A novel murine *P*-450 gene, *Cyp4a14*, is part of a cluster of *Cyp4a* and *Cyp4b*, but not of *CYP4F*, genes in mouse and humans

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Genomic clones for *Cyp4a12* and a novel member of the murine *Cyp4a* gene family were isolated. The novel gene, designated *Cyp4a14*, has a GC rich sequence immediately 5′ of the transcription start site, and is similar to the rat *CYP4A2* and *CYP4A3* genes. The *Cyp4a14* gene spans approximately 13 kb, and contains 12 exons; sequence similarity to the rat *CYP4A2* gene sequence falls off 300 bp upstream from the start site. In view of the known sex-specific expression of the rat *CYP4A2* gene, the expression and inducibility of *Cyp4a14* was examined. The gene was highly inducible in the liver when mice were treated with the peroxisome proliferator, methylclofenapate; induction levels were low in control animals and no sex differences in expression were observed. By contrast, the Cyp4a12 RNA was highly expressed in liver and kidney of control male mice but was expressed at very low levels in liver and kidney of female mice. Testosterone treatment increased the level of this RNA in female liver slightly, and to a greater extent in the kidney of female mice. In agreement with studies on the cognate RNA, expression of Cyp4a12 protein was male-specific in the liver of control mice and extremely high inducibility of Cyp4a10 protein, with no sex differences, was also demonstrated. In view of the overlapping patterns of inducibility of the three *Cyp4a* genes, we investigated whether the three genes were co-localized in the genome. Two overlapping yeast artificial chromosome (YAC) clones were isolated, and the three *Cyp4a* genes were shown to be present on a single YAC of 220 kb. The *Cyp4a* genes are adjacent to the *Cyp4b1* gene, with *Cyp4a12* most distant from *Cyp4b1*. The clustering of these two gene subfamilies in the mouse was replicated in the human, where the *CYPA411* and *CYP4B1* genes were present in a single YAC clone of 440 kb. However, the human *CYP4F2* gene was mapped to chromosome 19. Phylogenetic analysis of the *CYP4* gene families demonstrated that *CYP4A* and *CYP4B* are more closely related than *CYP4F*.

INTRODUCTION

The cytochrome *P*-450 (*CYP*) genes constitute a superfamily of haem–thiolate proteins which catalyse the oxidation of a wide variety of substrates, including endogenous lipids and xenobiotics. The *CYP4A* gene subfamily was originally discovered as fatty acid ω-hydroxylase enzymes, which are induced by peroxisome proliferators [1–3]. Several members of this family are now known [4]; the *CYP4A* gene family has two known members in humans [5,6], and four members in rats [2,7–9]. However, the gene family has been less well characterized in mouse; two cDNAs have been cloned in this laboratory [6]. Genes from the *CYP4B* and *CYP4F* family have been cloned [10,11], and are able to hydroxylate fatty acids [12,13]; thus the three mammalian *CYP4* gene families metabolize fatty acids. It is clear that this gene family is of ancient evolutionary lineage; *CYP4* genes are well known in invertebrates [14,15] and phylogenetic analysis indicates that the *CYP4* gene family clusters with families 102, 118, 52 and 110 [4], which are prokaryotic fatty acid hydroxylases.

Metabolism of fatty acids and eicosanoids by the *CYP4A* family is believed to be very important in lipid homoeostasis [1] and signalling. The metabolism of arachidonic acid to 20-hydroxyeicosatetraenoic acid by CYP4A proteins is believed to regulate blood pressure in the kidney [16,17]. Moreover, the rapid induction of CYP4A protein is known to be crucial for peroxisome proliferation in the liver [18–20]. The recent identification of leukotriene B_4 as an agonist of peroxisome proliferator activated receptor α [21–23] suggests a role for CYP4A proteins in the turnover of this agonist, and hence a physiological role in inflammation.

Several members of the rat *CYP4A* gene family are highly induced by peroxisome proliferators [1,6,8]. However, it is unclear how many *Cyp4a* family members exist in the mouse, or how many of these are inducible. There is conflicting evidence about the sex-specific expression of the Cyp4a10 and Cyp4a12 RNAs and protein; specifically, several authors have reported that an antibody raised against CYP4A1 detects a sex-specific expression of a murine Cyp4a protein in the male kidney [24,25], whereas RNAse protection data show no sex-specific expression of Cyp4a10 RNA in the kidney [6]. This conflicting evidence may be due to the differing specificities of antibodies but may also be due to the presence of additional mouse *Cyp4a*s. These observations are more difficult to interpret in view of the observation that there are strain differences in expression of murine hepatic Cyp4a proteins [25,26], although, since in these experiments an antibody raised against rat *CYP4A1* was used, it is not clear which murine

Abbreviations used: AP, adaptor-specific primer; CYP, cytochrome *P*-450; GSP, gene-specific primer; MCP, methylclofenapate; ORF, open reading frame; YAC, yeast artificial chromosome.

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The genomic sequence data reported in this paper have been deposited with GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with accession numbers Y11638, Y11639, Y11640, Y11641 and Y11642.

Cyp4a protein(s) are expressed in a male-specific manner. It is also unclear what phenomenon gives rise to differential male-specific regulation of these genes in liver and kidney in the various mouse strains. A more detailed knowledge of the murine *Cyp4a* gene family is essential for understanding the regulation and physiology of *Cyp4a* genes in mice or for establishing strategies for analysing the *Cyp4a* gene family in transgenic mice. The aim of this study was to characterize the murine *Cyp4a* gene family in detail, both to understand the co-ordinate regulation of these genes, and as a prelude to undertaking gene knock-out studies. We now report the cloning of a novel murine *Cyp4a* gene, describe the genomic structure of the mouse *Cyp4a* locus and demonstrate that the *CYP4A* and *CYP4B* genes are clustered in both mouse and human.

MATERIALS AND METHODS

Animals

Mice were fed a standard laboratory diet (Harlan Teklad) *ad libitum*. Groups of four male and four female NMC mice were treated by intraperitoneal injection with corn oil vehicle, testosterone (20 mg/kg body weight) or methylclofenapate (MCP) (10 mg/kg body weight) daily for ten days.

Cloning of the mouse *Cyp4a* genes

A mouse (C_{57}Bl6 × CBA) genomic library in λFIXII (Stratagene) was screened with a mixture of riboprobes for the mouse *Cyp4a10* and *Cyp4a12* cDNAs [6]. Phage (λ) clones were purified to homogeneity and characterized using standard protocols [27]. Briefly, 500000 clones were screened and phage lifts were done on Hybond-N+ membranes as described in the Amersham Hybond Handbook (Amersham). DNA was fixed by baking the membranes at 80 °C for 2 h. Prehybridization was carried out for approx. 2 h and hybridization was performed overnight in hybridization buffer [0.5 M sodium phosphate, pH 7.2, 1 % (w/v) BSA, 7 % (w/v) SDS and 1 mM EDTA] at 65 °C in a Techne HB-1D Hybridiser. Thereafter, membranes were rinsed in 2 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate)/0.1 % SDS at room temperature and washed 3 times in 40 mM sodium phosphate/0.1 % (w/v) SDS buffer, pH 7.0 at 65 °C for 15 min each. Membranes were then exposed to autoradiographic film at −80 °C. Initial screening yielded two distinct classes of overlapping clones (see Figure 1). One class of clone corresponded to the *Cyp4a12* gene, as determined by sequence analysis (Figure 2A). The other class of clone was subcloned for analysis and the 5′ most 2.2 kb *Xba*I subclone was used to rescreen the λFIXII library, yielding 15 independent clones. These were characterized and representative clones spanning the *Cyp4a14* gene are shown in Figure 1. An oligonucleotide (5′ CAA AGG CTT CTG GAA TTT AT 3′) covering bases 358–377 of the *Cyp4a14* open reading frame (ORF), corresponding to the region of the rat *CYP4A3* where there is a 9 bp deletion compared with *CYP4A2*, was end-labelled with [γ-^{32}P]ATP and hybridized to all murine *Cyp4a14* clones containing this region, using the tetramethylammonium-chloride-based wash method of [28].

A PromoterFinder DNA Walking kit (Clontech Laboratories) was used to walk upstream of exon 1 of the *Cyp4a14* gene. The primary PCR reaction was performed using a gene-specific primer (GSP)1 (5′-GAG ATA CCA TCC AAG TAC CTT GTA G-3′) and an adaptor-specific primer (AP)1 (5′-GTA ATA CGA CTC ACT ATA GGG C-3′) in 50 μl of Master Mix (Perkin–Elmer) containing 1 μl of AP1 (10 mM), 1 μl of GSP1 (10 mM), 1 μl of Advantage *Tth* polymerase mix (× 50) (Clontech Laboratories) and 1 μl of each of the five DNA libraries. For the secondary PCR reaction, bands were excised from the gel and re-amplified using a second GSP (GSP2) (5′-CCC ATG GTT AGT AGT TTC TGG ATC GAG-3′) and a second AP (AP2) (5′-ACT ATA GGG CAC GCG TGG T-3′) in the same mixture, except for the AP2 and GSP2 primers and 1 μl of gel-purified DNA. PCR was performed in a DNA Thermal Cycler 480 (Perkin–Elmer) according to the manufacturer's instructions. The PCR reaction cycle was: (94 °C for 25 s, 72 °C for 4 min) for 7 cycles, 32 cycles of (94 °C for 25 s, 67 °C for 4 min) followed by a 4 min extension at 67 °C. The positive band was then gel-purified and cloned into the pGEM-T vector (Promega).

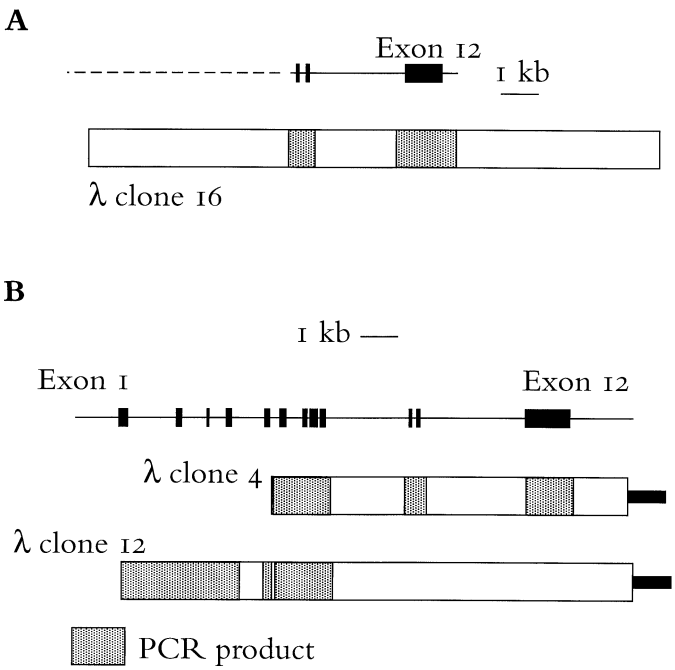

Figure 1 Genomic clones of mouse *Cyp4a* genes

Cartoon of genomic clones and PCR products isolated from mouse libraries. (**A**) The *Cyp4a12* gene. (**B**) The *Cyp4a14* gene. The phage clones (λ clone 16, λ clone 4 and λ clone 12) and a PCR clone, are represented by the lower, horizontal boxes; stippled regions have been sequenced on both strands, plain regions have not and the narrow black box represents further regions of the clone. The continuous upper, horizontal line represents characterized regions of the gene, the dotted region represents an area which has not been characterized and the vertical black boxes represent the position of exons. The scale is indicated by a line which represents one kb.

Phage were subcloned into pGEM7, pGEM5 (Promega) and pZErO (Invitrogen) vectors and DNA was prepared with DNA miniprep columns (Qiagen), according to the manufacturer's instructions. Plasmid minipreps were sequenced with an ABI373A automated DNA sequencer, primarily using oligonucleotide directed sequencing. DNA was sequenced on both strands and the sequence was analysed using the method of Bonfield et al. [29], GCG, CLUSTALW [30], Phylip [31] and WWW-based software packages.

Yeast artificial chromosome (YAC) screening and characterization

Mouse and human YAC libraries were provided as pools by the Medical Research Council Human Genome Mapping Project Resource Centre. The murine libraries [32] were screened by PCR for *Cyp4a14*, 5′ CCA CAG GGA CAT GCA GAT TAG 3′ and 5′ CAC ACA GAG CTC GGA AGA CC 3′, giving a

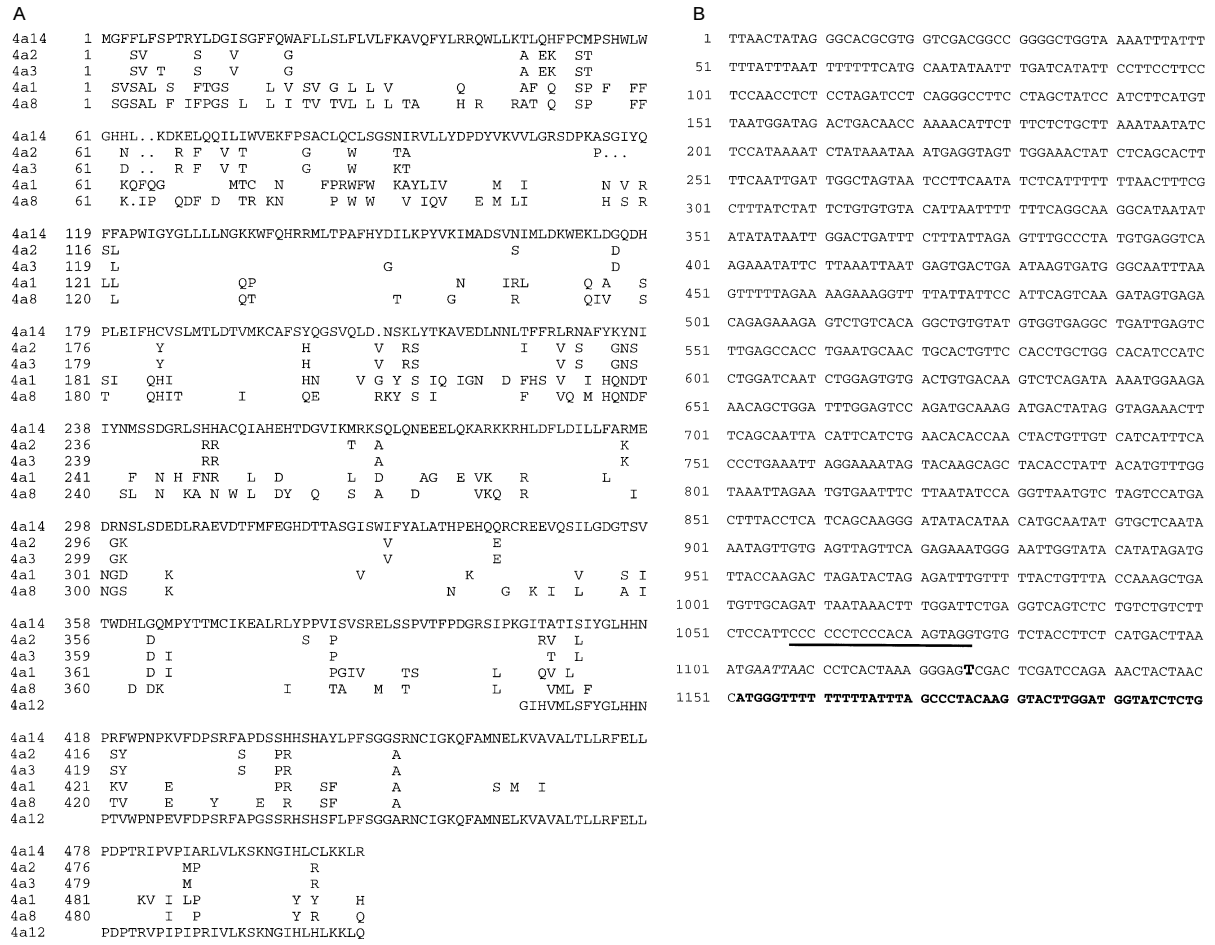

Figure 2 Sequence of mouse *Cyp4a12* and *Cyp4a14* genes

(**A**) The deduced amino acid sequence of the *Cyp4a14* gene (4a14), aligned with rat genes *CYP4A2* (4a2), *CYP4A3* (4a3), *CYP4A1* (4a1) and *CYP4A8* (4a8) and the deduced amino acid sequence of *Cyp4a12* (4a12). The sequence of the rat genes is indicated where there is a divergence from the *Cyp4a14* sequence. (**B**) The sequence of exon 1 and the 5′ of *Cyp4a14*. The coding sequence is indicated in bold, the transcription start site as a large size bold letter, a GAATTAA site upstream of the transcription start site in italics and a site conserved between rat and mouse *CYP4A* genes is underlined.

529 bp PCR product, and 5′ GGT CAT GAC ACT ACA GCC AGT 3′ and 5′ CAG GAA GAC TTT CCT CGG TC 3′, giving a 441 bp PCR product. For *Cyp4a12*, the oligonucleotides used were 5′ CTG GAA GCC CAG CAG AAG GTG 3′ and 5′ TGA GTC CTA TGA AAG AGT GCC 3′, with a 430 bp PCR product. The *Cyp4a*10 oligonucleotides were 5′ GAT CAG CTG CAG GAT GAG GGA G 3′ and 5′ CCT CAG CAC GTA GGT CCT TGT C 3′, giving a 217 bp product. The *Cyp4b1* oligonucleotides were 5′ GCA TTG GGC AAC AGT TTG CC 3′ and 5′ CCC AGG CAT TGA ATC ATT CC 3′, giving a 315 bp product. The human libraries were screened with probes for *CYP4A11* (5′ TCC TGT CTG CCC ATA TCC TG 3′ and 5′ CGG GCT TAG ATT ATG GTG CG 3′), *CYP4B1* (5′ GCC TTC AGG AGG CTT GTA GT 3′ and 5′ TTT GGT GGT ACC CAG CCA TA 3′) and *CYP4F2* (5′ CGG AAC CCA TCA CAA CCC AGC 3′ and 5′ CTG GGC CCT GCC GAG AAG GGA A 3′), giving PCR products of 300, 300 and 264 bp respectively. Individual positive YAC clones were identified and DNA was prepared from large scale cultures isolated as described (Bio-Rad CHEF Genomic DNA Plug kit). Pulsed field gel electrophoresis was carried out using the transverse alternating field electrophoresis system.

Mapping of human *CYP4F* gene

DNA from a series of monochromosomal somatic human–rodent cell hybrids based on mouse or hamster cell lines were provided by the Medical Research Council HGMP [33]. DNA was analysed by PCR.

RNA analysis

RNA was extracted from various organs using the method of Bell et al. [6]. RNase protections were as described previously [6]; the probe for *Cyp4a14* was a 237 bp antisense transcript (bases 1474–1711) of the 3′ non-coding region of *Cyp4a14*. The areas of radioactivity on dried RNase protection gels were analysed on a Bio-Rad GS-250 molecular imager. The oligonucleotide 5′-GAG ATA CCA TCC AAG TAC CTT GTA G-3′ was used for primer extension analysis.

Protein analysis

Microsomes were prepared from mouse liver and kidney by the method of Ashley et al. [34]. Briefly, tissue samples, cooled on

ice, were homogenized, using a motor-driven homogenizer and a Teflon pestle, in 3 vol. of 0.15 M NaCl/10 mM potassium phosphate, pH 7.4. The suspension was centrifuged at 10000 ***g*** for 10 min at 4 °C and the supernatant was centrifuged at 100000 ***g*** for 1 h at 4 °C to sediment microsomes. Microsomal pellets were resuspended and repelleted, followed by resuspension in a small volume of 0.1 M potassium phosphate/20 % (v/v) glycerol, pH 7.4, and were stored at −20 °C. Protein content, estimated by the Lowry method, and Western blotting were carried out as described previously [35]. An antibody against rat *CYP4A1* was prepared by cloning the 1350 bp *Pst*I + *Eco*RI fragment (829–2179 bp) from CYP4A1 [3], which was cloned, in frame, into pRSETc (Invitrogen). The entire 487 bp of the mouse *Cyp4a-12* cDNA previously described [6] was subcloned into the *Eco*RI and *Bam*HI sites of pRSETa. The expression vectors were transformed into *Escherichia coli* BL21 (DE3) and the expression of recombinant protein was induced by the addition of isopropyl thiogalactoside. The proteins were purified by Ni^{2+}-affinity chromatography, were found to be > 95 % pure by SDS/PAGE and were injected, in an emulsion of Freund's incomplete adjuvant, into rabbits. After multiple booster injections, the rabbits were bled and sera were obtained. Optimal conditions for Western blotting were determined empirically. Specific immunoreactivity was detected, using enhanced chemiluminescence (Amersham), on photographic film or by PhosphorImager (Bio-Rad) with a chemiluminescent screen.

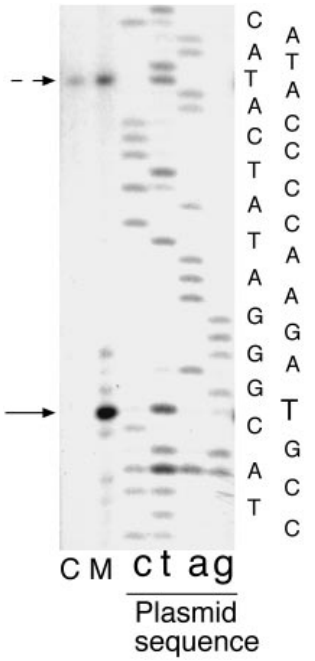

Figure 3 Primer extension analysis of the *Cyp4a14* gene

Male C57Bl6 mice were treated with 25 mg/kg body weight MCP (M) or corn oil vehicle (C) for 4 days and 30 μg of liver RNA was used for primer extension analysis, as described in the Materials and methods section. A subclone of λ clone 12 was used for sequencing with the same oligonucleotide primer (Plasmid sequence, lanes c, t, a and g) and were run in parallel on a denaturing 10% polyacrylamide gel. The sequence is indicated on the right side of the gel and the position of the major and minor primer extension products are indicated on the left by a solid and dashed arrow respectively. The bold nucleotide (T) indicates the transcription start site.

RESULTS

We have previously reported the isolation of two mouse *Cyp4a* cDNAs, *Cyp4a10* and *Cyp4a12* [6]. Screening of a mouse λ library with these clones yielded two classes of clones (Figure 1). One class of clones hybridized strongly to the *Cyp4a12* cDNA and partial analysis revealed a sequence similar to the previously reported *Cyp4a12* cDNA; the deduced peptide sequence contained 103 amino acids. Although there are three differences in nucleic acid sequence between the genomic and cDNA sequence, the deduced amino acid sequences are identical, demonstrating that this clone contains part of the mouse *Cyp4a12* gene (Figure 2A). The genomic sequence for this gene has been deposited with GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with accession numbers Y10221 and Y10222. The intron/exon junctions of this clone are analogous to the positions of exon boundaries 10–12 in *Cyp4a14* (see below). The sequence of exon 12 contains a stop codon followed by a consensus poly(A) addition site downstream from the stop codon, indicating that exon 12 is the final exon of this gene.

However, the other class of clone contained exon sequence from the 3′ half of a *Cyp4* distinct from the previously described *Cyp4a10* and *Cyp4a12* cDNAs. The library was rescreened to obtain clones covering the entire ORF of the gene and representative clones are shown in Figure 1(B). After extensive screening of the library, it was not possible to isolate a λ clone extending further 5′ and a PCR-based approach was used to clone a further 1.2 kb of upstream flanking sequence. The deduced sequence of the ORF encodes a protein of 506 amino acids (Figure 2A), with a molecular mass of 58720 Da (58307 Da for *Cyp4a10*) and, in common with other *Cyp4a* genes, a basic pI of 9.1. This protein encodes a novel cytochrome *P*-450 which has 71 % and 82 % identity with *Cyp4a10* and *Cyp4a12* respectively and is therefore a member of the *Cyp4a* family. In consultation with the *P*-450 nomenclature group [4] the gene was named *Cyp4a14*. The deduced amino acid sequence of the protein is highly similar to the rat *CYP4A2* and *CYP4A3* genes, at 88.3 % and 89.3 % respectively. *CYP4A2* and *CYP4A3* differ by a 3 amino acid deletion after residue 113 of *CYP4A2*; this deletion is not present in *Cyp4a14*. Fifteen independent genomic clones containing exon 3 were screened with an oligonucleotide probe spanning the region of the deletion under conditions of high stringency. However, all of the clones hybridized to the probe (results not shown) indicating an identical sequence in this region. Comparison of the sequence of *Cyp4a14* with other Cyp4a proteins shows that there are distinct regions of sequence similarity within the *CYP4A* subfamily (Figure 2A). In addition to the region between amino acids 296–360 (exon 8), residues 441–506 (exon 12) and 125–167 (exon 4) show high similarity.

The start site of transcription was determined by primer extension analysis (Figure 3). A 25 bp oligonucleotide was annealed to liver RNA from control and MCP-treated C57Bl6 mice and extended with reverse transcriptase. A parallel sequencing reaction with the same primer on a genomic subclone was used to calibrate the exact position of the start site at 25 bp upstream of the initiation methionine codon (Figure 3). The principal extension product was more abundant in RNA from the livers of mice treated with MCP and a less abundant but slightly longer primer extension product was also inducible. However, primer extension with an additional primer detected only the major start site (results not shown) and failed to detect the minor band (Figure 3), which is, therefore, an artefact specific to the primer and only the major, highly inducible band relates to the *Cyp4a*14 gene. The sequence of the *Cyp4a14* gene immediately before the transcription start site is shown in Figure 2(B). A GAATTAA box is present 17 bp upstream of the transcription start site and a 19 bp sequence upstream of this is

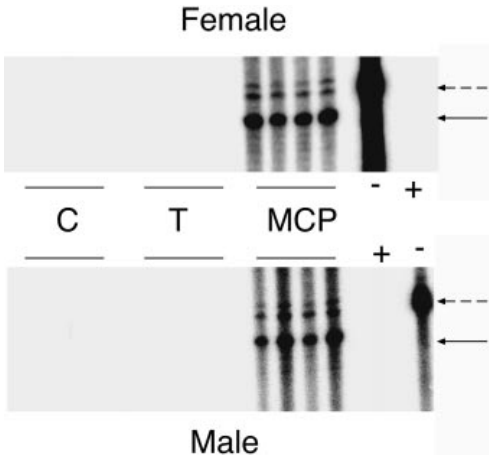

Figure 4 RNase protection analysis of Cyp4a14 RNA

A typical RNase protection with the *Cyp4a14* probe with liver RNA. RNase protection assays were performed using an antisense *Cyp4a14* probe as described in the Materials and methods section. Groups of four male or female mice were treated for 10 days with 10 mg/kg body weight MCP, 20 mg/kg testosterone (T) or corn oil vehicle (C). RNA was isolated from the liver and kidney of each animal and 15 μg of RNA was subjected to protection analysis. Each protection assay had lanes of yeast tRNA with (+) and without (−) RNase A. The position of undigested probe is shown by a dashed arrow and the position of the protected fragment by an unbroken arrow. The results of the protection assay are presented in Table 1. Each protection assay was performed at least twice.

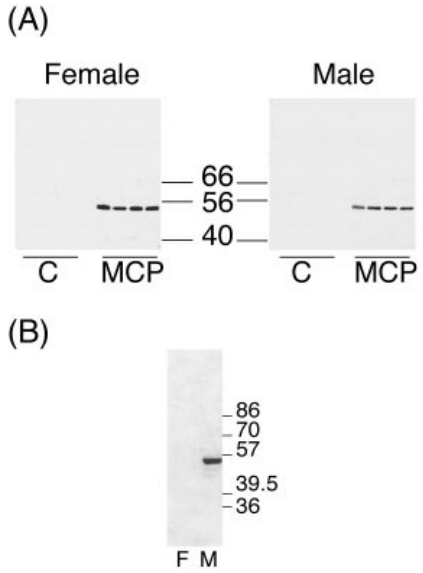

Figure 5 Western blotting for the Cyp4a10 and Cyp4a12 proteins

Liver microsomes from (**A**) groups of four male or four female NMC mice previously treated with 10 mg/kg body weight MCP or corn oil vehicle (C) for ten days or (**B**) untreated female (F) or male (M) mice were used. In (**A**) 10 μg of microsomal protein was loaded into each lane and in (**B**) 18 and 40 μg of protein was loaded for male and female tissue respectively. Samples were separated by SDS/PAGE (7.5% gels) and blotted with a primary antibody raised against recombinant (**A**) *CYP4A1* or (**B**) *Cyp4a12* and detected using enhanced chemiluminescence. The positions of molecular-weight markers (kDa) are indicated.

highly conserved between rat and mouse *Cyp4a14* genes. A putative poly(A) signal (AAATGAA) was detected in exon 12 at position 2163 (results not shown), with a poly(A) site at position 2222.

Regulation of *Cyp4a* genes

In view of the high similarity of *Cyp4a14* to *CYP4A2*, which shows male-specific regulation in the rat [36], the regulation of *Cyp4a* genes was examined in both male and female mice. The probe for *Cyp4a14* contained 237 bp of the 3′ non-coding region (nucleotides 1474–1711). In view of the high specificity of the RNase protection and the relatively high divergence between *Cyp4a* genes in the 3′ non-coding region (present work and [24]), it is likely that this probe will be specific for *Cyp4a14*. The probe for *Cyp4a12* has been described previously [6].

Groups of four male and four female NMC mice were treated with corn oil vehicle, testosterone (20 mg/kg body weight) or

Table 1 RNAse protection analysis of the (a) Cyp4a-14 and (b) Cyp4a-12 RNAs

RNase protection assays were performed using antisense probes as described in the Materials and methods section, and the legend to Figure 4. Groups of four male or four female mice were treated for 10 days with 10 mg/kg body weight MCP, 20 mg/kg body weight testosterone (T), or corn oil vehicle (C). RNA was isolated from the liver and kidney of each animal and 15 μg of RNA was subjected to protection analysis. The results were quantified (Bio-Rad GS-250 PhosphorImager) and represent the means ± S.D. Each assay was performed at least twice. The units are arbitrary and (a) and (b) are not comparable.

(a)		Liver			Kidney		
		C	T	MCP	C	T	MCP
	Male	1 ± 1	2 ± 1	1580 ± 66	10 ± 2	8 ± 3	140 ± 25
	Female	1 ± 2	1 ± 1	1370 ± 80	9 ± 1	7 ± 2	180 ± 45

(b)		Liver			Kidney		
		C	T	MCP	C	T	MCP
	Male	9200 ± 1400	4050 ± 100	4100 ± 1200	830 ± 160	1180 ± 130	750 ± 250
	Female	2 ± 1	100 ± 50	4500 ± 1900	5 ± 2	380 ± 15	030 ± 5

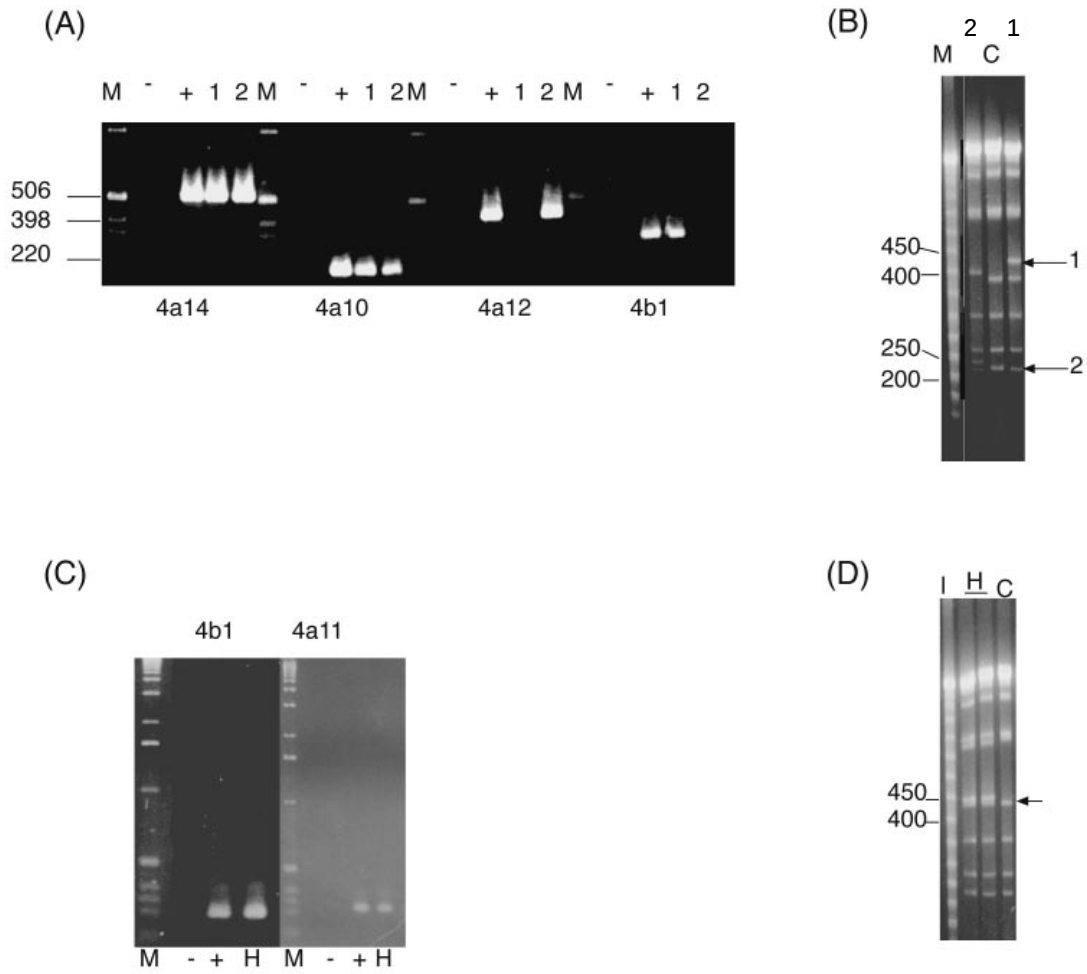

Figure 6 Linkage of *CYP4A* and *CYP4B* gene subfamilies in man and mouse

YAC clones were isolated as described in the Materials and methods section. Mouse YAC DNA was purified and (**A**) analysed by PCR for the presence of the *Cyp4a14* (4a14), *Cyp4a10* (4a10), *Cyp4a12* (4a12) and *Cyp4b1* (4b1) genes and (**B**) by pulsed field gel electrophoresis. Human YAC DNA was purified and (**C**) analysed by PCR for the *CYP4B1* (4B1) and *CYP4A11* (4A11) genes and (**D**) by pulsed field gel electrophoresis. The transverse alternating field electrophoresis system was used for pulsed field electrophoresis and the run parameters were, stage 1: 370 mA constant current, 45 s pulse for 12 h; stage 2: 390 mA constant current, 30 s pulse for 6 h and stage 3: 390 mA constant current, 45 s pulse for 18 h. Abbreviations: M, markers (kb); — control without DNA; + total genomic DNA, 1, mouse YAC clone 1; 2, mouse YAC clone 2; H, human YAC clone; C, yeast DNA; λ, multimerized λ ladders. The YAC insert DNA is indicated with arrows.

MCP (10 mg/kg body weight) daily for ten days. RNA from each of the animals was analysed by RNase protection (Figure 4). As shown in Table 1(a), Cyp4a14 RNA is expressed at extremely low levels in control liver of both male and female mice and at similar levels in control kidney, with no significant difference between male and female mice. Treatment of mice with testosterone had no effect on the level of Cyp4a14 RNA in liver or kidney in either male or female mice. However, treatment of mice with MCP caused a significant induction of Cyp4a14 in the livers and kidneys of both male and female mice; the induction in liver is over 1000-fold. We examined the expression of Cyp4a14 RNA in spleen, lung, brain, gut and gonads in both control and MCP-treated mice. Levels of Cyp4a14 were undetectable in all other tissues examined, except in the spleen where there was a small level of induction (results not shown). Thus there were no sexual differences the expression of *Cyp4a14* and the RNA was highly inducible by peroxisome proliferators in liver and kidney.

By contrast, the Cyp4a12 RNA was highly sex-specific in control animals, with high level expression in male liver and kidney and low levels in females (Table 1b). Treatment of mice with MCP led to a high level induction of Cyp4a12 in female liver, but slightly reduced the level of Cyp4a12 RNA in control males. In kidney, MCP treatment had no effect on levels in males and only led to a very small induction of RNA in female mice. Treatment of mice with testosterone had distinct effects; testosterone slightly reduced the level of Cyp4a12 RNA in male liver and had a minimal effect in male kidney. While testosterone had only a small effect on induction of *Cyp4a12* in the liver of female mice, it led to a much larger induction in the kidney. Thus testosterone and MCP have distinct patterns of induction of Cyp4a12 in female liver and kidney.

Expression of Cyp4a-10 and Cyp4a-12 proteins

There is conflicting evidence about the sex-specific expression of the Cyp4a10 and Cyp4a12 RNA and protein; specifically, several authors have reported that an antibody raised against Cyp4a1 detects a sex-specific expression of a murine Cyp4a protein in the male kidney [24–26], whereas our RNase protection data show no sex-specific expression of Cyp4a10 RNA in the

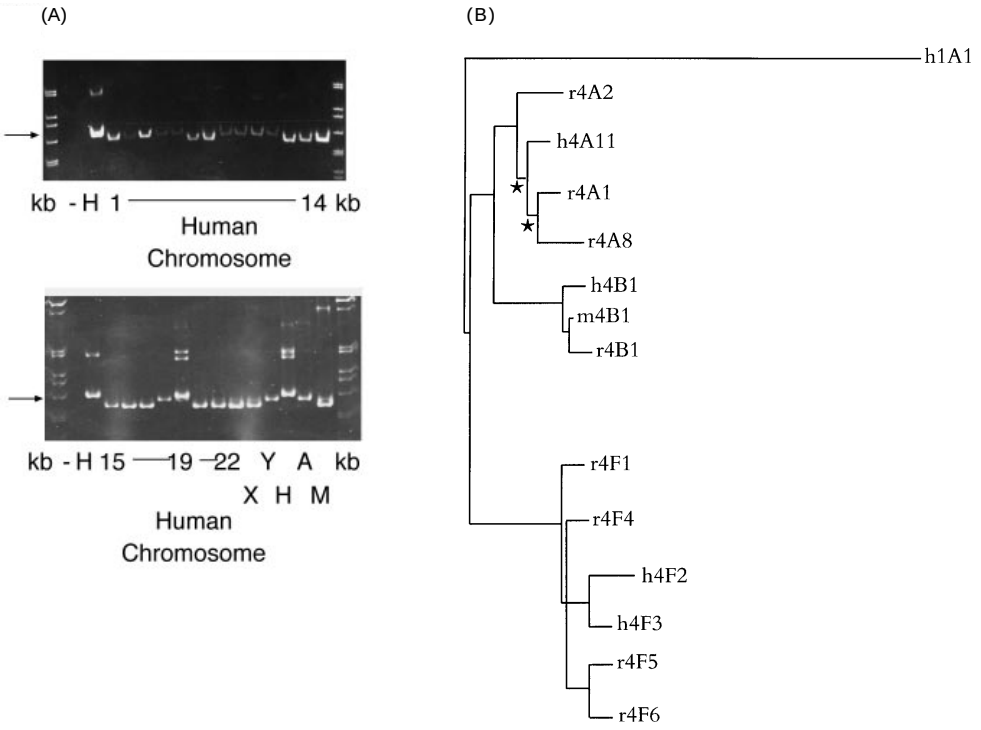

Figure 7 Mapping and distance analysis of the *CYP4F2* gene

(**A**) Monochromosomal human–rodent cell hybrids were analysed by PCR for the *CYP4F2* gene. Each cell line is numbered according to the human chromosome which it contains; —, PCR reaction with no template DNA; H, human genomic DNA; M, mouse DNA; A, hamster DNA; kb, BRL 1 kb marker. The human *CYP4F2* PCR product is indicated with an arrow. (**B**) Human (h), mouse (m) and rat (r) proteins of the *CYP4A*, *CYP4B* and *CYP4F* families were aligned and analysed with the PHYLIP ProtPars [31] and CLUSTALW [30] computer programs, using the human *CYP1A1* as the outgroup. A typical output from CLUSTALW analysis is shown. Bootstrap analysis shows that the relationship is strongly supported by the data ($P > 0.95$), with the exception of the branches marked *. The relative distance from the branch point is indicated by the length of the horizontal lines, corrected for multiple changes at a single position [31].

kidney [6]. An antibody was raised against recombinant rat CYP4A1 protein (amino acids 275–509) and failed to detect significant levels of Cyp4a proteins in the liver of control male or female mice (Figure 5A). By contrast, a protein, detected as a single band, was highly inducible in the liver of animals treated with MCP. In view of the difference in relative molecular mass (413 Da) and the lesser similarity between Cyp4a10 and Cyp4a14 and the distinct patterns of expression from *Cyp4a12* (see below), it is apparent that this antibody is specific for the murine Cyp4a10 and this pattern of expression is entirely consistent with previous reports from this laboratory on the expression of the rat CYP4A10 RNA [6]. A recombinant construct expressing 141 amino acids of Cyp4a12, corresponding to amino acids 316–456 of CYP4A1, was used to prepare an antibody, which detected a protein that was highly expressed in the liver of untreated male NMC mice, but not female mice (Figure 5B), in agreement with transcript levels of Cyp4a12.

Genomic structure of the *Cyp4a* locus

The three murine *Cyp4a* genes are inducible by peroxisome proliferators, suggesting common mechanisms of gene regulation. We therefore investigated whether the *Cyp4a* genes are clustered. A single copy sequence from the 3′ non-coding region of the *Cyp4a14* gene was mapped to 1p34–p12 on chromosome 4 (N. G. Copeland, unpublished work), at the same position as the murine *Cyp4a-12* [6]. In order to characterize this linkage further, pools of a YAC library were screened with PCR primers specific for the *Cyp4a12* and *Cyp4a14* genes, yielding two independent positive clones. These are 58-B-12 from the ICRF library (clone 2) [32], and I-31-E-2 (clone 1). DNA was prepared from the YAC clones and analysed by PCR (Figure 6). Negative contols (no DNA, or an irrelevant YAC from an *Arabidopsis thaliana* library) and a positive control (total mouse DNA) were prepared for each reaction and each result was confirmed by PCR with an independent set of primers for each *Cyp4* gene (results not shown). Clone 2 contained the *Cyp4a14*, *Cyp4a10* and *Cyp4a12* genes, whereas clone 1 contains the *Cyp4a14* and *Cyp4a10* genes, but not the *Cyp4a12* gene. This demonstrates that the three murine genes are physically linked on chromosome 4. Pulsed field gel electrophoresis of the mouse YAC clones revealed that the insert sizes of clone 1 and clone 2 are 440 and 220 kb, respectively (Figure 6B).

Co-localization of the *Cyp4* gene families in the mouse

The *Cyp4a*, *Cyp4b* and *Cyp4f* families share similar tissue-specific patterns of expression in the liver and kidney and so it is possible that common elements regulate the expression of these gene families. We investigated whether these genes were co-localized on the chromosome. PCR with primers derived from the mouse *Cyp4b1* cDNA sequence [10] revealed that the *Cyp4b1* gene is present in YAC clone 1, but not clone 2 (Figure 6A).

Linkage of the human *CYP4* gene families

A human YAC DNA library was screened with PCR primers specific for *CYP4A11* and one positive clone was isolated (4x35-G5). This clone contains an insert of 440 kb (Figure 6D) and PCR with multiple independent sets of primers demonstrated the

presence of the *CYP4A11* and *CYP4B1* genes (Figure 6C); however, *CYP4F2* was not detected in this clone (results not shown), despite the use of multiple primer pairs. The *CYP4F2* gene was mapped using the HGMP monochromosomal rodent–human cell hybrids to human chromosome 19 (Figure 7A). Thus the *CYP4A* and *CYP4B* subfamilies are linked on chromosome 1 in the human [6,37] but the *CYP4F2* gene is on a different chromosome. We therefore undertook phylogenetic analysis of the *CYP4* gene sub-families using maximum parsimony [31] and neighbour-joining methods [30]. The CLUSTALW algorithm and the most parsimonious tree from Phylip (ProtPars) [31] gave similar trees (Figure 7B), which were strongly supported by the results; the *CYP4A* and *CYP4B* families cluster together, apart from the *CYP4F* family.

DISCUSSION

While screening a mouse genomic library with *Cyp4a10* and *Cyp4a12* probes, we cloned a novel member of the murine *Cyp4a* gene family, now designated *Cyp4a14*. This is the third member of the *Cyp4a* gene family and it is highly similar to the orthologous rat genes, *CYP4A2* and *CYP4A3*. The rat *CYP4A2/3* genes are highly similar (97% amino acid identity), but differ by a three amino acid deletion in *CYP4A2*. We were unable to detect any evidence for a similar gene in mice with a nine base pair deletion and conclude that the duplication of genes leading to *CYP4A2* and *CYP4A3* in the rat occurred after the rat/mouse divergence. However, in contrast with the *CYP4A* genes of human, guinea-pig [6] and rabbit [4], the rat genes are highly similar to the mouse, indicating that three members of the *CYP4A* gene family were common to mouse and rat before divergence.

The mouse *Cyp4a14* gene shows high similarity to the rat *CYP4A2* gene sequence, with consistent similarity at the nucleotide level (87%) throughout the gene, although it is noteworthy that the amino acid identity with *CYP4A3* is lower at the N-terminal half of the protein (86%), compared with the C-terminal half (93%). In addition, the sequence similarity of the 5′ non-coding sequence before the gene is relatively high ($\sim$ 84–87%) for approximately 300 bp upstream of the transcription start site, but drops off sharply thereafter. This site is close to the position of a direct repeat in the *CYP4A2* gene which runs from -440 to -817 and it is tempting to speculate that this repeat may have had some role in the duplication of the *CYP4A2*/3 genes in the rat. In common with the rat *CYP4A2* gene, the mouse gene has a GAATTAA motif 17 bp upstream from the transcription initiation site and a conserved sequence (18/19) at -50. This sequence is highly conserved between rat and mouse and we have previously shown that the corresponding sequence in *CYP4A1* is required for basal levels of transcription of the *CYP4A1* gene in primary hepatocytes (D. R. Bell and C. Elcombe, unpublished work); it is likely to have a similar role for this gene.

It has been reported that the rat *CYP4A2* gene is regulated in a sex-specific manner in the rat liver and kidney [36]; however, the *CYP4A3* gene is not regulated in a sex-specific manner. There is evidence that there are sexually regulated Cyp4a proteins in the mouse [25,26,38], from Western-blotting approaches using antibodies raised against CYP4A1, although the identity of these genes is not clear [6]. We therefore sought to investigate the expression and induction of the *Cyp4a14* gene. *Cyp4a14* was highly inducible in the liver and kidney of male and female mice after treatment with the peroxisome proliferator, MCP. The high induction (over 1000-fold) was associated with very low basal levels of expression of this gene in both male and female mice. There was no sexual differentiation of response and no response to testosterone. These results are consistent with the observations of Lee et al. [21], who used a rat *CYP4A2* probe to show a highly inducible RNA, presumably the *Cyp4a14* RNA, in mouse liver. While the present manuscript was in preparation, we became aware that there were several reports of the mouse *Cyp4a14* arising from anonymous cDNA sequencing (accession numbers AA123331, W20947, AA070718, AA060595, W36713, W41435, AA061737, W17564, AA106365, W13618 and AA098524). The large number of sequences probably denotes a moderate level of expression of this gene in extrahepatic tissues.

In contrast, the Cyp4a12 RNA was highly expressed in male liver and kidney; indeed, MCP treatment reduced the level of this transcript in male liver. In the female, testosterone and MCP had quite distinct effects, depending on the organ. Thus in female liver, MCP led to high levels of induction and testosterone had little effect, whereas in kidney, the converse was true. This is consistent with other studies suggesting distinct modes of regulation of *CYP4A2* in liver and kidney [36]. In agreement with these data, obtained at the level of protein, an antiserum against Cyp4a12 detected a protein with a molecular mass of approx. 52 kDa, which was male-specific in NMC mouse liver. An antibody raised against recombinant Cyp4a1 protein showed very low levels of protein in control mice, with high inducibility by peroxisome proliferators and no evidence of sexual dimorphism. Thus we demonstrate that *Cyp4a12* is sexually dimorphic in mice and that *Cyp4a10* and *Cyp4a14* are not sexually regulated, although the latter has a rat homologue, *CYP4A2*, which is sexually dimorphic. The evolution of the *Cyp4a* gene family which led to such distinct regulation of closely related genes bears further scrutiny.

Mouse *Cyp4a* genes have previously been localized to mouse chromosome 4 using both heterologous and homologous probes [6,8,24] and the *Cyp4a14* gene additionally mapped to 1p34–p12 on chromosome 4. Isolation of YAC clones containing the three *Cyp4a* genes formally demonstrated that the three murine *Cyp4a* genes are physically linked and that these genes are linked to the *Cyp4b* subfamily. This work confirms and extends our data [6] and those of Nhamburo et al. [37], who localized the human *CYP4B1* gene to human chromosome 1. It has been suggested recently that the expansion of gene families provides a measure of evolutionary relatedness [39]. The linkage of these gene families in both mouse and human thus suggests a relatively recent divergence of the *CYP4A* and *CYP4B* subfamilies and a more ancient divergence of *CYP4F*, which is on human chromosome 19. In agreement with this hypothesis, phylogenetic analysis suggests that the *CYP4F* genes are more ancient than the *CYP4A/B* families. Of interest, in *Drosophila*, the *CYP4* gene sub-families are also scattered throughout the chromosome [15]. The clustering of genes is known to be associated with co-ordinate regulation of the cluster, mediated via global effects on chromatin [40]. These effects are also known to be important in the liver [41], but are poorly characterized in the case of the *CYP* gene family. It will be interesting to determine if long-range cis-acting elements play a role in regulation of the cytochrome *P*-450 gene family.

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