FUNCTIONAL PROPERTIES OF A RAINBOW TROUT CYP3A27 EXPRESSED BY RECOMBINANT BACULOVIRUS IN INSECT CELLS

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ABSTRACT:
Cytochrome P450 3A27 (CYP3A27) is highly expressed in liver and intestine of rainbow trout (Oncorhynchus mykiss). In many animal species, the intestine and liver are responsible for the first-pass metabolism of a wide range of xenobiotics. To help determine its physiological role, the catalytic capabilities of CYP3A27 protein were examined. An open reading frame of CYP3A27 in pFastBac donor plasmid was transferred to the baculovirus genome (bacmid) through Tn7 site-specific transposition in DH10Bac competent cells. The CYP3A27 cDNA was positioned under the control of the polyhedrin promoter of the Autographa californica nuclear polyhedrosis virus. The recombinant baculovirus containing a full-length CYP3A27 cDNA (Bv-3A27) was then transfected into Spodoptera frugiperda (Sf9) insect cells for overexpression of CYP3A27 protein. The expressed CYP3A27 protein (714 pmol/mg total protein) exhibited a maximum CO-reduced spectrum at 450 nm at 72 h postinfection after addition of 1 µg/ml exogenous hemin. The expressed CYP3A27 protein comigrated with the purified trout LMC5 cytochrome P450 (P450) and was recognized by anti-P450 LMC5 IgG on Western blot analysis. The expressed CYP3A27 protein was reconstituted with human NADPH-cytochrome P450 reductase and cytochrome b5. The reconstitution system showed catalytic activities for the 6β-, 2β-, and 16β-hydroxylation of testosterone at 1.42, 0.043, 0.034 nmol/min/nmol CYP3A27, respectively, and the dehydration of nifedipine at 50 pmol/min/nmol CYP3A27. The present results demonstrated that the baculovirus system is useful for the production of the functional aquatic CYP3A form and that CYP3A27 has the capability to metabolize steroid hormone as reported for mammalian CYP3A forms.

The cytochromes P450 are a heme-containing superfamily of enzymes that play important roles in the phase I metabolism of foreign compounds, such as toxic pollutants and drugs. Some of cytochrome P450s catalyze the oxidation of endogenous substrates, including steroid hormone, fatty acids, and prostaglandins (Guengerich, 1991; Porter and Coon, 1991; Waxman, 1999). The cytochrome P450 3A (CYP3A) family is involved in the oxidation of a broad range of structurally diverse foreign compounds and endogenous steroid hormones in mammals (Guengerich, 1999). This family has been distinguished from other cytochromes P450 in that they are the most abundantly expressed forms in liver and intestine (Kaminsky and Fasco, 1992; Kolars et al., 1994) and the major cytochrome P450 form involved in the metabolism of xenobiotics (Guengerich, 1999) and the 6β-hydroxylation of steroids (Waxman et al., 1985, 1988).

It is reported that there are at least 17 families of cytochrome P450s in mammals, which may contain 50 to 60 different cytochrome P450 genes in a given species (Nelson et al., 1996; Nelson, 1999). Several members of the CYP3A subfamily have been cloned, including four forms from humans (CYP3A4, CYP3A5, CYP3A7, and CYP3A43), five forms from rats (CYP3A1, CYP3A2, CYP3A9, CYP3A18, and CYP3A23), and four forms from mice (Cyp3a11, Cyp3a13, Cyp3a16, and Cyp3a25) (Nelson et al., 1996; Nelson, 1999). Prediction of the precise function of a single cytochrome P450 isoform after its purification and subsequent assays with different substrates has been a complex problem due to the difficulties of purification that arise from the lipophilic character of the membrane-bound cytochrome P450 forms and the overlapping substrate specificities between different cytochrome P450s. The accumulated cytochrome P450 sequence data cannot rule out the possibility that a single band on SDS-polyacrylamide gel electrophoresis (PAGE) after cytochrome P450 purification may contain more than one form, proving the complexity of isolation of closely related cytochrome P450 forms. Because there are no clear protocols available to separate one specific isoform from closely related CYP3A forms in microsomes, cDNA-directed heterologous expression systems have been applied to obtain a large amount of pure enzyme source for the study of catalytic properties (Buters et al., 1994; Gonzalez and Korzekwa, 1995; Lee et al., 1995).

There have been growing issues in aquatic contamination resulting from a rapid industrial development and the increased use of agricultural chemicals. Rainbow trout has been an extremely useful model for the toxicology and cancer study because of their sensitivity to chemicals (Bailey et al., 1996; Buhler and Wang-Buhler, 1998).
Seven different cytochrome P450 families with 11 cDNAs have been reported from the rainbow trout (Buhler and Wang-Buhler, 1998). In a previous study, we reported the cloning of a new CYP3A form, CYP3A27, from rainbow trout (Lee et al., 1998). CYP3A27 was obtained by cDNA library screening using the antibodies generated against purified trout cytochrome P450 LMC5, which displayed significant activity against steroids and reacted with the antibodies of human CYP3A4 (Miranda et al., 1989, 1991). Because members of the CYP3A family in mammals are involved in the steroid hormone metabolism and their expressions are affected by endocrine-disrupting chemicals (Kliewer et al., 1998; Buhler et al., 2000; Masuyama et al., 2000), the characterization of CYP3A forms from fish may provide a critical basis for the environmental monitoring. The aim of this study is to determine the catalytic activity of a rainbow trout CYP3A27 protein after baculovirus expression of the CYP3A27 cDNA. This is the first report of a functional fish CYP3A expression in Spodoptera frugiperda (Sf9) cells. A functional study of CYP3A27 gene would provide useful information for the comparative metabolism as well as for the environmental toxicology.

Materials and Methods

Materials. Testosterone, 1α,2-dilauroyl-sn-glycerol-3-phosphatidyl cholines, 1α,2-dioleoyl-sn-glycerol-3-phosphatidyl cholines, and phosphatidyl serine, 1α-phosphatidylcholine (dioleoyl), Lubrol PX, nifedipine, hemin chloride, and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). Nifedipine metabolite was from Sigma/RBI (Natick, MA). 2β,6β, 7α-, and 16β-Hydroxysterosterone were from Steraloids (Wilton, NH). X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside), DH5α Escherichia coli competent cells, DH10Bac competent cells, SF90-III SFM, pSPORT1 vector, pFastBac vector, genticamy cin, ampicillin, kanamycin, restriction endonucleases, and all Bac-to-Bac Baculovirus Expression System were from Invitrogen (Carlsbad, CA). The Sf9 insect cell was a gift from Dr. G. F. Rohrmann (Department of Microbiology, Oregon State University, Corvalis, OR). Human NADPH-cytochrome P450 oxidoreductase and cytochrome bs2 were purchased from Oxford Biomedical Research (Oxford, MI). 125I-Protein A was obtained from ICN Radiochemicals (Irvine, CA). Immobilon-P membrane was from Millipore Corporation (Bedford, MA), and reagents used in SDS-PAGE were from Amersham Biosciences (Piscataway, NJ). All other chemicals and organic solvents for HPLC were of the highest grade from commercial sources.

Constructions of Recombinant Baculovirus. The pSPORT1 containing CYP3A27 cDNA and the pFastBac vector were digested with EcoRI and NotI. The digested DNA fragment containing the CYP3A27 open reading frame and the linearized pFastBac vector was isolated after agarose gel electrophoresis and ligated into the pFastBac vector through EcoRI and NotI sites. The recircularized pFastBac plasmid was used to transform DH5α E. coli competent cells, and colonies containing the recombinant construct (pFastBac-3A27) were identified with restriction analysis. CYP3A27 cDNA in the pFastBac vector was used under the control of the polyhedrin promoter and this recombinant donor vector was transposed into DH10Bac competent cells that contain the bacmid DNA and the helper plasmid. The bacmid DNA contains a mini-F replicon, a kanamycin resistance marker, and a segment DNA encoding the laezu peptide from a PUC-based cloning vector. Because the insertion of a short segment containing the Tn7 attachment site for the bacterial transposon Tn7 in the N terminus of the laezu gene on the bacmid DNA does not disrupt the reading frame of the laezu peptide, the propagation of recombinant bacmid in E. coli DH10Bac was identified by white color in the presence of a chromogenic substrates (Blu-gal). The site-specific transposition of CYP3A27 cDNA fragment into the bacmid DNA, a baculovirus shuttle vector, was obtained by transposing a mini-Tn7 element from pFastBac-3A27 donor plasmid to the mini-attTn7 attachment site on the bacmid when the Tn7 transposition functions were provided by helper plasmid (Luckow et al., 1993). Before isolating the recombinant bacmid DNA, the candidate colonies were streaked in the presence of X-gal to ensure they were truly white. The isolated Bac-3A27 was confirmed with PCR by using CYP3A27-specific primers (forward primer: 5’ TCT TCT ACC CTG CTG AGC 3’ and reverse primer: 5’ GAA ACT CTG GAG GAT CTC 3’ for 95 base pairs of PCR product) and M13/pUC forward and reverse primers that exist near the insertion sites on bacmid DNA (forward primer: 5’ TGT AAA ACG ACG GCC AGT 3’ and reverse primer: 5’ CAG GAA ACA GCT ATG ACC 3’ for 4.1 kb of PCR product). The scheme of the procedures is diagrammed in Fig. 1.

Transfection and Amplification of Bv-3A27. Sf9 cells were maintained in SF-900II SFM containing 50 μg/ml ampicillin in sterile disposable plastic flasks at 27°C. Sf9 cells (1 × 10^6 cells/ml) in 2 ml of SF-900 SFM containing penicillin/streptomycin at 0.5× final concentration (50 units/ml penicillin and 50 μg/ml streptomycin) were seeded into the flat-bottomed cell wells (well diameter 22 mm) and placed in the incubator at 27°C for 2 h to allow cells to attach. Cells were washed twice with SF-900 SFM without antibiotics and attached Sf9 cells were transfected with the 3 μg of Bv-3A27 via Cell FECTIN Reagent in SF-900 SFM without antibiotics (in total volume 0.8 ml). After 5 h of infection, the transfection mixture was removed and SF-900 SFM containing 50 μg/ml ampicillin (total volume 1.5 ml) was added for 72 h. The supernatant solution was collected at 72 h post-transfection and added in the prepared Sf9 cells at 2 × 10^6 cells/ml for 5 days. After 5 days for the Bv-3A27 amplification, the supernatant was removed and 5 ml of the supernatant was used to infect 45 ml of Sf9 cells seeded at a density of 2 × 10^6 cells/ml for 5 days. The amplified Bv-3A27 supernatant was harvested and used for CYP3A27 protein expression analysis.

Expression of CYP3A27 in Sf9 Cells. Sf9 insect cells were maintained in SF-900II SFM containing 50 μg/ml ampicillin to a density of 2 × 10^6 cells/ml. The amplified Bv-3A27 at a multiplicity of infection of about 1 plaque-forming unit/cell [1 ml of Bv-3A27 to 9 ml of cells (v/v) ratio] was used to infect cells. After 24 h infection, 0 to 3 μg/ml hemin chloride [50/50 (v/v), 0.1 N NaOH/100% ethanol] was added to compensate for low endogenous level of heme in insect cells (Gonzalez et al., 1991). The infections were allowed for 24 to 96 h postinfection. Cells were harvested, washed twice with cold phosphate-buffered saline, and lysed with 15 to 20 strokes in a tightly fitting glass Dounce homogenizer in homogenation buffer (0.1 M potassium phosphate buffer, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethysulfonyl fluoride, and 20% glycerol). The cell lysates were centrifuged at 100,000g for 60 min to separate membrane fractions from soluble fraction. The membrane fraction was resuspended in the cytochrome P450 buffer (0.1 M potassium phosphate buffer, pH 7.4, 1 mM EDTA, and 20% glycerol) and stored at -80°C until use. CYP3A27 protein expression was verified with Western blotting. Total cytochrome P450 content was measured by CO difference spectrometry in samples collected at different times (Omura and Sat0, 1964).

Western Blot Analysis of Expressed CYP3A27 Protein. Proteins (10 μg/lane) were separated on 7.5% acrylamide gels containing SDS. The proteins were transferred to a nitrocellulose membrane. The membranes were blocked with 2% bovine serum albumin in 10 mM Tris/HCl, pH 7.4, including 0.9% NaCl (TBS). Rabbit anti-rainbow trout LMC5 IgG (Miranda et al., 1989) was used as a primary antibody (20 μg IgG in 100 ml of TBS containing 2% bovine serum albumin and 0.05% Tween 20). The purified LMC5 protein (Miranda et al., 1989) was used as a positive control. 125I-Labeled protein A (20 μCi in 100 ml of TBS-Tween 20) was used for the detection of LMC5 IgG. The protein bands were visualized by autoradiography on X-OMAT film (Eastman Kodak, Rochester, NY). The bands on X-ray film were scanned by using a Scanlet 4C scanner (Hewlett Packard, Palo Alto, CA) and presented with PowerPoint software (Microsoft, Redmond, WA).

Reconstitution and Enzyme Activity Assays. Cytochrome P450 content was measured spectrally by using Cary 219 (Omura and Sat0, 1964). All enzyme activity was reconstituted with incubation mixtures that included 50 μmol of human NADPH-cytochrome P450 oxidoreductase, 20 pmol of cytochrome b5, sodium cholate (50 mM in final), and 1:1:1 ratio of lipid mix 20 μmol/glycerol (1α,2-dilauroyl-sn-glycerol-3-phosphatidyl cholines; 1α,2-dioleoyl-sn-glycerol-3-phosphatidyl cholines; and phosphatidyl serine). The reconstituted mixture was incubated for 10 min at room temperature and then substrate and MgCl2 (30 mM in final) were added. Reactions were preincubated for 3 min at 30°C in a water bath and were initiated with NADPH (1 mM in final). All assays were performed in triplicate at 37°C for 30 min and total reaction volume was adjusted to 0.1 ml by using buffers (0.1 M potassium phosphate or 50 mM potassium HEPES, pH 7.7) and H2O.
Testosterone (250 μM) hydroxylation was assayed in various different mixtures to see the dependence of fish CYP3A27 for the optimal enzyme activity. Different mixtures include with and without MgCl₂ (30 mM), cytochrome b₅ (2-fold excess of CYP3A27), sodium cholate (50 mM), different buffers, and lipid mix. The reactions were stopped with the addition of 100 μl of methanol and vigorously vortexed for 2 min followed by centrifugation at 10,000 g for 15 min. Testosterone metabolites were analyzed by an HPLC system (Shimadzu, Kyoto, Japan) equipped with a UV/VIS detector (SPD-10V VP) and a prodigy column (Prodigy 3, 150 × 4.6 mm; Rancho Palos Verdes, CA). The detection method was slightly modified from Purdon and Lehman-McKeeman, 1997. The mobile phase for pump A was 5% tetrahydrofuran and 95% water, for pump B 100% methanol. Products were eluted at a flow rate of 0.5 ml/min in the following gradient system: 0 to 1 min (30% B), 1 to 10 min (30–60% B), 10 to 22 min (60–65% B), 22 to 28 min (65–80% B), 28 to 30 min (80 to 90% B), 30 to 32 min (90% B), 32 to 34 min (90–30% B), and 34 to 36 min (30% B). Data were collected via a system controller (SCL-10A VP; Shimadzu, Kyoto, Japan) and analyzed using CLASS-VP 4.3 software.

The reaction for the oxidation of nifedipine (200 μM in final) was the same as described above. The reactions were stopped by the addition of 100 μl of CH₃OH. The nifedipine metabolite was analyzed by HPLC with a Phenomenex Luna C₁₈ column (5 μm, 250 × 4.6 mm; ANSYS Technologies, Inc., Lake Forest, CA) (Shimada and Guengerich, 1989). Detection was at 254 nm and solvent flow was 1 ml/min of 65% methanol:35% water for 15 min. Retention time of oxidized nifedipine and nifedipine was 7.7 and 11.5 min, respectively. A metabolite was analyzed through a standard curve.

Results

Construction and Expression of Bv-3A27. Four different candidate colonies selected on the basis of white color were verified with PCR product (945 bp from gene specific primers and 4.1 kilobases from M13 forward and reverse primers) and isolated Bv-3A27 was transected into the S9 cells to express CYP3A27 protein. The expression of CYP3A27 from four different candidates was screened by Western blotting with LM55 polyclonal antibodies after 72 h postinfection. The selected virus (Bv-3A27) was optimized with different heme concentrations and different infection times. Maximal spectrally active protein was obtained with 1 μg of hemin/ml in 72 h postinfection (Fig. 2). The addition of hemin did not cause cell toxicity, but it was essential for the production of a spectrally active cytochrome P450 form. The CO difference spectrum was used to quantify the amount of active CYP3A27 protein, and the spectrum showed a peak maximum at 450 nm (Fig. 3). The level of spectrally active CYP3A27 expression was 714 pmol/mg total protein and the portion of CYP3A27 expression represented around 2.3% of total cellular protein. Protein concentration was determined by Coomassie Plus Protein Assay Reagent (Pierce Chemical, Rockford, IL) using bovine serum albumin as standard (Redinbaugh and Campbell, 1985). The expression of CYP3A27 protein was increased with infection time analyzed by Western blotting (Fig. 4), but the optimum infection...
Cells at $2 \times 10^6$/ml were infected with the recombinant baculovirus containing CYP3A27 cDNA with the indicated amount as described under Materials and Methods. Different amount of hemin chloride was added to compensate for low level of heme in insect cells. Cells in aliquots of 20 ml of culture were harvested at the indicated time points. The total membrane fraction was obtained as Methods. P450 content was analyzed by CO difference spectra in 0.1 M potassium phosphate buffer, pH 7.4, 1 mM EDTA, and 20% glycerol.

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The membrane fraction was isolated from hemin chloride (1 $\mu$g/ml)-fortified SF9 cells. Cells either uninfected or infected with the baculovirus containing trout CYP3A27 cDNA. The isolated membrane fraction was resuspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol and 1 mM EDTA in final concentration. The difference spectrum was recorded between 400 and 500 nm.

The baculovirus system has provided an abundant amount of functional cytochrome P450s without the cDNA modification (Asseffa et al., 1989; Barnes et al., 1994; Buters et al., 1994; Lee et al., 1995). Variable amounts of expressed cytochromes P450 were obtained from the baculovirus expression system, including 50 to 200 pmol/mg cell protein of CYP2D6 (Paine et al., 1996), 1.5 nmol/mg cell protein of CYP2E1 (Patten and Koch, 1995), 107 pmol/mg microsomal protein of CYP2E4 (Lee et al., 1995), and 460 pmol/mg cell protein of CYP3A4 (Buters et al., 1994). The present expressed trout CYP3A27 protein exhibited 714 pmol/mg total protein determined from a maximum CO-reduced spectrum at 450 nm after 72 h postinfection (Fig. 5, A and B). Lipid mix and MgCl$_2$ were helpful to get increased activity, but their contributions to total activity were not significant (Fig. 5, A and B). The activity of baculovirus expressed CYP3A27 was similar to those of mammalian CYP3A forms based on 6β-hydroxylase activity of testosterone and nifedipine dehydrogenation. The observed catalytic activities of Bv-CYP3A27 for the 6β-, 2β-, and 16β-hydroxylation of testosterone were 1.428, 0.043, and 0.034 nmol/min/nmol CYP3A27, respectively (Table 1).

Without the addition of sodium cholate, cytochrome $b_5$, and MgCl$_2$ there was no detectable nifedipine metabolite (data not shown). However, 50 mM potassium HEPES buffer, pH 7.7, with the addition of every component including sodium cholate, cytochrome $b_5$, and MgCl$_2$ showed weak dehydronifedipine formation at 50 and 33 pmol/min/nmol cytochrome P450, respectively (Table 1). The fraction isolated from noninfected SF9 cells with the same reconstitution components did not produce any metabolites. There were no catalytic activities when the spectrally active Bv-CYP3A27 protein was incubated in the absence of NADPH.

The characterization of CYP3A forms have been extensively investigated in mammals due to the important roles in the xenobiotic and steroid metabolism (Gonzalez and Korzekwa, 1995; Guengerich, 1999). The study of trout CYP3A form was initiated from the purification of LMC5 P450, which showed catalytic activity against testosterone and progesterone (Miranda et al., 1989) and cross-reacted with human CYP3A4 antibodies (Miranda et al., 1991). Previous research suggested that there is more than one CYP3A form in trout (Lee et al., 1998). It is possible, therefore, that the purification of specific CYP3A27 from the trout liver did not separate completely this isoform from the other CYP3A forms. CYP3A27 protein was expressed in COS-7 cells in our laboratory, but the expression level was too low to determine CO difference spectrum; however, Western blot analysis showed the recognition by LMC5 antibodies generated from cytochrome P450 LMC5 and the same migration with LMC5 expressed in COS-7 cells in our laboratory, but the expression level was too low to determine CO difference spectrum; however, Western blot analysis showed the recognition by LMC5 antibodies generated from cytochrome P450 LMC5 and the same migration with LMC5 P450 on the SDS-PAGE (data not shown).

The baculovirus system has provided an abundant amount of functional cytochrome P450s without the cDNA modification (Asseffa et al., 1989; Barnes et al., 1994; Buters et al., 1994; Lee et al., 1995). Variable amounts of expressed cytochromes P450 were obtained from the baculovirus expression system, including 50 to 200 pmol/mg cell protein of CYP2D6 (Paine et al., 1996), 1.5 nmol/mg cell protein of CYP2E1 (Patten and Koch, 1995), 107 pmol/mg microsomal protein of CYP3A4 (Lee et al., 1995), and 460 pmol/mg cell protein of CYP3A4 (Buters et al., 1994). The present expressed trout CYP3A27 protein exhibited 714 pmol/mg total protein determined from a maximum CO-reduced spectrum at 450 nm after 72 h postinfection after the addition of 1 $\mu$g of exogenous hemin per milliliter of culture. SF9 insect cells express zero background of cytochrome P450s; therefore,
catalytic activity study may not require the complete purification of expressed cytochrome P450. Although the expressed CYP3A forms from different laboratories have been purified by column procedures, the catalytic activities of the purified enzyme were strongly affected by several reconstitution factors (Brian et al., 1990; Buters et al., 1994; Lee et al., 1995; Yamazaki et al., 1995; Ingelman-Sundberg et al., 1996). Furthermore, the optimum condition for metabolism was not consistent because of different reconstitutions in each laboratory such as different amount of NADPH-cytochrome P450 reductase, cytochrome b5, lipid compositions, divalent cations, and other unknown factors (Brian et al., 1990; Buters et al., 1994; Lee et al., 1995; Yamazaki et al., 1995; Ingelman-Sundberg et al., 1996). Therefore, we used membrane fraction obtained from the Bv-CYP3A27-infected SF9 cells together with human NADPH-cytochrome P450 reductase and cytochrome b5 to determine CYP3A27 enzyme activity.

The optimum condition for the spectrally active form of CYP3A27 was determined by different amounts of hemin addition (1–3 μg/ml) combined with different cell harvest time. The expression level was increased with the increased infection time, however, spectrally active forms of CYP3A27 was sharply decreased after 72 h postinfection. The staining of trypan blue dye provided a good indication together with infection time, resulting in 30 to 50% cell staining for the spectrally active form in any different m.o.i. and different time infection. The higher amount of hemin produced strong absorption at 420 nm instead of 450 nm (data not shown). The overexpression of CYP3A27 may cause an unbalanced environment in the cell, affecting normal folding and targeting processes. The abundant amount of hemin addition also may affect the other proteins that require endogenous hemin for their functions. Therefore, the optimum condition for

## FIG. 5. Effects of various reconstitution components on CYP3A27 activity.

All reactions include 10 pmol of CYP3A27, 40 pmol of human NADPH-cytochrome P450 oxidoreductase, and 250 μM testosterone in 0.1 ml of reaction. All values are means of triplicate experiments ± S.D. The amount of cytochrome b5 used is 20 pmol/reaction. Detail procedures were described under Materials and Methods. Testosterone 6β-hydroxylation (A) and testosterone 2β-hydroxylation (B). The contribution of each component in reconstitutions is correlated in both 6β- and 2β-hydroxysteroid metabolism.

## TABLE 1

<table>
<thead>
<tr>
<th>Substrate Metabolite</th>
<th>Specific Activity</th>
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<tr>
<td></td>
<td>LMC5P450</td>
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<tr>
<td>Testosterone</td>
<td>6β-Hydroxytestosterone</td>
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<td>Testosterone</td>
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<td>Testosterone</td>
<td>16β-Hydroxytestosterone</td>
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<tr>
<td>Nifedipine</td>
<td>Dehydronifedipine</td>
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N.D., Not determined.

* Data for LMC5 P450 is from Miranda et al. (1989). LMC5 P450 is a purified protein from the liver microsomes of male rainbow trout.

* Bv-CYP3A27 is the protein obtained from CYP3A27 cDNA directed expression in insect cells. All assays were performed in triplicate at 37°C for 30 min as described under Materials and Methods. All values are means of triplicate experiments.
the functional expression of CYP3A27 in Sf9 cells was determined at 72 h postinfection to be 1 μg/ml of hemin addition.

Microsomal enzyme activities from fish liver microsomes are generally much lower than with mammalian microsomes (Buhler and Rasmussen, 1968; Miranda et al., 1989). In the current investigation with the microsomal fraction from Bv-3A27-infected insect cells, testosterone metabolism by Bv-CYP3A27 was slightly enhanced by different lipid mixes as described under Materials and Methods. The catalytic activity of Bv-3A27-expressed trout CYP3A27 was not improved with the additions of different amount of L-α-phosphatidylcholine (dioleoyl), and Lubrol PX (data not shown). These components are used in the reconstitution system in studies with mammalian CYP3A enzymes (Buters et al., 1994). A similar use of the membrane fraction from a baculovirus expression system containing a human cytochrome P450 form and purified NADPH-cytochrome P450 reductase and cytochrome b$_5$ has been reported (Richter et al., 2002).

Reconstitution of the enzyme, however, may require purified CYP3A27 protein together with purified NADPH-cytochrome P450 reductase, cytochrome b$_5$, and an appropriate lipid mix to obtain maximum activity. There is some concern about how the cytochrome P450 in membrane fraction can efficiently interact with the purified NADPH-cytochrome P450 reductase that is in a soluble form. The addition of sodium cholate with lipid mix increased the activity significantly in this study (Fig. 5, A and B). This increased activity with sodium cholate could be from the increased interaction of the added exogenous lipid and the insect membrane as well as from the increased interaction of cytochrome P450 and substrate.

Purified fish cytochrome P450s has been successfully reconstituted with rat NADPH-cytochrome P450 reductase (Klotz et al., 1983; Goksoyr, 1985). Replacing the rat NADPH-cytochrome P450 reductase with scup reductase stimulated the catalytic activity of the scup cytochrome P450A (Klotz et al., 1986). This result suggested that the nature of the cytochrome P450 reductase and cytochrome b$_5$ can affect catalytic activity. In the current study, human NADPH-cytochrome P450 oxidoreductase and cytochrome b$_5$ were successfully applied in the trout CYP3A27 reconstitution system, resulting in the formation of 6β-hydroxytestosterone as a major metabolite. The 6β-hydroxytestosterone is the major metabolite formed by both mammalian CYP3A forms and by the purified trout LMCS P450 (Miranda et al., 1989). CYP3A-type cytochromes P450 have been a problem because very low enzyme activity results upon reconstitution (Guengerich et al., 1986; Guengerich, 1999), although other cytochromes P450 are highly active in the similar reconstitution system (Disterath et al., 1985; Knodell et al., 1987). Various mixtures containing different type of lipids for acidic phospholipid system (Eberhart and Parkinson, 1991; Imaoka et al., 1992), GSH (Giilam et al., 1993), and detergents, cytochrome b$_5$, and divalent cations (Imaoka et al., 1992) have been successfully used to increase the activity of expressed CYP3A forms. It was reported that the divalent cations may be involved in the stimulation of electron transfer from cytochrome b$_5$ to cytochrome P450 and the rate of cytochrome P450 or cytochrome b$_5$ reduction (Tamura et al., 1990; Yamazaki et al., 1995). The major testosterone metabolite obtained from the purified LMCS P450 together with rat NADPH-cytochrome P450 oxidoreductase and rabbit cytochrome b$_5$ was 6β-hydroxytestosterone with 0.270 pmol/min/ml cytochrome P450 (Miranda et al., 1989), but the present reconstitution system showed 6β-hydroxytestosterone formation to be 1.428 nmol/min/mmol cytochrome P450 (Table 1). The increased activity may be derived from different components used in the reconstitution system or from the use of detergent in this study. Formation of 6β- and 2β-hydroxytestosterone showed similar requirements for the individual reconstitution components to obtain maximum CYP3A27 activity (Fig. 5, A and B).

The 6β-hydroxylation of testosterone, progesterone, and cortisol represents the major steroid metabolites formed by CYP3A forms in mammalian systems (Waxman et al., 1985, 1988; Guengerich, 1999). In this report, expressed trout CYP3A27 yielded 6β-hydroxylation of testosterone as the major metabolite. Altered levels of CYP3A expressions induced by environmental contamination may modify formation of the endogenous hormones, for example causing different metabolism of the male hormone testosterone. Therefore, the authors speculate that the growing xenobiotics issues in aquatic species could involve in the altered levels or activities of CYP3A forms.

Baculovirus expressed trout CYP3A27 protein successfully catalyzed a low level of nifedipine oxidation. Amino acid comparisons and immunological studies between trout CYP3A27 and human CYP3A4 were reported (Miranda et al., 1991; Lee et al., 1998) and the present observation of nifedipine metabolism could further support a similar structure function relationship between human CYP3A4 and trout CYP3A27. CYP3A27 is the first designated CYP3A form in the aquatic species (Lee et al., 1998) and the precise functional study requires cdNA directed expression of this gene to remove other background cytochrome P450s from the trout liver microsomes. In this study, we report the heterologous expression and the functional study of CYP3A27 gene by using recombinant baculovirus system.

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