Complementary DNA Sequence of 3-Methylcholanthrene-Inducible P-450 from the Rat Lung

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KIKUCHI, H., SAGAMI, I., FUJII, H., OHMACHI, T. and WATANABE, M. Complementary DNA Sequence of 3-Methylcholanthrene-Inducible P-450 from the Rat Lung. Tohoku J. Exp. Med., 1990, 160 (4), 323-332 — Cytochrome P-450MC was induced in pulmonary microsomes of 3-methylcholanthrene-treated rats and also at low level in that of isosafrole-treated rats. Cytochrome P-450d was not detected in the lungs of 3-methylcholanthrene- or isosafrole-treated rats by the method of Western blot analysis with a polyclonal antibody raised against cytochrome P-450c which is equally effective against P-450d, nor by the method of Northern hybridization probed with pcP450mc3 (P-450d probe). Complementary DNA of P-450MC was isolated from rat pulmonary cDNA library and the nucleotide sequence of pulmonary cDNA was compared with that of hepatic P-450c cDNA reported by Yabusaki et al. There was no gross change in nucleotide sequences of cDNA between pulmonary P-450MC and hepatic P-450c.

Cytochrome P-450s metabolize various exogenous and endogenous compounds (Nebert and Gonzalez 1987). Many P-450 isozyme types have been demonstrated to have the different substrate specificity and tissue distribution. Rat hepatic MC-inducible P-450 is involved in the hydroxylation of arylhydrocarbon carcinogens, including benzo(a)pyrene (Thakker et al. 1977).

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The abbreviations used are: MC, 3-methylcholanthrene; ISF, isosafrole; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl-0.015 M sodium citrate (pH 7.0); pfu, plaque forming unit; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; P-450c, hepatic cytochrome P-450 (low spin type) of rats treated with MC; P-450d, hepatic cytochrome P-450 (high spin type) of rats treated with MC; An attempt to standardize the nomenclature of diverse P-450 isozymes has been made (Nebert et al. 1987). P-450c and P-450d are termed P450IA1 and P450IA2, respectively. P-450MC denotes pulmonary cytochrome P-450 of rats treated with MC.

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Since the lung is a portal of entry for various environmental chemicals which are metabolized by cytochrome P-450, studies on pulmonary P-450 are particularly important. Thus, a number of researchers have studied the pulmonary P-450. There are some conflicting points among the results of their studies. Sagami and Watanabe (1983) suggested the presence of a form of rat pulmonary P-450MC different from hepatic P-450c. But Robinson et al. (1986) showed that the rat pulmonary P-450MC is structurally identical to the hepatic P-450c on the basis of several criteria. Degawa et al. (1987) and Goldstein and Linko (1984) demonstrated the absence of P-450d in the pulmonary tissue of MC-treated rats by the method of Western blot analysis. But Kimura et al. (1986) showed the induction of P-450 (equivalent to rat P-450d) in the pulmonary tissue of MC-treated mice by the method of Northern hybridization.

In this report, we compared the cDNA sequence of rat pulmonary P-450MC with that of hepatic P-450c, and showed the absence of P-450d-mRNA in the pulmonary tissue of MC-treated rats by the method of Northern hybridization.

**Materials and Methods**

**Preparation of microsomes**

Male Buffalo rats weighing 200-250 g were intraperitoneally treated with MC (25 mg/kg body weight) daily for 3 days or with ISF (150 mg/kg body weight) daily for 3 days. Liver tissue was homogenized in a 10 mM potassium phosphate buffer (pH 7.25) containing 150 mM KCl and 1 mM EDTA in a Potter-Elvehjem type homogenizer. Homogenization of pulmonary tissue was carried out with an Ultra-Turrax homogenizer (Tamura et al. 1981). The homogenate was centrifuged at 9,000 × g for 20 min. The supernatant fraction was centrifuged at 105,000 × g for 60 min. The pellet was suspended in a 100 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol, and stored at −80°C under N₂ gas until use.

**Preparation of anti-P-450c serum**

Rat hepatic P-450c was purified according to a method previously reported (Sagami and Watanabe 1983). The specific content of purified P-450c was 17.1 nmol/mg protein. The purified P-450c (0.27-1.07 mg) was mixed with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI, USA), and was injected intradermally 5 times into female New Zealand White rabbits to obtain the anti-P-450c serum.

**Western blotting**

Microsomal proteins were solubilized in SDS, resolved in 7.5% polyacrylamide gel as described in a previous report (Ohmachi et al. 1985) and then transferred to a nitrocellulose sheet (Thomas et al. 1984). Antigenic compounds reactive with anti-P-450c serum were immunostained with anti-rabbit IgG (Fc) alkaline phosphatase conjugate, using nitro blue tetrazolium and 5-bromo-4-chloro-3-indoly1 phosphate as the color development substrates. The reaction procedures were followed according to instructions recommended by the supplier (Promega Biotech, Madison, WI, USA).

**Preparation of poly(A)⁺ RNA**

The rats were treated with MC or ISF, and were sacrificed 16 hr later by decapitation. Tissues were quickly removed and frozen immediately at −80°C until use. RNA was prepared by the method of Fraizer et al. (1983). Poly(A)⁺RNA was isolated from total
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RNA by affinity chromatography with oligo(dT)-cellulose (Pharmacia, Uppsala, Sweden) as described by Maniatis et al. (1982).

Northern blot hybridization

Poly(A)^+ RNA was treated with 0.75 M glyoxal, 50% dimethylsulfoxide, and resolved on 1.1% agarose gel. After electrophoresis, RNA was transferred to a nitrocellulose sheet (Schleicher and Schuell GmbH, Dassel, FRG) by the capillary method (Maniatis et al. 1982). Prehybridization was performed in a prehybridization mixture [0.2% ficol, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 1% SDS, 50 mM Tris-HCl (pH 7.5), 1 M NaCl, 10% dextran sulfate and 100 μg/ml yeast RNA] at 65°C for 18 hr. The labeled probe was mixed with 0.3 ml of yeast RNA (10 mg/ml) and was denatured at 100°C for 5 min. The denatured probe was added to the prehybridization mixture and incubated for 18 hr. The hybridized filter was washed three times with 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 1% SDS at 65°C for 30 min. The washed filter was exposed at -80°C with an intensifying screen for 18 hr.

Construction of λgt11 cDNA library

Double-stranded-cDNA was synthesized using 2 μg of poly(A)^+ RNA from MC-treated rat pulmonary tissue, according to the procedure described by Watson and Jackson (1985), and was treated with T7 DNA polymerase to fill in any ragged end on the cDNA. The double-stranded-cDNA was then methylated with 4 units of EcoRI methylase (New England Biolabs, Beverly, MA, USA), and was ligated with 0.5 μg of EcoRI linker (Pharmacia P-L Biochemicals, Milwaukee, WI, USA). The ligated cDNA was digested with 75 units of EcoRI and was fractionated on a column (0.2 × 25 cm) of Sephacryl S-1000 (Pharmacia P-L Biochemicals, Milwaukee, WI, USA). Fractions containing DNA longer than 500 bp were pooled, and the recovered DNA was ligated to the EcoRI cleaved λgt11 DNA (Promega Corporation, Madison, WI, USA). The ligated DNA was packed in phage particles using an in vitro packaging system (Stratagene, San Diego, CA, USA). The pulmonary cDNA library contained 6 × 10^6 pfu independent clones, and the mean length of inserted cDNA was 1.01 ± 0.53 kb (n = 8).

Other materials and methods

The probe of mouse P450 (pPMI-2-16) and rat P-450d (pcP450mc3) were kindly provided by Dr. M. Negishi (NIEHS, NIH, USA) and Dr. Y. Fujii-Kuriyama (Tohoku University, Japan), respectively.

The DNA probe was labeled by the multi-priming method (Feinberg and Vogelstein 1983). Nucleotide sequences of DNA were determined by the dideoxy-termination method (Sanger et al. 1977).

RESULTS AND DISCUSSION

Tissue specificity of P-450 induction

Sagami and Watanabe (1983) purified P-450 from MC-treated rat lungs and found an immunological cross-reactivity to hepatic P-450c. The antibody against rat hepatic P-450c made a precipitation line with purified pulmonary P-450MC on the agar gel when subjected to Ouchterlony double diffusion analysis. Using the immunological properties of P-450, MC-inducible P-450 species in pulmonary tissue were determined. On the lane of pulmonary microsomes of Fig. 1 (lane 3), there is a single band that an equivalent molecular weight to hepatic P-450c (M.W. 56,000 Da). Fig. 1 also shows that P-450d was not induced either
Measurement of the amount of P-450 mRNA

Fig. 2 shows the results of Northern blot hybridization. In the pulmonary tissue of MC-treated rats, the mRNA of P-450MC was induced. On lane 3 of Fig. 2, there is a single band of P-450MC (2.9 kb) probed with L-6 which was isolated from pulmonary cDNA library (see below). However, the mRNA of P-450d (2.1 kb) was not detected in the pulmonary mRNA of MC-treated rats (on lane 9) using P-450d-cDNA (pcP450mc3) as a probe. Only a faint band of cross-hybridization to P-450c (2.9 kb) can be seen on lane 9. Though ISF (preferable inducer of P-450d) had been administered to the rats, the mRNA of P-450d was not detected in the pulmonary tissue (Fig. 2, lane 8). Pasco et al. (1988) also reported that P-450d mRNA was not detected in the lung, even in the animals treated with high dose of TCDD. However, Kimura et al. (1986) demonstrated the induction of P_{3}450-mRNA in the lungs of TCDD-treated mice by slot blot analysis probing with a 3'-specific portion of P_{3}450 cDNA. TCDD is also known as a strong inducer of P-450c and P-450d in hepatic tissue. The discrepancy between the result of our study and that of Kimura et al. may be attributed to the difference between
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Since P-450c and P-450d are 75% homologous at the nucleotide level, these probes cross-hybridize with each other under these hybridization conditions. When the Northern blot filter was probed with P-450d (the right side panel of Fig. 2, lane 7-12), there were strong signals of P-450d (2.1 kb) on the lane of MC-treated rat liver, compared to P-450c (2.9 kb). Hence there is much more mRNA of P-450d than of P-450c in the liver. Recently, Pasco et al. (1988) reported the accumulation of P-450d mRNA in the livers of MC-treated rats, due to the stability of this mRNA. But the result of Western blot analysis (Fig. 1, lane 6) shows almost equal signal-intensity of P-450d and P-450c. Steward et al. (1985) reported that ISF stabilized P-450d protein and maintained the rate of synthesis of P-450c protein in hepatocyte culture, though the induced levels of P-450c and P-450d mRNA by the ISF-treatment were not so high compared to those by the MC-treatment. Thus, there must also occur translational control as described by Pasco et al. (1988).

Isolation of P-450 cDNA

Taking the advantage of the immunological cross-reactivity described above,
cDNA library was screened by the immunological detection of fusion proteins with anti-P-450c serum. Out of $2 \times 10^5$ clones of λgt11 cDNA library, four positive clones were isolated, but only one clone (L-6) hybridized with the probe of mouse P₃₅₀ (pPMI-2-16) (data not shown). The nucleotide length of L-6 (1.5 kb) is too short to cover the whole mRNA (2.9 kb) of pulmonary P-450MC (Fig. 2). Therefore, the 5'-end of the L-6 DNA fragment (from 5'-end to Pvu II site) was isolated and used as the labeled probe for the screening of the same library. Ten positive clones were isolated from $10^6$ clones, and a partial restriction enzyme map was determined as shown in Fig. 3. The positive clones were divided into two classes by the method of restriction enzyme mapping. One group, including #2, has a pattern of Pst I/Pvu II sites identical to the cDNA of hepatic P-450c (Yabusaki et al. 1984). The other two clones (#19 and #24) showed different patterns.

Comparison of the pulmonary P-450MC with the hepatic P-450c in DNA sequence

Since clone #2 had the longest nucleotide length (2.6 kb), clone #2 was sequenced and compared with the cDNA nucleotide sequence of hepatic P-450c (Fig. 4). As the N-terminal amino acid of purified rat hepatic P-450c was reported to be proline by Yabusaki et al. (1984), the numbering of amino acid sequence of pulmonary P-450MC was started from proline (nucleotide 86-88). The nucleotide sequence of pulmonary P-450MC is almost identical to that of hepatic P-450c (Yabusaki et al. 1984). In the protein coding region, there is only one amino acid change from the hepatic one. Nucleic acid 241 (A in clone #2) was reported to be a G in the sequence of clone pAU157 (nucleotide 261) of Yabusaki et al. (1984). This base change results in an amino acid change from isoleucine
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Fig. 4. Nucleotide sequence of pulmonary P-450MC (clone #2). The sequence is numbered, based on the most upstream nucleotide of the pulmonary P-450MC cDNA. Differences of nucleotide (nucleotide position 134, 241, 1514, 1681, 1952, 2286 and 2288), insertion (nucleotide position 1902) and three bases-deletion (nucleotide position 72) from hepatic P-450c (Yabusaki et al. 1984) are designated by underlining. Only the different nucleotides of the hepatic P-450c sequence reported by Yabusaki et al. (1984) are indicated above the pulmonary one. The asterisks (***) show the stop codon (TAG).
to methionine in pAU157. But the genomic DNA sequence of rat P-450c showed the same sequence as that of Fig. 4 (Sogawa et al. 1984; Hines et al. 1985). Within the 5'-noncoding region of the gene, there is a three-base deletion (CAG) in #2. This site is just the junction between exon 1 and exon 2. But the end of intron 1 contains the three-base repetition of CAG, which may cause a mis-splicing between exons 1 and 2. Two additional differences were noted in the protein coding region where the C at position 134 and the T at position 1514 was reported as a T and as a C in the sequence of Yabusaki et al. (1984), respectively. But these changes do not alter the translation product. All other discrepancies lie within the 3'-noncoding region of the gene (nucleotide 1681, 1902, 1952, 2286 and 2288). These discrepancies may be attributable to the strain difference of the rats (Buffalo against Sprague-Dawley) or polymorphism among individual rats. The result of genomic Southern hybridization (Sogawa et al. 1984) suggested that the gene number of P-450c is one per haploid genome of the rat. Therefore, pulmonary P-450MC must be transcribed from the same structural gene as the hepatic one.

Clones #19 and #24 shown to contain the introns

The restriction enzyme maps of clone #19 and #24 show patterns different from that of hepatic P-450c. The sequencing of #19 and #24 revealed that these clones contained the introns. Clone #19 has intron 3, 5 and 6. Clone #24 has intron 4 (Fig. 5). If the triplet codon is aligned from the frame of exon 5 into intron 5, the stop codon TGA appears within intron 5. Since the total RNA was prepared from the whole homogenate of frozen tissue, clone #19 may be immature mRNA of P-450c.

In the case of clone #24, the clone has a poly(A)-tail in the middle of intron 4. There is a poly(A) addition signal (AATAAA) beside the stop codon (TAA) in intron 4. Therefore, clone #24 is a case of heterogeneity of mRNA due to a

![Fig. 5. Gene structures of clones #2, #19 and #24. Clones #19 and #24 were sequenced. The nucleotide sequences were compared and were aligned to clone #2. I-3, I-4, I-5, I-6 show the intron-3, 4, 5, 6, respectively.](image-url)
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different site of a poly(A) addition signal. The protein of this clone is too short to have any enzyme activity, because the C-terminal part of P-450 protein has the heme binding domain (exon 7).

In either case, clones #19 and #24 would not be translated into active proteins. Since eighth out of ten positive clones show the hepatic P-450c type sequence, and Fig. 2 shows the nucleotide length of pulmonary P-450MC mRNA to be the same as that of the hepatic one, there is no evidence of tissue specific differential splicing of P-450 mRNA.

From these results, we conclude that the pulmonary mRNA of P-450MC is transcribed from a single gene, and is spliced in the same manner as the hepatic P-450c. Therefore, the difference between P-450 characteristics in the liver and the lung in the protein studies (Sagami and Watanabe 1983) may be attributed to post-translational events, such as protein processings, phosphorylations (Tang and Chiang 1986) or glycosylations (Haugen and Coon 1976; Imai et al. 1980).

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References


