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FLUORESCENCE *IN SITU* HYBRIDIZATION ANALYSIS OF CHROMOSOMAL LOCALIZATION OF THREE HUMAN CYTOCHROME P450 2C GENES (CYP2C8, 2C9, AND 2C10†) AT 10q24.1

Kiyoshi INOUE,[1,*] Johji INAZAWA,[2] Yasuhiko SUZUKI,[1]
Tsutomu SHIMADA,[1] Hiroshi YAMAZAKI,[1] F. Peter GUENGERICH,[3]
and Tatsuo ABE[2]

[1]*Osaka Prefectural Institute of Public Health, Nakamichi, Higashinari-ku, Osaka 537, Japan*
[2]*Department of Hygiene, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602, Japan*
[3]*Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, TN 37232, U.S.A.*

Summary Chromosomal localization of three human cytochrome P450 genes belonging to the CYP2C subfamily (CYP2C8, 2C9, and 2C10) was identified by fluorescence *in situ* hybridization (FISH). An original MP-8 clone was used as a DNA probe for the assignment of the CYP2C10 gene, while two cDNA probes, a 1.37 kb fragment of CYP2C8 and a 1.19 kb fragment of CYP2C9, were obtained after amplifying the predicted fragments (MP-20 and MP-4 clones, respectively) by polymerase chain reaction using a single human liver cDNA library. The results showed that three human CYP2C8, 2C9, and 2C10 cDNAs were located at the same subchromosomal region, 10q24.1.

Key Words fluorescence *in situ* hybridization, CYP2C subfamily, chromosome 10, chromosomal localization, polymerase chain reaction

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*To whom correspondence should be addressed.

†The coding sequences of the genes termed CYP2C9 and CYP2C10 differ in only two amino acids, 358 and 417 (Ged *et al.*, 1988; Srivastava *et al.*, 1991). The CYP2C10 sequence corresponds to the first cDNA we isolated from this family (Umbenhauer *et al.*, 1987). We reported that the two cDNAs now termed 2C9 (MP-4) and 2C10 (MP-8) differed considerably in their 3′ non-coding sequences, and oligonucleotide probes were used to identify both groups of sequences in the mRNA of a single liver sample (Ged *et al.*, 1988). It is conceivable that the existence of the two cDNA clones (within an expression library generated from a single individual) is an artifact of the library construction, or that the sequences recognized by the probes are parts of other genes. Nevertheless, P450 2C9 and P450 2C10 are treated here as the products of individual genes. When proteins purified from the liver are considered here, they are designated P450 2C9/10 because no amino acid sequence analysis was done in the regions where differences occur (Ged *et al.*, 1988; Srivastava *et al.*, 1991).

INTRODUCTION

The human liver microsomal cytochrome P450 (P450) enzymes encoded by the CYP2C subfamily are known to catalyze the oxidation of mephenytoin and to exhibit genetic polymorphism of metabolism in humans (Wedlund *et al.*, 1984; Kupfer and Preisig, 1984). Shimada *et al.* (1986) first isolated two similar forms of human liver P450 enzymes responsible for (*S*)-mephenytoin 4-hydroxylation, termed P-450_{MP-1} and P-450_{MP-2}. Furthermore, Umbenhauer *et al.* (1987) and Ged *et al.* (1988) have isolated four cDNA clones that contained nearly full-length DNA sequences, from a bacteriophage λgt11 library prepared from a single human liver using anti-$P450_{MP-1}$ as a probe, and designated them as MP-12 (CYP2C8), MP-20 (CYP2C8), MP-4 (CYP2C9), and MP-8 (CYP2C10). Recently, CYP2C9 and CYP2C10 enzymes have been reported to catalyze tolbutamide methyl hydroxylation and hexobarbital 3′-hydroxylation (Brian *et al.*, 1989; Srivastava *et al.*, 1991; Yasumori *et al.*, 1991), and CYP2C8 enzyme is found to be one of the benzo-(*a*)pyrene 3-hydroxylases (Yun *et al.*, 1992). Although the human P450 gene(s) encoding the CYP2C subfamily was mapped to 10q24.1-10q24.3 by a panel of human-rodent somatic cell hybrids and isotopic *in situ* hybridization (Okino *et al.*, 1987; Riddell *et al.*, 1987; Meehan *et al.*, 1988), the exact localization of this multigene family remains unclear.

In the present study, we mapped the human CYP2C subfamily genes (CYP2C8, 2C9, and 2C10) by fluorescence *in situ* hybridization (FISH) combined with replication R-bands on prometaphase chromosomes (Inoue *et al.*, 1992). An original MP-8 clone was used as a DNA probe to assign the CYP2C10 gene. A 1.37-kb and a 1.19-kb fragment of CYP2C8 and 2C9 respectively, were amplified separately from the liver cDNA library by the polymerase chain reaction (PCR) and subcloned into the pBluescriptIIKS(+) vector. The amplified products were confirmed by DNA sequencing, and used to assign the chromosomal localization by FISH.

MATERIALS AND METHODS

Synthesis of oligonucleotide primers and PCR (CYP2C8 and 2C9). In order to obtain the CYP2C cDNA probes, two CYP2C cDNAs corresponding to clones MP-20 (CYP2C8) and MP-4 (CYP2C9) were amplified from a liver cDNA library by PCR. Oligonucleotide (20-mer) primers were chemically synthesized on an Applied Biosystems 380A DNA synthesizer (Foster City, CA) using cyanoethyl phosphoramidite chemistry according to the published CYP2C8 and CYP2C9 cDNA sequences (Brian *et al.*, 1989; Ged *et al.*, 1988). After detritylation and alkaline deprotection, the crude material was used for all applications without further purification. Primer pairs from CYP2C8 cDNA included: 5′-TCGAAGC-

TTTTTTCACTCTGGAGACAGAG-3′ and 5′-TCCCTGCAGAGTGACCTGAA-CAACTCTCC-3′, positions 19 to 38 of the CYP2C8 cDNA plus a *Hin*dIII cleavage site (underlined) and positions 1607 to 1588 plus a *Pst*I cleavage site (underlined), respectively. For the CYP2C9 cDNA, we used: 5′-GCAGTTAACATTTTGGGA-TGGGGAAGAGG-3′ and 5′-ACCGGTACCAATGTGAGATGACAGGTGAG-3′, positions 77 to 96 plus a *Hpa*I cleavage site (underlined) and positions 1263 to 1244 plus a *Kpn*I cleavage site (underlined). PCR (Saiki *et al.*, 1985) was performed with 200 ng DNA (liver cDNA library), 2.5 μl each of oligonucleotide primer (20 μM), 10 μl of 1 mM dNTPs, 5 μl of 10×PCR buffer and 1 U *Taq* DNA polymerase in a final volume of 50.4 μl (ATTO Zymoreactor AB-1800). The reaction was performed under the following conditions: denaturation at 92°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. After 35 cycles of amplification, the products underwent a final extension at 72°C for 5 min. A portion of each sample (1 μl) was analyzed by electrophoresis through a 1% agarose gel containing 1 μg/ml ethidium bromide.

Subcloning and sequencing of the PCR products (CYP2C8 and 2C9). According to the restriction sites of the primers, the amplified DNA fragments by PCR were digested with *Hin*dIII and *Pst*I in CYP2C8 and *Kpn*I and *Hpa*I in CYP2C9, respectively. The PCR products from CYP2C8 and CYP2C9 were ligated with the pBluescriptIIKS(+) vector and transformed into *Escherichia coli* strain XL-1 using a Gene Pulser apparatus for the electro-transformation *E. coli* (Bio-Rad). The transformants were selected on agar plates containing ampicillin (50 μg/ml) and X-Gal/IPTG. The 1.37 kb fragment of CYP2C8 was subcloned into the *Hin*d-III and *Pst*I sites of pBluescriptIIKS(+), and the 1.19 kb fragment of CYP2C9 was subcloned into the *Kpn*I and *Sma*I sites of pBluescriptIIKS(+). These fragments were sequenced using the AutoRead™ Sequencing kit according to the directions of the supplier (Pharmacia, Uppsala, Sweden).

Subcloning of CYP2C10. An M13 phage containing the original MP-8 cDNA described by Brian *et al.* (1989) was infected with *E. coli* JM103. The collected phage DNA was digested with *Eco*RI and the resulting fragment (1.6 kb) was subcloned into the plasmid vector pUC119 as described by Brian *et al.* (1989).

Chromosome preparation and fluorescence in situ hybridization. Metaphase chromosome spreads were obtained from normal human lymphocyte cultures and synchronized with thymidine as previously described (Viegas-Pequignot and Dutrillaux, 1978; Inoue *et al.*, 1992). R-banding was induced by treating cultures with 5-bromodeoxyuridine (BrdU) during the late S-phase. Slides were aged 1–2 days, baked at 65°C for at least 3 h, and kept at −20°C until use.

FISH was performed according to a method recently described for high-resolution mapping of single-copy small genes directly on banded chromosomes (Inazawa *et al.*, 1991; Lemieux *et al.*, 1992; Takahashi *et al.*, 1992; Inoue *et al.*, 1992). Briefly, chromosomes were denatured in 70% formamide in 2×SSC (1× SSC is 0.15 M sodium chloride/0.015 M sodium citrate, pH 7) at 70°C for 2 min

followed by dehydration in ethanol. The CYP2C8 cDNA probe (containing the 1.37-kb cDNA fragment cloned into pBluescriptIIKS(+)), the CYP2C9 cDNA probe (containing the 1.19-kb cDNA fragment cloned into pBluescriptIIKS(+)) and a CYP2C10 cDNA probe (1.6 kb) were labeled by nick translation using biotin-16-dUTP (Boehringer, Mannheim) for 1.5 h at 15°C. The biotinylated probes were denatured at 75°C for 10 min. The probe concentration on the slides was 500 ng in 10 μl of the hybridization mixture (50% formamide, 10% dextran sulfate, 2×SSC, and 10% bovine serum albumin). Hybridization proceeded for 16–18 h at 37°C. After being washed with 50% formamide/2×SSC, 2×SSC, and 1×SSC for 37°C for 15 min each, the slides were treated with 4% Block Ace™ (Dainippon Pharmaceutical, Osaka) at 42°C for 10 min to block fluorochrome background noise. We detected small single-copy genes (1.37-kb insert in CYP2C8 cDNA, 1.19-kb insert in CYP2C9 cDNA, and 1.6-kb insert in CYP2C10 cDNA), by amplification with avidin-conjugated fluorescein isothiocyanate (FITC-avidin) (Boehringer, Mannheim) and a biotinylated anti-avidin antibody (Vector, Burlingame, CA) (Pinkel *et al.*, 1986; Inoue *et al.*, 1992). Finally, the chromosomes were stained with propidium iodide (1 μg/ml, Sigma, St. Louis, MO). Slides were mounted with a fluorescence anti-fade solution containing 1% DABCO (1,4-diazabicyclo[2,2,2]octane, Sigma) and observed under a fluorescence microscope (filter combinations: B-2A, excitation 450–490 nm, barrier 520 nm; B-2E, excitation 450–490 nm, barrier 520–560 nm). The G-banding pattern on the same metaphase was delineated by means of a different filter combination (UV-2A, excitation 400–440 nm, barrier 470 nm). Photographs were taken using Fujichrome 400D film (800 ASA).

RESULTS AND DISCUSSION

CYP2C8 and CYP2C9 cDNAs (Ged *et al.*, 1988) from a human liver cDNA library were amplified by PCR. The primer pairs, positions 19 to 38 and 1607 to 1588 were selected to amplify a 1589 bp region of CYP2C8 and those from positions 77 to 96 and 1263 to 1244 of 1187 bp of CYP2C9 were also used. The product of CYP2C9 yielded fragments of the expected size. However, that of CYP2C8 was 1.37-kb long because of a *Pst*I site at position 1383. In order to confirm the CYP2C8 and CYP2C9 sequences in the amplified cDNA fragments, the fragments were subcloned into the vector pBluescriptIIKS(+). The nucleotide sequences obtained from the subclones showed complete identity with the corresponding published sequences (Ged *et al.*, 1988).

We performed FISH mapping using these biotin-labeled probes to assign the human CYP2C gene location. The procedure allows the simultaneous identification of the G-banded chromosome pattern and hybridization signals on the same metaphase preparation by simply changing the filters (filter combination B-2A and UV-2A, Nikon). A detailed distribution analysis showed that the hybridization

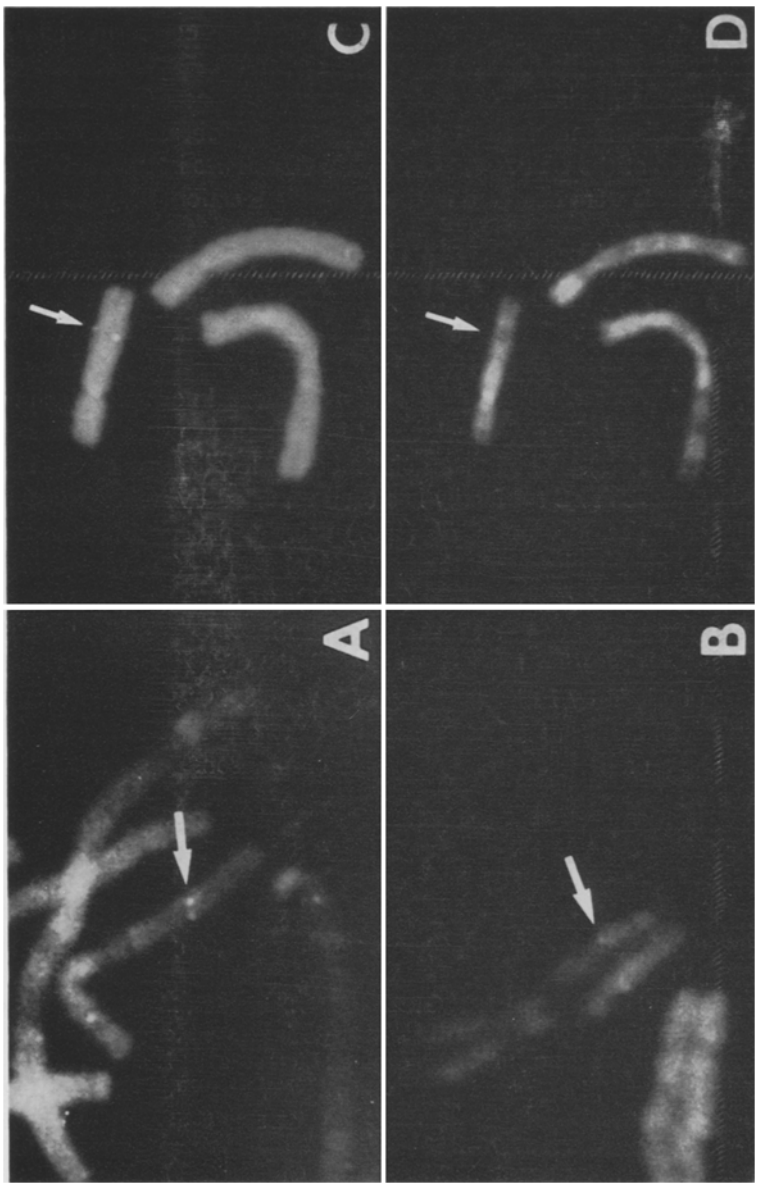

Fig. 1. Fluorescence *in situ* hybridization mapping of three human CYP2C cDNAs on chromosome 10q24.1 A, B: Partial metaphases with R-bands, observed with filter combination B-2A. A, CYP2C8 cDNA and B, CYP2C9 cDNA. C: Partial metaphases hybridized with CYP2C10 cDNA. D: G-banding of the same chromosomes shown in C. Fluorescent signals on chromosome 10q24.1 are indicated by white arrows.

site in the three probes was the same: 10q24 (Fig. 1). Among 100 metaphases examined with the CYP2C8 probe, 12 (12%) exhibited double fluorescent signals on only one of the chromatids. One hundred metaphases were also examined with CYP2C9 and CYP2C10 cDNA probes. Five (5%) and two (2%), respectively, of the metaphases exhibited complete symmetrical double fluorescent signals on both chromatids. Sixteen cells for CYP2C9 and 17 for CYP2C10 showed either single or double fluorescent signals on each chromatid. None of the other chromosomes showed consistent fluorescent signals in these probes. We further examined the localization of the CYP2C8, 2C9, and 2C10 cDNAs in the 10q24 region. For CYP2C8, the signals were observed in the following location; 8.7% at 10q23.3, 82.6% at 10q24.1, and 8.7% at 10q24.2, respectively. CYP2C9 signals were observed in 86.0% at 10q24.1, 6.0% at 10q24.2, and 8.0% at 10q24.3. CYP2C10 signals were found in 4.5% at 10q23.3, 72.2% at 10q24.1, 18.2% at 10q24.2, and 4.5% at 10q24.3, respectively. Thus, the localization of the three CYP2C cDNA examined was on human chromosome 10q24.1.

At present, 11 families comprising 19 subfamilies are known in the human cytochrome P450 gene superfamily, of which 17 have been mapped in the human genome (Nelson *et al.*, 1993). The human chromosome localization of the human P450 gene encoding the CYP2C subfamily has been reported by investigators from three different laboratories. Okino *et al.* (1987) using a panel of human-rodent somatic cell hybrids, have assigned the human P450 1 cDNA (designated Hpl-1), which is nealy identical with clone MP-20 (CYP2C8), to human chromosome 10. Riddell *et al.* (1987) and Meehan *et al.* (1988) using both somatic cell hybridization and isotopic *in situ* hybridization have also assigned the CYP2C9 to 10q24.1-10q-24.3. Our results obtained by FISH mapping of CYP2C8, 2C9, and 2C10 cDNAs confirmed the previous studies, further suggest that the genes for members of the CYP2C subfamily lie on a limited region of human chromosome 10 at q24.1.

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