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Sex-Related Differences in the Expression of Cytochrome P450 in Hamsters: cDNA Cloning and Examination of the Expression of Three Distinct CYP2C cDNAs

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SUMMARY

Sex-related differences in the expression of cytochrome P450 isozymes in hamsters were investigated. Three distinct cDNA clones (assigned as *CYP2C25*, *CYP2C26*, and *CYP2C27*) were isolated from liver cDNA libraries from male and female hamsters, using rat *CYP2C11* cDNA as a probe. Sequence analysis revealed that these three forms were very similar to each other (>90% identity of nucleotide sequences) and belong to the *CYP2C* gene subfamily. The *CYP2C25*, *CYP2C26*, and *CYP2C27* cDNA clones consisted of an open reading frame encoding 490 amino acids. CYP2C25, CYP2C26, and CYP2C27 proteins expressed in *Saccharomyces cerevisiae* catalyzed the hydroxylation of tolbutamide and the *N*-demethylation of aminopyrine and benzphetamine. Only CYP2C25 showed testosterone hydroxylase (16β) activity. Northern blot analyses with specific oligonucleotide probes demonstrated that expression of CYP2C27 was male specific and male predominant in kidneys and livers, respectively. No sex-related difference was observed in the expression of CYP2C25 and CYP2C26. These results support our previous findings that sex-related differences in the expression of liver microsomal cytochromes P450 occur in hamsters.

Cytochromes P450 comprise a superfamily of ubiquitous monooxygenases associated with the metabolism of a broad group of structurally unrelated compounds, including drugs, chemical carcinogens, environmental pollutants, and endogenous substrates (1). The expression of cytochromes P450 is regulated by many endogenous and exogenous factors (2). Sex hormones regulate the expression of some forms of cytochrome P450 by modulating growth hormone secretion (3–5). Forms of cytochrome P450 whose expression are regulated by sex hormones have been proven to be present in liver microsomes of rats and mice (6–10).

Because of the relatively high basal levels of drug-metabolizing enzymes (11) and because of efficient activation of chemical carcinogens including polycyclic aromatic hydrocarbons (12), hamsters have been used as a useful experimental animal for understanding the mechanisms involved in chemical carcinogenesis (13), tumorigenesis (14), and drug metabolism.

Information regarding cytochromes P450 in hamster livers is limited at present. Forms of cytochrome P450 inducible by 3-MC and acetone have been purified (15, 16) and cDNA clones belonging to the 1A, 2A, and 3A subfamilies have been isolated (17).

Previously, we reported results supporting the hypothesis that there is a form of cytochrome P450 that is expressed in a sex-related manner (18). We found sex differences in the oxidative metabolism of testosterone and erythromycin and in the amount of protein that is cross-reactive with anti-cytochrome P450-male (CYP2C11) antibodies. In the present study, we obtained cDNA clones coding for hamster CYP2C protein and analyzed the expression of these forms of cytochrome P450 to provide direct evidence for the sex-related differences in CYP2C expression in the hamster.

Materials and Methods

Animals. Male and female Syrian hamsters were obtained from Sankyo Experimental Animals (Tokyo, Japan) and killed by decapitation. Livers and other organs were immediately excised and used for the preparation of RNA and microsomes.

Materials. Restriction enzymes, S1 nuclease, and other DNA-modifying enzymes were obtained from Takara Shuzo (Kyoto, Japan), radioactive reagents and random primer labeling kit from Amersham (Aylesbury, UK), λZap cDNA synthesis kit and GIGAPACKII GOLD from Stratagene (Los Angeles, LA), nylon and nitrocellulose membranes from Schleicher & Schuell (Dassel, Germany), sequencing kit from United States Biochemical (Cleveland, OH), and oligo(dT)-cellulose from New England Biolabs (Beverly, MA). The partial cDNA clone pcP450(M-1)-4 (19), which encodes rat CYP2C11, was kindly supplied by Dr. Yoshiaki Fujii-Kuriyama of Tohoku University. The β-actin probe prepared from a human β-actin pseudogene was kindly

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ABBREVIATIONS: 3-MC, 3-methylcholanthrene; kb, kilobase(s); HPLC, high performance liquid chromatography; TLC, thin layer chromatography.

supplied by Dr. Takeo Kakunaga of Osaka University. A cDNA library prepared from livers of 3-MC-pretreated male Syrian hamsters was kindly donated by Satoru Ohgiya of Government Industrial Development Laboratory-Hokkaido, Agency of Industrial Science and Technology. Yeast *Saccharomyces cerevisiae* AH22 (*a leu2 his4 can1 cir*$^+$) and yeast expression vector pAAH5 were kindly supplied by Dr. Ryo Sato of Osaka University. Other chemicals were of the highest grade commercially available.

Construction and screening of cDNA libraries. Total RNA was prepared from the livers of untreated male and female hamsters by the guanidinium thiocyanate method. Poly(A)$^+$ RNA was enriched from the total RNA by oligo(dT)-cellulose column chromatography (20). cDNA was synthesized from the mRNA of female hamsters and an *Eco*RI adaptor was ligated. cDNA thus synthesized was fractionated by sucrose density gradient (5–25%) centrifugation. Judging from Southern blot analysis using the rat *CYP2C11* cDNA fragment [*Eco*RI-*Pst*I fragment containing most coding region and an entire 3′ untranslated region (1.6 kb) of pcP450(M-1)-4], the fractions in which *CYP2C* cDNA was presumably abundant were pooled, ligated into λZap cloning vector, and packaged *in vitro*. Two cDNA libraries, one prepared with λgt11 for 3-MC-pretreated males and the other prepared with λZap for untreated females, were screened by the plaque hybridization method, using the *Eco*RI-*Pst*I fragment (1.6 kb) of pcP450(M-1)-4 as a probe. Rescreening of both libraries was performed using the *Eco*RI fragment (1.0 kb) of *CYP2C27* cDNA (a cDNA clone isolated in this study) as a probe. As a result of this screening, three distinct cDNA clones containing the entire coding region were obtained (assigned as *CYP2C25*, *CYP2C26*, and *CYP2C27*).[1] The characteristics of these clones are described in Results and Discussion. Phage DNA of positive plaques was purified, and then the insert cDNA fragments in λgt11 were subcloned into pUC119 and those in λZap were subcloned into pBluescript SK(−) by *in vitro* excision. The nucleotide sequences of the positive clones were determined by the dideoxy method. The nucleotide sequences were analyzed with GENETYX (version 8.0; SDC Software, Tokyo, Japan).

Construction of yeast expression plasmids and preparation of microsomes from transformed yeast cells. cDNA fragments containing the entire coding region were constructed in pUC119 or pBluescript vector and then inserted between the yeast alcohol dehydrogenase 1 promoter and terminator regions of the yeast expression vector pAAH5, to construct expression plasmids. The strategy for construction of expression plasmid pAM1 for CYP2C25 is shown in Fig. 1A. The *Eco*RI fragment containing the carboxyl-terminal half of *CYP2C25* cDNA was inserted into the *Eco*RI site of pUC119 to yield pHSM1C. To facilitate the construction of expression plasmids, the *Eco*RI-*Hinc*II fragment prepared from pHSM1C was then inserted between *Eco*RI and *Hinc*II sites of pBluescript KS(−). The resulting plasmid, pHSM1C(H), was digested with *Eco*RI, and then the *Eco*RI fragment containing the amino-terminal half of *CYP2C25* cDNA was inserted to yield pHSM1. The pHSM1 plasmid contained the entire coding region and 13 and 134 base pairs of 5′ and 3′ untranslated sequences, respectively. Expression vector pAAH5 was digested with *Hind*III and blunt-ended to ligate with the *Sma*I-*Hinc*II fragment prepared from pHSM1. The orientation of the inserted *CYP2C25* fragment was confirmed by restriction enzyme analysis. The strategy for CYP2C26 (Fig. 1B) is almost the same as that for CYP2C25. The *Eco*RI fragment containing the carboxyl-terminal half of *CYP2C26* cDNA was inserted into the *Eco*RI site of pUC119 to yield pHSM2C. To shorten the 3′ untranslated sequence in pHSM2C, the *Xba*I fragment prepared by the *Eco*RI/*Xba*I double-digestion of pHSM2C was inserted into pHSM2C, to yield pHSM2C(X). The plasmid pHSM2C(X) was digested with *Eco*RI and dephosphorylated, and then the *Eco*RI fragment containing the amino-terminal half of *CYP2C26* cDNA was inserted to yield pHSM2. The pHSM2 plasmid was digested with *Sal*I, partially digested with *Eco*RI, filled in, and then ligated to a blunt-ended pAAH5 vector to yield pAM2. Fig. 1C shows the strategy for CYP2C27. Different from the others, the full-length cDNA clone was isolated from a female library that was constructed with λZap vector. The fragment containing the entire coding region was subcloned into pBluescript SK vector by *in vitro* excision. The resulting plasmid, pHSM3, was digested with *Xba*I, filled in, and then ligated into a blunted pAAH5 vector to yield pAM3. The expression plasmids thus obtained were used to transform *S. cerevisiae* strain AH22 by the lithium acetate method (21). Transformed yeast cells were selected on synthetic agar plates in the absence of leucine. Transformed yeast cells were grown to stationary phase in 20 ml of SD medium [0.17% yeast nitrogen base without amino acids (Difco, Detroit, MI), 2% dextrose, 20 μg/ml histidine]. The culture was then introduced into 1 liter of YPD medium [1% yeast extract (Difco), 2% peptone (Difco), 2% dextrose]. Yeast cells were grown until the absorbance at 600 nm reached about 1.0 and then were collected by centrifugation, and the microsomal fraction was prepared by the method of Oeda *et al.* (22). The concentrations of cytochrome P450 and protein were determined as reported by Omura and Sato (23) and Lowry *et al.* (24), respectively.

Western blot analysis of yeast microsomes. Western blot analysis of cytochrome P450 expressed in yeast microsomes was carried out according to the method of Nagayama *et al.* (25), using antibodies to cytochrome P450-male (CYP2C11). The immune complexes were identified with a goat anti-rabbit IgG and a peroxidase-antiperoxidase conjugate, followed by visualization with 3,3′-diaminobenzidine and hydrogen peroxide.

Assay for the activity of cytochrome P450-dependent monooxygenases. A typical incubation mixture contained 100 mM potassium phosphate, pH 7.4, yeast microsomes containing 0.3 nmol of cytochrome P450, 1.0 unit of rat NADPH-cytochrome P450 reductase, 0.1 mM EDTA, an NADPH-generating system (0.5 mM $NADP^+$, 5 mM glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, and 5 mM $MgCl_2$), and a substrate. The concentrations of substrates in the incubation mixture were 5, 5, 1, and 0.6 mM for aminopyrine, benzphetamine, tolbutamide, and testosterone, respectivity. The concentration of [4-^{14}C]testosterone for the TLC analysis was 7.2 μM. The *N*-demethylase activities for aminopyrine and benzphetamine were assayed in a final volume of 1.0 ml by measuring formaldehyde production during incubations according to the method of Nash (26). Testosterone hydroxylase activity was assayed in a final volume of 0.5 ml by measuring the amounts of hydroxylated metabolites by HPLC according to the method of Hayashi *et al.* (27). Testosterone hydroxylase activity was also examined by the TLC method (28). Briefly, the reaction mixture was extracted with benzene and then chromatographed on a TLC plate that was developed two times with solvent (chloroform/ethyl acetate/ethanol, 4:1:0.7, v/v). The TLC plate was analyzed with a Bio Image analyzer (Fuji Film, Tokyo, Japan). Tolbutamide hydroxylase activity was assayed in a final volume of 0.2 ml by measuring the hydroxylated metabolite by HPLC. HPLC was performed using an Inertsil ODS-2 column (5 μm, 4.6 × 250 mm; GL Sciences Inc., Tokyo, Japan), with a mobile phase consisting of 30% (v/v) acetonitrile and 1% (v/v) acetic acid, at a flow rate of 1 ml/min. The eluate was monitored at 236 nm with UV detector (SPD-10A; Shimadzu Corp., Kyoto, Japan). Phenacetin was used as an internal standard.

Synthesis of oligonucleotide probes. Oligonucleotides used in this research were synthesized with a DNA synthesizer (model 381A; Applied Biosystems, Foster City, CA). The sequences of the synthesized probes used for Northern blot analysis were as follows: oli-2C25 for CYP2C25, 5′-GGAATTGACAGAATGGGAA-3′; oli-2C26 for CYP2C26, 5′-GGACTTCATTGAATGGGAA-3′; oli-2C27 for CYP2C27, 5′-AGACTTGATTGAATGAGAA-3′. These probes were at corresponding regions, which were located at positions 1577–1595 in an aligned nucleotide sequence (see Fig. 2). The oligonucleotides were labeled at the 5′ end with [γ-^{32}P]ATP, using MEGALABEL (Takara Shuzo, Kyoto, Japan). The specific activities were >2 × 10^6 cpm/pmol.

Northern and Southern blot hybridization. Total RNA (20 μg)

[1] The nucleotide sequences of CYP2C25, CYP2C26, and CYP2C27 appear in the EMBL, GenBank, and DDBJ databases with accession numbers of X63022, D11435, and D11436, respectively.

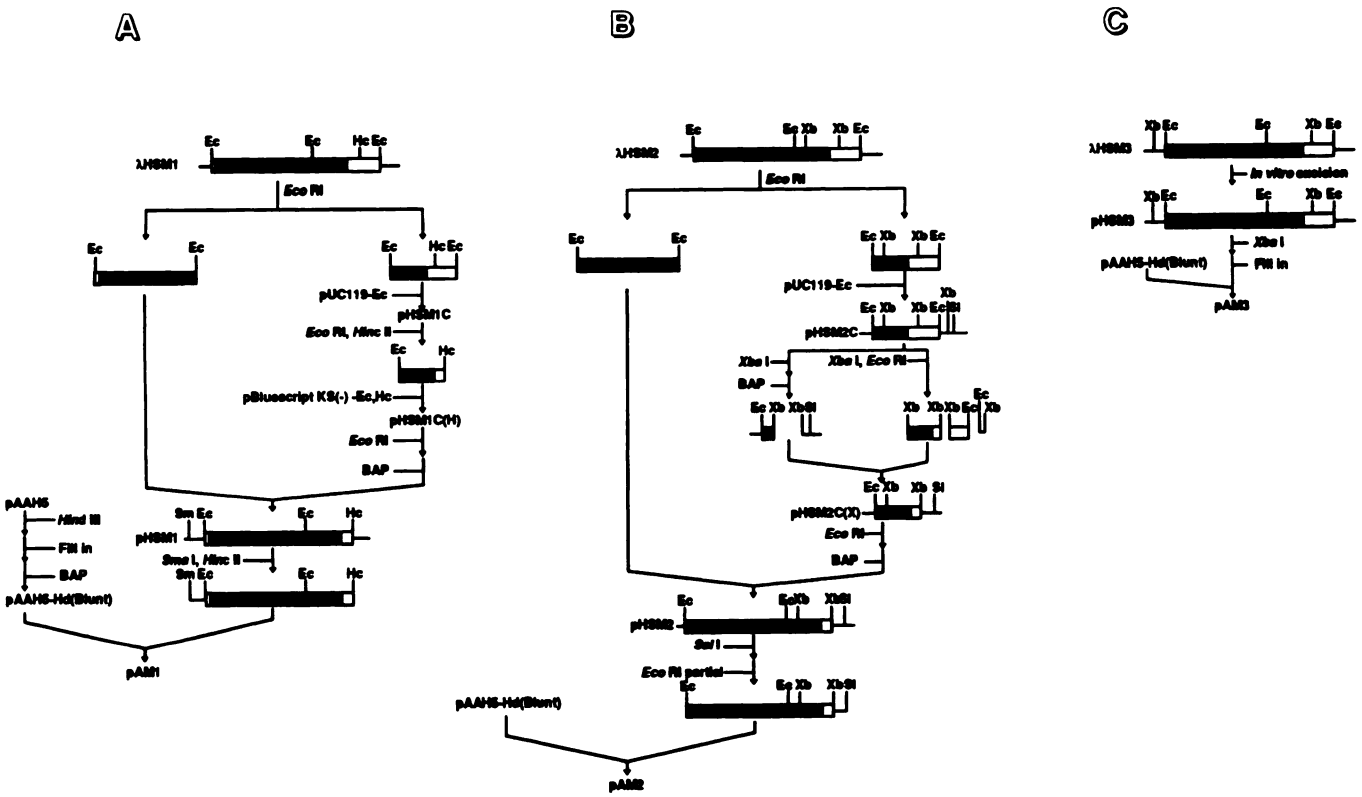

Fig. 1. Construction of the expression plasmids pAM1 (A), pAM2 (B), and pAM3 (C) for CYP2C25, CYP2C26, and CYP2C27, respectively. *Solid boxes*, protein-coding regions; *open boxes*, untranslated regions; *thin lines*, vector DNA. *Ec*, *Eco*RI; *Hc*, *Hin*cII; *Hd*, *Hin*dIII; *Sl*, *Sal*I; *Sm*, *Sma*I; *Xb*, *Xba*I; *BAP*, bacterial alkaline phosphatase.

was separated in a 0.8% agarose gel containing 2.2 M formaldehyde and was transferred onto a nylon membrane. In the case of Southern blot hybridization, subcloned plasmids (2 ng) were slot-blotted onto a nitrocellulose membrane. When we used oligonucleotide probes, the membranes were prehybridized for 4 hr at 42° in 0.5 M sodium phosphate, pH 7.2, containing 7% sodium dodecyl sulfate, 1% bovine serum albumin, and 1 mM EDTA, essentially following the method of Church and Gilbert (29). The hybridization was performed under the same conditions over 12 hr. The membranes were washed for 15 min at 42° in 0.1 M sodium phosphate, pH 7.2, containing 1% sodium dodecyl sulfate and 1 mM EDTA. When we used the β-actin probe, hybridization and washing were carried out at 55°. The other conditions were identical to those described for oligonucleotide probes. The β-actin probe, which was used to monitor the quantity of total RNA blotted, was an approximately 1.7-kb DNA fragment prepared from a human β-actin pseudogene (30). Autoradiography was carried out for 3–10 days at −80°, with intensifying screens.

S1 nuclease protection assay. For the S1 nuclease protection assay, a 146-nucleotide single-strand DNA encompassing positions 1203–1297 of CYP2C27 was prepared. The 51-nucleotide segment in this probe, which is located 3′ downstream of *CYP2C27* cDNA, must not show any hybridization bands, because this segment came from the M13 vector and will be cut off by S1 nuclease. The probe labeled at the 5′ end with [γ-^{32}P]ATP was hybridized at 42°. The S1 nuclease digestion was performed using 10 units/20 μg of hamster hepatic total RNA at 30° for 40 min.

Other methods. NADPH-cytochrome P450 reductase was purified from liver microsomes of phenobarbital-pretreated rats by the method of Taniguchi *et al.* (31). The activity of NADPH-cytochrome P450 reductase was measured using cytochrome *c* as an electron acceptor. One unit of reductase was defined as the amount of enzyme that catalyzed the reduction of 1 μmol of cytochrome *c*/min.

Results

Isolation and classification of cDNA clones. Liver cDNA libraries from untreated female hamsters and male hamsters pretreated with 3-MC were screened with a cDNA fragment of *CYP2C11* as a probe. Both libraries were rescreened using the *Eco*RI fragment of a cDNA clone (*CYP2C27*) obtained in this study as a probe. Seventy-seven positive clones in total were isolated from both cDNA libraries. Judging from restriction enzyme maps, most of the positive clones were classified into three forms and designated as *CYP2C25* (48 clones), *CYP2C26* (14 clones), and *CYP2C27* (12 clones). The numbers in parentheses indicate the numbers of cDNA clones that showed the same restriction enzyme map. The remaining three clones differ from each other and could not be classified into these three forms. The sequences of these clones are currently being analyzed in our laboratory. Restriction enzyme recognition sites of these cytochromes P450 were similar but were distinct from each other at some sites (data not shown).

Sequence analysis of three cDNA clones and comparison of nucleotide and amino acid sequences. The nucleotide and deduced amino acid sequences of the three forms are shown in Figs. 2 and 3, respectively. The longest cDNA clones belonging to *CYP2C25*, *CYP2C26*, and *CYP2C27* consisted of 1822, 1824, and 1809 nucleotides, respectively. All clones contained an open reading frame of 1470 nucleotides and encoded a polypeptide of 490 amino acids. The estimated molecular weights were 55,965, 55,723, and 55,766 for CYP2C25, CYP2C26, and CYP2C27, respectively. A typical poly(A)$^+$ addition signal and poly(A)$^+$ tail were also seen at corresponding positions in all forms. Table 1 shows a comparison of the

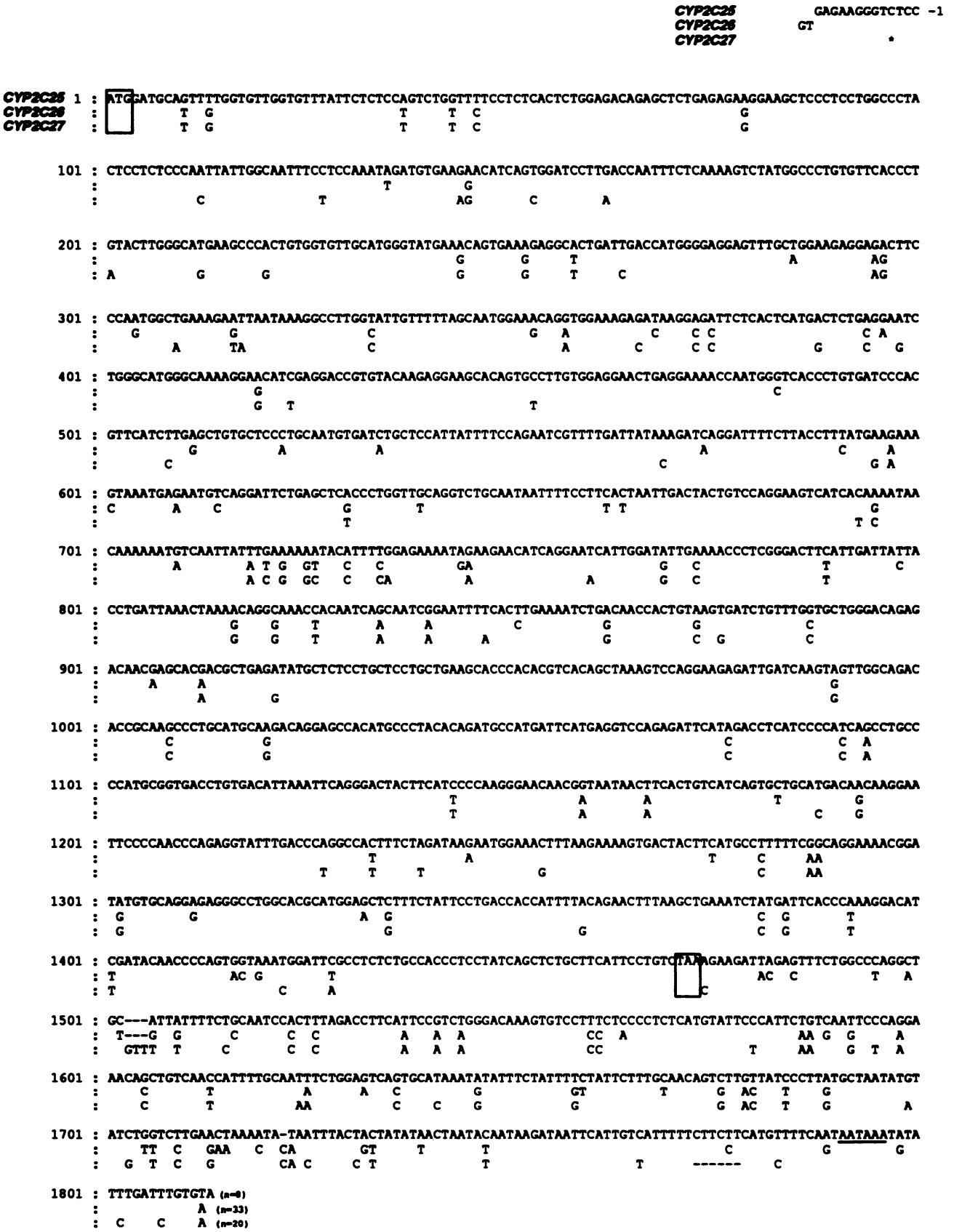

Fig. 2. Alignment of the nucleotide sequences of *CYP2C25*, *CYP2C26*, and *CYP2C27* cDNAs. The complete nucleotide sequence of *CYP2C25* is displayed and aligned with those of *CYP2C26* and *CYP2C27*. Only nucleotides that are not identical to those of *CYP2C25* are shown for *CYP2C26* and *CYP2C27*. Initiation and stop codons are *boxed*. A polyadenylation signal is *underlined*. *, 5′ end of *CYP2C27*. *Dashes*, insertions and deletions.

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                    10        20        30        40        50        60        70        80        90
CYP2C25   1 MDAVLVLVFILSSLVFLSLWRQSSERRKLPPGPTPLPIIGNFLQIDVKNISGSLTNFSKVYGPVFTLYLGMKPTVVLHGYETVKEALIDHGEEFAGRGDF
CYP2C26        FV       C FL          G                     D                                A                S
CYP2C27        FV       C FL          G          L    F     D                     R  A       A                S

        101 PMAERINKGLGIVFSNGNRWKEIRRFSLMTLRNLGMGKRNIEDRVQEEAQCLVEELRKTNGSPCDPTFILSCAPCNVICSIIFQNRFDYKDQDFLTFMKK
             V   V           S    T                S                              G                    K
                 Y               D       A  S      S                                                 T        E

        201 VNENVRILSSPWLQVCNNFPSLIDYCPGSHHKITKNVNYLKKYILEKIEEHQESLDIENPRDFIDYYLIKLKQANHNQQSEFSLENLTTTVSDLFGAGTE
            L   A       F       L          R    I  IRS LS   MK      VA               G Y       P   A       A
                                           T       IRS LS    K   T  VA               G Y      T    A   R   A

        301 TTSTTLRYALLLLLKHPHVTAKVQEEIDQVVGRHRKPCMQDRSHMPYTDAMIHEVQRFIDLIPISLPHAVTCDIKFRDYFIPKGTTVITSLSSVLHDNKE
                                               N                           TN                     I          S
                                               N                           TN                     I          S

        401 FPNPEVFDPGHFLDKNGNFKKSDYFMPFSAGKRICAGEGLARMELFLFLTTILQNFKLKSMIHPKDIDTTPVVNGFASLPPSYQLCFIPV   490
                                         T   M                          LV          L
                             K           T   M                          LV             L
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Fig. 3. Alignment of the deduced amino acid sequences of CYP2C25, CYP2C26, and CYP2C27. The complete amino acid sequence of CYP2C25 and those of CYP2C26 and CYP2C27 different from CYP2C25 are displayed. A conserved cysteinyl residue is *boxed*. Other conserved residues and regions are *underlined*, as discussed in the text.

TABLE 1

Comparison of the nucleotide and deduced amino acid sequences of members of the *CYP2C* gene subfamily in hamsters and rats

The upper values represent percentage identity of nucleotides and the lower values percentage identity of deduced amino acids. The values for nucleotides were obtained by calculations including both coding and noncoding regions. Rat CYP2C nucleotide and amino acid sequences were taken from the following references: 2C6 (32), 2C7 (33), 2C11 (19), 2C12 (34), 2C13 (35), 2C22 (36), 2C23 (37), and 2C24 (38).

	Identity									
	Hamster		Rat							
	2C26	2C27	2C6	2C7	2C11	2C12	2C13	2C22	2C23	2C24
						%				
Hamster	91.8	91.8	84.0	80.2	72.1	73.4	73.7	64.8	60.7	71.5
2C25	90.4	90.2	81.2	79.8	74.5	68.8	69.8	62.9	57.7	72.5
2C26		94.1	81.2	80.6	71.5	72.9	73.8	65.2	61.3	70.9
		93.6	82.1	79.3	74.4	70.8	70.6	60.6	58.7	74.1
2C27			84.5	80.1	72.6	72.4	74.5	65.7	61.5	71.2
			82.5	77.9	73.5	69.3	71.1	61.0	57.8	72.7

sequence of each hamster cDNA with other *CYP2C* cDNAs from hamsters and rats (19, 32–38). The three hamster *CYP2C* cDNAs showed high levels of identity. The identities were 91.8–94.1% at the nucleotide sequence level and 90.2–93.6% at the deduced amino acid sequence level. Comparison of the hamster cDNAs with rat *CYP2C* showed an overall nucleotide sequence identity between 60.6 and 84.5%.

Expression of hamster CYP2C forms in yeast cells. To confirm that the newly isolated cDNA clones code for cytochrome P450 and to determine whether the encoded cytochromes P450 are catalytically active, it was necessary to express these cDNAs. For these purposes, the recombinant plasmids pAM1 (for CYP2C25), pAM2 (for CYP2C26), and pAM3 (for CYP2C27) and vector pAAH5 were introduced into the yeast *S. cerevisiae* AH22. The yeast strains carrying these plasmids were designated as AH22/pAM1, AH22/pAM2, AH22/pAM3, and AH22/pAAH5. The expression of cytochrome P450 protein in these strains of yeast was examined by Western blot analysis using antibodies raised against cytochrome P450-male (CYP2C11). As shown in Fig. 4, proteins immunoreactive with the antibodies were detectable in all recombinant yeasts except AH22/pAAH5, a negative control. The apparent molecular weights of the expressed proteins were slightly different from the calculated molecular weights. The molecular weight of the expressed CYP2C27 protein should be slightly greater than that of CYP2C26, whereas the actual

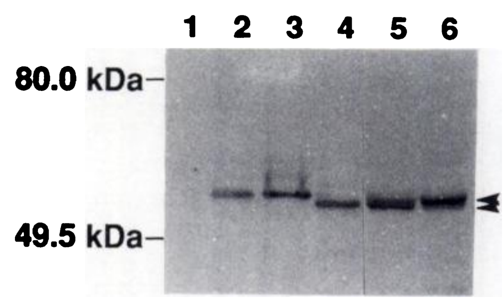

Fig. 4. Immunoblot analysis of microsomes from transformed yeast cells and from hamster livers with anti-cytochrome P450-male (CYP2C11) antibodies. *Lane 1*, AH22/pAAH5; *lane 2*, AH22/pAM1 (CYP2C25); *lane 3*, AH22/pAM2 (CYP2C26); *lane 4*, AH22/pAM3 (CYP2C27); *lane 5*, male hamster livers; *lane 6*, female hamster livers. *Arrowheads*, two immunoreactive bands in hamster microsomes. Each lane contained 20 μg of microsomal proteins.

molecular weight of CYP2C26 was greater than that of CYP2C27 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The reason for this difference is not clear as yet. The presence of holo-cytochrome P450 protein in the microsomal fractions of the recombinant yeasts was examined by reduced carbon monoxide difference spectra. An absorption peak at 450 nm was observed in the spectra of the yeast strains transformed with recombinant expression plasmids (data not shown). Based on the reduced carbon monoxide difference

spectra, the quantity of cytochrome P450 in microsomes from the recombinant yeasts was estimated to be 0.109, 0.136, and 0.078 nmol/mg of protein for AH22/pAM1, AH22/pAM2, and AH22/pAM3, respectively.

Monooxygenase activity of the expressed enzymes. The catalytic activities of microsomes prepared from recombinant yeasts were examined. As shown in Table 2, all of the hamster CYP2C forms catalyzed the *N*-demethylation of aminopyrine and benzphetamine and the hydroxylation of tolbutamide. Although these forms of cytochrome P450 showed high levels of identity in the primary structure, we found substantial differences in the catalytic properties of these forms of cytochrome P450. CYP2C25 and CYP2C26 showed high *N*-demethylase activities for both aminopyrine and benzphetamine. CYP2C27 showed low levels of activity for benzphetamine, in contrast to high activity for aminopyrine. CYP2C25 was the only form that showed testosterone hydroxylase activity. Identification of the testosterone metabolite formed by CYP2C25 was carried out by two methods, HPLC and TLC. The testosterone metabolite formed was different from authentic 2α,2β,6β,7α,16α-hydroxytestosterone, 1-dehydroxytestosterone, androstenedione, and testosterone, as judged by chromatographic behavior. The testosterone metabolite formed by CYP2C25 co-migrated with 16β-hydroxytestosterone.

Gender-dependent expression of cytochrome P450 in hamster livers and extrahepatic tissues. To estimate the relative amounts of mRNA for individual forms of cytochrome P450, Northern blot analysis was carried out with 5′-end-labeled oligonucleotides oli-2C25, oli-2C26, and oli-2C27 as probes. The specificities of these probes were examined by Southern blot analysis. Two nanograms of plasmid DNA containing an insert cDNA were slot-blotted onto a nitrocellulose membrane. Each oligonucleotide probe hybridized specifically with the corresponding cDNA (data not shown). Northern blot analysis for the expression of each form of cytochrome P450 was performed with 20 μg of total RNA prepared from livers, kidneys, lungs, and brains of male and female hamsters. The quantity of total RNA blotted was also monitored by the intensity of hybridized β-actin mRNA bands. The results are shown in Fig. 5. When oli-2C27 was used as a probe, hybridized bands were detected clearly in livers of both sexes and kidneys of males, whereas no hybridized bands were observed in the other tissues tested. This result indicates that the expression of CYP2C27 in kidneys is male specific. The intensities of bands in livers were also much higher in males than in females; the relative intensities of hybridized bands were 5-fold higher in males than in females, as measured with a Bio Image analyzer (Fuji Film). When oli-2C25 and oli-2C26 were used as probes, bands near 18 S rRNA were detected in livers of both sexes. No apparent sex-related differences in the expression of these forms of cytochrome P450 were observed. The expression of CYP2C25, CYP2C26, and CYP2C27 was reexamined by S1 nuclease protection assay. It has been thought that this assay shows higher selectivity than Northern blot analysis. When probes for CYP2C25 and CYP2C26 were used, the visualized bands, which corresponded to protected probes, were recognized at comparable levels in both sexes; no apparent sex differences were seen (data not shown). When probes for CYP2C27 were used, protected bands were seen in both sexes, although very faint bands existed around the predicted bands (Fig. 6). The

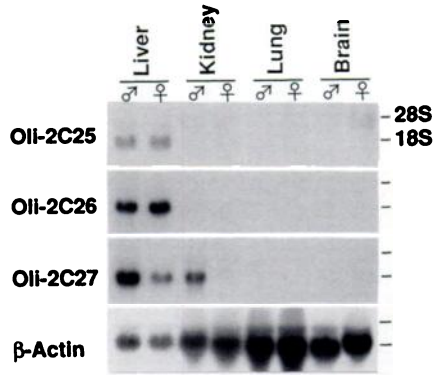

Fig. 5. Northern blot analysis of hamster CYP2C forms. Twenty micrograms of total RNA from various tissues from 8-week-old male and female hamsters were used. Typical results from three repeated experiments are shown.

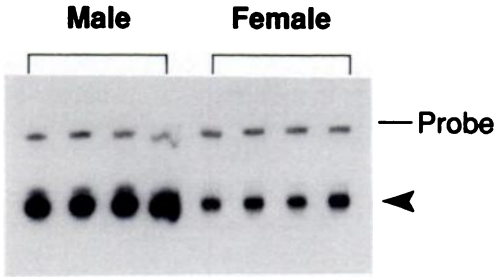

Fig. 6. S1 nuclease protection analysis of the relative amounts of mRNAs coding for CYP2C27 in male and female hamsters. A DNA probe encompassing positions 1203–1297 of *CYP2C27* was used. After being digested with S1 nuclease, RNA-DNA hybrids were electrophoresed on a polyacrylamide gel under denaturing conditions, and then the polyacrylamide gel was dried and exposed to X-ray films for 72 hr. *Arrowhead*, band corresponding to the protected DNAs. Total RNA from individual animals was applied to each lane.

TABLE 2

Drug- and steroid hormone-metabolizing activities of microsomes from recombinant yeast cells

Each value represents the mean of duplicate determinations.

	Activity			
	AH22/pAM1 (CYP2C25)	AH22/pAM2 (CYP2C26)	AH22/pAM3 (CYP2C27)	AH22/pAAH5
	nmol/min/nmol of P450			
Aminopyrine *N*-demethylation	4.92	4.48	2.85	—[a]
Benzphetamine *N*-demethylation	3.17	3.98	0.18	ND[b] (<0.09)
Tolbutamide 4-hydroxylation	0.075	0.096	0.057	ND (<0.002)
Testosterone 16β-hydroxylation	0.415	ND	ND	ND (<0.02)

[a] —, not calculated because of the low content of cytochrome P450 in AH22/pAAH5 yeast.
[b] ND, not detectable.

intensity of the major bands in males was considerably higher than that of the bands in females. Relative intensities of visualized bands for CYP2C27, estimated by densitometer (Quick Scan R & D, Helena Laboratories, Beaumont, TX), were about 5-fold higher in males than in females.

Developmental changes in the level of expression of hamster cytochrome P450. It is well known that the expression of most sex-specific cytochromes P450 changes with development after birth. To determine whether this occurs for hamster cytochromes P450, the level of expression was measured, by Northern blot analysis, periodically for 8 weeks after birth. As shown in Fig. 7, the expression of CYP2C25, CYP2C26, and CYP2C27 mRNA was found to be developmentally altered. In the case of CYP2C25 and CYP2C26, the levels of mRNA were not detectable at 1 week after birth and then increased up to about 3 weeks. The levels of both forms were suppressed. A similar pattern of developmental change in the level of mRNA was seen for CYP2C27 in female hamsters. However, a different pattern was seen for CYP2C27 in males. The level of mRNA for CYP2C27 in males increased rapidly up to 4 weeks and then gradually up to 8 weeks. As a result, a significant sex-related difference in CYP2C27 levels was observed beginning 4 weeks after birth.

Discussion

In the present study, we isolated three cDNA clones for *CYP2C*, indicating that there are multiple forms of the *CYP2C* gene subfamily in hamsters, as has been seen in rats, rabbits, and humans (17). Interestingly, all of the isolated cDNAs showed very high identity, i.e., 91.8–94.1% at the nucleotide sequence level and 90.2–93.6% at the deduced amino acid sequence level. This is the first report to show that one animal species possesses three very similar CYP2C forms. The mechanism for evolution of the hamster CYP2C subfamily is unclear but it is presumably the result of species-specific gene duplication and gene conversion events, as has been reported for rat CYP2A (39). Recently, conserved and variable regions of cytochrome P450 have been proposed by comparison of aligned sequences, based on the crystal coordinates of cytochrome P450cam (CYP101) (40). We found that some of the proposed sequences were also present in hamster CYP2C forms (Fig. 3). The most conserved motif (FxxGxxxCxG) (41), including a cysteinyl residue, the thiol group of which serves as the fifth ligand of heme iron, was located at positions 428–437. A putative recognition motif (RRxS) for phosphorylation by cAMP-dependent kinase was located at positions 124–127. The phosphorylation of rat phenobarbital-inducible cytochrome P450 protein at corresponding positions has been proven *in vivo* and *in vitro* (42, 43). This motif has been found specifically in forms of the *CYP2* gene family and thus can be regarded as a family-specific regulatory element (42). No phosphorylation of cytochrome P450 in hamster liver has been reported, but regulation by phosphorylation might be possible for the hamster CYP2C proteins, as has been observed in other animal species. In addition, a threonine-serine cluster and a tryptophan residue were also seen at residues 295–306 and position 120, respectively, in the hamster CYP2C forms.

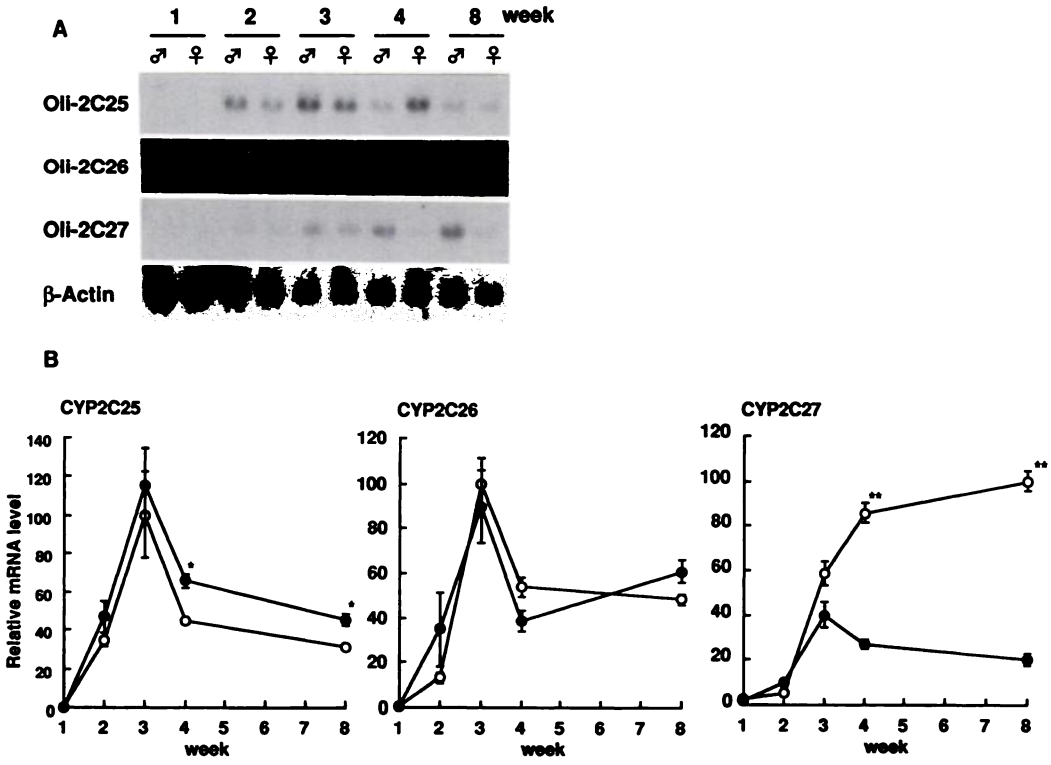

Fig. 7. Changes in the levels of mRNA for CYP2C forms during development. Total RNA was prepared from the livers of male and female hamsters at 1, 2, 3, 4, and 8 weeks after birth. Twenty micrograms of total RNA fraction were subjected to Northern blot analysis. Typical results are shown in A. The intensity of hybridized bands was measured with a Bio Image analyzer (Fuji Film) and is plotted in B. Values are the mean ± standard error from four male (○) and female (●) hamsters. *, Significant difference between male and female hamsters, $p < 0.005$; **, $p < 0.0001$. The expression levels at 3 weeks (CYP2C25 and CYP2C26) or 8 weeks (CYP2C27) in male hamsters were assigned as 100%.

In a previous paper, we showed results supporting the hypothesis that there are male- and female-predominant forms of cytochrome P450 in hamster liver microsomes (18). We found proteins that were cross-reactive with anti-cytochrome P450-male (CYP2C11) antibodies in Western blot analysis. This was the first reported result demonstrating the existence of a sex difference in cytochrome P450 in an animal species other than rats or mice. The purpose of the present study was to establish the sex-related difference in the expression of CYP2C in hamsters at a molecular level. Based on the results of the Northern blot analysis and S1 nuclease protection assay, it appeared that the mRNA level of CYP2C27 was higher in males than in females. Furthermore, by Western blot analysis (Fig. 4) the CYP2C27 protein expressed in yeast co-migrated with the male-predominant protein, the fastest migrating immunoreactive band in liver microsomes from male hamsters, as reported previously (18). The results of the present study indicate that sex-related differences also exist in the hamster CYP2C subfamily, and the male-predominant cytochrome P450 would be CYP2C27. Recently, Teixeira and Gil (44) reported that hamster lithocholic acid 6β-hydroxylase is a cytochrome P450 belonging to the *CYP3A* gene subfamily, designated as *CYP3A10*; its expression was male specific. In addition, Henderson *et al.* (45) have demonstrated the presence in rabbit kidneys of a male-dominant form of cytochrome P450 that cross-reacted with anti-CYP3A sera. Thus, it seems likely that the sex-related difference is not restricted to rats and mice.

With respect to the change of expression level during development, it was of interst to note that the expression of CYP2C25 and CYP2C26 but not CYP2C27 was changed similarly. Although a large number of studies have focused on the mechanism responsible for the sex-related difference in cytochrome P450, a clear explanation is still lacking. To explore this sex difference in CYP2C27 further, we are currently investigating the effects of sex hormones and growth hormone on the expression level of CYP2C27.

Studies in various tissues showed that only CYP2C27 was expressed in the kidney of male hamsters, but not in female ones. Regarding the physiological functions of renal cytochrome P450 in rats, immunological studies have indicated that the renal arachidonic acid epoxygenases belong to the *CYP2C* gene family (46). Therefore, it is expected that CYP2C27 has significant physiological functions in kidneys.

Another point of interest is the catalytic properties of the CYP2C isozymes. This is the first report to characterize members of the hamster CYP2C subfamily. In spite of their high levels of identity in primary structure, the expressed enzymes showed different substrate specificities.

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References

1. Guengerich, F. P. Reaction and significance of cytochrome P-450 enzymes. *J. Biol. Chem.* **266**:10019–10022 (1991).
2. Gonzalez, F. J., S.-Y. Liu, and M. Yano. Regulation of cytochrome P450 genes: molecular mechanisms. *Pharmacogenetics* **3**:51–57 (1993).
3. Kamataki, T., K. Maeda, Y. Yamazoe, T. Nagai, and R. Kato. Sex difference of cytochrome P-450 in the rat: purification, characterization, and quantitation of constitutive forms of cytochrome P-450 from liver microsomes of male and female rats. *Arch. Biochem. Biophys.* **225**:758–770 (1983).
4. Kamataki, T., M. Shimada, K. Maeda, and R. Kato. Pituitary regulation of sex-specific forms of cytochrome P-450 in liver microsomes of rats. *Biochem. Biophys. Res. Commun.* **130**:1247–1253 (1985).
5. Yamazoe, Y., M. Shimada, T. Kamataki, and R. Kato. Effects of hypophysectomy and growth hormone treatment on sex-specific forms of cytochrome P-450 in relation to drug and steroid metabolisms in rat liver microsomes. *Jpn. J. Pharmacol.* **42**:371–382 (1986).
6. Gonzalez, F. J. The molecular biology of cytochrome P450s. *Pharmacol. Rev.* **40**:243–288 (1988).
7. Waxman, D. J. Interactions of hepatic cytochrome P-450 with steroid hormones: regioselectivity and stereospecificity of steroid metabolism and hormonal regulation of rat P-450 enzyme. *Biochem. Pharmacol.* **37**:71–84 (1988).
8. Noshiro, M., and M. Negishi. Pretranslational regulation of sex-dependent testosterone hydroxylase by growth hormone in mouse liver. *J. Biol. Chem.* **261**:15923–15927 (1986).
9. Noshiro, M., M. Lakso, K. Kawajiri, and M. Negishi. *Rip* locus: regulation of female-specific isozyme (I-P45016α) of testosterone 16α-hydroxylase in mouse liver, chromosome localization, and cloning of P450 cDNA. *Biochemistry* **27**:6434–6443 (1988).
10. Noshiro, M., and M. Negishi. Genetic regulation of estrogen-dependent repression of female-specific testosterone 16α-hydroxylase (I-P45016α) in male mouse liver: murine *Ripr* locus. *Biochemistry* **27**:6444–6448 (1988).
11. Litterst, C. L., E. G. Mimnaugh, R. L. Reagan, and T. E. Gram. Comparison of *in vitro* drug metabolism by lung, liver, and kidney of several common laboratory species. *Drug Metab. Dispos.* **3**:259–265 (1975).
12. Mass, M. J., and D. Kaufman. A comparison between the activation of benzo[*a*]pyrene in organ cultures and microsomes from the tracheal epithelium of rats and hamsters. *Carcinogenesis (Lond.)* **4**:297–303 (1983).
13. Homburger, F. Chemical carcinogenesis in Syrian hamsters: a review through 1976. *Prog. Exp. Tumor Res.* **23**:100–129 (1979).
14. Henry, M. C., C. D. Port, R. R. Bates, and D. G. Kaufman. Respiratory tract tumors in hamsters induced by benzo[*a*]pyrene. *Cancer Res.* **33**:1585–1592 (1973).
15. Chiang, J. Y. L., and A. W. Steggles. Identification and partial purification of hamster microsomal cytochrome P-450 isoenzymes. *Biochem. Pharmacol.* **32**:1389–1397 (1983).
16. Puccini, P., S. Menicagli, V. Longo, A. Santucci, and P. G. Gervasi. Purification and characterization of an acetone-inducible cytochrome P-450 from hamster liver microsomes. *Biochem. J.* **287**:863–870 (1992).
17. Nelson, D. R., T. Kamataki, D. J. Waxman, F. P. Guengerich, R. W. Estabrook, R. Feyereisen, F. J. Gonzalez, M. J. Coon, I. C. Gunsalus, O. Gotoh, K. Okuda, and D. W. Nebert. The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol.* **12**:1–51 (1993).
18. Miura, T., M. Komori, M. Iwasaki, K. Kurozumi, K. Ohta, S. Ohmori, M. Kitada, and T. Kamataki. Sex-related difference in oxidative metabolism of testosterone and erythromycin by hamster liver microsomes. *FEBS Lett.* **231**:183–186 (1988).
19. Yoshioka, H., K. Morohashi, K. Sogawa, T. Miyata, K. Kawajiri, T. Hirose, S. Inayama, Y. Fujii-Kuriyama, and T. Omura. Structural analysis and specific expression of microsomal cytochrome P-450(M-1) mRNA in male rat livers. *J. Biol. Chem.* **262**:1706–1711 (1987).
20. Sambrook, J., E. F. Fritsch, and T. Maniatis. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).
21. Ito, H., Y. Fukuda, K. Murata, and A. Kimura. Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153**:163–168 (1983).
22. Oeda, K., T. Sakaki, and H. Ohkawa. Expression of rat liver cytochrome P-450MC cDNA in *Saccharomyces cerevisiae*. *DNA* **4**:203–210 (1985).
23. Omura, T., and R. Sato. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**:2379–2385 (1964).
24. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
25. Nagayama, S., R. Kitamura, T. Yokoi, Y. Kawaguchi, N. Kasai, N. Takeichi, H. Kobayashi, and T. Kamataki. Occurrence of autoimmune antibodies to liver microsomal proteins in association with fulminant hepatitis in the LEC strain of rats. *Biochem. Biophys. Res. Commun.* **179**:1134–1140 (1991).
26. Nash, T. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.* **55**:416–421 (1953).
27. Hayashi, S., M. Noshiro, and K. Okuda. Isolation of a cytochrome P-450 that catalyzes the 25-hydroxylation of vitamin D3 from rat liver microsomes. *J. Biochem. (Tokyo)* **99**:1753–1763 (1986).
28. Waxman, D. J. P450-catalyzed steroid hydroxylation: assay and product identification by thin-layer chromatography. *Methods Enzymol.* **206**:462–476 (1991).
29. Church, M. G., and W. Gilbert. Genomic sequencing. *Proc. Natl. Acad. Sci. USA* **81**:1991–1995 (1984).
30. Nakajima-Iijima, S., H. Hamada, P. Reddy, and T. Kakunaga. Molecular structure of the human cytoplasmic β-actin gene: interspecies homology of sequences in the introns. *Proc. Natl. Acad. Sci. USA* **82**:6133–6137 (1985).
31. Taniguchi, H., Y. Imai, T. Iyanagi, and R. Sato. Interaction between NADPH-

cytochrome P-450 reductase and cytochrome P-450 in the membrane of phosphatidylcholine vesicles. *Biochim. Biophys. Acta* **550**:341–356 (1979).
32. Gonzalez, F. J., S. Kimura, B.-J. Song, J. Pastewka, H. V. Gelboin, and J. P. Hardwick. Sequence of two related P-450 mRNAs transcriptionally increased during rat development. *J. Biol. Chem.* **261**:10667–10672 (1986).
33. Kimura, H., H. Yoshioka, K. Sogawa, Y. Sakai, and Y. Fujii-Kuriyama. Complementary DNA cloning of cytochrome P-450s related to P-450(M-1) from the complementary DNA library of female rat livers. *J. Biol. Chem.* **263**:701–707 (1988).
34. Zaphiropoulos, P. G., A. Mode, A. Strom, C. Moller, C. Fernandez, and J.-A. Gustafsson. cDNA cloning, sequence, and regulation of a major female-specific and growth hormone-inducible rat liver cytochrome P-450 active in 15β-hydroxylation of steroid sulfates. *Proc. Natl. Acad. Sci. USA* **85**:4214–4217 (1988).
35. McClellan-Green, P. D., M. Negishi, and J. A. Goldstein. Characterization of a cDNA for rat P-450g, a highly polymorphic, male-specific cytochrome in the P-450IIC subfamily. *Biochemistry* **28**:5832–5839 (1989).
36. Nagata, K., H. Sasamura, M. Miyata, M. Shimada, Y. Yamazoe, and R. Kato. cDNA and deduced amino acid sequences of a male dominant P-450Md mRNA in rats. *Nucleic Acids Res.* **18**:4934 (1990).
37. Cook, E. A., W. A. Groenewegen, I. S. Gloger, J. R. Piggott, K. E. Suckling, and C. R. Wolf. cDNA and deduced amino acid sequence of a novel cytochrome P-450 from female rat liver mRNA with high homology to P-450 IIC family. *Nucleic Acids Res.* **18**:7156 (1990).
38. Zaphiropoulos, P. G. cDNA cloning and regulation of a novel rat cytochrome P450 of the 2C gene subfamily (P450IIC24). *Biochem. Biophys. Res. Commun.* **180**:645–651 (1991).
39. Matsunaga, T., K. Nagata, E. J. Holsztynska, D. P. Lapenson, A. J. Smith, R. Kato, H. V. Gelboin, D. J. Waxman, and F. J. Gonzalez. Gene conversion and differential regulation in the rat P-450IIA gene family: purification, catalytic activity, cDNA and deduced amino acid sequence, and regulation of an adult male-specific hepatic testosterone 15α-hydroxylase. *J. Biol. Chem.* **263**:17995–18002 (1988).
40. Nelson, D. R., and H. W. Strobel. Secondary structure prediction of 52 membrane-bound cytochromes P450 shows a strong structural similarity to P450cam. *Biochemistry* **28**:656–660 (1989).
41. Black, S. D., and M. J. Coon. Comparative structure of P-450 cytochromes, in *Cytochrome P-450* (P. R. Ortiz de Montellano, ed.). Plenum Publishing Corp., New York, 161–216 (1986).
42. Pyerin, W., and H. Taniguchi. Phosphorylation of hepatic phenobarbital-inducible cytochrome P-450. *EMBO J.* **8**:3003–3010 (1989).
43. Koch, J. A., and D. J. Waxman. Posttranslational modification of hepatic cytochrome P-450: phosphorylation of phenobarbital-inducible P-450 forms PB-4 (IIB1) and PB-5 (IIB2) in isolated rat hepatocytes and *in vivo*. *Biochemistry* **28**:3145–3152 (1989).
44. Teixeira, J., and G. Gil. Cloning, expression, and regulation of lithocholic acid 6β-hydroxylase. *J. Biol. Chem.* **266**:21030–21036 (1991).
45. Henderson, C. J., A. R. Scott, C. S. Yang, and C. R. Wolf. Testosterone-mediated regulation of mouse renal cytochrome P-450 isoenzymes. *Biochem. J.* **266**:675–681 (1990).
46. Capdevila, J. H., S. Wei, J. Yan, A. Karara, H. R. Jacobson, J. R. Falck, F. P. Guengerich, and R. N. DuBois. Cytochrome P-450 arachidonic acid epoxygenase: regulatory control of the renal epoxygenase by dietary salt loading. *J. Biol. Chem.* **267**:21720–21726 (1992).

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