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Isolation of Human CYP4F2 Genomic DNA and Its 5' End Regulatory Region Structure

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Human cytochrome P450 4F2 shows high regioselectivity in ω-hydroxylation of stearic acid and leukotriene B_4. As a first step of its regulation study, human cytochrome P450 4F2 genomic DNA was isolated from liver of a person who was administered clofibrate for 10 years. From Southern hybridization, restriction enzyme digestion and sequencing experiments, isolated genomic DNA fragment was found to contain around 32 Kb DNA and more than 20 Kb of 5' end regulatory region. Sequences of the structural gene region revealed exon 1 and exon 2. Further regulation studies would elucidate the feedback mechanisms of the oxidative degradation of fatty acids, inflammatory response and the clearance of leukotriene B4 in the liver. Furthermore, regulation study of this gene could explain the species difference in responses to peroxisome proliferator and help in the safety evaluation of peroxisome proliferating chemicals to human being.

Key words : Human cytochrome P450 4F2, Leukotriene B4, Genomic DNA cloning, 5' end regulatory region

INTRODUCTION

Microsomal P450 monooxygenase superfamily play an important role in the oxidative metabolism of a wide variety of lipophilic compounds, including endogenous substrates such as steroids, fatty acids, and prostaglandins as well as foreign chemicals such as drugs and carcinogens. Among these, cytochrome P 450 4 family members function to hydroxylate the terminal ω-carbon of saturated fatty acids, cholesterol, arachidonic acid, prostaglandin and leukotrienes. The newly found CYP 4F subfamily members are known to ω-hydroxylate long chain saturated (C_{16}-C_{24}) and unsaturated fatty acids. To the present, about six CYP 4F subfamily members are isolated: CYP 4F1 from rat liver, CYP 4F2 from human liver, CYP 4F3 from human neutrophil, and CYP 4F4, 4F5, 4F6 from rat brain (Kawashima and Strobel, 1995). Among these, CYP 4F2 is found to have potential leukotriene B_4 (LTB_4) and stearic acid ω-hydroxylation activity (Hardwick and Chen, 1996).

The peroxisome proliferator-activated receptors (PPARs) are a group of transcription factors that regulate gene expression of enzymes associated with lipid homeostasis, including fatty acid degradation (Wahli and Green, 1994). PPARs are members of the nuclear hormone-receptor family named by virtue of their ability to mediate a pleiotropic response to peroxisome proliferators (PPs, Isseman and Green, 1990). These receptors can be activated by hypolipidaemic fibrates such as clofibrate (CF), some arachidonic acid metabolites, and various fatty acids. So far, three subtypes have been identified (α, β/δ or FAAR and γ) from *Xenopus,* rodents and humans. Transcriptional regulation by PPARs is achieved through PPAR-RXR (where RXR is the receptor for 9-*cis* retinoic acid) heterodimers which bind to DNA motifs termed PPAR-response elements (PPREs) in the promoter of their target genes.

Recently, Devchand *et al.* (1996) published that LTB_4 was an activator and a natural ligand of PPARα. In many inflammatory disease, the turnover of the dihydroxy fatty acid LTB_4 determines the extent and duration of inflammation (Ford-Hutchinson, 1990). LTB_4 induces a complex cascade of molecular and cellular events that ultimately recruit cells from the immune system to the site of injury and produce an inflammation. It is inactivated through metabolic degradation by the microsomal ω- and peroxisomal β-oxidation pathways (Jedlitschky *et al.*, 1991). Catabolism of LTB_4 occurs primarily in cells of the immune system at the site of inflammation (Koller *et al.*, 1987 and Minakami *et al.*, 1990) and also in hepatocytes, liver being the principle organ for clearance of LTB4 from blood (Keppler *et al.*, 1992).

Therefore, understanding the regulation of this gene including determination of the PPRE of human CYP

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4F2 gene and its response to PPs is very important to control the inflammatory diseases like rhematoid arthritis, lupus and psoriasis. In these regards, we tried to clone the genomic DNA of human CYP 4F2 gene and analyzed its 5' end regulatory region.

MATERIALS AND METHODS

Materials

The human CF liver from an individual administered CF for 10 years was obtained from the National Disease Tissue Exchange. SuperCos I and Gigapack III Gold packaging Extract was purchased from Stratagene (La Jolla, CA, USA) for genomic cloning. For probe labelling, DECAprime II (Ambion; Austin, TX, USA) system was used. [α-^{32}P] dCTP (3000Ci/mmol) were purchased from Amersham International (Chicago, IL, USA). Restriction enzymes, T_4 DNA polymerase and other enzymes were obtained from NEB (New England Biolabs; Berverly, MA, USA). DNA marker for pulsed field electrophoresis and other general DNA marker and antibiotics were purchased from Sigma (St. Louis, MO, USA).

Genomic DNA isolation and size fractionation

Human genomic DNA was isolated from liver of an individual who was administered CF for 10 years. Liver was homogenized with Potter-Elvehjem homogenizer and treated with proteinase K (final concentration of 1 mg/ml) and 10% SDS. The resultant lysate was loaded onto 10~40% sucrose density gradient and each fraction was collected, dialyzed against TE buffer and analyzed with FIGE (Field Inversion Gel Electrophoresis, BioRad; Hercules, CA, USA). Purified high molecular DNA (containing around 150 Kb DNA fragment) was partial digested with *Mbo* I, size-fractionated with 1.25-5 M NaCl gradient and dialyzed against TE buffer. Fractions containing DNA fragments of 35 Kb to 45 Kb were collected and used for genomic library construction.

Genomic library construction

Construction of genomic library was performed as described in the Stratagene instruction manual with slight modification. Insert DNA fragments were dephosphorylated and ligated to SuperCos I digested with *Xba* I, dephosphorylated and digested with *Bam*H I, *in vitro* packaged and transfected into *E. coli* XL1-Blue MR. And plated on nylon membrane covered on LB medium containing kanamycin without amplification. To verify the insert, plasmid isolation and digestion with *Not* I was carried out.

Probe preparation using CYP 4F2 cDNA

Various probes encompassing part or full length of CYP 4F2 cDNA were prepared by single or double digestion with restriction enzymes such as *Eco*R I-*Hinc* II, *Eco*R I-*Sac* I, *Sac* I-*Bst*X I, *Bst*X I, *Bst*X I-*Pst* I, *Pst* I-*Eco*R I and *Eco*R I-*Xho* I. Each probe was purified with agarose gel electrophoresis and labelled with [α-^{32}P] dCTP by random priming DNA labelling method (Ambion). These probes were used to confirm whether cloned DNA fragments contained entire CYP 4F2 genomic DNA also for the and mapping of cloned DNA by southern hybridization.

Screening of genomic library

Original genomic library spreaded on nylon membrane at the density of 50,000 colonies/150-mm agar plate was duplicated on another nylon membrane. Duplicated nylon membranes were screened by colony hybridization (Maniatis *et al.*, 1988) using 2385 bp full length CYP4F2 cDNA. Hybridization was done at 42°C for 18 hrs and the filters were washed at 65°C for 30 min three times in 0.1X SSCPE (1X SSCPE: 0.12 M NaCl; 0.015 M sodium citrate; 0.01 M potassium phosphate; 0.001 M Na_2EDTA), and 0.1% SDS. Positive colonies were selected, cosmid DNA was isolated and analyzed by restriction enzyme digestion and dot hybridization or southern hybridization at the same condition of colony hybridization with probes prepared above.

Cloning and sequencing of the 5'-flanking region and structural gene region including first two exons of the human CYP 4F2 gene

A 6 kb *Eco*R V-*Hin*d III DNA fragment was largest 5'-flanking DNA fragment that hybridized to 260 bp 5' end cDNA probe (EcoR I-*Hinc* II fragment). For cloning convenience, 5.4 kb *Bgl* II-*Hin*d III 5'-flanking region and 3.9 kb *Hin*d III-*Bgl* II structural gene region were subcloned into pBluescript II KS. Sequencing of these plasmids was carried out using a Sequenase kit (United States Biochemical Corp.; OH, Cleveland, USA) following the manufacturer's instruction.

RESULTS AND DISCUSSION

Genomic library screening

Around 5×10^5 colonies, which cover about more than 99% human genome were obtained from genomic library construction experiment without any amplification. This genomic library was screened with a full length CYP 4F2 cDNA probe (2.24 kb *Eco*R I-Xho I fragment, Fig. 1 and Fig. 2). Two hundred twenty three colonies were detected from first screening, which were screened sequentially by 5' end cDNA fragment (260 bp *Eco*R I-*Hinc* II fragment), cDNA fragment (413 bp *Bst*X I-*Bst*X I fragment) located in the

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    AATTCGCATGGCACCATGAGCGACTCTACAATGACCTGCATGCGCTCCAGCGCCGATAACTGTGGCTCCCCCGAAGCGGCCTCCGTGCTTGGTCTGCTTGGACTCCTGCTGGGCCGTGTC  120
1                                MetThrCysMetArgSerSerAlaAspAsnCysGlySerProGluAlaAlaSerValLeuGlyLeuLeuGlyLeuLeuLeuGlyArgVal

    GAGTTCTACATGCATTGGCAATCGCTTCTCAAGGCTATCAAGCAGTTCCCATCACCTACCTTCCACTGGTTCTTTGGGCACAAGCAGTTTCAAGGTTACAAAGTACTACAGCACATTACG  240
31  GluPheTyrMetHisTrpGlnSerLeuLeuLysAlaIleLysGlnPheProSerProThrPheHisTrpPhePheGlyHisLysGlnPheGlnGlyTyrLysValLeuGlnHisIleThr

    ACATTTGAGGTGACTTCCCCATCTGTTTATCCACGATTGTACTCCTGTAGCAAAGCCTACGAAATAGTGATTGCCCCTGTCACAATGAAGGCAATGCACGCTAGATCAGATCCCCACGCC  360
71  ThrPheGluValThrSerProSerValTyrProArgLeuTyrSerCysSerLysAlaTyrGluIleValIleAlaProValThrMetLysAlaMetHisAlaArgSerAspProHisAla

    CCTGTCGTATACACAGAGCAAGCTAATTGGATCGGATATGAGCTCCTCTTGCTGAATGGACATCCCTGCTTCCCGCAGGGGCCACTGCTCACACCAGAGTTGGCGTATGCCTTTCTGATT  480
111 ProValValTyrThrGluGlnAlaAsnTrpIleGlyTyrGluLeuLeuLeuLeuAsnGlyHisProCysPheProGlnGlyProLeuLeuThrProGluLeuAlaTyrAlaPheLeuIle

    GGCTATGTAATTTACGTGGCTCACTGCATTGGAGTGTTGCTCGATATATCGCCCCCGCAGGGACGTCCAGACTACTATATAGTTTTCTTTCACCATCTCTCCTTGATCCAGCTACAGGCA  600
151 GlyTyrValIleTyrValAlaHisCysIleGlyValLeuLeuAspIleSerProProGlnGlyArgProAspTyrTyrIleValPhePheHisHisLeuSerLeuIleGlnLeuGlnAla

    GTCATGAAGTGTGTCTTCAGCCACAATTGCAGTGTTCATGTGGATGGAAATTACAAGAGCTATATCCAGGCCATTGGGAACTTGAATGACCTCTTTACCTCCCGTGTGAGGAACATCTTT  720
191 ValMetLysCysValPheSerHisAsnCysSerValHisValAspGlyAsnTyrLysSerTyrIleGlnAlaIleGlyAsnLeuAsnAspLeuPheThrSerArgValArgAsnIlePhe

    CATCAGAATGATACCATCTATAATTTTTCTTCCAATGGCCACTTGTTCAACCGTGCTTGTCTTTTTGCCCCTGATCCGACACCTGCTGTGTGGAGGCTACCGACTGCTCCGCTGCACCCC  840
231 HisGlnAsnAspThrIleTyrAsnPheSerSerAsnGlyHisLeuPheAsnArgAlaCysLeuPheAlaProAspProThrProAlaValTrpArgLeuProThrAlaProLeuHisPro

    GCCGCAGCGCTGCACAACGTCAGGAAGCCAACACGTTTGGAACTTCTCCACATAGGGTCACTTGCCAGAATCGAGAATGGGGACAGCATGTGTGCCATGGCCCTAGGTGCTGAGGTGGAC  960
271 AlaAlaAlaLeuHisAsnValArgLysProThrArgLeuGluLeuLeuHisIleGlySerLeuAlaArgIleGluAsnGlyAspSerMetCysAlaMetAlaLeuGlyAlaGluValAsp
                                                                                                    *       *

    ACATTAATGTTCGAGGGTCATGACACCACAACAAGTCGAGTCTACTGGATATTCTATCCTCTGGCGACACACACTAAGCACAAACGAAGATGCAAAGAGGATGATCACAGCGTGCTGCAG 1080
311 ThrLeuMetPheGluGlyHisAspThrThrThrSerArgValTyrTrpIlePheTyrProLeuAlaThrHisThrLysHisLysArgArgCysLysGluAspAspHisSerValLeuGln
      *

    GATGGGTCCTCCATTACCTGGGATCACCTGGACCAGATTCCCTTCGCTACCATGTGTATTAAGGAGCCCCTGAGGTTGGACCCATCACAACCCAGCCGTGTGGCCGGACCCTGGAGGTCT 1200
351 AspGlySerSerIleThrTrpAspHisLeuAspGlnIleProPheAlaThrMetCysIleLysGluProLeuArgLeuAspProSerGlnProSerArgValAlaGlyProTrpArgSer

    ATGACCCTTTCGCTTAGACCCAAAGAACTCAAGGGAGGTCACCTCTGGCTTTTATTCCCTTCTCAGCACCACATCGGGCAGGCGTTGGATGGCCCAGAGGTGTTCGACCCATCCAGGTTT 1320
391 MetThrLeuSerLeuArgProLysGluLeuLysGlyGlyHisLeuTrpLeuLeuPheProSerGlnHisHisIleGlyGlnAlaLeuAspGlyProGluValPheAspProSerArgPhe

    GCGCTAGACTATCCCGGACACCGCAACTCATTCCTGCCCTTCTCAGGACGAGCGAGGAACTGCATTGGGAAACAATTTGCTATGAGTGAGATGAAGGTGATTGTGGCCCTGACCCTCCTC 1440
431 AlaLeuAspTyrProGlyHisArgAsnSerPheLeuProPheSerGlyArgAlaArgAsnCysIleGlyLysGlnPheAlaMetSerGluMetLysValIleValAlaLeuThrLeuLeu

    GCTTTGAGCTACTGCCAGCCCCCACCAAGGTCCCCTGCCCCTTAACACGATTCGTGTGGAAGTCCAAAAATGGTATCTACCTGTATCTCAAGAAGCTCCACTACTTCCGTTGTGAAGCTT 1560
471 ArgPheGluLeuLeuProAlaProThrLysValProCysProLeuThrArgPheValTrpLysSerLysAsnGlyIleTyrLeuTyrLeuLysLysLeuHisTyrPheArgCysGluAla

    CCGAAATCTGAAATGAGGTTCACTGGCAGAAAGCTGAGATGGTTTTGTGACTAGCCTTCTTCACCAGAGTGCTTCAGAGACTCCTCTCATCCTCTCTTAAGTACAGATCACCCTTCTCAG 1680
511 ProLysSerGluMetArgPheThrGlyArgLysLeuArgTrpPheCysAspEnd

    CACTGGAATATTCCTCTGCTTTAAAGGGAGCACCCTTCCATTACCCCCTCTTCTAAAAGCCTTCCCTTTTACAAATGATCATATGAGATCATCCAAGTCCACTGAAAAACTCCAAGATAA 1800
    TTTCCCATCTCAATATTCCTTACTCCATCTAACCTACTAAGTCCCTTTTGAATTATGAGGAATAATTCAATTTGTTCCATGGGCTCCAAAACTCAAGGCCTGAGCTCTATTGTGAAACCT 1920
    TTATTCAGCCTAATATCATCTTCACAAGACCTTAGACTCCATGGCTCTGACTAACCTGAAATGCGATCGCTCCTCCGACGGTTATATTTAATTAAGACTCTCGACTGCTGCACTGCACTG 2040
    CTTGAAACGCTGCGTTCGCTTGTTCAAAGGGCTGATCGTTTACTGGTTCAGACTGATCGAATAATCTAACGCTACGATCTAAGACTGACCCAATAAGGCTCACTGCTGCTCGATCAATAA 2160
    ACTGACTACTCCTAGCTCGAATAACGCGTACTAGTGGCTAATAATATAATCTCTACGTCAATAACTAC-poly A                                                2228
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Fig. 1. Complete nucleotide and deduced amino acid sequence of the human CYP 4F2 cDNA. The cytochrome p450 4 family conserved region is shaded. P450 conserved residues surrounding the heme-binding cysteine are boxed.

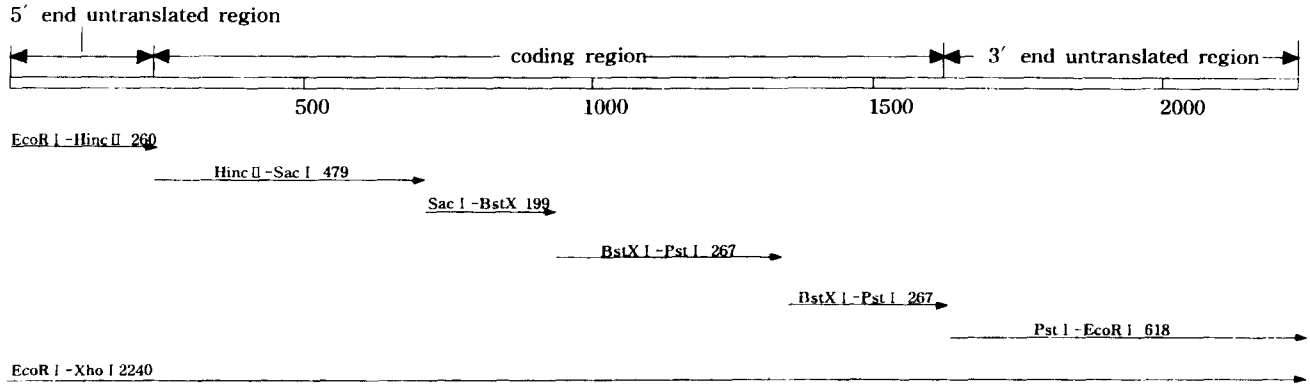

Fig. 2. Restriction map of the human liver CYP 4F2 cDNA and its fragments to prepare the probes for genomic library screening, southern and dot blot hybridization analysis. Upper figure is the restriction map of CYP 4F2 cDNA with *Eco*R I site(located in 5' end) added for insertion of cDNA into pBluescript vector. The 5' end non-translated region, open reading frame and 3' end nontranslated region are indicated wth arrow. Probes encompass full length cDNA sequentially.

middle of CYP 4F2 cDNA and 3' end cDNA fragment (618 bp *Pst* I-*Eco*R I). From these sequential screening, one clone (clone 4) was selected for further analysis.

Gene mapping by restriction enzyme digestion and southern hybridization

Various combinations of restriction enzyme digestion or single enzyme digestion were used to get the restriction enzyme map of clone 4. These digests were loaded onto 0.7% agarose gel and run for 16 hrs at 40 V. After running, DNA fragments were transferred to nylon membrane, probed sequentially with

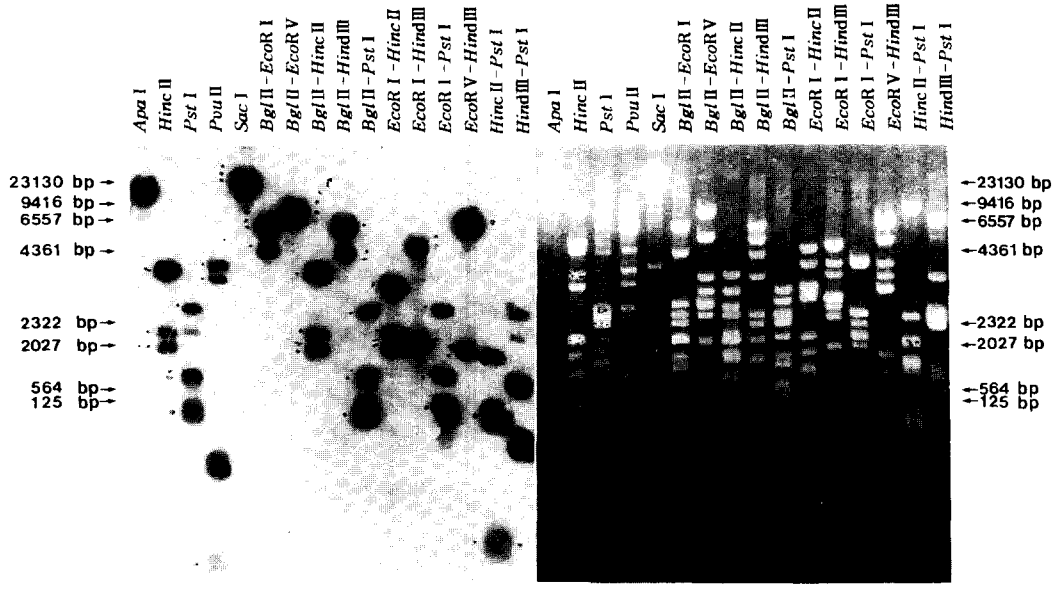

Fig. 3. Southern blot analysis of clone 4 with 2240 bp *Eco*R I-*Xho* I full length CYP 4F2 cDNA fragment as a probe. Clone 4 containing around 32 kb insert DNA was digested with various combination of restriction enzymes or single enzyme as indicated in figure, separated in an 0.7% agarose gel, blotted to nylon membrane, and probed with ^{32}P labelled full length 2240 bp *Eco*R I-*Xho* I CYP 4F2 cDNA fragment, fragments representing cDNA sequentially from 5' end to 3' end as shown Fig. 2. From these sequential southern blot analysis, subcloning and restriction enzyme digestion analysis, complete resriction enzyme map of CYP 4F2 genomic DNA was obtained.

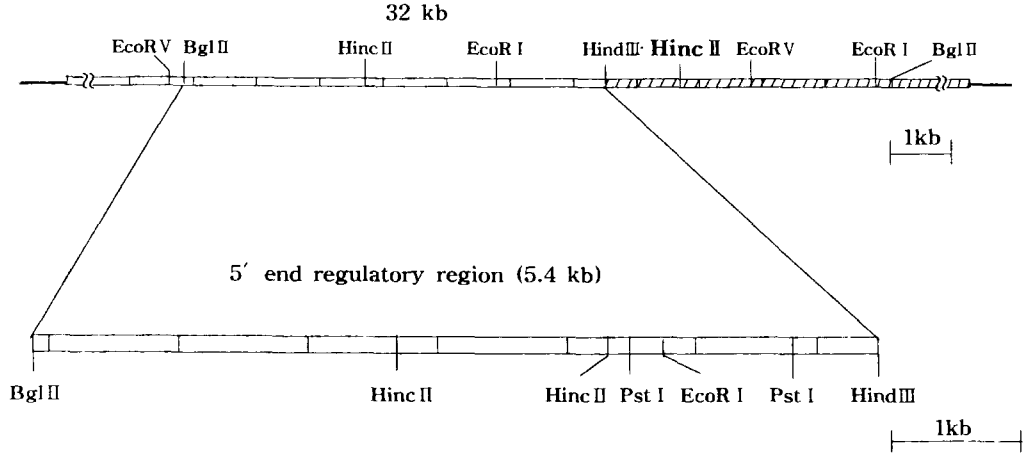

Fig. 4. Restriction enzyme map of human liver CYP 4F2 genomic DNA (clone 4). Upper figure is the restriction map of CYP 4F2 genomic DNA, first part of *Eco*R V to *Hind* III describes the 5' end regulatory region and second part of *Hind* III to *Bgl* II denotes the structural gene region (dashed bar). Cloned CYP 4F2 genomic DNA contains around 32 kb DNA fragment with strong hybridization signal with probes of *Eco*R I-*Hinc* II cDNA fragment to *Bst*X I-*Bst*X I cDNA fragment including more than 20 kb of 5' end regulatory region. Sequencing of *Hind* III-*Hinc* II and *Hinc* II-*Hinc* II fragments of structural gene region showed the exon 1 and exon 2. Second exon starts at the 259th nucleotide of cDNA and ends at the 403th nucleotide. Restriction map of 5' end regulatory region is enlarged in lower figure. Solid line denotes vector DNA.

2240 bp full length cDNA fragment (*Eco*R I-*Xho* I, Fig. 3), fragments covering 5' end cDNA to 3' end cDNA such as 260 bp *Eco*R I-*Hinc* II, 479 bp *Hinc* II-*Sac* I, 199 bp *Sac* I-*Bst*X I, 413 bp *Bst*X I-*Bst*X I, 267 bp *Bst*X I-*Pst* I, 618 bp *Pst* I-*Eco*R I cDNA fragment and complete restriction enzyme map was generated. From this study, this clone 4 was found to contain 32 kb DNA fragment showing strong southern hybridization signal with probes of *Eco*R I-*Hinc* II cDNA fragment to *Bst*X I-*Bst*X I fragment and other portion of clone 4 contain the 5' end regulatory region (more than 20 kb). From clone 4, two *Hind* III-*Bgl* II DNA fragment were subcloned into pBluescript II KS and digested with restriction enzyme for confirmation of map ob-

tained above (Fig. 4). Two maps were identical.

Sequencing of *Hind* III-*Bgl* II DNA fragment

Partial sequencing of *Hind* III-*Bgl* II DNA fragment containing structural gene region was carried out to confirm the clone obtained. From this partial sequencing, putative exon I and exon 2 could be located and their exon-intron junction sequences were found to obey its rule, that is in the beginning of intron, GT can be located and at the end of intron, CAG are found. Second exon starts at the 259th nucleotide of cDNA, which 6 nucleotides starting from is overlapped with *Hinc* II recognition site (bold letters in Fig. 4) and ends at the 403th nucleotide. From this result, we can confirm that clone 4 isolated from human genomic library is the gene fragment corresponding to CYP 4F2 cDNA. A previous study indicated that human CYP 4F2 shows high regioselectivity in the ω-hydroxylation of stearic acid and LTB_4 (Hardwick and Chen, 1996). It has been known that LTB_4 determines the extent and duration of inflammation (Hen- derson, 1994). Therefore, catabolism of LTB_4 at the site of inflammation is important to control the inflammation state (Shak and Goldstein, 1985). LTB_4 is primarily catabolized in cells of the immune system at the site of inflammation and also in hepacocytes, liver being the principle organ for clearance of LTB_4 from blood circulation (Keppler *et al.*, 1992). Recently, LTB_4 was found to be a natural ligand of peroxisome proliferator-activated receptor (PPARα), one of the group of transcription factors (nuclear hormone receptor family) that regulate gene expression of enzymes associated with lipid homeostasis, including fatty acid degradation (Wahli *et al.*, 1996). In the mean time, in liver, degradation of fatty acids including LTB_4 and detoxification of various xenobiotics are achieved by the ω- and β-oxidation pathways. ω-Oxidation is the first step of catabolism of fatty acids and mediated by cytochrome P450 4 family. Hence, regulation study of human liver cytochrome P450 4F2 gene, especially in the light of LTB_4 catabolism, is crucial to understand the fatty acid catabolism. In connection with PPARα-RXR heterodimer, expression study of cytochrome P450 4F2 will be helpful to get the insights of seemingly different disorders such as obesity, hyperlipidaemia and cardiovascular disease, and inflammatory conditions like rheumatoid arthritis, lupus and psoriasis (Wahli *et al.*, 1996). Furthermore, hypolidaemic drugs such as clofibrate and its structural analogues (Cohen and Grasso, 1981), phathalate and adipidate plasticizer, such as di(2-ethylhexyl) phthalate (DEHP) and di(2-ethylhexyl)adipate (Reddy and Lalwani, 1983) and a number of chlorinated hydrocarbons, such as trichloroethylene and perchloroethylene (Goldsworthy and Popp, 1987), branched-chained alkanes, such as 2,2,4-trimethylpentane (Elcombe *et al.*, 1987), phenoxyacetic acid and other classes of herbicides, steroids, food flavors and natural products are known to cause peroxisome proliferation and liver tumors in rodents. This is the reason that these chemicals are called peroxisome proliferators (PPs). But there are marked species differences in response to them; certain species, e. g., guinea pigs and monkeys, are relatively nonresponsive or resistant to the induction of hepatic peroxisomes. Analysis of PPARα knockout(-/-) mice indicates that the effects of PPs are mediated by PPARα (Gonzalez *et al.*, 1995). Even though hPPARα gene is cloned from human (Gonzalez *et al.*, 1993), there is no report that some of the human fatty acid metabolizing genes are induced by the interaction with hPPARα and human peroxisome is proliferated after clofibrate has been administrated for a long time (Sundberg *et al.*, 1995). Probably, human CYP 4F2 could be the candidate for analysis of species difference in response to PPs. Therefore, these future results (regulation study of CYP 4F2 gene) would also help in the safety evaluation of these chemicals to human beings.

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