Expression of UDP-Glucuronosyltransferase cDNA in *Saccharomyces cerevisiae* as a Membrane-Bound and as a Cytosolic Form

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ABSTRACT: The mouse clone UDPGT$_m$-1 encodes a UDP-glucuronosyltransferase enzyme which was isolated from a λgt11 cDNA library constructed with phenobarbital-induced liver mRNA [Kimura, T., & Owens, I. S. (1987) *Eur. J. Biochem. 168*, 515–521]. In order to establish substrate specificity, UDPGT$_m$-1 was inserted into the yeast vector pEVP11 and expressed in *Saccharomyces cerevisiae* strain AH$_{22}$. Cells transformed with the expression unit pUDPGT$_m$-1c (insert in correct orientation with respect to promoter) stably transcribe the transferase cDNA. Consistent with the presence of mRNA, pUDPGT$_m$-1c-transformed AH$_{22}$ cells synthesize a transferase protein with $M_r \simeq 51\,000$ by Western immunoblot analysis. The membrane-bound transferase expressed in yeast in glycosylated as indicated by its enhanced electrophoretic mobility in a SDS-polyacrylamide gel following endoglycosidase H treatment and detection by Western immunoblot analysis. A survey, using 12 aglycons in an assay with microsomes from cells which express the protein, shows preferential glucuronidation of naphthol and estrone followed by *p*-nitrophenol. Testosterone, phenolphthalein, dihydrotestosterone, androsterone, and 4-methylumbelliferone are conjugated at an intermediate level. There is barely detectable glucuronidation of 3-hydroxy- and 9-hydroxybenzo[*a*]pyrene and no detectable conversion of morphine or lithocholic acid. The truncated cDNA (lacking the putative membrane-insertion signal-peptide coding sequence, but with a newly adapted translation-start codon) is ligated into pAAH5 and is expressed as a cytosolic transferase form in the protease-deficient ZA521 strain of *S. cerevisiae*. The $M_r \simeq 51\,000$–$52\,000$ is similar to that seen in microsomes from AH$_{22}$ cells where the protein is presumably processed as it is inserted into the membrane. This cytosolic form expresses similar catalytic activity toward naphthol and 3-hydroxybenzo[*a*]pyrene, very much lower activity toward estrone, *p*-nitrophenol, and phenolphthalein, and detectable activity toward lithocholic acid when compared to the membrane-bound transferase in AH$_{22}$ cells. A comparison of the catalytic activity as the membrane-bound form when expressed in AH$_{22}$ versus ZA521 shows that the relative substrate profile is similar, but higher levels of the transferase protein and activities are seen in AH$_{22}$ than in ZA521. The transferase as a membrane-bound form glucuronidates at least three substrates traditionally classified as type I, one classified as type II, and at least four steroid aglycons. In contrast, the enzyme, when distributed into the yeast cytosol, has a more restricted substrate range and catalyzes most of these substrates at a much lower rate.

The membrane-bound UDP-glucuronosyltransferase (transferase)[1] system is composed of an undetermined number of isozymes responsible for glucuronidating numerous endogenous and exogenous lipophilic compounds. Glucuronidation, in practice, enhances water solubility and, thus, excretability of certain lipophiles from the cellular milieu. This process which occurs primarily in the liver is a major detoxifying system in the body.

In order to understand the development and regulation of the broad glucuronidating capacity of the liver, it is necessary first to determine the number of transferase isozymes and the substrate preference of each form. Biochemically, the transferases have been categorized, primarily, on the basis of the developmental profile of conjugation of certain substrates and the induction of activity toward particular aglycons following treatment with a prototypic effector compound (Wishart, 1978a,b; Beck et al., 1973). The number of isoforms which account for these collective activities is unknown, as transferases often have overlapping substrate specificity. Reports of purification studies show that certain forms catalyze chemically similar aglycons (Matern et al., 1982; Kirkpatrick et al., 1984; von Meyerinck et al., 1985) while other forms metabolize a broad range of acceptor substrates (Bock et al., 1982; Mackenzie et al., 1984). Although purification studies have advanced our understanding of this family of enzymes, certain limitations of this approach are essentially insurmountable, e.g., the possible detergent effects on the activity of purified enzyme and the difficulty of separating similar proteins with high retention of activity. Hence, the aims of this study are (a) to develop an expression system to establish the substrate specificity of the murine UDP-glucuronosyltransferase encoded by the cDNA clone, UDPGT$_m$-1 (Kimura & Owens, 1987), and (b) to compare the effect on activity of a truncated derivative of UDPGT$_m$-1 (lacking the membrane-insertion signal-peptide coding sequence, but with a newly adapted translation-start codon) which targets the protein to the cytosol rather than the endoplasmic reticulum. Recent reports of expression of rat transferase cDNAs in COS

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[1] Abbreviation: transferase, UDP-glucuronosyltransferase.

cells (Mackenzie, 1986a,b, 1987) have appeared.

The mouse full-length cDNA encoding $UDPGT_m$-1 was isolated and characterized with respect to regulation of the 2200-base mRNA (Kimura & Owens, 1987). This clone has a high level of sequence similarity to a 2000-base mRNA which is also regulated by the same agents (Kimura & Owens, 1987), suggesting that the two mRNAs represent a subfamily of transferases. The acquisition of information which would allow one to define the substrate specificity of the $UDPGT_m$-1 clone is a step toward eventually understanding the relationship of the chemical compounds catalyzed by the members of this subfamily. The accumulation of data on subsets of transferases should provide insight into the evolution of this family of enzymes and the development of this broadly based conjugating system.

Since *Saccharomyces cerevisiae* strain AH_{22} had been successfully used to express two endoplasmic reticulum bound proteins (Oeda et al., 1985; Murakami et al., 1985), it was of interest to determine whether this strain transformed with an appropriate expression vector containing $UDPGT_m$-1 would produce active transferase. In addition, yeast is reported to contain similar mechanisms for initiating protein synthesis (Sherman & Stewart, 1982) and for processing proteins (Drake et al., 1984; Novick et al., 1981; Schekman & Novick, 1982) as mammalian cells. The constitutive expression of a cDNA in transformed yeast avoids the complication of transient expression associated with transfections of mammalian cells or the task of developing stable transfectants of mammalian cells. Finally, the yeast provides another expression model and one which has no detectable constitutive glucuronidating activity.

Experimental Procedures

Materials

UDP–glucuronic acid, 1-naphthol, 4-methylumbelliferone, *p*-nitrophenol, phenolphthalein, testosterone, androsterone, and estrone were from Sigma Chemicals (St. Louis, MO); 3-hydroxy- and 9-hydroxybenzo[*a*]pyrenes were from the National Cancer Institute Chemical Repository; [^{14}C]UDP–glucuronic acid was from New England Nuclear (Boston, MA); deoxycytidine [α-^{32}P]triphosphate was from Amersham (Arlington Heights, IL).

Restriction enzymes and other reagents used in molecular biology techniques were from New England Biolabs (Beverly, MA), IBI Biochemicals (New Haven, CT), Bethesda Research Laboratory (Bethesda, MD), or Boehringer-Mannheim (Indianapolis, IN). *S cerevisiae* AH_{22} strain [*MATa, leu2-3,112 his4-519 can1* (cir$^+$)] (Oeda et al., 1985) was a gift from Dr. H. Ohkawa (Sumitomo Chemical Co., Hyogo, Japan); the protease-deficient strain of *S. cerevisiae*, ZA521 (*MATa ura3-52 leu2-3,112 bar1 pep4-Δ1::URA3*) (Ammerer et al., 1986), was a gift from Dr. V. MacKay (Zymogenetics, Seattle, WA), and pEVP11 was donated by Dr. P. Nurse (Imperial Cancer Research Fund Laboratory, London). The vector pAAH5 was from Dr. B. Hall (University of Washington, Seattle, WA). C57BL/6N mice were from the Veterinary Resources Branch, National Institutes of Health (Bethesda, MD).

Each yeast strain is a *Leu2* auxotroph and was grown in enriched YPD medium. Transformed yeast strains were cultivated in yeast minimal medium containing 0.01% of each amino acid (except leucine), 0.67% yeast nitrogen base (GIBCO Laboratories, Chagrin Falls, OH), and 1.0% dextrose to select by complementation of *Leu2* auxotrophy. pAAH5 contains *ADH1* promoter and terminator sequences from the alcohol dehydrogenase (*ADH1*) gene and the *Leu2* gene from *S. cerevisiae*, and pEVP11 contains *ADH1* promoter and terminator sequences from the alcohol dehydrogenase (*ADH1*) gene and the *Leu2* gene from *Schizosaccharomyces pombe*.

Methods

Construction of Expression Vectors. (*i*) *Vector Containing Full-Length cDNA.* The full-length insert encoding $UDPGT_m$-1 isolated from a λgt11 cDNA library, constructed according to Young and Davis (1983), was selected on the basis of hybridization to $UDPGT_r$-3 (Mackenzie, 1987). $UDPGT_m$-1 was sequenced as already described (Kimura & Owens, 1987). The insert was adapted with *Hin*dIII linkers and ligated into *Hin*dIII-digested pEVP11 and designated $pUDPGT_m$-1 (Figure 1A). $pUDPGT_m$-1 was transformed into *Escherichia coli* strain DH1 (F$^-$ recA1 gyrA96 thiol hsdR17 supE44λ$^-$). Plasmids were isolated from single colonies, grown overnight, and then subjected to restriction enzyme analysis to identify plasmids with correct and reversed orientation of the 5′ end of $UDPGT_m$-1 with respect to the *ADH1* promoter of pEVP11. The appropriate plasmids, prepared according to Maniatis et al. (1982), were used to transform AH_{22} cells according to the lithium procedure of Rothstein (1985). The full-length cDNA was similarly ligated into *Hin*dIII-digested pAAH5, and the recombinant vectors were used to transform DH1 cells to identify plasmids containing $UDPGT_m$-1 with the correct and reversed orientation with respect to the *ADH1* promoter of pAAH5. The appropriate plasmids were used to transform ZA521 cells.

(*ii*) *Removal of the Sequence Encoding the Membrane-Insertion Signal Peptide of $UDPGT_m$-1.* The amino acid sequence deduced from the coding sequence for the N-terminus of $UDPGT_m$-1 contains a motif consistent with a signal peptide (Watson, 1984) which targets the protein to the endoplasmic reticulum, i.e., a charged amino acid (lysine) within the first five residues, a sequence of eight hydrophobic amino acids, followed by a helix-breaking residue or a large residue (usually glutamine) which is four to eight residues before the cleavage site (Figure 1B, line 1). Digestion of the cDNA with *Pst*I results in the removal of codons for the 12 N-terminal amino acids including glutamine. The 5′ end of the newly truncated cDNA contains only the *G* of the CAG codon for glutamine after the formation of a blunt end by the exonuclease activity of T_4 DNA polymerase.

The oligomer 5′ATAAGCTTAT3′ was synthesized and ligated to the blunt-ended fragment to provide the methionine codon ATG and a *Hin*dIII restriction site (Figure 1B, line 2). The translation initiation codon, ATG, in effect, replaced the CAG for glutamine such that the newly truncated $UDPGT_m$-1 allowed the expression of a protein lacking a membrane-insertion signal peptide. Also, $UDPGT_m$-1 is truncated such that its 5′ end should encode an additional 4 to 5 amino acids toward the N-terminus of the predicted natural cleavage site of the protein (Watson, 1984) at a cysteinyl residue which is in both position 16 and position 17.

The adaptations at the 5′ end of $UDPGT_m$-1 were confirmed by subcloning the truncated clone into the *Hin*dIII site of the M13mp10 vector. The vector was then transformed into *E. coli* cells (strain JM103), and single-stranded DNA was purified and sequenced through the modified region according to the method of Sanger et al. (1977). The truncated and newly adapted $UDPGT_m$-1 was ligated to *Hin*dIII-digested pAAH5 (Figure 1B). $pUDPGT_m$-$1_c\Delta$ (the truncated insert in the *c*orrect orientation) and $pUDPGT_m$-$1_r\Delta$ (the truncated clone in the *r*eversed orientation) were transformed into *S. cerevisiae* strain ZA521.

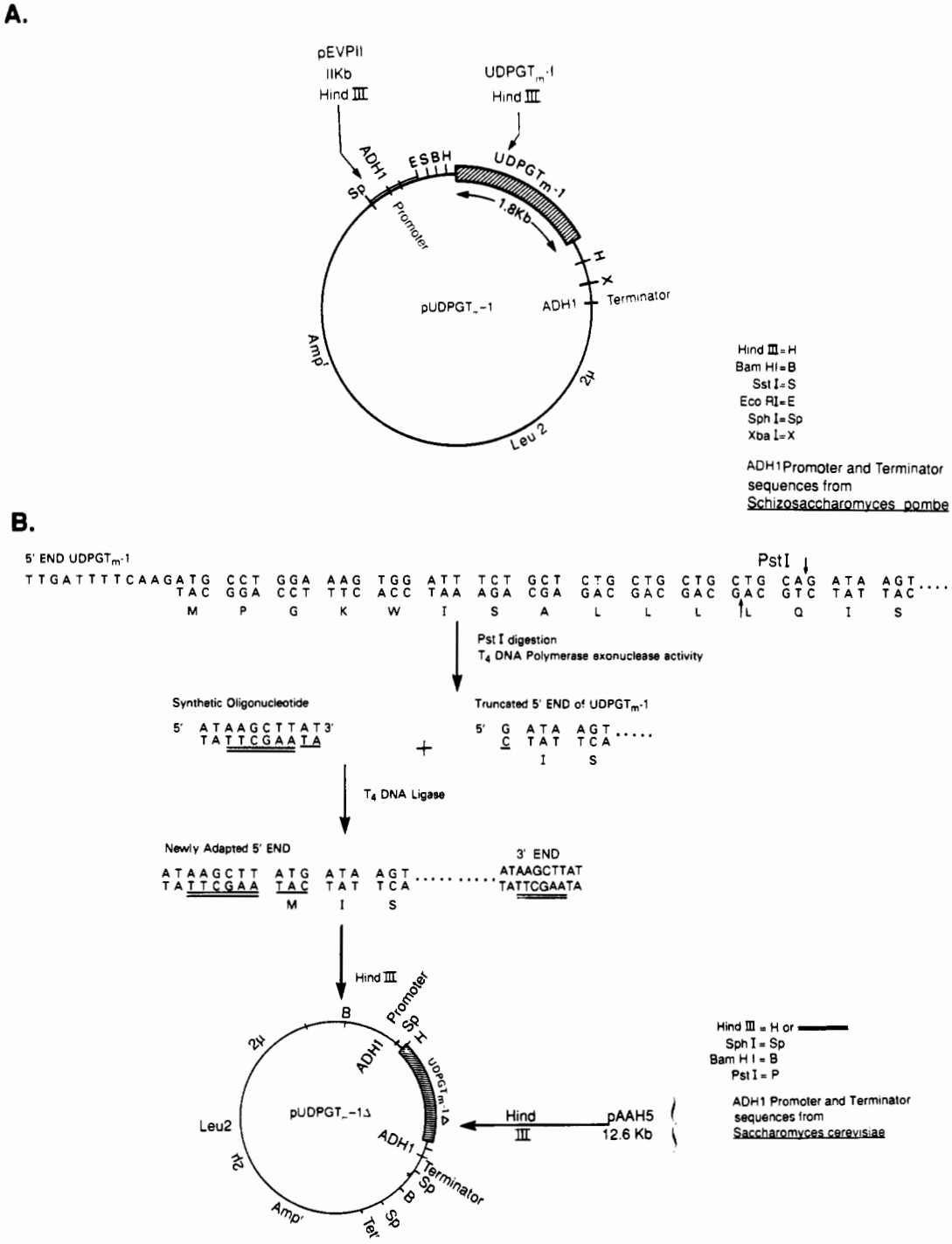

FIGURE 1: (A) Construction of the yeast expression system with pEVP11 and the UDPGT$_m$-1 insert. pEVP11 was digested with *Hin*dIII and ligated to UDPGT$_m$-1 which was adapted with *Hin*dIII linkers to generate an expression system. The expression unit is designated pUDPGT$_m$-1. (B) Construction of the yeast expression system with pAAH5 and the 5'-truncated UDPGT$_m$-1. Lines 1 and 2 depict the removal of the signal-peptide coding sequence from the 5' end of UDPGT$_m$-1 by *Pst*I digestion and the formation of its blunt end by T$_4$ DNA polymerase exonuclease activity. Line 3 depicts the establishment of a new translation start codon (Met) at the 5' end of UDPGT$_m$-1 by using a synthetic oligonucleotide which contains a *Hin*dIII restriction site. Finally, the 5'-truncated UDPGT$_m$-1 is ligated to pAAH5 at the *Hin*dIII cloning site to generate an expression unit designated pUDPGT$_m$-1$_c$Δ.

Analysis of Expressed Products. (*i*) *Northern Blot.* Poly(A+) RNA from each wild type and transformed yeast was isolated (Hinnebusch & Fink, 1983; Aviv & Leder, 1972) and electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde and blotted onto Zetabind membrane (0.45-μm pore size). The blots were hybridized to ^{32}P-labeled UDPGT$_m$-1, processed, and autoradiographed (Church & Gilbert, 1984). The insert was labeled by nick-translation in the presence of [α-^{32}P]dCTP.

(*ii*) *Western Immunoblot.* Microsomes and 100000*g* supernatants from each of the yeast systems were prepared according to published procedures (Oeda et al., 1985). Appropriate supernatants and microsomes from yeast and microsomes from 3-methylcholanthrene-treated C57BL/6N mice were solubilized in 0.1 M potassium phosphate, pH 7.5, 0.2 M KCl, 2% cholate, and 0.5% Zwittergent (buffer A). After sample preparation, the proteins were electrophoresed in a 7.5% SDS-polyacrylamide gel (Laemmli, 1970) and blotted onto a nitrocellulose filter in 25 mM Tris (unbuffered), 0.19 M glycine, and 20% methanol using 100 mA of current for 16 h. The filter was blocked in 5% reconstituted powdered milk in 50 mM Tris, pH 7.8, and 0.154 M NaCl (TBS). The

blocked filter was exposed overnight to a 1:100 dilution of goat anti-mouse transferase IgG [50% $(NH_4)_2SO_4$ saturation fraction] (Mackenzie et al., 1984) in TBS containing 1% bovine serum albumin. After the filter was washed in two changes of TBS, the second antibody, rabbit anti-goat IgG conjugated to horseradish peroxidase, was exposed to the filter for 3 h. After two washes in TBS, the filter was exposed to 4-chloro-1-naphthol for color development catalyzed by the specifically bound peroxidase.

(*iii*) *Treatment of Microsomes with Endoglycosidase H.* Microsomes from pUDPGT$_m$-1c-transformed AH_{22} cells (0.65 mg) and untreated mice (60 μg) were solubilized in buffer A containing 0.5 mg/L leupeptin, 0.7 mg/L pepstatin, 1.0 mM phenylmethanesulfonyl fluoride (PMSF), and 1.0 mM EDTA (buffer B). The solubilized yeast microsomes were then preadsorbed with 0.6 mL of carrier-fixed α_2-macroglobulin at 4 °C with gentle shaking for 30 min. The carrier had been previously washed with 3 volumes of buffer B. Similarly, the mouse microsomes were preadsorbed with 0.06 mL of α_2-macroglobulin. The microsomal content was recovered after pelleting the carrier in a microfuge. The yeast preparation was then divided into two aliquots: 0.1 unit of endoglycosidase H was added to 0.3 mg of solubilized microsomes, and the equivalent volume of 10 mM sodium phosphate, pH 7.0, was added to the other 0.3-mg sample. The preparations were incubated at 37 °C for 10 h. Similarly, mouse microsomes were divided into three aliquots such that one 20-μg aliquot was treated with 0.01 unit of endoglycosidase H. The preadsorption of solubilized microsomes with carrier-fixed α_2-macroglobulin removes a broad range of interfering protease activity. The samples were then subjected to SDS–polyacrylamide gel electrophoresis, and Western immunoblot analysis was carried out as already described.

Yeast Microsomal Glucuronidation Assays Using [^{14}C]*-UDP–Glucuronic Acid.* Appropriate microsomal preparations and 10000*g* supernatant from yeast were assayed for the glucuronidation of naphthol, 4-methylumbelliferone, *p*-nitrophenol, 3-hydroxy- and 9-hydroxybenzo[*a*]pyrene, phenolphthalein, morphine, testosterone, dihydrotestosterone, androsterone, estrone, and lithocholic acid according to published procedures (Bansal & Gessner, 1980) but with modifications. Protein samples contained in 100 mM potassium phosphate, pH 7.7, and 20% glycerol were activated by sonication in 0.05 mg of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) per milligram of protein. Activated protein (0.3 mg) was added to 10 μM aglycon, 50 μM [^{14}C]UDPGA (0.25 μCi), 50 mM Tris, pH 7.7, 4 mM $MgCl_2$, 1.0 mM PMSF, 0.5 mg/L leupeptin, and 0.7 mg/L pepstatin in a total volume of 0.15 mL and incubated at 24 °C for 16 h. Half of each sample was digested with β-glucuronidase (55 units), pH 7.0, at 37 °C for 3 h. Untreated and β-glucuronidase-treated reaction mixtures were analyzed by thin-layer chromatography as described (Bansal & Gessner, 1980). Each aglycon was converted to the β-glucuronide with mouse microsomes for comparison (data not shown). The plates were exposed to film for 20–30 days.

Finally, the individual areas representing β-glucuronides and the blank reaction (without aglycon) were scraped from the TLC plates and counted in a scintillation cocktail mix. Counts representing β-[^{14}C]glucuronides were corrected for background.

Microsomal Glucuronidation Using Conventional Methodology. Naphthol (0.5 mM) glucuronidation was carried out in the presence of 100 mM Tris, pH 7.5, 2.0 mM UDP–glucuronic acid, and 5 mM $MgCl_2$ with CHAPS-activated yeast microsomes. The reaction product was extracted with chloroform, and the aqueous phase was used to determine the fluorescence of naphtholylglucuronide according to Bock and White (1979). Yeast microsomal estrone transferase activity was attempted by using the published procedures of Rao et al. (1977).

Protein estimations were determined by the BCA method (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as the standard.

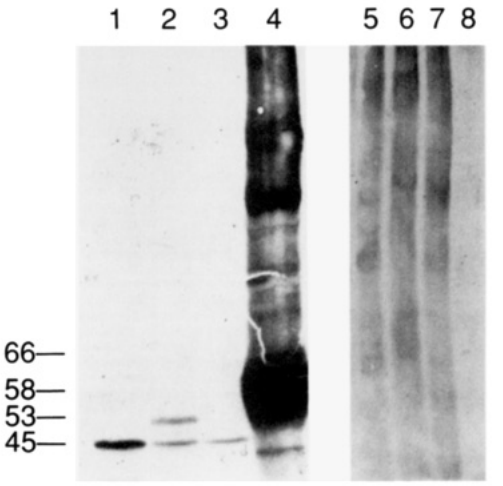

FIGURE 2: Western immunoblot of transferase protein in microsomes from mouse, wild-type, pUDPGT$_m$-1$_r$-transformed, and pUDPGT$_m$-1$_c$-transformed AH_{22} cells. Microsomes were isolated from mouse and yeast as described under Methods. Microsomes from a 3-methylcholanthrene-treated mouse (10 μg, lanes 4 and 8) and yeast (100 μg, lanes 1–3, and 200 μg, lanes 5–7) were electrophoresed and blotted, and color was developed as detailed under Methods. Yeast protein samples are as follows: pUDPGT$_m$-1$_r$-transformed cells (lanes 1 and 5); pUDPGT$_m$-1$_c$-transformed cells (lanes 2 and 6); wild-type cells (lanes 3 and 7). Lanes 1–4 were treated with goat anti-mouse transferase IgG, and lanes 5–8 were treated with goat preimmune IgG. Molecular weight markers are egg albumin (45K), glutamic dehydrogenase (53K), catalase (58K), and albumin (66K).

Results and Discussion

In order to identify a system which synthesizes a high level of transferase protein in an environment free of endogenous enzyme but compatible with expression of catalytic activity, the cDNA was inserted into pEVP11 in both orientations and then used to transform *S. cerevisiae* AH_{22} cells.

AH_{22} cells transformed with pUDPGT$_m$-1$_c$ or pUDPGT$_m$-1$_r$, as well as wild type, were monitored for the expression of transferase-specific mRNA and protein. Messenger RNA, expressed only in cells transformed with pUDPGT$_m$-1$_c$, was easily detected (data not shown).

Western Immunoblot Analysis of Transformed Yeast. In order to examine whether the cells synthesize transferase protein, microsomes were isolated from yeast transformed with either of the recombinant plasmids, as well as from the wild-type cells. Only microsomal proteins synthesized in the cells containing pUDPGT$_m$-1c (Figure 2, lane 2) have a transferase-immunoreactive band at approximately $M_r \simeq$ 51 000 by Western analysis. Proteins expressed by the wild type and by pUDPGT$_m$-1$_r$-containing cells (Figure 2, lanes 3 and 1, respectively) do not include this transferase-positive band. All strains contain a nonspecific immunoreactive protein with a molecular mass less than that of transferase. The microsomes isolated from 3-methylcholanthrene-treated mouse (Figure 2, lane 4) show a very broad positive band in the region of $M_r \simeq$ 51 000–54 000, which is due to multiple transferase proteins which react with this antibody preparation (Mac-

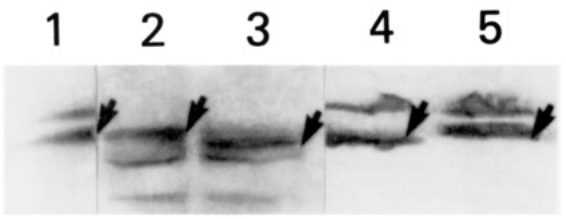

FIGURE 3: Western immunoblot of endoglycosidase H treated microsomes from pUDPGT$_m$-1$_c$-transformed AH_{22} cells and mouse. Microsomes from untreated mouse liver (20 μg per lane) and from AH_{22} cells (300 μg per lane) were solubilized and preadsorbed with carrier-fixed α_2-macroglobulin as described under Methods. The microsomal preparations were as follows: mouse (lanes 1, 4, and 5) and yeast (lanes 2 and 3). The contents of lanes 3 and 4 were treated with 0.1 and 0.01 unit of endoglycosidase H, respectively, as described under Methods. The samples were then electrophoresed and blotted, and color was developed as detailed under Methods.

kenzie et al., 1984). A blot containing microsomes from the same sources [pUDPGT$_m$-1$_r$-transformed, pUDPGT$_m$-1$_c$-transformed, wild-type cells, and mouse (Figure 2, lanes 5, 6, 7, and 8, respectively)] does not show immunoreactivity with preimmune serum.

Previously, careful characterization of the anti-mouse transferase IgG (Mackenzie et al., 1984) raised against a homogeneous protein preparation indicated that the antibody is specific for transferase. In the present study, the molecular mass of the presumed transferase protein ($M_r \simeq 51\,000$) expressed only in yeast transformed with pUDPGT$_m$-1$_c$ is consistent with the value of the antigen (Mackenzie et al., 1984).

In an attempt to clarify the nonspecific immunoreactive band in the wild-type and transformed cells, the antibody preparation was preadsorbed onto a Western blot which contained 3.9 mg of microsomal protein from wild-type cells or a blot without microsomes. Each membrane was blocked as described under Methods. The blot which contained microsomes caused the removal of all transferase-immunoreactive γ-globulin, while the antibody preparation exposed to the blot without microsomes retained transferase immunoreactivity, as well as reactivity against the nonspecific protein. This suggests that the protein in wild-type cells is reacting with the transferase-specific γ-globulin in the preparation. The lack of an immunoreactive component in preimmune serum is consistent with this interpretation.

The expression of membrane-bound transferase activity in the protease-deficient ZA521 strain was assessed after transformation with pAAH5 containing UDPGT$_m$-1 in either orientation. Microsomes isolated from cells having undergone each of the transformations, as well as wild type, were subjected to Western blot analysis as already described. Transferase protein is synthesized only in cells transformed with the plasmid containing UDPGT$_m$-1 in the correct orientation (data not shown). The level of the transferase protein was significantly less than that expressed in the transformed AH_{22} cells (Figure 2, lane 2). The nonspecific immunoreactive microsomal protein in ZA521 was similar to that seen in Figure 5 (lanes 5 and 7).

Evidence for Glycosylation of the Yeast Membrane-Bound Transferase Protein. The deduced amino acid sequence (Kimura & Owens, 1987) shows that the protein has two consensus sequences (residues 316 and 483) for N-linked glycosyl moieties. Endoglycosidase H treatment of solubilized microsomes from pUDPGT$_m$-1c-transformed AH_{22} cells and subsequent analyses by SDS–polyacrylamide gel electrophoresis and Western immunoblot indicate that the membrane-bound transferase is glycosylated. As seen in Figure 3 (lane 3, arrow), the yeast-expressed transferase protein has increased electrophoretic mobility after endoglycosidase H treatment compared to the untreated protein (Figure 3, lane 2, arrow). The more rapidly migrating nonspecific but immunoreactive protein in these cells is not affected by endoglycosidase H treatment. Similarly, the transferase protein $M_r \simeq 51\,000$ in noninduced mouse microsomes shows increased mobility after endoglycosidase H treatment (Figure 3, lane 4, arrow) compared to untreated microsomes (Figure 3, lanes 1 and 5, arrow). The blot also shows that the transferase protein $M_r \simeq 54\,000$ in the mouse microsomes (Figure 3, lane 4, upper band) is not affected by endoglycosidase H treatment when compared to the same band in lanes 1 and 5. At least one form of rat transferase encoded in UDPGT$_r$-3 (Mackenzie, 1987) does not contain a consensus sequence for glycosylation via an asparagine linkage.

Screen for UDP–Glucuronosyltransferase Activity. The family of transferase enzymes conjugates numerous compounds which contain an appropriate substituent group. We chose 12 commonly used substrates representative of 3 different types. Microsomes with specific immunoreactive protein from AH_{22} cells preferentially glucuronidate naphthol and estrone at a rate approximately 2-fold greater than that for the next best aglycon, *p*-nitrophenol (Figure 4, panel A, region of the arrow). A third subset of substrates, testosterone, phenolphthalein, dihydrotestosterone, androsterone, and 4-methylumbelliferone, is conjugated at a rate about half that of *p*-nitrophenol. There is a slight activity toward 3-hydroxy- and 9-hydroxybenzo[*a*]pyrene and no detectable glucuronidation of morphine and lithocholic acid. The relative glucuronidation rates are depicted in Figure 4, panel C.

All the products are sensitive to β-glucuronidase as indicated by the loss of the radioactive areas in Figure 4 (panel B, region of the arrow). This result indicates that the products are β-glucuronides. Further, there was no glucuronide formation by microsomes from pUDPGT$_m$-1$_r$-transformed cells (data not shown).

As naphthol and estrone are the best substrates tested, we attempted to determine whether we could, in fact, detect glucuronidation by the traditional assays described under Methods for these two aglycons. With either 0.05 or 0.5 mg of microsomal protein from pUDPGT$_m$-1$_c$-transformed cells, we observed 4 and 60 ΔF units, respectively, of naphtholylglucuronide according to Bock and White (1979), after a 60-min incubation at 37 °C. These results generated specific activities of 1.33 and 2 ΔF min^{-1} (mg of protein)$^{-1}$. We could not detect estrone glucuronidation in a traditional assay. We could detect, however, borderline activity for *p*-nitrophenol and phenolphthalein with 1 mg of microsomal protein in a 16-h incubation at room temperature. Microsomes from wild-type cells cause no change in fluorescence.

Microsomal transferase activity expressed in the ZA521 strain transformed with pAAH5 containing UDPGT$_m$-1 (in the correct orientation) produced the same relative substrate profile (data not shown) as that shown in Figure 4 (panels A and C) except that the specific activity was about one-third that seen in transformed AH_{22} cells.

Synthesis of Cytosolic UDPGT$_m$-1. The role of the putative N-terminus signal peptide in targeting transferase to the endoplasmic reticulum was examined by cleaving the nucleotide sequence encoding this peptide as described in Figure 1B. The truncated cDNA, inserted into pAAH5 and designated pUDPGT$_m$-1$_c\Delta$, is expressed in the ZA521 strain. It was expected that the removal of the 12 amino acids at the N-terminus would cause the protein to distribute to the cytosol. The use of the strain 2A521 which has decreased protease

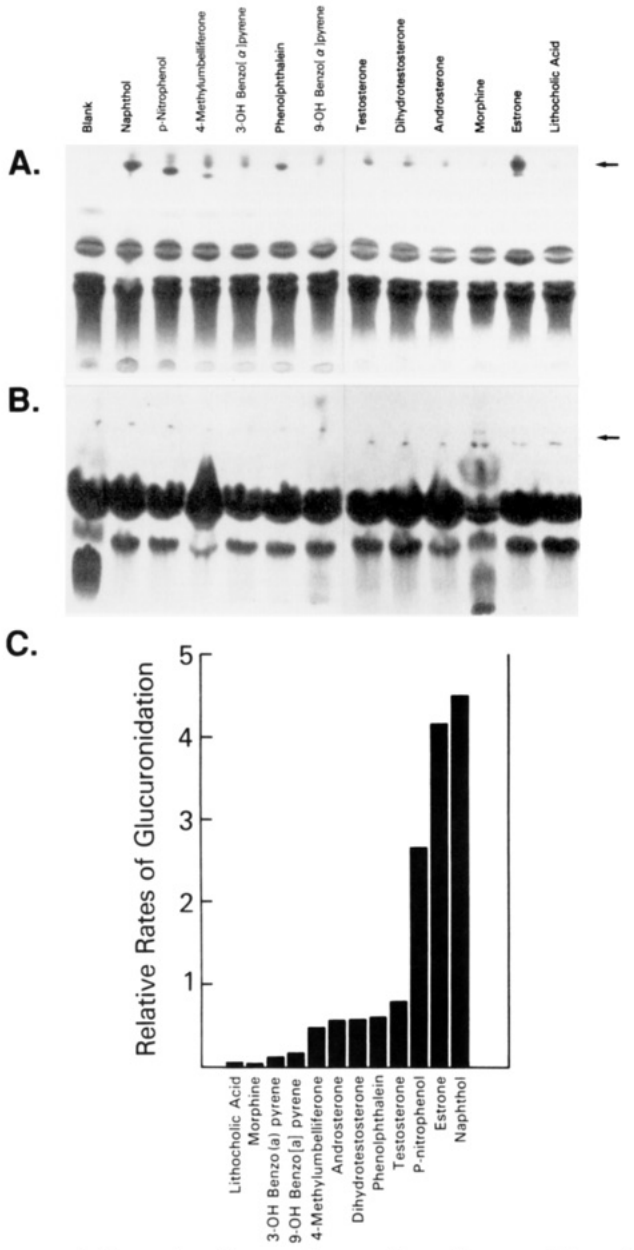

FIGURE 4: Expression of yeast microsomal transferase activity and sensitivity of product to β-glucuronidase. Microsomes were from pUDPGT$_m$-1$_c$-transformed AH$_{22}$ cells. Twelve different transferase substrates (as listed above each lane) were examined for suitability as an aglycon using microsomes (400 μg) from pUDPGT$_m$-1$_c$-transformed AH$_{22}$ cells (panel A, see arrow) according to the procedure detailed under Methods. Half of each reaction mixture represented in panel A was treated with β-glucuronidase (panel B) for 3 h at 37 °C as described under Methods and then applied to the TLC plates and chromatographed the same as in panel A. Relative rates of glucuronidation of 12 different aglycons (panel C). The radioactive areas representing β-glucuronides and the blank as shown in panel A were scraped and counted in liquid scintillation. The counts were corrected for background. Relative rates represent net cpm (×10^{-3}) incorporated into β-glucuronide under identical conditions (described under Methods) for each aglycon.

activity (Dr. V. Mackay, Zymogenetics) should reduce the risk that the soluble cytosolic form of the protein is proteolytically digested.

As shown by the Western immunoblot technique, the transferase protein (with M_r = 51 000–52 000) distributes into the cytoplasm (Figure 5, lane 2) and not the endoplasmic reticulum (Figure 5, lane 6). The wild-type ZA521 cells (Figure 5, lanes 1 and 5) or pUDPGT$_m$-1$_r$Δ-transformed cells (Figure 5, lanes 3 and 7) do not contain the presumed

FIGURE 5: Subcellular distribution of transferase in strain ZA521 *S. cerevisiae* following removal of the signal-peptide coding sequence. Lanes 1–3 represent yeast supernatant protein (300 μg), and lanes 5–7 contain the corresponding microsomal protein (300 μg). Protein samples are from wild-type (lanes 1 and 5), pUDPGT$_m$-1$_c$Δ [truncated insert with correct orientation with respect to the promoter sequence (lanes 2 and 6)], and pUDPGT$_m$-1$_r$Δ [truncated with *r*everse orientation (lanes 3 and 7)]. 3-Methylcholanthrene-treated mouse microsomes (10 μg) are contained in lane 4. Western immunoblot analysis was carried out as described under Methods, and molecular weight markers are the same as described in the legend to Figure 2.

transferase protein band located at $M_r \simeq 51\,000$ in supernatant or microsomes.

A comparison of the mobility in a SDS–polyacrylamide gel of the transferase synthesized from the full-length clone (Figure 2, lane 2) which is located in the microsomes reveals that the molecular mass is similar to the protein synthesized from the truncated cDNA (Figure 5, lane 2). The cytosolic form is predicted, as already described, to contain four to five amino acids at the N-terminus which would not be present in the naturally cleaved membrane-bound form. On the other hand, the membrane-bound form is shown to be glycosylated.

The microsomes from the ZA521 strain of *S. cerevisiae*, like AH$_{22}$, contain one nonspecific immunoreactive protein with the anti-transferase IgG but only in wild-type and pUDPGT$_m$-1$_r$Δ-transformed cells (Figure 5, lanes 5 and 7). The cytosol of these two strains contains two additional nonspecific proteins at $M_r \simeq 60\,000$. It appears that the synthesis of the cytosolic form of transferase in pUDPGT$_m$-1cΔ-transformed cells interferes with the synthesis of these proteins.

We have also seen this interference with the nonspecific protein in microsomes isolated from (a) ZA521 cells transformed with pAAH5 containing UDPGT$_m$-1 in the correct orientation, (b) AH$_{22}$ cells transformed with pAAH5 containing a human transferase cDNA in the correct orientation, and (c) AH$_{22}$ cells transformed with pEVP11 containing the human cDNA clone in the correct orientation. The presence of the nonspecific protein(s) is, therefore, variable.

The observation that the truncated protein distributes in the cytosol supports the claim that the first 12 amino acids at the N-terminus of UDPGT$_m$-1 contain a membrane-targeting signal. Further, it provides evidence that all membrane-bound transferases necessarily contain a targeting signal peptide at the N-terminus as suggested in published reports (Mackenzie, 1986a,b, 1987; Kimura & Owens, 1987; Jackson et al., 1987; Iyanagi et al., 1986).

Upon examination of the cytosol for transferase activity using the same level of immunoreactive protein from pUDPGT$_m$-1$_c$Δ-transformed cells as used with the membrane-located enzyme, we detected similar activity toward

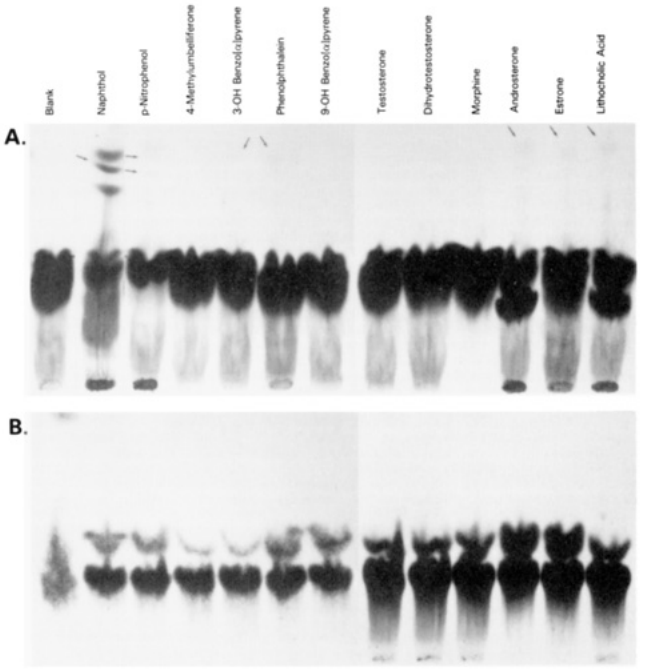

FIGURE 6: Expression of yeast cytosolic transferase activity following removal of signal-peptide coding sequence. The 12 substrates (used for microsomal activity in Figure 3) were examined for glucuronidation by cytosolic transferase following transformation of yeast with pUDPGT$_m$-1$_c\Delta$ (panel A) and with pUDPGT$_m$-1$_r\Delta$ (panel B). Cytosolic protein (450 μg, without activation) was used in each assay as detailed under Methods for microsomes. The arrows indicate the region containing β-glucuronides from the respective substrate.

naphthol and 3-hydroxybenzo[*a*]pyrene, very much lower activity toward *p*-nitrophenol, phenolphthalein, estrone, and androsterone, and detectable activity toward lithocholic acid (Figure 6, panel A, arrows) when compared with membrane-bound activity in AH_{22} cells. There is complete loss of activity toward 4-methylumbelliferone and testosterone. Figure 6 (panel B) represents the results with cytosol from pUDPGT$_m$-1$_r\Delta$-transformed cells.

The observation that three bands (Figure 6A, lane 2) of product appear with naphthol conjugation using the cytosolic form of transferase expressed in ZA521 is consistent with the formation of three bands when the same assay is carried out with mouse microsomes as control studies (data not shown) to verify the authenticity of each glucuronide. It is not known why one prominent band with evidence of two faint bands forms when membrane-bound transferase in AH_{22} cells was assayed (Figure 4). The *S. cerevisiae* strains, ZA521 and AH_{22}, differ at least by the deletion of certain protease genes in ZA521 (Ammerer et al., 1986) which should enhance the stability of soluble, cytosolic transferase.

The relative profile of substrate preference of the membrane-bound form expressed in either AH_2 or ZA521 cells does not appear to differ; however, the specific activity for each substrate is significantly less when expressed in ZA521.

The lack of an effect of the cytosolic location of the transferase on the metabolism of naphthol and 3-hydroxybenzo[*a*]pyrene and the marked reduction in activity for most of the other substrates signal several possibilities. Overall, the location of the enzyme in the membrane appears to enhance and broaden substrate activity. The kinetic restraint that intact microsomes have on the catalytic activity of transferase measured in vitro is well-known. Although the restraint appears to slow the rate of catalysis requiring an activation step for full expression of activity, the importance of the membranous environment may be to impose and stabilize the secondary structure at the active site via the interaction of the enzyme with structural protein(s) and/or the interactions of enzyme with phospholipids. The distribution of the enzyme to the cytosolic compartment could, therefore, destabilize secondary structure and cause a collapse in the conjugating capacity.

Glycosylation of the protein via asparagine linkage which is associated with the membrane is another factor which may contribute to more extensive glucuronidation by the enzyme. Since activity toward the preferred substrate naphthol is hardly changed, it can be said that there is no strict requirement for the membrane location of transferase.

UDPGT$_m$-1 was initially isolated on the basis of its hybridization to the rat UDPGT$_r$-3 clone (Mackenzie, 1987). The nucleotide and deduced amino acid sequences for UDPGT$_m$-1 are shown to be approximately 87% similar to UDPGT$_r$-3. Although the two clones have a high degree of similarity, UDPGT$_r$-3 (lacking a possible asaparagine-linked glycosyl moiety) preferentially glucuronides testosterone, dihydrotestosterone, and β-estradiol, and it does not catalyze estrone, androsterone, and type I substrates (such as naphthol, *p*-nitrophenol, and 4-methylumbelliferone). These two transferases appear to have maintained a core catalytic activity involving endogenous substrates, but evolved in the case of the mouse form, to include exogenous chemicals. The transferase glucuronidates at least three type I (3-methylcholanthrene-inducible substrate activities), one type II (phenobarbital-inducible substrate activity), and four steroid aglycons. The results of this study suggest that the types of substrates may not be distinctly categorized on the basis of enzyme inducibility but that some common property of the chemical compounds (not yet considered) is more important to the affinity of a particular enzyme for aglycons.

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Registry No. UDP-glucuronosyltransferase, 9030-08-4; naphthol, 1321-67-1; estrone, 53-16-7; *p*-nitrophenol, 100-02-7; testosterone, 58-22-0; phenolphthalein, 77-09-8; dihydrotestosterone, 521-18-6; androsterone, 53-41-8; 4-methylumbelliferone, 90-33-5; 3-hydroxybenzo[*a*]pyrene, 13345-21-6; lithocholic acid, 434-13-9; 9-hydroxybenzo[*a*]pyrene, 17573-21-6.

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