Species-specific induction of cytochrome *P*-450 4A RNAs: PCR cloning of partial guinea-pig, human and mouse *CYP4A* cDNAs

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PCR was used to demonstrate the presence of a conserved region and to clone novel members of the cytochrome *P*-450 4A gene family from guinea pig, human and mouse cDNAs. This strategy is based on the sequences at nucleotides 925–959 and at the haem binding domain (nucleotides 1381–1410) of the rat *CYP4A1* gene. Murine *Cyp4a* clones showed high sequence identity with members of the rat gene family, but *CYP4A* clones from human and guinea pig were equally similar to the rat/mouse genes, suggesting that the rat/mouse line had undergone gene duplication events after divergence from human and guinea-pig lines. The mouse *Cyp4a-12* clone was localized to chromosome 4 using interspecific backcross mapping, in a region of synteny with human chromosome 1. The assignment of the human *CYP4A11* gene to chromosome 1 was confirmed by somatic cell hybridization. An RNAase protection assay was shown to discriminate between the murine *Cyp4a-10* and *Cyp4a-12* cDNAs. Treatment of mice with the potent peroxisome proliferator methylclofenapate (25 mg/kg) induced *Cyp4a-10* RNA in liver, and to a lesser extent in kidney; there was no sex difference in this response. *Cyp4a-12* RNA was present at high levels in male control liver and kidney samples, and was not induced by treatment with methylclofenapate. However, *Cyp4a-12* RNA was present at low levels in control female liver and kidney RNA, and was greatly induced in both organs by methylclofenapate. Guinea pigs were exposed to methylclofenapate (50 mg/kg), but there was no significant induction of the guinea-pig *CYP4A13* RNA. These findings are consistent with a species difference in response to peroxisome proliferators between the rat/mouse and the guinea pig.

INTRODUCTION

Chronic administration of hypolipidaemic drugs such as clofibrate, methylclofenapate and nafenopin, and the structurally distinct WY-14,643 [4-chloro-6-(2,3-dimethylbenzamino)-2-pyrimidinylthiolacetic acid], cause liver enlargement in the rat and mouse, with proliferation of the smooth endoplasmic reticulum and peroxisomes [1]. The proliferation of smooth endoplasmic reticulum is associated with the induction of a cytochrome *P*-450 with specificity towards the ω-hydroxylation of lauric acid [2]. This cytochrome *P*-450 has been purified from clofibrate-treated rat liver [3], and cloning of the cDNA identified the first member of a new *P*-450 family, the *CYP4A1* gene [4–6].

It has been proposed that the induction of the CYP4A1 protein plays an important and mechanistic role in peroxisome proliferation [7–9]. In agreement with this hypothesis, the induction of CYP4A1 RNA precedes the induction of peroxisomal enzyme RNAs after administration of the potent peroxisome proliferator methylclofenapate *in vivo* [10]. *In vitro* [11] studies on primary cultures of rat hepatocytes confirmed and extended these data by showing that CYP4A1 RNA was induced in the presence of the protein synthesis inhibitor cycloheximide. In a study of the comparative tissue distribution and induction of CYP4A1 and acyl-CoA oxidase, induction of CYP4A1 RNA was found to be limited to the liver and kidney, which are known to be sites of peroxisome proliferation [12].

The cytochrome *P*-450 4 gene family is known to be present and inducible in the cockroach [13], and both peroxisome proliferation and lauric acid hydroxylases are known to be inducible in organisms as evolutionarily distinct as rats, plants [14] and yeast [15]. In this context, the non-responsive nature of guinea pigs and primates to treatment with potent peroxisome proliferators, as measured by induction of lauric acid hydroxylases [16,17] (or peroxisome proliferation [18,19] is surprising. There is no evidence for inducibility of human *CYP4A* genes [20,20a]. One possible explanation of this marked species difference resides in the fact that single amino acid changes in cytochrome *P*-450 primary structure may dramatically alter the catalytic activity of specific *P*-450 enzymes [21]. The *CYP4A* gene family in guinea pigs and primates may not efficiently catalyse the ω-hydroxylation of lauric acid.

In order to address this issue, we have cloned CYP4A cDNAs from mouse, guinea pig and human, and studied the chromosomal localization of the mouse and human genes. We have also studied the expression of genes in all three species and measured the induction of *P*-450 4A RNAs in the mouse and guinea pig.

MATERIALS AND METHODS

Animals

Groups of four male and female CFLP mice (25 g; obtained from Bantin and Kingman) were dosed daily by intraperitoneal

Abbreviation used: RFLP, restriction fragment length polymorphism.

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The nucleotide sequence data of *Cyp4a-10*, *Cyp4a-12*, *CYP4A11* and *CYP4A13* have been deposited with the EMBL, DDBJ and GenBank Nucleotide Sequence Databases under accession nos. X71478, X71479, X71480 and X71481 respectively.

injection with methylclofenapate (synthesized by Lancaster Synthesis Ltd., WhiteLund, Morecambe, Lancashire, U.K.), 25 mg/kg body weight, for 4 days. Groups of four Dunkin–Hartley guinea pigs (400 g) were dosed daily by intraperitoneal injection with methylclofenapate, 50 mg/kg body weight, for 4 days. Controls were dosed with the vehicle, corn oil (5 ml/kg), by intraperitoneal injection. Food and water were provided *ad libitum*. Animals were killed with an overdose of Sagatal, and organs were removed and frozen in liquid N_2 prior to storage at −20 °C. Human liver samples were obtained from surplus tissue from resection during transplants.

Cloning of CYP4A cDNAs

RNA was isolated by lithium chloride precipitation from guanidine thiocyanate [22]. Total RNA was reverse-transcribed using the cDNA synthesis kit from Amersham, according to the manufacturer's instructions. First-strand cDNA was extracted with phenol/chloroform (1:1, v/v), and used directly for PCR. Oligonucleotides for PCR (restriction sites in italics) were (a) *AAGCTTGAATTC*TTGTCTGACAAGGACCTACGTGCTGAGGTGGAT/CAC, and (b) *AAGCTTGGATCC*CATCTCACTCATAGCAAATTGTTTCCCAAT, based on bases 925–959 and 1381–1410 respectively of the rat *CYP4A1* sequence. The PCR cycle was 94 °C, 1 min; 50 °C, 1 min; 72 °C, 1 min, for 25 cycles, with 5 mM Mg^{2+} in the PCR buffer. PCR products were gel-purified, then restricted with *Bam*HI and *Eco*RI and cloned into pGEM7 (Promega). Plasmid DNAs were purified by banding in CsCl, and sequenced using the Sequenase kit. Individual clones were sequenced on both strands, and three independent isolates of each cytochrome *P*-450 were sequenced. Sequence analysis utilized the SERC Daresbury GCG package [23]. The clones were named after consultation with David R. Nelson of the *P*-450 nomenclature group [6].

RNAase protection assay

The RNAase protection assay was performed as described previously [10], except that hybridization was at 45 °C, and RNAase T_1 was omitted from the hybridization cocktail. Sense and anti-sense transcripts of CYP4A were transcribed from the T7 and SP6 promoters respectively. Autoradiograph signals in the linear range of film response were quantified by densitometry, and the results were normalized for transcript length. Known amounts of RNA probe of defined identical specific activity were co-electrophoresed with the protected fragments for quantification of RNA fragments.

Murine interspecific backcross mapping

Interspecific backcross progeny were generated by mating C57BL/6J × *Mus spretus* F_1 females and C57BL/6J males as described [24]. A total of 205 N_2 mice were used to map the *Cyp4a* locus (see the Results section for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer and hybridization were performed essentially as described [25]. All blots were prepared with a Zetabind nylon membrane (AMF-Cuno). The probe (the 487 bp *Cyp4a-12* cDNA described in the Results section) was labelled with [α-^{32}P]dCTP using a nick translation kit (Boehringer Mannheim); washing was done to a final stringency of 0.8 × SSC/0.01 % SDS at 65 °C. Fragments of 16.0, 9.4, 8.4, 6.2, 3.9, 3.3, 3.2, 1.8 and 1.2 kb were detected in *Hin*dIII-digested C57B16J DNA and fragments of 7.4, 4.2, 3.9, 3.1, 1.8 and 1.2 kb were detected in *Hin*dIII-digested *M. spretus* DNA. The presence or absence of the 7.4 and 3.1 kb *M. spretus*-specific *Hin*dIII fragments, which co-segregated, was followed in backcross mice.

A description of the probes and restriction fragment length polymorphisms (RFLPs) for the loci linked to *Cyp4a-12*, including *Jun*, *Lmyc1* and *Lck*, has been reported previously [26]. Recombination distance was calculated using the computer program SPRETUS MADNESS [27]. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

Human/mouse hybrid DNA mapping

Human lung type II pneumocytes (A549) deficient in hypoxanthine phosphoribosyltransferase, and mouse B82 cells, an L cell subclone deficient in thymidine kinase, were used to prepare hybrid cells as described previously [28]. Parent and hybrid cells were grown up in large quantities and cell pellets were frozen prior to analysis.

The human chromosome content of the hybrid cells was determined by analysing cell pellet homogenates for the presence of specific isoenzymes previously assigned to each human chromosome by starch gel electrophoresis [29,30]. The cell pellets were also used to isolate high-molecular-mass DNA from parent and hybrid cells. Cells were resuspended in a small volume of lysis buffer (100 mM Tris, pH 8, 50 mM spermidine, 100 μg of proteinase K/ml). After homogenization and incubation at 37 °C for 1 h, the sample was extracted with an equal volume of phenol. The aqueous layer was treated with 100 μg of RNAase A/ml prior to a second phenol extraction. DNA was precipitated from the aqueous layer with ethanol.

The human *CYP4A11* gene was detected by PCR; primers were derived from the cloned *CYP4A11* sequences (see below) CACCACAGCCAGTGGGATCTCCTGG and GGTTCTGGTGAAGGCCATAAATGG. Reaction conditions for PCR were 65 °C, 1 min; 72 °C, 2 min; 95 °C, 1 min, for 30 cycles. A human-specific PCR product of approx. 1300 bp was detected by electrophoresis on an agarose gel. The identity of the human *CYP4A11* gene product was confirmed by sequence analysis of the PCR product (results not shown).

RESULTS

Oligonucleotides for PCR were based on conserved sequences specific to the *CYP4A* gene family [31], the region at amino acids 303–332 of rat CYP4A1, and the haem binding region. Successful PCR of first-strand cDNA from the guinea pig or human required the presence of a high concentration of Mg^{2+} (5 mM) in the PCR buffer. Electrophoresis revealed the presence of the expected fragment of 487 bp, which was dependent on the presence of first-strand cDNA (results not shown). PCR products were subcloned into pGEM7, and > 20 clones from each species were screened to identify distinct clones. At least three independent isolates of each *P*-450 gene were sequenced to obviate artefacts arising from potential errors arising during the PCR process.

One *CYP4A* gene was identified in guinea pig and human, and two distinct genes were identified in the mouse. The cDNA and deduced amino acid sequences of the clones are shown in Figures 1(a) and 1(b) respectively. One of the mouse gene sequences is truncated due to the presence of a *Bam*HI site in the cDNA. The mouse genes are highly similar to known rat genes (Table 1): one shows high similarity to rat *CYP4A1*, and is named *Cyp4a-10*.

(a)

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      958
4A1   ACATTTATGT TCGAGGGTCA TGACACCACA GCCAGTGGAG TCTCCTGGAT
4a-10 acactcatgt tcgagggcca tgacaccaca gccagtggag tctcctggat
4a-12 actttcatgt tcgagggcca tgacaccaca gctagtggta tctcctggat
4A11  acattcatgt tcgagggcca cgacaccaca gccagtggga tctcctggat
4A13  accttcatgt ttgagggcca tgacaccaca gccagtggta tctgctggac

4A1   CTTCTATGCT CTGGCCACAC ACCCTAAGCA CCAACAAAGA TGCAGAGAGG
4a-10 cttctatgct ctggccacac accctgatca ccaacagaga tgcagagagg
4a-12 cttctatgct ttggccacaa atcctgaaca tcaacagaga tgcaggaagg
4A11  tctgtatgct ctggccacac accccaagca tcaggagagg tgccgggaag
4A13  cttgtatgct cttgctacac accccagcca tcaacagagg tgcagggaag

4A1   AAGTTCAGAG TGTCCTGGGG GATGGGTCCT CCATTACCTG GGATCACCTG
4a-10 aagttcagag cctcctgggg gatggatcc
4a-12 agatccaaag tctcctagga gatgggactt ctatcacctg gaatgacctg
4A11  agatccatgg cctcctgggt gatggagcct ccatcacctg gaaccacctg
4A13  aggtccagaa cctcctggga gataaaggct ccattacttg ggaacacctt

4A1   GACCAGATTC CCTACACCAC CATGTGTATC AAGGAGGCCC TGAGGCTTTA
4a-12 gacaagatgc cctatactac catgtgcatc aaggaggccc tgaggatcta
4A11  gaccagatgc cctacacaac catgtgcatt aaggaggcac tgagactcta
4A13  gaccagatgt cctataccac catgtgcatc aaggaggccc tgaggctcta

4A1   CCCACCTGTT CCAGGCATTG TCAGAGAACT CAGCACATCT GTCACCTTCC
4a-12 ccctcctgta ccaagtgtga gcagagagct cagctcacct gtcacctttc
4A11  cccaccggtg ccaggcatta gcagagagct cagcactccc gtcactttcc
4A13  tccaccagtt ccatcagttg gcagagagct cagcaaacct gttaccttcc

4A1   CTGATGGGCG CTCTTTACCC AAGGGTATCC AAGTCACACT CTCCATTTAT
4a-12 cagatggacg ttctttaccc aaaggtatcc atgtcatgct gtccttttat
4A11  ctgatgggcg ctccttgccc aaagggatca cggtcatgct ctccatttat
4A13  ctgatggaaa atctttaccc aaaggtatta acgtcatact ctcttttcat

4A1   GGTCTCCACC ACAACCCGAA GGTGTGGCCA AACCCAGAGG TGTTTGACCC
4a-12 ggccttcatc acaacccaac tgtgtggcca aatccagagg tgtttgatcc
4A11  ggccttcacc acaacccaaa agtgtggccc aacccagagg tgtttgaccc
4A13  ggccttcacc ataacccgaa ggtgtggcca aatcctgaga tatttgaccc

4A1   TTCCAGGTTT GCACCAGACT CTCCCCGACA CAGCCACTCA TTCCTGCCCT
4a-12 ttctcgattt gcaccagggt cttcccggca cagccactca ttcctgccct
4A11  ttcccgtttt gcaccgggtt ctgctcaaca cagccacgct ttcctgccct
4A13  ttcccgattt gcacctgatt cttccagaca cagccactca ttcctgccct

                          1380
4A1   TCTCAGGAGG AGCGAGGAAC TGC
4a-12 tctcaggagg agcaaggaac tgc
4A11  tctcaggagg atcaaggaac tgc
4A13  tctcaggagg accaaggaac tgc
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(b)

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          1                                                  50
Consensus TfMFEGHDTT ASGisWifYA LAThPeHQqr CReEvqslLG Dg.SiTWdhL

4a-10     TLMFEGHDTT ASGVSWIFYA LATHPDHQQR CREEVQSLLG DGS
4A11      TFMFEGHDTT ASGISWILYA LATHPKHQER CREEIHGLLG DGASITWNHL
4a-12     TFMFEGHDTT ASGISWIFYA LATNPEHQQR CRKEIQSLLG DGTSITWNDL
4A13      TFMFEGHDTT ASGICWTLYA LATHPSHQQR CREEVQNLLG DKGSITWEHL

          316
4A1       TFMFEGHDTT ASGVSWIFYA LATHPKHQQR CREEVQSVLG DGSSITWDHL
4A2       TFMFEGHDTT ASGISWVFYA LATHPEHQER CREEVQSILG DGTSVTWDHL
4A3       TFMFEGHDTT ASGISWVFYA LATHPEHQER CREEVQSILG DGTSVTWDHL
4A8       TFMFEGHDTT ASGISWIFYA LATNPEHQQG CRKEIQSLLG DGASITWDDL
4A4       TFMFEGHDTT ASGVSWIFYA LATHPEHQHR CREEIQGLLG DGASITWEHL

          51                                                 100
Consensus DqmpYTTMCI KEALRlYpPV psvsReLS.p VTFPDGrSlP KGitv.lsiy

4A11      DQMPYTTMCI KEALRLYPPV PGISRELSTP VTFPDGRSLP KGITVMLSIY
4a-12     DKMPYTTMCI KEALRIYPPV PSVSRELSSP VTFPDGRSLP KGIHVMLSFY
4A13      DQMSYTTMCI KEALRLYPPV PSVGRELSKP VTFPDGKSLP KGINVILSFH

4A1       DQIPYTTMCI KEALRLYPPV PGIVRELSTS VTFPDGRSLP KGIQVTLSIY
4A2       DQMPYTTMCI KEALRLYSPV PSVSRELSSP VTFPDGRSIP KGIRVTILIY
4A3       DQIPYTTMCI KEALRLYPPV PSVSRELSSP VTFPDGRSIP KGITTTILIY
4A8       DKMPYTTMCI KEALRIYPPV TAVSRMLSTP VTFPDGRSLP KGITVMLSFY
4A4       DQMPYTTMCI KEALRLYPPV PSVTRQLSKP VTFPDGRSLP KGVILFLSIY

          101                                          141
Consensus GLHhNPkvWp NPevFDPsRF aPdS.rHSH. fLPFSGGaRN C

4A11      GLHHNPKVWP NPEVFDPSRF APGSAQHSHA FLPFSGGSRN C
4a-12     GLHHNPTVWP NPEVFDPSRF APGSSRHSHS FLPFSGGARN C
4A13      GLHHNPKVWP NPEIFDPSRF APDSSRHSHS FLPFSGGPRN C

                                                       456
4A1       GLHHNPKVWP NPEVFDPSRF APDSPRHSHS FLPFSGGARN C
4A2       GLHHNPSYWP NPKVFDPSRF SPDSPRHSHA YLPFSGGARN C
4A3       GLHHNPSYWP NPKVFDPSRF SPDSPRHSHA YLPFSGGARN C
4A8       GLHHNPTVWP NPEVFDPYRF APESSRHSHS FLPFSGGARN C
4A4       GLHYNPKVWQ NPEVFDPFRF APDSAYHSHA FLPFSGGARN C
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Figure 1 Alignment of the cDNA (a) and deduced amino acid (b) sequences of guinea-pig, human and mouse *CYP4A* cDNA clones

CYP4A clones from guinea pig, human and mouse were obtained by PCR of first-strand liver cDNA, as described in the Materials and methods section. The PCR products were cloned into pGEM7 and sequenced on both strands. (**a**) The DNA sequences of *CYP4A1*, *Cyp4a-10*, *Cyp4a-12*, *CYP4A11* and *CYP4A13* were aligned using the program 'GAP'. The first and last bases of the rat *CYP4A1* cDNA are numbered. The first two bases of this sequence are derived from the 5′ oligonucleotide primer, and the sequence ends immediately before the 3′ primer. (**b**) The deduced amino acid sequences of guinea pig (4A13), human (4A11) and mouse (4a-10 and 4a-12) CYP4A clones are aligned with rat CYP4A1, CYP4A3, CYP4A8 and rabbit CYP4A4, and a consensus sequence (Consensus) has been generated using the program 'PROFILE'. The amino acid sequences were derived directly from the sequences in (**a**), and the numbering of the residues in the CYP4A1 protein is indicated. Nucleic acid sequence analysis was performed on the Genetics Computing Group software at Daresbury.

Table 1 Similarity of Cyp4a protein sequences

Similarity between the rat Cyp4a proteins and those of the guinea-pig, human and mouse were calculated using the programme 'GAP'.

	Identity (%)				
	Rat				Rabbit
	4A1	4A2	4A3	4A8	4A4
Cyp4a-10	93	84	84	81	86
Cyp4a-12	84	82	81	93	82
CYP4A13	83	78	77	79	80
CYP4A11	87	80	80	83	85

The other class of mouse clone shows highest similarity to the rat *CYP4A8* gene [32], and is named *Cyp4a-12*. The guinea-pig *CYP4A13* gene was significantly less similar to the rat *CYP4A* genes, but was most similar to rat *CYP4A1*. The human clone, *CYP4A11*, was not clearly identifiable as a particular homologue (Table 1).

Chromosomal mapping of the mouse *Cyp4a-12* gene

The chromosomal location of the murine *Cyp4a-12* gene was determined by interspecific backcross analysis using progeny derived from matings of (C57BL/6J × *Mus spretus*)F_1 × C57BL/6J mice. This interspecific backcross mapping panel has been typed for over 1100 loci that are well distributed among all the autosomes as well as the X chromosome. C57BL/6J and *M. spretus* DNAs were digested with several enzymes and analysed by Southern blot hybridization for informative RFLPs using a murine *Cyp4a-12* probe. The 7.4 kb and 3.1 kb *M. spretus Hin*dIII RFLPs were used to follow the segregation of the *Cyp4a-12* locus in backcross mice. The mapping results indicated that *Cyp4a-12* is located in the distal region of mouse chromosome 4, linked to *Jun*, *Lmyc1* and *Lck* (Figures 2a and

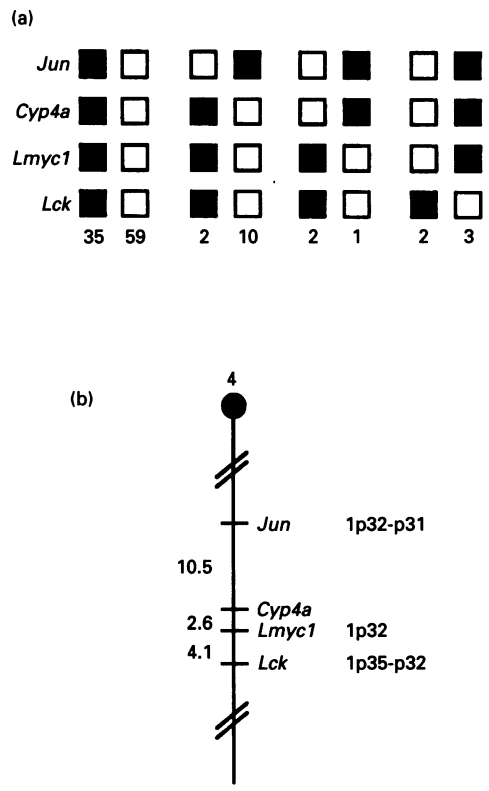

Figure 2 *Cyp4a* maps in the distal region of mouse chromosome 4

Cyp4a was localized to mouse chromosome 4 by interspecific backcross analysis. The segregation patterns of *Cyp4a* and flanking genes in 114 backcross animals that were typed for all loci are shown at the top of the Figure. For individual pairs of loci, up to 122 animals were typed (see the text). (**a**) Each column represents the chromosome identified in the backcross progeny that was inherited from the C57BL/6J × *M. spretus* F_1 parent. The closed boxes represent the presence of a C57BL/6J allele and open boxes represent the presence of an *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. (**b**) A partial chromosome 4 linkage map showing the location of *Cyp4a* in relation to linked genes is shown. Recombination distances between loci in cM are shown to the left of the chromosome and the positions of loci in human chromosomes, where known, are shown to the right. References for the map positions of loci mapped in this study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by the William H. Welch Medical Library of the John Hopkins University (Baltimore, MD, U.S.A.).

2b). Although 114 mice were analysed for every marker and are shown in the segregation analysis, up to 122 mice were typed for some pairs of markers. Each locus was analysed in pairwise combinations for recombinant frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analysed for each pair of loci and the most likely gene order are: centromere–*Jun*–(12/114)–*Cyp4a-12*–(3/114)–*Lmyc1*–(5/122)–*Lck*. The recombination frequencies [expressed as genetic distances in centiMorgans (cM); means ± S.E.M.] are *Jun*–(10.5 ± 2.9)–*Cyp4a-12*–(2.6 ± 1.5)–*Lmyc1*–(4.1 ± 1.8)–*Lck*.

Chromosomal localization of the human *CYP4A11* gene

The location of the human cytochrome *P*-450 *CYP4A11* gene was determined using somatic cell hybridization techniques. First, a method was developed which would distinguish between the human and mouse genes. Oligonucleotide primers were prepared which spanned the first 330 bases in the human cDNA sequence. This region is known to span two intron sequences in the rat *4A1* gene sequence. These primers amplified a specific product of 1300 bp from human genomic DNA, but failed to give any product from mouse DNA. The amplified human PCR product was confirmed as the human *CYP4A11* gene by partial sequence analysis (results not shown). Thus it was possible to specifically detect the presence of the human *CYP4A11* gene against the murine *Cyp4a* background.

Table 2 Segregation of the *CYP4A* gene in human × mouse hybrid clones

Hybrid clones were grown up in large quantity and tested for the presence or absence of chromosome specific enzymes or for the presence of the *CYP4A* gene by PCR, as described in the Materials and methods section. The presence or absence of the trait is marked by + or − respectively. Chromosomes 3 and 22 were not tested.

		Cytochrome/enzyme No. of hybrid clones			
Chromosome	Enzyme	+/+	+/−	−/+	−/−
1	Peptidase C	5	0	0	9
1	Adenylate kinase 2	5	0	0	9
X	Glucose-6-phosphate dehydrogenase	3	1	2	6
2	Malate dehydrogenase 1	4	0	4	4
4	Phosphoglucomutase 2	4	2	1	7
5	Hexosaminidase B	2	2	2	6
6	Malic enzyme 1	3	2	2	6
7	β-Glucuronidase	4	1	3	5
8	Glutathione reductase	3	2	1	7
9	Adenylate kinase 1	3	3	2	8
10	Glutamate oxaloacetate transaminase	2	4	3	5
11	Lactate dehydrogenase A	1	3	5	3
12	Lactate dehydrogenase B	2	2	3	6
13	Esterase D	4	0	3	7
14	Nucleoside phosphorylase	1	4	4	6
15	Hexosaminidase A	2	2	3	8
16	Adenosine phosphoribosyltransferase	2	3	4	5
17	Thymidine kinase	5	0	14	0
18	Peptidase A	1	3	2	7
19	Glucose phosphate isomerase	3	2	2	8
20	Adenosine deamidase	1	4	5	4
21	Superoxide dismutase	2	3	3	7

Sixteen primary hybrid clones were analysed for the presence or absence of the *CYP4A11* gene using PCR. Hybrids were scored as plus or minus according to the presence or absence of the PCR product. These hybrids were also analysed for their human chromosome content using isoenzyme analysis. The results (Table 2) show that the human *CYP4A11* gene segregates with the genes for peptidase C and adenylate kinase 1 previously assigned to human chromosome 1. All other chromosomes tested segregated independently from the *CYP4A11* gene (chromosomes 3 and 22 were not examined).

Expression and induction of murine *P*-450 *4A* genes

In view of the relatively high sequence similarity (84%) between the murine *Cyp4a* clones, an RNAase protection assay was evaluated for discrimination between the *Cyp4a-10* and *Cyp4a-12* genes (Figure 3a). Sense transcripts from the *Cyp4a-10* and *Cyp4a-12* cDNAs were hybridized to the anti-sense probe; the RNAase protection assay successfully discriminated between these transcripts under the conditions used.

Outbred male and female CFLP mice were treated with the

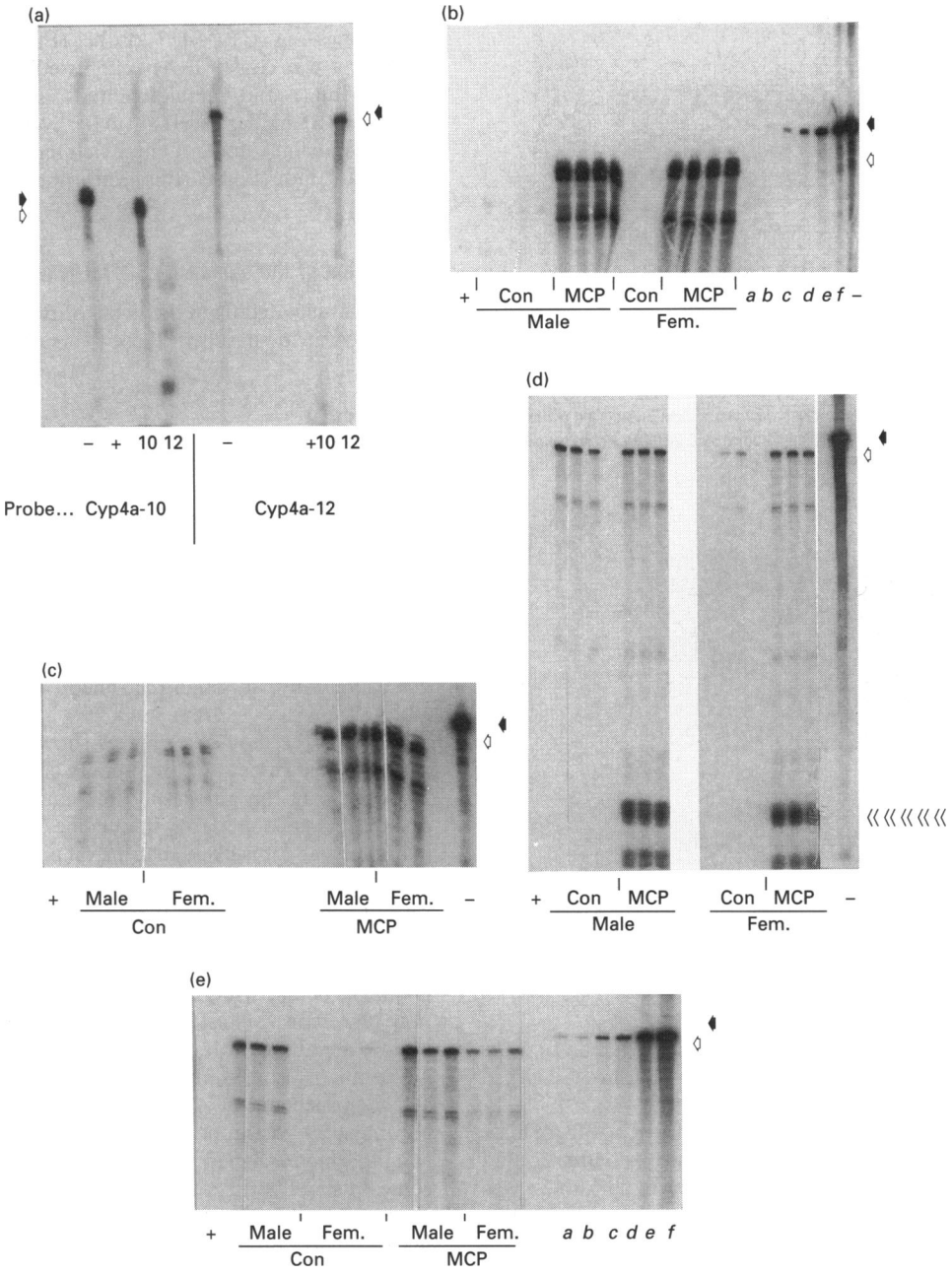

Figure 3 Expression and induction of *Cyp4a-10* (b, c) and *Cyp4a-12* (d, e) genes in male and female mice

RNAase protection assays were performed using sense and anti-sense transcripts from murine *Cyp4a* clones, as described in the Materials and methods section. Groups of four male and female (Fem.) mice were dosed daily with corn oil vehicle (Con) or with 25 mg of methylclofenapate/kg body weight (MCP) for 4 days, and RNA was isolated from each liver and kidney, as described in the text. The RNA probes were shown to be specific (**a**), and were used to detect *Cyp4a-10* (**b**, **c**) and *Cyp4a-12* (**d**, **e**) transcripts in 30 μg of total RNA. RNA samples from 4 mice per group are shown in (**b**), and from 3 mice per group in (**c**), (**d**) and (**e**). The position of full-length probe is indicated by a filled arrow, and the position of the protected fragment by an open arrow. The RNA probe was subjected to the RNAase protection procedure with 30 μg of yeast tRNA in the presence (+) or absence (−) of RNAase. (**a**) The indicated probe was incubated with sense transcripts of *Cyp4a-10* or *Cyp4a-12* and subjected to an RNAase protection assay. Known amounts of RNA transcripts were included for determination of the linear range of autoradiography, and are labelled *a–f* (see **b** and **e**). The position of a low-molecular-mass band which is detected by the *Cyp4a-12* probe is indicated by chevrons (**d**). Each protection assay was repeated at least once.

potent peroxisome proliferator methylclofenapate. The amounts of both Cyp4a RNAs were determined in control and treated liver and kidney RNAs (Figures 3b and 3c).

Cyp4a-10 was expressed at very low levels in the liver RNA of control mice: although *Cyp4a-10* RNA was detected after prolonged exposure of autoradiographs (results not shown), this level of expression could not be quantified. There was no gross difference in the levels of this RNA between male and female mice (Figure 3b). However, *Cyp4a-10* RNA was present at low levels in the kidneys of control animals. There was no significant difference between the levels of this RNA in male and female mouse kidneys.

Treatment of mice with methylclofenapate led to a large induction (> 35-fold) of *Cyp4a-10* in liver RNA, and also in

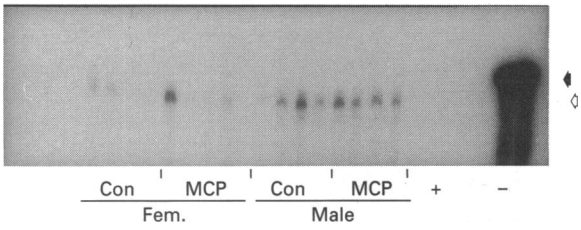

Figure 4 Expression of *CYP4A13* in male and female guinea pigs

Groups of four male and female guinea pigs were dosed daily by intraperitoneal injection of corn oil (Con) or methylclofenapate (50 mg/kg) (MCP) for 4 days, and RNA was isolated from each liver. A 30 μg portion of RNA from each sample was analysed by RNAase protection assay as described in the legend to Figure 3. + and – represent probe incubated with 30 μg of yeast tRNA throughout the protection assay, respectively with and without the addition of RNAase A. The filled arrow represents the position of full-length probe, and the open arrow represents the position of the protected fragment.

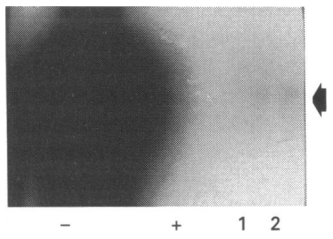

Figure 5 Expression of *CYP4A11* in human liver

RNA was isolated from two human livers (1 and 2), and 30 μg of RNA was analysed by RNAase protection assays as described in the legend to Figure 3. + and – represent probe incubated with 30 μg of yeast tRNA throughout the protection assay, respectively with and without the addition of RNAase A. The filled arrow represents the position of the protected fragment.

kidney RNA, where the levels were slightly higher than in liver. There was no effect of sex on the induction of *Cyp4a-10* RNA in either liver or kidney.

Cyp4a-12 was expressed at high levels in liver RNA from male control mice, but at lower levels in female mice (Figures 3d and 3e). The level of *Cyp4a-12* RNA in control male kidney was similar to that in control male liver, but the level of this RNA in control female kidney was lower than in control female liver (Figures 3d and 3e). The level of *Cyp4a-12* RNA in control mice is therefore regulated in a sex-specific fashion, with the level in male mice being greater than that in female mice. The administration of methylclofenapate led to no change in the male liver, but gave a large induction of *Cyp4a-12* RNA in the female liver, although the absolute levels of this RNA were similar in male and female treated livers. In kidney, treatment with methylclofenapate led to a marginal increase in the levels of *Cyp4a-12* RNA in the male, but gave a similar absolute level and a large fold induction in female mice (Figures 3d and 3e). There are, therefore, marked sex differences in the induction of *Cyp4a-12* RNA in the mouse, which may be attributable to large differences in the basal level of expression of this gene between male and female.

Expression of the guinea-pig *CYP4A13* gene

Guinea pigs were treated with vehicle or methylclofenapate at a dose of 50 mg/kg body weight for 4 days. RNA from the livers of these animals was analysed by RNAase protection assay with the guinea-pig *CYP4A13* probe (Figure 4). The *CYP4A13* transcript was constitutively expressed in liver RNA from both control male and female animals, although this RNA was expressed at higher levels in male liver. Treatment of animals with a maximal dose of methylclofenapate did not lead to a significant induction of this transcript in either male or female guinea pigs.

Expression of the human *CYP4A11* gene

RNA was isolated from the livers of two donors; *CYP4A11* was expressed at low levels in these livers (Figure 5).

DISCUSSION

A highly conserved region of the *CYP4A* gene family was postulated after comparison of members of the rat *CYP4A* and human *CYP4B* [33] gene families revealed an 18-amino-acid region starting at 300 amino acids. Oligonucleotide probes based on this region and the conserved haem binding domain amplified successfully *CYP4A* cDNAs from mouse, human and guinea pig. These findings provide further circumstantial evidence to support the ubiquity of this conserved region in the *CYP4A* gene subfamily, although the functional role of this sequence is still unknown.

Whereas the mouse sequences showed high identity with the rat genes, the guinea-pig and human clones were more divergent. In particular, the guinea-pig and human clones do not show greater sequence similarity to any one of the four rat genes in this subfamily, and it is likely that the rat and mouse gene duplication events occurred separately after divergence from the guinea-pig and human line. In this regard, a current hypothesis that the guinea-pig-like rodents (Caviomorpha) branched off before the separation of rat-like rodents (Myomorpha) from the primates [34] is particularly illuminating. However, the current sequences may represent a subset of the *CYP4A* genes from a species, and a more definitive evolutionary comparison awaits sequencing of all members of the guinea-pig, human and mouse *CYP4A* genes.

The placement of *Cyp4a-12* in the distal region of mouse chromosome 4 (Figure 2) confirms and extends previous studies [35,38], which assigned the murine *Cyp4a* gene cluster to chromosome 4 using hybridization with a rat probe. We have compared the interspecific map of chromosome 4 with a composite mouse linkage map that reports the map location of many uncloned mouse mutations (compiled by M. T. Davisson, T. H. Roderick, A. L. Hillyard and D. P. Doolittle, and provided by GBASE, a computerized database maintained by The Jackson Laboratory, Bar Harbor, ME, U.S.A.). *Cyp4a-12* mapped in a region of the composite map that lacks mouse mutations with a phenotype that might be expected for an alteration in this locus (results not shown).

Finally, this distal region of mouse chromosome 4 shares a region of identity with human chromosome 1p. In particular, genes flanking *Cyp4a* in the mouse, including *Jun* and *Lmyc1*, have been placed on human chromosomes 1p32-p31 and 1p32 respectively. The data obtained from the human/mouse hybrid cell analysis does indeed show that the human *CYP4A11* gene segregated with chromosome 1 enzyme markers. All other chromosomes tested segregated independently. It is thus evident that mouse chromosome 4 and human chromosome 1 share a region of synteny.

The peroxisome proliferator methylclofenapate was selected for induction studies in view of its potency, and its long half-life in the species studies. Previous studies on the induction of

peroxisome proliferation with perfluorinated compounds have been confounded by sex differences in metabolism of the inducer [36,37]. There is clear evidence for differential tissue-specific regulation of members of the *CYP4A* gene family in rat kidney [38], and so an RNAase protection assay was established which differentiated between the murine *Cyp4a-10* and *Cyp4a-12* clones. The high specificity of the RNAase protection assay has been defined [39], and it is therefore likely that these probes will not cross-react with other members of the *Cyp4a* family.

The constitutive level of *Cyp4a-10* RNA is low in liver and kidney, but this transcript is highly inducible in both tissues after treatment with the peroxisome proliferator. This is consistent with previous studies showing induction of lauric acid hydroxylase (the enzyme activity of the rat CYP4A1) in mouse liver [16,17]. Our previous studies have shown that the rat CYP4A proteins show cell-specific localization within the kidney [12]. If the murine *Cyp4a-10* shows a similar distribution, then the levels of expression of this protein in specific kidney cells may be higher than in the liver.

There is a report of a sex difference in the hepatic induction of the rat *CYP4A* genes after administration of clofibrate [40]. It was therefore surprising to note that there was no sex difference in either control levels or induced levels of the *Cyp4a-10* gene after administration of methylclofenapate. These data may be explained by a species difference in female susceptibility to peroxisome proliferation, or may reflect differential pharmacokinetics of the peroxisome proliferators in the two studies.

The *Cyp4a-12* gene exhibited distinct regulation; this gene was expressed constitutively at high levels in male liver and kidney, and did not show any induction after treatment of male mice with methylclofenapate. By contrast, the *Cyp4a-12* gene was expressed at low levels in both kidney and liver of female mice, and treatment of these animals with methylclofenapate induced this RNA to levels comparable with those in the male. Sex differences in the expression of murine renal *Cyp4a* proteins and lauric acid hydroxylase have been described in detail [41,42], although those studies were unable to detect a sex difference in the levels of *Cyp4a* RNA using a Northern blot with the rat *CYP4A1* probe. However, the data on the expression of the murine Cyp4a proteins [41,42] are in agreement with our results on the relative levels of expression of *Cyp4a-10* and *Cyp4a-12* RNAs in control mouse kidney (Figure 3). This sex difference in the expression of *Cyp4a-12* has parallels in the rat *CYP4A* gene family: *CYP4A2* RNA is present at higher levels in male liver and kidney, compared with the female, and testosterone increases *CYP4A2* RNA levels in both the liver and kidney of hypophysectomized female rats [40]. The rat *CYP4A8* gene is most similar to mouse *Cyp4a-12*, but no data are available on the sex-specific regulation of this gene; however, it is intriguing to note that it is regulated in the prostate by castration, and by treatment with testosterone [32].

In contrast to the great inducibility of *CYP4A* genes in the rat and mouse, treatment of guinea pigs with high doses of methylclofenapate (twice that administered to the mouse) failed to elicit a marked induction of *CYP4A13* RNA in either male or female livers (Figure 4). While there may also be additional guinea-pig *CYP4A* genes which are as yet uncloned, this finding is in agreement with earlier studies on the induction of lauric acid hydroxylase in the guinea pig, which have failed to show significant induction of *CYP4A* family enzymes and proteins [16,17]. Although the present study has not directly addressed the pharmacokinetics of methylclofenapate, a previous study has shown that this compound is poorly metabolized [43]. These data demonstrate species-specific induction of *CYP4A* genes, and therefore suggest that there is an intrinsic difference in sensitivity to peroxisome proliferators between the rat/mouse and the guinea pig.

A human *CYP4A11* cDNA was cloned and used in RNAase protection analysis. This was used to demonstrate the presence of the cognate RNA in two human livers, although this RNA was expressed at low levels. However, it was not possible to evaluate the inducibility of the human *CYP4A11* gene.

In summary, our results confirm the existence of a conserved peptide in the *CYP4A* gene subfamilies of the guinea pig, human and mouse; whereas the mouse cDNAs are highly related to their rat homologues, the guinea-pig and human cDNAs do not show appreciably greater similarity to any particular member of the rat/mouse *CYP4A* subfamily. The mouse *Cyp4a-12* gene was localized to chromosome 4 by interspecific backcross mapping, and the human *CYP4A11* gene was mapped to chromosome 1. Whereas both members of the mouse *Cyp4a* gene family are inducible after treatment of mice with a peroxisome proliferator, methylclofenapate, the guinea-pig *CYP4A13* cDNA clone was not induced by treatment of guinea pigs with methylclofenapate. These cDNAs may be a useful tool for gaining an understanding of the molecular basis for species differences in response to toxic agents.

After the submission of this manuscript, the sequences of the human *CYP4A11* (accession no. L04751) and mouse *Cyp4a-10* (X69296) genes were published in Genbank by Dr. C. N. A. Palmer and Dr. C. J. Henderson respectively.

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