG2 is weak and the induction by DEX is low in the absence of GR. Meanwhile, the level of UGT1A1 expression was increased by DEX in the presence of GR. The affinity for binding of DEX to the GR is in the sub-nanomolar range, therefore it is possible that the high expression of UGT1A1 at some μM DEX may be due to activation of a non-specific non-GR mediated mechanism. However, this induction by DEX-GR was inhibited by RU486, a GR antagonist. This clarified that the elevation of UGT1A1 is dependent on a DEX-GR complex. It has been reported that DEX induces PXR and RXR expression in human hepatocytes, which indicates a synergistic increase in the expression of CYP3A4 by PXR activators [35]. In addition, transcription of the CYP2C9 gene is regulated by recognition of GRE (−1684/−1648)

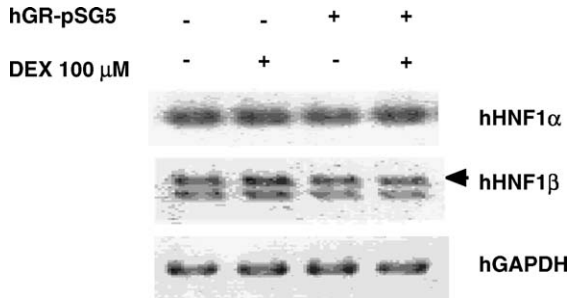

Fig. 6 – The expression levels of HNF1α and HNF1β in the presence or absence of GR and 100 μM DEX determined by RT-PCR.

and CAR element (−1856/−1783) by GR and CAR, respectively [29]. The orphan nuclear factor CAR controls UGT1A1 and other factors involved in drug metabolism and BIL clearance [36]. In the present study, RIF, a PXR activator, did not stimulate the transcription of UGT1A1, therefore, the signal of DEX might not pass through PXR, but DEX may be directly bound to GR.

PBREM was found in the CYP2B gene, which is induced by PB. PBREM (−3483/−3194) in human UGT1A1 has been found to be an important module for stimulating the transcription of UGT1A1 [14]. PBREM contains PXR (SXR) for induction by steroids. We could not find induction by β-Est, esterone or ethynylestradiol, because our vector DNA of UGT1A1 did not contain PBREM [13]. Recently, it has been reported that a novel distal enhancer module (xenobiotic-responsive enhancer module (XREM)) regulated by PXR/CAR is essential for maximal induction of CYP2B6 gene expression [37]. Meanwhile, in this study, we used the promoter region, which did not contain PBREM. Our promoter region (−3174/+14) was well regulated by DEX, as demonstrated by the results obtained using deletion mutants [13] and those of this study. We confirmed that the region for enhancing the luciferase reporter gene is PE, which does not contain GRE but the element for HNF1 (PE2). We showed the relation of GR with the HNF1 element on UGT1A1. GR did not raise the level of HNF1 but increased the binding of HNF1 protein to the element as shown in Fig. 5B. This may not depend on the amount of HNF1 (Fig. 6) but on the higher affinity of HNF1 in the stimulated NE. The binding of HNF1 to the probe could be promoted by unknown proteins associated with HNF and produced after

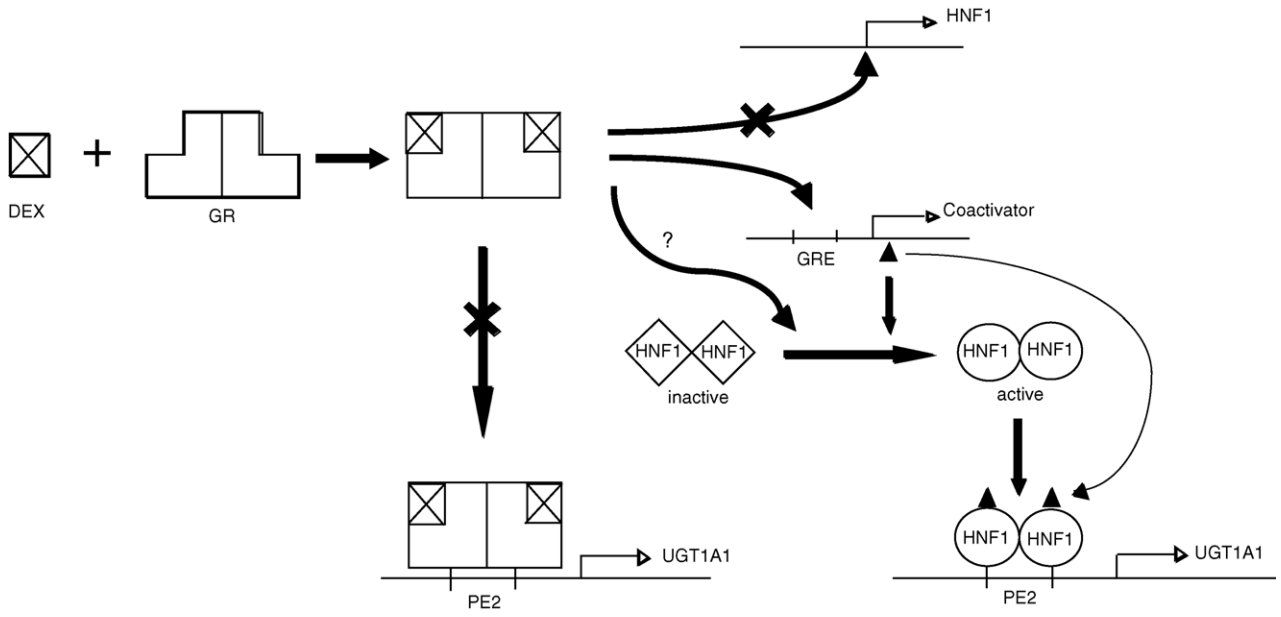

Fig. 7 – Model of the induction of the PE2 site (HNF1 element) of UGT1A1 by GR. DEX-GR does not bind directly to the HNF1 (PE2) site but indirectly affects the expression of UGT1A1 through HNF1 or co-regulators.

stimulation of GR (lane 10 in Fig. 5). It is possible that the activation by phosphorylation of HNF1, like the activation of NF-κB through I-κB, occurs as shown in the model in Fig. 7. DEX-GR complex did not stimulate the production of HNF1 and was not contained in the binding complex of HNF1 element and HNF1. The associated proteins or protein kinase as coactivators may be produced by stimulation by DEX-GR, and those proteins should indirectly mediate the stimulation of GR. The coactivator may promote the UGT1A1 expression through protein–protein interaction with HNF1. The possibility remains that the DEX-GR complex activates the latent inactive HNF1 without new transcriptional production of HNF1.

There have been reports of the synergistic stimulation of GRE and HNF1 element by phanylalanine hydrolase [38] and IGFBP [39]. The synergistic stimulation of PXR and GRE was also reported for CYP2C9 [24] and CYP3A4 [25]. HNF1 is expressed in the liver and the deletion of HNF1 is lethal. HNF1 is related to the transcription of many liver proteins such as albumin and fibrinogen, whose genes have the element for binding HNF1α and HNF1β. HNF1 also plays a role in the expression of UGT1A1 in mice, and a mutation in the HNF1 gene causes hyperbilirubinemia in mice [40,41]. We [13] and [40] have shown that the HNF1 sequence in UGT1A1 is important for the expression of UGT1A1, and in this paper we showed that GR induced UGT1A1 expression through the HNF1 site in the UGT1A1 promoter region. The mechanism of induction by DEX at the HNF1 site in the PE region (−97/−54) of the UGT1A1 promoter remains to be resolved.

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