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Characterization of a Rat Liver Cytochrome P-450$_{UT-H}$ cDNA Clone and Comparison of mRNA Levels with Catalytic Activity

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SUMMARY

A rat liver cDNA library was prepared using the expression vector bacteriophage λgt11 and plaques were screened using polyclonal antibodies raised to purified rat liver cytochrome P-450$_{UT-H}$, the major enzyme involved in debrisoquine 4-hydroxylation, bufuralol 1′-hydroxylation, and sparteine Δ^5-oxidation. A clone was selected which contained a 1.3-kb insert. The *Escherichia coli* β-galactosidase fusion protein had a molecular weight greater than that of native β-galactosidase (and reacted with anti-P-450$_{UT-H}$ after electrophoresis) and was also shown to compete with microsomal P-450$_{UT-H}$ for anti-P-450$_{UT-H}$, partially relieving catalytic inhibition by anti-P-450$_{UT-H}$ in rat liver microsomes. Hybrid selection experiments with the cloned cDNA also support the view that the insert is related to P-450$_{UT-H}$. mRNA electrophoresis/hybridization experiments indicated that the 1.3-kb cDNA probe recognized primarily only a single size class of mRNA (2.0 kb) in rat liver. mRNA blotting and *in vitro* translation/immunoprecipitation experiments both indicated that levels of P-450$_{UT-H}$ mRNA are similar in male and female Sprague-Dawley rats. Dark Agouti strain rats of both sexes contained significantly less P-450$_{UT-H}$ mRNA than did Sprague-Dawley rats and the females had approximately one-half the level of the males. These results are consonant with sex and strain differences in measured levels of P-450$_{UT-H}$ and bufuralol 1′-hydroxylase and sparteine Δ^5-oxidase activities. Analysis of genomic DNA indicated that several DNA restriction fragments hybridized to this partial length cDNA; no differences were found between the rat strains and sexes. The results suggest that the basis for the variation in P-450$_{UT-H}$ and its activities among rat strains and sexes is at the level of mRNA concentrations.

P-450 enzymes catalyze the oxidation of a wide variety of xenobiotic substrates (1, 2). One area of interest in this broad field is the elucidation of differences in P-450s which are responsible for interindividual variations in rates of oxidation of drugs. The most widely studied instance of genetic polymorphism of oxidative drug metabolism in humans involves the 4-hydroxylation of debrisoquine (3). About 5–10% of most Caucasian populations examined to date fall into the "poor metabolizer" segment of an apparently bimodal distribution. The incidence of defective metabolism varies with race. At least 15 reactions involving oxidation of other drugs have also been linked to the debrisoquine 4-hydroxylase locus (4).

The availability of an appropriate animal model might facilitate studies on the debrisoquine 4-hydroxylase polymorphism. Al-Dabbagh *et al.* (5) examined *in vivo* debrisoquine clearance in seven strains of rats and reported that only the DA strain was deficient. The same group (6) later reported that only female DA rats were deficient debrisoquine hydroxylators; males were categorized as "extensive metabolizers." Breeding studies using hybrids between DA and Lewis (extensive metabolizer phenotype) strains, together with analysis of the F_2 progeny, were interpreted to mean that the defective metabolism observed in the maternal DA strain is a simple Mendelian recessive trait (data for the sex specificity and cross-breeding experiments were not presented). Küpfer *et al.* (7) found that debrisoquine yielded a typical substrate-induced heme purturbation spectrum when added to liver microsomes isolated from Lewis but not DA rats.

Our laboratory purified a form of P-450 from liver microsomes of untreated SD rats on the basis of its ability to catalyze debrisoquine 4-hydroxylation (8). Reconstitution of catalytic activity with the purified enzyme (P-450$_{UT-H}$) and the extensive inhibition (>90%) of activity in rat liver microsomes by anti-P-450$_{UT-H}$ indicate that this protein is the major catalyst of debrisoquine 4-hydroxylation in rats. Purified P-450$_{UT-H}$ also efficiently catalyzed a number of other reactions which had been linked with debrisoquine 4-hydroxylation in clinical studies. The difference in the amount of immunochemically detectable P-450$_{UT-H}$ between liver microsomes isolated from male SD and female DA rats was approximately 10-fold, consonant with the observed difference in debrisoquine 4-hydroxylase

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ABBREVIATIONS: P-450, liver microsomal cytochrome P-450; DA, dark Agouti; SD, Sprague-Dawley; IPTG, isopropylthio-β-D-galactoside; NaDodSO$_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetracetate.

activity (8). In the same studies we did not see any substantial difference in P-450$_{UT-H}$ levels or debrisoquine 4-hydroxylase activity between male and female SD rats at any age. Rat P-450$_{UT-H}$ shows immunochemical cross-reaction with P-450$_{DB}$, the human debrisoquine 4-hydroxylase (9–11).

Available data with the rat model suggest that the major mode of regulation of debrisoquine 4-hydroxylase and related activities involves changes in the level of P-450$_{UT-H}$ (8). When we examined human liver microsomes, we found a significant correlation between debrisoquine 4-hydroxylase activity and the extent of immunochemical reactivity with anti-rat P-450$_{UT-H}$ (9) but not anti-human P-450$_{DB}$ (10), a result we interpret as suggesting that mutations in the active site of P-450$_{DB}$ may underlie the polymorphism in debrisoquine 4-hydroxylation in humans. In order to address these hypotheses, we have isolated and characterized a cDNA related to P-450$_{UT-H}$. We also report here the use of this probe, as well as other methods, in measuring mRNA coding for P-450$_{UT-H}$. The differences in sex and strain related to expression of P-450$_{UT-H}$ are also considered.

Materials and Methods

Preparations. SD rats weighing approximately 200 g were purchased from Harlan Industries, Indianapolis, IN; after animals were killed by decapitation, livers were removed and frozen rapidly by immersion in liquid nitrogen. Livers from DA strain rats of approximately the same age were supplied (shipped frozen in dry ice) by Dr. A. Küpfer, University of Bern, Switzerland. These frozen livers were used as sources of enzymes and RNA. Microsomes were prepared as described elsewhere (12). P-450$_{UT-H}$ was purified as described previously (8). Anti-P-450$_{UT-H}$ preparations were raised in rabbits and have been described elsewhere (8–10); anti-β-galactosidase was purchased from Cappel Laboratories (West Chester, PA). RNA was isolated from rat liver using guanidine thiocyanate/guanidine hydrochloride as described by Chirgwin et al. (13) without CsCl treatment—RNA preparations were judged to be intact as judged by agarose electrophoresis and visualization of the ribosomal RNA bands (14).

Assays. Protein concentrations in microsomal preparations were estimated using Pierce BCA reagent, using the procedures suggested by the supplier (Pierce Chemical Co., Rockford, IL). The samples (2–20 μg of protein) were heated with the reagent for 30 min at 60°, and bovine serum albumin ($E^{1\%}_{278}$ = 6.67) was used as a standard. In our experience this procedure gives estimates similar to those obtained with the method of Lowry *et al.* (15), but the sensitivity is improved and buffer components commonly utilized in purification of P-450s produce less interference. P-450 concentrations were estimated from ferrous-carbon monoxide versus ferrous difference spectra (16).

Bufuralol 1′-hydroxylation was assayed as described (10, 11). In some of the work, the HPLC system of Haefelfinger (17) was used with detection of product by absorbance (254 nm) or fluorescence ($F_{250/300}$) measurements. Later, an HPLC system modeled on a procedure for separation of quinidine metabolites was found to give much better resolution and did not expose silica-based columns to alkali (18). An Altex 0.46 × 25 cm Ultrasphere cyanopropyl column (Altex Division, Beckman Instruments, Berkeley, CA) was used following a 1-cm octadecylsilyl guard column: the (isocratic) mobile phase was 50 mM potassium phosphate buffer (pH 4.75)/CH_3CN/tetrahydrofuran, 65:30:5 (v/v), used at a flow rate of 1.5 ml min^{-1} (the buffer should stand overnight and then be passed through a 0.45-μm filter prior to use to prevent precipitation of salts). The system cleanly separated 1′-hydroxybufuralol (t_R 1.7 min) and the substrate (t_R 6.5 min). Sparteine Δ^5-oxidation was assayed as described elsewhere (10, 19).

Immunochemical quantitation of P-450$_{UT-H}$ levels in microsomes was done using immunoblotting as described elsewhere (20). We used equine liver alcohol dehydrogenase as an internal standard in individual gel lanes when making quantitative comparisons (21). For these studies we purchased goat antirabbit IgG from Research Biogenics Inc. (Bastrop, TX) and used this preparation at a dilution of 1/200; Boehringer-Mannheim (Indianapolis, IN) peroxidase/rabbit antiperoxidase complex was used at a dilution of 1/500; 4-chloro-1-naphthol was used in place of 3,3′-diaminobenzidine (22).

Recombinant cDNA library construction and screening. The reaction mixture for first strand cDNA synthesis contained 40 μg of poly(A)$^+$ SD adult female rat liver in mRNA/ml, 100 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 140 mM KCl, 100 μg of oligo(dT)/ml, 2 mM methylmercuric hydroxide, 20 mM β-mercaptoethanol, 0.5 unit of RNasin/μl, 800 units of reverse transcriptase/ml, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, and 1 mM dTTP. Second strand synthesis was carried out using RNase H and DNA polymerase I as described by Gubler and Hoffman (23). Following treatment with *Eco*RI methylase and ligation and kinase treatment of *Eco*RI linkers, the cDNA was ligated into the expression vector bacteriophage λgt11 (24). Packaging of λgt11 yielded a rat liver library containing more than 2×10^5 inserts.

Escherichia coli Y1090 was infected with recombinant λgt11 and plated on soft agar. The plates were incubated for 5 h at 42°. Dry nitrocellulose filter disks previously soaked in IPTG were placed on the plates and incubated for 12 h at 37°. The filters were removed and incubated in 1% (w/v) gelatin for 1 hr at room temperature. The filters were then incubated with primary antibody (1/200 dilution) that had previously been absorbed with an *E. coli* lysate covalently attached to Sepharose 4B (Pharmacia, Piscataway, NJ) for 12 hr at room temperature. The filters were then incubated with goat anti-rabbit IgG serum (1/200 dilution) for 1 hr at room temperature and finally with peroxidase/rabbit antiperoxidase complex (1/500 dilution). The filters were developed with H_2O_2 and 4-chloro-1-naphthol (22). Positive clones were removed and replated, with screening as previously described. A clone which was repeatedly positive was grown and the bacteriophage λgt11 was purified essentially as described by Yamamato *et al.* (25). The insert was sized and isolated after electrophoresis on a 1% (w/v) agarose gel. The 1.3-kb insert was subcloned into the plasmid pUC8. Plasmids were isolated by centrifugation in CsCl gradients containing ethidium bromide (14).

***In vitro* translation of RNA and immunoprecipitation of specific translation products.** Total rat liver RNA was translated in a rabbit reticulocyte lysate system (Bethesda Research Laboratories, Gaithersburg, MD) with [^{35}S]methionine (9). Following translation, $NaDodSO_4$ was added to a concentration of 4% (w/v) and the sample was heated for 5 min in a boiling water bath. The sample was diluted first with an equal volume of water and then with 4 volumes of 2.5% (w/v) Triton X-100, so that the final detergent concentrations were 2% Triton X-100 and 0.4% $NaDodSO_4$. This mixture was incubated overnight with anti-P-450$_{UT-H}$ (80-μg IgG fraction) followed by absorption to protein A-Sepharose CL-4B (50 mg) for 3 hr at 4°. The immunoprecipitates were washed five times with 1 ml of 50 mM Tris-HCl buffer (pH 7.4) containing 0.1% (w/v) Triton X-100, 0.02% $NaDodSO_4$, 150 mM NaCl, and 5 mM EDTA. The antibody-antigen complex was dissociated from the protein A-Sepharose CL-4B beads by heating the sample in a boiling water bath for 5 min in the presence of 2% (w/v) $NaDodSO_4$. Total translation products and immunoprecipitates were analyzed by $NaDodSO_4$-polyacrylamide gel electrophoresis and subsequent fluorography (26). Relative band intensity was measured by densitometry using a Kontes Fiber-Optic instrument (Kontes, Vineland, NJ) in the transmittance mode.

Hybrid selection of RNA. Linearized plasmid DNA (25 μg in 50 μl of H_2O) was denatured by boiling for 10 min and immediately cooled on ice. The solution was made 0.5 M in NaOH and kept at room temperature for 20 min. It was neutralized by the addition of 0.5 volume of a solution containing 1 M NaCl, 0.3 M sodium citrate, 0.5 M Tris-HCl (pH 8.0), and 1 M HCl. The sample was mixed well and immediately chilled on ice. The sample was then applied to nitrocellulose paper. The dried filters were baked at 80° for 2 hr, boiled in water for 1 min, and then prehybridized for 8 hr at 42° in 10 mM sodium

piperazine (*N,N'*-bis[2-ethane]sulfonate) buffer (pH 7.0) containing 0.4 M NaCl, 0.4 mM EDTA, 30% (v/v) formamide, and 0.5 mg of yeast tRNA/ml. Hybridization was carried out in 250 μl of the above solution containing 100 μg of poly(A)+ RNA for 16 hr. The filters were washed (using a vortex device) once in 30 mM sodium citrate buffer (pH 7.0) containing 0.3 M NaCl and 0.05% (w/v) $NaDodSO_4$ at room temperature, three times in 1.5 mM sodium citrate buffer (pH 7.0) containing 15 mM NaCl and 0.1% $NaDodSO_4$ at room temperature, and once at 50° in the latter buffer. Finally, the filters were washed in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA at 60°. The filters were then boiled in 300 μl of H_2O containing 10 μg of calf liver tRNA for 90 sec and frozen in a dry ice/ethanol bath. The eluted RNA was extracted once with a mixture of $CHCl_3$/phenol (1:1, v/v) and then once with $CHCl_3$. The RNA was then precipitated with 0.2 M potassium acetate buffer (pH 7.0) and 2.5 volumes of ethanol. The RNA was dissolved in 10 μl of H_2O and translated in a rabbit reticulocyte lysate system (Promega Biotec, Madison, WI) with [^{35}S]-methionine.

RNA transfer blotting analysis. RNA was heat-denatured at 65° in 20 mM sodium 3-[*N*-morpholino]propanesulfonate buffer (pH 7.0) containing 50% formamide, 2 M formaldehyde, and 0.5 mM EDTA and electrophoresed in a 1.25% agarose/formaldehyde gel (14). The RNA was then blotted to Gene Screen sheets (New England Nuclear, Boston, MA) in a manner similar to that described for blotting of DNA (14, 27). The 1.3-kb insert was nick-translated (14) and hybridized to the transferred RNA for 36 hr at 42° in a solution containing 40% formamide, 0.04% polyvinyl-pyrrolidone (*M*, 40,000), 0.04% bovine serum albumin, 0.04% Ficoll (*M*, 400,000), 0.75 M NaCl, 0.075 M sodium citrate, 1% $NaDodSO_4$, and calf thymus DNA (500 μg/ml). Following hybridization, the membrane was washed twice at room temperature for 5 min with 30 mM sodium citrate buffer (pH 7.0) containing 0.3 M NaCl. The membrane was then washed twice at 65° for 30 min with 30 mM sodium citrate buffer (pH 7.0) containing 0.3 M NaCl and 0.5% $NaDodSO_4$. The membrane was washed twice more (30 min) with 1.5 mM sodium citrate buffer (pH 7.0) containing 1.5 mM NaCl. Relative levels of P-450$_{UT-H}$ mRNA were estimated by densitometry (see below).

For quantitation of mRNA, varying amounts of RNA (2-fold series of dilutions) were applied to a nylon (Gene Screen Plus) membrane in a "dot" apparatus; the sheet was hybridized with the ^{32}P-labeled DNA probe and washed under the same conditions. Autoradiograms were scanned using a densitometer and levels of the specific mRNA were estimated by plotting peak area versus amount of RNA applied. For normalizing the RNA for any degradation, we hybridized the same samples (in the dot apparatus) with an end-labeled (^{32}P) oligonucleotide (50-mer) complementary to the 5' end of human serum albumin mRNA (28), which is highly similar to rat serum albumin.

Genomic DNA transfer blotting analysis. Genomic DNA was prepared (along with total RNA) from male SD, male DA, and female DA rats according to the method of Chirgwin *et al.* (13); the DNA was collected as a viscous solution at the interface between the CsCl and the homogenate. This solution was extracted once with an equal volume of a mixture of phenol and $CHCl_3$ (1:1), extracted once with $CHCl_3$, and then precipitated with 2 volumes of ethanol. The DNA was dissolved in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, treated with proteinase K (50 μg/ml) in 0.1% (w/v) $NaDodSO_4$, and reextracted with phenol/$CHCl_3$ and $CHCl_3$ as above. The purified DNA was then precipitated (as fibers) with 2 volumes of ethanol. DNA (20 μg) was digested (to completion, as judged by kinetic analysis) with various restriction enzymes using standard procedures and then electrophoresed in a 1% agarose gel. Blotting (27) was carried out using nylon sheets (Gene Screen Plus) according to the manufacturer's instructions. The similarity of banding patterns in the different DNAs (see Fig. 7) and the lack of nonspecific fragments are consistent with the integrity of the DNA used.

Results

Production of fusion polypeptides. Lysogens were prepared with native bacteriophage λgt11 and λgt11 that contained the 1.3-kb insert. The lysogens were grown in the presence or absence of IPTG, pelleted prior to solubilization, electrophoresed in the presence of $NaDodSO_4$ (26), and then transferred to nitrocellulose. Fig. 1A shows the results obtained when the nitrocellulose sheet was developed using anti-β-galactosidase. The induction of native β-galactosidase and the hybrid fusion protein are apparent; also, the fusion protein has a higher molecular weight than native β-galactosidase. In Fig. 1B the induced native β-galactosidase and the fusion protein were visualized after transfer to nitrocellulose using P-450$_{UT-H}$, and only the fusion protein reacted.

In other experiments which are not presented, antibodies were further purified by electrophoresing approximately 2 nmol of P-450$_{UT-H}$ in a $NaDodSO_4$-polyacrylamide gel (7.5%, w/v), electroblotting the protein to a sheet of nitrocellulose, adsorbing rabbit anti-P-450$_{UT-H}$ sera to the sheet, and, after washing the sheet extensively with 20 mM potassium phosphate buffer (pH 7.4) containing 0.15 M KCl, eluting the *M*, 50,000 zone of the nitrocellulose sheet (detected by staining only the edges of the sheet with the immunoperoxidase technique) three times with 0.5 M glycine-HCl buffer (pH 3.0). After each elution (2 min shaking) the eluate was adjusted to pH 7.0. The combined antibodies were used in immunoblotting experiments with the

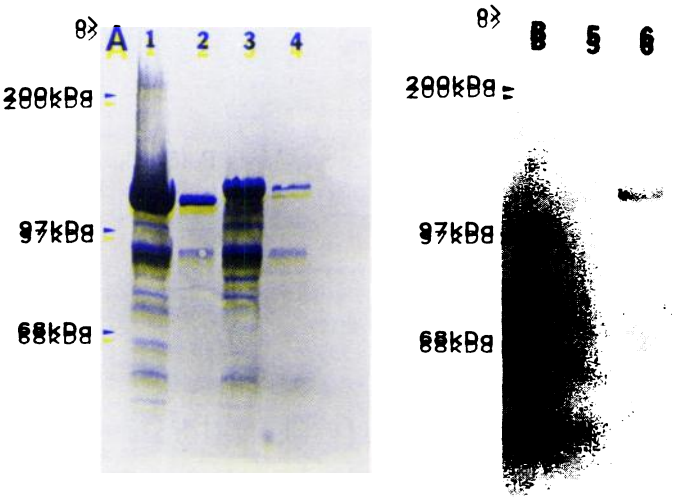

Fig. 1. Immunoelectrophoresis of bacteriophage λgt11 fusion proteins. Lysogens containing native λgt11 and recombinant λgt11 were incubated with shaking at 32° until the A_{600nm} was approximately 0.3. The cultures were then transferred to 44° for 15 min and split into two parts. IPTG was added to a final concentration of 2 mM to one half of each culture; the cultures were then incubated at 37° with shaking for 2 hr. The bacteria were pelleted and solubilized for analysis by $NaDodSO_4$-polyacrylamide gel electrophoresis (26) (7.5% acrylamide, w/v), and the electrophoresed proteins were transferred to nitrocellulose for immunoblotting. In A the nitrocellulose sheet was treated with anti-β-galactosidase, followed by goat anti-rabbit IgG, peroxidase-antiperoxidase complex, and H_2O_2/4-chloro-1-naphthol. Lysogens formed using native λgt11 are shown in lanes *1* and *3*. Lane *1* shows lysogens were induced with IPTG, whereas lane *2* shows uninduced lysogens. Lysogens formed using recombinant λgt11 are shown in lanes *3* and *4*. Lane *3* refers to recombinant lysogens which were induced with IPTG; lane *4* refers to recombinant lysogens which were not induced. In B a nitrocellulose sheet was treated with anti-P-450$_{UT-H}$. Lane *5* shows IPTG-induced lysogens formed using native λgt11, which produces native β-galactosidase. Lane *6* shows IPTG-induced lysogens formed using recombinant λgt11 which produces a β-galactosidase fusion protein containing part of P-450$_{UT-H}$. The origin (o) and positions of the molecular weight markers are indicated.

various lysogen preparations described above and detected the partial P-450$_{UT-H}$/*E. coli* β-galactosidase fusion protein produced by expression of the 1.3-kb insert (results not shown).

Finally, the ability of the fusion protein to disrupt binding of anti-P-450$_{UT-H}$ to microsomal P-450$_{UT-H}$ was examined (Fig. 2). In Fig. 2A, liver microsomes isolated from male SD rats were titrated with anti-P-450$_{UT-H}$ to find an amount which would reduce bufuralol 1′-hydroxylation to 20% of the uninhibited activity.[1] Microsomes and anti-P-450$_{UT-H}$ were mixed at the concentrations under which the 20% rate of activity was measured, and aliquots of this mixture were mixed with varying amounts of lysate protein isolated from lysogens containing the 1.3-kb putative P-450$_{UT-H}$ insert or a cDNA insert for the mitochondrial enzyme D-β-hydroxybutyrate dehydrogenase as a control. The lysogens containing the D-β-hydroxybutyrate dehydrogenase sequence did not have a significant effect upon the bufuralol 1′-hydroxylase activity of the microsomes. However, the lysogen containing the 1.3-kb insert under consideration increased the observed catalytic activity from 17% to 46% (Fig. 2B). Thus, the 1.3-kb insert appears to contain a portion of the P-450$_{UT-H}$ sequence which binds antibodies inhibitory to P-450$_{UT-H}$ and removes them from the microsomal enzyme to "rescue" the activity. Since the insert is not long enough to code for the entire protein, some epitopes related to the inhibitory antibodies are probably not present and full restoration of activity would not be expected. Two other possible explanations for the lack of complete rescue are that 1) the epitopes may not completely adopt their native configurations in the fusion protein, and 2) the fusion protein may bind nonproductively to NADPH-P-450 reductase to block P-450$_{UT-H}$.

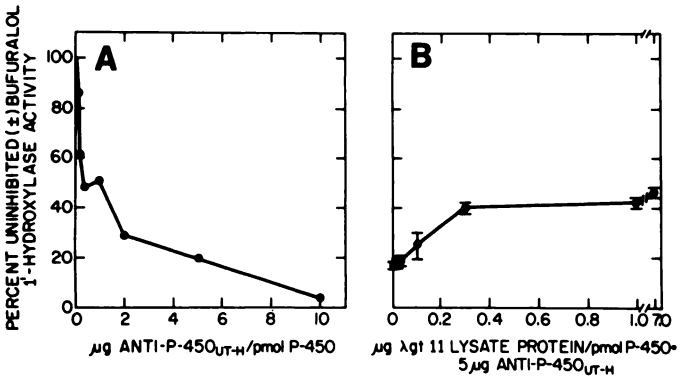

Fig. 2. "Rescue" of microsomal P-450$_{UT-H}$ from anti-P-450$_{UT-H}$ inhibition by P-450$_{UT-H}$/β-galactosidase fusion protein. In A, rat liver microsomes were incubated with varying amounts of anti-P-450$_{UT-H}$ (IgG fraction) using the general procedure described elsewhere (9–11), and residual bufuralol 1′-hydroxylase activity was measured. In B, anti-P-450$_{UT-H}$ was added to rat liver microsomes at the ratio of 5 mg of IgG/nmol of P-450 and aliquots of this mixture were mixed with varying amounts of the λgt11 protein lysate containing the P-450$_{UT-H}$/β-galactosidase fusion protein (solubilized by passage through a French pressure cell) and incubated at 23° for 30 min. Bufuralol 1′-hydroxylase activity was then measured in each sample, as described under Materials and Methods. Results in B are expressed as means ± standard deviations (range) of duplicate incubations and are set relative to the catalytic activity measured in microsomes which were mixed with λgt11 protein lysate but not anti-P-450$_{UT-H}$ [the λgt11 protein lysate had only a slight stimulatory effect on catalytic activity (<25% maximum at any levels shown)].

[1] Although purified P-450$_{UT-H}$ has been reported to have relatively high bufuralol 1′-hydroxylase activity (10) and anti-P-450$_{UT-H}$ has been reported to inhibit human liver microsomal bufuralol 1′-hydroxylase (10, 11), the inhibition of rat liver microsomal bufuralol 1′-hydroxylase activity by anti-P-450$_{UT-H}$ had not been reported previously.

Characterization of cDNA. Bacteriophage λgt11 containing the cDNA insert was digested with *Kpn*I followed by *Dra*I in order to establish the orientation of the DNA shown in the restriction map in Fig. 3. The cDNA insert was subcloned into pUC8 and restriction mapping was done with whole plasmids and cDNA inserts. Partial sequencing also established the position of the *Dra*I site as being 168 bases from a run of 33 As at the 3′ end of the cDNA clone.

Hybrid-selected RNA translation. The 1.3-kb insert was subcloned into the plasmid pUC8, amplified by growth in *E. coli*, and isolated for use in the hybrid selection of RNA. The total translation products directed by the mRNA selected by this plasmid were used for immunoprecipitation with the specific P-450$_{UT-H}$ antibody. One band was obtained with the same apparent monomeric molecular weight as P-450$_{UT-H}$ (Fig. 4). The recovery of labeled P-450$_{UT-H}$ following immunoisolation could also be competed with mature (unlabeled) P-450$_{UT-H}$, indicating that the mRNA selected by this procedure codes for P-450$_{UT-H}$.

***In vitro* translations.** Total RNA fractions isolated from liver prepared from male and female DA and SD rats were translated in a rabbit reticulocyte lysate system with [^{35}S] methionine as the radioactive precursor. Immunoprecipitates were recovered from total translation products and analyzed by NaDodSO$_4$-polyacrylamide gel electrophoresis (26) and subsequent fluorography. To estimate the relative levels of P-450$_{UT-H}$, the intensity of bands on film were measured by scanning densitometry. The data presented in Fig. 5 clearly indicate that more functional mRNA for P-450$_{UT-H}$ was found in SD than in DA rats. A notable sex difference exists in the DA strain, with males having more translatable P-450$_{UT-H}$ mRNA than females.

Characterization of P-450$_{UT-H}$ mRNA levels by blotting analysis. Northern blots of total RNA isolated from male and female DA and SD rats were hybridized to the 1.3-kb insert which had been labeled by nick-translation (Fig. 6). A single major band was found in all samples, corresponding to a size of approximately 2000 base pairs. The size is more than large enough to code for a protein the size of P-450$_{UT-H}$ (8). This result strongly indicates that higher levels of mRNA are found in SD than in DA rats. Other results obtained using dot blots are consistent with this observation (results not shown).

The integrity of the RNA samples was estimated using an oligonucleotide probe (50-mer) prepared against serum albumin (28) and RNA dot blot analysis (original data not shown). The results indicated that the relative levels of serum albumin RNA in the samples for the male SD, female SD, male DA, and female DA rats were 100, 84, 76, and 61, respectively. The values obtained for the levels of P-450$_{UT-H}$ mRNA have been

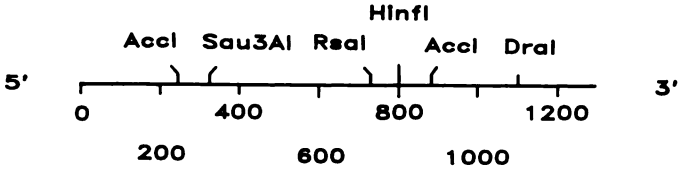

Fig. 3. Restriction endonuclease map of P-450$_{UT-H}$ clone. Plasmid DNA (pUC8) with and without insert and purified P-450$_{UT-H}$ cDNA were digested with restriction enzymes and electrophoresed on 1% agarose gels. Bacteriophage λgt11 containing the cDNA insert was digested with *Kpn*I followed by *Dra*I in order to establish the orientation of the DNA. Partial sequencing also established the position of the *Dra*I site as being 168 bases from a run of 33 adenyl moieties.

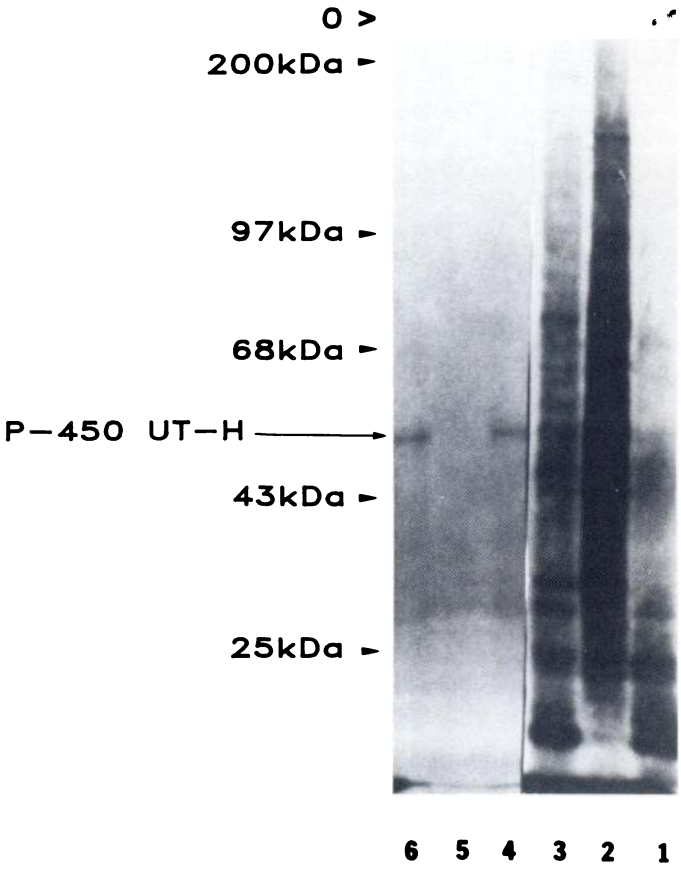

Fig. 4. Translation of hybrid-selected RNA. Rat liver poly(A)$^+$ RNA (100 μg) was hybridized to nitrocellulose filters containing 25 μg of linearized plasmid DNA containing the P-450$_{UT-H}$ cDNA clone. Hybridized RNA was released and translated in a rabbit reticulocyte lysate system in the presence of 5 μg of tRNA. Lane *1* shows the total translation products of the rabbit reticulocyte lysate using 5 μg of transfer RNA and should be compared with lane *3*, which shows the total translation products of hybrid-selected RNA, and lane *2*, which shows the total translation products of total rat liver RNA. Translation products were analyzed for P-450$_{UT-H}$ by immunoisolation and autoradiography (lanes *4–6*). Lane *4* shows the immunoisolation of P-450$_{UT-H}$ from the rabbit reticulocyte lysate when hybrid-selected RNA was used. In lane *5*, the immunoisolation was done in the presence of 250 pmol of added P-450$_{UT-H}$. Lane *6* shows the immunoisolation of P-450$_{UT-H}$ when total rat liver RNA was used in the reticulocyte system. →, the position of migration of purified P-450$_{UT-H}$ on the gel; the origin (*O*>) and molecular weight markers are also shown.

normalized using the values obtained for serum albumin. The levels of functional P-450$_{UT-H}$ mRNA determined by *in vitro* translation were normalized in two ways. The first method utilized the same amount of RNA from SD and DA rats in each reaction and the standardization factor obtained for serum albumin was applied in determining relative levels. In the second method an equal amount of trichloroacetic acid-precipitable radioactivity was used to normalize the *in vitro* translations prior to immunoprecipitation (Fig. 5).

Direct correlations of the levels of functional P-450$_{UT-H}$ mRNA and total P-450$_{UT-H}$ mRNA with the level of P-450$_{UT-H}$ detected by antibodies prepared against the purified protein and enzyme activities measured in microsomes of male and female DA and SD rats are summarized in Table 1.

Analysis of genomic DNA from SD and DA rats. DNA from SD male, DA male, and DA female rats was digested with *Hae*II, *Pst*I, *Bam*HI, and *Taq*I, subjected to agarose gel electro-

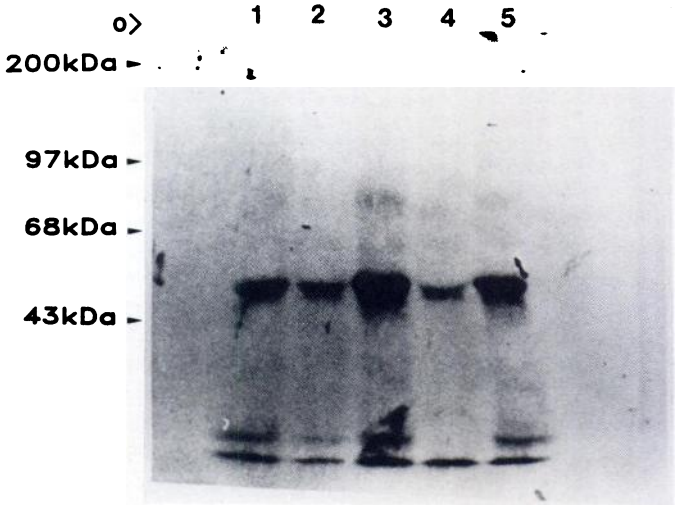

Fig. 5. *In vitro* translation of liver RNA prepared from male and female SD and DA rats. RNA samples were translated in a rabbit reticulocyte lysate system and protein products (1.0×10^6 cpm radioactivity from each) were analyzed for P-450$_{UT-H}$ content after immunoprecipitation. Immunoprecipitates were electrophoresed using NaDodSO$_4$-polyacrylamide gels (26) (7.5% acrylamide, w/v) and subjected to autoradiography. The lanes refer to RNA isolated from male DA (lane *1*), female DA (lane *2*), male SD (lane *3*), male SD (which had been competed with 100 pmol of purified P-450$_{UT-H}$ prior to immunoprecipitation) (lane *4*), and female SD rats (lane *5*). The origin of (*o*>) and position of molecular weight markers are indicated.

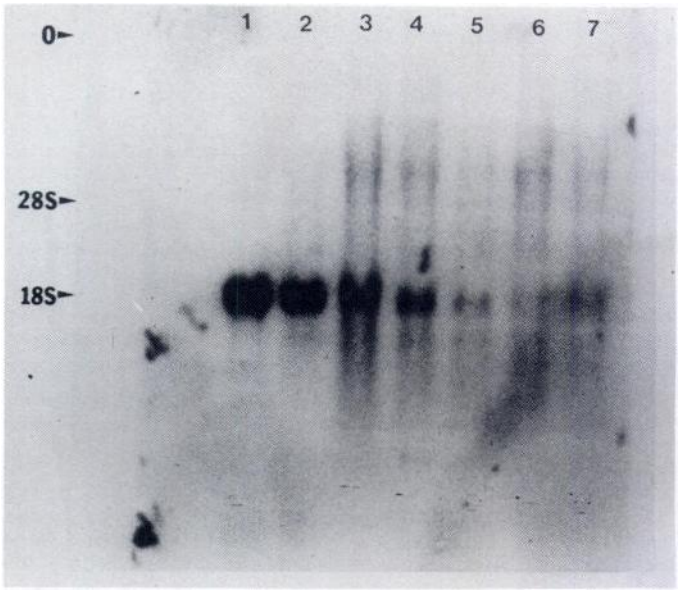

Fig. 6. Size and relative quantities of P-450$_{UT-H}$ mRNA as determined by RNA transfer blotting (Northern) analysis. RNA was electrophoresed in a 1.25% agarose-formaldehyde denaturing gel and transferred to nitrocellulose paper, which was hydridized with the ^{32}P-labeled 1.3-kb P-450$_{UT-H}$ cDNA insert for 36 hr (14). The filters were washed as described and then exposed to X-ray film at −70° for 36 hr. Positions of migration of 18 S and 28 S rRNA are marked, as is the origin. The profiles of 28 S and 18 S RNA bands following staining were taken as a crude measure of RNA integrity. Also, each of *these* particular RNA samples was found to be functional in *in vitro* translation systems (see below). The various lanes indicate experiments done with RNA isolated from a single male SD rat (lane *1*), a single female SD rat (lane *2*), two individual male DA rats (lanes *3* and *4*), and individual female DA rats (lanes *5–7*). The SD rat results are typical of those obtained with several other samples.

TABLE 1

Comparison of hepatic samples from male and female SD and DA rats

Measurement	Male SD	Female SD	Male DA	Female DA
Total microsomal P-450 (nmol/mg of protein)	0.90 ± 0.05	1.06 ± 0.10	1.07 ± 0.21	0.92 ± 0.14
Microsomal bufuralol 1′-hydroxylase activity (nmol of product formed/min/mg of protein)	2.11 ± 0.15	2.23 ± 0.15	0.59 ± 0.05	0.32 ± 0.09
Microsomal sparteine Δ^5-oxidase activity (pmol of product formed/min/mg of protein)	53 ± 2	32 ± 3	22 ± 6	6.0 ± 0.4
Microsomal P-450$_{UT-H}$ (nmol/mg of protein)	0.22 ± 0.04	0.18 ± 0.020	0.086 ± 0.010	0.040 ± 0.005
In vitro translation of P-450$_{UT-H}$ (relative)[a,b]	100[a]	74[a]	41[a]	23[a]
	100[b]	83[b]	32[b]	17[b]
mRNA (2.0 kb) hybridizing with cloned DNA (relative)[c]	100	96	59	26

[a,b] The values for *in vitro* translation of P-450$_{UT-H}$ refer to: *a*, electrophoresis of equal trichloroacetic acid-precipitable radioactivity from each incubation reacted with anti-P-450$_{UT-H}$ and *b*, equal amounts of RNA (based on A_{260nm} used in the *in vitro* translation reactions, and normalized to the amount of binding of mRNA to a ^{32}P-labeled oligonucleotide probe (50-mer) of human serum albumin cDNA (28).

[c] Values were normalized to the amount of binding of mRNA to a ^{32}P-labeled oligonucleotide (50-mer) of human serum albumin cDNA (28).

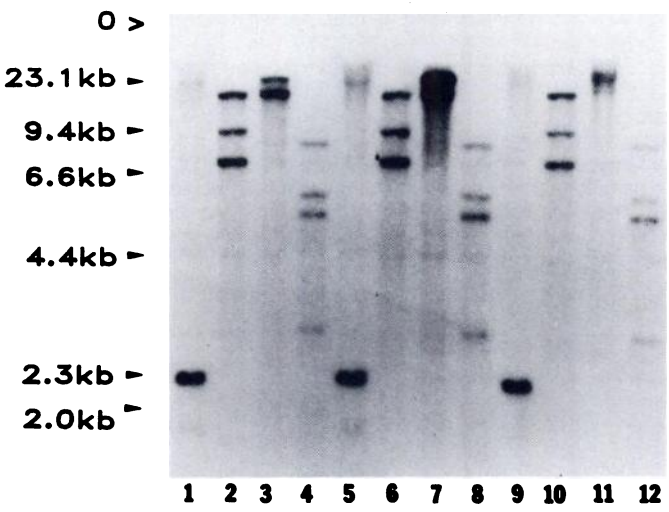

Fig. 7. Blot-hybridization analysis of restriction enzyme-digested rat liver genomic DNA. High molecular weight nuclear DNA from SD male rats (lanes *1–4*), DA male rats (lanes *5–8*), and DA female rats (lanes *9–12*) were digested with *Hae*II (lanes *1*, *5*, and *9*), *Pst*I (lanes *2*, *6*, and *10*), *Bam*HI (lanes *3*, *7*, and *11*), and *Taq*I (lanes *4*, *8*, and *12*), subjected to agarose gel electrophoresis, partially depurinated, and blotted to nylon (Gene Screen Plus). After hybridization with P-450$_{UT-H}$ cDNA, genomic DNA fragments were visualized by autoradiography. HindIII-digested λ phages were electrophoresed in parallel and the mobility and length (in kb) of the resultant fragments are indicated to the *left*.

phoresis, partially depurinated, and blotted to nylon (Fig. 7). Blot hybridization was then performed with the nick-translated cDNA insert. The insert hybridized with multiple fragments from each restriction cut. Summation of the major fragments that reacted with the P-450$_{UT-H}$ cDNA revealed a total of as much as 40–50 kb of DNA using some restriction endonucleases. This indicates that P-450$_{UT-H}$ may be coded by a multigene family. The results do not indicate the existence of any fragments in SD rat DNA that are not present in DA rat (male or female) DNA. In other experiments which are not presented, no differences were seen with the endonucleases *Eco*RI and *Msp*I. The results also indicate that the gene for P-450$_{UT-H}$ is not amplified in SD rats.

Discussion

A 1.3-kb clone related to rat liver P-450$_{UT-H}$ was isolated from a bacteriophage λgt11 expression library. The hybrid fusion protein which was produced was larger than β-galactosidase and reacted with antibody prepared against purified P-450$_{UT-H}$ in immunoblotting experiments. The λgt11 protein lysate containing the P-450$_{UT-H}$/β-galactosidase fusion protein was capable of partially reversing the inhibition of microsomal bufuralol 1′-hydroxylase by anti-P-450$_{UT-H}$. These results, together with those involving hybrid-select translation, provide strong evidence that the isolated 1.3-kb insert codes for P-450$_{UT-H}$ or a closely related protein.

A strain difference was shown to exist in both catalytic activity and concentration of P-450$_{UT-H}$ between DA and SD rats. A large strain difference in P-450$_{UT-H}$ mRNA levels was demonstrated by hybridization of a 1.3-kb cDNA clone to resolved RNA, as well as *in vitro* translation followed by immunoprecipitation, electrophoresis, and autoradiography of P-450$_{UT-H}$. A sex difference within the DA strain was shown to exist in catalytic activity, P-450$_{UT-H}$ content, and mRNA levels. We estimate that the content of P-450$_{UT-H}$ is 2.5-fold higher in male and female SD rats than in male DA rats and approximately 5-fold higher in SD than DA female rats. The good correlation between catalytic activity, P-450$_{UT-H}$ levels, *in vitro* translation of P-450$_{UT-H}$, and mRNA levels suggests that the strain and sex differences in P-450$_{UT-H}$ result from regulation at the mRNA level.

An abstract report suggests that debrisoquine 4-hydroxylase activity can be induced by administration of polycyclic hydrocarbons (29). However, neither we nor others have observed an increase in either debrisoquine 4-hydroxylase activity or levels of immunochemically detectable P-450$_{UT-H}$ (8, 30). Another abstract report (6) suggests that the deficiency of debrisoquine 4-hydroxylase activity is highly sex-linked in DA strain rats. However, our results (Table 1 and Ref. 8) indicate no major sex difference in levels of P-450$_{UT-H}$ (or its catalytic activities or mRNA levels) in SD rats, and the difference between males and females within the DA strain is only 2- to 3-fold.[2]

The mechanisms which underlie the differences in the steady state levels of mRNA in the various rats are unknown, but mRNA levels appear to be responsible for differences in amounts of P-450$_{UT-H}$ and catalytic activities related to the

[2] A recent report (31) suggests that DA female rats differ from Fischer female rats in having a lower V_{max} for a low K_m form of bufuralol 1′-hydroxylase and actually having a higher V_{max} (DA > Fischer) for a higher K_m form of the enzyme. Our comparisons (made with SD rats) are not consistent with such a view, for the 7-fold difference in bufuralol 1′-hydroxylation rates (Table 1, cf. female values, SD > DA) was observed with a relatively high bufuralol concentration (200 μM).

enzyme. To date we have found only small ontogenic changes in P-450$_{UT-H}$ concentrations and no effects of typical chemical modulators of P-450 enzyme levels (8, 30). A modest sex effect is seen, but only in DA strain rats (Table 1). Thus, P-450$_{UT-H}$ seems more refractory to environmental influences than any of the other nine rat liver P-450 enzymes we have studied in our own laboratory (22, 32). The strain and sex differences might be attributable to differential rates of transcription, but more complex explanations are possible involving RNA processing, translocation, and stability. At this point we should again emphasize that total (spectrally detectable) levels of P-450 were nearly identical in the various rats studied here (Table 1).

Finally, it has been shown that the cDNA clone recognizes a number of DNA fragments in Southern blot analysis, and that as much as 40–50 kb of DNA contains sequences which hybridize with the cDNA probe, suggesting that more than one gene related to P-450$_{UT-H}$ may be present. However, the gene family appears to be less complex than encountered to date in some of the other P-450 genes (33, 34). All fragments which were generated by restriction endonuclease treatment of SD rat DNA and hybridized with our probe were present in DA rat DNA. Thus, no evidence of a polymorphism was found, consistent with the positive results regarding the mRNA levels. The potential size of the gene family (Fig. 7) requires the caveat that the cDNA we have characterized may code for a protein which is closely related to the catalytically active P-450$_{UT-H}$ but may not be identical. However, this cDNA should serve as a further basis for addressing the regulation of the catalytic activity in rats and humans.

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