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# Genetic Polymorphism in the 5'-Flanking Region of Human *CYP1A2* Gene: Effect on the CYP1A2 Inducibility in Humans[1]

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**A genetic polymorphism was identified in the 5'-flanking region of human *CYP1A2* gene, and its effect on the transcriptional activation of the *CYP1A2* gene was investigated. Nucleotide sequence analysis revealed the existence of a point mutation from guanine (wild type) to adenine (mutated type) at position −2964 in the gene. This point mutation was detected by a polymerase chain reaction-restriction fragment length polymorphism method using *Dde*I or *Bsl*I restriction enzyme, and was proven to be genetically inherited. Allele frequency in 116 Japanese subjects showed 0.77 and 0.23 for the wild and mutated types of allele, respectively. The point mutation caused a significant decrease of CYP1A2 activity measured by the rate of caffeine 3-demethylation in Japanese smokers ($p<0.05$). Gel retardation analysis showed the existence of protein bound to the polymorphic locus. These results suggest that this polymorphism is a causal factor of decreased CYP1A2 inducibility.**

**Key words: caffeine metabolism, cigarette smoking, induction, PCR-RFLP.**

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CYP enzymes play an important role in the metabolism of endogenous and exogenous substrates, including drugs and various chemical carcinogens. CYP1A2 is responsible for the *O*-deethylation of phenacetin, the 3-demethylation of caffeine, and the metabolic activation of various carcinogens such as 2-aminofluorene, Trp-P-2, and PhIP (*1-3*). Several studies on the CYP1A2-specific metabolism of caffeine *in vivo* (*4*) and the microsomal metabolism of phenacetin (*5*) or caffeine (*6*), and the immunodetection of human CYP1A2 protein (*3, 5, 7*) showed that this enzyme was expressed in the human liver at different levels in different individuals. In fact, large interindividual differences in the contents of CYP1A2 mRNA (*8*) and the capacity to metabolize carcinogens such as 2-acetylaminofluorene, 2-naphthylamine, and 4-aminobiphenyl (*9-11*) have been reported in humans. These observations suggest that the interindividual variation can be ascribed to differences in transcriptional regulation of this enzyme.

Halogenated hydrocarbons, such as TCDD, and PAHs are potent inducers of CYPs in the *CYP1* gene subfamily (*12*). The 3MC-responsive region in the human *CYP1A2* gene is located as far as about 2.9 kb upstream (*13*) as analyzed by a transient transfection experiment. Recently, positive and negative regulatory *cis*-elements were reported to exist in the human *CYP1A2* gene (from −2352 base to −2094 base) (*14*) in breast carcinoma MCF-7 cells.

In the present study, we analyzed the nucleotide sequences of the 5'-flanking region of human *CYP1A2* gene in Japanese subjects whose CYP1A2 activity had been estimated by determination of caffeine metabolism. We found a genetic polymorphism in the 5'-flanking region of the CYP1A2 gene, which resulted in decreased induction levels of CYP1A2.

## MATERIALS AND METHODS

*Materials*—Taq DNA polymerase and T4 DNA polymerase were purchased from Takara (Kyoto), and T4 polynucleotide kinase and restriction enzymes were purchased from Toyobo (Osaka). Sequencing kit was obtained from United States Biochemicals (Cleveland, OH). [$\gamma$-$^{32}$P]dATP (5,000 Ci/mmol), [$\alpha$-$^{35}$S]dATP (1,000 Ci/mmol), and a random primer labeling kit were from Amersham (Aylesbury, UK). Other chemicals were of the highest grade commercially available.

*Analyses of Caffeine Metabolism in Humans*—One hundred and sixteen healthy unrelated Japanese subjects aged 18-66 years (32.2±9.9 years) were recruited from

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Abbreviations: CYP, cytochrome P450; EM, extensive metabolizer; 3MC, 3-methylcholanthrene; MR, molar ratio; PAH, polycyclic aromatic hydrocarbon; PCR, polymerase chain reaction; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; PM, poor metabolizer; RFLP, restriction fragment length polymorphism; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; 17X, 1,7-dimethylxanthine; Trp-P-2, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]-indole; 17U, 1,7-dimethyluric acid; 137X, 1,3,7-trimethylxanthine.

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Otsuka Assay Laboratories (Otsuka Pharmaceutical) and Faculty of Pharmaceutical Sciences, Hokkaido University. Their informed consent was obtained. The subjects were composed of 50 smokers (41 males and 9 females) and 66 non-smokers (21 males and 45 females). Only volunteers who reported no history of liver and kidney abnormalities or who were not taking medications that might have affected caffeine metabolism were selected as participants. Subjects refrained from consuming methylxanthine-containing foods and beverages from midnight until finishing the test. At 8 a.m. or later in the morning of the test, the subjects received a 9-ounce cup of coffee prepared from 3.6 g of instant coffee containing 114 mg of caffeine, voided 4 h after consuming the coffee, and provided a 1-h urine specimen at the end of the 5th hour following dosing. The urine specimens were immediately frozen, then stored at −20°C until analysis. Caffeine and its metabolites were extracted from the urine samples according to Butler *et al.* (*4*) and analyzed by HPLC using a program that allowed the separation of caffeine and all of its metabolites from each other and other urinary constituents as described previously by Nakajima *et al.* (*15*). The urinary molar ratio of (17U+17X)/137X was employed as the index of a CYP1A2 activity, because this ratio reflects caffeine 3-demethylation activity in this caffeine phenotyping procedure, which was investigated in detail by Butler *et al.* (*4*) and Nakajima *et al.* (*15*).

*Preparation of Genomic DNA and Oligonucleotide*—Peripheral blood samples (1 ml) were obtained from individuals. Genomic DNA was extracted from peripheral lymphocytes with phenol-chloroform followed by ethanol precipitation (*16*). Oligonucleotides for polymerase chain reaction (PCR) and for gel retardation analysis were synthesized with a DNA synthesizer (Model 381A, Applied Biosystems, Foster City, CA).

*PCR and Sequence Analysis of Amplified DNA Fragments*—Genomic DNA samples (1 $\mu$g) were added to the PCR mixtures consisting of 20 mM Tris-HCl buffer (pH 8.4), 25 mM KCl, 0.05% Tween 20, 0.1 mg·ml$^{-1}$ gelatin, 2 mM $MgCl_2$, 0.4 $\mu$M oligonucleotide primers, and 250 $\mu$M dNTPs. Thirty cycles of amplification were performed using a programmable heat block (Perkin Elmer, Norwalk, CT) under the following conditions: denaturation at 94°C for 1.5 min, annealing at 56°C for 2 min, and extension at 72°C for 2 min. The amplified DNA fragments using R1 (5′-AGAAAGGAACACAACAGGGACT-3′) and R3 (5′-CAGGTCTCTTCACTGTAAAGTTA-3′) primers were blunt-ended with T4 DNA polymerase, phosphorylated with T4 polynucleotide kinase, and subcloned into the blunt-ended M13mp18 vector (*17*). The nucleotide sequences of the amplified DNA fragments were determined by the dideoxy chain-termination method (*18*) from selected fragments inserted into the M13mp18 vector.

*Detection of Genetic Polymorphisms in the 5′-Flanking Region*—The genotypes of the *CYP1A2* gene ascribed to the *Dde*I and *Bsl*I sites in the 5′-flanking region were identified by RFLPs of a PCR-amplified DNA fragment using R2 (5′-GCTACACATGATCGAGCTATAC-3′) and the R3 primers. PCR was performed under the conditions described above. The amplified DNA fragment including the polymorphic site was digested with *Dde*I or *Bsl*I, and subjected to electrophoresis in a 2% agarose gel.

*Gel Retardation Analysis*—Double-stranded 12 mer nucleotides, dDdeW (5′-GCCTCTCGGATT-3′/3′-CGGAGAGCCTAA) and dDdeM (5′-GCCTCTCAGATT-3′/3′-CGGAGAGTCTAA), were used as probes. These probes were kinated with a T4 polynucleotide kinase in the presence of [$\gamma$-$^{32}$P]dATP. Gel retardation experiments were carried out by incubating a labeled probe (5 fmol) with 8 $\mu$g of a nuclear extract protein prepared from human liver as described by Gorski *et al.* (*19*) in a buffer containing 20 mM Hepes (pH 7.9), 40 mM KCl, 0.5 mM DTT (dithiothreitol), 0.1 mM EDTA, 1 mM $MgCl_2$, 4% Ficoll, 2 $\mu$g poly (dI-dC) at room temperature for 15 min. The samples were loaded onto a native polyacrylamide gel, and electrophoresis was carried out in 1×EPB (6.7 mM Tris, 1 mM EDTA, 3.3 mM sodium acetate, pH 7.5) at 130 V for 4 h. The unlabeled double-stranded dDdeW or dDdeM was added to the incubation mixture as a competitor.

*Statistics*—The results are expressed as the mean±SD throughout the text. Statistical analysis was performed by ANOVA and Fisher's LSD method. The normality of distribution and the homogeneity of variances were checked by Kolmogorov-Smirnov test and Bartlett test, respectively. Differences were considered significant when $p < 0.05$.

## RESULTS

*Identification of Polymorphic Mutation in the 5′-Flanking Region of CYP1A2 Gene*—To investigate whether individual differences occurred in the nucleotide sequence of the *CYP1A2* gene, the sequence of the 5′-flanking region of the *CYP1A2* gene (from −2526 to −3172 base) was analyzed. Genomic DNAs were obtained from two poor metabolizers (PM), who showed the urinary MR [(17U+17X)/137X by the caffeine test] of 2.4 and 3.2, and one EM with MR of 13.1 among non-smokers, and from one PM (MR, 1.8) and one EM (MR, 25.1) among smokers. These subjects are typical PMs and EMs, respectively (*15*). Four differences in the nucleotide sequence of 5′-flanking region between the present data and the data of Quattrochi and Tukey (*13*) were found. As a result, replacement of guanine by adenine at position −2964 upstream from the transcriptional start site was found in two PMs of the five subjects (Fig. 1). The homozygous wild (guanine type), the homozygous mutant (adenine type), and the heterozygous mutant were tentatively termed as the w/w, m/m, and w/m type, respectively. As shown in Fig. 2, the w/w type genomic DNA having guanine at position −2964 generated a *Bsl*I restriction enzyme site, while the m/m type having adenine at the same position produced a *Dde*I restriction enzyme site. For genotyping of the mutation, PCR-amplification was carried out using the oligonucleotide primers indicated as the R2 and the R3 in Fig. 1. On *Dde*I digestion, the amplified DNA fragments from the w/w type gene gave only a single undigested band of 596 bases; the fragments from type m/m gave bands of 464 and 132 bases; type w/m gave three bands of 596, 464, and 132 bases (Fig. 3). On *Bsl*I digestion, the fragments from the type w/w gene gave bands of 343, 132, 93, and 28 bases; type m/m gave bands of 475, 93, and 28 bases; and type w/m gave five bands of 475, 343, 132, 93, and 28 bases (Fig. 3). These results were consistent with our sequence analyses shown in Fig. 1.

To confirm whether the polymorphic mutation was genetically inherited, a family study was performed in

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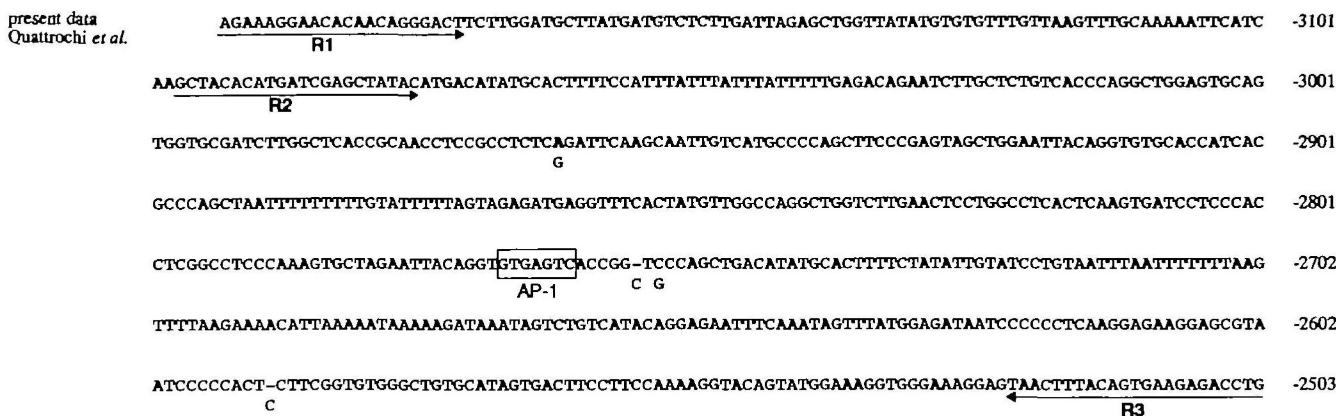


Fig. 1. **Nucleotide sequence of the 5′-flanking region (from −2503 to −3194) of the human *CYP1A2* gene.** The primers indicated as R1 and R3 in the figure were used for PCR, and the amplified DNA fragments were sequenced as described in "MATERIALS AND METHODS." Nucleotide numbering follows Quattrochi and Tukey (*13*). Only nucleotides that are not identical with the present data are shown for nucleotides reported by Quattrochi *et al.* (*23*). The sequence of the 5′-flanking region from +12 to −2578 was reported by Nakajima *et al.* (*15*). The AP-1 consensus sequence is boxed. The bold letter indicates the site of polymorphic point mutation from guanine (wild type) to adenine (mutated type) at position −2964 in the *CYP1A2* gene.

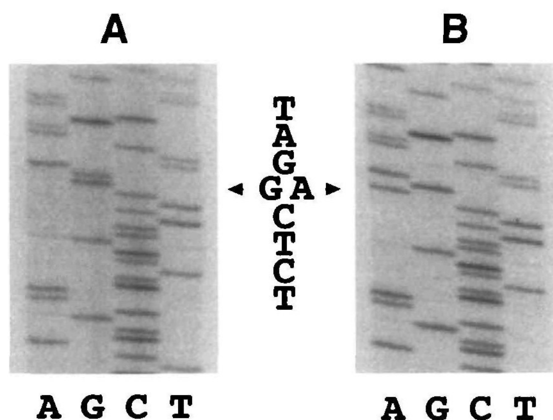


Fig. 2. **Nucleotide sequence of the 5′-flanking region around the polymorphic *Dde*I and *Bsl*I sites.** The genomic DNAs from subjects of the type w/w (A) and the type m/m (B) were prepared from peripheral blood samples. The primers indicated as R1 and R3 in Fig. 1 were used for PCR. The amplified DNA fragments were sequenced as described in "MATERIALS AND METHODS." The polymorphic site is shown by arrows.

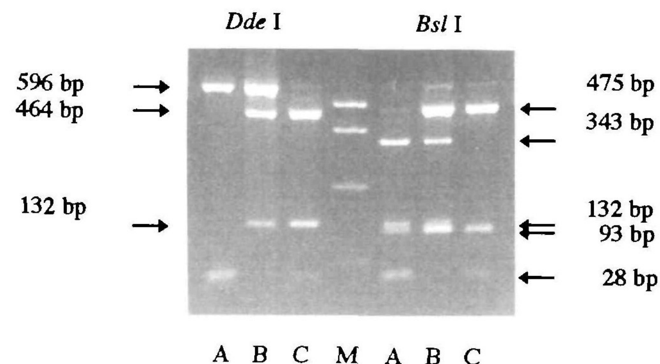


Fig. 3. **Diagnostic PCR-RFLPs for the genetic polymorphism in the 5′-flanking region of the human *CYP1A2* gene.** The primers indicated as R2 and R3 in Fig. 1 were used for PCR amplification. The PCR products were digested with *Dde*I or *Bsl*I, then subjected to agarose gel electrophoresis. Lanes A, B, C, and M are from type w/w, type w/m, type m/m DNAs and size markers, respectively.

eight pedigrees of Japanese (data not shown). No discrepancy was found with the assumption of genetic polymorphism at a single gene locus with autosomal codominant transmission. Allele frequencies in 116 healthy Japanese subjects were 0.77 and 0.23 for the wild and mutated allele, respectively.

As shown in Fig. 1, other point mutations which differed from the sequence reported by Quattrochi and Tukey (*13*) were found at the positions −2758, −2756, and −2592 in these five subjects.

*Relationship between Genetic Polymorphism of CYP1A2 Gene and Urinary MR of (17U+17X)/137X in In Vivo Caffeine Metabolism*—The urinary MR of (17U+17X)/137X was selected for the index of the CYP1A2 activity resulting from the caffeine 3-demethylation activity in a caffeine phenotyping test (*4*). The mean MR of (17U+17X)/137X was significantly higher ($p<0.005$) in smokers ($11.18\pm4.60$) than in non-smokers ($8.63\pm3.37$). The induction of CYP1A2 activity by smoking was consistent with our previous study (*15*). To investigate the effect of the mutation at −2964 in the *CYP1A2* gene on the inducibility of the CYP1A2 activity, a relationship between MR of the CYP1A2 phenotype and genotype was examined in 66 non-smokers and 50 smokers (Table I). In non-smokers, the mean MRs in w/w, w/m, and m/m types were $8.88\pm2.83$ ($n=38$), $8.19\pm4.24$ ($n=25$), and $9.13\pm1.49$ ($n=3$), respectively. No significant difference in the mean MR was observed between w/w type ($8.88\pm2.83$) and non-w/w type (w/m type and m/m type, $8.29\pm4.03$). In smokers, the mean MRs in w/w, w/m, and m/m types were $12.24\pm4.56$ ($n=31$), $9.77\pm4.01$ ($n=16$), and $7.79\pm5.11$ ($n=3$), respectively. The w/m type and m/m type each showed lower values of the mean MR than the w/w type, but due to the small number of subjects, the difference was not significant. The mean MR was significantly higher ($p<0.05$) in w/w type ($12.24\pm4.56$) than in non-w/w types ($9.46\pm4.17$) in smokers. In w/w type, the mean MR in smokers was significantly higher ($p<0.001$) than that in non-smokers. On the other hand, no significant difference



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TABLE I. **Relationship between genetic polymorphism at position −2964 in the *CYP1A2* gene and the urinary molar ratio of (17U+17X)/137X in *in vivo* caffeine metabolism.**

| Smoking status | Number of subjects | Genotype[a] | MR[b] | Comparison |
|---|---|---|---|---|
| Non-smokers | 38 | w/w | 8.88±2.83 | w/w vs. w/m+m/m: NS; non-smoker w/w vs. smoker w/w: $p<0.001$ |
| | 25+3 | w/m+m/m | 8.29±4.03 | non-smoker vs. smoker w/m+m/m: NS |
| | (25 | w/m | 8.19±4.24) | |
| | (3 | m/m | 9.13±1.49) | |
| | 66 | all | 8.63±3.37 | non-smoker all vs. smoker all: $p<0.005$ |
| Smokers | 31 | w/w | 12.24±4.56 | w/w vs. w/m+m/m: $p<0.05$ |
| | 16+3 | w/m+m/m | 9.46±4.17 | |
| | (16 | w/m | 9.77±4.01) | |
| | (3 | m/m | 7.79±5.11) | |
| | 50 | all | 11.18±4.60 | |

[a]The wild (w) and mutated (m) type have guanine and adenine at position −2964, respectively. [b]MR: Urinary molar ratio of (17U+17X)/137X in *in vivo* caffeine metabolism. NS: Not significant.

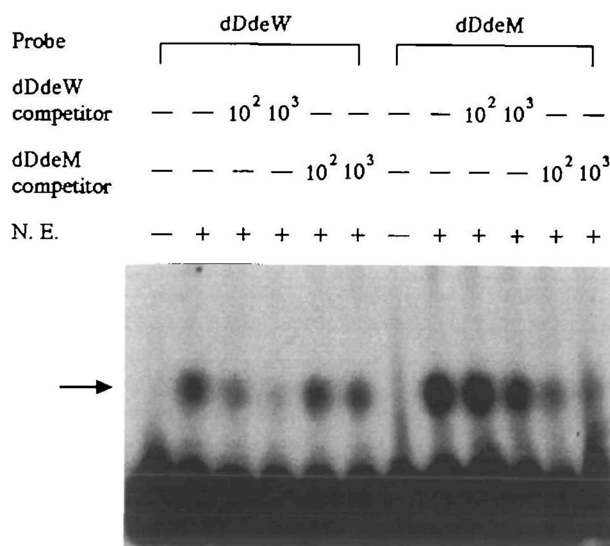


Fig. 4. **Gel retardation analysis with the polymorphic site of DNAs of the type w/w and the type m/m.** Double-stranded nucleotides (dDdeW and dDdeM) were labeled as described in "MATERIALS AND METHODS," and incubated with 8 μg of nuclear extract (N.E.; indicated by symbol +) from a human liver. Unlabeled competitor ($10^2$- or $10^3$-fold), dDdeW or dDdeM, was added to the reaction mixture as indicated on each lane.

was observed between smokers and non-smokers in non-w/w types. No relationship between the genotype and the sex or age of subjects was observed (data not shown).

*Gel Retardation Analysis of DNA Fragment Containing Polymorphic Site*—To investigate the effects of genetic polymorphism in the 5′-flanking region on the transcriptional regulation of the *CYP1A2* gene, we examined if a factor(s) able to bind to this region was present in the nuclear extract of human livers. The results of gel retardation analysis with the two types of DNA (type w/w and m/m) are shown in Fig. 4. As expected, a factor(s) was present which reacted with each DNA fragment. The formation of a complex with the dDdeW was inhibited by the presence of 100- and 1,000-fold molar excesses of the unlabeled dDdeW competitor, but not by unlabeled dDdeM competitor. On the other hand, the formation of the complex with the dDdeM was inhibited by the presence of excess amounts of the unlabeled dDdeM competitor, but not by the unlabeled dDdeW competitor (Fig. 4).

## DISCUSSION

Human CYP1A2 is known to be responsible for the metabolic activation of numerous promutagens and carcinogens. In the present study, we focused on the relationship between the genetic polymorphism of the *CYP1A2* gene and the inducibility of CYP1A2. In humans, large interindividual differences were previously reported in the activity of phenacetin *O*-deethylase (*20*), metabolism of carcinogens such as 2-acetylaminofluorene and 4-aminobiphenyl (*9, 11*), and the content of immunoreactive CYP1A2 protein (*3, 7*). When a polymorphic locus exists in the regulatory region of a gene, the difference can be explained by a change in the expression level of mRNA. This type of polymorphism in the *CYP* gene was previously reported in human CYP2E1 (*21*). In view of the variation in the content of the CYP1A2 mRNA in unrelated individuals (*8*), the interindividual differences in the activity and the content of immunoreactive CYP1A2 are expected to be due to a mutation(s) in the regulatory region of the gene.

In our previous study, we determined the nucleotide sequences of exon, exon-intron junction, and the 5′-flanking region (up to −2578) of the *CYP1A2* gene in several genomic DNA samples from Japanese subjects (*15*). Mutations were found which differed from sequences previously reported (*8, 13, 22, 23*). In the present study, a novel genetic polymorphism, a point mutation from guanine (type w/w) to adenine (type m/m) at −2964 base, in the 5′-flanking region of the human *CYP1A2* gene was found.

Cigarette smoking has been demonstrated to stimulate phenacetin metabolism (*24*), increase the amount of immunoreactive CYP1A2 in human liver microsomes (*5*), decrease the plasma half-lives of caffeine *in vivo* (*25*), and increase the molar ratio of caffeine metabolites as an index of CYP1A2 activity (*15*), indicating that human CYP1A2 is inducible by cigarette smoking. Such evidence that CYP1A2 is induced by smoking was confirmed by the present study. The presence of the mutation significantly decreased the induction of human CYP1A2 by smoking. This is the first report demonstrating the effect of the genetic polymorphism of the *CYP1A2* gene on the *in vivo* transcriptional activation of CYP1A2.

It was suggested that a 3MC-responsive region in the human *CYP1A2* gene was located at about 2.9 kb upstream of the gene (*13*). It is likely that the mutation found in the

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present study is the same as that reported previously. Cigarette smoking is known to induce CYP1A2 in the human liver as described above, but not in the lung (*26, 27*). These data suggest the existence of a liver-specific factor(s) that regulates CYP1A2 expression.

Gel retardation analysis clarified the presence of a factor(s) that reacted with the oligonucleotide probe regardless of the mutation site. The binding profiles with these oligonucleotide probes differed between the wild and the mutated types, suggesting that the binding of these proteins was specific for each DNA sequence. Since each unlabeled oligonucleotide probe competed in binding of a factor to own oligonucleotide probe, there appear to be at least two factors that bind specifically to the wild and the mutated oligonucleotide. A similar point mutation (adenine to cytosine) in the 5'-flanking region has been reported to human *CYP2E1* gene (*21*). In the previous report, it was shown that the binding profiles in gel retardation analysis differed between the wild and the mutated types, indicating the possibility of binding of different transcriptional factors to each allele. Hayashi *et al.* (*21*) also reported that the mutated allele increases transcription by up to 10-fold. Furthermore, it has been reported that hepatic mRNA content of CYP2E1 in the mutated allele was 3-fold higher than that in the wild type (*28*). However, many other studies showed that the mutated allele has no effect (*29-31*). Therefore, the effects of the mutated allele on the expression and the activity of CYP2E1 have been the subject of controversy. In contrast, the present study using *in vivo* caffeine metabolism indicated that the point mutation in the 5'-flanking region in human CYP1A2 might influence CYP1A2 activity.

It was reported that a region located at −2532/−2423 contained a xenobiotic-responsive element-like sequence that might contribute to the overall induction by 3MC. The induction was observed by the transient transfection of the upstream region of the gene using HepG2 cell line (*32*). Recently, *cis*-elements involved in the positive and negative regulations of the *CYP1A2* gene have been clarified using breast carcinoma MCF-7 cells (from −2352 to −2094) (*14*). Thus, the expression of CYP1A2 appears to be regulated by multiple elements including the region shown in the present study.

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