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Sequence of a novel cytochrome CYP2B cDNA coding for a protein which is expressed in a sebaceous gland, but not in the liver

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The major phenobarbital-inducible rat hepatic cytochromes *P*-450, CYP2B1 and CYP2B2, are the paradigmatic members of a cytochrome *P*-450 gene subfamily that contains at least seven additional members. Specific oligonucleotide probes for these genomic members of the CYP2B subfamily were used to assess their tissue-specific expression. In Northern-blot analysis a probe specific to gene 4 (which is designated now as CYP2B12) hybridized to a single mRNA present in the preputial gland, an organ which is used as a model for sebaceous glands, but did not hybridize to mRNA isolated from the liver or from five other tissues of untreated or Aroclor 1254-treated rats. The cDNA sequence for the CYP2B12 RNA was determined from overlapping cDNA clones and contained a long open reading frame of 1476 bp. The nucleotide sequence of the CYP2B12 cDNA was 85% similar to the sequence of the CYP2B1 cDNA in its coding region and was different from any CYP2B cDNA characterized until now. The cDNA-derived primary structure of the CYP2B12 protein contains a signal sequence for its insertion into the endoplasmic reticulum and the putative haem-binding site characteristic of cytochromes *P*-450. A part of the potential haem pocket of CYP2B12 was identical with a similar structure in a bacterial protocatechuate dioxygenase. In immunoblot analysis of preputial-gland microsomes, antibodies against CYP2B1 recognized a single abundant protein with a lower apparent molecular mass than that of CYP2B1. Our results demonstrate that the CYP2B12 protein has the potential to be enzymically active and are the first demonstration that a member of the CYP2B subfamily is expressed exclusively and at high levels in an extrahepatic organ.

INTRODUCTION

The hepatic cytochromes *P*-450 (*P*-450) are members of a superfamily of endoplasmic-reticulum-bound mono-oxygenase enzymes that catalyse the metabolism of a wide variety of endogenous and exogenous compounds, including steroids and polycyclic aromatic hydrocarbons [1]. Several of these *P*-450 enzymes are inducible by xenobiotics. About 20 distinct forms of rat liver microsomal *P*-450 have been purified and extensively characterized [1–4]. In recent years, numerous *P*-450 cDNAs and genomic clones have been isolated and sequenced, and the various *P*-450 genes were categorized into families and subfamilies based on sequence similarities [5,6]. The major phenobarbital-inducible *P*-450 genes, recently designated CYP2B1 and CYP2B2 [7], belong to a subfamily containing several other *P*-450 genes or pseudogenes [8].

The high degree of sequence similarity among members of the CYP2B subfamily has complicated studies on the expression of the individual *P*-450 enzymes in this subfamily. Recently specific oligonucleotide probes have been employed to determine the expression of the CYP2B1 and the CYP2B2 genes separately [9]. In the work presented here we have utilized specific oligomer probes corresponding to the variable regions within the partially sequenced genomic members of the CYP2B subfamily [8] in Northern-blot analyses to investigate whether other members of the cytochrome CYP2B gene subfamily are expressed in the rat. We now report that one of these only partially characterized genes, which was originally termed gene 4 [8] and which we designate now as 'CYP2B12', is expressed constitutively at relatively high levels in the preputial gland, an organ which is known to be involved in the metabolism of steroids [10] and which is often used as a model for sebaceous glands [11]. We also report the complete coding sequence of the corresponding mRNA which establishes that it has the potential to code for an enzymically active *P*-450.

EXPERIMENTAL

Animals

Male and female Sprague–Dawley rats of different ages were obtained from Interfauna, Tuttlingen, Germany. The animals were kept in a temperature-controlled animal facility with a constant dark/light cycle. The animals were either treated with Aroclor 1254 (500 mg/kg body wt.) in corn oil or with corn oil alone (controls) 24 h before they were killed.

Isolation or RNA and Northern blotting

Total RNA was prepared from various organs as described [12]. The integrity of the RNA was verified by gel electrophoresis. Polyadenylated [poly(A)$^+$]RNA was purified by two cycles of oligo(dT)–cellulose (Type III; Colaborative Research, Waltham, MA, U.S.A.) chromatography. Northern-blot experiments, including the hybridization of oligonucleotides to RNA, were performed as described by Giachelli *et al.* [9]. The oligomer

Abbreviations used: *P*-450, cytochrome *P*-450; the terms '*P*-450 form' and '*P*-450' enzyme are used interchangeably; the recently devised cytochrome *P*-450 nomenclature [7] is used to designate individual *P*-450 forms, including CYP2B12 described in the present paper (D. W. Nebert & D. Nelson, personal communication); CYP2B1 corresponds to *P*-450 forms previously designated PB-4 [4], b [3] and PB-B [2], and cytochrome CYP2B2 to the forms designated PB-5 [2], e [3] and PB-D [2]; poly(A)$^+$, polyadenylated; 1 × SSC, 0.15 M-NaCl/0.015 M-sodium citrate.

The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL Data Bank under the accession number X63545.

probes were end-labelled with ^{32}P to a specific radioactivity of 3×10^6 c.p.m./pmol of oligomer. The same blots were hybridized repeatedly to the different oligomer probes, after washing with 0.1 × SSC (1 × SSC is 0.15 M-NaCl/0.015 M-sodium citrate) (containing 0.3% SDS) at 70 °C.

Preparation of a preputial gland cDNA library and CYP2B12 cDNA isolation

Double-stranded cDNA was synthesized from preputial-gland poly(A)$^+$ RNA of untreated male rats by using a cDNA synthesis kit (Boehringer, Mannheim, Germany). This synthesis employed oligo(dT)-primed first-strand synthesis by reverse transcriptase and second-strand synthesis using RNAase H and DNA polymerase. The ends of the resulting cDNA were 'polished' by using T4 polymerase. The cDNA was cloned into *Bam*H1-linearized pSP 65 vector using non-phosphorylated *Bam*H1/blunt-end adaptors as described by Haymerle *et al.* [13]. The resulting cDNA was transfected into *Escherichia coli* C600 and the resulting cDNA library was screened by colony hybridization using as a probe the CYP2B1 cDNA insert of the vector pSV450 [14].

cDNA clones complementary to the 5′ end of the CYP2B12 were obtained using the recently described 5′ RACE protocol [15]. The oligodesoxynucleotides employed in this method were synthesized on the basis of DNA sequence information obtained from the CYP2B12 cDNAs originally isolated from the total preputial-gland cDNA library (see above). Primer 1 was complementary to bases 951–969 and primer 2 was complementary to bases 911–929 of the CYP2B12 sequence displayed in Fig. 3 (below). First-strand synthesis was performed with the Boehringer cDNA synthesis kit in the presence of [^{32}P]dATP using primer 1 for starting the first-strand synthesis. The resulting cDNA–RNA hybrid was fractionated using a Sephacryl S-1000 column (Pharmacia). Fractions containing the first one-third of incorporated radioactivity were pooled, and the cDNA–RNA hybrid was tailed using terminal transferase (Boehringer, Mannheim, Germany) and dATP. PCR (30 cycles) was performed by using the modified oligo(dT) primer, which contains restriction sites for *Xho*I, *Sal*I and *Cla*I [15] and primer 2. The resulting cDNA was fractionated by agarose-gel electrophoresis. The major DNA band was isolated from the gel by using DE-81 paper (Whatman). The purified cDNA was cleaved with *Sal*I and inserted into an *Sma*I/*Sal*I-linearized pSP 65 vector. The resulting DNA was transfected into *E. coli* C600, and the resulting cDNA library was screened with the CYP2B1 cDNA probe.

DNA sequencing

The cDNAs were sequenced in both directions by the Sanger method [16] combined with the exonuclease III/mung-bean nuclease deletion technique [17] in order to obtain cDNAs which were successively shortened from either their 5′ or their 3′ end. In this way we obtained overlapping sequence information. In some cases the cDNAs were subcloned into the vector Bluescript SKII+ (Stratagene), which contains suitable restriction sites for the exonuclease III deletion technique. cDNA inserts in this vector are marked with the additional designation SK$^+$. Two cDNA clones (clones 20 and 21) isolated from the total preputial-gland cDNA library and three independent cDNA clones obtained from the preputial-gland RNA by applying the RACE technique were sequenced. The DNA sequences of the latter three cDNA clones were aligned and, on the basis of literature data [18], it was assumed that the correct CYP2B12 cDNA sequence at a certain position is given when the sequence of at least two cDNA inserts coincides at this position. The sequencing strategy is given in Fig. 2.

RNAase A and nuclease S1 protection analysis

This RNAase A analysis was performed as described previously [19] using as a probe a CYP2B12 *in vitro*-synthesized RNA transcript which covered base-pairs −28 to +927 of the CYP2B12 cDNA (+1 marks the beginning of the open reading frame) and an additional 207 bp which were derived from the vector pSP 65, in which a part of the CYP2B12 cDNA had been inserted and which had been used for transcription.

Preparation of microsomal protein and its analysis

Liver and preputial-gland homogenates were prepared by homogenizing the tissues in KBS (110 mM-KCl/10 mM-sodium phosphate, pH 7.4), containing the proteinase inhibitors aprotinin (60 units/ml) and phenylmethanesulphonyl fluoride (0.1 mM). Microsomes were prepared from the homogenate by centrifugation at 10000 *g* for 20 min and subsequent centrifugation of the supernatant at 100000 *g* for 1 h. The 100000 *g* pellet was resuspended in the homogenization medium containing 20% (v/v) glycerol and 1 mM-dithiothreitol. This suspension was shock-frozen in liquid N_2 and stored at −70 °C.

SDS/PAGE was performed as described by Laemmli [20], with the following modifications: the stacking gel was 5% acrylamide and 0.1% bisacrylamide in 0.058 M-Tris/phosphate, pH 6.7, containing 0.1% SDS. The separating gel was 10% acrylamide/0.1% SDS/0.37 M-Tris/HCl, pH 8.3. The running buffer had twice the concentration of Tris/glycine given in [20] to facilitate the resolution of *P*-450s [21]. The transfer of proteins to nitrocellulose sheets and the immunological detection of proteins was performed as described previously [22]. Purified cytochromes CYP2B1 and CYP2B2, and the IgG against purified cytochrome CYP2B1 were generously provided by Dr. D. Waxman [4]. These antibodies were used at a 1:1000 dilution. Antibodies against short peptide segments of either cytochrome CYP2B1 or CYP2B2 were generated as described by Oesch *et al.* [23] and were used at a 1:200 dilution.

The *P*-450-dependent oxidation of testosterone or androstenedione was determined as described in [24,25], the *O*-dealkylation of pentoxyresorufin and benzoxyresorufin as described in [26], and NADPH:cytochrome *c* reductase as described in [27].

RESULTS

Design of oligomer probes complementary to members of the CYP2B subfamily

We previously isolated several partial genomic clones which hybridized to a CYP2B2 cDNA under conditions of moderate stringency [8]. Partial DNA sequence analysis of these genomic clones [8] revealed a strong sequence similarity to the genes for cytochrome CYP2B1 and CYP2B2 in regions corresponding to exons 7 and 8 and the intron between these exons. To determine whether these various members of the CYP2B subfamily were at all expressed in the rat, we selected oligodesoxynucleotide probes which were each complementary to only one of the various members of the CYP2B subfamily. A short stretch of the DNA sequence of the members of the CYP2B subfamily covering the region complementary to the various oligomer probes is given in Table 1. The probe, which is complementary to the CYP2B12 (the designation of this gene in [8] was gene 4), had four mismatches to the gene coding for cytochrome CYP2B2 and seven mismatches to CYP2B1. There were, however, only two mismatches between CYP2B8 (which corresponds to gene 2 described in [8]) and the oligomer probe for the CYP2B12. In order to test the specificity of the oligomer probes for their complementary *P*-450 genes, the probes were hybridized to dot-blots of DNA isolated from the genomic clones corresponding to

Table 1. Sequence comparison of the members of the P4502B family in the regions complementary to the oligomer probes

The sequence complementary to the oligomer probe for each member is underlined. Bases identical with he gene 4 (CYP2B12) genomic sequence are marked with a dash (–). Note that the CYP2B2 probe would also hybridize to the gene-3 product. The start of the gene 4 (CYP2B12) sequence in this Table corresponds to base-pair 1126 in the cDNA sequence given in Fig. 2.

IIB gene	Sequence
Gene 4*	TGCAGATCTGACCCCGATTGGTCTACCA
2B1	-T------TGT---T-----AG-----
2B2	--------TG----A------T-----
Gene 2*	----------GT----------
Gene 3*	----------TG----A------T-----
Gene 5*	-T-T--CG---T-T-----G-----G--

* The designation of these genes is taken from [8].

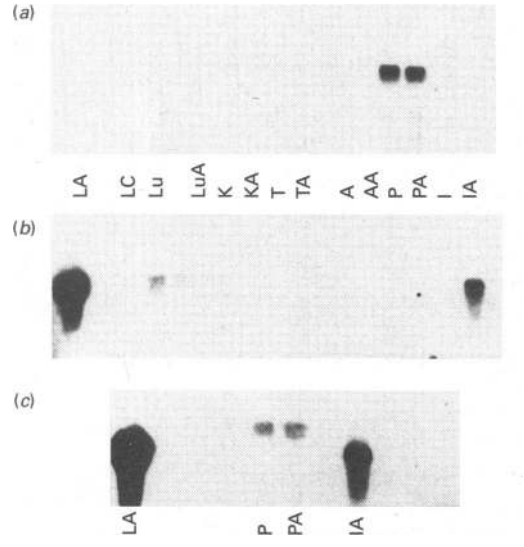

Fig. 1. Northern-blot analysis of tissue RNA with CYP2B probes

RNA was isolated from several tissues, subjected to Northern blotting and hybridized to several probes. (*a*) oligomer probe for CYP2B12; (*b*) oligomer probe for CYP2B1; (*c*) cDNA for CYP2B1 (covering nucleotides 1–1268 of the coding region). the same blot was used in the experiments displayed in (*a*) and (*b*). The following RNA samples were analysed: L, liver; Lu, lung; K, kidney; T, testis; A, adrenal gland; P, preputial gland; I, intestinal mucosa. The letter A following the designation of the tissue indicates that this tissue was derived from Aroclor 1254-treated animals. For all samples 15 μg of RNA was analysed. For the experiment displayed in (*c*), the hybridization and washing conditions were 6 × SSC and 55 °C.

the various CYP2B genes given in Table 1. In this analysis we found that each oligomer probe recognized only the gene which was completely complementary to it (results not shown). It should be noted that our probes for CYP2B1 and CYPB2 were targeted to a different region of these genes than were the probes used by Giachelli & Omiecinski [9], since their CYP2B1 probe would hybridize to other members of the CYP2B subfamily.

Oligomer probe for CYP2B12 hybridizes to preputial-gland RNA

The oligomer probe for the CYP2B12 was used in the Northern-blot analysis of total RNA from several tissues (Fig. 1*a*). Before the transfer of the RNA from the gel to the nylon membrane, the RNA was stained with ethidium bromide and revealed by u.v. illumination in order to verify that equal amounts of RNA had been loaded on to the gel. The CYP2B12 probe yielded an intense signal for the RNA samples isolated from the preputial gland of untreated rats and a signal of approximately equal intensity for the RNA sample isolated from the preputial gland of Aroclor 1254-treated animals. No RNA complementary to this oligonucleotide was found in the total RNA prepared from liver, lung, kidney, testis, adrenal gland and intestinal mucosa (Fig. 1*a*). The same Northern blot was then hybridized to the oligonucleotide corresponding to CYP2B1 (Fig. 1*b*). As expected, this oligonucleotide yielded a very strong signal for the total hepatic RNA, derived from Aroclor 1254-treated rats, and no signal for hepatic RNA isolated from untreated rats. Furthermore, RNA complementary to the CYP2B1 probe was detected in the analysis of RNA isolated from the lung of untreated or Aroclor 1254-treated rats, as well as in RNA isolated from the intestinal mucosa of Aroclor 1254-treated animals. The total RNA derived from the other tissues did not contain detectable RNA complementary to the CYP2B1 oligomer probe. When we used in the Northern-blot analysis a CYP2B1 cDNA instead of a CYP2B1 oligomer probe (Fig. 1*c*), we found that, under conditions of moderate stringency, this cDNA recognized a single RNA band in the preputial-gland RNA isolated from Aroclor 1254-treated or untreated animals (Fig. 1*c*). This RNA had the same size as the RNA detected by the CYP2B12 oligomer probe and was distinctly larger than the hepatic or the intestinal RNA species that hybridized to the CYP2B1 oligomer probe or to the CYP2B1 cDNA.

Oligomer probes complementary to the *P*-450-gene 2 and 5 (designation according to [8]) did not yield any detectable signals with the preputial-gland RNA and with the RNA analysed in the experiment displayed in Fig. 1*a* (results not shown).

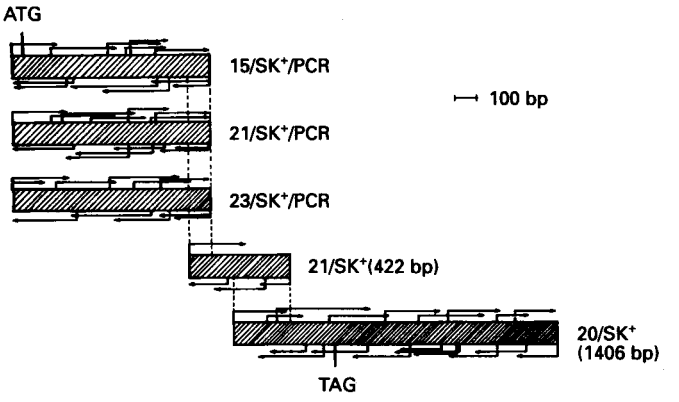

Fig. 2. Sequencing strategy for the CYP2B12 cDNA

Two overlapping CYP2B12 cDNA inserts (clones 20 and 21) which had been obtained from the total preputial-gland cDNA library (constructed in pSP 65) were subcloned into the vector Bluescript pBS to yield the plasmids 20/SK$^+$ and 21/SK$^+$. Similarly the three CYP2B12 cDNA inserts, which had been obtained in pSP 65 by using the 5′ RACE technique, were ligated into this vector to yield the plasmids 15/SK$^+$/PCR, 21/SK$^+$/PCR, and 23/SK$^+$/PCR. All cDNA clones were sequenced in both directions by using the Sanger [16] method combined with the exonuclease III/mung-bean nuclease deletion technique. Arrows indicate the sequence stretch covered in each sequencing run.

Isolation of CYP2B12 cDNA clones and CYP2B12 cDNA sequence

A preputial-gland cDNA library was screened with a ^{32}P-labelled CYP2B1 cDNA probe, and positive clones were isolated

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GGAGTGTTCACTGAAGTCAACAGTGGTTAGACCAGGACCATGGAATTCGGTGTCTTGCTCCTCCTCACTCTCACT
A---CA-G----------T--C-------C--------------GCC-A--A------------TG-----CTC

GTGGGCTTCTTGCTATTCTTAGTCAGCCAAAGTCAGCCAAAGACCCATGGGCACTTGCCACCAGGACCTCGTCCC 150
------------T--C----------   GG-A--C------T---G---CA----C------------------

CTGCCCTTCTTGGGGAACCTCTTACAGATGAACAGAAGAGGCTTTCTGAATTCTTTCATGCAGCTTCAAGAAAAA
--T---C--------------C-G---T--G-----G-G---C-C--C-----C------------G-------

TATGGAGATGTGTTCACAGTACACCTGGGACCAAGGCCCGTGGTCATATTGTGTGGGACAGACACCATAAGAGAG 300
----------------------------------------T--------GC-A-------------------AG---

GCTCTGGTGGACCAAGCTGAAGCTTTCTCTGGTCGGGGAACAGTTGCTGTGCTCCATCCAGTTGTACAGGGATAT
---------G---------G-A-----------------A-C------A-TG-G---A-CT-CA---A----

GGTGTGATCTTTGCCACTGGGGAACGTTGGAAGACCCTTCGGCGATTCTCTCTGGTCACCATGAAAGAATTCGGA 450
---------------A---------C------G---------------------CT-------G---C--T--G

ATGGGAAAGCGGAGTGTAGATGAGAGGATAAAGGAGGAGGCCCAGTGTTTGGTGGAGGAGCTGAAGAAATACAAG
---------A-------G--A--AC----TC-------A-----A--------------A---CG-----C-C--

GGAGCCCCCCTGAACCCCACCTTCCTCTTCCAATCCATCGGCAGCAAACACCATCTGCTCCATTGTCTTTGGAGAG 600
--------A---G-T-----------------G-G----A----C----T---------------G---------

CGCTTTGACTACAAAGACCACCAGTTCCTGCATCTGCTGGATCTAGTCTATAAGACATCTGTCCTTATGGGCTCC
-------------C-----G-----------GC---T----G--GT----CCG---C-T-TC---CC-AA-T--A

TTGTCCAGCCAGGTGTTTGAGCTTTACTCTGGCTTCCTGAAATACTTTCCTGGTGCCCACAAACAAATCTTCAAA 750
--C------------------T-C-T------G---------------------------G--------C----

AACCTACAGGAAATGCTCAACTACATTGGCCATATTGTGGAGAAGCACAGGGCAACCTTGGACCCCAGTGCTCCA
-----C--------C---G-T-----------------------------C-----A-----A--C------

CGAGATTTCATCGATACTTACCTTCTGCGCATGGAGAAGGAGAAGTCCAACCATCACACAGAGTTCAATCACCAG 900
-----C--------C-----------------------------------G-----C------------C----TG--

AACCTTGTGATCTCTGTGCTCTCTCTCTTCTTTGCTGGCACCGAGACCACCAGCACCACGCTCCGTTGCACCTTC
-----CA-------CC-------------------------T-------G---------A-----C-ATGGT---

CTGATCATGCTCAAGTACCCCCACGTTGCAGAGAAAGTCCAAAAGGAGATTGATCAGGTGATTGGCTCACACCGG 1050
---C-G-----------------T--C-------------------------------------C----------

CTACCAACCCCTGATGATCGTACCAAAATGCCATATACTGATGCAGTCATCCATGAGATTCAGAGATTTGCAGAT
----------T------C--C-GT----------C----------T-----C----------G---T-----

CTGACCCCGATTGGTCTACCACACAGAGTCACCAAAGACACCGTGTTCCGAGGGTACCTGCTTCCCAAGAACACT 1200
---TGT---T-----AG-------------------------A-------------------------------

GAGGTGTACCCCATCCTGAGTTCAGCTCTCCATGACCCACGGTACTTTGAACAACCAGACACTTTCAATCCTGAG
--A-------------------------------------A---------C--C-------GC----------A

CACTTCCTGGATGCCAATGGGGCACTGAAGAAGAGTGAAGCTTTTCTGCCCTTCTCTACAGGAAAGCGCATTTGT 1350
-----------------------------A--------------CA----------C------------------

CTTGGAGAAGGCATTGCCCGAAATGAATTGTTCATTTTCTTCACCGCCATCCTGCAGAACTTCACTTTGGCAAGC
-----C---------------------------C-C---------A-------C---------T--G--T-----

CCTGTGGCTCCTGAGGACATTGACCTCACTCCCATTAACATTGGTGTAGGCAAAATACCCTCTCCATACCAGATA 1500
-A-T-------CA----------------G----AGG-G-G---CA-T--A--------TC-AA-G--------C

AACTTCCTGTCCCGTTGAATAGGTTGAGAGAGAAGAGTCAATGGAATGACGTGTCCCCATTGCCGTACCCTATGA
TG----TCAG-T--G---
ATGGAATAGTAAAACCTAATGAAGTGGCTTTCTTGTTACATAGACAGAGAGACTTCTGGAGGCCACAGCTTGTTG 1650
TGAGACACCTCTCTCATGCTTGTTCACAGCCATAGTCCCTACTTGTTAGGAATCTAGACTCCAGGATCTGTGGGA
ACAGACCAGAGATGGTTTCTCTTGAGTTGGCTCCATTCTAATCCCATGTGGTGTGTTGGGGGGTCATGTTTGAGG 1800
ACCCCCTTCGTAGATGAGTAACAGACCCTATGATGGCATCACAGTGCTAGTGAGTAGTGGGGAACAAAATTATGC
TGTGAGTCTAGAAGAGCAACCCCCTCTCCTGCTTCTGTTTCAATTCAGATGAAATTCATATAACGAAGCTGACAA 1950
AATCTTTTAATTTTGTATTCAGGCCAGAGGCTCATGTAGTTCACACTGACCTCAAAATCCTATATACATCCAAAG
ATGTCTTGAAATCTCGATCTCCTGACCAGTGCTGGCATCACAGCCACATGGACGTATACCCAGATTTTAAAGTGA 2100
CTATATCATAATGTTTATATTTTAATCTCCTTTAATTTATTTGCAATATTACAACTTCACTATCTAATTCCAGGT
ATTTTGTCACACTTAACCAACACCACCCAAAAGCCCCAACCTATTCAAAGATCAATCCCTCATCCCCTCTCTCCA 2250
TAACCTCCCCTGCACTCAACACCCACCGTGCCACTTTTGTCACACTTAACCAACACCACCAAAAGCCCCAACCTA
TTCAAAGATCAATCCCTCATCCCCTCTCTCCATAACCTCCCCTGCACTCAACACCCACCGTGCCACTTTTGTCTG 2400
ACTTTTGTGTCATTGGACTGTCCTGAATTGTGTGTCTCATAGAAAAACAACGACATGATGTTTAATGCTGGCTGA
CTTTTATGCCACACCCAACTTCTCAGGCCCCTGACTGTGGCCTGCACAGTACAG
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Fig. 3. DNA sequence of the CYP2B12 cDNA and comparison with the CYP2B1 cDNA sequence

The cDNA sequence of the CYP2B12 cDNA, which was obtained as indicated in Fig. 2, is displayed in the upper line. Below is the cDNA sequence of the CYP2B1 cDNA. A dash in this sequence indicates identity with the CYP2B12 cDNA sequence and blanks indicate deletions of the CYP2B1 sequence when compared with the CYP2B12 sequence. Both sequences are only compared in the coding region of both cDNAs. The initiation and the termination codons for protein biosynthesis are given in **bold** type. Note that the three partial CYP2B12 cDNAs, which were obtained by using the 5′ RACE protocol, had different lengths at their 5′ ends. Therefore the cDNA sequence information obtained from base-pairs −39 to −22 relies only on the DNA sequence information obtained from either one or two CYP2B12 cDNA clones which had been generated by using the 5′ RACE technique.

and partially sequenced. The resulting sequences were aligned with the CYP2B1 cDNA sequence. From this comparison we concluded that two cDNA inserts (clones 20/SK$^+$ and 21/SK$^+$) covered a region of the CYP2B1 cDNA which started with base-pair +869 of the CYP2B1 cDNA (+1 corresponds to the first residue of the initiation codon) and that clone 20/SK$^+$ contained

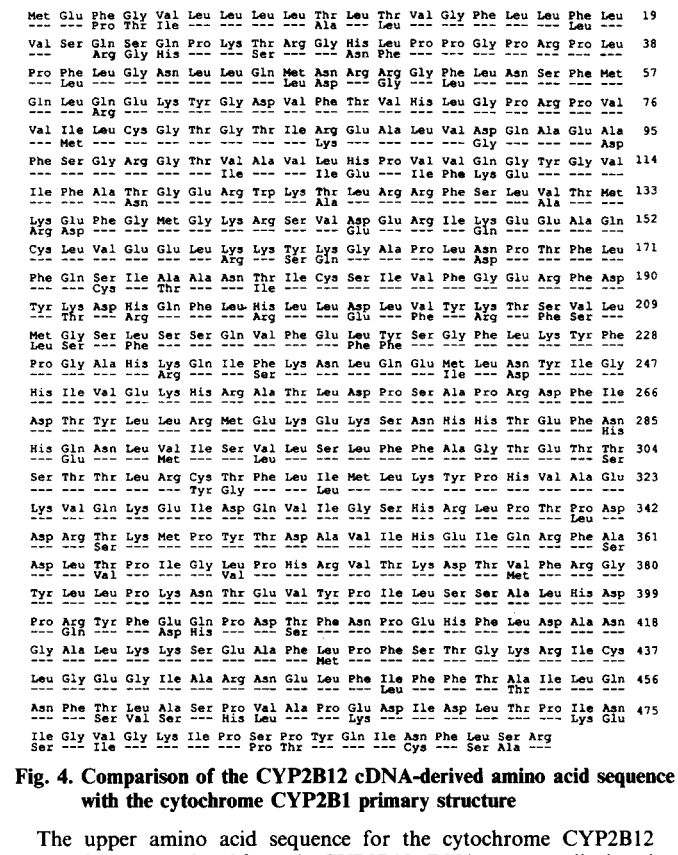

Fig. 4. Comparison of the CYP2B12 cDNA-derived amino acid sequence with the cytochrome CYP2B1 primary structure

The upper amino acid sequence for the cytochrome CYP2B12 protein was translated from the CYP2B12 cDNA sequence displayed in Fig. 3. The lower amino acid sequence gives the cDNA deduced primary structure of cytochrome CYP2B1. Dashes in this sequence indicate identity with the deduced primary structure of the cytochrome CYP2B12 protein. A blank in the CYP2B1 amino acid sequence (corresponding to amino acid residue 21 of the CYP2B12 protein sequence) indicates a deletion of one amino acid.

1013 bp of untranslated region (the region covered by the clones 20/SK$^+$ and 21/SK$^+$ in the CYP2B12 cDNA is given in the legend to Fig. 2). In order to obtain cDNAs which encompassed the initiation codon of the CYP2B12 cDNA, we applied the recently described 5′ RACE protocol (see the Experimental section). Because this RACE protocol involved 30 cycles of the PCR, which potentially could lead to the misincorporation of nucleotides into the resulting cDNAs [18], we isolated and sequenced three independent cDNA clones which hybridized to the CYP2B1 cDNA.

The DNA sequencing strategy for obtaining the CYP2B12 cDNA sequence is given in Fig. 2. The CYP2B12 cDNA sequence, which was obtained by sequencing clone 20/SK$^+$, clone 21/SK$^+$ and the three cDNA clones which were synthesized using the RACE protocol, is given in Fig. 3. It was found that two partial CYP2B12 cDNA clones which were obtained via the 5′ RACE protocol differed from the final consensus CYP2B12 cDNA sequence by either one (clone 15 PCR) or two (clone 21 PCR) base exchanges, whereas the third clone (23 PCR) displayed the correct CYP2B12 cDNA sequence. Fig. 3 also shows the comparison of the CYP2B12 cDNA sequence with the CYP2B1 cDNA sequence. The sequence of the CYP2B12 cDNA was also compared with the DNA sequence of the recently characterized genomic clone which represented only a part of the CYP2B12 sequence [8]. It was found (results not shown) that the CYP2B12 cDNA differed from the partial CYP2B12 genomic sequence only by 2 bp in a region covering exons 7 and 8 of the partial CYP2B12 genomic sequence (this region corresponds to base-pairs +969 to +1299 of the CYP2B12 cDNA). We think that these small DNA sequence differences are due to polymorphisms that are apparently not uncommon in the CYP2B subfamily [28,29] and that our cDNA sequence represents the CYP2B12 cDNA.

The CYP2B12 cDNA sequence contained 39 bp of 5′ untranslated region which differ from the CYP2B1 cDNA untranslated region in seven residues. The long open reading frame of the CYP2B12 cDNA starts with a ATG codon which is in an optimal sequence context [30] for the initiation of protein biosynthesis (which is a purine three bases upstream and a desoxyguanosine residue four bases downstream from the start of the open reading frame). The open reading frame of the CYP2B12 cDNA has the potential to encode a protein of 492 amino acids, which is one amino acid longer than cytochrome CYP2B1, followed by 1013 bp of 3′ untranslated region. The sequence similarity between CYP2B1 and CYP2B12 in the coding region was 85% at the nucleotide level. These differences between the CYP2B12 cDNA and the CYP2B1 cDNA translate into some differences in the primary structure of these two *P*-450 proteins (Fig. 4). Few amino acid differences are present, however, in the regions corresponding to amino acid residues 245–284, 331–399 and 413–458 of the cytochrome CYP2B12 protein. It is important to note that the latter region of this protein contains a cysteine residue (Cys^{437}) that is conserved in all forms of *P*-450 and which represents the putative haem-binding site for these enzymes [5].

Another conserved feature in the primary sequences of the various *P*-450 species is an *N*-terminal segment, containing a highly hydrophobic core region, that mediates co-translational insertion of the nascent polypeptide into the endoplasmic-reticulum membrane, anchors the protein in the membrane and ensures that downstream portions of the protein are exposed on the cytoplasmic face of the membrane [31,32]. In general, these *N*-terminal segments contain a negatively charged residue preceding the hydrophobic core and several positively charged residues following it. The *N*-terminal segment of the CYP2B12 product conforms to this consensus, with a glutamic acid residue at position 2, a 15-amino-acid hydrophobic core, a lysine residue at position 26, and histidine residues at positions 28 and 30.

RNAase A and nuclease S1 analysis of hepatic and preputial-gland mRNA

In the Northern-blot analysis of preputial-gland RNA (Fig. 1) we had obtained a single RNA band which hybridized to the CYP2B12 oligomer probe and to a CYP2B1 cDNA. In order to verify that this band represents a single CYP2B RNA species, we performed a RNAase A and nuclease S1 protection analysis (Fig. 5) using a CYP2B12 cDNA transcript which covered base-pairs −28 to +927 (+1 indicates the start of the coding region of this cDNA). Whereas nuclease S1 is known to cut a heteroduplex only in those regions which contain at least three contiguous mismatches between the probe and the cellular RNA, the enzyme RNAase A will often recognize single-base-pair mismatches. Both nuclease S1 and RNAase A yielded large amounts of a single protected fragment in the analysis of 1.2 μg of poly(A)$^+$ preputial-gland RNA, but fragmented the probe in the analysis of hepatic poly(A)$^+$ RNA from Aroclor 1254-treated animals. This result shows that preputial-gland RNA, but not the liver RNA, contains the CYP2B12 RNA and, more important, the absence of partially protected fragments in the analysis of preputial-gland RNA demonstrates that the preputial gland does not express an RNA which is as highly related to the CYP2B12 RNA as is CYP2B1 and 2B2 or other hepatic CYP2B mRNAs. The result that the size of the protected probe in the analysis of

Fig. 5. Nuclease S1 and RNAase A analysis of hepatic and preputial-gland RNA

The probe which covered base-pairs −28 to +927 of the CYP2B12 cDNA and 207 bp derived from the vector pSP65, which was used for the transcription reaction, was synthesized *in vitro* in the presence of [^{32}P]UTP and hybridized either to 1.2 μg of preputial-gland RNA from Aroclor 1254-treated animals (PA) or 1.2 μg of hepatic RNA from Aroclor-treated animals (LA). The resulting duplex was treated with nuclease S1 or RNAase A as described in [19]. The digests were analysed by denaturing gel electrophoresis, followed by autoradiography with an intensifier screen for 16 h.

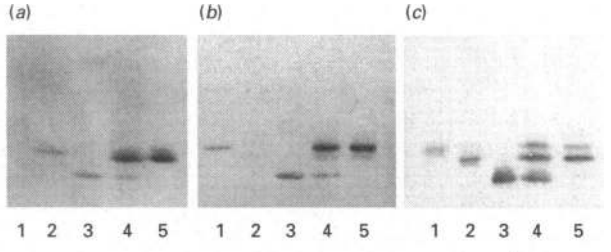

Fig. 6. Immunoblot analysis of preputial-gland microsomes and hepatic microsomes

Microsomal protein was subjected to immunoblotting and allowed to react with antibodies against peptide fragments which ae unique to cytochrome CYP2B1 (*a*) or to cytochrome CYP2B2 (*b*). In the experiment displayed in (*c*), polyclonal antibodies directed against purified cytochrome CYP2B1 were used. Protein samples applied were: 1, purified cytochrome CYP2B2; 2, purified cytochrome CYP2B1; 3, preputial-gland microsomes; 4, mixture of preputial-gland microsomes and hepatic microsomes from Aroclor 1254-treated animals; 5, hepatic microsomes from Aroclor 1254-treated animals; 0.1 μg of purified proteins, 12.5 μg of hepatic protein and 50 μg of preputial-gland protein were analysed.

preputial-gland RNA is slightly smaller than the size of the *in vitro*-synthesized transcript probe is explained by the presence of pSP 65 plasmid sequences (207 bp) which were part of the probe. These sequences are not protected by the cellular RNA.

Preputial gland contains a single cytochrome CYP2B1-related protein

Immunoblot analysis (Fig. 6) of microsomal protein from the rat preputial gland yielded an intense signal with antibodies against purified cytochrome CYP2B1 (*P*-450 PB4) (Fig. 6*c*). The mobility of this protein was greater than those of the two proteins recognized by these antibodies in the hepatic microsomes from Aroclor 1254-induced male rats, which co-migrate with purified cytochrome CYP2B1 and cytochrome CYP2B2 (*P*-450 PB5). By comparison with molecular-mass-standard proteins, we estimated that the apparent molecular mass of the CYP2B12 protein was, by 2500 Da, smaller than the apparent molecular mass of the CYP2B1 protein. To exclude the possibility that the CYP2B12 protein was proteolytically degraded during the preparation of the microsomes, we analysed the total preputial-gland protein which had been obtained by boiling the intact preputial glands in SDS sample buffer. The analysis of this protein by SDS/PAGE followed by immunoblotting using anti-CYP2B1 antibodies revealed that the size of the CYP2B12 protein in this sample was identical with that of the CYP2B12 protein determined in the microsomal protein.

It was conceivable, albeit unlikely, that the antibodies against purified cytochrome CYP2B1 reacted with a *P*-450 in the preputial gland which did not belong to the CYP2B subfamily. To exclude this possibility further, we used for the immunoblot analysis antibodies that were targeted against the small hypervariable regions of either cytochrome CYP2B1 or cytochrome CYP2B2. As previously reported [23], antibody D (Fig. 6*a*), which was directed against a region of cytochrome CYP2B1 (amino acids 362–380) where this protein differs from cytochrome CYP2B2 in two out of 19 amino acid residues, recognized purified CYP2B1 (Fig. 6*a*, lane 2), as well as the corresponding protein in liver microsomes, but did not react with CYP2B2 or any other hepatic *P*-450. Strikingly this antibody also recognized the cytochrome CYP2B1-related protein in the preputial gland (Fig. 6*a*, lane 3). Antibody C2 (Fig. 6*b*), which was directed against a region of cytochrome CYP2B2 (amino acids 329–361) where this mono-oxygenase differs from cytochrome CYP2B1 in four out of 33 amino acid residues, recognized, as previously reported [23], purified cytochrome CYP2B2 and mainly this protein in hepatic microsomes, but also the cytochrome CYP2B1-related protein in the preputial gland. These findings, using the antibodies which distinguish CYP2B1 and CYP2B2 and, hence, are sensitive to very small differences in the primary structure of the antigen, provide strong evidence that the protein recognized by these antibodies in the preputial gland is a cytochrome CYP2B protein. Immunoblot analysis of microsomes from the preputial glands of male rats of various age groups (30–40 g, 120–140 g and 350–450 g) and of mature females using an antiserum directed against purified cytochrome CYP2B1, revealed that the CYP2B12 protein was found in all age groups and in both sexes (results not shown).

P-450-dependent enzyme activities in preputial gland microsomes

Microsomes were isolated from the preputial gland and the liver of Aroclor 1254-treated animals and analysed for *P*-450-dependent enzyme activities. Hepatic microsomes catalysed the hydroxylation of testosterone at various positions (2β, 6β, 7α, 16α and 16β). The specific enzyme activity for the hydroxylation of this substrate at the 16β position and for the formation of androstenedione was 0.32 and 1.9 nmol/min per mg of protein respectively. Because these two testosterone metabolites are mainly formed by *P*-450 of the 2B family, we expected that they would be also formed by the cytochrome CYP2B12 protein found in the preputial gland. Preputial-gland microsomes did not, however, metabolize testosterone to any metabolites which can be identified in our assay. We estimate from the sensitivity of this assay that the capability of the preputial-gland microsomes to form 16β-hydroxytestosterone is at least 20-fold lower than the capacity of hepatic microsomes to form this metabolite. Since we used u.v. detection of the testosterone metabolites at 240 nm, species arising from the reduction of testosterone at the Δ^4

position are not readily detected. Hepatic microsomes, but not preputial-gland microsomes, metabolized androstenedione to several metabolites which could be detected in our assay. These metabolites were not further identified.

We also measured the *O*-dealkylation of pentoxyresorufin and benzoxyresorufin which are good substrates for cytochrome CYP2B proteins. Hepatic microsomes from Aroclor 1254-treated rats catalysed the dealkylation of pentoxyresorufin with a specific enzyme activity of 2330 pmol/min per mg of protein. Preputial-gland microsomes showed no activity with pentoxyresorufin and with benzoyresorufin. In order to establish whether preputial-gland microsomes contained an NADPH:cytochrome *P*-450 reductase enzyme activity, we determined the reduction of cytochrome *c*, which is catalysed by this enzyme. The NADPH:cytochrome *c* reductase enzyme activity of preputial-gland microsomes was 16 nmol/min per mg of protein and of hepatic microsomes 125 nmol/min per mg of protein.

DISCUSSION

We have shown here that the preputial gland, which serves as a model for sebaceous glands, contains rather high amounts of an RNA species which hybridized to an oligonucleotide probe which was complementary to, and specific for, the previously characterized [8] partial genomic clone which represents CYP2B12. This probe did not hybridize to RNA isolated from other organs of untreated and Aroclor 1254-treated rats (Fig. 1). The conclusion that the CYP2B12 is not expressed in the liver is strengthened by our highly sensitive RNAase A analysis (Fig. 5), which did not yield a fully protected CYP2B12-cDNA-derived transcript probe in the analysis of hepatic RNA.

The conclusion that CYP2B12 is expressed in the preputial gland is confirmed by our finding that our CYP2B12 cDNA sequence, which was obtained by the sequence analysis of preputial-gland cDNAs, had only two mismatches to the partial genomic CYP2B12 sequence in a region corresponding to exons 7 and 8 of this gene.

By sequencing overlapping CYP2B12 cDNA clones we obtained 2528 bp of contiguous sequence. Thus the CYP2B12 RNA is distinctly larger than the CYP2B1 and the CYP2B2 RNA, which have a size of 1750 bp. This result agrees with the findings of our Northern-blot analysis (Fig. 1*c*), from which we estimate that the size of the CYP2B12 RNA is approx. 2600 to 3000 bp. The sequence similarity between the CYP2B12 protein and the CYP2B1 protein is 80%, establishing that these proteins are indeed encoded by genes of the same subfamily [33]. The sequence of the CYP2B12 cDNA is also distinct (results not shown) from CYP2B2 and from the CYP2B3 cDNA which had been isolated from rat liver [34]. Low levels of expression of the CYP2B8 in liver have been recently described [35]. However, this gene is clearly different from CYP2B12.

The DNA sequence data presented (Fig. 3) show that the long open reading frame of the CYP2B12 cDNA starts with a ATG codon which is in an optimal context to function as an initiation codon for protein biosynthesis and that this long open reading frame has the potential to code for a cytochrome CYP2B12 protein which is one amino acid residue longer than cytochrome CYP2B1 (Fig. 4). The presence in the preputial gland of a protein that almost certainly represents the CYP2B12 protein was shown by immunoblot analysis (Fig. 6) using site-directed antibodies that were highly specific for CYP2B proteins [23]. The highly sensitive RNAase A analysis (Fig. 5) revealed that the preputial-gland RNA does not contain another CYP2B RNA that would have yielded partially-RNAase A-protected fragments, strengthening the conclusion that the protein which we have detected by our immunoblot analysis (Fig. 6) is encoded by the CYP2B12 RNA. The substantial difference in the electrophoretic mobilities of cytochrome CYP2B1 and the CYP2B12 protein, despite their similar molecular masses as determined from their primary structure (55800 versus 55900 Da) is reminiscent of the difference in the electrophoretic mobilities of the closely related cytochromes CYP1A1 and CYPIA2, which have almost the same molecular masses.

The immunoblot analysis also shows that the CYP2B12 protein is an abundant component of the preputial-gland microsomal fraction, because even though cytochrome CYP2B1 antibodies would be expected to react better with cytochrome CYP2B1 than with cytochrome CYP2B12, the signal obtained with these antibodies in the immunoblot of preputial-gland microsomes (50 μg) was similar to the signal obtained for cytochrome CYP2B1 in 12.5 μg of hepatic microsomes. We can estimate, therefore, that the expression of the cytochrome CYP2B12 protein in the preputial gland compares favourably with the extremely high expression of cytochrome CYP2B1 in the liver of Aroclor 1254-treated animals.

The cDNA-derived primary structure shows that the CYP2B12 protein contains a potential haem-binding site and a signal sequence for the insertion of this protein into the endoplasmic reticulum. On the basis of these observations we think that the cytochrome CYP2B12 protein is a functional *P*-450.

In principle the substrate specificity of the cytochrome CYP2B12 protein is best determined by the heterologous expression of the CYP2B12 cDNA in transfected cells. To this end we have reconstructed the full-length CYP2B12 cDNA from the partial cDNA inserts of clones 20, 21 and 23 PCR, inserted it into a mammalian expression vector and introduced this construct into V79 hamster lung fibroblasts. However, until now we have failed to express this reconstructed CYP2B12 cDNA, even at the RNA level, in these cells. In the same experiment, and using the same mammalian expression vector, we expressed a CYP2B1 cDNA successfully. In a first attempt to define the substrate specificity of the cytochrome CYP2B12 protein, we assayed several *P*-450-catalysed reactions in preputial-gland microsomes. These microsomes did not catalyse the hydroxylation of testosterone and androstenedione, which are good substrates for a large variety of *P*-450 isoenzymes. These results appear to be inconsistent with the data of Gustafson & Steinberg [10], who found that preputial glands have the capacity to metabolize androstenedione to several metabolites. However, these metabolites were mainly found to be formed by the action of the androstenedione 5α-reductase and would not absorb at a wavelength of 240 nm, which was used in our detection system (the study by Gustafson & Steinberg [10] employed radioactively labelled androstenedione). In addition, Gustafson & Steinberg [10] performed their assay with intact glands, whereas we used microsomal protein.

Also under our conditions preputial-gland microsomes did not catalyse the *O*-dealkylation of pentoxyresorufin and benzoxyresorufin, which are good substrates for cytochrome CYP2B1, but not for cytochrome CYP2B2. However, all these enzyme assays were performed under conditions which were optimal for hepatic *P*-450 species. It is conceivable that the cytochrome CYP2B12 protein has special requirements for optimal performance, such as cofactors (NADH instead of NADPH) or certain metal ions. It is also possible that our isolation procedure for microsomes, which is known to preserve hepatic *P*-450 enzyme activities, does not preserve these activities in microsomes derived from the preputial gland, which is a proteinase-rich organ. The absence of a detectable *P*-450 enzyme activity towards substrates which are metabolized by a wide variety of hepatic *P*-450 is not due to a lack of NADPH:cytochrome *P*-450 reductase

in the preputial gland, since we have shown the presence of significant amounts of this enzyme in this sebaceous gland.

The findings of Lindberg & Negishi [36] may, however, shed light on the unusual observation that the CYP2B12 protein does not oxidize substrates which are known to be metabolized by several members of the P450IIB family. These authors demonstrated, by site-directed mutagenesis, that the residue 209 (Phe) of coumarin 7α-hydroxylase mainly determines the substrate specificity of this enzyme. Certain amino acid substitutions at this residue will cause this enzyme to become a steroid 15α-hydroxylase. More recently, Iwasaki *et al.* [37] demonstrated that this residue forms a part of the haem-binding pocket of the coumarin 7α-hydroxylase and is involved in the substrate binding. Residue 209 of the coumarin 7α-hydroxylase corresponds to residue 207 of CYP2B1, which is also a phenylalanine residue. In CYP2B12 not only is this phenylalanine residue replaced by serine, but, in the region of amino acid residues 204–211, five amino acid substitutions between CYP2B1 or B2 and CYP2B12 are noted (see Fig. 4). Also in this region, CYP2B12 displays only low similarity to other mammalian *P*-450 (results not shown). However, even though no natural mammalian *P*-450 contains serine at the position corresponding to residue 207 of CYP2B12 or to residue 209 of the coumarin 7α-hydroxylase, the replacement of phenylalanine by serine at this position in the latter enzyme by site-directed mutagenesis still results in an enzymically active *P*-450 [38]. By comparison with other protein primary structures stored in the EMBL databank (update 1990), we found that, within this region, amino acid residues 204–209 of CYP2B12 are completely identical with residues 25–30 in the β-subunit of the protocatechuate 3,4-dioxygenase (EC 1.13.11.3), but not to any other protein primary structure. It is noteworthy that the β-subunit of this enzyme contains a ferric iron ligand which, unlike that in *P*-450 species, is ligated by two histidyl and two tyrosyl side chains. This region of the dioxygenase most likely forms part of the pocket of this protein in which the iron is contained [39]. To our knowledge this is the first demonstration that the potential haem pocket of a dioxygenase is identical with a site in a *P*-450 with a similar function. Outside this region of identity these two enzymes show no obvious similarity.

It should be noted that, until now, no substrate has been found for the recently described *P*-450 of the nasal mucosa [40] which, on the basis of its cDNA-derived primary structure, should be enzymically active.

The substrate cyclophosphamide, which is activated to cytotoxic species mainly by members of the CYP2B family [41], is a possible subject for future studies. This substrate could be of special interest, since sebaceous glands are tightly associated with hair follicles, and cyclophosphamide treatment of patients and of animals results in severe alopecia (hair loss). However, it remains to be seen whether this substrate is metabolized by the cytochrome CYP2B12 protein and whether this protein is also present in sebaceous cells or other cell types of the skin. In this respect it should be noted that the expression of CYP2B1-related proteins has been detected in the rat skin [42].

In conclusion, we have shown the expression of a novel cytochrome CYP2B mRNA and protein in the preputial gland, an organ which serves as a model for sebaceous glands. Unlike all other CYP2B genes, which are all expressed in the liver, the CYP2B12 is expressed exclusively in an extrahepatic tissue. On the basis of the CYP2B12 cDNA-derived amino acid sequence, it is highly likely that the cytochrome CYP2B12 protein is a functional *P*-450.

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