Characterization of a Cloned Human Dihydrotestosterone/Androstanediol UDP-Glucuronosyltransferase and Its Comparison to Other Steroid Isoforms‡

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ABSTRACT: A human cDNA, UDPGTh-3, encoding a dihydrotestosterone/5α-androstane-3α,17β-diol UDP-glucuronosyltransferase (transferase) has been isolated and characterized. The nucleotide sequence of UDPGTh-3 encodes a 530 amino acid protein with a typical membrane insertion–signal peptide, a membrane-anchoring domain, and three potential asparagine-linked glycosylation sites. Alignment shows that this encoded isozyme is 96% identical to an apparent estriol-metabolizing isoform, HLUG4 [Coffman, B. L., *et al.*, (1990) *Arch. Biochem. Biophys. 281*, 170–175]. The udpgth-3 isozyme is 78% identical to two other steroid isoforms, HLUG25 (udpgth-1) [Jackson, M. R., *et al.* (1987) *Biochem. J. 242*, 581–588; Ritter, J. K., *et al.* (1992) *Biochemistry 31*, 3409–3414] and udpgth-2 [Ritter, J. K., *et al.* (1990) *J. Biol. Chem. 265*, 7900–7906]. udpgth-2 and udpgth-1 metabolized parallel substrates (stereospecific estriols, 3,4-catechol estrogens, and the bile salt hyodeoxycholate), except that udpgth-2 was 100-fold more effective than udpgth-1. The mRNA encoding udpgth-3 is 2.4 kb in size and is present in liver, prostate, and testis; the mRNA encoding udpgth-2 is located in liver and kidney, whereas that for udpgth-1 is liver-specific. Each of the liver mRNA species encoding udpgth-3, udpgth-2, or udpgth-1 was induced 2.5–3-fold by phenobarbital treatment of the *Erythrocebus patas* monkey. In 16 human liver mRNA samples, the message encoding udpgth-3 was generally uniformly expressed and that for udpgth-1 exhibited wide variations in its level, whereas that for udpgth-2 was barely detectable in nine samples and not detectable in the others. Three samples contained no message for either isoform. Substrate turnover by udpgth-3 is ranked as follows: phenolphthalein > 5α-androstane-3α,17β-diol > 5α-dihydrotestosterone = 4-hydroxybiphenyl > phenolsulfonphthalein (phenol red) > phenolphthalin. Genes encoding udpgth-3, udpgth-2, and udpgth-1 mapped to human chromosome 4 with genomic DNA from human/mouse and human/hamster somatic cell hybrids; the genes encoding udpgth-1 and udpgth-2 mapped specifically to band 4q28. udpgth-3 exhibited similar K_m values both for 5α-dihydrotestosterone (10 μM) and for its metabolite, 5α-androstane-3α,-17β-diol (12.5 μM). Although the role of glucuronidation in the regulation of 5α-dihydrotestosterone levels is not known, the location of the message for this isoform in target tissues, testis and prostate, indicates that the isoform is, most likely, important in the control of hormonal levels and, thus, in 5α-dihydrotestosterone action. Furthermore, a critical role for udpgth-3 is suggested in light of the absence of its messenger RNA but the presence of that for four other transferase isoforms examined in the liver of a patient with benign prostate hyperplasia, a condition associated with depressed glucuronidation of dihydrotestosterone.

5α-Dihydrotestosterone (DHT) has been shown to be the active androgen in both rats and humans (Cunha *et al.*, 1987) for the development of external male genitalia, the prostate, and facial and body hair. DHT is, therefore, responsible for critical external virilization processes and for internal androgen-dependent reactions of the prostate. Serious virilization syndromes (varying degrees of pseudovaginal perineoscrotal hypospadias), as well as mild syndromes (female hirsutism, lack of acne in males), reflect abnormal levels and metabolism of DHT (Wilson, 1985). This androgen is formed in the prostate *via* the DHT-dependent 5α-reductase conversion of testosterone, which is synthesized and secreted by the testis. The DHT dependency of the reductase has the effect of high androgen levels, amplifying androgen action (Mowszowicz *et al.*, 1984).

In an attempt to account for androgen action, many studies have compared the level of DHT with hormonal action in the prostate. The results have been complicated by the apparent rapid 3α-reduction of DHT producing 5α-androstane-3α,-17β-diol, which is often present as a glucuronide (Horton *et al.*, 1982). As a consequence, the glucuronide of the metabolite is considered an indirect measure of the active androgen in hirsute women and normal men (Lobo *et al.*, 1983). Other reports (Vermeulen *et al.*, 1991) have shown, however, that women with and with out virilism can have elevated serum levels of 5α-androstane-3α,17β-diol glucuronide; the adrenal gland in women is the major source of the precursors, androstenedione and dehydroepiandrosterone, for forming the diol. It is believed that the fraction of the diol derived from DHT is related to virilization in women (Vermeulen *et al.*, 1991).

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Reports concerning tissues involved in the glucuronidation of DHT have also generated conflicting results. The formation of the androstanediol glucuronide is reportedly low in human genital skin and rat prostate and does not take place in human abdominal skin or human prostate (Rittmaster *et al.*, 1989). Earlier studies in normal men have indicated that androstanediol glucuronide formation occurs primarily in peripheral tissues with very little hepatic synthesis (Morimoto *et al.*, 1981). In rats, the prostate selectively generated androstanediol 17-glucuronide from DHT and essentially no DHT glucuronide; the liver generated both androstanediol 3-glucuronide and DHT glucuronide (Rittmaster *et al.*, 1988). It is also reported that the rat liver is 800 times more active for androstanediol glucuronidation than is the rat prostate (Rittmaster *et al.*, 1989). These results suggest that DHT can either undergo conversion to androstanediol and glucuronidation in the rat prostate or undergo secretion by prostate and uptake by the liver for conversion and glucuronidation. Studies have, therefore, generated conflicting results with respect to glucuronidation of this androgen and its diol metabolite.

Although studies in humans (Rittmaster *et al.*, 1989) indicate that the liver is a major site for the glucuronidation of both DHT and androstanediol, similar to the predominant site for glucuronidation in rats, it is possible that glucuronidation by the prostate is important for exquisite regulation of DHT in the target tissue and, thus, normal hormonal effects. In this study, we demonstrate that udpgth-3 selectively glucuronidates a single category of endogenous compounds, DHT and 5α-androstane-3α,17β-diol, with similar affinities, as measured by K_m values. The demonstration that the isozyme is located in liver, prostate, and testis indicates that it, most likely, plays a critical role in regulating the level of the potent androgen, DHT, in target tissue.

MATERIALS AND METHODS

Materials. UDP-glucuronic acid and all aglycons tested for substrate activity were obtained from Sigma Chemical Co. (St. Louis, MO), Fluka Chemical Corp. (Ronkonkoma, NY), or Aldrich Chemical Co. (Milwaukee, WI). [^{14}C]UDP-glucuronic acid was from Du Pont–New England (Boston, MA), [α-^{32}P]deoxycytidine triphosphate was from Amersham (Arlington Heights, IL), and [^{35}S]methionine was from ICN Biomedicals (Costa Mesa, CA). Restriction enzymes and other reagents used in molecular biology techniques were from New England Biolabs (Beverly, MA), Pharmacia LKB Biotechnology Inc. (Piscataway, NJ), IBI Biochemicals (New Haven, CT), Bethesda Research Laboratories (Bethesda, MD), or Boehringer Mannheim (Indianapolis, IN). The pSVL vector and the oligo-labeling kit were from Pharmacia, and COS-1 cells were from the American Type Culture Collection (Rockville, MD). The Bluescript plasmids and XL-1 Blue cells were from Stratagene (La Jolla, CA). Poly(A+)RNA from human prostate and testis was from Clontech (Palo Alto, CA). The Erase-a-Base kit was from Promega (Madison, WI). Tissue culture supplies were from Mediatech (Washington, DC) or Biofluids (Rockville, MD).

Nucleotide Sequencing of the UDPGTh-3 cDNA Clone. The clone, UDPGTh-3, was isolated from a human liver λgt11 cDNA library [supplied by Dr. Anil Jaiswal (Fox Chase Cancer Center, Philadelphia, PA)] by hybridization to the 3′ 700-bp *Eco*RI fragment of UDPGTh-2 (Ritter *et al.*, 1990), which was radiolabeled by priming with random hexamers in the presence of [α-^{32}P]deoxycytidine triphosphate as part of a kit protocol. Some 14 candidate clones were isolated. The entire UDPGTh-3 cDNA was sequenced in both directions by the direct plasmid dideoxy protocol, as previously described (Ritter *et al.*, 1990), using the Bluescript vector.

Isolation of RNA from Different Human Tissues. Total RNA was isolated from normal human liver, lung, kidney, brain (cerebral cortex), and skin (leg) and from liver biopsies of both untreated and phenobarbital-treated *Erythrocebus patas* monkey according to the guanidinium isothiocyanate method of Chirgwin *et al.* (1979). The treated monkey was maintained on drinking water which contained phenobarbital (15 mg %). Both human and monkey liver mRNA was affinity purified by chromatography through oligo(dT)–cellulose (Collaborative Research, Inc.) with an intermediate heat step. RNA was isolated from the normal tissue surrounding a diseased specimen taken from surgical patients 1, 2, and 8 represented in Figure 9.

Northern Blot Analysis of the mRNA Species Encoding UDPGTh-3, UDPGTh-2, and *UDPGTh-1.* The Northern blot technique was used to carry out four different studies as follows: (A) a filter containing human total RNA (20 μg) from liver, lung, kidney, skin, and brain (cerebral cortex) or poly(A+)RNA (5 μg) from human testis and prostate for the determination of the tissue distribution of the mRNA encoding UDPGTh-3; (B) a triplicate set of filters, each containing 2.0 μg of human liver mRNA, to study the relative size and abundance of the mRNAs encoding UDPGTh-1, UDPGTh-2, and UDPGTh-3; (C) a triplicate set of filters, each containing 2.0 μg of liver mRNA isolated from an untreated and from a phenobarbital-treated monkey, to determine whether this agent induces steroid transferase synthesis; and, finally, (D) a triplicate set of filters containing 20 μg of total RNA isolated from 16 different human liver specimens to assess the fluctuations in each of the steroid transferase mRNAs. In each case, the RNA was electrophoresed through a 1.0% agarose gel containing 2.2 M formaldehyde and transferred to a Zetabind membrane (AMF-CUNO, Meriden, CT) according to the manufacturer's instructions. Specific probes were radiolabeled by the random hexamer primer technique in the presence of [α-^{32}P]dCTP and one of the following DNA fragments: 5′ 412-bp *Eco*RI/*Acc*I fragment of UDPGTh-1 (Ritter *et al.*, 1992a); 3′ 700-bp *Eco*RI fragment of UDPGTh-2 (Ritter *et al.*, 1990); 5′ 667-bp *Nde*I fragment of UDPGTh-3; a 660-bp fragment of the human cyclophilin cDNA (McKinnon *et al.*, 1987); or the human β-actin cDNA. Northern blots were hybridized according to the method of Church and Gilbert (1984). Results of the hybridizations with either the cyclophilin or β-actin probe were used to normalize, as indicated, for the amounts of RNA applied to the gel and, ultimately, to the filters. In the case of the prostate and testis, β-actin, instead of cyclophilin, was used for the normalization since both of these tissues contain 1–2 orders of magnitude more cyclophilin than the liver. Each filter was exposed to X-ray film in order to develop and print radiographs.

Expression of the UDP-Glucuronosyltransferase Encoded by pUDPGTh-3 in COS-1 Cells. UDPGTh-3 was subcloned into the pSVL expression plasmid, with the sense strand downstream of the promoter element, to yield the unit pUDPGTh-3. The recombinant unit was transfected into COS-1 cells as already described (Ritter *et al.*, 1990). Synthesis of the encoded transferase protein was assessed 68 h after the transfection with incubation in regular medium, 1 h in methionine-free medium, and finally 4 h in [^{35}S]-methionine (100 μCi/mL), as previously described (Ritter *et*

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UDPGTh-3  MSLKWTSVFL LIQLSCYFSS GSCGKVLVWP TEYSHWINMK TILEELVQRG HEVTVLTSSA 60
HLUG4     .......                                                       53

UDPGTh-3  STLVNASKSS AIKLEVYPTS LTKNDLEDSL LKILDRWIYG VSKNTFWSYF SQLQELCWEY 120
HLUG4                             y                                   a  113

UDPGTh-3  YDYSNKLCKD AVLNKKLMMK LQESKFDVIL ADALNPCGEL LAELFNIPFL YSLRFSVGYT 180
HLUG4                               r    p g pvf       s  l    v  rs i r   173

UDPGTh-3  FEKNGGGFLF PPSYVPVVMS ELSDQMIFME RIKNMIHMLY FDFWFQIYDL KKWDQFYSEV 240
HLUG4     i                       i                                       233

UDPGTh-3  LGRPTTLFET MGKAEMWLIR TYWDFEFPRP FLPNVDFVGG LHCKPAKPLP KEMEEFVQSS 300
HLUG4                                                           l         293

UDPGTh-3  GENGIVVFSL GSMISNMSEE SANMIASALA QIPQKVLWRF DGKKPNTLGS NTRLYKWLPQ 360
HLUG4                                                                     353

UDPGTh-3  NDLLGHPKTK AFITHGGTNG IYEAIYHGIP MVGIPLFADQ HDNIAHMKAK GAALSVDIRT 420
HLUG4                                           d                         413

UDPGTh-3  MSSRDLLNAL KSVINDPVYK ENVMKLSRIH HDQPMKPLDR AVFWIEFVMR HKGAKHLRVA 480
HLUG4                             a                                       473

UDPGTh-3  AHNLTWIQYH SLDVIAFLLA CVATVIFIIT KFCLFCFRKL AKTGKKKKRD 530
HLUG4                         w                     k           523
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FIGURE 1: Deduced amino acid sequences of UDPGTh-3 and alignment with that of HLUG4. The nucleotide sequence was determined by the dideoxy nucleotide method of Sanger et al. (1977) as described in Materials and Methods. The deduced amino acid data are based on the open reading frame of that sequence as determined by the IBI Pustell program. The dots at the amino terminus represent the missing amino acid residues in the HLUG4 clone. The dashed lines above and the solid line below udpgth-3 represent the membrane insertion–signal peptide and the membrane-anchoring domain, respectively.

al., 1990). For comparison, the expression of the pUDPGTh-2 (Ritter *et al.*, 1990) recombinant plasmid was determined simultaneously. The [^{35}S]methionine-labeled transferases were solubilized, immunocomplexed with anti-mouse transferase IgG, and detergent-washed as already described (Mackenzie *et al.*, 1984). Immunocomplexes were detached and electrophoresed through a 7.5% polyacrylamide gel and processed for autoradiography as indicated (Ritter *et al.*, 1990).

Assay for Glucuronidation by the UDPGTh-3-Encoded UDP-Glucuronosyltransferase Using [^{14}C]UDP-Glucuronic Acid. After transfection with pUDPGTh-3 and incubation for 48 h, COS-1 cell homogenates were analyzed for glucuronidating activity by utilizing a modification of a published procedure (Bansal *et al.*, 1980; Ritter *et al.*, 1990) and testing some 80 different aglycons. The list of 75 potential substrates tested is already reported (Ritter *et al.*, 1990). In addition, the compounds listed in Figure 5, phenohydantoin, and 5α-androstane-3β,17β-diol were analyzed for glucuronidation with the transfected cell homogenate. Each reaction (0.075 mL) contained 10 or 100 μM aglycon, 50 μM [^{14}C]UDP-glucuronic acid (2.5 μCi/μmol), 50 mM Tris-HCl (pH 7.7), 4 mM $MgCl_2$, 1.0 mM phenylmethanesulfonyl fluoride, and 1.0 μg/mL leupeptin. The reaction mixtures were incubated for 16 h at room temperature and analyzed by thin-layer chromatography (TLC). The determinations of K_m values for DHT and 5α-androstane-3α,17β-diol were carried out with 0.2 mM [^{14}C]-UDP-glucuronic acid (5.0 μCi/μmol) and incubated for 3 h at 37 °C. The amount of product was quantified by scanning the plates on an Ambis radioanalytical system Mark II (Ambis, San Diego, CA).

Chromosomal Localization of the Genes Encoding UDPGTh-1, UDPGTh-2, and UDPGTh-3 by Southern Blot Analysis of Human/Rodent Cell Hybrids. A panel of human–hamster somatic cell hybrid clones (27 primary clones and 14 subclones) and human–mouse hybrids (14 primary clones and 40 subclones) has been described (McBride *et al.*, 1982). Genomic DNA from individual cell hybrid clones was digested with *Eco*RI and analyzed by Southern blot hybridization with ^{32}P-radiolabeled cDNA probes under stringent conditions (McBride *et al.*, 1989), with final washes in 0.1× SSC/0.2% SDS at 55 or 65 °C allowing for about 10% or 0% divergency, respectively. Subfragments of the UDPGTh-1 cDNA used as probes included a 5′ 412-bp *Eco*RI/*Acc*I fragment, the 831-bp *Acc*I/*Eco*RI middle coding region, and the 3′ 714-bp *Eco*RI/*Eco*RI fragment. A 1869-bp full-length UDPGTh-2 and a 5′ 667-bp *Nde*I fragment of UDPGTh-3 were also used as probes. All probes were labeled with [α^{32}P]dCTP by random hexamer primed synthesis.

In Situ Hybridization. Metaphase spreads were prepared using peripheral blood lymphocytes from a normal male after BrdUrd synchronization as previously described (Gnarra *et al*, 1990). Chromosomal DNA was processed as published (Singh *et al.*, 1977) prior to hybridization with tritium-labeled probes (Harper & Saunders, 1981). Following autoradiography, the spreads were treated with 0.25% Wright stain diluted in phosphate buffer (pH 6.8) and photographed. The slides were destained, stained with Hoecht 33258 (150 μg/mL) in deionized water, and taken through a standard process to generate banding. Metaphases were rephotographed after restaining in Wright stain (Bhatt *et al.*, 1988). The probes for *in situ* hybridization were full-length cDNAs of UDPGTh-1 and UDPGTh-2 in the Bluescript SK plasmid and were labeled by nick-translation in the presence of both [^{3}H]-dTTP and [^{3}H]dCTP. The specific activity for each probe was (1–3) × 10^7 CPM/μg of DNA. Standard wash conditions for *in situ* hybridization were used.

RESULTS AND DISCUSSION

Isolation of the Human Liver Clone, UDPGTh-3. The 2092-bp cDNA clone was isolated by hybridization at intermediate stringency to the 3′ *Eco*RI fragment of UDPGTh-2 (Ritter *et al.*, 1990). The clone was sequenced by using a series of nested overlapping deleted subclones as already described (Ritter *et al.*, 1990). The salient features of UDPGTh-3 are a 1590-bp open reading frame, a 17-bp 5′ untranslated (UT) region, and a 485-bp 3′ UT region which contains a LINE1 repetitive sequence. The deduced 530 amino acid sequence encoded by UDPGTh-3 and its comparison to a very similar steroid UDP-glucuronosyltransferase isozyme

Table I: Comparison of Deduced Amino Acid Sequence Encoded by UDPGTh-3 to That of UDPGTh-2 and UDPGTh-1

	UDPGTh-3							
	amino-terminal residue differences				carboxyl-terminal residue differences			
	1–100	101–200	201–300	% identity	301–400	401–531	% identity	overall identity
UDPGTh-2	34	29	18	73	11	24	85	78
UDPGTh-1	31	29	17	74	13	21	85	79

(Coffman *et al.*, 1990) are shown in Figure 1. The deduced structure of udpgth-3 contains a typical hydrophobic membrane insertion–signal peptide bracketed by both a positive lysine residue and an α-helix-breaking residue (dashed line above sequence), a membrane-anchoring domain consisting of a hydrophobic sequence bracketed by charged residues (solid underline), and three potential asparagine-linked (NXS/T) glycosylation sites at amino acid residues 65, 316, and 483.

Amino acid alignment of udpgth-3 with other cDNA-encoded UDP-glucuronosyltransferase isozymes shows that it is highly homologous (96%) to the human liver HLUG4 encoded-transferase (Coffman *et al.*, 1990). Figure 1 shows that the first 84 amino acid residues are identical and that 13 of 22 differences occur between residues 145 and 177. Further, there are other long stretches of identity between the two molecules. The alignment also indicates that HLUG4 is missing the seven amino-terminal codons (Figure 1, dots). The HLUG4 cDNA is thought to encode an estriol-specific isoform because of its deduced amino acid sequence identity (between residues 17 and 41) with that of the amino-terminal portion of a purified estriol transferase (Coffman *et al.*, 1990). Although the amino acid data for UDPGTh-3 and HLUG4 are identical between 17 and 41, UDPGTh-3 does not encode the purified estriol isoform (Coffman *et al.*, 1990; see below) since pUDPGTh-3-transfected COS-1 cell homogenates lacked estriol glucuronidating activity. HLUG4 may, therefore, encode the estriol isoform even though complete sequence comparisons between the purified and the HLUG4-encoded isoforms were not possible. The amino acid differences between 145 and 177, most likely, account for the differences in substrate specificity between the UDPGTh-3- and HLUG4-encoded isoforms. The high level of homology between UDPGTh-3 and HLUG4 points out (a) the complications of predicting substrate specificity of UDP-glucuronosyltransferases on the basis of partial nucleotide or peptide sequence data and (b) the structural basis for the copurification of isoforms often seen in purification studies.

The protein, udpgth-3, is more distantly related to two other human transferase isoforms, udpgth-1 and udpgth-2, which exhibited low and high turnover rates, respectively (Ritter *et al.*, 1992a), for the glucuronidation of the same three categories of substrates. Those compounds are stereospecific estriols, 3,4-catechol estrogens, and the bile salt hyodeoxycholate. udpgth-3 is 78% identical to udpgth-1 and udpgth-2 overall (Table I) with differences of 74 and 73% in the first 300 amino acids, respectively. A consideration of 100-residue segments from the amino termini of udpgth-1/udpgth-2 shows a distribution of 31/34, 29/29, and 17/18 differences for the first 300 residues. Both the unique 290 amino-terminal residues of the six isoforms encoded by the *UGT1* locus (Ritter *et al.*, 1992b) and a chimeric study (Mackenzie, 1990b) with two different isoforms demonstrate that the 298 amino-terminal residues determine the substrate specificities of transferase isoforms. Further, udpgth-3, like many previously reported studies on transferases, is more conserved in the carboxyl-terminal region with respect to other isoforms; it is 98% identical (Figure 1) to HLUG4 and 85% identical (Table I) to both udpgt-1 and udpgt-2 in the 230 amino acids of the carboxyl-terminal regions. It is interesting to note that udpgth-1 and udpgth-2 differ by some 55 amino acid residues of the first 300 but appear to have the same substrate specificity (Ritter *et al.*, 1992a), whereas udpgth-3 and the HLUG4-encoded isoforms differ in this region by only 18 residues (Figure 1) and apparently have different substrate specificities. These considerations provide additional evidence that a few amino acid residues can determine substrate specificity. This is noted in two human bilirubin isoforms which are 66% different in the amino-terminal regions (Ritter *et al.*, 1991). These results are, therefore, consistent with a few changes or even a single amino acid change (polymorphism) caused by a few point mutations or a single mutation determining substrate selection; in effect, a simple mechanism may explain the complement of transferase isozymes seen in human microsomes. A single amino acid difference accounts for two different substrate activities in the case of two highly related mouse cytochrome P-450 monooxygenases (Iwasaki *et al.*, 1991).

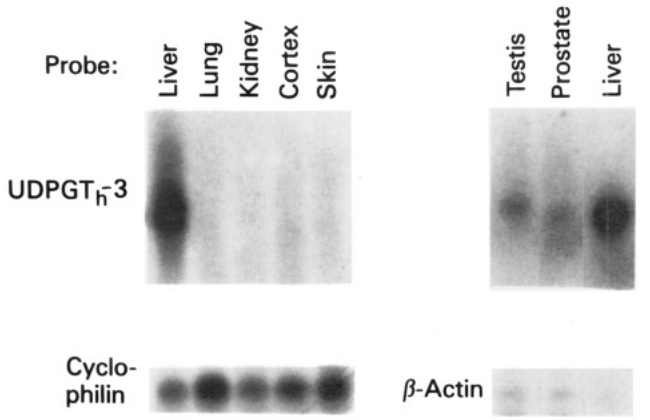

FIGURE 2: Tissue distribution of mRNA encoding UDPGTh-3. Total RNA isolated from human liver, lung, kidney, brain (cerebral cortex), and skin (leg) and poly(A+)RNA from prostate and testis were analyzed by Northern blot analysis as described in Materials and Methods. First, the UDPGTh-3-specific probe, the 5′ 667-bp *Nde*I fragment, was radiolabeled and hybridized to the Northern filter; subsequently, a 660-bp fragment of either the human cyclophilin or human β-actin cDNA was hybridized according to Materials and Methods. The normalization for the amount of RNA applied to the filter was made by using the results of either the cyclophilin or β-actin probe.

Tissue Distribution of the Messenger RNA Encoding UDPGTh-3. Initial Northern blot analysis showed that the message coding for UDPGTh-3 is present in liver and not in lung, kidney, brain (cerebral cortex), or skin (leg) (Figure 2). By comparison the message for UDPGTh-2 is present in liver and kidney (Ritter *et al.*, 1992a), and that for UDPGTh-1 is liver-specific (Ritter *et al.*, 1992a). After substrate activity determinations showed that the UDPGTh-3-encoded isoform is active toward DHT and its major metabolite (see Figure 5 below), the analysis was extended to include testis and prostate, two major DHT target tissues. Both of these tissues express UDPGTh-3 mRNA (Figure 2) although at levels 1–2 orders of magnitude lower than liver. This is in agreement with published biochemical data (Rittmaster *et al.*, 1989) showing that the liver is by far the organ with the greatest capacity for glucuronidating these endogenous substrates. The distribution of the message in prostate indicates a possible direct role of this isoform in controlling DHT action in target tissue.

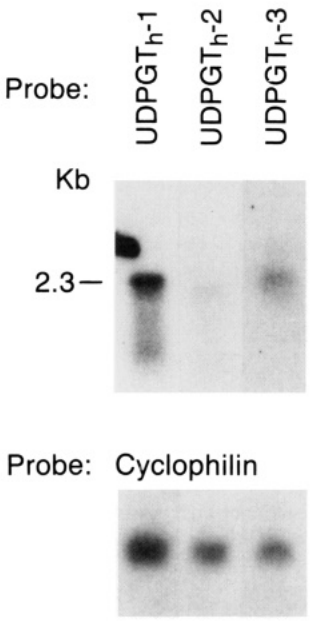

FIGURE 3: Relative abundance in liver of the mRNAs encoding the three steroid isozymes, udpgth-1, udpgth-2, and udpgth-3. Triplicate Northern filters were made as described above using human liver mRNA, prepared as described in Materials and Methods. A filter was hybridized to either the UDPGTh-1-specific 5′ *Eco*RI/*Acc*I 412-bp fragment, the UDPGTh-2-specific 3′ *Eco*RI 700-bp fragment, or the UDPGTh-3-specific 5′ *Nde*I 667-bp fragment. The fragments were radiolabeled, hybridized, and normalized for RNA content as described in Materials and Methods and the legend for Figure 2.

Comparisons of Size and Relative Abundance of the mRNA Species Encoding UDPGTh-1, UDPGTh-2, and UDPGTh-3 in Liver. In an attempt to establish the relationship between three different members of the steroid-metabolizing family of the transferase superfamily, UDPGTh-3 was compared to the previously described UDPGTh-1- and UDPGTh-2-encoded isoforms (Ritter *et al.*, 1992a) with respect to several parameters. Messenger RNA was isolated from normal human liver, electrophoresed, and analyzed by hybridization to UDPGTh-3-, UDPGTh-2-, and UDPGTh-1-specific probes as described in Materials and Methods. According to the Northern blot analysis (Figure 3), the mRNA coding for UDPGTh-3 (2.4 kb) is slightly larger in size than either the UDPGTh-1 (2.3 kb) or UDPGTh-2 (2.0 kb) mRNA. Although the open reading frames for the three messages specify peptides between 528 and 530 amino acids, the 3′ UT regions are 230, 469, and 485 bp for UDPGTh-2, UDPGTh-1, and UDPGTh-3, respectively. The UDPGTh-1 is also encoded by two other message versions of sizes 3.6 and 5.6 kb (data not shown) which are detectable when Northern blots are sufficiently overexposed. The differences among the UDPGTh-1 messenger RNA species are due to 3′ UT extensions, with the 2.3-kb version by far the most abundant followed by the 3.6-kb species. The relative abundance in liver of the mRNAs coding for UDPGTh-1, UDPGTh-3, and UDPGTh-2 is 2.4:1.3:1, respectively.

Immunocomplexes of UDP-Glucuronosyltransferases following Transfection of either the pUDPGTh-3- or the pUDPGTh-2 Unit into COS-1 Cells and with [^{35}S]Methionine Labeling. To establish whether UDPGTh-3 encodes a transferase protein following transfection of pUDPGT-3 into COS-1 cells, [^{35}S]methionine-labeled and solubilized cells were immunocomplexed with goat anti-mouse transferase IgG (Mackenzie *et al.*, 1984; Ritter *et al.*, 1990) as described in Materials and Methods and as shown in Figure 4. In order to compare the molecular weights of udpgth-3 and udpgth-2,

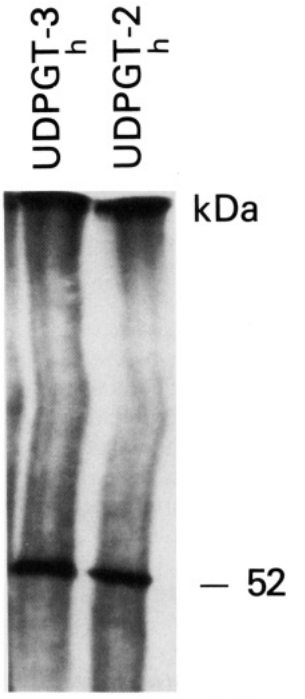

FIGURE 4: Immunocomplexes of pUDPGTh-3- and pUDPGTh-2-transfected COS-1 cells following [^{35}S]methionine labeling. Cells were transfected with pUDPGTh-3 or pUDPGTh-2, radiolabeled with [^{35}S]methionine, and immunocomplexed with anti-mouse transferase IgG. The labeled isozymes were analyzed by SDS–polyacrylamide gel electrophoresis as described in Materials and Methods. The pUDPGTh-2-encoded protein, previously demonstrated (Ritter et al., 1990) to have a molecular mass of 52 kDa when expressed in these cells, was immunocomplexed for comparison.

the pUDPGTh-2 expression unit was subjected to the same analysis. In Figure 4, it is shown that udpgth-3 has a molecular mass similar to that of udpgth-2, which was previously shown to be 52 kDa (Ritter *et al.*, 1990). Both encoded proteins have potentially three glycosylation sites, and it was demonstrated that udpgth-2 is glycosylated during expression in COS-1 cells (Ritter *et al.*, 1990). Previously, it was shown that udpgth-1, which contains one potential glycosylation site, has approximately a 3-kDa lower molecular mass than that of udpgth-2 (Ritter *et al.*, 1992a). It is predicted (Watson, 1984) that, after signal peptide cleavage, the mature proteins, udpgth-3 and udpgth-2, would contain 514 and 512 amino acids, respectively.

Determination of Substrate Specificity of the UDPGTh-3-Encoded Isozyme. COS-1 cells were analyzed for aglycon specificity 48 h after transfection with pUDPGTh-3 according to details outlined in Materials and Methods. The compounds tested for glucuronide formation by the transfected cell homogenate included the 75 chemicals previously tested as aglycons for udpgth-2 (Ritter *et al.*, 1990; Table I), the compounds listed in Materials and Methods (pertaining to the assay), and those included in Figure 5. The substrate preference of the udpgth-3 isoform was shown to be phenolphthalein > 5α-androstane-3α,17β-diol > 4-hydroxybiphenyl = 5α-dihydrotestosterone > phenolsulfonphthalein (phenol red) > phenolphthalin. The amounts of product formed are given in the legend to Figure 5. It should be pointed out that udpgth-2 compared to udpgth-1 is 100-fold more active for parallel substrates, stereospecific estriols, 3,4-catechol estrogens, and the bile salt hyodeoxycholate (Ritter *et al.*, 1990). With hyodeoxycholate as the best substrate for each, the relative rates of catalysis for udpgth-2 and udpgth-1 were 11 and 0.15 pmol of glucuronide formed/μg of protein/16 h, respectively. In this study, we used an identical expression

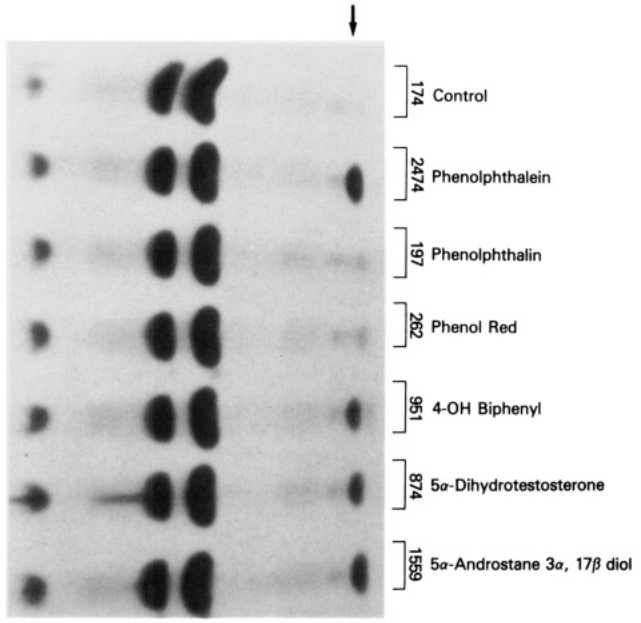

FIGURE 5: Substrate specificity of the UDPGTh-3-encoded UDP-glucuronosyltransferase following expression in COS-1 cells. pUDPGTh-3-transfected cells were assayed for the production of glucuronides (arrow) using 100 μM aglycon and 50 μM [^{14}C]UDP-glucuronic acid, as described in Materials and Methods. Reactions to generate glucuronide were incubated for 16 h at room temperature and then analyzed by TLC chromatography; the plate was scanned as described in Materials and Methods and exposed to X-ray film for print. The aglycons and the radioactivity (in CPM) incorporated into glucuronides are recorded at the top of the chromatogram. The specific activities (pmol of glucuronide/μg of protein/16 h) for the products formed are as follows: phenolphthalein, 3.20; phenolphthalin, 0.03; 4-hydroxybiphenyl, 1.08; phenol red, 0.12; 5α-dihydroxytestosterone, 0.98; 5α-androstane-3α,17β-diol, 1.93. CPMs in the control assay were subtracted from those with aglycons.

system and assay conditions and the best substrate (phenolphthalein) for udpgth-3; the isoform yielded 3.2 pmol of glucuronide/μg of protein/16 h. These calculations suggest that udpgth-3 is less active than udpgth-2, but far superior to udpgth-1.

Structural Similarities of Preferred Substrates. In Figure 6, the chemical structures of the preferred substrates of udpgth-3 and their relative effectiveness (designated by the number of + signs) are shown. Although there are few chemicals to consider in the comparisons, the best substrate, phenolphthalein, contains two aromatic rings, each with a hydroxyl group. This structure is considered ideal. The endogenous substrates indicate a requirement for a 5α-hydrogen as demonstrated by the negative results with testosterone. It is possible that the 4-hydroxybiphenyl has a structure and configuration similar to that of phenolphthalein in terms of suitability for fit at the active site of the isozyme. A degree of hydrophobicity of the substrate is apparently important since the phenolsulfonphthalein with two negative charges is a poor substrate compared to phenolphthalein.

Determination of Kinetic Properties of udpgth-3. In order to determine the relative affinity of the enzyme for the endogenous substrates, the effects of different concentrations of either DHT or 5α-androstane-3α,17β-diol on the velocity of the conjugation reaction were examined using pUDPGTh-3-transfected COS-1 cell homogenate. In Figure 7, Lineweaver–Burk plots show that the isoform has a similar K_m for DHT (10 μM) and its metabolite, 5α-androstane-3α,-17β-diol (12.5 μM). The V_{max}, however, is 2-fold higher for the diol (27.8 pmol/h) than for the active androgen (13.2 pmol/h). Its location in prostate and the restricted substrate specificity for this androgen and its metabolite would argue that this enzyme is involved in the regulation of DHT levels in this target organ. The higher turnover value for 5α-androstane-3α,17β-diol than for DHT but the same K_m value may explain why many studies found evidence for diol glucuronide formation but often failed to detect DHT glucuronide. The K_m of 10 μM DHT appears to allow for a significant androgen concentration. The relatively high K_m of udpgth-3 for DHT may be physiologically relevant. It is possible that DHT reaches relatively high levels in the prostate to support the DHT dependency of 5α-reductase which, in turn, leads to an amplifying effect on DHT action (Rittmaster *et al.*, 1989). One reported (Rittmaster *et al.*, 1989) K_m value using rat liver homogenate for DHT glucuronidation is 1.1 μM and that for 5α-androstane 3α,17β-diol is 14.2 μM. In parallel with our results, the turnover was greater (68-fold) for the diol than for DHT (Rittmaster *et al.*, 1989). The higher K_m for DHT with the pUDPGTh-3-transfected COS-1 homogenate compared with the study using rat liver homogenate may represent a different enzyme from this report or our results could represent cell-specific enzyme modifications and/or environmental differences.

Phenobarbital Induction of mRNAs Encoding UDPGTh-3, UDPGTh-2, and UDPGTh-1. In order to determine whether the messenger RNA encoding either of the cDNA clones, UDPGTh-3, UDPGTh-2, or UDPGTh-1, is regulated by phenobarbital, poly(A+)RNA was isolated from the liver biopsies of an untreated and a phenobarbital-treated *E. patas* monkey and analyzed by Northern blot. The agent was shown to induce all three mRNAs 2.5–3-fold when the RNA content on the filter was normalized by that for cyclophilin (Figure 8).

Variations in the Liver mRNA Levels Encoding UDPGTh-3, UDPGTh-2, and UDPGTh-1 among 16 Different Human Specimens. Among 16 different human samples (Figure 9), it can be seen that the message levels have parallel as well as independent fluctuations. The message for the androgen isoform is generally more uniformly expressed in the majority of the samples; it is similar in concentration to that of UDPGTh-1. Overall, the UDPGTh-2 message was barely detectable in nine samples and not detectable in the others, demonstrating a very low constitutive level of this isoform, whereas that for UDPGTh-1 and UDPGTh-3 is present in substantial amounts.

Since the message levels may be explained by the clinical history of the patient, we provide the following information: Patient 1, who was female, had non-A,non-B (as defined a decade ago) chronic active hepatitis and underwent a right lobe hepatectomy to remove a metastatic gastrinoma. The chronic hepatitis is likely, therefore, to have caused the depletion of liver mRNAs. Patient 2 (male), who contained normal message levels for UDPGTh-1 and UDPGTh-2 and no detectable level of UDPGTh-3, underwent a suprapubic prostectomy for benign prostate hyperplasia (BPH), suggesting that there is a relationship between the specific lack of message encoding UDPGTh-3 and BPH. (Also, there were normal levels of the messenger RNA encoding the two bilirubin transferase cDNAs, HUG-Brl and HUG-Br2, in patient 2; data not shown.) The observation that BPH patients have reduced DHT glucuronide has been reported (Brochu *et al.*, 1987). The patient also underwent a right hepatic lobectomy to remove a metastatic colorectal cancer. Sections of normal male livers (3–7) were removed from those donated to transplant patients. Although these individuals were considered normal, high levels of 6β-testosterone hydroxylase

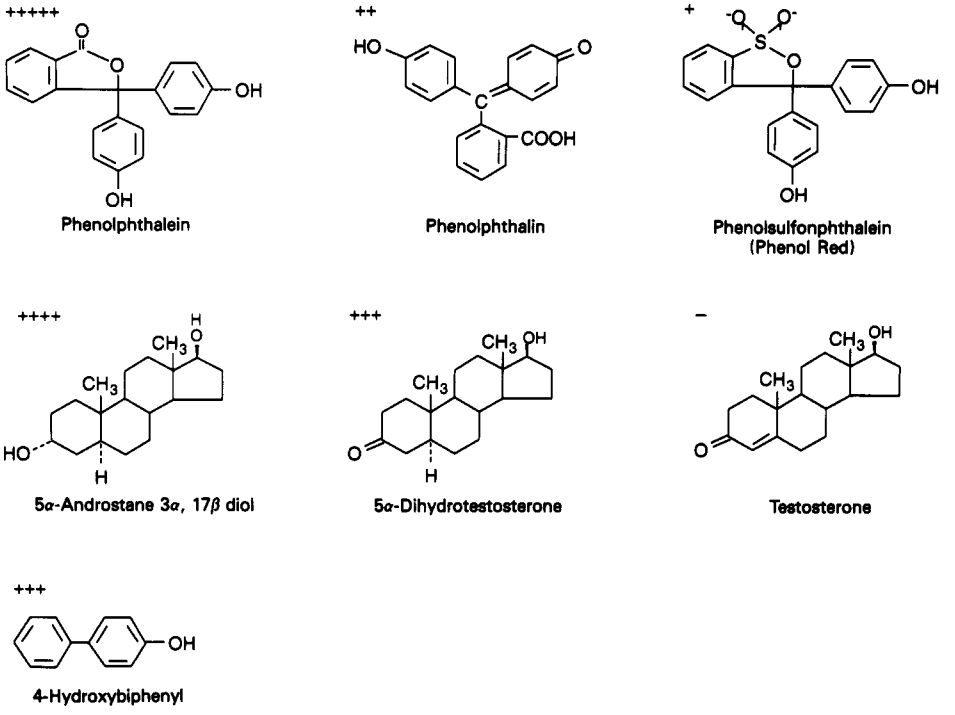

FIGURE 6: Chemical structures of the aglycons glucuronidated by udpgth-3. The relative effectiveness of each compound is represented by the number of plus signs (+) above the structure.

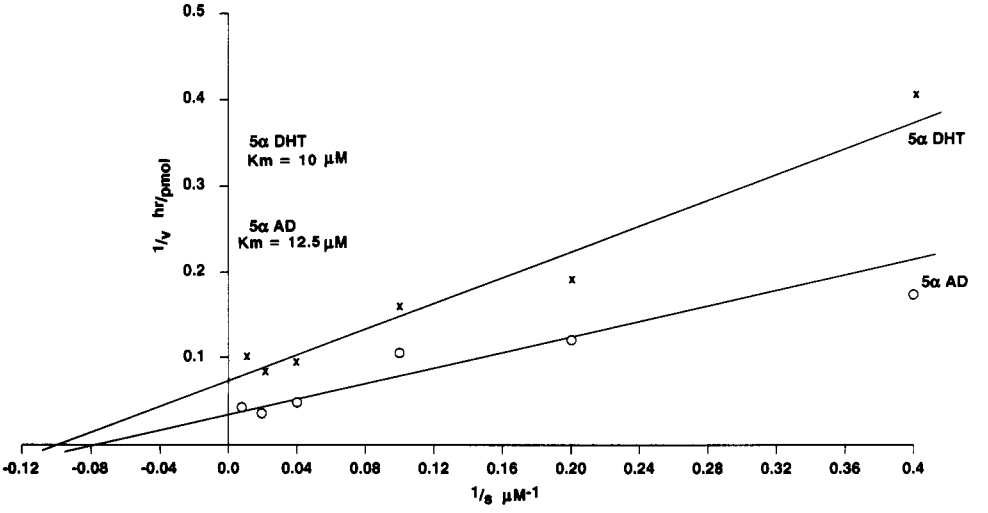

FIGURE 7: Lineweaver–Burk plots of 5α-dihydrotestosterone and 5α-androstane-3α,17β-diol concentration versus reaction velocity of glucuronidation using pUDPGTh-3-transfected COS-1 cells. The homogenate of pUDPGTh-3-transfected COS-1 cells (after 48 h of incubation) was analyzed for either 5α-dihydrotestosterone glucuronide or 5α-androstane-3α,17β-diol glucuronide formation as described in Materials and Methods, except that 2.5, 5.0, 10.0, 25.0, 50.0, and 100 μM DHT or 5α-androstane-3α,17β-diol were used with 0.2 mM [^{14}C]UDP-glucuronic acid (5.0 μCi/μmol), and incubations were for 3 h at 37 °C.

(CYPIIIA) activity were detected in samples 3–5. Patient 8 (male), who both smoked three packs of cigarettes and consumed at least three hard alcoholic drinks daily, underwent a right lobe hepatectomy for a metastatic adenocarcinoma. Specimens 9–16 were from supposedly normal individuals who succumbed due to trauma.

Localization of Genes Encoding Steroid-Metabolizing Isozymes to Chromosome 4. Probes for UDPGTh-1, UDPGTh-2, and UDPGTh-3 could all be localized to human chromosome 4 by Southern blot hybridization with DNAs from a panel of human–rodent somatic cell hybrids (Table II); the genes segregated discordantly (>19%) with all other human chromosomes. A complicated pattern of hybridizing bands was detected with each of these probes. It is possible that more than one hybridizing band was detected in some restriction digests with these probes due to the presence of restriction sites within the cDNA or intronic sequences spanned by the probes. However, most of the complexity resulted from cross-hybridization of each probe with several highly homologous members of a multigene family, and this interpretation was confirmed using subfragments of the cDNAs as probes. Of interest was the fact that cosegregation was observed for all hybridizing bands detected with each probe, indicating that all of the closely related sequences were present on chromosome 4. Weak cross-hybridization was also observed with a small number of rodent bands, and the number and the intensity of these cross-hybridizing bands increased when the stringency was decreased to permit 20–25% sequence divergence. In contrast, there was little change in the pattern of hybridization of human sequences, but most of these bands and the cross-hybridizing rodent bands could be eliminated when the stringency of washes was increased to 65 °C in 0.1×

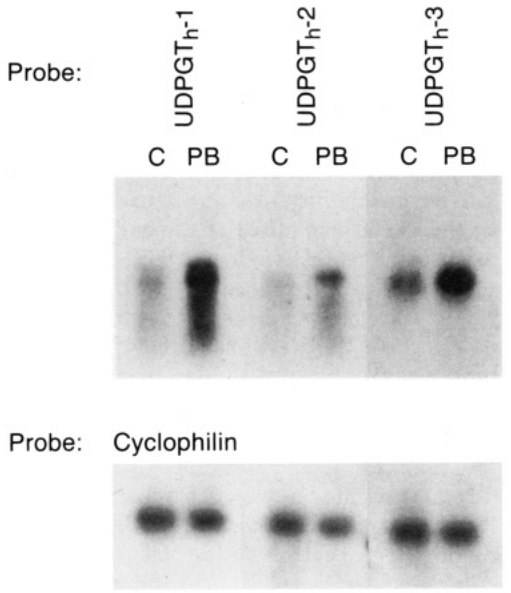

FIGURE 8: Northern blot analysis of liver mRNA isolated from an untreated and a phenobarbital-treated monkey using UDPGTh-1-, UDPGTh-2-, and UDPGTh-3-specific probes. Messenger RNA (2.0 μg/lane) was electrophoresed, blotted, and hybridized to specific probes for UDPGTH-1, UDPGTh-2, and UDPGTh-3 as described in Materials and Methods. In order to normalize for the amount of RNA blotted onto each of the three filters, each was hybridized with the cyclophilin probe.

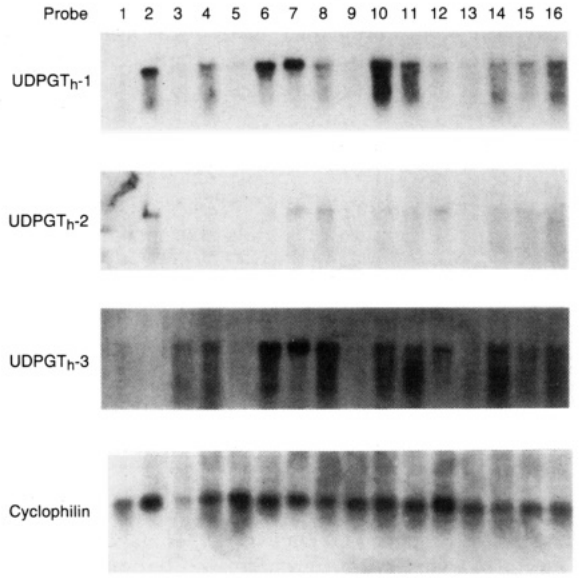

FIGURE 9: Northern blot analysis of liver total RNA from 16 individuals using UDPGTh-1-, UDPGTh-2-, and UDPGTh-3-specific probes. Total RNA was isolated from 16 individual liver specimens, and Northern blots were prepared according to Materials and Methods. In each lane, 20 μg of total RNA was applied for the analysis. The specific probes and the normalization with cyclophilin were the same as described in the legend for Figure 7.

SSC. For example, five hybridizing bands (3.1, 4.7, 5.5, 6.8, and 10.2 kb) were observed in *Eco*RI digests using the 412-bp 5′ UDPGTh-1 cDNA probe, but only the 3.1-kb band persisted at high stringency (65 °C wash). A single band also persisted in restriction digests with 11 other enzymes, whereas 3–7 were found when the blots were washed at 55 °C. The results suggested that small families of these genes in humans were more closely related to each of the three cloned cDNAs than to the rodent homologue indicating that some expansion of these gene families has probably occurred more recently than the evolutionary divergence of man and rodents. After hybridization with the full-length UDPGTh-2 cDNA probe, the blots were washed at the highest stringency (65 °C), and all four *Eco*RI bands (2.3, 5.4, 6.5, and 8.5 kb) segregated concordantly only with chromosome 4. The 5′ UDPGTh-3 cDNA probe identified four major *Eco*RI bands (1.7, 1.8, 10.4, and 16.5 kb), which also segregated with chromosome 4 when the blots were washed under standard conditions.

Table II: Segregation of the UDP-Glucuronosyltransferase Genes Encoding UDPGTh-1, UDGPTh-2, and UDPGTh-3 with Human Chromosome 4[a]

	gene/chromosome				
human	+/+	+/−	−/+	−/−	% discordancy
1	28	21	6	40	28
2	24	25	2	44	28
3	33	16	2	44	19
4	49	0	0	46	0
5	22	27	3	43	32
6	28	21	20	26	43
7	17	32	22	24	57
8	23	26	13	33	41
9	25	24	7	39	33
10	15	34	4	42	40
11	29	20	6	40	27
12	26	23	13	33	38
13	19	30	16	30	48
14	19	30	23	23	56
15	19	30	27	19	60
16	19	30	17	29	49
17	32	17	25	21	44
18	31	18	19	27	39
19	20	29	8	38	39
20	28	21	12	34	35
21	33	16	29	17	47
22	20	29	12	34	43
X	29	20	19	27	41

[a] The UDP-glucuronosyltransferase gene encoding UDPGTh-1 was detected as 3.1-, 4.7-, 5.5-, 6.7-, and 10.2-kb bands in *Eco*RI-digested human–rodent somatic cell hybrid DNAs after Southern hybridization, as described in Materials and Methods. The UDPGTh-2 gene was detected as 2.3-, 5.4-, 6.5-, and 8.5-kb bands; the UDPGTh-3 gene was detected as 1.7-, 1.8-, 10.4-, and 16.5-kb bands. Detection of the human bands is correlated with the presence of each human chromosome in the group of somatic cell hybrids. Discordancy represents the presence of the gene in the absence of the chromosome (+/−) or the absence of the gene despite the presence of the chromosome (−/+), and the sum of these numbers divided by total hybrids examined (×100) represents percent discordancy.

The UDPGTh-1 and UDPGTh-2 were further regionally localized to band 4q28 by *in situ* hybridization using the full-length cDNA probes; the results using UDPGTh-1 are shown in Figure 10. Nearly identical results were obtained using the probe for UDPGTh-2 (data not shown) based on an examination of 112 metaphases containing 222 grains (2.0 grains/metaphase). There were 95 grains (43%) present on chromosome 4 with a peak of 29 (30.5%) present on band 4q28. In contrast, 33 grains were found on chromosome 5 distributed randomly over its entire length. Interpretation of these results is complicated by the fact that each probe recognizes a small multigene family, and some cross-hybridization of each probe with the other gene can also be anticipated. Nevertheless, the fact that a single peak of hybridization was observed with each probe at the same location strongly suggests that both genes and their multigene families are present at this locus on 4q28. A preliminary publication of the chromosomal location of UDPGTh-1 has appeared (Sheen *et al.*, 1990), and more recently, Monaghan *et al.* (1992) reported the localization of the gene encoding HLUG25 (UDPGTh-1) to human chromosome 4 on the basis of PCR amplification of DNAs from a panel of human–rodent somatic cell hybrids using oligonucleotide-specific primers for this sequence.

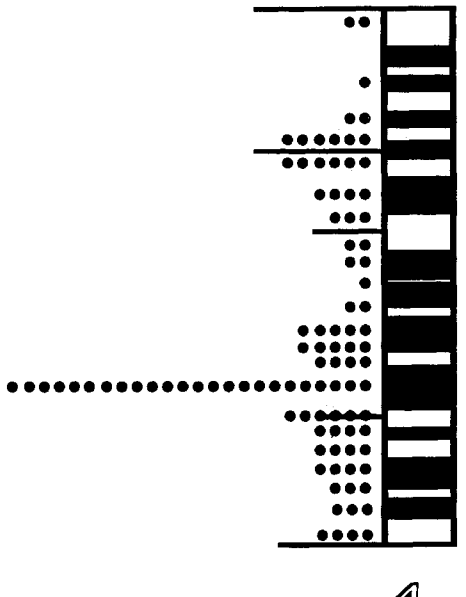

FIGURE 10: *In situ* hybridization. The ideogram shows the distribution of grains on human chromosome 4 after hybridization with the UDPGTh-1 full-length cDNA probe. A total of 90 metaphases containing 190 grains (2.1 grains/metaphase) were examined, and 97 grains (51%) were found on chromosome 4. A peak of 24 grains (24.7% of those on chromosome 4) was present on band 4q28, and an additional 10 grains were found on the two adjacent bands (4q27 and 4q31.1) for a total of 35% of the grains on this chromosome. The background grains on other human chromosomes were distributed randomly.

In contrast, the *UGT1* locus which encodes both bilirubin and phenol isozymes exists on the short arm of chromosome 2 (Harding *et al.*, 1990). The *UGT1* locus is a complex of at least seven genes linked through four common exons in the 3′ region, but they are independently synthesized and regulated by having seven nested exons 1 with individual promoter elements in each 5′ region (Ritter *et al.*, 1992b). The locus is predicted to generate seven overlapping primary transcripts due to alternative transcription initiation, whereby the lead exon 1 in each transcript is differentially spliced to the common exons to produce seven distinct and mature mRNAs. The steroid isoforms, as evident from the sequence comparisons, here do not appear to share exons. A rat testosterone transferase gene contains a typical exon/intron arrangement (Mackenzie *et al.*, 1990a).

Of the extensive number of endogenous chemicals tested, DHT and its metabolite, 5α-androstane-3α,17β-diol, were the only endogenous compounds glucuronidated by the udpgth-3 isoform. As indicated, the isomer, 5α-androstane-3β,17β-diol, was not a substrate. Thus, the human udpgth-3 isoform is distinguished from two different rat transferases, encoded by UDPGTr-3 and UDPGTr-2. Both glucuronidate male and female steroid hormones, dihydrotestosterone, testosterone, and β-estradiol; the $UDPGT_r$-2 protein also conjugates 4-hydroxybiphenyl, chloramphenicol, and 4-methylumbelliferone (Mackenzie, 1987). This apparent lack of overlap in substrate activity for other categories of endogenous compounds may signal that glucuronidation by the udpgth-3 isoform is important for the regulation of the level of DHT, either directly through its glucuronidation or indirectly by glucuronidation of its metabolite.

Although it is not known how androgen levels in the body are regulated, the presence of this isoform in prostate and testis suggests that DHT may, in fact, undergo inactivation in the testis and prostate *via* glucuronidation. It is known that DHT is made in the target tissue, prostate, by the reduction at the 5α-position of testosterone, an androgen which is made and secreted by the testis (Wilson, 1985). The presence of the udpgth-3 isoform in the target tissue may allow for exquisite regulation of the androgen at the site of action. On the other hand, it is possible that 3α-reduction converts the androgen to the inactive metabolite, 5α-androstane-3α,17β-diol, thus removing the amplifying effect of the androgen-dependent 5α-reductase in the synthesis of more DHT. The liver, judged by its high level of message encoding udpgth-3, has a greater capacity for glucuronidating DHT and its metabolite. Whereas the low level in target tissue could provide exquisite regulation, the very high level of udpgth-3 in liver may more crudely regulate androgen levels.

It is also possible that other transferases which glucuronidate DHT and 5α-androstane-3α,17β-diol exist in humans. It is noteworthy that prostate cancer patients (Belanger *et al.*, 1991) have DHT levels comparable to or higher than that of normal males, but do not have detectable levels of DHT glucuronides in the serum; glucuronides of 5α-androstane-3α,17β-diol are normal. Benign prostatic hyperplasia is also characterized by elevations in DHT levels in association with reduced glucuronidation (Brochu *et al.*, 1987). These abnormal glucuronidation rates of DHT in the two patient populations suggest that the isoform(s) responsible for its metabolism is important for normal regulation of this androgen. As suggested by the lack, specifically, of message encoding udpgth-3 in the patient with BPH (Figure 9, patient 2), it is of interest to screen other BPH patients as well as prostatic cancer victims to determine whether there is a relationship between either a low level or the lack of message encoding UDPGTh-3 and the respective disorders. With RNA isolated from appropriate specimens from patients with prostate cancer and/or benign prostatic hyperplasia, one can now make these determinations by using UDPGTh-3-specific probes. Furthermore, the distribution of the message encoding udpgth-3, its substrate specificity, and kinetic parameters indicate that this isoform could necessarily account for much of the biochemical data accumulated on 5α-dihydrotestosterone metabolism.

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