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Stable Expression of Two Human UDP-Glucuronosyltransferase cDNAs in V79 Cell Cultures

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SUMMARY

Two human liver UDP-glucuronosyltransferase cDNA clones (HLUGP1 and HLUG25) were individually inserted into the eukaryotic expression vector pKCRH2. Each recombinant plasmid was cotransfected with a SFVneo vector, thereby allowing establishment of several V79 cell lines retaining the exogenous UDP-glucuronosyltransferase cDNA after selection with G418 (Geneticin). Southern blot analysis suggested that the cDNAs were integrated into the host cell genome. Northern blot and immunoblot analyses indicated that the cDNAs were correctly transcribed and translated for the production of functional enzymes. The established recombinant V79 cell lines stably expressed the UDP-glucuronosyltransferase activities towards 1-naphthol (HLUGP1) and hyodeoxycholic acid (HLUG25) at levels 10–20-fold higher than with transient expression, and in the range found in human liver. These high levels of expression of UDP-glucuronosyltransferase activity allowed the determination of apparent kinetic constants and substrate specificities of glucuronidation in the genetically engineered cell lines. HLUG25 cDNA encoded an isoform with restricted specificity towards the 6-OH group of the bile acid hyodeoxycholic acid. The other steroids, bile acids, endobiotics, and xenobiotics tested as substrates were glucuronidated in various samples of human liver microsomes, but not by this isoenzyme. This study, allowing the expression of individual UDP-glucuronosyltransferases in heterologous cells with no endogenous transferases, offered a unique solution for the characterization of UDP-glucuronosyltransferase functional heterogeneity.

UDPGTs (EC 2.4.1.17) are a family of enzymes that metabolize xenobiotics such as drugs, carcinogens, and environmental pollutants, as well as endobiotics such as steroids, bile acids, and bilirubin (1). This group of enzymes carries out beneficial metabolic activities by detoxification of xenobiotics and endobiotics but can also catalyze the conversion of carboxylic substrates into reactive acylglucuronides, which are possibly involved in immunological reactions and other side effects (2). An understanding of the role of UDPGTs in beneficial and detrimental reactions could be realized with the isolation of individual forms and characterization of their catalytic activities.

The multiple molecular forms of UDPGT exhibit closely related properties such as molecular size and structure, immunological characteristics, and overlapping substrate specificities. The multiplicity and common properties, as well as the strong lipid dependence of UDPGTs, make the separation and purification of the various isoenzymes difficult, particularly from human tissue. Several cDNAs encoding rat, mouse, and human UDPGT isoforms have been isolated (3–10). Expression of these cDNAs in mammalian cell culture provides a unique opportunity to study the catalytic properties of each individual isoform. We (11–13) and others (3, 4, 7) previously reported the transient expression of human and rat UDPGT cDNAs in COS-7 cells. However, expression of human UDPGTs in a stable cell line presents several advantages, such as (a) allowing reproducible measurement of catalytically active protein, avoiding the large intertransfection variations, (b) providing a useful tool for the production and analysis of glucuronides metabolites, and (c) supplying a recipient cell line for other drug-metabolizing enzymes to define the role of the various enzymatic systems in the detoxification-toxification processes.

In this report, the full length cDNAs for HLUGP1 (12) and HLUG25 (9), placed under the control of the Simian virus early gene promotor (SV40), were used to transform lung Chinese hamster fibroblasts (V79) together with a selectable plasmid vector encoding the neomycin phosphotransferase gene. The derived cell lines stably expressed each of two enzymatically active human UDPGT isoforms, a UDPGT glucuronidating "short and planar phenols" (as defined in Refs. 11 and 12) and a UDPGT glucuronidating the bile acid hyodeoxycholic acid. The effectiveness of this approach for the identification and characterization of these individual members of the UDPGT multigene superfamily is discussed.

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ABBREVIATIONS: UDPGT, UDP-glucuronosyltransferase; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; kb, kilobases.

Materials and Methods

UDPGT clones and cloning vectors. cDNA clones encoding the complete coding sequence of two human liver UDPGTs, HLUGP1 (12) and HLUG25 (9), were subcloned into the *Hind*III sites of the eukaryotic expression vector pKCRH2 (which was obtained from S. Shibara, Freidrich Miescher Institut, Basel, Switzerland), as previously reported (12, 13).

Recombinant plasmids were grown in *Escherichia coli* JM109, and plasmids with cDNAs in the correct orientation for transcription from the SV40 early gene promotor were identified by restriction mapping. The expression plasmid SFVneo was a kind gift from Dr. H. Garoff (E.M.B.L., Heidelberg, FRG) and was grown in *E. coli* JM109 in the presence of 50 μg/ml ampicillin. DNA for transfections was prepared by the Triton-lysozyme method and purified by centrifugation on a cesium chloride/ethidium bromide equilibrium density gradient (14). HLUGP1 and HLUG25 were linearized with *Pst*I (Pharmacia-LKB, St. Quentin en Yvelines, France) and SFVneo with *Bam*HI (Pharmacia-LKB) before transfection. The probes used for hybridization of Northern and Southern blots were prepared by digestion with *Hind*III (Pharmacia-LKB) of the pKCRH2 recombinant vectors containing UDPGT cDNAs, followed by separation on agarose gels and purification by electroelution. The restriction fragments were ^{32}P radiolabeled, using a Pharmacia random primer oligolabeling kit, to a specific activity 5×10^8–10^9 dpm/μg of DNA. The probes were further purified from unincorporated labeled nucleotides by gel filtration chromatography on a Nick column (Pharmacia-LKB).

Cell cultures and transfection. V79 Chinese hamster lung fibroblasts were a kind gift from Dr. Marzin (Institut Pasteur, Lille, France). They were cultured in Dulbecco's modified Eagle's medium (GIBCO, Cergy-Pontoise, France) supplemented with 10% (v/v) Nu-serum (Sochibo, Velizy-Villacoublay, France), 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Transfections were carried out by the calcium phosphate/glycerol shock procedure (15), as previously described (11–13). In addition, following the transfection procedure, the cells were treated with 5 mM sodium butyrate (optimum concentration) for 24 hr, which increased the initial level of expressed activity by at least 3-fold (data not shown). Briefly, 40 μg of either linearized plasmid UDPGT cDNA and 10 μg of SFVneo plasmid DNA were applied to 50% confluent V79 cells in a 90-mm-diameter Petri dish. The cultures were split 1:5 24 hr after transfection, and G418 (Geneticin; GIBCO) (1 mg/ml)-containing medium (optimum concentration, data not shown) was applied 1 day later. Colonies appeared 1 week later and were either picked individually or pooled 2 weeks after addition of the selection medium. They were then grown as mass cultures for further studies. Cells were rinsed in sterile ice-cold phosphate-buffered saline, pH 7.4 (GIBCO), harvested, centrifugated at 1000 × *g* for 10 min, washed in phosphate-buffered saline, centrifugated again, and stored as pellets at −80° before further analysis.

Enzyme assays. The cells were disrupted by gentle homogenization of the cell pellets in distilled water and were analyzed immediately for UDPGT activities towards a variety of substrates. Human liver microsomes were prepared from post-mortem liver samples, as previously described (16). Homogenization of the human liver samples was performed in 0.25 M sucrose, 1 mM Tris·HCl buffer, pH 7.4, with a Potter-Elvejhem apparatus (3000 rpm, three strokes), to give a 10% (of the original weight) homogenate, which was assayed immediately for UDPGT activities.

UDPGT activity towards 1-naphthol was assayed by the conventional method using [1-^{14}C]naphthol (58 mCi/mmol; Amersham, les Ulis, France) (17). Morphine glucuronidation was measured by the method of Svensson *et al.* (18) and clofibric acid glucuronidation according to that of Hamar-Hansen *et al.* (19). Bilirubin UDPGT activity was evaluated by the method of Heirwegh *et al.* (20). UDPGT activities towards estriol, β-estradiol, estrone, androsterone, testosterone, chenodeoxycholic acid, lithocholic acid, ursodeoxycholic acid, hyocholic acid, cholic acid, hyodeoxycholic acid, serotonin, and chloramphenicol were measured by the thin layer chromatographic method of Bansal and Gessner (21), modified as previously described (11–13). The incubation mixture contained 0.05–0.1 mg of cell or microsomal protein, 0.5 mM substrate dissolved in methanol, 7 μM UDP-[^{14}C]glucuronic acid (304 mCi/mmol; Dupont de Nemours NEN Division, Dreiech, FRG), and 0.1 mM UDP-glucuronic acid (Boehringer, Mannheim, FRG), in 100 mM Tris-maleate, 10 mM $MgCl_2$, 2 mg/ml bovine serum albumin buffer, pH 7.4. The incubation was carried out for 20–30 min at 37°.

The protein concentration of tissue samples was measured by the method of Bradford (22) (Bio-Rad protein assay kit; Bio-Rad, Paris, France), using bovine serum albumin as standard.

Protein analysis. The production and characterization of the polyclonal antibody preparation of anti-rat liver testosterone/4-nitrophenol UDPGT (RAL1) are described elsewhere (23). Electrophoresis of proteins in 7.5% polyacrylamide gels in the presence of SDS was performed as described by Laemmli (24). The separated proteins were transferred onto nitrocellulose membranes, and immunological detection was carried out using anti-goat IgG alkaline phosphatase conjugate as a secondary antibody (Sigma, St Louis, MO) and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt, as substrate. Details are given in Ref. 23.

Immunochemical methods. Cells were grown under the conditions described above, on microscope slides placed in Petri dishes. The cells were washed with 0.1 M phosphate buffer, pH 7.4, and fixed with 2% (v/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at 4° for 20 min.

The immunocytochemical localization of UDPGT was carried out according to an indirect immunoperoxidase method, amplified with a nickel salt (25). Cells were first preincubated with 0.1 M phosphate buffer, pH 7.4, containing 0.5% Triton X-100 (v/v) and 1% (v/v) pre immune rabbit serum, for 20 min. They were then incubated with the antiserum RAL1 (23) at different concentrations (1, 0.5, or 0.025%, v/v, in 0.1 M phosphate buffer, pH 7.4, containing 0.5% v/v, Triton X-100) for 2 hr at room temperature. After washing with phosphate buffer, peroxidase-conjugated rabbit anti-sheep IgG immunoglobulin (Institut Pasteur, Paris, France), diluted to 1% (v/v) in the same buffer, was added for 45 min. Slides were rinsed and placed into cold 0.1 M phosphate buffer, pH 7.4. The immunocytochemical reaction was developed using 0.01% (w/v) 3,3′-diaminobenzidine, 0.02%, (w/v) $Ni(NH_4)_2(SO_4)_2$, and 0.003% (v/v) H_2O_2 in 0.1 M phosphate buffer, pH 7.4. Nuclear counterstaining was performed using nuclear fast red (Merck, Darmstadt, FRG). Slides were dehydrated and mounted for light microscopy observation.

Nucleic acid analysis. High molecular weight genomic DNA from the cultured cells was prepared by the proteinase K-RNase A method (14) and analyzed by Southern blotting (26). Briefly 10 μg of DNA were cleaved with *Eco*RI (Pharmacia-LKB), electrophoresed on a 0.7% agarose gel, and transferred to Hybond N. Blotted DNA was hybridized to the ^{32}P-labeled *Hind*III fragment of either HLUGP1 or HLUG25, including the total coding sequence of the two clones (see Refs. 9 and 12). Hybridization conditions were the following: 6× SSC (1× SSC = 0.15 M sodium chloride, 0.015 M sodium citrate), 5× Denhardt's solution (0.1%, w/v, bovine serum albumin, 0.1%, w/v, Ficoll, 0.02%, w/v, polyvinylpyrolidone), 100 μg of salmon sperm DNA/ml solution, and 0.1% (w/v) SDS, at 65° overnight. Membranes were washed twice at room temperature for 5 min in 2× SSC, 0.1% (w/v) SDS, twice at 65° for 30 min in the same solution, twice at 65° for 30 min in 0.02× SSC, 0.1% SDS (w/v), and twice at room temperature in 0.1% SDS. Autoradiography was carried out by exposure to Kodak X-ray films at −80°, with intensifying screens.

Total RNA of the cultured cells was prepared by the guanidium thiocyanate-phenol-chloroform extraction method (27). Cells from five 90-mm-diameter culture dishes were denaturated in 4 M guanidium thiocyanate, 25 mM sodium citrate, 0.5% (w/v) sarcosyl, 0.1 M 2-mercaptoethanol, pH 7.0. Sodium acetate (2 M), pH 4.0, and phenol-chloroform/isoamyl alcohol (49:1, v/v) were added sequentially to the homogenate, and the mixture was cooled to 0°. The RNA present in

the aqueous phase was precipitated with isopropanol and sedimented by centrifugation at 1000 × *g* for 20 min. The precipitation procedure was repeated and the RNA pellet was washed with ethanol, resuspended in sterile water, and stored at −80°. The 260/280 nm absorbance ratio (>1.9) indicated a low level of protein contamination. Hybridization conditions were the same as described above for DNA.

Kinetic constants determination and statistical analysis. Apparent kinetic constants (K_m, V_{max}) were calculated by linear least-squares regression analysis of values from double-reciprocal plots corresponding to at least six different concentrations of substrate or UDP-glucuronic acid. Incubations were performed on nonactivated human liver microsomes or homogenates and cell homogenates, as described above. The concentration of hyodeoxycholic acid varied from 0.05 to 2 mM for a constant concentration of UDP-glucuronic acid of 0.11 mM (0.25 μCi). The glucuronic acid concentration then varied from 0.01 to 2 mM (0.25 μCi) for a constant concentration of hyodeoxycholic acid of 0.5 mM.

Results and Discussion

Stable expression of cloned human UDPGT activities in cell cultures. The ability to introduce isolated DNA into cultured mammalian cells by DNA-mediated gene transfer techniques is a powerful tool for analyzing eukaryotic gene expression. In our experiment, stable integrants of two UDPGT cDNAs in the host cell (V79) genome were obtained by cotransfection of each cDNA with a neomycin resistance vector and selection with the neomycin analog G418. Preliminary experiments showed that no detectable UDPGT activity was found towards the substrate hyodeoxycholic acid and that the glucuronidation of 1-naphthol was at the level of the detection limit in nontransfected V79 cells. Consequently, these cells were considered suitable recipients for our purpose. Other cell lines such as Chinese hamster ovary, Fao (rat hepatoma), and HepG2 (human hepatoma) were found to possess high levels of basal UDPGT activities (see Refs. 28 and 29). Furthermore, V79 cells also had successfully served as host cells in previous transfection experiments for cytochrome P-450 cDNA species (30, 31). Resistant colonies were either pooled (V79HLUGP1) or individually isolated (V79HLUG25C1-8) and were grown in mass culture. Enzymatic activity in these various cell lines was evaluated towards 1-naphthol and hyodeoxycholic acid, which were previously shown to be representative substrates for the UDPGT isoforms encoded by HLUGP1 and HLUG25 cDNAs, respectively (12, 13). The expression of enzyme activities clearly shows that the UDPGT proteins in cells transformed by either cDNA were catalytically active (Figs. 1 and 2). Eight cell lines were obtained from the V79 cells transfected with HLUG25, exhibiting specific activities from 0 to 25 pmol of hyodeoxycholate glucuronide formed/min/mg of protein. This activity was in the range of the highly variable values found in human liver (50 to 973 pmol/min/mg of protein, corresponding to four samples tested (Table 1), because the latter values were obtained using microsomes rather than homogenates, where the enzyme would be purified approximately 5-fold.[1] Further, endobiotic glucuronide formation by human liver microsomes in the absence of added exogenous substrate complicates the examination of UDPGT substrate specificity (see Fig. 1, *lane b*). The activity in transfected V79 cells was substantially higher (approximately 14-fold) than that previously measured during transient expression assays in COS-7 cells (13). A similar result

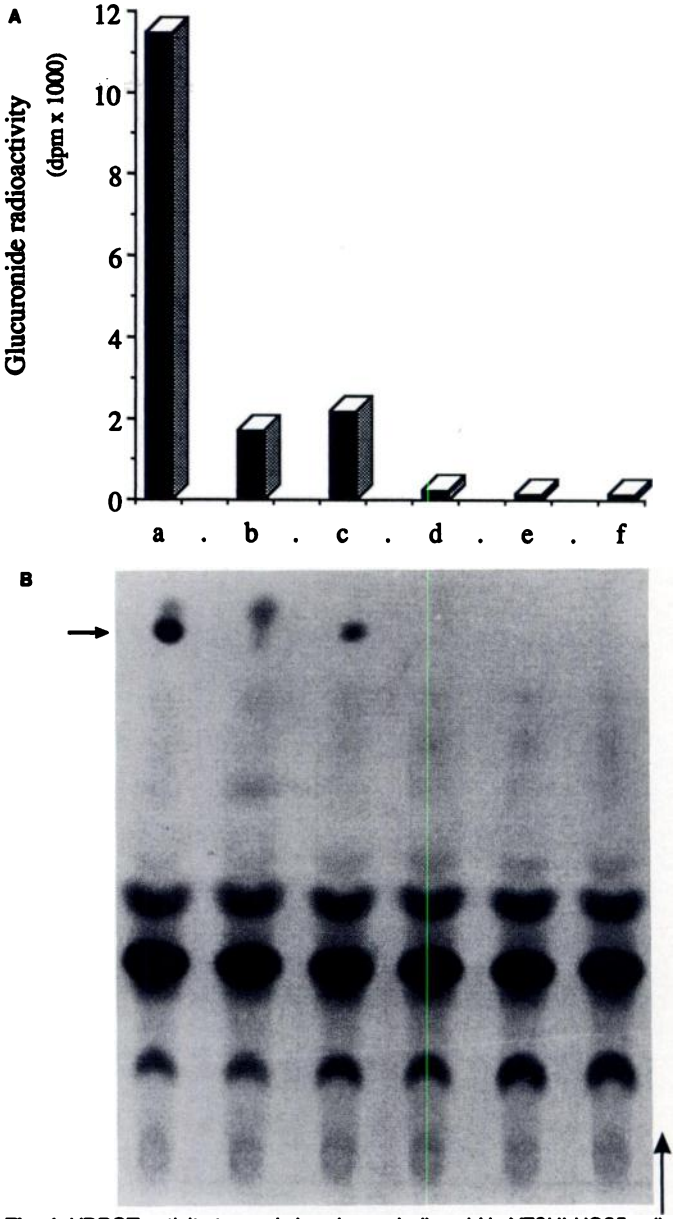

Fig. 1. UDPGT activity towards hyodeoxycholic acid in V79HLUG25 cells and in human liver microsomes. A, Quantitation of the radioactivity incorporated in the glucuronides formed and separated as shown below. *Lanes a* and *b*, human liver microsomes; *lanes c* and *d*, V79HLUG25 cells; *lanes e* and *f*, V79 control cells. Two measurements were performed on transformed cells from three separate plates. SD was less than 15%. Three measurements were performed on one batch of human microsomes. SE was less than 8%. B, Thin layer chromatographic analysis of hyodeoxycholic acid glucuronides. Cell homogenates (0.1 mg) or human microsomes (0.05 mg) were assayed as described in Materials and Methods, using a final concentration of UDP-glucuronic acid of 0.11 mM (0.25 μCi), and the radiolabeled products of the reaction were separated by thin layer chromatography. Hyodeoxycholate glucuronide migrated with an R_F value of 0.8, indicated by a *horizontal arrow*. In control assays (*lanes b, d,* and *f*), the aglycone substrate was omitted from the incubation mixture. Mobile phase migration was from the *bottom* to the *top* (*vertical arrow*).

was obtained for the V79HLUGP1 cell line, which exhibited a 1-naphthol UDPGT activity of 6.3 ± 0.9 nmol/min/mg of cell protein (Fig. 2). This level of activity was also comparable to that found in human liver microsomes (7 ± 3.4 nmol/min/mg of protein) (32). Thus, these new cell lines may serve as valuable

[1]Unpublished observations.

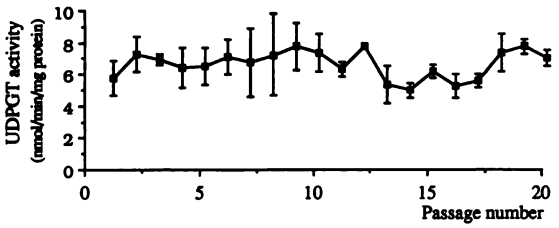

Fig. 2. Stability of the expression of UDPGT activity towards 1-naphthol in V79HLUGP1. UDPGT activity is expressed as nmol/min/mg of cell protein. Results are the mean of at least two determinations on cells harvested from two different plates. After selection, the cells were grown in the absence of G418. *Numbers on the x axis*, the number of cell passages, starting with the first cell sample harvested after selection (i.e., three passages).

TABLE 1

Substrate specificity of HLUG25 stably expressed in V79 cell line, compared with glucuronidation in microsomes of various human samples

Glucuronidation of the substrates was evaluated by a general thin layer chromatography assay, as described in Materials and Methods.

Substrates	UDPGT activity					
	Control V79 cells homogenate	V79HLUG25 cells homogenate	Human liver microsomes			
			1[a]	2[a]	3[a]	4[a]
	pmol/min/mg of protein					
Estriol	ND[b]	ND	240	525	183	288
β-Estradiol	ND	ND	11	25	3	26
Estrone	ND	ND	ND	ND	5	7
Androsterone	ND	ND	13	96	41	73
Testosterone	ND	ND	11	ND	5	15
Chenodeoxycholic acid	ND	ND	6	22	9	12
Lithocholic acid	ND	ND	7	15	3	ND
Ursodeoxycholic acid	ND	ND	22	10	ND	9
Hyocholic acid	ND	ND	62	22	35	131
Cholic acid	ND	ND	14	ND	4	ND
Hyodeoxycholic acid	ND	25	390	973	154	50
Serotonin	ND	ND	91	10	3	7
Chloramphenicol	ND	ND	16	ND	ND	ND

[a] Sample number.
[b] ND, nondetectable, specific activity less than 2 pmol/min/mg of protein.

systems in toxicological and metabolic studies, in association with scarce and variable human material. The stability of expression was also checked. In V79HLUGP1, no significant loss of activity was observed after 20 passages, i.e., 60 days of culture (Fig. 2). No significant difference was found when G418 was added or omitted from the medium after selection of the colonies (data not shown). Similar results were obtained with V79HLUG25, in which the initial activity was also maintained after 20 passages (data not shown). In addition, HLUGP1 was also stably expressed in NIH3T3 cells and, again, activities of 3.5 ± 1 nmol of 1-naphthol glucuronide formed/min/mg of protein were consistently detected and retained after more than 10 passages (40 days in culture).

Immunochemical detection and analysis of newly expressed UDPGT proteins. Homogenates of the V79 stable cell lines were electrophoresed on SDS-polyacrylamide gels and analyzed by immunoblotting. They were probed with anti-rat liver UDPGT antibodies, which were previously shown to react with human UDPGT isoforms (23), and, for comparison, human liver microsomes were blotted in parallel (Fig. 3, *lane a*). The presence of the UDPGT proteins could be detected by Western blotting, further indicating their higher level in the cell population exhibiting stable expression. UDPGT transiently expressed in COS-7 cells could only be shown by using [^{35}S]methionine labeling of cellular proteins and selective immunoprecipitation of UDPGT proteins (12, 13). The RAL1 antibodies, which exhibit a broad specificity towards several isoforms, recognized two polypeptide bands in V79 cells transformed with HLUGP1 (Fig. 3, *lane c*). The molecular weight of the M_r 55,000–56,000 polypeptide is identical to that of the transiently expressed protein in COS-7 cells, indicating the synthesis of a functional human phenol UDPGT of the correct size (12). It is not clear whether the smaller band (M_r 54,000–55,000) results from differential processing of the mRNA or differential maturation of the same protein. Two human liver-purified phenol-conjugating UDPGTs displayed a subunit molecular weight of 53,000 and 54,000 (33). Iyanagi *et al.* (5) similarly observed a second phenol UDPGT isoenzyme in rat liver microsomes that was recognized by two specific antipeptide antibodies, indicating a similar primary sequence. These results favor the hypothesis of differential glycosylation of a unique protein. In addition, the RAL1 antibodies reacted with a polypeptide band of M_r 52,000 in the V79 cells transformed with HLUG25 cDNA (Fig. 3, *lane d*), which corresponds to the mature and enzymatically active UDPGT isoform conjugating hyodeoxycholic acid (13). In homogenates of nontransformed control V79 cells, no immunoreactive polypeptide could be detected, as expected from the undetectable UDPGT activities found in this cell line (Fig. 3, *lane b*).

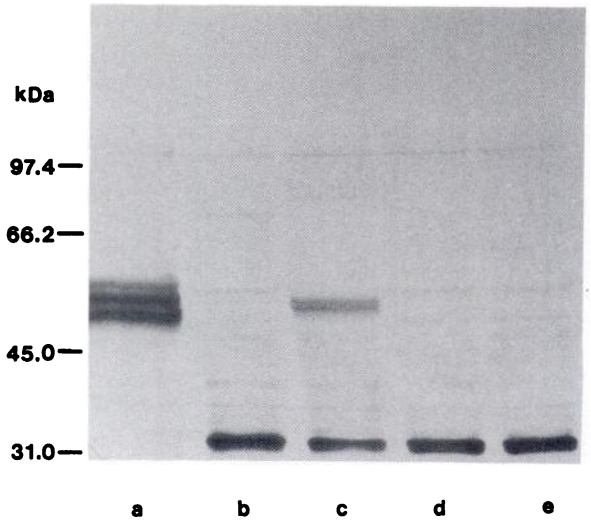

Fig. 3. Immunoblot analysis of expressed UDPGT protein in transformed cell lines. Cell homogenates were electrophoresed on SDS-7.5% polyacrylamide gels and probed with RAL1 antibody preparation. *Lane a*, human liver microsomes, 10 μg; *lane b*, V79 control cells, 50 μg; *lane c*, V79HLUGP1 cells, 50 μg; *lanes d* and *e*, V79HLUG25 cells, 50 μg.

The transformed cell lines were also analyzed by immunohistochemical techniques. The morphology of transformed cultured cells did not show any visible alterations. Immunoreactive UDPGT was intensely stained in both recombinant cell lines (Fig. 4, B and C), when compared with control V79 cells (Fig. 4A), where no UDPGT protein could be detected. The strongest staining could only be observed inside the cells, in an area in the vicinity of the nucleus, suggesting that the UDPGT protein might be expressed in its expected subcellular localizations, the endoplasmic reticulum and the nuclear membrane. As expected, the V79HLUG25 cell population appears homogeneous (Fig. 4C), whereas islets of positive UDPGT cells could be observed

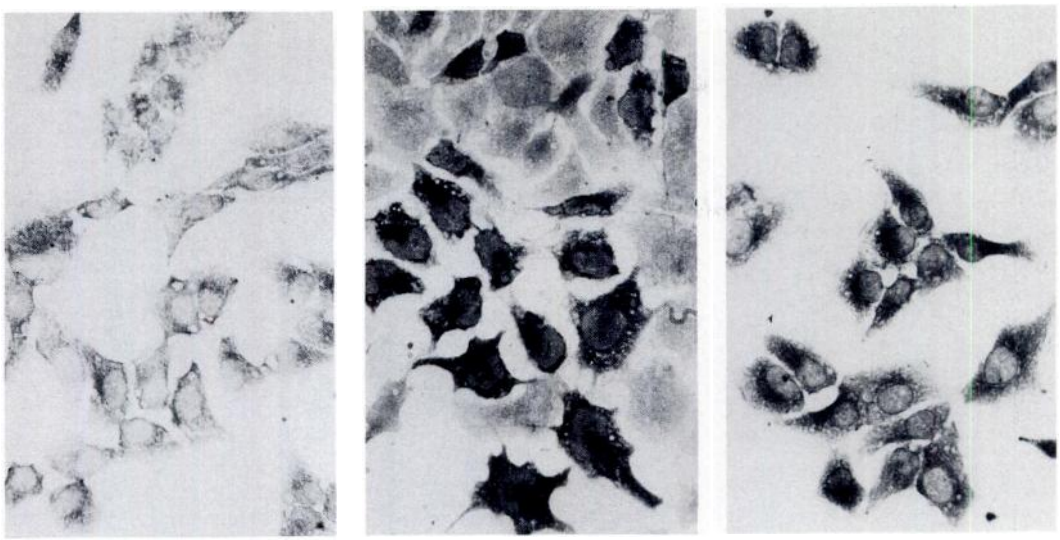

Fig. 4. Immunohistoperoxidase staining of V79 transformed cell lines. A, V79 control cells; B, V79HLUGP1 cells; C, V79HLUG25 cells. Cells were probed with RAL1 antibody preparation, as described in Materials and Methods. Magnification, 400×.

in V79HLUGP1 (Fig. 4B). Subcloning of the latter cell line is currently being performed in our laboratory.

Examination of the UDPGT mRNAs expressed in the recombinant cell lines. Total mRNA was isolated to determine whether the UDPGT cDNAs were correctly transcribed in the transformed V79 stable cell lines. The RNA was electrophoresed on 0.8% (w/v) agarose gels, under denaturating conditions, and hybridized with cDNAs containing the complete sequence of either HLUGP1 or HLUG25 (Fig. 5). Control V79 cell mRNA did not apparently contain any hybridizing mRNA (Fig. 5, *lanes a, b, f,* and *g*), confirming that these cells were suitable for the gene transfer experiment. One homogeneous transcript was detected in each of the two recombinant lines, V79HLUGP1 (Fig. 5A) and V79HLUG25 (Fig. 5B). The sizes of the transcripts are in agreement with those calculated from the expression vectors.

Integration of UDPGT cDNA into the host cell genome. Gene constructs were examined to assess the integration of the foreign DNA into the V79 host cell genome. G418-selected cell colonies were grown in mass culture, and their DNA was analyzed by Southern blot hybridization (Fig. 6). High molecular weight genomic DNA from V79HLUGP1 and V79HLUG25 cells was digested with *Eco*RI and hybridized with the appropriate probe (Fig. 6). No hybridizing fragment was observed in V79 control cell DNA probed with either of the radiolabeled UDPGT cDNAs. The digested chromosomal DNA from V79HLUGP1 cells (Fig. 6A, *lane c*) showed a hybridizing fragment of about 2.1 kb. This corresponded to the expected size of the *Eco*RI restriction fragment derived from this UDPGT cDNA (12). Southern blot analysis of V79HLUG25 cell DNA showed a unique hybridizing fragment of about 1.2 kb (Fig. 6B, *lane f*), which is the expected size of the *Eco*RI restriction fragment from HLUG25 cDNA (13). These results strongly suggested that the UDPGT cDNAs were stably integrated into the host cell DNA.

Substrate specificity of HLUG25 cDNA-encoded UDPGT isoform. Expression of closely related forms with overlapping substrate specificities, and their subsequent char-

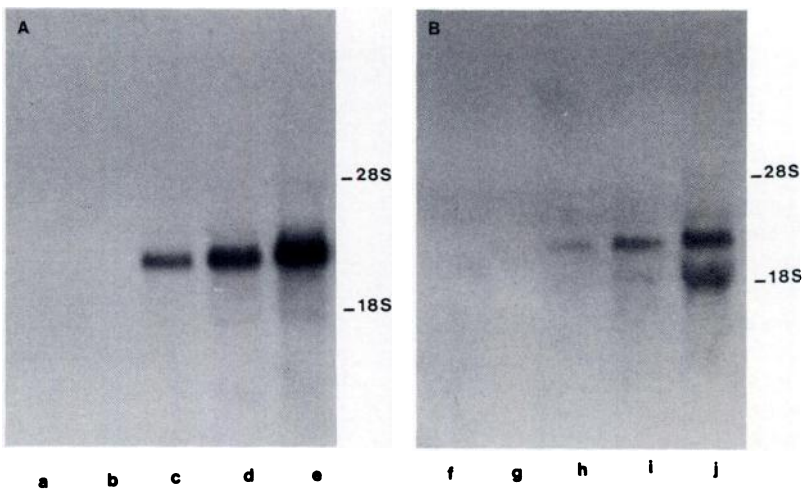

Fig. 5. Northern blot analysis of total mRNA from transformed V79 cell lines. Total mRNA from HLUGP1 (A) and HLUG25 (B) was hybridized with the *Hind*III fragment from the corresponding UDPGT cDNA. *Lanes a* and *b* plus *f* and *g*, 5 and 10 μg of V79 mRNA; *lanes c, d,* and *e*, 2, 5, and 10 μg of V79HLUGP1 mRNA; *lanes h, i,* and *j*, 2, 5, and 10 μg of V79HLUG25 mRNA, respectively.

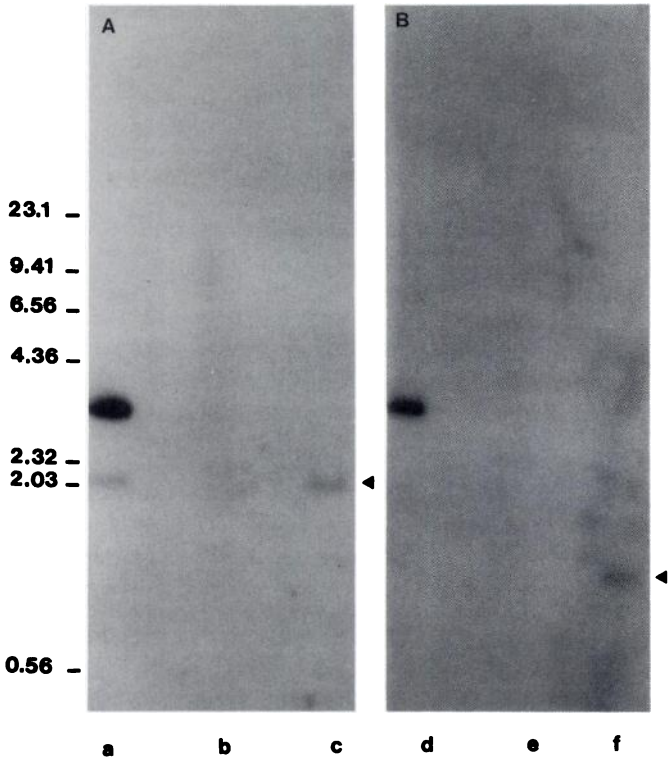

Fig. 6. Southern blot analysis of *Eco*RI-digested chromosomal DNA from transformed V79 cell lines. DNA from V79HLUGP1 (A) and V79HLUG25 (B) was hybridized with the *Hind*III fragment from the corresponding UDPGT cDNAs. *Lane a*, pKCRH2 HLUGP1, 10 ng; *lanes b* and *e*, V79 DNA, 10 μg; *lane c*, V79HLUGP1 DNA, 10 μg; *lane d*, pKCRH2 HLUG25, 10 ng; *lane f*, V79HLUG25, 10 μg. Size markers are given as kb. *Arrowheads*, restriction fragments.

acterization, will allow the analysis of the contribution of each of the enzymes to the metabolism of carcinogens and drugs in intact cells, in the absence of other UDPGTs. This expression system can also be used in a general procedure to identify the substrate specificity of an UDPGT cDNA of unknown function. Accordingly, we had previously determined, using transient expression assays (13), that HLUG25 cDNA encoded an isoform conjugating hyodeoxycholic acid. However, the study of the substrate specificity of the isoform was impaired in this system by the low level of activity (1.8 pmol/min/mg of cell protein). The newly engineered V79HLUG25 cell line, exhibiting a much higher level of stable catalytic activity, was an appropriate tool to achieve that goal. Several steroids and bile acids, mono-, di-, or trihydroxylated at various positions of the steroid nucleus, were tested as potential substrates, in order to determine on which site (3α-OH, 6α-OH, -COOH) hyodeoxycholic acid was glucuronidated in V79HLUG25 cells (Table 1). (a) Androsterone and lithocholic acid, which are a 3α-monohydroxylated steroid and a bile acid, respectively, were not substrates of the encoded isoform. (b) The methyl ester of hyodeoxycholic acid was glucuronidated at a similar rate as hyodeoxycholic acid itself (21 ± 6 pmol/min/mg of cell protein). (c) The radiolabeled glucuronide formed by human microsomes or by V79HLUG25 cells incubated with hyodeoxycholic acid and radiolabeled UDP-glucuronic acid exhibited the same chromatographic behavior (data not shown) when analyzed by high pressure liquid chromatography (34). It has been established unequivocally by several groups that the 6α-glucuronide of hyodeoxycholic acid is formed in human kidney and liver microsomes (35–37). The three lines of evidence described above strongly suggested that HLUG25 cDNA encoded a UDPGT isoform responsible for this reaction.

It is interesting to note that UDPGT activity towards hyocholic acid (3α-, 6α-, 7α-trihydroxylated bile acid), which could be easily measured in the four samples of human microsomes tested (22–131 pmol/min/mg of protein), could not be detected in V79HLUG25 cells (Table 1). Such results could not be explained by the sensitivity of the assay, because a low rate of activity of 2 pmol/min/mg of protein was still detectable under the experimental conditions used. It is tempting to speculate that the 7α-hydroxy group hinders access to the 6α site and that, in human microsomes, the glucuronidation of hyocholic and hyodeoxycholic acids does not occur at the same hydroxy position. However, direct structural analysis of the glucuronides formed is required to elucidate these points.

In addition, the formation of glucuronides from other steroids and bile acids, or from various unrelated substrates (serotonin, chloramphenicol), could not be found in V79HLUG25 recombinant cells, when evaluated by the general method of Bansal and Gessner (21). This result confirmed previous indirect evidence that 17β- (testosterone, β-estradiol), 3α- (androsterone, lithocholic acid), and 3- (estrone) hydroxylated steroids are glucuronidated by separated isoforms with restricted specificity towards one position of the steroid nucleus. Indeed, recently two different UDPGT cDNAs encoding transferases catalyzing the glucuronidation of estriol have been identified (38, 39). Moreover, V79HLUG25 cells were not able to glucuronidate either bilirubin, morphine, or clofibric acid (data not shown).

It was possible to determine the kinetic constants of the expressed UDPGT in stable V79HLUG25 transformed cells. To our knowledge, this report presents the first characterization of the catalytic properties of recombinant UDPGT stably expressed in eukaryotic cells lines. The apparent V_{max} of UDPGT towards hyodeoxycholic acid in recombinant cells was 14 times lower than that obtained in one sample of human microsomes, and glucuronidation efficiency (evaluated by the ratio of V_{max}/K_m) was also reduced (Table 2). A closer comparison arises from the evaluation of these kinetic constants in liver homogenates. In the latter case, the V_{max} values obtained in cells correspond well to the levels found in human liver (Table 2). In addition, the K_m values of UDPGT towards hyodeoxycholic acid or towards UDP-glucuronic acid were identical in cells and in human liver, further indicating similar

TABLE 2

Apparent kinetic constants of hyodeoxycholic acid glucuronidation in V79HLUG25 cells and in human liver

UDPGT activity was measured towards hyodeoxycholic acid, as described in Materials and Methods, using varying concentrations of hyodeoxycholic acid (0.05–2 mM) or of UDP-glucuronic acid (0.01–4 mM).

	K_m	V_{max}	V_{max}/K_m
	mM	nmol/min/mg of protein	
V79HLUG25 cells			
Hyodeoxycholic acid	0.27	0.036	0.13
UDP-glucuronic acid	0.12	0.040	0.34
Human liver microsomes			
Hyodeoxycholic acid	0.25	0.51	2.04
UDP-glucuronic acid	0.21	0.86	4.09
Human liver homogenate			
Hyodeoxycholic acid	0.28	0.097	0.34
UDP-glucuronic acid	0.12	0.103	0.85

catalytical properties of native and recombinant expressed enzymes.

Finally, a broader question arises concerning the physiological function of 6α-*O*-glucuronidation. Several authors (35–37) suggested that it could constitute a metabolic response to toxic hydrophobic bile acids, especially lithocholate and some dihydroxy bile acids, which accumulate in some pathological conditions such as cholestasis. The bile acid would be committed to excretion by 6α-hydroxylation, which in turn would trigger 6α-*O*-glucuronidation. The recombinant cells and probes described in this paper represent novel tools to investigate, on a molecular basis, the physiopathological significance of 6α-OH-glucuronidation. Similarly, V79HLUGP1 cells are currently being used to characterize the glucuronidation of therapeutic agents in humans.

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