Genetic polymorphisms in the 5'-flanking region of human UDP-glucuronosyltransferase 2B7 affect the Nrf2-dependent transcriptional regulation.

Genetic polymorphisms in the 5'-flanking region of human UDP-glucuronosyltransferase 2B7 affect the Nrf2-dependent transcriptional regulation

Short title: *UGT2B7*2* affects the regulation by Nrf2

Akiko Nakamura, Miki Nakajima, Eriko Higashi, Hiroyuki Yamanaka, and Tsuyoshi Yokoi

Drug Metabolism and Toxicology, Division of Pharmaceutical Sciences, Graduate School of Medical Science, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan

To whom all correspondence should be sent:

Tsuyoshi Yokoi, Ph.D.,

Drug Metabolism and Toxicology

Division of Pharmaceutical Sciences

Graduate School of Medical Science

Kanazawa University

Kakuma-machi, Kanazawa 920-1192, Japan

Tel / Fax +81-76-234-4407

E-mail: tyokoi@kenroku.kanazawa-u.ac.jp

Objectives Human UGT2B7 plays important roles in the metabolism of some clinical drugs, carcinogens, and steroid hormones. The molecular mechanisms of the inducible expression of UGT2B7 in response to xenobiotics have not been fully clarified. We sought to investigate whether the UGT2B7 is under the control of NF-E2 p45-related factor 2 (Nrf2), a key transcriptional factor regulating the expression of cytoprotective enzymes.

Methods HepG2, HuH7, HLE, and Caco-2 cells were treated with sulforaphane (SFN), and the UGT2B7 mRNA levels were determined by real-time RT-PCR. These cells were genotyped for the *UGT2B7*2* (H268Y) allele using the PCR-RFLP method. Luciferase analyses and gel shift analyses were performed to identify the responsive regions for the Nrf2 signaling.

Results The UGT2B7 mRNA was induced by SFN in HepG2 and HuH7 genotyped as *UGT2B7*1/*1* but not in HLE and Caco-2 cells genotyped as *UGT2B7*2/*2*. In HepG2 cells, the UGT2B7 protein level and morphine glucuronosyltransferase activity were also significantly induced by SFN. The induction was prominently decreased with siRNA for Nrf2. In the 5'-flanking region (-2.5 kb) of the *UGT2B7*2* allele, a 324-bp insertion at -2067 and 12 SNPs simultaneously existed. Luciferase analyses and gel shift analyses revealed that an antioxidant responsive element (ARE) at -1170 was responsible for the transactivation by Nrf2. In addition, a region from -990 to -858 on the *UGT2B7*1* allele was also responsible for the transactivation by Nrf2. Abrogation of the Nrf2-dependent transactivation of the *UGT2B7*2* allele was due to the SNP -900A>G.

Conclusions UGT2B7 is transcriptionally regulated by Nrf2, but the mechanism is hindered by polymorphisms in the promoter region of *UGT2B7**2. The allele specific mechanism may cause variability of the glucuronidation in response to oxidative stress.

Keywords: gene regulation; genetic polymorphism; Nrf2; UDP-glucuronosyltransferase; UGT2B7

Introduction

The glucuronidation reaction catalyzed by UDP-glucuronosyltransferase (UGT) enzymes is responsible for the clearance of numerous endogenous substrates, clinical drugs, and carcinogens [1]. Based on evolutionary divergence and homology, human UGTs are classified into two families, UGT1 and UGT2. The UGT2 family is further divided into two subfamilies, UGT2A and UGT2B [2]. For the UGT2B subfamily, seven active isoforms, UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15, UGT2B17, and UGT2B28, have been identified in humans [2]. UGT2B7 appears to be of particular significance in the metabolism of xenobiotics and endobiotics. For example, it catalyzes the glucuronidation of morphine, nonsteroidal anti-inflammatory drugs (NSAIDs), clofibric acid, valproic acid, 2-acetylaminofluorene, benzo[*a*]pyrene, retinoids, estrogens, and bile acids [3]. UGT2B7 is predominantly expressed in the liver but also in extrahepatic tissues such as the gastrointestinal tract, kidney, breast, and brain [3]. Previous reports demonstrated that some transcriptional factors such as hepatocyte nuclear factor 1 [4], octamer transcription factor-1 [4], caudal-related homeodomain protein 2 [5], and farnesoid X receptor [6] are involved in the basal expression of UGT2B7. However, the inducible expression of UGT2B7 in response to stimuli has not been fully clarified.

Mammalian cells are inevitably exposed to environmental insults, such as pollutants, chemicals, and natural toxins. These compounds exert their biological effects by perturbation of the cell redox homeostasis, a condition defined as oxidative stress. Oxidative stress is associated with the etiology of many diseases, including cancer, neurodegenerative disease, cardiovascular diseases, inflammation, and autoimmune diseases. When cells are exposed to oxidative stress, the expression of metabolizing enzymes and antioxidant proteins such as NAD(P)H:quinone oxidoreductase 1 (NQO-1) [7], heme oxygenase-1 (HO-1) [8], glutathione *S*-transferases (GST) [9] and γ-glutamylcysteine synthetase (γ-GCS) [10] is induced in order to restore the redox balance and boost xenobiotic detoxification. Such induction would be commonly regulated by an NF-E2 p45-related factor 2 (Nrf2), a member of the cap'n'collar (CNC) family of transcriptional factors [11]. Under basal conditions, Nrf2 is sequestered in

the cytoplasm by binding to the actin-associated protein Kelch-like ECH-associated protein 1 (Keap1) which facilitates proteasomal degradation of Nrf2 [12]. When oxidative or xenobiotic stimuli occur, Nrf2 is dissociated from Keap1 and accumulates in the nucleus. After dimerization with small Maf proteins [13], the Nrf2/Maf heterodimer binds to the antioxidant responsive element (ARE) and transactivates the target genes. Although it is recognized that UGTs are also up-regulated by Nrf2, the limited evidence is as follows: knock out or knock in technology revealed that mouse UGT1A [14, 15] and human UGT1A1 [16] would be regulated by Nrf2.

In the present study, we focused on the regulation of human UGT2B7 in response to stimuli. It has been reported that the UGT2B7 mRNA was increased by the treatment with *tert*-butylhydroquinone (tBHQ), an activator of Nrf2 [17]. A computer-assisted homology search revealed potential AREs on the *UGT2B7* gene. This background prompted us to investigate whether the human *UGT2B7* gene is regulated through an Nrf2-ARE pathway. In a polymorphic allele *UGT2B7*2* (802C>T, H268Y), several single nucleotide polymorphisms (SNPs) simultaneously exist in the 5'-flainking region. We investigated whether the genetic polymorphisms in the 5'-flaning region affect the Nrf2-dependent regulation of UGT2B7.

Materials and Methods

Materials

L-Sulforaphane (SFN) was purchased from Alexis (San Diego, CA). Morphine hydrochloride was purchased from Takeda Chemical Industries (Osaka, Japan). Morphine 3-glucuronide was a generous gift from Dr. Kazuta Oguri, Kyushu University (Fukuoka, Japan). Rabbit anti-human UGT2B7 polyclonal antibody was purchased from BD Gentest (Woburn, MA). Anti-human Nrf2 antibodies (C-20) and (H-300), which recognize the C- and N-terminus of Nrf2 protein, respectively, were from Santa Cruz Biotechnology (Santa Cruz, CA). Stealth Select RNAi for Nrf2 and Stealth RNAi negative control were from Invitrogen (Carlsbad, CA). Restriction enzymes were from Takara (Shiga, Japan), TOYOBO (Osaka,

Japan), and New England Biolabs (Beverly, MA). All primers and oligonucleotides were commercially synthesizes at Hokkaido System Sciences (Sapporo, Japan). All other reagents were of the highest grade commercially available.

Cells and culture conditions

Human hepatoma cell line HepG2 and human colon carcinoma cell line Caco-2 were obtained from American Type Culture Collection (Manassas, VA). Human hepatoma cell lines HLE and HuH7 were obtained from the Japan Collection of Research Biosources (Tokyo, Japan) and RIKEN BioResource Center (Ibaraki, Japan), respectively. HLE and HuH7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). HepG2 and Caco-2 cells were cultured in DMEM supplemented with 10% FBS and 0.1 mM nonessential amino acids (Invitrogen). The cells were maintained at 37°C under an atmosphere of 5% CO_2-95% air.

Sequence analysis and genotyping of *UGT2B7* gene

Genomic DNA was extracted from the HepG2, HuH7, HLE and Caco-2 cells using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). Genomic DNA samples from 176 European-American, 163 African-American, and 54 Japanese subjects were prepared previously [18]. Written informed consent was obtained from all subjects and the Human Studies Committee of Washington University School of Medicine (St. Louis, MO) and the Ethics Committee of Kanazawa University (Kanazawa, Japan) approved the analysis. The genotyping of the SNP 802C>T (H268Y) in the *UGT2B7*2* allele (http://www.ugtalleles.ulaval.ca) was performed with a PCR-RFLP method [19]. The 5'-flanking region from -2515 to -7 of the human *UGT2B7* gene was amplified by polymerase chain reaction (PCR). Throughout this manuscript, the base A in the initiation codon ATG is denoted +1 and the base before A is numbered -1. Genomic DNA samples (0.1 μg) were added to PCR mixtures (25 μl) consisting of 1 × PCR buffer [67 mM Tris-HCl buffer (pH

8.8), 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 0.02% gelatin], 1.5 mM $MgCl_2$, 0.4 μM primers, 250 μM dNTPs, and 1 U of Taq DNA polymerase (Greiner Japan, Tokyo, Japan). After an initial denaturation at 94°C for 3 min, the amplification was performed by denaturation at 94°C for 30 s, annealing at 54°C for 30 s and extension at 72°C for 3.5 min for 35 cycles. The final extension step was performed at 72°C for 5 min. The forward primer and the reverse primer were 5'-tatctcatattaagtgttttc-3' and 5'-atcctcgagcaatgcaatg-3', respectively. In the reverse primer, the *Xho* I site (underlined) was adapted for the cloning of the PCR product. Nucleotide sequences of the PCR product were confirmed by DNA sequencing using Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing kit or Thermo Sequenase Cy5 Dye Terminator Cycle Sequencing kit with a Long-Read Tower DNA sequencer (GE Healthcare Bio-Sciences, Piscataway, NJ).

Real-time RT-PCR analysis

Total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan) and cDNA was synthesized from total RNA using Rever Tra Ace (TOYOBO) according to the manufacturer's protocol. Human GAPDH mRNA was quantified by real-time RT-PCR using the Smart Cycler (Cepheid, Sunnyvale, CA) as described previously [20]. UGT2B7 and HO-1 mRNA levels were quantified under the same condition. The forward and reverse primers for UGT2B7 were 5'-agttggagaatttcatcatgcaacaga-3' and 5'-tcagccagcagctcaccacaggg-3', respectively. The forward and reverse primers for human HO-1 were 5'-atagagcgaaacaagcaga-3' and 5'-tagagctgtttgaacttgg-3', respectively.

SDS-PAGE and Western blot analysis

Nuclear extracts and cytoplasm were prepared according to the method described previously [21]. The cytoplasm (20 μg) or nucleus (20 μg) were separated with 7.5% SDS-PAGE and transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA). The membranes were probed with rabbit anti-Nrf2 antibody (H-300). Membrane fractions were prepared from HepG2 cells as described previously [22] with slight modifications.

Briefly, the cells were suspended in a small amount of TBS buffer (25 mM Tris-HCl (pH 7.4), 138 mM NaCl, 2.7 mM KCl) and disrupted by freeze-thawing three times. To the suspension, cold H_2O was added for osmotic lysis so that there were 100 mg of cells/8 ml of H_2O and stirred for 10 min. After the centrifugation at 41,000 *g* for 2 h, the pellets were suspended in TGE buffer (10 mM Tris-HCl, 20% glycerol, 1 mM EDTA (pH 7.4)). The membranes (50 μg) were separated by 7.5% SDS-PAGE and transferred onto a nitrocellulose membrane (Protran, Schleicher & Schuell BioScience, Keene, NH). The membranes were probed with rabbit anti-human UGT2B7 polyclonal antibody. Anti-rabbit IgG conjugated IRDye680 (LI-COR Biosciences, Lincoln, NE) were used as a second antibody. Protein bands were visualized and the densities were quantified with Odyssey Infrared Imager (LI-COR Biosciences).

Morphine glucuronosyltransferase activity

The incubation mixture (0.2 ml total volume) contained 50 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, 3 mM UDPGA, 25 μg/ml alamethicin, 1 mg/ml membrane preparation, and 5 mM morphine. The reaction was initiated by the addition of UDPGA. After the incubation at 37°C for 2 h, the reaction was terminated by boiling at 100°C for 5 min. After the removal of protein by centrifugation at 10,000 *g* for 5 min, a 50 μl portion of the supernatant was subjected to HPLC. The analytical column was a Develosil C30 (4.6 x 150 mm; 5 μm) column (Nomura Chemical, Aichi, Japan) and the mobile phase was 50 mM sodium dihydrogen phosphate (pH 4.5). The flow rate was 1.2 ml/min and the column temperature was 35°C. The morphine 6-glucuronide was fluorometrically detected (excitation: 210 nm; emission: 350 nm), and the morphine 3-glucuronide was monitored at 220 nm by a UV detector. The quantification of the metabolite was performed by comparing the HPLC peak height to that of the authentic standard.

Transfection of siRNA

HepG2 cells were transfected with the siRNA by using Lipofectamine RNAiMAX

(Invitrogen). Briefly, to each well in the 6-well plates containing 500 μl of DMEM without FBS, 30 pmol of the siRNA and 5 μl of Lipofectamine RNAiMAX were added and incubated for 15 min at room temperature. To the mixture, trypsinized suspensions of HepG2 cells (2.5 x 10^5 cells in 2.5 ml in culture medium) were added. After the incubation for 24 h, the cells were treated with 10 μM SFN or 0.1% DMSO for 48 h.

Human Nrf2 expression plasmid and reporter constructs

Human Nrf2 cDNA was amplified by PCR using cDNA from human normal kidney with the forward primer 5'-caacacacggtccacagc-3' and the reverse primer 5'-ggtcaaatcctcctaaatc-3'. The PCR fragment was cloned into the pTARGET vector (Promega, Madison, WI).

Double-stranded oligonucleotide containing the consensus ARE on the human *NQO-1* gene (5'-gatcagtcacagtgactcagcagaatct-3') adapted with a *Bgl* II site was cloned into the pGL3-tk vector. A construct with a double insertion was used as a positive control. The PCR fragments of the 5'-flanking region (-2517 to -7) of the *UGT2B7* gene amplified as described above using genomic DNA samples from two subjects with either *UGT2B7*1/*1* or *UGT2B7*2/*2* were cloned into the pGL3-basic vector (Promega) after the treatment with Klenow Fragment (Takara) and the digestion with *Xho* I. Using these plasmids, various plasmids were constructed by digestion with restriction enzymes and ligation. The plasmids mutated in an ARE were constructed by site-directed mutagenesis with a QuikChange II XL Site-Directed Mutagenesis Kits (Stratagene, La Jolla, CA). The used primer was 5'-gaaactacattatttGCTGAGGACgttccttgtggggtcctctag-3', in which the core ARE is indicated by capital letters and mutated nucleotides are underlined. To construct reporter plasmids, two copies of the fragment containing each ARE or the SNP of -900A or -900G and -1112C or -1112T on the *UGT2B7* gene, which were obtained by digestion with the appropriate restriction enzymes, were subcloned into the pGL3-tk vector. Nucleotide sequences of all constructs were verified by DNA sequencing.

Transfection and luciferase assay

HepG2 cells were seeded into 24-well plates at 1 x 10^5 cells/well. Transfection was performed using Tfx-20 reagent (Promega). Briefly, the transfection mixes consisted of 150 ng of pGL3 plasmid, 5 ng of phRL-TK plasmid (Promega), and 100 ng of human Nrf2 expression plasmid (or control vector). In some cases, 5 pmol of the siRNA-Nrf2 (or siRNA-control) were co-transfected and the cells were treated with 10 μM SFN or 0.1% DMSO for last 12 h. The cells were harvested 48 h after transfection and lysed to measure the luciferase activity using a Dual Luciferase Reporter Assay System (Promega). The relative luciferase activities were normalized with the *Renilla* luciferase activities.

Gel shift assay

Double-stranded oligonucleotides were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (TOYOBO) and purified by Microspin G-50 columns (GE Healthcare Bio-Sciences). The oligonucleotide sequences are shown in Table 1. The labeled probe (40 fmol, ~20,000 cpm) was applied to each binding reaction in 25 mM HEPES-KOH (pH 7.9), 0.5 mM EDTA, 10% glycerol, 50 mM KCl, 0.5 mM dithiothreitol, 0.5 mM (*p*-amidinophenyl) methanesulfonyl fluoride, 1 μg of poly(dI-dC), 5 μg of salmon sperm DNA, and 8 μg of the nuclear extracts from 10 μM SFN-treated HepG2 cells with a final reaction volume of 15 μl. To determine the specificity of the binding to the oligonucleotides, competition experiments were conducted by co-incubation with 10-, 50-, and 200-fold excesses of unlabeled competitors. For super-shift experiments, 2 μg of anti-Nrf2 antibodies or normal rabbit IgG were pre-incubated with the nuclear protein on ice for 30 min. The reactions were incubated on ice for 15 min and then loaded on 4% acrylamide gel in 0.5 x Tris-borate EDTA buffer. The gels were dried and exposed to film for 12 h. The DNA-protein complexes were detected with a Fuji Bio-Imaging Analyzer BAS 1000 (Fuji Film, Tokyo, Japan).

Statistical analyses

Data are expressed as mean ± SD. Comparison of two groups was made with two-tailed Student's *t* test. Comparison of multiple groups was made with ANOVA followed by Dunnett or Tukey test. A value of $P < 0.05$ was considered statistically significant.

Results

Sequence analysis of the 5'-flanking region of the *UGT2B7*1* and *UGT2B7*2* alleles

By genotyping of the SNP at 802C>T causing an amino acid substitution of H268Y, the allele frequencies of the *UGT2B7*2* were found to be 54.0% in 176 European-Americans, 31.3% in 163 African-Americans, and 32.4% in 54 Japanese, being consistent with previous studies [23-25]. The sequences of the 5'-flanking region of the *UGT2B7*1* (-7 to -2191) and *UGT2B7*2* (-7 to -2515) alleles were analyzed. Compared with the sequence of the *UGT2B7*1* allele, the *UGT2B7*2* alleles had a 324-bp insertion at -2067, which region included the Alu element-like sequence (Fig. 1a). In addition, 12 SNPs were found, of which 6 SNPs up to ~1.3 kb were identical with those reported by Duguay *et al.* [26]. These SNPs and the insertion were completely linked on the *UGT2B7*2* allele, being consistent with a sequence (AC111000.3) found in NCBI database. To find potential Nrf2 binding sites, the consensus ARE sequence 5'-TMAnnRTGAYnnnGCRwwww-3' [27] in which the core sequence is underlined was used to search ARE on the 5'-flanking region of *UGT2B7* gene. A computer-assisted homology search identified four putative AREs in up to 2.5 kb of the *UGT2B7* gene (Fig. 1a). These AREs at -739, -1080, -1170, and -2043 were termed ARE1, ARE2, ARE3, and ARE4, respectively. In the *UGT2B7*2* allele, several SNPs were around the ARE1, ARE2, and ARE3, and a SNP was found in ARE4. Therefore, we sought to investigate whether the polymorphic mutations in the 5'-flanking region of the *UGT2B7* gene might affect the Nrf2-dependent transactivation.

Effects of SFN treatment on UGT2B7 expression in cell lines

By the genotyping of the SNP at 802C>T, the HepG2 and HuH7 cells were found to be

*UGT2B7*1/*1*, whereas the HLE and Caco-2 cells were *UGT2B7*2/*2*. The sequence in the 5'-flanking region of the *UGT2B7* gene in the HepG2 and HuH7 cells was consistent with the sequence of the *UGT2B7*1* allele described above, except for heterozygous SNPs of -903G>C and -1073C>T in HepG2 and homozygous SNP of -804G>C in HuH7 cells. The sequence in the 5'-flanking region of the *UGT2B7* gene in the HLE and Caco-2 cells was completely consistent with the sequence of the *UGT2B7*2* allele described above. When these cell lines were treated with 10 μM SFN for 24 h, accumulation of Nrf2 to the nucleus was observed (Fig. 1b). Under this condition, the UGT2B7 mRNA was significantly ($p < 0.01$) increased in HepG2 (2.0-fold) and HuH7 (1.3-fold) cells, but not in Caco-2 and HLE cells (Fig. 1c). In contrast, HO-1 mRNA was significantly increased in all cell lines. The induction of UGT2B7 in HepG2 cells was confirmed at the protein level (Fig. 1d), and the enzymatic activity using morphine as a substrate (Fig. 1e). These results suggest that the UGT2B7 is induced by SFN in a *UGT2B7*1* allele specific manner.

Effect of siRNA for Nrf2 on the SFN-dependent induction of UGT2B7

To investigate whether Nrf2 is responsible for the induction of UGT2B7 by SFN, we sought to knock down the endogenous Nrf2 in HepG2 cells. The siRNA-Nrf2 prominently decreased the expression level of Nrf2 in the nucleus (Fig. 2a). Although the treatment with SFN for 48 h resulted in 4.0-fold induction of the UGT2B7 mRNA, the siRNA-Nrf2 significantly ($p < 0.001$) diminished the induction (Fig. 2b). Under this condition, the induction of HO-1 mRNA was also significantly diminished (data not shown). These results suggested that Nrf2 plays a critical role in the SFN-dependent induction of UGT2B7.

Identification of the Nrf2-responsive region in the *UGT2B7* promoter

To investigate whether AREs on the *UGT2B7* gene were functional for the Nrf2-dependent transactivation, luciferase assay was performed with reporter plasmids containing two copies of each ARE in HepG2 cells (Fig. 3a). We confirmed that the luciferase activity of pGL3-cARE used as a positive control was increased (2.4-fold) by the

overexpression of Nrf2. Among four reporter plasmids containing each putative ARE, only the plasmid containing ARE3 showed prominent (18-fold) transactivation by the overexpression of Nrf2. The SNP at -2029T>C in the plasmid pGL3-ARE4 did not affect the basal and Nrf2-dependent transcriptional activities (data not shown). The luciferase activity of the plasmid containing ARE3 was also increased (2.8-fold) by the treatment with SFN (Fig. 3b), but that of plasmids containing the ARE1, ARE2, and ARE4 did not (data not shown). The transfection of siRNA-Nrf2 decreased the basal activity of pGL3-ARE3 to 57% and completely abolished the transactivation by SFN. These results suggest that ARE3 would be responsible for the Nrf2-dependent transactivation of the human UGT2B7 by SFN.

Binding of Nrf2 to the ARE on the *UGT2B7* gene

To examine whether the Nrf2 can bind to the ARE3, gel shift assays were performed (Fig. 4). When the ^{32}P-labeled cARE was incubated with the nuclear extract prepared from vehicle-treated HepG2 cells, two bands were detected (Fig. 4a, lane 1). When it was incubated with the nuclear extracts from the SFN-treated cells, the density of the lower band was increased (lane 5). The band density was diminished with both anti-Nrf2 antibodies (C-20) and (H-300), but the super-shifted band was observed only with the anti-Nrf2 antibody (C-20), in agreement with a previous study [28] reporting that the super-shifted band was not observed with the anti-Nrf2 antibody (H-300). These results indicate that the lower band contain Nrf2 complexes. When the 2B7ARE3 was used as a probe, the specific band was observed with the nuclear extracts from the SFN-treated HepG2 cells (Fig. 4b). The band was competed out by unlabeled cARE and 2B7ARE3 and was super-shifted with the anti-Nrf2 antibody (C-20). These results suggest that Nrf2 binds to ARE3 on the human *UGT2B7* gene.

Nrf2-dependent transactivation of *UGT2B7*1* and *UGT2B7*2*

To compare the transactivities of the 5'-flanking region of *UGT2B7*1* and *UGT2B7*2*, luciferase assays were performed with a series of reporter plasmids (Fig. 5a). The basal promoter activities of pGL3*1c, pGL3*1d, pGL3*2a, pGL3*2b, pGL3*2c, and pGL3*2d

were almost the same, and the luciferase activities were decreased by the overexpression of Nrf2. In contrast, the basal activity of pGL3*1a and pGL3*1b showed higher activities than the other plasmids, and the luciferase activities were slightly increased by the overexpression of Nrf2. As shown in Fig. 5b, the basal activities of pGL3*1e and pGL3*2e were similar, and the luciferase activities were not affected by overexpression of Nrf2. The basal activity of pGL3*1f was higher than that of pGL3*2f, and the activities of both plasmids were increased by the coexpression of Nrf2. The extent of activation was higher in pGL3*1f (4.1-fold) than in pGL3*2f (2.5-fold). The Nrf2-dependent activation of pGL3*1f was significantly diminished with the mutation in ARE3 (pGL3*1g), and that of pGL3*2f was completely abolished with the mutation in ARE3 (pGL3*2g). These results suggest that ARE3 was indispensable in the Nrf2-mediated UGT2B7 promoter activation. Since the basal and Nrf2-activated activities of the pGL3*1h and pGL3*2h plasmids were similar with those of the pGL3*1f and pGL3*2f plasmids, respectively, it is suggested that the region from -1408 to -1174 containing two SNPs was not responsible for the transactivity. Unexpectedly, the pGL3*1i lacking ARE3 showed activation (2.4-fold) by the overexpression of Nrf2, whereas pGL3*2i did not. The differences between the two plasmids are two SNPs of -1112C>T and -900A>G. In the *UGT2B7*1* allele, a sequence at -906 to -898 TGAGTC<u>A</u>GA is similar to the core sequence of ARE. The underlined nucleotide is the site of SNP -900A>G in the *UGT2B7*2* allele. To investigate the effects of the SNPs on the transcriptional activity, luciferase assays were performed with reporter plasmids containing the polymorphic sites (Fig. 5c). The basal activities of pGL3*1k and pGL3*2k were similar, and neither plasmid was affected by the overexpression of Nrf2. The basal activity of pGL3*1l was higher (3.7-fold) than that of pGL3*2l. By the overexpression of Nrf2 the luciferase activity of pGL3*1l was substantially (1.8-fold) increased, but that of the pGL3*2l was marginally increased (1.2-fold). These results suggest that the SNP of -900A>G diminished the basal as well as the Nrf2-dependent transcriptional activities of UGT2B7.

Effects of the SNP at -900 to the binding of nuclear protein to UGT2B7 promoter

We performed gel shift assays to investigate whether Nrf2 binds to the region around -900 and the SNP -900A>G might affect the binding (Fig. 6a and b). Using a probe of 2B7*1-900, a shifted band was observed (Fig. 6a), and its density was increased with the nuclear extracts of the SFN-treated cells. Although the shifted band was not super-shifted with the anti-Nrf2 antibodies (C-20 and H-300), it was competed out by unlabeled cARE and 2B7*1-900. In contrast, no shifted band was observed by using the probe of 2B7*2-900 (Fig. 6b). These observations suggest that the SNP at -900 might decrease the binding ability of a nuclear protein that can bind to the ARE-like sequence.

Discussion

Earlier studies to understand the regulatory control of UGT2B7 [4-6] have focused on the constitutive expressions, whereas the regulation on the inducible expression has received less attention. In this study, we found that Nrf2 is involved in the regulation of UGT2B7. The extent of the induction of UGT2B7 via Nrf2 was comparable to those of known targets of Nrf2 such as NQO-1 and GST [9]. Thus, we could add the UGT2B7 to the list of the Nrf2-target genes. This is a first study to demonstrate that UGT2B7 is inducible in response to stimuli.

In the knock down experiment, the siRNA for Nrf2 could prominently diminished the induction, but the abrogation was incomplete. This might have been due to faint Nrf2 that failed to be knocked down (Fig. 2). In addition, the possibility could not be excluded that another mechanism independent of Nrf2 might be involved, since SFN has been reported to modify cell cycle regulation, histone deacetylase activity, and signaling pathways [29]. Moreover, it is known that Nrf2 and AhR can coordinately regulate target genes [30]. tBHQ, another activator of Nrf2, can also activate AhR [31]. We found two putative XREs (CACGC) in the *UGT2B7*2* promoter at the 324-bp insertion region, but not in the *UGT2B7*1* promoter up to -2.1 kb. We confirmed that the CYP1A1 mRNA level, a typical target of AhR, was not changed by SFN treatment in HepG2 cells (data not shown). Thus, the induction of UGT2B7 by SFN would be independent of AhR.

In the mouse *Gst Ya* gene, it has been reported that two adjacent AREs are synergistically responsible for the induction by tBHQ [32]. Accordingly, we expected that the close ARE2 and ARE3 on the human *UGT2B7* gene might act synergistically, but only the ARE3 was functional (Figs 3a and 5b) with no synergism. The core ARE sequences (TGAYnnnGC) of ARE2 and ARE3 are perfectly matched. Therefore, the flanking sequence of core ARE (TMAnnRTGAYnnnGCRwwww, underlined) would make a contribution to the function of ARE, in agreement with a previous study reporting that mutations in the flanking nucleotides affected the function of ARE in mouse *Nqo1* gene [7]. Approximately 180-bp sequences around ARE3 in the *UGT2B7*2* allele were identical with those in the *UGT2B7*1* allele. The reporter plasmids containing ARE3 in the *UGT2B7*2* allele (pGL3*2f and pGL3*2h) showed the transactivation by Nrf2 (Fig. 5b). Nonetheless, UGT2B7 mRNA was not induced by SFN in the HLE and Caco-2 cells genotyped as *UGT2B7*2/*2* (Fig. 1c). The chromatin structure, epigenetic events, and/or other unknown factor(s) may also be associated with the UGT2B7 regulation.

As shown in Fig. 5c, we found that the SNP -900A>G had a role in the repression of the Nrf2-dependent transactivation as well as basal promoter activity of UGT2B7. Duguay *et al.* [26] had also reported that this SNP plays an important role in the basal promoter activity. In this study, we firstly found that this SNP is associated with the decreased binding of some transcriptional factor(s). Although we did not identify the factor(s), it was surmised to be AP-1 because the sequence around -900 on the *UGT2B7*1* promoter includes a consensus AP-1 binding site, TGA(G/C)TCA, and the SNP -900A>G looses the putative AP-1 binding site. Although the gel shift analyses could not substantiate the binding of Nrf2 to this region, the luciferase analyses clearly demonstrated that the SNP -900A>G decreased the Nrf2-dependent transactivation.

Using an expression system of UGT2B7.1 and UGT2B7.2 in HEK293 cells, Coffman *et al.* [33] have reported that the amino acid substitution H268Y had an insignificant effect on the catalytic activity toward morphine and other opioids. In contrast, it has been reported that the amino acid substitution decreased the intrinsic clearance (Vmax/Km) for the

glucuronidation of AZT [34] or aldosterone [35]. We should take notice of the fact that the SNP causing the amino acid change is linked with the polymorphism in the 5'-flanking region, which might affect the expression level. However, it has been reported that the *UGT2B7*2* allele had no effect on the protein level in human liver microsomes [19]. The effects of the *UGT2B7*2* allele on the in vivo glucuronidation of drugs were also controversial. Holthe *et al.* [24] reported that no relationship was found between the *UGT2B7*2* genotype and the ratio of morphine 3-glucuronide/morphine in serum. In contrast, recent reports indicated that the *UGT2B7*2* allele was associated with the decreased clearance of morphine [36] and the increased serum concentration of mycophenolic acid [37]. Collectively, the clinical significance of the *UGT2B7*2* allele remains obscure. Here we found that the polymorphism in the 5'-flanking region of the *UGT2B7*2* allele abrogated the Nrf2-dependent transcriptional activity. This finding implies that oxidative stress from diet or environment would differently affect the glucuronidation of some drugs or hormones in an allele-dependent manner. Thus, the regulation mechanism may be one of the factors causing interindividual variability in the expression levels and enzymatic activities of UGT2B7. In vivo studies are needed to evaluate the clinical significance of the Nrf2-dependent induction of UGT2B7 in response to oxidative stress in association with the polymorphism.

UGT2B7 catalyzes the glucuronidation of tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) [38] and genotoxic 4-hydroxyestradiol [39], playing a role in their detoxification. It has been reported that the UGT2B7 level was significantly decreased or absent in human breast cancer in comparison to normal tissue [40], whereas the 4-hydroxyestradiol level is higher in breast cancers than in normal tissue [41]. Epidemiological studies suggested that high levels of isothiocyanates in human urine were associated with reduced breast cancer risk [42]. Since the isothiocyanates including SFN activate Nrf2, the up-regulation of UGT2B7 by Nrf2 would be one of the mechanisms in the chemopreventive effects of isothiocyanates.

In summary, we found that Nrf2 transcriptionally activated the human *UGT2B7* gene and polymorphic mutations in the promoter region on the *UGT2B7*2* allele interfered with

the regulation. The allele specific mechanism might explain the variability of glucuronosyltransferase activity of clinical drugs, environmental carcinogens, and steroid hormones in response to oxidative stress.

Acknowledgements

We acknowledge Brent Bell for reviewing the manuscript. None of the authors has any conflict of interest.

References

1 Tukey RH, Strassburg CP. Human UDP-glucuronosyltransferases: metabolism, expression, and disease. *Annu Rev Pharmacol Toxicol* 2000; **40**:581-616.

2 Mackenzie PI, Bock KW, Burchell B, Guillemette C, Ikushiro S, Iyanagi T, *et al*. Nomenclature update for the mammalian UDP glycosyltransferase (*UGT*) gene superfamily. *Pharmacogenet Genomics* 2005; **15**:677-685.

3 Radominska-Pandya A, Little JM, Czernik PJ. Human UDP-glucuronosyltransferase 2B7. *Curr Drug Metab* 2001; **2**:283-298.

4 Ishii Y, Hansen AJ, Mackenzie PI. Octamer transcription factor-1 enhances hepatic nuclear factor-1α-mediated activation of the human UDP glucuronosyltransferase 2B7 promoter. *Mol Pharmacol* 2000; **57**:940-947.

5 Gregory PA, Gardner-Stephen DA, Rogers A, Michael MZ, Mackenzie PI. The caudal-related homeodomain protein Cdx2 and hepatocyte nuclear factor 1α cooperatively regulate the UDP-glucuronosyltransferase 2B7 gene promoter. *Pharmacogenet Genomics* 2006; **16**:527-536.

6 Lu Y, Heydel JM, Li X, Bratton S, Lindblom T, Radominska-Pandya A. Lithocholic acid decreases expression of UGT2B7 in Caco-2 cells: a potential role for a negative farnesoid X receptor response element. *Drug Metab Dispos* 2005; **33**:937-946.

7 Nioi P, McMahon M, Itoh K, Yamamoto M, Hayes JD. Identification of a novel Nrf2-regulated antioxidant response element (ARE) in the mouse NAD(P)H:quinone oxidoreductase 1 gene: reassessment of the ARE consensus sequence. *Biochem J* 2003; **374**:337-348.

8 Balogun E, Hoque M, Gong P, Killeen E, Green CJ, Foresti R, *et al*. Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. *Biochem J* 2003; **371**:887-895.

9 Morimitsu Y, Nakagawa Y, Hayashi K, Fujii H, Kumagai T, Nakamura Y, *et al*. A sulforaphane analogue that potently activates the Nrf2-dependent detoxification

pathway. *J Biol Chem* 2002; 277:3456-3463.

10 Jeyapaul J, Jaiswal AK. Nrf2 and c-Jun regulation of antioxidant response element (ARE)-mediated expression and induction of γ-glutamylcysteine synthetase heavy subunit gene. *Biochem Pharmacol* 2000; **59**:1433-1439.

11 Moi P, Chan K, Asunis I, Cao A, Kan YW. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the β-globin locus control region. *Proc Natl Acad Sci USA* 1994; **91**:9926-9930.

12 McMahon M, Itoh K, Yamamoto M, Hayes JD. Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. *J Biol Chem* 2003; **278**:21592-21600.

13 Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, *et al*. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem Biophys Res Commun* 1997; **236**:313-322.

14 Thimmulappa RK, Mai KH, Srisuma S, Kensler TW, Yamamoto M, Biswal S. Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. *Cancer Res* 2002; **62**:5196-5203.

15 Iida K, Itoh K, Kumagai Y, Oyasu R, Hattori K, Kawai K, *et al*. Nrf2 is essential for the chemopreventive efficacy of oltipraz against urinary bladder carcinogenesis. *Cancer Res* 2004; **64**:6424-6431.

16 Yueh MF, Tukey RH. Nrf2-Keap1 signaling pathway regulates human UGT1A expression in vitro and in transgenic UGT1 mice. *J Biol Chem* 2007; **282**:8749-8758.

17 Münzel PA, Schmohl S, Heel H, Kälberer K, Bock-Hennig BS, Bock KW. Induction of human UDP glucuronosyltransferases (UGT1A6, UGT1A9, and UGT2B7) by t-butylhydroquinone and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in Caco-2 cells. *Drug Metab Dispos* 1999; **27**:569-573.

18 Nakajima M, Fukami T, Yamanaka H, Higashi E, Sakai H, Yoshida R, *et al*.

Comprehensive evaluation of variability in nicotine metabolism and *CYP2A6* polymorphic alleles in four ethnic populations. *Clin Pharmacol Ther* 2006; **80**:282-297.

19 Court MH, Krishnaswamy S, Hao Q, Duan SX, Patten CJ, Von Moltke LL, *et al*. Evaluation of 3'-azido-3'-deoxythymidine, morphine, and codeine as probe substrates for UDP-glucuronosyltransferase 2B7 (UGT2B7) in human liver microsomes: specificity and influence of the UGT2B7*2 polymorphism. *Drug Metab Dispos* 2003; **31**:1125-1133.

20 Tsuchiya Y, Nakajima M, Kyo S, Kanaya T, Inoue M, Yokoi T. Human CYP1B1 is regulated by estradiol via estrogen receptor. *Cancer Res* 2004; **64**:3119-3125.

21 Schreiber E, Matthias P, Muller MM, Schaffner W. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res* 1989; **17**:6419.

22 Kurkela M, Garcia-Horsman JA, Luukkanen L, Morsky S, Taskinen J, Baumann M, *et al*. Expression and characterization of recombinant human UDP-glucuronosyltransferases (UGTs). UGT1A9 is more resistant to detergent inhibition than other UGTs and was purified as an active dimeric enzyme. *J Biol Chem* 2003; **278**:3536-3544.

23 Hirota T, Ieiri I, Takane H, Sano H, Kawamoto K, Aono H, *et al*. Sequence variability and candidate gene analysis in two cancer patients with complex clinical outcomes during morphine therapy. *Drug Metab Dispos* 2003; **31**:677-680.

24 Holthe M, Rakvåg TN, Klepstad P, Idle JR, Kaasa S, Krokan HE, *et al*. Sequence variations in the UDP-glucuronosyltransferase 2B7 (*UGT2B7*) gene: identification of 10 novel single nucleotide polymorphisms (SNPs) and analysis of their relevance to morphine glucuronidation in cancer patients. *Pharmacogenomics J* 2003; **3**:17-26.

25 Sawyer MB, Innocenti F, Das S, Cheng C, Ramírez J, Pantle-Fisher FH, *et al.* A pharmacogenetic study of uridine diphosphate-glucuronosyltransferase 2B7 in patients receiving morphine. *Clin Pharmacol Ther* 2003; **73**:566-574.

26 Duguay Y, Báár C, Skorpen F, Guillemette C. A novel functional polymorphism in the

uridine diphosphate-glucuronosyltransferase 2B7 promoter with significant impact on promoter activity. *Clin Pharmacol Ther* 2004; **75**:223-233.

27 Wasserman WW, Fahl WE. Functional antioxidant responsive elements. *Proc Natl Acad Sci USA* 1997; **94**:5361-5366.

28 Wang W, Jaiswal AK. Nuclear factor Nrf2 and antioxidant response element regulate NRH:quinone oxidoreductase 2 (NQO2) gene expression and antioxidant induction. *Free Radic Biol Med* 2006; **40**:1119-1130.

29 Myzak MC, Dashwood RH. Chemoprotection by sulforaphane: keep one eye beyond Keap1. *Cancer Lett* 2006; **233**:208-218.

30 Köhle C, Bock KW. Coordinate regulation of Phase I and II xenobiotic metabolisms by the Ah receptor and Nrf2. *Biochem Pharmacol* 2006; **73**:1853-1862.

31 Gharavi N, El-Kadi AO. *tert*-Butylhydroquinone is a novel aryl hydrocarbon receptor ligand. *Drug Metab Dispos* 2005; **33**:365-372.

32 Friling RS, Bergelson S, Daniel V. Two adjacent AP-1-like binding sites form the electrophile-responsive element of the murine glutathione *S*-transferase Ya subunit gene. *Proc Natl Acad Sci USA* 1992; **89**:668-672.

33 Coffman BL, King CD, Rios GR, Tephly TR. The glucuronidation of opioids, other xenobiotics, and androgens by human UGT2B7Y(268) and UGT2B7H(268). *Drug Metab Dispos* 1998; **26**:73-77.

34 Barbier O, Turgeon D, Girard C, Green MD, Tephly TR, Hum DW, *et al*. 3'-Azido-3'-deoxythimidine (AZT) is glucuronidated by human UDP-glucuronosyltransferase 2B7 (UGT2B7). *Drug Metab Dispos* 2000; **28**:497-502.

35 Girard C, Barbier O, Veilleux G, El-Alfy M, Bélanger A. Human uridine diphosphate-glucuronosyltransferase UGT2B7 conjugates mineralocorticoid and glucocorticoid metabolites. *Endocrinology* 2003; **144**:2659-2668.

36 Darbari DS, van Schaik RH, Capparelli EV, Rana S, McCarter R, van den Anker J. UGT2B7 promoter variant -840G>A contributes to the variability in hepatic clearance of morphine in patients with sickle cell disease. *Am J Hematol* (in press).

37 Lévesque E, Delage R, Benoit-Biancamano MO, Caron P, Bernard O, Couture F, *et al*. The impact of UGT1A8, UGT1A9, and UGT2B7 genetic polymorphisms on the pharmacokinetic profile of mycophenolic acid after a single oral dose in healthy volunteers. *Clin Pharmacol Ther* 2007; **81**:392-400.

38 Ren Q, Murphy SE, Zheng Z, Lazarus P. *O*-Glucuronidation of the lung carcinogen 4-(methylnitrosamino)-1- (3-pyridyl)-1-butanol (NNAL) by human UDP-glucuronosyltransferases 2B7 and 1A9. *Drug Metab Dispos* 2000; **28**:1352-1356.

39 Lepine J, Bernard O, Plante M, Tetu B, Pelletier G, Labrie F, *et al*. Specificity and regioselectivity of the conjugation of estradiol, estrone, and their catecholestrogen and methoxyestrogen metabolites by human uridine diphospho-glucuronosyltransferases expressed in endometrium. *J Clin Endocrinol Metab* 2004; **89**:5222-5232.

40 Gestl SA, Green MD, Shearer DA, Frauenhoffer E, Tephly TR, Weisz J. Expression of UGT2B7, a UDP-glucuronosyltransferase implicated in the metabolism of 4-hydroxyestrone and all-*trans* retinoic acid, in normal human breast parenchyma and in invasive and *in situ* breast cancers. *Am J Pathol* 2002; **160**:1467-1479.

41 Castagnetta LA, Granata OM, Traina A, Ravazzolo B, Amoroso M, Miele M, *et al.* Tissue content of hydroxyestrogens in relation to survival of breast cancer patients. *Clin Cancer Res* 2002; **8**:3146-3155.

42 Fowke JH, Chung FL, Jin F, Qi D, Cai Q, Conaway C, *et al*. Urinary isothiocyanate levels, brassica, and human breast cancer. *Cancer Res* 2003; **63**:3980-3986.

Figure legends

Fig. 1. Human *UGT2B7* genotype and induction by SFN. (a) Schematic representation of the polymorphisms in the 5'-flanking region of the human *UGT2B7* gene and the sequences of putative AREs. The sequence of the *UGT2B7*2* allele was consistent with a sequence from the NCBI database (AC111000.3). Numbers indicate the nucleotide position when the A in the initiation codon ATG is denoted + 1 and the base before A is numbered -1. *Right*, the core ARE sequence is underlined. The nucleotides that are consistent with the consensus ARE are shown with bold letters. Effects of sulforaphane (SFN) treatment on (b) translocation of Nrf2 and (c) UGT2B7 or HO-1 mRNA levels in four cell lines. HepG2, HuH7, HLE, and Caco-2 cells were treated with 10 μM SFN or vehicle alone (0.1% DMSO) for 24 h. The Nrf2 protein level in the nucleus or cytoplasm was determined by Western blot analysis. The expression level of UGT2B7 and HO-1 mRNA was determined by real-time RT-PCR and normalized with the GAPDH mRNA levels. Effects of SFN treatment on (d) UGT2B7 protein level and (e) morphine glucuronosyltransferase activity in HepG2 cells. The cells were treated with 10 μM SFN or vehicle alone (0.1% DMSO) for 24 or 48 h. The UGT2B7 protein level in the membrane fraction was determined by Western blot analysis. Each column represents the mean ± SD of triplicate determinations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with control.

Fig. 2. Effect of Nrf2 knock down on UGT2B7 mRNA induction in HepG2 cells. (a) Western blot analysis of Nrf2 in HepG2 nucleus. The cells were transfected with siRNA at 10 nM. After 24 h, the cells were treated with 10 μM SFN for 6 h. (b) Expression level of UGT2B7 mRNA determined by real-time RT-PCR and fold induction by SFN. After 24 h transfection with siRNA, the cells were treated with 10 μM SFN for 48 h. To normalize RNA loading and PCR variations, the UGT2B7 mRNA levels were corrected with the GAPDH mRNA levels. Each column represents the mean ± SD of triplicate determinations. ***, $P < 0.001$ compared with control (vehicle).

Fig. 3. Luciferase assays of constructs containing two copies of putative AREs on the *UGT2B7* gene. (a) Reporter activities of the constructs co-transfected with human Nrf2 expression vector. After 48 h transfection, the cells were harvested and assayed for the luciferase activities according to the manufacturer's protocol. (b) Effect of Nrf2 knock down on transactivation through ARE3 by SFN. siRNA was co-transfected with reporter plasmids into HepG2 cells. After 36 h, the cells were treated with 10 μM SFN for 12 h. Each column represents the mean ± SD of three independent experiments. Control, pTARGET empty vector. ***, $P < 0.001$ compared with control. ††, $P < 0.01$ and †††, $P < 0.001$ compared with DMSO treatment.

Fig. 4. Gel shift analysis of binding of Nrf2 to ARE3 on the *UGT2B7* gene. Oligonucleotide probes of (a) consensus ARE and (b) 2B7ARE3 labeled with ^{32}P were incubated with nuclear extracts prepared from HepG2 cells treated with 0.1% DMSO or 10 μM SFN. The sequences of the probes are shown in Table 1. Cold oligonucleotides were used as a competitor in 10-, 50-, and 200-fold molar excess. For super-shift analyses, 2 μg of anti-Nrf2 antibodies (α-Nrf2) or normal rabbit IgG (IgG) were pre-incubated with the nuclear extracts on ice for 30 min. The *lower* arrow indicates the position of the Nrf2-dependent shifted band, and the *upper* one indicates the super-shifted complex by anti-Nrf2 antibodies. Due to the different exposure times, the band intensities in the panels (a) and (b) could not be compared.

Fig. 5. Promoter activities of UGT2B7 constructs and effects of coexpression of Nrf2. A series of reporter constructs containing the 5'-flanking region of the *UGT2B7* gene were transiently transfected into HepG2 cells with the expression plasmid for human Nrf2. After 48 h, the cells were harvested and assayed for the luciferase activities according to the manufacturer's protocol. Each column represents the mean ± SD of three independent experiments. Control, pTARGET empty vector. **, $P < 0.01$ and ***, $P < 0.001$.

Fig. 6. Gel shift analysis for the sequence around the SNP of -900A>G. Oligonucleotide probes of (a) 2B7*1-900 and (b) 2B7*2-900 labeled with ^{32}P were incubated with nuclear extracts prepared from HepG2 cells treated with 0.1% DMSO or 10 μM SFN. The sequences of the probes are shown in Table 1. Cold oligonucleotides were used as a competitor in 10-, 50-, and 200-fold molar excess. For super-shift analyses, 2 μg of anti-Nrf2 antibodies or normal rabbit IgG were pre-incubated with the nuclear extracts on ice for 30 min. The arrow indicates the position of the shifted band. Due to the different exposure times, the band intensities in the panels (a) and (b) could not be compared.

Table 1. Oligonucleotide sequences used for the gel shift analyses.

Probe	Sequence	Position
cARE[a]	5'-gatcttttatgctgagtcatggttt-3'	
2B7ARE3	5'-acattatttgctgagtcagttccttgtgggg-3'	-1174/-1144
2B7*1-900	5'-tgtgaggaagtgagtcAgagaacaagctaa-3'	-916/-887
2B7*2-900	5'-tgtgaggaagtgagtcGgagaacaagctaa-3'	-916/-887

[a]From Balogun *et al*. [8].

The polymorphic sites are indicated by capital letters.

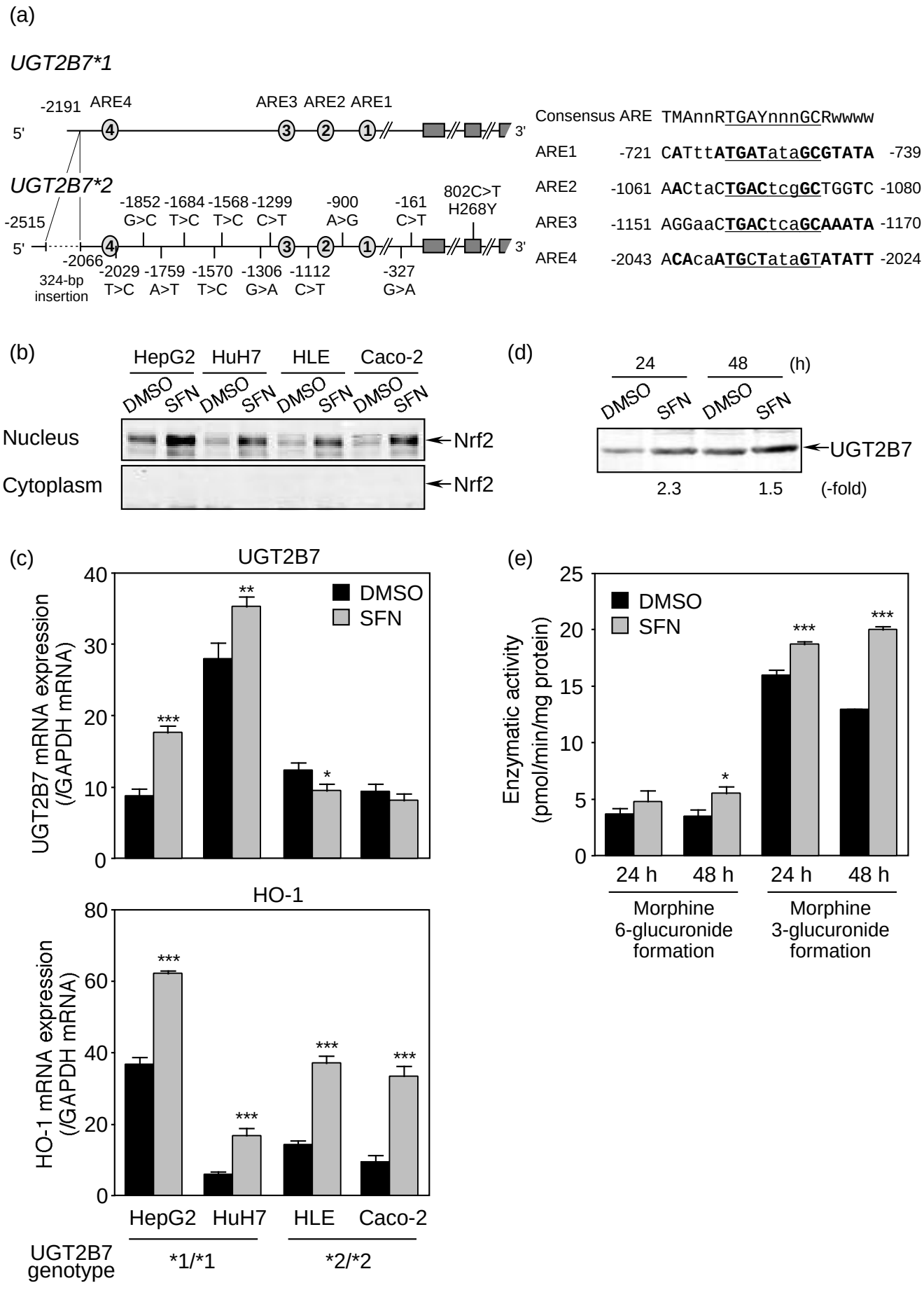
(a)
UGT2B7*1
-2191
ARE4
ARE3 ARE2 ARE1
5'
3'
UGT2B7*2
-2515
-2066
324-bp insertion
-1852 G>C
-1684 T>C
-1568 T>C
-1299 C>T
-900 A>G
-161 C>T
802C>T H268Y
-2029 T>C
-1759 A>T
-1570 T>C
-1306 G>A
-1112 C>T
-327 G>A
Consensus ARE TMAnnRTGAYnnnGCRwwww
ARE1 -721 CATttATGATataGCGTATA -739
ARE2 -1061 AACtaCTGACtcgGCTGGTC -1080
ARE3 -1151 AGGaaCTGACtcaGCAAATA -1170
ARE4 -2043 ACAcaATGCTataGTATATT -2024
(b)
HepG2 HuH7 HLE Caco-2
DMSO SFN DMSO SFN DMSO SFN DMSO SFN
Nucleus
Cytoplasm
Nrf2
Nrf2
(d)
24 48 (h)
DMSO SFN DMSO SFN
UGT2B7
2.3 1.5 (-fold)
(c)
UGT2B7
UGT2B7 mRNA expression (/GAPDH mRNA)
DMSO
SFN
HO-1
HO-1 mRNA expression (/GAPDH mRNA)
HepG2 HuH7 HLE Caco-2
UGT2B7 genotype
*1/*1
*2/*2
(e)
Enzymatic activity (pmol/min/mg protein)
24 h 48 h 24 h 48 h
Morphine 6-glucuronide formation
Morphine 3-glucuronide formation

Fig. 2. Nakamura et al.

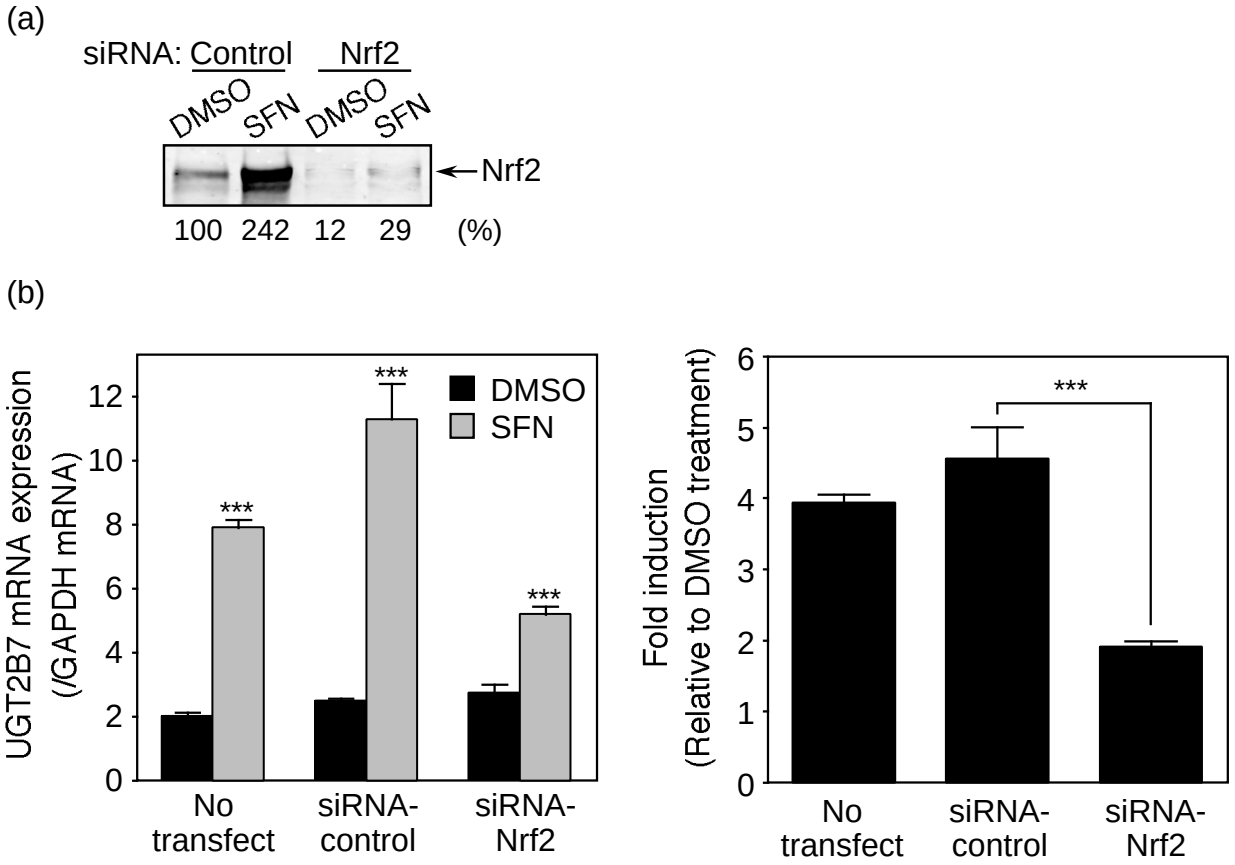

Fig. 3. Nakamura et al.

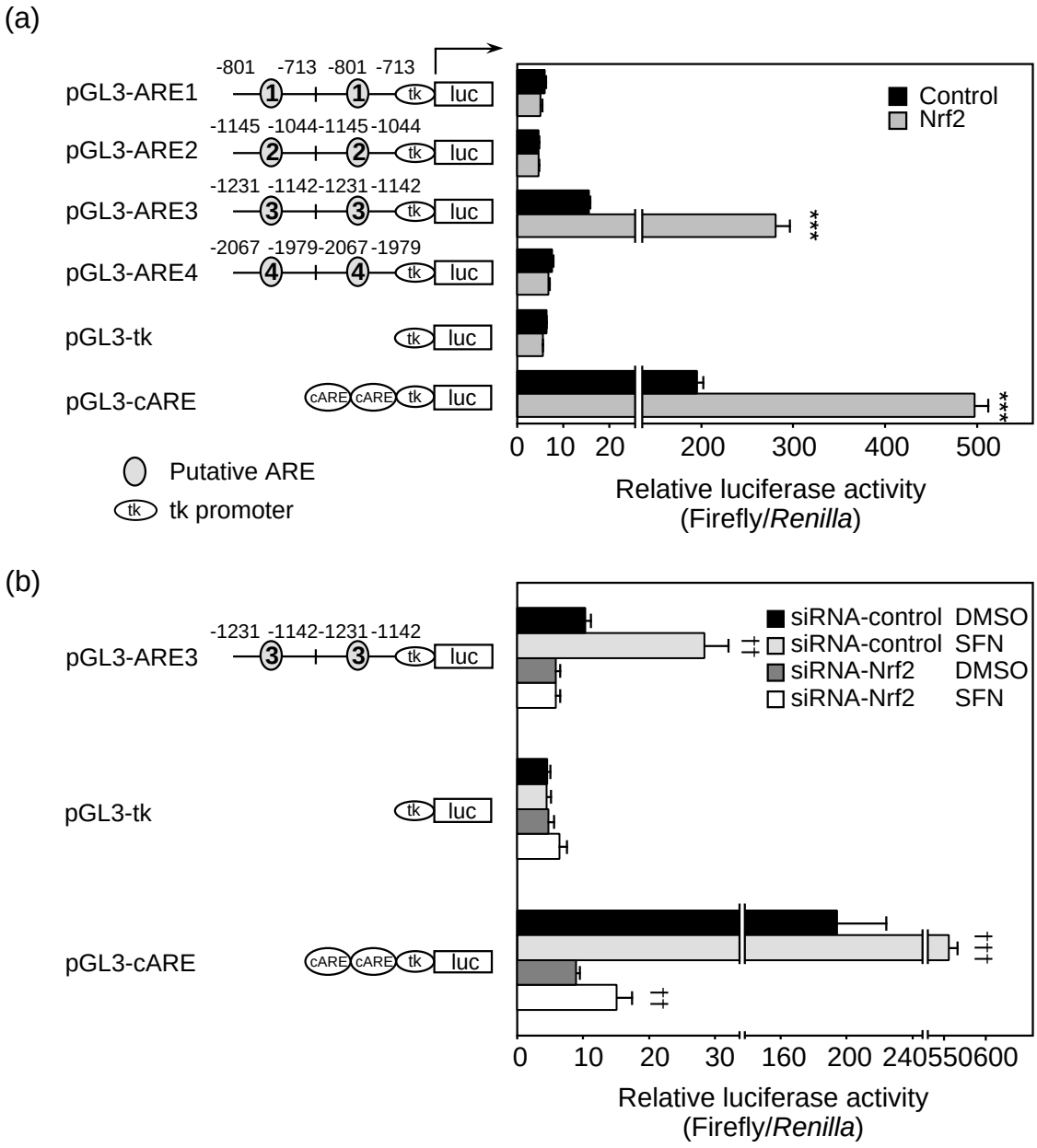

Fig. 4. Nakamura et al.

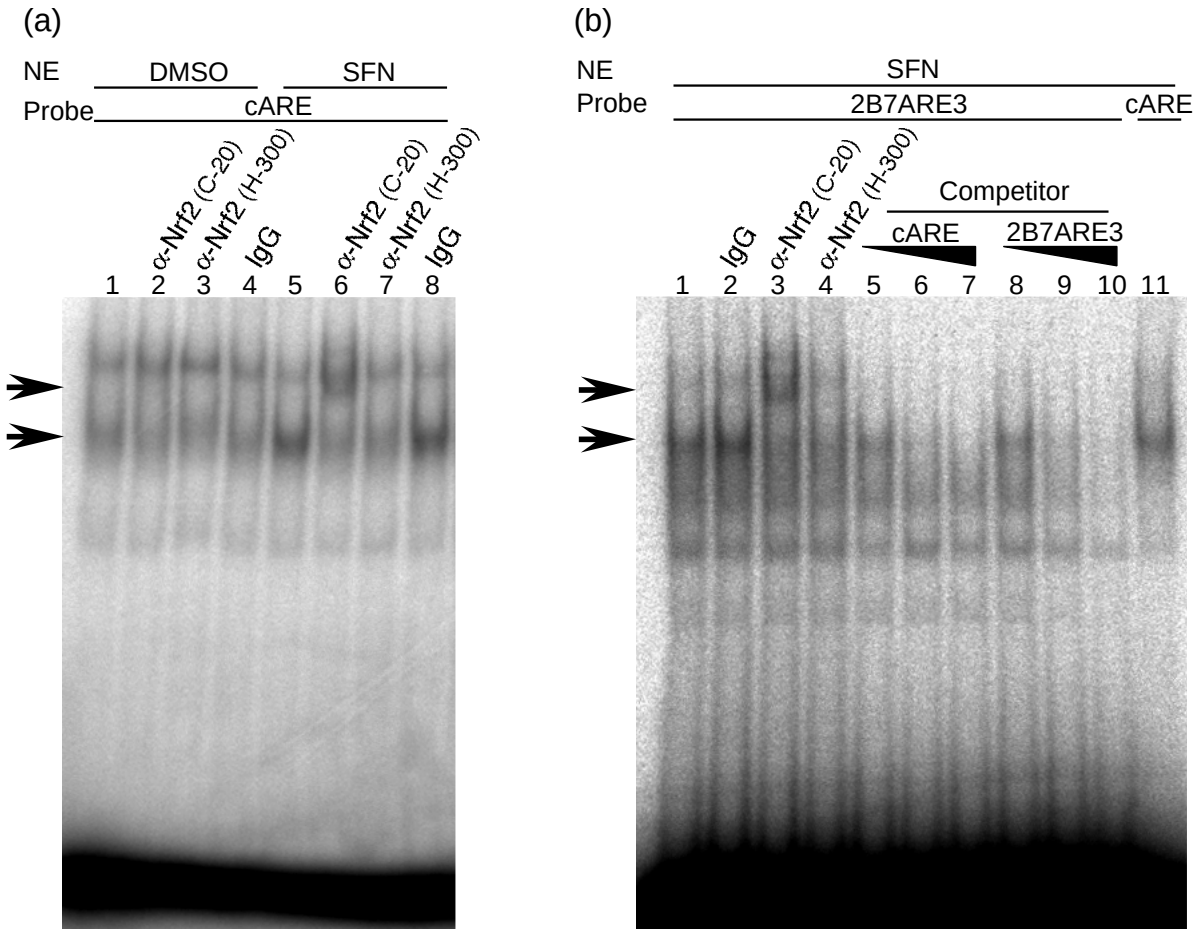

Fig. 5. Nakamura et al.

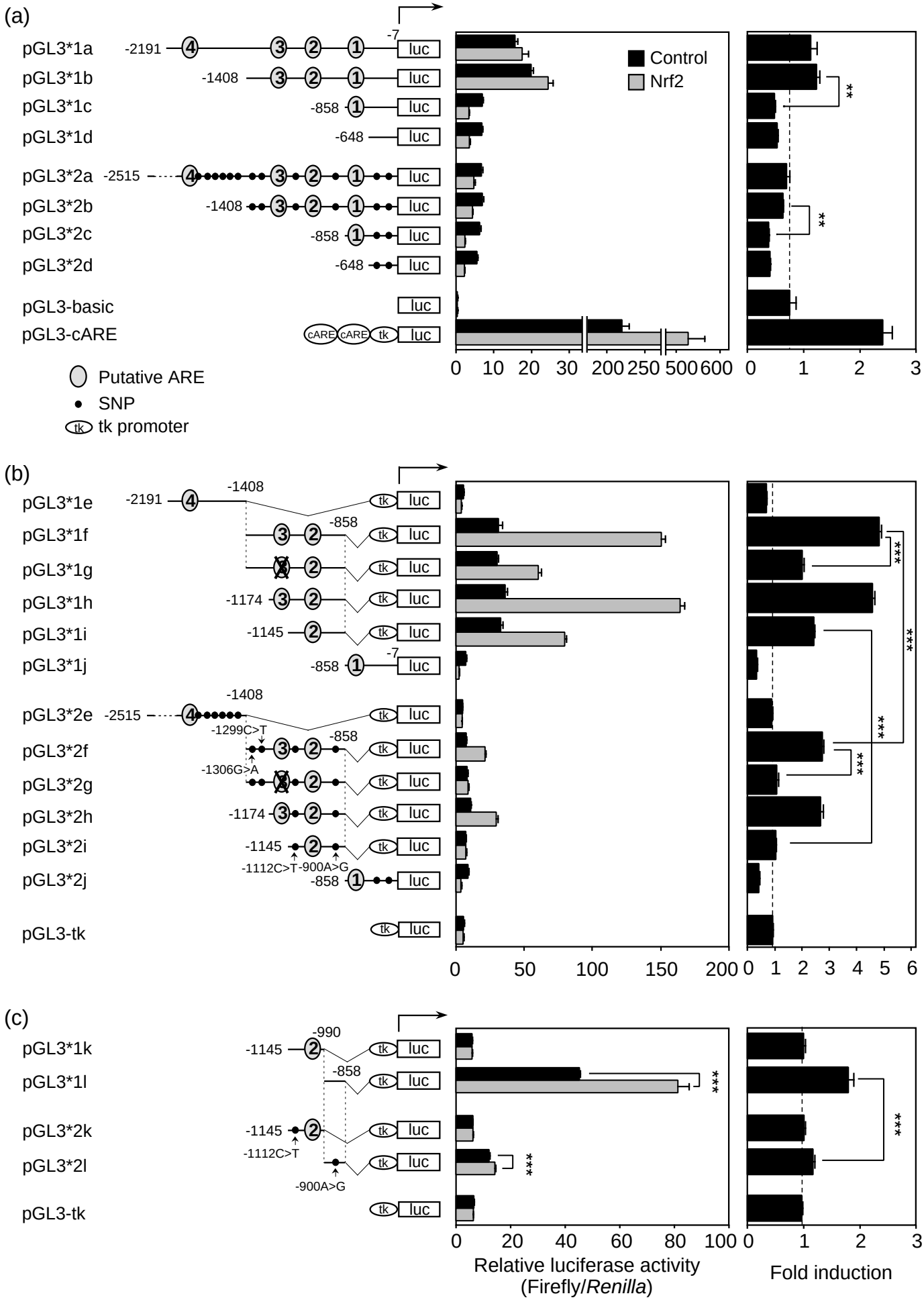

Fig. 6. Nakamura et al.