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Homologous Unequal Cross-Over within the Human *CYP2A* Gene Cluster as a Mechanism for the Deletion of the Entire *CYP2A6* Gene Associated with the Poor Metabolizer Phenotype[1]

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To clarify the molecular mechanisms involved in the generation of the *CYP2A6* gene deletion (E-type variant), we analyzed the *CYP2A7* gene, which is located in the 5′-flanking region of the *CYP2A6* gene, from individuals with the E-type variant and compared it with the sequences of wild type *CYP2A7* and *CYP2A6* genes. The 3′-downstream sequence (up to 324 bp from the *Sac*I site in exon 9) of the *CYP2A7* gene of the E-type variant is identical to that of the wild *CYP2A7* gene. However, the 3′-downstream sequence (starting from 325 bp from the *Sac*I site in exon 9) of the *CYP2A7* gene of the E-type variant is identical to that of the wild *CYP2A6* gene, indicating that the 3′-downstream region of *CYP2A7* and the 3′-downstream region of *CYP2A6* linked directly eliminating the whole *CYP2A6* gene. PCR analysis using primers specific to the *CYP2A7* gene and the *CYP2A6* and *CYP2A7* genes confirmed that all DNA samples obtained from 7 individuals carrying the E-type variant possessed the same break points. These results indicate that the breakpoint of the *CYP2A6* gene deletion lies in the 3′-downstream region of the *CYP2A7* and *CYP2A6* genes.

Key words: coumarin, genetic polymorphism, gene analysis, pharmacogenetics, RFLP.

Cytochrome P450 (CYP) is a heme-containing enzyme responsible for the metabolism of exogenous compounds such as drugs, environmental pollutants, and dietary chemicals, and endogenous compounds such as steroids, fatty acids, and prostaglandins (*1*). Among the forms of CYP, CYP2A6 has been recognized as a form with unique catalytic properties. CYP2A6 was first shown to catalyze coumarin 7-hydroxylation (*2-5*). Studies by us have shown that it also catalyzes SM-12502 S-oxidation (*6*) and cotinine 3′-hydroxylation (*7*). This molecular form of CYP is also capable of metabolically activating genotoxins including aflatoxin B_1 (*8*), NDEA (*9*), and the tobacco-specific NNK (*10*). It has been noted that there is great interindividual variation in the activity of the coumarin 7-hydroxylase *in vivo* (*11-13*). Recently, Nunoya *et al.* (*14, 15*) found new variant alleles that account for the large interindividual variations of CYP2A6 activity in a Japanese population using SM-12502 as a new probe drug. The disposition of SM-12502 was investigated in the plasma or urine from 28 healthy Japanese volunteers after a single intravenous administration of SM-12502. Three of the 28 Japanese were phenotyped as PM and 25 were EM (*15*).

The *CYP2A* gene cluster contains five tandemly arranged genes including three complete genes, *CYP2A6, CYP2A7*, and *CYP2A13*, and two pseudogenes, *CYP2A7PC* and *CYP2A7PT*, on chromosome 19q13.2 (*16, 17*). The *CYP2A7* and *CYP2A13* genes have DNA sequences similar to that of the *CYP2A6* gene (*2, 16*), while no substrates for CYP2A7 or CYP2A13 have been reported.

We proposed in a previous paper that there are at least five variants, namely A, B, C, D, and E, as judged by *Sac*I-RFLP. Among them, the E-type variant shows extremely low S-oxidase activity to SM-12502. The E-type variant was also shown to be caused by the whole deletion of the *CYP2A6* gene (*15*). Moreover, the *Sac*I-D-type seemed to be a significant factor in the poor metabolic capacity of SM-12502 in human liver microsomes (*14*). The frequency of *CYP2A6* gene deletions (D-type and E-type) in the Japanese population is 4.5% (*15*). The frequency of the *Sac*I-E-type variant is 3.2%. We also observed that 6.4-, 4.5-, and 2.6-kb fragments, which are present in the wild type gene, are absent in the E-type variant of *Sac*I-RFLP. The lack of a fragment about 13-kb in size as compared with the wild type allele was also noted in *Sph*I-RFLP.

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Abbreviations: CYP, cytochrome P450; dATP, deoxyadenosine 5′-triphosphate; dCTP, deoxycytidine 5′-triphosphate; dGTP, deoxyguanosine 5′-triphosphate; dNTP, deoxynucleotide 5′-triphosphates; dTTP, deoxythymine 5′-triphosphate; EM, extensive metabolizer; NDEA, *N*-nitrosodiethylamine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PM, poor metabolizer; SM-12502, (+)-*cis*-3,5-dimethyl-2-(3-pyridyl)thiazolidin-4-one hydrochloride.

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In the present study, we analyzed the sequence of the *CYP2A7* gene, which locates in the 5′-flanking region of the *CYP2A6* gene, from individuals with the E-type variant and compared it with the sequences of the *CYP2A7* and *CYP2A6* genes from wild type individuals. Break points in the gene were found to be present at about 300 bp 3′-downstream from the stop codon of the *CYP2A7* and *CYP2A6* gene, generating the deletion of the entire CYP2A6.

MATERIALS AND METHODS

Materials—Restriction endonuclease was obtained from Toyobo (Osaka), Takara Shuzo (Kyoto), or New England Biolabs (Beverly, MA), nylon membranes (Nytran NY13) were from Schleicher & Schuell (Dassel, Germany), lambda FIXII, Pfu DNA polymerase, and Gigapack III Gold Packaging Extracts were from Stratagene (La Jolla, CA), T4 DNA polymerase was from Takara Shuzo (Kyoto), Sequenase Ver. 2 was from United States Biochemical (Cleveland, OH), and Ampli Taq DNA Polymerase was from Perkin Elmer Cetus (Norwalk, CT). All other chemicals and solvents were of the highest grade commercially available.

Synthesis of Oligonucleotide—Oligonucleotides were synthesized with a DNA synthesizer (Model 381A; Applied Biosystems, Foster City, CA). The sequences and locations of the synthesized CYP2A oligonucleotides are shown in Table I.

Preparation of Genomic DNA and Southern Blot Analyses—Genomic DNA was prepared from peripheral lymphocytes by phenol-chloroform extraction followed by ethanol precipitation (*18*). The DNA preparations (10 μg) were digested with restriction endonucleases. The digested DNA was subjected to electrophoresis in 0.6% agarose gels. The gels were treated with 0.5 M NaOH containing 1.5 M NaCl, neutralized with 0.5 M Tris-HCl buffer (pH 8.0) containing 1.5 M NaCl, and equilibrated with 0.3 M sodium citrate containing 3 M NaCl prior to the transfer of the DNA to nylon membranes. The membranes were baked at 80°C for 2 h, prehybridized at 65°C for 2 h, and then hybridized at 65°C for 8 h. Hybridization was performed in reaction mixtures containing the 1.6 kb fragment of a ^{32}P-labeled human *CYP2A6* cDNA, 50 mM Tris-HCl buffer (pH 8.0), 1 M NaCl, 11.5 mM EDTA, 0.1% SDS, 0.1% ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA, and 0.1 mg/ml of denatured salmon sperm DNA. The membranes were washed with 7.5 mM sodium citrate containing 75 mM NaCl and 0.1% SDS at 50°C for 20 min. Hybridized bands were visualized on X-ray film. Human *CYP2A6* cDNA was obtained as previously described (*14*).

Construction and Screening of a Genomic DNA Library Prepared from Human Peripheral Lymphocytes—Genomic DNA from subjects PM3 and EM3 was extracted from peripheral lymphocytes with phenol-chloroform followed by ethanol precipitation (*18*). The examination of *CYP2A6* genotypes and phenotypes as judged by the *in vivo* metabolism of SM-12502 has already been reported (*15*). Subject EM3 was judged to show the wild type phenotype and to be a homozygote of the wild genotype or a heterozygote of wild/mutant genotypes. On the other hand, subject PM3 showed the PM phenotype and was judged to be a homozygote of the *CYP2A6* gene deletion. Genomic DNAs from subjects PM3 and EM3, partially digested with *Sau*3AI, were fractionated by a sucrose density gradient (10-38%) centrifugation. Fractions containing fragments with mean lengths of 15-kb were partially filled-in with dGTP and dATP, and ligated to a lambda FIXII vector, which had been digested with *Xho*I and partially filled-in with dTTP and dCTP (Stratagene, CA, USA). The ligated product was packaged *in vitro* using Gigapack III Gold Packaging Extracts. This library was screened by the plaque hybridization method using the 1.6-kb fragment of human CYP2A6 cDNA as a probe. Phage DNAs were purified from positive plaques. The inserted DNA fragments were digested with various restriction enzymes, and subcloned into pUC18, M13mp18, or M13mp19. The nucleotide sequences of the positive clones were analyzed by the dideoxy method with Sequenase Ver. 2 or an Applied Biosystems Model 373A DNA sequencer following the protocol provided by the manufacturer.

PCR Analysis of the 5′-Flanking Region of the CYP2A6 Gene—To confirm that the E-type variant contains the 5′-flanking region of the *CYP2A6* gene, PCR amplification was carried out. Primers 2A6-kaS1, 2A6-kaAS1, 2A6-kuS1, and 2A6-kuAS1 were designed and used to amplify the 5′-flanking region of the *CYP2A6* gene (Table I). PCR was performed in a reaction mixture containing 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, each dNTP (200 μM), primer (0.5 μM), genomic DNA (500 ng), and 1 U of Ampli Taq DNA polymerase in a final volume of 50 μl. Thirty cycles of amplification were performed using a DNA Thermal Cycler under the following conditions: denaturation at 94°C for 2 min, annealing at 48°C for 2 min, and extension at 72°C for 2 min. The PCR products were subjected to electrophoresis in 2% agarose gels.

Analysis of the Break Points That Generate the CYP2A6 Gene Deletion—To confirm that the break points for the *CYP2A6* gene deletion are located in the 3′-flanking region of the *CYP2A7* gene of a PM (E-type), PCR amplification was performed. For this purpose, primers 2A6-delS and 2A6-delAS were designed to amplify the 3′-flanking region of both the *CYP2A6* and *CYP2A7* genes (Table I). PCR reactions were carried out as described above except for denaturation at 94°C for 1 min and annealing at 56°C for 1 min. PCR products in PM showing different electrophoresis patterns compared with EM were subcloned into pMOS-Blue T-vector. The nucleotide sequences of the positive clones were sequenced using an Applied Biosystems Model 373A DNA sequencer.

TABLE I. **Oligonucleotides used in this study.**

Primer	Sequence	Position[a]
2A6-delS	5′-TTCGCGGAAGAGGCGGGTA-3′	209-227
2A6-delAS	5′-GATCTGTGTTCAAAGATTTTCTG-3′	549-571
2A6-kaS1	5′-TTGGGAGGAGGCTGAAAATC-3′	–
2A6-kaAS1	5′-TGTGACCTATGTGCCTCCC-3′	–
2A6-kuS1	5′-TCTCTCAACACCTATCTCAAT-3′	–
2A6-kuAS1	5′-GAGCCGAAATCCCACCAC-3′	–

[a]Numbers correspond to the location described in Fig. 3.

RESULTS

Isolation and Analysis of the CYP2A7 Gene—To further explore the molecular basis of *Sac*I-E-type RFLP associat-

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ed with the *CYP2A6* gene deletion and the PM phenotype (*15*), we isolated clones for *CYP2A7* from the genomic library constructed from the DNA of subjects EM3 (*Sac*I-C-type) and PM3 (*Sac*I-E-type) and analyzed the sequences. Clones 13, 1E, and 18E were isolated from subjects PM3, EM3, and EM3, respectively. The restriction maps are shown in Fig. 1. The sequences of each exon in the three clones showed more than 99% identity to the *CYP2A7* cDNA reported by Yamano *et al.* (*2*).

Analysis of Deletion of the 5′-Flanking Region of the CYP2A6 Gene—Probes b and c were isolated by PCR on the basis of the structure of the 5′-flanking region of the *CYP2A6* gene (Fig. 2a, Table I). When genomic DNAs were digested with *Sac*I and hybridized with probes b and c, subject 932, who showed *Sac*I-E-type RFLP, lacked a 5.6 kb fragment and a 6.4 kb fragment as compared with subject 934 (*Sac*I-B-type) and subject 943 (*Sac*I-C-type) (Fig. 2, b and c). To confirm the possibility that the *Sac*I-E-type RFLP is caused by the deletion of the 5′-flanking region of the *CYP2A6* gene, we amplified regions a and b (Fig. 2a) as described in "MATERIALS AND METHODS." The results of analyses of regions a and b are shown in Fig. 2, d and e. Subjects 932, 567, 570, PM1, PM2, and PM3 (*Sac*I-E-type) showed deletions of region a (243 bp fragment). On the other hand, Subjects EM2 (*Sac*I-A-type), 943, 569, EM1, and EM3 (*Sac*I-C-type) contained region a. Subjects 889 and 932 (*Sac*I-E-type) lacked region b (264 bp fragment). On the other hand, Subjects 893 and 943 (*Sac*I-C-type) contained region b. Regions a and b are located at about 11 and 9 kb upstream from the *CYP2A6* exon1, respectively. These results suggest that *Sac*I-E-type lacks more than 9 kb of the 5′-flanking region of the *CYP2A6* gene.

Analysis of the CYP2A7 Gene from Subjects EM3 and PM3—The *CYP2A7* gene is reported to be located in the upstream region of the *CYP2A6* gene (*16*). As shown in Fig. 1, we isolated clones for the *CYP2A7* gene, clone 13 from subject PM3 and clones 1E and 18E from subject EM3. The sequences from exon 1 to exon 9 in clones 13, 1E, and 18E were identical to those of the *CYP2A7* gene. Some base substitutions and deletions between subjects EM3 and PM3 were seen in the region from the stop codon on the 3′-downstream side of *CYP2A7*. As the entire sequences of the *CYP2A6* and *CYP2A7* genes have not yet been reported, we also isolated clone 23E for the *CYP2A6* gene from subject EM3 and analyzed the sequence of the region from the stop codon to the 3′-downstream region of the *CYP2A6* gene. Figure 3 shows the comparison of nucleotide sequences of clones 13 (PM), 1E, 18E, *CYP2A7* (M33317), *CYP2A6v2* (U22027), 23E, and *CYP2A6* (M33318) in the region from exon 9 to the 3′-downstream region. The sequence of clone 13 (PM) from 1 to 324 (Fig. 3) is identical to the corresponding region of 1E, 18E (*CYP2A7*) from EM3 and *CYP2A7* (M33317), but not to the corresponding region of 23E (*CYP2A6*) from EM3, *CYP2A6v2* (U22027), and *CYP2A6* (M33318). On the other hand, the 3′-downstream sequence from 325 is completely homologous to the corresponding sequence of 23E (*CYP2A6*) from EM3 and *CYP2A6* (M33318), and not to the corresponding sequence

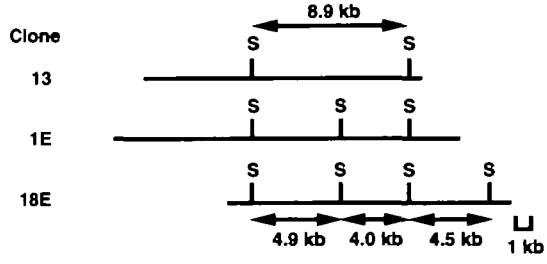

Fig. 1. **Organization of the human *CYP2A7* gene from EM and PM subjects.** The restriction sites within these DNA fragments are denoted by vertical lines. S, *Sac*I. Clones 13, 1E, and 18E were from subjects PM3, EM3, and EM3, respectively.

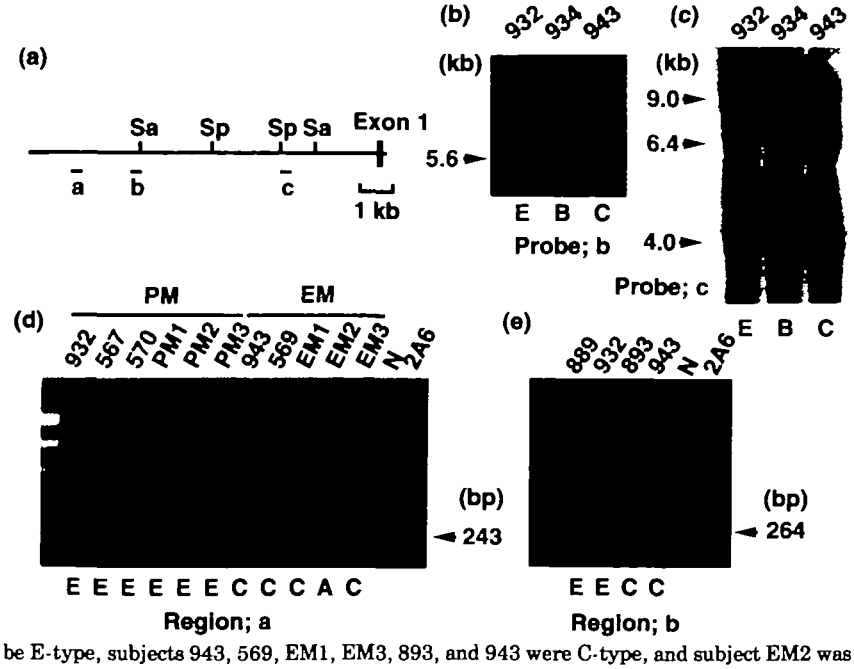

Fig. 2. **Analysis for the deletion of the 5′-flanking region in the human *CYP2A6* gene by PCR and Southern blot.** (a) The structure of the 5′-flanking region of the human *CYP2A6* gene (2A6-7E) (*15*). Restriction sites within this DNA fragment are denoted by vertical lines. Sa, *Sac*I; Sp, *Sph*I. The regions (a and b) were used for PCR analysis of the 5′-flanking region in the *CYP2A6* gene. (b) *Sac*I-RFLPs detected with human genomic DNAs using the b fragment as a probe (276 bp fragments). (c) *Sac*I-RFLPs detected with human genomic DNAs using c fragment as a probe (347 bp fragments). *Sac*I-digested DNAs isolated from subjects 932 (E-type), 934 (B-type), and 943 (C-type) were electrophoresed in a 0.6% agarose gel, transferred onto a nylon membrane, and hybridized using ^{32}P-labeled b and c fragments as probes. (d) The 243 bp PCR products were amplified using 2A6-kuS1 and 2A6-kuAS1 primers for the 5′-flanking region in the *CYP2A6* gene. (e) The 264 bp PCR products were amplified using 2A6-kaS1 and 2A6-kaAS1 primers for 5′-flanking region in the *CYP2A6* gene. The PCR products were subjected to electrophoresis in a 2% agarose gel. Subjects 932, 567, 570, PM1, PM2, and PM3 were judged to be E-type, subjects 943, 569, EM1, EM3, 893, and 943 were C-type, and subject EM2 was A-type. Lane N, without DNA; lane 2A6, *CYP2A6* gene.

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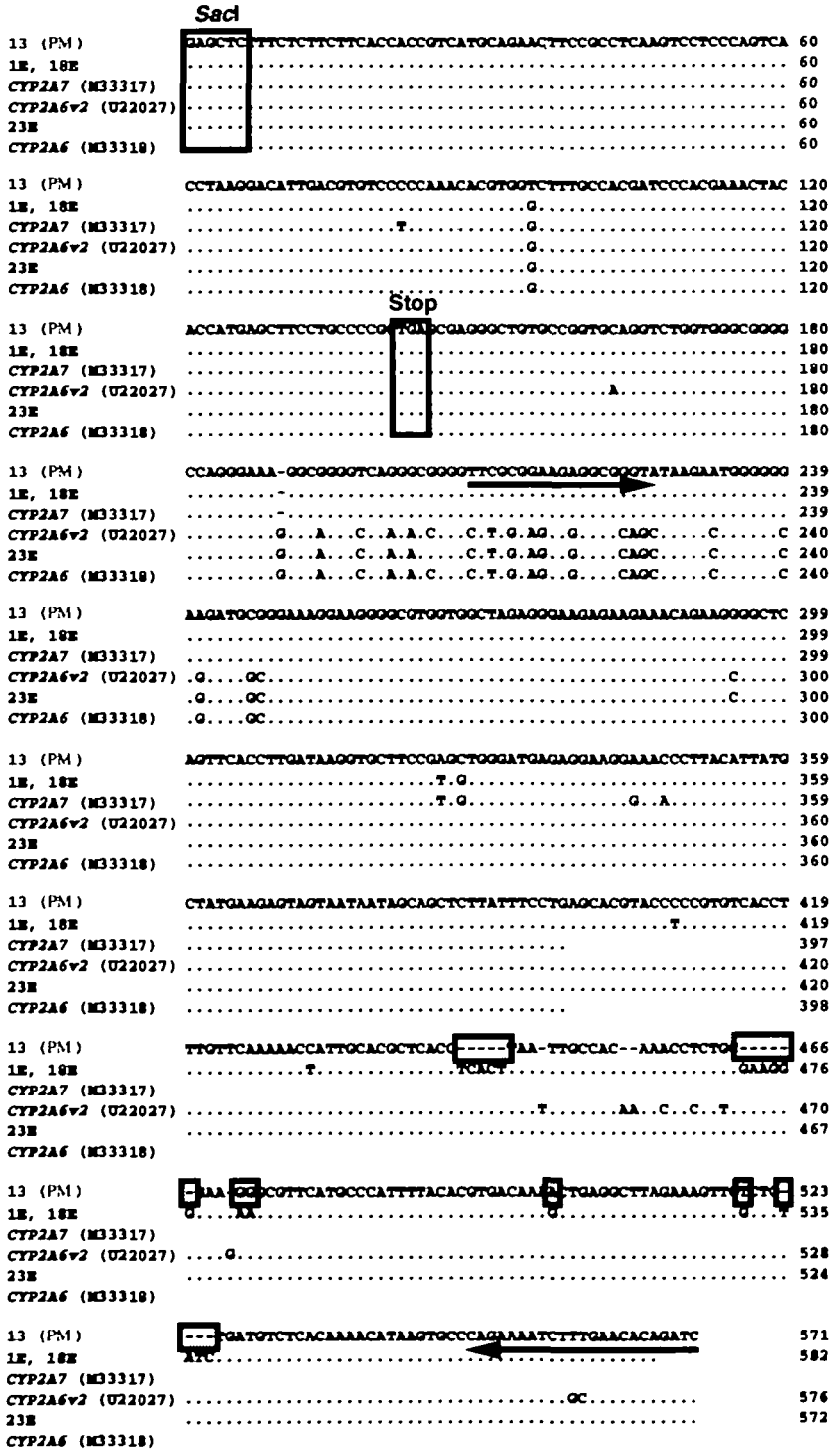

Fig. 3. **Alignment of sequences of clones 13, 1E, 18E, *CYP2A7*, *CYP2A6v2*, 23E, and *CYP2A6* including exon 9 and the 3′-flanking region.** Clones 13, 1E, 18E, and 23E were isolated from PM3, EM3, EM3, and EM3, respectively. The following DNA sequences (GenBank accession no.) were used: M33318, M33317, and U22027 for *CYP2A6*, *CYP2A7*, and *CYP2A6v2*, respectively. The sequences start from the *Sac*I site in exon 9. Only nucleotides that are not identical to sequences of clone 13 are shown for clones 1E, 18E, *CYP2A7*, *CYP2A6v2*, 23E, and *CYP2A6*. Insertions and deletions are denoted by dashes. The hatched box for clone 13 shows the PM-specific region. Arrows indicate the location and direction of primers for the PCR shown in Fig. 4.

of 1E and 18E (*CYP2A7*) from EM3, *CYP2A7* (M33317), and *CYP2A6v2* (U22027). The alignment shows that the former region of clone 13 is homologous to *CYP2A7* and that the latter region is identical to *CYP2A6*. The breakpoint of the *CYP2A6* gene deletion probably lies within this region (325-538 in Fig. 3).

PCR Analysis for a Chimeric CYP2A7/CYP2A6 Gene Associated with the Deletion of the Entire CYP2A6 Gene— If our hypothesis concerning the cause of the *CYP2A6* gene deletion is correct, the generation of the chimeric *CYP2A7/CYP2A6* gene associated with the deletion of entire *CYP2A6* gene can be expected. To confirm the result in Fig. 3, we designed a set of primers, one specific for the *CYP2A7* gene sequence (TTCGCGGAAGAGGCGGGTA in Fig. 3) and the other for the *CYP2A6* and *CYP2A7* gene sequence (5′-GATCTGTGTTCAAAGATTTTCTG-3′ in Fig. 3). In the wild type *CYP2A7* gene, these primers yield a PCR product 379 bp long. On the other hand, a PCR product of 364 bp is expected for the chimeric *CYP2A7/CYP2A6* gene. When 16 DNA samples with known *Sac*I-

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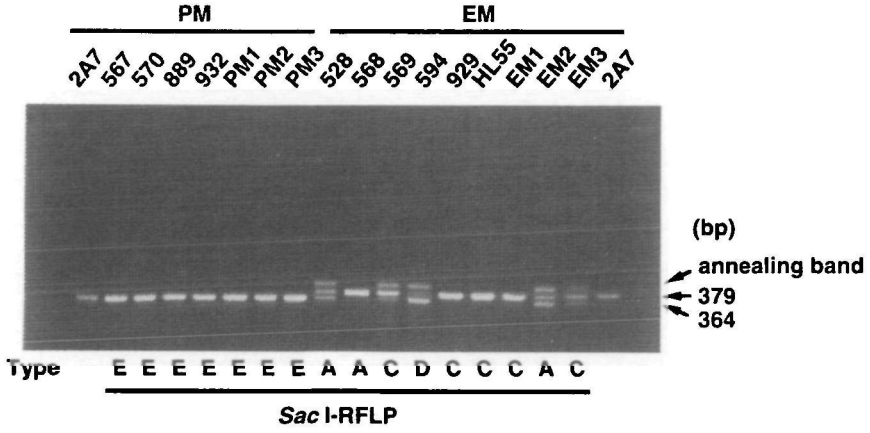

Fig. 4. **PCR analysis for a chimeric *CYP2A7/CYP2A6* gene associated with the deletion of the entire *CYP2A6* gene.** PCR products were amplified using 2A6-delS and 2A6-delAS primers for the 3′-flanking region of *CYP2A6* and *CYP2A7*. These primers are shown by arrows in Fig. 3. Wild type and chimeric *CYP2A7* genes show 379 and 364 bp fragments, respectively. Subjects 567, 570, 889, 932, PM1, PM2, and PM3 were classified as E-type, subjects 528, 568, and EM2 were A-type, subjects 569, 929, HL55, EM1, and EM3 were C-type, and subject 594 was D-type. Lane 2A7, *CYP2A7* gene.

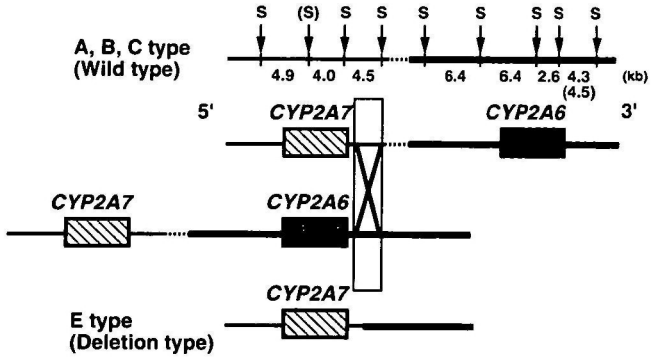

Fig. 5. **The predicted mechanism for generating the chimeric *CYP2A7/CYP2A6* gene associated with the deletion of the entire *CYP2A6* gene.** The chimeric *CYP2A7/CYP2A6* gene was generated by unequal crossing-over between two wild type alleles in the 3′-flanking region of the *CYP2A7* and *CYP2A6* genes.

RFLP patterns were examined for the presence of the chimeric gene with this set of primers, DNAs from 7 individuals classified as E-type (*CYP2A6* deletion type) produced 364 bp fragments (Fig. 4). DNAs obtained from the other 9 individuals classified as other types produced 379 bp fragments or 379 and 364 bp fragments. These results indicate that the breakpoint to generate the *CYP2A6* gene deletion lies in the 3′-downstream region (325-538 in Fig. 3) of the *CYP2A7* and *CYP2A6* genes, and shows the *Sac*I-E-type to possess a breakpoint in the same region.

In PCR analysis of the chimeric *CYP2A7/CYP2A6* gene, the allele produced a fragment bigger than 379 and 364 bp (Fig. 4). It appears that this fragment was produced by the annealing of 379 and 364 bp fragments. Agarose gel electrophoresis of the PCR products of subjects 528, 569, 594, EM2, and EM3 showed three fragments, while heat denaturation of the DNA prior to polyacrylamide gel electrophoresis resulted in the disappearance of the unknown fragment as seen in subjects PM1, PM2, PM3, 567, 570, 889, and 932.

DISCUSSION

In this study, we demonstrate the possibility that the chimeric *CYP2A7/CYP2A6* gene occurs as a result of a homologous unequal cross-over between the *CYP2A7* and *CYP2A6* genes (Fig. 5). Furthermore, we showed that the break points that produce the PM-associated deletion allele as indicated by *Sac*I-E-type RFLP are located in the 3′-downstream region (325-538 in Fig. 3) of the *CYP2A7* and *CYP2A6* genes. The 5′-end break point occurs between the 3′-downstream region of the *CYP2A7* gene and the 3′-end break point of the *CYP2A6* gene, probably because of the high homology in these regions. These data indicate that the deletion of the entire *CYP2A6* gene (*Sac*I-E-type) occurs due to an unequal recombination between two homologous sequences, either by chromosome misalignment followed by an unequal cross-over or by the formation of a loop structure on a single chromosome. Gaedigk *et al.* (*19*) proposed that the 3′-end break point for the *CYP2D6(D)* deletion is located 536 bp downstream from exon 9 of the *CYP2D6* gene. Recently, Steen *et al.* (*20*) reported that the mechanism involved in the deletion of the *CYP2D6(D)* gene is a homologous unequal cross-over involving a 2.8 kb repeated region that flanks the 3′-downstream regions of the *CYP2D7* and *CYP2D6* genes. Thus, the mechanism involved in the entire *CYP2A6* gene deletion resembles that of the *CYP2D6(D)* gene deletion.

CYP2A6 is capable of metabolically activating various procarcinogens, while the role of CYP2A6 in human cancer susceptibility is still unclear (*21*). The relationship between the *CYP2A6* gene deletion and susceptibility to procarcinogens is under examination in our laboratory.

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