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Cloning, Sequencing and Tissue Distribution of Rat Flavin-Containing Monooxygenase 4: Two Different Forms Are Produced by Tissue-Specific Alternative Splicing

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ABSTRACT

The nucleotide sequence of rat flavin-containing monooxygenase 4 (FMO4) mRNA was obtained by reverse transcription-polymerase chain reaction (RT-PCR) and 5′/3′ terminal extension. Complete cDNA was amplified, cloned, and sequenced from the mRNA obtained from rat kidney and brain. Two different transcripts (short and long) stemming from the splicing of an internal region of 189 bases pair, corresponding to exon 4 were identified. This alternative splicing seems to be specific of the brain. The long cDNA encodes a protein of 560 amino acids with a predicted molecular mass of 63,395 Da. The short cDNA encodes a protein of 497 amino acids with a predicted molec-ular mass of 55,871 Da. Both of these encoded sequences contain the NADPH- and FAD-binding sites and a hydrophilic carboxyl terminus. These sequences are 80 and 79% identical to the sequences of human and rabbit FMO4. By Northern blotting and/or RT-PCR, the long-form FMO4 mRNA was detected in the rat kidney, intestine, and liver and the short form particularly in the brain. For the first time, the expression of FMO4 protein was demonstrated. By Western blotting using the two different forms of FMO4 antibodies, a long FMO4 protein was detected in the rat kidney, whereas in the rat brain, only the short form of FMO4 was observed.

Microsomal flavin-containing monooxygenases (FMOs) [dimethylaniline monooxygenase (N-oxide forming); EC 1.14.13.8] catalyze the FAD-, NADPH- and O_2-dependent oxidation of a large number of structurally diverse compounds, including drugs, pesticides, and industrial chemicals containing a soft nucleophile (Ziegler, 1988). The FMOs convert many xenobiotics into more polar substances as a prelude to excretion. In certain cases, the FMOs can also catalyze the formation of reactive metabolites capable of binding to cellular macromolecules. Several endogenous compounds such as trimethylamine, methionine, or cysteamine (Sausen and Elfarra, 1990; Duescher et al., 1994; Lang et al., 1998) have been identified as substrates for FMOs, but the physiological role of some of these enzymes has not been determined.

To date, a maximum of five distinct FMO isoforms have been identified. Based on the cDNA sequence, they were classified into five subfamilies (FMO1 to 5) (Lawton et al., 1994). Orthologous sequences share at least 80% of amino acid identity, whereas homologous FMOs are 52 to 57% identical. The FMO isoforms differ in their tissue distribution or substrate specificity. The expression of recombinant proteins has greatly aided the characterization of FMOs isoforms. So, the cDNAs of FMO1, FMO2, FMO3, and FMO5 isoforms of various animal species have been expressed successfully in several expression systems (Lawton et al., 1991, 1993; Itoh et al., 1997) and high levels of expression have been observed in Escherichia coli (Atta-Asafo-Adjei et al., 1993; Lawton and Philpot, 1993; Lomri et al., 1993; Overby et al., 1995; Falls et al., 1997).

In contrast to these successes, the expressions of rabbit or human FMO4 in E. coli, yeast, or COS-1 cells have always failed for unknown reasons (Burnett et al., 1994; Phillips et al., 1995; Itagaki et al., 1996). The comparison of the human (Dolphin et al., 1992) and rabbit (Burnett et al., 1994) FMO4 transcripts with those encoding all known FMO isoforms, revealed that the transcripts of the coding regions of FMO4 are 60 to 75 nucleotides longer than the transcripts of the coding regions of other isoforms. This has been suspected to

ABBREVIATIONS: FMO, flavin-containing monooxygenase; FAD, flavin adenine dinucleotide; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; MMLV-RT, Moloney murine leukemia virus reverse-transcriptase; bp, base pair(s); SSC, standard saline citrate; KLH, keyhole limpet hemocyanin; ORF, open reading frame; GST, glutathione S-transferase.

be caused by a shift in the stop codon to the 3′-end of the consensus position. The supplementary *C*-terminal extremity could be associated with the incapacity to express the FMO4s in the in vitro expression systems, because the expression of a truncated FMO4 that does not contain the supplementary C-terminal extremity has been obtained in *E. coli* without real difficulty.

The fact that no purified FMO4 enzyme has been obtained up to now explains that no FMO4 antibodies are available, the tissue expression of FMO4 protein could not be recognized, and the catalytic activity of this protein could not be characterized. There are also no endogenous or exogenous substrates known for FMO4 at the present time. Nevertheless, the distribution of mRNA suggests that FMO4 plays some functional role in the kidney (Burnett et al., 1994) and brain (Blake et al., 1996).

In this article, we report for the first time the complete sequence of the cDNA encoding for rat FMO4, the existence of a tissue specific alternative splicing, and the tissue-specific expression of two FMO4 variants.

Materials and Methods

Animals. Male or female Oncins France strain A/Sprague-Dawley rats (200 g) were obtained from a commercial breeder (IFFA-CREDO, L'Arbresle, France). Food and water were made available to rats ad libitum until sacrifice. Rats were killed by decapitation. Various organs (kidney, brain, liver, lung, intestine, adrenal gland, ovary, testis, uterus) were removed quickly, frozen in liquid nitrogen and stored at −80°C until analysis.

Standard Procedures. Standard molecular biology techniques were carried out essentially as described by Sambrook et al. (1989). Poly(A)$^+$ RNA was purified from a fresh adult rat liver (100 mg) with streptavidin-coated paramagnetic particles (PolyATract mRNA isolation system; Promega, Charbonnières, France). Total RNA were isolated from rat liver, kidney, lung, brain, adipose tissue, and muscle by a single extraction with an acid guanidinium thiocyanate/phenol/chloroform solution according to Chomczynski and Sacchi (1987). Oligonucleotides were manufactured by OligoExpress (Paris, France). Sequencing was performed by Genome Express (Grenoble, France).

Amplification of an Internal Fragment of the cDNA Encoding the Rat FMO4. The first strand cDNA template was synthesized from rat kidney mRNA (50 ng) in the presence of oligo(dT)$_{15}$ (100 pmol), 200 units of Moloney murine leukemia virus reverse-transcriptase RNase H$^-$ (MMLV-RT; Promega) in 20 μl of standard reverse transcription buffer (50 mM Tris-HCl, pH 8.3, 3 mM MgCl$_2$, 75 mM KCl, 10 mM dithiothreitol, and 200 μM concentrations of each deoxynucleotide triphosphate) at 37°C for 1.5 h and then at 65°C for 10 min.

The sense primer FMO4-S1 (5′-TGCTGAGCACTTTGACCTCC-3′) and the antisense primer FMO4-AS1 (5′-CTTCCACTGTCCCATCT-TCAAAG-3′) were designed in the most highly conserved sequence from human [GenBank accession number Z11737 (FMO4-S1 from base 282 to 301 and FMO4-AS1 from base 955 to 933)] and rabbit (GenBank accession number L10392) FMO4 cDNA. The resulting cDNA was amplified by PCR using 20 pmol of FMO4-S1 and FMO4-AS1 primers, 1 unit of *Taq* DNA polymerase (Promega) in 50 μl of 3 mM MgCl$_2$ PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, and 200 μM concentrations of each deoxynucleotide triphosphate). The amplification was performed at 94°C for 2 min, 35 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 90 s, followed by a final extension at 72°C for 10 min. The PCR products were subjected to electrophoresis on agarose gel (1%). The major product (700 bp) was gel-purified, subcloned into pSTBlue-1 vector (Perfectly

Blunt cloning kit; Novagen, Madison, WI), and sequenced (clones F4-1 to F4-3).

Amplification of FMO4 cDNA Ends. To obtain the cDNA ends, the SMART RACE cDNA amplification kit (BD Clontech, Palo Alto, CA) was used according to the manufacturer's recommendations. The specific primers (FMO4-AS2, FMO4-AS3, FMO4-S2) used were designed from sequences of clones F4-1 to F4-3.

The 5′ first-strand cDNA template was synthesized from rat kidney mRNA (50 ng) in the presence of a modified oligo(dT)$_{30}$ primer and a SMART II oligonucleotide in a standard reverse transcription buffer containing 200 units of MMLV-RT at 42°C for 1.5 h. The PCR was performed with anchor primer and mix of polymerases (Advantage cDNA PCR kit; BD Clontech), using antisense primer FMO4-AS2 (25 pmol, 5′-CCCCGAAGGCATCTGGAATCCTG-3′, from base 550 to 528). The amplified products were further submitted to PCR performed using antisense primer FMO4-AS3 (25 pmol, 5′-GCTCT-GTCCTGTTTGCCCTCTGTCTCTG-3′, from base 413 to 386) and commercial primer, according to the manufacturer's recommendations. The major product (800 bp) was gel-purified and subcloned into pSTBlue-1 vector and sequenced.

The 3′ first-strand cDNA template was obtained from rat kidney mRNA (50 ng) in the presence of a (dT)$_{30}$-anchor primer in a standard reverse transcription buffer containing 200 units of MMLV-RT at 42°C for 1.5 h. The PCR was performed in similar conditions, using sense primer FMO4-S2 (25 pmol, 5′-GAGAGTTCTGCCCTC-CTGCTTTGTAAGCTG GAATC-3′, from base 731 to 763) to generate the 3′ ends of rat FMO4, respectively. The major PCR product (1400 bp) was gel-purified, subcloned into pSTBlue-1 vector, and sequenced.

Amplification of the Full-Length cDNA Encoding Rat FMO4. A (dT)$_{15}$ primed first-strand cDNA template was synthesized from rat kidney or brain mRNA (50 ng) in the presence of oligo(dT)$_{15}$, in the same conditions as described above. Sense FMO4-S4 (5′-GACCGAAACTTCTTCCTGTTG-3′) and antisense FMO4-AS4 oligonucleotides (5′-AACCTGC CCTATGGAGACTG-3′) designed from the 5′- and 3′-ends nonencoding sequences (from base −222 to base −201 and from base 1778 to 1759), respectively, were used to perform the amplification, by PCR, of the complete coding region. After an initial denaturation step at 94°C for 2 min, 1.25 units of *Pfu* DNA polymerase (Promega) were added and the reaction was incubated for 35 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 4 min and for an additional 10 min at 72°C. The amplified products were further submitted to PCR performed using sense FMO4-S5 (5′- GA-CAACTTCAGACCTTCACCC-3′) and antisense FMO4-AS5 (5′-AACTTCCTCCATTGCCAAAC-3′) primers in the same conditions (from base −125 to −105 and from base 1710 to 1691, respectively). The resulting products (1600 and 1800 bp) were gel-purified, cloned into pSTBlue-1, and sequenced (clones F4L-4 to -9 and clones F4C-10 to -12).

Amplification of a Region of the *FMO4* Gene. Genomic DNA was isolated from Sprague-Dawley rat kidney by use of a commercial DNA isolation kit (DNeasy tissue kit; QIAGEN, Valencia, CA). A region of the *FMO4* gene was amplified by PCR using the sense FMO4-S6 (5′-AGAGGAGTAGTGACTTCGGCG-3′) and the antisense FMO4-AS6 (5′-TGTGACAACTTCCCATTGGC-3′) primers (from base 417 to 437 and from base 709 to 690, respectively). Reaction mixtures containing approximately 200 ng of genomic DNA as template were incubated in the presence of Advantage polymerase mix (BD Clontech) for 25 cycles at 94°C for 45 s, 58°C for 45 s, and 72°C for 4 min, followed by an additional 10 min at 72°C. The PCR products were gel purified and sequenced directly.

Northern Blotting. Twenty micrograms of total RNA isolated from each organ were separated by electrophoresis on agarose gel (1.2%) containing formaldehyde (2 M) and then transferred to nylon membrane (Hybond N; Amersham Biosciences AB, Umeå, Sweden). The blot was washed in 5× SSC for 15 min at room temperature and baked at 80°C for 2 h. The membrane was prehybridized for 4 h at 42°C and then hybridized with a rat FMO4 coding region probe

(*Eco*RI/*Eco*RI, bases 280 to 981 of long FMO4 cDNA) at 42°C for 24 h. The probe was labeled by a random primer method (Prime-a-Gene labeling system; Promega). The hybridized membrane was washed for two cycles of 10 min in 2× SSC (0.1% SDS), then two cycles of 15 min in 1× SSC (0.1% SDS) at room temperature, and at 55°C for two cycles of 15 min in 0.1× SSC (0.1% SDS). The membrane was subjected to autoradiography for 48 h at −80°C. The membrane was stripped with a 0.01× SSC (0.5% SDS) at 100°C. The removal of the probe was verified by autoradiography at −80°C. The blot was subsequently hybridized with a actin cDNA probe.

Semiquantitative RT-PCR. Total RNA were extracted from various organs (liver, kidney, lung, uterus, intestine, whole brain homogenate, brain stem, cerebellum, cortex, hypothalamus, hippocampus, adrenal glands, testis, and ovary) by a double extraction, beginning with a manual extraction with an acid guanidinium thiocyanate/phenol/chloroform solution followed by an extraction on column (total RNA isolation system, Promega). Concentrations of total RNAs were evaluated spectrophotometrically from absorbance at 260 nm. The 260/280 ratios were between 1.8 and 2.0. The reverse transcription of 0.5 μg of total RNA was performed in the presence of oligo(dT)$_{15}$ (500 pmol) in 20 μl of standard reverse transcription buffer, as described above. Two microliters of resulting cDNA were amplified by PCR using specific primers FMO4-S4 and FMO4-AS7 (5′-GGCATCTGGAATCCTGTACT-3′, from base 543 to 524) in 50 μl of standard PCR buffer. The amplification was performed at 94°C for 90 s, 24 to 35 cycles of 94°C for 30 s, 61°C for 45 s, and 72°C for 60 s. The semiquantification of FMO4 transcripts was estimated by the number of amplification cycles necessary to obtain a similar signal density.

Production of Specific Antibodies Raised against Rat FMO4 Protein. Two oligopeptides, the one corresponding to amino acid residues 126 to 140 of deduced long FMO4 protein (P1), the other one corresponding to amino acid residues 409 to 423 of deduced long FMO4 protein (P2) were selected. These peptides have reasonable hydrophilic characters and specific sequences to rat FMO4 compared with known FMOs. The sequences of these peptides were EVVTETEGKQDRAVFY and YKKEELIKRGVIKDIS for peptides 1 and 2, respectively; the terminal tyrosines were not part of the rat FMO4 sequence but were added to facilitate coupling to the carrier protein. The oligopeptides were synthesized by Neosystem (Strasbourg, France). The composition of peptides was verified by mass spectrometry and amino acid analysis. Purity of the peptides was verified by reversed-phase high-performance liquid chromatography using a C-18 column (Neosystem). The oligopeptides were coupled to the carrier protein of keyhole limpet hemocyanin (KLH) by Neosystem. The peptides-KLH were used as an immunogen to immunize rabbits. The initial injection consisted of 400 μg of oligopeptide 1 or 2/KLH mixed with an equal volume of Freund's complete adjuvant (Sigma, St. Louis, MO); the immunogen was injected intradermally dispersed among 20 to 30 sites on the back of the rabbit. Intradermal booster injections of 200 μg of oligopeptide 1 or 2/KLH mixed with an equal volume of Freund's incomplete adjuvant (Sigma) were administered at 14, 28, and 56 days after the initial injection. Animals were bled 10 days after the last booster injection. Sera were tested for their reactivity to the recombinant rat long FMO4 by Western blotting.

Heterologous Expression in *E coli* The long and short forms of rat FMO4 were expressed in the glutathione *S*-transferase (*Schistosoma japonicum*) gene fusion vector, pGEX-6P3 (Amersham Biosciences AB). The coding sequence corresponding to the long and short FMO4 were amplified, by PCR, from clones F4L-4 and F4C-12, using *Pfu* DNA polymerase and specific primers FMO4S-*Bam*HI (5′-ATAGGATCCATGGCCAAAGAAAGTGGCAG-3′) and FMO4AS-*Sal*I (5′-ACAGTCGACTCATGAGTTCTGGGGGATGTAC-3′), including *Bam*HI and *Sal*I restriction sites in the extremities, respectively. After an initial denaturation step at 94°C for 2 min, 1.25 units of *Pfu* DNA polymerase were added and the reactions were incubated for 5 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 4 min, for 25 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 4 min and then for

an additional 10 min at 72°C. The gel-purified PCR products (1680 or 1490 bp) were digested by 10 units of *Bam*HI and *Sal*I restriction enzymes (Roche Diagnostics, Meylan, France) and ligated into the expression vector pGEX-6P3 linearized by the same enzymes. The clones, named pGEX-F4L and pGEX-F4C, were obtained and verified by sequencing.

E. coli BL21 cells were transformed with pGEX-F4L or pGEX-F4C and grown at 37°C in Terrific Broth (Sigma, St. Louis, MO) supplemented with ampicillin (100 μg/ml) to an absorbance of 0.6 to 0.8 at 600 nm. Isopropyl-β-D-thio-galactopyranoside was then added at a final concentration of 0.1 mM and the cells were further incubated for 5 h at 37°C. Cells were harvested by centrifugation at 10,000*g* for 10 min and resuspended in buffer A (140 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7.3) containing 1 mM EDTA, 5 mM dithiothreitol, 500 μM phenylmethylsulfonyl fluoride, and 100 μg/ml lysozyme. After an incubation for 30 min at 4°C, the resuspended cells were disrupted by sonication (five 30-s pulses separated by 30-s periods of cooling). The solutions rFMO4-L or rFMO4-C, treated with 1.5% lauroyl-sarcosine were gently stirred for 1 h at 4°C, then centrifuged at 100,000*g* for 1 h at 4°C. The resulting supernatants, after the addition of Triton X-100 (2%), were purified onto a glutathione Sepharose 4B (Sigma) column and equilibrated with buffer A containing 0.5% Triton X-100. Then, the column was washed with five column volumes of the same buffer, then with five column volumes of buffer B (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.2% Triton X-100, pH 8.0). Prescission-protease (Amersham Biosciences AB) (50 units) was added. After an incubation for 24 h at 4°C under gentle agitation, the cleavage result was eluted.

The various fractions (sonicates, solubilisates, and eluates) were analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970), and also by Western blotting (Towbin et al., 1979) using antibodies raised against P1 or P2 and by the determination of the GST activity. In the GST activity assay, glutathione and 1-chloro-2,4-dinitrobenzene serve as substrate for GST to yield a product detectable at 340 nm (Amersham Biosciences AB).

In Vitro Transcription/Translation. The in vitro transcription/translation of long and short FMO4s was performed by use of a commercial kit (TNT T7 Quick for PCR DNA; Promega). The PCR fragments were produced from clones pGEX-F4L and pGEX-F4C in the presence of primers T7-FMO4S (5′-TTATAATACGACT-CACTATAGGGAGCCACCATGGCCAAGAAAGTGGCAGTG-3′) and FMO4AS-*Sal*I. The PCR products (1680 bp for long FMO4 and 1491 bp for short FMO4) were gel-purified, then added (150 ng of PCR product) in the translation reactions, according to the manufacturer's recommendations. After an incubation for 1 h at 30°C, the reaction products were analyzed by Western blot in the presence of antibodies raised against P1 or P2.

Analysis of Proteins Expression. Microsomes were prepared from rat liver, lung, kidney, brain, intestine, and uterus samples by differential centrifugation as described previously (Moroni et al., 1995). Proteins concentrations were determined by the method of Bradford (1976) using serum bovine albumin as a standard. Microsomal proteins (30 μg) were loaded, and immunoblottings were performed as described above. Membranes were blocked and then incubated with antibodies anti-P1 (1:5000) or anti-P2 (1:2500). After washing, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences AB), as the secondary antibody. FMO4 was visualized by enhanced chemiluminescence on Hyperfilm (Amersham Biosciences AB).

Results

PCR-Based Cloning of cDNA. RT-PCR performed with rat kidney mRNA with sense primer FMO4-S1 and antisense primer FMO4-AS1, as described under *Materials and Methods*, generated a 670-bp fragment on an ethidium bromide-

stained agarose gel (results not shown). This 670-bp fragment was sequenced and compared with human (Dolphin et al., 1992) and rabbit (Burnett et al., 1994) FMO4 cDNAs. The results showed 82 and 83% homology with human and rabbit cDNAs, respectively.

To obtain the cDNA ends, the SMART RACE cDNA amplification kit was used, as described in the methodology section. 3′ and 5′-PCR products were obtained and sequenced. The 5′-PCR product (~700 bp) contained a putative ATG start codon and 322 bp of the 5′-untranslated region. The 3′-PCR product (~1200 bp) contained the remainder of the protein-coding region and 365 bp of an apparent 3′-untranslated region.

Sequencing of Two Different cDNAs Encoding the Long and Short FMO4. RT-PCR performed with rat kidney mRNA, with specific primers FMO4-S4 and FMO4-AS4 designed from the 5′- and 3′-ends nonencoding sequences of rat FMO4, generated an 1800-bp fragment. The 1800-bp product thus obtained was cloned and sequenced from three different Sprague-Dawley rat kidney mRNAs. The three clones showed very limited differences (from 1 to 3 bases). The consensus nucleotide sequence (Fig. 1) for the rat FMO4 cDNA obtained by alignment of these three sequences contains 322 bases at the 5′-flanking region followed by an open reading frame of 1680 bases, terminated with a TGA stop codon, and 380 bases at the 3′-flanking region (Fig. 1). The obtained nucleotide sequences showed 81 and 80% homology with human (Dolphin et al., 1992) and rabbit (Burnett et al., 1994) FMO4 cDNAs.

The RT-PCR performed with rat brain mRNA under the same conditions generated two PCR products (1800 bp and 1650 bp, respectively). After gel purification, these fragments were subcloned. Three clones containing the 1800-bp fragment and three clones containing the 1650-bp fragment were obtained from three different rat brain mRNAs and sequenced. The sequences of three clones containing the 1800-bp fragment were identical to the consensus sequence deduced from the kidney. The comparison of three 1650-bp sequences with the consensus sequence revealed the presence of a 189-bp deletion corresponding to the nucleotides from 132 to 321 of rat FMO4 cDNAs (Fig. 1). To assure the presence of these two transcripts, total RNAs from 10 rat brains and 10 rat kidneys were analyzed by PCR using the specific primers FMO4-S4 and FMO4-AS7 framing the 189-bp deletion. Whereas the RT-PCR performed with rat kidney total RNA generated only a single fragment (765 bp) on an ethidium bromide-stained agarose gel, two PCR products (765 bp and 576 bp) were always detected in the RT-PCR product obtained from the rat brain total RNA (Fig. 2).

With the exception of the 189-bp deletion, the sequences of two transcripts (long and short) are totally superimposable; the 5′- and 3′-flanking regions are similar. Whereas the ORF of the long transcript consists of 1680 bp, the ORF of the short transcript contains 1491 bp (Fig. 1). The amino acid sequences derived from the consensus long and short sequences are shown in Fig. 1. The sequence of long FMO4 encoded 560 amino acid residues, with a predicted molecular mass of 63,395 Da and a theoretical pI of 9.4. It showed 80 and 79% identity with the sequences of FMO4 from the human (Dolphin et al., 1992) and rabbit (Burnett et al., 1994). The sequence of short FMO4 encoded 497 amino acid

residues, with a predicted molecular weight of 55,871 Da and a theoretical pI of 9.7.

Long and Short FMO4 Transcripts Are Produced by Alternative Splicing. PCR amplification in the presence of FMO4-S6 and FMO4-AS6 primers was performed with rat kidney and brain cDNA (Fig. 3). A single 293-bp fragment was amplifed from rat kidney cDNA, whereas two PCR products (293 and 104 bp) were amplified from rat brain cDNA. The same amplification using the rat genomic DNA generated a single PCR fragment containing about 2200 bp (Fig. 3). After gel purification, this fragment was directly sequenced. Comparison of the sequences obtained from amplification of genomic DNA with the transcript sequence obtained from the long FMO4 cDNA revealed the presence of two intronic sequences framing the 189-bp region specific of the long transcript in the FMO4 gene. The first intron, found between bases 132 and 133 of the long transcript cDNA, was composed of 1360 bp. It contained all the characteristic sites of intron (5′- GT. . . . . CTTCTAAC(-21). . . . TATAG -3′). The other intron, found between bases 321 and 322 of the long transcript cDNA, contained 541 bp. The 189-bp region specific to the long transcript corresponded to a unique exon that was spliced out only in the short transcript.

Tissue Distribution of Long and Short FMO4 mRNAs in the Rat. Tissue distribution of long and short mRNA in various rat tissues was examined by Northern blot (Fig. 4) and RT-PCR (Fig. 2). Expression of mRNAs encoding FMO4 obtained in the male and female rat liver (lanes 1 and 2), kidney (lanes 3 and 4), lung (lanes 5 and 6), brain (lanes 7 and 8), muscle (lanes 9 and 10), adipose tissue (lanes 11 and 12), adrenal gland (lanes 13 and 14), testis (lane 15), ovary (lane 16), and intestine (lanes 17 and 18) is shown in Fig. 4. The probe for FMO4 specifically hybridized a 2.8-kilobase transcript in the intestine and kidney samples (Fig. 4). FMO4 mRNA levels were higher in the kidney compared with the intestine. No FMO4 mRNA signal was detected in other tissues, although the mRNAs integrity was confirmed by hybridization with an actin cDNA probe. Because the Northern blot sensitivity was lower than the RT-PCR sensitivity, the presence of the FMO4 mRNA was studied using a semi-quantitative RT-PCR, in the presence of FMO4-S4 and FMO4-AS7, framing the 189-bp deletion. The number of PCR cycles necessary to obtain a similar band signal intensity on an ethidium bromide-stained agarose gel was used as an index of the mRNA abundance (Fig. 2). The kidney was again found to contain a high level of FMO4 mRNA, needing only 24 PCR cycles to demonstrate its constitutive expression. The intestine and liver contained a more limited amount of FMO4 mRNA (28 PCR cycles were necessary) followed by uterus and adrenal gland (30 PCR cycles), then the whole-brain homogenate (32 PCR cycles), which was followed by testis, ovary, and lung (34 PCR cycles). In the rat brain, the constitutive expression of short form FMO4 mRNA was higher in the brain stem and hypothalamus (30 PCR cycles) than that observed in the hippocampus, cortex, or cerebellum (34 PCR cycles). Among the various organs tested, the PCR performed with the cDNA obtained either from whole brain homogenate or the homogenates of individual specific areas of the brain generated both transcripts (765 and 576 bp). The long-form FMO4 mRNA transcript was always more abundant than the short-form FMO4 mRNA transcript (Fig. 2).

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GATAGCAGAAGAAGCAACGGGCTTTTGTGTTGAACTGAGAGTTTGAGCAAAGTCAACAAAGTCAGTAGCACTCTGTTTCCTCCTTAGGACAAGGCTCTGAGACA
AACTTCTTCCTGTTGTTTTATAAAACAAACTCCACCTTTGACCTCTCACCTTTGGCTTCTCACCTGCTCTATGCTTGCAAGACAGGGCACGGCGGAGATCCGACAACT
TCAGACCTTCACCCAGAAATTCAAGTTGTTCTTCTCGGTGAAGGCCGTTTGTCCCTGGTTGAATGCCACCCAGGTGTTGACAACCCAAGGGAATCCACAGAGCGCACC
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1	ATG	GCC	AAG	AAA	GTG	GCA	GTG	ATT	GGA	GCT	GGA	GTG	AGT	GGC	CTG	TCC	TCA	ATC	AAG	TGC
	M	A	K	K	V	A	V	I	G	A	G	V	S	G	L	S	S	I	K	C	20
61	TGC	TTG	GAC	GAG	AAC	CTG	GAG	CCT	ACC	TGT	TTT	GAG	AGG	AGT	AGT	GAC	TTC	GGC	GGA	CTG
	C	L	D	E	N	L	E	P	T	C	F	E	R	S	S	D	F	G	G	L	40
121	TGG	AAG	TTT	GCT	GAA	GCA	TCT	GAG	GAT	GGA	ATG	ACC	AGG	GTT	TAC	AGG	TCA	CTA	GTG	ACA
	W	K	F	A	E	A	S	E	D	G	M	T	R	V	Y	R	S	L	V	T	60
181	AAT	GTC	TGC	AAG	GAA	ATG	TCA	TGT	TAC	AGT	GAC	TTC	CCA	TTC	CAC	GAA	GAT	TAC	CCC	AAT
	N	V	C	K	E	M	S	C	Y	S	D	F	P	F	H	E	D	Y	P	N	80
241	TTC	ATG	AGC	CAT	GAG	AAA	TTC	TGG	GAC	TAT	CTC	AGA	GAA	TTT	GCT	GAG	CAC	TTC	GGC	CTC
	F	M	S	H	E	K	F	W	D	Y	L	R	E	F	A	E	H	F	G	L	100
301	CTG	AAA	TAT	ATT	CGG	TTT	AAG	ACT	ACT	GTG	CGG	AGT	GTA	ACT	AAG	CGT	CCA	GAC	TTC	TCT
	L	K	Y	I	R	F	K	T	T	V	R	S	V	T	K	R	P	D	F	S	120
361	GAA	ACT	GGC	CAA	TGG	GAA	GTT	GTC	ACA	GAG	ACA	GAG	GGC	AAA	CAG	GAC	AGA	GCT	GTA	TTT
	E	T	G	Q	W	E	V	V	T	E	T	E	G	K	Q	D	R	A	V	F	140
421	GAT	GCT	GTT	ATG	GTT	TGC	ACT	GGG	CAA	TTC	CTG	AGC	CCA	CGT	TTA	CCT	CTG	GAG	TCC	TTC
	D	A	V	M	V	C	T	G	Q	F	L	S	P	R	L	P	L	E	S	F	160
481	CCT	GGA	ATC	CAT	AAG	TTT	AAA	GGA	CAG	ATC	CTA	CAC	AGC	CAA	GAG	TAC	AGG	ATT	CCA	GAT
	P	G	I	H	K	F	K	G	Q	I	L	H	S	Q	E	Y	R	I	P	D	180
541	GCC	TTT	CGG	GGC	AAG	CGC	ATC	CTG	GTG	GTT	GGT	CTT	GGA	AAC	ACT	GGC	GGG	GAT	GTC	GCA
	A	F	R	G	K	R	I	L	V	V	G	L	G	N	T	G	G	D	V	A	200
601	GTG	GAG	CTC	AGT	GGA	ATA	GCA	GCA	CAG	GTG	TTT	CTC	AGC	ACT	AGG	ACT	GGT	GCC	TGG	GTT
	V	E	L	S	G	I	A	A	Q	V	F	L	S	T	R	T	G	A	W	V	220
661	CGC	AGC	CGC	TCC	TCA	GTT	GGT	GGC	TAC	CCT	TTG	AAT	ATG	ATG	CAG	ACA	AGA	TGG	CGC	AAC
	R	S	R	S	S	V	G	G	Y	P	L	N	M	M	Q	T	R	W	R	N	240
721	TTC	CTC	GCA	CGA	GTT	CTG	CCC	TCC	TGC	TTT	GTA	AGC	TGG	AAT	CAA	GAG	AGG	CAA	ATG	AAT
	F	L	A	R	V	L	P	S	C	F	V	S	W	N	Q	E	R	Q	M	N	260
781	AAA	ATA	TTT	AAC	CAT	GAG	AAC	TAT	GGA	TTA	AGC	ATT	GCA	AAA	GGG	AAA	AAA	CCA	AAA	TTT
	K	I	F	N	H	E	N	Y	G	L	S	I	A	K	G	K	K	P	K	F	280
841	ATT	GTG	AAT	GAT	GAA	CTA	CCA	ACT	TGT	ATC	CTC	TGT	GGG	AAA	ATC	ACC	ATG	AAA	ACC	AGC
	I	V	N	D	E	L	P	T	C	I	L	C	G	K	I	T	M	K	T	S	300
901	GTG	AAG	GAT	TTA	ACA	GAA	TCC	TCT	ATC	GTC	TTT	GAA	GAT	GGG	ACA	ATA	GAA	GCC	AAC	ATT
	V	K	D	L	T	E	S	S	I	V	F	E	D	G	T	I	E	A	N	I	320
961	GAT	GTA	GTG	ATC	TTC	ACT	ACA	GGA	TAT	GAA	TTT	TCT	TTT	CCC	TTT	TTT	GAA	GAG	CCT	CTC
	D	V	V	I	F	T	T	G	Y	E	F	S	F	P	F	F	E	E	P	L	340
1021	AAA	AGC	CTT	TGC	ACA	AAG	AAG	GTC	ATA	CTA	TAC	AAG	CGA	GTG	TTT	CCC	CCA	AAT	TTG	GAG
	K	S	L	C	T	K	K	V	I	L	Y	K	R	V	F	P	P	N	L	E	360
1081	AGA	TCA	ACA	TTA	GCC	ATC	ATT	GGC	CTG	ATC	AGC	CTT	ACT	GGG	TCT	ATC	TTA	GTG	GGC	ACA
	R	S	T	L	A	I	I	G	L	I	S	L	T	G	S	I	L	V	G	T	380
1141	GAG	TTC	CAA	GCA	CGG	TGG	GCC	ACG	AGA	GTA	TTC	AAA	GGG	CTC	TGT	AAT	ATA	CCT	CCA	TCT
	E	F	Q	A	R	W	A	T	R	V	F	K	G	L	C	N	I	P	P	S	400
1201	CAG	AAA	CTG	ATG	GCC	GAA	GCC	ATT	AAA	AAG	GAA	GAG	CTC	ATT	AAA	AGG	GGT	GTG	ATT	AAA
	Q	K	L	M	A	E	A	I	K	K	E	E	L	I	K	R	G	V	I	K	420
1261	GAT	ACC	AGC	CAA	GAC	AAA	CTC	GAC	TTC	ATT	TCC	TAC	ATG	GAT	GAG	CTC	ACT	CAG	TGC	ATA
	D	T	S	Q	D	K	L	D	F	I	S	Y	M	D	E	L	T	Q	C	I	440
1321	GGC	GCG	AAG	CCC	AAT	ATT	CCA	CTT	CTG	TTC	CTT	AAG	GAC	CCA	AGA	TTG	GCG	TGG	GAA	GTT
	G	A	K	P	N	I	P	L	L	F	L	K	D	P	R	L	A	W	E	V	460
1381	TTC	TTT	GGA	CCA	TGT	ACC	CCT	TAC	CAA	TAT	CGC	CTA	ATG	GGC	CCT	GGA	AGA	TGG	GAC	GGA
	F	F	G	P	C	T	P	Y	Q	Y	R	L	M	G	P	G	R	W	D	G	480
1441	GCC	AGA	AAT	GCC	ATC	CTG	ACC	CAG	TGG	GAC	AGG	ACT	GTG	AAA	CCA	CTA	AAG	ACT	CGA	ACT
	A	R	N	A	I	L	T	Q	W	D	R	T	V	K	P	L	K	T	R	T	500
1501	GTT	CCT	AAG	TCC	CAG	GAG	CCT	GCC	TCC	CTC	TCA	CGT	TAC	TTA	AAA	ACT	TGG	GGG	GCA	CCT
	V	P	K	S	Q	E	P	A	S	L	S	R	Y	L	K	T	W	G	A	P	520
1561	GTC	CTG	ATT	GTC	TCT	CTT	TTA	CTT	ATC	TAC	AAG	TCT	TCA	CTG	TTT	CTT	GAA	CTG	GTG	CAA
	V	L	I	V	S	L	L	L	I	Y	K	S	S	L	F	L	E	L	V	Q	540
1621	AGT	AAG	CTG	CAA	GGA	AGG	TTT	TCC	CCT	TCC	CGA	ATA	CTG	TGG	TAC	ATC	CCC	CAG	AAC	TCA
	S	K	L	Q	G	R	F	S	P	S	R	I	L	W	Y	I	P	Q	N	S	560
1681	TGA																			
	*																			

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1681 TGA GTTGGTCTCCAGTTTGGCAATGGAGGAAGTTATCTGGGACATCCCTTAAACAGACTAGTGCTGCTCTCAAACACTGGATCAGTCTCCATAGGGCAGGTTGTC
     ATAAAGCAAGGCTGCCTCTTATCGCTTTTTCATGCATTTATTTGTGCCCTTTGACCTTTCTACTATGTAATGACATAGTACAAACCTCTCACCAGAAGCTGCCACTGTG
1895 TCCTTGAGCCCTCCAGCCTCCAAATCATGAACAAAATAAACTTCATTTACCCATTTTATGTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Fig. 1. Nucleotide sequence of rat FMO4 cDNA and deduced amino acid sequence of rat FMO4. The open reading frame starts at nucleotide 1 and goes to nucleotide 1680. Deleted region caused by an alternative splicing; the stop codon and the putative polyadenylation signal are underlined. The rat FMO4 sequences have been entered in the GenBank libraries with accession numbers AF458416 and AF458417.

Reactivity of Antipeptide Antibodies. cDNAs encoding long and short FMO4 were cloned into *E. coli* expression vectors (pGEX-6P3). The IPTG-dependent expression of pGEX-FMO4 in the *E. coli* BL21 was controlled by the measurement of the glutathione *S*-transferase activity in the sonicates. Low GST activity was detected in the sonicates containing long FMO4 or short FMO4. The presence of recombinant proteins was analyzed by Western blot using the antibodies raised against P1 or P2. Only the analysis of the sonicate containing the GST-long FMO4 fusion protein demonstrated the presence of a band, recognized by both antibodies, at the expected molecular mass (~90 kDa). No band migrating at about 85 kDa was detected in the sonicate containing short FMO4. In contrast, numerous bands of lower molecular mass were recognized by both antibodies in the sonicates containing either the long or the short FMO4 (result not shown). These lower bands seem to be the result of a strong proteolysis during the IPTG-dependent expression. The short FMO4 not having been obtained, only the

long FMO4 was purified. The proteins were solubilized from inclusion bodies of *E. coli* using *N*-lauroyl-sarcosine (1.5%), because the induced GST-long FMO4 fusion protein was insoluble in Triton X-100 (2%). This step may have compromised the ability to recover functional long FMO4. Solubilized fusion proteins were bound on a glutathione Sepharose 4B. The long FMO4 protein was eluted by cleavage with Prescission Protease, which keeps GST remaining on the column. The analysis of the purified long FMO4 by Western blot using antibodies raised against P1 or P2 revealed the presence of a band migrating at ~64 kDa (Fig. 5A, lane 1) along with several other bands with lower molecular mass, seeming to be the result of the proteolysis as described above.

Because of the strong proteolysis observed during the heterologous expression, the long and short FMO4 were produced by in vitro transcription/translation (Fig. 5B). Using immunoblot analysis, the long FMO4 (*lane 1*) obtained by in vitro transcription/translation, and the recombinant long FMO4 were recognized at the same molecular mass (~64
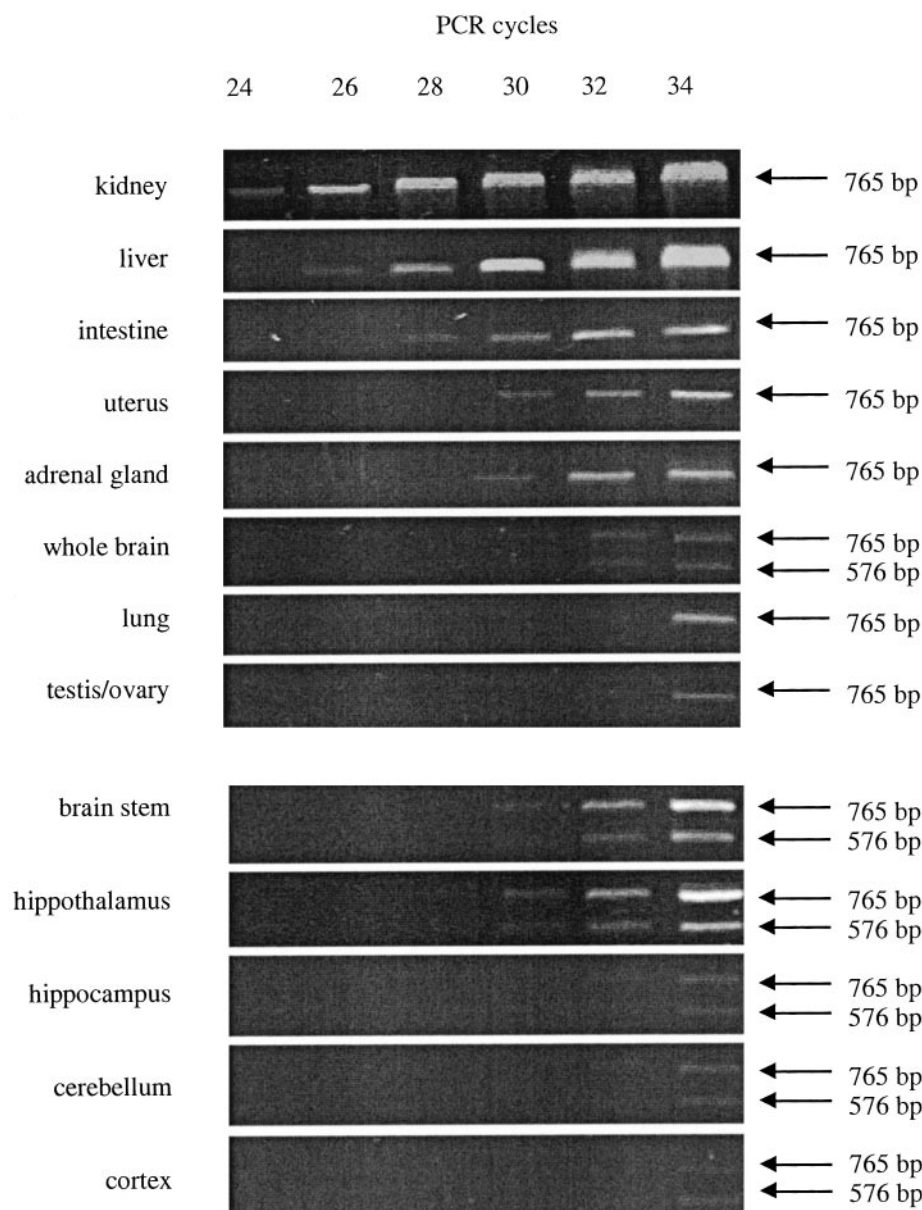

Fig. 2. Analysis of FMO4 mRNA distribution by RT-PCR using primers FMO4-S4 and FMO4-AS7 in the presence of cDNA (2 μl) produced by the reverse transcription of total RNA (0.5 μg in a total volume of 20 μl) from different rat tissues. Kidney, liver, intestine, adrenal gland, testis, whole brain, and specific brain areas are from male rats. PCR products were analyzed after 24, 26, 28, 30, 32, and 34 PCR cycles.

kDa) by employing the antibodies raised against P1 or P2. Immunoblot analysis of the short FMO4 obtained by in vitro transcription/translation using the antibodies raised against P1 or P2 revealed the presence of a single immunoreactive protein migrating at ~57 kDa (*lane 4*).

Expression of Long FMO4 in Kidney and Intestine and Short FMO4 in the Brain. Microsomes were prepared from kidney, intestine, uterus, liver, lung and brain of rats, and the constitutive expression of either forms of FMO4 was analyzed by Western blotting using antibodies raised against P1 (Fig. 5B) or P2 (Fig. 4A). A 64-kDa protein corresponding to the long FMO4 (Fig. 5B, lane 1) was detected in the rat kidney (Fig. 5, A, lane 2, and B) and intestine (result not shown). This 64-kDa protein was not detected in the rat brain (Fig. 5, A, lane 3, and B) or uterus (result not shown). On the other hand, a faint 57-kDa band corresponding to the short FMO4 was recognized in the rat brain (Fig. 5, A, lane 3, and B). Neither the 64-kDa protein nor the 57-kDa protein were detected in the liver or in the lung (results not shown).

Discussion

In this study, a cDNA clone encoding a protein of 560 amino acids was obtained from rat kidney by RT-PCR and 5'/3' terminal extension, using oligonucleotides designed from the sequences of FMO4s previously published. The deduced amino acid sequence revealed high homology with the FMO4s of humans (80%) (Dolphin et al., 1992) and rabbits (79%) (Burnett et al., 1994), whereas the homology was lower compared with the sequences of rat FMO1 (50%) (Itoh et al., 1993) or rat FMO3 (51%) (Lattard et al., 2001). The designa-

tion "rat FMO4" for this cDNA is in agreement with the actual nomenclature based on the comparison of the amino acid sequence of the mammalian FMOs (Lawton et al., 1994).

A 1800-bp cDNA containing an ORF of 1680 bp was obtained from rat kidney mRNA. The sequence surrounding the proposed initiation codon GCACCATGG is favorable for translational initiation of vertebrate mRNAs (Kozak, 1987). A polyadenylation signal (AATAAA) was found 22 bases upstream of a polyadenylated tail. From rat brain mRNA, two different transcripts (1800 and 1650 bp, respectively) encoding both long and short forms of FMO4 were amplified, cloned, and sequenced. The long transcript was identical to that previously sequenced in the rat kidney. The short transcript, encoding a protein of 497 amino acid residues, is the result of a 189-bp deletion, corresponding to the nucleotides from 132 to 321 of rat kidney FMO4 cDNAs. The constitutive presence of these two transcripts in the rat brain was confirmed by a PCR amplification of rat brain total RNA using specific primers framing the 189-bp deletion. Furthermore, the expression of short-form transcript seems to be specific in the rat brain. The constitutive expression of both forms of FMO4 transcripts is found in all cerebral regions tested.

The amplification of the corresponding region of genomic DNA revealed the presence of a single *FMO4* gene encoding the long form FMO4. The short FMO4 seems to be caused by an alternative splicing specific of the brain. The deleted 189-bp region corresponds exactly to the exon, which corresponds to exon 4 present in the human FMO4 gene (GenBank accession number AL031274). Exon 4 of the rat FMO4 has some intronic characteristics. The sequence AG observed at
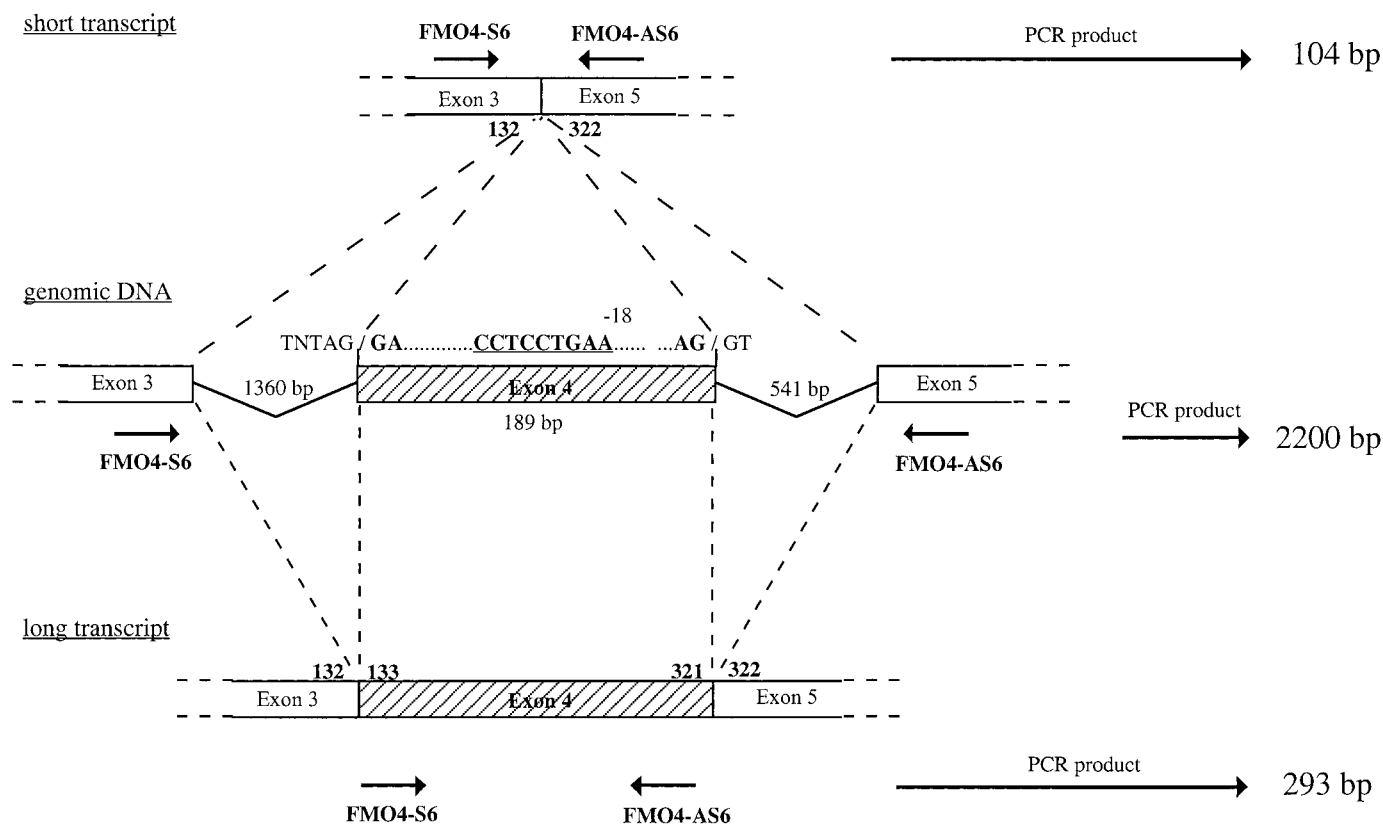

Fig. 3. Splicing pattern of FMO4 mRNA in rat. Exons (from exon 3 to exon 5) are designated by boxes and introns by lines. Alternative spliced exon is schematized by a hashed box. The boundaries of exon 4 are marked and the putative polypyrimidine tract at the 3' end of exon 4 are underlined.

the 3′ extremity of the deleted region corresponds to a potential acceptor site (Mount, 1982; Shapiro and Senapathy, 1987; Stephens and Schneider, 1992) for alternative splicing, and the sector rich in pyrimidine bases at 18 bases upstream could be considered as a potential branch site. On the other hand, the 5′ extremity of the exon 4 contains GA instead of GT, which is characteristic of a 5′ splice donor (Mount, 1982; Shapiro and Senapathy, 1987; Stephens and Schneider, 1992). However recent studies of the thioredoxin reductase 1 genes (Osborne and Tonissen, 2001) related the presence of the 5′ splice donor GC instead of GT, which is probably involved in the alternative splicing of the 5′ region. The occurrence of this modified splice signal could represent a potential regulation toward formation of the short form FMO4. The sequence of the exon 4 of the human *FMO4* gene is similar to the rat FMO4 exon 4. The existence of such an alternative splicing in human tissues should be explored.

Similar to the forms of FMO described previously, the long sequence exhibits the presence of characteristic FAD- and NADPH-binding sites, beginning at residues 9 and 191, respectively. As for the previously described forms of FMO4 (Dolphin et al., 1992; Burnett et al., 1994), the deduced protein is approximately 28 residues longer than other FMO isoforms and has a calculated molecular mass of ~64 kDa. These additional residues present in FMO4 are contained in a single block located at the COOH terminus and may have resulted from the loss of the stop codon commonly present in all other FMO isoforms. The translation continues to the

following stop codon, localized 84 nucleotides farther on. Because of this extension, rat FMO4 terminates with an additional hydrophilic peptide consisting of 19 extra amino acids not present in other FMO isoforms (Ozols, 1991). The 189-bp deletion does not modify the reading frame of the short sequence and corresponds to the loss of 63 amino acid residues (from residue 44 to 117) of the long sequence. Despite this deletion, putative FAD- (amino acids from 9 to 14) and NADPH- (amino acids from 128 to 133) binding domains were preserved. Nevertheless, the hydrophilic region localized usually between these two sites is not found in the short FMO4 form, perhaps expressed constitutively in the brain. Such exclusion of exon 4 in the short FMO4 form may constitute a significant structural and functional change.

The corresponding mRNA was found, by Northern blot, only in the rat kidney and intestine only. The constitutive expression of FMO4 mRNA in the kidneys of rabbit, guinea pig, or rat kidney had been already suggested by Northern blot studies using a rabbit cDNA probe (Burnett et al., 1994). In the rat kidney FMO1 (Itoh et al., 1993) and FMO3 (Lattard et al., 2001) are also found. The presence of FMO4 mRNA was detected in the liver of the guinea pig but not in the rat liver (Burnett et al., 1994). Our results obtained both by Northern blot and RT-PCR confirm that the expression of FMO4 mRNA in the rat liver is, for the best, extremely limited. FMO4 mRNAs (both short and long transcripts) were detected in the rat brain by RT-PCR only. This observation is coherent with the presence of FMO4 transcripts, detected by RT-PCR in the rabbit brain (Blake et al., 1996). All the tested cerebral regions contained these mRNA but the expression was higher in the hypothalamus and brain stem. The alternative splicing of the mRNA encoding for mRNA seems to be specific to the brain.

To date, the tissue expression of the corresponding FMO4 protein has not been reported. The production of rabbit or human recombinant proteins has always failed (Burnett et al., 1994; Phillips et al., 1995; Itagaki et al., 1996), with the exception of a truncated human FMO4 (Itagaki et al., 1996). Because of these difficulties, probably linked to a strong proteolysis, the production of even a limited amount of FMO4 necessary to immunize rabbit has never been achieved. Con-
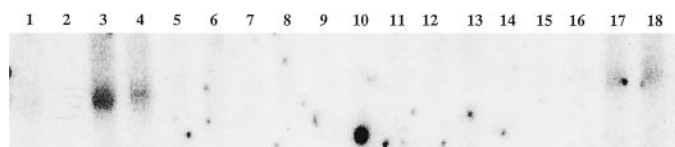

Fig. 4. Analysis of FMO4 mRNA distribution by Northern blot. Samples of total RNA (20 μg) isolated from liver (male, lane 1; female, lane 2), kidney (male, lane 3; female, lane 4), lung (male, lane 5; female, lane 6), brain (male, lane 7; female, lane 8), muscle (male, lane 9; female, lane 10), adipose tissue (male, lane 11; female, lane 12), adrenal gland (male, lane 13; female, lane 14), testis (lane 15), ovary (lane 16), and intestine (male, lane 17; female, lane 18) were electrophoresed in agarose (1.2%) containing formaldehyde (2 M), transferred to nylon membranes, and hybridized with a ^{32}P-labeled rat FMO4 coding region probe (bases 280–981).
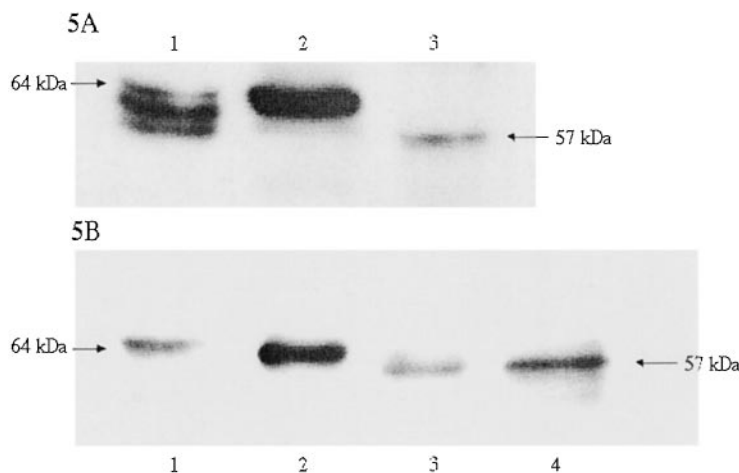

Fig. 5. Analysis of FMO4 protein distribution by Western blot using an anti-rat FMO4 peptide P2 antibody (A) or an anti-rat FMO4 peptide P1 antibody (B). Microsomal proteins (30 μg) from rat kidney (lane 2) and rat brain (lane 3) and 1 μg of recombinant long FMO4 (A, lane 1) or long FMO4 produced by in vitro transcription/translation (B, lane 1) and short FMO4 produced by in vitro transcription/translation (lane 4) were loaded. Immunoblottings were described under *Materials and Methods*.

sequently, no production of FMO4 antibodies has been reported. Our attempts to produce rat FMO4 were also partially unsuccessful. Only the long FMO4 was produced, in very small quantities and strongly degraded. Consequently, we could produce antibodies against only two rat FMO4 peptides. The limited amount of available recombinant long FMO4 was, however, sufficient to verify the recognition of the rat FMO4 by employing the two antibodies raised against the peptide fragments of long-form FMO4. The reactivity of these antibodies against long and short protein obtained by in vitro transcription/translation was also verified. The absence of cross-reactivity toward other FMO isoforms is confirmed by the absence of signal in Western blot between 40 and 70 kDa in rat liver and lung. Indeed FMO3, FMO1, and probably FMO5 are highly expressed in the liver (Lattard et al., 2001), and FMO2 is found in the lung (Lattard et al., 2002). The use of these two antibodies demonstrated the expression of a long FMO4 protein in the rat kidney and intestine but not in the rat brain. On the other hand, the expression of a short FMO4 protein in the rat brain was demonstrated and confirmed by both antipeptide antibodies.

Because a large number of psychotropic and neurotoxic agents are metabolized by FMO, the presence of FMO in the brain has been suspected for a long time. Metabolism of FMOs substrates (thiobenzamide, methimazole, dimethylaniline, or benzydamine) has already been reported in the rat brain (Duffel and Gillepsie, 1984; Bhamre and Ravindranath, 1991; Bhamre et al., 1993; Kawaji et al., 1994). But the expressed isoforms have not yet been identified. The expression of FMO1 (Itoh et al., 1993) or/and FMO2 (Bhamre et al., 1993) were suggested, but not confirmed. In the other hand, the presence of FMO4 transcript in the rabbit brain was reported by Blake et al. (1996). The expression, described for the first time in this study, of a short FMO4 protein in the rat brain produced by tissue specific alternative splicing, and the expression of a long FMO4 protein in the kidney, could be of great interest. Nevertheless, further investigations concerning the catalytic properties of these proteins are necessary.

Acknowledgments

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