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Simultaneous Expression of Guinea Pig UDP-Glucuronosyltransferase 2B21 and 2B22 in COS-7 Cells enhances UDP-Glucuronosyltransferase 2B21-Catalyzed Morphine-6-Glucuronide Formation

YUJI ISHII,[1] AYA MIYOSHI, RYOICHI WATANABE, KAZUOKI TSURUDA, MINORU TSUDA, YUKI YAMAGUCHI-NAGAMATSU, KUNIHIRO YOSHISUE, MITSUKO TANAKA, DAISUKE MAJI, SATORU OHGIYA, and KAZUTA OGURI

Graduate School of Pharmaceutical Sciences, Kyushu University, Maidashi, Higashi-ku, Fukuoka, Japan (Y.I, A.M., R.W., K.T., M.T., Y.Y.-N., K.Y, M.T., D.M., K.O.); and Hokkaido National Industrial Research Institute, Sapporo, Japan (S.O.)

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ABSTRACT

Although UDP-glucuronosyltransferases (UGTs) act as an important detoxification system for many endogenous and exogenous compounds, they are also involved in the metabolic activation of morphine to form morphine-6-glucuronide (M-6-G). The cDNAs encoding guinea pig liver UGT2B21 and UGT2B22, which are intimately involved in M-6-G formation, have been cloned and characterized. Although some evidence suggests that UGTs may function as oligomers, it is not known whether hetero-oligomer formation leads to differences in substrate specificity. In this work, evidence for a functional hetero-oligomer between UGT2B21 and UGT2B22 is provided by studies on the glucuronidation of morphine in transfected COS-7 cells. Cells transfected with UGT2B21 cDNA catalyzed mainly morphine-3-glucuronide formation although M-6-G was also formed to some extent. In contrast, cells transfected with UGT2B22 cDNA did not show any significant activity toward morphine. When UGT2B21 and UGT2B22 were expressed simultaneously in different ratios in COS-7 cells, extensive M-6-G formation was observed. This stimulation of M-6-G formation was not observed, however, when microsomes containing UGT2B21were mixed with those containing UGT2B22 in the presence of detergent. Furthermore, this effect was not very marked when human UGT1A1 and UGT2B21 were coexpressed in COS-7 cells. This is the first report suggesting that UGT hetero-oligomer formation leads to altered substrate specificity.

Glucuronidation is well known as one of the detoxification pathways for both exogenous and endogenous compounds. However, in rare cases, metabolic activation is also possible, such as formation of an active metabolite of morphine, morphine-6-glucuronide (M-6-G) (Shimomura et al., 1971). Glucuronidation is catalyzed by UDP-glucuronosyltransferases (UGTs), members of a superfamily of glycosyltransferases that are found in the endoplasmic reticulum membrane (Mackenzie et al., 1997). Two UGT gene families involved in glucuronidation have been described on the basis of evolutionary divergence: UGT1 and UGT2. The N-terminal region of UGT is now believed to be an important determinant of substrate specificity (Mackenzie et al., 1997, and references therein).

M-6-G is very potent after intracerebroventricular injection into mice, whereas morphine-3-glucuronide (M-3-G) is a metabolite with no analgesic activity (Shimomura et al., 1971). The pharmacological significance of M-6-G in patients has been widely recognized (Säwe et al., 1985) and the presence of endogenous morphine is also well known (Oka et al., 1985). In vivo and in vitro studies on morphine glucuronidation have revealed that the formation of M-6-G differs markedly from species to species (Kuo et al., 1991). Although the urinary excretion of M-6-G in rats and mice was too small to

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[1] Present address: Department of Environmental Medicine, Institute of Community Medicine, University of Tsukuba, Tennodai, Tsukuba, Ibaraki, Japan.

ABBREVIATIONS: M-6-G, morphine-6-glucuronide; UGT, UDP-glucuronosyltransferase; M-3-G, morphine-3-glucuronide; UDP-GlcA, UDP-glucuronic acid; Ptd-choline, L-α-phosphatidyl choline; PVDF, polyvinylidene difluoride; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; bp, base pair(s); RACE, rapid amplification of cDNA ends; ORF, open reading frame; AP1, adapter primer 1; CMV, cytomegalovirus.

be determined, the ratios of UGT activities toward the 3- and 6-hydroxyl groups of morphine in liver microsomes of mice, rats, guinea pigs, rabbits and humans were approximately 300:1, 90:1, 4:1, 40:1, and 6:1, respectively (Yue et al., 1990; Kuo et al., 1991). Recently, Nagano et al. (2000) demonstrated that the formation of M-3-G and M-6-G is comparable when brain homogenate of rat was incubated with an endogenous level of [^{3}H]morphine.

UGT isoforms involved in M-3-G formation have been purified from experimental animals (Bock et al., 1979; Mackenzie et al., 1984; Puig and Tephly, 1986; Ishii et al., 1993; Oguri et al., 1996), although the UGT isoform involved in M-6-G formation remains to be purified. On the basis of the substrate specificity of expressed cloned UGT isoforms, human UGT2B7 and monkey UGT2B9 were shown to catalyze the glucuronidation of morphine at the 6-hydroxyl and 3-hydroxyl groups (Coffman et al., 1997; Green et al., 1997). However, guinea pigs exhibit rather a high rate of M-6-G formation compared with humans (Kuo et al., 1991).

In this work, we have investigated guinea pigs in an attempt to purify the UGT isoforms involved in M-6-G formation using ω-(β-carboxypropionylamino)octyl Sepharose 4B, chromatofocusing, and UDP-hexanolamine Sepharose 4B column chromatography. This gave us an active preparation with two UGT isoforms, UGT55K and UGT59K, which are difficult to separate. The oligomeric behavior of UGT has been discussed and Koiwai et al. (1996) have demonstrated in vitro that a dominant negative effect is observed in the Crigler Najjar syndrome type-II that is probably caused by hetero-oligomer formation of the wild-type and an inactive mutant type of UGT1A1. Meech and Mackenzie (1997) have shown the importance of the N-terminal domain in the dimerization of UGT2B1, whereas Ikushiro et al. (1997) have demonstrated that there is a protein-protein interaction between the UGT2B1 and UGT1A subfamily isoforms leading to the formation of hetero-oligomers. However, the determination of the substrate specificity associated with the formation of hetero-oligomers has so far been only superficial. In this study, we have cloned UGT2B21 and UGT2B22 cDNAs, which encode UGT55K and UGT59K. Nucleotide sequences of UGT2B21 and UGT2B22 have been deposited in the DDBJ/GenBank/EMBL database with accession numbers AB034987 and AB03988. We demonstrate here that extensive M-6-G formation was observed only when UGT2B21 and UGT2B22 were expressed simultaneously in COS-7 cells. This is the first report suggesting that UGT hetero-oligomer formation results in a substrate specificity that is different from the corresponding homo-oligomer.

Experimental Procedures

Materials. UDP-glucuronic acid (UDP-GlcA) was purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). Morphine hydrochloride was purchased from Takeda Chemical Ind. Co., Ltd. (Osaka, Japan). Egg yolk L-α-phosphatidyl (Ptd)-choline was obtained from Sigma Chemical Co. (St. Louis, MO). Morphine-3-glucuronide (M-3-G) and morphine-6-glucuronide (M-6-G) were synthesized by the method described previously (Yoshimura et al., 1968). Emulgen 911 was kindly donated by Kao Chemical, Ltd. (Tokyo, Japan). PBE94, Polybuffer-74, and Polybuffer-96 were obtained from Pharmacia LKB (Uppsala, Sweden). Polyvinylidene difluoride (PVDF) membrane was obtained from Millipore (Bedford, MA). Restriction endonucleases were from Toyobo (Tokyo, Japan), New England Biolabs (Beverly, MA), and Takara (Tokyo, Japan). Oligonucleotides were synthesized and supplied by Sawady Technology (Tokyo, Japan). An alkaline phosphatase-labeled rabbit antibody to goat IgG was purchased from Cappel, ICN Pharmaceuticals (Aurora, OH). Other reagents were commercially available.

Purification of UDP-Glucuronosyltransferases in Guinea Pig Liver That Catalyze Morphine-6-Glucuronide Formation. Two male Hartley guinea pigs (250 g body weight) were obtained from Kajitani Laboratory Animal (Fukuoka, Japan). The animals were fasted for 20 h before sacrifice and then the liver was removed and perfused with physiological saline. The microsomes were prepared and solubilized with Emulgen 911 as described previously (Oguri et al., 1996). The recovered protein in the supernatant amounted to 45 to 55%. All buffers used in the purification procedures contained 1 mM dithiothreitol, 0.05% Emulgen 911, and 20% glycerol unless otherwise specified.

Solubilized microsomes (160 mg of protein) from guinea pig liver underwent chromatography on an ω-(β-carboxypropionylamino)octyl Sepharose 4B column (34 × 40 mm, 37 ml) as described previously (Ishii et al., 1993). Elution was carried out as described previously (Oguri et al., 1996). Most UGT activity toward the 6-hydroxyl group of morphine was eluted as a single peak at a KCl concentration of 360 mM and coeluted with the activity toward the 3-hydroxyl group of morphine.

The peak fractions of UGT from the ω-(β-carboxypropionylamino)-octyl Sepharose 4B column were pooled and dialyzed against 25 mM tris-acetate buffer, pH 8.9. The dialyzed fraction was subjected to chromatofocusing according to Puig and Tephly (1986) with slight modifications as follows. A chromatofocusing column (0.9 × 40 cm) was packed with 27 ml of degassed Polybuffer-exchanger 94 and equilibrated with 50 volumes of 25 mM Tris-acetate buffer, pH 8.9, containing 175 μg of Ptd-choline/ml. Approximately 6 mg of protein from a previous column was applied; then, a pH 8.9 to 5.5 gradient elution was applied using the following buffer: 7% Polybuffer-74, 3% Polybuffer-96, and 100 μg of Ptd-choline/ml, adjusted to pH 5.5 with 30% acetic acid. UGT activities toward both the 3- and 6-hydroxyl groups of morphine were coeluted as a broad peak with an approximate p*I* of 7.8.

Fractions with a p*I* of 7.8 were pooled, and dialyzed against 25 mM Tris-acetate buffer, pH 8.4, with 20% glycerol, 0.05% Emulgen 911, 0.5 mM dithiothreitol and 100 μg of Ptd-choline/ml of [buffer D]. Approximately 2 mg of protein from the dialyzed fraction was subjected to chromatography on a UDP-hexanolamine Sepharose 4B column (1.5 × 2.5 cm, 4.4-ml gel) equilibrated previously with buffer D and washed successively with 6 volumes of buffer D, 10 volumes of buffer D containing 25 mM KCl, and 10 volumes of buffer D containing 50 μM UDP-GlcA and 25 mM KCl. Then, UGT was eluted with 10 volumes of buffer D containing 3 mM UDP-GlcA and 25 mM KCl. Major UGT activity was obtained by this elution. To determine the N-terminal sequences of the 55- and 59-kDa proteins, termed UGT55K and UGT59K, in the purified preparation, these were electroblotted onto a PVDF membrane according to Matsudaira (1987) and stained with Coomassie Brilliant Blue R-250. Each band corresponding to UGT55K and UGT59K was cut out and subjected individually to a fully automated analysis using an 473A Protein Sequencer (Applied Biosystems, Foster City, CA)

***Staphylococcus aureus* V$_8$ protease Digestion and Analysis of the Peptides.** The UGT55K- and UGT59K-enriched fractions were obtained by ω-(β-carboxypropionylamino)octyl Sepharose 4B column chromatography and chromatofocusing as described above. UGT55K and UGT59K in the fractions exhibiting an approximate pI of 7.5 on the 1st chromatofocusing were subjected to two-dimensional gel electrophoresis using nonequilibrium pH gradient gel electrophoresis (O'Farrell et al., 1977) and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The proteins were stained with Coomassie Brilliant Blue R-250. UGT55K and UGT59K had p*I* values of 7.4 and 8.4, respectively. The gel slices containing UGT55K and UGT59K were subjected individually to SDS-PAGE and di-

gested, in situ, with *S. aureus* V_8 protease (Wako, Tokyo, Japan) according to the method of Kennedy et al. (1988) with an improved separation gel and running buffer (Okajima et al., 1993). The fragments in the gel were electroblotted onto a PVDF membrane as described above. Each band was cut out and sequenced as described above.

Probe Synthesis for Screening UGT55K cDNA. Polymerase chain reaction (PCR) was performed using DNA prepared from a λgt11 Hartley guinea pig liver 5′-stretch cDNA library (CLONTECH, Palo Alto, CA). Two groups of mixed PCR primers, including the degenerate codons, were synthesized as shown in Fig. 1, based on the amino- and carboxyl-terminal sequence of the peptide. PCR was performed using a GeneAmp PCR reagent kit with AmpliTaq DNA polymerase (PerkinElmer-Applied Biosystems, Chiba, Japan). The PCR reaction mixture contained 1× PCR buffer, 4 μM each of the mixed sense primers I and II, 8 μM the mixed antisense primer, 200 μM dNTPs, and 10 ng of λgt11–DNA of the Hartley guinea pig liver cDNA library in a final volume of 50 μl. PCR was carried out for 5 cycles (94°C, 1 min; 47°C, 30 s; 72°C, 30 s), 35 cycles (94°C, 30 s; 55°C, 30 s; 72°C, 30 s) and 72°C, 9 min with 2.5 units of AmpliTaq DNA polymerase. The amplified PCR products of 61 base pairs (bp) were subcloned into the *Sma*I site of the M13 mp18 vector and sequenced using the Applied Biosystems dye-primer DNA sequencing system. The confirmed nucleotide sequence that corresponds to the amino acid sequence of the peptide was labeled by [α-^{32}P]dCTP (Amersham Pharmacia Biotech, Tokyo, Japan) during PCR as the probe for screening the cDNA library. The concentration of dCTP in the PCR was reduced to 4 μM and [α-^{32}P]dCTP (total activity, 3.7 MBq) was included in a final volumes of 20 μl.

Isolation of UGT2B21 cDNA which encodes UGT55K. Two cDNA clones, pGUGT1 and pGUGT2, containing the UGT2B21 cDNA were isolated from a λgt11 Hartley guinea pig liver 5′-stretch cDNA library (CLONTECH) using the above ^{32}P-labeled PCR products as a probe. Once amplified, the phage (1.25×10^6 plaque-forming units) was screened with the 61-bp ^{32}P-labeled probe as described above. Plaque hybridization was carried out as described previously (Mizukami et al., 1983). pGUGT1 and pGUGT2, which carry UGT55K cDNA (1.0-kilobase pair insert), were isolated and sequenced using the Applied Biosystems dye-primer and dye-terminator DNA sequencing system. To determine the 5′- and 3′-ends of UGT55K cDNA, 5′- and 3′-rapid amplification of the cDNA ends (RACE) was performed. mRNAs were also isolated from male Hartley guinea pig liver using a Bio-Mag mRNA purification kit (Perseptive Biosystems, Framingham, MA) and quantified by measuring the optical density at 260 nm. 5′- and 3′-RACEs were carried out using a Marathon cDNA amplification kit (CLONTECH). For the 5′-RACE of UGT2B21, the adapter primer AP1 (5′-CCA TCC TAA TAC GAC TCA CTA TAG GGC-3′) and antisense primer 1 (5′-TTT CAC CAC TTA ACC CTG AC-3′), which corresponds to nucleotide positions +591 to +610 (complement) of newly cloned guinea pig UGT55K cDNA, were designed from the sequence of pGUGT2. The first PCR was performed using primers AP1 and 1 and PCR was performed according to the instructions in the manufacturer's manual. For the seminested 2nd PCR, antisense primer 2 (5′-ACC CAG TAT TTA ACC ACG TG-3′), which corresponds to +279 to +298 (complement) of newly cloned guinea pig UGT55K cDNA, was designed from the sequence of pGUGT2. This was performed using primers AP1 and 2 and the product containing the UGT55K initiation codon was sequenced. 3′-RACE of UGT55K was performed from oligo(dT)$_{20}$-P7 primed cDNA with primer P7 (5′-CGC CAG GGT TTT CCC AGT CAC GAC-3′) and sense primer 3 (5′-TGG TGG CTG AGA TAC TAC AC-3′), which corresponds to the nucleotide position of +473 to +492 of the newly cloned guinea pig UGT55K cDNA, were designed from the sequence of pGUGT2. Sense primer 4 (5′-ACT CCA CTG CAA ACC TGC C-3′), which corresponds to the nucleotide position of +834 to +852 of the newly cloned guinea pig UGT55K cDNA, were designed from the sequence of pGUGT2. Seminested PCR was performed with primers 4 and P7 and the 1036-bp product was sequenced. The UGT55K cDNA has been named UGT2B21 by the UGT nomenclature committee. Nucleotide sequences of UGT2B21 and UGT2B22 have been deposited in the DDBJ/GenBank/EMBL database with accession numbers AB034987 and AB03988.

Construction of Expression Plasmid for UGT2B21. To obtain UGT2B21 cDNA containing the full length of the open reading frame (ORF), sense primer 5 (5′-GGG CTC GAG ATG AAA AGG ATT TTG GCT TT-3′) and antisense primer 6 (5′-CAT CTT GTC ATG ACT CTG CC-3′), which correspond to nucleotide positions +1 to +20 and +1647 to +1666 (complement) of the newly cloned guinea pig UGT2B21 cDNA, were designed. The first PCR was performed using primers 5 and 6 from oligo(dT)$_{20}$-P7 primed cDNA using a proofreading enzyme, Ex-*Taq* DNA polymerase (Takara, Tokyo, Japan). For the seminested 2nd PCR, antisense primer 7 (5′-CCC TCT AGA GGT AAA TGA AAT TGT CAC AC-3′), which corresponds to +1615 to +1636 (complement) of newly cloned guinea pig UGT2B21 cDNA, was designed. Seminested PCR was performed with primers 5 and 7. The underlined parts of primers 5 and 7 represent the *Xho*I and *Xba*I restriction sites facilitated for subcloning. The product was digested with *Xho*I and *Xba*I and subcloned into the correspond sites of pSVL SV40 mammalian expression vector (Amersham Pharmacia Biotech). The 1.6-kilobase pair insert was confirmed by sequencing.

Isolation of UGT2B22 cDNA which Encodes UGT59K. A cDNA clone pGUGT3, containing the UGT59K cDNA, was isolated from λZAPII 3-methylcholanthrene-treated Hartley guinea pig liver cDNA library using a 492-bp UGT2B21 cDNA (+121 to +612) as a probe. Our preliminary data suggested that there were no significant changes in M-6-G formation activity between untreated and 3-methylcholanthrene-treated Hartley guinea pig liver. The *Eco*RI-adapted double- stranded cDNA library was constructed from a size fractionated poly(A$^+$) RNA of 3-methylcholanthrene-treated Hartley guinea pig liver (Ohgiya et al., 1993). The double-stranded cDNAs were ligated with *Eco*RI-cleaved λZAPII DNA (Stratagene, La Jolla, CA). After in vitro packaging with Gigapack II gold (Stratagene), the phage was plated out on host strain *Escherichia coli* XL1-Blue MRF. Screening was carried out with the 492-bp UGT2B21 cDNA. The probe was labeled during PCR with fluorescein-dUTP, instead of dCTP, using enhanced chemiluminescence probe-amp reagents (Amersham Pharmacia Biotech). Unamplified phage (3×10^5 plaque-forming units) was screened with the fluorescein-labeled 492-bp probe and this was carried out using the enhanced chemiluminescence detection system according to the company's instruction man-

Mixed sense primer I

```
5'-ATT CTT CAT ATT CCT TTT GT-3'
     C   C   C   C   C   C
     A   A       A   A
         G           G
```

Mixed sense primer II

```
5'-ATT TTA CAT ATT CCT TTT GT-3'
     C   G   C   C   C   C
     A           A   A
                 G
```

Mixed antisense primer

```
5'-CG TTT TTC IGC TTG AAA TCC-3'
    T C   C       C   G   C
                        A
                        G
```

Fig. 1. A pair of mixed oligonucleotide primers corresponding to the internal amino acid sequence of UGT55K for PCR.

ual (Amersham Pharmacia Biotech). pGUGT3 which carried UGT59K cDNA (2.5-kilobase pair insert) was isolated. In vivo excision was performed with ExAssist helper phage. The resulting clone in pBluescriptSK$^+$ was sequenced as described above. To determine the 5′-end 77 bases missing from pGUGT3 for UGT59K cDNA, 5′-RACE was performed as described above. For 5′-RACE of UGT59K, the adapter primer AP1 and antisense primer 8 (5′-TCC AAT TCC TTA GGT AGA GG-3′), which corresponds to nucleotide positions +859 to +878 (complement) of newly cloned guinea pig UGT59K cDNA, were designed from the sequence of pGUGT3. The first PCR was performed using primers AP1 and 8 and PCR was performed according to the manual instructions. For the seminested 2nd PCR, antisense primer 9 (5′-CAG GAT CTG CCA AGA GGA C-3′), which corresponds to +439 to +457 (complement) of newly cloned guinea pig UGT59K cDNA, was designed from the sequence of pGUGT3. This was performed using primers AP1 and 9 and the product containing UGT59K initiation codon was sequenced. The UGT nomenclature committee named the UGT59K cDNA UGT2B22.

Construction of Expression Plasmid for UGT2B22. The *Pst*I site that is facilitated for subcloning was introduced just upstream of the initiation codon by PCR using 5′-RACE product as a template. After digestion with *Pst*I and *Sph*I, this was then subcloned into pBluescriptSK$^+$-pGUGT3 digested with the same restriction enzymes. The resulting pBluescriptSK$^+$ that carried UGT2B22 with a full length of ORF was digested with *Hinc*II and ligated to *Xba*I linker. After digestion with *Xba*I, UGT2B22 cDNA was subcloned into pSVL-SV40 vector at the same restriction site.

The expression plasmid for UGT2B22 was also constructed using pTARGET vector (Promega, Madison, WI). The construct was designed by deleting 883 bp of the 3′-untranslated region from the UGT2B22 cDNA. PCR was carried out using sense primer 10 (5′-CTC GAG ATG TCC TTG AAA TGG ATC TC-3′) which corresponds to nucleotide positions +1 to +20 and antisense primer 11 (5′-TCT AGA CCA ATT CTG ATG CCA TGC AC-3′) which corresponds to nucleotide positions +1602 to +1621 (complement) using Ex-*Taq* DNA polymerase (Takara, Tokyo, Japan) with pSVL-UGT2B22 as template. The underlined areas represent restriction sites for *Xho*I and *Xba*I, respectively. The products were TA-cloned using pTARGET vector.

Transient Expression of UGT2B21, UGT2B22 and Human UGT1A1 in COS Cells. COS-7 cells (JCRB9127) were obtained from the Japanese Collection of Research Bioresources (JCRB) through the Health Science Research Resources Bank and maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen). For transfection, confluent COS cells were split in a ratio of 1:6 and cells were allowed to grow until 80 to 90% confluent. Then, the medium was changed to a serum-free one after washing with phosphate-buffered saline. Transfections were performed with expression plasmids in nonserum medium using a polyamine liposome reagent, Trans-IT LT1 (Mirus, Madison, WI), according to the instructions in the manufacturer's manual. The expression plasmid of human UGT1A1 in pCMV5 vector was kindly provided by Dr. Behnaz Mojarrabi and Prof. Peter I Mackenzie of Flinders Medical Center (Adelaide, Australia). Expression plasmids were purified by Wizard PureFection kits (Promega, Madison, WI) and the cells were incubated at 37°C in a 5% CO_2 atmosphere. Five hours after transfection, the medium was changed to Dulbecco's modified Eagle's medium with 10% fetal bovine serum and incubation continued under the same conditions until harvesting took place. Cells were harvested at 72 h after transfection and microsomes were prepared.

Assays. Glucuronidation of morphine was assayed according to the method reported previously (Kuo et al., 1991) with slight modifications (Ishii et al., 1993) for 16 h at 37°C. All the assay was performed in the presence of 5 mg of bovine serum albumin in a final volume of 0.3 ml. For blank, UDP-GlcA was excluded from the incubation mixture. The substrate and cosubstrate concentration in the incubation mixture for the glucuronidation reaction used were submaximal, namely 5 mM morphine and 2 mM UDP-GlcA. Protein was measured by the method of Lowry et al. (1951), and that in the sample with the high detergent concentration was determined according to the procedure of Bensadoun and Weinstein (1976). Bovine serum albumin was used as a standard.

Immunoblotting. Protein separated by SDS-PAGE was electroblotted onto PVDF membrane and reacted with goat anti-mouse low p*I* form UGT antibody (Mackenzie et al., 1984) as a primary antibody. Immunochemical staining was performed after reaction with alkaline phosphatase-labeled secondary antibody.

Results

Purification of UDP-Glucuronosyltransferases That Catalyze Morphine-6-glucuronide Formation. The UGT activity for the morphine 6-hydroxyl group in Emulgen 911 solubilized liver microsomes of guinea pig was eluted as a single peak from the ω-(β-carboxypropionylamino)octyl Sepharose 4B column using buffer A (25 mM Tris-HCl buffer, pH 7.4, containing 5 mM $MgCl_2$ and 100 μg of Ptd-choline/ml) supplemented with 360 mM KCl. Further purification was performed using chromatofocusing and UDP-hexanolamine affinity chromatography to give a major fraction containing UGT activity toward the morphine 6-hydroxyl group as well as the 3-hydroxyl group. Activity toward 4-hydroxybiphenyl was also present in this fraction. Two protein bands with molecular masses of 55 and 59 kDa were observed after analysis by SDS-PAGE with band intensities of ~3:1 (Fig. 2). The N-terminal 15-residue sequences of UGT55K and UGT59K were GKVLV WPMEF SHWMN and GNVLV WPMEY SHWMN, respectively. These N-terminal sequences are highly homologous with previously reported UGTs. UGT55K and UGT59K had p*I* values of 7.4 and 8.4, respectively, on two-dimensional PAGE. Attempts to separate these two forms were made and UGT55K was purified to apparent homogeneity (data not shown). However, the M-6-G formation activity of the purified UGT55K was quite low and may possibly reflect denaturation during the purification proce-

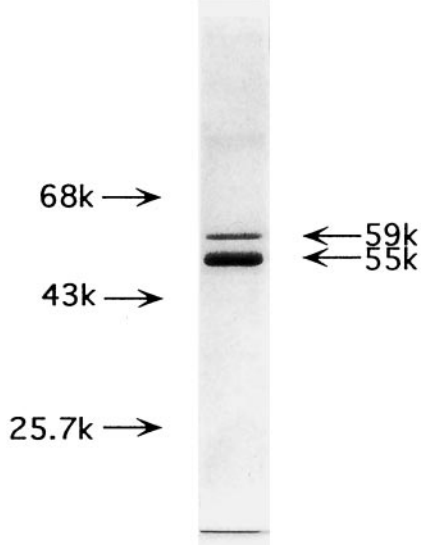

Fig. 2. SDS-PAGE of fractions containing UGT55K and UGT59K purified from guinea pig liver with activities toward morphine 3- and 6-hydroxyl groups. Purification of the UGTs is described in the text. Specific eluate (2 μg of protein) with UDP-GlcA from a UDP-hexanolamine Sepharose 4B affinity column was subjected to 10% SDS-PAGE and stained with Coomassie blue. K, kilodaltons.

dure. Thus, it was not possible to identify which form was responsible for M-6-G formation. These results raise the question: do UGT55K and UGT59K form oligomers that contribute to the formation of M-6-G?

cDNA Cloning of UGT2B21 and UGT2B22 That Encode UGT55K and UGT59K. Two new cDNAs, which encode UGT55K and UGT59K, were isolated and identified as new members of the UGT2B subfamily. These were named UGT2B21 and UGT2B22, respectively, by the UGT nomenclature committee. The 1825-bp nucleotide of UGT2B21 cDNA has been deposited in the DDBJ/EMBL/GenBank database and the deduced 528 amino acid sequences are shown in Fig. 3 aligned with UGT2B22. The deduced primary sequence of UGT2B21 corresponds exactly to the N terminus and internal peptide sequences of UGT55K, which were determined by peptide mapping and protein sequencing (Table 1). There was a one-base difference, G313T, between pGUGT2 and PCR products covering the UGT2B21 ORF, which results in a one-residue difference, V62L. It is possible that the mutation was introduced during PCR. However, it could be caused by differences between animals because we used conventional guinea pigs. The mature form of UGT2B21 is suggested to consist of 507 amino acids on the basis of the N terminus of UGT55K. The signal peptide is estimated to be 21 amino acids in length. There are four putative Asn-*N*-glycosylation sites. A poly(A)$^+$ addition signal was observed at +1800 to +1805. The 2520-bp nucleotide of UGT2B22 cDNA has been deposited in the DDBJ/EMBL/GenBank database and the deduced 529 amino acid sequences are shown in Fig. 3 aligned with UGT2B21. All the peptide sequences determined after peptide mapping of UGT59K corresponded to the UGT2B22 primary sequence (Table 1). The mature form of UGT2B22 is suggested to be 506 amino acids long on the basis of the N-terminal sequence of UGT59K. Thus, the signal peptide is estimated to be 23 amino acids long. There are three putative *N*-glycosylation sites. A poly(A)$^+$ addition signal was observed at +1908 to +1913.

A comparison of the predicted primary sequence with other members of the UGT2B subfamily indicates that UGT2B21 has approximately 70% homology with rat UGT2B12 (Green et al., 1995), rabbit UGT2B13 (Tukey et al., 1993), and rabbit UGT2B14 (Tukey et al., 1993), whereas UGT2B22 has 67% homology with rabbit UGT2B13 (Tukey et al., 1993). However, UGT2B21 and UGT2B22 have a 76.5% homology, the highest when UGT2B21 was compared with the UGT isoforms known at present.

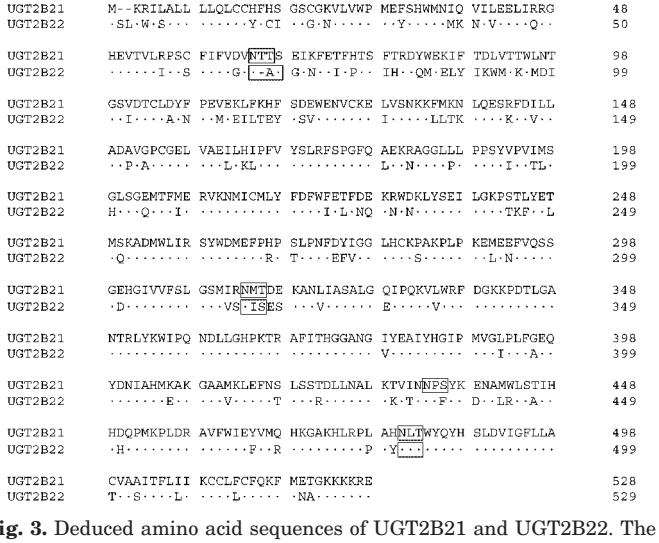

Fig. 3. Deduced amino acid sequences of UGT2B21 and UGT2B22. The nucleotides sequences of UGT2B21 and UGT2B22 have been deposited in the DDBJ/GenBank/EMBL database with accession numbers AB034987 and AB03988. Amino acid sequences of UGT2B21 and UGT2B22 are aligned and the residues are numbered on the right side. Dots represent identical residues. The potential *N*-glycosylation sites are boxed.

M-6-G Formation by UGT2B21 and UGT2B22 Expressed in COS-7 Cells. To examine morphine glucuronidation activity, UGT2B21 and UGT2B22 were transiently expressed in COS-7 cells. Morphine 3-glucuronidation was catalyzed by microsomes from COS-7 cells transfected with the UGT2B21 cDNA. Morphine 6-glucuronidation by UGT2B21 was also detected but the formation ratio M-3-G/M-6-G was 10:1. This is very different from that of 4:1 in guinea pig liver microsomes (Kuo et al., 1991). In contrast, UGT2B22 was without activity toward morphine.

Because the purified morphine UGT preparation from guinea pig liver microsomes contained both the UGT55K and UGT59K proteins, simultaneous expression of UGT2B21 and UGT2B22 was carried out. In accordance with the reports describing possible dimer or tetramer formation (Koiwai et al., 1996), COS-7 cells were transfected with different molar ratios of expression plasmids, namely, UGT2B21/UGT2B22 4:0, 3:1, 2:2, 1:3, and 0:4. Differences in the ratio of UGT2B21 and UGT2B22 in COS-7 cell microsomes were observed (Fig. 4). The 55- and 59-kDa bands were observed when COS-7 cells were transfected with UGT2B21 and UGT2B22 cDNA, respectively. COS-7 cell microsomes, with UGT2B21 and UGT2B22 expressed in different ratios, were subjected to an analysis of their UGT activity toward the 3- and 6-hydroxyl groups of morphine (Figs. 5 and 6). When the molar ratio of UGT2B21- and UGT2B22-expression plasmids was 2:2, the M-6-G formation observed was 4.5-fold higher than that of the UGT2B21 transfected alone (4:0). When UGT2B21 and UGT2B22 were transfected in the ratio 1:3, the ratio of M-3-G: M-6-G formed was 1:1. A M-3-G/ M-6-G ratio of 4:1, similar to that observed with guinea pig liver microsomes (Kuo et al., 1991), was found when UGT2B21 and UGT2B22 were transfected into COS-7 cells at a molar ratio of 3:1. In addition, of the dual-transfections tested, M-3-G formation activity was highest in cells transfected with UGT2B21/UGT2B22 equal to 3:1. Furthermore, the ratio of UGT2B21/UGT2B22 protein expressed in these transfected cells was similar to that in the purified preparations from guinea pig

TABLE 1

N-terminal and internal amino acid sequences of UGT55K and UGT59K that correspond UGT2B21 and UGT2B22

Internal amino acid sequences were determined after peptide mapping of UGT55K and UGT59K. The unidentified residues are shown as X.

	Corresponding residues[a]
UGT2B21	
GKVLV WPMEF SHWMN	22–36
ILHIP FVYSL RFSPG FQAEK RAGGL LLPPXYV	163–193
FVQSS GEHGI VVFSL GSMIR XMTD	294–317
UGT2B22	
GNVLV WPMEY SHWMN	24–38
NVXKE IVSNK KLLTK LQESK FDVLL ADP	125–152
NFVQS SGDHG IVVFS LGSMV SXISE	294–318
XIYHG IPMVG XPLFA EQYDN	383–402

[a] Amino acid positions of UGT2B21 and UGT2B22 (Fig. 3).

liver that formed M-6-G (Fig. 2). These data strongly suggest that the ratio of UGT2B21 and UGT2B22 in COS-7 cell microsomes is a determinant of the extent of M-6-G formation.

Because the expressed level of UGT2B22 in COS-7 cells was not comparable with that of UGT2B21, a new UGT2B22 expression plasmid consisting of UGT2B22 cDNA truncated by 883 bp from the 3′-untranslated region, downstream of the cytomegalovirus (CMV) promoter in pTARGET (Promega, Madison, WI), was constructed. The amount of UGT2B22 protein expressed in COS-7 cells transfected with this construct was 30 times that of cells transfected with the original pSVL-UGT2B22 construct (Fig. 7A). However this protein showed no activity toward morphine either in the presence or absence of Brij 58 (data not shown). Furthermore, COS-7 cell microsomes from cells transfected with pSVL-UGT2B21 alone, when mixed with microsomes containing an equivalent amount of UGT2B22 in the presence of detergent, did not lead to altered ratios of formation of M-6-G and M-3-G (Fig. 7B). In addition, Fig. 8 shows the coexpression of human UGT1A, which does not exhibit morphine glucuronidation (Senafi et al., 1994), with UGT2B21. Coexpression of UGT1A1 with UGT2B21 altered both M-3-G and M-6-G formation activities, slightly increasing them; however, the effects were not as marked as cotransfection with UGT2B22. Taken together, these data indicate that the enhancement of UGT2B21-catalyzed M-6-G formation by UGT2B22 is only observed when UGT2B21 and UGT2B22 are expressed simultaneously.

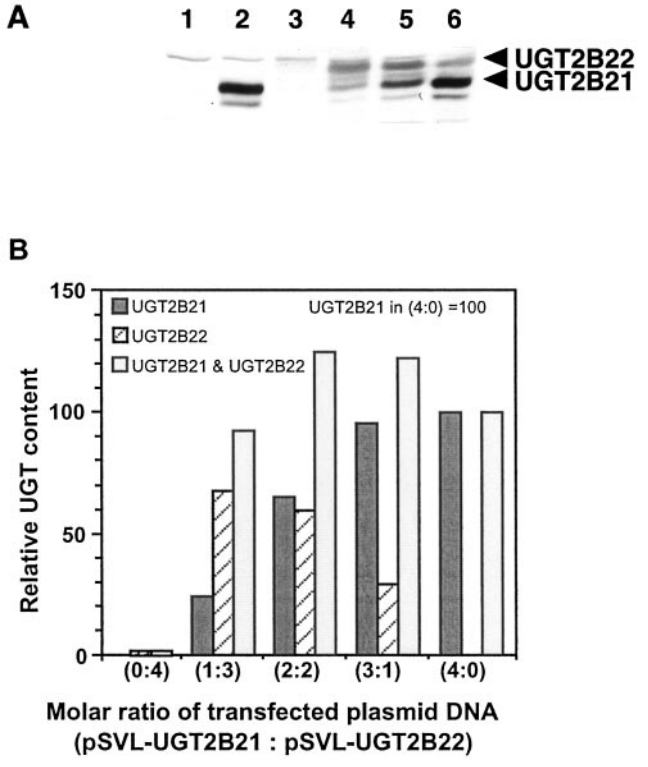

Fig. 4. Immunoblot analysis of UGT2B21 and UGT2B22 expressed in COS-7 cell microsomes. COS-7 cells were transfected with pSVL expression vector for UGT2B21 and UGT2B22 at different molar ratios. Assuming 10 μg of pSVL-UGT2B21 DNA is equivalent to 2.37 pmol, the molar ratio of the expression plasmid for UGT2B21 and UGT2B22 was calculated. A total of 9.48 pmol of pSVL expression plasmid was used for the transfection to 80% confluent COS-7 cells in a flask (175 cm^2). pSVL-UGT2B21 and pSVL-UGT2B22 were transfected at ratios of 4:0, 3:1, 2:2, 1:3, and 0:4. For control transfection, pSVL (9.48 pmol) was used. Cells were harvested at 72 h after transfection and microsomes were prepared. A, microsomal protein (50 μg) from COS-7 cells UGT2B21 and/or UGT2B22 was subjected to SDS-PAGE (7.5%) and immunoblotted using an anti-UGT antibody. Lane 1, control (pSVL). The molar ratios of expression plasmid used for the transfection of UGT2B21 and UGT2B22 are 4:0 (lane 2), 0:4 (lane 3), 1:3 (lane 4); 2:2 (lane 5), and 3:1 (lane 6). Arrows represent the probable mature protein of UGT2B21 and UGT2B22. B, the band intensity of UGT2B21 and UGT2B22 was estimated by analysis using NIH Image software (http://rsb.info.nih.gov/nih-image/) after scanning data into a computer. The filled bar represents the amount of UGT2B21; the hatched bar represents the amount of UGT2B22. The open bar represents the total of UGT2B21 and UGT2B22. The contents are shown as a percentage with respect to UGT2B21 in UGT2B21/UGT2B22 (4:0).

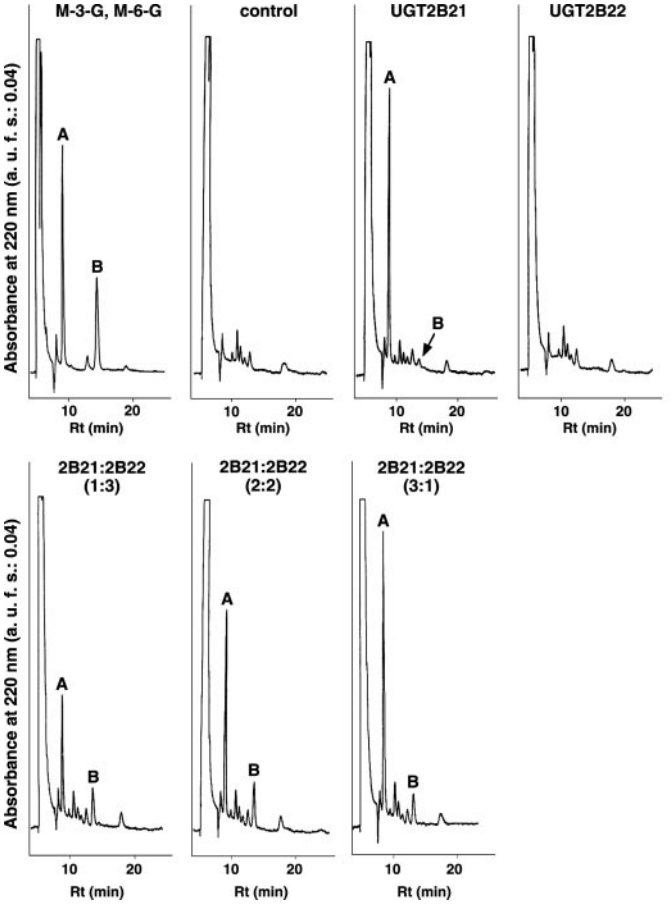

Fig. 5. High-performance liquid chromatography chromatogram of morphine glucuronide formed by expressed UGT2B21 and UGT2B22. Transfection of COS-7 cells with different ratios of UGT2B21 and UGT2B22 as described in the legend to Fig. 4. Incubation was performed with COS-7 cell microsomal protein (100 μg) with 5.0 mM morphine and 2.0 mM UDP-GlcA in the presence of 0.85 mM saccharic acid-1,4-lactone for 16 h. Of the final 400-μl reaction mixture, 100 μl was subjected to high-performance liquid chromatography using a system equipped with a Nova Pak C18 cartridge (4 μ), the column was eluted with 100 mM phosphate and 5 mM SDS (pH 2.1 with NaOH)/methanol/acetonitrile (79:10:11,v/v/v). Peaks A and B represent M-3-G and M-6-G, respectively. In the panel labeled 'M-3-G and M-6-G', 400 ng each of M-3-G and M-6-G were used for calibration. The panel labeled 'UGT2B21 and UGT2B22' represents the chromatogram from microsomes of single transfected cells (4:0) and (0:4), respectively. 2B21:2B22 (1:3), 2B21:2B22 (2:2), and 2B21:2B22 (3:1) represent those from microsomes of COS-7 cells transfected with UGT2B21 and UGT2B22 expression plasmids with a molar ratio of 1:3, 2:2, and 3:1, respectively.

Discussion

UGT2B21 and UGT2B22, which are involved in the formation of the potent morphine glucuronide, M-6-G, have been investigated. Purification of morphine UGT in guinea pig liver, which is responsible for M-6-G formation, produced a preparation containing UGT55K and UGT59K with an approximate band ratio of 3:1 on SDS-PAGE (Fig. 2). Other reports document the presence of more than one type of protein in purified UGT preparations. These include the preparation of testosterone UGT and androsterone UGT from Wistar rat liver (Matsui and Nagai, 1986). Purified pig phenol UGT contained three bands on SDS-PAGE with a ratio 1:2:1 (Hochman et al., 1983). Because a purified UGT preparation containing a single protein species that was active in the formation of M-6-G could not be obtained, the cDNAs

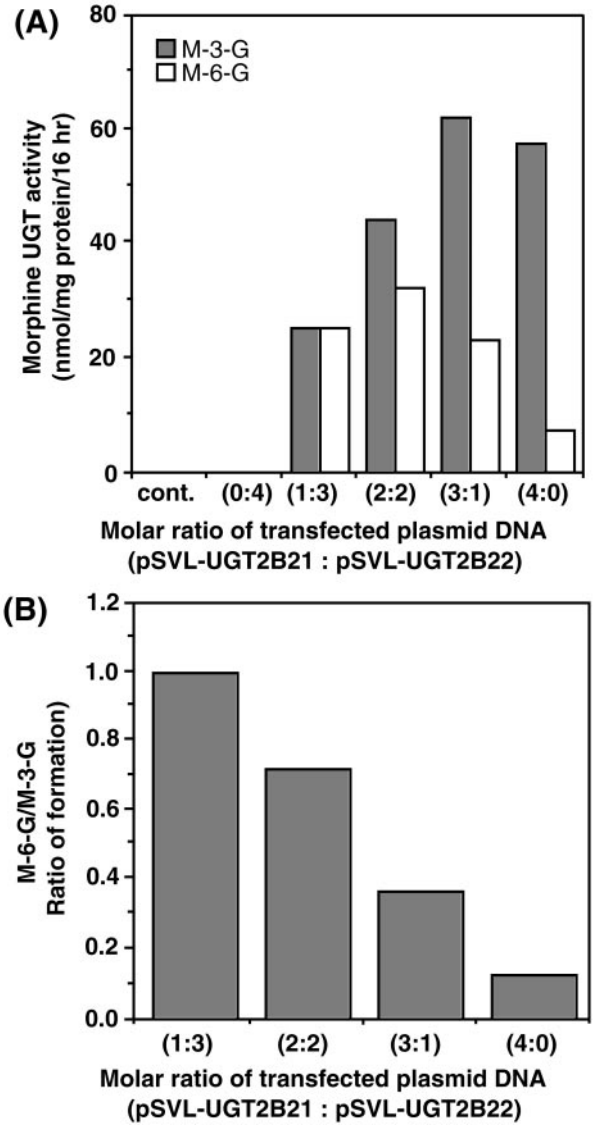

Fig. 6. Morphine UGT activity in microsomes of COS-7 cells transfected with UGT2B21 and/or UGT2B22. Effect of the molar ratio of the expression plasmids for UGT2B21 and UGT2B22 on the formation of M-3-G and M-6-G from morphine was examined. The transfection and assay are described in the legends to Figs. 4 and 5. A, morphine UGT activities toward the 3- and 6-hydroxyl group of the sample are shown in closed and open columns, respectively. B, the ratio of glucuronide formation (M-6-G/M-3-G) was calculated from A.

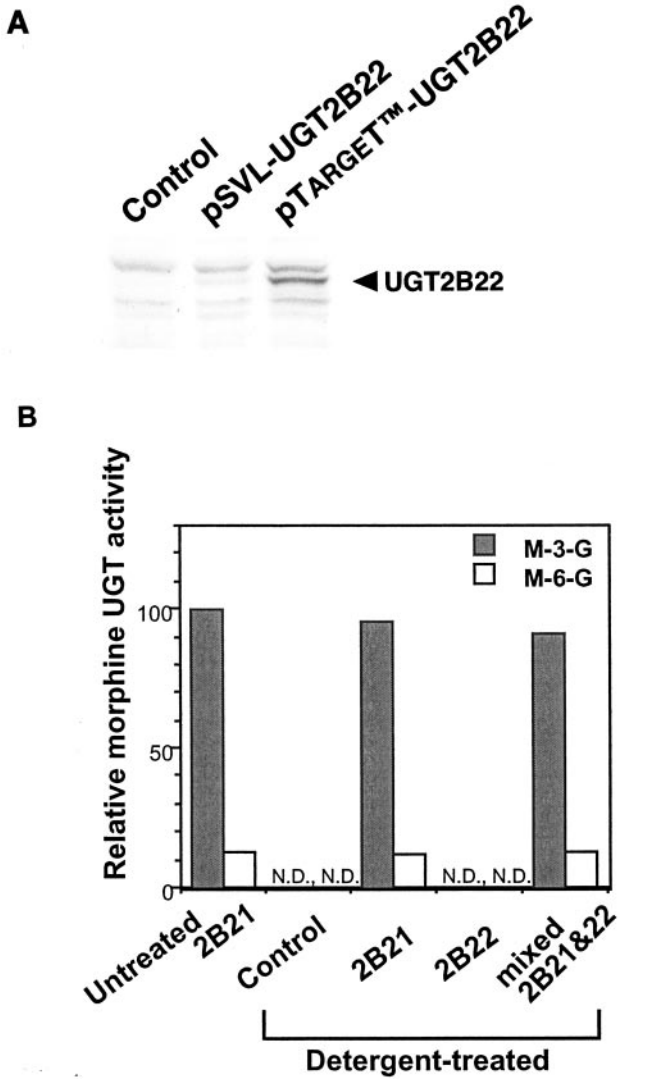

Fig. 7. Morphine UGT activity in mixed microsomes of COS-7 cells separately transfected with UGT2B21 and UGT2B22. A, the UGT2B22 expression levels were compared between the microsomes obtained from COS-7 cells transfected with pSVL-UGT2B22 and pTARGET-UGT2B22. Each expression vector (30 μg), pSVL-SV40 (control), pSVL-UGT2B22, and pTARGET-UGT2B22 was used for the transfection to 80% confluent COS-7 cells in a flask (175 cm^2) using Trans-IT LT1 as described under *Experimental Procedures*. Microsomal protein (50 μg of protein) from the COS-7 cells of each transfection was subjected to 7.5% SDS-PAGE and immunoblotted with anti-UGT antibody. B, morphine UGT activity was compared using mixed microsomes of COS-7 cells that were separately transfected with UGT2B21 and UGT2B22. In "control", the microsomes (254 μg of protein) from COS-7 cells transfected with pSVL were used. "2B21", the UGT2B21-expressed COS-7 cell microsomes (100 μg of protein), which is shown as 2B21:2B22 (4:0) in Fig. 5, were mixed with the microsomes (154 μg of protein) from COS-7 cells transfected with pSVL. "2B22", the UGT2B22-enriched microsomes (154 μg of protein) shown in Fig. 7A, which contained equivalent an amount of UGT2B22 to the UGT2B21 in "2B21", was mixed with the microsomes (100 μg of protein) from COS-7 cells transfected with pSVL. "2B21&22 mixed", the UGT2B21-microsomes (100 μg of protein) were mixed with the UGT2B22-enriched microsomes (154 μg of protein) to yield a ratio of UGT2B21 to UGT2B22 of 1:1. The total volume of 254 μg of microsomal protein was adjusted to 50 μl with 1.15% KCl. Then, the microsomes were treated with an equal amount of 0.3% Brij 58 in 1.15% KCl for 15 min on ice. "untreated 2B21", UGT2B21-expressed microsomes (100 μg of protein) in 50 μl were treated with 50 μl of 1.15% KCl. The morphine UGT activity was measured as described in the text. The values represent the average of triplicate assays of the percentages for the M-3-G formation in "untreated 2B21". N.D., not detectable.

encoding UGT55K and UGT59K were isolated based on their peptide sequence. The predicted primary sequences of UGT2B21 and UGT2B22 corresponded exactly to the N terminus and internal sequences of UGT55K and UGT59K, respectively. The difference in their migration on SDS-PAGE is also supported by immunoblotting (Fig. 4), although the number of possible glycosylation sites is suggested to be four and three, respectively.

UGT2B21 exhibits glucuronidation activity toward both the 3-hydroxyl and 6-hydroxyl groups of morphine. The formation ratio M-3-G/M-6-G (10:1) is similar to that of UGT2B7, a predominant human UGT responsible for M-6-G formation (Coffman et al., 1997). The homology of UGT2B21 to UGT2B7 (Ritter et al., 1990) and UGT2B9 (Bélanger et al., 1997), which are involved in M-3-G and M-6-G formation in humans and monkeys, was 68.6 and 69.0%. However, UGT2B21 has a somewhat higher homology with rat UGT2B12 (70%), rabbit UGT2B13 (69.8%), and rabbit UGT2B14 (70%), which have not been shown to form M-6-G (Tukey, 1993; Green et al., 1995). In contrast, UGT2B22 did not exhibit any glucuronidation activity toward morphine. It is interesting that high expression of UGT2B22 is observed when cotransfected with UGT2B21. It is likely that coexpression with UGT2B21 could help UGT2B22 expression. Possibly, UGT2B22 is stabilized via formation of hetero-oligomers with UGT2B21. However, as UGT2B22 can be expressed at high levels, with the CMV promoter after deleting a major part of the 3′-untranslated region, in the absence of UGT2B21, the stability of UGT2B22 remains to be investigated.

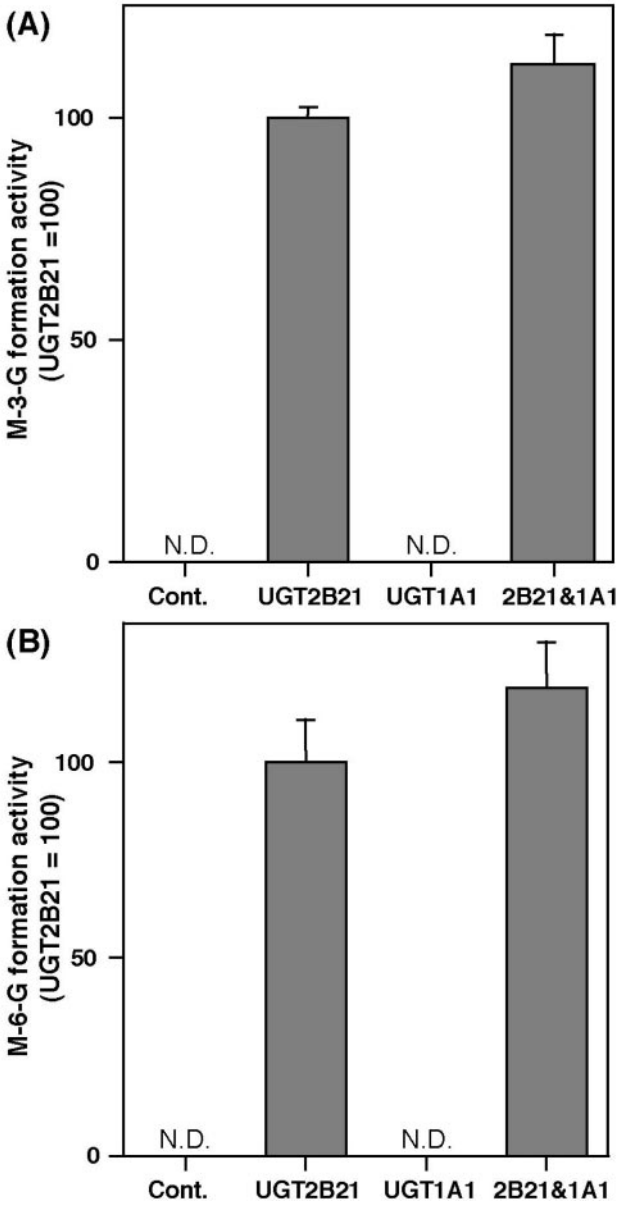

Fig. 8. Effect of simultaneous expression of UGT1A1 on morphine glucuronidation activity of UGT2B21. COS-7 cells were transfected with 30 μg of pSVL-UGT2B21 DNA and pCMV5-human UGT1A1 DNA. For UGT2B21 or UGT1A1 single expression, 30 μg of pSVL vector DNA was included to adjust the DNA amount to 60 μg. The combined plasmids were used for the transfection to 80% confluent COS-7 cells in a flask (175 cm^2). For control transfection, pSVL (60 μg) was used. Cells were harvested at 72 h after transfection and microsomes were prepared. Determination of morphine UGT activity of COS-7 cell microsomes expressing UGT2B21 and/or UGT1A1 was performed as described in the legend to Fig. 5. The values are given as a percentage (means ± S.D., triplicate assays) of the M-3-G or M-6-G formation activity in COS-7 cell microsomes expressing UGT2B21.

Because guinea pig liver shows an M-3-G/M-6-G formation ratio of 4:1, UGT2B21 with an M-3-G/M-6-G formation ratio of 10:1 cannot be the sole contributor to this activity. Therefore, cotransfection experiments with UGT2B21 and UGT2B22 were carried out using different molar ratios of their expression plasmids. Simultaneous expression of UGT2B21 and UGT2B22 resulted in increased M-6-G formation. The M-6-G formation depended on the ratio of UGT2B21 and UGT2B22 coexpressed in COS-7 cell microsomes. However, only a slight effect was observed when UGT1A1 was cotransfected with UGT2B21. The enhancement of M-6-G formation activity of UGT2B21 by UGT2B22 is fine-tuned. These data strongly support the hypothesis that extensive M-6-G formation in guinea pig liver is catalyzed by UGT2B21 and UGT2B22 hetero-oligomers. Koiwai et al. have shown that a dominant negative effect could be explained by the hetero-oligomeric behavior of wild-type and a mutant-type UGT1A1 in the Crigler Najjar-type II syndrome (Koiwai et al., 1996). Homo-oligomer formation of UGT2B1 (Meech and Mackenzie, 1997) and hetero-oligomer formation of the UGT2B1 and UGT1A family have also been reported (Ikushiro et al., 1997). Radiation inactivation studies suggest that the size of the active UGT in the membrane is comparable with the dimer or tetramer (Peters et al., 1984; Gschaidmeier and Bock, 1994). Gschaidmeier and Bock (1994) suggested that monoglucuronidation of phenols may be catalyzed by a dimeric form of UGT, whereas diglucuronidation is catalyzed by a tetramer. It is likely that the ratio of UGT2B21 and UGT2B22 alters the substrate site recognition of morphine. The present data suggest that UGT2B21 and UGT2B22 form active hetero-oligomers to catalyze extensive M-6-G formation. As far as we know, this is the first report suggesting that UGT hetero-oligomer formation results in a substrate specificity that is different from their homo-oligomers. Hetero-oligomer formation for dopamine and somatostatin receptors has resulted in enhanced functional activity (Rocheville et al., 2000). The catalytic properties of some forms of cytochrome P450 are changed by interaction with other isoforms (Backes et al., 1988). Recently, Taura et al. reported that CYP1A1 could interact with epoxide hydrolase and UGT isoforms (Taura et al., 2000).

Most studies on the substrate specificities of UGT isoforms use cells transfected with a single UGT cDNA. In this situation, only homo-oligomers would be formed. However, co-transfections with different UGT cDNAs could yield many different combinations of hetero-oligomer. If these hetero-oligomers have altered substrate specificities, the potential for a limited number of UGT gene products to glucuronidate a vast array of chemicals is enormous and should provide important information for understanding the physiological function of UGT isoforms and their polymorphisms. Further investigations are necessary to elucidate the potential for UGT hetero-oligomer formation and its impact on catalytic activities.

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Address correspondence to: Prof. Kazuta Oguri, Ph.D., Graduate School of Pharmaceutical Sciences, Kyushu University 62, 3–1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. E-mail: oguri@xenoba.phar.kyushu-u.ac.jp